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CHARACTERIZATION OF GENES CONFERRING V FACTOR INDEPENDENCE IN *HAEMOPHILUS PARAINFLUENZAE* AND *HAEMOPHILUS DUCREYI*

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A thesis submitted to the Faculty of Medicine, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements of Doctor of Philosophy

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ABSTRACT

CHARACTERIZATION OF GENES CONFERRING V FACTOR INDEPENDENCE IN *HAEMOPHILUS PARAINFLUENZAE* AND *HAEMOPHILUS DUCREYI*

WINDSOR, Helen Marie, PhD., University of the Witwatersrand, 1994.

Haemophilus influenzae and *Haemophilus parainfluenzae* are obligate human parasites that form part of the flora of the mucous membranes. They are normally present in the mouth and upper respiratory tract of healthy individuals. *H. influenzae* is known as a major pathogen in children while *H. parainfluenzae* is an opportunistic pathogen which is also found in the urogenital tract. *H. ducreyi*, however, has only been isolated from genital ulcers in patients suffering from chancroid. The various species of the genus *Haemophilus* are characterised by their requirements for two growth factors, X factor or haemin and V factor or nicotinamide adenine dinucleotide (NAD). According to present taxonomic criteria and laboratory practice, isolates belonging to the genus *Haemophilus* that grow in the absence of NAD cannot be identified as *H. influenzae* or *H. parainfluenzae.* However, in 1989 four unusual clinical strains of *H. parainfluenzae* were isolated which were found to be V factor-independent.

The aim of this study was to identify and locate the genes coding for V factor independence in these *H. parainfluenzae* isolates and to

compare them with the genes conferring V factor independence in other *Haemophilus* species. In addition, attempts were made to elucidate the biochemical basis for the V factor independence.

The location of the genes coding for V factor independence in the *H. parainfluenzae* isolates was determined by transferring these genes to a plasmid-free *H. influenzae* strain by DNA transformation and analysing the DNA from the resultant transformants for the presence of plasmids by agarose gel electrophoresis. All V factor-independent transformants were found to carry a single plasmid which was of the same size (5.4 kb). Spontaneous loss of V factor independence occurred with a low frequency (0.1 to 0,2% of the progeny of a single clone) in both *H. parainfluenzae* and *H. influenzae* Rd and in all V factor-dependent clones the small plasmid was absent. This was also confirmed by Southern blotting and DNA hybridization with a digoxigenin-labelled plasmid originating from one of the V factor-independent *H. parainfluenzae* isolates. Similarly, plasmid 'curing' could be induced by mitomycin C with a rate of between 1 and 6% of the surviving clones. Because of the instability of the genes conferring V factor independence and the fact that all V factor-independent transformants acquired a plasmid, it was concluded that the genes conferring V factor independence were located on the small 5.4 kb plasmid.

As one of the unusual *H. parainfluenzae* isolates was recovered from a genital ulcer in a mixed culture with a *H. ducreyi* strain, it was hypothesized that *H. ducreyi* could have been the source of this

plasmid since this species does not require V factor for growth. Two reference and three clinical strains of *H. ducreyi* from different geographical locations were tested to determine whether the same plasmid was present in this species. Although no plasmids were detectable after agarose gel electrophoresis of DNA preparations from the original *H. ducreyi* strains, all the V factor-independent transformants obtained, when this species was used as a donor and a plasmid-free *H. influenzae* strain was used as a recipient, were found to carry a single plasmid which was of the same size as those found in the unusual *H. parainfluenzae* isolates. DNA hybridization revealed that the genes coding for V factor independence in *H. ducreyi* were located both extrachromosomally and integrated into the chromosome whereas in *H. parainfluenzae* this plasmid was present only extrachromosomally. A comparison of the digestion patterns of the plasmids from the *H. parainfluenzae* and *H. ducreyi* strains using thirteen endonucleases and subsequent restriction mapping of the plasmids demonstrated that no detectable differences could be found between the plasmids originating from the two *Haemophilus* species. Because of its small size, unique restriction sites and efficient transformability this plasmid, with its easily selectable marker, has the potential to become a good cloning vector.

The biochemical basis of the V factor independence was studied by comparing the growth in a chemically defined medium (CDM) of plasmid-free, V factor-dependent *H. influenzae* and V factor-independent transformants that had acquired the plasmid conferring V factor independence from *H. parainfluenzae* or *H. ducreyi.* The only difference

was shown to be the ability of the V factor-independent clones to utilize nicotinamide (NAm) as a pyridine nucleotide precursor. As most commercial complex media contain NAm, the strains which appear to be V factor-independent when grown on these media are, in fact, V factor-dependent in a CDM. These results indicate the necessity of using a medium which is free of pyridine precursors for the precise determination of the growth factor requirements.

Although the frequency of occurrence of V factor-independent *H. parainfluenzae* strains is presently low, these strains have the potential to become more common because of the extrachromosomal nature of the genes conferring V factor independence.

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

Helen Windsor

Helen Windsor

18th day of December, 1994.

To Harry, Julia and Marina with love

and to the memory of

George Gordon Baikie (1912-1968)

and Albert Gordon Baikie (1925-1975)

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PUBLICATIONS

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WINDSOR, H., GROMKOVA, R. & KOORNHOF, H. (1994). Prevalence of V factor-independent *Haemophilus parainfluenzae* in Johannesburg. *Southern African Journal of Epidemiology and Infection* **9**, 45-47.

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CHAPTER 1 :

REVIEW OF THE PATHOGENICITY, GENETICS AND TAXONOMY OF THE GENUS *HAEMOPHILUS* AND OF NAD BIOSYNTHESIS IN THE FAMILY *PASTEURELLACEAE*

1.1 ECOLOGY AND PATHOGENICITY OF SOME SPECIES OF THE GENUS *HAEMOPHILUS*

The members of the genus *Haemophilus* are obligate parasites that form part of the indigenous flora of the mucous membranes of the mouth and upper respiratory tract of humans and animal species, and may also be recovered from the urogenital and intestinal tracts. They are responsible for a variety of diseases in these hosts ranging from acute and chronic respiratory infections, conjunctivitis and meningitis to genital ulcers and occasional cases of endocarditis and abscess formation. Members of the genus are Gram-negative, aerobic or facultatively anaerobic coccobacilli and for *in vitro* growth one or both of two growth factors are required. These are known as X factor and V factor and are discussed in further detail in sections 1.3.1 & 1.3.2. It is this requirement for growth factors, which can be supplied by lysed erythrocytes, that has given these organisms their generic name of blood-loving or *Haemophilus*

Haemophili have been isolated from humans, pigs, sheep and various fowl species as well as healthy dogs, cats, cattle, rabbits, rats, mice and monkeys and there is reason to believe that most mammalian and avian species can be included in the list of carriers. With the exception of humans, pigs and sheep, detailed information on the carrier rates is lacking (Kilian, 1976). It appears as if natural carriage of the individual species is strictly related to specific hosts - with the possible exception of *Haemophilus parainfluenzae*, where organisms

closely resembling *H. parainfluenzae* have been isolated from monkeys, pigs, rabbits and rats. The habitats of the 16 *Haemophilus* species listed in the 1st edition of Bergey's Manual of Systematic Bacteriology are shown in Table 1 (Albritton, 1982 ; Kilian & Biberstein,1984). Two of the species, *Haemophilus pleuropneumoniae* and *Haemophilus avium* have recently been transferred to the genera *Actinobacillus* and *Pasteurella*, respectively, but they have still been included for discussion in this thesis (Pohl *et al.*, 1983; Mutters *et al.*, 1985).

Nearly all of these organisms are found as commensal inhabitants in healthy hosts but a few have only been isolated from infection sites. They are *Haemophilus aegyptius*, the causative agent of conjunctivitis and Brazilian purpuric fever; *Haemophilus ducreyi*, the aetiological agent of chancroid and *A. pleuropneumoniae* and *Haemophilus paragallinarum*, the causative agents of necrotizing pleuropneumonia in pigs and infectious coryza in poultry, respectively .

The human upper respiratory tract provides at least two different ecosystems as regards haemophili. The pharynx gives shelter to both X factor-dependent and X factor-independent species whereas only X factor-independent species have been isolated from the oral cavity (Kilian, 1976). The oral *Haemophilus* species, which very rarely encompass *Haemophilus influenzae*, amount to an average of 4×10^{7} bacteria ml^{-1} in the saliva which represents about 10% of the total flora (Sims,1970). These species include *H. parainfluenzae*, *Haemophilus parahaemolyticus*,

Table 1. The natural hosts and sites of isolation of species of the genus *Haemophilus* and some closely related species

Species	First described	Host	Site
H. influenzae	1892	Human	Nasopharynx
H. aegptius	1883	Human	Eye; cojunctivitis
H. haemolyticus	1919	Human	Nasopharynx
H. haemoglobinophilus	1903	Dogs	Preputial sac
H. ducreyi	1889	Human	Genital ulcers; chancroid
H. parainfluenzae	1922	Human	Oral cavity, nasopharynx
H. parahaemolyticus	1953	Human	Oral cavity, nasopharynx
H. paraphrohaemolyticus	1971	Human	Oral cavity
A. pleuropneumoniae	1964	Pigs	Respiratory tract; pneumonic lesions
H. paracuniculus	1979	Rabbits	Gastrointestinal tract
H. aphrophilus	1940	Human	Dental plaque, gingiva
H. paraphrophilus	1968	Human	Oral cavity, pharynx
H. segnis	1977	Human	Dental plaque, oral cavity
H. parasuis	1969	Pigs	Upper respiratory tract
H. paragallinarum	1969	Poultry	Respiratory tract; infectious coryza
P. avium	1977	Poultry	Respiratory tract

Haemophilus paraphrohaemolyticus, *Haemophilus aphrophilus*, *Haemophilus paraphrophilus* and *Haemophilus segnis* of which the latter three are found predominantly in dental plaque.

1.1.1 *Haemophilus influenzae*

H. influenzae was first isolated in 1892 by Pfeiffer who thought it was the aetiological agent of influenza, hence the generic name which has remained with this organism long after it was discovered that it did not cause influenza. However, it has a wide range of pathogenic potential and today two contrasting patterns of *H. influenzae* infections have been identified. The first and most serious are invasive infections such as meningitis, septic arthritis, epiglottitis and cellulitis in which bacteraemia is a prominent feature and the second category includes less serious but numerically more common infections that occur as a result of contiguous spread of *H. influenzae* within the respiratory tract eg. otitis media, sinusitis, conjunctivitis and bronchopneumonia (Moxon, 1990). Clinical isolates of *H. influenzae* are often found to be encapsulated and this has been found to correlate with the virulence of the isolate. In 1931, Pittman described six antigenically distinct capsular types of *H. influenzae*, designated a to f. Up to 80 % of healthy people are colonized from infancy onwards with unencapsulated strains of *H. influenzae* and the less virulent infections listed above are usually, but not invariably, caused by these unencapsulated strains. However virtually all the isolates from meningitis and epiglottitis cases are serotype b strains and these infections occur mainly in children less

than 2 years old. In the United States there were, before the institution of mass immunization against *H. influenzae* type b infections, an estimated 10, 000 cases of meningitis per year caused by *H. influenzae* (Kilian, 1991). The relatively few cases of meningitis in the older age group are caused by unencapsulated strains and usually occur in persons who have suffered anatomical trauma or are immunocompromised. In a survey of children in Cape Town, South Africa, Hussey *et al.* (1994) found that children hospitalised with tuberculosis had an overall carriage rate of *H. influenzae* of 66 %. This was 20 % higher than in a comparable control group of healthy children and the authors speculate that this may be due to some degree of immunosuppression as a result of the tuberculosis infection.

The identification of *H. influenzae* is based on its requirement for both X factor and V factor for growth. In his taxonomic study of the genus *Haemophilus*, Kilian (1976) further differentiated between the strains of *H. influenzae* using three biochemical criteria - indole production, urease and ornithine decarboxylase activity. He proposed the division of this species into five biotypes based on these biochemical differences and he found a positive correlation between the biotype, the capsular serotype of Pittman and the source of the strains. 85 % of the strains isolated from meningitis, epiglottitis and blood cultures could be assigned to biotype I, whereas all but one of the strains from conjunctivitis were assigned to biotypes II and III, which also contained most of the respiratory strains. This system of biotyping *H. influenzae* has since been enlarged and now recognises 8 different biotypes (Gratten, 1983; Sottnek & Albritton,1984). Although the vast majority of strains from invasive infections belong to

biotype I, it now appears as if the distribution of the biotypes and serotypes of clinical isolates of *H. influenzae* varies significantly when comparisons are made worldwide (Musser *et al.*, 1990). In South Africa Rowji *et al.* (1989) found that 58 % of *H. influenzae* serotype b isolates from blood, cerebro-spinal fluid and throat specimens were biotype II and only 29 % were biotype I. Some authors have shown that biotype IV has been associated with urogenital infections in the United States and in France but other authors in England, Australia and the United States have not reported a predominance of biotype IV strains as the causative agent of genital and neonatal sepsis (Wallace *et al.*, 1983; Casin *et al.*, 1988; Kleiman *et al.*, 1983; Harper & Tilse, 1991). Other reports on infections caused by *H. influenzae* have shown that age does not significantly alter the distribution of biotypes involved in noninvasive infections and that, in patients with cystic fibrosis, biotype I strains are isolated more often and biotype II strains are isolated less often in comparison with the frequency of isolation of strains from the normal respiratory tract (Watson *et al.*, 1985;1988). More sensitive tools than biotyping have become available for epidemiological purposes such as subtyping on the basis of outer membrane proteins, lipopolysaccharides or isoenzymes (Musser *et al.*, 1990) but they need to be more extensively exploited.

1.1.2 *Haemophilus aegyptius*

H. aegyptius causes acute contagious conjunctivitis in tropical climates and it has never been isolated from healthy individuals. It was first

described by Koch in 1883 and then isolated by Weeks in 1886 and hence is still sometimes referred to as the Koch-Weeks bacillus (Albritton, 1982). However, both genetic transformation results and a 78 % DNA homology between *H. influenzae* and *H. aegyptius* appear to indicate that these two species are the same (Leidy *et al.*, 1965; Pohl, 1981; Albritton *et al.*, 1984b). In his study of 426 *Haemophilus* species, Kilian (1976) included the *H. aegyptius* strains with those of *H. influenzae* on the basis of similarities in biochemical results and classified them as *H. influenzae* biotype III. Subsequently, in 1984, an apparently new syndrome, Brazilian purpuric fever (BPF), was reported and the causative agent has been described as *H. influenzae* biogroup aegyptius (Brenner *et al.*,1988). This infection is characterised by young children initially having conjunctivitis and then developing a potentially fatal form of sepsis very similar to meningococcaemia but with the absence of meningitis. The strain isolated from BPF cases has been shown to be distinct from other strains of *H. influenzae* and *H. aegyptius* in its unique total cellular protein profile and isoenzyme pattern as well as containing a characteristic 24 megadalton (Mda) or 36 kilobase (kb) plasmid. However, despite the high association between this plasmid and BPF, there is no evidence to prove its direct role in pathogenisis (Swaminathan *et al.*, 1989).

1.1.3 *Haemophilus parainfluenzae*

In 1922 Rivers described an influenzae - like bacillus that required only the heat-labile growth factor (later termed V factor) and 6 years later

Russell & Fildes first reported disease in man due to *H. parainfluenzae* - a case of endocarditis (Albritton, 1982). Today it is regarded as an organism of low pathogenicity that is ubiquitous in the human oral cavity and pharynx and may be present in the normal vaginal and urethral flora. However, because of refinements in the isolation and identification of *H. parainfluenzae* and other human haemophili, it is apparent that they cause infection more commonly than was previously believed. These improved identification methods have also led to the questioning of whether *H. paraphrohaemolyticus* and *H. parahaemolyticus* are sufficiently different from *H. parainfluenzae* to warrant them being classified as separate species (Kilian, 1976; 1991). Albritton (1982) states that " a number of species have been created by taxonomists based on limited phenotypic traits of uncertain significance without regard to clinical relevance " and he queries the reliability of the few reports of infections caused by these species.

H. parainfluenzae, as an opportunistic pathogen in the human mouth and pharynx, may cause pharyngitis, epiglottitis, otitis media, conjunctivitis, dental abscesses, jaw infections and infections following human bites or finger sucking. It has also been reported to cause endocarditis, peritonitis, pneumonia, septic arthritis, osteomyelitis, meningitis, epidural and brain abscesses and urinary tract and genital infections (Hand, 1990; Auten *et al.*, 1991). The occurrence of endocarditis caused by *H. parainfluenzae* appears to have increased presumably due to improved isolation techniques but similarly many earlier cases of meningitis and other infections which have been ascribed to

H. parainfluenzae can probably be explained by misidentification of *H. influenzae* isolates if, for example, the porphyrin test (see section 1.3.1) was not used to identify the isolates (Albritton, 1982).

H. parainfluenzae constitutes at least 74 % of the isolates of *Haemophilus* species from the pharynx of healthy children and adults (Kuklinska & Kilian, 1984) and it is commonly detected in the upper and lower respiratory tract secretions of chronic bronchitics but is generally considered to be non-pathogenic. Kilian (1976) proposed the differentiation of *H. parainfluenzae* into 3 biotypes using the same biochemical tests as for *H. influenzae*. This biotyping has now been enlarged to include 8 different biotypes (Sturm, 1986; Doern & Chapin 1987). Taylor *et al* (1992) examined *H. parainfluenzae* isolates from respiratory secretions of 36 healthy adults and 128 patients with chronic bronchitis at monthly intervals over a period of one year. Biotypes I and II constituted most of the isolates both from the oropharynx of controls (75 %) and chronic bronchitics (90 %). Biotypes IV, V, VI, VII and VIII were isolated less frequently (< 5 %) in all subjects and biotype III was twice as common in healthy subjects as in bronchitis patients (16 % vs 7 %). These authors conclude that as little difference was observed in the frequencies of isolation of the different biotypes in the presence or absence of acute bronchitis, specific biotypes do not appear to play a recognisable role in the pathogenesis of this infection.

1.1.4 *Haemophilus ducreyi*

H. ducreyi is the aetiological agent of chancroid and even though it was first recognized as being distinct from syphilis over 140 years ago there is still relatively little known about this organism (Albritton, 1989). Although chancroid is considered an uncommon sexually transmitted disease (STD) in Europe and North America, the number of reported cases has increased every year since 1984 with over 4000 cases reported in the United States in 1990. It is however very common in third world countries where it is recognized as the commonest cause of sexually acquired genital ulceration accounting for 68 % of cases in the Transvaal, South Africa (Dangor *et al.*,1989), 62% in the Gambia (Mabey *et al.*, 1987), 60 % in Nairobi, Kenya (Plummer *et al.*, 1985), 42 % in Swaziland (Meheus *et al.*, 1983) and 37 % in Thailand (Taylor *et al.*, 1984). The current importance of chancroid lies in the epidemiological association of this and other genital ulcer infections with the acquisition and transmission of human immunodeficiency virus (HIV) (Kreiss *et al.*, 1989; Plummer *et al.*, 1990).

One of the reasons for the lack of information on *H. ducreyi* is that it is notoriously difficult to isolate as it needs enriched media for *in vitro* growth. It is the only human *Haemophilus* species that requires only X factor but it also needs 5 % foetal calf serum and IsoVitalex or some similar vitamin additive. The addition of vancomycin (3 $\mu g\ ml^{-1}$) to the media is useful as it helps inhibit certain Gram - positive flora that are also associated with genital ulcers (Hammond *et al.*, 1978b; Nsanze

et al., 1984; Dangor *et al.*, 1992). However, it may suppress the growth of some clinical strains as well as some of the older laboratory isolates such as the type strain, CIP 542, isolated in 1954 which are sensitive to vancomycin necessitating the use of other media for the isolation of these strains. The optimum incubation temperature is about 35 °C and an atmosphere with 5 % CO_2 is required for growth. The colonies are usually slow growing and at least 48 hours incubation time is needed. The colonies are distinctively cohesive and can be pushed intact across the agar plate but this property makes it virtually impossible to obtain a homogeneous suspension in liquid media for further analysis. Apart from the distinctive patterns seen using the Gram stain, there are few reliable biochemical tests that can be used to identify *H. ducreyi* as many conflicting reports about its enzymatic activities have appeared in the literature (Morse, 1989). The classic biochemical activities of *H. ducreyi* are production of alkaline phosphatase and the reduction of nitrate but some reports even show negative results for the latter test. Recently monoclonal antibodies against *H. ducreyi* have been developed and these may be used in immunofluorescence assays but the specificity of this technique still needs to be improved (Hansen & Loftus, 1984; Karim *et al.*, 1989). Enzyme-linked immunosorbent assay (ELISA)-based antigen detection has recently been reported by Roggen *et al.* (1993) but the future of this technique, like those mentioned above, remains to be evaluated in clinical trails. There is a great need for a simple, inexpensive diagnostic test that could be used in the low - resource communities where chancroid is prevalent (Jonasson, 1993).

At present the exact mode of infection by *H. ducreyi* is being examined by various researchers. It is known that *H. ducreyi* penetrates the skin through minor abrasions or by invasion of the epithelial cells. After 2 - 7 days a small tender papule develops which becomes pustular and erodes to form an ulcer with ragged edges and a necrotic base. These lesions vary in size and may give rise to satellite ulcers but the disease never spreads systemically. Another characteristic feature of chancroid is the painful swelling of the lymph nodes and their spontaneous rupture. Abeck *et al.* (1992) have studied the binding of *H. ducreyi* to extracellular matrix proteins and found that this binding is associated with the expression of pili by the bacteria. There are, however, conflicting reports about the ability of *H.ducreyi* to invade epithelial cells and to cause lysis. Shah *et al.* (1992) state that the epithelial cell cultures used by them engulfed the bacterial cells and that this caused the lysis of the epithelial cells and the release of *H. ducreyi* but Alfa (1992) used transmission electron microscopy to show that *H. ducreyi*, although closely associated with cells of human foreskin in tissue culture, did not penetrate the cell membrane. They did report, however, that *H. ducreyi* had a cytopathic effect on the foreskin cell line. Lammel *et al.* (1992) have also reported on the growth of *H. ducreyi* in cells of genital origin and they state that after 2h *H. ducreyi* cells from a known virulent strain were found in the cytoplasm whereas an avirulent strain, CIP 542, took a few hours longer to appear internally in the cytoplasm of the tissue culture cells. After 5 days the cells infected with the clinical isolate of *H ducreyi* appeared to be degenerating while those infected with strain CIP 542 appeared healthy

despite the presence of intracellular bacterial cells. An extra-cellular cytotoxin produced by *H. ducreyi* cells during exponential growth has been reported by Purvén & Lagergård (1992) but Lammel *et al.* (1992) state that this product is only cytotoxic for epithelial cell lines that are not of genital origin. They speculate that this cytotoxic product may function by damaging epithelial cells thus allowing the bacteria to establish infection in the epithelium with subsequent interactions with subepithelial cells such as foreskin fibroblasts. Another important subject receiving attention at present is the role of the lipopolysaccharides (LPS) present in the outer membranes of *H. ducreyi* which have been shown to be important factors in the virulence of these pathogens. The toxicity of the LPS resides in the lipid A region of the molecule and the core structure shows similarities with that found in *H. influenzae* (Jonasson, 1992).

There are a number of unanswered questions regarding *H. ducreyi*. These include the molecular basis of pathogenesis, the virulence factors responsible for infection, whether there is an asymptomatic carrier state, its effect on the host's immune system, its metabolic pathways, its genetic relatedness to other *Haemophilus* species and its taxonomic position within the family *Pasteurellaceae* in general. Little is known about the genetics of *H. ducreyi* except for some of the genes that encode resistance to antimicrobial agents (McNicol & Ronald, 1984).

1.2 GENETICS OF THE GENUS *HAEMOPHILUS*

Bacteria and yeasts are the only known organisms naturally able to take up naked DNA from the surrounding environment and to incorporate it into their genome and hence alter their own genotype. This is known as genetic transformation. Only a few bacterial species appear to be naturally transformable and these include some of the species of *Haemophilus*. In their natural environments some cells in the population act as donors by releasing DNA by autolysis while others take up the DNA and incorporate it to produce a population with a vast array of new nucleotide sequences and different phenotypes. During the past 40 years the natural transformation systems of *H. influenzae* and *H. parainfluenzae* as well as the genomes and plasmids of these organisms have been studied in detail.

1.2.1 DNA transformation

In 1950 Alexander and Leidy demonstrated the occurrence of transformation in *H. influenzae* using capsulation as a genetic marker. Since that time, most studies on DNA transformation in *Haemophilus* have been carried out with a single highly transformable non-encapsulated avirulent strain, Rd, derived from a capsular type d *H. influenzae* strain isolated by Alexander and Leidy in 1951. This strain is plasmid-free but it has an inducible defective prophage. Much work has been done on the molecular events that accompany transformation, and several steps in transformation that are of

significance have been identified : (i) development of competence or the ability to take up DNA (ii) recognition and uptake of transforming DNA (iii) integration of DNA in the recipient genome and (iv) expression of the genetic marker (Smith *et al.*, 1981).

In *H. influenzae* competence develops when cell division is blocked under conditions that allow continued protein synthesis. By transferring exponentially growing cells into a defined non-growth medium it is possible to generate cultures that are essentially 100% competent for transformation (Herriott *et al.*, 1970b). This is also achieved if the cells grow in nutritionally rich media under static aerobic conditions (Gromkova & Goodgal, 1979; Gromkova *et al.*, 1989b). Competence- and uptake-deficient mutants of both *H. influenzae* and *H. parainfluenzae* have been used by researchers to try to elucidate the phenomenon of transformation. Competence development involves the synthesis of several new inner and outer membrane proteins and also two normally cytoplasmic proteins become membrane-associated. Studies on cellular changes that occur during development of competence have provided evidence that competence is associated with the appearance of vesicles on the outer membrane (Kahn *et al.*, 1979; Kahn & Smith, 1984). These vesicles, called transformasomes, protect the DNA from the action of non-specific nucleases during the uptake and transport of the DNA into the cell's interior during transformation (Kahn *et al.*, 1983).

