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CHARACTERISATION OF PLASMIDS CONFERRING AMPICILLIN RESISTANCE IN SOUTH AFRICAN ISOLATES OF *HAEMOPHILUS DUCREYI*

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ABSTRACT

Characterisation of plasmids conferring ampicillin resistance in South African isolates of *Haemophilus ducreyi*.

Fifty-two strains of *Haemophilus ducreyi* from various geographic regions of southern Africa (Botswana, Lesotho, Namibia, Gauteng, Natal and Transkei) isolated between 1988 to 1994 were tested for susceptibilities to five antimicrobial agents and characterized according to their plasmid content and ampicillin-resistance genes.

Minimum inhibitory concentrations (MIC) of the antimicrobial agents were determined by the agar dilution method. All isolates were found to be resistant to ampicillin (MIC_{90}, > 128 μg/ml) owing to the production of β-lactamase. The majority of the strains exhibited resistance also to chloramphenicol (MIC_{90}, 16 μg/ml), kanamycin (MIC_{90}, 8 μg/ml), streptomycin (MIC_{90}, 32 μg/ml) and tetracycline (MIC_{90}, 16 μg/ml).

Plasmid DNAs extracted from the isolates by an alkaline lysis procedure were compared by agarose gel electrophoresis. Five plasmid profiles were observed. Strains from each geographic region showed several plasmid profiles. An unusual plasmid size combination of 5.6-, 4.5-, 3.9- and 3.0-kilobases (kb) was detected in strains originating only in Botswana (1992) and Gauteng (1994).

The *H.ducreyi* plasmids conferring ampicillin resistance were identified by transformation into a plasmid-free *H.influenzae* Rd strain. Three sizes of plasmid of 10.6-, 9.3- and 5.6-kb were recovered from the ampicillin-resistant β-lactamase-positive transformants. After restriction endonuclease digestion with *Ava* I, *Bam* HI, *Pst* I and *Pvu* II, four plasmid types were observed on agarose gels. Although two of the plasmids had an identical size of 10.6 kb, they show different restriction

patterns indicating DNA rearrangement.

A procedure for the transformation of *H.ducreyi* cells with homologous *H.ducreyi* plasmid DNA was developed by applying electroporation. Optimal conditions for transformation of clinical and reference strains were determined using various parameters, including growth of the cells, the expression time, the composition of the electroporation buffer, the electroporation conditions and the effect of plasmid size and plasmid DNA concentration. Electroporation using 9- to 10-kV/cm with pulse lengths of 15- and 10-msec respectively resulted in optimal transformation frequencies ranging from 3×10^{-4} to 10^{-6}. Agitation of the cells before or after electroporation improved the transformation frequency by 25- and 22-fold respectively. Three plasmid genes conferring resistance to ampicillin, kanamycin and sulphonamides were transformed successfully to *H.ducreyi* recipients. All plasmids transferred to *H.ducreyi* by electroporation remained intact and extrachromosomal.

This is the first demonstration of transformation of *H.ducreyi* plasmids to homologous cells. The availability of transformable plasmids would allow the development of *H.ducreyi* vectors that may be useful in cloning genes involved in pathogenesis or other important functions.

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science (medicine) in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

Geraldine Leong

May-Yong Geraldine Leong

27th day of March 1996

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DEDICATION

To Ivan

Thanks for your friendship during our rehabilitation
and for teaching me how to play chess

and

In memory of my favorite authors,

Roald Dahl (1919-1990)

British writer of novels, short stories, film scripts and childrens books

and

James Alfred Wight (1916-1995)

Yorkshire Dales veterinarian and writer

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1.0. INTRODUCTION

1.1. Historical Background

Chancroid or soft chancre is a sexually transmitted disease (STD) which was first described by Bassereau in France in 1852. He observed that the exudate from some genital ulcers would induce similar cutaneous ulcers at another site of the patient following autoinoculation. Thirty seven-years later, Ducrey (1889) identified microscopically chains of short, gram-negative pleomorphic rods of 0.5 μm by 1.0 to 1.5 μm, aligned in a distinctive 'railroad track' or 'school of fish' arrangement (Figure 1.1.), from ulcers of patients following autoinoculation. Ducrey (1889) consistently observed this organism, *Haemophilus ducreyi*, in cleaned soft chancres, in pus from aspirated buboes and in the deep portions of abscesses.

Ducrey was unsuccessful in culturing these bacteria; this was accomplished by several investigators just before the turn of the twentieth century. The first convincing isolations have been credited to Istamanoff and Akspianz in 1897 (Himmel, 1901; Davis, 1903; Ritchie, 1904), who reported cultures in a medium containing macerated human skin in agar. Later, Lenglet reported isolations in 1898 of organisms that appear to be *H.ducreyi* by using similar human skin agar containing blood. Bezacon, Griffon and Le Sourd isolated *H.ducreyi* on blood agar alone in 1900 and the organism, after serial passages, was able to produce soft chancres when reinoculated into humans. Subsequently, other investigators were able to produce disease experimentally in humans, apes, chimpanzees and rabbits following inoculation with the bacillus or with bubo pus obtained from cases of chancroid (Sullivan, 1940).

H.ducreyi is classified in the genus *Haemophilus* on the basis of morphological characteristics, a requirement for haemin and a guanine-plus-cytosine (G+C)

content of the deoxyribonucleic acid (DNA) (Kilian, 1976). The requirement for haemin was confirmed by demonstrating the absence of enzymes involved in the conversion of δ-aminolevulinic acid to protoporphyrin (Hammond *et al.*, 1978a). The quantitative haemin requirement of *H.ducreyi* is considerably higher than that reported for other haemin-requiring *Haemophilus* species with *H.ducreyi* requiring 25 to 50 μg/ml compared to 1 to 10 μg/ml required by the other haemin-dependent *Haemophilus* species (Albritton *et al.*, 1981). Deoxyribonucleic acid hybridization studies have shown *H.ducreyi* strains to belong to a highly homogeneous group with homology values of 85 to 100% (Casin *et al.*, 1985). Casin *et al.* (1985) and Albritton (1989) demonstrated that the level of DNA homology between *H.ducreyi* and other *Haemophilus* spp., or several members of the genera *Actinobacillus* and *Pasteurella* was not significant, nor could Albritton *et al.* (1981) confirm significant competition for homospecific transformation in *Haemophilus influenzae* recipients. Recently from the analysis of the ribosomal ribonucleic acid (rRNA) sequences of the type strain of *H.ducreyi* (CIP 542), Rossau *et al.* (1991) inferred that *H.ducreyi* is related to the *Pasteuraellaceae* branch of the gamma subdivision of the *Proteobacteria*. They pointed out that the comparison of rRNA molecular sequences is a very important tool for studying the evolutionary relationships of organisms.

1.2. Clinical Features of Chancroid

Chancroid is a sexually transmitted disease characterised by single but more commonly multiple painful genital ulcerations which have a sloughy purulent base surrounded by ragged undermined edges, with a distinctive red margin. The ulcer may, in addition to exhibiting a necrotic purulent exudate, have a granulomatous base which bleeds on scraping (Lagergård, 1995). There is minimal inflammation of the surrounding skin (Ronald and Plummer, 1985; Lagergård, 1995). Typical ulcers are usually localised on mucosal and moist areas of the genitals (Figure 1.2.) (Lagergård, 1995). Following contact with an infected partner, initial lesions

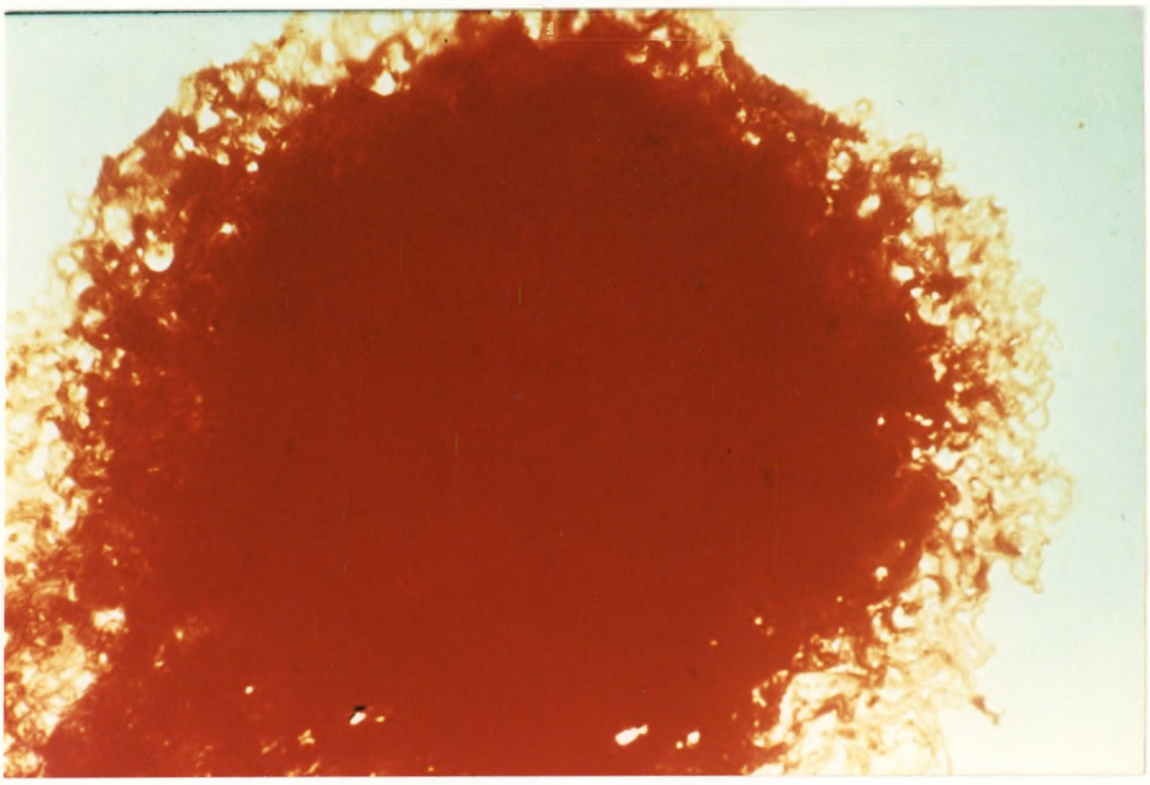


Figure 1.1. Gram stain of *H.ducreyi* colony. Magnification 1000 X.



Figure 1.2. Clinical presentation of a typical chancroid lesion.

usually appear within 4-7 days (Clarridge, Shawar and Simon, 1990). Without treatment, the ulcers may persist for 1-3 months and are frequently complicated by fluctuant lymph glands and draining inguinal abscesses (Ronald and Plummer, 1985). The rate of resolution of the disease has been shown to depend on a number of factors, including the site of primary ulceration, size and number of lesions and the degree of involvement of associated lymph nodes (Ballard, 1993). Chancroid does not spread systemically (Morse, 1989), even in individuals with AIDS, in whom the lesions may be extensive (Lagergård, 1995).

1.3. Epidemiology

Genital ulcerative diseases are prevalent in all countries of the world (Morse, 1989). Recent studies on the aetiology of genital ulcer disease show differences that may partly reflect differences in study material, but also genuine geographical differences in relative rates of specific genital pathogens (Morse, 1989).

Chancroid is endemic and a major cause of sexually transmitted genital ulcer disease in tropical countries, including Kenya (Tyndall *et al.*, 1993, 1994), Thailand (Taylor *et al.*, 1985a; Sarafian *et al.*, 1991a) and southern Africa (Meheus *et al.*, 1983; Abeck *et al.*, 1988; Dangor *et al.*, 1988a). The disease is rare in Europe and is also less common in the United States, although outbreaks have occurred since 1981 (Coutinho, 1994).

The epidemiology of chancroid is poorly understood because of the lack of typing methods that would permit differentiation among strains of *H.ducreyi* (Sarafian *et al.*, 1991b). Difficulties in establishing a clinical and laboratory diagnosis can interfere with an accurate identification of chancroid and its aetiological agent (Parsons *et al.*, 1989). *H.ducreyi* is a fastidious organism that requires enriched media and other defined growth conditions for successful isolations and has few distinguishing biochemical features. Nonetheless, isolation of *H.ducreyi* in culture

is required in order to establish a definitive diagnosis of chancroid (Sturm *et al.*, 1987). Recently, there has been an increased interest in the development of nonculture methods for the detection and identification of *H.ducreyi* and for the diagnosis of chancroid in general (Johnson *et al.*, 1994). *H.ducreyi* strains have been characterised phenotypically by outer membrane protein (OMP) profiles (Taylor *et al.*, 1985a), enzyme profiles (Van Dyck and Piot, 1987), plasmid profiles (Sarafian and Knapp, 1992; Brunton *et al.*, 1982; Handsfield *et al.*, 1981) and ribotypes (Sarafian *et al.*, 1991b; Brown and Ison, 1993) as markers which have been used for the epidemiological study of *H.ducreyi.* In addition, enzyme immunoassays for detecting serum IgG antibody to *H.ducreyi* (Duncan *et al.*, 1994) could be used for epidemiological studies.

Taylor *et al.* (1985a) found five different OMP patterns by analysing proteins in the range of 26 to 61 kilodaltons from 100 strains of *H.ducreyi* isolated in Thailand. Of the strains, 98% could be categorised into three patterns, however these differences are usually small (Taylor *et al.*, 1985a).

Using the API-ZYM system, which included 95 different substrates, Van Dyck and Piot (1987) determined the enzyme profiles of 200 strains of *H.ducreyi* isolated from four geographical areas. The best type distribution showing the highest discrimination of strains was found by the combination of two enzyme substrates L-alanyl-L-phenylalanyl-L-proline and L-ornithine. Significant differences were shown between isolates from Asia, Africa and Europe by the two enzyme substrates, but not between strains isolated in the two African cities of Nairobi and Johannesburg (Van Dyck and Piot, 1987).

It is possible to characterise strains of *H.ducreyi* phenotypically by plasmid content (Sarafian and Knapp, 1992). The plasmid profiles of the *H.ducreyi* isolates are analysed to gain insight into the distribution and extent of temporal changes in

H.ducreyi strain populations (Sarafian and Knapp, 1992), and may indicate the relative efficacy of regimens for the treatment of chancroid (Motley *et al.*, 1992). Antimicrobial susceptibilities of *H.ducreyi* isolates appear to vary according to geographical location (Taylor *et al.*, 1985a). The difference in antimicrobial susceptibilities among the isolates may be due either to intrinsic differences in the susceptibilities of isolates from different geographical areas or in the procedures used to determine susceptibility. Motley *et al.* (1992) observed an association between the plasmid content and antimicrobial susceptibility of *H.ducreyi* isolates. Isolates with the 5.7 Mdal β-lactamase plasmid were less susceptible to erythromycin, spectinomycin and trimethoprim than were isolates that possessed the 3.2 Mdal β-lactamase plasmid. Handsfield *et al.* (1981) observed that plasmids isolated from *H.ducreyi* in their study varied according to geographic origin of the strains.

A recent development in nucleic acid analysis, ribotyping, is based on restriction fragment length polymorphism (RFLP) of rRNA genes (Sarafian *et al.*, 1991b). RFLP and plasmid analysis were used to evaluate an outbreak of *H.ducreyi* in San Francisco between May 1989 and May 1991 (Flood *et al.*, 1993). RFLP analysis may prove useful as a tool in distinguishing strains of *H.ducreyi* in an endemic area or during an outbreak, as an adjunct to traditional epidemiologic methods (Flood *et al.*, 1993).

In a seroepidemiological study performed by Duncan *et al.* (1994), in Ethiopian women, they observed that a difference in the distribution of seropositivity rates may reflect past exposure to *H.ducreyi* infection. Such differences in seropsitivity rates may reflect an evolution over time in the prevalence of chancroid and in the geographical distribution of the disease (Duncan *et al.*, 1994).

1.4. Treatment

For the treatment of chancroid, the geographical origin of the isolate is an important determinant for the success of therapy, since antimicrobial susceptibilities vary from place to place. A major public health concern relates to studies in Africa which have shown that chancroid is a significant co-factor in the transmission of the human immunodeficiency virus (HIV) (Wasserheit, 1992).

Early in the antibiotic era, *H.ducreyi* was susceptible to almost all available drugs. However, increasing resistance has been documented among isolates from around the world (Aldridge, Cammarata and Martin, 1993). Most isolates now produce β-lactamase (Schmid, 1986) with increasing resistance to the sulphonamides and trimethoprim (Plourde *et al.*, 1992), tetracyclines, chloramphenicol (Dangor *et al.*, 1990), streptomycin and kanamycin (Rajan and Sng, 1982; Taylor *et al.*, 1985a). Erythromycin is a widely available, reasonably inexpensive and well-tolerated drug (Boyd, 1989). It is taken 500 mg orally, four times a day for seven days (Tyndall *et al.*, 1994). Azithromycin (1 g) orally as a single dose has been included in the Centers for Disease Control (CDC) treatment guidelines (along with ceftriaxone and erythromycin) based on its excellent *in vitro* activity and pharmacokinetics which result in prolonged high concentrations in genital tissues and recorded clinical efficacy (Krohn, 1991). However, treatment trials with single doses of azithromycin and ceftriaxone have proved inadequate in persons with HIV and some experts recommend multidose erythromycin therapy to treat HIV infected patients with chancroid (Levine *et al.*, 1994). Seven days after initiation of therapy, reduced tenderness, absence of purulence and partial reepithelisation should be apparent (Ronald *et al.*, 1992).

1.5. Genetics and Mechanisms of Antimicrobial Resistance

Failure of cases of chancroid to respond clinically to therapy can either be the result of disease caused by *H.ducreyi* strains which are resistant to the antimicrobial

agent or to concomitant HIV infection. The spread of plasmid-mediated resistance has been observed to penicillin, ampicillin, sulphonamides, streptomycin, kanamycin, tetracycline and chloramphenicol (McNicol and Ronald, 1984) (Table 1.1.).

1.5.1. Resistance to ampicillin

During an outbreak of chancroid in Winnipeg, Canada, β-lactamase producing strains of *H.ducreyi* were isolated (Hammond *et al.*, 1978b). Ampicillin-resistance was later found to be due to the presence of an 5.7 megadalton (Mdal) plasmid, designated pJB1 (Brunton *et al.*, 1979). Resistance to ampicillin [minimal inhibitory concentration (MIC) ≥ 128 μg/ml] was shown to be due to the production of β-lactamase of the TEM-1 type, the enzyme associated with the transposon Tn2, a subclass of Tn4 (MacLean, Bowden and Albritton, 1980). Heteroduplex studies of this plasmid demonstrated that it carries 100% of the transposable ampicillin-resistance sequence, TnA (Brunton, Bennett and Grinsted, 1981). Two additional β-lactamase producing plasmids have subsequently been identified (Handsfield *et al.*, 1981; Totten *et al.*, 1982). A larger plasmid, 7.0 Mdal, isolated in Seattle, Washington, from a *H.ducreyi* strain recovered from a patient in the Philippines and a smaller 3.2 Mdal plasmid identified in *H.ducreyi* isolated from a patient who was infected in Brazil. Totten *et al.* (1982) studied the β-lactamase producing plasmids and found that the 5.7- or 7.0-Mdal plasmid had the whole ampicillin transposon inserted on the plasmid, whereas the 3.2 Mdal plasmid had only part of TnA on its plasmid.

Recently, two different β-lactamase plasmids of 3.51 Mdal containing the *bla* ROB-1 gene, a Bush group 2b enzyme (Bush, 1989b), and a TEM-1 β-lactamase plasmid were found within the same isolate of *H.ducreyi* from Thailand in 1985. Isoelectric-focusing data suggest that when these two particular plasmids are together, only the TEM-1 β-lactamase is expressed (MacLean *et al.*, 1992).

1.5.2. Resistance to sulphonamides and aminoglycosides

In 1981, *H.ducreyi* isolates from Kenya were found to be resistant to sulphonamides (Nsanze *et al.*, 1981). A 4.9 Mdal nonconjugative plasmid encoding sulphonamide resistance was found in clinical isolates of *H.ducreyi* from Kenya and Atlanta (Albritton *et al.*, 1982). This plasmid was found alone or in combination with a 5.7- or 7.0-Mdal ampicillin-resistance plasmid (Albritton *et al.*, 1982).

In Singapore, treatment failure following streptomycin therapy has been observed since 1980 (Rajan and Sng, 1982), and susceptibility studies have shown that a large percentage of these strains were resistant to both streptomycin and kanamycin. Sanson-Le Pors, Casin and Collatz (1985) observed that resistance to both these aminoglycoside antibiotics was mediated by a 4.7 kb plasmid and due to the synthesis of two aminoglycoside phosphotransferases (APH). Willson *et al.* (1989) characterised a 4.8 kb plasmid from *H.ducreyi* (plasmid pLS88) which encoded resistance determinants for sulphonamides and streptomycin related to those of the enteric plasmid RSF1010 and for kanamycin related to Tn903. The original source of plasmid pLS88 was a clinical strain of *H.ducreyi* isolated in Thailand by Taylor *et al.* (1985a).

1.5.3. Resistance to trimethoprim

Trimethoprim in combination with a sulphonamide has been effective in the treatment of chancroid in spite of the high prevalence of sulphonamide-resistant strains of *H.ducreyi* (Morse, 1989). However, clinical resistance to trimethoprim has been reported in Thailand (Taylor *et al.*, 1985b) and more recently in Africa. The genetic mechanism of trimethoprim resistance in these strains is unknown (Morse, 1989), although Van Dyck *et al.* (1994) has proposed that resistance may be due to several mechanisms: plasmid coding for a novel dihydrofolate reductase enzyme that may become incorporated into the chromosome via transposons or to

the overproduction of dihydrofolate reductase.

