CLONING AND CHARACTERIZATION OF THE CAPSULE TRANSPORT GENE REGION FROM *Haemophilus paragallinarum*

by

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Submitted in fulfillment of the requirements for the degree

Magister Scientiae in Biochemistry

in the faculty of Natural Science and Agriculture, Department of Microbiology and Biochemistry, University of the Free State, Bloemfontein, South Africa.

November 2001

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Dr. E van Heerden

Acknowledgements

I would like to express my sincerest gratitude to the following people and institutions:

Dr J. Albertyn for his guidance, friendship and patience through this study. I appreciated the opportunities he granted me to develop my laboratory- and teaching skills, I found his passion for molecular biology inspiring.
Dr J. Boyce at the Monash university, Australia, for his contribution to my study.
Botma Visser for sparking my interest in molecular biology.
All my colleagues and friends at the Department of Microbiology and Biochemistry, UFS, for their interest and encouragement.
In particular by best friend Sanet Nel for always being there.
Ewald Albertse for his friendship and valued contribution to my thesis with respect to all the scanned images.
Cornelia Casaleggio for all her expert advice and stimulating ideas concerning our field of study.
My parents for believing in me and always encouraging me through difficult times. Thank you Mom and Dad for your love and support, I know you have sacrificed much for the sake of my studies.
My sister for always being curious about my work.
The National Research Foundation (NRF) and the Department of Microbiology and Biochemistry, UFS, for financial support.

The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' (I found it!) but 'That's funny ...'"

Isaac Asimov

This thesis is dedicated to my parents and my sister

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Chapter 1

Introduction

Haemophilus paragallinarum causes an acute respiratory disease of chickens known as infectious coryza (IC), a disease first recognized as a distinct entity in the late 1920's. Since the disease proved to be infectious and primarily affected nasal passages, the name "infectious coryza" was adopted (Blackall, 1989). Infectious coryza may occur in both growing chickens and layers. The major economic effect of the disease is an increased culling rate in meat chickens and a reduction in egg production (10-40%) in laying and breeding hens. The disease is limited primarily to chickens and has no public health significance (Yamamoto, 1991). The most common clinical signs are a nasal discharge, conjunctivitis, and swelling of the sinuses and face. Various sulfonamides and antibiotics are useful in alleviating the severity and course of infectious coryza; however, none of the therapeutic agents has been found to be bactericidal. Relapse often occurs after treatment is discontinued, and the carrier state is not eliminated (Yamamoto, 1991). All the commercially available bacterins against IC, consist of inactivated broth cultures of a combination of two or three different serotypes. Although vaccines against IC have been used in South Africa since 1975, it became apparent in the 1980s that the vaccines were becoming less effective in controlling the disease (Bragg *et al.*, 1996). This could have been due to the emergence of a previously unknown serovar, or even serogroup and the possibility of changes in the population dynamics. Vaccine efficiency is therefore a problem and an alternative to available vaccines is needed.

Capsules have long been associated with virulence properties of bacteria. The role that the capsule play in the virulence of bacterial species related to *H. paragallinarum* has been investigated by several workers (Kroll *et al.*, 1988; Inzana *et al.*, 1993; Boyce and Adler, 2000). Mutation, deletion or allelic exchange of gene/s involved in the transport of capsule polysaccharides in related species like *Haemophilus influenza*, *Actinobacillus pleuropneumoniae* and *Pasteurella multocida*, resulted in organisms with reduced virulence. The noncapsulated mutants of *Actinobacillus pleuropneumoniae* reported by Inzana *et al.* (1993) showed extreme stability and induced

a protective immune response without any symptoms of disease. This not only proves the capsule's involvement in virulence of bacteria but also offers the opportunity to investigate the possibility of producing live vaccines.

The aim of this study was an attempt to understand the genetic organization of the capsular genes of *H. paragallinarum* in comparison to related HAP organisms and the possibility of producing a mutant lacking the capsule.

The goals were:

1. Isolation and cloning of the capsule transport gene locus.
2. Sequencing and characterization of the locus
3. Transplacement of a gene/s to produce a noncapsulated mutant of *H. paragallinarum.*

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Chapter 2

Literature review

2.1 Infectious coryza

Haemophilus paragallinarum is the causative agent of infectious coryza (IC) and has a major economic effect on the poultry industry in South Africa. Although the disease is limited primarily to chickens and has no public health significance, an increased culling rate in meat chickens and a reduction in egg production (10-40%) in laying and breeding hens result in money losses for chicken farmers (Yamamoto, 1991). The most common clinical signs are a nasal discharge, conjunctivitis, and swelling of the sinuses and face. Birds may develop swelling of the wattles and diarrhea. Decreased feed and water consumption retards growth in young stock and reduces egg production in laying flocks. Involvement of the lower respiratory tract may be due to synergism between *H. paragallinarum* and other respiratory tract pathogens (Blackall, 1989). Lesions associated with the disease reflect an acute catarrhal inflammation of the upper respiratory tract. Typically, a mucoid sinusitis occurs, with sloughing, edema, and congestion of the sinus mucosa as well as an infiltration of the mucosa with mast cells. It has been suggested that these mast cells, along with heterophils and macrophages, cause the characteristic lesions of coryza (Sawata *et al.*, 1985).

2.1.1 Therapy with selected drugs

A number of sulfonamides are useful as therapeutic agents, but relapse often occurs after treatment is discontinued (Yamamoto, 1991). Sulphachlorpyrazine, a combination of sulphachorpyrazine and dihydrostreptomycin, and sulphadimidine prevents the spread of the disease during the period of medication. A combination of chlortetracycline and sulphodimethylpyrimidine was found to be less effective. None of the compounds used could cure all infected birds of infection (Buys, 1972).

Sulphathiazole (Delaphane and Stuart, 1941), sulphadime thoxine (Mitrovic, 1967), and chlortetracycline plus sulphadimethosine (Kato, 1967) were chemotherapeutically active against infectious coryza in chickens. Streptomycin (Bornstein and Samberg, 1955) has also been found to be effective in the treatment of infectious coryza against a number of resistant strains. Erythorymycin thiocyanate (poultry formula) afforded clinical relief to a significant number or infected birds (Page, 1962b). Streptomycin and spectinomycin-erythromycin combinations could not contain the infection, but there was a marked decrease in the number of clinically affected birds (Hanley *et al.,* 1968). Buys (1972) showed that sulphachlorpyrazine, sulfhadimidine and dihydrostreptomycinsulfate can be used to good effect to counteract the more severe symptoms of infectious coryza, if recovery rates and percentage of birds showing aerocystitis are considered. The drugs used did not cure the birds from infection but economic losses could be limited.

2.1.2 Inactivated vaccines

Commercial bacterins prepared from chicken embryos or broth may be autogenous or may contain strains of 2 to 3 different serotypes. The products, inactivated with formalin or merthiolate, must contain at least 10^8 CFU/ml to be effective. They may contain adjuvants, stabilizers, or saline diluents. Bacterins injected subcutaneously in birds 10-20 weeks of age yield optimal results when injected 2-3 weeks prior to the expected natural outbreak i.e. 2 injections given approximately 3 weeks apart before 20 weeks of age seem to result in better performance of layers than a single injection. When administered to growing birds the bacterin reduces losses from complicated respiratory disease. Chickens vaccinated with the chicken embryo product at 16 weeks of age maintained a significant degree of immunity against challenging for up to 27 weeks (Yamamoto, 1991). Although vaccines against IC have been used in South Africa since 1975 it became apparent in the 1980s that the vaccines were becoming less effective in controlling the disease (Bragg *et al.,* 1996).

This could be due to the emergence of a previously unknown serovar, or even serogroup of *H. paragallinarum* in South Africa, as two newly emerge serovars in

Australia were described by Eaves *et al.* (1989) and Blackall *et al.* (1990a). Another explanation is the possibility of changes in the population dynamics, resulting in changes in relative abundance of serovars between which there is no cross protection. It was shown by Bragg *et al.* (1996) that there is a need for an ongoing system for monitoring of these population dynamics of *H. paragallinarum*, in order to ensure sustained vaccine efficiency.

2.2 Haemophilus paragallinarum

H. paragallinarum is a gram negative, polar staining, non-motile bacterium. In 24-hr cultures, it appears as short rods, or coccobacilli 1-3µm in length and 0.4-0.8µm in width, with a tendency for filament formulation. The organism undergoes degeneration within 48-60 hours, showing fragments and indefinite forms (Yamamoto, 1991).

2.2.1 Classification of *Haemophilus paragallinarum*

The genus *Haemophilus*, was created for bacteria "growing best (or only) in the presence of hemoglobin and in general requiring blood serum or ascetic fluid" and appeared to be a natural home for these organisms. Hence, the independent proposals of Elliot and Lewis (1934) and Delaplane *et al.* (1934) of the name "*Haemophilus gallinarum*" for the causative agent of infectious coryza were readily accepted. The earliest examination of the growth factor requirements of the avian *haemophili* appears to be the little recognized work of McGaughey (1932), which demonstrated that these strains required only V factor (nicotinamide adenine dinucleotide, NAD). In contrast, Delaplane *et al.* (1938) reported that their isolates required both X (hemin) and V (NAD) factors. The designation went unchallenged until Page (1962a) renewed interest in the disease. He made a number of *Haemophilus* isolations from cases of infectious coryza and found that none required hemin for growth, but all required NAD. This led to the proposal and general acceptance of a new species, *H. paragallinarum* for organisms requiring only NAD (Yamamoto, 1991). *H. gallinarum* and *H. paragallinarum* are identical in all other growth characteristics and disease-producing potential. Unfortunately, strains designated *H.*

gallinarum by early workers are no longer available for study of their hemin requirement by more accurate procedures (Rimler, 1979). NAD-independent *H. paragallinarum* was isolated from chickens in Natal, South Africa (Mouahid *et al.*, 1992). Horner *et al.* (1992) have also reported on the isolation of NAD-independent organisms from chickens suffering from typical infectious coryza symptoms in Natal. The work of Bragg *et al.* (1993) corroborates the observations of Mouahid *et al.* (1992), who identified NAD-independent South African isolates of *H. paragallinarum*, by means of DNA/DNA hybridization, and argues against the suggestion of Horner *et al.* (1992) that the NAD-independent isolates, made from chickens with symptoms of infectious coryza in Natal cannot be classified as *H. paragallinarum* based purely on the NAD independence of these isolates.

The argument was based on the work of Gromkova and Koornhof (1990) who reported NAD independence in 4 organisms biochemically indistinguishable from *H. parainfluenza*, except for the fact that they were capable of growth without NAD. As such, they could not be classified as *H. parainfluenza* according to the current taxonomic criteria. It was later established by Windsor *et al.* (1991) that these isolates carried a small 5,25kb plasmid which, if lost or removed, rendered the isolates NAD dependent. It can therefore be concluded from their work that *H. parainfluenza* is capable of acquiring a small plasmid, which renders the isolate NAD independent. These isolates must, however, still be classified as *H. parainfluenza* in spite of the naturally occurring NAD independence. Based on the *H. parainfluenza* studies of Gromkova and Koornhof (1990), a crude DNA extract of NAD-independent *H. paragallinarum* was used to transform NAD-dependent isolates rendering it NAD-independent. It was then suggested that the genes conferring the independence were located on a plasmid, analogously to the plasmid located in *H. parainfluenza* (Bragg *et al.*, 1993)

2.2.2 Growth conditions

The reduced form of NAD (NADH) (1.56-25µg/ml media) or its oxidized form and sodium chloride (NaCl) (1-1.5%) are essential for growth of *H. paragallinarum* (Rimler *et al.*, 1977). Chicken serum (1%) is required by some strains. Brain heart infusion

(BHI), tryptose agar, and chicken meat infusion are some basal media to which supplements are added. The pH of these media varies from 6.9-7.6. Other sources of NAD include yolk from chicken embryos, fresh yeast extract and chicken or sheep serum (Yamamoto, 1991). The organism may be maintained on blood agar plates by weekly passages. Young cultures maintained in a candle jar would remain viable for 2 weeks at 4°C. A number of bacterial species excrete NAD and have been used as "feeder" cultures to support growth of *H. paragallinarum* (Page, 1962a). The organism is commonly grown in an atmosphere of carbon dioxide; however, it is not an essential requirement, since the organism is able to grow under reduced oxygen tension or anaerobically (Elliot and Lewis, 1934; Page, 1962a). The minimum and maximum temperatures of growth are 25 and 45°C respectively, the optimal range being 34-42°C. The organism is commonly grown at 37-38°C. Tiny dewdrop colonies up to 0.3mm in diameter develop on suitable media. In obliquely transmitted light, mucoid (smooth) iridescent and rough non iridescent and other intermediate colony forms have been observed (Page *et al*., 1963).

2.2.3 Biochemical characterization

All the recognized taxa of the avian *haemophili* contain both ubiquinone and dimethylmenaquinone as respiratory quinones (Mutters *et al.*, 1985; Piechulla *et al.*, 1985). The cellular fatty acids of the avian *haemophili* have received little attention. Jantzen *et al.* (1981) examined one strain of *H. paragallinarum* among many strains of other members of the genera *Haemophilus, Actinobacillus* and *Pasteurella* and found that all the strains had remarkably similar fatty acid profiles. Blackall and Yamamoto (1989) used standardized SDS-PAGE to show that isolates of *H. paragallinarum* possess similar protein profiles with no correlation between protein profiles and pathogenicity. A medium has been developed for fermentation pattern investigation, consisting of phenol red broth enriched with NaCl (1%), NADH (25µg/ml), chicken serum (1%), and carbohydrates (1%). Using this broth and a standard test procedure, a large number of strains from various parts of the world were tested and found to produce acid in fructose, glucose, and mannose; none fermented trehalose or galactose. Acid from other sugars was variable or did not occur (Hinz and Kunjara; Rimler *et al*., 1977). Hydrogen sulfate

and indole are not produced, gelatin is not liquefied and litmus and methylene blue milk does not changed color (Bornstein and Samberg, 1954). Nitrates are reduced (Clark and Godfrey, 1961) and catalase activity is absent (Page, 1962a).

2.2.4 Serologic classification and immunogenic properties

Little is known about antigens and other substances in *H. paragallinarum* that are responsible for pathogenicity and immunogenicity of these organisms. Antigens participating in an agglutination reaction have been studied by various workers (Page, 1962a; Sawata *et al.*, 1978 and 1979) for serologic typing of the organisms.

2.2.4.1 Hemagglutinins of *Haemophilus paragallinarum*

Hemagglutination of *H. paragallinarum* was first demonstrated by Kato *et al.* (1965) using a serotype 1 strain and freshly collected chicken erythrocytes (RBC). The hemagglutinating antigen was correlated with immunogenicity of the serotype 1 organisms, and hemagglutination-inhibition (HI) antibodies were used to evaluate the efficiency of vaccines. The original serological classification of *H. paragallinarum* was performed by Page (1962a), who used an agglutination test to recognize three serovars; A, B, and C. Other methods described by Hinz (1980) recognized six serovars and Kume *et al.* (1983) recognized three serogroups and seven serovars. Neither Hinz nor Kume's schemes have been widely used, and the Page scheme continues to be the most commonly used serotyping scheme for *H. paragallinarum*. Blackall *et al.* (1990b) used the Page scheme to identify a range of Australian and non-Australian isolates of *H. paragallinarum* and found all the isolates studied could not be classified, being either nonagglutinable or auto-agglutinating. Yamaguchi *et al.* (1989) reported that isolates of *H. paragallinarum* show hemagglutinating activity if hyaluronidase treated cells and formaldehyde-fixed chicken erythrocytes are used.

More than one type of hemagglutinin was found for *H. paragallinarum* serotype 1 organisms. Sawata *et al.* (1984) found the presence of 3 types of hemagglutinin, which were designated HA-L, HA-HL, and HA-HS. The HA-L hemagglutinin was defined as

heat-labile, trypsin-sensitive, hyaluronidase-resistant, and active against glutaraldehyde (GA)-fixed RBC. The HA-HL hemagglutinin was defined as heat-labile and trypsin-resistant, whereas HA-HS hemagglutinin was defined as heat-stable and trypsin resistant (Sawata *et al.*, 1984). Kume *et al.* (1983) illustrated that the HA-L hemagglutinin is the only one responsible for immunogenicity among the 3 types of hemagglutinins.

In a study of the hemagglutinating activity of *H. paragallinarum* serotype 2, Sawata *et al.* (1982) found a correlation between the heat-labile, trypsin-sensitive L antigen that could be divided into 3 factors: serotype 1-specific L1, serotype 2-specific L2, and common L antigen L3, which is shared by serotypes 1 and 2. The HA-HS was also found to be a common antigen shared by both serotypes and may be located on the surface of the cells. Kume *et al.* (1980) investigated the relationship between protective activity and antigenic structure of *H. paragallinarum* serotypes 1 and 2. Their results showed that a close relationship exists between protective activities and phenotypic form and also between protective activities and L antigens. Kume *et al.* (1980) also found that unencapsulated organisms were lacking in their pathogenicity, HA activity, HI antibody and NT agglutinin (anti serotype-specific agglutinin)- producing ability and protective activity. Protection was found to be correlated with serotype-specific antigens L1 and L2 and the antigens treated by trypsinization or heating at 121°C lost their HA activity, HI antibody- and agglutinin-producing ability. It was shown by Yamaguchi *et al.* (1993) that the HA antigen of *H. paragallinarum* does indeed play an important role in pathogenicity and immunogenicity, based upon work done on a serovar C mutant strain lacking a hemagglutinating antigen. Immunity induced by bacterins is serotype specific (Rimler *et al.*, 1977; Kume *et al.*, 1980) and closely correlates with serotype specific L and HA-L antigens (Sawata *et al.*, 1979). Conversely, the findings of Rimler and Davis (1977) that live organisms induce a broader-based cross-protection between serotypes suggest that a common antigen may be important in conferring immunity. The discrepancy in these two observations might be due to differences in quality of the immunogen (bacterin vs. live organism), inoculation methods, challenge strain, dose, etc (Kume *et al.*, 1980). Irrespectively of this question, one cannot disregard the importance of immunotype specificity in bacterin preparation.

Since dissociation of *H. paragallinarum* is a common phenomenon (Sawata *et al.*, 1979), care should be taken in selecting the proper colony morphology, use the proper medium, and incubation period to obtain the most product. The relationship between the various antigens examined by different workers is not quite clear. We do not know whether type 1 HA, HA-L, and the purified hemagglutinin of Iritani *et al.* (1980) are the same or different antigens. It seems probable that there is not just one protective antigen. A number of different antigens, outer membrane proteins, polysaccharides and lipopolysaccharides are all likely to be involved. The final consideration on the nature of protective antigens is that, inactivated vaccines confer less cross-protection than natural infections. This implies that there are protective antigens produced by *H. paragallinarum in vivo*, which are either not produced or produced in greatly reduced amounts under *in vitro* conditions (Blackall, 1989). These studies show that there is a great need to develop live, stable, attenuated strains that would be safe to use in non-immune hosts, would provide improved protection against clinical disease and development of lesions, and would be practical for commercial development.

2.2.4.2 Virulence associated factors

Information on the factors associated with the virulence of *H. paragallinarum* is limited. Sawata *et al.* (1979) suggested that the L and HA-L antigens located in the outer membrane were responsible for adherence to the sinus mucosal surface. Sawata *et al.* (1982) also suggested that the hyaluronic acid capsule might be the primary structure associated with attachment, rather than the HA-L and L antigens and Kume *et al.* (1980) showed that the virulence factor(s) could be dissociated from protective factor(s). Variants possessing both capsular material and L antigen caused coryza and induced immunity; those not possessing a capsule but having L antigen were not pathogenic and were immunogenic, and those lacking both capsule and L antigen were nonpathogenic and non-protective. It is important to have a clear idea of the gram negative cell wall structure that precedes the capsule, before the biosynthesis and transport of capsular polysaccharides as well as the role that the capsule plays in the virulence of bacteria can be investigated.

2.3 The bacterial cell wall

The cell wall of bacteria is chemically unique and of immense practical importance. Because of the concentration of dissolved solutes inside the bacterial cell, a considerable turgor pressure develops, estimated at 2 atmospheres in a bacterium like *Escherichia coli*. To withstand these pressures, bacteria contain cell walls, which also function to give shape and rigidity to the cell. Gram positive and gram negative cells differ markedly in the appearance of their cell walls, the gram negative cell wall is a multi layered structure and quite complex (Brock *et al.*, 1994).

2.3.1 Structure and function

In the cell wall, the peptidoglycan is the layer that is primarily responsible for strength of the wall, sharing a chemical similarity in both gram positive and –negative bacteria (Fig. 1). Peptidoglycan is a thin sheet composed of two sugar derivatives and a small group of amino acids, which are connected to form a repeating structure, the glycan tetrapeptide. The sheet in the glycan chains formed by the sugars are connected by peptide cross-links formed by the amino acids. The shape of the cell is thought to be determined by the lengths of the peptidoglycan chains and by the manner and extent of cross-linking of the chains. This two-dimensional web can readily bear stress in any direction. In gram negative bacteria, only about 10% of the wall is peptidoglycan, the remainder being present in an outer wall layer outside the peptidoglycan layer, called the outer-membrane or lipopolysaccharide (LPS) layer (Fig. 1) (Brock *et al.*, 1994).

This layer is effectively a second lipid bilayer, but is not constructed solely of phospholipids as in the cytoplasmic membrane, but also contains polysaccharide and protein. The lipids and polysaccharides are intimately linked in the outer layer to form specific lipopolysaccharide structures (Fig. 1). The polysaccharide portion of the lipopolysaccharide is formed by sugars connected in four- or five membered sequences, which often are branched. When the sugar sequences are repeated, the long polysaccharide is formed. The repeating oligosaccharide units protrude like minute fibers from the outer membrane surface. Since they represent the outer surface of the cell and

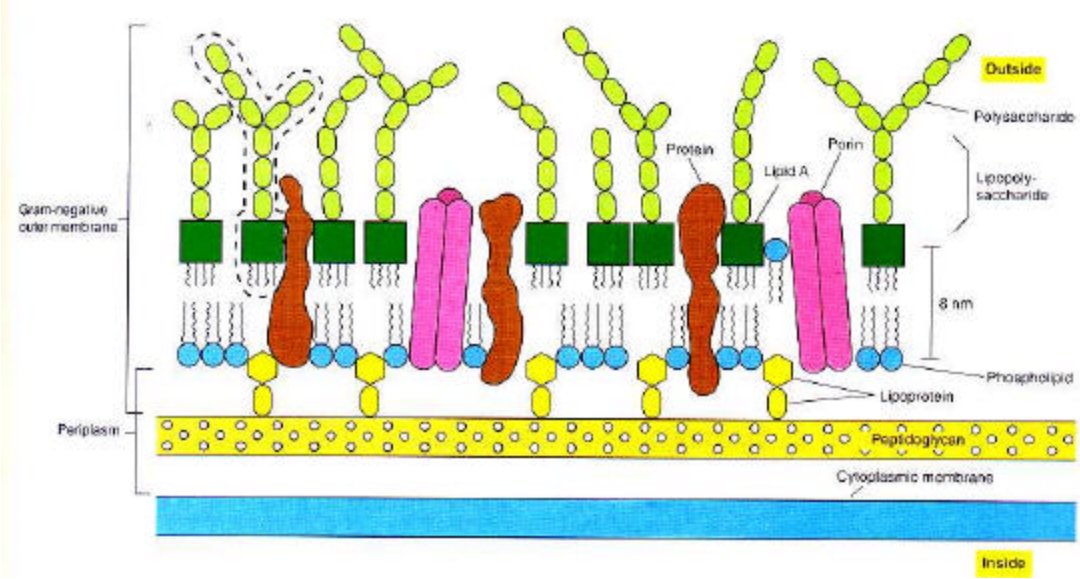

Figure 1: Arrangement of lipopolysaccharide, lipid A, phospholipid, porins and lipoprotein in the gram negative bacterium *Salmonella*. Dashed lines circle the O antigen. (Brock *et al.,* 1994)

are composed of specific carbohydrate structures, these fibers are intensely immunogenic – hence the term O antigen that is applied to the fibers. Production of antibodies directed against O antigens represents a primary defense mechanism used by vertebrates against bacterial infection. Bacteria have responded evolutionarily by being able to change O antigen structure by rapid genetic change. Consequently, there exist hundreds of different serotypes of bacteria, each with a different O antigen-repeating unit (Matthews and Van Holde, 1996). The outer membrane layer of gram negative bacteria is frequently toxic to animals. The toxic properties are associated with part of the LPS layer. The term endotoxin is frequently used to refer to this toxic component. The lipid portion of the lipopolysaccharide, referred to as lipid A (Fig. 1), is not a glycerol lipid, but instead the fatty acids are connected by ester amine linkage to a disaccharide composed of N-acetylglycosamine phosphate. Fatty acids commonly found in lipid A include caproic, lauric, myristic, palmitic and stearic acids. In the outer membrane, the LPS associates with various proteins to form the outer half of the unit membrane structure. A lipoprotein complex is found on the inner side of the outer membrane; a small protein that serves as an anchor between the outer membrane and peptidoglycan (Brock *et al.,* 1994).

2.3.2 Porin proteins

Unlike the cytoplasmic membrane, the outer membrane of gram negative bacteria is relatively permeable to small molecules, even though it is basically a lipid bilayer. Proteins called porins (Fig. 1) are present in the outer membrane and serve as membrane channels for the entrance and exit of hydrophillic low-molecular-weight substances. Several porins have been identified and both specific and non-specific classes are known. Non-specific porins form water filled channels through which small substances of any type can pass. By contrast, some porins are highly specific because they contain a specific binding site for one or more substances. Structural studies have shown that most porins are proteins containing three identical sub-units. Porins are transmembrane proteins and associate to form small membrane holes about 1nm in diameter. Apparently a mechanism exists for opening and closing the pores, because resistance to certain antibiotics is related to porin structure. The outer membrane layer is not permeable to protons as well as enzymes or other larger molecules. In fact, one of the main functions of the outer layer may be to keep certain enzymes, which are present outside the cytoplasmic membrane, from diffusing away from the cell (Brock *et al.*, 1994).

2.3.3 Periplasmic proteins

These enzymes are present in a region called the periplasm (Fig. 1). This space between the outer surface of the cytoplasmic membrane and the inner surface of the LPS containing outer membrane, occupies a distance of some 12-15nm and is gel-like in consistency, presumably because of the abundance of periplasmic proteins found there. The periplasm of gram negative bacteria generally contains three types of proteins: *hydrolytic enzymes,* which function in the initial degradation of food molecules, *binding proteins,* that begin the process of transporting substrates, and *chemoreceptors,* which are proteins involved in the chemotaxis response. Periplasmic binding proteins function to bind a substance and bring it to the membrane-bound carrier. These processes are probably not linked to the proton gradient but may instead use ATP as energy source. Wang *et al.* (1998) showed that O antigen is synthesized separately on a lipid carrier and

the mature O antigen is then transferred to core lipid A to form LPS before being translocated to the outer membrane (Brock *et al.,* 1994).