Work focused on the specificity of the DNA uptake mechanism in cells has shown that *Haemophilus* and *Neisseria* species recognize their own DNA at the cell surface. Although foreign DNA does bind to the surface of the competent cell, only DNA from the same or closely related species is able to penetrate into the cells and this phenomenon has been used to determine relatedness within the genus (Leidy *et al.*, 1959 ; Albritton *et al.*, 1984b & 1986). In *H. influenzae* an 11 basepair (bp) sequence on the donor DNA identifies this DNA to the cell (Danner *et al.*, 1980). It has been estimated that there are about 600 uptake sites in the *Haemophilus* genome or one site per 4000 bp. If the donor DNA is sonicated and broken into very small pieces of less than 300 bp, the chance of these recognition sequences being intact are low and experimental work has verified this as few or no transformants are obtained from such small pieces of DNA.

The uptake of DNA involves 3 steps : (a) the reversible binding between a DNA uptake site and a cell surface receptor (b) the formation of an irreversible site-receptor complex and (c) the uptake of the entire DNA duplex in a DNase-resistant form (Smith *et al.*, 1981). Under saturating DNA conditions, binding and uptake are estimated by these authors to be complete within one minute. The amount of DNA that is bound to the competent cells depends on the number of cell surface receptors which vary from four to eight per cell (Deich & Smith, 1980). There is also variation in the amount of DNA that is bound per receptor as electron microscopy has shown that there may be a large number of small DNA particles associated

with a single transformasome or a large DNA molecule may even fold back onto the same vesicle a number of times. The protein responsible for binding to the donor DNA has been identified as one of the membrane-associated proteins that are synthesised during competence induction in *H. influenzae* (Deich & Smith, 1980). Several other polypeptides have since been identified using uptake-deficient mutants that lack one or other of these polypeptides and these latter proteins appear to permit the donor DNA to penetrate the wall and inner membrane structure of the cell.

Competent cells of *H. influenzae* tightly bind double-stranded DNA in a form resistant to DNase and it is as intact, duplex molecules that donor DNA is taken into the cells (Goodgal,1982). Although transformation by single-stranded DNA with competent cells of *H. influenzae* has been reported, the efficiency of transformation is about 50 % of that obtained with double-stranded DNA. The donor DNA is protected from the cellular exonucleases and restriction endonucleases both by the formation of the polypeptide-DNA complex as well as being physically isolated from the bulk of the cell's cytoplasm by the transformasome. During integration one strand of the donor DNA is released from the transformasome and incorporated into the host chromosome while the other strand and the displaced host strand are degraded and released as waste. Using the transformation-deficient mutants of *H. influenzae*, *rec* 1 and *rec* 2, three loci on the *H. influenzae* genome have been shown

to be involved in this integation of donor DNA (Barcak *et al.*, 1989; Larson *et al*, 1991).

Although phage, plasmid and chromosomal DNAs all seem to be taken up by the same mechanism in naturally competent cells, transformation with extrachromosomal or plasmid DNA is characteristically very inefficient - fewer than 1 in 10 000 plasmids become established in both *H. influenzae* and in *H. parainfluenzae* compared to 1 in 10 to 1 in 100 chromosomal markers (Stuy, 1979; Gromkova & Goodgal, 1981; Notani *et al.*, 1981). Plasmid transformation efficiencies are dramatically increased when plasmids with chromosomal DNA inserts are used (Setlow *et al.*, 1981; Balganesh & Setlow, 1985, Stuy & Walter, 1986a). These authors suggest that this is probably due to the presence of uptake-specific sites on insert DNA and recombination between the homologous segments on plasmid and chromosome. It is hypothesised that double-stranded circular plasmid DNA cannot leave the transformasomes without first being broken up. These single-stranded fragments are then reconstituted in the cytoplasm by the homologous regions using the cell's replication enzymes. Stuy & Walter (1986a) and Pifer (1986) have both proposed that plasmid transformation may occur due to the occasional 'leak' of double-stranded DNA from the transformasomes. They state that the recovery of plasmids from cells transformed by linear DNA reveals that no information is lost from the ends of linear molecules during plasmid establishment despite the fact that it is known that normally one strand of DNA is

completely degraded and the other strand is at least partially degraded during entry into the cytoplasm. They speculate that this low frequency 'leakage' is the principal route of plasmid homology when the plasmid bears no homolgy to recipient DNA. Pifer refers to this as illegitimate transformation.

Various methods have been proposed to try to increase the efficiency of plasmid DNA transformation. Gromkova & Goodgal (1979) found that plasmid DNA transformation was stimulated more than 1000-fold by the addition of either 10mM Ca^{2+} or 20mM Mg^{2+} to the transformation mixture while chromosomal DNA efficiency was only increased 20 times by the addition of these divalent cations. They also found that the effect of $Mg^{2+}$and Ca^{2+}on DNA uptake was independent of the molecular structure of DNA, since both covalently closed circular and linear plasmid DNA were stimulated by these divalent cations showing that linearization itself was not essential for DNA uptake. In 1986, Stuy and Walter showed that the addition of 30% glycerol to the competent cells after uptake of the plasmid DNA and before plating for transformants, increased the number of transformed cells by 100 times. This large increase resulted from the release of double-stranded plasmid molecules from the transformasomes due to the presence of the glycerol which appears to disrupt the membrane proteins. This method did not increase the efficiency of transformation by either chromosomal or linearized plasmid DNA.

As plasmids from *H. influenzae* and *H. parainfluenzae* are widely used as cloning vectors in modern molecular experiments, researchers have had to overcome the problem of low transformation efficiency by using either cloned chromosomal fragments in the vectors, by preparing artificially competent cells using Ca^{2+} cations or by employing heat-shock treatment or glycerol stimulation (Barcak *et al*, 1991). A new method that has been used to introduce genes from plasmid DNA into *Haemophilus* cells is voltage shock transformation or electroporation which allows for the transfer of plasmid, but not chromosomal, DNA into cells unable to become competent or to transform naturally (Mitchell *et al.*, 1991; Setlow & Albritton, 1992).

1.2.2 Plasmids and antibiotic resistance

Plasmid-mediated ampicillin resistance was first shown in *H. influenzae* in 1973 (Elwell *et al.*,1975). These authors demonstrated the presence of a 30 Mdal (45 kb) plasmid carrying a complete transposon A (TnA) sequence coding for TEM β-lactamase production which had previously been found on plasmids isolated from enteric strains. They also found a smaller 3 Mdal (4.5kb) plasmid which did not carry the entire TnA sequence and they speculated that these TnA segments had been incorporated into indigenous, phenotypically "silent" *Haemophilus* plasmids. Since then double and triple resistant strains have emerged and research has shown two main groups of antibiotic resistance plasmids in *Haemophilus* species. The first group of plasmids are large (45 kb), conjugative and carry various combinations of

genes specifying β-lactamase, tetracycline resistance and chloramphenicol acetyltransferase on transposons. Similar or identical large plasmids have been isolated from both *H. parainfluenzae* and *H. ducreyi*. The plasmids of the second group are smaller (5 - 10 kb), specify only β-lactamase production and are prevalent in *Neisseria gonorrhoeae, H. ducreyi* and *H. parainfluenzae* (Brunton *et al.*, 1986b). There have also been reports of conjugative plasmids in *H. ducreyi* that do not encode antibiotic resistance but that promote the intra- and interspecific spread of the small resistance plasmids (Deneer *et al.*, 1982) as well as reports of phenotypically cryptic plasmids in *H. parainfluenzae* (Mann & Rao, 1979).

In the late 1970's resistant *H. influenzae* strains were reported which did not appear to harbour plasmids after being lysed and electrophoresed on agarose gels (Stuy, 1979; Roberts & Smith, 1980). Roberts & Smith showed that many of the strains did carry plasmids when two different lysing methods were used. They also showed that all these "plasmid-free" strains could transfer a plasmid to the plasmid-free recipient strain, *H. influenzae* Rd after conjugation. In 1980 Stuy suggested that in many instances the conjugative antibiotic resistance plasmids were chromosomally integrated and in 1984 Murphey-Corb *et al.* showed this to be true using restriction enzyme analysis and Southern transfer of chromosomal DNA. One consistent observation is that conjugative plasmids not detectable by conventional plasmid isolation techniques can be detected after they have been transferred to other strains.

The large conjugative antibiotic resistance plasmids in *H. influenzae* from different geographic areas have been shown by Roberts *et al.* (1980) and Laufs *et al.* (1981) to contain a common core of about 27 Mdal (41 kb). These core sequences are 70 % - 90 % homologous even in sequences carrying different resistance determinants. Laufs and his co-workers have also isolated a 27 Mdal plasmid that contains no antibiotic resistant determinants but that is 80 % homologous to the large conjugative plasmid of *H. influenzae*. In 1984 Albritton *et al.* reported that large conjugative tetracycline and tetracycline-chloramphenicol resistance plasmids from *H. ducreyi* strains isolated in Kenya and the United States were also highly related to an *H. influenzae* β-lactamase plasmid. The main differences between these plasmids were due to the integration of additional transposons. This supports the hypothesis that these plasmids could have simultaneously arisen in several parts of the world as a result of independent transposition events of resistance genes onto different but closely related indigenous phenotypically cryptic *H. influenzae* plasmids. Scheifele and Fussell (1981; 1982) have shown that β-lactamase-producing strains of *H. parainfluenzae* are far more prevalent in the oropharynx of children than β-lactamase-producing strains of *H. influenzae*. As these strains both harbour similar plasmids and are able to transfer them between each other, these authors proposed that *H. parainfluenzae*, as a common commensal, may be serving as a reservoir of antibiotic resistance plasmids that could be acquired by the pathogenic *H. influenzae* strains.

Similarly, Brunton *et al.* (1986b) have suggested that the small cryptic plasmids found relatively commonly in *H. parainfluenzae* may be the direct progenitors of the β-lactamase-encoding plasmids found in *H. ducreyi* and in *N. gonorrhoeae*. Although these authors were unable to prove this by producing a recombinant β-lactamase-encoding plasmid in the laboratory using the TnA gene as an insert in a cryptic *H. parainfluenzae* plasmid, Martel *et al.* (1987) have since reported the isolation of three *H. parainfluenzae* strains recovered from the urogenital tract that carry a 3.2 Mdal (4.9 kb) β-lactamase-encoding plasmid as well as other cryptic plasmids. These former plasmids have been shown to be identical to a similar plasmid from *N. gonorrhoeae* by restriction endonuclease digestion and by hybridization with probes for the TEM-1 β-lactamase gene and for fragments of TnA.

1.2.3 Restriction endonucleases in *Haemophilus* species.

Restriction endonucleases digest double stranded DNA after recognizing specific nucleotide sequences by cleaving two phosphodiester bonds, one within each strand of the duplex DNA. These restriction enzymes form part of the restriction-modification system of bacterial cells to provide protection against invasion of the cell by foreign DNA. Protection against self-digestion is achieved by the presence of specific DNA methyltransferases which transfer methyl groups to adenine or cytosine residues. All restriction endonucleases and their corresponding DNA modification methyltransferases have

been classified according to gene and protein structure, cofactor dependence and specificity of binding and cleavage into classes I, II and III. Of these three types of enzymes, class II restriction endonucleases are the most useful due to their absolute sequence specificity for both binding and cleavage reactions. These enzymes are powerful tools for the dissection of DNA and they are essential for the construction of recombinant DNA molecules and for DNA sequence determination. A large portion of our knowledge of these enzymes and the way they function are due to work carried out on the endonucleases of *Haemophilus* species. The first class II restriction enzyme to be purified was *Hin*d II from *H. influenzae* Rd (Smith & Wilcox, 1970; Kelly & Smith, 1970) and since then at least 8 more enzymes have been described from different *Haemophilus* species, making it one of the genera that is used most often in the commercial production of restriction enzymes.

1.2.4 Chromosomal mapping, DNA fingerprinting and the use of DNA probes for the detection of *Haemophilus* species

The physical maps of the chromosomes of a few strains of *H. influenzae* and of *H. parainfluenzae* have been published (Lee *et al.*, 1989; Kauc *et al.*, 1989; Kauc & Goodgal, 1989b; Butler & Moxon, 1990). The *H. influenzae* strains mapped include both the laboratory strain, Rd, as well as an encapsulated type b strain isolated from a case of meningitis. The sites of certain ribosomal RNA operons essential for growth, the loci for restriction endonuclease production, antibiotic resistance and outer

membrane protein synthesis as well as twelve potential virulence loci have been identified. Although several genetic maps have been published there is still variation between the maps from different researchers but there is agreement that *H. influenzae* Rd has a circular genome that is between 1834 and 1980 kb in size while the virulent *H. influenzae* type b strain has a slightly larger (2110 kb) genome.

The use of restriction enzyme digests of chromosomal DNA (or fingerprinting) is becoming a popular method for the characterisation and classification of isolates for epidemiological studies. Research on the genomes of non-typable *H. influenzae* isolates have shown that these are among the most genetically diverse organisms known (Stull *et al.*, 1988; Loos *et al.*, 1989; Forbes *et al.*, 1991). However, this is not true of all members of the *Pasteurellaceae*, as Borr and her co-workers (1991) have shown that the 12 serotypes of *A. pleuropneumoniae* share a large number of co-migrating fragments even though each pattern is unique. Ribotyping or the hybridization of restriction endonuclease-digested chromosomal DNA with rRNA from *Escherichia coli* has been used to differentiate among strains of *H. ducreyi* (Sarafian *et al.*, 1991) and to compare strains of *H. influenzae* biogroup aegyptius (Irino *et al.*, 1988).

Another area where research is being conducted at present on the genomes of *Haemophilus* species is in the search for different DNA fragments from the various species that could be used as probes in

the specific detection of these species in clinical specimens. A 5 kb *H. influenzae* *Hind*III - *Bgl*II DNA fragment coding for clinical beta-lactam resistance through alteration of penicillin-binding proteins has been used as a probe in clinical trials in Canada to detect both encapsulated and non-encapsulated *H. influenzae* strains in sputum, blood and CSF specimens (Malouin *et al.*, 1988). In similar research a 1.5 kb DNA fragment from *A. pleuropneumoniae* has been shown to hybridize with all 12 seroypes of *A. pleuropneumoniae*, thus enabling Sirois and his co-workers (1991) to detect strains of this organism in clinical specimens from infected swine using the polymerase chain reaction (PCR). Similarly, both DNA fragments from *H. ducreyi* which encode *H. ducreyi*-specific proteins, and rRNA-derived oligonucleotides from the *H. ducreyi* type strain, have been used as probes to detect this organism without cross reacting with other organisms from the urogenital tract (Parsons *et al.*, 1989; Rossau *et al.*, 1991). The latest test involves the use of PCR to detect *H. ducreyi* in clinical specimens (Johnson *et al.*, 1994).

1.3 TAXONOMY OF THE GENUS *HAEMOPHILUS*

The genus *Haemophilus* was created in 1920 by the American Committee on Classification and Nomenclature to include a number of haemophilic organisms (Winslow *et al.*, 1920). Presently, the authoritative Bergey's Manual of Systematic Bacteriology places the genus *Haemophilus* in the family *Pasteurellaceae* and it includes 16 species in this genus with an additional 3 species *incertae sedis* (Mannheim, 1984; Kilian & Biberstein, 1984). Since then *Haemophilus pleuropneumoniae* has been transferred to the genus *Actinobacillus* (Pohl *et al.*,1983) and *Haemophilus avium* to the genus *Pasteurella* (Mutters *et al.*, 1985) but they have still been included for discussion in this thesis.

Morphologically, haemophili are Gram-negative, nonmotile, non-spore-forming coccobacilli – although stained organisms can vary microscopically from small coccobacilli to long filaments. They are all aerobic or facultatively anaerobic and for *in vitro* growth one or both of two supplements are required. These are known as X factor and V factor. Table 2 illustrates how the requirement for X and V factors is used to differentiate between the species of the genus *Haemophilus.*

1.3.1 The requirement for X factor

In 1921 Thjötta and Avery introduced the terms X factor and V factor to refer, respectively, to the heat-stable substance needed only by the

Table 2. X and V factor requirements of *Haemophilus* species

Characteristic	*H. influenzae*	*H. aegyptius*	*H. haemolyticus*	*H. haemoglobinophilus*	*H. ducreyi*	*H. parainfluenzae*	*H. parahaemolyticus*	*H. paraphrohaemolyticus*	*A. pleuropneumoniae*	*H. paracuniculus*	*H. aphrophilus*	*H. paraphrophilus*	*H. segnis*	*H. parasuis*	*H. paragallinarum*	*P. avium*
X factor requirement *	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–
V factor requirement	+	+	+	–	–	+	+	+	+	+	–	+	+	+	+	+

* As determined by the porphyrin test.

influenzae bacillus and the heat-labile vitamin-like substance needed by both it and the influenzae-like bacillus later classified as *Haemophilus parainfluenzae*. Fildes established the identity of X factor as haemin in 1921 and today X factor is usually considered to be protoporphyrin IX but in some instances the iron-containing protoheme. Blood or blood derivatives including haemin are the traditional sources of X factor. The customary 5% of blood used in blood and chocolate agar is ample. When crystalline haemin is used, the required amounts have been shown to vary between 0.1 and 10 μg ml^{-1} for *Haemophilus influenzae* (Evans *et al.*, 1974) and 25 and 200μg ml^{-1} for *Haemophilus ducreyi* (Hammond *et al.*, 1978a). Lwoff & Lwoff (1937a) and White & Granick (1963) showed that haemin is used by *H. influenzae* in the biosynthesis of the

iron-containing respiratory enzymes, cytochrome, cytochrome oxidase, catalase and peroxidase. Although haemin is required for growth under aerobic conditions, growth is possible without haemin under anaerobic incubation (Gilder & Granick, 1947; Evans *et al.*, 1974). It is the enzymatic ability to incorporate iron in the protoporphyrin molecule that divides the species of the genus *Haemophilus* into those that do not require haemin (X factor) and those which do (Table 2). Because of the importance of this characteristic, a definitive test to determine whether an isolate can synthesize porphyrins from δ-aminolaevulinic acid has been devised (Biberstein *et al.*, 1963; Kilian, 1974). The haemin-independent isolates excrete porphobilinogen and porphyrins which fluoresce in the ultra violet light range.

1.3.2 The requirement for V factor

V factor is a heat - labile coenzyme or vitamin (hence the name V) and is usually described as β - nicotinamide adenine dinucleotide (NAD). Several studies have shown that nicotinamide mononucleotide (NMN), nicotinamide riboside (NR) and NAD phosphate (NADP) can also function as V factor for the growth of the various V factor-dependent *Haemophilus* species (Lwoff & Lwoff, 1937b; Gingrich & Schlenk, 1944). NAD biosynthesis in *Haemophilus* is described in more detail in sections 1.4.1 and 1.4.2

In the routine laboratory culture of members of the genus *Haemophilus*, V factor is either present in chocolate agar plates where the NAD has been liberated from blood cells by heating and subsequent lysis of the erythrocytes or it is added to the media such as brain heart infusion broth at a concentration of about 2.0 μg NAD ml^{-1} [*H. influenzae* requires 0.2 - 1.0 μg NAD ml^{-1} and *H. parainfluenzae* requires 1-5 μg NAD ml^{-1} (Evans *et al.*, 1974)]. V factor-dependent *Haemophilus* species do not grow on blood agar plates but in some laboratories media such as blood agar which is deficient in V factor are used and the *Haemophilus* colonies then cluster around a feeder strain such as *Staphylococcus aureus* which releases excess NAD (Niven & Le Blanc, 1992). This phenomenon is known as satellitism and was first described by Grassberger in 1897.

1.3.3 Should X and V factor dependency be the sole criteria for classification of species of the genus *Haemophilus* ?

According to the present definition of the genus the demonstrable need of X or V factor on the part of a Gram-negative rod or coccobacillus would qualify that organism as a member of the genus *Haemophilus.* Conversely, the absence of such needs would exclude a bacterium from the genus (Kilian & Biberstein, 1984). Although *Haemophilus aphrophilus* was originally described as a haemophilic organism that required X factor and increased CO_2 (Khairat, 1940), subsequent research has shown that this requirement for X factor may be lost on subculture and that *H. aphrophilus* will give a positive result

to the porphyrin test showing that it has the enzymes needed for the biosynthetic pathway for haemin synthesis (Kilian, 1976). The growth factor requirements for the other species in the genus appear to be stable but the validity of these growth factors as primary generic criteria is currently being questioned in the light of DNA homology studies.

Similarly, DNA homology studies in which 25 strains of *H. ducreyi* from diverse geographic origins were compared, showed that these strains formed a homogeneous DNA hybridization group but the type strain, CIP 542^T was shown to be only distantly related to 17 other *Haemophilus* species (Casin *et al.*, 1985). Similar results were obtained by De Ley *et al.* (1990) using DNA : rRNA hybridization techniques. Both Albritton (1989) and Morse (1989) question the inclusion of *H. ducreyi* in the genus *Haemophilus* but Albritton states that it shares many morphological, structural and metabolic features with members of this genus. Both authors agree that the taxonomy of *H. ducreyi* needs further study to ensure clarification.

Recent results of DNA homology studies of the family *Pasteurellaceae* have shown that species with common growth factor requirements do not form a homogeneous group suggestive of a common genus (Pohl, 1981) and it appears as if the taxonomic significance of V factor has been over-emphasised in the past (Mannheim, 1981). Subsequent discussion has challenged the use of V factor requirement as a genetic criterion and has resulted in the transfer of the species, *Haemophilus pleuropneumoniae* to the genus *Actinobacillus* as it was found to be

more related to *Actinobacillus lignieresii* than to *H. influenzae* (Pohl *et al.*, 1983). These authors also proposed the division of the species into two biotypes, biotype 1 being V factor-dependent and biotype 2 consisting of the V factor-independent strains. The latter strains were first described by Bertschinger & Seifert in 1978 and caused porcine necrotizing pleuropneumonia in Switzerland but were physiologically similar to *H. pleuropneumoniae.* There have also been proposals for the transfer of *Haemophilus avium* to the genus *Pasteurella* (Mutters *et al.*, 1985) and the splitting of this species into three *Pasteurella* species and for the transfer of *Actinobacillus actinomycetemcomitans* to the genus *Haemophilus* even though it is both X and V factor-independent (Potts *et al.*, 1985) - both based on the results of DNA-DNA hybridization studies.

Since 1989 strains of *Haemophilus paragallinarum* which are V factor-independent, but which are 89% DNA-DNA related to the type strain of *H. paragallinarum* which is V factor-dependent, have been isolated from chickens in South Africa (Mouahid *et al.*, 1991; Horner *et al.*, 1992). Similarly in 1989, Gromkova isolated a strain of V factor-independent *H. parainfluenzae* which, together with subsequent isolates from the same geographical area, exhibited all the biochemical characteristics of *H. parainfluenzae* biotype II. Genetic homology between these unusual strains and a reference strain of *H. parainfluenzae* was demonstrated by DNA transformation (Gromkova *et al.*, 1989a; Gromkova & Koornhof, 1990).

