PLASMID-MEDIATED ANTIBIOTIC RESISTANCE IN *HAEMOPHILUS DUCREYI*

A Thesis Presented to the

Department of Medical Microbiology

Faculty of Medicine

University of Manitoba

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

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by

Ian William Maclean

June, 1980

PLASMID-MEDIATED ANTIBIOTIC RESISTANCE IN *HAEMOPHILUS DUCREYI*

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IAN WILLIAM MACLEAN

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

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ACKNOWLEDGEMENTS

I would like to extend my appreciation to Dr. Bill Albritton for the time he spent with me and the guidance he offered as my advisor. Special thanks is given to Leslie Slaney, Peter Bertram and Dr. Jim Brunton for their technical expertise, helpful advice and friendship.

Financial assistance was received by way of a University of Manitoba Student Fellowship.

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ABSTRACT

Three of 21 isolates of Haemophilus ducreyi, the bacterial agent which causes the sexually transmitted disease chancroid, were found to be resistant to ampicillin and tetracycline. Using a chromogenic cephalosporin substrate they were shown to produce a B-lactamase.

When these three strains were screened for plasmid DNA, a single 6 x 10^6 dalton (6 Mdal) band of plasmid DNA was seen on agarose gel electrophoresis. This plasmid was purified by density gradient centrifugation and transformed into an ampicillin, tetracycline sensitive Escherichia coli strain C600. Transformants were ampicillin but not tetracycline resistant and produced a B-lactamase similar in substrate profile to that of the H. ducreyi B-lactamase. This suggested that the ampicillin resistance gene resides on this plasmid (designated pJB1), while the tetracycline resistance gene was integrated into the chromosome.

Using the restriction endonuclease HincII, the H. ducreyi plasmid was digested and the digestion pattern compared to the digestion pattern from plasmids containing the ampicillin transposons Tn1, Tn2, and Tn3. These are three previously described transposable pieces of DNA which carry genes coding for TEM-type B-lactamases. The H. ducreyi plasmid was shown to contain at least 60% of the ampicillin transposon Tn1 or Tn2.

Conjugation experiments were carried out to determine whether

or not the plasmid pJB1 could transfer itself from the resistant *H. ducreyi* to a sensitive recipient. No such transfer took place, suggesting the plasmid is non-self transmissible. However, strains of *H. ducreyi* were shown to accept and donate large conjugative plasmids originally isolated in *H. influenzae*.

Studies were carried out to characterize the B-lactamase produced by *H. ducreyi*. The substrate profile suggested a TEM-type B-lactamase. The isoelectric point was similar to that of the B-lactamase mediated by the plasmid RSF1030 which carries the ampicillin transposon Tn2. This type of B-lactamase is designated TEM-1. Using the technique of tandem crossed immunoelectrophoresis with antiserum prepared against partially purified B-lactamase from the transformant *E. coli* C600 (pJB1) the B-lactamases from RSF1030 (Tn2), RP4 (Tn1) and an ampicillin resistant *H. influenzae* were shown to be immunologically similar to the B-lactamase coded by the *H. ducreyi* plasmid pJB1.

INTRODUCTION

Bacteria resistant to penicillin have been around longer than penicillin has been in clinical use (Pollock, 1967). The resistance in some cases is due to cell wall modifications while in others it is due to the production of the enzyme B-lactamase, which inactivates penicillins and cephalosporins by hydrolysis of their B-lactam bond (Davies, 1979). Originally, the gene coding for B-lactamase production was found as part of the chromosome while today it is frequently found inserted into a plasmid or extrachromosomal piece of DNA (Slocombe and Sutherland, 1973; Heffron et al, 1975b).

Plasmids carrying drug resistance genes were first recognized in 1957 among enteric bacteria, specifically Shigella spp. causing bacillary dysentery (Watanabe, 1963). These bacteria were resistant to chloramphenicol, tetracycline, streptomycin and sulfonamide. Plasmid-mediated ampicillin resistance was not recognized until approximately 1965, when an E. coli with a resistance factor (R factor) was shown to transfer ampicillin resistance and B-lactamase production to a sensitive strain by transfer of the R factor (Datta and Kontomichalou, 1965).

By 1972, plasmid-mediated ampicillin resistance due to the production of a variety of B-lactamases was found throughout the Enterobacteriaceae (Richmond and Sykes, 1973). In 1975, clinical isolates of ampicillin resistant H. influenzae were found to

contain large 30 Mdal plasmids (Elwell et al, 1975). These plasmids were shown to contain a 3 Mdal piece of DNA which could transpose from one plasmid to another, inserting itself into the recipient plasmid (Heffron et al, 1975a; DeGraaff et al, 1976). The transposable piece of DNA carried the gene for B-lactamase production and is designated the ampicillin transposon or TnA. A smaller 4.1 Mdal plasmid which mediated ampicillin resistance in H. parainfluenzae was shown to carry only one-third of the TnA (Gromkova and Goodgal, 1977; Roberts et al, 1977). In 1977, two small plasmids from Neisseria gonorrhoeae were shown to carry 30-40% of TnA (Roberts et al, 1977).

There are a number of ampicillin transposons identified at this time carrying genes which code for either a TEM-1 or a TEM-2 type B-lactamase (Matthew and Hedges, 1976; Campbell et al, 1977). These two B-lactamases have a similar substrate profile but differ in their isoelectric point.

When three of 21 strains of H. ducreyi were found to be resistant to ampicillin and tetracycline and to produce a B-lactamase, plasmid-mediated drug resistance was suspected (Hammond et al, 1978). We undertook to characterize the B-lactamase produced by these ampicillin resistant strains and to determine the genetic basis of the ampicillin and tetracycline resistance.

LITERATURE REVIEW

A. B-Lactamases

1. Introduction

Fleming, in 1929, was the first to report specifically on the growth inhibiting factor produced by a mold identified as Penicillium rubrum (Fleming, 1929). In a recent paper, Selwyn has described a number of instances where the antibacterial factor of Penicillium molds was acting but its effect was not recognized (Selwyn, 1979). Some of these studies dated back to 1871. The antibiotic penicillin has therefore been around a long time and it has been in clinical use since about 1940.

Fleming noted the lysis of his cultures of Staphylococcus by penicillin. Today it is believed there are a number of penicillin-binding proteins which have important physiological roles in the cell. It is these proteins which make an organism sensitive to the antibacterial action of penicillin and its derivatives (Tomasz, 1979). One such action is the breakdown of the cell wall structure, resulting in lysis of the organism.

Abraham and Chain (1940) followed up on Fleming's observation that some bacteria of the coli-typhoid group were not inhibited by penicillin. They used a Bacillus coli and were able to extract a substance which destroyed the growth inhibiting property of penicillin. The substance, an enzyme, was given the name penicillinase. Kirby (1944) isolated a number of penicillinase producing strains of

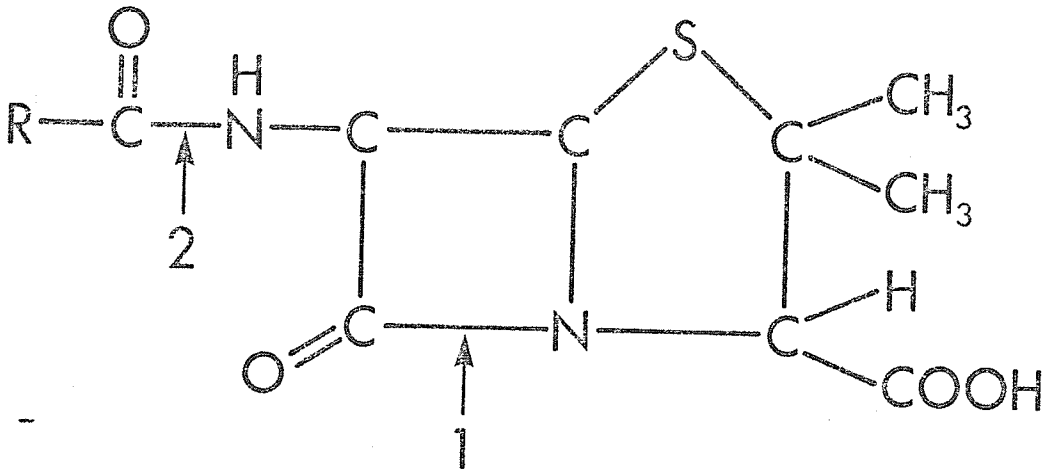
Staphylococcus aureus from patients who had never received penicillin. S. aureus made resistant to penicillin by serial passage in the presence of the antibiotic did not produce a penicillinase.

Work continued on penicillinases with the efforts of Benedict et al (1945) and Lepage et al (1946), who initiated the studies on the penicillinases produced by the organisms of the genus Bacillus. It was not until the mid-1960's, with the increased use of ampicillin, that interest was renewed in the penicillinases of gram-negative organisms. Today it is known that penicillinase producing strains are widely distributed among bacterial species but there is no evidence that penicillinases are formed by organisms other than bacteria (Citri and Pollock, 1966).

The term penicillinase denotes an enzyme which catalyzes the hydrolysis of the amide bond in the B-lactam ring of 6-amino-penicillanic acid and its N-acyl derivatives (Figure 1). Because the enzyme also breaks the B-lactam bond of 7-amino-cephalosporanic acid, the more general term B-lactamase is used rather than penicillinase or cephalosporinase. No bond other than the amide bond in the intact nucleus of penicillin or cephalosporin is broken by the enzyme. When the B-lactam bond is broken the antibiotic is no longer active against bacteria.

There are two other enzymes which are known to destroy or reduce the effect of penicillin and its derivatives (Sykes and Matthew, 1976). One is an amidase and the other is an esterase (Figure 1). They are produced by a number of bacteria. Because

PENICILLINS



CEPHALOSPORINS

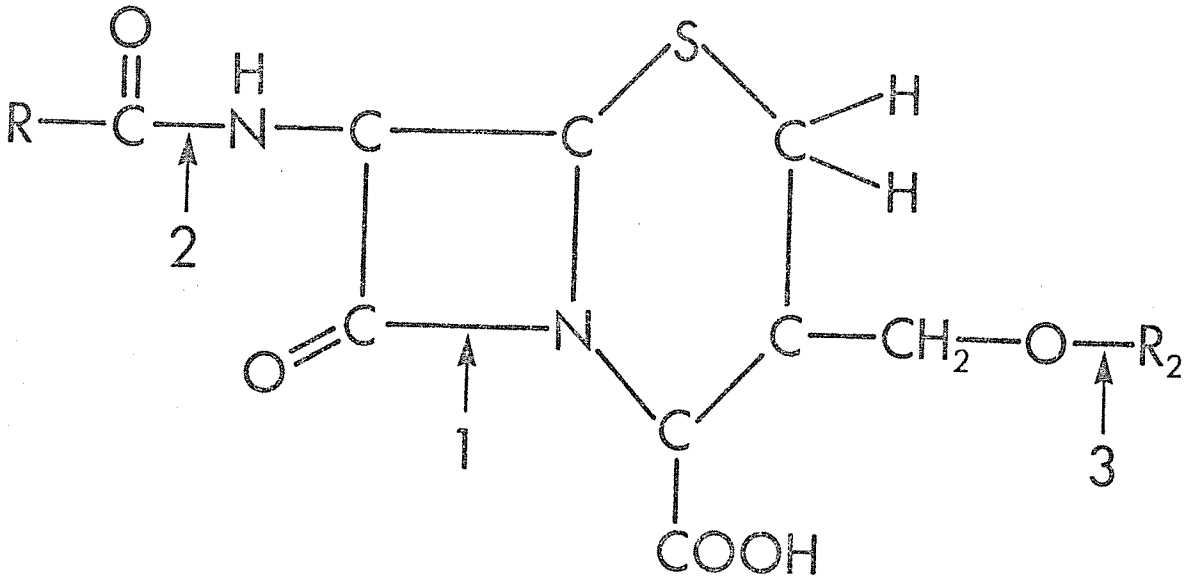


Fig. 1 Sites for enzymatic attack on penicillins and cephalosporins. 1. B-lactamase
2. amidase
3. esterase

they cleave only the side chains from the penicillin or cephalosporin and leave the nucleus intact, the antibiotic can sometimes maintain antibacterial activity.

2. Characterization of B-Lactamases

B-lactamases are characterized by a number of criteria as outlined by Sykes and Matthew (1976).

a) Substrate profile: Substrate profile refers to the hydrolytic activity of a B-lactamase against a variety of B-lactam substrates. The profiles are generally expressed as ratios related to a value of 100 for a chosen substrate (usually benzyl-penicillin). (e.g. a profile of penicillin 100 and ampicillin 200 indicates an enzyme with a rate of hydrolysis of ampicillin twice that of penicillin.)

b) Inhibition of enzyme activity:

i. B-lactam inhibitors: Penicillins such as cloxacillin and methicillin which are generally stable towards B-lactamases can act as inhibitors of certain types of B-lactamases. This is due to competition between the stable penicillin and the hydrolyzable penicillin or cephalosporin for the active sites on the B-lactamase molecule. Some B-lactamases are strongly inhibited while for others the inhibition is reduced. Therefore, the degree of inhibition has been used in the classification of B-lactamases.

ii. Non-B-lactam inhibitors: Inhibition of B-lactamases by both chloride ions and p-chloromecuribenzoate (pCMB) has been used in the classification of B-lactamases (Richmond and Sykes, 1973). At a concentration of 0.5 μM, pCMB completely inhibits B-lactamases containing cysteine residues.

c) Analytical isoelectric focusing: Isoelectric focusing is a procedure which allows direct visualization of the B-lactamase on a thin-layer polyacrylamide gel. The gel is stained with chromogenic cephalosporin (O'Callaghan et al, 1972), which is specific for B-lactamases. Purification of the enzyme is not necessary and the procedure is quite sensitive. The B-lactamases align themselves at their isoelectric point which helps differentiate enzymes similar by other biochemical and immunological methods of characterization.

d) Immunological studies: Antiserum raised against one B-lactamase can react with other B-lactamases and any cross-reaction observed with methods such as inhibition of enzyme activity (Jack and Richmond, 1970) and immunoelectrophoresis (Ross and Boulton, 1972).

e) Molecular weight determination: The molecular weight of any protein and more specifically, any B-lactamase, can be determined by a number of procedures (Andrew, 1965; Weber and Osborn, 1969). This method of protein

characterization is not that useful in differentiating B-lactamases as some enzymes with similar molecular weights have different biochemical characteristics (Matthew, 1979). It is therefore the least effective method for characterizing B-lactamases.