2.4 Bacterial capsules

2.4.1 Biological significance

Capsules have long been associated with virulence properties of bacteria. Polysaccharide capsules are ubiquitous structures found on the cell surface of a broad range of bacterial species. The polysaccharide capsule often constitutes the outermost layer of the cell; as such, it may mediate direct interactions between the bacterium and it's immediate environment and has been implicated as an important factor in the virulence of many animal and plant pathogens (Cross, 1990; Moxon and Kroll, 1990; Roberts 1995). Capsules are important determinants of the behavior of bacteria within the animal host. To survive within the host, bacteria must be able to evade a diverse array of defense mechanisms that include complement mediated bacteriolysis, uptake and killing by phagocytes as well as cell-mediated immune mechanisms (Cross, 1990). Capsules may inhibit host bactericidal defenses not only by impeding the binding of antibody, but also by impeding the efficient fixation of complement on the surface of bacteria. This, in turn, may block the uptake and killing of bacteria by phagocytes via the complement receptors, or the formation of lytic pores by the membrane attack of complement at the cell membrane (Howard and Glynn, 1971). Because of their importance in the virulence of many bacteria and their usefulness as vaccines (Lee, 1987) for prevention of bacterial infections, capsules have been the subject of intensive investigation (Boulnois and Roberts, 1990).

2.4.2 Structure

Capsular polysaccharides are highly hydrated molecules that contain over 95% water (Costerton *et al.,* 1981). They are composed of repeating single units joined by

glucosidic linkages. They can be homo- or heteropolymers and may be substituted by both organic and inorganic molecules. Any two monosaccharides may be joined in a number of configurations as a consequence of the multiple hydroxyl groups within each monosaccharide unit that may be involved in the formation of a glycosidic bond. As a result of this, capsular polysaccharides are an incredibly diverse range of molecules that may differ not only by monosaccharide units, but also in how these units are joined together. The introduction of branches into the polysaccharide chain and substitution of both organic and inorganic molecules yield additional structural complexity (Roberts, 1996). Production of a capsule starts with the synthesis of the sugar components of the polysaccharides and their activation by conversion to nucleotide derivatives. Sugar biosynthesis and activation are generally considered to be cytoplasmic-based activities. In contrast, the subsequent polymerization is catalyzed by an inner membrane bound transferase complex. These transferases are poorly defined, with an unknown number of components and a catalytic mechanism which remains obscure. Polymerization is generally believed to involve a lipid carrier (undecaprenol or polyprenol phosphate) on which monosaccharides or oligosaccharides are assembled. Whether or not this lipid functions as a carrier for all capsule polysaccharides is not clear (Barr and Rick, 1987). Since many capsular polysaccharides are found associated with phosphatic acid, the endogenous acceptor might also contain this phospholipid. This phospholipid has been suggested to function as an anchor for extracellular polysaccharide in the outer membrane (Schmidt and Jann, 1982). The final stage of capsule production is the translocation of the polysaccharide to the cell surface and its organization into a capsule.

2.4.3 Genes involved in capsule formation

Many encapsulated gram negative bacteria, including *Escherichia coli*, *Neisseria meningitidis* and *Haemophilus influenzae*, possess clusters of genes responsible for capsule biosynthesis organized in operons. These operons often contain genes encoding (i) enzymes required for sugar nucleotide precursor synthesis, (ii) glycosyltransferases for polymerizing the exopolysaccharide, and (iii) proteins implicated in polysaccharide export (DeAngelis *et al.*, 1998). Studies by Frosch *et al.* (1992) demonstrated a broad

homology between the capsule gene loci of different encapsulated gram negative bacterial species. This homology includes proteins of the capsular polysaccharide transport system of *N. meningitidis*, *H. influenzae* and *E. coli*, indicating a common evolutionary origin of the molecular mechanisms of encapsulation. The capsules of *E.coli* have been grouped into two classes on the basis of physical, chemical and biochemical criteria (Jann and Jann, 1982). The type II capsules resembles those of *H. influenzae* and *N. meningitidis*. These capsules characteristically have molecular weights of less than 50 000 and frequently contain 2-keto-3-deoxy-D-mannooctulonic acid (KDO) and/or NeuNAc. In contrast, the type I capsules have molecular weights in excess of 100 000 and resemble those produced by *Klebsiella* sp (Boulnois and Roberts, 1990).

2.4.4 Capsule polysaccharide transport

The export of capsular polysaccharides in gram negative bacteria from their site of synthesis on the inner face of the cytoplasmic membrane onto the bacterial surface presents a unique challenge to the micro-organism. It requires the translocation of a high molecular weight, negatively charge macromolecule across two lipid bilayers. Understanding this process offers potential benefits in terms of engineering polysaccharides of biomedical importance in bacteria and in designing new anti-microbials that inhibit this process. In contrast to protein secretion, our understanding of how capsular polysaccharide transport is achieved is currently scant (Arrecubieta *et al.*, 2001). The expression of capsular polysaccharides (or K antigens) in *E.coli* offers an experimentally tractable system in which to try to understand the mechanisms of polysaccharide transport. The ABC transporter-dependent systems for polymerization and export of group 2 capsules take place on the cytoplasmic face of the plasma membrane and involve the sequential action of glycosyltransferases that elongate the polysaccharide at the non-reducing end. The nascent polysaccharide is transported across the plasma membrane by an ABC-2 (ATP-binding cassette) transporter. In the case of group 2 capsules, this comprises the KpsM (the transmembrane component) and KpsT (the ATPase component) illustrated in Figure 2 (Whitfield and Roberts, 1999). A working model for the action of this transporter has been proposed (Bliss and Silver,

1996). Studies on the biosynthesis of the K5 capsule have indicated that biosynthesis of group 2 capsules involves a hetero-oligomeric membrane-bound complex on the plasma membrane (Rigg *et al.*, 1998). This consists of the proteins (KfiA-D) required for the polymerization of the K5 polysaccharide, together with the KpsC, M, S and T proteins that are involved in the translocation of group 2 polysaccharide across the plasma membrane (Fig. 2). Analysis of mutants lacking individual Kps proteins indicated that the KpsC, M, S and T proteins play critical roles in the formation and stabilization of the biosynthetic/export complex on the plasma membrane. The conservation of these Kps proteins in *E. coli* expressing group 2 capsules (Roberts, 1996) is consistent with the biosynthetic complex being a conserved feature in the biosynthesis of group 2 capsules. The formation of such a complex would permit the spatial co-ordination of polymer initiation, elongation, attachment of phosphatidyl-Kdo (Fig. 2) and export. Complex formation could optimize the efficiency of the assembly process by increasing the effective concentrations of the proteins at a single site (Whitfield and Roberts, 1999).

Moving high-molecular-weight polymers across the outer membrane represents a significant practical and conceptual problem, and translocation remains a major open question in capsule assembly. Translocation of *E.coli* group 1 and 2 capsules to the cell surface occurs at specific sites where the plasma and outer membranes appear to come into close opposition, when examined by electron microscopy (Whitfield and Valvano, 1993; Roberts, 1996; Jann and Jann, 1997). The nature of these sites (termed 'membrane-adhesion sites' or 'Bayer junctions') has been controversial (Whitfield and Valvano, 1993) but growing evidence indicates the widespread involvement of periplasmic 'scaffolds', comprising translocation machinery that transfers various molecules in or out, across the outer membrane. Such machinery would form a transient link between the two membranes. The translocation of group 2 capsular polysaccharide to the cell surface is mediated by the KpsE and KpsD proteins (Fig. 2), in a process that also requires porin proteins (Bliss and Silver, 1996; Roberts, 1996). The KpsE and D proteins appear to be linked to the biosynthetic/export complex located on the plasma membrane to form a multiprotein 'capsule assembly complex'. Osmotic shock releases cytoplasmic components of this complex into the periplasm, a characteristic property of complexes associated with areas of adhesion between the plasma and outer membrane

(Rigg *et al.*, 1998). Therefore, the group 2 capsule assembly complex may form at such sites and result in a direct continuum between the cytoplasm and the cell surface. The recent observations that the KpsT protein (Fig. 2) may be transiently exposed in the periplasm (Bliss and Silver, 1996) is consistent with a direct link between the cytoplasmic components of the capsule assembly complex and the periplasm. A scenario in which biosynthesis and translocation are part of a linked dynamic process, involving multiple protein-protein interactions, would explain the pleiotropic nature of mutations in

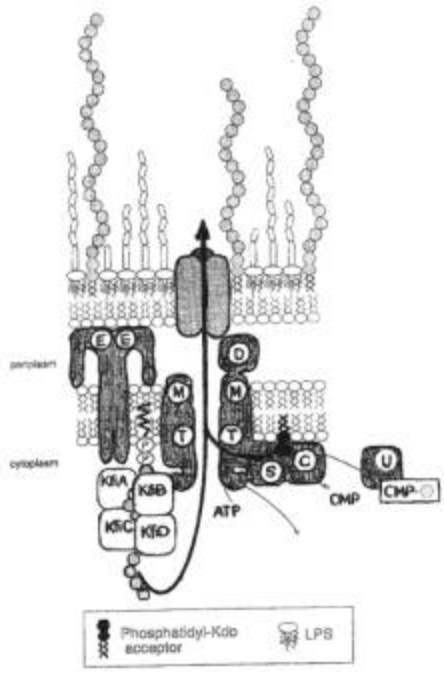

Figure 2: A model for assembly of the K5 capsule demonstrating the ABC-transporter-dependent systems for polymerization and export. Proteins involved in polymer synthesis (KfiABCD) interact with a "scaffold" comprising KpsCMST to form a biosynthesis-export-translocation complex on the plasma membrane. The polymer is shown growing on an undecaprenyl purophosphate carrier before being transferred to a phosphatidyl-Kdo. (Whitfield and Roberts, 1999)

certain *kps* genes. The ability of conserved Kps proteins to export chemically different group 2 polysaccharides from the cytoplasm suggests that the nascent polysaccharide molecules carry some common export motif that is recognized in order to engage the translocation apparatus (Roberts, 1996).

2.4.5 Capsules of related HAP species

Three species from the genera *Haemophilli, Actinobacilli* and *Pasteurella* (HAPs) of the family Pasteurellaceae; *Haemophilus influenzae, Actinobacillus pleuropneumoniae* and

Pasteurella multocida show considerable homology between the genes responsible for polysaccharide export. *P. multocida* is the etiological agent of fowl cholera, bovine haemorrhagic septicemia and atrophic rhinitis in pigs. Many strains of *P. multocida* express a capsule on their surface (Chung *et al.*, 1998). The composition of the *P. multocida* capsule has been investigated in several serotypes and the capsular material of *P. multocida* serotype A strains tested has shown to contain hyaluronic acid, a polymer of D-glucuronic acid and N-acetyl-D-glucosamine (Fig. 3) (Rosner *et al.*, 1992). Hyaluronic acid appears to be responsible for most of the suggested virulence properties of the capsule.

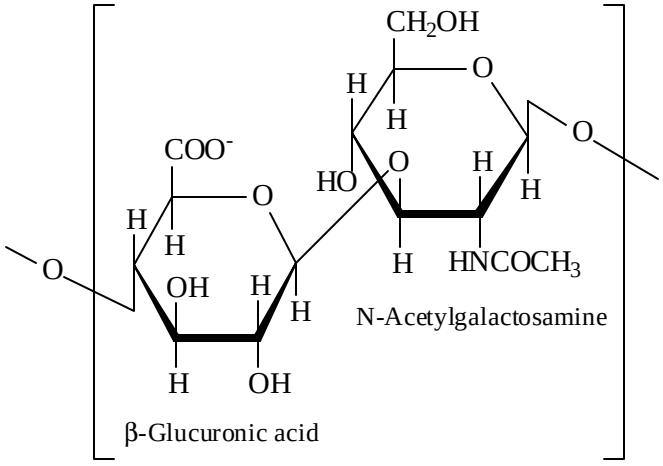

Figure 3: Hyaluronic acid

In general, hyaluronic acid is believed to influence the physical properties of the capsule and probably prevents phagocytosis by phagocytic cells, perhaps by mimicking host antigens, since the acid is naturally present in the host tissues (Christensen and Bisgaard, 1997). Studies done by DeAngelis *et al.* (1998) showed that wild-type cells treated with hyaluronidase became complement-sensitive and were more readily phagocytosed in comparison with untreated microbes.

2.4.6 *Pasteurella multocida* capsule locus and postulated protein products

The entire capsule locus of *P. multocida* has been cloned and sequenced. According to Chung *et al.* (1998) the locus is divided into three regions (Fig. 4). Region 1 comprises four open reading frames (ORFs) which are involved in the transport of the capsule polysaccharide to the surface. Region 2 comprises five ORFs whose postulated protein products are involved in the biosynthesis of the polysaccharide capsule. Region 3 comprises two ORFs whose postulated protein products show similarity to proteins that are involved in the phospholipid substitution of the polysaccharide capsule.

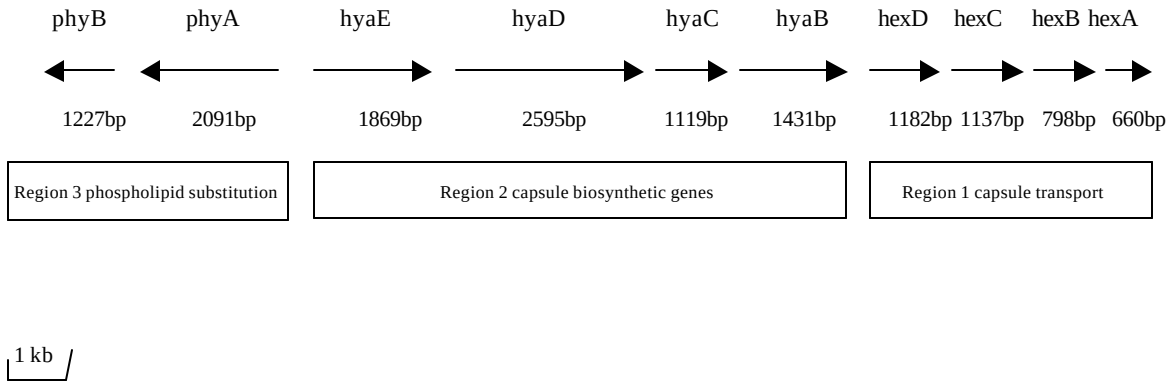

Figure 4: Genetic map of the capsule locus in *P. multocida*. The arrows indicate the direction of transcription of the ORFs. The organization of the locus into three regions is indicated by solid boxes. (Chung *et al.,* 1998; Townsend *et al.,* 2001)

2.4.6.1 Capsule transport genes

The deduced protein products of the four ORFs, *hexA*, *hexB*, *hexC* and *hexD* (*hex* for hayluronic acid export) coded for by region 1, show similarity to the export proteins of *H. influenzae* (Kroll *et al.,* 1990) as well as corresponding proteins from *A. pleuropneumoniae* and *E.coli* (Roberts, 1996). Overlapping of stop and start codons of *hexC*, *hexB* and *hexA* of *P. multocida* as well as *bexC*, *bexB* and *bexA* of *H. influenzae*,

suggest that at least these three genes, if not all four, are translationally coupled (Kroll *et al.*, 1990). In comparison to the *bexABC* and *D* genes of *H. influenzae* (Kroll *et al.*, 1990), Chung *et al.* (1998) stipulated that HexA possesses the typical ATP-binding domains A and B (Walker motifs) and is therefore predicted to be the ATP-binding component of the capsule exporter. HexB is highly hydrophobic, suggesting that it form an integral inner membrane component of the polysaccharide exporter. Transposon mutagenesis of *bexC* done by Kroll *et al.* (1990) suggested that this gene might be a periplasmic protein. Prediction of protein subcellular localizaion of the HexC protein performed with PSORT (Nakai and Kahehisa, 1991), suggested an inner membrane protein, possibly with a periplasmic domain, concurring with the transposon mutagenesis data on BexC (Chung *et al.*, 1998). The N-terminus of BexC having phosphatase activity suggests that the protein is either excreted into the periplasm with cleavage of an N-terminal leader peptide or anchored in the bacterial inner membrane by an uncleaved N-terminal domain to protrude into the periplasm. It is therefore a candidate for a periplasmically orientated component of a capsular polysaccharide exporter. HexD shows similarity to the BexD and CtrA proteins from *H. influenzae* and *N. meningitidis*, respectively. Based on the similarity between HexD and CtrA, it is believed that HexD is an outer membrane protein with porin properties (Frosch *et al.*, 1992). In addition, there is a signal peptidase II consensus sequence LXYC (Von Heijne, 1989) at positions 24-27 within HexD, suggesting that this is a lipoprotein.

2.4.6.2 Capsule biosynthesis genes

The four ORFs, *hyaB*, *hyaC*, *hyaD* and *hyaE* (*hya* for hyaluronic acid) hypothetically encodes proteins that are involved in the formation of precursor activated sugar monomers and the assembly of the capsular polysaccharide polymer (Chung *et al.*, 1998). The deduced protein HyaB shows similarity to known glycosyl transferases in region 2 of other bacterial species. At least two types of glycosyl transferases have been proposed, those that transfer multiple sugar residues (processive) to the acceptor and those that transfer only a single sugar residue (non-processive) to the acceptor (Saxena *et al.*, 1995). According to Saxena *et al.* (1995), hydrophobic cluster analysis showed that processive

glycosyl transferases possess two conserved domains, A and B, while non-processive glycosyl transferases possess only domain A. In the *P. multocida* glycosyl transferase HyaB, there exist the conserved aspartate residues of domain A and no consensus motif of domain B, suggesting that the putative glycosyl transferase is a non-processive enzyme, responsible for adding one of the monomer monosaccharides. In addition *hyaC* encodes a deduced protein HyaC that shows a highly conserved alignment with UDP-glucose dehydrogenase (Chung *et al.,* 1998). The presence of this gene in the capsule locus of *P. multocida* was expected as the enzyme UDP-glucose dehydrogenase catalyses the conversion of UDP-glucose to UDP-glucuronic acid, a monomeric precursor of the *P. multocida* hyaluronic acid capsule. No function could as yet be ascribed to either HyaB or HayE (Chung *et al.,* 1998).

2.4.6.3 Phospholipid substitution

While region 1 and 2 genes are transcribed in the same direction, region 3 consists of two genes *phyA* and *phyB* (*phy* for phospholipid substitution of hyaluronic acid) that are transcribed in the opposite direction. The phospholipid substitution of capsular polysaccharides at the reducing end has been shown to act as a hydrophobic anchor for the polysaccharide capsule in the outer membrane (Kuo *et al.,* 1985). These encoded proteins PhyA and PhyB are significantly similar to proteins shown to be involved in phospholipid substitution of capsular polysaccharides in *E. coli* and *N. minigitidis* (Chung *et al.,* 1998).

2.4.6.4 Promoter elements

A search of the entire sequence of *P. multocida* revealed only a single putative promoter upstream of *hyaE* in region 2. This promoter has a -10 TATAAT 156bp upstream of the start of *hyaE* and a -35 box ATCACA (with 2bp mismatch to the consensus) exactly 17bp upstream of the -10 box. There appear to be no other sequence resembling σ^{70} or other promoter elements in the *cap* locus of *P. multocida*. Regions 1 and 2 thus appear to

constitute a single transcriptional unit. The promoter sequence for region 3 do not resemble any known σ^{70} promoter elements (Chung *et al.,* 1998).

2.4.7 Construction of acapsular mutants

Genetically defined acapsular mutants have been shown to have reduced virulence in a number of organisms (Boyce *et al.,* 2000a). The role of the capsule has been investigated at molecular level by Boyce *et al.* (2000b). A mutant defective in the export of the *P. multocida* capsule was constructed by allelic exchange. Using the sequence of the *P. multocida cap* locus, a DNA fragment was constructed with a *tet*(M) insertion within the capsule export gene *hexA.* Virulence assays in mice indicated that acapsular *P. multocida* B:2 were 10^6 fold less virulent than their encapsulated counterparts (Boyce and Adler, 2000). This data proves that the capsule is a major virulence factor in *P. multocida*. The reduction in virulence is due primarily to the rapid removal of the acapsular bacteria from the blood and other organs, and this removal is likely due to an increased susceptibility to phagocytosis (Boyce and Adler, 2000). Similar studies have been conducted on the *bexA* gene of *H. influenzae* (Kroll *et al.,* 1988). A frame shift mutation engineered at a restriction site within the ORF resulted, when introduced into the *cap* locus in the chromosome, in the expression of a mutant phenotype. From their study of this mutant, Kroll *et al.* (1988) deduced that the *bexA* gene product is necessary for the successful export of capsule polysaccharides to the surface.

Current *A. pleuropneumoniae* vaccines are known to be inadequate, particularly against the development of lesions and chronic infections (Higgins *et al.,* 1985; Nielsen, 1976 and Nielsen, 1984). Attenuated vaccines, though lacking extracellular toxins, do provide some protective immunity, indicating that somatic antigens may also contribute to protection (Nielsen, 1979). Clonal, non-iridescent mutants of *A. pleuropneumoniae* were isolated by Inzana *et al.* (1993) following chemical mutagenesis with ethyl methanesulfonate, showing the absence of any detectable capsule. Using a severe, intratracheal challenge that kills or causes acute lesions of pleuropneumonia in nonimmune pigs challenged in order to test the limits of the protective efficacy of these mutants and to ensure that all challenges were reproducible and identical in dose. All

the pigs immunized twice with 2 X 10^9 CFU mutant strain in PBS were significantly protected against challenge and showed 100% mortality.

These noncapsulated mutants reported by Inzana *et al.* (1993) have several advantages that make them ideal live vaccine candidates. (i) They are highly efficacious and appear to provide protection similar to that induced by the parent against homologous and heterologous serotype challenge. (ii) They are safe and cannot persist or cause substantial infection in the lung and the release of recombinant, mutagenic agents into the environment is not a factor. (iii) They are extremely stable and have never reverted to the encapsulated phenotype *in vivo* or *in vitro*. (iv) They do not induce antibodies against the capsule, and immunized pigs can therefore be distinguished from infected ones. (v) They can replicate and induce a protective immune response when administered subcutaneously without any adjuvant, therefore, inconvenient and expensive aerosol or intranasal immunization is not required, and lesions or side effects due to adjuvants do not occur. (vi) A commercial preparation of this type of vaccine would be less costly than current killed vaccines, making it economically feasible. (Inzana *et al.*, 1993).

2.5 An alternative to commercial infectious coryza vaccines

Despite the widespread use of vaccines against IC, the disease remains a serious problem in the poultry industry, not only in South Africa but also in many other parts of the world. Currently all vaccines used against IC consist of bacterins produced by inactivated cultures of the different serovars of *H. paragallinarum*. It has been well established that there is no cross protection between different serogroups (Rimler *et al.*, 1977; Kume *et al.*, 1980). Furthermore, serogroup C strains appeared to be less immunogenic than the serogroup A strains (Kume *et al.*, 1980) and provided incomplete cross protection among different serovar C strains. Another serious problem with the use of inactivated vaccines in layers is the need to vaccinate the birds by infection of the vaccine subcutaneously. Handling of birds in production for the purpose of vaccination result in a drop in egg production, which can be larger than when influenced by the disease. Thus, the birds can only be vaccinated up to 18 weeks of age. The average layer birds will be in production

for more than 70-80 weeks. Therefore, after 18 weeks of age these birds cannot be vaccinated again and protection afforded by the vaccines decrease with increasing age of the birds.

There is an urgent need for a live vaccine against IC, not only in South Africa, but worldwide. There are growing concerns with injecting poultry with inactivated vaccines that contain adjuvants. Although negative effects of the adjuvants are kept to a minimum, they cannot be completely prevented. The development of a live vaccine would provide better protection, as there is substantial evidence that live viral vaccines provide better protection than the inactivated alternative. Another significant advantage of a live vaccine is that it can be administered to the birds via the drinking water or by spraying. This means that the birds need not be handled and can be vaccinated throughout their productive lives, greatly improving the levels of production. The successful development of such vaccines would capture large market shares in the poultry industry in any country and would therefore be of huge economic interest.

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Chapter 3

Isolation, cloning and characterization of the capsule transport genes of *Haemophilus paragallinarum*

3.1 Abstract

Capsules from a range of pathogenic bacteria are key virulence determinants. Three of the HAP organisms *Haemophilus influenzae*, *Actinobacillus pleuropneumoniae* and *Pasteurella multocida* are virulent pathogenic bacteria that show considerable homology between the genes responsible for capsule polysaccharide export. Degenerate primers designed from analysis of a multiple sequence alignment of the capsule transport genes of the three HAPs were used to amplify a 2.6kb fragment representing a segment of the *Haemophilus paragallinarum* capsule transport gene locus. This fragment was used as a digoxigenin labeled probe to isolate the complete *H. paragallinarum* capsule transport gene locus from digested genomic DNA. The DNA was sequenced and analysis revealed the presence of four genes which showed high homology with the transport genes of all three HAPs on both nucleotide and protein level. The four genes designated *hctA*, *B*, *C* and *D* (for *H. paragallinarum* capsule transport genes) are 648bp, 798bp, 1164bp and 1188bp in size respectively. The postulated protein products of these genes form an ABC-transporter system responsible for transport of the capsule polysaccharides to the cell surface.

3.2 Introduction

The capsules of many bacteria have been implicated as virulence factors through enhanced survival due to the evasion of host immune response by capsulated strains. The presence of a surface capsule protects the bacteria from phagocytosis and also against the bactericidal action of complementation (Robbins *et al.*, 1980). To begin understanding the role of the capsule in virulence, most researchers sought to identify the genes involved in capsular polysaccharide export and biosynthesis. The genetic organization of the group II capsule gene loci of *H. influenzae* type b (Kroll *et al.*, 1989; Kroll, 1992), *E. coli* K1 and K5 (Boulnois *et al.*, 1987; Jann and Jann, 1990), *P. multocida* M1404 (B:2) (Boyce *et al.*, 2000) and *A. pleuropneumoniae* serotype 5a (Ward and Inzana, 1997) have been determined and is very similar. In each of these species, a central, DNA segment necessary for capsular polysaccharide biosynthesis is flanked by DNA encoding proteins for capsule export. Substantial homology occurs in the genes required for capsular polysaccharide export among these species, suggesting a common evolutionary origin (Frosch *et al.*, 1991). For this study, the similarities among *H. influenzae*, *A. pleuropneumoniae* and *P. multocida* capsular transport genes were considered on nucleotide as well as protein sequence, in view of the fact that these species also belong to the family Pasteurellaceae. The capsule transport gene organization of *H. influenzae* and *A. pleuropneumoniae* are similar to that of *P. multocida* (Boyce *et al.*, 2000; Ward and Inzana, 1997) with the gene groups named *bexABCD*, *cpxABCD* and *hexABCD* respectively.

The nucleotide sequence for each organism was obtained from the Genbank database [(http://wwww.ncbi.nlm.nib.gov/); *H. influenzae* - X54987, *A. pleuropneumoniae* - U36397 and *P. multocida* - AF067175] and subjected to a multiple sequence alignment using DNAssist as analytical aid. Conserved areas from each gene, were selected and degenerate primers designed in both forward and reverse orientation for use in PCR on *H. paragallinarum* genomic DNA. These degenerate primers served as the starting block for isolating and cloning the *H. paragallinarum* capsule transport gene locus.

3.3 Materials and Methods

3.3.1 Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are shown in Table1. *Haemophilus paragallinarum* strain 1742 was grown in TM/SN media (1% Biosate peptone, 1% NaCl, 0.5% glucose, 0.1% starch and 0.0005% thiamine solution, Oleic acid-albumin complex and chicken serum as supplements) described by Blackall and Yamamoto (1990), in which 1.5% agar was used to solidify the media if required. In liquid culture the organisms were grown without aeration and on solid media in a candle jar at 37°C. *Escherichia coli* strain Sure®2 (Strategene) were grown with aeration in Luria-Bertani (LB) broth (Sambrook *et al.*, 1989) under selective pressure with 60µg/ml ampicillin in liquid and solid media when required.