From the above-mentioned studies it appears that V factor dependency is no longer a primary generic feature of *Haemophilus* but it is rather a feature common among various members of the family *Pasteurellaceae.*

1.4 NICOTINAMIDE ADENINE DINUCLEOTIDE BIOSYNTHESIS AND PYRIDINE NUCLEOTIDE CYCLE METABOLISM

Nicotinamide adenine dinucleotide (NAD) is an essential co-enzyme in cellular oxidation-reduction reactions in both prokaryotic and eukaryotic cells. Most organisms are able to synthesize NAD *de novo* utilizing dihydroxyacetone phosphate and aspartate, formate and aspartate or tryptophan (Foster & Moat, 1980). Although the former pathway appears to be the most common one, in all these pathways quinolinic acid (QA) is a key intermediate and it is now recognized as a precursor involved in all known biosynthetic pathways to NAD. In 1958 this pathway was studied in human erythrocytes and yeast cells by Preiss and Handler. They isolated the intermediates and identified the enzymes involved in the conversion of nicotinic acid (NA) to NAD. This is now referred to as the Preiss-Handler pathway. In 1963 Andreoli *et al* working on *Escherichia coli* showed that QA not NA is the precursor in the *de novo* pathway to NAD. In these organisms dihydroxyacetone phosphate condenses with aspartic acid to form QA and this is converted to nicotinic acid mononucleotide (NAMN) in the presence of 5'-phosphoribosyl pyrophosphate (PRPP). The NAMN reacts with adenosine triphosphate (ATP) to form nicotinic acid adenine

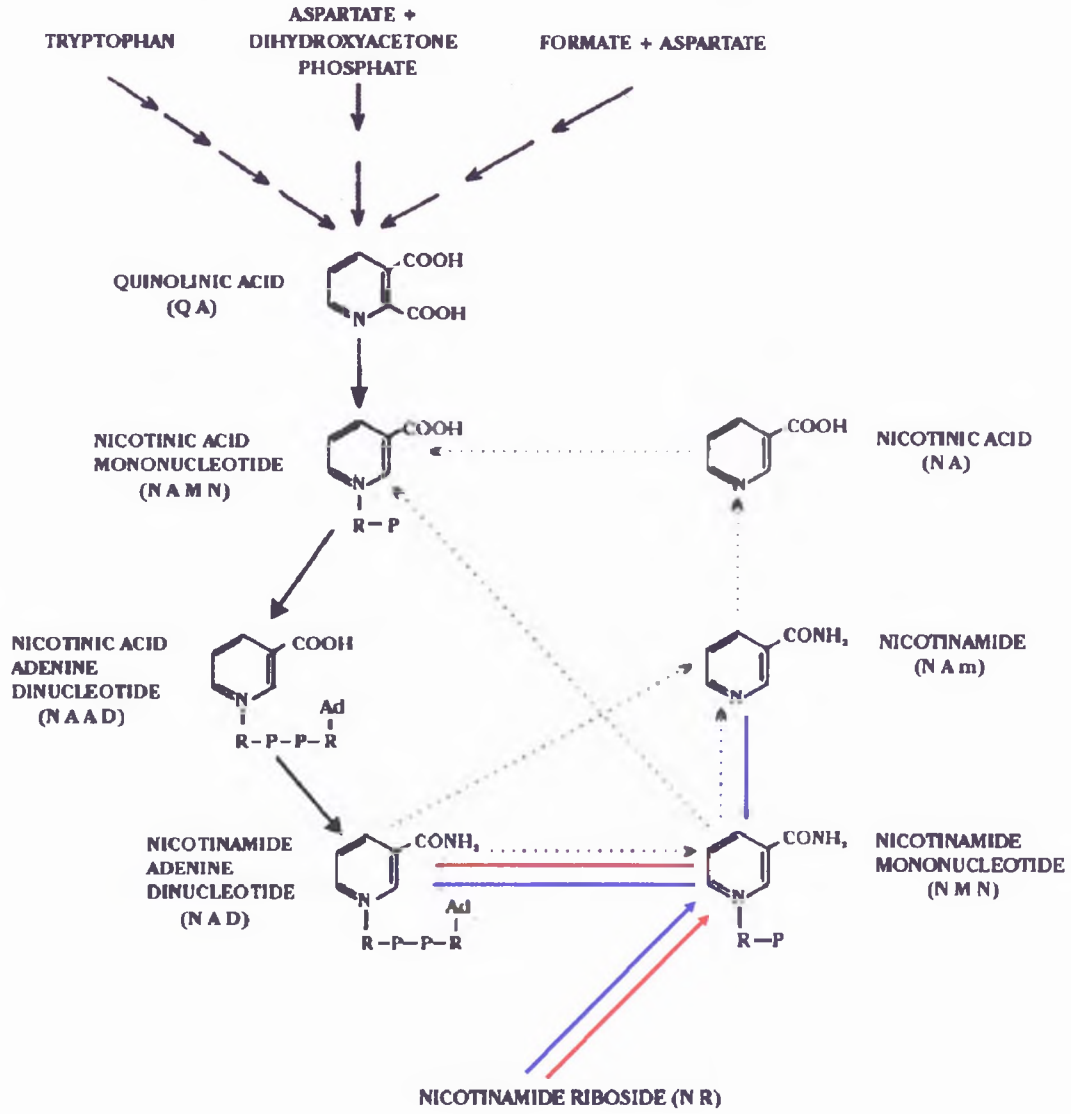


Figure 1. NAD metabolism in aerobic bacteria.

The solid black arrows indicate the Preiss-Handler pathway and the dotted pathways represent the pyridine nucleotide cycles. The blue and red arrows represent the proposed pathways for NAD biosynthesis in V factor-independent and V factor-dependent *Haemophilus* species, respectively.

Abbreviations : R, ribose ; P, phosphate ; Ad, adenine.

dinucleotide (NAAD) which forms NAD with the addition of an amino group from glutamine (Foster & Moat, 1980; Cynamon *et al.*,1988). This is illustrated in Figure 1. This *de novo* intracellular biosynthetic pathway of NAD does not exist in *Haemophilus* species that require exogenous NAD for growth.

In addition to the *de novo* pathways, several salvage pathways known as the pyridine nucleotide cycles have been described that allow some cells to utilize pre-formed pyridine bases such as NA, nicotinamide (NAm), or nicotinamide mononucleotide (NMN) when they are available (Hillyard *et al.*, 1981). These pyridine bases may be obtained from the breakdown of intracellular NAD or from exogenous sources.

1.4.1 Pyridine nucleotide metabolism in V factor-dependent *Haemophilus* species

In the case of *Haemophilus* several studies on V factor-dependent species have shown that NMN, nicotinamide riboside (NR) and NAD phosphate (NADP) can function as a growth factor in the place of β - NAD whereas NAm, NA, NAAD, NAMN, QA and α - NAD cannot support the growth of these organisms (Lwoff & Lwoff, 1937b; Schlenk & Gingrich, 1942; Gingrich & Schlenk, 1944; O'Reilly & Niven, 1986a; Cynamon *et al.*, 1988). This illustrates that these members of the genus *Haemophilus* are not able to synthesize NAD *de novo* from low molecular weight compounds nor do they use a pyridine nucleotide cycle as do some other bacteria but they can use

certain exogenous pyridine precursors available to them as a source of NAD. It can also be concluded that these organisms possess only a limited capacity for the uptake and metabolism of both pyridine nucleotides and precursors (Niven & O'Reilly, 1990). These authors also suggest that the ability of NADP to support growth of *Haemophilus* species should be re-examined in the light of their finding that commercial NADP may contain both NMN and NR. Only pyridine compounds that possess an intact pyridine-ribose bond in the β - configuration and a carboxamido group on the pyridine ring can serve as V factor (Kahn & Anderson, 1986). These authors also state that the inability of the highly polar NAD molecule to diffuse across various biological membranes suggests the possibility that the functioning of NAD as V factor for *H. influenzae* would require the hydrolysis of NAD to a smaller transportable fragment. Their studies have shown the presence of a nucleotide pyrophosphatase that catalyses the degradation of NAD to NMN and adenosine monophosphate (AMP). Similarly, O'Reilly and Niven (1986b) and Cynamon and his co-authors (1988) have found that NAD is also degraded by extra cytoplasmic enzymes in *Haemophilus parasuis* and *H. parainfluenzae*, respectively, to NMN or NR. Further work by these three groups of authors has shown that cell fractions from these V factor-dependent *Haemophilus* species can synthesize NAD from NMN or NR in the presence of ATP showing that NAD must be resynthesized inside the cell. There are only two enzymes involved in the synthetic pathway to NAD viz. NR kinase and NMN adenylyltransferase with the former enzyme catalyzing the synthesis of NMN from NR and the latter catalyzing the synthesis of

NAD from NMN. This is illustrated in Figure 1. It thus appears as if V factor dependency can be explained entirely on the basis of the limited capacities of these *Haemophilus* species for pyridine compound metabolism.

1.4.2 Pyridine nucleotide metabolism in V factor-independent *Haemophilus* species and related organisms

In 1973 Kasarov and Moat studied the cell extracts of *Haemophilus haemoglobinophilus*, a V factor-independent *Haemophilus* species to determine the pathway used by this organism to synthesize NAD. Although *H. haemoglobinophilus* could not utilize NA, QA or nicotinate adenine dinucleotide as a growth factor, the cell extracts could synthesize NAD from NAm with NMN as the only intermediate. This provided evidence that this organism cannot synthesize NAD via the *de novo* or the Preiss-Handler pathway but used the same pathway as that found in some mammalian systems and in one other bacterium, *Lactobacillus fructosus*. The authors speculated that this might be the pathway used by other V factor-independent *Haemophilus* and *Pasteurella* species.

There are several *Pasteurella* species including *Pasteurella multocida, Pasteurella haemolytica* and *Pasteurella ureae* which also require NAm for growth but which have been shown not to utilize NA or QA (Koser *et al.*, 1941; Berkman, 1942; Wessman 1965; 1966; 1970; 1972). These organisms thus appear to resemble *H. haemoglobinophilus*. Further

work by Berkman showed that *P. multocida* could grow equally well if NAD and NADP were substituted for NAm which is not known to be possible in the case of *H. haemoglobinophilus*.

The isolation of a V factor-independent *Haemophilus pleuropneumoniae* strain and its subsequent transfer to the genus *Actinobacillus* as *A. pleuropneumoniae* biotype 2 (Pohl *et al*, 1983) has given researchers a new organism to investigate. Niven and Lévesque (1988) grew this new biotype on a chemically defined medium as most commercial complex media contain NAm or NA. They used a modified version of the medium developed in 1970 by Herriott *et al*. for *H. influenzae* and various pyridine nucleotide precursors were added to examine their ability to support growth. NAD, NMN, NR and NAm could all serve as a growth factor, whereas QA, NA, NAMN and NAAD could not. This demonstrated that *A. pleuropneumoniae* biotype 2, like biotype 1, could not synthesize NAD *de novo* but it also showed that this organism although described as V factor-independent could exhibit V factor (NAD, NMN or NR)-dependent growth. The only difference between the two biotypes was that NAm could support the growth of biotype 2 whereas it could not be used by biotype 1 as a pyridine nucleotide precursor. This suggests that the genetic differences between the V factor-dependent and the V factor-independent strains may not be as significant as had been previously thought - but entails only the presence or absence of the one enzyme, NAm phosphoribosyltransferase. Niven and Lévesque proposed that NAm could be included in the group of compounds that

can serve as V factor and then both biotypes could be described as V factor-dependent.

Niven and O'Reilly (1990) in their review of V factor and the taxonomy of the genus *Haemophilus* and related organisms conclude that the family *Pasteurellaceae* may consist of V factor-dependent and V factor-independent members and that these definitions reflect only the ability of members of this family to use NAm or not. Indeed further research may show that the differences between V factor-dependent and V factor-independent strains are not sufficient to warrant continued use of the V factor terminology in its present form. They propose that if NAm is included with NAD, NMN, NR and NADP in the group of V factor compounds, an organism could be V factor-dependent if it can use one, but not necessarily all, of these compounds as growth factors. In that case it may be conceivable that all members of the family *Pasteurellaceae* would then be V factor-dependent and V factor would then have the potential to serve as a familial criterion of considerable taxonomic significance.

CHAPTER 2:

IDENTIFICATION OF THE GENES CONFERRING V FACTOR INDEPENDENCE IN *HAEMOPHILUS PARAINFLUENZAE*

2.1 INTRODUCTION

In 1989, Gromkova and her co-workers isolated a naturally occurring V factor-independent *H. parainfluenzae* strain in mixed culture with a strain of *H. ducreyi* from a genital ulcer (Gromkova *et al.*, 1989a). Three further clinical strains recovered from unrelated sources were isolated in our laboratory and Gromkova and Koornhof (1990) showed that, with the exception of V factor requirement, these four strains all exhibited the biochemical characteristics of *H. parainfluenzae* biotype II. These isolates were able to grow on blood agar as well as in brain heart infusion (BHI) broth and agar without the addition of NAD and produced satellitism when streaked on unsupplemented BHI agar inoculated with a typical V factor- requiring *H. parainfluenzae* strain.

The genetic relationship between these isolates and a standard strain of *H. parainfluenzae* was determined by testing transforming activities of two chromosomal markers, streptomycin resistance and nalidixic acid resistance. The clinical isolates were efficient donors and recipients in transformation. These authors also demonstrated the transfer of the genes conferring V factor independence to the typical V factor-requiring *H. parainfluenzae* 14 and to *H. influenzae* Rd.

Since V factor-independent strains of *H. parainfluenzae* had not been previously reported, further work was then carried out as part of the present study to identify the genes conferring V factor independence in

these unusual *H. parainfluenzae* strains and to investigate their stability as it was possible that the genes coding for this property were located extrachromosomally on a plasmid. An attempt was also made to assess the frequency of occurrence of V factor-independent *H. parainfluenzae* in patients from the same geographical area. As previous work had shown that the genes conferring this independence in *H. parainfluenzae* could be transferred to *H. influenzae* by DNA transformation (Gromkova and Koornhof, 1990), it was of interest to determine whether V factor-independent *H. influenzae* strains occurred in nature.

2.2 MATERIALS AND METHODS

2.2.1 Bacterial strains and media

A V factor-independent strain of *H. parainfluenzae*, designated 78, was recovered from a male patient with a genital ulcer who was attending a clinic for sexually transmitted diseases at Carletonville, near Johannesburg (Gromkova *et al.*, 1989a). Three strains of V factor-independent *H. parainfluenzae*, designated 9, 15 and 19, were isolated from paediatric patients at Baragwanath Hospital, Johannesburg (Gromkova & Koornhof, 1990). These were recovered from a sputum specimen, a purulent skin lesion and a throat swab, respectively.
H. influenzae Rd and *H. parainfluenzae* 14 (Boss) were provided by G. Leidy, Columbia University, New York.

Streptomycin-resistant strains, used as donors in DNA transformation, were obtained by selecting spontaneous mutants on antibiotic-containing plates.
Twenty-two *H. parainfluenzae* and five *H. influenzae* strains were isolated from healthy children at a crèche in Johannesburg.
Three *Escherichia coli* strains carrying plasmids pBR 322, pUC 18 and p7F12 were used as a source of plasmid DNA of known size.

The *Haemophilus* strains were grown in Brain Heart Infusion (BHI) broth and on BHI agar (Becton Dickinson). These media were

supplemented with 10 μg haemin ml^{-1} (Sigma) for the *H. influenzae* strains while the V factor-dependent *H. influenzae* and *H. parainfluenzae* strains had 2 μg NAD ml^{-1} (Sigma) added as well. The concentration of streptomycin used for the selection of mutants and transformants was 100 μg ml^{-1}. In some experiments chocolate agar was also used for growth of *Haemophilus* strains.

2.2.2 DNA preparation

Bacterial DNA was extracted by the method described by Marmur (1961). Plasmid DNA was prepared by an alkaline lysis method based on that described by Sambrook *et al.* (1989) but with the addition of a phenol-chloroform extraction step (Appendix A). DNA concentration was determined by measuring absorbance at 260nm.

2.2.3 DNA transformation

Competence was induced by the static aerobic procedure (Gromkova & Goodgal, 1979; Gromkova *et al.*, 1989b). For the transformation of *H. parainfluenzae* cells, 100 μl purified DNA at a saturating concentration of 1 μg ml^{-1} was added to 100μl competent cells and the mixture was incubated for 60 min at 37°C with gentle shaking. After the addition of 800 μl BHI broth supplemented with 10mM $MgSO_4$ and 2 μg NAD ml^{-1}, pancreatic DNase (Sigma) at a concentration of 10 μg ml^{-1} was added. For expression of the genes, the mixture was incubated at 37°C for 4 h and then diluted and plated in quintuplicate on BHI agar

unsupplemented with NAD for selection of transformants. Each transformation experiment was repeated three times and control cultures with no added DNA were tested each time to check for spontaneous mutants. For the transformation of *H. influenzae* cells, the same method was used but the time of uptake was extended from 60 min to 90 min and the BHI broth was supplemented with only 1mM $MgSO_4$ as well as NAD and haemin.

2.2.4 Plasmid Characterization

The purified plasmid DNA was separated by agarose gel electrophoresis in 0.8 % SeaKem GTG agarose (FMC Corporation) using Tris-EDTA-acetate (TEA) buffer in a Bio-Rad Mini-Sub DNA electrophoresis cell. After 90 min at 90 V the gels were stained with ethidium bromide (Sigma) at a concentration of 0.6 $\mu g\ ml^{-1}$. The size of the plasmid conferring V factor independence was determined by comparison with *E. coli* plasmids of known molecular size.

2.2.5 Selection of spontaneous V factor-dependent clones

A single colony from a BHI agar plate unsupplemented with NAD was subcultured into 10 ml BHI broth in the absence of NAD and was grown overnight by shaking at 37°C. The culture was diluted and plated on chocolate agar for single colonies. After overnight incubation at 37°C about 1000 colonies were examined for NAD requirement by

inoculating 1ml BHI broth unsupplemented with NAD and 1ml BHI broth supplemented with NAD.

2.2.6 Mitomycin C treatment and plasmid 'curing'

A single colony from a BHI agar plate unsupplemented with NAD was subcultured into 10 ml BHI broth in the absence of NAD and was grown overnight by shaking at 37°C. The culture was diluted 1 : 50 in BHI broth without NAD and grown at 37°C with shaking to an optical density at 650nm (OD_{650}) of 0.1. Mitomycin C (Sigma) was added at a concentration of 0.2μg ml^{-1} to *H. parainfluenzae* and at a concentration of 0.1μg ml^{-1} to *H. influenzae* and incubation was continued with OD_{650} readings at 1h or 2h intervals to determine if lysis could be induced.

When plasmid 'curing' was being examined, the culture was grown as described above and 5 hours after the addition of mitomycin C an aliquot was diluted, plated onto chocolate agar and incubated at 37°C. After 24h the size and number of colonies were noted and recorded. Approximately 1000 colonies were examined for their NAD requirement as described in section 2.2.5.

2.2.7 Plasmid 'curing' with ethidium bromide

A single colony from a BHI agar plate unsupplemented with NAD was subcultured into 10 ml BHI broth in the absence of NAD and

was grown overnight by shaking at 37°C. Tubes with 0.9 ml BHI broth containing ethidium bromide at a final concentration between 0.78 and 100$_{\mu}$g ml^{-1} were inoculated with 0.1 ml of the culture and incubated overnight at 37°C. Cultures that showed minimal inhibition of growth were diluted and plated onto chocolate agar for single colonies. About 1000 colonies were tested for NAD requirement as described in section 2.2.5.

2.2.8 Southern blotting and DNA hybridization

Total bacterial DNA was separated by gel electrophoresis (section 2.2.4) and transferred to a nylon membrane (Amersham Hybond N) by Southern blotting (Southern, 1975). Hybridization was carried out using a non-radioactive digoxigenin-labelled plasmid as a probe as described by the suppliers, Boehringer Mannheim (Appendix B). In all experiments the plasmid conferring V factor independence in *H. parainfluenzae* 9 was used to prepare the probe. This plasmid DNA was purified by caesium chloride gradient centrifugation and linearized by digestion with *Eco* R1 before labelling. Both restriction enzymes and molecular weight markers were supplied by Boehringer Mannheim.

2.2.9 Isolation of V factor-independent strains from clinical specimens

Specimens were obtained during a three month period between March and June, 1993 from patients with respiratory tract infections, meningitis and septicaemia at three hospitals in the Johannesburg area (Table 7). For primary isolation the specimens were plated on heated blood (chocolate) agar supplemented with 300μg ml^{-1} bacitracin. Bacteria from isolated colonies were Gram-stained and examined microscopically. Those which were Gram-negative were tested for growth on five types of media : (i) Brain heart infusion agar supplemented with 10 μg ml^{-1}haemin and 2 μg ml^{-1} NAD ; (ii) BHI agar with only 2 μg ml^{-1} NAD (iii) BHI agar with only 10 μg ml^{-1} haemin; (iv) unsupplemented BHI agar and (v) horse blood agar. The strains able to grow in the absence of V factor were further examined for their biochemical characteristics according to the methods described by Kilian (1976). The biochemical properties of these isolates were compared to those of reference strains of *H. parainfluenzae*, *H. influenzae* and *H. aphrophilus*.

2.3 RESULTS

2.3.1 Presence of plasmids in V factor-independent *H. parainfluenzae*

In order to study whether or not the genes conferring V factor independence were located extrachromosomally, DNA preparations from the four naturally occurring V factor-independent *H. parainfluenzae* isolates were examined for the presence of plasmids by agarose gel electrophoresis. Numerous extrachromosomal bands were present in these preparations as can be seen in Figure 2a, suggesting that each *H. parainfluenzae* strain contained several plasmids. To see whether the genes coding for V factor independence resided on one of these plasmids, the transforming activity of plasmid DNA preparations and total bacterial DNA were compared using V factor independence as a putative plasmid marker and streptomycin resistance (Str^r) as a known chromosomal marker. The results shown in Table 3 show that plasmid DNA was more effective than bacterial DNA in transforming *H. parainfluenzae* 14 competent cells to V factor independence whilst bacterial DNA was about 4000-fold more effective than plasmid DNA in transforming the same competent cells to Str^r. Transformation experiments were repeated using three different batches of competent cells and these yielded similar results, supporting the conclusion that the genes coding for V factor independence were probably plasmid-linked.

Table 3. Transformation of *H. parainfluenzae* 14 by bacterial and plasmid DNA from V factor - independent *H. parainfluenzae* *

DNA donor	Total bacterial DNA		Plasmid DNA	
	V factor indep.	Str ^{r}	V factor indep.	Str ^{r}
H. parainfluenzae 9 Str ^{r}	8.1×10^4	1.8×10^6	5.5×10^5	4.4×10^2
H. parainfluenzae 15 Str ^{r}	6.2×10^4	1.4×10^6	3.3×10^5	3.2×10^2
H. parainfluenzae 19 Str ^{r}	8.9×10^4	2.1×10^6	4.8×10^5	3.9×10^2
H. parainfluenzae 78 Str ^{r}	9.1×10^4	1.9×10^6	5.2×10^5	4.8×10^2

* Number of transformants per 5×10^8 colony forming units per ml

2.3.2 Presence of plasmids in V factor-independent transformants of *H. influenzae* Rd.

To provide further evidence that V factor independence was controlled by genes located on a plasmid, an attempt was made to transfer these genes to a plasmid-free recipient. However, the standard, highly transformable *H. parainfluenzae* 14 is known to carry several plasmids (Mann & Rao, 1979) and was therefore not a suitable recipient, nor were 22 local strains of *H. parainfluenzae* recovered from healthy children since none of them was plasmid-free (data not presented). Previous studies had shown that V factor independence could be transmitted by transformation to the standard, highly transformable

H. influenzae Rd strain (Gromkova & Koornhof, 1990), which is plasmid-free. Results from transformation experiments using plasmid DNA from the V factor-independent *H. parainfluenzae* isolates as donor DNA and *H. influenzae* Rd competent cells as recipients are given in Table 4. Five V factor-independent transformants from each donor DNA were examined for the presence of plasmids and all transformants were found to carry a single plasmid. Figure 2b shows agarose gel electrophoresis of one representative V factor-independent transformant from each group (lanes 2 to 5) with DNA from the recipient, the plasmid-free *H. influenzae* Rd strain, as a control (lane 1) and plasmid DNA of known molecular size from three different *E. coli* strains also shown (lanes 6 to 8). The single band present in lane 1 corresponds to the chromosomal DNA. The three extrachromosomal

Table 4. Transformation of *H. influenzae* Rd by plasmid DNA from V factor-independent *H. parainfluenzae* *

DNA donor	V factor indep.	Str ^{r}
H. parainfluenzae 9 Str ^{r}	1.8 x 10 ^{4}	8
H. parainfluenzae 15 Str ^{r}	2.1 x 10 ^{4}	9
H. parainfluenzae 19 Str ^{r}	9.8 x 10 ^{3}	7
H. parainfluenzae 78 Str ^{r}	1.9 x 10 ^{4}	9

* Number of transformants per 5 x 10 ^{8} colony forming units per ml

bands present in each of the V factor-independent *H. influenzae* Rd transformants (lanes 2 to 5) correspond to the three molecular forms of plasmid DNA : covalently closed circular, open circular and a dimer of closed circular DNA. The plasmids originally harboured by the four V factor-independent *H. parainfluenzae* isolates, which were recovered from unrelated sources, appear to be of identical size. By comparing the electrophoretic mobility of the three *E. coli* plasmids of known size and the mobility of the plasmids from the V factor-independent *H. influenzae* Rd transformants, these latter plasmids were estimated to be 5.25 kb in size (see section 5.3.2).

To exclude the possibility of accidental co-transfer of a small highly transformable plasmid with the genes coding for V factor independence, 10 transformants selected for a chromosomal genetic marker (Str^r) were tested for the presence of plasmids. None of them was found to carry a small plasmid. These results show that only transformants selected for V factor independence acquired the plasmid and indicate that the genetic element coding for V factor independence is associated with the plasmid.

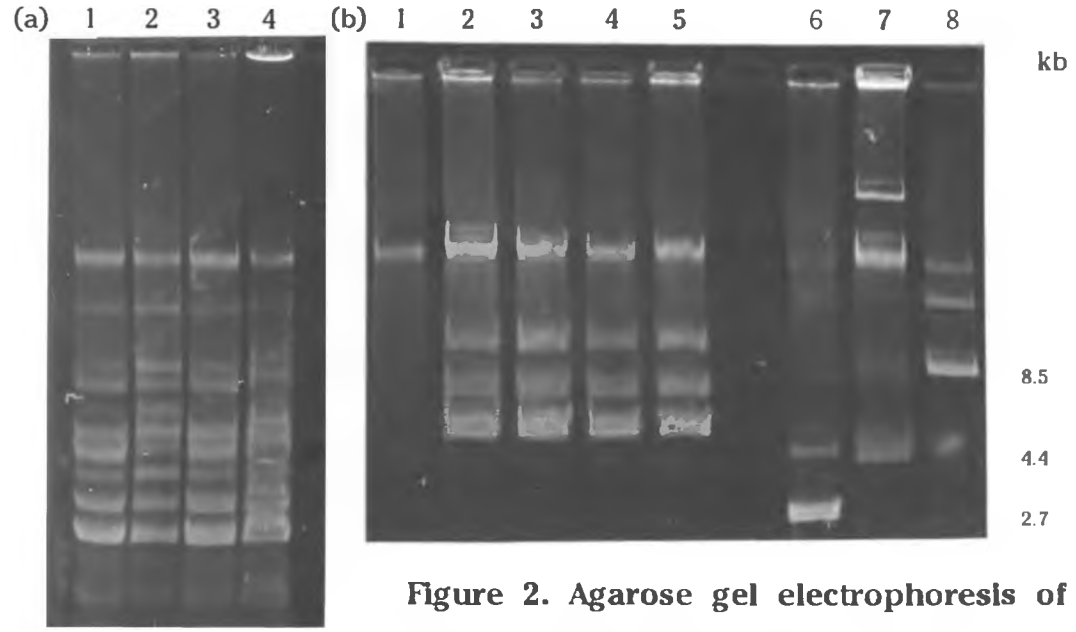
2.3.3 Stability of V factor independence in *H. parainfluenzae* and in *H. influenzae*

Since plasmid genes are known to be less stable than chromosomal genes, the four naturally occurring V factor-independent *H. parainfluenzae* isolates and the V factor-independent transformants

of *H. influenzae* Rd were examined for their ability to revert spontaneously to V factor dependence after overnight incubation at 37°C. The results presented in Table 5 show that spontaneous loss of V factor independence occurred with a low frequency in both *H. parainfluenzae* and *H. influenzae* hosts. The colony size of the V factor-dependent clones was smaller than that of the V factor-independent clones when plated on chocolate agar. Four V factor-requiring clones obtained from V factor-independent transformants of *H. influenzae* Rd were examined for the presence of plasmids by gel electrophoresis. The results shown in Figure 2c show that the loss of V factor independence corresponds with the loss of the small 5.25 kb plasmid.

