University of Glasgow

Sequence variation and conservation in virulence-related genes and expression of adenylate cyclase toxin of *Bordetella pertussis*

Erica Ruth Packard

Presented for the degree of Doctor of Philosophy in the
Division of Infection and Immunity,
Institute of Biomedical and Life Sciences,
University of Glasgow

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DECLARATION

Unless otherwise stated, this thesis is the original work of the author

Erica Packard

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ABBREVIATIONS

AC	Adenylate cyclase
ACV	Acellular vaccine
Amp	Ampicillin
APS	Adenosine 5′ phosphosulphate
ATP	Adenosine triphosphate
BapC	Bordetella autotransporter protein C
BLAST	Basic local alignment tool
BG	Bordet-Gengou
BrkA	Bordetella resistance to killing protein A
BrkB	Bordetella resistance to killing protein B
BSA	Bovine serum albumin
Bvg	Bordetella virulence gene
CAA	Casamino acids
CaM	Calmodulin
cAMP	Adenosine 3′, 5′-cyclic monophophate
cfu	Colony forming unit
CL	Cyclodextrin liquid
CyaA	Adenylate cyclase toxin
DII	Division of Infection and Immunity
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNT	Dermonecrotic toxin
dNTP	Deoxynucleoside triphosphate
EDTA	Ethylenediaminetetraacetic acid

EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
FHA	Filamentous haemagglutinin
Fim	Fimbriae
x *g*	gravity
g/mg/µg/ng	Gram/milligram/microgram/nanogram
gfp	Green fluorescent protein
h	Hour
HPA	Health Protection Agency
IU	International units
l/ml/µl	Litre/millilitre/microlitre
LB	Luria Betrani
LC	LightCycler
LD_{50}	50% lethal dose
M/mM/µM	Molar/millimolar/micromolar
MgAC	Magnesium acetate
min	Minute
MLST	Multilocus sequence typing
MTT	3-(4,4-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide
MVGST	Multilocus virulence gene sequence typing
NCTC	National Collection of Type Cultures
OD_{xxxnm}	Optical density at xxx_{nm} wavelength
OMP	Outer-membrane protein
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis

PICU	Paediatric intensive care unit
PMSF	Phenylmethanesulphonyl fluoride
PPi	Pyrophosphate
PPiase	Inorganic pyrophosphatase
Prn	Pertactin
Ptx	Pertussis toxin
rpm	Revolutions per minute
RSIL	Respiratory and Systemic Infections Laboratory
SDS	Sodium dodecyl sulphate
sec	Seconds
SS	Stainer-Scholte
T	Type strain
TBE	Tris-borate-EDTA
TCF	Tracheal colonisation factor
TCT	Tracheal cytotoxin
TE	10mM Tris, 1mM EDTA
Tet	Tetracycline
Tm	Melting temperature
U	Units
vag	Virulence-activated gene
Vag8	Virulence-activated gene 8
vrg	Virulence-repressed gene
WCV	Whole-cell vaccine

ABSTRACT

The aetiological agent of pertussis, *Bordetella pertussis*, is still a major cause of disease today and occurs even in countries that have had vaccination programmess implemented since about the 1950s. Several countries have reported increasing levels of pertussis in recent years and one possible reason for this is that an antigenic shift has occurred in the *B. pertussis* population since the introduction of pertussis whole-cell vaccines. Changes have been described in sequence types of two virulence-related genes, encoding pertactin (*prnA*) and pertussis toxin S1 subunit (*ptxA*), amongst *B. pertussis* from several countries and the United Kingdom (UK). *B. pertussis* population was expanded in this study by investigating other virulence-related genes. An already established multilocus sequence typing (MLST) scheme (based on a combination of alleles of *ptxA*, pertussis toxin S3 subunit (*ptxC*) and tracheal colonisation factor (*tcfA*) genes) was applied to the UK *B. pertussis* population. The same predominating *tcfA* allele was found in all time periods studied, however, there has been a shift in predominating sequence types in the UK population relating to *ptxC* and *prnA* types. The predominating alleles found in recent isolates were not seen in isolates from the pre-vaccination era or from the era just after the introduction of whole-cell vaccine. New, multilocus virulence gene sequence typing (MVGST) schemes are proposed in this study. These include the *prnA* gene and another polymorphic gene encoding fimbrial antigen 2 and these schemes are compared to the MLST scheme. Increasing the number of gene targets increased the discriminatory power of the typing scheme. All schemes identified sequence types in recent *B. pertussis* isolates that were not found in the pre-vaccination era or the era just after the introduction of vaccination. However, no increased incidence of pertussis has been described for the UK. It is accepted that particular sequence types found within the UK *B. pertussis* population and other *B. pertussis* populations may not be identical, and that there may be unknown genes that are affecting pertussis levels within different countries. Other genes were investigated, namely those encoding adenylate cyclase toxin, virulence-activated gene 8, bordetella resistance to killing protein A, bordetella autotransporter protein C, outer-membrane protein Q, but they showed insufficient variation to be useful for inclusion in the MVGST schemes.

A novel conductimetry tool was used to develop a rapid procedure for detecting adenylate cyclase (AC) enzymic activity from adenylate cyclase toxin (CyaA)

preparations and the optimisation of this assay is described. The conductimetry assay was found to be useful for determining the phenotypic phase of *Bordetella* strains as CyaA is expressed only in the virulent (Bvg^+) phase. However, the conductimetry assay was not sufficiently sensitive for use as a diagnostic tool. Two recent (1999) isolates of *B. pertussis* were found to be different from other isolates due to the presence of a 6 bp insertion between *cyaA* and its accessory gene *cyaC*. These isolates were screened for differences in AC activity by the conductimetry assay and for differences in cytotoxicity. No clear differences were found between these two isolates and a laboratory strain of *B. pertussis* laboratory strain, BP338 strain, which was considered to be a typical strain and representative of the *B. pertussis* population. Further studies should be carried out to determine if this 6 bp insertion has an effect on the phenotype of the strains and their virulence.

Novel plasmid constructs were made containing the *cyaA* promoter region inserted upstream of promoterless reporter genes, namely the *lux* operon and *gfp*, and these were maintained in *Escherichia coli*. A plate-mating method was used to transfer the *lux* construct from the donor *E. coli* strain to the recipient *B. pertussis* BP338 strain but the plasmid was not maintained in *B. pertussis* possibly due to the *Hin*dIII restriction/modification system known to be present in this species.

1.0 Introduction

1.1 DESCRIPTION OF *Bordetella* spp.

1.1.1 Definition of the genus *Bordetella*

Bordetella is a member of the family *Alcaligenaceae* to which *Alcaligenes* and *Achromobacter* also belong (von Wintzingerode *et al.*, 2001). Comparison of 16S rRNA gene sequences has shown that *Bordetella* forms a distinct cluster separated from *Alcaligenes* and *Achromobacter* within the β-Proteobacteria. The *Bordetella* are Gram-negative, catalase positive, asaccharolytic coccobacilli with a G+C content of 60-69 mol %. Bacterial growth is aerobic but one species grows anaerobically under conditions favouring (respiratory) nitrate and selenate reduction (von Wintzingerode *et al.*, 2001; see below). Seven species are isolated from humans and warm-blooded animals while one species is an environmental organism (see below).

1.1.2 Members of the genus *Bordetella*

The genus *Bordetella* consists of eight species, of which *Bordetella pertussis* the causative agent of whooping cough (pertussis) is the type species. The first description of the characteristic symptoms of pertussis was by de Baillou in 1640 who reported an epidemic in Paris in 1578 (Gerlach *et al.*, 2001) but the organism was not isolated until 1906 by Bordet and Gengou. The organism was originally classified as *Haemophilus pertussis* due to its growth requirement for blood but was found not to need the growth factors X (haemin) and V (nicotinamide adenine dinucleotide). Moreno-Lopez (1952; cited by Parton, 2004) later reclassified the organism in its own genus, *Bordetella*.

B. parapertussis was isolated originally from a mild case of human pertussis (Bradford and Slavin, 1937, cited by R. Parton) and later from lambs showing signs of pneumonia (Cullinane *et al.*, 1987). *B. bronchiseptica* was first isolated from the respiratory tracts of dogs by Ferry (1910, cited by Parton, 2004) and is also now known to infect many mammals, including humans, although infection in humans is relatively uncommon and usually occurs in immunocompromised hosts (Woolfrey and Moody, 1991). Some cases of humans infected with *B. bronchiseptica* have been related to contact with infected animals (Gueirard *et al.*, 1995). When *B. bronchiseptica* causes bacteraemia in humans there is evidence of a primary respiratory tract infection, which probably provides access to secondary haematogenous growth (Cookson *et al.*, 1994).

B. avium is usually isolated from turkey poults and other birds with respiratory disease (Kersters *et al.*, 1984). *Bordetella hinzii*, a species similar to *B. avium*, can cause secondary or opportunistic infections in poultry (Vandamme *et al.*, 1995). Bacteremia caused by *B. hinzii* has been described in an immunocompromised patient without evidence of respiratory infection (Cookson *et al.*, 1994).

B. holmesii isolates were originally classified as CDC non-oxidiser group 2 but were re-named when they were found to conform to the description of the family *Alcaligenaceae* and the genus *Bordetella* (Weyant *et al.*, 1995). The first isolates were from the blood of patients that had underlying clinical conditions but, more recently, this species has been isolated from the sputum of immunocompromised patients and from nasopharyngeal specimens of patients suspected to have pertussis (Tang *et al.*, 1998; Yih *et al.*, 1999). The prevalence of *B. holmesii* carriage in asymptomatic individuals is unknown, therefore it cannot be presumed that *B. holmesii* is the causative agent of respiratory infection in man (Yih *et al.*, 1999). The species *B. trematum* has been isolated from human wound and ear infections and these isolates were originally described as atypical *Bordetella* or as unidentified organism (Vandamme *et al.*, 1996).

The recently described *B. petrii* is distinct from the other seven known members of the genus. To date *B. petrii* is the first member of the genus to be isolated from the environment and capable of anaerobic growth. It was originally isolated from a mixed dechlorinating culture and the organism is able to reduce selenate to its elemental form, selenium (von Wintzingerode *et al.*, 2001). *B. petrii* is suggested to be closely related to an environmental progenitor of bordetellae and further characterisation of this species may provide interesting information on the early evolution of members of this genus (Gerlach *et al.*, 2001).

1.2 *B. pertussis* the organism

B. pertussis is a small (0.2-0.5 μm x 0.5-2 μm) Gram-negative, aerobic, non-motile, bacterium. Growth of *B. pertussis* was originally achieved in 1906 by Bordet and Gengou using a glycerol-potato extract medium without peptone and containing blood. The Bordet-Gengou (BG) medium, with minor modifications, is still used today for cultivation of the organism. *B. pertussis* forms tiny (0.5 mm in diameter) convex pearl-like glistening colonies

when grown on BG medium and each colony is surrounded by a narrow zone of haemolysis. After 5-6 days of incubation the diameter of the colonies increase to 2-3 mm (Roberts and Parton, 2001). The organism is a strict aerobe with an optimum growth temperature of 35-37°C, it does not ferment carbohydrates and its energy is primarily obtained from oxidation of amino acids (Roberts and Parton, 2001). *B. pertussis* has simple nutritional requirements and can be cultured in a simple buffered salts medium containing several amino acids, an organic sulphur source and several growth factors. The amino acids required for its growth are glutamic acid or proline and growth factors are nicotinic acid (an essential vitamin for *B. pertussis*), glutathione and ascorbic acid. Of the *Bordetella* species, *B. pertussis* is the most fastidious. It is sensitive to unsaturated fatty acids or residual detergents that may be found on laboratory glassware. *B. pertussis* is also known to release these fatty acids into the growth medium (Frohlich *et al.*, 1996). Media used for the growth of *B. pertussis* are usually supplemented with substances, such as blood and starch, which can absorb these growth inhibitors.

Traditionally, *B. pertussis* virulence factors have been divided into two groups: adhesins and toxins. In addition, the organism produces other factors to cause disease. Figure 1.2.1 depicts the organism and its virulence factors, which are described in detail in the following sections.

1.3 ADHESINS AND AUTOTRANSPORTERS

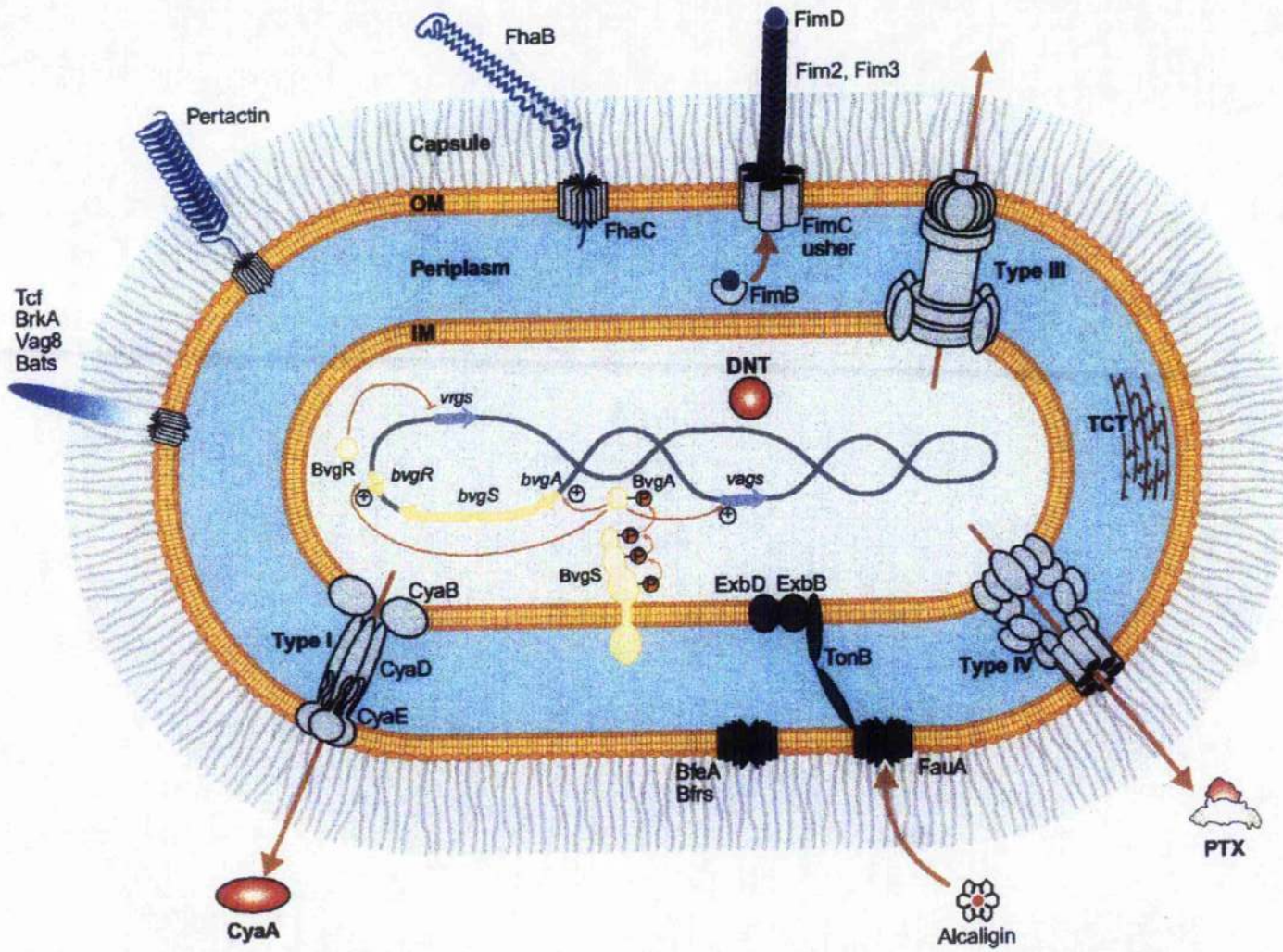
1.3.1 Filamentous haemagglutinin

Filamentous haemagglutinin (FHA) is expressed by *B. pertussis* and similar proteins are also expressed by *B. parapertussis*, *B. bronchiseptica* and *B. avium*. Filamentous haemagglutinin is the major adhesin of *B. pertussis*, and is synthesised as a 367 kDa protein (Domenighini *et al.*, 1990). The structural gene is *fhaB*, and the accessory gene *fhaC* is required for its secretion and activation (Locht *et al.*, 1993).

Makhov *et al.* (1994) investigated the structure of the *B. pertussis* FHA molecule by combining electron microscopy and circular dichroism spectroscopy with computational analyses of its amino acid sequence. The FHA molecule was found to be 50 nm in length with a globular head consisting of 2 domains, a 35 nm long shaft, and a small flexible tail.

Figure 1.2.1. *B. pertussis* virulence factors. *B. pertussis* a Gram-negative organism with inner and outer membranes (IM and OM). The adhesins are shown in blue and the toxins are shown in red. Adapted from Locht *et al.* (2001). The key lists the abbreviated virulence factors that will be described in this section.

Key:
Fha – Filamentous haemagglutinin
Fim – Fimbriae
TCT – Tracheal cytotoxin
DNT – Dermonecrotic toxin
PTX – Pertussis toxin
CyaA – Adenylate cyclase toxin
Tcf – Tracheal colonisation factor
BrkA – *Bordetella* resistance to killing protein A
Vag8 – Virulence-activated gene 8 protein
bvg – *bordetella* virulence gene
vrgs - virulence-repressed genes
vags – virulence-activated genes
Bats – Bordetella autotransporters
TypeI/III/IV- secretion systems
Exb/TonB/FauA/Bfe - Iron uptake systems


FhaB
FimD
Fim2, Fim3
Pertactin
Capsule
OM
FhaC
FimC
usher
Periplasm
FimB
Type III
Tcf
BrkA
Vag8
Bats
IM
DNT
vrgs
TCT
BvgR
bvgR
bvgS
bvgA
BvgA
vags
BvgS
ExbD
ExbB
CyaB
Type I
CyaD
CyaE
TonB
Type IV
BteA
Bfrs
FauA
PTX
CyaA
Alcaligin

Biosynthesis of FHA is dependent on the outer-membrane associated accessory protein, FhaC. Interactions between the FHA precursor and FhaC are highly specific and involve an amino-proximal secretion domain on FHA. FhaC is able to form channels through which FHA is believed to cross the outer membrane in an extended conformation (Locht *et al.*, 2001). A newly identified autotransporter, SphB1 (see section 1.7), was found to proteolytically process the FHA precursor to release the N-terminal 220kDa 'mature' protein as it reaches the cell surface (Coutte *et al.*, 2001). Haemagglutinating and adherence activities have been associated almost exclusively with this 220kDa species (Domenighini *et al.*, 1990).

FHA has multiple binding activities; there are at least two separate sites involved in separate adhesion reactions, the Arginine-Glycine-Aspartic acid (RGD) motif and the haemagglutination site, which are located at opposite ends of the molecule. FHA binds specifically to sulphated glycolipids but not to gangliosides or other neutral glycolipids, suggesting that epithelial cell surface sulphated glycolipids function as receptors for FHA. The molecule may utilise sulphated glycolipids and proteoglycans commonly found on the surface of human cells and tissues to initiate infection. The specificity that FHA has for binding to these molecules is responsible for its haemagglutinating activity (Hannah *et al.*, 1994). Interaction of its RGD sequence with CR3 integrins triggers invasion of cells without generating an oxidative burst, allowing survival of *B. pertussis* within the macrophage (Locht *et al.*, 1993). Both FHA and fimbriae are involved in the adhesion of *B. pertussis* to laryngeal epithelial cells but only FHA is involved in the adherence to bronchial epithelial cells (van den Berg, 1999). FHA has the ability to mediate the attachment of *B. pertussis* to neutrophils but does not block phagocytosis (Weingart and Weiss, 2000).

FHA is probably involved in the first steps of *B. pertussis* pathogenesis as the *fha* genes are activated together with *bvg* genes (see section 1.6). Within a few minutes following environmental changes that allow virulence expression, BvgA activates its own expression and the expression of the *fha* genes. The *fhaC* gene is co-transcribed with the fimbriae accessory genes in a common polycistronic operon (Locht *et al.*, 1993). This suggests that the *B. pertussis* fimbriae, along with FHA, may act during the initial steps of infection. It was demonstrated using a *B. bronchiseptica fhaB* deletion strain in a rat respiratory model

that FHA is absolutely required for the colonisation of the trachea (Cotter *et al.*, 1998). In order for bacteria to resist the clearing action of the mucociliary escalator, efficient adherence to ciliated tracheal epithelial cells is required, and FHA may provide this. This molecule was not required for establishment of tracheal colonisation in anaesthetised animals in which the mucociliary clearance and lower airway protection were compromised. FHA has been demonstrated to provide protection against *B. pertussis* infection. Mice that were immunised with FHA by intraperitoneal or intramuscular inoculation, then challenged with an aerosol of *B. pertussis*, had significantly reduced bacterial colonisation in the lungs and trachea (Kimura *et al.*, 1990). FHA is currently included in multicomponent acellular pertussis vaccines.

1.3.2 Fimbriae

Bordetella species express fimbriae of at least four serotypes, Fim2, Fim3, FimX and FimA. In *B. pertussis* and *B. bronchiseptica* the locus that is required for secretion and assembly of fimbriae (*fimB*, *fimC* and *fimD*) is located between *fhaB* and *fhaC* (Mattoo *et al.*, 2000). *B. pertussis* fimbriae are composed of a major subunit, either Fim2 or Fim3, and a minor subunit FimD (Geuijen *et al.*, 1997). Expression of *fim2* and *fim3* genes are positively regulated by the *bvg* locus and by phase variation (this operates at the level of individual *fim* genes). Phase variation is characterised by switching between a high and a low level of expression of a particular *fim* gene. A region located 100 bp upstream of the *fim3* gene is involved in this regulation (Willems *et al.*, 1990) where a stretch of Cytosines (Cs) upstream of the putative –10 box on the *fim* promoter would be subject to slip-strand mispairing during chromosome replication. Analysis of the *fim3* genes in the + or - phases have revealed that insertions and deletions of 1-5 bp in this stretch of Cs are responsible for fimbrial phase variation (Willems *et al.*, 1990) but this differs for *fimX* due to the stretch of Cs being shorter suggesting that a deletion had occurred. The *fim3* promoter region was found to be conserved in the other *B. pertussis* fimbrial genes *fim2* and the silent gene *fimX*. The *B. pertussis* fimbrial operon also contains a pseudogene, *fimA*, at its 5' end (Boschwitz *et al.*, 1997). This gene is expressed by *B. bronchiseptica* in a *bvg*-regulated manner. It was suggested that *fim3*, *fim2* and *fimX* were derived from an ancestor of *fimA* that was duplicated to positions outside the *fim* operon. The differences in host range between *B. pertussis* and *B. bronchiseptica* may be due to differences in *fimA* expression.

The major fimbrial subunit of *B. pertussis* is able to bind to the sulphated glycosaminoglycans: heparan sulphate, chondroitin sulphate and dextran sulphate. Mutations in the *fim* or *fha* genes decreased binding of *B. pertussis* to heparan sulphate (Geuijen *et al.*, 1996). Proteins that are able to bind to heparin (this molecule is a polysaccharide structurally similar to heparan sulphate) contain local accumulations of positively-charged amino acid residues bounded by negatively-charged amino acid residues. Two such regions, H1 and H2, important for binding to heparin, have been found in Fim2 and there were regions outside of H1 and H2 that may also be important for binding to sulphated sugars (Geuijen *et al.*, 1998). Major heparin-binding sites identified in Fim2 are part of the epitopes recognised by human antibodies, suggesting that the heparin-binding regions are exposed at the fimbrial surface and are immunodominant (Geuijen *et al.*, 1998). Comparison of the heparin-binding regions of Fim2 with Fim3 sequence showed that homologous regions were present. Since *B. pertussis* fimbriae show weak serological cross-reactivity, the differences in primary structure in the heparin-binding regions of Fim2 and Fim3 may affect antibody binding but not heparin binding, allowing the bacteria to evade antibody mediated host defences by switching fimbrial gene expression.

Immunoelectron microscopy has located the minor subunit, FimD, at the fimbrial tip (Geuijen *et al.*, 1997). *B. pertussis* FimD interacts with the integrin very late antigen-5 (VLA-5) on monocytes, which activates CR3 resulting in enhanced binding of *B. pertussis* to the CR3 receptor via FHA (Hazenbos *et al.*, 1995). Similar to the major fimbrial subunits, FimD also binds to the sulphated sugar heparin.

The role of fimbriae in the pathogenesis of *Bordetella* spp. has been investigated using mutant strains. A *B. bronchiseptica* mutant that was unable to express fimbriae was defective in its ability for efficient establishment and persistent colonisation of the rat trachea (Mattoo *et al.*, 2000). The role of FimD in the colonisation of the mouse respiratory tract was studied using fimbrial and FHA mutants of *B. pertussis* (Geuijen *et al.*, 1997). Mice were infected intranasally with the wild type strain or one of the mutant strains. Compared to the FHA and FimD$^+$ mutants (the major subunits were not expressed, only the minor subunit was expressed), the FimD$^-$ mutant (which did not express major or minor subunits) showed the lowest level of colonisation with the most pronounced defect in the mouse

trachea. However, the FimD⁻ mutant was found to produce 75% less FHA than the wild-type and this may have been partly responsible for the difference in colonisation behaviour. The FHA mutant was far less affected in its colonisation ability than FimD⁻ mutant so it was assumed that colonisation defects were due to the lack of the minor subunit (Geuijen *et al.*, 1997). *B. pertussis* fimbriae were found to confer a high level of protection against challenge with *B. pertussis,* demonstrated by a reduction in colonisation by the bacteria in the mouse nasopharynx, trachea and lungs following vaccination with a preparation containing both fimbriae (Willems *et al.*, 1998).

1.3.3 Autotransporters

A number of *B. pertussis* virulence factors are classified as autotransporter proteins and use the type V secretion system (Henderson and Nataro, 2001). This secretion system depends on the protein moiety that mediates export through the outer membrane being contained within the precursor of the secreted protein itself. There are three distinct domains for the majority of autotransporters, an amino-terminal leader peptide, the surface-localised mature protein (passenger domain) and a carboxy-terminal domain (β domain) that mediates secretion through the outer membrane (Henderson *et al.*, 1998). It is believed that these three domains confer all the requirements for transport through the inner and outer membranes. From the whole-genome sequences of *B. pertussis*, *B. parapertussis* and *B. bronchiseptica*, many other putative autotransporters have now been identified amongst these *Bordetella*. To date, genes with potential to encode 21 of these proteins (Parkhill *et al.*, 2003) are known. *B. bronchiseptica* encodes the greatest number of autotrasnsporters. The other two species, *B. pertussis* and *B. parapertussis*, have fewer autotransporter genes and more autotransporter pseudogenes (non-functional) than *B. bronchiseptica*. The autotransporters that have been best characterised (pertactin, tracheal colonisation factor, virulence-activated gene 8, bordetella resistance to killing protein A) and one that is of particular interest to this study (bordetella autotransporter protein C) are described below and shown Fig. 1.3.3.1.

Figure 1.3.3.1: Schematic diagram showing similarities between the autotransporter proteins, pertactin (Prn), tracheal colonisation factor (Tcf), bordetella resistance to killing protein A (BrkA), virulence-activated gene 8 (Vag8) and bordetella autotransporter protein C (BapC). Numbering relates to the amino acid position and Arginine-Glycine-Aspartatic Acid (RGD) sites are indicated. Pertactin is also shown with its immunodominant region 1 $(GGXXP)_x$ and immunodominant region 2 $(PQP)_x$.

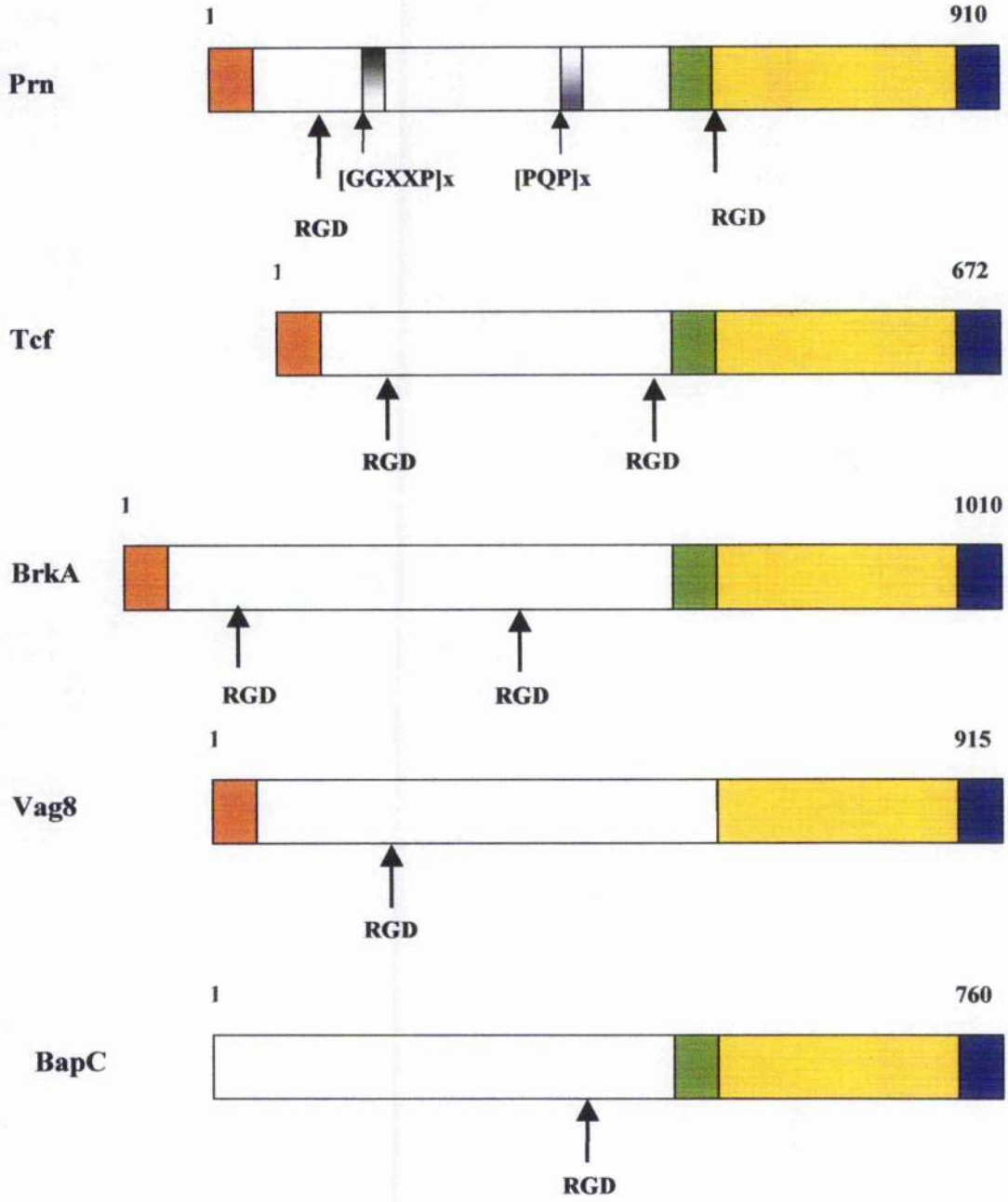
Key:

Signal sequence

Putative cleavage site

C-terminal region (30 kDa)

Outer membrane localisation signal


Prn
1
910
[GGXXP]x
[PQP]x
RGD
RGD
Tcf
1
672
RGD
RGD
BrkA
1
1010
RGD
RGD
Vag8
1
915
RGD
BapC
1
760
RGD

1.3.3.1 Pertactin

Pertactin (Prn) was demonstrated to be capable of functioning as an adhesin for mammalian cells such as Chinese hamster ovary (CHO) cells and this attachment involved the participation of an RGD-containing sequence within this molecule (Leininger *et al.*, 1991). The name pertactin was proposed for this protein due to its function, [per (pertussis) and tactin ('tactus' to touch)] (Leininger *et al.*, 1991). A Prn mutant of *B. pertussis* adhered 30-40% less well than the wild-type strain to CHO and HeLa cells. This decrease in adherence rather than complete inhibition supports the role of other factors such as FHA and fimbriae involved in *B. pertussis* adherence to these cells. The gene, *prnA*, which encodes Prn is controlled by the *bvg* locus (Charles *et al.*, 1989). The 93 kDa protein precursor of *B. pertussis* is cleaved at the carboxy terminus to generate a mature protein of 69 kDa, known as the passenger domain (Charles *et al.*, 1994). The site where cleavage is proposed to occur is at Aspartatic acid (D) 631 (Passerini de Rossi et al., 1999). The processed mature passenger domain remains noncovalently associated with the 30 kDa carboxy-terminal domain (β domain) (Henderson and Nataro, 2001). Pertactin is also expressed by *B. parapertussis* and *B. bronchiseptica* and a similar cleavage event also occurs to generate mature passenger proteins of 68 kDa and 70 kDa, respectively (Charles *et al.*, 1994).

Pertactin contains two repeat regions, region 1 Glycine-Glycine-X-X-Proline $(GGXXP)_x$ at positions 939-1014 and region 2 P-Glutamine-P $(PQP)_x$ at positions 1878-1943 (Fig. 1.3.3.1; Charles *et al.*, 1989). These repeat regions of Prn have been shown to be immunodominant as demonstrated by monoclonal antibody mapping. Charles *et al.*, (1991) identified that region 2 was more immunogenic than region 1 and a B cell epitope, recognised by mice and humans, was identified within region 1. Investigation of *prnA* sequence variation between *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* determined that sequence variation was mainly found in region 2 (Boursaux-Eude and Guiso, 2000). This is consistent with the fact that there is a lack of cross-protection between the Prn molecules of these three species.

Pertactin is the only *Bordetella* autotransporter for which the crystal structure has been determined (Emsley *et al.*, 1996). The mature protein comprises a 16-stranded parallel β helix with a V-shaped cross-section. Several loops from the helix protrude, each containing

sequence motifs associated with the biological activity of the protein. One of the loops is formed from the GGXXP repeats (immunodominant region 1). Pertactin was shown to be protective when adult female BALB/c mice were immunised intranasally with *B. pertussis* Prn and then challenged by aerosol exposure to *B. pertussis*. The mice that had been immunised with this antigen were greatly enhanced in their ability to clear *B. pertussis* from their lungs (Roberts *et al.*, 1993). Pertactin is included in some of the multicomponent acellular vaccines that are currently in use (van Loo *et al.*, 2002). Several authors have described sequence variation between the immunodominant region 1 of Prn molecules expressed by circulating *B. pertussis* isolates and this is reported in section 1.13.2. In an intranasal challenge of mice with *B. pertussis*, a Dutch whole-cell vaccine preparation of a strain expressing *prnA*(1) afforded less protection against *B. pertussis* strains expressing *prnA*(2) or *prnA*(3) than against a strain expressing *prnA*(1) (King *et al.*, 2001).

1.3.3.2 Tracheal colonisation factor

Transposon *phoA* insertions were used to identify virulence-activated genes (*vags*) of *B. pertussis* strains. One such strain, SK34, contained a gene interrupted by the transposon that was designated *vag-34*. This gene was characterised and renamed tracheal colonisation factor (*tcfA*) (Finn and Stevens, 1995). In mouse studies, a *tcfA* mutant strain SK34 was significantly less able to colonise and persist in the tracheas when compared to *B. pertussis* strain 18323^{T}. However, the number of bacteria isolated from the lungs of mice infected with SK34 and 18323^{T} did not differ significantly (Finn and Stevens, 1995). The mutant strain SK34, was defective in producing four outer-membrane proteins. Three of these (92 kDa, 90 kDa and 60 kDa) were only visible on immunoblots and the fourth (30 kDa) was visible on SDS-PAGE. *In vitro* translation and transcription data revealed that TcfA migrates as two bands of approximately 90 kDa. However, from its derived amino acid sequence a mass of 68 kDa is predicted. The 68 kDa form of TcfA contains 10% proline residues and it is known from previous Prn studies that the presence of proline residues affect migration of the protein in SDS-PAGE. Therefore, the four proteins found correspond to the precursor forms (92 and 90 kDa), mature protein (60 kDa) and the C-terminal β domain (30 kDa). After cleavage of its signal sequence, the resulting 64kDa mature protein is translocated across through the outer membrane via its C-terminal tail. Proteolytic cleavage occurs at D393 releasing the 34 kDa passenger domain from the 30 kDa β domain,

which would then remain embedded in the outer membrane (Fig. 1.3.3.1; Finn and Stevens, 1995; Passerini de Rossi *et al.*, 1999). The 34 kDa passenger domain contains an RGD motif implicated in integrin binding (Fig. 1.3.3.1; Henderson and Nataro, 2001). Unlike Prn the majority of the N-terminal domain is released from the *B. pertussis* surface although culture supernates of *B. pertussis* were found to contain only the 60 kDa mature form, indicating that outer-membrane associated and secreted versions of passenger domain exist (Finn and Stevens, 1995). Western blotting showed that Tcf was not present in *B. parapertussis* or *B. bronchiseptica* and these results agree with the sequencing of the *Bordetella* genomes, which revealed that *tcfA* is not present in *B. parapertussis* and is present only as a pseudogene in *B. bronchiseptica* (Parkhill *et al.*, 2003). Tracheal colonisation factor has been shown to exhibit protective activity. Mice that were immunised with attenuated *Vibrio cholerae*, used as a live oral vaccine, expressing *B. pertussis* Tcf showed protection against *B. pertussis* infection of the trachea, with approximately 10-fold reduction in colonisation by the bacteria but not of the lungs (Chen *et al.*, 1998).

1.3.3.3 Bordetella resistance to killing protein A

B. pertussis mutants were generated using insertion Tn5 *lac* to interrupt genes and those that were not deficient in expressing any of the previously characterised *vags* were further investigated. One such *B. pertussis* mutant strain, BPM2041, possessed all the known *vags* but was 10 times less virulent in mice, suggesting that a previously unidentified virulence factor gene had been interrupted (Fernandez and Weiss, 1994). BPM2041 was more susceptible to killing than BP338, the parent strain, by normal human serum via the classical complement pathway, and this Tn5-interrupted locus was called *brk* (bordetella resistance to killing). The *brk* locus was found to contain two divergently transcribed open reading frames, labelled *brkA* and *brkB* (Fernandez and Weiss, 1994). It was demonstrated that it is BrkA that primarily mediates serum resistance. A *B. pertussis* BrkA deletion mutant strain behaved identically to BPM2041 as did another strain that had most of *brkA* and *brkB* deleted. The BrkB mutant strain was not statistically significantly reduced in its capacity to survive in human serum (Fernandez and Weiss, 1998). *B. pertussis* mutant strains that were deficient in expressing other virulence factors such as FHA, dermonecrotic toxin (DNT), pertussis toxin (PT), adenylate cyclase toxin (CyaA), Tcf and virulence-activated gene 8

(Vag8), were unaffected, indicating that they played a negligible role, in serum resistance (Fernandez and Weiss, 1998).

Bordetella resistance to killing protein A has a predicted molecular mass of 103 kDa and BrkB has a molecular mass of 32 kDa, (Fernandez and Weiss, 1994). There is a 42 amino acid signal sequence that is required for BrkA to traverse the inner membrane (Fig. 1.3.3.1; Oliver *et al.,* 2003a). Bordetella resistance to killing protein A is cleaved at D731 during secretion to yield a 73 kDa N-terminal passenger domain and a 30 kDa C-terminal β domain (Passerini de Rossi *et al.,* 1999). The BrkA junction, situated between residues Glycine (G) 601 to Alanine (A) 692, confers stability to the BrkA passenger domain. Deletion of this junction renders BrkA susceptible to degradation by outer membrane proteases and the junction also appeared to be important for folding of the passenger domain (Oliver *et al.,* 2003b). Sequence conservation of this junction is found in many autotransporters, although the conserved domain is not found in Tcf. The minimum translocation unit necessary to mediate the secretion of the BrkA passenger domain to the bacterial surface is the 42 amino acid signal peptide, and a 30-39 amino acid region preceding the β domain, together with the β domain (Oliver *et al.,* 2003a). It was previously suggested that BrkB may aid in the secretion of BrkA, but BrkA appears to be present in its processed form in BrkB mutant strains (Fernandez and Weiss, 1994). The other suggestion for the function of BrkB is that it serves to counteract the damage caused by complement attack.

Database searching for homologues of BrkA and BrkB revealed that the best match for BrkA was Prn and the best match for BrkB was a protein encoded by an *E. coli* open reading frame of unknown function (Fernandez and Weiss, 1994). Most striking similarity between BrkA and Prn was found at their C-terminal ends where they are 54.5% identical over the last 300 amino acids and share a C-terminal outer membrane localisation motif. The proteolytic processing sites are conserved and BrkA also has two RGD-motifs although the spacing of the two RGD sequences is not shared between the two proteins (Fig. 1.3.3.1). Positioning and alignment of the first RGD is similar between the two proteins whereas the positioning and alignment of the second RGD sequence is not (Fernandez and Weiss, 1994). The *brk* locus is present in *B. parapertussis* and *B. bronchiseptica*, although *brkA* is considered to be a pseudogene in both species (Parkhill *et al.,* 2003). The *B. pertussis* type

strain, 18323^T, was found to be more serum sensitive than BP338 and, when an extra copy of the *brk* locus was added to a *B. pertussis* isolate, there was an increase of 2-5 fold in serum resistance. A *B. holmesii* strain, which was isolated from a septicaemic patient, was reported to exhibit serum resistance equivalent to that of *B. pertussis* BP338 (Fernandez and Weiss, 1998). To date, it is not known whether or not *B. holmesii* possesses BrkA (Gerlach *et al.*, 2001). The advantage given to *B. pertussis* expressing BrkA may be that resistance to killing by the antibody-dependent pathway of complement enables the organism to colonise previously infected or vaccinated individuals (Fernandez and Weiss, 1998).

1.3.3.4 Virulence-activated gene 8

Like TcfA and BrkA, virulence-activated gene 8 (Vag8) is the product of a gene positively-controlled by the *bvg* locus, and was initially discovered by transposon Tn*phoA* mutagenesis. A 95kDa protein, Vag8, was identified to be the fourth member of the autotransporter family (Finn and Amsbaugh, 1998). A *B. pertussis* mutant strain, SK8, defective for Vag8, was significantly less able to colonise and persist in the lungs and tracheas of mice when compared to *B. pertussis* strain 18323^T (Finn and Amsbaugh, 1998). This protein showed greatest homology with the C-terminal ends of Prn, BrkA, and Tcf, with amino acid identity of their transport domains ranging from 33-44%. Virulence-activated gene 8 also contains an RGD motif (Fig. 1.3.3.1). The N-terminus of this polypeptide possesses a predicted 37 amino acid signal sequence and the cleavage of this would generate a mature protein of 91 kDa (Finn and Amsbaugh, 1998). However, with Vag8, this cleavage event may not occur as the predicted size corresponds to that of the size of the band visualised on SDS-PAGE (Finn and Amsbaugh 1998). Virulence-activated gene 8 had been detected in whole-cell lysates of *B. bronchiseptica* but not those of *B. parapertussis,* although the complete gene is present in the *B. parapertussis* genome (Parkhill *et al.*, 2003). Higher expression of Vag8 was detected in *B. pertussis* compared to *B. bronchiseptica.*

Virulence-activated gene 8 appears to be involved in the type III secretion system in *B. bronchiseptica.* Genes encoding components of the type III secretion system and its potential translocated proteins have been identified in *B. bronchiseptica,* ovine *B. parapertussis*, human *B. parapertussis* and *B. pertussis.* Previously this secretion system appeared to only be expressed in *B. bronchiseptica* and ovine *B. parapertussis* strains (Yuk

et al., 1998; 2000), but it is now thought to be also expressed by *B. pertussis* and human *B. parapertussis* isolates (Hennuy *et al.*, 2002). *B. bronchiseptica* contains a *bvg*-regulated extracytoplasmic sigma factor (ECF), TrS, which controls the expression of virulence factors including the type III secretion system. Virulence-activated gene 8 was identified to be also regulated by TrS and analysis of the *vag8* promoter region revealed the presence of three putative sites where ECFs might bind. Western blot analysis of culture supernate from a *B. bronchiseptica* Vag8-deficient strain showed that type III effector proteins were not secreted, indicating that *vag8* was required for type III secretion (Foreman-Wykert *et al.*, 2002).

1.3.3.5 Bordetella autotransporter protein C

Bordetella autotransporter protein C (BapC) is a newly identified member of the autotransporter family. The *B. pertussis bapC* gene sequence was originally submitted to GenBank as a putative autotransporter protein gene (accession number AF081494) named *bap5* (Blackburn, 2000) and later submitted independently as *bapC* (AJ277634). The potential role of *bapC* was identified using a *B. pertussis bapC* mutant in *in vitro* and *in vivo* studies. The mutant strain was less able to colonise the respiratory tract in a mouse model when compared to the parent strain and was more susceptible to complement-mediated killing *in vitro* than the parent strain (Bokhari, 2002). Bordetella autotransporter protein C has properties in common with the other members of the autotransporter family, particularly BrkA (Blackburn, 2000; Bokhari, 2002). Bordetella autotransporter protein C is predicted to be 79.5 kDa. No N-terminal signal sequence was detected but the C-terminal domain is characteristic of the autotransporter protein family (Fig. 1.3.3.1; Blackburn, 2000). Removal of this domain would yield a mature passenger protein of 49 kDa and the predicted protein sequence also has an RGD motif (Fig. 1.3.3.1). Bordetella autotransporter protein C was found to be regulated by the *bvg* locus (Bokhari, 2002) and is predicted to be also encoded by *B. parapertussis* and *B. bronchiseptica* (Parkhill *et al.*, 2003). To date there is no information on the protective abilities of BapC.

1.4 TOXINS

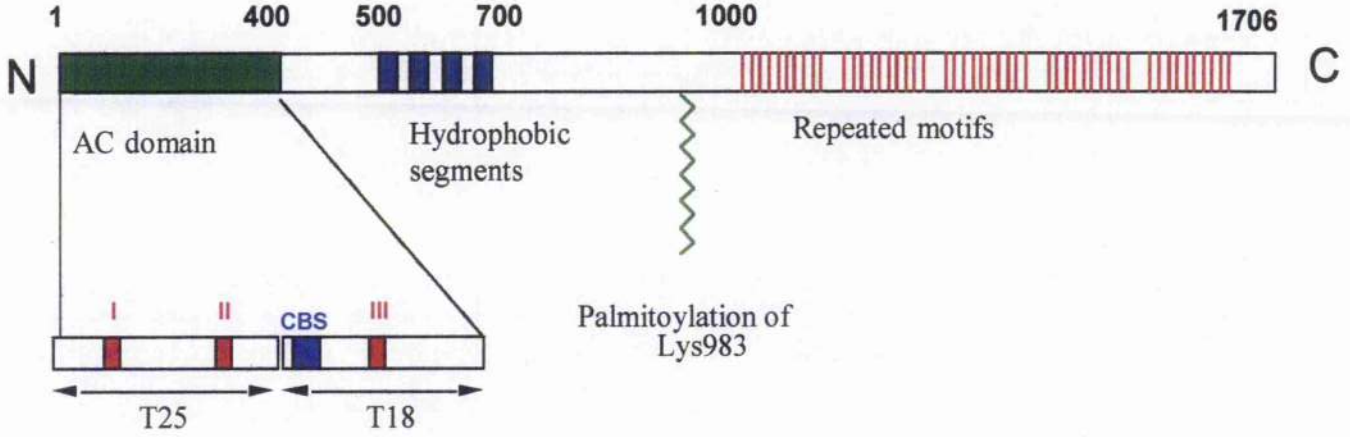
1.4.1 Adenylate cyclase toxin

The gene *cyaA*, controlled by the regulatory locus *bvg*, encodes adenylate cyclase toxin (CyaA) comprised of 1706 amino acids. The *cya* operon is composed of *cyaA* and three accessory genes *cyaB*, *D* and *E*, that are located downstream and are necessary for the secretion of CyaA (Goyard and Ullmann, 1991). The product of the *cyaC* gene is required to activate the CyaA protoxin to enable it to carry out its biological activities. Adenylate cyclase toxin, originally described by Hewlett *et al.* (1976), is one of the major virulence factors secreted by *B. pertussis* and is also expressed by *B. parapertussis* and *B. bronchiseptica*. It is a bifunctional protein with cytotoxic and haemolytic activities (Ladant and Ullmann, 1999). Adenylate cyclase toxin is constructed in a modular fashion with its enzymic (AC) domain located within the first 400 residues with the haemolytic domain taking up the 1306 residues at the C-terminal end (Fig. 1.4.1.1).

The enzymic and haemolytic functions are able to act independently of one another. Khelef *et al.*, (1992) showed that *B. pertussis* mutants without the AC enzymic activity retained full haemolytic activity. Adenylate cyclase toxin exhibits three important characteristics. Firstly, it is an important virulence factor. A *B. pertussis* CyaA mutant, BP348, was unable to cause lethal infection in an infant mouse model at a dose that was 10,000 times greater than the wild type LD_{50} (Weiss *et al.*, 1984). Secondly, AC enzymic activity of CyaA is activated up to 1000 fold by the eukaryotic protein calmodulin (CaM), in a dose dependent manner (Wolff *et al.*, 1980). Lastly, CyaA delivers its AC domain to the eukaryotic cell cytosol where high levels of cAMP, a known inhibitor of phagocyte function, are produced from the substrate ATP which paralyses the target cell and may defend *B. pertussis* against phagocytic attack (Confer and Eaton, 1982).

Within the AC domain there are two subdomains designated T25 and T18 (Fig. 1.4.1.1). The T25 domain, residues 1-224, contains the catalytic site where ATP is bound and boxes I and II are the regions that have been shown by mutation studies to be involved in catalysis. T18 lies between residues 225-299 and this subdomain contains the calmodulin binding site and box III, a site that is also involved in catalysis (Mock and Ullmann, 1993). The regions involved in catalysis were located initially by comparing the catalytic domain of *B. pertussis*

Figure 1.4.1.1: Schematic diagram of *Bordetella pertussis* adenylate cyclase toxin (CyaA). Numbering represents the amino acid residues. The enzymic (AC) domain, residues 1-400, is depicted with two subdomains (T25 and T18). Boxes I-III are regions that are involved in catalysis and CBS denotes the calmodulin-binding site. The C-terminus (400-1706) is depicted here with hydrophobic segments, modification site (palmitoylation of lysine 983) and calcium binding sites in the repeated region. Adapted from Ladant and Ullmann (1999).


1
400
500
700
1000
1706
N
C
AC domain
Hydrophobic
segments
Repeated motifs
I
II
CBS
III
Palmitoylation of
Lys983
T25
T18

CyaA and the central part of the *Bacillus anthracis* adenylate cyclase, which revealed a high degree of homology (Glaser *et al.*, 1991).

Lysine (L) 58 and L65 are critical residues for the expression of enzymic activity. Other critical residues are Aspartatic acid (D) 188, D190, Histidine (H) 298 and Glycine (G) 301. When these residues were altered, the ability of CyaA to bind to ATP analogues was diminished (Glaser *et al.*, 1991). Situated between T25 and T18 is an epitope insertion site where it is possible to insert exogenous peptides without compromising the function of CyaA (Ladant *et al.*, 1992).

The C-terminal domain, residues 400-1706 (Fig. 1.4.1.1), has several features that aid in its ability to mediate the binding and internalisation of the toxin into eukaryotic target cells. There is a pore-forming region with four hydrophobic segments situated between residues 500-700 (Fig. 1.4.1.1) (Ladant and Ullmann, 1999). An amphipathic and potentially α-helical segment of CyaA that includes glutamate residues 509 and 516 is important for AC translocation across the target cell membrane and this segment modulates the formation of CyaA channel membranes and their cation selectivity (Osickova *et al.*, 1999). The haemolytic domain also displays structural characteristics that link CyaA to the bacterial Repeats in ToXin (RTX) family (Coote, 1996). These toxins are characterised by a series of tandem repeats that are rich in G and D residues. This repeated motif domain is made up of 42 copies of the sequence GGXGXGXDXLX. As with many other RTX toxins, the haemolytic activity of CyaA, although weak, is attributed to its ability to form cation-selective channels in cell membranes. This repeat region is also the main calcium-binding site of the protein. RTX toxins are post-translationally activated by a modification that is mediated by an accessory protein (Ludwig and Goebel, 1999). Palmitoylation of CyaA occurs at L983 (Fig. 1.4.1.1) mediated by the accessory protein CyaC. This modification is required for the toxic and haemolytic properties of CyaA (Hackett *et al.*, 1994) as well its protective properties (Betsou *et al.*, 1993). C-terminal modification and the repeat portion (last 800 amino acid residues) of CyaA are crucial for the immunoprotective activity of the entire molecule (Betsou *et al.*, 1995a).

Entry of the AC domain into the target cell is rapid and proceeds without a lag phase. This suggested that it occurs by direct penetration of the plasma membrane (Ladant and Ullmann, 1999). Cyclic AMP generation parallels intracellular AC accumulation, therefore the activation of the bacterial enzyme must be rapid, occurring coincidently with the entry of the AC domain (Friedman *et al.*, 1987). Protein-protein interactions between CyaA molecules, which have been indicated to be involved in haemolysis, appear to be also involved in the internalisation of the AC domain (Iwaki *et al.*, 2000). High-affinity calmodulin binding is not required for the efficient delivery of the AC domain of CyaA into target cells (Heveker and Ladant 1997). Intoxication is thought to be a two-step process. Firstly CyaA binds to the cell surface, which requires the integrity of the carboxy-terminal moiety of the protein and palmitoylation of L983. The second step is the actual translocation of the AC domain of the bound CyaA through the plasma membrane of the cell and this step is temperature-dependent (Ladant and Ullmann, 1999). A stretch of 15 amino acids located at the C-terminus of CyaA appears to be necessary for toxin insertion into target cell membranes. This stretch of amino acids is distinct from the C-terminal signal sequence required for the secretion of CyaA toxins via the ABC secretion system (Bejerano *et al.*, 1999). The cytotoxic activity of CyaA is calcium-dependent and the translocation process occurs only at concentrations greater than 0.1 mM (Rose *et al.*, 1995). Calcium-binding properties of CyaA have been analysed and it has been shown that there are probably two classes of binding sites that have different affinities. The first class may consist of 3-5 high-affinity calcium-binding sites, and the second class consists of about 45 low-affinity calcium-binding sites (Rose *et al.*, 1995). Calcium-binding to the high affinity sites appeared to be critical for membrane-binding capability and haemolytic activity of the toxin. Binding to the low-affinity sites may be involved in the cytotoxic activity of CyaA (Rose *et al.*, 1995) and has been demonstrated to induce a steep increase in the channel-forming activity of CyaA (Knapp *et al.*, 2003).

Comparisons between CyaA and EF (oedema factor from *Bacillus anthracis*) have been made regarding their activation by CaM. CyaA has 100-fold higher affinity for CaM than EF (Shen *et al.*, 2002). Oedema factor and CyaA have about 3 orders of magnitude higher AC activity than the AC activity of host cells, therefore entry of EF and CyaA should raise cAMP concentrations of host cells to levels that disrupt cellular function. The affinity of EF

for CaM is low at resting calcium concentrations in target cells so that EF may be minimally activated. Oedema factor becomes tightly associated with CaM and is fully active only when intracellular calcium is elevated from 20-50 nM to 1 μM (Shen *et al.,* 2002). Adenylate cyclase toxin has a 100-fold higher affinity for CaM than EF and CyaA is optimally activated at resting calcium concentrations. Oedema factor activity may be regulated by the intracellular calcium concentration while CyaA is fully active regardless of the intracellular calcium concentration of its host cell (Shen *et al.,* 2002).

The role that CyaA may have in the pathogenicity of *B. pertussis* has been investigated. Adenylate cyclase toxin may be involved in *B. pertussis*-induced lung inflammation and in apoptotic death of alveolar macrophages. The resulting apoptosis may play a role in the initiation of infection (Gueirard *et al.,* 1998). Further studies have been carried out investigating *B. pertussis* induced apoptosis of macrophages mediated by the organism. It was determined that both domains of CyaA, AC and haemolysin, were necessary to induce macrophage apoptosis as a mutated protein containing only the haemolysin domain was unable to induce apoptosis (Bachelet *et al.*, 2002). Monocytes infected with wild-type *B. pertussis in vitro* have higher levels of cAMP and release lower amounts of TNF (enhance anti-microbial immune defences, mediate apoptosis and modulate Hsp70 expression), O_2^- (acts as defence mechanism against pathogens) and Hsp70 (induction of Hsp70 during phagocytosis may trigger protective functions) compared to monocytes infected with a *B. pertussis* CyaA mutant (Njamkepo *et al.*, 2000). This relationship that occurs between CyaA expression and various host cell activation/adaptive pathways during *B. pertussis* monocyte interaction demonstrates how *B. pertussis* may subdue host cells during infection (Njamkepo *et al.*, 2000). Through the action of CyaA, a decrease in intracellular ATP secondary to a rise in cAMP contributed to the mitochondrial dysfunction that determines that apoptotic fate of macrophages (Bachelet *et al.*, 2002). There is a correlation between the ability of CyaA to induce macrophage death and the internalisation of the protein through a vesicular pathway that requires actin polymerisation. Once successfully transported, CyaA accumulates in intracellular vesicles in macrophages leading to apoptosis (Khelef *et al.*, 2001). Harvill *et al.* (1999) compared wild-type and *cyaA* deletion strains of *B. bronchiseptica* in immunocompetent and immunocompromised mice. It was found that CyaA targets one or more aspects of the innate immune response, one of which is probably

neutrophils. This was further investigated and it was demonstrated that CyaA inhibits both Fc receptor-mediated attachment and phagocytosis of *B. pertussis* by neutrophils (Weingart *et al.*, 2000). Adenylate cyclase toxin binds specifically to eukaryotic target cells via $\alpha_M\beta_2$ integrin (CD11b/CD18) and this interaction is required for several activities including the intracellular delivery of the AC domain into leukocytes, cAMP production and CyaA-induced cell death (Gucrmonprez *et al.*, 2001). Zaretzky *et al.* (2002) state that there are studies being carried out investigating CyaA delivery from *B. pertussis* to target cells. It has been noted that FHA *B. pertussis* mutants secrete much more CyaA into the culture supernate than wild-type *B. pertussis* strains and the presence or absence of FHA was the only known virulence determinant (compared to Prn, endotoxin and pertussis toxin) that was associated with the release of CyaA (Zaretzky *et al.*, 2002). From purification studies it was also demonstrated that there is a direct physical interaction between CyaA and FHA. This interaction of CyaA and FHA may help to increase the local concentration of CyaA on the outer membrane of *B. pertussis* and adherence to host cells mediated by FHA may be coupled to delivery of CyaA to target host cell (Zaretzky *et al.*, 2002).

Purified active CyaA exhibited protective activity in mice against intranasal challenge with *B. pertussis* 18323^T and the protective efficacy was similar to the protective efficacy of whole-cell pertussis vaccine (Guiso *et al.*, 1991; Hormozi *et al.*, 1999). The CyaA molecule has been investigated for its potential inclusion in future generations of acellular pertussis vaccines (MacDonald-Fyall, 2002). It was reported that when mice were immunised with CyaA alone a strong IgG response was produced and this was boosted by a second vaccine. Also, when CyaA was combined with a protective antigen mixture (pertussis toxin (PTX), FHA and Prn) containing those commonly used in acellular vaccine preparations, anti-PTX, -FHA, and -Prn IgG levels were also raised compared to mice with just the antigen mixture alone (MacDonald-Fyall, 2002). Recently, it was demonstrated that CyaA has adjuvant activity promoting Th2 (T helper type 2) and Tr1 (T regulatory type 1) responses and the enhancement of antigen-specific IL-10-producing cells also occurs (Ross *et al.*, 2004). Due to the ability of CyaA to deliver its AC domain to the eukaryotic cell cytosol, it has also been investigated for its use as a multi-purpose vaccine. *B. pertussis* CyaA was expressed in recombinant form in *E. coli* with a lymphocytic choriomeningitis virus (LCMV) nucleoprotein $CD8^+$ T cell epitope inserted between residues 224 and 225 (Saron *et al.*,

1997). Cytotoxic T cells stimulated by the recombinant CyaA were able to recognise virus-infected target cells and protected mice against challenge with the virus. These data encourage the use of CyaA for the development of safe non-replicative anti-viral vaccines (Saron *et al.*, 1997). Guermonprez *et al.*, (1999) demonstrated that recombinant CyaA is processed intracellularly for presentation of epitopes to $CD8^+$ T cells, which is important for the design of recombinant $CD8^+$ T cell-specific vaccines. The binding interaction of CyaA to eukaryotic target cells via $\alpha_M\beta_2$ integrin (CD11b/CD18) could be specifically exploited so that detoxified mutants of CyaA might be able to deliver pharmacologically active molecules to $CD11b^+$ cells, without effecting other cell types (Guermonprez *et al.*, 2001).

1.4.2 Pertussis Toxin

Pertussis toxin (PTX), an AB toxin, is structured as a hexamer composed of five dissimilar subunits, designated S1-S5 relative to their electrophoretic mobilities in denaturing gels (Locht and Keith, 1986). The subunit S1 (A moiety) contains an enzymic adenosine diphosphate (ADP) ribosylation activity and subunits S2-S5 (B moiety) contain target cell receptor binding activity (Locht *et al.*, 2001). Mature proteins contain 234 amino acids for S1, 199 amino acids for S2 and S3, 110 amino acids for S4 and 100 amino acids for S5. The total molecular weight for the holotoxin was calculated to be 104,950 (Locht and Keith, 1986). All the genes for the PTX subunits are closely linked in an operon and are probably expressed in a precise ratio, which is similar to that of other AB_5 toxins such as, cholera and *E. coli* heat-labile toxins (Locht and Keith, 1986). Directly downstream of the PTX structural genes there is a region that encodes proteins necessary for the transport of PTX designated the pertussis toxin liberation, *ptl*, locus (Weiss *et al.*, 1993). The secretion of PTX with the Ptl system uses the Type IV secretion pathway (Burns, 2003).

Pertussis toxin structural and export operons are also present in *B. parapertussis* and *B. bronchiseptica* (Parkhill *et al.*, 2003). The S2 subunit is present as a pseudogene in *B. parapertussis* but there are no identifiable pseudogenes of the toxin in *B. bronchiseptica* (Parkhill *et al.*, 2003). Lack of PTX expression in *B. bronchiseptica*, and also in *B. parapertussis*, is due to differences in the promoter regions of the operons. Parkhill *et al.* (2003) compared the sequence preceding the PTX S1 subunit gene, *ptxA*, of these species and found that 62% of the base changes were due to changes in *B. pertussis*, while the

sequence was conserved in the other two species. Several of these changes occur within the RNA polymerase or BvgA binding sites (see section 1.6) and the effect of these was to increase the similarity to the σ^{70} or putative BvgA binding consensus sequence. It is suggested that recent mutations in *B. pertussis* have acted to increase the regulated expression of PTX in this organism.

Pertussis toxin is important in the pathogenesis of *B. pertussis* as it has been described as both a toxin and an adhesin. Using a PTX *B. pertussis* mutant strain, BP357, in infant mouse model studies it was determined that PTX is an important virulence factor due to the fact that the LD_{50} for the mutant strain was more than 1,000 times higher than that of the wild-type *B. pertussis* strain (Weiss *et al.*, 1984). The toxin is involved in a number of *in vivo* activities, such as histamine sensitisation, insulin secretion, lymphocytosis promotion and immunopotentiating effects (Locht and Keith, 1986). The molecular action of PTX involves three steps: binding of the toxin to its receptors via the B oligomer, retrograde transport to the endoplasmic reticulum, membrane translocation of the S1-subunit and intracellular expression of the ADP-ribosyl-transferase activity catalysed by the S1 subunit. (Locht and Antoine, 1999). Pertussis toxin was investigated for its potential as a colonisation factor and it was found that its enzymic activity rather than its cell binding activity was necessary for full colonisation in the mouse model (Carbonetti *et al.*, 2003). Several of the toxin's biological effects can be reproduced in the mouse model by injection of PTX and some of these effects are long lasting (Munoz *et al.*, 1981). For these reasons PTX has been suggested to have a central role in pertussis pathogenesis and in some way being responsible for the prolonged course of the disease and for the symptoms that persist after infection has cleared (Pittman, 1984). However, the precise role of PTX and its main site of action in the host still remain obscure (Hewlett, 1997a). Chemically-detoxified PTX (PTXd) has been demonstrated to be protective in the mouse model against intracerebral and aerosol challenges of *B. pertussis* (Sato and Sato, 1984) and this molecule has been included in all acellular pertussis vaccine preparations (Hewlett, 1997a). More recently, plasmid DNA expressing PTX S1 subunit was evaluated for immunogenicity and for the ability to induce protection against PTX challenge or *B. pertussis* infection in mice. This DNA vaccination induced anti-PTX IgG antibody production, inhibited leukocytosis-promoting activity and induced protection against intracerabral challenge of *B. pertussis* (Kamachi *et al.*, 2003).

1.4.3 Dermonecrotic Toxin

Dermonecrotic toxin (DNT) was originally referred to as heat-labile toxin as its activity was destroyed by heating at 60°C for 10 min. This toxin is lethal when injected intravenously or intraperitoneally into animals and the most characterised action of this toxin is its ability to induce skin lesions in animals when injected intradermally (Endoh *et al.*, 1990). Dermonecrotic toxin is a member of a family of bacterial cytotoxins that are all large polypeptides and affect regulation of cell growth or division (Pullinger *et al.*, 1996).

DNT is expressed by *B. pertussis*, *B. parapertussis*, *B. bronchiseptica* and *B. avium* and the DNTs from each species were compared on a genetic level. Similarity was found between DNTs of *B. pertussis*, *B. parapertussis* and *B. bronchiseptica*, but there were genetic and biological differences between these DNTs and the *B. avium* DNT (Walker and Weiss, 1994). The toxin is another virulence factor that is controlled by the regulatory locus *bvg* (section 1.6). Avirulent phase *B. bronchiseptica* strains express some DNT but in much reduced amounts compared to expression by wild-type strains (Pullinger *et al.*, 1996). Dermonecrotic toxin is known to induce nuclear division without subsequent cell division, resulting in bi- or multi-nucleation and stimulation of stress fibre formation. This toxin binds to cells through its N-terminal region consisting of 54 amino acids (Matsuzawa *et al.*, 2002) which recognises an unknown cell surface receptor which may be uncommon because only a few cell lines are sensitive to DNT. The production of *B. bronchiseptica* DNT is necessary for the production of lesions in swine, turbinate atrophy and pneumonia developed by pigs infected with wild-type DNT^+ *B. bronchiseptica* strains (Brockmeier *et al.*, 2002). *B. bronchiseptica dnt* mutants were reduced in their ability to cause turbinate atrophy and pneumonia, but were not completely avirulent (Pullinger *et al.*, 1996). Pigs infected with the mutants did experience clinical signs of sneezing, coughing and inflammation in the nasal cavity. The role of DNT in the pathogenesis of *B. pertussis* is still unclear. However, the dermonecrotising activity appears to be due to a specific constrictive effect on vascular smooth tissue muscle. This effect on similar tissues in the respiratory tract could induce a local inflammatory reaction and could account for some of the pathology of pertussis (Parton, 2004).

1.4.4 Tracheal Cytotoxin

Tracheal cytotoxin (TCT) was discovered to cause ciliostasis and ciliated cell extrusion in hamster tracheal organ cultures and inhibition of DNA synthesis in hamster trachea epithelial cell cultures (Goldman *et al.*, 1982). The toxin is known to be expressed by *B. pertussis*, *B. parapertussis*, *B. bronchiseptica* and *B. avium*. Purified and biologically-active TCT contains amino acids and amino sugar residues consistent with a peptidoglycan structure and was found to be a fragment of peptidoglycan released from the *B. pertussis* cell (Cookson *et al.*, 1989). The structural features of TCT were defined using fast atom bombardment mass spectrometry and its primary structure was determined to be N-acetylglucosaminyl-1,6-anhydro-N-acetylmuramylalanyl-γ-glutamyldiamino-pimelylalanine (Cookson *et al.*, 1989).

B. pertussis endotoxin (section 1.4.5) plays an important role in combination with TCT to damage the airway epithelium. Tracheal cytotoxin and endotoxin were synergistic in the induction of IL-1α mRNA and protein (this is an essential intermediate in the toxicity pathway), and production of nitric oxide (Flak and Goldman, 1999; Flak *et al.*, 2000). This production of nitric oxide results in the depletion of intracellular iron, which inhibits a number of iron–containing enzymes that are important for DNA synthesis (Heiss *et al.*, 1994). Flak *et al.*, (2000) have suggested that these factors act together to activate a localised antibacterial nitric oxide response from epithelial cells. *B. pertussis* may have evolved to take advantage of this system by releasing a large amount of peptidoglycan pieces which would lead to over-stimulating the defence system to autotoxic levels, resulting in ciliated cell damage compromising the natural airway mechanism and the corresponding coughing reaction becomes an efficient means for disease transmission.

1.4.5 Endotoxin

Bordetella spp. endotoxins differ from one another in their structure. The endotoxin of *B. pertussis* is referred to as lipo-oligosaccharide (LOS) (Brodeur *et al.*, 1993). The structure of LOS is comprised of a lipid A domain with a branched chain core oligosaccharide and this is referred to as LPS-B. The addition of a trisaccharide to this produces LPS-A (Chaby and Caroff, 1988; Caroff *et al.*, 2000). The two LPS phenotypes can be distinguished electrophoretically, either wild-type (Lps AB) or phase variant (Lps B) (Peppler, 1984). The wild-type pattern consists mainly of an electrophoretically slower migrating LPS-A band

with lesser amounts of the faster migrating, LPS-B band. The phase variant LPS consists of a single faster migrating band, indistinguishable from the wild-type band LPS-B. *B. parapertussis* and *B. bronchiseptica* endotoxins consist of smooth type lipopolysaccharide (LPS) with higher molecular weight O-polysaccharide components (Parton, 2004).

The LPS expression of the *Bordetella* species and its dependency on growth temperature was analysed by oxidative silver staining of proteinase-K treated bacteria separated by Tricine-SDS-PAGE (van den Akker, 1998). Three *B. pertussis* strains were compared for their LPS expression as shown by their profiles on Tricine-SDS-PAGE. *B. pertussis* grown at 37°C contained the wild-type pattern and the organism grown at 25°C contained only the expected phase variant LPS-B band. There were no differences between profiles and it was also found that BvgAS was not involved in the biosynthesis of its LPS (van den Akker, 1998). There were differences in the profiles visualised using Tricine-SDS-PAGE for ovine and human *B. parapertussis* strains. These differences were host-specific and profile differences were apparent for those grown at 37°C compared to *B. parapertussis* grown at 25°C. For some *B. bronchiseptica* strains, LPS expression was subject to phase variation controlled by BvgAS, unlike *B. pertussis*, and this was not host restricted (these strains were isolated from pig and dog hosts). *B. avium* strains had similar LPS expression profiles when grown at their host natural body temperature (41°C), however these profiles differed from each other when the organism was grown at other culture temperatures of 25°C and 37°C. Modulation by temperature shift did not affect the LPS expression profiles of *B. holmseii*, *B. hinzii* (except for one human isolate in which an additional LPS was expressed at 41°C) and *B. trematum* (van den Akker, 1998). *B. petrii* had not been described at the time of publication of the above study and there is no information on its LPS structure or its expression.

The *B. pertussis* genetic locus, *wlb*, consisting of 12 genes (*wlbA-L*), was found to be required for LPS-A biosynthesis. This locus was also found in *B. parapertussis* and *B. bronchiseptica* (Allen *et al.*, 1998). Deletion of the *wlb* locus from *B. pertussis* and *B. bronchiseptica* followed by complementation studies had revealed that this locus was solely required for LPS-A synthesis and the biosynthesis of LPS-B was unaffected (Allen *et al.*, 1998). *B. parapertussis* does not express LPS-A and this may be possibly due to the

presence of a mutation in the gene *wlbH* (Allen *et al.*, 1998). Allen *et al.* (1998) also demonstrated that deletion of the *wlb* locus in *B. parapertussis* and *B. bronchiseptica* prevented O-antigen biosynthesis and it was proposed that there is a gene or genes present within *wlb* locus that is involved in its biosynthesis. The effect of wild-type and *wlb* deletion strains of *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* in mouse respiratory tract infection models were compared (Harvill *et al.*, 2000). All three mutants were defective in the colonisation of the respiratory tract of mice. *B. pertussis* LOS molecules were required for efficient nasal colonisation. In *B. parapertussis* they were required for initial colonisation of the lungs and in *B. bronchiseptica* they were required for extended survival in the lower respiratory tract of normal mice but not in mice lacking adaptive immunity (Harvill *et al.*, 2000).

Adjacent to the *wlb* locus is the *wbm* locus that is responsible for the biosynthesis of the O-antigen. The *wbm* deletion *B. parapertussis* and *B. bronchiseptica* mutants differed in their ability to colonise the respiratory tract of mice. The *B. parapertussis* mutant was severely defective in its ability to colonise the trachea and lungs, whereas the *B. bronchiseptica* mutant showed almost no defect (Burns *et al.*, 2003). When the ability of serum complement to kill *Bordetella* was also investigated, wild-type *B. parapertussis* and *B. bronchiseptica* were unaffected by high concentrations of naïve-rabbit, -rat, or –mouse serum while the *wbm* deletion mutants were sensitive to very low concentrations of serum. It was suggested that the O-antigen provides protection from the bactericidal activities of serum complement in *B. parapertussis* and *B. bronchiseptica* (Burns *et al.*, 2003).

B. pertussis LOS has the properties of toxicity, pyrogenicity and adjuvanticity characteristic of endotoxin (Parton, 2004). It also has the ability to induce antiviral activity, B-cell mitogenicity and polyclonal B-cell activation (Chaby and Caroff, 1988). *B. pertussis* LOS in the whole-cell pertussis vaccine is considered to contribute to the vaccine's toxicity and reactogenicity, therefore it was suggested that LOS should be excluded from acellular pertussis vaccines (Robinson *et al.*, 1985).

1.5 OTHER POTENTIAL VIRULENCE FACTORS

1.5.1 Outer-membrane protein P

B. pertussis produces a 40 kDa porin protein which complexes in the outer membrane to form a channel for anionic molecules and this porin resembles the derepressible porin protein P of *Pseudomonas aeruginosa* (Armstrong *et al.*, 1986). The structural gene for the major *Bordetella pertussis* porin, OmpP was identified and sequenced by Li *et al.* (1991). Outer-membrane protein P is expressed by Bvg^+ and Bvg^- *B. pertussis* strains and Southern blot analysis had also revealed the presence of the gene in *B. parapertussis* and *B. bronchiseptica*.

1.5.2 Outer-membrane protein Q

The gene *ompQ* encodes a porin-like outer-membrane protein Q (OmpQ), which has a molecular mass of 39.1 kDa (Finn *et al.*, 1995). From mouse model studies it has been demonstrated that a mutant *B. pertussis* 18323^T strain lacking OmpQ was not adversely affected in its ability to survive after aerosol challenge in the mouse model of infection and was not affected in its ability to survive *in vitro* (Finn *et al.*, 1995). The lack of effect was unexpected as Finn *et al.* (1995) had suggested that OmpQ may have an important role in allowing the bacterium to have access to essential nutrients. In the mouse model system, it may be that other outer membrane proteins are able to substitute for OmpQ or the protein may not be involved in the infectious process. Outer-membrane protein Q is expressed along with other Bvg^+ proteins and it was suggested that the protein may play a role in the establishment of a carrier state. The gene *ompQ* was present in *B. parapertussis* and *B. bronchiseptica* but was not found to be present in *B. avium* (Finn *et al.*, 1995).

1.5.3 Bordetella intermediate phase protein

Bordetella intermediate phase protein (BipA), a surface-expressed protein, is encoded by the gene *bipA*. The protein is expressed by *B. pertussis*, ovine *B. parapertussis* and *B. bronchiseptica* strains but not by human *B. parapertussis* strains (Fuchslocher *et al.*, 2003). This can be attributed to *bipA* being a pseudogene in human *B. parapertussis* strains. Reverse transcriptase PCR analysis indicated that *bipA* was expressed maximally under semi-modulating (Bvg^i phase) conditions and that expression was controlled at the level of transcription (Stockbauer *et al.*, 2001). However, levels of BipA expression differ in ovine

B. parapertussis strains compared to *B. pertussis* and *B. bronchiseptica* strains due to sequence differences present at positions predicted to be involved in BvgA–mediated transcriptional activation (Fuchslocher *et al.,* 2003). The C-terminus of BipA is exposed on the surface of *B. bronchiseptica* and, consistent with this topology, the predicted amino acid sequence of N-terminal 500 amino acids shares significant similarity with the membrane localisation domains of EHEC (enterohaemorrhagic *Escherichia coli*) and EPEC (enteropathogenic *E. coli*) intimin and *Yersinia* invasin. The fact that *bipA* is absent from some *B. bronchiseptica* strains and is not expressed or expressed at very low levels, for example in ovine *B. parapertussis* strains, suggests that the potential role that BipA plays in the infectious cycle is either not essential or can be compensated for by other genes (Fuchslocher *et al.,* 2003). However, BipA could play some important role that is unique to the strains that express it.

1.5.4 Vir90

A potential virulence factor of *B. pertussis*, Vir90, has been described recently (Passerini de Rossi *et al.,* 2003). The protein sequence displayed closest homology with a number of ferrisiderophore receptors from Gram-negative bacteria. Open reading frames of *vir90* were also found in *B. parapertussis* and *B. bronchiseptica* genomes. This protein is encoded by a gene of 2322 bp, which is highly conserved between *B. pertussis* and *B. bronchiseptica* (99% homology) and between *B. pertussis* and *B. parapertussis* (98% homology). The *vir90* promoter was scanned for regulatory regions; putative –10 and –35 sites, BvgA binding sites and a putative Fur box overlapping the –10 site were found (Passerini de Rossi *et al.*, 2003). The expression of *vir90* was demonstrated to increase when *B. pertussis* was grown in low iron growth conditions and its expression was iron regulated. Therefore, it was suggested that Vir90 may play a role in the uptake of iron.

1.6 REGULATION OF *B. pertussis* VIRULENCE FACTORS

B. pertussis can alternate between virulent and avirulent states either by phase variation or by phenotypic modulation. Inactivation of the bordetella virulence genes locus, *bvgAS*, by mutation (phase variation), or repression of its products (BvgAS) by the presence of modulating signals (phenotypic modulation), results in the loss of virulence gene expression (Cotter and DiRita, 2000). Phase variation was first described by Leslie and Gardner (1931,

cited by Roberts and Parton, 2001) as a stepwise degradative process from phase I, fresh virulent isolates, to phase IV, avirulent isolates. Isolates that are phase IV no longer express the virulence factors described previously, except for TCT and LOS. These mutants occur at the frequency of 1 per 10^3 to 10^6 organisms due to spontaneous mutation in *bvgAS* (Weiss *et al.,* 1983). Goldman *et al.* (1984) described phase variation as an ordered gradual process composed of several non-random and inter-dependent events. Phenotypic modulation, originally described by Lacey (1960, cited by Parton, 2004), is completely reversible and it has been suggested that it could be used as a mechanism to escape host defences (Weiss and Walker, 1984). The C (cyanic) avirulent, Bvg^-, mode occurs when grown in modulating conditions *in vitro* such as high concentrations of magnesium sulphate or nicotinic acid and the X (xanthic) virulent, Bvg^+, mode occurs when there are no such modulating signals (Roberts and Parton, 2001).

