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Characterisation and secretion mechanism of *Bordetella pertussis* autotransporter proteins

by

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Abbreviations

ACV	Acellular vaccine
AL	Alhydrogel
ATP	Adenosine triphosphate

BLAST	Basic local alignment
BG	Bordet-Gengou
BrkA	*Bordetella* resistance to killing A
BSA	Bovine serum albumin
Bvg	*Bordetella* virulence gene
CAA	Casamino acids
CL	Cyclodextrin liquid
Da	Dalton
DNA	Deoxyribonucleic acid
DNT	Dermonecrotic toxin
FHA	Filamentous haemagglutinin
GSP	General secretory pathway
h	Hour
HLT	Heat-labile toxin
IPTG	Isopropyl ß-D-thiogalactopyranoside
kDa	KiloDalton
Kpsi	Kilo-pounds per square inch
KV	KiloVolt
l	Litre
LB	Luria Bertani
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
M	Molar
mM	Millimolar
min	Minute
ml	Millilitres
μl	Microlitres
MOPS	Morpholinopropane-sulphonic acid solution
Mw	Molecular weight
Ni-NTA	Nickel-nitrilotriacetic acid
nM	Nanomolar

°C	Degrees Celsius
OMP	Outer-membrane protein
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PRN	Pertactin
PTX	Pertussis toxin
P.30	30 KDa processed form of pertactin
P.69	69 KDa processed form of pertactin
P.93	93 KDa unprocessed form of pertactin
RGD	Arginine-Glycine-Aspartic acid
RNA	Ribonucleic acid
RT-PCR	Reverse transcription-polymerase chain reaction
s	Seconds
SDS	Sodium dodecyl sulphate
Sec	Secretory
TCF	Tracheal colonisation factor
TCT	Tracheal cytotoxin
Vag	Virulence-activated gene
Vag8	Virulence-activated gene 8
Vrg	Virulence-repressed gene
WCV	Whole-cell vaccine
g	Gravity
X-GAL	5-bromo-4 chloro-3-indolyl-ß-D-galactosidase

Abstract

The identification and characterisation of new virulence determinants of *B. pertussis* is providing important information for understanding the colonisation and survival strategies of the microorganism. *B. pertussis* deploys a range of surface-associated components to enable its successful colonisation of the host. Bap-5 has been identified as a new member of the *B. pertussis* autotransporter family of proteins that includes PRN, BrkA, TCF and Vag-8, largely due to its homology at the C-terminus and some other similar regions such as the RGD (integrin-binding) and SGXG (glycosaminoglycan-binding) motifs. The *bap-5* gene also exists in *B. bronchiseptica* and *B. parapertussis*. Characteristic upstream regulatory sequences such as a ribosome-binding site were not seen in *bap-5*, but a potential heptameric BvgA-binding motif was identified. The expression of Bap-5 was confirmed by RT-PCR and Western blotting and was shown to be *bvg* dependent. Although Bap-5 does not possess a typical signal sequence like pertactin (PRN), its surface localisation was confirmed by agglutination and immunofluorescence assays.

A potential role for Bap-5 in infection was studied by generating Bap-5 deficient mutants in two strains of *B. pertussis*. An allelic exchange procedure with the suicide vector pSS1129 carrying the *bap-5* gene disrupted with a kanamycin-resistance cassette was used. PCR and Southern blotting confirmed the replacement of the wild-type *bap-5* gene with the mutated version. Moreover, SDS-PAGE and Western blotting of outer-membrane preparations of *B. pertussis* Taberman wild-type and its Bap-5-deficient mutant showed a clear difference in their outer-membrane profile at ~79.9kDa presumably

representing the unprocessed form and bands at ~65 kDa and ~16 kDa may represent the processed forms of the protein.

The Bap-5 characterisation studies showed that the Taberman Bap-5-deficient strain was less able than the parent strain to colonise the lower respiratory tract of mice and adhesion studies (*in vitro*) showed that the Taberman parent was better in adhering to certain cell types than the Bap-5-deficient mutant. The study also showed that a Bap-5-deficient mutant was less able to resist complement-mediated killing by normal human serum. A rabbit antibody to Bap-5 had the ability to potentiate bacterial killing by complement. Thus, Bap-5 appears to have properties in common with other members of the *B. pertussis* autotransporter protein family such as PRN and BrkA.

The second part of the project dealt with the processing and secretion of PRN in a range of protease-deficient strains of *E. coli* to determine the involvement of particular protease(s) in the processing and secretion of PRN and other autotransporter proteins. The results showed that the processing and release of PRN does not appear to rely on a specific protease. However, secretion was defective in the most protease-deficient *E. coli* strain lacking several periplasmic and OM-located proteases.

The ability of the recombinant detergent-extracted and re-natured C-terminal domain (P.30) of PRN and of the native mature domain (P.69) to function as protective immunogens was compared. The study showed that the P.30 protein did not have any protective effect, under the conditions tested, against *B. pertussis* colonisation of the respiratory tract of mice whereas the P.69 protein proved to be an excellent protective antigen, in agreement with previously reported data.

1. Introduction

1.1 The genus *Bordetella*

The genus *Bordetella* is comprised of small, aerobic (with the exception of the most recently identified species *B. petrii*), non spore forming, Gram-negative minute cocco-bacilli (0.3-0.5 μm x 0.5-2.0 μm). Members of the genus *Bordetella* are pathogenic bacteria that can cause respiratory infections in mammalian as well as avian species. The genus was named in honour of Jules Bordet, who identified the organism initially from a patient with whooping cough in 1906. The GC content of these bacteria is in the range of 61 and 68 mol% (Gerlach *et al.*, 2001) and their optimal growth temperature is 35°-37°C (Parton, 1998).

1.2 Bordetellae and their close relatives

Bordetella species are closely related to bacteria classified in the genera *Alcaligenes* and *Achromobacter*. Phenotypic differentiation between these genera is virtually impossible and has caused several misclassifications in the past (Busse and Auling, 1992; Vandamme *et al.*, 1996). However, genotypically all three genera are clearly distinct by comparative 16S RNA sequence analysis, amplified ribosomal DNA restriction analysis and DNA-RNA hybridisation studies (De Ley *et al.*, 1986; Vandamme *et al.*, 1996). These studies also indicated that *Bordetella* species are more closely related to *Achromobacter* species, due to the high GC content in their DNA, than to members of the genus *Alcaligenes*. *Achromobacter* species are environmental organisms and some of them are facultative pathogens, which may cause uncommon but serious nosocomial epidemics (Granowitz and Keenholtz, 1998). Due to the close relationship of *Bordetella* spp to some environmental bacteria, it is likely that the pathogenic species have evolved from environmental relatives and have acquired relevant virulence traits to become successful and obligate colonisers and pathogens of mammals and birds.

1.3 Species of *Bordetella*

The following species have been identified and characterised in the genus *Bordetella.*

1.3.1 Classical species

These are *B. pertussis*, *B. parapertussis* and *B. bronchiseptica.* They agglutinate erythrocytes from a variety of mammals and appear to be obligate mammalian pathogens. Humans are the only hosts for *B. pertussis*, whereas humans and sheep are hosts of *B. parapertussis* and wild and domesticated animals are the hosts of *B. bronchiseptica.*

B. pertussis

Due to the requirement for blood in laboratory media, *B. pertussis* was first classified as *Haemophilus pertussis* (Hornibrook, 1940). In 1952, after several changes of its name, it was classified in its own genus. It causes the highly infectious and acute childhood disease known as whooping cough (pertussis) with severe clinical manifestations (section 1.5) (Hewlett, 1995).

B. parapertussis

B. parapertussis is responsible for a mild form of whooping cough in humans (Khelef *et al.*, 1993). It has been also isolated from healthy and pneumonic sheep. Several surveys indicated that between 5-25% of pertussis-like cases in some countries in Europe may be caused by this pathogen (Mertsola, 1985).

B. bronchiseptica

This organism was first isolated by Ferry (1910) from the respiratory tract of dogs and is a cause of infection in a wide range of domestic as well as wild animals and birds

(Charles *et al.*, 1994). It is the cause of kennel cough in dogs, atrophic rhinitis in swine and snuffles in rabbits. It appears to be more of a respiratory tract commensal and opportunist pathogen, sometimes associated with cases of septicaemia in immnuo-compromised patients (Woolfrey and Moody, 1991; Parton, 1999).

The genetic diversity between these three *Bordetella* species is quite limited and all three cause upper respiratory tract disease which involves the interaction of the bacteria with ciliated tracheal epithelial cells, resulting in ciliostasis and killing of the ciliated cells (Gerlach *et al.*, 2001).

1.3.2 Recently described species

B. avium

B. avium associates with ciliated tracheal epithelium and is a causative agent of infections of upper respiratory tract. However, the pathogenic potential of *B. avium* appears to be confined to birds, causing avian bordetellosis and turkey coryza (Skeeles *et al.*, 1988).

B. hinzii

B. hinzii is the name given to a *B. avium*-like group of organisms isolated from the respiratory tracts of turkeys and chickens (Vandamme *et al.*, 1995). It was reported as the causative agent of a fatal septicaemia in man and other cases indicate that *B. hinzii* may cause disease in humans under certain conditions e.g. in HIV and cystic fibrosis patients (Cookson *et al.*, 1994; Funke *et al.*, 1996; Kattar *et al.*, 2000).

B. holmesii

B. holmesii belongs to a group of isolates originally identified from human blood

cultures (Weyant *et al.*, 1995). It is also isolated from sputum and, with increasing incidence, from nasopharyngeal specimens of otherwise healthy young people with a cough (Mazengia *et al.*, 2000). As for *B. hinzii*, the pathogenic potential of *B. holmesii* is not clear. Both *B. holmesii* and *B. hinzii* appear to be opportunists in man and have been isolated mainly from compromised patients. No alternative host other than man has been reported for *B. holmesii* (Parton, 1999).

B. trematum

The seventh member of this family is *B. trematum* which was isolated from wounds and infected ears in humans but not from the respiratory tract (Vandamme *et al.*, 1996). It was originally described as atypical *Bordetella* or unidentified organism. At present the pathogenic potential of this species is not clear.

B. petrii

Recently, a bacterium designated as strain SE1111R, was proposed to represent a novel *Bordetella* species, *Bordetella petrii* spp. nov. It was isolated from an anaerobic dechlorinating bioreactor consortium enriched from river sediment (Wintzingerode *et al.*, 2001). Although it represents the first type of *Bordetella* having an environmental origin and being an anaerobic organism, detailed analysis of chemotaxonomic, biochemical and genetic characters e.g. by comparative 16S-rDNA analysis have classified it as a new member of the genus *Bordetella* (Wintzingerode *et al.*, 2001).

Studies on the relatedness of *B. pertussis, B. parapertussis* and *B. bronchiseptica* by multilocus enzyme electrophoresis, nucleic acid hybridisation analyses and other comparisons have suggested that they are in fact subtypes of a single genomic species, whereas *B. avium, B. holmesii, B. hinzii, B. trematum* and perhaps *B. petrii* each form true

genomic species (Vandamme *et al.*, 1995, 1996; Weyant *et al.*, 1995; Wintzingerode *et al.*, 2001).

1.4 Pertussis and its causative agent

1.4.1 Epidemiology of pertussis

Bordetella pertussis is the causative agent of whooping cough and is responsible for one of the ten most common causes of death from infectious diseases. The World Health Organisation (WHO:http://www.who.int/vaccines-diseases/diseases/pertussisvaccine.html) has reported 50 million cases and 350,000 deaths world-wide per year, mainly among unvaccinated children in third world countries. In the USA, where there is a high vaccine uptake, 9500 cases were reported in 1994-95. In the UK, 2390 cases were reported in 1996, despite a vaccine uptake of 95 % (Miller *et al.*, 1992). However, it is widely believed that the actual number of cases is much higher. The organism is highly communicable with attack rates of 50-100 % depending upon the intensity of exposure. It is believed that transmission of *B. pertussis* occurs via aerosol droplets, expelled by severe coughing from the respiratory tract of infected individuals to the susceptible hosts. It has been shown that whether immunisation is practised or not, the disease is endemic. In most countries an epidemic occurs approximately every 3-4 years, possibly due to an increasing number of susceptible individuals in the population reaching a threshold level. The organism is not thought to survive in the environment for a prolonged period, and so transmission must occur from one individual to another. Recent evidence suggested that adults and adolescents even when they have mild or unrecognised disease constitute an important reservoir of infection (Black, 1997; Cherry, 1999). Physicians and researchers have known for decades that the pathogen destroys the ciliated cells in the epithelial lining

of the respiratory tract. The hair-like cilia sweep away mucus but, when they die, coughing provides the only way to clear the airway. The result likely contributes to the intense gasping cough that not only gives the disease its name but also spreads the bacterium to other victims (Strauss, 1999).

1.4.2 Clinical disease

Early in this century, Mallory and Hornor characterised pertussis infection as a non-invasive bacterial colonisation of the ciliated cells in the respiratory epithelium. The bacterium damages the epithelium and, more recently, this has been confirmed by nasal biopsies taken from children with whooping cough. The consequent impairment of mucociliary transport is thought to be responsible for the paroxysmal coughing and choking which characterises the illness (Soane *et al.*, 2000).

Pertussis is characterised by three phases. Following exposure in a susceptible individual, there is an incubation period of 5 to 7 days prior to the development of symptoms. Non-specific symptoms, similar to those of the common cold, lasting approximately 7 days, constitute the catarrhal phase, during which the isolation rate of the organism may be as high as 90 %. This gradually becomes the paroxysmal phase as the dry non-productive cough becomes a paroxysmal cough with mucus secretion and vomiting (Wardlaw and Parton, 1988). Coughing is an attempt to clear the respiratory secretions, and is followed by inspiration against the still-narrowed glottis to produce the characteristic whoop, from which the alternative name "whooping cough" is derived. Other characteristic signs during this phase include leukocytosis, lymphocytosis, weight loss and occasional hypoglycaemia. The recovery rate of the organism is much reduced by the time of the paroxysmal phase. Since the organism is only rarely isolated from the blood of a patient, it has been suggested that most of the clinical findings may be due to

toxin release and dissemination. The paroxysmal phase lasts at least 2 weeks. The third stage of the disease is the convalescent stage which can last for weeks or even months and eventually leads to a gradual disappearance of the severe symptoms (Gerlach *et al.*, 2001). A high percentage of all reported cases of pertussis require hospitalisation, especially in those infants around 6 months of age. The case fatality rate due to pertussis has been estimated as up to 0.2 % in USA and the majority (84 %) of these deaths were in infants aged about 6 months (Cherry *et al.*, 1988, 1996; Hewlett, 2000). The impairment of the respiratory tract defences also makes the individual more susceptible to secondary infections with other bacteria. Secondary infections such as otitis media and pneumonia are relatively frequent, especially in infants and may result from impairment of clearance mechanisms by the bacterium. Central nervous disturbances, in the form of seizures and encephalopathy may also occur and are thought to be due to hypoxia or possibly to bacterial toxins. There is evidence that *B. pertussis* inhibits the immune responses within the respiratory tract. Recent studies have suggested that *B. pertussis* has the capability of inducing mucin gene expression in infected bronchial tissues, to which *B. pertussis* can bind and colonise the host effectively and thereby subvert the host innate defences (Bechler *et al.*, 2000).

1.4.3 Diagnosis

Clinical diagnosis of pertussis is often possible due to the characteristic and prolonged paroxysmal coughing in the disease. However clinical features tend to vary with age and immune status. A pronounced leukocytosis is also suggestive of pertussis. The WHO definition of pertussis requires a minimum of 21 days of paroxysmal coughing with laboratory confirmation or epidemiological linkage (WHO, 1991). Laboratory methods include direct detection of *B. pertussis* by culture, direct fluorescent antibody test

(DFA test) and PCR methods, or indirect diagnosis by serology (Muller *et al.*, 1997; Hallander, 1999; Kerr and Mathews, 2000).

1.4.4 Antimicrobial therapy

Several antibiotics including erythromycin, tetracycline, chloramphenicol, trimethoprim-sulpamethoxazole are effective but erythromycin is usually considered a better choice for treating pertussis patients (Hoppe, 1992). Antibiotic treatment in the early (catarrhal or early paroxysmal) stages proves to be more effective in ameliorating the symptoms of pertussis (Cherry *et al.*, 1988).

1.4.5 Virulence factors

Bordetella species produce a variety of surface-associated and secreted molecules (Table 1.1). These products are presumed to be involved in adhesion to eukaryotic tissues, evasion of host defences and toxicity towards the host. Some of these secreted products, including pertussis toxin, adenylate cyclase toxin, pertactin and filamentous haemagglutinin have been most extensively studied in *B. pertussis*. However the fastidious nature of this species in the laboratory in some cases has led to use of *E. coli* recombinant strains and *Salmonella* spp. for their expression and investigating their role in virulence (Shareck and Cameron, 1984; Fairweather *et al.*, 1990; Makoff *et al.*, 1990; Strugnell *et al.*, 1992; Charles *et al.*, 1994; Anderson *et al.*, 1996).

1.4.5.1 Adhesins

Pertussis is a multifactorial disease process that is dependent on the well-orchestrated action of numerous virulence factors of *B. pertussis*. The ability of bacteria to gain access to the respiratory tract by developing its association to the ciliated cells is provided by an

array of adhesins. Adhesins may be considered the first instrument of developing *B. pertussis* infection as they are required to bring *B. pertussis* into close contact with the target cells for their toxic effect to be induced (Cundell *et al.*, 1994).

1.4.5.1.1 Fimbriae

Fimbriae are long, thread-like surface structures found on the bacterial surface at up to 500 copies per cell. *B. pertussis* fimbriae (Willems *et al.*, 1993) are proteinaceous appendages that protrude from the cell surface. They are usually ~200 nm in length and ~5 nm in diameter (Zhang *et al.*, 1985). The main body of fimbriae is composed of a major subunit protein, Fim2 or Fim3, of 22.5 kDa and 22 kDa, respectively. The tips of both types (Fim2 and Fim3) of fimbriae are occupied by a minor 40-kDa fimbrial protein called FimD (Geuijin *et al.*, 1997).

The structural genes for Fim2 and Fim3 are scattered on the chromosome, likewise the gene for another fimbrial subunit, FimX (Stibitz and Garletts, 1992). The *fimX* gene is not expressed in *B. pertussis* because of a deletion in its promoter region (Willems *et al.*, 1993). Genes *fimB-D* are required for export and assembly of fimbrial subunits (Fim2 and Fim3) and are located between the structural gene for FHA (*fhaB*) and a gene for FHA secretion *fhaC* (Section 1.8.1.2) (Willems *et al.*, 1993). The arrangement of the genes is *fimABCD*, which is like other fimbrial operons in other organisms, with the first gene encoding the major fimbrial subunit. However the *fimA* (Fim1) gene is not expressed in *B. pertussis* because of deletion at its 5' end, whereas it is intact in *B. parapertussis* and *B. bronchiseptica* and it is expressed in *B. bronchiseptica* (Willems *et al.*, 1993; Boschwitz *et al.*, 1997).

It has been shown that *B. pertussis fim* mutants are more adherent to Vero cells than wild type *B. pertussis* but less adherent to tracheal rings prepared from the primate *Papio*

anubis (Funnel and Robinson, 1993). The purified fimbriae bind to sulphated sugars, which are commonly found in the respiratory tract (Geuijen *et al.*, 1996). A *B. pertussis* strain unable to produce fimbriae due to a mutation in *fimB* was less able to colonise the nasopharynx, trachea and lungs of intranasally-infected mice when compared with its wild-type parent and the effect was more pronounced in the trachea (Geuijen *et al.*, 1997). However, the mutation also reduces the production of FHA by 75 %. A strain with a mutation in *fhaC*, which inhibited the production of FHA specifically, was less affected for respiratory tract colonisation, as was a strain unable to produce Fim2 and Fim3 (Geuijen *et al.*, 1997). A fimbrial mutant Δ*fimBCD* of *B. bronchiseptica* appeared to be altered in its ability to cause respiratory tract infection in the rat trachea, although adhesion to cell lines e.g. human epithelial laryngeal (Hep-2) cells was not affected (Mattoo *et al.*, 2000). It appears that the binding of fimbriae to non-ciliated cells including monocytes may promote phagocytosis. Fimbriae on *B. pertussis* interact with very late antigen-5 (VLA-5) on monocytes which activates complement receptor 3 (CR3), which is a receptor for FHA (section 1.4.5.1.2), reflecting their synergistic action during infection.

The initial translocation of fimbrial components across the cytoplasmic membrane is dependent on the normal type II export system (1.7.3.2). However, further export from the periplasm to the cell exterior is mediated by a specific two-component system consisting of a periplasmic chaperone and an usher (1.7.3.6), which is an outer membrane located pore which serves as the assembly platform (Jones *et al.*, 1996).

Table 1.1: Virulence factors in *Bordetella* species

Virulence Factor	*B. pertussis*	*B. parapertussis*	*B. bronchiseptica*	*B. holmesii*	*B. hinzii*	*B. avium*	*B. trematum*
Pertussis toxin (PTX)							
Genes	+	+	+	-	-	-	-
Expression	+	-	-	-	-	-	-
Adenylate cyclase toxin (ACT)							
Genes	+	+	+	-	-	-	-
Expression	+	+	+	-	-	-	-
Tracheal cytotoxin (TCT)							
Genes	+	+	+	-	-	+	-
Expression	+	+	+	-	-	+	-
Dermonecrotic toxin (DNT)							
Genes	+	+	+	-	-	+	-
Expression	+	+	+	-	-	+	-
Filamentous haemagglutinin (FHA)							
Genes	+	+	+	-	-	-	?
Expression	+	+	+	-	-	-	?
Pertactin (PRN)							
Genes	+	+	+	-	-	-	?
Expression	+	+	+	-	-	-	?
Fimbriae (FIM)							
Genes	+	+	+	?	?	+	?
Expression	+	+	+	?	?	+	?
Tracheal colonisation factor (TCF)							
Genes	+	+	+	+	?	?	?
Expression	+	-	-	-	?	?	?
Bordetella-resistance to killing (BrkA)							
Genes	+	+	+	?	?	-	?
Expression	+	?	some strains	?	?	-	?
Type III secretion							
Genes	+	?	+	?	?	?	?
Expression	some strains	?	some strains	?	?	?	?
Motility	-	-	+	-	+	+	+
Genes	-/+	-/+	+	?	+	+	+

+ = Present
- = Absent
? = Not known
+/-= Present in some species

Table adapted from Gerlach *et al.*, 2001

Purified fimbriae are protective immunogens in mice and induce agglutinating antibodies (Ashworth *et al.*, 1982; Robinson *et al.*, 1986, 1989; Gustafsson *et al.*, 1996; Willems *et al.*, 1998). This is consistent with the finding that, to some extent, sero-specific protection occurs when children are immunised with whole-cell vaccine preparations (Mooi *et al.*, 1998). The five-component acellular pertussis vaccine (APVs) containing Fim2 and Fim3 is very efficacious in humans infants (Ashworth *et al.*, 1982; Robinson *et al.*, 1986, 1989; Gustafsson *et al.*, 1996)

1.4.5.1.2 Filamentous haemagglutinin

The closely-related *Bordetellae* species, namely *B. pertussis, B. parapertussis* and *B. bronchiseptica,* produce a similar array of virulence factors. Among these, FHA is regarded as the dominant attachment factor (Arico *et al.*, 1993; Locht *et al.*, 1993), and has been shown to promote attachment to a variety of eukaryotic cell types and tissues and appears to interact with complementary receptors through a number of different mechanisms (Hannah *et al.*, 1994).

In *B. pertussis*, the mature 220 kDa, form of FHA derives from a 370 kDa FhaB precursor by an as yet uncharacterised proteolytic removal of a large 150 kDa C-terminal portion (Domenighini *et al.*, 1990; Renauld-Mongenie *et al.*, 1996). FHA is a large filamentous protein with dimensions of 2 nm x 40-100 nm, quite distinct from *B. pertussis* fimbriae (Relman *et al.*, 1989) and is both surface-associated and secreted from virulent bacteria (Hannah *et al.*, 1994). Most *B. bronchiseptica* isolates produce and secrete FHA at significantly lower levels than *B. pertusssis*. This may reflect differences in strength of their respective FHA promoters, in the primary structures or function of the FhaC accessory proteins, or in the overall cell envelope structure (Locht *et al.*, 1993).

The fate of the carboxy terminal domain is unlike that of PRN, BrkA and TCF

(section 1.8) and, following cleavage it may be probably degraded in the periplasm. However, It has also been suggested that the C-terminal domain of precursor FhaB anchors FHA in the cell envelope, perhaps by spanning the periplasmic space and in this way linking the inner and outer membranes (Domenighini *et al.*, 1990). Proline rich repeats have been identified in the carboxy terminal domain of FhaB (Domenighini *et al.*, 1990). Such a proline rich region exists in the mature PRN protein, close to the putative C-terminal processing site (Charles *et al.*, 1989). These regions are thought to be characteristic of proteins that act as anchors and span the periplasm (Domenighini *et al.*, 1990).

A noteworthy feature of FHA is its high level of secretion by *B. pertussis* (Locht *et al.*, 1993). FHA represents the most abundant polypeptide in the culture supernatant of *B. pertussis* grown *in vitro*. It is also associated with the bacterial outer membrane. Coating of the bacterial outer surface with FHA is thought to be responsible for self-agglutination of the bacteria by FHA-FHA (intramolecular) homotypic interactions (Menozzi *et al.*, 1994). It has also been suggested that FHA (and perhaps, pertussis toxin as well) released from *B. pertussis* in the respiratory tract could bind to other bacteria by a process which is known as 'piracy of adhesins' allowing these other bacteria to efficiently adhere to cilia and promoting secondary bacterial infections (Tuomanen, 1993).

The multiple binding activities of the mature 220 kDa protein have been the focus of many studies. FHA is recognized by lactose-containing glycolipids on ciliated respiratory epithelial cells (Tuomanen *et al.*, 1988). It also binds to sulphated carbohydrates of sulphatides and proteoglycans on the surface of epithelial cells or in the extracellular matrix (Brennan *et al.*, 1991). In addition, it possesses an Arg-Gly-Asp tri-peptide (RGD motif), which is a recognition sequence for the members of the integrin family. This RGD site within the mature protein is recognized by the β-3 leukocyte response integrin, in

concert with an integrin-associated protein (Ishibashi *et al.*, 1994). A recent study has suggested that *B. pertussis* FHA may promote invasion of human respiratory cells through the interaction of its RGD sequence with host cell $\alpha_5\beta_1$ integrin (Ishibashi *et al.*, 2001). As the dominant adhesin, differences in FHA-mediated function might contribute to differences in *Bordetella* host species tropism.

A number of *in vitro* adhesion assays using primary cell lines and tissues of various types and from a variety of species have indicated that FHA is the major *B. pertussis* adhesin (Funnell and Robinson, 1993; Bassinet *et al.*, 2000). However studies with a *B. pertussis* FHA mutant using the mouse model of infection have suggested that FHA is far less important *in vivo* (Roberts *et al.*, 1991; Khelef *et al.*, 1994; Geuijen *et al.*, 1997). In some cases this might have been due to the mutants being created by an internal in-frame deletion in *fhaB* which might have allowed the production of truncated FHA that possibly would have retained some activity. On the other hand an *fhaC* mutant (deficient in surface exposed FHA) exhibited a slight, transient defect in colonisation of the nasal cavity and trachea of mice (Geuijen *et al.*, 1997). *B. bronchiseptica* FHA is required for tracheal colonisation of rats, but it is insufficient for colonisation when expressed ectopically in the Bvg^- phase (Cotter *et al.*, 1998). FHA is absolutely required for the colonisation of the tracheas of conscious but not anaesthetised rats by *B. bronchiseptica.* The use of anaesthesia in mice may be the factor responsible for the relative lack of effect of FHA mutations observed in some, but not all, of the experiments with *B. pertussis* (Cotter *et al.*, 1998).

B. pertussis FHA is also highly immunogenic in humans and a protective antigen in animal models (Amsbaugh *et al.*, 1993). Most monoclonal antibodies generated against FHA from *B. pertussis* cross-react with FHA from *B. bronchiseptica*, indicating shared epitopes (Menozzi *et al.*, 1994). FHA is one of the main components of most APVs

currently in use in humans (Mills *et al.*, 1998; Mahon *et al.*, 1996; Guiso *et al.*, 1999).

The other adhesins, along with some other functionally different virulence factors of *B. pertussis,* are described in section 1.8 under the heading of members of *B. pertussis* autotransporter family.

1.4.5.2 Toxins

After successful colonisation, different toxins play their role in completing the pathogenic cycle of *B. pertussis* and establishing the full spectrum of whooping cough (pertussis). For example, tracheal cytotoxin is thought to be the cause of ciliostasis and eventually death of columnar epithelial cells, producing local lesions on the mucosa, accumulation of mucus and debris, and hence eliciting the paroxysmal cough. Adenylate cyclase toxin and probably pertussis toxin help to protect the organisms from clearance by phagocytic cells and may stimulate secretion of fluid and mucus.

1.4.5.2.1 Pertussis toxin

Pertussis toxin (PTX) is an exotoxin and a major virulence factor of *B. pertussis* (Locht and Keith, 1986) and in its toxoided form is the main component of all acellular pertussis vaccines (Castro *et al.*, 2001). It has a remarkable range of biological activities *in vivo* and *in vitro*, resulting from its action on many different cell types and tissues and hence is implicated as having a fundamental role in the pathogenesis of pertussis.

PTX is a complex protein of 105 kDa comprised of 5 subunits that are the products of five genes (*s1-s5*), present in the ratio of 1:1:1:2:1 and together constitute a complex with ADP-ribosylating activity. The crystal structure of PTX reveals one or more intrachain disulphide bonds that stabilise each of the subunits in the mature toxin (Stein *et al.*, 1994).

al., 1994). PTX is an AB_5 toxin; with S1 being the enzymatic subunit of the toxin and the B oligomer being the pentamer that binds to the surface receptors on eukaryotic cells and translocates the toxic subunit across the cell membrane. Some of the properties of PTX are completely dependent upon the binding of the B-oligomer to cell surfaces. These include haemagglutination and T-cell mitogenicity (Ui, 1988; Kaslow and Burns, 1992) and at least some of its adjuvant activities (Roberts *et al.*, 1995; Ryan *et al.*, 1997). The other, harmful, effects depend on the activities of the holotoxin. Upon receptor-binding, PTX is taken into the eukaryotic cell by receptor-mediated endocytosis. PTX interferes with cellular signalling processes across the membrane by ADP-ribosylating the α-subunit of various regulatory G proteins (Krueger and Barbieri, 1995). This results in a variety of consequences including inhibition of chemotactic detection by leukocytes, lymphocytosis and increased vascular permeability (Craig, 1988).

PTX is secreted as an assembled protein complex and novel machinery is needed (Weiss, 1994). This export pathway has been termed type IV secretion (section 1.7.3.4). Most proteins are presented to the translocation apparatus in the form of a long slender like structure, which is secreted in an unfolded state and folds following secretion. However, pertussis toxin is an exception. The export of a functional toxin complex prevents energy from being wasted on producing subunits that do not become part of a functional toxin (Weiss, 1994).

Most of the PTX is secreted but some remains surface-associated, as depicted by electron-microscopy (Ashworth *et al.*, 1985). The surface located PTX may take part in adhesion of *B. pertussis* to eukaryotic cells (Tuomanen, 1988) as evident by 50 % decrease in binding of a *B. pertussis* PTX^- mutant to human respiratory ciliated cells (Relman *et al.*, 1989). PTX has also been shown to play role in adherence of *B. pertussis* to non-ciliated epithelial cells (Relman *et al.*, 1989). The S2 or S3 subunit binding to the macrophages

enhances the binding of *B. pertussis* to the CR3 integrin mediated by FHA. The activation of CR3 and augmentation of FHA-binding is a property that PTX shares with *B. pertussis* fimbriae (section 1.4.5.1.1). PTX (B oligomer) enables *B. pertussis* to bind to eukaryotic cells including human respiratory ciliated cells and macrophages (Sandros and Tuomanen, 1993). The nature of the lectin-like interaction between the B-subunits and the eukaryotic cell mimics the relationship between the leukocyte surface and the capillary wall that occurs during early diapedesis (Sandros and Tuomanen, 1993). The B subunit targets PTX to eukaryotic cells by binding to glycoconjugates, preferentially glycoproteins (Brennan *et al.*, 1988; Tyrrell *et al.*, 1989; Saukkonen *et al.*, 1992; Armstrong *et al.*, 1994).

In a coughing rat model of pertussis, a wild type *B. pertussis* strain produced a significant number of coughing paroxysms where as PTX^- mutant, a phase IV strain and a *B. parapertussis* strain, none of which produced PTX, did not (Parton *et al.*, 1994). PTX has been shown to be involved in lethal *B. pertussis* infection of infant mice although mutations affecting the production of PTX did not affect the growth of *B. pertussis* in murine lungs (Weiss *et al.*, 1984; Weiss and Goodwin, 1989). However, in the adult non-lethal mouse model, a *B. pertussis* PTX^- mutant strain inflicted a less severe pulmonary inflammation as compared to its wild-type strain (Khelef *et al.*, 1994).

The exact role of PTX in the pathogenesis of whooping cough and its main site of action in the host, whether locally in the respiratory tract or systemically, perhaps in the central nervous system remains obscure (Hewlett, 1997), although its absence in *B. parapertussis* implies that it may have only a minor role in causing the typical whooping cough symptoms (Parton, 1996). Despite the uncertainty of the role of PTX in pathogenesis, toxoided PTX (PTD), is included in all acellular vaccines due to successful provision of protection in mouse models involving either intracerebral or aerosol challenge with *B. pertussis* (Sato *et al.*, 1984; Sato and Sato, 1988, 1990). Chemical detoxification

of PTX however is known to reduce its immunogenicity and more immunogenic, genetically detoxified, recombinant PTD (rPTD) is now available (Rappuoli *et al.*, 1992a, 1992b).

1.4.5.2.2 Adenylate cyclase toxin

A second important protein toxin of *B. pertussis* called adenylate cyclase toxin (ACT) or CyaA, is a bifunctional protein of molecular weight 200 kDa comprising of 1,706 amino acids (Hewlett *et al.*, 1976). *B. parapertussis* and *B. bronchiseptica* have ACT activity similar to that of *B. pertussis* but the toxins are antigenically distinct (Gueirard and Guiso, 1993). CyaA exhibits a number of striking features: (Locht *et al.*, 2001). It is secreted by virulent bacteria, (section 1.7.3.1), it is activated by the eukaryotic calcium-binding protein calmodulin (CAM), and it can enter eukaryotic cells, where upon after activation by endogenous CAM, it catalyses high level synthesis of cyclic 3'-5' AMP (cAMP), an important signalling molecule, which in turn alters cellular physiology due to the depletion of ATP

CyaA is constructed in a modular fashion: the ATP-cyclizing and CAM-activated catalytic adenylate cyclase domain (AC) is located in the 400 amino-proximal residues, whereas the carboxy-terminal 1306 residues of CyaA are responsible for the pore-forming and haemolytic phenotype of *B. pertussis*. The haemolysin domain (residues 400-1706) displays structural characteristics that link CyaA to a family of bacterial toxins known as the RTX (repeat in toxin) family, the prototype of which is the *E. coli* α- haemolysin. The haemolysin domain mediates the binding and internalisation of the toxin into eukaryotic cells (Ladant and Ullman, 1999). The toxin is synthesized as an inactive precursor and then converted to its active form by CyaC-mediated palmitoylation of Lys983. This palmitoylation is required for binding to eukaryotic cells and for the formation of a pore

through which the catalytic subunit can penetrate into the cell (Betsou *et al.*, 1993).

Immune effectors cells such as neutrophils, monocytes, macrophages and natural killer cells are thought to be the primary targets. ACT has also been suggested to play a role in mucus secretion to produce the excessive fluid as seen in clinical pertussis cases. The mouse model of infection using ACT-deficient mutants has suggested its importance as a virulence factor in the infectious process. ACT-deficient mutants have reduced ability to cause lethal infection in infant mice and to colonise the lungs of older mice (Weiss and Goodwin, 1989; Khelef *et al.*, 1994). Recent studies have confirmed that phagocytic cells are the primary target of ACT. It can induce apoptosis in mouse alveolar macrophages *in vitro* and *in vivo* (Gueirard *et al.*, 1998). Comparison of the effects of wild-type and ACT$^-$ mutant *B. bronchiseptica* strains during infection of normal and immunodeficient mice has suggested that neutrophils are critical in providing the early defence against infection and that ACT was responsible for overcoming this arm of the innate immune system (Harvill *et al.*, 2000). Other studies have suggested that the expression of ACT inhibits uptake and possible destruction of *B. pertussis* by human respiratory epithelial cells (Bassinet *et al.*, 2000) and by human neutrophils after opsonisation (Weingart and Weiss, 2000).

ACT is not included in the current acellular pertussis vaccines but it has been shown to be a protective antigen in number of studies. The purified AC enzymic moiety of ACT was shown to protect mice against *B. pertussis* colonisation of the respiratory tract and brain after sublethal intranasal and lethal intracerebral challenge, respectively (Guiso *et al.*, 1991). Anti ACT-antibodies have been shown to protect mice against lethal intranasal infection with *B. pertussis* (Brezin *et al.*, 1987). Other studies suggested that immunisation with either native ACT or recombinant active (acylated) ACT will promote more rapid clearance of *B. pertussis* from the respiratory tract than in controls or in mice immunised with inactive (non-acylated) recombinant ACT (Betsou *et al.*, 1993; Hormozi *et al.*, 1999).

1.4.5.2.3 Tracheal cytotoxin

The paraoxysmal cough, a distinctive characteristic of pertussis has been attributed to the elaboration of the tracheal cytotoxin (Cookson *et al.*, 1989). Goldman and colleagues purified tracheal cytotoxin (TCT), a peptidoglycan fragment, from culture supernates of *B. pertussis* and the material caused progressive cytotoxic changes in ciliated cells of hamster tracheal organ cultures (Flak and Goldman, 1999).

TCT is an unusual, low molecular weight (921 Da) toxin consisting of muramyl peptide containing N-acetylglucosamine, N-acetylmuramic acid, alanine, glutamic acid and diaminopimelic acid in the molar ratio 1:1:2:1:1 (Goldman, 1988; Cookson *et al.*, 1989). TCT and related muramyl peptides have been associated with diverse biological activities such as ciliostasis, pyrogenicity, adjuvanticity, and arthritogenicity, induction of slow-wave sleep and stimulation of IL-1 production which causes increase in body temperature. The abnormal release of large amounts of TCT by *Bordetella* spp. causes an exaggerated response that results in respiratory tract pathology (Flak *et al.*, 2000). The cytopathic effects of TCT have been related to its triggering effect on the production of IL-1 which stimulates nitric oxide (NO) production leading to epithelial cell damage. The cytopathic effects were initially thought to be specific for ciliated cells; however in another study both ciliated and non-ciliated human nasal epithelial cells were damaged by the toxin (Soane *et al.*, 2000). TCT, even at very low concentrations, also impairs neutrophil functions and may thereby contribute to survival of bordetellae *in vivo* (Cundell *et al.*, 1994). There is no information so for available which can categorise the TCT molecule as an immunogen.

1.4.5.2.4 Dermonecrotic toxin

B. pertussis produces a highly lethal toxin called dermonecrotic toxin (DNT), also known as heat-labile toxin (HLT) (Pullinger *et al.*, 1996), which is thought to induce inflammation, vasoconstriction and necrotic lesions around the areas where *B. pertussis* colonise in the respiratory tract. The lethal toxin is a 102 kDa protein composed of four subunits, two of 24 kDa and two of 30 kDa. DNT belongs to the same toxin family as the cytotoxic necrotising factor (CNF1 and 2) of *E. coli* which affects mammalian cell division or cell differentiation (Pullinger *et al.*, 1996). Purified DNT has also been found to stimulate DNA and protein synthesis in mammalian cells without cell division, leading to polynucleation. It also induces the assembly of actin stress fibres and tyrosine phosphorylation of focal adhesion kinase (Horiguchi *et al.*, 1994). DNT is believed to cause these effects by glutamine 63-deamidation of the small GTP-binding protein, RhoA, involving residues 1136 to 1451 of DNT (Schmidt *et al.*, 1999). Glutamine 63 is essential for GTP hydrolysis by Rho. Deamidation of glutamine by DNT inhibits the GTPase activity of Rho and renders the Rho protein constitutively active. Rho GTPases are regulators of actin cytoskeleton and act as molecular switches to trigger several intracellular signalling pathways.

DNT is a cytoplasmic component of the bacterium and does not seem to be released by actively-growing cells (Nikai *et al.*, 1985; Walker *et al.*, 1994). A *B. pertussis* transposon insertion mutant deficient in DNT production was found to be unaltered in its ability to cause a lethal intranasal infection in infant mice (Weiss and Goodwin, 1989). However, studies on other *Bordetella* species in their natural hosts have suggested that DNT does appear to play a role both in disease process and colonisation. In *B. bronchiseptica,* DNT seems to be involved in producing the turbinate atrophy associated

with atrophic rhinitis in pigs as a DNT-deficient mutant was associated with significantly less atrophy and pneumonia compared with wild-type parent. A *B. bronchiseptica* DNT$^-$ mutant was also unable to colonise the nasal cavity as efficiently as the wild-type parent (Brockmeier *et al.*, 2000). Another study with a *B. avium* DNT$^-$ mutant has suggested that the mutant was less virulent and persisted for a shorter period of time in young turkeys, and was also less adherent to turkey ciliated tracheal cells *in vitro* (Temple *et al.*, 1998). The protective nature of DNT in toxoided form in humans and animals remains to be addressed. On the other hand, it has been suggested that it is not an essential component for protection as it is destroyed or removed during whole cell and acellular vaccine preparation, respectively. Moreover, convalescent sera lack anti-DNT antibodies (Wardlaw and Parton, 1988). Thus, DNT is a complicated toxin which may have a significant, but as yet unclear role in the alteration of the host physiological reactions in response to *B. pertussis* infection (Babu *et al.*, 2001).

1.4.5.2.5 Lipopolysaccharide

The lipopolysaccharide (LPS) constitutes an integral part of the outer-membrane of *B. pertussis,* in common with other Gram-negative bacteria. The LPS of *B. pertussis*, but not that of *B. bronchiseptica* and *B. parapertussis,* lacks an O-polysaccharide side-chain characteristic of the LPS of the enteric bacteria and because of this it is sometimes referred to as lipooligosaccharide (Chabby and Caroff, 1988; Martin *et al.*, 1992; Le Blay *et al.*, 1994 ; Preston *et al.*, 1996).

LPS has the usual properties of endotoxins such as general toxicity, pyrogenicity and adjuvanticity as well as some unusual properties such as ability to induce resistance to mouse adenovirus infection and the ability of the polysaccharide component to induce B-cell mitogenicity and polyclonal B cell activation in mice (Chabby and Caroff, 1988).

Endotoxins mediate most of their diverse biological effects by triggering cytokine production by monocytes. More recently, LPS has been shown to act synergistically with tracheal cytotoxin to induce inflammation in the respiratory mucosa by inducing nitric-oxide (NO) production and release by tracheal cells and thereby poisoning the activity of adjacent ciliated epithelial cells (Flak and Goldman, 1999; Caroff *et al.*, 2000; Flak *et al.*, 2000).

LPS probably causes much of the reactogenicity of the whole-cell pertussis vaccine and one of the main aims in pertussis vaccine development has been to eliminate its adverse effects from the new generation of acellular pertussis vaccines. Nevertheless, LPS may have a role in inducing immunity to pertussis infection. It was reported that anti-LPS antibodies may be bactericidal in the presence of complement (Ackers and Dolby, 1972). The presence of smooth LPS in *B. bronchiseptica* correlated with the resistance to killing *in vitro* by antibody and complement and antimicrobial peptides whereas *B. pertussis*, lacking the *O*-polysaccharide side-chains, was more sensitive to these agents (Banemann *et al.*, 1998; Byrd *et al.*, 1998). The role of LPS in *B. pertussis, B. bronchiseptica* and *B. parapertussis* has been investigated by deleting the *wbl* locus required for the synthesis of polysaccharide domains consisting of lipid A and the core oligosaccharide (Harvill *et al.*, 2000). Mutants of all three species were found to be defective in colonising the trachea but, in addition, the *B. pertussis* mutant was also less able to colonise the nasal cavity than the wild-type strain. The other interesting observation was that the *wbl* mutation rendered the normal resistant strains of *B. bronchiseptica* and *B. parapertussis,* sensitive to killing by normal rabbit serum, whereas *B. pertussis* wild-type and mutant were equally killed (Harvill *et al.*, 2000). Thus the role of LPS seems to be different in different *Bordetella* species in determining host-pathogen interaction.

1.5 Regulation of Virulence

Like many other bacterial pathogens, the expression of virulence factors in *B pertussis* is controlled by growth conditions. These virulence factors include cell surface proteins e.g. adhesions, and several extracellular toxins that inhibit host defences and induce damage to host tissues. The virulence genes of *B. pertussis* are scattered around the chromosome and not grouped together in distinct mobile elements. However, some of the virulence genes are clustered together, such as *bvgAS*, *fha* and *fim* genes. In many instances the structural genes are linked to the accessory genes. This is the case for PTX, a toxin composed of five subunits, the structural genes of which are organised in an operon and this is followed by an additional seven accessory genes, named *ptl* genes, that are involved in the secretion of toxin (section 1.8.2.1) (Weiss *et al.*, 1993). Two important phenomena in the regulation of virulence genes are phase variation and phenotypic modulation. Phase variation indicates a reversible alteration in the genotype caused by frame shift mutations in which the virulent bacteria simultaneously lose the ability to synthesize toxins and other factors associated with pathogenicity (Weiss and Falkow, 1984). Differential erythromycin tolerance has been shown to be a phase marker caused by extensive alterations in the surface properties between the virulent and a non-virulent strain, and this confers susceptibility to the virulent (phase I) strain (Weiss and Falkow, 1984). The natural emergence of phase variants in the later stages of infection implies that phase changes could be a defence mechanism to escape immune detection, like *Salmonella* flagellar phase variation where the change of antigenic type helps the bacteria to evade the immune system. Non-virulent phase variants arise in a population at a frequency of 10^{-3} to 10^{-6}. The other phenomenon is termed phenotypic modulation and was first observed by Lacey in 1960. It is characterised by lack of expression of virulence factors, except the tracheal cytotoxin, at low temperature (25°C) or in the presence of *in vitro* modulators such

as nicotinic acid, sulphate ions (SO^-_4) and chlorate ions (ClO^-_4) (Lacey, 1960; Scarlato *et al.*, 1990).

Genetic analysis has shown that both phenotypic modulation and phase variation are under the control of a single genetic locus, the *bvg* locus (Weiss *et al.*, 1983; Arico *et al.*, 1989; Stibitz and Yang, 1991). The *bvg* locus occupies ~5 kb of the total genome and codes for the BvgA and BvgS proteins and another gene downstream *bvgR* codes for the protein, BvgR (Arico *et al.*, 1989; Stibitz and Yang, 1991; Sindt *et al.*, 1994). The *bvg* locus has been shown to regulate production of the virulence determinants of both *B. pertussis* and *B. bronchiseptica* organism, by controlling the expression of virulence-activated genes (*vags*) and virulence-repressed genes (*vrgs*) (Stibitz *et al.*, 1988; McGillivray *et al.*, 1989; Scarlato *et al.*, 1993; Fernandez and Weiss, 1994; Finn and Amsbaugh., 1998). BvgS is a 135 kDa periplasmic sensor histidine kinase. It consists of a periplasmic input domain and several cytoplasmic domains, the linker, transmitter, receiver and the C-terminus. The BvgS periplasmic domain senses external stimuli (perhaps via dimerization) and triggers a series of phosphorylation steps starting with autophosphorylation in the transmitter domain at a conserved histidine residue. This is followed by the transfer of the phosphate group to a conserved aspartic acid in the receiver domain, which transfers it to a histidine at the C–terminal domain (Roy *et al.*, 1990; Uhl and Miller, 1995; Beier *et al.*, 1996). BvgA, a response regulator, is a 23 kDa cytoplasmic protein comprising an N-terminal receiver and a C-terminal output domain, which contains a helix-turn-helix (HTH) DNA binding motif. BvgA is activated by the transfer of the phosphate group from the C-terminus of BvgS to a conserved aspartic acid in the BvgA N-terminal receiver domain. Upon phosphorylation, BvgA positively regulates the *bvgAS* and other virulence factor promoters, including *bvgR,* by binding to the heptameric target sequence TTTCCTA (Karimova and Ullmann, 1997). It has also been shown that BvgA is

able to dimerize in solution and this may represent a possible means to enhance its DNA-binding property (Scarlato *et al.*, 1990). Transcription of the *bvgR* gene produces a repressor of *vrg* gene transcription (Karimova and Ullmann, 1997).

