TRANSFERABLE ANTIBIOTIC RESISTANCE IN THE GENUS *HAEMOPHILUS*

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by

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ABSTRACT

Plasmid-mediated conjugation, as a means for the dissemination of antibiotic-resistance determinants, was examined in the genus Haemophilus. Four self-transmissible antibiotic-resistance plasmids, ranging in size from 32 x 10^6 to 38 x 10^6 daltons (32-38 Mdal), were independently isolated from clinical strains of H. influenzae, H. parainfluenzae, and H. ducreyi of diverse geographic origins. Restriction endonuclease analysis of the plasmids revealed a high degree of structural similarity, suggestive of a common origin with the possibility of active transfer between these species. Intra- and interspecific membrane filter matings showed that, of ten Haemophilus species tested as possible recipients, transfer was only possible between the three species in which the plasmids were initially isolated. Comparison of efficiency of transfer showed that intraspecific matings were more efficient than interspecific matings, although H. ducreyi proved to be a highly efficient recipient yet a poor donor in interspecific transfer. Conjugative transfer of large plasmids was not possible to Escherichia coli nor to Neisseria gonorrhoeae isolates, although a small, non-conjugative ampicillin-resistance plasmid could be mobilized from N. gonorrhoeae to three Haemophilus species. The conjugative process itself in Haemophilus was found not to require the participation of the host recombination system and was sensitive to variations in the ratio of donor

to recipient cells in the mating mixture.

A means was also sought which would account for the dissemination of non-conjugative R-plasmids in Haemophilus species, particularly H. ducreyi. A clinical isolate of H. ducreyi was found to harbor three plasmids: a 23.5 Mdal phenotypically cryptic plasmid, a 7.0 Mdal ampicillin-resistance plasmid, and a 4.9 Mdal sulfonamide resistance plasmid. The two smaller plasmids were transferable by conjugation to Haemophilus and E. coli recipients but only if the 23.5 Mdal plasmid was co-resident in the cell, indicating that this plasmid had mobilizing capabilities. H. influenzae transconjugants which had acquired both the 23.5 Mdal plasmid and one of the R-plasmids could subsequently re-transfer the R-plasmid to other Haemophilus recipients. In addition, the 23.5 Mdal plasmid could mobilize for transfer other non-conjugative R-plasmids previously isolated from H. ducreyi, H. parainfluenzae, and N. gonorrhoeae. Restriction endonuclease analysis of the 23.5 Mdal plasmid and a 27 Mdal derivative plasmid containing an ampicillin-resistance transposon suggested it was different from the 24.5 Mdal mobilizing plasmid common to N. gonorrhoeae.

These sets of data indicate that conjugation is an important and efficient means for the dissemination of both large and small R-plasmids within Haemophilus. Large plasmids probably had a common origin and are self-transferable between at least three species of Haemophilus. Small plasmids are normally non-conjugative, but can sometimes be mobilized for conjugative transfer through the use of a plas-

mid mobilization system discovered in *H. ducreyi*.

INTRODUCTION

The successful treatment of bacterial infections with antibiotics and other antimicrobial agents has long been hampered by the apparent ease with which organisms resistant to these drugs have emerged. In many cases the resistance was due to the production by the bacterium of a specific protein which either inactivated the antibiotic directly or altered the cell's metabolic machinery so that the antibiotic was no longer effective against it (Davies and Smith, 1978). In addition, the gene encoding this protein was frequently found to reside not on the cell's chromosome but on a discrete segment of "extrachromosomal" DNA - small and circular in form and able to replicate autonomously from the chromosome - known as a plasmid (Lederberg, 1952; Hayes, 1970). Only recently has it been discovered that the concept of "plasmid-borne resistance genes" is somewhat of an oversimplification - these resistance genes may in fact be part of even smaller genetic elements known as transposons. Transposons are linear segments of DNA consisting of only three or four genes, one of which is the actual resistance gene. While they are usually found as part of a larger plasmid genome, they have the unique ability to excise from one particular site on a genome, transpose to a different site on the same genome or to another unrelated genome, and integrate into this new site (Kleckner, 1977; Calos and Miller, 1980). Thus the

genes conferring antibiotic resistance in bacteria can be envisaged as existing on two levels; as part of transposable genetic elements and, on the larger scale, as part of individual plasmids.

Amongst the enteric bacteria such as Shigella, Salmonella, and Escherichia, resistance to many new, clinically useful antibiotics inevitably arose soon after the introduction of these drugs into widespread use and usually spread quickly to various other enteric species (Watanabe, 1963, 1966). The many different types of resistance plasmids (R-plasmids) subsequently isolated could broadly be divided into two major groups (Helinski, 1976): large plasmids, greater than 25-30 x 10^6 daltons (25-30 Mdal) in size, which frequently had the ability to promote their own transfer from cell to cell by the sexual means of genetic exchange known as conjugation, and small plasmids of less than 10 Mdal which were unable to mediate their own conjugative intercell transfer. The conjugative ability of the large plasmids was assumed to be the reason for the rapid spread of antibiotic resistance among the enteric species of bacteria (Richmond, 1969; Watanabe, 1971).

Other genera of bacteria such as Haemophilus however, displayed a different pattern in the acquisition of antibiotic-resistance traits. Several species of Haemophilus are pathogenic for humans and some, such as H. influenzae, are responsible for a number of serious early childhood infections. H. influenzae was uniformly susceptible to ampi-

cillin for many years until suddenly in 1974 the first ampicillin-resistant strains were isolated (Kahn et al., 1974). From this point, the isolation rate of resistant H. influenzae increased dramatically and today the number of ampicillin-resistant H. influenzae may approach 18% of the total isolates in the United States (Thornsberry, 1980). In addition, the ampicillin-resistance phenotype has subsequently spread both to H. ducreyi, which causes the sexually transmitted disease chancroid, and H. parainfluenzae (Elwell et al., 1974; Brunton et al., 1979). A similar pattern was followed in the development of tetracycline and chloramphenicol resistance within these species of Haemophilus and possibly in the future for sulfonamide resistance as well although this has so far been confined to the species H. ducreyi.

Resistant species of Haemophilus were found to possess the two size classes of R-plasmids; ampicillin-resistance was mediated by conjugative plasmids of 30 Mdal in H. influenzae but by small non-conjugative plasmids of between 4 and 5 Mdal in H. parainfluenzae (Elwell et al., 1975; Albritton and Slaney, 1980). Ampicillin-resistant H. ducreyi may contain plasmids of either 5.7 or 7.0 Mdal, neither of which is conjugally self-transferable (Brunton et al., 1979; W. Albritton, unpublished observations). Tetracycline and chloramphenicol resistance has thus far only been found to be mediated by plasmids greater than

30 Mdal in size in H. influenzae, H. parainfluenzae and H. ducreyi (Albritton and Slaney, 1980). Sulfonamide resistance in H. ducreyi however, is encoded by a non-conjugative plasmid of 4.9 Mdal (unpublished data). Molecular hybridization studies have demonstrated that the large Haemophilus R-plasmids isolated in different parts of the world are remarkably homologous with one another but not with enteric plasmids of the same size and carrying the same resistance determinants (Laufs and Kaulfers, 1977; Roberts et al., 1980). Small ampicillin-resistance plasmids of Haemophilus origin however, show a considerable amount of DNA base sequence homology with similarly sized plasmids of Neisseria gonorrhoeae and the transposon carrying the ampicillin-resistance gene in both these sets of plasmids is probably identical to the transposon found on enteric plasmids (Brunton et al., 1980; MacLean et al., 1980). Furthermore, the actual base composition of both large and small Haemophilus R-plasmids is very similar to that of the Haemophilus chromosome but quite unlike the composition of the Escherichia coli chromosome (De Graaff et al., 1976; Brunton et al., 1979). These findings have suggested that the large conjugative plasmids seen in Haemophilus species are in fact indigenous to this genus and that only the transposable elements carrying the drug-resistance genes had their origins in species of another genus. The origin of the small Haemophilus R-plasmids may represent a similar situation but here there may have been some exchange of complete plasmids with

genera such as Neisseria or certain enteric bacteria.

In order to fully understand how antibiotic resistance plasmids have become established in Haemophilus species, where they originated, and how they could spread in the future, it becomes necessary to examine the mechanisms whereby the exchange of plasmids can occur in this genus. Spread of large R-plasmids by conjugative means has been well described in Haemophilus but only within the species H. influenzae; little is known of the interspecific or intergeneric host range of such plasmids and little has been done to determine the actual efficiency of conjugative plasmid transfer.

The factors behind the apparently rapid spread of small R-plasmids are somewhat less clear however. Since plasmids of this type cannot initiate their own conjugative transfer, there are presumably only three other means by which they could successfully spread within a cell population; transformation, transduction, and mobilization. Transformation of H. influenzae or H. parainfluenzae with purified plasmid DNA can occur although with relatively poor efficiency (Gromkova and Goodgal, 1979; Albritton et al., 1981), yet H. ducreyi, in which small R-plasmids appear to be most common, has never been shown to be transformable. Phage-mediated transduction has never been shown to operate as a means of plasmid transfer in any species of Haemophilus although bacteriophage have been isolated in several strains of H. influenzae and H. parainfluenzae (Boling et al., 1973;

Gromkova and Goodgal, 1977). Finally, the mobilization of small R-plasmids by co-resident conjugative plasmids is probably an important system for plasmid dissemination within N. gonorrhoeae and the Enterobacteriaceae (Smith, 1977; Roberts et al., 1978), but has as yet not been demonstrated to occur in Haemophilus. Thus, while it is possible that some small Haemophilus R-plasmids could have originated in genera other than Haemophilus or, on the other hand, that certain Haemophilus plasmids were the progenitors of R-plasmids in other genera, there remains the problem of demonstrating an efficient means by which such intergeneric transfers could have taken place.

Over the past five years, a large number of antibiotic-resistant Haemophilus strains have been acquired by this laboratory, usually as clinical isolates from the Health Sciences Center in Winnipeg, Manitoba (Albritton et al., 1978) but also from other parts of the world. These strains have carried both large conjugative and small non-conjugative plasmids specifying single or multiple resistance to ampicillin, tetracycline, chloramphenicol, and sulfonamide. Thus in order to trace the possible spread of drug-resistance plasmids and provide some insight into the continuing evolution of such plasmids in this genus, a number of parameters were examined.

1) The interspecific host range of conjugative Haemophilus plasmids.
2) The efficiency of conjugative transfer both intra-

and interspecifically.

3) The stability of large plasmids once introduced into a new host species.
4) The overall structural relatedness of plasmids determining the same resistance pattern but isolated from different species.
5) The means by which small non-conjugative Haemophilus plasmids may be transferred intra- and interspecifically.

LITERATURE REVIEW

A) Plasmid-mediated resistance to antibiotics

1) Historical perspectives:

The discovery in 1946 by Lederberg and Tatum that the bacterium Escherichia coli possessed a highly developed form of sexuality - exchanging genetic information in an organized manner - marked a major breakthrough in the field of microbial genetics (Lederberg and Tatum, 1946). In the early 1950's, the classic work of William Hayes established the existence of at least two sexually differentiated types of E. coli - males or donor cells and females or recipient cells - which could conjugate in unlike pairs (Hayes, 1953). The donor cells were unique in that they possessed an autonomous genetic unit separate from the E. coli chromosome, the so-called F- or fertility-factor, which was physically transferred from donor to recipient cells and was solely responsible for the genetic character of "maleness". The term "plasmid" was coined by Lederberg (1952) to refer to all such extrachromosomal genetic systems having the capacity for autonomous replication.

While plasmids were at first only of academic interest (Hayes, 1970), their practical relevance in the area of medical microbiology was brought to light in 1963 in a comprehensive review by Watanabe of earlier work by various Japanese researchers (Watanabe, 1963). In 1955 a single strain of Shigella dysenteriae was isolated from a patient

with bacillary dysentery who did not respond to treatment with antibiotics. This strain was found to be highly resistant to sulfonamide, streptomycin, chloramphenicol, and tetracycline. From 1955 to 1959, many more such multiply resistant Shigella strains were isolated, most being resistant to at least two of these drugs but up to 10% showing resistance to all four. Epidemiological studies revealed several unique features (see Mitsuhashi, 1977):

i) both drug-sensitive and multi-resistant Shigella could occasionally be isolated from different patients during the same epidemic.

ii) sometimes both sensitive and resistant strains of the same taxonomic type were isolated from the same patient.

iii) when patients who had been excreting sensitive Shigella were treated with a single antibiotic only, they frequently began to excrete Shigella which were resistant to all four drugs.

iv) most patients carrying multi-resistant Shigella also had multi-resistant E. coli with the same drug resistance pattern.

These findings suggested that the multi-resistance trait might be transferable not only between Shigella strains but also between Shigella and E. coli in the gastrointestinal tract. Ochiae in 1959 and Akiba in 1960 succeeded in demonstrating the transfer of the multi-resistance character between Shigella and E. coli in vitro (see Watanabe, 1963).

The transferable multi-resistance characters were termed resistance factors or simply R-factors (Watanabe, 1963). It soon became apparent that the R-factors had several properties in common with the E. coli F-factor, most importantly, the ability to promote their own intercell transfer by conjugation.

During the 1960's, R-factors in various species of Enterobacteriaceae were identified with increasing frequency, first in England (Datta, 1962) and then in many other parts of the world (Watanabe, 1966). Concurrent with the increased frequency of isolation of multi-resistant bacteria was the finding that the spectrum of R-factor mediated antibiotic resistance was increasing as well. In addition to streptomycin, chloramphenicol, sulfonamide, and tetracycline resistance, strains resistant to kanamycin and neomycin appeared (Lebek, 1963) and later, to penicillin due to the production of a β-lactamase enzyme (Anderson and Datta, 1965). In some cases, single R-factors carrying resistance determinants for as many as eight antibiotics were identified (Anderson, 1968; Kontomichalou et al., 1967). How this could be occuring was unknown but several theories arose based on earlier work by Watanabe et al. (1963, 1964). He provided evidence that the Shigella R-factors were actually comprised of two basically independent elements - the genetic determinants for drug resistance (R-determinants) and an element responsible for the conjugal transfer of the R-determinant, termed the resistance transfer factor (RTF).

Structurally, these were thought to be organized such that multiple R-determinants, each one specifying resistance to a single antibiotic, were linked to a larger segment of DNA (the RTF) to form a circular molecule. This association was not necessarily a stable one - a complete R-factor could dissociate into R-determinants and RTF either spontaneously or upon conjugal transfer (Watanabe, 1971). However, both these elements could be maintained together in the cell as independent circular replicative units which could at any time reassociate into the original composite R-factor. It was felt that such a mechanism could have played a role in the apparent continual acquisition of more and more drug-resistance determinants by R-factors (Watanabe, 1971).

2) Variety of plasmids

Since the initial discovery of extrachromosomal elements carrying drug-resistance determinants as well as having sex factor (or conjugative) ability, a variety of different plasmids have been identified, all having one property in common - the stable existence in an autonomous extrachromosomal state. Essentially, plasmids can be broadly categorized based on their ability to promote their own conjugal transfer from cell to cell (Helinski, 1976). Non-conjugative plasmids are usually small in size, containing less than 10 x 10^6 daltons (10 Mdal) of DNA, and are not able to self-transfer between cells. Conjugative plasmids contain in excess of 20-25 x 10^6 daltons (20-25 Mdal) of DNA and are self-transmissible within a cell population. These para-

meters are not absolute - the smallest known conjugative plasmid is approximately 17 Mdal (Crosa et al., 1975) while some naturally occurring large plasmids do not necessarily have conjugative abilities.

Plasmids usually confer upon their host cells some unique phenotypic trait which is lacking in plasmid-free cells and which is lost when the plasmid itself is lost. A considerable number of properties may be plasmid-encoded (see Reanney, 1976; Harwood, 1980), some of which, under certain environmental conditions, will give the cell a selective advantage over cells lacking them. But from a clinical if not historical standpoint, the most significant of these properties is the antibiotic-resistance trait.

3) Mechanisms of antibiotic resistance

The biochemical basis for plasmid-mediated drug resistance varies from one antibiotic to another although theoretically, only seven different mechanisms whereby resistance could arise are thought possible (Davis and Maas, 1952). In actuality, only four general mechanisms of resistance have rigorously been shown to be plasmid-mediated in bacterial systems (Davies and Smith, 1978) although examples of all seven mechanisms are known (see Koch, 1981). These are:

i) alteration of the target site in the cells so that binding of the antibiotic to the site is reduced or eliminated. The only well-established instance where such a mechanism is plasmid-determined is seen with macrolide

(erythromycin) - lincosamide (lincomycin) resistance in staphylococci and streptococci (Weisblum et al., 1971) Here, a plasmid-encoded methylase modifies the ribosomal 23S RNA so as to prevent the binding of the antibiotic to its normal target site - the 50S ribosomal subunit.

ii) a change in the cellular membrane transport system such that the permeability of the cell to the antibiotic is altered. Resistance to tetracycline, for example, results not from a failure of the drug to penetrate the cell (Franklin and Higginson, 1969), but from an enhanced outflow of the antibiotic such that a lethal internal concentration is never attained. At least two plasmid-encoded membrane-bound proteins have been implicated in this mechanism of resistance (Chopra and Howe, 1978).

iii) detoxification or inactivation of the antibiotic. The breakdown of penicillin and its derivatives, for instance, is by a plasmid-encoded β-lactamase enzyme (Richmond and Sykes, 1973). This enzyme splits the β-lactam ring of the antibiotic to penicilloic acid derivatives which have no antibacterial action. Another means of direct detoxification is through an enzyme-catalyzed modification; for example, the acetylation of chloramphenicol by a plasmid-borne chloramphenicol acetyltransferase (Davies and Smith, 1978).

iv) providing the cell with a replacement for the metabolic step that is inhibited. Sulfonamides for example, exert their antimicrobial effect by inhibiting dihydropteroic acid

synthesis and therefore stopping folic acid synthesis and bacterial growth. Resistance is associated with the production of a new plasmid-encoded dihydropteroic acid synthase isoenzyme which is insensitive to sulfonamide (Wise and Abou-Donia, 1975).

4) Genetics of antibiotic resistance

It is not sufficient to state merely that the genes responsible for the antibiotic resistance phenotype of many bacteria are located on plasmids. Frequently these genes are found as part of a unique class of genetic elements known as transposons (for reviews see Cohen, 1976; Kleckner, 1977; Calos and Miller, 1980), which are themselves structural components of plasmids or other replicons. In 1964, Kondo and Mitsuhashi observed that the E. coli phage P1 could receive an antibiotic-resistance gene originally located on a plasmid (Kondo and Mitsuhashi, 1964). Later, Iyobe et al. (1969, 1970) described the spontaneous integration of a plasmid-borne chloramphenicol-resistance gene into various sites on the E. coli chromosome and the subsequent movement of this resistance trait onto a second plasmid introduced into the cell. Also, Datta et al. (1971) observed the apparent exchange of the β-lactamase gene between two unrelated plasmids present in the same cell, while Richmond and Sykes (1972) reported that this gene could also integrate into the E. coli chromosome. Finally, Hedges and Jacob (1974) concluded that the structural gene for the β-lactamase enzyme was carried by a discrete sequence which could

translocate or transpose from one location to another within the same, or to another, genome; hence, it was termed a transposon and since it was the first one so discovered, it was designated TnA or Tnl. Currently, more than 20 different transposons have been identified (Calos and Miller, 1980), carrying resistance genes for various antibiotics or specifying other phenotypic traits. Some of the more widely studied transposons are listed in Table 1.

All transposons have in common the ability to move from one position on a procaryotic genome to another by a mechanism that is recA-independent - that is, translocation events do not require the involvement of the system responsible for general homologous recombination in bacteria since they occur quite efficiently in strains where homologous recombination has been eliminated by mutation ($recA^-$) (Rubens et al., 1976). One of the best characterized transposons, Tn3, has been shown to consist of a 3.2 Mdal DNA sequence bounded on either end by a 38 base pair sequence in an inverted repeat formation (Ohtsubo et al., 1979). The 4957 base pairs of the transposon itself code for three polypeptides (Heffron et al., 1979; Chou et al., 1979): a "transposase" which is required for the transposition event, a repressor protein which regulates the transposase gene, and the β-lactamase enzyme which confers ampicillin resistance. The two - 38 base pair ends of the transposon are of critical importance since they are thought to be recognized by the transposase and possibly other proteins involved in transposition (Calos

Table 1. Transposable Elements in Procaryotic Cells[a]

Transposon	Phenotypic Trait Conferred	Length (Base Pairs)	Reference
TnA { Tn1, Tn2, Tn3 }	Ap^r[b]	4,957	Hedges and Jacob, 1974; Kopecko and Cohen, 1975
Tn4	Ap^r, Sm^r, Su^r	20,500	Kopecko et al., 1976
Tn5	$kanamycin^r$	5,400	Berg et al., 1975.
Tn7	$trimethoprim^r$ $spectinomycin^r$	14,000	Barth et al., 1976.
Tn9	Cm^r	2,638	Gottesman and Rosner, 1975
Tn10	Tc^r	9,300	Kleckner et al., 1975
Tn1721	Tc^r	10,900	Schmitt et al., 1979
Tn501	$mercuric\ ion^r$	7,800	Bennett et al., 1978
Tn951	lac^+	16,600	Cornelis et al., 1978
Tn1681	heat stable enterotoxin production	2,088	So et al., 1979

[a] Modified from Calos and Miller, 1980.

[b] Abbreviations used: Ap, ampicillin; Sm, streptomycin; Su, sulfonamide; Tc, tetracycline; Cm, chloramphenicol.

and Miller, 1980). The structure of these inverted repeat ends varies considerably from one transposon to another; in some cases they themselves may actually be distinct genetic units capable of independent transposition events. The translocatability of at least one transposon, Tn9, is due solely to the fact that the DNA segments flanking it are themselves transposable (Starlinger and Saedler, 1976; Calos and Miller, 1980). The precise mechanism of transposition and the role of the transposase protein is still unclear, although several models have been proposed which account for most of the experimental findings (Grindley and Sherratt, 1979; Shapiro, 1979; Harshey and Bukhari, 1981; for review see Bukhari, 1981).

Finally, it is known that the inverted repeat termini of transposons can serve as "portable regions of homology" (Kleckner et al., 1977) - sites where general homologous recombination can take place so as to join together two unrelated DNA segments that lack extensive nucleotide sequence homology. Thus, transposons could mediate the association and dissociation of resistance-determinant portions of R-plasmids as originally observed by Watanabe (1963, 1964) and may have played a major role in plasmid evolution (Cohen, 1976; Kopeko et al., 1976).

B) Techniques in plasmid biology

1) Isolation of plasmid DNA

Intracellularly, plasmid DNA takes the form of a covalently closed circular (CCC) duplex molecule, formed when

a linear, double stranded DNA molecule circularizes and closes upon itself so that the free ends may covalently bond to one another (Clowes, 1972). The torsional stresses inherent in such a structure give rise to a "supercoiled" configuration in which the DNA helix is twisted around itself (Bauer, 1978). This configuration has been demonstrated for the F-factor (Freifelder, 1968) and for a variety of R-plasmids (Nisioki et al., 1970; Kontomichalou et al., 1970; Silver and Falkow, 1970). The supercoiled plasmid molecule can be converted to a "relaxed" form simply by breaking one of the two strands of the DNA duplex. This has the effect of relieving torsional stress by providing a point of free rotation and allowing the supertwisted molecule to unwind into the so-called open circular form (Clowes, 1972).

The covalently closed circular aspect of plasmid structure has been exploited chiefly in the isolation and purification of plasmid DNA from bulk chromosomal DNA. Most such methods involve as the initial step the formation of spheroplasts followed by their lysis with detergents so that both chromosomal and plasmid DNA are gently released (Clewell and Helinski, 1969; Guerry et al., 1973). Slow centrifugation of this lysate results in a supernatant containing a mixture of intact CCC-DNA, plasmid molecules which have been accidentally nicked to the open circular form, and linear fragments of chromosomal DNA. Radloff et al., (1967) had observed that linear and open circular DNA binds more

of the intercalating dye ethidium bromide (EtBr) than does CCC-DNA. Hence, since intercalation of EtBr causes extension of the DNA duplex thereby lowering its density, the density of CCC-DNA will be lowered to a lesser degree than that of non-CCC DNA (Bauer and Vinograd, 1968). Separation of these two forms of DNA can then be achieved by centrifugation through a cesium-chloride density gradient; CCC-DNA, because of its higher density, will migrate to a point further down the gradient than will non-CCC-DNA.

Where pure preparations of plasmid DNA are not required, rapid, less expensive methods have been devised which allow one to detect simply the presence or absence of CCC-DNA in a given strain (Meyers et al., 1976; Sox et al., 1978). Essentially, cellular lysis and slow centrifugation are performed as before but the mixture of the various DNA configurations is electrophoresed through an agarose gel. Separation in this case is on the basis of molecular size - the greater the molecular mass, the slower the rate of migration through the gel (Aaij and Borst, 1972). Therefore plasmids of different sizes can be separated from one another and from any linear and open circular DNA since all would migrate to different points in the gel over a set length of time.

2) Characterization of plasmids

Generally, two approaches have been applied to the study of the structure of R-plasmids and the relationship between them; genetic analysis and physiochemical characterization (Helinski, 1973; Clowes, 1972). Early genetic studies were

aimed primarily at mapping the relative location of genes determining the three general properties of R-factors - replicative autonomy, sexuality, and antibiotic resistance. The use of classical phage transduction techniques led Watanabe and Fukasowa (1961) to propose that R-factors were composed of two linked units: a sex factor unit which controlled replication and conjugal transfer and a unit of drug resistance genes. Transductional analysis of one particular multiple resistance plasmid, R222, showed that three of the drug resistance determinants were closely linked to one another but not to a fourth determinant (see Watanabe, 1963). Such linkage relationships were consistent with a circular genetic map for this R-factor. More recently, complementation analysis and deletion mapping have been used to define and map the genes required for the conjugal transfer of the F-factor (Ohtsubo et al., 1970; Ippen-Ihler et al., 1972).

A second form of genetic analysis involves the study of the interrelationship of two plasmids co-habitating the same cell. Scaife and Gross (1962) and Echole (1963) have observed that two different plasmids may be introduced into the same host cell by selecting for properties encoded by both plasmids. However, when selective pressure is subsequently removed, one of two things may result; either both plasmids may continue to stably co-exist in the cell for prolonged periods, in which case they are said to be compatible with one another, or, one of the two plasmids is

lost from the cell over the next few generations leaving the other to exist alone, in which case the two plasmids are said to be incompatible. Since incompatibility is thought to reflect similarities in the replicative machinery of plasmids (Timmis, 1979), two plasmids which cannot stably co-exist are thought to be closely related to one another in a genetic as well as physical sense. Consequently, by genetic analysis of incompatibility properties, a natural classification scheme has been devised for certain Enterobacteriaceae and Pseudomonadaceae plasmids which are mutually incompatible; that is, plasmids closely related to one another as judged by their mutual incompatability are placed into a common grouping (Datta, 1979).