3. Assay of B-lactamases

When penicillin is hydrolyzed by a B-lactamase the corresponding penicilloic acid is produced in stoichiometric amounts. A penicilloic acid has two acidic groups where the parent compound has only one and it is a stable substance which can be readily assayed.

Cephalosporins are more complicated as following hydrolysis an unstable cephalosporanic acid is produced. This breaks down to produce a compound which has two additional acidic groups. However, not all cephalosporins follow this pattern. Cephaloridine for example is decomposed to products with two acidic groups and one basic group.

a) Methods of assay:

i. The most widely used method to determine breakdown products of penicillins and cephalosporins is the iodometric assay. Basically, an excess of iodine buffered to pH 4.0 is used to stop the reaction. The hydrolysis products of the B-lactam antibiotic react with the iodine. The remaining iodine can be measured by titration with sodium thiosulphate

(Perret, 1954), or by measuring the decrease in optical density at 490 mm (Sargent, 1968). Other methods, such as a decrease in absorbance at 620 nm of a starch-iodine complex have also been used (Sykes and Nordstrom, 1972).

ii. The concentration of residual penicillin or cephalosporin can be determined. Hydroxylamine has been used as it reacts with the intact B-lactam giving a hydroxamic acid which forms a chromogen with ferric ions (Hamilton-Miller et al, 1965). Destruction of the B-lactam antibiotic would be seen as a decrease in absorbance at 490 nm. Hydrolysis of cephalosporins can be monitored directly as they absorb in the 250-260 nm range (O'Callaghan et al, 1968). Finally, a biological assay using suitably sensitive organisms can be used to determine residual cephalosporins and penicillins.

b) Interpretation of the assay: When the assay has been completed the enzyme activity is expressed in units. One B-lactamase unit is defined as equivalent to one μ mole of benzylpenicillin hydrolyzed per minute at 30°C, pH 7.0. The temperature and pH have been varied by many authors to produce optimum conditions for their particular B-lactamase. This practice sometimes makes comparisons between various B-lactamases difficult.

4. B-Lactamases of Gram-Positive Bacteria

Citri and Pollock (1966) reviewed the available information on B-lactamases produced by Bacillus cereus, B. licheniformis and S. aureus.

The B-lactamases of gram-positive bacteria are invariably found to be liberated into the growth medium. Recent work with B. licheniformis showed three distinct cellular locations for the same B-lactamase (Traficante and Lampen, 1977). The B-lactamase was found as an extracellular hydrophilic enzyme and as a hydrophobic cell-bound form(s) located on the outer surface of the plasma membrane of the protoplast and at the same time associated with vesicular membrane components. Aiyappa et al (1977) purified a penicillinase-releasing protease which cleaved 24 amino acid residues from the hydrophobic, membrane bound B-lactamase, converting it to the extracellular form.

There appears to be no permeability or accessibility barrier between the B-lactamases of gram-positive organisms and the penicillin substrates. Studies have shown that sonicated and intact cells have similar rates of hydrolysis of the substrate suggesting that gram-positive B-lactamases have unrestricted access to the substrate.

The substrate profile of the B-lactamases from gram-positive bacteria (Table I) indicates good activity against the penicillins with very low activity against cloxacillin and the cephalosporins. However, under certain conditions the profile may change. Pollock

Table I. Comparison of the Substrate Profiles of B-Lactamases from *Bacillus cereus*, *B. licheniformis* and *Staphylococcus aureus*[1]

	Substrate profile				
	Penicillin	Ampicillin	Cloxacillin	Cephalosporin C	Cephaloridine
Bacillus cereus	100	120	0.5	0	3
Bacillus licheniformis	100	64	0	15	20
Staphylococcus aureus	100	120	0	0.5	10

[1] (Richmond and Sykes, 1973)

(1963) has shown that with the addition of specific antiserum to *B. subtilis* B-lactamase the rate of hydrolysis of methicillin was increased ten-fold. Kuwabara (1970) isolated two separate B-lactamases from a strain of *B. cereus*, produced under specific conditions. One was a penicillinase while the other was a cephalosporinase and was Zn^{2+} dependent. These two enzymes may be similar to the hydrophilic and hydrophobic B-lactamases described by Traficante and Lampen (1977).

The genetic regulation of B-lactamase synthesis in gram-positive bacteria was recently reviewed (Imsande, 1978). It has long been known that gram-positive organisms must be induced to produce B-lactamase, usually by exposure to penicillin (Citri and Pollock, 1966). Saz and Lowry (1964) reported on two non-penicillin-like cyclic peptides which acted as inducers and substrates for a staphylococcal B-lactamase. This work was never substantiated, suggesting that B-lactamases are induced only by penicillins or cephalosporins and that these are the only substrates for the enzyme.

The position of the B-lactamase gene in gram-positive bacteria has been well worked out. In *Bacillus* spp. the gene is part of the chromosome, while in *Staphylococcus* spp. the B-lactamase gene resides on a plasmid (Rush et al, 1969; Sherratt and Collins, 1973).

The origin of B-lactamases in gram-positive organisms is unclear. Approximately 40% of the 260 amino acid residues of the *Bacillus* spp. and *Staphylococcus* spp. B-lactamases are found in both

suggesting a common origin for these two enzymes (Imsande, 1978). Pollock (1967) states that there are enough penicillin producing fungi in the natural environment that B-lactamase producing bacteria would have a selective advantage. Modern day use of penicillins and cephalosporins was obviously not the cause for the evolution of the B-lactamase gene and the enzyme for which it codes, as a strain of B. licheniformis grown from spores stored since 1689 produced a B-lactamase similar to the gram-positive B-lactamases seen today (Sneath, 1962). Candidates for a possible B-lactamase ancestor would be enzymes involved in the formation of the peptidoglycan of the bacterial cell wall as these reactions are specifically inhibited by penicillin.

5. B-Lactamases of Gram-Negative Bacteria

A B-lactamase designated TEM was the first B-lactamase to be purified from a gram-negative organism (Datta and Richmond, 1966). Since that time using a variety of techniques, a number of B-lactamases from gram-negative organisms such as Pseudomonas aeruginosa (Labia et al, 1977), Bacteroides fragilis (Britz and Wilkinson, 1978), and Neisseria gonorrhoeae (Eriquez and D'Amato, 1979) have been purified.

The B-lactamases of gram-negative bacteria are for the most part, produced constitutively. There is no need to induce their formation with penicillins or cephalosporins and there is no increase in rate of formation in the presence of these antibiotics (Richmond and Sykes, 1973). This property differs from the B-lactamases of gram-

positive bacteria which normally must be induced with penicillin.

Another point of difference is that gram-negative B-lactamases are not released extracellularly. They are located in the periplasmic space which is between the peptidoglycan and the outer cell wall (Curtis et al, 1972). The B-lactamase is bound to this region of the cell and not released into the medium. In some gram-negative bacteria the outer cell wall can act as a permeability barrier such that antibiotics are restricted in their access into the cell and are more easily inactivated by the strategically placed B-lactamases (Richmond and Sykes, 1973). The periplasmic position of the gram-negative B-lactamases eliminates the need for copious amounts of the enzyme as are usually produced by the gram-positive bacteria.

There are a number of reviews on gram-negative B-lactamases with the first having been written by Jack and Richmond (1970). They tested 46 B-lactamases from enteric bacteria by various criteria and found eight distinct B-lactamases. Two types specifically hydrolyzed penicillin while two others were cephalosporinases. The remaining four had activity against both penicillins and cephalosporins. Only three of the eight types of B-lactamases described were shown to be plasmid-mediated while the others presumably originated from genes carried on the chromosome.

The next major review appeared in 1973 (Richmond and Sykes, 1973). It described five classes of B-lactamases based on substrate profile and activity of the enzyme after addition of cloxacillin and pCMB (Table II). The TEM B-lactamase was placed in class III (Table III).

Table II. Overall Classification of B-lactamases from Gram-Negative Bacteria on the Basis of Their Substrate Profiles[2] and Relative Activities[1]

Enzyme class	Enzyme type	Substrate Profile					
		Penicillin	Ampicillin	Carbenicillin	Cloxacillin	Cephaloridine	Cephalexin
I	a	100	0	0	ND	8000	620
	b	100	0	0	ND	350	80
	c	100	150	ND	ND	2000	ND
	d	100	10	0	0	600	80
II	a	100	180	45	ND	< 20	0
	b	100	160	ND	0	< 20	0
III	a	100	180	10	0	140	< 10
IV	a	100	120	10	<10	150	0
	b	100	125	45	20	50	<10
	c	100	170	50	20	50	0
V	a	100	950	ND	200	120	ND
	b	100	300	ND	200	50	ND
	c	100	100	60	0	20	<10
	d	100	180	80	0	40	<10

ND indicates not determined.

[1] (Richmond and Sykes, 1973).

[2] Substrate prifile refers to the rate of hydrolysis of the antibiotic by the enzyme relative to a value of 100 for penicillin.

Table III. Characteristics of Class III Enzymes[1]

Enzyme type	Host species	Substrate Profile						Inhibited by		R-factor
		Penicillin	Ampicillin	Carbenicillin	Cloxacillin	Cephaloridine	Cephalexin	Cloxacillin	pCMB	
a	R-factor-mediated	100	180	10	0	140	10	Sensitive	Resistant	RTEM

[1] Richmond and Sykes, 1973.

This class had a wide substrate specificity with approximately equal rates of hydrolysis of penicillins and cephalosporins. The enzyme was sensitive to inhibition by cloxacillin but resistant to the effect of pCMB. The gene coding for TEM B-lactamase was plasmid-mediated, causing the TEM B-lactamase to be widely disseminated throughout the family Enterobacteriaceae.

Hedges et al (1974a) studied B-lactamases determined by 29 ampicillin-resistance plasmids. Two groupings were recognized. The most predominant were the TEM-type B-lactamases mediated by plasmids with a wide range of plasmid compatibility groups. The ability of the structural gene for the TEM B-lactamase to translocate from one plasmid to another was given as one reason for its spread among plasmids. The other type of B-lactamase studied was an enzyme which hydrolyzed oxacillin at a higher rate than penicillin. This B-lactamase was found in nine of the 29 cases studied.

In 1975 the technique of isoelectric focusing was used to further differentiate the TEM-type B-lactamases into two groups (Matthew et al, 1975). One group was found to have an isoelectric point of 5.4 while the other had an isoelectric point of 5.6. Both had identical substrate profiles. An interesting point was that all strains tested, including supposed B-lactamase negative organisms, had bands appearing at an isoelectric point of 8.3. These were considered to be chromosomally-mediated B-lactamases. Unexplained satellite bands were also apparent near the main B-lactamase band. Matthew and Hedges (1976) designated the TEM B-lactamases with an

isoelectric point of 5.4 as TEM-1 and 5.6 as TEM-2. The oxacillin type B-lactamases had three different isoelectric points and were named OXA-1, OXA-2 and OXA-3.

The most recent review covering all gram-negative B-lactamases appeared in 1976 (Sykes and Matthew, 1976). Here the B-lactamases were divided by whether or not the B-lactamase gene was chromosomally-located or plasmid-located. The majority of chromosomally-located B-lactamases were cephalosporinases with the one exception of a broad spectrum B-lactamase produced by a Klebsiella strain. The plasmid-located B-lactamases were of the TEM and OXA variety.

Finally, Matthew (1979) examined a number of plasmid-mediated B-lactamases. Included were six additional enzymes not known at the time of the Sykes and Matthew review. Four of the six had originated from Pseudomonas- plasmids. Of the 363 gram-negative B-lactamases examined, 77.4% were of the TEM type with 15.4% of the OXA variety. The Pseudomonas enzymes were rare but exceptional as only they hydrolyze carbenicillin at rates equal to benzylpenicillin.

A partial amino acid sequence of the TEM-1 B-lactamase has recently been reported (Ambler and Scott, 1978). When compared to the sequences of the gram-positive B-lactamases there appeared to be 30-35% identity. One point of interest was that the TEM-1 B-lactamase did not show similarity with the NH_2 terminal region of the membrane bound form of the B-lactamase from B. licheniformis which has an unusual amino acid composition. This question was answered by Sutcliffe (1978). He sequenced the gene coding for the TEM-1 B-

lactamase and found that it encodes for a protein of 286 amino acid residues which is 23 amino acid residues longer than the mature enzyme. These extra residues may be similar to the 24 residues cleaved from the membrane B-lactamase of B. licheniformis to form the extracellular B-lactamase (Aiyappa et al, 1977).

There are a wide range of gram-negative bacteria which can produce a B-lactamase (Sykes and Matthew, 1976) with some organisms able to produce two distinct B-lactamases (Letarte et al, 1978). The TEM B-lactamase was initially restricted to the Enterobacteriaceae but it is now found in such gram-negative organisms as H. influenzae (Elwell et al, 1975) and N. gonorrhoeae (Elwell et al, 1977a).

B. Plasmid-mediated Antibiotic Resistance

1. Introduction

The bacterial cell may contain a wide variety of genetic material. Some of this is its own chromosomal DNA but at times it may contain the DNA of an infecting virus or the DNA of a sex factor, colicin factor or drug resistance factor.