Studies on the *bvg* locus have revealed that its transcription is controlled by a 350 bp DNA fragment having four promoters. Three of them, P1, P2 and P3, are involved in the transcription of the *bvg* locus, while the fourth, P4, is involved in the synthesis of an anti-sense RNA. Under non-inducing conditions, the promoter P2 is active and maintains a low level of the regulatory protein BvgA and BvgS which is not enough to activate the virulence genes. Under inducing conditions, BvgA is activated by phosphorylation, following which it activates the promoters P1, P3, P4 and the other promoters of the virulence-activated genes (*vag*). Expression of these genes represents the Bvg^+ phenotype. The antisense RNA coded from P4 positively regulates P1, P2 and P3 by hybridization to their 5' untranslated regions. Hybridization inhibits the formation of secondary structure in the mRNAs, thereby favouring their interaction with the ribosome. This causes a 50-fold increase in their rate of translation (Scarlato *et al.*, 1990). The Bvg^- phase is defined by the absence of *vag*-encoded factors and occurs when the *bvg* locus is deleted or when BvgAS activity is suppressed by modulating conditions. It is also involved in the regulation of the virulence-repressed genes (*vrg*) (Beattie *et al.*, 1993), namely *vrg6, vrg18, vrg24, vrg53 and vrg73.* The first four of these *vrg* genes mentioned are regulated by BvgR through a conserved repressor binding site.

The Bvg^+ phase is thought to be necessary and sufficient for the colonisation of animal respiratory tracts (Cotter and Miller, 1994; Martinez *et al.*, 1998), while the Bvg^- phase appears not to play a role in virulence (Akerley *et al.*, 1995). However there is some indication of the presence of a Bvg^i (intermediate phase) and its expression *in vivo* but the significance of this phase is not completely understood.

The product of one such *bvgi* gene, *bilA* (*Bordetella* intimin-like), has been recently identified in *B. bronchiseptica* whose closest homologue is intimin expressed by enteropathogenic *E. coli*. The product of *bilA* has been shown to be involved in colonisation in a rabbit model (Stockbauer *et al.*, 2001). The *bvg*-repressed genes of *B. bronchiseptica* appear to be involved in survival outside the host (Mcmillan *et al.*, 1996), whereas their function in *B. pertussis* is unknown. However, it has been shown that BvgR-mediated regulation of gene expression contributes to respiratory infection in mice because a mutant of *B. pertussis* with a mutation in the *bvgR* gene failed to colonise mice as well as the wild-type (Merkel *et al.*, 1998). The virulence factors of *B. pertussis* have been shown to be expressed differentially, when their time course of expression was analysed in laboratory conditions. The results indicated that the adhesins are the first to be expressed in order to colonise the respiratory tract of the host and toxins are expressed late when they are needed (Scarlato *et al.*, 1993; Kinnear *et al.*, 2001).

Several lines of evidence suggest that the mechanism of regulation by the *bvg* locus is different for different virulence loci. Other work has also implicated the requirement of additional factors or the differential effects of DNA topology (Graeff *et al.*, 1995), for selective activation of some promoters. Given the varied aspects of BvgA-mediated transcriptional activation, the *bvg* regulon has proved to be an excellent system for the study of differential and timely regulation of target promoters to optimise the infection process.

1.6 Vaccines

Whooping cough is an important example of a highly contagious and dangerous disease which can be controlled by the availability of effective vaccination. In fact, there are still several pockets of disease around the world, significantly in developing nations,

where the death toll remains high due to non-availability of proper vaccination. Effective killed whole-cell vaccines against *B. pertussis*-mediated disease have been used in developed countries for more than 50 years and have led to dramatic reductions in morbidity and mortality. Despite extensive research on virulence factors and potential vaccine candidates, there has been no consensus as to the molecular nature of the antigens which confer protective immunity.

The protection afforded by the whole-cell vaccine comes at the cost of its associated reactogenicity ranging from local and systemic reactions to permanent brain damage and death (Cherry *et al.*, 1988; Griffith, 1989; Hodder and Mortimer, 1992). These problems of reactogenicity have acted as a stimulus for the development of non-toxic efficacious vaccines in the form of acellular vaccines. The research is focused on the identification of defined protective antigens that would be efficacious and non-toxic (Cherry and Hewlett, 1990). Various pertussis virulence factors have been shown to be more or less protective when used in an acellular vaccine e.g. pertussis toxoid, filamentous haemagglutinin, pertactin, and fimbriae (Trollfors *et al.*, 1995). The safety and the extent of immunogenicity afforded by these vaccines paved the way for their consideration as candidates for booster immunization of older children and adults to control pertussis more effectively in populations (Keital and Edwards, 1995: Cherry, 1998).

The major hindrance in the area of better vaccine development has been the shortcomings associated with various animal models available for assessing mechanisms of virulence and immunity, although the mouse models, using intracerebral, intranasal and aerosol challenge have provided much useful information (Sato and Sato, 1988). The intracerebral mouse protection test has provided a reliable estimate of the potencies of WCPVs for children but is unreliable for assessing the potency of APVs (Robinson and Funnell, 1992). Thus the identification and characterisation of the protective antigens of *B.*

pertussis has been slow and is still underway. The other emerging problem regarding the use of vaccines is that there is evidence to suggest that they have acted as a driving force in microevolution of *B. pertussis,* leading to resurgence in the incidence of pertussis in the last ten years in several countries with an historical low incidence, attributable to high vaccine uptake. Several sequence polymorphisms in the S1 subunit gene of PTX and within the *prn* gene were found which led to slightly altered protein sequences compared with the corresponding factors present in the vaccine preparations. Protective immunity against strains expressing such sequence variants might be reduced. It has been suggested that vaccination programmes have caused a selective force leading to an antigenic shift in important antigens which gradually may have decreased vaccine efficacy, allowing the re-occurrence of pertussis epidemics (Mills, 2001).

1.7 General mechanism for secretion in Gram-negative bacteria

1.7.1 Outer membrane assembly

The outer membrane of Gram-negative bacteria protects it from the surrounding harsh environment. At the same time, the embedded proteins fulfil a number of tasks that are crucial to the bacterial cell, such as solute and protein translocation, as well as signal transduction (Koebnik *et al.*, 2000). The outer membrane functions as an exclusion barrier with selective permeability which allows the control of substances moving in and out of the cell. In Gram-negative bacteria, the major porin is involved in such control. This porin consists of complexes of a monomer of approximately 40 kDa that assembles in the outer membrane and allows the passage of ionic molecules (Armstrong and Parker, 1986; Li, 1991). The *B. pertussis* major porin has been characterised (Hannah, 1994). The porin prevents passage of hydrophobic and large hydrophilic molecules into the cell, and numerous proteins, peptides, carbohydrates and hydrophobic drugs are actively exported

by other means (secretory pathways) from the cytoplasm into the extra-cellular medium (Dinh *et al,* 1994).

1.7.2 Surface-located outer membrane proteins

Nearly all bacterial virulence factors are either located on the bacterial surface or secreted. In the past few years, there has been an explosion of information identifying bacterial factors that are needed as accessories to transport virulence factors to the cell surface and into the surrounding environment (Finlay and Falkow, 1997). Pathogenic bacteria produce virulence factors that cross the bacterial cell envelope from cytoplasm to extracellular milieu where they promote disease. In some cases export is a single energy-coupled translocation due to a secretion (Sec) apparatus associated with both inner and outer membranes of Gram-negative bacteria. In other cases, secretory intermediates accumulate in the periplasm and two distinct, independently functioning processes are responsible for transport across the two membranes of the cell (Hancock, 1991).

1.7.3 Protein translocation to the cell exterior / pathways of secretion

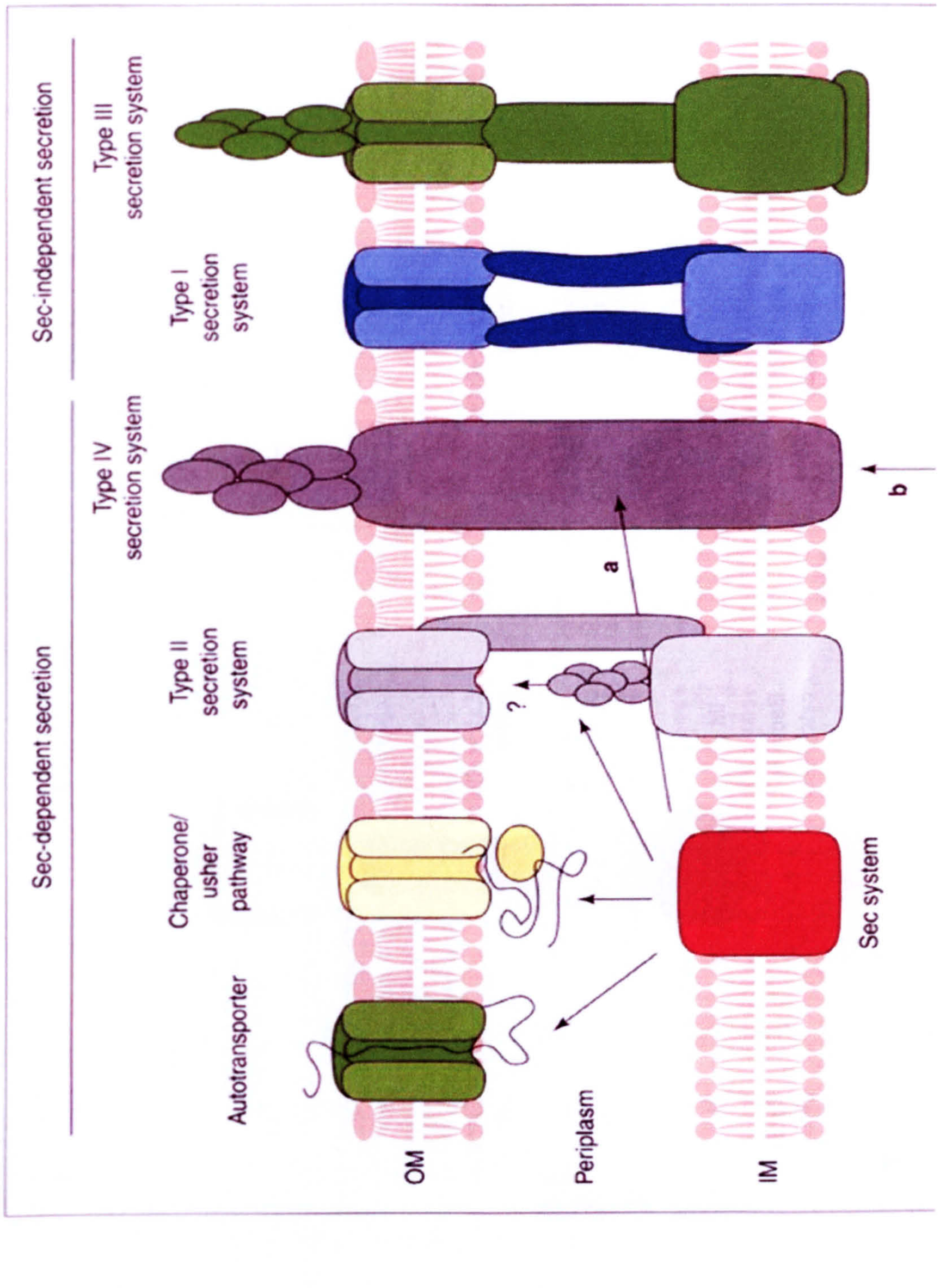
The mechanisms by which proteins traverse bacterial membranes vary considerably (Sandkvist, 2001). In Gram-negative bacteria, six non-homologous protein export systems have been identified (Figure 1.1) allowing them not only to construct their membrane and cell envelope but also to secrete an array of toxins, adhesins and hydrolytic enzymes. The secretion systems vary in a number of important properties, including complexity, dependence on a protein signal sequence (Sec), and whether the secretion across the inner and outer membrane is achieved in a single step. Of these systems, only one, the type II secretion system is essential for cell viability (Finlay and Falkow, 1997).

At the moment it is not understood why certain proteins are secreted via one pathway

Fig. 1.1: Shows the different secretion pathways used by Gram-negative bacteria for exporting their protein products

Among the six major protein-secretion pathways of Gram-negative bacteria, four depend on the Sec system for protein transport across the inner membrane (IM). Autotransporters (also known as type V secretion systems) mediate the transport of a passenger domain across the outer membrane (OM). Type VI (chaperone/usher) pathway requires a chaperone termed an usher and an outer membrane protein, for the secretion of the proteins. The type II secretion systems mediate the transport of extracellular enzymes and toxins, involving 12-16 proteins, most of which are associated with the inner membrane (IM). Four inner membrane proteins are proposed to form a pilus-like structure that could function as a piston to push protein through the OM pore (indicated by an arrow). Type IV secretion systems transport a variety of substrates, some of which e.g. pertussis toxin, require a Sec system for secretion (a), whereas others like the T-DNA-protein complexes of *Agrobacterium tumefaciens* are transported directly from the cytosol (b). The type I and type III secretion pathways are Sec-independent. Type I systems secrete toxins, proteases, lipases and S-layer proteins directly into the extracellular milieu, whereas type III secretion systems mediate delivery of virulence proteins into the host cell. Extracellular appendages are associated with several type III and type IV systems.

The figure and legend is taken from Buttner and Bonas, (2002).


Sec-dependent secretion
Sec-independent secretion
Type III secretion system
Type I secretion system
Type IV secretion system
Type II secretion system
Chaperone/ usher pathway
Autotransporter
a
b
?
OM
Periplasm
IM
Sec system

and not another. It is possible that the choice of the secretion pathway for individual proteins is determined in part by the function they perform at the extracellular site where they are delivered, as suggested by Lorry (1998).

1.7.3.1 Signal sequence independent pathway (Type I)

The type I secretion pathway (Fig 1.1) is wholly independent of the general secretory pathway (GSP) formed by the products of the *sec* genes and hence is known as a Sec-independent pathway. This system is relatively simple, involving protein secretion directly from the cytoplasm to the exterior of the bacterial cell without a periplasmic intermediate stage (Kerr, 1999). Sec-independent proteins mediate their own transport via several accessory proteins that form a transmembrane channel through which the secreted molecule moves (Femlee *et al.*, 1985).

The prototype member of the Sec-independent pathway is the *E.coli* α-hemolysin. Proteins like adenylate cyclase toxin from *B. pertussis*, alkaline protease from *Pseudomonas aeruginosa*, leukotoxin from *Pasteurella haemolytica*, and proteases of the plant pathogen *Erwinia chrysanthemi,* are also exported via this pathway (Finlay and Falkow, 1997).

The proteins targeted through the type I pathway have no classical amino-terminal signal sequences and are translocated with the assistance of accessory proteins (membrane traffic wardens) encoded by the structural genes, which recognise a signal at the C-terminus of the proteins. The clustering of structural gene(s) and secretory apparatus genes has allowed cloning of several complete enzyme secretion systems into *E.coli*, aiding their molecular analysis (Salmond and Reeves, 1993). *B. pertussis* CyaA toxin is secreted via a type 1 secretion system, which does not involve a cleavable signal peptide and a periplasmic intermediate, but requires the accessory proteins CyaB, CyaD and CyaE.

1.7.3.2 General secretory pathway (Type II)

The type II secretion system is thought to be the major export pathway in most Gram-negative organisms (Salmond and Reeves, 1993). Several virulence factors use a type II secretion system for their translocation in Gram-negative organisms (Pugsley, 1993). The type II secretion system is a two-step process that has best been studied for the pullulanase enzyme, PulA, from *Klebsiella oxytoca* (Henderson *et al.*, 1998; Hueck, 1998). Type II secreted proteins first pass across the inner membrane via the Sec system of the general secretory pathway 'GSP' (Fig 1.1) and is dependent on several Sec proteins (Sec A, B, D, E, F). These proteins are all probably involved both in recognition of the signal sequence and in a subsequent interaction with the inner membrane protein SecY, which may form the part of a specific translocation channel (Henderson *et al.*, 1998).

Transport across the outer membrane is subsequently mediated by a dedicated secretion apparatus composed of 13 or 14 different gene products (Pugsley, 1993; Filloux and Hardie, 1998; Henderson *et al.*, 1998). Like the type I secretion system, one component of this system has a consensus ATP-binding site (Walker box), which indicates that energy for export comes from this high energy nucleotide. These systems also encode a peptidase that cleaves off a small N-terminal sequence which plays a role in delivery of these proteins to the Sec machinery (Perlman and Halvorson, 1983; Finlay and Falkow, 1997). At least 11 proteins and one RNA species are known to be directly involved in this secretion pathway in *E. coli* (Economou, 1998).

This pathway is the carrier for many of the *B. pertussis* proteins like the autotransporter proteins pertactin, tracheal colonisation factor, etc (section 1.8) as well as the major *B. pertussis* adhesin FHA and fimbriae across the inner membrane and into the periplasmic space (section 1.4.5.1). Type II secretion pathway is also presumed to be

involved in transporting the pertussis toxin sub-units into the periplasmic space (section 1.4.5.2.1).

1.7.3.3 Contact-dependent pathway (Type III)

More recently, a third major highly-conserved export system, which plays an active role in the specific secretion of virulence factors in both human and animal pathogens, has been identified (Salmond and Reeves, 1993). The recent interest in type III (Fig 1.1) secretion system (TTSS) emerges largely from the evidence that host-pathogen interaction influences the regulation of the secretion products, with the result that secreted proteins are often delivered directly into the target cells. This triggering of secretion in response to the presence of host cells, termed contact-dependent secretion, helps to ensure that effectors are secreted only when required (Lewthwaite *et al.*, 1998; Beuzon *et al.*, 1999; Corneils and Gigsegem, 2000). This can make the systems difficult to study. Because of the nature of the TTSS, secreted proteins are often not produced in sufficient quantities to be readily detectable in culture supernatants, and are secreted only when the right environmental conditions are in place. The type III secretion apparatus is composed of approximately 20 proteins (d'Enfert, 1993; Bergmann *et al.*, 1994; Allaoui *et al.*, 1994, 1995), most of which are located in the inner membrane, and assembly of these structures requires a cytoplasmic, probably membrane-associated ATPase (Hueck, 1998).

This type of export system is responsible for secretion of Yops (*Yersinia* outer membrane proteins; prototype) in *Yersinia* species (Michiels *et al.*, 1990; Wattiau *et al.*, 1996), *Salmonella* and *Shigella* invasion and virulence factors, (Galan *et al.*, 1992; Allaloui *et al.*, 1995; Shea *et al.*, 1996), EPEC signal transduction molecules, and virulence factors in several plant pathogens including *Pseudomonas solanacearum* and *Erwinia* species (Finlay and Falkow, 1997). Secretion of bacterial pathogenic proteins by type III pathway

and their injection into the cytosol of animal or plant cell initiates a sophisticated biochemical cross talk between pathogen and host. The injected proteins often resemble eukaryotic factors with signal transaction functions and are capable of interfering with eukaryotic signalling pathways (Hueck, 1998; Kerr, 1999).

More recently, homologues to the proteins secreted by TTSS in Gram-negative bacteria have been identified in *B. bronchiseptica* (BscN), (Yuk *et al.*, 1999) and in *B. pertussis* (BpeI) (Kerr, 1999), and are under the control of the *bvgAS*-locus. The TTSS of *B. bronchiseptica* interferes with the action of the components of the innate and acquired immune systems, enabling the long term persistence of *B. bronchiseptica* in the trachea of rodents (Yuk *et al.*, 2000). TTSS may modulate immune responses during infection by inhibiting NF-κB activation by TNFα. The role of TTSS in *B. pertussis* is yet to be determined. In most cases reported to date, the G + C mol % contents of TTSS genes in various Gram-negative bacteria are lower than the surrounding genome, an indication of acquisition by horizontal transmission from a host with a lower G + C mol % content. Even when the difference in G + C mol % content does not appear to be significant, closer analysis provides support for the notion of gene transfer. In *B. bronchiseptica,* G + C mol % levels in the TTSS genes are only 2-3% lower than overall G + C mol % contents for these organisms (Winstanley *et al.*, 2000).

1.7.3.4 Type IV secretion

This pathway is primarily involved in mobilisation of DNA either between bacteria or from bacteria to plant cells; however, recently the type IV system has also been found to transport proteins, such as *B. pertussis* PTX and *Helicobacter pylori* CagA antigen (Burns *et al.*, 1999; Segal *et al.*, 1999; Stein *et al.*, 2000). While the DNA is thought to be transferred as a DNA-protein complex in one single step across the cell envelope, PTX

appears to be transported to the periplasmic compartment in a Sec-dependent (type II) manner and then translocated across the outer membrane in a separate step (Fig 1.1). Interaction between components of the pertussis toxin liberation (Ptl) system and a region of the S1 subunit appears to be essential for secretion of the assembled toxin (Craig-Mylius *et al.*, 2000). Only the holotoxin and not the S1 subunit in the absence of B oligomer is exported (Farizo *et al.*, 2000). Recent findings have suggested that disulphide bond forming enzymes or Dsb proteins are required for assembling the toxin's subunits in the periplasm (DsbA) whereas another enzyme DsbC is necessary for extracellular secretion of the toxin (Stenson and Weiss, 2002). Translocation of the holotoxin across the outer membrane is mediated by an operon of seven genes (*ptlB* to *ptlH*) that are located downstream from the *ptx* operon and are co-transcribed with the *ptx* structural genes from the *ptx* promoter (Weiss *et al.*, 1993; Ricci *et al.*, 1996; Craig-Mylius and Weiss, 1999; Burns, 1999; Farizo *et al.*, 2000). They have extensive homology with the outer membrane complex responsible for the transfer of DNA from the plant pathogen *Agrobacterium tumefaciens*. In the latter system, the tumour inducing T-DNA is secreted in the form of a complex of protein that coats the DNA (Weiss, 1994; Ricci *et al.*, 1996).

1.7.3.5 Autotransporters / Type V secretion

The autotransporters are a growing family of extracellular proteins, found in many Gram-negative bacteria that have many different functions but appear to have the same mechanism of export (Jose *et al.*, 1995; Loveless and Saier, 1997; Henderson *et al.*, 1998). As the name implies, secretion through the autotransporter pathway does not require any accessory factors. The proteins containing the N-terminal signal sequence are first translocated across the cytoplasmic membrane via the Sec machinery. The signal recognition particles (SRP), the soluble nucleoprotein complex, binds to hydrophobic

targeting signals that are found both in presecretory and integral membrane proteins (Kurzchalia *et al.*, 1986) The signal sequence of type V proteins, essential for moving the pre-protein to the periplasm, is often highly unusual, being characterized by 10-15 additional residues present after the amino terminal methionine and often preceding an unusually high number of positive charges compared with conventional amino-terminal export signals. Nevertheless, it has been suggested that the majority of these proteins are initially transported by the inner membrane Sec system (Blobel and Doberstein, 1975; Henderson *et al.*, 1998). In the cases of many proteins which do not have typical signal peptides, the first transmembrane segment can be the alternative choice for crossing the inner membrane (Friedlander and Blobel, 1985). Once the signal sequence is cleaved, the respective C-terminal domains of these secreted proteins are thought to insert into the outer membrane forming a pore that allows transport of the N-terminal portion (passenger domain) to the cell surface. The mature portion of the protein can then be released from this pore structure by proteolytic cleavage.

Table 1.2 shows the diversity of functions associated with the autotransporter passenger domains of bacteria in Gram-negative bacteria. Some of the members of this diverse family are immunoglobulin A (IgA) proteases from *Neisseria gonorrhoeae* (Klauser *et al.*, 1993), and *Haemophilus influenzae* (Poulsen *et al.*, 1989), the AIDA-I adhesin from *E. coli* (Maurer *et al.*, 1997; Suhr *et al.*, 1996), IcsA from *Shigella flexneri* (Suzuki *et al.*,1995) which is involved in intracellular spread, Tsh a temperature-sensitive hemagglutinin from an avian *E.coli* strain (Provence *et al.*,1995) and tracheal colonization factor (Finn and Stevens, 1995), the adhesin, pertactin (Charles *et al.*, 1994) and serum resistance protein BrkA (Fernandez and Weiss, 1994) from *Bordetella pertussis* (section 1.8). All of these proteins are grouped together by the following characteristics in their

respective organism:

They usually contain an N-terminal signal sequence. Most of the mature proteins are proteolytically processed into an approximately 30-kDa C-terminal domain and a much larger N-terminal domain. The C-terminal domains are predicted to form amphipathic β-barrels in the outer membrane through which the N-terminal passenger domain is translocated.

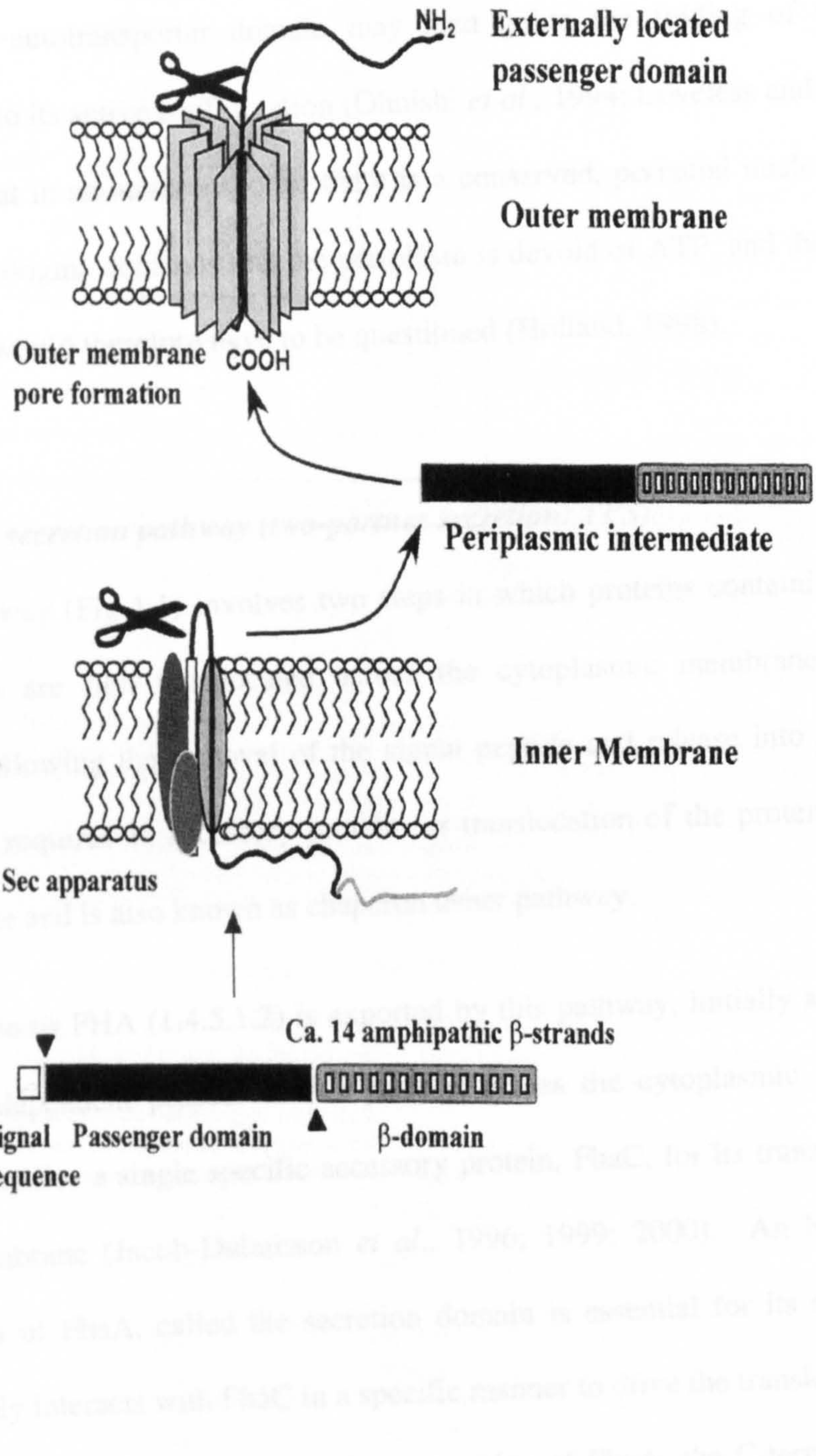
In the proposed model of autotransporter secretion (Fig 1.2) (Klauser *et al.*, 1993), an N-terminal signal sequence enables translocation across the cytoplasmic membrane. Once the protein is in the periplasm, the signal sequence is cleaved and the C-terminal domain then inserts itself into the outer membrane. It presumably forms a pore through which the N-terminal domain is exported by the formation of hairpin loop. Cleavage of the N-terminal domain is thought to occur after translocation through the outer membrane, either autoproteolytically or by another protease (Egile *et al.*, 1997). There is no evidence of autoproteolytic processing in the case of any of the autotransporter proteins known, with the exception of IgA1 protease (Jose *et al.*, 1995). C-terminal domains of autotransporter proteins exhibit sequence similarity whereas the N-terminal virulence factors are generally non-homologous except in cases of functionally similar proteins from related bacteria (Jose *et al.*, 1995). The antiparallel β-strands are considered to be amphipathic and interspersed with hairpin turns and loops (Suzuki *et al.*, 1995).

The hydrophobic sides of the β-strands presumably face the lipid bilayer while the hydrophilic faces comprise an aqueous pore. Secretion of the N-terminal domain takes place by an energy-independent unspecified mechanism as it passes through the β-barrel channel in a denatured or partially denatured state (Yanagida *et al.*, 1986). In some cases

Fig. 1.2: Shows the model for the secretion of autotransporter proteins in Gram-negative bacteria

The model presented shows the steps involved in the processing of an autotransporter precursor protein into its processed constituents i.e. passenger domain and C-terminal domain. The N-terminal signal peptide is cleaved after targeting of the precursor protein to the Sec apparatus, located in the inner membrane of Gram-negative bacteria. The periplasmic intermediate then travels to the outer membrane where its C-terminal domain forms a β-barrel pore through which its passenger domain traverses to reach the outer-membrane (surface) of the bacteria. Once exposed on the outer-membrane, the passenger domain can be cleaved off or remain attached to its C-terminal domain.

Model is taken from Klauser *et al.*, (1993).


NH2
Externally located passenger domain
Outer membrane
Outer membrane pore formation
COOH
Periplasmic intermediate
Inner Membrane
Sec apparatus
Ca. 14 amphipathic β-strands
Signal sequence
Passenger domain
β-domain

the secreted proteins remain attached to the bacterial surface whereas some proteins are released into the external medium. The junctional/linker region between the passenger domain and its autotransporter domain may then guide the folding of the exported virulence factor to its active conformation (Ohnishi *et al.*, 1994; Loveless and Saier, 1997). It is assumed that in an autotransporter there is a conserved, potential nucleotide binding motif, however, dogma demands that the periplasm is devoid of ATP, and the significance of such a motif would therefore have to be questioned (Holland, 1998).

1.7.3.6 Type VI secretion pathway (two-partner secretion: TPS)

This pathway (Fig 1.1) involves two steps in which proteins containing N-terminal signal peptides are first translocated across the cytoplasmic membrane via the Sec machinery. Following the removal of the signal peptide and release into periplasm, the mature protein requires an accessory protein for translocation of the proteins through the outer membrane and is also known as chaperon usher pathway.

B. pertussis FHA (1.4.5.1.2) is exported by this pathway, initially a signal peptide (22 residues)-dependent process allows passage across the cytoplasmic membrane and thereafter it requires a single specific accessory protein, FhaC, for its translocation across the outer membrane (Jacob-Dubuisson *et al.*, 1996; 1999; 2000). An N-proximal 115 residue region of FhaA, called the secretion domain is essential for its secretion. This region probably interacts with FhaC in a specific manner to drive the translocation of FhaA through the outer membrane. During the maturation of FhaA, the C-terminal domain of the precursor FhaB is cleaved. The function of this cleaved carboxy-terminus (150 KDa) in this instance is probably to prevent the formation of a hairpin structure within the

Table 1.2: Autotransporter proteins and their predicted functions in Gram-negative bacteria

Organism	Protein	Function
Bordetella spp.	Pertactin	Adhesin
	BrkA	Serum resistance
	Tcf	Adhesin
	Vag8	Adhesin?
Dichelobacter nodusus	BprV	Elastase?
	BprB	Elastase?
	AprV2	Elastase?
	BprX	Elastase?
Escherichia coli	Esp	Proteolytic toxin
	Pet	Proteolytic toxin
	Sat	Proteolytic toxin
	Tsh	Hemagglutinin/hemoglobin binding
	Pic	Mucinase
	AIDA-I	Adhesin
	TibA	Adhesin
	Ag43	Biofilm formation/Adhesin
Haemophilus influenzae	IgA1 protease	Cleavage of IgA1
	Hap	Adhesin/protease
	Hia	Adhesin
	Hsf	Adhesin
Helicobacter mustelae	Hsr	S layer?
Helicobacter pylori	VacA	Toxin
	BabA	Adhesin
Moraxella catarrhalis	UspA1	Adhesin
	UspA2	Serum resistance
	UspA2h	Adhesin
Neisseria spp.	IgA1 protease	Cleavage of IgA1
Pasteurella haemolytica	Ssa1	Protease
Pseudomonas aeruginosa	EstA	Esterase
Pseudomonas fluorescens	PspA	Protease
	PspB	Protease
Rickettsiales	rOmpA	Adhesin
	rOmpB	S layer/adhesin
Salmonella enterica serovar Typhimurium	ApeE	Esterase
Serratia marcescens	PrtS	Protease
	PrtT	Protease
	Ssp-H1	Protease
	Ssp-H2	Protease
Shigella flexneri	SepA	Protease/inflammation/invasion
	Pic	Mucinase
	SigA	Proteolytic toxin
	IcsA	Mediator of intracellular motility
Xeno-rhabdus luminescens	PlaA	Lipase

? = Not definite
The table is adapted from Henderson *et al.*, (2001)

periplasmic space and possibly to aid presentation of the N-terminal domain to FhaC (Renauld-Mongenie, 1996). The C-terminal domain may also prevent premature interaction between the N-terminal region and FhaC during export of these proteins towards the outer membrane (Renauld-Mongenie, 1996). The C-terminal domain of precursor FhaB is probably therefore an intramolecular chaperone of FHA (Jacob-Dubuisson *et al.*, 1996).

1.8 Members of the *Bordetella pertussis* autotransporter family

Recent work has shown that the *vag*-encoded virulence proteins PRN, TCF, BrkA, Vag-8 and most recently Bap-5 are a family of proteins that have structural homology at their C-termini and also share common properties such as RGD and SGXG motifs, although they may be functionally different. Figure 1.3 shows the major structural similarities among the autotransporter proteins of *B. pertussis*.

1.8.1 Pertactin

Pertactin (PRN) is one of a family of closely related proteins expressed by the bordetellae. Pertactins are detected as polypeptide bands of molecular weight of 69 kDa (P.69) in *B. pertussis*, P.70 in *B. parapertussis* and P.68 in *B. bronchiseptica* in SDS-PAGE profiles of their outer membrane-enriched fractions. Sequencing of these relevant genes has shown that they all contain an open reading frame encoding larger precursor polypeptides of similar size. Thus it appears that the structure of these genes is highly conserved in the bordetellae. In fact, the carboxy-terminal region is the most highly conserved region between the pertactins from the three *Bordetella* species suggesting the functional importance of this region (Charles *et al.*, 1994).

Fig.1.3: Shows some of the important structural features of autotransporter proteins of *B. pertussis*

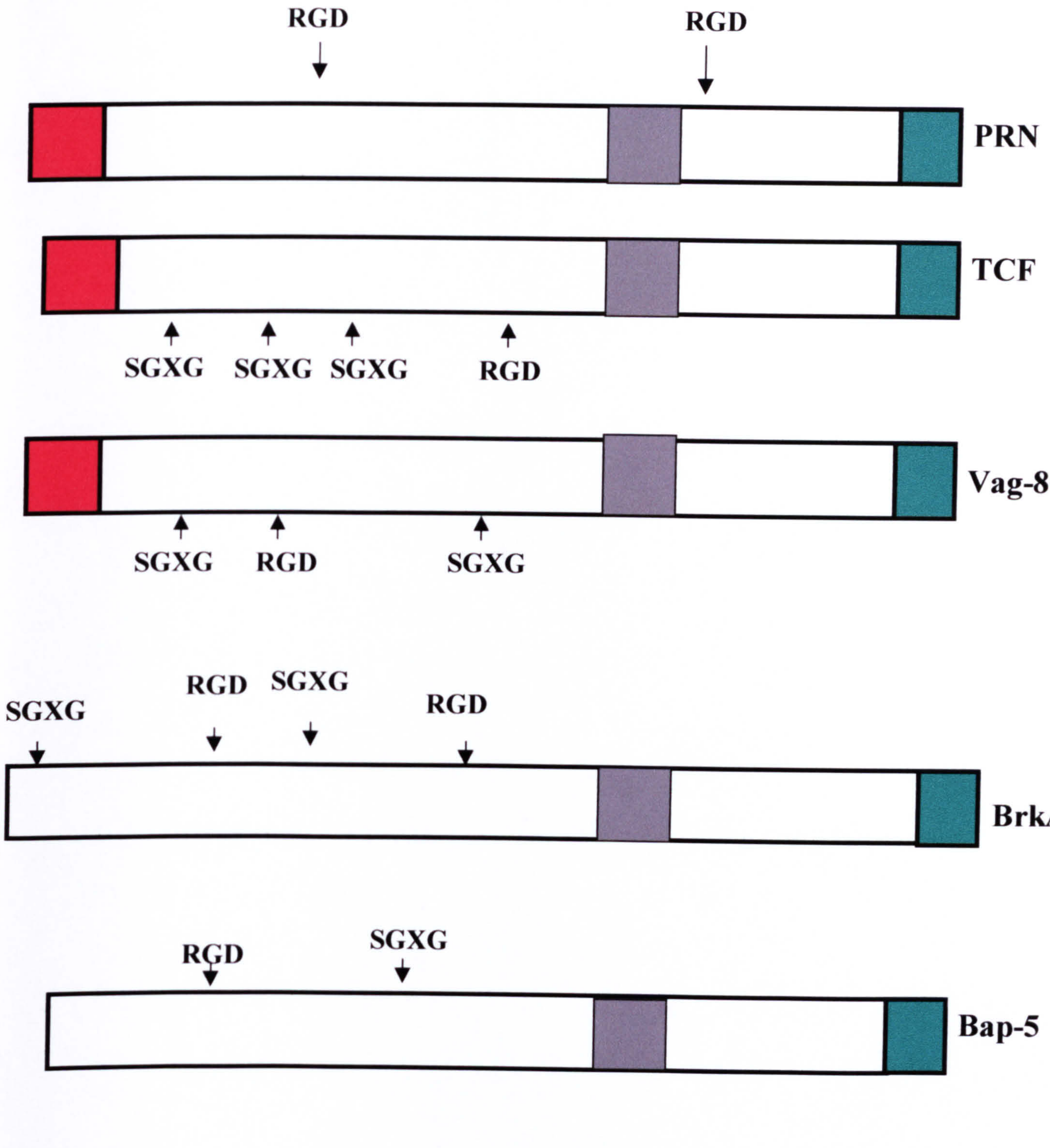
= **Outer membrane localisation signal**

= **Putative cleavage site**

= **Signal sequence**

RGD = **Integrin-binding motif**

SGXG = **Glycosaminoglycan binding motif**


RGD
RGD
PRN
SGXG
SGXG
SGXG
RGD
TCF
SGXG
RGD
SGXG
Vag-8
SGXG
RGD
SGXG
RGD
BrkA
RGD
SGXG
Bap-5

The full-length *prn* gene of *B. pertussis* comprises an open reading frame of 2730 bp capable of expressing a mature protein of 93.4 kDa. The expression of the pertactin gene family is under the control of the *bvg* global regulatory system in the bordetellae. The *bvg*-regulated promoters express poorly in the absence of the *bvg* locus, hence expression of the full length genes in a heterologous host requires the use of a functional promoter (Charles *et al.*, 1994). The precursor protein contains a 34 amino acid, amino-terminal signal sequence and a 30 kDa carboxy-terminal (β-barrel) sequence thought to be involved in translocation of the mature pertactin/P.69 onto the surface of *B. pertussis*.

The PRN cleavage event is thought to occur at a protease recognition site Lys-Arg at position 597 in the sequence (Capiau *et al.*, 1990). The β-helix of PRN is thought to be the longest helix reported to date. Several loops protrude from the helix that are associated with the biological function of pertactin including the major immunodominant region and the cell-binding motif (Fig: 1.4) (Emsley *et al.*, 1996).

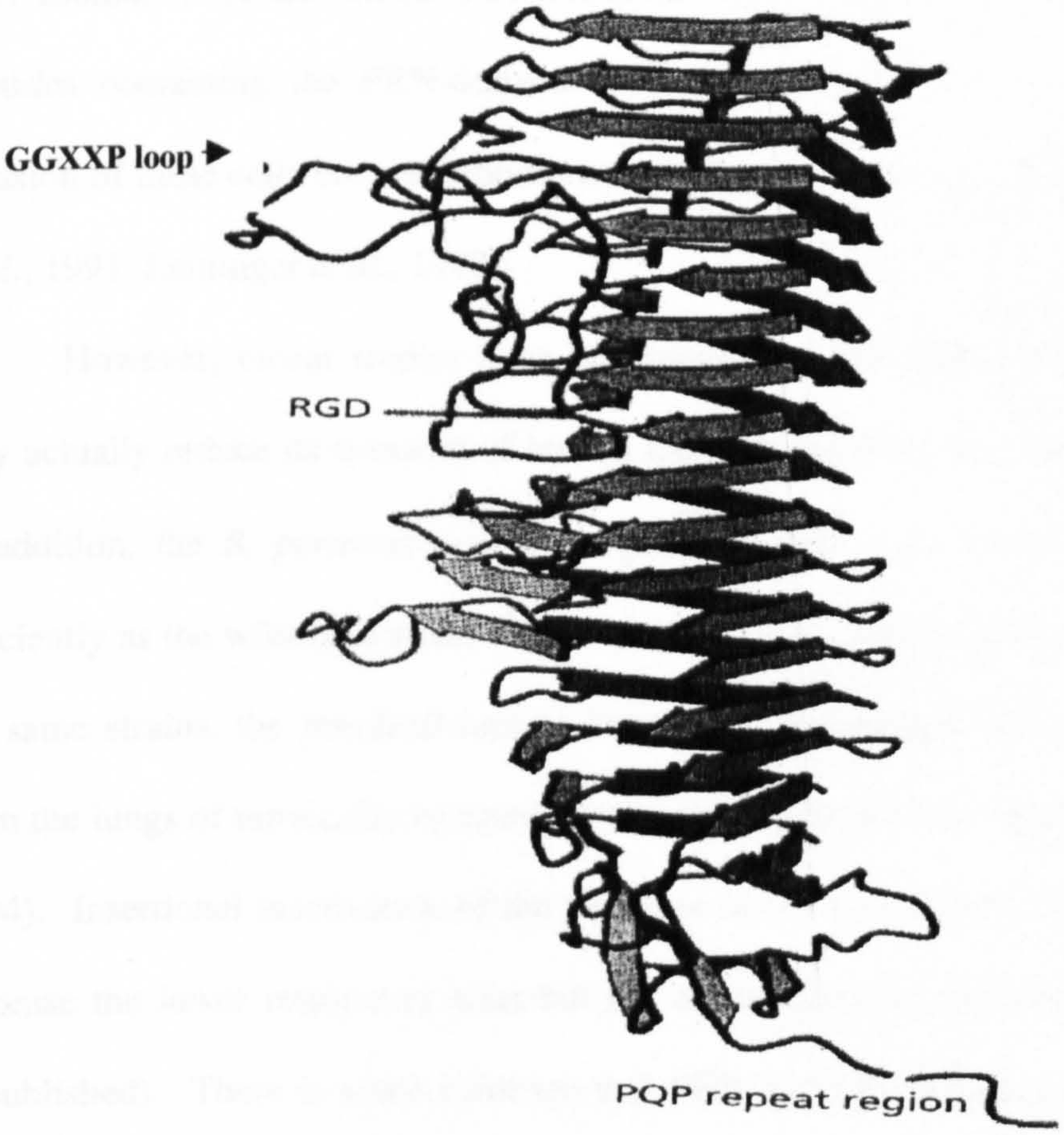
An interesting feature of the PRN molecule is the presence of two regions of direct repeats and in each case the motif is repeated five times (Charles *et al.*, 1989). The first repeat is located towards the N-terminus of the mature protein, adjacent to the RGD motif and has the sequence Gly-Gly-X-X-Pro (GGXXP). The second repeat is located near the C-terminus and has the sequence of Pro-Gln-Pro (PQP). Epitope mapping using a number of pertactin-specific monoclonal antibodies has shown that both of the repeat regions are immunodominant B- cell epitopes with PQP as a major partner (Charles *et al.*, 1991).

PRN/P.69 is a surface-associated protein of *B. pertussis* that is involved in the adherence process of the bacterium to eukaryotic cells (Leininger *et al.*, 1991; Roberts *et al.*, 1991). The mature PRN as well as its autotransporter domain contain one RGD motif. This provided the preliminary information that PRN may act as an adhesin. Chinese hamster ovary cells (CHO) were shown to interact with PRN in microtitre plate wells that

Fig. 1.4: shows the crystal structure of pertactin (P.69)

The three dimensional structure of *B. pertussis* pertactin (P.69) determined by X-ray crystallography showing the functionally important regions of the molecule and ß helix. The figure shows the positions of the immunodominant repeat regions i.e. GGXXP and PQP, of the pertactin molecule. It also shows the RGD peptide (integrin-binding site)

Figure is taken from Emsley *et al.*, (1998).


GGXXP loop
RGD
PQP repeat region

were previously coated with mature purified PRN. Moreover a *B. pertussis* PRN⁻ mutant exhibited reduced adherence to epithelial cells e.g. CHO, Hep-2; HeLa cells (Leininger *et al.*, 1991). It has also been noted that a *B. bronchiseptica* PRN⁻ mutant were less adherent to macrophages and porcine-ciliated respiratory cells (Forde *et al.*, 1999). *B. pertussis* PRN⁻ mutants were also found to be less invasive for cultured epithelial cells and synthetic peptides containing the PRN-derived RGD sequence were found to be inhibitory to invasion of these cells but not peptides containing the FHA-based RGD sequence (Roberts *et al.*, 1991; Leininger *et al.*, 1992).

However, recent studies have suggested that expression of PRN by *B. pertussis* may actually reduce its invasion of human tracheal epithelial cells (Bassinet *et al.*, 2000). In addition, the *B. pertussis prn* or *prn-fhaB* mutants were found to colonise mice as efficiently as the wild-type strain (Roberts *et al.*, 1991), although in a separate study using the same strains, the *prn-fhaB* mutant but not the *prn* mutant was cleared more rapidly from the lungs of intranaslly infected mice compared to the wild-type strain (Khelef *et al.*, 1994). Insertional inactivation of the *prn* gene in *B. bronchiseptica* reduced its ability to colonise the lower respiratory tract but not the nasal cavity of mice (Prof. M. Roberts, unpublished). There is some evidence that PRN and FHA functionally interact (section 1.4.5.1.2) (Arico *et al.*, 1993). Natural and recombinant preparations of purified pertactin/P.69 have been shown to induce protective immune responses against *B. pertussis* in experimental models (Shahin *et al.*, 1990; Roberts *et al.*, 1992). In addition, PRN has been identified as a critical antigen for improving the efficacy of acellular whooping cough vaccines in humans.

P.69 is exposed on the surface of *B. pertussis* (Roberts *et al.*, 1991). Mature P.69 was detected at the cell surface of *E. coli* TG1 only when the full length *prn* gene was used. The presence of PRN signal sequence alone, without the intact C-terminal domain,

did not appear to be sufficient to direct the mature PRN protein to the *E. coli* cell surface. It is not clear whether the P.30 carboxy terminal domain plays an active role in targeting P.69 to the cell surface like the C-terminal domains of secreted virulence factors produced by other Gram-negative bacteria such as the IgA-proteases of *Neisseria gonorrhoeae* and *Haemophilus influenzae* and elastase of *Pseudomonas aeruginosa* have been shown to play an active role in targeting these proteins. Comparison of the P.93, P.69 and P.30 amino acid sequences with those of IgA proteases, elastase and haemolysin did not reveal any significant homology (Charles *et al.*, 1994).

1.8.2 Tracheal colonisation factor

A virulence-associated factor, known as tracheal colonisation factor (TCF) is produced by strains of *B. pertussis*, but not *B. parapertussis* and *B. bronchiseptica* (Finn and Stevens, 1995). This protein is encoded by the *tcfA* gene. The derived amino acid sequence contains a RGD and three SGXG motifs and is a proline-rich protein (Finn and Stevens, 1995). Tracheal colonisation factor is a member of the autotransporter family defined upon the basis of the C-terminal domain that is highly conserved among members of this family. Western blot analysis and protein staining (immunofluorescence) data indicate that most of the apparent 90 kDa form of TCF is cell associated, whereas the apparent 60 kDa form can be found as a major band in the supernatant fraction of cultures. The C-terminal 30 kDa tail of TCF has 50 % identity over 300 amino acids to the full length precursors of the PRN proteins. The similarity in the region extends to the proposed proteolytic cleavage site identified in PRN. The ability of purified TCF to protect against *B. pertussis* infection has not been reported so far. However its immunogenicity has been indicated by intranasal immunisation of mice with an attenuated *Vibrio cholerae* strain expressing TCF, which reduced the ability of *B. pertussis* to colonise the trachea (Chen *et*

al., 1998).