The *bvg* locus sequence was described by Arico *et al.* (1989). The predicted products of this locus are homologous to a family of regulatory proteins that transmit sensory signals using a conserved two-component motif. Originally *bvgA, B* and *C* were described. *bvgA* encodes a transcriptional activator and, in the absence of modulating conditions, it was proposed that BvgC activated BvgA by phosphorylation (Arico *et al.,* 1989). It was later discovered that BvgB and BvgC were part of one larger protein and this was termed BvgS (for sensor) (Stibitz and Yang, 1991). BvgA, a 23 kDa protein, is located within the cytoplasm and BvgS, a 135 kDa protein, has transmembrane topology (Fig. 1.2.1). BvgS acts as a sensor of conditions in the external environment and transduces those signals to the inside of the cell where communication with BvgA by phosphorylation results in the expression of *bvg*-associated factors (Fig. 1.2.1) (Stibitz and Yang, 1991). BvgAS uses a four-step Histidine (H)-Aspartic acid (D)-Histidine-Aspartic acid phosphotransfer signalling mechanism (Cotter and DiRita, 2000). The first three phosphotransfer domains are contained on BvgS and the last is contained on BvgA. When BvgS is activated, it autophosphorylates at H729 in the transmitter domain. This phosphoryl group is transferred to D1023 in the receiver domain and then to H1172 in the histidine phosphotransfer domain or the phosphoryl group can be transferred from the BvgS receiver to water to form inorganic phosphate. The phosphorylated histidine phosphotransfer domain is the substrate for the phosphorylation of D54 in the BvgA receiver. Phosphorylated BvgA can bind DNA via its helix-turn-helix

domain, and it functions as a transcriptional activator and repressor, resulting in the expression of distinct phenotypic phases (Cotter and DiRita, 2000). The amount of BvgA protein detected in the lysates of *B. pertussis* decreases significantly in cells grown under modulating conditions or in cells that have *bvg* locus mutations (Stibitz and Yang, 1991). Similar regulation of virulence factors is also known to occur in *B. parapertussis*, *B. bronchiseptica* and *B. avium* (Parton, 2004).

Transposon Tn*phoA* mutagenesis was used to facilitate isolation of gene fusions between *phoA* and *B. pertussis* genes encoding secreted proteins. By isolating these Tn *phoA* fusions controlled by modulating signals, genes that were activated or repressed by BvgAS were identified (Knapp and Mekalanos, 1988). This work led to the discovery of virulence-repressed genes (*vrgs*) and virulence-activated genes (*vags*), (Fig. 1.2.1). The products of *vrgs* are considered to be related to C-mode products. The virulent and avirulent phases or modes are now referred to as Bvg^+ and Bvg^-, respectively.

A temperature switch (from 25 to 37°C) induces the expression of a number of *bvg*-regulated genes in two temporal steps (Scarlato *et al.*, 1991). The first step of activation involves the P1 and P4 promoters at the *bvg* locus and the *fhaB* gene promoter. The second step of activation includes the *bvg* P3 promoter and the promoters of the *ptx* and *cyaA* genes. The fact that FHA is synthesised before PTX and CyaA suggests that the differential activation of adhesins and then toxins is part of the infection process and results from a selective advantage (Scarlato *et al.*, 1991). That is, the adhesins such as FHA are necessary for adherence to eukaryotic cells in the first stage of infection and the expression of the toxins may not be required until *B. pertussis* is at the correct anatomic site of the upper respiratory tract. Early and late promoters are recognised with high and low affinity by phosphorylated BvgA, respectively (Zu *et al.*, 1996). Recognition of the late-activated promoters requires a larger amount of phosphorylated BvgA compared to the early promoters.

The *bvg* locus was thought to only consist of *bvgS* and *bvgA* until Merkel and Stibitz (1995) reported the discovery of a third component of the locus, *bvgR*, in *B. pertussis*, which is responsible for repression of genes. The *bvgR* locus lies immediately downstream of *bvgAS*

and is convergently transcribed relative to *bvgAS*. The *bvgR* promoter was found to be significantly stronger than the *fha* promoter and such a high level of expression was considered unusual for a transcriptional regulator (Merkel *et al.*, 2003). Results of the study suggested that the *bvgR* transcript is very unstable and is rapidly degraded in the cell after synthesis. BvgR (as well as FHA) is expressed early and this may be required to repress the expression of a gene product(s) that may interfere with colonisation of the host by factors such as FHA (Merkel *et al.*, 2003).

There are three known phase phenotypes: Bvg^+, Bvg^- and Bvg^i. The Bvg^+ phase is characterised by the expression of Bvg-activated virulence factors and the lack of expression of the Bvg^- phenotype. In the Bvg^- phase, Bvg-activated virulence factors are not expressed and a specific set of proteins that are unique to *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* are expressed. *B. pertussis* grown in submodulating conditions of nicotinic acid or magnesium sulphate express the Bvg^i phenotype (Cotter and DiRita, 2000). This phase is characterised by the absence of expressed Bvg^- proteins, the presence of some of the Bvg-activated proteins and the presence of proteins that are unique to this phase, such as BipA (Cotter and DiRita, 2000). These distinct phases may be expressed at different stages of infection by the organism, or outside the host during transmission or for survival in the environment (Cotter and DiRita, 2000; Coote, 2001).

1.7 COMPARISON OF *B. pertussis*, *B. parapertussis* AND *B. bronchiseptica* GENOMES

During the course of this PhD study the complete genome sequences of *B. pertussis* (Tohama-I), *B. parapertussis* (12822, human strain) and *B. bronchiseptica* (RB50, rabbit strain) were published (Parkhill *et al.*, 2003). The *B. bronchiseptica* genome is the largest of the three genomes, ca. 5.3 Megabases (Mb) with 5007 predicted genes. The *B. parapertussis* genome is ca. 4.8 Mb with 4404 genes and the *B. pertussis* genome is ca. 4.1 Mb with 3816 genes. Parkhill *et al.* (2003) estimated the time of divergence of the three species based on the pairwise synonymous substitution rates for approximately 3000 genes that are common to the three species. Estimated times to the last common ancestors were 0.27-1.4 million years, for *B. bronchiseptica* and *B. parapertussis*, 0.7-3.5 million years *B. bronchiseptica* and *B. pertussis* and 0.8-4.0 million years for *B. parapertussis* and *B.*

pertussis. *B. pertussis* is known to be a clonal species and this and other evidence has suggested a much more recent origin (Musser *et al.*, 1986; Gerlach *et al.*, 2001). The suggestion of recent origin can also be related to the fact that the first recorded clinical description of the highly characteristic disease, pertussis, was in 1578. The genome data suggest that the genetic uniformity in *B. pertussis* reflects a recent bottleneck (due to the human population not increasing at a rate to support a larger *B. pertussis* population, the allelic diversity is reduced) rather than recent descent from *B. bronchiseptica*. Another possible explanation is that *B. bronchiseptica* might be sufficiently genetically diverse that isolates exist which are much more similar to the last common ancestor of *B. pertussis* than *B. bronchiseptica* RB50 strain.

Analysis of the genomes revealed that 114 genes were unique to *B. pertussis*, 50 genes were unique to *B. parapertussis* whereas *B. bronchiseptica* had over 600 genes that were not found in either *B. pertussis* or *B. parapertussis*. Twenty-three genes that were found in *B. pertussis* and *B. parapertussis* were not found to be present in *B. bronchiseptica*. From the predicted functions of the products of the genes not present in the *B. bronchiseptica* genome it appeared that very few would be involved in pathogenicity or in specifying host range. The type of genes lost by *B. pertussis* or *B. parapertussis* with respect to *B. bronchiseptica* indicated that many of them were involved in membrane transport, small molecule metabolism, regulation of gene expression and synthesis of surface structures. *B. bronchiseptica* has over 1000 genes in common with *B. parapertussis* and 100 genes in common with *B. pertussis*. Three hundred and fifty-eight genes in *B. pertussis* and 200 genes in *B. parapertussis* appeared to have been inactivated by insertion of IS elements, in-frame stop codons or frameshifts. This has occurred to a lesser extent in *B. bronchiseptica* as it only has 19 pseudogenes.

The distinct traits of each species have been suggested to be due to independent mechanisms of large-scale gene inactivation and loss in *B. pertussis* and *B. parapertussis*. Their limited host range, compared to *B. bronchiseptica*, could be due to the loss of host-interaction mechanisms. Increased virulence for humans of *B. pertussis* could be due to an over-expression or constitutive expression of virulence factors that are subject to tighter temporal or environmental control in *B. parapertussis* and *B. bronchiseptica*. *B. pertussis* may have

followed this evolutionary path due to opportunities for increased transmission rate provided by the increase in size and density of human populations (Parkhill *et al.*, 2003).

Antoine *et al.* (2000) searched the *B. pertussis* genome that was available at the time to identify new potential virulence factors. A suicide vector was developed for rapid gene inactivation by homologous recombination and generation of transcriptional fusions between the interrupted genes and a promoterless *lacZ*. The genes identified included putative virulence factors. Two genes that code for putative proteins homologous to FHA were found. These authours described a gene, *adhS*, encoding a putative signal peptide bearing protein, which was homologous to a salivary streptococcal adhesin. Five other autotransporters (*phg*, *aidB*, *sphB1*, *sphB2* and *sphB3*) were also identified. Expression levels of *sphB2* and *sphB3* were very low or undetectable, however *phg*, *aidB*, and *sphB1* were better expressed. A serine protease homologue, *sphB1*, was also identified to be another *vag*. The completion of the *B. pertussis* genome has thus led to the discovery of other novel molecules and will help to identify other putative virulence factors and protective proteins.

1.8 CLINICAL ASPECTS OF PERTUSSIS

1.8.1 Pertussis symptoms

B. pertussis infection is transmitted via respiratory secretions from an infected individual to a new host under conditions of close contact and is highly contagious during the first week of illness. During this stage up to 90% of susceptible family contacts of an index household case develop pertussis (Hodder and Mortimer, 1992). The clinical symptoms of pertussis can be split into three phases, catarrhal, paroxysmal and convalescent. Following exposure there is an incubation period that varies between 7 and 13 days. Non-specific symptoms then develop, which last approximately 7 days, and this is known as the catarrhal phase. These symptoms consist of nasal congestion, low-grade fever and mild cough (Hodder and Mortimer, 1992). The catarrhal phase gradually becomes the paroxysmal phase as the non-productive cough becomes a paroxysmal cough with mucus secretion and vomiting. The paroxysmal phase may last for 1-6 weeks. The action of coughing attempts to clear the viscous respiratory secretions and is often followed by inspiration against a narrowed glottis, which produces the characteristic whoop. Physical damage can result from the paroxysmal

coughing, such as rupturing the frenulum of the tongue, or cause spontaneous pneumothorax, inguinal hernia and rectal prolapse (Kerr and Matthews, 2000). The other characteristic signs of the paroxysmal phase include leukocytosis, lymphocytosis, weight loss, occasional hypoglycaemia and, rarely, encephalopathy (Kerr and Matthews, 2000). Central nervous complications are relatively frequent and convulsions can also occur. During the convalescent phase the cough gradually starts to subside but it can be reactivated by subsequent, unrelated infection.

Age is an important determinant of disease severity and prognosis. Infants under the age of one year have the highest mortality rate (Hodder and Mortimer, 1992). Seventy-nine percent of reported deaths from pertussis in the United States (US) occurred in infants under one year of age. This age group is also more likely to be hospitalised than older individuals and to suffer complications such as pneumonia, and central nervous system manifestations. During 1980-1989, of the pertussis patients under 1 yr in the US, 69% were hospitalised, 3% had convulsions, 0.9% had encephalopathy and 0.6% died (Kerr and Matthews, 2000). During 1995-1997 in the United Kingdom (UK), 65% of pertussis hospital admissions were infants less than 1 year of age (van Buynder *et al.,* 1999). The clinical symptoms of pertussis in adults and adolescents are generally considered to be milder.

A recent study has confirmed that vaccination status significantly changes the clinical presentation of pertussis and that this disease is more severe in unvaccinated individuals (Tozzi *et al.,* 2003). Clinical presentation was found not to vary with the background incidence of pertussis i.e., during epidemic periods, pertussis symptoms were not more severe. The recommended World Health Organisation (WHO) case definition of pertussis for surveillance purposes includes spasmodic coughing for 2 weeks. As a result of this strict definition the impact of pertussis may be underestimated in vaccinated children and in older age groups. Tozzi *et al.* (2003) recommended that children with prolonged cough should always be considered for diagnosis of pertussis, even those that have been vaccinated.

1.8.2 Treatment of pertussis

Antibiotic treatment in the catarrhal or early paroxysmal stages can ameliorate the symptoms of pertussis and eliminate the infection. Treatment of patients in later stages of infection may

have no clinical benefit but it could potentially limit the spread of the organism to susceptible contacts (Parton, 2004). There are several antimicrobial agents that have been reported to be effective *in vitro* against *B. pertussis*, such as ampicillin, chloramphenicol, tetracycline and erythromycin (Hodder and Mortimer, 1992). However, these antibiotics have not been equally effective against pertussis infection *in vivo*. Ampicillin was ineffective in eradicating *B. pertussis* from the nasopharynx and patients that were treated with tetracycline or chloramphenicol had slower eradication of *B. pertussis* than those treated with erythromycin. Erythromycin is still the antibiotic of choice for treating pertussis infection (Hodder and Mortimer, 1992). Patients receiving treatment become culture-negative within a few days but, if therapy is not continued for the recommended two weeks, culture results may become positive again. Recent reports have described US *B. pertussis* isolates that were resistant to erythromycin (Hill *et al.*, 2000; Wilson *et al.*, 2002; Bartkus *et al.*, 2003). In a study of 1,030 US *B. pertussis* isolates that were screened for erythromycin resistance using the disk diffusion assay, it was determined that five (0.5%) of these isolates displayed a heterogeneous phenotype after 5-7 days of incubation (Wilson *et al.*, 2002). *B. pertussis* isolates are normally grown for 3-5 days using this assay, therefore potentially missing detection of resistant isolates. As a result of the study it was recommended to grow isolates for an extended period of 5-7 days. Even though the number of erythromycin-resistant *B. pertussis* isolates known are small it is important to screen future isolates to determine if this number will increase.

1.9 PERTUSSIS VACCINATION

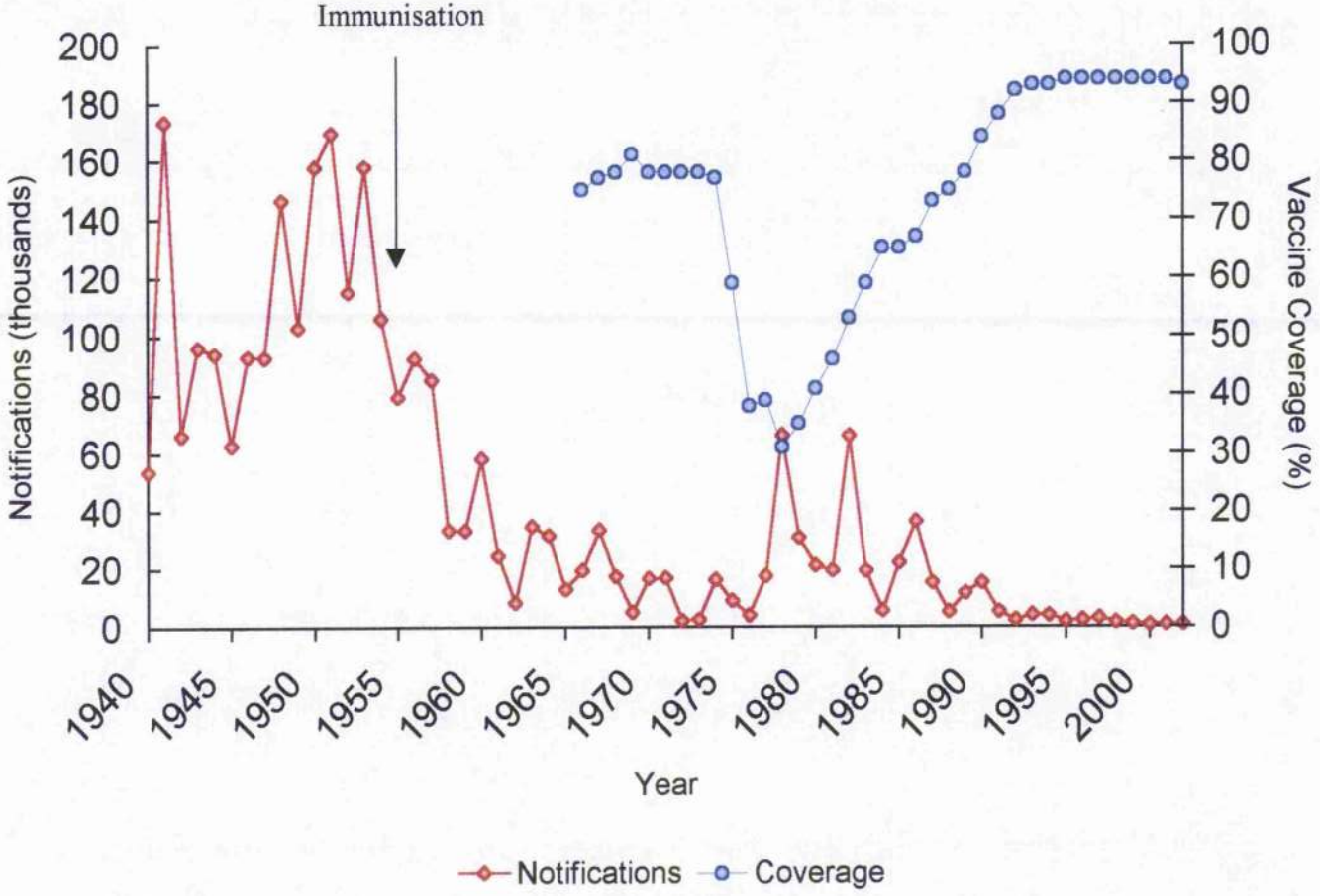
Pertussis vaccination is considered to be more effective at preventing disease rather than infection due to the fact that the interepidemic period has not increased since the introduction of vaccination (Crowcroft and Britto, 2002). Pertussis vaccines used world-wide are generally whole-cell vaccines (WCVs). Vaccine potency, reactogenicity and the immune response that they produce, as well as the vaccination schedules, vary from country to country. Currently, worldwide pertussis vaccination of children is about 80% (http://www.who.int/vaccines/en/pertussis.shtml).

The composition of many vaccines is unknown. However, the World Health Organisation recommendation is that WCVs must contain Fim2 and Fim3, by selection of the appropriate

B. pertussis strains (WHO, 1990). A US vaccine was found to contain 0-0.66 μg FHA/dose and 0.02-0.68 μg total PTX/dose (0-0.28 μg PTX/dose is biologically active). The Wellcome vaccine was found to contain 1.6 μg FHA/dose, 4.7 μg Fim2/dose and 0.3 μg PTX/dose (biologically active). The amount of Fim3 in this vaccine was estimated to be similar to that of Fim2 (Robinson *et al.*, 1985). Whole-cell vaccines were also found to contain the virulence factor CyaA. The AC enzymic activity of CyaA was measured in several commercial vaccines and it was found that all of the vaccines tested from one particular manufacturer had significant AC activity (Hewlett *et al.*, 1977). The original observation of AC activity in a WCV was thought to be due to the association of CyaA with the whole organism and the possible secretion of the enzyme into the medium of the vaccine during storage and handling. The authors analysed the same vaccine again which was stored at 4°C and found that it contained enzymic activity equivalent to 10% of the value measured 5 years previously (Hewlett *et al.*, 1977). The current UK whole-cell vaccines contain less than $2x10^{10}$ killed *B. pertussis* cells in a 0.5 ml dose, with a potency of more than 4 international units by comparison with the International Standard for Pertussis Vaccine in the intracerebral mouse protection test (Roberts and Parton, 2001). The pertussis component is usually combined with diphtheria and tetanus toxoids (DTP) and absorbed to aluminium hydroxide and with thiomersal as a preservative (Roberts and Parton, 2001). Local and systemic reactions have been associated with the administration of WCV. Local reactions include redness, swelling, pain and fever and these are reported to increase in frequency and severity with increasing number of doses. Systemic reactions that have been described were drowsiness, fretfulness, vomiting, anorexia, persistent or unusual crying but these were significantly less frequent with increasing dose number (Cody *et al.*, 1981).

Before the introduction of pertussis immunisation in the UK in the 1950s, the number of notifications per year for England and Wales exceeded 100,000 (http://www.hpa.org.uk/topics_az/whoopingcough/gen_info.htm) (Fig. 1.9.1). Between 1948 and 1960 there were case reports describing vaccine complications ranging from encephalopathy and coma to permanent neurological injury and death. However clinical trials of immunisation prior to 1960, carried out by the Medical Research Council, involved over 36,000 children of which there were no reported cases of encephalopathy (Baker,

Figure 1.9.1: Pertussis notifications and vaccine coverage for England and Wales from 1940-2000 (source: Immunisation Department, Health Protection Agency Communicable Disease Surveillance Centre, London)


Immunisation
Notifications (thousands)
Vaccine Coverage (%)
Year
Notifications
Coverage
0
20
40
60
80
100
120
140
160
180
200
0
10
20
30
40
50
60
70
80
90
100
1940
1945
1950
1955
1960
1965
1970
1975
1980
1985
1990
1995
2000

2003). Vaccine acceptance was over 80% in 1972 and the number of pertussis notifications was 2069.

In January 1974 there was a published report from the Hospital for Sick Children, Great Ormond Street describing a potential link between pertussis vaccination and permanent brain damage. This and other adverse publicity given to the vaccine resulted in a drop in vaccine uptake, which led to two epidemics, 1977-1979 and 1981-1983 (Baker, 2003). There were 200,000 extra pertussis notifications and 100 deaths in the 1970s and the 1980s. No significant link was ever established between the pertussis WCV and brain damage (Cody *et al.*, 1981). By the late 1980s immunisation rates were at pre-1974 levels (Baker, 2003). Currently, vaccine coverage is ca. 95% with pertussis at historically low levels (Fig. 1.9.1). In 1991 an accelerated vaccination schedule was introduced and babies were vaccinated at 2, 3 and 4 months rather than 3, 5 and 10 months of age.

One positive result from the above health scare has been investigations worldwide into the development and use of the less reactogenic acellular pertussis vaccines (ACVs). Field trials of various ACVs have been carried out in Sweden, Italy, Germany and Senegal. To date PTX, in the toxoided form, is the one component present in every ACV tested (Hewlett, 1997a). The monocomponent ACV consisted of PTX alone, which was toxoided by hydrogen peroxide treatment. Pertussis toxoid and FHA were used for the two component ACV and the addition of Prn was used for the three component ACVs. The five component vaccines contained the same antigens that were used for the three component ACV with the addition of Fim2 and Fim3. Most of the vaccines contained chemically-toxoided PTX (using formaldehyde, glutaraldehyde, or hydrogen peroxide) and one was genetically toxoided (Hewlett, 1997b). A genetically toxoided PTX molecule has been prepared by substituting two amino acids in the S1 subunit (Roberts and Parton, 2001). The latter was considered to be an improvement over the chemically toxoided PTX as this treatment can alter the structure and epitopes of molecules and reduce immunogenicity, thus requiring larger amounts of the antigen (Roberts and Parton, 2001).

Many of these trials differed in their design and in the composition of the vaccines used but several general conclusions have been drawn from the vaccine trials overall (Hewlett,

1997b). Use of ACVs results in fewer local adverse reactions than those associated with WCVs. However, as with WCVs, the frequency of local adverse reactions increases with the number of doses. Acellular vaccines also result in significantly lower rates of infrequent, but more severe, systemic reactions such as hypotonic-hyporesponsive episodes and febrile convulsions (Grant and Cherry, 2002). Acellular vaccines provide some level of protection against pertussis and there was a general trend that the more components an ACV contained the greater the protection (Hewlett, 1997b). Most current WCVs have greater efficacy than all ACVs except the five-component ACV (Grant and Cherry, 2002). The five-component ACV and WCV used in an Italian study were equally efficacious against typical pertussis (Olin *et al.*, 1997). The first ACV was developed in Japan, and these vaccines were licensed for immunisation of children aged two years or more in 1981 and in 1989 they were licensed for use in infants aged more than 3 months. The first US acellular pertussis vaccine was licensed for use in 1991 (http://www.who.int/vaccines/en/pertussis.shtml). Due to the results of a recently published UK study, an ACV was introduced in the UK as a pre-school booster in November 2001 (Crowcroft *et al.*, 2003). Using ACVs also offers the opportunity to extend pertussis prevention throughout the community to include vaccination of adolescents and adults, as WCVs have unacceptable side-effects when given past early childhood (Campins–Marti *et al.*, 2001). For implementation of booster vaccines across the population, it has been recommended that pertussis booster should be incorporated with tetanus and diphtheria toxoids and administered every 10 years (Heininger, 2001).

1.10 DIAGNOSTIC TESTS AVAILABLE FOR DETECTING *B. pertussis*

1.10.1 Culture

Culture is considered the 'gold standard' for diagnosing pertussis infection due to its specificity. However, it is slow and has low sensitivity compared to other diagnostic techniques, in particular polymerase chain reaction, where comparative studies have been undertaken (van der Zee *et al.*, 1993; Muller *et al.*, 1997; Lind-Brandberg *et al.*, 1998; Kosters *et al.*, 2001). Success of isolating *B. pertussis* from clinical specimens is dependent on several aspects. The sensitivity is dependent on collection methods, devices used, transport and enrichment media, conditions and duration of transport, choice of media, and incubation conditions and duration. *B. pertussis* should preferably be isolated from nasopharyngeal aspirates (NPAs) rather than nasopharyngeal swabs (NPSs). It has been

reported that there is a 15% gain in isolation rate when using NPAs (Guiso, 1997). The use of transport and enrichment media can increase the isolation of *B. pertussis* from clinical samples (Guiso, 1997). For transportation of NPSs, Regan and Lowe medium containing half-strength charcoal agar, horse blood and the antibiotic cephalexin (which supresses growth of normal nasopharyngeal flora) is the recommended transport medium (Regan and Lowe, 1977). The medium that is suitable for transportation of NPAs samples is Casamino acids solution held at 4°C (Cassiday *et al.*, 1994).

Isolation of *B. pertussis* is optimal at the end of the incubation period, during the catarrhal stage and at the beginning of the paroxysmal phase (Muller *et al.*, 1997). During this stage, isolation of *B. pertussis* may be as high as 90% (Kerr and Matthews, 2000). For primary isolation of *B. pertussis*, the Regan and Lowe medium is recommended, but for laboratory cultivation cyclodextrin solid medium is considered to be a better growth medium for *B. pertussis* than Bordet-Gengou medium. This medium contains heptakis (2,6-*O*-dimethyl) beta-cyclodextrin (MeCD), which is known to be a significant growth stimulant for *B. pertussis* (Aoyama *et al.*, 1986). Isolation rate was determined to be better with this medium and it has a longer shelf-life than BG medium. Positive cultures obtained later in infection may be a marker of delay in the clearance of *B. pertussis* from the respiratory epithelium with greater tissue damage and a more severe presentation of pertussis (Tozzi *et al.*, 2003).

1.10.2 Direct fluorescent antibody

Direct fluorescent antibody (DFA) test allows the direct detection of *B. pertussis* or *B. parapertussis* or their components in clinical samples. The DFA test uses fluorescein-labelled antibodies to *B. pertussis* and *B. parapertussis* to detect the bacteria in clinical specimens by fluorescence microscopy. Background staining has been reported to be problematic for this technique when using monoclonal and polyclonal reagents (Tilley *et al.*, 2000). The morphology of the organism can be difficult to verify as *B. pertussis* is small and the presence of cell artefacts also hinders the process (Tilley *et al.*, 2000). Low sensitivity for the DFA reagents has been reported, ranging from 28-50% (Tilley *et al.*, 2000). This test is also prone to false-positives (Ewanowich *et al.*, 1993; Ieven and Gossens 1997), which can be attributed to the presence of other bacterial species (unencapsulated *Haemophilus influenzae*, *Proteus mirabilis* and *Staphylococcus epidermidis*) being significantly cross-

reactive with commercial DFA reagent (Ewanowich *et al.,* 1993). Ewanowich *et al.* (1993) also reported a high proportion of samples, 88.4%, DFA positive, but culture negative.

1.10.3 Serology

There are a variety of serological assays that have been used to confirm diagnosis of pertussis, such as agglutination, complement fixation and enzyme-linked immunosorbent assays (ELISA) (Roberts and Parton, 2001). Of these assays, ELISA has been used as a base method for serology (Hallander, 1999). It is used to determine serological response to vaccination or infection by measuring changes in antibody levels induced by exposure to relevant antigens and also to compare antibody levels after immunisation with different vaccines or in a single serum test for diagnostic purposes (Hallander, 1999). Diagnostic sensitivity is dependent on the timing of sample collection, on the choice of antigen and the class of immunoglobulin (Hallander, 1999).

Serology has been used to measure anti-pertactin (anti-Prn), anti-filamentous haemagglutinin (anti-FHA) and anti-pertussis toxin (anti-PTX) antibodies in populations (Garcia-Corbeira *et al.,* 2000; Van der Wielen *et al.,* 2003). Of these, the most widely used antigens are PTX and FHA (Hallander, 1999). There are three different classes of antibodies (IgG, IgA and IgM) to specific *B. pertussis* antigens that can be used (Hodder and Mortimer, 1992). High IgA titres are considered to be indicative of infection and high IgM titres are not found in infected, previously immunised patients. Tests for IgG antibodies to PTX and to FHA are both very sensitive in typical cases of pertussis (Hallander, 1999). After infection with *B. pertussis*, antibodies can only be detected relatively late in the process of the disease, that is 1-2 weeks after the beginning of the symptoms in the non-vaccinated infected individuals (Muller *et al.,* 1997). Pertussis toxin antibody response can be considered as a specific marker for pertussis as an antibody response to FHA also occurs after infection with *B. parapertussis* and potentially after exposure with nonencapsulated *H. influenzae*. IgG antibody responses to Prn have also been investigated for use in diagnostic serology (Hallander, 1999) where antibody responses have been demonstrated both in individuals with pertussis and individuals with parapertussis. Prn IgG antibody determinations were recommended to be used alongside PTX and FHA assays in vaccinated individuals rather than alone (Trollfors *et al.,* 2003).

Differences in the distribution of antibodies to PTX, FHA and Prn were found amongst various age groups. An increase of anti-FHA and anti-PTX levels occurs with increasing age and levels of antibodies to the latter antigen peak in the adolescent/young adult (15-24 years) age group (Garcia-Corbeira *et al.*, 2000; Van der Weilen *et al.*, 2003). It has been observed that there is a decrease in anti-Prn levels with older age groups (Van der Weilen *et al.*, 2003). Pertussis immunity is known to wane after vaccination or infection, therefore the increase of anti-PTX in the adolescent and young adult age groups may be due to repeated exposure to *B. pertussis,* which would suggest a widespread circulation of the organism in the population (Garcia-Corbeira *et al.*, 2000). This would also suggest that this age group could be the main reservoir of infection and for these reasons vaccine boosters should be administered to individuals of this age (Van der Wielen *et al.*, 2003). A recent study has investigated the seroepidemiology of pertussis by examining the distribution of high titre anti-PTX IgG antibodies amongst the UK population (Nardone *et al.*, 2004). It was determined that there was an important circulation of serologically-defined pertussis infection in the UK population that often remains unreported or undiagnosed. In 1996 the incidence of pertussis was higher than that reported, 2232 per 100,000 versus 6 per 100,00 (Nardone *et al.*, 2004).

1.10.4 Polymerase chain reaction

Due to problems associated with other previously-described diagnostic tests such as lack of sensitivity, specificity and time delay, there is a need for a rapid, specific and sensitive method for detecting *B. pertussis* from clinical specimens. Polymerase chain reaction (PCR) in particular is a specific and sensitive method. Comparison of PCR with culture and DFA revealed that the three techniques had similar specificities (99.3%, 100% and 94.6% respectively) but differed in their sensitivity. PCR was the most sensitive (95%), culture was 36% and DFA had the lowest sensitivity ranging from 5.2-11.4% (Tilley *et al.*, 2000). Several diagnostic PCR assays have been developed to target the following genes, *ptx*, *cyaA*, outer membrane porin and insertion sequences (Douglas *et al.*, 1993; Mastrantonio *et al.*, 1996; van der Zee *et al.*, 1996a; Muller *et al.*, 1997; Farrell *et al.*, 1999, 2000; Fry *et al.*, 2004). Thorough investigation and optimisation of PCRs that amplify a portion of the genes that encode insertion sequences, IS*481* and IS*1001*, has been carried out by several groups (Glare *et al.*, 1990; van der Zee *et al.*, 1993; Backman *et al.*, 1994; Farrell *et al.*, 1999;

Reischl *et al.*, 2001; Kosters *et al.*, 2001; Sloan *et al.*, 2002; Podder, 2003). The target sequences IS*481* and IS*1001* can be used to determine if *B. pertussis* or *B. parapertussis* are present, respectively.

There are disadvantages with utilising IS*481* as a diagnostic target as it has been reported that an IS*481*-like sequence is present in *B. holmesii* (Loeffelholz *et al.*, 2000; Kosters *et al.*, 2001). Loeffelholz *et al.* (2000) tested *B. holmesii* isolates along with *B. pertussis* isolates using IS*481* PCR, and they both gave positive results and the level of sensitivity for *B. holmesii* was similar to that of *B. pertussis*. Direct sequencing of the IS*481* amplified product revealed that, in all six *B. holmseii* strains, there was the presence of two IS*481* alleles that differed by two nucleotides (Reischl *et al.*, 2001). Restriction fragment length polymorphism analysis has revealed that there are more than 50 copies of IS*481* in *B. pertussis* and only 8-10 copies in *B. holmesii* suggesting that IS*481* sequences in *B. pertussis* have been present longer. IS*481* may have been inherited from an ancestor common to both species and the fact that they can colonise the same anatomic site, the upper respiratory tract, suggests the opportunity for horizontal transfer of this element. Podder (2003) has utilised the reported sequence differences between IS*481* of *B. pertussis* and *B. holmesii* and developed a PCR assay that discriminated between each species. Recent work has also demonstrated the isolation of IS*481* from human *B. bronchiseptica* isolates (Gladbach *et al.*, 2002). Sequence analysis of the 200 bp fragment from the human *B. bronchiseptica* isolate revealed 97% homology with the consensus sequence which was derived from the two IS*481* GenBank sequences that are available. This information questions whether IS*481* should still be considered as an optimal PCR target for diagnosis of *B. pertussis* infection.

The promoter region of *ptx* can be used to detect and discriminate *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* due to this region being significantly different between the three species (Nygren *et al.*, 2000). Pertussis toxin promoter has been used as target to detect *B. pertussis* from clinical samples in several studies (Houard *et al.*, 1989; Mastrantonio *et al.*, 1996). Insertion sequence *481* and *ptx* promoter have been used together as PCR tests for detection of *B. pertussis* (Houard *et al.*, 1989; Farrell *et al.*, 2000; Qin *et al.*, 2002). Some clinical samples can be IS*481* negative and positive for the presence

of *ptx* promoter. This may be related to the other *Bordetella* species having the *ptx* promoter and not the insertion element IS*481*. A third target has been used, the pertussis specific porin gene (PO), if there was no consensus between the results of the other two PCR tests (Qin *et al.*, 2002).

There is the possible danger regarding an over reliance on PCR, which may lead to the over-diagnosis of pertussis (Lievano *et al.*, 2002). During a pertussis epidemic in New York, unvaccinated children that were exposed to PCR-confirmed cases of pertussis did not become ill, and vaccinated children did become ill. *B. pertussis* may not have been the primary or only cause of the outbreaks that were investigated. A positive PCR result due to transient colonisation with *B. pertussis* or a false-positive (contamination of PCRs were found) would lead to misdiagnosis of pertussis in persons with cough illness due to other agents.

1.10.5 Radioactive and conductimetric assays

The presence of AC enzymic activity in clinical specimens from pertussis-infected individuals has been suggested for use in diagnosis of pertussis (von Koenig *et al.*, 1989; Confer *et al.*, 1990; Scheftel *et al.*, 1992). Confer *et al.* (1990) developed a novel method to assay adenylate cyclase activity of nasopharyngeal swabs by determining the conversion of radioactive ATP to cAMP using a commercial kit. This assay took 24 h from initiation of assay to completion of the assay. The swabs were incubated overnight in 1 ml of modified Stainer-Scholte broth before the assay was carried out. In a separate study, trials with clinical material showed that 90% of modified Stainer-Scholte broths inoculated with nasal swabs required incubation for 72 h for measurable AC activity (von Koenig *et al.*, 1989), and thus differed from the AC assay used by Confer *et al.* (1990) that only required 24 h. The detection limit for this radioactive assay was measured by viable counting and was determined to be 3-6 x 10^4 cfu ml^{-1} for nasal swabs that had been incubated in SS liquid medium (von Koenig *et al.*, 1989). A similar method involved the collection of NPSs, which were immediately used for inoculation of Stainer-Scholte media and for bedside inoculation on to BG agar (Scheftel *et al.*, 1992). Inoculated Stainer-Scholte medium was incubated for 18 h at 37°C and the culture supernate was assayed for AC activity using a radio-competition kit. All AC-positive samples were isolated from infants under 3 months. There

was 100% correlation between AC positive and culture positive samples (Scheftel *et al.*, 1992). Scheftel *et al.* (1992) reported that *B. pertussis* was not isolated from any of the 36 AC negative samples. These assays that detected the presence of AC used radioisotopes and were time-consuming as well as expensive. A novel conductimetric assay that is rapid and inexpensive has been used to detect AC enzymic activity (Lawrence *et al.*, 2002). This assay involves a continuous recording of adenylate cyclase activity by the conductimetric determination of enzyme-coupled pyrophosphate cleavage. Features of this conductimetric assay will be reported and discussed later.

1.11 TYPING METHODS FOR DISCRIMINATION OF *B. pertussis* ISOLATES.

Various techniques have been used to study *B. pertussis* populations but some of these techniques are not easily compared between laboratories. Standardisation of techniques would allow isolates from different geographic regions to be compared and spread of individual isolates to be traced. Analysis and comparison of *B. pertussis* populations from different countries is especially important for determining effectiveness of different vaccination schedules and vaccines used. Mooi *et al.* (2000) have proposed that there should be a standardisation of techniques to study *B. pertussis*, including serotyping, DNA fingerprinting (PFGE) and typing of the pertussis toxin S1 subunit, pertactin genes and other genes that encode virulence factors. These typing techniques and others will be described.

1.11.1 Serotyping

Three different serotypes are used to differentiate *B. pertussis* isolates, serotype 1, 2 and 3 and isolates may be serotype 1; 1,2; 1,3 or 1,2,3 (Roberts and Parton, 2001). Comparison of serotyping results between laboratories is difficult due to the different sera and assays used. Assays include slide agglutination tests, traditionally with antisera raised in rabbits and made monospecific by absorption with heterologous strains (Parton, 2004). Mooi *et al.* (2000) recommended that serotyping should be carried out using monoclonal antibodies with a standard protocol, due to the lack of uniformity of different polyclonal sera preparations. Serotype antigens 2 and 3 are fimbrial antigens located on the major fimbrial subunits (section 1.4.2; Mooi *et al.*, 2000). Serotype antigen 1 (agglutinogen 1) is common to all fresh isolates and is located on LPS. Serotyping is not very discriminatory and does not provide much useful information for epidemiological studies of *B. pertussis* populations.

However, it is still useful as fimbrial antigens are potentially important in protection (Roberts and Parton, 2001).

1.11.2 Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) has been considered to be the gold-standard for typing *B. pertussis* strains due to its discriminatory power. Fragments of genomic DNA are generated using rarely-cutting restriction enzymes and are analysed using PFGE to produce a fingerprint for that organism. This technique has been used in epidemiological investigations to identify outbreak-associated isolates, determine genetic variation between vaccine isolates and recent clinical isolates, and transmission of *B. pertussis* isolates (Brennan *et al.*, 2000; Weber *et al.*, 2001; Hardwick *et al.*, 2002; De Schutter *et al.*, 2003; Kourova *et al.*, 2003; Peppler *et al.*, 2003). A study of French and Russian *B. pertussis* isolates revealed that there had been a shift in PFGE type with time as the PFGE profiles of vaccine strains and pre-vaccination era isolates differed from those of isolates that were circulating at the time (Weber *et al.*, 2001; Kourova *et al.*, 2003). A Canadian study demonstrated that there was a uniformity of PFGE types within epidemiologically-linked clusters of pertussis cases (Brennan *et al.*, 2000). Beall *et al.* (1995) described the PFGE technique for typing *B. pertussis* isolates using two possible combinations of restriction enzymes, *Xba*I with *Spe*I or *Dre*I (Beall *et al.*, 1995). The restriction endonucleases *Spe*I and *Dre*I were useful for further discrimination when *Xba*I profiles showed minor differences. Results also demonstrated detectable differences in profiles after repeated subculture of *B. pertussis* isolates. This was reported for all three enzymes.

Mooi *et al.* (2000) have recommended that DNA fragments to be analysed by PFGE should be cut with two enzymes: *Xba*I and *Spe*I. There has been no standardisation of this method and there are problems relating to reproducibility. Hardwick *et al.* (2002) reported a PFGE technique using only *Xba*I and compared results between five laboratories. A major source of variability within a laboratory was derived from the preparation of DNA from different cultures rather than from use of different gel preparations or use of different equipment. Comparison of PFGE profiles between laboratories is hindered by the unknown reproducibility of DNA fragment sizes (partial digestion) and of the profiles. Their standardised procedure produced PFGE profiles that were sufficiently reproducible to allow

each laboratory to define each of the test profiles by molecular sizes of its composite fragments. The profiling results from each laboratory were readily compared. The PFGE technique has also been applied to a small set of *B. holmesii* strains and the restriction enzymes employed were *Xba*I and *Spe*I. The limited number of band patterns that were observed suggested that the *B. holmesii* genome may be highly conserved or that further investigation would be required as the restriction enzymes that were used may not be those that would optimally discriminate the *B. holmesii* strains (Mazengia *et al.*, 2000).

1.11.3 IS*1002* restriction fragment length polymorphism

Another approach to study population structure of closely-related bacteria is based on the insertion sequence (IS)-associated DNA polymorphism. There are two main insertion elements found scattered along the *B. pertussis* genome, IS*481* and IS*1002*. For this method, chromosomal *B. pertussis* DNA is digested with a restriction endonuclease, and the resulting fragments are separated by gel electrophoresis. A DNA probe comprised of an internal fragment of IS*1002* is obtained by PCR, labelled and then hybridised to the separated DNA fragments under high stringency conditions. Applying this method using the high copy number IS*481* (>80 copies) resulted in a complex fingerprint pattern that was not easily analysed (van der Zee *et al.*, 1996b). Insertion sequence *1002* (4-8 copies) restriction fragment length polymorphism (RFLP) fingerprinting produces patterns that can be easily analysed and has been used to study population structure and epidemiology of *B. pertussis* isolates. For Dutch strains there was a shift in population structure over time. Van der Zee *et al.* (1996b) speculated that this shift in population structure may have been due to vaccine-driven evolution. There was a large overlap of RFLP types between Dutch and German *B. pertussis* populations, which may have been due to the close proximity of the countries. *B. pertussis* isolates from more distant countries such as Canada and Japan, showed less overlap of RFLP types amongst their *B. pertussis* populations. This study was further expanded to type a large number of Dutch *B. pertussis* isolates from 1949-1996 (van Loo *et al.*, 1999). Significant differences in DNA types between populations from five time periods were observed. The most pronounced difference was between the pre-vaccination group of isolates and the ensuing time group. Van Loo *et al.* (1999) suggested that these differences were driven by whole-cell pertussis vaccination in The Netherlands. However, random drift and changes in the human population unrelated to vaccination cannot be disregarded. A

comparison of the Dutch *B. pertussis* isolates from 1996 (Dutch epidemic year) did not reveal major shifts in DNA type or genetic diversity and there was no evidence for introduction or expansion of a particular strain (van Loo *et al.*, 1999).

1.11.4 Multilocus enzyme electrophoresis

Multilocus enzyme electrophoresis (MLEE) has been used to determine genetic diversity and structures in natural populations of a variety of bacterial species (Selander *et al.*, 1986). The genetic diversity and relationships within and amongst *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* was determined by investigating the electrophoretic variation in 15 metabolic enzymes (Musser *et al.*, 1986). Each isolate was characterised by its combination of alleles at the 15 enzyme loci, and distinctive profiles corresponding to unique multilocus genotypes were designated as electrophoretic types (ETs). Fourteen ETs were found amongst the 60 isolates investigated. The MLEE results have demonstrated that the *Bordetella* spp. populations are clonal. Musser *et al.* (1986) have shown that *B. pertussis* strain 18323^{T} is atypical and that it is probably more closely related to *B. bronchiseptica* isolates than *B. pertussis* isolates. Only three ETs were found amongst 23 *B. pertussis* isolates investigated. However, this was increased to four ETs in a later study with 18 *B. pertussis* isolates (van der Zee *et al.*, 1997). In contrast, IS*1002* RFLP typing had revealed 45 DNA types amongst 213 *B. pertussis* isolates investigated. The latter method has revealed a faster molecular clock probably due to the transposition of and recombination between IS elements (van Loo *et al.*, 1999).

Van der Zee *et al.* (1997) determined the distribution of three different IS elements (IS*481*, IS*1002* and IS*1001*) within different multilocus genotypes of *B. pertussis*, *B. parapertussis* and *B. bronchiseptica*. *B. bronchiseptica* showed the highest degree of genetic diversity and isolates were subdivided in three distinct clusters of ETs. Both MLEE and distribution of IS*1001* showed that *B. parapertussis* was more closely related to particular *B. bronchiseptica* strains that were grouped in cluster C (strains predominantly isolated from pigs) than other ETs of *B. bronchiseptica*. This is suggested to reinforce the notion that *B. parapertussis* is another lineage of *B. bronchiseptica*. Ovine and human *B. parapertussis* isolates were closely related but were distinct and showed differences in genetic diversity. Based on MLEE and IS typing, ovine *B. parapertussis* show a greater degree of divergence

than human *B. parapertussis* isolates. *B. pertussis* and *B. parapertussis* may have derived from distinct clones of *B. bronchiseptica* because the ETs of *B. pertussis* and *B. parapertussis* are distantly related and both species contain different IS elements. Genetic diversity of *B. pertussis* is relatively large compared to that of *B. parapertussis*.

1.11.5 Repetitive element PCR fingerprinting

There are three known repetitive elements that may be present on bacterial genomes, repetitive extragenic palindromic (REP), enterobacterial repetitive intergenic consensus (ERIC) and BOX (conserved DNA element found within the intergenic regions of Gram-positive bacterial chromosomes) (Stern *et al.*, 1984; Hulton *et al.*, 1991; Martin *et al.*, 1992). REP and ERIC were originally described in *Escherichia coli* and *Salmonella typhimurium* (Stern *et al.*, 1984; Hulton *et al.*, 1991), and BOX was originally described in *Streptococcus pneumoniae* (Martin *et al.*, 1992). REP, ERIC and BOX sequences have been utilised as PCR targets for DNA fingerprinting of several bacterial pathogens (Versalovic *et al.*, 1991; van Belkum *et al.*, 1996; Sechi *et al.*, 1998; Jersek *et al.*, 1999; Zavaglia *et al.*, 2000). Consensus outward facing primers have been designed to target these repetitive elements and are used in PCR to amplify the region between neighbouring elements. This process generates DNA fingerprints that enable the differentiation of eubacterial species and strains. Gillings and Holley (1997) have shown that ERIC-PCR patterns can also be readily generated from eukaryotes and bacteriophage even when using the recommended standard PCR annealing temperature of 52°C.

The discriminatory powers of three DNA commonly used fingerprinting techniques to type *B. pertussis* were compared; PFGE, randomly amplified polymorphic DNA (RAPD)-PCR and ERIC-PCR (Moissenet *et al.*, 1996). The technique RAPD-PCR involves the amplification of arbitary DNA targeted by short random primers producing a DNA fingerprint. Of the three techniques, PFGE was considered to be the optimal method to discriminate *B. pertussis* isolates. ERIC- and RAPD-PCR fingerprinting methods were found to be poor in discriminating the isolates. A search of the *B. pertussis* Tohama-1 genome using Basic Local Alignment Sequence Tool (BLAST) (Altschul *et al.*, 1990) during this PhD study revealed that ERIC sequences were not present. The ERIC-PCR annealing temperature used in the above study was lower than the standard 52°C annealing

temperature. Moissenet *et al.* (1996) used an initial annealing temperature of 26°C followed by 40°C.

1.11.6 Multilocus sequence typing

Maiden *et al.* (1998) described a molecular technique that could be used to type bacterial pathogens causing disease locally or globally. Multilocus enzyme electrophoresis was adapted to produce the method, multilocus sequencing typing (MLST), that assigned alleles directly from nucleotide sequences of internal fragments of housekeeping genes rather than comparing electrophoretic mobilties of the enzymes they encode. This allelic profile is assigned a sequence type (ST). Multilocus sequence typing has several advantages over MLEE, firstly more variation would be detected resulting in greater discrimination between strains. Multilocus sequence typing data can be compared easily between laboratories compared to MLEE data, which is difficult to compare between different laboratories. The sequence typing data is submitted and stored in an expanding database (http://www.mlst.net), which has produced a powerful tool for global epidemiology (Maiden *et al.*, 1998). Multilocus sequence typing was originally described for *Neisseria meningiditis* and *Streptococcus pneumoniae* (Maiden *et al.*, 1998; Enright and Spratt 1998). This method is used for several applications, including tracking the emergence of antibiotic-resistant pathogens, association of particular genotypes with virulence and antigenic characteristics, and global spread of disease caused by novel variants (Urwin and Maiden 2003). The data have also been used in evolutionary and population analyses that estimate recombination and mutation rates and investigate evolutionary relationships among bacteria that are classified as belonging to the same genus.

1.12 SEQUENCE TYPING OF *B. pertussis* VIRULENCE GENES

Sequencing of virulence-associated *B. pertussis* genes has similar advantages to the MLST technique described in section 1.11 with regard to the fact that results from different laboratories could be easily compared and such a method can be informative for clonal organisms such as *B. pertussis*. Several sequence typing studies of *B. pertussis* isolates have been reported. The genes that encode the virulence factors pertussis toxin S1 subunit (*ptxA*) and pertactin (*prnA*) have been demonstrated to contain polymorphic regions that can be used to type *B. pertussis* isolates (Mooi *et al.*, 1998). *B. pertussis* is known to be clonal

from MLEE studies, see section 1.11.4, and sequencing of its housekeeping genes (van Loo *et al.*, 2002). Therefore using virulence-related genes that are under adaptive pressure will potentially show greater variation than that exhibited by its housekeeping genes. Targets chosen for study of the epidemiology of circulating strains by van Loo *et al.* (2002) included genes coding for surface proteins and pertussis ACV components. The following genes were sequenced: pertactin (*prnA*), subunits of pertussis toxin, (*ptxA, B, C, D* and *E*), fimbriae (*fim2* and *fim3*), filamentous haemagglutinin (*fhaB*), tracheal colonisation factor (*tcfA*), outer membrane proteins (*ompP* and *ompQ*), bordetella intermediate phase protein (*bipA*), bordetella resistance to killing protein A (*brkA*) and virulence-activated gene 8 (*vag8*). Only the genes *ptxA*, *ptxC*, *tcfA*, *fim2* and *prnA* showed variation in *B. pertussis* isolates. Van Loo *et al.* (2002) characterised strains from The Netherlands, Finland, Italy, United States and Japan using a MLST scheme based on three of these genes: *ptxA*, *ptxC* and *tcfA*. Further information relating to this scheme will be reported and discussed later.

Sequence analyses from different countries can be directly compared to determine if *B. pertussis* populations world-wide are the same or if they differ in some or all gene targets. However, agreement is required with regard to the gene target and its regions that are analysed for an individual typing scheme. The results of sequence typing studies relating to shift in *B. pertussis* populations and how this may relate to the resurgence in pertussis are described in sections 1.13.2 and 1.13.3.

1.13 EPIDEMIOLOGY OF PERTUSSIS

1.13.1 World-wide epidemiology of pertussis

B. pertussis causes 20-40 million cases of pertussis worldwide each year. Ninety percent of these cases are from developing countries and there are an estimated 200,000–400,000 fatalities, mainly in infants (http://www.who.int/vaccines/en/pertussis.shtml). The majority of the most severe cases of pertussis and fatalities occur in young infants but pertussis can cause disease at any age. In Western countries, about 10-12% of all pertussis cases have been reported from individuals more than 15 years old. Incidence of pertussis is cyclic with a peak approximately every 4 years as seen in the data from the UK (Fig. 1.9.1). Cycles are caused by the changing number of susceptible individuals and the rate of transmission by *B. pertussis*. After a peak, the number of susceptible people is low and there is little

transmission. As immunity wanes and newborn infants enter the community, there is an increase in the number of susceptible individuals, resulting in greater transmission and a new peak (Robbins, 1999).

1.13.2 Genetic and antigenic shifts in *B. pertussis* population

Comparisons of currently circulating isolates with pre-vaccination era isolates and vaccine strains used in various countries have revealed that a shift in the *B. pertussis* population has occurred over time. Sequence typing of several virulence factors along with PFGE and IS*1002* RFLP have revealed this trend (see sections 1.11.2, 1.11.3 and 1.12). A shift in pertactin (*prnA*) and pertussis toxin S1 subunit (*ptxA*) gene types in *B. pertussis* strains circulating in the pre-vaccination era compared to recent isolates has occurred and it was suggested, after a study in The Netherlands, that these shifts were driven by vaccination with Dutch WCV (Mooi *et al.*, 1998). The emergence and subsequent dominance in circulating strains of *prnA* and *ptxA* types not found in the WCV were subsequently described in several other countries, including US, Finland, Poland, and Italy (Mastrantonio *et al.*, 1999; Mooi *et al.*, 1999; Cassiday *et al.*, 2000; Gzyl *et al.*, 2002). The situation for *B. pertussis* isolates from the UK differs regarding *ptxA* variation in that the same *ptxA* allele was found in two of three WCV strains and in recently circulating isolates (Fry *et al.*, 2001).

There was no apparent shift in *prnA* type until the early 1980s; that is, the vaccine strains, pre-vaccination isolates and strains isolated after implementation of vaccination all contained *prnA*(1) in The Netherlands, Finland, US, UK and Poland (Mooi *et al.*, 1998; 1999; Cassiday *et al.*, 2000; Fry *et al.*, 2001; Gzyl *et al.*, 2002). No Polish isolates were available from the 1980s so it is not possible to determine when non-*prnA*(1) alleles appeared in this country. There were differences between the *B. pertussis* populations from these countries with regard to the *ptxA* alleles that were present during the pre-vaccination era to the 1980s. Dutch isolates during this period contained *ptxA*(2) or *ptxA*(3) and the country's vaccines contain both *ptxA* alleles (Mooi *et al.*, 1998). The US isolates from a similar time period were *ptxA*(1), *ptxA*(2) and *ptxA*(3) and the US vaccines (that are known) contain *ptxA*(2) (Cassiday *et al.*, 2000). The Finnish *B. pertussis* population contained only *ptxA*(2) and its vaccines contain the same *ptxA* alleles as the Dutch vaccines (Mooi *et al.*, 1999). *B. pertussis* isolates from UK and Poland contained *ptxA*(1) and *ptxA*(2) (Fry *et al.*, 2001; Gzyl

et al., 2002). The Polish vaccine strains are *ptxA*(2) whereas the UK vaccine strains are *ptxA*(1) and *ptxA*(2).

The presence of *prnA*(2) and *prnA*(3) was found in 16-24 (20-30%) Dutch isolates from 1981-1988 (Mooi *et al.*, 1998). From 1989 onwards, the frequency of *prnA*(2) and *prnA*(3) increased, 204 of 227 (90%) Dutch isolates were either of these *prnA* alleles. During 1993-1996, the predominance of *prnA*(2) and *prnA*(3) alternated in successive years (Mooi *et al.*, 1998). It should be noted that, when The Netherlands had the unexpected epidemic in 1996, *prnA*(3) was predominant. The frequency of vaccine alleles *ptxA*(2) and *ptxA*(3) decreased after the introduction of vaccination, *ptxA*(1) was not present until 1975-1985 and this allele was in 150 of 170 (88%) Dutch isolates during 1990-1996 (Mooi *et al.*, 1998). The presence of the non-vaccine *prnA*, *prnA*(2), isolates became apparent in the US *B. pertussis* population during 1975-1987. This shifted until 64 of 92 (70%) US isolates were *prnA*(2) during the 1990s. A shift in *ptxA* types was also apparent with a decrease in *ptxA*(2) and an increase of *ptxA*(1) until 1989-1999 when 72 of 92 (78%) were *ptxA*(1) (Cassiday *et al.*, 2000). During 1990-1996 four *prnA* alleles (*prnA*(1)-*prnA*(4)) were present in the Finnish *B. pertussis* isolates with the non-vaccine type, *prnA*(2), as the predominant type found in 31 of 43 (72%) isolates (Mooi *et al.*, 1999). From 1982 onwards, all Finnish isolates contained the non-vaccine *ptxA* type, *ptxA*(1) (Mooi *et al.*, 1999). During the 1990s, Polish isolates were predominantly, 11 of 19 (58%), the vaccine *prnA* type, *prnA*(1), and the predominance of *ptxA*(1) shifted until all Polish isolates from the 1990s (including 2000) were *ptxA*(1) (Gzyl *et al.*, 2002). A shift in *prnA* type amongst UK isolates was apparent after 1982. During 1982-1985 and 1998-1999, 21 of 86 (24%) isolates and 56 of 105 (53%) of isolates were non-*prnA*(1), respectively. The frequency of *ptxA*(1) amongst the UK *B. pertussis* population increased until 1990-1999 when all isolates were *ptxA*(1) (Fry *et al.*, 2001). A similar study was also carried out on recent Italian *B. pertussis* isolates. Italy differs from the other countries in that it has a history of low vaccination. Even with this history of low vaccination, similar *ptxA* and *prnA* types were found amongst the recent Italian isolates and these differed from the alleles found in the Italian vaccine strains. The vaccine strains were *prnA*(1) and *ptxA*(2) or *prnA*(5) and *ptxA*(3). All Italian strains from 1993-1995 were *ptxA*(1). Of these isolates, 65 of 129 (51%) were *prnA*(3), 53 (41%) were *prnA*(2), 8 (6%) were *prnA*(1) and 3 (2%) were *prnA*(5) (Mastrantonio *et al.*, 1999).

The *prnA* and *ptxA* trends in the different *B. pertussis* populations studied are very similar in that these populations had similar *prnA* and *ptxA* types during the pre-vaccination era and the alleles that are now found are also similar. The polymorphic regions of *prnA* and *ptxA* regions interact directly with the immune system. Mooi *et al.* (1998) suggested that the expansion of particular *prnA* and *ptxA* types is not a consequence of variation in these particular molecules only but is due also to changes in other bacterial antigens which affect strain fitness and which may be linked to particular *ptxA* and *prnA* types. An MLST scheme adapted for typing *B. pertussis* isolates had demonstrated that predominant MLST types that were present in the pre-vaccination era were found only at very low frequencies or not at all amongst recent isolates. A predominant type, MLST-5, was present in the Netherlands, US and Japan and this MLST type was not apparent in *B. pertussis* isolates until 1990s (van Loo *et al.*, 2002). Determining whether a shift in MLST type has occurred in the UK *B. pertussis* population has been investigated during this PhD and the results will be reported and discussed later.

1.13.3 Resurgence in pertussis

Pertussis has been increasing recently in several countries that have had vaccination programmes implemented since the 1950s. Several explanations have been put forward for the resurgence of pertussis in a vaccinated population. These include improved surveillance, changes in case definitions and diagnostic techniques. It has also been suggested that it is possibly due to demographic changes, waning vaccine-induced immunity, changes in vaccine effectiveness, changes in vaccine coverage and adaptation of *B. pertussis* population to vaccine-induced immunity. Countries that have reported an increase in pertussis cases include The Netherlands, Canada, Australia, Poland, US and Taiwan. In 1996, The Netherlands had an unexpected pertussis epidemic that showed an incidence that was five-fold higher than previous epidemics (Mooi *et al.*, 1998). In 1996, there were 2,771 reported cases of pertussis compared with 319 cases reported during the previous year (de Melker *et al.*, 1997). The study of age distribution of pertussis in The Netherlands had shown that there was a significant decrease in the proportion of patients aged less than 1 year (21% in 1989 to 7% in 1996) and a significant increase in the proportion of patients aged 1-4 years (21% in 1989 to 30% in 1996). There was also a decrease from 1989 to 1995 in those infected with pertussis aged 5-9 years but, by 1996, this incidence had increased to 39%. A

sequencing study of *prnA* and *ptxA* allelic variation in the Dutch *B. pertussis* population revealed that, during 1996, isolates were predominantly non-*prnA* and non-*ptxA* vaccine types, *prnA*(3) and *ptxA*(1). From a previous study it was known that the Dutch whole-cell vaccine strain expressing *prnA*(1) was not as protective against *B. pertussis* strains expressing *prnA*(2) or *prnA*(3) (King *et al.*, 2001). It has been suggested that this shift may have played a part in the resurgence of pertussis (Mooi *et al.*, 1998). However, a shift has also been demonstrated in the UK and Finnish *B. pertussis* populations but this has not been accompanied by resurgence in pertussis levels (Mooi *et al.*, 1999; Fry *et al.*, 2001).

In Canada, the incidence of pertussis has increased four-fold, from 5 per 100,000 in the late 1980s to about 20 per 100,000 in recent years (Hoey, 2003). In Australia, since the 1990s, pertussis notification rates have increased noticeably from record low levels that were seen during the 1970s and 1980s (Menzies *et al.*, 2003). A Polish study had noted that, during the 1990s, there was an increase in morbidity and an unexpectedly large number of pertussis notifications were observed in 1997 and 1998 (Gzyl *et al.*, 2002). This Polish study had noted that there was increase in pertussis. However, vaccine *prnA* types were still predominant amongst recent *B. pertussis* isolates. The US had reported an increase in pertussis incidence since the 1980s, from 0.75 per 100,000 in 1980 to 2.74 per 100,000 in 1998 (Yih *et al.*, 2000) and the highest number of pertussis cases in the US since 1967 occurred in 1996 (Cassiday *et al.*, 2000). It was suggested that, unlike The Netherlands, much of this increase in the US occurred amongst patients aged 10-19 years. Similar to The Netherlands, this increase was suggested to be related to the shift in its *B. pertussis* population from the predominance of vaccine *prnA* and *ptxA* alleles to those that are non-vaccine types. In Taiwan, pertussis vaccination has been carried out since 1955 and, during 1971-1991, the annual numbers of reported pertussis cases were all in single digits (Lee *et al.*, 2003). Since a pertussis outbreak in 1992 there has been an upward trend in the number of cases.

Several studies relating to waning vaccine-induced immunity have reported an increase in the pertussis incidence amongst adults and adolescents (Yih *et al.*, 2000; Guris *et al.*, 1999; Gilberg *et al.*, 2002; Hoey 2003). *B. pertussis* infection in adults produces a wide spectrum of symptoms thus making the correct diagnosis of a suspected pertussis case difficult,

leading to potential under-reporting of the disease and providing a reservoir of infection to the unvaccinated population. It is mainly accepted that atypical or asymptomatic symptoms of pertussis infection occur within these individuals but in one study with reported cases in adolescents and adults, 83% and 87% respectively, had paroxysmal coughing and many had whooping, apnoea and vomiting at the time of diagnosis (Yih *et al.,* 2000). An investigation of the predominance of *Chlamydia pneumoniae* amongst adults that had a cough for more than two weeks revealed that pertussis infection was more predominant, 18.5% versus 6.5% in controls, and dual infections were also found (Miyashita *et al.,* 2003). There is evidence of transmission of *B. pertussis* from symptom-free adults and adolescents to susceptible children. Asymptomatic *B. pertussis* infection was detected amongst household members of infants and children with pertussis (De Schutter *et al.,* 2003). There was homogeneity of isolates recovered from contacts and the primary case of infection within a family. It was suggested that well-vaccinated adolescents and adults play an important role in the epidemiology of pertussis by acting as reservoirs of spread to incompletely vaccinated and non-vaccinated children but the mechanism for this transmission is unclear.

1.13.4 Pertussis epidemiology in the UK

The UK *B. pertussis* population has demonstrated a shift from vaccine *prnA* types to non-vaccine *prnA* types, as seen in several other countries. The UK *B. pertussis* population differs from others with regard to its *ptxA* trend. It is unique in the fact that the recent toxin type is the same as that found in its whole-cell pertussis vaccine strains as well as in pre-vaccination isolates (Fry *et al.,* 2001). Resurgence of pertussis in various countries has been described above but this situation has not been seen in the UK. In fact, pertussis incidence is at historically low levels. A pertussis epidemic took place in the UK during the late 1970s and the early 1980s but this was due to a decrease in the vaccine uptake rate (Fig. 1.9.1; see section 1.9). An enhanced surveillance programme in the UK during 1995-1997 was undertaken to monitor the pertussis situation in England and Wales. From this study it was found that pertussis is increasingly affecting younger, unvaccinated children and to a lesser extent adolescent and young adults (van Buynder *et al.,* 1999). The proportion of notifications occurring in patients 15 years of age and older since 1991 has increased from 4.4% to 9.3% and for those 6 months and younger notifications increased from 6.3% in 1991 to 19.1% in 1997 (van Buynder *et al.,* 1999). It was also found that there was a

continuing significant and under-reported mortality associated with pertussis in the very young age group.

A recent UK study aimed to determine whether parents or siblings were the source of pertussis infection for infants too young to be protected by vaccination (Crowcroft *et al.*, 2003). It was found that pertussis was a more frequent cause of admission to the paediatric intensive care unit (PICU) than previously thought. For most of the infants in this study, the symptom presentation was atypical therefore pertussis was not suspected and the case would have not been otherwise investigated or notified as pertussis. Under-notification and under ascertainment of severely-affected infants requiring admission to the PICU was also described (Crowcroft *et al.*, 2003). This study highlighted the potential carriage of *B. pertussis* by individuals. There were household contacts that had no symptoms but these individuals had either PCR-positive results or anti-PTX IgG levels indicating recent infection. There could be several explanations for these indications of pertussis infection such as false PCR positives, carriage of *B. pertussis*, modification of disease through vaccination and subclinical infection with immunological boosting. Highly sensitive diagnostic methods such as PCR may be detecting carriage in individuals, if it does occur, which might explain the persistence of the infection in the community despite high vaccination coverage. Even though overall levels of pertussis have declined in recent times, and are greatly reduced compared to those of the pre-vaccination era, it still remains a significant cause of mortality and severe morbidity in the very young.

1.14 AIMS AND OBJECTIVES

The main aim of this study was to investigate whether the adenylate cyclase toxin gene and other virulence-related genes of *Bordetella pertussis* could be used as potential epidemiological markers. A sequencing study has been previously carried out on *B. pertussis* isolates from the United Kingdom (UK) dating from the pre-vaccination era to present day using pertactin and pertussis toxin S1 genes as markers and similar studies have been carried out in several other countries. Several virulence-related genes are to be investigated for the presence of polymorphic regions and, once identified, the allele frequencies of individual genes should be determined for the UK *B. pertussis* population from the pre-vaccination era to present day (1920-2002). Gene marker sequence typing studies are to be applied to UK *B. pertussis* isolates to determine whether a genetic shift has occurred in the *B. pertussis* population from the pre-vaccination era to present day and if circulating isolates are of the same gene types as the *B. pertussis* whole-cell and acellular vaccine strains.

The virulence factor, adenylate cyclase toxin has been described previously as target for diagnostic tests using conventional radioisotopic assays or for polymerase chain reactions. A novel conductimetric assay that has been developed within the Division of Infection and Immunity detects the presence of adenylate cyclase by its enzymic activity. It is important to determine whether this assay could be used in a diagnostic setting. This involves developing and optimising a quick and easy protocol for detecting enzymic (AC) activity from *B. pertussis* cells and determining the sensitivity of the assay. To aid with the optimisation of the conductimetry assay as a diagnostic test, constructs containing the adenylate cyclase toxin promoter inserted upstream of promoterless reporter genes, *lux* (this operon codes for the enzyme luciferase and the substrate luciferin that are required for the light emitting reactions) and green fluorescent protein (*gfp*), will be developed to determine when the optimal expression of adenylate cyclase toxin gene occurs during the growth of the organism.

2.0 Materials and Methods

2.1 BACTERIAL STRAINS AND CULTURE CONDITIONS

2.1.1 *Bordetella* strains

Bacterial strains used in the conductimetry and sequencing analysis studies are described in Table 2.1.1.2.1.1.1. Other studies were performed using a panel of 152 United Kingdom (UK) clinical isolates of *B. pertussis*, from 1920 to 2002 (Table 2.1.1.2), and all isolates in this study were assumed to be epidemiologically unrelated. The three strains used in the preparation of one of the UK whole-cell pertussis vaccines (Medeva Pharma), were CN2992, CN5476 and CN3099. In addition, the isolate Tohama-I (components of which are used in acellular pertussis vaccines in the UK and other countries) and the type strain of *B. pertussis* (18323^T, $NCTC10739^T$) were also characterised. Appendices 6-8 list further information on the UK isolates. The years of isolation and numbers of clinical isolates in the panel are listed in Table 2.1.1.2. Not all isolates were examined for all targets and data from a previous study (Fry *et al.*, 2001) were used to aid the selection of subsets of isolates containing representatives of all known *Bordetella* isolates. These isolates were obtained from the Division of Infection and Immunity (DII), University of Glasgow; Glasgow Veterinary School, University of Glasgow; the Respiratory and Systemic Infection Laboratory (RSIL), Health Protection Agency, Colindale, London; the National Collection of Type Cultures (NCTC), Colindale, London; and the Wellcome Bacterial Collection held by NCTC.

2.1.2 Growth of *Bordetella*

Isolates of *Bordetella pertussis*, *B. parapertussis*, *B. bronchiseptica*, *B. avium*, *B. trematum*, *B. hinzii* and *B. holmesii* were grown either on (i) Bordet-Gengou (BG) agar (Appendix 1.1) in a humidified environment at 37°C for up to 3 days or (ii) on blood charcoal agar (Media Services, CPHL, HPA) at 37°C with 5% CO_2 for up to 5 days. To obtain visible colonies, *B. avium*, *B. bronchiseptica*, *B. trematum* and *B. hinzii* required incubation for only one day, *B. parapertussis* required two days and *B. holmesii* required three days. Some *B. pertussis* isolates required incubation for up to five days. Bacterial growth on solid media was used for inoculation into liquid medium, preparation of cell lysates for conductimetry analysis or for preparation of DNA. To check for haemolysis *Bordetella* spp. were grown on BG sandwich plates. This medium was prepared by pouring a layer of BG agar without blood and, once set, a thin layer of BG agar with blood was

Table 2.1.1.1. *Bordetella* species used for conductimetry analysis

***Bordetella* species**	**Strain**	**Information**	**Source**
B. pertussis	BP338	Tohama-I derivative, Nal^R	Division of Infection and Immunity, originally from Prof. A. Weiss
B. pertussis	BP347	BP338 derivative (Bvg^-)	Division of Infection and Immunity, originally from Prof. A. Weiss
B. pertussis	BP348pRMB1	CyaA overexpressor	Division of Infection and Immunity, Brownlie *et al.* (1988)
B. pertussis	NCTC10739		RSIL
B. parapertussis	NCTC5952		RSIL
B. parapertussis	NCTC10853		RSIL
B. bronchiseptica	NCTC452		RSIL
B. bronchiseptica	NCTC458		RSIL
B. bronchiseptica	BBC17	Pig isolate	Glasgow Vet School, Prof M. Roberts
B. avium	NCTC12033		RSIL
B. avium	NCTC12034		RSIL
B. hinzii	NCTC13199		RSIL
B. hinzii	NCTC13200		RSIL
B. holmesii	NCTC12912		RSIL
B. holmesii	NCTC13202		RSIL
B. trematum	NCTC12995		RSIL
B. trematum	NCTC13203		RSIL

Table 2.1.1.2. UK isolates of *Bordetella pertussis* characterised by gene sequencing

Year of isolation	No. of isolates per year	No. of isolates used for sequencing of:								
		ptxC	*tcfA*	*fim2*	*cyaA*	*ompQ*	*bapC*	*brkA*	*vag8*	*cyaApr**
1920	1	1	1	1	1	1	1	1	1	1
1941	4	4	4	1	1	1	1	1	1	1
1942	2	2	2	1	1	1				1
1943	1	1	1	1	1	1				1
1944	2	2	2	1	1	1				1
1946	1	1	1	1	1	1				1
1947	1	1	1	1	1	1				1
1948	2	2	2	1	1	1				1
1949	2	2	2	1	1	1				1
1950	2	2	2	1	1	1	1	1	1	1
1954	2	2	2	1	1	1				1
1956	3	3	3	1	1	1				1
1963	2	2	2	1	1	1				1
1964	1	1	1	1	1	1	1	1	1	1
1966	1	1	1	1	1	1				1
1967	1	1	1	1	1	1				1
1977	4	4	4	1	1	1				1
1978	2	2	2	1	1	1	1	1	1	1
1979	2	2	2	1	1	1				1
1982	6	6	6	1	1	1				1
1983	7	7	7	2	2	2	1	1	1	2
1984	2	2	2	1	1	1				1
1985	2	2	2	1	1	1				1
1998	24	21	21	18	6	6	1	1	1	6
1999	40	30	30	26	8	8	2	2	2	12
2002	35	34	34	12	4	4				4
Total no. of isolates	138	138	138	80	42	42	9	9	9	46

* adenylate cyclase toxin promoter region.

poured on top. For liquid culture, *B. pertussis* was inoculated from BG plates into Stainer-Scholte (SS) liquid medium or Cyclodextrin Liquid (CL) medium (Appendices 1.2 and 1.3) and grown for three days (unless stated otherwise) at 37°C with shaking at 150-200 rpm. The volume of liquid medium inoculated varied, either a loopful of bacteria in 5 ml liquid medium in a universal or approximately 1.5 ml of bacterial culture to 250 ml of liquid medium in a 500 ml flask. Bacterial growth in liquid culture was used for growth curve analysis, preparation of urea extracts and for use in conductimetry analysis.

2.1.3 Storage of *Bordetella*

Long-term storage of *Bordetella* spp. was achieved by either (i) suspension of bacteria in 1% (v/v) Casamino acids (CAA) solution (Appendix 1.4) containing 20% (v/v) glycerol and storage at -70°C or (ii) suspension in nutrient broth with 15% (v/v) glycerol and storage on pre-autoclaved Embroidery beads (2mm, Ellis and Farrier Ltd) at -80°C. From some of the suspensions, bacterial pellets were obtained by centrifugation and stored at -80°C for subsequent DNA extraction. All media were sterilised by autoclaving at 15 p.s.i (121°C) for 15 min.

2.1.4 *Escherichia coli* strains

Escherichia coli strains (Table 2.1.4.1) used for maintenance and cloning of *lux* (operon encodes for luciferase and luciferin) and *gfp* (green fluorescent protein) reporter plasmids (section 2.15) are listed in Table 2.2.2.1. *E. coli* isolates were obtained from Stratagene and the Division of Infection and Immunity (DII), University of Glasgow.

2.1.5 Growth of *E. coli*

E. coli strains were grown on Luria Bertrani (LB) agar (Appendix 1.5) or on SGAL™/LB Agar Blend (Sigma), prepared according to manufacturer's instructions, if blue and white colony selection was required for identification of successfully transformed cells, with the appropriate antibiotics. The resulting white colonies potentially contained the plasmid with insert due to disruption of the plasmid-encoded β-galactosidase. Inoculated plates were incubated overnight at 37°C and inoculated LB broth (Appendix 1.5) was incubated overnight at 37°C with shaking at 150-200 rpm. All media were sterilised by autoclaving at 15 p.s.i (121°C) for 15 min.

Table 2.1.4.1. *E. coli* strains used

Strain	Genotype	Source
XL-1 Blue MRF' Supercompetent cells	Δ(*mrcA*)*183* Δ(*mcrCB-hsdSMR-mrr*) *173*, *endA1*, *supE44*, *thi-1*, *recA1*, *gyr96*, *relA1*, *lac* [F' *proAB* *lacI*^{q}*ZΔM15*, Tn*5* (Kan^{r})]	Stratagene
XL-2 Blue	*recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17*, *supE44*, *relA1*, *lac* [F' *proAB* *lacI*^{q}*ZΔM15* Tn*10*(Tet^{r}) Amy Cam^{r}]	Stratagene
SM10 λpir	*thi-1*, *thr*, *leu*, *tonA*, *lacY*, *supE*, *recA*::RP4-2-Tet::Mu, Kan^{r}, λpir	Division of Infection and Immunity

2.1.6 Storage of *E. coli*

Stocks of *E. coli* were prepared by adding 300 µl of 70% (v/v) glycerol (sterile-filtered) to 700 µl of an *E. coli* overnight culture in LB broth and stored at -80°C.

2.2 DNA EXTRACTION

2.2.1 Genomic DNA extraction

Genomic DNA for use as template in the polymerase chain reaction (PCR) was extracted from *Bordetella* spp. and *E. coli* using (i) an in-house (DII) boiling protocol or (ii) a commercial kit (Nucleon BACC2 genomic DNA extraction kit, Amersham Biosciences).

(i) A loopful of bacterial cells from a BG plate was suspended in 100 µl of Double Processed Tissue Culture Water (Sigma) in a 1.5 ml microfuge tube to produce a turbid suspension. The suspension was placed in a heating block set to 100°C and incubated for 10 min to allow lysis. Suspensions were placed at -20°C for 5 min to snap-freeze then allowed to thaw and these suspensions were centrifuged at 6,700 x *g* for 10 min (Biofuge pico, Heraeus). The resulting supernate (containing DNA template) was transferred to a fresh tube and stored at -20°C. A volume of 1 µl of this DNA solution was used as template in subsequent PCR reactions.

(ii) Genomic DNA was extracted with the Nucleon genomic DNA extraction kit BACC2 according to the manufacturer's instructions (Amersham Biosciences). A previously-prepared *B. pertussis* cell pellet was suspended in lysis solution, Reagent B. A volume of 2.5 µl of 50 µg ml^{-1} of RNase A was added to the suspension and this was incubated for 30 min at 37°C. The lysed suspension was de-proteinised using sodium perchlorate followed by the extraction of DNA using chloroform and Nucleon® resin. DNA was precipitated with ice-cold 95% ethanol and the resulting pellet was washed twice with ice-cold 70% ethanol. The pellet was resuspended in 50 µl of nuclease-free water (Promega) and the DNA concentration determined using a GeneQuant II spectrophotometer (Amersham Biosciences) at A_{260} and stored at -20°C. The amount of genomic DNA used as template in subsequent PCR reactions was 100 ng.

