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Analysis and expression of important vaccine antigens of *Bordetella pertussis*

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**Analysis and expression of important vaccine
antigens of *Bordetella pertussis***



**A thesis submitted in fulfilment of the requirements for the
award of the degree**

Doctor of Philosophy

from

**The University of Wollongong
Department of Biological Sciences**

by

Adam M. Smith

Declaration

This thesis is submitted in accordance with the regulations of the University of Wollongong in fulfilment of the degree of Doctor of Philosophy. It does not include any material previously published by another person except where due reference is made in the text. The experimental work described in this thesis is original work and has not been submitted for a degree to any other University or Institution.

Adam Smith

Acknowledgments

Well, here it is, and this would not have been possible without support from a myriad of people.

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Abbreviations

Amp	Ampicillin
APS	Ammonium persulfate
bp	nucleotide base pairs
Cm	Chloramphenicol
Cp	Cephalexin
°C	Degrees Celsius
dH ₂ O	Sterile glass distilled water
DMF	N,N-dimethylformamide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
g	Gram
g	Gravity
HRP	Horse radish peroxidase
IPTG	Isopropyl-β-D-thiogalactopyranoside
Kan	Kanamycin
kb	Kilobases
kD	Kilodalton
L	Litres
LB	Luria broth
M	Molarity
m	Milli
μ	Micro
n	Nano
NBT	Nitro blue tetrazolium
NaI	Nalidixic acid

OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
Rif	Rifampicin
SDS	Sodium dodecyl sulfate
TEMED	NNN'N'-Tetramethylethylenediamine
Tet	Tetracycline
T _{H1}	Type 1 helper T cell population
T _{H2}	Type 2 helper T cell population
Tp	Trimethoprim
Tris. Base	Tris[hydroxymethyl] aminomethane
Tris. HCl	Tris[hydroxymethyl] aminomethane hydrochloride
Triton-X	t-octylphenoxypolyethoxyethanol
Xgal	5-bromo-4-chloro-3-indolyl- β -D- thiogalactopyranoside

Abstract

Bordetella pertussis is the causative agent of whooping cough, a contagious childhood respiratory disease. Increasing public concern over the safety of current whole-cell vaccines has led to decreased immunisation rates and a subsequent increase in the incidence of the disease. The preparation of safer vaccines is at present concentrated on the production of detoxified virulence factors such as pertussis toxin (PT), filamentous haemagglutinin (FHA), pertactin and serotype specific fimbriae for inclusion in acellular vaccine preparations. This study analyses the expression of several of these antigens in different genetic backgrounds.

A permanently avirulent *Bordetella bronchiseptica* strain was previously engineered to constitutively produce PT (Walker *et al.*, 1991). An *in vivo* cloning technique, based on the principles of conjugal mating and chromosome transfer was employed to transfer the PT expression locus of this strain to virulent and avirulent strains of *B. bronchiseptica*. This transfer was confirmed by Southern hybridisation. An analysis of PT secretion in isogenic virulent and avirulent strains of *B. bronchiseptica* revealed that the PT produced was cell-associated, and not secreted to the growth medium. This evidence further suggests that *B. bronchiseptica* does not possess functional PT secretion (*ptl*) genes. Therefore, to achieve a PT expression and secretion system suitable for vaccine purposes in *Bordetella bronchiseptica*, functional *ptl* genes of *B. pertussis* are also required.

The use of "natural mutants" of *Bordetella pertussis* overexpressing immunogenic proteins as whooping cough vaccine strains may have a higher level of public acceptance than both the traditional whole cell preparation and the genetically engineered recombinant acellular vaccines

currently under formulation. Mutants naturally overexpressing various candidate vaccine components have been created by Commonwealth Serum Laboratories (CSL Ltd.) via continuous subculture. These three strains (CSL 127S, CSL 137S and CSL 1237S) were genetically characterised by amplifying relevant regions of the genome, cloning these fragments into plasmid vectors and performing DNA sequencing analyses. *B. pertussis* CSL 137S, producing large amounts of type 3 fimbriae was found to possess an extra cytosine residue in the “fimbrial C stretch” of the promoter when compared to its parental strain. *B. pertussis* CSL 127S, which overexpresses type 2 fimbriae as well as FHA also has an altered C stretch. It contains one less cytosine than its parental strain. The upregulation in FHA could not be explained in terms of promoter differences. When the parental strain and CSL 127S were compared, the promoter regions of the FHA gene and the FHA/fimbrial accessory gene cluster were genetically identical. The third candidate vaccine strain (CSL 1237S) presented a more puzzling expression pattern. A moderate increase in the expression of most virulence factors analysed compared to its parental strain suggested a possible favourable mutation in the BvgAS promoter region. This however was not the case, with no differences being observed at any of the promoters analysed.

The two strategies for vaccine antigen expression investigated here may eventually lead to the production of readily manufactured and therefore more affordable whooping cough vaccine preparations.

Chapter One Literature Review

1.1 *Bordetella pertussis* - A General Perspective

1.1.1 Whooping Cough

Pertussis, or whooping cough is a highly contagious disease of the human respiratory tract which is particularly severe in infants. The disease is characterised by bronchopneumonia, paroxysmal coughing and the distinctive "whooping" intake of air, and if left untreated can lead to neurologic damage or death. This is especially pertinent in developing countries where appropriate medical assistance is often unavailable and disease progression is unimpeded by the lack of suitable antimicrobials. The readily transmissible nature and severity of the disease in these countries has seen worldwide deaths from whooping cough increase to over 350 000 per year (Strebel *et al.*, 1994). This serious disease is caused by the obligate human pathogen, *Bordetella pertussis*, a Gram-negative coccobacillus originally isolated in 1906 by Bordet and Gengou.

The normal course of infection begins with the bacteria entering the host via the airways, contained within airborne droplets derived from the cough of an infected individual. The pathogens proceed down the respiratory tract, adhering to ciliated epithelial cells in the trachea and nasopharynx. Once attachment is initiated, the bacteria begin to replicate and colonise adjacent areas. Toxins secreted by the microorganism damage the epithelial lining, resulting in the loss of ciliated cells which induces the characteristic coughing. These toxins also allow the bacterium to evade the host immune response by interfering with clearance mechanisms. Halting ciliary function, short circuiting host G protein

signalling apparatus and inhibiting immune cell function by upregulating cAMP levels are all examples of the strategies employed by the various toxins of *Bordetella pertussis*.

The incidence of whooping cough has been greatly reduced since the advent of pertussis vaccines in the 1940's. These "whole cell" vaccines consist simply of chemically or heat-killed *Bordetella pertussis* cells. These vaccines, as part of the Diphtheria, Tetanus, Pertussis (DTP) immunisation regime proved extremely effective at preventing the symptoms of whooping cough. Unfortunately, a loose association with rare neurologic complications and deaths has led to a decline in public confidence, a subsequent reduction in immunisation rates, and an inevitable worldwide increase in the incidence of the disease. The other constituents of the DTP vaccine are purified, inactivated diphtheria and tetanus "toxoids". These single component vaccines have attained a comparatively less controversial reputation, and there is currently much research being carried out in an attempt to bring the pertussis component into line with the less reactogenic constituents of the vaccine.

1.1.2 The Genus *Bordetella*

Historically, the bacteria currently classified within this Genus have had a very dynamic and controversial taxonomic existence, not always being classified together. It was Lopez (1952) who proposed that a new group, the Genus *Bordetella* be created to house the then three species, *B. pertussis*, *Bordetella parapertussis* and *Bordetella bronchiseptica*. More recent molecular evidence, including DNA sequencing analysis, multilocus enzyme electrophoresis and RFLP typing has justified the grouping of these three species into the same genus (Table 1.1) (Arico *et*

al., 1987; van der Zee *et al.*, 1997). *B. pertussis* is exquisitely tuned to a single environment and will only infect humans. *B. parapertussis* causes a milder “pertussis-like” disease state in humans and has also been found associated with ovine respiratory infections (Cullinane *et al.*, 1987). Most other mammals can be colonised by *B. bronchiseptica*, and many commercially important diseases are caused by this versatile organism (Goodnow, 1980). The most notable being an association with atrophic rhinitis in swine which costs the meat industry considerable profits and kennel cough in dogs (Switzer, 1956; Thompson *et al.*, 1976). The bird pathogen *Bordetella avium*, a later addition to the genus (Table 1.1), causes turkey coryza and other respiratory diseases of fowl (Kersters *et al.*, 1984).

Table 1.1 The four most studied members of the Genus *Bordetella* comparing characteristic proteins produced by each species. Modified from Gross *et al.*, 1989; Parton, 1996.

	<i>B. pertussis</i>	<i>B. parapertussis</i>	<i>B. bronchiseptica</i>	<i>B. avium</i>
Pertussis Toxin	+	–	–	–
Adenylate Cyclase	+	+	+	–
FHA	+	+	+	–
Fimbriae	+	+	+	+
Pertactin	+	+	+	+
Tracheal Cytotoxin	+	+	+	+
Lipopolysaccharide	+	+	+	+
Dermonecrotic Toxin	+	+	+	+
Flagella	–	–	+	+
Urease	–	+/–	+	–

More recently, a number of new members have been proposed. *Bordetella hinzii* is the name assigned to a novel species recently isolated from a bacteremic individual with HIV/AIDS (Cookson *et al.*, 1994). Associated with human septicemia, another species, designated *Bordetella holmesii* has been identified (Weyant *et al.*, 1995). The name *Bordetella trematum*

has recently been proposed for a novel species isolated from human wounds and ear infections (Vandamme *et al.*, 1996). Although none of these new species were associated with respiratory tract infections they display characteristics indicative of the Genus. Since its initial discovery, *Bordetella hinzii* has been isolated from the respiratory tract of infected poultry (Vandamme *et al.*, 1995).

1.2 Genetic Control of Virulence - Turn On's and Turn Off's

Pathogenic bacterium colonise and multiply by exploiting their environment to the fullest extent. *Bordetella pertussis* accomplishes this aim with prodigious efficiency. The method of achieving this is for the bacterium to have some level of control over the production of the specific factors which enable it to infect its host. There is a very coevolutionary link between the exceedingly strict regulation of the virulence determinants of *B. pertussis* and the highly evolved interaction it possesses with the human respiratory tract.

1.2.1 *Bordetella* Virulence Gene Regulation System

It was realised many years ago that *Bordetella pertussis* existed in more than one phase (Leslie and Gardner, 1931). "Phase variation" occurs when spontaneous mutations during DNA replication give rise to avirulent variants. This process is generally irreversible and occurs at a frequency of one in a thousand to one in a million cells. This produces phase-locked avirulent bacteria which do not have the ability to invade and colonise the host. However, if when culturing wild type *Bordetella pertussis*, the growth conditions are altered, the bacteria undergo a totally reversible

conversion to one of principally two states, called X and C modes (Lacey, 1960). Termed "antigenic modulation", this phenomenon, may be important in the survival of this microorganism. This landmark study also recognised a third state (I mode), a phase of growth intermediate between X and C modes, and "an infinitude of other antigenic states".

Research since has dealt almost entirely with the two extreme modes of growth, virulent (X) and avirulent (C) phases. New research on the related species *B. bronchiseptica* has given us new insight into the intricacies of antigenic modulation. Due to the expression of a set of genes under modulating growth conditions which are easily monitored, *B. bronchiseptica* may be used as a model for other members of the Genus. An intermediate phase *B. bronchiseptica* mutant similar to Lacey's I mode, has recently been isolated which expresses phenotypes characteristic of both virulent and avirulent states (Cotter and Miller, 1997).

The members of the Genus *Bordetella* share a genetic locus which encodes a type of biological "switch" enabling these species to oscillate principally between two very different phenotypic states depending on whether they are inside a suitable host or in the outside environment. Species of *Bordetella* encounter vast differences between these two environmental extremes. For a pathogenic bacterium which has coevolved with its host to live in a specific niche, the environment outside a host is extremely harsh. The drying atmosphere and fluctuating temperatures make it a very inhospitable place. Under these conditions, the *Bordetellae* switch to avirulent phase. Proteins required for host invasion are not produced, instead, a set of genes which allow the organism to best deal with such difficult conditions are expressed. There

is considerable energy saved in not producing virulence factors, a point substantiated in the laboratory by the considerable difference in growth rates between virulent and avirulent organisms. The warm, moist, surroundings of the human respiratory tract is a more obliging place for the bacterium to thrive. In these physically favourable conditions, a different subset of genes are expressed. The genes for a number of adhesins, such as filamentous haemagglutinin (FHA), serotype specific fimbriae, and a 69 kDa outer membrane protein known as pertactin are expressed, as are the genes encoding more harmful products involved in evasion of the host immune system, pertussis toxin, adenylate cyclase toxin/haemolysin and dermonecrotic toxin.

In the laboratory, the virulence control switch can be manipulated to bring *Bordetella* species into avirulent phase by the addition of sulfate ions or nicotinic acid to the culture medium, or by reducing the culture temperature from 37°C to 25°C.

The expression of virulence factors in *Bordetella* species is coordinately regulated by what was originally termed the *vir* locus (Weiss and Falkow, 1984) and is now known as the *Bordetella* virulence gene locus (*bvg*) locus. This gene locus encodes two proteins which enable the bacterium to “sense” the prevailing environmental conditions, and then “act” accordingly by controlling the expression of specific genes and gene loci. The sensor protein is known as BvgS and the activator, BvgA. Together they constitute a two-component signal transduction system and make up part of a signal transduction superfamily (Parkinson and Kofoed, 1992). This type of system is utilised by many prokaryotes, and recently described in some eukaryotes (Choi and Greenberg, 1991; Parkinson and Kofoed, 1992; Chang *et al.*, 1993, Ota and Varshavsky, 1993) in response to many

different environmental stimuli.

The genes encoding BvgA and BvgS are contained within an operon (Arico *et al.*, 1989). The original sequencing data unfortunately contained mistakes, confusing the exact arrangement of the operon (Figure 1.1). The discovery and sequencing of a third gene, *bvgR* has confirmed the true operon structure (Merkel *et al.*, 1998a). While *bvgA* and *bvgS* are transcriptionally linked, *bvgR* is transcribed separately from the opposing DNA strand (Figure 1.1).

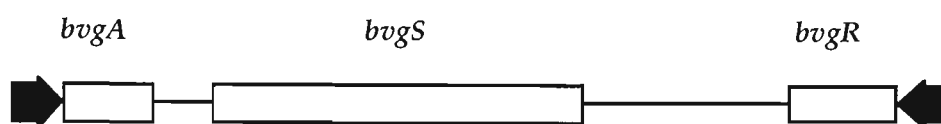


Figure 1.1 Schematic representation of the operon structure of the *Bordetella* virulence gene regulation system. Genes encoding BvgA, BvgS and BvgR (open boxes) and promoter regions (filled arrows) are indicated. The genes for *bvgA* and *bvgS* are transcriptionally linked. *bvgR* is transcribed separately in the opposite direction.

Transcription of *bvgAS* is controlled by three self regulated promoters, P_1 , P_3 and P_4 and one *bvg* independent promoter, P_2 (Figure 1.2) (Roy *et al.*, 1990; Scarlato *et al.*, 1990). Transcription from P_2 is constitutive, thereby maintaining low level production of intracellular BvgAS proteins. While P_1 , P_2 and P_3 are all involved in the transcription of *bvgAS*, P_4 produces an anti-sense RNA strand complimentary to transcripts expressed from the other three promoters. The exact *in vivo* function of this promoter remains a mystery, however it may be involved with stabilisation of the sense transcripts via the prevention of secondary structure formation. Hybridisation between mRNA from P_1 and P_4 may lead to a more efficient interaction with ribosomes (Scarlato *et al.*, 1990).

The gene encoding FHA is transcribed from a region just upstream of these four promoters (Scarlato *et al.*, 1990) at P_{fha} (Figure 1.2). There has since been discovered a *bvg*-independent FHA promoter, P_{fha^*} which expresses low levels of the FHA precursor protein, FhaB (Steffen *et al.*, 1996; Boucher *et al.*, 1997).

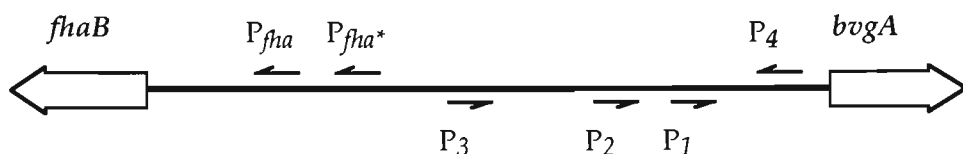


Figure 1.2 Promoter organisation of the *bvg/fha* intergenic region. The *bvgA* and *fhaB* genes (open boxes) are shown. Direction of transcription from each promoter is indicated by arrows. Modified from Scarlato *et al.*, 1990 and Coote, 1991.

1.2.2 BvgA

The transcriptional activator, BvgA, which can directly and indirectly affect the expression of a number of virulence controlled genes is a 23 kDa cytoplasmic protein. The two domain structure of BvgA (Figure 1.3), consisting of a receiver at the N-terminus and a C terminal helix-turn-helix (HTH) motif is characteristic of response regulators (Arico *et al.*, 1989; Stibitz and Yang, 1991). It is this HTH module which facilitates specific DNA sequence binding at the promoters of *bvg*-activated genes (Boucher *et al.*, 1994). Although not part of the HTH domain, the last 20 amino acid residues are also required for binding, probably by stabilising the HTH~DNA binding interaction (Boucher *et al.*, 1994; Kahn and Ditta, 1991).

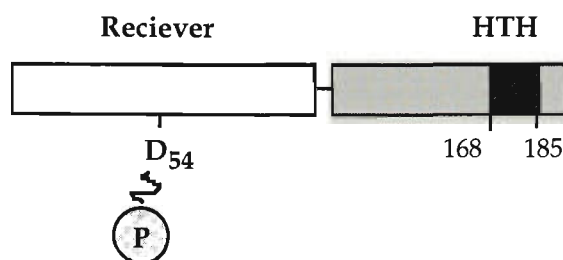


Figure 1.3. Domain structure of the 209 amino acid transcriptional activator BvgA. The receiver domain (open box) is shown, with the phosphorylation site (Aspartic acid, D₅₄) indicated. The C-terminal domain (light shading) incorporating the helix-turn-helix domain (dark shading; amino acids 168-185) is also shown. Modified from Uhl and Miller, 1995

The binding of DNA by proteins is a common phenomenon within cells, and the C terminal HTH domain of BvgA shares sequence homology with a number of different protein families. As expected, this domain is homologous to corresponding areas of other response regulators such as FixJ, UhpA and NarL (Kahn and Ditta, 1991). Other DNA binding proteins such as LuxR from *Vibrio fischeri* (Choi and Greenberg, 1992) as well as the C termini of selected bacterial σ factors (Helmann and Chamberlin, 1988) also share homology to this evolutionally well conserved domain.

Direct control over the transcription of a number of *bvg*-activated genes by BvgA is achieved via binding to specific sequences at target promoter regions (Roy *et al.*, 1989; Roy and Falkow, 1991). The heptameric sequence specific BvgA binding site, TTTCCTA or TTTGGTA, first proposed by Roy and Falkow is present as either direct or inverted repeats upstream of various *bvg*-regulated genes (Roy and Falkow, 1991). The importance of these repeats has since been confirmed at a number of virulence activated promoters by DNase I protection studies (Boucher and Stibitz, 1995; Karimova *et al.*, 1996; Zu *et al.*, 1996; Marques and Carbonetti, 1997).

1.2.3 BvgS

In a process that is not yet fully understood, changes in the extracellular environment trigger a histidine kinase in the transmitter domain of BvgS to autophosphorylate (Uhl and Miller, 1994). Following a series of intramolecular phosphorylation events, the phosphoryl group is eventually transferred to BvgA (Uhl and Miller, 1994).

The 135 kDa sensory component of the signal transduction system, BvgS is often termed an unorthodox sensor protein because of the number of extra functional domains it contains. The membrane spanning nature of this protein predicts it possess both periplasmic and transmembrane domains, and sequence homology studies confirm the absolutely conserved histidine kinase domain (Arico *et al.*, 1989; Stibitz and Yang, 1991). However, BvgS also contains a linker section and regions usually reserved for response regulators such as BvgA, a receiver domain and a C terminal output domain (Figure 1.4) (Arico *et al.*, 1989; Stibitz and Yang, 1991). The deletion of these “extra” domains has been shown to make BvgS inoperable (Arico *et al.*, 1989; Uhl and Miller, 1994; Beier *et al.*, 1995).

Experiments with BvgS-PhoA fusion proteins have shown the N-terminal region is located in the periplasm (Stibitz and Yang, 1991). Mutations in the linker region render the bacterium incapable of sensing the environment providing evidence that this region acts as a molecular relay between periplasmic and C terminal domains located in the cytoplasm (Miller *et al.*, 1992; Manetti *et al.*, 1994).

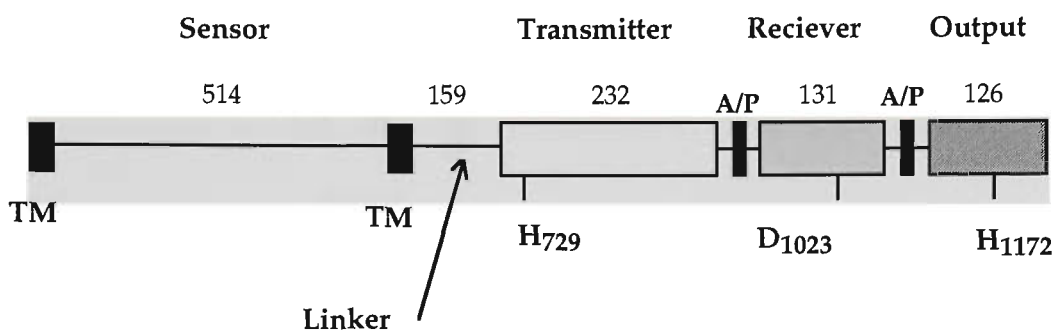


Figure 1.4 Domain structure of the 1236 amino acid protein BvgS. The 514 aa sensor domain (single line), the 159 aa linker region (single line), 232 aa transmitter domain (open box), 131 aa receiver domain (lightly shaded box) and the 126 aa C-terminal output (heavily shaded box) regions are shown. Phosphorylation sites (Histidine, H₇₂₉; Aspartic acid, D₁₀₂₃; and Histidine, H₁₁₇₂) are indicated. The short membrane spanning domains (TM; black boxes) are also depicted. Modified from Uhl and Miller, 1996a

The transmitter region of BvgS is necessary and sufficient for autophosphorylation (Uhl and Miller, 1994) however transfer of this phosphoryl group to the C-terminal (output) domain can only be effected via the receiver domain (Uhl and Miller 1996a). Not only does the receiver domain positively regulate the phosphotransfer to the C terminal output domain, it also has the ability to reverse this transfer, thereby rephosphorylating itself and postponing BvgA activation (Figure 1.5). It can also totally dephosphorylate BvgS from the receiver itself, thereby completely halting the activation of BvgA (Figure 1.5) (Uhl and Miller 1996a). It is the output domain (particularly His₁₁₇₂) of the C terminal which is responsible for the phosphorylation of BvgA (Uhl and Miller 1996b).

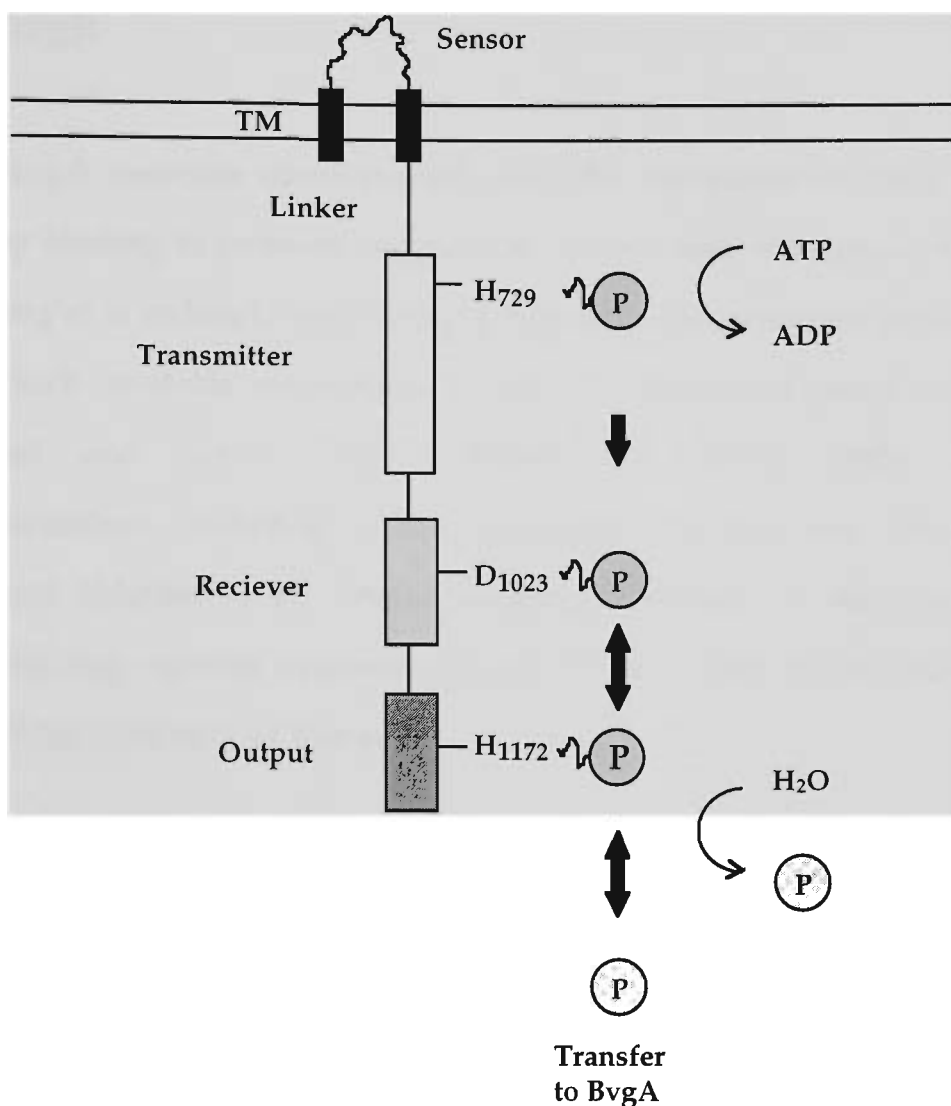


Figure 1.5 The BvgS-BvgA phosphorylation cascade model. The sensor domain receives an environmental signal that causes the autophosphorylation of the histidine (H₇₂₉) residue of the transmitter domain (open box). The phosphoryl group is then transferred to an aspartic acid residue (D₁₀₂₃) of the receiver domain (lightly shaded box). The phosphoryl group can then be relayed to the histidine (H₁₁₇₂) residue of the C-terminal output (heavily shaded box), or can react with water to dephosphorylate the BvgS molecule. The output domain then donates the phosphoryl group to BvgA, leading to the transcriptional initiation of virulence genes. The short membrane spanning domains (TM; black boxes) are also depicted. Modified from Cotter and Miller, 1997.

1.2.4 BvgR

While BvgA exercises direct control over the expression of *bvg*-activated genes by binding to promoter sequences, control over virulence repressed genes (*vrg*'s) is indirect, implicating a Bvg activated repressor protein. The gene which controls repression of the *bvg* repressed genes has been identified and termed *bvgR* (Merkel and Stibitz, 1995). Further characterisation, including DNA sequence analysis has since been performed (Merkel *et al.*, 1998), revealing mistakes in the previously published *bvg* operon sequence (Arico *et al.*, 1989) which may have delayed the discovery of this gene.

The DNA sequence of the 5' end of each of the five *bvg* repressed genes (*vrg6*, *vrg18*, *vrg24*, *vrg53* and *vrg73*) has brought to light a conserved consensus sequence element present in the coding region of four of these genes (Beattie *et al.*, 1990; Beattie *et al.*, 1993). Using a southwestern analysis at *vrg6*, this sequence was recognised by the *vrg*-repressor (BvgR) a 34 kDa protein (Beattie *et al.*, 1993). Although unusually situated in the coding sequence, homology of the 32 base pair conserved BvgR binding site among *bvg* repressed genes is restricted to the DNA level. None of the native promoter regions of any of the *bvg* repressed genes seem necessary for modulative repression to occur (Beattie *et al.*, 1993).

The *bvgR* gene lies downstream of and adjacent to *bvgAS* (Merkel and Stibitz, 1995). Further characterisation led to the identification of the exact location of the *bvgR* gene (Fig 1.1) (Merkel *et al.*, 1998a). Transcribed from its own BvgA regulated promoter, *bvgR* lies 43 base pairs downstream of *bvgS* and is read in the opposite direction. Transcriptional analysis has shown that this open reading frame produces a predicted protein of 32

kDa, close to the molecular weight (34 kDa) obtained by Beattie and associates (Merkel *et al.*, 1998a).

The new corrected sequence analysis also allowed *bvgR* to be placed in a gene family in which none of the members have been assigned a function. Although there are now 19 homologous genes in this family, *bvgR* is the only one for which a role has been elucidated (Merkel *et al.*, 1998a). A follow up study showed that mutants lacking *bvgR* are less efficient than the wild type in the mouse aerosol challenge model, demonstrating that BvgR regulation of the *bvg*-repressed genes makes a significant contribution to infection in mice (Merkel *et al.*, 1998b).

1.2.5 Transcriptional Regulation by BvgA

As the final part of an intricate phosphorelay process, BvgS phosphorylates BvgA. When compared to its unphosphorylated counterpart, this activated "BvgA \approx P" has a far greater affinity for virulence factor promoter BvgA binding sites (Boucher *et al.*, 1994; Boucher and Stibitz, 1995; Karimova *et al.*, 1996; Steffen *et al.*, 1996). Indeed, BvgA must be phosphorylated for *bvg* activated promoters to become fully operational in *in vitro* transcription assays (Steffen *et al.*, 1996; Boucher *et al.*, 1997). Although BvgA independent transcription of the *fhaB* gene has been observed, expression from this alternative promoter, P_{fha}* only occurs at low levels (Steffen *et al.*, 1996; Boucher *et al.*, 1997).

It was theorised that the positioning of BvgA \approx P at a promoter region may induce BvgA oligomerisation (Scarlatto *et al.*, 1990) and raise the affinity of the response regulator for the target DNA (Boucher *et al.*, 1994). Recent

research has demonstrated that BvgA \approx P binds cooperatively to *bvg*-activated promoters, forming multimers along the promoter template (Boucher and Stibitz, 1995; Karimova *et al.*, 1996; Zu *et al.*, 1996; Boucher *et al.*, 1997). Initially, high affinity binding occurs far upstream from the transcriptional start site, then weaker sites are progressively bound until the site adjacent to the RNA polymerase recognition sequence is reached.

1.2.6 Involvement with RNA Polymerase

When bound to the promoter DNA at the correct start site, RNA polymerase catalyses the initiation of transcription. This complex enzyme is composed of four types of subunit, α , β , β' , and σ . The α subunit is generally the portion of RNA polymerase that interacts with transcription factors at positively regulated prokaryotic promoters (Ishihama, 1992; Busby and Ebright, 1994). The σ subunit is involved in promoter recognition, interaction with transcriptional activators, DNA unwinding and setting up initiation complex (Ishihama, 1993). Much research into the important roles played by these two RNA polymerase subunits is currently underway.

The α and major σ factor of *B. pertussis* have both been cloned and characterised (Carbonetti *et al.*, 1994; Steffen *et al.*, 1997). The major σ factor of *B. pertussis* is larger than the *E. coli* σ^{70} and has been designated σ^{80} (Steffen *et al.*, 1997). The *B. pertussis* RNA polymerase σ^{80} subunit confers enhanced expression of *fhaB* in *E. coli* (Steffen *et al.*, 1997). The authors suggest that the role played by σ^{80} may be in positioning the RNA polymerase/BvgA \approx P complex at the correct site along the promoter template. By interacting directly with BvgA \approx P, the RNA polymerase σ subunit is provided with an interface point through which it can initiate

transcription (Steffen *et al.*, 1997).

Studies on response regulator molecules related to BvgA such as LuxR from *Vibrio fischeri* and UhpA from *Escherichia coli* have demonstrated that while still able to bind target DNA, transcription from promoters regulated by these proteins is severely hindered by C terminal deletions (Choi and Greenberg, 1991; Webber and Kadner, 1995). Similar experiments carried out on BvgA have shown that a deletion of as little as two amino acids resulted in an avirulent phenotype (Stibitz, 1998). When this truncated *bvg* gene was presented on a plasmid (in *trans*), in otherwise wild type *B. pertussis* strains, a massive inhibition of growth was observed (Stibitz, 1998). Mutations found to remedy the slowed growth were traced to the α subunit of RNA polymerase, further suggesting that an interaction between RNA polymerase and the C terminal of BvgA is required for the transcriptional activation of virulence associated genes in *B. pertussis* (Stibitz, 1998).

In the absence of BvgA \approx P, RNA polymerase binds upstream of the *in vivo* transcription start sites of the P_{fha} promoter leading to low level expression from the BvgA independent promoter, P_{fha}* (Steffen *et al.*, 1996; Boucher *et al.*, 1997). However the addition of BvgA \approx P allows transcription to be initiated at the normal *in vivo* site. This is further evidence that BvgA acts upon *bvg*-activated promoters by correctly positioning RNA polymerase for transcription (Steffen *et al.*, 1996; Boucher *et al.*, 1997).

Recent DNase I protection assays at *bvg*-activated promoters suggest that the promoter DNA flexes to loop around the BvgA \approx P-RNA polymerase complex (Boucher and Stibitz, 1995; Boucher *et al.*, 1997; Zu *et al.*, 1996).

This may increase the binding affinity of the weak secondary BvgA \approx P and the RNA polymerase and stabilise the entire transcriptional machinery (Boucher *et al.*, 1997).

1.2.7 Differential Activation of Virulence Factors

Expression of *bvg*-activated genes has been shown to proceed in two stages, (Scarlato *et al.*, 1991; Scarlato and Rappuoli, 1991). This differential activation of "early" and "late" promoters allows the bacterium to produce adhesin molecules soon after entering the host, while the "second wave" *bvg*-activated genes are not expressed until attachment is achieved.

The activation of PT requires the presence of a threshold concentration of BvgA \approx P (Scarlato *et al.*, 1991). There is actually a ten fold higher concentration of BvgA \approx P required to initiate transcription at the P_{ptx} promoter than either P_{fha} or *bvg* P₁ (Zu *et al.*, 1996).

Demonstration of the functional difference between early and late expressers came when mutations in the C-terminal of BvgA allowed normal levels of FHA expression while totally abolishing PT expression (Stibitz, 1994). This region has been proposed as the region of the BvgA molecule that interacts with RNA polymerase (Stibitz, 1994; Stibitz, 1998).

A mutation leading to an increase in expression of the RNA polymerase α subunit results in a phenotype lacking the production of PT and adenylate cyclase toxin/haemolysin, but expressing normal amounts of FHA (Carbonetti *et al.*, 1994). The authors proposed that much of the intracellular BvgA became bound to the excess α subunit and while there

was still enough free BvgA to activate the P_{fha} promoter, the titration was not sufficient to turn on expression of the late promoters.

Binding studies at the P_{ptx} promoter have found an initial high affinity binding site far upstream of the -35 promoter motif (Boucher and Stibitz, 1995). This region includes a 20 bp direct repeat which in turn encompasses heptameric inverted repeats (Boucher and Stibitz, 1995; Marques and Carbonetti, 1997). Genetic analysis of this region has demonstrated that it is the heptanucleotide repeats that are crucial for promoter activation (Marques and Carbonetti, 1997). Due to the distance of these repeats from the actual start site, P_{ptx} may require BvgA \approx P to multimerise along the promoter template with weaker sites progressively bound until the site adjacent to the RNA polymerase recognition sequence is reached.

A similar analysis has been undertaken at the P_{fha} promoter (Boucher *et al.*, 1997) at which the binding of BvgA \approx P to an extent mirrors that at the P_{ptx} promoter. Again a high affinity site is bound initially, including an inverted repeat region homologous to that at P_{ptx} , however it lies further downstream than the corresponding element at the P_{ptx} promoter (Zu *et al.*, 1996; Boucher *et al.*, 1997). These BvgA \approx P binding sites have been calculated to be 7-8 turns of helix upstream from P_{fha} and *bvg* P_1 and 14 turns upstream from P_{ptx} (Zu *et al.*, 1996). The architecture of the *bvg* P_1 promoter, another early promoter is very similar to that of P_{fha} and interacts with BvgA \approx P and RNA polymerase in a similar manner (Zu *et al.*, 1996). Since the expression of PT is temporally linked to that of adenylate cyclase toxin/haemolysin it is interesting that the transcriptional machinery of these two toxins is alike. The P_{cya} promoter has been shown to behave in a manner which parallels that of P_{ptx}

(Karimova *et al.*, 1996).

Differences in BvgA binding sites at the promoters of the two classes of virulence genes suggest specificity of BvgA~RNA polymerase interactions may control differences in transcription at these promoters (Boucher and Stibitz, 1995; Karimova *et al.*, 1996; Zu *et al.*, 1996; Boucher *et al.*, 1997; Karimova and Ullmann, 1997).

1.2.8 "Bvgⁱ"

Some of the recent research findings involving the coordinate regulation by the *bvg* locus has been performed in the closely related species *B. bronchiseptica*. While the *bvg*-repressed genes of *B. pertussis* are seldom studied and little is known about their function, *B. bronchiseptica* has an array of important functional *bvg*-repressed genes including motility, iron scavenging, urease and phosphatase activity which are at least in part repressed by the *bvg* locus (Akerley *et al.*, 1992; West *et al.*, 1997; Giardina *et al.*, 1995; McMillan *et al.*, 1996; Chhatwal *et al.*, 1997).

Due to this expression of functional genes under modulating growth conditions, *B. bronchiseptica* is sometimes used as a model for *B. pertussis*. An intermediate phase *B. bronchiseptica* mutant has recently been isolated which expresses phenotypes characteristic of both Bvg⁺ and Bvg⁻ states (Cotter and Miller, 1997). Further investigation using polyclonal antisera from animals infected by the intermediate mutant revealed that under growth conditions described as semi-modulating, *B. bronchiseptica* expressed elevated levels of a novel class of gene products. The phase locked "Bvgⁱ" mutant has a C to T transition in the *bvgS* gene resulting in the threonine at position 733 of wildtype BvgS being altered

to a methionine. The close proximity of this mutation to the putative phosphorylation site (His⁷²⁹) in the transmitter domain is thought to affect kinase activity (Cotter and Miller, 1997).

1.2.9 DNA Topology

Another factor theorised to affect transcription is the actual structure of the DNA itself. The topology of DNA relates to changes in the structure of supercoiled DNA which is maintained in a constant state of flux by DNA gyrase and topoisomerase I. Stability and structure of the RNA transcripts, and the presence of DNA-bound proteins such as histones and histone-like proteins are also involved.

Many genes can be affected by changes in DNA topology, including those controlled by signal transduction systems (Higgins, 1988; Dorman, 1991). Histone-like proteins have been implicated in the regulation of virulence factors in a number of pathogens by changing the local DNA topology in response to environmental signals, altering the activity of topology-sensitive promoters (Dorman, 1991).

With respect to *B. pertussis*, Scarlato and coworkers (1993) found evidence to suggest that transcriptional regulation of the pertussis toxin gene may require alterations in the local DNA topology. A far more comprehensive study has since demonstrated that *B. pertussis* virulence gene expression is sensitive to DNA topology related changes (Graeff-Wohlleben *et al.*, 1995). Drugs which inhibit DNA gyrase activity were added to the growth medium, and the expression of various virulence factors monitored. Most of the *bvg*-activated promoters assayed were downregulated in the presence of such drugs (Graeff-Wohlleben *et al.*, 1995). Although this

study implicates a role for DNA topology in the regulation of virulence factors in *B. pertussis*, the data was obtained under *in vitro* conditions and the *in vivo* situation has not yet been elucidated.

1.2.10 Bvg Accessory Factor

The temporally differential regulation employed by BvgAS to control virulence factor expression in *Bordetella pertussis* may require the presence of accessory factors. In *E. coli*, the activation of either *fhaB* or *bvgAS* merely requires the presence of *bvgAS* in *trans* (Stibitz *et al.*, 1988; Miller *et al.*, 1989; Roy *et al.*, 1989). It was thought that this was not sufficient however when trying to express pertussis toxin or adenylate cyclase toxin (Miller *et al.*, 1989; Roy *et al.*, 1989; Goyard and Ullmann, 1991). When investigating the factors affecting the expression of PT, it was found that the activation of the *bvg* locus may depend on the topology of the DNA (Scarlato *et al.*, 1993). The authors also alluded to the possibility that the action of an as yet undiscovered auxiliary protein may be required to activate expression from the *ptx* promoter (Scarlato *et al.*, 1993). Stibitz (1994) also proposed a model suggesting an accessory protein, which interacts with the C-terminus of BvgA, may be required for *cya* and *ptx* activation (Stibitz, 1994). This protein has been characterised and termed the Bvg accessory factor (Baf) (DeShazer *et al.*, 1995). These workers showed that the expression of a *ptx-lacZ* fusion in *E. coli* requires BvgAS and Baf in *trans*. The expression from P_{ptx} in *E. coli* has been demonstrated without the presence of Baf, thereby contradicting the previous work and questioning the need for Baf (Uhl and Miller, 1995).

1.3 Virulence Factors of *Bordetella pertussis*

The set of genes expressed by *B. pertussis* which allow it to invade and persist inside the host are termed virulence factors or virulence determinants. These include toxins which enable the bacterium to evade the host immune system and adhesins which facilitate attachment to target host cells. At present the consensus with regard to the strategy for developing recombinant whooping cough vaccines seems to include a few purified immunogens in an acellular, component vaccine. Many of the virulence factors of *B. pertussis* have been considered for this purpose. Since the direct cause of whooping cough symptoms is attributed to pertussis toxin, it is widely accepted that this antigen constitute the main component in any new vaccine (Pitman, 1984). Other candidate antigens include FHA, serotype-specific fimbriae, adenylate cyclase and pertactin.