Viral DNA can replicate autonomously in the cytoplasm or as an integrated part of the host chromosome. This type of genetic element was given the name episome (Hayes, 1968). With the demonstration of a large number of genetic elements which did not integrate into the chromosome the word plasmid was coined (Hayes, 1968). Such genetic elements as the sex factor, colicin factor and resistance factor are plasmids as they replicate independently and only infrequently

integrate into the chromosome.

Sex factors which are found in E. coli strains promote conjugation and chromosome transfer between bacterial strains which possess the factor and similar strains which do not.

Colicins are bactericidal agents which are produced by some species of the Enterobacteriaceae and kill other strains of this species. Certain classes of colicin determinants could promote their own transfer by conjugation to recipient bacteria while others could not do so, but could be transferred if a sex-factor was added to the donor organism.

2. Resistance Transfer Factors in the Enterobacteriaceae

Resistance transfer factors were first noted by Japanese workers in 1957 when large numbers of multiply-drug resistant Shigella strains causing dysentery were isolated (Watanabe, 1963). It was shown that these organisms could transfer the resistance genes to other Shigella as well as E. coli. The mechanism of transfer proved to be conjugation.

The antibiotics involved were streptomycin, sulfonamide, chloramphenicol and tetracycline. Resistance to these drugs could be transferred together or as numerous combinations, indicating the association between the resistance genes and the resistance transfer factor was not always stable (Hayes, 1968).

Anderson (1965), using a triple-cross technique, demonstrated that many drug sensitive strains of Salmonella had only the transfer

factor which could couple with a non-transmissible drug resistance determinant from a second organism with the combined piece of DNA being transferred to a third recipient organism. It was suggested that such a mechanism could be going on in the gut which would give rise to a large variety of R factors and resistant organisms.

The increase in drug resistant S. typhimurium was followed in Great Britain from 1962-1965 (Anderson, 1968). Phage type 29 was shown to predominate among the resistant bacterial population. The reservoir for this resistant organism was found to be cattle raised by intensive farming methods where overcrowding and heavy use of antibiotics to control infection caused it to be selected over sensitive strains. The organisms were spread to farm workers and the general population by unpasteurized milk from infected cows.

Following the introduction of ampicillin into clinical use in the early 1960's, transferrable ampicillin resistance quickly arose (Slocoambe and Sutherland, 1973). This resistance was due to the production of a broad-spectrum B-lactamase active against both penicillins and cephalosporins. In 1965 Datta showed transfer of ampicillin resistance due to a B-lactamase gene being carried on a large R factor (Datta and Kontomichalou, 1965).

3. Transposable Antibiotic Resistance

Hedges et al (1974) studied a number of ampicillin resistance plasmids and found that virtually all of the plasmids had been originally isolated from members of the Enterobacteriaceae. How the

gene for TEM B-lactamase came to be on such a variety of plasmids was soon explained.

It was found that a 1.7 - 3.9 Mdal piece of DNA could remove itself from one plasmid and insert into another plasmid (Hedges and Jacobs, 1974b). This piece of DNA carried the gene for TEM-type B-lactamase production. Hedges and Jacobs (1974b) designated DNA sequences with transposition potential as transposons and the transposon marked by ampicillin resistance gene(s) as transposon A.

Continued research demonstrated that recombinant plasmids which acquired ampicillin resistance had an identical insertion of a 3.2 Mdal piece of DNA which was bounded by short inverted repeat sequences (Heffron et al, 1975a; Rubens et al, 1976). This small piece of DNA was the transposon A or TnA. The insertion of TnA into a plasmid was shown to be at a number of non-random sites, some of which would cause loss of host gene function.

Transposons are named numerically as they are found. Tn1, Tn2 and Tn3 are all ampicillin transposons identified and characterized by three separate authors (Campbell et al, 1977). Tn4 to Tn10 are transposons carrying drug resistance genes other than for ampicillin.

The ability of the TnA to move from one plasmid to another explained the rapid emergence of ampicillin resistance among the Enterobacteriaceae. No recombination was required as the TnA simply inserted itself at a certain site on the recipient plasmid. With such indiscriminant transfer of DNA it was only a matter of time before ampicillin resistance mediated by a TEM B-lactamase spread

out from the enteric bacteria and into other gram-negative organisms of clinical importance.

4. Plasmids in Gram-Negative Organisms Other than Enteric Bacteria

Khan et al (1974) was the first to describe a case of meningitis caused by a strain of H. influenzae which did not respond to ampicillin. The organism isolated produced a B-lactamase.

Elwell in 1975 reported ampicillin resistance in H. influenzae which was associated with a 30 Mdal plasmid (Elwell et al, 1975). This plasmid was further characterized by DeGraaff et al (1976) where it was shown to contain all of the ampicillin transposon.

A small plasmid from H. parainfluenzae was shown to possess only about 30% of the TnA (Roberts et al, 1977). This small plasmid was non-conjugative while the larger plasmid of H. influenzae was conjugative (Elwell et al, 1975).

Two disconcerting reports appeared in Lancet describing B-lactamase producing N. gonorrhoeae (Phillips, 1976; Ashford et al, 1976). One strain had originated in West Africa while the other was associated with a U.S. serviceman returning from the Far East.

Work by Elwell et al (1977) showed that two small plasmids were involved in carrying the TEM B-lactamase gene in N. gonorrhoeae. A 4.4 Mdal plasmid was isolated from Far East strains while a 3.2 Mdal plasmid was found in strains from West Africa. These two plasmids were non-conjugative but could be mobilized and transferred to recipient organisms by a large 25 Mdal plasmid which was found in

both ampicillin sensitive and resistant N. gonorrhoeae (Sox et al, 1978). The two small plasmids were shown to be highly homologous to the small plasmid from H. parainfluenzae and possessed about 40% of the TnA.

Only strict surveillance and follow-up of contacts has prevented ampicillin resistant N. gonorrhoeae from gaining a foothold in North America. However, this is not the case with isolates of H. influenzae. Approximately 9% of clinical isolates of this organism in Winnipeg are resistant to ampicillin with others being resistant to tetracycline and chloramphenicol (Albritton et al, 1978). This probable spread of ampicillin resistance from the enteric bacteria to N. gonorrhoeae and H. influenzae is probably due to wide use of ampicillin and the increased number of B-lactamase resistance plasmids.

C. Antibiotic Susceptibility of Haemophilus ducreyi

H. ducreyi is believed to be the causative agent in the sexually-transmitted disease chancroid. It is a small gram-negative coccobacillus which requires the X-factor hemin but not the V-factor NAD for growth (Kilian, 1976). In normal culturing practice the organisms are grown on chocolate agar plus added enrichment media.

There are a number of papers reporting antibiotic sensitivities of H. ducreyi but no drug resistance patterns, including resistance to penicillin, were noted (Thayer et al, 1955; Reymann, 1949; Mortara, 1944). However, these studies were done before plasmid-mediated drug resistance became widespread.

Kerber et al (1969) described the treatment of 90 cases of chancroid in Vietnam. Only 30% of the ulcers healed with tetracycline, while 89% healed with sulfisoxazole. When both drugs were used there was a 100% cure rate.

Marmur (1972) followed this with a drug study on 67 patients who failed to respond to the combination of tetracycline and sulfisoxazole therapy. Kanamycin was the antibiotic used to clear up the resistant lesions.

Genital ulcers which did not respond to streptomycin but healed with a five-day course of cephalothin were reported by Hart (1975). Kerber suggests the use of low doses of antibiotics for long periods of time by Vietnamese prostitutes may be the reason for development of drug-resistant chancroid in Vietnam.

From late 1975 to early 1977, 21 isolates of H. ducreyi were recovered in Winnipeg by culturing swabs from genital ulcers on chocolate agar plus 3 µg/ml vancomycin (Hammond et al, 1978). These organisms were tested against a wide array of antimicrobial agents to determine their minimum inhibitory concentration. Three of the 21 were found to be resistant to 128 µg/ml ampicillin and one of these three was also resistant to 32 µg/ml tetracycline (Hammond et al, 1978). All three were shown to produce a B-lactamase.

Patients in Winnipeg were treated with sulfisoxazole, tetracycline and doxycycline (unpublished data). Failures to heal in each group were associated with non-compliance, presence of lesions $\geq$ 1 cm in diameter and presence of large inguinal buboes.

MATERIALS AND METHODS

A. Bacterial Strains and Plasmids

The organisms used in this study are listed in Table IV. Included are the plasmids used for molecular weight standards. The plasmid RSF1030 carries the gene coding for a TEM-1 B-lactamase while the plasmid RP4 carries the gene coding for a TEM-2 B-lactamase (Matthew and Hedges, 1976).

B. Growth Media

1. H. ducreyi

Antibiotic sensitive strains were grown on chocolate agar (CA, Appendix Ia). Ampicillin resistant strains were grown on CA plus 5-10 µg/ml ampicillin. The plates were incubated in a humid incubator with 5% CO_2 at 35°C.

2. H. influenzae

Antibiotic sensitive organisms were grown on plain CA while resistant organisms had the appropriate antibiotic added to the medium (5-10 µg/ml ampicillin, 4 µg/ml tetracycline, 4 µg/ml chloramphenicol, 50 µg/ml nalidixic acid and 1000 µg/ml streptomycin).

3. E. coli

a) General growth conditions: The liquid medium used was L-broth (Appendix Ib). The solid medium was MacConkey agar without crystal violet. Resistant strains had

Table IV. Bacterial Strains and Plasmids

Strain	Plasmid Complement (x 10^6 daltons)	Phenotype[3]
A. *H. ducreyi*		
54198	6.0	Amp Tet
54207	6.0	Amp Tet
54211	6.0	Amp Tet
35000 Sm[1]	-	Sm
35000 Sm Amp[2]	30.0	Sm Amp
35000 Sm Chl Tet[2]	34.0	Sm Chl Tet
54205 Chl Tet[2]	34.0	Chl Tet
B. *H. influenzae*		
B031	30.0	Amp
R230	34.0	Chl Tet
B003 Nal[1]	-	Nal
R087 Sm[1]	-	Sm
B003 Nal Amp[2]	30.0	Nal Amp
C. *E. coli*		
C600	-	-
C600 (pJB1)	6.0	Amp
C600 (RSF1010:Tn1)Ap101	8.7	Amp
C600 (RSF1010:Tn1)Ap111	8.7	Amp Sm
C600 (RSF1010:Tn3)Ap230	8.7	Amp
C600 (RSF1010)	5.5	Su Sm
W1485-1 (RSF1030)	5.5	Amp

D. Plasmids for molecular weight standards

Plasmid	Molecular weight (x 10^6 daltons)
R1 drd 19	62
RP4	34
Sa	23
RSF1010	5.5

[1] These strains were made resistant by passage on agar containing increasing amounts of the antibiotic.

[2] These strains were recipients of a large plasmid which carried a resistance marker.

[3] Abbreviations: Su (Sulfonamide), Amp (Ampicillin), Chl (Chloramphenicol), Sm (Streptomycin), Tet (Tetracycline), Nal (Nalidixic Acid). Designates resistance to these antibiotics.

25 µg/ml ampicillin added to the media.

b) Partial B-lactamase purification: The various strains of E. coli were grown in the low phosphate broth of Neu and Chou (1967) (Appendix Ic).

C. Antimicrobial Susceptibility

1. Agar Dilution

This method was used to determine the minimum inhibitory concentrations of ampicillin and tetracycline for the H. ducreyi strains. The antibiotics were incorporated in CA decreasing in doubling dilutions from 128 µg/ml to .06 µg/ml. The organisms were scraped from CA plates, suspended in broth, vortexed and allowed to settle. The supernatant was adjusted to approximately 10^8CFU/ml (.5 MacFarland Standard) and samples applied to the surface of the plates with a Steers-Foltz replicator. The plates were incubated for 24 hours in a CO_2 incubator at 35-37°C. The end point was taken as $\geq$ 80% reduction in growth when compared to the growth on plain CA.

2. Microtitre Plate Dilution

This method was used to determine the minimum inhibitory concentrations of ampicillin and tetracycline for various E. coli strains. Colonies were picked from a plate, inoculated into L-broth and grown for three to five hours. The broth was diluted to 10^5CFU/ml. The antibiotics were serially diluted in broth from 128 µg/ml to .06 µg/ml. The cells and antibiotics were mixed by adding 0.1 ml of each to wells

in a microtitre plate. The plate was incubated at 37°C for 18 hours and growth or no growth observed. Growth was defined as visual cloudiness of the broth in the well, as compared to the clear control well.

D. B-Lactamase Assay and Determination of Substrate Profile

1. B-lactamase Assay

The B-lactamase assay used was a modification of the procedure of Sawai et al (1978). It measures the breakdown products of the penicillins and cephalosporins following hydrolysis by the B-lactamase. The composition of the iodine reagent is given in Appendix II. The assay took place at 30°C in a .1 M Na_2HPO_4 - KH_2PO_4 buffer pH 7.0. The B-lactamases were prepared by sonication of the organisms. The cell debris was discarded following centrifugation at 48,000 g for 30 minutes with the supernatant being used as the crude B-lactamase.

The procedure for the assay was to pre-incubate 100 µmoles of the antibiotic in the phosphate buffer for ten minutes. To this was added enough B-lactamase to hydrolyze 100 µmoles of penicillin G in about ten minutes. The final volume would be 4.0 ml. At various times 80 µl of this solution was removed and added immediately to a solution consisting of 1.25 ml iodine reagent and 1.17 ml phosphate buffer making a final volume of 2.5 ml. This was left for at least ten minutes and not longer than 20 minutes at which time the absorbance was measured at 490 nm in a Unicam SP-1800 spectrophotometer.

The inhibition studies were carried out in the same manner, except the enzyme was pre-incubated for ten minutes with either .5 uM pCMB or 10 $\frac{\mu Moles}{\Lambda}$ of cloxacillin. At this time 100 µmoles of

penicillin G was added to the reaction mix.

The controls for the assay consisted of the antibiotic incubated with no enzyme and the enzyme incubated with no antibiotic added. No decrease in absorbance at 490 nm over a period of 120 minutes was seen with these controls.