2.2.2 Plasmid purification

Plasmid DNA (pGEM®-T Easy (Promega), pSB395 and pPROBE-AT', Table 2.2.2.1) was extracted from *B. pertussis* and *E. coli* using the QIAprep® Spin Miniprep kit (Qiagen) according to the manufacturer's protocol. *E. coli* was grown at 37°C overnight in 5 ml of LB with 12.5 μg ml^{-1} of tetracycline or 150 μg ml^{-1} of ampicillin. *B. pertussis* was grown at 37°C for 3 days in 10 ml of SS and 12.5 μg ml^{-1} of tetracycline. Purity of *B. pertussis* cultures was ensured by using the Gram stain method. Bacterial cells were pelleted by centrifugation for 10 min at 4,000 x *g* (Megafuge 1.0, Heraeus). Pellets were resuspended in 250 μl of buffer P1. A volume of 250 μl of lysis solution, buffer P2, was added and the solution was mixed gently for a few sec. Buffer N3 (350 μl) was added to neutralise the reaction and the tube was inverted several times. Tubes were centrifuged for 10 min at 11,200 x *g* (Biofuge pico, Heraeus) and the resulting supernate was decanted into QIAprep columns which were then centrifuged to allow binding of DNA to the column. A volume of 500 μl of buffer PB was added to the column to remove any endonucleases. The second wash step, addition of 0.75 ml of buffer PE, was to remove any traces of salts and the column was centrifuged for one minute at 11,200 x *g*. The column was centrifuged for a further minute at the same speed to remove any further traces of buffer PE. The plasmid DNA was eluted by the addition of 50 μl of buffer EB and the DNA was stored at -20°C. Purified plasmid was not quantified throughout this study. Instead the intensity of the purified plasmid band on 1% (w/v) agarose gel gave an indication of how much plasmid was present and this determined the volume added in further assays.

2.3 Restriction Digests

Suitable restriction enzymes were selected to digest the plasmids pGEM®-T Easy (Promega), pSB395 and pPROBE-AT' (Table 2.2.2.1) as well as the insert *cyaA* promoter region, amplified with primers CyaA1/CyaA6 or CyaA1/CyaA8 (section 2.4). The program Webcutter 2.0 (www.firstmarket.com/cutter/cut2.html) was used to analyse the amplified *B. pertussis cyaA* promoter region for the presence of restriction sites, thus determining which enzymes would be suitable for the digestion of this region. Any restriction enzyme that digested this region was disregarded. Purified plasmid preparations isolated from *B. pertussis* and *E. coli* were digested using appropriate enzymes for use in ligation reactions or to isolate an insert that the plasmid already contained. The restriction enzymes used

Table 2.2.2.1. Plasmids used.

Plasmid	Comment	Source/Reference
pGEM®-T Easy	High copy number plasmid. Contains T7 and SP6 promoters flanking a multiple cloning region within the α-peptide coding region of β-galactosidase. An insertion within this region allows recombinant clones to be identified by blue/white screening. Amp^r	Promega
pSB395	broad host range mobilisable (*oriV*, *oriT*) medium copy number plasmid. Contains the promoterless *luxCDABE* operon. Tet^r	Provided by Dr M. Lynch, Winson *et al.*, 1998
pPROBE-AT'	broad host-range plasmid derived from the *B. bronchiseptica* plasmid pBBR1. Contains the promoterless *gfp* gene. Amp^r	Provided by Dr M. Lynch, Miller *et al.*, 2000

during this study were *Kpn*I, *Sac*I, *Pst*I and *Eco*RI (Promega). The volume of purified plasmid added was 10 µl, with 3 µl of buffer H (90 mM Tris-HCl, 10 mM magnesium chloride, 50 mM sodium chloride, used with *Pst*I and *Eco*RI) or buffer J (10 mM Tris-HCl, 7 mM magnesium chloride, 50 mM potassium chloride and 1 mM dithiothreitol, used with *Kpn*I and *Sac*I) and 1 µl (10 Units (U)) of appropriate restriction enzyme to a total reaction volume of 30 µl. The digest reaction was incubated at 37°C overnight. To ensure that digestion had been successful the reactions were analysed by electrophoresis on a 1% (w/v) agarose gel with a 1 kb ladder (Invitrogen Ltd) (section 2.6).

2.4 Primer design

Oligonucleotide primers were designed to target eight genes: *ptxC*, *tcfA*, *fim2*, *vag8*, *ompQ*, *cyaA*, *bapC* and *brkA*. GenBank sequences were analysed for suitable sequences to be selected for use as primers for PCR amplification and sequencing of (i) a small region of polymorphism (*ptxC*, *tcfA*, *fim2*, *vag8* and *ompQ*), (ii) a large section of a gene for investigation of polymorphism (*cyaA*) or (iii) a complete open reading frame (*bapC* and *brkA*). Once primers were selected they were analysed using the internet program NetPrimer (http://www.premierbiosoft.com), thus ensuring that the selected primer sequence did not contain any potential secondary structures such as hairpins, cross-dimers and self-dimers. NetPrimer also calculated the melting temperature of each designed primer. Primers (50 nmol and desalted) were obtained from Invitrogen Ltd. The primers were resuspended in 10 mM Tris–HCl, 1 mM EDTA (TE) pH 8.0 buffer (Sigma) to give a stock concentration of 1 µg μl^{-1}. Table 2.4.1 shows the primers used in this study. Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990; http://www.ncbi.nlm.nih.gov) was used to check that the primer sequences would target the desired *B. pertussis* gene sequence. Using the BLAST option available through the Sanger website (http://www.sanger.ac.uk), it was possible to check that primer sequences were unique for the appropriate target and would not amplify any other *B. pertussis* sequences. BLAST was also used to check for potential nucleotide variation of the adenylate cyclase toxin gene (*cyaA*) between *B. pertussis* strains, as the available genome sequence is of Tohama-I and the GenBank sequence is of the atypical *B. pertussis* strain 18323^T ($NCTC10739^T$).

Table 2.4.1. Oligonucleotides designed for this study for amplification and sequencing of the target genes of *B. pertussis*. Sequences that are highlighted in red indicate restriction sites that have been engineered into the primer sequences: *Kpn*I (GGTACC); *Hin*dIII (AAGCTT) and *Bgl*II (AGATCT). The position of the primer is according to the numbering of the sequences of the following GenBank accession numbers: *cyaA*, Y00545; *tcfA*, U16754; *vag8*, U90124; *ptxC*, M13223; *brkA*, U12276; *bapC*, AF081494; *ompQ*, U16266; *fim2*, Y00527 and *luxC* AF403784.

Primer name	Sequence (5'-3')	Gene	Position	Source of information
CyaA1F	TCGTGGGCAAGCAGGACCGC	*cyaA*	3586-3605	E. Packard
CyaA1R	GCCGCCAACCAGGGTGTCGT	*cyaA*	4136-4117	E. Packard
CyaA2F	ACGACACCCTGGTTGGCGGC	*cyaA*	4117-4136	E. Packard
CyaA2R	CCTGGATGGATCATGGCGGA	*cyaA*	4636-4617	E. Packard
CyaA3F	TCCGCCATGATCCATCCAGG	*cyaA*	4617-4636	E. Packard
CyaA3R	CACCACGTTCTCGATACCGG	*cyaA*	5186-5167	E. Packard
CyaA4F	CCGGTATCGAGAACGTGGTG	*cyaA*	5167-5186	E. Packard
CyaA4R	TGCCCAAGCTCAGGAATACG	*cyaA*	5496-5477	E. Packard
CyaA5F	CGTATTCCTGAGCTTGGGCA	*cyaA*	5477-5496	E. Packard
CyaA5R	GCGCCAGTTGACAGCCAGGG	*cyaA*	6098-6079	E. Packard
CyaA1	GGTACCGGCGAAGCTTAGGGCC CAGCTGCA	*cyaA*	287-303	M. Lynch
CyaA8	CTCGAGATCTACGCCATCGGCC	*cyaA*	1396-1376	M. Lynch
CyaA6	ACGCCCAAGATCTTTGGTGGC	*cyaA*	1165-1146	M. Lynch
LUXCr	GGATTGCACTAAATCATCACTTT CG	*luxC*	1007-983	E. Packard
CyaApF	CATCATGGTTGCGCCGGAAT	*cyaA*	570-589	E. Packard
CyaApR	GATTGAACGGATTCGCACCG	*cyaA*	958-939	E. Packard
CyaPins-S	CGATCGCGATCGCGTTCG(FLUO)	*cyaA*	685-696	E. Packard
CyaP-A	(RED640)GCTTGCTCGCTTATTTA TCTCCCTTGAAG(PHOSPHATE)	*cyaA*	698-726	E. Packard
PtxS3F	CAGCCCGTATGAAGGCAGGT	*ptxC*	3433-3452	E. Packard
PtxAJK8 S3R	TCTTTCAAGGGATTCATTCGCG	*ptxC*	3700-3679	F. Mooi
PtxS3-3270F	TTTATCGCGAAACTTTCT	*ptxC*	3270-3287	N. Fry
PtxS3-3658R	biotin-TGGACAGGCGAACAG	*ptxC*	3658-3644	N. Fry
PtxS3-3610FS	GGCAGCGTCGATATG	*ptxC*	3610-3624	N. Fry
Fim2-632F	biotin-TGGGTGCGAACGAGGCGA	*fim2*	632-649	N. Fry
Fim2-900R	CCGGCCGGGCTCCTTGAG	*fim2*	900-885	N. Fry
Fim2-720RS	GTAGCGCATCGTGAC	*fim2*	720-706	N. Fry
OmpQ-1377F	GCCTATGTCGTGACGCTG	*ompQ*	1377-1394	N. Fry.
OmpQ-1533R	biotin-AGAAGCGCTGGGTCA	*ompQ*	1533-1519	N. Fry

OmpQ-1444FS	GGTATATGAAAGGCTACGA	*ompQ*	1444-1462	N. Fry
TcfAF1	ACGCCAGCCTGCCAAGACG	*tcfA*	4-22	E. Packard
TcfAR1	GGCTGCGCTTGAAATCCTCC	*tcfA*	529-510	E. Packard
TcfAF	TTCTTGCGCGTCGTGTCTTC	*tcfA*	270–289	F. Mooi
TcfAR4	TTCGGAGTTTTCGTCATTGCG	*tcfA*	813–792	F. Mooi
TcfAF4	CTCCGGTTGCGAAGCCAGGT	*tcfA*	577-597	E. Packard
TcfAR5	GATTCAAGCCTCCAGCCGAC	*tcfA*	1032-1014	E. Packard
TcfAF2	TCGTCTGGCGGACATACCCC	*tcfA*	688–707	F. Mooi
TcfAR3	GCGGTTGCGGACCTTCAT	*tcfA*	1339–1322	F. Mooi
TcfAF3	AAACGGTGGCAACGGTGG	*tcfA*	1233-1250	F. Mooi
TcfAR2	CCTTGGCGTCGTCCACCT	*tcfA*	1867-1850	F. Mooi
TcfAM	GCGGTGGCTGGCTACACC	*tcfA*	1693-1710	F. Mooi
TcfAR6	TACCAGGCGTAGCGATAGC	*tcfA*	2345-2327	E. Packard
BapC1F	GATTCGGTCCTGCGTGGTTC	*bapC*	219-238	E. Packard
BapC1R	ACGGCCCCATCCTCTACGCT	*bapC*	748-729	E. Packard
BapC2F	TCAATGGCGAGGCGAACATC	*bapC*	694-713	E. Packard
BapC2R	TCATCTCGAACAGGCCGCTT	*bapC*	1371-1352	E. Packard
BapC3F	TGCCGAAAGCGACGGAGAAT	*bapC*	1301-1320	E. Packard
BapC3R	GTCGAGCTGCTGCTTCTGGG	*bapC*	1790-1771	E. Packard
BapC4F	TGGTATGCGGAAGGCAATGC	*bapC*	1683-1702	E. Packard
BapC4R	AGGTGCCGCCTTCGTCCTTG	*bapC*	2244-2225	E. Packard
BapC5F	ACGTTGCACGACGGCTGGT	*bapC*	2130-2149	E. Packard
BapC5R	ATGCGCCGCTACCAGGTG	*bapC*	2548-2531	E. Packard
92KF	GATTGATATCGAGGCGGCCAAG	*vag8*	3-24	F. Mooi
92KR4	AACCACCAGCCCCTGTGC	*vag8*	684-667	F. Mooi
92KF7	AGGAGGAGGCACGCGTTTG	*vag8*	495-513	F. Mooi
92KR2	TACTCTCGCCGTCGACCTCC	*vag8*	1249-1230	F. Mooi
92KF5	ATCGATATCGATGGCGGCTG	*vag8*	2425-2444	F. Mooi
92KR	TTCACCAGCTGTAGCGATACCC	*vag8*	2838-2817	F. Mooi
BrkAF	GTCGTTTCCTTCGCGTCACC	*brkA*	1139-1158	F. Mooi
BrkAR5	ATCGGCTCCATCCCTTCCCC	*brkA*	1709-1690	E. Packard
BrkAF2	CCATCGATGCCACCGAGG	*brkA*	1607-1624	F. Mooi
BrkAR4	TCGACACCGTGGTTCCGTCC	*brkA*	2221-2202	E. Packard
BrkAF7	ACAGTCAGCGTGCAGGGCGA	*brkA*	2113-2132	E. Packard
BrkAM2	AATGGTGGAATGCTGCGGAG	*brkA*	2766-2746	F. Mooi
BrkAM1	GACACGCCGCTGAAGCTGAT	*brkA*	2647-2666	F. Mooi
BrkAR3	ATCCTCCGCCAGGCTGTAGC	*brkA*	3291-3272	E. Packard
BrkAF3	CATACCCAGGGGCAGGGC	*brkA*	3199-3216	F. Mooi
BrkAR1	GTGTCGAGATAGTAGCCGCCAT	*brkA*	3710-3689	E. Packard
BrkAF6	CCTGCTCGGCTACACCTATGC	*brkA*	3591-3611	E. Packard
BrkAR6	CCTGGCGGGGTTTTTCATTG	*brkA*	4270-4251	E. Packard

Oligonucleotide primers to target a 2512 bp C-terminal region of the *cyaA* gene encoding the immunodominant moiety (Betsou *et al.*, 1995a) were designed from the available sequence (Y00545) described by Glaser *et al.* 1988. Five primer pairs targeting bases 3586-6098 (Table 2.4.1) were used to amplify this region (regions I-IV, Fig. 2.4.1).

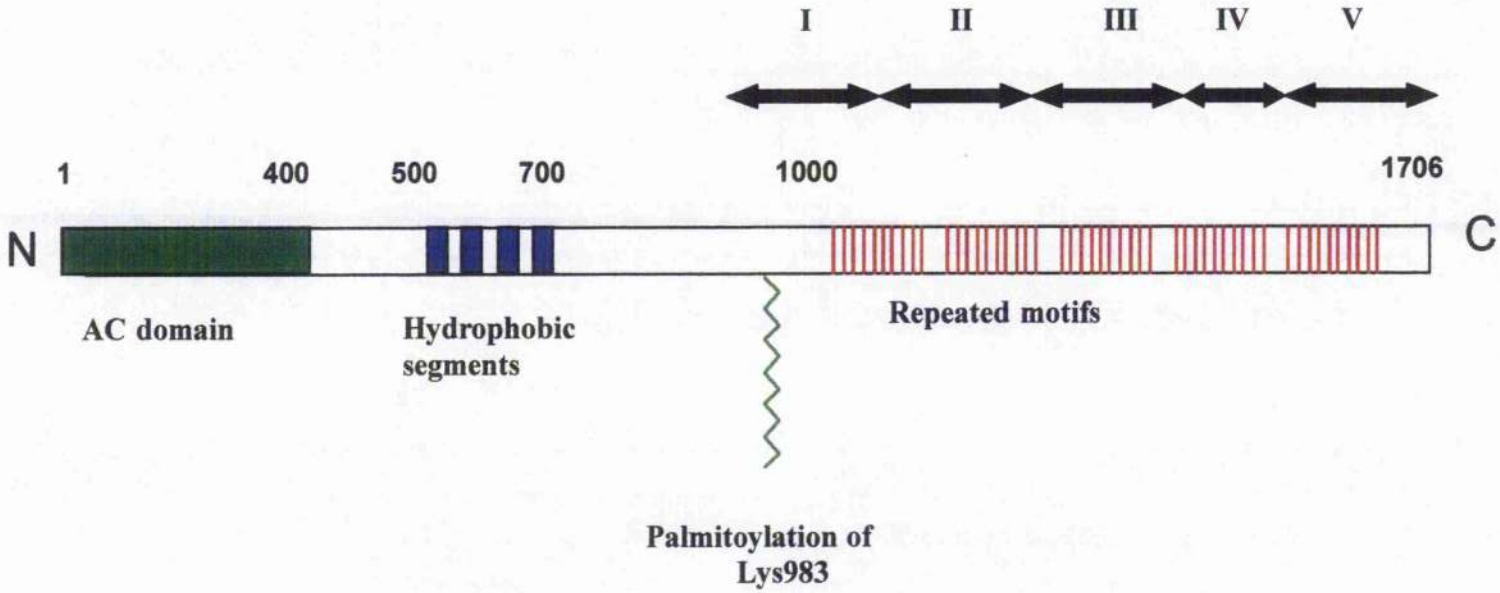
A section of the *cyaA* gene including the promoter region used to study gene expression with a *lux* reporter for bioluminescence studies was amplified with primers CyaA1 and CyaA6/CyaA8 targeting bases 287-1165/1396 (Y00545) (Table 2.4.1). Primers, CyaApF and CyaApR, were later designed to target polymorphisms found within the region 570-958 previously determined from sequencing data of the *cyaA* promoter.

The complete open reading frame was sequenced for *bapC* (2280 bp in total) with primers BapC1F and BapC5R targeting bases 219 – 2548 (AF081494). The primers (BrkAF and BrkAR6) targeted nucleotides 1139-4270 (U12276) (van Loo *et al.*, 2002; this study) amplifying 3033 bp of the *brkA* gene in total (Table 2.4.1). Three described polymorphic sites of *vag8* were targeted by the following primers 92KF and 92KR4, 92KF7 and 92KR2, 92KF5 and 92KR, and the primers used are those previously described (van Loo *et al.*, 2002). The *ptxC* primers (PtxS3F and PtxAJK8S3R) targeted bases 3433-3700 (van Loo *et al.*, 2002; this study).

Primers TcfAF1 and TcfAR6 targeted bases 3-2345 of *tcfA* (U16754). The primers TcfAR3 and TcfAF2 were used to amplify a region, which was previously determined to be polymorphic (van Loo *et al.*, 2002). Primers TcfAF4 and TcfAR5 were designed to clarify some *tcfA* sequences queried as *tcfA*(2) or *tcfA*(5) alleles, and bases 578-1032 were amplified. The open reading frame of *tcfA*, bases 3-2345, was amplified for two *B. pertussis* isolates that were designated *tcfA*(6), to check for further polymorphisms and for submission of the sequence to GenBank.

Primers for amplification and sequencing of *ptxC*, *fim2* and *ompQ* for Pyrosequencing (see section 2.10 for further details) were designed by N. Fry using the program OLIGO plus Pyrosequencing software (Table 2.4.1). Primers were ordered from MWG-Biotech AG and resuspended in TE buffer pH 8.0 (Sigma) to give a stock concentration of 100 pmoles μl^{-1} and then stored at -20°C. One of each set of the primers was biotinylated at the 5'-end.

Figure 2.4.1: Schematic of the *B. pertussis* adenylate cyclase toxin (CyaA), showing the enzymic (AC) domain (amino acids 1-400), the hydrophobic membrane spanning domain, the site of post-translational modification and the 42 calcium-binding nonapeptide repeats. Numbering represents amino acid residues. Arrows indicate the regions (I-V) of CyaA corresponding to the regions of the *cyaA* gene amplified by the five primer pairs (CyaA1F-CyaA5R, Table 2.4.1) (adapted from Ladant & Ullmann, 1999).


I
II
III
IV
V
1
400
500
700
1000
1706
N
C
AC domain
Hydrophobic segments
Repeated motifs
Palmitoylation of Lys983

Primers ptxS3-3270F and biotin-ptxS3-3658R targeting bases 3270-3658 (M13223) were used to amplify *ptxC* for analysis by Pyrosequencing. The sequencing primer of the amplified *ptxC* product was ptxS3-3610FS. The *fim2* primers (biotin-fim2-632F and fim2-900R) targeted bases 632-900 (Y00527) and the sequencing primer, fim2-720RS, was used. Outer-membrane protein Q primers (ompQ-1377F and biotin-ompQ-1533R) were used for amplification, targeting bases 1377-1533 (U16266) and ompQ-1444FS was used as the sequencing primer.

2.5 POLYMERASE CHAIN REACTION (PCR)

All PCR reaction mixtures contained 1.5 mM $MgCl_2$ and 200 µM of each deoxynucleotide in a final volume of 50 µl. In addition, mixtures designed to amplify *cyaA* and *tcfA* contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.0 µM of each primer, (except for *cya*-2F and -2R, 0.1 µM), 5% (v/v) dimethyl sulphoxide (Sigma) and 1 U of *Taq* DNA polymerase (Invitrogen). Other target reaction mixtures contained HotStarTaq™ DNA polymerase with Q-solution (Qiagen), and 1.0 µM of each primer (Invitrogen), except for *bapC*-5F and -5R (0.1 µM). Template DNA (ca. 100 ng or 1 µl of boiled extract) was added and reaction mixtures containing no added DNA served as negative controls. Triplicate reactions were prepared for products that were to be sequenced by the dideoxy method. Amplification was performed using a DNA Engine (MJ Research) with the following conditions: predenaturation for 10 min at 94°C, then 30 cycles of denaturation for 2 min at 94°C, annealing for 2 min at 60°C, extension for 1 min at 72°C and a final extension step of 10 min at 72°C.

2.6 AGAROSE GEL ELECTROPHORESIS

Agarose gels (2%, w/v) were used for electrophoretic analysis of PCR product sizes predicted to be between 156 and 762 bp, 1% (w/v) agarose gels were used for analysis of digested products. PCR product (5 µl) was mixed with 5 x Tris-borate-EDTA (TBE, 1 x TBE contains 89 mM Tris, 89 mM Boric acid and 0.2 mM EDTA) DNA loading buffer (Appendix 2.1) in a v:v ratio of 1:1 prior to loading into the wells of agarose gels made up in TBE buffer. Volumes ranging from 10 µl – 15 µl of digested vector was applied to the gel. A volume of 30 µl of digested vector containing insert was applied and aliquoted between two wells due to the volume being too large for one well. Molecular weight

markers (100 bp and 1 kb ladders, Invitrogen) were used according to the manufacturer's instructions, 0.1 µl of desired marker per mm of lane width (0.5 µg) was added to loading buffer. Agarose (type II-A medium EEO, Sigma or Gibco Low-Grade) was dissolved in 1 x TBE buffer (Appendix 2.2). Gel preparation and electrophoresis was done in one of two ways (i) the solution was allowed to cool (approx 50°C) and ethidium bromide (Sigma) was added to a final concentration of 1 µg ml^{-1}. Once the gel had set, it was immersed in a tank containing 1 x TBE buffer. Samples were applied and electrophorised at a voltage of 100V until the marker dye had migrated an appropriate distance to enable visualisation of the DNA. (ii) The gel was prepared without the addition of ethidium bromide. Once the products had migrated a reasonable distance on the gel, it was stained in 1 x TBE containing 1 µg ml^{-1} ethidium bromide for approx 20 min. After electrophoresis DNA was visualised either by (i) being placed on a Dual Intensity Transilluminator and viewed using a UVP Gel Documentation System ImageStore 5000 Version 7.12 (Ultra Violet Products Ltd, Cambridge) or (ii) viewed using a transilluminator with the Polaroid MP-4 system and black and white 667 film (Polaroid).

2.7 EXTRACTION OF DNA FROM AGAROSE GEL

Once amplified plasmid DNA containing the CyaA1-6/1-8 insert (amplified with primers CyaA1 and CyaA6/8) had been purified and digested with appropriate restriction enzymes, the restriction fragments were visualised using gel electrophoresis. The digested insert was isolated and purified using the QIAquick® gel extraction kit (Qiagen) following the protocol described for gel extraction using a microcentrifuge. Plasmid DNA that had been digested was run in triplicate on a 1% agarose gel. The digested inserts were excised from the gel and pooled together in sterile 1.5 ml microfuge tubes. Gel slices in the microfuge tubes were weighed and three volumes of Buffer QG to 1 volume of gel (assuming 100 mg=100 µl) were added. Tubes were incubated at 50°C for 10 min in a heating block. Every 2 min, the tubes were vortexed to aid gel fragments to dissolve. One gel volume of isopropanol was added to the sample and this mixture was added to a QIAquick spin column. Columns were centrifuged for 1 min at 11,200 x *g* (13,000 rpm) (Biofuge pico, Heraeus). To remove any remaining traces of agarose 0.5 ml of buffer QG was added and the column was centrifuged for 1 min. A volume of 750 µl of Buffer PE was added and the column was centrifuged as before, the flow-through was discarded and the column was

spun for a further minute to remove traces of Buffer PE. DNA was eluted with 50 µl of Buffer EB and stored at -20°C.

2.8 DIGESTED PLASMID AND PCR PRODUCT PURIFICATION

Digested plasmid for use in ligation reactions was purified using the QIAquick® gel extraction kit (Qiagen) and amplified PCR products were purified using (i) QIAquick® gel extraction kit (Qiagen) or (ii) the Montage PCR_{96} Filter Plates (Millipore).

(i) Digested plasmid or PCR products were purified using QIAquick® gel extraction kit according to the manufacturer's protocol for PCR product purification using a microcentrifuge. The binding buffer, buffer PB, was added to PCR products at a 5:1 (v:v) ratio. This mix was placed in a QIAquick column and centrifuged for 1 min at 11,200 x g (Biofuge pico, Heraeus). A volume of 750 µl buffer PE was added to the column and centrifuged for 1 min at 11,200 x *g*. The flow-through was discarded and the column was centrifuged for a further minute to remove traces of the buffer. The column was placed in a 1.5 ml microfuge tube and the DNA was eluted by adding 30 µl of buffer EB and centrifuged for one minute. The purified PCR products and digested plasmid were stored at -20°C.

(ii) PCR products were also purified using Montage PCR_{96} Filter Plates (Millipore) according to manufacturer's instructions using a MultiScreen Vacuum Manifold (Millipore). Triplicate PCR reactions were pooled and added to each well. The plate was placed under vacuum pressure 20 p.s.i for 10 min. A volume of 50 µl of nuclease-free water (Promega) was added to the wells and placed on a rotary shaker for 20 min. DNA was quantified using a GeneQuant II spectrophotometer (Amersham Biosciences) at A_{260} and stored at -20°C. Template DNA (100 ng) was used in sequencing reactions.

2.9 SEQUENCING BY THE DIDEOXY METHOD

Sequence polymorphism was investigated in regions of eight genes (*ptxC*, *tcfA*, *fim2*, *cyaA*, *vag8*, *ompQ*, *bapC* and *brkA*) using various subsets of the 152 *B. pertussis* isolates. Sequences for all of the eight gene targets were determined for the four vaccine strains and

the type strain (18323^T). The four vaccine strains and the type strain were included in this study for comparative purposes.

2.9.1 Sequencing PCR reactions

Sequencing of *brkA*, *ptxC*, *tcfA* and *vag8* was as described by van Loo *et al.* (2002), with minor modifications (this study). The two genes *bapC* and *cyaA* were sequenced as described in this study. Nucleotide sequences determined by the dideoxynucleotide method used the Dye Terminator Cycle Sequencing Quick Start Kit (Beckman Coulter) and the products analysed on a CEQ 8000 Genetic Analysis System (Beckman Coulter). Sequencing PCR reactions were carried out according to manufacturer's recommendations. The reaction mixture contained 8 µl of Dye Terminator Cycle Sequencing Quick Start Kit master mix (Beckman Coulter), 100 ng of purified DNA and a final concentration for each primer of 3.2 µM in a total volume of 20 µl. The control DNA template, pUC18, was included for each set of sequencing reactions (supplied with Dye Terminator Cycle Sequencing Quick Start kit). The primers used were the same as those used for the original PCR reaction (see Table 2.4.1). The sequencing PCR parameters were 30 cycles of 96°C for 20 sec, 50°C for 20 sec and 60°C for 4 min followed by holding at 4°C until products were taken for ethanol precipitation.

2.9.2 Ethanol precipitation

Ethanol precipitation of the sequencing PCR products was carried out according to the manufacturer's instructions, either in individual 1.5 ml microfuge tubes or in a 96-well plate depending on the number of reactions to be sequenced. For plate and tube methods, 5 µl of stop solution (1.5 M NaOAc pH 5.2, 50 mM Na_2EDTA pH 8.0, 4 µg glycogen) was added to each reaction followed by 60 µl of 95% ice-cold ethanol. For the tube protocol, samples were centrifuged for 15 min at 19,280 x *g* (Allegra 21 R Centrifuge and rotor F3602, Beckman Coulter). Plates were inverted several times to mix the sequencing reactions with stop solution and ethanol, incubated at -20°C for 10 min, then centrifuged for 30 min at 1238 x *g* (rotor S2096, Beckman Coulter). The 95% ethanol was carefully removed and the pellets washed twice with 200 µl of 70% ice-cold ethanol. For the tube method, samples were centrifuged for 2 min at 19,280 x *g* and the plate was centrifuged for 5 min at 1238 x *g*. All centrifugations were carried out at 4°C. Samples were vacuum dried

for 20 min and resuspended in 35 µl of sample loading solution (deionised formamide, supplied with Dye Terminator Cycle Sequencing Quick Start Kit, Beckman Coulter).

2.9.3 Sequence analysis

Sequence analyses were performed using software packages BioNumerics and Kodon (Applied Maths, Kortrijk, Belgium) and the program BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). For all analyses, sequence data from forward and reverse primers were combined and aligned manually. New sequence data were aligned with the available sequence(s) for *B. pertussis* from GenBank, *cyaA* Y00545, *tcfA*(1) U16754, *tcfA*(2) AJ009785, *tcfA*(3) AJ420991, *tcfA*(4) AJ507643, *tcfA*(5) AJ420992, *brkA* U12776, *vag8*(1) U90124 and *bap5*(1) AF081494.

2.9.4 Gene nomenclature and numbering

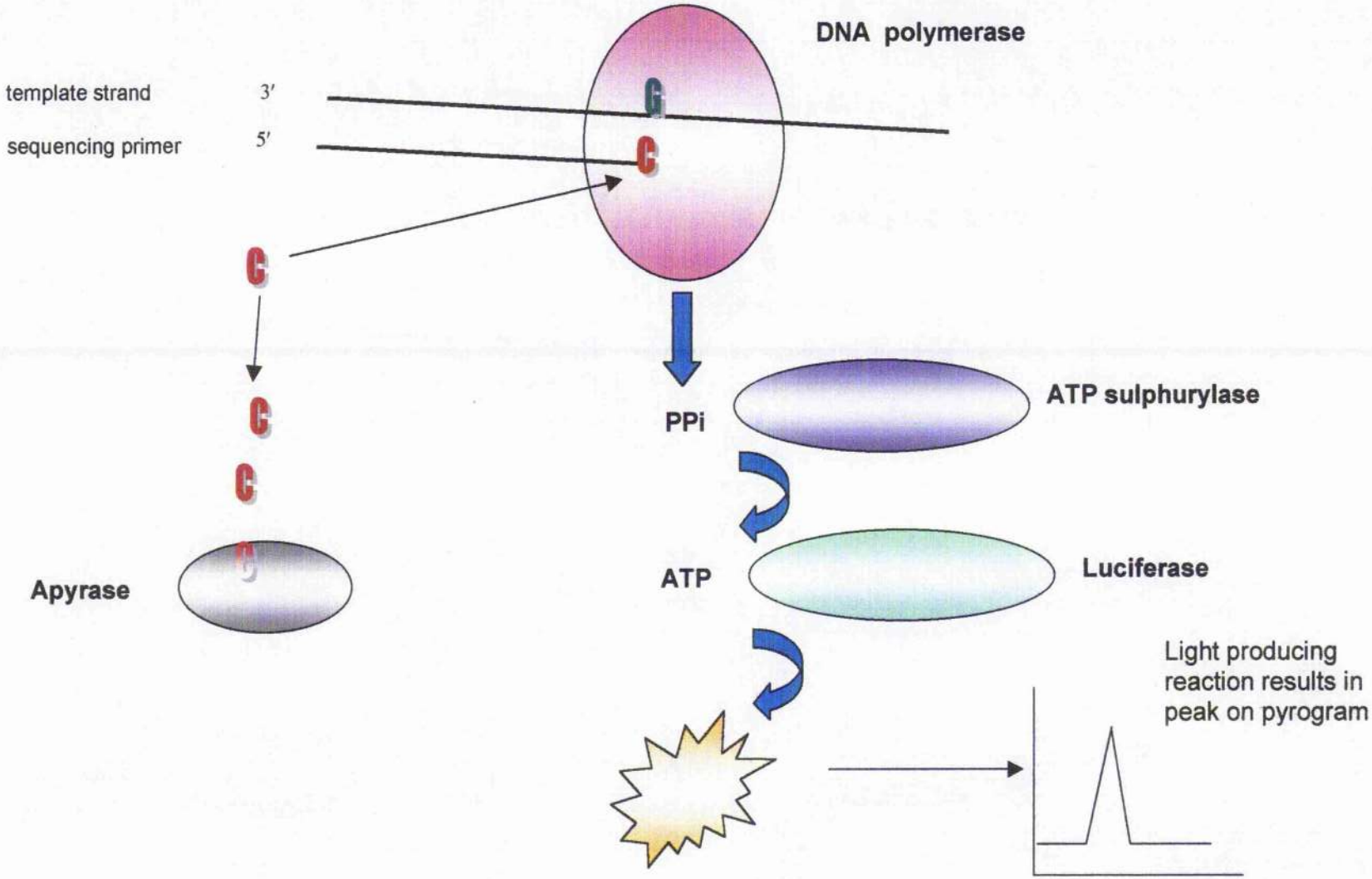
The description of the pertactin and pertussis toxin S1 gene variants, first described by Mooi and colleagues (Mooi *et al.*, 1998; Mooi *et al.*, 1999; Mooi *et al.*, 2000), is as defined by Fry *et al.* (2001). Other gene variants described by van Loo *et al.* (2002), or novel variants found in this study, follow the format of Fry *et al.* (2001), i.e., gene designation followed by allele number in parenthesis. Throughout this study, the location of numbered positions on the genes is with respect to the GenBank reference sequence indicated.

2.10 PYROSEQUENCER

2.10.1 Pyrosequencer technology

For single nucleotide polymorphism (SNP) analysis, pyrosequencing standard protocols (Pyrosequencing AB) were used. This method is based on the technology originally described by Ronaghi *et al.* (1998) and is described in Pyrosequencing technical note 101. The sequencing primer was hybridised to single stranded DNA template, which was incubated with a mixture of four enzymes: DNA polymerase, ATP sulphurylase, luciferase and apyrase, and the substrates, adenosine 5' phosphosulphate (APS) and luciferin (Fig. 2.10.1.1). The first of the four dNTPs was incorporated into the DNA strand by the action of DNA polymerase if it was complementary to the base in the template strand. This incorporation was accompanied by the release of pyrophosphate (PPi). ATP sulphurylase

Figure 2.10.1: Pyrosequencing reaction containing the four-enzyme (DNA polymerase, ATP sulphurylase, luciferase and apyrase) mixture with substrates (ATP and luciferin) and sequencing primer annealed to template DNA. Once a complementary nucleotide to the template strand is incorporated, the reaction shown on the opposite page takes place resulting in the production of light. This light results in a peak on the pyrogram, (source: Pyrosequencing technical information) and sequence information is obtained from the peaks on pyrograms.


DNA polymerase
template strand
3′
sequencing primer
5′
G
C
G
C
C
G
Apyrase
PPi
ATP sulphurylase
ATP
Luciferase
Light producing reaction results in peak on pyrogram

converts PPi to ATP in the presence of the substrate APS, the ATP drives the conversion of luciferin to oxyluciferin by the enzymic action of luciferase, and generates visible light in amounts that are proportional to the amount of ATP. This light is detected by a charge coupled device (CCD) camera and is visualised as a peak in a Pyrogram (Fig. 2.10.1.1). The height of each peak in the Pyrogram is proportional to the number of nucleotides incorporated. The enzyme apyrase continuously degrades the ATP and unincorporated dNTPs (Fig. 2.10.1.1). This switches the light off and regenerates the reaction solution, before the next dNTP is added.

Three gene targets (*fim2*, *ompQ* and *ptxC*) had previously been investigated by van Loo *et al.* (2002) for polymorphic regions. Each gene contained a single nucleotide polymorphism defining two alleles. The single nucleotide polymorphism of *ptxC* was a target for the MLST scheme and this polymorphism was investigated for the UK *B. pertussis* isolates. Neither *ompQ* nor *fim2* were included in the MLST scheme due to limited variation. However, for this study it was informative to investigate if the same *ompQ* and *fim2* alleles occurred in the UK *B. pertussis* population at similar or different frequencies. As the alleles and the position of the polymorphisms were known, the real-time sequencing technique, Pyrosequencing was applied to detect the allelic frequency of the three genes within the UK *B. pertussis* population as this method is much more rapid than conventional dideoxy sequencing. Three *B. pertussis* isolates, CN137, NCTC10739^T and BP711 were selected for the initial investigation of Pyrosequencer technology to determine whether the two known *ptxC* alleles present amongst these three isolates would be detected. It was found that the two sequencing techniques were completely concordant and Pyrosequencing was then used to determine the allelic frequency of these three genes in further *B. pertussis* isolates.

2.10.2 Preparation of Pyrosequencer reactions

Single PCR reactions were prepared in place of triplicate reactions required for conventional sequencing. Target reaction mixtures contained HotStarTaq™ DNA polymerase with Q-solution (Qiagen), and 10 pmoles of each primer (MWG-Biotech AG) in a final reaction volume of 50 µl. Template DNA (ca. 100 ng) was added and reaction mixtures containing no added DNA served as negative controls. Polymerase chain reactions for *ptxC*, *ompQ* and *fim2* were optimised by comparing different annealing temperatures of 50°C, 55°C and 58°C. The optimal temperatures were 50°C for *ptxC* and

55°C for *ompQ*. For *fim2* amplification, an annealing temperature of 58°C was used. Amplification was performed using a DNA Engine (MJ Research) with the following conditions: predenaturation for 10 min at 94°C, then 30 cycles of denaturation for 2 min at 94°C, annealing for 2 min at 50-58°C, extension for 1 min at 72°C and a final extension step of 10 min at 72°C.

Typically, 20 µl of biotinylated PCR product from a 50 µl PCR reaction was immobilised onto streptavidin-coated beads and converted to single-stranded DNA template by denaturation. The biotinylated PCR product is a result of amplification with one of the primer pair being labelled with biotin and biotin is known to bind to streptavidin. After washing, removal of the unmodified strand (amplified by the non-biotinylated primer) and neutralisation, the sequencing primer (0.35 µM final concentration) was annealed to the template. Samples were incubated at 80°C for 2 min, allowed to cool to room temperature and analysed using the PSQ™ 96MA System (Pyrosequencing AB, Uppsala, Sweden). Results were analysed using the Pyrosequencer software and Appendix 9 shows examples of Pyrograms for the three targets.

2.11 LIGHTCYCLER ANALYSIS

2.11.1 Target for LightCycler melting curve analysis

During this PhD study, a 6 base pair insertion was identified between the two genes *cyaA* and *cyaC* after sequencing in two *B. pertussis* isolates, 99K45 and PICU475. The initial sequencing of *B. pertussis* isolates for *cyaA* promoter variation was carried out in DBS Genomics, Durham University. Forty-six isolates, four vaccine strains and the type strain were screened for the presence of this 6 bp insertion using melting curve analysis of the LightCycler (LC) (Roche) assays. The LC assay combines real-time PCR with product detection using fluorogenic hybridisation probes that use fluorescence resonance energy transfer (FRET) to achieve rapid PCR results. One feature of using this assay is the capacity to determine the melting point analysis of PCR products, which allows the differentiation of distinct amplified sequences.

2.11.2 LightCycler Assays

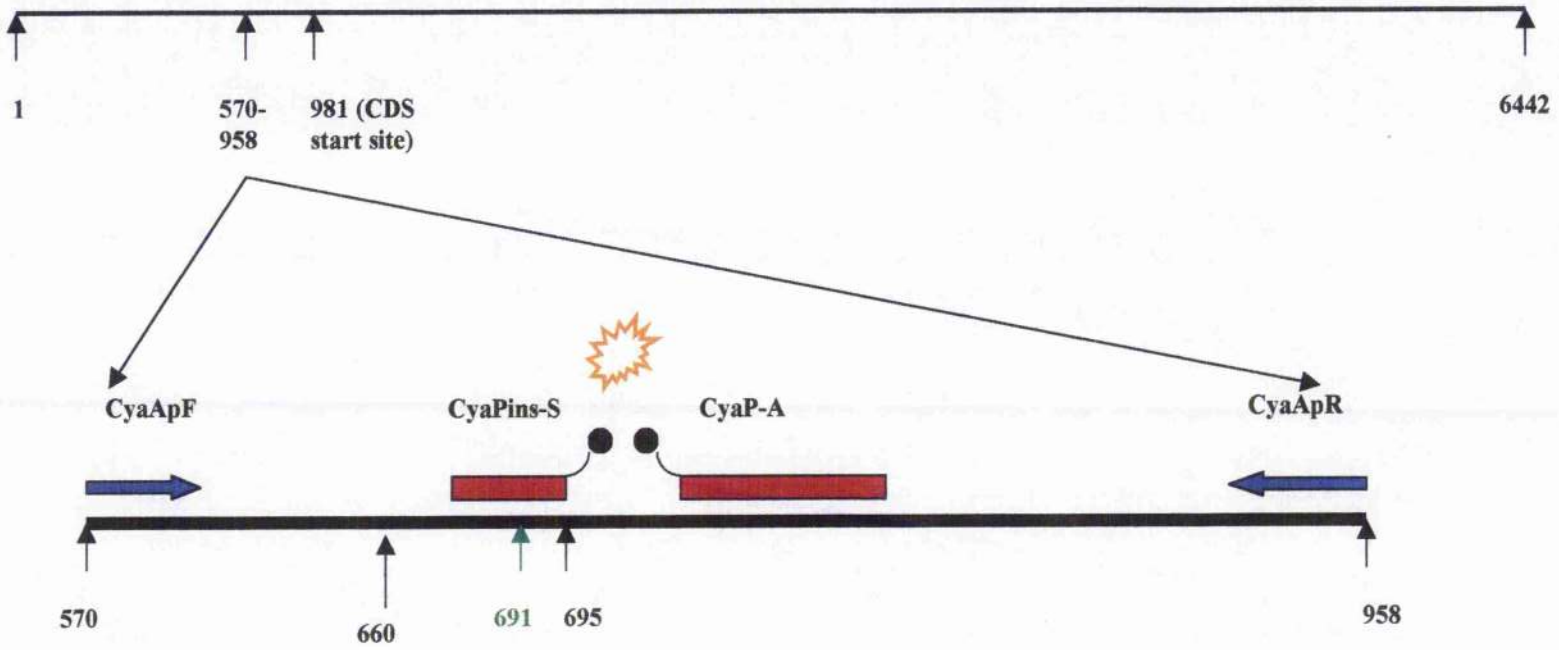
The primers, CyaApR and CyaApF, were used for amplification of a 388 bp section of *cyaA* in the LC assay. Suitable probe sequences that are fluorescently labelled were

designed from the GenBank sequence Y00545. Probes were designed to be situated on the same strand and as far from the extending primer as possible, which delays the displacement of the probe by polymerase allowing maximum hybridisation time. The spacing between the fluorophores must be ideally be between one to four bases as the proximity of the fluorophores allows FRET to be monitored by the LightCycler. The probes used here only had one base between them (CyaPins-S: 683-696, CyaP-A: 698-726). The long anchor probe (CyaP-A) desgined to detect the wild-type sequence is labelled at the 5' end with LightCycler Red 640 and the sensor probe (CyaPins-S) designed to target the insertion sequence and the single nucleotide difference between the type strain and the other *B. pertussis* isolates is labelled with fluorescein at the 3' end (Fig. 2.11.2.1). The melting temperature of the probes CyaPins-S and CyaP-A (Table 2.4.1) (PROLIGO, France) were checked using a Tm calculator ensuring that the melting temperature (Tm) of the probes was about 5°C higher than that of the CyaApR and CyaApF primers. This ensured that the probes have enough time to bind to the target sequence before being displaced by the DNA polymerase.

To ensure that the PCR mix and parameters were suitable for use in the hybridisation probe assay they were initially optimised by carrying out a SYBR green I assay. This would ensure that suitable amplification was occurring without wasting any probe material. A final reaction volume of 20 µl contained 2 µl of SYBR green 1 mix (Roche, 10 µl of solution A added to the vial containing solution B), 2.5 mM $MgCl_2$, with or without 5% (v/v) DMSO (Sigma), 1 µM of each primer CyaAPf and CyaAPr (Invitrogen), and 25 ng of DNA template. An initial denaturation step at 94°C for 10 min was followed by 45 cycles of denaturation at 94°C for 10 sec, annealing at 60°C for 10 sec, and extension at 72°C for 16 sec. This was followed by Melt (95°C 5 sec ramp rate 20°C/sec, 40°C 10 sec ramp rate 20°C/sec and 95°C 0 sec ramp rate 0.5°C/sec) then Cool programmes. Optimisation had determined that 5% (v/v) DMSO in the reaction mix was essential for successful amplification to occur.

For melting curve analysis assays, the final volume 20 µl contained 2 µl LC-FastStart DNA Master Hybridisation probes mix (Roche, 60 µl of MixB was added to the vial containing

Figure 2.11.2.1: Schematic diagram of adenylate cyclase toxin gene (*cyaA*) with the 388 bp region (bases 570-958) amplified by primers CyaApF and CyaApR (as indicated on diagram) shown in greater detail and the probes that target the sequence internal to that region. The sensor probe CyaPins-S, targets bases 683-696, will anneal to sequences containing the 6 bp insertion at position 691 (highlighted in green) or the single nucleotide difference at position 695. The anchor probe, CyaP-A, will anneal to the wild-type sequence targeting nucleotides 698-726. CyaPins-S is donor probe labelled with fluorescein at its 3' end and CyaP-A is the acceptor probe labelled with LightCycler Red-640 at its 5' end. All numbering is relative to that of *cyaA* GenBank sequence Y00545.The arrow that is situated at position 660 indicates the start of the overlap of *cyaC* GenBank sequence (bases 1-91, M57286) with Y00545.


1
570-
958
981 (CDS
start site)
6442
CyaApF
CyaPins-S
CyaP-A
CyaApR
570
660
691
695
958

MixA), 2.5 mM $MgCl_2$, 5% (v/v) DMSO, 0.5 μM of each primer CyaAPf and CyaAPr, 0.25 μM of probes CyaPins-S and CyaP-A, and 2 μl of 1 ng μl^{-1} DNA. *B. pertussis* strains CN137 (wild-type), NCTC10739^T (single nucleotide difference) and PICU475 (6 bp insertion) were used as positive controls and a reaction with no DNA served as negative control. The initial denaturation step at 94°C for 10 min was followed by 45 cycles of denaturation at 94°C for 10 sec, annealing at 60°C for 10 sec, and extension at 72°C for 16 sec. This was followed by Melt (95°C 5 sec ramp rate 20°C/sec, 40°C 10 sec ramp rate 20°C/sec and 95°C 0 sec ramp rate 0.5°C/sec) then Cool programmes. Melting curve analysis was carried out using LightCycler software and the data are converted to melting peaks allowing the easy distinction of wild-type from mutant species.

2.12 DETERMINATION OF PROTEIN CONCENTRATION

To compare the enzymic activity or cytotoxic activity of CyaA preparations from *B. pertussis* cells it was essential that the preparations were compared at the same protein concentrations. The protein concentration of *B. pertussis* urea extracts was quantified using the protein assay dye reagent concentrate (Bio-Rad). The working reagent was prepared by diluting the concentrate 1 in 5 with sterile distilled water, which was then filtered. Bovine serum albumin (BSA, Sigma) was prepared to a stock concentration of 100 mg ml^{-1} in the same buffer that the urea extracts were prepared (section 2.13.3). The concentration of BSA used as a positive control in the protein concentration assay was 1 mg ml^{-1}. Four-fold dilutions of the BSA and the urea extracts were prepared across the 96-well plates to the final dilution of 1 in 512. The final concentration of BSA was 0.488 μg ml^{-1}. A blank column contained buffer alone. A volume of 20 μl of each dilution was added to 200 μl of the diluted Bio-Rad protein assay dye reagent. Plates were incubated at room temperature for 5 min before the absorbance at 620nm was measured in a plate reader (Rosys anthos 2001). A graph of BSA concentration against A_{620nm} was plotted using the software Microcal™ Origin™ version 5.0 (Microcal Software, Inc. Northampton, USA). Protein concentrations of each urea extract were determined from the standard curve.

2.13 SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS

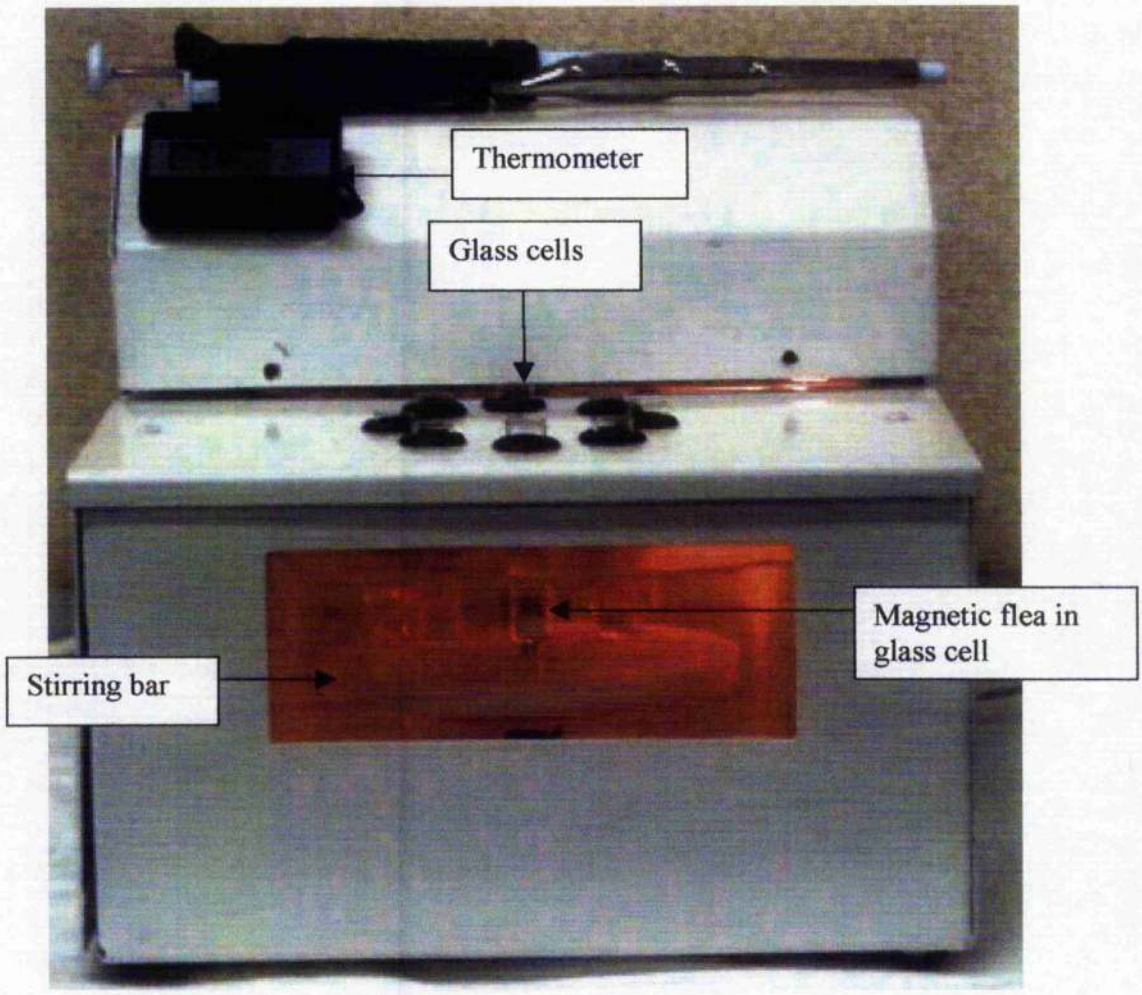
The presence of CyaA in the urea extracts of *B. pertussis* and the comparison of the intensity of the CyaA band of different urea extracts was visualised using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Gel cassette preparation and Mini-PROTEAN® 3 Electrophoresis Module assembly were carried out as described by the manufacturer's instructions (Bio-Rad). The resolving gel containing 7.5% acrylamide (Appendix 4.1) was poured between two glass plates and 100% ethanol was used as an overlay. The gel was allowed to set for approximately 1 hour at room temperature. Stacking gel containing 4% acrylamide (Appendix 4.2) in a 1 ml volume was poured on top of the resolving gel and a comb was placed within the stacking gel, which was then allowed to set. A volume of 10 µl of 2 x sample loading buffer (Appendix 4.3) was added to 10 µl of urea extract (various concentrations) and to 10 µl of marker (SeeBlue® Plus2 Pre-Stained Standard, Invitrogen). The samples, except marker, were placed in a boiling bath for 5 min. Samples were run for 30 min at 200V in 1 x electrode buffer (Appendix 4.6). The gel was removed and stained with Coomassie blue (Appendix 4.4) overnight on a rotating platform for protein visualisation. The stain was replaced with destain solution (Appendix 4.5) until the background was decolourised.

2.14 CONDUCTIMETRY

2.14.1 Conductimetry apparatus

The adenylate cyclase (AC) enzymic activity in clinical specimens containing *B. pertussis* has been used as a target in diagnostic tests. A conductimetry assay developed in-house (DII) to detect *B. pertussis* AC enzymic activity was investigated for its potential as a diagnostic test for pertussis. The assay for AC enzymic activity measured a change in the electrical conductance of the reaction solution. The conductimetry equipment was developed originally by Mezna and Lawrence (1994) and consists of an eight glass-cell system. It has been modified recently for control from a computer with automatic balancing and calibration (Lawrence *et al.*, 2002). The enclosed water bath where the glass cells are suspended is allowed to heat to, and maintained, at 37°C and the stirrer inside maintains constant water temperature (Figure 2.14.1.1).

Figure 2.14.1.1: Conductimetry apparatus. Apparatus consists of enclosed water bath with eight glass cells suspended above a stirring bar. Within each glass cells there is a magnetic flea. Thermometer indicates when the water is heated to 37°C and this is kept constant throughout assay.


Thermometer
Glass cells
Magnetic flea in glass cell
Stirring bar

The sampling period is 1 s per cell, cycling through the cells, from cell 1 through to cell 8, with each cell containing a magnetic flea. This allows continuous mixing of the contents of each cell. The conductivity of incomplete assay mixtures (2 ml) is allowed to balance before the reactions are initiated by addition of enzyme. Data processing of results includes blank subtraction (removes background noise) and on-screen line drawing to measure tangents of progress curves and total change.

2.14.2 Conductimetry assay of adenylate cyclase activity

The conductimetry assay was described by Lawrence *et al.* (2002). Buffer (Appendix 5.1) containing only 10 mM Bicine and 1.5 mM magnesium acetate (MgAc) was degassed by heating for 1-2 min to >90°C and placed under vacuum pressure for ca. 30 sec. Buffer was allowed to cool to room temperature before the addition of ATP. A volume of 2 ml of buffer, 1 µl of 1 mg ml^{-1} calmodulin (CaM) (Sigma) and 1 µl of 0.5 unit μl^{-1} of inorganic pyrophosphatase (PPiase) (Sigma) were added to each glass cell. Balancing of the cells was allowed to occur before addition of samples containing AC enzyme. After three readings, 2 µl of 43 ng μl^{-1} of purified recombinant adenylate cyclase toxin (rCyaA) was added as a positive control (provided by Dr B. Orr, University of Glasgow). The following reaction occurs in the cells where the buffer and reagents are present.

$$(ATP.Mg)^{2-} + Bicine^{-} \xrightarrow[\text{Calmodulin}]{\text{AC enzyme}} cAMP^{-} + (BicineH) + (PPiMg)^{2-} \xrightarrow{\text{Inorganic pyrophosphatase}} 2Pi^{2-} + Mg^{2+}$$

This equation predicts no change in conductance unless the pyrophosphate product is cleaved to release the magnesium ion from chelation (Lawrence *et al.*, 2002). It is the release of the independently mobile ions, Pi^{2-} and Mg^{2+}, that produces the change in conductance that is measured by the assay. Total conductance change and tangents to reaction curves were measured by an on-screen drawing line drawing routine.

2.14.3 Optimisation for AC enzymic activity of rCyaA and native CyaA assay

For the optimisation of rCyaA and native CyaA assay, ATP and CaM dose response curves were carried out. Commercial CaM (Sigma) from bovine brain was compared with CaM prepared in-house from porcine testicular tissue (Lawrence *et al.*, 2002). Both stock

preparations of CaM were at concentrations of 1 mg ml^{-1}. To determine the optimal CaM concentration for routine use, the effect of different amounts of CaM (Sigma and in-house) had on the rate of reaction were compared. Rates of reaction of 2 µl and 1 µl of 1mg ml^{-1} of the CaM (Sigma) were compared with CaM prepared in-house as well as 2 µl-10 µl of 0.1 mg ml^{-1} and 2 µl and 1 µl of 0.01 mg ml^{-1} of CaM (Sigma) being compared. In order to determine the optimal concentration of ATP in the assays, buffer was prepared with Bicine and MgAc but without ATP. Various concentrations of ATP (0.2 mM to 1.4 mM) and buffer were then added to the glass cells along with the standard amounts of CaM and PPiase before cell balancing was carried out. Reactions were started by the addition of rCyaA and the reaction rates of various ATP concentrations were compared.

2.14.4 Effect of ATP, CaM and PPiase on conductimetry assays

Conductimetry assays were carried out without each of the following reagents ATP, CaM or PPiase to determine the effect that this would have on the enzymic rate of reaction of rCyaA and native CyaA. Buffer was prepared as described in 2.12.2 except that ATP was omitted. Various combinations of the reagents were added to the 2 ml of buffer in the glass cells to allow reaction rates to be investigated without one of these reagents.

2.14.5 Detection level of rCyaA in conductimetry assays

Assay protocol was followed as described in 2.12.2. The detection level of rCyaA in the conductimetry assay was determined by adding decreasing concentrations of rCyaA ranging from 0.216 mg ml^{-1} to 0.864 µg ml^{-1} to the glass cells. At the lowest limit of detection, the tangents of reaction were compared with and without CaM to ensure that the low activity was due to CaM-dependent AC activity.

2.14.6 Calculation of enzymic activity

To determine the total change value (arbitrary units) of the conversion of 1 mM substrate (ATP) that would be used to calculate the initial rate of reaction, the following assay was carried out. Buffer was prepared with Bicine and MgAc only. Adenosine triphosphate was added separately to the conductimetry cells. Final concentrations of ATP in the cells ranged from 0.1 mM to 1 mM. A volume of 2 ml of buffer, an appropriate volume of 100 mM ATP, 1 µl of 1 mg ml^{-1} CaM (Sigma) and 1 µl of 0.5 U $µl^{-1}$ of PPiase (Sigma) was added to

each glass cell before balancing. To start the reaction 2 µl of 0.216 mg ml^{-1} rCyaA was then added. For a substrate concentration of 1 mM giving a total change of x units and an initial slope of y units per minute, the initial rate was: initial rate = (y/x) µmol/ml/min (Lawrence *et al.*, 2002).

2.14.7 Testing of inhibitory reagents

Commercial nonionic detergents (PIERCE and Novagen) and urea (Sigma) were investigated as potential lysis agents of *Bordetella* spp. (Table 2.14.7.1). Before the nonionic detergents and urea could be used as lysis agents, they had to be tested to determine if they affected the conductimetry assay by inhibiting or enhancing the reaction. Reaction rates of spiked samples containing 2 µl of rCyaA in the presence of various amounts of nonionic detergents, ranging from 2 µl to 300 µl (0.05%-1.5%) in 2 ml were compared with the rate of reaction of 2 µl of rCyaA alone. Recombinant adenylate cyclase toxin was added to cells which contained concentrations of urea ranging from 0.25 M to 8 M urea and the rates of reaction were compared to that of the positive control. To determine if there was a volume effect regarding the addition of larger volumes of lysate (300 µl) an assay was carried out using 2 µl – 300 µl of a non-inhibiting solution (assay buffer) containing 2 µl of rCyaA. The rates of reactions were compared to that of the addition of 2 µl CyaA alone.

2.15 CONDUCTIMETRY OF ADENYLATE CYCLASE TOXIN ENZYMIC ACTIVITY OF IN *B. pertussis*

2.15.1 Optimisation of preparation of *B. pertussis* cell lysates

The method developed for the detection of CyaA activity from lysed cells of *Bordetella* spp. was adapted from the manufacturer's instructions supplied with B-PER™ (PIERCE) reagent. Initially, assays were carried out using the *B. pertussis* strain BP348pRMB1, an AC-overexpressor (Brownlie *et al.*, 1988). BP348pRMB1 was grown on BG plates and incubated for 72 h at 37°C. Cell growth was used to prepare a turbid suspension of freshly-grown bacteria in saline and 200 µl was spread on BG agar and incubated overnight at 37°C to obtain a confluent layer of growth. Suspensions of bacteria from this overnight growth were prepared to 2 x 10^9 cfu ml^{-1} in saline, initially by comparison with an opacity rod, which was then later determined to be equivalent to ca. OD_{600nm} 0.46.

Table 2.14.7.1. Potential lysis agents for *B. pertussis*.

Lysing agent	**Description**	**Source**
Urea	Powder, dissolved in deionised distilled water	Sigma
B-PER™ Bacterial Protein Extraction Reagent	Nonionic	PIERCE
BugBuster™ Protein Extraction Reagent	Nonionic	Novagen
Triton X-100	Nonionic, 10% solution	PIERCE
Triton X-114	Nonionic, 10% solution	PIERCE
Brij 35	Nonionic, 10% solution	PIERCE
Brij 58	Nonionic, 10% solution	PIERCE
Tween-20	Nonionic, 10% solution	PIERCE
Tween-80	Nonionic, 10% solution	PIERCE
NP40	Nonionic, 10% solution	PIERCE

Four nonionic detergents, Triton X-100, NP40, Brij 35 and Brij 58 were used for the optimisation of preparation of bacterial cell lysates.

1. A volume of 300 µl of nonionic detergent alone was tested to determine if the detergent would have an effect on the reaction curves of enzymic reactions.
2. Bacterial suspensions were frozen (-70°C for 5 min) then allowed to thaw at room temperature. Cells were pelleted by centrifugation for 10 min at 6,600 x *g* (Biofuge pico, Heraeus), and the supernate was tested for AC activity to determine if enzymic activity was lost during this step.
3. Pellets were resuspended in 300 µl of the four different nonionic detergents and then tested for AC activity.
4. Pellets that were resuspended in the nonionic detergents were mixed at 4°C on a rotary shaker for different times (10 min, 30 min and 1h), then tested for AC activity to determine if length of mixing time had an effect on the lysis of bacterial cells. A control containing 2 µl of rCyaA in 300 µl of nonionic detergent was allowed to mix for 30 min at 4°C and tested for AC activity. This control would determine if AC activity decreased during the mixing step.
5. The nonionic detergents that were known not to cause inhibition of the assay were used in the *B. pertussis* lysis protocol. BP348pRMB1, AC over-expressor, and NCTC10739^T were tested for AC activity using the conductimetry assay as described in 2.15.5. Tangents of reactions were compared to determine which condition provided optimal lysis as shown by detection of highest level of AC activity.

2.15.2 Detection of AC activity from *B. pertussis* grown in SS medium

B. pertussis was grown in 5 ml volumes of SS shaking at 150-200 rpm for 48 h at 37°C. The resulting culture, supernate and cell pellet, which was resuspended in 1 ml of assay buffer (no ATP) were tested for AC activity as *B. pertussis* is known to secrete CyaA into the growth medium. Conductimetry assay was carried out as described in 2.14.5, except that 300 µl of each sample was added to the glass cells to start the reaction. A method for desalting SSX medium was investigated because of the inhibitory properties of this medium (see section 3.7.3), using PD-10 desalting columns (Amersham Biosciences) and following the manufacturer's instructions. The gel in the PD-10 column was equilibrated with 25 ml of the conductimetry buffer (no ATP), then 2.5 ml of the culture was added to

column and the flow-through was discarded. Uninoculated SS alone was also added to one column. Elution buffer (3.5 ml) was added and 3 ml of eluted sample was collected. The presence of CyaA in the desalted samples (5 µl–200 µl) was detected using the conductimetry assay.

2.15.3 Preparation of *B. pertussis* urea extracts

The amount of CyaA expressed at different times during growth of *B. pertussis* BP338 was determined. The extraction of CyaA from *Bordetella* was adapted from the method described by Westrop *et al.* (1994). The growth curve of BP338 had previously been determined. Volumes of 250 ml of SS liquid medium were inoculated with BP338 to an OD_{600nm} of 0.01 and at 32, 56 and 73 h and the resulting cultures were Gram stained to ensure that no contaminating bacteria were present. The cultures were pelleted by centrifugation at 9,000 x *g* for 20 min (centrifuge Sorvall® RC-5B with rotor Sorvall® GSA). The resulting cell pellets were stored at -20°C. For the production of urea extracts, cell pellets were thawed and resuspended in 2 ml of 10 mM Tricine pH 8, 0.5 mM EDTA pH 8, 0.5 mM EGTA pH 8, 4 M urea and 1 mM PMSF (Sigma). Suspensions were stirred for 1 h at 4°C on a rotary shaker and then centrifuged for 30 min at 15,000 x *g* (centrifuge Sorvall® RC-5B and rotor Sorvall® SS-34). The protein concentrations of urea extracts were quantified using a Bradford's assay described in 2.12 and urea extracts were stored at -20°C. Adenylate cyclase toxin enzymic activity of the urea extracts was determined by the protocol described in section 2.15.5.

The supernates from cultures in CL, from which the cells were harvested for production of urea extracts, were desalted using PD-10 columns as described in 2.15.2. This would detect AC activity that may have been secreted into the medium. A positive control containing 2 µl of crude rCyaA in 2.5 ml of conductimetry assay buffer was added to a PD-10 column. This would determine if AC activity was lost during the desalting process. Spiked samples containing desalted supernate and 2 µl of crude rCyaA was also tested for AC activity. These would show the presence of any inhibitory products.

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2.15.4 Concentration of urea extracts

Amicon® Ultra centrifugal filter device columns (Millipore) with a 30,000 molecular weight cut-off were used to concentrate urea extracts of *B. pertussis* BP338 and 99K45. Approximately 2 ml volumes of urea extract were added to the columns and centrifuged at 4,000 x *g* for 4 min at 4°C (Multifuge 3S-R, Hereaus). The urea extracts were concentrated to about 500 µl. Volumes of 10 µl of concentrated samples were compared to 10 µl of unconcentrated samples for CyaA content by SDS-PAGE. Remaining samples were stored at -20°C.

2.15.5 Conductimetry assay of *B. pertussis* CyaA

The assay procedure is described in section 2.14.2. After degassing the buffer, it was allowed to cool before the addition of ATP. A volume of 2 ml of buffer, 1 µl of 1 mg ml^{-1} CaM (Sigma) and 1 µl of 0.5 U μl^{-1} PPiase was added to each glass cell. Balancing was allowed to occur before addition of *B. pertussis* lysate or other *Bordetella* spp. lysate. Once balanced, 300 µl of the lysate was added to glass cells. A volume of 2 µl of 43 ng μl^{-1} of purified rCyaA and/or 300 µl BP338 lysate were added as positive controls and, for some assays, 300 µl BP347 lysate was included as a negative control.

2.16 CYTOTOXICITY ASSAY

Two *B. pertussis* isolates were found to contain a 6 bp insertion between *cyaA* and *cyaC*. The gene *cyaC* activates the adenylate cyclase protoxin enabling the molecule to carry out several biological activities one of which is its cytotoxicity activity. The 6 bp insertion is situated very close to *cyaC* relating to the start of the *cyaC* GenBank sequence, which may affect the expression of this gene. Whether this sequence variation has an effect on CyaA cytotoxic activity was therefore examined. The cytotoxic activity of *B. pertussis* urea extracts were determined using the CellTitre 96® Non-Radioactive Cell Proliferation Assay (Promega). The assay is dependent on the reduction of the tetrazolium salt (3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide) by the mitochondrial dehydrogenase of viable cells to form an insoluble purple formazan product (MAXline™ Microplate Readers, application note 5). Mouse macrophage-derived (J774.2) cells were suspended in phenol red-free RPMI medium (Gibco) supplemented with 10% foetal calf serum (Gibco), 200 mM L-glutamine, 100 µg ml^{-1} streptomycin and 100 U ml^{-1} penicillin.

Cell viability was checked using Trypan Blue exclusion. Live cells were counted using a haemocytometer, then the suspension was pelleted by centrifugation at 250 x *g* (Multifuge 3S-R, Hereaus) for 5 min. The pellet was resuspended in an appropriate volume of phenol red-free RPMI medium to give a final concentration of 5 x 10^5 cells ml^{-1}. Suspended cells (50 µl) were added to a 96-well plate and then incubated at 37°C for 1 h. Meanwhile, the urea extracts were prepared to a starting concentration of 1500 µg ml^{-1}. Doubling dilutions of the urea extracts were prepared in phenol red-free RPMI medium. A volume of 50 µl of phenol red-free media and 1% Triton X-100 (Sigma) were added to the cells serving as a negative control and a positive control, respectively. A volume of 50 µl of diluted urea extracts were added to the cells, with final concentrations ranging from 750 µg ml^{-1} to 3 µg ml^{-1}, and incubated at 37°C for 2 h. A volume of 15 µl of MTT dye (Promega) was added to each well that contained cells with CyaA and this plate was further incubated at 37°C for 2 h. Following this incubation, 100 µl of solubilisation/stop solution (Promega) was added to each well and this was incubated overnight at 37°C. The plate was read using a plate reader (Rosys anthos 2001) at OD_{540nm}. Percentage cell killing was calculated using the following formula:

$$\text{Percentage killing} = 1 - \left(\frac{\text{test sample } OD_{540nm} - \text{positive control } OD_{540nm}}{\text{negative control } OD_{540nm} - \text{positive control } OD_{540nm}} \right) \times 100$$

Protein concentration was plotted against percentage killing to determine the protein concentration required to give 50% killing of the macrophages using software Microcal™ Origin™ version 5.0.

2.17 CONSTRUCTION OF BIOLUMINESCENT VECTORS CONTAINING THE *cyaA* PROMOTER

Constructs containing a promoterless bioluminescent reporter gene, *lux* or *gfp*, with the *cyaA* promoter region inserted upstream were developed in order to determine when in the growth cycle *cyaA* is optimally expressed and the effect of different growth conditions (including modulating signals) on expression. Information from these assays would aid in the optimisation of the conductimetry assay for use as a diagnostic tool. Bioluminescent reporters provide the ability to quantify gene expression at high sensitivity over a large dynamic range in real time and non-destructively. Some reporter systems have the problem

of background expression due to host enzymes but with bioluminescent reporters this is much less of a disadvantage as naturally bioluminescent bacteria are rare outside the marine environment (Winson *et al.*, 1998).

The *lux* genes that are required for luminescence are arranged in a single operon, *luxCDABE*. The reporter *lux* genes were derived from *Photorhabdus* (*Xenorhabdus*) *luminescens*, these genes are functional at temperatures as high as 45°C and therefore more suitable than the working temperatures of those derived from *Vibrio harveyi* (<37°C) and *V. fischeri* (<30°C) (Winson *et al.*, 1998). Winson *et al.* (1998) reported the use of lucifersases to report on gene expression throughout the bacterial culture growth curve using an automated photometer/luminometer (Lucy 1, Anthos Labtech, Salzberg, Austria). This would allow real-time determination of *cyaA* expression throughout the *B. pertussis* growth. The gene, *gfp*, encoding green fluorescent protein is originally from the jellyfish *Aequorea victoria* which can be produced expressed in prokaryotic and eukaryotic cells (Chalfie *et al.*, 1994). Exogenous substrates and cofactors are not required for this fluorescence thus allowing its use for monitoring gene expression and protein localisation in living cells. Unlike *lux* expression, *gfp* expression cannot be monitored in a real-time fashion, *cyaA* expression could only be detected by measuring the fluorescence intensity at different time points.

Two plasmids were used for the cloning study: pSB395 (containing a promoterless *lux* operon) and pPROBE-AT' (containing a promoterless *gfp*) (Table 2.2.2.1). Plasmid pSB395 contains an *Eco*RI site upstream of *luxC* that was used for promoter cloning. The plasmid pPROBE-AT' contains several unique restriction sites, *Kpn*I and *Sac*I sites were chosen for restriction analyses.

2.17.1 Ligation

Ligation of purified *cyaA* PCR product (amplified with primers CyaA1 and CyaA6/CyaA8) with the commercial A-T cloning vector pGEM®-T Easy (Promega) was carried out following the manufacturer's protocol. Standard reactions and positive control reactions were prepared. The standard reactions contained Rapid Ligation Buffer, 5 ng of pGEM®-T Easy vector, 2 µl of the cleaned PCR product, 3 U of T4 DNA ligase, and sterile deionised

water was added to give a final volume of 10 µl. The positive reaction was prepared as that described for the standard reaction but 2 µl (8 ng) of control insert DNA was used in place of the PCR product. Reactions were incubated overnight at 4°C and then stored at -20°C.

Ligation of purified *cyaA* PCR product (amplified with primers CyaA1 and CyaA6/CyaA8) with the vectors pPROBE-AT' or pSB395 digested with *Kpn*I/*Sac*I and *Eco*RI respectively, was carried out using the Fast-Link™ DNA Ligation and Screening Kit (Epicentre). The manufacturer's protocol was followed for ligation of insert DNA with cohesive ends. The total reaction volume (15 µl) contained 1 x Fast-link ligation buffer, 1 mM ATP, 2 µl of digested and purified vector DNA, 7 µl of digested and purified insert DNA and 1 µl (2 U) of Fast-Link DNA ligase. The last reagent added to the ligation reaction was DNA ligase. Reactions were incubated overnight at room temperature. These ligation reactions were used to transform competent *E. coli* strain SM10 λ pir.

2.17.2 Preparation of electroporation-competent cells

E. coli cells were used to inoculate 10 ml LB and grown overnight at 37°C, shaking at 150-200 rpm. The overnight culture was diluted 1 in 100 in 500 ml of fresh LB in a 2 l dimpled flask and was incubated at 37°C, shaking at 150-200 rpm until an OD_{600nm} of 0.5-0.7 was obtained. The flasks were chilled on ice for 30 min and the cells were then harvested by centrifugation at 2,500 x *g* for 15 min at 4°C (centrifuge Sorvall® RC-5B with rotor Sorvall® GSA). The cell pellets were resuspended in 500 ml of chilled sterile distilled water and cells were pelleted as before. The pellet was resuspended in 10 ml of cold 10% (v/v) glycerol. Aliquots of 40 µl were added to 1.5 ml microfuge tubes which were snap frozen in liquid nitrogen and then stored at -70°C for up to approximately six months.

2.17.3 Electroporation

A volume of 1 µl of plasmid preparation or ligation reaction was added to 40 µl of *E. coli* competent cells and this was placed in a Gene Pulser® (E. coli Pulser®) Cuvette (Bio-Rad) with a 0.2 cm electrode gap. The mixture was gently shaken to the bottom of the electroporation cuvette, which was then placed in a Bio-Rad Gene Pulser™ connected to a Bio-Rad Pulse Controller. The following conditions were used: 2.5 kV, 200 Ohms and 25 µFD (Faraday). Luria Bertani broth (0.75 ml) was then added to the cuvette and this was

incubated at 37°C with shaking at 150-200 rpm for 1 h. Aliquots of 200 µl volumes were then spread on to LB plates containing the appropriate antibiotics and incubated overnight at 37°C. Resulting single colonies were re-streaked on to LB agar with appropriate antibiotics and incubated overnight. Plasmid and glycerol stocks were prepared from the resulting colonies.

2.17.4 Bacteriophage (ffmΦ4) preparations

The bacteriophage ffmΦ4 (provided by Dr M. Lynch) was prepared for use in plate mating assays (section 2.15.4). Bacteriophage ffmΦ4 lyses *E. coli* cells but *B. pertussis* is resistant to ffmΦ4. The *E. coli* strain SM10 λ pir containing plasmid pSB395 was grown overnight in 5 ml of LB at 37°C with shaking at 150-200 rpm. The culture was diluted 1 in 100 in fresh LB broth and this was prepared as four replicates to produce enough phage stocks. The resulting culture was grown for 2 h at 37°C shaking at 200 rpm. Bacteriophage ffmΦ4 (50 µl) was added to the culture and this was incubated at 37°C shaking for ca. 2 h until the culture became much less turbid. This was due to lysis of *E. coli* cells by the phage. A few drops of chloroform were added to kill any remaining non-lysed cells. The chloroform and lysed culture were mixed by swirling. The chloroform layer was allowed to settle and the top layer was aspirated in to a fresh tube. *B. pertussis* BP338 (100 µl) and *E. coli* SM10 λ pir pSB395 (100 µl) suspensions were spread on to BG agar and LB agar respectively. Aliquots (20 µl) of each phage preparation were spotted on to both plates. The presence of plaques was checked on the plates after 24 h for *E. coli* and 72 h for *B. pertussis*. This ensured that the fresh phage preparation was effective in lysing *E. coli* cells and that *B. pertussis* was resistant to the phage. The bacteriophage stocks were stored at 4°C.

2.17.5 Conjugation

The first method that was used for plate mating *E. coli* with *B. pertussis* was based on a method communicated by Dr M. Lynch. *B. pertussis* BP338 and *E.coli* SM10 λ pir, a DNA mobilising strain, transformed with the vector pSB395 were grown on BG and LB and 12.5 µg ml^{-1} of tetracycline (pSB395 confers tetracycline resistance) respectively. Colonies of *B. pertussis* were inoculated in 5 ml of SSX medium and grown at 37°C for 72 h. Colonies of *E. coli* were inoculated in 5 ml of LB and 12.5 µg ml^{-1} of tetracycline and incubated at 37°C overnight. Five x 1 ml of each inoculated broth was centrifuged for 1 min at 11,200 x

g (Biofuge pico, Heraeus). The supernates were aspirated and pellets were resuspended in 1 ml of growth media. Suspensions were centrifuged for 1 min at 11,200 x *g* (Biofuge pico, Heraeus). Pellets were resuspended in 100 µl of growth media and mixed in a 1:1 ratio of donor:recipient. Aliquots (10 µl) of this mixture were placed on freshly-made dry BG agar and were incubated at 37°C for 24 h. The bacterial growth resulting from each aliquot of bacterial mix was scraped from the plate and suspended in 100 µl of LB. A volume of 100 µl of bacteriophage (ffmΦ4) preparation was added to the resuspended cells and gently pipetted to mix the suspension. The suspensions were incubated at room temperature for 30 min and then spread on to dry BG plates containing the following selective antibiotics, 12.5 µg ml^{-1} of tetracycline and 40 µg ml^{-1} of cephalexin (chosen to select against *E. coli*). The only bacteria that should be present after the plate mating should be *B. pertussis* containing pSB395. Plates were incubated at 37°C for 72 h. Resulting cultures were checked by the Gram stain method. Plates were checked the following day and contaminating *E. coli* cells were present. It was assumed that the *E. coli* had outgrown *B. pertussis* even in the presence of cephalexin. Therefore the above protocol was altered to include the method described by Stibitz and Carbonetti (1994).