1.3.1 Pertussis Toxin

A member of the A-B bacterial toxin superfamily, pertussis toxin (PT) is a 106 kDa, hexameric protein comprising five distinct subunits (Tamura *et al.*, 1982). PT is an ADP ribosyltransferase (116) with the ability to cause a plethora of effects on host cells. It is the A protomer or S1 subunit which possesses enzymatic capabilities, catalysing the transfer of the ADP-ribose moiety of NAD⁺ to a family of G proteins, which are involved in vital signal transduction pathways within the host cell. This short circuits the cell signalling machinery of surrounding epithelial and immune system cells by breaking down G protein interactions (Katada *et al.*, 1983). The B oligomer comprises the remaining subunits, S2, S3, S4 and S5 make up the B oligomer in the ratio 1:1:2:1 (Tamura *et al.*, 1982). This region of the

protein facilitates attachment of the toxin to host cells, delivering the toxic action of the S1 subunit (Tamura *et al.*, 1982).

The genes encoding the pertussis toxin subunits are clustered together in an operon typical of many other bacterial toxins (Figure 1.6). Genetic analysis has revealed that each subunit is translated separately with an amino-terminal signal sequence which is cleaved during transport to the periplasm where the holotoxin is then assembled and secreted (Locht and Keith, 1986; Nicosia *et al.*, 1986).

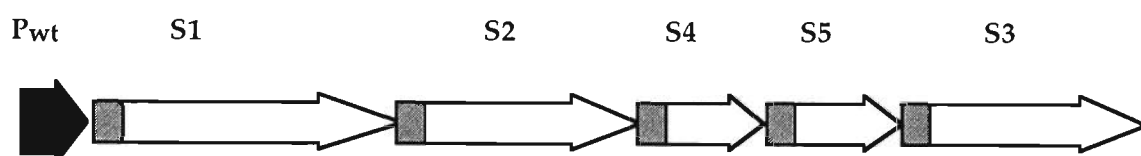


Figure 1.6 Schematic diagram of the pertussis toxin operon. The genes coding the PT subunits S1-S5 are represented by open arrows. Signal sequences are demonstrated as shaded boxes. The wild type pertussis toxin promoter is shown as a filled arrow. The arrows indicate the direction of transcription of the genes.

The major hurdle yet to be overcome in the preparation of acellular, component vaccines is the inability to obtain purified antigens in large amounts. The incompatibility of PT gene expression signals with respect to *Escherichia coli* make the production of PT in this organism difficult to achieve (Nicosia *et al.*, 1987; Burnette *et al.*, 1988). There are also problems associated with obtaining high yields of purified PT from virulent *B. pertussis*. These include the slow growth rate, fastidious nutritional requirements, and the inherent low PT production levels of the bacterium. There is also the risk that the co-purification of low levels of other virulence regulated toxins may occur in vaccine preparations. Of the *Bordetella* species, only *B. pertussis* produces PT. A cryptic PT operon

is present in *B. parapertussis* and *B. bronchiseptica*; base pair mutations in the promoter region leave these operons transcriptionally silent (Arico and Rappuoli, 1987).

An expression system for pertussis holotoxin has been the goal of a number of groups, particularly stimulated by the cloning and sequencing of the PT operon. The S1 subunit gene has upstream regions analogous to *E. coli* ribosomal binding sites. The remaining subunits, however, contain novel ribosome binding site sequences upstream of the structural genes, which bear no resemblance to the *E. coli* consensus ribosome binding site. This, and the fact that the PT operon is positively regulated by the *bvg* locus, are major reasons why pertussis holotoxin is yet to be expressed in *E. coli*. Expression of PT subunits has been achieved in *E. coli* and *Bacillus subtilis* (Burnette *et al.*, 1988; Runeburg-Nyman *et al.*, 1987; Saris *et al.*, 1990). Unfortunately, expression of PT subunits in *E. coli* caused the majority of the protein to congregate in inclusion bodies, and not be secreted into the periplasm (Burnette *et al.*, 1988). The other major problem with expression of subunits for vaccine purposes is their inability to assemble into holotoxin *in vitro*. The individual PT subunits have been shown to induce antibody responses in immunised mice. However, these mice were not protected when challenged intracerebrally with *B. pertussis* cells (Burnette *et al.*, 1988). The B oligomer has been assembled *in vitro* and mice immunised with this preparation were able to neutralise the toxic effects of PT (Burnette *et al.*, 1992). Simultaneous expression of all five PT subunits has been achieved in *E. coli* and *Salmonella typhimurium aroA* (Dalla Pozza *et al.*, 1997; Dalla Pozza *et al.*, 1998). While this was initially promising, varying levels of expression and post translational processing, together with a lack of protection in orally immunised mice indicates that these strains would not be useful as

vaccine strains.

Efficient expression of pertussis holotoxin, has to date only been achieved in *Bordetella* species. The major concern here is that PT production takes place in only virulent strains of *B. pertussis*. The copurification of other toxins and thus contamination of vaccine preparations may therefore occur. Lee and associates have used recombinant plasmids to express PT in *B. parapertussis* and *B. bronchiseptica* (Lee *et al.*, 1989). Yields were comparable to those of *B. pertussis* and although the majority of the PT produced was localised in the periplasm, some PT was detected in the culture supernatant. *B. parapertussis* and *B. bronchiseptica* have also been used to produce genetically altered detoxified pertussis toxin (Pizza *et al.*, 1989; Nencioni *et al.*, 1990). Bacterial strains engineered to produce a safe pertussis "toxoid" are very good candidates for the production of acellular vaccines. Overexpression and secretion of genetically detoxified pertussis toxin has been achieved in *B. pertussis* (Zealey *et al.*, 1992) by using allelic exchange techniques to introduce multiple copies of a genetically altered PT operon into the chromosome. Fully inducible production of PT in *B. bronchiseptica* has resulted in improved growth rates and PT yields when compared to *B. pertussis* (Suarez *et al.*, 1997). Expression of PT was achieved in a permanently avirulent strain of *B. bronchiseptica* using mini-transposons to clone a promoterless PT operon in front of a strong constitutive *B. bronchiseptica* promoter (Walker *et al.*, 1991a). Unfortunately, the toxin was not secreted to the growth medium, making this strain less than suitable as a vaccine expression system. Likely explanations for this lack of secretion may include (i) the secretion of PT in *B. bronchiseptica*, as it is in *B. pertussis* (Weiss *et al.*, 1993), is regulated by the *bvg* locus resulting in only virulent strains being capable of PT secretion, (ii) the strain of *B. bronchiseptica* used does not possess the

entire genetic machinery to secrete PT, or (iii) that it does possess PT secretion genes, but the genes are cryptic or the gene products serve an alternate function. If the necessary PT secretion genes are intact, the coordinate regulation by the *bvg* locus may result in only virulent phase *B. bronchiseptica* secreting the toxin. When the *B. pertussis* PT operon was sequenced (Locht and Keith, 1986; Nicosia *et al.*, 1986), information for the first 1252 base pairs of the *B. pertussis* *ptl* (pertussis toxin liberation) operon was also obtained. The *ptl* operon of *B. pertussis* has been shown to direct the secretion of PT to the culture supernatant (Weiss *et al.*, 1993), a finding confirmed in part by Covacci and Rappuoli (1993), who determined that PT secretion was dependent on at least one of these genes.

The entire *B. pertussis* *ptl* operon has recently been cloned and sequenced (Weiss *et al.*, 1993). There was originally thought to be eight open reading frames (ORFs) present in the operon, designated *ptlA-ptlH* (Weiss *et al.*, 1993). Evidence for the presence of a ninth gene, *ptlI*, has since been found (Figure 1.7) (Farizo *et al.*, 1996).

Partial *ptl* sequence data of *B. parapertussis* and *B. bronchiseptica* can be compared to the equivalent genomic region of *B. pertussis* (Locht and Keith, 1986; Nicosia *et al.*, 1986; Weiss *et al.*, 1993). If one focuses on the *ptl* region of *B. pertussis* and *B. bronchiseptica* encompassing the first 1252 bp, including *ptlA*, *ptlB* and the first 624 bp of *ptlC* (Locht and Keith, 1986; Nicosia *et al.*, 1986; Weiss *et al.*, 1993), there are 60 base differences in this region. However almost all of these are concentrated within and immediately upstream of the *ptlA* gene. It has been postulated that *ptlA* of *B. pertussis* is not actually a trans-acting gene, but a cis-acting promoter (Weiss *et al.*, 1993). If *ptlA* is the promoter region, then mutations here

Pertussis Toxin

Pertussis Toxin Liberation

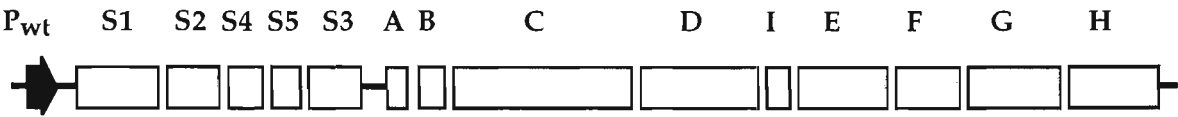


Figure 1.7 Schematic representation of the *ptx/ptl* operon. Pertussis toxin subunit genes (open boxes; S1-S5) and pertussis toxin liberation genes (shaded boxes; A-I) are indicated. The *ptx/ptl* promoter (filled arrow; P_{wt}) is also depicted.

may render the bacterium incapable of transcribing the *ptl* operon. The homology between *B. pertussis* and *B. bronchiseptica* *ptlB* and *ptlC* genes is very high, with only 5 base differences in 924 base pairs. Fusion proteins expressed in *E. coli* have been used to raise polyclonal antibodies against the predicted PtlA, B, C, E, F, G and H proteins (Johnson and Burns, 1994). Immunoreactive bands in western blots of *B. pertussis* whole cell extracts were only detected using polyclonal antibodies against PtlE, F and G. The same antibodies raised against PtlE and F failed to detect immunoreactive bands in *B. bronchiseptica* whole cell extracts (Johnson and Burns, 1994). Replacement of the *B. bronchiseptica* *Pptx* promoter with the *B. pertussis* *Pptx* promoter results in detectable expression of PtlF in western blots (Kotob *et al.*, 1995). This suggested that the *ptlA* region is not the promoter of the *ptl* operon and that *Pptx* actually drives the transcription of the complete *ptx/ptl* region. This was confirmed when the 11 kb mRNA for the entire *ptx/ptl* operon was isolated from *B. pertussis* (Baker *et al.*, 1995). The introduction of the *B. pertussis* *Pptx* promoter into *B. bronchiseptica* and *B. parapertussis* via homologous recombination results in the expression and secretion of the toxin (Hausman *et al.*, 1996).

1.3.2 Adenylate Cyclase Toxin/Haemolysin

The adenylate cyclase-haemolysin of *Bordetella pertussis* is a bifunctional protein belonging to the RTX (repeat in toxin) family. This assemblage also includes the much studied α -haemolysin of *E. coli*, together with haemolysins and leukotoxins of various pathogenic Gram-negative bacteria including *Pasteurella haemolytica*, and *Actinobacillus* species (Welch, 1991). Both the adenylate cyclase, and the haemolytic activities have been shown to be essential for the initiation of *B. pertussis* infection (Khelef *et al.*, 1992). Adenylate cyclase-haemolysin is a secreted polypeptide chain consisting of 1706 amino acid residues (Glaser *et al.*, 1988a), with a molecular weight of between 175 and 220 kDa (Rogel *et al.*, 1989; Leusch *et al.*, 1990; Gentile *et al.*, 1990).

The adenylate cyclase activity of this protein is located within the first 400 amino acids at the amino terminal end and is activated by calmodulin (Glaser *et al.*, 1988a; Ladant *et al.*, 1989). Although the high affinity of adenylate cyclase toxin/haemolysin for calmodulin was initially thought to be involved in the translocation of the toxin into host cells, recent evidence has proven this to be incorrect (Heveker and Ladant, 1997). The calmodulin is however, crucial for the enzyme once inside the cell, where it catalyses the formation of ultraphysiologic levels of cyclic AMP (Confer and Eaton, 1982; Greenlee *et al.*, 1982).

The remaining 1300 residues contains an independently functional haemolysin (Glaser *et al.*, 1988a; Sakamoto *et al.*, 1992) among other structurally and functionally important domains. The 200 residue hydrophobic region is inserted into the membrane of target host cells as the initial step of delivery into the eukaryotic cell (Hanski, 1994). The

repeat domain of adenylate cyclase toxin/haemolysin contains 41 repeats of the glycine and aspartic acid rich regions indicative of the RTX family. Homology with other RTX members suggest that this region is involved in host cell recognition and calcium binding (Ludwig *et al.*, 1988; Boehm *et al.*, 1990). In fact, each repeat motif binds a single calcium ion, leading to a major conformational change and possibly the translocation of the catalytic portion of the molecule into host cells (Rose *et al.*, 1995). There is also a specialised secretion signal domain which interacts with accessory proteins required for the entry of adenylate cyclase toxin/haemolysin into host cells (Glaser *et al.*, 1988b).

The gene encoding the adenylate cyclase toxin/haemolysin (*cyaA*) is contained within an operon, together with a number of other factors involved in activating and secreting the toxin (Glaser *et al.*, 1988a) (Figure 1.8).

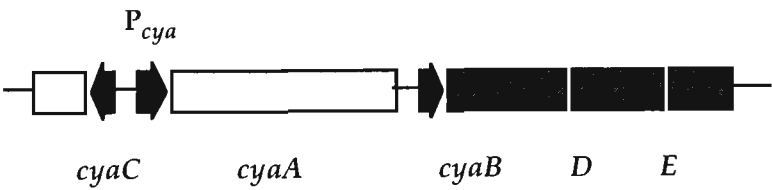


Figure 1.8 Operon Structure of the genes encoding adenylate cyclase toxin/haemolysin and accessory proteins. The adenylate cyclase gene (lightly shaded box; *cyaA*) and accessory genes (heavily shaded boxes; B, C, E) are indicated. The adenylate cyclase gene promoter (filled arrow; P_{cya}) is also depicted. Sizes of promoters indicates relative levels of transcription. Modified from Goyard *et al.*, 1993.

Secretion will only proceed with the expression of *cyaB*, *cyaD*, and *cyaE* (Glaser *et al.*, 1988b). The final member of the *cya* operon, *cyaC*, is required to activate adenylate cyclase toxin/haemolysin, and is involved in post

translational modification of the toxin (Barry *et al.*, 1991). This protein causes the acylation of adenylate cyclase toxin/haemolysin resulting in the addition of a palmitoyl fatty acid residue to Lys⁹⁸³ of the polypeptide chain (Hackett *et al.*, 1994).

Host cell invasion by adenylate cyclase toxin/haemolysin is not effected via a receptor mediated endocytotic pathway (Gordon *et al.*, 1989), and instead uses a specialised calcium and temperature dependent entry system (Hanski, 1994).

The use of a detoxified form of adenylate cyclase toxin/haemolysin in acellular vaccine preparations has been advocated by a number of groups (Hewlett *et al.*, 1977; Arcinega *et al.*, 1991; Guiso *et al.*, 1991). Immunisation with adenylate cyclase toxin/haemolysin has been shown to be protective against bacterial colonisation in animal models (Novotny *et al.*, 1985; Guiso *et al.*, 1989; Guiso *et al.*, 1991). Humans infected with *B. pertussis* are known to produce antibodies against adenylate cyclase toxin/haemolysin to fight the infection (Arcinega *et al.*, 1991). The interaction between adenylate cyclase toxin/haemolysin and aspects of the human immune system is currently the focus of many research groups. Invasion and survival of *B. pertussis* into many different immune cells is now well documented (Saukkonen *et al.*, 1991; Steed *et al.*, 1991; Freidman *et al.*, 1992; Masure, 1992). The inhibition of antigen dependent T cell proliferation by *B. pertussis* has recently been demonstrated and necessitates the presence of adenylate cyclase toxin/haemolysin (Boschwitz *et al.*, 1997).

The role which adenylate cyclase toxin/haemolysin plays in apoptosis has been demonstrated *in vitro* and *in vivo* in a number of different cell

types (Khelef *et al.*, 1993; Khelef and Guiso, 1995; Gueirard *et al.*, 1998). It has been proposed that this may be a method by which *Bordetella pertussis* might escape the antibacterial effects of macrophages (Gueirard *et al.*, 1998). Alternatively, it could be an action facilitated by the immune system to remove the bacteria from their possibly safer intracellular niche and expose it to the rigours of the extracellular environment, patrolled by killer T cells and antibodies.

1.3.3 Dermonecrotic Toxin

Although known for many years (Bordet and Gengou, 1909), this toxin has not been as extensively studied as other *Bordetella* virulence determinants. This may be due in part, to difficulty in the purification process. Termed heat labile toxin because it becomes inactive at 56°C, it was given its current name due to its ability to cause necrotic skin lesions when injected subcutaneously into mice (Iida and Okonogi, 1971; Livey and Wardlaw, 1984). This injection is lethal when given intravenously. Dermonecrotic toxins are produced by *Bordetella* species and are regulated by the *bvg* locus (Bordet and Gengou, 1909; Endoh *et al.*, 1986; Endoh *et al.*, 1988; Gentry-Weeks *et al.*, 1988). The toxin is not secreted to the extracellular environment and has been localised to the cytoplasm (Cowell *et al.*, 1979). This and its very low expression levels are probably the major purification problems. The latest studies agree on a molecular weight of 140 kDa and contrary to previous reports, a single polypeptide chain (Horiguchi *et al.*, 1990; Zhang *et al.*, 1991). The gene encoding the *B. pertussis* dermonecrotic toxin was sectionally cloned and sequenced (Walker and Weiss, 1994). The toxin can be placed by homology and functionality into a family of toxins including the cytotoxic necrotising factors of *E. coli*, botulinum C3 toxin and *Pasteurella multocida* toxin

(Hoshijima *et al.*, 1990; Rozengurt *et al.*, 1990; Falbo *et al.*, 1993; Oswald *et al.*, 1994). All members of this family induce dormant cells to begin DNA synthesis, leading to either increased cell division or multinuclear cells, as is the case with dermonecrotic toxin. Although dermonecrotic toxin is considered a virulence factor, strangely, mutants deficient in dermonecrotic toxin do not exhibit any differences in virulence in mouse studies (Weiss and Goodwin *et al.*, 1989). The exact role of dermonecrotic toxin in the pathogenesis of *B. pertussis* infection is still unknown, however recent technological advances which have allowed the purification of the toxin and the cloning of its gene may allow further insight into the molecular mechanisms of dermonecrotic toxin to be obtained.

1.3.4 Tracheal Cytotoxin

Unique among *Bordetella* virulence factors, tracheal cytotoxin is the only toxin expressed in both virulent and avirulent growth phases. The major biological activity endowed by this toxin is the damage to ciliated respiratory epithelial cells (Cookson *et al.*, 1989a). Tracheal cytotoxin is a very small molecule and is actually a disaccharide-tetrapeptide derivative of the cell wall peptidoglycan layer. The structure of this 921 Da monomeric compound is *N* - acetylglucosaminy - 1, 6 - anhydro - *N* - acetylmuramyl - (L) - alanyl - g - (D) - glutamyl - *meso* - diaminopimelyl - (D) - alanine (Cookson *et al.*, 1989b). Both structurally and functionally, tracheal cytotoxin falls into the muramyl peptide family. Gram-negative bacteria produce a layer of peptidoglycan, however only *Bordetella* and *Neisseria* release fragments extracellularly, in the form of muramyl peptides, normally during exponential growth (Sinha and Rosenthal, 1980; Cookson and Goldman, 1987).

The destruction of cilia and ciliated epithelial cells by tracheal cytotoxin causes ciliostasis and forces the infected individual to cough persistently in order to remove mucus, a task ordinarily performed by ciliated cells. It has been demonstrated that the disaccharide moiety of tracheal cytotoxin is not required for this cytotoxicity (Luker *et al.*, 1993). The lactyl tetrapeptide portion of the molecule is responsible for its full toxic activity (Luker *et al.*, 1995). The toxicity conferred by tracheal cytotoxin is indirect, being caused by the induction of host cells to produce interleukin-1 (Heiss *et al.*, 1993). This activates host cell nitric oxide synthase leading to high levels of nitric oxide radicals (Heiss *et al.*, 1994). It is still not certain whether it is the tracheal cytotoxin or the interleukin-1 which stimulates nitric oxide synthase. The nitric oxide acts by destroying iron dependent enzymes, eventually inhibiting mitochondrial function and DNA synthesis in nearby host cells (Heiss *et al.*, 1993). Since *Bordetella pertussis* is attached to the ciliated epithelial cells, it is these which undergo the most damage. Tracheal cytotoxin also has a toxic effect on other cells, impairing neutrophil function at low concentrations and conferring toxic activity in larger quantities (Cundell *et al.*, 1994). The production of tracheal cytotoxin by *B. pertussis* evolutionally, may have been merely as a by-product or breakdown product of peptidoglycan manufacture, however it is now an integral component in the pathogenesis of *B. pertussis* infection.

1.3.5 Filamentous Haemagglutinin

The critical step in *Bordetella pertussis* infection is attachment of the pathogen to host cells. The filamentous haemagglutinin (FHA) (Figure 1.9) is consistently referred to as the major adhesin of this bacterium. It is a 220 kDa surface associated protein secreted to the extracellular

environment to facilitate adherence to ciliated respiratory epithelial cells, thereby initiating the pathogenic cycle.

FHA has a filamentous structure, supported by electron microscopy studies giving the dimensions of the molecule as 2 nm wide and 45-50 nm long (Arai and Sato, 1976; Makhov *et al.*, 1994). An extensive study by Makhov and coworkers (1994) has revealed the structure of FHA to be a polypeptide chain folded into a monomeric hairpin (Figure 1.9). The hairpin comprises head, shaft and tail regions. Whilst the head contains the N and C termini, and the tail the important RGD sequence, the shaft is composed of tandem 19-residue repeat regions R1 (38 cycles) and R2 (13 cycles) which maintain the structural integrity of the molecule.

This protein is unusual in a number of respects. Firstly, the gene for FHA, *fhaB* has a coding potential of 367 kDa (Delisse-Gathoye *et al.*, 1990; Domenighini *et al.*, 1990). The precursor protein, FhaB, is later cleaved in

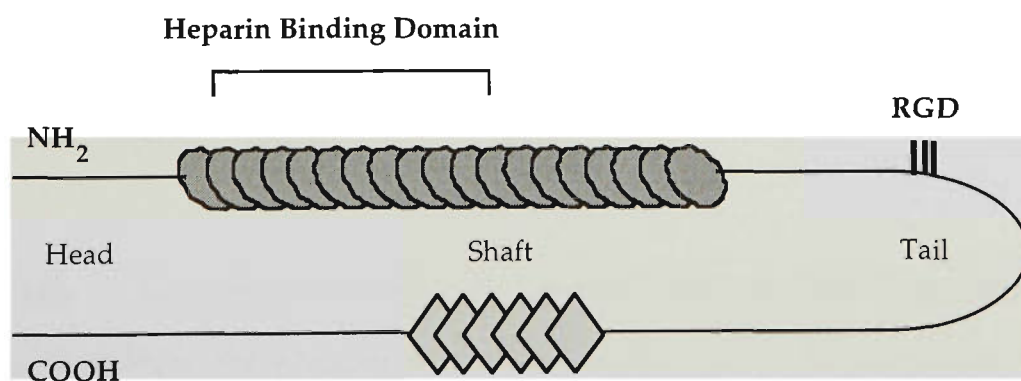


Figure 1.9 Diagrammatic representation of the model of the mature FHA polypeptide, illustrating the hairpin structure and the positioning of repeat regions. The RGD site implicated in integrin binding is also shown. Modified from Makhov *et al.*, 1994; Brennan and Shahin, 1996.

a complex post-translational maturation process. Filamentous haemagglutinin also possesses a number of possible binding sites and functions. A further interesting point about FHA is the level of regulation involved in producing and secreting the polypeptide.

Binding Activities

The different domains of FHA (Figure 1.10) encompass various binding capabilities, including integrin mediated attachment to phagocytes, lectin-like binding to sulfated sugars found in the extracellular matrix (ECM) and epithelial cells, and a carbohydrate recognition domain which allows attachment to ciliated cells present in the respiratory epithelium. The main target binding site for *B. pertussis* is the ciliated epithelium of

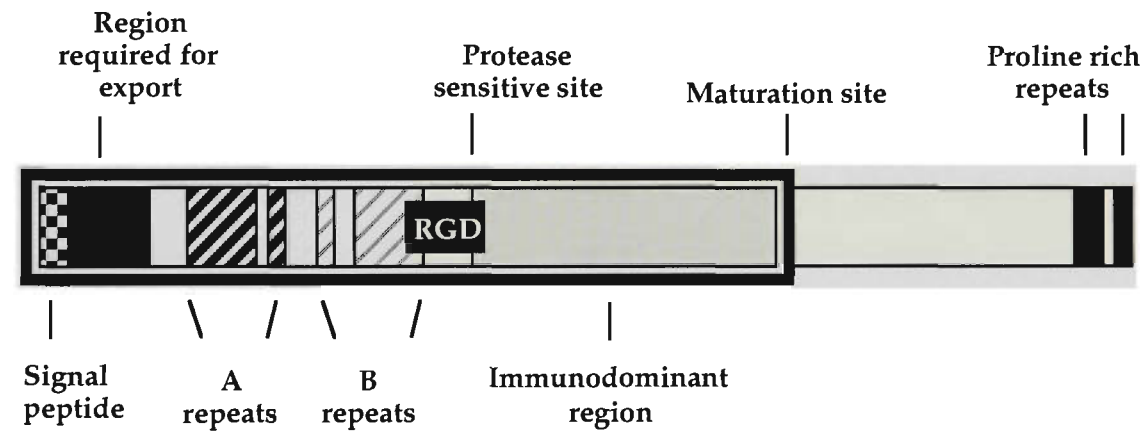


Figure 1.10 The domain structure of the precursor molecule, FhaB demonstrates the complexity of FHA. The precursor must undergo a cleavage at the maturation site to release the mature protein (black rectangle). The signal peptide (checked box) is shown, as is the region that exhibits homology to ShlA and HpmaA (filled box). Both sets of repeat regions (A, thick striped boxes; B, thin striped boxes) are indicated. Proline rich repeat units are designated. The immunodominant region is represented by the lightly shaded box. There is also a site which is easily degraded by proteases. The RGD site is also shown. Modified from Lochter *et al.*, 1993.

the respiratory tract (Tuomanen and Weiss, 1985). Attachment to cells in this region is mediated by the carbohydrate binding capacity of FHA (Tuomanen *et al.*, 1988; Relman *et al.*, 1989), which has a unique affinity for glycolipids and ciliated cells (Prasad *et al.*, 1993). This carbohydrate recognition domain was narrowed down to amino acid residues 1141 to 1279 using monoclonal antibodies (Prasad *et al.*, 1993). Recent studies of this region have enabled high level, soluble expression in *E. coli* and further characterisation of this 18 kDa polypeptide, designated fragment A (Liu *et al.*, 1997). Anti-fragment A antibodies were shown to inhibit binding of *B. pertussis* to ciliated rabbit cells in the same fashion as antibodies elicited by whole FHA, raising speculation that with further investigation, the more easily obtained fragment A may replace FHA in component vaccines against whooping cough (Liu *et al.*, 1997).

The heparin binding activity of FHA may allow *B. pertussis* to bind to non-ciliated cells as demonstrated using WiDr cells, HeLa cells, and Vero cells (Urisu *et al.*, 1986; Sato *et al.*, 1981) or perhaps facilitate extracellular matrix interactions. The lectin-like binding of FHA to heparin and dextran sulfate was characterised when it was shown that binding to epithelial cells could be inhibited by the addition of heparin (Menozzi *et al.*, 1994). Hannah and associates (1994) extended this research by revealing that FHA also binds specifically to sulfated, but not uncharged glycolipids and mapped the heparin binding domain to residues 442-863 of the amino terminal end of FHA.

The tail region of FHA includes a binding moiety specific for complement receptor type 3 (CR3, $\alpha_M\beta_2$, CD11b/CD18) integrins present on the surface of macrophages (Relman *et al.*, 1990). FHA contains an RGD (arginine-glycine-aspartic acid) sequence which is present in a number of other

bacterial adhesins and is a region of attachment in many eukaryotic integrin binding proteins (Ruoslahti and Pierschbacher, 1986; Hynes, 1982). It has been determined that it is this RGD tripeptide which facilitates binding to CR3 (Relman *et al.*, 1990). *B. pertussis* may use this protein as a dock, which may initiate entry into phagocytes without the associated oxidative burst. It has since been shown that the binding process involves the formation of a leukocyte signal transduction complex which upregulates CR3 binding activity, suggesting that FHA could regulate the binding activity of its own receptor. This complex is composed of a receptor protein called leukocyte response integrin (LRI) and integrin-associated protein (CD47) (Ishibashi *et al.*, 1994). These authors suggest that the carbohydrate/glycoconjugate binding capacity of FHA may also be involved in the primary stages of the CR3-LRI-CD47 complex formation. This hypothesis was formulated using the example of leukocyte-endothelial cell recognition (Butcher, 1991) whereby a transitory state of attachment is mediated by lectins and this weak connection is strengthened by CR3-dependent binding. Hazenbos and associates found that a macrophage surface fibronectin receptor, very late antigen-5 (VLA-5) may also be involved and proposed that binding and cross-linking of VLA-5 by *B. pertussis* activates CR3, facilitating attachment to FHA (Hazenbos *et al.*, 1993). Further study led to the finding that VLA-5 was acting as a ligand for the minor fimbrial subunit FimD (Hazenbos *et al.*, 1995b). These authors suggest that fimbriae and FHA may act cooperatively to bind to macrophages, leading to uptake and intracellular survival.

The advantages gained by *B. pertussis* in binding to “professional phagocytes” are concerned with evasion of the immune system and may be threefold. Severing cellular communication links to phagocytic cells by

obstructing a vital receptor site (CR3, VLA-5 or C4BP) may reduce the impact of the immune system. Secondly, by attaching to CR3, *B. pertussis* has taken the preliminary step in entry and persistence in phagocytes, and “hand-to-hand combat” using CR3 as a site from which to deliver bacterial toxins at close range may kill macrophages and neutrophils more economically (Saukkonen *et al.*, 1991; Steed *et al.*, 1991). Finally, a recent addition to the *B. pertussis* versus human immune system story is the finding that FHA binds the serum protein C4BP (Berggård *et al.*, 1997). This protein is known as a regulator of complement activation and acts to inhibit the classical complement pathway, ceasing the formation of the membrane attack complex. Whether *B. pertussis* is able to utilise this binding capacity remains unclear, but once again demonstrates the extent and complexity with which *B. pertussis* interacts with our immune system.

Genetic Regulation and Biogenesis

Being a virulence determinant of *Bordetella pertussis*, FHA undergoes a high level of genetic regulation. The *bvg* locus controls the expression from *fhaB* depending on the prevailing environmental conditions. When *B. pertussis* is in suitable conditions for colonisation, FHA is one of the first proteins produced, detected in a matter of minutes (Scarlato *et al.*, 1991). The chromosomal geography of the genes encoding BvgAS and FHA may be a factor in this fast reaction time. They are physically adjacent, further suggesting direct interaction between BvgA and *fhaB*. (Stibitz *et al.*, 1988). Production of FHA is also dependent on the expression of at least one accessory protein, FhaC, the gene for which lies downstream of *fhaB* in a cluster of accessory genes involved in the export of both FHA and fimbriae (Figure 1.11) (Locht *et al.*, 1992; Willems *et al.*, 1992). The outer membrane protein FhaC is thought to interact with FHA

allowing for secretion of the adhesin (Willems *et al.*, 1994). As the role played by FHA in the pathogenesis of whooping cough becomes more clear, more as yet undiscovered factors (Jacob-Dubuisson *et al.*, 1996) involved in the expression and secretion of FHA may be found.

Post Translational Maturation

The DNA sequence of the structural gene *fhaB* was determined independently by two groups (Delisse-Gathoye *et al.*, 1990; Domenighini *et al.*, 1990). The gene consists of approximately 10.1 kb and encodes a large protein of 367 kDa termed FhaB. This precursor must undergo a series of modifications which are still only partially understood before mature FHA is secreted to the extracellular environment.

Toward the N-terminus of FhaB lies a domain of approximately 115 residues, bearing homology to regions of the haemolysins of *Serratia marcescens* and *Proteus mirabilis*, and which are required for secretion of these proteins (Delisse-Gathoye *et al.*, 1990). Secretion of each of these proteins is also dependent on the presence of a homologous accessory protein (FhaC in *B. pertussis*) (Willems *et al.*, 1994). An FhaC-like outer membrane protein, HMWB has since been found in *Haemophilus influenzae*, where its function is also to aid in the export of an adhesin, HMWA (Barenkamp and St Geme, 1994). The precursors of FHA and HMWB share an extra region of homology comprising 22 amino acid residues, followed by a positively charged region and a hydrophobic segment. This acts as an atypical signal peptide and is cleaved from the molecule prior to export (Delisse-Gathoye *et al.*, 1990). The C-terminal third of the precursor protein is also removed leaving mature FHA comprising the N-terminal 220 kDa of FhaB. Although not part of the mature protein, the C-terminal plays an important role in secretion

(Renauld-Mongénie *et al.*, 1996) possibly as a type of intramolecular chaperone to stabilise the protein and prevent incorrect folding, allowing the required secretory interactions to proceed. The latest model of FHA export put forward by Jacob-Dubuisson *et al.* (1996), involves the HMWA homologous N-terminus of FhaB binding to a “pilot” protein which transports the precursor to the translocase on the inner membrane. The unusual signal peptide is then cleaved as the protein is translocated to the periplasmic space. The 115 residue, haemolysin-homologous domain then interacts with FhaC. As the N-terminal portion is passing across the outer membrane, the 150 kDa C-terminal section can be cleaved liberating mature FHA, which then folds into the rigid hairpin structure (Figure 1.9) proposed by Makhov *et al.* (1994).

1.3.6 Serotype Specific Fimbriae

Fimbriae, also known as pili and agglutinogens, are long filamentous protrusions which extend from the bacterial cell surface and facilitate a variety of binding capabilities. The fimbriae of *B. pertussis* incorporate both major and minor subunits. The major subunits form the fimbrial strand, being grouped into pentameric repeat units, each 13 nm in length and comprising 2 full helical turns (Steven *et al.*, 1986). This finding has been supported and extended by more recent research (Heck *et al.*, 1996) which also obtained an accurate value of 5.7 nm for the diameter of *B. pertussis* serotype 3 fimbriae.

Bordetella pertussis produces two distinct types of fimbriae, serotype 2 and serotype 3 (also called serotype 6). These are constructed using major subunits, expressed from the *fim2* and *fim3* genes respectively, and minor fimbrial subunit, encoded by *fimD* (Willems *et al.*, 1992; Loch *et al.*, 1992).

Major subunits are stacked to form the long filamentous structure characteristic of fimbriae and the minor subunit is located at the tip (Geuijen *et al.*, 1997).

Major Subunits

The major fimbrial subunits which form the structural helices, Fim2 and Fim3 are small proteins of 22 kDa and 22.5 kDa respectively. The *fim2* and *fim3* genes which encode these proteins have both been cloned and sequenced (Livey *et al.*, 1987; Mooi *et al.*, 1990) and exhibit considerable homology at both the nucleotide and amino acid level. Two other pseudogenes related to the major fimbrial subunits have also been found in *Bordetella pertussis*, *fimX* and *fimA* (Pedroni *et al.*, 1988; Willems *et al.*, 1992). The *fimX* gene encodes for a protein of approximately 20 kDa which is expressed at very low levels if at all (Riboli *et al.*, 1991). Using CAT fusion assays, these authors were able to demonstrate transcription from the *fimX* promoter in *B. bronchiseptica*, also showing that it was *bvg*-regulated. The other silent fimbrial gene, *fimA*, is located in the cluster of fimbrial and FHA accessory genes (Figure 1.11) (Willems *et al.*, 1992). This is a region of DNA with sequence homology to *fim2*, *fim3* and *fimX*, however the 5' end of the gene corresponding to the N-terminal region of FimA is not present in *B. pertussis*. Recently, it was found that *B. bronchiseptica* expresses a fully intact version of FimA (Boschwitz *et al.*, 1997). The low level expression of FimA seen in *B. bronchiseptica* is *bvg*-regulated. It has been postulated that the two functional major fimbrial subunit genes and the pseudogene *fimX* may have arisen via gene duplication. The discovery of *fimA*, its chromosomal location within the fimbrial operon, and its viability in *B. bronchiseptica* suggest that *fimA* may actually be the original, ancestral major fimbrial subunit from which *fim2* and *fim3* arose (Willems *et al.*, 1992; Boschwitz *et al.*,

1997).

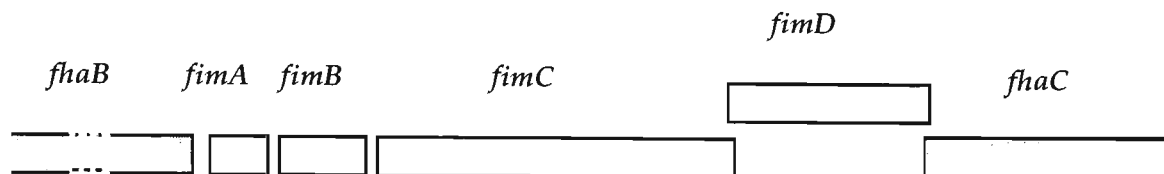


Figure 1.11 Diagrammatic representation of the *fim/fha* gene cluster. Names are given above genes. Genes involved in FHA production are shown in shaded boxes and those involved in fimbrial production are shown in open boxes. Modified from Willems *et al.*, 1994

Since the discovery of the minor subunit and adhesin FimD, the role of the major subunits in adherence has been somewhat downgraded to them almost being regarded as merely the scaffolding on which sits the important adhesin molecule. Recently it has been shown however, that the major fimbrial subunit has an affinity for sulfated sugars (Geuijen *et al.*, 1996), an ability shared by the other principal adhesin molecule, filamentous haemagglutinin. The association between *B. pertussis* fimbriae and derivatives of heparan sulfate is highly dependent on both the number of sulfate groups present and their placement around the disaccharide molecule (Geuijen *et al.*, 1996). The localisation of two heparin binding regions (H1 and H2) has since been fully characterised (Geuijen *et al.*, 1998). These regions share sequence and structural homology to each other and to similar heparin binding sites of fibronectin, a eukaryotic signalling molecule.

The major antigenic domains present on Fim2 and Fim3 fimbriae have been identified using monoclonal antibodies to screen various synthetic peptides (Pearce *et al.*, 1994). These epitopes were again confirmed as highly immunogenic when they were exposed to, and recognised serum

antibody from, whooping cough infected patients (Williamson and Matthews, 1996). A number of these antigenic epitopes in fact correspond to the recently identified heparin binding domains (Geuijen *et al.*, 1998).

Minor Subunit and Accessory Proteins

The other genes found in the fimbrial cluster (Figure 1.11) are designated *fimB*, *fimC*, and *fimD* (Willems *et al.*, 1992; Loch *et al.*, 1992). Both these studies performed homology searches on the proteins predicted by the newly discovered operon and found similarities to proteins essential for fimbrial biogenesis in both *Escherichia coli* (Pap) and *Klebsiella pneumonia* (Mrk). The genetic organisation of the entire cluster/operon is very similar to the *mrk* operon (Allen *et al.*, 1991), perhaps suggesting a common origin. Another similar fimbrial gene cluster has also been found in *Haemophilus influenzae* type b (van Ham *et al.*, 1994). An even greater level of homology to *fimB*, *fimC* and *fimD* is demonstrated by these *hif* genes. The most interesting aspect of this report was the inference that the entire cluster represents an ancestral mobile genetic element and was originally inserted via a transposition event. This was suggested because, in the regions flanking the cluster, was made the discovery of repetitive extragenic palindromic (REP) sequences and the remnants of a duplicated section of the unrelated *pur* operon. These two genetic phenomena taken together are indicative of the occurrence of transposition. Using this as a hypothesis, we can imply that the ancestral *Bordetella*, *Haemophilus*, and *Klebsiella* species may have obtained fimbrial genes from one another.

The predicted amino acid sequence of FimB is homologous to the PapD superfamily of periplasmic chaperones (Locht *et al.*, 1992; Willems *et al.*, 1992). A putative signal peptide sequence probably directs the newly

translated FimB to the periplasm, where the signal sequence is cleaved, leaving a mature polypeptide of approximately 24 kDa (Locht *et al.*, 1992; Willems *et al.*, 1992). Southern hybridisation of *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* disclosed regions homologous to *fimB* (Willems *et al.*, 1992) in these closely related species, suggesting that all three may utilise a similar process to escort fimbrial proteins to the cell surface.

FimC is a protein with predicted homology to many outer membrane fimbrial accessory proteins, including PapC, MrkC and HifC all of which are essential for the biogenesis of fimbriae in their respective organisms. The molecular weight of this predicted protein is approximately 91 kDa (after signal peptide cleavage) (Locht *et al.*, 1992; Willems *et al.*, 1992). Based on homology, it was proposed that FimC is probably located in the outer membrane, involved in fimbrial transportation and anchorage (Locht *et al.*, 1992; Willems *et al.*, 1992).