1.8.3 *Bordetella* resistance to killing (BrkA)

Fernandez and Weiss (1994) identified an insertional mutant of *B. pertussis* which was at least 10-fold more susceptible to serum killing than the wild-type and was less virulent in mice. The *brk* locus encoding this serum resistance function encodes two divergently transcribed open reading frames (ORFs), termed *brkA* and *brkB* (Fernandez and Weiss, 1994). Within the 300 bases which separate the two ORFs are putative sites for BvgA binding. The BrkA precursor lacks a typical N-terminal signal sequence and it is presumed to cross the inner membrane with the assistance of the inner membrane protein BrkB. It has been reported that *brkB* has homology with ORFs of unknown function identified in *E. coli* and *Mycobacterium leprae* and is predicted to be a cytoplasmic membrane protein (Fernandez and Weiss, 1994). The 103 kDa BrkA precursor is processed to yield a 73 kDa mature protein and a ~30 kDa C-terminal autotransporter domain (Fernandez and Weiss, 1994). The recombinant BrkA C-terminal domain can form pores in lipid bilayers (Shanon and Fernandez, 1999). The gene products *brkA* and *brkB*, regulated by the *bvg*AS locus, appear to be essential for resistance to killing by the classical (antibody-dependent) complement pathway contributing to a substantial amount of resistance (section 1.9). BrkA and BrkB also separately reduce the susceptibility of *B. pertussis* to antimicrobial peptides such as the defensins that are an important feature of the mucosal immune system (Fernandez and Weiss, 1994, 1996). Recent evidence suggested that a *B. pertussis* BrkB mutant was less able to survive in human serum, but this was not found to be statistically significant difference compared to the BrkB wild-type strain (Fernandez and Weiss, 1998).

BrkA shows 29 % identity to PRN and has two RGD motifs in addition to conserved

proteolytic processing site and an outer membrane-targeting signal (Fig 1.3) and like PRN, a role in adhesion to epithelial cells and invasion of eukaryotic cells has also been suggested for BrkA (Fernandez and Weiss, 1994, 1996). Despite the similarities, a pertactin mutant was not as sensitive to serum killing as the BrkA or BrkB mutants. BrkA protein has most homology with PRN at its C-terminus but there is no amino terminal signal sequence (Fernandez and Weiss, 1994).

In addition to two RGD motifs, there are also two SGXG glycosaminoglycan (heparin) binding motifs which may also mimic the FHA-heparin interaction but could allow efficient binding of the highly glycosylated C1 inhibitor (Barnes and Weiss, 2001), or have a role in inhibiting the polymerisation of C9 and hence preventing bacterial lysis. By preventing activation of the complement cascade (Fig 1.6) beyond C4, BrkA protects the bacterium against all the effects of complement activation products such as upregulation of the immune response by C4a, C3a and C5a ; opsonisation of pathogens by C3b and iC3b; and direct killing of Gram-negative bacteria by the membrane attack complex (Barnes and Weiss, 2001). In Southern blot analysis, *brkAB* sequences were found in *B. bronchiseptica* and *B. parapertussis* but not in *B. avium*. Clinical isolates of *B. pertussis* and *B. parapertussis* were serum resistant, and wild-type strains possessing an additional copy of the *brkA* locus were 2 to 5-fold more resistant to serum killing (Fernandez and Weiss, 1998). Although BrkA confers on *B. pertussis* a resistance to killing by human serum, interestingly, loss of BrkA in *B. bronchiseptica* did not confer sensitivity to complement mediated killing (Rambow *et al.*, 1998). This may be explained by the fact that *B. bronchiseptica* is inherently more resistant to complement than *B. pertussis* (sections 1.4.5.2.5, 1.9) (Henderson and Nattaro, 2001). A BrkA mutant of *B. pertussis* was found to be 10-fold less virulent in intranasally-infected infant mice (Weiss and Goodwin, 1989; Fernandez and Weiss, 1994). There is no information regarding the

ability of BrkA to function as a protective immunogen and it is not part of any current acellular vaccines.

1.8.4 Virulence- activated gene-8 (Vag-8)

B. pertussis expresses a *bvg*-regulated 95-KDa protein called Vag-8. This *bvg*-regulated protein is the fourth member of the *Bordetella* autotransporter family to be identified (Finn and Amsbaugh, 1998). The other members of this family, PRN, TCF and BrkA, are expressed as large precursor proteins, and cleavage of a C-terminal 30-kDa fragment results in the mature protein. The N-terminus of Vag-8 possesses a predicted 37 amino acid signal sequence, the cleavage of which yields a predicted polypeptide of 91 kDa. It is not clear whether this ~91 kDa detected on the surface of *B. pertussis* represents either a Vag-8 precursor that is not processed to remove the 30 kDa C-terminal autotransporter domain or the mature protein with the predicted molecular weight of 61kDa which migrates anomalously at ~91 kDa on SDS-PAGE like PRN and TCF. The highest degree of sequence similarity in Vag-8 with other autotransporters of *B. pertussis* was found in the carboxy-terminal portion of this protein. Vag-8 like other well known *B. pertussis* autotransporters i.e. PRN, TCF and BrkA, also contains an RGD site and two SGXG motifs (Fig 1.3). The original transposon Tn*pho-vag8* mutant was found to be reduced in its ability to colonise the respiratory tract of mice but a later derived non-polar *vag8* mutant colonised the respiratory tract normally (Finn and Amsbaugh, 1998). The Vag-8 deficient mutant colonised the trachea and lungs of mice as efficiently as the isogenic parent. Southern blot analysis indicates that strains of *B. bronchiseptica* and *B. parapertussis* carry sequences similar to *vag-8*, yet expression of the protein has not been detected in *B. parapertussis* (Finn and Amsbaugh, 1998). Antibodies to Vag-8 recognised a protein of 95 kDa in a whole-cell lysates of *B. pertussis* and *B. bronchiseptica* but not in

B. parapertussis. In addition, supernatant fractions of strains of *B. pertussis* do not contain a protein which reacts with antibodies raised to Vag-8 (Finn and Amsbaugh, 1998).

1.8.5 *B. pertussis* autotransporter protein-5 (Bap-5)

A PCR amplicon MR30 produced by Prof. M. Roberts (Department of Veterinary Pathology, University of Glasgow) using primers directed to the region encoding the C-terminal domain of pertactin on *B. pertussis* genomic DNA, was later identified as a gene for fifth member of *B. pertussis* autotransporter family (Blackburn, 2000). The gene sequence was deposited in Genbank under accession no. AF081494. Because of its sequence similarity to *prn* and genes encoding three other autotransporters, namely TCF, BrkA and Vag-8, the product of *bap-5* was designated as Bap-5 (*Bordetella* autotransporter protein-5).

The predicted open reading frame (ORF) of the pertactin-like sequence was determined by amino acid homology with PRN and BrkA which begins at a serine residue encoded at nucleotide position 300 (Appendix II). This information leads to an assumption that a methionine upstream from this sequence (nucleotide position 261) may be the possible translational start. The presence of an upstream stop codon in this particular reading frame also infers that the open reading frame begins at this point. There are, however, alternative potential translational start sites downstream and it would require further investigation to ascertain the true ATG/GTG translational start codon. There is no translational stop codon until position 2538 within the expected reading frame, which is consistent with previously described *B. pertussis* autotransporters as it is immediately preceded by a consensus outer-membrane localization motif. There is a potential transcriptional terminator in the form of mRNA secondary hairpin structure located downstream of the predicted ORF (Blackburn, 2000).

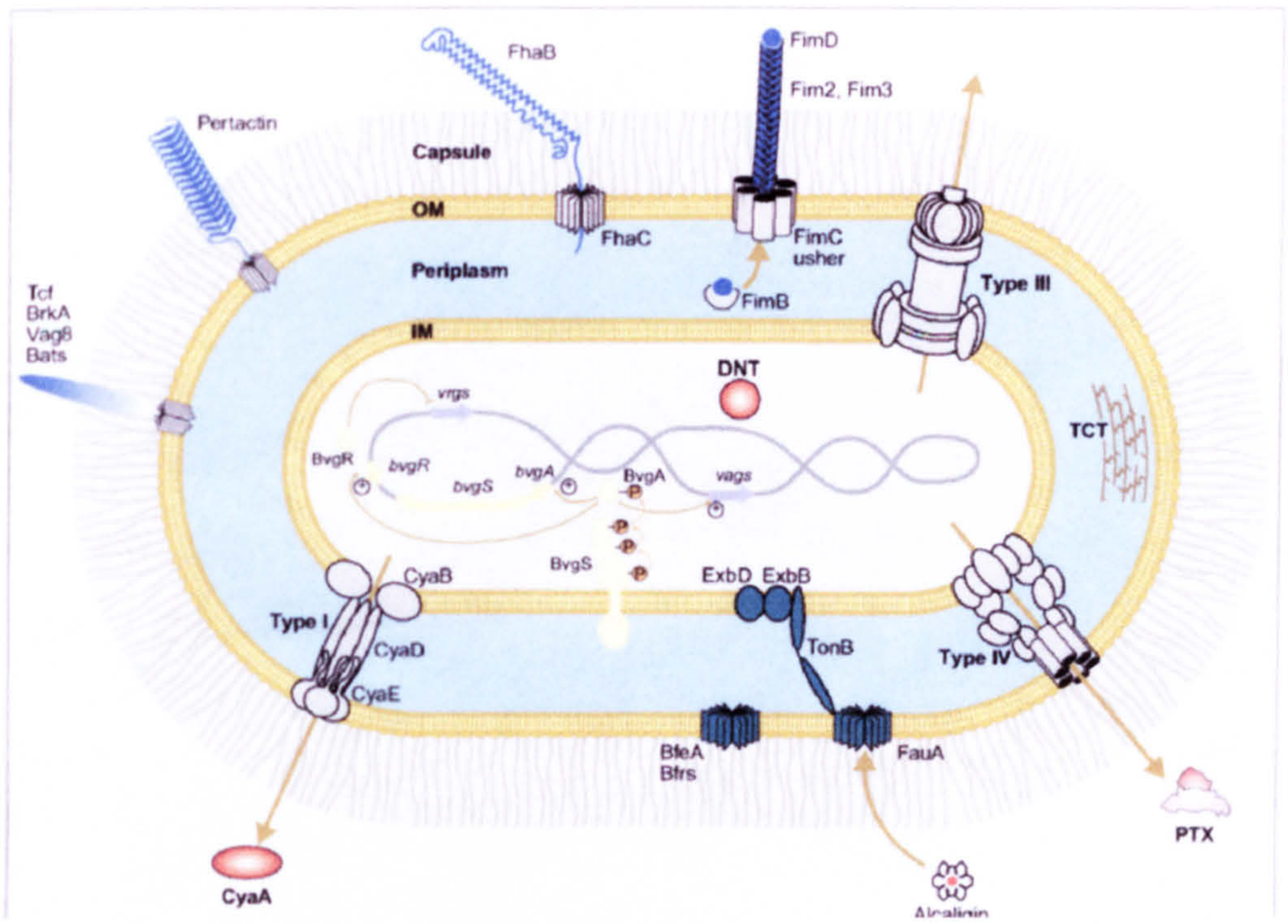
Sequence analysis has shown the presence of two integrin-binding motifs (RGD), a glycosaminoglycan-binding site (SGSG), a proteolytic processing site (ALSKRLGEL) and an outer-membrane localization signal (FHLGYRYRW, position 2510-2537) (Appendix II). No typical signal sequence or upstream regulatory elements (e.g. ribosome binding site or promoter sequences) have been identified in *bap-5* gene. The predicted molecular weight of Bap-5 is 79.9 kDa using the first ATG translational start codon. The processing at the predicted proteolytic cleavage site yields a predicted mature N-terminus of 49 kDa leaving a 30- kDa β-barrel forming C-terminus. Protein sequence analysis has shown that Bap-5 shares the highest similarity at its C-terminus with that of PRN. It was indicated by Southern blotting, using a *bap-5* specific probe, that a *bap-5*-like sequence also existed in *B. parapertussis* and *B. bronchiseptica* and preliminary evidence indicated its expression as measured by RT-PCR in *B. pertussis* (Blackburn, 2000). The final work of P. Blackburn was the cloning of a disrupted *bap-5* gene (with a kanamycin resistance cassette insertion) in the pGEMT Easy vector and I took over the project from this stage onwards.

Genes encoding several other putative autotransporters, in addition to the above mentioned autotransporters, were identified in the genome of *B. pertussis* (Dubuisson *et al.*, 2000). Several of them have significant sequence similarities to proteases, whereas others are more related to adhesins. Whether any of them plays a role in *B. pertussis* virulence remains to be investigated e.g. the gene products of five of them (Phg, AidB, SphB1, SphB2 and SphB3) are serine protease homologues. It has been reported that expression levels of the *sphB2* and *sphB3* genes were very low or undetectable, while the *phg*, *aidB* and *sphB1* genes were better expressed. In addition, *sphB1* was strongly activated by *bvg*, making it a new *vag* gene (Locht *et al.*, 2001). Figure 1.5 shows an overall view of the various adhesins and toxins produced by *B. pertussis*.

Fig. 1.5*: B. pertussis virulence factors*

Diagram showing the export of the various virulence factors of *B. pertussis* and their corresponding secretion mechanisms. Adhesins such as Fim, FHA, autotransporter proteins e.g. PRN, TCF, BrkA, Vag8, other *Bordetella* autotransporters (Bats) and toxins namely PTX and CyaA (ACT) are transported across the outer membrane via different secretory pathways. It also indicates the import of the siderophore (alcaligin) and the regulatory systems BvgA, BvgS and BvgR controlling the release of virulence factors and uptake of siderophores.

Figure is taken from Locht *et al.*, (2001).


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

1.9 General defence strategies deployed by *B. pertussis*

Gram-negative bacteria can employ its range of surface-associated or released proteins to escape, delay or overcome the immune effector mechanisms deployed by the host against it. A summary of this strategy employed by *B. pertussis* is given in the Discussion section, Table 8.1. One of the main immune effector mechanisms to effectively kill the microorganism and relevant to the present study will be discussed here i.e. complement-mediated killing.

The complement system is a series of proteins that act in a defined sequence (Fig 1.6) to promote immune clearance by opsonising or killing micro-organisms. Antigen-antibody complexes on the surface of a micro-organism can activate the classical pathway of complement, a part of the acquired immune system, by providing a binding site for C1 deposition. This will lead to a cascade of proteolytic activation steps that lead to deposition of C4b, C2a, C3b and C5b on the bacterium. Finally, after a series of binding steps the formation of the membrane attack complex (C5b-9) occurs which promotes the lysis of the bacterium.

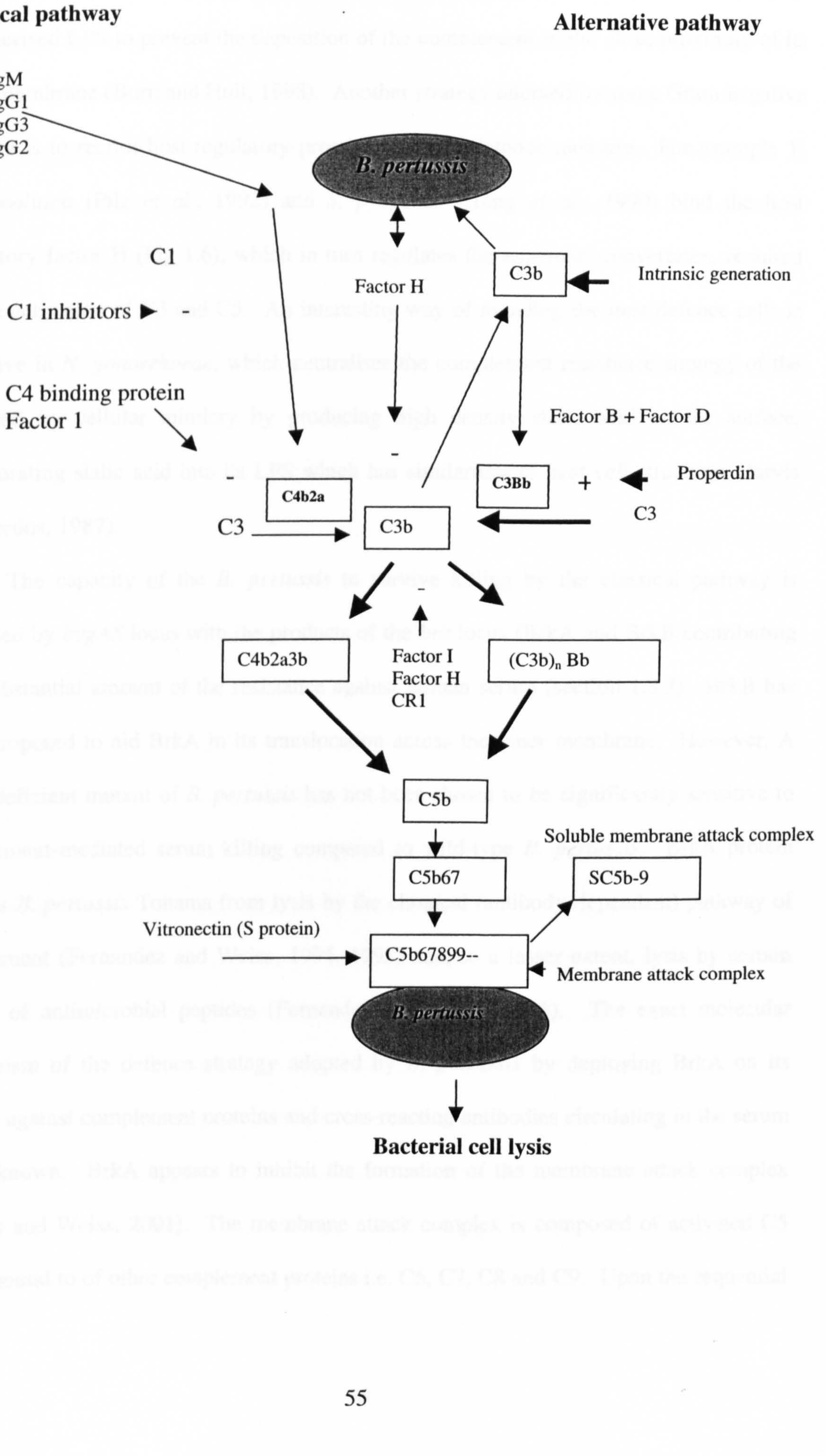
The complement cascade can be activated by carbohydrates on bacterial surfaces (such as lipopolysaccharide, LPS), that can activate the alternate pathway (Taylor, 1992; Moffit and Frank, 1994). Serum mannose-binding lectin (MBL), is able to trigger complement activation through both the classical (Ikeda *et al.*, 1987; Kawasaki *et al.*, 1989) and alternative pathways (Schweinle *et al.*, 1989), upon binding to pathogens possessing mannose, leading to direct killing.

There are various mechanism operative in Gram-negative bacteria to interfere or disrupt the activity of the complement system at many steps in the cascade (Joiner, 1988; Cooper, 1991; Frank, 1992). For example, *Pseudomonas aeruginosa* produces proteases

Fig. 1.6: Activation of complement leading to formation of the lytic membrane attack complex.

In the immune individual, the classical pathway of complement activation occurs following binding of specific antibody to outer membrane proteins (OMPs) and polysaccharide antigens on the surface of *B. pertussis* whereas, in the non-immune individual, the alternative complement pathway is activated following binding of C3b to the bacterial cell surface. For both pathways of complement activation, the final common pathway is formation of a lytic membrane attack complex, which punches a hole in the outer membrane of the organism. Deposition of complement on the cell surface also induces phagocytosis but the relative contribution of antibody, complement and phagocytes to immunity *in vivo* is unknown. + indicates a factor that enhances formation of complement complex and - indicates a factor that inhibits components of the complement cascade.

Figure has been taken and modified from Pollard and Frasch (2001).


Classical pathway
Alternative pathway
IgM
IgG1
IgG3
IgG2
B. pertussis
C1
C1 inhibitors
Factor H
C3b
Intrinsic generation
C4 binding protein
Factor 1
Factor B + Factor D
C4b2a
C3Bb
Properdin
C3
C3b
C4b2a3b
Factor I
Factor H
CR1
(C3b)n Bb
C5b
C5b67
Soluble membrane attack complex
SC5b-9
Vitronectin (S protein)
C5b67899--
Membrane attack complex
B. pertussis
Bacterial cell lysis

specific for C1q and C3 (Hong and Ghebrehiwet, 1992), enteric *E. coli* produces a highly polymerised LPS to prevent the deposition of the complement in the close proximity of its outer membrane (Burn and Hull, 1998). Another strategy adopted by some Gram-negative bacteria is to recruit host regulatory proteins as a self-defence measure. For example *Y. enterocolitica* (Pilz *et al.*, 1992) and *S. pyogenes* (Hong *et al.*, 1990) bind the host regulatory factor H (Fig 1.6), which in turn regulates the enzymes, convertases, required for the activation of C3 and C5. An interesting way of avoiding the host defence cells is operative in *N. gonorrhoeae*, which neutralises the complement resistance strategy of the host cell by cellular mimicry by producing high density sialic acid on the surface, incorporating sialic acid into its LPS which has similarities to host cell structures (Jarvis and Verdos, 1987).

The capacity of the *B. pertussis* to survive killing by the classical pathway is regulated by *bvgAS* locus with the products of the *brk* locus (BrkA and BrkB contributing to a substantial amount of the resistance against human serum (section 1.8.3). BrkB has been proposed to aid BrkA in its translocation across the inner membrane. However, A BrkB-deficient mutant of *B. pertussis* has not been shown to be significantly sensitive to complement-mediated serum killing compared to wild-type *B. pertussis*. BrkA protein protects *B. pertussis* Tohama from lysis by the classical (antibody-dependent) pathway of complement (Fernandez and Weiss, 1994, 1998) and, to a lesser extent, lysis by certain classes of antimicrobial peptides (Fernandez and Weiss, 1996). The exact molecular mechanism of the defence strategy adopted by *B. pertussis* by deploying BrkA on its surface against complement proteins and cross-reacting antibodies circulating in the serum is not known. BrkA appears to inhibit the formation of the membrane attack complex (Barnes and Weiss, 2001). The membrane attack complex is composed of activated C5 (C5b) bound to of other complement proteins i.e. C6, C7, C8 and C9. Upon the sequential

binding of C5b to C6 and C7, the complex becomes (C5b-7) lipophilic and can insert itself into the membrane. Once the complex is membrane inserted, C8 and C9 bind to it followed by the addition of more C9 molecules to form poly-C9 tailing. The poly-C9 forms the pore in the membrane of Gram-negative bacteria (*B. pertussis*) and leads to bacterial lysis and death (Fig 1.6). Alternative to this, C5b-7 complex can bind to a soluble complement regulatory protein, protein S, prior to membrane insertion and form a complex, SC5b-7. This complex is defective in associating with the membrane but can bind C8 and C9, leading to the formation of the soluble, inactive SC5b-9 complex (Kolb and Muller, 1975). The formation of SC5-9 is often measured to determine activation of the terminal complement cascade (Rinder *et al.*, 1999). However, previous results suggested that BrkA inhibits the deposition of C3 and also C4 or promotes the degradation of C4 after its deposition on the bacterial surface (Barnes and Weiss, 2001). The interaction of two proteoglycan SGXG motifs of BrkA with the C1 inhibitor is another potential mechanism of action, as it can allow the efficient binding of the highly glycosylated C1 inhibitor on bacterial surface (Barnes and Weiss, 2001). Recruitment of the C4 binding protein is another possible mechanism by which BrkA would result in a decrease in the C4 deposition on the bacterial surface.

LPS can mediate either protection or susceptibility to complement killing e.g. in enteric bacteria (*E. coli*) the long highly polymerised polysaccharide (O-chain) of the LPS on smooth strains protects the bacteria from complement while rough strains or mutants lacking the sugar repeats are killed (Frank, 1992; Taylor, 1992; Moffit and Frank, 1994). The LPS of *B. pertussis* was reported to have a simple structure consisting of lipid A, core polysaccharide and a single O-chain trisaccharide (Lebbar *et al.*, 1994; Allen et al., 1998). However many new genes and some unexpected genes (e.g. the capsule biosynthesis locus) have been identified with the completion of genome sequence of *B. pertussis,* which may

lead to a new outlook on the organism and the manner in which it interacts with the innate immune system (Preston and Maskell, 2002).

1.10 Protective capacity of *B. pertussis* autotransporters

One of the main objectives of any research on a pathogenic organism is to explore its surface architecture and attempt to exploit that information against it. That could be a achieved by the better understanding of the virulence factors of the infectious organism. Most of the members, if not all, of the *B. pertussis* autotransporter family characterised to date have some role in *B. pertussis*-associated virulence mechanisms as evident from the animal studies. Purified pertactin (P.69), one of the well-characterised autotransporter proteins, has an ability to provide protection against *B. pertussis* in the mouse respiratory tract (Roberts *et a*l., 1991). The protective properties of mature PRN (P.69) have been well documented across the *Bordetella* spp. Immunisation with vaccines containing PRN, including recombinant PRN, and attenuated *S. typhimurium* expressing PRN, provides protection against *B. pertussis* infection in mice and *B. bronchiseptica* in mice and pigs (Charles *et al.*, 1991; Roberts *et al.*, 1992; Mills *et al.*, 1993, 1998; Boursaux-Eude *et al.*, 1999; Guiso *et al.*, 1999). However the ability of the *B. pertussis* purified TCF, BrkA and Vag-8 autotransporter proteins in providing protection against the colonisation of *B. pertussis* in the respiratory tract of mice is less clear (Fernandez and Weiss, 1994; Finn and Stevens, 1995; Finn and Amsbaugh, 1998). Also there is a lack of knowledge regarding the use of the autotransporter C-terminal domains in vaccine preparations. The protective capacity of the C-termini of *B. pertussis* autotransporter proteins is difficult to assess as they have not yet been shown to be surface exposed (Charles *et al.*, 1994). P.69 is an important component of the new range of multicomponent acellular vaccines but there is no evidence to indicate whether its C-terminus has a role in protection. A study has

indicated that mice immunised intranasally with an attenuated *Vibrio cholerae* strain expressing TCF, reduced the ability of *B. pertussis* to colonise the trachea (Chen *et al.*, 1998).

The importance of presenting *B. pertussis* antigens in the correct form has been studied and it appears that the best response occurs in the mouse models when outer membrane complexes or microspheres are used (Hamstra *et al.*, 1995; Shahin *et al.*, 1995). Hamstra *et al* (1995) reported that an outer membrane complex (protein-detergent micelles) containing a 32 kDa protein (now confirmed as the TCF C-terminus) was protective in an intracerebral mouse protection tests. The 92 kDa (presumably Vag-8) protein described by Hamstra *et al* (1995) was protective only when non-protective levels of pertussis toxin were present. Monji *et al.*, (1986), suggested a role for a 30 kDa protein (possibly the BrkA C-terminus) in *B. pertussis* outer membrane preparations which potentiated an immune response to *Haemophilus* type B capsular polysaccharide, indicating a role of the C-terminal domains as adjuvants. The purified C-terminal domains of TCF and BrkA from *B. pertussis* outer membranes were not found to be protective in the intracerebral mouse protection test (Blackburn, 2000).

1.11 Aims and objectives

The main objective of the project was to characterise the function, expression, and *bvg*-regulation of the newly identified gene *bap-5,* which encodes Bap-5, an autotransporter protein of *B. pertussis,* by generating a Bap-5 deficient mutant. It was of interest to determine the role of this outer membrane protein, which shares many common features with other *B. pertussis* virulence associated proteins, to provide a better understanding of the pathogenesis of the disease, pertussis. Once constructed, a Bap-5-deficient mutant could be tested for virulence in a mouse model of pertussis and studies could be performed to determine its possible role in adhesion and invasion of mammalian cells and in serum resistance.

Part of the project involved investigating the processing of the autotransporters into a mature form. The *B. pertussis* autotransporter PRN is processed into a mature form P.69 from the precursor P.93. However, it is not clear how the mature PRN (P.69) is detached from its ~30 kDa autotransporter C-terminal domain. The proteolytic activity of PRN is not known and the primary and tertiary structure of PRN does not reveal any proteolytic domain. The possibility of involvement of unknown outer membrane proteases for the release of PRN from its autotransporter domains, as occur with other Gram-negative bacterial autotransporters, was investigated. The study was designed to provide information regarding the processing and secretion of the PRN to the outer-membranes and into the culture supernate of various protease-deficient strains of *E. coli.* This would have implications towards an understanding of the processing of the autotransporter protein in *B. pertussis* and, possibly, the pathogenicity of the organism.

2. Materials and Methods

2.1 General bacteriological procedures

2.1.1. Source, storage and media for bacteria

The details of *E. coli* strains used in this study are given in Tables 2.1 and 2.2. *Bordetella* strains used in the study are given in the table 2.4. The different media used in the study were Luria Bertani (LB) broth (Sigma) Luria Bertani agar (Sigma); Bordet-Gengou agar (Sigma). The compositions of the following media and buffers used during the study are given in Appendix I: Casamino acids (CAA), Cyclodextrin liquid (CL), Stainer-Scholte and SOC. All media were sterilised by autoclaving at 15 p.s.i. (121°C) for 15 min unless stated. Heat-labile ingredients such as antibiotics (Table 2.3) and vitamin supplements were sterilised by filtration through a sterile 0.22 μm filter (Gelman Sciences, USA) and were added to the growth medium at appropriate concentrations. Glassware was sterilised by heating to 160°C for 2 h.

For routine long term storage, the *E. coli* strains were kept at -70°C in LB broth supplemented with 50% glycerol (final concentration, v/v) whereas *Bordetella* strains were resuspended in 1% CAA containing 20% (v/v, final concentration) glycerol and stored at -70°C. Occasionally, long term storage was performed by freeze drying to increase the shelf life of bacterial strains (*E. coli* and *B. pertussis*).

2.1.2 Growth of *E. coli*

E. coli strains were grown routinely overnight at 37°C on LB agar. Where necessary, 500 ml of LB broth in a 2L-dimpled flask was inoculated from such plates or with 5 ml of liquid culture grown overnight in a universal bottle in LB medium. The flasks were incubated overnight or for the stated period of time at 37°C with shaking at 150-200 rpm.

Table 2.1: The *E. coli* strains used in this study

Strain	Genotype/Phenotypes	Source/Remarks
E. coli M15p(REP4)	Nal^S, Str^S, Rif^S, Thi^-, Ara^+, Gal^+, Mtl^-, $RecA^+$, Uvr^+, Lon^+	Qiagen; Recombinant protein expression strain
E. coli TOP10F'	F'{*lacI*^{q}, *Tn10*(Tet^R)} *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC), ϕ80*lacZ*ΔM15, Δ*lacX*74, *deoR*, *recA*1, *araD*139 Δ(*ara-leu*)7639, *galU*, *galK*, *rspL*(Str^R), *endA*1 *nupG*	Invitrogen; General transformation strain
E. coli DH5α	*supE*44, Δ*lacU*169 (ϕ80*lacZ*ΔM15) *hsdR*17, *recA*1, *endA*1, *gyrA*96, *thi*-1, *relA*1	Infection and Immunity, University of Glasgow; General transformation strain
E. coli JM109	*recA*1*supE*44, *endA*1, *hsdR*17, *gyrA*96, *relA*1, *thi* Δ(*lac-pro*AB)	Promega; General transformation strain
E. coli SM10.λ_{PIR}	*recA*::RP4-2-Tc::Mu, Km^R, *λpir*, *thi*, *thr*, *leu*, *tonA*, *lacY*, *supE*	Infection and Immunity, University of Glasgow; DNA mobilising strain

Table 2.2: The protease-deficient strains of *E. coli* used in the study

Strain designation	**Relevant genotypes**	**Relevant protease-deficiency associated with each individual strain and their described roles**
KS272(parent)	F *ΔlacX 74, galE, galK, thi, rpsL(strA), Δpho(pvuII)*	Parent strain
SF110	*ompT, degP*	OmpT: A trypsin like outer membrane protease which cuts between lysine-arginine, arginine-arginine, arginine-valine, arginine-alanine
SF120	*ompT, degP, ptr*	DegP: possesses a proteolytic and general chaperonic activity; located in periplasm and may be associated with envelopes.
HM101	*tsp, eda*	Eda: An aldolase enzyme which was disrupted with a tetracycline resistance marker for selection purposes.
HM111	*ompT, tsp, eda*	Tsp: a C-terminal specific periplasmic protease; cleaves by endoproteolytic cleavage and cuts between alanine-leucine, alanine-lysine, alanine-arginine and valine -serine
HM112	*ptr, tsp, eda*	Ptr (Protease III): a metalloprotease mainly found in periplasm and perhaps associated with envelopes; cleaves between phenylalanine-tyrosine, tyrosine-leucine, tyrosine-isoleucine
HM120	*degP, ompT, tsp, eda*	
HM130	*degP, ptr, ompT, tsp, eda*	

Table 2.3: The antibiotics used in the study

Antibiotic	Concentration μg/ml
Ampicillin (Ap)	100
Chloramphenicol (Cm)	20
Kanamycin (Km)	40
Nalidixic acid (mono-sodium salt) (Nal)	40
Streptomycin (Sm)	100
Tetracyclin (Tet)	40

2.1.3 Growth of *B. pertussis*

Growth of *B. pertussis* requires special media and a long incubation time (bacterial duplication time is approximately 4-6 h at 35-37°C). *B. pertussis* was grown on BG agar containing 15% (v/v) defibrinated horse blood (E and O Laboratories, Scotland) and 1% (v/v) glycerol. Plates were placed in a humidified box and incubated for 2 -3 days at 37°C. The *B. pertussis* colonies can be maintained for a period of 7-10 days on BG agar plate stored inverted at room temperature or at 4°C in a humified condition. Where necessary, a large inoculum, such as loopful of bacteria from a plate grown to confluence, was added into 10 ml of CL medium in a 25 ml dimpled flask. After 24-36 h, this culture was used to inoculate 500 ml of fresh CL medium in 2L dimpled flask which was incubated for 2-3 days at 37°C with shaking at 150-200 rpm.

2.1.4 Growth of other *Bordetella* strains

Routinely *B. bronchiseptica*, *B. parapertussis* and *B. avium* were grown for 24-48 h at 37°C on BG agar plates. Where necessary, 500 ml of CL broth in 2L dimpled flasks were inoculated from such BG plates and incubated for 24-48 h at 37°C with shaking at 150-200 rpm.

2.1.5 Spontaneous antibiotic resistant strains of *B. pertussis*

During the genetic manipulations of bacteria, it is often desirable to use strains that can be easily selected, for example on antibiotic-containing plates. The most commonly used antibiotics for generating spontaneous antibiotic resistant strains of *B. pertussis*, are streptomycin and nalidixic acid. In order to raise spontaneous antibiotic resistant strains for the above mentioned antibiotics, a 10 ml volume of a mid-log phase culture was centrifuged and resuspended in 0.1-0.2 ml of fresh Stainer-Scholte medium. Aliquots

Table 2.4: The *Bordetella* strains used during the study

Strain	Genotype/ Phenotype	Source/Remarks
B. pertussis Taberman	Parent	Isolated from a child post-mortem. Ruchill Hospital, Glasgow (1979)
B. pertussis Taberman I	Sm^R and Nal^R derivative of BP Taberman Parent	This study
B. pertussis Taberman II	Bap-5-insertional mutant of Taberman I	This study
B. pertussis Tohama	Parent	Division of Infection and Immunity, University of Glasgow
B. pertussis Tohama I	Sm^R and Nal^R derivative of BP Tohma parent.	This study
B. pertussis Tohama II	Bap-5 insertional mutant of Tohama I parent	This study
B. pertussis 18-323	Wild-type	Challenge strain for intracerebral mouse protection tests. Manclark, C. Centre for Biologics Evaluation and Research, Bethesda, MD 20892 U.S.A.
B. pertussis Tohama (BP338)	Derivative of BP Tohama (Nal^R). Parent of BrkA mutant 2041 and BP 347.	Weiss, A. Dept of Molecular Genetics, University of Cincinnati
B. pertussis Tohama (BP2041)	Deletion mutant of BrkA	Weiss, A. Dept of Molecular Genetics, University of Cincinnati
B. pertussis Tohama (BP2041)	Sm^R and Nal^R derivative of BP2041, created for making a BrkA and Bap-5 double mutant	This study
B. pertussis Tohama (BP347)	Avirulent mutant. Tn insertion of Tohama. Bvg^- (Vir^-)	Weiss, A. Dept of Molecular Genetics, University of Cincinnati
B. pertussis BBC29	Wild-type	Prof. M. Roberts, Dept of Veterinary Pathology, University of Glasgow
B. pertussis BBC30	Deletion mutant of PRN	Prof. M. Roberts, Dept of Veterinary Pathology, University of Glasgow
B.parapertussis NCTC5952	Wild-type	Division of Infection and Immunity, University of Glasgow
B.bronchiseptica 214	Wild-type	Division of Infection and Immunity, University of Glasgow
B. avium 4480	Wild-type	R. Rimler, Ames, Iowa

(100 µl, 50 µl) of the bacterial suspension were then spread on BG agar plates containing 100 µg/ml of streptomycin or 40 µg/ml of nalidixic acid and incubated at 37°C for 4-5 days under humidified conditions to allow the growth of the spontaneous resistant strains.

2.1.6 Bacterial conjugation and gene replacement

Conjugation between *E. coli* and *B. pertussis* is a common way to introduce DNA into the *B. pertussis* chromosome. The structure of the most widely used conjugative plasmid pRTP1 (return to pertussis) in *Bordetella* has been described previously (Stibitz *et al.*, 1986). A derivative of this plasmid, pSS1129 (Stibitz and Yang, 1991) was used to create *B. pertussis* mutants. Bacterial conjugation was carried out by plate mating on BG agar plates between *E. coli* SM10.λ_{PIR} (donor strain) carrying the mutated *bap-5* gene on pSS11229 and *B. pertussis* recipient strains (Taberman 1 and Tohama 1). One freshly transformed colony of donor *E. coli* strain SM10.λ_{PIR} was grown in LB broth at 37°C for 4-5 h to reach an OD_{600}~0.4. *B. pertussis* was grown in Cyclodextrin liquid (CL) medium (10 ml) until the OD_{600}~0.5 (~early logarthmic phase). The concentration was adjusted by comparison with 5th International Reference of Opacity (Perkins *et al.*, 1973) to 10 opacity units i.e. ~10^9 CFU/ml. Dilutions of *E. coli* SM10.λ_{PIR} cultures were then prepared containing ~10^7 or 10^5 CFU/ml and centrifuged at 14000 rpm for 1 min and, in parallel 1 ml culture of *B. pertussis* containing ~10^9 CFU/ml was centrifuged at 14000 rpm for 1 min. The pellets were resuspended gently and washed twice in their respective media and pelleted again at the same speed as above for 5 minutes. Pellets of both donor and recipient strains were resuspended in 100 µl of CL medium and mixed together in a 1.5 ml eppendorf tube at different donor: recipient ratios i.e. 1:1, 1:2, 1:3. The mixture was plated on BG agar

plates supplemented with 10mM $MgCl_2$, which is known to facilitate conjugation, in the form of 10 µl drops at various spots and incubated at 37°C for 14 h (Stibitz and Yang, 1991).

The bacterial cells were then collected with the help of loop, in Stainer-Scholte medium, washed and resuspended in fresh Stainer-Scholte medium. Bacterial suspensions obtained were then plated (100 µl) onto BG agar plates supplemented with nalidixic acid and kanamycin and incubated at 37°C for 4-5 days. This step selected for the incorporation of the suicide plasmid into the recipient chromosome via a single cross over event. To select for loss of the plasmid and a second recombination event, the exconjugants were plated on BG agar plates supplemented with streptomycin and incubated at 37°C for 4-5 days. This selected for the loss of plasmid which codes for sensitivity to streptomycin due to the presence of the native ribosomal proteins (*rspL*). The survivors of the streptomycin selection were further analysed for the loss of the plasmid and retention of kanamycin resistance by streaking on to a BG plate containing kanamycin and streptomycin together and incubating for 3-4 days at 37°C. Further confirmation for the loss of integrated plasmid was achieved by their inability to grow on BG agar plates containing ampicillin (a marker on the suicide plasmid) (Stibitz, 1994).

2.2 DNA extraction

2.2.1 Genomic DNA

For routine isolation of genomic DNA from *B. pertussis,* Promega's Wizard® genomic DNA purification kit was used. Bacteria from 1ml volumes of an overnight

culture grown to OD_{600nm}~ 0.6-0.7 were harvested by centrifugation at 13,000 rpm for 2 min in a 1.5 ml eppendorf and the supernate was discarded. To the pellet was added 600 µl of nuclei lysis solution (Promega) and the mixture was pipetted gently to completely mix and the sample was then incubated at 80°C for 5 min and then cooled at room temperature. 3 µl of RNase solution was then added and, after thorough mixing, the sample was incubated at 37°C for 30 min and then cooled to room temperature. After cooling, 200 µl of protein precipitation solution (Promega) was added, the mixture vortexed and incubated on ice for 5 min. The sample was centrifuged at 13,000 rpm for 3 min and the supernate was carefully transferred to an eppendorf tube containing 600 µl of isopropanol and mixed thoroughly. The sample was centrifuged at 13,000 rpm for 2 min and to the pellet was added 600 µl of 70% ethanol. The sample was then mixed, and centrifuged for 2 min at 13,000 rpm. The supernate was removed and the pellet was air-dried to completely remove traces of ethanol. The DNA pellet was rehydrated by adding 100 µl of rehydration solution (Promega) for 1 h at 65°C or occasionally overnight at 4°C. The DNA was aliquoted and stored at -20°C.

2.2.2 Plasmid DNA

The QIAprep® Miniprep purification system (Qiagen) was used for plasmid DNA isolation according to the manufacturer's protocol. In brief, a 5ml volume of an overnight *E. coli* culture was centrifuged at 10,000 x *g* for 10 min (Biofuge, Rotor: HFA 14.2). The resultant bacterial pellet was resuspended in 250 µl of Buffer P1 and lysed using 250 µl of Buffer P2 (alkali-detergent solution) for 5 min at room temperature. The macromolecules were then precipitated using 350 µl of Buffer N3 (chaotropic solution) and centrifuged at 15,000 x g for 10 min (Biofuge, Rotor: HFA 14.2). The supernate was added to a QIAprep® mini column and centrifuged at 10,000 x *g* for 1min. The flow-through was

discarded and the column was then washed with 500 µl of Buffer PB (trace nuclease removal) and centrifuged at 10,000 x *g* for 1min. The flow-through was discarded and the final wash was performed with 750 µl of Buffer PE (containing ethanol) with a further centrifugation at 10,000 x g for 1 min. An additional centrifugation was performed following flow-through removal to ensure thorough removal of ethanol. Finally, the DNA was eluted by centrifugation after addition of 40-80 µl of sterile distilled preheated (65°C) water. The detail of plasmids used during the study is given in Table 2.5.

2.3 Agarose gel electrophoresis

2.3.1 Sample preparation

The sample DNA (5-30 µl) was mixed with 6 x DNA loading buffer (Appendix I) in a 5:1 (vol/vol) ratio prior to loading into the wells. Molecular weight markers (1 Kb ladder, Gibco BRL, UK) were utilised according to manufacturer's instructions.

2.3.2 Gel preparation

Pre-weighed agarose (type II-A medium EEO, Sigma), was suspended in 0.5 x Tris-borate-EDTA (TBE) buffer (Appendix I) and heated until the agarose solution became clear (completely dissolved). The solution was allowed to cool to the extent that it was still warm and ethidium bromide (Bio-Rad, UK) was added to a final concentration of 0.5 µg/ml. A gel tray was prepared by taping the edges with adhesive tape and the gel was cast to the desired thickness. Upon setting, the gel was immersed in 0.5 x TBE buffers containing ethidium bromide (0.5 µg/ml) in a horizontal submarine electrophoresis tank (E-C Apparatus Corporation, USA). A power pack (model SL3655, Scotlab, UK) was

used to provide a constant voltage corresponding to 1-5 volts/cm. Electrophoresis was carried out until the marker dye in the loading buffer migrated an appropriate distance.

2.3.3 Visualisation of DNA

The ethidium bromide-stained DNA was visualised using a UV transilluminator (model TM-40, UVP Inc., California, USA). Images were stored electronically as appropriate using the Gel Documentation System-Image Store 5000, version 7.2 (Ultra Violet Products, Cambridge, UK) as well as printed using a video graphic printer (model UP-860, Sony). Electronic images were edited using Adobe Photoshop 3.0 and images labelled with Microsoft PowerPoint 4.0

2.3.4 Gel extraction procedure

For DNA purification purposes, a Qiaex®II purification kit (Qiagen) was used according to the manufacturer's instructions. The band of interest was excised from the agarose gel and solubilised in Buffer QX1 (usually 3 volumes of buffer to one volume of gel). Qiaex resin (10-15 µl) was introduced and the mixture incubated at 50°C for 10 min. Following centrifugation for 1 min at 10,000 x *g* the supernate was removed. The pellet was then washed once with 500 µl of Buffer QX1 and twice with 500 µl of Buffer PE, with centrifugation for 1 minute at 10,000 x g. The resin/DNA pellet was air-dried, 5-25 µl of sterile distilled water was added and, after incubation for 5 min at 50°C, the resin was removed by centrifugation as before and the eluted DNA was retained.

Table 2.5: Plasmids used or created during this study

Plasmid Name	Comments	Source/Remarks
pET11a(BCT1)	*brkA* C-terminal region cloned into pET11a: expression construct for BrkA C-terminus	P. Blackburn, Div. of Infection and Immunity University of Glasgow
pET33b(PCT1)	*prn* C-terminal region cloned into pET33b: expression construct for Prn C-terminus	P. Blackburn, Div. of Infection and Immunity University of Glasgow
pET33b(TCT1)	*tcf* C-terminal region cloned into pET33b: expression construct for Tcf C-terminus	P. Blackburn, Div. of Infection and Immunity University of Glasgow
pET33b(Bp5CT1)	*bap-5* C-terminal region cloned into pET33b: expression construct for Bap-5 C-terminus	P. Blackburn, Div. of Infection and Immunity University of Glasgow
pQE-60(Bp5ATG1)	*bap-5* cloned into pQE-60: full length expression construct for Bap-5 (from the 1st predicted translational start site, ATG1) (Appendix II)	P. Blackburn, Div. of Infection and Immunity University of Glasgow
pGEMT(Bap-5 Km^r)	*bap-5* gene disrupted with kanamycin resistance cassette in pGEMT Easy vector	P. Blackburn, Div. of Infection and Immunity University of Glasgow
pBAD/gIIIA (PCTlink)	pertactin C-terminal and linker region (PCT+link) cloned into pBAD/gIIIA: expression construct of Prn C-terminus and linker fused to a signal sequence	P. Blackburn, Div. of Infection and Immunity University of Glasgow
41869p	Full length pertactin gene (p.93) cloned into a broad-host range expression vector pMMB66EH	M. Roberts, Dept of Veterinary Pathology, University of Glasgow
pSS1129	Suicide vector used for gene replacement in *B. pertussis*	Stibitz, S. (1994)
(pBap5-Kana)	Suicide vector containing mutated *bap-5* gene disrupted by insertion of kanamycin resistance cassette	This study

2.4 Quantification of DNA

The concentration of DNA was estimated by measuring the absorbance at 260 nm in a 1-cm quartz cuvette and by applying the equation:

Concentration of DNA (ng/µl) = A_{260nm} x 50 x dilution factor

2.5 Restriction enzyme reactions

All reactions were performed according to the manufacturers' instructions (Gibco BRL, New England Biolabs or Promega). Total reaction volumes of 20 µl and 30 µl were commonly used. Where appropriate, the enzymes were heat-inactivated (65°C for 15 min) and the DNA was purified using the Qiaex®II kit, often in conjunction with agarose gel electrophoresis if isolation of a single linear restriction product was required.

2.6 Polymerase chain reaction

The PCR conditions were optimised, where necessary, according to the orthogonal array method described by Cobb *et al.* (1994). The annealing and elongation thermal parameters were then adjusted to obtain optimal conditions. A Hybaid thermal cycler (model: Touchdown, Hybaid Ltd, Middlesex, UK) was used for all reactions. The products of the PCR were stored at -20°C or used immediately.

2.6.1 Primers

During primer design, care was taken to avoid potential internal secondary structure and, where possible, a GC clamp was engineered at the 3' end of the primer. Additionally, primers were checked to avoid overlap and possible dimerisation, potential for secondary structure formation and for compatible Tm values. Tm values were calculated according to

the equation: $Tm\ (°C) = 4(G + C) + 2(A + T) - 5°C$

Primers (50 nmol, desalted and deprotected) were obtained from Gibco BRL (Paisley, UK) and resuspended in sterile distilled water to give final concentrations of 50 pmol /µl or 0.5 pmol /µl for PCR. The nucleotide sequences are taken from the *bap-5* gene sequence (Appendix II).