1.5.4. Resistance to chloramphenicol and tetracycline

Handsfield *et al.* (1981) described three isolates of *H.ducreyi* from the United States and one from the Philippines that were resistant to chloramphenicol (MICs, 16 μg/ml). In southern Africa, chloramphenicol-resistant strains of *H.ducreyi* were reported with MICs of 8 μg/ml (Dangor, Miller and Koornhof, 1988b). Sanson-Le Pors *et al.* (1982) demonstrated the presence of chloramphenicol acetyltransferases (CAT) in a chloramphenicol-resistant clinical isolate of *H.ducreyi.* Roberts, Actis and Crosa (1985), demonstrated that the CAT determinant was located on a 34 Mdal conjugative plasmid. They also found tetracycline-resistance genes on the plasmid. The recognition of linked tetracycline-chloramphenicol resistance suggest that multiple-resistance plasmids may become more prevalent (Albritton *et al.*, 1984).

Tetracycline-resistant strains have been found in every geographical area surveyed, with the percentage of resistant strains varying from 16 to 100% (Albritton *et al.*, 1984). Both plasmid and chromosome-mediated tetracycline resistance have been described in *H.ducreyi.*

Plasmid-mediated tetracycline resistance is associated with conjugative plasmids of 30 Mdal (Albritton *et al.*, 1984) and 34 Mdal (Roberts, Actis and Crosa, 1985). Albritton *et al.* (1984) demonstrated conjugative matings, with the 30 Mdal plasmid, using as recipients other strains of *H.ducreyi* or strains of *H.influenzae.* The 34 Mdal plasmid isolated by Roberts, Actis and Crosa (1985) was shown to hybridise with a 1.8 kb Tet M probe, a tetracycline-resistance determinant belonging to the class M group.

Tetracycline-resistant strains without apparent plasmids were unable to transfer resistance by conjugation, and no difference in resistance levels between strains with or without demonstrable plasmids were detected (McNicol and Ronald, 1984). Johnson, Biddle and De Witt (1989) studied a tetracycline-resistant, ampicillin-resistant *H.ducreyi* isolate that harboured TetM integrated into the chromosome of an isolate of *H.ducreyi.* The organisation of TetM in *H.ducreyi* was unusual and apparently involved the duplication of the TetM structural gene and perhaps also adjoining portions of the transposon (Johnson, Biddle and De Witt, 1989).

1.5.5. Other plasmids of *Haemophilus ducreyi*

Three novel plasmids with molecular masses of 1.8-, 2.6- and 2.8-Mdal were observed in 29 *H.ducreyi* isolates collected in Thailand in 1984, and in a strain isolated in San Francisco, California (Sarafian *et al.*, 1991b). Weak hybridisation of the 2.6 Mdal plasmid with the *bla* probe using the 4.4 Mdal plasmid of *Neisseria gonorrhoeae* was observed (Sarafian *et al.*, 1991b). These isolates appear to be unique, as the number and diversity of the plasmids present in each of these isolates distinguish them from strains previously described.

1.5.6. Mobilization of non-conjugative antibiotic resistance plasmids

Deneer *et al.* (1982) reported the presence of a 23.5 Mdal phenotypically cryptic, self transferrable plasmid in a clinical isolate of *H.ducreyi* from Kenya which contained two other plasmids. The 23.5 Mdal plasmid was capable of mobilizing a small co-resident 7.0 Mdal ampicillin-resistance plasmid and a 4.9 Mdal sulphonamide-resistance plasmid in conjugative matings to *H.influenzae* and *Escherichia coli* recipients (Deneer *et al.*, 1982). The recipient received both the 23.5 Mdal and either the 7.0 Mdal or the 4.9 Mdal plasmid (Deneer *et al.*, 1982). The conjugative plasmid could also mediate the transfer of gonococcal ampicillin-resistance plasmids. This plasmid was shown to share homology with the ampicillin-resistance plasmids of *H.ducreyi* (McNicol, Albritton and Ronald, 1983).

The 23.5 Mdal conjugative plasmid from *H.ducreyi* was mapped by McNicol, Albritton and Ronald (1986) by restriction endonuclease digestion and its molecular size was estimated to be 21.7 Mdal. An *Ori* T site was located which is required for *in trans* mobilization of DNA (McNicol, Albritton and Ronald, 1986). Another conjugative plasmid of 30 Mdal that carries the tetracycline-resistance determinant, was shown by Albritton *et al.* (1984) to transfer tetracycline resistance in conjugative matings with tetracycline-susceptible laboratory strains of *H.ducreyi.* Three additional tetracycline-resistant strains not exhibiting a 30 Mdal plasmid on initial screening were able to transfer tetracycline resistance in conjugative matings with a tetracycline-susceptible *H.ducreyi* strain (Albritton *et al.*, 1984) and transconjugants from these matings acquired the 30 Mdal plasmid.

1.5.7. Molecular relationships among the plasmids

As mentioned previously, Albritton *et al.* (1982) characterised a 4.9 Mdal plasmid that specified sulphonamide resistance in *H.ducreyi* isolates. Electron microscopic heteroduplex analysis showed that this plasmid was 80% related to RSF1010, a plasmid specifying linked resistance to streptomycin and sulphonamides which is found in a wide variety of species, including *E.coli* and several *Salmonella* species (Brunton, Clare and Meier, 1986). The sulphonamide-resistance plasmid has not been found in other *Haemophilus* species (McNicol and Ronald, 1984).

Albritton *et al.* (1984) reported conjugative tetracycline and tetracycline-chloramphenicol-resistance plasmids in *H.ducreyi.* These plasmids were closely related to an *H.influenzae* β-lactamase plasmid, as judged by restriction endonuclease digestion patterns and their mutual incompatibility (Albritton *et al.*, 1984). Roberts, Actis and Crosa (1985) examined chloramphenicol-resistant *Haemophilus parainfluenzae* and *H.ducreyi* strains and showed that their CAT genes were related to the enteric type II class and to the *H.influenzae* CAT genes. Their data also suggest that all three *Haemophilus* species have a common ancestral

Table 1.1. Plasmids of *H.ducreyi*.

Plasmid type	Mass (Mdal/kb)	Resistance determinant	Reference
Antimicrobial resistance	4.9/7.5	Su^r	Albritton *et al.* (1982)
	3.1/4.8	Su^r Km^r	Sanson-Le Pors, Casin and Collatz (1985)
	3.1/4.8	Su^r Str^r Km^r	Willson *et al.* (1989); Dixon, Albritton and Willson (1994)
	34/52[a]	Tc^r Cm^r	Albritton *et al.* (1984); Marshall *et al.* (1984); Roberts, Actis and Crosa (1985)
	34/52[a]	Tc^r	Roberts (1989)
	30/46[a]	Tc^r	Albritton *et al.* (1984)
	5.7/8.8	Amp^r	Brunton *et al.* (1979); MacLean, Bowden and Albritton (1980); Totten *et al.* (1982)
	7.0/10.8	Amp^r	Totten *et al.* (1982); Brunton *et al.* (1982)
	3.2/4.9	Amp^r	Totten *et al.* (1982)
	3.5/5.4	Amp^r	MacLean *et al.* (1992)
Conjugative	23.5/36	None	Deneer *et al.* (1982)
Novel	1.8/2.8		Sarafian *et al.* (1991a); Sarafian and Knapp (1992)
	2.6/4[b]		Sarafian *et al.* (1991a) Sarafian and Knapp (1992)
	2.8/4.3		Sarafian *et al.* (1991a) Sarafian and Knapp (1992)

kb kilobase
r resistant
a conjugative plasmid
b showed weak hybridisation with the *bla* probe
Amp ampicillin; Cm chloramphenicol; Km kanamycin; Str streptomycin; Su sulphonamides; Tc tetracycline

source for the CATs. A 24.5 Mdal phenotypic cryptic plasmid, which mediates the conjugal transfer of R plasmids, has also been described in *N.gonorrhoeae* (Roberts and Falkow, 1977). Both the gonococcal 24.5 Mdal plasmid and the *H.ducreyi* 23.5 Mdal plasmid were able to mobilize ampicillin-resistance plasmids of *H.ducreyi* and *N.gonorrhoeae* (Morse, 1989). In spite of similar size and function, restriction mapping and hybridisation studies showed that these were different plasmids (McNicol, Albritton and Ronald, 1986). However, some homology is present which is located within the transfer operon of the plasmid (McNicol, Albritton and Ronald, 1986).

Homology studies of the ampicillin-resistance plasmids of *H.ducreyi* with DNA from other species have been performed by several investigators. The G+C content of the 5.7 Mdal plasmid of *H.ducreyi* of 41 mol% is close to that found in *H.influenzae* and *H.ducreyi* chromosomal DNA (38 to 39%) (Kilian, 1976) and to that of the *H.influenzae* 30 Mdal plasmid RSF0885 (40%) and the *N.gonorrhoeae* 3.2 Mdal plasmid (41%) (Roberts, Elwell and Falkow, 1977). The 7.0 Mdal plasmid of *H.ducreyi* is homologous to and carries the entire sequence of the 4.4 Mdal `Asian' plasmid of *N.gonorrhoeae* (Anderson *et al.*, 1984; Brunton, Bennett and Grinsted, 1981). On the other hand, the 7.0- and 5.7-Mdal plasmids of *H.ducreyi* differ only in the presence of a 1.3 Mdal insertion sequence in the former, which is also present in the 4.4 Mdal gonococcal plasmid (Anderson *et al.*, 1984). The 5.7- and 3.2-Mdal *H.ducreyi* plasmids differ only by the presence of the entire Tn2/TnA sequence in the former; the 3.2 Mdal plasmid contains only the right-hand 40% of the Tn2 sequence (Anderson *et al.*, 1984). The 3.2 Mdal *H.ducreyi* plasmid is identical to the 3.2 Mdal `African' plasmid of *N.gonorrhoeae* (Anderson *et al.*, 1984). This strongly suggests that the gonococcal plasmids could have been introduced to *N.gonorrhoeae* from *H.ducreyi* or other *Haemophilus* species (Brunton *et al.*, 1982).

Restriction endonuclease and hybridization studies confirm that the 5.4 kb plasmid of *H.ducreyi*, which contains the *bla* ROB-1 gene, is similar to the 5.4 kb ROB-1 β-lactamase plasmid from *Actinobacillus pleuropneumoniae* (MacLean *et al.*, 1992).

Cryptic plasmids which could serve as direct progenitors of the *H.ducreyi* plasmid pJB1 (5.7 Mdal) have been found relatively commonly in *H.parainfluenzae* (Brunton, Clare and Meier, 1986). Hybridisation studies showed that all of the non-TnA fragments of pJB1 are homologous to the cryptic plasmid found in *H.parainfluenzae* (Brunton, Clare and Meier, 1986).

2.0. CHARACTERISATION OF CLINICAL ISOLATES OF *HAEMOPHILUS DUCREY*

2.1.1. Growth Characteristics and the Culture of *Haemophilus ducreyi*

Pure cultures of *H.ducreyi* have a distinctive colony morphology (Sottnek *et al.*, 1980). Colonies are generally pinpoint size at 24 h and increase to 1 to 2 mm in diameter after 48 to 72 h of incubation at 35°C (Lubwama *et al.*, 1986; Morse, 1989). The colonies are nonmucoid, raised, compact and granular and have a tan, yellowish or grey-yellow colour (Morse, 1989). Grown on solid media (Figure 2.1.) they vary in size, perhaps due to the peculiar cohesiveness of the organism (Parsons *et al.*, 1989). Electron microscopic studies suggest these features of *H.ducreyi*, namely the `railroad track' arrangement and cohesiveness, may be related to areas of intercellular adhesion (Morse, 1989). This may explain the characteristic observation that colonies of *H.ducreyi* can be pushed intact across the surface of solid media with an inoculating loop and that uniform suspensions of the organism are difficult to obtain (Morse, 1989), as they remain tightly agglutinated when suspended in liquid (Oberhofer and Back, 1982; Parsons *et al.*, 1989). Although most *H.ducreyi* colonies will be detected after 48 h of incubation, cultures should be held for 5 days before being discarded (Ronald and Plummer, 1985).

The accurate diagnosis of chancroid depends on the ability to culture *H.ducreyi* (Sturm *et al.*, 1987). A major advance in the ability to isolate *H.ducreyi* was the development of a selective medium consisting of an enriched chocolate agar, comprising of either a gonococcal or Mueller-Hinton agar base, supplemented with 5% foetal calf serum (FCS) (Sottnek *et al.*, 1980), L-glutamine in a concentration of 0.01%, which is supplemented by IsoVitaleX (Van den Berghe, 1987), haemoglobin or chocolatised horse blood (Hammond *et al.*, 1978a) and 3 mg/l of vancomycin to inhibit the growth of certain gram-positive flora associated with

genital ulcers (Hammond *et al.*, 1978c). Recently, new media for the growth of *H.ducreyi* have been reported. These include: transport medium for *H.ducreyi*, where FCS and IsoVitaleX are substituted by 5% Fildes extract (Dangor *et al.*, 1992) and clear broth and plate media, using catalase as a source of haeme (Totten and Stamm, 1994).

Optimum growth conditions for fresh clinical isolates and laboratory strains of *H.ducreyi* are incubation at 33 - 35°C, for 48 h under microaerophilic conditions in an anaerobic jar with 5% CO_2 (Sturm and Zanen, 1984a) and a water saturated atmosphere which enhances the growth of the organism (Hammond *et al.*, 1978a; Sottnek *et al.*, 1980; Lubwama *et al.*, 1986).

Nsanze *et al.* (1984) reported that by using a single solid medium and optimal conditions, the isolation rate of *H.ducreyi* from presumptive chancroid ulcerations is estimated to be 60% to 70%, with higher rates achieved if two media are used.

2.1.2. Biochemical Activity

H.ducreyi is a fastidious organism with very limited biochemical activity because it is asaccharolytic (Morse, 1989; Clarridge, Shawar and Simon, 1990), making it difficult to identify except on the basis of morphological and cultural characteristics. The important characteristics of *H.ducreyi* are listed in Table 2.1.

Although no unique colonial or biochemical characteristics have been demonstrated, *H.ducreyi* can be differentiated from the other human-haemin-requiring species of *Haemophilus* by its slow growth and lack of requirement for nicotinamide adenine dinucleotide (NAD, V-factor) (Ronald and Albritton, 1984).

Non-culture methods of detection of *H.ducreyi* have also been developed, such as those using DNA probes specific for *H.ducreyi* (Parsons *et al.*, 1989). The recent



Figure 2.1. **Colonial morphology of *H.ducreyi* on Nsanze medium after 48 h growth in microaerophilic conditions at 35°C with humidity.**

development of a polymerase chain reaction (PCR) assay (Johnson *et al.*, 1994) appears to be useful, especially since culture sensitivity is low. However, DNA probes do not have the specificity necessary for detecting clinical strains as other species of the *Pasteurellaceae* family give weak positive reactions. Applying PCR, using primers selected from sequences of an anonymous fragment of DNA cloned from *H.ducreyi*, no amplification was observed for bacteria other than *H.ducreyi* with the exception of a single strain of *H.parainfluenzae*, which gave inefficient amplification of a 500 bp fragment (Johnson *et al.*, 1994). The sensitivity of PCR was also reduced by the presence of *Taq* inhibitors in the specimen.

Table 2.1. Major biochemical characteristics of *H.ducreyi*.

Characteristics	Reaction
Porphyrin biosynthesis	-
Nitrate reduction	+[v]
Catalase	-[v]
Oxidase	+[v]
Voges-Proskauer	+[v]
Indole production	-
Urease	-[v]
Ornithine decarboxylase	-
Arginine dihydrolase	-
Alkaline phosphatase	+
H_2S production	-
Deoxyribonuclease	-
Sodium polyanethole-sulfonate	(a)

(a) Zone of inhibition with an average size of 15 mm (Shawar, Sepulveda, Clarridge, 1990)
v variable reactions
Extracted from Albritton (1989)

2.1.3. Antimicrobial Susceptibility

The first report of antimicrobial susceptibility testing of *H.ducreyi* dealt with 19 isolates from an epidemic in Winnipeg, Canada (Hammond *et al.*, 1978b). Most of these isolates were moderately or very susceptible to the antibiotics tested: 84% were moderately susceptible to ampicillin; 94% were susceptible to tetracycline; all were susceptible to cephalothin, kanamycin, chloramphenicol and sulphonamides. Three isolates resistant to penicillin and ampicillin were the first *H.ducreyi* strains reported that produced β-lactamase (Hammond *et al.*, 1978b). More recently most *H.ducreyi* isolates have been found to be β-lactamase positive, from 47% in the Netherlands (Sturm, 1987) to nearly 100% in other countries (Nsanze *et al.*, 1981; Taylor *et al.*, 1985a; Dangor *et al.*, 1988a).

Many strains of *H.ducreyi* exhibit resistance to one or more antimicrobial agents including sulphonamides, trimethoprim, tetracycline, chloramphenicol, streptomycin, kanamycin, penicillin, ampicillin, doxycycline, spectinomycin, gentamicin and thiamphenicol (Morse, 1989; Dangor *et al.*, 1990). In many parts of Africa, the penicillins, tetracyclines, sulphonamides and most recently trimethoprim have become ineffective (McNicol and Ronald, 1984; Plourde *et al.*, 1992). Most isolates of *H.ducreyi* worldwide are susceptible to erythromycin which remains the recommended treatment for chancroid, however strains have been encountered in Singapore and Thailand which have MICs of 4 μg/ml (Sng *et al.*, 1982; Knapp *et al.*, 1993).

Tetracycline resistance is widespread in Thailand (MIC_{50} > 32 μg/ml), France (MIC_{50}, 32 μg/ml), The Netherlands (MIC_{50}, 16 μg/ml) and South Africa (MIC_{50}, 16 μg/ml) (Dangor *et al.*, 1990).

Canadian and African isolates of *H.ducreyi* have been shown to be susceptible to kanamycin (Slootsman *et al.*, 1983), however MICs of ≥ 16 μg/ml have been

reported from South Africa (Dangor *et al.*, 1988a), France (Sanson-Le Pors, Casin and Collatz, 1985) and Thailand (Taylor *et al.*, 1985a).

Although *H.ducreyi* isolates from Belgium have been shown to be susceptible to chloramphenicol (Slootsman *et al.*, 1983) and thiamphenicol has been used successfully to treat the disease in Zimbabwe (Latif, 1982), chloramphenicol-resistant strains producing chloramphenicol acetyltransferases (Sanson-Le Pors *et al.*, 1982) have been reported from South Africa (Dangor *et al.*, 1988a), Thailand (Taylor *et al.*, 1985a), The Netherlands (Sturm, 1987), France (Sanson-Le Pors *et al.*, 1982) and the Philippines (Handsfield *et al.*, 1981).

Trimethoprim resistance has been documented in Thailand (Taylor *et al.*, 1985b) and in Nairobi (Plummer *et al.*, 1983) with MICs > 32 µg/ml being frequently detected in the United States (Schmid, 1986). Fourteen percent of isolates from South Africa exhibited *in vitro* resistance to trimethoprim (MIC, 4 µg/ml) (Dangor *et al.*, 1988a). Susceptibility to sulphamethoxazole has been reported in *H.ducreyi* isolates in Kenya, South Africa and The Netherlands (Dangor *et al.*, 1990), whereas isolates in Thailand had MICs > 160 µg/ml for most strains (Taylor *et al.*, 1985b). The combination of trimethoprim and sulphonamide is synergistic against *H.ducreyi*, unless high-level sulphonamide-resistance is present (Schmid, 1990).

In this study, antimicrobial susceptibilities of 52 *H.ducreyi* clinical isolates collected during 1988 to 1994 from various regions in southern Africa was investigated.

2.2. MATERIALS AND METHODS

2.2.1. Bacterial Strains

The reference strains *H.ducreyi* ATCC 27722 and CIP 542 were obtained from the American Type Culture Collection, USA and the Pasteur Institute in Paris,

respectively. A total of 52 clinical isolates of *H.ducreyi* were studied (Table 2.2.). Twenty-three isolates acquired in Botswana, Lesotho, Natal and Transkei were collected in Carletonville, Gauteng in 1992 when migrant gold miners returned from their homes; 7 isolates collected in 1994 in Gauteng; 4 isolates from Windhoek, Namibia and 18 isolates collected in 1988 in Gauteng. All isolates were collected by the STD reference centre, SAIMR except for the isolates from Windhoek, Namibia which were provided by the Namibian Ministry of Health and Social Services.

2.2.2. Media (Appendix A)

The media used for *H.ducreyi* in these studies were Nsanze agar medium (Nsanze *et al.*, 1984), chocolate agar and serum-free medium/charcoal agar (Lockett *et al.*, 1991). Other media used was a supplemented Brain Heart Infusion (sBHI) broth. Supplemented BHI broth with the addition of 15% glycerol (Merck, Germany) was used for the storage of isolates at -70°C.

Isolates grown on agar plates were incubated for 48 h at 35°C under microaerophilic conditions, in an anaerobic jar (GasPak, BBL, Microbiology systems, Becton Dickinson, Cockeysville, USA) with 5% CO_2 (GasPak, BBL) and high humidity.

2.2.3. Examination of Colony and Cell Morphology

Colony morphology was examined from cultures grown on Nsanze or chocolate agar medium. Cell morphology and the arrangement of the bacterial cells were observed microscopically after the bacteria were heat fixed onto a glass slide and Gram stained.

Table 2.2. Clinical isolates of *H.ducreyi* used in this study.