Conditions for culture were the same as those described previously, however *B. pertussis* was incubated in SS for 48 h instead of 72 h and *E. coli* SM10 λ pir was grown in 10 ml of LB instead of 5 ml. The resulting OD_{600nm} for *E. coli* SM10 λ pir culture was 1.0 and for *B. pertussis* 0.8. Five x 1 ml of each inoculated broth was centrifuged for 1 min at 11,200 x *g* (Biofuge pico, Heraeus). The supernates were aspirated and pellets were resuspended in a total of 1 ml of growth media. Suspensions were centrifuged for 1 min at 11,200 x *g* (Biofuge pico, Heraeus). Pellets were resuspended in 100 µl of growth media and mixed in a 1:10 mix of donor:recipient. Aliquots (10 µl) of the mixture were added to freshly-made dry BG agar containing 10 mM magnesium sulphate ($MgSO_4$) and were incubated at 37°C for 7 h. The bacterial growth resulting from each aliquot of bacterial mix was scraped from the plate and suspended in 100 µl of LB. A volume of 100 µl of phage preparation was added to the resuspended cells and gently pipetted to mix the suspension. The suspensions were incubated at room temperature for 30 min and then spread on to dry BG containing the following selective antibiotics, 12.5 µg ml^{-1} of tetracycline and 40 µg ml^{-1} of cephalexin. Plates were incubated at 37°C for 72 h. Presumptive *B. pertussis* colonies were

subcultured on to BG with 12.5 μg ml^{-1} of tetracycline and incubated for 72 h. Resulting cultures were Gram-stained to ensure that it was *B. pertussis* present. Glycerol stocks and plasmid preparations were prepared from the *B. pertussis* cultures.

2.17.6 Bioluminescence assay

Colonies of *B. pertussis* BP338 containing pSB395 with the cyaA1-6 insert (pSB1-6), as a result of conjugation, were investigated for their ability to produce light using a luminometer, Lucy-1. *B. pertussis* wild-type BP338 and BP338 pSB1-6 colonies were inoculated in 5 ml SS liquid medium and incubated for 48 h at 37°C, shaking at 150-200 rpm. The bacteria were diluted to OD_{600nm} 0.05 in fresh SS liquid medium in 96-well white microplates. White or black microplates are available for use with Lucy-1, however white plates were chosen due to the requirement of increased light output. The plate was placed in Lucy-1 and assayed for 72 h to determine luminescence of the bacteria.

3.0 Results

3.1 SEQUENCE VARIATION IN *B. pertussis* VIRULENCE-ASSOCIATED GENES

Sequence variation was determined in regions of eight virulence-associated genes (*ptxC*, *tcfA*, *fim2*, *brkA*, *vag8*, *ompQ*, *bapC* and *cyaA*) in a selection of historical and recent UK isolates of *B. pertussis*. The regions that were sequenced for determination of genotypic variation were either the open reading frame (*bapC* and *brkA*), polymorphic sites determined by previous studies (*ptxC*, *tcfA*, *fim2*, *vag8*, and *ompQ*) or a region determined to contain sequence variation by a BLAST search of the Tohama-I genome (BX470248) and the GenBank sequence (Y00545) (*cyaA*). The three *B. pertussis* strains used to produce the UK whole-cell pertussis vaccine (CN2992, CN5476 and CN3099), the strain used to produce components for the acellular pertussis vaccine (Tohama-I) and the type strain 18323^T (NCTC10739^T) of *B. pertussis* were included for comparative purposes.

3.1.1 Electrophoretic analysis of PCR products

Amplified PCR products to be sequenced were separated on 2% (w/v) agarose gels to confirm successful amplification of a product of expected size. For each gene target, amplified products of the expected size were obtained with the primer pairs listed in Table 2.4.1 (Figs. 3.1.1.1a and 3.1.1.1b).

3.1.2 Summary of frequency of gene polymorphisms in UK *B. pertussis* isolates

Sequence information on eight genes, *ptxC*, *tcfA*, *cyaA*, *fim2*, *ompQ*, *vag8*, *brkA* and *bapC*, and also on the promoter region of *cyaA*, *cyaApr*, was obtained with one or a combination of the following methods: LightCycler hybridisation assay, conventional dideoxy DNA sequencing or Pyrosequencing. The sequencing results are summarised in the Table 3.1.2.1. The sequence information for genes *ptxA* and *prnA* has been described in previous studies (Fry *et al.*, 2001; Packard *et al.*, 2004). Table 3.1.2.1 shows the overall frequency of each allele of each gene target together with the appropriate GenBank accession numbers. The variation reported for each gene will be described individually.

Figure 3.1.1.1a. Electrophoretic analysis following amplification of *B. pertussis* gene targets bordetella resistance to killing protein A (*brkA*), virulence-activated gene 8 (*vag8*) and tracheal colonisation factor (*tcfA*).

Lane	PCR primers	Gene target	Product size predicted/obtained
M	100bp ladder (Invitrogen)		not applicable (n/a)
1	BrkAF and BrkAR5	*brkA*	570 bp
2	BrkAF2 and BrkAR4	*brkA*	614 bp
3	BrkAF7 and BrkAM2	*brkA*	653 bp
4	BrkAM1 and BrkAR3	*brkA*	644 bp
5	BrkAF3 and BrkAR1	*brkA*	511 bp
6	BrkAF6 and BrkAR6	*brkA*	679 bp
7	92KF and 92KR4	*vag8*	681 bp
8	92KF7 and 92KR2	*vag8*	754 bp
9	KF5 and 92KR	*vag8*	413 bp
10	TcfAF and TcfAR4	*tcfA*	543 bp
11	TcfAF2 and TcfAR3	*tcfA*	651 bp
12	TcfAF1 and TcfAR1	*tcfA*	525 bp
13	TcfAF and TcfAR5	*tcfA*	762 bp
14	TcfAF2 and TcfAR3	*tcfA*	576 bp
15	TcfAF3 and TcfAR3	*tcfA*	634 bp
16	TcfAM and TcfAR6	*tcfA*	652 bp
17	TcfAF4 and TcfAR5	*tcfA*	380 bp

Figure 3.1.1.1b. Electrophoretic analysis following amplification of *B. pertussis* gene targets adenylate cyclase toxin gene (*cyaA*), pertussis toxin S3 subunit (*ptxC*), outer-membrane protein Q (*ompQ*), fimbrial antigen 2 (*fim2*) and bordetella autotransporter protein C (*bapC*).

Lane	PCR primers	Gene target	Product size predicted/obtained
M	100 bp ladder (Invitrogen)		n/a
18	CyaA3F and CyaA3R	*cyaA*	569 bp
19	CyaA5F and CyaA5R	*cyaA*	621 bp
20	CyaA4F and CyaA4R	*cyaA*	329 bp
21	CyaA2F and CyaA2R	*cyaA*	519 bp
22	CyaA1F and CyaA1R	*cyaA*	550 bp
23	CyaApF and CyaApR	*cyaA*	388 bp
24	PtxS3f and PtxAJK8 S3R	*ptxC*	247 bp
25	ptxS3-3270F and ptxS3-3658R	*ptxC*	388 bp
26	ompQ-1377F and ompQ-1533R	*ompQ*	156 bp
27	fim2-632F and fim2-900R	*fim2*	268 bp
28	BapC1F and BapC1R	*bapC*	529 bp
29	BapC2F and BapC2R	*bapC*	677 bp
30	BapC3F and BapC3R	*bapC*	489 bp
31	BapC4F and BapC4R	*bapC*	561 bp
32	BapC5F and BapC5R	*bapC*	418 bp

Figure 3.1.1.1a

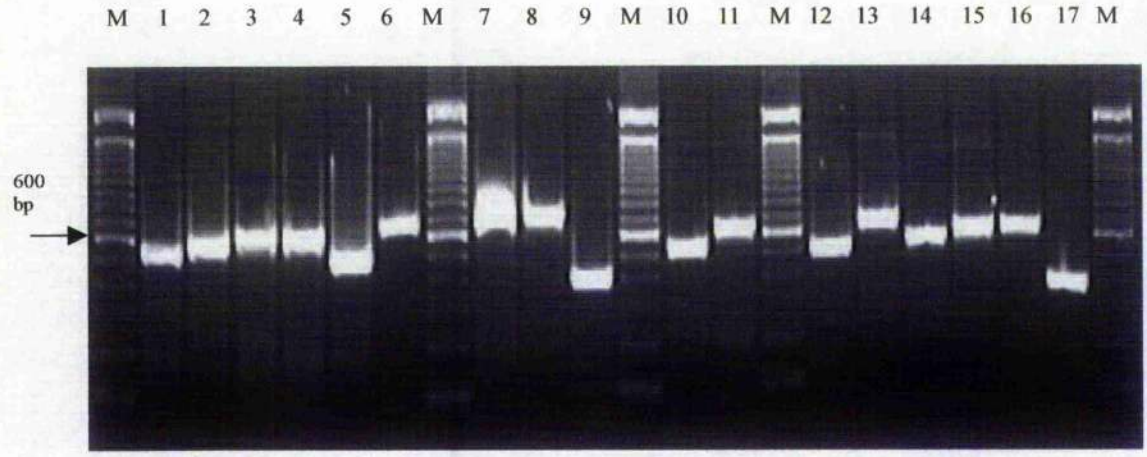


Figure 3.1.1.1b

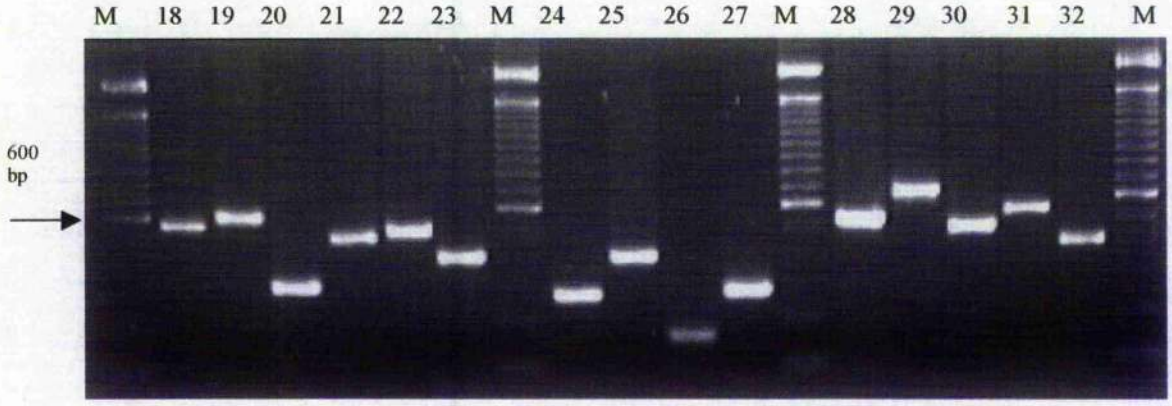


Table 3.1.2.1. Frequency of gene polymorphisms in United Kingdom isolates of *B. pertussis*.

Gene	Allele	Number of isolates	Frequency (%)	GenBank Accession no.	*B. pertussis* strain
ptxA		136			
	ptxA(1)	122	89.7	AJ006155	287
	ptxA(2)	14	10.3	AJ006157	Tohama
ptxC		138			
	ptxC(1)	83	60.1	M13223	3779
	ptxC(2)	55	39.9	AJ420987	NK
tcfA		138			
	tcfA(1)	0	0	U16754	18323^T
	tcfA(2)	124	89.8	AJ009785	B596
	tcfA(3)	2	1.4	AJ420991	NK
	tcfA(4)	9	7.1	AJ507643	NK
	tcfA(5)	1	0.7	AJ420592	NK
	tcfA(6)*	2	1.4	AY375533	DCH154
cyaA		42			
	cyaA(1)	0	0	Y00545	18323^T
	cyaA(2)*	42	100	BX470248	Tohama-I
ompQ		42			
	ompQ(1)	0	0	U16266	18323^T
	ompQ(2)	42	100	AJ420990	NK
vag8		9			
	vag8(1)	0	0	U90124	18323^T
	vag8(2)	9	100	AJ420993	NK
brkA		9	100	U12276	Tohama
bapC		9			
	bapC(1)	9	100	AF081494	Taberman
	bapC(2)*	0	0		18323^T
prnA		138			
	prnA(1)	72	52.1	AJ011091	B391
	prnA(2)	62	44.9	AJ011092	B345
	prnA(3)	4	3	AJ011093	B343
fim2		80			
	fim2(1)	60	75	Y00527	Wellcome 28
	fim2(1)	20	25	AJ420988	NK
cyaApr		46			
	cyaApr(1)	0	0	Y00545	18323^T
	cyaApr(2)*	43	93.4	BX470248	Tohama-I
	cyaApr(3)*	2	6.6		PICU475

* Alleles identified in this study, NK= not known.

3.1.3 Pertussis toxin S1 (*ptxA*) and S3 (*ptxC*) genes

The sequence variation for the pertussis toxin S1 subunit gene (*ptxA*) has been reported elsewhere (Fry *et al.*, 2001; Packard *et al.*, 2004) and is to be included here as it is one of the MLST targets. Five *ptxA* types (*ptxA*(1)-(6)) are found amongst *B. pertussis* isolates world-wide. There are two *ptxA* types found amongst UK *B. pertussis* isolates, *ptxA*(1) and *ptxA*(2). The summary table (Table 3.1.2.1) shows the frequency of both *ptxA* alleles found in UK isolates from 1920-2002 with their respective GenBank numbers. The majority of isolates (n=122; 89.7%) were *ptxA*(1) (AJ006155) and 10.3% (n=14) were *ptxA*(2) (AJ006157). For trend analysis of each virulence-related gene, the isolates have been divided into seven groups according to the year of isolation: six groups of approximately 10 years from 1920-1999 and the most recent isolates, 2002, as a separate group (Fig. 3.1.3.1). These time groups were chosen for convenience and to facilitate comparison of the UK *B. pertussis* population with other populations (see section 4.2). Isolates were not available for all years and it should be noted that within some of the decades only a few years are represented. For example, the groups titled 1990-1999 only contain isolates from 1998 and 1999. For actual dates of isolation see Table 2.1.1.2 and Appendix 6. Figure 3.1.3.1 depicts the *ptxA*(1) and *ptxA*(2) trends throughout this time period.

There has been an increasing predominance of *ptxA*(1) with the concurrent decrease of *ptxA*(2) until all *B. pertussis* isolates were *ptxA*(1). Both *ptxA* alleles were found from 1920-1989 and from 1990-2002 only *ptxA*(1) was found amongst UK isolates. The three whole-cell vaccine strains were *ptxA*(1) or *ptxA*(2) (CN3099 and CN5476 were *ptxA*(1) and CN2992 was *ptxA*(2)) and the ACV strain as *ptxA*(2). The *B. pertussis* type strain was *ptxA*(4). The published genome sequence of *B. pertussis* strain Tohama-I (BX470248) contained *ptxA*(2) .

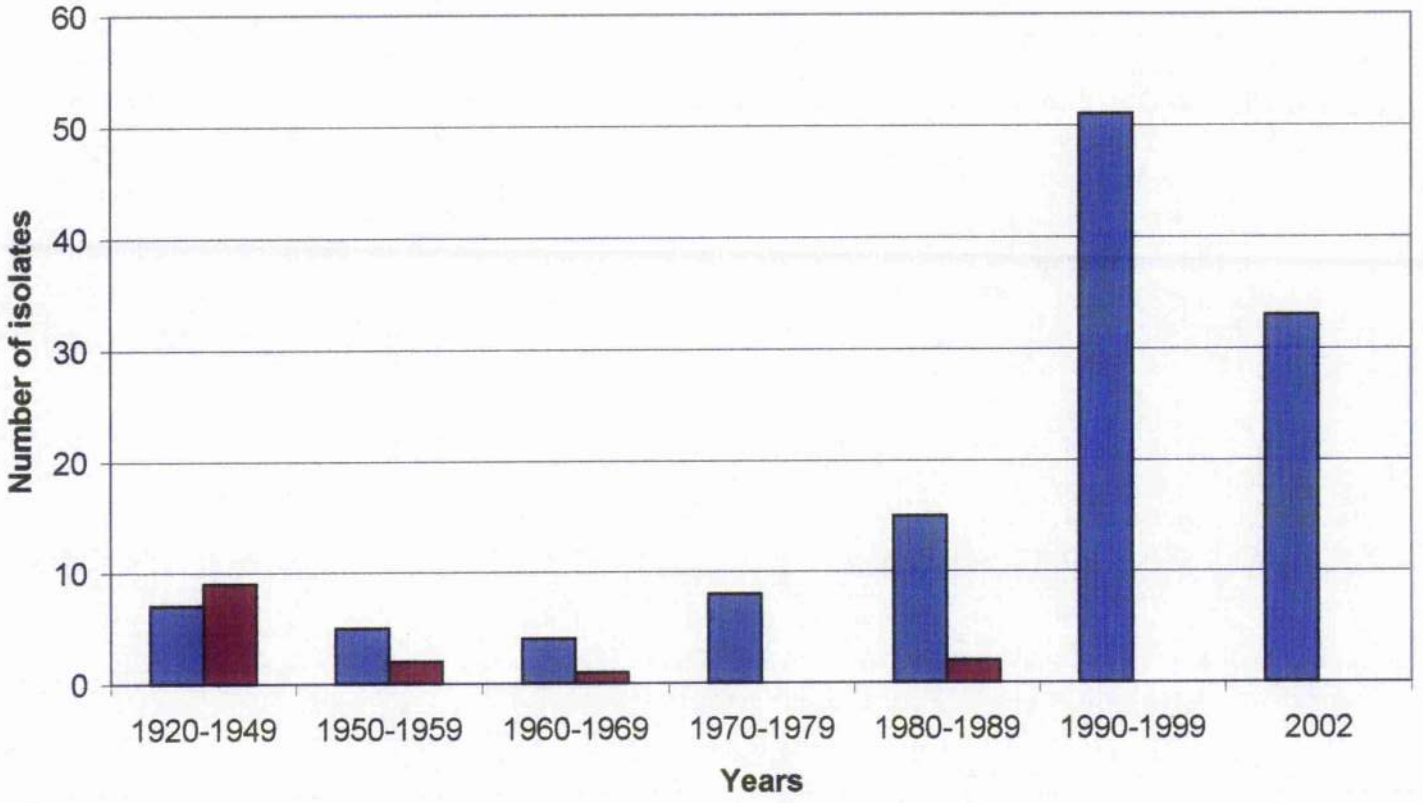
The pertussis toxin S3 subunit gene (*ptxC*) alleles had previously been defined by a single nucleotide polymorphism at position 3625 (M13223, van Loo *et al.*, 2002): *ptxC*(1) (TGC, Cys) and *ptxC*(2) (TGT, Cys). Pertussis toxin subunit C sequences of 138 UK isolates were obtained by Pyrosequencing as well as by conventional dideoxy sequencing (see Appendix 9 for examples of Pyrograms). Pertussis toxin subunit C sequences of three *B. pertussis* isolates were obtained originally by conventional sequencing and these were also analysed by

Figure 3.1.3.1: Trends in pertussis toxin S1 subunit (*ptxA*) alleles in UK isolates of *B. pertussis* from 1920-2002. Information from previous studies (Fry *et al.*, 2001; Packard *et al.*, 2004).

Key:

ptxA(1)

ptxA(2)


60
50
40
30
20
10
0
Number of isolates
1920-1949
1950-1959
1960-1969
1970-1979
1980-1989
1990-1999
2002
Years

Pyrosequencing to ensure that both methods were able to detect the same alleles of these isolates. Both techniques were found to agree and the isolates were sequenced routinely for *ptxC* variation by Pyrosequencing. Only the two known *ptxC* allele types were identified in the UK isolates. The summary table (Table 3.1.2.1) shows the frequency of each allele, 60.1% (n=83) for *ptxC*(1) and 39.9% (n=55) for *ptxC*(2) together with respective GenBank numbers M13223 and AJ420987. The trends in *ptxC* allelic variation found amongst UK isolates from 1920-2002 are shown in Fig. 3.1.3.2. As before, the isolates have been divided into seven time groups: six groups of approximately 10 years from 1920-1999 and the most recent isolates, 2002, as a separate group (Fig. 3.1.3.2). These time groups were chosen as not all years had representative isolates (see Appendix 7). When the results were analysed according to date of isolation, it was determined that there has been an apparent shift in the predominant allele of *ptxC* (Fig. 3.1.3.2). From 1920 until 1998, all isolates (n=53) were *ptxC*(1). During 1998 and 1999, 22 of 51 (43%) isolates were *ptxC*(2) (Fig. 3.1.3.2). Of 34 isolates from 2002, 33 were *ptxC*(2) and one was *ptxC*(1). The four vaccine strains and the type strain were all *ptxC*(1) (Appendix 8). The published genome sequence was of *B. pertussis* strain Tohama-I which was *ptxC*(1).

3.1.4 Tracheal colonisation factor gene (*tcfA*)

Nucleic acid sequence of the tracheal colonisation factor (*tcfA*) gene was determined by conventional sequencing for 138 isolates, four vaccine strains and the *B. pertussis* type strain between bases 688-1339 (U16754). Five *tcfA* (*tcfA*(1)-(5)) alleles based on the variation found within this region have been found amongst other *B. pertussis* populations (van Loo *et al.*, 2002) (Table 3.1.2.1). Of the five sequence types previously described for this region four, *tcfA*(2), *tcfA*(3), *tcfA*(4) and *tcfA*(5), were found amongst the UK *B. pertussis* population (Fig. 3.1.4.1). A novel *tcfA* type, designated *tcfA*(6), not previously reported, was also found amongst the UK isolates (Fig. 3.1.4.1). Sequencing of the isolates containing this novel allele was repeated to ensure that this was not due to sequencing errors. The allele *tcfA*(6) has a 15 bp deletion resulting in bases missing between nucleotides 932–946 (U16754) (Fig. 3.1.4.1). This *tcfA* allele sequence was deposited in GenBank under accession number AY375533. Allele *tcfA*(1) contains a 75 bp fragment not found in alleles *tcfA*(2-4 and 6) (Fig. 3.1.4.1), and is not represented in the UK clinical isolates or the four

Figure 3.1.3.2: Trends in pertussis toxin S3 subunit (*ptxC*) alleles in UK isolates of *B. pertussis* from 1920-2002.

Key:

ptxC(1)

ptxC(2)

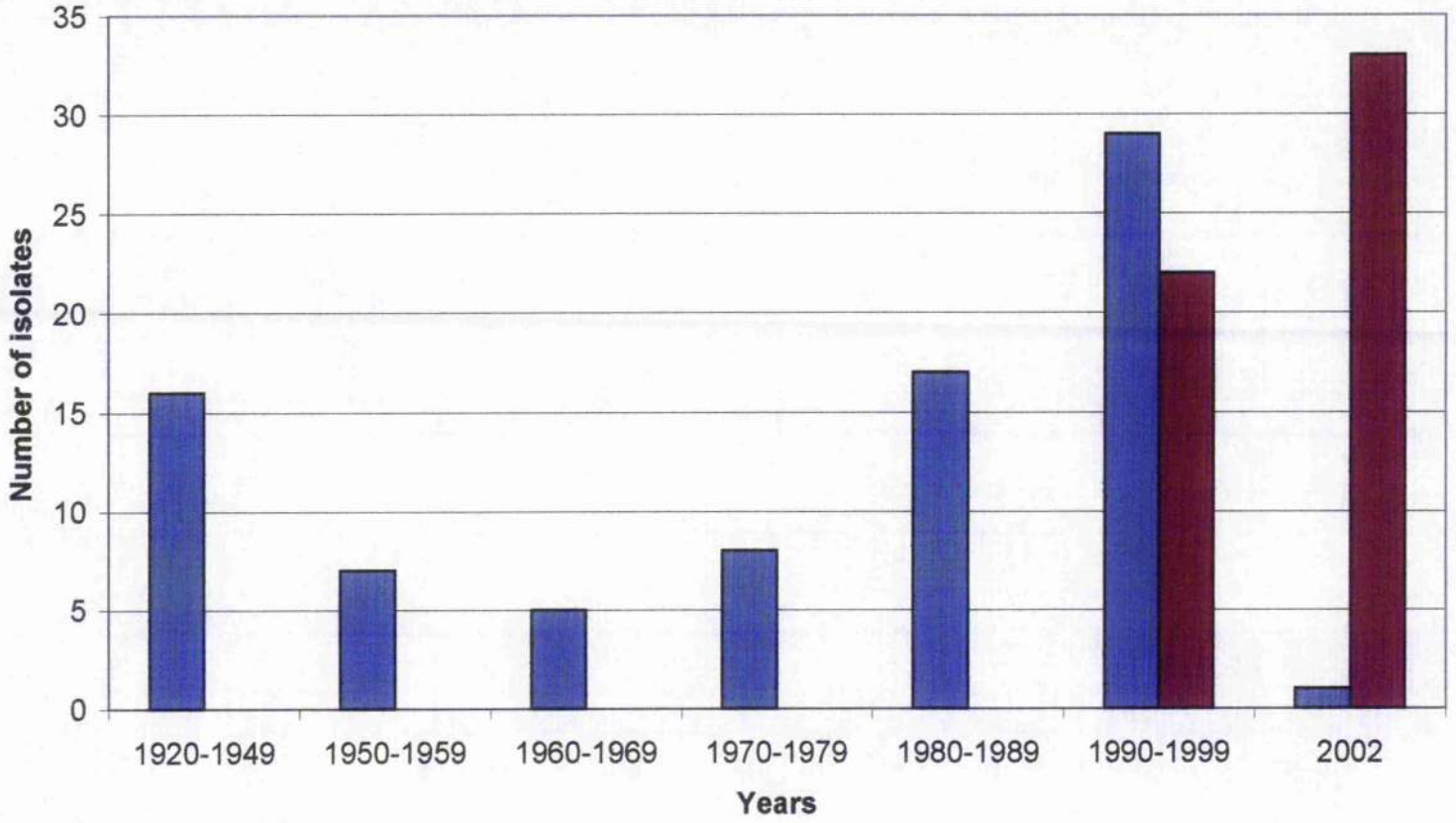
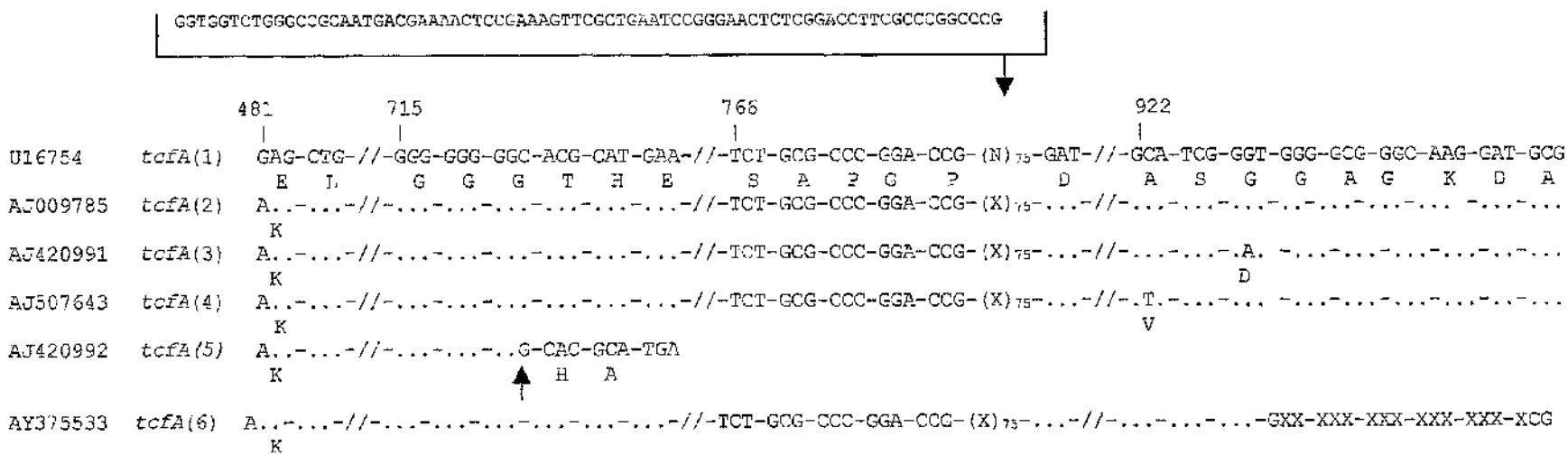

35
30
25
20
15
10
5
0
Number of isolates
1920-1949
1950-1959
1960-1969
1970-1979
1980-1989
1990-1999
2002
Years

Figure 3.1.4.1: Primary structure of a c. 467 bp region of the *B. pertussis* tracheal colonisation factor gene (*tcfA*) showing the six types described to date, designated *tcfA*(1) to *tcfA*(5) (van Loo *et al.*, 2002) and *tcfA*(6) (this study). Numbering is with respect to *tcfA*(1), U16754. Nucleotide polymorphisms are shown and associated amino acid changes are indicated beneath the relevant codon. Dots indicate identity, and codons are separated by dashes. In *tcfA*(1), $(N)_{75}$ indicates a region of 75 bp, which is absent from the remaining alleles. Deletions are indicated by X. The arrow below *tcfA*(5) indicates an inserted G, which changes the reading frame and results in premature translational termination two codons downstream.


GGTGGTCTGGGCCGCAATGACGAAAACTCCGAAAGTTCGCTGAATCCGGGAACTCTCGGACCTTCGCCCGGCCCG
481
715
766
922
U16754 tcfA(1) GAG-CTG-//-GGG-GGG-GGC-ACG-CAT-GAA-//-TCT-GCG-CCC-GGA-CCG-(N)75-GAT-//-GCA-TCG-GGT-GGG-GCG-GGC-AAG-GAT-GCG
E L G G G T H E S A P G P D A S G G A G K D A
AJ009785 tcfA(2) A..-...-//-...-...-...-...-...-...-//-TCT-GCG-CCC-GGA-CCG-(X)75-...-//-...-...-...-...-...-...-... -...-...
K
AJ420991 tcfA(3) A..-...-//-...-...-...-...-...-...-//-TCT-GCG-CCC-GGA-CCG-(X)75-...-//-...-...-.A. -...-...-...-...-...-...
K D
AJ507643 tcfA(4) A..-...-//-...-...-...-...-...-...-//-TCT-GCG-CCC-GGA-CCG-(X)75-...-//-.T.-...-... -...-...-...-...-...-...
K V
AJ420992 tcfA(5) A..-...-//-...-...-..G-CAC-GCA-TGA
K H A
AY375533 tcfA(6) A..-...-//-...-...-...-...-...-...-//-TCT-GCG-CCC-GGA-CCG-(X)75-...-//-...-...-...-GXX-XXX-XXX-XXX-XXX-XCG
K

vaccine strains. The *tcfA*(1) allele has only been found in two of eight American *B. pertussis* isolates from the pre-vaccination era (van Loo *et al.*, 2002) and this is the same as the *tcfA* GenBank sequence (U16754) of *B. pertussis* strain 18323^{T} originally deposited by Finn and Stevens (1995). The *tcfA* alleles *tcfA*(2-4 and 6) also differ from one another by nonsilent nucleotide differences, whereas *tcfA*(5) is predicted to encode a truncated Tcf protein by virtue of a nucleotide addition which alters the reading frame to create a premature translational stop codon.

The summary table (Table 3.1.2.1) displays the overall frequencies of each *tcfA* allele found in the UK *B. pertussis* population from 1920-2002 together with their respective GenBank numbers. The allele *tcfA*(2) was predominant amongst the UK *B. pertussis* population (124 of 138; 89.9%). To determine if there has been shift in *tcfA* variation, the *tcfA* sequence types were grouped according to the period of isolation (Fig. 3.1.4.2). There appears to have been no particular trend, but different *tcfA* alleles have appeared throughout time period studied. However, these were found only at low frequency. Therefore, *tcfA*(2) has been the predominant allele in all time groups (Fig. 3.1.4.2). During 1950-1959, 1960-1969 and 2002, the only allele detected was *tcfA*(2). The two isolates that had the *tcfA* allele *tcfA*(3) were from a pertussis case in paediatric intensive care isolated in 1999 (Crowcroft *et al.*, 2003), and from an unvaccinated individual who was less than two months old, in 1999. Nine isolates from 1970-1999 were *tcfA*(4). The *tcfA*(5) isolate was from 1942. The two strains with the novel *tcfA* type, *tcfA*(6), were isolated in 1983. All UK vaccine strains were *tcfA*(2) and the type strain was *tcfA*(1) (Appendix 8). The genome sequence of Tohama-I carried the *tcfA*(2) allele.

3.1.5 Adenylate cyclase toxin gene (*cyaA*)

Sequence variation was investigated using conventional dideoxy sequencing in 42 UK isolates of *B. pertussis*, together with the four vaccine strains and the type strain NCTC10739^{T} in the region (2512 bp) of the *cyaA* gene encoding the immunodominant moiety. The CyaA protein that this gene encodes is being investigated for possible inclusion in future generation pertussis ACVs and for its use in multipurpose vaccines. Therefore, it is important to determine its potential for antigenic variation. Variation or lack of variation in the *B. pertussis cyaA* gene has not been described previously.

Figure 3.1.4.2: Trends in tracheal colonisation factor (*tcfA*) alleles in UK isolates of *B. pertussis* from 1920-2002.

Key:

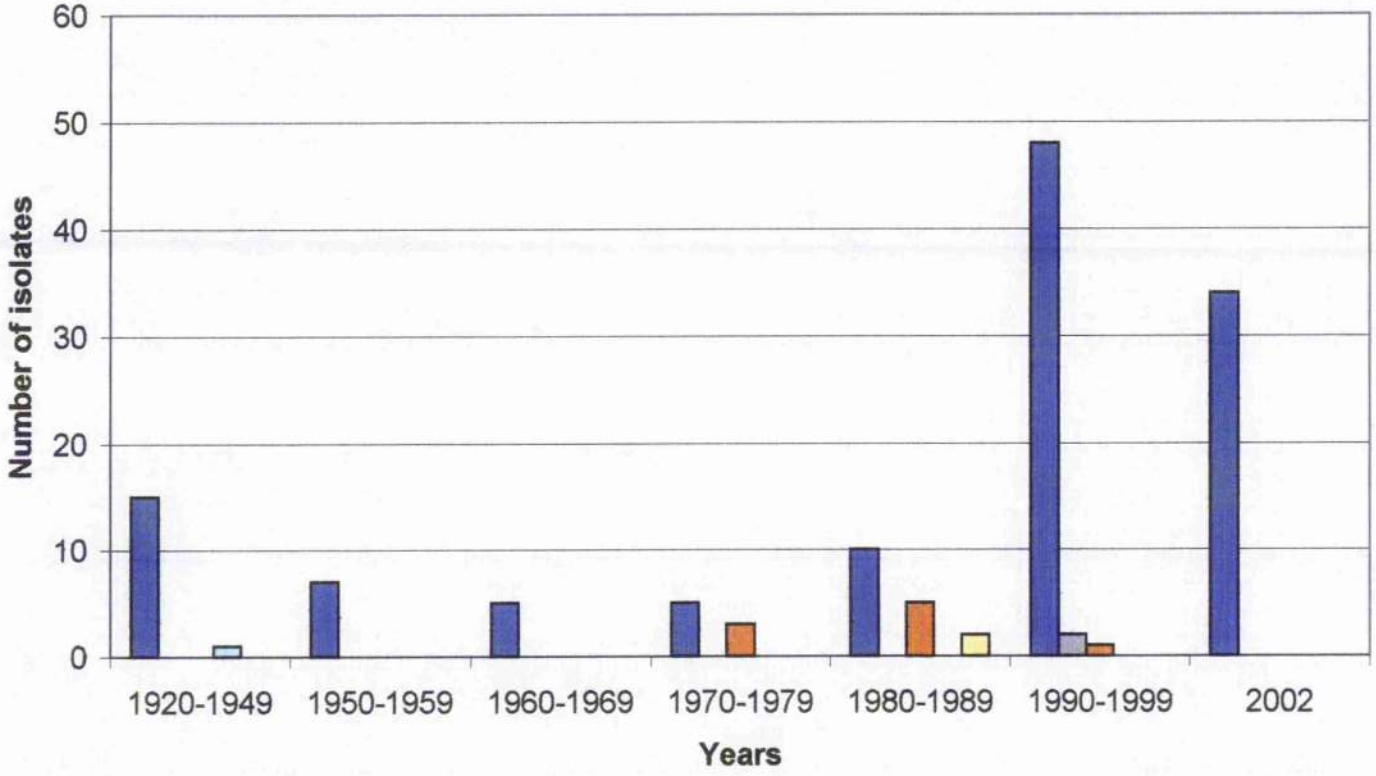
tcfA(2)

tcfA(3)

tcfA(4)

tcfA(5)

tcfA(6)


60
50
40
30
20
10
0
Number of isolates
1920-1949
1950-1959
1960-1969
1970-1979
1980-1989
1990-1999
2002
Years

Only one allelic type was detected amongst UK *B. pertussis* isolates from 1920-2002 (Table 3.1.2.1). The allelic type of *cyaA* found in the type strain of *B. pertussis* sequenced in this study was designated *cyaA*(1). The two allele types, *cyaA*(1) and *cyaA*(2) differ by only a single nucleotide at position 4403 corresponding to a synonymous mutation; *cyaA*(1) has the codon CTA (Leu) and *cyaA*(2) has the codon CTG (Leu). Comparison of the *cyaA* sequence from the type strain (NCTC10739^T) determined in this study with that of the same strain obtained from GenBank (Y00545) (Glaser *et al.*, 1988) showed one other nucleotide difference at position 3981 (Y00545). This difference was a C nucleotide rather than the G nucleotide present at this position in the GenBank sequence. In our study, sequencing from forward and reverse primers covering this region was done in triplicate with concordant results. All other isolates analysed in this study, including the four vaccine strains, and 42 isolates of *B. pertussis* isolated in the United Kingdom from 1920-2002 belonged to the distinct allelic type designated *cyaA*(2) (Table 3.1.2.1, Appendix 8). The genome sequence of Tohama-I was also found to be *cyaA*(2). Thus, no polymorphism was found in the isolates and there was no allelic variation between the vaccine strains and the isolates in a potentially important, immunodominant region of *cyaA*.

3.1.6 Outer-membrane protein Q gene (*ompQ*), virulence-activated gene 8 (*vag8*), bordetella resistance to killing protein gene A (*brkA*) and bordetella autotransporter protein C gene (*bapC*).

Sequence polymorphism of *ompQ* was investigated in 42 UK isolates chosen from different time periods and in the four vaccine strains. Alleles of *ompQ* were determined by Pyrosequencing and examples of pyrograms are shown in Appendix 9. This real-time sequencing method was used as *ompQ* variation had been determined previously by van Loo *et al.* (2002) and was defined as a single nucleotide polymorphism. Pyrosequencing offers a more rapid method for determining single nucleotide variation amongst samples than conventional sequencing. There are two known *ompQ* alleles (van Loo *et al.*, 2002) and only one of these was found in the UK *B. pertussis* population. The two alleles of *ompQ* are defined by a nonsilent single nucleotide polymorphism at position 1465 (U16266), *ompQ*(1) (CTG, Leu), found only in the type strain, and *ompQ*(2) (CCG, Pro) found to be present in all 42 UK isolates and the four vaccine strains (Appendix 8, Table 3.1.2.1). The *B. pertussis* Tohama-I genome sequence was determined to be *ompQ*(2).

Nine isolates selected from each of the different time periods, the four vaccine strains and the type strain (NCTC10739^T) were investigated for variation in (all or part of) the *vag8*, *brkA* and *bapC* genes by conventional DNA sequencing. Two alleles of *vag8* have been reported by van Loo *et al.* (2002), therefore the same polymorphic sites were targeted to directly compare allelic variation of the strain panel used for this PhD study and Dutch *B. pertussis* isolates. There was no sequence variation in *vag8* between UK isolates and the UK vaccine strains, but there were three single nucleotide differences in this gene between the type strain and the other strains (Appendix 8). These differences occurred at positions 510, 782 and 2697 (U90124) (CGT, Arg; GTC, Val; CGC, Arg) *vag8*(1), in the type strain, and (CGC, Arg; GCC, Ala; CGT, Arg) *vag8*(2) in all other strains and the Tohama-I genome sequence.

The recently described putative autotransporter gene, *bapC*, has not been investigated for sequence variation. This gene encodes an autotransporter protein, which is a potential virulence factor and protective antigen. Therefore it is important to investigate its potential for antigenic variation. There was a single nucleotide difference at position 2159 (AF081494), (CCT, Pro) *bapC*(1) in all of the UK isolates, the four vaccine strains and the Tohama-I genome sequence, compared with (CCC, Pro) *bapC*(2) in the type strain (Appendix 8, Table 3.1.2.1).

The gene *brkA* that encodes an autotransporter has been investigated for sequence variation and none was found amongst Dutch *B. pertussis* isolates (van Loo *et al.*, 2002). It was considered informative to determine if this was also observed amongst UK *B. pertussis* isolates. No sequence variation was found in *brkA* between the UK isolates, the four vaccine strains, the Tohama-I genome sequence or the type strain (Appendix 8, Table 3.1.2.1).

3.2 MULTILOCUS SEQUENCE TYPING

Multilocus sequence types (MLST-1 to -9) based on point mutations in the three genes *ptxA*, *ptxC*, and *tcfA* have been described by van Loo *et al.* (2002). This scheme was applied to 136 UK *B. pertussis* isolates from 1920-2002 and the four vaccine strains. The various combinations of the three genes *ptxA*, *ptxC*, and *tcfA* that contribute to different MLSTs are listed in Table 3.2.1.

Table 3.2.1. Multilocus sequence types (MLSTs) of *B. pertussis* defined by alleles of genes coding for pertussis toxin S1 subunit (*ptxA*), S3 subunit (*ptxC*), and tracheal colonisation factor (*tcfA*) (van Loo *et al.*, 2002; this study).

MLST	**Alleles**
MLST-1	*ptxA*(3), *ptxC*(1), *tcfA*(2)
MLST-2	*ptxA*(2), *ptxC*(1), *tcfA*(2)
MLST-3	*ptxA*(1), *ptxC*(1), *tcfA*(2)
MLST-4	*ptxA*(1), *ptxC*(1), *tcfA*(3)
MLST-5	*ptxA*(1), *ptxC*(2), *tcfA*(2)
MLST-6	*ptxA*(1), *ptxC*(1), *tcfA*(4)
MLST-7	*ptxA*(1), *ptxC*(1), *tcfA*(5)
MLST-8	*ptxA*(2), *ptxC*(2), *tcfA*(2)
MLST-9	*ptxA*(1), *ptxC*(1), *tcfA*(1)
MLST-10*	*ptxA*(1), *ptxC*(1), *tcfA*(6)
MLST-11*	*ptxA*(2), *ptxC*(1), *tcfA*(5)

* Novel MLSTs found in this study.

The MLSTs listed are based on those previously described by van Loo *et al.* (2002) and this list has been expanded to include two novel MLSTs based on allelic combinations found during this study. MLST-10 had not been previously described and includes the discovery of a new *tcfA* allele, *tcfA*(6), and MLST-11 is based on a unique combination of previously described alleles.

3.2.1 Temporal analysis of multilocus sequence types of *Bordetella pertussis* in the UK

The temporal trends in MLST frequencies in the UK were examined by dividing the panel of *B. pertussis* isolates into seven time periods: 1920-1949, 1950-1959, 1960-1969, 1970-1979, 1980-1989, 1990-1999 and 2002 (Fig. 3.2.1.1). As explained earlier, isolates were not available for all years and, for ease of describing the trends and for comparison with other *B. pertussis* populations, the isolates were split into these time groups. Seven distinct MLST types were identified in a collection of 136 UK isolates: MLST-2, 3, 4, 5, 6, 10 and 11.

The earliest available UK isolate from 1920 was MLST-2. Three MLSTs, MLST-2, MLST-3 and MLST-11, were observed prior to the widespread introduction of pertussis vaccination in the UK in the 1950s. Of the 23 isolates from the pre-vaccination era, 10 were MLST-2 (*ptxA*(2), *ptxC*(1), *tcfA*(2)) and 12 were MLST-3 (*ptxA*(1), *ptxC*(1), *tcfA*(2)) while an isolate from 1942 was a novel MLST type, MLST-11 (*ptxA*(2), *ptxC*(1), *tcfA*(5)) (Fig. 3.2.1.1). MLST-2 persisted into the time period 1980-1989 but has not been seen in isolates from the panel since 1985 (there were no isolates available from 1986 to 1997). MLST-3 was found in all time periods studied, the most recent of which was from 2002. Nine isolates from 1970-1999 were MLST-6 (*ptxA*(1), *ptxC*(1), *tcfA*(4)) (Fig. 3.2.1.1). Another novel MLST type was identified in two clinical isolates from 1983, namely MLST-10 defined as *ptxA*(1), *ptxC*(1), *tcfA*(6). MLST-4 (*ptxA*(1), *ptxC*(1), *tcfA*(3)) was seen only in two isolates, from 1999.

In the recent isolates from 2002, only two MLST types were found, MLST-5 (n=31), detected only from 1990 onwards, and a single representative of the MLST-3 type (Fig. 3.2.1.1). It is important to note that MLST-5 was not present in the four UK vaccine strains. Two of the three UK WCV strains were MLST-3 (CN5476 and CN3099) and the other was

Figure 3.2.1.1: Temporal trends in the frequency of multilocus sequence types (MLSTs) in the UK *B. pertussis* population.

Key:

MLST-2 ■ **Vaccine strains**: (CN2992, Tohama-I)

MLST-3 ■ **Vaccine strains**: (CN3099, CN5476)

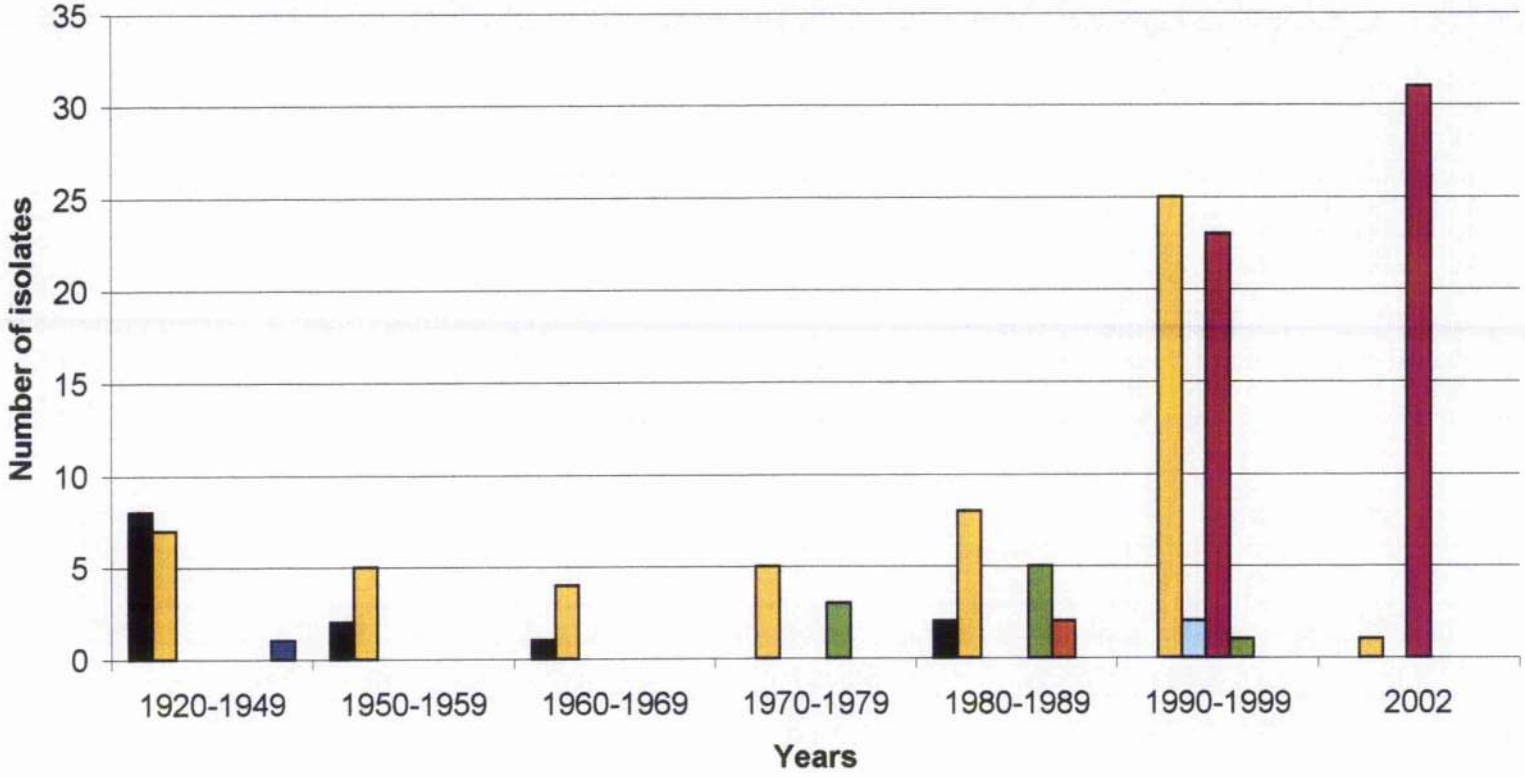
MLST-4 ■

MLST-5 ■

MLST-6 ■

MLST-10 ■

MLST-11 ■


35
30
25
20
15
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5
0
Number of isolates
1920-1949
1950-1959
1960-1969
1970-1979
1980-1989
1990-1999
2002
Years

MLST-2 (CN2992) (Fig. 3.2.1.1, Appendix 8). The ACV strain, Tohama-I, was MLST-2 and the type strain of *B. pertussis* was MLST-9 (Appendix 8).

3.2.2 Correlation of MLST with pertactin (*prnA*) types

The variation found in the pertactin (*prnA*) gene amongst UK isolates has been described elsewhere (Fry *et al.*, 2001; Packard *et al.*, 2004). In previous work, the *prnA* gene was used alongside the *ptxA* gene as markers to determine if allelic shift has occurred amongst *B. pertussis* isolates world-wide (section 1.13.2). Several *prnA* alleles, have been described, *prnA*(1-8) (Mooi *et al.*, 2000), *prnA*(9) (AF218785) and *prnA*(11) (AJ507642).

These alleles differ by the number of repeats found in the molecule's immunodominant regions 1 and 2 or, with *prnA*(7) a point mutation upstream of region 2. Three known *prnA* alleles, *prnA*(1-3), have been identified amongst 138 UK *B. pertussis* isolates within the panel (Table 3.1.2.1). The *prnA* trends are depicted in Fig. 3.2.2.1 and it is shown that a shift in *prnA* type has occurred over time. All isolates and the four vaccine strains from 1920-1979 were *prnA*(1) and the non-vaccine *prnA* alleles were first identified in isolates from the 1980s. The predominant allele amongst recent UK isolates from 1990-1999 and 2002 is the non-vaccine *prnA* allele, *prnA*(2). The *B. pertussis* Tohama-I genome sequence contained *prnA*(1) and the type strain was *prnA*(6).

Multilocus sequence types of 136 UK *B. pertussis* isolates and the four vaccine strains were compared to their previously determined *prnA* types and the distribution of these three *prnA* types within the various MLSTs. MLST-2 (n=13), -6 (n=9), -10 (n=2) and -11 (n=1) isolates were all *prnA*(1). MLST-3 isolates (n=12) were *prnA*(1) during the pre-vaccination era but contained representatives of all three UK *prnA* (*prnA*(1)-*prnA*(3)) alleles after 1980. However, a greater number of MLST-3 isolates were associated with *prnA*(1) (Fig. 3.2.2.2). Of the two MLST-4 types, one was *prnA*(2) and the other was *prnA*(3). The majority of MLST-5 isolates (n=29) were linked with *prnA*(2), and one was *prnA*(3). Two of the three WCV strains were MLST-3 *prnA*(1) and the other was MLST-2 *prnA*(1). The ACV strain was MLST-2 *prnA*(1) and the type strain of *B. pertussis* was MLST-9 *prnA*(6).

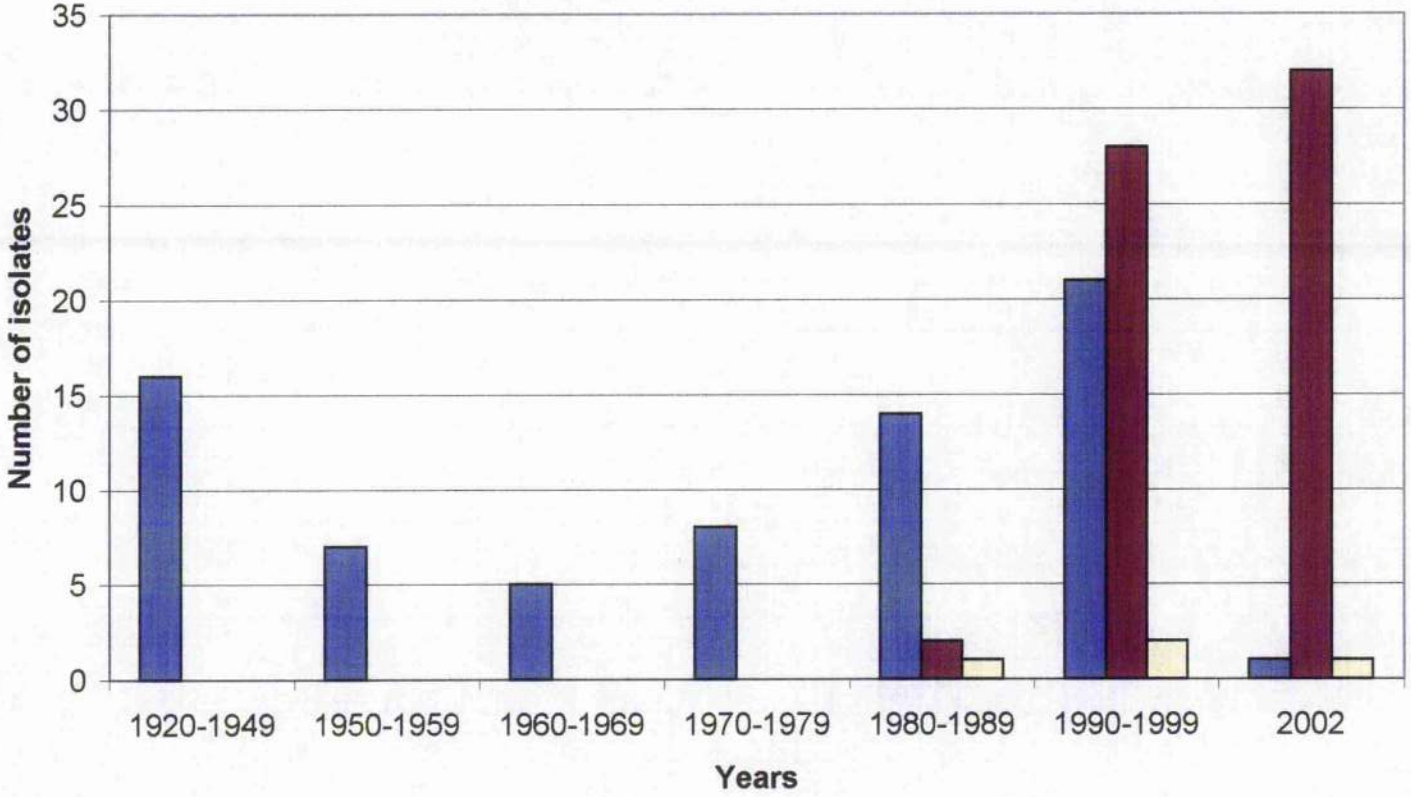
Figure 3.2.2.1: Trends in pertactin (*prnA*) alleles in UK isolates of *B. pertussis* population from 1920-2002. Information from previous studies (Fry *et al.*, 2001; Packard *et al.*, 2004).

Key:

prnA(1)

prnA(2)

prnA(3)


35
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25
20
15
10
5
0
Number of isolates
1920-1949
1950-1959
1960-1969
1970-1979
1980-1989
1990-1999
2002
Years

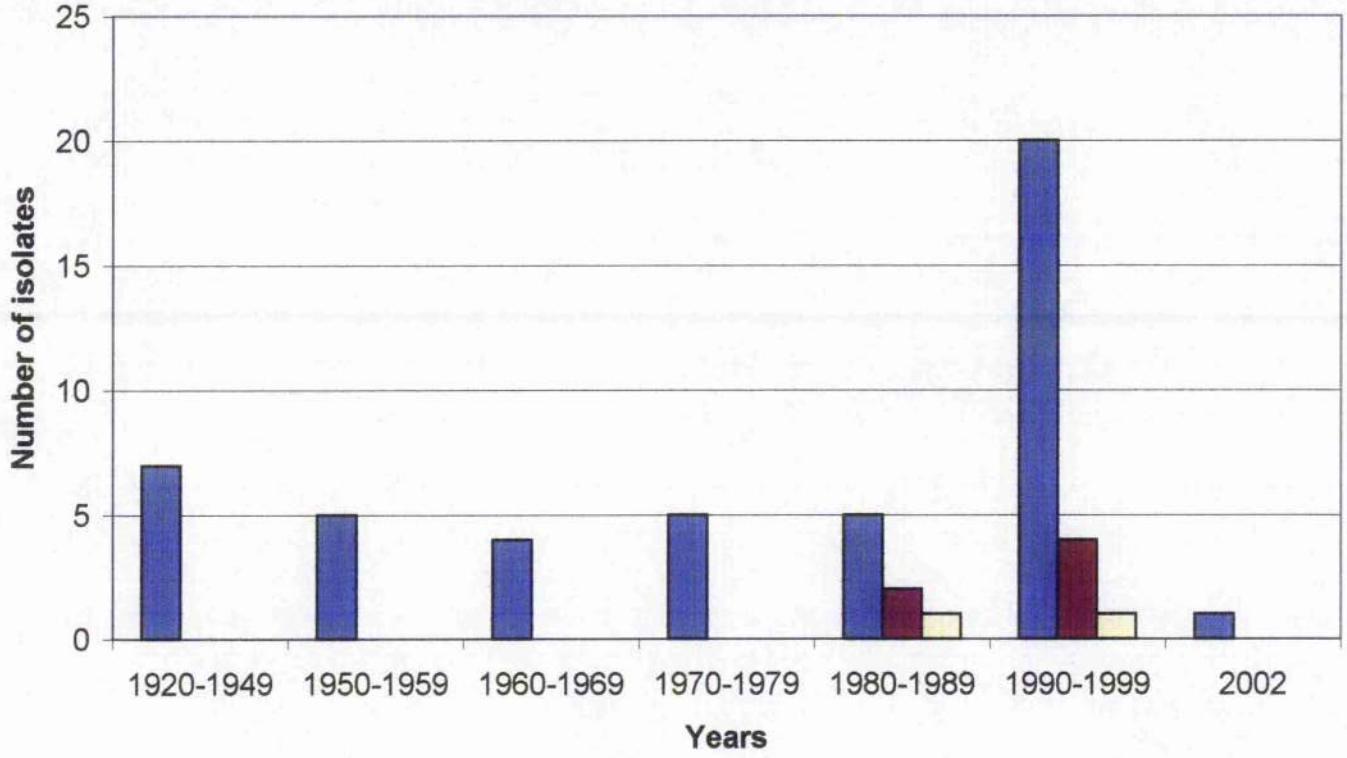
Figure 3.2.2.2: Variation in pertactin (*prnA*) type in MLST-3 isolates from the UK *B. pertussis* population.

Key:

prnA(1)

prnA(2)

prnA(3)


25
20
15
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5
0
Number of isolates
1920-1949
1950-1959
1960-1969
1970-1979
1980-1989
1990-1999
2002
Years

3.3 POLYMORPHISM IN THE GENE ENCODING FIMBRIAL ANTIGEN 2 (*fim2*)

Variation in the *fim2* gene has been described previously in Dutch *B. pertussis* isolates (van Loo *et al.*, 2002). Two *fim2* alleles were reported in Dutch isolates from the pre-vaccination era. After the introduction of vaccination only the *fim2*(1) allele was apparent. In the present study, polymorphism was investigated for eighty UK *B. pertussis* isolates from 1920-2002 and was detected at position 701 (Y00527) using the real-time sequencing technique Pyrosequencing. This method was used for detection of the single nucleotide polymorphism amongst UK isolates as it is much more rapid for attaining such results than dideoxy sequencing. The two alleles found in the UK *B. pertussis* population were *fim2*(1) (AGA, Arg) and *fim2*(2) (AAA, Lys) (Table 3.1.2.1), in agreement with those found by van Loo *et al.* (2002). Table 3.1.2.1 shows that 60 of 80 isolates (75%) were *fim2*(1) and 25% were *fim2*(2). The trends in *fim2* allelic variation found amongst the UK isolates from 1920-2002 is shown in Fig. 3.3.1. The isolates have been divided into five time groups: two groups of approximately 30 years from 1920-1985, isolates from 1998, isolates from 1999 and the most recent isolates, from 2002, as separate groups (Fig. 3.3.1). These time groups were chosen as isolates were not available for all years. Allele *fim2*(2) was only found among UK isolates from 1998-1999 (Fig. 3.3.1). The four vaccine strains, the Tohama-I genome sequence and the *B. pertussis* type strain were *fim2*(1) (Appendix 8). During 1998, 9 of 18 *B. pertussis* isolates were *fim2*(2) and in 1999, 11 of 26 isolates were *fim2*(2) . Interestingly, this allele was only linked to isolates that were serotype 1,2, determined by a previous study (Fry *et al.*, 2001), and all were MLST-3. The allele *fim2*(1), conversely, was associated with isolates that were all possible combinations of serotypes (1, 1,2, 1,2,3, 1,3).

3.4 INCLUSION OF FIMBRIAL ANTIGEN 2 (*fim2*) AND PERTACTIN (*prnA*) GENES IN MULTILOCUS VIRULENCE GENE SEQUENCE TYPING (MVGST) SCHEME

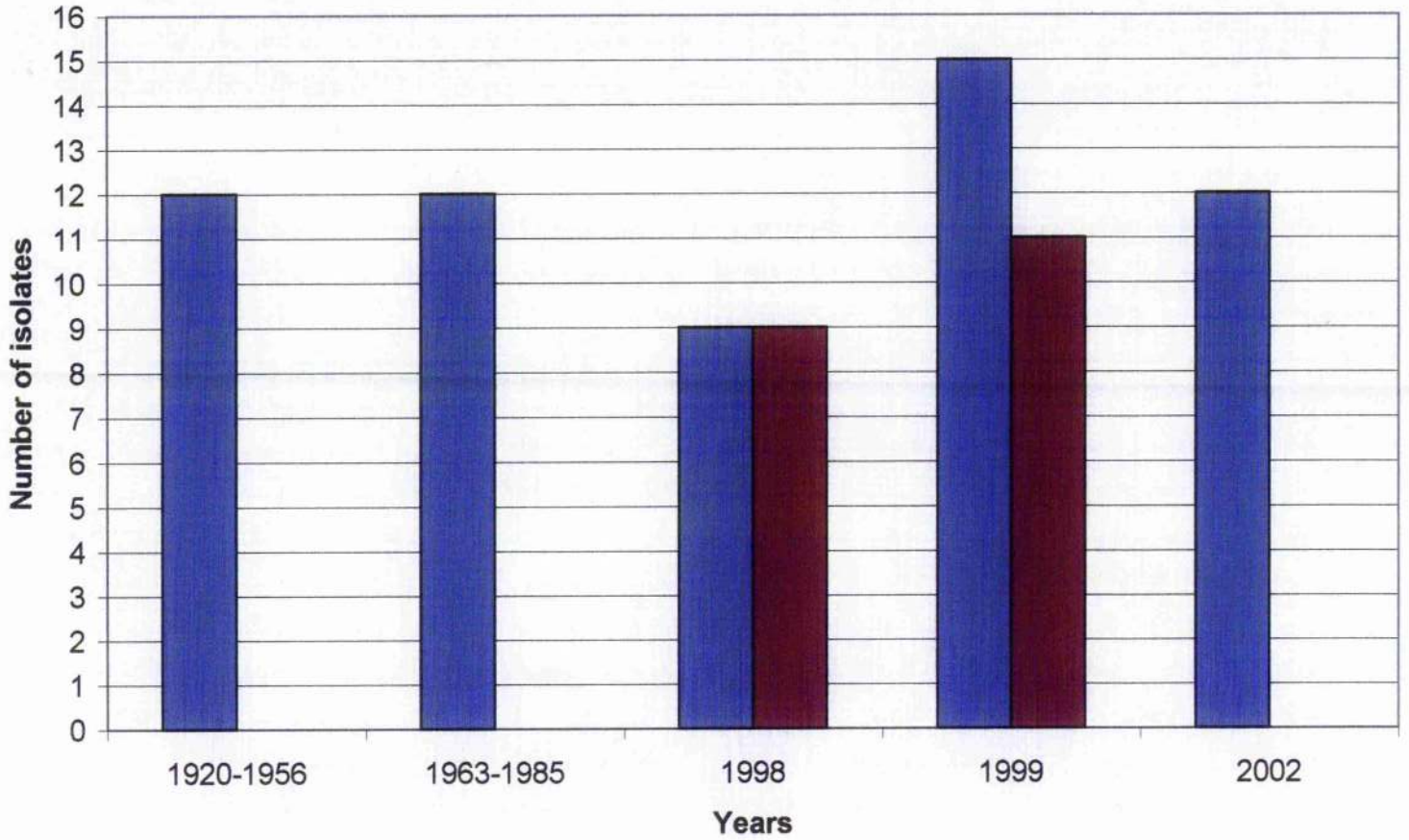
The MLST scheme developed by van Loo *et al.* (2002) was based on the allelic variation of the three gene targets *ptxA*, *ptxC* and *tcfA*, with each gene exhibiting a degree of variation suitable for use as gene markers. However, two other genes, *fim2* and *prnA*, had also demonstrated variation and therefore had potential for use in strain discrimination. The genes *fim2* and *prnA* were not included in the original MLST scheme because only Dutch *B. pertussis* isolates from the pre-vaccination era had shown variation in *fim2* and the variation

Figure 3.3.1: Trends in fimbrial antigen 2 (*fim2*) alleles in UK isolates of *B. pertussis* from 1920-2002.

Key:

fim2(1)

fim2(2)


16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
Number of isolates
1920-1956
1963-1985
1998
1999
2002
Years

of *prnA* was not deemed suitable. This is due to *prnA* variation not being a result of single nucleotide polymorphisms, but due to insertion and deletion of repeat units and this is a process that is expected to occur relatively frequently as well as reversibly (van Loo *et al.*, 2002). However, the variation of *prnA* had been used in previous sequence typing studies (see section 1.13.2) as it was considered a suitable gene target, and was therefore included here. It should also be noted that the known alleles of the MLST gene marker *tcfA* also differ due to the presence of an insertion and a deletion as well as single nucleotide differences. Sequence variation of *fim2* was only observed during the pre-vaccination era for Dutch isolates but *fim2* variation may differ with different *B. pertussis* populations. This was shown for the UK *B. pertussis* population as variation was observed during 1998-1999. Inclusion of *fim2* in a sequence typing scheme would further discriminate the same MLST isolates from the same time periods as well as between *B. pertussis* populations.

Three variations of the van Loo *et al.* (2002) MLST scheme, designated here as Multilocus Virulence Gene Sequence Typing (MVGST), are described in this section. The first scheme, MVGST(1), is based on a combination of sequence types of *ptxA*, *ptxC*, *tcfA* and *prnA*, MVGST(2) is based on a combination of sequence types of *ptxA*, *ptxC*, *tcfA* and *fim2* and MVGST(3) is based on a combination of sequence types of *ptxA*, *ptxC*, *tcfA*, *prnA* and *fim2*. There were seven MLST types found within the UK *B. pertussis* population using the original scheme (section 3.2). Sequence variation of *prnA* was known for 138 *B. pertussis* isolates and *fim2* variation was known for 80 UK *B. pertussis* isolates. The inclusion of *prnA* expanded the original scheme to eleven types. The inclusion of *fim2* resulted only in six types but not all isolates had been investigated for *fim2* variation. The inclusion of both *prnA* and *fim2* yielded ten sequence types which is a lower number of sequence types than that found with the MVGST(1) scheme, again due to the smaller number of isolates investigated for *fim2* variation. The temporal trends of particular types of *B. pertussis* according to the different MVGST schemes are described later. As before, the isolates were divided into seven time groups: six groups of approximately 10 years from 1920-1999, and the most recent isolates, 2002, as a separate group. The trends for each of the MLST schemes were examined for these same time groups, allowing comparison between the original MLST scheme and the three MVGST schemes described here.

The integration of *prnA* sequence types alone, in MVGST(1), yielded the greatest number of sequence types found amongst UK *B. pertussis* isolates but this can be partially attributed to *prnA* variation being known for a greater number of isolates than *fim2* variation. Table 3.4.1 lists the eleven combinations of MVGST(1) types found amongst the UK *B. pertussis* population. The MVGST(1) trends are depicted in Fig. 3.4.1.

There were three MVGST(1) sequence types found during the pre-vaccination era, MVGST(1)–1 to -3 and this was similar to that seen with the original MLST scheme where three types were found during the same time period. Two sequence type MVGST(1)-1 (*ptxA*(2), *ptxC*(1), *tcfA*(2), *prnA*(1)) and MVGST(1)-2 (*ptxA*(1), *ptxC*(1), *tcfA*(2), *prnA*(1)) were present during 1920-1949 at similar frequencies whereas MVGST(1)-2 dominated during the 1950s and 1960s. Lastly, MVGST(1)-3 (*ptxA*(1), *ptxC*(1), *tcfA*(5), *prnA*(1)) was seen only in one *B. pertussis* isolate, from the period 1920-1949. The four UK vaccine strains were MVGST(1)-1 or MVGST(1)-2. During the 1970s and 1980s when the pertussis vaccination rate in the UK had declined, there was the appearance of sequence types not found during 1920-1969 (Fig. 3.4.1). During the 1970s, two sequence types were apparent, MVGST(1)-2 and MVGST(1)-4 (*ptxA*(1), *ptxC*(1), *tcfA*(4), *prnA*(1)), with the vaccine strain type, MVGST(1)-2, predominating. From 1980s onwards this scheme is apparently more discriminatory than the original MLST scheme. Six sequence types were found during 1980-1989 compared to the four types found using the original scheme. Two predominating types were found, MVGST(1)-2 and MVGST(1)-4 with MVGST(1)-1 found in very few isolates (Fig. 3.4.1). Three types not seen previously were present amongst isolates from 1980-1989: MVGST(1)-5 (*ptxA*(1), *ptxC*(1), *tcfA*(2), *prnA*(2)), MVGST(1)-6 (*ptxA*(1), *ptxC*(1), *tcfA*(2), *prnA*(3)) and MVGST(1)-7 (*ptxA*(1), *ptxC*(1), *tcfA*(6), *prnA*(1)) (Fig. 3.4.1). Seven sequence types were found with this scheme (MVGST(1)) amongst *B. pertussis* isolates from the 1990s compared to four types found using the original scheme. The sequence type, MVGST(1)-2 was one of the predominating sequence types. Three types, MVGST(1)-4, -5 and -6, were present at low frequencies and there was the appearance of three types not seen previously, MVGST(1)-8, -9 and -10 (Fig. 3.4.1). MVGST(1)-8 (*ptxA*(1), *ptxC*(2), *tcfA*(2), *prnA*(2)) was a sequence type predominating during this time period and was present in a greater number of UK *B. pertussis* isolates than MVGST(1)-2. The two sequence types, MVGST(1)-9 (*ptxA*(1), *ptxC*(1), *tcfA*(3), *prnA*(2))

Table 3.4.1. Multilocus virulence gene sequence types (1) (MVGST(1)) of UK isolates of *B. pertussis* defined by alleles of genes coding for pertussis toxin S1 subunit (*ptxA*), S3 subunit (*ptxC*), tracheal colonisation factor (*tcfA*) and pertactin (*prnA*).

Sequence types	**Alleles**
MVGST(1)-1	*ptxA*(2), *ptxC*(1), *tcfA*(2), *prnA*(1)
MVGST(1)-2	*ptxA*(1), *ptxC*(1), *tcfA*(2), *prnA*(1)
MVGST(1)-3	*ptxA*(1), *ptxC*(1), *tcfA*(5), *prnA*(1)
MVGST(1)-4	*ptxA*(1), *ptxC*(1), *tcfA*(4), *prnA*(1)
MVGST(1)-5	*ptxA*(1), *ptxC*(1), *tcfA*(2), *prnA*(2)
MVGST(1)-6	*ptxA*(1), *ptxC*(1), *tcfA*(2), *prnA*(3)
MVGST(1)-7	*ptxA*(1), *ptxC*(1), *tcfA*(6), *prnA*(1)
MVGST(1)-8	*ptxA*(1), *ptxC*(2), *tcfA*(2), *prnA*(2)
MVGST(1)-9	*ptxA*(1), *ptxC*(1), *tcfA*(3), *prnA*(2)
MVGST(1)-10	*ptxA*(1), *ptxC*(1), *tcfA*(3), *prnA*(3)
MVGST(1)-11	*ptxA*(1), *ptxC*(2), *tcfA*(2), *prnA*(3)

Figure 3.4.1: Temporal trends in the frequency of multilocus virulence genes sequence types (1) (MVGST(1)) types in the UK *B. pertussis* population (135 *B. pertussis* isolates).

Key:

MVGST(1)-1 **Vaccine strains**: (CN2992, Tohama-I)

MVGST(1)-2 **Vaccine strains**: (CN3099, CN5476)

MVGST(1)-3

MVGST(1)-4

MVGST(1)-5

MVGST(1)-6

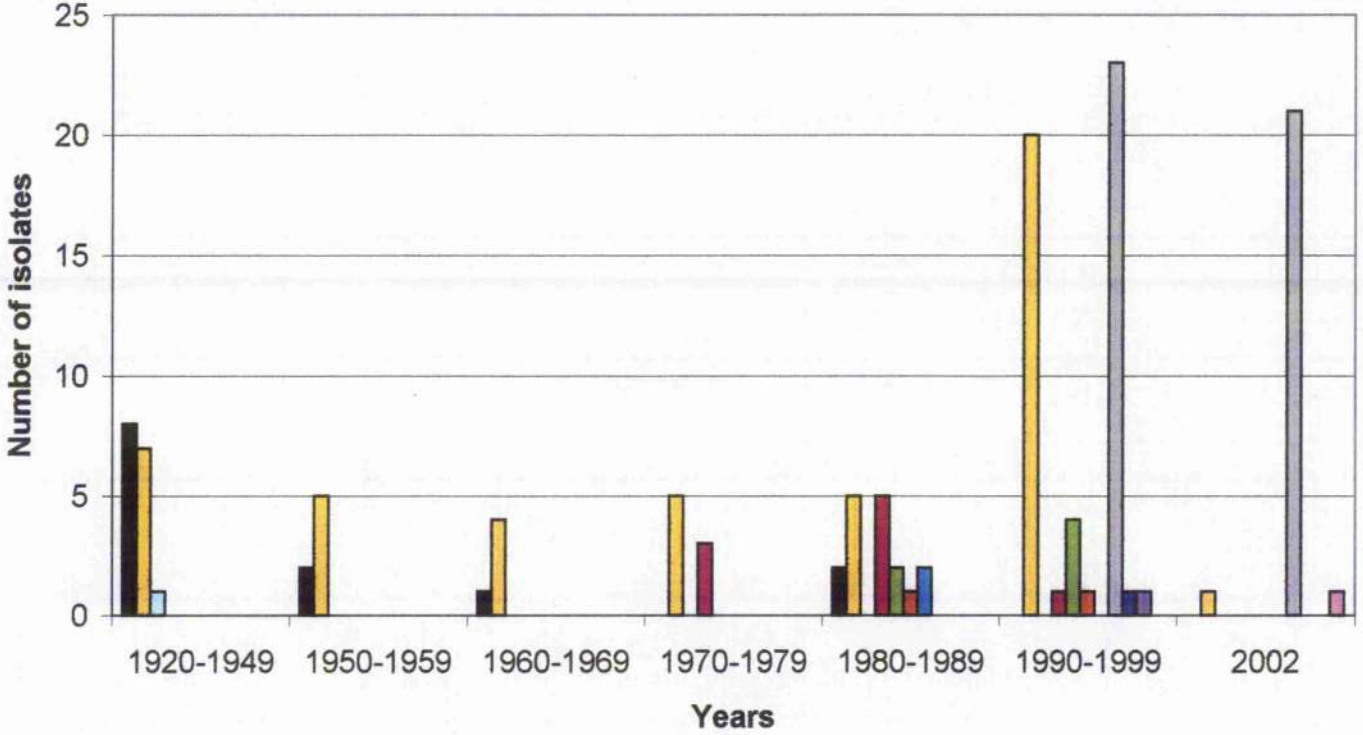
MVGST(1)-7

MVGST(1)-8

MVGST(1)-9

MVGST(1)-10

MVGST(1)-11


25
20
15
10
5
0
Number of isolates
1920-1949
1950-1959
1960-1969
1970-1979
1980-1989
1990-1999
2002
Years

and -10 (*ptxA*(1), *ptxC*(1), *tcfA*(3), *prnA*(3)), were detected in a lower number of *B. pertussis* isolates. During the most recent time period examined, 2002, three sequence types (MVGST(1)-2, -8 and -11) were identified, compared with two with the original MLST (Fig. 3.4.1). One of these types was not seen in previous time periods, MVGST(1)-11 (*ptxA*(1), *ptxC*(2), *tcfA*(2), *prnA*(3)). The predominating type was MVGST(1)-8 and MVGST(1)-2 and -11 were each found in one *B. pertussis* isolate.

The scheme MVGST(2) based on the gene variation of *ptxA*, *ptxC*, *tcfA* and *fim2*, yielded the least number of sequence types compared to MVGST(1) and MVGST(3). Regarding greater variation found with the MVGST(1) scheme this was presumably related to a smaller number of isolates having been investigated for *fim2* variation. The six MVGST(2) types that were found amongst *B. pertussis* isolates are described in Table 3.4.2. The MVGST(2) trends from 1920-2002 are shown in Fig. 3.4.2. Two types were found amongst *B. pertussis* isolates from 1920-1949, MVGST(2)-1 (*ptxA*(2), *ptxC*(1), *tcfA*(2), *fim2*(1)) and MVGST(2)-2 (*ptxA*(1), *ptxC*(1), *tcfA*(2), *fim2*(1)) with MVGST(2)-1 found in a slightly greater number of isolates (Fig. 3.4.2). The four UK vaccine strains were MVGST(2)-1 or -2. Only these two types were found until the 1970s when there was the appearance of MVGST(2)-3 (*ptxA*(1), *ptxC*(1), *tcfA*(4), *fim2*(1)). The vaccine strain types, MVGST(2)-1 and -2, were found amongst isolates from 1980s. During 1990-1999, there were four types found, MVGST(2)-2, -4 (*ptxA*(1), *ptxC*(1), *tcfA*(2), *fim2*(2)), -5 (*ptxA*(1), *ptxC*(1), *tcfA*(3), *fim2*(1)) and -6 (*ptxA*(1), *ptxC*(2), *tcfA*(2), *fim2*(1)). Two types, MVGST(2)-4 and -6, were the predominating types during the 1990s (Fig. 3.4.2). During 2002, only one type, MVGST(2)-6, was apparent.