Table 5. Spontaneous loss of V factor independence in *H. parainfluenzae* and in *H. influenzae* Rd transformants

Strain	% loss of V factor independence	Strain	% loss of V factor independence
H. parainfluenzae 9	0.12	*H. influenzae* 9	0.15
H. parainfluenzae 15	0.14	*H. influenzae* 15	0.21
H. parainfluenzae 19	0.16	*H. influenzae* 19	0.13
H. parainfluenzae 78	0.19	*H. influenzae* 78	0.22



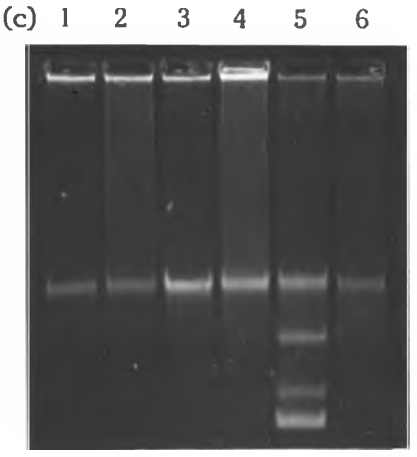


Figure 2. Agarose gel electrophoresis of plasmid DNA preparations from:

(a) V factor-independent *H. parainfluenzae* strains. Lanes: 1, isolate 9; 2, isolate 15; 3, isolate 19; 4, isolate 78.

(b) V factor-independent *H. influenzae* Rd transformants. Lanes: 1, plasmid-free *H. influenzae* Rd used as transformation recipient; 2 to 5, transformants of *H. influenzae* Rd using *H. parainfluenzae* isolates 9, 15, 19 and 78, respectively, as DNA donors; 6 to 8, plasmid markers pUC18, pBR322 and p7F12, respectively.

(c) V factor-dependent clones obtained from V factor-independent *H. influenzae* Rd transformants. Lanes: 1 to 4, spontaneous V factor-dependent clones of *H. influenzae* Rd transformants by *H. parainfluenzae* DNA from isolates 9, 15, 19 and 78, respectively; 5, V factor-independent *H. influenzae* Rd transformant (*H. parainfluenzae* 9 as DNA donor); 6, isogenic plasmid-free *H. influenzae* Rd used as transformation recipient.

2.3.4 Treatment of V factor-independent *H. parainfluenzae* and *H. influenzae* with mitomycin C

Each of the four naturally occurring V factor-independent *H. parainfluenzae* isolates exhibited spontaneous lysis after overnight incubation in BHI broth and the V factor-dependent clones did not show loss of this lytic activity. Assuming that the presence of a prophage may be responsible for this, an attempt was made to induce lysis by treatment with mitomycin C. This was not successful which suggests that these strains may carry non-inducible prophages. However this was not proved as no phage-sensitive indicator strain could be found which would produce phage plaques when 22 *H. parainfluenzae* and 5 *H. influenzae* strains from healthy children were tested.

Although lysis was not stimulated by mitomycin C there was an increase in the number of small sized colonies visible when determining the survival rate of the treated cultures. About 0.1 to 0.5 % of the bacteria survived using the mitomycin C concentrations of 0.2μg ml^{-1} for *H. parainfluenzae* and 0.1μg ml^{-1} for *H. influenzae*. The results in Table 6 show that the loss of V factor independence in both *H. parainfluenzae* and *H. influenzae* Rd was enhanced 4- to 40- fold after treatment with mitomycin C as compared to the spontaneous rate shown in Table 5. The 'curing' effect of mitomycin C was stronger in *H. parainfluenzae* than in *H. influenzae* and this was shown to be so even when higher (0.2μg

Table 6. 'Curing' of V factor independence in *H. parainfluenzae* and in *H. influenzae* Rd by mitomycin C *

Strain	% loss of V factor independence	Strain	% loss of V factor independence
H. parainfluenzae 9	3.17	*H. influenzae* 9	1.30
H. parainfluenzae 15	6.05	*H. influenzae* 15	1.00
H. parainfluenzae 19	4.42	*H. influenzae* 19	0.59
H. parainfluenzae 78	4.13	*H. influenzae* 78	0.82

* Mitomycin C concentration was 0. 2μg ml^{-1} for *H. parainfluenzae* and 0.1μg ml^{-1} for *H. influenzae* Rd. The survival rate of treated *H. parainfluenzae* and *H. influenzae* Rd strains was 0. 1 to 0. 5 %.

ml^{-1}) and lower (0.05μg ml^{-1}) concentrations of mitomycin C were used. Four V factor-requiring clones obtained after mitomycin C treatment were examined for the presence of plasmids and were found to be plasmid-free (data not presented).

The frequency of loss of V factor independence in *H. parainfluenzae* and in *H. influenzae* Rd was not enhanced by treatment with ethidium bromide which has been found to be an effective plasmid 'curing' agent in many bacterial systems (Bouanchaud *et al.*, 1969).

2.3.5 Identification of the plasmids conferring V factor independence in *H. parainfluenzae*

Several plasmids were visible in the four naturally occurring V factor-independent *H. parainfluenzae* isolates and thus hybridization with the plasmid from a V factor-independent transformant of *H. influenzae* Rd was used to provide direct evidence which of the plasmids in *H. parainfluenzae* carried the genes conferring V factor-independence. Figure 3a shows the electrophoretic mobility of total bacterial DNA from the four V factor-independent *H. parainfluenzae* isolates (lanes 1 to 4) and the corresponding V factor-dependent clones obtained after spontaneous loss of the plasmid conferring V factor independence (lanes 5 to 8). The presence of multiple extrachromosomal bands in both the wild type strains and the V factor-dependent clones indicates that each strain carries several plasmids and shows that the plasmid conferring V factor independence cannot be directly identified by gel electrophoresis. However, when DNA was transferred by Southern blotting and hybridized with the labelled probe (Figure 3b) only the plasmid that was homologous to the plasmid conferring V factor independence was visible in lanes 1 to 4. The three extrachromosomal bands present in each of the lanes correspond to the three molecular forms of plasmid DNA. The size of this plasmid in the four naturally occurring V factor-independent *H. parainfluenzae* strains was the same as that found in the V factor-independent *H. influenzae* Rd transformants viz. 5.25 kb (Figure 2b). This plasmid was not present in DNA from the V

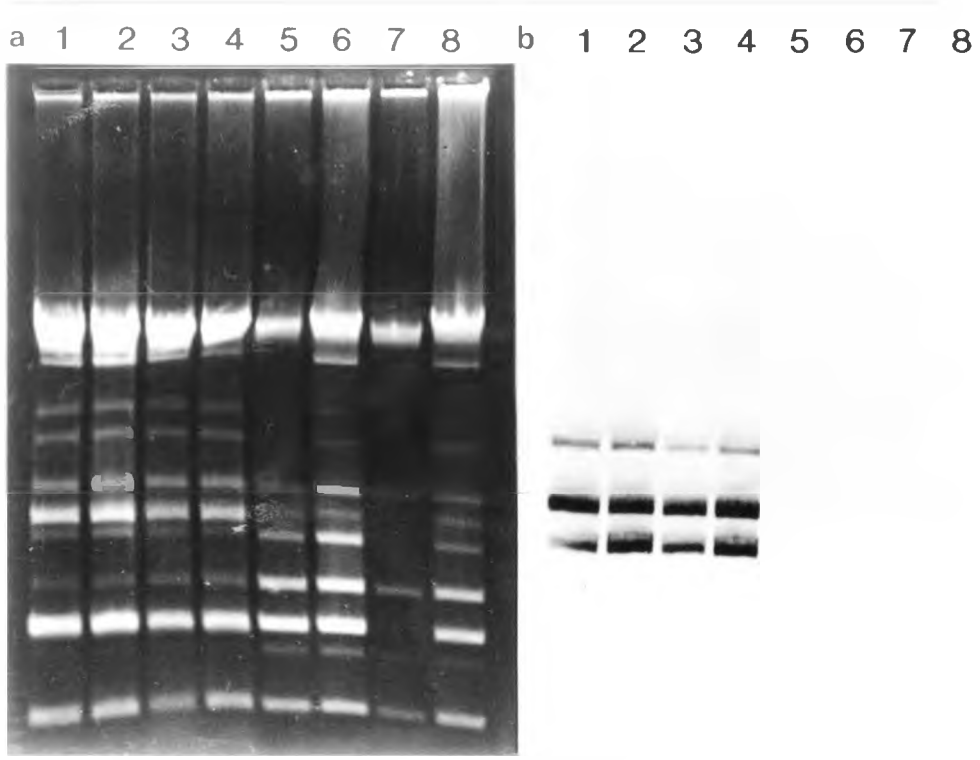


Figure 3: Total bacterial DNA preparations from V factor-independent *H. parainfluenzae* strains and from corresponding V factor-dependent clones

(a) Agarose gel electrophoresis of DNA from *H. parainfluenzae* isolates 9, 15, 19 & 78 (lanes 1 to 4) and from the clones of *H. parainfluenzae* isolates 9, 15, 19 & 78 that had spontaneously reverted to V factor dependence (lanes 5 to 8). (b) Southern hybridization of the gel shown in (a) with a digoxigenin - labelled probe from the plasmid conferring V factor independence in *H. parainfluenzae* 9.

factor-dependent clones (lanes 5 to 8) indicating that this plasmid carries the genes conferring V factor independence. Figure 3b also shows that the chromosomal DNA from both the V factor-independent *H. parainfluenzae* isolates and from the V factor-dependent clones was not able to hybridize with the probe.

2.3.6 Prevalence of V factor-independent *H. parainfluenzae* in Johannesburg

A total of 533 strains (281 *H. parainfluenzae* and 252 *H. influenzae*) were isolated from patients at three hospitals in the Johannesburg area. As shown in Table 7, the majority of the strains in this clinical survey were recovered from the upper respiratory tract. Two of the 281 *H. parainfluenzae* strains, designated 356 and 698, respectively, were found to be V factor-independent. Comparison of the growth and biochemical characteristics of these isolates with those of typical *H. parainfluenzae, H. influenzae* and *H. aphrophilus* strains are presented in Tables 8 and 9. *H. aphrophilus* was included for comparison as it is the only human *Haemophilus* species that does not require either V factor or X factor for growth. However, unlike 356 and 698, *H. aphrophilus* is urease, oxidase, ornithine decarboxylase and hydrogen sulphide negative and it requires CO_2 to enhance its growth. The only difference between the two unusual isolates and a typical *H. parainfluenzae* strain is the ability of the former to grow on blood agar and on BHI agar unsupplemented with V factor. The remaining biochemical properties

corresponded to a typical *H. parainfluenzae* biotype II strain. On blood agar isolates 356 and 698 produced green discolouration similar to that of α-streptococci and both were also shown to be β-lactamase positive.

The genetic relationship between the two unusual V factor-independent isolates and a typical *H. parainfluenzae* strain was determined by DNA transformation (section 2.2.3). The chromosomal streptomycin resistance marker from these isolates was transformed to *H. parainfluenzae* 14 with an efficiency similar to that of a homologous strain. Transformation of the same marker between *H. aphrophilus* NCTC 5906 and *H. parainfluenzae* 14 was not efficient. When DNA preparations of the two V factor-independent *H. parainfluenzae* strains were examined by agarose gel electrophoresis, each of the strains carried several plasmids similar to those seen in the earlier isolates (Figure 2a). Transformation of the genes conferring V factor independence to a plasmid-free *H. influenzae* Rd strain demonstrated that these genes were located on a plasmid. This plasmid was of the same size as that found in the four V factor-independent *H. parainfluenzae* strains isolated in 1989 (Figure 2b).

Table 7: Sources of *H. parainfluenzae* and *H. influenzae* isolates

SPECIES	Number of strains from SAIMR laboratory* at :			TOTAL†
	Baragwanath Hospital	Johannesburg Hospital	Hillbrow Hospital	
H. parainfluenzae	170	90	21	281
H. influenzae	157	71	24	252

* SAIMR laboratories are those of the South African Institute for Medical Research

† *H. parainfluenzae* strains were all respiratory tract isolates. *H. influenzae* strains were 92 % respiratory tract isolates, 4 % from CSF and 4 % from blood.

Table 8: Growth characteristics of V factor-independent isolates and typical *H. parainfluenzae, H. influenzae* and *H. aphrophilus* strains

Media	Unusual *H. parainfluenzae* strains		*H. parainfluenzae* 14*	*H. influenzae* Rd*	*H. aphrophilus* NCTC 5906*
	Isolate 356	Isolate 698			
BHI agar with X and V factors	+	+	+	+	+
BHI agar with V factor	+	+	+	–	+
BHI agar with X factor	+	+	–	–	+
Unsupplemented BHI agar	+	+	–	–	+
Horse blood agar	+	+	–	–	+

* *H. parainfluenzae* 14 and *H. influenzae* Rd are reference strains and *H. aphrophilus* NCTC 5906 is the type strain.

Table 9. Biochemical characteristics of the V factor-independent isolates and typical *H. parainfluenzae*, *H. influenzae* and *H. aphrophilus* strains

Characteristics	Isolates 356 & 698	*H. parainfluenzae* 14 *	*H. influenzae* Rd *	*H. aphrophilus* NCTC 5906 *
V factor requirement	–	+	+	–
Porphyrin	+	+	–	+
Indole	–	–	–	–
Ornithine decarboxylase	+	+	+	–
Hydrogen suphide	+	+	–	–
Urease	+	+	+	–
Oxidase	+	+	+	–
Alkaline phosphatase	+	+	+	+
Nitrate reduction	+	+	+	+
Glucose; acid	+	+	+	+
Lactose; acid	–	–	–	+
Sucrose; acid	+	+	–	+
Ribose; acid	–	–	+	+
CO_2 enhances growth	–	–	–	+

* *H. parainfluenzae* 14 and *H. influenzae* Rd are reference strains and *H. aphrophilus* NCTC 5906 is the type strain.

2.4 DISCUSSION

The isolation from natural sources of four unusual *H. parainfluenzae* strains that were able to grow in the absence of V factor raised the question of the genetic basis of this property. Gromkova & Koomhof (1990) suggested that the difference in the transformation efficiencies of the marker for V factor independence and those of two chromosomal genetic markers might be due to the fact that the genes coding for V factor independence were extrachromosomal.

Further comparisons in this study of the transformation frequencies of V factor independence and a known chromosomal marker, streptomycin resistance (Str^r), with total bacterial DNA and plasmid DNA preparations from the unusual *H. parainfluenzae* strains used as donors and the two highly transformable reference strains, *H. parainfluenzae* 14 and *H. influenzae* Rd as recipients, provided further evidence in support of the plasmid hypothesis. The transformation to V factor independence of *H. parainfluenzae* 14 cells by total bacterial DNA was about 20-fold less efficient than transformation by the chromosomal Str^r marker but marker efficiencies of two chromosomal markers in *H. parainfluenzae* and *H. influenzae* do not normally vary by more than a factor of two in intraspecific transformation (Goodgal & Gromkova, 1973). However, previous studies have demonstrated that a plasmid conferring ampicillin resistance in *H. parainfluenzae* was about 100-fold less efficient in transformation than the chromosomal Str^r marker when CsCl gradient-purified plasmid RSF0885 DNA was

used (Gromkova & Goodgal, 1979). Also the plasmid DNA preparations were more efficient than total bacterial DNA in transforming *H. parainfluenzae* 14 cells to V factor independence while the plasmid DNA preparations transformed the chromosomal Str^r marker far less efficiently than bacterial DNA. (Table 3). The residual transforming activity to Str^r in the plasmid preparations was probably due to incomplete removal of chromosomal DNA (Tables 3 & 4). It should be borne in mind that the *H. parainfluenzae* plasmid preparations used in the transformation experiments each contained several plasmids, as visible in Figure 2a, and if purified plasmid DNA containing only one plasmid species had been used, higher transforming efficiencies to V factor independence would have been expected because of the lack of competition from other plasmid DNA molecules for DNA binding sites.

Evidence that the genes conferring V factor independence were plasmid-linked was provided by the finding that after transforming the plasmid-free *H. influenzae* Rd to V factor independence, all transformants acquired a small 5.25 kb plasmid as shown in Figure 2b. Further proof that these genes were extrachromosomal was provided by the Southern blot and DNA hybridization of total bacterial DNA from the original *H. parainfluenzae* isolates with a labelled plasmid conferring V factor independence (Figure 3b). The lack of homology between the probe and the chromosomal DNA from all *H. parainfluenzae* strains indicates that the genes conferring V factor independence are not located on the chromosome.

There have been no previous reports of the extrachromosomal location of genes involved in NAD biosynthesis. The only genes that have so far been found to be located on plasmids in *Haemophilus* species are those conferring drug resistance and conjugal transfer. In most bacterial systems, genes coding for metabolic functions that are essential for the survival of the cells do not reside on plasmids but on the chromosome. However, this does not apply to *H. parainfluenzae*, as wild-type *H. parainfluenzae* strains are usually not able to synthesize V factor. It appears unlikely that the genes located on this small plasmid code for *de novo* synthesis of NAD because in other bacteria the *de novo* synthesis of NAD involves at least six enzymatic steps (Figure 1).

Although the size of this plasmid was similar to that of plasmid RSF0885 conferring ampicillin resistance (de Graaff *et al.*, 1976), the plasmid coding for V factor independence transformed cells with a higher frequency than the former plasmid (Gromkova & Goodgal, 1979). This was probably due to the more efficient expression of the marker for V factor independence than that of the β-lactamase marker. Because of its high transformability and the presence of a biological marker that can be easily expressed and selected, the plasmid conferring V factor independence has the potential to become a good cloning vector in *Haemophilus* species.

The relative instability of V factor independence in both *H. parainfluenzae* and *H. influenzae*, which is due to the loss of the 5.25

kb plasmid (Figures 2c & 3b), is characteristic of plasmid DNA. It is also of interest that the loss of the plasmid occurred both spontaneously as well as due to the chemical action of mitomycin C. Mitomycin C is an antibiotic that has bactericidal and mutagenic effects on bacteria (Iijima & Hagiwara, 1960) due to structural changes it makes in the bacterial DNA (Waring, 1968). It has the ability to selectively block the synthesis of host DNA but to permit viral DNA synthesis and so it has been used extensively as an effective prophage inducer (Otsuji *et al.*, 1959). It is also able to induce the production of some colicins (Hardy & Meynell, 1972). Although it has been widely used in *Pseudomonas* species for 'curing' catabolic plasmids (Chakrabarty, 1972; Williams & Worsey, 1976) there are no previous reports on the use of mitomycin C as a plasmid 'curing' agent in the genus *Haemophilus*.This 'curing' is probably due to selective inhibition of plasmid DNA synthesis without inactivation of the host DNA. In *H. influenzae* the plasmid 'curing' by mitomycin C appears to be less efficient than in *H. parainfluenzae* (Table 6). This was probably due to the stronger bactericidal effect of mitomycin C on *H. influenzae*.

The frequency of occurrence of V factor-independent *H. parainfluenzae* was found to be low (0.7 %) and none of the 252 *H. influenzae* strains examined in the clinical survey were found to be V factor-independent. However, the two V factor-independent strains that were isolated in 1993 were recovered from the respiratory tracts of adult male patients from Baragwanath Hospital and three of the four V factor-independent strains of *H. parainfluenzae* isolated

in 1989 were also recovered at this hospital but these were from paediatric patients. The similarity in size of the plasmids conferring V factor independence, despite the fact that they originated from six *H. parainfluenzae* strains isolated from unrelated sources, suggests that they may have a common origin. It is possible that the plasmids have been acquired by the different strains independently by genetic exchange with an organism able to synthesize V factor. However since these isolates were all recovered in or near Johannesburg the clonal distribution of a single unusual V factor-independent *H. parainfluenzae* strain cannot be excluded. In this regard it is interesting that these isolates all belong to the same *H. parainfluenzae* biotype as well as all being β-lactamase positive (Gromkova & Koomhof, 1990) and that each strain carries several plasmids with similar electrophoretic mobility (Figure 2a). From an ecological perspective, these strains may have a survival advantage as a result of their ability to grow in the absence of V factor. As both *H. parainfluenzae* and *H. influenzae* share a common ecological niche in the human respiratory tract, it is possible that genetic exchange of this plasmid may occur in nature. It has been demonstrated that some wild-type strains of *H. influenzae* are transformable (Rowji *et al.*,1989) and, because of the greater clinical significance of *H. influenzae*, it is important that clinical microbiologists are aware of the possibility of recovering V factor-independent strains.

CHAPTER 3 :

STUDY OF THE GENES CONFERRING V FACTOR INDEPENDENCE IN *HAEMOPHILUS DUCREYI*

3.1 INTRODUCTION

The isolation of naturally occurring V factor-independent *H. parainfluenzae* strains and the location of the genes conferring V factor independence on a 5.25 kb plasmid in these strains (Chapter 2; Windsor *et al.*, 1991), raised the question of the origin of this small, highly transformable plasmid. As one of the *H. parainfluenzae* isolates was recovered from a genital ulcer in a mixed culture with a typical *H. ducreyi* strain (Gromkova *et al.*, 1989a), it was hypothesized that *H. ducreyi* could have been the source of this plasmid. *H. ducreyi* is the only human *Haemophilus* species that requires X factor for growth but does not require V factor (Lwoff & Pirosky, 1937). Although the growth factor requirements in *H. ducreyi* have been used as major taxonomic and diagnostic criteria, no information is available on the location and the characterization of the genes coding for the biosynthesis of these growth factors due to the lack of suitable methods for genetic analysis in *H. ducreyi.*

The present study was undertaken to investigate whether the same plasmid was present in various *H. ducreyi* strains from different geographical locations and to determine whether this plasmid, if present, could be transferred to *H. parainfluenzae* and *H. influenzae* by DNA transformation.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial strains and media

The strains used in this study are listed in Table 10. The *H. ducreyi* strains were grown on the enriched agar medium described by Nsanze *et al.* (1984) in 5 % CO_2 at 35°C for 24 - 48 h except for *H. ducreyi* CIP 542^T that was grown on chocolate agar. This was necessary as *H. ducreyi* CIP 542^T is sensitive to vancomycin which is present in the former agar medium. The *H. parainfluenzae* 14 and *H. influenzae* Rd strains were grown on supplemented BHI agar and broth as described in section 2.2.1. The V factor-independent *H. parainfluenzae* and *H. influenzae* transformants were always grown in the absence of V factor.

3.2.2 DNA Preparation and plasmid characterization

Total bacterial DNA was extracted by the method described by Marmur (1961) and plasmid DNA was prepared by the methods of Sambrook *et al.*(1989), Birnboim and Doly (1979), Meyers *et al.* (1976), Hansen and Olsen (1978) and Abeck *et al.* (1988). DNA concentration was determined by measuring absorbance at 260nm. Both total and plasmid DNA preparations were separated by agarose gel electrophoresis as described in section 2.2.4.

Table 10. *H. ducreyi* **strains used**

Strain *	Country of origin	Source *
H. ducreyi CIP 542 Type strain	Vietnam	ATCC
H. ducreyi ATCC 27722	--	ATCC
H. ducreyi 124	South Africa	SAIMR
H. ducreyi Y78 †	South Africa	SAIMR
H. ducreyi C147	Kenya	L. Slaney, Manitoba

* ATCC: American Type Culture Collection, Rockville, Maryland, USA; CIP: Collection de l'Institut Pasteur, Paris, France; SAIMR: South African Institute for Medical Research, Johannesburg

† Isolated in mixed culture with *H. parainfluenzae* 78 from a genital ulcer (section 2.2.1)

3.2.3 DNA transformation

Competence induction and transformation was carried out as described in section 2.2.3 using the two recipient strains, *H. parainfluenzae* 14 and *H. influenzae* Rd. Ampicillin-resistant transformants were selected after the addition of ampicillin (Sigma) to the BHI agar to give a final concentration of 10μg ml^{-1}.

3.2.4 Selection of spontaneous V factor-dependent clones

The technique used for the selection of V factor-dependent clones is described in section 2.2.5.

3.2.5 DNA restriction enzyme digestion and hybridization.

Restriction enzyme digestions using *Hpa* I and *Pst* I were carried out as described by Sambrook *et al.* (1989) using 3 units of enzyme per 18μl of DNA sample and digesting overnight at 37°C. Both restriction enzymes and molecular weight markers were supplied by Boehringer Mannheim. Digested and undigested plasmid and total bacterial DNA were separated by gel electrophoresis and transferred to a nylon membrane (Amersham Hybond N) by Southern blotting. Hybridization was carried out using, as a probe, the plasmid conferring V factor independence in *H. parainfluenzae* 9 which had been linearized and digoxigenin-labelled as described by the suppliers, Boehringer Mannheim (Appendix B).

3.3 RESULTS

3.3.1 Analysis of *H. ducreyi* for the presence of plasmids

As previous study had shown that the genes conferring V factor independence in four unusual clinical isolates of *H. parainfluenzae* were located on a 5.25 kb plasmid, attempts were made to investigate whether these genes were also located extrachromosomally in *H. ducreyi*. DNA from two reference strains (CIP 542^T and ATCC 27722) and from three clinical isolates of *H. ducreyi* (Table 10) was extracted by the method of Sambrook *et al.*(1989) and examined for the presence of plasmids by agarose gel electrophoresis (Figure 4a). No extrachromosomal bands were visible in strains CIP 542^T, ATCC 27722 or C147 but a 8.5 kb plasmid was present in the DNA preparations from the two clinical isolates, 124 and Y78 (lanes 3 & 4). Antibiotic susceptibility tests have shown that these two clinical strains are resistant to ampicillin while the other three strains are ampicillin-sensitive. Previous research on 38 *H. ducreyi* strains recovered from patients at the same clinic as isolates 124 and Y78, showed that all 38 strains were β-lactamase positive and 25 of these carried a 5.7 Mdal (8.5 kb) plasmid (Abeck *et al.*, 1988).