Gauteng, 1988					
D16	Y5	Y10	Y70	YD29	Y56
Y76	Y78	Y88	Y109	Y156	Y163
Y165	Y179	Y119	Y182	Y193	Y195
Botswana, 1992					
CH137	CH216	P15	P77	P116	
Lesotho, 1992					
CH57	CH90	CH128	CH138	CH161	CH246
P157	P167				
Natal, 1992					
CH87	CH122	CH145	CH242	CH248	P2
P6	P97	P143			
Transkei, 1992					
CH247					
Gauteng, 1994					
HD1	HD2	HD4	HD5	HD6	HD7
HD8					
Windhoek, Namibia, 1994					
WD793	WD794	G802	G723		

2.2.4. Biochemical Characteristics

The biochemical characteristics of *H.ducreyi* strains were examined according to a scheme proposed by Kilian (1976). The following tests, which are described in Appendix A.7., were performed for the detection of:

Porphyrin biosynthesis; indole; urease; ornithine decarboxylase; nitrate reduction; alkaline phosphatase; hydrogen sulphide (H_2S); catalase; oxidase; sodium polyanethole-sulfonate (SPS) disk susceptibility test; β-lactamase.

Isolates were streaked on BHI agar (BBL, Microbiology systems), BHI containing 2 µg/ml NAD and BHI containing NAD and 10 µg/ml haemin to test for the presence of contaminants and other *Haemophilus* species. *H.parainfluenzae* and *H.influenzae* were used as positive controls. Both haemin (BDH chemicals Ltd, Poole, UK), prepared by heating a stock of 10 mg/ml at 65°C, and NAD (Sigma Chemical Company, USA), prepared as a filter sterilized stock of 2 mg/ml, were stored at -20°C.

Gram staining was performed on suspicious colonies, with *H.ducreyi* ATCC 27722 used as a control.

2.2.5. Antimicrobial Susceptibility Testing

2.2.5.1. Preparation of inocula

An 0.5 McFarland standard was prepared using *H.ducreyi* ATCC 27722. From a 48 h culture on Nsanze medium, 30 colonies were suspended into 2 ml of quarter-strength Ringers solution. The cells were dispersed by the method described by Jardine (1990). In this method, cells were drawn up a 26 G needle 10 times for even dispersal and allowed to settle afterwards for 15 min. Dilutions from 10^{-1} to 10^{-6} were made from the suspension to find the appropriate concentration of cells to be used as a standard.

Twenty colonies of each *H.ducreyi* isolate was removed from solid media and the cells dispensed as described above. Cells were allowed to settle for 10 min before being compared and adjusted to the 0.5 McFarland standard, to produce approximately 10^6 cells/ml. Standardization of the density of the inoculum is essential if variation in results is to be avoided.

2.2.5.2. Medium and antibiotics for antimicrobial susceptibility testing

Minimum inhibitory concentrations of the antimicrobial agents were determined

using the agar dilution method by using a gonococcal agar base medium with 10 g/l haemoglobin (Difco, Detroit, Michigan, USA), 1% IsoVitaleX (BBL) and 5% FCS (Flow laboratories, Scotland). The following antibiotics used were prepared according to the manufacturers instructions: ampicillin (Sigma), chloramphenicol (SAIMR), kanamycin monosulphate (Sigma), streptomycin sulphate (Sigma) and oxytetracycline HCl (Tedro products, Johannesburg, SA). Serial two-fold dilutions of each antibiotic within the range from 0.5 to 128 µg/ml were prepared. Control plates were prepared by replacing the antibiotic with sterile water. The antibiotic was incorporated into the molten agar and evenly distributed by swirling the plate. When the agar had set the plates were stored overnight at 4°C.

2.2.5.3. Inoculation of the antibiotic plates

Prior to inoculation, plates were dried at 37°C. Plates were inoculated with the *H.ducreyi* isolates, using a 36 pin multipoint inoculator (Mast laboratories, England), from the lowest to the highest concentration for each antibiotic. Two reference strains of *H.ducreyi*, ATCC 27722 and CIP 542, were used as controls for each run of susceptibilities. Control plates with no antibiotics were inoculated at the beginning, end and between changes of different antibiotics. The inocula were allowed to dry and the plates incubated at 35°C for 48 h in an anaerobic jar containing 5% CO_2 and moisture. The MIC was defined as the lowest concentration of the antibiotic showing no growth, a light hazy growth or 1-3 colonies.

2.3. RESULTS

2.3.1. Media

Fifty-two clinical isolates grew on Nsanze medium recovered from chancroid-infected patients and showed characteristics typical for *H.ducreyi*. Yellowish-grey colonies, varying from pinpoint to approximately 2 mm in diameter were observed. Cohesiveness of the *H. ducreyi* colonies was apparent as they could be pushed

intact across the surface of the agar. Growth of *H.ducreyi* strains on serum-free (charcoal) medium was similar to growth on Nsanze medium except the average size of the *H.ducreyi* colonies was smaller (0.5-1 mm).

The clinical isolates were able to grow in sBHI broth although growth was weaker than the growth of *H.ducreyi* ATCC 27722 in sBHI broth. Growth of *H.ducreyi* in sBHI broth was more pronounced with agitation at 200 revolutions per minute (rpm) resulting in 10^9 colony-forming units (CFU)/ml as compared to static growth of 10^7-10^8 CFU/ml.

Gram-stained *H.ducreyi* colonies showed gram-negative rods appearing as short chains in the distinct 'railroad track' formation.

2.3.2. Biochemical Reactions

The results obtained from the biochemical tests which are summarized in Table 2.3. demonstrate the inactivity unique to these organisms. All clinical isolates hydrolyse β-lactamase, tested positive for alkaline phosphatase and oxidase and were negative in the porphyrin test. Results were inconsistent for the nitrate reduction test as 55% of the isolates showed reduction of nitrates to nitrites. Negative catalase, indole, urease, carbohydrate utilization and hydrogen sulphide tests confirm positive identification of *H.ducreyi*. All isolates showed zones of inhibition around SPS disk of approximately 15 to 16 mm in diameter after 48 h.

2.3.3. Antimicrobial Susceptibilities of the *Haemophilus ducreyi* Clinical Isolates

The susceptibilities of 52 *H.ducreyi* isolates to ampicillin, chloramphenicol, kanamycin, streptomycin and tetracycline are presented in Tables 2.4., 2.5., B.1. and B.2. The reference strain ATCC 27722 was found to be susceptible to the following antibiotics: ampicillin, MIC < 0.5 μg/ml; chloramphenicol, MIC < 0.5

µg/ml; kanamycin, MIC 4 µg/ml; streptomycin, MIC 16 µg/ml; and tetracycline, MIC < 0.5 µg/ml.

H.ducreyi isolates from 1988 had MICs ranging from 32 to > 128 µg/ml for ampicillin. An increase in resistance to kanamycin and streptomycin was evident from strains isolated from the Gauteng migrant workers. Chloramphenicol and tetracycline resistance also increased in 1994. Strains from Windhoek which were isolated separately from the South African strains had low levels of resistance to kanamycin and streptomycin.

Table 2.3. Summary of biochemical characteristics of *H.ducreyi*.

Biochemical test	Reference strain ATCC 27722	isolates (n=52)
Porphyrin biosynthesis	-	-
Nitrate reduction	+	28/52 +
Catalase	-	-
Oxidase	+	+
Indole production	-	-
Urea hydrolysis	-	-
Ornithine decarboxylase	-	-
Alkaline phosphatase	+	+
H_2S production	-	-
β-lactamase hydrolysis	-	+

n = number of isolates

Table 2.4. Susceptibility of 52 *H.ducreyi* isolates to antimicrobial agents.

	Ampicillin	Chloramphenicol	Kanamycin	Streptomycin	Tetracycline
MIC_{50}	>128	<0.5	2	16	4
MIC_{90}	>128	16	8	32	16
Range	32 - >128	<0.5 - 32	<0.5 - >128	<0.5 - >128	<0.5 - 32

Table 2.5. Susceptibility of the ***H.ducreyi*** isolates from the various geographical regions to five antimicrobial agents from 1988 to 1994.

Geographic region	Number of isolates	MIC µg/ml	Ampicillin	Chloramphenicol	Kanamycin	Streptomycin	Tetracycline
Gauteng	18	50	>128	< 0.5	0.5	8	4
1988		90	>128	0.5	4	16	8
Botswana	5	50	>128	< 0.5	2	16	8
1992		90	>128	8	16	16	8
Lesotho	8	50	>128	< 0.5	1	16	4
1992		90	>128	4	8	16	8
Natal	9	50	>128	<0.5	4	16	4
1992		90	>128	16	64	32	8
Transkei	1	90	>128	0.5	8	32	8
1992							
Gauteng	7	50	>128	16	4	64	8
1994		90	>128	32	>128	> 128	16
Windhoek	4	50	64	8	< 0.5	8	16
1994		90	>128	16	8	16	16

Isolates from the various regions had similar MICs for tetracycline and for ampicillin. Isolates from Natal showed increased resistance to chloramphenicol, kanamycin and streptomycin. Gauteng isolates from 1994 showed resistance to all antibiotics tested.

2.4. DISCUSSION

All *H.ducreyi* isolates were identified on the basis of colony morphology and the results of biochemical tests. The colonial appearance of *H.ducreyi* is as described by other investigators (Sottnek *et al.*, 1980; Nobre, 1982; Oberhofer and Back, 1982; Sturm and Zanen, 1984a; Kraus, Morse and Sottnek, 1991; Totten and Stamm, 1994) and the diagnostic importance of simple observations such as colonial cohesiveness and the 'railroad track' arrangement of cells in Gram-stained smears of these colonies are valued.

The various media that were used supported the growth of *H.ducreyi* with the best growth obtained using Nsanze medium. Growth on the serum-free medium produced pinpoint colonies and in broth, the characteristic clumping of *H.ducreyi* cells was eliminated. Clumping on agar media makes quantitation of bacterial suspensions difficult and methods to reduce clumping have been devised (Totten and Stamm, 1984). Growth of *H.ducreyi* in broth was initiated in a static environment as *H.ducreyi* is microaerophilic (Oberhofer and Back, 1982). Totten and Stamm (1994) grew *H.ducreyi* in broth incubated at 35°C with shaking at 200 rpm for 24 h. They observed that the speed of agitation affected the growth curve since it changes the amount of oxygen in the broth. Agitation at 200 rpm resulted in a one-to-two fold increase in the number of cells per ml.

H.ducreyi has few demonstrable biochemical characteristics (Ronald and Albritton, 1984) because of its fastidious growth requirements. *H.ducreyi* strains have a broad range of phosphatase activity, including alkaline phosphatase, acid

phosphatase and phosphoamidase (Albritton, 1989). Although *H.ducreyi* is described as catalase negative and oxidase positive (Kilian and Biberstein, 1984), conflicting reports concerning these enzymes exist and results have depended on the methods and reagents used (Shawar, Sepulveda and Clarridge, 1990). Catalase activity has been uniformly negative and oxidase activity is generally negative when N,N-dimethyl-*p*-phenylenediamine oxalate is used and positive when N,N,N',N'-tetramethyl-1.4-phenylenediamine dihydrochloride is used (Nobre, 1982). The slide method was used to test for catalase production and gas production was not detected (Kilian, 1976; Sottnek *et al.*, 1980; Clarridge, Shawar and Simon, 1990; Shawar, Sepulveda and Clarridge, 1990). Sturm and Zanen (1984b) were unable to detect catalase activity after dropping 5% H_2O_2 on colonies of *H.ducreyi* but observed a positive catalase reaction in the test tube. The detection of cytochrome oxidase appears to depend on the substrate used for the test (Shawar, Sepulveda and Clarridge, 1990). Positive results were observed with tetramethyl-*p*-phenylenediamine dihydrochloride, a finding which is in agreement with previous studies using the same substrate (Nobre, 1982; Lubwama *et al.*, 1986; Shawar, Sepulveda and Clarridge, 1990).

The porphyrin test is the preferred method for demonstrating the dependence of *H.ducreyi* strains on exogenous haemin (factor X) (Hammond *et al.*, 1978b; Albritton *et al.*, 1981). Several investigators have reported the failure of some strains to reduce nitrate (Hammond *et al.*, 1978c; Sturm and Zanen, 1984b). This failure has been attributed either to incubation time or the test used to measure this enzyme activity (Sng *et al.*, 1982). Oberhofer and Back (1982) reported negative nitrate reduction by the Minitek test system, while conventional nitrate broth supplemented with 20% rabbit serum yielded positive test for nitrate reduction for all their strains. Indole production and urease activity have not been convincingly demonstrated, although one report (Sottnek *et al.*, 1980) demonstrated three strains with weak urease activity. The SPS disk susceptibility test is useful for

differentiating *H.ducreyi* from similar organisms, as only *H.ducreyi*, *Gardnerella vaginalis* and *Capnocytophaga* species show a zone of inhibition ≥ 14 mm (Shawari, Sepulveda and Clarridge, 1990).

H.ducreyi is a fastidious organism that presents special problems in susceptibility testing. Preparation of a suitable standard inoculum suspension was difficult because of the peculiar cohesive nature of the colonies. Methods used to overcome this problem have included mechanical agitation with a vortex mixer (Hammond *et al.*, 1978b; Bilgeri *et al.*, 1982; Slootsman *et al.*, 1983; Taylor *et al.*, 1985b), ultrasonication (Jones, Hafiz and Duerden, 1986; Dangor *et al.*, 1988a) and by passing the suspension through a 28 G needle (Jardine, 1990; Dangor, Radebe and Ballard, 1993). The recommendations of the National Committee for Clinical Laboratory Standards for antimicrobial susceptibility testing of fastidious organisms cannot be applied to *H.ducreyi*, since this organism requires haemin and supplementation with other nutrients for growth (Dangor *et al.*, 1990). Furthermore, the cohesiveness of colonies and the agglutination in suspension renders standardization of inoculum size difficult. In addition, the slow and differing growth rates of isolates results in the failure to predictably reproduce the logarithmic growth phase (Dangor *et al.*, 1990).

Direct detection of resistance mechanisms was the approach taken for antimicrobial susceptibility testing where the resistance mechanisms were deduced from the resistance phenotype characterized by conventional testing of a range of agents (Courvalin, 1992). The reported resistance phenotype was then predicted from the resistance mechanisms (Brown, 1994).

The antimicrobial susceptibility studies were compared with previous studies in South Africa (Bilgeri *et al.*, 1982; Abeck *et al.*, 1988; Dangor *et al.*, 1988a). All clinical isolates in this study produced β-lactamase, as with investigations in 1988

(Abeck *et al.*, 1988; Dangor *et al.*, 1988a). The 1982 study (Bilgeri *et al.*, 1982) showed 98% of *H.ducreyi* isolates produced β-lactamase. Although all strains in the 1988 studies produced β-lactamase, the MIC_{50} for penicillin of 16 μg/ml was lower than for the 1982 study (Bilgeri *et al.*, 1982) of 128 μg/ml. The isolates collected from Windhoek had MIC_{50} of 64 μg/ml and the remainder had MIC_{50} of 128 μg/ml.

Comparing the 1988 and 1994 Gauteng *H.ducreyi* isolates, an increase in resistances to all antibiotics tested was evident. Resistances to the antibiotics from isolates taken from migrant workers returning from their homes in different regions show a similar pattern. There was an increase in the kanamycin resistance of strains from MIC_{50}, 4 μg/ml (Dangor *et al.*, 1988a) to 8 μg/ml with a range of < 0.5 - > 128. The MIC for chloramphenicol was stable with the previous study by Dangor *et al.* (1988a) of 16 μg/ml and a range of < 0.5 - 32. The first report of emerging resistance to chloramphenicol and thiamphenicol in South Africa was reported by Dangor, Miller and Koornhof (1988b). Tetracycline resistance has been reported by the previous investigators (Bilgeri *et al.*, 1982; Abeck *et al.*, 1988; Dangor *et al.*, 1988a) to increase from an MIC_{90} of 16 μg/ml to 64 μg/ml and 128 μg/ml respectively. However, in this study MIC_{90} was 16 μg/ml with a range of < 0.5 - 32. This decrease could be the result of decreased prescription of this antibiotic. Streptomycin resistance has not been observed by the previous investigators, but comparing the results from 1988 and 1994, there has been an increase in resistance to this antibiotic.

In general, isolates from developing countries such as South Africa and more increasingly from industrialized societies have been found to be resistant to penicillin, tetracyclines and sulphonamides as a result of the use of these antimicrobials for clinical treatment (Abeck *et al.*, 1988).

3.0. PLASMID PROFILES AND THE CHARACTERISATION OF THE AMPICILLIN-RESISTANCE PLASMID OF *HAEMOPHILUS DUCREYI*

3.1. Introduction

β-Lactam antibiotics are among the most frequently-prescribed antibiotics worldwide. They inhibit enzymes involved in cell wall biosynthesis and cell division. The most common mechanism by which clinically-important bacteria become resistant to β-lactam antibiotics is by the acquisition of plasmids coding for the production of β-lactamases (Saunders, Hart and Saunders, 1986). These are enzymes that hydrolyze β-lactam antibiotics to inert and ineffective agents (Bush, 1989a). The enzymes inactivates the drug before it penetrates the cell wall and reaches the cytoplasmic membrane-located penicillin-binding-protein (PBP) targets (Foster, 1983). The plasmid-specified β-lactamases of gram-negative bacteria are expressed constitutively and are located in the periplasmic space between the cytoplasmic and outer membranes (Foster, 1983).

The TEM enzyme was the first β-lactamase coded by a plasmid to be recognized in gram-negative bacteria (Datta and Kontomichalou, 1965). There are two subtypes called TEM-1 and TEM-2 which differ in sequence by a single amino acid (Ambler and Scott, 1978; Sutcliffe, 1978), resulting in a change in isoelectric point (pI) but no major change in kinetic properties.

Another enzyme, ROB, with a broad TEM-like substrate specificity has been isolated from *H.influenzae* type *b* (Rubin *et al.*, 1981). It differs from TEM in having a different pI and a faster rate of ampicillin hydrolysis (Foster, 1983).

One of the objectives of these studies was to compare the plasmid profiles of strains isolated in different geographical regions and to identify the plasmids

conferring ampicillin resistance in *H.ducreyi.* This was accomplished by transferring the ampicillin resistance genes to a plasmid-free *H.influenzae* Rd strain by DNA transformation and testing the transformants for the production of β-lactamase and for the presence of plasmids.

3.2. MATERIALS AND METHODS

3.2.1. Bacterial Strains

H.ducreyi clinical strains used are listed in Table 2.2.

Haemophilus influenzae Rd, obtained from G.Leidy, University of Columbia, New York, USA, is a transformable rough derivative of the capsular type d strain originally isolated by Alexander and Leidy (1951). The cultures were maintained at -70°C in sBHI broth with 10% glycerol (Merck).

3.2.2. Media

H.influenzae Rd was grown on chocolate agar (Appendix A.2.) and on BHI agar and in BHI broth (BBL, Becton Dickinson and Co.) supplemented with 10 µg/ml haemin and 2 µg/ml NAD. Ampicillin-, streptomycin- and kanamycin-resistance transformants were grown on chocolate agar supplemented with either 5 µg/ml ampicillin (Sigma), 100 µg/ml streptomycin sulphate (Sigma), 100 µg/ml kanamycin monosulphate (Sigma) or 100 µg/ml sulphamethoxazole (SAIMR).

3.2.3. *Haemophilus ducreyi* Plasmid Extraction

Plasmid extraction was performed according to a modified procedure of Abeck *et al.* (1988). *H.ducreyi* isolates were grown on Nsanze medium containing the appropriate antibiotic. Colonies from two plates were suspended in 5 ml of TAE (50 mM tris (hydroxymethyl) aminomethane (Tris)-HCl, 20 mM sodium acetate, 20 mM ethylenediaminetetra-acetate (EDTA)) buffer, pH 8.0, and centrifuged in a J-21 Beckman centrifuge (Beckman instruments Pty Ltd, California, USA) at

7000 rpm for 20 min at 4°C in a JA-14 rotor. The pellet was resuspended in 0.5 ml of 35% (w/v) sucrose dissolved in TE buffer (50 mM Tris-HCl, 20 mM EDTA, pH 8.0). The suspension was lysed with 1.2 ml buffer (3% sodium lauryl sulphate (SDS) in 50 mM Tris-HCl, pH 12.6) and the lysate heated at 70-80°C for 5 min. This disrupts base-pairing, causing the linear chromosomal DNA to denature. The pH of the mixture was reduced by the addition of 1 ml of 2 M Tris-HCl, pH 7.0. Chromosomal DNA was precipitated by the addition of 1 ml ice-cold 4 M potassium acetate-2 M acetic acid and the mixture stored on ice for 1 h. The precipitate was sedimented at 7000 rpm for 30 min in a JA-14 rotor at 4°C. The supernatant was transferred to another tube and extraction of proteins with a phenol: chloroform: 3-methyl-Butan-1-ol (25:24:1) mixture was performed several times until no precipitate was present at the interface. Any remaining traces of phenol were removed by a chloroform extraction. DNA was precipitated by the addition of 0.6 vol isopropanol (Merck). The precipitate was collected by centrifugation and either resuspended in 20 µg/ml ribonuclease A (RNase A) (Sigma) (heat-treated at 80°C for 10 min to inactivate contaminating deoxyribonuclease, if present) in TE (10 mM Tris-HCl, 1 mM EDTA) buffer, pH 8.0, and incubated at 37°C for 1 h or purified further on a caesium chloride (CsCl) gradient (Sambrook, Fritsch and Maniatis, 1989; Appendix A.8.). To the RNA free solution 0.2 M sodium acetate and 2 vol of absolute ethanol (Merck) was added to precipitate the nucleic acid at -20°C overnight. The DNA precipitate was recovered by centrifugation and the DNA was resuspended in TE (10 mM Tris, 1 mM EDTA) buffer, pH 7.6, and stored at -20°C.