2. Substrate Profile

Once the B-lactamase assay was completed the change in absorbance at 490 nm was plotted against the time of removal of the hydrolysis products from the reaction mixture (Figure 2). From this plot the approximate time required for a given amount of B-lactamase to hydrolyze 100 µmoles of the antibiotic could be determined. By setting the time to hydrolyze 100 µmoles of penicillin G to the relative value of 100 the other times can be compared to it and the substrate profile produced. Inhibition by cloxacillin was defined as greater than 20% increase in the time required to hydrolyze 100 µmoles of penicillin G as compared to penicillin G alone.

E. Partial Purification of B-Lactamase

The organism used for this procedure was E. coli C600 (pJB1) which was produced by transformation of E. coli C600 with the purified plasmid pJB1 isolated from H. ducreyi.

The cells were grown to early stationary phase (9-12 hours) in 1 liter volumes of the low phosphate broth of Neu and Chow (1967). The lysis procedure was the method of Lindquist and Nordstrom (1970)

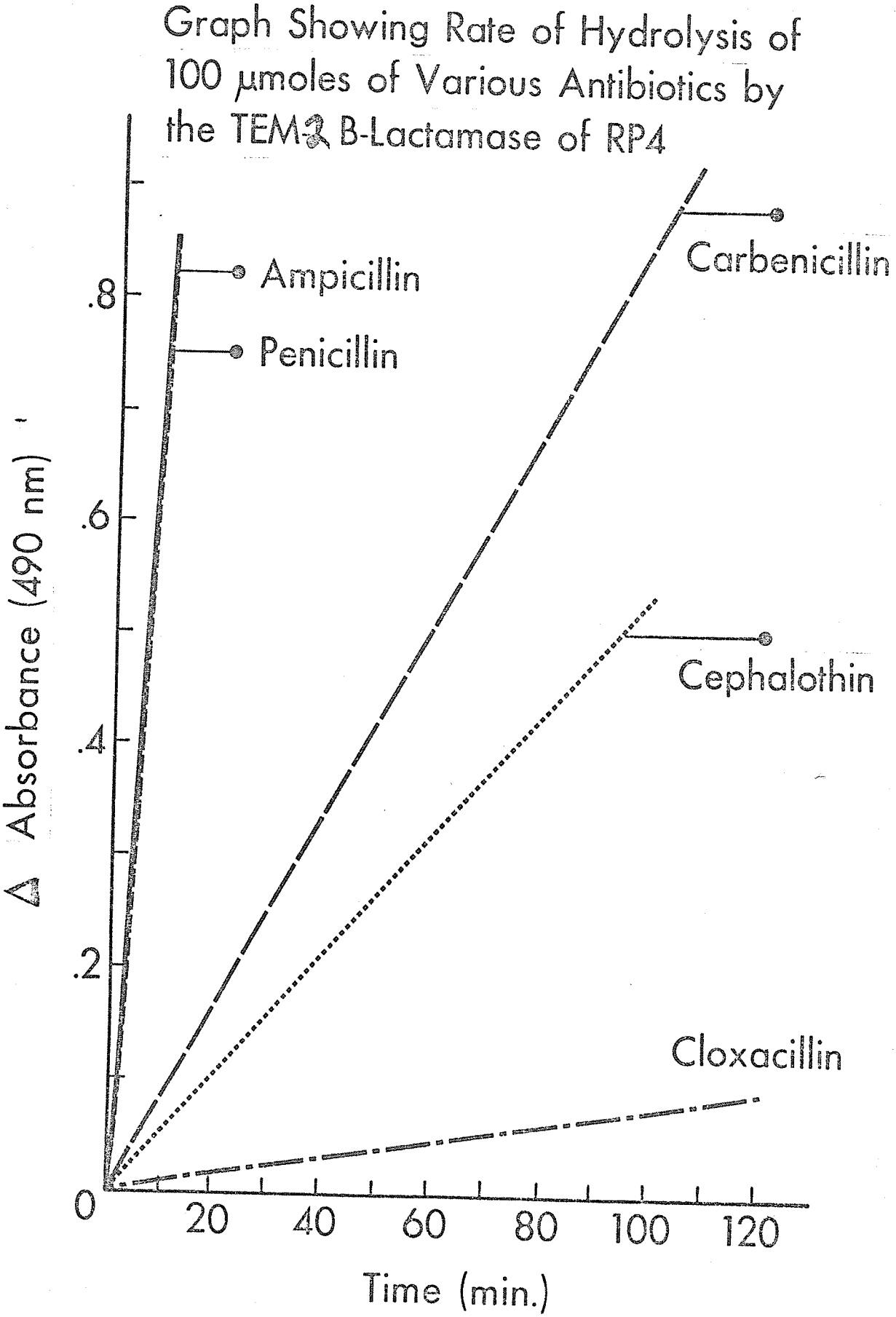

Graph Showing Rate of Hydrolysis of 100 μmoles of Various Antibiotics by the TEM-2 B-Lactamase of RP4
Ampicillin
Penicillin
Carbenicillin
Cephalothin
Cloxacillin
Δ Absorbance (490 nm)
0
.2
.4
.6
.8
20
40
60
80
100
120
Time (min.)

Fig. 2

for the release of periplasmic enzymes. The supernatant was applied to a column containing DEAE-Sephadex in .01 M TRIS pH 8.0. The B-lactamase was batch eluted from the column with .4 M NaCl. Fractions of high activity were pooled and lyophilized. Upon resuspension in 3 ml of .01 M TRIS pH 8.0, it was run on a G-75 Sephadex column. Fractions of high activity were again pooled. At the conclusion of this step the B-lactamase from E. coli C600 (pJB1) had increased in specific activity from 40 units/mg to 479 units/mg protein with 50% recovery. Approximately six other proteins were present as determined by polyacrylamide gel electrophoresis.

F. Isoelectric Focusing

This technique was similar to that of Matthew et al (1975). Ready-made PAG ampholine gels with a pH range of 3.5 - 9.5 were purchased from LKB (Sweden). The gels were run on a LKB 2117 multiphor apparatus.

The gel was prefocused for one hour at which time 1 cm squares of filter paper soaked in the B-lactamase solution were placed on top of the gel. The B-lactamase samples had been partially purified by elution from a DEAE-Sephadex column. At the end of one hour of focusing the B-lactamases, the pH gradient was measured with a surface electrode and then the gel was immersed in a solution of chromogenic cephalosporin. The red colour produced upon reaction of the B-lactamase with the chromogenic cephalosporin was allowed to develop for 20 minutes at which time a photograph was taken. One gel was stained

for more than 60 minutes to look for chromosomally-mediated B-lactamases.

G. Preparation of Immune Serum

A lysozyme preparation of E. coli C600 (pJB1) was lyophilized and resuspended to give 6 mg/ml protein. 0.1 ml (25 units B-lactamase activity) was mixed with Freund's adjuvant and injected into the footpad of young rabbits. At weekly intervals for four weeks, 0.1 ml of antigen was injected intravenously. The rabbits were bled two weeks following the last injection.

H. Immunoelectrophoresis

Immunoelectrophoretic procedures were carried out on a LKB 2117 multiphor apparatus according to the method of Weeke (1973). For crossed immunoelectrophoresis, 5 - 10 µl of partially-purified B-lactamase from E. coli C600 (pJB1) was run in the first dimension for 20-30 minutes at 10 v/cm. The upper part of the gel not containing any enzyme was cut away and 12 ml of 1% agarose with 1 ml antiserum was poured. The B-lactamase was then run in the second dimension for 16 hours at 2 v/cm. The gel was stained for B-lactamase activity by immersion in a solution of chromogenic cephalosporin. After photographs had been taken, the gel was washed with saline and distilled water, dried, and stained for protein with Coomassie Brilliant Blue R250 (.25% in 7% acetic acid).

Tandem crossed immunoelectrophoresis was run using B-lactamase

from E. coli C600 (pJB1) as the reference enzyme and B-lactamases from B031, RP4 and RSF1030 as the sample enzymes to determine immunogenic relatedness. The technique was the same as described above, except two sample wells 1 cm apart were used instead of one well for crossed immunoelectrophoresis. Again, the gels were stained first for B-lactamase activity and then protein.

I. Plasmid Screening

The preparation of cleared lysates was by the method of Meyers et al (1976). For cultures of H. ducreyi and H. influenzae five plates of chocolate agar were swabbed with organisms and then scraped 18 hours later. Cultures of E. coli were grown in 30 ml of L-Broth for 18 hours at 37°C. All cells were washed in TES buffer (50 mM NaCl, 5 mM EDTA, 30 mM TRIS, pH 8.0).

Using Meyers' method, the cells were lysed with 10% sodium dodecyl sulfate (SDS), the protein was extracted with buffered phenol and the DNA precipitated with 95% ethanol. The DNA was resuspended in .2 ml of TES buffer and .05 ml of tracking dye (bromophenol blue (0.07%), SDS (7%), and glycerol (33%), in water). The plasmid DNA samples were now ready for agarose gel electrophoresis.

J. Agarose Gel Electrophoresis

Samples (1-25 µl) of ethanol precipitated DNA with tracking dye were subjected to electrophoresis on .7% agarose gels in TRIS-BORATE buffer (89 mM TRIS, 2.5 mM disodium EDTA and 8.9 mM boric

acid). Electrophoresis was carried out on a vertical slab gel with the samples placed in wells made with a lucite comb. The gels were run at 60 mA and 100 v until the tracking dye had reached the bottom of the gel. The gel was then placed in a solution of ethidium bromide (EtBr) in water (.4 µg/ml) and stained for 15-30 minutes.

The gels were made to fluoresce with a shortwave UV transilluminator and then photographed with a Polaroid camera using type 55 pos/neg film.

K. Calculation of Plasmid Molecular Weight

The molecular weight of the plasmid was estimated by plotting the logarithm of the relative distance migrated of standard plasmid DNA preparations through the gel, versus the logarithm of the plasmid molecular weight. The distance migrated by the test plasmid is known and when plotted on the graph, the molecular weight can be determined. Care was taken to not overload the sample as this affected the rate of migration and, therefore, the molecular weight estimation.

L. Purification of Plasmid DNA

This procedure was a modification of the method of Clewell and Helinski in which Triton X-100 (0.1% final concentration) was substituted for Brij 58 (Elwell et al, 1975). The cleared lysates of H. ducreyi or E. coli had 1 g of cesium chloride added per 1 ml of cleared lysate. To this was added .2 ml of 10 mg/ml ethidium bromide. The refractive index was adjusted with TES to approximately 1.3990 for

H. ducreyi and 1.3945 for *E. coli*. The tubes were spun at 100,000 g for 40 hours. The plasmids were drip fractionated from the bottom of the tube. The EtBr was extracted with CsCl-saturated isopropyl alcohol and the remaining plasmid dialyzed overnight against 50 mM TRIS pH 8.0. The purified plasmid DNA was stored at -20°C.

M. Restriction Endonuclease Digestions

The restriction endonucleases PstI, HincII, BamHI and HindIII were purchased from New England Biolabs. The reaction buffers for each enzyme are listed in Appendix III. The amount of purified plasmid DNA used depended upon the number of fragments produced by the digestion procedure and the concentration of plasmid DNA. Usually 2-15 µl of DNA was added to 1 µl of enzyme, 3 µl of buffer and made to 30 µl with distilled H_2O. The digestion reactions were carried out at 37°C for 90 minutes and stopped by adding tracking dye to a final concentration of 20%. The digests were run on .7% to 1.5% agarose gels, stained with ethidium bromide and photographed with the Polaroid camera.

The endonucleases PstI, HincII and BamHI were used to digest the various plasmids. HindIII was used to digest λ DNA into 7 fragments of known molecular weights from which molecular weight estimates of the plasmid fragments were made (Murray and Murray, 1975).

N. Transformation of *E. coli* C600

The procedure followed was that of Cohen et al (1972). *E. coli* C600 was grown for three hours in 30 ml of L-Broth and then washed

with .03 M $CaCl_2$. 0.1 ml of purified plasmid DNA (pJB1) was added to 0.2 ml of competent cells, incubated for one hour at 0°C and then heat pulsed at 42°C for two minutes for maximum uptake of DNA. L-Broth (2.7 ml) was added and the cells incubated for 3.5 hours at 37°C to allow expression of the resistance genes. At the end of this time, 0.1 ml aliquots were spread on MacConkey agar plates containing 25 µg/ml ampicillin, 8 µg/ml tetracycline or a combination of both. The plates were incubated for 18 hours at 37°C. Transformants were checked for B-lactamase production, plasmid content and minimum inhibitory concentrations to ampicillin and tetracycline. Controls consisted of competent cells with no DNA added and DNA with no cells added.

O. Genetic Mating Experiments

The object of these procedures was to determine if H. ducreyi strains could act as donors and recipients of plasmid DNA. The donor and recipient strains used are listed in Table V.

The method was to take fresh cultures of donor and recipient strains and suspend each in 1 ml of broth to 10^8CFU/ml. They were then poured in a 3 ml syringe and filtered onto a .45 µm membrane filter. The filter was placed culture side up on plain CA and incubated overnight. The next day the filter was vortexed in 1 ml of broth and .01 or .1 ml aliquots spread onto appropriate selective media. This included 2-5 µg/ml ampicillin, 4 µg/ml tetracycline and chloramphenicol, 1000 µg/ml streptomycin or 50 µg/ml nalidixic acid. Individual colonies were picked and passed a number of times on the selective

media. As well, the supposed transcipients were checked for plasmid content and B-lactamase production.