Various plasmid extraction procedures, namely those of Bimboim and Doly (1979), Meyers *et al.* (1976), Hansen and Olsen (1978) and Abeck *et al.* (1988) were also tried to ensure that the "plasmid-free" strains did not, in fact, carry plasmids. No plasmids were detected in the strains CIP 542, ATCC 27722 or C147 using any of the above procedures.

3.3.2 Transformation of V factor independence from *H. ducreyi* to *H. parainfluenzae* and *H. influenzae*

Two highly transformable V factor-dependent *Haemophilus* strains - *H. parainfluenzae* 14 and *H. influenzae* Rd were examined for their ability to be transformed to V factor independence by total bacterial DNA from five *H. ducreyi* strains. The results presented in Table 11 show that total bacterial DNA from all five *H. ducreyi* strains was efficient in transforming *H. parainfluenzae* 14 competent cells to V factor independence and DNA from four *H. ducreyi* strains was active in transforming *H. influenzae* Rd to V factor independence. In general *H. parainfluenzae* 14 was a more efficient recipient than *H. influenzae* Rd. This was probably due to the presence of an exclusion mechanism in *H. influenzae* that inhibits plasmid transformation (Gromkova & Goodgal, 1981). The transformation experiments were repeated several times using different batches of competent cells and different DNA preparations. Similar efficiencies of transformation were obtained when different batches of competent cells were used, however, the efficiency varied more significantly using different batches of DNA. There were no transformants in the absence of donor DNA indicating that spontaneous mutations to V factor independence did not occur in either *H. parainfluenzae* 14 or *H. influenzae* Rd competent cells.

Although it was not possible to transform *H. influenzae* Rd directly to V factor independence with *H. ducreyi* CIP 542^{T} DNA, it was possible

Table 11. Transformation of *H. parainfluenzae* 14 and *H. influenzae* Rd to V factor independence by total bacterial DNA from *H. ducreyi* *

DNA donor	Recipient strain	
	H. parainfluenzae 14	*H. influenzae* Rd
H. ducreyi CIP 542 ^{T}	30	0 †
H. ducreyi ATCC 27722	2.5×10^{4}	17
H. ducreyi 124	8.8×10^{3}	15
H. ducreyi Y78	2.1×10^{4}	32
H. ducreyi C147	5.6×10^{4}	84

* Number of transformants per 5×10^{8} colony forming units per ml

† Transformants of *H. influenzae* Rd were obtained indirectly using DNA from *H. parainfluenzae* 14 transformants

to introduce these genes into the *H. influenzae* Rd strain using DNA prepared from a V factor-independent transformant of *H. parainfluenzae* 14 obtained when this strain was transformed with *H. ducreyi* CIP 542^{T} DNA.

Since two of the clinical strains of *H. ducreyi* showed the presence of the 8.5 kb plasmid conferring ampicillin resistance (Figure 4a), it was of interest to investigate whether the genes conferring V factor independence and the gene coding for β-lactamase production were linked. Fifty V factor-independent transformants of *H. influenzae* Rd were tested for β-lactamase production and the same

number of transformants selected for β-lactamase production were examined for V factor independence. None of the transformants showed co-transformation of the unselected marker suggesting that these genes were not linked.

3.3.3 Presence of plasmids in V factor-independent transformants of *H. influenzae* Rd

H. influenzae Rd is a plasmid-free strain and thus it was suitable for testing whether the genes conferring V factor independence were plasmid-linked. Five V factor-independent transformants of *H. influenzae* Rd obtained with DNA from each *H. ducreyi* donor strain were examined for the presence of plasmids and all transformants were found to carry a single plasmid. Figure 4b shows agarose gel electrophoresis of one representative V factor-independent transformant obtained with DNA from each *H. ducreyi* strain (lanes 2 to 6). DNA from the plasmid-free *H. influenzae* Rd recipient strain is shown in lane 1 and in lane 7 a V factor-independent transformant of *H. influenzae* Rd using *H. parainfluenzae* 15 as a source of donor DNA, is presented. This is one of the transformants from the four naturally occurring V factor-independent *H. parainfluenzae* strains (Figure 2b). The single band present in lane 1 corresponds to the chromosomal DNA. The three extrachromosomal bands present in each of the V factor-independent *H. influenzae* Rd transformants (lanes 2 to 7) correspond to the following molecular forms of plasmid DNA : covalently closed circular, open circular and a

dimer of closed circular DNA. By comparing the electrophoretic mobility of the plasmids from the V factor- independent *H. influenzae* Rd transformants using both *H. ducreyi* and *H. parainfluenzae* as sources of donor DNA, these plasmids all appear to be of the same size. The plasmid acquired from *H. parainfluenzae* has previously been shown to be 5.25 kb in size (section 2.3.2 ; Windsor *et al.*, 1991).

The plasmid recovered from *H. influenzae* Rd transformants selected for β-lactamase production was of identical size (8.5kb) to the plasmid recovered in the original ampicillin-resistant isolates of *H. ducreyi* (Figure 4a). This finding, as well as the lack of linkage between the genes conferring V factor independence and β-lactamase production and the recovery of the 5.25kb plasmid from V factor-independent transformants with donor DNA from both ampicillin-sensitive and ampicillin-resistant strains, indicated that the plasmids conferring V factor independence and β-lactamase production are independent.

Spontaneous loss of V factor independence in *H. influenzae* Rd transformants using *H. ducreyi* donor DNA occurred with a low frequency (1-2 colonies per 1000 tested per strain) and similar low levels of spontaneous loss of V factor independence occurred in *H. influenzae* Rd transformants with *H. parainfluenzae* donor DNA (Table 5). None of the 50 V factor-dependent clones tested showed the presence of the 5.25 kb plasmid as was the case with the V factor-dependent clones of *H. influenzae* Rd transformants using *H. parainfluenzae* donor DNA (Figure 2c).

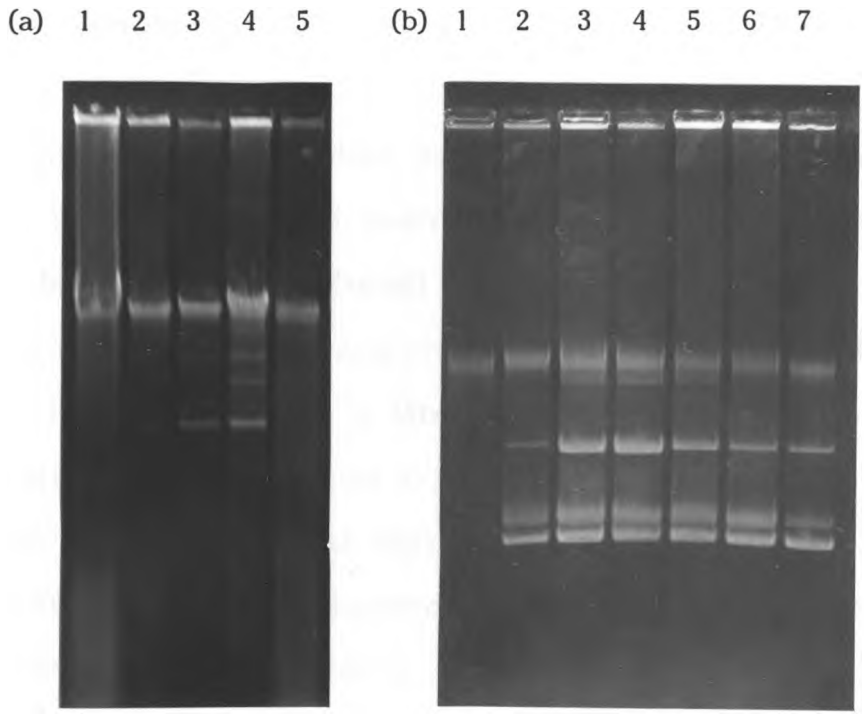


Figure 4. Agarose gel electrophoresis of plasmid DNA preparations from:

(a) *H. ducreyi* strains. Lane 1, Type strain CIP 542 ^{T}; lane 2, reference strain ATCC 27722; lanes 3 to 5, clinical isolates 124, Y78 & C147, respectively.

(b) V factor-independent *H. influenzae* Rd transformants. Lane 1, plasmid-free *H. influenzae* Rd strain used as transformation recipient ; lanes 2 to 6, transformants of *H. influenzae* Rd using *H. ducreyi* strains CIP 542 ^{T}, ATCC 27722, 124, Y78 & C147, respectively, as DNA donors; lane 7, transformant of *H. influenzae* Rd using V factor-independent *H. parainfluenzae* 15 as DNA donor.

3.3.4 Detection of the plasmid conferring V factor independence in *H. ducreyi*

Although V factor-independent transformants from the five *H.ducreyi* strains all carried the 5.25 kb plasmid conferring V factor independence, the inability to detect this plasmid in the DNA preparations of the five strains of *H. ducreyi* using agarose gel electrophoresis needed further study. Hybridization with a labelled plasmid conferring V factor independence was carried out to investigate whether this plasmid was present in either an autonomous state or integrated on the chromosome using both digested and undigested total bacterial DNA from the five *H. ducreyi* strains.

The results in Figure 5 show that undigested bacterial DNA from four *H. ducreyi* strains, ATCC 27722, 124, Y78 and C147 reveals the presence of two bands that hybridize with the probe (lanes 2 to 5). The band corresponding to the larger DNA moiety represents the position of the chromosomal DNA, while the other band is located extrachromosomally. The size of this piece of DNA is the same as that of the undigested covalently closed circular plasmid conferring V factor independence from *H. parainfluenzae* 9 (lane 20). No extrachromosomal band that hybridized with the probe was detected in *H. ducreyi* CIP 542^T (lane 1). Similar results were obtained with several duplicate DNA preparations. In addition, *H. ducreyi* CIP 542^T, unlike the other *H. ducreyi* strains, showed only weak hybridization reaction at

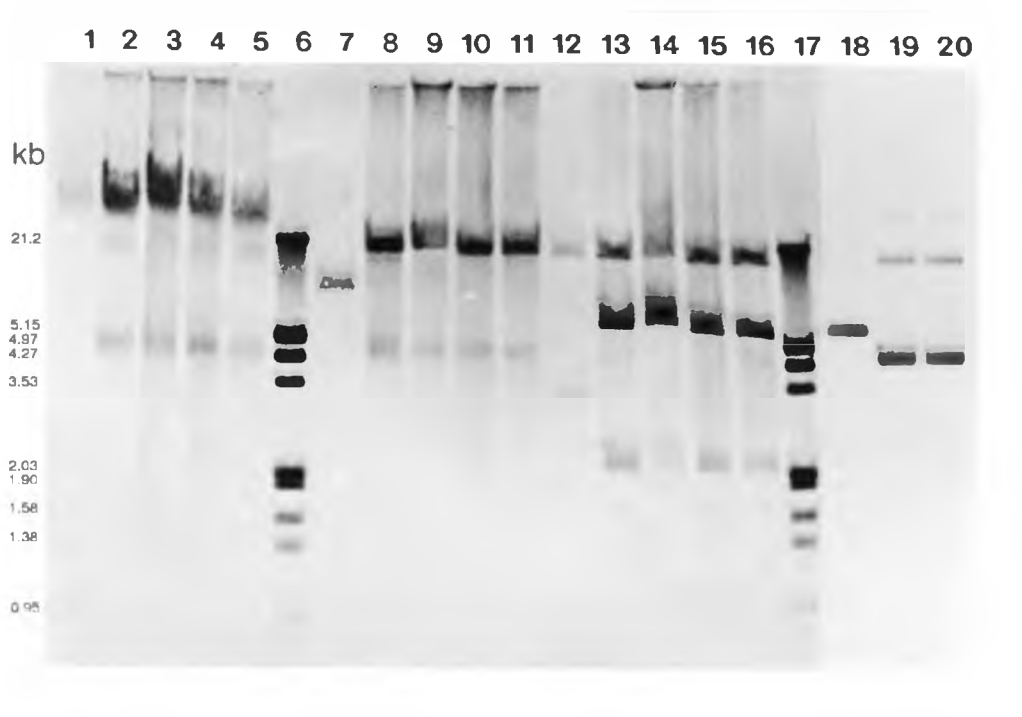


Figure 5. Southern blot and DNA hybridization of total DNA preparations from *H. ducreyi* strains and from purified plasmid preparations of V factor-independent *H. parainfluenzae* 9 with a probe from the plasmid conferring V factor independence in *H. parainfluenzae* 9.

Lanes 1 to 5, undigested DNA from *H. ducreyi* CIP 542 ^{T}, ATCC 27722, 124, Y78 & C147, respectively ; lanes 7 to 11, DNA shown in lanes 1 to 5 after digestion with *Hpa* I ; lanes 12 to 16, DNA shown in lanes 1 to 5 after digestion with *Pst* I ; lanes 6 & 17, λ \ *Eco* RI & *Hin*d III molecular weight standard ; lane 18, plasmid DNA from *H. parainfluenzae* 9 after digestion with *Pst* I ; lane 19, plasmid DNA shown in lane 18 treated with *Hpa* I ; lane 20, undigested plasmid DNA shown in lane 18.

the position of the chromosomal DNA despite the fact that the same concentration of DNA was used for all strains.

When total bacterial DNA from *H. ducreyi* strains was digested with *Hpa* I, a restriction enzyme that does not cleave the plasmid conferring V factor independence (lane 19) but was active against chromosomal DNA, the position of the extrachromosomal band that hybridized with the probe remained the same as in the undigested DNA (lanes 8 to 11). However, the size of the chromosomal fragment that hybridized with the probe was smaller than in the case of undigested DNA. In all four *H. ducreyi* strins, ATCC 27722, 124, Y78 and C147 (lanes 8 to 11) this fragment was of similar size (18kb) while in *H. ducreyi* CIP 542^T (lane 7) the fragment that hybridized with the probe was smaller (12 kb). The restriction enzyme, *Pst* I that cut the plasmid conferring V factor independence once (lane 18) was able to linearize the plasmids present extrachromosomally in the four *H. ducreyi* strains, ATCC 27722, 124, Y78 and C147 (lanes 13 to 16) to give one linear piece of DNA of 5.3 kb in size. This enzyme also produced two chromosomal segments of 17 kb and 2.2 kb that hybridized with the probe. However, *H. ducreyi* CIP 542^T produced only two bands after digestion with *Pst* I (lane 12) which are 17 kb and 3.4 kb in size. These results suggest that in *H. ducreyi* the genes conferring V factor independence are present integrated into the chromosome as well as in the form of an autonomous plasmid in four of the five strains tested.

3.4 DISCUSSION

The finding that V factor-independent transformants of plasmid-free *H. influenzae* Rd with *H. ducreyi* DNA acquired a small 5.25kb plasmid and that the clones that have spontaneously lost V factor independence show the absence of this plasmid, suggests that the genes coding for V factor independence in *H. ducreyi* are, like those in *H. parainfluenzae,* plasmid-linked. However, this plasmid was not detectable by agarose gel electrophoresis in the original *H. ducreyi* strains (Figure 4a) and it is unlikely that this was due to the lack of suitable methods for plasmid DNA extraction because plasmids coding for drug resistance were easily detectable (Figure 4a, lanes 3 & 4). A possible reason for the inability to recover the plasmid conferring V factor independence in *H. ducreyi* is the presence of this plasmid in a low copy number or the integration of the plasmid into the chromosome. Hybridization with a labelled plasmid conferring the same phenotypic function in *H. parainfluenzae* was necessary to detect the presence of an autonomous plasmid coding for V factor independence in four of the five *H. ducreyi* strains. Although extrachromosomal plasmid DNA was not detected in *H. ducreyi* CIP 542^{T} after hybridization, V factor-independent transformants carrying detectable plasmids were recovered when this strain was used as a DNA donor (section 3.3.2). However the frequency of transformation was much lower than that with DNA from the other *H. ducreyi* strains tested.

The detection of hybridization between the undigested chromosomal DNA from all five *H. ducreyi* strains and the labelled plasmid suggests that the plasmid is probably also integrated into the chromosome. This is in contrast with the finding that the labelled plasmid did not hybridize with the chromosomal DNA from the V factor-independent *H. parainfluenzae* isolates (Figure 3b) nor with the chromosomal DNA from *H. parainfluenzae* 14, *H. influenzae* Rd or *E. coli* pUC 18 when DNA from each of these strains was used as a negative control (data not presented). Usually plasmids that carry transposons or sequences homologous to chromosomal sequences have the potential to be integrated into the chromosome (Setlow *et al.*, 1984; Balganesh & Setlow, 1985; Kauc & Goodgal,1989a). The lack of sequencing data of the plasmid coding for V factor independence does not allow for speculation about the mechanism of plasmid integration in *H. ducreyi*. However, treatment of total bacterial DNA with *Hpa* I and with *Pst* I has allowed the identification of the chromosomal fragments adjacent to the plasmid and provides evidence, based on the size of the fragments, which suggests that the plasmid is integrated into the chromosome of *H. ducreyi*. This probably occurs at a unique site as the fragments of four of the five strains are of the same size. In all four *H. ducreyi* strains, ATCC 27722, 124, Y78 and C147 digestion with *Hpa* I produces one piece of chromosomal DNA, 18kb in size, which hybridizes with the probe and thus contains the integrated plasmid. Furthermore *Pst* I, an enzyme that linearizes the purified plasmid, yields two bands, 17 kb and 3.4 kb in size, of digested chromosomal DNA that hybridize with the probe. Each of the above

mentioned pieces have a segment of the integrated plasmid after digestion. In *H. ducreyi* CIP 542^T the fragments that hybridize with the probe are smaller (*Hpa* I - 12kb ; *Pst* I - 17 kb & 2.2 kb) and the difference in the size of these fragments can be explained by variation in the size of the flanking DNA. This strain was first isolated in Hanoi, Vietnam in 1954 and it would be of interest to test other Asian isolates for their ability to hybridize with the labelled probe. This method could be used for the identification of clones that are similar to the reference *H. ducreyi* CIP 542^T strain or to the more recent clinical isolates of *H. ducreyi*. *H. ducreyi* CIP 542^T is one of the few *H. ducreyi* strains that have been reported to be nonpathogenic when inoculated intradermally in rabbits (Hammond *et al.*, 1978c) and to produce no cytopathic effects in tissue culture (Alfa, 1992).

The transformation results show that transfer of the genes coding for V factor independence to a plasmid-free recipient by DNA transformation is a reliable method for demonstrating that these genes are plasmid-linked. Similar results have been reported with large multiresistance plasmids in *H. influenzae* and *H. parainfluenzae* (Bendler, 1976; Stuy, 1979; Stuy, 1980; Roberts & Smith, 1980; Murphey-Corb *et al.*, 1984). These authors found that "plasmid-free" strains were efficient donors in conjugation experiments and yielded transconjugants that all carried drug resistance plasmids detectable by agarose gel electrophoresis. However in the original strains the plasmids were not found extrachromosomally as some of these plasmids were integrated into the chromosome.

No data on extrachromosomal genes conferring metabolic functions in *H. ducreyi* are available. The only plasmid genes that have been previously described are those conferring drug resistance (Brunton *et al.*, 1979; Totten *et al.*, 1982; Albritton *et al.*,1982) or conjugal transfer (Deneer *et al.*, 1982). There are also no published reports on the chromosomal integration of small plasmids in *H. ducreyi*. However a large 30 Mdal plasmid conferring tetracycline resistance has been found to be present in either the integrated or autonomous state (Albritton *et al.*,1984; Johnson *et al.*, 1989). There are also no data on the genes coding for metabolic functions in *H. ducreyi* having been transferred to other *Haemophilus* species by DNA transformation.

These results show that the plasmids conferring V factor independence originating from two reference and three clinical strains of *H. ducreyi* isolated at different times and in different geographical areas (Table 10) are of similar size (Figure 4b) and share a degree of homolgy with the plasmid from *H. parainfluenzae* when it was used as a probe in DNA hybridization experiments (Figure 5). This is of interest as *H. ducreyi* is a species that is classified as V factor-independent and *H. parainfluenzae* is known taxonomically as a V factor-dependent species. However, some strains, after acquiring the plasmid have had their phenotype converted to V factor independence (Windsor *et al.*, 1991). Exchange of plasmids between related or unrelated bacterial species is well documented. *H. influenzae*, *H. parainfluenzae* and *Neisseria gonorrhoeae* have all been found to harbour genetically

related plasmids conferring drug resistance (Anderson *et al.*, 1984; Brunton *et al.*, 1986; Roberts 1989). Because of their small size and transformability one may speculate that the mechanism of transfer of the plasmids conferring V factor independence in nature is probably DNA transformation. However, the original source of these plasmids is unknown.

CHAPTER 4:

ATTEMPTS TO LOCATE THE GENES CONFERRING V FACTOR INDEPENDENCE IN OTHER *HAEMOPHILUS* SPECIES

4.1 INTRODUCTION

Three of the sixteen *Haemophilus* species listed in the latest edition of Bergey's Manual are classified as V factor-independent (Kilian & Biberstein, 1984). These are the human species, *H. ducreyi* and *H. aphrophilus* and the canine species, *H. haemoglobinophilus*. However, during the past few years there have been reports of the isolation of V factor-independent strains of some of the V factor-dependent *Haemophilus* species. Initially, in 1978 Bertschinger described an organism causing porcine necrotizing pleuropneumonia in Switzerland (Pohl *et al.*, 1983) which resembled *H. pleuropneumoniae* in many physiological properties except that the former organism was V factor-independent. These organisms have now been transferred to the genus *Actinobacillus* and are known as *A. pleuropneumoniae* biotypes 1 and 2, the former being V factor-dependent and the latter being V factor-independent (Pohl *et al.*, 1983). Similarly, since 1989 chickens in South Africa have been found to be suffering from a respiratory disease called infectious coryza which is caused by strains of *H. paragallinarum* closely related to the type strain except that these South African isolates are V factor-independent (Mouahid *et al.*, 1992; Horner *et al.*,1992) and as mentioned in Chapter 2, Gromkova and her co-workers (1989a; 1990) have isolated and subsequently characterized four V factor-independent clinical isolates of *H. parainfluenzae* which has stimulated the research presented in this thesis.

Since the genes coding for V factor independence in these *H. parainfluenzae* strains and in *H. ducreyi* were found to be plasmid-linked (chapters 2 & 3), it was of interest to examine V factor-independent strains from other *Haemophilus* species for the presence of similar plasmids. In this chapter data are presented on the examination of nine V factor-independent strains from four species, *H. aphrophilus*, *H. haemoglobinophilus*, *A. pleuropneumoniae* and *H. paragallinarum*. Attempts were made to detect plasmids in these strains and to study whether the plasmids, if present, shared some homologous regions with the plasmid conferrring V factor independence in *H. parainfluenzae* and *H. ducreyi*.

4.2 MATERIALS AND METHODS

4.2.1 Bacterial strains and media

The strains used and their sources are listed in Table 12.

Table 12. *Haemophilus* **and** *Actinobacillus* **strains used**

Strain*	Source*	V factor phenotype
H. aphrophilus NCTC 5906^T	NCTC	V factor-independent
H. haemoglobinophilus NCTC 1659^T	NCTC	V factor-independent
A. pleuropneumoniae ATCC 27088^T	ATCC	V factor-dependent
A. pleuropneumoniae P500	R. Nielsen, Copenhagen	V factor-independent
A. pleuropneumoniae P600	R. Nielsen, Copenhagen	V factor-independent
H. paragallinarum 0083	R. Horner, Natal, RSA	V factor-dependent
H. paragallinarum 05586	R. Horner,Natal, RSA	V factor-independent
H. paragallinarum 08823	R. Horner,Natal, RSA	V factor-independent
H. paragallinarum 01554	R. Horner,Natal, RSA	V factor-independent
H. paragallinarum 08074	R. Horner,Natal, RSA	V factor-independent
H. paragallinarum 09472	R. Horner,Natal, RSA	V factor-independent

* NCTC National Collection of Type Cultures, London; ATCC American Type Culture Collection, Maryland.

H. aphrophilus was grown on chocolate agar at 37°C in 5% CO_2 for 48 h or in 20 ml unsupplemented BHI broth in a sealed McConkey bottle without shaking at 37°C for 48 h. *H. haemoglobinophilus* was grown on chocolate agar and on BHI agar and broth supplemented with 10μg haemin ml^{-1} at 37°C for 24 h. The V factor-independent strains of *A. pleuropneumoniae* and *H. paragallinarum* were grown on unsupplemented BHI agar and broth at 37°C for 48 h but the V factor-dependent strains of these two species were grown in the same media supplemented with 2μg NAD ml^{-1} and incubated for 48 h. The *H. paragallinarum* isolates were grown in the presence 5% CO_2 as this enhances the growth of these organisms. V factor-dependent strains were included in this study as they were used as controls in the DNA hybridization experiments.

4.2.2 DNA preparation and transformation

Total bacterial and plasmid DNA was extracted as described in Appendix A. 500ml BHI broth cultures with 100μg ml^{-1} chloramphenicol added 8 hours after inoculation were also used to try to amplify plasmid DNA. All these DNA preparations were separated by agarose gel electrophoresis as in section 2.2.4. Competence induction and DNA transformation was carried out as described in section 2.2.3 using *H. parainfluenzae* 14 and *H. influenzae* Rd as recipient cells and the V factor-independent strains listed in table 12 as the DNA donors.