In general though, the genetic analysis of plasmids has yielded little information on their fine structure and molecular organization. Fortunately, because of their relatively small size and ease with which they can be isolated, plasmids have proven to be amenable to study by some of the recently developed techniques of molecular genetics (see Broda, 1979). The type II restriction endonucleases, for example, comprise a class of over 150 enzymes (Roberts, 1976) isolated from a variety of procaryotic organisms, which have the unique ability to recognize specific DNA base sequences and cleave the DNA duplex at those sequences. Because the recognition sequences may be only from 4 to 6 base pairs in length and are different for most enzymes (Szalay et al., 1979), it is highly likely that any given segment of DNA will contain at least one cleavage site for at least one

such enzyme. Thus, the endonuclease Eco Rl cleaves the F-factor into 10 fragments (Skurray et al., 1976) which can be separated electrophoretically on an agarose gel; the larger fragments migrating at a slower rate. Such fragmentation patterns can be used as a simple test of plasmid relatedness (Thompson et al., 1974; Causey and Brown, 1978). Two plasmids producing a highly similar cleavage pattern (ie. many common bands on an agarose gel) when treated with the same restriction enzyme would, by inference, have the same recognition sequences located the same distance apart on the genomes. Therefore, it may be concluded that two such plasmids are probably highly homologous at the DNA base sequence level.

A more precise means of assessing DNA base sequence relatedness involves the relatively more sophisticated techniques of molecular hybridization and heteroduplex mapping. Nucleic acid hybridization as originally applied to plasmid DNA (Brenner et al., 1969; Guerry and Falkow, 1971), first involves the separation of the two strands of a DNA duplex followed by the mixing of this denatured plasmid DNA with denatured DNA from a different plasmid. Under conditions which allow the DNA strands to reanneal, two types of products will be formed depending on the degree of sequence homology between the two plasmids. If the two denatured plasmids have no nucleotide sequences in common, then complete duplexes can only be reformed from the two DNA strands originally bonded together before denaturation - non-com-

plementary strands cannot reanneal. If, on the other hand, the two denatured plasmids are highly similar at the base sequence level, then some of the renatured duplexes will consist of one strand from one of the plasmids and the complementary strand from the second plasmid - a heteroduplex. The degree of complementariness will determine the extent of heteroduplex formation. This technique can be further refined through the use of electron microscopy to show precisely which parts of the two plasmids are homologous and which are dissimilar. This type of heteroduplex mapping (Davis et al., 1971; Sharp et al., 1972) involves simply the observation of renatured heteroduplex molecules under the electron microscope. Areas of nucleic acid homology will be seen as double stranded regions of DNA whereas those areas where the two DNA strands are dissimilar can readily be detected as single stranded loops. Regions of non-homology as short as 50 base pairs can be detected in this way (Broda, 1979).

C) Distribution of R-plasmids

1) Enterobacteriaceae

R-factors are widely distributed among members of the Enterobacteriaceae and much of the current knowledge of plasmid structure and function has come from a detailed study of these plasmids. As mentioned before, R-factors were first demonstrated in Shigella by various Japanese groups and subsequently by workers in many other countries (Anderson, 1968). Since then, R-factors have been found in

many species of Enterobacteriaceae including Salmonella (Datta, 1962), Escherichia, Klebsiella, Citrobacter (Salzmoar and Klemm, 1966), Proteus (Mitsuhashi et al., 1967), and others; not only from human sources but from animal isolates as well.

These R-factors represent a truly heterogeneous group in terms of drug resistance patterns, size, conjugal properties, and other features (see Novick, 1974). However, from the point of view of the resistance determinants themselves, several patterns may be discerned. The ampicillin resistance transposon TnA, for example, can be found distributed on a diverse group of both large and small plasmids which may otherwise have little else in common (Yamada et al., 1979; Heffron et al., 1975). The ubiguity of this genetic element has been attributed to its transposability properties (Keffron et al., 1975). Another widely dispersed resistance pattern is that of linked streptomycin-sulfonamide resistance, commonly found associated with non self-transferable plasmids in the 5.5-6.3 Mdal size range (Barth and Grinter, 1974; Guerry et al., 1974). Despite the fact that small Sm-Su^r plasmids can be isolated from many different species of Enterobacteriaceae, most of the plasmids are highly homologous and are thought to have a common evolutionary origin (Barth and Grinter, 1974; Grinter and Barth, 1976). Recently however, a different class of Sm-Su^r plasmids have been isolated from E. coli which are smaller in size and show little homology with the previously described prototype Sm-Su^r plasmids (Van Treeck et al., 1981). Other

resistance determinants encoded by transposable elements can also be found represented in various combinations on almost all size classes of Enterobacteriaceae plasmids (see for example, Ike et al., 1981).

At present, most conjugative Enterobacteriaceae plasmids have been placed into some 23 different incompatibility (Inc) groups (Datta, 1979), and generally plasmids within any one Inc group show similarities both in terms of DNA sequence homology (Falkow et al., 1974) and certain other features (see Datta, 1979). The small non-conjugative plasmids may also be placed into incompatibility groups but interestingly, none of them have been found to fall into any of the same Inc groups identified for conjugative plasmids (Datta, 1979). This is not simply a function of the conjugal transfer genes since the few large non-conjugative plasmids which have been found all fall into the same Inc groups as conjugative ones (Datta and Hedges, 1973). Instead, naturally occurring small plasmids have thus far been placed into only two large groups (Datta, 1979). The large number of incompatibility groupings seen for Enterobacteriaceae plasmids illustrates the great diversity in terms of structure and function seen among these plasmids in general; a diversity not often encountered among plasmids of other genera (see below).

2) Haemophilus

The genus Haemophilus contains both members which are pathogenic for humans and others which are important in

veterinary medicine (Young, 1978). H. influenzae type b is a primary agent of childhood infections including meningitis, acute epiglottitis, and otitis media and may represent an important pathogen in adults as well (Weinstein, 1970). Infections due to H. aphrophilus or H. parainfluenzae are rarer but still represent a serious clinical problem (Sutter et al., 1970; Krishnaswami et al., 1972). H. ducreyi is thought to be the causative agent of the sexually transmitted disease chancroid which remains a problem in tropical and sub-tropical areas. Due to the nature of its transmission however, it has a world-wide distribution with sporadic outbreaks occurring in areas where it may not have been previously observed (for example, Hammond et al., 1978, 1980). Most other species of Haemophilus are primarily associated with infections in animals; for example H. haemoglobinophilus (dogs), H. suis and H. pleuropneumoniae (swine), H. gallinarum (fowl) (see Killian, 1976).

Plasmid-mediated antibiotic resistance in Haemophilus first appeared in 1974 when a β-lactamase producing strain of H. influenzae resistant to ampicillin was isolated from a child with meningitis (Kahn et al., 1974). This was quickly followed by reports from other parts of the world documenting similar cases of ampicillin-resistant H. influenzae (Clymos and Harper, 1974; Tomeh et al., 1974; Sykes et al., 1975), illustrating not only the sudden emergence but the seemingly rapid spread of this particular resistance determinant. The β-lactamase enzyme responsible for this resistance was

found to be very similar to the TEM-type β-lactamase known to be widely distributed amongst the Enterobacteriaceae (Farrar and O'Dell, 1974; Medeiros and O'Brien, 1975). Elwell et al. (1975) showed that this β-lactamase gene could be carried on a large 30 Mdal plasmid in H. influenzae and that this large plasmid contained the same ampicillin resistance transposon (TnA) found on various enteric plasmids. Subsequently, Laufs et al. (1979) showed that the β-lactamase gene could also be carried on a small plasmid of approximately 4.4 Mdal in H. influenzae but that this plasmid contained only about 40% of the complete TnA transposon. Earlier, Thorne and Farrar (1975) had demonstrated that ampicillin resistance was transferable from one strain of H. influenzae to another by a mechanism which required cell-to-cell contact and was unaffected by the presence of deoxyribonuclease. This mechanism was presumed to be conjugation and was mediated by the large 30 Mdal plasmid described by Elwell et al. (1975).

Between 1975 and 1979, reports of plasmid-mediated resistance to other antibiotics and also multiple resistance in H. influenzae began to appear with increasing frequency. A kanamycin-resistance plasmid of unknown size but apparently self-transmissible was identified by Dang Van et al. (1975); large, self-transmissible plasmids specifying tetracycline resistance were isolated by Elwell et al. (1977b) and Kaulfers et al. (1975); and, conjugative plasmids were isolated which carried multiple resistance determinants for ampi-

cillin-tetracycline and tetracycline-chloramphenicol (Jahn et al., 1979; Albritton and Slaney, 1980) as well as for ampicillin-tetracycline-chloramphenicol (Bryan, 1978; Roberts et al., 1980). Interestingly, all multiply resistant H. influenzae so far reported have carried tetracycline resistance as one of the determinants (Albritton and Slaney, 1980) and all such plasmids have been of the large (> 30 Mdal) size class.

Studies into the molecular relatedness of the various large H. influenzae plasmids isolated in different parts of the world have thus far been rather limited in scope. Laufs and Kaulfers (1977) for instance, found that a 30 Mdal ampicillin resistance (Ap^r) plasmid isolated in West Germany had nearly 100% of its nucleotide sequence in common with a 30 Mdal Ap^r plasmid from the United States, as judged by nucleic acid hybridization studies, but had only about 70% homology with similarly sized tetracycline (Tc) and tetracycline-chloramphenicol (Tc-Cm) resistance plasmids. Saunders et al. (1978) found that a large Tc^r plasmid from England was about 65-70% homologous to a large Ap^r plasmid from the United States and a $Tc\text{-}Cm^r$ plasmid from the Netherlands. Roberts et al. (1980) compared a number of large conjugative $Tc\text{-}Cm^r$ plasmids from North America and Europe by hybridization techniques and found them to be from 61 to 92% homologous to one another and also about 75% homologous to a large Ap^r plasmid from the U.S.A. Finally, using the restriction endonuclease method of relatedness determination, Harkness and Murray (1978) found three large

Ap^r plasmids to be quite similar while Albritton and Slaney (1980) found a similar degree of relatedness between several large Ap^r, Ap-Tc^r, and Tc-Cm^r plasmids. This suggested to Albritton and Slaney (1980) and others (Laufs and Kaulfers, 1977; Harkness and Murray, 1978) that most Haemophilus R-plasmids of the 30-40 Mdal size range shared a common "core" sequence and that only the addition of various resistance determinants resulted in the apparently slight differences in sequence homology or restriction enzyme cleavage patterns. In addition, Albritton and Slaney (1980) presented evidence to suggest that the limited number of conjugative plasmids which they studied belonged to at least two incompatibility groups, indicating again that perhaps large Haemophilus plasmids are more closely related as a group than are many of the Enterobacteriaceae plasmids which may fall into numerous incompatability groups (Datta, 1979).

Plasmid-determined antibiotic resistance in other species of Haemophilus has not to date approached the diversity seen in H. influenzae. An H. parainfluenzae clinical isolate carrying a 4.1 Mdal Ap^r plasmid (labelled RSF0885) was identified in 1975 (Elwell et al., 1975; Gromkova and Goodgal, 1977) but the plasmid only carried a portion of the complete TnA gene sequence. Shaw et al. (1978) described a strain of H. parainfluenzae which was resistant to Cm and Tc and which could transfer both these resistance traits to E. coli by co-growth on agar surfaces, but wherein no plasmid DNA of any size could be detected. Multiple

antibiotic resistance in H. parainfluenzæ is uncommon (W. Albritton, personal communication) and only the occasional strain has been identified in which Tc^r or $Ap\text{-}Tc^r$ is mediated by a large plasmid (B. Mallory and W. Albritton, unpublished data).

In H. ducreyi, ampicillin resistance has been shown to be mediated only by small plasmids of either 7.0 or 5.7 Mdal (Brunton et al., 1979; and, unpublished data), both of which carry the complete TnA gene sequence. These two plasmids are essentially identical at the base sequence level except that the larger plasmid carries an additional 1.3 Mdal segment of DNA not present on the smaller plasmid (W. Albritton, personal communication). Likewise, sulfonamide resistance in H. ducreyi has recently been shown to be mediated by a small 4.9 Mdal plasmid which is highly related to enteric Su^r plasmids (Albritton et al., manuscript in preparation). Large conjugative plasmids have also been isolated in H. ducreyi but these have been shown to confer only Tc^r or multiple resistance to Tc and Cm (Brunton et al., 1978).

As for other members of the genus Haemophilus, there has as yet been no report of plasmid-mediated drug resistance although a number of Haemophilus species do harbor small plasmids to which no phenotypic trait can be assigned. Such small cryptic plasmids - ranging in size from less than 1 to about 3 Mdal - are quite common in H. parainfluenzae isolates (Mann and Rao, 1979). A large majority of drusensitive H. parainfluenzae strains may carry as many as

three or four cryptic plasmids. H. influenzae isolates however, very infrequently carry small cryptic plasmids (Albritton and Slaney, unpublished observations) and only two reports have documented the presence of large cryptic plasmids in this species - one of 20 Mdal (Harkness and Murray, 1977) and one of 26 Mdal (Saunders et al., 1978). In addition, cryptic plasmids of 0.9 and 1.2 Mdal have been observed in an isolate of H. haemolyticus (Mann and Rao, 1979) and up to three small plasmids have been detected in some H. pleuropneumoniae species which are resistant to Ap, Tc, Sm or Su although no correlation has yet been made between a particular plasmid and any drug-resistance determinants (unpublished data). Cryptic plasmids have not been demonstrated in other Haemophilus species although this may be a reflection on the need for more detailed studies of some of the less well characterized species.

In terms of the actual intracellular state of Haemophilus plasmids, several reports have suggested that some large R-factors may exist as integrated components of the host chromosome, much like the chromosomally-integrated (episomal) state sometimes seen for the F-factor (Hayes, 1970). Bendler (1976) constructed a strain of H. influenzae in which the chromosome contained a physically integrated linear segment of DNA which encoded ampicillin-resistance and had a calculated molecular weight of about 30×10^6. Subsequent work (W. Albritton and L. Slaney, personal communication) demonstrated that this strain could conjugally donate ampicillin-resistance to Haemophilus recipients and that these reci-

pients now contained a non-integrated plasmid of 30 Mdal. This indicated that Bendler's original strain in fact contained a complete conjugative Ap^r plasmid in a chromosomally-integrated state and that this plasmid could excise from the chromosome in response to some unknown signal. Finally, Stuy (1980) studied 23 strains of H. influenzae resistant to Ap or Tc but which showed no visible plasmid when screened by agarose gel electrophoresis and found that at least 18 of them carried a conjugative plasmid integrated into the chromosome. Stuy also suggested that a few cells in every population always excised these plasmids so that conjugative transfer to other cells could conceivably always take place. Most such recipients however, would not maintain these plasmids in a free state but would instead integrate them into the chromosome. There is little evidence in support of this premise other than Stuy's (1979, 1980) observation that many drug-resistant H. influenzae isolated appeared to contain chromosomally-located resistance genes since plasmids could not be detected. Roberts and Smith (1980) pointed out however, that failure to detect a free plasmid by one method of screening does not necessarily mean that such a plasmid does not exist. In any event, the occurrence and distribution of chromosomally-integrated large plasmids in H. influenzae is presently unclear and their existence in other species of Haemophilus has to date not been reported. Also, there is no evidence to suggest that small R-plasmids can undergo a similar transition from a free to a chromosomally-integrated state or vice versa.

3) Neisseria

In N. gonorrhoeae only resistance to ampicillin has been shown to be plasmid mediated. Gonococci bearing this resistance trait, the so-called penicillinase (β-lactamase)-producing N. gonorrhoeae or PPNG, were first isolated in 1976 in the Phillipines and in England (Ashford et al., 1976; Phillips, 1976) and subsequently, on all continents (Siegel et al., 1978). The TEM-type β-lactamase gene is found on one of two small plasmids - a 4.4 Mdal plasmid if the PPNG strain originated in the Far East, or a 3.2 Mdal plasmid if it originated in West Africa (Siegel et al., 1978; Roberts et al., 1978). Both these plasmids carry about 40% of the complete TnA transposon (Elwell et al., 1977) and in fact have about 70% of their base sequences in common (Roberts et al., 1977). In addition, they are very closely related to the 4.1 Mdal Ap^r plasmid RSF0885 from H. parainfluenzae (Roberts et al., 1977) which also lacks the complete TnA sequence, and have about 60% sequence homology with the small 5.7 Mdal H. ducreyi Ap^r plasmid (Brunton et al., 1980). However, neither of the two PPNG Ap^r plasmids appear to be closely related to a small 2.6 Mdal cryptic plasmid which is found in virtually all gonococcal isolates (Roberts et al., 1977, 1979).

A highly significant feature of the PPNG system is that the 4.4 Mdal Ap^r plasmid can be conjugally transferred to other gonococcal recipients but only if another plasmid, a large 24.5 Mdal cryptic plasmid, is co-resident in the cell

(Eisenstein et al., 1977; Roberts and Falkow, 1977). Similar mobilization of the 3.2 Mdal Ap^r plasmid has not been reported although this has not been rigorously tested since both the 24.5 Mdal and the 3.2 Mdal plasmids have never been observed to naturally occur in the same cell (Siegel, 1978). Presumably, this type of transfer system has been an important factor in the dissemination of the 4.4 Mdal Ap^r plasmid among N. gonorrheae species (Roberts et al., 1978; Siegel et al., 1978).

D) Transferability of plasmids

1) Transformation

The adaptability of microorganisms to stressful situations can very often be ascribed to the presence of an efficient means for the exchange of genetic material (Bennett and Richmond, 1978) and three types of gene transfer systems have been described: transformation, transduction, and conjugation (see Hayes, 1970). In no instance however, have all three mechanisms been shown to naturally operate within the same species (Stanier et al., 1970).

Transformation involves the uptake by competent cells of naked, unencapsulated DNA and the incorporation of the DNA into the genome of the host cell by the normal genetic recombination processes (Hayes, 1970). The occurrence of transformation has been demonstrated in a number of bacterial genera and species (Spizizen, 1966), including Pneumococcus, Bacillus, Neisseria, Haemophilus, and most recently,

in E. coli which, although not naturally transformable, can be treated in such a way that the cell membrane allows the entry of DNA (Cohen and Chang, 1972). While most studies of the transformation process have centered on the use of fragmented, linear chromosomal DNA, covalently closed circular plasmid DNA may also be taken up by competent cells, although in this case recombination with the host genome is not seen and the plasmid is maintained intracellularly in the CCC form.

In the genus Haemophilus, as yet only two species - H. influenzae and H. parainfluenzae - have rigorously been shown to be transformable with either chromosomal or plasmid DNA. An early report (Leidy et al., 1959) suggested that H. aegyptius might also be transformable but this has not been expanded upon. Similarly, H. suis was reported to be transformable but only with chromosomal DNA prepared from H. suis (Leidy et al., 1956). Both H. influenzae and H. parainfluenzae however, are able to take up chromosomal DNA purified from other Haemophilus species, including H. parahaemolyticus (Stuy, 1976), H. haemolyticus, and H. aegyptius (Deich et al., 1978). Both high and low molecular weight plasmid DNA may be transformed into H. influenzae or H. parainfluenzae but, in general, transformation with plasmid DNA is considerably less efficient than transformation with chromosomal fragments. Albritton et al. (1981), for example, found that transformation of H. influenzae with chromosomal DNA was between 10^4 and 10^5 times more efficient than transformation with large, 30-34 Mdal plasmids. Gromkova and Goodgal (1979)

were able to optimize conditions for low molecular weight plasmid transformation of H. parainfluenzae but still found at best a 100-fold difference between this and the efficiency of chromosomal DNA transformation. Notani has speculated (N. Notani, J. Setlow, D. McCarthy, N. Clayton, unpublished data), that the inefficient transformation of Haemophilus with plasmid DNA may be due partly to the poor uptake by competent cells of the DNA, and partly because even those plasmid molecules which are taken up are not always established as functional replicons. Albritton et al. (1981) however, found that the efficiency of transformation by large plasmid DNA could be increased as much as 1000-fold if the competent recipient cell already contained a plasmid incompatible (ie. highly homologous) with the incoming plasmid. As this enhancement was not seen in strains unable to mediate generalized recombination and was somewhat reduced if the recipient contained a compatible plasmid, it was suggested that possibly recombination between the incoming plasmid and the plasmid resident in the recipient was essential for efficient transformation.

In absolute terms however, most authors agree that while transformation may have played some role in originally establishing certain drug-resistance determinants in Haemophilus and other genera, it is probably not an efficient enough mechanism to account for the subsequent rapid spread of these determinants both intra- and interspecifically

(Bendler, 1976; Gromkova and Goodgal, 1977; Stuy, 1979). This is especially true for species such as H. ducreyi in which transformation as a means of gene transfer is not known to operate, yet which have still managed to acquire a number of drug-resistance determinants.

2) Transduction

The possible role of transduction in both the establishment and spread of antibiotic-resistance determinants is even less clear. Transduction is a process whereby a segment of bacterial DNA is encapsulated in a bacteriophage particle in place of the phage's own DNA, and then transferred to another cell infected by this phage (Hayes, 1970). A large number of R-factors of enteric bacteria retain this form of transfer as an option (see Jones and Sneath, 1970) even though one of the other two mechanisms of genetic exchange may be available to them as well. However, only one situation exists where transduction is the only known mechanism of plasmid transfer between strains. Multi-resistant strains of Staphylococcus aureus frequently carry small plasmids of 2.7 and 3.0 Mdal specifying Tc^r and Cm^r respectively (Novick and Bouanchaud, 1971). These plasmids can only be transferred from cell to cell by a transducing phage native to this species of Staphylococcus (Novick and Richmond, 1965; Novick et al., 1974).

Among the Haemophilus species, only three different bacteriophage have been isolated from H. influenzae; HP1 and S2 which are very homologous with one another (Boling et al.,

1973) and N3 (Samuels and Clarke, 1969) which is different in a number of properties (Jablonska and Piekarowicz, 1980). Bacteriophage N3 can only infect one particular strain of H. influenzae, called strain JC9, while HP1 and S2 can infect all strains of H. influenzae except strain JC9 (Piekarowicz and Glover, 1972). In addition, JC9 harboring phage N3 in a lysogenic state is known to be very poorly transformable with both chromosomal (Piekarowicz and Swinska, 1977) and plasmid (Albritton et al., 1981) DNA. Although the HP1, S2 and N3 phage are infective, there have been no reports that they are capable of acting as transducing particles for either chromosomal or plasmid-borne genes.

Only one report has appeared concerning the presence of bacteriophage in other Haemophilus species. Gromkova and Goodgal (1977) detected the presence of a lysogenic bacteriophage in a mitomycin C-treated strain of H. parainfluenzae. They showed that this phage was able to incorporate chromosomal and possibly even plasmid-borne drug-resistance genes but were unable to prove that the phage could actually transduce these genes to other strains of H. parainfluenzae.

In general, because of the lack of detectable bacteriophage in most Haemophilus species, the extremely limited host range of known phage, and the lack of evidence that these phage have transducing capabilities, it must tentatively be concluded that phage-mediated transduction is not a major form of gene transfer within this genus. This leaves only one other means of plasmid transfer to be considered.

3) Conjugation

i) Transfer systems

Conjugation is a specialized form of gene transfer which requires some type of direct cell-to-cell contact and is always plasmid-dependent. Thus, conjugative ability is a property attributable to an individual plasmid rather than to the bacterial cell in which the plasmid resides. While the different stages of conjugative transfer are probably similar among the various Enterobacteriaceae plasmids, there can be important underlying genetic differences which serve to distinguish one plasmid transfer system from another (Willetts, 1977). It has been noted for example, that plasmids in any given incompatibility group usually have similar conjugation systems (Gorai et al., 1979). Plasmids of one group can be distinguished from those of another by the production of different, morphologically-distinct sex pili (Bradley, 1980) - long appendages probably necessary for the formation of stable cell-to-cell contacts - and this probably reflects certain key differences in the transfer genes of each particular plasmid. To date, plasmids of only four incompatibility groupings - Inc F, I, N, and P - have been studied in detail (Willetts, 1977) and their conjugation systems have been found to be phenotypically and genetically distinct.

The F-factor, the best characterized conjugative plasmid, remains the prototype of the Inc F conjugation system which includes such naturally occurring R-plasmids as R1 and R100

as well as some large colicin-producing plasmids (Willetts, 1977). The 19 known genes required to effect conjugative transfer of the F-factor are clustered within a single stretch of DNA - the transfer operon - which spans some one-third of the entire F genome (Willetts and Skurray, 1980). At least 13 of these genes are needed for the synthesis of the sex pilus itself; the remainder serve in a regulatory capacity or function during the actual transfer of DNA (Clark and Warren, 1979). Transcription of the transfer operon, and hence plasmid transfer itself, requires the products of one of these "tra" genes which in turn is repressed by the co-operative action of the products of two other plasmid-borne genes (Willetts, 1977b). Hence, the presence of these latter two gene products results in a repression of plasmid transfer as is seen with most of the Inc F plasmids with the exception of F itself. The F-factor lacks one of these genes and so is in a constantly "de-repressed" state; in other words, transfer of F can proceed whenever a suitable recipient is present (Rowbury, 1977).

The actual mechanics of conjugative transfer in the F-factor system have been reviewed in detail by many authors (Curtiss, 1969, 1977; Hayes, 1970; Rowbury, 1977; Clark and Warren, 1979; Willetts and Skurray, 1980) and essentially involves three stages (Broda, 1979b): collision of donor and recipient cells leading to a stable pairing mediated by the sex pilus, the preparation and actual transfer of plasmid DNA, and finally, the establishment of the transferred

plasmid in a covalently closed circular form within the recipient.