Table 2.6: Primers used during the study

Primer designation	Primer sequence
bap-5 Forward 5'-3'	ATGGCACCTCGCCTTCGATTCGCGTCCAAG From nucleotide 286
bap-5 Reverse 5'-3'	AGGTGGAACGTCCAAGGCAAGGTCAGCTTG From nucleotide 2518
bap-5 NTS Forward 5'-3'	ATGGGTGCAAATAACGTCGCTGTG From nucleotide 336
bap-5 NTS Reverse 5'-3'	GTCGTAGTAATACTCGACTGCATCG From nucleotide 791

2.6.2 HotStarTaq™ method

Hot-start PCR was performed according to the HotStarTaq™ PCR kit (Qiagen) manufacturer's instructions. The following master mix was prepared in a thin-walled 0.5 ml tube immediately before use (per reaction):

Master mix per reaction	µl
10 x PCR buffer (containing 1.5 mM $MgCl_2$)	2.5
5 x Q solution	5.0

dNTP mix (10 mM of each)	0.5
HotstarTaq DNA polymerase	0.2
Distilled water	15.3
<u>Added individually in tubes</u>	<u>µl</u>
5' primer (50 pmol/µl)	0.5
3' primer (50 pmol/µl)	0.5
Template DNA (100ng-1 µg)	0.5
Total volume in each PCR tube	**25**

Q-solution changes the melting behaviour of the DNA and is particularly useful when amplifying GC rich templates. The following thermo-cycling parameters were used:

Initial activation step:	15 min	95°C
and 30 cycles of		
Denaturation:	60 sec	94°C
Annealing:	60 sec	60°C
Extension:	1 min	72°C

For PCR products longer than 1 Kb the extension time was increased by 1 min per Kb DNA. For amplification of the full length *bap-5* gene (2.2 Kb) the extension time was optimised at 3 min.

Final extension step	10 min	72°C

2.7 Cloning protocol

2.7.1 DNA preparation

Vector and insert DNA were subjected to restriction endonuclease digestion to

obtain complimentary cohesive overhangs. The resultant fragments were purified from the agarose gel using the Qiagen purification kit. After gel purification, the resultant vector was treated with alkaline dephosphorylase (calf intestine, Promega) at 37°C for 45 min, then heated to 65°C for 10 min to inactivate dephosphorylase and then re-purified by passing through a Qiagen mini-centrifuge column at 10,000g. DNA concentrations were estimated according to section 2.5 and occasionally DNA samples (insert and vector) were compared with the 1 Kb ladder (Gibco, BRL) the concentration of whose individual bands are given by the manufaturer. Insert: vector ratios of 1:1 and 3:1 were commonly used for ligation.

2.7.2 Ligation strategies

Ligation reactions were performed in a total volume of 10 µl using 3 units of T4 DNA ligase (Promega, USA) and Promega ligase buffer or occasionally Boehringer ligase, using their specified buffers according to the manufacturer's instructions. After incubation at 16°C for 18h (Promega ligase) or 4°C for 18h (Boehringer ligase), the ligation was terminated by heating the reaction mix to 70°C for 10 min and the products were stored at -20°C until use.

2.8 Transformation

2.8.1 Preparation of electroporation-competent cells

To a 2L dimpled flask containing 500 ml of LB medium, 5 ml of an overnight culture of *E. coli* was added. The flask was incubated at 37°C on the shaker until an OD_{600nm} of 0.6-0.8 was obtained and thereafter it was chilled on ice. Bacterial cells were

harvested at 7000 g for 15 min in a Sorvall superspeed (rotor GS-3). The resultant bacterial pellet was washed and resuspended in 500 ml of cold sterile distilled water and centrifuged as mentioned above. The cell pellet was again resuspended in 250 ml of cold sterile water, centrifuged as above and resuspended in 10 ml of cold sterile water containing 10% (v/v) glycerol. Following a final centrifugation, the cells were resuspended in 2.0 ml of cold sterile glycerol 10% (v/v) and 100 µl aliquots were snap frozen in liquid nitrogen. Cells were kept cold (on ice) at all times during the procedure and were stored at -70°C.

2.8.2 Electroporation procedure

Electroporation cuvettes (0.2 cm) (Flowgen, UK)) and the safety chamber were chilled at -20°C. To the chilled cuvette, 48 µl of competent cells and 2 µl of DNA (plasmid preparation) were added. The mixture was mixed using a sterile yellow tip, shaken to the bottom of the cuvette and the cuvette placed in a Bio-Rad Gene Pulser set at 2.5 KV, 25 µFD and was connected to a Bio-Rad pulse controller set to 200Ω. The cells were pulsed once for 4-5 msec. Immediately following electroporation, 1 ml of pre-warmed (37°C) SOC medium (Appendix I) was added to the cells and thoroughly mixed and then incubated in a water bath at 37°C without shaking for 1 h.

2.9 Southern blotting

The detailed composition of all solutions is given in Appendix I. Unless otherwise stated, all reagents were supplied by Boehringer Mannheim. The DNA samples to be analysed were electrophoresed on an ethidium bromide-stained agarose gel. The gel was then depurinated by soaking it in 0.25M hydrochloric acid for 15 min and then rinsed thoroughly in dH_2O twice. The gel was allowed to float in denaturing solution for 45 min

with gentle shaking followed by rinsing it twice with dH_2O. Then it was soaked for 30 min in neutralising solution (Appendix I). The gel was placed on the top of a wick made up of 3MM Whatmann filter paper saturated with 20 x SSC (Appendix I) and then blotted overnight by capillary action onto positively-charged nylon membrane (Boehringer). After dismantling the blotting set up, the positions of wells were marked to determine the orientation of the gel later on. The nylon membrane was removed and washed in 2 x SSC to remove any agarose particles and wrapped in cling film. DNA was then crosslinked to the membrane with the exposure of each side of the membrane for 1 min using a UV cross linker (Spectrolinker XL-1000, Spectroline, New York, USA). The membrane was then prehybridised for 4-6 h in a rolling hybridisation oven (model HB-1D, Techne, Cambridge, UK) with at least 20ml of prehybridising solution (Appendix I). The temperature of the oven was set at 65°C for prehbridisation. This temperature was maintained for the subsequent hybridisation and washing steps (see below).

2.9.1 Preparation of digoxigenin-labelled probes

The digoxigenin (Dig) labelled probes were synthesised using a Dig-labelling PCR kit (Boehringer Mannheim). The PCR kit is provided with the Dig-labelled mix, which is a mixture of lithium salts of dNTPs and digoxigenin-11-dUTP. This nucleotide mixture can be added (200 μM each in final concentration) directly to the PCR reaction and the Dig-labelled nucleotides will be incorporated into the PCR product. Hotstar Taq polymerase was used under similar conditions to those in section 2.6.2, using the primer sequences *bap-5* Forward and *bap-5* Reverse (Table 2.6) which cover the full length *bap- 5* gene (Appendix II). The PCR product can be cleaned after agarose gel extraction as described in section 2.3.2. The contents of the labelling tube were then added to 8 ml of

prehybridisation solution (Appendix I) and stored at -20°C. Denaturation of the probe was performed for 10 min in a boiling water bath followed by immediate chilling on ice just before use.

2.9.2 Probe hybridisation

The prebridisation was performed at 60°C for 3-4 h in prehybridisation mix (Appendix I). The prehybridising solution was then discarded and the denatured probe immediately added to the roller tube carrying the DNA samples cross-linked to the nylon membrane. Hybridisation was performed overnight (12-14 h) at 60°C. The probe was poured into a universal tube and stored at –20°C. The membrane was washed at the hybridisation temperature for 2 x 15 min in 50 ml of 2 x SSC / 0.1% SDS (w/v) at room temperature (low stringency washing), followed by 2 x 15 min washes in 50 ml 0.5 x SSC / 0.1% SDS (w/v) at 65°C (high stringency washing).

2.9.3 Chemiluminescence detection

Detection was performed according to the protocol described in the DIG system User's Guide for Filter Hybridisation (Boehringer Mannheim). The washed membrane was equilibrated for 1-2 min in wash buffer (Appendix I). Antibody blocking solution (Appendix I) was then incubated with the membrane for 30-60 min. The antibody (anti-Dig-conjugated to alkaline phosphatase) was centrifuged for 5 min to remove any aggregates and then diluted to 1 in 5,000 (2 µl per 10 ml) in the antibody blocking buffer and the membrane was then incubated for 30 min in at least 20 ml of antibody solution at room temperature. The membrane was then washed for 2 x 15 min in washing buffer and then equilibrated in detection buffer (Appendix I) for 5 min. The alkaline phosphatase substrate used (NBT)) was diluted to 1 tablet in 10 ml of detection buffer and this was

dripped onto the membrane, avoiding air bubbles, prior to sealing within a plastic sheet, for 5 min. The excess substrate was removed using a damp paper towel on the top plastic surface. The plastic was heat-sealed and then left at room temperature for 15-45 min in the dark.

2.10 Protein analysis

2.10.1 Fractionation of bacterial cells and collection of concentrated supernate

The composition of all reagents and buffers can be found in Appendix I or otherwise stated in the text. Bacteria cells were harvested from 500 ml of broth culture at 5000 g for 15 min in a Sorvall superspeed centrifuge (rotor GS-3). The pellet of bacteria was resuspended in 50-ml of envelope buffer (10 mM sodium phosphate, pH 7.2). The cells were disrupted using a mechanical cell disrupter (One Shot, Constant Systems, Warwick, UK) to 15 K p.s.i or occasionally, using a cell sonicator (Sonics and Materials; probe SM 05) for 3 X 45 sec while keeping the cell container on ice. The resultant lysate was centrifuged at 2500 x *g* for 5 min in a Sorvall superspeed centrifuge (rotor SS34) to pellet large debris and unbroken cells. The supernate was centrifuged in a Sorvall ultracentrifuge (OTD-COMBI) at 100,000 rpm for 60 min at 4°C. The resulting supernate contained the soluble content of the cytoplasm and periplasm. The pellet obtained, containing cell envelopes, was rinsed in envelope buffer and the tubes dried upside down on tissue paper for 10 min. It was then resuspended in 0.5 ml of envelope buffer containing sodium N-laurylsarcosinate (sarcosyl) 0.5% (w/v) and then mixed thoroughly by frequent pipetting and brief vortexing after every 5 min for 30 min to differentially solubilise the cytoplasmic membrane proteins. In some experiments, protease inhibitor cocktail (Sigma) was also included at this stage. A further centrifugation was performed at

100,000 rpm for 1h at 4°C as described previously. The final supernate contained the sarcosyl-soluble inner membrane proteins and the final pellet contained the sarcosyl insoluble outer membrane. The outer membrane-enriched fractions were resuspended in 0.5-1 ml of envelope buffer (Appendix I). The 10-20 ml volume of the bacterial culture was spun down at 7000 x g for 10 min in a Sorval superspeed centrifuge (SS34) at 4°C. The supernate fraction was transferred carefully into another tube without disturbing the pellet. The clear supernate fraction collected was passed through a 0.2 μm filter to remove any particulate matter or cells. 4.5 ml of supernate was then poured into a spin column (Greiner, 30,000 M.W. cut-off) and centrifuged at 7500 x g for 15 min at 4°C in centrifuge ((Jouan: MR1822, 80mM). The concentrated material was collected and the column reused for further centrifugation steps. The centrifugation process was repeated with further volumes 4-6 times until the desired concentration (~40 times) from an initial volume of about 10-20 ml was achieved.

2.10.2 Expression of recombinant proteins

The expression of the different recombinant proteins in various *E. coli* strains was regulated either by isopropyl β-D- thiogalactopyranoside (IPTG from Sigma) inducible promoters or L-arabinose inducible promoters.

Overnight cultures (5 ml) of *E. coli* strains (Table 2.1 and 2.2) containing expression constructs (Table 2.5) were used to inoculate 2L dimpled flasks containing 500 ml volumes of LB with appropriate antibiotics. The cultures were then incubated at 37°C with shaking until an OD_{600nm} of 0.7-0.9 was obtained. Either IPTG (1mM final concentration) or 0.2% (w/v) of L-arabinose was added to the culture and incubated at 37°C continued on the shaker for further 3 h. Cells were harvested at 5,000 x g for 15 min in a Sorvall superspeed centrifuge (rotor GS-3).

2.10.3 Urea extraction of inclusion bodies

After the expression of recombinant protein, bacterial cells were harvested as described above. The cell pellets were resuspended in 20 ml of Buffer A (Appendix II) and incubated with lysozyme (Sigma) 1µg/ml and DNaseI (protease free, Sigma; 0.5 µg/ml) and protease inhibitor cocktail 2µg/ml (Sigma) for 1 h on ice. The cell suspension was disrupted using a cell sonicator and the resultant lysate was centrifuged at 17,000 x *g* in a Sorvall Superspeed centrifuge (rotor SS34) for 30 min to allow inclusion bodies to be pelleted. The pellets were resuspended in 10 ml of Buffer B (Appendix I) and inclusion bodies were solubilised, with tube rotation, for 4 h at room temperature or overnight at 4°C, until the suspension became clear. A final ultracentrifugation step of 100,000 x *g* for 3 h was performed and the supernate, containing urea-soluble protein, was retained and stored at -20°C.

2.10.4 Small-scale affinity purification (His-tagged proteins)

Purification of histidine-tagged proteins (Blackburn, 2000) was performed with the Ni-NTA spin kit (Qiagen) according to the manufacturer's instructions. Briefly, a Ni-NTA column was set up by pouring the column with Ni-NTA agarose 1-2 ml equilibrated with 5 ml of Buffer A for 20-30 minutes by allowing the buffer to pass drop by drop. The flow-through was discarded. To the equilibrated column, 2 ml of urea-soluble protein in Buffer A (Appendix I) was added and allowed to stand for 2 min to interact with the Ni-NTA agarose. It was then allowed to flow through drop by drop. The column was then washed two times with 5 ml of Buffer B (Appendix I). The protein bound to the resin was again washed with 5 ml of Buffer C (Appendix I) twice. Finally it was eluted twice with 500 µl of Buffer D (Appendix I) and the eluates were pooled.

2.10.5 Protein estimation

2.10.5.1 Lowry (modified) procedure for membrane proteins (Folin-Ciocalteu)

The composition of all the stock solutions relevant to this procedure is given in Appendix I. To make the working solution, 100 parts of solution A were mixed with 1 part of reagent B to form reagent C, the alkaline copper reagent. Sample volumes of 1ml containing approximately 10-100 μg/ml of protein, along with known protein standard BSA ranging from 20-200 μg/ml, were mixed with 3 ml of reagent C and the tubes were incubated at room temperature for 10-60 min. The samples were then mixed vigorously with 0.3 ml of Folin-Ciocalteu reagent diluted in distilled water (1vol/1vol) and incubated for 45 min at room temperature. The absorbance at 660 nm was determined with the help of a microtiter plate reader and protein concentrations estimated from the standard curve.

2.10.5.2 Standard procedure for soluble proteins (Bio-Rad)

Dye reagent was prepared by diluting 1 part of dye reagent concentrate with 4 parts of double distilled water according to the manufacturer's instructions. This was filtered through a Whatmann No1 filter paper to remove particulates. This reagent is stable for about 2 weeks when kept at room temperature. Dilutions of protein standard (BSA) and the protein solution to be tested were prepared. The linear range of this assay is 50-500 μg/ml. Protein solutions were normally assayed in duplicate or triplicate. 20 μl of each standard or sample solution, containing different protein concentrations, was pipetted into separate microtitre plate wells. The diluted dye reagent (200 μl) was then added to each well. The sample and reagent were thoroughly mixed using a multichannel pipete to dispense the reagent. The samples were incubated at room temperature for a

maximum of 1 h and absorbance was measured at 595 nm. The protein concentration in the test sample was estimated from the standard curve.

2.10.6 Sample preparation for electrophoresis

50 µl of sample was mixed with 10 µl of 6x protein loading dye (Appendix I). Samples were stored at -20°C until loading. Just before loading on to the polyacrylamide gel, samples were heated in a boiling water bath for 10 min. A 10 kDa protein ladder (Gibco BRL) or Bio-Rad precision protein (stained and unstained) standards were used according to the manufacturer's instructions.

2.10.7 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The method for SDS-PAGE was adopted from Laemmli (1970) and gels were set up in a vertical electrophoresis gel tank (model SE600, Hoefer Scientific Instruments, California, USA). The composition of all solutions can be found in Appendix I. Glass plates were assembled according to the manufacturer's instructions. Resolving gels, containing 10-14% acrylamide, were poured in between the assembled plates until the gel was 4 cm below the top of the plates and 100% ethanol was used as an overlay. The gel remained undisturbed for approximately 1 h at room temperature until completely set. The ethanol was poured off and the gel surface washed with distilled water. A stacking gel containing 6% acrylamide was poured onto the polymerised separating gel and a comb was placed onto the gel solution. Again the gel was left for approximately 1 h at room temperature until set. The comb was removed and wells were immediately washed once with 1x electrode buffer and filled with 1 x electrode buffer and assembled into the electrophoresis gel tank. The lower buffer reservoir was stirred with a magnetic stirrer during vertical gel electrophoresis. After addition of samples to the wells the gel was run

at 20 mA until the tracer dye entered the separating (lower) gel and thereafter the current was increased to 50 mA. The gel was removed when the tracker dye reached the bottom of the separating gel (approximately 4 h). Occasionally the gel was allowed to run at constant current 20 mA overnight (14 h). The gel was removed from the plates and stained or blotted as appropriate. For staining, coomassie blue stain (Appendix I) was utilised, for a minimum of 3 h or overnight, on a rotating platform. The stain was then replaced with de-stain solution (Appendix I) until the background became transparent.

2.10.8 Western blotting

2.10.8.1 Blotting protocol

To transfer proteins resolved by SDS-PAGE onto the immobilising nitrocellulose membrane (Hybond-C, Amersham Pharmacia Biotech) the method of Towbin *et al* (1979) was followed. In short, the stacking gel was cropped and the separating gel was rinsed gently in electrode buffer (Appendix I) for 5 min. The blot was then assembled and placed in the electroblotting apparatus (Trans-Blot Cell, Bio-Rad, USA) containing electroblotting buffer (Appendix I) with magnetic stirring. The transference of the proteins from the gel to the membrane was carried out at constant 50 volts for 1-2 h and occasionally at 30 volts overnight.

2.10.8.2 Development of blots

Once proteins were transferred onto the membrane, it was equilibrated for 1-2 minutes in electroblotting buffer at room temperature. To see if the proteins were transferred effectively, the membrane was immersed into Ponceau S solution (Sigma, 0.1% w/v Ponceau S and 5% v/v Acetic acid) for 1-2 min. The stained background was removed

by destaining in water for 2 min and the position of the molecular weight markers marked with indelible ink if unstained protein standards were used. The membrane was then destained in phosphate-buffered saline (PBS) for 10 min and incubated with blocking buffer (Appendix I) for 1 h at room temperature with gentle agitation. The primary antibody was diluted appropriately in blocking buffer and incubated with the membrane for 1 h at room temperature on a rotating platform. The membrane was then washed for 15 min in 2 changes of PBS at room temperature. Horseradish peroxidase-conjugated secondary antibody (anti-mouse or anti-rabbit as appropriate, Scottish Antibody Production Unit, UK) diluted 1:1000 (v/v) in blocking buffer was added to the membrane and incubated for 1h at room temperature. Again the membrane was washed for 2 x 15 minutes in PBS at room temperature. The membrane was then immersed in freshly prepared 3, 3'diaminobenzidine (DAB) (Sigma) 0.05% w/v in PBS for approximately 1-2 min and the staining reaction was stopped by washing the membrane with distilled water.

2.11 RNA analysis

2.11.1 Extraction of total RNA from B. pertussis

The RNeasy mini kit (Qiagen) was used for the extraction of total RNA from *B. pertussis* cells according to the manufacturer's instructions. The bacterial cells were harvested from 1 ml volume of mid-log phase cultures in CL medium. Occasionally bacteria were collected from heavily inoculated BG-plates (24-48h growth) and resuspended in 1 ml volume of sterile PBS in 1.5 ml eppendorf. The number of cells was adjusted to ~$1x10^9$/ml using an opacity rod. The pellets were collected after centrifugation at 10,000 x g for 5 min at 4°C and the supernates were discarded. Cells were then resuspended thoroughly in 100 µl of TE buffer (10mM Tris + 1mM EDTA, pH 8.0) containing 400 µg/ml of lysozyme, vortexed for 5 s and incubated at room temperature for

5 min. Then, 350 µl RLT buffer (chaotropic reagent, protects RNA) was added to the sample and vortexed vigorously. After centrifugation for 2 min at 10,000 x g, the supernate was collected carefully without disturbing the pellet. To the lysate was added 250 µl of absolute ethanol and then mixed well by pipetting. The sample was then added to the RNeasy minispin column in a 2 ml collection tube. The minispin column was then centrifuged for 15 s at 10,000 x g and the flow through was discarded. Then 700 µl RW1 buffer (washing buffer) was pipetted onto the RNeasy mini column and centrifuged for 15-s at 10,000 x g. The RNeasy column was transferred into a new 2 ml collection tube, and 500 µl buffer RPE was pipetted onto it and the tube was centrifuged for 15 s at 10,000 x g to wash off the column-bound RNA. An additional 500 µl of RPE buffer was added and centrifuged at 10,000 x g for 2 min to dry the RNeasy spin-column membrane completely. The RNeasy spin column was placed in a new 2 ml collection tube and centrifuged at 10,000 x g for 1 min. The RNeasy column was then transferred to a new 1.5 ml diethylpyrocarbonate (DEPC: RNase inhibitor) treated collection tube and 30-40 µl of RNase-free water was added directly onto the RNeasy membrane. The column was centrifuged for 1 min at 10,000 x g. The eluted RNA was then stored at -70°C in 10 µl aliquots until used. For running agarose gels with RNA samples, the procedure was the same as in section 2.3 with the exception that all the buffers were made in DEPC-treated water and morpholinopropane-sulphonic acid (MOPS) buffer (Appendix I) was used instead of TE buffer. The gel tank was also treated with DEPC-treated water.

2.11.2 Removing the co-purified contaminating DNA

This step was adopted to remove any trace of genomic DNA co-purified along with total RNA. The procedure was used for removal of DNA prior to RT-PCR. All solutions were made up in DEPC-treated water and RNase free tips and tubes were used

PROCEDURE

In brief the following reagents were added to the DEPC-treated eppendorfs

Total RNA (with contaminating DNA)	≤ 1 μg
10 x DNase buffer (Appendix I)	= 2 μl
RNase inhibitor (Promega; RNasin)	= 10 units
RNase free DNase (Qiagen)	= 0.5 kunitz units
Total volume of reaction set with RNase free water	= 20 μl

The samples were incubated at 37°C in a water bath for 30 min and the reaction was stopped by adding 5 mM EDTA, mixed thoroughly and left at room temperature for 5 min. Finally, the samples were incubated at 65°C for 5 min to inactivate DNase completely. Occasionally, samples were spun down through the mini columns provided with the RNeasy mini kit and the RNA was collected by eluting it with RNase-free water (section 2.11.1). The samples were stored at –70°C in aliquots.

2.11.3 Reverse transcription-PCR

The reverse transcription-PCR (RT-PCR) was performed using the Omniscript Kit (Qiagen, UK) according to the manufacturers' instructions using a two step reaction i.e. cDNA synthesis and PCR. The following components were assembled in a thin-walled 0.5 ml tube, on ice for cDNA synthesis

RNA template (section 2.11.2)	4-6 μl
10x reverse transcriptase buffer	2 μl

5mM dNTP's	2 µl
RNasin	1 µl
3'-5' primer (50p/mole stock; Reverse primer)	1 µl
Total volume adjusted with RNase free water	20 µl

The above reaction was thermocycled as follows:

First strand cDNA synthesis

1 cycle	39°C	60 min
1 cycle	93°C	2 min

cDNA kept in aliquots of 4 µl in 0.5 ml PCR tubes at –20°C.

Second strand cDNA synthesis (PCR amplification)

The second strand synthesis was carried out using the standard PCR procedure with the exception of a slight modification (section 2.6.2). 3 µl of cDNA template obtained from the previous step was added to the PCR tube after the initial step of activation of HotStar Taq i.e. 95°C for 15 min.

Master mix	µl
10X PCR Buffer (containing 15 mM $MgCl_2$)	2.5
5x Q solution	5.0
dNTP mix (10 mM of each)	0.5
Hotstar Taq DNA polymerase	0.5
Distilled water	13.5
Added individually in tubes	
5' primer (50 pmol/µl)	0.5
3' primer (50 pmol/µl)	0.5

Template cDNA (100 ng-1 μg)	3.0
Total volume in each PCR tube	**25 μl**

The following thermo-cycling parameters were used:

Initial activation step:	15 min	95°C	
Denaturation:	30 sec	94°C	**40 Cycles**
Annealing:	60 sec	60°C	
Extension:	1 min	72°C	
Final extension step	10 min	72°C	

The products were analysed by agarose gel electrophoresis according to section 2.3

2.12 Antibody production

Rabbit anti-Bap-5 antibody was raised by Dr. D. Xing, Department of Bacteriology, NIBSC, London, against the purified full length Bap-5 protein prepared during the study. His-tagged Bap-5 cloned in pQE-60 (Bp5ATG1) expression construct (Table 2.5) was expressed in *E. coli* M.15 (Table 2.1) and purified (section 2.10.4). The purified samples of Bap-5 (8 mg/ml) were run on 10% SDS-PAGE and the protein was transferred to nitrocellulose membrane. After the transfer it was then briefly stained in ponceau S (Sigma) to locate the band. The protein bands were removed with a sterile knife and the membrane strip washed twice with sterile PBS. After running several gels to obtain a sufficient amount of protein, the nitrocellulose membrane was chopped into very fine pieces in a sterile petri dish and then sonicated in 3-4 ml of PBS to obtain an injectible solution (Diano *et al.*, 1987). The material obtained was then sent to Dr D. Xing for the production of antiserum.

For the production of antibody, three female New Zealand Albino rabbits, each weighing approximately 2.5 Kg, were used. Before injection, blood samples were taken from each rabbit. The antigen solution was mixed in equal proportion with Freund's incomplete adjuvant such that each 0.5 ml contained approximately 1 mg of antigen. 0.5 ml volumes were injected subcutaneously using a 21 gauge needle at 3 different sites. Four weeks later, the first booster was given exactly in the same way as above. Also a blood sample was taken at that occasion. The second dose was given after a further 4 weeks and the rabbits were test bled at this occasion. Two weeks after the second boosting, the rabbits were terminally bled and serum was collected from each rabbit and pooled. The detail of other antibodies used during the study is given in Table 2.7.

2.13 *In vivo* tests

2.13.1 Mouse protection test using P.30 and P.69 antigens

The construct expressing pertactin C-terminal domain (pET 33b PCT1; Table 2.5) was expressed in *E. coli* M.15 (Table 2.1) and the protein extracted from inclusion bodies by solubilisation in 8M urea (Blackburn, 2000; Prof. M. Roberts, Department of Veterinary Pathology, University of Glasgow). The crude urea extracts of P.30 were dialysed against 1 M urea and then re-folded by making gradual dilution using a re-foldase buffer (Prof. N. Isaacs, Department of Chemistry, University of Glasgow). To prepare the vaccine doses re-natured P.30 or purified P.69 (recombinant P.69 purified from *Pichia pastoris*) kindly provided by Prof. M. Roberts, Department of Veterinary Pathology, University of Glasgow, 100 μg/ml of protein was mixed with an equal volume of alhydrogel $Al(OH)_3$. Thus each 0.2-ml volume contained 10 μg of protein and 10 μg of alhydrogel. A control for the experiment was to replace the antigen solution with an equivalent volume of PBS added to an equal volume of alhydrogel.

Table 2.7: Antibodies used during the study

Antibody designation	Used for	Source
Rabbit polyclonal anti-whole Bap-5	Western blots and immunofluorescence, agglutination and bactericidal assays	This study
Mouse polyclonal anti-whole Bap5	Western blots	P. Blackburn, Div. of infection & immunity, University of Glasgow
Mouse polyclonal anti-Bap-5 N-terminus (NTS)	Western blots	P. Blackburn, Div. of infection & immunity, University of Glasgow
Mouse polyclonal anti-Bap-5 C-terminus	Western blots	P. Blackburn, Div. of infection & immunity, University of Glasgow
Mouse monoclona anti-BrkA C-terminus (P28)	Western blots	J.B. Hertz, Copenhagen, Hvidovre Hospital
Rabbit polyclonal anti-whole *B. pertussis*	Agglutination test and ELISA-based adhesion studies	J.B. Hertz, Copenhagen, Hvidovre Hospital
Rabbit polyclonal anti-PRN (mature,p.69)	Western blots, agglutination tests and bactericidal assays	M. Roberts, Dept of Vet. Pathology, University of Glasgow
Mouse monoclonal anti-PRN linker-region (BBO5)	Western blots	M. Roberts, Dept of Vet. Pathology, University of Glasgow
Mouse monoclonal anti-TCF C-terminal (49.3)	Western blots	J.B. Hertz, Copenhagen, Hvidovre Hospital
FITC conjugated goat anti-rabbit antibody	Immunofluorescence	SIGMA

2.13.1.1 Immunisation

Randomised groups of female CD1 mice (Harlan Olac, Bicester, Oxfordshire, UK) aged 3-4 weeks (4 mice per group), were injected subcutaneously, under light halothane anaesthesia, with 10 μg of test antigen per mouse. One group of mice was vaccinated with renatured P.30 antigen whereas another group received the same amount of purified pertactin (P.69), a well-known component of acellular vaccines. A third group of mice received only alhydrogel in PBS. For each test group, a second dose, as above, was-administered three weeks later. The weight of each group of mice was recorded prior to the vaccination and thereafter at regular intervals to check for any toxicity because of the antigen preparation.

2.13.1.2 Intranasal challenge

B. pertussis 18-323 was grown as a lawn on BG plates in a humidified box at 37°C for 24h. The resultant growth was suspended in 1% (w/v) casamino acids solution (CAA) (Appendix I) and adjusted to 10 opacity units using the 5th International Reference of Opacity (Perkins *et al.*, 1973) i.e. approx. $2x10^9$ CFU/ml. Two weeks after the second vaccination the challenge suspension of *B. pertussis* 18323 containing ~$2x10^6$ CFU/ml was prepared and a sublethal dose of $1x10^5$ CFU in 50 μl volume was instilled intranasally to each mouse under light halothane anaesthesia. The weight of each group of mice was recorded prior to challenge as well as at regular intervals afterwards. Four mice/group were sacrificed at each sampling time on days 0, 3, 9 and 14 and the lungs and trachea were separately removed aseptically into sterile universal bottles. The nasal cavities of each mouse were located carefully by detaching the head of the animal first and then flushing sterile casamino acid through the nasal cavity (1ml each); the nasal washes were collected in sterile tubes.

2.13.1.3 Bacterial counts

The nasal washes were collected in volume of 1 ml CAA and 100 µl volumes of undiluted samples were cultured on BG agar. Organs (lungs and trachea) were homogenised in 10 ml of CAA with a sterile homogeniser (Silverson machines, UK). 100 µl volumes from undiluted samples and 1:10 and 1:100 dilutions in 1% (w/v) CAA were cultured on BG agar plates and incubated at 37°C in a tightly covered humidified box. Colonies were counted after 3-4 days.

2.13.2 Virulence tests

Randomised groups of female CD1 mice (Harlan Olac, Bicester, Oxfordshire, UK) aged 3 weeks were allowed to acclimatise for 3-4 days. They were divided into different challenge groups, which were categorised according to experiment number. In experiment I mice were challenged intranasally with *B. pertussis* strains 18323 (standard mouse virulent strain), Taberman I strain (clinical isolate of *B. pertussis*) and the Bap-5 deficient mutant of Taberman. In experiment II mice were challenged with *B. pertussis* strain BBC29 and its PRN-deficient mutant strain BBC30 as well as Taberman and its derivative Bap-5-deficient mutant strain as in experiment I. Each group comprised five mice and each mouse was challenged intranasally with *B. pertussis* containing ~$1x10^5$ CFU in 50 µl of (PBS), under halothane anaesthesia. The body weights of the mice were monitored regularly for 7 days. Mice were killed on day 7 with an overdose of CO_2 and viable counts of *B. pertussis* in lungs, tracheas and nasal washings assayed as described above.

2.14 Statistical analysis

The number of colony forming units per lung, trachea and nasal washes was extrapolated from the colony counts obtained on BG agar plates. Nominal values of 10

and 10^7 were given to counts outside the detection limits <100 and >$3x10^6$ per organ respectively. The log_{10} counts were calculated and the means, standard deviation and standard error values of each group were calculated in Excel. The data was analysed by applying the Student's *t*-test and one way of analysis of variance (ANOVA).

2.15 *In vitro* tests

2.15.1 Agglutination test

This test was initially carried out to identify surface-exposed Bap-5 in *B. pertussis* using antibodies to Bap-5 (anti-whole Bap-5). The Bap-5 deficient mutant was used as a negative control. The antibodies to other antigens like PRN and whole *B. pertussis* were also used in the studies (Table 2.6). The procedure involved the collection of bacteria from BG plates that had been incubated for 48-72 h at 37°C. One ml of a thick suspension was obtained by spinning down ~7 ml volume of culture containing ~$2x10^9$ CFU/ml of the various *B. pertussis* parent and mutant strains and re-suspending the pellets in 1 ml of PBS. A 100 µl volume from the thick suspension was added to individual wells in a 96 well u-shaped plate (Greiner, UK) in quadruplicate. Thereafter, 50 µl volumes of antisera were added to each well. The samples in each well were mixed thoroughly with a multichannel pipette and plate was placed on a rotating shaker for 1 h at room temperature. The plate was then incubated at 37°C for 3-4 h and thereafter left at 4°C for 24-48 h. At the end of the experiment, agglutination or button formation at the bottom of each well as well as the type of agglutination was observed.

2.15.2 Fluorescence microscopy

Immunofluorescence was carried out to identify Bap-5 on the surface of *B. pertussis*. Again, a Bap-5 deficient mutant was used as a negative control. Rabbit anti

whole Bap-5 serum and flourescein isothiocyanate conjugated-alkaline phosphate (FITC-AP) anti-rabbit IgG whole molecule (Sigma) were used in the assay.

2.15.2.1 Staining of bacteria for immunofluorescence

One ml volume from a *B. pertussis* culture grown for 48 h in cyclodextrin liquid medium (OD_{600}=1.0) or in some experiments *B. pertussis* grown on BG agar plates, was re-suspended to the desired volume in PBS to make a thick suspension. The *B. pertussis* suspension was standardised to ~$2x10^8$ CFU/ml using the Opacity rod. The cells were centrifuged at 8,000 x g for 5 min in 1.5 ml eppendorf. The pellet obtained was rinsed carefully with PBS (pH=7.4) and fixed with acetic acid: ethanol (3:1, v/v) for 5 min at room temperature. The tubes were centrifuged for 2 min at 8,000 x g and the pellet obtained was rinsed with PBS twice and then left inverted on a clean paper towel for ~5-10 min at room temperature to let the pellet dry or occasionally left at 4°C, overnight. The cells were then incubated with Hoechst 33258 stain (1 in 200 v/v ratio from the stock of 5mg/ml in 70% ethanol, in sterile PBS) which stains the nuclear material of bacteria, for 30-60 min at room temperature. The cells were then washed with PBS 3-4 times for 1 min and finally once for 10 min.

2.15.2.2 Immunofluorescence

12 well multitest slides (ICN, Biomedicals) were coated with 0.1 % poly-lysine (Sigma) in PBS overnight at 4°C. 5-10 µl volume of the suspension containing the stained bacterial cells was seeded into each well and the slide was covered and left for 10-15 at room temperature. It was then rinsed gently 2-3 times with 0.1% Tween 20 in PBS in a series of coplin jars for 1-2 min to detach the loosely-bound bacteria. Blocking was carried out in BSA (2% w/v) in PBS for 45 min in coplin jar at 37°C or overnight at 4°C. The

slides were then shifted into a coplin jar containing rabbit anti-Bap-5 serum 1:1000 (v/v) in 2 % BSA in PBS for 2 h at 37°C. The slide was then washed 3-times for 5 min in a series of coplin jars containing sterile 0.1% Tween 20 in PBS. The slide was then immersed in coplin jar-containing anti rabbit FITC-AP conjugate diluted in 2% BSA in PBS (1:80) for 1 h at 37°C. Then the slide was washed 3 times for 5 min in 0.1% Tween 20 in PBS. The slide was kept in a clean petri plate and to each well was added a tiny drop of glycerine and then the slide was carefully covered with the coverslip without introducing air bubbles into the wells. The slide was exposed for a fixed time (~18 msec) and viewed under the Zeiss Axioskop immunofluoresence microscope using the magnification of 63 X 20 (1260) .

2.15.3 Serum killing assay

2.15.3.1 Serum collection of normal human serum

Serum was obtained from the blood samples of volunteers from the Division of Infection and Immunity who were not actively engaged in working with *B. pertussis*. The blood samples were initially collected and allowed to clot for 1 h at 37°C. They were then placed on ice for 1-2 h to retract and the clear serum was collected after centrifugation at 10,000 x g for 5 min without disturbing the clot. The serum was centrifuged again to remove any remaining particles and the supernate was collected. The serum was pooled and distributed in small aliquots and stored at –70°C.

2.15.3.2 Bactericidal assays with normal human serum

B. pertussis cells were harvested from BGA plates incubated at 37°C for 24-48 h and suspended at ~$2x10^9$ CFU/ml by comparison with Opacity rod, in pre-warmed (37°C) Stainer and Scholte (SS) medium. A volume of 100 µl of bacterial suspension was

transferred to an eppendorf containing 350 µl of PBS (pH=7.4) and 50 µl of normal or heat inactivated (30 min at 56°C) control serum. The tubes were incubated at 37°C in a water bath for 45 min. The tube was then shifted to ice for 5 min to stop the complement reaction. A 20 µl aliquot of the mixture was added to 180 µl PBS with 10 mM EDTA (inactivates complement activity). Ten-fold serial dilutions were made in SS medium. The dilutions were then plated on BG agar, and incubated at 37°C. Bacterial colony forming units were counted after 3-4 days. Survival was calculated as a percentage, when the number of survivors from the normal serum-treated samples was compared to the number of survivors obtained from the heat-inactivated serum control (non-killing control) (Fernandez and Weiss, 1994; Barnes and Weiss, 2001). Statistical analysis was performed using Student's *t*-test. In some experiments, serum killing assays were carried out by adding an external source of complement to the heat-inactivated human serum. Guinea pig serum (5 % v/v) (Sigma) in PBS (pH=7.4) was used as a source of complement.

2.15.3.3 Bactericidal assays with rabbit anti-$_r$Bap-5 serum

The anti-$_r$Bap-5 serum was used at a final concentration of 2% v/v, as this concentration caused negligible reduction in CFU/ml of *B. pertussis* due to agglutination. The bacterial suspension was adjusted to contain approximately $2x10^8$ CFU in 100 µl in SS medium. 50 µl of anti-$_r$Bap-5 serum was added to the bacteria and the mixture incubated for 10 min at 37°C. This allowed the bacteria to be pre-sensitized for the next reaction. 25 µl of normal or heat inactivated (56°C at 30 min) guinea pig serum (5% v/v) was added to the samples and the total volume was brought to 500 µl by adding sterile PBS (pH=7.4). The samples were incubated at 37°C for 60 min. The reaction was halted by placing the sample on ice for 5 min and then by adding 10 mM EDTA (final concentration). Ten-fold

serial dilutions in SS medium were plated in 100 µl volumes on BG agar plates and bacterial colonies were counted after 4-5 days. In some antibody-mediated killing experiments anti-PRN (P.69) antibody (Table 2.7) was used as a control. In some other experiments, EGTA and $MgCl_2$ (10mM final concentration of each) was incubated with the sample to prevent the killing of bacteria by the classical pathway (Fernandez and Weiss, 1994).

2.15.4 Adhesion studies

2.15.4.1 ELISA-based protocol for adhesion studies

Tissue culture cell lines (Table 2.8) were seeded (3×10^5) in a 100 µl volume in flat bottomed 96-well (Costar) tissue culture plate and incubated at 37°C under 5% CO_2 for 48 h. The cells formed confluent monolayers during this period. The medium was decanted from the micro-plates and cells were washed three times with pre-warmed (37°C) 10mM PBS (pH=7.4) using a multichannel pipette to get rid of unattached cells. 100 µl of *B. pertussis* suspension containing ~10^7 bacteria was then added to each well and plates were incubated at 37°C under 5% CO_2 for 90 min. In some experiments, plates were incubated at 4°C to determine the effect of low temperature on adhesion. The plates were washed three times with pre-warmed (37°C) PBS to remove non-adherent *B. pertussis*. 100 µl of 4% paraformaldehyde in PBS was added to each well and fixation was carried out at 4°C for 60 min. After washing with PBS 2-3 times, 100 µl of 1% (v/v) hydrogen peroxide (Sigma) in methanol was added to each well and plates were incubated at room temperature for 10 min to inactivate (exhaust) any residual endogenous peroxidase activity. Blocking was carried out at 4°C overnight or one hour at 37°C in 2% BSA. After washing as above, 100 µl of rabbit polyclonal anti whole-*B. pertussis* serum (Hertz serum)

(1:10,000) was added to each well and plates were incubated for 2 h at 37°C. The primary antibody was removed by vigorous tapping of the plate on the thick layer of paper towels and then 3-4 washes with PBS and 0.1% Tween 20. Thereafter, 100 µl of anti rabbit-conjugate (SAPU) diluted 1 in 1000 in 2% BSA in PBS was added to each well and plates were incubated for 2 h at 37°C. Wells were washed with PBS 3-4 times as above and then 100 µl of *o*-phenylenediamine (0.4 mg/ml) in 10 mM citrate phosphate buffer (pH= 5.0) containing 0.02% hydrogen peroxide was added to each well and the plates were incubated at room temperature for 15 min. The reaction was terminated by adding 50 µl of 3M sulphuric acid. The absorbance of reaction was measured at 492 nm with a microtitre plate reader. The increase in absorbance is directly proportional to the number of *B. pertussis* adherent to the target cells (Roberts *et al.*, 1996). In some experiments, *B. pertussis* Taberman and its derivative Bap-5 mutant strains were first incubated with rabbit anti-whole anti-$_r$Bap-5 serum for 1 h at 37°C prior to start the adhesion assay, to see if anti-$_r$Bap-5 serum could reduce *B. pertussis* adhesion to different target cell lines. The bacterial cells treated with anti-$_r$Bap-5 were then centrifuged and standardised to same number of cells as above ~10^7 bacteria/100µl and used along with untreated bacterial cells in adhesion assays as above.

Table 2.8: Tissue culture cell lines and their growth media used during the adhesion studies

Type of cells	Description	Media +Supplements	Reference
HeLa 229	Human non respiratory epithelial cells	DMEM + 10% FCS + 2mM glutamine	Bassinet *et al.*, 2000
A549	Human (Caucasian) lung-carcinoma epithelial cells	F12K + 10% FBS + 2mM glutamine	Ishibashi *et al.*, 2001
Hep-2	Human laryngeal epithelial cells	DMEM + 10% FCS + 2mM-glutamine	Ewanowich *et al.*, 1989
Caco-2	Human colon carcinoma cell line	DMEM + 10% FCS + 2mM-glutamine	Schipper *et al.*, 1994
P338D-1	Murine macrophage cell line	L15 + 10% FCS + 2mM glutamine	J.Immunol,1975; 114:894
RAW 264.7	Murine macrophage cell line	L15 + 10% FCS + 2mM glutamine	Forde *et al.*, 1999

FCS= Foetal calf serum

FBS= Foetal bovine serum

3. Results

3.1: Expression of *bap-5* and construction of *bap-5*-defective mutants

The role of *bap-5* (2280 bp) identified by Blackburn (2000), in virulence of *B. pertussis,* was studied by RT-PCR to show if the gene was, in fact, expressed and by generating knockout mutants in the gene. This would lead to inactivation of the gene in question and possibly a decrease in virulence of the pathogen if Bap-5 was an important virulence factor.

3.1.1 Expression of *bap-5*

A preliminary RT-PCR result during this study indicated that *bap-5* appeared to be expressed in *B. pertussis* (Taberman I strain) by using a set of primers (*bap-5* forward + NTS REV; Appendix II) which amplify a product of 505 bp from the 3' region of the gene spanning the N-terminal region of the protein. RT-PCR was done initially using the protocol outlined by Blackburn (2000) i.e. by isolating total RNA using an acid-phenol extraction method and subjecting it to a single tube reaction for cDNA synthesis (Promega). But using the same conditions and procedural steps, the RT-PCR was not reproducible. Further study used total RNA whose quality was confirmed by the undegraded nature of the ribosomal RNA bands seen in Fig. 3.1. Total RNA was isolated using the RNeasy mini kit (section 2.11.1, Qiagen). Expression of *bap-5* was confirmed by performing RT-PCR (section 2.11.3) with *bap-5*-specific primers which were designed to amplify the 3' region of about 505 bp (Appendix II, shown by arrows). The primers *bap-5* forward and NTS-REV were designed (Table 2.6). The reverse transcription (Fig. 3.2) was carried out in two steps, using NTS-REV primer for making cDNA in the first step and then subjecting the cDNA to PCR as described in section 2.11.3. Appropriate controls for the experiment were included to eliminate any possibilities of DNA being carried over in

the RNA preparation and eventually an amplified PCR product was obtained. (section 2.11.2). Fig. 3.2, lane A shows the first control reaction without added reverse transcriptase. Another control was included in which no sample RNA was added, to eliminate the possibility of any genomic DNA cross contamination from any of the procedural steps or ingredients of the reaction involved (Fig. 3.2, lane C). The results reproducibly showed that a transcript of the expected size i.e. 505 bp was obtained with *B. pertussis* (Taberman I) as shown in Fig. 3.2, lane B indicating that *bap-5* was expressed. There is one anomaly i.e. lane B shows an additional, smaller band of ~100 bp. This could have been due to contamination in the experimental tube but the result was reproducible and moreover there was no hint of this band in the control reactions. One possible explanation is the presence of another, smaller transcript further inside the targeted region towards the 3' end that may have co-amplified with the desired product.

3.1.2 Generation of *bap-5*-defective mutants of *B. pertussis*

The suicide vector pSS1129, used for create allelic replacement of the wild-type *bap-5* gene with its mutated version, had been used previously in *B. pertussis* (Stibitz, 1994). The salient features of this construct are that it has an origin of transfer from plasmid RP4, and, provided that *tra* functions are supplied *in trans*, the vector will be transferred into the recipient bacterium via conjugation. The vector, having a ColE1 origin of replication, is unable to replicate in *B. pertussis*. It has the *E. coli rspL* gene which codes for RspL protein and which is responsible for the streptomycin-sensitive (Sm^S) phenotype in strains normally resistant to streptomycin (Sm^R) due to mutation in the chromosomal *rspL* homologue. This phenotype conferred by the plasmid was checked by transforming the vector into the *E. coli* Sm^R strain JC3272 to show that it became Sm^s. Therefore, transformation of *B. pertussis* with the plasmid carrying a gene disrupted by an antibiotic-

Fig. 3.1: Agarose gel (0.7%) showing RNA samples extracted from *B. pertussis*

B. pertussis grown to log phase (OD_{600nm}=0.7) in CL medium and RNA was extracted from the 2 x 10^9 bacterial cells using the RNeasy mini-kit as described in section 2.11.1.

A = RNA preparation from *B. pertussis* Taberman I

B = RNA preparation from *bap-5* mutant Taberman II

Fig. 3.2: RT-PCR results depicting the expression of *bap-5* in *B. pertussis*.