Table 1: Bacterial strains and plasmids used in the isolation, cloning and characterization of the capsule gene locus of *H. paragallinarum*

Strain/plasmid	Relevant characteristic	Reference or source
Strains		
Hp 1742	NAD-independent, C3, wild type	Field isolate (Bragg *et al*., 1993)
E.coli Sure®2	For high-efficiency cloning of DNA with secondary structures. F'-episome, recA- and blue-white screening	Strategene
Plasmids		
pGemT-Easy	For cloning of PCR products, Amp^r	Promega
pGem3Z	Standard cloning vector, Amp^r	Promega
pGhct-p	2.6kb insert in pGemT-Easy, contains *hct***BC* and parts of *hctA* and *D*	This study
pGhct-c	6.15kb *Hind*III insert in pGem3Z, contains part of Region 2 and the entire Region 1 (*hctABCD*)	This study

* *Haemophilus paragallinarum* capsule transport gene

3.3.2 Identification of *Haemophilus paragallinarum* by means of PCR

There are several complications to the isolation and identification of *H. paragallinarum*. It requires complex media and additives for growth, is a relatively slow growing organism *in vitro*, which means that other organisms can overgrow and mask the presence of *H. paragallinarum* and it is not the only hemophilic organism that occurs in chickens (Blackall and Yamamoto, 1990). Identification of *H. paragallinarum* was made easy by Chen *et al.* (1996) with the design of PCR primers ensuring amplification of a 500bp DNA fragment when *H. paragallinarum* is used as template. The PCR test is consistently negative with all closely related organisms, making this an ideal identification method possible within hours.

In order to confirm the identity of *H. paragallinarum* strains, sequence specific primers HP-1F and HP-1R (Table 2) were used in a PCR procedure in 50µl volume. The reaction mixture consisted of a 10X dilution of reaction buffer (100mM Tris-HCl, 15mM $MgCl_2$, 500mM KCl, pH 8.3), 0.2mM dNTP mixture, 2pmol of each primer and 5U of *Taq* polymerase (Roche Molecular Biochemicals). A single *H. paragallinarum* colony was used as template to amplify the 500bp fragment under reaction conditions starting with an initial denaturation at 94°C for 10min, followed by 25 cycles of 94°C for 30sec, 55°C for 30sec, 72°C for 1min and a final cycle at 72°C for 5 min.

3.3.3 Preparation and analysis of genomic- and plasmid DNA

Genomic DNA was prepared from twenty 5ml liquid cultures of *H. paragallinarum* grown for 16 hours (Towner, 1991). The cells were harvested by centrifugation at 3000g for 10min at 4°C and weighed. The pellet was washed in TE-buffer (10mM Tris-HCl, 1mM EDTA) pH 8 and centrifuged again at 3000g for 5min at 4°C. The pellet was resuspended in resuspension buffer (50mM Tris-HCl, pH8, 0.7mM sucrose) and lysozyme (20mg/ml) was added before the suspension was incubated on ice for 5min. 0.5M EDTA (pH8) and 10% SDS were added, gently mixed and placed on ice for 5min. After the addition of digestion buffer (1% SDS, 50mM Tris-HCl pH8, 0.1M EDTA, 0.2M

NaCl, 0.5mg/ml proteinase K), the suspension was incubated at 55°C for 3-16 hours with mild shaking. Biophenol (pH calibrated) was added to the lysate and incubated a further 3 hours at 25°C with constant inversion. Cell debris was removed by centrifugation at 4000g for 10min and the supernatant mixed with 5M NaCl and placed on ice for 5min. Genomic DNA was precipitated with 10ml 100% ethanol, spooled and washed in 70% ethanol. After drying, the pellet was suspended in TE-buffer and incubated at 50°C for 1 hour or kept at 4°C overnight before use.

Plasmid DNA was isolated by a rapid alkaline lysis method described by Sambrook *et al.* (1989) and resuspended in TE-buffer with 10µg/ml RNase.

Genomic- and plasmid DNA were analyzed by restriction enzyme digestion. Plasmid DNA was digested with *Eco*RI or *Hind*III for 1 hour, while genomic DNA was digested using either *Bam*HI, *Eco*RI, *Hind*III, PstI or *Xba*I for 3-16 hours . All the enzymes used in these digestions were obtained form Roche Molecular Biochemicals and used with the appropriate buffers as specified by the manufacturer.

3.3.4 PCR analysis and cloning techniques

PCR analysis was performed in the Perkin-Elmer geneamp 2400 thermocycler, with reaction chemicals obtained form Roche Molecular Biochemicals. *H. paragallinarum* genomic DNA from strain 1742 was used as template and PCR reactions were carried out in 50µl volumes. The reaction mixtures consisted of a 10X dilution of reaction buffer (100mM Tris-HCl, 15mM $MgCl_2$, 500mM KCl, pH 8.3), 2pmol of each degenerate primer (Table 2) in different combinations, 0.2mM dNTP mixture and 5U of *Taq* polymerase. The reaction conditions consisted of an initial denaturation cycle of 94°C for 5min followed by 25 cycles of 94°C for 30sec, 45°C for 30sec, 72°C or 2min and a final elongation cycle of 72°C for 5min.

Table2: Degenerate oligonucleotides used for the partial amplification of the capsule transport gene locus and sequence specific primers used for sequence analysis of *Haemophilus paragallinarum*

Name	Sequence
HctD-1F	5'- GAT AAA GAT WTW GTH TAT GTR TCR AAT GCA CC -3'
HctD-1R	5'- GGT GCA TTC GAC ACA TAT AC -3'
HctC-1F	5'- GCB TCY GAT ATT TAT RTT TCD SAA TCD AG -3'
HctC-1R	5'- CYA AAT AMA RTT GYT GGC GAT C -3'
HctB-1F	5'- ATG ATG TGG CGH AAT GCD TC -3'
HctB-1R	5'- AAC ATT TCY GWR CCR TGA ATC ATY GG -3'
HctA-1R	5'- ATY TTR GTT TCW CAT AGC CCG WVT -3' G
HctA-1F	5'- ATT TTA GTT TCT CAT AGT CCA ACC G -3'
Hcs-1F	5'- GCA AGA ATT TAT GCC GTT CG -3'
Hcs-1R	5'- CGG ACA GGT TCG GTA CC -3'
Hcs-2F	5'- GGA TTT GCT TGC AGT ACA G -3'
Hcs-3F	5'- GCT TGC GTC CAA AGT GGC -3'
Hcs-4F	5'- GTG CGA CTG ACT CGT GG -3'
Hcs-5F	5'- GTA TCT AAT GCA CCA TTG GC -3'
Hdp-1R	5'- TGA GAT CTC CAA GGT AGC TTA ACG G -3'
Hdp-2R	5'- GGC TGA CGG TGA TGT GCA AG -3'
HdpA-1F	5'- TGA GAT CTC TTC TAT TGA TGA GGC -3'
HdpA-1R	5'- TGA GAT CTC GTG GTC TAC ACT AAT C -3'
HP-1F	5'- TGA GGG TAG TCT TGC ACG CGA AT -3'
HP-1R	5'- CAA GGT ATC GAT CGT CTC TCT ACT -3'

The same reaction constituents and conditions applied for amplification of the partial *H. paragallinarum* capsule transport gene locus and producing a DNA probe for screening.

DNA fragments were recovered from gels or purified after digestion with the GFX™-PCR DNA and gel band purification kit obtained from Amersham Pharmacia Biotech. Purified fragments were cloned into the vector pGemT-Easy with T4 DNA ligase in 2X Rapid ligation buffer (60mM Tris-HCl pH7.8, 20mM $MgCl_2$, 20mM DTT, 2mM ATP, 10% polyethylene glycol) according to the manufacturers instructions. Prior

to ligation the vector pGem3Z was linearized by virtue of *Hind*III sites and dephosphorylated with calf intestine alkaline phosphatase (0.1U/µl), ligation then followed in a 10X dilution of ligation buffer (660mM Tris-HCl, 50mM $MgCl_2$, 10mM DTT, 10mM ATP, pH 7.5). Ligation into pGemT-Easy was performed at 4°C for 16 hours and 14°C for 16 hours in the case of pGem3Z.

Escherichia coli strain Sure®2 was grown to early log phase at 18°C in SOB-media (2% bacto-tryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$ pH 6.7-7.0) as described by Hanahan (1983). The cells were harvested by centrifugation at 2500g for 10min at 4°C and resuspended in ice-cold TB media (10mM Pipes, 55mM $MnCl_2$, 15mM $CaCl_2$, 250mM KCl). After repeating the centrifugation, the pellet was gently resuspended in TB, and DMSO was added with gentle swirling to a final concentration of 7%. The suspension was incubated in an ice bath for 10 min, dispensed in 200µl aliquots and chilled by immersion in liquid nitrogen (Inoue *et al.*, 1990).

Recombinant plasmids were incubated with the competent cells for 30min on ice, followed by heat-shock of 30sec at 42°C. Cells were incubated in 800µl LB- or SOB media, supplemented with 20mM glucose, for 1 hour at 37°C with aeration. After centrifugation at 4000g the cells were resuspended in 100µl SOB media and plated on LB media solidified with 1.5% agar and containing ampicillin. pGhct-p transformants were identified by blue-white selection and pGhct-c transformants by screening with a digoxigenin labeled probe.

3.3.5 Probe labeling and screening methods

The 2.6kb fragment used for labeling was amplified from *H. paragallinarum* genomic DNA with primers HctD-1F and HctA-1R (Table 2) under conditions as described previously. This fragment was prepared as the Hct-probe by random primed labeling with digoxigenin using the DIG labeling and detection kit (Roche Molecular Biochemicals). Southern blot analysis were used as a method to identify fragments in

digested genomic DNA possibly representing the transport gene locus and colony screening for identification of pGhct-c clones among other transformants.

3.3.6 Construction of a mini-library

Genomic DNA was digested with either *Bam*HI, *Eco*RI, *Hind*III, *Pst*I or *Xba*I and the fragments separated by agarose-gel electrophoresis with TAE buffer (40mM Tris-HCl, 1mM EDTA). After photographing the gel for documentation, DNA was nicked in the GS gene linker UV chamber (BIO-RAD) or by increasing the exposure period to the UV light from the transilluminator to 5 minutes.

3.3.6.1 Blotting techniques

The DNA was transferred to a magnacharge nylon transfer membrane (Micron separations, Inc.) by one-hour downward capillary transfer as described by Chomczynski (1992). Prior to transfer the gel was placed in denaturation solution (0.4M NaOH, 1.5M NaCl) for 30min. The schematic presentation of the setup for the downward alkaline transfer is shown in Figure 5. The bottom base was formed by a 2-3cm high stack of paper towels sequentially covered with five sheets of blotting paper (Whatman no.3), magnacharge nylon membrane and the agarose gel. The gel was covered with three sheets of Whatman paper and two more sheets forming a connection (bridge) between the gel stack and tray containing the transfer solution (8mM NaOH, 3M NaCl pH 11.4). Transfer was complete within 1 hour, where after the membrane was soaked in neutralizing solution (0.2M sodium phosphate buffer, pH 6.7-6.8) for 10min. The membrane was linked in the GS gene linker™ (BIO-RAD) and submitted to hybridization with the Hct-probe.

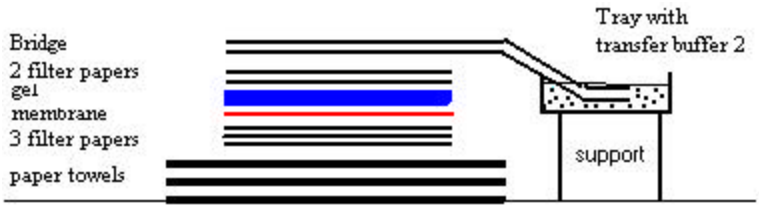

Figure 5: Schematic presentation of the downward alkaline transfer (Chomczynski, 1992)

Colony blotting was performed on transformants grown for 16 hours on LB plates with ampicillin to screen for the presence of pGhct-c clones. Blotting proceeded as described by the DIG systems users guide for filter hybridization (Roche Molecular Biochemicals). Colonies were lifted from the growth media and fixed on a magnacharge nylon membrane (micron separations. Inc.) in the GS gene linker™ (BIO-RAD). The membrane was submitted to denaturation in 10% SDS and denaturation solution (0.5M NaOH, 1.5M NaCl) followed by neutralization (1M Tris-HCl pH 7.5, 1.5M NaCl) and washing in 2X SSC until all the cell debris was removed.

3.3.6.2 Hybridization

Hybridization and colorimetric detection were performed as described in the Nucleic Acid detection kit (Roche Molecular Biochemicals) at a temperature of 68°C for both pre-hybridization and hybridization followed by washing under low stringency conditions.

3.3.7 Sequencing and analysis

Plasmid constructs pGhct-p and pGhct-c were used as templates for sequencing. Sequencing was performed with the ABI Prism™ Big Dye terminator™ V3.0 cycle sequencing ready reaction kit (Perkin-Elmer biosystems). PCR was performed on the

Eppendorf mastercycler personal thermal cycler with sequence specific primers (25 cycles; 10sec 94°C, 5sec 50°C, 4min 60°C). DNA was precipitated with 75% ethanol and 0.03M NaOAc. After drying in the SpeedVac concentrator SVC 100H (Savant), formamide and 25mM EDTA were added. About 30-50% of each reaction was loaded onto a 4% acrylamide gel, separated at 1.6kv and data collected on an ABI Prism 377 DNA sequencer (Perkin-Elmer biosystems). Data was analyzed using Sequencing analysis V3.3. Sequences were reverse complemented and compared by using Sequence Navigator V 1.0.1 and assembled using Auto-assembler V1.4.0 and DNAssist V1.0.2.

3.4 Results

3.4.1 Partial amplification of the *Haemophilus paragallinarum* capsule transport gene region.

Genomic DNA was isolated from *H. paragallinarum* strain 1742 and used as template in an attempt to amplify segments of the *H. paragallinarum* capsule transport genes. Figure 6 shows the isolated genomic DNA separated on a 0.8% agarose gel. The *Eco*RI, *Hind*III digested lambda DNA indicated in Figure 6A was used as DNA marker in chapters 3 and 4, shown in the lanes marked M on each gel.

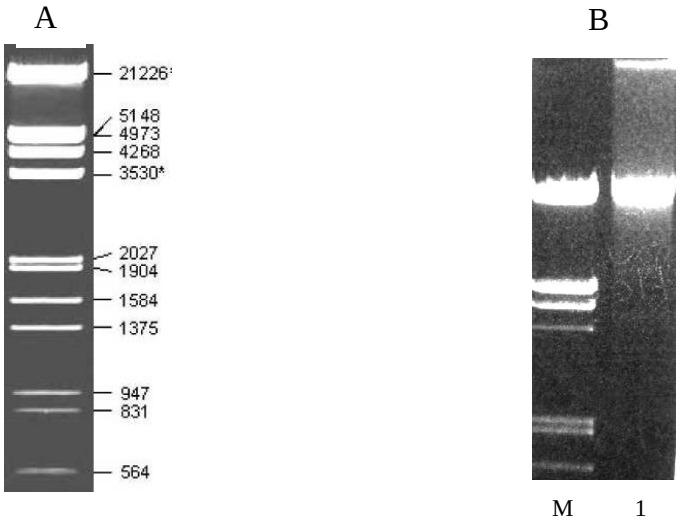

Figure 6: (A) *Eco*RI, *Hind*III digested lambda DNA. (B) Genomic DNA of *H. paragallinarum*. Lane 1 represents the isolated DNA from strain 1742 (350µg/µl)

The capsule transport gene sequences of the related HAP organisms, *H. influenzae* (*bex* genes), *A. pleuropneumoniae* (*cpx* genes) and *P. multocida* (*hex* genes) was obtained from Genbank and submitted to a multiple sequence alignment performed with DNAssist V 1.0.2. Primers were designed (Table 2) from areas in these aligned gene sequences where the sequence stayed relatively conserved among the three species. Six primers were designed with the position of each indicated in Figure 7; HctD-1R, within the D

transport genes (Figure 7a), HctC-1F and HctC-1R within the C transport genes (Figure 7b), HctB-1F and HctB-1R within the B transport genes (Figure 7c) and HctA-1R within the A transport genes (Figure 7d).

Figure 7: Position of degenerate primers on the multiple sequence alignment of the capsule transport genes of the HAP organisms

Figure 7a: Forward primer HctD-1F on the alignment of the D transport genes

```
hexD  1                ATGAAACCAATAAAAAC---ATTATCTATTTCTTTTATCACAACGCTCATCCTCG 52
bexD  1                ATGAGAAATTATCCATT---AAAAGCGGTCTGTGCGGTGTTAATGTTGGGGCTGT 52
cpxD  1    AGAGAAATTAGATGAAACTCATCAAACTTAGATTACTCCTTTCTTTGGGGCTGGTTGCTAGTTTGG 66

hexD  53   CTGGCTGTCATAGCATGCCAACTTCGGGACCCGCTCAAAATCATATTATTGGGTTAAAAAAACCAC 118
bexD  53   CAGCTTGTTCTTCGTTACCCACTTCAGGGCCAACCGATTCGGCTGTATTAGAGATAAACCAAGGTG 118
cpxD  67   CTGCCTGCTCAAGCTTACCCACTTCAGGCCCTAGCCATAGTGCGATTTTAGAGGCTAATTCCCAGA 132

hexD  119  AAAATGA----GAGCTTGC--CAAGTGTTGATGTGATTGAAATGAATGATAAAGTAGCACACACTT 178
bexD  119  CTGATGCTTCTGAATTAGCAGCAAAAGTTAATGTAATTGAATTAAATGAGTCGTTAGTGCAGCAAA 184
cpxD  133  ACTCAGAT---AAACCTTTACCGGAAGTTAATTTAGTGGAGTTAGATAATGGCTTAGTTCAGCAGT 195

hexD  179  TGTTTAAACAAAAGCAATCGCAATCCTTTACTCAATTCAAACAGCAAAATAGCAAC------TATG 238
bexD  185  TATATGCAGCTCAACAAAGCCAGCGTTTTTCAGGTTTTGCAGATGTACGTGGCAATGGTGGTTATG 250
cpxD  196  TGTATCAGACTCAGCAAAGTCAGCAATTTTCCGGCTTTTTAGGCACGGCTGGCGGTGCTGGATATG 261

hexD  239  CAGATATCATTAATGTTGGCGATACATTAGATGTGTTGATTTGGGAAGCCGCACCCGCTATTTTAT 304
bexD  251  CCGGTGCTGTAAATGTGGGGGATGTATTAGAGATTTCCATTTGGGAAGCGCCTCCTGCAGTATTAT 316
cpxD  262  CCGGTGCGGTCAATGTGGGGGATGTTCTTGAAATTTCAATTTGGGAAGCGCCACCGGCAGTGTTGT 327

hexD  305  TCGG--CAGTGTTCTGTCACAAACTGGTAGTGGCGGTGCAAATCTCACGACATTACCCGAACAAAT 368
bexD  317  TTGGTACAACCTTTAGTAGTGAA--GGACAGGGCAGTGGTCATGTTACGCAATTACCTTCACAAAT 380
cpxD  328  TTGGCGGTACTTTTAGTTCTGAA--GGGCAAGGTAGCGGGCATTTAACGCAATTACCGGCGCAAAT 391

hexD  369  TGTTGCACGCAATGGAAAAATCACAATCCCATTTTTAGGCCCTATCTTAGTAAAAGGCAAAACACC 434
bexD  381  CGTGAATAAAAATGGTACGGTGACTGTACCATTTGTAGGAAATATCAGTGTTGCAGGTAAGACACC 446
cpxD  392  GGTTAACCAAAACGGTACGGTTACTGTGCCGTTTGTGGGTAATATTCGTGTTGCAGGTAAAACACC 457

hexD  435  AGAGCAAATTCAAAGAGATATTGCT-CATGCTTTATCCTCCTTAGCAAATAAACCTCAGGTGATTG 499
bexD  447  AGAGGCTATTCAAGCTCAAATTGTAGCAAGCCTTAGCCGTAA-AGCGAATCAGCCACAAGCCGTAG 511
cpxD  458  GGAAGCGATTCAGTCTCAAATTGTTGGGGCATTGCAACGTAA-AGCGAATCAGCCACAAGTATTAG 522

hexD  500  TTCGTTTAAATAAAAATAACTCAAAAAATGTCACCATTCTCCGCCAAGGCAATAGTGTCAGAATGC 565
bexD  512  TTAAAATTGCTAACAATAATTCATCAGATGTCACTGTTATTCGTCAAGGTAGTGCTGTGCGTATGC 577
cpxD  523  TAAAAATTGCGAATAATAACTCTGCGGATGTTACGGTTATTCGTCAGGGTAACAGTATTCGTATGC 588

hexD  566  CATTAACATCACAAGGGGAACGTGTACTTGATGCTATTGCCGCAGTGGGCGGTGCCACAGAAAATC 631
bexD  578  CATTAACTGCAAATGATGAACGTGTTTTAGATGCGGTGGCAGCTATAGGTGGTTCAACAGGAAATA 643
cpxD  589  CGCTGAGTGCGAATAACGAACGTGTGTTAGATGCTGTTGCAGCAGTAGGCGGTACAACTGAAAATA 654

hexD  632  TACAAGATATTTCCGTTCAACTTACACGTGGTAAAGAAGTAAAAATGCTTTCCTTAGAAAAATTAG 697
bexD  644  TTGAAGACGTGACAGTTCAGCTTACTCGAGGAAATCAAGTGAAAACATTGGCATTTGAAACCTTAA 709
cpxD  655  TTGAAGACGTTACCGTAAAATTAACTCGTGGCTCGCAAGTCAAAACATTAGCGTTTGAAACTCTAA 720

hexD  698  CCACTCATCCTGAAGAAAACATTCTGCTACGTTCAAATGATGTCGTTACATTACTGAATAAACCTC 763
bexD  710  TTGCCGATCCTAAGCAAAATATTGTATTACGTGCAGGGGATGTGGTTTCACTTTTAAATACGCCAT 775
cpxD  721  TTTCCGATCCGGCGCAAAATATTATGTTACGTGCCGGCGATGTCGTTTCGTTGCTAAACACGCCTT 786

hexD  764  TTAGTTTCACAGGATTAGGTGCCCTCGGGACAAATAAACAGGTGAAATTCTCTGCGAATGGCTTAA 829
bexD  776  ATAAATTCACAGGGCTAGGTGCTGTAGGTAATAACCAACAGTTACGTTTTTCTAGCAGTGGTATTA 841
cpxD  787  ATAGCTTTACCGGTTTAGGTGCGGTGGGTAACAACCAGCAAATGAAATTCTCAAGTAAAGGAATTA 852

hexD  830  CACTCGCAGAAGGCATCGGAGAAATGGGTGGACTATTAGATAACCGTGCTGATCCTAAAGGGGTTT 895
bexD  842  CGCTTGCTGAAGCGATTGGTAAAATGGGTGGTTTGATTGATACGCGTTCTGACCCACGAGGTGTCT 907
cpxD  853  CGCTTGCCGAAGCTATCGGTAAGATGGGTGGCCTAATTGATACTCGTTCGGATCCGAGAGGGTAT 918
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hexD  896   TTGTCTTCCGTTATATTCCATTTAATAAATTAT-CTCTCGCAGAACAAACTAAATGGAAAGCTCGT 960
bexD  908   TTGTATTCCGTTATATGCCGTTTGCACAACTAGACTCTAA-AGCACAGCAAGAATGGGCGGCTAAG 972
cpxD  919   TCGTCTTCCGTCATGTGCCTTTTTCTCAATTAA-GTTTAGATCAGCAAACACAATGGGGAGCGAAA 983

hexD  961   GGCTATGATAACGACATGGAGATTCCAACAGTTTATAGTGTCAATCTACTTAATCCAAACGCGTTG 1026
bexD  973   GGGTATGGTAATGGCATGGAAGTGCCAACAGTATATCATGCGAATTTATTACAACCGGAAACCATG 1038
cpxD  984   GGCTATGGTATGGGTATGGATGTACCGACGGTTTATCGTGTGAATTTACTTGAGCCGCAATCACTG 1049

hexD  1027  TTTTGGTTACAACGTTTCCCTATTCAAGATAAAGATTTAGTTTATGTATCAAATGCACCAATGGCA 1092
bexD  1039  TTTTTATTACAACGTTTCCCTATCCAAGATAAAGATATTGTATATGTGTCGAATGCACCTCTTTCA 1104
cpxD  1050  TTTTTATTACAACGCTTCCCGATGCAAGATAAAGATATTGTCTATGTATCAAATGCACCGTTGTCC 1115
                          HctD-1R  5'- GATAAAGATWTWGTHTATGTRTCRAATGGACC -3'

hexD  1093  GAATTCCAAAAATTCTTAAAATTAGTGTTTTCTATCACCTCTCCTGTTACGGGTACACTTCATAAT 1158
bexD  1105  GAATTCCAAAAATTCTTGAGAATAATTTTCTCGATTACCTCGCCGGTAACGAGTACGACCAATACG 1170
cpxD  1116  GAATTCCAAAAATTCTTGAGAATGATTTTCTCGATTACTTCGCCGGTTACAAGTACGACTAATGCT 1181

hexD  1159  ATTA-ATGTAATTAAAAATTTATAA       1182
bexD  1171  ATCAGAAGTTATTAA                1185
cpxD  1182  ATTCGTGCCTATTAATATATTGAATTTATAA 1212
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Figure 7b: Forward primer HctC-1F and reverse primer HctC-1R on the alignment of the C transport genes