Other groups of plasmids have not yet been well characterized in terms of the genetics or mechanics of their transfer functions. The conjugative Haemophilus R-plasmids for example, exhibit several significant differences when compared to the F-factor system. Transfer of the F-factor can occur equally well if donors and recipients are grown in liquid media or if they are grown together on a solid agar surface (Curtiss et al., 1979). Transfer of plasmids between Haemophilus cells however, will not occur in liquid media (Stuy, 1979; Albritton and Slaney, 1980) but will only take place if the donor and recipient cells are forced into close contact either by filtration onto a membrane filter (Thorne and Farrar, 1975), co-growth on a solid agar surface (L. Slaney, personal communication), or similar methods (Stuy, 1979). Furthermore, preliminary electron microscopic examinations of Haemophilus donor cells have not revealed any cellular appendages analogous to the sex pili seen in other systems (P. Bertram, M.Sc. Thesis, University of Manitoba, 1980; and, unpublished data), although detailed studies are necessary in this regard. Thus, it appears that the transfer genes of large Haemophilus plasmids may be organized quite differently from those in plasmids of enteric origin.

ii) Plasmid host range

The host range of a particular plasmid denotes the extent to which that plasmid can transfer to, and be maintained

in, other species which may or may not be closely related to the original donor species. Among the Pseudomonas and enteric plasmids at least, the host range has been observed to vary greatly and no pattern is recognized. The F-factor for example, can be transferred quite efficiently from E. coli to E. coli, somewhat less efficiently from E. coli to Salmonella (Zinder, 1960), Shigella (Schneider and Falkow, 1964), Pasteurella and certain other species (see Jones and Sneath, 1970), and not at all to most other species or genera. Some R-plasmids can show both more restricted and very much broader host ranges than the F-factor. The self-transmissible plasmids from Pseudomonas aeruginosa provide a good example of this; some such as R91 (Jacoby et al., 1978) are apparently restricted to transfer between strains of P. aeruginosa whereas others such as RP4 (Ingram et al., 1973) can be transferred widely among Gram negative bacteria including Pseudomonas, Acinetobacter, Erwinia, Klebsiella, Escherichia, Rhizobium, Aeromonas, Salmonella, and others (Sykes and Richmond, 1970; Jacoby and Shapiro, 1977; Harwood, 1980). The apparent promiscuity of the Inc P-group plasmids, particularly RP4, has led to speculations that they could have been responsible in the past for genetic exchanges between otherwise unrelated bacteria and thus the establishment of drug-resistance genes in certain species (Thomas, 1981).

The conjugative Haemophilus plasmids have not been particularly well studied in terms of their host range, however. Transfer of various 30-40 Mdal plasmids between

strains of H. influenzae has been well documented although the frequency of such transfer has often differed greatly; Van Klingeren et al. (1977) observed transfer frequencies of from 10^{-3} to 10^{-6} transconjugants per donor to less than 10^{-8} depending on which of three H. influenzae strains were used as recipients; Stuy (1979) observed frequencies of from 10^{-2} for isogenic matings to 10^{-4}-10^{-8} for matings between H. influenzae strains of different serotypes. As for matings conducted between other species of Haemophilus, Stuy (1979) could not detect transfer of a large conjugative Ap^r plasmid from H. influenzae to a strain of H. parainfluenzae or to strains of H. parahaemolyticus or H. aegyptius. Sykes et al. (1975) was able to transfer an Ap^r plasmid of unknown size from H. influenzae to an H. parainfluenzae recipient. Similarly, Stuy (1980) found that a Tc-Cm^r plasmid resident in a strain of H. parainfluenzae was transferable to an H. parainfluenzae recipient at frequencies of 10^{-7}-10^{-8} but could not be transferred to an H. influenzae Rd strain. Albritton and Mallory (personal communication) however, were able to detect several large R-plasmids in H. parainfluenzae which were transferable to H. influenzae recipients. Finally, Brunton et al. (1979) was able to transfer a large Ap^r plasmid from H. influenzae to H. ducreyi and vice versa but the actual frequency of the transfer was not reported.

Many attempts have also been made to conjugatively transfer large Haemophilus plasmids to E. coli since it was known that small Haemophilus R-plasmids could be introduced by

transformation into E. coli and maintained in a covalently closed circular state (Brunton et al., 1979, 1980). Dang Van et al. (1975) first observed the transfer of a kanamycin-resistance plasmid of unreported size from H. influenzae to E. coli where it apparently existed in a CCC form but this has remained unconfirmed. Van Klingeren et al. (1977) was able to transfer the Tc- and Cm-resistance of an H. influenzae strain carrying a 38 Mdal plasmid to E. coli recipients at frequencies of 10^{-6}-10^{-7} but CCC DNA was never observed in the transconjugants, nor were the transconjugants able to re-transfer these resistance markers. Laufs and Kaulfers (1977) were able to transfer ampicillin resistance from an H. influenzae strain with a 30 Mdal plasmid to E. coli with the unusually high frequency of $3x10^{-1}$ but did not report if the plasmid could be detected in the transconjugants. Similar results were seen by Shaw et al. (1978) with a Tc-Cm^r H. parainfluenzae donor and an E. coli recipient and again CCC DNA could not be detected in the transconjugants nor could resistance be re-transferred. Most authors have concluded that large Haemophilus plasmids are not stably maintained in E. coli recipients but the resistance determinants themselves may be rescued by transposition onto the E. coli chromosome.

The nature of the barriers preventing successful plasmid transfer between bacterial species or genera has remained unclear. Presumably, this could entail a combination of factors including surface exclusion (the system which pre-

vents donors from mating with donors) (Sheehy et al., 1972), absence of proper cell surface receptors (Jones and Sneath, 1970), environmental conditions (Burman, 1977), host cell restriction-modification systems (Arber, 1974), inability to replicate within the new host (Broda, 1979b), or, an inability of the host cell to transcribe or translate the foreign plasmid DNA, ie. lack of gene expression (Ehrlich and Sgaramella, 1978).

iii) Mobilization

Small plasmids of less than 10 Mdal as stated before, lack the genes - the transfer operon - necessary for conjugal self-transmissibility. Indeed, the smallest known conjugative plasmid, with a mass of 17 Mdal (Crosa et al., 1975), must be composed almost entirely of transfer-related genes. However, some non-conjugative plasmids may nevertheless be conjugally transferred within a cell population by a process known as mobilization (Rowbury, 1977; Clark and Warren, 1979) which requires that a large, conjugative plasmid be co-resident in the cell with the small plasmid. An early example of such a transfer system was the "delta" system of Anderson (Anderson and Lewis, 1965). A strain of Salmonella typhimurium was found to be able to transfer resistance to Sm, Ap, and Tc but, unlike other R-factor systems, these resistance determinants were not linked to one another - some transconjugants were resistant to only one antibiotic while some were resistant to all and some could re-transfer their resistance while others could not. It was later found that the Ap- and Sm-resistance markers

were on small, separate non self-transferable plasmids of about 6 Mdal each (Clowes, 1972) and that isolates which could transfer either of these resistance determinants also carried a 60 Mdal conjugative plasmid termed a transfer factor and labelled "delta". Conversely, Ap- or Sm-resistant strains lacking the transfer factor were unable to transfer these resistance determinants.

A similar situation is seen with the non-conjugative ColE1 plasmid, a 4.2 Mdal enteric plasmid encoding colicin production, which can be very efficiently mobilized for conjugative transfer by the co-resident F-factor (Veltkamp and Stuitje, 1981) as well as by R-plasmids belonging to the Inc I incompatibility group (Hardy, 1975). Currently, a large number of small, naturally-occurring plasmids carrying a variety of drug-resistance determinants are known to be mobilizable by one or more conjugative plasmids of enteric origin (see for example, Smith, 1977).

However, this means of transfer for small, normally non self-transmissible plasmids is clinically most significant in the transfer of ampicillin resistance between strains of Neisseria gonorrhoeae. As mentioned before, the penicillinase-producing N. gonorrhoeae (PPNG) frequently harbors a 24.5 Mdal cryptic plasmid in addition to the 4.4 Mdal Ap^r plasmid (Siegel et al., 1978; Roberts et al., 1978). Strains containing these two plasmids are able to conjugally transfer the small Ap^r plasmid to other N. gonorrhoeae recipients; this transfer being promoted by the large 24.5 Mdal

conjugative plasmid. The transconjugants in turn, may harbor just the small Ap^r plasmid, or both the large and small plasmids (Baron et al., 1977) in which case they are able to re-transfer the small plasmid. Suitable recipients in such matings include E. coli and other species of Neisseria in addition to N. gonorrhoeae (Eisenstein et al., 1977; Roberts and Falkow, 1977; Sox et al., 1978). E. coli recipients however have never been reported to receive or maintain the 24.5 Mdal plasmid although the small Ap^r plasmid can stably exist in a covalently closed circular form and can even be re-transferred by mobilization if a suitable conjugative plasmid such as the F-factor is now introduced (Sparling et al., 1978).

The actual mechanism whereby small plasmids may be mobilized has been most extensively studied in the ColE1-F-factor system and several models have been proposed (Warren et al., 1978; Broome-Smith, 1980; Nordheim et al., 1980; Veltkamp and Stuitje, 1981), including a mathematical analysis of the mobilization process (Levin and Rice, 1980). It has been found that the entire transfer operon of the F-factor with the exception of three genes is required for efficient mobilization of ColE1 (Willetts, 1980). However, concurrent transfer of the F-factor to the recipient cell during the mobilization event i s not required, indicating that there is no physical association ("cointegrate" formation) between the F mobilizing plasmid and the ColE1 plasmid (Veltkamp and Stuitje, 1981). In other words, mobilization

requires that the F-factor supply a number of diffusible gene products, the structural proteins for the sex pilus for instance, which are not encoded by ColE1 itself. Yet the role of ColE1 in mobilization is not a passive one; mutants of ColE1 have been isolated which are not mobilizable by F or by other conjugative plasmids (Warren and Sherratt, 1977; Inselburg and Ware, 1979). A region of DNA encompassing about 30% of the ColE1 genome has ben mapped (Dougan et al., 1978) and found to be essential for the mobilization of this plasmid. Presumably, the genes within this region substitute for the three genes of the F transfer operon originally found not to be required for ColE1 mobilization (Willetts, 1980). As well, a second smaller region of the ColE1 genome is also necessary for efficient mobilization (Warren et al., 1978). This region includes a special site with which the ColE1-encoded mobility proteins must interact and also a site at which the plasmid is enzymatically nicked to the open circular form - this being a prerequisite to conjugal transfer (Clark and Warren, 1979).

The process by which ColE1 is mobilized however, is not applicable to all plasmid mobilization systems. Kilbane and Malamy (1980) have delineated four general mechanisms whereby small plasmids may be mobilized by larger conjugative ones and suggest that all mobilization systems should fall into one of these classes. However, the authors emphasize that which pathway of mobilization is followed is largely dependent on which conjugal mobilizing plasmid is used.

Some small plasmids for example, may be mobilized by one mechanism if the F-factor is used but by an entirely different mechanism and at possibly different frequencies if a different R-plasmid acts as the mobilizing plasmid.

Class I mobilizing systems are typified by the ColE1-F-factor system wherein mobilization does not require a physical association of the two plasmids but instead requires that various transfer proteins be supplied via the cytoplasm. The "delta" system of Anderson is another example of this mechanism as is probably the mobilization of Ap^r plasmids by the 24.5 Mdal Neisseria gonorrhoeae plasmid. Mobilization in Class II systems however, is thought to proceed via a homology-dependent, recA-mediated fusion of the large mobilizing plasmid with the smaller plasmid. This cointegrate structure then simply transfers as a single large plasmid and once in the recipient, it may either dissociate into the large and small plasmid components or may remain and replicate as the larger cointegrate molecule. The mobilization of tumor-inducing plasmids of Agrobacterium tumefaciens by R-plasmids such as RP4 is thought to proceed by such a mechanism (Hooykaas et al., 1980). Class III mobilization also requires a fusion of plasmids but here the cointegrate structure can be formed by a recA-independent mechanism and is very unstable, undergoing dissociation as soon as transfer is completed. Finally, in Class IV systems, conintegrate formation is again involved and again is recA-independent but here is mediated by the presence of a transposon on

either the large conjugative plasmid or on the smaller one. It is possible that the transposon functions as a portable region of homology (see Section A, part 4), allowing recombination to take place between the two plasmid units and thus form the cointegrate structure. These cointegrates are, in general, unstable and readily dissociate into their component plasmids after transfer.

E) Origin and Evolution of Plasmid-mediated Drug Resistance

It has been suggested that drug-resistance determinants may have existed in the pre-antibiotic era (Broda, 1979). Evans et al. (1968) for example, found that some E. coli strains isolated before the use of ampicillin nevertheless carried R-factors specifying ampicillin-resistance while Smith (1967) reported that a strain of E. coli isolated before 1937 contained an R-factor encoding streptomycin and tetracycline resistance. These reports indicate that chemotherapy with antibiotics was not necessarily directly related to the development of antibiotic resistance determinants. There seems to be little doubt however, that the subsequent rapid spread of R-plasmids in bacteria has been a direct consequence of the powerful selective pressures exerted by the widespread and liberal use of antibiotics in the last few decades, both in human and veterinary medicine (Anderson, 1965, 1966, 1968; Richmond, 1969; Broda, 1979; O'Brien et al., 1980).

Heffron et al (1975) has speculated that while the replication and transfer regions of R-plasmids may comprise a

heterogeneous class with diverse origins, the actual drug-resistance determinants may simply represent a limited pool of transposable elements, some with a common origin. The ampicillin-resistance transposon TnA, for instance, has been detected in R-plasmids belonging to at least 14 different incompatibility groups (Hedges et al., 1974), on four continents, and on plasmids in representatives of at least 17 different taxospecies, including H. influenzae and N. gonorrhoeae (Datta, 1977). In all these cases, the β-lactamase product of the TnA genes has been shown by analytical isoelectric focusing to be virtually identical (Mathews and Hedges, 1976). Thus, the genes specifying many phenotypic characteristics of plasmids may have arisen and evolved independently from the plasmid "core" regions.

The precise origins of the antibiotic resistance determinants themselves however, has remained unclear. The possibility has been considered that resistance genes may have arisen in the microorganisms that actually produce the antibiotics, where they would perform a protective role (Davis et al., 1977). Benveniste and Davis (1973) have shown that antibiotic-modifying enzymes of certain Actimomycetes species are similar to those responsible for plasmid-mediated drug resistance in some enteric bacteria. Various soil microrganisms may have been forced to acquire such genes simply in order to co-exist with the antibiotic-producing species. While no known plasmid can transfer directly between enteric bacteria and Actinomycetes, such gene ex-

change may not be impossible if taxonomically intervening species are used as stepping stones (Jones and Sneath, 1970). Thus, extrachromosomal elements may have existed in bacteria well before true R-plasmids first appeared and the development of plasmid-mediated drug resistance was simply a reflection of the unique ability of transposable genetic elements to excise from one genome and integrate into another. Presumably, the evolution of R-plasmids is an ongoing event and even today new combinations of resistance genes and transfer factors may be arising due to the transposable nature of certain phenotypic traits (Watanabe, 1971; Cohen and Kopeko, 1976; Cohen et al., 1978).

MATERIALS AND METHODS

A) Bacterial Strains and Plasmids

The organisms used throughout this study and the plasmids they contain are listed in Table 2. These organisms are all either clinical isolates, reference strains, or strains made resistant to certain antibiotics in the laboratory. Not included in this table are the various transconjugants arising through the conjugative mating of plasmid-carrying donor strains and plasmid-free recipient strains.

The plasmids used as molecular weight standards are included. These plasmids were all purified from *E. coli* C600 host strains.

B) Growth Media and Conditions

1) Solid media

All *Haemophilus* species were routinely grown on chocolate agar (CA, Appendix I), and incubated in a humid incubator with 5-10% CO_2 at 35°C. Strains known to be drug-resistant were grown on chocolate agar supplemented with the appropriate antibiotics (10 µg/ml ampicillin, 4 µg/ml tetracycline, 4 µg/ml chloramphenicol, 5 µg/ml nalidixic acid, 5 µg/ml novobiocin, 50 µg/ml rifampin, 100 µg/ml sulfonamide, 1000 µg/ml streptomycin).

E. coli strains were grown either on CA or on MacConkey agar without crystal violet (Gibco). Antibiotics were included where necessary at the above concentrations except

Table 2. Bacterial Strains and Plasmids

Strain	Phenotype[a]	Plasmid Complement (Mdal)	Source
H. ducreyi			
HD35000[b]	Sm^r	-	clinical isolate (CI)-Winnipeg
HD35000[b]	Nal^r	-	CI - Winnipeg
HD9468	Tc^r, Ap^r, Su^r	7.0, 4.9	CI - Kenya
HD147	Tc^r, Ap^r, Su^r	23.5, 7.0, 4.9	CI - Kenya
HD9265	Tc^r, Ap^r	32, 7.0	CI - Kenya
V1157	Tc^r, Cm^r, Ap^r	38, 7.0	CI - Seattle
H. influenzae			
HI2265	Tc^r, Cm^r	35	CI(sputum), Wpg.
HI1008[b]	Sm^r	-	CI(blood) - Wpg.
HI1008[b]	Rif^r	-	CI(blood) - Wpg.
HI5327[b]	Sm^r	2.6, 1.8(cryptic)	CI(sputum)- Wpg.
9(N3)[b]	Sm^r	-	J. Bendler
Rd[b]	Sm^r	-	J. Setlow
Rd[b]	Nov^r	-	J. Setlow
rec-1 Rd[b]	Sm^r	-	J. Setlow
rec-2 Rd[b]	Sm^r	-	J. Setlow
H. parainfluenzae			
HP1007	Tc^r	33, 2.0	CI(urethral)-Wpg.
HP R[b]	Sm^r	2.2, 1.7, 1.2 (cryptic)	J. Setlow
HP R[b]	Rif^r	2.2, 1.7, 1.2 (cryptic)	J. Setlow
HP T[b]	Sm^r	2.2 (cryptic)	ATCC 7901
HP T[b]	Rif^r	2.2 (cryptic)	ATCC 7901

Table 2. (cont'd)

H. pleuropneumoniae			
HN001[b]	Sm^r	-	ATCC 27088
HN002[b]	Sm^r	-	ATCC 27089
HN015[b]	Sm^r	2.9, 2.2 (cryptic)	CI (swine)
H. aphrophilus			
HA002[b]	Sm^r	-	ATCC 19415
HA005[b]	Sm^r	-	NCTC 11098
NA003[b]	Sm^r	-	NCTC 5906
H. parahaemophilus			
HPA002[b]	Sm^r	-	ATCC 29241
H. gallinarum			
HC006[b]	Sm^r	-	ATCC 14385
H. haemoglobinophilus			
HH002[b]	Sm^r	-	ATCC 19416
HH72-164[b]	Sm^r	-	CI-S. Biberstein
HH77-40[b]	Sm^r	-	CI-S. Biberstein
H. suis			
HS001[b]	Sm^r	-	NCTC 4557
H. haemolyticus			
HM001[b]	Sm^r	-	NCTC 10659
H. paraphrohaemolyticus			
HPH001[b]	Sm^r	-	ATCC 29237

Table 2. (cont'd)

E. coli			
C600	r^-m^+, thi^-, Sm^r	-	R. Gill
EC007	Ap^r, Tc^r, Km^r	34 (RP4)	J. Brunton
EC003	Ap^r, Km^r, Cm^r, Su^r, Sm^r	62 (Rldrd19)	J. Brunton
N. gonorrhoeae			
22209	Ap^r	24.5, 4.4, 2.6	CI - Winnipeg
88557	Ap^r	3.2, 2.6	CI - Winnipeg

Plasmids used as molecular weight standards:

Plasmid	Size (Mdal)
Rldrd19	62
RP4	34
Sa	23
RSF1010	5.5
pMB8	1.8

[a] Abbreviations used: Ap, ampicillin, Tc, tetracycline; Cm, chloramphenicol; Sm, streptomycin; Km, kanamycin; Su, sulfonamide; Nal, nalidixic acid; Rif, rifampin; Nov, novobiocin; rm, restriction-modification; thi, thiamine.

[b] These strains were made resistant by continual passage on chocolate agar containing increasing concentration of the antibiotic.

for ampicillin, 20 µg/ml; tetracycline, 8 µg/ml; and chloramphenicol, 8 µg/ml. Wellcotest agar (Burroughs-Wellcome) supplemented with 100 µg/ml sulfonamide was used in sulfonamide-resistance transfer studies with E. coli recipients.

2) Liquid media

For genetic mating studies or plasmid DNA purification, all Haemophilus species, with the exception of H. ducreyi, were grown in liquid media consisting of brain heart infusion broth (Gibco) supplemented with hemin and nicotinamide adenine dinucleotide (Appendix I). Where appropriate, this sBHI broth had antibiotics added at the concentrations listed above. Incubation was at 35°C in a 5-10% CO_2 atmosphere. H. ducreyi strains could only be grown on the solid media described previously.

E. coli strains were grown in brain heart infusion broth (BHI) without supplements, and incubated at 37°C.

C) Biochemical tests

It was sometimes necessary to verify that Haemophilus reference strains or clinical isolates received from outside our institution were actually the species indicated, or that transconjugants isolated in matings between two different biotypes of one Haemophilus species were actually the biotype expected. Haemophilus species were differentiated based on a limited number of biochemical characteristics as delineated by Killian (1976). Hemin (X-factor) or nicotinamide adenine dinucleotide (V-factor) requirements were

tested by observing satellite growth around standard X- and V-factor impregnated filter strips (BBL Taxo Strip) on GC agar base (Appendix I). The porphyrin test (Killian, 1974) was used to confirm X-factor requirement. Other biochemical properties were tested by inoculating API - 20E strips (Analytab Products, Plainview, N.Y.) with a dense suspension (in physiological saline) of organisms from an 18 h chocolate agar plate. Incubation and interpretation of results were as outlined by the manufacturer.

Biotyping of H. influenzae followed the protocol outlined by Albritton, et al.(1978) except that only three properties - indole production, urease activity, and ornithine decarboxylase activity - were used to assign the H. influenzae species to one of the five biotypes.

D) Antimicrobial susceptibility

1) Disk diffusion

Resistance or sensitivity to the antibiotics ampicillin, tetracycline, chloramphenicol, and sulfonamide was determined by the disk diffusion method. Most strains were grown to log phase in sBHI broth and diluted to 10^8 colony-forming units per ml (CFU/ml) (#64 red filter, 640-700 mμ, Klett-Summerson photometer). H. ducreyi strains were grown 18 h on CA, scraped into sBHI broth, and diluted to 10^8 CFU/ml. This inoculum was swabbed onto the surface of a plain CA plate and standard filter paper disks (Pfizer) impregnated with antibiotics (Ap 10 μg, Tc 30 μg, Cm 30 μg, Su 300 μg) were dropped onto the plate. After overnight

incubation, the zone of growth inhibition surrounding each disk was measured. Susceptible strains were taken as having zone diameters of greater than: Ap 20 mm, Tc 25 mm, Cm 25 mm, Su 12 mm. Strains having zone diameters less than these values were considered to be resistant.

2) Presence of the β-lactamase enzyme

Strains showing resistance to ampicillin either by disk diffusion susceptibility testing or by the ability to grow on CA containing ampicillin, were further tested to verify the presence of a β-lactamase enzyme. This was done by placing 2 or 3 drops of a chromogenic cephalosporin solution (Appendix II) onto filter paper and emulsifying into this with a platinum loop, a small scraping of cells taken from an overnight growth on chocolate agar. Immediate formation of a red-purple color in the area where the cells had been spread indicated the presence of a β-lactamase (O'Callaghan et al., 1972). This test had the advantage of simplicity and immediate verification that ampicillin resistance was mediated by the activity of a β-lactamase.

E) Plasmid screening

The method used to rapidly screen bacterial strains for the presence or absence of plasmids was that described by Meyers et al. (1976). Various other methods were tried in an attempt to reduce the time required for screening (Sox et al., 1978; Williams et al., 1979) but none achieved the reproductibility nor the quality of the Meyers method.

Fresh (18 h) cultures of Haemophilus species were suspended in 1.0 ml broth and swabbed onto the surface of five CA plates. After incubation, cells were scraped off the plates into TES buffer (50 mM NaCl, 5 mM EDTA, 30 mM TRIS-HCl, pH 8.0), pelleted, then frozen at -20°C until used. Cultures of E. coli were grown with shaking in 30 ml BHI broth for 18 h at 37°C, pelleted, and frozen at -20°C.

For the preparation of cleared lysates, cells were first thawed and resuspended in TRIS-sucrose buffer (25% sucrose in 10 mM TRIS-HCl, 1 mM EDTA, pH 8.0), then lysed with 10% sodium dodecyl sulfate (SDS) in the presence of 0.25 M TRIS, pH 8.0 and 0.25 M EDTA, pH 8.0. E. coli strains were pre-treated with a 5 mg/ml solution of lysozyme (Guerry et al., 1973) in order to enhance subsequent SDS lysis. After lysis, NaCl was added to a final concentration of 1 M in order to precipitate the higher molecular weight chromosomal DNA. Most of the chromosomal DNA was pelleted by centrifugation at 35,000 x g., while the covalently closed circular plasmid DNA remained in the supernatant. Ribonucleic acid was degraded by addition of RNase and protein extracted with buffered phenol. Finally, plasmid DNA was precipitated by addition of 95% cold ethanol and incubation at -20°C. Centrifugation at 20,000 x g. gave a pellet of DNA which was air-dried and re-dissolved in 0.2 ml of TES buffer and 0.05 ml of tracking dye (0.07% bromophenol blue, 7% SDS, 33% glycerol in water). Samples of this plasmid DNA were then analyzed by agarose gel electrophoresis.

F) Agarose gel electrophoresis

Agarose (Seakem, Marine Colloid, Inc.) at a concentration of 0.7% was dissolved by heating in TRIS-borate buffer (89 mM TRIS-HCl, 2.5 mM disodium EDTA, 8.9 mM boric acid) and poured onto the bed of a horizontal gel electrophoresis apparatus (Aquebogue Machine and Repair Shop, Aquebogue, N.Y.). Wells were formed by inserting a 12-tooth lucite comb and allowing the agarose to harden. Samples (20-30 µl) of ethanol-precipitated DNA with tracking dye were placed into the wells and electrophoresed at 50 mA and 85-90 V for 3-4 h or until the tracking dye had migrated to the bottom of the gel. The gel was then placed in a 0.4 µg/ml solution of ethidium bromide (EtBr) in water and stained for 30-45 minutes. The agarose gel was then placed on a longwave UV transilluminator so that the plasmid bands could be visualized due to the fluorescence of the DNA-bound EtBr. Gels were photographed with a Polaroid MP4 camera equipped with an orange filter and using Kodak type 55 pos/neg film.

G) Calculation of plasmid molecular weight

The molecular weight of an unknown plasmid can be determined by comparing the relative distance of its migration through an agarose gel to the distance migrated by a series of plasmids of known molecular weight. Since the rate of migration of covalently closed circular DNA is inversely related to the logarithm of its mass, a linear

curve results when the relative migration of standard plasmid DNA is plotted versus the logarithm of the plasmid molecular weight (Meyers et al., 1976). Plotting the distance migrated by the unknown plasmid on this curve gives a reasonably accurate estimation of its molecular weight. If care is taken not to overload the gel with plasmid DNA, molecular weight estimates by this method compare to within 5% of the values determined by more accurate methods such as electron microscopic contour-length measurements (Meyers et al., 1976).