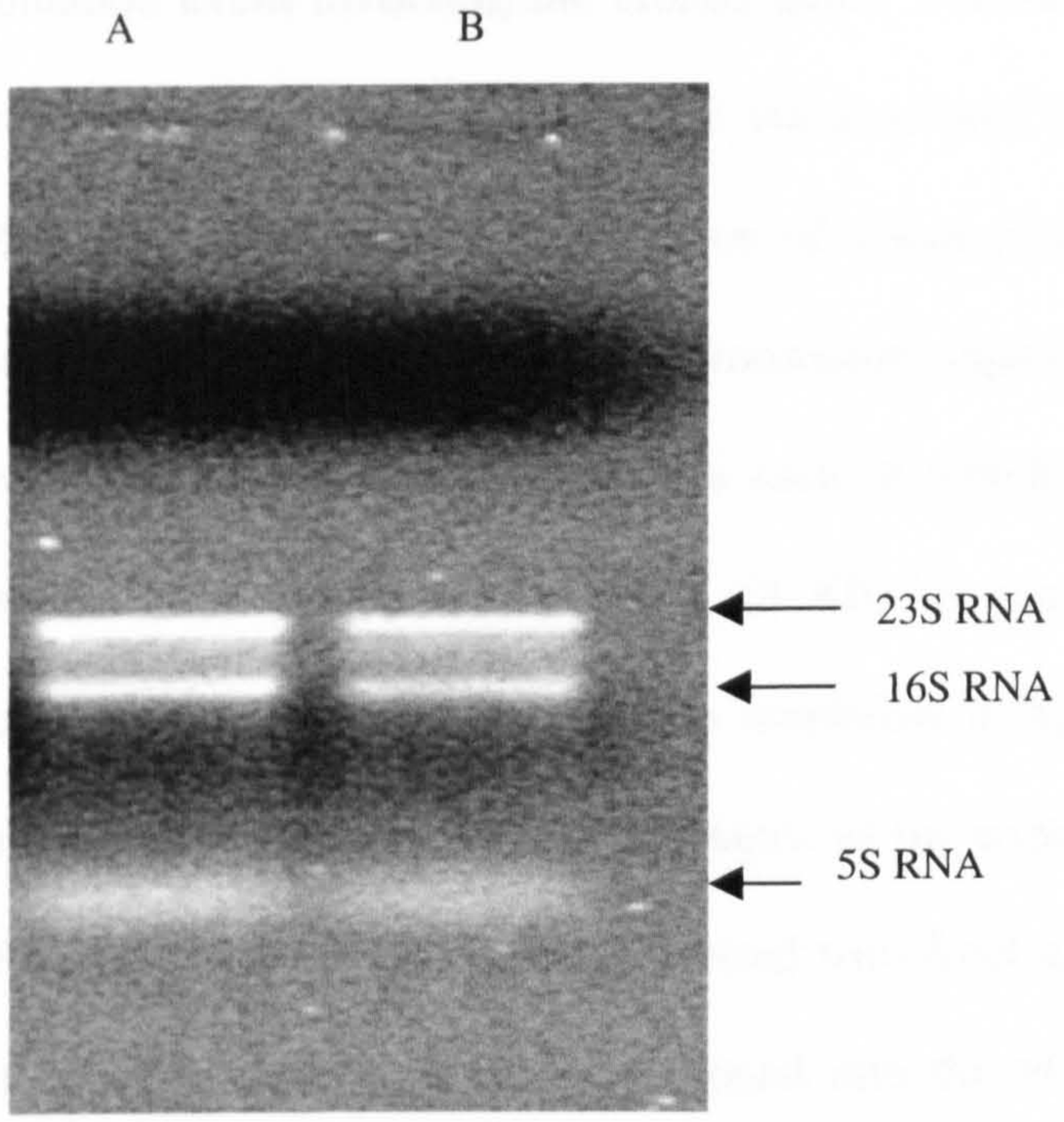
RT-PCR was performed using the RNA isolated from *B. pertussis* Taberman I as above according to the procedure given in section 2.11.3

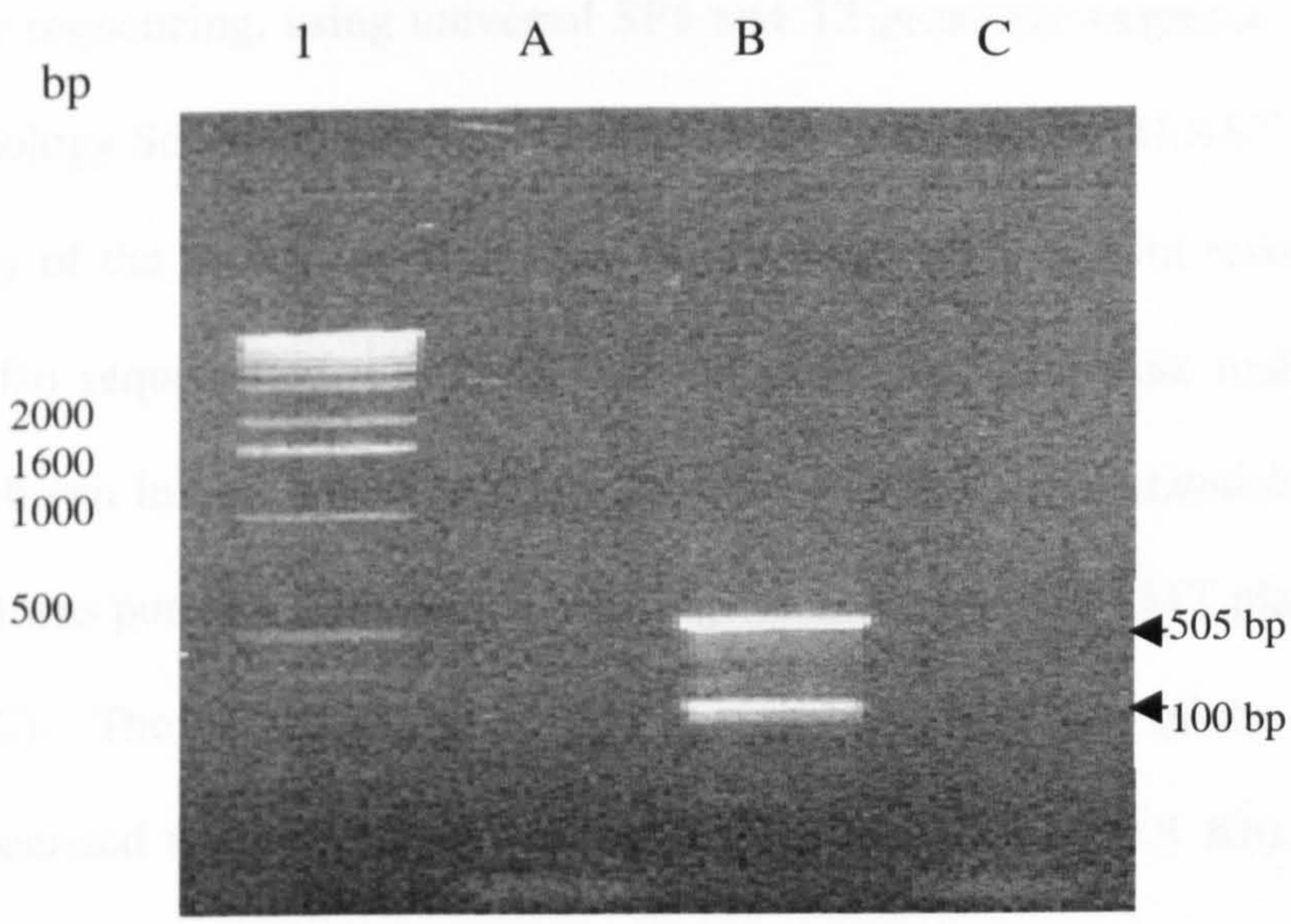
1 = 1Kb marker

A = No added reverse transcriptase (control)

B = RT-PCR reaction with *B. pertussis* Taberman I RNA

C = No added RNA sample (control)


A
B
23S RNA
16S RNA
5S RNA


1
A
B
C
bp
2000
1600
1000
500
505 bp
100 bp

resistance marker can take place via an insertion of the plasmid into the chromosome by a single homologous recombination event involving the cloned gene. Further selection in the presence of streptomycin promotes the loss of plasmid via a second recombination event. The *bap-5* gene (2.28 Kb) was disrupted by insertion of a kanamycin resistance cassette (km^R) carrying its own promoter and transcription termination sequences. The km^R cassette was initially amplified from pUC4K using primers each of which contained an *Nco*I site and the final product was cloned into pGEMT (3 Kb) to generate 4.3 Kb pGEMT(KANA) (Blackburn, 2000). The *bap-5* gene was amplified using primer sets carrying *Bgl*II sites to allow cohesive ligation in the later stages of the experiment. The km^R was purified from pGEMT(KANA) after cutting the plasmid with *Nco*I and inserted at the *Nco*I restriction site of *bap-5* (Appendix II) already cloned into the pGEMT vector (Fig. 3.3, lane A, band at 6.5 Kb), leaving *bap-5* flanking regions of 542 bp and 1738 bp, enough for homologous recombination to take place for the construction of the *B. pertussis* *bap-5* mutant (Blackburn, 2000). The orientation of the cloned sequence in pGEMT was confirmed by sequencing, using universal SP6 and T7 promoter sequence primers by the Molecular Biology Sequencing Unit of the University of Glasgow. BLAST search showed 100% identity of the insert with *bap-5* and to the inserted kanamycin resistance cassette, which was also sequenced. A summary of the steps involved while making the *bap-5* construct is shown in Fig. 3.3. The *Bgl*II fragment carrying the mutated *bap-5* gene (3.5 Kb fragment) was purified from the gel after digestion from the pGEMT plasmid (Fig. 3.3, lane B and C). The next step was to ligate it into the *Bgl*II site of the suicide vector pSS1129, linearised (section 2.7.1) with *Bgl*II (Fig. 3.4, lane D, 9.8 Kb). The ligation (section 2.7.2) of the mutated *bap-5* gene into the suicide vector was achieved after many attempts (Fig. 3.3, lane E, 13.3 Kb). The plasmid construct obtained (Fig. 3.3, lane E) was then transformed by electroporation into an *E. coli* mobilising strain SM10.λ_{PIR}.

Fig. 3.3: Agarose (0.7 %) gel showing the summary of the scheme for *bap-5* mutant construction

1 = Super-coiled DNA ladder

A= Whole plasmid pGEMT *(bap-5::km^R*) (6.5 Kb) (Table 2.5)

B = *Bgl*I digestion releasing the mutated *bap-5::km^R* (3.5 Kb) fragment from pGEMT plasmid (3 Kb)

C = Purified *bap-5:: km^R* fragment

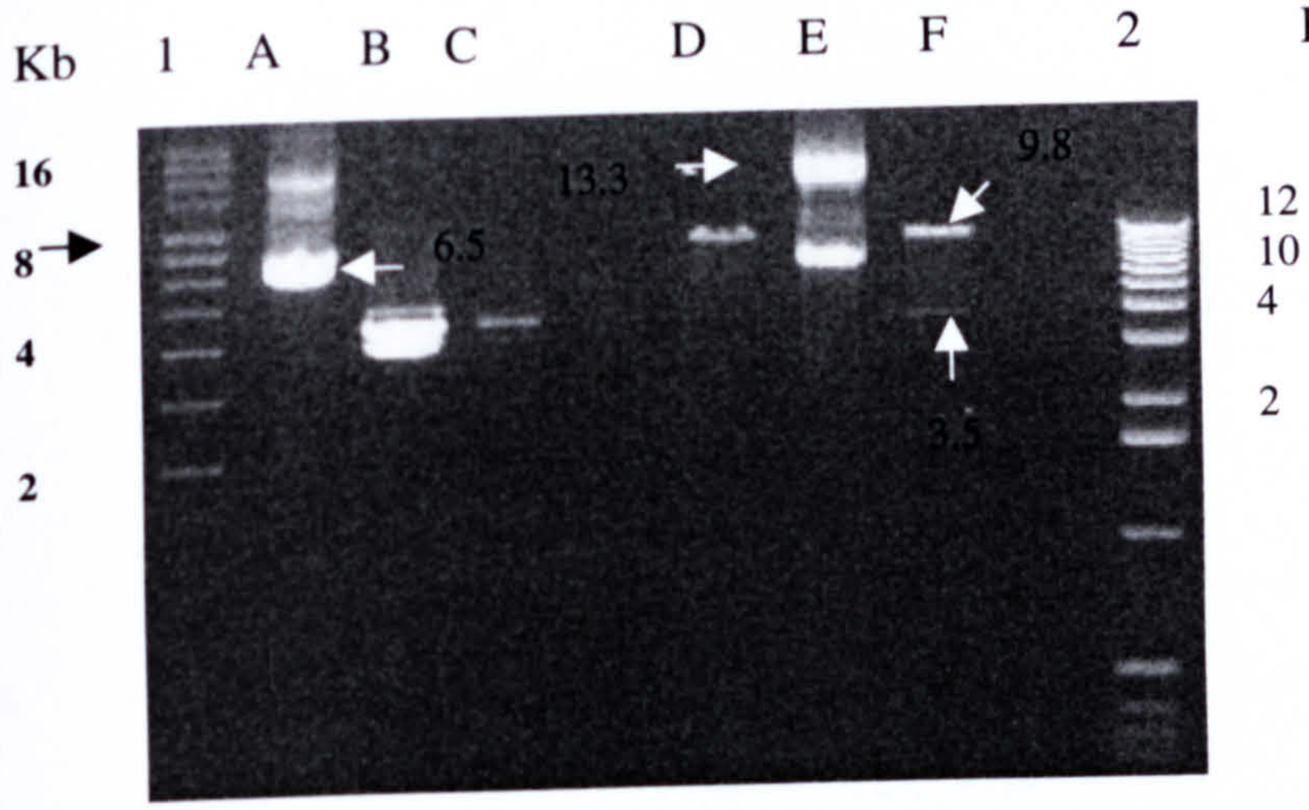
D = Suicide vector pSS1129 (9.8 Kb)

E = Whole plasmid pSS1129 (pBap5-Kana) (13.3 Kb) (Fig 3.3, Table 2.5)

F = Plasmid pSS1129 (pBap5-Kana) digested with *Bgl*II to release the 3.5 Kb *bap-5::km^R* fragment

2 =1 Kb DNA ladder

Arrows indicate sizes of the DNA fragments (Kb)


Kb
1
A
B
C
D
E
F
2
Kb
16
8
4
2
6.5
13.3
9.8
3.5
12
10
4
2

The plasmid (Fig 3.4, plasmid construct pBap5-Kana) was isolated and digested with *Bgl*II releasing mutated *bap-5* (3.5 Kb) and suicide vector (9.8 Kb), which showed the successful ligation of the mutated *bap-5* in the suicide vector (Fig. 3.4, lane F). Spontaneous Nal^R and Sm^R mutants of *B. pertussis* were isolated for subsequent selection purposes in both Taberman I and Tohama I strains by the procedure described in section 2.1.5. Conjugation between the *E. coli* mobilising strain SM10.λ_{PIR} and *B. pertussis* (Taberman I or Tohama I) was carried out according to the procedure given in section 2.1.6. The exconjugants were plated onto the first selection BG agar containing Km (20 μg/ml), Nal (40 μg/ml) and cephalexin (40 μg/ml). Cephalexin was included to further minimise the carry over of *E. coli* as *B. pertussis* can survive due to an inherent resistance to this antibiotic. The survivors of this selection (single cross-over) were assumed to be *B. pertussis* which contained the plasmid integrated into the chromosome. Thereafter the survivors of the single cross-over were restreaked onto BG agar containing Sm (100μg/ml) to select against the plasmid. The survivors obtained after this selection were assumed to be bacteria that had lost the integrated plasmid due to a double cross-over event. This loss occurred due to the homologous recombination between the regions of *bap-5* present on the plasmid and on the host chromosome. A summary of the molecular mechanism of gene replacement by this strategy is given in Fig. 3.5.

PCR was performed on the selected colonies from the final selection to show that the mutated *bap-5* gene had successfully replaced its wild-type counterpart in the *B. pertussis* chromosome. PCR was carried out using the *bap-5* specific primers *bap-5* forward and *bap-5* reverse (Table 2.6; Appendix II, shown by arrows) which cover the full length *bap-5* gene starting from the first methionine (presumed start site). The PCR results (Fig. 3.6) using genomic DNA extracted (section 2.2.) from *B. pertussis* Taberman I strain and its *bap-5* defective mutant, as well as *B. pertussis* Tohama I and its *bap-5* defective

Fig. 3.4: Map of suicide construct pBap5-Kana

The suicide vector pSS1129 was used to create pBap5-Kana. The *bap-5* gene was disrupted by insertion of a kanamycin resistance gene at a *Nco*I site 501bp from the predicted translational start site. The vector has a ColEI origin of replication which is not functional in *B. pertussis*. It has an origin of transfer (*oriT*) from the RP4 plasmid. It also contains the *E. coli rspL* gene which encodes streptomycin sensitivity and an ampicillin resistance gene. The mutated *bap-5* gene was cloned into pSS1129 at a *Bgl*II site to make the pBap5-Kana construct.

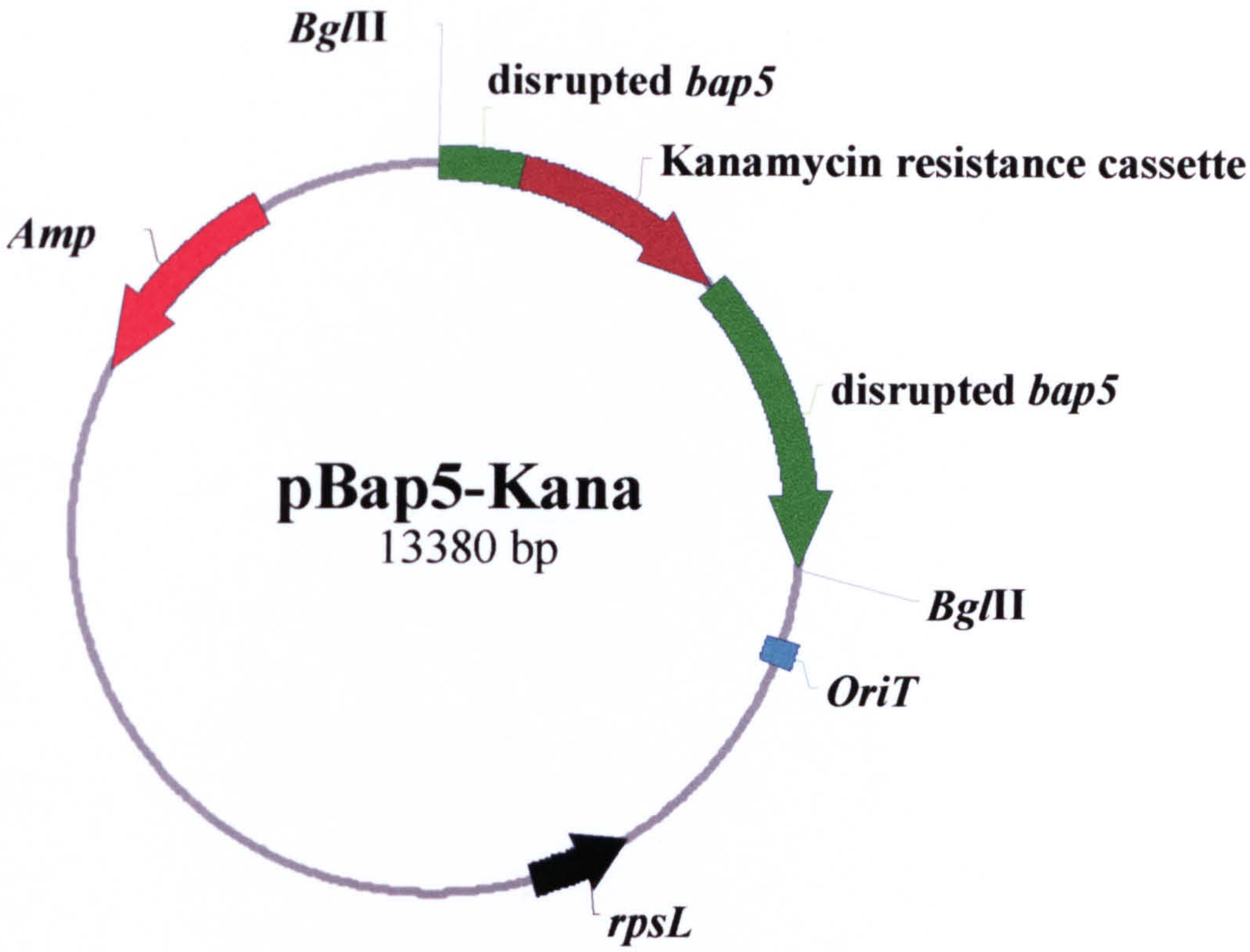

BglII
disrupted bap5
Kanamycin resistance cassette
Amp
disrupted bap5
pBap5-Kana
13380 bp
BglII
OriT
rpsL

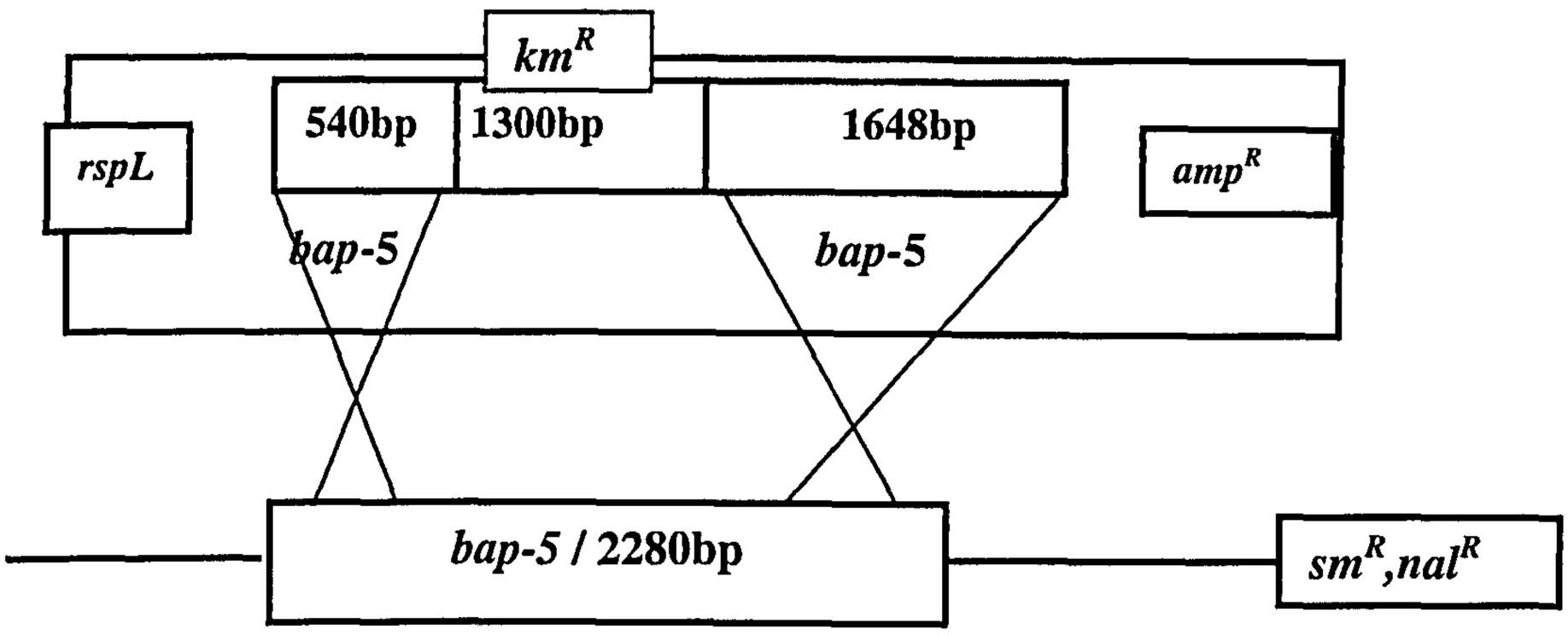
Fig. 3.5: Strategy adopted for replacement of *bap-5* gene with *bap-5* mutated with km^R (*bap-5::*km^R)

Single cross-over selection was made by selecting for the plasmid (pBap5-Kana) using antibiotics nalidixic acid and kanamycin.

pSS1129 + *bap-5 ::* km^R

↓

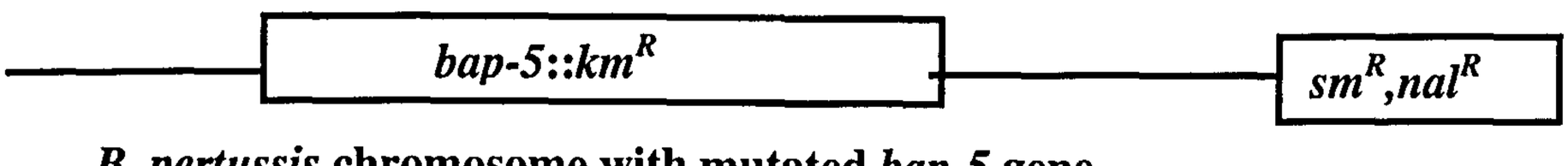
pBap5-Kana



B. pertussis **chromosome**

Double cross-over selection was made by selecting against the plasmid using the antibiotic streptomycin.

↓



B. pertussis **chromosome with mutated** ***bap-5*** **gene**

Fig. 3.6: Agarose (0.8%) gel showing the PCR products of the wild type and mutated versions of *bap-5*

PCR was performed on chromosomal DNA extracted as described in section 2.2.1; using primers *bap-5*-forward and *bap-5* reverse which amplify the whole gene (Table 2.6.1, Appendix II)

1 = 1 Kb ladder

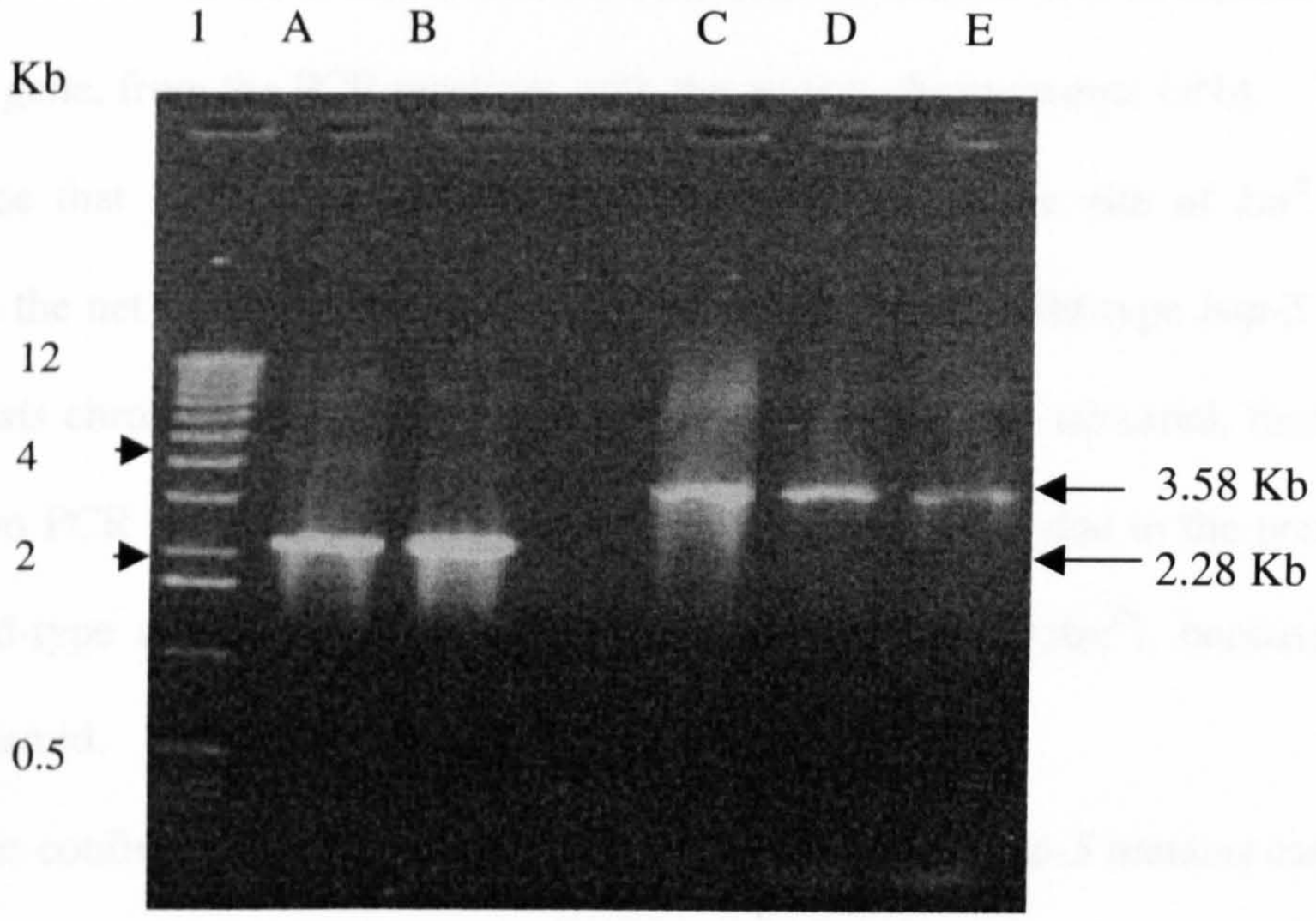
A = *bap-5* gene amplified using DNA from *B. pertussis* Taberman I

B = *bap-5* gene amplified using DNA from *B. pertussis* Tohama I

C = *bap-5*::km^R (mutated *bap-5*) amplified from plasmid construct pBap5-Kana.

D= *bap-5*::km^R amplified using DNA from *B. pertussis* Taberman II mutant

E = *bap-5*::km^R gene amplified using DNA from *B. pertussis* Tohama II mutant


1
A
B
C
D
E
Kb
12
4
2
0.5
3.58 Kb
2.28 Kb

mutant, clearly indicated that *bap-5:: km*^{R} was present in the chromosome of the *B. pertussis* mutant strain.

Lanes A and B in Fig. 3.6 show the PCR product (2.28 Kb) from the parent *B. pertussis* strains (Taberman I and Tohama I) and lanes D and E show the PCR products (3.58 Kb) from the *B. pertussis* strains with mutated *bap-5* (Taberman II and Tohama II). Lane C shows the PCR product from the cloned mutated *bap-5* gene in the suicide vector pBap5-Kana. There was no evidence of a 2.28 Kb band in lanes D and E, representing the native *bap-5* gene, from the PCR reactions with the mutant chromosomal DNA. This was clear evidence that cross-overs had occurred on each side of the site of *km*^{R} cassette insertion and the net result was the complete replacement of the wild-type *bap-5* allele in the *B. pertussis* chromosome. If such a double cross-over had not occurred, there would have been two PCR products obtained from the *B. pertussis* strain due to the presence of both the wild-type allele (*bap-5*) and the mutated allele (*bap-5::km*^{R}), because of the integrated plasmid.

Further confirmation of the successful construction of the *bap-5* mutants came from the Southern blotting procedure. Genomic DNA from the parents and *bap-5* mutant strains was digested with restriction enzyme *Sac*I (Fig. 3.7). This enzyme has no restriction sites in *bap-5* or in the *km*^{R} locus. The rationale for doing this was that, on probing the digested DNA with a Dig-labelled whole *bap-5* specific probe, a size difference would be seen between the parent and mutant strains. Southern blot analysis showed that the Dig-labelled *bap-5* probe hybridised to an approximately 5.2 Kb fragment in both parent DNA preparations, but to an approx. 6.5 Kb fragment in both mutant strains (Fig. 3.8). Lanes A and B represent the *Sac*I digested genomic DNA from the *B. pertussis* Taberman I and Tohama I strains respectively probed with Dig-labelled *bap-5* gene (section 2.9.1). Lanes C and D represent the *bap-5* mutant strains, Taberman II and Tohama II, respectively,

Fig. 3.7: Agarose (1%) gel showing the genomic DNA of *B. pertussis* strains digested with *Sac*I and used for Southern blotting.

Chromosomal DNA extracted by the procedure described in section 2.2.1, was subjected to restriction digestion with *Sac*I which does not cut either the *bap-5* gene or the kanamycin resistance cassette and the fragments were separated on a 1 % agarose gel.

A = *B. pertussis* Taberman I

B = *B. pertussis* Tohama I

C = *B. pertussis* Taberman II, *bap-5* mutant

D = *B. pertussis* Tohama II, *bap-5* mutant

1 = Dig-labelled DNA molecular weight marker

Fig. 3.8: Southern blot showing *bap-5* parent and mutant genomic DNA digested with *Sac*I and probed with a *bap-5* specific probe.

Chromosomal DNA was digested and separated as in Fig. 3.7 and it was then transferred to nylon membrane and probed with a Dig-labelled *bap-5* gene fragment prepared as described in section 2.9.1.

A = *B. pertussis* Taberman I, wild type

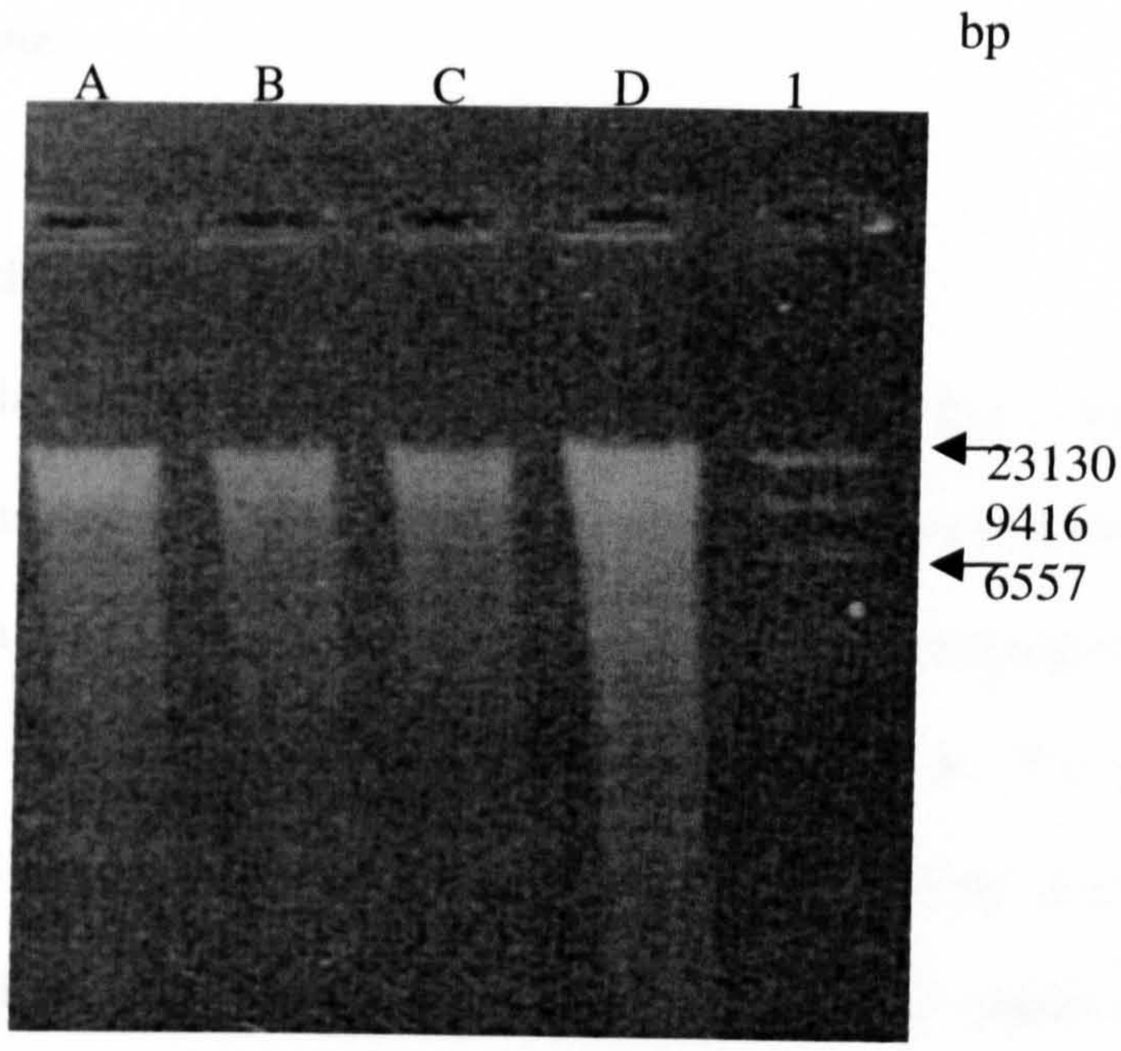
B = *B. pertussis* Tohama I, wild type

C = *B. pertussis* Taberman II, *bap-5* mutant

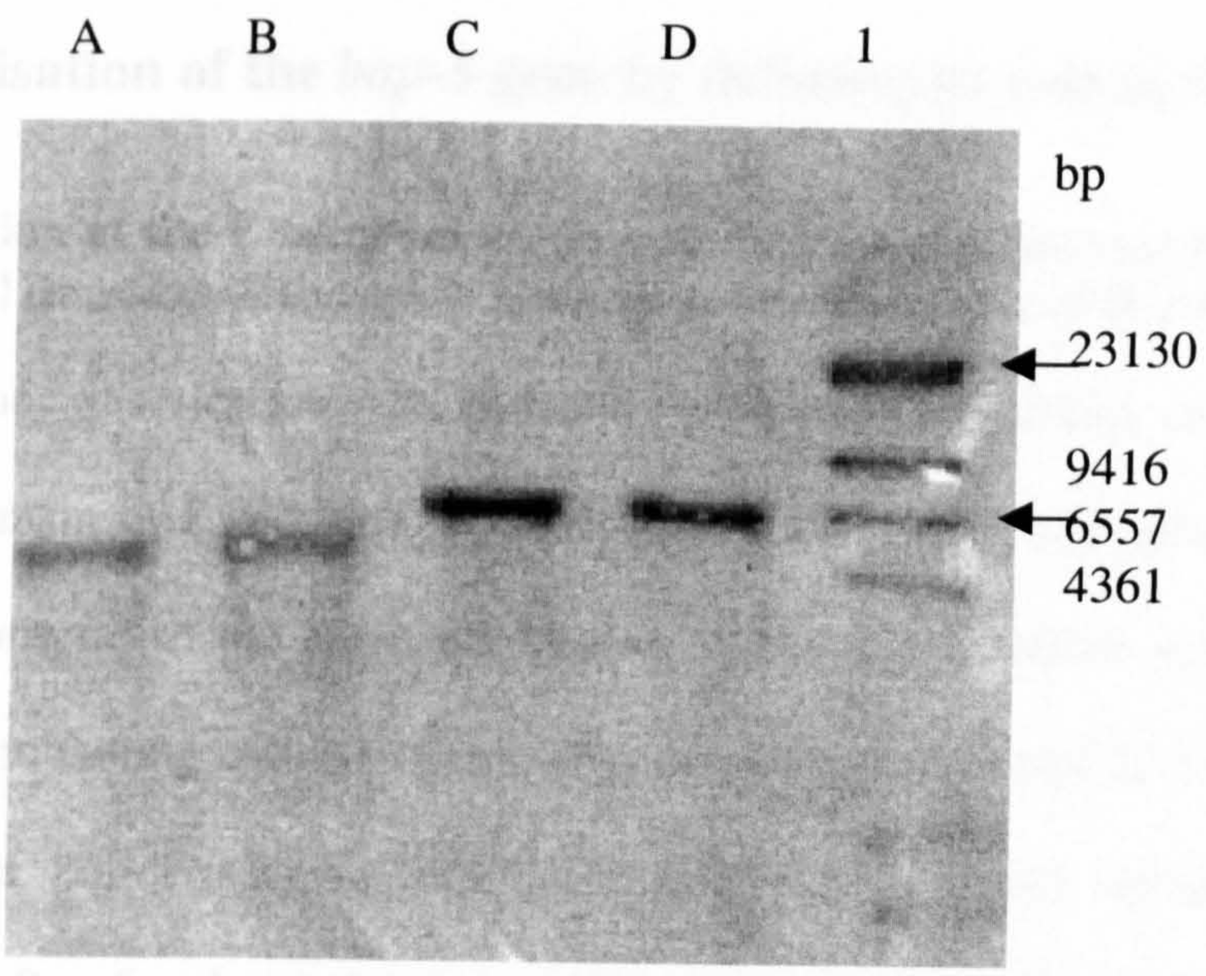
D = *B. pertussis* Tohama II, *bap-5* mutant

1 = Dig-labelled DNA molecular weight marker

3.7



3.8



containing the kanamycin resistance cassette insertion in *bap-5*. The difference of 1.3 Kb between the parent strains and the *bap-5* mutant strains was expected from the insertion of the ~1.3 Kb km^R cassette.

3.1.3 Characterisation of the *bap-5*-defective mutants

The growth kinetics of *B. pertussis* Taberman I and its *bap-5*-defective mutant Taberman II were measured over 72 h by noting the optical density at 600 nm (OD_{600nm}) at intervals. The bacteria were grown in 100 ml volumes of cyclodextrin liquid (CL) medium inoculated with 2 ml of a culture grown in CL medium for 48 h. The growth curves showed that the *bap-5* mutagenesis had not affected the growth of the *B. pertussis bap-5* mutant (Taberman II) in any significant way (Fig. 3.9). The *bap-5* mutant of *B. pertussis* Tohama I, however, appeared to be abnormal. It produced non-haemolytic colonies on BG agar and grew more rapidly than the parent strain in the CL medium (not shown) hence was not included in the subsequent study.

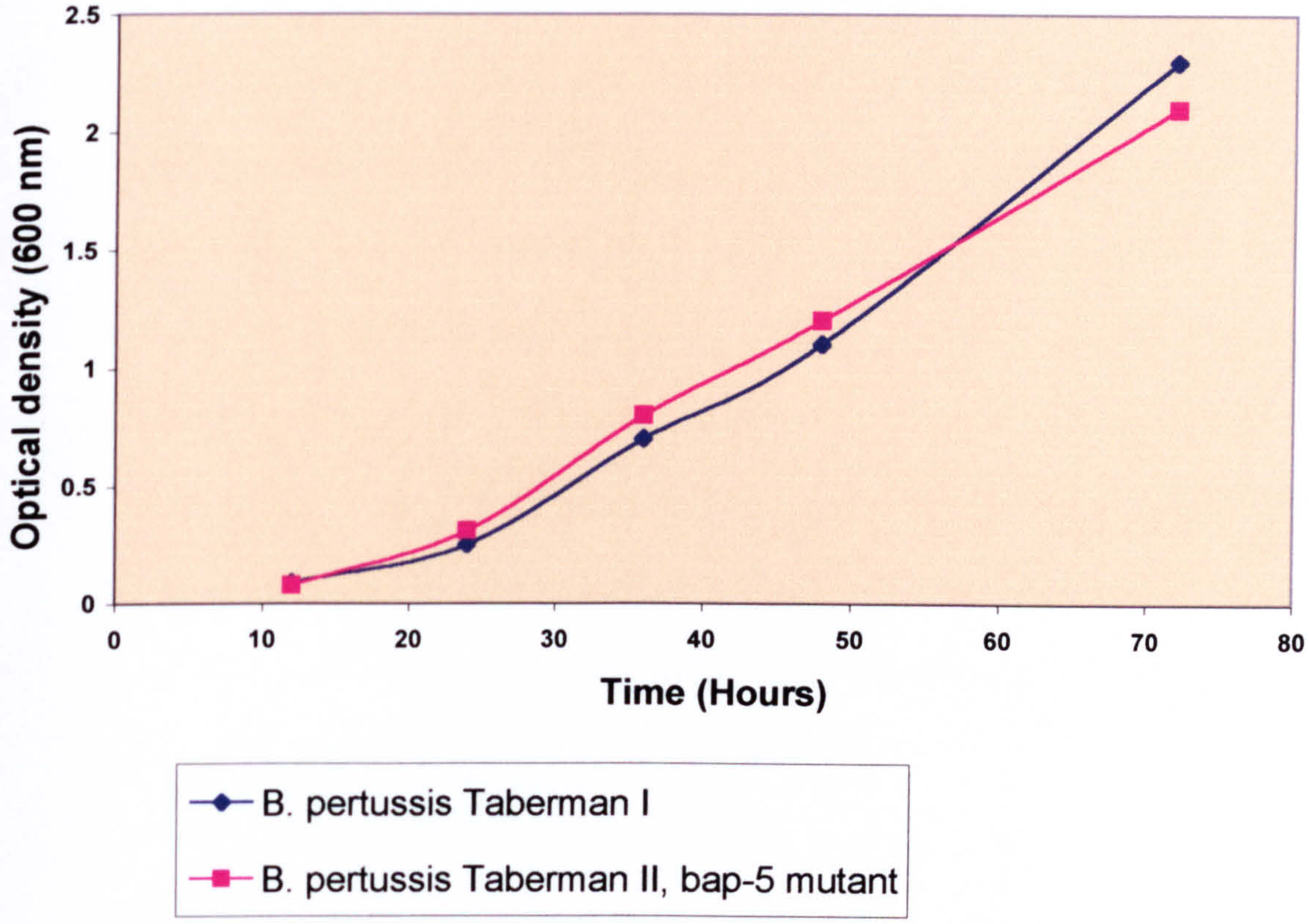
3.2: Characterisation of the *bap-5* gene by defining its role in *B. pertussis*

3.2.1 Identification of the C-terminal domain of Bap-5 and cross reactivity with the C-terminal domains of the other autotransporter proteins of *B. pertussis*

This section was designed to generate information regarding the C-terminal, autotransporter domain of Bap-5 that, by analogy with the other transporters, would be involved in the transport of the passenger domain of the Bap-5 protein across the outer membrane. Immunoblotting with a polyclonal mouse antiserum (Table 2.7) raised against recombinant whole Bap-5 protein (Blackburn, 2000) cross-reacted strongly with the recombinant whole Bap-5 and a slight cross reaction was noted with the whole cells of *B.*

Fig. 3.9: Growth curve of *B. pertussis* Taberman I and its *bap-5*-defective mutant.

Bacteria were grown in CL medium and the OD at 600_{nm} was measured at regular intervals.


Optical density (600 nm)
2.5
2
1.5
1
0.5
0
0
10
20
30
40
50
60
70
80
Time (Hours)
B. pertussis Taberman I
B. pertussis Taberman II, bap-5 mutant

pertussis Taberman I (not shown). This same antiserum did not show any cross-reaction either with the outer membrane-enriched fractions from *B. pertussis* Taberman I, or with recombinant, urea-extracted C-terminus of Bap-5 (result not shown). Moreover, a mouse polyclonal serum against the recombinant C-terminus of Bap-5 (Table 2.7), raised by Blackburn, (2000) did not prove to be a useful tool for specifically identifying the C-terminus of Bap-5 because it cross-reacted with other autotransporter C-terminal domains. Fig. 3.10 shows an immunoblot of crude urea extracts containing recombinant C-termini of Bap-5 (lane A), PRN (lane B), TCF (lane C) and BrkA (lane D) when reacted with the mouse anti-C-terminal Bap-5 serum. However, it appeared that the mouse anti-Bap-5 serum did cross-react with two components in the preparations of the recombinant C-termini of Bap-5 (lane A) and PRN (lane B) and one component in the preparation of TCF (lane C) although not with the recombinant C-terminal domain of BrkA (lane D).

Another line of investigation was to search for cross-reactivity between the Bap-5 C-terminal region and the C-termini of the other autotransporters i.e. TCF, BrkA and PRN, using their C-terminus-specific antibodies. This would also be useful in determining the closest significantly homologous autotransporter to Bap-5 from the above mentioned three. Fig. 3.11 shows the SDS-PAGE profiles of urea-solubilised crude extracts of the recombinant C-termini of the different *B. pertussis* autotransporter proteins expressed in *E. coli* (M15) and extracted and purified as the His-tagged (2.10.4) constructs (Blackburn, 2000) (section 2.1.2, Table 2.5). Blots of these purified recombinant C-termini were incubated with mouse monoclonal antibodies to the C-termini of BrkA (antibody P28) and TCF (antibody 49.3) as well as with a mouse polyclonal antibody to the C-terminal region of PRN (Table 2.7) raised by Blackburn, (2000). Fig. 3.12 shows all the recombinant C-termini screened with the monoclonal antibodies to BrkA (C-terminal). The antibody

Fig. 3.10: Immunoblot indicating the cross-reaction between the mouse polyclonal anti-Bap-5 (C-terminus) antibody and various purified recombinant C-terminal domains of *B. pertussis*

Antiserum raised against recombinant C-terminal domain of Bap-5 (section 2.12, Table 2.7) was used in a western blot against recombinant preparations of the various autotransporter C-terminal domains purified as outlined in section 2.10.4.

A= Purified recombinant C-terminus of Bap-5

B= Purified recombinant C-terminus of PRN

C= Purified recombinant C-terminus of TCF

D= Purified recombinant C-terminus of BrkA

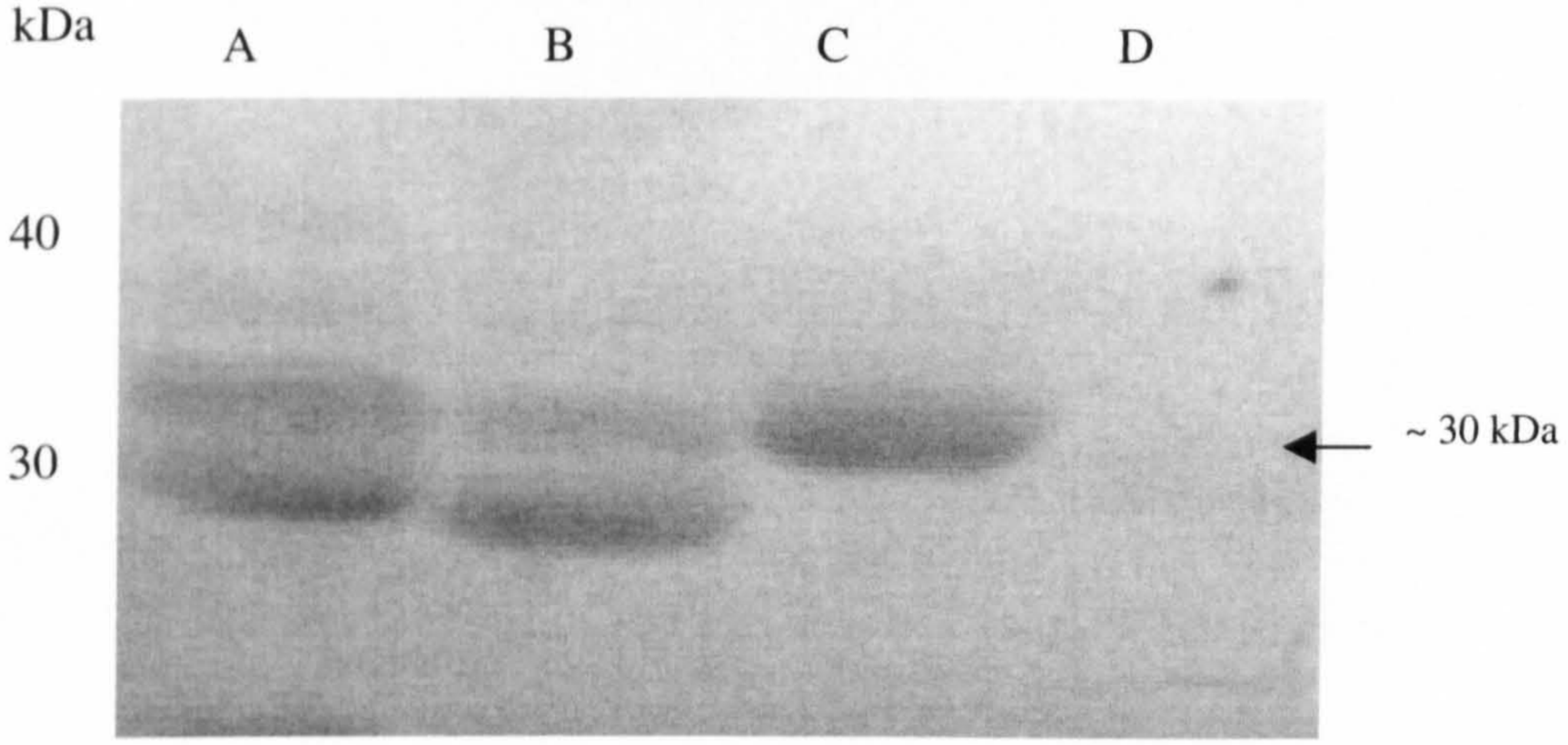

kDa
A
B
C
D
40
30
~ 30 kDa

Fig. 3.11: SDS-PAGE of the purified recombinant C-terminal domains of the different *B. pertussis* autotransporters.