Table V. Plasmid Transfers

Donor		Recipient
H. duc 54198 Amp[1] (6.0)[2]	x	H. duc 35000 Sm
H. duc 54198 Amp (6.0)	x	H. inf R087 Sm
H. inf B031 Amp (30)	x	H. duc 35000 Sm
H. duc 35000 Amp, Sm (30)	x	H. inf B001 Nal
H. inf R230 Chl, Tet (34)	x	H. duc 54205
H. inf R230 Chl, Tet (34)	x	H. duc 54198 Amp (6.0)
H. duc 54205 Chl, Tet (34)	x	H. duc 35000 Sm
H. duc 54205 Chl, Tet (34)	x	H. inf R087 Sm

[1] Abbreviations: Amp (Ampicillin), Chl (Chloramphenicol), Sm (Streptomycin), Tet (Tetracycline), Nal (Nalidixic Acid). Designates resistance to these antibiotics.

[2] Molecular weight of plasmid (x 10^6 daltons).

RESULTS

A. Antimicrobial Susceptibility of *H. ducreyi*

From the initial susceptibility tests done by Hammond et al (1978), it was seen that three strains of *H. ducreyi* were resistant to ampicillin and one of those was also resistant to tetracycline. These tests were repeated on the same organisms and the results shown in Table VI.

It can be seen that three strains are resistant to ampicillin as was found by Hammond et al (1978). However, all three of these strains are also resistant to tetracycline in the range 8-32 µg/ml. When a loopful of each of the three ampicillin-tetracycline resistant strains was emulsified in .05 ml of chromogenic cephalosporin, the colour would change from yellow to red within a couple of minutes. This indicates production of B-lactamase by these three strains.

B. Substrate Profile

The substrate profile for the B-lactamases is given in Table VII. The *H. ducreyi* B-lactamase shows good activity against penicillin and ampicillin with a much lower rate of hydrolysis of cephalothin. It is inhibited by cloxacillin but does not show inhibition in the presence of pCMB. This type of substrate profile is suggestive of a TEM B-lactamase. Using the same assay methods, the B-lactamases coded by the plasmids RSF1030 (TEM-1) and RP4 (TEM-2) appear very similar to the *H. ducreyi* B-lactamase. This also suggests the *H.*

Table VI. Minimum Inhibitory Concentrations of *H. ducreyi* for Ampicillin and Tetracycline

Strain Number	Minimum Inhibitory Conc (µg/ml)	
	Ampicillin	Tetracycline
35000	1.0	0.5
35001	1.0	0.5
35199	0.5	0.5
36652	1.0	0.5
78118	1.0	1.0
78226	1.0	0.5
82038	0.5	0.25
54182	0.5	0.25
54183	1.0	0.5
54189	1.0	0.5
54198[a]	> 128	8.0
54201	1.0	0.25
54202	2.0	1.0
54204	1.0	0.25
54205	1.0	0.5
54207[a]	> 128	32
54209	1.0	0.25
54211[a]	> 128	16
54213	1.0	0.5
54220	1.0	0.25
54239	0.5	0.5

[a] These strains contained a plasmid and produced a B-lactamase.

Table VII. Substrate Profile of Crude

B-Lactamase from Various Sources

Source of B-Lactamase	Relative Rate of Hydrolysis[1]: Penicillin	Ampicillin	Carbenicillin	Cloxacillin	Cephalothin	Inhibited by[2]: Cloxacillin	pCMB
H. ducreyi	100	100	9	1	11	S	R
H. influenzae	100	100	11	1	11	S	R
E. coli C600 (pJR1)	100	87	8	1	9	S	R
E. coli (RP4)	100	92	9	1	9	S	R
E. coli (RSF1030)	100	97	8	1	10	S	R

[1] The rates of hydrolysis of ampicillin, carbenicillin, cloxacillin and cephalothin are relative to the rate of hydrolysis of penicillin which was set at 100.

[2] The B-lactamase is sensitive (S) or resistant (R) to inhibition by these compounds.

ducreyi B-lactamase is a TEM-type B-lactamase. There also appears to be no apparent change in substrate specificity when the B-lactamase was derived from H. ducreyi or the transformant E. coli C600 (pJB1).

C. Isoelectric Focusing

The isoelectric focusing pattern for several B-lactamases is shown in Figure 3. The isoelectric point for the B-lactamase from the transformant E. coli C600 (pJB1) was similar to the isoelectric point of the RSF1030 TEM-1 B-lactamase and the B-lactamase from the ampicillin resistant H. influenzae strain B031. The B-lactamase from RP4, a TEM-2 type B-lactamase focused at a slightly higher isoelectric point. Satellite bands as noted by others (Brive et al, 1977) were seen both above and below the main band. Although the gels were stained with chromogenic cephalosporin for up to one hour, no chromosomal B-lactamases were seen in the pH range of 7-9.

D. Immunoelectrophoresis

Crossed immunoelectrophoresis of partially purified B-lactamase from E. coli C600 (pJB1) with the homologous antiserum showed five precipitin lines. By staining first for B-lactamase activity with chromogenic cephalosporin the B-lactamase precipitin line was identified (Figure 4).

The tandem crossed immunoelectrophoresis technique allowed us to show immunological similarity between the B-lactamases from E. coli

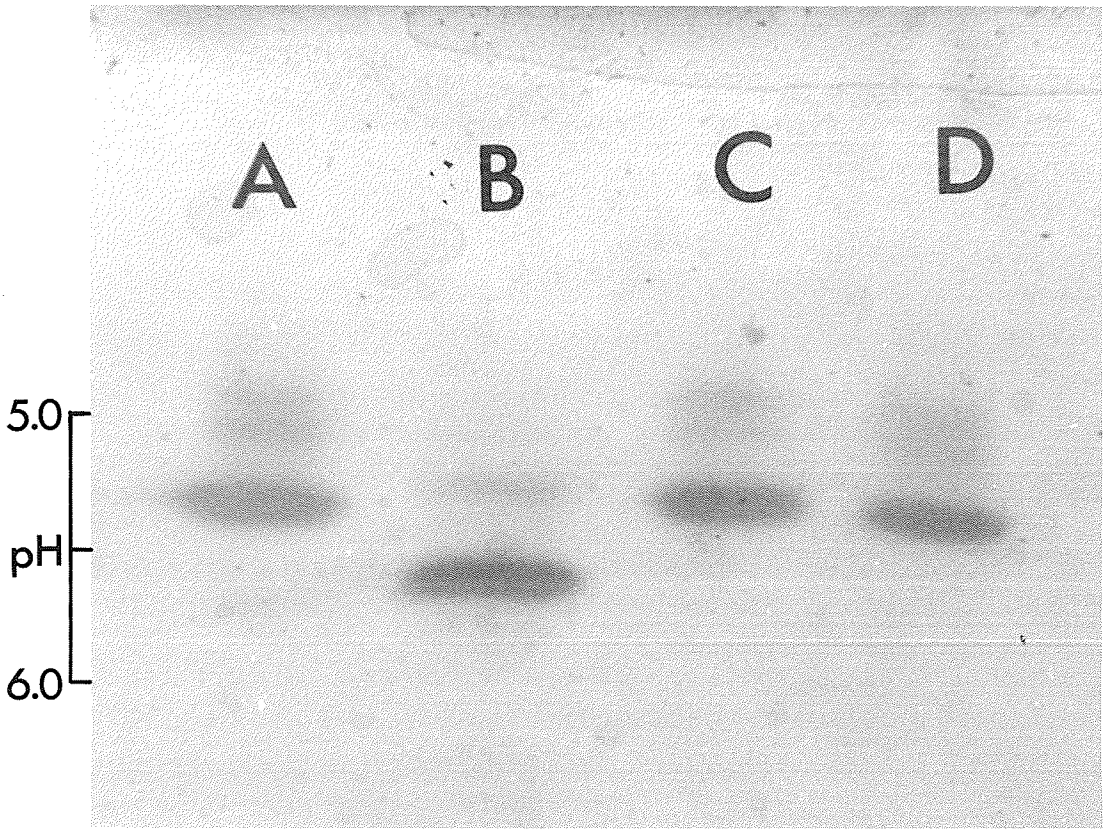


Figure 3. Partially purified B-lactamases from the following strains were isoelectrically focused and then stained with chromogenic cephalosporin.

A. *E. coli* C600 (pJR1)
B. *E. coli* (RP4)
C. *E. coli* (RSF1030)
D. *H. influenzae* B031

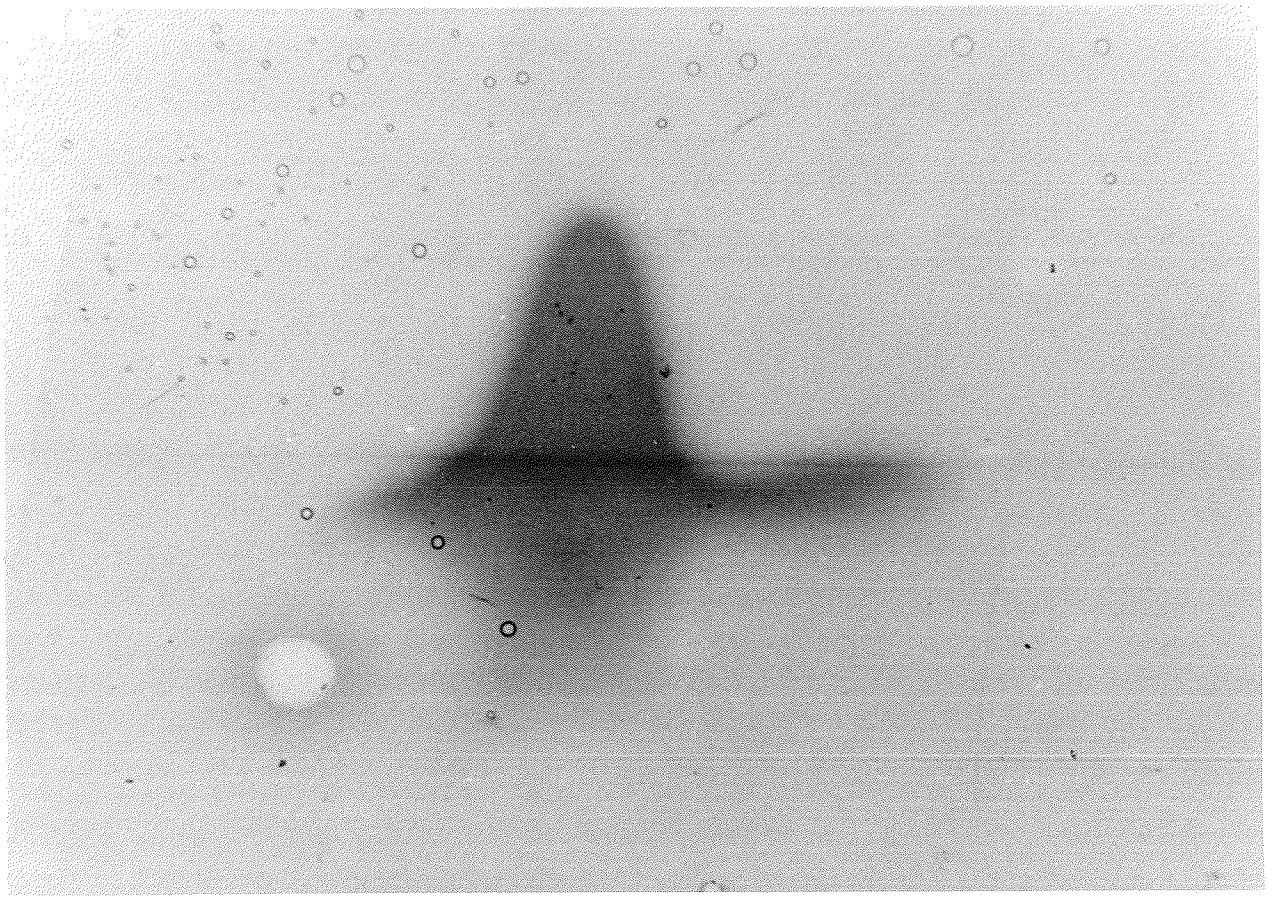


Figure 4. Crossed immunoelectrophoresis of B-lactamase from E. coli C600 (pJB1) stained with chromogenic cephalosporin. The B-lactamase was electrophoresed into agar containing antiserum against the B-lactamase from E. coli C600 (pJB1) producing the precipitin line seen.

C600 (pJB1) and each of the B-lactamases from H. influenzae B031, RP4 and RSF1030 (Figure 5). The E. coli C600 (pJB1) B-lactamase was used as the reference enzyme and run with one of the other B-lactamases as the test enzyme. The antiserum was prepared against the E. coli C600 (pBJ1) B-lactamase and the E. coli C600 (pJB1) B-lactamase could not be distinguished immunologically by this method from the B-lactamases of H. influenzae B031, RP4 and RSF1030.

E. Agarose Gel Electrophoresis of Cleared Lysates

The 21 strains of H. ducreyi were screened for the presence of plasmid DNA. The only plasmids seen were those found in the three ampicillin-tetracycline resistant strains (Figure 6). None of the other strains were shown to contain any plasmid DNA.

The plasmid DNA was purified and run in an agarose gel with known molecular weight standards (Figure 7). The molecular weight calculated from its electrophoretic mobility was 6.0 Mdal This was confirmed by contour length measurements of the plasmid nicked with DNAse which gave an estimate of 5.9 Mdal (Brunton et al, 1979). All three plasmids from the three resistant strains were of the same molecular weight. The plasmid from H. ducreyi 54198 was designated pJB1.