3.2.4. Plasmid Preparation from *Haemophilus influenzae* Transformants

Plasmids were extracted from *H.influenzae* Rd by a modification of the alkaline lysis procedure of Birnboim and Doly (1979). An overnight culture was grown in 10 ml of sBHI broth containing 10 µg/ml ampicillin (Sigma). The culture was harvested by centrifugation and the cells resuspended in 200 µl of plasmid buffer

(50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0) after which 400 µl of SDS/NaOH solution (0.2 NaOH, 1% SDS (w/v)) was added and the contents mixed by inversion. The mixture was place on ice for 10 min for cell lysis to occur before the addition of 300 µl, 3 M sodium acetate (pH 4.8). After mixing the contents thoroughly by inversion, the mixture was incubated on ice for 1 h. The precipitated, denatured chromosomal DNA was sedimented by centrifugation at 4°C and the supernatant containing plasmid DNA was precipitated with 2 vol of ethanol. After 1 h of incubation at -20°C the nucleic acids were sedimented by centrifugation at 4°C. The pellet was resuspended in TE buffer (pH 8.0) and RNA was removed by the addition of 20 µg/ml heat-treated RNase A (Sigma). After incubation at 37°C for 1 h the DNA was resuspended in 300 µl of TE buffer. An equal volume of a phenol: chloroform: 3-methyl-Butan-1-ol (25:24:1) mixture was added and the tube inverted several times to mix the contents. The mixture was centrifuged and the upper aqueous layer containing the plasmid DNA was transferred to a clean tube. The procedure was repeated until no protein was present at the interface. The plasmid DNA was concentrated by the addition of 0.3 M sodium acetate and 2 vol of absolute ethanol. The DNA precipitate collected after centrifugation was resuspended in 100 ml TE buffer, pH 7.6.

3.2.5. Transformation of *Haemophilus influenzae* Rd

Transformation of *H.influenzae* Rd with *H.ducreyi* resistance plasmids was performed by using the method of Gromkova, Rowji and Koornhof (1989).

3.2.5.1. Preparation of competent cells

H.influenzae Rd was streaked from a frozen stock onto a fresh chocolate agar plate and incubated overnight at 37°C. One colony was transferred into sBHI broth. The culture was incubated in an Erlenmeyer flask by shaking at 200 rpm for 18 h at 37°C. Next day the culture was diluted 1:50 into 5 ml of fresh sBHI broth and placed in sterile 90 mm petri dishes. The cells were incubated without shaking at 37°C until an optical density reading of 0.6-0.8 at 650 nm (OD_{650}) was obtained.

3.2.5.2. Transformation procedure

All transformation experiments were performed with freshly prepared competent cells. To 1 ml of competent *H.influenzae* cells, $MgCl_2$ was added to a final concentration of 0.001 M. One hundred µl aliquots of cells were dispensed into 5 ml test tubes with the addition of 20 µg/ml of plasmid DNA. The transformation mixtures were shaken at 80 rpm, at 37°C for 90 min, in a G 24 Environmental incubator shaker (New Brunswick Scientific, Edison, N.J., USA), for the uptake of DNA by the cells. For the expression of the genetic markers, 0.5 ml of sBHI broth was added and the mixtures were incubated for 4 h, at 37°C with agitation at 200 rpm. Controls for the level of competence consisted of competent cells with 20 µg/ml *H.influenzae* Rd chromosomal DNA containing a streptomycin resistance marker which was obtained from Dr.R.Gromkova (University of the Witwatersrand, Johannesburg). To estimate the frequency of mutation, controls of competent cells without the addition of DNA were used.

3.2.5.3. Selection of transformants

To select for transformants 1 ml of diluted bacterial culture was placed in a petri dish followed by the addition of 20 ml of molten sBHI agar containing the appropriate antibiotic. The plates were incubated at 37°C for 48 h. All experiments were repeated twice. *H.influenzae* that grew in the ampicillin-containing sBHI agar were picked up with an inoculating needle and streaked onto fresh chocolate agar. After 24 h incubation at 37°C the colonies were tested for β-lactamase activity.

3.2.6. Restriction Endonuclease Analysis of Plasmid DNA

Restriction enzymes (Table 3.1.) obtained from Boehringer Mannheim GmbH (Germany) were used as prescribed by the manufacturers. The addition of 5 µl loading/stop buffer (0.1% bromophenol blue, 1% SDS, 100 mM EDTA, 50% glycerol) terminated the reaction. The incubation mixture was heated at 65°C for 10 min for the inactivation of the following restriction endonucleases: *Ava* I, *Hind*

II and *Hind* III. Fragments containing cohesive ends which might have hybridized during the incubation are denatured by this procedure.

3.2.7. Molecular Weight Markers

The plasmids used as molecular weight markers, pUC18 (2.7 kb), pBR322 (4.4 kb), p7F12 (8.5 kb) and pDA37 (15.3 kb), were extracted from laboratory-derived strains of *E.coli* by the alkaline lysis procedure of Birnboim and Doly (1979). DNA molecular weight markers VII and X were obtained from Boehringer Mannheim GmbH (Appendix A.9.).

3.2.8. Agarose Gel Electrophoresis of Plasmid DNA

The DNA was loaded onto agarose gels (SeaKem, FMC) in TAE buffer (40 mM Tris-HCl, 5 mM sodium acetate, 1 mM EDTA, pH 7.6) and electrophoresed on a horizontal Bio-Rad mini-sub system (Bio-Rad, Richmond, California, USA) at 60 V. The gel was stained with 0.5 µg/ml ethidium bromide (EtBr) and the band pattern viewed by UV-light. Size determinations of DNA were performed by comparing with the mobility of molecular weight markers by the method of Southern (1979).

Table 3.1. Recognition sequences of restriction endonucleases used in this study.

Restriction enzyme	Recognition sequence
Ava I	C↓(T,C) C G (A,G) A C
Bam HI	G↓G A T C C
Pst I	C T G C A↓G
Pvu II	C A G↓C T G
Hind II	G T (T,C)↓(A,G) A C
Hind III	A↓A G C T T

A	Adenine	C	Cytosine
G	Guanine	T	Thymine
↓	Site of cleavage		

3.3. RESULTS

3.3.1. Plasmid Profiles of *Haemophilus ducreyi*

The data shown in Table 3.2. indicate five different types of plasmid profiles (Figure 3.1.) among the 52 *H.ducreyi* isolates. Some differences in the distribution of *H.ducreyi* isolates were observed based on their plasmid profiles. Most isolates from all geographical regions possess the 9.3 kb plasmid (75%), while some isolates harbour the 10.6 kb plasmid (15%). In addition, two strains from Botswana, *H.ducreyi* CH137 and P116, and three strains from Gauteng (1994), *H.ducreyi* HD4, HD6 and HD8 contain a plasmid profile of 5.6-, 4.5-, 3.9- and 3.0-kb. Two strains, from Gauteng (1988) and Botswana, Y88 and P77 respectively, carry a 9.3- and 7.5-kb plasmid. An isolate from Natal (CH87) harboured a 9.3- and a 4.5-kb plasmid. Strains lacking plasmids were not detected.

Table 3.2. Geographic distribution of 52 isolates of *H.ducreyi* by year of isolation and plasmid content.

Geographic origin	Year of isolation	No. of isolates	Plasmid Content[a]
Gauteng	1988	17	9.3
		1	9.3; 7.5
Botswana	1992	1	9.3; 7.5
		2	10.6
		2	5.6; 4.5; 3.9; 3.0
Lesotho	1992	6	9.3
		2	10.6
Natal	1992	5	9.3
		3	10.6
		1	9.3; 4.5
Transkei	1992	1	10.6
Gauteng	1994	4	9.3
		3	5.6; 4.5; 3.9; 3.0
Windhoek[b]	1994	4	9.3

a. Molecular masses of plasmids are given in kilobases (kb)
b. Windhoek, Namibia

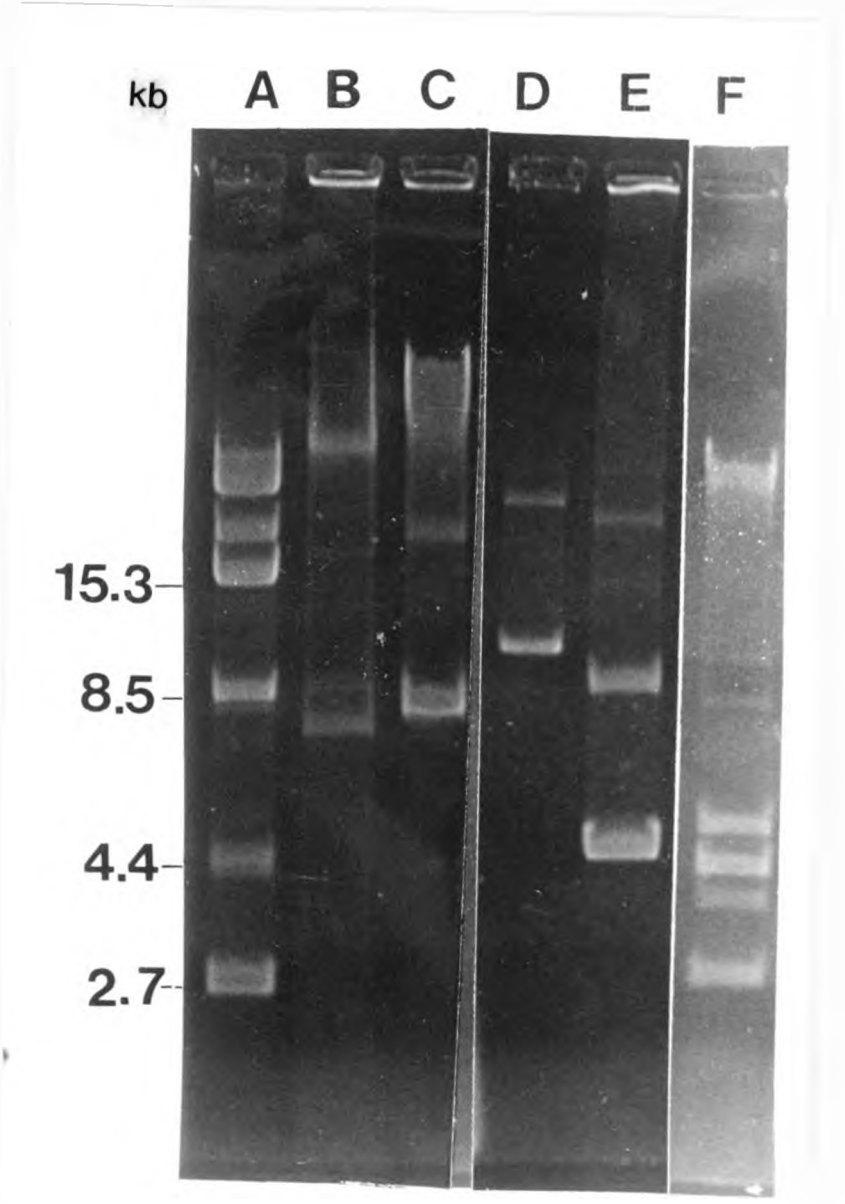


Figure 3.1. **0.8% Agarose gel electrophoresis profiles of plasmids. Lanes B to F, plasmid DNA from isolates Y88, CH138, CH247, CH87 and P116 respectively. Lane A represents the *E.coli* molecular weight markers with their sizes in kb's.**

3.3.2. Identification of the Ampicillin-Resistance Plasmids

These plasmids were identified by transformation. Ampicillin-resistant transformants were obtained by transforming plasmid DNA extracted from the clinical isolates of *H.ducreyi* into plasmid-free *H.influenzae* Rd. The frequencies of transformation of the plasmids harbouring the ampicillin-resistance gene and the streptomycin-resistance gene of homologous chromosomal DNA were determined and the results from representative plasmids from each size is presented in Table 3.3. The transformation frequencies of *H.ducreyi* ampicillin-resistance genes were at least 10^4 lower than the frequencies of the streptomycin-resistance genes of homologous chromosomal DNA. The ampicillin-resistance transformants were examined for β-lactamase production and for the presence of plasmids. All ampicillin-resistant transformants were β-lactamase producers. The results presented in Figure 3.2. show that β-lactamase production is mediated by the 5.6-, 9.3- and 10.6-kb plasmids.

3.3.3. Comparison of Ampicillin-Resistance Plasmids by Restriction Endonuclease Digestion

Since bacteria of the same species may have different plasmids of similar molecular mass, restriction endonuclease digestions were conducted to determine whether the ampicillin-resistance plasmids harboured by the *H.ducreyi* clinical isolates were similar. The ampicillin-resistance plasmids were digested with the restriction enzymes listed in Table 3.1. and their digestion products observed by agarose gel electrophoresis. A comparison of the restriction endonuclease patterns of the *H.ducreyi* plasmids is shown in Figure 3.3. The four ampicillin-resistance plasmids were designated pCH138 (9.3 kb), pP116 (5.6 kb), pP15 (10.6 kb) and pCH128 (10.6 kb).

Table 3.3. Transformation frequencies of *H.influenzae* Rd with *H.ducreyi* ampicillin-resistance plasmids.

Donor DNA	Genetic marker	Transformation frequency (TF)	Number of CFU
H.ducreyi plasmids			
pCH137	Amp^r	9.1×10^8	10^9
pCH138	Amp^r	1.0×10^8	10^9
pP15	Amp^r	7.1×10^9	10^9
Controls			
H.influenzae Rd (Chromosomal)	Str^r	10^3	10^9
No DNA		0	10^9

r resistance
Amp ampicillin
Str streptomycin

Table 3.4. Ampicillin-resistance plasmids recovered from *H.ducreyi* strains from different geographical regions.

Geographic region	No. of isolates	plasmids		
		5.6 kb	9.3 kb	10.6 kb
Gauteng (1988)	18		18	
Botswana	5	2	1	2
Lesotho	8		6	2
Natal	9		6	3
Transkei	1			1
Gauteng (1994)	7	3	4	
Windhoek	4		4	

Recovery of plasmids in the ampicillin-resistance transformants and their sizes.

Two plasmids of the same size of 10.6 kb were observed when cut with *Bam* HI (7.6- and 2.5-kb) and a third *Bam* HI site of 9 kb was revealed in pP15. All plasmids have a common *Pst* I fragment of 3.1 kb and *Ava* I fragment of 4.5 kb. In addition, the 9.3 kb and 10.6 kb plasmids have a common *Bam* HI fragment of 2.5 kb. The 5.6 kb and 9.3 kb plasmids showed identical *Pst* I sites of 6.1 kb. *Pvu* II cut the 10.6 kb plasmid into three sizes of 4.8- 6.4- and 8.3-kb. Cleavage of the plasmids with *Hind* II and *Hind* III was not observed.

3.3.4. Recovery of the 4.5 kb Plasmid

The 4.5 kb kanamycin-resistance plasmid from a clinical isolate of *H.ducreyi*, Hd4 (resistant to ampicillin and kanamycin), was recovered by extracting the plasmid from a 0.8% agarose gel using the Geneclean II kit (BIO 101 Inc., California, USA) and transforming the plasmid into *H.influenzae* Rd. Kanamycin-resistant transformants were selected on 100 µg/ml kanamycin-containing, chocolate agar. The transformants were also resistant to 100 µg/ml streptomycin and 100 µg/ml sulphamethoxazole.

3.4. DISCUSSION

The plasmid profiles of *H.ducreyi* isolates were compared to those obtained in previous studies undertaken in southern Africa. Plasmids of 6.1-, 8-, 8.9- and 9.9-kb (Thomson and Bilgeri, 1982) and of 8.8-, 7.5- and 10.8-kb (Abeck *et al.*, 1988) have been identified previously from *H.ducreyi* isolates on the subcontinent. Comparing the plasmid profiles with the previous studies, an increase in the number of plasmids, with an increase in the level of antimicrobial resistance (as discussed in Chapter 2) in the past 4 - 6 years was evident. Plasmids identified in this study include the 9.3-, 10.6-kb and the 7.5 kb plasmids, the 9.3- with 4.5-kb plasmids and the 5.6-, 4.5-, 3.9- and 3.0-kb plasmids.

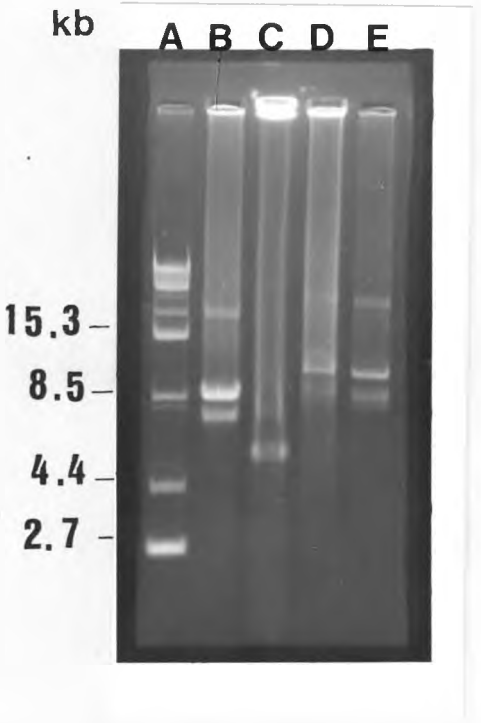


Figure 3.2. Agarose gel electrophoresis of plasmid DNA extracted from *H.influenzae* Rd transformants. Lanes: A, *E.coli* molecular weight markers; *H.influenzae* Rd transformants using *H.ducreyi* plasmid DNA: B, pCH138 (9.3 kb); C, pP116 (5.6 kb); D, pP15 (10.6 kb); E, pCH128 (10.6 kb).

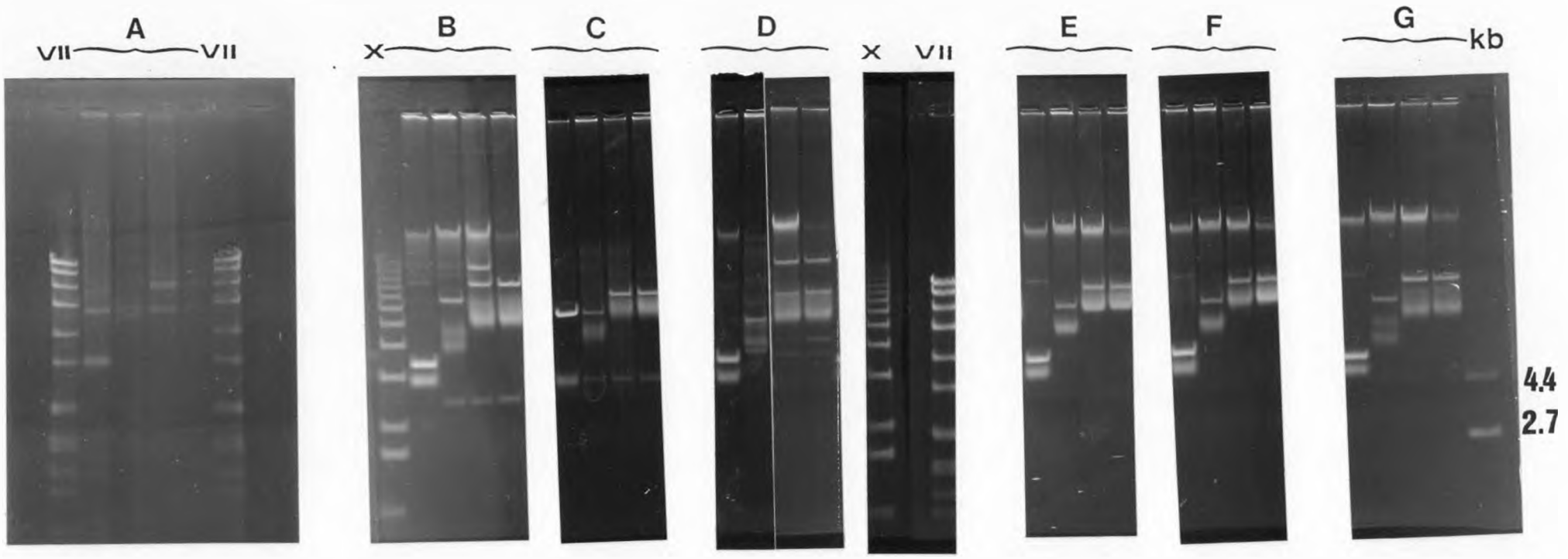


Figure 3.3. Electrophoresis on 1% agarose gels after digestion of plasmid DNA, extracted from the *H.influenzae* Rd transformants. Plasmids pP116, pCH138, pP15 and pCH128 were digested with A, *Ava* I; B, *Bam* HI: C, *Pst* I; D, *Pvu* II; E *Hind* II; F, *Hind* III respectively. The molecular weight markers VII and X were used. Lane G represents the untreated plasmid DNA.

The plasmids which were recovered from the clinical isolates of *H.ducreyi* seldom varied according to the geographic regions, except for the 5.6-, 4.5-, 3.9- and 3.0-kb plasmid combination which were present in only two regions. One can speculate that migrant mine workers moving from Botswana to Gauteng (1994) could have spread the strains carrying the plasmid combination.