Much of the more recent research into the fimbriae of *B. pertussis* has concentrated on the minor fimbrial subunit and primary adhesin, FimD. Lying directly upstream of *fimC*, the *fimD* gene encodes a fimbrial adhesin protein with a molecular weight of approximately 40 kDa (Willems *et al.*, 1993). Like its genomic neighbours, *fimD* exhibits homology to genes in the fimbrial clusters of *H. influenzae* (*hifE*), *K. pneumoniae* (*mrkD*) and *E. coli* (*fimH*) (Klemm and Christiansen, 1987; Gerlach *et al.*, 1989; Loch *et al.*, 1992; Willems *et al.*, 1992; van Ham *et al.*, 1994). A *fimD* homologue is also present in *B. parapertussis* and *B. bronchiseptica* (Willems *et al.*, 1993). In fact, the corresponding gene in *B. bronchiseptica* only differs by a single, albeit non-silent base pair change. There are 20 different amino acids in the FimD polypeptide of *B.*

parapertussis resulting from 34 base pair differences.

This sequence data is evidence that FimD is not involved in *Bordetella* host specificity and that the similar FimD adhesins of *B. pertussis* and *B. bronchiseptica* may interact with receptors which are highly conserved between mammals and humans. The differences present in the FimD adhesins of *B. pertussis* and *B. parapertussis* may create an evolutionary advantage for both by eliminating binding site competition while sharing the same host.

Binding studies involving fimbrial mutant strains lacking FimD have demonstrated that *Bordetella pertussis* binds to human monocytes via FimD (Hazenbos *et al.*, 1995a). Known to interact with *B. pertussis* fimbriae, VLA-5 has since been confirmed as the FimD receptor on the surface of monocytes (Hazenbos *et al.*, 1995b). The construction of what the authors call “true” fimbrial mutants, devoid of both major and minor fimbrial subunits allowed further investigation (Geuijen *et al.*, 1997). A mouse colonisation procedure was utilised to show that FimD plays a vital role in colonisation. The Fim2⁻/3⁻; FimD⁻ mutant colonised the lungs and trachea of mice significantly less efficiently than wild type, FHA⁻ mutant, and Fim2⁻/3⁻; FimD⁺ mutant strains (Geuijen *et al.*, 1997). These researchers also demonstrated the binding of FimD to the sulfated sugar heparin, also a possible target molecule for FHA and major fimbrial subunits (Menozzi *et al.*, 1991; Menozzi *et al.*, 1994; Geuijen *et al.*, 1996).

Genetic Regulation

Like filamentous haemagglutinin, fimbrial expression in *B. pertussis* is regulated at a number of levels. A virulence factor employed to enable the bacteria to attach to host cells, the expression of fimbriae is controlled

by the *bvg* locus. The correct processing and secretion of fimbrial subunits however, requires the expression of a number of accessory genes, which are located in the fimbrial operon (Figure 1.11). *B. pertussis* produce two distinct fimbriae: serotype 2 and serotype 3. Some strains (serotype 2,3) express both simultaneously (Ashworth *et al.*, 1982). The serotype expressed depends on the level of transcription of fimbrial major subunit genes. Alteration of serotype involves switching promoter activation between the two fimbrial subunit genes in a process termed fimbrial phase variation (Willems *et al.*, 1990). The genes encoding the major fimbrial subunits, *fim2* and *fim3* with which the fimbriae are constructed, have been fully characterised (Livey *et al.*, 1987; Mooi *et al.*, 1990) as have the homologous but transcriptionally inactive *fimX* and *fimA* genes (Pedroni *et al.*, 1988; Loch *et al.*, 1992; Willems *et al.*, 1992). Sequence data has revealed a high level of homology between all of these genes which extends into the promoter regions, unveiling some interesting features of fimbrial promoter architecture (Figure 1.12).

The most noticeable aspect of the fimbrial promoters is the "C stretch", a long run of cytosine residues which has been implicated in fimbrial phase variation (Willems *et al.*, 1990). Insertion or deletion of extra cytosine residues in this C stretch is the source of fimbrial phase variation (Willems *et al.*, 1990). It is thought that the distance between the putative -10 box and the activator (BvgA) binding site is vital for transcriptional activation, and the alteration in the number of C's in the stretch changes this distance (Willems *et al.*, 1990). The reason for the lack of transcription from the *fimX* pseudogene may be the short C stretch in the promoter region.

CTG**TTTCCC**ACATCGGAATCAGCCCTCCCCCCCCCCCCCTAAGACCT**TAAGAT**CGTGGCTCCATAA *fim2* (W28)

CTG**TTTCCC**ACATCGGAATCAGCCCCCCCCCCCCCCCCCTAAGACCT**TAAGAT**CGTGGCTCCATAA *fim2* (BPSA1)

CAA**ATTCCC**ACACAACCATCAGCCCTCCCCCCCCCCC-GGACCTGAT**TATTCT**GATGCCGACGCCA *fim3* (W28)

CAA**ATTCCC**ACACAACCATCAG----CCCCCCCCCCC-GGACCTGAT**TATTCT**GATGCCGACGCCA *fim3* (BPSA1)

CAA**ATTCCT**ACACATCCATCAGCCCCCCC-----GAGGCG**TCTAATA**ATCTTGCACACA *fimX* (Tohama)

CAA**ATTCCT**ACACATCCATCAGCCCCCCC-----GAGGCG**TCTAATA**ATCTTGCACACA *fimX* (BPSA1)

Figure 1.12 Promoter regions of the three fimbrial genes found in *B. pertussis*. The -10 boxes and putative activator (BvgA) binding sites are indicated in bold. Dashes have been introduced to increase homology. Differences between strains in the equivalent promoter regions are underlined. The strain from which each sequence was derived is named in parentheses. Sequence of *fim2* (Wellcome 28 strain) is from Livey *et al.* (1987). Modified from Willems *et al.*, 1990 and Riboli *et al.*, 1991.

The expression of the minor subunit genes, or accessory genes is also required for the correct delivery and presentation of *B. pertussis* fimbriae at the cell surface (Locht *et al.*, 1992; Willems *et al.*, 1992). A mutational analysis of the *fim/fha* gene cluster has provided evidence that *fimC*, *fimD*, and *fhaC* are translationally linked (Willems *et al.*, 1993). These three genes overlap slightly and there is a putative ribosome binding, Shine Dalgarno site directly ahead of the first gene of the triplet, *fimC*. Further evidence to support this is the hairpin loop structure formed by the mRNA just prior to the end of the *fimC* gene (Willems *et al.*, 1993). The translation of *fimD* may require the complete translation of *fimC*, thereby preventing the construction of the hairpin loop and permitting continuous ribosomal attachment and translation of *fimD* (Willems *et al.*, 1993).

1.3.7 Pertactin

Also known by the aliases p.69 and OMP 69 because of its presumed molecular weight from electrophoretic mobility in SDS PAGE, pertactin is actually a 60 kDa outer membrane protein involved in bacterial adherence (Makoff *et al.*, 1990; Leininger *et al.*, 1991). Similar molecules are produced by other members of the genus, p.70 in *B. parapertussis* (Li *et al.*, 1990) and p.68 in *B. bronchiseptica* (Montaraz *et al.*, 1985).

The crystal structure of pertactin has been elucidated, revealing a 16 stranded parallel β -helix with a V-shaped cross section (Emsley *et al.*, 1996). The secondary structure of pertactin contains two immunodominant direct repeat regions. The first, (Gly.Gly.Xaa.Xaa.Pro)₅ is located directly after the important RGD tripeptide and the other, (Pro.Gln.Pro)₅ thought to be the major immunoprotective epitope, lies toward the C-terminal end (Charles *et al.*, 1988; Charles *et al.*, 1991).

Pertactin is transcribed from the *prn* gene, which encodes a 93.5 kDa polypeptide comprising 910 amino acids (Charles *et al.*, 1988). This precursor, termed p.93, later undergoes the removal of a 34 amino acid N-terminal signal peptide (Makoff *et al.*, 1990) and cleavage of a 30 kDa polypeptide (p.30) from the C-terminus. Although the precise role of p.30 is unclear, it is detected in outer membrane fractions and is likely to be involved in the export of pertactin to the outer membrane (Charles *et al.*, 1994). In a comparison of the *prn* gene sequences of *B. pertussis*, *B. parapertussis* and *B. bronchiseptica*, the precursors of the pertactins were found to be extremely homologous and interestingly, the most highly conserved region is the C-terminal, suggesting the importance of p.30 to these organisms (Li *et al.*, 1992).

The mechanism by which pertactin promotes adherence to eukaryotic cells is unknown and no receptor has been found. The amino acid sequence revealed an arginine-glycine-aspartic acid (RGD; 225-227) motif, a known integrin binding moiety present in many bacterial adhesins such as FHA, and eukaryotic extracellular matrix (ECM) proteins including fibronectin and vitronectin (Relman *et al.*, 1990; Hynes, 1987). It is easy to assume that this region facilitates binding to mammalian cells. Mutants deficient in pertactin expression adhered 30-40% less well to CHO (Chinese hamster ovary) cells and HeLa cells (Leininger *et al.*, 1991). Synthetic peptides containing RGD derived from pertactin were found to inhibit pertactin binding to epithelial cells and reduce *B. pertussis* entry into HeLa cells (Leininger *et al.*, 1991; Leininger *et al.*, 1992). However, a recent study has found no role for pertactin as a mediator of invasion (Everest *et al.*, 1996). These researchers used site-directed mutagenesis (aspartic acid to glutamic acid) to obtain a pertactin molecule with an RGE site rather than RGD. Strains expressing the mutation displayed no difference in their ability to promote adhesion to HEp-2 or CHO cells. Most of the studies performed on this intriguing protein have used either heterologous bacterial species or mutated *B. pertussis* strains in the so far fruitless search for its role in the pathogenesis of disease. It would be advantageous that the role of pertactin in wild type *B. pertussis* be determined, leading to a greater understanding of whooping cough and the vaccines which prevent it.

Expression of pertactin in wild type *B. pertussis* is very low, hence production to high levels for use in vaccines is currently a goal of many laboratories. Many strategies have been used to achieve this aim including expression in *E. coli* (Makoff *et al.*, 1990); in *Salmonella typhimurium* *aro* mutants (Strugnell *et al.*, 1992); in the industrial yeast

Pichia pastoris (Romanos *et al.*, 1991); in recombinant *B. pertussis* strains (Loosmore *et al.*, 1995); and in a baculovirus-based expression system (Charles *et al.*, 1993).

It has been demonstrated by a number of groups that pertactin is an immunoprotective antigen, being used in subunit and live oral vaccines to protect mice from respiratory challenge with virulent *B. pertussis* (Shahin *et al.*, 1990; Romanos *et al.*, 1991; Novotny *et al.*, 1991; Strugnell *et al.*, 1992; Roberts *et al.*, 1993). Many human vaccine trials suggest the inclusion of pertactin in future preparations is imperative. Although the role of pertactin in vaccine based immunoprotection is unknown, preparations including pertactin have attained promising vaccine efficacy results (Marwick, 1996; Poland, 1996). Recently, the Dutch whole cell vaccine has come under fire as the incidence of whooping cough rose in The Netherlands despite high immunisation rates. The cause was traced to mutations in the *B. pertussis* strains circulating in the community. Mutations in the genes encoding pertussis toxin and pertactin were discovered, indicating the importance of these proteins in eliciting protective immunity (de Melker *et al.*, 1997; Mooi *et al.*, 1998).

1.3.8 Lipopolysaccharide

The lipopolysaccharide (LPS) molecule of *Bordetella pertussis* is smaller than many other bacterial LPS structures and is therefore often referred to as a lipooligosaccharide (LOS). The role which LPS plays in the pathogenesis of *B. pertussis* infection is unclear, however biological activities exhibited by *B. pertussis* LPS include antigenic and immunomodulating properties (Amano *et al.*, 1990; Watanabe *et al.*, 1990). The most common action associated with LPS is the potent

endotoxin activity (Chaby and Caroff, 1988).

Present in most Gram negative bacteria, LPS generally comprises lipid A, core oligosaccharide and a long polysaccharide O antigen. The structure of LPS molecules expressed at the surface of *Bordetella* species varies greatly. The structure of *B. pertussis* LPS deviates from the norm in that it lacks the O antigen. *Bordetella pertussis* actually produces two types of LPS, designated A and B (Peppler, 1984). These molecules possess differing electrophoretic mobilities due to the presence of an extra trisaccharide moiety on LPS-A (Caroff *et al.*, 1990). All of the species of *Bordetella* express different LPS molecules, which may be a factor in the high level of species specificity demonstrated within this Genus (van den Akker, 1998). *Bordetella parapertussis* strains isolated from humans and sheep display distinct LPS profiles (van den Akker, 1998) adding to the host specific features of this species (Porter *et al.*, 1995; van der Zee *et al.*, 1996). The LPS produced by *B. bronchiseptica* is similar to that of *B. parapertussis*, as they both express temperature dependent O antigen (Di Fabio *et al.*, 1992; van den Akker, 1998). The charged O antigen of *B. bronchiseptica* is thought to be the element which confers protection against antimicrobial peptides since *B. pertussis*, which lacks O antigen, is not protected (Baneman *et al.*, 1998). *B. bronchiseptica* also produces host specific LPS molecules, with isolates from dogs having more heterogeneous LPS structure than isolates from pigs (van den Akker, 1998). A human isolate of *B. bronchiseptica* displays a different LPS profile than that of a rabbit isolate (Le Blay *et al.*, 1997; Gueirard *et al.*, 1998). Subsequent isolation of *B. bronchiseptica* from the same patient over two years has shown a variation in LPS profiles during the course of infection (Gueirard *et al.*, 1998).

Transposon mutagenesis has allowed the identification of a huge gene cluster encoding LPS biosynthesis in *B. pertussis* (Allen and Maskell, 1996). Many of these genes are similar to polysaccharide and LPS synthesis genes present in other bacteria, however the *B. pertussis* LPS operon is arranged in a unique manner (Allen and Maskell, 1996).

Unfortunately, our knowledge of the *Bordetellae* LPS remains limited and further research is necessary before we are able to define the involvement of this molecule in both the pathogenesis of diseases caused by this Genus, and the immunogenicity and adjuvanticity of vaccines targeted against those diseases.

1.3.9 Tracheal Colonisation Factor

Originally discovered with the aid of a transposon mutated strain which was deficient in four outer membrane proteins (Finn *et al.*, 1991), tracheal colonisation factor is a *bvg*-regulated protein, exclusively produced among the *Bordetellae* by *B. pertussis*. The gene encoding tracheal colonisation factor, *tcfA* was first mapped using a *TnphoA* insertional mutation and was termed virulence activated gene #34 (*vag* 34) (Finn *et al.*, 1991). The original *TnphoA* mutants were ten fold less able to colonise and persist in the trachea of mice, but were not significantly disadvantaged in the lungs (Finn and Stevens, 1995). The *tcfA* gene has now been cloned and sequenced, with the derived amino acid sequence predicting a 68.6 kDa precursor, the first 39 amino acids of which comprise a likely signal peptide, leaving a 64.4 kDa protein (Finn and Stevens, 1995). The C-terminal half of tracheal colonisation factor exhibits over 50% homology to the C-terminal of the precursors of the pertactins of *Bordetella* species, including the Lys-Arg putative proteolytic cleavage

site. Another *Bordetella* protein, the serum resistance factor BrkA also possesses a C terminal with significant homology to that of tracheal colonisation factor (Fernandez and Weiss, 1994). The N-terminal half of tracheal colonisation factor contains 3 RGD motifs and has a surprisingly high 16.5% proline content, which may affect migration in SDS PAGE gels, perhaps explaining the discrepancy between the predicted 68.6 kDa protein and the presumed ~90 kDa seen in PAGE gels (Finn and Stevens, 1995). This demonstrates another similarity to pertactin and the authors have hypothesised that the maturation processes between the two proteins may also share common features. Thus, their model of tracheal colonisation factor secretion involves the precursor molecule (68.6 kDa) being transported to the periplasm after the cleavage of the typical signal peptide. The remaining 64.4 kDa precursor is then translocated across the outer membrane, like pertactin, assisted by its own C-terminal region. Cleavage at the Lys-Arg proteolysis site then occurs, liberating the mature 34 kDa tracheal colonisation factor protein. As a consequence of the limited research literature on tracheal colonisation factor, the elucidation of the exact role played by this protein in the pathogenesis of pertussis will require subsequent scientific scrutiny.

1.4 Whooping Cough Vaccines: Past Indiscretions, Recent Trials and Future Directions

Humankind has eternally struggled with the blight of pathogenic microorganisms. The most successful prophylactic treatment against disease to date has been immunisation. The complete eradication of smallpox was achieved using an extensive worldwide vaccination program and many other diseases have been controlled by immunisation,

including diphtheria, tetanus, measles, polio, tuberculosis and whooping cough. Recent data suggests that many of these diseases are reemerging. As expected, developing countries have high incidences of these and many other preventable diseases because the costs of immunisation are prohibitive. Whooping cough has returned to the fore in many western countries recently. Interestingly, this is not because of the cost or the accessibility of vaccination, but mainly due to a trend in public opinion that has led to a decrease in immunisation rates.

1.4.1 Whole Cell Vaccines

When *Bordetella pertussis* was identified as the microbe which caused whooping cough (Bordet and Gengou, 1906) vaccination against this scourge became an immediate priority (Evans and Maitland, 1937). Killed whole *B. pertussis* cells were found to be protective in a number of studies and whole cell preparations were manufactured for human use quite early this century (1914 in the USA and 1920 in Australia). These vaccines were crude, and efficacy and reactogenicity testing was not very sophisticated so it was not until the 1940's and 1950's that whole cell pertussis vaccines were routinely used in government immunisation programs in these and other countries.

With the advent of such vaccination regimes, the incidence of whooping cough in western countries decreased dramatically. With a vaccine efficacy consistently quoted at between 80% and 100%, and immunisation rates upwards of 80% of the population, the disease was virtually wiped out in many countries. This situation remained unchanged until probably the 1970's when medical practitioners and the public (particularly in the United Kingdom) became aware of allegations about

the vaccine causing brain damage. Vaccine coverage in the UK dropped almost instantly from 80% to below 30% and whooping cough resumed the epidemic proportions of pre-immunisation days. Unfortunately this unconfirmed, media driven hysteria slowly filtered out to the rest of the western world, reducing worldwide public acceptance of whole cell pertussis vaccines and causing an increase in the incidence of whooping cough.

Side effects

The mild side effects known to be caused by pertussis immunisation can be categorised into two classes. Local side effects occur at the site of injection and include erythema, tenderness and swelling. This occurs in up to 50% of vaccinees (Cherry *et al.*, 1988). Systemic reactions such as fever, vomiting, diarrhoea and general irritability are also very common (Cherry *et al.*, 1988).

More severe side effects including febrile seizures, atypical high-pitched crying and persistent screaming occur far less frequently at ~3% (Cody *et al.*, 1981; Feery *et al.*, 1985). Convulsions and hypotonic-hyporesponsive episodes have also been associated with pertussis vaccination but only occur in approximately 0.06% of vaccinees (Harris *et al.*, 1995).

Contrary to popular folklore, whole cell pertussis vaccines do not cause infantile spasms (Cherry *et al.*, 1989), sudden infant death syndrome (Beal, 1990) or epilepsy (Howson and Fineberg, 1992). Another unfortunate side effect attributed to these preparations is the alleged association with encephalopathy and death. These "symptoms" occur so rarely that it is very difficult to prove that either can be connected to the vaccine. This has been the topic of many law suits in the USA and in the UK (reviewed

in Griffith, 1989). The extreme rarity of these events will ensure that this conundrum is never fully resolved one way or the other.

The current Australian pertussis vaccine is an aluminium phosphate adsorbed whole cell preparation manufactured by CSL Ltd. This highly efficacious preparation is usually administered as a component of the DTP "Triple Antigen®" intramuscular injection given to infants at 2, 4, 6 and 18 months of age as recommended by the National Health and Medical Research Council. This vaccine can cause mild to moderate side effects, documented by Feery (1985) as local reaction at the site of injection (~50%), fever (~20%) and irritable crying (30%). This benchmark Australian study has since been confirmed and updated in a study with 531 immunisations (Harris *et al.*, 1995) which documented just a single hypotonic/hyporesponsive episode and no convulsions or other major side effects.

1.4.2 Acellular Vaccines

The decline in public confidence and usage of the highly efficacious but inherently reactogenic whole cell pertussis vaccines, made it imperative to find a more "user friendly" product which still retained the protective qualities of the traditional vaccines. Japan was the first country to create acellular vaccines and introduced them with success, into their immunisation program in 1981 (Sato *et al.*, 1984). These original component vaccines contained formalin detoxified purified pertussis toxin and demonstrated a decrease in side effects. The Swedish efficacy trial (Ad Hoc Group, 1988) tested the protection conferred by two Japanese vaccines. The two component (pertussis toxoid and filamentous haemagglutinin) vaccine induced a greater level of protection against

culture confirmed pertussis than the single component (toxoid only) vaccine (Storsaeter *et al.*, 1990) demonstrating the advantage of multicomponent vaccine preparations.

1.4.3 The Role of Cell Mediated Immunity

One of the major hindrances in developing superior vaccines, is the limited understanding of the mechanisms involved in either natural infection based immunity, or that conferred by vaccination. Which components of the human immune system become primed to fight *B. pertussis* infection after vaccination? The response elicited by the immune system can be divided into two broad sections, cell mediated (Th1) and humoral (Th2) responses, based upon cytokine release and cell types activated. Defence against intracellular pathogens is usually dependent on the activation of a cell mediated Th1 response. *B. pertussis* has been shown to invade an increasing number of cell types (Ewanovich *et al.*, 1989; Bromberg *et al.*, 1991; Saukkonen *et al.*, 1991) and is now considered a facultative intracellular pathogen. Intracellular bacteria may be protected from host clearance mechanisms resulting in the infection persisting. It is therefore astute to assume that cell mediated immunity (CMI) may play some role in eliminating *B. pertussis* infection, at the very least by destroying bacteria surviving within phagocytes. Recent studies have demonstrated the importance of CMI in natural and vaccine based immunity (Petersen *et al.*, 1992; Redhead *et al.*, 1993). Immunisation of mice with whole cell vaccine or recovery from natural *B. pertussis* infection induces a Th1 response, with the activation of CD4⁺ T lymphocytes secreting interleukin-2 (IL-2) and interferon gamma (IFN- γ), whereas immunisation with an acellular vaccine elicits a Th2 response, with the production of antibodies, and the secretion of IL-5 (Redhead *et*

al., 1993). Further illustrating the important role of cytokines, secretion of IL-12 by macrophages stimulated by infection or whole cell vaccination, in part due to the presence of lipopolysaccharide, is also involved in the induction of the Th1 response (Mahon *et al.*, 1996). This production of IL-12 does not occur after acellular vaccination, however when exogenous IL-12 is included as an adjuvant with a component vaccine in the murine respiratory challenge model, the Th2 response switches to a Th1 response and raises the protective efficacy and level of bacterial clearance to that of whole cell vaccine (Mahon *et al.*, 1996). It has also been demonstrated that administration of exogenous IL-12 and IFN- γ can reduce infection by *B. pertussis* by augmenting a Th1 response to promote intracellular killing by activated macrophages and destroy *B. pertussis* infected phagocytes (Torre *et al.*, 1996).

The Th2 response elicited after immunisation with an acellular vaccine, results in delayed bacterial clearance in the murine respiratory challenge model. In contrast, natural infection or immunisation with killed whole cells, inducing a Th1 response, was associated with rapid clearance (Redhead *et al.*, 1993; Barnard *et al.*, 1996). Similar studies in humans have shown that a mixed Th1/Th2 response is generated (Ryan *et al.*, 1997). Independent studies analysing the CMI responses of children in the Mainz and Rome vaccine efficacy trials, revealed that acellular vaccines induce Th1 responses (Zepp *et al.*, 1996; Ausiello *et al.*, 1997) which are equivalent to responses after natural infection (Zepp *et al.*, 1997).

The differences seen in CMI responses to vaccination of mice and humans remains the topic of much debate, and further study is required to resolve this paradox. The importance of immunisation route and genetic background of the mice in priming the desired cellular immune

response has been highlighted (Barnard *et al.*, 1996), and these may, in part, explain the differences between the human and murine responses.

The efficacy of whole cell pertussis vaccines has traditionally been evaluated using the Kendrick murine intracerebral challenge test (Kendrick, 1947). This does not mimic the natural infection process at all, but is an effective measure of the protective capability of a whole cell vaccine. Acellular vaccines cannot be tested using this method and unfortunately vaccine trials have shown no correlation between antibody responses and vaccine efficacy (Greco *et al.*, 1996; Gustafsson *et al.*, 1996; Simondon *et al.*, 1997).

Recent research to emerge from studies into the mechanisms of protective immunity is the evidence that the murine respiratory challenge test is an excellent model for acellular and whole cell vaccine efficacy in humans, and that humoral and cell mediated immunity play complementary roles in vaccine elicited protection (Mills *et al.*, 1998). This extensive work, using samples from recent field trials in Italy (Greco *et al.*, 1996) and Sweden (Gustafsson *et al.*, 1996) has established a highly significant correlation between protection in the murine respiratory challenge model and vaccine efficacy in infants. Using knockout mice deficient in different aspects of the immune system, Mills and associates found that both humoral and cell mediated responses are involved in vaccine based protective immunity. The initial response may be antibody mediated and is involved in restricting the extent of infection and minimising the damage to epithelial and immune cells. Antibodies directed against the various adhesins and toxin molecules produced by the bacteria would be most effective at this stage. To achieve complete bacterial clearance however, also requires the induction of a cell mediated

immune response. This two-phase process is consistent with the finding that antibodies directed against *B. pertussis* antigens in children immunised with acellular or whole cell vaccines waned dramatically after immunisation (Zepp *et al.*, 1996; Cassone *et al.*, 1996). The CMI responses in these children were much longer lasting and in some cases became more intense, even after antibody titres had fallen to below detectable limits (Zepp *et al.*, 1996; Cassone *et al.*, 1996). An unusual finding to arise from the latter study was the tentative synergistic link between humoral and cell mediated immunity. Subjects demonstrating positive CMI induction exhibited higher antibody titres than those which failed to elicit a CMI response. This is unusual in that it belies the dichotomy of the human immune response, however it may simply be characteristic of a mixed Th1/Th2 response, or that some children did not elicit good responses, whether humoral or cell mediated.

1.4.4 Recent Vaccine Trials

The potential shown by the vaccines in Japan and Sweden spurred a number of vaccine companies in the US and Europe to establish research programs aimed at the development of "new generation" vaccines. The last decade has seen a number of candidate vaccines trialed with quite varied results (Table 1.1). However the trend lies clearly toward acellular vaccines, containing at least three components, pertussis toxoid, FHA and pertactin and possibly also fimbriae. Although trials have demonstrated that acellular vaccines are less reactogenic than whole cell vaccines, the complete elimination of adverse reactions seems impossible.

With the advent of large scale trials came a need to uniformly define pertussis infection. A special WHO meeting (WHO, 1991) developed a

standard pertussis case definition consisting of culture or serologic confirmation of *B. pertussis* infection or household contact with positive individuals, plus 21 days or more of paroxysmal coughing. This definition has been used in most of the trials and is a good indicator of moderate to severe pertussis but does not include milder (culture confirmed) infection of less than 21 days. For this reason, many investigators also cite protection data with respect to mild pertussis with cough for greater than 7 days.

In the Göteborg, Sweden pertussis vaccine study, infants were given either an Amvax monocomponent vaccine containing 40 µg of PT along with diphtheria and tetanus toxoids (DT) (n=1670), or a DT only vaccine (n=1665) at 3, 5 and 12 months of age (Trollfors *et al.*, 1995). The protective efficacy conferred against WHO case definition pertussis was calculated as 71%. Protection against milder pertussis however was significantly lower at only 53%. The method of PT detoxification in this preparation was hydrogen peroxide treatment.

A large household contact trial was performed in Mainz, Germany with a total of 22 503 infants receiving either a three component SmithKline Beecham (SKB-3) vaccine, a German licensed whole cell vaccine (Behring), or a DT control vaccine (Schmitt *et al.*, 1996). The immunisation schedule was three doses at 3, 4 and 5 months. The efficacy of the acellular SKB-3 and whole cell vaccines against WHO case defined pertussis was 89% and 97% respectively.

Concurrently run double blind trials in Stockholm and Rome used equivalent immunisation schedules (2, 4 and 6 months), the same Connaught whole cell vaccine, and DT controls. The Italian trial (Greco *et*

al., 1996) compared the SKB-3 vaccine (n=4 481) used in the trial in Mainz, with a three component vaccine produced by Chiron-Biocrine (n=4 452). The latter preparation contains a genetically detoxified form of PT (rPT) which remains in native form (as distinct from chemically treated PT) allowing for a reduced dose. The acellular vaccines demonstrated equal efficacy against WHO defined pertussis, 84% and against milder pertussis, 71%. The efficacy of the SKB-3 vaccine was similar to that observed in the Mainz trial. The 4 348 vaccinees given the whole cell vaccine showed a remarkably high incidence of pertussis. The US licensed Connaught vaccine afforded only 36% efficacy against typical pertussis and only 23% against mild disease (Greco *et al.*, 1996). This contrasts markedly with the Behring vaccine used in the Mainz trial.

The same Connaught whole cell vaccine (n=2 001) was used in the Swedish trial (Gustafsson *et al.*, 1996), again exhibiting surprisingly low efficacy, 48% and 41% for typical and mild pertussis respectively. A two component SmithKline Beecham vaccine (SKB-2), administered to 2 538 subjects was 59% protective against WHO defined pertussis and 42% protective against milder pertussis. A five component vaccine produced by Connaught given to 2 551 infants was 85% and 78% protective against WHO defined and mild pertussis respectively.

A case-control study performed in Munich, Germany (Liese *et al.*, 1997) compared a two component Connaught/Biken vaccine (n=12 710) with the German licensed Behring whole cell vaccine (n=3 200) and DT controls (n=2 100). A total of four doses were administered, at 2, 4 and 6 months, with a booster at 15-25 months. This study defined a pertussis case as any cough of 21 or more days plus a positive culture or household contact. The acellular and whole cell vaccines achieved efficacies of 82%

and 96% respectively.

Table 1.1 Contents of, and efficacy induced by vaccines in recent trials.

Site	Vaccine *	Contents (µg/dose)	Efficacy #
Göteborg	Amvax (1)	PT(40)	71
Mainz	SmithKline Beecham (3)	PT(25); FHA(25); PRN(8)	89
	Behring	whole cell	97
Stockholm	SmithKline Beecham (2)	PT(25); FHA(25)	59
	Connaught-Canada (5)	PT(10); FHA(5); PRN(3); FIM(5)	85
	Connaught-Canada	whole cell	48
Rome	SmithKline Beecham (3)	PT(25); FHA(25); PRN(8)	84
	Chiron Biocine (3)	PT(5); FHA(2.5); PRN(2.5)	85
	Connaught-Canada	whole cell	36
Munich	Connaught US/Biken (2)	PT(23.4); FHA(23.4)	82
	Behring	whole cell	96
Senegal	Pasteur Merieux (2)	PT(25); FHA(25)	85
	Pasteur Merieux	whole cell	96
Erlangen	Wyeth-Lederle/Takeda (4)	PT(3.5); FHA(35); PRN(2); FIM(0.8)	82
	Lederle	whole cell	91
Sweden	Chiron Biocine (3)	PT(5); FHA(2.5); PRN(2.5)	1.38
	Past Mer-Conn (5)	PT(20); FHA(20); PRN(3); FIM(5)	0.85
	Evans (Wellcome)	whole cell	1.0

* Bracketed numbers represent number of vaccine components

Efficacy values are measured as percentages, except for the final row. These are instead measured as relative risk of protection against pertussis in comparison to the whole cell vaccine, which was assigned a risk of 1.0.

The Senegal pertussis trial compared a two component acellular (n=1 847) and a whole cell vaccine (n=1 772), both produced by Pasteur Merieux (Simondon *et al.*, 1997). A three dose schedule was adopted, with immunisations at 2, 4 and 6 months. The absolute efficacy of these preparations were estimated at 85% and 96% respectively using the WHO case definition. The protection afforded by both vaccines dropped markedly to 31% and 55% against the milder protocol case definition adopted in this study.

The trial in Erlangen, Germany compared a four component vaccine from Lederle-Praxis/Takeda to a Lederle whole cell vaccine and a DT control (Stehr *et al.*, 1998). Subjects were immunised with either the acellular vaccine (n=4 273) or the whole cell vaccine (n=4 259) at 3, 4.5, 6 and 15-18 months of age, or the DT only combination (n=1 739) at 3, 4.5 and 15-18 months. Specific efficacy against a modified WHO definition of 21+ days of cough with paroxysms, whoop or posttussive vomiting was 83% for the acellular vaccine and 93% for the whole cell vaccine. Against mild pertussis, protection was 72% and 83% respectively. The acellular vaccine also exhibited moderate efficacy (31%) against *B. parapertussis* infection.

In a huge, nationwide trial in Sweden (Olin *et al.*, 1997) over 80 000 infants were immunised in a randomised trial comparing the two component SmithKline Beecham DTP vaccine (n=20 465), the three component Chiron Biocine DTP (n=20 412), the Pasteur-Merieux-Connaught five component DTP (n=20 491), and a UK licensed whole cell vaccine from Evans Medical (n=20 467). Apart from the unusually low efficacy demonstrated by the whole cell vaccine in two recent trials (Greco *et al.*, 1996; Gustafsson *et al.*, 1996), preliminary results of this latest Swedish study are consistent with data from previous trials. Specific efficacy analyses were not performed, however case numbers and relative risk (DTwP assigned to 1.0) calculations are available. They demonstrate that the five component acellular vaccine (relative risk to DTwP: 0.85) elicited the greatest protection against WHO defined pertussis, followed closely by the whole cell vaccine (1.0) with the three component acellular vaccine, with a relative risk of 1.38, affording less protection. Against mild disease, the relative risk calculations were 1.0, 1.40 and 2.55 for the whole cell, five component and three component vaccines respectively. The

further analysis of the outcomes of this trial should confirm and extend our knowledge of vaccines against pertussis.

In a study which assessed adverse events within 72 hours after vaccination with either whole cell DTP vaccine or two different acellular vaccines (Connaught and Lederle), the acellular vaccines were approximately three times less reactogenic (Rosenthal *et al.*, 1996). However, apart from the Connaught whole cell vaccine, the excellent efficacy, reduced occurrence of severe side effects and long lasting protection reported after immunisation with whole cell vaccines reaffirm that they still constitute a valid alternative to acellular formulations.

1.4.5 Future Vaccine Development

With all the time spent on research into mechanisms of immunity, efficacy and reactogenicity of vaccines against *Bordetella pertussis*, the bacterium itself has evolved and reemerged. Recent data from the Netherlands reported a dramatic increase in the incidence of pertussis in that country in 1996, despite high levels of immunisation (de Melker *et al.*, 1997). Many factors might have explained this epidemic, however an extensive study has traced the problem to *B. pertussis* itself (Mooi *et al.*, 1998). The population structure of this bacterium in the Netherlands has changed. The antigenic variation of the protective antigens pertactin and the S1 subunit of pertussis toxin was analysed using circulating clinical isolates collected over a period of 47 years. Three different variants each of pertactin and S1 were discovered and the presence of these variants show temporal shifts. Polymorphism in pertactin was found to occur in the midst of a repeat region (GlyGlyXaaXaaPro)₅ termed region 1. The mutations in the S1 subunit were similarly confined to a small region of

the polypeptide. The mutations in S1 and pertactin are concentrated in regions defined as T cell and B cell epitopes respectively (Peppoloni *et al.*, 1995; Charles *et al.*, 1991).

The Dutch whole cell pertussis vaccine introduced in the 1950's was originally a clinical isolate and therefore matched the circulating population, and was effective in preventing whooping cough. The ancestral pertactin molecule (A) remained unchanged until 1981, when both of the other variants (B and C) emerged. These mutated forms circulated in the population at low levels until 1989. Fluctuations in the population structure occurred until the present situation was reached, with C being the dominant polymorph and A, the form contained within the whole cell vaccine, only representing 10% of the population. A similar temporal shift was seen in the S1 variants with 90% of the present isolates containing a different S1 molecule to that produced by the whole cell vaccine strain. The temporal trends in both the pertactin and S1 polymorphs, clearly suggest that *Bordetella pertussis* is under selective pressure caused by human immunisation, and is undergoing vaccine driven evolution. There are major implications of this study, concerned with the whole cell vaccines currently in use worldwide. This new evidence may also explain in part, the poor performance of the US Connaught licensed whole cell vaccines in recent trials (Greco *et al.*, 1996; Gustafsson *et al.*, 1996). The *B. pertussis* strain used in this Connaught vaccine may be divergent enough from the strains circulating in Sweden and Italy that they do not confer high levels of protection. The impact on acellular vaccines may be even greater because by their very nature they confer a narrower field of protection. In fact some licensed acellular vaccines contain variants which might only protect 10% of the Dutch population.

This evidence reinforces the fact that this organism has probably infected humans for thousands of years and is exquisitely fine tuned to inhabit the human respiratory tract. This was achieved via an evolutionary process. The twentieth century had caught up with this pathogen somewhat, with the advent of antimicrobials and immunisation, and it was on the way out. Now it is again evolving and reemerging as a dangerous pathogen.

Together with the attention to vaccine reactogenicity, the induction of appropriate immune response mechanisms and raising the protective efficacy of future anti-pertussis vaccines, we must also consider the rapid evolution of *Bordetella pertussis*, which we are actually facilitating, in the development of future whooping cough vaccines.

Chapter Two Materials and Methods

2.1 Bacterial strains, plasmids and media

Recipes for all media and buffers used in this study are listed in Appendix II. Bacterial strains used in this study are listed in Table 2.1. *Escherichia coli* strains were grown on Z agar (Walker and Pemberton, 1988) or in LB liquid media (Sambrook *et al.*, 1989). *Bordetella pertussis* and *Bordetella bronchiseptica* strains were grown on Bordet Gengou agar supplemented with 10% defibrinated horse blood. When liquid media was required, these species were grown in modified SS medium (Stainer and Scholte, 1970).

Liquid cultures of *Bordetella* strains assayed for PT secretion were grown in SS medium supplemented with 2% (2,6-Di-O-methyl)- β -cyclodextrin (M β CD). Bacteria containing plasmids were routinely grown in media supplemented with appropriate antibiotics, selecting for plasmid retention. Antibiotic concentrations used were as follows: ampicillin, 100 μ g/mL; cephalexin, 100 μ g/mL; kanamycin, 50 μ g/mL; nalidixic acid, 50 μ g/mL; rifampicin, 100 μ g/mL and trimethoprim, 50 μ g/mL. Agar containing X-gal (0.004%) and IPTG (40 μ M) was used for blue/white selection of possible clones. All bacterial strains were grown at 37°C. Plates were incubated in a Qualtex Solidstat Incubator. Liquid cultures were aerated by shaking in a BioLine 4610 Shaking Incubator or a Paton 461 Orbital Shaker. Plasmids used in this study are listed in Table 2.2

Table 2.1 Bacterial strains used in this study

Bacterial Strain	Relevant Characteristics #	Reference or Source
<i>Bordetella pertussis</i>		
Tohama I	serotype 2 Hly ⁺	Kasuga <i>et al.</i> , 1954
CSL 127	serotype 2 Hly ⁺	CSL Ltd.
CSL 127S	serotype 2 Hly ⁺	CSL Ltd.
CSL 137	serotype 3 Hly ⁺	CSL Ltd.
CSL 137S	serotype 3 Hly ⁺	CSL Ltd.
CSL 1237	serotype 2, 3 Hly ⁺	CSL Ltd.
CSL 1237S	serotype 2, 3 Hly ⁺	CSL Ltd.
<i>Bordetella bronchiseptica</i>		
BB7865	Hly ⁺ Sm ^r	Monack <i>et al.</i> , 1989
BB7866	Hly ⁻ Sm ^r $\Delta bvgS$	Monack <i>et al.</i> , 1989
5376	Fim ⁺ FHA ⁺ Hly ⁺	Walker <i>et al.</i> , 1991a
5377	Hly ⁻ derivative of 5376	This study
ATCC 10580	Hly ⁻	Walker <i>et al.</i> , 1991a
ATCC 10580:: <i>Tn</i> <i>fusPT1</i>	Hly ⁻ <i>Tn</i> <i>fusPT</i> Kan ^r	Walker <i>et al.</i> , 1991a
ATCC 10580:: <i>Tn</i> <i>fusPT1</i> (pR751::Tn813)	Hly ⁻ <i>Tn</i> <i>fusPT</i> Kan ^r	This study
BB7865 Rif ^r	Rif ^r Nal ^r derivative of BB7865	This study
BB7866 Rif ^r	Rif ^r Nal ^r derivative of BB7866	This study
5376 Rif ^r	Rif ^r derivative of 5376	This study
5377 Rif ^r	Rif ^r derivative of 5377	This study
5376 Rif ^r Nal ^r	Nal ^r derivative of 5376 Rif ^r	This study
5377 Rif ^r Nal ^r	Nal ^r derivative of 5377 Rif ^r	This study
BB7865 Rif ^r :: <i>Tn</i> <i>fusPT1</i>	<i>Tn</i> <i>fusPT</i> Kan ^r Rif ^r Nal ^r	This study
BB7866 Rif ^r :: <i>Tn</i> <i>fusPT1</i>	<i>Tn</i> <i>fusPT</i> Kan ^r Rif ^r Nal ^r	This study
5376 Rif ^r Nal ^r :: <i>Tn</i> <i>fusPT1</i>	<i>Tn</i> <i>fusPT</i> Kan ^r Rif ^r Nal ^r	This study
5377 Rif ^r Nal ^r :: <i>Tn</i> <i>fusPT1</i>	<i>Tn</i> <i>fusPT</i> Kan ^r Rif ^r Nal ^r	This study
<i>Escherichia coli</i>		
Q358 pR751::Tn813	Tp ^r	Bowen and Pemberton, 1985
XL1-Blue MRF' Kan	Kan ^r competent cells	Stratagene
INV α F'	competent cells	Invitrogen

Abbreviations; r, resistant to; Kan, kanamycin; Tp, trimethoprim; Sm, Streptomycin; Fim, fimbriae production; FHA, filamentous haemagglutinin production; Hly, haemolytic activity; *Tn**fusPT*, recombinant PT expression locus; $\Delta bvgS$, non-functional *bvgS* gene.