```
bexC  1                                   ATGACAACCGAAAATGCGGCAATACCGACGAAAAA 35
cpxC  1     AGGATAAAATATGGAAACAACTATTACGGCAAGTCCGACAGAAAAACTACAAAAACCGGTTAAACA 66
hexC  1                                ATGATCGAAACAAAAATACAAAAAATCAATTTAAAAA 38

bexC  36    GAAGAAATCATTCTGGAAGAAAATGAAACCGCTTTTTGGATTAACTGTATTGATTCCAACAGCCTT 101
cpxC  67    GAAAAAAAGTTGGTTAAAAAAGCTTAATCCGTTATTTTGGGTAACTGTAGCGATTCCTACGGTATT 132
hexC  39    ATGGTCCCGAAAATTTCGAAAAATAAGTACACTCTTTAAATACATGGTAATACTTCCAACATGTTG 104

bexC  102   TTCTGCGGTATACTTTGGACTGTTTGCGTCTGATATTTATGTTTCAGAATCTAGTTTTGTAGTACG 167
cpxC  133   ATCAGCCTTTTATTTCGGTTCTGTTGCTTCCGATATTTATATTTCGGAATCAAGCTTCGTTGTAAG 198
hexC  105   CTCACTCTTTTATTTTAGTTTATGGGCCTCTGATATTTATATTTCTCAATCGAGTTTTGTTGTGCG 170
                        HctC-1F  5'- GCBTCYGATATTTATRTTTCDSAATCDAG -3'

bexC  168   TTCTCCTCGTAGTCAGTCTTCTTTAAGCGGAGTTGGTGCATTGTTGCAAAGTACGGGTTTTTCTCG 233
cpxC  199   ATCTCCTCAAAATCAGACCGCTTTAACCGGTGTCGGTGCCTTATTACAAGGTTCCGGATTTTCTCG 264
hexC  171   CACGCCAAAAAATCAAGCCGCATTAAGTGGTGTCGGTGCCCTCTTACAAAGCTCTGGCTTTGCTCG 236

bexC  234   TTCACAGGATGATACTTACTCTGTGCAAGAGTATATGCGTTCACGTACAGCACTATCCGCATTAGA 299
cpxC  265   AGCTCAAGATGATACTTATACCGTACAAGAATATATGCATTCTCGTACGGCACTAGAACAGTTAAT 330
hexC  237   CGCACAAGATGATACTTATACTGTACAAGAATTCATGCGTTCACGTTCTACGCTAGAATTATTAGA 302

bexC  300   GCAAGGTTTGCCTCTTCGCACTTTTTATTCTGAAAAAGGCGATTTATTAAGCCGCTTTAATGGGTT 365
cpxC  331   GAAAGACTTGCCAATACGTGAATTACTATGAGAATCAAGGCGATATTATCGCTCGCTTTAATGGATT 396
hexC  303   AAAAAGTATCCCTATCCGCCAGTTTTATGAGGATAAAGGTGATTTATTTAGCCGCTTTAATCCTCT 368

bexC  366   TGGTTTGAATGATACACAAGAGGCATTCTATCGTTATTTAAAGAGCGTTTGAGTGTTGATGTGGA 431
cpxC  397   TGGTTTAAATAATAGTAAAGAAGCGTTTTATAAATATTTCCGAGATCGCTTAAGTGTGGACTTTGA 462
hexC  369   AAACATTTTCTCGGAACAAGAAGCGTTTTATCAATACTTTAGTAAAAAGCTTTCTGTTAATTTTGA 434

bexC  432   TTCTATTTCTGGCATTGCAACATTGCGAGTGCATGCTTTTGATGCTGAAGAAGGTTATCAAATCAA 497
cpxC  463   CTCTGTTTCCGGTATCGCCAGCTTACGTATTCGAGCATTTAACGCGGAAGAGGGGCAACAAATTAA 528
hexC  435   TTCAGTATCAGGCATTGCTACGCTCAATATCCGGGCATTCGATCCGAAAGAAGCACAACAAATTAA 500

bexC  498   TGAGCGCTTATTGAAAGAAGGTGAATCATTAATCAATAGATTAAATGAGCGTGCAAGAAAAGATAC 563
cpxC  529   TCAAAAATTACTTGCCGAAGGTGAAACGCTTATTAACCGTTTAAACGAACGTGCAAGAAAAGATAC 594
hexC  501   CCAAGAATTGTTAAAACAAGGTGAATATCTTATCAATCGCCTCAATGAACGTGCGCGAAAAGATAC 566

bexC  564   GATTGAATTTGCTGAACAGGCCGTAAAAGATGCAGAGAAAAATGTGAATGAAACTGCACAAGCATT 629
cpxC  595   CATTTCATTTGCGGAACAAGCGGTTACAGAAGCGGAAAATAATGTAAACGAAACGGCAAATGCTTT 660
hexC  567   GGTTATGTTTGCTGAACTTGCAGTCAGTGAAGCAGAAAAAAAAGTGACAGAAACTTCTTCTGCATT 632
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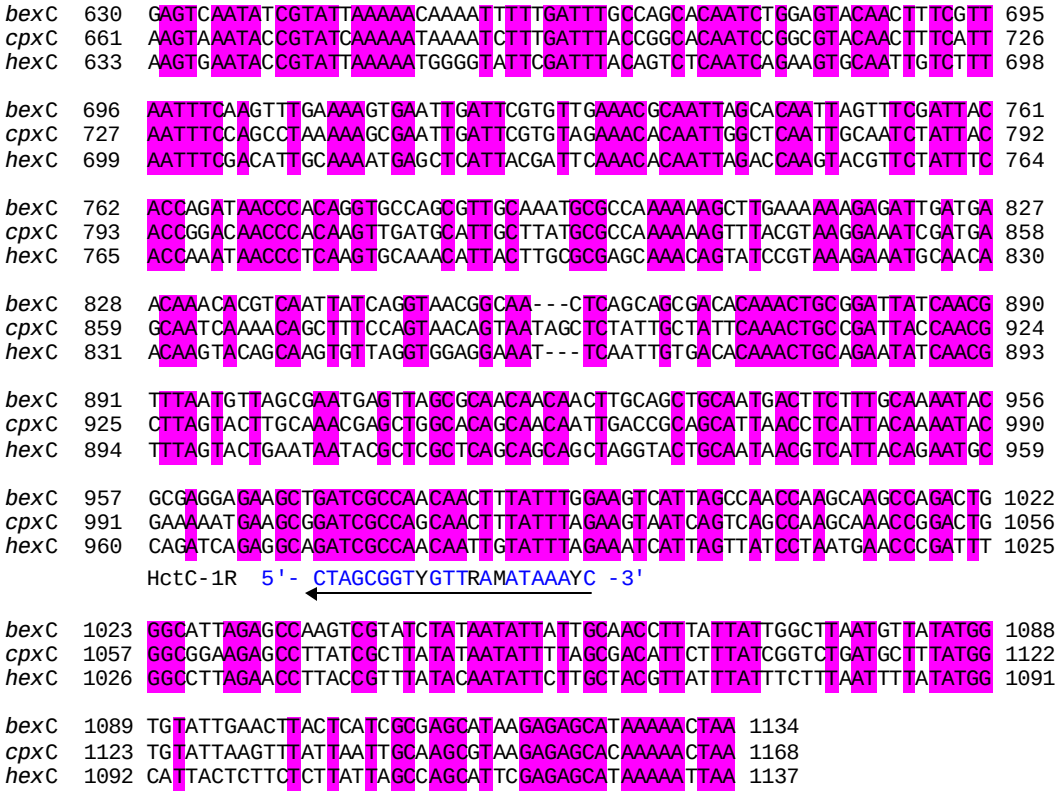
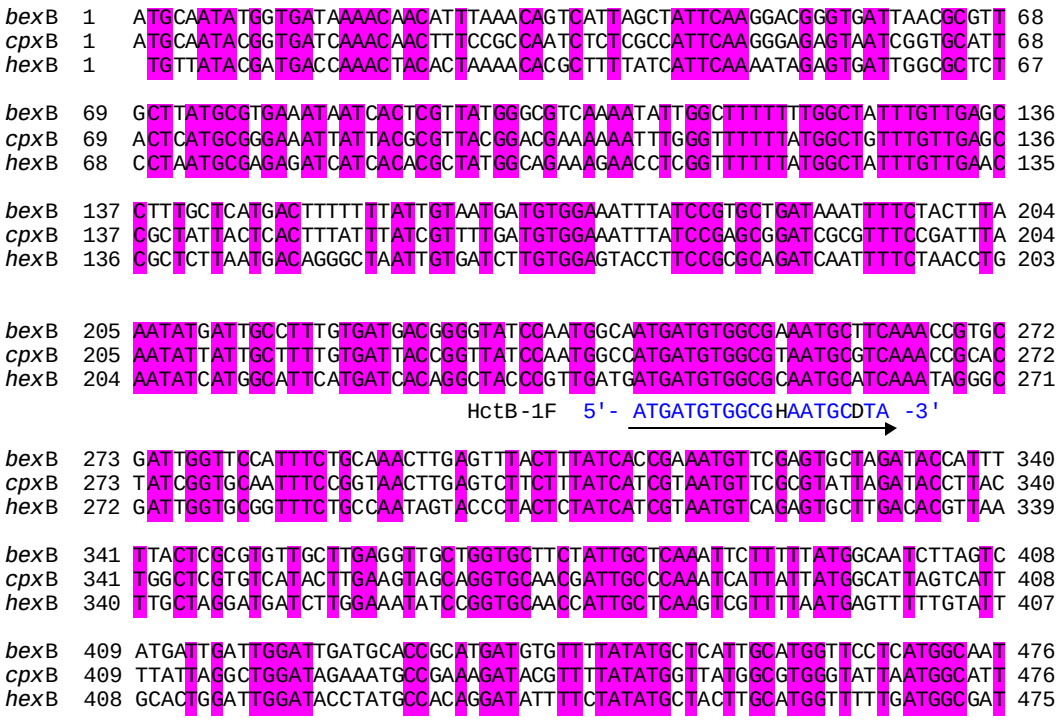


Figure 7c: Forward primer HctB-1F and reverse primer HctB-1R on the alignment of the B transport genes



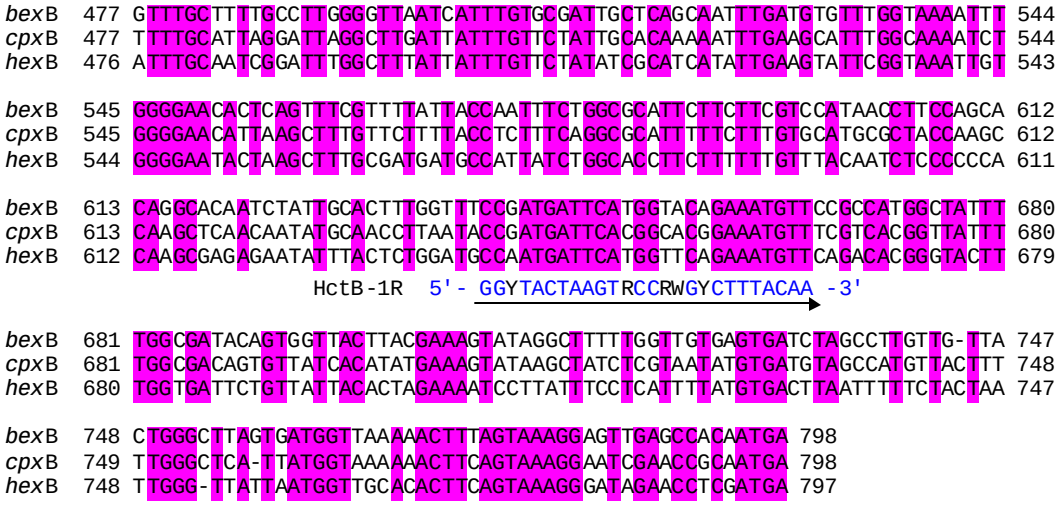
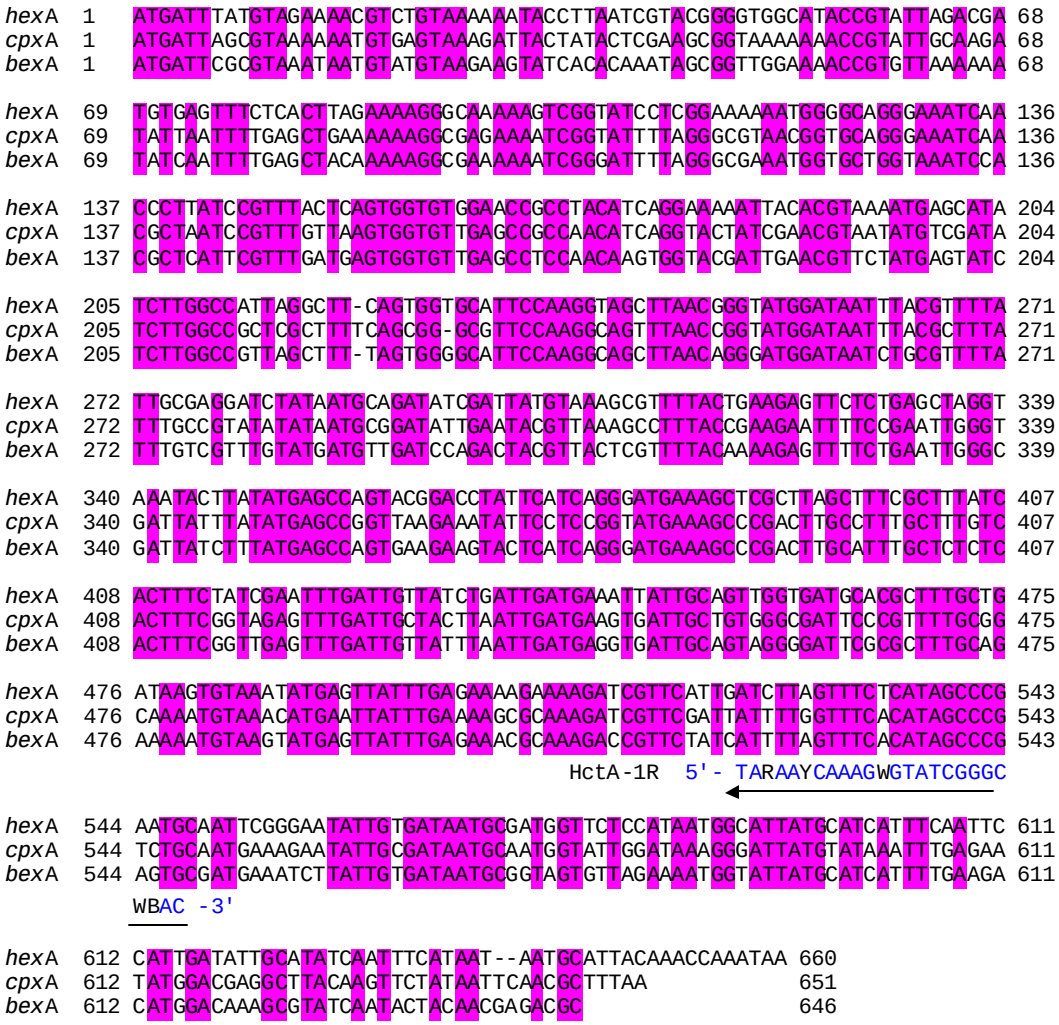


Figure 7d: Reverse primer HctA-1R on the alignment of the A transport genes



Theoretically a number of fragments differing in size will be amplified by using these primers in different combinations. Figure 8 shows the proposed fragments roughly estimated by comparison to the corresponding genes of the HAP organisms.

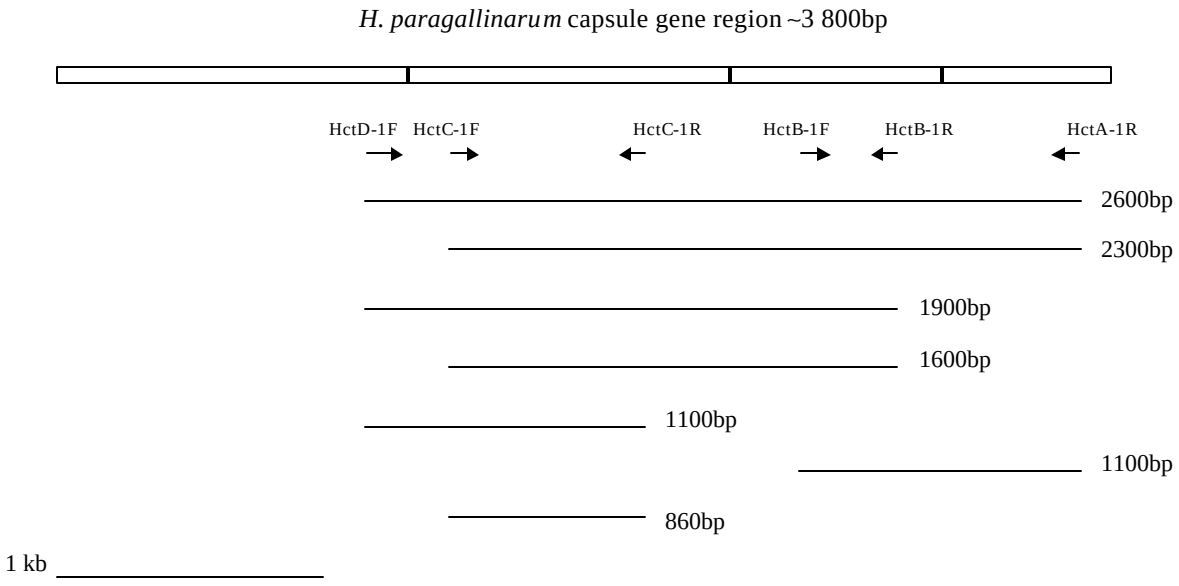
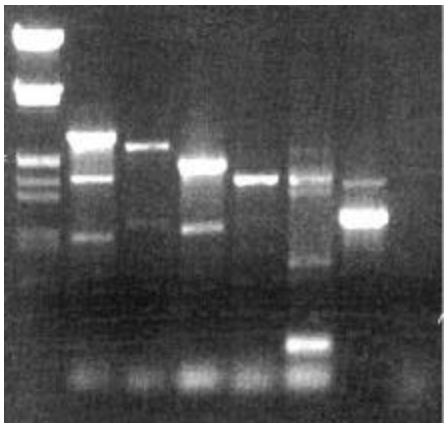


Figure 8: Proposed fragments to be amplified by the degenerate primers indicating the postulated position where each primer will anneal.

The PCR performed with oligonucleotide combinations (Fig. 9), showed amplification of a number of the proposed fragments corresponding to the predicted sizes (Fig. 8). In lanes 1, 2, 3, 4 and 6 the predicted fragments of ~2600bp, ~2300bp, ~1900bp, ~1600bp and ~1100bp respectively were obtained (Fig.9). Lanes 5 and 7 showed either non-specific or no amplification. This might be an indication that the HctC-1R oligonucleotide was non-specific for amplification in both these reactions. More than one band is visible in some lanes because the PCR was performed at a low annealing temperature of 45°C, allowing a certain amount of non-specific binding to occur in the case of oligonucleotides with a high melting temperature.



M 1 2 3 4 5 6 7

Figure 9: Amplification of segments of the *H. paragallinarum* capsule transport gene region. The different degenerate oligonucleotides were used in the following combinations: HctD-1F & HctA-1R (lane 1), HctC-1F & HctA-1R (lane 2), HctD-1F & HctB-1R (lane 3), HctC-1F & HctB-1R (lane 4), HctD-1F & HctC-1R (lane 5), HctB-1F & HctA-1R (lane 6) and HctC-1F & HctC-1R (lane 7).

The estimated ~2600bp fragment amplified by the oligonucleotides HctD-1F and HctA-1R (Fig. 9, lane 1), was cloned into the vector pGemT-easy and designated pGhct-p. This fragment was sequenced and sequence analysis revealed a 51% homology with the capsule transport genes of the HAP organisms (*H. influenzae*, *A. pleuropneumoniae* and *P. multocida*). This high degree of homology among the four species indicates that the sequenced 2638bp insert in the pGhct-p plasmid construct partially represent the capsule transport gene region of *H. paragallinarum*.

3.4.2 Amplification and labeling of the Hct- probe

The remaining 1000bp representing the *hctD* gene as well as ~100bp representing the remainder of the *hctA* gene of the postulated *H. paragallinarum* capsule transport region (Fig. 8) was isolated from the genome. The 2638bp fragment in the pGhct-p construct was used as a digoxigenin labeled probe to isolate the remainder of the capsule transport

gene region. The 2638bp fragment was amplified from *H. paragallinarum* genomic DNA with the primer pair HctD-1F and HctA-1R (Table 2). The PCR was performed in duplicate to generate ample DNA product for labeling (Fig. 10). The PCR products were purified from the gel, pooled and labeled using the DIG labeling and detection kit according to the manufacturers instructions (Roche Molecular Biochemicals).

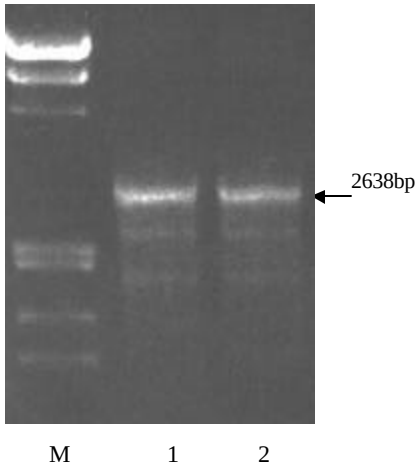


Figure 10: Amplification of a 2 638bp fragment for digoxigenin labeling, with oligonucleotides HctD-1F and HctA-1R from the genome of *H. paragallinarum*

The amount of the labeled DNA, designated Hct-probe, was determined by comparison of the intensity of the spots of a serial dilution of the Hct-probe (Fig. 11) to that of a labeled control (supplied by the manufacturer). In this case the amount of labeled probe DNA was estimated as 5 000pg/µl (5ng/µl). Multiplying this value with the volume of the labeling reaction mixture (50µl) provides the total amount of labeled DNA (250ng) from which the amount of labeled DNA per hybridization volume was calculated.

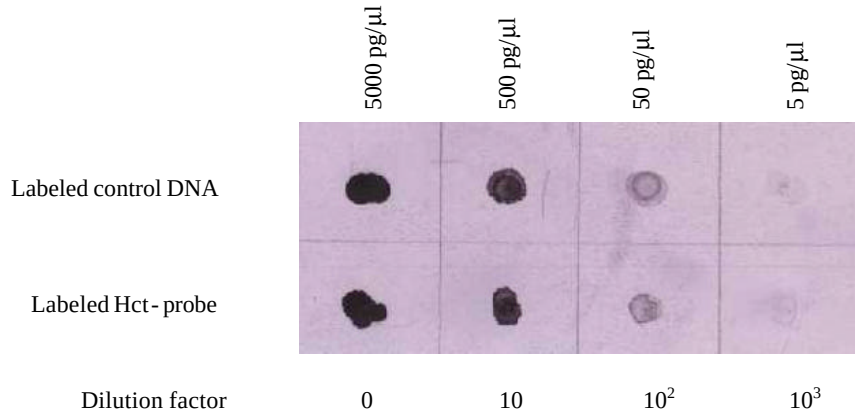


Figure 11: Determination of the labeling efficiency of the Hct-probe. The total amount of labeled DNA estimated as 250ng.

3.4.3 Construction of a mini-library to isolate the complete capsule transport gene region

Cloning DNA, by whatever method, gives rise to a population of recombinant DNA molecules, often in plasmid or phage vectors, maintained either in bacterial cells or as phage particles. A collection of independent clones is termed a clone bank or library. The term genomic library is often used to describe a set of clones representing the entire genome of an organism. However only 3.8kb of the genome is of interest in this study, therefore a mini-library was constructed.

Haemophilus paragallinarum genomic DNA was digested with five different restriction enzymes (Fig. 12A), transferred to a nylon membrane and hybridized with the digoxigenin labeled Hct-probe under stringent conditions. Colorimetric detection of possible hybridization showed the presence of a fragment in the *Hind*III digestion, estimated at ~6.15kb (Fig 12B, lane 3). Hybridization is also visible in lanes 1, 2, 4 and 5 at a position of ~21.3kb, which corresponds to the relative position of undigested genomic DNA on a 0.8% agarose gel (Fig. 6B).

The fragment was cloned and colony hybridization was used as a screening method for positive clones, performed with the Hct-probe. A total of 93 colonies were

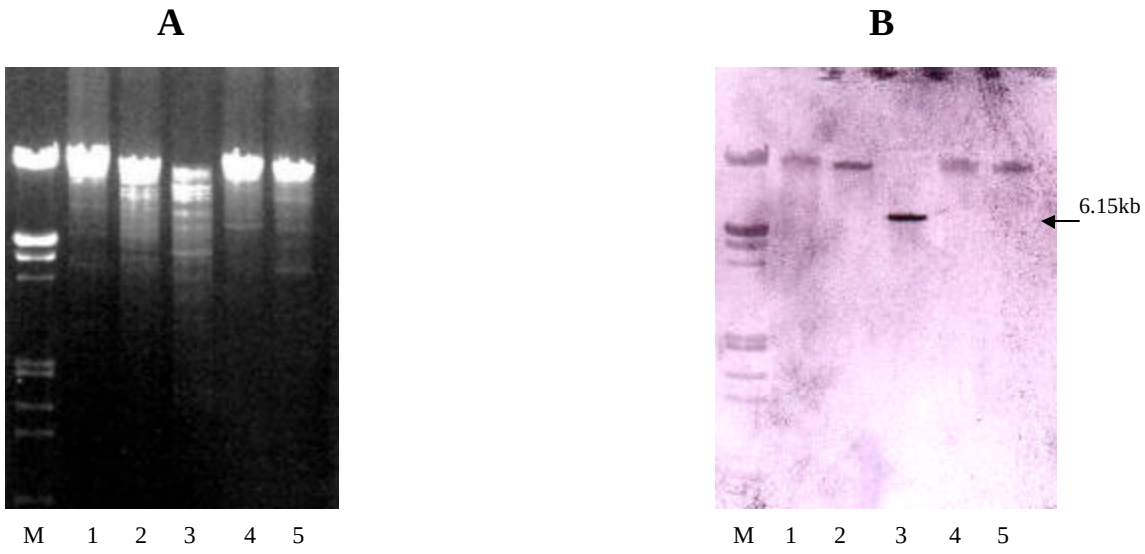


Figure 12: Southern blot analysis of digested genomic DNA hybridized with the Hct-probe under stringent conditions. (A) shows the genomic DNA digested with *Bam*HI (lane 1), *Eco*RI (lane 2), *Hind*III (lane 3), *Pst*I (lane 4) and *Xba*I (lane 5) for 3 hours. (B) shows hybridization with a fragment ~6 .15kb in the *Hind*III digestion.

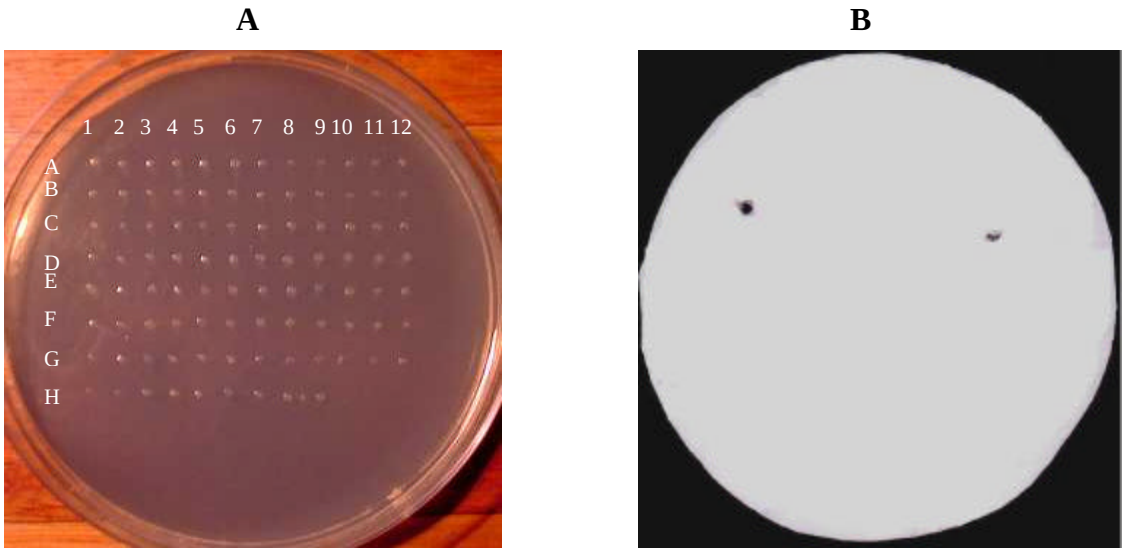


Figure 13: Colony hybridization and screening for clones containing the ~6.15kb fragment. (A) The 93 transformants that were visible within one day of transformation transferred to a grid pattern on a LB plate with 60µg/ml ampicillin. (B) Hybridization of two colonies (B3 and C12) with the Hct-probe under stringent conditions

visible within one day of transformation (Fig. 13A) and two colonies, B3 and C12, showed hybridization with the Hct-probe under stringent conditions (Fig. 13B)

Plasmid extracted from 16-hour cultures of B3 and C12 and digested with *Hind*III revealed the presence of the ~6.15kb band as well as a band of 2743bp in sized representing the pGem3Z plasmid backbone (Fig. 14). Both these plasmid constructs were submitted to sequencing with universal oligonucleotides Sp6 and T7 to determine whether the isolates show any homology with the capsule transport gene regions of the HAP organisms and the capsule biosynthesis gene region of *P. multocida*. The capsule biosynthesis gene region of *P. multocida* was the only sequence used in the alignment, because this is the only HAP organism of which the specified gene region has been characterized (Chung *et al.*, 1998). Sequencing with these two oligonucleotides also gave an indication of the orientation in which the ~6.15kb fragment was ligated into the vector.