In this study five strains were found to harbour multiple plasmids of sizes 3.2-, 2.8-2.6- and 1.8-Mdal (4.9-, 4.3-, 4- and 2.8-kb respectively) which appear to be similar to the plasmids of *H.ducreyi* strains isolated recently in Thailand (Sarafian *et al.*, 1991a) and in San Francisco, USA (Sarafian and Knapp, 1992). Sarafian *et al.* (1991a) observed that the 3.2 Mdal/4.9 kb plasmid present in these isolates did not hybridize with the *bla* probe, prepared from a 4.4 Mdal β-lactamase-specifying plasmid of *N.gonorrhoeae*, therefore demonstrating that it was unrelated to the 3.2 Mdal β-lactamase plasmid found in *H.ducreyi* and *N.gonorrhoeae* strains described by Anderson *et al.* (1984). Later, MacLean *et al.* (1992) analysed the strains from Thailand. They demonstrated that the ROB-1 β-lactamase is expressed in an isolate of *H.ducreyi* from Thailand and that the *bla* ROB-1 gene for this enzyme is carried on a 5.4 kb plasmid. Furthermore they reported that the 5.4 kb plasmid was found in conjunction with the antibiotic-resistance plasmid pLS88 (4.8 kb) and two small cryptic plasmids. The 2.8 Mdal (4.3 kb) plasmid observed in the majority of these strains may be the kanamycin-resistance plasmid described previously (Sanson-Le Pors, Casin and Collatz, 1985; Willson *et al.*, 1989), as in this study the plasmid when transformed to *H.influenzae* conferred resistance to kanamycin, streptomycin and sulphonamide.

One of the most surprising observations in transformation of gram-negative bacteria is the specificity of the DNA uptake system for homologous DNA (Smith, Danner and Deich, 1981). Foreign DNAs can bind to the competent cell surface in large amounts but are absorbed poorly (Smith, Danner and Deich, 1981). The poor

uptake of plasmid DNA into cells reflects the lack of specific base sequences on the plasmid DNA (Notani *et al.*, 1981), as the same sequence required for the efficient uptake of chromosomal DNA (Danner *et al.*, 1980) is also required for the efficient uptake of plasmid DNA (Smith, Danner and Deich, 1981). It has been reported (Bendler, 1976) that plasmid DNA transfers its antibiotic-resistance markers at least 10^{-4} fold less frequently than chromosomal DNA transfers its auxotrophic markers. This low efficiency applies even to plasmids that bear the *Haemophilus* uptake sequence (Smith, Danner and Deich, 1981).

All *H.ducreyi* strains examined in this study were ampicillin resistant and the genes coding for this resistance were located on plasmids. This was demonstrated, by first direct examination of *H.ducreyi* strains for the production of β-lactamases and secondly by transforming the ampicillin-resistance gene to a plasmid-free *H.influenzae* Rd and demonstrating that all transformants have acquired plasmids. At least four different types of plasmids were identified. From the 52 isolates, 39 strains (75%) have the 9.3 kb plasmid, 8 strains (15%) have the 10.6 kb plasmid and 5 strains (10%) have the 5.6 kb plasmid. These plasmids are similar in size to the ampicillin-resistance plasmids previously described (McNicol and Ronald, 1984; MacLean *et al.*, 1992). Although a 3.9 Mdal/6.1 kb ampicillin-resistance plasmid has been described in 1982 (Thomson and Bilgeri, 1982), it is uncommon in Africa (Anderson *et al.*, 1984; McNicol and Ronald, 1984). The acquisition of this plasmid could be by conjugative transfer from *N.gonorrhoeae* isolates which carry a 3.2 Mdal (4.9 kb) β-lactamase plasmid, which is also predominant in Africa (Roberts, Elwell and Falkow, 1977; Anderson *et al.*, 1984). Plasmid exchange between *Haemophilus* and *Neisseria* species has been suggested by the isolation of an identical ampicillin-resistance plasmid in *H.parainfluenzae* and *N.gonorrhoeae* (Brunton, Clare and Meier, 1986).

The majority of clinical isolates possessed the 9.3 kb plasmid coding for the production of β-lactamase. This plasmid was designated pJB1 by Brunton *et al.* (1979) and the first reported isolation of this plasmid was at the 1978 epidemic in Winnipeg, Canada (Hammond *et al.*, 1978b). This plasmid is well distributed and was observed frequently over a period of time in this study as well as by other investigators (Thomson and Bilgeri, 1982; Abeck *et al.*, 1988). Brunton, Bennett and Grinsted (1981) demonstrated that this plasmid possessed an intact TnA which could be transposed to co-resident plasmids. The plasmid was shown to code for TEM-1 type β-lactamase (MacLean, Bowden and Albritton, 1980).

The 10.6 kb plasmid contains the complete TnA sequence (McNicol and Ronald, 1984). The finding that there are two different β-lactamase encoding plasmids of a similar size of 10.6 kb suggests the possibility of an rearrangement of nucleic acids, resulting in a third *Bam* HI site in the plasmid DNA, as other investigators (Brunton *et al.*, 1982) have reported only two *Bam* HI sites. An enzyme like TEM-1 may tolerate amino acid changes better than other enzymes that modify drugs because its structure is braced by disulphide and salt bridges (Jelsch *et al.*, 1993) and because β-lactamases have a single site for binding to their substrate (Jacoby, 1994).

The 5.6 kb ampicillin-resistance plasmid was found only in strains from Botswana and Gauteng. As mentioned previously, the ampicillin-resistance plasmid carries the ROB-1 β-lactamase gene and three other ROB-1 β-lactamase plasmids have been described previously (MacLean *et al.*, 1992). A 4.1 kb ROB-1 plasmid from *Pasteurella* strains (Livrelli *et al.*, 1988), a 4.4 kb plasmid, R_{rob}, in *Haemophilus* strains and at least one isolate of *P.haemolytica* (Livrelli, Peduzzi and Joly, 1991) and a 5.4 kb plasmid possessing the *bla* ROB-1 gene in *Actinobacillus pleuropneumoniae* (Medeiros, Levesque and Jacoby, 1986). All of these plasmids are related, as measured by DNA hybridization (Medeiros, Levesque and Jacoby,

1986; Livrelli, Peduzzi and Joly, 1991) and they express a ROB-1-type β-lactamase. It has also been suggested that this form of ampicillin resistance has an animal reservoir and that conditions fostering its prevalence in animal strains may play a role in the spread of resistance to human pathogens (Medeiros, Levesque and Jacoby, 1986).

Hind II and *Hind* III do not cut the plasmid DNA. This is due either to the lack of recognition sites on the plasmid DNA or because of the modification of these sites by specific methylases. Since both *Hind* II and *Hind* III enzymes are derived from *H.influenzae* Rd (Smith and Wilcox, 1970), which was used as a recipient in transformation, the lack of digestion of the plasmids was most probably due to methylation of the plasmid DNA. It is known that restriction endonuclease are active only against unmethylated heterologous and not homologous DNA (Roy and Smith, 1973).

4.0. TRANSFORMATION OF *HAEMOPHILUS DUCREYI* BY ELECTROPORATION

4.1. Introduction

Studies on the pathogenicity, physiology and genetics of *H.ducreyi* have been hindered by the lack of efficient methods for gene transfer, in particular, DNA transformation. Although several members of the genus *Haemophilus* are known to possess a natural competence-dependent transformation mechanism, attempts to induce competence in *H.ducreyi* under different growth conditions or to promote uptake by inducing protoplast formation have been unsuccessful (Dr.R.Gromkova, unpublished results).

4.1.1. Background

The use of an electric field to reversibly render cells permeable has been termed 'electroporation' (Neumann *et al.*, 1982; Potter, Weir and Lieder, 1984) and the procedure has found widespread application in biology. The first application of the electric pulse technique to transfer genetic material was reported by Auer, Brandner and Bodemer (1976) who demonstrated transfer of SV 40 DNA and of mammalian cell RNA into human red blood cells. Subjecting membranes to a high-voltage electric field results in their temporary breakdown and the formation of pores (Kinosita and Tsong, 1977a,b), which permits the exchange of intracellular and extracellular components. Kinosita and Tsong (1977a) observed that biomembranes are transiently made more permeable by the action of short electric pulses above a certain field strength, without damaging the membrane structures. The size of these pores could be varied in a controlled manner: increasing the field intensity, the pulse duration, or decreasing the ionic strength all resulted in the formation of larger pores (Kinosita and Tsong, 1977a). The permeability induced is reversible, and the original membrane resistance and impermeability are restored, provided the magnitude or duration of the electric field does not exceed a critical limit,

otherwise the pore size expands, resulting in the release of intracellular components (Kinosita and Tsong, 1977b).

In 1982, the first electroporative gene transfer, with subsequent actual gene expression was reported (Neumann *et al.*, 1982). Plasmid DNA carrying the thymidine kinase (*tk*) gene, from the herpes virus, was introduced into *tk*-deficient mammalian culture cells by the application of short electric pulses. Stable transformants surviving in the HAT (hypoxanthine, aminopterin and thymidine) selection medium, in which only cells expressing the *tk* gene will grow, proved the direct gene transfer and subsequent expression of the gene.

Electroporation has become a valuable technique for the transfer of nucleic acids into adherent or suspended eukaryotic cells (electro-transfection) and prokaryotic cells (electro-transformation), and is an excellent alternative for many cell types which cannot be transfected or transformed by using chemical or biological methods (Shigekawa and Dower, 1988).

A rapidly growing number of reports on gene transfer by electroporation, demonstrates the applicability of this technique not only for mammalian, but also for plant, bacterial and unicellular cells.

4.1.2. Mammalian Cells

Studies on the control of gene expression in eukaryotes rely heavily on the ability to induce the integration and stable expression of cloned genes in mammalian cells (Potter, 1988). Chu, Hayakawa and Berg (1987) demonstrated electroporation-mediated transfection can yield efficiencies greater than those obtained by calcium phosphate precipitation methods, with electroporation resulting in an increase in transient expression, ranging up to 50-fold. These workers applied electroporation to a large variety of mammalian cells from different species and tissue types.

4.1.3. Plant Protoplasts

Electro-transfection of monocot and dicot protoplasts has been performed using exponential or square wave pulses. Fromm, Taylor and Walbot (1985) developed a method for the electroporation of DNA into carrot, tobacco and maize protoplasts. They observed that with the electrical introduction of genes into plant protoplasts, a gene can be transferred into a cell and its expression analysed within hours. The advantages of the method are convenience, low cell toxicity, efficiency and applicability to a wide range of plant protoplasts (Fromm, Taylor and Walbot, 1985). Langridge, Li and Szalay (1987) by introducing labelled plasmid DNA into chloroplasts indicated that, in addition to mediating DNA uptake into protoplasts, electroporation may also be used to introduce nucleic acids directly into isolated subcellular organelles. Thus, electroporation may now be applied for the genetic modification of genes involved in photosynthesis which are transcribed in the chloroplasts of economically important crop plants.

4.1.4. Unicellular Organisms

Hashimoto *et al.* (1985) succeeded in introducing plasmid DNA into intact *Saccharomyces cerevisiae* cells by applying electric field pulses under optimal electrical conditions. Since the method was not accompanied by cell fusion, it provided a novel method for transformation of yeast cells (Hashimoto *et al.*, 1985).

Two groups have successfully used electroporation to introduce DNA into *Trypanosoma brucei.* Eid and Sollner-Webb (1987) used plasmid DNA containing the promoter from a *T.brucei* rRNA gene and attempted to monitor electroporation both by uptake of radiolabelled DNA and by increased transient RNA expression. At about the same time, Gibson, White and Borst (1987) introduced whole mini-chromosomes isolated from *T.congolense* into *T.brucei* by electroporation. The foreign chromosomes apparently survived in the recipient parasite for several generations in the absence of selective pressure. If DNA transformation and

expression in trypanosomes after electroporation becomes reproducible, it should be a great help in elucidating the highly complex and unusual transcription systems in these organisms (Potter, 1988).

4.1.5. Bacteria

Since the majority of bacterial species are not naturally competent, this prompted the search for new techniques for introducing DNA into bacterial cells. A method developed by Mandel and Higa in 1970, used calcium for inducing the formation of protoplasts. They subsequently were able to introduce bacteriophage DNA into protoplast of bacterial cells. Later, Cohen, Chang and Hsu (1972) further developed this procedure by demonstrating that the calcium chloride treatment could be used to transfer plasmid DNA into *E.coli* cells. However, since many bacteria of interest are recalcitrant to the above transformation techniques, this initiated the development of electro-transformation which is rapidly gaining ground today as a simple and efficient technique to transform many species (Solioz and Bienz, 1990).

Two bacterial species, *E.coli* (Calvin and Hanawalt, 1988; Dower, Miller and Ragsdale, 1988; Fiedler and Wirth, 1988) and *Campylobacter jejuni* (Miller, Dower and Tomkins, 1988) have been studied extensively. Recent examples include *Legionella pneumophila* (Pope, Dhand and Cianciotto, 1994), which causes legionnaires' disease; *Clostridium perfringens*, a source of food poisoning in humans and animals caused by a potent enterotoxin (CPE) (Melville, Labbe and Sonenshein, 1994); *Bacillus thuringiensis*, where the *B.thuringiensis* toxin gene was introduced into wild-type *B.cereus*, *B.brevis* and *B.subtilis* which were used in toxicity assays against the caterpillar of *Heliothis assulta* (Sun *et al.*, 1994); and the transformation of *Bartonella bacilliformis*, an intracellular parasite of human erythrocytes (Grasseschi and Minnick, 1994).

4.1.6. Important Parameters of Electroporation

Although electroporation is effective in producing pores in a wide variety of cell types, each situation requires slightly different conditions that depend on the special characteristics of the target cell (Potter, 1988).

Several parameters are relevant to successful electroporation. Of these, the field strength, the duration of the current pulse and the electroporation buffer are the most important, and these factors interact in a compensatory manner (Dower, Miller and Ragsdale, 1988; Potter, 1988; Shigekawa and Dower, 1988). Two different types of electric field pulses are used for membrane electropermeabilization: exponential decay or square wave (Potter, 1988; Shigekawa and Dower, 1988). The voltage of a square wave is raised to a given amplitude which is maintained for a specified time, and then returned to zero (Figure 4.1.). The voltage of an exponential pulse is raised to an initial peak amplitude, then allowed to decay exponentially (Figure 4.2.). Thus a power supply can be either discharged directly across the sample (square wave) (Potter, 1988; Shigekawa and Dower, 1988) or it can be used to store charge in a capacitor which is subsequently discharged across the sample (exponential decay pulse) (Neumann *et al.*, 1982; Fromm, Taylor and Walbot, 1985; Chu, Hayakawa and Berg, 1987).

Other experimental conditions also play a role in the efficiency of transformation. These include the specific conditions used for transformation (such as nutrients, ionic strength, purity of reagents and temperature) and the time necessary for the expression of the genetic marker. It is known from previous studies that the expression time depends on the nature of the genetic marker.

4.1.7. Capacitors and Resistance-Capacitance (*RC*) Circuits

The electric pulse used in electroporation is generated when the charged capacitor is suddenly discharged through the electroporation cuvette containing the cells and

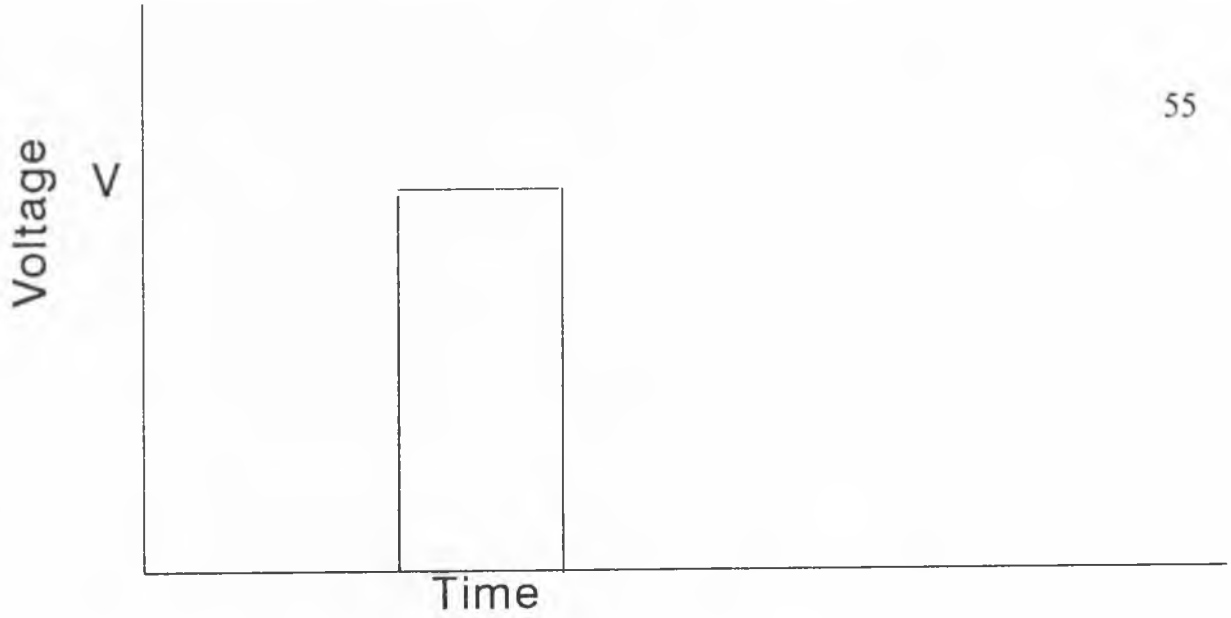


Figure 4.1. Square wave pulse.

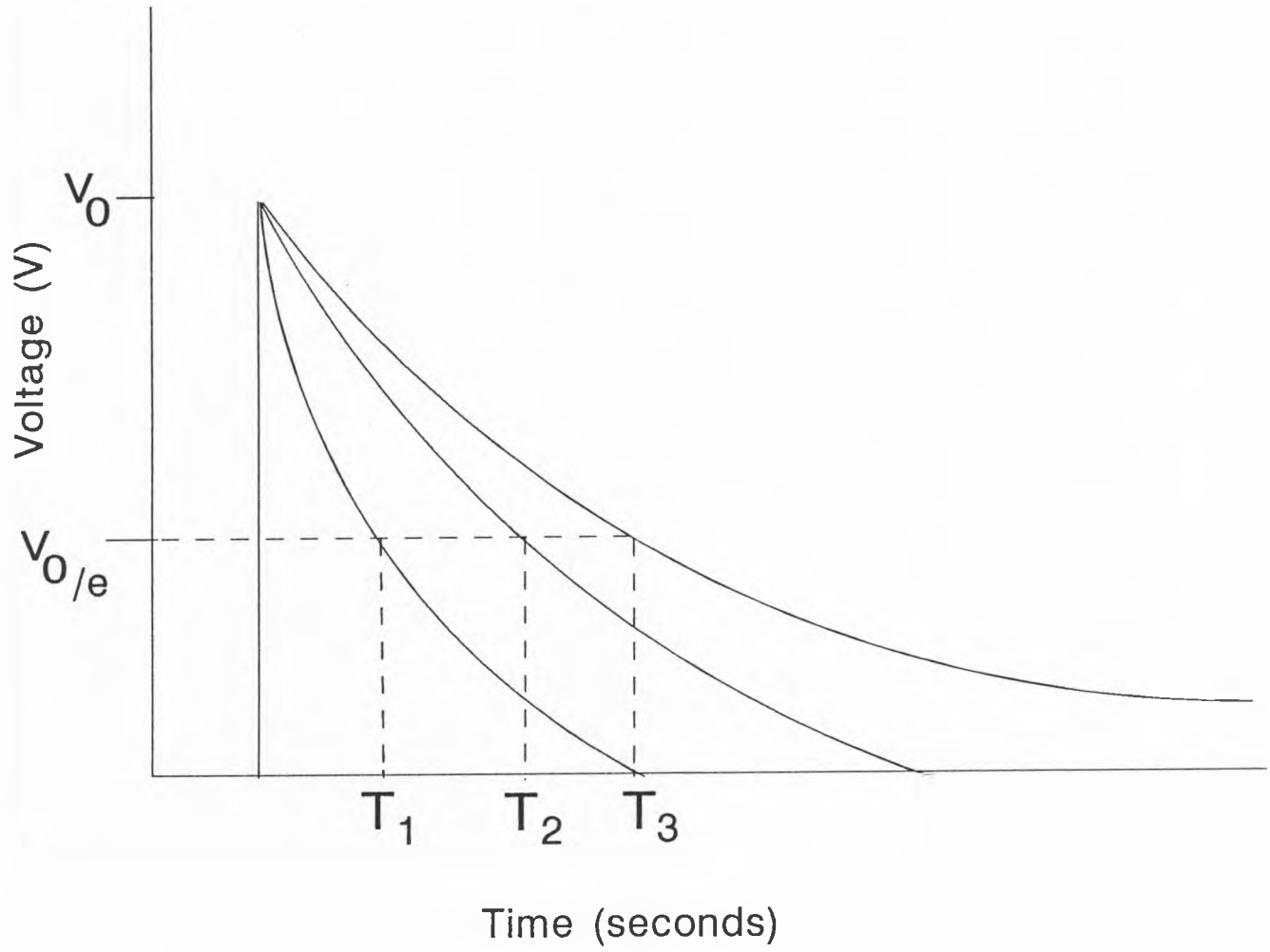


Figure 4.2. Exponential decay waveform.

DNA (Fromm *et al.*, 1987; Dower, Miller and Ragsdale, 1988). The ionic composition of the solution in the electroporation cuvette, the geometry of the cuvette and electrodes determines the electrical resistance of the sample (Fromm *et al.*, 1987; Dower, Miller and Ragsdale, 1988; Shigekawa and Dower, 1988). The resistance and ionic strength of the medium are inversely related; increasing the ionic strength of the medium results in a lower resistance. Discharge of a given size capacitor into a medium of higher ionic strength (lower resistance) will produce a pulse with a shorter time constant (τ). Two dimensions of the sample chamber affect resistance: the path length through the sample solution (interelectrode distance) and the cross-sectional area of the sample solution. Increasing the interelectrode distance or reducing the cross sectional area of the solution at the electrode surface will increase resistance.