When the sequence typing scheme MVGST(3), based on variation of all five genes, *ptxA*, *ptxC*, *tcfA*, *prnA* and *fim2* known to exhibit sequence variation amongst the UK *B. pertussis* population, was used, ten types were identified and these are listed in Table 3.4.3. Figure 3.4.3 shows the MVGST(3) trends found amongst UK *B. pertussis* isolates. Two sequence types were found amongst *B. pertussis* isolates from 1920-1969, MVGST(3)-1 (*ptxA*(2), *ptxC*(1), *tcfA*(2), *prnA*(1), *fim2*(1)) and MVGST(3)-2 (*ptxA*(1), *ptxC*(1), *tcfA*(2), *prnA*(1), *fim2*(1)) (Fig. 3.4.3). The four UK vaccine strains were also MVGST(3)-1 or -2. During 1970-1979 MVGST(3)-2 and -3 (*ptxA*(1), *ptxC*(1), *tcfA*(4), *prnA*(1), *fim2*(1)) were present.

Table 3.4.2. Multilocus virulence gene sequence types (2) (MVGST(2)) of UK isolates of *B. pertussis* defined by alleles of genes coding for pertussis toxin S1 subunit (*ptxA*), S3 subunit (*ptxC*), tracheal colonisation factor (*tcfA*) and fimbrial antigen 2 (*fim2*).

Sequence types	**Alleles**
MVGST(2)-1	*ptxA*(2), *ptxC*(1), *tcfA*(2), *fim2*(1)
MVGST(2)-2	*ptxA*(1), *ptxC*(1), *tcfA*(2), *fim2*(1)
MVGST(2)-3	*ptxA*(1), *ptxC*(1), *tcfA*(4), *fim2*(1)
MVGST(2)-4	*ptxA*(1), *ptxC*(1), *tcfA*(2), *fim2*(2)
MVGST(2)-5	*ptxA*(1), *ptxC*(1), *tcfA*(3), *fim2*(1)
MVGST(2)-6	*ptxA*(1), *ptxC*(2), *tcfA*(2), *fim2*(1)

Figure 3.4.2: Temporal trends in the frequency of multilocus virulence genes sequence types (2) (MVGST(2)) types in the UK *B. pertussis* population (80 *B. pertussis* isolates).

Key:

MVGST(2)-1 ■ **Vaccine strains**: (CN2992, Tohama-I)

MVGST(2)-2 ■ **Vaccine strains**: (CN3099, CN5476)

MVGST(2)-3 ■

MVGST(2)-4 ■

MVGST(2)-5 ■

MVGST(2)-6 ■

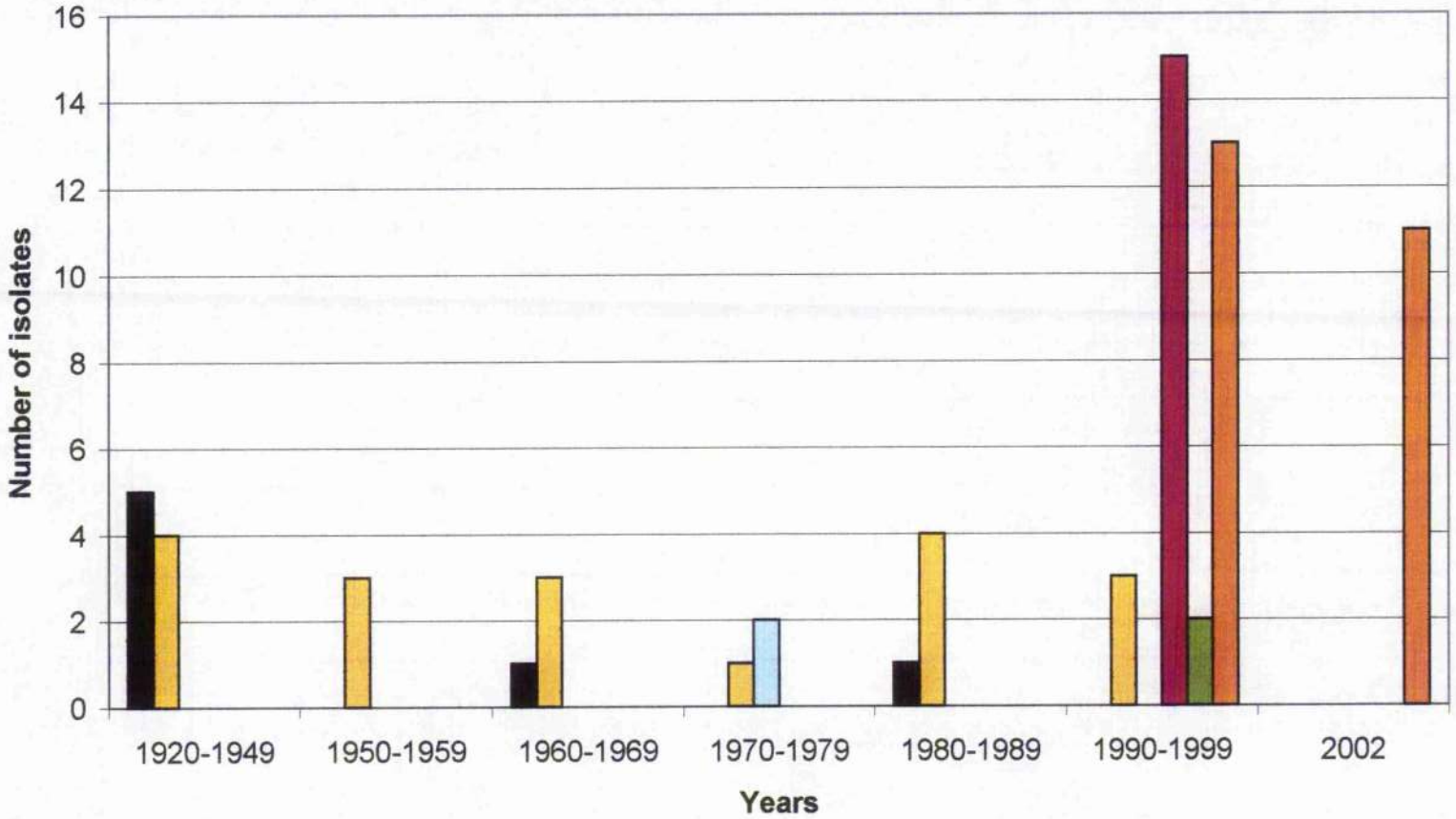

16
14
12
10
8
6
4
2
0
Number of isolates
1920-1949
1950-1959
1960-1969
1970-1979
1980-1989
1990-1999
2002
Years

Table 3.4.3. Multilocus virulence gene sequence types (3) (MVGST(3)) of UK isolates of *B. pertussis* defined by alleles of genes coding for pertussis toxin S1 subunit (*ptxA*), S3 subunit (*ptxC*), tracheal colonisation factor (*tcfA*), pertactin (*prnA*) and fimbrial antigen 2 (*fim2*).

Sequence types	**Alleles**
MVGST(3)-1	*ptxA*(2), *ptxC*(1), *tcfA*(2), *prnA*(1), *fim2*(1)
MVGST(3)-2	*ptxA*(1), *ptxC*(1), *tcfA*(2), *prnA*(1), *fim2*(1)
MVGST(3)-3	*ptxA*(1), *ptxC*(1), *tcfA*(4), *prnA*(1), *fim2*(1)
MVGST(3)-4	*ptxA*(1), *ptxC*(1), *tcfA*(2), *prnA*(2), *fim2*(1)
MVGST(3)-5	*ptxA*(1), *ptxC*(1), *tcfA*(2), *prnA*(3), *fim2*(1)
MVGST(3)-6	*ptxA*(1), *ptxC*(1), *tcfA*(2), *prnA*(1), *fim2*(2)
MVGST(3)-7	*ptxA*(1), *ptxC*(1), *tcfA*(2), *prnA*(2), *fim2*(2)
MVGST(3)-8	*ptxA*(1), *ptxC*(2), *tcfA*(2), *prnA*(2), *fim2*(1)
MVGST(3)-9	*ptxA*(1), *ptxC*(1), *tcfA*(3), *prnA*(3), *fim2*(1)
MVGST(3)-10	*ptxA*(1), *ptxC*(1), *tcfA*(3), *prnA*(2), *fim2*(1)

Figure 3.4.3: Temporal trends in the frequency of multilocus virulence genes sequence types (3) (MVGST(3)) in the UK *B. pertussis* population (80 *B. pertussis* isolates).

Key:

MVGST(3)-1 **Vaccine strains**: (CN2992, Tohama-I)

MVGST(3)-2 **Vaccine strains**: (CN3099, CN5476)

MVGST(3)-3

MVGST(3)-4

MVGST(3)-5

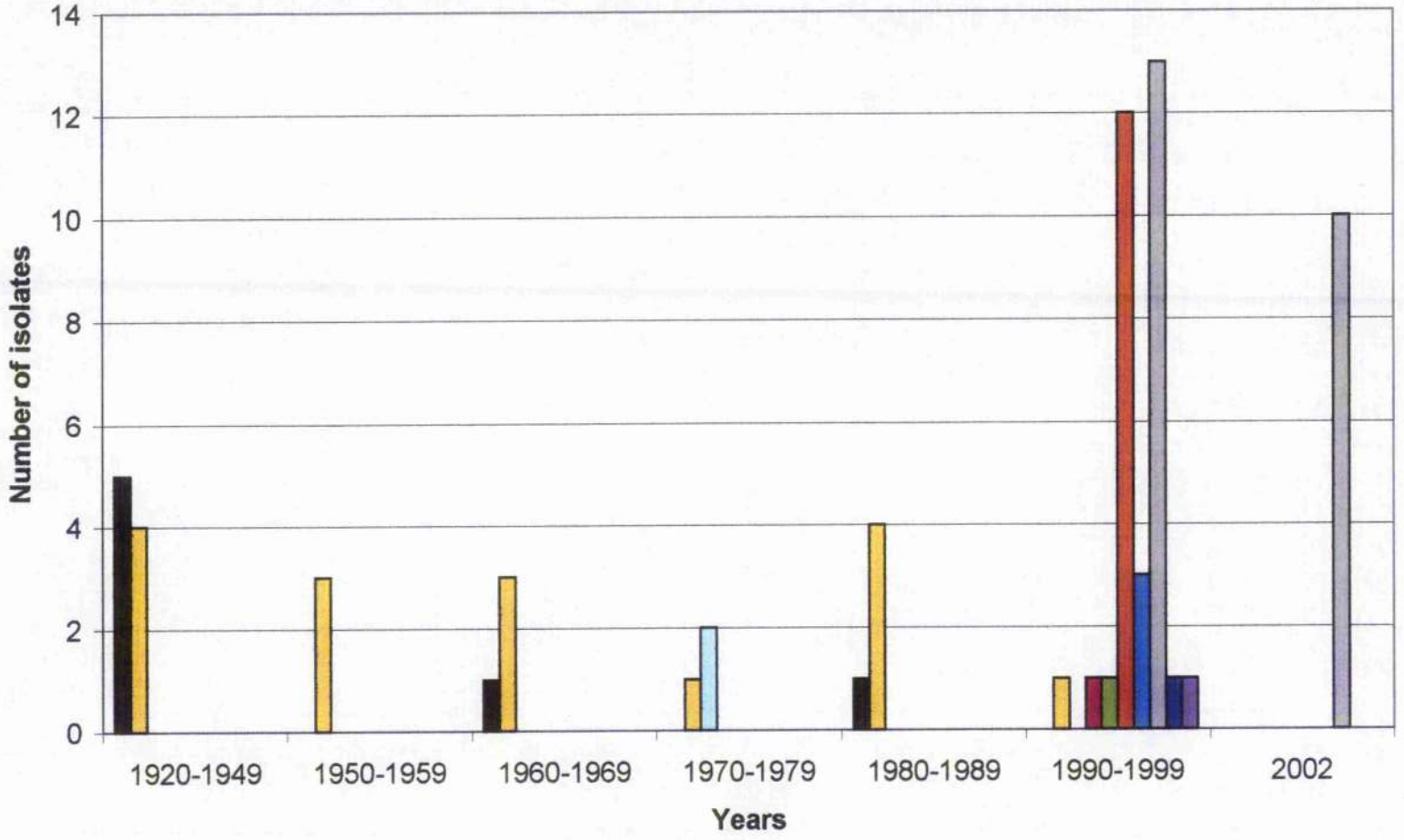
MVGST(3)-6

MVGST(3)-7

MVGST(3)-8

MVGST(3)-9

MVGST(3)-10


14
12
10
8
6
4
2
0
Number of isolates
1920-1949
1950-1959
1960-1969
1970-1979
1980-1989
1990-1999
2002
Years

The vaccine strain types, MVGST(3)-1 and -2, were found amongst isolates from 1980-1989.

This method was found to be the most discriminatory for isolates from 1990-1999 compared to MVGST(1), MVGST(2) and the original MLST. Eight sequence types were found amongst isolates from this time period. The sequence type, MVGST(3)-2 was present in a low number of isolates and MVGST(3)-4 to -10 were present during this time period and were not apparent in other years (Fig. 3.4.3). Sequence types MVGST(3)-4 (*ptxA*(1), *ptxC*(1), *tcfA*(2), *prnA*(2), *fim2*(1)), MVGST(3)-5 (*ptxA*(1), *ptxC*(1), *tcfA*(2), *prnA*(3), *fim2*(1)), MVGST(3)-9 (*ptxA*(1), *ptxC*(1), *tcfA*(3), *prnA*(3), *fim2*(1)) and MVGST(3)-10 (*ptxA*(1), *ptxC*(1), *tcfA*(3), *prnA*(2), *fim2*(1)) were each found only in one *B. pertussis* isolate. MVGST(3)-7 (*ptxA*(1), *ptxC*(1), *tcfA*(2), *prnA*(2), *fim2*(2)) was also found in a small number of isolates. Two types predominated: MVGST(3)-6 (*ptxA*(1), *ptxC*(1), *tcfA*(2), *prnA*(1), *fim2*(2)) and MVGST(3)-8 (*ptxA*(1), *ptxC*(2), *tcfA*(2), *prnA*(2), *fim2*(1)) amongst isolates from the 1990s. During the most recent time period examined, 2002, all isolates were MVGST(3)-8 (Fig. 3.4.3).

It was found that with the inclusion of *prnA* the number of sequence types increased compared to using only *ptxA*, *ptxC* and *tcfA*, thus discriminating amongst *B. pertussis* isolates that belonged to a particular MLST according to the original scheme. All three schemes showed that a shift in UK *B. pertussis* population occurred between the pre-vaccination era and the present day. A larger number of sequence types were identified amongst isolates from the 1980s and 1990s with the MVGST(1) and MVGST(3) schemes compared to the number found using the original MLST scheme. However, each scheme has demonstrated that novel sequence types were present in *B. pertussis* isolates from 2002 that were not found amongst isolates from the pre-vaccination era or the immediate post-vaccination era and which also differed from the sequence types of the four UK vaccine strains.

3.5 *cyaA* PROMOTER

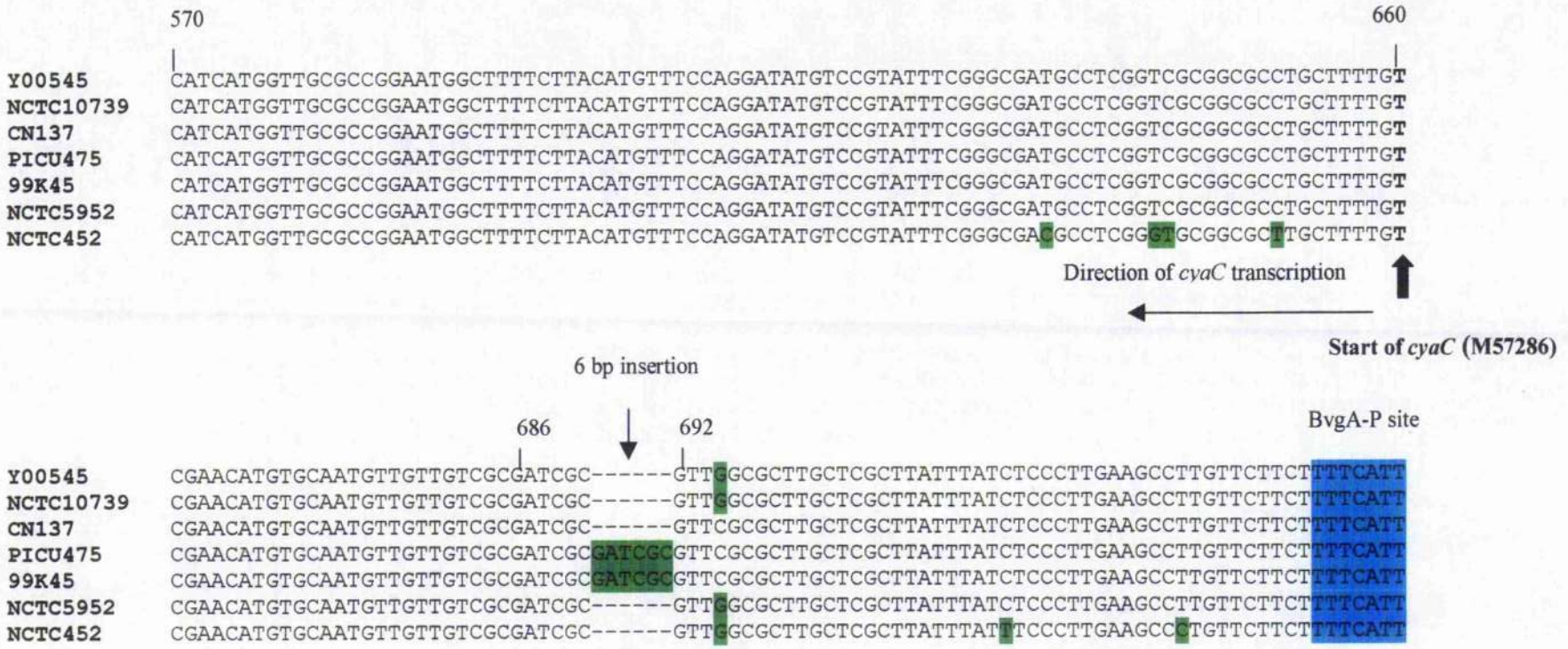
3.5.1 Polymorphism in the *cyaA* promoter region

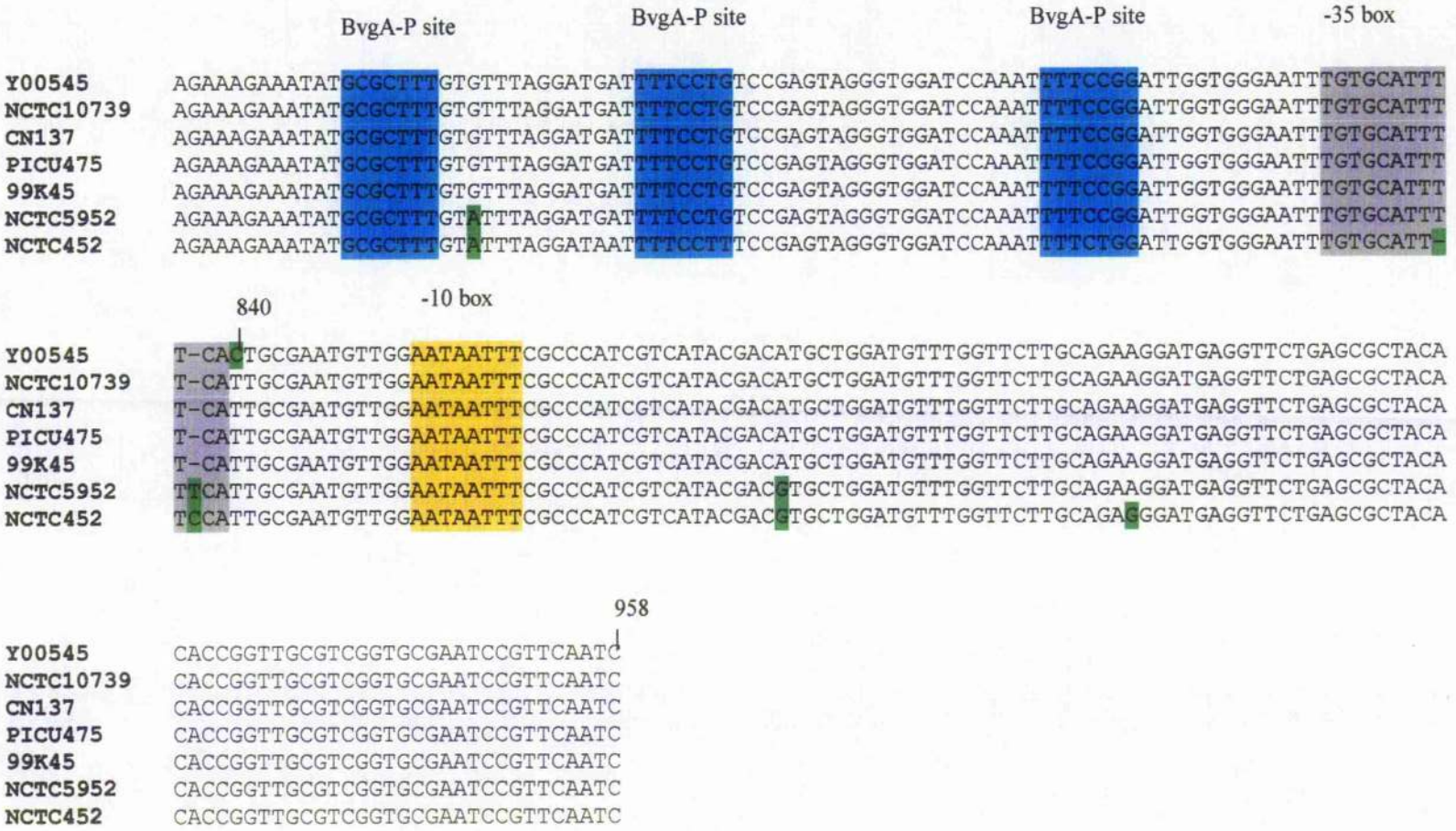
One of the objectives of this investigation was to study the expression of the *cyaA* gene that

encodes the virulence factor CyaA under different growth conditions. For this, the promoter region of the *cyaA* gene was successfully cloned upstream of the promoterless *lux* operon contained on the plasmid, pSB395, and the promoterless *gfp* gene contained on the plasmid pPROBE-AT', which would be used as reporters for *cyaA* expression. The type strain of *B. pertussis* has been previously described to have three times more adenylate cyclase enzymic (AC) activity and to express three times more CyaA protein than the strain Tohama-I (Guiso *et al.,* 1991). It was therefore desirable to determine if sequence variation existed in the promoter region of *cyaA* between the type strain and other isolates that might explain the difference in expression.

The region was amplified using primers CyaA1 and CyaA6, which targeted bases 287-1165 (Y00545), containing the *cyaA* promoter region. This amplified region was sequenced for ten UK *B. pertussis* isolates (including the non-UK *B. pertussis* type strain, NCTC10739^T) from different time periods and *B. pertussis* Tohama-I derivative strain BP338 that has been used in conductimetry analysis and is the host strain for the *lux* and *gfp* plasmid cloning work (section 3.9). The four vaccine strains were not included in the original investigation for sequence variation. The available GenBank sequence for *cyaA* gene is of the *B. pertussis* type strain (18323^T) and sequencing results were aligned with this control sequence for analyses. The type strain NCTC10739^T was sequenced for this region to ensure that this was the same as that of the submitted GenBank sequence. There was a single nucleotide difference between the GenBank sequence Y00545 and that of strain NCTC10739^T identified at position 840 and this latter sequence was designated *cyaApr*(1). Strain BP338 was also sequenced in this region and a single difference was found between BP338 sequence and NCTC10739^T at nucleotide position 695 (Fig. 3.5.1.1). Only one of the ten isolates in the panel was *cyaApr*(1). Seven were of the same *cyaApr* sequence type as that of BP338, *cyaApr*(2). There were two isolates, PICU475 and 99K45, of the panel of 10 isolates, that contained a 6 bp insertion at nucleotide position 692 (Y00545) which lies between *cyaA* and the *cyaC* gene upstream of *cyaA* promoter. It should be noted that the 6 bp insert does not actually overlap with the *cyaC* GenBank sequence (M57286) as this insertion is situated approximately 31 bases from the start of *cyaC* (Fig. 3.5.1.1). However, it is situated close to the start of the *cyaC* sequence. This sequence type was designated *cyaApr*(3). Sequencing of strains 99K45 and PICU475 for the same region using primers

Figure 3.5.1.1. Alignment of the adenylate cyclase toxin (*cyaA*) promoter region of *B. pertussis* strains (NCTC10739^{T}, CN137, PICU475 and 99K45) with the GenBank sequence of the type strain (Y00545), *B. parapertussis* strains (NCTC5952), and *B. bronchiseptica* strain (NCTC452). The areas highlighted in green are differences in sequence compared to that of the *cyaA* promoter sequence predominantly found amongst isolates and this is represented by CN137. The -10 and -35 areas are highlighted in yellow and grey respectively. The binding sites of phosphorylated BvgA (BvgA-P) predicted by Karimova *et al.*, 1996 are highlighted in blue. The numbering on the diagram is relative to that of Y00545. The bold letter and arrow at position 660 indicates the start of the accessory gene (*cyaC*) GenBank sequence M57286. The overlap between M57286 and the sequence amplified is bases 1-91 (M57286).


570
660
Y00545 CATCATGGTTGCGCCGGAATGGCTTTTCTTACATGTTTCCAGGATATGTCCGTATTTCGGGCGATGCCTCGGTCGCGGCGCCTGCTTTTGT
NCTC10739 CATCATGGTTGCGCCGGAATGGCTTTTCTTACATGTTTCCAGGATATGTCCGTATTTCGGGCGATGCCTCGGTCGCGGCGCCTGCTTTTGT
CN137 CATCATGGTTGCGCCGGAATGGCTTTTCTTACATGTTTCCAGGATATGTCCGTATTTCGGGCGATGCCTCGGTCGCGGCGCCTGCTTTTGT
PICU475 CATCATGGTTGCGCCGGAATGGCTTTTCTTACATGTTTCCAGGATATGTCCGTATTTCGGGCGATGCCTCGGTCGCGGCGCCTGCTTTTGT
99K45 CATCATGGTTGCGCCGGAATGGCTTTTCTTACATGTTTCCAGGATATGTCCGTATTTCGGGCGATGCCTCGGTCGCGGCGCCTGCTTTTGT
NCTC5952 CATCATGGTTGCGCCGGAATGGCTTTTCTTACATGTTTCCAGGATATGTCCGTATTTCGGGCGATGCCTCGGTCGCGGCGCCTGCTTTTGT
NCTC452 CATCATGGTTGCGCCGGAATGGCTTTTCTTACATGTTTCCAGGATATGTCCGTATTTCGGGCGACGCCTCGGGTGCGGCGCTTGCTTTTGT
Direction of cyaC transcription
Start of cyaC (M57286)
6 bp insertion
686
692
BvgA-P site
Y00545 CGAACATGTGCAATGTTGTTGTCGCGATCGC------GTTGGCGCTTGCTCGCTTATTTATCTCCCTTGAAGCCTTGTTCTTCTTTTCATT
NCTC10739 CGAACATGTGCAATGTTGTTGTCGCGATCGC------GTTGGCGCTTGCTCGCTTATTTATCTCCCTTGAAGCCTTGTTCTTCTTTTCATT
CN137 CGAACATGTGCAATGTTGTTGTCGCGATCGC------GTTCGCGCTTGCTCGCTTATTTATCTCCCTTGAAGCCTTGTTCTTCTTTTCATT
PICU475 CGAACATGTGCAATGTTGTTGTCGCGATCGCGATCGCGTTCGCGCTTGCTCGCTTATTTATCTCCCTTGAAGCCTTGTTCTTCTTTTCATT
99K45 CGAACATGTGCAATGTTGTTGTCGCGATCGCGATCGCGTTCGCGCTTGCTCGCTTATTTATCTCCCTTGAAGCCTTGTTCTTCTTTTCATT
NCTC5952 CGAACATGTGCAATGTTGTTGTCGCGATCGC------GTTGGCGCTTGCTCGCTTATTTATCTCCCTTGAAGCCTTGTTCTTCTTTTCATT
NCTC452 CGAACATGTGCAATGTTGTTGTCGCGATCGC------GTTGGCGCTTGCTCGCTTATTTATTTCCCTTGAAGCCTGTTCTTCTTTTCATT


BvgA-P site
BvgA-P site
BvgA-P site
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NCTC10739 AGAAAGAAATATGCGCTTTGTGTTTAGGATGATTTTCCTGTCCGAGTAGGGTGGATCCAAATTTTCCGGATTGGTGGGAATTTGTGCATTT
CN137 AGAAAGAAATATGCGCTTTGTGTTTAGGATGATTTTCCTGTCCGAGTAGGGTGGATCCAAATTTTCCGGATTGGTGGGAATTTGTGCATTT
PICU475 AGAAAGAAATATGCGCTTTGTGTTTAGGATGATTTTCCTGTCCGAGTAGGGTGGATCCAAATTTTCCGGATTGGTGGGAATTTGTGCATTT
99K45 AGAAAGAAATATGCGCTTTGTGTTTAGGATGATTTTCCTGTCCGAGTAGGGTGGATCCAAATTTTCCGGATTGGTGGGAATTTGTGCATTT
NCTC5952 AGAAAGAAATATGCGCTTTGTATTTAGGATGATTTTCCTGTCCGAGTAGGGTGGATCCAAATTTTCCGGATTGGTGGGAATTTGTGCATTT
NCTC452 AGAAAGAAATATGCGCTTTGTATTTAGGATAATTTTCCTTTCCGAGTAGGGTGGATCCAAATTTTCTGGATTGGTGGGAATTTGTGCATT-
840
-10 box
Y00545 T-CACTGCGAATGTTGGAATAATTTCGCCCATCGTCATACGACATGCTGGATGTTTGGTTCTTGCAGAAGGATGAGGTTCTGAGCGCTACA
NCTC10739 T-CATTGCGAATGTTGGAATAATTTCGCCCATCGTCATACGACATGCTGGATGTTTGGTTCTTGCAGAAGGATGAGGTTCTGAGCGCTACA
CN137 T-CATTGCGAATGTTGGAATAATTTCGCCCATCGTCATACGACATGCTGGATGTTTGGTTCTTGCAGAAGGATGAGGTTCTGAGCGCTACA
PICU475 T-CATTGCGAATGTTGGAATAATTTCGCCCATCGTCATACGACATGCTGGATGTTTGGTTCTTGCAGAAGGATGAGGTTCTGAGCGCTACA
99K45 T-CATTGCGAATGTTGGAATAATTTCGCCCATCGTCATACGACATGCTGGATGTTTGGTTCTTGCAGAAGGATGAGGTTCTGAGCGCTACA
NCTC5952 TTCATTGCGAATGTTGGAATAATTTCGCCCATCGTCATACGACGTGCTGGATGTTTGGTTCTTGCAGAAGGATGAGGTTCTGAGCGCTACA
NCTC452 TCCATTGCGAATGTTGGAATAATTTCGCCCATCGTCATACGACGTGCTGGATGTTTGGTTCTTGCAGAGGGATGAGGTTCTGAGCGCTACA
958
Y00545 CACCGGTTGCGTCGGTGCGAATCCGTTCAATC
NCTC10739 CACCGGTTGCGTCGGTGCGAATCCGTTCAATC
CN137 CACCGGTTGCGTCGGTGCGAATCCGTTCAATC
PICU475 CACCGGTTGCGTCGGTGCGAATCCGTTCAATC
99K45 CACCGGTTGCGTCGGTGCGAATCCGTTCAATC
NCTC5952 CACCGGTTGCGTCGGTGCGAATCCGTTCAATC
NCTC452 CACCGGTTGCGTCGGTGCGAATCCGTTCAATC

CyaA1 and CyaA6 was repeated. The primers CyaApR and CyaApF were designed to amplify a smaller region of 388 bp targeting bases 570-958 (Y00545) and this was also sequenced.

The amplified regions were sent to DBS Genomics, University of Durham; MBSU, University of Glasgow for sequencing and this was also carried out in RSIL to ensure that sequencing error had not occurred. The results from the repeated sequencing were concordant with that of the original sequencing. *B. parapertussis* (NCTC5952) and *B. bronchiseptica* (NCTC452) were also sequenced for this region as these two species are known to express the gene *cyaA*. Sequence differences were found between all three *Bordetella* species (Fig. 3.5.1.1). However, neither the *B. parapertussis* strain nor the *B. bronchiseptica* strain contained the 6 bp insertion. There is overlap between the GenBank sequences of *cyaA* and *cyaC* and the gene *cyaC* starts within Y00545 at position 660 and the 6 bp insertion is situated at position 691 (Fig. 3.5.1.1).

3.5.2 Screening the *cyaA* promoter region in *B. pertussis* isolates with LightCycler assay

The LightCycler (LC) hybridisation probe assay was used as an alternative to conventional sequencing to screen 42 isolates, the four vaccine isolates and the type strain for the presence of the 6 bp insertion between *cyaA* and *cyaC*. The LightCycler was used as an alternative to conventional sequencing to ensure that this 6 bp insertion in PICU475 and 99K45 was not a sequencing error and it is also much more rapid. Due to the differences in sequences of the *cyaA* promoters, there were differences in melting temperatures of each sequence type, thus making this target suitable for screening with the LC hybridisation probe assay. Different sequence types are indicated by different melting curves. The primers used, CyaApR and CyaApF, targeted bases 570-958 (Y00545) amplifying a total of 388 bp for the LC assay. Figure 3.5.2.1 shows an example of LightCycler melting curve analysis.

There were three positive controls, the *B. pertussis* type strain (*cyaApr*(1)), CN137 (*cyaApr*(2)), PICU475 (*cyaApr*(3)), included in each LC assay performed to ensure that all three sequence types were capable of being detected easily. The three sequence types, *cyaApr*(1)-(3), were easily discriminated from one another by melting curve analysis

Figure 3.5.2.1: Melting curve analysis of LightCycler assay of *B. pertussis* isolates PICU287, 519, 522, 523 and 524. The yellow cursor indicates the melting curve of the *B. pertussis* type strain NCTC10739^{T}, the green cursor highlights the melting curves of the PICU287, 519, 522, 523 and 524 isolates and the blue cursor highlights the melting curve of PICU475 which is known to contain the 6 bp insertion.

Key:

Negative control

NCTC10739^{T}

CN137

PICU475

PICU287

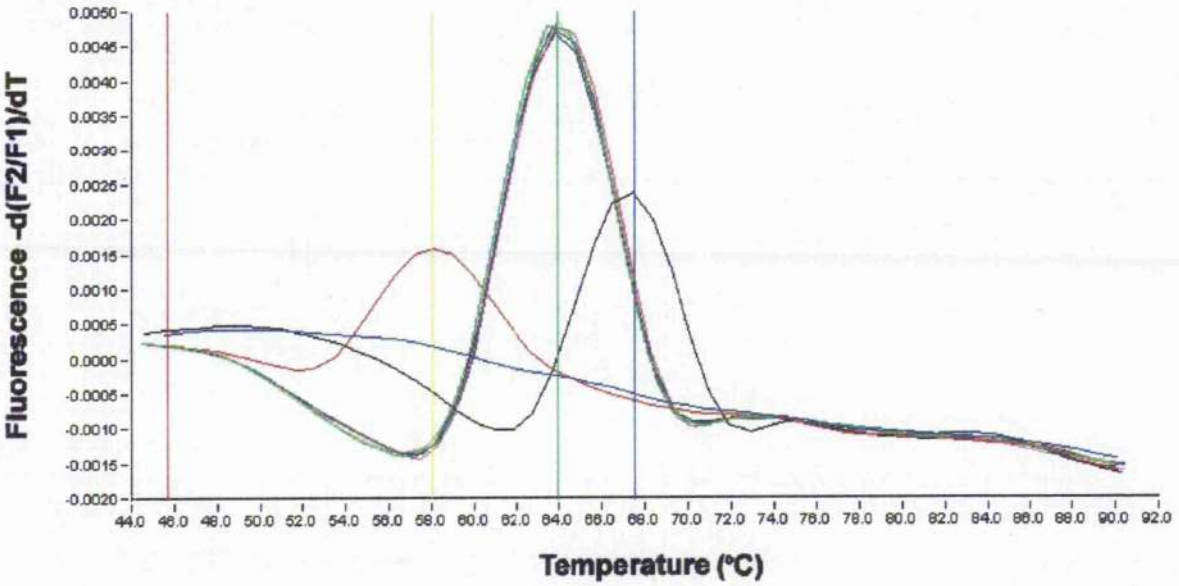
PICU519

PICU522

PICU523

PICU524

File: C:\LightCycler3\Users\EPackard\Data\Ericatest28Mar03.ABT Program: Melt Run By: EPackard
Run Date: Mar 28, 2003 13:19 Print Date: March 28, 2003



Digital Filter: Enabled Calculation Method: Polynomial

Degrees to Average: 8.3 Red cursor Tm = 46.6469 Yellow cursor Tm = 58.0157 Green cursor Tm = 63.9055

Color Compensation: Off Blue cursor Tm = 67.5433

resulting in three individual peaks. The peak indicated with the yellow cursor represents that of the type strain (positive control) and it has a melting temperature of 58°C. The peaks indicated with the green and yellow cursors represent the wild-type *B. pertussis* isolates (CN137 as positive control) and PICU475 (positive control) respectively with melting temperatures of 63.9°C and 67.5°C respectively. Table 3.1.2.1 summarises the frequency of the *cyaApr* alleles found amongst UK isolates. No further sequence variation was found in this region and only the two isolates, 99K45 and PICU475, which had the known 6 bp insertion, were confirmed using the LC assay. Three alleles have been identified, *cyaApr*(1)-(3) and only the *B. pertussis* type strain was *cyaApr*(1). The majority of UK *B. pertussis* isolates, including the four vaccine strains, were *cyaApr*(2) (43 of 46; 93.4%). The other UK isolates, 99K45 and PICU475, were *cyaApr*(3) (6.6%). The Tohama-I genome sequence was *cyaApr*(2). Four other PICU isolates (PICU287, PICU519, PICU522, PICU523 and PICU524) were screened using the LightCycler assay to check for the 6 bp insertion to determine if *B. pertussis* isolated from other young infants admitted to paediatric intensive care units (PICUs) infected with pertussis had this sequence variation present between the genes *cyaA* and *cyaC*. None of the other PICU isolates contained this sequence variation. There were no family contact samples of PICU475 containing *B. pertussis* available for similar analyses to determine if the family contacts also had *B. pertussis* isolates with this 6 bp insertion.

The two isolates PICU475 and 99K45 were MLST-4 but they differed in their *prnA* types: PICU475 was *prnA*(2) and 99K45 was *prnA*(3). This MLST type is based on the *tcfA* allele *tcfA*(3). Moreover, these strains are the only two isolates of the selected panel of 138 isolates, vaccine strains and the type strain that have the *tcfA*(3) allele. In view of this finding, it was desirable to determine if there was a link between isolates that were *tcfA*(3) and the 6 bp insertion situated between *cyaA* and *cyaC*. Other *tcfA*(3) isolates (n=3) were provided by Prof F. Mooi, National Institute of Public Health and the Environment, Bilthoven, The Netherlands. The region of interest was amplified using CyaApR and CyaApF primers, already used for the LC assay, and the amplified DNA was sent to the sequencing unit at DBS Genomics, University of Durham. Sequencing data were analysed using BioNumerics (Applied Maths). None of these isolates contained the 6 bp insertion. Therefore, there is no linkage between *tcfA*(3) and the presence of the 6 bp insertion.

3.6 CONDUCTIMETRY ASSAYS OF rCyaA

The enzymic (AC) activity of adenylate cyclase toxin has been used as a diagnostic tool in radioisotopic assays that detect cAMP levels in clinical samples from pertussis patients or *B. pertussis* cultures. A novel conductimetric assay developed by Dr A. Lawrence (University of Glasgow) has been used to detect AC activity from recombinant CyaA (rCyaA). The method of preparation of rCyaA will be described briefly as this was used to optimise the assay. It was kindly supplied by Dr B. Orr. The CyaA protoxin (containing the AC activity) and CyaC (which is required for activation of cytotoxic activity of CyaA) were expressed on separate compatible plasmids, pGW44 and pGW54, respectively (MacDonald-Fyall, 2002). The *E. coli* strain BL21/DE3 was the host strain for these expression vectors. Urea extracts of BL21/DE3 expressing active CyaA were prepared and rCyaA was extracted and purified using a combination of two protein purification methods, DEAE sepharose (anionic exchange) chromatography and phenyl sepharose (hydrophobic interaction) chromatography. This section will report the optimisation of the conductimetry assay measuring AC activity from rCyaA, its detection limit and the effect of inhibitory substances. It will also describe the development of a protocol for the measurement of AC activity from *B. pertussis* urea extracts and cell lysates and the optimisation of this assay.

3.6.1 Optimisation of rCyaA and native CyaA assay protocols

There are two reagents, ATP and CaM, used in this assay for detection of AC activity from CyaA that affect the rate of reaction. It was important for this investigation to determine the concentration of each reagent that provided the optimal reaction rate of AC activity. The reagent pyrophosphatase (PPiase) was not considered to be a rate-limiting reagent due to the rate of reaction not being affected by the amount that is added, as the conversion of ATP to cAMP with the release of PPi is the rate-limiting step. It is only when very active stock rCyaA is used that there may not be enough PPiase to catalyse the second reaction optimally. One way to overcome this is to ensure that dilutions of stock rCyaA are used. The optimal ATP concentration was determined by measuring AC enzymic activities with ATP concentrations ranging from 0.2-1.4 mM ATP. The optimal concentration of ATP was found to be 0.5 mM for assays detecting AC activity from rCyaA as increasing the concentration above this level did not affect the reaction rate. The optimal CaM concentration was determined in a similar way with both CaM from a commercial source

(Sigma) and CaM prepared in-house. The optimal amount was determined to be 1µl of 1mg ml^{-1} of either CaM source but due to supply limitations, the commercial preparation was preferred.

The optimal ATP concentration for assays of native CyaA was determined as described above. The optimal concentration of ATP was found to be 1 mM to be used for assays detecting AC activity from *B. pertussis* cell lysates prepared in the non-ionic detergent Triton X-100. The optimal CaM concentration was also determined as described above. This was determined to be 1 µl of 1 mg ml^{-1} of either preparation and again the commercial preparation was preferred.

3.6.2 Determination of AC enzymic activity

To calculate the AC enzymic activity in international units (IU) per mg of protein, the total conductance change must be known. Total conductance change in arbitrary units for a substrate concentration of 1 mM (ATP) was 2200 (Fig. 3.6.2.1) and this value was used in calculating the specific activities of AC.

Example of calculation for determining AC activity expressed in international units (IU) mg^{-1}: Stock preparation of rCyaA concentration was 0.216 mg protein ml^{-1}. This was used at a 1 in 10 dilution (0.0216 mg protein ml^{-1}) and 2 µl were added to a final volume of about 2 ml therefore in the conductimetry cell there was a final concentration of 0.0216 µg protein ml^{-1} of CyaA.

initial reaction rate = (y/x) µmol/ml/min

initial reaction rate = tangent of reaction **(y)** (containing 0.0216 µg ml^{-1}) (units per min) / total change value **(x)** (arbitrary units)

For example:

= 26 **(y)** / 2200 **(x)**

= 0.0118 µmoles/ml/min

To convert µmoles/ml/min to µmoles/min/µg protein

= 0.0118 µmoles/ml/min / 0.0216 µg protein ml^{-1}

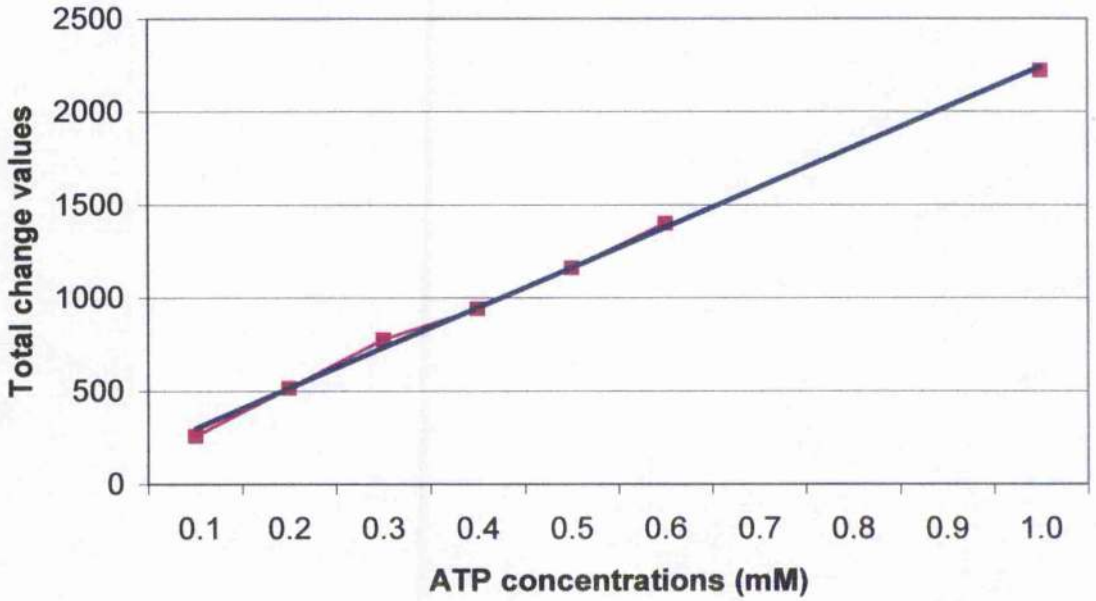
= 0.55 µmoles/min/µg protein

Figure 3.6.2.1: Standard curve for total conductance change (arbitrary units) for ATP concentrations from 0.1 mM to 1 mM.

Key:

Average

Linear (Average)


2500
2000
1500
1000
500
0
Total change values
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
ATP concentrations (mM)

To convert μmoles/ml/min to IU (international units)/mg
= 550 IU mg^{-1} protein

3.6.3 Determination of detection limit of AC assay

This was determined by testing various dilutions of recombinant CyaA in the optimised reaction to give logarithmic scale of tangents of the reaction curves. At the lowest dilutions, it was necessary to determine if the reaction was genuine and not due to background. This was achieved by determining the rate of reactions with and without CaM. No reaction occurred when CaM was absent. With this information, the detection level for AC activity of purified recombinant CyaA in the cell was 0.864 ng protein ml^{-1}.

3.6.4 Requirement for ATP, CaM and PPiase in AC assays

Reactions in the conductimetric assay containing different reagents and the effect it had on the enzymic reaction of purified CyaA were compared. A reaction that contained CaM and ATP but no PPiase showed a negative reaction (Fig. 3.6.4.1). With reactions that contained no ATP, the progress curves remained at the baseline and no increase in conductivity took place (Fig. 3.6.4.2). Reactions that did not contain CaM showed only a slight increase in conductivity (Fig. 3.6.4.2). Thus rCyaA had very low AC activity in the absence of CaM, but AC activity was greatly activated by CaM. Therefore the assays were CaM-specific, as expected.

3.6.5 Effect of different lysis agents on AC enzymic activity

The AC enzymic activity of *B. pertussis* CyaA has been detected in the culture supernate and is also known to cell-associated. Hewlett *et al.* (1976) reported that up to 20% of AC activity is detected in the culture supernate whereas the remaining activity is cell-associated with 91-93% of it being extracytoplasmic. Initially, a suspension of whole-cells of *B. pertussis* BP348pRMB1 freshly grown on BG agar was originally tested for AC activity in the conductimetry assay. No AC activity was detected after adding whole cells to the conductimetry cell, which implied that any CyaA on the cell surface was at undetectable levels. Therefore it was important for this study to develop a protocol for the lysis of *B. pertussis* cells that would maintain sensitivity of the assay and minimise the introduction of inhibitory substances. Urea and several nonionic detergents were investigated for their

Figure 3.6.4.1: Enzymic (AC) activity without the addition of pyrophosphatase (PPiase). Conductimetry assay determining the effect of PPiase on AC activity. The first and last cells were the blank controls consisting of buffer with CaM and PPiase and no recombinant adenylate cyclase toxin (rCyaA). Cells 2-4 contained all reagents and rCyaA. Cells 5-7 contained assay buffer with CaM and CyaA with no PPiase.

Key:

1 — Blank
2 — rCyaA+ATP+CaM+PPiase
3 — rCyaA+ATP+CaM+PPiase
4 — rCyaA+ATP+CaM+PPiase
5 — rCyaA+ATP+CaM
6 — rCyaA+ATP+CaM
7 — rCyaA+ATP+CaM
8 — Blank

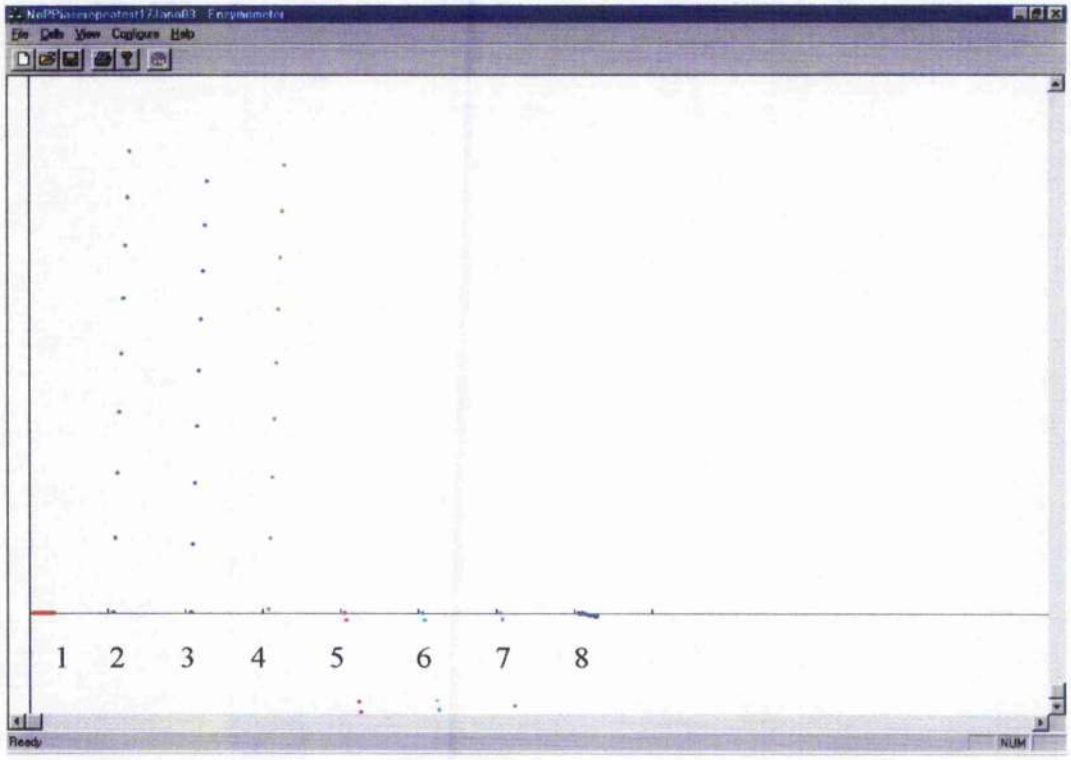
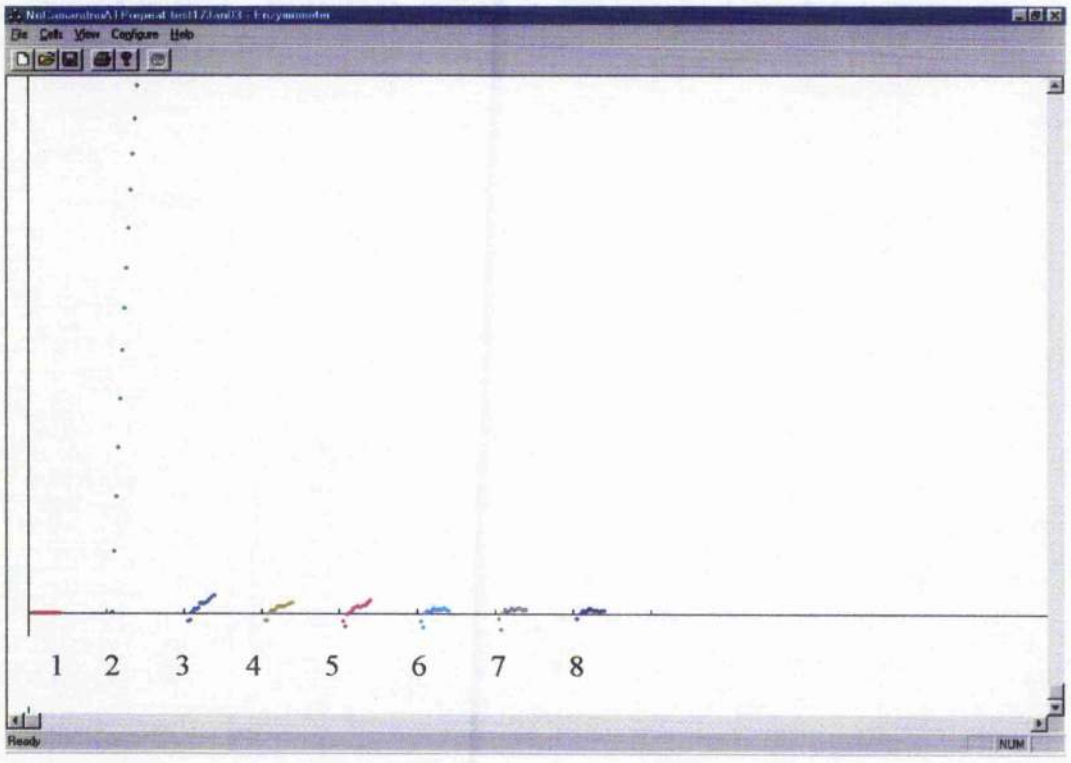

File Cells View Capture Help
1 2 3 4 5 6 7 8
Ready
NUM

Figure 3.6.4.2: Enzymic (AC) activity without the addition of calmodulin (CaM) or ATP. Conductimetry assay determining the effect of CaM and ATP on AC activity. The first cell was the blank control as for Fig. 22a. Cell 2 contained the positive control, assay buffer with all reagents and rCyaA. Cells 3-5 contained assay buffer with ATP, PPiase and rCyaA but no CaM. Cells 6-8 contained assay buffer with CaM, PPiase and rCyaA but no ATP.

Key:
1 — Blank
2 — rCyaA+ATP+CaM+PPiase
3 — rCyaA+ATP+PPiase
4 — rCyaA+ATP+PPiase
5 — rCyaA+ATP+PPiase
6 — rCyaA+CaM+PPiase
7 — rCyaA+CaM+PPiase
8 — rCyaA+CaM+PPiase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1 2 3 4 5 6 7 8
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potential as lysis agents of *B. pertussis*. It was important to determine the potential inhibitory effect of these agents in conductimetry assays.

The maximum concentration of urea that could be present in the glass cell without any noticeable inhibition of AC enzymic activity was 0.25 M (Fig. 3.6.5.1). Two commercially-available agents that contain nonionic detergents, B-Per® Bacterial Protein Extraction Reagent and BugBuster™ Protein Extraction Reagent were also tested. Both of these detergents caused inhibition even at low amounts (2 µl). The seven nonionic detergents tested (Table 6) did not inhibit the AC enzymic activity (the effects of Tween-20 are shown in Fig. 3.6.5.2) over the concentration range of 0.01% to 1.5% and with a final concentration of 43.2 ng ml^{-1} of rCyaA. By comparing the tangents of the progress curves, it was determined that AC activities were approximately similar and therefore no significant inhibition had occurred. It was also determined that there was no volume effect when adding different volumes of a known non-inhibiting reagent.

3.7 CONDUCTIMETRY ASSAY OF AC ENZYMIC ACTIVITY IN *B. pertussis* SAMPLES

3.7.1 Optimisation of *B. pertussis* CyaA protocol

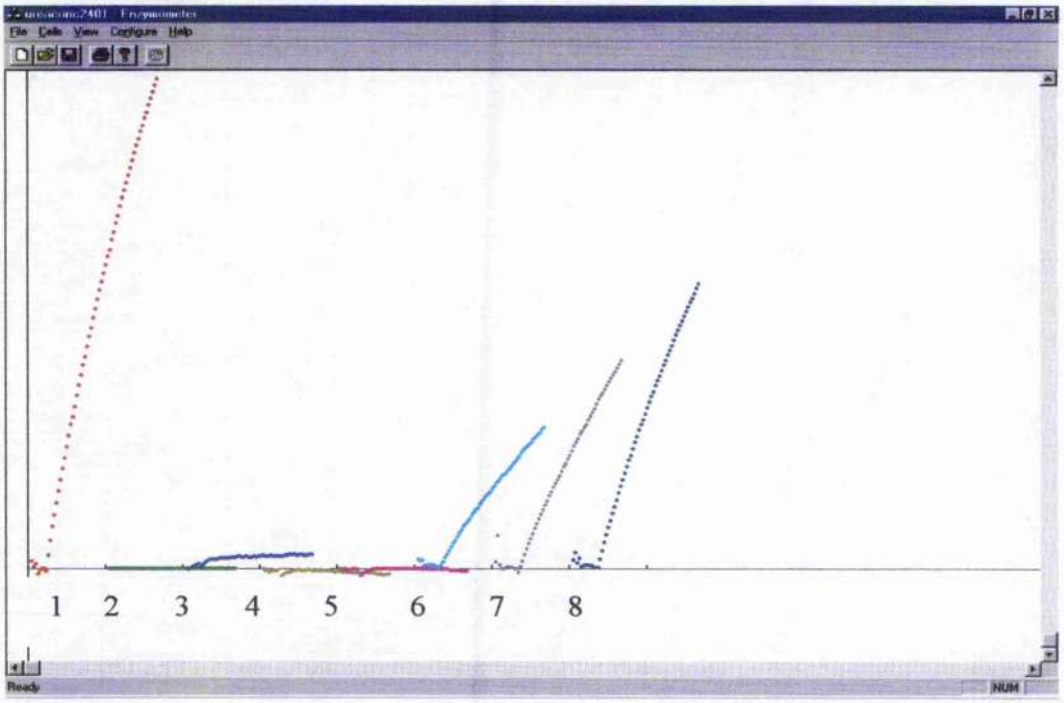
The seven nonionic detergents, for which no inhibition of AC activity was found, were further investigated to determine which one provided optimal lysis of *B. pertussis* for detection of enzymic activity. Initially, lysis agents were tested on strain BP348pRMB1, an overexpressor of AC (Brownlie *et al.*, 1988), then tested on strain NCTC10739^{T} (reported to express AC activity 3-fold greater than that of Tohama-I) and strain BP338. Cell suspensions of 2 x 10^{9} cfu ml^{-1} of *B. pertussis* strain BP348pRMB1 were prepared in saline and lysed with the seven different non-ionic detergents.

To aid in lysis of the cells, the cell suspension was maintained in detergent at 4°C for different lengths of time (10 min, 30 min, 1 h) on a rotary shaker. It was found that the inclusion of the mixing stage did improve lysis and 30 min was the time chosen for mixing of cells with detergent.

Figure 3.6.5.1: Effect of urea concentration of enzymic (AC) activity of recombinant adenylate cyclase toxin (rCyaA). The first cell contained the positive control (rCyaA). Cell 2 contained the blank control consisting of buffer alone. Cells 3-8 contained decreasing concentrations of urea, with final concentrations ranging from 8M to 0.25 M urea.

Key:

1 — Positive control
2 — Blank
3 — 8M urea
4 — 4M urea
5 — 2 M urea
6 — 1 M urea
7 — 0.5 M urea
8 — 0.25 M urea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Ready
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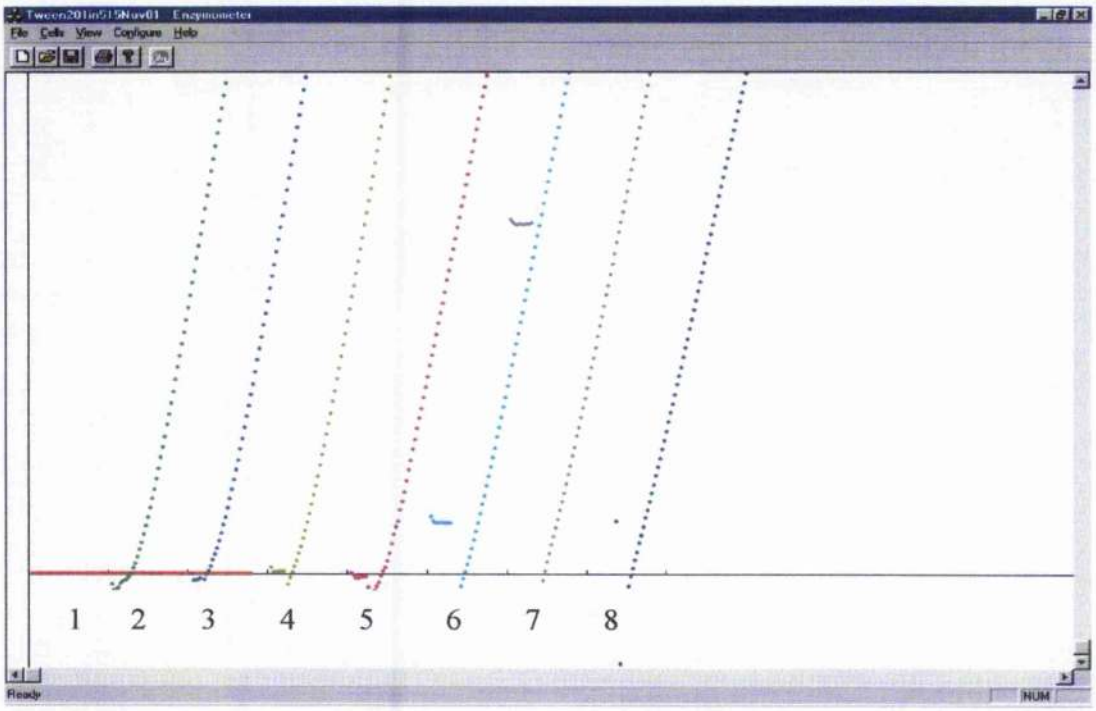
Figure 3.6.5.2: Effect of a nonionic detergent (Tween-20) on enzymic activity. Cell 1 was the blank control. Cell 2 contained the positive control, 2µl of rCyaA alone. Cell 3-8 contained increasing concentrations of Tween-20. The final concentrations in cells 3-8 ranged from 0.01%-1.5%.

Key:

1 — Blank
2 — Positive control
3 — 0.01% Tween-20
4 — 0.05% Tween-20
5 — 0.1% Tween-20
6 — 0.25% Tween-20
7 — 1% Tween-20
8 — 1.5% Tween-20


Tween20in515Nov01 - Enzymometer
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1 2 3 4 5 6 7 8
Ready
NUM

In some assays, an extra control was included consisting of 86 μg protein of positive control rCyaA added to 300 μl of nonionic detergent (Tween-20, Tween-80 or Brij 35) which was also mixed for 30 min at 4°C. This control was included in the assays to determine if any AC activity was lost during this mixing stage of the lysis protocol. It was tested in three assays and it was found that there was a loss of about 30% of AC activity. For example, the positive control had a tangent of 116 while that with Brij 35 had a tangent of 82, after the mixing step.

Table 3.7.1.1 lists the non-ionic detergents investigated, the final concentration of each lysing agent in the conductimetry cell, the number of lysed cells of *B. pertussis* strains in the conductimetry cell and the tangent value of each progress curve. This table was derived after different volumes of cell suspension (10-300 μl), lysed with non-ionic detergent, were added to the conductimetry cell to determine the optimal amount of BP348pRMB1 lysate and NCTC10739^T lysate to add as the source of AC activity in the assay. Three of the non-ionic detergents, Triton X-100, Brij-58 and NP-40, appeared to provide the best lysis of *B. pertussis* cells according to the tangent values of the AC activity progress curves. The tangent values of each progress curve for these three detergents did not differ greatly. However, from the comparison of the activity of BP348pRMB1 and NCTC10739^T lysates, Triton X-100 was found to provide optimal lysis of *B. pertussis* cells. As expected, strain BP348pRMB1, the recombinant overexpressor strain, showed greater AC activity than the type strain NCTC10739^T.

By adding different numbers of *B. pertussis* lysed by Triton X-100, the detection limit of AC enzymic activity from the AC overexpresser (BP348pRMB1) was equivalent to c. 10^7 cfu in 2 ml in the conductimetry cell and the detection limit of AC activity from the type strain (NCTC10739^T) was c. 10^8 cfu in 2 ml in the conductimetry cell. The protocol was optimised and is described as follows: *B. pertussis* cells from an overnight lawn plate on BG agar were suspended in saline to an OD_{600nm} 0.46 (approximately 2 x 10^9 cfu ml^{-1}). Suspensions were centrifuged for 10 min at 6,600 x *g* (Biofuge pico, Heraeus). Pellets were resuspended in 300 μl of 10% Triton X-100 and suspensions were allowed to mix for 30 min at 4°C. The mixed suspensions were used immediately for the conductimetry assays. This method of lysing *B. pertussis* cells with Triton-X100 developed in this investigation was not considered

Table 3.7.1.1. Effect of non-ionic detergents on AC activity of *B. pertussis* cell suspensions

Nonionic Detergent	**Strain**	**Final concentration of lysis agent in cell**	**No. of bacterial cells in conductimetry cell**	**Tangent**
Triton X-100	BP348pRMB1	0.05%	$3.3x10^7$	4
		1.5%	$1x10^9$	40
	NCTC10739^T	0.5%	$3.3x10^8$	5
		1.5%	$1x10^9$	13
Brij 58	BP348pRMB1	0.05%	$3.3x10^7$	1.3
		1.5%	$1x10^9$	35
	NCTC10739^T	0.5%	$3.3x10^8$	4
		1.5%	$1x10^9$	9
Brij 35	BP348pRMB1	0.05%	$3.3x10^7$	1.5
		1.5%	$1x10^9$	16
	NCTC10739^T	0.5%	NT	NT
		1.5%	NT	NT
Triton X-114	BP348pRMB1	0.05%	$3.3x10^7$	3
		1.5%	$1x10^9$	18
	NCTC10739^T	0.5%	$3.3x10^8$	5
		1.5%	$1x10^9$	11
NP40	BP348pRMB1	0.05%	$3.3x10^7$	2
		1.5%	$1x10^9$	38
	NCTC 10739^T	0.5%	$3.3x10^8$	0
		1.5%	$1x10^9$	18
Tween-20	BP348pRMB1	0.05%	$3.3x10^7$	0
		1.5%	$1x10^9$	8
	NCTC10739^T	0.5%	NT	NT
		1.5%	NT	NT
Tween-80	BP348pRMB1	0.05%	$3.3x10^7$	0
		1.5%	$1x10^9$	8
	NCTC10739^T	0.5%	NT	NT
		1.5%	NT	NT

NT = not tested, this is due to a low tangent value for AC activity of BP348pRMB1, the AC over-expresser strain, as a result of inefficient lysis of the *B. pertussis* cells. Therefore this detergent was not tested for its ability to lyse *B. pertussis* strain NCTC10739^T.

sensitive enough for diagnostic use directly from clinical specimens due to the large number of bacterial cells required for detection of AC enzymic activity.

Using the optimised lysis method, lysed cells of *B. pertussis* BP338 strain grown in two different ways, on BG agar and in SS liquid medium, were compared as sources of AC activity to be detected by the conductimetry assay. This would determine whether there was an optimal method of growing the bacteria to be used as a source of AC activity. *B. pertussis* BP338 was grown either on BG agar for 72 h at 37°C or inoculated into 5 ml SS liquid medium (Appendix 1.2) and grown for 72 h at 37°C with shaking at 150-200 rpm. After incubation, lawn plates of *B. pertussis* cells were prepared by restreaking from BG agar and incubated overnight. Alternatively, a volume of 1.25 ml of culture grown in SS was used to inoculate 5 ml of fresh SS liquid medium and incubated overnight at 37°C with shaking at 150-200 rpm. Bacteria grown on the lawn plates were suspended to 2 x 10^9 cfu ml^{-1} in saline and lysed with Triton X-100 as described on the previous paragraph. Bacteria from the overnight SS liquid medium were centrifuged and suspended to 2 x 10^9 cfu ml^{-1} in saline and lysed as described previously with Triton X-100. A volume of 300µl of lysed cells was analysed for AC activity using the conductimetry assay. Cells that were grown on BG agar were found to have greater AC activity than cells grown in SS liquid medium, tangents of progress curves were 55 and 3 respectively. An explanation for this large difference in AC activity is difficult to determine, but may relate to CyaA accumulating in cells grown on BG lawn plates and the possibility that in SS liquid medium, a significant proportion of CyaA is secreted and lost.

3.7.2 Requirement for ATP, CaM and PPiase in *B. pertussis* lysate AC assays

B. pertussis strain BP338 lysates were used and reactions that did not contain one of the reagents, ATP, CaM or PPiase, were compared. This would determine if the enzymic activity detected by the assay was CyaA activity or background enzymic reactions from the *B. pertussis* cell lysate. The strain BP338 is a derivative of Tohama-I and is a widely used laboratory strain. BP338 was favoured in place of the *B. pertussis* type strain NCTC10739^T, as it is likely to be more representative of *B. pertussis* isolates. BP338 cells from an overnight lawn plate grown on BG agar were suspended in saline to a suspension of 2 x 10^9 cfu ml^{-1} and the cells were lysed with the nonionic detergent Triton X-100. A volume of 300

µl of cell lysate was used immediately as a source of AC activity. The reaction containing CaM and ATP, but no PPiase, still showed a progress curve, although the tangent of the reaction was 15 compared to a tangent of 25 for the positive control in this experiment, which contained ATP, CaM and PPiase (Fig. 3.7.2.1). This implied that the BP338 cell lysate contained PPiase activity. With the reaction that contained CaM and PPiase, but no ATP, the progress curves remained at the baseline, i.e. there was no increase in conductivity (Fig. 3.7.2.1). Reactions that contained ATP, PPiase and no CaM had a much slower rate of reaction, the tangent of which was 5 compared to a tangent of 25 for the positive control reaction that contained all reagents (Fig. 3.7.2.1). This implied that BP338 cell lysate also had some CaM-like stimulating activity because the tangent was greater than that obtained with rCyaA in the absence of CaM (Fig. 3.6.4.2).

In another experiment using a lysate of *B. pertussis* strain BP347 lysate, reactions that did not contain one of the reagents, ATP, CaM or PPiase, were compared. The *B. pertussis* strain BP347 is a mutant strain that is known not to express virulence-activated genes including CyaA. This strain was included in assays as a negative control for the detection of AC activity. BP347 cells from an overnight lawn plate grown on BG agar were suspended in saline to a suspension of 2 x 10^9 cfu ml^{-1} and the cells were lysed using the nonionic detergent Triton X-100. A volume of 300 µl of cell lysate was used immediately. The reaction that contained BP347 lysate with ATP, PPiase and CaM had a progress curve that was slightly raised above the baseline, tangent = 1 (Fig. 3.7.2.2). The BP347 reaction that did not contain CaM had a reaction rate marginally faster that the reaction containing no ATP, but noticeably lower that BP347 reaction with CaM, ATP and PPiase (Fig. 3.7.2.2). The reaction lacking PPiase had a rate similar to that of the complete reaction, again indicating that BP347 had endogenous PPiase activity. There was no slight raise above the baseline for the BP347 lysate reaction that did not contain ATP (Fig. 3.7.2.2).

The work indicated that, the conductimetric assay was suitable for specific measurement of AC activity as the activity detected in the BP338 lysate in the absence of ATP was negligible. However, a noticeable reaction occurred if CaM was excluded which might suggest that the assay was unsuitable for specifically measuring CaM-dependent CyaA. AC activity in the BP347 lysate was negligible in the absence of CaM indicating that the assay is

Figure 3.7.2.1: Enzymic (AC) activity of *B. pertussis* BP338 cell lysate without the addition of calmodulin (CaM), ATP or pyrophosphatase (PPiase). The first cell, and cells 7 and 8 contained the blank control. Cell 2 contained the positive control. The third cell contained assay buffer with all reagents and BP338 cells lysed in Triton X-100. Cells 4, 5 and 6 contained assay buffer with all but one of the regents, CaM, ATP and PPiase respectively.

Key:

1 — Blank
2 — rCyaA+ATP+CaM+PPiase
3 — BP338+ATP+CaM+PPiase
4 — BP338+ATP+PPiase
5 — BP338+CaM+PPiase
6 — BP338+ATP+CaM
7 — Blank
8 — Blank

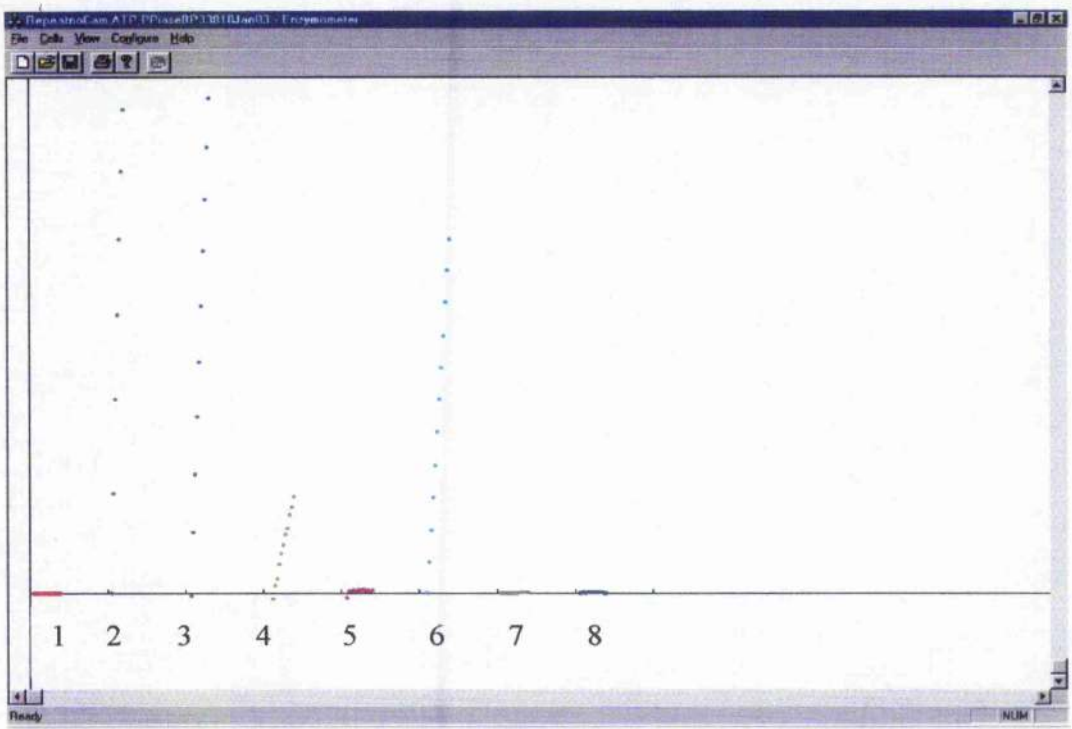
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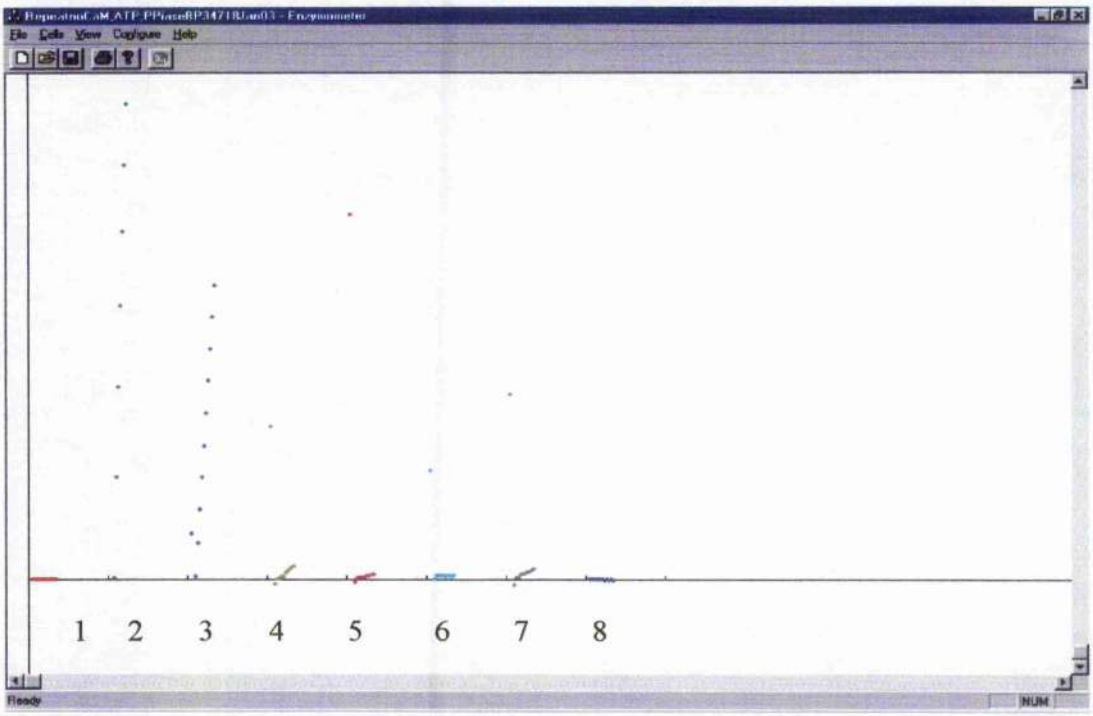
Figure 3.7.2.2: Enzymic (AC) activity using BP347 cell lysate without the addition of calmodulin (CaM), ATP or pyrophosphatase (PPiase). The first and last cells contained the blank control. Cells 2 and 3 contained that described for Fig. 3.7.2.1. Cell 4 contained assay buffer with all reagents and BP347 cells lysed in Triton X-100. Cells 5, 6 and 7 contained everything except one reagent, CaM, ATP and PPiase respectively.

Key:

1 — Blank
2 — rCyaA+ATP+CaM+PPiase
3 — BP338+ATP+CaM+PPiase
4 — BP347+ATP+CaM+PPiase
5 — BP347+ATP+PPiase
6 — BP347+CaM+PPiase
7 — BP347+ATP+CaM
8 — Blank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suitable for detection of CyaA even if the cell lysate contains some CaM-like molecule.