4.2.3 DNA restriction enzyme digestion and hybridization

Digestion was carried out as described by Sambrook *et al.* (1989). Total bacterial DNA and digested plasmid DNA was transferred to a nylon membrane by Southern blotting and DNA hybridization was carried out as described in Appendix B.

4.3 RESULTS

4.3.1 Presence of plasmids in V factor-independent strains from other *Haemophilus* species

Both plasmid and total bacterial DNA preparations of each of the strains were examined for the presence of plasmids by agarose gel electrophoresis. The only strains in which extrachromosomal DNA was detected were the V factor-independent *H. paragallinarum* strains. Figure 6a shows the electrophoretic mobility of DNA preparations from these five *H. paragallinarum* strains as well as that from the wild type strain, *H. paragallinarum* 0083 (lane 1). All five V factor-independent *H. paragallinarum* strains tested (lanes 2 to 6) carried one plasmid which was slightly larger than the 5.25 kb plasmid found in *H. parainfluenzae* and *H. ducreyi* (lane 7). It was estimated that this *H. paragallinarum* plasmid is 6.3 kb in size. None of the other DNA preparations of the strains examined, including that of the wild type, V factor-dependent *H. paragallinarum* 0083, showed the presence of plasmids in either untreated or chloramphenicol-treated cells. However some of the DNA preparations of *H. ducreyi* also appeared to be plasmid-free after agarose gel electrophoresis (section 3.3.1 & Figure 4a) but DNA hybridization revealed that the plasmids conferring V factor independence were present in these strains both extrachromosomally (Figure 5) in what is assumed to be a low copy number, as well as integrated into the chromosome (section 3.3.4).

4.3.2 Transformation of *H. parainfluenzae* 14 and *H. influenzae* Rd to V factor independence

Transformation of the genes coding for V factor independence from *H. parainfluenzae* and *H. ducreyi* to the recipient strains, *H. parainfluenzae* 14 and *H. influenzae* Rd had previously shown that these genes were plasmid-linked, even when no extrachromosomal DNA was visible after gel electrophoresis of the DNA from the *H. ducreyi* strains (sections 3.3.2 & 3.3.3). Similar transformation experiments were carried out using donor DNA from all nine V factor-independent strains listed in Table 12. *H. parainfluenzae* 14 competent cells were transformed to V factor independence by donor DNA from both *H. aphrophilus* and *A. pleuropneumoniae* P500. However the frequency of transformation was very low (beteween 8 and 20 per 5 x 10^8 cfu per ml^{-1}). No V factor-independent transformants of *H. parainfluenzae* 14 were obtained using DNA from *H. haemoglobinophilus, A. pleuropneumoniae* P600 or any of the *H. paragallinarum* strains. The transformation experiments were repeated at least five times using different batches of competent cells and different total bacterial and plasmid DNA preparations. Similar low efficiencies of transformation were obtained on different occasions with DNA from both strains that did produce transformants.

No V factor-independent transformants of *H. influenzae* Rd were obtained using DNA from any of the nine donor strains listed in Table 12, in several transformation experiments. As had been the case

with *H. ducreyi* CIP 542 ^{T} (section 3.3.2), it was only possible to introduce these genes into *H. influenzae* Rd indirectly using DNA prepared from the V factor-independent *H. parainfluenzae* 14 transformants obtained when this latter strain was transformed with DNA from *H. aphrophilus* and *A. pleuropneumoniae* P500.

Although the five V factor-independent *H. paragallinarum* strains carry visible plasmids, it was not possible to transfer these plasmids to *H. parainfluenzae* 14 or to *H. influenzae* Rd by DNA transformation. Attempts to transform these plasmids to the wild-type, plasmid-free strain, *H. paragallinarum* 0083, were also made but these were not successful due to problems experienced with competence induction in this strain.

4.3.3 Comparison of the plasmids from different DNA donors in V factor-independent transformants of *H. influenzae* Rd

Five of each of the V factor-independent transformants of *H. influenzae* Rd obtained with donor DNA from *H. aphrophilus* and *A. pleuro-pneumoniae* P500 were tested for the presence of plasmids and all these transformants were found to carry a single plasmid. By comparing the electrophoretic mobility of the plasmids in these transformants and those in transformants to V factor independence using donor DNA from *H. parainfluenzae* and *H. ducreyi*, it was found that the plasmids in all the V factor-independent transformants are of the same size irrespective of the original

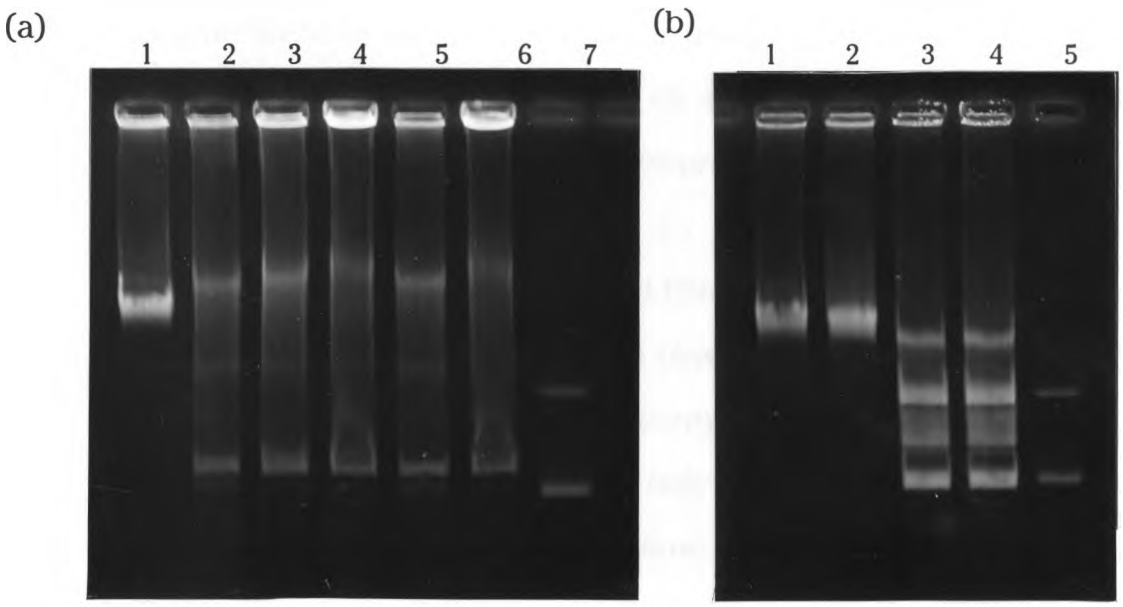


Figure 6. Agarose gel electrophoresis of total and plasmid DNA preparations from:

(a) *H. paragallinarum* **strains**. Lane 1, wild type strain 0083; lanes 2 to 6 clinical V factor-independent isolates 05586, 08823, 01554, 08074 and 09472, respectively; lane 7 transformant of *H. influenzae* Rd using *H. parainfluenzae* 15 as DNA donor showing the plasmid conferring V factor independence.

(b) V factor-independent *H. influenzae* **Rd transformants and the DNA donor strains.** Lanes 1 & 2 , total bacterial DNA from *H. aphrophilus* and *A. pleuropneumoniae* P500, respectively; lanes 3 & 4, transformants of *H. influenzae* Rd using plasmid DNA originating from *H. aphrophilus* and *A. pleuropneumoniae* P500; lane 5 transformant of *H. influenzae* Rd using *H. ducreyi* ATCC 27722 as DNA donor showing the plasmid conferring V factor independence.

source of the DNA. The plasmid conferringV factor independence in *H. parainfluenzae* and *H. ducreyi* has been shown to be 5.25 kb in size (sections 2.3.2 & 3.3.3). Figure 6b shows that although the DNA donor strains, *H. aphrophilus* and *A. pleuropneumoniae* P500, appear to be plasmid-free (lanes 1 & 2), the V factor-independent transformants obtained with plasmid DNA originating from these two organisms (lanes 3 & 4) carry a single plasmid of the same size as that found in the transformant with *H. ducreyi* ATCC 27722 DNA (lane 5) used for comparison in this gel. Not only were these plasmids of the same size, they also displayed the same restriction enzyme digestion pattern with various endonucleases including *Hae* II, *Hae* III, *Dpn* I and *Rsa* I (data not presented).

4.3.4 DNA hybridization of V factor-independent strains from other *Haemophilus* species

Hybridization with the digoxigenin-labelled plasmid conferring V factor independence in *H. parainfluenzae* 9 was carried out using plasmid DNA from the V factor-independent transformants of *H. parainfluenzae* 14 and *H. influenzae* Rd obtained with plasmid DNA originating from *H. aphrophilus* and *A. pleuropneumoniae* P500. Homology between the probe and plasmid DNA from the transformants was detected.

Hybridization was also carried out using both digested and undigested total bacterial DNA from the strains listed in Table 12 to determine whether a similar plasmid could be detected in either an autonomous

state or integrated into the chromosome. Despite the fact that the V factor-independent transformants of *H. parainfluenzae* 14 with DNA from *H. aphrophilus* and *A. pleuropneumoniae* P500 acquired a plasmid that appears to share homology with regions of the labelled plasmid from *H. parainfluenzae* 9, no homology between the same probe and the undigested and digested DNA from these strains was detected. Negative results were also obtained in plasmid DNA preparations from both untreated and chloramphenicol-treated cells. These results were confirmed using different hybridization conditions as well as by increasing the concentrations of the labelled plasmid in the probe. At high concentrations (>10µl probe ml^{-1} in the hybridization solution) there was a degree of homolgy visible in the chromosomal DNA but this was also present in the negative control of the DNA from V factor-dependent *A. pleuropneumoniae* ATCC 27088 ^{T} showing that non-specific binding had taken place.

There was also no hybridization between the probe and the total bacterial DNA or the plasmid DNA preparations from *H. haemoglobinophilus, A. pleuropneumoniae* P600 and *H. paragallinarum*. The plasmid present in the V factor-independent isolates of *H. paragallinarum* does not show homology with the plasmid conferring V factor independence in *H. parainfluenzae* and *H. ducreyi*. Only when high concentrations of the probe was used did some non-specific binding occur in all strains (including strain 0083).

4.4 DISCUSSION

In this preliminary study into the presence of plasmids in several V factor-independent strains it was found that only the V factor-independent *H. paragallinarum* isolates had detectable extrachromosomal DNA after gel electrophoresis of total bacterial and plasmid DNA preparations. However it was not possible to demonstrate whether the genes conferring V factor independence are located on this plasmid due to the lack of a suitable recipient for transformation. In addition, the inability to detect homolgy between the labelled plasmid from *H. parainfluenzae* 9 and the plasmids visible in all five V factor-independent *H. paragallinarum* isolates shows that either these plasmids confer V factor independence using genes unrelated to those on the plasmid found in the human isolates of *H. parainfluenzae* and *H. ducreyi* or these former plasmids may code for some other unknown phenotype.

The remaining strains appeared to be plasmid-free but transformation of the plasmids conferring V factor independence was demonstrated with two strains (*H. aphrophilus* and *A. pleuropneumoniae* P500) although with a low frequency. This could be due to the fact that the relatedness between the donor DNA species and *H. parainfluenzae* and *H. influenzae* is too distant, that the plasmid conferring this phenotype is present in a low copy number or that the plasmid was destroyed by the action of endonucleases after uptake. However, comparison of the size of the plasmids visible in all the V

factor-independent transformants examined, showed that the plasmids were of the same size as those originating in *H. parainfluenzae* and *H. ducreyi* and that they shared a similar restriction enzyme digestion pattern with the plasmids from the other V factor-independent transformants. Also the transformants of both *H. parainfluenzae* 14 and *H. influenzae* Rd obtained using donor DNA from *H. aphrophilus* and *A. pleuropneumoniae* P500 hybridized with the probe. These preliminary results suggest that the plasmids conferring V factor independence in *H. ducreyi*, *H. aphrophilus* and *A. pleuropneumoniae* biotype 2 are similar to those in the V factor-independent *H. parainfluenzae* isolates.

However, DNA hybridization with the labelled plasmid conferring V factor independence in *H. parainfluenzae* 9 failed to detect the presence of this plasmid exrachromosomally or integrated into the chromosome in either *H. aphrophilus* or *A. pleuropneumoniae* P500. Similarly after digestion with various restriction enzymes there were no fragments of chromosomal DNA which hybridized with the probe. This could be because the plasmid conferring V factor independence in these species is present in such low copy number that it is not detectable or because the plasmid DNA had been destroyed by endonucleases during lysis and DNA extraction.

Neither *H. haemoglobinophilus* nor *A. pleuropneumoniae* P600 had any visible plasmids nor were they efficient DNA donors in transforming the genes for V factor independence to either *H.*

parainfluenzae 14 or *H. influenzae* Rd. In addition these strains did not hybridize with the probe from *H. parainfluenzae* 9. The inability to obtain transformants with DNA from *H. haemoglobinophilus* was probably due to the low level of DNA homology between this species and *H. parainfluenzae* and *H. influenzae* (Pohl, 1981; de Ley *et al.*, 1990).

This brief study shows that some strains of *H. parainfluenzae*, *H. ducreyi*, *H. aphrophilus* and *A. pleuropneumoniae* harbour a plasmid conferring V factor independence which may have a common origin, but its source remains unknown. This plasmid was not, however, detected in *H. haemoglobinophilus*.

CHAPTER 5:

CHARACTERIZATION OF A PLASMID CONFERRING V FACTOR INDEPENDENCE IN *HAEMOPHILUS PARAINFLUENZAE* AND *HAEMOPHILUS DUCREYI*

5.1 INTRODUCTION

It has been demonstrated in chapters 2 and 3 that four unusual *H. parainfluenzae* isolates and five *H. ducreyi* strains all carried a small plasmid coding for V factor independence. These plasmids were of identical size (5.25 kb) and shared regions of genetic homology as labelled plasmid DNA from one of these strains (*H. parainfluenzae* 9) hybridized with the plasmids from the other V factor-independent *H. parainfluenzae* strains as well as with extrachromosomal DNA from four of the *H. ducreyi* strains (Figures 3 & 5).

In order to study the similarities between these plasmids from *H. parainfluenzae* and *H. ducreyi*, plasmid DNA was digested using different restriction enzymes and then DNA hybridization was carried out. The cleavage sites of nine restriction enzymes were mapped and their location in the plasmids coding for V factor independence in both *Haemophilus* species were compared.

5.2 MATERIALS AND METHODS

5.2.1 Bacterial strains and media

The strains used were the same as those listed in sections 2.2.1 and 3.2.1. The V factor-independent *H. influenzae* Rd transformants were grown in BHI broth supplemented with 10 μg haemin ml^{-1} (Sigma) without the addition of NAD.

5.2.2 Plasmid DNA extraction and purification

Since each of the original V factor-independent *H. parainfluenzae* isolates carried several plasmids and the plasmid conferring V factor independence was not visible in *H. ducreyi*, the transformants of plasmid-free *H. influenzae* Rd that had acquired the plasmid conferring V factor independence from the four *H. parainfluenzae* and five *H. ducreyi* strains were used as a source of plasmid DNA. The extraction procedure was based on the alkaline lysis method described by Sambrook *et al.* (1989) and the plasmid DNA was further purified by caesium chloride gradient centrifugation in a Beckman L8 - 55 ultracentrifuge (Appendix A).

5.2.3 Restriction enzyme digestion and construction of restriction map of the plasmids

Purified plasmid DNA was digested for 2h as described by Sambrook *et al.* (1989) using endonucleases and the corresponding incubation buffers supplied by Boehringer Mannheim. Double and triple digests with different combinations of the enzymes were used when the plasmid DNA was fragmented so that a map of the restriction sites could be compiled. Digested plasmid DNA was separated by gel electrophoresis in 1 % SeaKem GTG agarose (section 2.2.4). The fragments of DNA were compared and their sizes determined before and after hybridization using both non-labelled and digoxigenin-labelled molecular weight markers (Boehringer Mannheim).

5.2.4 DNA hybridization

Plasmid DNA fragments were transferred to a nylon membrane (Amersham Hybond N) by Southern blotting and hybridization was carried out using a non-radioactive digoxigenin-labelled plasmid as a probe with reagents supplied by Boehringer Mannheim (see Appendix B). In all experiments the plasmid conferring V factor independence in *H. parainfluenzae* 9 was used to prepare the probe. This plasmid DNA was purified by caesium chloride gradient centrifugation and linearized by digestion with *Eco* R1 before labelling.

5.3 RESULTS

5.3.1 Comparison of the pattern of digestion of plasmids from *H. parainfluenzae* and *H. ducreyi*

Various restriction enzymes were used to compare the patterns of digestion of the plasmids conferring V factor independence in four *H. parainfluenzae* and five *H. ducreyi* strains. These endonucleases each recognized different hexanucleotide, pentanucleotide or tetranucleotide sequences in the DNA. Table 13 lists the restriction enzymes used and the number of recognition sites found on the plasmids from both *H. parainfluenzae* and *H. ducreyi.* Although most of the digested DNA fragments were visible on the agarose gel after staining with ethidium bromide, better resolution of the smaller fragments was achieved after hybridization. Eleven of the restriction enzymes were not active against any of the plasmids conferring V factor independence indicating that the plasmids had no recognition sites for these enzymes. The other thirteen restriction enzymes all cleaved the plasmids and each of the nine plasmids exhibited the same pattern of digestion with each enzyme tested indicating that the plasmids had a similar number of recognition sites for each of the thirteen different enzymes, regardless of the source of the donor DNA.

Table 13. Restriction enzymes used to compare the plasmids conferring V factor independence in *H. parainfluenzae* and *H.ducreyi*

Restriction enzyme	No. of recognition sites
Hind II, *Hind* III, *Hpa* I, *Kpn* I, *Pvu* II, *Sac* I, *Sau* I, *Sma* I, *Sph* I, *Sty* I, *Xba* I	None
Bam HI, *Bgl* II, *Eco* RI, *Msp* I, *Pst* I	One
Acc I, *Hae* II,	Two
Dpn I, *Hinf* I, *Nde* II	Three
Hae III	Four
Rsa I, *Taq* I	Multiple

This is illustrated in Figure 7 which presents the patterns of digestion of the plasmids conferring V factor independence from four *H. parainfluenzae* (lanes 2 to 5) and five *H. ducreyi* strains (lanes 6 to 10) treated with *Rsa* I, an enzyme which recognises tetranucleotide sequences. This enzyme produces six fragments in the plasmids from both donor species. Similar multiple fragments were obtained after digestion with *Taq* I. Figure 8 summarizes these results by showing the patterns of digestion of one representative plasmid from *H. parainfluenzae* and one from *H. ducreyi* after digestion with four different restriction enzymes: *Pst* I (lanes 3 & 4), *Hae* II (lanes 6 & 7), *Dpn* I (lanes 8 & 9) and *Rsa* I (lanes 11 & 12).

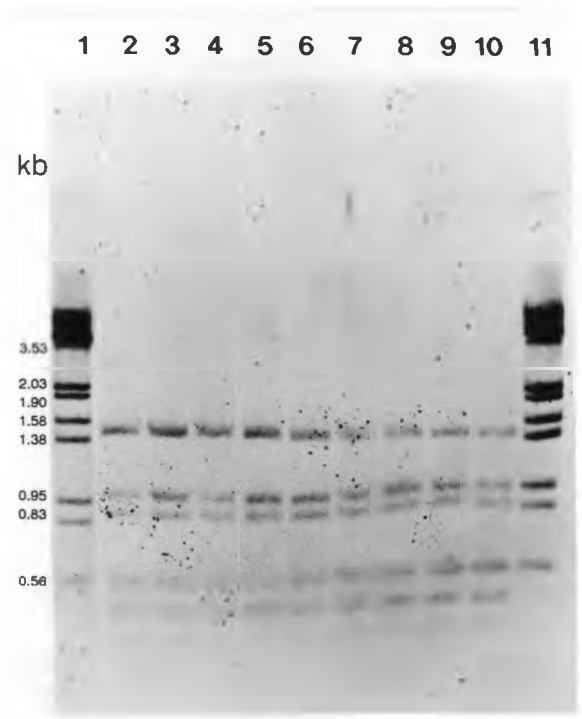


Figure 7. Southern hybridization of *Rsa* I-digested plasmid DNA preparations from *H. parainfluenzae* and *H. ducreyi* with a probe from the plasmid conferring V factor independence in *H. parainfluenzae* 9.

Lanes 2 to 5 plasmid DNA from *H. parainfluenzae* 9, 15, 19 & 78, respectively; lanes 6 to 10 plasmid DNA from *H. ducreyi* CIP 542, ATCC 27722, 124, Y78 & C147, respectively; lanes 1 & 11 λ\ *Eco* RI & *Hind* III molecular weight standard.

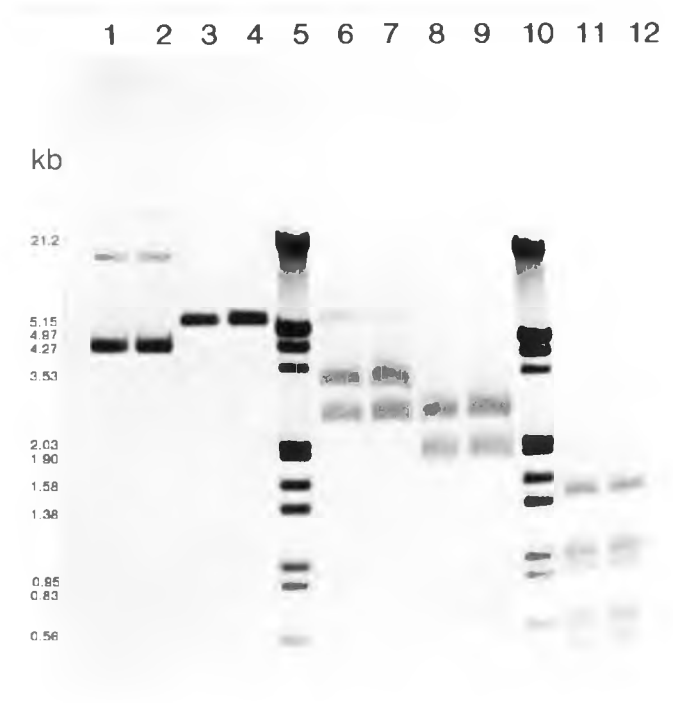


Figure 8. Digestion patterns of plasmids conferring V factor independence in *H. parainfluenzae* and *H. ducreyi* after hybridization with a probe from the plasmid conferring V factor independence in *H. parainfluenzae* 9.

Plasmid DNA in lanes 1, 3, 6, 8 & 11 originated from *H. parainfluenzae* 9 and in lanes 2, 4, 7, 9 & 12 from *H. ducreyi* ATCC 27722. Undigested DNA (lanes 1 & 2); plasmid DNA after digestion with *Pst* I (lanes 3 & 4); *Hae* II (lanes 6 & 7); *Dpn* I (lanes 8 & 9); *Rsa* I (lanes 11 & 12); λ\ *Eco* RI & *Hind* III molecular weight standard (lanes 5 & 10).

These results demonstrate that the plasmids from the two donor species were not only identical in size but that they exhibit the same pattern of digestion for all thirteen of the restriction enzymes. Figures 7 and 8 also show the presence of homology between the plasmid DNA fragments and the labelled plasmid from *H. parainfluenzae* 9 used as a probe in DNA hybridization.

5.3.2 Restriction map of the plasmid conferring V factor independence in *H. parainfluenzae* and *H. ducreyi*

The restriction map presented in Figure 9 was compiled after comparison of the size of the fragments on the Southern blots following multiple digests with different combinations of restriction enzymes. Plasmid DNA from one representative plasmid from *H. parainfluenzae* (*H. parainfluenzae* 9) and one from *H. ducreyi* (*H. ducreyi* ATCC 27722) was used. As stated in the previous section, even though most of the fragments were visible on the agarose gel after staining with ethidium bromide, better resolution of the smaller fragments was achieved after hybridization and also the nylon membrane provided a more stable and accurate record of the digests than the agarose gel did. The restriction sites of only nine of the enzymes listed in Table 13 are shown on the map as the enzymes which had multiple recognition sites viz. *Rsa* I and *Taq* I were not used in the mapping since the number and size of fragments generated after multiple digests with these enzymes made the sizing of the fragments difficult. The cleavage sites of *Nde* II and *Dpn* I

were not determined because incompatibility of the incubation buffers needed in digests with *Nde* II and *Dpn* I meant that no double or triple digests using these enzymes were successful due to the inhibition of digestion of the other enzyme(s) in the mixture.

The sizes of the fragments of plasmid DNA generated after multiple digests with the nine different restriction enzymes were the same, irrespective of the original host, and therefore the restriction maps of the plasmids from both donor species appear to be identical.

The size of this plasmid based on the sum of the fragments obtained during mapping is calculated to be, on average, 5.4 kb. This is slightly larger than the original estimate of 5.25 kb (sections 2.3.2 and 3.3.3). Previously a comparison was made of the covalently closed circular plasmid DNA from *H. influenzae* Rd transformants with *H. parainfluenzae* and *H. ducreyi* donor DNA and that of plasmids of known size from *E. coli*. The measurement of the size of the plasmid using the sum of the sizes of digested fragments after cleaving with different enzymes is likely to be more accurate than using a comparison of the mobility of closed circular molecules after agarose gel electrophoresis.