Gene transfer of *H.ducreyi* DNA has been performed (Deneer *et al.*, 1982; McNicol, Albritton and Ronald, 1983; Albritton *et al.*, 1984) with the bacterium, containing a plasmid capable of transferring resistance in conjugative matings, as the donor and either *H.influenzae*, *E.coli* or *H.ducreyi* as the recipient. The major objective of this study was to attempt to transform *H.ducreyi* plasmid genes into reference and clinical strains of *H.ducreyi* by electroporation and to determine the optimal conditions for transformation.

4.2. MATERIALS AND METHODS

4.2.1. Bacterial Strains and DNA

The bacterial strains and plasmids are listed in Table 4.1. The DNA concentration was measured at OD_{260} with an LKB Ultrospec 4050 spectrophotometer (1 OD_{260} unit = 50 μg/ml).

Chromosomal DNA extracted from a nalidixic acid-resistant mutant of *H.ducreyi* ATCC 27722 was obtained from Dr.R.Gromkova.

Table 4.1. ***H.ducreyi*** **strains and plasmids used.**

Strain	Plasmid	Molecular mass (kb)	Phenotype
ATCC 27722	plasmid-free		
CIP 542	plasmid-free		
C147 (Kenya)[a]	plasmid-free		
CH128	pCH128	10.6	amp^r
CH138	PCH138	9.3	amp^r
CH137	pCH137	5.7	amp^r
HD124	pHD124	7.5	su^r
HD4	pHD4	5.1	kan^r

r resistance
a from L.Slaney, Manitoba, Canada
amp ampicillin; kan kanamycin; su sulphonamides

4.2.2. Electroporation Buffers

Three buffers were used: 10% sterilized glycerol (Merck), 1 mM filter sterilized 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethansulfonic acid (HEPES) (Merck) with 10% glycerol and filter-sterilized phosphate, sucrose and glycerol (PSG) buffer (2.43 mM K_2HPO_4, 0.57 mM KH_2PO_4, 272 mM sucrose and 15% glycerol, pH 7.4)

4.2.3. Preparation of Recipient Cells Grown on Solid Media

A suspension of *H.ducreyi* was plated directly onto Nsanze medium or chocolate agar. Cells from one plate were resuspended in 1.5 ml ice-cold electroporation buffer to form a thick suspension. The cells were dispersed by pushing them through a 26 G hypodermic needle ten times (Jardine, 1990) followed by centrifugation and washing four times with low ionic strength cold electroporation buffer. Electroporation at high voltages requires a cell suspension of very low conductivity. To achieve this, the ionic strength of the suspension was reduced by extensive washing. After the final centrifuge step, the cells were resuspended in 0.2 ml of electroporation buffer and kept on ice.

4.2.4. Preparation of Recipient Cells Grown in Broth

Concentrated suspensions of *H.ducreyi* were diluted 1:50 in 10 ml aliquots of sBHI broth (Appendix A.4.). The aliquots were incubated for 48 h at 35°C without agitation after which cultures appeared evenly dispersed with no clumping. This was achieved by several subcultures of *H.ducreyi* in broth, followed by storage at -70°C in 15% glycerol. Broth cultures showed no presence of clumping, but settled to the bottom of the tube. To acquire a cell suspension of very low conductivity, the cells were washed more stringently than cells grown on solid media, as the surface area of the cells were exposed to higher quantities of nutrients and salt than cultures grown on solid media. The cultures were chilled on ice for 10 min followed by centrifugation at 10 000 g for 15 min at 4°C. The pellet was resuspended to the initial volume in ice-cold PSG electroporation buffer and centrifuged as before. In the next wash, the volume of electroporation buffer was reduced by half, with subsequent washes (four more times) performed in sterile 1.5 ml Eppendorf tubes (to minimize the loss of cells). After the final wash, the cells were resuspended in 0.2 ml of electroporation buffer (a 50 fold concentration) and kept on ice prior to electroporation.

4.2.5. Electroporation Equipment

The exponential decay pulses were generated by a Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, California, USA). This is a capacitor discharge machine that uses individual disposable cuvettes for holding the cell suspension during the shock. An electroporation cuvette (Bio-Rad) with a 0.2 cm electrode gap was used. The equipment, circuit and electrodes are described in more detail in Appendix C.

The power supply internal to the Gene Pulser apparatus charges the chosen capacitor to the selected voltage. The microprocessor monitors this charging process and automatically discharges the capacitor through the sample via an electronic switch mechanism when the precise voltage has been reached. The pulse

decays exponentially through time and is defined by the initial field strength and the time constant.

4.2.6. Electro-transformation Procedure

Forty µl of washed cells were transferred to a cold 1.5 ml Eppendorf tube and 1 to 2 µl of DNA, in a low ionic strength medium such as TE or distilled H_2O, was added to a final concentration of 100 ng/ml to 1 µg/ml. The mixture was mixed well and transferred to a chilled sterile disposable 0.2 cm electroporation cuvette (Bio-Rad). The cuvette was placed in the electroporation chamber and the appropriate pulse applied. Immediately following electroporation, 0.36 ml of sBHI broth, warmed to 37°C, was added to the cells and the mixture transferred to a sterile 5 ml tube or 1.5 ml Eppendorf tube. All experiments included controls comprising of cell-DNA mixtures that were not subjected to electroporation and cells which were electroporated in the absence of added plasmid DNA. The samples were incubated at 35°C for 4 h to allow for expression of the antibiotic resistance gene (shaking the tubes at 225 rpm during this incubation period improved recovery). The cells were diluted appropriately in BHI broth and plated (0.1 ml) in duplicate on Nsanze medium or chocolate agar containing either 10 µg/ml ampicillin, 100 µg/ml kanamycin or on serum-free medium (Appendix A.6.) containing 100 µg/ml sulphamethoxazole to screen for transformants. The plates were incubated for 48 h, except for *H.ducreyi* CIP 542 which was incubated for 72 h.

Transformation frequency was determined by dividing the number of transformants by the number of viable cells. Viability was assessed by determining the number of CFU per ml on plates without antibiotics.

Plasmid DNA was extracted from the *H.ducreyi* transformants and resolved on 0.8% agarose gels as described in Section 3.2.8.

4.3. RESULTS

4.3.1. Preliminary Trial

Preliminary experiments were performed using *H.ducreyi* ATCC 27722 as a recipient and the ampicillin-resistance plasmid, pCH138 (9.3 kb) as the donor DNA in order to determine the optimal conditions for electroporation. Experimental conditions which were varied include the type of electroporation buffer and the electrical parameters used.

Three low ionic buffers were compared. Bacteria suspended in the glycerol medium tended to aggregate together and settle to the bottom of the tube, even after dispersal using the needle and syringe technique. Bacteria suspended in the HEPES medium also settled to the bottom, with a tendency to aggregate. However, bacteria suspended in the PSG medium were evenly suspended and the buffer was accordingly used in subsequent experiments.

In optimizing the conditions for electroporation of *H.ducreyi* ATCC 27722, the effect of several variables on transformation frequency and cell viability were determined. The recipient bacteria were initially subjected to electroporation at a field strength of 12.5 kV/cm and a pulse length of 10 msec (400 Ω, 25 μFd). The electroporated bacteria were incubated in sBHI broth at 35°C for 24 h, for expression of the antibiotic marker, prior to plating onto ampicillin-containing medium. Transformation frequency of 5.1×10^5 was obtained with a survival ratio of 1.0. No loss of viability was observed relative to the control cells without DNA.

Various field strengths and pulse lengths of the Bio-Rad electroporation apparatus were examined. Approximately 10^7 CFU of *H.ducreyi* and 1 μg of pCH138 plasmid DNA were mixed in electroporation solution. Aliquots (40 μl) were electroporated at different field strengths and time constants to determine optimal conditions for transformation. Field strengths in the range from 9 kV/cm to

12.5 kV/cm and pulse lengths from 5 msec to 15 msec were utilized with the capacitor held constant at 25 μFd.

The results presented in Table 4.2. show that parameters in the range of 9 kV/cm and 10 kV/cm with pulse lengths of 15 msec and 10 msec respectively, were the most efficient for transformation of *H.ducreyi* with the ampicillin-resistance plasmid. The higher field strengths and longer pulse lengths were not as effective in transformation of *H.ducreyi*. No negative effect was displayed on the viability of the cells.

H.ducreyi cells subjected to electroporation in the absence of plasmid DNA did not mutate spontaneously to ampicillin-resistance and the addition of plasmid DNA without applying an electric pulse did not result in transformants.

Table 4.2. Effect of voltage and pulse length on transformation frequency of *H.ducreyi* ATCC 27722 with plasmid pCH138.

Field strength (kV/cm)	Time constant (msec)	Transformation frequency[a]	Survival[b]
9.0	10	6.9×10^{-5}	1.1
9.0	15	1.3×10^{-4}	1.1
10.0	10	1.4×10^{-4}	1.0
10.0	15	4.5×10^{-5}	1.3
11.25	5	1.2×10^{-4}	1.1
11.25	10	8.1×10^{-6}	1.1
12.5	10	2.2×10^{-5}	1.3

a The number of CFU was 10^8/ml. The cells were grown in sBHI broth. Plasmid concentration was 1 μg/ml. The expression time was 4 h.

b Survival ratio = number of CFU after electroporation/number of CFU before electroporation.

4.3.2. Expression of the Genetic Marker

It is known that the duration of the expression time of the genes during transformation depends upon the nature of the genetic marker. Transformed cells incubated at different times (1 to 24 h) were compared. Ampicillin-resistance transformants were recovered after 1 h of incubation with improvement in the number of transformants with increasing time (Table 4.3.). The number of transformants appeared to stabilize after 4 h of incubation.

Table 4.3. **Transformation frequency of the ampicillin-resistance gene of plasmid pCH138 in *H.ducreyi* ATCC 27722 after varying times for expression.**

Time (h)	Transformation frequency
1	5.0×10^{-5}
1.5	7.2×10^{-5}
2	7.6×10^{-5}
4	1.2×10^{-4}
24	1.9×10^{-4}

Electroporation was performed at 10 kV/cm, 10 msec.

4.3.3. The Effect of Cell Concentration, DNA Dosage and the Size of Plasmid DNA on Transformation Frequency

The effect of shaking cells at 200 rpm before or after electroporation was investigated. Shaking cells prior to electroporation did not affect the number of transformants, although the yield of cells increased from 10^7 to 10^9 cells/ml after the washing procedure. Agitation of electroporated cells before plating onto solid media increased the number of transformants recovered but not the transformation frequency. The results of the effect of agitation of the recipient cells using the 4.8 kb kanamycin-resistance plasmid is presented in Table 4.4.

Table 4.4. **Effect of agitation on the transformation frequency (TF) of the 4.5 kb kanamycin-resistance plasmid in *H.ducreyi* ATCC 27722.**

Agitation (200 rpm)	No. Transformants /plate	Viability	TF
Before electroporation	625	8.3×10^{8}	7.5×10^{7}
After electroporation	560	2.8×10^{8}	2.0×10^{-6}
None	25	2.6×10^{7}	9.6×10^{7}

Electroporation was performed at a field strength of 10 kV/cm and a pulse length of 10 msec.

The effect of plasmid DNA concentration on the transformation frequency was assessed using DNA concentrations from 0.25 to 1.0 µg/ml. A concentration-dependent increase in transformation frequency, approaching a level of saturation with increasing DNA from 0.75 µg/ml of transforming DNA per ml (Figure 4.3.) was observed.

In order to evaluate the effect of the plasmid DNA size on transformation frequency, three ampicillin-resistance plasmids ranging in sizes from 5.6 kb to 10.6 kb were transformed into plasmid-free *H.ducreyi* recipients (Table 4.5. and Figure 4.4.). A decrease in the frequency of transformation as the size of the plasmid increased was observed.

4.3.4. Chromosomal DNA

Attempts to transform *H.ducreyi* chromosomal DNA using the nalidixic acid-resistance gene as a genetic marker to *H.ducreyi* ATCC 27722 were unsuccessful. Parameters of field strength 10 kV/cm and pulse lengths of 5 and 10 msec were used to electroporate both intact and broken chromosomal DNA. Higher voltages resulted in arcing of the electric current. The electroporated cells were incubated

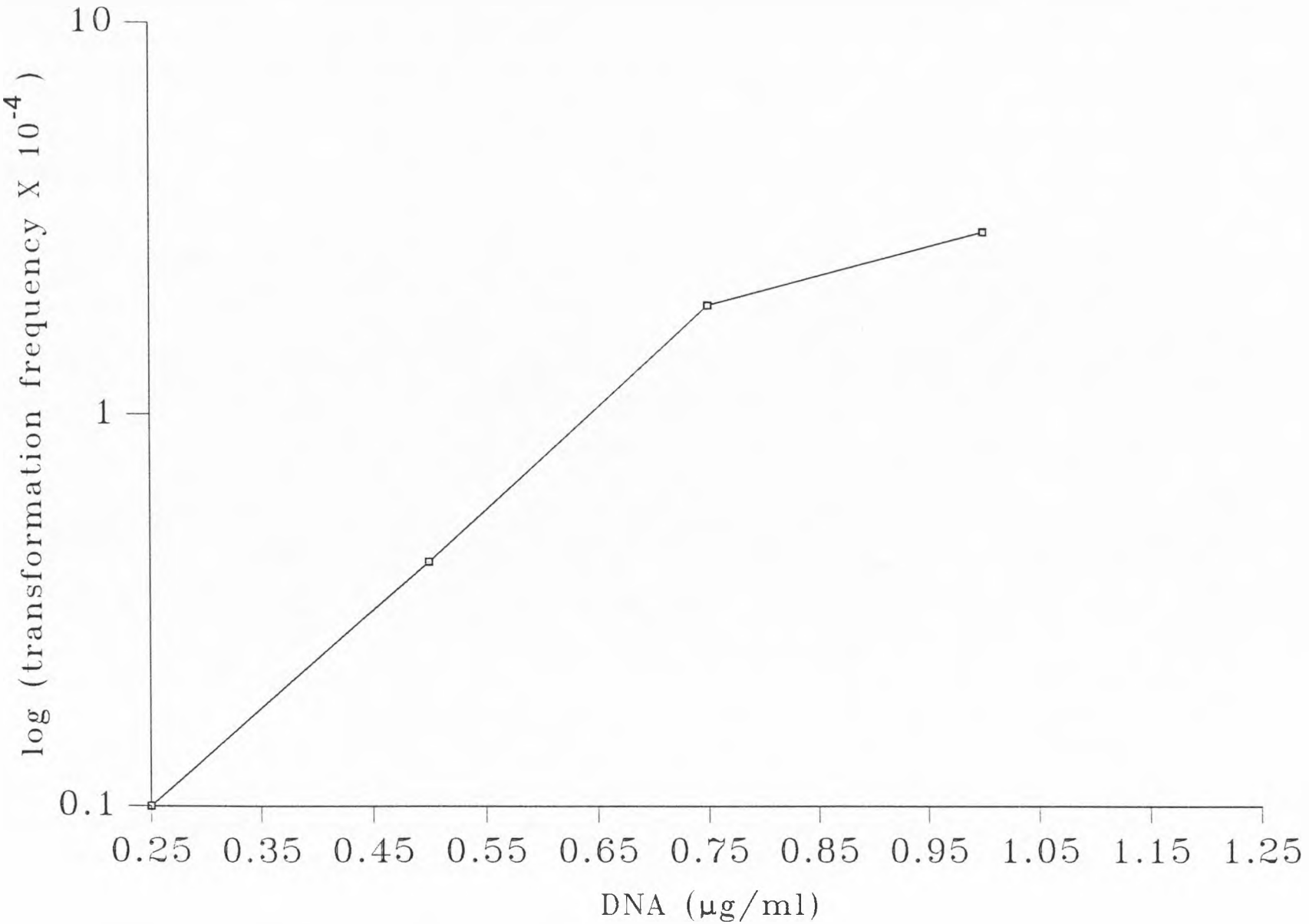


Figure 4.3. Effect of DNA concentration on transformation of plasmid pCH138 to *H.ducreyi* ATCC 27722 by electroporation. Cells were grown in sBHI broth. The expression time was 4 h.

Table 4.5. Effect of plasmid size on transformation frequency.

Ampicillin-resistance plasmids	Size	Transformation frequency
pCH128	10.6	3.0×10^{-4}
pCH138	9.3	3.6×10^{-4}
pCH137	5.6	7.0×10^{-3}

Electroporation was performed at a field strength of 10 kV/cm and a pulse length of 10 msec.

for the expression of the genetic marker at 24 h before plating onto Nsanze medium containing 5 µg/ml nalidixic acid. No transformants were observed.

4.3.5. Transformation of Different Drug-Resistance Plasmids

The transformation frequency of kanamycin- and sulphonamide-resistance plasmids was lower when compared to ampicillin-resistance plasmids (Table 4.6.). Electroporation did not damage the plasmids as intact plasmids were viewed after plasmid extraction and gel electrophoresis (Figure 4.5.).

4.3.6. Transformation of Different *Haemophilus ducreyi* Strains

The type strain, CIP 542 was electroporated and tested for transformation of the ampicillin-resistance plasmids. Both solid and liquid media were used for growth of the strain. CIP 542 cells electroporated at 10 kV/cm, 10 msec showed transformation frequencies of 6.4×10^{-4} and 2.2×10^{-4} when cells were prepared by the broth and agar methods respectively. This strain grew more slowly, formed smaller colonies and thus required a 72 h incubation time after plating for transformants. Electroporation of the clinical strain of *H.ducreyi*, C147 (plasmid-free) as recipient to the ampicillin-resistance plasmid (9.3 kb), kanamycin-resistance plasmid (4.8 kb) and the sulphonamide-resistance plasmid (7.5 kb) were performed (Table 4.7.).

Table 4.6. Transformation frequencies (TF) of different antimicrobial-resistance plasmids.

Resistance marker	Size of plasmid (kb)	Approximate TF
ampicillin	9.3	10^{-4}
kanamycin	4.5	10^{-6} to 10^{-7}
sulphonamides	7.5	10^{-5}

To establish the reproducibility of the transformation procedure, each experiment was repeated at least three times. Only slight variations in the transformation frequencies were observed.

Table 4.7. Transformation frequency of *H.ducreyi* clinical strain C147.

Recipient	plasmid size (kb)	phenotype	TF
C147	4.5	kan^r	9.0×10^{-6}
	7.5	su^r	3.6×10^{-5}
	5.6	amp^r	5.9×10^{-4}
	9.6	amp^r	1.7×10^{-4}

Electroporation was performed at a field strength of 10 kV/cm and at a pulse length of 10 msec.

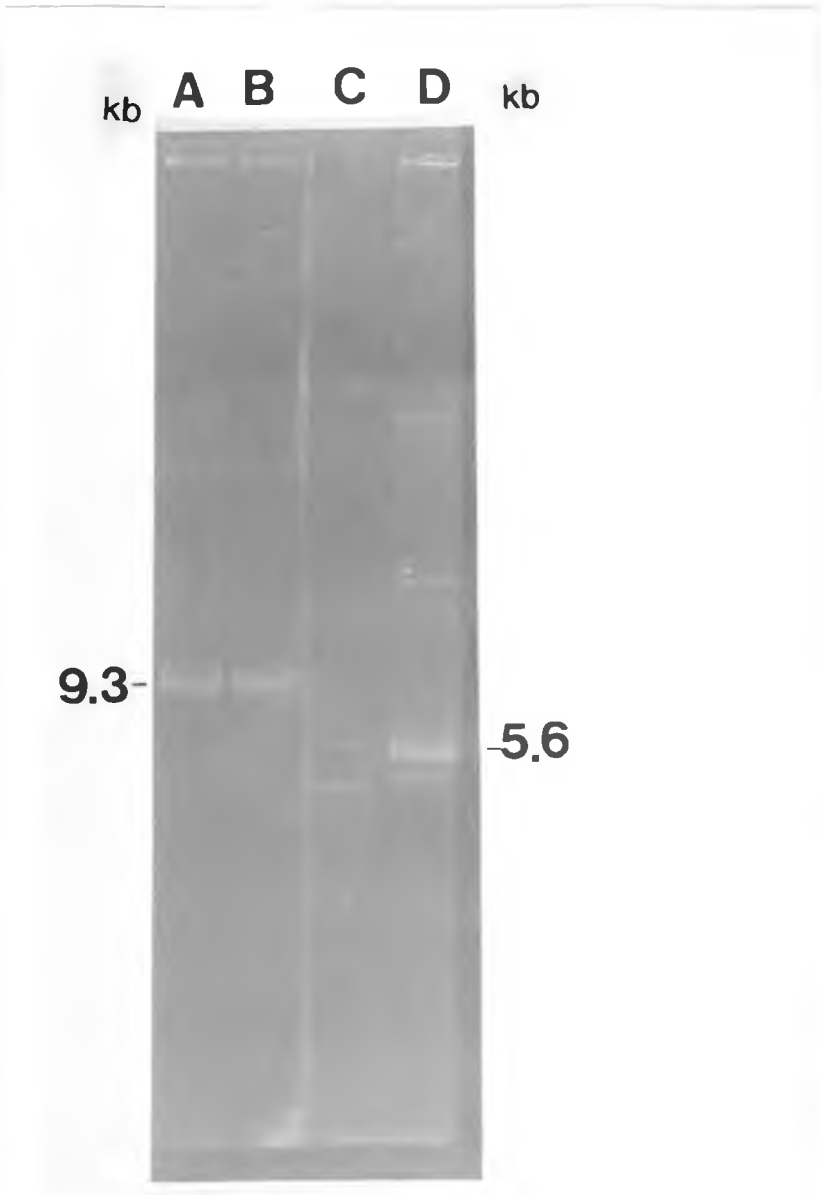


Figure 4.4. **Agarose gel electrophoretic patterns of two ampicillin-resistance plasmids isolated from *H.ducreyi* transformants and plasmids from donor strains. Lane A and C are plasmids from the donor strains and corresponding lanes B and D are the transformants respectively.**

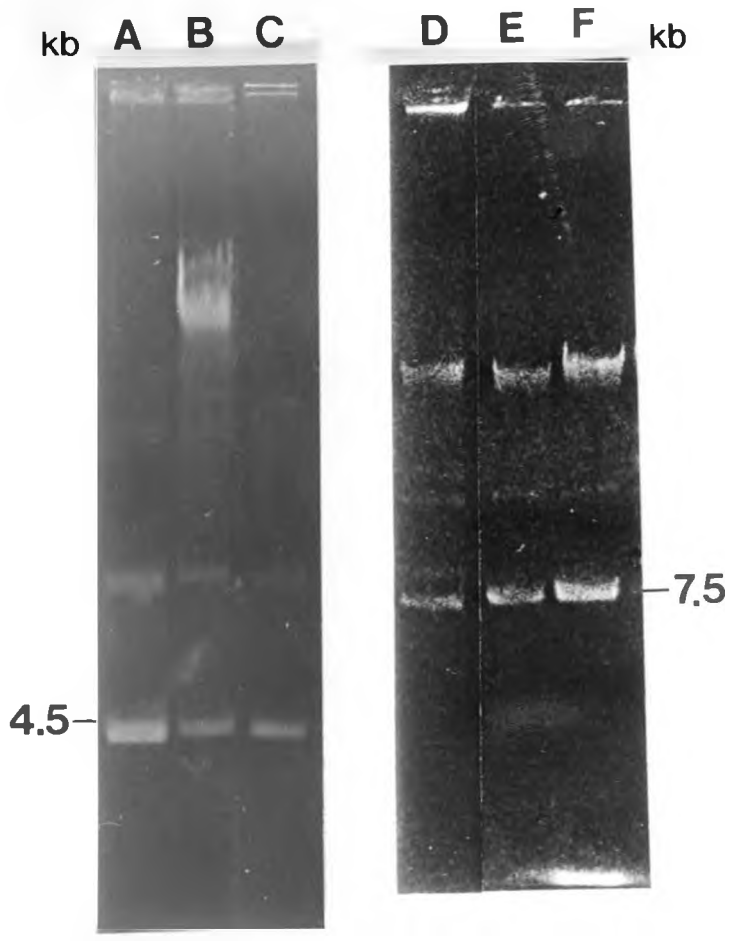


Figure 4.5. Agarose gel electrophoresis of (A) the kanamycin-resistance plasmid (4.5 kb) and (D) the sulphonamide-resistance plasmid (7.5 kb). Transformants C147 (Lanes B and E) and reference strain ATCC 27722 (Lanes C and F).