Table 2.2 Plasmids used in this study

Plasmid	Relevant Characteristics #	Reference or Source
pCRII / pCR2.1	Amp ^r , Kan ^r ,	Invitrogen
pCR Script	pBluescript based, Amp ^r	Stratagene
pAS5	partial <i>ptlH</i> gene in pCRII	This study
pAS6	fimbrial accessory promoter of CSL 127S in pCR Script	This study
pAS7	fimbrial accessory promoter of CSL 127S in pCR Script	This study
pAS8	<i>P_{fim2}</i> of CSL 127S in pCRII	This study
pAS9	<i>P_{fim2}</i> of CSL 127S in pCR Script	This study
pAS10	<i>bvg/fha</i> intergenic region of CSL 127S in pCR Script	This study
pAS12	<i>P_{ptx}</i> of CSL 127S in pCRII	This study
pAS14	<i>P_{fim3}</i> of CSL 127S in pCR2.1	This study
pAS16	<i>bvg/fha</i> intergenic region of CSL 127U in pCR Script	This study
pAS17	<i>bvg/fha</i> intergenic region of CSL 127U in pCR Script	This study
pAS18	<i>P_{ptx}</i> of CSL 127U in pCRII	This study
pAS19	<i>P_{ptx}</i> of CSL 127U in pCR2.1	This study
pAS20	<i>P_{fim2}</i> of CSL 127U in pCRII	This study
pAS21	<i>P_{fim2}</i> of CSL 127U in pCR Script	This study
pAS22	<i>P_{fim3}</i> of CSL 127U in pCR2.1	This study
pAS24	fimbrial accessory promoter of CSL 127U in pCR Script	This study
pAS25	fimbrial accessory promoter of CSL 127U in pCR Script	This study
pAS26	<i>bvg/fha</i> intergenic region of CSL 137S in pCR Script	This study
pAS28	<i>P_{ptx}</i> of CSL 137S in pCR Script	This study
pAS29	<i>P_{ptx}</i> of CSL 137S in pCRII	This study
pAS30	<i>P_{fim2}</i> of CSL 137S in pCR Script	This study
pAS32	<i>P_{fim3}</i> of CSL 137S in pCR2.1	This study
pAS33	<i>P_{fim3}</i> of CSL 137S in pCR2.1	This study
pAS34	fimbrial accessory promoter of CSL 137S in pCR II	This study
pAS36	<i>bvg/fha</i> intergenic region of CSL 137U in pCR Script	This study
pAS38	<i>P_{ptx}</i> of CSL 137U in pCR Script	This study
pAS40	<i>P_{fim2}</i> of CSL 137U in pCRII	This study
pAS42	<i>P_{fim3}</i> of CSL 137U in pCR2.1	This study
pAS43	<i>P_{fim3}</i> of CSL 137U in pCR2.1	This study
pAS44	fimbrial accessory promoter of CSL 137U in pCRII	This study
pAS45	fimbrial accessory promoter of CSL 137U in pCR Script	This study
pAS46	<i>bvg/fha</i> intergenic region of CSL 1237S in pCRII	This study
pAS47	<i>bvg/fha</i> intergenic region of CSL 1237S in pCR Script	This study
pAS48	<i>P_{ptx}</i> of CSL 1237S in pCR Script	This study
pAS50	<i>P_{fim2}</i> of CSL 1237S in pCR Script	This study

pAS52	<i>P_{fim3}</i> of CSL 1237S in pCR2.1	This study
pAS54	fimbrial accessory promoter of CSL 1237S in pCRII	This study
pAS56	<i>bvg/fha</i> intergenic region of CSL 1237U in pCR Script	This study
pAS58	<i>P_{ptx}</i> of CSL 1237U in pCR Script	This study
pAS60	<i>P_{fim2}</i> of CSL 1237U in pCRII	This study
pAS62	<i>P_{fim3}</i> of CSL 1237U in pCR2.1	This study
pAS64	fimbrial accessory promoter of CSL 1237U in pCRII	This study
pR751::Tn813	<i>tra⁺ tnpA⁺ tnpR⁻</i>	Bowen and Pemberton, 1985
pMW132	<i>bvg⁺ Kan^r</i>	Walker <i>et al.</i> , 1991a
pMW127	<i>Tn_{fusPT} Kan^r</i>	Walker <i>et al.</i> , 1991a

Abbreviations; r, resistant to; Amp, ampicillin; Kan, kanamycin; Tp, trimethoprim; *Tn_{fusPT}*, recombinant PT expression locus; *tra*, conjugative transfer genes; *tnpA*, transposase; and *tnpR*, resolvase; *bvg*, expresses functional *bvg* genes.

2.2 Isolation and purification of DNA

2.2.1 Chromosomal DNA Extraction

Genomic DNA of *Bordetella pertussis* strains was obtained using the method of Priefer and associates (1984). Cells were grown to confluence on Bordet Gengou agar and suspended in 25 mL of 1.0 M NaCl in a sterile 50 mL centrifuge tube. The tube was then shaken on ice for 60 minutes and centrifuged at 12 000 g for 10 minutes in a Sorvall RC-5 superspeed centrifuge or a Beckmann J2-MC. The pellet was then washed with 25 mL of chilled TES buffer and the centrifugation repeated. The supernatant was discarded and the cells concentrated in 5 mL of cold TE (chromosomal) buffer. Preliminary degradation of bacterial cell walls was carried out with the addition of lysozyme (chicken egg white, Sigma) in TE to a final concentration of 0.2 mg/mL, followed by a 15 minute incubation at room temperature. The addition of SDS to 1 % (w/v) resulted in solubilisation of cellular membranes and following a brief

room temperature incubation, a protease solution (Pronase E, Sigma) in TE was added to a final concentration of 500 $\mu\text{g}/\text{mL}$ to induce protein degradation. The preparation, now extremely viscous, was swirled intermittently over a three hour period to achieve optimal protein digestion. Cellular proteins were removed with 5 mL of TE saturated phenol. Vigorous shaking facilitated transfer of proteins from the aqueous phase to the phenol phase. Centrifugation at 12 000 g for 10 minutes separated the phases. The aqueous phase was transferred to 1.5 mL microcentrifuge tubes and a further phenol extraction performed. Trace levels of phenol were removed from the solution by two ether extractions. The addition of 1 mL of diethyl ether, followed by violent shaking and centrifugation for 1 minute at 16 000 g allowing the removal of the organic phase, produces a fraction containing nucleic acids. A second ether extraction was performed and the nucleic acids were precipitated by the addition of one tenth volume of 3 M sodium acetate (pH 7.0) followed by 2.5 volumes of chilled ethanol. The precipitate was pelleted by centrifugation at 16 000 g for 5 minutes, washed with cold 70% ethanol and allowed to air dry. The nucleic acids were then dissolved in an appropriate volume of sterile distilled water containing ribonuclease A (bovine pancreas, Sigma) at a concentration of 20 $\mu\text{g}/\text{mL}$.

2.2.2 Plasmid DNA Extraction

Phenol Extraction

This procedure, modified from Naumovski and Friedberg (1982), was routinely used for the screening of possible clones by restriction enzyme analysis. Freshly grown cells were removed from selective media using a sterile loop and resuspended in 0.4 mL of TE buffered sucrose in a sterile 1.5 mL microcentrifuge tube. Lysozyme (chicken egg white, Sigma) was

added to approximately 1 mg/mL and allowed to breakdown the bacterial cell walls at room temperature for 30 minutes. A solution containing Triton X-100 was then added (0.3 mL) to solubilise cellular membranes. After incubation at 65°C for 10 minutes, the cell debris was spun down at 16 000 g for 30 minutes. The supernatant was placed into a fresh tube and 0.5 mL of TE saturated phenol added with vigorous shaking. Following centrifugation at 16 000 g for 15 minutes in an Eppendorf 5415C centrifuge, the aqueous (upper) layer was placed in a fresh microfuge tube and 1 mL of diethyl ether added, again with vigorous shaking. The phases were separated after centrifugation for 1 minute at 16 000 g, and the organic (upper) layer was discarded. A further ether extraction followed, ensuring that all trace levels of phenol were removed. The plasmid DNA was then precipitated, along with cellular RNA, by the addition of 0.1 volume of 3 M sodium acetate and 2.5 volumes of pre-chilled absolute ethanol. Centrifugation at 16 000 g for a period of 5 minutes yielded a pellet of nucleic acid which was then washed with chilled 70% ethanol, and allowed to air dry. A total of 50 µL of dH₂O was then added, together with ribonuclease A (bovine pancreas, Sigma) to a final concentration of 100 µg/mL, and incubated for 30 minutes at 37°C to resuspend the pellet and degrade any RNA contaminating the DNA sample.

QIAprep Spin Plasmid Kit

These 1.5 mL spin columns contain a silica gel which binds plasmid DNA which is then washed, and eluted with water. The manufacturers instructions were adhered to when extracting this "sequencing quality" plasmid DNA. Bacterial cells were grown overnight, then 1.5 mL aliquots were spun at 16 000 g for 30-60 seconds and the pellet resuspended in 250 µL of buffer P1. The cells were then subjected to lysis by the addition of 250 µL of buffer P2. Precipitation of cellular debris was achieved by adding

350 μ L of buffer N3 and centrifugation at 16 000 g for 10 minutes. The impure plasmid preparation was then loaded onto a small Qiaprep column, spun for 60 seconds to allow the plasmid DNA to bind to the silica matrix. The bound DNA was then washed with 750 μ L of PE buffer. The column was centrifuged to accelerate washing flow rate and the flow through liquid was discarded. The spin was then repeated to remove all traces of PE buffer. The DNA was then eluted by placing 50 μ L of dH₂O on the membrane inside the column. After 1 minute the column was again centrifuged and the eluant collected in a fresh microfuge tube.

Qiagen midi columns (Tip 100) are larger and rely on gravity, making them markedly slower than the Spin Preps. However, these were used when a greater amount of DNA was required, again the manufacturer's protocol was followed.

Pharmacia Biotech Flexi-Preps

The principle of this kit is similar to the Qiagen spin preps, however the DNA binding material is a slurry of "Sephaglas"TMFP. The bacterial cells are lysed and the cellular debris precipitated. To the remaining supernatant is added a small amount of Sephaglas which reversibly binds the plasmid DNA. The matrix is then washed, and eluted with water leaving a high quality plasmid preparation. The manufacturer's instructions were followed closely throughout this extraction. Bacterial cells were grown overnight, then 1.5 mL aliquots were spun at 16 000 g for 30-60 seconds and the pellet fully resuspended in 200 μ L of Solution I. The cells were then lysed with the addition of 200 μ L of Solution II and the cellular debris precipitated with 200 μ L of Solution III. After 5 minutes centrifugation at 16 000 g, the supernatant was removed to a new microfuge tube and 0.7 volumes of isopropanol added. A room

temperature, 1 minute incubation followed by a 5 minute full speed spin in an Eppendorf 5415C centrifuge, pelleted the plasmid preparation. The crude DNA pellet was then resuspended in 150 μ L of Sephaglas slurry by vortexing for a minute. The supernatant was separated from the plasmid-Sephaglas complex by centrifuging at 16 000 g for 15 seconds and aspirating the supernatant. The complex was then washed with 200 μ L of Wash buffer and recentrifuged. Again the supernatant was removed and 300 μ L of 70% ethanol added. The tube was briefly vortexed, spun and the ethanol removed. This step was repeated to ensure the removal of all traces of ethanol and the complex was resuspended in 50 μ L of dH₂O. The plasmid DNA detaches from the matrix, and after 5 minutes room temperature incubation, the Sephaglas beads are spun down and the aqueous plasmid solution is transferred to a fresh microfuge tube.

2.3 Amplification and Cloning of *Bordetella* Chromosomal DNA

2.3.1 Polymerase Chain Reaction

Relevant regions of *Bordetella pertussis* DNA were amplified using the polymerase chain reaction (PCR). This technique, uses a thermostable DNA polymerase to replicate DNA defined and flanked by specific oligonucleotide primers. The reaction involves many rounds of strand separation, primer annealing and strand extension to create copies of the target DNA. Oligonucleotide primers used in PCR amplifications in this study are listed in Table 2.3. All PCR procedures were carried out in either 0.1 or 0.5 mL microcentrifuge tubes in either a Perkin Elmer GeneAmp9600, or a Hybaid Thermal Reactor. The components of a typical PCR are described below in Table 2.4.

Table 2.3 Oligonucleotide primers used for PCR and DNA sequencing in this study

Name	Oligonucleotide Sequence	Supplier
bvg/fha 5'	5'-CCCGGGATGAGGACTTTGTTGTACAT-3'	Biotech
bvg/fha 3'	5'-GGATCCCCCCTGTACGGTTCGTGTTTCAT-3'	Biotech
PT 5'	5'-GAATTCGCCAAGCTGAAGTAGCAGCG-3'	Biotech
PT 3'	5'-GGATCCCGAATTGCCCCGAGTGCAACGCAT-3'	Biotech
fim 2 5'	5'-GATTCACGCTGATGCGCCGGCCCGG-3'	Biotech
fim 2 3'	5'-GGATCCCCGCGTTGGAAAGGGATTTGCAT-3'	Biotech
fim 3 5'	5'-GAATTCGACGGCGCCCGCCGAACCGG-3'	Biotech
fim 3 3'	5'-GGATCCCCAGGGTATGAAAATTGGACAT-3'	Biotech
fim access 5'	5'-GAATTCTGAAACCAACAAATAGGTAG-3'	Biotech
fim access 3'	5'-GGATCCAGCCGCGCGGCCAGCGCGCCAT-3'	Biotech
GBF fim3 5'	5'-TGCAACAAGAACGAAGACGCGGCCGCCCCG-3'	Biotech
GBF fim3 3'	5'-TGGCAGTACGGGCGAGGCGGCAAGGATAAG-3'	Biotech
fim 2 BIG 5'	5'-ACGCTGATGCGCCGGCCCGCGCCGCATGG-3'	Biotech
fim 2 BIG 3'	5'-ATGGTGCCGGTGATGACGATGGTGCCGTCG-3'	Biotech
PT secrtn 5'	5'-GGCCAGACCGGTTTCGGGCAAGACCACA-3'	Biotech
PT secrtn 3'	5'-TCATGGCGCCGGGAGGCCATCCCGGTA-3'	Biotech
Universal	5'-GTAAACGACGGCCAGT-3'	Pharmacia
Reverse PUCR-2	5'-TTCACACAGGAAACAGCTATGACC-3'	Biotech

Table 2.4 Components of a typical PCR and their concentration

Reagent	Final Concentration
sterile dH ₂ O	to final volume
PCR Buffer (10X)	1 X
dGTP	0.2 mM
dATP	0.2 mM
dTTP	0.2 mM
dCTP	0.2 mM
DNA template	1 ng
DNA polymerase	2.5 Units
5' primer	0.25 µM
3' primer	0.25 µM

Since each PCR involves different templates and different primers, the concentrations of each of the components may have been varied to achieve optimisation of the reaction. After spot centrifugation, 100 µL of mineral oil was layered on top of the aqueous reaction mixture to prevent evaporation. The tube was then placed in the thermal cycler and subjected to a temperature cycling regime as set out in Table 2.5. Variations to the basic temperature and time parameters were employed to optimise product formation.

Table 2.5 General guide to temperature cycles used for the PCR.

Step	Temperature	Time	Cycles
1.1	96°C	3 min.	1
1.2	55°C	1 min.	
1.3	72°C	2 min	
2.1	96°C	40 sec.	30
2.2	55°C	50 sec.	
2.3	72°C	1 min.	
3	72°C	5 min.	1

The DNA polymerases used in this study were *Taq*, from the thermophilic bacterium *Thermus aquaticus* (Boehringer Mannheim, Qiagen or Perkin Elmer) and *Pfu* from *Pyrococcus furosius* (Stratagene, both native and cloned). Mismatches can occur in a PCR when the polymerase inserts an incorrect base. These “*Taq* errors” can be reduced by using a thermostable DNA polymerase which has 3'-5' proofreading capabilities such as *Pfu*. To ensure accuracy, much of the work in this study was done using *Pfu* to decrease the chances of a *Taq* error occurring. As an absolute safeguard against errors, if a difference between promoter DNA sequences was found, an independent PCR and cloning was performed, and the new clone sequenced.

2.3.2 Invitrogen TA Cloning System

This PCR specialised cloning system takes advantage of an inherent property of *Taq* DNA polymerase. Products amplified by this thermostable enzyme possess an extra adenine overhang at each end. The vector used in this strategy (pCR II or pCR2.1) has complimentary thymine overhangs to facilitate a more efficient “sticky-end” ligation.

Amplification products generated using *Taq* DNA polymerase were

cloned into either pCRII or pCR2.1 and transformed into *Escherichia coli* INVαF' competent cells. The procedure used was essentially that specified by the manufacturer.

Ligation

The ligation reaction was initiated by adding 1 to 2 μL of the PCR product DNA to be cloned to a microfuge tube containing 2 μL of precut pCRII/pCR2.1 (50 ng), 1 μL of 10X ligation buffer, 1 μL (4 Units) of T4 DNA ligase and dH₂O to a final volume of 10 μL. This mixture was then incubated at 12°C overnight and then stored on ice before the transformation reaction proceeded.

Transformation

A vial of competent INVαF' cells supplied with the kit were thawed on ice and 2 μL of 0.5 M β-mercaptoethanol was added. A 1 μL aliquot of the ligation mix was added and allowed to transform on ice for 30 minutes. The reaction was then heat shocked at 42°C for 60 seconds and placed on ice for 2 minutes. Warmed SOC medium (450 μL) was added to the transformation which was then incubated with shaking at 37°C for 60 minutes and plated onto selective Z agar containing ampicillin and kanamycin, together with X-gal for blue/white selection of possible clones. No IPTG was required because the cloning vector does not encode the *lacI^q* repressor.

2.3.3 Stratagene pCR Script Cloning Procedure

Thermostable DNA polymerases which have 3' to 5' proofreading ability such as *Pfu* or *Vent*, generate "blunt-ended" products/fragments, thereby making cloning/ligation a more difficult task. The pCR Script system

employs a deft combination of ligase and the rare cutter restriction endonuclease *Srf* I to maximise the cloning efficiency. Re-ligation of the vector without an insert results in the formation of an *Srf*I restriction site. Also present in the ligation mixture, this eight base pair cutter immediately reopens the vector (leaving blunt ends), increasing the chances of insert : vector ligation.

Amplification products generated using *Pfu* DNA polymerase were ligated into pCR Script and transformed into supercompetent *E. coli* XL1-Blue MRF'Kan (Stratagene) cells. The protocol followed was essentially that specified by the manufacturer.

Ligation

Prior to cloning the blunt ended PCR product into the pCR Script vector, a purification step was required. To the PCR product, an equal volume of 4 M ammonium acetate was added, followed by 2.5 volumes of 100% ethanol. The DNA was then centrifuged at 10 000g for 20 minutes and the pellet washed with 70% ethanol and air dried. The product was then resuspended in dH₂O to the original volume. Between 2 and 4 µL of this reprecipitated DNA product was mixed with 1 µL of pCR Script (10 ng), 1 µL of 10x reaction buffer, 0.5 µL of ATP, 1µL (5 Units) of *Srf*I, 1 µL (4 Units) of T4 DNA ligase and dH₂O to a final volume of 10 µL. This mixture was then incubated at room temperature for 60 minutes, followed by a 10 minute inactivation step at 65°C.

Transformation

Supercompetent *E. coli* cells supplied with the kit were thawed on ice. A 40 µL aliquot was placed into a 15 mL centrifuge tube and 0.7 µL of 0.5 M β-mercaptoethanol was added. Following incubation on ice for 10

minutes with gentle rocking, 2 μ L of the ligation mix was added and allowed to transform on ice for 30 minutes. The reaction was then heat shocked at 42°C for 45 seconds and placed on ice for 2 minutes. Warmed SOC medium (450 μ L) was added to the transformation which was then incubated with shaking at 37°C for 60 minutes and plated onto ampicillin selective Z agar containing X-gal and IPTG for blue/white selection of possible clones.

2.4 Detection and Analysis of DNA

2.4.1 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to detect the presence of DNA and determine the size of restriction fragments. An appropriate amount of restriction enzyme digested DNA was added to 5 μ L of loading buffer and applied to 1% agarose/TAE gels. Electrophoresis took place at 50 V in a Bio Rad Mini Sub™ DNA Cell powered by a Bio Rad Power Pack 300 for an appropriate period of time. The gel was then stained in an aqueous 1 μ g/mL solution of ethidium bromide and the intercalated DNA viewed under ultra-violet light (UV Transilluminator, UVP). Typically, lambda bacteriophage DNA (Boehringer-Mannheim) digested with *Hind* III restriction enzyme or 1kb Ladder (Gibco BRL) were used as molecular weight markers, to determine the size of DNA fragments. The fluorescent DNA bands were documented using a Polaroid MP-4 LC land camera and Polaroid 667 film. Alternatively, a Novaline gel documentation system was used.

2.4.2 Elution of DNA from Agarose Gels

Occasionally, certain separated bands of DNA were recovered from agarose gels for further analysis. This was done as an extra purification step, or to separate low molecular weight PCR products from excess primers prior to cloning. The DNA of interest was electrophoresed through 0.7% agarose gel and the fragment to be eluted excised using a sterile scalpel. The piece of agarose containing the fragment was then subjected to the Bresa-Clean™ procedure (Bresatec). This system involves melting the gel in buffer and adding a silica based matrix. The silica beads reversibly bind the DNA now in solution and the complex is washed and then eluted from the matrix with sterile dH₂O. The band of agarose containing the DNA to be retrieved was excised and placed in a microcentrifuge tube containing 3 volumes of Bresa-Salt™. Incubation at 55°C for 5-10 minutes with occasional vortexing melts the gel sliver, forming a homogeneous solution. Addition of 6 µL of Bresa-Bind™ silica matrix and incubation for five minutes at room temperature allows the DNA to bind the matrix. The silica-DNA complex was then pelleted and washed with a volume of Bresa-Wash™ equal to the initial amount of salt used. The wash solution was then removed by centrifugation, the complex was dried and resuspended in dH₂O. Incubation at 55°C for 5 minutes, breaks the interaction between the DNA and the silica matrix and the DNA is released into solution. The silica is then pelleted and the DNA solution transferred to a fresh tube.

2.4.3 Southern Hybridisation Analysis of DNA

The transfer of DNA onto a nylon membrane for detection of specific fragments is based on the technique pioneered by Southern (1975).

Preparation

The DNA to be analysed was electrophoresed through a 0.7% agarose gel and visualised using ethidium bromide intercalation. The gel was then placed in a glass dish and submerged in 0.25 M HCl. Two washes in the acid solution, of 10 minutes duration were performed, followed by a brief rinse in dH₂O. The gel was then subjected to 2 washes of 10 minutes in a solution containing 1.5 M NaCl and 0.5M NaOH. This was followed by a neutralisation step consisting of 4 washes of 10 minutes in a solution of 1 M Tris.HCl and 1.5 M NaCl (pH 8.0).

Transfer

The Southern transfer apparatus was set up as follows. A wick was constructed by positioning a large strip of filter paper across a glass plate spanning a large glass dish. The gel was lowered onto the wick without the introduction of air bubbles. Gel sized pieces of filter paper and nylon membrane (Boehringer Mannheim, positively charged) were soaked in 2X SSC. The membrane was lowered onto the gel, followed by 5 pieces of filter paper. A stack of tissue paper 7 cm high was positioned atop the filter paper and a weight of 1 kg applied. The set-up was surrounded with cling wrap to prevent "short circuiting" of the blot. The glass dish was filled with 10X SSC and the DNA was allowed to transfer overnight, after which the membrane was cross-linked for 3 minutes at 254 nm using a Stratagene UV Stratalinker 1800 to fix the DNA onto the membrane.

Probe Labelling

The DNA to be used as a hybridisation probe was labelled using nick translation. This involves incubating the probe DNA with a mixture of the enzymes DNase I, which "nicks" the sequence and DNA polymerase I, which fills in the gaps created by the DNase I. Some of the nucleotides

thus reincorporated are radiolabelled, in this case with ^{32}P and are therefore detectable on X-ray film.

The DNA to be labelled (approx. 1 μg) was placed on ice in a microfuge tube. Added to the tube was a mixture of dCTP, dGTP and dTTP at final concentrations of 25 μM each. An aliquot of 50 μCi of radiolabelled [α - ^{32}P]-dATP (Bresatec, ICN) was then added to a final concentration of 3 μM . The solution was made up to 40 μL with water and the buffered enzyme mixture (Gibco BRL) was added (200 pg DNase I; 2 Units DNA polymerase I). Following a 1 hour incubation at 15°C, the reaction was halted by the addition of 5 μL of a 200 mM EDTA solution (pH 8.0). Labelled DNA was separated from unincorporated nucleotides by passing the reaction through an STE buffer equilibrated Sephadex® G50 (Pharmacia) column. Constructed using a 1 mL syringe and glass wool, the spin column allows the passage of the large plasmid DNA, whilst retarding the movement of the free nucleotides as they remain in the pores of the Sephadex® beads.

Hybridisation

The membrane was placed in a Hybaid incubation bottle and 5 mL of prehybridisation solution was added. This solution contains various blocking reagents and detergents. The membrane was prehybridised at 65°C in a Hybaid Mini Oven Mk II for 6 hours and the denatured ^{32}P labelled probe was added. The probe was allowed to hybridise to the membrane overnight at 65°C. To remove any non-specific binding, the blot was washed twice at 42°C for 5 minutes with 0.2% SDS in 5X SSC. This wash step was then repeated with 0.2% SDS in 2X SSC and 0.1% SDS in 1X SSC. The blot was removed from the bottle and allowed to dry on paper towel.

Detection

After the membrane was completely dry, it was wrapped neatly in cling wrap and exposed to X-ray film (Fuji) for an appropriate period of time, dependant on the radioactive strength of the probe and the degree of binding. The film was then developed in a darkroom. This was done by immersion in X-ray developer (GBX, Kodak) for 1-2 minutes, rinsed with water and held in X-ray fixer (GBX, Kodak) until transparency became evident. The film was rinsed thoroughly with water and air dried before viewing hybridised bands.

2.4.4 Restriction Endonuclease Analysis of DNA

The DNA to be digested (usually 5-10 μL) was added to a microfuge tube together with 2 μL of an appropriate 10X restriction buffer. The volume was made up to 19 μL with dH_2O and 1 μL (10 Units) of restriction enzyme (Boehringer-Mannheim) added. The tube was then incubated for at least 60 minutes at 37°C. Deactivation of the enzyme was then achieved by incubation of the tube at 65°C for 10 minutes.

2.4.5 Ligation of DNA

Appropriate amounts of the DNA to be ligated were combined with dH_2O (to a volume of 35 μL) in a sterile microfuge tube at 60°C and incubated for 5 minutes. The tube was then incubated for 30 minutes each at 37°C, room temperature and 10°C, then placed on ice. After the addition of 4 μL of 10X ligation buffer and 1 μL (2 Units) of T_4 DNA ligase (Boehringer-Mannheim), the tube was stored at 12°C overnight. The ligase enzyme was then deactivated at 65°C for 10 minutes.

2.4.6 Competent Cell Preparation

Cells to be made chemically competent were inoculated into 200 mL of LB and incubated with agitation at 37°C until an optical density of approximately 0.5 at OD₅₆₀ was attained. Following centrifugation at 5 000 g for 10 minutes at 4°C, the supernatant was discarded and the cells placed on ice for 10 minutes. The cells were resuspended in 100 mL of chilled 0.1 M MgCl₂ and centrifuged again under the same conditions. The supernatant carefully poured off. The pellet was resuspended in 10 mL of chilled 0.1 M CaCl₂ and stored on ice.

2.4.7 Transformation of DNA

Transformation of plasmid DNA into chemically competent cells was accomplished using the method of Cohen (1972). To 200 µL of competent cells on ice, either 5 µL of uncut plasmid DNA or 20 µL of a ligation mixture was added. The cells were placed on ice for 60 minutes before a 5 minute heat shock step was carried out at 37°C. The cells were pelleted at 10 000 g for 1 minute and the supernatant discarded. The pellet was carefully resuspended in 0.5 mL of LB and incubated without shaking at 37°C for 60 minutes. The suspension was then aliquotted out onto selective agar plates and incubated at 37°C until transformant colonies appeared.

2.4.8 DNA Sequence Analysis

Sequencing Reactions

Plasmids containing *B. pertussis* promoters were sequenced using either an ABI 373 or 377 system. The plasmid DNA to be sequenced was

extracted using either the Qiagen Spin-Prep or the Pharmacia Flexi-Prep methods. The amount of DNA required for each sequence reaction ranged between ~200 ng and 1 µg. Sequencing reactions were performed in 20 µL volumes either in 0.1 mL microfuge tubes in a Perkin Elmer GeneAmp9600 thermal cycler or in 0.5 mL tubes in a Hybaid Thermal Reactor. Sequencing reactions were set up using 8 µL of sequencing reaction mix (Perkin Elmer), 5 ng of oligonucleotide primer and 500 ng of plasmid template DNA. A total of 25 cycles of either of the cycling parameters listed in Table 2.6 were used. Alternative 1, with a longer denaturation step was used especially when the GC content of DNA to be sequenced was particularly high.

Table 2.6 Alternative parameters used for sequencing reactions

Step	Alternative 1	Alternative 2
Denaturation	96°C; 55 sec.	95°C; 10 sec.
Annealing	50°C; 15 sec.	50°C; 5 sec.
Polymerisation	62°C; 4 min.	60°C; 4 min.

The 20 µL sequencing reaction was then precipitated with the addition of 2 µL of 3 M sodium acetate (pH 4.6) and 50 µL of chilled ethanol. Incubation at -20°C for 10 minutes accelerated this process. Centrifugation at 16 000 g for 20 minutes pelleted the DNA which was then washed with 250 µL of 70% ethanol and air or vacuum dried. Resuspension of the pellet took place in 5 µL of formamide loading buffer. The sample was then heated to 90°C for 2 minutes to promote denaturation and then placed on ice prior to loading onto the gel.

Sequencing Gel Electrophoresis

Polyacrylamide sequencing gels were made using 4% acrylamide (19:1 acrylamide: bis-acrylamide; Bio-Rad; Amresco) buffered with 1X TBE and

containing 6 M urea. The gel solution was exposed to amberlite ion exchange resin, filtered through a 0.2 μm cellulose nitrate filter (Schleicher & Schuell) and degassed for 10 minutes prior to polymerisation with ammonium persulfate and TEMED and injected into an ABI casting apparatus. After a curing time of 2 hours, the gel was pre-run for 1 hour until the gel temperature was maintained at a constant 48°C. Samples (2 μL) were loaded onto the gel with the aid of a shark tooth comb and gel loading pipette tips. The gel was then run for 10 hours on an ABI 373 or 377 DNA sequencer at a constant 37 W (~2350 V, 16 mAmp).

2.5 Microbiological Techniques

2.5.1 Bacterial Conjugation

The bacterial strains to be mated were grown on appropriate medium after which one loopful of each strain was resuspended in 500 μL of sterile 0.7% saline solution. This mixture was plated out onto non-selective medium and incubated overnight at 37°C, allowing the strains to mate. A loopful of the resultant growth was resuspended in 1 mL of a sterile 0.7% saline solution, tenfold serially diluted and plated out onto selective agar.

2.5.2 Replica Plating

Patching onto replica agar plates was performed using sterile wooden toothpicks. Individual toothpicks were used to remove (pick) individual bacterial colonies from growth plates and patch them onto replica plates in sets of 50 or 100 colonies per plate.

2.6 Characterisation of Recombinant Bacterial Strains

2.6.1 Polyacrylamide Gel Electrophoresis

Newly formed recombinant bacterial strains were characterised and compared to parental strains using a polyacrylamide gel electrophoresis (PAGE) system modified from Laemmli (1970), western blot analysis and enzyme linked immunosorbent assay (ELISA) to examine protein expression.

Sample Preparation

An appropriate number of cells ($\sim 10^6$ cells/mL) were resuspended in 100 μ L of a sterile 0.7% sodium chloride solution in a microfuge tube. To this, an equal volume of PAGE cracking buffer was added and the tube incubated at 95°C for 10 minutes. To aid in the disintegration of cell debris, the solution was subjected to a brief period of sonication using a Branson Sonifier 250 until opacity was sufficiently diminished.

Electrophoresis and Staining

A homogeneous 10 or 15% polyacrylamide (35:0.8 acrylamide: bis-acrylamide, Bio-Rad) running gel was used to resolve proteins, whilst a 4% stacking gel allowed for concentration and even running of samples. Protein samples were electrophoresed in a Bio-Rad Mini Protean II gel apparatus powered by a Bio-Rad Power Pack 300. The gel was run at 100 V for 1-2 hours in Tris/Glycine buffer, after which the protein bands were stained for 3 hours using Coomassie Brilliant Blue. Excess stain was then removed by washing in PAGE Destain for 4 hours or overnight.

2.6.1 Western Blot Analysis

Following the electrophoretic separation of proteins by PAGE, transfer onto nitrocellulose (western blotting), a process developed by Burnette (1981), was performed.

Transfer onto Nitrocellulose

After the stacking gel had been removed, the gel, together with a piece of nitrocellulose (pore size: 0.45 μ m, Bio-Rad) or nylon membrane (positively charged, Boehringer Mannheim) of equal size was equilibrated for 15 minutes in western buffer. Five sheets of filter paper, pre-soaked in the same buffer were individually placed onto the anode of the transfer apparatus, a Bio-Rad Trans-Blot® Semi-Dry transfer cell. These were followed by the nitrocellulose membrane, the gel and another five filter papers. Air bubbles were removed after the addition of each layer. The cathode was placed on top of the stack and a constant voltage of 15 V was passed through the system for 20 minutes.

Detection of Proteins

Immediately following transfer, the nitrocellulose was placed in a blocking solution (5% skim milk powder in PBS) and rocked gently at room temperature for 60 minutes. The blocker was discarded and an appropriate dilution of the primary antibody (a cocktail of anti-pertussis toxin monoclonal antibodies E19, E205 and E251 (Walker *et al.*, 1991b) diluted appropriately in PBS) was applied. After at least 60 minutes incubation time, the antibody solution was discarded and the membrane washed in PBS (3 x 10 minutes) to remove all traces of unbound antibody. The secondary antibody solution, an anti-mouse alkaline phosphatase conjugate (Boehringer Mannheim), was added and incubation took place

for a further 60 minutes. This solution was discarded and another 3×10 minute washes in PBS followed. A chemiluminescent detection system based on Lumigen PPD or Lumi-Phos 530 (Boehringer Mannheim) was used. The manufacturer's suggested protocol was strictly adhered to when using this system as an alkaline phosphatase substrate.

2.6.3 Enzyme Linked Immuno-Sorbent Assay (ELISA)

Bordetella culture samples were taken at the end of log-phase growth ($\sim 10^9$ cells/mL). The cells were separated from the medium by centrifugation at 10 000g for 10 minutes and heat killed at 65°C for 15 minutes. An appropriate dilution (1/5) of each sample was prepared for the ELISA assay. Supernatant and cellular fractions were tested for the presence of pertussis toxin using a non-competitive, simultaneous sandwich assay.

Plates used for the detection of PT or FHA were coated with antibodies against these proteins. Plates used in the detection of serotype 2 or 3 fimbriae or pertactin were coated with *B. pertussis* cells. Horse radish peroxidase (HRP) conjugated monoclonal antibodies (mAb), specific for each antigen were used as the secondary antibody. The *B. pertussis* samples were added to plate wells, together with the HRP conjugated mAb and incubated for 1 hour at room temperature. If present in the sample, the antigen is bound specifically by the HRP conjugated mAb and the immobilised antibody simultaneously. On the addition of NBT substrate, HRP catalyses the formation of a coloured product. This reaction was allowed to proceed until the absorbance of a positive control well reached a fixed level, when sulfuric acid (0.25 M, final concentration) was added to stop the reaction. The absorbances, indicative of the amount

of antigen present were then measured spectrophotometrically at 450 nm using a Bio-Rad Model 3550 Microplate Reader. Pre-coated ELISA plates were supplied by CSL Ltd.

Chapter Three Results

Part A. Pertussis Toxin Expression in *B. bronchiseptica*

The current strategy for the vaccination against whooping cough is to immunise with a component vaccine, consisting of detoxified and purified virulence determinants from *B. pertussis*. Included in all such vaccines produced thus far has been pertussis toxoid. Therefore the expression of large amounts of genetically detoxified PT is required. The expression of PT in *B. pertussis* itself is inherently low and this bacterium is also slow-growing and nutritionally fastidious. Production of PT in *E. coli* has also not been achieved to an acceptable extent due to the incompatibility of gene expression signals, inclusion body formation and posttranslational processing problems. Therefore, the basis of this work is to use the closely related species, *B. bronchiseptica*, to express and secrete PT. This species is faster growing and less fastidious than *B. pertussis* and due to the high homology to *B. pertussis* was thought to be an excellent candidate PT production species.

3.1 Characterisation of Original Strains

The strain from which the PT expression locus was to be cloned, ATCC 10580::Tn*fusPT1* is a permanently avirulent strain of *B. bronchiseptica*, genetically modified to produce large amounts of pertussis toxin (Walker *et al.*, 1991a). It was constructed by cloning a promoterless PT operon into a suicide vector where it was randomly transposed into the chromosome of an avirulent *B. bronchiseptica* strain under the control of a strong constitutive promoter (Walker *et al.*, 1991a). The PT production status of

this strain was analysed using polyacrylamide gel electrophoresis and western blotting techniques. It was found that the PT expression levels of ATCC 10580::Tn*fusPT1* had remained unchanged since the original study (Walker *et al.*, 1991a) (result not shown). Although the permanent loss of virulence in this strain is due to deletions in of the *bvg* locus, the avirulent (non-haemolytic) status of ATCC 10580::Tn*fusPT1* was confirmed by culturing on Bordet Gengou agar.

In this study, isogenic *bvg*-positive and *bvg*-negative strains of *B. bronchiseptica* were used to compare PT expression and secretion levels. Thus, the virulent *B. bronchiseptica* strain BB7865 and the isogenic avirulent strain BB7866 (Monack *et al.*, 1989) were tested for haemolytic activity. To obtain a second set of isogenic strains, it was necessary to create a *bvg*-negative derivative of the virulent (haemolytic) *B. bronchiseptica* strain 5376. This was achieved by continued subculture of this strain on Bordet Gengou agar. Eventually, large, flat, non-haemolytic spontaneous avirulent mutant colonies were isolated and one designated *B. bronchiseptica* 5377 (result not shown). Naturally, a non-haemolytic phenotype may also result from a mutation in the *cya* operon encoding adenylate cyclase and associated accessory proteins. Proof that the non-haemolytic phenotype displayed by 5377 was caused by a spontaneous mutation in the *bvg* operon was therefore required. It was demonstrated that *B. bronchiseptica* 5377, was genuinely avirulent when the addition of pMW132 (Walker *et al.*, 1991a) (Appendix III) converted the phenotype to haemolytic (results not shown), thereby complementing the mutation. This plasmid contains the genes encoding the *bvg* two component signal transduction system.

3.1.1 Isolation of Spontaneous Resistance Mutants

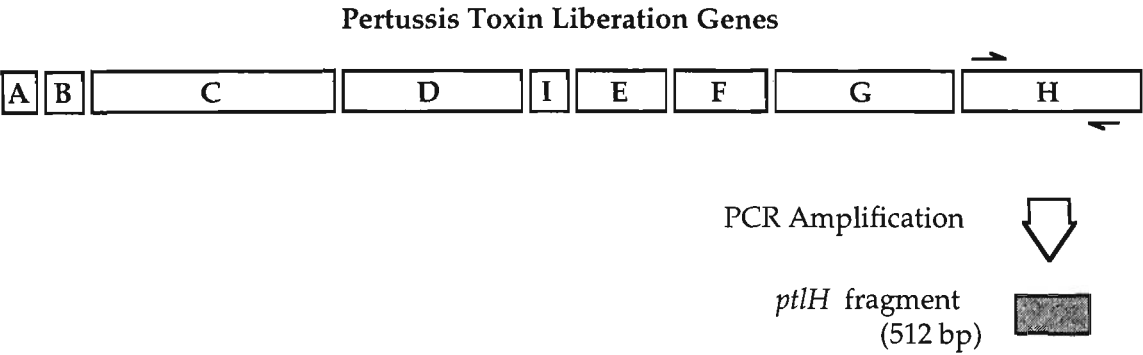
To aid in the selection of transconjugants in subsequent mating experiments, all four recipient strains (the virulent *B. bronchiseptica* 5376 and BB7865, and the isogenic avirulent strains 5377 and BB7866) were subjected to passaging on agar containing rifampicin until spontaneous resistant mutant colonies arose. The ease with which spontaneous rifampicin resistant mutants were isolated, created the possibility of this phenomenon occurring in subsequent matings. Therefore the rifampicin resistant recipient strains were also passaged on agar containing nalidixic acid to allow for double resistance mutants to be used in further matings. During this process, it was found that BB7865, and BB7866, possessed a natural resistance to nalidixic acid. Rifampicin/nalidixic acid double resistance mutants of BB7865, BB7866, 5376 and 5377 were isolated. These strains, designated BB7865 Rif^r, BB7866 Rif^r, 5376 Rif^r Nal^r, and 5377 Rif^r Nal^r were used as recipients in subsequent conjugation experiments. The donor strain, *B. bronchiseptica* ATCC 10580::Tn*fusPT1* was found to be sensitive to both of these antibiotics.