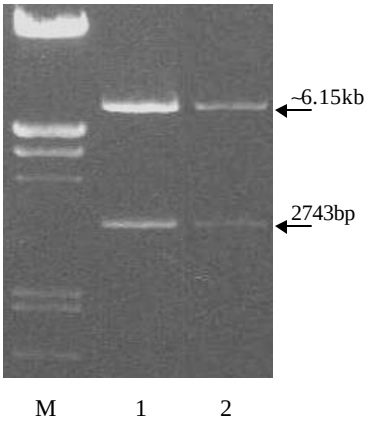


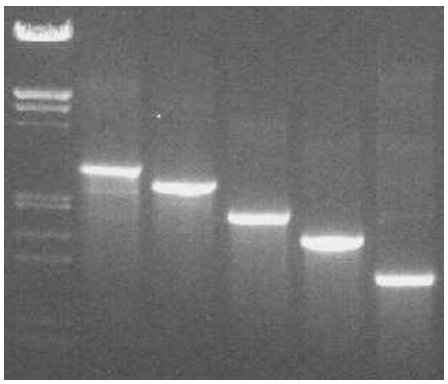
Figure 14: *Hin*dIII restriction enzyme digestion profile of clones containing the ~6.15kb fragment in pGem3Z. DNA digested with *Hind*III from clone B3 is visible in lane 1 and DNA from C12 digested with the same enzyme in lane 2.

Analysis of a 630bp sequence obtained from the sequencing reaction with oligonucleotide T7 using the B3 plasmid as template, showed 52% homology with the capsule biosynthesis gene region of *P. multocida* as well as 51% homology in a multiple sequence alignment with the capsule transport genes *bexD*, *cpxD* and *hexD* representing

the transport genes of *H. influenzae*, *A. pleuropneumoniae* and *P. multocida* respectively. Similar homologies were obtained when a 620bp sequence of the C12 plasmid, sequenced with oligonucleotide SP6 was submitted to the same alignments.

The high degree of homology with the capsule transport gene regions of the HAP organisms indicated that the ~6.15kb fragment is suitable for further characterization. The sequencing results also indicate that the ~6.15kb fragment was ligated into clones B3 and C12 in different orientations. In the case of clone B3 the fragment was inserted into pGem3Z in such a way that the oligonucleotides T7 functioned as forward primer and oligonucleotide SP6 served as reverse primer, with the opposite being true for clone C12. For facilitation of sequencing analysis clone B3 was used for further study.

A PCR performed on clone B3 with the oligonucleotides indicated in Table 2 (except HctC-1R) was a convenient way to determine which part or parts of the Hct-probe features in the ~6.15kb fragment. The PCR showed amplification in each reaction



M 1 2 3 4 5

Figure 15: Amplification of the Hct-probe sequence within the ~6.15kb fragment, using clone B3 as template. Oligonucleotide combinations HctD-1F & HctA-1R (lane 1) amplified a ~2600bp fragment, HctC-1F & HctA-1R (lane 2) amplified a ~2300bp fragment, HctD-1F & HctB-1R (lane 3) amplified a ~1900bp fragment, HctC-1F & HctB-1R (lane 4) amplified a ~1600bp fragment) and HctB-1F & HctA-1R (lane 5) amplified a ~1100bp fragment.

with the primer combinations used and the estimated sizes of the products indicated in Figure 15. The ~2600bp fragment amplified with oligonucleotides HctD-1F and HctA-1R in lane 1 indicated that the entire probe hybridized with the ~6.15kb fragment and that the 2600 nucleotide sequence is also present within this fragment

The position of the Hct-probe sequence in the ~6.15kb fragment was determined with PCR using synthesized oligonucleotides HctD-1R and HctA-1F (Table 2) as well as universal oligonucleotides SP6 and T7. The two oligonucleotides HctD-1R and HctA-1F have the same annealing position as HctD-1F and HctA-1R (Table 2) respectively, but differing in orientation. The degenerate nucleotides have been replaced and HctD-1R consists of less nucleotides than HctD-1F to accommodate a suitable melting temperature. Clone B3 was used as template in this PCR (Fig. 16) and oligonucleotides HctD-1R was used in combination with T7 (lane 1) and HctA-1F in combination with SP6 (lane 2). Amplification of 2 bands were visible, a ~1500bp band in lane 1 representing the segment upstream and a ~2050bp band in lane 2 indicating the segment downstream from the Hct-probe sequence. These results and the high degree of homology with the transport genes of the HAP organisms, proves that the ~6.15kb fragment represents the entire *H. paragallinarum* capsule transport region and clone B3 was designated pGhct-c.

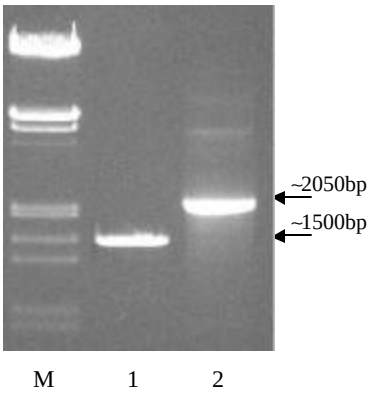


Figure 16: Amplification of the regions up- and downstream from the Hct-probe sequence within the ~6.15kb fragment. Amplification of a~1500bp fragment when oligonucleotide HctD-1R was used in combination with T7 (lane 1). A ~2050bp fragment amplified by oligonucleotides HctA-1F and SP6 (lane 2).

3.4.4 Nucleotide sequence and analysis of *Haemophilus paragallinarum* capsulation DNA.

The nucleotide sequence of the ~6.15kb *Hind*III restriction fragment of pGhct-c was determined with primer walking. Analysis of the complete sequence revealed that the *H. paragallinarum* capsule transport gene region is 3792bp in length comprising four ORFs representing the four capsule transport genes designated *hctDCBA* (Fig. 17). The most 5', *hctD*, contains 1188 nucleotides and terminates at a TGA stop codon, encoding a putative protein of 395 amino acids. The next open reading frame starts at the third base of the *hctD* stop codon and represents the 1164bp *hctC* gene, encoding a putative protein of 387 amino acids. The third base of the stop codon at the 3'- end of *hctC* is the first base of the ATG at the start of *hctB*, 798 nucleotides in length and coding for a putative protein of 265 amino acids. *hctB* terminates with a TGA stop codon where it overlaps with *hctA*, the nucleotides ATGA forming part of the start codon. *hctA* contains 648 nucleotides, encodes a putative protein of 215 amino acids and terminates at a TAG stop codon. Downstream of *hctA* all reading frames in both directions are closed with multiple stop codons. Part of an open reading frame is present upstream of *hctD*, which showed considerable homology with the *P. multocida hyaA* biosynthesis gene.

Physicochemical property analysis of the gene cluster with DNAssist revealed a clear bias toward codons rich in nucleotides A and T (37% GC) consistent with the 39.1% GC content of the *H. influenzae* capsule gene cluster and 37% GC content of the genome overall reported by Kroll *et al.* (1990) and Roy & Smith (1973) respectively. It also correlates well with the calculated GC contents of the *A. pleuropneumoniae* (40%) and *P. multocida* (37%). The gene lengths and region size correlates well with those of the HAP organisms as indicated in Table 3.

A

```
-167          …GA TAA GTG TTG ATA TAA ATA AAA TTT CCC GAG TCT TTA  -130

-129  AAA AAT TGG AAT TAT TTT TAT AAA AAA GTT TTC TAC AGG AAA TTG  -85

 -84  AGC AAA AAT TAA TAA TTA TCT ATG ATA ATT ACT CAC TTT TAA TAG  -40

                                                        hctD
 -39  AAA AAT CAT GAT CAA AAA CAA AAT AAT TAA GGT AAA ACT ATG CGT    6
                                                        Met Arg    2

   7  AAA TCG CTG ATT GCA GTA AGT TAC TGC TTA TTA TTA ATG TCT TGG   51
   3  Lys Ser Leu Ile Ala Val Ser Tyr Cys Leu Leu Leu Met Ser Trp   17

  52  TCT TAT TTG CCA AAT TCA GGA CCG AGC AAA GGC AAT ATT GAG GTA   96
  18  Ser Tyr Leu Pro Asn Ser Gly Pro Ser Lys Gly Asn Ile Glu Val   32

  97  GTC AAT AAA CAG AAA TCC AAT GAG GAT TTG CTT GCA GTA CAG TTG  141
  33  Val Asn Lys Gln Lys Ser Asn Glu Asp Leu Leu Ala Val Gln Leu   47

 142  ATC GAG GTG AAT AAT AAA GTT GCG GAA AGT ATG TTT AAT CAA CAA  186
  48  Ile Glu Val Asn Asn Lys Val Ala Glu Ser Met Phe Asn Gln Gln   62

 187  CAC CCT CAA TCA TTT TTG CAG TTT CCT TCA TCA AAA GCA CAT TAT  231
  63  His Pro Gln Ser Phe Leu Gln Phe Pro Ser Ser Lys Ala His Tyr   77

 232  CAT GGG GTA GTT AAA TGC TGG TGT TTA CTT GAT ATT ACT CTC TGG  276
  78  His Gly Val Val Lys Cys Trp Cys Leu Leu Asp Ile Thr Leu Trp   92

 277  GAA GCA CCC GCC AGC AAC TTT GTT TGG CAG TGT GTT GAA TCA AGC  321
  93  Glu Ala Pro Ala Ser Asn Phe Val Trp Gln Cys Val Glu Ser Ser  107

 322  CGG TGT GTC GGG CGG ACA AAG CAC TCA CTT ACC GGA ACA GGT GGT  366
 108  Arg Cys Val Gly Arg Thr Lys His Ser Leu Thr Gly Thr Gly Gly  122

 367  TAT AGC AAT GGA AGA ATA ACC ATT CCT TTT GTT GGT GCA TTA AAA  411
 123  Tyr Ser Asn Gly Arg Ile Thr Ile Pro Phe Val Gly Ala Leu Lys  137

 412  GTA GCA GGG AAA ACA CCG GAG CAG ATC CAA TCT GAA ATT GTT GGA  456
 138  Val Ala Gly Lys Thr Pro Glu Gln Ile Gln Ser Glu Ile Val Gly  152

 457  CGT TTA CAA GCA ATT GCC AAT CAA CCA CAA GCA GTG GTG CGA ATT  501
 153  Arg Leu Gln Ala Ile Ala Asn Gln Pro Gln Ala Val Val Arg Ile  167

 502  GTG AAG AAT AAT TCT GCT AAT GTG ACG GTT TTA ACT AAA TCG ACT  546
 168  Val Lys Asn Asn Ser Ala Asn Val Thr Val Leu Thr Lys Ser Thr  182

 547  ACT ATT CGA ATG GCT TTA ACT GCT TAC GGT GAA CGA AGT GTT AGA  591
 183  Thr Ile Arg Met Ala Leu Thr Ala Tyr Gly Glu Arg Ser Val Arg  197

 592  TGC TAT TGC GGC AGC AGG TGG AGC CGG TGG TAT GTG CAA AGA TGT  636
 198  Cys Tyr Cys Gly Ser Arg Trp Ser Arg Trp Tyr Val Gln Tyr Cys  212

 637  TTC AGT GCG ACT GAC TCG TGG GAA ATC AGG GTG CAA ACG ATT TCT  681
 213  Phe Ser Ala Thr Asp Ser Trp Glu Ile Arg Val Gln Thr Ile Ser  227

 682  TTA GCC AGG ATT AAC GGA GGG AGC CAC AGG CAA AAT ATC CTA TTA  726
 228  Leu Ala Arg Ile Asn Gly Gly Ser His Arg Gln Asn Ile Leu Leu  242

 727  CGT TCC GGC GAT GTA GTA ACG TTA TTA AAT AAT CCA CTT TCT TTC  771
 243  Arg Ser Gly Asp Val Val Thr Leu Leu Asn Asn Pro Leu Ser Phe  257
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 772  ACT GCA ATG GGT GCG GTA GGA AAT AGT AAA GAA ATT CGT TTT TCG  816
 258  Thr Ala Met Gly Ala Val Gly Asn Ser Lys Glu Ile Arg Phe Ser  272

 817  GCA GAA GGT TTA ACT TTA GCA GAA GCA ATC GGT CGT TTA GGT GGA  861
 273  Ala Glu Gly Leu Thr Leu Ala Glu Ala Ile Gly Arg Leu Gly Gly  287

 862  TTG AAT GAT GAT CGT GCA GAT CCA AGA GGA GTA TTT ATC TTT CGT  906
 288  Leu Asn Asp Asp Arg Ala Asp Pro Arg Gly Val Phe Ile Phe Arg  302

 907  TAT GTT CCA TTT GAA GAA ATG CCC TTA AGT AAA CAA AAT GAA TGG  951
 303  Tyr Val Pro Phe Glu Glu Met Pro Leu Ser Lys Gln Asn Glu Trp  317

 952  CAA GCC AAG GGG TAT CAC AAC GGA ATG AAA ATT CCA ACA GTA TAT  996
 318  Gln Ala Lys Gly Tyr His Asn Gly Met Lys Ile Pro Thr Val Tyr  332

 997  CAA GCG AAT TTA CTT GAA CCT CAA TCA ATG TTT TGG ATT CAA CAA 1041
 333  Gln Ala Asn Leu Leu Glu Pro Gln Ser Met Phe Trp Ile Gln Gln  347

1042  TTT CCA ATT AAA GAT AAA GAT ATT GTT TAT GTA TCT AAT GCA CCA 1086
 348  Phe Pro Ile Lys Asp Lys Asp Ile Val Tyr Val Ser Asn Ala Pro  362

1087  TTG GCT GAA TAC CAA ATT TAT TCG TAT GAT TTA CGC CAC CGT TGC 1131
 363  Leu Ala Glu Tyr Gln Ile Tyr Ser Tyr Asp Leu Arg His Arg Cys  377

1132  AAC TAC ACC GCC GGT TTC AAC TGT AAA CAA GTG TTA ATA ATC TGT 1176
 378  Asn Tyr Thr Ala Gly Phe Asn Cys Lys Gln Val Leu Ile Ile Cys  392

                    hctC
1177  AGG GGG AGA TGA  TG GAA CAA AAT GTA GTA GTT CAA TCG AAA GAA 1220
 393  Arg Gly Arg ***
                     Met  Glu Gln Asn Val Val Val Gln Ser Lys Glu  406

1221  CAA CTG AGA AAG TTA AAA CAG TGG TTG CGA AAA ATT AAT CTG TTA 1265
 407  Gln Leu Arg Lys Leu Lys Gln Trp Leu Arg Lys Ile Asn Leu Leu  421

1266  TTT TTA CTG ACG GTG ATT ATT CCG ACT TTT TGT TCG TTA TTT TAT 1310
 422  Phe Leu Leu Thr Val Ile Ile Pro Thr Phe Cys Ser Leu Phe Tyr  436   Region A1
                                                                        (421-432)
1311  TTT TCT ATT TGG GCT TCC GAT GTT TAT ATT TCG GAG TCC AGT TTT 1355
 437  Phe Ser Ile Trp Ala Ser Asp Val Tyr Ile Ser Glu Ser Ser Phe  451

1356  ATT GTG CGT TCT TCT CGT GCT CAG GCA TCG CTC GGA GGT ATG GGG 1400
 452  Ile Val Arg Ser Ser Arg Ala Gln Ala Ser Leu Gly Gly Met Gly  466   Region A2
                                                                        (458-475)
1401  GCT TTA TTG CAG AGT ATC GGT TTT GCT CGT TCG CAA GAT GAT ACT 1445
 467  Ala Leu Leu Gln Ser Ile Gly Phe Ala Arg Ser Gln Asp Asp Thr  481

1446  TTT ACG GTG CAA GAA TTT ATG CGT TCG CGT AAT GCG TTG ACA ACA 1490
 482  Phe Thr Val Gln Glu Phe Met Arg Ser Arg Asn Ala Leu Thr Thr  496

1491  TTG GAA AGT GAG TTA CCG GTG AGA AAA TTT TAT GAA GAT GAA GGG 1535
 497  Leu Glu Ser Glu Leu Pro Val Arg Lys Phe Tyr Glu Asp Glu Gly  511

1536  GAT TTT TTC AGC CCG TTT AAT CCG TTA GGT TTT TTT AAT GAA CAG 1580
 512  Asp Phe Phe Ser Pro Phe Asn Pro Leu Gly Phe Phe Asn Glu Gln  526

1581  GAA TTG TTT TAT CAA TAT TTT CGT AAA CAT TTG ATG ATT AAT ATC 1625
 527  Glu Leu Phe Tyr Gln Tyr Phe Arg Lys His Leu Met Ile Asn Ile  541

1626  GAT TCT TTA TCT GGG TAT TGC TAC TTT ACA GGT TCC GTG GGT TTA 1670
 542  Asp Ser Leu Ser Gly Tyr Cys Tyr Phe Thr Gly Ser Val Gly Leu  556

1671  ATG GCT GAC CTC CGG CAC CAA CAA GAA TTA AAT GGA AGC CAT TAT 1715
 557  Met Ala Asp Leu Arg His Gln Gln Glu Leu Asn Gly Ser His Tyr  571
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1716  TGC CAT TTT GGC GGG AAA CCA TTT AGT GGA ATA AAC TCA ATG ATC  1760
 572  Cys His Phe Gly Gly Lys Pro Phe Ser Gly Ile Asn Ser Met Ile  586

1761  GTG CAC GTA AAG ATA CAA TTA CTT TGC GGA ACA ATC GGT AAT GAA  1805
 587  Val His Val Lys Ile Gln Leu Leu Cys Gly Thr Ile Gly Asn Glu  601

1806  GCA GAA AAA TAT TTG TCT GAA ACC TCG ACA GCC TTA AGC CAA TAT  1850
 602  Ala Glu Lys Tyr Leu Ser Glu Thr Ser Thr Ala Leu Ser Gln Tyr  616

1851  CGT GTA AAA AAT GGG ATA TTT GAT ATT GGG GCA CAA TCT GAA TCG  1895
 617  Arg Val Lys Asn Gly Ile Phe Asp Ile Gly Ala Gln Ser Glu Ser  631

1896  ATT TTA ACT TTA GTG CAG AAG TTG CAG GAT GAA CTG ATT GCC ATT  1940
 632  Ile Leu Thr Leu Val Gln Lys Leu Gln Asp Glu Leu Ile Ala Ile  646

1941  CAG ACG CAA CTT GAT CAG GTG AGG GGC GTT ATC TCC GGA TAC CCT  1985
 647  Gln Thr Gln Leu Asp Gln Val Arg Gly Val Ile Ser Gly Tyr Pro  661

1986  CAG GTT AAA GTG TTA AAG GCA AGG CAA TTT GAA AGT ATT CGT GAA  2030
 662  Gln Val Lys Val Leu Lys Ala Arg Gln Phe Glu Ser Ile Arg Glu  676

2031  AGA AGT GGC ACA ACA ATT GAA TCC GGG GTT TTT GAG GGG AAA CCA  2075
 677  Arg Ser Gly Thr Thr Ile Glu Ser Gly Val Phe Glu Gly Lys Pro  691

2076  TTC TTT AAC AAC ACA ATC AGC AGA GTA CCA GCC GTT AAT TTA GAT  2120
 692  Phe Phe Asn Asn Thr Ile Ser Arg Val Pro Ala Val Asn Leu Asp  706

2121  GAA ACC TTG GCA AAA CAG CAA TTA ACA GCT GCA ATG TCT TGC GTT  2165
 707  Glu Thr Leu Ala Lys Gln Gln Leu Thr Ala Ala Met Ser Cys Val  721

2166  ACA AGT GGC AAA GAA GAA GCT GGA AGA CAA CAG CTT TAT CTG GAA  2210
 722  Thr Ser Gly Lys Glu Glu Ala Gly Arg Gln Gln Leu Tyr Leu Glu  731

2211  ATT ATT GCT AAA CCT AGC CAT CCA GAT TTA GCA TTG GAA CCG CAC  2255
 737  Ile Ile Ala Lys Pro Ser His Pro Asp Leu Ala Leu Glu Pro His  751

2256  CGT TTG TAC AAT ATT TTG GCA ACT TTG ATT CTT GGA TTA GTT ATT  2300
 752  Arg Leu Tyr Asn Ile Leu Ala Thr Leu Ile Leu Gly Leu Val Ile  766   Region A3
                                                                        (753-777)
2301  TAT GGC GTT TCA ACT TTA TTA TTA GCC GGT GTG AGA GAG CAT AAG  2345
 767  Tyr Gly Val Ser Thr Leu Leu Leu Ala Gly Val Arg Glu His Lys  781

            hctB
2346  AAC TGA  TG CAG TAT GGT GAA CAA ACT TCG TTA AAA GAT TCA TTT  2389
 782  Asn ***
             Met  Gln Tyr Gly Glu Gln Thr Ser Leu Lys Asp Ser Phe  795

2390  ACT ATC CAA GGA CGG GTG TTG AAA GCG TTG TTG TTG CGT GAA ATT  2434
 796  Thr Ile Gln Gly Arg Val Leu Lys Ala Leu Leu Leu Arg Glu Ile  810

2435  ATC ACT CGT TAT GGT CGT AAA AAT TTA GGC TTT TTG TGG GTT GTT  2479
 811  Ile Thr Arg Tyr Gly Arg Lys Asn Leu Gly Phe Leu Trp Val Val  825

2480  CGT GAG CCA TTT TTG ATG AGC CTA GTT ATT GTG GTA ATG TGG CAT  2524
 826  Arg Glu Pro Phe Leu Met Ser Leu Val Ile Val Val Met Trp His  840

2525  TTT TTT CGT GCT GAT CGC TTT TCA ACA TTA AAC ATT GTT GCT TTT  2569
 841  Phe Phe Arg Ala Asp Arg Phe Ser Thr Leu Asn Ile Val Ala Phe  855

2570  GCA ATG ACG GTT ATC CAT TAT TAT GGA TGT GGC GTA ATG CTT CTA  2614
 856  Ala Met Thr Val Ile His Tyr Tyr Gly Cys Gly Val Met Leu Leu  870
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2615  ACC GTG CAA TTA GCG GGA ATG GAT TCC AAT ATC CCA TTA CTT TTA  2659
 871  Thr Val Gln Leu Ala Gly Met Asp Ser Asn Ile Pro Leu Leu Leu  885

2660  TCA CGT AAT GTA CGT CCT CTT GAT ACG CTT TTT TCT CGT ATG ATT  2704
 886  Ser Arg Asn Val Arg Pro Leu Asp Thr Leu Phe Ser Arg Met Ile  900

2705  TTG GAG ATT GCT GGT GCG ACT GTA GCA CAA ATT GTG ATG TTA GTG  2749
 901  Leu Glu Ile Ala Gly Ala Thr Val Ala Gln Ile Val Met Leu Val  915

2750  ATT TTA ATT GCT ATT GAT TGG ATC GGC TTG CCA AAT GAT GTG TTG  2794
 916  Ile Leu Ile Ala Ile Asp Trp Ile Gly Leu Pro Asn Asp Val Leu  930

2795  TAT ATG CTT TTT GCT TGG TTC TTA ATG GCA CTG TTT GCC ATT GGT  2839
 931  Tyr Met Leu Phe Ala Trp Phe Leu Met Ala Leu Phe Ala Ile Gly  945

2840  TTA GGT TTA ATT ATT TGT GCT ATT TCT TAT TAT TTA GAG TTT TTC  2884
 946  Leu Gly Leu Ile Ile Cys Ala Ile Ser Tyr Tyr Leu Glu Phe Phe  960

2885  GGT AAA ATT TGG GGA ACA TTA TCT TTT GTG ATG TTT CCT ATT TCC  2929
 961  Gly Lys Ile Trp Gly Thr Leu Ser Phe Val Met Phe Pro Ile Ser  975

2930  GGT GCA TTC TTT TTA GTG AAT AGT TTG CCA AAC AAT CTG CAA TCT  2974
 976  Gly Ala Phe Phe Leu Val Asn Ser Leu Pro Asn Asn Leu Gln Ser  990

2975  ATT TTG CTT TGG TTT CCA ATG GTT CAC GGT ACG GAA ATG TTT CGT  3019
 991  Ile Leu Leu Trp Phe Pro Met Val His Gly Thr Glu Met Phe Arg 1005  Region B
                                                                      (990-1009)
3020  CAC GGT TAT TTT GGT TCT TCA GTT ATT ACA ATG GAA TCA CCG AGT  3064
1006  His Gly Tyr Phe Gly Ser Ser Val Ile Thr Met Glu Ser Pro Ser 1020

3065  TAT TTA TTT ATT TGT GAT TTG GTG ATG TTA TTA ATC GGT CTA CTG  3109
1021  Tyr Leu Phe Ile Cys Asp Leu Val Met Leu Leu Ile Gly Leu Leu 1035

                                                    hctA
3110  ATG GTG GGT AGT TTT AGT AAT AGG ATT AAT GCA AGA  TG ATT AGT  3153
1036  Met Val Gly Ser Phe Ser Asn Arg Ile Asn Ala Arg  ***
                                                      Met  Ile Ser 1050

3154  GTA GAC CAC GTT TAT AAA AAA TAT CAA ACA CGG ACA GGT TCG GTA  3198
1051  Val Asp His Val Tyr Lys Lys Tyr Gln Thr Arg Thr Gly Ser Val 1065

3299  CCC GTA TTA AAT GAT ATT AAT TTT AGC CTT ACC AAA GAA GAA AAA  3243
1066  Pro Val Leu Asn Asp Ile Asn Phe Ser Leu Thr Lys Glu Glu Lys 1080

3244  ATT GGT ATT TTA GGT CGC AAC GGA GCA GGA AAA TCA CCA TTA ATT  3288
1081  Ile Gly Ile Leu Gly Arg Asn Gly Ala Gly Lys Ser Pro Leu Ile 1095  Region C1
                                                                      (1080-1092)
3289  CGT TTA ATG AGT GGT GTT GAA GCT CCA ACT TCA GGA ATA ATT CGA  3333
1096  Arg Leu Met Ser Gly Val Glu Ala Pro Thr Ser Gly Ile Ile Arg 1110

3334  CGA GAA ATG AGC ATT TCT TGG CCA TTA GCC TTT AGC GGT GCA TTC 3378
1111  Arg Glu Met Ser Ile Ser Trp Pro Leu Ala Phe Ser Gly Ala Phe 1125

3379  CAA GGT AGC TTA ACG GGA ATG GAT AAT TTA CGC TTC ATT TGT CGT  3423
1126  Gln Gly Ser Leu Thr Gly Met Asp Asn Leu Arg Phe Ile Cys Arg 1140  Region C2
                                                                      (1131-1138)
3424  ATT TAT AAT GCT GAT ATT AAT TAT GTT ACT GAA TTT ACG GAA TCC  3468
1141  Ile Tyr Asn Ala Asp Ile Asn Tyr Val Thr Glu Phe Thr Glu Ser 1155

3469  TTT TCC GAA TTG GGC AAT TAT TTA TAT GAG CCT GTA AAA AAT TAT  3513
1156  Phe Ser Glu Leu Gly Asn Tyr Leu Tyr Glu Pro Val Lys Asn Tyr 1170

3514  TCT TCA GGA ATG AAA GCA CGC TTA GCT TTT GCA TTG TCG TTA TCC  3558
1171  Ser Ser Gly Met Lys Ala Arg Leu Ala Phe Ala Leu Ser Leu Ser 1185
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3559  GTT GAG TTT GAT TGC TAT CTC ATT GAT GAA GTG ATT GCC GTT GGA  3603
1186  Val Glu Phe Asp Cys Tyr Leu Ile Asp Glu Val Ile Ala Val Gly  1200

3604  GAT TCT CGT TTT AGT GAT AAA TGT CGC TAT GAA CTT TTT GAA AAA  3648
1201  Asp Ser Arg Phe Ser Asp Lys Cys Arg Tyr Glu Leu Phe Glu Lys  1215

3649  CGC AAA GAT CGT TCC ATT ATT TTA GTT TCT CAT AGT CCA ACC GCT  3693
1216  Arg Lys Asp Arg Ser Ile Ile Leu Val Ser His Ser Pro Thr Ala  1230

3694  ATT AGA CAA TAT TGT GAT AAT GCA AAA GTA TTA GAT AAA GGA AAA  3738
1229  Ile Arg Gln Tyr Cys Asp Asn Ala Lys Val Leu Asp Lys Gly Lys  1245

3739  TTG TTA GAT TTC TCT TCT ATT GAT GAG GCT TAT CAA TAT TAT AAT  3783
1246  Leu Leu Asp Phe Ser Ser Ile Asp Glu Ala Tyr Gln Tyr Tyr Asn  1260

3784  CAG ACA TAG AGG TTA GAT TTT AAA ATA AAA TAA CGT TAC TTT CTT  3883
1261  Gln Thr ***                                                 1260

3829  GCT TTA TCA TAA ATT TCA ATG GCT ATA GTT AAG TTC GAA ATA AAT  3873

3874  CAA GGT AAC AAG CTG AAT ACA GTG AAA AAT AGC ACT TTT TAT GCC  3918

3919  AAG GT…
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B

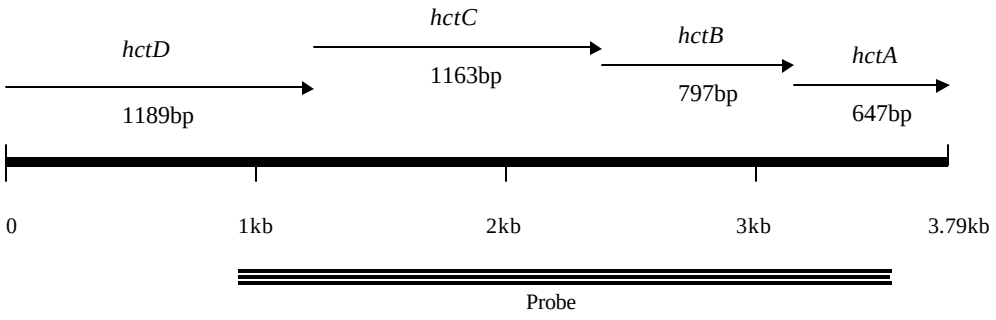

Figure 17: (A) Nucleotide sequence of the capsule transport region. 3923 nucleotides of the sequence are shown, from arbitrary points 167bp upstream of *hctD* to 131bp downstream of *hctA*. The four long open reading frames are indicated as *hctD, hctC, hctB* and *hctA* , in each case from the first ATG, with the translated peptide sequence beneath. The underlined regions are referred to in the text. (B) Genetic map of the capsule transport gene region of *H. paragallinarum.* The locations and directions of transcription of the four open reading frames *hctDCBA* are indicated. The 2638bp fragment used as the DNA probe in Figures 12 and 13 is also shown.

Table3: Comparison of the gene region and capsule gene sizes of *H. paragallinarum* and the HAP organisms

Bacterial species	**Region size**	**Individual genes and corresponding lengths**			
		bexA	*bexB*	*bexC*	*bexD*
H. influenzae	3770bp	654bp	798bp	1134bp	1182bp
		cpxA	*cpxB*	*cpxC*	*cpxD*
A. pleuropneumoniae	3812bp	651bp	798bp	1167bp	1212bp
		hexA	*hexB*	*hexC*	*hexD*
P. multocida	3781bp	660bp	798bp	1137bp	1182bp
		hctA	*hctB*	*hctC*	*hctD*
H. paragallinarum	3792bp	648bp	798bp	1164bp	1188bp

Blast searches of the combined, non-redundant nucleotide and protein databases at the National Center for Biotechnology Information (NCBI) indicated that *H. paragallinarum hctDCBA* were highly homologous at both the nucleotide and amino acid levels to *H. influenzae bexDCBA* (Kroll *et al.*, 1990), *A. pleuropneumoniae cpxDCBA* (Ward and Inzana, 1997), *P. multocida hexDCBA* (Chung *et al.*, 1998) and *N. meningitidis ctrABCD* (Frosch *et al.*, 1992). Protein homology is indicated in Table 4.

The postulated HctDCBA protein sequences were also submitted to a multiple sequence alignment with the corresponding proteins of the HAP organisms. The predicted HctA protein showed 69.3% identity and 80.9% similarity with the A proteins of the HAP organisms (Fig. 18A). HctA contains the ATP-binding domains A (GXLGRXGXGKS) and B (XXDNLRFI) (Walker *et al.*, 1982) at amino acids 1080-1092 and 1131-1138 respectively (Regions C1 & C2, Fig. 17A), which are conserved in the *bexA* and *cpxA* homologues (Kroll *et al.*, 1990; Higgins, 1992; Fath and Kolter, 1993; Ward and Inzana, 1997). The homology shown in Figure 18A as well as the high degree of similarity between homologous proteins in Table 4, supports the speculation that *hctA* might encode an ATP-binding protein component of a polysaccharide export apparatus.

HctB showed 43.4% identity and 75.8% similarity with corresponding proteins of the HAPs (Fig. 18B) and in comparison to the literature is predicted to be a hydrophobic protein over most of its length, containing at least six potential membrane-spanning

Table 4: Comparison of proteins encoded by ORFs in *H. paragallinarum* 1742 with known proteins in the NCBI database

ORF (size bp)	Protein (size aa)	Similar proteins (bacterial species)	Accession no.	% Identity	% Similarity
hctA (648)	HctA (215)	BexA *(H. influenzae*)	P10640	77.2	85.1
		CpxA (*A. pleuropneumoniae*)	U36397	78.6	85.6
		CtrD (*N. meningitidis*)	P32106	78.6	87.4
		HexA (*P. multocida*)	AF067175	75.3	85.6
hctB (798)	HctB (265)	BexB (*H. influenzae*)	P19391	57.7	86
		CpxB (*A. pleuropneumoniae*)	U36397	57.7	84.9
		CtrC (*N. meningitidis*)	P32106	58.1	86.7
		HexB (*P. multocida*)	AF067175	58.5	82.3
hctC (1164)	HctC (387)	BexC (*H. influenzae*)	P22930	43.7	67.2
		CpxC (*A. pleuropneumoniae*)	U36397	43.9	65.6
		CtrB (*N. meningitidis*)	P32106	38.7	58.1
		HexC (*P. multocida*)	AF067175	49.9	71.8
hctD (1188)	HctD (395)	BexD (*H. influenzae*)	P22236	42.5	63.0
		CpxD (*A. pleuropneumoniae*)	U36397	42.5	65.0
		CtrA (*N. meningitidis*)	P32106	41.5	62.5
		HexD (*P. multocida*)	AF067175	43.0	68.1

A

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CpxA  1    MISVKNVSKDYYTRSGKKTVLQDINFELKKGEKIGILGRNGAGKSTLIRLLSGVEPPTSGTIER  64
BexA  1    MIRVNNVCKKYHTNSGWKTVLKNINFELQKGEKIGILGRNGAGKSTLIRLMSGVEPPTSGTIER  64
HctA  1    MISVDHVYKKYQTRTGSVPVLNDINFSLTKEEKIGILGRNGAGKSPLIRLMSGVEAPTSGIIRR  64
HexA  1    MIYVENVCKKYLNRTGWHTVLDDVSFSLRKGQKVGILGKNGAGKSTLIRLLSGVEPPTSGKITR  64

CpxA  65   NMSISWPLAFQRAFQGSLTGMDNLRFICRIYNADIEYVKAFTEEFSELGDYLYEPVKKYSSGMK  128
BexA  65   SMSISWPLAFSGAFQGSLTGMDNLRFICRLYDVDPDYVTRFTKEFSELGDYLYEPVKKYSSGMK  128
HctA  65   EMSISWPLAFSGAFQGSLTGMDNLRFICRIYNADINYVTEFTESFSELGNYLYEPVKNYSSGMK  128
HexA  65   KMSISWPLGFSGAFQGSLTGMDNLRFIARIYNADIDYVKRFTEEFSELGKYLYEPVRTYSSGMK  128

CpxA  129  ARLAFALSLSVEFDCYLIDEVIAVGDSRFAAKCKHELFEKRKDRSIILVSHSPSAMKEYCDNAM  192
BexA  129  ARLAFALSLSVEFDCYLIDEVIAVGDSRFAEKCKYELFEKRKDRSIILVSHSPSAMKSYCDNAV  192
HctA  129  ARLAFALSLSVEFDCYLIDEVIAVGDSRFSDKCRYELFEKRKDRSIILVSHSPTAIRQYCDNAK  192
HexA  129  ARLAFALSLSIEFDCYLIDEIIAVGDARFADKCKYELFEKRKDRSLILVSHSPNAIREYCDNAM  192

CpxA  193  VLDKGIMYKFENMDEAYKFYNSTL    216
BexA  193  VLENGIMHHFEDMDKAYQYYNET     215
HctA  193  VLDKGKLLDFSSIDEAYQYYNQT     215
HexA  193  VLHNGIMHHFNSIDIAYQFHNNALQTK 219
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B

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CpxB  1    MQYGDQTTFRQSLAIQGRVIGALLMREIITRYGRKNLGFLWLFVEPLLLTLFIVLMWKFIRADR  64
BexB  1    MQYGDKTTFKQSLAIQGRVINALLMREIITRYGRQNIGFFWLFVEPLLMTFFIVMMWKFIRADK  64
HexB  1    MLYDDQTTLKHAFIIQNRVIGALLMREIITRYGRKNLGFLWLFVEPLLMTGLIVILWSTFRADQ  64
HctB  1    MQYGEQTSLKDSFTIQGRVLKALLLREIITRYGRKNLGFLWVVREPFLMSLVIVVMWHFFRADR  64

CpxB  65   VSDLNIIAFVITGYPMAMMWRNASNRTIGAISGNLSLLYHRNVRVLDTLLARVILEVAGATIAQ  128
BexB  65   FSTLNMIAFVMTGYPMAMMWRNASNRAIGSISANLSLLYHRNVRVLDTIFTRVLLEVAGASIAQ  128
HexB  65   FSNLNIMAFMITGYPLMMMWRNASNRAIGAVSANSTLLYHRNVRVLDTLIARMILEISGATIAQ  128
HctB  65   FSTLNIVAFAMTVIHYYGCGVMLLTVQLAGMDSNIPLLLSRNVRPLDTLFSRMILEIAGATVAQ  128

CpxB  129  IIIMALVILLGWIEMPKDTFYMVMAWVLMAFFALGLGLIICSIAQKFEAFGKIWGTLSFVLLPL  192
BexB  129  ILFMAILVMIDWIDAPHDVFYMLIAWFLMAMFAFALGLIICAIAQQFDVFGKIWGTLSFVLLPI  192
HexB  129  VVLMSFCIALDWIPMPQDIFYMLLAWFLMAIFAIGFGFIICSISHHIEVFGKLWGILSFAMMPL  192
HctB  129  IVMLVILIAIDWIGLPNDVLYMLFAWFLMALFAIGLGLIICAISYYLEFFGKIWGTLSFVMFPI  192

CpxB  193  SGAFFFVHALPSQAQQYATLIPMIHGTEMFRHGYFGDSVITYESISYLVICDVAMLLFGLIMVK  256
BexB  193  SGAFFFVHNLPAQAQSIALWFPMIHGTEMFRHGYFGDTVVTYESIGFLVVSDLALLLLGLVMVK  256
HexB  193  SGTFFFVYNLPPQAREYLLWMPMIHGSEMFRHGYFGDSVITLENPYFLILCDLIFLLIGLLMVA  256
HctB  193  SGAFFLVNSLPNNLQSILLWFPMVHGTEMFRHGYFGSSVITMESPSYLFICDLVMLLIGLLMVG  256

CpxB  257  NFSKGIEPQ 265
BexB  257  NFSKGVEPQ 265
HexB  257  HFSKGIEPR 265
HctB  257  SFSNRINAR 265
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C

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CpxC  1    METTITASPTEKLQKPVKQKKS-WLKKLNPLFWVTVAIPTVLSAFYFGSVASDIYISESSFVVR  63
BexC  1           MTTENAAIPTKKKKS-FWKKMKPLFGLTVLIPTAFSAVYFGLFASDIYVSESSFVVR  56
HexC  1           MIETKIQKNQFKKWSRKFRKISTLFKYMVILPTCCSLFYFSLWASDIYISQSSFVVR  57
HctC  1         MEQNVVVQSKEQLRKLKQWLRKINLLFLLTVIIPTFCSLFYFSIWASDVYISESSFIVR  59

CpxC  64   SPQNQTALTGVGALLQGSGFSRAQDDTYTVQEYMHSRTALEQLMKDLPIREYYENQGDIIARFN  127
BexC  57   SPRSQSSLSGVGALLQSTGFSRSQDDTYSVQEYMRSRTALSALEQGLPLRTFYSEKGDLLSRFN  120
HexC  58   TPKNQAALSGVGALLQSSGFARAQDDTYTVQEFMRSRSTLELLEKSIPIRQFYEDKGDLFSRFN  121
HctC  60   SSRAQASLGGMGALLQSIGFARSQDDTFTVQEFMRSRNALTTLESELPVRKFYEDEGDFFSPFN  123

CpxC  128  GFGLNNSKEAFYKYFRDRLSVDFDSVSGIASLR---IRAFNAEEGQQIN-QKLLAEGETLINRL  187
BexC  121  GFGLNDTQEAFYRYFKERLSVDVDSISGIATLR---VHAFDAEEGYQIN-ERLLKEGESLINRL  180
HexC  122  PLNIFSEQEAFYQYFSKKLSVNFDSVSGIATLN---IRAFDPKEAQQIN-QELLKQGEYLINRL  181
HctC  124  PLGFFNEQELFYQYFRKHLMINIDSLSGYCYFTGSVGLMADLRHQQELNGSHYCHFGGKPFSGI  187
```