4.4. DISCUSSION

For most bacteria, electroporation is the only experimental method currently available for efficient introduction of plasmid DNA. The results presented here demonstrate that electroporation can be used for genetic transformation of both reference and clinical strains of *H.ducreyi*.

During these studies the composition of the electroporation buffer used to resuspend *H.ducreyi* was investigated. The electroporation medium composition is an important parameter affecting transformation efficiency. The buffer containing 10% glycerol and that containing HEPES and glycerol previously used for experiments with *E.coli* cells (Dower, Miller and Ragsdale, 1988) was not able to disperse the aggregation of *H.ducreyi* cells. If electroporated, the aggregated clump would be recognized by the applied voltage as a single large membrane-bound structure, rather than as a collection of individual cells due to intracellular communication within the aggregate (Knight and Scrutton, 1986). The PSG buffer used by other investigators (Miller, Dower and Tomkins, 1988; Shigekawa and Dower, 1988; Mitchell *et al.*, 1991; Setlow and Albritton, 1992) was found to be most suitable for electroporation of *H.ducreyi* as cells remained dispersed and was thus used for all experiments. Electroporation treatment in a higher-ionic-strength medium, eg. isotonic saline leads to the implantation of small pores whereas in a lower-ionic-strength medium, eg. isotonic sucrose, leads to larger pores where identical electroporation conditions are used (Tsong, 1989).

The degree of membrane permeabilization achieved depended primarily on the amplitude (electric field intensity) and duration (time constant) of the discharge wavelength. Within the range tested, both field strength and pulse length exerted an effect on the transformation frequency. Transformation frequencies presented in this study demonstrate a complementary relationship between field strength and

time constant. At the higher field strengths, transformation frequencies decreased, which is contradictory to the theory of the inverse relationship of field strength to cell length (Potter, 1988; Shigekawa and Dower, 1988) as the size of *H.ducreyi* cells (0.5 by 1.0 μm) is much smaller than *E.coli* cells (0.5 by 2.0 μm) whose optimum transformation frequencies have previously been achieved at field strengths higher than 11 kV/cm (Calvin and Hanawalt, 1988; Dower, Miler and Ragsdale, 1988). The characteristic clumping of *H.ducreyi* cells could explain the maximum transformation frequency and the lack of a lethal effect at a lower field strength and the survival of electroporated *H.ducreyi.* The lethal effect of electroporation on *H.ducreyi* as with *C.jejuni* (Miller, Dower and Tomkins, 1988) was not observed even when the highest voltages of 12 kV/cm were applied. These observations are in contrast to those obtained with mammalian cells (Chu, Hayakawa and Berg, 1987), plant protoplasts (Fromm *et al.*, 1987) and other bacterial cells (Dower, Miller and Ragsdale, 1988; Dunny, Lee and LeBlanc, 1991) where the high frequency of gene transfer is related to a decrease in cell viability.

A decrease in the transformation frequency with an increase in plasmid size was observed in these studies which is in agreement with the findings of Dunny, Lee and LeBlanc (1991); Kim, Ray and Johnson (1992); Metzler, Zhang and Chen (1992) and Siguret *et al.* (1994). The fewer number of molecules of the larger plasmids and the breakage of plasmid DNA molecules in the recipient could be related to the lower number of transformants (Kim, Ray and Johnson, 1992). These findings contrast with the findings of other authors who observed that plasmids of 20 kb transform with the same molar efficiency as plasmids of 3- to 5-kb in *E.coli* (Bio-Rad Laboratories, 1988; Powell *et al.*, 1988) and plasmids from 9.8- to 30-kb transform with the same molar efficiency in *Lactococcus lactis* (McIntyre and Harlander, 1989). The interpretation of the above results remains unclear.

Increased number of transformants have been observed with increasing DNA concentration (Dower, Miller and Tomkins, 1988; Leonardo and Sedivy, 1990; Dunny, Lee and LeBlanc, 1991; Kim, Ray and Johnson, 1992). The electroporation system appears to be quite sensitive to both DNA and cell concentration as transformants could be obtained with very small concentrations of DNA (in picogram range) (Dower, Miller and Ragsdale, 1988). Raising cell concentration by agitation (Totten and Stamm, 1994) before or after electroporation increased the number of transformants 25- and 22-fold respectively. The recipient cells in this study demonstrated signs of saturation with increasing DNA concentration. Saturation, previously observed with streptococci (Powell *et al.*, 1988) and *Enterococcus faecalis* (Dunny, Lee and LeBlanc, 1991) could be the result of fewer permeabilized cells in the bacterial preparation, greater sensitivity of the cells to small amounts of deleterious contaminants (eg. phenol, SDS or EDTA) that could enter the cell during electroporation (Shigekawa and Dower, 1988) or due to a lower concentration of cells. Thus it is possible to improve electroporation by encouraging the interaction of cells and DNA by increasing the cell and/or DNA concentration (Shigekawa and Dower, 1988).

Little is known about how DNA molecules enter cells during electroporation (Potter, 1988). Conductivity studies have shown that transient pores are formed in the lipid bilayer as a result of the electroshock and resealed within milliseconds (Kinosita and Tsong, 1977a; Neumann *et al.*, 1982). The longevity of the reversibly electropermeabilized membrane state is explained by Neumann *et al.* (1982) and has been studied systematically by measuring the uptake of normal impermeable molecules (drugs, dyes) added to the cells at different times after pulse application (Jacob *et al.*, 1981). A strong temperature dependence of the resealing has been documented in these studies. Potter, Weir and Leder (1984) observed that conducting electroporation at 0°C was 6- to 16-fold more effective than at 20°C, which is probably the result of the slower closing of the membrane

pores at 0°C. Therefore, electroporative gene transfer experiments are usually performed at 0-4°C. Incubation of DNA with the cells prior to electroporation has no effect on efficiency of uptake (Dower, Miller and Ragsdale, 1988) and the efficiency drops rapidly after electroporation unless the cells are immediately placed in a growth medium (Dower, Miller and Ragsdale, 1988; O'Hare *et al.*, 1989).

In experiments where the *H.ducreyi* recipients were electroporated in the presence of different antimicrobial-resistance plasmids a 10 to 100-fold difference in transformation frequencies of ampicillin-resistance and sulphonamide-or kanamycin-resistance transformants were observed. As plasmids screened for transformants were not damaged, the lower frequencies must therefore be due to some damage to the bacteria. Steele, Zhang and Shillitoe (1994) compared the effect of ampicillin and tetracycline on the efficiency of transformation of *E.coli* by electroporation and their results are consistent with the suggestion that cell wall damage can decrease transformation efficiency. This was shown by the decrease in the number of tetracycline-resistance transformants recovered after electroporation as compared to selection for ampicillin-resistance transformants. They suggested that if the cell membrane was damaged by electroporating in such a way as to prevent interactions with the drug-resistance protein, this might lead to the inability to recover tetracycline-resistant colonies after electroporation. Another possible explanation for the low frequency of transformation of some plasmid antibiotic-resistance genes is the requirement of some unknown conditions for the expression of the antibiotic-resistance genes.

Mitchell *et al.* (1991) attempted to transform *H.influenzae* chromosomal DNA by electroporation into non-competent *H.influenzae* Rd but was unsuccessful. Short fragments of endonuclease-digested genomic DNA have been electroporated into *Alcaligenes eutrophus* (Taghavi, van der Lelie and Mergeay, 1994) and

A.tumefaciens (Charles, Doty and Nester, 1994). Although plasmid DNA was transformed into *H.ducreyi* efficiently, attempts to transform chromosomal DNA were unsuccessful. However, after cloning *H.influenzae* genes (Setlow and Albritton, 1992) or *H.ducreyi* genes (Hansen *et al.*, 1992) in compatible plasmid vectors it is possible to transform these genes into *H.ducreyi* cells. The demonstration that *H.ducreyi* plasmids can be transformed into *H.ducreyi* by electroporation opens the possibility for developing cloning vectors from homologous plasmids that would be more efficient in transformation due to the better expression of the selective marker. In addition, the possibility to transform the avirulent strains would allow for the study of the genes involved in the pathogenicity of *H.ducreyi*.

5.0. CONCLUSION

Clinical isolates of *H.ducreyi* recovered in southern Africa during the period between 1988 to 1994 have shown a significant increase in the level of antibiotic resistance. β-lactam agents are among the most frequently prescribed antibiotics worldwide. Thus it is not surprising that the most dramatic increase observed was that of ampicillin resistance among clinical isolates investigated in these studies. Ampicillin-resistant β-lactamase-negative strains of *H.ducreyi* were not detected here although such strains have previously been described from other *Haemophilus* species. These results confirm the conclusion of other authors that the prescribing of antimicrobials should be given serious considerations so as to minimize the prevalence of resistance.

The majority of *H.ducreyi* isolates examined in these studies harboured the ampicillin-resistance 9.3 kb plasmid (75%) and 15% of the isolates contained the ampicillin-resistance 10.6 kb plasmid. A plasmid combination of an ampicillin-resistance plasmid of 5.6 kb in conjunction with a 4.5 kb aminoglycoside (kanamycin and streptomycin) and sulphonamide-resistance plasmid and two plasmids of 3.9- and 3.0- kb was observed in isolates from Botswana (1992) and in Gauteng (1994). A similar plasmid profile has been described recently by other investigators in isolates obtained from Thailand and San Francisco, USA (Sarafian *et al.*, 1991a; Sarafian and Knapp, 1992). This plasmid combination could represent an emerging profile among *H.ducreyi* clinical isolates. This demonstrates the diversity of plasmid profiles and permits the identification of several strains. Although plasmid profiles are useful in epidemiologic studies, they provide only limited information about strain populations. Strains used in this study may be more diverse than has been described when applying two or more typing systems. Current studies (Sarafian *et al.*, 1991b; Brown and Ison, 1993) suggest the use of ribotyping or DNA hybridization in association with plasmid profiles to be more

relevant, as many strains have identical plasmid profiles.

Studies conducted here using restriction endonuclease digestion of the ampicillin-resistance plasmids revealed the presence of four different types of plasmids. The finding that two of the plasmids of identical size (10.6 kb) show different restriction fragments indicates that some rearrangement of the nucleic acid sequences has occurred. Further investigations involving restriction digestions and DNA homology studies may reveal more differences or similarities showed by these plasmids.

This study describes the development of an efficient method for electroporation of *H.ducreyi* cells and demonstrates for the first time the transfer of *H.ducreyi* plasmids to homologous reference and clinical strains. Three plasmid genes coding for ampicillin, aminoglycoside and sulphonamide resistance, were transformed successfully into *H.ducreyi.* The ampicillin-resistance genes were the most efficient in transformation indicating differences in the ability of the genes to be expressed. The recovery of electroporated bacteria may be influenced by the antimicrobials used to select for transformants as demonstrated by the higer number of ampicillin-resistance transformants than for the two other antimicrobials. In addition, it was found that the small ampicillin-resistance plasmids transformed with higher frequencies. The availability of a standard method for genetic transformation will provide a tool for developing *H.ducreyi* cloning vectors that may be useful for the investigation of *H.ducreyi* on the molecular level and it will provide the opportunity to examine the expression of chromosomal genes in the organism. This may lead to a better understanding of the pathogenicity and other properties of this important human pathogen.

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APPENDIX A (MEDIA AND TECHNIQUES)

A.1. Nsanze agar /1l (Nsanze *et al.*, 1984)

(A)	Goncoccoal agar base (Gibco BRL, UK)	36 g
	Distilled water	600 ml
	Autoclave at 121°C for 15 min	
(b)	Haemoglobin powder (Difco, Detroit, Michigan, USA)	10 g
	Distilled water	400 ml
	Mix rapidly on magnetic stirrer. Autoclave at 121°C for 15 min	

Final Medium

Mix solutions (A) with (B). Cool to 50°C.

Add 10 ml IsoVitaleX (BBL, Microbiological systems).

Add 3 mg vancomycin (Eli Lilly, SA).

Add 50 ml foetal calf serum (Sterilab, Delta Products, SA) (heat inactivated at 56°C for 1 h).

Note: If the haemoglobin forms a deposit, do not transfer the deposit.
Mix well and distribute into sterile petri dishes.

A.2. Chocolate agar /2l

(A)	Columbia agar base (Oxoid, Hampshire, UK)	80 g
	Autoclave at 121°C for 15 min.	
(B)	Citrated horse blood (SAIMR)	100 ml

Cool (A) to 60°C; add (B). Heat to 80°C. The suspension must be dark chocolate in colour. Cool to 50°C and distribute into sterile petri dishes.

A.3. Serum-free medium (Lockett *et al.*, 1991)

This consists of a gonococcal agar base (Gibco), to which 1% haemoglobin (Difco) and 1% IsoVitaleX (BBL) was added. Serum was substituted with 0.2% activated charcoal (Sigma).

A.4. Supplemented BHI broth for the growth of *H.ducreyi*

This consists of Brain Heart Infusion broth (Difco), supplemented with 5% FCS (Flow laboratories, Scotland), 1% IsoVitaleX (BBL) and 100 mg/ml haemin (Sigma).

A.5. Media for the storage of *H.ducreyi* and *H.influenzae* at -70°C

To autoclaved BHI broth add 5% FCS, 100 µg/ml haemin (BDH Chemicals Ltd, England), 1% IsoVitaleX and 15% sterile glucose (Merck) for storage of *H.ducreyi* or 10 µg/ml haemin, 2µg/ml NAD (Sigma) and 15% sterile glucose for the storage of *H.influenzae*.

A.6. Serum-free medium for the growth of sulphonamide resistant *H.ducreyi* strains

Sulphonamide-resistance *H.ducreyi* strains were grown on Mueller-Hinton agar (Difco) supplemented with 50 ml/l laked horse blood (SAIMR), 0.1% glucose (Holpro), 0.01% L-glutamine (Hopkins and Williams) and 100 mg/ml haemin (Sigma).

A.7. Biochemical test

A.7.1. Porphyrin test / test for X-factor requirements.

A heavy suspension of 48 h *H.ducreyi* culture was added to 0.5 ml enzyme substrate, δ-aminolevulinic acid (ALA-Porphyrin test, SAIMR). Tubes were incubated at 35°C for 18-24 h and examined for red fluorescence, under a UV light, indicating the presence of porphyrins. *H.influenzae* and *H.parainfluenzae*

were used as negative and positive controls respectively.

A.7.2. Indole test.

The indole test is based on the development of a bright red colour complex when indole reacts with the aldehyde group of *p*-dimethylaminobenzaldehyde, the active chemical in Ehrlich's reagent. The peptone water inoculated with the isolate was incubated at 35°C for 48 h, after which Ehrlich's reagent was added.

A.7.3. Urease test.

The ability to degrade urea by means of the enzyme urease was determined. The urea slant was inoculated with the isolate and incubated at 35°C for 48 h. A positive reaction occurs when the substrate urea is split into its products and the presence of ammonia creates an alkaline environment, which causes the phenol red to turn a deep pink colour.

A.7.4. Ornithine decarboxylase test.

The ability to decarboxylate (remove a carboxyl group, COOH) ornithine was demonstrated by the addition of a loopful of isolate into medium containing a brom-cresol purple indicator and the amino acid ornithine, layered with mineral oil to create an anaerobic environment. In a positive reaction, indicated by a colour change, from yellow to purple, the decarboxylase enzyme will split off the appropriate carboxyl group, creating an alkaline pH in the medium.

A.7.5. Nitrate reduction test.

The reduction of nitrates was performed by adding a heavy inoculum, from a 48 h culture, into nitrate broth medium and incubating at 35°C for 48 h. The ability to reduce nitrates to nitrites was determined by the addition of two reagents: Nitrate (sulfanilic acid) followed by Nitrate II (α-naphthylamine). Following reduction, the addition of the two solutions will immediately produce a cherry red

colour, indicating the presence of nitrites.

A.7.6. Alkaline phosphatase test.

An agar slant containing phenolphthalein phosphate was inoculated with a loopful of isolate and incubated at 35°C for 48 h. After exposure to ammonia, phosphate-positive colonies turned pink due to free phenolphthalein.

A.7.7. Hydrogen sulphide test.

A piece of lead (II) acetate paper strip (Merck) was attached to the inside of a tube containing a sBHI broth culture of the isolate. The culture was incubated at 35°C for 24 to 48 h. H_2S production was indicated by a black ferrous sulphide precipitate on the paper strip.

A.7.8. Catalase test.

The ability to degrade hydrogen peroxide, by producing the enzyme catalase was determined. A loopful of inoculum was added to 3% of $2H_2O_2$ on a glass slide. A positive reaction is indicated by the formation of bubbles of free oxygen gas.

A.7.9. Oxidase test.

The presence of cytochrome oxidase was determined using filter paper saturated with N,N,N',N'-tetramethyl-1.4-phenlyenediammonium-dichloride (Merck). A portion of a colony to be tested was smeared onto the filter paper. A positive reading was observed by the presence of a violet colour.

A.7.10. SPS disk susceptibility test.

An isolate was spread evenly onto fresh Nsanze medium. A disk containing 0.001 g of sodium polyanethole-sulfonate (SPS) was placed on the surface of each test plate, which was incubated at 35°C for 48 h in microaerophilic conditions, with moisture. Zones of inhibition around the disk of > 12 mm was considered to

indicate susceptibility.

A.7.11. β-lactamase test.

Cultures were screened for β-lactamase production by streaking colonies, grown on Nsanze medium, onto β-lactamase agar (Bacto-agar containing 3 mg/ml penicillin and 5 ml/l phenol red, pH 8.6). Bacteria which produced β-lactamase were identified by turning yellow after a few minutes due to hydrolysis of its β-lactam bond.

A.8. Caesium Chloride gradient (Sambrook, Fritsch and Maniatis, 1989)

DNA was resuspended in 7.5 ml of TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.6) to which 7.5 g of CsCl (Merck) was added. The mixture was shaken, until the CsCl was dissolved completely and transferred to a Beckman polyallomer ⅝ X 3 inch centrifuge tube. 0.75 ml of 10 mg/ml ethidium bromide (EtBr) (Sigma) was added. The tubes were sealed with liquid paraffin, balanced and centrifuged in the Beckman LK-55 ultra centrifuge at 40,000 rpm for 40 h at 15°C in the fixed angle 50 Ti rotor. After centrifugation, the tubes were illuminated with a UV lamp to detect the presence of fluorescent bands of DNA-EtBr. The upper bands consist of chromosomal DNA and linear or nicked plasmid DNA, and the lower band(s) consist of covalently closed circular plasmid DNA. The bands were collected by inserting a hypodermic needle just below the band. Ethidium bromide was eliminated by the addition of an equal volume of 5 M NaCl saturated isopropanol and then leaving the mixture until the two phases separated. The upper layer, containing the isopropanol and EtBr was discarded and the procedure repeated until the EtBr (red colour) was no longer visible.