3.1.2 Genetic Analysis of *B. bronchiseptica* for the Presence of Pertussis Toxin Secretion Genes

With the failure of *B. bronchiseptica* ATCC 10580::Tn*fusPT1* to secrete PT in an earlier study (Walker *et al.*, 1991a), and the characterisation of the *ptl* genes of *B. pertussis* which are required for pertussis toxin secretion (Weiss *et al.*, 1993) it was important to determine whether *B. bronchiseptica* possesses an intact *ptl* operon. Most of the 60 base differences in the *ptlA-ptlC* region of *B. pertussis* and *B. bronchiseptica* are situated around the *ptlA* gene (Locht and Keith, 1986; Nicosia *et al.*,

1986; Weiss *et al.*, 1993). The homology between *B. pertussis* and *B. bronchiseptica* *ptlB* and *ptlC* genes is very high, with only 5 base differences in 924 base pairs. Although *B. bronchiseptica* Ptl proteins are not detected in immunoassays, only low levels of three of the Ptl proteins could be detected in *B. pertussis* (Johnson and Burns, 1994). Replacement of the *B. bronchiseptica* *Pptx* promoter with the *B. pertussis* *Pptx* promoter results in detectable expression of PtlF in western blots (Kotob *et al.*, 1995) suggest that *B. bronchiseptica* may contain functional *ptl* genes, and only the promoter was non-functional. It was assumed that homologous sequences at both ends of the operon would be indicative of a complete *ptl* operon. Thus, *B. bronchiseptica* chromosomal DNA was probed for the presence of the *ptlH* gene at the 3' end of the operon. The *EcoRI* restricted DNA of three strains of *B. bronchiseptica* and one strain of *B. pertussis* underwent Southern hybridisation analysis (Figure 3.1). The PCR amplified 0.5 kb *ptlH* fragment was radiolabelled and used as a probe. It was found that all strains tested exhibited equal binding, suggesting that the genome of *B. bronchiseptica* contains sequences homologous to the *ptlH* gene of *B. pertussis*.

A



B

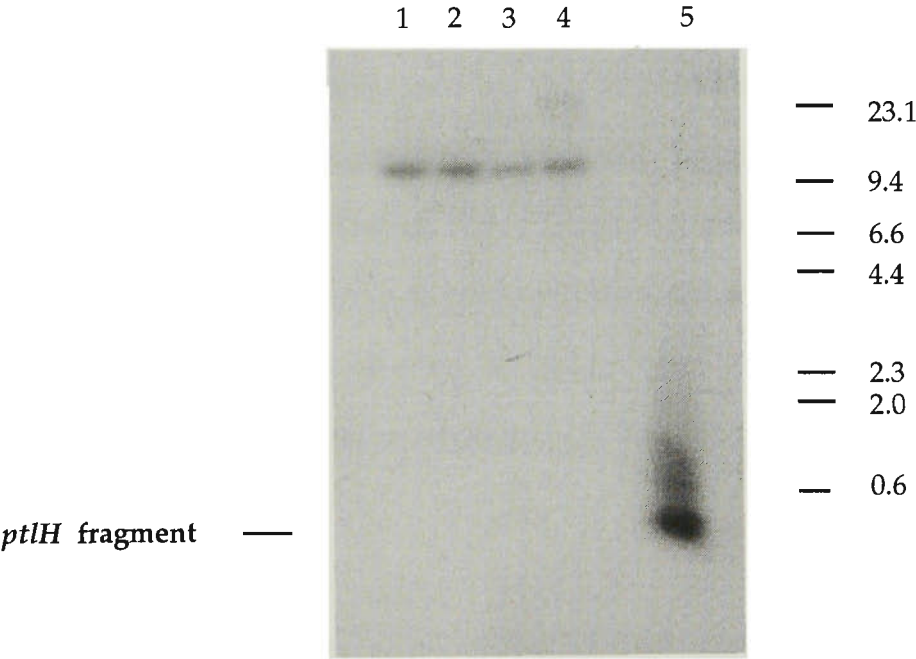


Figure 3.1 Genetic analysis of pertussis toxin secretion genes. (A) Diagrammatic representation of the *ptl* operon and amplification of the *ptl* gene fragment (*ptlH*) which is essential for PT secretion in *B. pertussis*. Specific oligonucleotide primers (arrowheads) were designed for the amplification of the 512 bp fragment (shaded box) using PCR technology. Primers used for this procedure, PTSECRTN 5' and PTSECRTN 3' are listed in Table 2.3. (B) Southern hybridisation of *Eco*RI digested chromosomal DNA of three *B. bronchiseptica* strains, ATCC 10580 (lane 1), 5377 (lane 2), BB7866 (lane 3) and *B. pertussis* Tohama I (lane 4) using the ³²P labelled 0.5 kb *ptlH* fragment as a probe (lane 5). Molecular weight markers (kb) are indicated.

3.2 *In vivo* Cloning of the Pertussis Toxin Producing Locus

The cloning of the PT expression locus of ATCC 10580::Tn*fusPT1*, encompassing the PT operon, the *B. bronchiseptica* promoter (P_{Bb}) and the kanamycin resistance gene was first attempted using cosmid cloning techniques. A number of kanamycin resistant clones were obtained, however whilst all contained the kanamycin resistance gene, none of these contained sequences homologous to the recombinant PT operon as determined by Southern hybridisation analyses (results not shown). Possibly the genetic locus containing the PT operon controlled by P_{Bb} is either unstable in *E. coli* or produces gene products which are lethal to *E. coli*. With the failure of cosmid cloning to isolate the PT expression locus for transfer to *B. bronchiseptica* strains, an *in vivo* technique was developed.

Two mating experiments were required for the *in vivo* cloning of the Tn*fusPT* PT expression locus. Firstly, pR751::Tn813 was conjugally transferred from *E. coli* Q358 (pR751::Tn813) into *B. bronchiseptica* ATCC 10580::Tn*fusPT1*. In the second mating, chromosome transfer between *B. bronchiseptica* ATCC 10580::Tn*fusPT1* (pR751::Tn813) and the rifampicin and nalidixic acid resistant derivatives of *B. bronchiseptica* strains occurred. Transconjugants containing the PT expression locus were identified by dual antibiotic selection for transfer (kanamycin resistance) in the recipient strains (rifampicin resistance).

3.2.1 Transfer and Integration of pR751::Tn813

Escherichia coli Q358 (pR751::Tn813) (Bowen and Pemberton, 1985)

harbours the trimethoprim resistant, broad host range plasmid pR751 (Jobanputra and Datta, 1974), containing the cointegrate-forming transposon, Tn813 (Diver *et al.*, 1983). Transposon Tn813, is a transposase positive (*tnpA*⁺), resolvase negative mutant (*tnpR*⁻) derivative of the mercury resistant transposon Tn21 (Grinstead *et al.*, 1982). Plasmid pR751 (48 kb) possesses transfer genes (*tra*) and an origin of transfer (*oriT*), allowing for the conjugal transfer of the plasmid DNA between donor and recipient strains. The entire plasmid then integrates into the recipient chromosome via the transposase of Tn813. Since the resolvase capability of this entity is non-functional due to a deletion mutation (Diver *et al.*, 1983), the plasmid DNA remains part of the recipient chromosome. The *tra* genes and *oriT* of pR751, now resident on the bacterial genome, thus transform the chromosome into a high frequency recombinant. This enables the principal of chromosome transfer to occur in subsequent conjugation experiments.

The strain carrying the PT expression locus, *Bordetella bronchiseptica* ATCC 10580::Tn*fusPT1* was mated with *Escherichia coli* Q358 pR751::Tn813 (Figure 3.2). The resultant growth from this mating was plated onto SS-X agar containing kanamycin and trimethoprim to select for *B. bronchiseptica* transconjugants (ATCC 10580::Tn*fusPT1* containing pR751::Tn813). This mating selected for the transfer of the conjugative plasmid into the PT expression strain of *B. bronchiseptica*. The frequency of transfer of this mating was calculated as 3.2×10^{-1} transconjugants per recipient cell (Table 3.1). A single transconjugant from this mating was tested separately, on urease agar, SS-X agar supplemented with cephalexin, and Bordet Gengou agar. After it was confirmed that this clone was a non-haemolytic, urease positive *B. bronchiseptica* strain, it was designated ATCC 10580::Tn*fusPT1* (pR751::Tn813) and used in

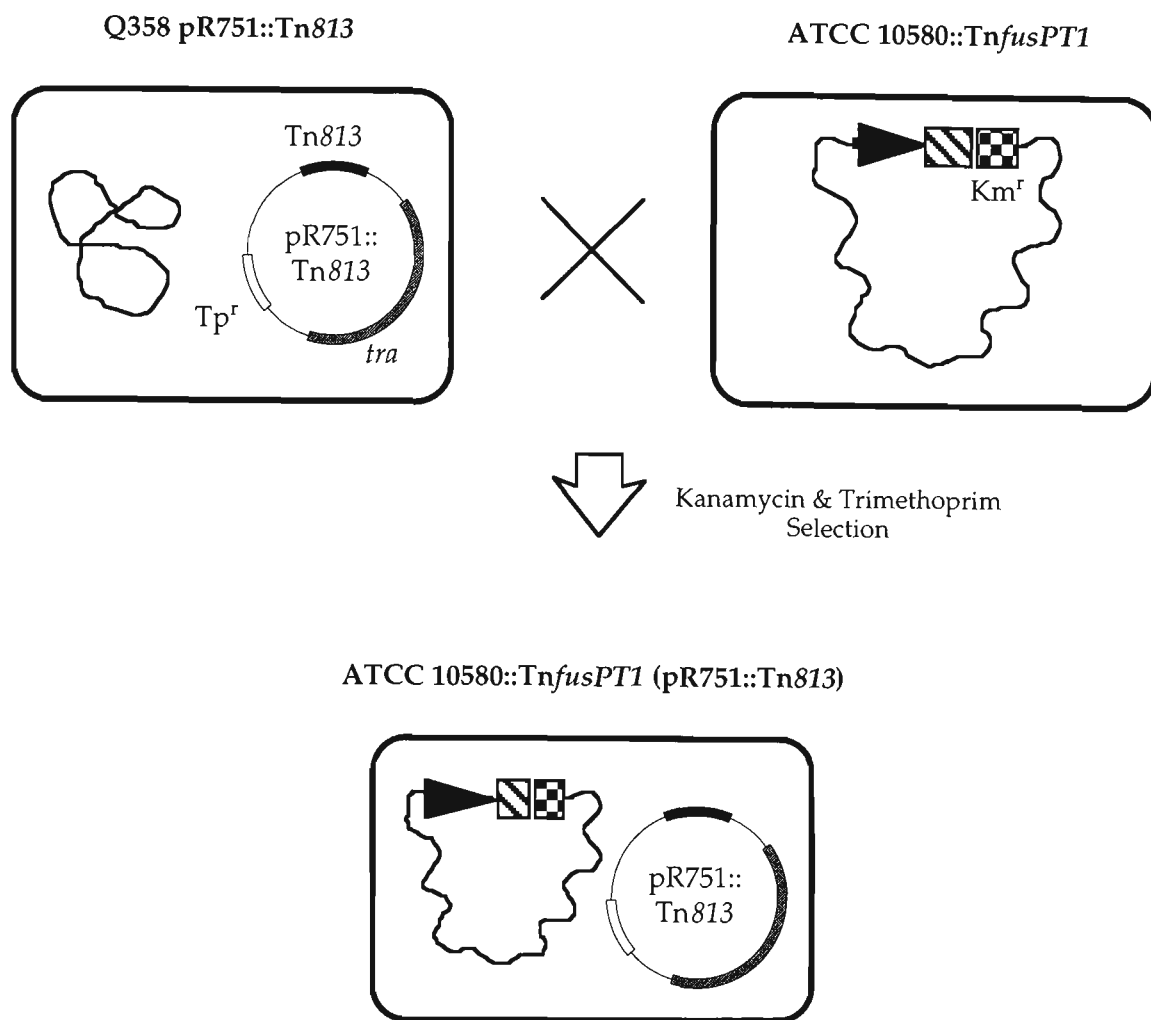


Figure 3.2 Transfer of pR751::Tn813 into ATCC 10580::Tn813. The *Escherichia coli* strain, Q358 (pR751::Tn813) contains the conjugative plasmid pR751, harbouring the cointegrate forming transposon Tn813 (filled box). When this strain is mated with ATCC 10580::Tn813, products encoded by the *tra* genes of pR751 (shaded box) induce the transfer of the plasmid to the *B. bronchiseptica* strain. The transposase enzyme encoded by Tn813 catalyses cointegrate formation of pR751::Tn813 with the chromosome of ATCC 10580::Tn813. This forms a permanent genomic cointegrate due to the defective resolvase gene of Tn813. The trimethoprim resistance gene is represented by an open box. The PT expression locus of ATCC 10580::Tn813 consisted of the promoter P_{BB}, (filled arrowhead), the PT operon (striped box), and the kanamycin resistance gene (checked box).

subsequent experiments. The newly transferred plasmid is capable of cointegrative transposition into the bacterial chromosome, mediated by the transposase enzyme of Tn813.

3.2.2 Chromosome Transfer and Homologous Recombination of the Pertussis Toxin Production Locus

Now integrated into the chromosome, pR751 still has the ability to induce conjugation. The transfer of part of the ATCC 10580::Tn*fusPT1* chromosome into a recipient can be achieved using the integrated pR751 as a catalyst for this transfer. Two virulent and two avirulent *B. bronchiseptica* strains were each mated with ATCC 10580::Tn*fusPT1* (pR751::Tn813) (Figure 3.3) and the resultant growth was plated onto SS-X agar with kanamycin and rifampicin selection. As would be expected for this mechanistically difficult chromosome transfer event, transconjugants arose at a frequency in the order of 10^{-6} - 10^{-8} kanamycin resistant transconjugants per recipient cell (Table 3.1). Due to the promiscuous nature of the conjugative plasmid, the transfer of trimethoprim resistance occurred at a much higher rate, in the order of 10^{-1} transconjugants per recipient cell (Table 3.1).

The high number of rifampicin spontaneous mutants formed in the initial mutation experiments led to an extra precaution when selecting transconjugants. When the donor, ATCC 10580::Tn*fusPT1* (pR751::Tn813) was mated with each of the four *B. bronchiseptica* recipient strains, there was a distinct possibility that many of the transconjugants were in fact merely spontaneous rifampicin resistant mutants of the donor strain. To select transconjugants (*B. bronchiseptica* Rif^r Nal^r::Tn*fusPT1*), single Kan^r Rif^r colonies growing on selection plates were replica plated onto SS-X

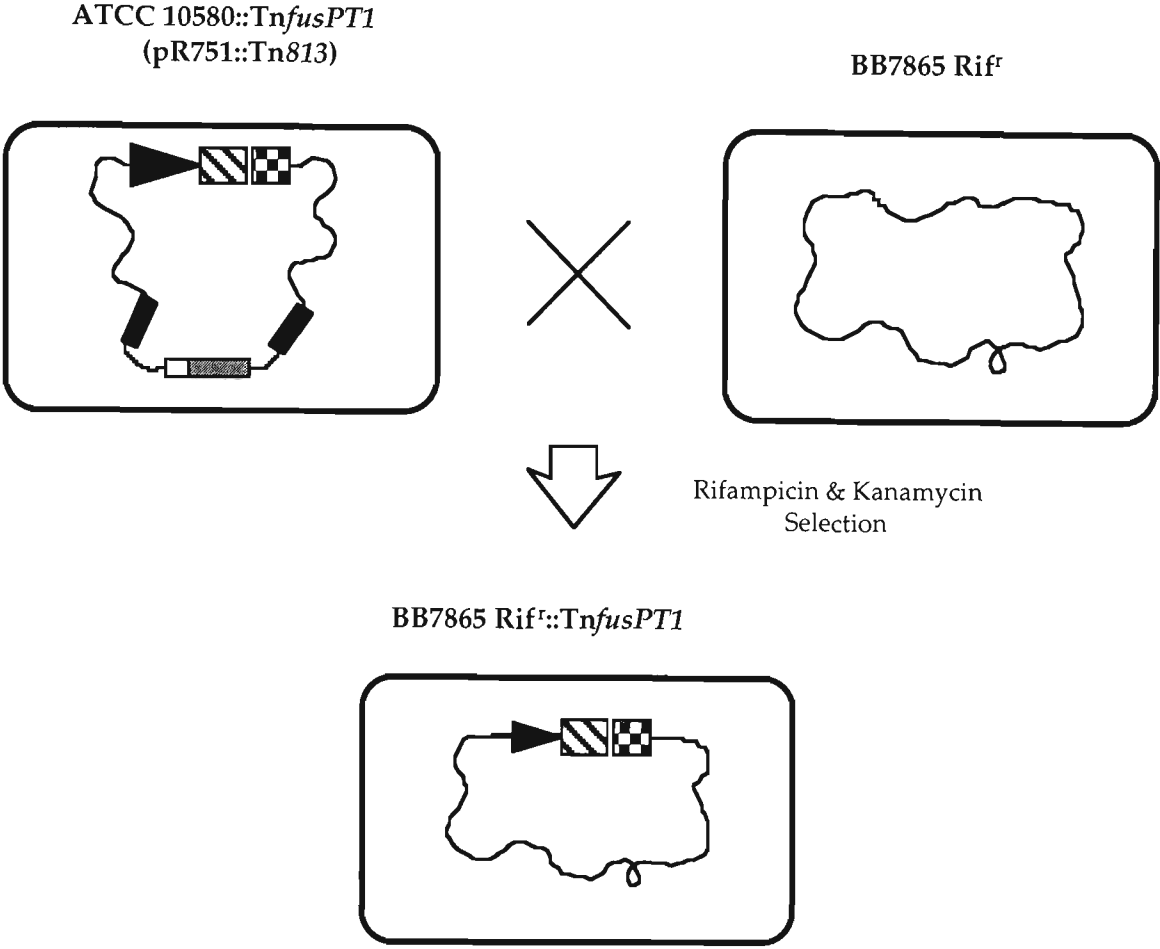


Figure 3.3 *In vivo* transfer of the PT expression locus of *Bordetella bronchiseptica* ATCC 10580::Tn_{fusPT1}. The newly formed strain, designated ATCC 10580::Tn_{fusPT1} (pR751::Tn813) was conjugally mated with the *B. bronchiseptica* strains (eg. BB7865 Rif^r). The *tra* genes of pR751, once within the chromosome, promotes chromosome transfer. The transferred section of donor DNA then undergoes homologous recombination to become integrated into the recipient genome. The PT expression locus is thus transferred to the recipient strain. The PT expression locus of ATCC 10580::Tn_{fusPT1} consisted of the promoter P_{Bb}, (filled arrowhead), the PT operon (striped box), and the kanamycin resistance gene (checked box).

agar with kanamycin and rifampicin selection and onto SS-X agar with nalidixic acid selection. As spontaneous double mutants are extremely rare (less than 1 in 10⁹), the colonies which were kanamycin, rifampicin and nalidixic acid resistant were selected for further study. The proportion

Table 3.1 Frequency of transfer data of the PT expression locus (Kan^r) and the conjugative plasmid pR751::Tn813 (Tp^r) from ATCC 10580::Tn*fusPT1* (pR751::Tn813) to each of the *B. bronchiseptica* recipient strains. Frequency of transfer is measured as transconjugant cells per recipient cell.

Recipient Strain	Plasmid Transfer	Chromosome Transfer
5376 Rif ^r Nal ^r	1.60 × 10 ⁻²	1.04 × 10 ⁻⁶
5377 Rif ^r Nal ^r	8.71 × 10 ⁻¹	7.92 × 10 ⁻⁸
BB7865 Rif ^r	2.50 × 10 ⁻²	1.52 × 10 ⁻⁶
BB7866 Rif ^r	6.60 × 10 ⁻¹	8.25 × 10 ⁻⁹

of transconjugants expressing resistance to nalidixic acid was ~50%. The colonies sensitive to nalidixic acid, were assumed to be spontaneous mutants of the donor strain. The Kan^r Rif^r Nal^r transconjugants were tested on SS-X agar with trimethoprim selection and exhibited 100% resistance to this antibiotic; presumably, these transconjugants also contain the plasmid pR751::Tn813 which encodes the trimethoprim resistance gene. Virulent and avirulent derivatives containing the mobilised PT expression locus retained the appropriate phenotype as determined by haemolytic activity on BG agar plates (results not shown). A selected Kan^r Rif^r Nal^r transconjugant from each of the four matings, designated *B. bronchiseptica* BB7865 Rif^r::Tn*fusPT1*, BB7866 Rif^r::Tn*fusPT1*, 5376 Rif^r Nal^r::Tn*fusPT1*, and 5377 Rif^r Nal^r::Tn*fusPT1*, was taken for further analysis using Southern hybridisation, PAGE and ELISA techniques.

3.3 Analysis of Recombinant Strains

3.3.1 Southern Hybridisation Analysis

To confirm the success of the *in vivo* cloning of the PT expression locus, Southern hybridisation analysis was performed. The chromosomal DNA of recombinant strains and their respective parental strains was digested with *Eco*RI restriction endonuclease and subjected to agarose gel electrophoresis. The subsequent restriction profile was then Southern transferred, and probed with ³²P labelled pMW127 DNA (Appendix III) (Walker *et al.*, 1991a). This plasmid contains the recombinant mini-transposon *Tn_{fusPT}* (Figure 3.1). The results presented in Figure 3.4 suggest that the PT expression locus, (P_{Bb} + *Tn_{fusPT}*, 10.5 kb band) was transferred from ATCC 10580::*Tn_{fusPT1}* to each of the two virulent and two avirulent strains of *B. bronchiseptica*. The 2.2 kb kanamycin resistance gene and the cryptic PT operon which is present in all *B. bronchiseptica* strains (Arico and Rappuoli, 1987) were also detected. The plasmid pMW127 contains an additional reactive band (Figure 3.4B, Lane 12) which represents the suicide plasmid into which the promoterless PT operon was cloned (pUT::*miniTn5/Km*) (Herrero *et al.*, 1990) to form pMW127. The PT expression system, including the constitutive promoter from ATCC 10580 (P_{Bb} + *Tn_{fusPT}*, lane 2, 10.5 kb) was cloned, by *in vivo* techniques, into virulent and avirulent strains of *B. bronchiseptica* as demonstrated in Figure 3.2 and Figure 3.3.

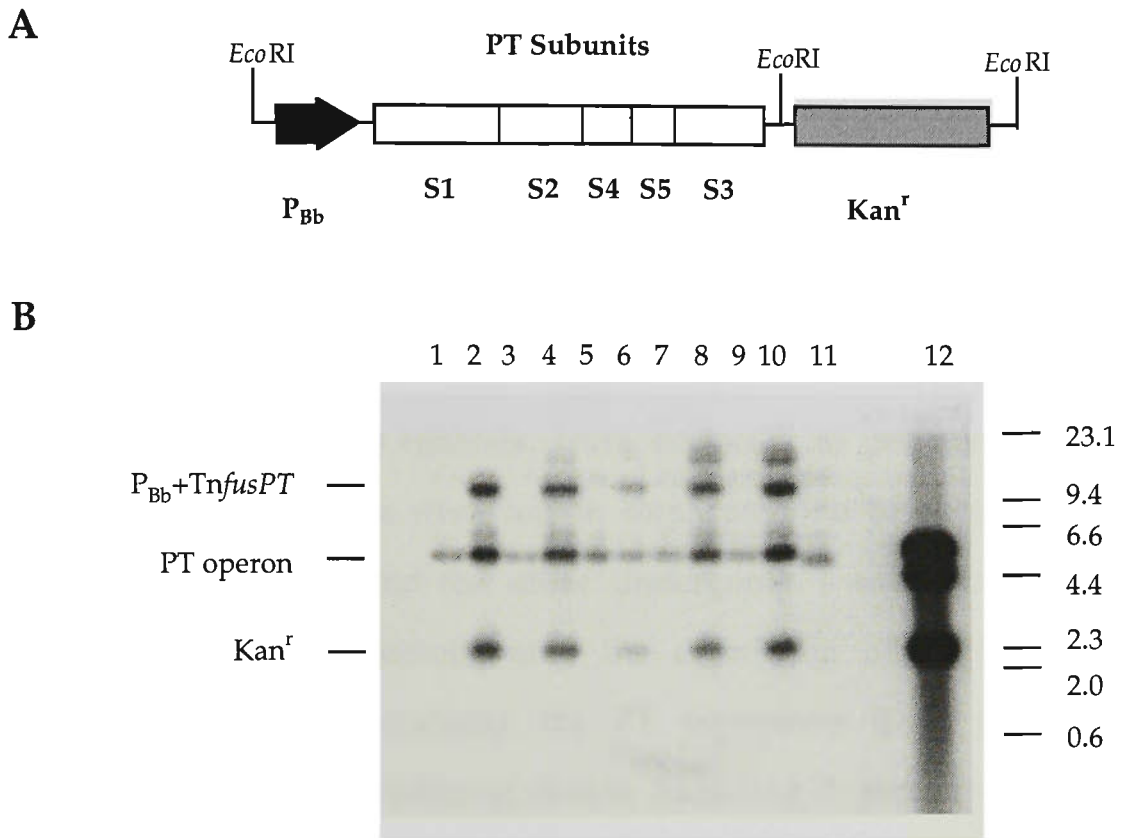


Figure 3.4 Diagrammatic representation of the PT expression locus (A). PT subunit genes S1 to S5 (open boxes), the kanamycin resistance gene (Kan^r ; shaded box) and the constitutive *B. bronchiseptica* promoter (P_{Bb} ; filled arrow) are indicated. Only *EcoRI* restriction enzyme sites are shown. (B) Southern hybridisation analysis of *B. bronchiseptica* strains before and after the transfer of the PT expression locus. *TnfnusPT* (incorporating the PT operon and kanamycin resistance gene) harboured on plasmid pMW127 (Walker *et al.*, 1991a) was used to probe the *EcoRI* digested genomic DNA of ATCC 10580 (lane 1), ATCC 10580::*TnfnusPT1* (lane 2), 5377 Rif^r Nal^r (lane 3), 5377 Rif^r Nal^r ::*TnfnusPT1* (lane 4), BB7866 Rif^r (lane 5), BB7866 Rif^r ::*TnfnusPT1* (lane 6), 5376 Rif^r Nal^r (lane 7), 5376 Rif^r Nal^r ::*TnfnusPT1* (lane 8), BB7865 Rif^r (lane 9), and BB7865 Rif^r ::*TnfnusPT1* (lane 10). lanes 11 and 12 contain *EcoRI* digested *B. pertussis* Tohama I and pMW127 DNA respectively. Sizes of markers (kilobases) are indicated.

3.3.2 Protein Analysis of Recombinant *B. bronchiseptica* Using PAGE and Western Blotting

The four recombinant *B. bronchiseptica* clones containing the PT expression locus were prepared for PAGE analysis. A sample of each recombinant strain, together with negative (parental strain) and positive (ATCC 10580::Tn*fusPT1*) controls, were subjected to polyacrylamide gel electrophoresis. The gels were run in duplicate, one being stained with Coomassie blue R250, and the other undergoing western transfer. The western blot analysis demonstrated the expression of PT by the new recombinant strains containing the PT expression locus (results not shown). All of the PT producing strains, including *B. pertussis* exhibited a band corresponding to the binding of the anti-S1 subunit monoclonal antibody, E19 (Walker *et al.*, 1991b) used as the primary antibody.

3.3.3 Analysis of *B. bronchiseptica* Strains for Pertussis Toxin Secretion by Enzyme Linked Immunosorbent Assay

Western blot analysis confirmed the transfer of the PT expression locus into two virulent and two avirulent strains of *B. bronchiseptica* was successful and that PT production was equivalent to that of the original strain. Supernatant and cellular fractions of *Bordetella* cultures were assayed for the presence of PT using ELISA techniques. Isogenic virulent and avirulent *B. bronchiseptica* strains and their PT producing derivatives were tested, as was *B. pertussis* Tohama I (Figure 3.5). Expression of PT by all *B. bronchiseptica* strains containing the PT expression locus was cell-associated, whereas the *B. pertussis* strain secreted large quantities of PT to the culture supernatant.

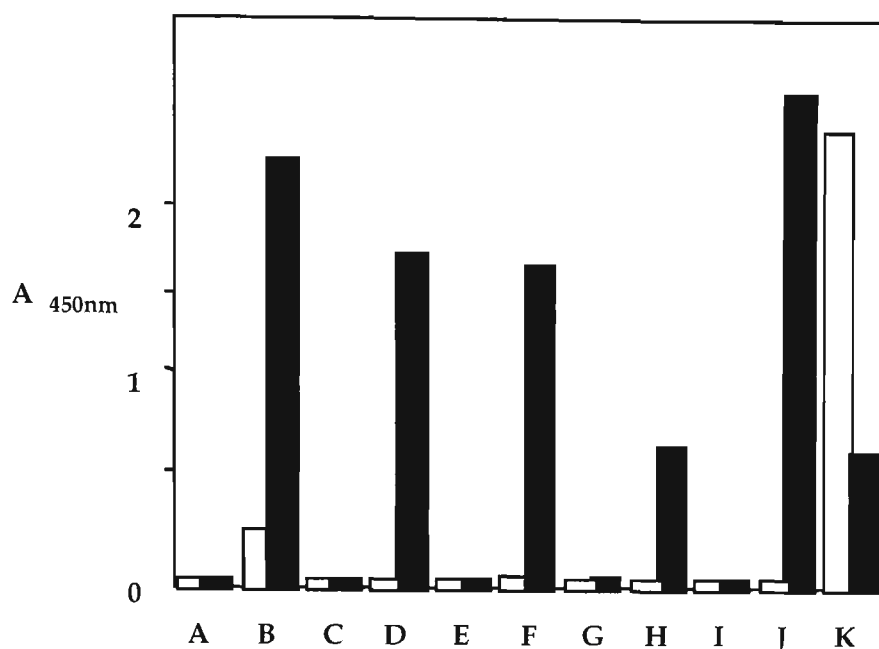


Figure 3.5 Analysis of pertussis toxin secretion by recombinant *B. bronchiseptica* strains. Absorbance at 450 nm corresponding to relative PT levels in cell-associated (filled bars) and culture medium fractions (open bars) of *B. bronchiseptica* strains (A) ATCC 10580, (B) ATCC 10580::Tn*fusPT1*, (C) 5377 Rif^r Nal^r, (D) 5377 Rif^r Nal^r::Tn*fusPT1*, (E) 5376 Rif^r Nal^r, (F) 5376 Rif^r Nal^r::Tn*fusPT1*, (G) BB7866 Rif^r, (H) BB7866 Rif^r::Tn*fusPT1*, (I) BB7865 Rif^r and (J) BB7865 Rif^r::Tn*fusPT1* were compared to equivalent fractions of (K) *B. pertussis* Tohama I using a PT-specific ELISA assay. In contrast to *B. pertussis* Tohama I which secreted a large proportion of the PT it produced to the growth medium, the PT produced by the recombinant *B. bronchiseptica* strains was cell-associated.

Thus, a system has been developed for the *in vivo* cloning and transfer of the PT expression locus of ATCC 10580::Tn*fusPT1*. The PT expression locus was transferred to isogenic *bug*-positive and *bug*-negative strains of *B. bronchiseptica* and the PT secretion levels of these strains was monitored. It was found that neither virulent nor avirulent *B. bronchiseptica* exhibited the ability to secrete PT, confirming that the *B. bronchiseptica* *ptl* genes are not expressed.

Part B. Genetic Analysis of *Bordetella pertussis* Virulence Factor Promoters

Research in the area of whooping cough vaccines is currently concentrated on the development of new generation subunit vaccines. A number of laboratories around the world are working on acellular vaccines, using component antigens purified from *B. pertussis*. Immunogenic antigens important in the protective efficacy of the current vaccine have recently been tested in human field trials of acellular vaccine preparations (Greco *et al.*, 1996; Gustafsson *et al.*, 1996). These antigens include pertussis toxin, filamentous haemagglutinin, serotype specific fimbriae and pertactin.

The current Australian whooping cough vaccine, developed by Commonwealth Serum Laboratories Australia Ltd. (CSL) consists of a heat killed whole-cell preparation of *Bordetella pertussis* included within the Diphtheria Tetanus Pertussis (DTP) Triple Antigen®. Research at CSL has been directed towards the selection of stable, virulent-phase *B. pertussis* vaccine strains capable of producing high levels of the desired vaccine antigens. In the endeavours to develop a more effective vaccine strain of *Bordetella pertussis*, CSL have, through continued subculture and selection, obtained non-recombinant naturally occurring mutant strains which overproduce PT, FHA, serotype specific fimbriae and pertactin.

Three of these strains, CSL 127S, CSL 137S and CSL 1237S (Table 3.2) represent clinically significant serotypes of *B. pertussis* and are excellent vaccine candidates for either a whole cell vaccine or for the production of antigens for new generation acellular vaccines. However the nature and

extent of the mutations resulting in antigen expression were not elucidated at the genetic level. Such mutations, once characterised, may be engineered into other *B. pertussis* vaccine strains to improve antigen yield.

3.4 Characterisation of Original CSL Strains

Three parental (wild-type) and three selected (overexpressing) strains were initially cultured on SS agar supplemented with cephalixin and Bordet Gengou agar to ensure their virulence status. All were found to be haemolytic *Bordetella pertussis* strains exhibiting small colony morphology indicative of virulent phase organisms (results not shown).

The antigen production of these strains was analysed using an ELISA. Data characterising the amount of antigen overproduction by the selected *B. pertussis* strains as compared with their respective parental strains were performed at CSL (Table 3.2). A problem arose at this point of the study when it was found that one of the parental strains, CSL 137 could not be analysed for virulence antigen expression. The strain, possibly after being passaged many times on agar plates during the selection process at CSL, seems to have lost the ability to grow in defined liquid SS medium. The antigen expression levels of the selected strain, CSL 137S cannot be compared to those of the parental strain, only those of the other CSL strains.

The selected *B. pertussis* strains demonstrate overexpression of different proteins which were therefore postulated to be the result of mutations at different genetic loci. This study aimed to determine the genetic basis of these mutations.

Table 3.2 Virulence factor production by CSL *B. pertussis* strains. All figures are concentrations measured in µg/mL except those for pertactin, which are concentrations measured in EIA units/mL. Abbreviations: ND, not detected.

Strain	PT	FHA	Fim2	Fim3	Pertactin
CSL 127	3.5	0.5	0.4	ND	12
CSL 127S	5.5	23.5	7.3	ND	14
CSL 137S	10.8	49	ND	6.8	351
CSL 1237	5.2	64	1.1	1.6	79
CSL 1237S	9.8	40	1.7	3.1	106

The chromosomal DNA from each strain was extracted, and using appropriate oligonucleotide primers, the relevant promoter and ribosome binding regions were amplified using PCR techniques. The amplified promoter DNA was then cloned into a plasmid cloning vector. The DNA sequence of the amplified promoter and ribosome binding region obtained from each PCR reaction was elucidated to determine the location of any differences (arising from mutations) between the selected and parental strains.

3.5 Analysis of CSL 127 Strains

Searching for an upregulation in the fimbrial production of potential vaccine strains, researchers at CSL Ltd. detected and isolated CSL 127S, a putative spontaneous mutant of the parental strain CSL 127. This strain exhibits an almost 20 fold increase in Fim2 production compared with the parental strain. An upregulation of FHA expression (47 fold) was also observed. Due to the linkage of the fimbrial and FHA accessory genes (*fimB-fimD, fhaC*), it was therefore hypothesised that the overexpression exhibited by this strain may be the result of a mutation in the promoter of the fimbrial/FHA accessory genes.

The fimbrial serotype expressed by *B. pertussis* is dependent upon the transcription from the major fimbrial subunit genes. Strain CSL 127 expresses serotype 2 fimbriae. The serotype can change *in vivo* by a switching of promoter activation termed fimbrial phase variation (Willems *et al.*, 1990). Sequence data of fimbrial genes, has revealed some interesting features of fimbrial promoter architecture (Figure 1.12) (Pedroni *et al.*, 1988; Loch *et al.*, 1992; Willems *et al.*, 1992). Alteration in the number of cytosine residues in the "C stretch" of the promoter is the source of fimbrial phase variation (Willems *et al.*, 1990). The distance between the putative -10 box and the BvgA binding site (Figure 1.12) may be crucial for the activation of transcription, and an alteration in the number of cytosine residues in the C stretch affects this distance (Willems *et al.*, 1990).

The genes encoding accessory proteins required for the correct delivery and presentation of both fimbriae and FHA, *fimC*, *fimD*, and *fhaC* are translationally linked (Willems *et al.*, 1993). All three of these genes overlap and there is a putative Shine-Dalgarno site directly upstream of *fimC*, the first of these genes. Translation of *fimD* may require the translation of *fimC* to prevent the creation of a hairpin loop just prior to the end of the *fimC* mRNA. This would allow continual ribosomal attachment and translation of *fimD* (Willems *et al.*, 1993; Willems *et al.*, 1994).

3.5.1 Amplification of CSL 127 Promoter Regions

Genomic DNA of both the parental (CSL 127) and selected (CSL 127S) strains was extracted. This DNA was then tested for purity and concentration then diluted to an appropriate level for use as template

DNA in PCR amplification reactions. Regions of DNA encompassing the putative promoters and ribosome binding sites of each of five relevant genes were amplified using PCR technology.

The bvg/fha Intergenic Region

The 470 base pair region between the *fhaB* gene and the *bvg* operon contains a number of virulence regulated and non-regulated promoters. The entire region was amplified using the oligonucleotide primers bvg/fha 5' and bvg/fha 3' (Figure 3.6).

The double stranded DNA PCR products shown in Figure 3.6 were then eluted from the gel to remove contaminating unincorporated nucleotides and excess primer DNA. The bvg/fha intergenic regions of CSL 127 and CSL 127S were amplified using *Pfu* DNA polymerase.

The Pertussis Toxin Gene Promoter Region

The 304 base pair region containing the CSL 127 and CSL 127S pertussis toxin gene promoter and Shine Dalgarno sequence was amplified using the oligonucleotide primers PT 5' and PT 3' (Figure 3.7).

The double stranded DNA PCR products shown in Figure 3.7 were then eluted from the gel to remove contaminating unincorporated nucleotides and excess primer DNA. The PT promoter regions of CSL 127 and CSL 127S were amplified using *Taq* DNA polymerase.

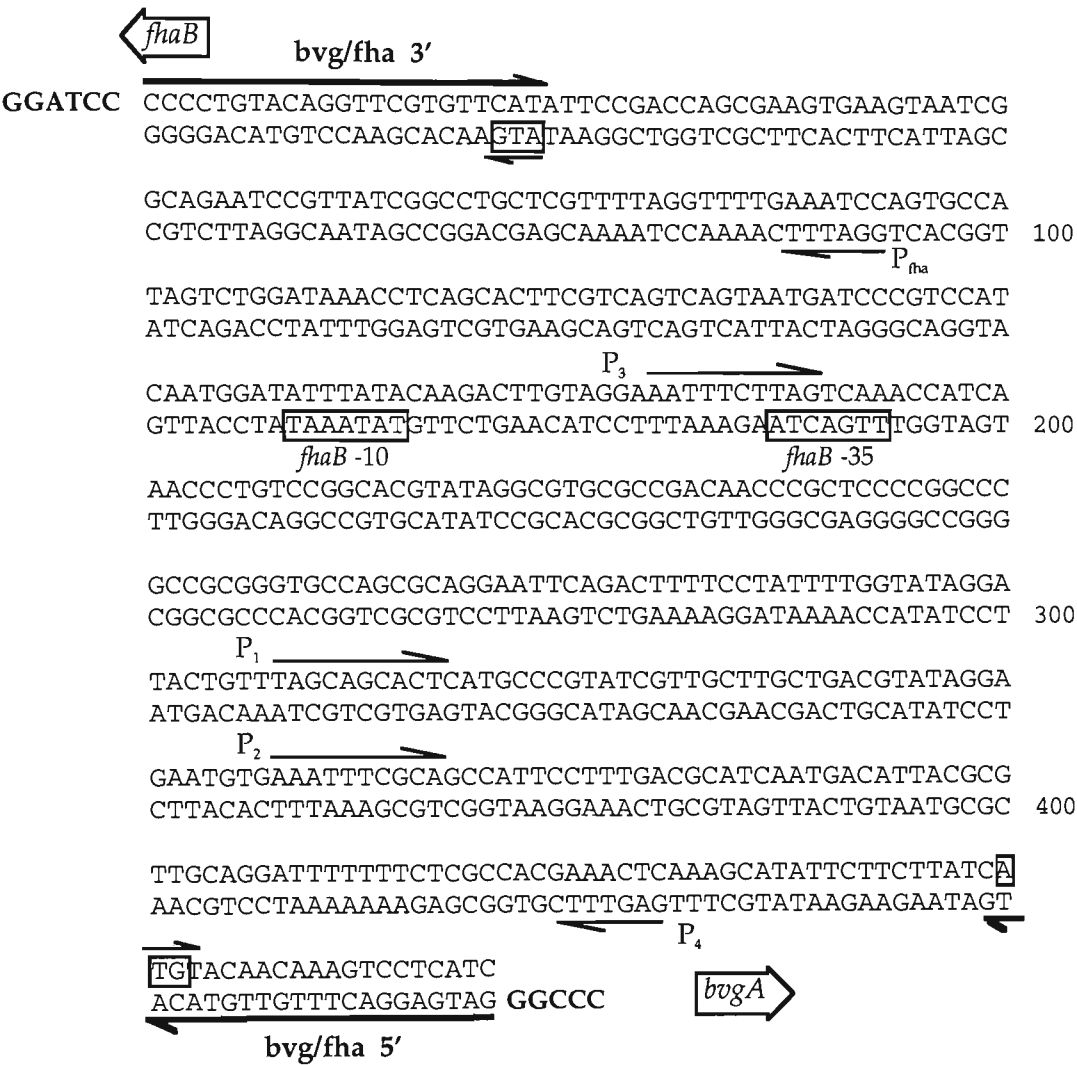
The Fimbrial Accessory Genes Promoter Region

The 800 base pair region containing the CSL 127 and CSL 127S fimbrial accessory gene promoter and Shine Dalgarno sequence was amplified using the fim access 5' and fim access 3' primers (Figure 3.8).