F. Restriction Endonuclease Digestion

The plasmid DNA from the three B-lactamase producing strains of H. ducreyi was purified and analyzed by electrophoresis of

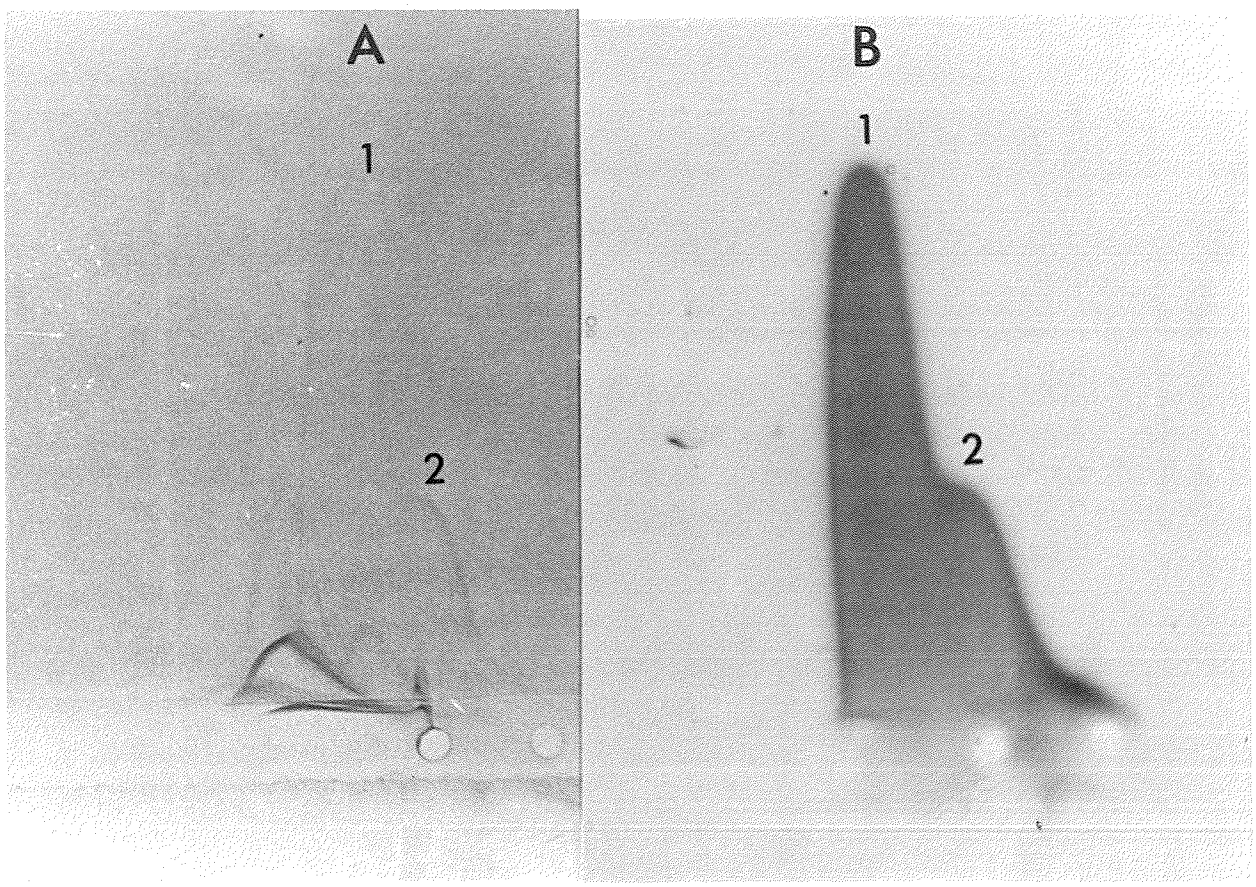


Figure 5. A: Tandem crossed immunoelectrophoresis of B-lactamases from (1) RSF1030 and (2) pJB1 stained for protein showing a number of precipitin lines and a line of identity between the two B-lactamases.

B: The same gel as in (A) stained with chromogenic cephalosporin. Similar patterns were obtained when the B-lactamases from the strain carrying RP4 or the H. influenzae B031 were run in tandem with the enzyme from E. coli C600 (pJB1).

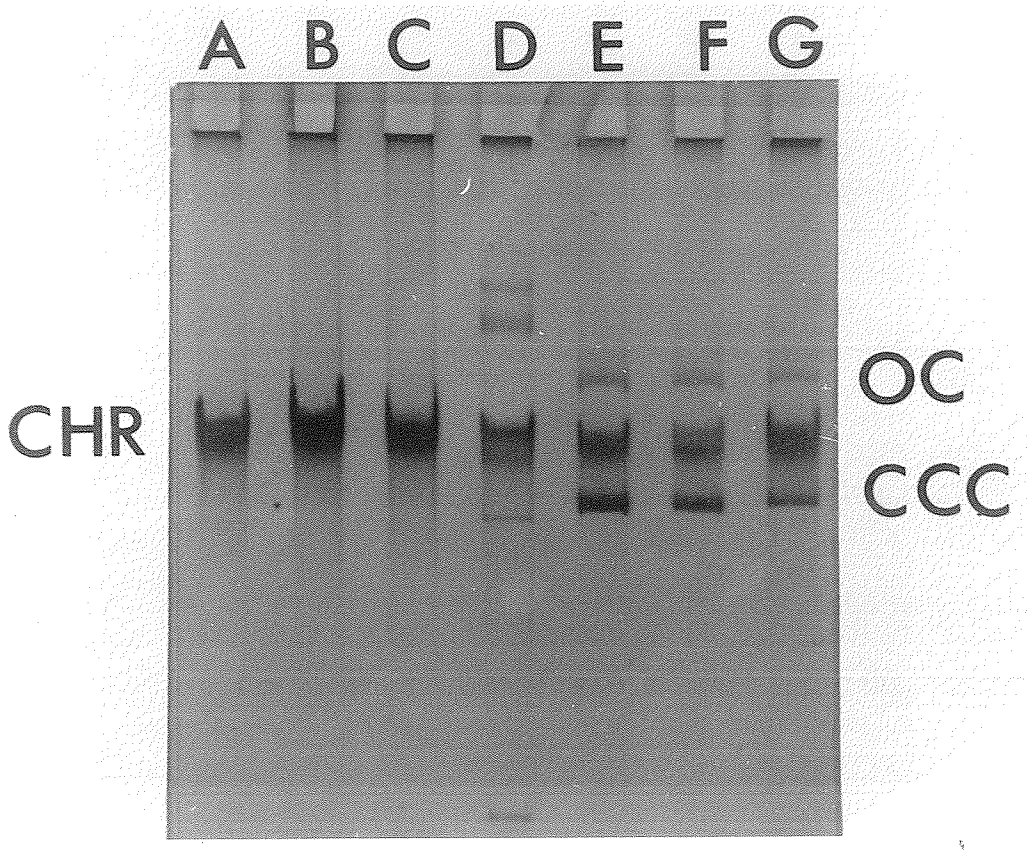


Figure 6. Agarose gel (.7%) of DNA screened from the following H. ducreyi strains isolated in Winnipeg. A. 54205, B. 35000, C. 36652, E. 54198, F. 54211, G. 54207. The first three antibiotic-sensitive strains (A,B and C) have only chromosomal (CHR) DNA while the last three antibiotic resistant strains (E,F and G) have chromosomal DNA as well as covalently closed circular (CCC) or plasmid DNA of about 6.0×10^6 daltons. Open circular (OC) forms of the plasmid are seen above the chromosome. Column D contains plasmids used as molecular weight standards.

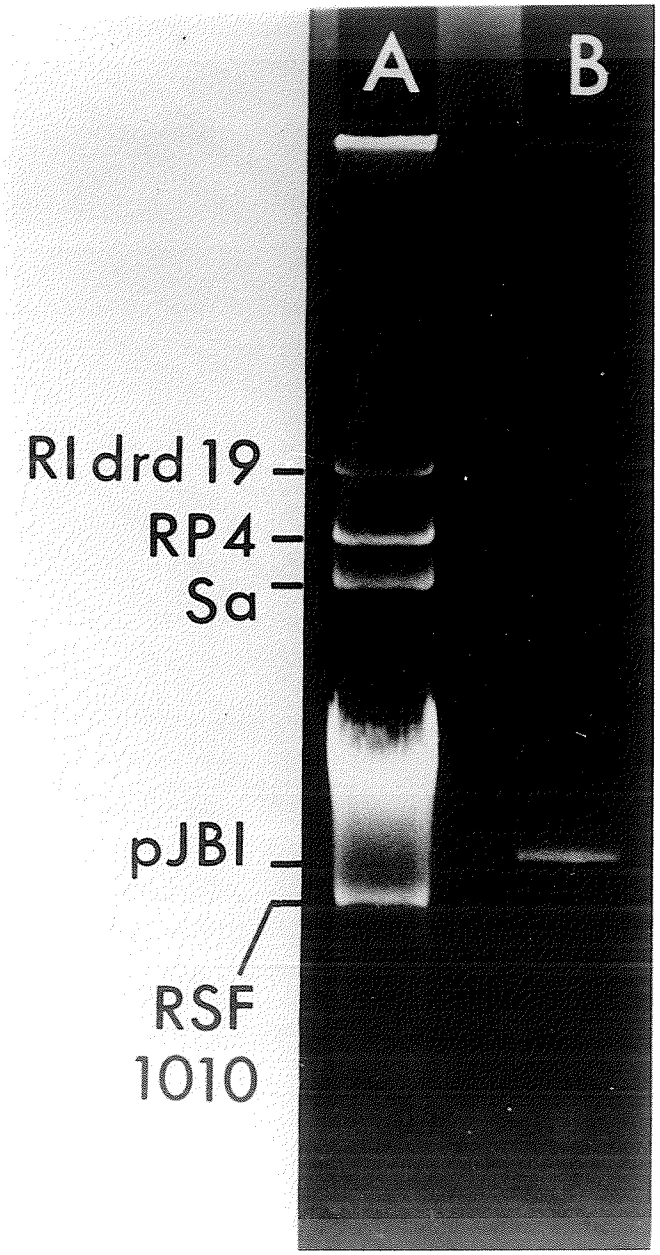


Figure 7. Agarose gel (.7%) of the purified plasmid pJB1 from H. ducreyi strain 54198.

A. Molecular weight standards Rldrd19, 62 x 10^6; RP4, 34 x 10^6; Sa, 23 x 10^6; RSF1010, 5.5 x 10^6.

B. pJB1 plasmid DNA with molecular weight of 6.0 x 10^6.

fragments produced by restriction endonuclease digestion (Figures 8 and 9). Identical fragmentation patterns were produced for the plasmid DNA isolated from all three strains for each enzyme used.

The digestion pattern produced by HincII was used to compare pJB1 DNA with the plasmids RSF1030, RSF1010::Tn1 Ap101, RSF1010::Tn1 Ap111, RSF1010, and RSF1010::Tn3 Ap230. The RSF1010::Tn1 plasmids differ from RSF1010 only in having the Tn1 transposon inserted in RSF1010 (Heffron et al, 1975a). The plasmid designated RSF1010::Tn1 Ap101 differs from the other designated RSF1010::Tn1 Ap111 only in the location of insertion of the transposon Tn1 (Heffron et al, 1975a). RSF1030 is a plasmid containing the whole of the ampicillin transposon Tn2 (Campbell et al, 1977). The remaining plasmid RSF1010::Tn3 Ap230 has the whole of the transposon Tn3 inserted into the plasmid RSF1010.

There are a number of fragments formed with HincII digestion as seen in Figure 10. The two fragments labelled 1 and 2 are common to pJB1, the two RSF1010::Tn1 plasmids and RSF1030. They are not present in RSF1010 or RSF1010::Tn3 Ap230. The molecular weight estimate of fragments 1 and 2 is 1.0 Mdal and 0.7 Mdal respectively. As well, there are three more fragments produced from RSF1010::Tn1 than from RSF1010, suggesting there are three HincII cleavage sites within Tn1 and Tn2 and these sites are present in pJB1.

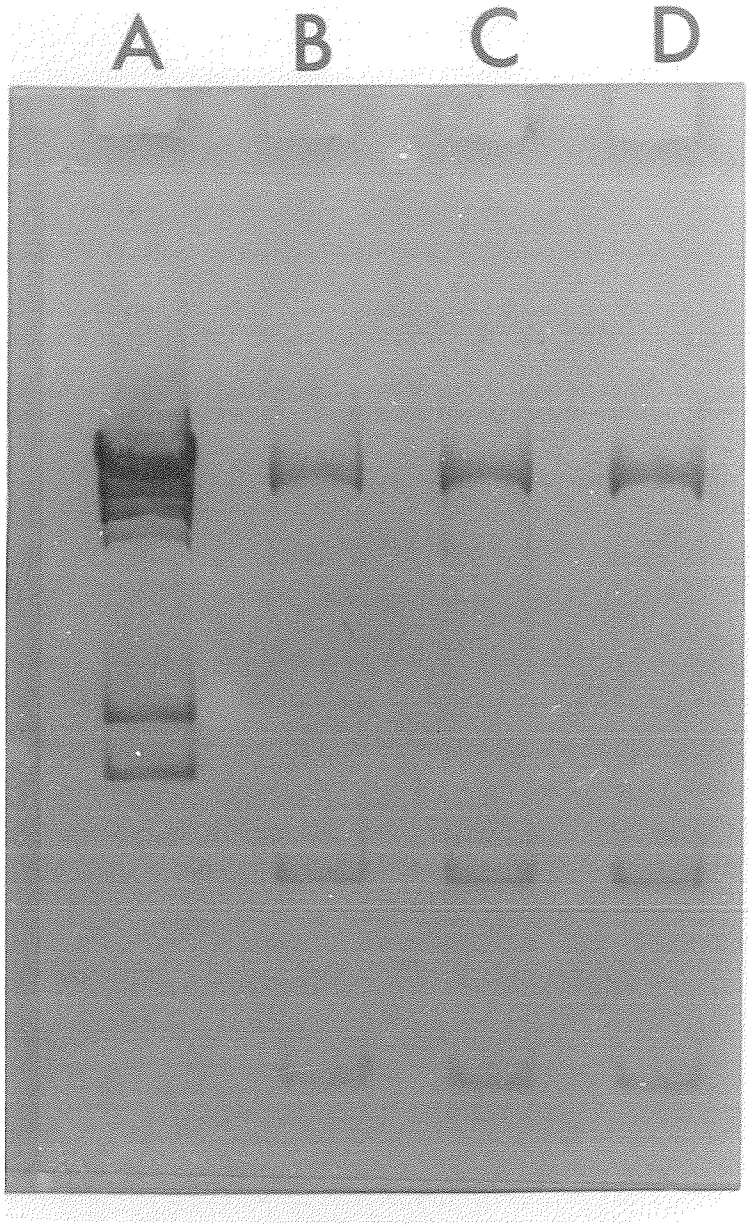


Figure 8. Agarose gel (1.5%) of the following: A. ↑ DNA digested with restriction endonuclease HindIII. B., C., and D. Plasmids from H. ducreyi strain 54198, 54211 and 54207 respectively digested with the restriction endonuclease HincII.