3.7.3 Detection of *B. pertussis* CyaA from SS medium

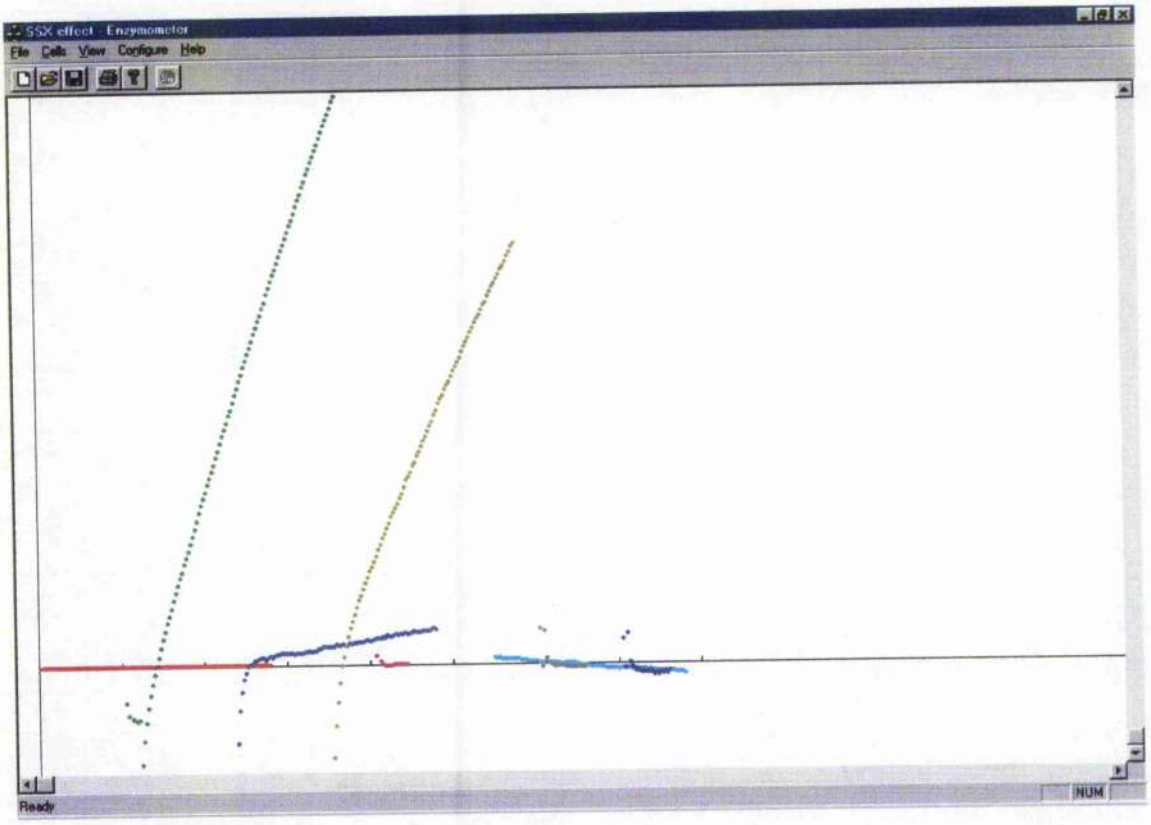
B. pertussis is known to secrete a proportion of CyaA into the culture supernate (Hewlett *et al.*, 1976; Brownlie *et al.*, 1985). Previous work, section 3.6.5, did not detect any AC activity in a whole-cell suspension prepared from BP348pRMB1 grown on BG agar. However, it was important to determine if growth in liquid media released more CyaA protein. A loopful of *B. pertussis* previously grown on BG agar was used to inoculate 5 ml of liquid (SS) medium, which was then incubated for approximately 72 h. This culture provided samples to be tested for AC activity using the conductimetry assay. Samples that were tested were 300 µl of the growth culture, 300 µl of pelleted cells resuspended in 1 ml of conductimetry buffer and 300 µl of SS medium alone. The progress curves are shown in Fig. 3.7.3.1. The positive control (final concentration in conductimetry cell was 10.8 ng protein ml^{-1} of rCyaA) exhibited AC activity of 510 IU mg^{-1} protein. There was little enzymic activity detectable with the cell pellet resuspended in buffer as the source of AC activity. The tangent of the reaction curve was equivalent to 1.3. Spiked samples (300 µl of the previously mentioned samples and 10.8 ng ml^{-1} of rCyaA) were also introduced for this assay to determine if there were any inhibitory effects from the samples. Only the spiked cell pellet resuspended in buffer showed AC activity equivalent to that of rCyaA alone (440 IU^{-1} mg) (Fig. 3.7.3.1) which suggested that inhibitory reagents were present in SS liquid medium.

Investigation of desalting the SS medium to remove inhibitory substances was carried out. PD-10 columns (Amersham) were used as a method to desalt the samples. *B. pertussis* NCTC10739^T was grown in SS medium for 72 h and the culture was desalted using the columns. Different volumes (5–200 µl) of the desalted culture were added to the conductimetry assay to determine AC activity of the samples. Activity was only observed with the addition of 200 µl of desalted sample but this was very low. In another assay, a loopful of *B. pertussis* NCTC10739^T grown on BG agar was used to inoculate CL liquid medium, which was then incubated for 72 h. The cultures were set up in duplicate, designated 1 and 2. The supernates from cultures grown in CL liquid medium were desalted using PD-10 columns and a volume of crude rCyaA was also placed through the column to

Figure 3.7.3.1: Enzymic (AC) activities in different fractions of *B. pertussis* cultures after growth in SS liquid medium. Cell 1 was the blank control. Cell 2 contained the positive control consisting of assay buffer with all reagents and recombinant adenylate cyclase toxin (rCyaA). Cells 3-8 contained the test samples, see key below for description.

Key:

1 — Blank
2 — Positive control
3 — Cell pellet from SS resuspended in assay buffer
4 — Cell pellet from SS resuspended in assay buffer spiked with rCyaA
5 — SS whole culture
6 — SS whole culture spiked with rCyaA
7 — SS medium, uninoculated
8 — SS medium, uninoculated spiked with rCyaA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determine whether AC activity was lost during the desalting process. The cells from the culture had already been pelleted for preparation of urea extracts. The positive controls in the assay contained final concentrations of 5 μg protein ml^{-1} of crude rCyaA alone and 5 μg protein ml^{-1} of crude rCyaA that had been through a PD-10 column. The supernates that had been desalted were tested alone and as spiked samples to ensure that no inhibitory substances remained. The progress curves of desalted supernates from cultures 1 and 2 are shown on Fig. 3.7.3.2. The rCyaA that had been through the desalting process had a similar activity to the rCyaA that had not, therefore no AC activity was lost during the desalting process. The spiked samples also had an activity similar to that of the positive control, therefore no inhibition was occurring with the desalted samples. No activity was observed with the culture supernates alone (Fig. 3.7.3.2).

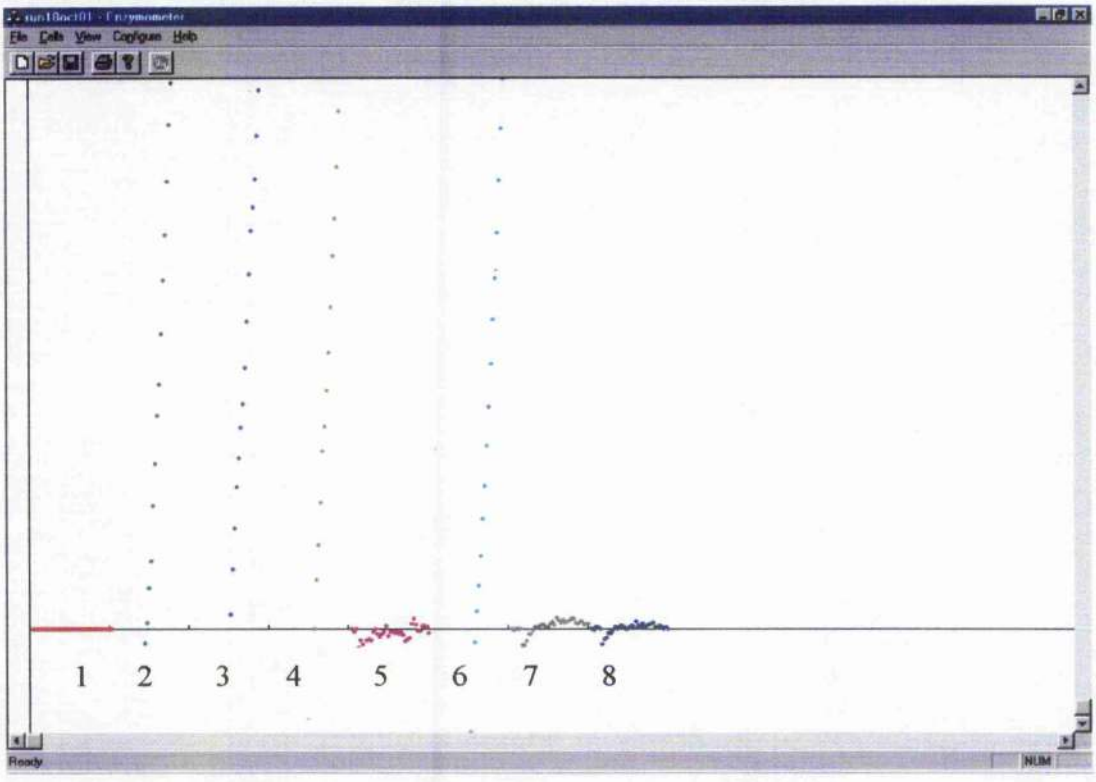
3.7.4 AC activity during growth curve of *B. pertussis*

To aid in the optimisation of the conductimetry assay it was important to determine at which point during the *B. pertussis* growth curve maximum AC activity was obtained as this information would increase the sensitivity of detection if the cells were to be grown from clinical specimens. For this assay, specimens of 1.6 ml of BP338 cells grown in 5 ml SS liquid medium was used to inoculate 250 ml of fresh SS liquid medium to attain a starting OD_{600nm} of 0.01. This culture was incubated for 73 h at 37°C, with shaking at 150-200 rpm. Throughout this time, OD_{600nm} values were taken to determine the time points of early log phase, late log phase and late stationary phase. Cells were pelleted from BP338 cultures at the three time points, early log phase (32 h), late log phase (56 h) and late stationary phase (73 h). Urea extracts of BP338 were prepared from cells from the three different growth phases. Preparation of urea extracts of *B. pertussis* cells was used here rather than *B. pertussis* cells lysed with Triton X-100 due to the inability to visualise CyaA by SDS-PAGE with the latter method. Samples produced a smear on gel and it was not possible to determine whether bands were present at 200 kDa or to compare the intensity of CyaA bands between samples to predict which crude sample would have greater AC activity in the conductimetry assay. The protein concentrations of the urea extracts by the Bradford's assay were: early log phase urea extract, 157 μg ml^{-1}; late log phase urea extract, 1791 μg ml^{-1}; and the stationary phase urea extract, 462 μg ml^{-1}. Two protein concentrations of the BP338 urea extracts were tested for AC enzymic activity, 100 μg ml^{-1} and 10 μg ml^{-1}; 300 μl of each

Figure 3.7.3.2: Enzymic (AC) activities in the desalted culture supernates from 72 h *B. pertussis* cultures grown in CL medium. The first cell contained assay buffer alone. Cell 2 contained assay buffer and all reagents with rCyaA as positive control. Cell 3 contained the positive control that has been placed through the PD10 column. Cells 5 and 7 contained desalted supernates alone and cells 4 and 6 contain spiked desalted supernates.

Key:

1 — Blank
2 — Positive control
3 — Positive control through PD10
4 — desalted supernate spiked no. 1
5 — desalted supernate no. 1
6 — desalted supernate spiked no. 2
7 — desalted supernate no. 2
8 — Blank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sample was added to the conductimetry cell. A positive control containing a final concentration of 54 ng protein ml^{-1} of rCyaA was included. Urea inhibition of AC activity detection was reported previously in section 3.6.5 where it was shown that the final maximum concentration of urea in the assay was 0.25 M before inhibition was demonstrated. Therefore, as the urea extracts were stored in a solution containing 4 M urea, it was important to include a sample containing a final concentration of 54 ng protein ml^{-1} of rCyaA with the sample maximum concentration of urea as in those urea extracts. The maximum concentration of urea that was determined to be present in the samples at 100 µg protein ml^{-1} of the early exponential phase urea extracts was 2.5 M urea. The urea concentration of urea extracts of cells from late exponential and stationary phases were 0.2 M and 1.1 M, respectively. The rCyaA with 2.5 M urea control had a reduction in AC activity compared to that of rCyaA alone (Fig. 3.7.4.1). Therefore, urea inhibition of AC activity was occurring. The activity of the urea extract from the late exponential phase could be calculated due to the lower urea concentration and was determined to be 0.97 IU mg^{-1} of protein. Activities of urea extracts from early exponential and late exponential phases were analysed at a lower protein concentration to reduce the urea concentration.

Due to the possibility of urea inhibition, urea extracts at 10 µg protein ml^{-1} were assayed as there would be no inhibitory effect at this dilution. The urea concentrations of early exponential, late exponential and stationary phases were 0.25 M, 0.02 M and 0.08 M, respectively. A positive control of rCyaA alone (final concentration 54 ng protein ml^{-1}) and rCyaA with the maximum amount of urea (final concentration 54 ng protein ml^{-1} and 0.25 M urea) were included (Fig. 3.7.4.2). Comparison of the AC activities of rCyaA alone with rCyaA and 0.25 M urea showed that there was no inhibitory effect of urea in this assay. Analysis of AC activities in urea extracts at 10 µg protein ml^{-1} over the growth curve of BP338, showed that activity was only detected from the stationary phase (ca. 73 h growth). The activity of BP338 urea extract from the stationary phase was 1.5 IU mg^{-1} protein. Therefore urea extracts of *B. pertussis* cells from the stationary phase should be used for comparison of AC activities and cytotoxic activities between *B. pertussis* strains (see section 3.9).

Figure 3.7.4.1: Enzymic (AC) activity of urea extracts of *B. pertussis* BP338 (100 µg protein ml^{-1}) from early exponential, late exponential and stationary phase. Cells 1 and 2 contained negative (assay buffer only) and positive (rCyaA with assay buffer and all reagents) controls respectively. Cells 3-6 contained urea extracts from the different growth phases of *B. pertussis* to test for AC activity.

Key:
1 — Blank
2 — Positive control
3 — Positive control (with urea)
4 — Early exponential phase BP338
5 — Late exponential phase BP338
6 — Stationary phase BP338

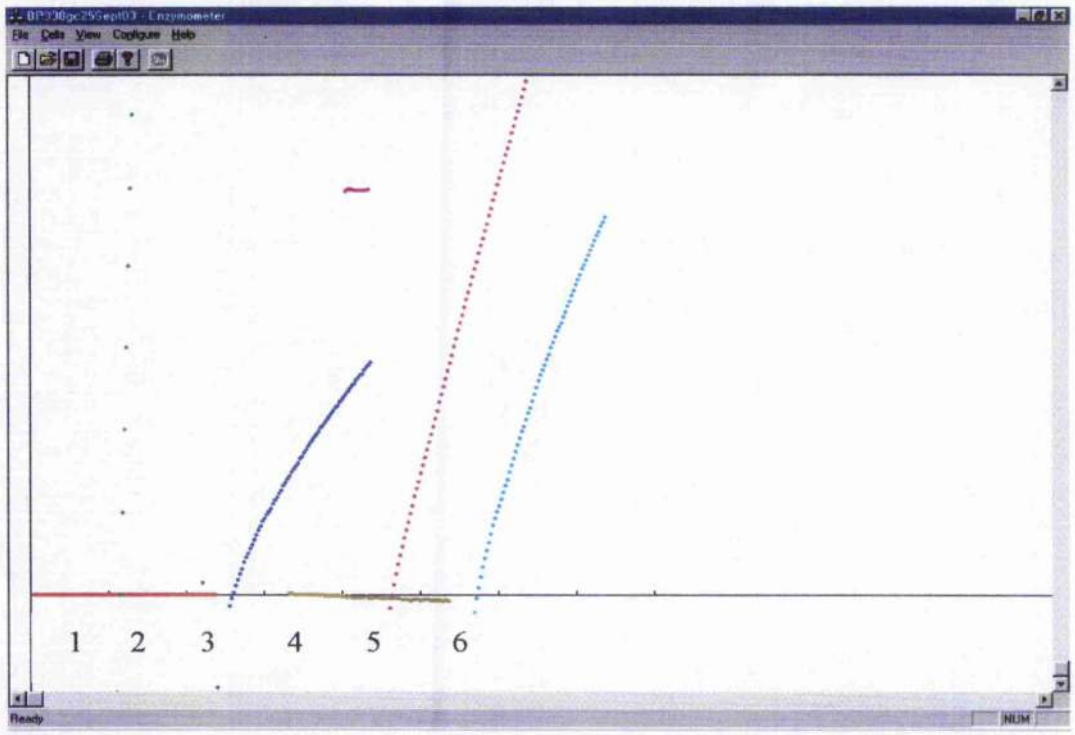
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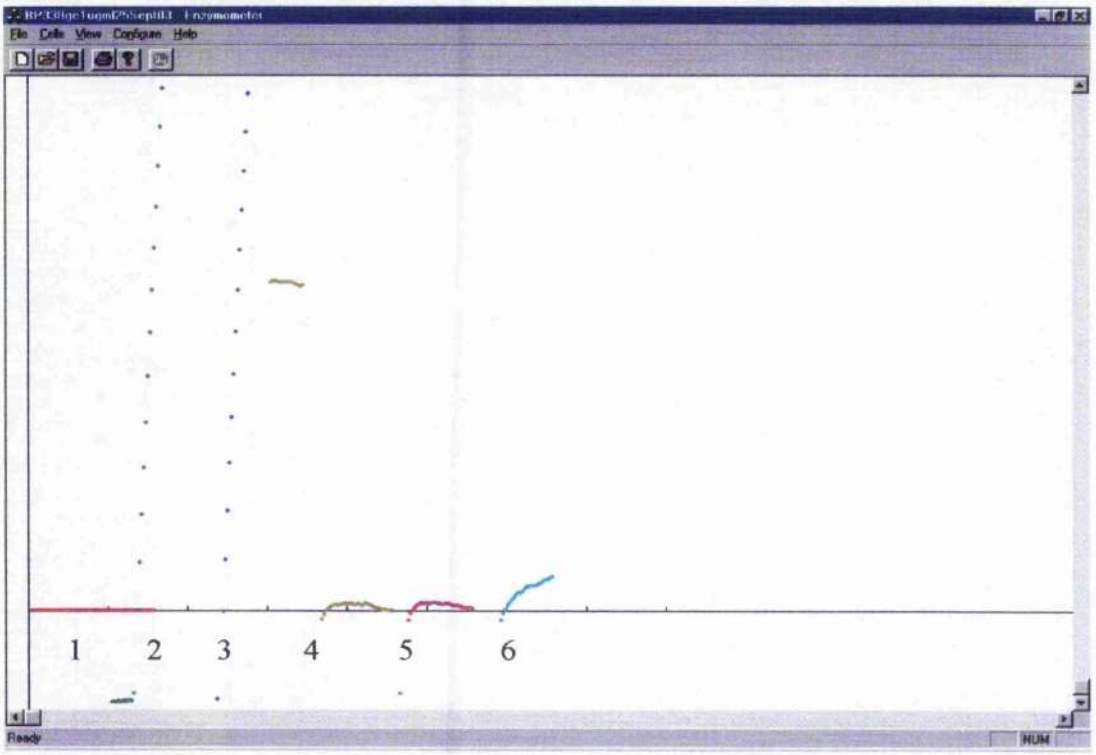
Figure 3.7.4.2. Enzymic (AC) activity of urea extracts of *B. pertussis* BP338 (10 μg protein ml^{-1}) from early exponential, late exponential and stationary phase. Cells 1 and 2 contained negative (assay buffer only) and positive (rCyaA with assay buffer and all reagents) controls respectively. Cells 3-6 contained urea extracts from the different growth phases of *B. pertussis* to test for AC activity.

Key:
1 — Blank
2 — Positive control
3 — Positive control (with urea)
4 — Early exponential phase BP338
5 — Late exponential phase BP338
6 — Stationary phase BP338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3.7.5 Detection of AC activity in other *Bordetella* species

Lysates of other *Bordetella* spp. namely *B. parapertussis*, *B. bronchiseptica*, *B. avium*, *B. hinzii*, *B. holmesii* and *B. trematum* were prepared using the method described in section 3.7.1, developed for lysing *B. pertussis* cells with Triton X-100. The *B. pertussis* strains BP338 and BP347 were included in the majority of assays as positive and negative controls, respectively. AC activity was detected only in *B. parapertussis* (NCTC5952, NCTC10853) and *B. bronchiseptica* (BBC17). It was noted that AC activity was not detected in *B. bronchiseptica* strains NCTC452 and NCTC458. The two *B. parapertussis* strains tested had different AC activities, the progress curve of NCTC5952 had a tangent of 31 and NCTC10853 had a tangent of 58 (Fig. 3.7.5.1). *B. parapertussis* NCTC10853 was also observed to be much more haemolytic on BG sandwich plates (indicative of the production of CyaA). In this assay it should be noted that the negative control *B. pertussis* strain, BP347, does appear to have activity and this may be due to the presence of contaminating AC activity or due to the fact that it has been demonstrated that the conductimetry can be to, some extent, non-specific.

Conductimetry assays determining the AC activity of *B. bronchiseptica* BBC17 had shown that there was a difference in AC activities of strains grown from different glycerol stocks, designated 1 and 2. The AC activity of *B. bronchiseptica* BBC17 (from glycerol stock 2) strain used for intranasal infection of mice and the passaged strain recovered from lung culture at seven days after challenge (M. Lynch, personal communication) were also investigated (Fig. 3.7.5.2). The tangent of the AC enzymic activity reaction curve of the original strain was 12, but, after isolation from the mouse lungs, the tangent of AC enzymic activity reaction curve was 58, about 21% increase in specific activity (Fig. 3.7.5.2). It was also noted that the strain that had been passaged in the mouse model was noticeably much more haemolytic on BG sandwich plates. This result emphasises that *Bordetella* expressing AC enzymic activity must express it most when in the appropriate host or may be selected for by host passage, which is reflected in the greater activity found in passaged isolate. Expression must decrease when the strain is propagated in a laboratory environment. AC activity was not detected in any of the strains of the following species *B. avium*, *B. hinzii*, *B. trematum* and *B. holmseii*. These species were also noted to not be haemolytic on BG sandwich plates.

Figure 3.7.5.1: Comparison of enzymic (AC) activities of *B. parapertussis* (NCTC5952 and NCTC10853) lysates with that of *B. pertussis* BP338. The blank control was in cell 1. *B. pertussis* BP338 was used as a positive control in cell 2. Cell 3 contained BP347 as a negative control. Cells 4 and 5 contained NCTC5952 and NCTC10853 respectively.

Key:

1 — Blank
2 — BP338
3 — BP347
4 — NCTC5952
5 — NCTC10853

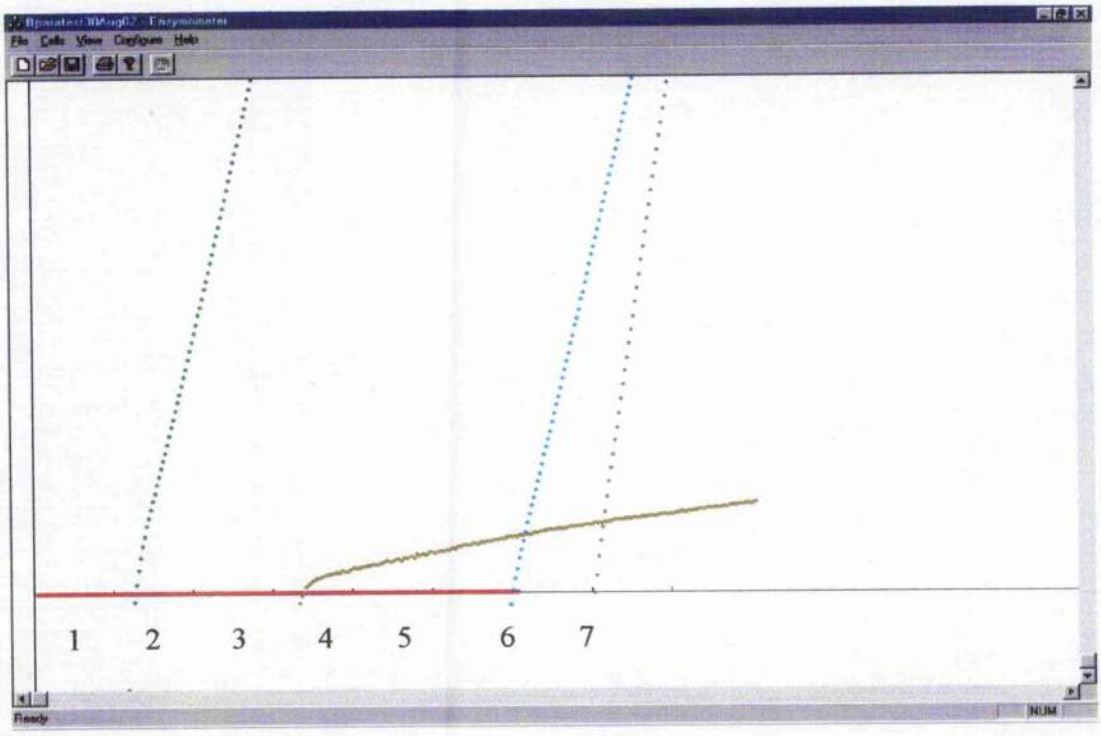
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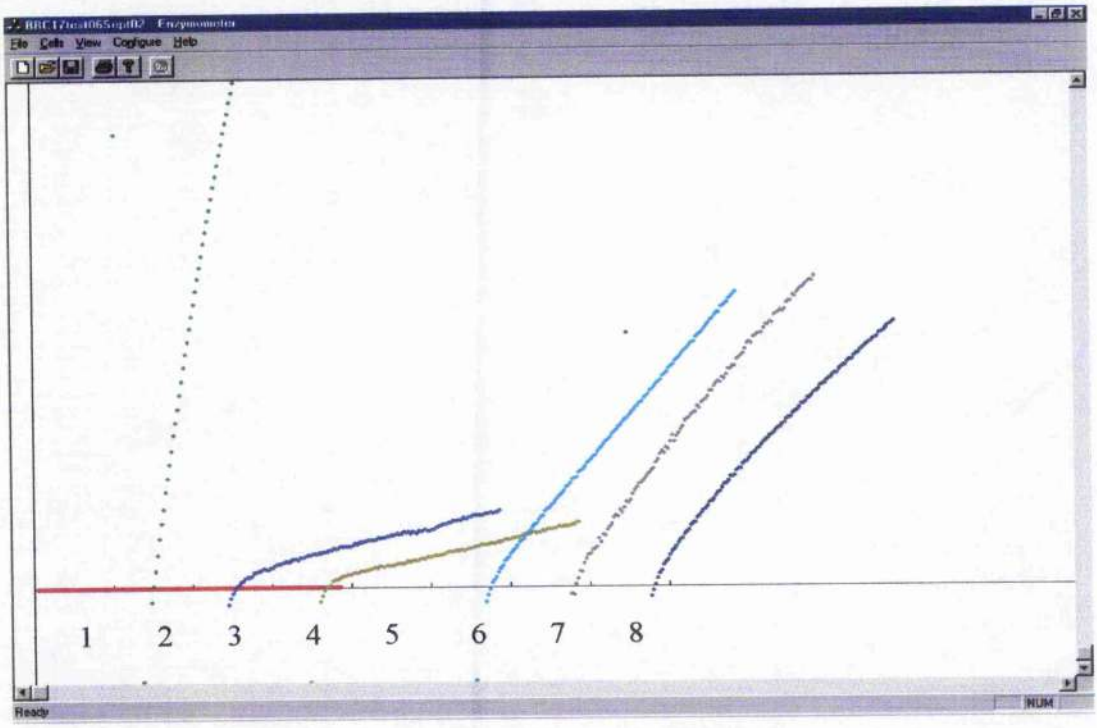
Figure 3.7.5.2: Comparison of enzymic (AC) activities of *B. bronchiseptica* isolates (BBC17 stocks (1) and (2)) with the activity of BBC17 from glycerol stock 2 passaged through the mouse. Cell 1 was the blank control. Cell 2 contained BBC17 from glycerol stock 2 (BBC17(2)) from mouse model. Cells 3-8 contained BBC17 from two different glycerol stocks (1 and 2).

Key:

1 — Blank
2 — BBC17 passaged
3 — BBC17 (1)
4 — BBC17 (1)
5 — BBC17 (1)
6 — BBC17 (2)
7 — BBC17 (2)
8 — BBC17 (2)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3.8 PHENOTYPIC ANALYSIS OF PICU475 AND 99K45

3.8.1 Comparison of AC activities of cell lysates 99K45, PICU475 and BP338

B. pertussis strains 99K45 and PICU475 from 1999 were the only two isolates from a panel of 46 to contain a 6 bp insertion between the *cyaA* and *cyaC* genes (see section 3.3). The presence of this 6 bp insertion was determined by sequencing and LightCycler melting curve analysis. It was desirable to determine if this insertion had any effect on expression of AC enzymic activity. The AC enzymic activity was compared between four *B. pertussis* strains: BP347, BP338, PICU475 and 99K45. Lysates of the four strains were prepared using the non-ionic detergent Triton X-100 from suspensions of 2 x 10^9 cfu ml^{-1} (see section 3.7.1) and these were immediately added to the conductimetry assay to determine differences in AC activity. The positive control rCyaA in the conductimetry cells was at a final concentration of 43 ng protein ml^{-1}. Strain 99K45 had increased CyaA activity compared to BP338 (tangents 48 and 23 respectively), and in another assay PICU475 had CyaA activity similar to that of BP338 (tangents 21 and 19 respectively) (Fig. 3.8.1.1). The tangent of the positive control, rCyaA, was slightly lower than that of previous assays, whereas the tangent of the BP338 preparation was similar to that obtained previously. Strain 99K45 did not appear more haemolytic on BG agar compared to BP338 and PICU475. Lysates of *B. pertussis* cell suspensions using the non-ionic detergent Triton X-100 could not be analysed by SDS-PAGE to visualise the presence and intensity of a CyaA band. It was important to visualise and quantify CyaA from these *B. pertussis* strains, therefore urea extracts of BP338, 99K45 and PICU475 were prepared.

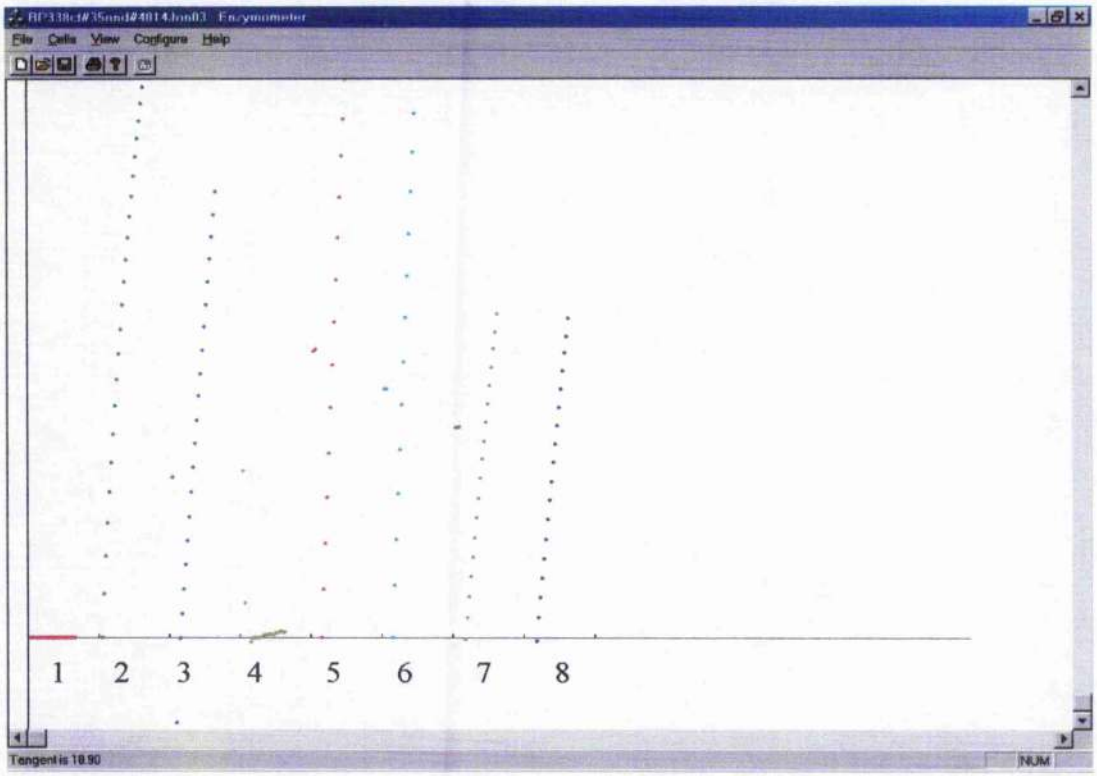
3.8.2 Comparison of AC activities of urea extracts of 99K45 and BP338

Previous analysis of the *B. pertussis* growth curve had determined that the greatest CyaA expression occurred from cells taken from stationary phase. Therefore *B. pertussis* strains BP338 and 99K45 were grown in 250 ml of SS liquid medium for 73 h at 37°C with shaking at 150-200 rpm. It was observed that the PICU475 strain did not grow as well in liquid (SS) medium as BP338 and 99K45, therefore urea extracts (see section 2.13.3) were prepared only for the latter two *B. pertussis* strains. The AC activity of the urea extracts were determined at 10 µg protein ml^{-1} by the conductimetry assay. Activity of the samples was low, their activities were about 1.5 IU mg^{-1} protein, which was similar to that previously found for the BP338 stationary phase urea extract from the growth curve analysis. This

Figure 3.8.1.1: Enzymic (AC) activities of lysates of *B. pertussis* strains BP338, BP347, 99K45 and PICU475. Cell 1 was the assay blank control consisting of assay buffer and reagents only. The positive control (rCyaA) was in cell 2. Cell 3 contained BP338 lysate used as the *B. pertussis* AC activity positive control and cell 4 contained BP347 lysate as the negative control for *B. pertussis* AC activity. Cells 5-8 contained the test samples, see key below for description.

Key:

1 — Blank
2 — Positive control
3 — BP338 lysate
4 — BP347 lysate
5 — 99K45 lysate
6 — 99K45 lysate duplicate
7 — PICU475 lysate
8 — PICU475 lysate duplicate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NUM

result does not agree with that of the previous assay with *B. pertussis* cells freshly grown on BG agar and lysed with Triton X-100, where 99K45 had greater AC activity. However, due to the low activity of these extracts it is probably not possible to accurately determine any differences in AC activity.

3.8.3 SDS-PAGE analysis of urea extracts

The AC activity of the urea extracts was low and it was required to make the *B. pertussis* urea extracts as active as possible and this was done by concentrating the samples. Urea extracts were concentrated using AMICOM® columns with 30,000 molecular weight cut-off. From the protein assay it was determined that the unconcentrated urea extract of 99K45 was 389 μg ml^{-1} and this was concentrated to 1945 μg ml^{-1}. The unconcentrated sample of BP338 was 719 μg ml^{-1} and that of the concentrated sample was 2170 μg ml^{-1}. SDS-PAGE analysis was used to visualise the presence and intensity of CyaA in unconcentrated and concentrated urea extracts of BP338 and 99K45. A volume of 10 μl of each urea extract was used and the intensity of bands at c. 200 kDa, indicating the presence of CyaA, were compared (Fig. 3.8.3.1). The concentrated urea extracts of BP338 and 99K45 had a greater band intensity of CyaA on the gel and these were used in the following cytotoxicity assay. These concentrated samples were not analysed for AC activity with the conductimetry assay due to the small volume available for the cytotoxicity assay.

3.8.4 Comparison of cytotoxic activities of 99K45 and BP338

Investigation of the potential effect of the 6 bp insertion on expression of AC or AC activity was inconclusive, as no clear differences in AC activity between BP338 and 99K45 were detected. As this insertion is situated very close to the *cyaC* gene (according to the GenBank sequences of *cyaA* and *cyaC*) it was of interest to determine if this would have any effect on the cytotoxic activity of CyaA by virtue of limitation of expression of *cyaC* whose product CyaC is required to activate pro-CyaA. The urea extracts of BP338 and 99K45 were investigated for differences in their cytotoxic ability by determining their effect on the killing of the mouse macrophage-like cell-line J774.2 by the MTT assay. The urea extracts of BP338 and 99K45 exhibited cell killing only at high protein concentrations, with that of 99K45 being more active than that of BP338 (Fig. 3.8.4.1). The 50% cell killing by the 99K45 extract was achieved at the concentration of 609 μg ml^{-1} whereas that of BP338 was

Figure 3.8.3.1: SDS-PAGE analysis of BP338 and 99K45 urea extracts unconcentrated and concentrated using AMICON® columns. Each lane contained 10 µl of concentrated or unconcentrated sample.

Lane	
M	SeeBluc® Plus2 Pre-Stained Standard (Invitrogen)
1	BP338 unconcentrated
2	BP338 concentrated
3	99K45 unconcentrated
4	99K45 concentrated

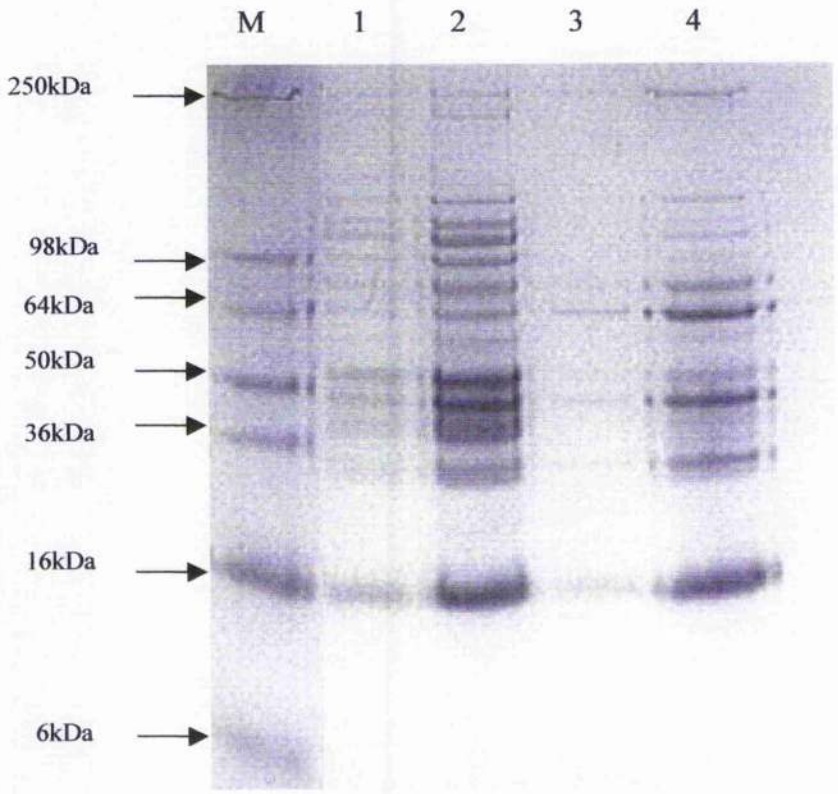
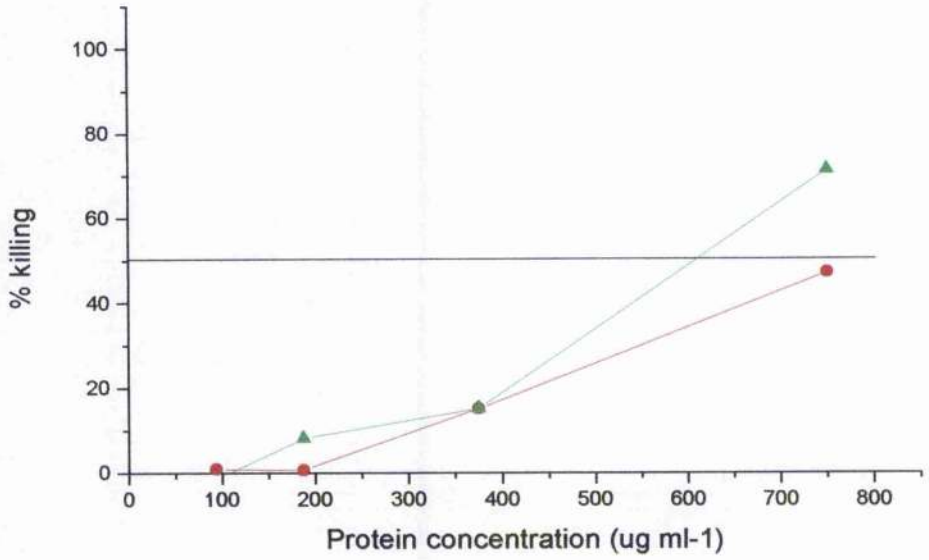

M
1
2
3
4
250kDa
98kDa
64kDa
50kDa
36kDa
16kDa
6kDa

Figure 3.8.4.1. Cytotoxicity assay of urea extracts of *B. pertussis* strains 99K45 and BP338 on J774.2 mouse macrophage cells. Each point on the opposite graph represents the mean of the two values.

Key:

BP338 ——

99K45 ——


100
80
60
40
20
0
% killing
0
100
200
300
400
500
600
700
800
Protein concentration (ug ml-1)

not able to kill 50% of cells in this assay (Fig. 3.8.4.1).

3.9 STUDY OF *cyaA* EXPRESSION WITH REPORTER GENES *lux* AND *gfp*

For investigation of the diagnostic potential of the conductimetry assay, it was important to determine when optimal expression of *cyaA* occurred during the growth of *B. pertussis*. Reporters such as *lux* (codes for luciferase and luciferin, which are required for the light-emitting reaction) and green fluorescent protein (*gfp*) genes provide the ability to study gene expression. The *lux* reporter, with the use of a luminometer, allows this to be done in a real-time fashion and quantitatively. The *gfp* reporter allows gene expression to be followed by fluorescence microscopy or fluorimetry. This system would supplement the previous work using selected growth curve time points for investigation of *cyaA* expression, by determining the AC promoter activity in more rapid and informative ways. The use of *lux* and *gfp* reporters would also facilitate studies on the expression of this essential virulence factor when *B. pertussis* was grown under a variety of different growth conditions such as in the presence of modulating factors, or in the intracellular environment.

3.9.1 Amplification of *B. pertussis cyaA*

The region of DNA that was amplified for use in cloning studies included the promoter region of *cyaA*. Primers CyaA1 and CyaA6 amplified the region including nucleotides 287-1165 (Y00545) whereas primers CyaA1 and CyaA8 targeted and amplified nucleotides 287-1396 (Y00545) (Fig. 3.9.1.1). Boiled lysates of *B. pertussis* strain BP338 were used as the source of DNA template. Visualisation of the resulting PCR products by gel electrophoresis ensured that the target region had been successfully amplified and were of the expected size (see Fig. 3.9.1.2). PCR products of the expected sizes (878 bp and 1109 bp respectively) were obtained with these primer pairs. Negative controls containing water in place of template DNA were included in the PCR reaction to ensure that no contaminating *B. pertussis* DNA was present (Fig. 3.9.1.2).

From gel lanes 1 and 3 containing the negative controls, it can be seen that no product is present thus confirming that the PCR has worked successfully. The sizes obtained were the same as those predicted from GenBank *cyaA* sequence (Y00545). The PCR products were purified and used for digestion and ligation reactions.

Figure 3.9.1.1: Schematic diagram of *cyaA* (Y00545) amplified by primers CyaA1 and CyaA6/8, the overlapping region of *cyaC* (M5726) is shown. Numbering relates to that of the GenBank sequences. The 6 bp insertion and CDS start sites are indicated. The black arrows indicate the direction of gene transcription. The BvgA-P binding sites and the –35 and –10 regulatory regions are indicated on *cyaA*. See Fig. 3.5.1.1 for nucleotide positions.

Key:

- **4 Bvg binding sites**
- **-35 box**
- **-10 box**

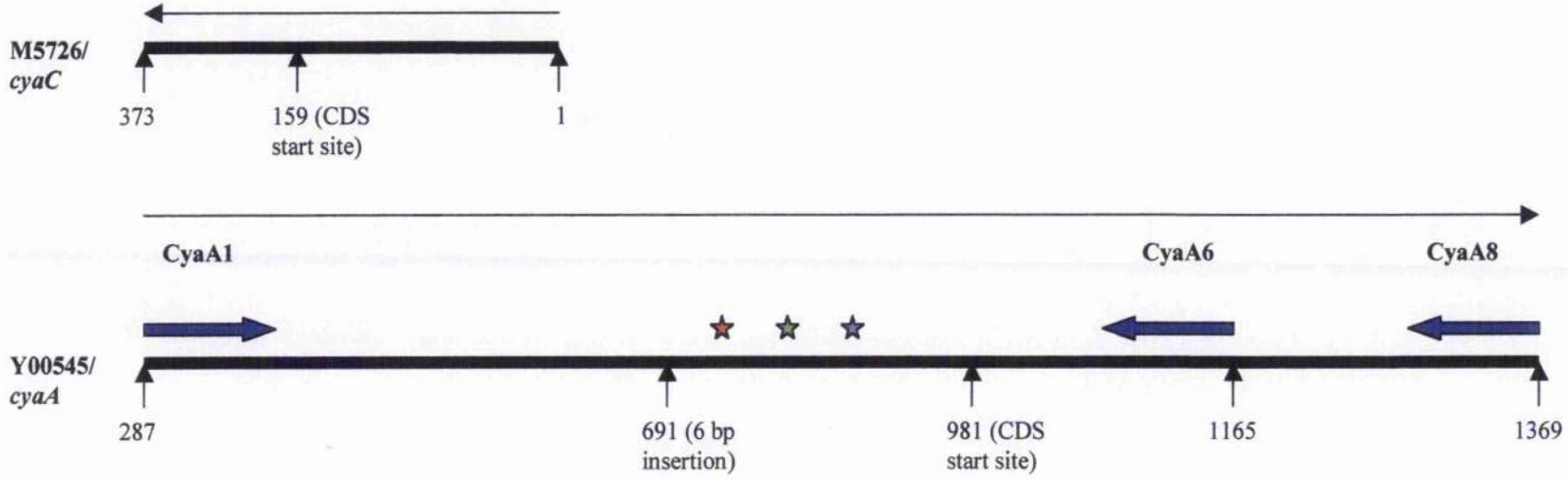
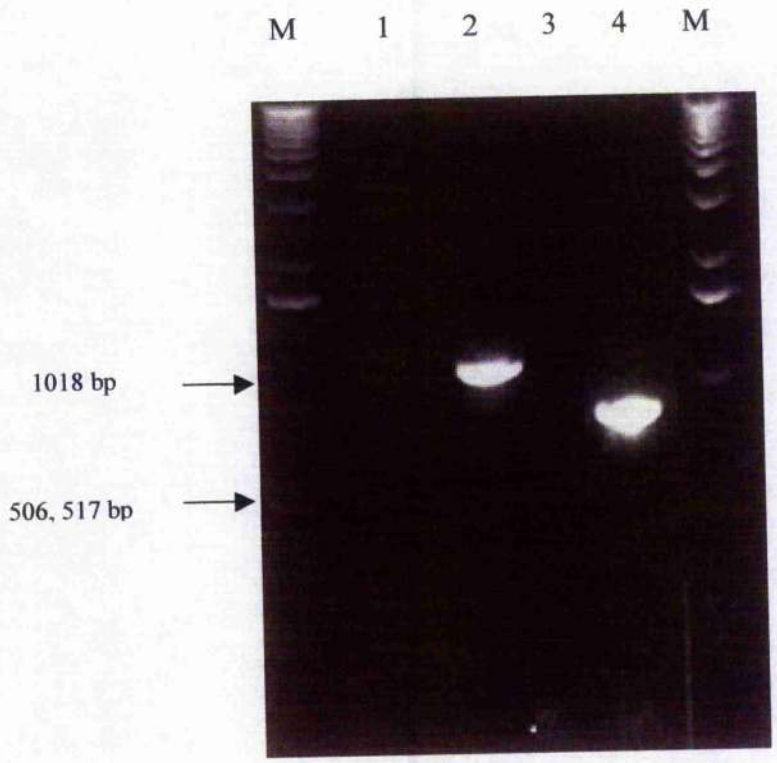

M5726/
cyaC
373
159 (CDS
start site)
1
CyaA1
CyaA6
CyaA8
Y00545/
cyaA
287
691 (6 bp
insertion)
981 (CDS
start site)
1165
1369

Figure 3.9.1.2. Electrophoretic analysis on 1% (w/v) agarose gel of adenylate cyclase toxin (*cyaA*) promoter amplified with primers CyaA1 and CyaA6 or CyaA1 and CyaA8.

Lanes		Predicted product size
M	1 kb ladder (Invitrogen)	n/a
1	negative control for CyaA1/CyaA8 PCR	n/a
2	CyaA1/CyaA8 PCR product	1109 bp
3	negative control for CyaA1/CyaA6 PCR	n/a
4	CyaA1/CyaA6 PCR product	878 bp


M
1
2
3
4
M
1018 bp
506, 517 bp

3.9.2 Cloning of *B. pertussis cyaA* into constructs

CyaA1-6 and cyaA1-8 PCR products were purified, then ligated into the pGEM®-T Easy vector (Promega). *E. coli* XL-2 cells were transformed with 1 µl of the ligation reaction product and grown on SGAL™/LB Agar Blend media containing 50 µg ml^{-1} of ampicillin for blue and white colony screening to determine which cells had been successfully transformed with the construct. Single white colonies were selected and subcultured on LB agar containing 150 µg ml^{-1} of ampicillin. The resulting colonies were grown in LB broth with 150 µg ml^{-1} of ampicillin for preparation of glycerol stocks and plasmid preparation.

For further cloning into the promoterless *gfp* vector, pPROBE-AT', the orientation of the cyaA1-6/1-8 insert in pGEM®-T Easy had to be determined. The enzyme *Pst*I was chosen as that would be predicted to produce a fragment of the size corresponding to cyaA1-6 and cyaA1-8 in one orientation, but not in the other as a *Pst*I site was included in primer CyaA1 (Fig. 3.9.2.1). If no fragment was present from digestion with *Pst*I then the cyaA1-6/1-8 insert was not in the desired orientation. The restriction enzymes that were chosen for cloning into pPROBE-AT' (Fig. 3.9.2.2) were the enzymes *Kpn*I/*Sac*I. The forward primer CyaA1 contains a *Kpn*I site and the pGEM®-T Easy contains a *Sac*I site, and both of these sites are present in the multi-cloning site of pPROBE-AT'.

The cyaA1-6 and cyaA1-8 inserts were removed from pGEM®-T Easy vector with restriction enzymes *Kpn*I and *Sac*I in a double digestion reaction. Electrophoretic separation of the digested fragment and vector was visualised on 1% (w/v) agarose gels. The insert was extracted and purified from the agarose gel for use in further cloning assays with the vector pPROBE-AT' (Fig. 3.9.2.2). The pPROBE-AT' was digested with the enzymes *Kpn*I and *Sac*I and this was visualised using gel electrophoresis to ensure that successful digestion had occurred. The digested vector was purified before ligation with the digested insert such that the cyaA1-6 or the cyaA1-8 fragment was inserted upstream of the *gfp* gene in the correct orientation. A volume of 1 µl of the ligation reaction was used to transform *E. coli* XL-2 and SM10 λ pir strains by electroporation. There were many *E. coli* colonies resulting from transformation with the ligation reaction after electroporation. Representative colonies were subcultured and used for plasmid preparation.

Figure 3.9.2.1: Desired orientation of cyaA1-6/cyaA1-8 insert as anti-sense to *lacZ* in pGEM®-T Easy vector (Promega), drawn using Redasoft™ Visual Cloning 2000.

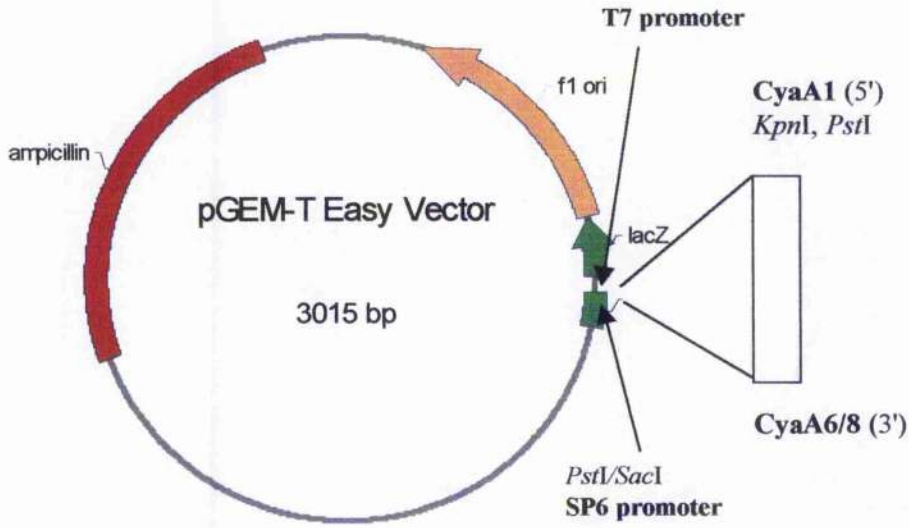
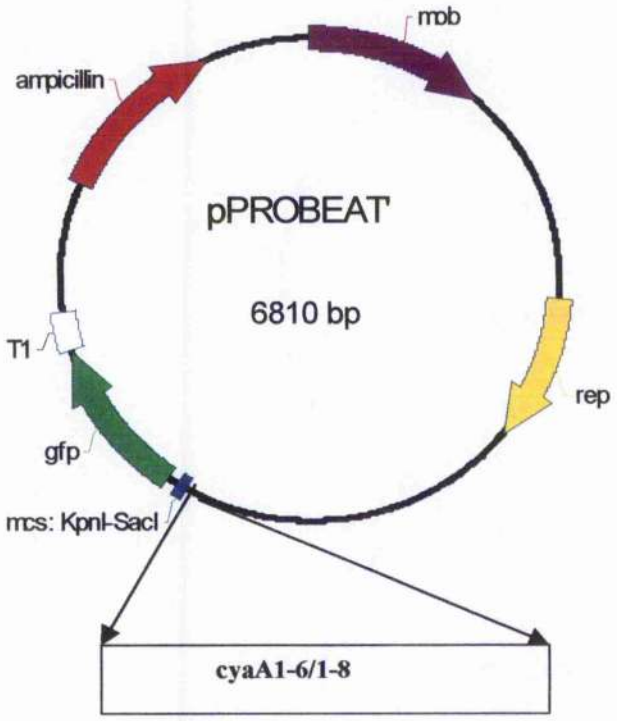

T7 promoter
f1 ori
CyaA1 (5')
KpnI, PstI
ampicillin
pGEM-T Easy Vector
lacZ
3015 bp
CyaA6/8 (3')
PstI/SacI
SP6 promoter

Figure 3.9.2.2. Promoterless *gfp* construct, pPROBEAT', showing multi-cloning site (mcs) with restriction sites used for cloning of *Kpn*I/*Sac*I-digested *cyaA* promoter product, drawn using Redasoft™ Visual Cloning 2000.


mob
ampicillin
pPROBEAT'
6810 bp
T1
rep
gfp
mcs: KpnI-SacI
cyaA1-6/1-8

Correct orientation of the cyaA1-6 or cyaA1-8 fragment was not required for cloning with the vector pSB395 as this vector only contains one restriction site, *Eco*RI (Fig. 3.9.2.3). The cyaA1-6 and cyaA1-8 inserts were removed from pGEM®-T Easy vector with restriction enzyme *Eco*RI. Electrophoretic analysis of the digestion reaction was visualised on 1% (w/v) agarose gels. The insert was extracted and purified from the agarose gel for use in further cloning assays with the *lux*-containing plasmid pSB395. The vector was digested and purified before further cloning by ligation of the digested pSB395 with the digested insert such that the cyaA1-6 or cyaA1-8 fragment was inserted upstream of the promoterless *lux* operon. A volume of 1 µl of the ligation reaction was used to transform *E. coli* XL-1 MRF' and SM10 λ pir strains by electroporation. Only two colonies were present after transformation of pSB395 containing the cyaA1-6 insert by electroporation. These were subcultured and used for plasmid preparation.

3.9.3 Confirmation of reporter constructs in *E. coli*

Purified plasmid preparations of putative cyaA1-6 and cyaA1-8 constructs in *E. coli* were checked by restriction analysis and PCR. Restriction analysis of pPROBE-AT' plasmid preparations from *E. coli* XL-2 and SM10 λ pir transformants was carried out to ensure that the construct was present with its insert. The ligation was successful only with the cyaA1-6 insert with pPROBE-AT' as determined by restriction analysis of the plasmid preparation. The plasmid preparations from cells suspected to contain pPROBE-AT' with cyaA1-8 insert contained only the vector, and no insert was visualised. Fig. 3.9.2.1 shows digestion of pPROBE-AT' constructs containing cyaA1-6 fragment from plasmid preparation of *E. coli* XL-2 cells. Lanes 1-12 contain digestion reactions of individual colonies resulting from transformation with pPROBE-AT' containing the cyaA1-6 ligation reaction. After double digestion with *Kpn*I/*Sac*I, the insert was seen as a band of 878 bp for cyaA1-6 (Fig. 3.9.3.1). The plasmid constructs could be used for transfer to *B. pertussis* to study the expression of *cyaA* under different growth conditions.

Ligation reactions of the cyaA1-6 insert and pSB395 were successful whereas ligation reactions with the cyaA1-8 insert were not; that is, colonies were present only after transformation of *E. coli* cells with the cyaA1-6 and pSB395 ligation reaction. Therefore, further studies with the *lux* plasmid were carried out using cyaA1-6. The nature of the *lux*

Figure 3.9.2.3. Promoterless *lux* construct, pSB395, with *Eco*RI restriction site used for cloning of *Eco*RI-digested *cyaA* promoter product, drawn using Redasoft™ Visual Cloning 2000.

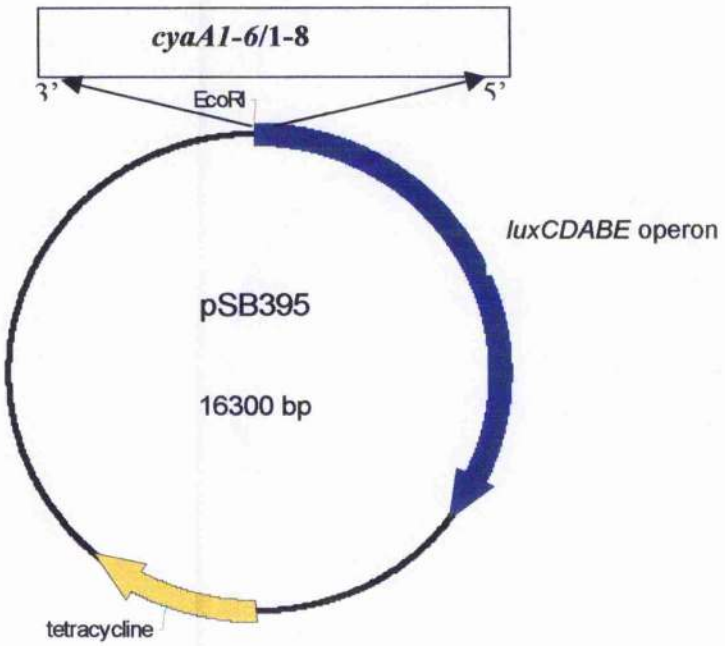
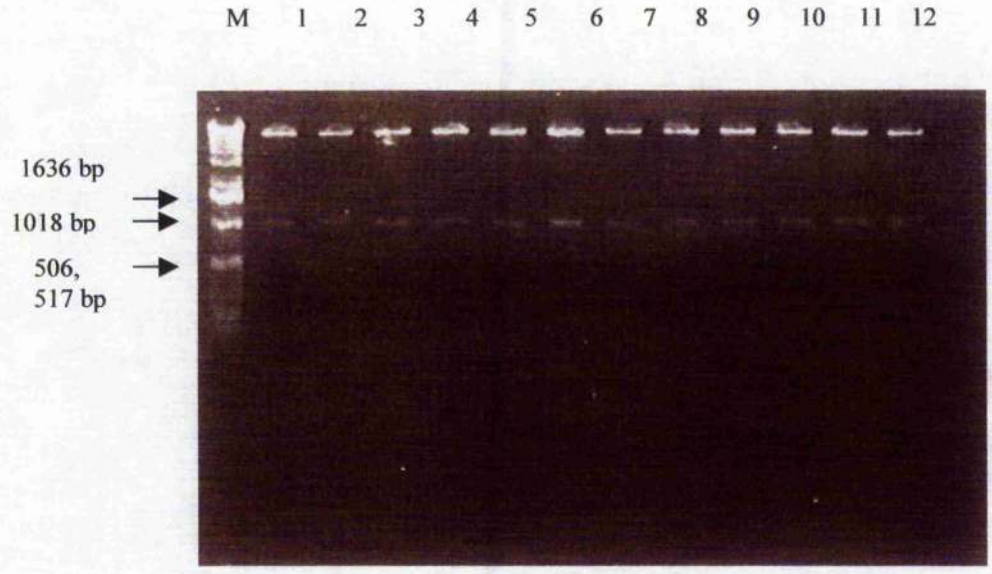

cyaA1-6/1-8
3'
EcoRI
5'
luxCDABE operon
pSB395
16300 bp
tetracycline

Figure 3.9.3.1: Electrophoretic analysis on 1% (w/v) agarose gel of digested plasmid preparations from *E. coli* XL-2 blue transformed cells containing pPROBE-AT' with cyaA1-6 insert. Purified plasmid (pPROBE-AT') preparations from individual colonies were digested with *Kpn*I and *Sac*I and then analysed for the presence of cyaA1-6 insert. The expected size is c. 878 bp.

Lanes	
M	1 kb ladder (Invitrogen)
1-12	Plasmid preparations double-digested with *Kpn*I and *Sac*I


M 1 2 3 4 5 6 7 8 9 10 11 12
1636 bp
1018 bp
506,
517 bp

plasmid, pSB395, with the cyaA1-6 insert in *E. coli* XL-1 and *E. coli* SM10 λ pir cells was also checked by PCR to ensure that the *cyaA* promoter was in the correct orientation to allow expression of the promoterless *lux* operon. To overcome the lack of orientation of the inserts in pSB395, a reverse primer was designed to target the *luxC* gene (the first gene of the *lux* operon) and the forward CyaA1 primer from the previous PCR amplification of this insert was used. Boiled lysates of the resulting *E. coli* colonies from transformation by electroporation were used as DNA templates. A negative control containing water in place of DNA template was included to ensure that no contaminating DNA was present.

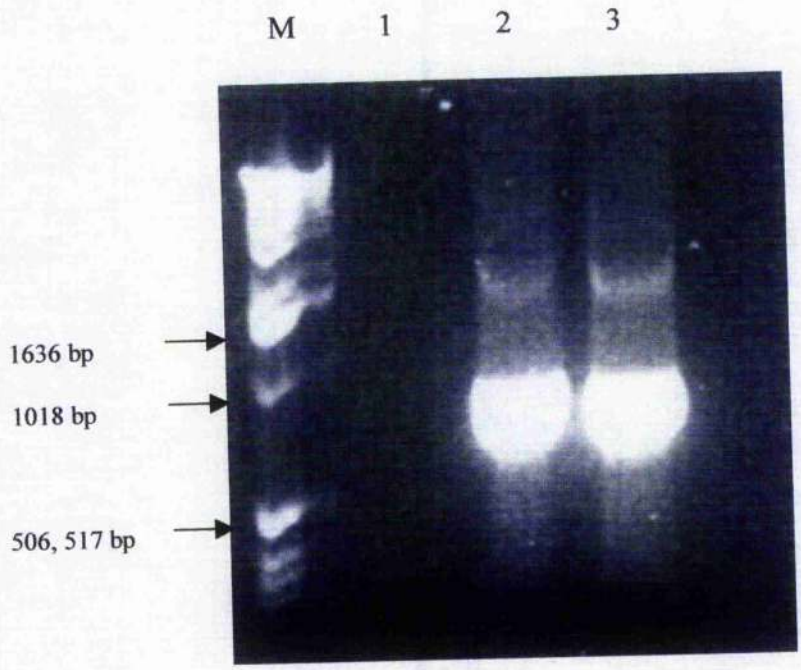
PCR reactions were analysed by agarose gel electrophoresis (Fig. 3.9.3.2). If a PCR product was present, it ensured that transformation had been successful and that the cyaA1-6 insert was present in the correct orientation for expression of the *lux* operon. PCR products of the correct size, 878 bp, were found for two of the transformed *E. coli* SM10 λ pir colonies resulting from electroporation with the ligation reaction (gel lanes 2 and 3, Fig. 3.9.3.2). The plasmid pSB395 containing the cyaA1-6 insert in the correct orientation could be used for transfer to *B. pertussis* to study the expression of *cyaA* in different growth conditions. The transformed *E. coli* SM10 λ pir containing pSB395 with cyaA1-6 insert was checked for luminescence using the luminometer Lucy-1. No light output was detected but this was expected due to *cyaA* expression occurring only under the control of the *bvg* locus, which is not present in *E. coli*.

3.9.4 Plate mating of *E. coli* and *B. pertussis*

The pSB395 plasmid containing the cyaA1-6 insert was introduced into *B. pertussis* BP338 from *E. coli* SM10 λ pir by conjugation. This method was also used as an attempt to introduce the *gfp* plasmid, pPROBE-AT' containing the cyaA1-6, from *E. coli* SM10 λ pir strain to *B. pertussis* BP338 but this was not successful. Therefore this method will be described for pSB395 with the cyaA1-6 insert. It was found through optimisation of the plate-mating method that the *E. coli* would easily outgrow *B. pertussis* and one way that this could be overcome was to increase the donor: recipient ratio and make the incubation time shorter. The protocol that was found to result in *B. pertussis* cells containing the plasmid pSB395 with the cyaA1-6 insert was a combination of two protocols methods, described by M. Lynch (personal communication) and Stibitz and Carbonetti (1994). The optimised

Figure 3.9.3.2: PCR products of *E. coli* SM10 λ pir cells containing the plasmid pSB395 with the cyaA1-6 insert in the correct orientation. DNA from boiled lysates of *E. coli* cells were amplified with primers CyaA1 and LUXCr. PCR products, pSB-16, were analysed on 1% (w/v) agarose gel and reactions that gave products of expected size were considered to contain the cyaA1-6 insert in the correct orientation to allow expression of the *lux* operon.

Lanes		Predicted product size
M	1 kb ladder (Invitrogen)	n/a
1	negative control	n/a
2	pSB1-6 PCR product	878 bp
3	pSB1-6 PCR product	878 bp


M
1
2
3
1636 bp
1018 bp
506, 517 bp

method is described in section 2.15.4. Briefly, the method involved preparing a donor:recipient (1:10) mix of *E. coli* and *B. pertussis* cultures. This bacterial mix was incubated on BG agar for 7-8 hours. The bacteriophage, ffm04, was then added to this mix to lyse *E. coli* cells. After conjugation, cultures were plated on BG containing cephalexin and tetracycline, which counterselected against *E. coli* donor cells and selected for *B. pertussis* carrying the pSB395 construct, respectively. Only six colonies resulted from the conjugation and these colonies were selected and individually subcultured on BG agar with 12.5 μg ml^{-1} of tetracycline. The resulting cultures were used for preparation of glycerol stocks and subcultured in SS liquid medium for plasmid preparation.

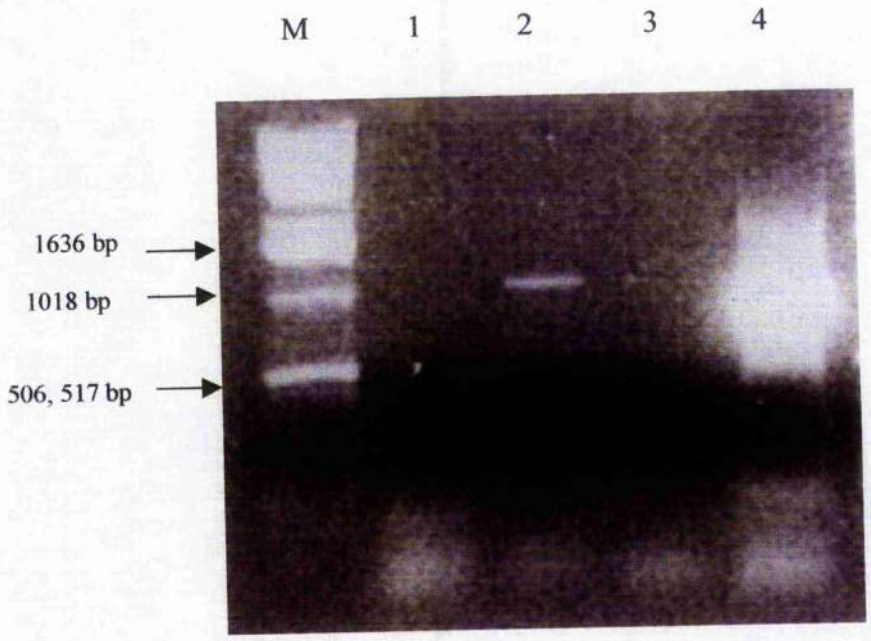
The six *B. pertussis*-like colonies containing pSB395 with the cyaA1-6 insert were grown in SS liquid medium and were Gram-stained to check if they were potential contaminants before plasmid preparation. Two colonies were clearly *B. pertussis* isolates from which plasmid preparations were made and PCR analysis was carried out. Boiled lysates of the *B. pertussis* colonies were used as DNA template and, if pSB395 with the cyaA1-6 insert was present, a PCR product would be obtained using CyaA1 and LUXCr primers. A negative control containing water in place of DNA template was included to ensure no contaminating DNA was present. The positive control consisting of boiled lysate of *E. coli* containing pSB395 with cyaA1-6 insert, which was also included to ensure that the PCR analysis had not failed due to technical error. Electrophoretic analysis on 2% (w/v) agarose gels was used to visualise the PCR products. From Fig. 3.9.4.1 it can be seen that only one of the two plasmid preparations has a PCR product that could be visualised on the gel, however the size of the band is higher than expected. The *E. coli* preparation that was used in the plate-mating has a PCR product of much greater intensity than that of *B. pertussis*.

3.9.5 Detection of bioluminescence from pSB395 with *cyaA* promoter insert in *B. pertussis*

Once the presence of the *cyaA* promoter inserted upstream of the *lux* reporter in the correct orientation was confirmed, glycerol stocks were prepared and used to prepare cell suspensions for bioluminescence assay using the luminometer Lucy1. *E. coli* with the *lux* construct containing the *cyaA* promoter and BP338 wild-type were included as controls as they were not expected to be bioluminescent. BP338 wild-type and BP338 with the *lux*

Figure 3.9.4.1. Electrophoretic analysis on 1% (w/v) agarose of purified plasmid from *B. pertussis* BP338 cells and *E. coli* SM10 λ pir cells containing the construct pSB1-6. The PCR product pSB1-6 consists of vector pSB395 and adenylate cyclase toxin (*cyaA*) promoter region amplified with primers CyaA1 and CyaA6. Purified plasmid from *E. coli* SM10 λ *pir* containing the construct pSB1-6 PCR was also included as a positive control. The purified plasmid preparations of confirmed *B. pertussis* cells and *E. coli* cell were amplified using LUXCr and CyaA1 primers to ensure that the vector and insert were present.

	Lanes	Predicted product size
M	1 kb ladder (Invitrogen)	n/a
1	negative control	n/a
2	*B. pertussis* pSB1-6 no.1	878 bp
3	*B. pertussis* pSB1-6 no.2	878 bp
4	*E. coli* pSB1-6	878 bp


M
1
2
3
4
1636 bp
1018 bp
506, 517 bp

construct, pSB1-6, were both haemolytic on BG sandwich plates. It was important to check that *B. pertussis* strains containing the *lux* plasmid with cyaA1-6 insert were as haemolytic as the wild-type strain so as to ensure that the introduction of the plasmid had not affected the expression of *cyaA* from the chromosome and it still was in the Bvg^+ phase. *B. pertussis* cells were grown in 5 ml of SS liquid medium before inoculation of fresh SS medium in the 96-well plates. However, the *B. pertussis* strain containing pSB395 with the *cyaA* promoter cloned upstream of the *lux* operon was not light-producing when assayed by the luminometer. Boiled extracts of *B. pertussis* pSB1-6 cells grown on BG agar for 72 h were re-checked using PCR with CyaA1 and *luxC* primers. No PCR products were visualised using gel electrophoresis. These findings will be discussed in Section 4.6.

4.0 Discussion

4.1 SEQUENCE VARIATION AND CONSERVATION

4.1.1 Strain collection used for sequencing

The strain collection used for determining genotypic variation of *B. pertussis* isolates during the course of this PhD study was carefully selected to be representative of the UK *B. pertussis* population from the pre-vaccination era to the present day, but with constraints due to strain availability. In the culture collection held at RSIL, isolates were not available for all years, or in some cases, few were available for a particular time period. The total number of *B. pertussis* isolates held at RSIL from 1920-2002 was 446. These isolates were from several collections held within the UK: CAMR, NCTC, Wellcome collection, University of Glasgow and University of Manchester. The strain collection is presumed to be representative of *B. pertussis* isolates from the UK, but there are only a few isolates available from Scotland, Wales and Northern Ireland. Only a limited number of isolates were available for a particularly interesting period, from 1974-1983 when there was a decline in vaccination uptake levels in the UK. Even though there was a limited number of isolates available from this period, unusual *tcfA* types and MLSTs were detected. No *B. pertussis* strains from 1986-1997 were available in this study therefore it is difficult to be precise about trends of genes such as *ptxC* where the shift in allele type was not apparent until 1998. Thus it remains unknown at present when the appearance of the *ptxC* allele, *ptxC*(2), actually occurred. This problem relating to gaps in the strain collection and limited numbers of isolates appears to have been encountered in other, similar studies that have been carried out elsewhere. Lack of isolates in the Dutch strain collection were apparent for the time period 1952-1965 (van Loo *et al.*, 2002). The MLST scheme developed by van Loo *et al.* (2002) was applied to only 10 Italian strains, 13 Japanese strains, 27 Finnish strains and 18 US strains. This US strain panel also included a number of isolates (n=10) from the pre-vaccination era. Therefore it is difficult to compare the MLST trends of these countries with those in the UK and The Netherlands that have used larger strain collections. The four vaccine strains and *B. pertussis* type strain NCTC10739^T were included in the present study as reference strains to help identify vaccine and non-vaccine allele types and to increase the probability of identifying polymorphic sites, as the type strain has been shown to be an atypical strain (Musser *et al.*, 1986; Gerlach *et al.*, 2001).

4.1.2 Isolate numbers chosen for individual targets

Different numbers of *B. pertussis* isolates were used for investigation of gene variation in different virulence factors (see section 2.1.1 for further information). Isolates stored in RSIL had previously been sequenced for *prnA* and *ptxA* as well as being serotyped (Fry *et al.*, 2001), therefore isolates that showed differences in these properties were known. The gene *cyaA* was the original target for investigation of sequence variation amongst UK *B. pertussis* isolates. Forty-seven of these isolates were selected from the strain collection held at RSIL and ranged in date of isolation from 1920 – 2002. They included strains with known *ptxA* and *prnA* differences as well as distinct serotypes. The subset also contained the four vaccine strains and the type strain to be used as reference strains and to determine if a shift in sequence types from the vaccine strains had occurred. The subset panel was considered to be representative of UK isolates. Limited variation was suspected after sequencing regions of the *cyaA* gene in only 20 *B. pertussis* isolates due to the lack of variation between the atypical type strain and these other isolates. However, another 27 isolates were sequenced.

The only difference in the *ompQ* gene reported previously was between the type strain and Dutch *B. pertussis* isolates (van Loo *et al.*, 2002). However, it was still important to determine whether this lack of variation also applied to UK isolates (discussed in section 4.1.6). The same set of isolates used for investigating *cyaA* variation were chosen for single nucleotide polymorphism sequencing of *ompQ* with the Pyrosequencer as this technique is much more rapid for identifying single nucleotide polymorphisms than conventional sequencing. Only a limited numbers of isolates (n=14) were chosen for *brkA* and *vag8* as previous sequencing studies had shown little variation (van Loo *et al.*, 2002), but it was still important to determine if any variation was present amongst UK *B. pertussis* isolates (discussed in section 4.1.6). Using the subset of 47 isolates was considered to be too time-consuming when limited variation has already been demonstrated in other *B. pertussis* populations. Regions of *bapC* were initially amplified and sequenced for five isolates to ensure that primers had amplified the desired region and to determine any potential regions of polymorphism. The number of *B. pertussis* isolates investigated for *bapC* variation was increased to 14 and showed that only a single nucleotide difference existed between the type strain and the other strains including the vaccine strains. These limited numbers (n=14) used isolates chosen from the time period 1920 to 1999 as well as the vaccine strains, for the investigation of *brkA*,

vag8 and *bapC* gene variation. It was intended that this subset would highlight any variation in sequence type throughout this time, if any change had occurred with time and if such change coincided with the introduction of whole-cell vaccination or the drop in vaccination rate and the resurgence of pertussis.

A larger number of UK *B. pertussis* isolates were selected for determining allelic frequencies of *ptxC*, *tcfA* and *fim2* genes in the different time periods. These three gene targets had previously demonstrated variation (van Loo *et al.*, 2002). Gene variation of *ptxC* and *tcfA* was investigated for 138 UK isolates in order that these allele types could be used in conjunction with the known variation of *ptxA* in the strains. The MLST scheme of van Loo *et al.* (2002) (based on these three genes) could then be applied to UK isolates and the data compared with the MLST trends found in *B. pertussis* populations in other countries. Eighty UK *B. pertussis* isolates, as well as the four vaccine strains and the type strain, were selected for investigation of *fim2* variation. Gene variation of *fim2* had been reported in Dutch isolates, but only from the pre-vaccination era (van Loo *et al.*, 2002) and it was important to determine if this was the case for UK isolates. An initial investigation of 42 isolates with the four vaccine strains and the type strain had revealed that *B. pertussis* isolates from 1998 and 1999 contained the two known *fim2* alleles, *fim2*(1) and *fim2*(2). The number of UK isolates was expanded to include more isolates from 1998 and 1999 as the initial investigation showed this was when variation occurred and that only serotype 1,2 isolates from this time period appeared to have the *fim2*(2) allele.

Due to the restricted availability of strains from different time periods and different geographic locations of the UK (Appendix 6), this study may provide only a limited insight into the epidemiology of *B. pertussis* in the UK from 1920-2002. For convenience, isolates were divided into seven time groups: 1920-1949, 1950-1959, 1960-1969, 1970-1979, 1980-1989, 1990-1999 and 2002. In fact, the actual dates of isolation within these groups were 1920, 1941-1949, 1950-1956, 1963-1967, 1977-1979, 1982-1985, 1998-1999 and 2002. For discussion of each virulence-related gene and the MLST scheme actual dates of isolation will be used. The number of isolates used here would have to be increased to determine if there is a link between a particular sequence type and vaccination status or severity of pertussis. However, valuable information regarding strain variation has resulted from this investigation and highlights

the fact that a shift in the genetic makeup of the UK *B. pertussis* population has occurred. Therefore it is important to continue surveillance of gene variation, particularly in relation to the introduction of the ACV that consists of limited antigenic components and the likelihood of its more widespread and increased use.

4.1.3 Pertussis toxin subunit S3 gene

A total of 138 UK *B. pertussis* isolates from the pre-vaccination (1920, 1941-1956), post-vaccination era (1963-1985) and recent (1998-1999, 2002) time periods were investigated for *ptxC* allelic variation. The two known *ptxC* alleles found in the UK and in other *B. pertussis* populations differed by a single silent nucleotide difference. There has been an apparent shift with time from *ptxC*(1) to *ptxC*(2); that is *ptxC*(1) was the only *ptxC* allele found in UK isolates from 1920-1985 and *ptxC*(2) became apparent from 1998 onwards, 22 of 51 (43%) from 1998-1999, and was the predominant allele in 2002, 33 of 34 (97%). Analysis of the published Tohama-I genome sequence (BX470248) was confirmed it contained the allele *ptxC*(1). The allele *ptxC*(1) was the only *ptxC* allele present during the pre-vaccination era and this has been reported for other countries, The Netherlands and the US (van Loo *et al.*, 2002). The allele *ptxC*(2) was present in Dutch isolates between 1990 and 1999 (32 of 85 isolates, 38%) and in the US *B. pertussis* population (7 of 10, 70%) from the same time period (van Loo *et al.*, 2002). The Japanese isolates from 1990-1999 had both *ptxC* alleles but the frequency of *ptxC*(2), 2 of 13 isolates (15%), was lower than that of UK, US and Dutch *B. pertussis* populations. Finnish and Italian *B. pertussis* populations differed from the other countries in that, from 1990-1999, only *ptxC*(1) was found.