Figure 3.6 Amplification of the *bvg/fha* intergenic regions of CSL 127 and CSL 127S.

A. Genetic organisation of the *bvg/fha* intergenic region (Arico *et al.*, 1989; Domenighini *et al.*, 1990). Open arrows represent *bvgA* and *fhaB* genes, with arrows indicative of the direction of translation. Promoter regions *bvg* P₁, P₂, P₃, and P₄ as well as P_{*fha*} are shown. Start codons for each gene are boxed. Putative -10 and -35 boxes for *fhaB* are designated. Oligonucleotide primers are represented by long arrows. The non-homologous section of the *bvg/fha* 5' primer (*Sma*I site) and the corresponding region of the *bvg/fha* 3' primer (*Bam*HI site) are shown in bold type. **B.** Agarose gel electrophoresis of amplified *bvg/fha* intergenic regions of CSL 127 (lane 1) and CSL 127S (lane 2). Amplified bands are indicated.

A

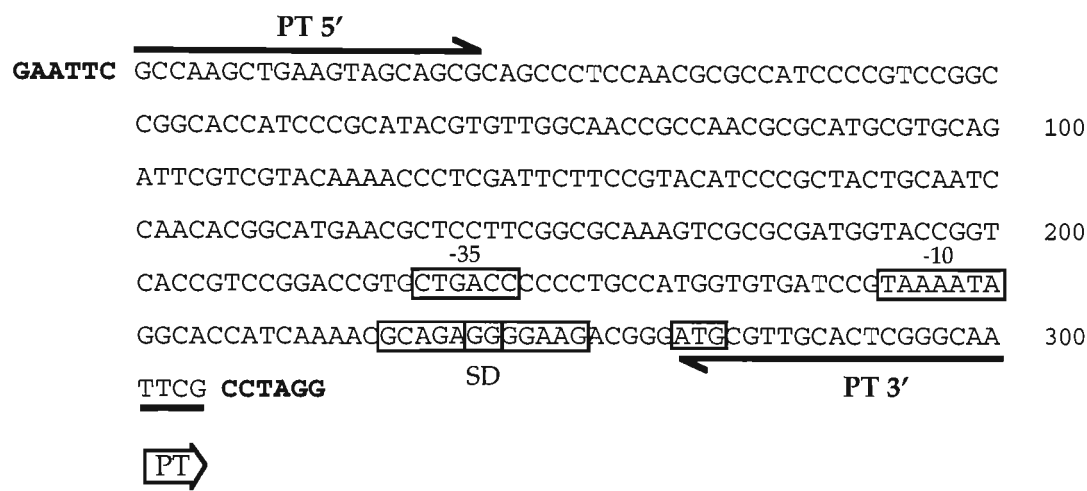


B



Figure 3.7 Amplification of the pertussis toxin genes promoter regions of CSL 127 and CSL 127S. **A.** Sequence and organisation of the P_{ptx} promoter (Locht and Keith, 1986; Nicosia *et al.*, 1986). Open arrow represents PT subunit genes, with the arrow indicative of the direction of translation. Putative -10 and -35 boxes are indicated, as are 2 putative overlapping consensus ribosome binding sites (SD, overlapping open boxes). Oligonucleotide primers are represented by long arrows with attached restriction enzyme (*Eco*RI and *Bam*HI) sites shown in bold type. The PT 3' primer includes the first 23 bp of the S1 subunit gene. **B.** Agarose gel electrophoresis of amplified PT promoter regions of CSL 127 (lane 1) and CSL 127S (lane 2). Amplified bands are indicated.

A



B

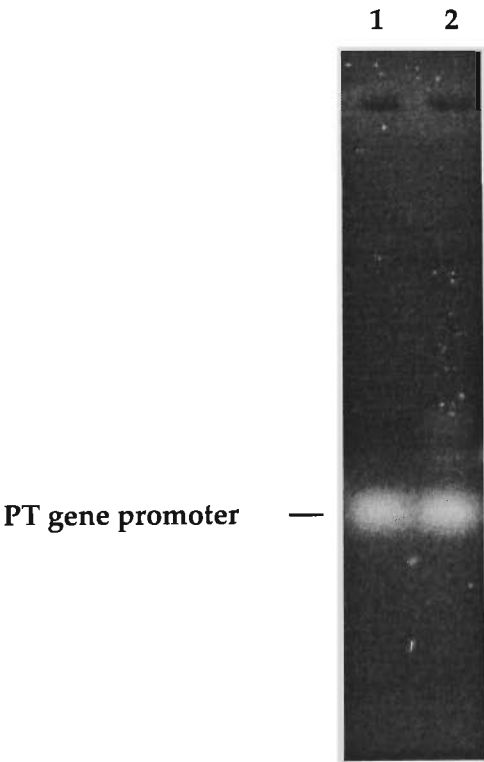


Figure 3.8 Amplification of the fimbrial accessory gene promoter regions of CSL 127 and CSL 127S. **A.** Nucleotide sequence of the region between the 3' end of *fhaB* and the 5' end of *fimB* (Willems *et al.*, 1992). Open arrows represent *fhaB* and *fimB* genes, with arrows indicative of the direction of translation. The putative -10 region (open) and BvgA binding sites (shaded) are boxed. The fim access 5' primer, incorporating the last 20 base pairs of the *fhaB* gene, is represented as a long arrow. The non-homologous section of the primer (*Eco*RI site) is shown in bold type. The fim access 3' primer, incorporating the first 23 base pairs of the *fimB* gene, is represented as a long arrow. The non-homologous section of the primer (*Bam*HI site) is shown in bold. Internal primers (+300Forward, -500Reverse) used for sequencing are also depicted. Inverted repeats (a, b, c, d) are shown above dotted lines. **B.** Agarose gel electrophoresis of amplified fimbrial/FHA accessory genes promoter regions of CSL 127 (lane 1) and CSL 127S (lane 2). Amplified bands are indicated.

The double stranded DNA PCR products shown in Figure 3.8 were then eluted from the gel to remove contaminating unincorporated nucleotides and excess primer DNA. The fimbrial accessory genes promoter regions of CSL 127 and CSL 127S were amplified using *Pfu* DNA polymerase.

The Type 2 Fimbriae Promoter Gene Region

The 203 base pair region containing the *fim 2* gene promoter and Shine Dalgarno sequence was amplified using the oligonucleotide primers fim2 5' and fim2 3' (Figure 3.9).

The double stranded DNA PCR products shown in Figure 3.9 were then eluted from the gel to remove contaminating unincorporated nucleotides and excess primer DNA. The *fim2* promoter regions of CSL 127 and CSL 127S were amplified using *Taq* DNA polymerase.

The Type 3 Fimbriae Promoter Gene Region

The 320 base pair region containing the *fim 3* gene promoter and Shine Dalgarno sequence was amplified using the oligonucleotide primers fim3 5' and fim3 3' (Figure 3.10).

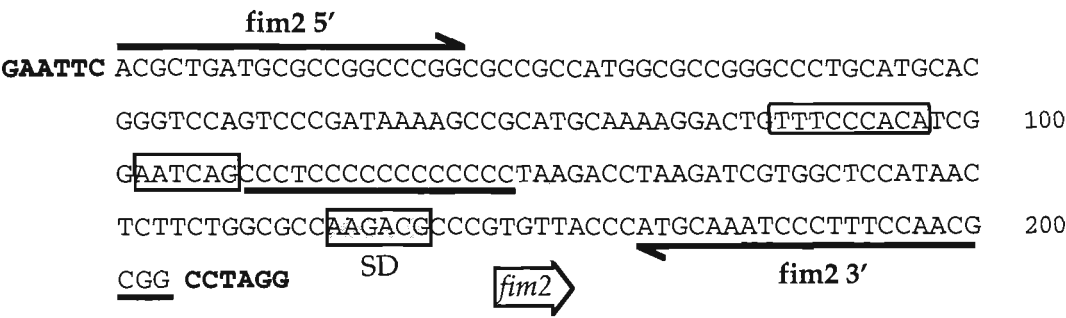
The double stranded DNA PCR products shown in Figure 3.10 were then eluted from the gel to remove contaminating unincorporated nucleotides and excess primer DNA. The *fim3* promoter regions of CSL 127 and CSL 127S were amplified using *Taq* DNA polymerase.

Figure 3.9 Amplification of the *fim2* gene promoter regions of CSL 127 and CSL 127S.

A. Genetic organisation and DNA sequence of the *fim2* gene promoter. (Livey *et al.*, 1987). The open arrow represents the *fim2* gene, with the arrow indicative of the direction of translation. Primers are represented by long arrowheads with attached restriction enzyme (*Eco*RI and *Bam*HI) sites shown in bold type. The *fim2* 3' primer incorporates the first 23 nucleotides of the *fim2* gene. The C-stretch is underlined and the putative ribosome binding site is indicated (SD, shaded box). The *fim* boxes (Riboli *et al.*, 1991) are also shown (open boxes).

B. Agarose gel electrophoresis of amplified *fim2* gene promoter regions of CSL 127 (lane 1) and CSL 127S (lane 2). Amplified bands are indicated.

A



B

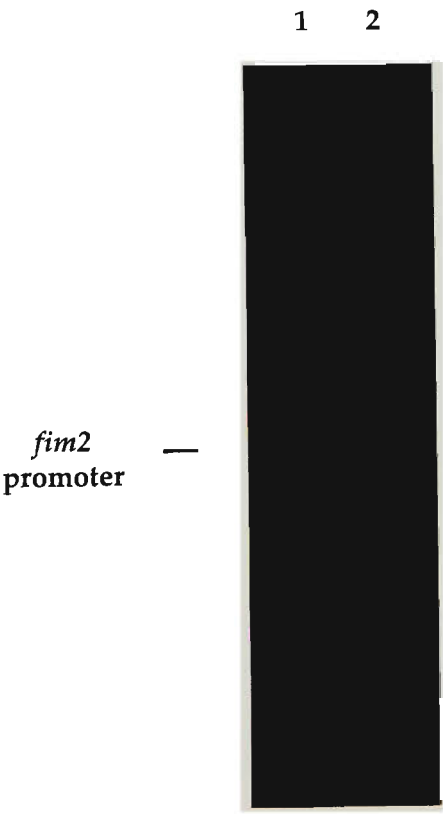
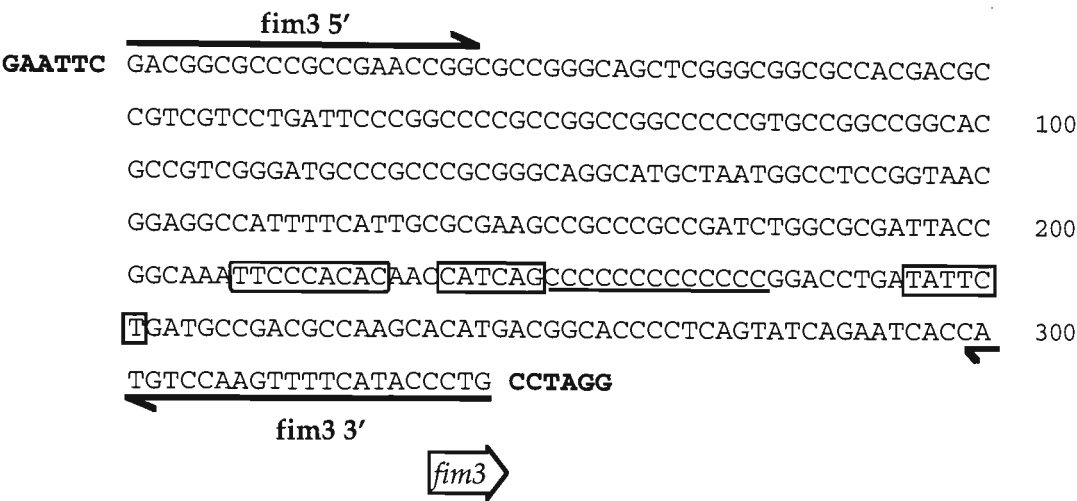


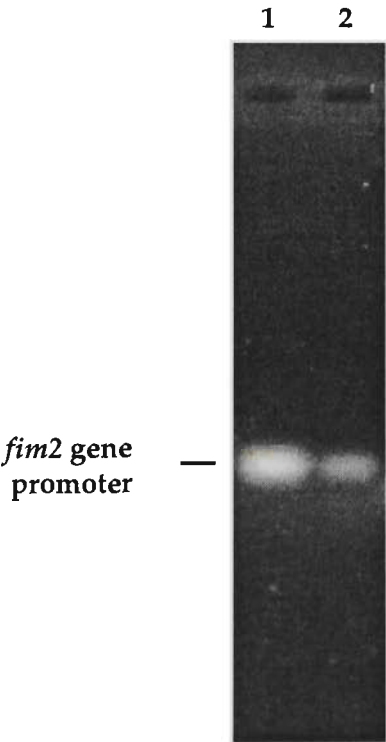
Figure 3.10 Amplification of the *fim3* gene promoter regions of CSL 127 and CSL 127S.

A. Genetic organisation and DNA sequence of the *fim3* gene promoter (Mooi *et al.*, 1990). The open arrow represents the *fim3* gene, with the arrow indicative of the direction of translation. Primers are represented by long arrows with attached restriction enzyme (*Eco*RI and *Bam*HI) sites shown in bold type. The *fim3* 3' primer incorporates the first 22 nucleotides of the *fim3* gene. The proposed -10 box is contained within a shaded box. The C-stretch is underlined. The *fim* boxes (Riboli *et al.*, 1991) are also shown (open boxes). **B.** Agarose gel electrophoresis of amplified *fim3* gene promoter regions of CSL 127 (lane 1) and CSL 127S (lane 2). Amplified bands are indicated.

A



B



3.5.2 Cloning of CSL 127 Promoter Region DNA

The PCR product of each relevant promoter region was cloned into one of the three cloning vectors used in this study. Products amplified using *Taq* DNA polymerase were cloned into pCRII or pCR2.1 due to the compatibility created by the A and T overhangs present on the PCR product and cloning vector respectively. When *Pfu* was used as the polymerising enzyme, products were cloned into the blunt-ended vector pCR Script. Ligations into pCRII and pCR2.1 were transformed into INV α F' cells and ligations involving pCR Script were transformed into competent XL1-Blue MRF' *Kan* cells.

The bvg/fha Intergenic Region

The amplified 470 base pair bvg/fha intergenic regions of CSL 127 and CSL 127S were cloned into pCR Script (Figure 3.11). In the interests of brevity, only the former of these clonings will be illustrated.

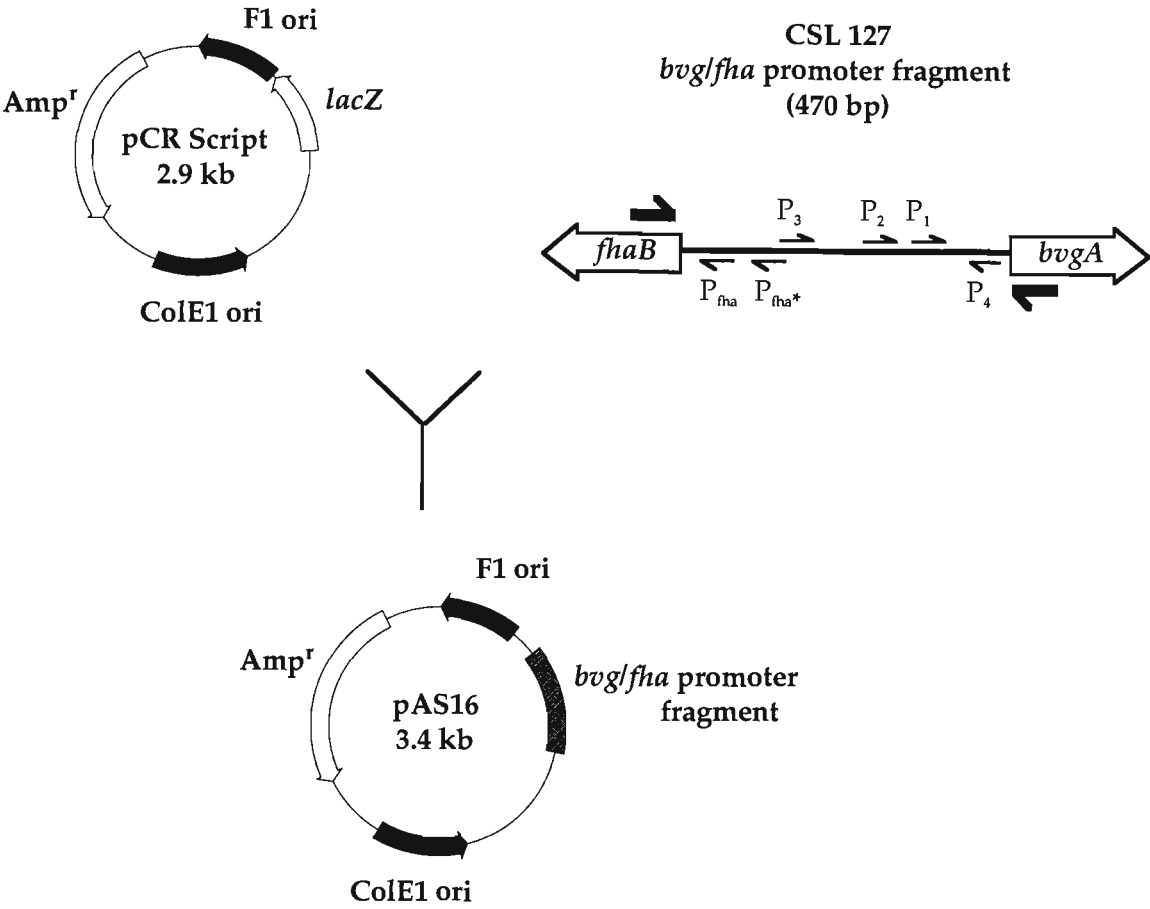
The cloning of the bvg/fha intergenic region of CSL 127 and CSL 127S as PCR products into pCR Script was confirmed by digestion with endonucleases *Eco* RV and *Sac* I. The restricted plasmids (pAS16 and pAS10) were then electrophoresed through a 1% agarose gel to separate the vector and insert fragments (Figure 3.11 B, lanes 1 and 2).

The Pertussis Toxin Gene Promoter Region

The amplified 304 base pair pertussis toxin gene promoter regions of CSL 127 and CSL 127S were cloned into pCRII (Figure 3.12 A). Again, only the former of these clonings will be illustrated.

Figure 3.11 Cloning of the *bvg/fha* intergenic regions of CSL 127 and CSL 127S. **A.** Amplification and cloning of the CSL 127 *bvg/fha* intergenic region into pCR Script. Genes are represented as open arrows demonstrating the direction of transcription. Promoter regions are indicated. Specific oligonucleotide primers (thick arrowheads) were used for the amplification of the *bvg/fha* intergenic region using PCR technology. The PCR product was ligated into the *SrfI* site of pCR Script. The *lacZ* gene (open arrow) and the ampicillin (Amp^r) resistance gene (open arrow) are indicated, as are F1 and ColE1 origins of replication (filled arrows) and the *bvg/fha* intergenic region (shaded box). **B.** Agarose gel electrophoresis of restricted pAS16 and pAS10, demonstrating release of the *bvg/fha* intergenic regions of CSL 127 (lane 1) and CSL 127S (lane 2) from pCR Script and pCRII respectively.

A



B

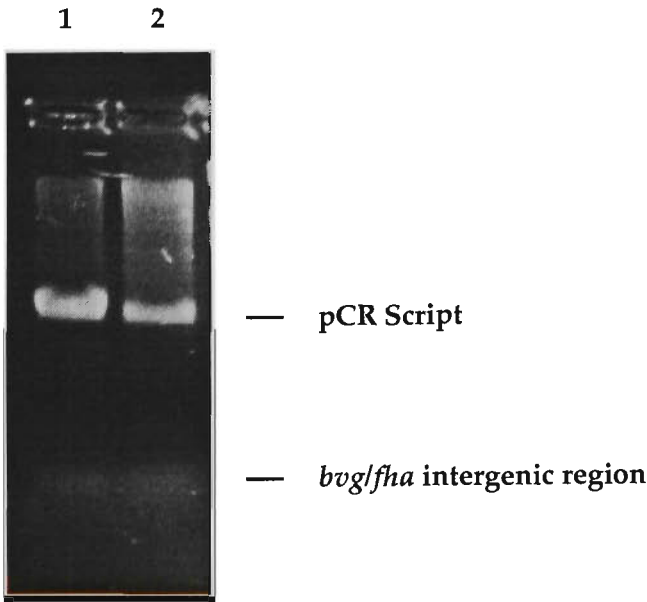
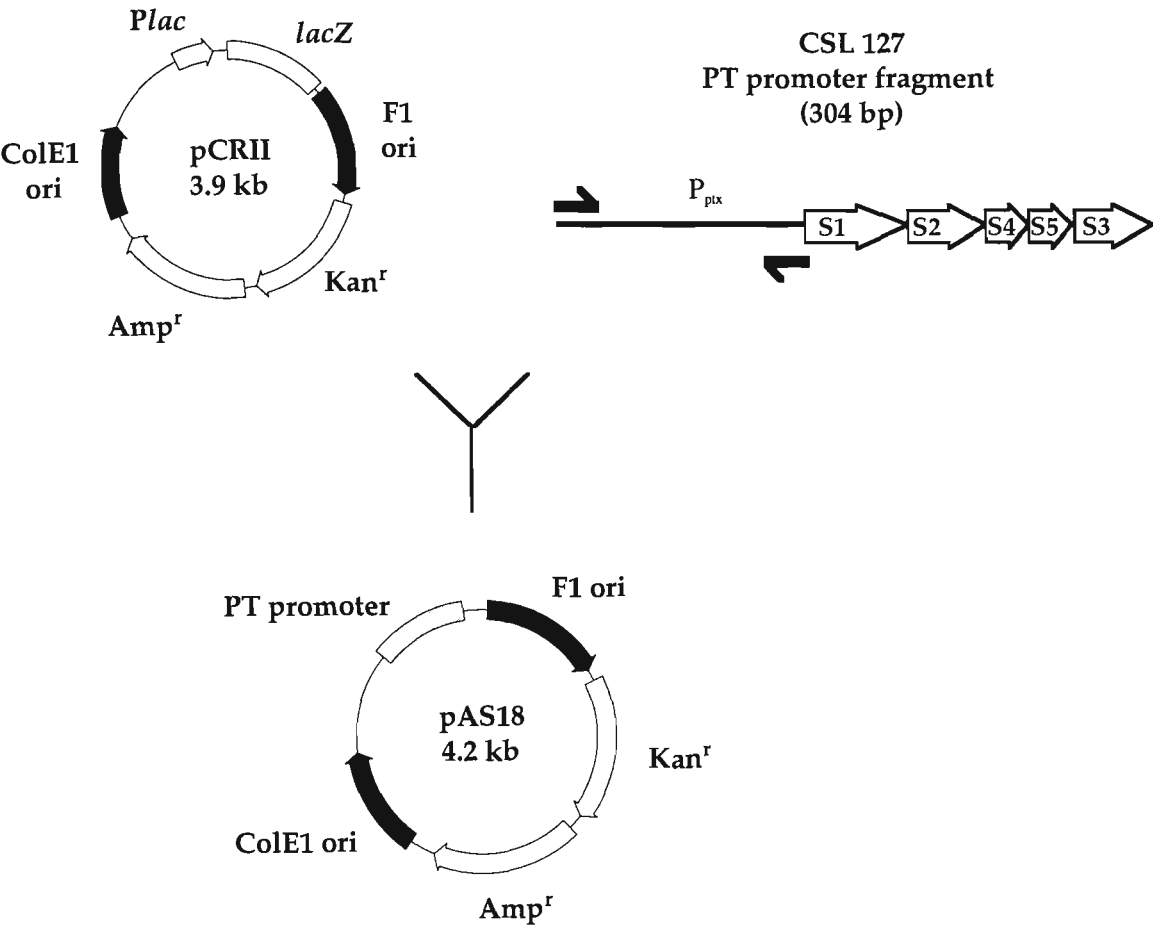
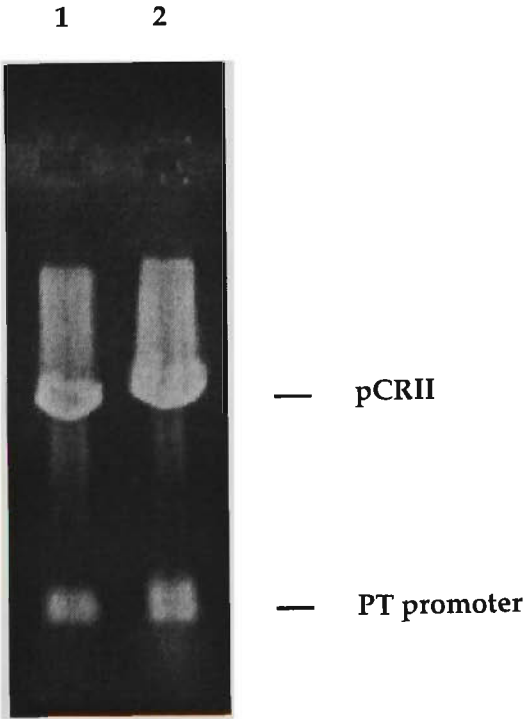


Figure 3.12 Cloning of the PT gene promoter regions of CSL 127 and CSL 127S. **A.** Amplification and cloning of the CSL 127 PT gene promoter region into pCRII. Genes are represented as open arrows demonstrating the direction of transcription. Primers used for the amplification of the PT promoter are represented by thick arrowheads. The PCR fragment was ligated into the dual *EcoRI* sites of pCRII. The *lacZ* gene (open arrow), the kanamycin (Kan^r) and ampicillin (Amp^r) resistance genes (open arrows) are indicated, as are F1 and ColE1 origins of replication (filled arrows) and the PT promoter (shaded box). **B.** Agarose gel electrophoresis of restricted pAS18 and pAS12, demonstrating release of the PT promoter regions of CSL 127 (lane 1) and CSL 127S (lane 2) from pCRII and pCR Script respectively.

A



B



The cloning of the PT gene promoter region of CSL 127 and CSL 127S as PCR products into pCRII was confirmed by digestion with endonuclease *Eco*RI. The restricted plasmids (pAS18 and pAS12) were then electrophoresed through a 1% agarose gel to separate the vector and insert fragments (Figure 3.12 B, lanes 1 and 2).

The Fimbrial Accessory Genes Promoter Region

The amplified 800 base pair fimbrial accessory genes promoter regions of CSL 127 and CSL 127S were cloned into pCR Script (Figure 3.13).

The cloning of the fimbrial accessory genes promoter region of CSL 127 and CSL 127S as PCR products into pCR Script was confirmed by digestion with endonucleases *Eco* RI and *Not* I. The restricted plasmids (pAS24 and pAS6) were then electrophoresed through a 1% agarose gel to separate the vector and insert fragments (Figure 3.13 B, lanes 1 and 2).

The Type 2 Fimbriae Gene Promoter Region

The amplified 203 base pair *fim2* gene promoter regions of CSL 127 and CSL 127S were cloned into pCRII (Figure 3.14).

The cloning of the *fim2* gene promoter region of CSL 127 and CSL 127S as PCR products into pCRII was confirmed by digestion with endonuclease *Eco* RI. The restricted plasmids DNA (pAS20 and pAS8) were then electrophoresed through a 1% agarose gel to separate the vector and insert fragments (Figure 3.14 B, lanes 1 and 2).

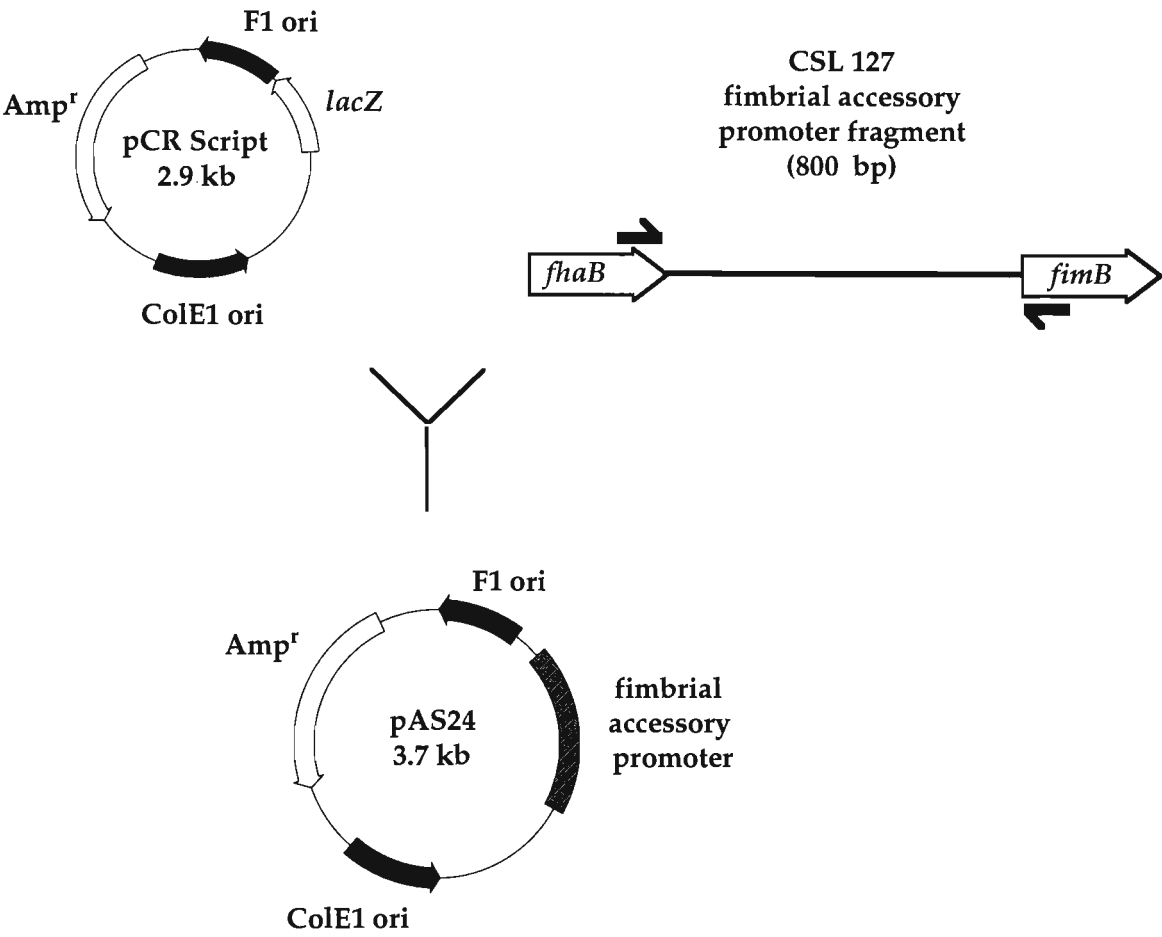
The Type 3 Fimbriae Promoter Gene Region

The amplified 320 base pair *fim3* gene promoter regions of CSL 127 (Figure 3.15) and CSL 127S were both cloned into pCR2.1.

The cloning of the *fim3* gene promoter region of CSL 127 and CSL 127S PCR products into pCR2.1 was confirmed by digestion with endonuclease *Eco* RI (Figure 3.15 A). The restricted plasmids (pAS22 and pAS14 respectively) were then electrophoresed through a 1% agarose gel to separate the vector and insert fragments (Figure 3.15 B, lanes 1 and 2).

Figure 3.13 Cloning of the fimbrial/FHA accessory genes promoter regions of CSL 127 and CSL 127S. **A.** Amplification and cloning of the CSL 127 fimbrial accessory genes promoter region into pCR Script. Genes are represented as open arrows demonstrating the direction of transcription. Oligonucleotide primers used for the amplification of the fimbrial accessory genes promoter are represented as thick arrowheads. The PCR product was ligated into the *SrfI* site of pCR Script. The *lacZ* gene (open arrow) and the ampicillin (Amp^r) resistance gene (open arrow) are indicated, as are F1 and ColE1 origins of replication (filled arrows) and the fimbrial/FHA accessory genes promoter region (shaded box). **B.** Agarose gel electrophoresis of restricted pAS24 and pAS6, demonstrating release of the fimbrial accessory genes promoter regions of CSL 127 (lane 1) and CSL 127S (lane 2) from pCR Script and pCRII respectively.

A



B

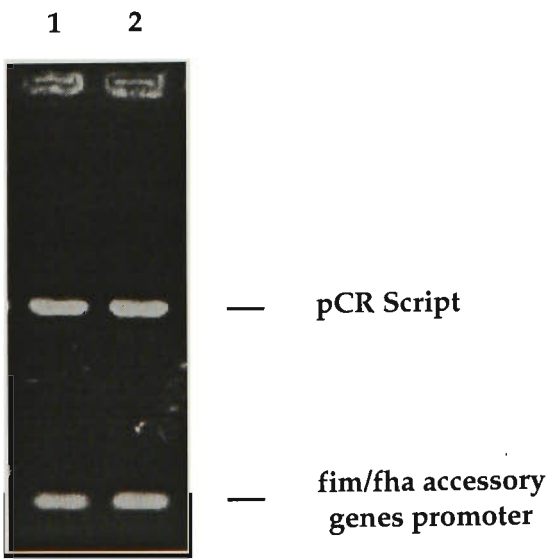
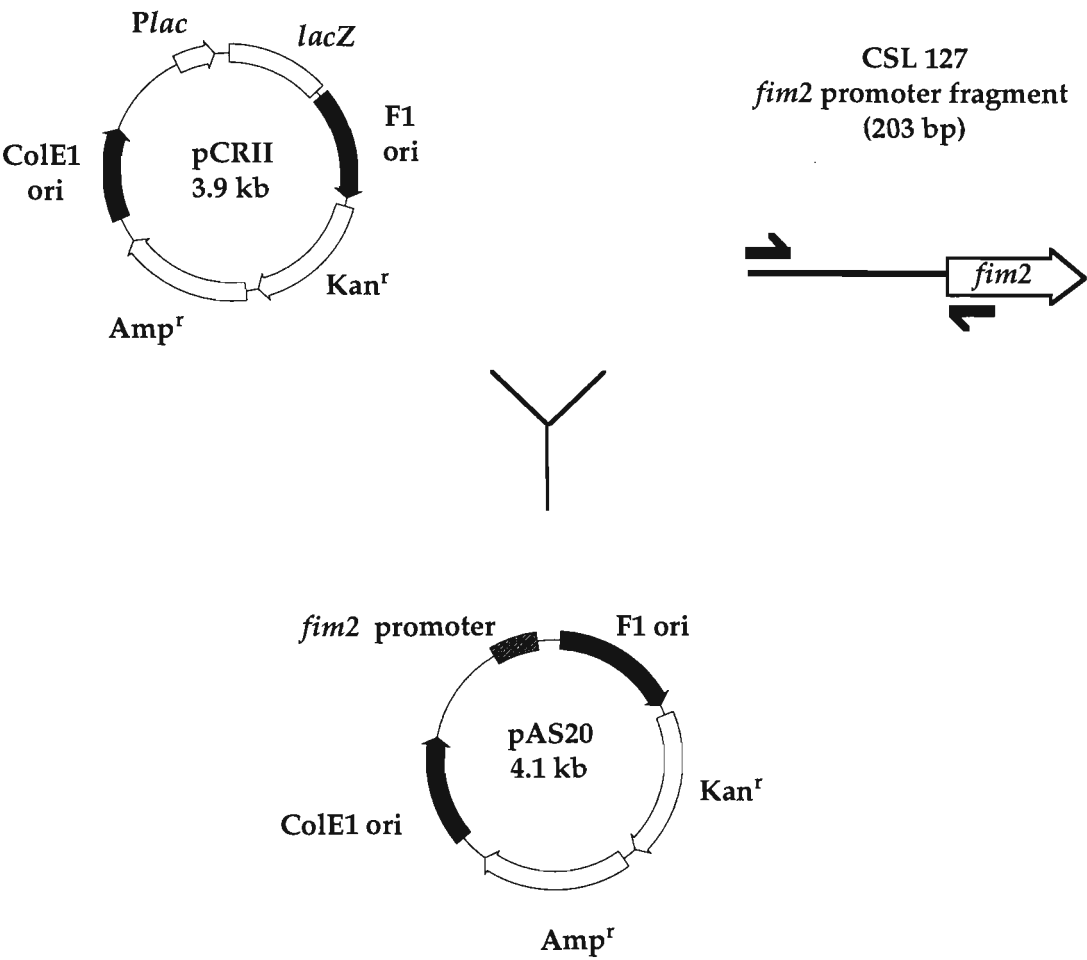


Figure 3.14 Cloning of the *fim2* gene promoter regions of CSL 127 and CSL 127S. **A.** Amplification and cloning of the CSL 127 *fim2* gene promoter into pCRII. Genes are represented as open arrows demonstrating the direction of transcription. Primers used for the amplification of the *fim2* gene promoter are represented by thick arrowheads. The PCR fragment was ligated inside the dual *Eco*RI sites of pCRII. The *lacZ* gene (open arrow), and the kanamycin (Kan^r) and ampicillin (Amp^r) resistance genes (open arrows) are indicated, as are F1 and ColE1 origins of replication (filled arrows) and the *fim2* gene promoter (shaded box). **B.** Agarose gel electrophoresis of restricted pAS20 and pAS8, demonstrating release of the PT promoter regions of CSL 127 (lane 1) and CSL 127S (lane 2) from pCRII and pCR Script respectively.

A



B

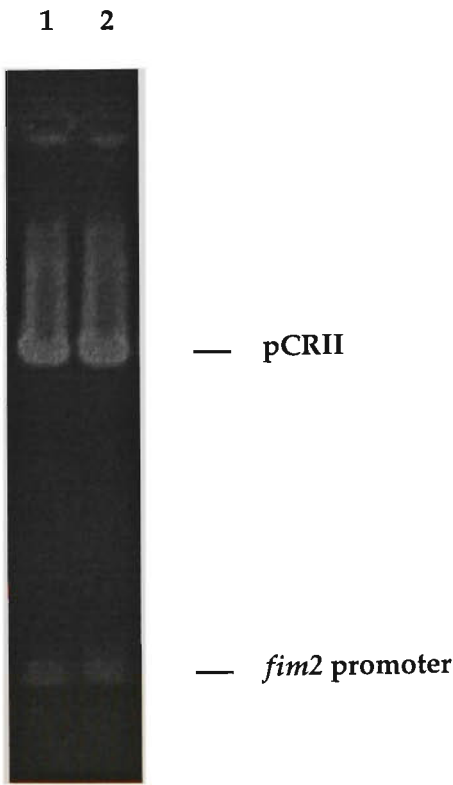
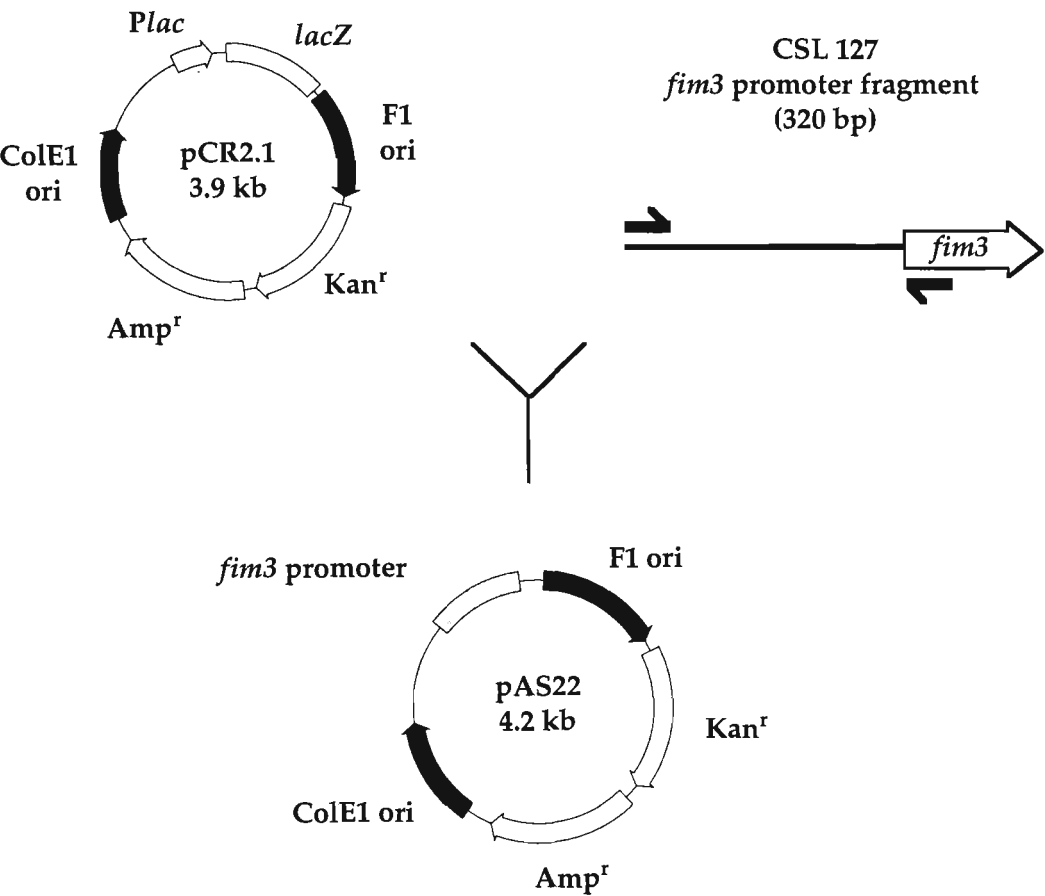
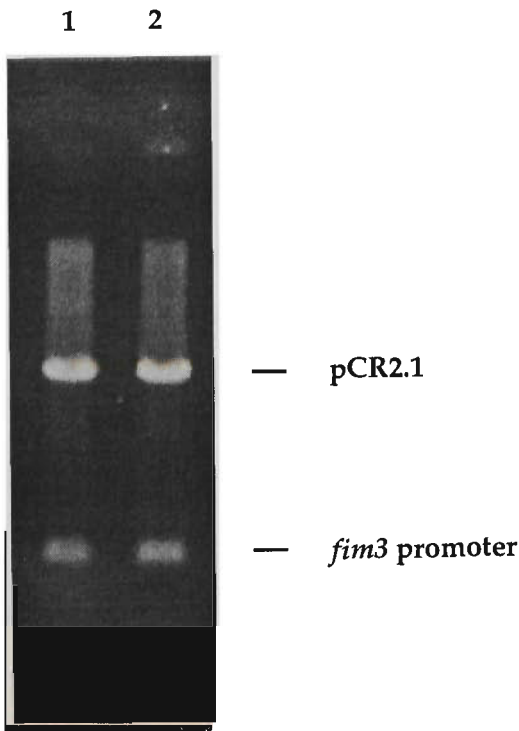


Figure 3.15 Cloning of the *fim3* gene promoter regions of CSL 127 and CSL 127S. **A.** Amplification and cloning of the CSL 127 *fim3* gene promoter into pCR2.1. Genes are represented as open arrows demonstrating the direction of transcription. Primers used for the amplification of the *fim3* gene promoter are represented by thick arrowheads. The PCR fragment was ligated inside the dual *Eco*RI sites of pCR2.1. The *lacZ* gene (open arrow), and the kanamycin (Kan^r) and ampicillin (Amp^r) resistance genes (open arrows) are indicated, as are F1 and ColE1 origins of replication (filled arrows) and the *fim3* gene promoter region (shaded box). **B.** Agarose gel electrophoresis of restricted pAS22 and pAS14, demonstrating release of the *fim3* promoter regions of CSL 127 (lane 1) and CSL 127S (lane 2) from pCR2.1.