H) Purification of plasmid DNA

Large plasmids were first conjugatively transferred (see below) to a plasmid-free recipient strain (usually HD35000); thus they could all be purified from the same background strain. The procedure used here was developed by Clewell and Helinski (1969) and modified by Elwell et al. (1975) and employs cesium chloride-dye buoyant density gradient ultracentrifugation of cleared bacterial cell lysates to achieve separation of covalently closed circular DNA from bulk chromosomal DNA. Cesium cloride (CsCl) at 1 gm/ml was dissolved into cleared lysates of Haemophilus or E. coli cells in Beckman polyallomar tubes. To this was added 0.2 ml of a 10 ug/ml solution of EtBr. The refractive index was adjusted with TES buffer to 1.3990 for Haemophilus and 1.3945 for E. coli preparations. Centrifugation was at 100,000 x g. for 40 h in a Beckman L-2 ultracentrifuge. The polyallomar tubes were then placed into a Beckman drip fractionator, illuminated with a hand-held UV light source to visualize the plasmid

band, and the plasmid DNA drip-collected from the bottom of the tube. The EtBr was extracted with CsCl-saturated isopropyl alcohol and the CsCl removed by exhaustively dialyzing the plasmid DNA solution against 50 mM TRIS, pH 8.0. This purified plasmid DNA was stored at -20^{o}C and not left for any great length of time at room temperature so as to minimize conversion of the covalently closed circular DNA to open circular forms. This DNA was sufficiently pure for subsequent restriction endonuclease digestion or for transformation purposes.

I) Restriction endonuclease digestion

The restriction endonuclease Hinc II was obtained from New England Biolabs while Pst I, Eco Rl, Hind III, Bam Hl, Sma I, and Hae II were purchased from Boehringer-Mannheim, Canada. The specific nucleotide sequence recognized by each of these enzymes as well as the point of cleavage of the DNA strand is given in Table 3. The reaction buffers for each enzyme are listed in Appendix III and are essentially as recommended by the supplier. Reaction mixtures usually consisted of 50 µl of purified plasmid DNA, 6 µl of a 10X buffer solution, 4 µl of distilled water, and 2 µl of the enzyme, all contained in 1.5 ml Eppendorf tubes. Incubation was for 90 minutes, usually at 37^{o}C except for Sma I digestions which were incubated at 25^{o}C. Reactions were stopped by adding 15 µl of a urea-bromophenol blue tracking dye (4.0 mol/l area, 50% (w/v) sucrose, 5.0 mMol/l

Table 3. Restriction Endonuclease Recognition Sequences[a]

Designation	Source	Sequence
Eco Rl	*E. coli* RY 13	5'___G↓pAATTC___3'
Hind III	*H. influenzae* Rd	5'___A↓pAGCTT___3'
Hae II	*H. aegyptius*	5'___PuGCGC↓pPy___3'
Hinc II	*H. influenzae*	5'___GTPy↓pPuAC___3'
Bam Hl	*Bacillus amyloliquefaciens*	5'___G↓pGATCC___3'
Pst I	*Providencia stuartii*	5'___CTCGA↓pG___3'
Sma I	*Serratia marcescens*	5'___CCC↓pGGG___3'

Nucleotide bases: C,cytosine; G,guanine; A,adenine; T,thymine; Pu,G or A; Py,C or T.

[a] Taken from Roberts, 1976.

EDTA, 0.1% (w/v) bromophenol blue, pH 7.0). The fragments generated by endonuclease digestion were separated by electrophoresis through 0.7% to 1.5% agarose gels and stained and photographed as described above.

The endonuclease Hind III was also used to digest phage lambda DNA which produced 7 fragments of known molecular weight (Murray and Murray, 1975) and which were used to calibrate the gel. Molecular weight estimates of endonuclease-digested plasmid fragments could then be made in the manner described for the estimation of covalently closed circular plasmid DNA.

J) Transformation of H. influenzae

Purified plasmid DNA was used to transform H. influenzae cells made competent by the MIV method of Steinhart and Herriot (1968), modified from the original Spencer and Herriot (1965) procedure. Essentially, 4 ml of an overnight broth culture of H. influenzae was inoculated into 40 ml of sBHI broth in a sidearm flask and agitated on a rotary shaker at 37°C. Cells were grown to 25-35 Klett units (#64 red filter, Klett-Summerson photometer) and monitored to ensure that their generation time was less than 1 h. Ten ml of cells were pelleted, washed once in MIV medium (Appendix IV); cells were dispensed into 18x50 mm glass test tubes and shaken for 100 minutes at 37°C. Finally, 200 µl of plasmid DNA was added to each tube and incubated a further 30 minutes to allow DNA uptake. Dilu-

tions of transformed cells were plated in molten sBHI agar, cooled, and incubated 90 minutes at 37°C to allow full expression of the plasmid-determined antibiotic-resistance characters. At this point, the agar base was overlaid with molten sBHI agar containing twice the desired final concentration of selective antibiotic (ie. ampicillin, 20 µg/ml to give final concentration of 10 µg/ml). When cooled, the plates were incubated overnight and any transformants which arose were removed with a sterile Pasteur pipette, dispersed in 1 ml of sBHI broth, then plated on antibiotic-supplemented CA for further testing. Transformants were checked by agarose gel electrophoresis to ensure they carried the plasmid in a covalently closed circular form.

K) Genetic mating procedure

The membrane filter technique for the conjugative transfer of antibiotic-resistance plasmids was originally described by Thorne and Farrar (1975). The method employed here is a modification of this technique which allows a quantitative assessment of conjugal transferability in terms of the frequency or efficiency of plasmid transfer. H. ducreyi donors and recipients were grown 18 h on fresh CA media containing the relevant antibiotics, scraped off, and resuspended in sBHI broth to a density of approximately 1×10^9 CFU/ml (#64 red filter, Klett-Summerson photometer). Other Haemophilus species or E. coli strains were grown overnight without stirring in 1.0 ml of sBHI broth, then diluted with 5.0 ml of fresh broth and grown with stirring

to a density of 1 x 10^9 CFU/ml. One ml of donor cells was mixed with 1.0 ml of recipient cells and collected by filtration onto a 0.2 µm polycarbonate membrane filter ("Unipore", Bio-Rad Laboratories). Where a large number of matings were to be done simultaneously, a 12-sample vacuum filtration chamber (Millipore) was used; otherwise the donor-recipient mixture was poured into a 5-ml syringe attached to a single Millipore filter unit. Filters were removed from the support unit, placed culture-side-up on the surface of a fresh plain CA plate, and incubated 8 h at 35°C, 5-10% CO_2. In some experiments, a 1 mg/ml solution of deoxyribonuclease (DNase - Sigma) was added at a final concentration of 100 µg/ml to both donors and recipients 10 minutes before mixing and was flooded over the cells on the membrane filter during the 8 h incubation period. After incubation, the filters were removed from the plate surface, placed into 2.0 ml of sBHI broth in a Bijou bottle, and vortexed vigorously to dislodge the cells from the filter. Use of poly-carbonate filters gave almost complete recovery of cells at this step whereas other types of filters (eg. Millipore type HA) retained a large portion of the initial inoculum. Cells were then diluted to at least 10^{-6} with sBHI broth and plated on CA containing selective antibiotics. Because of the genetic markers present on the chromosome of the donors and recipients, selection could be made for three types of cells in any given mating; transconjugants arising from a donor x recipient mating,

donor cells alone, and, transconjugants plus remaining recipients. E. coli transconjugants were selected on antibiotic-supplemented MacConkey agar (without crystal violet). Antibiotics were used at the concentrations listed previously in Section A with the exception that the concentration of ampicillin was increased to 20 µg/ml, tetracycline to 8 µg/ml, and chloramphenicol to 8 µg/ml when E. coli transconjugants were to be selected. As a control and to measure spontaneous mutation frequencies, donors and recipients separately were plated on the antibiotic plates used for transconjugant selection.

Because the numbers of all cells present at the end of an 8 h mating was known, the frequency or efficiency of conjugation could be calculated. The number of transconjugants formed was added to the number of recipients present at the end of the mating to give a value representative of the total number of cells present which were, or at one point had been, viable recipients[1]. This number was divided into the number of transconjugants formed during the mating period to give the frequency of conjugative transfer:

$$\text{Frequency (Efficiency)} = \frac{\text{Number of transconjugants after 8 h}}{\text{No. of transconjugants + No. of recipients}}$$

---

(1) In general, the number of transconjugants was so much less than the number of recipients, that addition of the number of transconjugants did not significantly alter the value of the denominator. Therefore, the denominator essentially represents the total number of recipients present in the mating mixture after 8 hours

Usually, any newly isolated transconjugants were checked in three respects to verify that in fact they were transconjugants:

- they were tested for certain biochemical properties (Section C) to ensure that they were the correct species or biotype (ie. that of the recipient).
- disk diffusion testing was performed to ensure that they possessed the expected antibiotic-resistance pattern. In the case of ampicillin-resistant transconjugants, only the presence or absence of the β-lactamase enzyme was determined by hydrolysis of a chromogenic cephalosporin as described earlier.
- they were screened by agarose gel electrophoresis to ensure that they carried the plasmid originally present in the donor strain.

L) Monitoring of plasmid stability

Transconjugants were tested for their ability to maintain plasmids under non-selective conditions by continual passage in drug-free media. Strains of H. influenzae or H. parainfluenzae were inoculated into 1.0 ml of sBHI broth (without antibiotics) and incubated as standing cultures. Everyday for a 3-week period, 0.001 ml was transferred to 1.0 ml of fresh sBHI broth. Every second day during this time, 0.1 ml of the culture was removed, diluted, and plated on both plain CA and on CA containing those antibiotics to

which resistance was plasmid-mediated. The percentage of cells in the culture still containing a plasmid (% R^+ cells) was calculated as:

$$\% \ R^+ = \frac{\text{plate count on antibiotic-supplemented CA}}{\text{plate count on plain CA}} \times 100$$

The rationale behind this was that loss of a plasmid would enable a cell to grow on plain CA but not on CA supplemented with antibiotics to which it was normally sensitive, whereas a cell harboring a plasmid could grow on both media.

RESULTS

Part I - Self-transferability of Large Haemophilus Plasmids

A) Characteristics of antibiotic-resistant strains

1) Biochemical characteristics and antibiotic resistance pattern.

The four plasmids used throughout the first part of this study were initially identified in clinical isolates of three species of Haemophilus, specifically, two strains of H. ducreyi, one of H. influenzae, and one of H. parainfluenzae. Biochemical characteristics of these strains are shown in Table 4. When compared to Killian's taxonomic scheme for the genus Haemophilus (Killian, 1976), it was seen that HI2265 was a biotype II H. influenzae while HP1007 was a biotype I H. parainfluenzae. Both the strains V1157 and HD9265 showed a nutritional requirement for X-factor (hemin) but not for V-factor (NAD), characteristic of the H. ducreyi species, and also exhibited the colonial morphology (cohesive colonies, remain intact when pushed across an agar surface) expected of this species.

The susceptibility of these strains to various antibiotics was examined by disk diffusion sensitivity testing; the results are shown in Table 5. Only the H. parainfluenzae strain HP1007 was resistant to a single antibiotic, tetracycline, while the other strains were multiply resistant to at least two and as many as three antibiotics.

Table 4. Biochemical Characteristics of Antibiotic-Resistant Clinical Isolates

Strain	Hemin (X-factor) requirement	NAD[a] (V-factor) requirement	Indole Production	Urease Activity	Ornithine decarboxylase Activity	Biotype
HI2265	+	+	+	+	-	II
HP1007	-	+	+	-	-	I
HD9265	+	-	-	-	-	-[b]
V1157	+	-	-	-	-	-[b]

[a] Nicotinamide Adenine Dinucleotide.

[b] Not applicable.

Table 5. Disk Diffusion Zone Size Diameters

	Zone size (mm) to:			
Strain	Ap[a]	Tc	Cm	Su
HI2265	38[b]	12	20	40
HP1007	32	14	48	35
HD9265	6	19	52	38
V1157	6	14	23	30

[a] Abbreviations used: Ap, ampicillin; Tc, tetracycline; Cm, chloramphenicol; Su, sulfonamide.

[b] Zone size breakpoints below which strains are considered resistant are: Ap-20mm, Tc-25mm, Cm-25mm, Su-12mm.

2) Plasmid profiles

Each of these four strains was seen to contain one or more plasmid bands upon agarose gel electrophoresis. A representative pattern is shown in Figure 1. H. ducreyi strain V1157 (Lane B) appeared to harbor only a single plasmid of low molecular weight (ie. migrating below the chromosomal DNA). No other plasmids could be detected, even if the amount of ethanol-precipitated DNA applied to the agarose gel was doubled (not shown). However, if this strain was used as a donor in conjugative matings with a second, plasmid-free H. ducreyi strain such as shown in lane G, and if tetracycline or chloramphenicol-resistance was the selected marker, then transconjugants could be isolated (Lane C) which contained a single high molecular weight plasmid (ie. migrating above the chromosomal DNA band). This plasmid was designated pV1157.

The remaining three strains however, all contained one large plasmid of high molecular weight and in some cases contained one or more small plasmids as well (see, for example, HD9265-Lane D, HP1007-Lane E). Only strain HI2265 (Lane F) contained a single large plasmid; no smaller plasmids could be detected in the isolate. The three large plasmids were designated pHD9265, pHP1007, and pHI2265. The smaller plasmids were not labelled. In general, what was known about each of these four strains and their plasmids at the time this work was initiated is summarized in Table 6.

Strains of H. influenzae or H. ducreyi intended for use as recipients in conjugative matings harbored no plasmids of

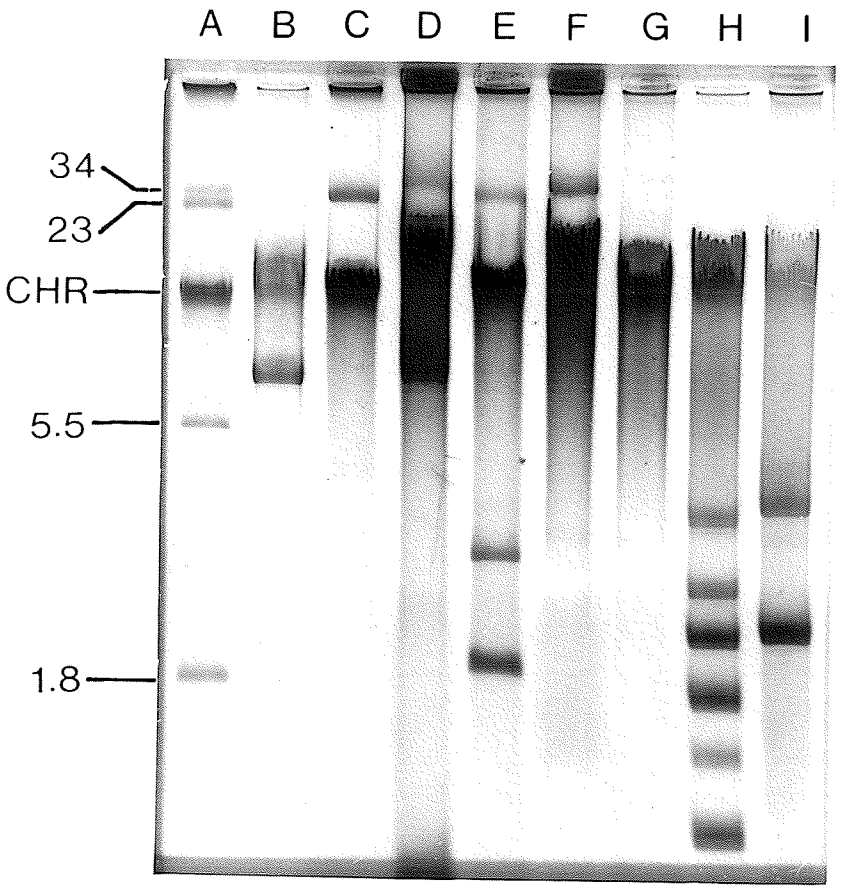


Figure 1. Agarose gel electrophoresis of representative Haemophilus isolates (0.7% gel). Lane A, molecular weight standards; B, H. ducreyi V1157; C, transconjugant from V1157 x HD35000; D, HD9265; E, HP1007; F, HI2265; G, HD35000 (plasmid-free); H, H. parainfluenzae strain R; I, H. parainfluenzae strain T. CHR-Chromosomal DNA.

any size (for example; Figure 1, Lane G). Prospective H. parainfluenzae recipients however, frequently were found to contain one or more low molecular weight plasmids which were phenotypically cryptic. Two examples are seen in lanes H and I. Both the H. parainfluenzae strains were sensitive to all antibiotics tested and showed no biochemical differences either when compared to each other or to standard reference strains. It was known however, that strain HP T lacked Type II restriction endonucleases while HP R contained these enzymes (J. Setlow, unpublished data). Since the majority of drug-sensitive H. parainfluenzae strains carry small cryptic plasmids (W. Albritton, L. Slaney, personal communication), it was decided not to attempt to isolate plasmid-free strains of H. parainfluenzae for use as recipients; instead, the H. parainfluenzae recipients used in this work were those shown in Figure 1, lanes H and I.

3) Molecular weight of large plasmids

The four high molecular weight plasmids were purified by CsCl-density gradient centrifugation and electrophoresed through a 0.7% agarose gel (Figure 2). Comparison with plasmids of known molecular weight gave the following size estimates for each plasmid:

pVl157 (from H. ducreyi) - 38 x 10^6 daltons (38 Mdal)

pHI2265 (from H. influenzae) - 35 x 10^6 daltons (35 Mdal)

pHP1007 (from H. parainfluenzae) - 33 x 10^6 daltons (33 Mdal)

pHD9265 (from H. ducreyi) - 32 x 10^6 daltons (32 Mdal)

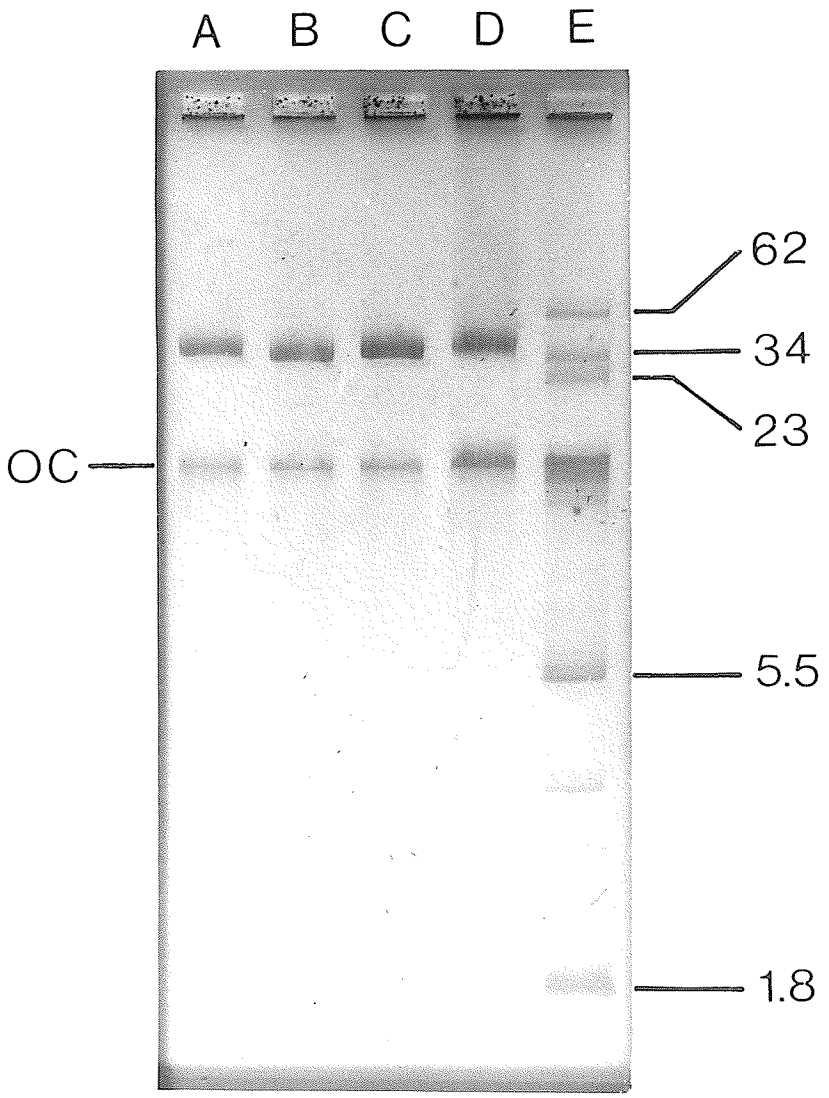


Figure 2. Agarose gel electrophoresis of purified plasmid DNA (0.7% gel). Lane A, pHPl007; B, pHD9265; C, pHI2265; D, pVl157; E, Molecular weight standards. OC - open circular form of plasmids.

Table 6. Characteristics of R-plasmids From Haemophilus Clinical Isolates

Host Species	Plasmid Complement (Mdal)	Resistance Encoded	Self-transferable Intraspecifically
H. influenzae HI2265	30[a]	Tc/Cm	+
H. ducreyi V1157	30	Tc/Cm	+
	7.0	Ap	-
H. ducreyi HD9265	30	Tc	+
	7.0	Ap	-
H. parainfluenzae HP1107	30	Tc	+
	2.0	Cryptic	-

[a]Accurate molecular weight determinations of the large plasmids present in these four strains had not been done before this study was initiated. These plasmids had previously been estimated to be in the 30-34 Mdal size range. A more accurate estimation of their molecular weight was undertaken here.

B) Restriction endonuclease analysis of large plasmids

Each of the four plasmids, after purification by density gradient centrifugation, were digested with several restriction endonucleases. The DNA fragments so produced were separated by electrophoresis in agarose gels and representative patterns are shown in Figures 3 to 6. In some cases, a fifth plasmid, RP4, was included for comparative purposes. This plasmid, originally isolated in Pseudomonas aeruginosa (Datta et al., 1971), has a mass of 34 Mdal and specifies ampicillin, tetracycline, and kanamycin resistance.

Size estimates can be made for each fragment by comparison with the cleavage products of Hind III-digested lambda DNA which are of known molecular weight. In this way, the amount of DNA common to all plasmids (based on the molecular weight of equal-sized fragments) can be calculated. This is shown in Table 7.

C) Self-transferability of large R-plasmids in Haemophilus

1) Interspecific host range

Previous work had demonstrated that these four 32-38 Mdal plasmids were conjugally self-transferable at least between strains of the Haemophilus species from which they were originally isolated (W. Albritton, personal communication). It was therefore necessary to determine if plasmid transfer was also possible to other members of the genus Haemophilus. Ten different Haemophilus species were tested for their ability to receive plasmids in conjugative membrane filter matings with each of the four plasmid-

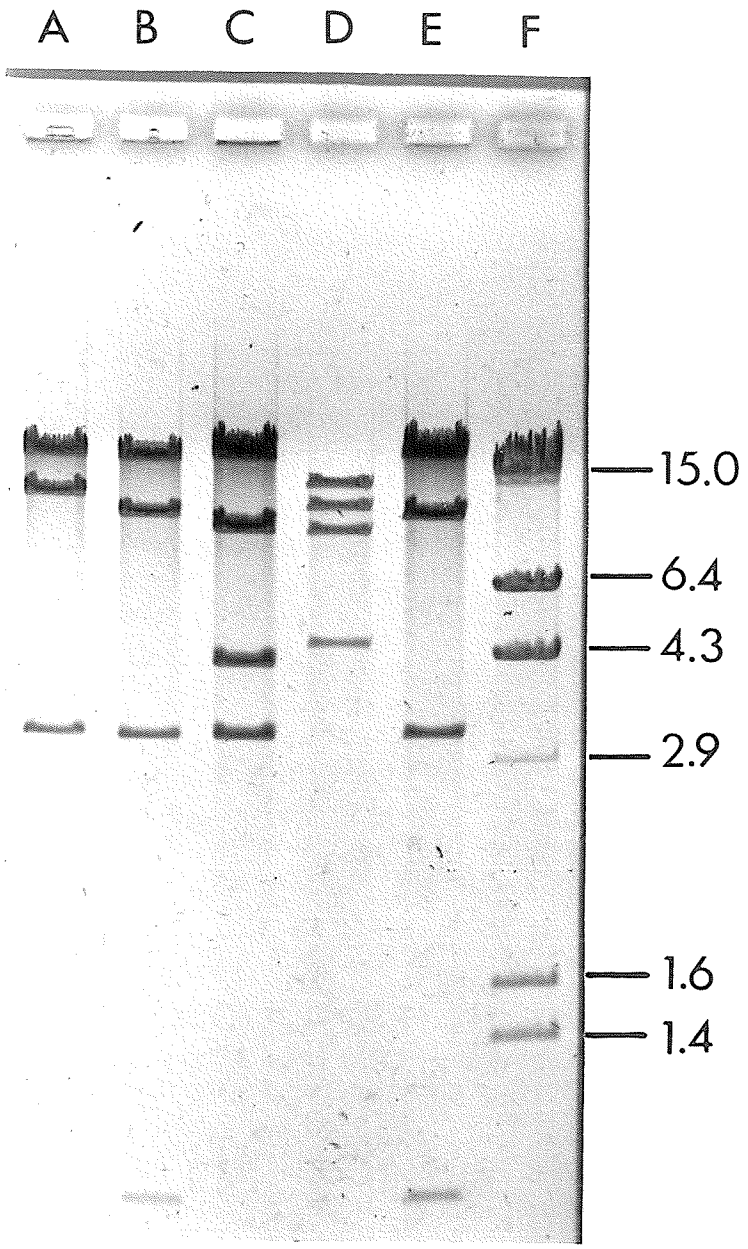


Figure 3. Agarose gel electrophoresis of Sma I-digested plasmid DNA (1.0% gel). Lane A, pHPl007; B, pHD9265; C, pHI2265; D, RP4; E, pVl157; F, lambda DNA digested with Hind III as molecular weight standard.

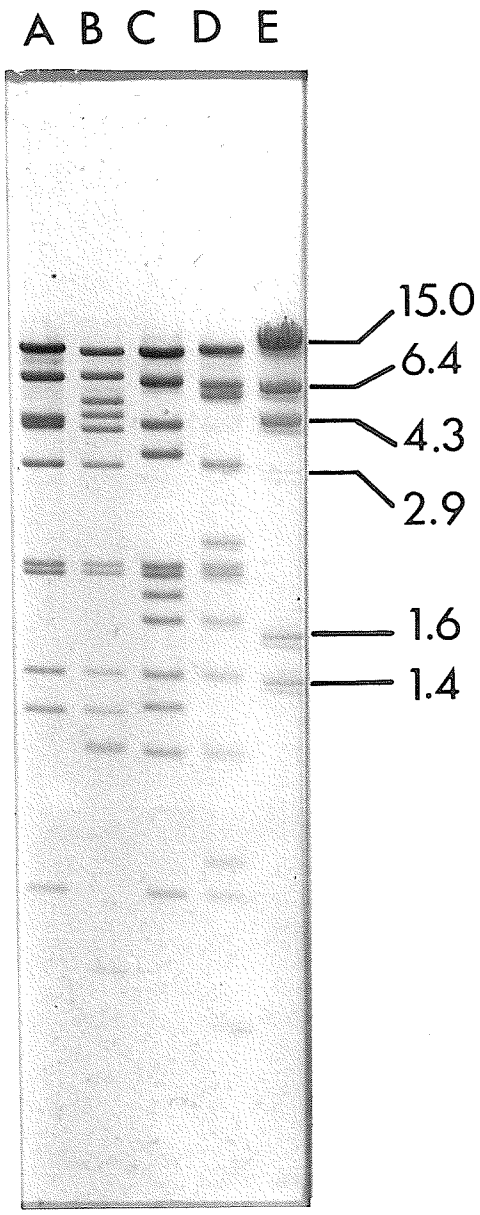


Figure 4. Agarose gel electrophoresis of Pst I-digested plasmid DNA (1.2% gel). Lane A, pHI2265; B, pVl157; C, pHD9265; D, pHPl007; E, lambda DNA digested with Hind III as molecular weight standard.