4.1.4 Tracheal colonisation factor gene

A total of 138 UK *B. pertussis* isolates from the pre-vaccination (1920, 1941-1956), post-vaccination era (1963-1985) and recent (1998-1999, 2002) time periods were investigated for *tcfA* allelic variation. The four vaccine strains and the type strain were also investigated. Of the five known *tcfA* alleles (van Loo *et al.*, 2002), *tcfA*(1)-*tcfA*(5), four alleles *tcfA*(2)-*tcfA*(5) were found amongst UK *B. pertussis* isolates. A novel *tcfA* allele, designated *tcfA*(6), was found during this study. The allele *tcfA*(1) contains a 75 bp segment not seen in the other alleles. There are single non-silent nucleotide differences between *tcfA*(2), *tcfA*(3) and *tcfA*(4). The allele *tcfA*(5) contains an extra G nucleotide resulting in the premature translational termination which leads to expression

of a truncated protein. The novel allele *tcfA*(6) found during this study contains a 15 bp deletion not present in the other alleles. All of these described differences occur within the sequence that encodes the N-terminal region of Tcf. The majority of UK isolates including the four vaccine strains and the reported Tohama-I genome sequence were *tcfA*(2). The type strain was *tcfA*(1) and *tcfA*(3)- *tcfA*(6) were found in a small number of isolates. The predominant *tcfA* allele type, *tcfA*(2), found in this study has also been reported in isolates from The Netherlands, Finland, Italy, Japan and the USA and *tcfA*(2) was predominant in all countries except Italy (van Loo *et al.*, 2002). Of ten Italian *B. pertussis* isolates studied, six (60%) were *tcfA*(3) and four (40%) were *tcfA*(2) . The allele *tcfA*(3) was not found in the US or Japanese *B. pertussis* populations. In the three other countries, the allele *tcfA*(3) has been reported in isolates from the period 1990-1999 only. This allele is apparently also more predominant in The Netherlands than in the UK, in that 27 of 85 (31.8%) Dutch *B. pertussis* isolates from 1990-1999 were *tcfA*(3), compared to 2 of 51 (4%) UK *B. pertussis* isolates from 1998-1999. Eight of nine UK *tcfA*(4) isolates were from 1977-1983, and the other was from 1999. The allele *tcfA*(4) has not been reported in *B. pertussis* isolates from The Netherlands, Finland, Italy, Japan, or the USA, but has been seen in at least one isolate from Australia (AJ507643). The UK isolate from 1942 that was *tcfA*(5) was unusual as this allele has previously been reported only in isolates from the United States from 1990-1999 (van Loo *et al.*, 2002). It may be significant that the unusual alleles (*tcfA*(4) and *tcfA*(6)), except for one isolate, occurred during the late 1970s to the mid 1980s, following a drop in vaccine uptake rate that resulted in two epidemic peaks of pertussis in the UK around 1979 and 1983.

4.1.5 Adenylate cyclase toxin gene

A total of 42 UK *B. pertussis* isolates from the pre-vaccination (1920, 1941-1956), post-vaccination era (1963-1985) and recent (1998-1999, 2002) time periods were investigated for allelic variation in *cyaA*. Sequence variation was investigated for the region that encodes the immunodominant moiety, bases 3586-6098 (Y00545) i.e. a total of 2512 bp were sequenced. The same region was also sequenced for the four vaccine strains and the type strain. Only two *cyaA* types were confirmed in this study and the type strain (NCTC10739^T) was the only strain found with allele type *cyaA*(1). This variation existed as a single silent nucleotide difference at position 4403 (Y00545) in the region that encodes the immunodominant moiety (Fig. 2.4.1). There was also a single nucleotide difference at position 3981 (Y00545) in the previously deposited sequence,

Y00545, when compared to the sequence data of NCTC10739^T for the same region. The strain used for the sequence data in Y00545 was *B. pertussis* strain 18323^T. These type strains may have existed as independent cultures for a considerable time, which may explain the single nucleotide difference. Another explanation for this discrepancy is that there was a sequencing error at position 3981 in the previously deposited sequence, Y00545. The *cyaA* sequence was deposited in GenBank in 1988 and the earlier sequencing techniques used may have led to this error. The *cyaA* sequence of NCTC10739^T was confirmed by sequencing in triplicate during this study. Since the *cyaA*(2) sequence is found in all other strains and is also present in the whole genome sequence of Tohama-I strain (Parkhill *et al.*, 2003), it should perhaps be regarded as the prototype sequence. However, amongst all the UK isolates and vaccine strains studied, no variation was found in the immunodominant region (Fig. 2.4.1). To my knowledge this is the first report of *cyaA* variation (Packard *et al.*, 2004).

Adenylate cyclase toxin protein is of interest because it has been shown to be protective against *B. pertussis* infection in the mouse (Hormozi *et al.*, 1999; Guiso *et al.*, 1991; MacDonald-Fyall, 2002) but is not included in any ACV preparation at present. Acellular vaccines have been investigated for their protective efficacy in field trials and some have had their efficacy compared to that of WCVs. The vaccines may contain one to five components consisting of detoxified PTX, Prn, FHA, Fim2 or Fim3. It was concluded from these trials that the greater the number of components an ACV contains, the greater protection afforded (Hewlett, 1997b). One five-component ACV was found to have efficacy equivalent to the WCV that it was compared with (Olin *et al.*, 1997). Such vaccines are less reactogenic than WCVs and are used as pre-school boosters in the UK or for the primary vaccination series in some other countries. These vaccines may be used in the future for routine boosting of adults and adolescents. The genes that encode some of these antigens have demonstrated sequence variation. King *et al.* (2001) demonstrated that the Dutch WCV expressing *prnA*(1) afforded less protection against *B. pertussis* strains expressing *prnA*(2) or *prnA*(3), at least in the mouse, and the introduction of ACVs may effect shifts in the *B. pertussis* population further resulting in the predominance of more non-vaccine sequence types. The CyaA protein has been shown to be protective, at least in mice, and its immunodominant region has been demonstrated to be stable which would make this antigen suitable as a component in any new generation of ACVs. However, countries that have currently licensed ACVs and

included them in their immunisation programmes may not be amenable to the incorporation of an extra component, such as CyaA as this would be expensive and time-consuming. However, CyaA has other potential in vaccine formulations. Its lack of variation in the immunodominant region and overall stability, together with its cell-invasive activity make it suitable for delivery of foreign epitopes to antigen-presenting cells. The molecule has been used as a non-replicative anti-viral vaccine. Recombinant adenylate cyclase toxin containing the lymphocytic choriomeningitis virus nucleoprotein $CD8^+$ epitope inserted in the AC domain between residues 224 and 225, protected mice against challenge with the virus (Saron *et al.*, 1997). Recombinant CyaA could therefore be used as a multivalent vaccine, boosting the immune response to pertussis ACV and, at the same time, delivering protective epitopes against other infections.

The immunodominant region of CyaA has been sequenced previously in *B. parapertussis* (AJ249835, BX470249) and *B. bronchiseptica* isolates (Betsou *et al.*, 1995b). The immunodominant region in *cyaA* of *B. bronchiseptica* exhibited much greater variation than in *B. pertussis* with 11 sequence types for this region having been deposited in the GenBank database. No further variation has been reported for *B. parapertussis cyaA* as there are only two GenBank entries, one of which is the genome sequence. Using the alignment tool BLAST no differences were found between these two GenBank entries. Greater variation in *B. bronchiseptica* isolates is not surprising as it has been shown by MLEE to be the most diverse of the *Bordetella* species whereas *B. pertussis* and *B. parapertussis* were shown to be clonal (Musser *et al.*, 1986). It is also thought that *B. pertussis* and *B. parapertussis* have evolved from *B. bronchiseptica* farily recently, explaining this lack of variation. However the genome data described by Parkhill *et al.* (2003) suggest that the genetic uniformity in *B. pertussis* reflects a recent bottleneck rather than recent descent from *B. bronchiseptica*.

4.1.6 Outer-membrane protein Q gene, virulence-activated gene 8, bordetella autotransporter protein C gene and bordetella resistance to killing protein A gene

A total of 42 UK *B. pertussis* isolates from the pre-vaccination (1920, 1941-1956), post-vaccination era (1963-1985) and recent (1998-1999, 2002) time periods were investigated for allelic variation in *ompQ*. Fourteen UK isolates from the pre-vaccination era (1920, 1941-1950), post-vaccination era (1964-1983) and recent (1998-1999) time periods were investigated for *brkA*, *vag8* and *bapC* variation. Variation was found only

between the type strain and the other isolates in *ompQ*, *vag8* and *bapC*. The variation found in *ompQ* was a single non-silent nucleotide difference, for *vag8* it was three single nucleotide differences, one of which was non-silent, and for *bapC* it was a single silent nucleotide difference. No variation was found in *brkA* in any of the strains examined by van Loo *et al.* (2002) and present data were concordant with this finding. However, a difference in serum resistance has been described between the type strain 18323^T and BP338 (Fernandez and Weiss, 1998). The type strain was found to be more serum sensitive than BP338 despite both expressing BrkA as shown by Western blotting. The reason for increased sensitivity of strain 18323^T is not known, but may be related to differences in the level of BrkA expression. Alternatively, sequence variation in *brkA* might have been expected, creating differences in serum resistance. As $NCTC10739^T$ is, in many respects, an atypical strain of *B. pertussis*, the *ompQ*(2) and *vag8*(2) sequences, which are also present in the genome sequence of the Tohama-I strain (Parkhill *et al.* 2003), should perhaps be regarded as the prototype sequences. This would not apply to *bapC* as the deposited GenBank sequence was designated as the allele *bapC*(1) and was found in all isolates except the type strain.

4.2 MLST TRENDS IN THE UK AND GLOBAL TRENDS

4.2.1 MLST trends

Previous studies have reported variation or lack of variation in the genes coding for five of the acellular vaccine components (*prnA*, *ptxA-E*, *fha*, *fim2*, *fim3*), and other surface-associated proteins, including *tcfA*, in isolates from The Netherlands, Finland, Italy, Japan, and the United States (van Loo *et al.*, 2002). The MLST scheme described by van Loo *et al.* (2002) is based on the allelic variation of three genes, *ptxA*, *ptxC* and *tcfA*. Van Loo *et al.* (2002) reported nine MLST types. This MLST scheme was applied to data from UK isolates in the present study to allow direct comparison between countries. Multilocus sequence type trends from the pre-vaccination era up to recent isolates have been reported only for Dutch and US *B. pertussis* isolates, whereas MLSTs for recent isolates have been reported only for Japan, Italy and Finland (van Loo *et al.*, 2002). The sequence type of vaccine strains has been reported only for The Netherlands.

A shift in MLST types in the UK *B. pertussis* population was found in the present study. Figure. 4.2.1.1a is a duplicate of the MLST data depicted in the results (Fig. 3.2.1.1), to allow ease of comparison between trends in the UK and other countries. During the UK

pre-vaccination era, MLST-2, MLST-3 and MLST-11 were present. Vaccination with UK WCVs was introduced in the 1950s and the MLST types of the vaccine strains were MLST-2 and MLST-3. MLST-2 and –3 were found in post-vaccination isolates and, during the decrease in UK vaccination rates in the 1970s, there was the appearance of unusual MLST types, MLST-6 and –10. A non-vaccine MLST type, MLST-5, was found to predominate in recent isolates (this study).

During the pre-vaccination era in the UK, US and The Netherlands different MLST types were found. In the Dutch *B. pertussis* population, MLST-1 and MLST-2 isolates were present (Fig. 4.2.1.1b; van Loo *et al.*, 2002), in the US population MLST-1, MLST-2 and MLST-9 were present (Fig. 4.2.1.1c; van Loo *et al.*, 2002) whereas the UK had MLST-2, –3 and the novel MLST-11 present (Fig. 4.2.1.1a; this study). All countries had MLST-2 as the predominating type in this period. Whole-cell vaccination was introduced and the four UK vaccine strains contained MLST-2 and MLST-3, whereas the Dutch vaccine strains contained MLST-1 and MLST-2. Multilocus sequence type data for *B. pertussis* strains isolated immediately after the introduction of whole-cell vaccination is only available for UK and The Netherlands and both populations contained MLST-2 and MLST-3 with the latter type predominating (Figs 4.2.1.1a and 4.2.1.1b). However, during 1977-1983 there was the appearance of unusual MLST types, MLST-6 and -10, in the UK and this coincided with the decrease in vaccination uptake.

Recent isolates from 1990-1999 have been investigated for Dutch, US, Finnish, Italian and Japanese populations (van Loo *et al.*, 2002). Each country differed in the MLST frequencies present during this time period (Fig. 4.2.1.1b-f). In the UK, MLST-3 and –5 predominated with MLST-4 and –6 present in very low frequencies. The Dutch isolates from the 1990s also contained similar MLST types but at different frequencies. The Dutch vaccine MLST types, MLST-1 and MLST-2, were present in Dutch isolates but at very low frequencies, 1 and 4%, respectively (van Loo *et al.*, 2002). MLST-3 was present, but at a lower frequency than that of UK isolates, 25% versus 49% for UK. MLST-4 and –5 were present at a greater frequency. Only 4% of recent UK isolates were MLST-4, whereas 31.8% of recent Dutch isolates were MLST-4 (Fig. 4.2.1.1a and b). The frequency of the non-vaccine MLST type, MLST-5, isolates in the UK and Dutch *B. pertussis* populations were similar, 42% and 38% respectively. With regard to US *B. pertussis* isolates from 1990-1999, MLST-5, an MLST type not found in the pre-

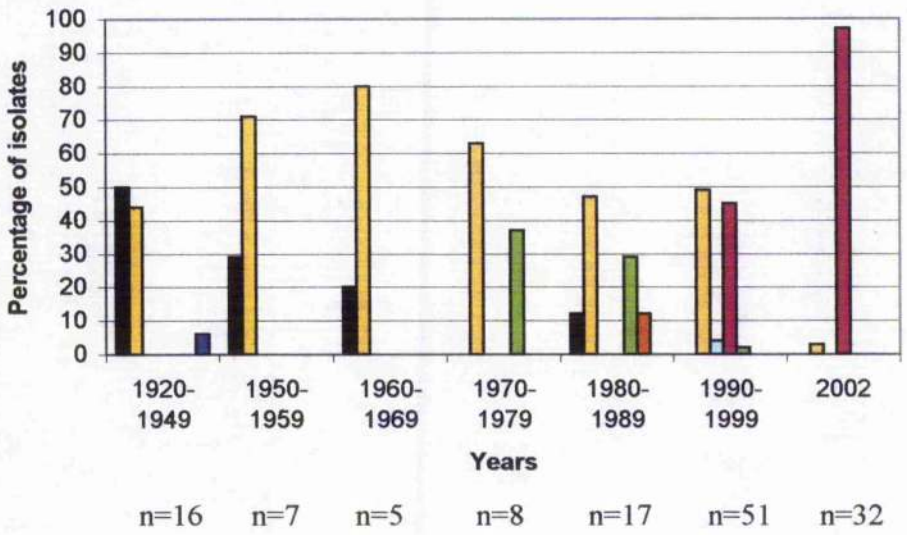
Figure 4.2.1.1a: Temporal trends in multilocus sequence types (MLSTs) in the UK *B. pertussis* population (this study). MLSTs of current UK whole-cell vaccine strains: ■ ■

Key (For Figures 4.2.1.1a-f):

MLST-1 ■
MLST-2 ■
MLST-3 ■
MLST-4 ■
MLST-5 ■
MLST-6 ■
MLST-7 ■
MLST-8 ■
MLST-9 ■
MLST-10 ■
MLST-11 ■

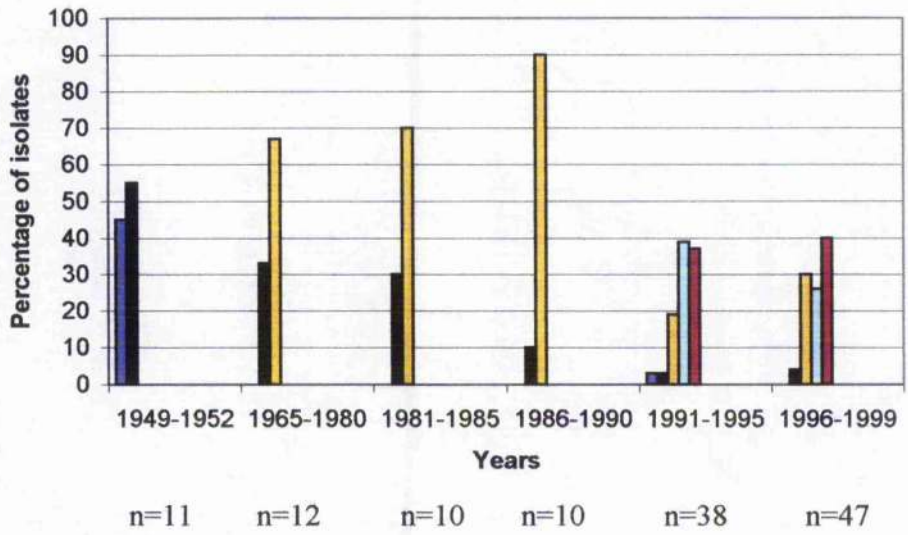
Figure 4.2.1.1b: Temporal trends in multilocus sequence types (MLSTs) in the The Netherlands *B. pertussis* population (van Loo *et al.*, 2002). MLSTs of current Dutch whole-cell vaccine strains: ■ ■

Figure 4.2.1.1a.



n=16 n=7 n=5 n=8 n=17 n=51 n=32

Figure 4.2.1.1b.



n=11 n=12 n=10 n=10 n=38 n=47

Figure 4.2.1.1c: Temporal trends in multilocus sequence types (MLSTs) in the United States *B. pertussis* population (van Loo *et al.*, 2002).

Figure 4.2.1.1d: Multilocus sequence types in the Japanese *B. pertussis* population (1990-1999) (van Loo *et al.*, 2002).

Figure 4.2.2.1c

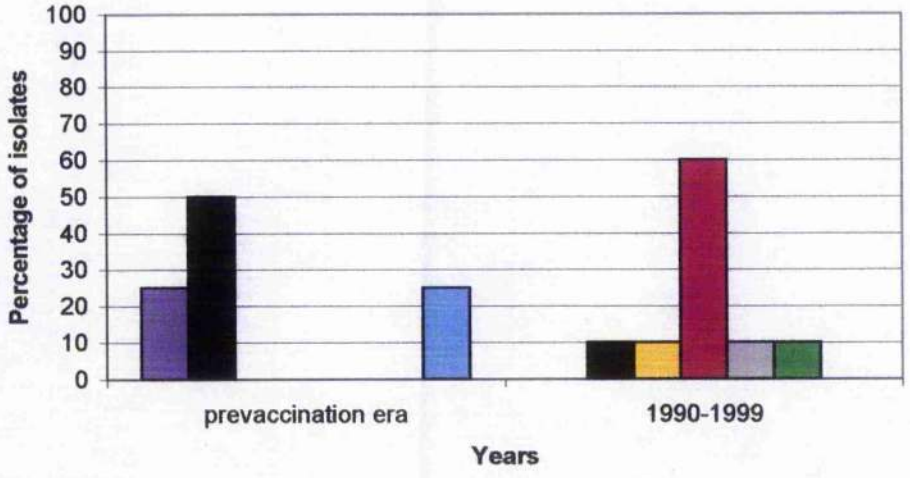


Figure 4.2.1.1d

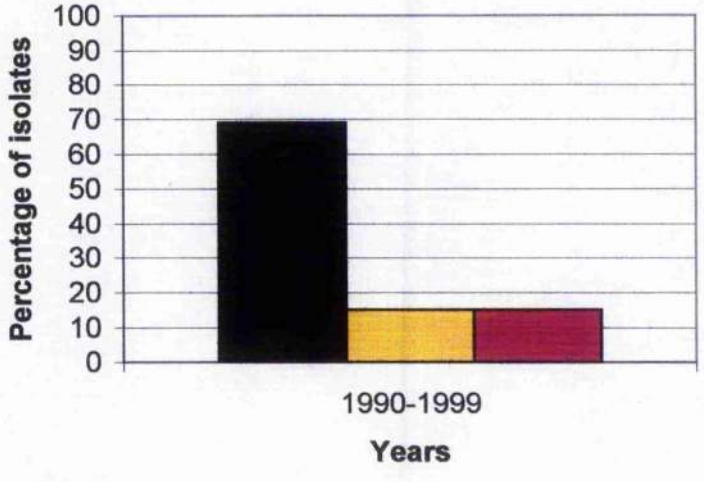


Figure 4.2.1.1c: Multilocus sequence types in the Italian *B. pertussis* population (1990-1999) (van Loo *et al.*, 2002).

Figure 4.2.1.1f: Multilocus sequence types in the Finnish *B. pertussis* population (1990-1999) (van Loo *et al.*, 2002).

Figure 4.2.1.1e

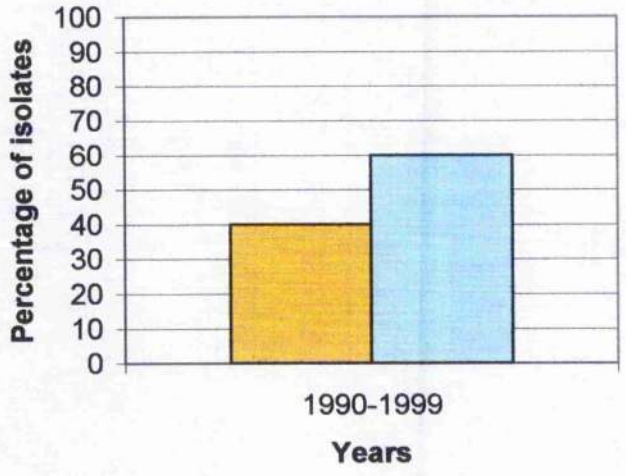
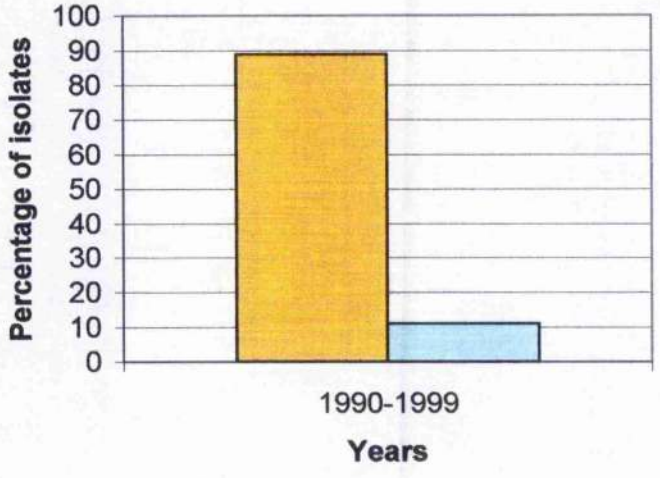


Figure 4.2.1.1f



vaccination era, was the predominating type, found in 60% of isolates (Fig. 4.2.1.1c) (van Loo *et al.*, 2002). This frequency of MLST-5 is slightly higher than that found for UK and Dutch isolates. MLST-2, -3, -7 and –8 were found in much lower frequencies (Fig. 4.2.1.1c). The latter two types were not found in recent UK or Dutch isolates.

Information for Japanese, Italian and Finnish *B. pertussis* isolates is incomplete as MLST types are only known for recent isolates from 1990-1999 (van Loo *et al.*, 2002). The Japanese isolates differ from other countries in that the predominating MLST type, MLST-2, found in about 69% of isolates (Fig. 4.2.1.1d; van Loo *et al.*, 2002), was found to predominate in other countries only during the pre-vaccination era. MLST-3 and MLST-5 were present equally but lower frequencies, 15%, and the MLST-5 frequency was lower than that seen for UK, Dutch and US isolates from the same time period. The recent Italian and Finnish isolates had only two MLST types, MLST-3 and MLST-4 (Fig. 4.2.1.1e and f) (van Loo *et al.*, 2002). These recent isolates differ markedly from those described for other countries as no MLST-5 isolates were found. Recent Italian isolates had a greater predominance of MLST-4 (60%) in contrast to the frequencies found in The Netherlands and UK. Finnish isolates showed a predominance of MLST-3 and had the greatest frequency of MLST-3 compared to other *B. pertussis* populations.

Van Loo *et al.* (2002) have noted that the resurgence of pertussis in The Netherlands since 1996 has coincided with the appearance of the new MLST types, MLST-4 and MLST-5. The US has also reported increasing levels of pertussis since the 1980s and, during 1990-1999, MLST-5 became predominant in its *B. pertussis* population. In the UK, MLST-4 and MLST-5 were detected from 1999 onwards. Although only two UK MLST-4 isolates were found, MLST-5 was the predominant type in the most recent time period (2002), which is similar to that reported for The Netherlands and US. In contrast to The Netherlands and US, there has been no resurgence of pertussis in the UK (Mooi *et al.*, 1998; Cassiday *et al.*, 2000; Fry *et al.*, 2001) and therefore no evidence that vaccine efficacy has been compromised by change in the antigenic make-up of circulating strains.

4.2.2 MLST and pertactin type

A significant correlation between MLST and *prnA* type was previously demonstrated by van Loo *et al.* (2002), although *prnA* was not included as a gene target in the MLST

scheme described by these authors. The reason given was that variation in the *prnA* gene occurs as a result of insertion and deletion of repeat units and this process is expected to occur relatively frequently and is reversible (van Loo *et al.*, 2002). However, they found a non-random association between the MLST of recent isolates from 1990-1999 and the *prnA* type for Dutch, Finnish, Italian, Japanese and US isolates. Recent Dutch MLST-2 isolates were all associated with *prnA*(1). The MLST-3 and -5 recent Dutch isolates were associated with the three *prnA* alleles, *prnA*(1)-*prnA*(3), but the majority of recent Dutch MLST-3 and-5 isolates, 61% and 93% respectively, were associated with *prnA*(2) (van Loo *et al.*, 2002). All MLST-4 isolates were associated with *prnA*(3). American *B. pertussis* isolates from 1990-1999 that were MLST-5 were all *prnA*(2). The recent Japanese *B. pertussis* MLST-2 isolates were wither *prnA*(1) or *prnA*(2) but the majority, 89%, were associated with *prnA*(1). Recent Finnish isolates differed from recent Dutch isolates in that there were four *prnA* alleles, *prnA*(1)-*prnA*(4), present. Its MLST-3 isolates were associated with all four alleles but *prnA*(2) predominated (84%). Similar to the Dutch isolates, all Finnish MLST-4 and Italian MLST-4 isolates were *prnA*(3).

During this study it was considered important to determine whether similar correlations existed between UK MLST and *prnA* types in recent isolates. UK MLST-3 isolates from 1998-1999 were associated with three *prnA* alleles, *prnA*(1)-*prnA*(3). The majority of MLST-3 isolates (90%) from this period were *prnA*(1) and this differs from Dutch and Finnish MLST-3 isolates (1990-1999) that were mainly associated with *prnA*(2). The UK MLST-4 isolates (both from 1999) were different from the previously reported MLST-4 isolates as they were *prnA*(2) or *prnA*(3). UK MLST-5 isolates were mainly *prnA*(2) (96%) and no UK MLST-5 isolates were *prnA*(1). There are some similarities between *B. pertussis* populations and their MLST and *prnA* types but there are also some differences such as the correlation between MLST-3 and *prnA* type for UK, Dutch and Finnish isolates. This difference can be related to when the shift in *prnA* type from *prnA*(1) to non- *prnA*(1) alleles occurred and this shift to predominating non-*prnA*(1) alleles has occurred later in the UK (Mooi *et al.*, 1998; 1999; Fry *et al.*, 2001).

4.3 FIMBRIAL ANTIGEN 2 AS A VIRULENCE MARKER

The *fim2* allele, *fim2*(2), was seen only in UK *B. pertussis* isolates from 1998 and 1999. Isolates from any other time period were all *fim2*(1). In 1998, 9 of 18 isolates were *fim2*(2) and in 1999, 11 of 26 isolates were *fim2*(2). This differs from the *fim2* variation

that has been described for the Dutch *B. pertussis* population. The *fim2*(2) allele was only apparent in The Netherlands during the pre-vaccination era and not in any recent isolates (van Loo *et al.*, 2002). Using a larger sample size, it would be possible to investigate whether *fim2*(2) is a potential marker for virulence. In the present study, there was an apparent 100% linkage between isolates that were *fim2*(2) and serotype 1,2. *B. pertussis* isolates that are serotype 1,2 have been linked to more severe cases of pertussis (van Buynder *et al.*, 1999). It has been reported that in UK *B. pertussis* isolates the proportion of those that were serotype 1,3 decreased steadily over the years 1995-1997 with a concomitant increase in proportion of isolates that were serotype 1,2 but, in 2002, the majority of isolates were serotype 1,3 (J. Duncan, personal communication). Serotype 1,2 was also related to the increase in rates of hospital admissions and complications associated with pertussis (van Buynder *et al.*, 1999). Those that were infected with isolates that were serotype 1,2 were twice as likely to be admitted to hospital as serotype 1,3 and seven times as likely as those with serotype 1,2,3. However, during 1998-1999 there was no overall increase in the number of pertussis cases in the UK (Fig. 4). Information regarding vaccination status of individuals from whom isolates were recovered was available only for those from 1998-1999. There was no relationship between vaccination status and *fim2* allele, but the sample size for 1998-1999 was small and it should be expanded to investigate a greater number of isolates to determine whether such a relationship existed. The allele *fim2*(2) has the potential to be used as a virulence marker for isolates. Using Pyrosequencing technology it would be possible to rapidly determine which isolates were *fim2*(2) and might indicate which patients will have more severe disease.

4.4 MLST LINK WITH VIRULENCE

A link with pertussis toxin promoter sequence variation and MLST type has been reported by Prof F. Mooi (Mooi, 2003). Multilocus sequence types are distinguished by allelic variation of *ptxA*, *ptxC* and *tcfA* genes. There has been the 'appearance' of MLST-5 (*ptxA*(1), *ptxC*(2), *tcfA*(2)) in the Dutch *B. pertussis* population during the 1990s and this was found to be the predominating type. Prior to this the predominating MLST type was MLST-3 (*ptxA*(1), *ptxC*(1), *tcfA*(2)). Since 1990 in The Netherlands, the frequency of MLST-5 has been increasing and this increase coincided with the epidemic that occurred in 1996 as well as the overall increase of pertussis. The difference between the two known *ptxC* alleles, *ptxC*(1) and *ptxC*(2), is only a single silent nucleotide difference

and this had led to the speculation that there may be other, unknown variation in virulence genes that has allowed MLST-5 strains to be more successful than other MLST types. An investigation of *ptx* promoter sequence variation amongst Dutch *B. pertussis* isolates led to the discovery of eleven *ptx* promoter alleles, *ptxp*1-*ptxp*11, and this variation occurred within the regulatory region of the promoter (Mooi, 2003). There was 100% linkage between *ptxp*3 and the *ptxC*(2) allele. It was also found that the frequency of the *ptxp*3 allele has increased from 1997 onwards and the allele *ptxp*1 had decreased during this time. Mooi and colleagues constructed isogenic strains to compare the two *ptxp* alleles in mouse respiratory studies (Mooi, 2003). The studies showed that *B. pertussis* isolates containing the *ptxp*3 allele were better colonisers of mice than isolates that were *ptxp*1. *B. pertussis* isolates with the *ptxp*3 allele appeared therefore to be more virulent than those with the *ptxp*1 allele and/or are transmitted more efficiently. It was also reported that isolates containing the *ptxp*3 allele occurred in the other countries (The Netherlands, Finland and Sweden) known to have the *ptxC*(2) alleles in their *B. pertussis* populations. However, Italian *B. pertussis* isolates did not have this *ptx* promoter allele, but these isolates were not *ptxC*(2). It would be informative to expand the current sequencing study of UK *B. pertussis* isolates to include the *ptx* promoter to determine if a similar link between MLST-5 isolates and *ptxp3* allele exists in the UK. If this *ptx* promoter allele does not exist in UK isolates that are *ptxC*(2) then it may partly explain why the UK pertussis levels are not increasing. However, if UK *B. pertussis* isolates have this pertussis toxin promoter allele in recent isolates it would conflict with the suggestion that recent Dutch isolates are potentially more virulent, thus leading to increases in pertussis levels. It would also emphasise the flaws associated with the published MLST scheme and indicate that other virulence target genes should be included to gather more information on isolates.

4.5 ADVANTAGES AND DISADVANTAGES OF THE *B. pertussis* MLST SCHEME

Characterisation of strains by nucleotide sequence analysis has allowed epidemiological tracking of shifts in the circulating population of *B. pertussis* of several countries. The MLST scheme devised by van Loo *et al.* (2002) based on the allelic variation of *ptxA*, *ptxC* and *tcfA* is not the one that is traditionally used which would be based on the allelic variation of an organism's housekeeping genes. This is because of the clonal nature of *B. pertussis*. However, the term MLST for the *B. pertussis* sequence typing scheme

described by van Loo *et al.* (2002) should perhaps be altered to prevent confusion between this scheme and the MLST scheme based on variation of an organism's housekeeping genes described by Maiden *et al.* (1998).

Although the MLST scheme by van Loo *et al.* (2002) has provided useful information about the changes in the *B. pertussis* populations of different countries with time, it does have its limitations as it is based on only three genes. Thus, there is no guarantee that an MLST-5 isolate in the UK is genotypically or phenotypically the same as an MLST-5 isolate from another country. There may be differences in other gene sequences and/or gene expression. The inclusion of further genes in this scheme would provide greater discrimination and potentially would be more informative. Such targets that have demonstrated sequence variation are *prnA* and *fim2*. The addition of the *prnA* and *fim2* alleles described here would increase the number of possible *B. pertussis* sequence types. The other genes, *cyaA*, *hapC*, *vag8*, *brkA*, *ompQ*, that were investigated for sequence variation revealed no polymorphism in UK isolates which precludes them from use as epidemiological markers.

Sequence typing schemes such as the van Loo *et al.* (2002) MLST scheme has allowed the comparison of data from different countries. This is useful to determine the shift in the *B. pertussis* population world-wide and whether, potentially, this relates to each country's vaccines and vaccination schedules. Acellular vaccines are increasingly being used as booster vaccines in children of school age or in place of the traditional WCVs for primary immunisation in some countries. Such vaccines are composed of a limited number (1-5) of specific antigens from *B. pertussis*. Vaccination schedules may differ, as well as the number of components in the ACVs. The component antigens will also be of particular amino acid sequence types. Typing schemes such as MLST are important as they will be a powerful tool in determining whether vaccination with ACVs will have an effect on the circulating *B. pertussis* population. The efficacy of the UK WCVs has not been hampered by the shift that has occurred within its *B. pertussis* population. With further monitoring it would be possible to find if ACVs cause further shifts and if this affects their efficacy.

4.6 MULTILOCUS VIRULENCE GENE SEQUENCE TYPING (MVGST) SCHEMES

It was mentioned in sections 3.2.2 and 3.3 that two other genes, *fim2* and *prnA*, had demonstrated variation suitable for inclusion in a gene typing scheme. Allelic variation of *prnA* had been used previously for sequence typing of *B. pertussis* isolates but was not considered suitable for the MLST scheme developed by van Loo *et al.* (2002). The *fim2* gene had also shown variation amongst Dutch isolates but the two *fim2* alleles were only found together during the pre-vaccination era and, for this reason, were not included in the MLST scheme by van Loo *et al.* (2002). In contrast, UK *B. pertussis* isolates had exhibited *fim2* allelic variation amongst recent isolates from 1998-1999. By inclusion of these genes in a multilocus virulence gene sequence typing (MVGST) scheme of *ptxA*, *ptxC*, *tcfA*, *prnA* and *fim2*, it was hoped to gain further information on UK *B. pertussis* isolates by providing more discriminatory power. Three different combinations, (1)-(3), of gene targets for this sequencing scheme were compared: MVGST(1) *ptxA*, *ptxC*, *tcfA* and *prnA*; MVGST(2) *ptxA*, *ptxC*, *tcfA* and *fim2*; and MVGST(3) *ptxA*, *ptxC*, *tcfA*, *prnA* and *fim2*. There was one problem in comparing these three techniques in that *prnA* variation had been investigated for a greater number of UK *B. pertussis* isolates than for *fim2*. Therefore the number of sequence types achieved with each scheme differed, due to differences in strain numbers. Seven MLST types from 136 strains, eleven MVGST(1) types from 136 strains, six MVGST(2) from 80 strains and ten MVGST(3) types from 80 strains were found amongst the UK *B. pertussis* population from 1920-2002. An equivalent number of UK *B. pertussis* isolates should be investigated for *fim2* variation to determine whether the number of MVGST(2) types would increase significantly. However, by the inclusion of *prnA* (MVGST(1)) or *prnA* and *fim2* (MVGTS(3)) in the original MLST scheme, the number of sequence types present in recent isolates from 1982 onwards increased compared to those found using the earlier MLST scheme. Six MVGST(1) types were found amongst 17 isolates from 1982-1985 compared to four MLST types found during this time period. Four MLST types and seven MVGST(1) types were found amongst 51 UK isolates from 1998-1999. During 2002 three MVGST(1) types were found amongst 31 UK isolates compared to two MLST types. With the MVGST(3) scheme, eight MVGST types were found amongst 33 isolates from 1998-1999 compared to four MLST types.

4.7 VARIATION IN *cyaA* PROMOTER REGION OF *B. pertussis* AND ITS EFFECT ON VIRULENCE

Although little variation was found in the immunodominant region of CyaA, three alleles were identified in a region of *cyaA* between bases 686-695 (Fig. 3.5.1.1). This *cyaA* promoter region was amplified with primers CyaA1 and CyaA6, targeting bases 287-1165 (Y00545), and used to study the expression of *cyaA* (section 3.9). The allele *cyaApr*(1) was found in the *B. pertussis* type strain NCTC10739^{T}. When this sequence was compared to that of the *cyaA* GenBank sequence, Y00545, there was a single nucleotide difference at position 840. The strain source of Y00545 was *B. pertussis* 18323^{T} and this strain is considered to be identical to NCTC10739^{T}. However, these strains may have been separated many years previously and could have evolved by mutation, which may explain this difference in *cyaA* sequence. Another explanation for this discrepancy is that there was a sequencing error due to old sequencing technology at position 840 in the previously deposited sequence, Y00545. This region for NCTC10739^{T} was sequenced again and the data were found to agree with the nucleotide difference at position 840. The second allele, *cyaApr*(2), was found amongst the majority of UK *B. pertussis* isolates. Of the 46 *B. pertussis* isolates examined only 99K45 and PICU475 were *cyaApr*(3). These two strains also differed from other isolates as they were the only two MLST-4 strains in the UK collection. It has been suggested that there is a 100% link between a particular pertussis toxin promoter allele and the *ptxC*(2) allele (Mooi, 2003). Therefore there was further investigation to determine if there was a 100% linkage between isolates that had *tcfA*(3) (MLST-4) and the 6 bp insertion between the *cyaA* and *cyaC* genes. *B. pertussis* isolates that were known to have the *tcfA*(3) allele were requested from Prof F. Mooi and sequenced for the *cyaA* promoter variation. Sequencing results indicated that there was no such linkage. This emphasises the problems that are currently associated with the MLST scheme for typing *B. pertussis* isolates. An isolate that is MLST-4 from the selected UK panel is not necessarily the same as an MLST-4 strain from elsewhere. Even so, these two isolates, 99K45 and PICU475, were known to be epidemiologically unrelated because they had different IS*1002* RFLP profiles (Neal, 2004).

The effect that the 6 bp insertion might have on enzymic or cytotoxic activity of CyaA was determined by phenotypic investigation of strains 99K45 and PICU475. Their AC enzymic activity was investigated initially with bacterial cell suspensions lysed by Triton

X-100 as a source of AC activity and compared to lysed cells of *B. pertussis* BP338. These assays showed that 99K45 had greater AC enzymic activity compared to PICU475 and BP338. Their tangents of the progress curves from the conductimetry assay were 48, 21 and 23, respectively. It was found that PICU475 did not grow as well as the other *B. pertussis* isolates in SS liquid medium and therefore it was not used for AC and cytotoxic activity measurements, due to the need to prepare urea extracts from stationary growth phase cells. The urea extract of 99K45 had a similar AC enzymic activity to that of strain BP338; both were determined to have an activity of 1.5 IU mg^{-1} protein. The urea extracts of strain 99K45 and BP338 were used for further comparison. 99K45 had slightly higher killing activity than BP338 in the MTT assay. Each sample was tested in duplicate but the cytotoxicity assay was carried out only once. These assays should be repeated with an improved urea extraction protocol using a larger culture volume (1 l in instead of 250 ml) and it should be investigated whether purified CyaA can be prepared rather than a crude extract to determine if this 6 bp insertion has a true effect on increasing the cytotoxic activities of CyaA. However with only one strain containing this 6 bp insertion able to grow well, it may be difficult to draw definitive conclusions. To prove linkage between the insertion and increased cytotoxic activity, several more *B. pertussis* isolates with this particular genotype would have to be investigated to determine if this 6 bp insertion had an effect.

The 6 bp insertion is situated upstream (position 692) from the RNA polymerase binding sites and the phosphorylated BvgA binding sites of *cyaA* (Fig. 3.9.1.1). The sequence of the 6 bp insertion was checked to ensure that it was not an extra BvgA binding site but the sequence was not similar to that of the BvgA binding site consensus sequence [TTTC(C/G)TA] (Cotter and Jones, 2003). When the region between *cyaA* and *cyaC* is viewed using the program Artemis (www.sanger.ac.uk) the 6 bp insertion is situated very close to the start of the *cyaC* gene (Fig. 3.9.1.1).

4.8 COMPARISON OF TECHNIQUES USED TO DETECT SEQUENCE VARIATION

There were three techniques (sequencing by dideoxy method, Pyrosequencing and melting curve analysis with LightCycler) used to determine sequence variation for the genes described in results sections 3.1 and 3.3. Conventional, dideoxy sequencing was used routinely for investigation of genotypic variation of *cyaA*, *tcfA*, *brkA*, *vag8* and

bapC. Pyrosequencing was used for the investigation of single nucleotide polymorphisms in *ompQ*, *fim2* and *ptxC*. Lastly, melting curve analysis was used for screening isolates for *cyaA* promoter variation. The main advantage of using LightCycler assays and Pyrosequencing over conventional sequencing is the fact that they are much more rapid.

For example, with Pyrosequencing of a 96-well plate containing a sample in each well for determining single nucleotide polymorphisms, the time taken from the purification of PCR products to determining the allelic variation of the samples using the software supplied with the equipment is approximately 2.5 h - 3 h. This differs from conventional sequencing, which requires one day from the purification of PCR products to receiving chromatograms for sequence analyses. The analysis of chromatograms requires further time. The LightCycler assay takes approximately one hour but fewer samples (maximum 32) can be analysed in one assay. Analysis of LightCycler melting curve results takes little time using the software supplied with the equipment. The advantage that conventional sequencing has over both of these techniques is related to the size of the region that can be sequenced.

Only single nucleotide polymorphisms were investigated using Pyrosequencing thus potentially missing any other variation that may have been present outside the known polymorphic site. This drawback also applies to the LightCycler as only a limited region is covered by hybridisation of specifically-designed probes. Targets that were used for LightCycler and Pyrosequencing were investigated initially by sequencing using the dideoxy sequencing method to determine polymorphic sites and to ensure that results would be concordant between methods. LightCycler and Pyrosequencing provide a powerful tool for screening hundreds of samples in a relatively short time.

4.9 CONDUCTIMETRY

4.9.1 Conductimetry assay as a tool to study rCyaA activity

The conductimetry assay was developed as a rapid and convenient method to determine the AC enzymic activity of different forms of rCyaA in studies to characterise their potential as immunogens and immunomodulators. This is an easy and rapid assay to detect the AC activity of rCyaA preparations. The detection limit of the conductimetry assay was determined in this study and found to be low, at approximately 1 ng protein (526 IU mg^{-1} protein) in a 2 ml volume in the conductimetry cell. The conductimetry

assay has several advantages over the radioisotopic assays that have been developed; for example it is more rapid, cheaper and technically less demanding. However, it is accepted that the radioactive assay can achieve a lower limit of detection (Lawrence *et al.*, 2002). Adenylate cyclase toxin is an unstable enzyme/toxin that aggregates and loses activity in aqueous solution. It is normally purified by a stepwise procedure involving ion-exchange and hydrophobic interaction chromatography in presence of a high concentration of urea. The conductimetry assay is convenient in that it allows the AC activity of rCyaA preparations to be determined quickly at each stage of purification. Different CyaA preparations at the same protein concentration can be compared, to determine which purification method is optimal. The method can also be used to determine the enzymic activity of different mutated forms of CyaA for use in immunogenicity studies.

It was found during this study that there was inter-assay variation of AC activity of the same rCyaA preparation at the same protein concentration. Thus without the inclusion of an internal standard, it would be difficult to compare the AC activity of one rCyaA preparation with that of another preparation on a different day. The assay was therefore standardised between conductimetry runs by including the same positive control of a known concentration and specific activity in every assay. It was also apparent that when the standard preparation of rCyaA was diluted in distilled water and freeze-thawed once, it lost approximately 50% of its AC activity. Therefore, dilutions of the stock preparation of rCyaA were prepared immediately prior to use in the conductimetry assay.

The eight glass cells within the conductimetry machine were suspended over a spinning magnet. Cell number 5 was positioned very close to the rotor and sometimes the spinning magnet within this cell would jump and not spin properly which resulted in unreliability of the AC activity of the sample in this cell. The magnets of the individual cells would occassionally not spin correctly and would have to be adjusted to allow correct spinning of the magnet. The machine that was used was an early design of apparatus by Dr A Lawrence (Division of Infection and Immunity, University of Glasgow). Further versions of equipment have been developed since and they may overcome the problem of the conductimetry cell 5 position in relation to the rotor and the potential 'jumping' of magnetic fleas.

4.10 CONDUCTIMETRY OF NATIVE CyaA

4.10.1 Conductimetry as a diagnostic tool

Currently there are several diagnostic tests for pertussis (see section 1.10). Of these PCR diagnostics is becoming one of the main tests, but it is suggested that it should be used alongside culture and serology. Routine PCR testing is a costly process. Thus, there is a need for a sensitive, specific, inexpensive diagnostic assay for laboratories that cannot afford to routinely use PCR assays. The presence of AC enzymic activity in clinical specimens from pertussis-infected individuals has been suggested for use in diagnosis of pertussis (von Koenig *et al.*, 1989; Confer *et al.*, 1990; Scheftel *et al.*, 1992). It was reported that there was a 100% correlation between AC positive samples and *B. pertussis* culture-positive patients (Scheftel *et al.*, 1992). However, these assays use radioisotopes and are technically demanding. Use of the novel conductimetric assay, that is simple, rapid and inexpensive, to detect AC enzymic activity was investigated for its diagnostic potential. Initially a method for lysing the *B. pertussis* cells had to be developed as whole cells, freshly grown on BG agar, did not exhibit much AC activity.

A small proportion of *B. pertussis* CyaA is known to be secreted into the culture medium but a large proportion (about 80%) is cell-associated (Hewlett *et al.*, 1976; Brownlie *et al.*, 1985). Urea and various non-ionic detergents were chosen as potential lysis agents to optimise the detection of AC activity from *B. pertussis* cells. Two commercial reagents and urea solution were found to cause inhibition at higher concentrations. Solubilisation of membranes involves hydrophobic interactions between surfactant chains and the lipid/protein content. Synthetic nonionic detergents offer a wide range of hydrophilic/lipophilic balance (HLB) values and are non-denaturing. Maximum membrane solubilisation occurs using a surfactant with an HLB of about 12.5 (Jones, 1999). Six of the seven nonionic detergents used in the present study had their HLB values listed by Jones (1999). The seven non-ionic detergents, Triton X-100, -114, Brij-35, Brij-58, Tween-20, Tween-80 and NP40, were found not to cause inhibition of AC activity in the conductimetry assay within a range of final concentrations of 0.05%-1.5% (v/v). Different numbers of *B. pertussis* BP348pRMB1 cells were lysed with the non-ionic detergents and were analysed to determine which would provide the best source of AC activity for conductimetry. It was found that the greater the number of cells used, the greater the AC activity detected, as would be expected. Four detergents, Triton X-100, -114, Brij-35 and NP40, which appeared to optimally lyse BP348pRMB1 cells, were also

used to lyse the type strain 18323^T. These four detergents, except Brij-58, had an HLB value close to 12.5. *B. pertussis* cells lysed with Triton X-100 were found to exhibit the greatest activity. Therefore, Triton X-100 with an HLB value of 13.5 gave optimal lysis of *B. pertussis* cells, for use in conductimetry.

The data indicated that the conductimetry assay was probably not sensitive enough to detect AC activity from *B. pertussis* cells lysed with Triton X-100 if this was to be done directly from a clinical sample. This was due to the fact that the lowest number of cells that the assay could detect was about 10^7 per conductimetry cell. This value is considerably higher than the detection level reported for the conventional radioactive assay described by von Koenig *et al.* (1989), which was determined to be 3-6 x 10^4 cfu ml^{-1}.

The specificity of the conductimetry assay for detecting AC activity from lysed *B. pertussis* cells was also investigated. In reactions that did not contain the reagent CaM, some AC activity was still apparent in the conductimetry assay. However, reactions that did not contain the reagent ATP were negative and the progress curve remained at the baseline. Therefore, lysed *B. pertussis* cells provide a good source of AC activity but there are either background reactions occurring that are ATP dependent, AC in the absence of CaM has some basal activity, or a molecule in *B. pertussis* lysate is able to substitute for CaM to some extent. In fact a CaM-like protein has been reported in *B. pertussis* and this CaM-like protein was determined to activate *B. pertussis* CyaA (Nagai *et al.*, 1994). Unlike the conductimetry assay that specifically detects AC activity from rCyaA that is CaM dependent, this has been demonstrated by this study (section 3.6.4), this assay may not be completely specific for detecting native CyaA AC activity from *B. pertussis* cells. Reactions of enzymic activity from rCyaA that did not contain CaM gave very low progress curves almost at baseline level.

4.10.2 Conductimetry screening for *Bordetella* spp.

The conductimetry assay has potential application as a tool for detecting gene expression in *B. pertussis*, *B. parapertussis* and *B. bronchiseptica*. Tu *et al.* (2001) developed a mini-Tn5 transposon derivative containing *cyaA'* encoding the N-terminal 400 amino acids of CyaA which provided the CyaA enzymic activity. This transposon was able to randomly insert the reporter gene *cyaA'* into the *B. bronchiseptica* chromosome.

However, the authors reported difficulty in screening *B. bronchiseptica* mutants containing the reporter using a radioactive assay. It would be beneficial for such screening to use the conductimetric assay, which would allow multiple isolates to be screened quickly, inexpensively and without the use of radioactive reagents.

B. bronchiseptica is known to express the *bvg*-repressed protein urease when grown in modulating conditions. Friedman *et al.* (2001) described the phenotypic evaluation of *B. bronchiseptica* cultures by urease activity and Congo red affinity. The conductimetry assay could be adapted to detect urease because the reactions of the enzyme produces ammonia from urea with the release of free ions. Urease in *B. bronchiseptica* has been found to be cell-associated and not detected in the culture supernates (McMillan *et al.*, 1996). Therefore, there is no need to take into account that some activity may not be present due to secretion into the culture medium as is the case with CyaA. Use of the conductimetry assay would allow the discrimination of Bvg^+ and Bvg^- strains of *B. bronchiseptica*. The ability to rapidly identify Bvg^+ strains would aid in the production of effective veterinary vaccines where the known virulence factor antigens need to be expressed in the Bvg^+ phase. The conductimetry assay could be further explored by determining if Bvg^i isolates can be detected with a modified urease or CyaA assay.

4.11 STUDY OF *cyaA* EXPRESSION WITH *lux* AND *gfp* REPORTER GENES

It was considered important to study the expression of the *cyaA* gene in *B. pertussis* to determine how this is affected by different growth conditions and during the different phases of growth. Constructs containing a promoterless reporter gene, *lux* or *gfp*, with the *cyaA* promoter region (cyaA1-6 or cyaA1-8) inserted upstream, were developed for determining the level of *cyaA* expression. The *lux* plasmid, pSB395, containing the cyaA1-6 insert was successfully introduced into *B. pertussis* by plate-mating. However, this transfer was never achieved with the *gfp* plasmid containing the cyaA1-6 insert. Studying *cyaA* expression using the *lux* operon is potentially useful as expression could be monitored in a real-time fashion using a luminometer, such as Lucy-1 machine.

The bioluminescence studies undertaken to investigate the expression of *cyaA* with the *lux* reporter gene found no light production. PCR analysis of the bacterial cells used for these studies revealed that the cyaA1-6 insert in the plasmid was not present, suggesting that the plasmid was unstable. The primers that were used to amplify the *cyaA* fragments

containing the promoter region contained several restriction sites within the sequence. The CyaA1 primer contained *Kpn*I, *Hin*dIII and *Pst*I restriction sites, whereas CyaA6 and CyaA8 both contained *Bgl*II restriction sites. *B. bronchiseptica* contains a restriction enzyme called *Bbr*I (*Hin*dIII) which recognises the sequence AAGCTT (Roberts, 1980). *B. pertussis* as well as *B. bronchiseptica* has a *Hin*dIII restriction system (Greenaway, 1980). Weiss and Falkow (1982) reported that they were not able to obtain *B. pertussis* transformants with plasmid DNA isolated from *E. coli*, but when using *B. pertussis* was used as the donor in conjugation experiments, the transfer frequency increased. Therefore, it was suggested that *B. pertussis* may also modify the *Hin*dIII site. The plasmid used in this study was known to contain a single *Hin*dIII site and this was isolated from *B. pertussis* and *E. coli* then digested with *Hin*dIII and *Bam*HI (included as a control enzyme to demonstrate that other enzymes can digest *B. pertussis* DNA). When the resulting digest profiles were compared, the digested plasmid profile from *E. coli* contained one more fragment than that from *B. pertussis*, consistent with the plasmid DNA isolated from *B. pertussis* not being cleaved at the *Hin*dIII site, thus indicating that the latter organism had modified the site. The reporter gene *gfp* does not have any *Hin*dIII sites, however the vector pPROBE-AT' contains a single *Hin*dIII restriction site. Further investigation revealed that the *lux* operon contains three *Hin*dIII restriction sites. This indicates that the *lux* reporter may not be useful for studying *B. pertussis cyaA* expression and suggests why there appears to have been the loss of plasmid from *B. pertussis* even though it was successfully transferred. The *B. bronchiseptica lux* construct containing cyaA1-8, insert designed by Dr M. Lynch, was also unstable. Only *B. bronchiseptica* colonies resulting directly from the plate-mating were light-emitting, but not when taken from glycerol stocks of the transconjugants (Dr M. Lynch).

There are several possibilties to overcome this problem of the restriction/modification system in *B. pertussis* and *B. bronchiseptica*. It would be valuable to find out from other *B. pertussis* research groups if there is a strain that has had this system mutated, thereby allowing the use of the *lux* operon that contains *Hin*dIII sites. Weingart *et al.* (1999) used *gfp* labelling of live *B. pertussis* to study phagocytosis of opsonised and nonopsonised bacteria. For this study, a construct containing *gfp* under the control of a constitutive promoter was developed. The resulting vector pGB5P1 consisted of pBBR1MCS-2 with the *gfp* mutant 2 gene cloned in as a *Bam*H1 and *Eco*R1 fragment.

A *Sau*3A fragment encoding a constitutive *B. pertussis* promoter was cloned upstream of the *gfp* gene to control its expression. This vector was successfully introduced into *B. pertussis* by electroporation. The vector was requested and provided by Prof A. Weiss, University of Cincinnati. However, the constitutive promoter is a *Sau*3A fragment and this site is known to exist within the fragment amplified by CyaA1 and CyaA6 primers used for *cyaA* cloning. The vector could be manipulated for future use as a plasmid for studying *gfp* expression in *B. pertussis*.

4.12 CONCLUSIONS

The investigation of polymorphism in several virulence-related genes amongst UK *B. pertussis* isolates from 1920-2002 has given insight into the trends within the UK population and has allowed the comparison of UK *B. pertussis* isolates with those from other countries. Only three of the eight genes (*ptxC*, *tcfA* and *fim2*) investigated were found to exhibit polymorphism suitable for use as markers to study these trends. Polymorphism was already known to exist in *prnA* and *ptxA* in the UK (Fry *et al.*, 2001) and other countries (Mooi *et al.*, 1998). There has been a shift in predominating sequence types in the UK population relating to *ptxC* and *prnA* types. The predominating alleles found in recent isolates were not seen in pre-vaccination era isolates or from the era just after the introduction of the pertussis whole-cell vaccine (WCV). For example, the *ptxC*(2) allele was not apparent until the 1998 and is now the predominant *ptxC* allele. Variation was shown within *tcfA* and *fim2* genes but was restricted to certain time periods and, there has been no clear shift in *tcfA* or *fim2* alleles. There was one dominant *tcfA* allele with other alleles present in very low frequencies. However, during the drop in UK vaccination uptake between 1974 and 1983, novel or unusual *tcfA* alleles were apparent but these have not been seen (except one *tcfA*(4) allele in 1999) since vaccination uptake returned to normal levels. Amongst UK isolates, *fim2* allelic variation was apparent only amongst isolates from 1998-1999. The reason for this is unknown as vaccination uptake was normal and pertussis levels were low at this time. Other countries have reported increases in the incidence of pertussis and this has been attributed to genetic shifts in the *B. pertussis* populations. However, the UK has experienced similar shifts but no increase in pertussis levels has been noted. In fact, pertussis levels in the UK are historically low. Such increases in pertussis elsewhere may be related to differences in efficacy of WCV preparations and vaccination programmes in different countries. Surveillance of isolates should continue, to monitor for sequence variation of virulence-related genes and, if possible, more historical isolates should be investigated. An acellular pertussis vaccine (ACV) composed of a small number of antigen components has recently been introduced to the UK as a pre-school booster. This may lead to further genetic and, possibly, antigenic shifts in the *B. pertussis* population thus potentially compromising the efficacy of the ACV.

During the sequence investigations, a 6 bp insertion was found situated between the gene that codes for the CyaA protoxin, *cyaA*, and its accessory gene, *cyaC*, in two recent *B. pertussis* isolates. Further investigation revealed that these were the only two such strains from a panel of 46 isolates. Due to the position of this insertion, it was of interest to determine the enzymic and cytotoxicity activities of the isolates but results of these phenotypic analyses were not conclusive. Further analysis regarding these two strains should be carried to determine whether this sequence variation has an effect on the biological activity of CyaA and how this relates to virulence of *B. pertussis*.

The novel conductimetry assay could not be made sufficiently sensitive for use as a diagnostic test for pertussis, but this assay was found to be a useful laboratory test. Individuals in the Division of Infection and Immunity and in other institutions are currently investigating the immunogenic and adjuvant properties of different forms of CyaA and this assay provides a quick and easy method of determining the AC activity of crude and purified preparations of CyaA. It has other potential applications such as determining the phenotypic phase of *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* cultures and the likelihood of whether or not other virulence-related properties of the Bvg^+ or the Bvg^- phase are being expressed.

The development of *lux* or *gfp* reporter genes with the *cyaA* promoter inserted upstream of the genes would have provided important information regarding the expression of the *cyaA* gene. However, problems were experienced with the stability of plasmid constructs in *B. pertussis*. This may be due to the modification and restriction system present in *B. pertussis* which targets the *Hin*dIII recognition sequence.

References

REFERENCES

Allen, A. G., R. M. Thomas, J. T. Cadisch, and D. J. Maskell. 1998. Molecular and functional analysis of the lipopolysaccharide biosynthesis locus *wlb* from *Bordetella pertussis, Bordetella parapertussis* and *Bordetella bronchiseptica*. Mol Microbiol **29:**27-38.

Altschul, S. F., W. Gish, W. Miller, E. W. Meyers, and D. J. Lipman. 1990. Basic Local Alignment Search Tool. J Mol Biol **215:**403-410.

Antoine, R., S. Alonso, D. Raze, L. Coutte, S. Lesjean, E. Willery, C. Locht, and F. Jacob-Dubuisson. 2000. New virulence-activated and virulence-repressed genes identified by systematic gene inactivation and generation of transcriptional fusions in *Bordetella pertussis*. J Bacteriol **182:**5902-5905.

Aoyama, T., Y. Murase, T. Iwata, A. Imaizumi, Y. Suzuki, and Y. Sato. 1986. Comparison of blood-free medium (cyclodextrin solid medium) with Bordet-Gengou medium for clinical isolation of *Bordetella pertussis*. J Clin Microbiol **23:**1046-1048.

Arico, B., J. F. Miller, C. Roy, S. Stibitz, D. Monack, S. Falkow, R. Gross, and R. Rappuoli. 1989. Sequences required for expression of *Bordetella pertussis* virulence factors share homology with prokaryotic signal transduction proteins. Proc Natl Acad Sci U S A **86:**6671-6675.

Armstrong, S. K., T. R. Parr, Jr., C. D. Parker, and R. E. Hancock. 1986. *Bordetella pertussis* major outer membrane porin protein forms small, anion-selective channels in lipid bilayer membranes. J Bacteriol **166:**212-216.

Bachelet, M., M. J. Richard, D. Francois, and B. S. Polla. 2002. Mitochondrial alterations precede *Bordetella pertussis*-induced apoptosis. FEMS Immunol Med Microbiol **32:**125-131.

Backman, A., B. Johansson, and P. Olcen. 1994. Nested PCR optimized for detection of *Bordetella pertussis* in clinical nasopharyngeal samples. J Clin Microbiol **32:**2544-2548.

Baker, J. P. 2003. The pertussis vaccine controversy in Great Britain, 1974-1986. Vaccine **21:**4003-4010.

Bartkus, J. M., B. A. Juni, K. Ehresmann, C. A. Miller, G. N. Sanden, P. K. Cassiday, M. Saubolle, B. Lee, J. Long, A. R. Harrison, Jr., and J. M. Besser. 2003. Identification of a mutation associated with erythromycin resistance in *Bordetella pertussis*: implications for surveillance of antimicrobial resistance. J Clin Microbiol **41:**1167-1172.

Beall, B., P. K. Cassiday, and G. N. Sanden. 1995. Analysis of *Bordetella pertussis* isolates from an epidemic by pulsed-field gel electrophoresis. J Clin Microbiol **33:**3083-3086.

Bejerano, M., I. Nisan, A. Ludwig, W. Goebel, and E. Hanski. 1999. Characterization of the C-terminal domain essential for toxic activity of adenylate cyclase toxin. Mol Microbiol **31:**381-392.

Betsou, F., P. Sebo, and N. Guiso. 1993. CyaC-mediated activation is important not only for toxic but also for protective activities of *Bordetella pertussis* adenylate cyclase-hemolysin. Infect Immun **61:**3583-3589.

Betsou, F., P. Sebo, and N. Guiso. 1995a. The C-terminal domain is essential for protective activity of the *Bordetella pertussis* adenylate cyclase-hemolysin. Infect Immun **63:**3309-3315.

Betsou, F., O. Sismeiro, A. Danchin, and N. Guiso. 1995b. Cloning and sequence of the *Bordetella bronchiseptica* adenylate cyclase-hemolysin-encoding gene: comparison with the *Bordetella pertussis* gene. Gene **162:**165-166.

Blackburn P. E. 2000. Characterisation of the virulence-related, outer memebrane proteins of *B. pertussis*. PhD thesis, University of Glasgow.

Bokhari S. H. 2002. Characterisation and secretion of *Bordetella pertussis* autotransporter proteins. PhD thesis, University of Glasgow.

Boschwitz, J. S., J. W. Batanghari, H. Kedem, and D. A. Relman. 1997. *Bordetella pertussis* infection of human monocytes inhibits antigen-dependent CD4 T cell proliferation. J Infect Dis **176:**678-686.

Boursaux-Eude, C. and N. Guiso. 2000. Polymorphism of repeated regions of pertactin in *Bordetella pertussis, Bordetella parapertussis*, and *Bordetella bronchiseptica*. Infect Immun **68:**4815-4817.

Brennan, M., P. Strebel, H. George, W. K. Yih, R. Tachdjian, S. M. Lett, P. Cassiday, G. Sanden, and M. Wharton. 2000. Evidence for transmission of pertussis in schools, Massachusetts, 1996: epidemiologic data supported by pulsed-field gel electrophoresis studies. J Infect Dis **181:**210-215.

Brockmeier, S. L., K. B. Register, T. Magyar, A. J. Lax, G. D. Pullinger, and R. A. Kunkle. 2002. Role of the dermonecrotic toxin of *Bordetella bronchiseptica* in the pathogenesis of respiratory disease in swine. Infect Immun **70:**481-490.

Brodeur, B. R., D. Martin, J. Hamel, R. D. Shahin, and C. Laferriere. 1993. Antigenic analysis of the saccharide moiety of the lipooligosaccharide of *Bordetella pertussis*. Springer Seminars in Immunopathology **15:**205-215.

Brownlie, R. M., J. G. Coote, R. Parton, J. E. Schultz, A. Rogel, and E. Hanski. 1988. Cloning of the adenylate cyclase genetic determinant of *Bordetella pertussis* and its expression in *Escherichia coli* and *B. pertussis*. Microb. Pathog. **4:**335-344.

Brownlie, R. M., R. Parton, and J. G. Coote. 1985. The effect of growth conditions on adenylate cyclase activity and virulence-related properties of *Bordetella pertussis*. J Gen. Microbiol **131:**17-25.

Burns D. L. 2003. Type IV transporters of pathogenic bacteria. Curr Opin Microbiol. **6**:29-34.

Burns, V. C., E. J. Pishko, A. Preston, D. J. Maskell, and E. T. Harvill. 2003. Role of Bordetella O antigen in respiratory tract infection. Infect Immun **71:**86-94.

Campins-Marti, M., H. K. Cheng, K. Forsyth, N. Guiso, S. Halperin, L. M. Huang, J. Mertsola, G. Oselka, J. Ward, C. H. Wirsing von Konig, and F. Zepp. 2001. Recommendations are needed for adolescent and adult pertussis immunisation: rationale and strategies for consideration. Vaccine **20:**641-646.

Carbonetti, N. H., G. V. Artamonova, R. M. Mays, and Z. E. Worthington. 2003. Pertussis toxin plays an early role in respiratory tract colonization by *Bordetella pertussis*. Infect Immun **71:**6358-6366.

Caroff, M., J. Brisson, A. Martin, and D. Karibian. 2000. Structure of the *Bordetella pertussis* 1414 endotoxin. FEBS Lett **477:**8-14.

Cassiday, P., G. Sanden, K. Heuvelman, F. Mooi, K. M. Bisgard, and T. Popovic. 2000. Polymorphism in *Bordetella pertussis* pertactin and pertussis toxin virulence factors in the United States, 1935-1999. J Infect Dis **182:**1402-1408.

Cassiday, P. K., G. N. Sanden, C. T. Kane, S. M'Boup, and J. M. Barbaree. 1994. Viability of *Bordetella pertussis* in four suspending solutions at three temperatures. J Clin Microbiol **32:**1550-1553.

Chaby, R., and M. Caroff. 1988. Lipopolysaccharides of *Bordetella pertussis* endotoxin. In: Wardlaw AC, Parton R (eds) Pathogenesis and Immunity in Pertussis. Chichester: John Wiley and Sons. 247-271.

Chalfie M, Y. Tu, G. Euskirchen, W. W. Ward, and D. C. Prasher. 1994. Green fluorescent protein as a marker for gene expression. Science **263**:802-5.

Charles, I., N. Fairweather, D. Pickard, J. Beesley, R. Anderson, G. Dougan, and M. Roberts. 1994. Expression of the *Bordetella pertussis* P.69 pertactin adhesin in *Escherichia coli*: fate of the carboxy-terminal domain. Microbiology **140:**3301-3308.

Charles, I. G., G. Dougan, D. Pickard, S. Chatfield, M. Smith, P. Novotny, P. Morrissey, and N. F. Fairweather. 1989. Molecular cloning and characterization of protective outer membrane protein P.69 from *Bordetella pertussis*. Proc Natl Acad Sci U S A **86:**3554-3558.

Charles, I. G., J. L. Li, M. Roberts, K. Beesley, M. Romanos, D. J. Pickard, M. Francis, D. Campbell, G. Dougan, M. J. Brennan, C. R. Manclark, M. Au Jensen, I. Heron, A. Chubb, P. Novotny, and N. F. Fairweather. 1991. Identification and characterization of a protective immunodominant B cell epitope of pertactin (P.69) from *Bordetella pertussis*. Eur J Immunol **21:**1147-1153.

Chen, I., T. M. Finn, L. Yanqing, Q. Guoming, R. Rappuoli, and M. Pizza. 1998. A recombinant live attenuated strain of *Vibrio cholerae* induces immunity against tetanus toxin and *Bordetella pertussis* tracheal colonization factor. Infect Immun **66:**1648-1653.

Cody, C. L., L. J. Baraff, J. D. Cherry, S. M. Marcy, and C. R. Manclark. 1981. Nature and rates of adverse reactions associated with DTP and DT immunizations in infants and children. Pediatrics **68:**650-660.

Confer, D. L. and J. W. Eaton. 1982. Phagocyte impotence caused by an invasive bacterial adenylate cyclase. Science **217:**948-950.

Confer, D. L., G. Zackrisson, T. Lagergard, and J. W. Eaton. 1990. Rapid diagnosis of pertussis. Scand. J Infect Dis **22:**175-177.

Cookson, B. T., A. N. Tyler, and W. E. Goldman. 1989. Primary structure of the peptidoglycan-derived tracheal cytotoxin of *Bordetella pertussis*. Biochemistry **28:**1744-1749.

Cookson, B. T., P. Vandamme, L. C. Carlson, A. M. Larson, J. V. Sheffield, K. Kersters, and D. H. Spach. 1994. Bacteremia caused by a novel Bordetella species, "*B. hinzii*". J Clin Microbiol **32:**2569-2571.

Coote, J. G. 1996. The RTX toxins of Gram-negative bacterial pathogens: modulators of the host immune system. Rev Med Microbiol **7**: 53-62.

Coote, J. G. 2001. Environmental sensing mechanisms in Bordetella. Adv. Microb. Physiol **44:**141-181.

Cotter, P. A. and V. J. DiRita. 2000. Bacterial virulence gene regulation: an evolutionary perspective. Annu. Rev Microbiol **54:**519-565.

Cotter, P. A. and A. M. Jones. 2003. Phosphorelay control of virulence gene expression in Bordetella. Trends Microbiol **11:**367-373.

Cotter, P. A., M. H. Yuk, S. Mattoo, B. J. Akerley, J. Boschwitz, D. A. Relman, and J. F. Miller. 1998. Filamentous hemagglutinin of *Bordetella bronchiseptica* is required for efficient establishment of tracheal colonization. Infect Immun **66:**5921-5929.

Coutte, L., R. Antoine, H. Drobecq, C. Locht, and F. Jacob-Dubuisson. 2001. Subtilisin-like autotransporter serves as maturation protease in a bacterial secretion pathway. EMBO J **20:**5040-5048.

Crowcroft, N. S., R. Booy, T. Harrison, L. Spicer, J. Britto, Q. Mok, P. Heath, I. Murdoch, M. Zambon, R. George, and E. Miller. 2003. Severe and unrecognised: pertussis in UK infants. Arch Dis Child **88:**802-806.

Crowcroft, N. S. and J. Britto. 2002. Whooping cough--a continuing problem. BMJ **324:**1537-1538.

Cullinane L. C., M. R. Alley, R. B. Marshall, and B. W. Manktelow. 1987. *Bordetella parapertussis* from lambs. New Zealand Veterinary Journal **35:**175.

de Melker, H. E., M. A. Conyn-van Spaendonck, H. C. Rumke, J. K. van Wijngaarden, F. R. Mooi, and J. F. Schellekens. 1997. Pertussis in The Netherlands: an outbreak despite high levels of immunization with whole-cell vaccine. Emerg Infect Dis **3:**175-178.

De Schutter, I, A. Malfroot, I. Dab, N. Hoebrekx, G. Muyldermans, D. Pierard, and S. Lauwers. 2003. Molecular typing of *Bordetella pertussis* isolates recovered from Belgian children and their household members. Clin Infect Dis **36:**1391-1396.

Domenighini, M., D. Relman, C. Capiau, S. Falkow, A. Prugnola, V. Scarlato, and R. Rappuoli. 1990. Genetic characterization of *Bordetella pertussis* filamentous haemagglutinin: a protein processed from an unusually large precursor. Mol Microbiol **4:**787-800.

Douglas, E., J. G. Cootc, R. Parton, W. McPheat. 1993. Identification of *Bordetella pertussis* in nasopharyngeal swabs by PCR amplification of a region of the adenylate cyclase gene. J Med Microbiol **38**:140-4.

Emsley, P., I. G. Charles, N. F. Fairweather, and N. W. Isaacs. 1996. Structure of *Bordetella pertussis* virulence factor P.69 pertactin. Nature **381:**90-92.

Endoh, M., M. Nagai, D. L. Burns, C. R. Manclark, and Y. Nakase. 1990. Effects of exogenous agents on the action of *Bordetella parapertussis* heat-labile toxin on guinea-pig skin. Infect Immun **58**:1456-1460

Enright, M. C. and B. G. Spratt. 1998. A multilocus sequence typing scheme for *Streptococcus pneumoniae*: identification of clones associated with serious invasive disease. Microbiology **144:**3049-3060.

Ewanowich, C. A., L. W. Chui, M. G. Paranchych, M. S. Peppler, R. G. Marusyk, and W. L. Albritton. 1993. Major outbreak of pertussis in northern Alberta, Canada: analysis of discrepant direct fluorescent-antibody and culture results by using polymerase chain reaction methodology. J Clin Microbiol **31:**1715-1725.

Farrell, D. J., G. Daggard, and T. K. Mukkur. 1999. Nested duplex PCR to detect *Bordetella pertussis* and *Bordetella parapertussis* and its application in diagnosis of pertussis in nonmetropolitan Southeast Queensland, Australia. J Clin Microbiol **37:**606-610.

Farrell, D. J., M. McKeon, G. Daggard, M. J. Loeffelholz, C. J. Thompson, and T. K. Mukkur. 2000. Rapid-cycle PCR method to detect *Bordetella pertussis* that fulfills all consensus recommendations for use of PCR in diagnosis of pertussis. J Clin Microbiol **38:**4499-4502.

Fernandez, R. C. and A. A. Weiss. 1994. Cloning and sequencing of a *Bordetella pertussis* serum resistance locus. Infect Immun **62:**4727-4738.

Fernandez, R. C. and A. A. Weiss. 1998. Serum resistance in bvg-regulated mutants of *Bordetella pertussis*. FEMS Microbiol Lett **163:**57-63.

Finn, T. M. and D. F. Amsbaugh. 1998. Vag8, a *Bordetella pertussis* bvg-regulated protein. Infect Immun **66:**3985-3989.

Finn, T. M., Z. Li, and E. Kocsis. 1995. Identification of a *Bordetella pertussis* bvg-regulated porin-like protein. J Bacteriol **177:**805-809.

Finn, T. M. and L. A. Stevens. 1995. Tracheal colonization factor: a *Bordetella pertussis* secreted virulence determinant. Mol Microbiol **16:**625-634.46.

Flak, T. A. and W. E. Goldman. 1999. Signalling and cellular specificity of airway nitric oxide production in pertussis. Cell Microbiol **1:**51-60.

Flak, T. A., L. N. Heiss, J. T. Engle, and W. E. Goldman. 2000. Synergistic epithelial responses to endotoxin and a naturally occurring muramyl peptide. Infect Immun **68:**1235-1242.

Foreman-Wykert, A. K., R. Deora, S. Mattoo, and J. F. Miller. 2002. An autotransporter is required for secretion of Type III effector proteins in *Bordetella bronchiseptica*. Abstract from 7th International Symposium on Pertussis, Hinxton, Cambridge.

Friedman, R. L., R. L. Fiederlein, L. Glasser, and J. N. Galgiani. 1987. *Bordetella pertussis* adenylate cyclase: effects of affinity-purified adenylate cyclase on human polymorphonuclear leukocyte functions. Infect Immun **55:**135-140.

Friedman, L. E., B. N. de Rossi, M. T. Messina, and M. A. Franco. 2001. Phenotype evaluation of *Bordetella bronchiseptica* cultures by urease activity and Congo red affinity. Lett Appl. Microbiol **33:**285-290.

Frohlich, B. T., M. d'Alarcao, R. S. Feldberg, M. L. Nicholson, G. R. Siber, and R. W. Swartz. 1996. Formation and cell-medium partitioning of autoinhibitory free fatty acids and cyclodextrin's effect in the cultivation of *Bordetella pertussis*. J Biotechnol. **45:**137-148.

Fry, N. K., S. Neal, T. G. Harrison, E. Miller, R. Matthews, and R. C. George. 2001. Genotypic variation in the *Bordetella pertussis* virulence factors pertactin and pertussis toxin in historical and recent clinical isolates in the United Kingdom. Infect Immun **69:**5520-5528.

Fry, N. K., O. Tzivra , Y. T. Li, A. McNiff, N. Doshi, P. A. Maple, N. S. Crowcroft, E. Miller, R. C. George, and T. G. Harrison. 2004. Laboratory diagnosis of pertussis infections: the role of PCR and serology. J Med Microbiol **53**:519-25.

Fuchslocher, B., L. L. Millar, and P. A. Cotter. 2003. Comparison of *bipA* alleles within and across Bordetella species. Infect Immun **71:**3043-3052.

Garcia-Corbeira, P., R. Dal Re, L. Aguilar, and J. Garcia-de-Lomas. 2000. Seroepidemiology of *Bordetella pertussis* infections in the Spanish population: a cross-sectional study. Vaccine **18:**2173-2176.

Gerlach, G., F. von Wintzingerode, B. Middendorf, and R. Gross. 2001. Evolutionary trends in the genus *Bordetella*. Microbes. Infect **3:**61-72.

Geuijen, C. A., R. J. Willems, and F. R. Mooi. 1996. The major fimbrial subunit of *Bordetella pertussis* binds to sulfated sugars. Infect Immun **64:**2657-2665.

Geuijen, C. A., R. J. Willems, M. Bongaerts, J. Top, H. Gielen, and F. R. Mooi. 1997. Role of the *Bordetella pertussis* minor fimbrial subunit, FimD, in colonization of the mouse respiratory tract. Infect Immun **65:**4222-4228.

Geuijen, C. A., R. J. Willems, P. Hoogerhout, W. C. Puijk, R. H. Meloen, and F. R. Mooi. 1998. Identification and characterization of heparin binding regions of the Fim2 subunit of *Bordetella pertussis*. Infect Immun **66:**2256-2263.

Gilberg, S., E. Njamkepo, C. Du, I, H. Partouche, P. Gueirard, C. Ghasarossian, M. Schlumberger, and N. Guiso. 2002. Evidence of *Bordetella pertussis* infection in adults presenting with persistent cough in a french area with very high whole-cell vaccine coverage. J Infect Dis **186:**415-418.

Gillings, M. and M. Holley. 1997. Repetitive element PCR fingerprinting (rep-PCR) using enterobacterial repetitive intergenic consensus (ERIC) primers is not necessarily directed at ERIC elements. Lett Appl. Microbiol **25:**17-21.