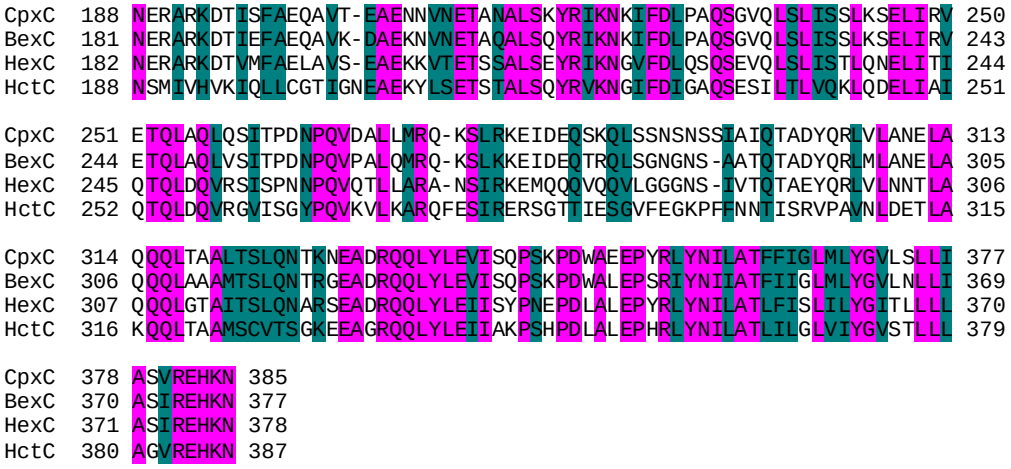

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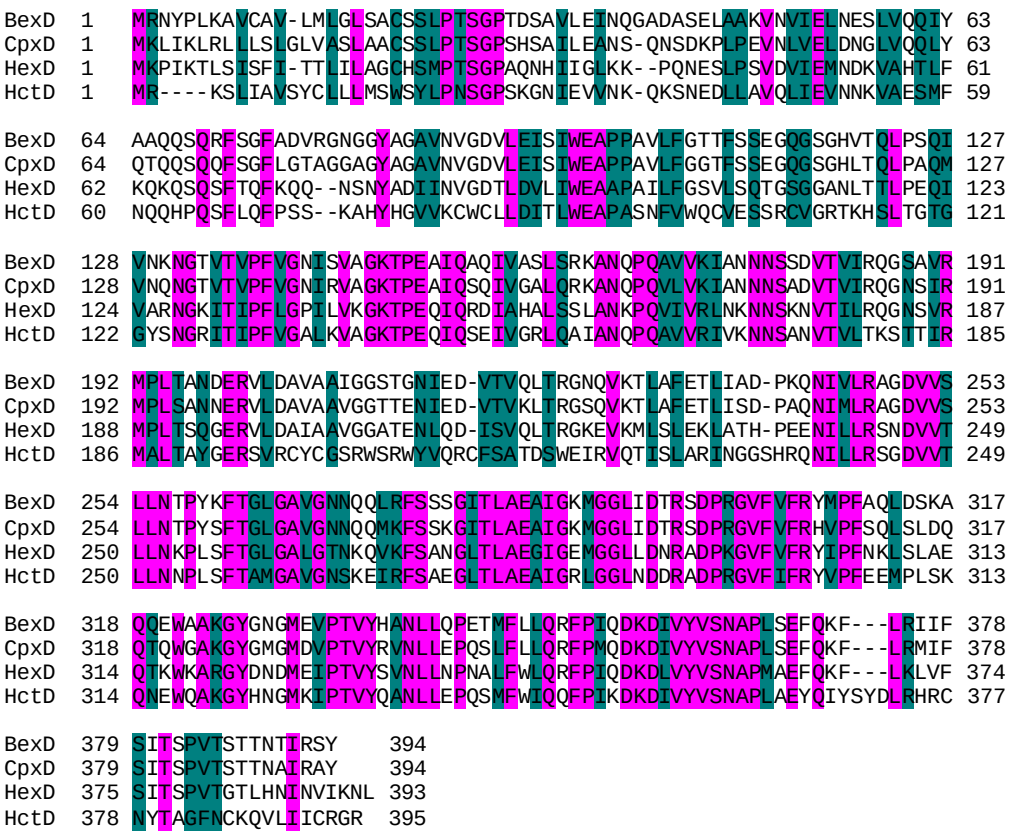

Figure 18: Multiple sequence alignment of the predicted Hct proteins with the corresponding proteins of the HAP organisms with Bex, Cpx and Hex representing the different proteins of *H. influenzae*, *A. pleuropneumoniae* and *P. multocida* respectively. (A), (B), (C) and (D) show the alignment of the HctA, HctB, HctC and HctD proteins with the HAP proteins respectively. The two colors distinguish between identity (pink) and similarity (green) among the proteins.

α- helical domains (Kyte and Doolittle, 1982; Kroll *et al.*, 1990;Ward and Inzana, 1997). A short relatively hydrophillic region starting at amino acid 990 (Region B, Fig. 17A) aligned with a similar region in OppB of *Salmonella typhinurium* (Hiles *et al.*, 1987) and BexB (Kroll *et al.*, 1990). Each in turn showing a marginal sequence similarity to a consensus thought to be involved in intermolecular interactions in the oligopeptide transporter (Dassa and Hofnung, 1985). Figure 17A shows the position of this sequence on HctB and Figure 19 shows an alignment of the relatively hydrophilic portions of HctB, BexB and OppB. HctB is therefore a candidate for an integral inner-membrane component of the putative polysaccharide exporter.

```
HctB  (207)    S I L L W F P M V H G T E M F R H G Y F
BexB  (208)    S I A L W F P M I H G T E M F R H G Y F
OppB  (209)    R T A R A K G L P M R R I I F R H A L K
```

Figure 19: Alignment of the relatively hydrophilic portions of HctB, BexB and OppB. The number in brackets is the position of the first amino acid in each sequence. Identities are shown, and the matches of the OppB sequence to the Dassa/Hofnung consensus are underlined.

The multiple protein sequence alignment of HctC with the respective C proteins of the HAPs (Fig. 18C), showed 34.6% identity and 56.8% similarity. Transposon mutagenisis of BexC from *H. influenzae* (Kroll *et al.*, 1990) and PSORT of HexC (Chung *et al.*, 1998) suggested an inner membrane protein, possibly with periplasmic domain. Ward and Inzana (1997) predicted the CpxC protein of *A. pleuropneumoniae* to be relatively hydrophilic with hydrophobic domains near the N and C-termini that may serve as membrane anchors. Three long hydrophobic stretches of amino acid sequence with membrane-spanning potential allowing the possibility of anchoring at more than one site, have been identified in BexC (Kroll *et al.*, 1990). Similar stretches of sequence are present in HctC at amino acids 421-432, 458-475 (Regions A1 & A2, Fig. 17A) at the

proposed N-terminal and 753-777 at the C-terminal (Region A3, Fig 17A). Considering this information and the facts known about the HctC homologues, this protein is also proposed to serve as the second component of a protein complex involved in polysaccharide export across the cytoplasmic membrane (Reizer *et al.*, 1992)

HctD showed 30.6% identity and 54.2% similarity with the predicted D proteins of the HAP organisms (Fig 18D). HctD also showed similarity of 63% with BexD, and 62.5% with CrtA from *H. influenzae* and *N. memingitidis* respectively (Table 4). CtrA from *N. meningitidis* is believed to be an outer membrane protein with porin properties (Frosch *et al.*, 1992), BexD and homologues to be outer membrane associated (Kroll *et al.*, 1990; Rosenow *et al.*, 1995) and mutations in the *bexD* gene coding for this corresponding protein accumulated polysaccharide in the periplasmic space (Bronner *et al.*, 1994). Based on its similarities with CtrA and BexD, HctD is probably an outer membrane protein involved in capsular polysaccharide transport across the outer membrane, possibly with porin properties.

This data is therefore consistent with the hypothesis that the *hctABCD* gene cluster encode proteins that form an export complex for the hyaluronic acid capsule.

3.5 Discussion

The capsule transport gene region of *H. paragallinarum* 1742 was cloned and characterized. The nucleotide sequence was determined and the transport region shown to be 3792bp in length. Analysis of the gene cluster revealed four ORFs, 648bp, 798bp, 1164bp and 1188bp respectively speculated to encode components of an ATP-driven capsule polysaccharide export apparatus. The genetic organization of loci encoding such transporters can be variable (Higgins *et al*., 1986), but typically there are four or five genes in one or a pair of transcriptional units. Examples include the *S. typhimurium* histidine transporter (Higgins *et al*., 1982), the *S. typhimurium* oligopeptide permease (Hiles *et al*., 1987), the capsule polysaccharide exporters in *H. influenzae* (Kroll *et al*., 1990), *A. pleuropneumoniae* (Ward and Inzana, 1997) and *P. multocida* (Chung *et al*., 1998). These transport systems normally consist of three or four membrane-associated domains or polypeptides, two hydrophobic integral membrane proteins and one or two hydrophilic ATP-binding proteins.

The overlapping stop and start codons in the *hct* genes evident in Fig. 17A indicate that these four genes are transcriptionally coupled. This gene organization correspond with the genes present in similar regions of the HAP organisms (Kroll *et al*., 1990; Ward and Inzana, 1997; Chung *et al*., 1998). The broad homology between the capsule gene loci of *H. paragallinarum* and those of different encapsulated gram negative bacterial species have been demonstrated in this study (Table 4; Fig. 18). This homology included proteins of the capsular polysaccharide export systems of *H. influenzae, A. pleuropneumoniae, P. multocida* and *N. meningitidis*, arguing a common evolutionary origin of the molecular mechanisms of encapsulation. The homology also indicates that the capsule transport genes of *H. paragallinarum* encode proposed proteins similar in function to those of the other gram negative bacterial species mentioned.

Clues to the possible function of the gene products have been provided by comparisons with known proteins whose sequences have been deposited with the NCBI databases and consensus and conserved regions within the transport genes of *H. paragallinarum* also considered. The conclusion has been made that the region encodes proteins forming an ABC-transporter system. HctA with its conserved ATP-binding

domains A and B (Fig. 17A) is proposed to be the ATP-binding protein component, while HctB is a candidate for an integral inner-membrane component. Hydrophobic domains in HctC (Fig. 17A) with the possible function of acting as membrane spanning anchors, supports the speculation that HctC acts as a second membrane associated protein in the transport complex. Lesser sequence similarities, although still significant, between the proposed HctD protein and BexD, CpxD, CtrA and HexD proteins (Table 4), support the speculations that *hctD* encodes a outer membrane protein with possible porin properties. This data is in accordance with findings by Kroll *et al*. (1990), Ward and Inzana (1997) and Chung *et al*. (1998), who suggested an ATP-dependent export system for the *H. influenzae, A. pleuropneumoniae* and *P. multocida* capsule polysaccharides. There is also evidence that the capsule transport system is a member of a superfamily designated 'ABC (ATP-binding cassette) transporters' (Higgins *et al*., 1990; Hyde *et al*., 1990)

These findings will greatly facilitate the investigation at molecular level of the role of the *H. paragallinarum* capsule in pathogenisis and immunity. However, confirmation of the importance of each gene product and elucidation of the function of each protein, will require characterization of the phenotypic impact of in-frame deletions or other mutations in the respective ORF.

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Chapter 4

Transplacement of the capsule transport gene region with a tetracycline resistance cassette

4.1 Abstract

Non-capsulated mutants of several gram negative bacterial species have been constructed in order to study the role of the capsule as virulence factor. In this study the genetic information of the capsule transport gene region was employed to construct an isogenic strain of *H. paragallinarum* in which the transport process have been disturbed. Two attempts were made to construct these mutants by insertion of a tetracycline resistance cassette into plasmids in which a transport gene/s have been deleted. Virtually all four the capsule transport genes were deleted (~2770bp) with PCR and replaced with a ~3.2kb tetracycline resistance cassette by virtue of introduced *Bgl*II restriction sites. The ~5.85kb linear PCR product (*hct*Δ::Tet^{+}) for transformation to *H. paragallinarum* was amplified with sequence specific oligonucleotide primers and represented the tetracycline resistance cassette flanked by a ~500bp segment of the biosynthesis gene region and *hctD* upstream and downstream by ~2100bp partially representing *hctA* and non-coding DNA. In the second deletion construct the final gene of region 1, *hctA*, was replaced with the tetracycline resistance cassette and amplified by PCR resulting in a ~6.66kb fragment with 606bp of the *hctA* gene replaced with the ~3.2kb tetracycline resistance cassette with *hctBC* ~1750bp flanking the upstream region and ~1700bp of non-coding DNA downstream from the 3'- *Bgl*II restriction site.

4.2 Introduction

Polysaccharide capsules are found on the surface of a wide range of bacteria. With gram negative bacteria, the capsule lies outside the outer membrane and is composed of highly hydrated polyanionic polysaccharides (Roberts, 1996). Capsules have a significant role in determining access of certain molecules to the cell membrane, mediating adherence to surfaces, and increasing tolerance of desiccation. Furthermore, capsules of many pathogenic bacteria impair phagocytosis (Shimoji *et al.,* 1994; Nilsson *et al.,* 1997; Smith *et al.,* 1999) and reduce the action of complement-mediated killing (Tu *et al.,* 1982; Snipes and Hirsh, 1986; Chae *et al.,* 1990). Thus capsules are likely to be major virulence determinant and genetically defined acapsular mutants of a number of organisms have been constructed by various techniques and showed reduced virulence (Chang *et al.,* 1996; Thakker *et al.,* 1998; Zuppardo and Siebeling, 1998; Favre-Bonte *et al.,* 1999; Smith *et al.,* 1999).