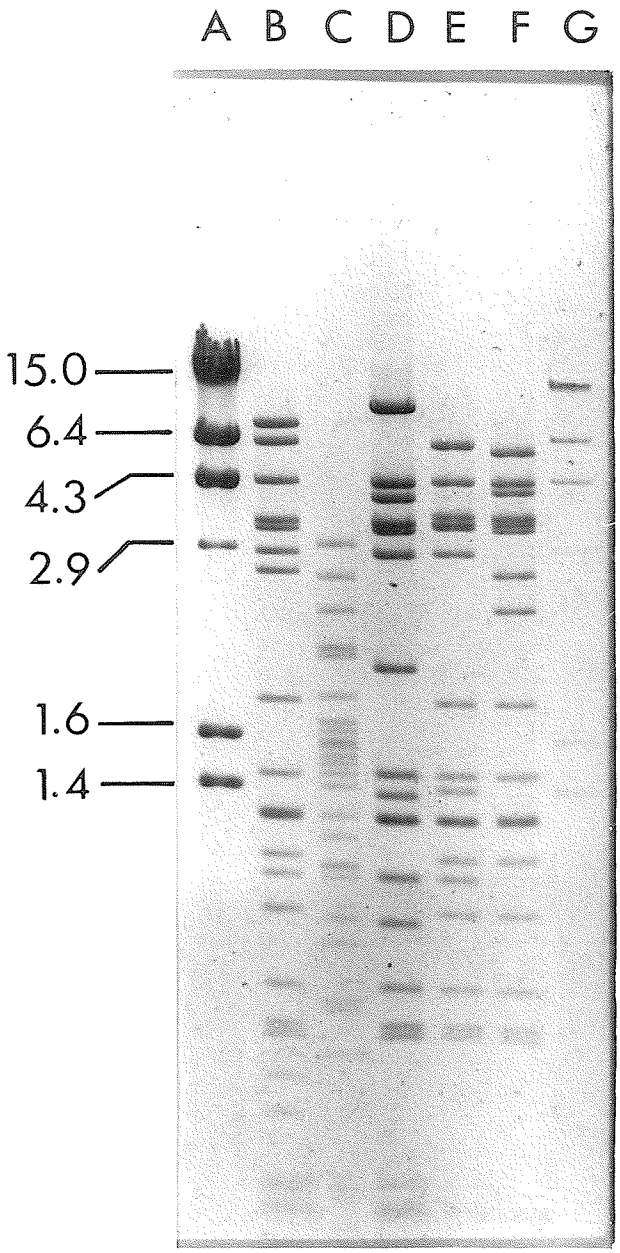


Figure 5. Agarose gel electrophoresis of Hinc II -digested plasmid DNA (1.0% gel). Lanes A and G, lambda DNA digested with Hind III as molecular weight standard; Lane B, pV1157, C, RP4; D, pHI2265; E, pHD9265; F, pHP1007.

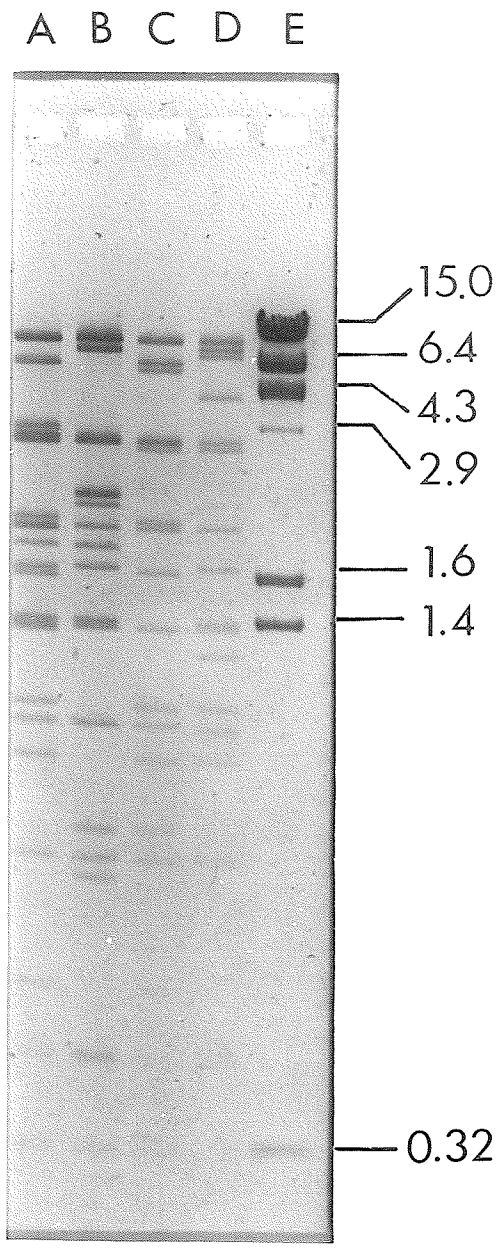


Figure 6. Agarose gel electrophoresis of Hae II-digested plasmid DNA (1.5% gel). Lane A, pV1157; B, pHI2265; C, pHD9265; D, pHP1007; E, lambda DNA digested with Hind III as molecular weight standard.

Table 7. Size Estimates for Restriction Endonuclease-cleaved Fragments of Plasmid DNA

Endonuclease Used	Combined Size (Mdal) of all Fragments[a]				Number of Common Fragments[b]	Combined Size (Mdal) of Common Fragments
	pHI2265(Tc/Cm^r)	pV1157(Tc/Cm^r)	pHD9265(Tc^r)	pHP1007(Tc^r)		
Hinc II	38.7 (18)	43.1 (21)	33.7 (18)	37.0 (17)	9	14.6
Sma I	35.4 (4)	34.9 (4)	32.9 (4)	33.4 (3)	1[c]	3.4
Pst I	37.5 (10)	44.8 (12)	38.6 (12)	40.3 (13)	4	17.9
Hae II	41.9 (16)	40.9 (18)	36.4 (16)	43.5 (16)	8	17.7

[a] Molecular weight of all fragments cleaved from one plasmid was determined using Hind III-digested lambda DNA as standard, and totalled. Bracketted numbers refer to number of fragments cleaved from one plasmid.

[b] Fragments were considered to be common if they migrated to the same point in an agarose gel. Only those fragments of identical size in all four plasmids were used.

[c] Only one fragment was considered to be common to all four Sma I-digested plasmids. The largest fragment was probably common as well or at least was significantly homologous in all cases, but this could not be verified because of the density of the band in this gel.

carrying strains. Matings were carried out at ratios of one donor to one recipient and transconjugants were selected on the basis of all plasmid-encoded traits. The results are shown in Table 8. Conjugal transfer of plasmids was possible to all strains of H. influenzae, H. parainfluenzae, and H. ducreyi tested as well as to two of three strains of H. haemoglobinophilus. The V1157, HP1007, and HD9265 donors were never observed to co-transfer their small, non-conjugative plasmids together with the large R-plasmids. There was some question however, as to the correct speciation of the two H. haemoglobinophilus strains unable to act as plasmid recipients. Although these strains were sent to us as H. haemoglobinophilus, certain biochemical differences became apparent when they were compared to an ATCC reference strain of H. haemoglobinophilus (Table 9). It is possible that these two strains may not in fact be H. haemoglobinophilus species although they could not be assigned to any other Haemophilus species based on the Killian classification scheme (Killian, 1976).

2) Efficiency of intraspecific vs. interspecific plasmid transfer

Conjugative plasmid transfer was quantitated in terms of frequency (or efficiency) of transconjugant formation. In this, and in subsequent parts of this study, only three plasmids were used - pHI2265, pHP1007, and pHD9265. Each of these plasmids was introduced by conjugation into a standard recipient strain, either H. influenzae strain Rd, H. ducreyi strain HD35000, or H. parainfluenzae strain Hp R, all of which carried a chromosomal streptomycin-resistance marker. These strains were then used as donors to secondary

Table 8. Interspecific Host Range of Conjugative *Haemophilus* Plasmids

Species Tested as Recipient	No. of Strains Tested[a]	Ability to Receive Plasmid From: HI2265 (pHI2265-Tc/Cm^r)	V1157 (pV1157-Tc/Cm^r)	HD9265 (pHD9265-Tc^r)	HP1007 (pHP1107-Tc^r)
H. influenzae	5	+(5/5)[b]	+(5/5)	+(5/5)	+(5/5)
H. parainfluenzae	5	+(4/5)	+(4/5)	+(4/5)	+(4/5)
H. ducreyi	2	+(2/2)	+(2/2)	+(2/2)	+(2/2)
H. haemoglobinophilus (clinical isolates)	2	+(2/2)	+(2/2)	+(2/2)	+(2/2)
H. haemoglobinophilus (ATCC reference strain	1	-[c]	-	-	-
H. pleuropneumoniae	3	-	-	-	-
H. suis	1	-	-	-	-
H. paraphrohaemolyticus	1	-	-	-	-
H. gallinarum	1	-	-	-	-
H. aphrophilus	3	-	-	-	-
H. paraphrophilus	1	-	-	-	-

[a] At least one strain of each species tested was an ATCC reference strain (see Table 2).

[b] Bracketted numbers refer to the number of strains able to receive plasmids out of the total number of strains of each species tested. In each case where transfer of resistance was found to occur, a plasmid band could also be visualized by agarose gel electrophoresis.

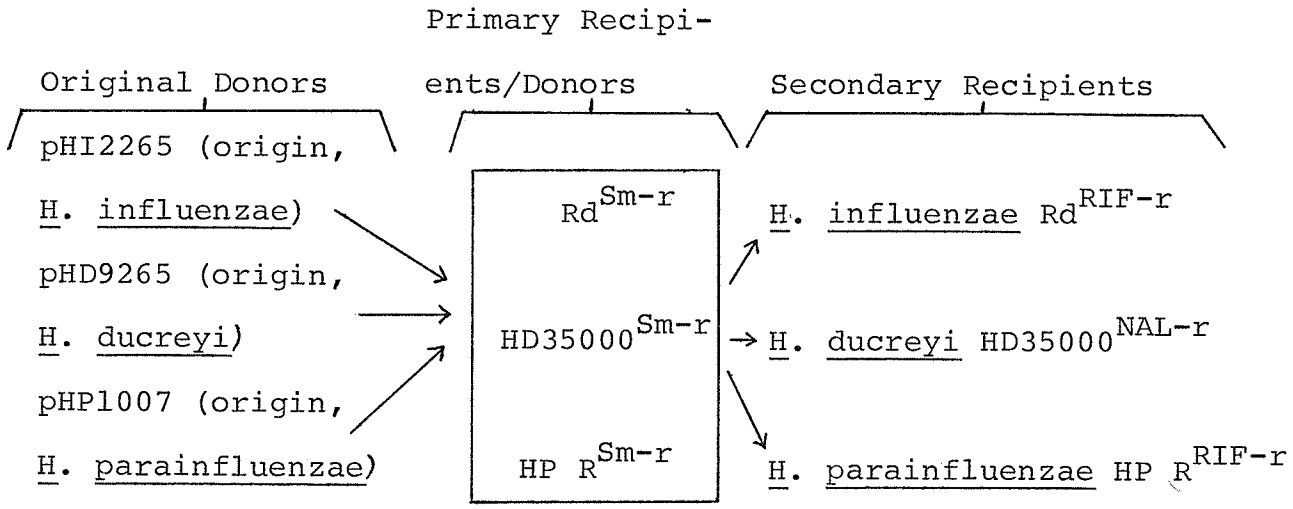
[c] Minus sign indicates transfer of resistance was not possible to these species.

Table 9. Biochemical Characteristics of *H. Haemoglobinophilus* Strains[a]

Strain	Source	Hemin Requirement	NAD Requirement	ONPG	Ornithine Decarboxylase	Urease	Indole	VP	Glucose	Inositol	Sucrose	Melibiose	Amylose	Mannose	Arabinose	Nitrate	Oxidase
HH72-164	Clinical Isolate	+	-	+	-	-	-	+	+	-	-	+	-	+	+	+	+
HH77-40	Clinical Isolate	+	-	+	-	-	-	+	+	-	-	+	-	+	+	+	+
ATCC 19416	Reference	+	-	-	-	-	+	-	+	+	+	-	-	+	-	+	+

[a]All tests except Hemin and NAD requirements were performed using the API-20E system.

recipients which were either Rd, HD35000, or HP R carrying either rifampin- or nalidixic acid-resistance chromosomal markers. This mating scheme may be diagrammed as follows, where the arrows indicate the direction of plasmid transfer:

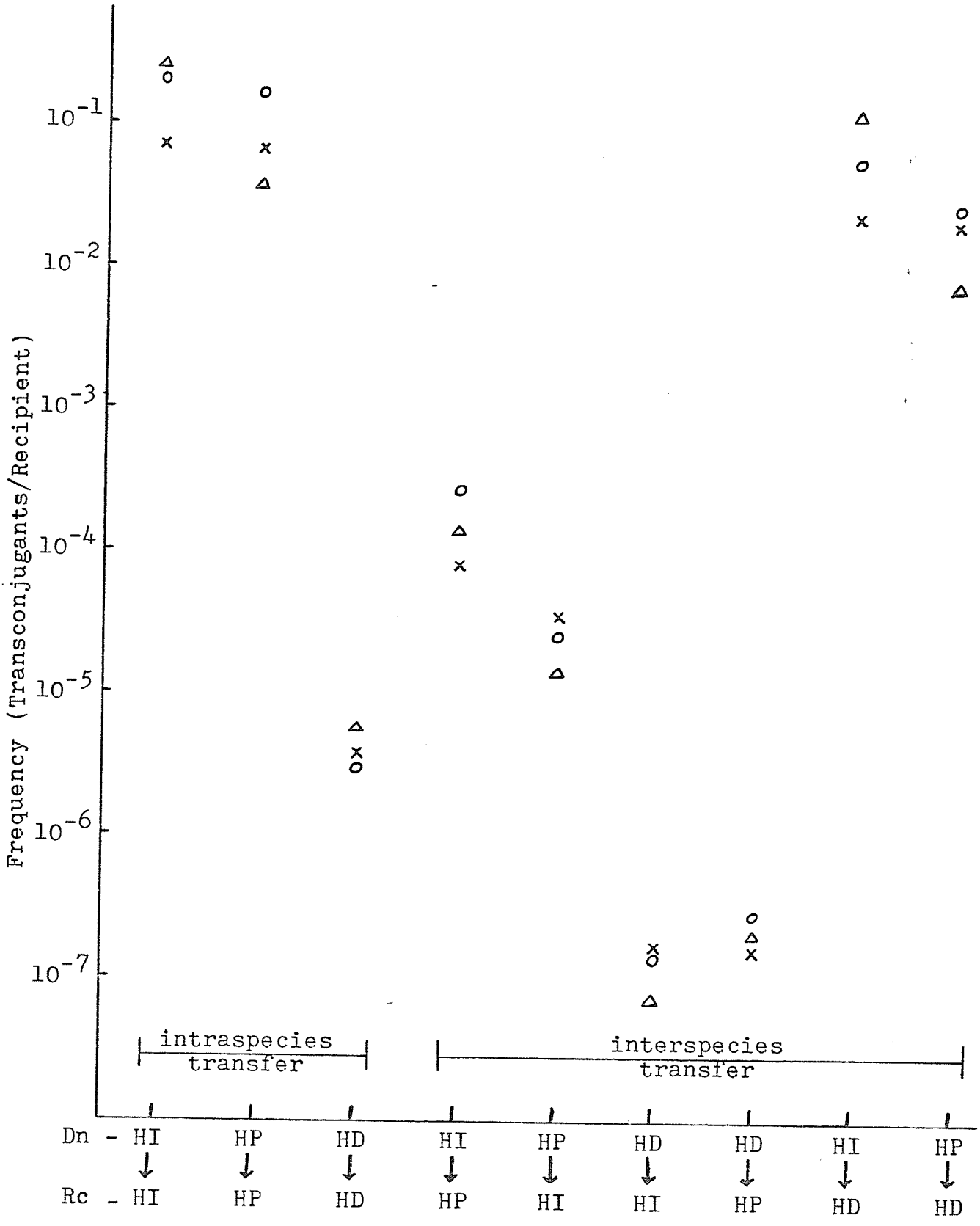


All matings to secondary recipients were quantitated so that comparisons could be drawn between intraspecific matings (both partners of same species) and interspecific matings (partners of different species), as well as between the three plasmids themselves in any given host. These results are graphically displayed in Figure 7. Furthermore, some matings were performed in which either partner was the H. haemoglobinophilus strain HH72-164. These results are shown in Table 10.

3) Stability of plasmids in different host species

Strains of H. influenzae or H. parainfluenzae containing pHI2265, pHD9265, or pHP1007 were monitored for the rate at which these plasmids were lost when selective pressure was removed. These results are shown in Table 11. Stability

Figure 7. Intra- and interspecific transfer frequencies of conjugative Haemophilus plasmids. Donor species were either H. influenzae Rd, H. parainfluenzae R, or H. ducreyi 35000, carrying one of three conjugative plasmids: pHI2265 (×), pHD9265 (○), or, pHP1007 (△). Recipients were either H. influenzae Rd, H. parainfluenzae R, or H. ducreyi 35000 carrying a different chromosomal drug-resistance marker for counterselection purposes. Matings were for 8h on membrane filters. The particular donor-recipient pair in each experiment is shown along the y-axis, where the arrow denotes the direction of plasmid transfer. The frequency of transfer for each one of the three plasmids in any given mating is shown along the x-axis, where frequency is expressed as the number of Tc^r or $Tc\text{-}Cm^r$ transconjugants formed per recipient. Intraspecies transfer refers to a mating in which both donor and recipients are of the same species, irregardless of which of the three plasmids is carried by the donor. Interspecies transfer refers to matings where partners are of different species. Abbreviations used: Dn - donor species; Rc - recipient species; HI - H. influenzae Rd; HP - H. parainfluenzae R; HD - H. ducreyi 35000.



(→) - Direction of plasmid transfer

Table 10. Plasmid Transfer Frequencies With H. Haemoglobinophilus HH72-164[a]

Plasmid Donor	Recipient			
	H. haemoglobinophilus HH72-164	H. influenzae Rd	H. parainfluenzae HP R	H. ducreyi HD35000
H. influenzae Rd (pHPl007)	4.3×10^{-5}	3.0×10^{-1}	9.1×10^{-5}	3.6×10^{-2}
H. parainfluenzae HP R (pHPl007)	3.6×10^{-6}	4.9×10^{-5}	5.6×10^{-2}	8.8×10^{-3}
H. ducreyi HD35000 (pHPl007)	5.0×10^{-7}	1.4×10^{-7}	4.1×10^{-7}	6.5×10^{-5}
H. haemoglobinophilus HH72-164 (pHPl007)	5.7×10^{-3}	1.9×10^{-4}	5.7×10^{-5}	1.7×10^{-4}

[a]Frequency expressed as number of transconjugants formed per output recipient.

Table 11. Stability of Conjugative Plasmids in *Haemophilus* Species

		% R^+ Cells Remaining After (Days):										
		2	4	6	8	10	12	14	16	18	20	22
pHI2265	in HI[a]	98	100	100	96	85	84	86	88	78	72	-
(origin: *H. influenzae*)	in HP	100	100	99	94	100	85	78	63	81	49	60
pHP1007	in HI	100	84	97	100	95	89	98	93	83	92	82
(origin: *H. parainfluenzae*)	in HP	83	92	100	97	100	94	-	-	-	-	-
pHD9265	in HI	100	98	100	80	78	79	68	58	57	38	-
(origin: *H. ducreyi*)	in HP	100	100	97	98	100	99	100	98	95	84	-

[a] Abbreviations used: HI, *H. influenzae*; HP, *H. parainfluenzae*.

of plasmids in H. ducreyi could not be tested in this way because this species did not grow well in liquid media. However, continuous subculturing of plasmid-containing H. ducreyi strains on plain CA did not affect the ease with which antibiotic-resistance colonies could subsequently be isolated.

D) Kinetics of plasmid transfer

1) Frequency of transfer vs. donor/recipient ratio

Because the frequency of plasmid transfer in other genera was known to be proportionately related to the ratio of donor cells to recipient cells (Cullum et al., 1978), it was decided to see if this was also the case in certain intra- and interspecific Haemophilus matings. Specifically, matings were performed in which the donor was either H. influenzae Rd or H. parainfluenzae R carrying the pHP1007 Tc^r plasmid, and the recipient was a plasmid-free strain of H. influenzae Rd or H. parainfluenzae R. Prior to each mating, the donor or recipient cell cultures were diluted in sBHI broth so that upon final mixing and filtration, the donor concentration vs. recipient concentration spanned a range from 10^3:1 to 1:10^3. In other words, each experiment involved seven separate filter matings such that one filter contained 1000 donors for every one recipient, another 100 donors per recipient, and so on until the last filter contained 1 donor per 1000 recipients. Actual donor/recipient ratios were calculated on the basis of numbers of donors or recipients present

at the end of an 8 h mating. The frequency of transconjugant formation as a function of varying donor/recipient ratio can be graphically illustrated as in Figures 8 and 9.

2) Effect of chromosomal rec 1 or rec 2 mutation on plasmid transfer.

The products of the rec 1 and rec 2 genes are necessary for generalized recombination in Haemophilus. It was therefore of interest to determine if these gene products were necessary for, or in any way altered, the self-transferability of plasmid DNA in Haemophilus. Matings were performed at varying donor/recipient ratios between wild-type H. influenzae Rd strains carrying pHP1007 and H. influenzae Rd rec 1 or rec 2 recipients. The results, shown in Figure 10, illustrate that the presence of the rec 1 or rec 2 mutation in the recipient had little apparent effect on the frequency of plasmid transfer through a range of donor/recipient ratios. Representative transconjugants isolated from these matings all showed the presence of a plasmid band of the expected molecular weight upon screening by agarose gel electrophoresis.

Since it was seen that H. influenzae rec 1 or rec 2 strains could act as recipients of plasmids, it was therefore possible to determine whether they could act as donors as well. Matings were performed as described above but essentially in reverse; H. influenzae rec 1 or rec 2 strains carrying the pHP1007 plasmid were used as donors to wild-type Rd recipients. Here again, as seen in Figure 11,

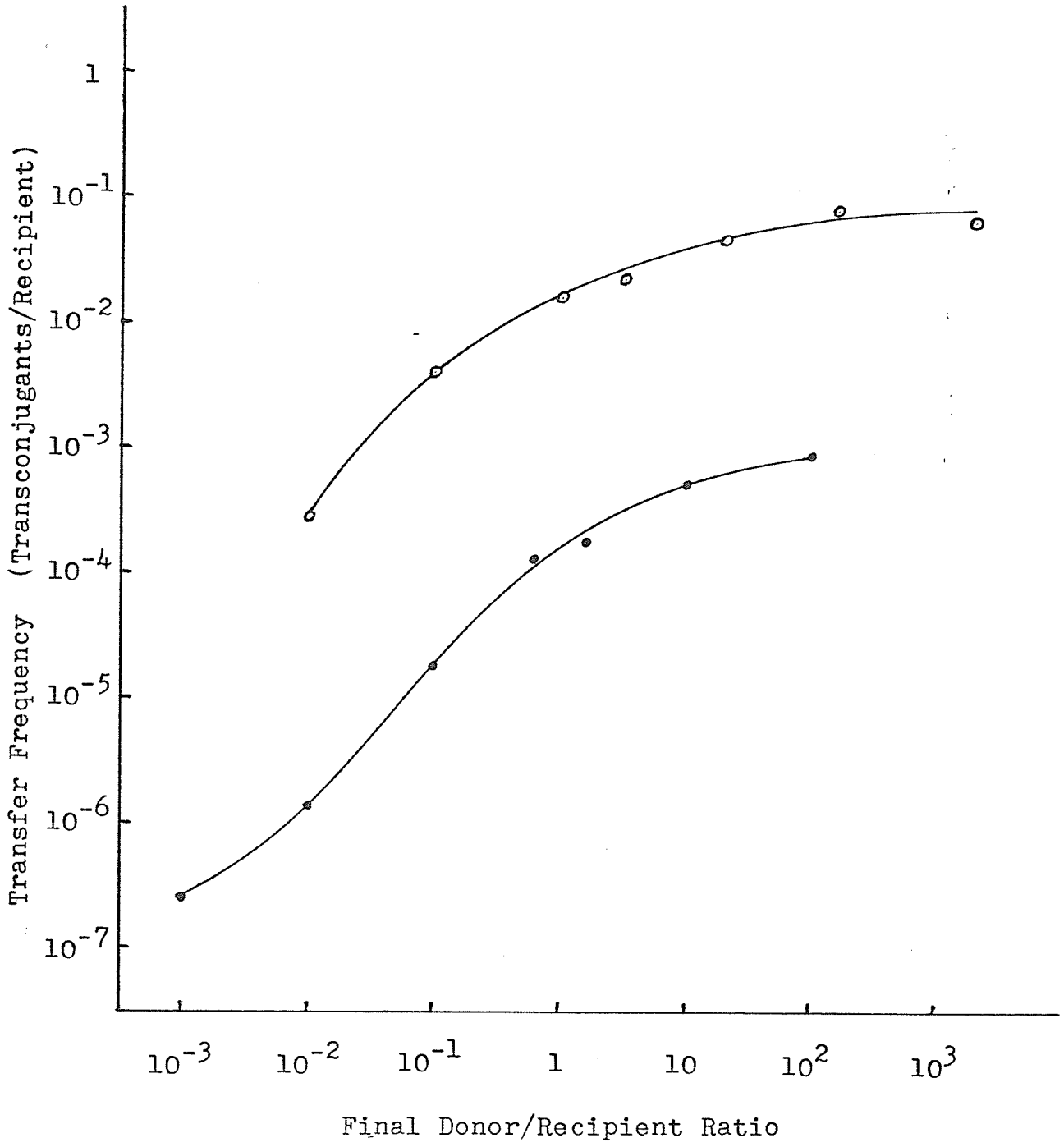


Figure 8. Frequency of plasmid transfer vs. donor/recipient ratio - H. parainfluenzae donors. Membrane filter matings (8h) with final donor to recipient cell ratios from 10^{-3}:1 to 10^{3}:1. Donors in both experiments were H. parainfluenzae R (Rif-r) carrying the plasmid pHPl007. Recipients were either H. parainfluenzae R (Str-r) (o—o) or H. influenzae Rd (Str-r) (●—●). Frequency of plasmid transfer expressed as the number of tetracycline-resistant transconjugants formed per recipient.