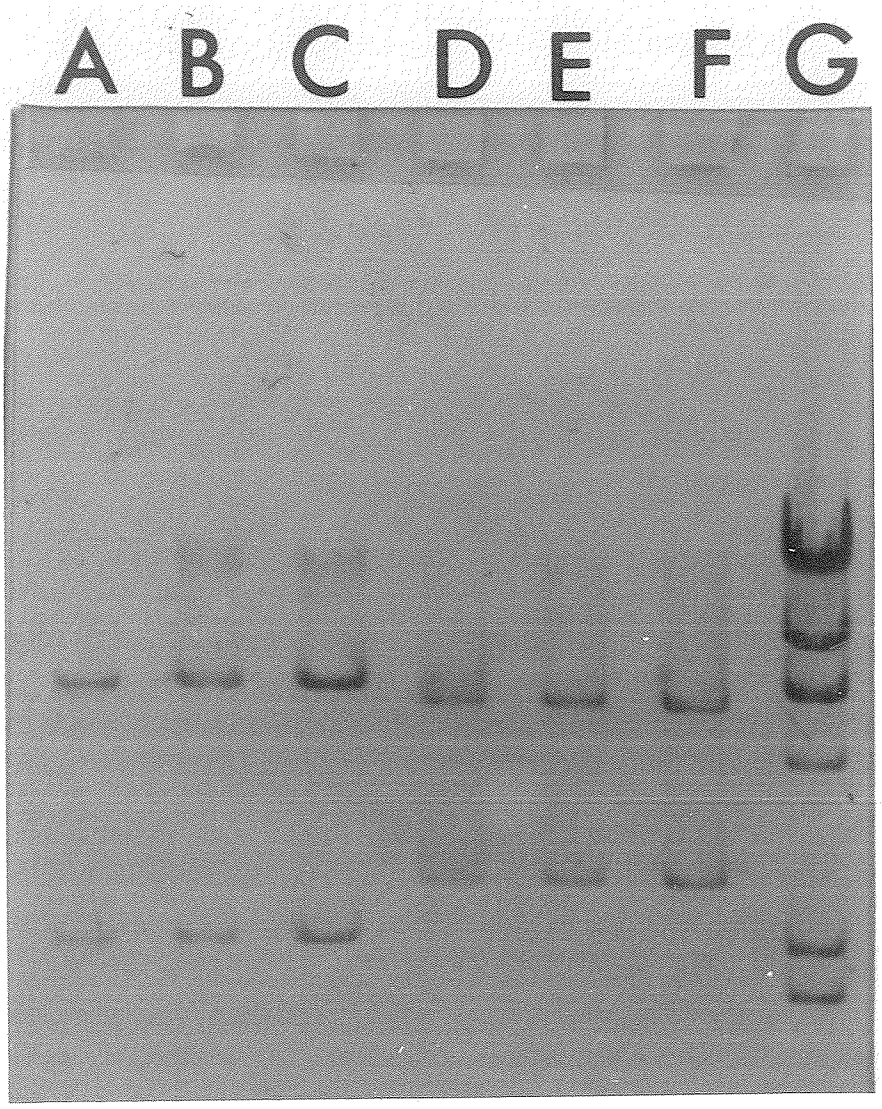


Figure 9. Agarose gel (.7%) of plasmid DNA from H. ducreyi strains 54198 (A and D), 54211 (B and E) and 54207 (C and F) digested with the restriction endonucleases BamHI (A,B, and C) and Pst (D,E, and F). DNA digested with the restriction endonuclease HindIII is in Column G.

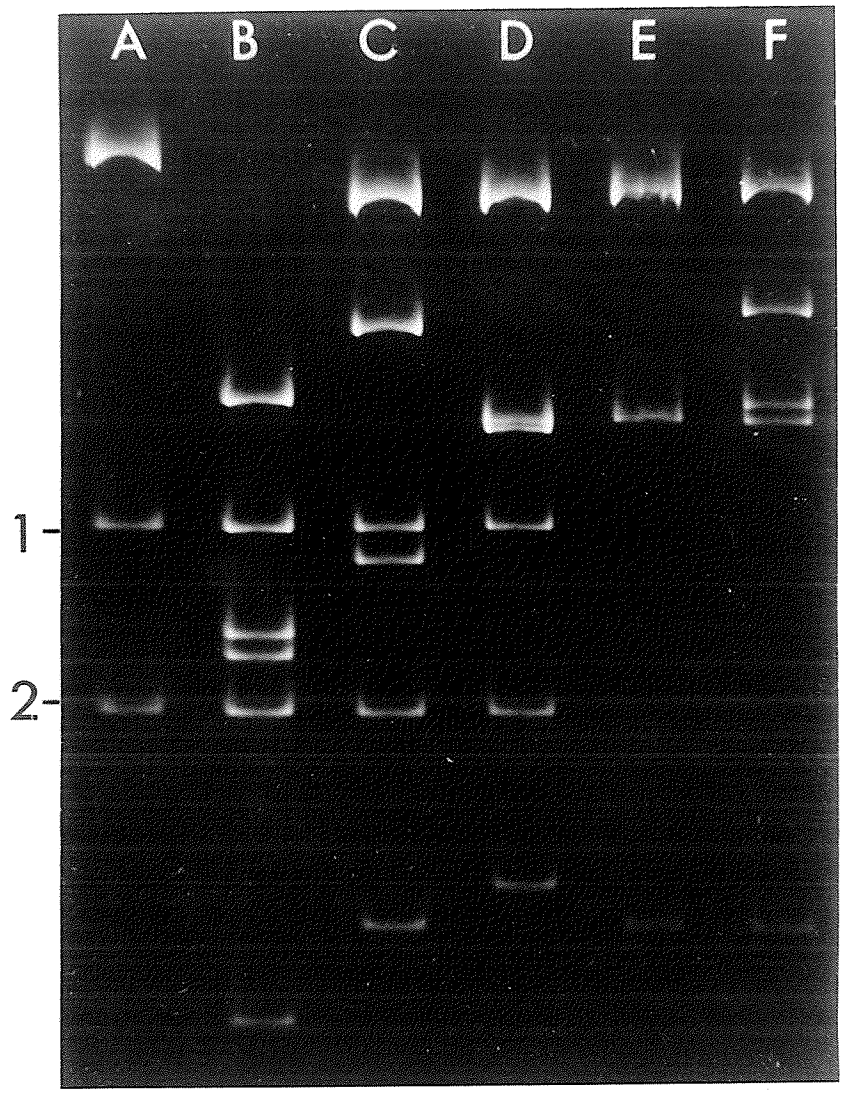


Figure 10. Agarose gel (1.5%) of HincII endonuclease digests. A. pJB1; B. RSF1030; C. RSF1010::Tn1 (Ap111); D. RSF 1010::Tn1 (AP101); E. (RSF1010); F. RSF1010::Tn3 (Ap230). Molecular weight estimates of fragments 1 and 2 are 1.0×10^6 and 0.7×10^6, respectively.

G. Transformation of *E. coli* C600

The purified plasmid DNA from the three *H. ducreyi* strains was successfully transformed into *E. coli* C600. The transformant was designated *E. coli* C600 (pJB1). When minimum inhibitory concentrations were done on the transformants, only ampicillin resistance was shown to be transferred while tetracycline sensitivity remained the same as the recipient *E. coli* C600.

Purified plasmid DNA from the transformants was digested with the restriction endonucleases HincII, BamHI and PstI. These fragmentation patterns were compared to the patterns from digests of *H. ducreyi* plasmid DNA and appeared to be identical.

H. Mating Experiments

The results are summarized in Table V. These experiments were undertaken to try and transfer the small plasmid from *H. ducreyi* to other *H. ducreyi* and *H. influenzae* strains. We were unable, after a number of attempts, to demonstrate transfer of this plasmid to recipient organisms.

Attempts to transfer a large plasmid into the B-lactamase producing strains of *H. ducreyi* also failed. It was hoped that the large plasmid could perhaps mobilize the small *H. ducreyi* plasmid such that it would transfer to a recipient organism.

Other strains of *H. ducreyi* were able to act as recipients of large plasmids from *H. influenzae* and in turn donate these large plasmids to recipient strains of *H. ducreyi* and *H. influenzæ*.

Table V. Plasmid Transfer Results

Donor		Recipient	Transcipient
H. duc 54198 Amp[1] (6.0)[2]	x	H. duc 35000 Sm	—
H. duc 54198 Amp (6.0)	x	H. inf R087 Sm	—
H. inf B031 Amp (30)	x	H. duc 35000 Sm	H. duc 35000 Amp Sm (30)
H. duc 35000 Amp, Sm (30)	x	H. inf B001 Nal	H. inf B001 Amp, Nal (30)
H. inf R230 Chl, Tet (34)	x	H. duc 54205	H. duc 54205 Chl, Tet (34)
H. inf R230 Chl, Tet (34)	x	H. duc 54198 Amp (6.0)	—
H. duc 54205 Chl, Tet (34)	x	H. duc 35000 Sm	H. duc 35000 Chl, Tet, Sm (34)
H. duc 54205 Chl, Tet (34)	x	H. inf R087 Sm	H. inf R087 Chl, Tet, Sm (34)

[1] Abbreviations: Amp (Ampicillin), Chl (Chloramphenicol), Sm (Streptomycin), Tet (Tetracycline), Nal (Nalidixic Acid). Designates resistance to these antibiotics.

[2] Molecular weight of plasmid (x 10^6 daltons).

DISCUSSION

A. Introduction

The purpose of these studies was to characterize the B-lactamase produced by ampicillin and tetracycline resistant strains of H. ducreyi and to determine the genetic basis of the antibiotic resistance in these same organisms.

B. Characterization of the B-lactamase from H. ducreyi

One of the first procedures carried out when examining an unidentified B-lactamase is to evaluate the hydrolytic activity of the enzyme against various penicillins and cephalosporins. From these data the substrate profile is determined and the B-lactamase can be grouped into one of a number of classes as suggested by Richmond and Sykes (1973).

The iodometric assay of Sawai et al (1978) was used for our evaluation of the B-lactamase produced by H. ducreyi. The assay originally used a Klett-Summerson colorimeter but was adapted by us for use in a Pye-Unicam Spectrophotometer. The reagents were easily obtained and when made up to the working solutions, were stable over long periods of time. The only drawback was that a precipitate was formed when hydrolyzed cephaloridine was mixed with the iodine reagent so this antibiotic was not included in the substrate profile.

There was no purification of the B-lactamases used in the substrate profiles other than discarding the cell material after

sonication and centrifugation. Along with the H. ducreyi B-lactamase the B-lactamases from RSF1030 and RP4 were included in the profile as they have been previously characterized as TEM-type B-lactamases (Matthew and Hedges, 1976). The B-lactamase from the ampicillin resistant H. influenzae strain was also assayed as previously described strains have been shown to harbour large plasmids, some of which code for a TEM B-lactamase (Saunders et al, 1978). Finally, the B-lactamase from the transformant E. coli C600 (pJB1) was assayed to look for any differences caused by host modification of the enzyme.

The B-lactamase from H. ducreyi proved to have a substrate profile similar to that of a TEM-type B-lactamase. It would fall under class III of Richmond and Sykes' (1973) grouping scheme. The rate of hydrolysis values from our substrate profile differed from those of Richmond and Sykes (1973) but were close to those of Matthew (1979). This demonstrates the need for inclusion of previously characterized enzymes to eliminate any discrepancies caused by different assay methods. The B-lactamase from H. influenzae B031 appears to be a TEM-type B-lactamase while the B-lactamase from the transformant E. coli C600 (pJB1) shows no major differences in substrate profile when compared to the original H. ducreyi B-lactamase.

Isoelectric focusing has been used to differentiate plasmid-mediated B-lactamases from gram-negative bacteria (Matthew, 1979). It is especially helpful when looking at the two TEM-B-lactamases which have identical substrate profiles but different isoelectric points (Matthew and Hedges, 1976). When the B-lactamases used in

the substrate profiles were isoelectrically focused, two main B-lactamase bands at different isoelectric points were seen. The B-lactamases derived from H. ducreyi, H. influenzae and RSF1030 were at one point while the B-lactamase of RP4 banded at a slightly higher isoelectric point. Although we could not get precise readings opposite each B-lactamase band, the bands did fall within the pH 5 - pH 6 range expected for TEM B-lactamases.

The isoelectric focusing pattern shows the B-lactamases of H. ducreyi and H. influenzae to be TEM-1 B-lactamases. This enzyme is the one most frequently encountered in gram-negative bacteria whose B-lactamases are plasmid-mediated (Matthew, 1979). Saunders et al (1978) earlier reported that all clinical isolates of ampicillin-resistant H. influenzae produced a TEM-1 B-lactamase so our results fit in with the present trend. However, no B-lactamase bands at pH 7.2 representing chromosomally-mediated B-lactamases as previously mentioned by these same authors were seen.

Satellite bands both above and below the main B-lactamase bands were noticed on the isoelectrically focused gels. The TEM-1 and TEM-2 satellite bands were not similar to each other. These bands which represent microheterogeneity of the TEM B-lactamases have been reported before (Matthew 1975; Brive et al, 1977). The latter author suggested the position of the satellite bands may vary, depending on the host organism in which the plasmid coding for the B-lactamase resides.