Purified recombinant C-terminal domains as in Fig. 3.10 (section 2.10.4) were subjected to SDS-PAGE (12 %) and stained with coomassie blue.

A= Purified recombinant C-terminus of Bap-5

B= Purified recombinant C-terminus of PRN

C= Purified recombinant C-terminus of TCF

D= Purified recombinant C-terminus of BrkA

Fig. 3.12: Immunoblot showing the reaction between the monoclonal anti-BrkA (C-terminus) antibody and different purified recombinant C-terminal domains of *B. pertussis*

Equal amounts of the protein (2.10.5) were subjected to SDS-PAGE (12%) as shown in Fig. 3.11 and blotted to Hybond-C membrane and probed with the mouse monoclonal P28 to the BrkA C-terminal domain (Table 2.7).

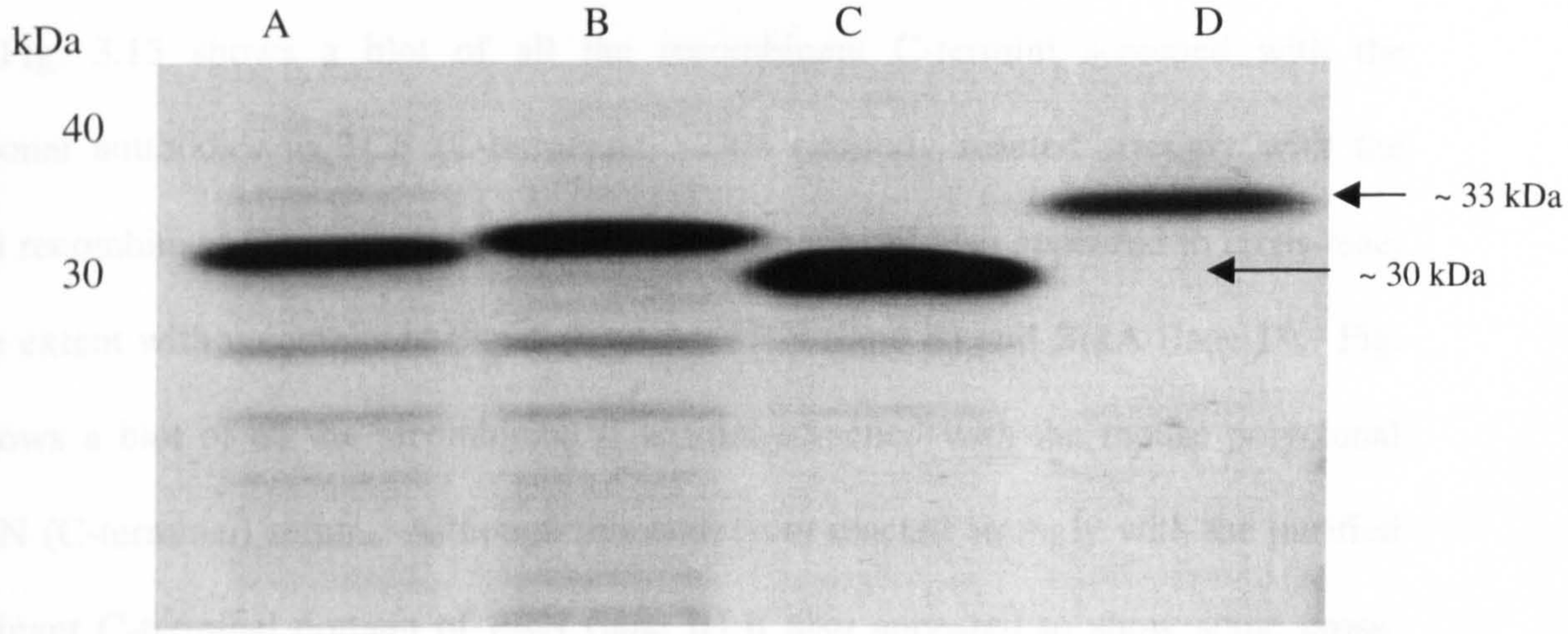
A= Purified recombinant C-terminus of Bap-5

B= Purified recombinant C-terminus of PRN

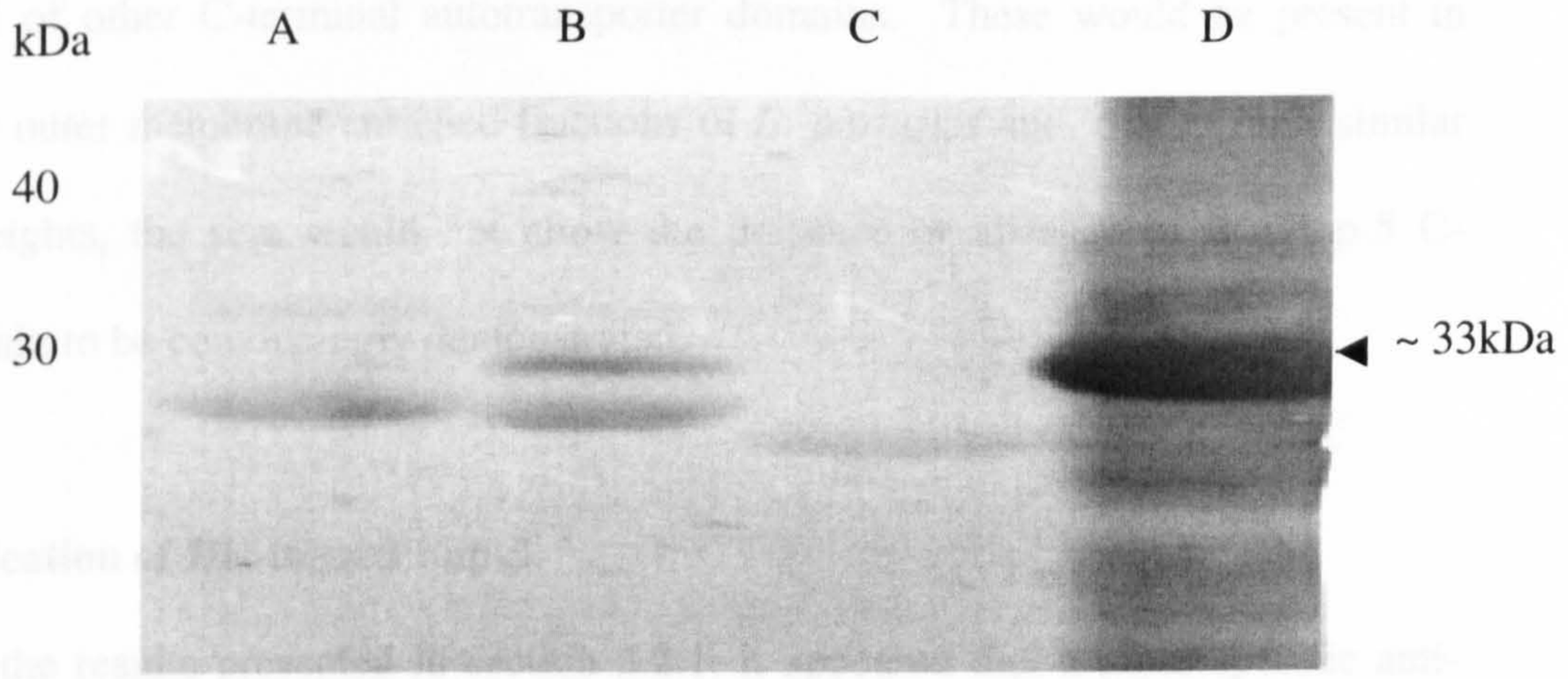
C= Purified recombinant C-terminus of TCF

D= Purified recombinant C-terminus of BrkA

3.11



3.12



reacted strongly with the purified recombinant C-terminal domain of BrkA (lane D) and it also appeared to cross-react to some extent with recombinant Bap-5 (lane A), PRN (lane B) and TCF (lane C).

Fig. 3.13 shows a blot of all the recombinant C-termini screened with the monoclonal antibodies to TCF (C-terminal). This antibody reacted strongly with the purified recombinant C-terminal domain of TCF (lane C) but also appeared to cross-react to some extent with recombinant Bap-5 (lane A), PRN (lane B) and BrkA (lane D). Fig. 3.14 shows a blot of all the recombinant C-termini screened with the mouse polyclonal anti-PRN (C-terminal) serum. Although this antiserum reacted strongly with the purified recombinant C-terminal domain of PRN (lane B) it also appeared to show some cross-reaction with recombinant Bap-5 (lane A) and TCF (lane C) and to a lesser extent with BrkA (lane D). The overall result suggested that it would be extremely difficult to distinguish the C-terminal region of Bap-5 in *B. pertussis* Taberman I, or its absence, in the *bap-5* mutant (Taberman II) with any of these antiserum as they seem to cross-react with the C-termini of other C-terminal autotransporter domains. These would be present in whole-cell or outer membrane-enriched fractions of *B. pertussis* and, due to their similar molecular weights, the sera would not allow the presence or absence of the Bap-5 C-terminal domain to be convincingly demonstrated.

3.2.2 Purification of His-tagged Bap-5

From the results presented in section 3.2.1, it appeared that a more specific anti-Bap-5 serum was needed for this study. Therefore a rabbit polyclonal anti-Bap-5 serum was prepared against the purified His-tagged recombinant full length Bap-5 expressed in *E. coli* expression strain M15 from the construct pQE60 (Blackburn, 2000). The full length Bap-5 was purified during this study according to the method given in section 2.10.4. Fig.

Fig. 3.13: Immunoblot showing the reaction between the monoclonal anti-TCF(C-terminus) antibody and different purified recombinant C-terminal domains of *B. pertussis*

Equal amounts of the protein (2.10.5) were subjected to SDS-PAGE (12%), blotted to Hybond-C membrane and probed with the mouse monoclonal (49.4) to the TCF C-terminal domain (Table 2.7).

A= Purified recombinant C-terminus of Bap-5

B= Purified recombinant C-terminus of PRN

C= Purified recombinant C-terminus of TCF

D= Purified recombinant C-terminus of BrkA

Fig. 3.14: Immunoblot showing the reaction between the mouse polyclonal anti-PRN (C-terminus) antibody and different purified recombinant C-terminal domains of *B. pertussis*

Equal amounts of the protein (2.10.5) were subjected to SDS-PAGE (12%), blotted to Hybond-C membrane and probed with a mouse monoclonal to the PRN C-terminal domain (Table 2.7).

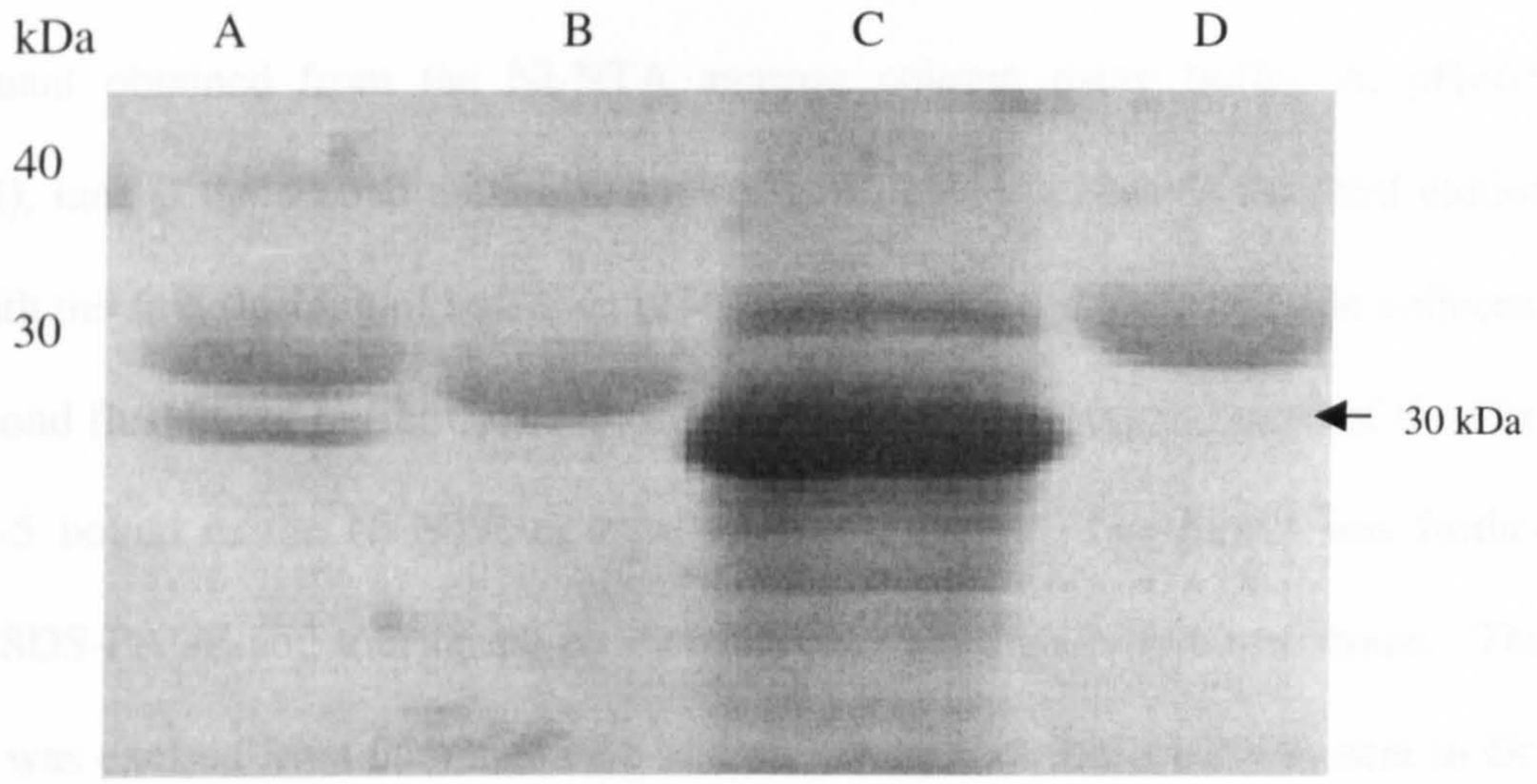
A= Purified recombinant C-terminus of Bap-5

B= Purified recombinant C-terminus of PRN

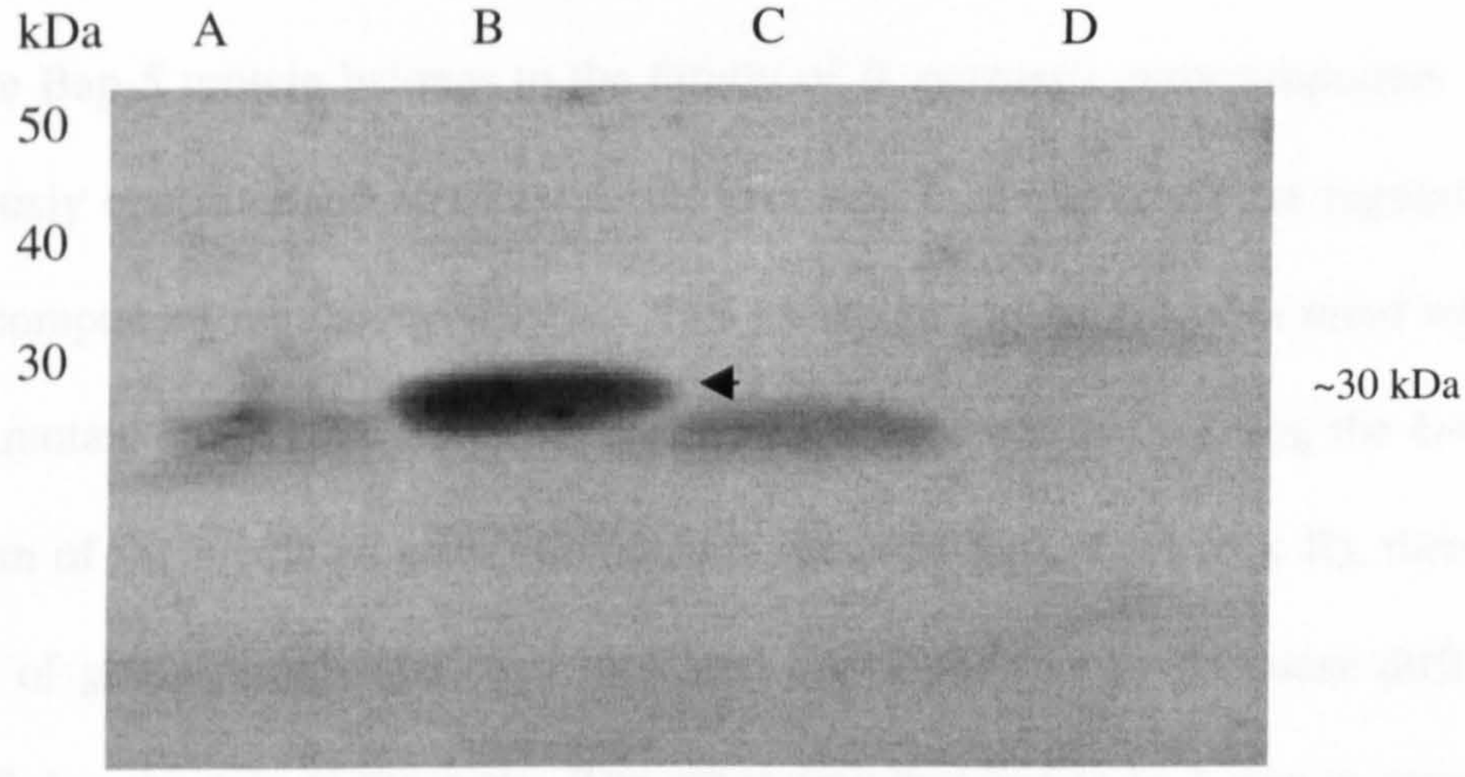
C= Purified recombinant C-terminus of TCF

D= Purified recombinant C-terminus of BrkA

3.13



3.14



3.15 (lane A) shows Bap-5, which runs at ~90 kDa on SDS-PAGE, in the urea-extract of the inclusion bodies obtained from the *E. coli* expression strain M15. Lane B represents the first eluant obtained from the Ni-NTA agarose column using buffer A, pH=6.0 (Appendix I), lane C the second elution using buffer B, pH=5.2, lane D the third elution collected with the first flushing of buffer C, pH=4.5 and lane E the fourth elution collected with the second flushing of buffer C, pH=4.5. The last elution uncoupled most of the His-tagged Bap-5 bound to the Ni-NTA agarose affinity column. The Bap-5 was further purified by SDS-PAGE and transferred by blotting onto a nitrocellulose membrane. The Bap-5 band was excised from the membrane and an extract (section 2.12) was sent to Dr, D. Xing, NIBSC, London to raise a rabbit antiserum. This anti-serum was used in subsequent work (section 3.3).

3.2.3. The role of Bap-*5* in *B. pertussis*

The Bap-5 protein belongs to the family of *B. pertussis* autotransporters and all of the previously characterised autotransporter members in *B. pertussis* are regulated by the Bvg two-component regulatory system. Two points had to be borne in mind when using the *bap-5* mutant strain. As the mutagenesis was carried out by inserting the km^R cassette downstream of the predicted translational start site (~510bp) (Appendix II), there was the possibility of getting transcription of truncated *bap-5* and that could cause difficulties in clearly defining the role of the gene. The other area that had to be borne in mind was the possible effect of the mutagenesis on neighbouring genes. The data base search of the flanking regions of *bap-5* using the *B. pertussis* genome sequence (BLAST search performed at http://www.sanger.ac.uk/projects/B_pertussis) indicated that a gene located 5'-upstream of *bap-5* is an amino acid transport gene and during a search for potential BvgA binding sites in the 5'-upstream region of *bap-5,* two potential BvgA-binding sites

Fig. 3.15: Stages in the purification of His-tagged full-length Bap-5.

Bap-5 prepared from the urea-solubilised inclusion bodies was allowed to interact with Ni-NTA agarose and then eluted with buffers of different pH values (2.10.4).

A = Urea extract of Bap-5

B = Buffer A elutate

(8M urea, 0.1M sodium phosphate, 0.01M Tris, pH 6.0)

C= Buffer B eluate

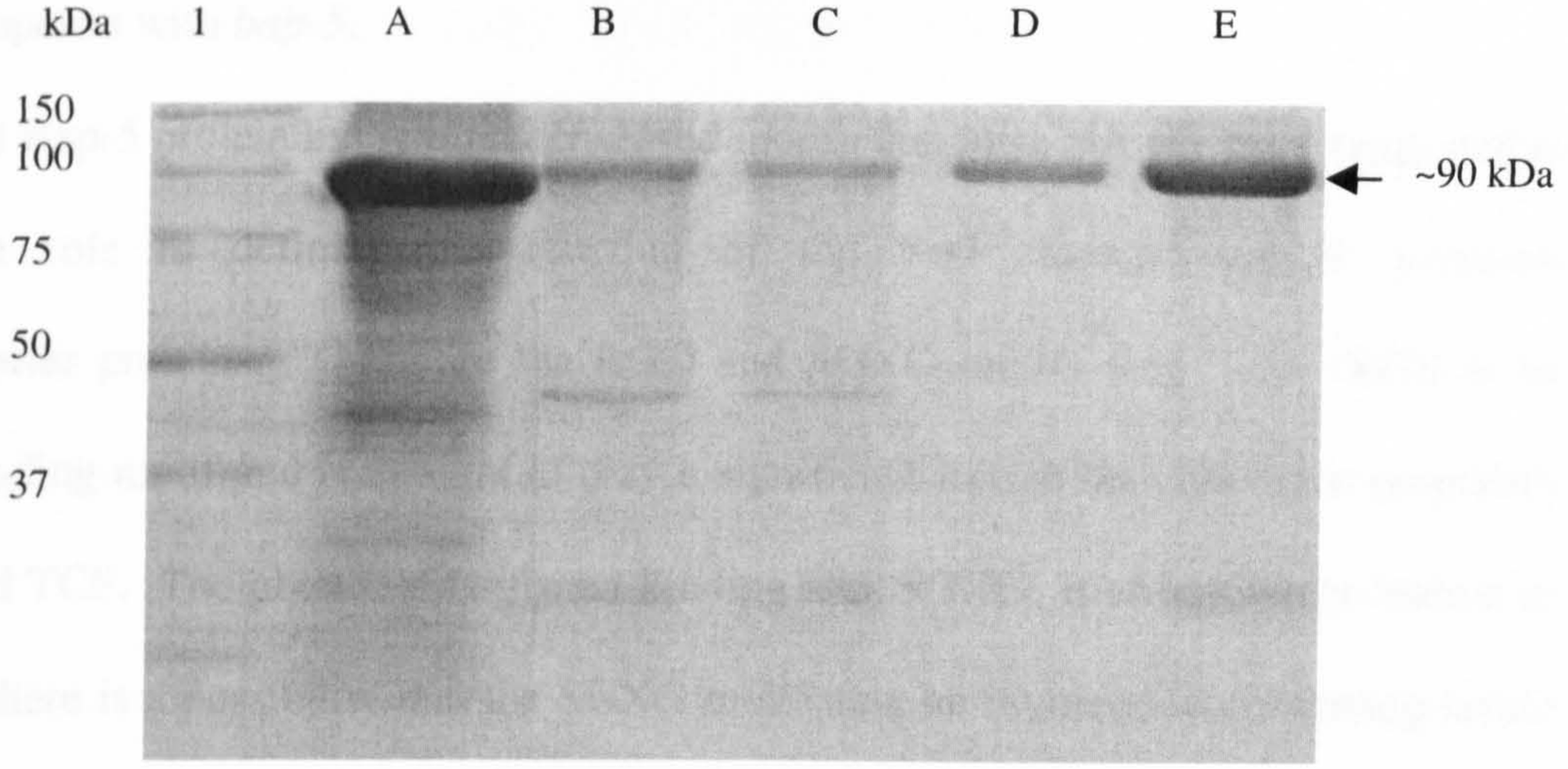
(8M urea, 0.1M sodium phosphate, 0.01M Tris, pH 5.2)

D = Buffer C eluate 1

(8M urea, 0.1M sodium phosphate, 0.01M Tris, pH 4.0)

E = Buffer C eluate 2

(8M urea, 0.1M sodium phosphate, 0.01M Tris, pH 4.0)


kDa
1
A
B
C
D
E
150
100
75
50
37
~90 kDa

(Appendix III, shown in red) at 844 bp and 936 bp from the predicted translational start site i.e. first methionine (ATG 1) were located from the *B. pertussis* genome sequence (Contig 1069) between the amino acid transport gene and *bap-5*. A gene located 3'-downstream was a hypothetical gene of unknown function, which did not appear to form a part of an operon with *bap-5*.

The Bap-5 protein has certain conserved motifs that have already been suggested to play some role in defining the function of the well characterized *B. pertussis* autotransporter proteins. These are the RGD and SGXG motifs (Fig. 1.3). RGD is an integrin-binding motif and is thought to play a significant role in the adherence properties of PRN and TCF. The glycosaminoglycan-binding site, SGXG, is an important feature of BrkA and there is a possibility that the SGXG motif may be involved in conferring serum resistance on *B. pertussis* against complement-mediated killing by the classical pathway (Fernandez and Weiss, 1994; Barnes and Weiss, 2001). Hence these properties were worthy of investigation in relation to the role of *bap-5* in *B. pertussis*. It also seemed desirable to see if the *bap-5* mutagenesis had produced any effects on the production of the already well-characterised autotransporter virulence factors of *B. pertussis* i.e. PRN and BrkA before going any further. Another aim was to study Bap-5 localisation in *B. pertussis*.

3.2.3.1: Expression of bap-5 under non-modulating (Bvg^+) and modulating (Bvg^-) conditions.

The expression of *bap-5* in the wild-type *B. pertussis* was investigated initially by RT-PCR as described in section 3.1 and in Fig. 3.2. As all the *B. pertussis* autotransporters (section 1.9) described to date were found to be *bvg*-regulated, and taking this consensus as a precedent, the expression of *bap-5* in a Bvg^- strain BP347, of *B. pertussis* Tohama was

examined. Also, any expression of *bap-5* in the *bap-5*-defective *B. pertussis* Taberman II and Tohama II mutants was checked. The *bap-5* forward and NTS REV primers (Table 2.6) for the *bap-5* gene were selected for RT-PCR. These were expected to amplify a 505 bp fragment from the 3' region of *bap-5,* as shown in Fig. 3.16; lane B i.e. starting from 286bp to 791bp (Appendix II) (section 3.1.2). The RT-PCR result (Fig. 3.16) once again indicated that *bap-5* is expressed (505 bp product) in wild-type *B. pertussis* Taberman I and Tohama I (lanes C and E) and also indicated that *bap-5* expression is being effectively blocked by the successful introduction of the km^R cassette into the corresponding *bap-5* locus of *B. pertussis* chromosome (lane D, Taberman II; lane F, Tohama II). The RT-PCR was repeated twice and one representative gel is presented (Fig. 3.16). Lanes A and B represent PCR reactions with the genomic DNA extracted from the *bap-5* mutant (505 bp+1300bp km^R cassette) and its wild-type *B. pertussis* strain, Taberman I (505 bp), respectively. There is again an anomaly in these results which was described before in section 3.1.1 (Fig. 3.2), in that a band of ~100 bp was present in both *bap-5* wild-type strains (lanes C, E) and in BP347 (lane G). This band was not present in the *bap-5* mutant RT-PCR products. Hence, the RT-PCR result could not rule out the possibility of a truncated *bap-5* transcript in *bap-5* mutants, which possibly may have occurred in the portion of the gene prior to the km^R cassette insertion site. The other important finding was the absence of desired product of correct size (505 bp) in Bvg^- BP347 strain (lane G). However, the absence of the desired product from BP347 strain suggests that *bap-5* is *bvg*-regulated but the appearance of the smaller product is harder to explain. After this stage, as already stated in section 3.1.3 there were some problems regarding the abnormal phenotype of the Tohama-derived *bap-5*-defective mutant so only *B. pertussis* Taberman I and its *bap-5* mutant Taberman II was used for further characterisation study.

Fig. 3.16: RT-PCR results with RNA prepared from *B. pertussis* wild-type strains, *bap-5* mutants and *B. pertussis* strain BP347 (Bvg⁻).

RT-PCR was performed according to the protocol given in section 2.11.3 using the RNA isolated as in section 2.11.

1 = 1 Kb ladder

A= PCR control using genomic DNA from *B. pertussis* Taberman II, *bap-5* mutant

B = PCR control using genomic DNA from *B. pertussis,* Taberman I

C = RT-PCR with the RNA from the *B. pertussis,* Taberman I

D = RT-PCR with the RNA from the *B. pertussis,* Taberman II, *bap-5* mutant

E = RT-PCR with the RNA from the *B. pertussis,* Tohama I

F = RT-PCR with the RNA from the *B. pertussis,* Tohama II, *bap-5* mutant

G = RT-PCR with the RNA from the *B. pertussis,* Tohama, BP347 (Bvg⁻)

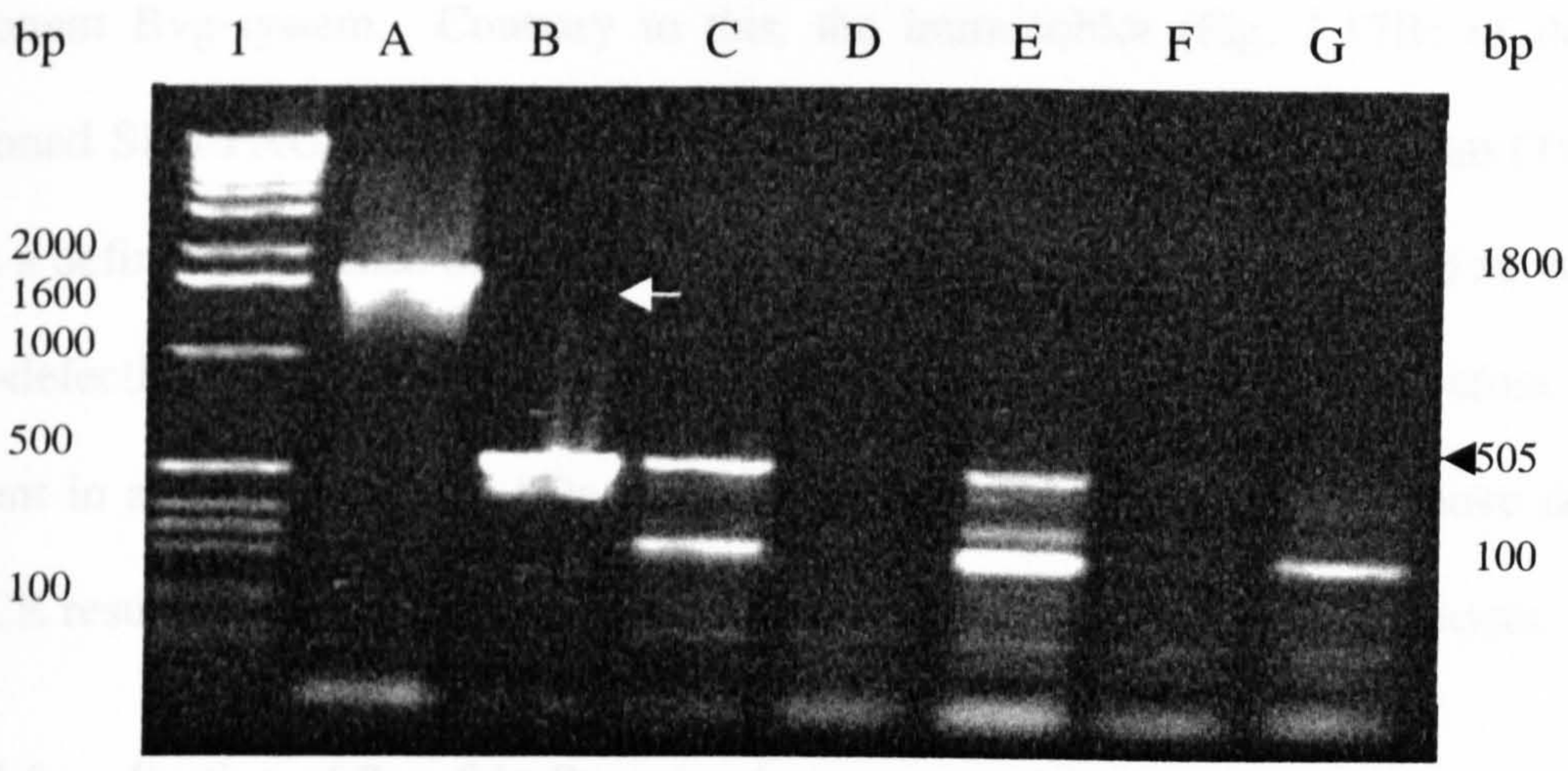

bp
1
A
B
C
D
E
F
G
bp
2000
1600
1000
500
100
1800
505
100

Fig. 3.17A shows the whole-cell protein profiles of *B. pertussis* Taberman I (lane A), its derived *bap-5*-defective mutant Taberman II (lane B) and modulated (growth in the presence of 40mM $MgSO_4$) Taberman I (lane C) as well as modulated *bap-5*-defective mutant Taberman II (lane D). The SDS-PAGE (Fig. 3.17A) profile of whole cells of parent and mutant strains look alike apart from the fact that modulated strains are deficient in number of protein bands, presumably representing virulence factors regulated by the two component Bvg-system. Contrary to this, the immunoblot (Fig. 3.17B) of the above mentioned SDS-PAGE gel screened with polyclonal rabbit anti-$_r$Bap-5 serum (Table 2.8) shows a definite difference between the *B. pertussis* Taberman I (lane A) and its derivative *bap-5*-defective mutant Taberman II (lane B) at ~90kDa. There is some cross reaction apparent in all 4 lanes at ~75 kDa. Thus the immunoblot confirms the above described RT-PCR results in particular regarding the *bvg*-regulation of *bap-5* in *B. pertussis*.

3.2.3.2 Localisation of Bap-5 in B. pertussis

3.2.3.2.1: Agglutination test

A agglutination procedure (section 2.15.1) was carried out using a rabbit polyclonal anti-whole-cell *B. pertussis* serum (1:10 of stock), rabbit anti-PRN antibody (anti-P.69) and rabbit polyclonal anti-whole $_r$Bap-5 specific antibody (section 2.12, Table 2.7). The agglutination test was carried out in 96-well u-shaped microplates using bacterial cells scraped from BG plates and suspended in PBS and the suspension standardized using an opacity rod. A dense fluffy type of agglutination was observed in cases wherever a positive (+++) reaction occurred with the anti-whole-cell *B. pertussis* serum, presumably due to the cross-linking of surface appendages such as FHA and fimbriae. However, in the case of rabbit anti-PRN and rabbit anti-whole $_r$Bap-5 sera, wherever positive (+) reactions were seen, a less dense, granular type of agglutination was noticed, presumably due to a

Fig. 3.17: SDS-PAGE showing the protein profile of the whole cell of *B. pertussis* in non-modulating and modulating conditions

Solubilised whole cells were prepared from bacteria grown in CL medium to OD_{600nm} ~ 1.0. The whole cell proteins were screened with rabbit anti-whole Bap-5 serum.

3.17A SDS-PAGE

A = *B. pertussis,* Taberman I

B = *B. pertussis,* Taberman II, *bap-5* mutant

C = *B. pertussis* Taberman I modulated by adding $MgSO_4$ to the growth medium

D = *B. pertussis* Taberman II, *bap-5* mutant modulated by adding $MgSO_4$ to the growth medium

3.17B Immunoblot

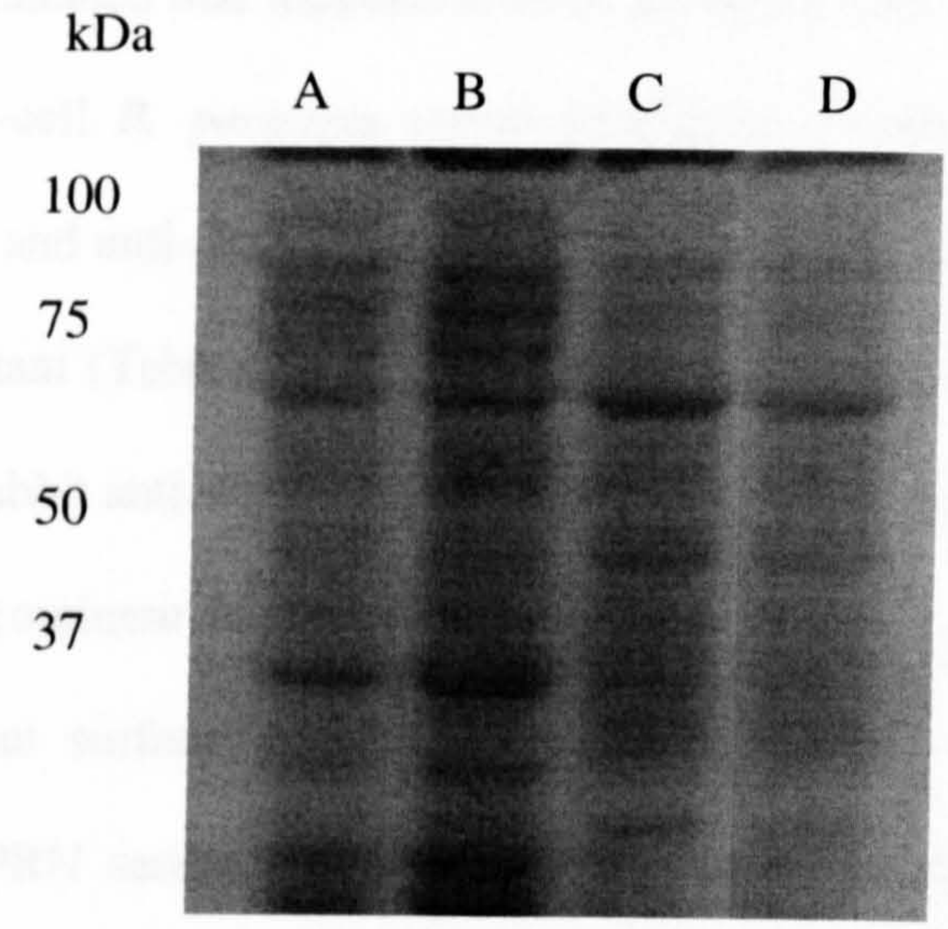
A = *B. pertussis,* Taberman I

B = *B. pertussis,* Taberman II, *bap-5* mutant

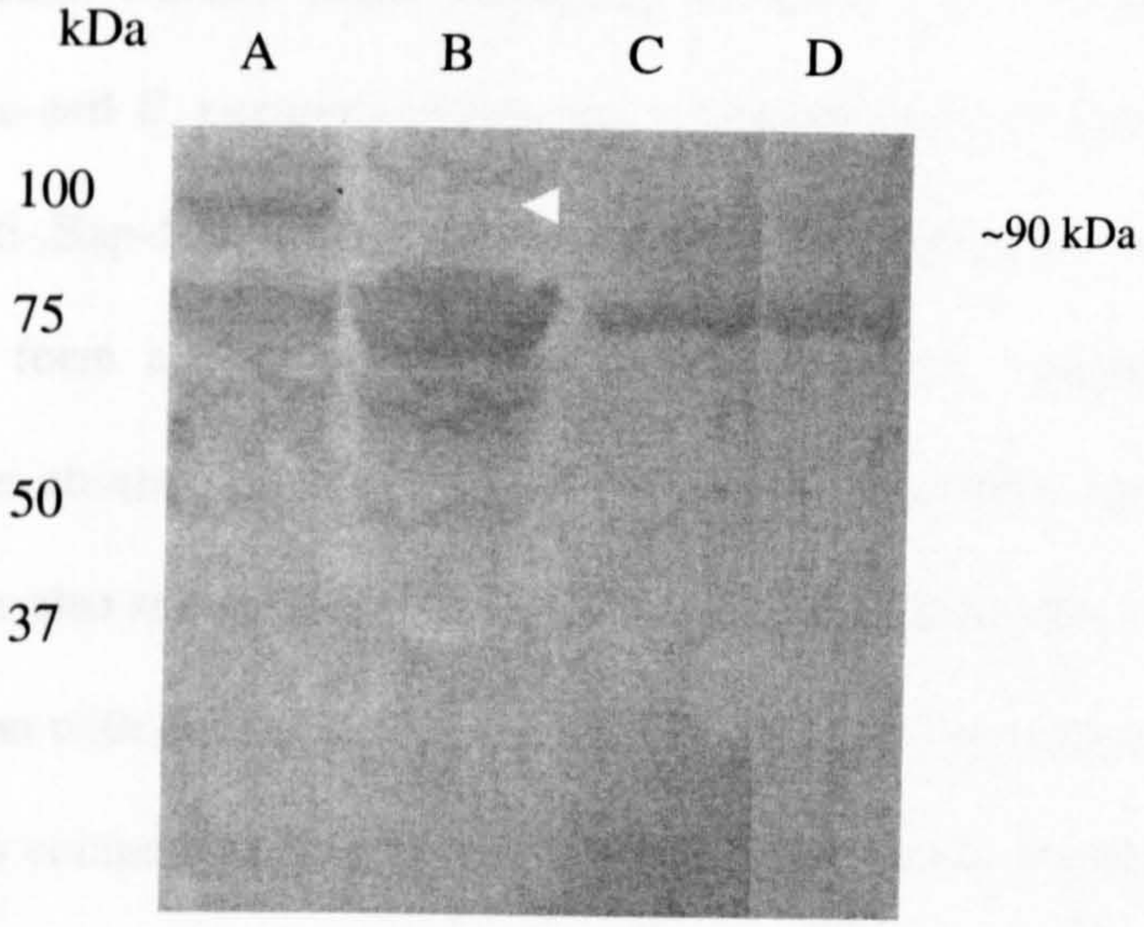
C = *B. pertussis* Taberman I modulated by adding $MgSO_4$ to the growth medium

D = *B. pertussis* Taberman II, *bap-5* mutant modulated by adding $MgSO_4$ to the growth medium

3.17A



3.17B



closer-association of bacteria created by antibodies to the outer-membrane proteins. The result was repeated twice and similar results were observed. Table 3.1 shows a summary of the results which indicated that incubation of *B. pertussis* Taberman I strain with rabbit polyclonal anti-whole-cell *B. pertussis* serum produced a fluffy type of agglutination, whereas the anti-PRN and anti-$_r$Bap-5 sera produced a granular type of agglutination. The *B. pertussis bap-5* mutant (Taberman II) behaved like its parent Taberman I strain except after incubation with rabbit anti-whole $_r$Bap-5 antibodies, where the *bap-5* mutant appeared to form a clear and prominent button which suggested lack of agglutination due to the absence of the relevant surface antigen i.e. Bap-5. The *bap-5* mutant did however agglutinate with anti-PRN serum. *B. pertussis* Tohama (BP338) and its derived *brkA* mutant (BP2041) strain also appeared to show a fluffy type of agglutination when incubated with anti-whole-cell *B. pertussis* serum whereas, on incubation with anti-PRN and anti-$_r$Bap-5 antisera, they showed the granular type of agglutination. *B. pertussis* BBC29 and its *prn* mutant BBC30 strain produced the fluffy type of agglutination on reacting with anti-whole-cell *B. pertussis* serum and a granular type of agglutination was observed with rabbit anti-$_r$Bap-5 antiserum. However, the *prn* mutant after incubation with anti-PRN, appeared to form a clear and prominent button which suggested a lack of agglutination due to the absence of relevant surface antigen i.e. PRN, unlike its parent strain BBC29. The table also shows the results with *B. bronchiseptica* BBC17 and its Bvg$^-$ derivative and its reaction with different antisera. As most of the outer-membrane proteins are regulated by the two component Bvg system (section 1.5), a lack of those proteins on the bacterial surface would be expected to produce a negative result on incubation with the antisera which was raised predominantly against them. The expected result was observed as there appeared to be no visible agglutination in the case of Bvg$^-$ BBC17 strain, whereas

Table 3.1: Results of agglutination assay

STRAINS	Anti whole-cell *B. pertussis* (Hertz)	Anti-PRN	Anti-whole Bap-5	Self-agglutination control, bacteria alone
B. pertussis Taberman I, wild-type	+++ F	+ G	+ G	-
B. pertussis Taberman II, *bap-5* mutant	+++ F	+ G	-	-
B. pertussis BP338	+++ F	+ G	+ G	-
B. pertussis BP2041, *brkA*-mutant	+++ F	+ G	+ G	-
B. pertussis BBC29	+++ F	+ G	+ G	-
B. pertussis BBC30, *prn* mutant	+++ F	-	+ G	-
B. bronchiseptica BBC17	+++ F	+ G	+ G	-
B. bronchiseptica) BBC17, Bvg⁻ mutant	-	-	-	-
E. coli M15 expressing recombinant (Bap-5)	-	-	++ G	-
B. pertussis Taberman I, modulated by adding $MgSO_4$ to the growth medium	+ G	-	-	-

- = No agglutination,but clear button formation
+++ = Strong agglutination
++ = Moderate agglutination
+ = Weak agglutination

F = Fluffy type of agglutination presumably due to cross-linking surface-appendages such as fimbriae and FHA

G = Granular type of agglutination presumably due to cross-linking via surface-exposed OMPs such as autotransporters of *B. pertussis*

the parent BBC17 strain appeared to show the fluffy type of agglutination on incubation with rabbit anti-whole-cell *B. pertussis* serum and the granular type of agglutination when incubated with anti-PRN and anti-$_r$Bap-5 antisera. This result showed close cross-reactivity between *B. pertussis* and *B. bronchiseptica bvg*-regulated OMPs. The Table also shows the cross reaction between *E. coli* M15 expressing the full length recombinant Bap-5 with anti-$_r$Bap5 serum but not the other sera. The *E. coli* strain expressing Bap-5 seemed to show a stronger agglutination with the anti-$_r$Bap-5 antiserum than the *B. pertussis* strains and *B. bronchiseptica* BBC17 strain. This result strongly suggested that Bap-5 is surface exposed in this *E. coli* strain, M15. The last result shows that *B. pertussis* Taberman I strain, when modulated by growing in the growth medium containing 40mM $MgSO_4$, gave some granular type of agglutination on incubation with rabbit anti-whole-cell *B. pertussis* serum only, which suggested that most of the surface agglutinogens are not present to react with their respective antiserum components. The last column represents the different bacterial cells resuspended in PBS alone (negative control).

3.2.3.2.2: Immunofluorescence

To prove that Bap-5 is surface-located, like many other virulence determinants of *B. pertussis*, an indirect immunofluorescence assay was carried out using rabbit anti-whole $_r$Bap-5 serum. The bacteria were stained with Hoechst 33272 stain and immobilised on poly L-lysine (Sigma) coated slides (Section 2.19.2) and were then exposed to different concentrations of rabbit anti-$_r$Bap-5 serum. An initial high fluorescence background problem was minimized by using the antiserum at a dilution of 1:200 and by incorporating extensive washing between each step. A FITC-conjugated goat anti-rabbit secondary antibody was used to detect any surface bound anti-$_r$Bap-5 antibody. Figs. 3.18A and 3.18C show *B. pertussis* Taberman I and Taberman II, its *bap-5* mutant strain visualised

Fig. 3.18: Immunofluorescence detection of Bap-5 antigen on the surface of *B. pertussis* using rabbit anti-$_r$Bap-5 antibody.