A



B



3.6 Analysis of CSL 137 Strains

A major problem with the analysis of the CSL 137 series was the fact that the parental strain, CSL 137 cannot be grown in defined liquid medium. Therefore the antigen expression levels of the selected strain, CSL 137S can only be compared to those of the other CSL strains. Strain CSL 137S was selected for the production of large amounts of serotype 3 specific fimbriae (Table 3.1). However, the production of other antigens is also high. This strain demonstrates the greatest expression levels of PT and pertactin of all the CSL strains. Due to the complex pattern of antigen expression levels and the lack of a parental strain comparison, it was difficult to assign a possible cause for the expression levels of this strain. It was hypothesised that perhaps a favourable mutation in the *bvg* promoter region might cause an overexpression of PT, fim3 and pertactin. The expression of FHA is usually tuned to the levels of BvgA. High levels of FHA are associated with an increased production of this transcriptional activator. The FHA production by CSL 137S could perhaps be described as “moderate” in comparison to other strains in the CSL series. Certainly it is moderate when compared to the high levels of expression of PT, fim3 and pertactin by this strain. The paradox of course being that why would the levels of FHA not increase by a level similar to (or greater than, considering the usual link in expression levels) the other virulence controlled proteins assayed. The promoter regions of the genes encoding FHA and BvgAS sit adjacent to one another on the genome. If there was a favourable mutation in the *bvg* promoter, which upregulated virulence antigen expression, it is possible that the same mutation could cause a detrimental effect to the transcription of *fhaB*, or at least effect *fhaB* promoter architecture in such a way that upregulation of FHA expression was not observed.

If no difference in the sequence of the *bvg/fha* intergenic regions of CSL 137 and CSL 137S is observed, then mutations at promoter regions of each of the overexpressed genes would be considered likely. Although this study is not analysing the pertactin promoter of these strains, alterations in the PT promoter (especially in the BvgA binding site or direct repeat regions) and *fim3* promoter (particularly in the C stretch) were considered possible outcomes.

3.6.1 Amplification of CSL 137 Promoter Regions

The relevant promoter regions of both the parental and selected CSL 137 strains were amplified using either *Taq* or *Pfu* DNA polymerase. This was achieved using the same procedures used for the amplification of the equivalent regions in the CSL 127 strains.

The CSL 137 and CSL 137S PCR products shown in Figure 3.16 were eluted from the gel to remove any unincorporated nucleotides or excess primer DNA and ligated into cloning vectors.

3.6.2 Cloning of CSL 137 Promoter Region DNA

The PCR product of each relevant promoter region was cloned into either of the three cloning vectors used in this study. Ligations into pCRII and pCR2.1 were transformed into INV α F' cells and ligations involving pCR Script were transformed into competent XL1-Blue MRF' *Kan* cells.

The bvg/fha Intergenic Region

The amplified 470 base pair *bvg/fha* intergenic regions of CSL 137 and CSL 137S were both cloned into pCR Script to form pAS36 and pAS26

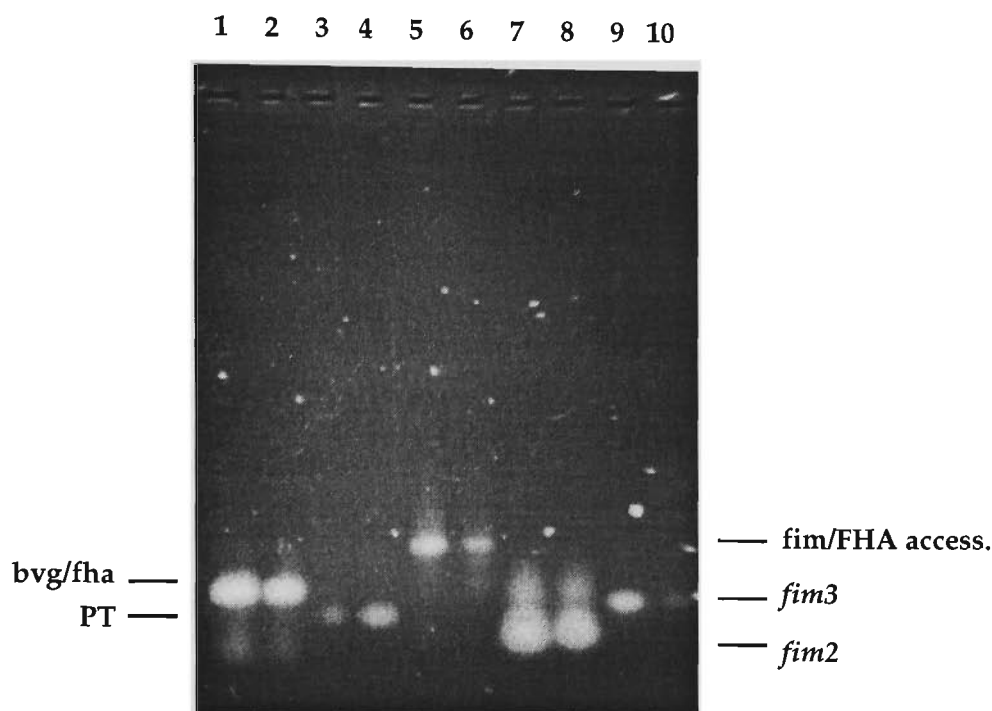


Figure 3.16 Amplification of the relevant gene promoter regions of CSL 137 and CSL 137S. Agarose gel electrophoresis of the PCR amplified promoter regions of CSL 137 (odd lanes) and CSL 137S (even lanes). The *bvg/fha* intergenic region (lanes 1 and 2), PT promoter (lanes 3 and 4), *fim*/FHA accessory genes promoter (lanes 5 and 6), *fim2* gene promoter (lanes 7 and 8) and *fim3* gene promoter (lanes 9 and 10) were all PCR amplified and run through a 1% agarose gel.

respectively (Figure 3.17). These clonings were confirmed by digestion with endonucleases *Eco* RV and *Sac* I. The restricted plasmid DNA was then electrophoresed through a 1% agarose gel to separate vector and insert fragments (Figure 3.17, lanes 1 and 2).

The Pertussis Toxin Gene Promoter Region

The amplified 304 base pair pertussis toxin gene promoter regions of CSL 137 and CSL 137S were both cloned into pCR Script to form pAS38 and pAS28 respectively. These clonings were confirmed by digestion with endonucleases *Eco* RI and *Not* I. The restricted plasmids were then

electrophoresed through a 1% agarose gel to separate vector and insert fragments (Figure 3.17, lanes 3 and 4).

The Fimbrial Accessory Genes Promoter Region

The amplified 800 base pair fimbrial accessory genes promoter regions of CSL 137 and CSL 137S were both cloned into pCR2.1 creating pAS44 and pAS34 respectively. These clonings were then confirmed by digestion with endonuclease *Eco* RI. The restricted plasmid DNA was then electrophoresed through a 1% agarose gel to separate the vector and insert fragments (Figure 3.17, lanes 5 and 6).

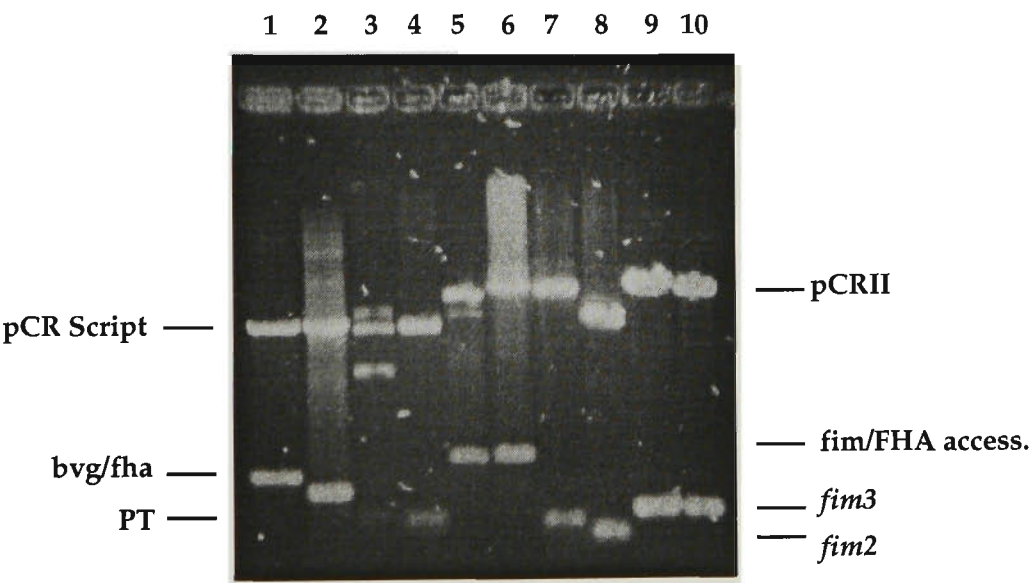


Figure 3.17 Cloning of the relevant promoter regions of CSL 137 and CSL 137S. Agarose gel electrophoresis of restricted plasmids, demonstrating release of the various cloned promoter regions of CSL 137 (odd lanes) and CSL 137S (even lanes) from pCR Script and pCR II/pCR2.1. The plasmids pAS36 and pAS26 (lanes 1 and 2), pAS38 and pAS28 (lanes 3 and 4), pAS44 and pAS34 (lanes 5 and 6), pAS40 and pAS30 (lanes 7 and 8) and pAS42 and pAS32 (lanes 9 and 10) were restricted using appropriate enzymes and run through a 1% agarose gel.

The Type 2 Fimbriae Gene Promoter Region

The amplified 203 base pair *fim2* gene promoter regions of CSL 137 and CSL 137S were cloned into pCR Script and pCR2.1 forming pAS40 and pAS30 respectively. The cloning of these PCR products was then confirmed by digestion with endonucleases *Eco* RI and *Not* I for pAS40 and *Eco* RI for pAS30. The restricted plasmids were then electrophoresed through a 1% agarose gel to separate the vector and insert fragments (Figure 3.17, lanes 7 and 8).

The Type 3 Fimbriae Promoter Gene Region

The amplified 320 base pair *fim3* gene promoter regions of CSL 137 and CSL 137S were both cloned into pCR2.1 creating pAS42 and pAS32 respectively. The cloning of the *fim3* gene promoter region of CSL 137 and CSL 137S PCR products into pCR2.1 was then confirmed by digestion with restriction endonuclease *Eco* RI. The digested plasmids were then electrophoresed through a 1% agarose gel to separate the vector and insert fragments (Figure 3.17, lanes 9 and 10).

3.7 Analysis of CSL 1237 Strains

Strain CSL 1237S which overexpresses a number of virulence determinants (Fim 3, Fim2, FHA and PT) with respect to the parental strain (Table 3.1), may have arisen via mutations in and around the promoter of the *bvg* locus. A mutation in a vital section of the *bvg* promoter would almost certainly affect the expression of most, if not all, virulence factors. Again, like that of the CSL 137 series, the expression of FHA is at a "normal" level, and in fact the levels of this protein in the selected strain CSL 1237S are decreased compared to the parental strain. Interestingly, the increase in expression of the other four antigens assayed

was similar, with CSL 1237S exhibiting a 1-2 fold increase in each, when compared to the parental strain. This small but equivalent increase may represent a favourable mutation in a less significant region of the *bvg* promoter, which, as may be the case in the CSL 137 series, is also detrimental to FHA production.

3.7.1 Amplification of CSL 1237 Promoter Regions

The relevant promoter regions of both the parental and selected CSL 1237 strains were amplified using either *Taq* or *Pfu* DNA polymerase. This was achieved using the same procedures used for the amplification of the equivalent regions in the CSL 127 and CSL 137 series.

The CSL 1237 and CSL 1237S PCR products shown in Figure 3.18 were eluted from the gel to remove any unincorporated nucleotides or excess primer DNA and ligated into cloning vectors.

3.7.2 Cloning of CSL 1237 Promoter Region DNA

The PCR product of each relevant promoter region was cloned into either of the two cloning vectors used in this study. Ligations into pCRII and pCR2.1 were transformed into INV α F' cells and ligations involving pCR Script were transformed into competent XL1-Blue MRF' *Kan* cells.

The bvg/fha Intergenic Region

The amplified 470 base pair *bvg/fha* intergenic regions of CSL 1237 and CSL 1237S were cloned into pCR Script and pCR II, to form pAS56 and pAS46 respectively (Figure 3.17). These clonings were confirmed by digestion with endonucleases *Eco* RV and *Sac* I. The restricted plasmid

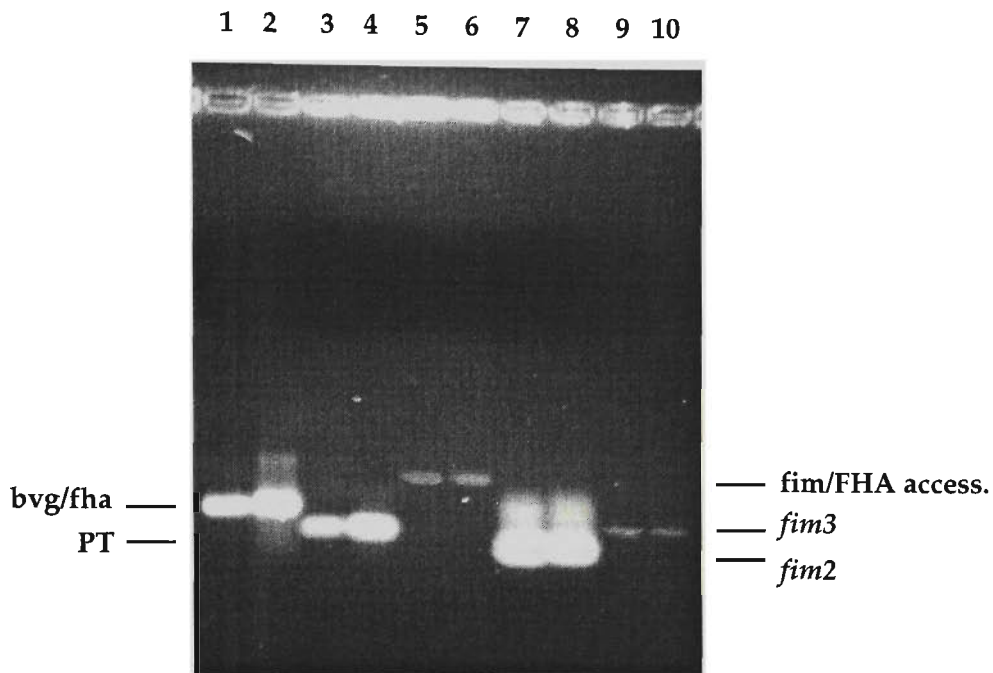


Figure 3.18 Amplification of the relevant gene promoter regions of CSL 1237 and CSL 1237S. Agarose gel electrophoresis of the PCR amplified promoter regions of CSL 1237 (odd lanes) and CSL 1237S (even lanes). The *bvg/fha* intergenic region (lanes 1 and 2), PT promoter (lanes 3 and 4), *fim*/FHA accessory genes promoter (lanes 5 and 6), *fim2* gene promoter (lanes 7 and 8) and *fim3* gene promoter (lanes 9 and 10) were all PCR amplified and run through a 1% agarose gel.

DNA was then electrophoresed through a 1% agarose gel to separate vector and insert fragments (Figure 3.19, lanes 1 and 2).

The Pertussis Toxin Gene Promoter Region

The amplified 304 base pair pertussis toxin gene promoter regions of CSL 1237 and CSL 1237S were both cloned into pCR Script to form pAS58 and pAS48 respectively. These clonings were confirmed by digestion with endonucleases *Eco* RI and *Not* I. The restricted plasmid DNA was then electrophoresed through a 1% agarose gel to separate vector and insert fragments (Figure 3.19, lanes 3 and 4).

The Fimbrial Accessory Genes Promoter Region

The amplified 800 base pair fimbrial accessory genes promoter regions of CSL 1237 and CSL 1237S were both cloned into pCR2.1 creating pAS64 and pAS54 respectively. These clonings were then confirmed by digestion with endonuclease *Eco* RI. The restricted plasmids were then electrophoresed through a 1% agarose gel to separate the vector and insert fragments (Figure 3.19, lanes 5 and 6).

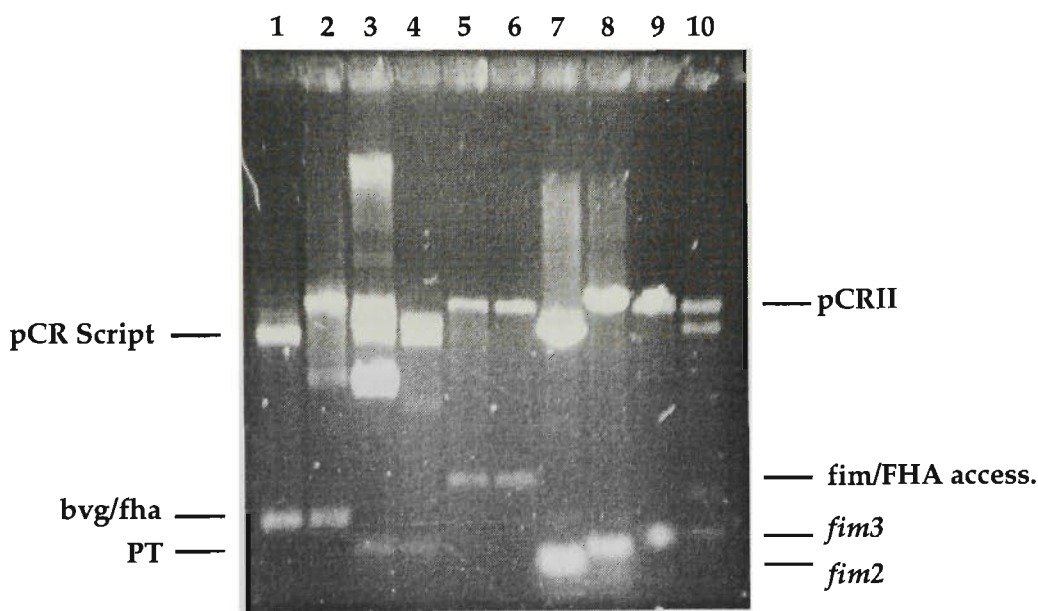


Figure 3.19 Cloning of the relevant promoter regions of CSL 1237 and CSL 1237S. Agarose gel electrophoresis of restricted plasmids, demonstrating release of the various cloned promoter regions of CSL 1237 (odd lanes) and CSL 1237S (even lanes) from pCR Script and pCR II/pCR2.1. The plasmids pAS56 and pAS46 (lanes 1 and 2), pAS58 and pAS48 (lanes 3 and 4), pAS64 and pAS54 (lanes 5 and 6), pAS60 and pAS50 (lanes 7 and 8) and pAS62 and pAS52 (lanes 9 and 10) were restricted using appropriate enzymes and run through a 1% agarose gel.

The Type 2 Fimbriae Gene Promoter Region

The amplified 203 base pair *fim2* gene promoter regions of CSL 1237 and

CSL 1237S were cloned into pCR Script and pCRII forming pAS60 and pAS50 respectively. The cloning of these PCR products was then confirmed by digestion with endonucleases *Eco* RI and *Not* I for pAS60 and endonuclease *Eco* RI for pAS50. The restricted plasmids were then electrophoresed through a 1% agarose gel to separate the vector and insert fragments (Figure 3.19, lanes 7 and 8).

The Type 3 Fimbriae Promoter Gene Region

The amplified 320 base pair *fim3* gene promoter regions of CSL 1237 and CSL 1237S were both cloned into pCR2.1 creating pAS62 and pAS52 respectively. The cloning of these PCR products into pCR2.1 was then confirmed by digestion with restriction endonuclease *Eco* RI. The digested plasmids were then electrophoresed through a 1% agarose gel to separate the vector and insert fragments (Figure 3.19, lanes 9 and 10).

3.8 Sequence Analysis of Promoter Regions

The plasmids containing all of the relevant promoter region DNA from each of the three parental and three selected CSL strains were DNA sequenced in an attempt to identify any genetic differences.

3.8.1 The *bvg/fha* Intergenic Region

Promoter region DNA from the *bvg/fha* intergenic regions of the six strains contained in plasmids pAS10, pAS16, pAS26, pAS36, pAS46 and pAS56 was sequenced. The data generated by the ABI Prism 377 sequencing software was transferred to MacVector where the sequences were aligned (Figure 3.20).

Initially, a difference was found in the *bvg/fha* intergenic region of CSL 1237S, the strain which shows a small increase in all virulence factors assayed except FHA (pAS46) (Figure 3.20). This difference is in a region close to the *bvg* P₄ promoter region that is conserved between the P₄ and the P_{*fha*} promoters (Scarlato *et al.*, 1990). This is also a region that shows a high degree of homology to the proposed Shine-Dalgarno ribosome binding sequence of the *B. pertussis* *fim2* and *fim3* genes (Livey *et al.*, 1987; Mooi *et al.*, 1990). The possibility of a change in the DNA sequence here causing a small but equivalent alteration in the expression of a number of virulence regulated genes was exciting. A cytosine to adenine transition in this region resulting in the increased expression of BvgA, via an upregulation of either transcription or translation, could theoretically increase the levels of virulence factor production. A decrease in FHA production might also be accounted for by an upregulation in the transcription from the *bvg* P₄ promoter. The production of *fhaB* mRNA may be affected by the excess sense strand sterically hindering the binding of transcriptional activators or RNA polymerase.

Due to the discovery of this difference, this region was amplified again in an independent PCR and cloned. This plasmid (pAS47) was then sequenced to determine whether the difference was a genuine genetic change or merely a DNA polymerase error. Unfortunately, the latter was true with the sequence exhibiting 100% homology to that of the parental gene sequences.

Figure 3.20 Aligned DNA sequence comparison from the *bvg/fha* intergenic region of each of the CSL strains. Primer sequences are underlined. The *Taq* error (see text) in the CSL 1237S sequence is shown as a C to A transition. Primers used for PCR are underlined.

CSL 127	<u>CCCCGTGTACAGGTTTCGTGTTTCATATTCCGACCAGCGAAGTGAAGTAATCG</u>
CSL 127S
CSL 137
CSL 137S
CSL 1237
CSL 1237S
CSL 127	GCAGAATCCGTTATCGGCCTGCTCGTTTTAGGTTTTGAAATCCAGTGCCA
CSL 127S
CSL 137
CSL 137S
CSL 1237
CSL 1237S
CSL 127	TAGTCTGGATAAACCTCAGCACTTCGTCAGTCAGTAATGATCCCGTCCAT
CSL 127S
CSL 137
CSL 137S
CSL 1237
CSL 1237S
CSL 127	CAATGGATATTTATACAAGACTTGTAGGAAATTTCTTAGTCAAACCATCA
CSL 127S
CSL 137
CSL 137S
CSL 1237
CSL 1237S
CSL 127	AACCCTGTCCGGCACGTATAGGCGTGCGCCGACAACCCGCTCCCCGGCCC
CSL 127S
CSL 137
CSL 137S
CSL 1237
CSL 1237S
CSL 127	GCCGCGGGTGCCAGCGCAGGAATTCAGACTTTTCCTATTTTGGTATAGGA
CSL 127S
CSL 137
CSL 137S
CSL 1237
CSL 1237S
CSL 127	TACTGTTTTAGCAGCACTCATGCCCGTATCGTTGCTTGCTGACGTATAGGA
CSL 127S
CSL 137
CSL 137S
CSL 1237
CSL 1237S
CSL 127	GAATGTGAAATTTTCGCAGCCATTCTTTGACGCATCAATGACATTACGCG
CSL 127S
CSL 137
CSL 137S
CSL 1237
CSL 1237S
CSL 127	TTGCAGGATTTTTTTTCTCGCCACGAAACTCAAAGCATATTCTTCTTATCA
CSL 127S
CSL 137
CSL 137S
CSL 1237
CSL 1237SA.....
CSL 127	<u>TGTACAACAAAGTCCTCATC</u>
CSL 127S
CSL 137
CSL 137S
CSL 1237
CSL 1237S

3.8.2 The Pertussis Toxin Gene Promoter Region

Promoter region DNA from the pertussis toxin gene of the six strains contained in plasmids pAS12, pAS18, pAS28, pAS38, pAS48 and pAS58

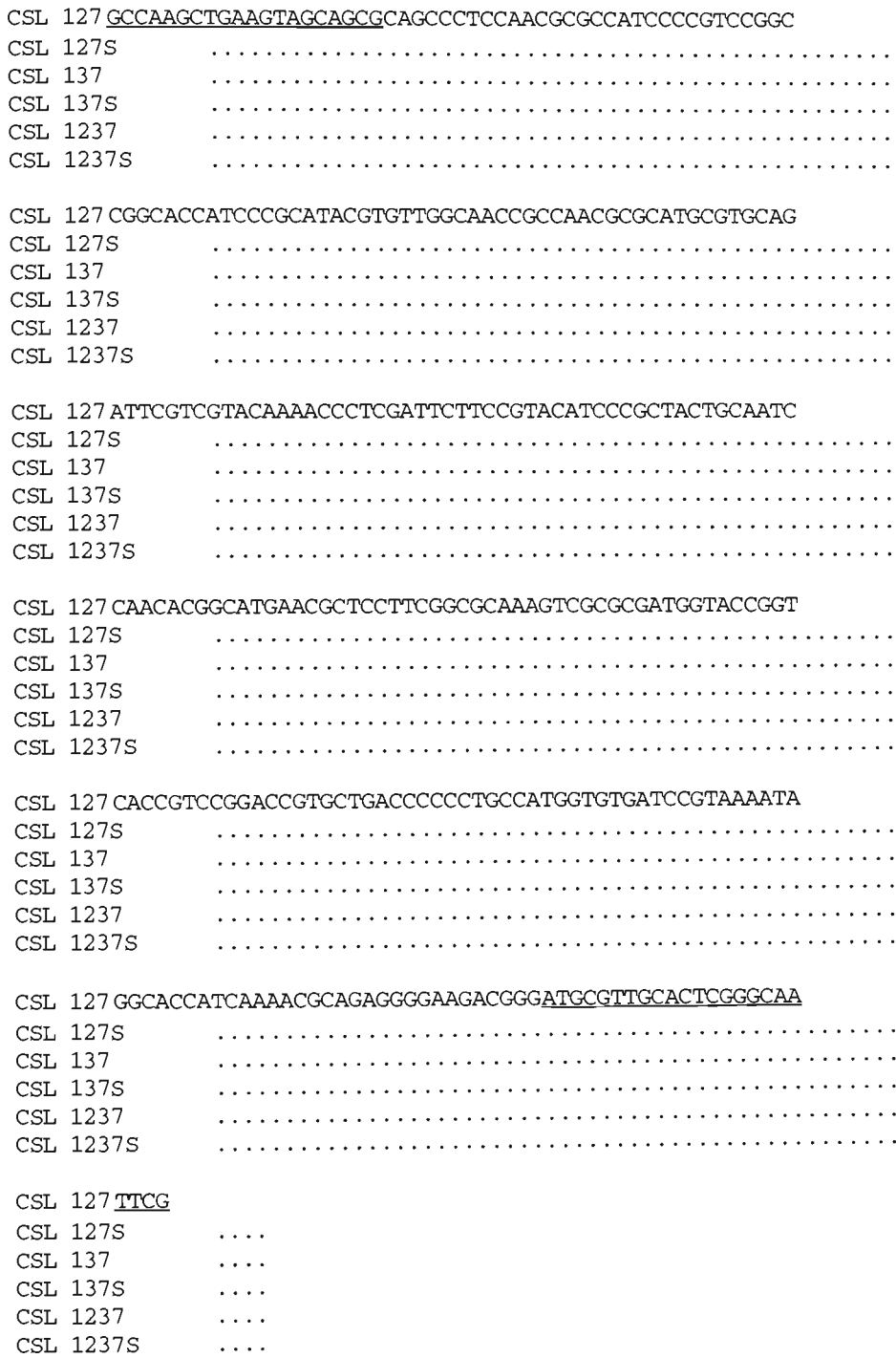


Figure 3.21 Aligned DNA sequence from the pertussis toxin gene promoter region of each of the CSL strains. Primers used for PCR are underlined.

was sequenced. The data generated by the ABI Prism 377 sequencing software was transferred to MacVector where the sequences were aligned (Figure 3.21).

The CSL strains exhibited 100% homology within the pertussis toxin gene promoter region. There was also no difference observed in this region between the CSL series and the original sequences published by Loch and Keith, (1986) and Nicosia *et al.* (1986).

3.8.3 The Fimbrial Accessory Genes Promoter Region

Promoter region DNA from the fimbrial accessory genes of the six strains contained in plasmids pAS6, pAS24, pAS34, pAS44, pAS54 and pAS64 was sequenced. The data generated by the ABI Prism 377 sequencing software was transferred to MacVector where the sequences were aligned (Figure 3.22).

Although it was hypothesised that there may be a difference in this region between strains CSL 127 and CSL 127S to explain the upregulation in both serotype 2 specific fimbriae and FHA, no difference was observed.

The region targeted as the fimbrial accessory genes promoter in this study was the 800 nucleotide base pairs between the *fhaB* gene (including the final 20 bp) and the *fimB* gene (including the first 23 bp). After these original oligonucleotide primers were designed and constructed and experiments initiated, it was proposed that *fimC*, *fimD*, and *fhaC* were translationally linked (Willems *et al.*, 1993; Willems *et al.*, 1994) indicating that the linkage of the fimbrial/FHA accessory genes was at the level of translation. Therefore the area of the genome targeted in this

CSL 127 TGAAACCAACAAATAGGTAGTCGCGGCCTGCCGCGGCTCGGCGCATGGGG

CSL 127S

CSL 137

CSL 137S

CSL 1237

CSL 1237S

CSL 127 ATTTCGCAGGGTTCTCATGCGCCGGCCAATGCCGGATAGCGGTGCAATTGC

CSL 127S

CSL 137

CSL 137S

CSL 1237

CSL 1237S

CSL 127 CGACCATTTCGCGCACC GCGCTCAAGGACGTAGGGTCGACGGCAGGCGGG

CSL 127S

CSL 137

CSL 137S

CSL 1237

CSL 1237S

CSL 127 ACAGTTTTTGACGTGAAACTGACCGAGTGTCGCGAGGCATTGAATGGTCA

CSL 127S

CSL 137

CSL 137S

CSL 1237

CSL 1237S

CSL 127 GCAAGTGGGATTGTTCCTTCGAATCTGGTGGCACGGTTGACTATACGTCGG

CSL 127S

CSL 137

CSL 137S

CSL 1237

CSL 1237S

CSL 127 GAAACCTGTTTTCGTATCGGGCCGATAGTCAGGGCGTCGAACAGGTACCG

CSL 127S

CSL 137

CSL 137S

CSL 1237

CSL 1237S

CSL 127 CAGACGAAAGCCGACAACGTGCAAGCCAATCTGGATGGTTCCGCTATTCA

CSL 127S

CSL 137

CSL 137S

CSL 1237

CSL 1237S

CSL 127 TTGGGCCCGCAACAAGGGTGCAGGCTGCTCAGACGTTTCTGGTATCGC

CSL 127S

CSL 137

CSL 137S

CSL 1237

CSL 1237S

CSL 127 AGACGGCTGGGTCGTCGACGTACGGGGCGACCCTGCGCTATCTGGCATGC

CSL 127S

CSL 137

CSL 137S

CSL 1237

CSL 1237S

CSL 127 TACATCCGTTCCGGGCGCTGGTTCCATTGTTGCGGGGAATCTCCGCAGTCA

CSL 127S

CSL 137

CSL 137S

CSL 1237

CSL 1237S

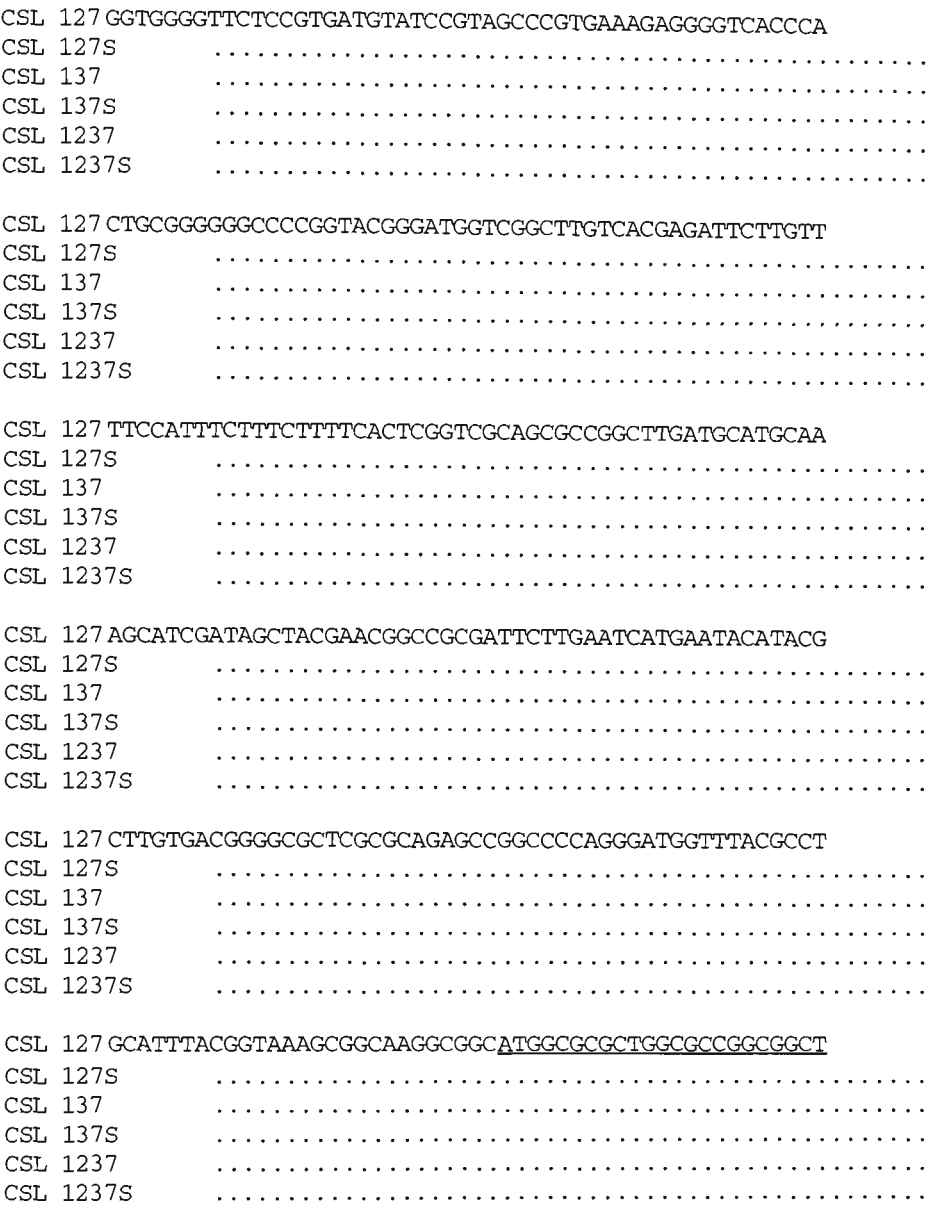


Figure 3.22 Aligned DNA sequence from the fimbrial accessory promoter region of each of the CSL strains. Primers used for PCR are underlined.

study as the *fim*/FHA accessory genes promoter region was potentially not the only area requiring sequence analysis. The other appropriate area where a mutation might cause an alteration in the expression of fimbriae and FHA may be the region between *fimB* and *fimC*. To determine if this was the case, oligonucleotide primers were designed to amplify the region of DNA immediately upstream of the *fimC* gene. It was thought that if there was a change in this region, it would be restricted to the strain

overexpressing both fimbriae and FHA. For this reason, only strains CSL 127 and CSL 127S were subjected to the amplification, cloning and sequencing process. A 473 base pair region was sequenced, revealing no difference between the parental strain and the selected strain (results not shown).

3.8.4 The Type 2 Fimbriae Gene Promoter Region

Promoter region DNA from the *fim2* gene of the six strains contained in plasmids pAS8, pAS20, pAS30, pAS40, pAS50 and pAS60 was sequenced. The data generated by the ABI Prism 377 sequencing software was transferred to MacVector where the sequences were aligned (Figure 3.23).

Due to the upregulation of fimbriae and FHA, it was hypothesised that a difference between CSL 127 and CSL 127S would be observed in either the fimbrial accessory genes promoter region, or the *fimC* upstream region. While no differences were detected in either of these regions, an alteration to the *fim2* gene promoter was discovered (Figure 3.23). This difference was traced to the C stretch, a region involved in fimbrial phase variation. While the *fim2* gene promoter of the parental strain CSL 127 contained 17 cytosine residues within the C stretch, the corresponding region of the overexpressing strain, CSL 127S contained only 16 residues. The 16 cytosine C stretch is equivalent to the original published sequence of the Wellcome 28 strain (Livey *et al.*, 1987). The results of this study may indicate that 16 residues is the optimum number for expression of type 2 fimbriae. The *fim2* expression of CSL 127 (which possesses 17 nucleotides within the C stretch) is 20 fold less than the selected strain with only 16 nucleotides. The CSL 1237 series strains contain 15 residue C stretches and display 4 and 6 fold less *fim2* expression compared to that of CSL 127S.

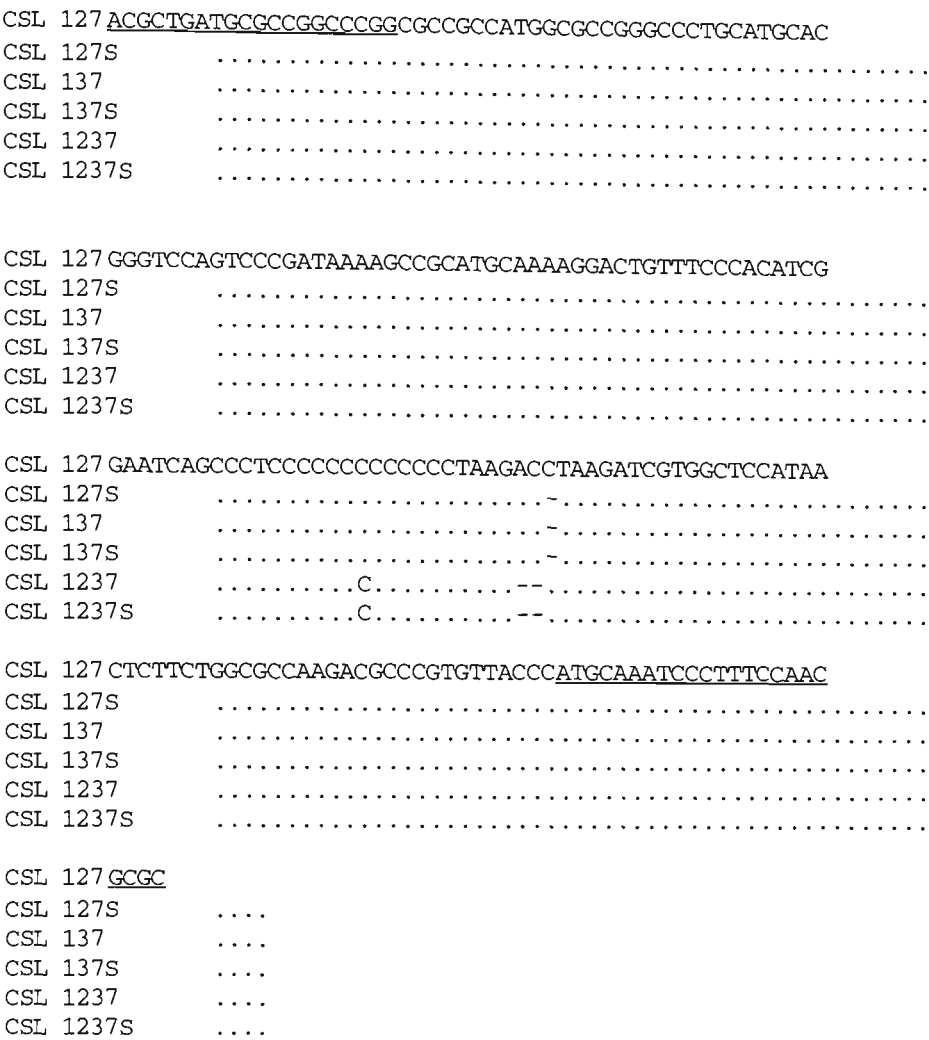


Figure 3.23 Aligned DNA sequence from the *fim2* promoter region of each of the CSL strains. Dashes have been introduced to increase alignment. Primers used for PCR are underlined. The differences in the sequence are displayed as T to C transitions in the CSL 1237 strains and dashes in the C stretch.