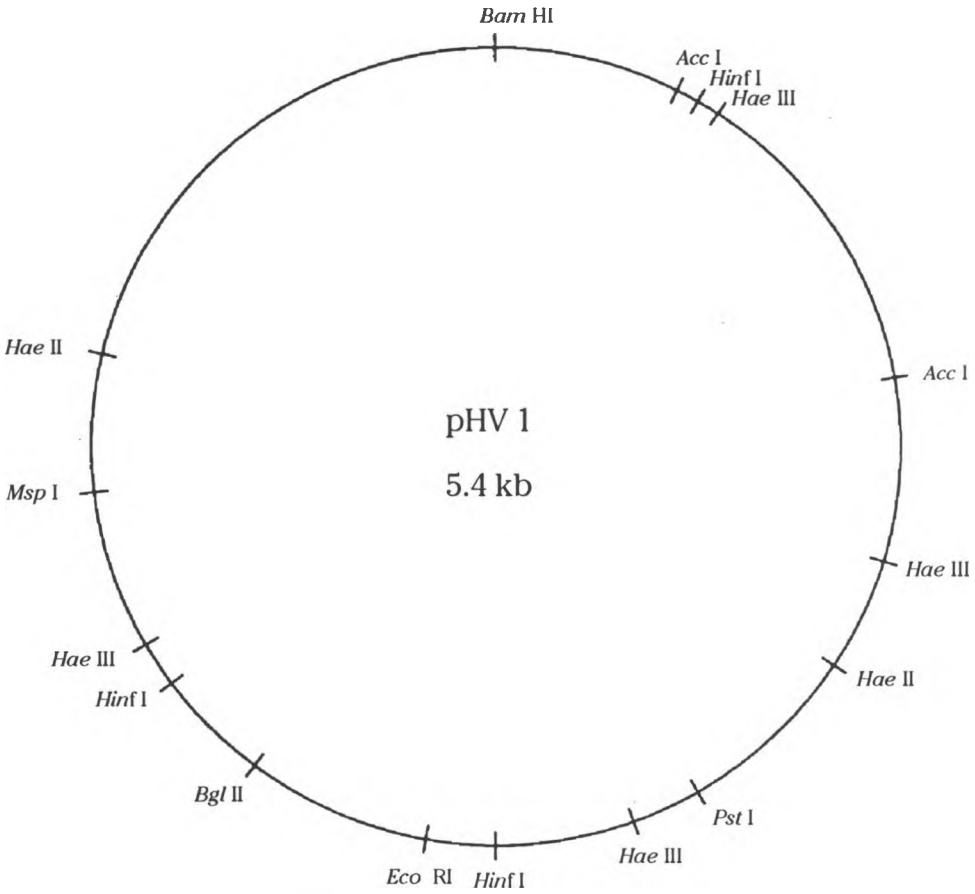


Figure 9. Restriction map of the plasmid conferring V factor independence in *H. parainfluenzae* and in *H. ducreyi*

5.4 DISCUSSION

The similarity in size and phenotypic function of the plasmids conferring V factor independence carried by two *Haemophilus* species, *H. parainfluenzae* and *H. ducreyi* suggested that these plasmids may be genetically related. The present study of the digestion patterns of these plasmids from four *H. parainfluenzae* and five *H. ducreyi* strains shows that they share the same number of recognition sites for thirteen restriction enzymes. The comparison of the size and number of fragments obtained after digestion with the enzymes that have tetranucleotide recognition sequences are most significant as the statistical chance of these enzymes cleaving DNA is higher (theoretically they should cut every 256 basepairs). There were six enzymes that recognized tetranucleotide sequences used in this study - *Dpn* I, *Hae* III, *Msp* I, *Nde* II, *Rsa* I and *Taq* I - and five of them cut the plasmid DNA from both *Haemophilus* species into three or more fragments with the same characteristic pattern as shown in Figure 7. In addition, the results of the DNA hybridization revealed that a labelled plasmid from *H. parainfluenzae* 9 used as a probe hybridized with the fragments of digested plasmid DNA even under conditions of high stringency and no detectable differences were found between the plasmid DNA from the different sources. These results demonstrate that the plasmids conferring V factor independence originating from both *H. parainfluenzae* and *H. ducreyi* are genetically related and have regions that are homologous. However complete homology can only be determined after DNA sequencing. Since the

restriction maps of the plasmids from *H. parainfluenzae* and *H. ducreyi* appear to be identical the plasmid from both species was designated pHV 1. Study on the mechanism of V factor independence conferred by the plasmids from *H. parainfluenzae* and *H. ducreyi* has revealed that these plasmids enable the bacterial host to utilize nicotinamide as a pyridine precursor (Chapter 6). This suggests that this function is probably coded from the same gene(s).

V factor independence is a genetic marker that can be easily expressed and selected and it has been found that the plasmid, pHV1, is highly transformable. Both these properties indicate that pHV1 has the potential to become a good cloning vector. Old and Primrose (1985) state that an ideal plasmid from which to develop a cloning vector would (i) be stably maintained in its host, (ii) be small, (iii) be present in high copy number, (iv) contain unique restriction sites for cloning purposes and (v) possess a selectable marker. Although some spontaneous V factor-dependent clones do occur, these are not of great significance as the % frequency is low – between 0.12 and 0.19 % (Table 5).

CHAPTER 6 :

DETERMINATION OF THE BIOCHEMICAL BASIS OF PLASMID-MEDIATED V FACTOR INDEPENDENCE

6.1 INTRODUCTION

The requirement for V factor has been used as a taxonomic criterion in the genus *Haemophilus* for over seventy years (Thjötta & Avery,1921). *H. influenzae*, *H. parainfluenzae* and several other members of the genus *Haemophilus* require V factor (Table 2) which is also described as β-nicotinamide adenine dinucleotide or NAD. Unlike most other bacteria, these species are not able to synthesize NAD *de novo* from low-molecular-weight compounds but use a limited number of exogenous pyridine nucleotides or precursors as a source of NAD. Subsequent studies have shown that in addition to NAD, nicotinamide riboside (NR), β-nicotinamide mononucleotide (NMN) and nicotinamide adenine dinucleotide phosphate (NADP) can serve as V factor while nicotinamide (NAm), nicotinic acid (NA) and quinolinic acid (QA) cannot (O'Reilly & Niven, 1986a ; Cynamon *et al.*, 1988). This has been described in Chapter 1.4 and the pyridine nucleotide metabolic pathway illustrated in Figure 1.

The only reported study on the biosynthesis of NAD in a V factor-independent *Haemophilus* species is the research of Kasarov and Moat (1973) using *H. haemoglobinophilus*. They revealed that synthesis from NAm via NMN appears to be the sole pathway of NAD formation in this organism. The authors speculated that this pathway, previously only recorded in some mammalian systems and in a *Lactobacillus* species, may be used by other *Haemophilus* and *Pasteurella* species that do not require V factor for growth. In 1988

Niven and Lévesque reported that *Actinobacillus pleuropneumoniae* biotype 2 (a V factor-independent porcine strain very similar to the species previously classified as *Haemophilus pleuropneumoniae*) was similarly able to utilize NAm as a pyridine nucleotide precursor. They also showed that this biotype, although described as V factor-independent, demonstrated NAD- and NMN-dependent growth when cultured in a chemically defined medium (CDM).

The identification of four V factor-independent *H. parainfluenzae* strains in this laboratory and the subsequent work which showed that the genes conferring V factor independence were plasmid-mediated in both these isolates and in reference and clinical strains of *H. ducreyi*, prompted this investigation into the biochemical basis of V factor independence. *H. ducreyi* is one of the two human *Haemophilus* species that does not require V factor for growth but very little is known about its metabolism as it is notoriously difficult to grow *in vitro*.

Two approaches have previously been used for studying the NAD biosynthetic pathway and the requirement for exogenous pyridine nucleotides in *Haemophilus* species. The first approach has been to examine cell-free extracts for their ability to synthesize NAD and to identify the enzyme activities required for this synthesis (Kasarov & Moat, 1973; Kahn & Anderson, 1986 ; Cynamon *et al.*, 1988). The second approach has been to test exogenous pyridine nucleotides or precursors for their ability to support growth in a CDM as was done by Niven and Lévesque (1988). As a CDM had been specifically

developed for the reference strain, *H. influenzae* Rd by Herriott *et al.* in 1970, V factor-independent transformants of *H. influenzae* Rd were used in these experiments. Although the plasmids conferring V factor independence were originally recovered from *H. parainfluenzae* and *H. ducreyi*, the use of Herriott's medium saved many extra hours of testing other defined media to see if there was a suitable CDM for these species. In previous research on the transformation of the plasmid conferring V factor independence (sections 2.3.2 and 3.3.2) *H. influenzae* Rd had been used as the recipient strain. It was shown that this plasmid was present in all the *H. influenzae* Rd transformants with the V factor-independent phenotype; thus, a comparison could be made of the growth characteristics of *H. influenzae* Rd before and after acquiring the plasmid from *H. parainfluenzae* and *H. ducreyi.* Using Herriott's medium it was possible to determine which exogenous pyridine compounds were utilized for growth.

6.2 MATERIALS AND METHODS

6.2.1 Bacterial strains and media

The V factor-independent transformants of *H. influenzae* Rd using donor DNA from the four *H. parainfluenzae* isolates described in section 2.2.1 and the five *H. ducreyi* strains listed in Table 10 were selected as described in sections 2.2.3 and 3.2.3. These V factor-independent transformants of *H. influenzae* Rd were grown in the absence of NAD in haemin-supplemented BHI broth and agar as well as in the CDM developed by Herriott *et al* (1970a). The following pyridine compounds supplied by Sigma were added to CDM to examine their ability to support the growth of the V factor-independent transformants of *H. influenzae* Rd : NAD, NMN, NAm, NA, nicotinic acid mononucleotide (NAMN), nicotinic acid adenine dinucleotide (NAAD) and QA.

6.2.2 Growth in a complex medium

Frozen BHI cultures of *H. influenzae* Rd or the V factor-independent transformants were diluted 1 : 50 in 10-ml aliquots of supplemented BHI broth in 250-ml flasks. These cultures were incubated for about 5 h at 37°C with agitation at 200 rpm on a gyratory shaker until they reached an optical density at 650 nm of 1.0. The cells were harvested by centrifugation, washed twice with 10 ml of CDM, and resuspended in 10 ml of CDM. Duplicate 20-ml aliquots of haemin-supplemented

BHI broth in 500-ml flasks, one with 5 μM* NAD added and one without any NAD were inoculated with 0.2 ml of washed and resuspended cells and incubated at 37°C with shaking at 200rpm. Samples were taken at different intervals and plated onto supplemented BHI agar to determine the number of colony forming units (CFU) per ml over a 20-hour period.

* 5 μM NAD ≡ 3 μg NAD ml^{-1}.

6.2.3 Growth in Herriott's chemically defined medium (CDM)

The inoculum was prepared as described in section 6.2.2. Aliquots of CDM in 500-ml flasks were inoculated with the resuspended cells (0.2 ml in 20 ml of CDM) and incubated for 5h in the absence of pyridine compounds. The filter sterilized pyridine compounds were added to the starved cells to give a final concentration of 5 μM, and incubated at 37°C with shaking at 200rpm. The number of CFU per ml was determined over a 20-hour period as described above.

6.3 RESULTS

6.3.1 Kinetics of growth of the V factor-independent transformants of *H. influenzae* Rd in a complex medium.

In order to study whether or not the presence of a plasmid conferring V factor independence had an effect on the kinetics of growth of *H. influenzae* Rd in a complex medium, one V factor-independent transformant of *H. influenzae* Rd obtained by using DNA from each of the four *H. parainfluenzae* and five *H. ducreyi* donor strains was examined for its ability to grow in BHI broth both in the presence and absence of NAD. Figures 10a and 10b present the growth curves of two representative transformants of *H. influenzae* Rd. Although the source of the plasmid in these transformants was different, their growth curves exhibited similar profiles. Because of their ability to grow equally well in BHI broth in the presence and absence of NAD, the transformants were designated as V factor-independent. The growth curves of the remaining seven transformants were similar to those presented in Figures 10a and 10b. The generation times of all transformants in unsupplemented and NAD-supplemented broth was in the range of 38 to 45 min and 40 to 51 min respectively. The generation time was determined using the linear portion of the growth curve. Unlike the transformants, the isogenic plasmid-free *H. influenzae* Rd was not able to grow in absence of NAD (Figure 10c). The generation time of this strain in NAD-supplemented broth was 45 min. It has been previously demonstrated that spontaneous loss of the plasmid conferring V factor independence in *H. influenzae*

Rd transformants occurs at a frequency of 0.1 to 0.2 % of the progeny of a single clone (Table 5). These results show that the segregation to V factor dependence does not appear to have an effect on the growth curves in the absence of NAD (Figures10a and10b).

6.3.2 Kinetics of growth of the V factor-independent transformants of *H. influenzae* Rd in CDM and the determination of the requirement for pyridine nucleotides

Growth of *H. influenzae* Rd and the nine *H. influenzae* Rd transformants was determined after a five hour starvation period to ensure that no growth precursors were present extra- and intracellularly at the start of the experiment. No increase in the optical density of the cells was observed during the starvation period showing that any growth that had occurred was minimal and all growth factors had been exhausted after 5 hours. All nine *H. influenzae* Rd transformants tested were able to grow in the presence of NAD, NMN and NAm and their growth curves showed similar profiles. The growth curves of two representative V factor-independent transformants using donor DNA from *H. parainfluenzae* and *H. ducreyi* and those of the plasmid-free *H. influenzae* Rd in the presence and absence of exogenous pyridine compounds are presented in Figures 11a, 11b and 11c respectively. Generation times of transformants in the presence of pyridine compounds were: 51 to 63 min in the case of NAD, 53 to 65 min using NMN and 55 to 63 min with NAm. The source of the plasmid does not appear to have an effect on the generation times of the different growth curves. Unlike the transformants, the isogenic

plasmid-free *H. influenzae* Rd failed to grow in the presence of NAm; however NAD and NMN did support its growth (Figure 11c). The generation time of *H. influenzae* Rd was 60 min in the presence of either NAD or NMN. Neither *H. influenzae* Rd nor the V factor-independent transformants were able to grow in unsupplemented CDM or in CDM containing NAAD, NAMN, NA or QA (data not presented in Figures 11a, 11b or 11c), suggesting that these strains were incapable of *de novo* NAD biosynthesis.

It is of interest to note that in CDM the *H. influenzae* Rd transformants exhibit V factor-dependent growth (Figures 11a and 11b), whereas, in BHI broth their growth appears to be V factor-independent (Figures 10a and 10b). This was probably due to the presence of NAm in the commercially produced BHI broth. The growth in CDM of two *H. influenzae* Rd transformants that have lost the small plasmid was also examined. The growth curves of these isolates (data not presented) were similar to those of the original plasmid-free *H. influenzae* Rd recipient strain (see Figure 11c). The correlation between the loss of the plasmid and the loss of the ability to utilize NAm for growth supports the conclusion that the genes coding for utilization of NAm are plasmid-linked.

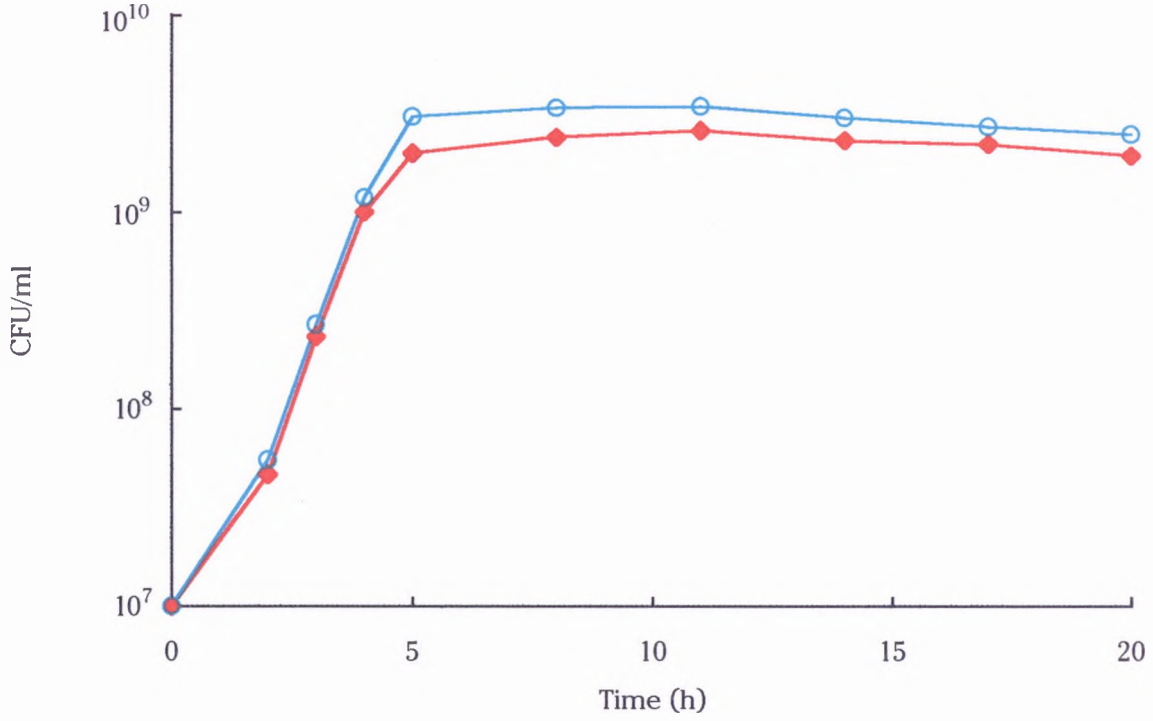


Figure 10a. Growth curves of a V factor-independent transformant of *H. influenzae* Rd with donor DNA from *H. parainfluenzae* 9 in BHI broth in the absence (–○–) and presence (–◆–) of 5 μM NAD

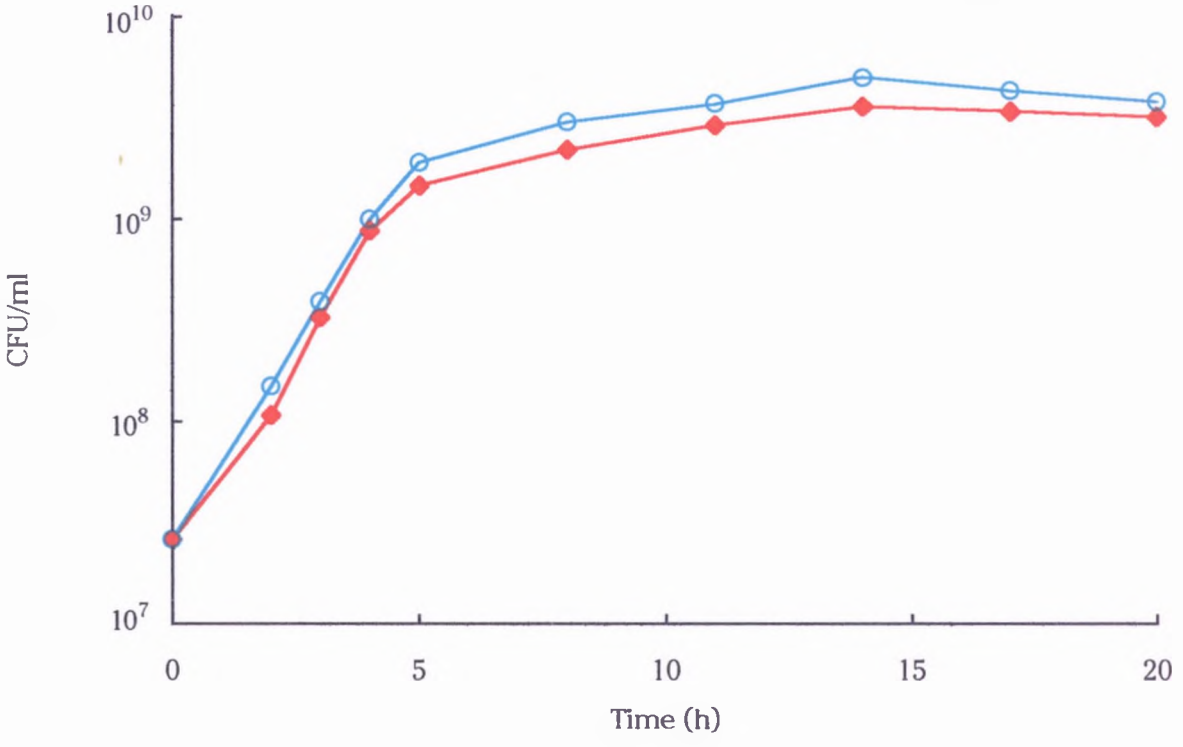


Figure 10b. Growth curves of a V factor-independent transformant of *H. influenzae* Rd with donor DNA from *H. ducreyi* ATCC 27722 in BHI broth in the absence (–○–) and presence (–◆–) of 5 μM NAD

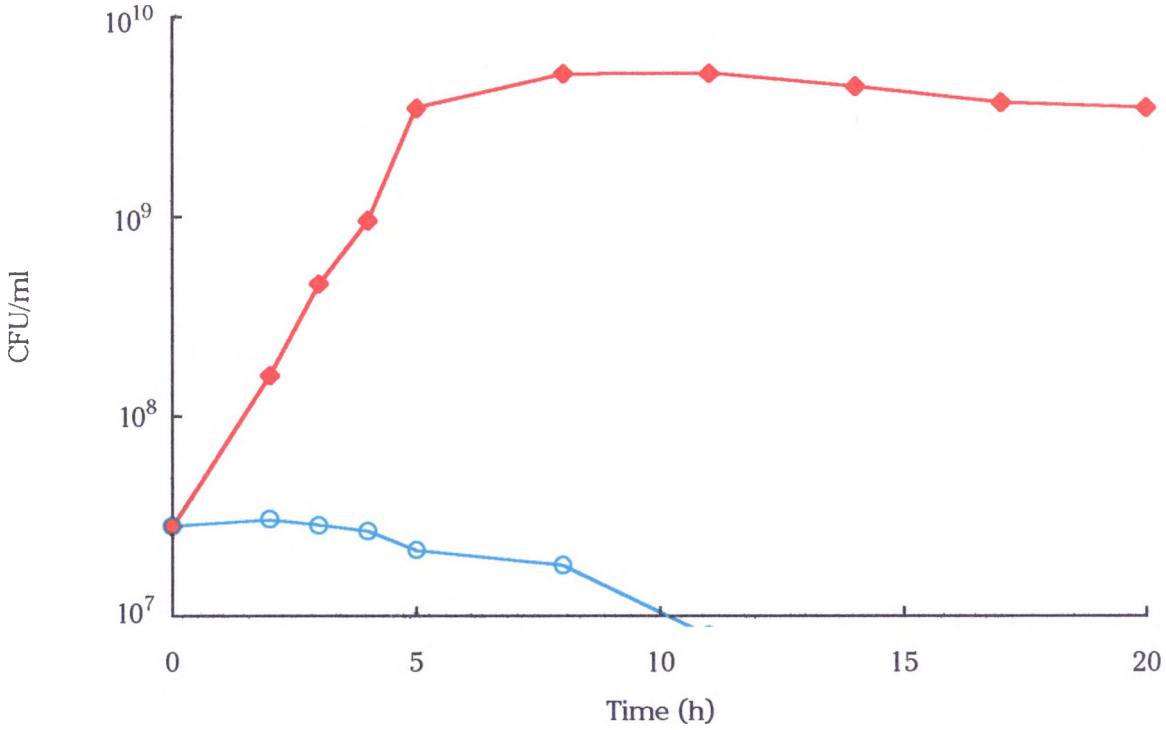


Figure 10c. Growth curves of the plasmid-free recipient strain *H. influenzae* Rd in BHI broth in the absence (–○–) and presence (–◆–) of 5 μM NAD

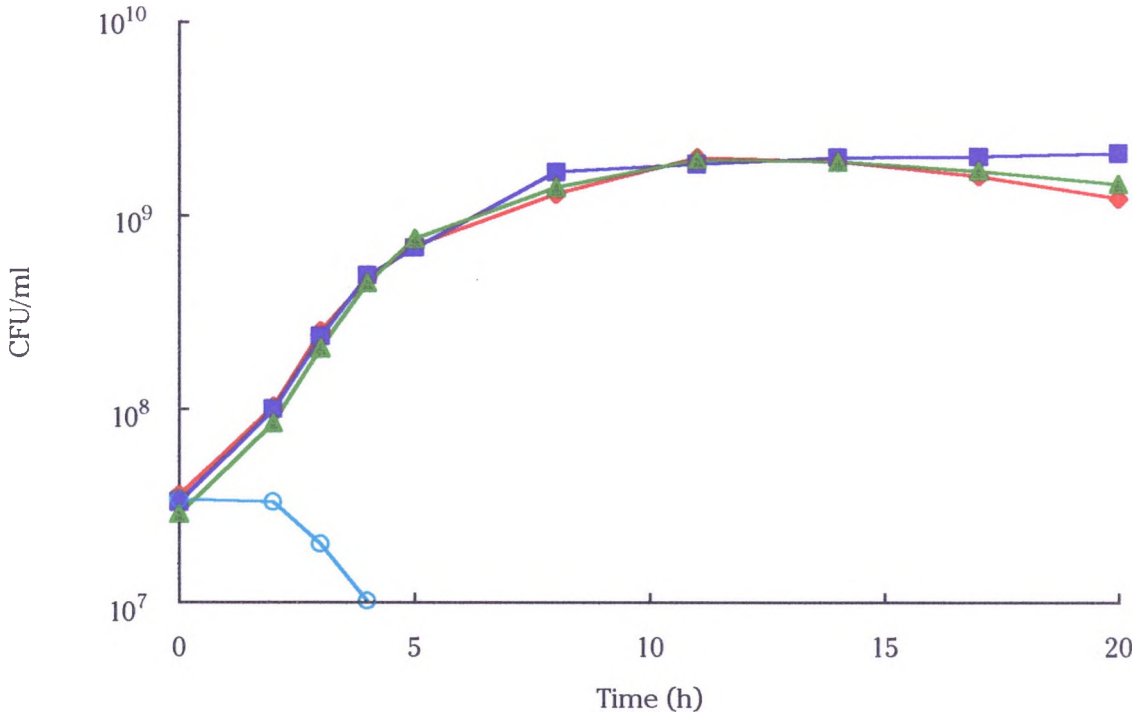


Figure 11a. Growth curves of a V factor-independent transformant of *H. influenzae* Rd with donor DNA from *H. parainfluenzae* 9 in CDM supplemented with NAD (–◆–), NMN (–■–), NAm (–▲–) or unsupplemented (–○–)