Non-capsulated mutants of *Actinobacillus pleuropneumoniae* were isolated following chemical mutagenises with ethyl methanesulfonate and used for intratracheal challenge of pigs. Challenge with the mutants resulted in no clinical disease or lesions and provided protection against infection with the wild-type strain (Inzana *et al.,* 1993). Boyce and Adler (2000) have constructed isogenic mutants of *Pasteurella multocida* serotype B strain M1404 by transplacement of the capsule transport gene, *cexA* to determine whether the capsule is instrumental to the virulence of the organism. Mutants were created by allelic replacement and tested for virulence by intraperitoneal challenge of mice, with results showing conclusively that the capsule is a crucial virulence determinant of *P. multocida*.

The virulence mechanisms of *H. paragallinarum* are not clearly understood at this time. Sawata and Kume (1983) suggested that the L and HA-L outer membrane antigens were responsible for adherence to the sinus mucosal surface and therefore responsible for pathogenicity of *H. paragallinarum*. Sawata *et al.* (1985) also suggested that the hyaluronic acid capsule might be the primary structure associated with attachment (rather than the L and HA-L antigens) as well as lesion formation. It has also been stated by Sawata *et al.* (1984) that the capsule protects against the bactericidal activity of fresh

chicken serum. The capsule has thus been implicated in virulence, since non-capsulated serotype B isolates have been regarded as non-virulent wild-type strains of *H. paragallinarum* (Kume *et al.*, 1980). No genetically defined acapsular strains have been constructed to allow unequivocal demonstration of the *H. paragallinarum* capsule as a virulence determinant. The nucleotide sequence of the capsule transport genes has been determined and by using this information, an attempt was made to construct an isogenic strain impaired in capsule export to investigate the contribution of the capsule to virulence of the *H. paragallinarum*.

4.3 Materials and methods

4.3.1 Strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 5. *Haemophilus. paragallinarum* cultures were grown in TM/SN media (Blackall and Yamamoto, 1990), supplemented with 0.0025% NAD if needed, at 37°C in a candle jar for solid cultures and in 10ml liquid cultures without aeration. *E.coli* strains Sure®2 (Strategene) and JM109 (Promega) were used for cloning and amplification of DNA. *Escherichia coli* cultures were grown at 37°C in Luria-Bertani (LB) media and transformed with plasmids as described by Inoue *et al.* (1990). Ampicillin (60µg/ml) and tetracycline (80µg/ml) were added to liquid and solid media if required.

Table 5: Bacterial strains and plasmids used in this study

Strains/plasmids	**Relevant characteristic**	**Reference or source**
Strains		
HP 0222	Wild type, NAD dependent	Field isolate (Bragg *et al.*, 1993a)
HP 1742	Wild type, C3, NAD independent	Field isolate (Bragg *et al.*, 1993a)
E.coli Sure®2	For high-efficiency cloning of DNA with secondary structures, recA$^-$ and blue-white screening	Strategene
E.coli JM109	β-galactosidase deficient, recA$^-$	Promega
Plasmids		
pGhct-c	6.15kb *Hind*III insert in pGem3Z contains part of region 2 and the entire region 1	This study
pBR*tet*(M)	3.2kb tetracycline resistance cassette *tet*(M) insert in pVB101	Dr. J.D. Boyce, Monash University, Australia
pGhct-Δ	pGhct-c with *hctB,C* and parts of *hctA,D* deleted, ligated at the *Bgl*II sites produced by PCR primers	This study
pGhctA-Δ	pGhct-c with *hctA* deleted by PCR, ligated at the *Bgl*II sites produced by PCR primers	This study
pGhct-Tet$^+$	3.2kb *tet*(M) cassette *Bam*HI insert cloned into pGhct-Δ	This study
pGhctA-Tet$^+$	3.2kb *tet*(M) cassette *Bam*HI insert cloned into pGhctA-Δ	This study
Native HP plasmid	Rendering NAD independence	Bragg *et al.*, 1993b

4.3.2 Capsule staining

Staining was performed on 16-hour cultures with nigrosine and crystal violet. A loop of organism was suspended in a drop of nigrosine on a microscope slide and heat fixed. The preperate of the bacteria was then covered with crystal violet and left for 1min before carefully rinsing with water. The slide was blotted dry and inspected under oil immersion.

4.3.3 Construction of the deletion plasmids pGhct-Δ and pGhctA-Δ

The deletions were performed on the pGhct-c plasmid by means of PCR with primers introducing *Bgl*II restriction sites on both the 5'- and 3' end of these products. The PCR reactions included 0.2mM dNTP mixture, 2pmol of each primer (Table 6), a 10X dilution of buffer system 1 (100mM Tris-HCl, 17.5mM $MgCl_2$, 500mM KCl, pH 8.3) and 2.6U Expand long template *Taq* polymerase (Roche Molecular Biochemicals) in a final volume of 50µl. The reaction conditions had an initial denaturation cycle at 93°C for 2min, followed by 10 cycles at 93°C for 10sec, 52°C for 30sec and 68°C for 3min. The reaction continued with a further 20 cycles at of 93°C for 10sec, 52°C for 30sec and 68°C for 3min, with 20sec added to the last step with every cycle. A final elongation cycle was performed at 68°C for 4min.

PCRs were performed on the Eppendorf mastercycler personal thermal cycler and the products separated by agarose-gel electrophoresis in TAE buffer (40mM Tris-acetate, 1mM EDTA). The PCR products were purified with the GFX™ PCR DNA and gel band purification kit (Amersham Pharmacia Biotech) and submitted to restriction analysis with *Hind*III for confirmation. Both PCR products were circularized by ligation with T4 DNA ligase in a 10X dilution of ligation buffer (660mM Tris-HCl, 50mM $MgCl_2$, 10mM DTT, 10mM ATP, pH 7.5) (Roche Molecular Biochemicals) and transformed to competent Sure2® *E.coli* cells (Strategene). Positive clones were selected on LB plates with 60µg/ml ampicillin.

Plasmid was extracted from transformants using the rapid alkaline lysis method described by Sambrook *et al.* (1989) and submitted to digestion with restriction enzymes *Hind*III and *Bgl*II to confirm the presence of the correct construct and to ensure that the *Bgl*II restriction sites were intact.

Table 6: Primers used for construction of pGhct-Δ and pGhctA-Δ, with the introduced *Bgl*II restriction sites underlined.

Product	**Primer**	**Sequence**
pGhct-Δ	Hdp-1F	5'- TGA GAT CTC CAA GGT AGT TTA ACG G -3'
	Hdp-1R	5'- TGA GAT CTC TGC TGC CGC AAT AG -3'
pGhctA-Δ	HdpA-1F	5'- TGA GAT CTC TTC TAT TGA TGA GGC -3'
	HdpA-1R	5'- TGA GAT CTC GTG GTC TAC ACT AAT C -3'

4.3.4 Preparation of the tetracycline resistance cassette *tet*(M) for cloning into the deletion plasmids.

The pBR*tet*(M) vector containing the ~3.2kb tetracycline cassette was submitted to restriction analysis with enzymes *Bam*HI or *Eco*RI from Roche Molecular Biochemicals to excise the cassette from the vector. The *Bam*HI digestion was purified from the gel with the GFX™ PCR DNA and gel band purification kit and cloned into the vector constructs pGhct-Δ and pGhctA-Δ with T4 DNA ligase in a 10X dilution of ligation buffer (660mM Tris-HCl, 50mM $MgCl_2$, 10mM DTT, 10mM ATP, pH 7.5). The ligations were performed at 14°C for 16 hours.

4.3.5 Construction of plasmids pGhct-Tet$^+$ and pGhctA-Tet$^+$ with *tet*(M) inserts.

The tetracycline resistance cassette was cloned into the deletion vectors pGhct-Δ and pGhctA-Δ as described previously and transformed to competent JM 109 *E.coli* cells

(Promega). Plasmid was extracted from the transformants according to the rapid alkaline lysis method of Sambrook *et al.* (1989). Possible pGhct-Tet$^+$ and pGhctA-Tet$^+$ constructs were submitted to restriction analysis with enzymes *Hind*III (Roche Molecular Biochemicals) and *Acc*65I (New England Biolabs), to confirm the orientation of the tetracycline cassette in the deletion plasmids.

4.3.6 PCR amplification of the *hct*Δ::Tet$^+$ and *hctA*Δ::Tet$^+$ constructs for transformation to *Haemophilus paragallinarum.*

The plasmid constructs pGhct-Tet$^+$ and pGhctA-Tet$^+$ were used as template to amplify *hct*Δ::Tet$^+$ and *hctA*Δ::Tet$^+$ respectively. PCRs were performed on the Eppendorf master cycler personal thermal cycler with the Expand long template kit (Roche Molecular Biochemicals) in a final volume of 50µl. In addition to the template DNA, the PCR reaction contained 2pmol of each of the two sequence specific primers Hcs-1F in combination with Hdp-2R or Hcs-2F in combination with Hdp-2R (Table 7), 0.2mM dNTP mixture, a 10X dilution of buffer system 1 (100mM Tris-HCl, 17.5mM $MgCl_2$, 500mM KCl, pH 8.3) and 2.6U of Expand long template *Taq* polymerase. The reaction conditions included an initial denaturation cycle at 93°C for 2min followed by 10 cycles at 93°C for 10sec, 51°C for 30sec and 68°C for 3min. The reaction continued with a further 20 cycles at 93°C for 10sec, 51°C for 30sec and 68°C for 3min, with 20sec added to the last step with every cycle and a final elongation cycle at 68°C for 4min.

PCR products *hct*Δ::Tet$^+$ and *hctA*Δ::Tet$^+$ were purified with the GFX™ PCR DNA and gel band purification kit and submitted to restriction analysis with enzymes *Hind*III and *Acc*65I.

Table 7: Primers used for amplification of *hct*Δ::Tet$^+$and *hctA*Δ::Tet$^+$

Product	**Primer**	**Sequence**
*hct*Δ::Tet$^+$	Hcs-1F	5'- GCA AGA ATT TAT GCC GTT CG -3'
	Hdp-2R	5'- GGC TGA CGG TGA TGT GCG AG -3'
*hctA*Δ::Tet$^+$	Hcs-2F	5'- GGA TTT GCT TGC AGT ACA G -3'

4.3.7 Transformation methods

Competent *E.coli* cultures were prepared according to the method described by Inoue *et al.* (1990). Plasmid constructs were incubated with the competent cells for 30min on ice, followed by heat-shock of 30sec at 42°C. Cells were incubated in 800µl LB- or SOB media (2% bacto-tryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$ pH 6.7-7.0), supplemented with 20mM glucose, for 1 hour at 37°C with aeration. After centrifugation at 4000g the cells were resuspended in 100µl SOB media and plated on LB media solidified with 1.5% agar and containing ampicillin or tetracycline. A concentration of 60µg/ml ampicillin was used to select for possible clones containing the pGhct-Δ and pGhctA-Δ constructs and 80µg/ml tetracycline to select for possible clones containing the pGhct-Tet^+ and pGhctA-Tet^+ constructs (Table5).

Competent *H. paragallinarum* cells were prepared according to the method described by Sambrook *et al.* (1989) with slight modifications. Single colonies of *H. paragallinarum* were inoculated into four tubes containing 10ml TM/SN media (1% Biosate peptone, 1% NaCl, 0.5% glucose, 0.1% starch and 0.0005% thiamine solution, Oleic acid-albumin complex and chicken serum as supplements) and incubated for 16 hours at 37°C without shaking. The cultures were aseptically transferred to ice-cold 50ml polypropylene tubes and cooled to 0°C by storing the tubes on ice for 10min. The cells were recovered by centrifugation at 4000g for 15min at 4°C in a JA-20 rotor. The media was discarded and the pellet washed twice with 15ml of sterile water. The washing steps were followed by resuspension of the pellet in 10% glycerol and centrifugation at 4000g for 15min at 4°C. The pellet was finally resuspended in 200µl 10% glycerol and dispensed into 50µl aliquots, kept on ice for immediate use.

Several methods were tested for the transformation of *H. paragallinarum*. An attempt was made to determine the transformation efficiency by transforming NAD dependent strain 0222 with the native HP plasmid (Bragg *et al.*, 1993b) and NAD independent strain F113 with *E.coli* plasmids pUC18 and pGem3Z. Transformation was performed by electroporation as described by Sambrook *et al.* (1989) with the pulse

applied with the Gene pulser™ (BIO RAD) set at 2.5kv, 25μF and 200ohms. The voltage setting was varied at either 1.5kv, 1.8kv, 2kv or 2.5kv in different attempts to transform *H. paragallinarum* with either 1ng, 2ng or 5ng of plasmid DNA. In the case of the NAD dependent strain 0222 cells were plated immediately after electroporation on TM/SN plates without any NAD. NAD independent strain 1742 was transformed with pUC 18 and pGem3Z, allowing a 1-hour recovery phase at 37°C with gentle shaking before the cells were plated out on TM/SN plates containing 60μg/ml ampicillin. The plates were incubated at 37°C in a candle jar.

Transformation of strain 1742 with the linear constructs *hct*Δ::Tet$^+$ and *hctA*Δ::Tet$^+$ was also performed by electroporation as described, with variation of a number of parameters. Electroporation was performed at four different voltage settings (1.5kv, 1.8kv, 2kv or 2.5kv) and 1μg, 3μg or 5μg of each construct was transformed in different reactions. The recovery phase lengths were also varied between 1, 1.5, 2, and 3 hours. The cells were plated on TM/SN plates, which contained 2.5μg/ml, 5μg/ml or 10μg/ml tetracycline for selection. The transformation method described by Gromkova and Goodgal (1979) for *H. parainfluenzae*, was also used to transform the linear constructs to *H. paragallinarum*. This method required incubation of 100μl of a 16-hour culture of cells with 0.5μg DNA at 37°C for 5 hours with gentle shaking. The cells were plated out on TM/SN plates containing 2.5μg/ml tetracycline.

4.4 Results

4.4.1 Capsule staining

Certain strains of *H. paragallinarum*, like serotype B isolates (Kume *et al.*, 1980) lack capsular material and have thus been regarded as non-virulent strains. To confirm the presence of a capsule in strain 1742 capsule staining was performed with nigrosine and crystal violet. In Figure 20 the short rods or cocco-bacilli shaped cells of *H. paragallinarum* are visible with the surrounding halo representing the capsule.

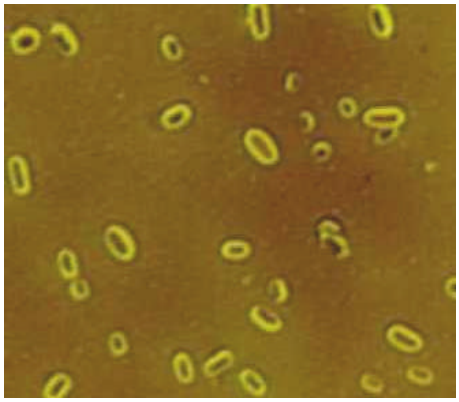

Figure 20: *H. paragallinarum* stained with nigrosine and crystal violet. The capsules are visible as transparent halos surrounding each cell.

4.4.2 Construction of the deletion plasmids pGhct-Δ and pGhctA-Δ

The objective of this part of the study was to generate a non-encapsulated strain of *H. paragallinarum* by replacing a capsule transport gene/s in the genome with a tetracycline resistance cassette through allelic transplacement. The pGhct-c plasmid construct (Table 5) was used as template to construct two deletion plasmids pGhct-Δ and pGhctA-Δ. In the case of deletion plasmid pGhct-Δ, virtually the entire transport region was deleted by means of PCR and pGhctA-Δ represents the construct in which only the *hctA* transport gene was deleted from the original pGhct-c. This process required a linear fragment that represents the functional tetracycline cassette flanked by regions present in the genome to allow homologous recombination *in vivo.* Two constructs were created for

this study; the first (*hct*Δ::Tet$^+$) contained the tetracycline cassette flanked on the 5'-terminal by a ~500bp segment of DNA (representing the *hctD* gene and a segment of a biosynthetic gene), and a ~2100bp region on the 3'- terminal end representing part of the *hctA* gene as well as a 3'- non-coding region. The second construct (*hctA*Δ::Tet$^+$) contained the tetracycline cassette with the 3'- terminal flanking region representing an approximately 1750bp non-coding region and the 5'- terminal flanking region of ~1700bp representing the *hctBC* and *D* genes. These final products were obtained by first deleting certain segments of DNA from the plasmid containing the capsule transport genes and flanking regions (pGhct-c) with PCR, replacing these deleted fragments with a tetracycline resistance cassette and finally generating the linear fragments with PCR.

Primers Hdp-1F and Hdp-1R (Table 6) introducing *Bgl*II restriction sites and amplifying in opposite directions on the pGhct-c template, were used to amplify pGhct-Δ. The PCR reaction resulted in a product of ~6.1kb in size (lane 1, Fig. 21A), representing the flanking regions of the capsule transport region and the pGem3Z plasmid backbone with a segment of 2770bp deleted (Fig. 22A). A segment of 606bp of the *hctA* gene was

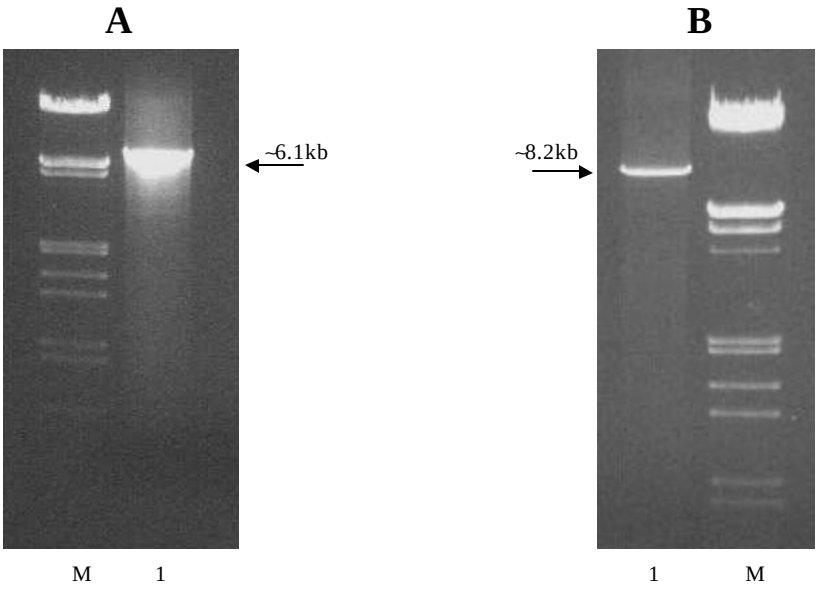

Figure 21: Amplification of deletion constructs pGhct-Δ and pGhctA-Δ. (A) pGhct-Δ amplified with primers Hdp-1F and Hdp-1R. (B) pGhctA-Δ amplified with primers HdpA-1F and HdpA-1R. Lane M in both A and B represent *Eco*RI and *Hind*III digested λ-DNA.

deleted (Fig. 22B) in a similar way with primer pair HdpA-1F and HdpA-1R (Table 5), resulting in a PCR product ~8.2kb in size (lane 1, Fig. 21B). This product also represents the flanking regions and pGem3Z plasmid backbone, but including the *hctB*, *C* and *D* genes.

Both PCR products (Fig. 21) were analyzed by restriction enzyme digestion to confirm that the desired constructs have been amplified. The ~6.1kb PCR product was digested into three fragments with sizes 2743bp, 2375bp, and 1005bp (Fig. 23A). The ~8.2kb PCR product also showed three bands after digestion with *Hind*III ranging in

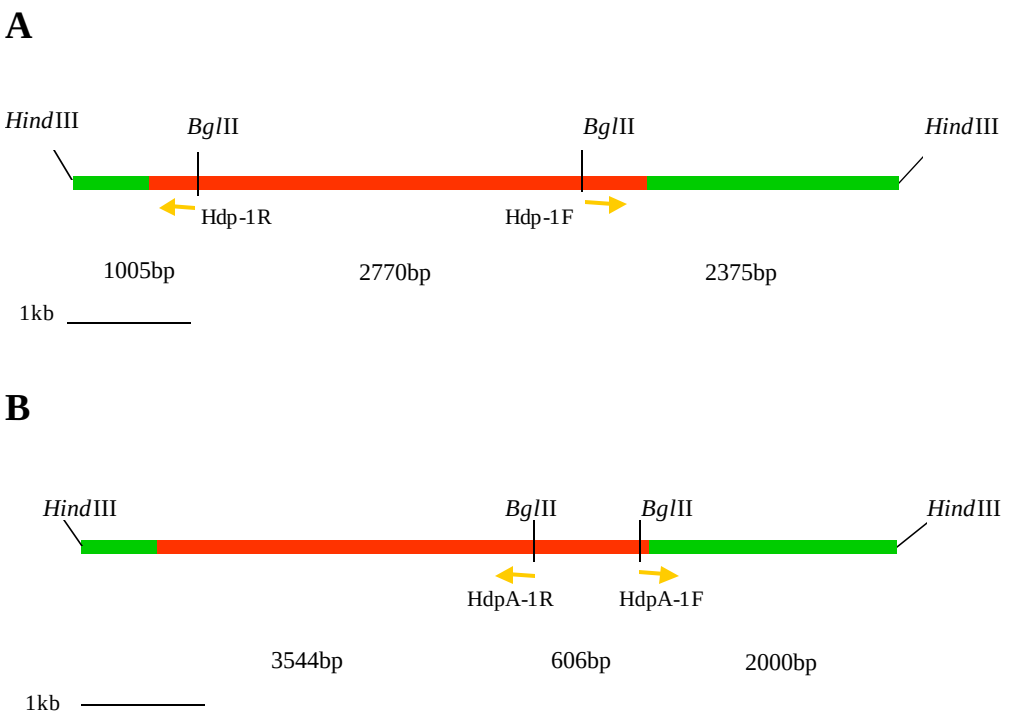

Figure 22: Restriction maps of the *Hind*III insert of pGhct-c modified by PCR to produce the pGhct-Δ and pGhctA-Δ deletion constructs. The red areas represent the capsule transport gene region (3793bp) and the green areas represent the flanking regions. The *Bgl*II restriction sites were introduced by the primers indicated as arrows. (A) 2770bps were deleted with primers Hdp-1F and Hdp-1R to produce pGhct-Δ (B) 606bps deleted with primers HdpA-1F and HdpA-1R to produce pGhctA-Δ. Fragment sizes correlates with the PCR products in Fig 21A and B if the 2743bp pGem3Z plasmid backbone, which is also amplified (not indicated in Fig. 22) is included in the calculation.

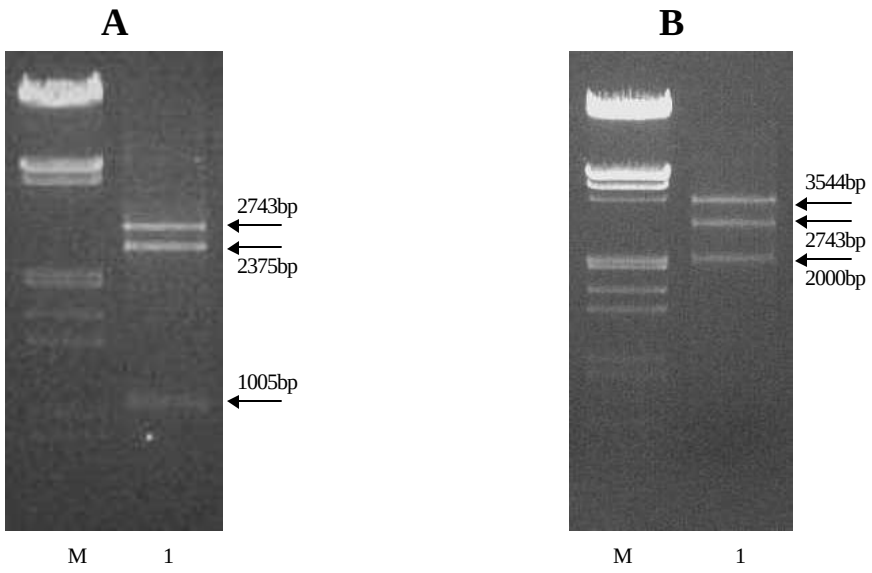

Figure 23: Restriction analysis of amplified pGhct-Δ and pGhctA-Δ with *Hind*III. (A) digestion profile of the ~6.1kb PCR product lane 1, representing the pGhct-Δ deletion construct. (B) digestion profile of the ~8.2kb PCR product lane 1, representing the pGhctA-Δ deletion construct. The 2743bp fragment present on both A and B, represent the pGem3Z plasmid backbone of the pGhct-c template.

sizes 3544bp, 2743bp and 2000bp (Fig. 23B). The band sizes in both digestion profiles confirmed that the desired segments were deleted.

The amplified pGhct-Δ and pGhctA-Δ deletion constructs were purified and recircularized by virtue of their generated *Bgl*II restriction sites. The circular pGhct-Δ and pGhctA-Δ plasmids were submitted to restriction analysis with *Hind*III and/or *Bgl*II. The digestion profile of the pGhct-Δ plasmid showed three bands of the same length than those in Figure 23A when digested with *Hind*III and *Bgl*II (Fig. 24A, lane2) and two bands 3380bp and 2743bp in size when digested with only *Hind*III (Fig. 24A, lane 1). The profile of pGhctA-Δ also indicated three bands corresponding to those in Figure 23B when digested with *Hind*III and *Bgl*II (Fig. 24B, lane 1) and two bands 5544bp and 2743bp when digested with *Hind*III (Fig. 24B, lane 2). The 2743bp band in both cases again representing the pGem3Z plasmid backbone. This data showed that the two deletion plasmids pGhct-Δ and pGhctA-Δ have been successfully constructed with intact *Bgl*II restriction sites present in both constructs.

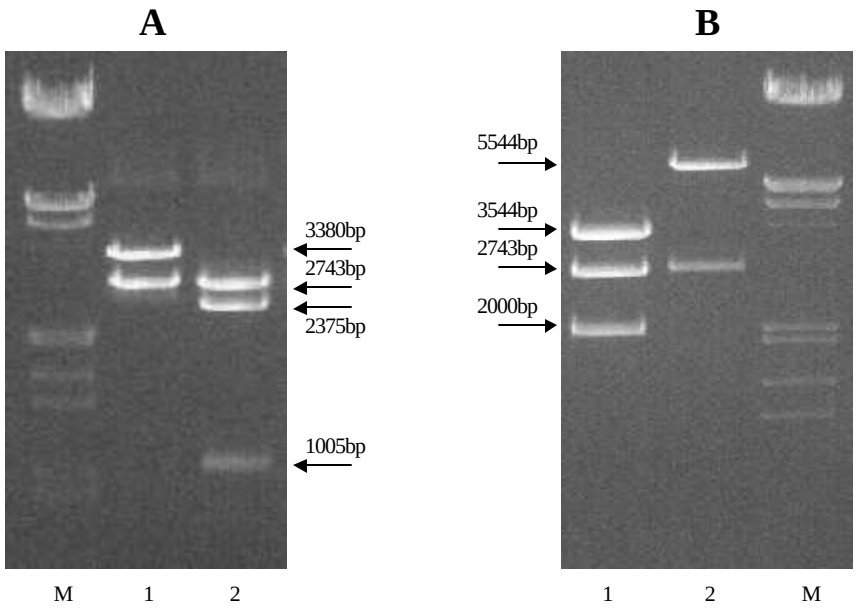

Figure 24: Restriction analysis of the pGhct-Δ and pGhctA-Δ deletion plasmids with *Hind*III and/or *Bgl*II. (A) two bands 3380bp and 2743bp were obtained when pGhct-Δ was digested with *Hind*III (lane1). The 2375bp and 1005bp bands (lane 2) represent the regions between the *Hind*III and *Bgl*II sites (Fig. 22A). (B) two bands 5544bp and 2743bp were visible when pGhctA-Δ was digested with *Hind*III (lane 2) and digestion with *Hind*III as well as *Bgl*II revealed 2 fragments, 3544bp and 2000bp (lane1) representing the regions flanking the deleted 606bp fragment (Fig. 22B).