The removal of the CsCl was performed by one of the three methods, the latter being preferred.

a) Dialysis of the sample in dialysis tubing in TE buffer (pH 7.6) for

48 h at 4°C.

b) Spin dialysis using the Ultrafree-MC, 30.000 NMWL filter-unit.

c) Using a VS 0.025 μm hydrophobic Millipore filter, floating shiny-side up on TE buffer (pH 7.6). One-hundred microlitres of DNA-CsCl sample was placed onto the filter for approximately 30 min. The CsCl passed through the filter during that period with the sample remaining on the filter.

A.9. Boehringer Mannheim molecular weight markers

DNA molecular weight marker VII

Catalogue number 1209 264

The mixture contains 15 fragments with the following number of kilobase pairs: 8.0; 7.1; 6.0; 4.8; 3.5; 2.7; 1.9; 1.85; 1.5; 1.4; 1.15; 1.0; 0.68; 0.49; 0.37.

DNA molecular weight marker X

Catalogue number 1498 037

The mixture contains 18 fragments with the following number of kilobase pairs: 12.216; 11.198; 10.180; 9.162; 8.144; 7.126; 6.108; 5.090; 4.072; 3.054; 2.036; 1.636; 1.018; 0.517; 0.396; 0.344; 0.298; 0.220; 0.154.

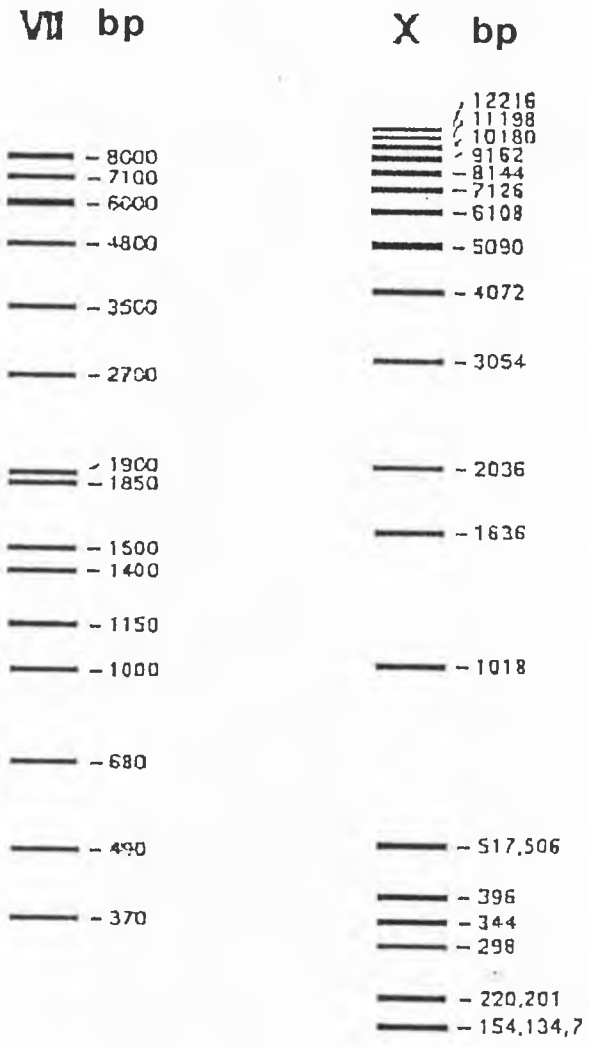


Figure A.1. Schematic of the fragment sizes for molecular weight markers VII and X, Boehringer Mannheim.

APPENDIX B (RESULTS OF INDIVIDUAL ANTIMICROBIAL SUSCEPTIBILITY TESTS)

Table B.1. Susceptibilities (MICs) of 52 *H.ducreyi* strains in various geographical regions to selected antimicrobial agents (µg/ml).

STRAINS	AMPICILLIN	CHLORAMPHENICOL	KANAMYCIN	STREPTOMYCIN	TETRACYCLINE
GAUTENG, 1988					
D16	> 128	0.5	8	16	32
Y5	128	< 0.5	0.5	8	0.5
Y10	64	< 0.5	4	16	4
Y70	> 128	< 0.5	< 0.5	< 0.5	4
YD29	> 128	< 0.5	< 0.5	2	4
Y56	64	< 0.5	4	16	4
Y76	128	< 0.5	< 0.5	4	4
Y78	> 128	8	< 0.5	4	4
Y88	> 128	< 0.5	2	16	8
Y109	> 128	8	2	8	8
Y156	> 128	< 0.5	0.5	4	2
Y163	> 128	< 0.5	0.5	8	0.5
Y165	128	< 0.5	< 0.5	8	0.5
Y179	128	< 0.5	< 0.5	8	0.5
Y119	> 128	< 0.5	< 0.5	8	< 0.5
Y182	64	< 0.5	< 0.5	8	0.5
Y193	> 128	< 0.5	0.5	8	4
Y195	32	< 0.5	0.5	8	4
BOTSWANA, 1992					
CH137	> 128	8	64	16	8
CH216	> 128	< 0.5	2	16	4
P15	> 128	< 0.5	4	16	8
P77	> 128	< 0.5	1	16	8
P116	> 128	8	64	16	8

STRAINS	AMPICILLIN	CHLORAMPHENICOL	KANAMYCIN	STREPTOMYCIN	TETRACYCLINE
LESOTHO, 1992					
CH57	> 128	< 0.5	1	2	4
CH90	> 128	2	8	16	4
CH128	> 128	< 0.5	8	16	8
CH138	> 128	< 0.5	8	16	4
CH161	> 128	4	1	8	8
CH246	> 128	1	8	32	8
P157	> 128	< 0.5	1	2	8
P167	> 128	32	1	16	2
NATAL, 1992					
CH87	> 128	< 0.5	64	2	8
CH122	> 128	< 0.5	4	32	4
CH145	> 128	8	4	16	4
CH242	> 128	16	4	16	8
CH248	> 128	< 0.5	4	32	4
P2	> 128	< 0.5	4	16	4
P6	> 128	< 0.5	4	2	2
P97	> 128	16	64	8	16
P143	> 128	16	4	16	2
TRANSKEI, 1992					
CH247	> 128	0.5	8	32	8
GAUTENG, 1994					
HD1	> 128	< 0.5	< 0.5	16	2
HD2	> 128	< 0.5	< 0.5	16	2
HD4	> 128	16	> 128	> 128	16
HD5	> 128	16	0.5	8	4
HD6	> 128	32	> 128	> 128	16
HD7	> 128	32	4	64	8

STRAINS	AMPICILLIN	CHLORAMPHENICOL	KANAMYCIN	STREPTOMYCIN	TETRACYCLINE
HD8	> 128	16	> 128	128	32
WINDHOEK, NAMIBIA, 1994					
WD793	> 128	8	2	4	16
WD794	64	8	< 0.5	8	16
G802	64	16	< 0.5	8	16
G723	> 128	1	8	16	4

Table B.2. Susceptibilities of 52 *H.ducreyi* isolates to selected antimicrobial agents.

MIC (µg/ml)	Ampicillin	Chloramphenicol	Kanamycin	Streptomycin	Tetracycline
< 0.5	0	29	12	1	1
0.5	0	2	6	0	5
1	0	2	5	0	0
2	0	1	4	5	6
4	0	1	11	4	18
8	0	7	7	14	14
16	0	7	0	20	6
32	1	3	0	4	2
64	5	0	4	1	0
128	4	0	0	1	0
> 128	42	0	3	2	0

APPENDIX C (PHYSICS OF ELECTROPORATION)

C.1. Gene Pulser

The Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, CA, USA), illustrated in Figure C.1., is a pulse generator using capacitor discharge to produce controlled exponential pulses for cell electroporation. Pulses of field strengths of up to 12.5 kV/cm can be generated in the Bio-Rad 0.2 cm electroporation cuvette. The unit provides a digital read-out of the voltage and capacitance settings and after a pulse is delivered, the actual voltage and resistance-capacitance (*RC*) time constant are automatically measured and may be displayed. The Gene Pulser apparatus contains a power supply and capacitors of 0.25, 1.0, 3.0 and 25.0 microfarads (μFd).

The Pulse Controller unit (Bio-Rad) is used with the Gene Pulser apparatus for electroporation of bacteria where pulses of very high field strength are applied to samples of small volume and high resistance.

C.2. Pulse Controller

The Pulse Controller is installed between the output of the Gene Pulser apparatus and the sample chamber (Figure C.1.). The multi-position switch on the front of the Pulse Controller determines which of six resistors (100, 200, 400, 600, 800, or 1000 Ω) is placed in parallel with the sample chamber. When this resistor is much smaller than the resistance of the sample, it is the primary determinant of the resistance of the circuit. This resistance and the size of the capacitor determine the length of the pulse. The approximate time constants obtained with various combinations of capacitors and resistors are shown in Table C.1.

The Pulse Controller greatly reduces the incidence of arcing at high voltages and protects the Gene Pulser circuit in the event that a high voltage, high current arc

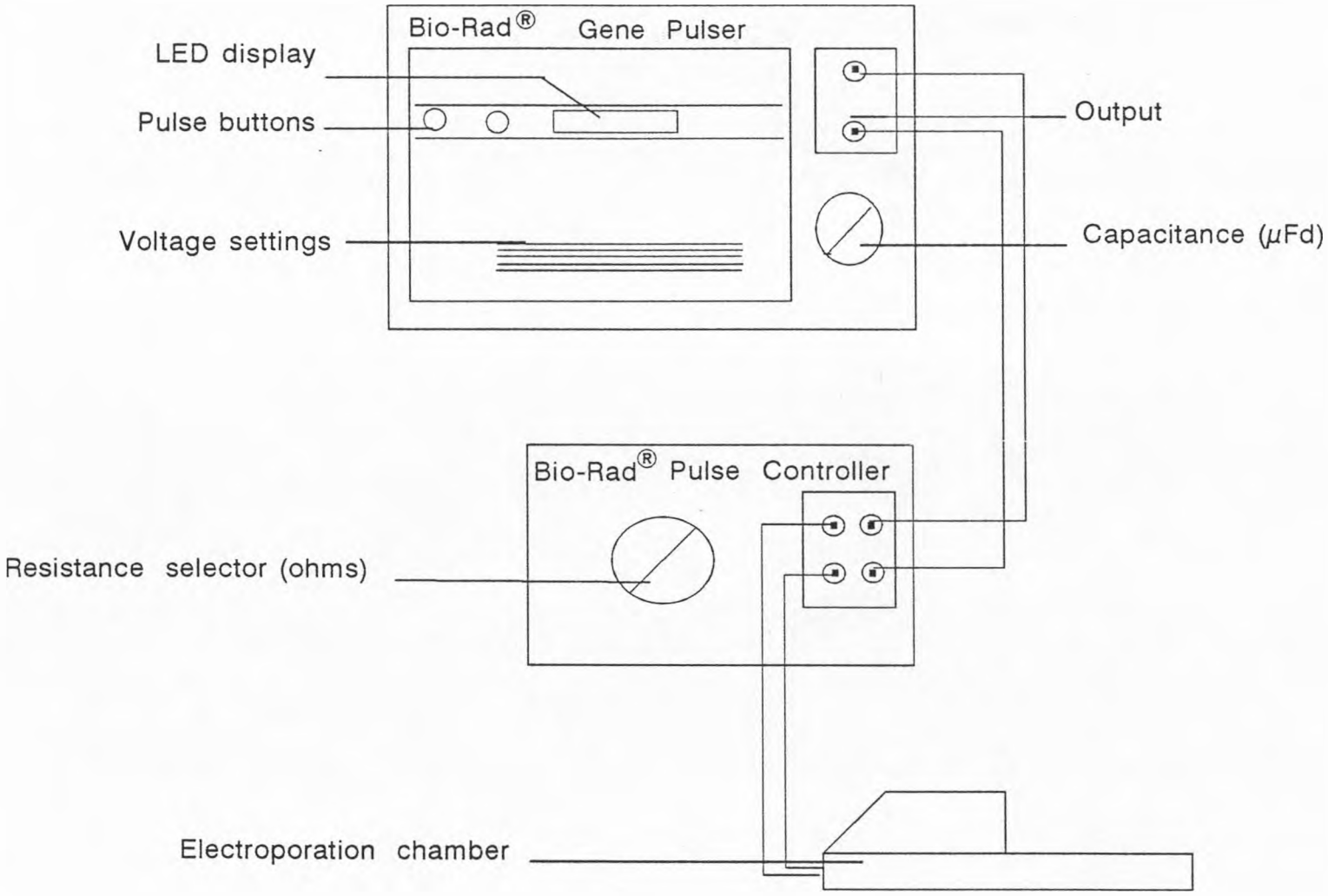


Figure C.1. Connections between the Gene Pulser apparatus, the Pulse Controller and the Electroporation chamber. (Extracted from Bio-Rad Laboratories, 1989)

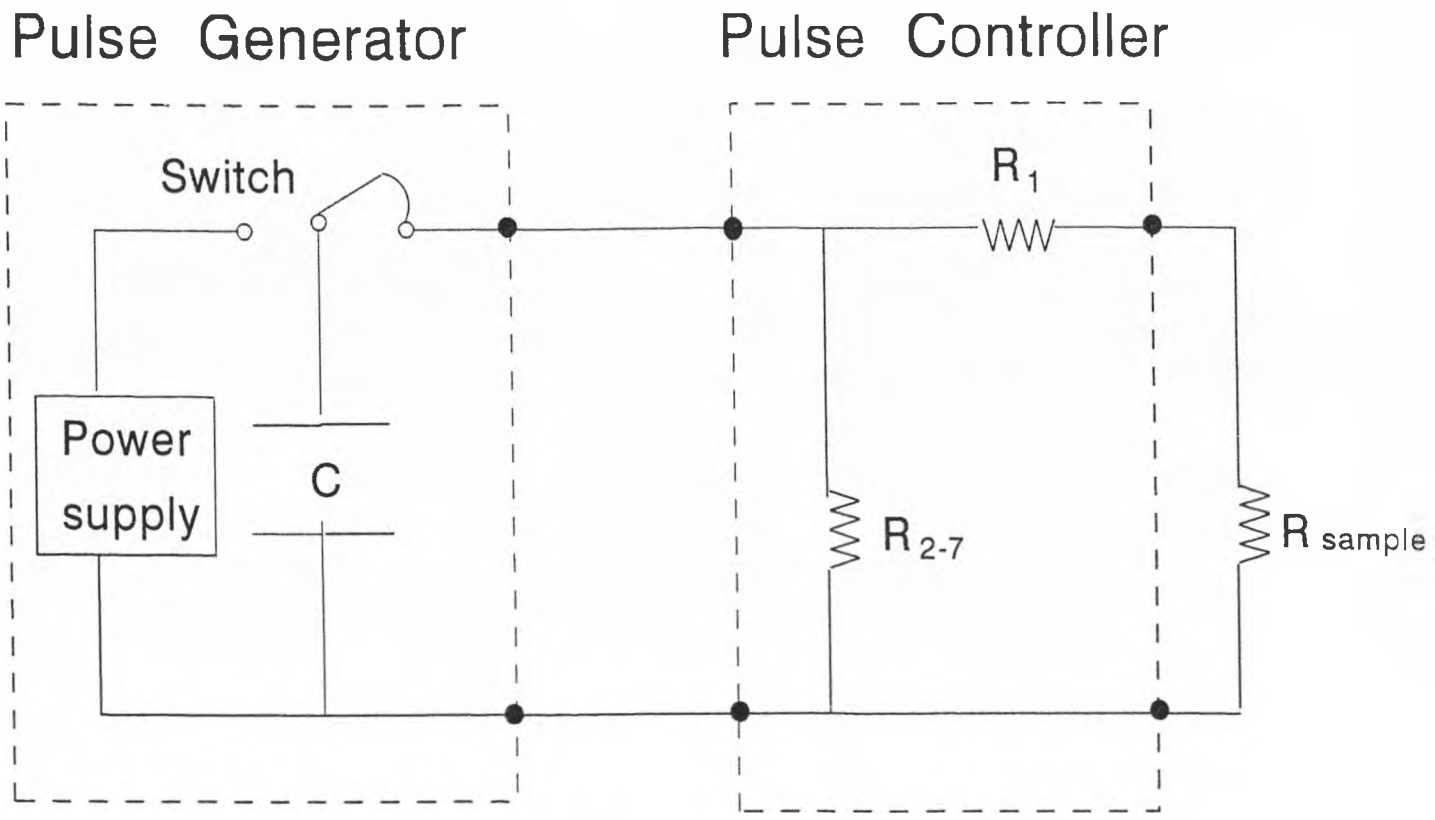


Figure C.2. Electrical circuit for bacterial electroporation. The pulse discharged from the capacitor, C, is directed through a pulse controlling circuit consisting of R_1 placed in series with the sample and one of six resistors, R_{2-7}, placed in parallel with the sample. (Extracted from Bio-Rad Laboratories, 1989)

does occur. The unit also allows the Gene Pulser apparatus to fire into a high resistance sample and provides the operator with electronic control of the time constant.

For bacterial electroporation, very high field strengths and small volumes are used, resulting in enormous energy densities that can cause arcing across the electrodes. To avoid this the Pulse Controller shunts part of the energy around the sample and through one of six resistors placed in parallel with the sample (Figure C.2.). With samples prepared as described (Sections 4.2.3. and 4.2.4.), the parallel resistance is much smaller than the resistance of the sample and determines the total resistance of the circuit. This allows control of the time constant by the choice of the parallel resistor (see Equation 3, Section C.3.).

An additional function of the Pulse Controller circuit is to place a 20 Ω resistor in series with the sample. This protects the Gene Pulser circuit by limiting the current should an arc occur. With a bacterial sample prepared as described, the resistance is about 5000 Ω; therefore, during normal operation (no arc) the voltage drop across the 20 Ω protective resistor will be less than 1%. However, if this circuit is used with samples of the much lower resistance typical of eukaryotic electroporation (20-200 Ω), the voltage lost across the protective resistor becomes highly significant.

C.3. Electrical Variables

The capacitor discharge circuit of the Gene Pulser apparatus generates an electrical pulse with an exponential decay waveform (Figure 4.2.). When the charge from the capacitor is directed to a sample placed between two electrodes, the voltage across the electrodes rises rapidly to a peak voltage (also known as the initial voltage, V_o), and declines over time as

$V_t = V_o[e^{-(t/\tau)}]$ Equation 1

where τ is the *RC* time constant, a convenient expression of the pulse length. According to Equation 1, τ is the time over which the voltage declines to 1/e (~37%) of the peak value.

The voltage gradient between the electrodes is also known as the electric field (E) and is calculated as the initial voltage divided by the distance between the electrodes,

$E = V/d$ Equation 2

where d is the distance (in cm) between the electrodes. The strength of the field and the size of the cells determine the voltage drop across each cell, and it is this voltage drop that seems to be the important manifestation of the voltage effect in electroporation.

The effective resistance placed in parallel with the electrodes is much lower than that of the sample and determines the time constant of the pulse. The time of the electric current pulse (the shock) is described by a decay time constant, τ, which corresponds to the time at which the voltage has dropped to approximately 37% of its original value. The time constant ($\tau = RC$) of the electric shock is determined by the product of the resistance, *R*, (both of the cell/DNA mixture and any parallel resistor) and the capacitance, *C*, of the circuit through which the electric field is being discharged.

The time constant is a measure of pulse length, and is defined as the time it takes for the initial voltage to drop by

$1/e(\tau = V_0/e)$ Equation 3

or by ~37%. The time constant may also be defined as

$\tau = RC$ Equation 4

where τ is measured in seconds, R is resistance in ohms (Ω), and C is capacitance in farads (Fd). The resistance of the sample depends on both its conductivity and its cross-sectional area.

For bacterial electroporation, using the Pulse Controller, part of the energy, resulting from the field strengths used and the volume of suspension, is shunted around the sample and through one of several high power resistors ($R_{2\text{-}7}$) that can be switched into the circuit in parallel with the sample. This greatly reduces the likelihood of striking an arc. The resistance of the samples (~5000 Ω) is usually much higher than that of the parallel resistor, $R_{2\text{-}7}$ (100 to 1000 Ω). In most cases, the total resistance of the circuit is determined primarily by the choice of parallel resistor, as follows,

$$\frac{1}{R_{total}} = \frac{1}{R_1+R_{sample}} + \frac{1}{R_{2\text{-}7}} \quad \text{Equation 5}$$

When $R_1 + R_{sample} >> R_{2\text{-}7}$, then $R_{total} \approx R_{2\text{-}7}$. Since it establishes the resistance of the circuit, $R_{2\text{-}7}$ can be chosen to obtain a particular pulse length as described in Equation 4. However, when the value of $R_{2\text{-}7}$ is closer to the resistance of the sample, the sample has a greater effect on the total resistance and the time constant.

R_1 is a small resistor placed in series with the sample to limit the current and protect the instrument should an arc occur. R_1 is much smaller than R_{sample} and, during a normal pulse (no arc), a negligible voltage loss will occur across R_1.

Table C.1. Approximate time constants produced with various combinations of capacitors and resistors.

Resistor (ohms)	Time constants (msec) with various capacitors			
	0.25 μFd	1.0 μFd	3.0 μFd	25 μFd
100	0.025[a]	0.1	0.3	2.5
200	0.05[a]	0.2	0.6	5
400	0.1	0.4	1.2	10
600	0.15	0.6	1.8	15
800[b]	0.2	0.8	2.4	20
1000[b]	0.25	1.0	3.0	25

a Pulses of less than 0.1 msec are displayed as 0.1 on the Gene Pulser apparatus LED display.

b With the larger resistors, the pulse length may be more greatly affected by the sample resistance. The actual pulse length may be about 20% lower than those shown in this table.

Extracted from Bio-Rad Laboratories (1989).