The B-lactamase from H. ducreyi was shown to be immunologically similar to both of the TEM B-lactamases and to the B-lactamase of H.

influenzae B031, using the technique of tandem crossed immunoelectrophoresis. This procedure is quite sensitive as the antigens (B-lactamases) are electrophoresed side-by-side in one direction and then electrophoresed into a gel containing the antiserum at right angles to the original direction. If the B-lactamases are similar a line of identity will be formed much in the same manner as that of the Ouchterlony double diffusion method.

No immunological cross-reaction has been shown between TEM antiserum and other B-lactamases (Jack and Richmond, 1970; Matthew et al, 1975). The two TEM B-lactamases have previously been reported to be immunologically similar (Sykes and Richmond, 1970; Matthew et al, 1975). The only difference between the two enzymes is the isoelectric point which could be due to a single amino acid substitution (Sutcliffe, 1978). This would not necessarily change the immunological reaction between the antiserum and the B-lactamase.

C. The Genetic Basis for Antibiotic Resistance in *H. ducreyi*

When antibiotic resistance suddenly appears in a specific species of bacteria which has traditionally been sensitive to antibiotics, plasmid-mediated drug resistance is one explanation. An example is the emergence of ampicillin resistance in *H. influenzae* (Khan et al, 1974) and *N. gonorrhoeae* (Phillips, 1976). On the whole, for these two organisms the resistant isolates possessed plasmids which carried the ampicillin resistance genes (Elwell et al, 1975; Elwell et al, 1977a). However, some of the sensitive isolates were

also found to contain plasmids which had no identifiable function (cryptic) in the cell (Elwell et al, 1977b; Sox et al, 1978). Therefore, to determine the genetic basis of antibiotic resistance in a given bacterial species, a number of sensitive and resistant isolates must be examined for the presence of plasmid DNA.

The gram-negative bacterium H. ducreyi was originally sensitive to various antibiotics (Reymann, 1949) until the more resistant cases of chancroid arose during the Vietnam War (Marmar, 1972). As these organisms are difficult to grow, it has not been until recently (Hammond et al, 1978) with consistent isolation of H. ducreyi from clinical cases of chancroid that a number of antibiotic sensitive and resistant strains were collected to screen for their plasmid DNA content.

Initially, 21 strains of H. ducreyi from Winnipeg were examined for plasmid DNA. A single plasmid of molecular weight 6 Mdal was seen in three isolates which were resistant to ampicillin and tetracycline. The other 18 sensitive strains did not harbour any detectable plasmid DNA. Since that time, a number of other isolates from various parts of the world have been screened for plasmid DNA (Figure 11). Six isolates of H. ducreyi from Atlanta, Georgia resistant to ampicillin and tetracycline each had two plasmids of molecular weight 4.2 and 6.0 Mdal. An isolate from Sweden resistant to ampicillin had a plasmid similar in size to the Winnipeg plasmid while three multiply resistant strains (ampicillin, tetracycline and chloramphenicol) from Seattle were found to harbour a plasmid of 7 - 10

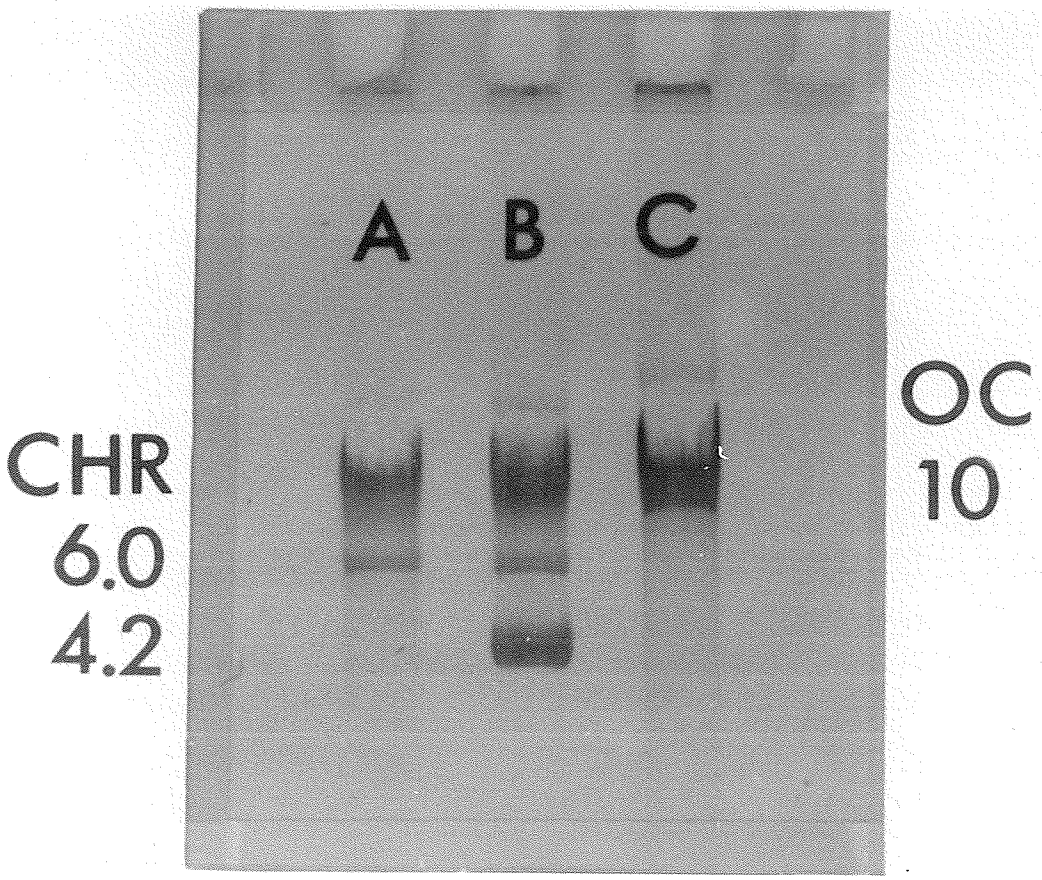


Figure 11. Agarose gel (.7%) of plasmids screened from H. ducreyi strains originating in A. Winnipeg, B. Atlanta and C. Seattle. The chromosomal band (CHR) is common to all, while plasmids of 6.0 x 10^6 daltons are seen in A and B, with plasmids of 4.2 x 10^6 and 10 x 10^6 daltons seen only in B and C respectively. Bands above the chromosomal DNA represent the open circle (OC) form of the plasmids.

Mdal . Three new sensitive isolates did not possess any plasmid DNA. This data would suggest that antibiotic resistance in isolates of H. ducreyi is likely to be plasmid-mediated.

Cryptic plasmids may play a part in the emergence of antibiotic resistance in an organism. They have the potential to act as recipients of transposable pieces of DNA carrying resistance genes. If such an event occurs a stable resistance factor would be produced as the host organism would recognize the plasmid as its own, even though it contained a small piece of foreign DNA.

Very few cryptic plasmids have been described for the Haemophilus spp. or N. gonorrhoeae. A 26 Mdal plasmid was found in an H. influenzae strain while two small plasmids with molecular weights of 2.7 and .75 Mdal were found in a single isolate of H. parainfluenzae (Elwell et al, 1977b). Recently cryptic plasmids of molecular weight 4.6, 2.0 and 1.7 Mdal were seen in strains of H. influenzae isolated in Winnipeg (L. Slaney, personal communication). The strain with the 2.0 Mdal plasmid was also found to contain a large conjugative 30 - 34 Mdal plasmid. A plasmid of 2.7 Mdal and a larger 25 Mdal plasmid associated with transfer ability but not antibiotic resistance have been described for isolates of N. gonorrhoeae (Baron et al, 1977). As stated earlier, no cryptic plasmids were seen in our isolates of H. ducreyi.

Another way in which an organism may acquire plasmid-mediated antibiotic resistance is by receipt of a plasmid by conjugation with the resistant donor organism. In our studies, H. ducreyi could act

as both a donor and recipient of plasmid DNA which had the proper information to allow conjugal transfer. The small H. ducreyi plasmid would not appear to have arrived by this method but it may have been part of a larger plasmid which was restricted with endonucleases to its present size, or it may have been mobilized from another strain.

The plasmid from H. ducreyi strain 54198 was designated pJB1. It was purified and used to transform an E. coli strain in hopes of determining which resistance genes resided on the plasmid. The resulting transformant was only resistant to ampicillin, suggesting the gene coding for the B-lactamase resides on the plasmid while the gene coding for tetracycline resistance might be integrated with the chromosomal DNA. As the characterization of the H. ducreyi B-lactamase identified a TEM-1 B-lactamase which is coded by an ampicillin transposon we looked to see if a TnA was on pJB1 using restriction endonucleases.

The analysis of plasmids with restriction endonucleases has been used before on plasmids purified from H. influenzae (Harkness and Murry, 1978) and N. gonorrhoeae (Sox et al, 1978). The plasmids were digested and the fragments electrophoresed on agarose gels such that common fragments could be identified and any relationship between the plasmids established. This procedure is precise as each restriction enzyme has its own sequence of nucleotide bases which it recognizes. Any change within the recognition site or change of position of the recognition site on the plasmid will produce a different fragmentation pattern (Roberts, 1976).

When the H. ducreyi plasmid and a number of other plasmids containing the whole of the transposons Tnl, Tn2 and Tn3 were cleaved with HincII it was seen that pJBl had two fragments in common with the plasmids carrying the transposon Tnl and Tn2. As RSF1010 by itself produced three fragments and RSF1010::Tnl gave six fragments then the transposon Tnl must have three HincII sites. These three sites appear to be on the pJBl and RSF1030 plasmids. RSF1010:Tn3 had only five fragments, suggesting two HincII cleavage sites. Heffron (1977) has shown that Tn3 does indeed have two HincII sites.

The two fragments from within the ampicillin transposon on pJBl amount to about 60% of the transposon Tn2. This fact is significant as a number of small plasmids isolated from H. influenzae (pVe445), H. parainfluenzae (RSF0885) and N. gonorrhoeae (pMR0200 and pMR0360) also contain portions of the ampicillin transposon Tn2 (Lanfs et al, 1979). The small plasmids of the latter three organisms are highly homologous ($\rangle$ 80% homology) and all have a similar guanine plus cytosine (G + C) content of about 41% (Laufs et al, 1979). The H. ducreyi plasmid pJBl also has a G + C content of 41% (Brunton et al, 1979) which again links this plasmid with the others. The one distinguishing point is that pJBl has at least 60% of the Tn2 while the other small plasmids have only 30-40% of the Tn2 (Laufs et al, 1979).

All of this information suggests that the small plasmids of H. parainfluenzae, H. influenzae, H. ducreyi and N. gonorrhoeae carrying the ampicillin transposon Tn2 may be related. The

transposon Tn2 may have integrated into a yet to be described cryptic plasmid with this new plasmid disseminating to the Haemophilus spp. and N. gonorrhoeae. The various sizes ranging from 3.2 - 6.0 Mdal could be due to restriction processes within the individual recipient organisms. Just how much these plasmids are related can be determined by carrying out DNA homology and DNA hybridization experiments between pJB1 and the other small plasmids of the Haemophilus spp. and N. gonorrhoeae.

D. Summary

1. Three of 21 strains of *H. ducreyi* isolated in Winnipeg were resistant to ampicillin and tetracycline.

2. Ampicillin resistance was due to the production of a TEM-1 type B-lactamase.

3. The gene coding for the B-lactamase resides on a 6.0 Mdal plasmid present in the three antibiotic resistant strains of *H. ducreyi*. The plasmid from one of these strains was designated pJB1.

4. Although this plasmid was non-conjugative, two strains of *H. ducreyi* were able to act as donors and recipients of conjugative plasmids common to antibiotic-resistant strains of *H. influenzae*.

5. The plasmid pJB1 contains at least 60% of the ampicillin transposon Tn2.

6. This plasmid may be similar to other small ampicillin resistance plasmids found in certain strains of *H. influenzae*, *H. parainfluenzae* and *N. gonorrhoeae*.

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APPENDIX I

Growth Media

a. Chocolate Agar

GC agar base	36 g
Bovine hemoglobin	10 g
Distilled H_2O	1,000 ml
CVA enrichment	10 ml

b. L-Broth pH 7.5

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
1M $MgSO_4$	1 ml
.1M $CaCl_2$	1 ml
40% glucose	30 ml
Distilled H_2O to	1,000 ml

c. Low phosphate broth pH 7.4

Tris	.12 M
NaCl	.08 M
Na_2SO_4	3 mM
KCl	.02 M
$MgCl_2$	1 mM
$CaCl_2$	.1 mM
Bacto-peptone	.5%
Glucose	.5%

APPENDIX II

Iodine Reagent

1. Stock Iodine Solution

Iodine	20.3	g
Potassium iodide	100.0	g
Distilled H_2O	500	ml

2. Acetate Buffer

Anhydrous sodium acetate	80	g
Adjust with acetic acid to pH 4.0		
Distilled H_2O make to	2	l

3. Iodine reagent

5 ml stock iodine solution
95 ml acetate buffer

5 ml of this reagent contains 40 umoles I_2

APPENDIX III

Buffers for Restriction Endonucleases:

A. Hind III Buffer

1M TRIS-HCl pH 7.4	1.0 ml
5.0M NaCl	1.2 ml
.6M $MgCl_2$	1.16 ml
5.0 mg/ml Bovine Serum Albumin	2.0 ml
Distilled H_2O make to	10 ml

B. Hinc II Buffer

1M TRIS-HCl pH 7.9	1 ml
.6M $MgCl_2$	1.1 ml
.6M B-mecaptoethanol	1.0 ml
5.0M NaCl	1.2 ml
5.0 mg/ml Bovine Serum Albumin	2.0 ml
Distilled H_2O make to	10 ml

C. Pst and Bam HI Buffer

.6M TRIS-HCl pH 7.4	1 ml
5M NaCl	1 ml
.6M $MgCl_2$	1 ml
5 mg/ml Bovine Serum Albumin	2 ml
Distilled H_2O make to	10 ml