B. pertussis from BG agar was stained with Hoechst stain as in section 2.15.2.1 and subjected to immunofluorescence as in section 2.15.2.2 and viewed under a Zeiss-Axioskop epifluorescence microscope.

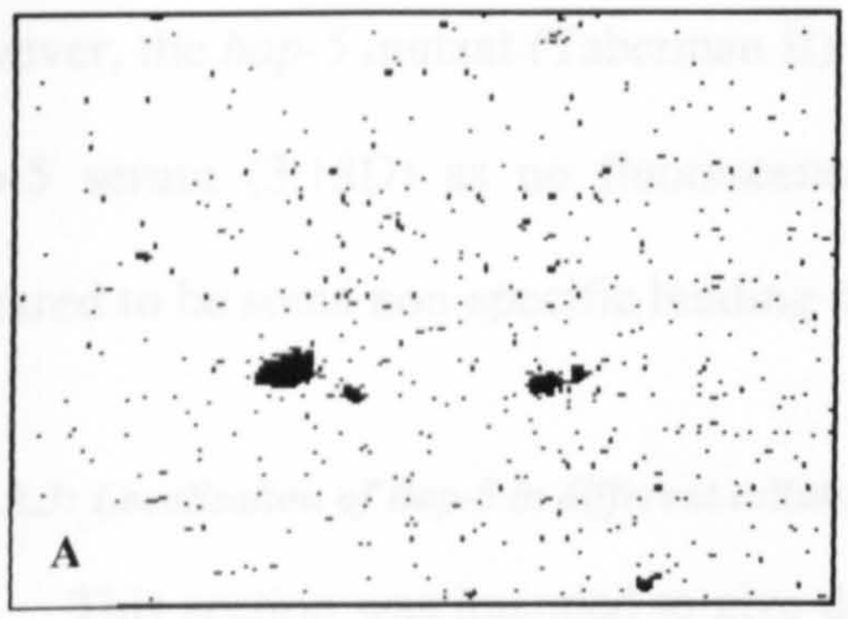
A = *B. pertussis* strain Taberman I as seen by phase contrast microscopy.

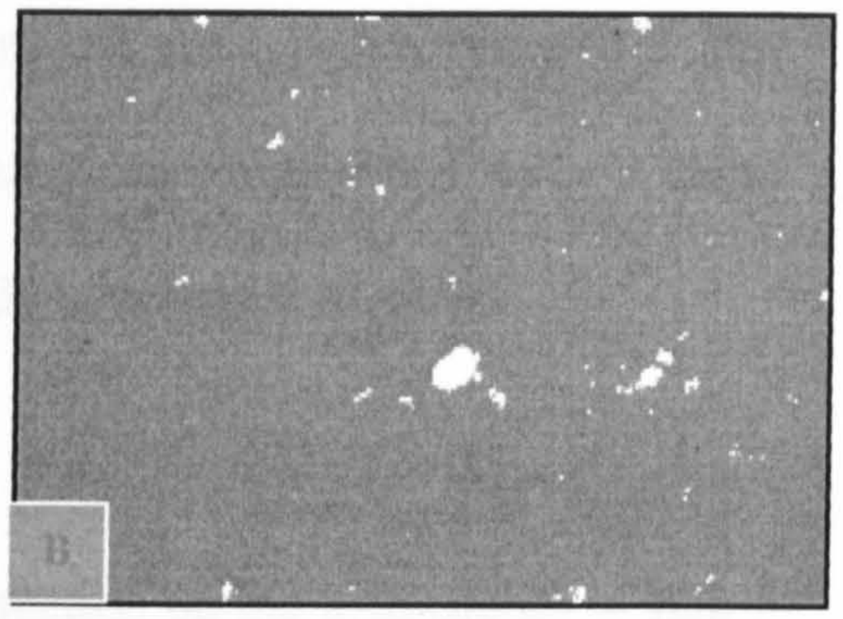
B = Image of the same field (A) viewed by the fluorescence microscopy.

C = *B. pertussis* Taberman II, *bap-5*-defective mutant, as seen by phase contrast microscopy.

D = Image of the same field (C) viewed by the fluorescence microscopy.

All the images were visualised with a 63 x neofluor Ph3 lens (magnification 63 x 20 = 1260).


A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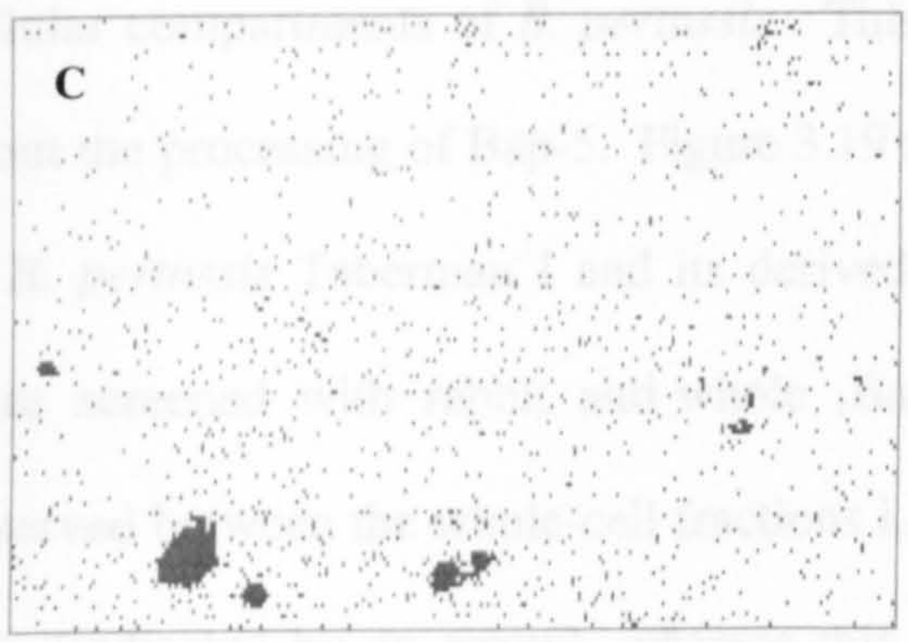
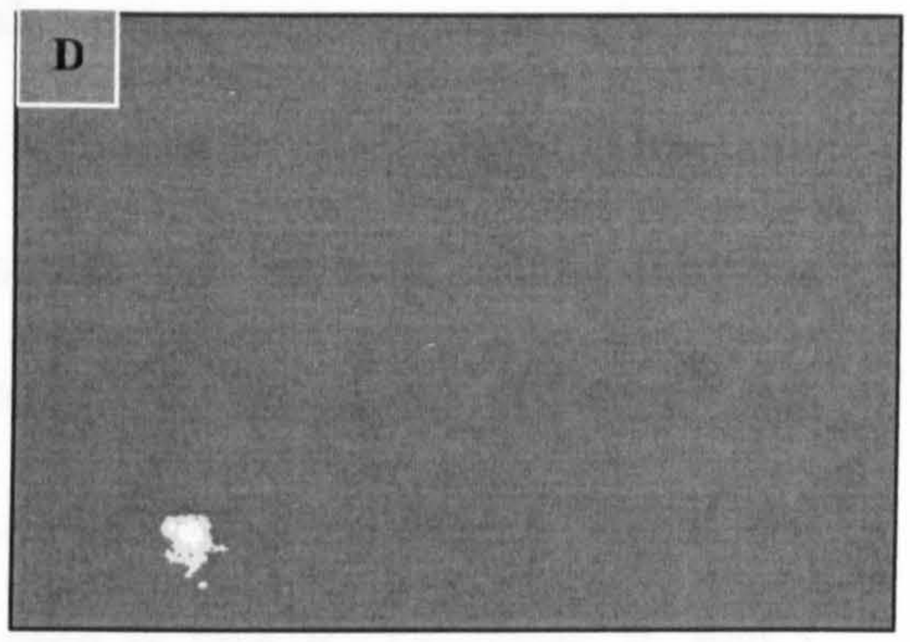

C


D

by phase-contrast microscopy. Figs 3.18B and 3.18D respectively show the same fields as 3.18A and 3.18C when visualised by fluorescence microscopy (magnification 1260). As shown in Fig. 3.18, it could be demonstrated that Bap-5 in *B. pertussis* (Taberman I) is surface exposed (3.18B) as the individual bacterial cells were clearly fluorescent. However, the *bap-5* mutant (Taberman II) of *B. pertussis* did not appear to react with anti-ᵣBap-5 serum (3.18D) as no fluorescence was detected from individual cells. There appeared to be some non-specific binding of the antibody to clumped bacteria.

3.2.3.2.3: Localisation of Bap-5 in different cellular fractions

This section was intended to give detailed information on the localisation of Bap-5 in *B. pertussis.* After showing by immunofluorescence and agglutination that Bap-5 appears to be surface-exposed, the next attempt was to identify Bap-5 in the different cellular compartments of *B. pertussis.* This would also presumably generate information about the processing of Bap-5. Figure 3.19 shows an immunoblot of the different fractions of *B. pertussis* Taberman I and its derived *bap-5* mutant Taberman II. These fractions were screened with rabbit anti-whole ᵣBap-5 antibody. A prominent difference was observed between the whole-cell fractions i.e. lane A (Taberman I) showing a band at ~90 kDa (indicated by an arrow), whereas this was not seen in lane B (Taberman II, *bap-5* mutant), a result similar to that shown in Fig. 3.17 previously. This band runs at a higher molecular weight than the predicted size of Bap-5 i.e. ~79 kDa and it may be that the protein runs anomalously on SDS-PAGE. This behaviour has been described for other autotransporters such as mature PRN, which has molecular weight of 61 kDa but runs at 69 kDa on SDS-PAGE. There was another difference noticed in the outer membrane-enriched fractions. In *B. pertussis* Taberman I, the outer membrane fraction showed a band at ~65 kDa (lane C, indicated by an arrow), which was absent in the *bap-5* mutant

Fig. 3.19: Immunoblot showing different fractions of *B. pertussis* Taberman I and its *bap-5* mutant, reacted with anti-whole$_r$Bap-5 serum

B. pertussis strains grown to same OD_{600nm}~1 in CL medium were used to isolate different cellular fractions, as described in section 2.10.1.

A = *B. pertussis* Taberman I, whole cell preparation

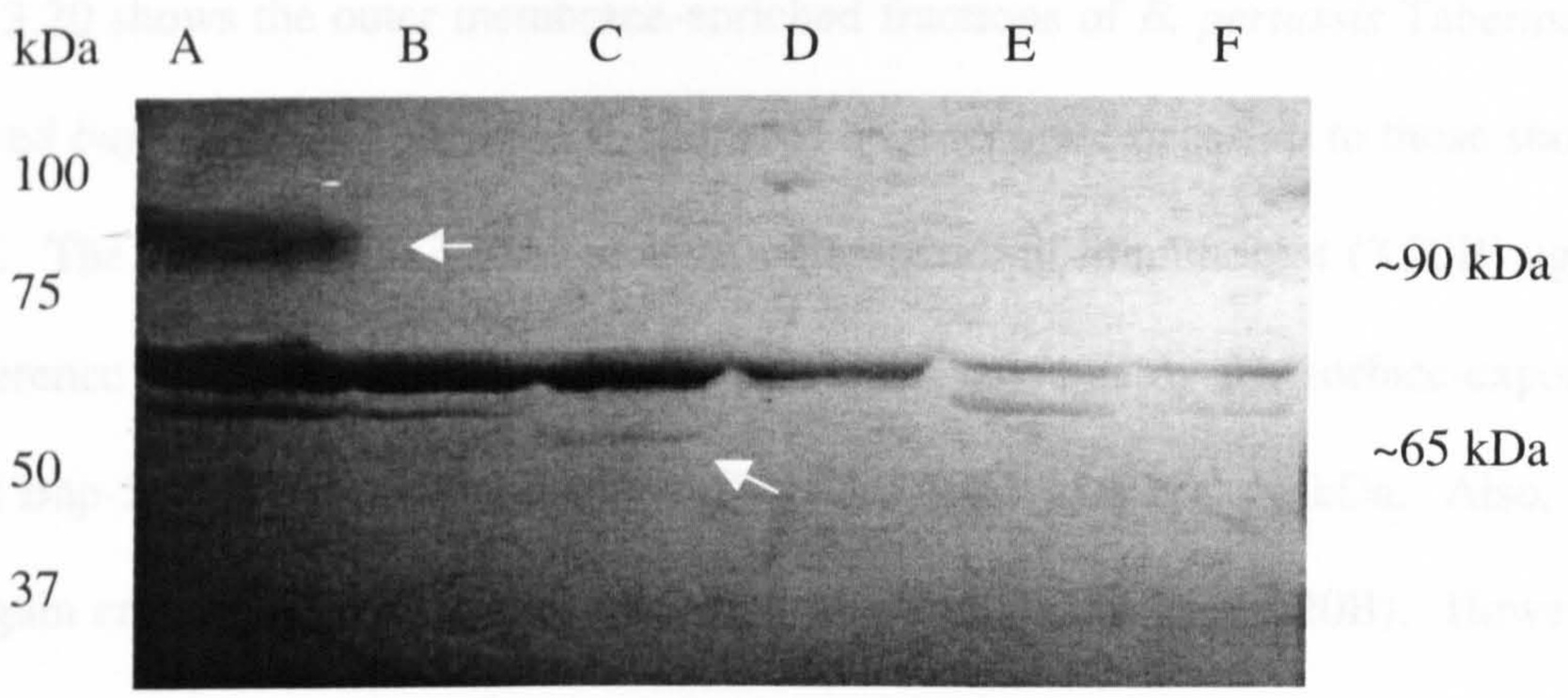
B = *B. pertussis* Taberman II, *bap-5* mutant whole cell preparation

C = *B. pertussis* Taberman I, outer membrane preparation

D = *B. pertussis* Taberman II, *bap-5* mutant outer membrane preparation

E = *B. pertussis* Taberman I, inner membrane preparation

F = *B. pertussis* Taberman II, *bap-5* mutant inner membrane preparation


kDa
A
B
C
D
E
F
100
75
50
37
~90 kDa
~65 kDa

(Taberman II, lane D). This may be the processed form of Bap-5 running at an approximately higher than expected size (predicted to be 49 kDa). The inner-membrane fractions of Taberman I and its derived *bap-5* mutant Taberman II showed similar profiles. The anti-whole $_r$Bap-5 serum cross-reacted strongly in all fractions with two bands at ~70-75 kDa, which may have represented cross-reactions with other autotransporter proteins e.g. mature BrkA protein (73 kDa).

Fig. 3.20 shows the outer membrane-enriched fractions of *B. pertussis* Taberman I and its derived *bap-5* mutant Taberman II, prepared on a separate occasion to those shown in Fig. 3.19. The SDS-PAGE (3.20A) and the corresponding immunoblot (3.20B) again show a difference between the two at ~90 kDa. This is presumably the surface-exposed unprocessed Bap-5. There are other minor differences at 65 kDa and 49 kDa. Also, the antiserum again cross-reacted strongly with bands at ~70-75 kDa (Fig. 3.20B). However the other major difference between Taberman I (Fig. 3.20A, lane A) and its derived *bap-5* mutant (Fig. 3.20 lane B) is at ~16 kDa which is consistently reproducible in SDS-PAGE gels stained with coomassie blue, but the difference is also present as a weak reaction in the immunoblot (Fig. 3.20B, lane B). This could possibly be the N -terminal portion of the Bap-5 being processed by some unknown outer membrane proteases or perhaps may represent the processing of the ~30 kDa C-terminal portion of Bap-5 which is not immediately obvious in SDS-PAGE gels or immunoblots.

Fig. 3.21 shows SDS-PAGE profiles of the outer membrane fractions of Taberman I and its derived *bap-5* mutant Taberman II (lanes A and B) run on a 16% polyacrylamide gel. The BrkA parent strain, BP338 and BrkA-deficient mutant BP2041 (lanes C and D) were included as controls for the outer membrane preparation as BrkA is known to be a surface-exposed protein. The ~16 kDa protein noticed in previous result (Fig. 3.20A) is

Fig. 3.20: SDS-PAGE and corresponding Immunoblot showing the outer membrane-enriched preparations of *B. pertussis* Taberman and its *bap-5* mutant reacting with anti whole $_r$Bap-5 serum

B. pertussis strains grown in CL medium were used to isolate different cellular fractions as in section 2.10.1

3.20A SDS-PAGE

A = Outer membrane preparation from *B. pertussis* Taberman I

B = Outer membrane preparation from *B. pertussis* Taberman II, *bap-5*-mutant

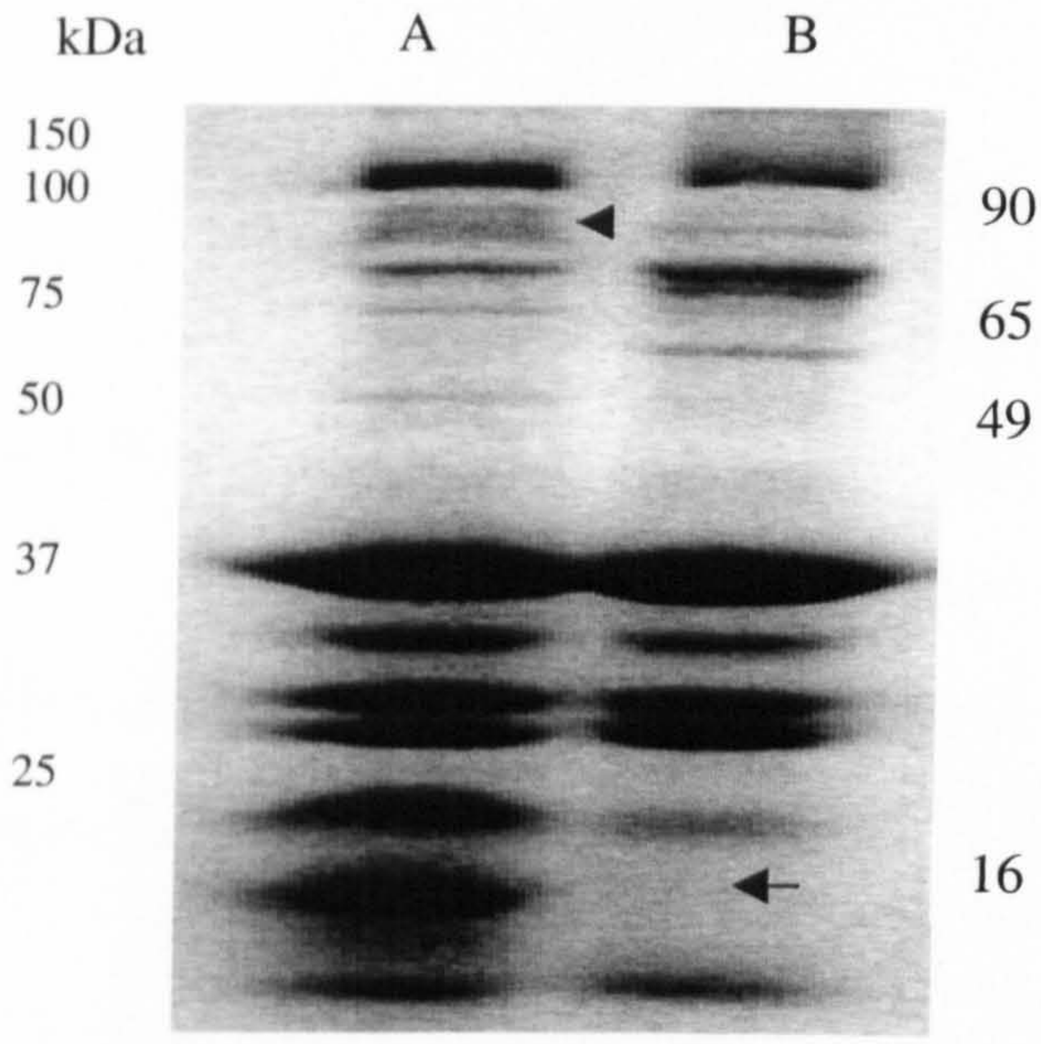
3.20B Immunoblot

1 = Protein ladder

A = Outer membrane preparation from *B. pertussis* Taberman I

B = Outer membrane preparation from *B. pertussis* Taberman II, *bap-5*-mutant

3.20A


kDa
A
B
150
100
75
50
37
25
90
65
49
16

3.20B

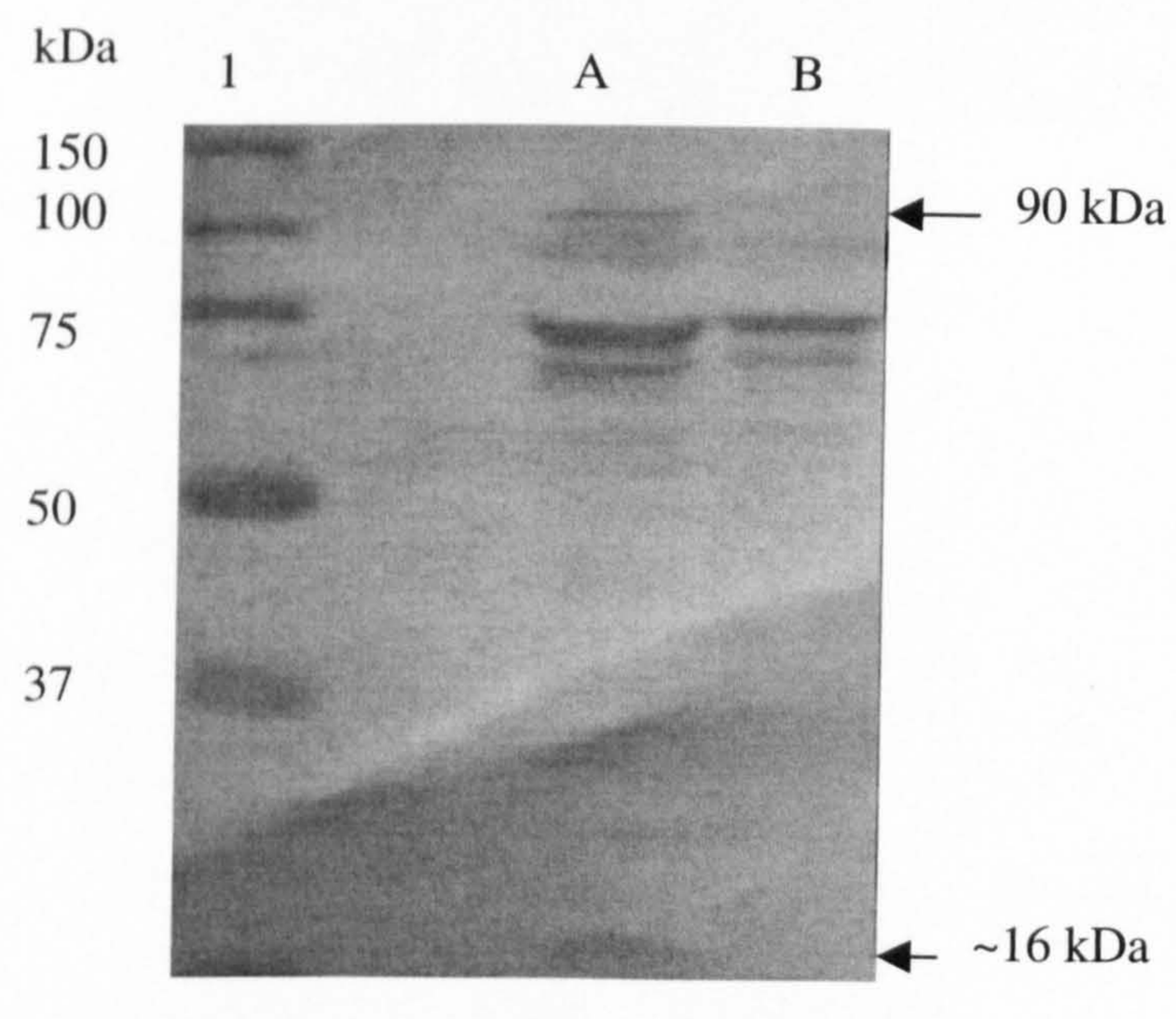

kDa
1
A
B
150
100
75
50
37
90 kDa
~16 kDa

Fig. 3.21: SDS-PAGE showing the outer membrane-enriched fractions of different *B. pertussis* strains and the effect of solubilisation temperature on the mobility of Bap-5

1 = Pre stained protein marker

A = Outer membrane preparation from *B. pertussis* Taberman I

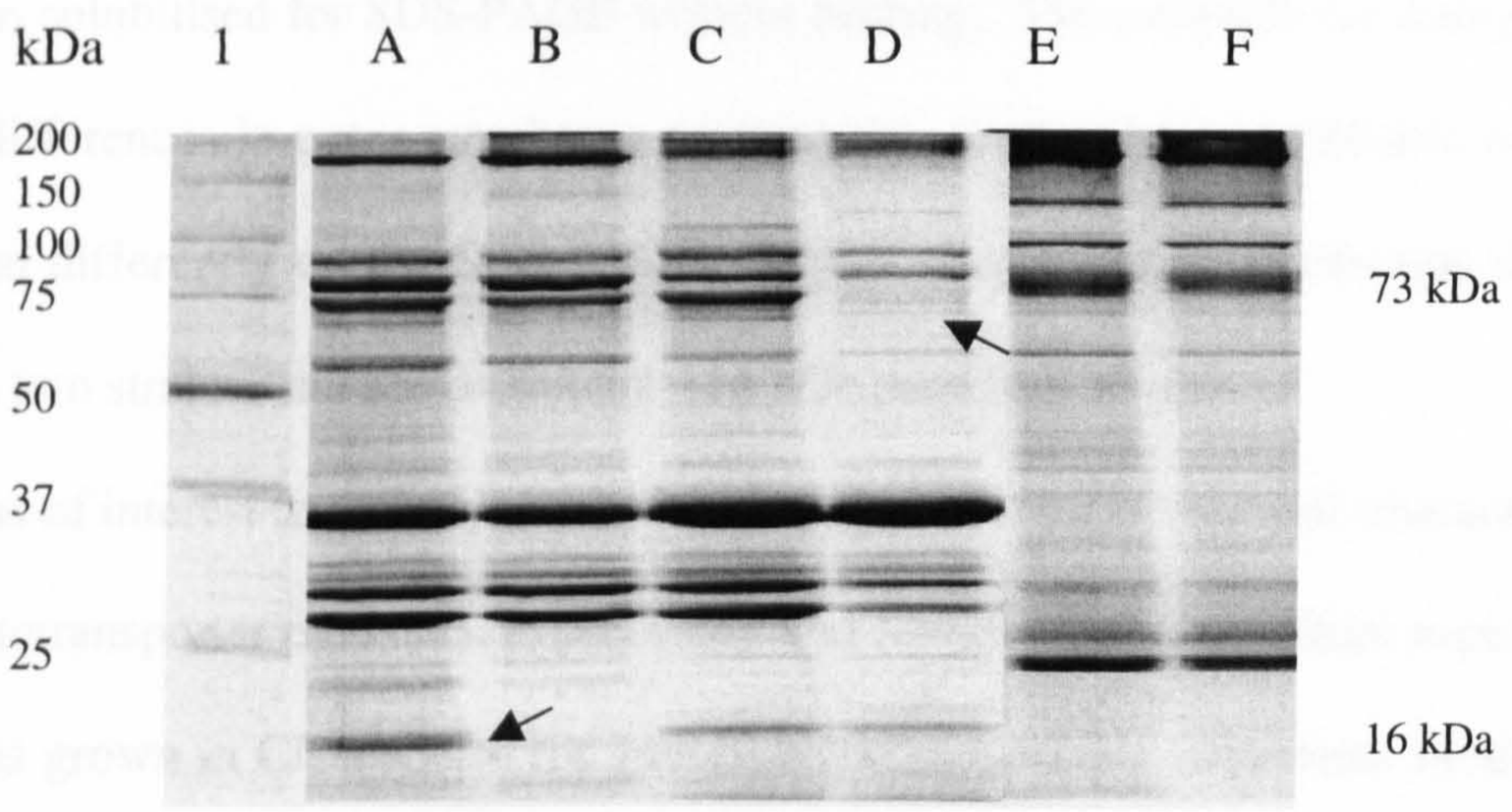
B = Outer membrane preparation from *B. pertussis* Taberman II, *bap-5*-mutant

C = Outer membrane preparation from *B. pertussis* BP338

D = Outer membrane preparation from *B. pertussis brkA* mutant (BP2041)

E = Outer membrane preparation from *B. pertussis* Taberman I solubilised without heat treatment

F = Outer membrane preparation from *B. pertussis* Taberman II, *bap-5*-mutant solubilised without heat treatment


kDa
1
A
B
C
D
E
F
200
150
100
75
50
37
25
73 kDa
16 kDa

present in lane A (Taberman I) and absent in its *bap-5* mutant lane B but there is no obvious difference at ~90 kDa and ~65 kDa. A difference at ~73 kDa (indicated by an arrow) can be noted between lane C (BP338) and lane D (*brkA* mutant, B2041), which may correspond to the processed BrkA protein. Also a difference in band-density at 30 kDa can be seen in the lanes C (bold band present) and D (fainter band present), which may reflect the absence of the 30 kDa autotransporter domain of BrkA protein. Lanes E and F show the profiles of outer-membrane preparations of *B. pertussis* Taberman I and its *bap-5* mutant when solubilised for SDS-PAGE without heating. The rationale for doing this was to see any differences in outer membrane proteins due to their heat modifiable nature and ability to run differently on the SDS-PAGE. In this case, there is no obvious difference between the two strains, and the prominent ~16 kDa protein is absent.

It was of interest to determine whether Bap-5, like most of the well-characterised *B. pertussis* autotransporter members, is processed and released into the culture supernate. *B. pertussis* was grown in CL medium for ~48-72 h (OD_{600nm}~1.2). Supernate samples were concentrated (~50 times) with centrifuge columns (centricons; Greiner, U.K) of 30,000 M.W. cut off size according to manufacturer's instructions (section 2.10.1). The concentrated supernate fractions of different *Bordetella* species grown in CL medium as specified in section 2.1.3 and 2.1.4 (Table 2.4), were run on SDS-PAGE and blots were screened with the rabbit anti-whole $_r$Bap-5 serum (Fig. 3.22). The SDS-PAGE profile (Fig. 3.22A) showed that there was no apparent difference between the culture supernates of Taberman I (lane A) and its *bap-5* mutant (lane B), whereas there was a difference between Tohama BP338 (BrkA parent) (lane C) and its *brkA* mutant strain BP2041 at the correct place i.e. ~73 kDa. Lanes E and F show *B. pertussis* BBC29 and its *prn* mutant BBC30. The latter supernate unfortunately did not concentrate well enough or proteins were degraded by proteolytic activity of some unknown nature. Similarly, the supernates

Fig. 3.22: SDS-PAGE (A) and its corresponding immunoblot (B) showing the concentrated culture supernates of various *Bordetella* species reacting with anti-whole $_r$Bap-5 serum

Bordetella species were grown in CL medium to OD_{600nm} ~1 and their supernatant fractions were concentrated as in section 2.10.1 and separated on 12% SDS-PAGE and also transferred onto nitrocellulose-membrane for immunoblotting.

1 = Protein ladder

A = *B. pertussis* Taberman I

B = *B. pertussis* Taberman II, *bap-5* mutant

C = *B. pertussis* BP338

D = *B. pertussis* BP2041, *brkA* mutant

E = *B. pertussis* BBC29

F = *B. pertussis* BBC30, *prn* mutant

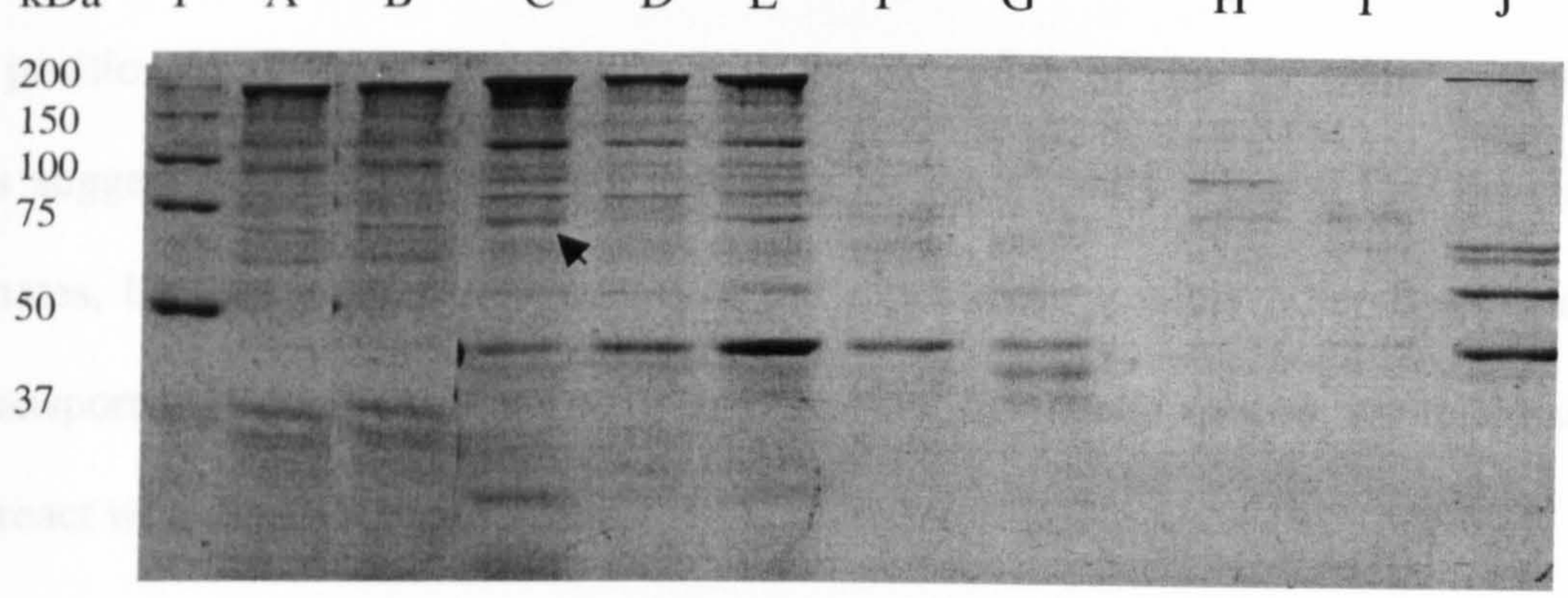
G = *B. pertussis* Taberman I, modulated by growing in the presence of $MgSO_4$.

H = *B. parapertussis* (NCTC 5952)

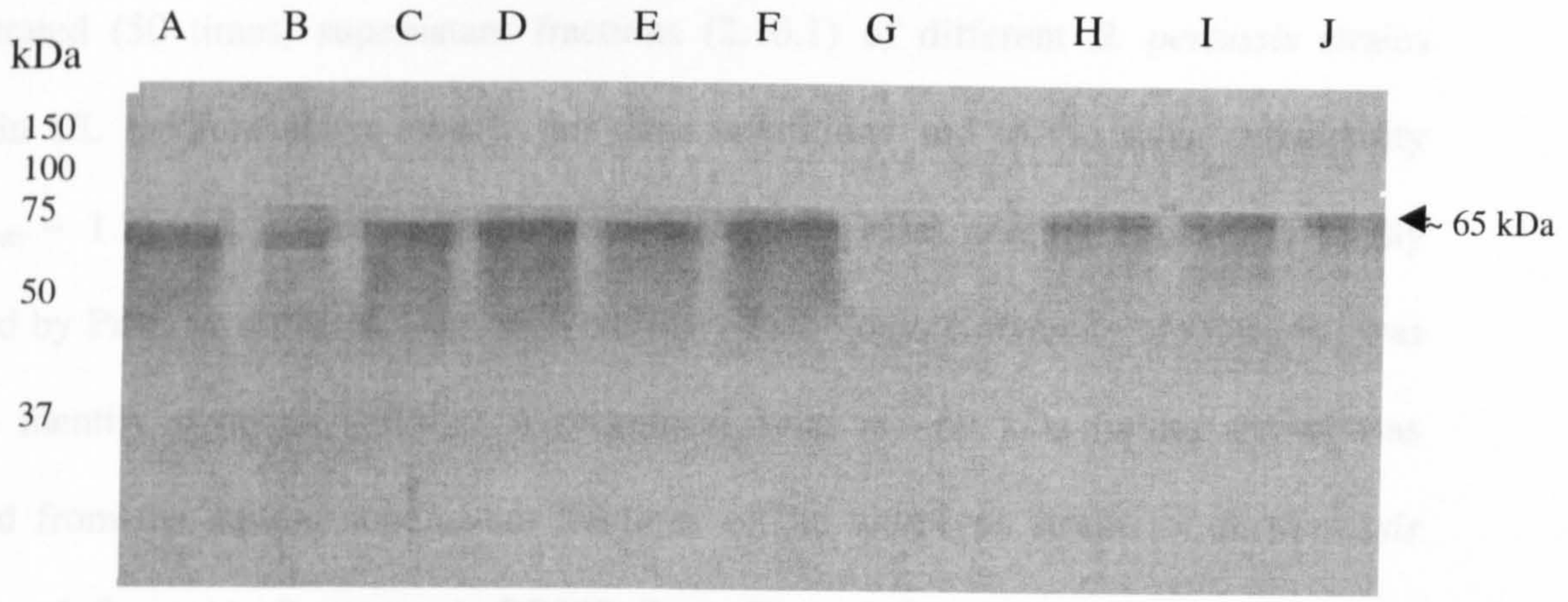
I = *B. avium* 4480

J = *B. bronchiseptica* BBC17

3.22A


kDa
1
A
B
C
D
E
F
G
H
I
J
200
150
100
75
50
37

3.22B


kDa
A
B
C
D
E
F
G
H
I
J
150
100
75
50
37
65 kDa

for *B. parapertussis* (lane H) and *B. avium* (lane I) showed that little protein was present. Lane G contains the supernate from Taberman I strain, modulated with $MgSO_4$ which shows that few proteins are present. Lane J shows *B. bronchiseptica* strain (BBC17). The corresponding immunoblot (Fig. 3.22B) of the gel shows consistent cross-reaction at the same position in all lanes i.e. ~65 kDa with the exception of lane G. Thus, overall the results suggest that a Bap-5 does not appear to be significantly released into the culture supernates, but that some component(s) of the supernates, possibly other Bvg-regulated autotransporters common to *B. pertussis* and the other *Bordetella* species, are released and cross-react with the anti-,Bap-5 serum.

3.2.3.3: Localisation of other B. pertussis autotransporter proteins

The production of other, well-characterised autotransporters in *B. pertussis* was also investigated by using their specific antisera in immunoblots of the various cell fractions of the different parent and mutant strains. Fig. 3.23 shows an immunoblot of concentrated (50 times) supernatant fractions (2.10.1) of different *B. pertussis* strains grown in CL medium under exactly the same conditions and to the same cell density (OD_{600nm} ~ 1.2). *B. pertussis* rabbit anti-PRN (P.69 kDa) serum (Table 2.7) kindly provided by Prof. M. Roberts, Dept. of Veterinary Pathology, University of Glasgow, was used to identify pertactin (PRN). A prominent band at ~69 kDa (white arrow) was observed from the culture supernatant fractions of the wild-type strains of *B. pertussis* Taberman I (lane A), *B. pertussis* BP338 (lane C), and *B. pertussis* BBC29 (lane E) showing that PRN is released from the cell surface. The *bap-5* mutant Taberman II (lane B) and *brkA* mutant, BP2041 (lane D) also reacted with rabbit anti-PRN serum. However, no band was seen with the concentrated culture supernatant fraction from the *prn* mutant BBC30 (lane F) and Taberman I modulated with $MgSO_4$ (lane G). Hence the result clearly

Fig. 3.23: Immunoblot of concentrated culture supernates of various *B. pertussis* strains screened with anti-PRN serum to identify pertactin

Bordetella pertussis strains were grown in CL medium, whereas *E. coli* strains expressing recombinant proteins were grown in LB medium, and their supernatant fractions were concentrated as in section 2.10.1 and separated on 12% SDS-PAGE and transferred onto the nitrocellulose-membrane for immunoblotting with anti-P.69 serum (Table 2.7).

1 = Protein ladder

A = *B. pertussis* Taberman I

B = *B. pertussis* Taberman II, *bap-5* mutant

C = *B. pertussis* BP338

D = *B. pertussis* BP2041, *brkA* mutant

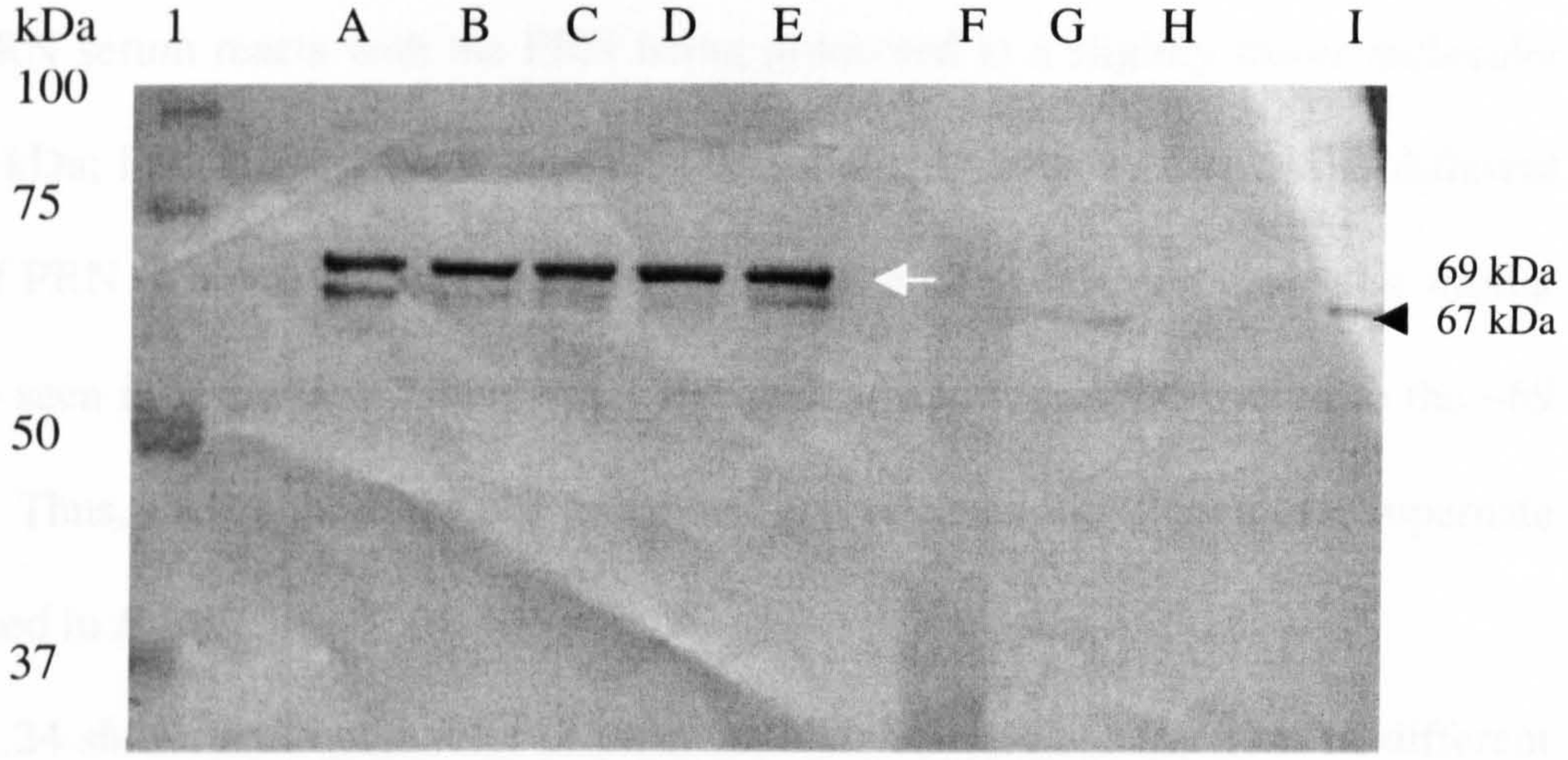
E = *B. pertussis* BBC29

F = *B. pertussis* BBC30, *prn* mutant

G = *B. pertussis* Taberman I, modulated by growing in the presence of $MgSO_4$.

H = Purified recombinant whole Bap-5

I = Supernatant fraction of *E. coli* KS272 expressing full length pertactin (p41869) (Table 2.5)


kDa
1
A
B
C
D
E
F
G
H
I
100
75
50
37
69 kDa
67 kDa

showed that the mutagenesis of *bap-5* did not affect the production of PRN in Taberman II, the *bap-5* mutant as was also the case with the *brkA* mutant, BP2041. The result also indicated that there was no cross-reaction between antiserum to P.69 and purified recombinant Bap-5 (lane: H). Lane I shows the culture supernate obtained when full length recombinant PRN (P.93) is expressed in *E. coli* (KS 272). It appeared (lane I) that rabbit anti-PRN serum reacts with the PRN being processed at a slightly lower molecular weight (~67 kDa; indicated by black arrow). This anomaly may be due to the different processing of PRN in a non native system (*E. coli*), but a cross-reacting band at a similar size was also seen in *B. pertussis* supernates, although at a lower concentration to the ~69 kDa protein. Thus, PRN appeared to be processed and released into the culture supernate when expressed in *E. coli*.

Fig. 3.24 shows an immunoblot of outer membrane-enriched fractions of different *B. pertussis* strains, grown under the same conditions and to the same cell density (OD_{600nm} ~1.2) as before. A mouse monoclonal anti-BrkA C-terminal antibody (P28) (Table 2.7) was used to screen these preparations. A prominent band at 31 kDa was observed from the culture supernatant fractions of the wild-type strains of *B. pertussis* Taberman I (lane A), Tohama BP338 (lane D) and BBC29 (lane F). Moreover, the *bap-5* mutant Taberman II (lane B) and *prn* mutant, BBC30 (lane G) have also reacted with the monoclonal anti-BrkA (C-terminus) serum. However, no band was seen with the outer membrane enriched fraction of the BrkA-deficient mutant BP2041 (lane E) and with the Taberman I modulated strain (lane C), although there seems to be some carry-over in lane C from the adjacent lane. However, the result shows that BrkA production is normal in the Taberman II, *bap-5* mutant and in the BBC30, *prn* mutant strains of *B. pertussis*.

Fig. 3.24: Immunoblot showing various *B. pertussis* strains screened with anti-BrkA (C-terminal) serum to identify BrkA in their outer membrane preparations

Bordetella pertussis strains were grown in CL medium to OD_{600nm} ~1.0, and their outer membrane –enriched fractions were prepared as described in section 2.10.1 and separated on 12% SDS-PAGE and transferred onto nitrocellulose membrane for immunoblotting with anti-BrkA-C-terminal serum (Table 2.7).

1 = Protein ladder

A = *B. pertussis* Taberman I

B = *B. pertussis* Taberman II, *bap-5* mutant

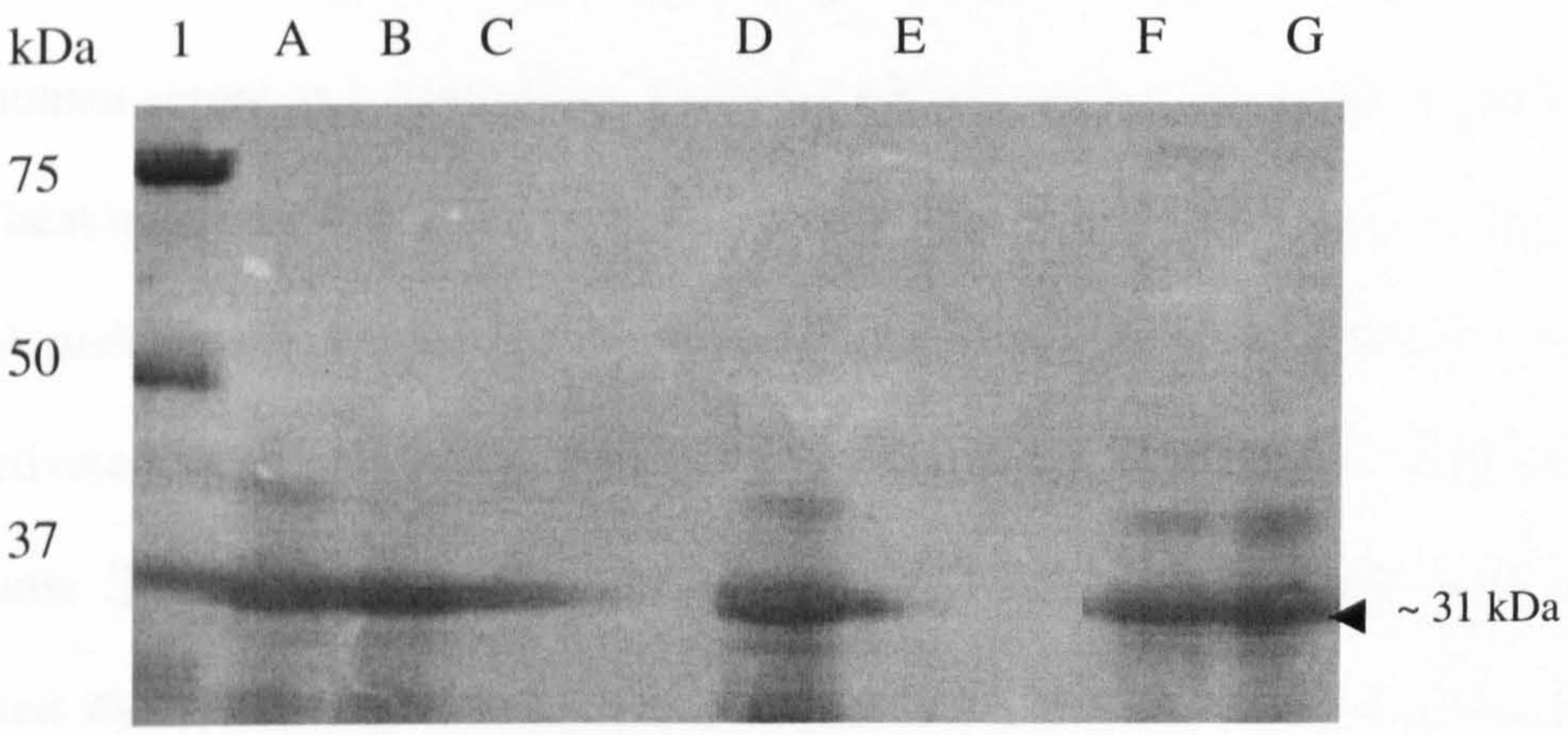
C = *B. pertussis* Taberman I, modulated by growing in the presence of 40 mM $MgSO_4$.