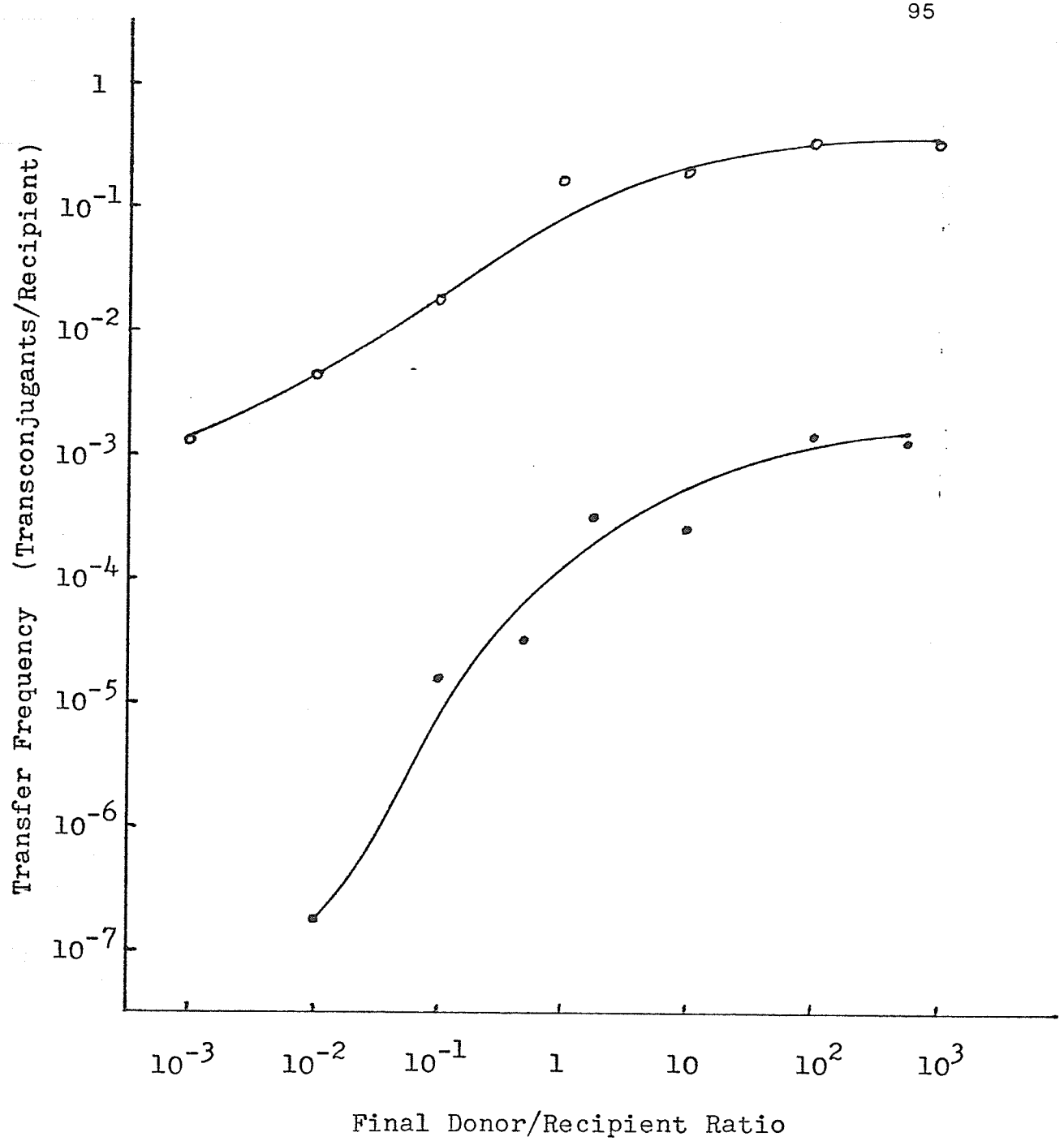


Figure 9. Frequency of plasmid transfer vs. donor/recipient ratio - H. influenzae donors. Membrane filter matings (8h) with final donor to recipient cell ratios from 10^{-3}:1 to 10^{3}:1. Donor in both experiments was H. influenzae Rd (Str-r) carrying the plasmid pHP1007. Recipients were either H. influenzae Rd (Nov-r) (o—o) or H. parainfluenzae R (Rif-r) (●—●). Frequency of plasmid transfer is expressed as the number of tetracycline-resistant transconjugants formed per recipient.

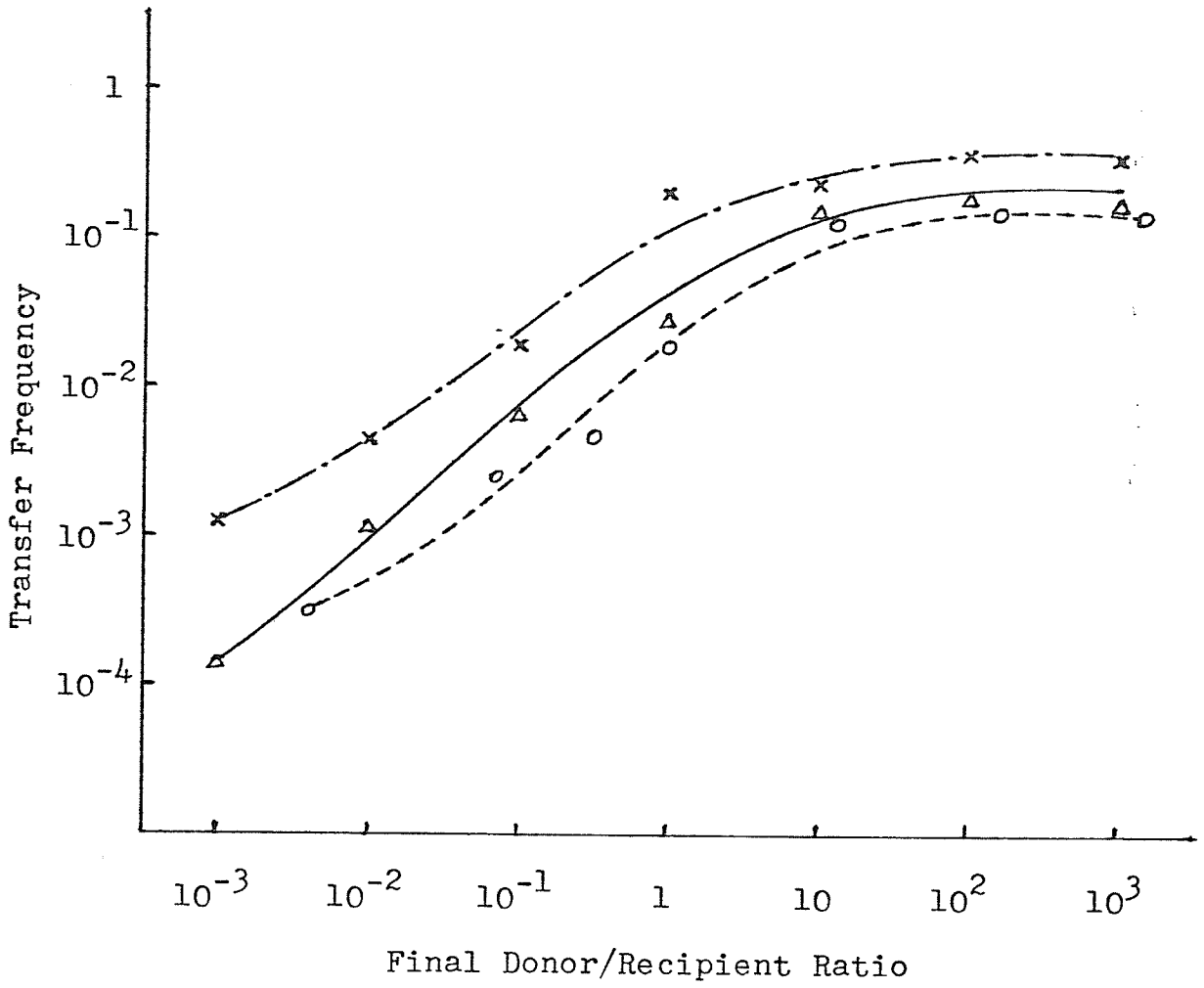


Figure 10. Frequency of plasmid transfer vs. donor/recipient ratio - recl, rec2 recipients. Donors in all experiments were wild-type H. influenzae Rd (Nov-r) carrying the plasmid pHP1007. Recipients were either H. influenzae recl (Str-r) (o----o), H. influenzae rec2 (Str-r) (Δ——Δ), or the wild-type H. influenzae Rd (Str-r) (x— -—x). Donor to recipient cell ratios were varied from 10^{-3}:1 to 10^{3}:1. Frequency of plasmid transfer is expressed as the number of tetracycline-resistant transconjugants formed per recipient.

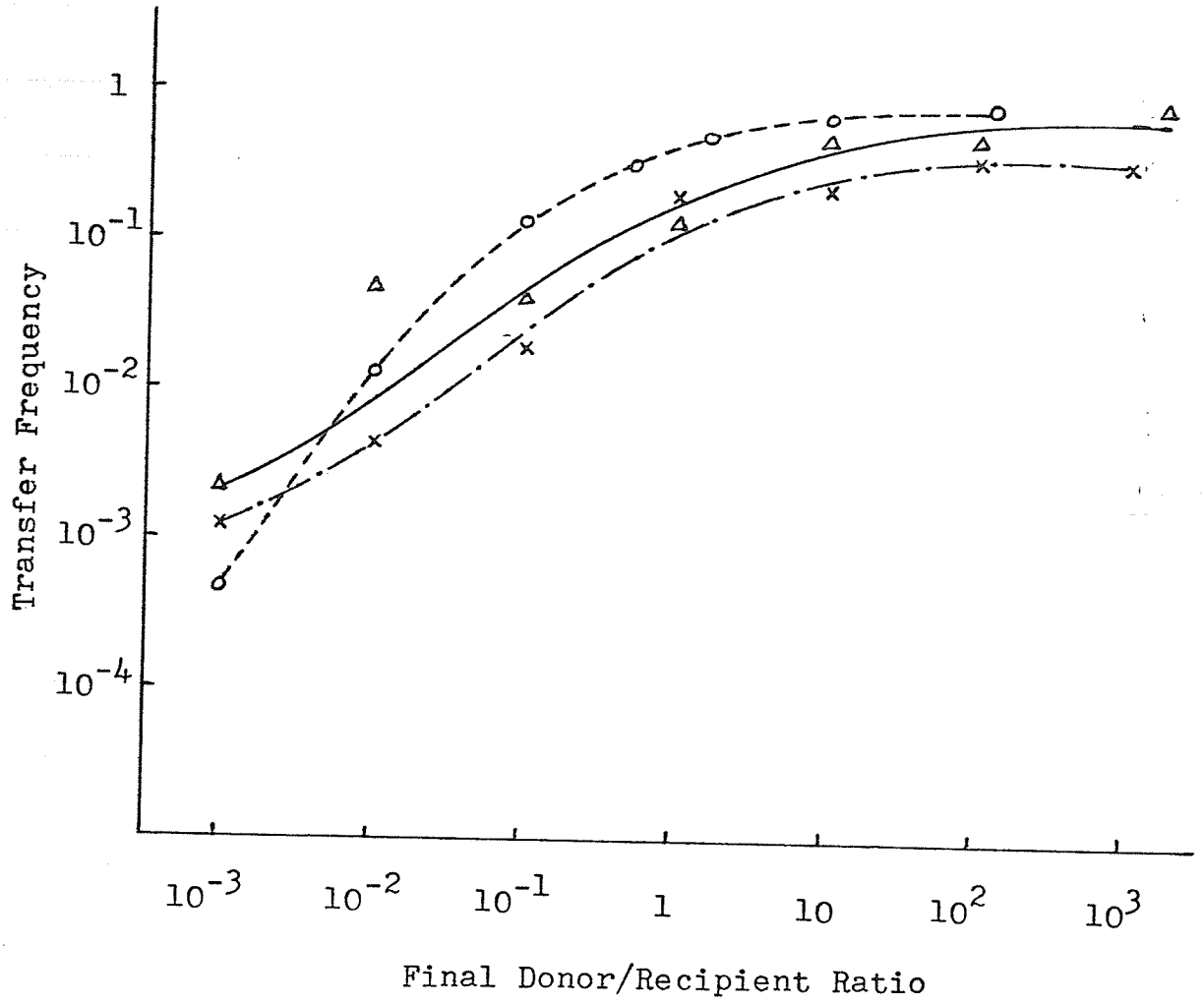


Figure 11. Frequency of plasmid transfer vs. donor/recipient ratio - recl, rec2 donors. Recipients in all experiments were wild-type H. influenzae Rd (Nov-r). Donors were either H. influenzae recl (Str-r) (o----o) or H. influenzae rec2 (Str-r) (Δ——Δ) carrying the plasmid pHP1007. These had been obtained from the mating experiment shown in Figure 10. Also used as a donor strain was wild-type H. influenzae Rd (Str-r) carrying pHP1007 (x— -—x). Donor to recipient cell ratios were varied from 10^{-3}:1 to 10^{3}:1. Frequency of plasmid transfer is expressed as the number of tetracycline-resistant transconjugants formed per recipient.

there was no appreciable difference in plasmid transfer frequency when recl or rec2 strains were used as donors as compared to a wild-type Rd strain carrying the same plasmid used as a donor.

E) Intergeneric transfer of large Haemophilus plasmids

1) Escherichia coli

An E. coli C600 strain which lacked DNA restriction endonuclease activity was used as a recipient in filter matings with Haemophilus species carrying the pHI2265 or the pHPl007 R-plasmids. Selection for linked Tc/Cm^r in the case of pHI2265 or just Tc^r in the case of the pHPl007 plasmid yielded E. coli transconjugants at frequencies indicated in Table 12. However, when these transconjugants were screened for the presence of plasmid DNA by agarose gel electrophoresis, no such bands of any size could be detected. This was true for over 50 such transconjugants screened. Furthermore, neither Tc/Cm^r nor Tc^r could ever be re-transferred from these E. coli transconjugants to other E. coli recipients or back to Haemophilus recipients in filter matings.

Attempts were then made to use Haemophilus species as recipients for large, self-transferable Enterobacteriaceae and Pseudomonas plasmids. Donor strains consisted of E. coli C600 containing either the 34 Mdal broad host range plasmid RP4 or the larger, 62 Mdal plasmid Rl, both of which carried multiple antibiotic resistance markers. Reci-

Table 12. Intergeneric Transfer Frequency - *E. coli* C600 Recipient

Donor Species	Frequency[a]
H. influenzae Rd (pHI2265)	4.8×10^{-7}
H. influenzae Rd (pHPl007)	1.1×10^{-7}
H. parainfluenzae R (pHI2265)	4.5×10^{-7}
H. parainfluenzae R (pHIl007)	1.1×10^{-6}
H. ducreyi HD35000 (pHI2265)	3.7×10^{-8}
H. haemoglobinophilus HH72-164 (pHI2265)	$< 10^{-9}$
H. haemoglobinophilus HH72-164 (pHPl007)	$< 10^{-9}$

[a] Frequency expressed as a number of *E. coli* transconjugants formed per output recipient. *E. coli* transconjugants selected on the basis of Tc^r (for pHPl007) or Tc/Cm^r (for pHI2265).

pients in standard membrane filter matings were either H. influenzae Rd, H. parainfluenzae R, or H. ducreyi HD35000. No transconjugants were ever isolated when selection was for any of the markers known to be present on the E. coli R-plasmids.

2) Neisseria gonorrhoeae

Experiments similar to those described above were carried out using N. gonorrhoeae T1 and T4 colonial variants as recipients for the pHI2265 or pHP1007 plasmids carried by H. influenzae Rd, H. parainfluenzae R, or H. ducreyi HD35000. Selection was on GC agar base containing antibiotics but in no case were any antibiotic-resistant N. gonorrhoeae transconjugants isolated.

Large plasmids encoding antibiotic resistance indigenous to N. gonorrhoeae have thus far not been encountered. However, certain penicillinase-producing N. gonorrhoeae (PPNG) are known to carry a small 4.4 Mdal R-plasmid encoding a β-lactamase enzyme which is transferable to other N. gonorrhoeae or E. coli recipients via mobilization by a co-resident 24.5 Mdal plasmid. Thus, filter matings were performed to determine if this small Ap^r plasmid could be mobilized to Haemophilus species as well. Figure 12 shows the results of a mating between a PPNG clinical isolate carrying a large 24.5 Mdal mobilizing plasmid plus two smaller plasmids of 4.4 and 2.6 Mdal (Lane A) and a H. influenzae HI5327 recipient carrying two small cryptic plasmids (Lane B). H. influenzae transconjugants from this mating could be isolated at frequencies of 1.5×10^{-6} and upon screening were found to contain three plasmids

(Lane C) - the two non-transferring cryptic plasmids of strain HI5327 and the 4.4 Mdal Ap^r plasmid from the PPNG strain. Biochemical characterization also verified that this transconjugant was the HI5327 strain. However, the 24.5 Mdal mobilizing plasmid was not visible in this transconjugant. Transfer of the 4.4 Mdal Ap^r plasmid from PPNG was also possible to H. ducreyi and H. parainfluenzae recipients but transfer frequencies were not obtained. Again, only the 4.4 Mdal plasmid was seen in the transconjugants (for example, Lane D); the 24.5 Mdal mobilizing plasmid was never detected. Furthermore, re-transfer of the small Ap^r plasmid was not possible from any of these Haemophilus transconjugants.

Part II - Mobilization of Small Haemophilus Plasmids

A) Identification and characterization of H. ducreyi strain HD147.

Strain HD147 (see Table 2) was one of several strains of H. ducreyi isolated from patients displaying symptoms of chancroid in Nairobi, Kenya (I. MacLean, M. Fast, A. Ronald, H. Nsanze, unpublished data). Disk diffusion susceptibility testing revealed that this strain was resistant to ampicillin, tetracycline and sulfonamide. A second H. ducreyi isolate, HD9468, had an identical antibiotic-resistant pattern. Screening of HD147 by agarose gel electrophoresis revealed the presence of three plasmid

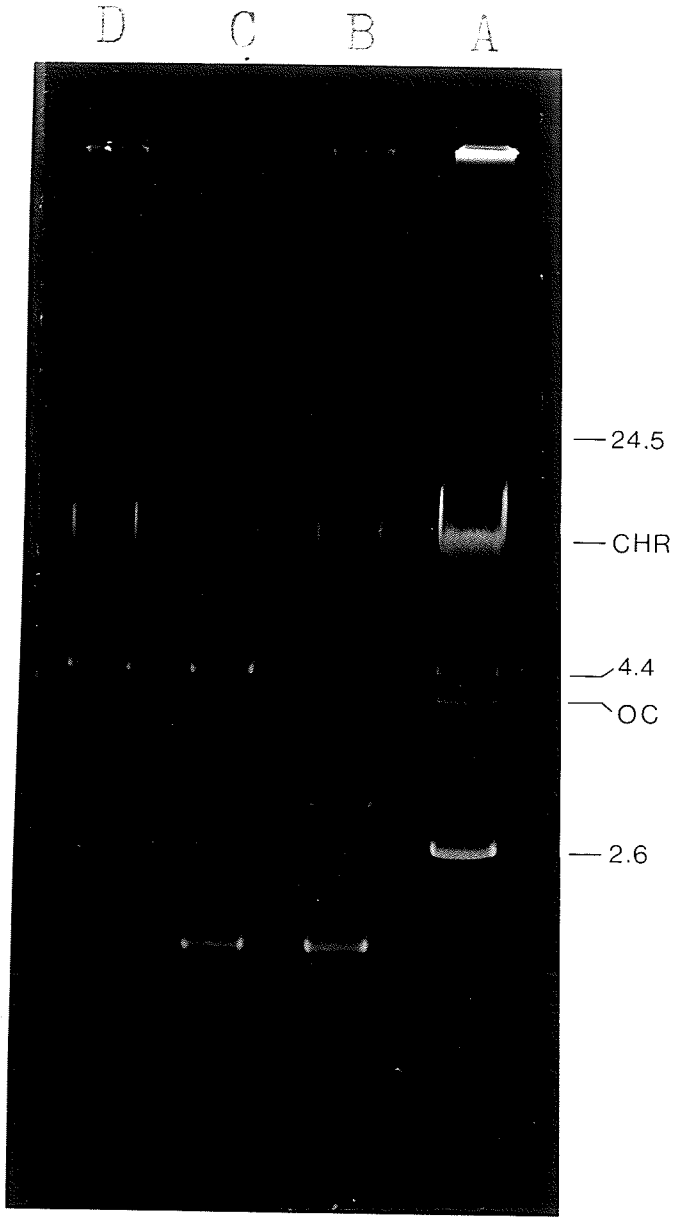


Figure 12. Agarose gel electrophoresis of Neisseria x Haemophilus transconjugants (0.7% gel). Lane A, N. gonorrhoeae strain 22209 showing 3 plasmids of 24.5 (faint band), 4.4, and 2.6 Mdal; B, HI5327 recipient strain carrying a cryptic plasmid; C, transconjugant of 22209 x HI5327; D, transconjugant of 22209 x H. ducreyi HD35000. CHR-chromosomal DNA, OC - open circular form of 2.6 Mdal plasmid of Lane A.

bands of 23.5, 7.0, and 4.9 Mdal. These plasmids were designated pHD147, pHD147-7, and pHD147-4, respectively. In contrast, strain HD9468 harbored only two plasmids of 7.0 and 4.9 Mdal. This 7.0 Mdal plasmid is identical in size to other H. ducreyi plasmids which are known to encode a β-lactamase enzyme which confers ampicillin resistance (W. Albritton, personal communication). Likewise, a plasmid of 4.9 Mdal has recently been shown to confer resistance to sulfonamide in H. ducreyi (W. Albritton, J. Brunton, L. Slaney, I. MacLean, manuscript in preparation).

B) Conjugative transfer of ampicillin resistance (Ap^r).

1) Transfer from HD147

H. ducreyi strain HD147 was used as a donor in membrane filter matings with a recipient strain of H. influenzae HI1008. H. influenzae transconjugants were selected on the basis of ampicillin resistance, tested for the presence of the β-lactamase enzyme, and screened by agarose gel electrophoresis. Figure 13 illustrates the plasmid complement of some of these transconjugants. Strain HD147 with its three plasmids is shown in Lane A while Lane B demonstrates the plasmid-free state of the HI1008 recipient. An Ap^r, β-lactamase producing transconjugant of HD147 x HI1008 is shown in Lane C and is seen to contain two plasmids of 23.5 and 7.0 Mdal (ie. pHD147 and pHD147-7). Neither tetracycline nor sulfonamide resistance was ever co-transferred when transconjugants were selected only on the basis of

ampicillin resistance. Repeated passage of the Ap^r strain shown in Lane C in ampicillin-free broth occasionally yielded Ap-senstive clones which had lost the 7.0 Mdal plasmid yet still retained the 23.5 Mdal plasmid (strain HI1008 (pHD147), Lane D); indicating that the ampicillin resistance determinant is in fact located on the 7.0 Mdal plasmid and that the 23.5 Mdal pHD147 plasmid is phenotypically cryptic. One HD147 x HI1008 mating yielded an Ap^r transconjugant which contained only a 7.0 Mdal plasmid (strain HI1008 (pHD147-7), Lane E). This was apparently a relatively rare event since only one such clone was observed in over 50 Ap^r H. influenzae transconjugants screened by agarose gel electrophoresis.

In contrast, similar matings using HD9468 as a donor failed to yield Ap^r transconjugants in repeated trials. Similarly, Ap-resistance could not be transferred from H. ducreyi strain V1157 or from HD9265, both of which carry a 7.0 Mdal Ap^r plasmid in addition to either a 38 Mdal conjugative Tc/Cm^r plasmid (pV1157) or a 32 Mdal Tc^r plasmid (pHD9265). Both these large plasmids are themselves easily transferable to H. influenzae recipients in membrane filter matings (see Part I, Section C).

2) Re-transfer of Ap^r from H. influenzae transconjugants.

Table 13 shows the frequency of Ap^r transfer from the original HD147 donor to recipient strains of H. influenzae H. ducreyi, or E. coli, as well as from Ap^r H. influenzae transconjugants to these same recipients. When Ap^r H. influenzae transconjugants harboring both the 23.5 and 7.0

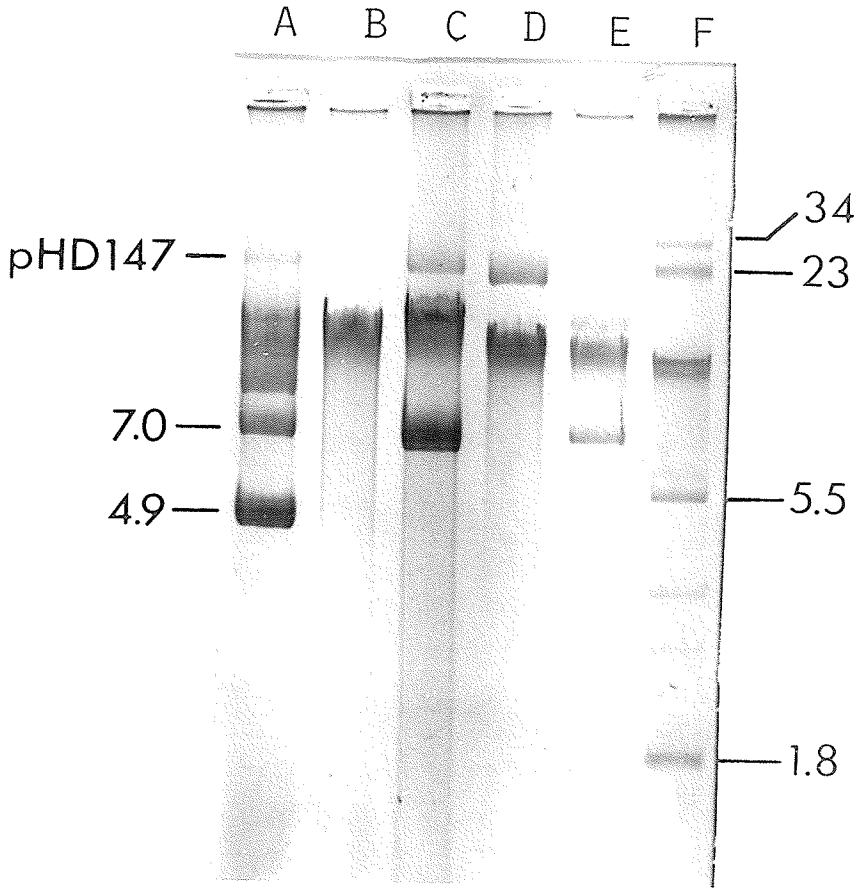


Figure 13. Agarose gel electrophoresis of Ap^r H. influenzae transconjugants (0.7% gel). Lane A, strain HD147 showing three plasmids of 23.5 (pHD147), 7.0 (pHD147-7), and 4.9 Mdal (pHD147-4); B, HI1008 recipient, C, Ap^r transconjugant of HD147 x HI1008; D, HI1008 (pHD147)-Ap-sensitive derivative of strain in Lane C; E, HI1008 (pHD147-7)-Ap^r transconjugant of HD147 x HI1008 containing only the 7.0 Mdal plasmid; F, Molecular weight standards.