Gladbach, S., S. Hanauer, U. Reischl, K. Wilson, and G. N. Sanden. 2002. Identification of IS*481* in *Bordetella bronchiseptica*: implications for *Bordetella* spp. Phylogeny and diagnosis of *Bordetella pertussis* infections by polymerase chain reaction assays. Abstract from 7th International Symposium on Pertussis, Hinxton, Cambridge.

Glare, E. M., J. C. Paton, R. R. Premier, A. J. Lawrence, and I. T. Nisbet. 1990. Analysis of a repetitive DNA sequence from *Bordetella pertussis* and its application to the diagnosis of pertussis using the polymerase chain reaction. J Clin Microbiol **28:**1982-1987.

Glaser, P., D. Ladant, O. Sezer, F. Pichot, A. Ullmann, and A. Danchin. 1988. The calmodulin-sensitive adenylate cyclase of *Bordetella pertussis*: cloning and expression in Escherichia coli. Mol Microbiol **2:**19-30.

Glaser, P., H. Munier, A. M. Gilles, E. Krin, T. Porumb, O. Barzu, R. Sarfati, C. Pellecuer, and A. Danchin. 1991. Functional consequences of single amino acid substitutions in calmodulin-activated adenylate cyclase of *Bordetella pertussis*. EMBO J **10:**1683-1688.

Goldman, S., E. Hanski, and F. Fish. 1984. Spontaneous phase variation in *Bordetella pertussis* is a multistep non-random process. EMBO J **3:**1353-1356.

Goldman, W. E., D. G. Klapper, and J. B. Baseman. 1982. Detection, isolation, and analysis of a released *Bordetella pertussis* product toxic to cultured tracheal cells. Infect Immun **36:**782-794.

Goyard, S. and A. Ullmann. 1991. Analysis of *Bordetella pertussis cya* operon regulation by use of *cya-lac* fusions. FEMS Microbiol Lett **61:**251-256.

Grant, C. C. and J. D. Cherry. 2002. Keeping pace with the elusive *Bordetella pertussis*. J Infect **44:**7-12.

Greenaway, P. J. 1980. The isolation of a restriction enzyme from *Bordetella pertussis*. Biochem. Biophys. Res. Commun. **95:**1282-1287.

Gueirard, P., A. Druilhe, M. Pretolani, and N. Guiso. 1998. Role of adenylate cyclase-hemolysin in alveolar macrophage apoptosis during *Bordetella pertussis* infection in vivo. Infect Immun **66:**1718-1725.

Gueirard, P., C. Weber, A. Le Coustumier, and N. Guiso. 1995. Human *Bordetella bronchiseptica* infection related to contact with infected animals: persistence of bacteria in host. J Clin Microbiol **33:**2002-2006.

Guermonprez, P., N. Khelef, E. Blouin, P. Rieu, P. Ricciardi-Castagnoli, N. Guiso, D. Ladant, and C. Leclerc. 2001. The adenylate cyclase toxin of *Bordetella pertussis* binds to target cells via the alpha(M)beta(2) integrin (CD11b/CD18). J Exp Med **193:**1035-1044.

Guermonprez, P., D. Ladant, G. Karimova, A. Ullmann, and C. Leclerc. 1999. Direct delivery of the *Bordetella pertussis* adenylate cyclase toxin to the MHC class I antigen presentation pathway. J Immunol **162:**1910-1916.

Guiso, N. 1997. Isolation, identification and characterization of *Bordetella pertussis.* Dev. Biol Stand. **89:**233-238.

Guiso, N., M. Szatanik, and M. Rocancourt. 1991. Protective activity of Bordetella adenylate cyclase-hemolysin against bacterial colonization. Microb. Pathog. **11:**423-431.

Guris, D., P. M. Strebel, B. Bardenheier, M. Brennan, R. Tachdjian, E. Finch, M. Wharton, and J. R. Livengood. 1999. Changing epidemiology of pertussis in the United States: increasing reported incidence among adolescents and adults, 1990-1996. Clin Infect Dis **28:**1230-1237.

Gzyl, A., E. Augustynowicz, L. van, I, and J. Slusarczyk. 2002. Temporal nucleotide changes in pertactin and pertussis toxin genes in *Bordetella pertussis* strains isolated from clinical cases in Poland. Vaccine **20:**299-303.

Hackett, M., L. Guo, J. Shabanowitz, D. F. Hunt, and E. L. Hewlett. 1994. Internal lysine palmitoylation in adenylate cyclase toxin from *Bordetella pertussis.* Science **266:**433-435.

Hallander, H. O. 1999. Microbiological and serological diagnosis of pertussis. Clin Infect Dis **28 Suppl 2:**S99-106.

Hannah, J. H., F. D. Menozzi, G. Renauld, C. Locht, and M. J. Brennan. 1994. Sulfated glycoconjugate receptors for the *Bordetella pertussis* adhesin filamentous hemagglutinin (FHA) and mapping of the heparin-binding domain on FHA. Infect Immun **62:**5010-5019.

Hardwick, T. H., B. Plikaytis, P. K. Cassiday, G. Cage, M. S. Peppler, D. Shea, D. Boxrud, and G. N. Sanden. 2002. Reproducibility of *Bordetella pertussis* genomic DNA fragments generated by XbaI restriction and resolved by pulsed-field gel electrophoresis. J Clin Microbiol **40:**811-816.

Harvill, E. T., P. A. Cotter, M. H. Yuk, and J. F. Miller. 1999. Probing the function of *Bordetella bronchiseptica* adenylate cyclase toxin by manipulating host immunity. Infect Immun **67:**1493-1500.

Harvill, E. T., A. Preston, P. A. Cotter, A. G. Allen, D. J. Maskell, and J. F. Miller. 2000. Multiple roles for Bordetella lipopolysaccharide molecules during respiratory tract infection. Infect Immun **68:**6720-6728.

Hazenbos, W. L., C. A. Geuijen, B. M. van den Berg, F. R. Mooi, and R. van Furth. 1995. *Bordetella pertussis* fimbriae bind to human monocytes via the minor fimbrial subunit FimD. J Infect Dis **171:**924-929.

Heininger, U. 2001. Pertussis: an old disease that is still with us. Curr. Opin. Infect Dis **14:**329-335.

Heiss, L. N., J. R. Lancaster, Jr., J. A. Corbett, and W. E. Goldman. 1994. Epithelial autotoxicity of nitric oxide: role in the respiratory cytopathology of pertussis. Proc Natl Acad Sci U S A **91:**267-270.

Henderson, I. R. and J. P. Nataro. 2001. Virulence functions of autotransporter proteins. Infect Immun **69:**1231-1243.

Henderson, I. R., F. Navarro-Garcia, and J. P. Nataro. 1998. The great escape: structure and function of the autotransporter proteins. Trends Microbiol **6:**370-378.

Hennuy, B., N. Kling, V. Caro, F. Rimlinger, and N. Guiso. 2002. The Type III secretion pathway is expressed by *Bordetella pertussis* and *Bordetella parapertussis* and is regulated by the two component *bvgAS* system. Abstract from 7th International Symposium on Pertussis, Hinxton, Cambridge.

Heveker, N. and D. Ladant. 1997. Characterization of mutant *Bordetella pertussis* adenylate cyclase toxins with reduced affinity for calmodulin. Implications for the mechanism of toxin entry into target cells. Eur J Biochem. **243:**643-649.

Hewlett, E. L. 1997a. Pertussis: current concepts of pathogenesis and prevention. Pediatr Infect Dis J **16:**S78-S84.

Hewlett, E. L. 1997b. Reemerging Infections: Recent developments in pertussis. Emerg Infect 2 **Chapter** 7:145-157.

Hewlett, E. L., C. R. Manclark, and J. Wolff. 1977. Adenyl cyclase in *Bordetella pertussis* vaccines. J Infect Dis **136 Suppl:**S216-S219.

Hewlett, E. L., M. A. Urban, C. R. Manclark, and J. Wolff. 1976. Extracytoplasmic adenylate cyclase of *Bordetella pertussis*. Proc Natl Acad Sci U S A **73:**1926-1930.

Hill, B. C., C. N. Baker, and F. C. Tenover. 2000. A simplified method for testing *Bordetella pertussis* for resistance to erythromycin and other antimicrobial agents. J Clin Microbiol **38:**1151-1155.

Hodder, S. L. and E. A. Mortimer, Jr. 1992. Epidemiology of pertussis and reactions to pertussis vaccine. Epidemiol. Rev **14:**243-267.

Hoey, J. 2003. Pertussis in adults. CMAJ. **168:**453-454.

Hormozi, K., R. Parton, and J. Coote. 1999. Adjuvant and protective properties of native and recombinant *Bordetella pertussis* adenylate cyclase toxin preparations in mice. FEMS Immunol Med Microbiol **23:**273-282.

Houard, S., C. Hackel, A. Herzog, and A. Bollen. 1989. Specific identification of *Bordetella pertussis* by the polymerase chain reaction. Res. Microbiol **140:**477-487.

Hulton, C. S., C. F. Higgins, and P. M. Sharp. 1991. ERIC sequences: a novel family of repetitive elements in the genomes of *Escherichia coli, Salmonella typhimurium* and other enterobacteria. Mol Microbiol **5:**825-834.

Ieven, M. and H. Goossens. 1997. Relevance of nucleic acid amplification techniques for diagnosis of respiratory tract infections in the clinical laboratory. Clin Microbiol Rev **10:**242-256.

Iwaki, M., K. Kamachi, and T. Konda. 2000. Stimulation of *Bordetella pertussis* adenylate cyclase toxin intoxication by its hemolysin domain. Infect Immun **68:**3727-3730.

Jersek B, P. Gilot, M. Gubina, N. Klun, J. Mehle, E. Tcherneva, N. Rijpens, and L. Herman. 1999. Typing of *Listeria monocytogenes* strains by repetitive element sequence-based PCR. J Clin Microbiol **37:**103-109.

Jones, M. N. 1999. Surfactants in membrane solubilisation. Int J Pharm. **177:**137-159.

Kamachi, K., T. Konda, and Y. Arakawa. 2003. DNA vaccine encoding pertussis toxin S1 subunit induces protection against *Bordetella pertussis* in mice. Vaccine **21:**4609-4615.

Karimova, G., J. Bellalou, and A. Ullmann. 1996. Phosphorylation-dependent binding of BvgA to the upstream region of the cyaA gene of *Bordetella pertussis*. Mol Microbiol **20:**489-496.

Kerr, J. R. and R. C. Matthews. 2000. *Bordetella pertussis* infection: pathogenesis, diagnosis, management, and the role of protective immunity. Eur J Clin Microbiol Infect Dis **19:**77-88.

Kersters, K., K. H. Hinz, A. Hertle, P. Segers, A. Lievens, O. Siegmann, and J. Deley. 1984. *Bordetella avium* sp-nov, isolated from the respiratory tracts of turkeys and other birds. International Journal of Systematic Bacteriology **34:**56-70.

Khelef, N., P. Gounon, and N. Guiso. 2001. Internalization of *Bordetella pertussis* adenylate cyclase-haemolysin into endocytic vesicles contributes to macrophage cytotoxicity. Cell Microbiol **3:**721-730.

Khelef, N., H. Sakamoto, and N. Guiso. 1992. Both adenylate cyclase and hemolytic activities are required by *Bordetella pertussis* to initiate infection. Microb. Pathog. **12:**227-235.

Kimura, A., K. T. Mountzouros, D. A. Relman, S. Falkow, and J. L. Cowell. 1990. *Bordetella pertussis* filamentous hemagglutinin: evaluation as a protective antigen and colonization factor in a mouse respiratory infection model. Infect Immun **58:** 7-16.

King, A. J., G. Berbers, H. F. van Oirschot, P. Hoogerhout, K. Knipping, and F. R. Mooi. 2001. Role of the polymorphic region 1 of the *Bordetella pertussis* protein pertactin in immunity. Microbiology **147:**2885-2895.

Knapp, O., E. Maier, G. Polleichtner, J. Masin, P. Sebo, and R. Benz. 2003. Channel formation in model membranes by the adenylate cyclase toxin of *Bordetella pertussis*: effect of calcium. Biochemistry **42:**8077-8084.

Knapp, S. and J. J. Mekalanos. 1988. Two trans-acting regulatory genes (*vir* and *mod*) control antigenic modulation in *Bordetella pertussis*. J Bacteriol **170:**5059-5066.

Kosters, K., M. Riffelmann, and C. H. Wirsing von Konig. 2001. Evaluation of a real-time PCR assay for detection of *Bordetella pertussis* and B. parapertussis in clinical samples. J Med Microbiol **50:**436-440.

Kourova, N., V. Caro, C. Weber, S. Thiberge, R. Chuprinina, G. Tseneva, and N. Guiso. 2003. Comparison of the *Bordetella pertussis* and Bordetella parapertussis isolates circulating in Saint Petersburg between 1998 and 2000 with Russian vaccine strains. J Clin Microbiol **41:**3706-3711.

Ladant, D., P. Glaser, and A. Ullmann. 1992. Insertional mutagenesis of *Bordetella pertussis* adenylate cyclase. J Biol Chem. **267:**2244-2250.

Ladant, D. and A. Ullmann. 1999. *Bordetella pertussis* adenylate cyclase: a toxin with multiple talents. Trends Microbiol **7:**172-176.

Lawrence, A. J., J. G. Coote, Y. F. Kazi, P. D. Lawrence, J. MacDonald-Fyall, B. M. Orr, R. Parton, M. Riehle, J. Sinclair, J. Young, and N. C. Price. 2002. A direct pyrophosphatase-coupled assay provides new insights into the activation of the secreted adenylate cyclase from *Bordetella pertussis* by calmodulin. J Biol Chem. **277:**22289-22296.

Lee, Y. S., C. Y. Yang, C. H. Lu, and Y. H. Tseng. 2003. Molecular epidemiology of *Bordetella pertussis* isolated in Taiwan, 1992-1997. Microbiol Immunol **47:**903-909.

Leininger, E., M. Roberts, J. G. Kenimer, I. G. Charles, N. Fairweather, P. Novotny, and M. J. Brennan. 1991. Pertactin, an Arg-Gly-Asp-containing *Bordetella pertussis* surface protein that promotes adherence of mammalian cells. Proc Natl Acad Sci U S A **88:**345-349.

Li, Z. M., J. H. Hannah, S. Stibitz, N. Y. Nguyen, C. R. Manclark, and M. J. Brennan. 1991. Cloning and sequencing of the structural gene for the porin protein of *Bordetella pertussis*. Mol Microbiol **5:**1649-1656.

Lievano, F. A., M. A. Reynolds, A. L. Waring, J. Ackelsberg, K. M. Bisgard, G. N. Sanden, D. Guris, A. Golaz, D. J. Bopp, R. J. Limberger, and P. F. Smith. 2002. Issues associated with and recommendations for using PCR to detect outbreaks of pertussis. J Clin Microbiol **40:**2801-2805.

Lind-Brandberg, L., C. Welinder-Olsson, T. Lagergard, J. Taranger, B. Trollfors, and G. Zackrisson. 1998. Evaluation of PCR for diagnosis of *Bordetella pertussis* and *Bordetella parapertussis* infections. J Clin Microbiol **36:**679-683.

Locht, C., and R. Antoine. 1999. *Bordetella pertussis* toxins. In: J. E., Alouf and J. H. Freer (eds) The Comprehensive Sourcebook of Bacterial Protein Toxins 2nd edition, 130-146.

Locht, C., R. Antoine, and F. Jacob-Dubuisson. 2001. *Bordetella pertussis*, molecular pathogenesis under multiple aspects. Curr. Opin. Microbiol **4:**82-89.

Locht, C., P. Bertin, F. D. Menozzi, and G. Renauld. 1993. The filamentous haemagglutinin, a multifaceted adhesion produced by virulent *Bordetella* spp. Mol Microbiol **9:**653-660.

Locht, C. and J. M. Keith. 1986. Pertussis toxin gene: nucleotide sequence and genetic organisation. Science **232:**1258-1264.

Loeffelholz, M. J., C. J. Thompson, K. S. Long, and M. J. Gilchrist. 2000. Detection of *Bordetella holmesii* using *Bordetella pertussis* IS481 PCR assay. J Clin Microbiol **38:**467.

Ludwig, A., and W. Goebel. 1999. The family of multigenic encoded RTX toxins. In: J. E., Alouf and J. H. Freer (eds) The Comprehensive Sourcebook of Bacterial Protein Toxins 2nd edition.

MacDonald-Fyall J. 2002. The protective and immunomodulatory properties of *Bordetella pertussis* adenylate cyclase toxin. PhD thesis, University of Glasgow.

Maiden, M. C., J. A. Bygraves, E. Feil, G. Morelli, J. E. Russell, R. Urwin, Q. Zhang, J. Zhou, K. Zurth, D. A. Caugant, I. M. Feavers, M. Achtman, and B. G. Spratt. 1998. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc Natl Acad Sci U S A **95:**3140-3145.

Makhov, A. M., J. H. Hannah, M. J. Brennan, B. L. Trus, E. Kocsis, J. F. Conway, P. T. Wingfield, M. N. Simon, and A. C. Steven. 1994. Filamentous hemagglutinin of *Bordetella pertussis*. A bacterial adhesin formed as a 50-nm monomeric rigid rod based on a 19-residue repeat motif rich in beta strands and turns. J Mol Biol **241:**110-124.

Martin, B., O. Humbert, M. Camara, E. Guenzi, J. Walker, T. Mitchell, P. Andrew, M. Prudhomme, G. Alloing, R. Hakenbeck, and . 1992. A highly conserved repeated DNA element located in the chromosome of *Streptococcus pneumoniae*. Nucleic Acids Res. **20:**3479-3483.

Mastrantonio, P., P. Spigaglia, H. van Oirschot, H. G. van der Heide, K. Heuvelman, P. Stefanelli, and F. R. Mooi. 1999. Antigenic variants in *Bordetella pertussis* strains isolated from vaccinated and unvaccinated children. Microbiology **145:**2069-2075.

Mastrantonio, P., P. Stefanelli, and M. Giuliano. 1996. Polymerase chain reaction for the detection of *Bordetella pertussis* in clinical nasopharyngeal aspirates. J Med Microbiol **44:**261-266.

Matsuzawa, T., T. Kashimoto, J. Katahira, and Y. Horiguchi. 2002. Identification of a receptor-binding domain of Bordetella dermonecrotic toxin. Infect Immun **70:**3427-3432.

Mattoo, S., J. F. Miller, and P. A. Cotter. 2000. Role of *Bordetella bronchiseptica* fimbriae in tracheal colonization and development of a humoral immune response. Infect Immun **68:**2024-2033.

Mazengia, E., E. A. Silva, J. A. Peppe, R. Timperi, and H. George. 2000. Recovery of *Bordetella holmesii* from patients with pertussis-like symptoms: use of pulsed-field gel electrophoresis to characterize circulating strains. J Clin Microbiol **38:**2330-2333.

McMillan, D. J., M. Shojaei, G. S. Chhatwal, C. A. Guzman, and M. J. Walker. 1996. Molecular analysis of the *bvg*-repressed urease of *Bordetella bronchiseptica*. Microb. Pathog. **21:**379-394.

Menzies, R., H. Wang, and P. McIntyre. 2003. Has pertussis increased in NSW over the past decade? An evaluation using hospitalisation and mortality data versus notifications 1988-2002. N. S W. Public Health Bull. **14:**71-76.

Merkel, T. J., P. E. Boucher, S. Stibitz, and V. K. Grippe. 2003. Analysis of *bvgR* expression in *Bordetella pertussis*. J Bacteriol **185:**6902-6912.

Merkel, T. J. and S. Stibitz. 1995. Identification of a locus required for the regulation of bvg-repressed genes in *Bordetella pertussis*. J Bacteriol **177:**2727-2736.

Mezna, M. and A. J. Lawrence. 1994. Conductimetric assays for the hydrolase and transferase activities of phospholipase D enzymes. Anal. Biochem. **218:**370-376.

Miller, W. G., J. H. J. Leveau, and S. E. Lindow. 2000. Improved *gfp* and *inaZ* broad-host-range promoter-probe vectors. Molecular Plant-Microbe Interactions **13:**1243-1250.

Miyashita, N., H. Fukano, K. Yoshida, Y. Niki, and T. Matsushima. 2003. *Chlamydia pneumoniae* infection in adult patients with persistent cough. J Med Microbiol **52:** 265-269.

Mock, M. and A. Ullmann. 1993. Calmodulin-activated bacterial adenylate cyclases as virulence factors. Trends Microbiol **1:**187-192.

Moissenet, D., M. Valcin, V. Marchand, E. Grimprel, P. Begue, A. Garbarg-Chenon, and H. Vu-Thien. 1996. Comparative DNA analysis of *Bordetella pertussis* clinical isolates by pulsed-field gel electrophoresis, randomly amplified polymorphism DNA, and ERIC polymerase chain reaction. FEMS Microbiol Lett **143:**127-132.

Mooi, F. R. Dutch isolates and the murine challenge model. Eupertstrain meeting, June 3rd 2004, Turku, Finland.

Mooi, F. R., H. Hallander, C. H. Wirsing von Konig, B. Hoet, and N. Guiso. 2000. Epidemiological typing of *Bordetella pertussis* isolates: recommendations for a standard methodology. Eur J Clin Microbiol Infect Dis **19:**174-181.

Mooi, F. R., Q. He, H. van Oirschot, and J. Mertsola. 1999. Variation in the *Bordetella pertussis* virulence factors pertussis toxin and pertactin in vaccine strains and clinical isolates in Finland. Infect Immun **67:**3133-3134.

Mooi, F. R., H. van Oirschot, K. Heuvelman, H. G. van der Heide, W. Gaastra, and R. J. Willems. 1998. Polymorphism in the *Bordetella pertussis* virulence factors P.69/pertactin and pertussis toxin in The Netherlands: temporal trends and evidence for vaccine-driven evolution. Infect Immun **66:**670-675.

Muller, F. M., J. E. Hoppe, and C. H. Wirsing von Konig. 1997. Laboratory diagnosis of pertussis: state of the art in 1997. J Clin Microbiol **35:**2435-2443.

Munoz, J. J., H. Arai, R. K. Bergman, and P. L. Sadowski. 1981. Biological activities of crystalline pertussigen from *Bordetella pertussis*. Infect Immun **33:**820-826.

Musser, J. M., E. L. Hewlett, M. S. Peppler, and R. K. Selander. 1986. Genetic diversity and relationships in populations of *Bordetella* spp. J Bacteriol **166:**230-237.

Nagai, M., M. Endoh , H. Danbara, and Y. Nakase. 1994. Purification and characterization of Bordetella calmodulin-like protein. FEMS Microbiol Lett **116**:169-74.

Nardone, A., R. G. Pebody, P. A. Maple, N. Andrews, N. J. Gay, E. Miller. 2004. Sero-epidemiology of Bordetella pertussis in England and Wales. Vaccine **22**:1314-9.

Neal, S. E. 2004. Genotypic diversity and epidemiological typing of *Bordetella pertussis*. PhD thesis. University of Glasgow.

Njamkepo, E., F. Pinot, D. Francois, N. Guiso, B. S. Polla, and M. Bachelet. 2000. Adaptive responses of human monocytes infected by *Bordetella pertussis*: the role of adenylate cyclase hemolysin. J Cell Physiol **183:**91-99.

Nygren, M., E. Reizenstein, M. Ronaghi, and J. Lundeberg. 2000. Polymorphism in the pertussis toxin promoter region affecting the DNA-based diagnosis of Bordetella infection. J Clin Microbiol **38:**55-60.

Olin, P., F. Rasmussen, L. Gustafsson, H. O. Hallander, and H. Heijbel. 1997. Randomised controlled trial of two-component, three-component, and five-component acellular pertussis vaccines compared with whole-cell pertussis vaccine. Ad Hoc Group for the Study of Pertussis Vaccines. Lancet **350:**1569-1577.

Oliver, D. C., G. Huang, and R. C. Fernandez. 2003a. Identification of secretion determinants of the *Bordetella pertussis* BrkA autotransporter. J Bacteriol **185:**489-495.

Oliver, D. C., G. Huang, E. Nodel, S. Pleasance, and R. C. Fernandez. 2003b. A conserved region within the *Bordetella pertussis* autotransporter BrkA is necessary for folding of its passenger domain. Mol Microbiol **47:**1367-1383.

Osickova, A., R. Osicka, E. Maier, R. Benz, and P. Sebo. 1999. An amphipathic alpha-helix including glutamates 509 and 516 is crucial for membrane translocation of adenylate cyclase toxin and modulates formation and cation selectivity of its membrane channels. J Biol Chem. **274:**37644-37650.

Packard, E. R., R. Parton, J. G. Cootc and N. K. Fry. 2004. Sequence variation and conservation in virulence-related genes of *Bordetella pertussis* isolates from the UK. J Med Microbiol. **53**: 355-365.

Parkhill, J., M. Sebaihia, A. Preston, L. D. Murphy, N. Thomson, D. E. Harris, M. T. Holden, C. M. Churcher, S. D. Bentley, K. L. Mungall, A. M. Cerdeno-Tarraga, L. Temple, K. James, B. Harris, M. A. Quail, M. Achtman, R. Atkin, S. Baker, D. Basham, N. Bason, I. Cherevach, T. Chillingworth, M. Collins, A. Cronin, P. Davis, J. Doggett, T. Feltwell, A. Goble, N. Hamlin, H. Hauser, S. Holroyd, K. Jagels, S. Leather, S. Moule, H. Norberczak, S. O'Neil, D. Ormond, C. Price, E. Rabbinowitsch, S. Rutter, M. Sanders, D. Saunders, K. Seeger, S. Sharp, M. Simmonds, J. Skelton, R. Squares, S. Squares, K. Stevens, L. Unwin, S. Whitehead, B. G. Barrell, and D. J. Maskell. 2003. Comparative analysis of the genome sequences of *Bordetella pertussis*, *Bordetella parapertussis* and *Bordetella bronchiseptica*. Nat. Genet. **35:**32-40.

Parton, R. 2004. Bordetella. Chapter 72. In: Topley and Wilson's Microbiology and Microbial Infections 10th Ed Edward Arnold (Publishers) Limited.

Passerini de Rossi, B. N., L. E. Friedman, C. B. Belzoni, S. Savino, B. Arico, R. Rappuoli, V. Masignani, and M. A. Franco. 2003. Vir90, a virulence-activated gene coding for a *Bordetella pertussis* iron-regulated outer membrane protein. Res. Microbiol **154:**443-450.

Passerini de Rossi, B. N., L. E. Friedman, F. L. Gonzalez Flecha, P. R. Castello, M. A. Franco, and J. P. Rossi. 1999. Identification of *Bordetella pertussis* virulence-associated outer membrane proteins. FEMS Microbiol Lett **172:**9-13.

Peppler, M. S. 1984. Two physically and serologically distinct lipopolysaccharide profiles in strains of *Bordetella pertussis* and their phenotype variants. Infect Immun **43:**224-232.

Peppler, M. S., S. Kuny, A. Nevesinjac, C. Rogers, Y. R. de Moissac, K. Knowles, M. Lorange, G. De Serres, and J. Talbot. 2003. Strain variation among *Bordetella pertussis* isolates from Quebec and Alberta provinces of Canada from 1985 to 1994. J Clin Microbiol **41:**3344-3347.

Pittman, M. 1984. The concept of pertussis as a toxin-mediated disease. Pediatr Infect Dis **3:**467-486.

Poddar, S. K. 2003. Detection and discrimination of *B. pertussis* and *B. holmesii* by real-time PCR targeting IS481 using a beacon probe and probe-target melting analysis. Mol Cell Probes **17:**91-98.

Pullinger, G. D., T. E. Adams, P. B. Mullan, T. I. Garrod, and A. J. Lax. 1996. Cloning, expression, and molecular characterization of the dermonecrotic toxin gene of *Bordetella* spp. Infect Immun **64:**4163-4171.

Qin, X., D. K. Turgeon, B. P. Ingersoll, P. W. Monsaas, C. J. Lemoine, T. Tsosie, L. O. Stapp, and P. M. Abe. 2002. *Bordetella pertussis* PCR: simultaneous targeting of signature sequences. Diagn. Microbiol Infect Dis **43:**269-275.

Regan, J. and F. Lowe. 1977. Enrichment medium for the isolation of Bordetella. J Clin Microbiol **6:**303-309.

Reischl, U., N. Lehn, G. N. Sanden, and M. J. Loeffelholz. 2001. Real-time PCR assay targeting IS481 of *Bordetella pertussis* and molecular basis for detecting *Bordetella holmesii. J* Clin Microbiol **39:**1963-1966.

Roberts, M., I. Cropley, S. Chatfield, and G. Dougan. 1993. Protection of mice against respiratory *Bordetella pertussis* infection by intranasal immunization with P.69 and FHA. Vaccine **11:**866-872.

Roberts, M., and R. Parton. 2001. *Bordetella pertussis.* In: M. Sussmann (ed) Mol Med Microbiol. 1565-1615.

Roberts, R. J. 1980. Restriction and modification enzymes and their recognition sequences. Nucleic Acids Res. **8:**r63-r80.

Robbins, J. B. 1999. Pertussis in adults: introduction. Clin Infect Dis **28 Suppl 2:**S91-S93.

Robinson, A., L. I. Irons, and L. A. Ashworth. 1985. Pertussis vaccine: present status and future prospects. Vaccine **3:**11-22.

Ronaghi M, M. Uhlen, and P. Nyren. 1998. A sequencing method based on real-time pyrophosphate. Science **28**:363-365

Rose, T., P. Sebo, J. Bellalou, and D. Ladant. 1995. Interaction of calcium with *Bordetella pertussis* adenylate cyclase toxin. Characterization of multiple calcium-binding sites and calcium-induced conformational changes. J Biol Chem. **270:**26370-26376.

Ross, P. J., E. C. Lavelle, K. H. Mills, and A. P. Boyd. 2004. Adenylate cyclase toxin from *Bordetella pertussis* synergizes with lipopolysaccharide to promote innate interleukin-10 production and enhances the induction of Th2 and regulatory T cells. Infect Immun **72:**1568-1579.

Saron, M. F., C. Fayolle, P. Sebo, D. Ladant, A. Ullmann, and C. Leclerc. 1997. Anti-viral protection conferred by recombinant adenylate cyclase toxins from *Bordetella pertussis* carrying a CD8+ T cell epitope from lymphocytic choriomeningitis virus. Proc Natl Acad Sci U S A **94:**3314-3319.

Sato, H. and Y. Sato. 1984. *Bordetella pertussis* infection in mice: correlation of specific antibodies against two antigens, pertussis toxin, and filamentous hemagglutinin with mouse protectivity in an intracerebral or aerosol challenge system. Infect Immun **46:**415-421.

Scarlato, V., B. Arico, A. Prugnola, and R. Rappuoli. 1991. Sequential activation and environmental regulation of virulence genes in *Bordetella pertussis*. EMBO J **10:**3971-3975.

Scheftel, J. M., J. Matis, U. Simeoni, P. Lutz, M. Haubensack, J. Geisert, and R. Minck. 1992. Use of the *Bordetella pertussis* adenylate cyclase assay in the diagnosis of whooping cough in infants and young children. Med Microbiol Lett **1**: 78-84.

Sechi, L. A., S. Zanetti, I. Dupre, G. Delogu, and G. Fadda. 1998. Enterobacterial repetitive intergenic consensus sequences as molecular targets for typing of *Mycobacterium tuberculosis* strains. J Clin Microbiol **36:**128-132.

Selander, R. K., D. A. Caugant, H. Ochman, J. M. Musser, M. N. Gilmour, and T. S. Whittam. 1986. Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. Appl. Environ. Microbiol **51:**873-884.

Shen, Y., Y. S. Lee, S. Soelaiman, P. Bergson, D. Lu, A. Chen, K. Beckingham, Z. Grabarek, M. Mrksich, and W. J. Tang. 2002. Physiological calcium concentrations regulate calmodulin binding and catalysis of adenylyl cyclase exotoxins. EMBO J **21:**6721-6732.

Sloan, L. M., M. K. Hopkins, P. S. Mitchell, E. A. Vetter, J. E. Rosenblatt, W. S. Harmsen, F. R. Cockerill, and R. Patel. 2002. Multiplex LightCycler PCR assay for detection and differentiation of *Bordetella pertussis* and *Bordetella parapertussis* in nasopharyngeal specimens. J Clin Microbiol **40:**96-100.

Stern, M. J., G. F. Ames, N. H. Smith, E. C. Robinson, and C. F. Higgins. 1984. Repetitive extragenic palindromic sequences: a major component of the bacterial genome. Cell **37:**1015-1026.

Stibitz, S. and N. H. Carbonetti. 1994. Hfr mapping of mutations in *Bordetella pertussis* that define a genetic locus involved in virulence gene regulation. J Bacteriol **176:**7260-7266.

Stibitz, S. and M. S. Yang. 1991. Subcellular localization and immunological detection of proteins encoded by the *vir* locus of *Bordetella pertussis*. J Bacteriol **173:**4288-4296.

Stockbauer, K. E., B. Fuchslocher, J. F. Miller, and P. A. Cotter. 2001. Identification and characterization of BipA, a Bordetella Bvg-intermediate phase protein. Mol Microbiol **39:**65-78.

Tang, Y. W., M. K. Hopkins, C. P. Kolbert, P. A. Hartley, P. J. Severance, and D. H. Persing. 1998. *Bordetella holmesii*-like organisms associated with septicemia, endocarditis, and respiratory failure. Clin Infect Dis **26:**389-392.

Tilley, P. A., M. V. Kanchana, I. Knight, J. Blondeau, N. Antonishyn, and H. Deneer. 2000. Detection of *Bordetella pertussis* in a clinical laboratory by culture, polymerase chain reaction, and direct fluorescent antibody staining; accuracy, and cost. Diagn. Microbiol Infect Dis **37:**17-23.

Tozzi, A. E., L. Rava, M. L. Ciofi degli Atti, and S. Salmaso. 2003. Clinical presentation of pertussis in unvaccinated and vaccinated children in the first six years of life. Pediatrics **112:**1069-1075.

Trollfors, B., T. Lagergard, E. Gunnarsson, and J. Taranger. 2003. Determination of pertactin IgG antibodies for the diagnosis of pertussis. Clin Microbiol Infect **9:**585-589.

Tu, X., I. Nisan, J. F. Miller, E. Hanski, and I. Rosenshine. 2001. Construction of mini-Tn5cyaA' and its utilization for the identification of genes encoding surface-exposed and secreted proteins in *Bordetella bronchiseptica*. FEMS Microbiol Lett **205:**119-123.

Urwin, R. and M. C. Maiden. 2003. Multi-locus sequence typing: a tool for global epidemiology. Trends Microbiol **11:**479-487.

van Belkum, A., M. Sluijuter, R. de Groot, H. Verbrugh, and P. W. Hermans. 1996. Novel BOX repeat PCR assay for high-resolution typing of *Streptococcus pneumoniae* strains. J Clin Microbiol **34:**1176-1179.

van Buynder, P. G., D. Owen, J. E. Vurdien, N. J. Andrews, R. C. Matthews, and E. Miller. 1999. *Bordetella pertussis* surveillance in England and Wales: 1995-7. Epidemiol. Infect **123:**403-411.

van den Akker, W. M. 1998. Lipopolysaccharide expression within the genus Bordetella: influence of temperature and phase variation. Microbiology **144:**1527-1535.

van den Berg, B. M., H. Beekhuizen, F. R. Mooi, and R. van Furth. 1999. Role of antibodies against *Bordetella pertussis* virulence factors in adherence of *Bordetella pertussis* and *Bordetella parapertussis* to human bronchial epithelial cells. Infect Immun **67:**1050-1055.

Van der Wielen, M., P. Van Damme, K. Van Herck, S. Schlegel-Haueter, and C. A. Siegrist. 2003. Seroprevalence of *Bordetella pertussis* antibodies in Flanders (Belgium). Vaccine **21:**2412-2417.

van der, Zee. A., C. Agterberg, M. Peeters, J. Schellekens, and F. R. Mooi. 1993. Polymerase chain reaction assay for pertussis: simultaneous detection and discrimination of *Bordetella pertussis* and *Bordetella parapertussis*. J Clin Microbiol **31:** 2134-2140.

van der, Zee. A., C. Agterberg, M. Peeters, F. Mooi, and J. Schellekens. 1996a. A clinical validation of *Bordetella pertussis* and *Bordetella parapertussis* polymerase chain reaction: comparison with culture and serology using samples from patients with suspected whooping cough from a highly immunized population. J Infect Dis **174:**89-96.

van der, Zee. A., S. Vernooij, M. Peeters, J. van Embden, and F. R. Mooi. 1996b. Dynamics of the population structure of *Bordetella pertussis* as measured by IS1002-associated RFLP: comparison of pre- and post-vaccination strains and global distribution. Microbiology **142:**3479-3485.

van der, Zee. A., F. Mooi, J. van Embden, and J. Musser. 1997. Molecular evolution and host adaptation of *Bordetella* spp.: phylogenetic analysis using multilocus enzyme electrophoresis and typing with three insertion sequences. J Bacteriol **179:**6609-6617

van Loo, I. H., K. J. Heuvelman, A. J. King, and F. R. Mooi. 2002. Multilocus sequence typing of *Bordetella pertussis* based on surface protein genes. J Clin Microbiol **40:**1994-2001.

van Loo, I. H., H. G. van der Heide, N. J. Nagelkerke, J. Verhoef, and F. R. Mooi. 1999. Temporal trends in the population structure of *Bordetella pertussis* during 1949-1996 in a highly vaccinated population. J Infect Dis **179:**915-923.

Vandamme, P., M. Heyndrickx, M. Vancanneyt, B. Hoste, P. De Vos, E. Falsen, K. Kersters, and K. H. Hinz. 1996. *Bordetella trematum* sp. nov., isolated from wounds and ear infections in humans, and reassessment of Alcaligenes denitrificans Ruger and Tan 1983. Int J Syst Bacteriol **46:**849-858.

Vandamme, P., J. Hommez, M. Vancanneyt, M. Monsieurs, B. Hoste, B. Cookson, C. H. Wirsing von Konig, K. Kersters, and P. J. Blackall. 1995. *Bordetella hinzii* sp. nov., isolated from poultry and humans. Int J Syst Bacteriol **45:**37-45.

Versalovic, J., T. Koeuth, and J. R. Lupski. 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res. **19:**6823-6831.

von Koenig, C. H. W., A. Tacken, and E. L. Hewlett. 1989. Detection of *Bordetella pertussis* by determination of adenylate cyclase activity. Eur J Clin Microbiol Infect Dis **8:**633-636.

von Wintzingerode, F., A. Schattke, R. A. Siddiqui, U. Rosick, U. B. Gobel, and R. Gross. 2001. *Bordetella petrii* sp. nov., isolated from an anaerobic bioreactor, and emended description of the genus Bordetella. Int J Syst Evol. Microbiol **51:**1257-1265.

Walker, K. E. and A. A. Weiss. 1994. Characterization of the dermonecrotic toxin in members of the genus Bordetella. Infect Immun **62:**3817-3828.

Weber, C., C. Boursaux-Eude, G. Coralie, V. Caro, and N. Guiso. 2001. Polymorphism of *Bordetella pertussis* isolates circulating for the last 10 years in France, where a single effective whole-cell vaccine has been used for more than 30 years. J Clin Microbiol **39:**4396-4403.

Weingart, C. L., G. Broitman-Maduro, G. Dean, S. Newman, M. Peppler, and A. A. Weiss. 1999. Fluorescent labels influence phagocytosis of *Bordetella pertussis* by human neutrophils. Infect Immun **67:**4264-4267.

Weingart, C. L., P. S. Mobberley-Schuman, E. L. Hewlett, M. C. Gray, and A. A. Weiss. 2000. Neutralizing antibodies to adenylate cyclase toxin promote phagocytosis of *Bordetella pertussis* by human neutrophils. Infect Immun **68:**7152-7155.

Weingart, C. L. and A. A. Weiss. 2000. *Bordetella pertussis* virulence factors affect phagocytosis by human neutrophils. Infect Immun **68:**1735-1739.

Weiss, A. A., and S. Falkow. 1982. Plasmid transfer to *Bordetella pertussis*: Conjugation and Transformation. Jnl Bac **152**: 549-552.

Weiss, A. A., F. D. Johnson, and D. L. Burns. 1993. Molecular characterization of an operon required for pertussis toxin secretion. Proc Natl Acad Sci U S A **90**:2970-4.

Weiss, A. A., E. L. Hewlett, G. A. Myers, and S. Falkow. 1983. Tn5-induced mutations affecting virulence factors of *Bordetella pertussis*. Infect Immun **42:**33-41.

Weiss, A. A., E. L. Hewlett, G. A. Myers, and S. Falkow. 1984. Pertussis toxin and extracytoplasmic adenylate cyclase as virulence factors of *Bordetella pertussis*. J Infect Dis **150:**219-222.

Westrop, G. D., G. Campbell, Y. Kazi, B. Billcliffe, J. G. Coote, R. Parton, J. H. Freer, and J. G. Edwards. 1994. A new assay for the invasive adenylate cyclase toxin of *Bordetella pertussis* based on its morphological effects on the fibronectin-stimulated spreading of BHK21 cells. Microbiology **140:**245-253.

Weyant, R. S., D. G. Hollis, R. E. Weaver, M. F. Amin, A. G. Steigerwalt, S. P. O'Connor, A. M. Whitney, M. I. Daneshvar, C. W. Moss, and D. J. Brenner. 1995. *Bordetella holmesii* sp. nov., a new gram-negative species associated with septicemia. J Clin Microbiol **33:**1-7.

WHO. 1990. Requirements for diphtheria, tetanus, pertussis and combined vaccines. WHO Technical Report Series 800, Annex 2: 87-151.

WHO. 1991. Meeting on case definitions of pertussis. Conference proceedings MIN/EPI/PERT91.1, 4-5.

Willems, R. J., J. Kamerbeek, C. A. Geuijen, J. Top, H. Gielen, W. Gaastra, and F. R. Mooi. 1998. The efficacy of a whole cell pertussis vaccine and fimbriae against *Bordetella pertussis* and *Bordetella parapertussis* infections in a respiratory mouse model. Vaccine **16:**410-416.

Willems, R., A. Paul, H. G. van der Heide, A. R. ter Avest, and F. R. Mooi. 1990. Fimbrial phase variation in *Bordetella pertussis*: a novel mechanism for transcriptional regulation. EMBO J **9:**2803-2809.

Wilson, K. E., P. K. Cassiday, T. Popovic, and G. N. Sanden. 2002. *Bordetella pertussis* isolates with a heterogeneous phenotype for erythromycin resistance. J Clin Microbiol **40:**2942-2944.

Winson, M. K., S. Swift, P. J. Hill, C. M. Sims, G. Griesmayr, B. W. Bycroft, P. Williams, and G. S. A. B. Stewart. 1998. Engineering the *luxCDABE* genes from *Photorhabdus luminescens* to provide a bioluminescent reporter for constitutive and promoter probe plasmids and mini-Tn5 constructs. Fems Microbiology Letters **163:**193-202.

Wolff, J., G. H. Cook, A. R. Goldhammer, and S. A. Berkowitz. 1980. Calmodulin activates prokaryotic adenylate cyclase. Proc Natl Acad Sci U S A **77:**3841-3844.

Woolfrey, B. F. and J. A. Moody. 1991. Human infections associated with *Bordetella bronchiseptica*. Clin Microbiol Rev **4:**243-255.

Yih, W. K., Lett SM, des Vignes FN, Garrison KM, Sipe PL, Marchant CD. 2000. The increasing incidence of pertussis in Massachusetts adolescents and adults, 1989-1998. J Infect Dis **182:**1409-16.

Yih, W. K., E. A. Silva, J. Ida, N. Harrington, S. M. Lett, and H. George. 1999. *Bordetella holmesii*-like organisms isolated from Massachusetts patients with pertussis-like symptoms. Emerg Infect Dis **5:**441-443.

Yuk, M. H., E. T. Harvill, P. A. Cotter, and J. F. Miller. 2000. Modulation of host immune responses, induction of apoptosis and inhibition of NF-kappaB activation by the Bordetella type III secretion system. Mol Microbiol **35:**991-1004.

Yuk, M. H., E. T. Harvill, and J. F. Miller. 1998. The BvgAS virulence control system regulates type III secretion in *Bordetella bronchiseptica*. Mol Microbiol **28:**945-959.

Zaretzky, F. R., M. C. Gray, and E. L. Hewlett. 2002. Mechanism of association of adenylate cyclase toxin with the surface of *Bordetella pertussis*: a role for toxin-filamentous haemagglutinin interaction. Mol Microbiol **45:**1589-1598.

Zavaglia, A. G., P. de Urraza, and G. De Antoni. 2000. Characterization of Bifidobacterium strains using box primers. Anaerobe **6:**169-177.

Zu, T., R. Manetti, R. Rappuoli, and V. Scarlato. 1996. Differential binding of BvgA to two classes of virulence genes of *Bordetella pertussis* directs promoter selectivity by RNA polymerase. Molecular Microbiology **21:**557-565.

Appendices

APPENDICES

Appendix 1: Media

1.1 Bordet-Gengou (BG) agar

Bordet-Gengou agar base (Difco)	30 g
1% (v/v) glycerol	3 ml
15% (v/v) defibrinated horse blood (E and O laboratories, Scotland)	150 ml

Made to 1 litre in distilled water, then autoclaved.

1.2 Stainer Scholte (SS) liquid medium

Solution A

L-glutamate	10.72 g
L-proline	0.24 g
Sodium chloride	2.5 g
Potassium di-hydrogen orthophosphate	0.5 g
Potassium chloride	0.2 g
Magnesium chloride.$7H_20$	0.1 g
Calcium chloride	0.02 g
Tris	6.1 g

Made to 1 litre in distilled water. Solution is prepared to pH 7.6 then autoclaved.

Solution B

L-cysteine	0.04 g
Iron sulphate.$7H_20$	0.01 g
Ascorbic acid	0.02 g
Nicotinic acid	0.004 g
Glutathione	0.1 g

Solution B was made to 10 ml in distilled water, sterile-filtered then added to 1 l of solution A.

1.3 Cyclodextrin Liquid (CL) medium

For CL medium, prepared solution A as described above and supplemented with casamino acids (10g) (Difco) and methyl-β-cyclodextrin 0.25 g. Autoclaved and added solution B as above.

1.4 Casamino acids (CAA) solution

Casein hydrolysate	10 g
Magnesium chloride	0.1 g
Calcium chloride	0.016 g
Sodium chloride	5 g

Made to 1 litre in distilled water, pH to 7.1, then autoclaved. Used for preparation of *Bordetella* glycerol stocks.

1.5 Luria Bertrani (LB) broth

Tryptone (Duchefa biochemie, The Netherlands)	10 g
Yeast (Duchefa biochemie, The Netherlands)	5 g
Sodium chloride	10 g

Add 1% (w/v) agar for solid media

Made up to 1 litre in distilled water, then autoclaved.

Appendix 2: DNA solutions

2.1 Loading buffer

20% glycerol	4 ml
5 x TBE (see below)	5 ml

Add bromophenol blue to colour.

Made to 20 ml in sterile filtered distilled water.

2.2 10 x TBE

Tris (Sigma)	108 g
Boric Acid	55 g
0.5 M EDTA pH 8.0	40 ml

Made to 1 litre in distilled water, used 1 x TBE for electrophoresis.

Appendix 3: Urea extraction

3.1 Buffer

100 mM Tricine pH 8	1 ml
0.5 M EDTA	10 µl
0.5 M EGTA	10 µl
8 M urea	5 ml
100mM PMSF(dissolved in ethanol)	100 µl

Made to 10 ml in deionised distilled water.

Appendix 4: Protein analysis solutions

4.1 7.5% acrylamide

30% Acrylamide-Bis (Bio-Rad)	1.25 ml
1M Tris pH 8.8	1.875 ml
10% (w/v) SDS (Fisher Scientific)	50 µl
10% (w/v) APS (Riedel de Haen, distributed by Sigma-Aldrich)	50 µl
TEMED	5 µl

Made to 5 ml in distilled water

4.2 4% stacking gel

30% Acrylamide-Bis	0.67 ml
1M Tris pH 8.8	1.875 ml
10% (w/v) SDS	50 µl
10% (w/v) APS	50 µl
TEMED	5 µl

Made to 5 ml in distilled water.

4.3 2 x loading buffer

Glycerol	8 ml
20% (w/v) SDS	16 ml
2-β-mercaptoethanol	4 ml
0.5 M Tris-HCl pH 6.8	10 ml
0.05% (w/v) bromophenol blue	2 ml

Made to 40 ml with distilled water.

4.4 Coomassie blue stain

Methanol	400 ml
Acetic acid	100 ml
Brilliant blue R (Sigma)	0.5 g

Made to 500 ml with distilled water.

4.5 Destain

Made according to that described for coomassie blue stain without the addition of Brilliant blue R.

4.6 10 x Electrode buffer

Tris	30 g
Glycine	144 g
SDS	10 g

Made to 1 litre with distilled water, used 1 x electrode buffer for electrophoresis.

Appendix 5: Conductimetry solution

5.1 Bicine buffer

100 mM Bicine pH 8	25 ml
100 mM Magnesium acetate	3.75 ml
100mM ATP pH 7	1.25 ml (0.5mM)/ 2.5 ml (1mM)

Made to 250 ml in deionised distilled water.

Appendix 6: UK *B. pertussis* isolates from 1920-2002 listed with their serotypes, genotypes and MLSTs

Isolate	Year	Serotype*	*ptxA**	*ptxC*	*tcfA*	MLST	*prnA**	*bapC*	*vag8*	*brkA*	*ompQ*	*fim2*	*cyaA*	*cyaA pr†*
NCTC10901	1920	1	*ptxA*(2)	*ptxC*(1)	*tcfA*(2)	MLST-2	*prnA*(1)	*bapC*(1)	*vag8*(2)	*brkA*	*ompQ*(2)	*fim2*(1)	*cyaA*(2)	*cyaApr*(2)
CN129	1941		*ptxA*(2)	*ptxC*(1)	*tcfA*(2)	MLST-2	*prnA*(1)							
CN132	1941		*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(1)							
CN134	1941		*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(1)							
CN137	1941	1,2,3	*ptxA*(2)	*ptxC*(1)	*tcfA*(2)	MLST-2	*prnA*(1)	*bapC*(1)	*vag8*(2)	*brkA*	*ompQ*(2)	*fim2*(1)	*cyaA*(2)	*cyaApr*(2)
CN141	1942		*ptxA*(2)	*ptxC*(1)	*tcfA*(5)	MLST-11	*prnA*(1)							
CN351	1942	1,2	*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(1)				*ompQ*(2)	*fim2*(1)	*cyaA*(2)	*cyaApr*(2)
CN741	1943	1	*ptxA*(2)	*ptxC*(1)	*tcfA*(2)	MLST-2	*prnA*(1)				*ompQ*(2)	*fim2*(1)	*cyaA*(2)	*cyaApr*(2)
CN909	1944	1,3	*ptxA*(2)	*ptxC*(1)	*tcfA*(2)	MLST-2	*prnA*(1)				*ompQ*(2)	*fim2*(1)	*cyaA*(2)	*cyaApr*(2)
CN1005	1944		*ptxA*(2)	*ptxC*(1)	*tcfA*(2)	MLST-2	*prnA*(1)							
CN1407	1946	1,2,3	*ptxA*(2)	*ptxC*(1)	*tcfA*(2)	MLST-2	*prnA*(1)				*ompQ*(2)	*fim2*(1)	*cyaA*(2)	*cyaApr*(2)
CN2055	1947	1,2	*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(1)				*ompQ*(2)	*fim2*(1)	*cyaA*(2)	*cyaApr*(2)
CN2420	1948	1,2	*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(1)				*ompQ*(2)	*fim2*(1)	*cyaA*(2)	*cyaApr*(2)
CN2455	1948		*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(1)							
CN2995	1949		*ptxA*(2)	*ptxC*(1)	*tcfA*(2)	MLST-2	*prnA*(1)							
CN2998	1949	1,2	*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(1)				*ompQ*(2)	*fim2*(1)	*cyaA*(2)	*cyaApr*(2)
CN3108	1950	1	*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(1)	*bapC*(1)	*vag8*(2)	*brkA*	*ompQ*(2)	*fim2*(1)	*cyaA*(2)	*cyaApr*(2)
CN3208	1950		*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(1)							
CN3701	1954	1,2	*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(1)				*ompQ*(2)	*fim2*(1)	*cyaA*(2)	*cyaApr*(2)
CN3704	1954		*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(1)							
CN4131	1956		*ptxA*(2)	*ptxC*(1)	*tcfA*(2)	MLST-2	*prnA*(1)							
CN4154	1956	1,3	*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(1)				*ompQ*(2)	*fim2*(1)	*cyaA*(2)	*cyaApr*(2)
CN4161	1956		*ptxA*(2)	*ptxC*(1)	*tcfA*(2)	MLST-2	*prnA*(1)							
CN5420	1963		*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(1)							
CN5447	1963	1,2,3	*ptxA*(2)	*ptxC*(1)	*tcfA*(2)	MLST-2	*prnA*(1)				*ompQ*(2)	*fim2*(1)	*cyaA*(2)	*cyaApr*(2)
CN5631	1964	1,2,3	*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(1)	*bapC*(1)	*vag8*(2)	*brkA*	*ompQ*(2)	*fim2*(1)	*cyaA*(2)	*cyaApr*(2)
NCTC10909	1966	1,0,3,0,0,6	*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(1)				*ompQ*(2)	*fim2*(1)	*cyaA*(2)	*cyaApr*(2)
NCTC10910	1967	1,(2),3,4,0,0	*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(1)				*ompQ*(2)	*fim2*(1)	*cyaA*(2)	*cyaApr*(2)

*Information provided by S Neal and J Duncan, RSIL, Health Protection Agency, London. † *cyaA* promoter region

Isolate	Year	Serotype*	*ptxA**	*ptxC*	*tcfA*	MLST	*prnA**	*bapC*	*vag8*	*brkA*	*ompQ*	*fim2*	*cyaA*	*cyaApr*†
77/19110	1977	1,2,3	*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(1)				*ompQ*(2)	*fim2*(1)	*cyaA*(2)	*cyaApr*(2)
77/24171	1977		*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(1)							
77/25171	1977		*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(1)							
Taberman	1977		*ptxA*(1)	*ptxC*(1)	*tcfA*(4)	MLST-6	*prnA*(1)							
MAN18335	1978	1,2,3	*ptxA*(1)	*ptxC*(1)	*tcfA*(4)	MLST-6	*prnA*(1)	*bapC*(1)	*vag8*(2)	*brkA*	*ompQ*(2)	*fim2*(1)	*cyaA*(2)	*cyaApr*(2)
MAN39424	1978	1,2	*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(1)							
MAN29836	1979	1,3	*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(1)							
MAN8002	1979	1,2	*ptxA*(1)	*ptxC*(1)	*tcfA*(4)	MLST-6	*prnA*(1)				*ompQ*(2)	*fim2*(1)	*cyaA*(2)	*cyaApr*(2)
DCH28	1982		*ptxA*(1)	*ptxC*(1)	*tcfA*(4)	MLST-6	*prnA*(1)							
DCH53	1982	1,3	*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(1)				*ompQ*(2)	*fim2*(1)	*cyaA*(2)	*cyaApr*(2)
DCH63	1982		*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(1)							
DCH64	1982		*ptxA*(1)	*ptxC*(1)	*tcfA*(4)	MLST-6	*prnA*(1)							
DCH88	1982		*ptxA*(1)	*ptxC*(1)	*tcfA*(4)	MLST-6	*prnA*(1)							
DCH89	1982		*ptxA*(1)	*ptxC*(1)	*tcfA*(4)	MLST-6	*prnA*(1)							
DCH131	1983		*ptxA*(1)	*ptxC*(1)	*tcfA*(4)	MLST-6	*prnA*(1)							
DCH132	1983	1,3	*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(2)				*ompQ*(2)	*fim2*(1)	*cyaA*(2)	*cyaApr*(2)
DCH154	1983		*ptxA*(1)	*ptxC*(1)	*tcfA*(6)	MLST-10	*prnA*(1)							
DCH164	1983	1,2	*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(3)	*bapC*(1)	*vag8*(2)	*brkA*	*ompQ*(2)	*fim2*(1)	*cyaA*(2)	*cyaApr*(2)
DCH166	1983		*ptxA*(1)	*ptxC*(1)	*tcfA*(6)	MLST-10	*prnA*(1)							
DCH171	1983		*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(2)							
DCH180	1983		*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(1)							
CN8410	1984	1,2	*ptxA*(2)	*ptxC*(1)	*tcfA*(2)	MLST-2	*prnA*(1)				*ompQ*(2)	*fim2*(1)	*cyaA*(2)	*cyaApr*(2)
DCH182	1984		*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(1)							
CN8486	1985		*ptxA*(2)	*ptxC*(1)	*tcfA*(2)	MLST-2	*prnA*(1)							
CN8540	1985	1,3	*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(1)				*ompQ*(2)	*fim2*(1)	*cyaA*(2)	*cyaApr*(2)
98K290	1998	1,3	*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)							
98K297	1998	1,3	*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)							
98K299	1998	1,3	*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)					*fim2*(1)		
98K300	1998	1,2	*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(1)	*bapC*(1)	*vag8*(2)	*brkA*	*ompQ*(2)	*fim2*(2)	*cyaA*(2)	*cyaApr*(2)
98K301	1998	1,2	*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(1)					*fim2*(2)		

Isolate	Year	Serotype*	*ptxA**	*ptxC*	*tcfA*	MLST	*prnA**	*bapC*	*vag8*	*brkA*	*ompQ*	*fim2*	*cyaA*	*cyaApr*†
98K302	1998	1,2	*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(1)							
98K304	1998	1,2	*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(1)							
98K311	1998	1,3	*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)					*fim2*(1)		
98K314	1998	1,3	*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)							
98K316	1998	1,2	*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(2)				*ompQ*(2)	*fim2*(2)	*cyaA*(2)	*cyaApr*(2)
98K318	1998	1,3	*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)					*fim2*(1)		
98K319	1998											*fim2*(1)		
98K320	1998	1,3	*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-5	*prnA*(2)				*ompQ*(2)	*fim2*(1)	*cyaA*(2)	*cyaApr*(2)
98K321	1998											*fim2*(2)		
98K322	1998	1,2	*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(1)				*ompQ*(2)	*fim2*(2)	*cyaA*(2)	*cyaApr*(2)
98K323	1998	1,2	*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(1)				*ompQ*(2)	*fim2*(2)	*cyaA*(2)	*cyaApr*(2)
98K325	1998											*fim2*(1)		
98K327	1998	1,2	*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(1)					*fim2*(2)		
98K328	1998	1,3	*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(3)				*ompQ*(2)	*fim2*(1)	*cyaA*(2)	*cyaApr*(2)
98K332	1998	1,2	*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(1)					*fim2*(1)		
98K335	1998	1,3	*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)							
98K337	1998	1,3	*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)					*fim2*(1)		
98K340	1998	1,2	*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(1)					*fim2*(2)		
98K351	1998	1,2	*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(1)					*fim2*(2)		
99K1	1999	1,2	*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(1)							
99K2	1999											*fim2*(2)		
99K3	1999	1,2	*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(1)					*fim2*(2)		
99K4	1999	1,3	*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)					*fim2*(1)		
99K6	1999	1,2	*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(1)							
99K8	1999											*fim2*(1)		
99K10	1999	1,2	*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(1)				*ompQ*(2)	*fim2*(2)	*cyaA*(2)	*cyaApr*(2)
99K11	1999	1,3	*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)							
99K13	1999											*fim2*(1)		
99K15	1999	1,3	*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)							
99K16	1999	1,2	*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(1)							
99K20	1999	1,2	*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(1)					*fim2*(2)		
99K22	1999											*fim2*(1)		

Isolate	Year	Serotype*	*ptxA**	*ptxC*	*tcfA*	MLST	*prnA**	*bapC*	*vag8*	*brkA*	*ompQ*	*fim2*	*cyaA*	*cyaApr*†
99K23	1999	1,3	*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(2)				*ompQ*(2)	*fim2*(2)	*cyaA*(2)	*cyaApr*(2)
99K25	1999	1,3	*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)					*fim2*(1)		
99K30	1999	1,2	*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(1)					*fim2*(2)		
99K31	1999											*fim2*(2)		
99K34	1999	1,2	*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(1)							
99K38	1999	1,2	*ptxA*(1)	*ptxC*(1)	*tcfA*(4)	MLST-6	*prnA*(1)							
99K39	1999	1,3	*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)				*ompQ*(2)	*fim2*(1)	*cyaA*(2)	*cyaApr*(2)
99K42	1999	1,3	*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)					*fim2*(1)		
99K44	1999	1,2	*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(1)					*fim2*(2)		
99K45	1999	1,3	*ptxA*(1)	*ptxC*(1)	*tcfA*(3)	MLST-4	*prnA*(3)	*bapC*(1)	*vag8*(2)	*brkA*	*ompQ*(2)	*fim2*(1)	*cyaA*(2)	*cyaApr*(3)
99K46	1999	1,2	*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(2)				*ompQ*(2)	*fim2*(2)	*cyaA*(2)	*cyaApr*(2)
99K48	1999											*fim2*(2)		
99K50	1999											*fim2*(2)		
99K51	1999	1,2	*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(1)							
99K55	1999											*fim2*(1)		
99K58	1999	1,3	*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)					*fim2*(1)		
99K62	1999		*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)				*ompQ*(2)	*fim2*(1)	*cyaA*(2)	*cyaApr*(2)
99K71	1999	1,3	*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)					*fim2*(1)		
99K72	1999		*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)					*fim2*(1)		
99K78	1999	1,3	*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)							
99K79	1999	1,3	*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(2)				*ompQ*(2)	*fim2*(1)	*cyaA*(2)	*cyaApr*(2)
PICU278	1999		*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)							
PICU475	1999		*ptxA*(1)	*ptxC*(1)	*tcfA*(3)	MLST-4	*prnA*(2)	*bapC*(1)	*vag8*(2)	*brkA*	*ompQ*(2)	*fim2*(1)	*cyaA*(2)	*cyaApr*(3)
PICU519	1999		*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)							*cyaApr*(2)
PICU522	1999													*cyaApr*(2)
PICU523	1999		*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)							*cyaApr*(2)
PICU524	1999													*cyaApr*(2)
BP436	2002	1,3	*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)							
BP442	2002	1,3	*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)							
BP463	2002	1,3	*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)							
BP568	2002	1,3	*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)							
BP584	2002	1,3	*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)							

Isolate	Year	Serotype*	*ptxA**	*ptxC*	*tcfA*	MLST	*prnA**	*bapC*	*vag8*	*brkA*	*ompQ*	*fim2*	*cyaA*	*cyaApr*†
BP602	2002	1,3	*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)							
BP609	2002	1,2	*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(1)							
BP610	2002	1,3	*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)							
BP618	2002	1,3	*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)							
BP632	2002	1,3	*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(3)							
BP637	2002	1,3	*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)							
BP650	2002	1,3	*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)							
BP706	2002	1,3	*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)					*fim2*(1)		
BP707	2002	1,3	*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)				*ompQ*(2)	*fim2*(1)	*cyaA*(2)	*cyaApr*(2)
BP710	2002	1,3	*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)				*ompQ*(2)	*fim2*(1)	*cyaA*(2)	*cyaApr*(2)
BP711	2002	1,3	*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)				*ompQ*(2)	*fim2*(1)	*cyaA*(2)	*cyaApr*(2)
BP831	2002	1,3	*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)				*ompQ*(2)	*fim2*(1)	*cyaA*(2)	*cyaApr*(2)
BP842	2002	1,3	*ptxA(1)*	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)							
BP849	2002	1,3	*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)					*fim2*(1)		
BP865	2002	1,3	*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)					*fim2*(1)		
BP873	2002	1,3	*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)					*fim2*(1)		
BP874	2002	1,3	*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)					*fim2*(1)		
BP1213	2002	1,3	*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)					*fim2*(1)		
BP1237	2002	1,3	*ptxA*(1)				*prnA*(2)					*fim2*(1)		
BP1240	2002	1,3	*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)							
BP1248	2002	1,3	*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)							
BP1251	2002	1,3	*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)							
BP1263	2002	1,3	*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)					*fim2*(1)		
BP1265	2002	1,3	*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)							
BP1266	2002	1,3	*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)							
BP1337	2002	1,3		*ptxC*(2)	*tcfA*(2)		*prnA*(2)							
BP1348	2002	1,3	*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5								
BP1373	2002	1,3	*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)							
BP1374	2002	1,3		*ptxC*(2)	*tcfA*(2)		*prnA*(2)							
BP1450	2002	1,3	*ptxA*(1)	*ptxC*(2)	*tcfA*(2)	MLST-5	*prnA*(2)							

Appendix 7: Background information on UK *B. pertussis* isolates from 1920-2002

Isolate	Patient data where known			
	Origin	Date of birth	Age	Vaccination status
NCTC10901	St Mary's Hospital, London			
CN129	Dr IE MacLean, St Mary's Hospital, London			
CN132	Dr Cruickshank, NW Hospital Group Lab, Hampstead			
CN134	Dr Cruickshank, NW Hospital Group Lab, Hampstead			
CN137	Dr Cruickshank, NW Hospital Group Lab, Hampstead			
CN141	Dr Cruickshank, NW Hospital Group Lab, Hampstead			
CN351	Dr Wright, NE Group Hospitals Lab			
CN741	Dr Ungar, Glaxo Ltd			
CN909	Prof GS Wilson, Oxford			
CN1005	E Topley EPHLS, Oxford			
CN1407	E Topley, EPHLS, Oxford			
CN2055	London School of Hygiene and Tropical Medicine			
CN2420	Dr Standfast, Lister Institute			
CN2455	Chemotherpay Dept, WRL			
CN2995	Dr Holt, CPHL, Colindale, London			
CN2998	Dr Holt, CPHL, Colindale, London			
CN3108	Dr Standfast, Lister Institute, Elstree			
CN3208	Dr Standfast, Lister Institute, Elstree			
CN3701	Prof CL Oakley, Medical School, Leeds			
CN3704	Prof CL Oakley, Medical School, Leeds			
CN4131	Lister Institute			
CN4154	Dr G Turner, School of Medicine, Leeds			
CN4161	Dr Standfast, Lister Institute			
CN5420	Hither Green Hospital via Mr G Turner, WRL			
CN5447	Hither Green Hospital via Mr G Turner, WRL			

PHL-Public Health Laboratory; CPHL-Central Public Health Laboratory

Isolate	Patient data where known			
	Origin	Date of birth	Age	Vaccination status
CN5631	Dr NW Preston			
NCTC10909	PHL, Manchester			
NCTC10910	PHL, Coventry			
77/19110	Prof GT Stewart, Ruchill Hospital, Glasgow			
77/24171	Prof GT Stewart, Ruchill Hospital, Glasgow			
77/25171	Prof GT Stewart, Ruchill Hospital, Glasgow			
Taberman	Prof GT Stewart, Ruchill Hospital, Glasgow			
MAN18335				
MAN39424				
MAN29836				
MAN8002				
DCH28				
DCH53				
DCH63				
DCH64				
DCH88				
DCH89				
DCH131				
DCH132				
DCH154				
DCH164				
DCH166				
DCH171				
DCH180				
DCH182				
CN8410				
CN8486				
CN8540				
98K290	Frimley Park Hospital, South Thames	20/01/92	5÷ years	Vaccinated
98K297	Alder Hey County Hospital	23/08/97	1-4 years	
98K299	Good Hope General Hospital, West Midlands	07/05/98	2-5 months	Vaccinated
98K300	Wrexham Maelor General Hospital, Wales	26/06/98	2-5months	Not vaccinated
98K301	Alder Hey County Hospital	15/07/98	2-5months	Vaccinated

Isolate	Patient data where known			
	Origin	Date of birth	Age	Vaccination status
98K302	Manchester Booth Hall Hospital	23/06/98	2-5months	Vaccinated
98K304	Conquest Hospital, South Thames	31/10/94	1-4 years	Vaccinated
98K311	Alder Hey County Hospital	14/01/98	6-11 months	Not vaccinated
98K314	Londonderry Altnaglevin Hospital	27/03/98	6-11 months	
98K316	Truro Public Health Lab	12/08/98	2-5 months	Not vaccinated
98K318	Manchester Trafford General Hospital	23/03/94	1-4 years	Vaccinated
98K319	Leeds Public Health Lab	27/11/97	6-11 months	Not vaccinated
98K320	Alder Hey County Hospital	21/01/95	1-4 years	Not known
98K321	South Tyneside District Hospital	09/02/93	5+ years	Vaccinated
98K322	Aberystwyth Bronglais Hospital, Wales	07/02/69	5+ years	Vaccinated
98K323	Aberystwyth Bronglais Hospital, Wales	25/02/97	1-4 years	Vaccinated
98K325	Oxford Public Health Lab	26/03/93	5+ years	Vaccinated
98K327	Portsmouth Public Health Lab	22/05/98	2-5 months	Vaccinated
98K328	Salisbury Public Health Lab	06/08/98	2-5 months	Vaccinated
98K332	Bristol Public Health Lab	03/10/98	<2 months	Not vaccinated
98K335	Poole Public Health Lab	24/07/98	2-5 months	Vaccinated
98K337	Manchester Booth Hall Hospital	08/09/98	2-5 months	Not vaccinated
98K340	Whiston Hospital, West Midlands	23/10/98	<2 months	Not vaccinated
98K351	Bristol Southmead Hospital	27/10/98	<2 months	Not vaccinated
99K1	Cardiff Public Health Lab	16/10/98	<2 months	Not vaccinated
99K2	Truro Public Health Lab	10/11/98	<2 months	Not vaccinated
99K3	Leeds Public Health Lab	28/11/92	5+ years	Vaccinated
99K4	Nottingham Public Health Lab	11/10/98	2-5 months	Vaccinated
99K6	Leeds General Infirmary	09/10/98	2-5 months	Not vaccinated
99K8	Merthyr Tydfil Prince Charles Hospital, Wales	06/07/87	5+ years	Not vaccinated
99K10	Southampton Public Health Lab	27/10/98	<2 months	Not vaccinated
99K11	Dumfries Royal Infirmary			
99K13	Chester Public Health Lab	30/10/98	<2 months	Not vaccinated
99K15	Altnagelvin area	01/11/98	<2 months	
99K16	Altnagelvin area	09/07/98	2-5 months	
99K20	Manchester Booth Hall Hospital	08/12/93	5+ years	Vaccinated
99K22	Crawley Hospital, London	16/11/98	2-5 months	

Isolate	Patient data where known			
	Origin	Date of birth	Age	Vaccination status
99K23	Poole Public Health Lab	25/12/98	<2 months	Not vaccinated
99K25	Gloucester Public Health Lab	11/02/94	1-4 years	Vaccinated
99K30	Lancaster Royal Infirmary	22/10/88	5+ years	Not vaccinated
99K31	Carlisle Public Health Lab	12/09/98	2-5 months	Vaccinated
99K34	Bristol Public Health Lab	24/11/98	<2 months	Not vaccinated
99K38	Cambridge Public Health Lab	24/12/98	<2 months	Not vaccinated
99K39	Furness General Hospital	26/10/90	5+ years	Vaccinated
99K42	Exeter Public Health Lab	14/11/98	2-5 months	Vaccinated
99K44	Portsmouth Public Health Lab	27/09/98	2-5 months	Vaccinated
99K45	Alder Hey County Hospital	08/01/99	<2 months	Not vaccinated
99K46	Manchester Booth Hall Hospital	28/01/99	<2 months	Not vaccinated
99K48	Epsom Public Health Lab, London	12/02/68	5+ years	Vaccinated
99K50	Nottingham Public Health Lab	19/12/98	<2 months	Not vaccinated
99K51	Alder Hey County Hospital	30/09/98	2-5 months	Vaccinated
99K55	Bishops Stortford Herts and Essex Hospital	29/01/99	<2 months	Not vaccinated
99K58	Norwich Public Health Lab	29/11/98	2-5 months	Vaccinated
99K62	Gloucester Public Health Lab	28/02/91	5+ years	Not vaccinated
99K71	Swindon Princess Margaret Hospital	21/02/96	1-4 years	Not vaccinated
99K72	Swindon Princess Margaret Hospital	10/01/99	2-5 months	Vaccinated
99K78	Furness General Hospital	05/02/99	<2 months	Not vaccinated
99K79	Nottingham Public Health Lab	23/12/53	5+ years	
PICU278	RSIL	22/04/99	2-5 months	
PICU475	RSIL	30/04/99	6-11 months	
PICU519	RSIL	17/05/64	5+ years	
PICU522	RSIL	01/07/99	2-5 months	
PICU523	RSIL	01/07/88	5+ years	
PICU524	RSIL	07/02/97	1-4 years	
BP436	Glamorgan Royal Hospital			
BP442	Farnborough Public Health Lab		2-5 months	
BP463	Nobles Isle of Man Hospital	15/02/95	5+ years	
BP568	Whittington Hospital	13/08/02		
BP584	Guernsey Princess Elizabeth Hospital	13/05/64	5+ years	

Isolate	Patient data where known			
	Origin	Date of birth	Age	Vaccination status
BP602	Whittington Hospital	15/03/02		
BP609	Stoke on Trent Public Health Lab	05/03/02		
BP610	Grantham Hospital	02/03/02		
BP618	RSIL			
BP632	Exeter Public Health Lab	23/01/02		
BP637	Birmingham Public Health Lab	14/03/02		
BP650	Withybush via CDSC			
BP706	Nottingham Public Health Lab	10/07/89	5+ years	
BP707	Nottingham Public Health Lab	10/12/93	5+ years	
BP710	Nottingham Public Health Lab	01/02/59	5+ years	
BP711	Nottingham Public Health Lab	06/07/95	5+ years	
BP831	Luton			
BP842	Luton			
BP849	Hereford			
BP865	Birmingham			
BP873	John Radcliffe			
BP874	John Radcliffe			
BP1213	Booth Hall			
BP1237	Bristol Royal Infirmary			
BP1240	Torbay			
BP1248	Bristol Royal Infirmary			
BP1251	Preston			
BP1263	Plymouth			
BP1265	James Paget			
BP1266	Portsmouth			
BP1337	Clatterbridge			
BP1348	Torbay			
BP1373	Stoke			
BP1374	Bristol Royal Infirmary			
BP1450	Bristol			

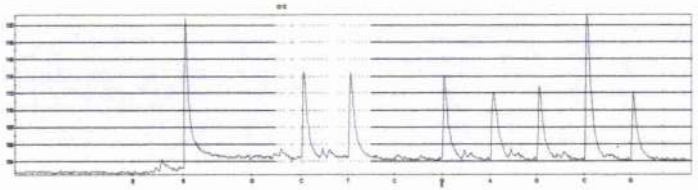
Appendix 8: UK vaccine strains and the type strain (18323^T) with their serotype, genotype and MLST information

Isolate	Vaccine	Serotype*	*ptxA**	*ptxC*	*tcfA*	MLST	*prnA**	*bapC*	*vag8*	*brkA*	*ompQ*	*fim2*	*cyaA*	*cyaApr*†
CN2992	WCV	1,2,3	*ptxA*(2)	*ptxC*(1)	*tcfA*(2)	MLST-2	*prnA*(1)	*bap5*(1)	*vag8*(2)	*brkA*	*ompQ*(2)	*fim2*(1)	*cyaA*(2)	*cyaApr*(2)
CN3099	WCV	1,2	*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(1)	*bap5*(1)	*vag8*(2)	*brkA*	*ompQ*(2)	*fim2*(1)	*cyaA*(2)	*cyaApr*(2)
CN5476	WCV	1,3	*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	MLST-3	*prnA*(1)	*bap5*(1)	*vag8*(2)	*brkA*	*ompQ*(2)	*fim2*(1)	*cyaA*(2)	*cyaApr*(2)
Tohama-I	ACV	1,2	*ptxA*(2)	*ptxC*(1)	*tcfA*(2)	MLST-2	*prnA*(1)	*bap5*(1)	*vag8*(2)	*brkA*	*ompQ*(2)	*fim2*(1)	*cyaA*(2)	*cyaApr*(2)
18323^T		1	*ptxA*(4)	*ptxC*(1)	*tcfA*(1)	MLST-9	*prnA*(6)	*bap5*(2)	*vag8*(1)	*brkA*	*ompQ*(1)	*fim2*(1)	*cyaA*(1)	*cyaApr*(1)

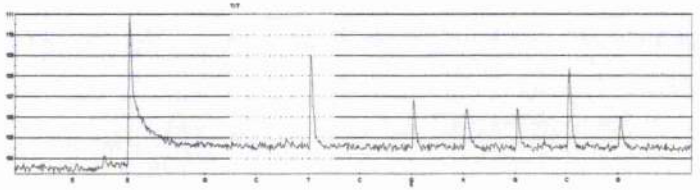
*Information provided by S Neal and J Duncan, RSIL, Health Protection Agency, London. † *cyaA* promoter region

Appendix 9: Pyrograms of *ptxC*(1), *ptxC*(2), *fim2*(1), *fim2*(2), *ompQ*(1) and *ompQ*(2) alleles. The base highlighted in red shows the single nucleotide polymorphism.

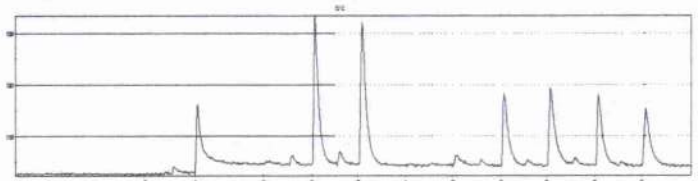
***ptxC*(1) allele**



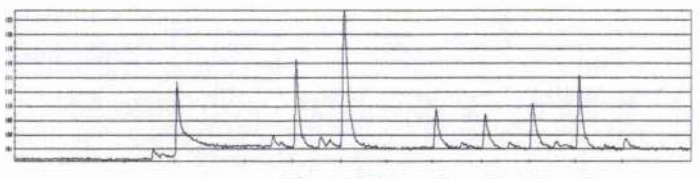
***ptxC*(2) allele**



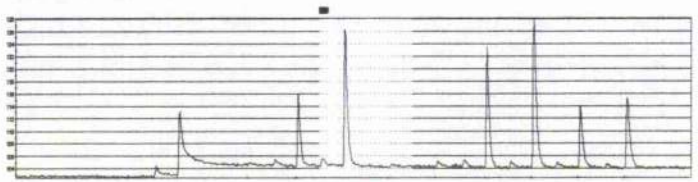
***fim2*(1) allele**



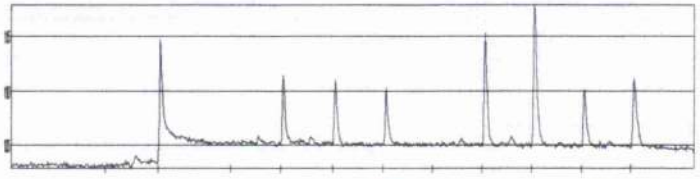
***fim2*(2) allele**



***ompQ*(1) allele**



***ompQ*(2) allele**



Appendix 10: Published work arising from this study.

1. **E. Packard, R. Parton, J. G. Coote and N. K. Fry**. 2002. *Bordetella pertussis* adenylate cyclase toxin: a potential epidemiological marker and diagnostic tool for pertussis infection. Clin Microbiol Infect 8: 289.

2. **Packard E. R., R. Parton, J. G. Coote, and N. K. Fry**. 2004. Sequence variation and conservation in virulence-related genes of *Bordetella pertussis* isolates from the Uk. J Med Microbiol. 53: 355-365.