3.8.5 The Type 3 Fimbriae Promoter Gene Region

Promoter region DNA from the *fim3* gene of the six strains contained in plasmids pAS14, pAS22, pAS32, pAS42, pAS52 and pAS62 was sequenced.

The data generated by the ABI Prism 373/377 sequencing software was transferred to MacVector where the sequences were aligned (Figure 3.24).

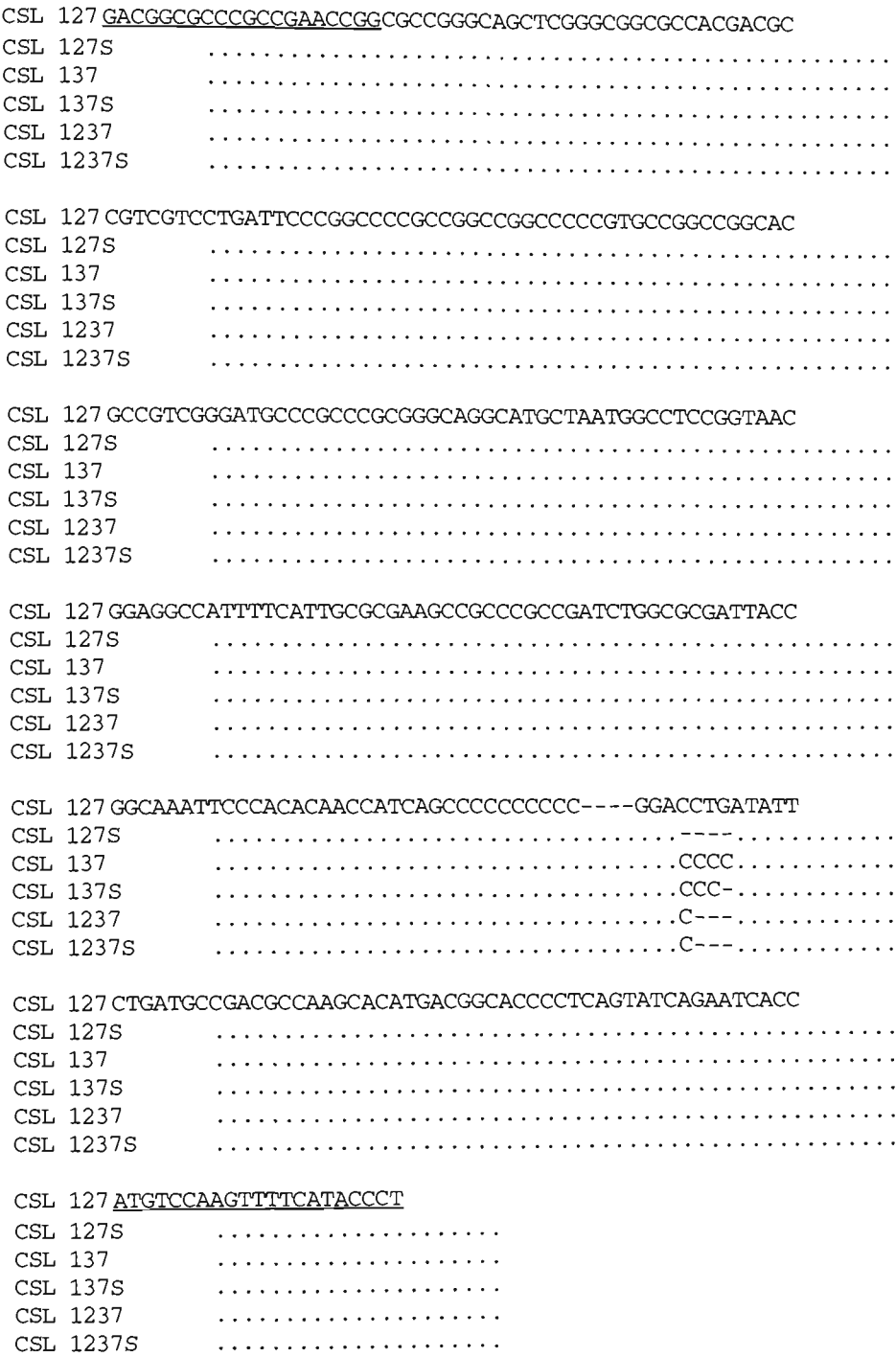


Figure 3.24 Aligned DNA sequence from the *fim3* promoter region of each of the CSL strains. Dashes have been introduced to improve alignment. Primers used for PCR are underlined. The differences in the sequence are displayed as extra cytosine residues or dashes in the C stretch.

The differences demonstrated in the *fim3* gene promoter (Figure 3.24) were not an unexpected result. The difference between the CSL 137 strains in the number of cytosine residues within the C stretch of the *fim3*

promoter was actually the most anticipated mutation among the entire CSL series.

However the exact changes observed were not predicted. Fimbrial phase variation involves genetic changes in the C stretch (Mooi *et al.*, 1990). Although data concerning fimbrial expression levels of the parental strain could not be obtained, an alteration in this “hot spot” seems to have led to an overexpression of type 3 fimbriae in CSL 137S. The number of nucleotides within the C stretch has previously been shown to be important in fimbrial expression levels (Mooi *et al.*, 1990; Riboli *et al.*, 1991). Strains which produce large amounts of type 3 fimbriae (variants of the Wellcome 28 strain) were found to contain 14 nucleotides in the C stretch, while this region in strains deficient in type 3 fimbriae (Tohama I) contained 13 or less nucleotides (Mooi *et al.*, 1990; Riboli *et al.*, 1991). The results of the current study with the non-engineered overexpressing mutant CSL 137S, somewhat contradict these previous observations. In this study, the overexpressing strain contains a 13 residue C stretch and the parental strain, a 14 residue C stretch. The implications of this difference lie in the area of the complexity of fimbrial expression and phase variation. Alterations in the C stretch region may not be the sole source of fimbrial phase variation.

Differences within the fimbrial promoter regions between parental and selected *B. pertussis* strains were observed, however not all were easily predictable. The upregulation in type 3 fimbriae expression in CSL 137S in comparison to its parent strain CSL 137 was found to be caused by a difference in the promoter region of the *fim3* gene. This difference was expected between these two strains because of the increase in production in only a single protein. The strain overexpressing type 2 fimbriae and

FHA (CSL 127S) with respect to its parental strain (CSL 127) was expected to exhibit changes in the fimbrial accessory genes, however this was not the case. Although the difference in FHA production in these strains cannot be fully explained, the expression of large amounts of *fim3* in the selected strain is attributable to a difference in the C-stretch of the promoter region of the *fim2* gene. The final pair of strains, the CSL 1237 series were predicted to possess DNA sequence differences with respect to one another within the *bvg/fha* intergenic region, due to altered expression levels of a number of virulence factors. The expression levels of the parental and selected strain were not greatly varied, and, perhaps reflecting this, no differences were found at the DNA level at the sites sequenced.

These studies offer an opportunity to further expand the knowledge of the mechanisms of how the virulence genes of *B. pertussis* are controlled. It is anticipated that the identified mutations can be incorporated into other strains to increase antigen production for the preparation of new generation acellular vaccines.

Chapter Four Discussion

Whooping cough, the severe childhood respiratory disease, is caused by the obligate human pathogen, *Bordetella pertussis* (Bordet and Gengou, 1906). The traditional whole cell vaccine used for the prevention of the disease is currently under scrutiny due to reported side effects. The subsequent decline in vaccine usage has inevitably led to a worldwide increase in the occurrence of whooping cough (Robinson *et al.*, 1985, Romanus *et al.*, 1987). The preparation of safer, less reactogenic vaccines is at present concentrated on the production of purified recombinant antigens for inclusion in acellular vaccine preparations. Recent vaccine trials have demonstrated that important antigens include pertussis toxin, filamentous haemagglutinin, pertactin and serotype specific fimbriae (Greco *et al.*, 1996; Gustafsson *et al.*, 1996). The production of these vaccine immunogens in a simple, cost-effective manner is one of the most important requirements in the development of future vaccine preparations.

The present study is focussed on the gene expression of different antigens of *Bordetella pertussis*, and more specifically, on the promoters of virulence genes. The first section of this thesis dealt with the cloning of a pertussis toxin expression locus, including a constitutive promoter. The latter part has explored the virulence gene promoter regions of a number of antigen overexpressing whooping cough vaccine strains.

A detoxified form of PT is considered to be an excellent candidate protective antigen for any acellular component vaccine preparation. The incompatibility of PT gene expression signals with respect to *Escherichia coli* make the production of PT in this organism difficult to achieve

(Burnette *et al.*, 1988; Nicosia *et al.*, 1987). Production of the toxin in virulent *B. pertussis* is hampered due to a number of factors, such as slow growth rates, fastidious nutritional requirements and a tightly regulated expression system. Another uncertainty here is the risk that co-purification of low levels of other virulence regulated toxins could occur in vaccine preparations. Expression and secretion of PT in organisms other than virulent phase *B. pertussis* has been attempted by a number of laboratories. Plasmids containing a *B. pertussis* PT operon introduced into *B. bronchiseptica* were found to produce levels of PT similar to those of *B. pertussis* (Lee *et al.*, 1989). When the location of expressed PT was examined in these recombinant *B. bronchiseptica* strains, most of the PT remained intracellular, however significant amounts were actually detected in the culture medium. A similar result was obtained when genetically altered pertussis toxoid was expressed in *B. parapertussis* and *B. bronchiseptica* (Pizza *et al.*, 1989). These studies suggested that the two species incapable of PT expression may in fact possess the ability to secrete the toxin, catalysing further research in this area. Expression of PT in ATCC 10580::Tn_{fus}PT1, an avirulent *B. bronchiseptica* strain, is controlled by the strong constitutive promoter, P_{Bb} (Walker *et al.*, 1991a). Production levels of PT were promising, however all of the toxin was located in the periplasm, making this an unsuitable vaccine expression strain.

One of the goals of this study was to determine whether *B. bronchiseptica* could secrete PT, using ATCC 10580::Tn_{fus}PT1 to develop a PT expression system in virulent and avirulent strains of *B. bronchiseptica*. The examination of PT secretion in these PT producing recombinant strains would determine if the secretion of PT is possible in *B. bronchiseptica*, and whether it is *bvg*-regulated.

The first strategy used to clone the PT expression locus of ATCC 10580::*Tn_{fusPT1}* was to construct a cosmid library. A number of potential clones were isolated, however Southern blot analysis using three different probes containing PT sequences failed to detect the PT expression locus. It was hypothesised that either one of the gene products of the expression locus may have been lethal to *E. coli*, or the expression locus DNA was unstable. The isolation of expression locus clones would have allowed for the characterisation and sequencing of the P_{Bb} promoter of the PT expression locus. It was envisaged that this promising promoter would then be used for the expression of other important whooping cough vaccine antigens.

With the failure of cosmid cloning experiments, the pertussis toxin expression locus of *B. bronchiseptica* ATCC 10580::*Tn_{fusPT1}*, incorporating P_{Bb}, was transferred into virulent and avirulent strains of *B. bronchiseptica* using an *in vivo* cloning technique. The *tra* genes of the plasmid pR751, when integrated into a chromosome, have the ability to induce conjugal transfer to a recipient bacterium. Transposon Tn813 contains a defective resolvase gene, therefore once it transposes into the chromosome it cannot resolve the cointegrate formed. *E. coli* Q358 (pR751::Tn813) was conjugally mated with the original avirulent *B. bronchiseptica* PT expressing strain, ATCC 10580::*Tn_{fusPT1}*. This permitted pR751::Tn813 to transpose into the chromosome of ATCC 10580::*Tn_{fusPT1}*, thereby facilitating chromosome transfer in subsequent conjugal mating experiments. Using this technique, the PT expression locus was successfully cloned *in vivo* into derivatives of virulent and avirulent *B. bronchiseptica* strains. The levels of PT expression in strains of *B. bronchiseptica* containing the PT expression locus were similar to the original strain. Secretion analysis demonstrated that all of the

recombinant *B. bronchiseptica* strains examined in this study failed to secrete PT into the culture medium, whereas *B. pertussis* Tohama I was found to secrete significant amounts of PT.

The discovery of the *B. pertussis* *ptl* operon, required for PT secretion, led to an investigation into the presence of the *ptl* operon in *B. bronchiseptica*. It had been established that *B. bronchiseptica* contains genes homologous to the *B. pertussis* *ptlA*, *ptlB* and part of *ptlC* (Arico and Rappuoli, 1987). Significantly, none of the 56 base pair differences detected in the sequenced region of the *B. bronchiseptica* *ptl* operon result in the introduction of a stop codon thereby truncating the putative open reading frames. Southern blot analysis demonstrated that *B. bronchiseptica* also contains sequences homologous to the 3' end of the *ptl* operon (*ptlH* gene). Although the *B. bronchiseptica* *ptlA*, *B* and *C* open reading frames so far examined remain essentially intact, and despite the presence of DNA sequences homologous to *ptlH*, this study has shown that *B. bronchiseptica* is unable to secrete PT. The reason for this has been traced to the *ptl* operon in *B. pertussis* being transcribed from the P_{ptx} promoter (Kotob *et al.*, 1995; Baker *et al.*, 1995). The P_{ptx} promoters in *B. parapertussis* and *B. bronchiseptica* are inactive.

The lack of PT secretion in *B. bronchiseptica* in this study confirm the findings of Kotob *et al.* (1995) and strongly suggest that the *ptl* genes of *B. bronchiseptica* are cryptic. This has since been further confirmed with the introduction of the *B. pertussis* P_{ptx} promoter into *B. parapertussis* and *B. bronchiseptica* strains (Hausman *et al.*, 1996). This resulted in the production and secretion of PT in *B. bronchiseptica* and in limited amounts in *B. parapertussis*. Yields were well below that of *B. pertussis*, however it was shown that *B. bronchiseptica* contains all the essential *ptl*

genes required for PT secretion (Hausman *et al.*, 1996).

Although this study and others have shown that the *ptl* genes of *B. bronchiseptica* are fundamentally intact, the lack of transcription from the P_{ptx} promoter of *B. bronchiseptica* abrogates expression of Ptl proteins in the strains used in this study. Therefore secretion of PT cannot be achieved in these strains without further genetic modification. The cloning technique developed in this study was utilised to obtain expression of pertussis toxin in both virulent and avirulent strains of *B. bronchiseptica*. Furthermore, this *in vivo* chromosome transfer system could be used in other transposon-tagged loci of *Bordetella* spp. to analyse transposon induced gene mutations across species and genera.

Apart from pertussis toxin, a number of other antigens have recently been included in vaccine trials, with promising results (Greco *et al.*, 1996; Gustafsson *et al.*, 1996). These antigens include filamentous haemagglutinin, serotype specific fimbriae and pertactin. The selected *B. pertussis* CSL strains used in this study demonstrate overexpression of these different proteins, the increased expression postulated to be the result of mutations within the promoter regions of the different loci. This investigation was aimed at providing evidence of these mutations in an effort to genetically characterise these *B. pertussis* strains.

The Australian licensed whole cell whooping cough vaccine, manufactured by CSL Ltd. is a heat killed preparation of *Bordetella pertussis*. Research methods at CSL have not utilised deliberate genetic modification, but have relied on continuous subculture and selection to produce vaccine strains expressing high levels of virulence antigens.

The selected strains, CSL 127S, CSL 137S and CSL 1237S represent excellent vaccine candidates for either a whole cell vaccine or for the production of antigens in new generation acellular vaccines. It may also be possible to engineer the beneficial mutations contained within overexpressing promoter regions into other *B. pertussis* vaccine strains to improve antigen yield.

The promoter regions of CSL 137 and CSL 137S exhibited a single genetic difference. Predictably, this change results from a difference in the number of cytosine residues within the C stretch of the *fim3* promoter of CSL 137S. This mechanism is employed by *B. pertussis* populations *in vivo* to alter the fimbrial serotype expressed. This fimbrial phase variation has been shown to be caused by a change in the number of cytosine residues in the C stretch of the promoter (Willems *et al.*, 1990). The activation of transcription is proposed to be dependent on the distance between the putative -10 box and the BvgA binding site. The alteration of the nucleotide number in the C stretch affects this distance, changing the expression of fimbriae (Willems *et al.*, 1990; Mooi *et al.*, 1990; Riboli *et al.*, 1991).

High level expression of type 3 fimbriae has been found to correlate with a sequence of 14 nucleotides in the C stretch of the *fim3* gene promoter (Willems *et al.*, 1990; Riboli *et al.*, 1991). The original published sequence of *fim3* was taken from the Tohama I strain. The promoter of the *fim3* gene of this strain contains 13 cytosine residues within the C stretch (Mooi *et al.*, 1990). However, this strain is considered a serotype 2 strain, and produces very low levels of type 3 fimbriae. A subsequent study determined that the Wellcome 28 strain, which produces large amounts of both serotype specific fimbriae, contains 14 residues within the C

stretch (Willems *et al.*, 1990). The *fim3* promoter sequence from another strain, BPSA1 has also been obtained (Riboli *et al.*, 1991). The C stretch of this strain contains only 10 cytosine residues. The *fim3* promoter of this strain was shown to be only 30% active (as determined by CAT assays) when compared to the *fim2* gene promoter. The authors suggest that the low level *fim3* production was likely due to the deletion of 4 residues in the C stretch region.

Although a difference in the C stretch regions of CSL 137 and CSL 137S was predicted, the nature of this change was somewhat unexpected. The results of the current study with the non-engineered overexpressing mutant CSL 137S, are somewhat contrary to previous observations. The strain expressing the highest levels in this study contains a run of 13 cytosine residues in the fimbrial C stretch, equivalent to that of Tohama I, which expresses only low levels of type 3 fimbriae. The parental strain from which this selected strain was obtained contained 14 cytosines, the number usually reserved for strong *fim3* gene promoters.

One possible rationalisation for the difference between the CSL 137 strains is that the control of expression of fimbrial subunits may actually be more complex than initially proposed. The original sequence of the *B. pertussis* *fim3* gene was analysed using the Tohama I strain (Mooi *et al.*, 1990). There was found to be 13 cytosines in the *fim3* C stretch of this strain, which does not produce serotype 3 fimbriae. CSL 137S exhibits a 13 residue C stretch, yet expresses high levels of *fim3*.

Willems *et al.* (1990) demonstrated that a loss of residues in the C stretch resulted in fimbrial phase variation, using a *B. pertussis* mutant strain deficient in the expression of both fimbrial subunit genes. Into this strain

(B52) they placed plasmids containing *fim3* genes along with the corresponding promoters. One promoter region contained 14 cytosines within the C stretch and the other contained only 9. The strain with the larger promoter expressed significantly higher levels of type 3 fimbriae. A difference of 5 residues might be expected to significantly alter transcription from the gene.

The discrepancies observed between previous studies and this current work may suggest that the expression of serotype 3 fimbriae is more complex than originally postulated. A comparison between CSL 137S and Tohama I *fim3* expression suggests that the length of the C stretch may not be the only factor affecting fimbrial serotype. They both contain 13 cytosine residues within the *fim3* C stretch yet exhibit markedly differing *fim3* expression profiles. It is postulated here that there may be different requirements for different *Bordetella* strains. There are a number of possible mechanisms which could bring about these differences. The transcriptional activator, BvgA may be different in different strains due to accumulated mutations that have altered its binding affinity for fimbrial promoters. These mutations may cause the binding capacity of BvgA to become stronger or weaker at one particular serotype fimbrial gene promoter while having no affect at the other. The same argument can also be made for the RNA polymerase of different strains. Perhaps there is more than one RNA polymerase (α or σ subunit difference) operating within *B. pertussis* which initiate transcription at different fimbrial subunit genes. The inference here is that the RNA polymerase displaying affinity for the *fim3* gene promoter of Tohama I is no longer functional, while in CSL 137S, this protein is fully operational. The RNA polymerase α subunit interacts with transcription factors and the σ subunit is involved in promoter recognition, interaction with transcriptional

activators, DNA unwinding and setting up initiation complex (Ishihama, 1992; Ishihama, 1993; Busby and Ebright, 1994). Mutations in the *B. pertussis* RNA polymerase have been shown to effect the transcription of virulence genes (Stibitz, 1998). Bacteria produce a range of σ factors for separate tasks. Specialised RNA polymerases (σ^F) are used by a number of species to control the expression of flagellar genes (Arnosti and Chamberlin, 1989; Arnosti, 1990; Akerley and Miller, 1993; Kapatral *et al.*, 1996). The flagellar-specific σ^F factor has been implicated in the regulation of flagellar genes of *B. bronchiseptica* (Akerley and Miller, 1993). Perhaps there is a corresponding fimbrial-specific σ factor controlling the expression of fimbriae that may recognise and bind certain fimbrial promoter architectures and not others. Mutations accumulated over time could cause these specific σ factors to become quite strain-specific. Along the same lines, there is also a possibility that an RNA polymerase designed for other tasks, such as the transcriptional control of heat shock or flagella genes may have mutated, allowing it to recognise some, but not all, fimbrial gene promoters. Again, this rogue RNA polymerase could become a strain-specific phenomenon after many generations.

Another possible explanation may be the presence of an undiscovered factor which, possibly via an uncharacterised binding activity, has a degree of control over the expression of major fimbrial subunit genes. In their analysis of the *fimX* pseudogene of *B. pertussis*, Riboli and associates (1991) alluded to the "C stretch" and the "fim box" being binding regions for two separate transcription factors which may interact to bring about fimbrial expression. They isolated a *fim3* gene promoter exhibiting constitutive expression. The loss of virulence regulation was traced to deletions in the fim box. These results and those of this study point to the fim box being involved with BvgA binding, while the C stretch may be

involved in the binding of a yet to be elucidated serotype determination factor and/or RNA polymerase.

Strain CSL 127S overexpresses serotype 2 fimbriae and FHA. There was an 18 fold increase in *fim2* production and 47 fold upregulation of FHA expression exhibited by this strain compared with the parental strain. Due to the linkage of the fimbrial and FHA accessory genes (*fimA-fimD* and *fhaC*), it was hypothesised that the overexpression exhibited by this strain may be the result of a mutation in the promoter of the fimbrial/FHA accessory genes or the *fimC* upstream region. This was not the case however, with no differences detected in either of these regions. The difference between CSL 127 and CSL 127S was an alteration to the *fim2* gene promoter, again located within the C stretch. The promoter of the parental *fim2* gene contains a 17 residue C stretch, while the equivalent region of the overexpressing strain was 16 residues long. The non-*fim2* producing serotype 3 CSL 137 strains both contain a 16 residue C stretch. The strains expressing both fimbrial types, the CSL 1237 series, both possess a *fim2* 15 cytosine C stretch and exhibit only approximately 20% the *fim2* expression of CSL 127S. The original published sequence demonstrated that the Wellcome 28 strain (serotype 2, 3) *fim2* gene promoter region contains a 16 residue C stretch (Livey *et al.*, 1987). Sequence data of the *fim2* promoter of BPSA1 revealed that this strain also possesses a 16 cytosine C stretch. The agreement of this work with previous studies may be further evidence that 16 residues is the optimum number for expression of serotype 2 fimbriae. Once again however, the number of residues within the C stretch does not seem to be the sole determinant of fimbrial serotype. The type 3 strains (CSL 137 and CSL 137S), which do not express *fim2* at all, contain the same number of residues as the type 2 overexpressing strain (CSL 127S). As was discussed

earlier, it may be that different strains have different positioning requirements for the expression of fimbriae. There may be another factor involved in the expression of the serotype 2 major fimbrial subunit gene. The precise identity of this factor is unknown, however it is proposed here that an RNA polymerase or an as yet undetermined serotype determination factor may be the cause of differential fimbrial expression from similar or identical promoter architectures in different strains. Whatever the factor involved, a simple mechanism can be employed to explain differences exhibited by different strains. The "activating factor" of one strain (CSL 127S) might recognise and bind to a *fim2* gene promoter containing a 16 residue C stretch, thereby initiating transcriptional machinery. Another strain may also possess a *fim2* gene promoter with a 16 residue C stretch (CSL 137S), however the activating factor in this strain, might only recognise promoters containing a 13 cytosine C stretch. In this case transcription would not be activated at the *fim2* gene, however expression of the *fim3* gene would proceed.

The strain exhibiting a minor increase in all virulence factors assayed except FHA (CSL 1237S) does not contain differences in any of the promoters analysed. Possible reasoning for the increase in a number of proteins may include an alteration to a membrane protein which may have slightly increased the efflux of periplasmic proteins.

The results of this study also suggest that the sensitivity to alterations in the C stretch differ between the two major fimbrial serotypes. Changes of one residue either side of the ideal number (16 in W28 strain, BPSA (Livey *et al.*, 1987; Riboli *et al.*, 1991) and CSL 127S) contained within the C stretch of the *fim2* gene promoter can markedly alter expression. An addition of a single cytosine in CSL 127 (17 residues) resulted in the

abolition of fim2 expression entirely. The removal of one residue (CSL 1237 series, 15 residues) caused a reduction of fim2 expression to only 20% of the original. At the *fim3* gene promoter however, a reduction of 2-3 residues still allows for substantial fim3 production. The CSL 1237 series produce moderate levels of fim3 even though the C stretch contains only 11 residues, which is down on the value of 14 indicative of high expression levels (Willems *et al.*, 1990; Riboli *et al.*, 1991) and the 13 obtained for the overexpressing strain (CSL 137S) in this work.

The differences among the promoter regions of the *B. pertussis* strains studied has once again demonstrated the intricate nature of the expression of virulence genes in this highly complex organism. Further research into the regulation and architecture of the promoter regions of virulence genes and the roles they play in the pathogenesis of whooping cough will eventually lead to the development of more suitable vaccines against this disease.

General Conclusion

The inherent side effects associated with whole cell whooping cough immunisation regimes has necessitated the development of new, less reactogenic recombinant acellular vaccines. Purified pertussis toxoid has been a major component in all recent vaccine trials. Thus, there is a need for a safe and efficient system for the high level expression of pertussis toxin. This work reports that *B. bronchiseptica* possesses sequences homologous to the 3' end of the *ptl* operon (*ptlH* gene). Also described is the development of a system designed for the transfer of the pertussis toxin expression locus from ATCC 10580::Tn*fusPT1* to other *B. bronchiseptica* strains. The transfer was successful, with this locus being mobilised to derivatives of virulent and avirulent strains of *B. bronchiseptica*. No loss in the expression levels of the toxin was observed after the transfer. The levels of PT secretion in these overexpressing strains was monitored and it was found that neither virulent nor avirulent *B. bronchiseptica* exhibited the ability to secrete PT, confirming that the *B. bronchiseptica* *ptl* genes are cryptic. The transfer system developed here has the potential to be used to clone other types of selectable transposon-tagged expression systems. Insertion of the entire *ptx/ptl* operon into *B. bronchiseptica*, again under the control of a P_{Bb}-like promoter, would allow for the constitutive expression and secretion of PT in avirulent *B. bronchiseptica*. This would create an ideal background for PT production for vaccine preparations.

Increasingly, vaccine manufacturers are including fimbriae and pertactin in acellular preparations in addition to the PT and FHA used in the original acellular vaccines. These multivalent pertussis vaccines confer a greater immune response and are no more reactogenic than their mono-

and divalent counterparts. Therefore there is a need for the production of large amounts of these antigens. This study has examined *B. pertussis* strains expressing increased levels of these immunogens. These results suggest that the control of fimbrial expression and phase variation may be more complicated than has been previously understood. Further work using a greater range of *B. pertussis* strains may confirm this observation. Increasing our level of understanding of the regulation of fimbrial expression may bring about safer, easier production methods for these immunologically important antigens, leading to less reactogenic, more affordable vaccines. Alternatively, the fimbrial production by whole cell vaccine strains could be improved with the introduction of overexpressing promoter regions, resulting in a net increase in the immunogenic response elicited.

Whether in *B. pertussis* or *B. bronchiseptica*, the development of high level expression and secretion systems for recombinant candidate antigens for use in vaccines against whooping cough, will ensure the development of safer, more protective vaccines in the future.

Appendices

Appendix I Publications and Conference Papers

Publications

Smith, A. M., and M. J. Walker. 1996. Transfer of a pertussis toxin expression locus to isogenic *bvg* positive and *bvg*-negative strains of *Bordetella bronchiseptica* using an *in vivo* technique. Microbial Pathogenesis 20:263-273.

Guzmán, C. A., T. Dalla Pozza, A. M. Smith, and M. J. Walker. 1998. New generation vaccines against whooping cough. Recent Research Developments in Microbiology 2: 481-497.

Smith, A. M., H. Yan, T. Dalla Pozza, and M. J. Walker. 1999. Identification, cloning and expression of the signal peptidase I of *Bordetella pertussis*. Manuscript submitted for publication.

Smith, A. M., and M. J. Walker. 1999. The Virulence Factors of *Bordetella pertussis*: A Matter of Control. Manuscript submitted for publication.

Smith, A. M., D. Drane, J. Bennet, and M. J. Walker. 1999. Analysis of the *fim2* gene promoter regions of three different serotype specific strains of *Bordetella pertussis*. Implications for the mechanism of fimbrial phase variation. Manuscript in preparation.

Smith, A. M., D. Drane, J. Bennet, and M. J. Walker. 1999. The promoter of a *Bordetella pertussis* strain expressing large amounts of serotype 3 specific fimbriae contains a truncated C stretch. Manuscript in preparation.

Conference Presentations

Smith, A. M., and M. J. Walker. 1993. Development of a system for high level expression in *Bordetella bronchiseptica* for vaccine purposes. Vaccines '93, Lorne, Australia.

Smith, A. M., and M. J. Walker. 1994. High level pertussis toxin expression in *Bordetella bronchiseptica* for vaccine purposes. Australian Society for Microbiology, Annual Scientific Meeting, Melbourne, Australia.

Smith, A. M., K. N. Timmis, and M. J. Walker. 1994. *In vivo* transfer system for the overexpression of pertussis toxin in *Bordetella* species for vaccine purposes. Sixth International Congress for Infectious Diseases, Prague, Czech Republic.

Smith, A. M., and M. J. Walker. 1995. Pertussis toxin secretion in *Bordetella bronchiseptica*? Australian Society for Microbiology, Annual Scientific Meeting, Canberra, Australia.

Appendix II Recipes And Buffers

Media

Bordet-Gengou Agar

This agar, used to determine haemolytic ability, consisted of sterilised Bordet Gengou agar base supplemented with glycerol to 10 g/L. After cooling to approximately 45°C, sterile defibrinated horse blood to 15% was added.

Z Agar

Z agar was used as the solid nutrient substrate for all *Escherichia coli* strains. This medium contained glucose 1 g/L; NaCl 10 g/L; CaCl₂.2H₂O 0.367 g/L; tryptone 10 g/L; yeast extract 5 g/L. Agar to 15% was then added to solidify the medium.

Stainer-Scholte Medium

Used as minimal medium for *Bordetella* spp., Stainer-Scholte medium has two components.

SS-X Base

The contents of this medium consisted of L-glutamate (monosodium salt) 10.72 g/L; L-proline 0.24 g/L; NaCl 2.5 g/L; KH₂PO₄ 0.5 g/L; KCl 0.2 g/L; MgCl₂.6H₂O 0.1 g/L; CaCl₂.2H₂O 0.026 g/L; Tris-Base 6.075 g/L. The pH of this solution was then adjusted to pH 7.6. After sterilisation, the solution is supplemented with an appropriate amount of SS-X 100x salts.

SS-X 100x Salts

This is a mixture of heat-sensitive components which are essential to the diet of *Bordetella*; L-cysteine 0.04 g/L; FeSO₄.7H₂O 0.01 g/L; ascorbic acid 0.02 g/L; nicotinic acid 0.004 g/L; glutathione 0.1 g/L. Components were filter sterilised

X-Gal Medium

X-Gal broth was prepared by adding 2 mL of a 2% X-gal in DMF solution and 200 µL of a 0.1 M solution of IPTG to 1 L of sterile Z agar.

Urea Agar

The presence of urease enzymes can be detected using this medium. To prepare this, sterilised urea agar base 25.2 g/L was cooled to 50°C and supplemented with 5 mL of a 40% filter sterilised urea solution.

Luria Broth (LB)

LB was used as the liquid nutrient medium substrate for all *E. coli* strains. It consisted of Tryptone 10 g/L; Yeast extract 5 g/l; NaCl 10 g/L.

LBMgMal

Cells to be transfected by bacteriophage lambda were grown in this medium. It contained Tryptone 10 g/L; Yeast extract 5 g/l; NaCl 10 g/L; Glucose 1g/L; Maltose 4 g/L; MgCl₂.6H₂O 1 g/L.

SOC Medium

SOC medium was used in conjunction with the TA Cloning™ Kit. It contained tryptone, 2%; yeast extract, 0.5%; sodium chloride, 10 mM; potassium chloride, 2.5 mM. A 2 M stock of Mg²⁺, comprised of 1 M MgCl₂ and 1 M MgSO₄ was filter sterilised, along with a 2 M glucose stock. These were added to the sterile medium just prior to use, and the medium was then filter sterilised.

Antibiotics

Medium was made selective by the addition of antibiotics, to final concentrations as shown below in the following table:

Antibiotic	Concentration (µg/mL)	
	for <i>E. coli</i>	for <i>B. bronchiseptica</i>
Ampicillin	100	200
Chloramphenicol	50	50
Cephalexin	50	100
Kanamycin	50	50
Nalidixic Acid	50	50
Rifampicin	100	100
Tetracycline	20	20
Trimethoprim	50	50

Agarose Gel Electrophoresis

Agarose Gel (1%)

Agarose 2.0 g
1X TAE buffer to 200 mL

solution was heated to dissolve agarose, cooled to 50°C and poured

TAE Buffer 10X

Tris.Base	48.44 g/L
Sodium acetate	27.22 g/L
EDTA	7.44 g/L
made up to volume with dH ₂ O and adjusted to pH 8.0	

DNA Loading Buffer

Ficoll 400	15%
Bromophenol blue	0.25% (w/v)
made up to volume with dH ₂ O	

Molecular Weight Markers (λ HindIII)

Bacteriophage lambda DNA	50 μ L
dH ₂ O	120 μ l
Restriction buffer 10x	20 μ L
HindIII restriction enzyme	10 μ L

This mixture was incubated at 37°C for 60 minutes, followed by 65°C for 10 minutes

DNA Extraction Buffers

TE Buffer (Plasmid DNA)

Tris.HCl	50mM
EDTA	10mM
made up to volume and adjusted to pH 8.0	

TE Buffer (Chromosomal DNA)

Tris.HCl	100mM
EDTA	25mM
made up to volume and adjusted to pH 8.0	

TES

Tris.HCl	100mM
EDTA	25mM
NaCl	150mM
made up to volume and adjusted to pH 8.0	

TE Buffered Sucrose

Tris.HCl	50mM
EDTA	50mM
Sucrose	15% (w/v)
made up to volume with dH ₂ O and adjusted to pH 8.5	

Triton-X Solution

Triton-X 100	0.1% (v/v)
Tris.HCl	50mM
EDTA	10mM
made up to volume with dH ₂ O and adjusted to pH 8.5	

TE saturated Phenol

TE	200 mL
Phenol (solid)	to 200 mL
8-Hydroxyquinoline	0.1%

Enzyme Incubation Buffers (10X)

Enzymes were supplied with incubation buffer concentrates by manufacturers.

T4 DNA ligase buffer

Tris.HCl	660mM	pH 7.5
MgCl ₂	50mM	
EDTA	1mM	
Dithioerythritol	10mM	
ATP	10mM	

4 Units of ligase was used per reaction.

Southern Blot Buffers

SSC Buffer 20x

NaCl	175.3 g/L
Sodium citrate	88.2 g/L
made up to volume with dH ₂ O and pH adjusted to 7.0	

*Protein Analysis***PBS**

NaCl	137mM
KCl	2.7mM
Na ₂ HPO ₄	8mM
KH ₂ PO ₄	1.5mM

made up to volume with dH₂O and pH checked at 7.4

Cracking Buffer

Tris.HCl	60mM
Bromophenol blue	0.01% (w/v)
Glycerol	10% (v/v)
SDS	1% (w/v)
β-mercaptoethanol	1% (w/v)

Running Buffer

Tris.Base	15 g/L
Glycine	72 g/L
SDS	5 g/L

made up to volume with dH₂O and pH checked at 8.3

Acrylamide Stock Solution (35:0.8)

Acrylamide	87.5 g
Bis	2.0 g

made up to 250 mL with dH₂O

Resolving Gel (15%)

Acrylamide stock (35: 0.8)	4.25 mL
1.5M Tris.HCl (pH 8.8)	2.5 mL
10% SDS	0.1 mL
dH ₂ O	3.4 mL
Fresh 10% APS	50 μL
TEMED	50 μL

Stacking Gel (4%)

Acrylamide stock (35: 0.8)1.1 mL	
0.5M Tris.HCl (pH 6.8)	2.5 mL
10% SDS	0.1 mL
dH ₂ O	6.3 mL
Fresh 10% APS	50 µL
TEMED	50 µL

Coomassie Stain

Coomassie Brilliant Blue R250	1 g/L
Methanol	50% (v/v)
Acetic acid	10% (v/v)

Destain

Methanol	5% (v/v)
Acetic acid	7.5% (v/v)

Western Buffer

SDS	0.037% (w/v)
Glycine	39mM
Methanol	20% (v/v)
made up to volume with dH ₂ O	

TBE Buffer

TBE buffer (x 10) for DNA sequencing contained

Tris-base	162 g/l
Boric acid	27.5 g/l
EDTA	9.5 g/l.

The pH of this solution is approximately pH 8 and was not adjusted.

Buffers For Qiagen Plasmid Extraction

Buffer P1

Buffer P1 contained RNase A, 100 mg/l; Tris-HCl, 6.06 g/l; EDTA, 3.72 g/l; pH 8.0, store at 4°C.

Buffer P2

Buffer P2 contained NaOH, 8.0 g/l; 20% SDS, 50 ml/l.

Buffer P3

Buffer P3 contained potassium acetate, 294.45 g/l.

Buffer QBT

Buffer QBT contained NaCl 43.83 g/l; MOPS, 10.46 g/l; ethanol, 15 ml; Triton X-100, 0.15%; pH 7.0.

Buffer QC

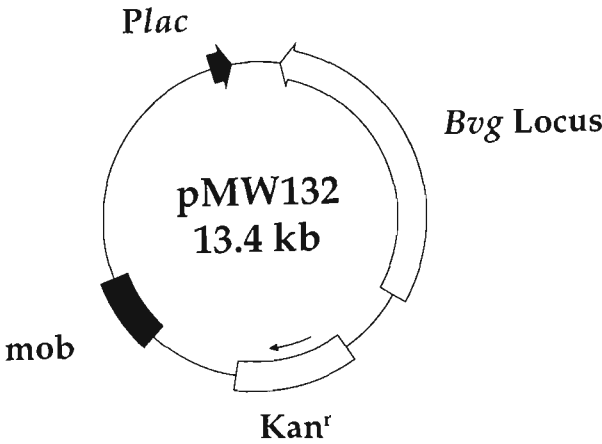
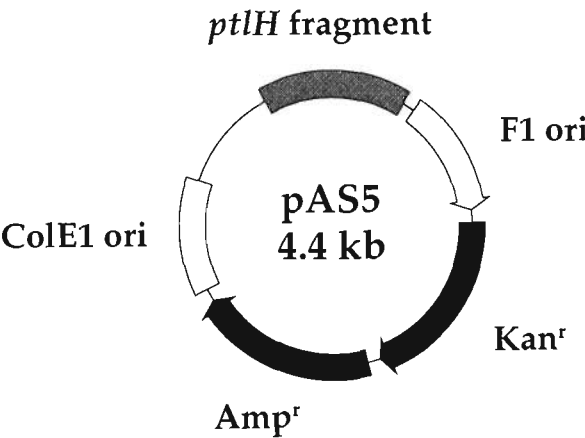
Buffer QC contained NaCl 1 M, 43.83 g/l; MOPS, 10.46 g/l; ethanol ,15%; pH 7.0.

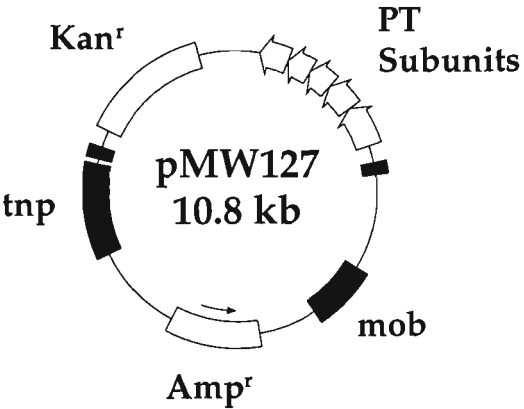
Buffer QF

Buffer QF contained NaCl, 73.05 g/l; Tris-HCl, 6.06 g/l; ethanol, 15%; pH 8.5.

Appendix III Plasmids Used in This Study

These are diagrammatic representations of plasmids used in this thesis that are mentioned, but not pictured within the text.





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