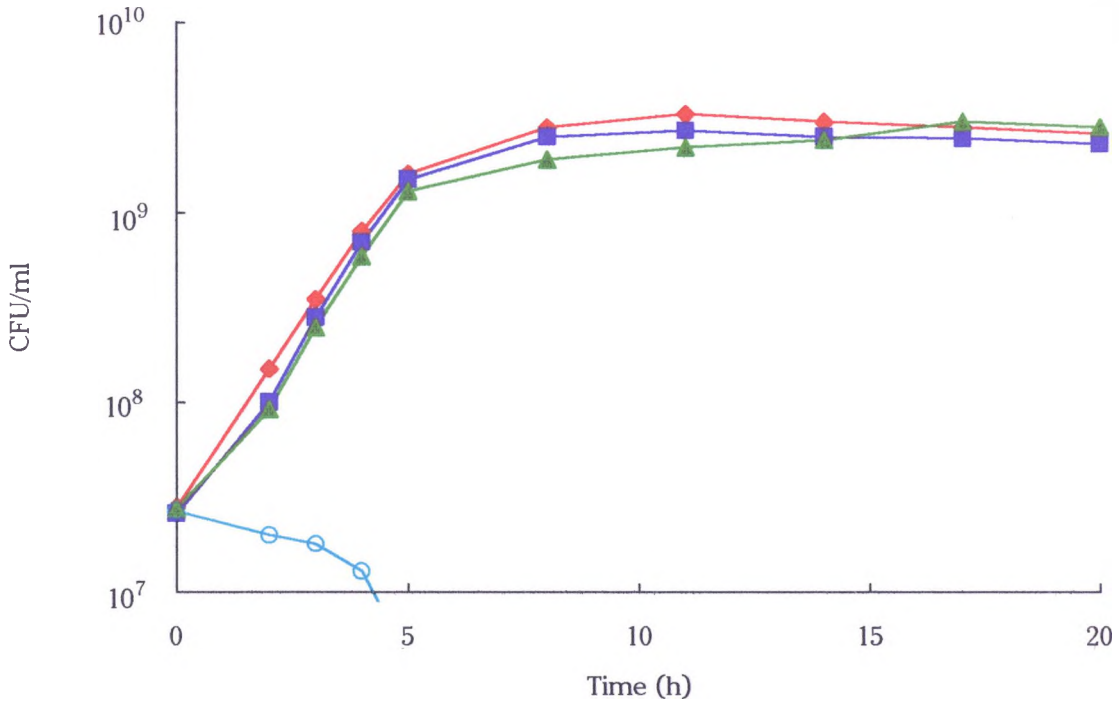


Figure 11b. Growth curves of a V factor-independent transformant of *H. influenzae* Rd with donor DNA from *H. ducreyi* ATCC 27722 in CDM supplemented with NAD (–◆–), NMN (–■–), NAm (–▲–) or unsupplemented (–○–)

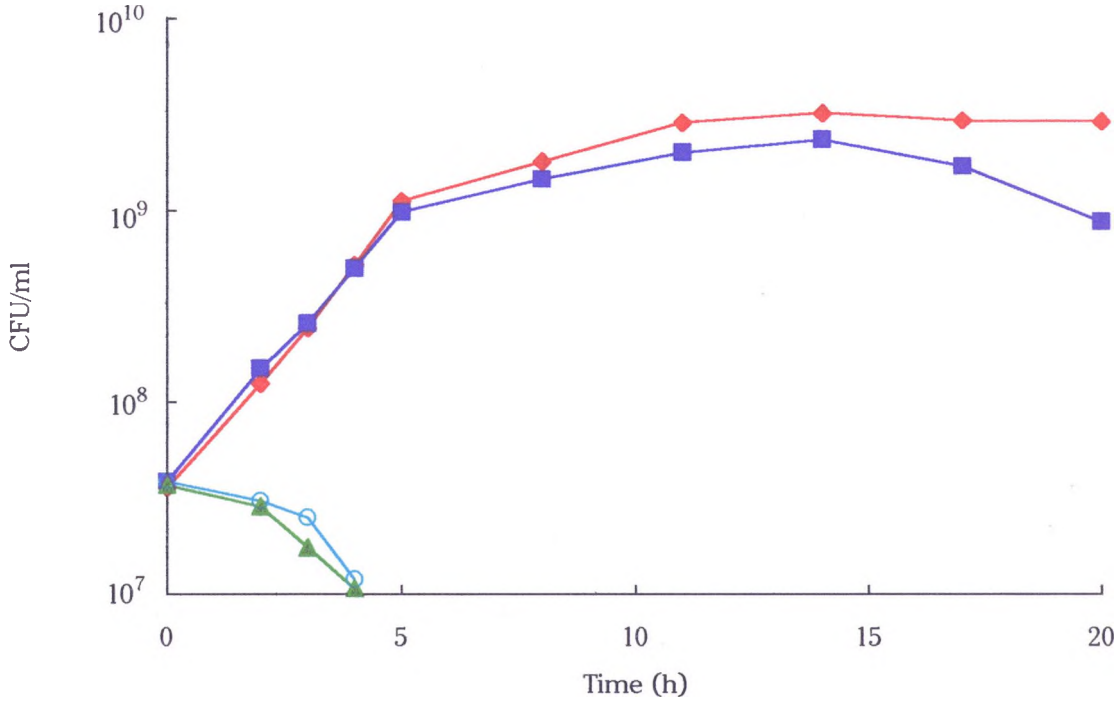


Figure 11c. Growth curves of the plasmid-free recipient strain *H. influenzae* Rd in CDM supplemented with NAD (–◆–), NMN (–■–), NAm (–▲–) or unsupplemented (–○–)

6.4 DISCUSSION

Investigation of the growth requirements of *A. pleuropneumoniae* biotype 2 in Herriott's CDM by Niven and Lévesque (1988) has provided evidence that this biotype shows both V factor-dependent and NAm-dependent growth. However in a complex medium *A. pleuropneumoniae* biotype 2 appears to be V factor-independent (Pohl *et al.*, 1983). This was probably due to the presence of NAm in the commercially produced phytone and proteose peptone medium used. Similarly, the results presented in this study show that the growth requirements of *H. influenz*ae Rd transformants that have acquired the small 5.4 kb plasmid from *H. parainfluenzae* and *H. ducreyi* donors are similar to those of *A. pleuropneumoniae* biotype 2 while the plasmid-free *H. influenzae* Rd (Figures 10c & 11c), like *A. pleuropneumoniae* biotype 1, is V factor-dependent. In the CDM both the *A. pleuropneumoniae* biotype 2 and the V factor-independent transformants of *H. influenzae* Rd were able to utilize NAm, NMN and NAD (Figures 11a & 11b) whereas NAAD, NAMN, NA and QA could not substitute as growth precursors showing, in fact, that these strains do exhibit V factor-dependent growth and also demonstrating that they are unable to synthesize NAD *de novo*. However, the isogenic, plasmid-free *H. influenzae* Rd, like *A. pleuropneumoniae* biotype 1, was able to utilize only NAD and NMN for growth indicating that in both systems the only difference in the growth of the V factor-dependent and the so-called "V factor-independent" strains is the ability of the latter to utilize NAm. This suggests that the genetic differences between the V factor-

dependent and the V factor-independent strains are not as significant as has been previously thought and involve only the presence or absence of one enzyme, NAm phosphoribosyltransferase and perhaps a NAm transport system. Other examples of V factor-independent bacteria of the family *Pasteurellaceae* that are able to utilize NAm for growth are *H. haemoglobinophilus*, *Pasteurella multocida*, *Pasteurella haemolytica* and *Pasteurella ureae* (Kasarov & Moat, 1973; Koser *et al.*, 1941; Wessman, 1966 & 1972). This illustrates that, within the family *Pasteurellaceae*, the requirement for exogenous pyridine nucleotides or precursors is not only confined to the genus *Haemophilus* but rather, may be characteristic of the family as a whole (Niven & O'Reilly, 1990).

No previous information is available about the location of the genes involved in NAD biosynthesis in members of the family *Pasteurellaceae*. Four unusual V factor-independent *H. parainfluenzae* isolates were the first bacterial strains in which the genes coding for V factor independence were found to be located on a small 5.4kb plasmid (Chapter 2; Windsor *et al.*, 1991). Subsequently a plasmid of a similar size also conferring V factor independence was recovered from *H. influenzae* Rd transformants with *H. ducreyi* DNA (Chapter 3; Windsor *et al.*, 1993a). In this study evidence is provided that the V factor independence is due to the ability of the cells to utilize NAm for growth and that the genes coding for this function are plasmid-linked in *H. parainfluenzae* and *H. ducreyi*. Because of the small size of this plasmid, NAD is most probably synthesized directly from NAm

using a pathway similar to that described for *H. haemoglobinophilus* (Kasarov & Moat, 1973). It would be of interest to determine whether the genes coding for V factor independence in other members of the family *Pasteurellaceae* are also plasmid-linked. Although no published information is available about the location of the genes coding for the utilization of V factor they appear to be of chromosomal nature because of their stability.

The recovery from natural sources of both V factor-dependent and V factor-independent isolates from species belonging to two different genera, *Actinobacillus* (Pohl *et al.*, 1983) and *Haemophilus* (Gromkova *et al.*, 1989; Gromkova & Koomhof, 1990; Mouahid *et al.*, 1991; Horner *et al.*, 1992) indicates that the V factor requirement may vary among strains of the same species and, with the present methods for determination, V factor is not suitable as a taxonomic criterion for either generic or species differentiation. In addition, the demonstration that in at least two *Haemophilus* species the genes conferring V factor independence are plasmid-linked (Windsor *et al.*, 1991; 1993a) suggests that other V factor-dependent species have the potential to become V factor-independent due to the mobility of extrachromosomal genes or conversely, some V factor-independent isolates may become V factor-dependent due to loss of the plasmid. Both of these phenomena have been demonstrated under experimental conditions with the transfer of the plasmid conferring V factor independence between both homologous and heterologous species

by DNA transformation and the loss of the plasmid in the naturally occurring *H. parainfluenzae* isolates both spontaneously and by chemical means.

In a recent review Niven and O'Reilly (1990) analysed the biochemical basis of the V factor requirement in three genera of the family *Pasteurellaceae*: *Haemophilus, Pasteurella* and *Actinobacillus* and concluded that a common characteristic of these genera is the lack of *de novo* NAD biosynthesis and the ability to grow in the presence of a few exogenous pyridine nucleotides or precursors. These authors propose the inclusion of NAm in the pyridine compounds that may serve as a V factor. This would allow the classification of NAm-dependent strains that are presently defined as V factor-independent to be classified as V factor-dependent and would result in the designation of all members of the family *Pasteurellaceae* as V factor-dependent. However, in view of the extrachromosomal nature of the genes coding for NAm utilization in at least two *Haemophilus* species, it might be more appropriate to preserve the old definition of V factor and merely to modify the method of determination of the V factor requirement. Both *A. pleuropneumoniae* biotype 2 (Niven & Lévesque, 1988) and the "V factor-independent" transformants of *H. influenzae* Rd used in this study have shown V factor-dependent growth when cultured in a defined medium. Therefore the use a commercial medium that does not contain either V factor or NAm for the determination of V factor requirements would be more suitable. However, from a practical

point of view, the present method for determination of the growth factor requirement on complex media could still be used for initial screening of human *Haemophilus* species. The majority of these species would be identified as V factor-dependent, but in a few cases clinical isolates that appear to be V factor-independent may exhibit the biochemical and morphological characteristics of a *Haemophilus* species. These strains should then be tested on a medium which is lacking in V factor and NAm for proper determination of the growth requirements. After the addition of the various nucleotide precursors the NAD, NMNand NAm utilization could be accurately determined. If this method had been used previously both the four unusual V factor-independent *H. parainfluenzae* isolates and *A. pleuropneumoniae* biotype 2 strains would be defined as V factor-dependent organisms that also possess the ability to utilize NAm as an additional nucleotide precursor.

It could be that other members of the family *Pasteurellaceae* that are presently designated V factor-independent would exhibit similar V factor-dependent growth if their growth requirements were determined as described above. If this proves to be the case, it would result in the designation of all members of the family *Pasteurellaceae* as V factor-dependent without having to alter the present definition of V factor to include a characteristic (viz. NAm utilization) that is known to be plasmid-linked in two *Haemophilus* species. It would also explain why Mannheim (1981) and Pohl (1981) found that, after comparing DNA relatedness among members of the family

Pasteurellaceae, V factor dependency was found to be spread over the whole range of the family and that it could not be used to characterize a natural genus.

CONCLUSIONS

The isolation of four naturally occurring, V factor-independent strains of *H. parainfluenzae* from unrelated sources prompted this research into the genetic and biochemical basis of V factor independence. The finding that the genes conferring V factor independence were located extrachromosomally on a 5.4 kb plasmid was the first report of genes involved in metabolic activity being plasmid-mediated in a *Haemophilus* species. Although the frequency of isolation of the unusual V factor-independent *H. parainfluenzae* strains is low, less than 1%, the fact that this plasmid can be transferred both intra- and inter-specifically indicates that it has the potential to spread to other *Haemophilus* species that are found in the same ecological niche. However naturally occurring *H. influenzae* strains were not found among 252 clinical isolates. Subsequent work showed that a similar plasmid conferring V factor independence was also present in five strains of *H. ducreyi* from different geographical regions. The restriction map of these plasmids originating from both *Haemophilus* species was indistinguishable and the plasmid was designated pHV1. However in *H. ducreyi*, unlike *H. parainfluenzae*, it appears to exist in both an autonomous and chromosomally integrated state. pHV1 could be transferred to both *H. influenzae* and *H. parainfluenzae* by DNA transformation. There have been no previous reports of genes coding for metabolic functions in *H. ducreyi* being transferred to other *Haemophilus* species. Earlier studies of the DNA homolgy between *H. ducreyi* and other *Haemophilus* species including *H. influenzae*

and *H. parainfluenzae* have shown that *H. ducreyi* appears to be only distantly related to these latter species (Casin *et al.*, 1985; de Ley *et al.*, 1990). However plasmids conferring drug resistance in *H. influenzae*, *H. parainfluenzae* and *H. ducreyi* have been reported to be genetically related (Brunton *et al.*, 1986a). The evidence that the plasmid coding for V factor independence can also be found in *H. aphrophilus* and *A. pleuropneumoniae* biotype 2 strains suggests that this plasmid appears not to be unique to just *H. ducreyi* and the unusual *H. parainfluenzae* isolates. Nevertheless the original source of the plasmid is unknown.

This plasmid, pHV1, is small, contains unique restriction sites and is highly transformable. It appears to be present in a high copy number in its host and it codes for a genetic marker, V factor independence, that can be easily expressed and selected. These properties all indicate that pHV1 has the potential to become a good cloning vector.

The investigation of the biochemical basis of the V factor independence mediated by the plasmid, pHV 1 has demonstrated that the genes on this plasmid code for the utilization of nicotinamide, a pyridine nucleotide precursor. There are no previous reports of the extrachromosomal location of genes involved in pyridine nucleotide biosynthesis in other bacterial systems. Nicotinamide is present in most commercial bacterial culture media, including brain heart infusion medium, but is not utilized for growth by typical *H. influenzae*, *H. parainfluenzae*, *H. parasuis* or *A. pleuropneumoniae* biotype 1 strains (Schlenk & Gingrich, 1942; O'Reilly & Niven, 1986a; Cynamon *et*

al., 1988). In complex media *H. influenzae* transformants that have acquired the 5.4 kb plasmid, pHV 1, appear to be V factor-independent but in CDM they exhibit both V factor- and NAm-dependent growth. It was concluded that the initial designation of these isolates as V factor-independent was incorrect. A similar conclusion was made by Niven and Lévesque (1988) with regard to the growth requirements of V factor-independent *A. pleuropneumoniae* biotype 2 in complex media and CDM.

Data provided by other authors and by this study showed that the V factor requirement varies among strains from the same species in at least two different genera, *Haemophilus* and *Actinobacillus*, suggesting that this requirement with its present definition and methods of determination is not suitable for either generic or species differentiation. Niven and O'Reilly (1990) proposed the inclusion of NAm as a V factor compound which they state could eventually result in all members of the family *Pasteurellaceae* being shown to be V factor-dependent. This would allow V factor dependency to be used to differentiate the other Gram-negative bacteria from members of the family *Pasteurellaceae* and thus it has the potential to serve as a familial criterion of considerable taxonomic significance. However, in view of the extrachromosomal nature of the genes coding for NAm utilization in at least two *Haemophilus* species, it would be more appropriate to preserve the old definition of V factor and merely to modify the method of determination of the V factor requirement. By using basal media that do not contain either V

factor or NAm it would be possible to ascertain which of the various nucleotide precursors could be utilized for growth. Thus all members of the family *Pasteurellaceae* may exhibit V factor-dependent growth because of their inability to synthesize NAD *de novo*.

However, to satisfy the taxonomist, the microbial physiologist and the clinical microbiologist, the question of the definition of V factor and its method of determination still need to be debated further.

APPENDIX A: DNA EXTRACTION AND PURIFICATION TECHNIQUES

Total bacterial DNA extraction

1. Spin down 50ml overnight BHI broth cultures in Beckman J2-21 centrifuge at 12,000 rpm for 10 min or scrape the colonies off the surface of 2 agar plates, resuspend in 5ml saline and centrifuge as described.

2. Resuspend the pellet in 3 ml saline and add 0.15 ml 20% SDS to give a final concentration of 1%. Incubate at 37°C for 1h.

3. Add 30 µl RNase (10 mg ml^{-1};Sigma), incubate at 37°C for 1h, then add 15µl Proteinase K (10 mg ml^{-1};Sigma) and continue incubation for a further 1h.

4. Mix with an equal volume phenol mixture (Phenol:chloroform: isoamyl alcohol ≡ 25:24:1) and spin at 3,500 rpm for 10 min. Repeat if necessary until the aqueous phase is no longer cloudy.

5. Remove aqueous phase and dialyse overnight at 4°C in 1x SSC.

   1 x SSC Solution (pH 7.0):

   150 mM Na Cl

   15mM Na Citrate

6. Remove from dialysis tubing and store at 4°C.

Plasmid DNA extraction

1. Spin down 100ml overnight BHI broth cultures in Beckman J2-21 centrifuge at 12,000 rpm for 10 min or scrape the colonies off the surface of 5 agar plates and resuspend in 5ml solution I and centrifuge as described above.

   Solution I:

   50 mM glucose

   25 mM Tris-HCl (pH 8.0)

   10 mM CDTA

2. Resuspend the bacterial pellets in 2ml solution I.

3. Add 4 ml solution II, mix gently and stand on ice for 10 min.

   Solution II:

   0.2N NaOH

   1% SDS

4. Add 3ml solution III, invert tubes to mix and stand on ice for 30-40 min.

   Solution III:

   60 ml 5M potassium acetate

   11.5 ml glacial acetic acid

   28.5 ml H_2O

5. Centrifuge at 12,000 rpm for 10 min and transfer supernatants to 15 ml polypropylene tubes. Add an equal volume phenol mixture (Phenol:chloroform:isoamyl alcohol ≡ 25:24:1), mix well for 20 min and spin at 3,500 rpm for 10 min.

6. Remove upper layers, transfer to clean polypropylene tubes and add 2 volumes cold absolute ethanol. Leave at –20°C for 1h, spin at 4°C at 8,000 rpm for 10 min, discard supernatants and dry pellets in vacuum desiccator.

7. Dissolve pellets in 100µl TE buffer, transfer to an Eppendorf microfuge tube, add 10 µl RNase (10 mg ml^{-1}) to each and incubate at 37°C for 45 min.

T E Buffer :

10mM Tris-HCl (pH 8.0)

1 mM EDTA

8. Precipitate plasmid DNA with 250µl cold absolute ethanol, leave at –20°C for at least 1h, spin at 4°C at 12,000 rpm for 10 min, dry pellets as above and dissolve DNA in 400µl sterile TE buffer. Store plasmid DNA at –20°C.

Caesium chloride gradient purification

1. Extract DNA as described above; total DNA (steps 1-4) and plasmid DNA (steps 1-6). Add dH_20 or TE buffer to DNA to make total volume of dissolved DNA equal to 7.5ml.

2. Weigh out 7.5g CsCl (Boehringer Mannheim) into Beckman polypropylene tubes, add 7.5 ml DNA solution and mix well until all CsCl is dissolved. Add 0.75ml ethidium bromide (10mg ml^{-1}) to each tube and invert to mix. After filling with mineral oil and sealing, balance

and spin for 40-45h at 42,000 rpm at 15°C in Ti 50 rotor in Beckman L8-55 ultracentrifuge.

3. Collect separated DNA bands by piercing through polypropylene tube with a hypodermic needle and collect the purified DNA in a 1ml syringe before transferring to an Eppendorf microfuge tube.

4. Remove ethidium bromide by mixing with an equal volume of NaCl-saturated iso-propanol. Repeat until DNA solution is colourless and then dialyse overnight in TE buffer at 4°C to remove CsCl.

APPENDIX B : NON-RADIOACTIVE DIGOXIGENIN-LABELLED HYBRIDIZATION

(All items marked B M are obtained from Boehringer Mannheim)

Labelling plasmid DNA with digoxigenin-11-dUTP for use as a probe

1. Denature 100µl *Eco* R1-digested CsCl-purified plasmid DNA from *H. parainfluenzae* 9 by heating at 95°C for 10min in boiling water and rapidly chilling on ice for 3min.

2. Add the following to the denatured DNA in a microfuge tube on ice:
   20µl Hexanucleotide mixture (B M)
   20µl dNTP labelling mixture (B M)
   50µl sterile dH_20
   10µl Klenow enzyme (B M)

3. Centrifuge briefly to mix and incubate overnight at 37°C.

4. Add 20µl 0.2 M EDTA (pH 8.0) to stop reaction and precipitate DNA with 25 µl 4M LiCl and 750µl chilled absolute ethanol. Mix well and leave at – 70°C for at least 1h.

5. Spin at 12,000 rpm at 4°C for 10min, wash pellet with 40µl 70% ethanol (chilled), spin again and dry.

6. Dissolve labelled DNA in 500µl TE buffer (page 143) and store at –20°C.

Southern blotting and hybridization

1. Separate DNA by gel electrophoresis in 1 % SeaKem GTG agarose (if digested DNA is known to fragment into small pieces, 1.5 % or 2% gel

can be used), stain with ethidium bromide (0.6μg ml^{-1}) and photograph.

2. Depurinate DNA in gel in 0.25M HCl for 20 min with gentle shaking, denature DNA in a solution of 1.5M NaCl and 0.5M NaOH for 30 min and then shake gently in neutralization buffer (1.5M Na Cl and 0.5M Tris-HCl - pH 7.4) for 30 min.

3. Transfer DNA from gel to a nylon membrane (Amersham Hybond N) by the capillary transfer method described by Southern (1975) using 20 x SSC (page 141) as the blotting buffer. Leave for about 16h for complete transfer of chromosomal DNA.

4. Mark one corner of membrane for orientation then carefully remove it from gel and paper towels. Bake at 120°C for 30min to bind DNA to membrane. Check gel under UV light to ensure no DNA is left in agarose.

5. Prehybridize membrane in sealed plastic bag with 50ml freshly made hybridization solution at 68°C for at least 1h. [All volumes given are for a membrane of 10 x 15 cm - volumes must be halved for the smaller 10 x 6.5 cm size]

Prehybridization / Hybridization Solution:

12.5ml	20 x SSC (page 141)	
50mg	Na lauroylsarcosine (Sigma)	Made up to 50ml with dH_20
50μl	20% SDS	and dissolved at 68° C.
500mg	blocking reagent (B M)	

6. Discard prehybridization solution and replace with 5ml of hybridization solution containing 30 µl freshly denatured probe DNA. Reseal plastic bag and incubate membrane overnight at 68°C. See notes (i) & (ii) below.

7. Wash membrane at room temperature with 50ml 2x SSC and 0.1% SDS for 5 min. Repeat. Wash at 68°C with 50ml 0.1 x SSC and 0.1% SDS for 15 min. Repeat.

Detection of digoxigenin-labelled hybrids

1. Wash membrane briefly in buffer 1 and then shake gently for 30 min in a solution of 0.5g blocking reagent (B M) dissolved in 100ml of buffer 1.

   Buffer 1:

   100mM Tris-HCl (pH 7.5)

   150mM NaCl

2. Rinse membrane in buffer 1 and add 40ml of diluted anti-digoxigenin antibody-conjugate (B M). Normally 150 mU ml^{-1} or 8µl in 40 ml buffer 1 is sufficient to give detectable results. Mix gently for 30 min.

3. Remove unbound antibody-conjugate by washing with 100ml of buffer 1 for 15 min. Repeat. Equilibrate membrane with 20ml buffer 2 for 3 min.

   Buffer 2:

   100mM Tris-HCl (pH 9.5)

   100mM NaCl

   50mM $MgCl_2$

4. Dilute colour detection reagents - 45μl nitro blue tetrazolium chloride (B M) and 35μl X phosphate (B M) in 10ml buffer 2. Seal membrane in plastic bag with 10 ml colour detection solution and store in dark.

5. Colour reaction can be stopped at any time by washing the membrane with TE buffer (page 143).

Notes:

(i) Hybridization solution containing digoxigenin-labelled probe can be frozen at –20°C and reused after renewed DNA denaturation at 95°C. Frozen hybridization solutions are stable for at least 12 months.

(ii) Parameters for hybridization can be varied depending on the DNA on the membrane. eg In the case of plasmid DNA known to be homologous with the probe, 5ml of hybridization solution with 50 μl labelled DNA was used and the time of hybridization reduced to 2 h.

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