Mdal plasmids were used as donors to sensitive H. influenzae recipients, transfer of Ap^r was seen but at frequencies somewhat higher than seen with the original host strain, HD147, as the donor. Even upon re-transfer of Ap^r, H. influenzae transconjugants again showed the presence of two plasmid bands of 23.5 and 7.0 Mdal when screened. Re-transfer of Ap^r was not detected however, when strain HI1008 (pHD147-7) containing just the 7.0 Mdal plasmid was used as a donor.

Matings between various Haemophilus donors and E. coli C600 recipients gave β-lactamase-producing E. coli transconjugants at frequencies similar to those seen earlier with H. influenzae recipients (Table 13). However, the presence of the 23.5 Mdal pHD147 plasmid could not be detected in these E. coli transconjugants and neither could Ap-resistance be re-transferred to other E. coli or Haemophilus recipients.

Transformation as the mode of gene transfer in these and in previous experiments could be ruled out for three reasons. The frequency of Ap-resistance transfer was not decreased when DNase was included in the matings in the manner previously described (Materials and Methods, Section K) nor was the frequency of transfer altered when either the donor or the recipient strain carried the recl or rec2 mutations (Table 13). Also, transfer was possible to the poorly-transformable H. influenzae strain 9(N3) at frequencies comparable to those seen with the wild-type Rd strain as a recipient (Table 13).

Table 13. Frequency of Ampicillin Resistance Transfer in Conjugative Matings[a]

Donor Strain	Plasmid Complement (Mdal)	Recipient Strain					
		H.ducreyi HD35000	H.influenzae Rd	H.influenzae 9(N3)	H.influenzae rec1	H.influenzae rec2	E.coli C600
HD147	23.5,7.0,4.9	1.5×10^{-4}	1.6×10^{-5}	2.0×10^{-6}	1.7×10^{-6}	9.1×10^{-7}	5.3×10^{-5}
Rd(pHD147, pHD147-7)	23.5,7.0	4.0×10^{-5}	1.2×10^{-4}	8.5×10^{-5}	6.0×10^{-4}	4.0×10^{-5}	4.1×10^{-5}
rec1(pHD147, pHD147-7)	23.5, 7.0	6.3×10^{-5}	3.9×10^{-5}	1.4×10^{-4}	-[b]	-	-
rec2(pHD147, pHD147-7)	23.5,7.0	4.5×10^{-5}	1.1×10^{-4}	2.2×10^{-4}	-	-	-
HI1008 (pHD147-7)	7.0	$< 10^{-9}$	$< 10^{-9}$	$< 10^{-9}$	$< 10^{-9}$	$< 10^{-9}$	$< 10^{-9}$
9(N3) (pHD147::TnA)	27.0	3.4×10^{-5}	1.1×10^{-5}	-	1.3×10^{-5}	1.8×10^{-5}	-

[a] Transfer frequency expressed as the number of transconjugants formed per output recipient. Results are the average of at least two trials.

[b] Not Determined.

C) Conjugative transfer of sulfonamide resistance (Su^r)

Since the original H. ducreyi isolate, HD147, was also resistant to sulfonamide, an attempt was made to transfer Su^r in standard membrane filter matings with E. coli C600 and H. influenzae recipients. Sulfonamide-resistant E. coli transconjugants could be isolated at frequencies of 10^{-3} to 10^{-4} and when screened by agarose gel electrophoresis, were found to contain only a single plasmid of 4.9 Mdal. Sulfonamide-resistant H. influenzae transconjugants arose at slightly higher frequencies of 10^{-2} to 10^{-3} but these were found to contain two plasmids of 23.5 and 4.9 Mdal. Retransfer of Su^r could not be detected from E. coli transconjugants but was observed at frequencies of 10^{-3} when Su^r H. influenzae transconjugants were used as donors to other H. influenzae recipients.

As a control, Su-resistance could not be transferred by membrane filter matings from HD9468 to either E. coli or H. influenzae recipients. This H. ducreyi strain had the same antibiotic resistance pattern as HD147 but contained only two plasmids of 7.0 and 4.9 Mdal.

D) Mobilization of other non-conjugants plasmids by pHD147

Since it was apparent that the 23.5 Mdal pHD147 plasmid could mobilize at least two different non-conjugative plasmids - a 7.0 Mdal Ap^r plasmid and a 4.9 Mdal Su^r plasmid - for conjugative transfer, it was questionned whether other small non-conjugative R-plasmids could be mobilized as well.

Four small R-plasmids, ranging in size from 3.2 to 5.7 Mdal and originally isolated from H. ducreyi, H. parainfluenzae, or N. gonorrhoeae, were tested. These plasmids were introduced individually into H. influenzae Rd by transformation and their ability to be mobilized by pHD147 was tested by a three-way mating procedure (Anderson, 1965b). This procedure involved essentially a standard membrane filter mating with three participants; the initial donor was strain HI1008 (pHD147) containing just the 23.5 Mdal mobilizing plasmid, the intermediate recipient was H. influenzae Rd containing one of the small plasmids to be tested, and the final recipient was E. coli C600. Selection was for Ap-resistant E. coli transconjugants. The results are shown in Table 14. All of the small Ap^r plasmids from Haemophilus and Neisseria were mobilizable by pHD147. Similar results are seen when H. influenzae is used as a final recipient (not shown).

E) Restriction endonuclease analysis of pHD147.

The mobilizing plasmid pHD147 was purified from HI1008 (pHD147) and incubated separately with each of the restriction endonucleases Pst I, Eco Rl, Hinc II, Hind III, Bam Hl, Sma I, and Hae II. Agarose gel electrophoresis of the reaction mixture revealed that this plasmid DNA was not cleaved by any of the enzymes used (see for example, Figure 14B, Lane A). In contrast, the 32 Mdal conjugative plasmid, pHD9265 introduced into, and purified from, the

Table 14. Mobilization of R-plasmids by pHD147 [a]

Plasmid	Size (Mdal)	Phenotype	Original Source	Mobilized by pHD147
pHD147-7	7.0	Ap^r	H. ducreyi	+
pJB1	5.7	Ap^r	H. ducreyi	+
RSF0885	4.1	Ap^r	H. parainfluenzae	+
p22209	4.4	Ap^r	N. gonorrhoeae	+
p88557	3.2	Ap^r	N. gonorrhoeae	+
pHD147-4	4.9	Su^r	H. ducreyi	+

[a] Three-way matings using E. coli C600 as final recipient, H. influenzae HI1008(pHD147), and H. influenzae Rd containing the indicated plasmid as intermediate recipient.

same background strain could be cleaved to yield the expected fragmentation pattern using these enzymes under the same reaction conditions. To ensure that bound protein was not interfering with the endonuclease reaction, pHD147 DNA was treated with Proteinase K prior to incubation with the restriction enzymes. This however, still failed to yield a fragmentation pattern.

F) Isolation of pHD147::TnA

From one mating of HD147 and H. influenzae strain 9(N3), a β-lactamase producing transconjugant was isolated which was found to carry only a single plasmid of 27 Mdal (Figure 14A, Lane C). This plasmid was transferable to Haemophilus recipients upon selection for ampicillin resistance. The frequency of this transfer was similar to that seen for the usual mobilization of the 7.0 Mdal plasmid by pHD147 (Table 13). For this reason it was suspected that the ampicillin resistance transposon TnA may have transposed from the 7.0 Mdal plasmid to the cryptic mobilizing plasmid pHD147 to yield a new phenotypically marked conjugative plasmid, pHD147::TnA. Because TnA was known to possess three internal cleavage sites for the Pst I endonuclease (Brunton et al., 1980), the plasmid pHD147::TnA was purified, digested with Pst I, and the fragments separated on an agarose gel (Figure 14B). It was seen that while digestion of pHD147 produced no cleavage fragments (Lane A), digestion of pHD147::TnA yielded two small fragments of 2.0 and 0.38 Mdal plus the

remainder of the 23.5 Mdal plasmid (Lane B). These two small fragments corresponded to Pst I-cleaved fragments from the 5.7 Mdal Ap^r plasmid pJB1 (Lane C) and the 7.0 Mdal Ap^r plasmid pHD147-7 (Lane D). Both these plasmids were known to carry the complete TnA gene sequence and hence these small fragments probably represented cleavage within the transposon TnA.

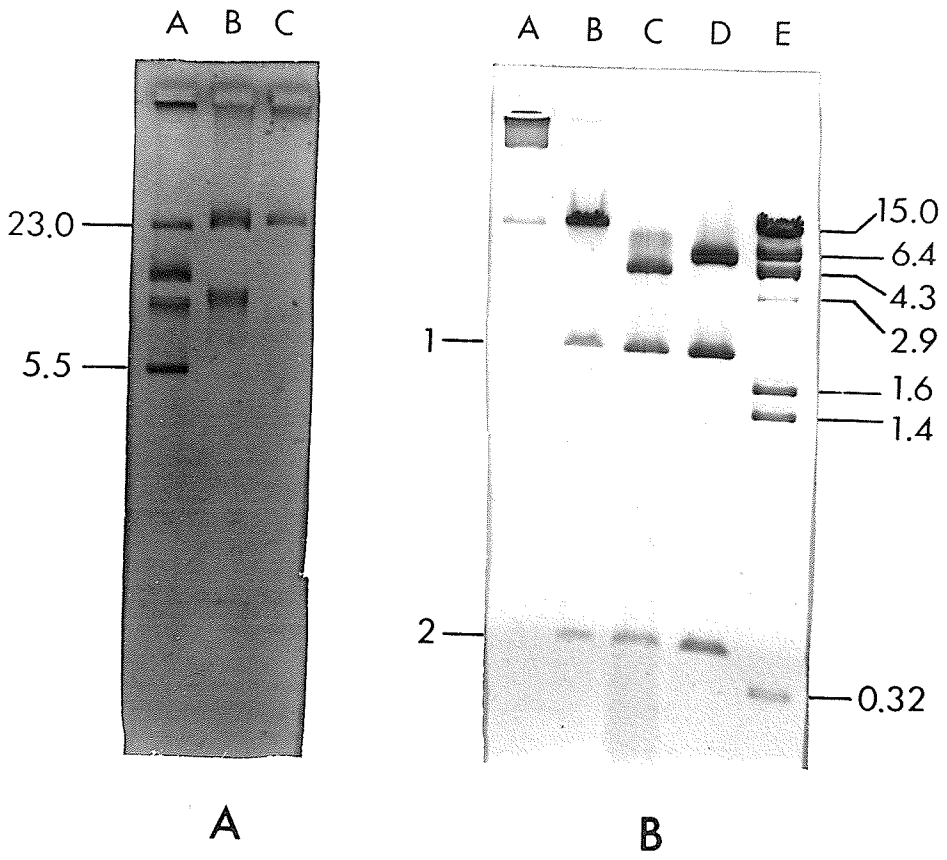


Figure 14A. Agarose gel electrophoresis of purified plasmid DNA (0.7% gel). Lane A, molecular weight standards; B, pHD147; C, pHD147::TnA.

14B. Agarose gel electrophoresis of PstI-digested plasmid DNA (1.2% gel). Lane A, pHD147. The lower band in this lane is the open circular form of the plasmid; B, pHD147::TnA; C, pJB1; D, pHD147-7, 7.0 Mdal plasmid of HD147; E, lambda DNA digested with Hind III as molecular weight markers. Numbers 1 and 2 denote, respectively, the 2.0 Mdal and 0.38 Mdal internal Pst I-cleaved fragments of TnA.

DISCUSSION

A) Introduction

This study was designed to address the following questions:

1) How did plasmid-mediated antibiotic resistance originate in the genus Haemophilus and why are such plasmids present in some Haemophilus species but not others? What mechanism can account for its sudden emergence and what can account for the presence of both large and small R-plasmids?

2) Are the large conjugative plasmids isolated from different species of Haemophilus but specifying the same resistance pattern structurally related to one another or are they totally different, and is there a difference based on the geographic origin of the host species?

3) Since the large R-plasmids of Haemophilus are conjugative, are there any limits within this genus or beyond it to which such self-transfer can be effected?

4) Is conjugation itself an efficient means for the spread of an antibiotic-resistance phenotype between the various Haemophilus species?

5) Finally, what is the mechanism for the dissemination of small, non-conjugative Haemophilus R-plasmids within a cell population, not just intraspecifically but amongst the various Haemophilus species as well?

B) Characterization of conjugative plasmids

Four plasmids which encoded either tetracycline resis-

tance (Tc^r) or linked tetracycline-chloramphenicol resistance ($Tc\text{-}Cm^r$) were initially studied. All were from 32 to 38 Mdal in size, were conjugally self-transferable, and were isolated from three species of Haemophilus of diverse geographic origins. The plasmid pHI2265 was identified in a Tc-Cm resistant clinical isolate of H. influenzae from Winnipeg, Manitoba while pHP1007 was a Tc-resistance plasmid identified in an H. parainfluenzae clinical isolate also from Winnipeg. The remaining two plasmids, pHD9265 (Tc^r) and pV1157 ($Tc\text{-}Cm^r$) were isolated from H. ducreyi strains from Nairobi, Kenya and Seattle, Washington, respectively.

While three of these plasmids (pHI2265, pHP1007, pHD9265) could be clearly visualized by agarose gel electrophoretic analysis of the original host strains, the plasmid pV1157 was unique in that it could not be detected in the covalently closed circular (CCC) form when the H. ducreyi V1157 host strain was screened by this method (see Figure 1, Lane B). This plasmid could only be visualized as a CCC molecule in the transconjugant arising from a conjugative mating between V1157 and a suitable recipient. This indicates that within the original V1157 host strain, this plasmid either existed in a form undetectable by agarose gel electrophoresis, possibly as a protein-bound relaxation complex (Clewell and Helinski, 1969; Kuperaztoch-Portnoy et al., 1974), or in fact, was fully integrated into the H. ducreyi chromosome as suggested by Stuy (1979, 1980) for several antibiotic-resistant "plasmid-free" H. influenzae isolates. However, unlike

Roberts and Smith (1980) we did not attempt other screening methods to try to visualize this plasmid within V1157. In any event, the intracellular state of pV1157 apparently did not affect its conjugative ability and reversion to its "non-CCC" or non-visualizable form was never observed in any of the Haemophilus species to which it was transferred. Apparently therefore, the host species itself may have no bearing on whether pV1157 is in the CCC or the "non-CCC" form and the factors controlling its intracellular state in H. ducreyi V1157 are presently unclear.

The four plasmids pHI2265, pHP1007, pHD9265 and pV1157 were further characterized at the DNA base sequence level through the use of sequence-specific restriction endonucleases followed by agarose gel electrophoresis. This method is based on a comparison of the number and size of fragments produced by cleavage of different circular plasmid DNA molecules with a specific endonuclease. It can be a powerful tool in determining if two plasmids are identical at the base sequence level or assessing the degree of any relatedness (Thompson et al., 1974). While perhaps not as accurate in estimating overall relatedness as some other methods such as DNA-DNA hybridization or heteroduplex mapping, the endonuclease method has the advantage of being rapid, easy to apply, relatively inexpensive, and requiring only minute amounts of purified DNA. Although this method is subject to some minor limitations (Causey and Brown, 1978), it has been successfully used in the comparative analysis of both large and small plasmids from a number of bacterial species (Foster and Foster, 1976; Grinter and Barth, 1976).

The fragmentation patterns generated from these four plasmids show considerable similarity when compared to each other but virtually none when compared to an R-plasmid (RP4) of similar size from Pseudomonas (see for example, Figures 3 and 5). From a total of 16 to 18 fragments produced from each plasmid by digestion with Hae II for instance, 8 fragments for a combined molecular weight of 17.7 Mdal, were common to all four plasmids. If these plasmids were assumed to be completely unrelated, then the maximum amount of common DNA should total only about 3 Mdal, which is the size of the tetracycline-resistance transposon common to all four plasmids. A similar situation was seen after digestion with Pst I and to a slightly lesser extent after Hinc II digestion. Digestion with Sma I apparently produces only one common fragment of 3.4 Mdal but this is presumably because the plasmids have only 3 (pHP1007) or 4 (pH12265, pHD9265, pV1157) recognition sites for this endonuclease compared with up to 21 sites for some of the other enzymes. The largest Sma I fragment of about 15 Mdal is probably also highly similar in these four plasmids and this would again yield a combined molecular weight of 17-18 Mdal as seen with the other enzyme digests.

It must be noted though, that while fragments of the same size cleaved from different plasmids are assumed to be highly related to each other, this can only be proven by the actual DNA sequencing of the restriction fragments. This was not attempted here. Also, the combined molecular weight of all fragments cleaved from any one plasmid should theoretically be equal to the size of that plasmid as determined by other means. In this case however, the size of the complete

plasmids determined in this way was, on average, about 10-12% higher than that determined previously by gel electrophoresis of purified plasmid DNA. This difference can be attributed to the small degree of error inherent in estimating molecular weight from a standard curve, which is compounded when the sizes of many small fragments must be determined.

Using restriction endonuclease analysis, it was difficult to assess whether the two H. ducreyi plasmids (pV1157, pHD9265) were more closely related to each other than to the plasmids from the other two Haemophilus species. Both the H. ducreyi plasmids showed an extra fragment of 0.95 Mdal upon Sma I digestion which was not present in the other two plasmids, but digestion with the other enzymes did not serve to highlight any greater degree of relatedness between any two plasmids compared to the rest.

The fact that these four plasmids are quite similar, although not identical, at the DNA base sequence level is all the more remarkable when one considers not only their diverse geographic origins but also the fact that they were isolated from three different Haemophilus species. Previous work, also using endonuclease cleavage patterns, has shown that 30 Mdal Ap^r plasmids isolated from only one species, H. influenzae, were very similar (Harkness and Murray, 1978). Albritton and Slaney (1980) also observed significant relatedness between several large H. influenzae R-plasmids carrying different drug-resistance determinants, which was consistent with the previously reported homology between large Haemophilus plasmids found by hybridization studies

(Jahn et al., 1979). However, no reports have thus far appeared concerning a comparison of large R-plasmids from as many as three different Haemophilus species. The apparent relatedness observed here implies that these four plasmids at one point shared a common ancestor, since it is unlikely that the similarity in endonuclease cleavage patterns could have arisen independently in all four plasmids. Since previous studies have shown that large Haemophilus plasmids have a DNA base composition similar to that of the Haemophilus chromosome (De Graaff et al., 1976), it is possible that such plasmids are indigenous to this genus rather than introduced from outside. Presumably, a cryptic "core" plasmid of approximately 25 Mdal and having conjugative abilities once existed in a species of Haemophilus and served as the ancestor of the conjugative R-plasmids seen today. Transposable drug-resistance elements, introduced from an external source either through illigitimate matings with species of other genera or by some other as yet unknown means, could have inserted in various combinations into this "core" plasmid. These newly-formed R-plasmids could have been disseminated to other species of Haemophilus and then distributed to various parts of the world. The slight structural variability seen among these plasmids today could have arisen in a number of ways: through insertion of transposons at different points on the plasmid genome; through deletions or inversions of DNA sequences occasionally caused by the excision of transposons (Calos and Miller, 1980); through the occasional

cell to cell transfer by means such as transformation which is known to alter plasmid structure (Sox et al., 1979); or through various other factors which are known to affect plasmid evolution (Cohen et al., 1978).

This scenario for the evolution of R-factors in Haemophilus depends on the ability of both the core plasmid and its offspring R-plasmids to transfer from cell to cell, not only within one species but also between species, since presumably a different core plasmid did not exist in each species of Haemophilus in which R-plasmids are seen today. Equally important is the requirement that such intercell transferability be relatively efficient in order to account for the rapid spread of plasmid-mediated resistance observed in this genus.

C) Interspecific conjugal transfer of R-plasmids

To get an indication of the interspecific host range of these four R-plasmids, ten species of Haemophilus were tested for their ability to act as recipients in conjugative matings with each of the three Haemophilus species carrying one of the plasmids. Transfer of all four plasmids could only be detected between species of H. influenzae, H. parainfluenzae, and H. ducreyi as well as to two strains which were sent to us as H. haemoglobinophilus but which were subsequently found not to give the biochemical reaction pattern expected of this species. Unfortunately, these latter two strains could not be speciated based on the tests available to us and so there is some doubt as to whether

plasmid transfer is actually possible to H. haemoglobinophilus, especially since the ATCC reference strain of H. haemoglobinophilus was found to be unable to accept large conjugative plasmids.

In the three species where plasmid transfer was unequivocally demonstrated to occur, all transconjugants from such matings were found to carry plasmids in the CCC form, and all were readily able to re-transfer the plasmids to any of these three species. Thus, continual transfer from one species to another had no apparent effect on the expression of plasmid-borne genes since conjugative ability was not lost and the resistance determinants were fully expressed in the new host - in other words, interspecific transfer of these plasmids did not result in any loss of plasmid function, an important requirement if the widespread distribution of R-plasmids is to be both effective and provide the cell population with a selective advantage.

In addition, stability studies indicated that the plasmids were readily maintained once introduced into a different host species and after removal of selective pressures. There was no real decline in the percentage of plasmid-carrying (R^+) cells after at least 10 days of unselected passage and even after 22 days a considerable number of cells in the population still harbored plasmids. This implies that continual selective pressure such as when antibiotics are present is not absolutely necessary to maintain R-plasmids in Haemophilus species. It is probable that once

introduced, R-plasmids are never completely lost from a cell population even in the absence of selective pressure; there will always remain a small number of cells carrying plasmids and these plasmids will still be transferable to sensitive strains. Also, there is no evidence that plasmids were any more stable in their species of origin than in hosts of a different Haemophilus species. This is to be expected if the premise of a single "core" plasmid being disseminated to other Haemophilus species is correct.

The results presented thus far imply that the host range of conjugative plasmids in Haemophilus is limited to only three species, namely H. influenzae, H. parainfluenzae, and H. ducreyi. However, this conclusion is subject to several qualifications. Firstly, it may be necessary to test recent clinical isolates as potential recipients rather than relying mostly on standard reference strains as was the case here. Among the species unable to act as plasmid recipients, only two clinical isolates were tested; both were H. pleuropneumoniae species but neither was able to accept conjugative plasmids. Secondly, in most cases only one strain of a particular species was tested and it may be of some benefit to include a number of strains before concluding that a species is unable to accept plasmids. Finally, not all of the known species of Haemophilus were tested due to the difficulties encountered in introducing appropriate chromosomal markers for counter-selection purposes.

Assuming that the host range is truly limited to these three Haemophilus species, it can be speculated that large

R-plasmids, or at least the R-plasmids studied here, will not appear in other species of Haemophilus in the near future. Furthermore, if the ancestral "core" plasmid still exists in its cryptic form in Haemophilus, it will probably be found in either H. influenzae, H. parainfluenzae, or H. ducreyi and not in some other species from which conjugal transfer would be difficult or impossible.

These results give no indication as to why the host range of these large Haemophilus plasmids is relatively restricted although this is by no means unusual when compared with certain enteric plasmids. A number of factors may be involved: cell surface incompatibilities such that stable mating pairs cannot form, degradation of incoming plasmids by the host restriction-modification system, lack of gene expression or plasmid replication within new hosts. It is always possible of course, that plasmid transfer is indeed occurring but at frequencies too low to be detected. This however, is unlikely since only one transconjugant need be formed from 10^9 recipient cells in order for transfer to be detected; at frequencies this low the value of possessing a conjugal transfer system is questionable.

D) Characteristics and efficiency of conjugation in Haemophilus

Conjugative gene transfer has never been thoroughly examined in Haemophilus, neither mechanistically, genetically, nor in terms of its ability to carry out the rapid, efficient, spread of antibiotic-resistance determinants within this

genus. While a detailed examination of conjugation in terms of the mechanics of the process or the genetics of the plasmid-borne transfer operon is beyond the scope of this work, several features have been looked at. To begin with, conjugation in Haemophilus does not require the participation of the system for generalized recombination (the rec system) in either the donor or the recipient, as shown by the fact that transfer is as efficient into or out of recl or rec2 host cells as it is in wild-type cells (see Figures 10 and 11). This is a distinguishing characteristic of conjugation in general and has been observed on all other conjugal transfer systems to date (Curtiss, 1969; Hayes, 1970); transformation on the other hand, requires a functioning host recombination system. In addition, the rec-independence of plasmid transfer in Haemophilus implies that there is probably no active recombination taking place with the host cell chromosome during transfer.

Secondly, it is known that, in other genera at least, the overall frequency of plasmid transfer can be related to the ratio of the number of donor cells to the number of recipient cells present in the final mating mixture (Cullum et al., 1978). Figures 8 and 9 indicate that transfer frequency, as measured in terms of the number of transconjugants produced per individual recipient cell, is highest when there are ten or more donor cells for every one recipient; frequency can decline by as much as 2 logs when the number of donor cells is lower than the number of recipients. This

suggests that at donor/recipient ratios beyond 1:1, a point is reached where every recipient able to receive a plasmid comes into contact with one or more donors capable of donating a plasmid. Thus, the frequency of transfer becomes a function of the ability of the recipient cell population to receive and maintain plasmids; the transfer frequency does not attain 1 - that is, when every recipient in the mating mixture receives a plasmid - probably because a small percentage of cells in any given recipient population will, for one reason or another, be unable to function as viable recipients. This effect of cell ratios on transfer frequency was evident for both intraspecific crosses as well as interspecific matings between H. influenzae and H. parainfluenzae, indicating that it is a general characteristic of conjugation in Haemophilus.