4.4.3 Tetracycline resistance cassette

The tetracycline resistance cassette derived from tn916 and carried in pVB101 plasmid (pBR322 vector) was kindly supplied by Dr. J. Boyce (Department of Microbiology, Monash University, Melbourne , Australia). The cassette consists of the tetracycline resistance gene ~3.2kb in size, containing its own promoter. Excision of the cassette from the pBR*tet*(M) vector was performed by virtue of *Bam*HI restriction sites (Fig. 25).

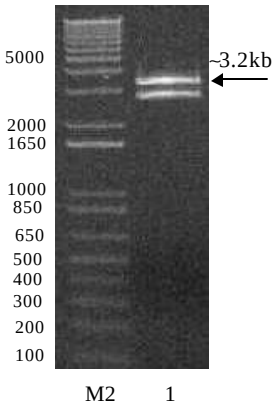

Figure 25: The tetracycline resistance cassette excised with *Bam*HI. The ~3.2kb band in lane 1 represents the cassette and lane M2 represents commercial 1 kb marker.

The ~3.2kb fragment in lane 1 represents the tetracycline cassette and the smaller band represents the plasmid backbone.

Restriction sites for numerous restriction enzymes are present within the cassette. *Hind*III and *Acc*65I were the two enzymes of choice for this study and position of their respective restriction sites within the tetracycline cassette are shown in Figure 26. Although *Hind*III restriction sites are present in the multiple cloning site of the pGem3Z plasmid backbone, no restriction sites for *Hind*III and *Acc*65I are present within the inserts of either of the deletion plasmids (pGhct-Δ and pGhctA-Δ). Therefore these two enzymes were used in the analysis of the deletion constructs after insertion of the tetracycline cassette.

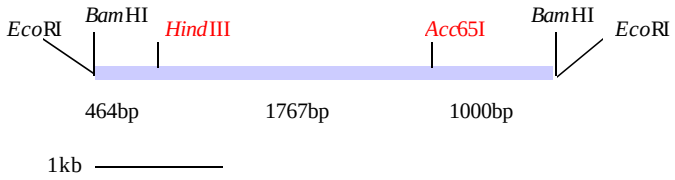

Figure 26: Restriction map of the ~3.2kb tetracycline resistance cassette indicating the positions of the restriction sites for enzymes *Acc*65I, *Bam*HI, *Eco*RI and *Hind*III.

4.4.4 Construction of plasmids pGhct-Tet$^+$ and pGhctA-Tet$^+$.

The *Bam*HI digested tetracycline cassette (Fig. 25) was cloned into the deletion plasmids pGhct-Δ and pGhctA-Δ by means of the introduced *Bgl*II restriction sites present on both plasmids. The final products were designated pGhct-Tet$^+$ and pGhctA-Tet$^+$ where the deleted capsule transport gene region was replaced with the tetracycline cassette in the case of pGhct-Tet$^+$ (Fig. 27A) and the deleted *hctA* gene was replaced with the cassette in the case of pGhctA-Tet$^+$ (Fig. 27B). The plasmids were submitted to restriction analysis with *Hind*III and/or *Acc*65I to confirm that the insertion was successful and to examine the orientation of the cassette in the positive clones. pGhct-Tet$^+$ was digested with

*Hind*III and showed the presence of the expected bands 5142bp, 2743bp and 1469bp in size (Fig. 28A, lane 2). pGhctA-Tet$^+$ was digested into four fragments 4008bp, 3000bp,

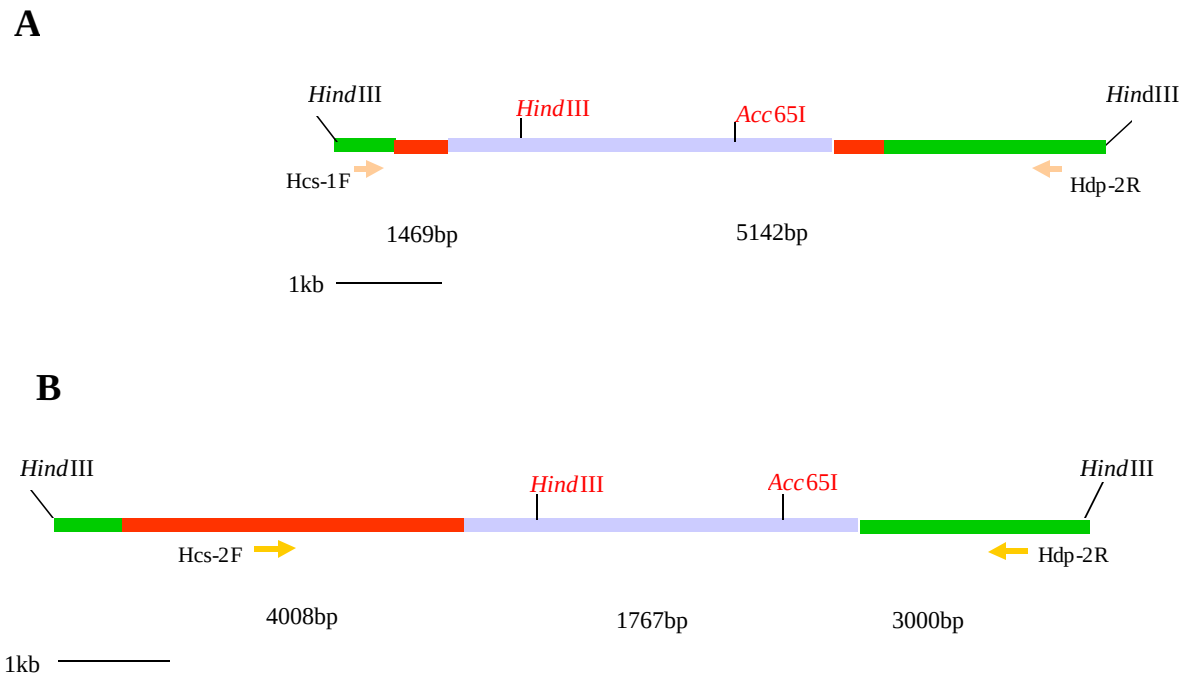

Figure 27: Restriction maps of the (A) pGhct-Tet$^+$ and (B) pGhctA-Tet$^+$ constructs. The red areas represent the remaining capsule transport gene region, the green areas the flanking regions and the purple areas represent the inserted tetracycline cassette. The arrows indicated the primers used to amplify the linear products *hct*Δ::Tet$^+$ and *hctA*Δ::Tet$^+$ revered to in the text.

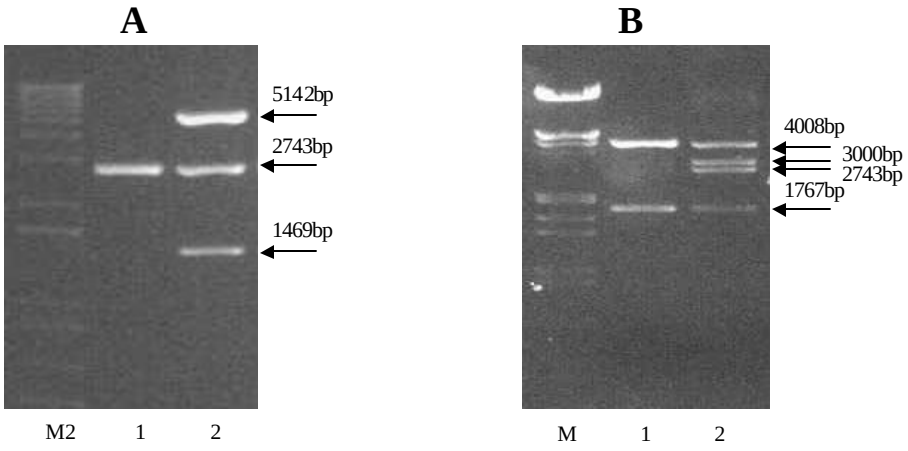

Figure 28: Restriction analysis of pGhct-Tet$^+$ and pGhctA-Tet$^+$. (A) pGhct-Tet$^+$ digested with *Hind*III (lane 2). (B) pGhctA-Tet$^+$ digested with both *Hind*III and *Acc*65I (lane 2).

2743bp and 1767bp by the combination of *Hind*III and *Acc*65I (Fig. 28A, lane 2). These results indicated that the tetracycline cassette was inserted into the pGhct-Δ and pGhctA-Δ deletion plasmids in the same orientation.

4.4.5 PCR amplification of the *hct*Δ::Tet$^+$ and *hctA*Δ::Tet$^+$ constructs for transformation to *Haemophilus paragallinarum*.

The constructed plasmids pGhct-Tet$^+$ and pGhctA-Tet$^+$ (Fig. 27) were used as templates for PCR to amplify the two linear constructs *hct*Δ::Tet$^+$ and *hctA*Δ::Tet$^+$ for transformation to *H. paragallinarum* cells. Primers Hdp-2R and Hcs-2F (Table 7) were used to amplify *hct*Δ::Tet$^+$ ~5.85kb in size (Fig. 29A) and primers Hdp-2R and Hcs-1F (Table 7) were used to amplify *hctA*Δ::Tet$^+$ ~6.66kb in size (Fig. 29B). The annealing site of each primer on the two constructs is indicated in Figure 27 as arrows. The PCR products were purified and submitted to restriction enzyme analysis with *Hind*III and

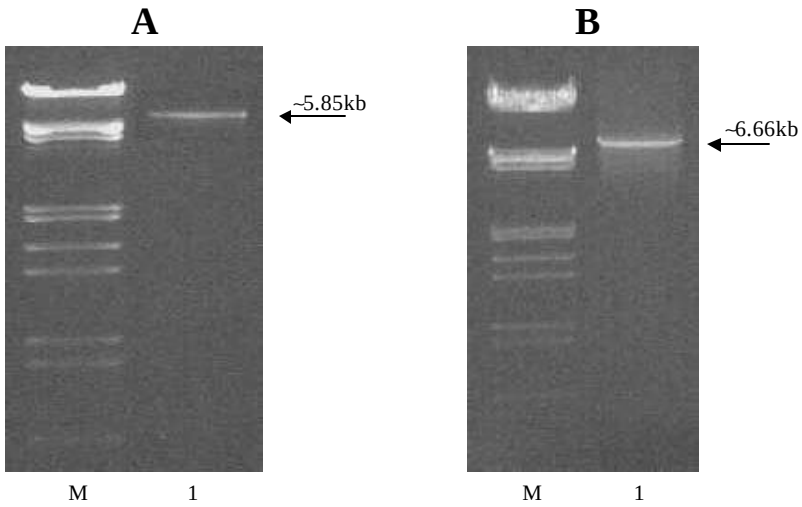

Figure 29: Amplification of linear constructs *hct*Δ::Tet$^+$ and *hctA*Δ::Tet$^+$. (A) *hct*Δ::Tet$^+$ amplified with primers Hcs-2F and Hdp-2R. (B) *hctA*Δ::Tet$^+$ amplified with primers Hcs-1F and Hdp-2R.

*Acc*65I. Digestion of *hct*Δ::Tet$^+$ with these two enzymes showed the presence of three bands 3124bp, 1767bp and 961bp in size (Fig. 30A). *hctA*Δ::Tet$^+$ digested with the same two enzymes were also digested into three fragments 2751bp, 2167bp and 1767bp in length (Fig. 30B).

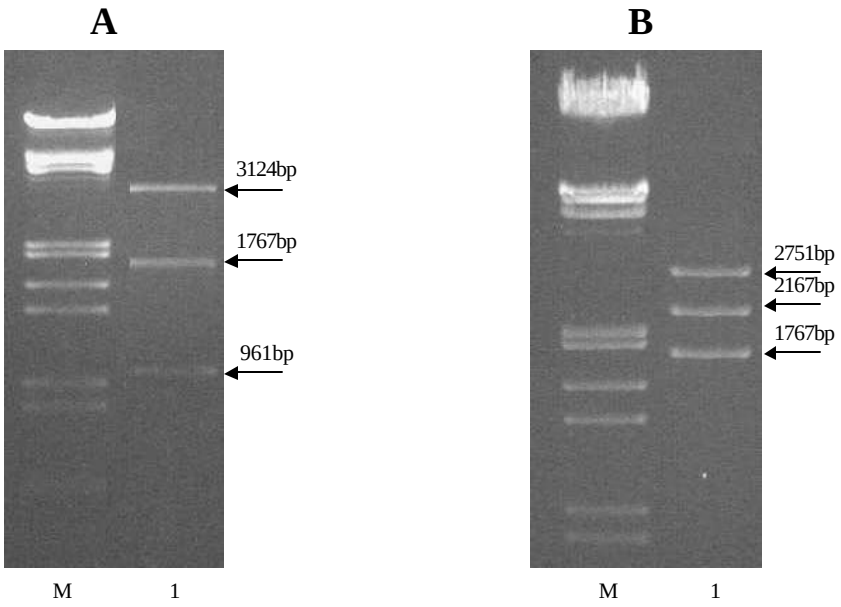

Figure 30: Digestion profiles of (A) *hct*Δ::Tet$^+$ and (B) *hctA*Δ::Tet$^+$ digested with a combination of *Hind*III and *Acc*65I. The 1767bp band present on both gels represents the segment of the tetracycline cassette between the restriction sites for *Hind*III and *Acc*65I (Fig. 26).

4.4.6 Transformation of *Haemophilus paragallinarum*

It was previously determined that the NAD-independence observed in some isolates of *H. paragallinarum* is mediated through a plasmid entity (Bragg *et al.*, 1993b). The molecular properties of this plasmid are currently under investigation to determine why it alleviates NAD-dependence in this bacterium. This native plasmid was used to determine the transformation efficiency of *H. paragallinarum* using electroporation. Transformation of this plasmid into NAD-dependent strain 0222 complements this phenotype reverting the strain to NAD-independence. However, the selective property of this phenotype (growth on media without NAD) together with the fastidious nature of *H.*

paragallinarum does not allow the accurate determination of transformation efficiency. As an alternative it was attempted to use the *E.coli* vectors pUC18 and pGem3Z, however these plasmids did not replicate in *H. paragallinarum* and thus also resulted in a futile attempt to determine the transformation efficiency.

Attempts were made to generate non-encapsulated *H. paragallinarum* mutants by allelic replacement with the amplified *hct* region deletion construct *hct*Δ::Tet^{+} and the *hctA* deletion construct *hctA*Δ::Tet^{+}. However, after repeated attempts with various transformation methods and variation of all the possible parameters, no tetracycline resistant 1742 *H. paragallinarum* organisms could be isolated.

4.5 Discussion

To confirm the role of the *hctABCD* genes in capsular polysaccharide export, attempts were made to generate isogenic mutants of *H. paragallinarum* in which the capsule polysaccharide transport was disturbed. Because a high level of transformation is required for the *in vivo* construction of null mutants, it was necessary to determine the transformation efficiency of *H. paragallinarum*. Transformation of NAD-dependent strain 0222 with the native HP plasmid was the only available method for this purpose but did not allow the accurate determination of the transformation efficiency, due to the low selective property and the fastidious nature of *H. paragallinarum*. Development of this method can be obtained through the insertion of antibiotic resistance genes (obtained form commercial vectors like pUC18 or pBR322) that will lead to resistance in the native HP plasmid. This can be performed by using either the available restriction sites on this plasmid or by inserting appropriate restriction sites incorporated into oligonucleotide primers and amplifying the construct using PCR. This will also confirm that these resistance genes are expressed by *H. paragallinarum* resulting in an antibiotic resistant phenotype.

To disrupt the capsule export process in *H. paragallinarum* two linear deletion constructs were created. In the first DNA fragment (*hct*Δ::Tet$^+$) all four the capsule transport genes *hctABCD* were deleted and replaced with the tetracycline resistance cassette (Fig. 29A). The second represented a fragment (*hctA*Δ::Tet$^+$) in which only the *hctA* transport gene was replaced with the same cassette (Fig. 29B). pGhct-Tet$^+$ and pGhctA-Tet$^+$ plasmids containing these constructs were transformed to *E.coli* cells rendering them tetracycline resistant and thus indicating that the cassette was functional. Deletions were constructed in different transport genes, because evidence exist that deletion of certain capsular genes might be lethal. Kroll *et al.* (1988) demonstrated that viable mutants of *H. influenzae* with mutations build into the *bexA* gene could be produced. Similar work done by Boyce and Adler (2000) showed that isogenic strains of *P. multocida* impaired in capsule export could be constructed through allelic exchange by inserting a tetracycline resistance cassette within the capsule export gene *hexA*. Export gene *cpxC* from *A. pleuropneumoniae* were deleted by Ward and Inzana (1997) resulting

in viable, non-capsulated cells. However, workers were unable to obtain viable cells when the B capsule transport genes of both *H. influenzae* and *A. pleuropneumoniae* were mutated (Kroll *et al.*, 1990; Ward and Inzana, 1997), concluding that mutations in both *bexB* and *cpxB* were lethal. Ward and Inzana (1997) were also unsuccessful in generating non-capsular *A. pleuropneumoniae* mutants by allelic replacement of *cpxA*. Mutations of the *bexD* transport gene *H. influenzae* resulted in accumulation of polysaccharides in the periplasmic space (Bronner *et al.*, 1994).

Replacing a chromosomal gene/s with an inactivated or otherwise modified gene/s is a complicated process requiring a double recombination event. To inactivate the transport gene/s of *H. paragallinarum*, a fragment of the gene/s was deleted with PCR from the genes contained on a plasmid (pGhct-c) and replaced with a tetracycline resistance cassette. This insertion disrupted the coding sequence of the transport gene/s usually completely destroying the function. From these plasmids, fragments were amplified (*hct*Δ::Tet$^+$ and *hctA*Δ::Tet$^+$) and these fragments were transformed to *H. paragallinarum* cells, because DNA ends are favored substrates of the recombination enzymes. With low but workable frequency, the transformed fragment recombines twice, once at each end, resulting in replacement of the chromosomal gene/s with the engineered one/s. Transplacement is experimentally quite simple and the efficiency of the reaction makes its use routine especially for yeast geneticists. Although this technique has been performed with some success on bacteria, like allelic transplacement of the *hexA* transport gene in *P. multocida* (Boyce and Adler, 2000), transplacement of the capsule transport genes of *H. paragallinarum* was unsuccessful.

Despite several attempts made in this study with different transformation methods, no viable cells were obtained when *H. paragallinarum* was transformed with either the *hct*Δ::Tet$^+$ or *hctA*Δ::Tet$^+$ constructs. A number of speculations have been made on why this anomaly occurred. The possibility exists that the recombination abilities of *H. paragallinarum* are in some way limited. No information is available on the recombination machinery, especially the enzymes involved, so it is not possible to conclude as to the role that it played in the failure to produce isogenic mutants of this organism. Ward and Inzana (1997) were also unable to produce viable cells of *A. pleuropneumoniae* when the transport gene *cpxA* was mutated. Since *H. paragallinarum*

transport gene *hctA* showed the highest identity (78.6%) on protein level with CpxA of *A. pleuropneumoniae* (Table 4), it could be possible that disruption of *hctA* in *H. paragallinarum* was also lethal explaining why transformation of neither the *hct*Δ::Tet$^+$ nor *hctA*Δ::Tet$^+$ constructs rendered viable cells.

The pBR*tet*(M) plasmid representing the tetracycline resistance cassette integrated in the vector pBR322 (Table 5) was transformed to *H. paragallinarum* cells however, no viable cells were obtained. This can be explained by assuming that the cassette was either not expressed, or that the *E.coli* vector pBR322 was unable to replicate in *H. paragallinarum*. Expression of the tetracycline resistance cassette in *H. paragallinarum* could thus not be confirmed. A possible solution for this problem could be to clone the tetracycline resistance cassette into the native HP plasmid by virtue of its *Bgl*II restriction site. However, characterization of the native plasmid is incomplete, so no information is currently available on the position of the *Bgl*II restriction site and whether it might disrupt an area/gene essential for plasmid function if used for cloning. Determination of the transformation efficiency of *H. paragallinarum* proved troublesome and no conclusive value could be established. It was possible that the transformation efficiency was too low for *in vivo* recombination, thus explaining why transformation was unsuccessful.

Although no isogenic strains of *H. paragallinarum* deficient in capsular export could be generated in this study, it marks the important first step toward incorporating defined mutants in the transport locus and evaluating *H. paragallinarum* capsule mutants in pathogenic and vaccine studies.

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Chapter 5

Concluding remarks

Capsules are found on the surface of a wide range of bacteria and have long been associated with their virulence properties. These polysaccharide structures have been the subject of intensive investigation because of their usefulness as vaccines for prevention of bacterial infections. Hyaluronic acid was shown to be the main component of the capsule material of several organisms belonging to the family Pasteurellaceae, including *H. influenzae, A. pleuropneumoniae* and *P. multocida*. In general, hyaluronic acid is believed to influence the physical properties of the capsule and probably prevents phagocytosis by mimicking host antigens. Many researchers sought to understand the role of the capsule in virulence by identifying the genes involved in capsular polysaccharide export and biosynthesis.

Haemophilus paragallinarum, as the causative agent of infectious coryza, is problematic for the poultry industry in South Africa and also world wide. The virulence factors of this organism are being researched to find a possible candidate for generating a live vaccine. The capsule has been identified and studied as a virulence factor since non-capsulated serotype B isolates are non-pathogenic. The capsule transport gene region of *H. paragallinarum* has been isolated, cloned and nucleotide sequence analysis showed it to be 3792bp in length. The region comprises four ORFs *hctABCD* with overlapping stop and start codons indicating coupled transcription. The genetic organization of this region corresponds to similar regions in the related HAP organisms *H. influenzae, A. pleuropneumoniae* and *P. multocida*. Homology studies supported the believe that the *H. paragallinarum* transport proteins form an ABC-transporter system, with an ATP-binding component, integral inner-membrane component, membrane spanning protein and outer membrane protein with porin properties. Furthermore, the high degree of protein homology of *H. paragallinarum* with related species argue a common evolutionary origin of molecular mechanisms of encapsulation.

Attempts to investigate the role of the capsule in virulence were performed by the construction of isogenic mutants of *H. paragallinarum* in which capsule export was disturbed. Constructs, in which all or some of the capsule transport genes were deleted and replaced with a tetracycline resistance cassette, were employed for transformation to wild-type *H. paragallinarum* cells. Through the process of transplacement, tetracycline resistant non-capsulated mutants were expected, but unfortunately no viable cells were obtained. These results were attributed to the possible lethality of the deletions, too low a transformation efficiency, the possible inability of *H. paragallinarum* to express the tetracycline resistance cassette and/or a foible recombination system.

The isolation and characterization of the capsule transport region will greatly facilitate investigation at molecular level of the role of the *H. paragallinarum* capsule in pathogenesis and immunity. For the further construction and study of isogenic mutants as possible live vaccines, the undefined aspects mentioned have to be resolved and characterized.

Chapter 6

Summary

Haemophilus paragallinarum causes an acute respiratory disease in chickens known as infectious coryza. Failure of commercially available vaccines to produce effective immunity against this disease, inspired research on producing a possible live vaccine by investigating the capsule as virulence factor

The capsule transport gene region of *H. paragallinarum* comprises four ORFs, *hctABCD*, 3792bp in total with overlapping stop and start codons. The two genes *hctA* (648bp) and *hctB* (798bp) showed considerably homology (>80%) on protein level with the postulated protein products of the corresponding genes in related organisms. These homologies as well as conserved regions within the genes support the speculation that *hctA* codes for an ATP-binding protein and *hctB* for an integral inner-membrane protein. Lesser homologies (>60%), although still significant, evident for both *hctC* (1164bp) and *hctD* (1188bp), aided the suggestion that HctC acts as an membrane associated protein with membrane spanning anchors and *hctD* encodes an outer membrane protein with porin properties. These proteins form an ABC-transporter system responsible for transporting *H. paragallinarum* capsular polysaccharides across the cell membrane.

This genetic information of the transport region of *H. paragallinarum* was employed to generate deletion constructs in which a gene/s were replaced with a tetracycline resistance cassette and transformed to *H. paragallinarum* cells. The entire region was replaced in one deletion construct (*hct*Δ::Tet^{+}) and only the *hctA* gene in a second construct (*hctA*Δ::Tet^{+}). The linear products were transformed to *H. paragallinarum* cells and through the process of transplacement, isogenic mutants lacking capsular material, which could be possible candidates for a live vaccine against infectious coryza, were expected. Unfortunately, no viable cells were obtained, but a number of factors must still be studied and optimized to enable the production of such isogenic strains.

Chapter 7

Opsomming

Die bakterium *H. paragallinarum* veroorsaak 'n akute lugweginfeksie in hoenders genaamd "infectious coryza". Kommersiële entstowwe verskaf nie meer effektiewe beskerming teen die siekte nie en alternatiewe moontlikhede word bestudeer. Die moontlikheid van 'n lewende entstof word oorweeg deur die kapsel van *H. paragallinarum* as 'n virulensiefaktor te bestudeer.

Die kapseltransport geenstreek bestaan uit vier oopleesrame, *hctABCD*, 3792 basispare in geheel, met begin- en termineringkodons wat oorvleuel. Die twee gene *hctA* (648bp) en *hctB* (798bp) is op proteïenvlak met die voorgestelde proteïne van verwante organismes vergelyk en het 'n ooreenkoms van meer as 80% getoon. Hierdie homologie, asook gekonserveerde streke binne elke geen, ondersteun die hipotese dat die *hctA*-geen vir 'n ATP-bindingproteïen kodeer en *hctB* vir 'n integrale membraanproteïen. 'n Laer homologie (>60%) bestaan tussen *hctC* (1164bp) en ander voorgestelde *C*-gene asook *hctD* (1188bp) in vergelyking met die *D*-gene van verwante spesies. Die vermoede dat HctC optree as 'n membraan-geassosieerde proteïen met ankers wat die membraan oorbrug, en dat *hctD* vir 'n buite-membraanproteïen met porieneienskappe kodeer, word deur die homologie-studies ondersteun. Al vier proteïne saam vorm 'n ABC-vervoerkompleksstelsel in *H. paragallinarum*, verantwoordelik vir die vervoer van kapsulêre polisakkariede na die seloppervlak.

Die genetiese inligting aangaande die transportgene van *H. paragallinarum* is gebruik om delesiekonstrukte saam te stel. In die konstrukte is 'n transportgeen of -gene met 'n tetrasiklienweerstand-kasset vervang en na *H. paragallinarum* selle getransformeer. In die eerste delesiekonstruk (*hct*Δ::Tet^{+}) is al die gene vervang en in die tweede konstruk (*hctA*Δ::Tet^{+}) is slegs die *hctA*-geen met die tetrasiklien-kasset vervang. Die liniêre produkte is na *H. paragallinarum* selle getransformeer en deur die proses van geenvervanging is isogeniese mutante sonder kapsulêre materiaal verwag. Bo alle verwagting was daar geen lewensvatbare bakterieë nie.

Hierdie was slegs die eerste stappe in die rigting van 'n lewende entstof en daar is steeds 'n aantal invloede wat ongedefinieerd is.