Finally, studies were performed to determine the relative efficiency of intraspecific vs. interspecific conjugal plasmid transfer in Haemophilus - again as measured in terms of the number of transconjugants formed per recipient - and how it varied for each of the three plasmids pHI2265, pHD9265, and pHP1007. A high frequency of plasmid transfer between a given pair of cells is taken to mean that conjugation is an efficient process between these two particular species. The data, as presented in Figure 7, suggests that the relative efficiency of conjugation varies greatly as a function of the particular Haemophilus species used as donors or recipients. The most efficient transfer of plasmids - in the

range of 10^{-1} to 10^{-2} - occurs between two strains which are of the same species. Frequency of transfer can decline by more than 3 logs in matings where strains were of two different species. This pattern of intraspecific matings being more efficient than interspecific matings held true for both H. influenzae and H. parainfluenzae species and was also seen when one of the presumptive H. haemoglobinophilus strains was used as plasmid donor or recipient (Table 10). With this particular species however, the intraspecific transfer frequencies were somewhat lower, on the order of 10^{-3}, than usually seen with the H. influenzae or H. parainfluenzae species. In contrast, H. ducreyi was somewhat anomalous in that when used as a recipient in interspecific matings, transfer frequencies of 10^{-2}-10^{-3} could be obtained, which is close to that normally seen for the intraspecific matings of the other species. When used as a plasmid donor to other species however, the frequency of plasmid transfer was quite low, dropping to about 10^{-7} compared to the usual 10^{-4}-10^{-5} seen for the interspecific matings of other species. Why H. ducreyi is able to act as a highly efficient plasmid recipient yet a poor donor of plasmids is currently unknown. It is possible that the processing of plasmid DNA as a necessary prelude to conjugative transfer is somehow altered in H. ducreyi hosts and this is manifested as a reduced capacity to donate plasmids.

The three conjugative plasmids studied here all behaved similarly in terms of transfer frequency; that is, for any given mating pair, there was little difference in transfer frequency if the donor carried pHI2265, pHD9265, or pHP1007 (see Figure 7), and therefore the differences in intra- vs.

interspecific transfer frequency observed here are a function of the particular host species being mated and not of the individual plasmids. This is further evidence that the three plasmids themselves are probably very closely related, at least as far as the organization and operation of their transfer operons is concerned and supports the structural similarities previously deduced from the restriction endonuclease analysis. This is again indicative of a single conjugative "core" plasmid being the ancestor of the present-day R-plasmids in Haemophilus.

Generally, these data suggest that plasmid-mediated conjugation may be a highly efficient form of gene transfer in this genus. Compared to previous reports from the literature, the intra- and interspecific transfer frequencies observed here are among the highest for Haemophilus matings. Transfer of large plasmids by transformation does not approach the frequency seen for conjugative transfer; Albritton et al. (1981) for example,were able to transform H. influenzae at a maximum frequency of 10^{-4} with pHI2265 plasmid DNA, which is considerably less than the intraspecific conjugal transfer frequency of almost 10^{-1} seen here. Even interspecific transfer frequencies of between 10^{-4} and 10^{-6} can be considered to be quite efficient by the usual transformation standards. Unfortunately, there is no way of knowing if the in vitro transfer frequencies, performed here under optimal conditions, are in any way indicative of the actual frequencies seen in vivo when the cell population may be under stress from host defenses, poor growth conditions, or other

factors. Burman (1977) and Cullum et al. (1978) have pointed out many of the factors which can affect the overall frequency of conjugal transfer among the enteric bacteria. No attempt was made to study all these parameters here, although one of these - the final donor/recipient cell ratio - was, as mentioned before, seen to have a dramatic effect on the final plasmid transfer frequency. It is probable therefore, that optimal conditions for conjugation in vivo are not often realized and that maximal transfer frequency is attainable only under controlled conditions. However, even if the efficiency of transfer was reduced by as much as 4 or 5 logs in intraspecific matings carried out under "natural" conditions, the frequency would still be equivalent to that seen for transformation. Thus conjugation is probably still a relatively efficient process in vivo and therefore an important one for the dissemination of drug-resistance genes within Haemophilus. In fact, the very existence of plasmids with conjugative abilities points to the importance of conjugation as a mode of gene transfer. Transfer-related genes can comprise from one-third to one-half of the total genetic capacity of a plasmid; it would be difficult to justify this added genetic burden if the presence of a transfer operon did not confer some important selective advantage, if not directly to the cell then to the population as a whole.

E) Intergeneric plasmid transfer

It has previously been shown that small Haemophilus Ap^r

plasmids, if introduced by transformation into E. coli, could be stably maintained in the CCC form (Brunton et al., 1979). If large Haemophilus R-plasmids could be conjugatively transferred to E. coli, maintained here, and be re-transferred back to Haemophilus, it would constitute evidence in favour of the theory that such plasmids had their origins in other genera, possibly Escherichia. This was found not to be the case however. Matings between E. coli recipients and Haemophilus donors carrying one of two large R-plasmids (pHI2265 or pHPl007) produced E. coli transconjugants resistant to the selected drug-resistance markers encoded by the plasmids, but in no case was a plasmid in the CCC form observed in these transconjugants. Furthermore, the resistance markers could not be re-transferred from the E. coli transconjugants, again indicating that either a complete plasmid was not present, or existed in a form other than a covalently closed circle and had been rendered non-conjugative. These results suggest that transfer of these plasmids from Haemophilus to E. coli was probably occurring but that the plasmids were unstable in E. coli hosts - possibly they were unable to replicate and subsequently lost or were in some way recognized as foreign and degraded. The E. coli C600 recipient used here lacked DNA restriction endonuclease activity so the former possibility may be more reasonable. Presumably however, the Haemophilus plasmids remained in E. coli host cells long enough to permit the drug-resistance transposon to excise from the plasmid and integrate into the E. coli

chromosome. This would account for the drug-resistance phenotype of the E. coli transconjugants as well as for their inability to re-transfer resistance since the plasmid itself is now lost. The low frequency of transconjugant formation supports this contention since transposition of the drug-resistance genes would probably not occur in every case of plasmid transfer, so in actuality, transposition rather than plasmid transfer is being measured here. One way to verify this theory would be to examine the chromosome of the transconjugant for the presence of transposons by the standard nucleic acid hybridization techniques. It is unknown why small Haemophilus plasmids are able to replicate and be maintained in E. coli while large R-plasmids are not; this is possibly a function of a basic difference in the replicative machinery of these two types of plasmids (ie. relaxed vs. stringent replication - Rowbury, 1977; Novick, 1980).

Likewise, conjugative plasmids present in E. coli such as the broad host range plasmid RP4 were not transferable to Haemophilus recipients as a complete plasmid, and neither could Haemophilus transconjugants be isolated which carried just the drug-resistance transposons known to be present on these plasmids. This indicates that a complete block exists in this direction of plasmid transfer, and is not simply a question of E. coli plasmids being unable to be maintained by Haemophilus hosts. The nature of this barrier is unknown; it may be due to the fact that pili are necessary to mediate cell to cell contacts before RP4 can conjugally transfer and

it is possible that the Haemophilus cell surface lacks the proper receptor sites for these pili, hence stable contacts cannot form. In any event, the observation that effective barriers exist which apparently prevent conjugal gene transfer between these two genera is further evidence that large Haemophilus R-plasmids did not originate in E. coli.

The interrelationship between Haemophilus and Neisseria is somewhat different however. Here also, we were unable to transfer large Haemophilus plasmids such as pHI2265 or pHP1007 to sensitive N. gonorrhoeae isolates, suggesting again the presence of effective barriers to prevent conjugal gene transfer in this direction. Ampicillin-resistant isolates of N. gonorrhoeae frequently have an efficient gene transfer system of their own however, involving a small 4.4 Mdal Ap^r plasmid and a larger 24.5 Mdal cryptic mobilizing plasmid (Roberts et al., 1978). Because the small Ap^r plasmids found in H. ducreyi share considerable sequence homology with the gonococcal Ap^r plasmids (Brunton et al., 1980), it has often been suggested that these two groups of plasmids had a common origin. The experiments performed here show that the 4.4 Mdal Ap^r plasmid from N. gonorrhoeae is transferable by conjugation to H. influenzae, H. parainfluenzae, or H. ducreyi recipients, although at relatively low frequencies. The Ap^r plasmid is stably maintained in the CCC form in Haemophilus transconjugants but the 24.5 Mdal mobilizing plasmid could not be detected; hence re-transfer of the Ap^r plasmid to other Haemophilus recipients or back to

Neisseria was not possible.

The value of this particular route of plasmid transfer is questionable. Although it demonstrates that Neisseria could have at one point donated plasmids to Haemophilus species, the transfer has in effect reached a dead-end since there can be no further dissemination of the small plasmids within Haemophilus and therefore no means to account for the widespread distribution of Ap^r plasmids in this genus and in H. ducreyi in particular. The remainder of this work describes one means by which small, non-conjugative Ap^r plasmids in H. ducreyi and possibly small plasmids conferring other phenotypes in Haemophilus in general could have been disseminated to the point seen today.

F) Mobilization of small plasmids in Haemophilus

Plasmid-mediated ampicillin resistance has been observed in many clinical isolates of H. ducreyi since it was first reported by Brunton et al. (1979), but the mechanisms behind the apparent spread of this resistance trait have remained unclear. The situation is unusual because large conjugative plasmids encoding Ap^r have not been identified in H. ducreyi; this resistance trait is only mediated by non-conjugative plasmids of 5.7 or 7.0 Mdal (Brunton et al., 1979; W. Albritton, unpublished data). Furthermore, neither transformation nor transduction have been shown to operate within this species, precluding these forms of genetic exchange as mechanisms in the dissemination of Ap-resistance.

We have demonstrated, in an African isolate of H.

ducreyi, the existence of a plasmid mobilization system consisting of a 23.5 Mdal cryptic mobilizing plasmid and two non-conjugative plasmids of 7.0 and 4.9 Mdal encoding ampicillin and sulfonamide resistance, respectively. Unlike other conjugative Haemophilus plasmids such as pV1157 or pHD9265, the H. ducreyi plasmid pHD147 has the ability to mobilize for conjugal transfer either of these small R-plasmids to sensitive H. ducreyi, H. influenzae or E. coli recipients. Haemophilus transconjugants acquiring Ap- or Su-resistance were found to have received both the 23.5 Mdal pHD147 plasmid and either the 7.0 or 4.9 Mdal R-plasmids. Both large and small plasmids were stably maintained in these transconjugants and as a result, either Ap- or Su-resistance was subsequently able to be re-transferred to other Haemophilus recipients. H. parainfluenzae was not tested as a possible recipient for the Ap^r or Su^r plasmids although it could probably function in this capacity, based on its ability to readily accept large conjugative R-plasmids. Ampicillin or sulfonamide resistant E. coli transconjugants, on the other hand, were not able to re-transfer these resistance markers; this was not unexpected since the 23.5 Mdal mobilizing plasmid was not stably maintained in these transconjugants and could not be detected by agarose gel electrophoresis.

The plasmid pHD147, in addition to being able to mobilize the two small Ap^r and Su^r plasmids originally found co-resident in H. ducreyi strain HD147, is also able to mobilize

other non-conjugative plasmids, including Ap^r plasmids originally isolated in H. parainfluenzae and N. gonorrhoeae, as well as a second Ap^r plasmid of 5.7 Mdal from H. ducreyi. Of these plasmids (see Table 14) neither pJB1, RSF0885, nor the 3.2 Mdal gonococcal plasmid have been reported to be mobilizable by other R-plasmids before. That all these small Ap^r plasmids are mobilizable by pHD147 should not be surprising if it is remembered that all are highly related to one another at the DNA base sequence level (Roberts et al., 1977; Brunton et al., 1980). Mobilization, at least in certain Enterobacteriaceae systems, requires not only the presence of a large mobilizing plasmid but also the products of several genes located on the small non-conjugative plasmid (Clark and Warren, 1979); hence it is reasonable to expect that these genes would be present on all these related Ap^r plasmids. What is perhaps more surprising is that the small Su^r plasmid (pHD147-4) of H. ducreyi is also readily mobilized by pHD147, since this plasmid is highly related to enteric plasmids and shows no sequence homology with small H. ducreyi Ap^r plasmids such as pJB1 (W. Albritton, J. Brunton, L. Slaney, I. MacLean, manuscript in preparation). This would imply that either no specific gene product is required from the non-conjugative plasmid in order for mobilization to occur or in fact that this product can vary in composition such that the genes encoding it on the Su^r plasmid are significantly different from similar genes present on the various Ap^r plasmids.

The mechanism of the actual mobilization process as it occurs in H. ducreyi is currently unclear. There is no direct evidence for the formation of stable co-integrate structures between pHD147 and the non-conjugative plasmids; plasmids greater than 30 Mdal - as would be expected for co-integrates - were never observed in any Haemophilus transconjugants. Also, the presence of the recl or rec2 mutation in either the donor or the recipient had no effect on the frequency of mobilization. In this regard, the H. ducreyi mobilization system resembles the "trans" acting Class I system of Kilbane and Malamy (1980), in which only a diffusible product from the mobilizing plasmid is required to effect transfer. As further evidence of this, one Ap^r transconjugant, HI1008 (pHD147-7), was found to carry just the 7.0 Mdal plasmid, implying that co-transfer of the 23.5 Mdal, pHD147 plasmid was not required. However, it is unknown how frequently transconjugants carrying just the single 7.0 Mdal plasmid arise; in repeated trials we succeeded in isolating only one such clone and hence this may be a relatively rare event. The possibility exists therefore, that the lack of a 23.5 Mdal plasmid in this one clone is simply the result of a post-conjugative loss of this plasmid and indeed, co-transfer of the mobilizing plasmid in the form of some type of co-integrate is always required. Were this the case, then mobilization of H. ducreyi plasmids would be similar to that described in Class III or IV systems by Kilbane and Malamy, wherein mobilization can occur via the rec-

independent formation of transitory co-integrates between the two plasmids followed by the dissociation of the co-integrate structure after transfer. In the Class IV system such co-integrates would be the result of transposon-mediated fusion and a transposon such as the TnA on the 7.0 Mdal plasmid would presumably be all that was required. Unfortunately, there is insufficient data to be able to distinguish between these three possibilities.

That the ampicillin-resistance transposon TnA is indeed capable of transposition is shown by the fact that one Ap^r transconjugant, 9(N3)(pHD147::TnA), was found to carry only a single plasmid molecule of about 27 Mdal. Restriction endonuclease analysis suggests that the plasmid pHD147::TnA now contains the entire 3.2 Mdal TnA transposon. The cryptic 23.5 Mdal mobilizing plasmid most likely acquired this sequence through a transposition event from the 7.0 Mdal Ap^r plasmid during transfer. The new pHD147::TnA plasmid retains the conjugative ability of the parent pHD147, indicating that transposition of TnA was not onto a region of the plasmid required for self-transferability. It is not known however, if the mobilizing capabilities of this plasmid have been affected by insertion of TnA. Nevertheless, pHD147::TnA, which carries a readily identifiable and selectable phenotypic marker should prove useful in any further characterization of the H. ducreyi mobilization system.

It should be mentioned that a mobilization system in any species of Haemophilus has not been previously reported

although various workers have observed the apparent transfer of small R-plasmids by conjugative means (Saunders and Sykes, 1977; Sparling et al., 1978). However, since no large conjugative plasmids could be detected in these strains and since transfer ability was rapidly lost upon storage, the suggestion was that a large, highly unstable sex factor was responsible for the conjugal transfer of the small R-plasmids. This was never proven however.

Finally, a plasmid mobilization system of this type in H. ducreyi is remarkably similar to the mobilization of the 4.4 Mdal Ap^r plasmid in N. gonorrhoeae. The mobilizing plasmid in Neisseria has a molecular size of 24.5 Mdal, compared to 23.5 Mdal for pHD147, is part of a Class I mobilization system according to the Kilbane and Malamy scheme (Sox et al., 1978), and can mobilize an Ap^r plasmid to other gonococci, Neisseria spp., E. coli (Eisenstein et al, 1977), as well as Haemophilus spp. The degree of structural relatedness between the 24.5 Mdal gonococcal plasmid and pHD147 is presently unknown. The H. ducreyi plasmid is unusual in that it appears to lack recognition sites for at least seven restriction endonucleases, and consequently is not cleaved by these enzymes. Whether this is an artifact of some type or is indeed genuine is not clear at this point. It should be noted that the plasmid pHD147::TnA, carrying the Ap^r transposon, can be cleaved by these enzymes but only sequences within the actual transposon are recognized; the bulk of the plasmid molecule remains

uncleaved as does the parent pHD147 plasmid (see Figure 14B). Only one enzyme, Alu I, has thus far been found to cleave pHD147, but the fragments produced are of such a low molecular weight that special electrophoretic techniques (ie. polyacrylamide gel electrophoresis) are needed to separate them. This has not been attempted here. In contrast, Tenover et al. (1980) has shown that the 24.5 Mdal gonococcol plasmid is readily cleaved by EcoRl, Hinc II, and Sma I, three enzymes which had no effect on pHD147. This suggests that these two plasmids may in fact be structurally quite distinct but nucleic acid hybridization experiments must be performed to verify this conclusion. It seems unreasonable to assume that two apparently closely related species, H. ducreyi and N. gonorrhoeae, which already share highly similar Ap^r plasmids would have each evolved totally unrelated mobilizing plasmids to perform essentially the same function. However, preliminary experiments designed to determine if pHD147 could mobilize either the 7.0 Mdal Ap^r plasmid or the 4.9 Mdal Su^r plasmid to gonococcal recipients failed, although no attempt was made to mobilize the normally indigenous gonococcal 3.2 or 4.4 Mdal Ap^r plasmids from Haemophilus to Neisseria in similar matings. Thus, it is possible that the mobilization system of H. ducreyi serves chiefly to disseminate small plasmids only within the genus Haemophilus and does not have the ability to cross generic lines, unlike the Neisseria mobilization system which is readily able to donate Ap^r plasmids to Haemophilus recipients. A more rigor-

ous test of this is necessary however, especially in terms of determining the intergeneric host range of the Haemophilus mobilization system.

G) Summary and Conclusions

The conclusions which may be drawn from this work may be stated as follows:

1) Four conjugative Haemophilus R-plasmids in the 32-38 Mdal size range and carrying similar resistance determinants appeared to be very closely related to one another despite having their origins in three different species of Haemophilus isolated in widely scattered parts of the world. Evidence for this comes from:

- restriction endonuclease cleavage patterns which revealed a high degree of similarity at the DNA base sequence level.
- conjugal transfer frequencies which showed no difference from one plasmid to another. Although only three of the plasmids were tested in this manner, preliminary experiments have shown that the fourth (pV1157) is transferable at similar frequencies.
-stability in different host species. All plasmids were retained to an equal extent upon removal of selective pressure, regardless of the host species used.

Thus, these four plasmids were probably all evolved from the same ancestral "core" plasmid which acted, in essence,

as a receptacle for drug-resistance transposons introduced from elsewhere. This was followed by intra- and interspecific dissemination within this genus by way of conjugal transfer. The ancestral core plasmid has not yet been satisfactorily identified in any species of Haemophilus. Based on the inability of large Haemophilus plasmids to establish themselves in E. coli and on the apparent inability of E. coli plasmids to successfully transfer to Haemophilus, it is unlikely that the Haemophilus core plasmid had its origins in Escherichia. This, and other previously reported evidence, suggests that in fact it was indigenous to the genus Haemophilus and that only the drug-resistance genes themselves had a foreign origin. Hence the evolution of conjugative, multi-resistance plasmids in Haemophilus can be viewed as a three-stage process; evolution of a cryptic, self-transferable core plasmid; acquisition of drug-resistance transposons by unknown means from an external source such as the Enterobactericeae; dissemination to, and further evolution in, other species of Haemophilus.

2) The host range of these four large plasmids appears to be limited to at least three species of Haemophilus - H. influenzae, H. parainfluenzae, and H. ducreyi - and possibly a fourth, H. haemoglobinophilus, although this is still unclear. This suggests that the ancestral core plasmid probably originated in one of these three species. Furthermore, the rather restricted host range of these R-plasmids suggests that they will have difficulty in becoming estab-

lished in other species of Haemophilus, particularly those of veterinary concern. Neither is it likely that conjugative Haemophilus R-plasmids will successfully establish themselves in genera such as Neisseria or Escherichia. However, there may be a system of plasmid transfer operating between Neisseria and Haemophilus based on the ability of small gonococcal Ap^r plasmids to be mobilized to Haemophilus recipients.

3) Conjugation as a means of transferring drug resistance within a cell population appears to be an efficient process in Haemophilus. It operates in a rec-independent manner in this genus and in terms of efficiency is subject to at least one of the same parameters (ie. a function of donor/recipient ratio) as are plasmids of other genera. This implies that even though there are certain mechanistic differences between the conjugative transfer of Haemophilus plasmids compared to other plasmids (ie. solid surface mating vs. liquid mating), from the broad point of view, conjugation as it takes place in Haemophilus is not unusual or unique.

Highest transfer frequencies are obtained in intraspecific crosses and, while plasmids are freely transferable between H. influenzae, H. parainfluenzae, and H. ducreyi, the frequency declines somewhat when mating partners are of different species. Whether such high frequencies occur under natural conditions is unknown; nevertheless, the data presented here implies that conjugation may be the most important means for the rapid dissemination of antibiotic-

resistance genes, particularly multiple drug resistance, within this genus.

4) A system also exists in H. ducreyi for the rapid, efficient dissemination of small, non-conjugative plasmids by conjugative means; that is, through mobilization by a co-resident conjugative plasmid. This is particularly important because other means for transfer of small plasmids are not known in this species; however the true significance of this system lies in the fact that it is not limited strictly to H. ducreyi - plasmids can be mobilized to H. influenzae and probably to H. parainfluenzae with subsequent re-transfer being possible as well. In other words, the host range of the mobilization system probably parallels that seen for the conjugative R-plasmids in Haemophilus and as such, this system would provide a means for the free exchange of non-conjugative R-plasmids between species of Haemophilus. Why the small Ap^r plasmids present in H. ducreyi have not yet appeared in H. influenzae or H. parainfluenzae is unknown; it may only be a question of time. Since the H. ducreyi mobilization system can mobilize for transfer other small R-plasmids found in Haemophilus, there is no reason not to expect that these plasmids will become distributed throughout this genus to an even greater extent than previously observed. At the very least, the presence of such a system provides a reasonable answer to the problem of how non-conjugative R-plasmids in this genus became distributed to the point seen today.

The fact that the H. ducreyi system can mobilize Ap^r plasmids originally derived from N. gonorrhoeae is also significant since it again implies that some type of genetic exchange system exsits between these genera. Although we have as yet been unable to mobilize H. ducreyi plasmids directly into gonococcal recipients, further work is required in this area. If this were possible, it would provide a rational explanation for the origin of gonococcal Ap^r plasmids; that is, as derivatives of the 7.0 or 5.7 Mdal H. ducreyi plasmids. Presumably, these plasmids could have been mobilized to Neisseria and modified after transfer by excision of small portions of the genome to give the smaller 4.4 and 3.2 Mdal plasmids seen today. The exact relationship between the 23.5 Mdal H. ducreyi mobilizing plasmid and the 24.5 Mdal gonococcal plasmid is unclear at this point but may also have a bearing on this model for the development of ampicillin resistance.

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Appendix I

Growth Media

a) GC agar base (Gibco)

Peptone	15.0 g
Cornstarch	1.0 g
Potassium phosphate, dibasic	4.0 g
Potassium phosphate, monobasic	1.0 g
Sodium chloride	5.0 g
Agar	10.0 g

b) Chocolate agar

GC agar base	36.0 g
Bovine haemoglobin	10.0 g
Distilled water	1000 ml
CVA enrichment (Gibco)	10 ml

c) sBHI

Brain heart infusion broth (Gibco)	38.0 g
Distilled water	1000 ml
Hemin (Sigma) - 0.1%	10 ml
Nicotinamide adenine dinucleotide - 0.03%	10 ml

Appendix II

Miscellaneous reagents

a) Chromogenic cephalosporin

Nitrocefin (Compound 87-312)[1]	0.005 g
Dimethylsulfoxide	0.5 ml
Phosphate buffer (0.1 M, pH 7.0)	
- store in dark at 5°C	9.5 ml

[1]Glaxo Research Ltd., Greenford, England.

Appendix III

Restriction endonuclease buffers

a) Eco Rl 10X buffer

2.0 M TRIS-HCl (pH 7.5)	5.0 ml
5.0 M NaCl	1.0 ml
0.6 M $MgCl_2$	0.83 ml
0.5% Bovine serum albumin	2.0 ml
Distilled water	1.17 ml

b) Pst I and Bam Hl 10X buffer

0.6 M TRIS-HCl (pH 7.4)	1.0 ml
5.0 M NaCl	1.0 ml
0.6 M $MgCl_2$	1.0 ml
0.6 M 2-mercaptoethanol	1.0 ml
0.5% Bovine serum albumin	2.0 ml
Distilled water	4.0 ml

c) Hind III 10X buffer

0.6 M TRIS-HCl (pH 7.4)	1.16 ml
5.0 M NaCl	1.20 ml
0.6 M $MgCl_2$	1.16 ml
0.5% Bovine serum albumin	2.0 ml
Distilled water	4.48 ml

d) Hinc II 10X buffer

1.0 M TRIS-HCl (pH 7.9)	1.0 ml
5.0 M NaCl	1.2 ml
0.6 M $MgCl_2$	1.16 ml
0.6 M 2-mercaptoethanol	1.0 ml
0.5% Bovine serum albumin	2.0 ml
Distilled water	3.64 ml

e) Hae II 10X buffer

0.6 M TRIS-HCl (pH 7.4)	1.0 ml
0.6 M $MgCl_2$	1.0 ml
0.6 M 2-mercaptoethanol	1.0 ml
0.5% Bovine serum albumin	2.0 ml
Distilled water	5.0 ml

f) Sma I 10X incubation buffer

1.0 M TRIS-HCl (pH 8.5)	1.5 ml
1.0 M KCl	1.5 ml
0.6 M Mg Cl_2	1.0 ml
Distilled water	6.0 ml

dilution buffer (dilute enzyme 1:2 prior to use)

1.0 M potassium phosphate (pH 7.0)	1.0 ml
0.1 M EDTA	1.0 ml
0.7 M dithiothreitol	1.0 ml
Distilled water	7.0 ml

Appendix IV

MIV Transformation Medium

To make medium MIV, add 1.0 ml of solutions 2, 3, 4, 5 and 6 to 100 ml of solution 1.

solution 1:

L-aspartic acid	4.0 g
L-glutamic acid	0.2 g
fumaric acid	1.0 g
NaCl	4.7 g
K_2HPO_4	0.87 g
KH_2PO_4	0.67 g
Tween 80	0.2 ml
Distilled water	850 ml

- adjust to pH 7.3 with 1 N NaOH
- make to 1000 ml with distilled water, autoclave

solution 2:

1 N HCl	10 ml
L-cystine	0.04 g
L-tyrosine	0.1 g

- dissolve at 37°C
- dilute to 100 ml with distilled water

L-arginine	0.06 g
L-phenylalanine	0.2 g
L-serine	0.3 g
L-alanine	0.2 g

- filter sterilize

solution 3:

$CaCl_2$ (anydrous)	1.11 g
Distilled water	100 ml

- autoclave

solution 4:

$MgSO_4$: 7 H_2O	1.23 g
Distilled water	100 ml

- autoclave

solution 5:

2, 2', 2" - nitriolotriethyl alcohol	4.0 ml
hemin	0.1 g
histidine	0.1 g
Distilled water	96.0 ml

- filter sterilize

solution 6:

Casein hydrolysate	5.0 g
Distilled water	100 ml

- autoclave