D = *B. pertussis* BP338

E = *B. pertussis* BP2041, *brkA* mutant

F = *B. pertussis* BBC29

G = *B. pertussis* BBC30, *prn* mutant


kDa
1
A
B
C
D
E
F
G
75
50
37
~ 31 kDa

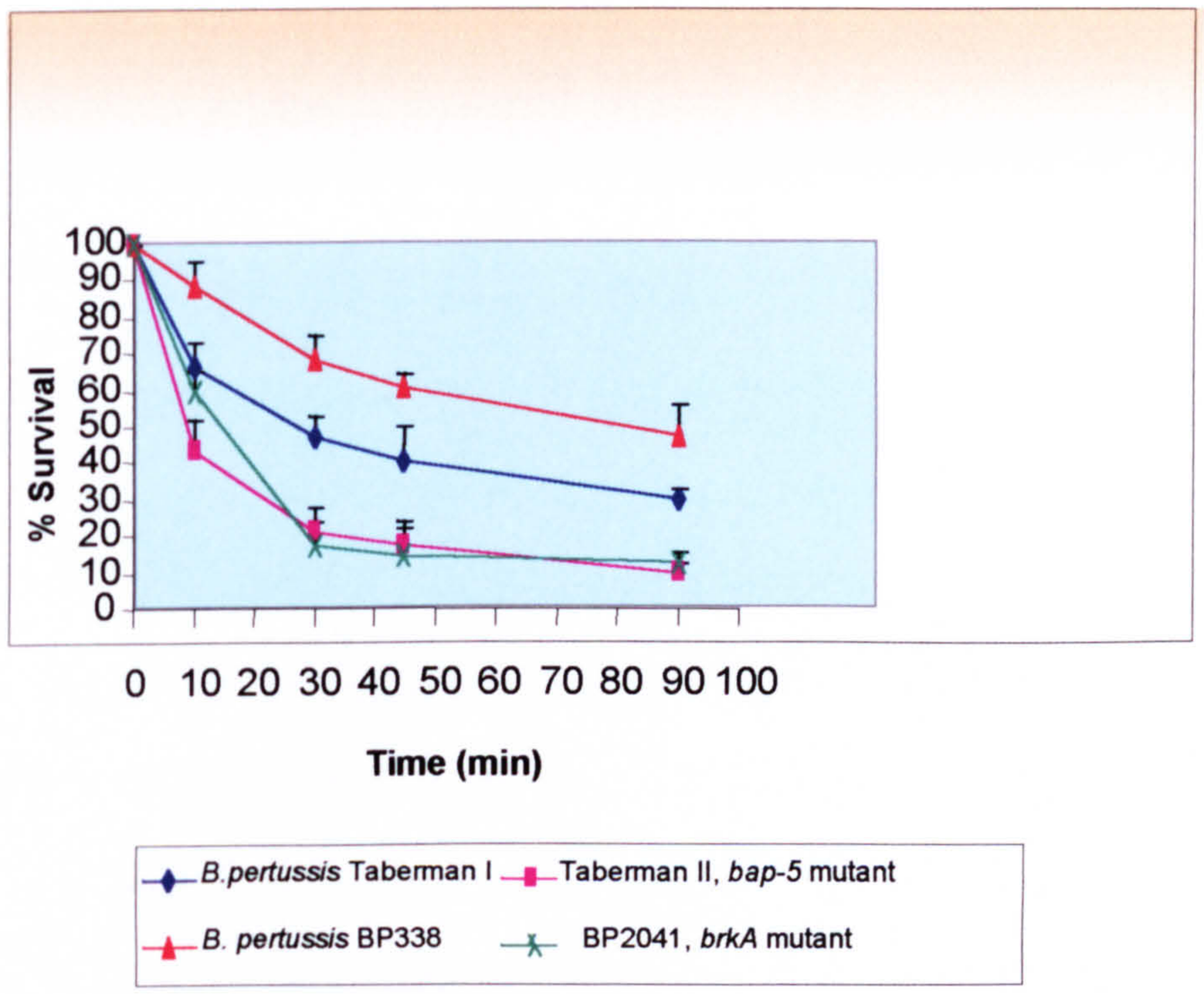
3.2.4. Serum killing assays

The serum killing assays (2.15.3) were carried out to determine whether Bap-5, due to its putative structural homology (Fig. 1.3) with the autotransporter protein BrkA (serum resistance factor), could also confer a serum resistance phenotype on *B. pertussis*. All the assays were performed using pooled normal human serum (section 2.19.3.1) obtained from volunteers with no recent history of pertussis. The incubation time for the assay was determined initially by doing a time-course study for killing of *B. pertussis* BP338 and its *brkA* mutant (BP2041) as well as *B. pertussis* Taberman I and its *bap-5* mutant Taberman II, by the human serum. The survival was calculated from the number of survivors from the normal human serum as a percentage of the number of survivors from heat-inactivated serum. The heat treatment (56°C for 30 min) removes the inherent complement activity of the serum. A preliminary comparison of the number of survivors from a PBS control and the heat-inactivated serum showed a slight but non-significant difference over the 90 min incubation time (Result not shown). This observation suggested that any decrease in bacterial counts due to agglutination by any antibodies present in the human serum, rather than killing, was negligible. A time-course experiment indicated that, after exposure to 10% normal human serum for 10 min, ~65% of *B. pertussis* Taberman I strain survived compared to only ~40% of its *bap-5* mutant Taberman II (Fig. 3.25). The result also showed that, after exposure to normal serum for 90 min, the Taberman I parent was still a better survivor (~30%) compared to its *bap-5* mutant (<10%). Strain BP338 and its *brkA* mutant BP2041 were controls in this experiment. Strain BP338, after exposure to normal human serum for 10 min appeared to survive better (>80%) compared to its *brkA* mutant (~55%). This difference increased slightly with time. After 90 min, about 50% of *B. pertussis* BP338 survived compared to 10% for the *brkA* mutant BP2041. Thus, these results show that *B. pertussis* Taberman I strain appeared to be more resistant to serum-

Fig. 3.25: Time course of killing of *B. pertussis* strains by normal human serum (10% v/v)

The results are the means of three separate experiments and bars indicate the standard deviations.

$$\text{Percentage of survival} = \frac{\text{No. of survivors from normal human serum}}{\text{No. of survivors from heat-inactivated serum}} \times 100$$


% Survival
100
90
80
70
60
50
40
30
20
10
0
0 10 20 30 40 50 60 70 80 90 100
Time (min)
B.pertussis Taberman I
Taberman II, bap-5 mutant
B. pertussis BP338
BP2041, brkA mutant

killing compared to its *bap-5* mutant Taberman II and that *B. pertussis* Tohama derivative BP338 seemed to be inherently more resistant compared to the *B. pertussis* Taberman I parent strain. From the time course result, an incubation time of 45 min was selected for the further serum killing assays as there was little change thereafter in the number of CFU of *B. pertussis* recovered from the normal serum.

The time course assay was repeated several times using different additional parameters to investigate the mechanisms whereby Bap-5 could confer the serum resistance phenotype on *B. pertussis*. Previous work suggested that the classical (antibody-dependent) pathway of complement activation is responsible for killing of *B. pertussis* (Barnes and Weiss, 1998). To block the classical pathway, the pooled normal human serum was treated with EGTA.$MgCl_2$ (final concentration 10mM of each) to render this pathway inactive by chelating calcium ions (Ca^{+2}), an essential component of this pathway. Fig. 3.26 shows the result taken from the average of 4 separate experiments. The shaded bars represent the survivors in the normal human serum after exposure for 45 min. The results presented in the form of solid bars show the number of survivors in normal human serum in the presence of EGTA.$MgCl_2$ after exposure for 45 min. The result shows that *B. pertussis* Taberman I (lane A, shaded) survives better than its *bap-5* mutant, (lane B, shaded) (***P*<0.001**) in agreement with the results presented in Fig. 3.25. Similarly the percentage survival after exposure to normal serum for 45 min of strain BP338 (lane C) was significantly more than that of its *brkA* mutant, BP2041 (lane D)(***P*< 0.05**). A slight discrepancy was seen regarding the difference in percentage survival compared to previous result (Fig. 3.25). The *brkA* mutant survived better here than the *bap-5* mutant although the reverse was the case in Fig. 3.25. Lanes E and F show *B. pertussis* BBC29 and its *prn* mutant, BBC30. The *prn* mutant, BBC30 has been reported to be slightly more serum-sensitive compared to its parent (BBC29) although, the effect was not found to be

Fig. 3.26: Classical pathway-mediated serum killing of *B. pertussis*

The results are the mean values and standard deviations of the combined results of 4 separate experiments. Shaded bars represent the percentage of survivors in 10 % normal human serum after exposure for 45 min. Solid bars represent the percentage of survivors in the presence of EGTA.$MgCl_2$ in the normal serum after exposure for 45 min. Student's *t*-test was used to compare the strains. ***P*<0.05, ****P*<0.0001.

$$\text{Percentage of survival} = \frac{\text{No. of survivors in normal human serum +/- EGTA.MgCl}_2}{\text{No. of survivors in PBS control}} \times 100$$

A= *B. pertussis* Taberman I

B= *B. pertussis* Taberman II, *bap-5* mutant

C= *B. pertussis* BP338

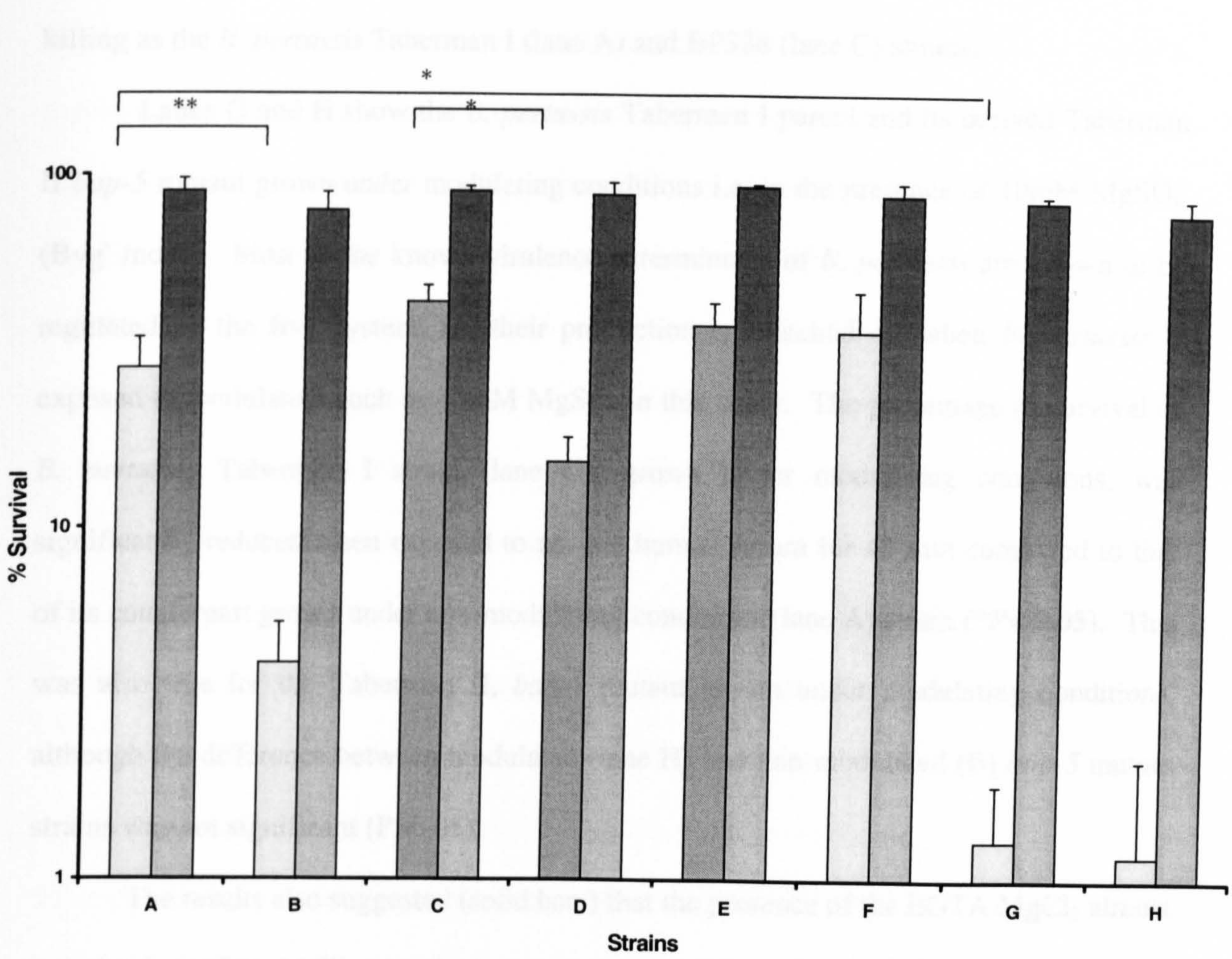
D= *B. pertussis* BP2041, *brkA* mutant

E= *B. pertussis* BBC29

F= *B. pertussis* BBC30 *prn* mutant

G= *B. pertussis* Taberman I, modulated by adding $MgSO_4$ to the growth medium

H= *B. pertussis* Taberman II, modulated by adding $MgSO_4$ to the growth medium


*
**
100
10
1
% Survival
A
B
C
D
E
F
G
H
Strains

statistically significant (Fernandez and Weiss, 1994). This study supported the previous work as lanes E shows that BBC29 does not seem to be significantly different, in terms of percentage of survival in normal serum, compared with its *prn* mutant BBC30 (lane F) ($P>0.05$). Both BBC29 and BBC30 (PRN^-) showed a similar degree of resistance to serum killing as the *B. pertussis* Taberman I (lane A) and BP338 (lane C) strains.

Lanes G and H show the *B. pertussis* Taberman I parent and its derived Taberman II *bap-5* mutant grown under modulating conditions i.e. in the presence of 40mM $MgSO_4$, (Bvg^- mode). Most of the known virulence determinants of *B. pertussis* are known to be regulated by the Bvg system, i.e. their production is switched off when *B. pertussis* is exposed to modulators such as 40mM $MgSO_4$ in this study. The percentage of survival of *B. pertussis* Taberman I strain (lane G), grown under modulating conditions, was significantly reduced when exposed to normal human serum for 45 min compared to that of its counterpart grown under non-modulating conditions (lane A) strain (***$P< 0.05$**). This was also true for the Taberman II, *bap-5* mutant grown under modulating conditions, although the difference between modulated (lane H) and non-modulated (B) *bap-5* mutant strains was not significant ($P>0.05$).

The results also suggested (solid bars) that the presence of the EGTA.$MgCl_2$ almost completely inhibited killing by the human serum (survival 95% to 99%) except in the case of the Taberman II *bap-5* mutant strain (lane B; solid bar; 80%) and the strains grown under modulating conditions (lanes G and H; solid bars; 80%). Overall, these results showed that, in addition to BrkA, Bap-5 also seems to play a role in conferring serum resistance on *B. pertussis* against the classical pathway-mediated killing of complement. It can be concluded that the presence of both BrkA and Bap-5 protect *B. pertussis* against the bactericidal activity of serum.

The complement proteins, the key players in killing *B. pertussis*, are very labile and their activity can be affected during the procedural steps involved in preparation of serum and storage. Thus, it was possible that the pooled normal human serum used in this work had sub-optimal levels of complement. An experiment was devised, therefore, to assess the killing ability of the pooled normal human serum (same lot as used in previous experiments) that had been de-complemented by heating to 56°C for 30 min and supplemented with a commercially-prepared source of complement (guinea pig serum, Sigma). The heat-inactivated human serum would act as a source of cross-reacting antibodies, whereas the guinea pig serum, which was presumably free of anti-*B. pertussis* antibodies, would provide the complement activity. The experiment (Fig. 3.27) was performed by adding the source of complement (5% v/v guinea pig serum) to the 10% v/v heat-inactivated human serum (section 2.15.3). It is worth mentioning here that *B. pertussis* Taberman II, *bap-5* mutant was found to be somewhat sensitive to complement alone compared to its Taberman I parent strain, which will be discussed in section 3.3.5. However, the *brkA* mutant BP2041 was similar to parent BP338 strain in its sensitivity to complement alone. A significant reduction was noticed in the percentage survival of the Taberman II *bap-5* mutant (lane B) compared to its parent *B. pertussis* Taberman I strain (lane A) (***P*<0.05**). As expected, *B. pertussis* BP338 (lane C) was found to be more resistant compared to its *brkA* mutant strain BP2041 (lane D) (* ***P*<0.05**). The percentage survival of *B. pertussis* BBC29 (lane E) was not found to be significantly different from its *prn* mutant BBC30 (lane F; $P>0.05$). The difference in survival between the non-modulated (lane A) compared to that of its modulated Taberman I strain counterpart (lane G) was statistically significant (***P*<0.05**). The modulated Taberman II, the *bap-5* mutant

Fig. 3.27: Effect of de-complemented normal human serum and added complement on the killing of different *B. pertussis* strains

The human serum (10% v/v) was de-complemented as described in section 2.15.3.2 and a commercially-prepared source of complement (guinea pig serum 5% v/v) was added to it. The results show the percentage survival after exposure for 45 min and are the means and standard deviations of 3 separate experiments. Student's *t*-test was used to compare the strains. * $P<0.05$

$$\text{Percentage of survival} = \frac{\text{No. of survivors in de-complemented normal human serum + guinea pig serum}}{\text{No. of survivors from PBS control}} \times 100$$

A= *B. pertussis* Taberman I

B= *B. pertussis* Taberman II, *bap-5* mutant

C= *B. pertussis* BP338

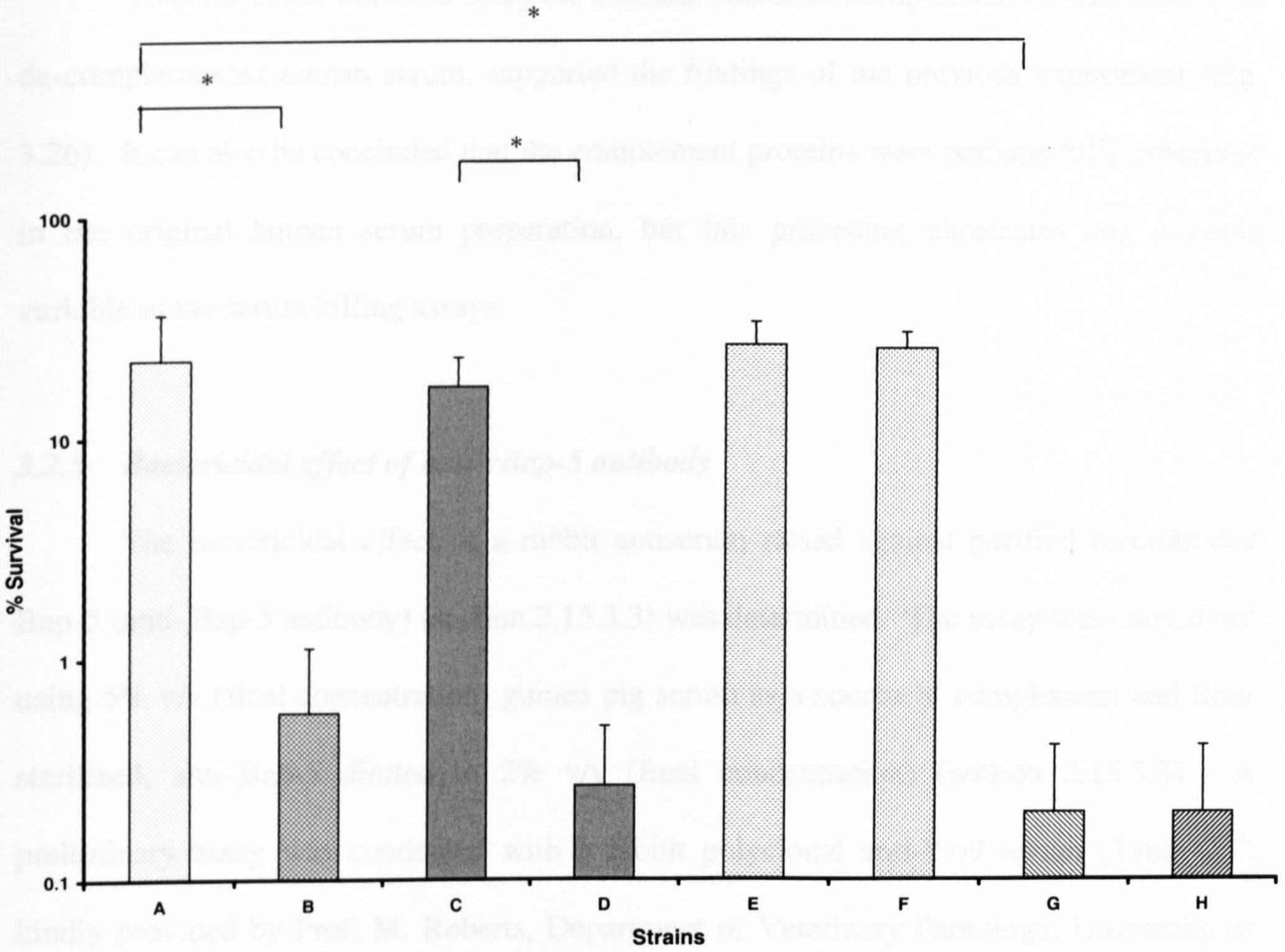
D= *B. pertussis* BP2041, *brkA* mutant

E= *B. pertussis* BBC29

F= *B. pertussis* BBC30 *prn* mutant

G= *B. pertussis* Taberman I, modulated by adding $MgSO_4$ to the growth medium

H= *B. pertussis* Taberman II, modulated by adding $MgSO_4$ to the growth medium


*
*
*
100
10
1
0.1
% Survival
A
B
C
D
E
F
G
H
Strains

(lane H) was also highly sensitive to serum killing in this assay but when compared to its non-modulated counterpart (lane B) which is also sensitive to serum killing, no significant difference was noticed ($P>0.05$).

Thus the result obtained using the external source of complement to supplement the de-complemented human serum, supported the findings of the previous experiment (Fig. 3.26). It can also be concluded that the complement proteins were perhaps fully preserved in the original human serum preparation, but this procedure eliminates one possible variable in the serum killing assays.

3.2.5 Bactericidal effect of anti-rBap-5 antibody

The bactericidal effect of a rabbit antiserum raised against purified recombinant Bap-5 (anti-rBap-5 antibody) (section 2.15.3.3) was determined. The assay was carried out using 5% v/v (final concentration) guinea pig serum as a source of complement and filter sterilized, anti-rBap-5 diluted to 2% v/v (final concentration) (section 2.15.3.3). A preliminary assay was conducted with a rabbit polyclonal anti-P.69 serum (Table 2.7) kindly provided by Prof. M. Roberts, Department of Veterinary Pathology, University of Glasgow, which had been reported to be bactericidal for *B. pertussis.*

The percentage of survival was determined from the number of survivors in the test serum (anti-P.69 serum + guinea pig serum) compared to bacteria treated with PBS alone after exposure for 45 min. Survival was also determined after treatment with anti-P.69 serum and de-complemented guinea pig serum. A 1% v/v final concentration of anti-P.69 serum was used, as higher concentrations of the antiserum alone caused agglutination. In fact, this 1% v/v concentration itself slightly reduced the bacterial counts (by 2-6%), compared to the PBS control (result not shown). The antiserum was de-complemented by heat treatment at 56°C for 30 min before use. The results (Fig 3.28) indicated that the -

Fig. 3.28: Bactericidal effect of anti-P.69 antibody on different *B. pertussis* strains with or without added complement.

The anti-P.69 serum was de-complemented as described in section 2.15.3.3 and the effect of added guinea pig serum (5% v/v), as a source of complement, was seen. The percentage of *B. pertussis* survivors was determined after exposure for 45 min. The solid bars show the percentage of survivors in anti-P.69 + de-complemented guinea pig serum. The shaded bars show the percentage of survivors in anti-P.69 + guinea pig serum. The results are the means and standard deviations of 3 separate experiments. Student's *t*-test was used to compare the strains. ***P*<0.05, ****P* <0.0001

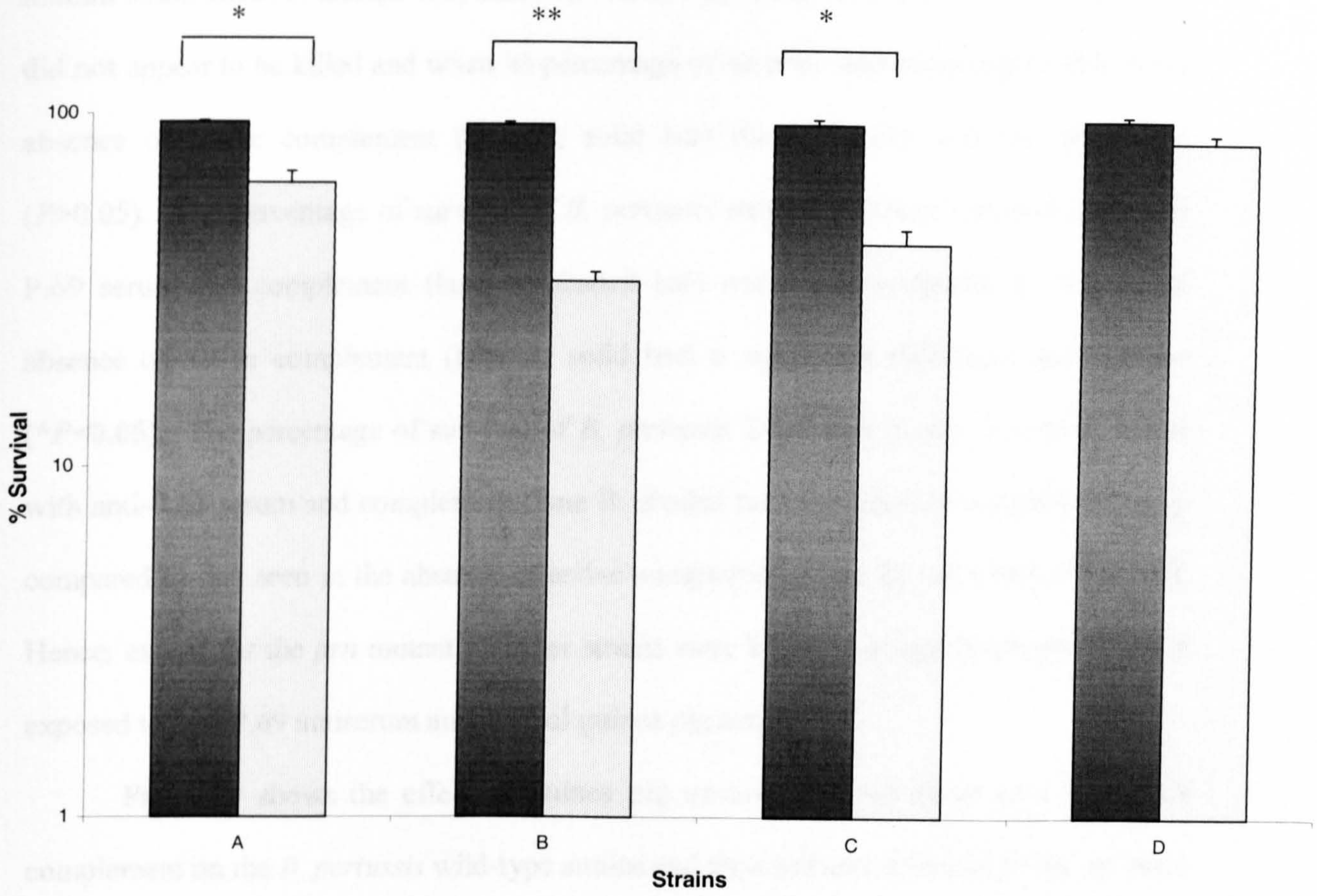
$$\text{Percentage of Survival} = \frac{\text{No. of survivors in anti-p.69 antibody + normal or de-complemented guinea pig serum}}{\text{No. of survivors in PBS control}} \times 100$$

A= *B. pertussis* Taberman I

B= *B. pertussis* Taberman II, *bap-5* mutant

C= *B. pertussis* BC29

D= *B. pertussis* BBC30, *prn* mutant


*
**
*
100
10
1
% Survival
A
B
C
D
Strains

anti-P.69 serum and complement had a bactericidal effect on the wild-type *B. pertussis* strain BBC29 (shaded bar; lane C) and when the percentage of survivors was compared to that in the absence of active complement (de-complements guinea pig serum; solid bar, lane C), a significant difference was observed (***P<0.05**) as expected. However, the *prn* mutant strain BBC30, treated with anti-P.69 serum and complement (lane D; shaded bars) did not appear to be killed and when its percentage of survival was compared to that in the absence of active complement (lane D; solid bar) the difference was not significant (P>0.05). The percentage of survival of *B. pertussis* strain Taberman I treated with anti-P.69 serum and complement (lane A; shaded bar) was when compared to that in the absence of active complement (lane A; solid bar) a significant difference was noticed (***P<0.05**). The percentage of survival of *B. pertussis* Taberman II, *bap-5* mutant treated with anti-P.69 serum and complement (lane B; shaded bar) also showed a significant drop compared to that seen in the absence of active complement (lane B; solid bar) (***P<0.05**). Hence, except for the *prn* mutant all other strains were killed to a significant extent when exposed to anti-P.69 antiserum and normal guinea pig serum.

Fig. 3.29 shows the effect of guinea pig serum (5% v/v) alone as a source of complement on the *B. pertussis* wild-type strains and their mutants deficient in one or other Bvg-regulated gene products. The percentage of survival was determined from the number of survivors in the unheated guinea pig serum (normal complement) (shaded bars) and compared with the number of survivors in the heat-inactivated guinea pig serum (56°C for 30 min, de-complement source of complement) (solid bars). The results suggests that the *bap-5* mutant (Taberman II; lane B) appears to be more sensitive (<60 % survival) to complement alone compared to its parent Taberman I strain (>90% survival) (lane A;***P<0.05**). However, with *B. pertussis* Taberman I, there was some killing by complement alone, but the viability of its *bap-5* mutant strain was reduced significantly

Fig. 3.29: Effect of unheated or de-complemented guinea pig serum on the viability of *B. pertussis*

The guinea pig serum used was either unheated or de-complemented by heat-treatment at 56°C for 30 min. The percentage of *B. pertussis* survivors was determined after exposure for 45 min. The shaded bars show the percentage of survivors in normal guinea pig serum. The solid bars show the percentage of survivors in de-complemented guinea pig serum. The results are the means and standard deviations of 3 separate experiments. Student's t-test was used to compare the strains. ***P*<0.05, ****P*<0.0001

$$\text{Percentage of Survival} = \frac{\text{No. of survivors in unheated or heated complement}}{\text{No. of survivors in PBS control}} \times 100$$

A= *B. pertussis* Taberman I

B= *B. pertussis* Taberman II, *bap-5* mutant

C= *B. pertussis* BP338

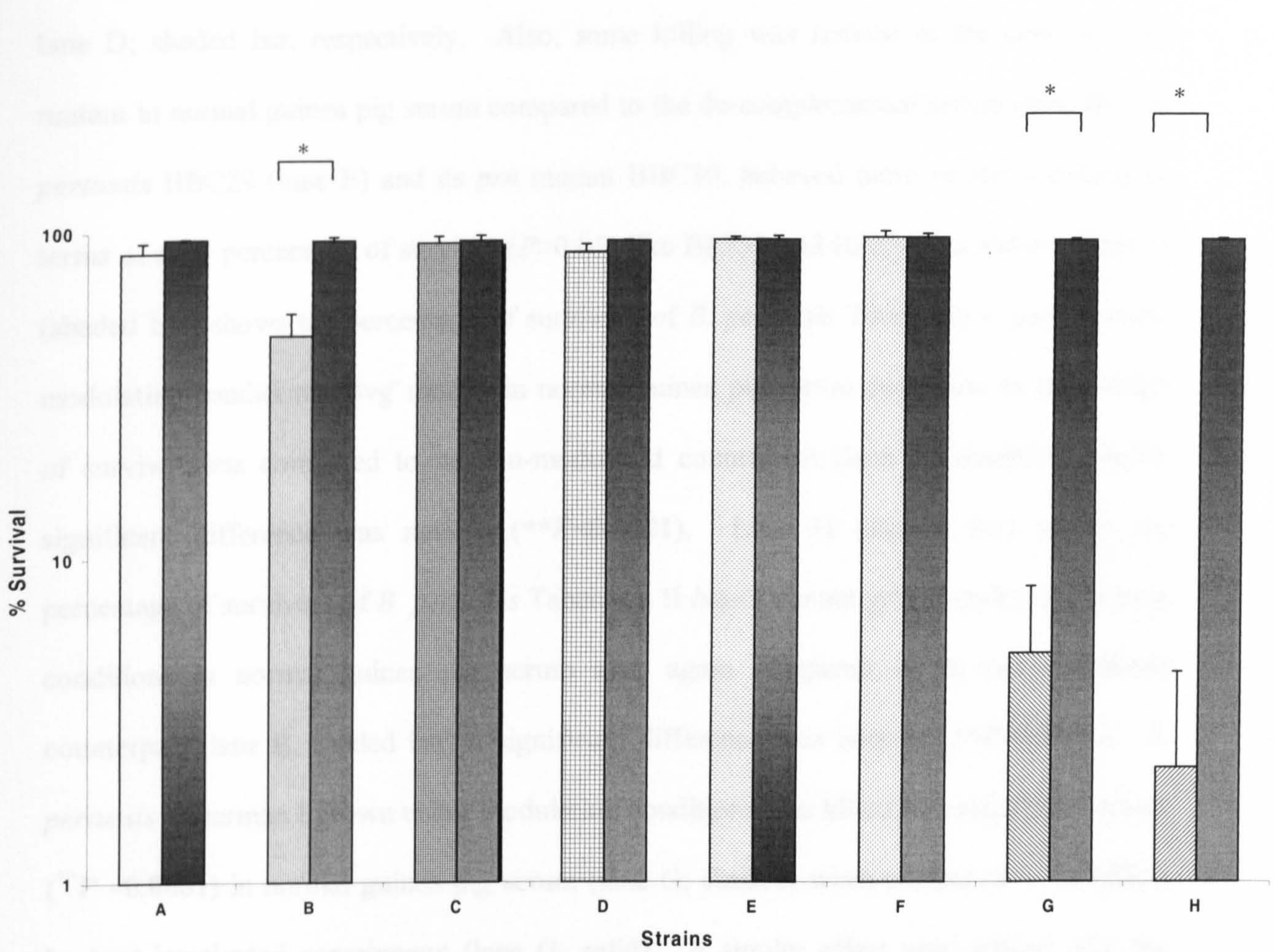
D= *B. pertussis* BP2041, *brkA* mutant

E= *B. pertussis* BBC29

F= *B. pertussis* BBC30 *prn* mutant

G= *B. pertussis* Taberman I, modulated by adding $MgSO_4$ to the growth medium

H= *B. pertussis* Taberman II, modulated by adding $MgSO_4$ to the growth medium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% Survival
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D
E
F
G
H
Strains

compared to its heat-inactivated complement control (lane B; solid bar) (***P<0.05**). However, the percentage of survivors of *B. pertussis* BP338 (lane C; solid bar) and its *brkA* mutant BP2041 (lane D; solid bar) in heat-inactivated guinea pig serum was not different ($P>0.05$) compared to the survivors in normal guinea pig serum i.e. lane C; shaded bar and lane D; shaded bar, respectively. Also, some killing was noticed in the case of *brkA* mutant in normal guinea pig serum compared to the de-complemented serum (lane D). *B. pertussis* BBC29 (lane E) and its *prn* mutant BBC30, behaved more or less similarly in terms of their percentage of survival ($P>0.05$) like BP338 and BP2041 as above. Lane G (shaded bar) shows the percentage of survivors of *B. pertussis* Taberman I, grown under modulating conditions (Bvg$^-$ mode), in normal guinea pig serum and when its percentage of survival was compared to its non-modulated counterpart (lane A, shaded) a highly significant difference was noticed (****P<0.0001**). Lane H (shaded bar) shows the percentage of survivors of *B. pertussis* Taberman II *bap-5* mutant grown under modulating conditions in normal guinea pig serum and, again compared to its non-modulated counterpart (lane B, shaded bar) a significant difference was noticed (****P<0.0001**). *B. pertussis* Taberman I grown under modulating conditions was killed to a significant extent (**$^{**}P$ <0.0001)** in normal guinea-pig serum (lane G; shaded) when compared to its killing by heat-inactivated complement (lane G; solid). A similar effect was noticed with the modulated strain of *B. pertussis* Taberman II, *bap-5* mutant (lane H).

To determine the percentage of complement-mediated killing and in order to check the possibility of any cross-reacting antibody in the source of complement, which could enhance killing of the *B. pertussis* by the classical pathway (antibody-dependent) of complement activation, EGTA.$MgCl_2$, an inhibitor of this pathway was included in the assay.

Fig. 3.30 show the effect of guinea pig serum in the presence (solid bars) and

Fig. 3.30: Effect of complement in the presence and absence of EGTA.$MgCl_2$ on *B. pertussis*

The effect of complement (guinea pig serum 5% v/v) alone was observed in the presence and absence of EGTA.$MgCl_2$, an inhibitor of the classical pathway-mediated killing of *B. pertussis* as described in section 2.15.3.3. The percentage of *B. pertussis* survivors was determined after exposure for 45 min. The shaded bars show the percentage of survivors in guinea pig serum in the absence of EGTA.$MgCl_2$. The solid bars show the percentage of survivors in guinea pig serum in the presence of EGTA.$MgCl_2$. The results are the means and standard deviations of 3 separate experiments. Student's t-test was used to compare the strains. ***P*<0.05, ****P*<0.0001

$$\text{Percentage of Survival} = \frac{\text{No. of survivors in normal Complement source +/- EGTA.MgCl}_2}{\text{No. of survivors in PBS control}} \times 100$$

A= *B. pertussis* Taberman I

B= *B. pertussis* Taberman II, *bap-5* mutant

C= *B. pertussis* BP338

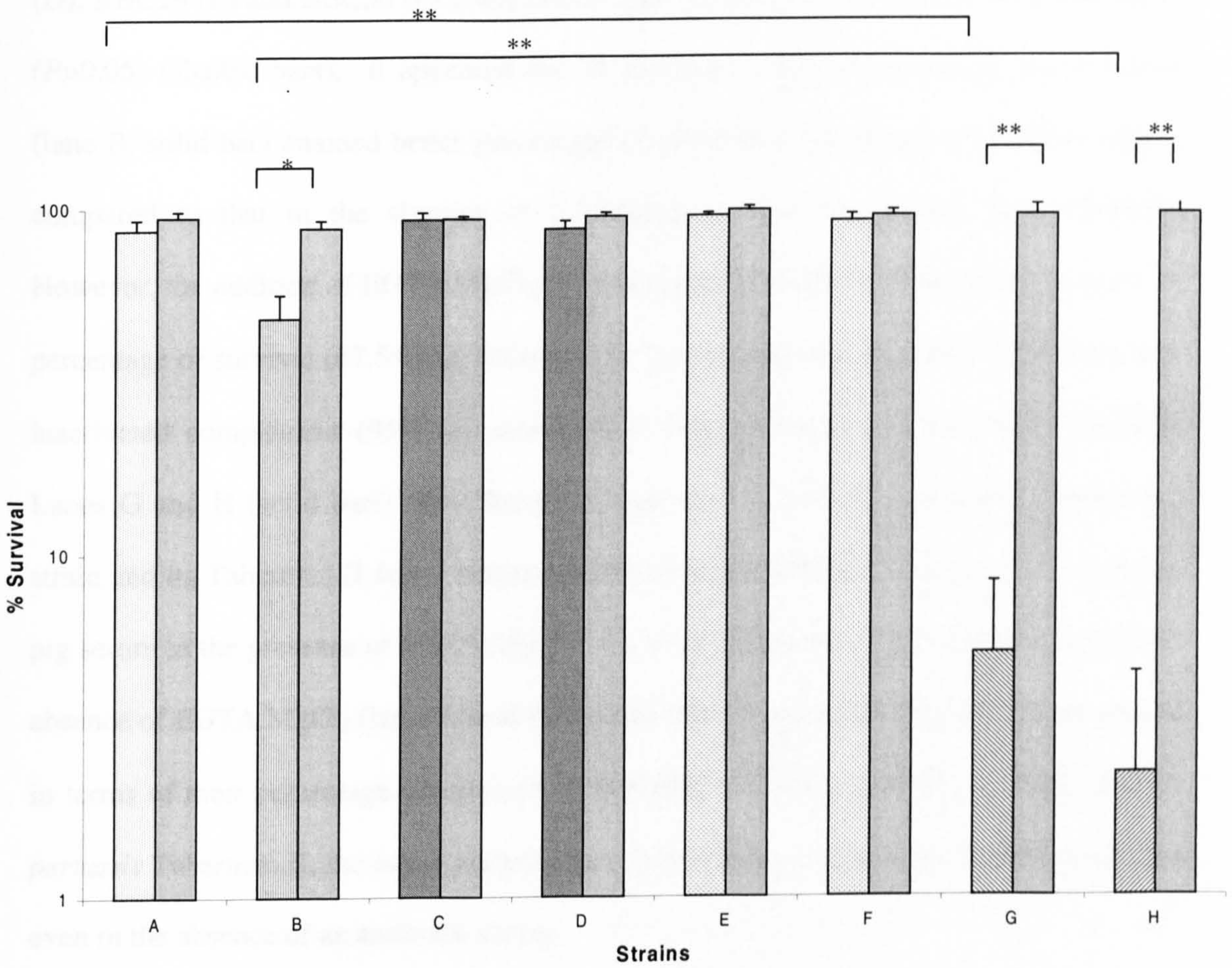
D= *B. pertussis* BP2041, *brkA* mutant

E= *B. pertussis* BBC29

F= *B. pertussis* BBC30 *prn* mutant

G= *B. pertussis* Taberman I, modulated by adding $MgSO_4$ to the growth medium

H= *B. pertussis* Taberman II, modulated by adding $MgSO_4$ to the growth medium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% Survival
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H
Strains

absence (shaded bars) of EGTA.$MgCl_2$ (10mM final concentration of each). The addition of EGTA.$MgCl_2$ to guinea pig serum did not make any significant difference to the percentage of survival of the *B. pertussis* strains i.e. Taberman I (A), BP338 (C), BP2041 (D), BBC29 (E) and BBC30 (F) compared to their survival in the absence of EGTA.$MgCl_2$ ($P>0.05$) (shaded bars). It appeared that *B. pertussis* strain Taberman II, *bap-5* mutant (lane B, solid bar) attained better percentage of survival in the presence of EGTA.$MgCl_2$ compared to that in the absence of EGTA.$MgCl_2$ (lane B, shaded bar) (**$P<0.05$**). However, the addition of EGTA.$MgCl_2$ to guinea pig serum did not appear to improve the percentage of survival (67.5%) of Taberman II, *bap-5* mutant to the extent seen in its heat-inactivated complement (95%) as described in Fig.3.29 (lane B, solid bar) (**$P<0.05$**). Lanes G and H (solid bars) show the percentage of survival of *B. pertussis* Taberman I strain and its Taberman II *bap-5* mutant grown under modulating conditions in the guinea pig serum in the presence of EGTA.$MgCl_2$ and when compared to their counterparts in the absence of EGTA.$MgCl_2$ (lanes G and H; shaded bars) a significant difference was noticed in terms of their percentage of survival (**$P<0.0001$**). Thus it can be concluded that *B. pertussis* Taberman II, the *bap-5* mutant strain is sensitive to complement-mediated killing even in the absence of an antibody source.

The next part of the experiment was carried out to determine if antibody to Bap-5 could potentiate bacterial killing in the presence of guinea pig serum as a source of complement and also to see the effect of added EGTA.$MgCl_2$ on the killing ability of the test serum (anti-$_r$Bap-5 antibody + guinea pig serum). The *B. pertussis* strains were exposed to guinea pig serum (5% v/v) and rabbit anti-$_r$Bap-5 serum (2% v/v) (Table 2.7) for 45 min. Western blotting results indicated in section 3.2.3.2 that the rabbit anti-$_r$Bap-5 antiserum could cross-react with perhaps other autotransporters such as BrkA (~73 kDa) protein. To minimise any effect due to the cross-reaction, the anti-$_r$Bap-5 serum was

absorbed with a suspension of the *B. pertussis* Taberman II, *bap-5* mutant (section 2.15.3.3). The percentage of survival was determined by calculating the number of *B. pertussis* survivors in absorbed anti-$_r$Bap-5 serum + normal guinea pig serum and the number of survivors in absorbed anti-$_r$Bap-5 serum + normal guinea pig serum + EGTA. $MgCl_2$.

Fig. 3.31 shows the percentage of survivors of *B. pertussis* in absorbed anti-$_r$Bap-5 serum + normal guinea pig serum (shaded bars) and in absorbed anti-$_r$Bap-5 serum + normal guinea pig serum + EGTA. $MgCl_2$ (solid bars). Lane A shows the percentage of survivors of *B. pertussis* Taberman I strain in the absence of EGTA.$MgCl_2$ (shaded bar) and when compared to that of in the presence of EGTA.$MgCl_2$ (solid bar), a significant difference was observed (***P<0.05**). Lane B indicates the percentage of survivors of *B. pertussis* Taberman II, the *bap-5* mutant strain in the absence of EGTA.$MgCl_2$ (shaded bar) and when compared to that of in the presence of EGTA.$MgCl_2$ (solid bar), a significant difference was seen (***P<0.05**). The percentage of survivors of the *bap-5* mutant in the presence of EGTA.$MgCl_2$ (77%) was not improved to the extent as it did in the case of *B. pertussis* Taberman I (93%). Surprisingly, the *bap-5* mutant was more sensitive to killing by the anti-$_r$Bap-5 serum and complement than the wild-type Taberman strain and this is presumably due to the sensitive nature of *bap-5* mutant to complement activity alone as seen by the results of Fig. 3.29 (lane B, shaded bar). However, there is one anomaly compared to the result seen in Fig. 3.30 in terms of the difference of percentage of survivors (solid bars) of *B. pertussis* Taberman II, *bap-5* mutant that can be due to the tube to tube variation of the activity of source of complement (guinea pig serum) used in these experiments. Lane C shows the percentage of survivors of *B. pertussis* BP338 in the absence of EGTA.$MgCl_2$ (shaded bar) and in the presence of EGTA.$MgCl_2$

Fig. 3.31: Bactericidal effect of the anti-$_r$Bap-5 serum + complement on different *B. pertussis* strains in the presence or absence of EGTA.$MgCl_2$

The rabbit anti-$_r$Bap-5 serum was de-complemented and absorbed with the *B. pertussis* Taberman II, the *bap5* mutant, to minimise any cross reaction with other closely-related surface antigens as described in section 2.15.3.3 and the effect of added guinea pig serum (5% v/v), as a source of complement, was seen. The percentage of *B. pertussis* survivors was determined after exposure for 45 min. The results are the means and standard deviations of 3 separate experiments. The shaded bars show the percentage of survivors in anti-$_r$Bap-5 + guinea pig serum. The solid bars show the percentage of survivors in anti-$_r$Bap-5 + guinea pig serum + EGTA.$MgCl_2$. Student's *t*-test was used to compare the strains. $^{*}P<0.05$, $^{**}P<0.0001$

$$\text{Percentage of Survival} = \frac{\text{Survivors in absorbed anti-}_r\text{Bap-5 serum + guinea pig serum +/- EGTA.MgCl}_2}{\text{Survivors in PBS control}} \times 100$$

A= *B. pertussis* Taberman I

B= *B. pertussis* Taberman II, *bap-5* mutant

C= *B. pertussis* BP338

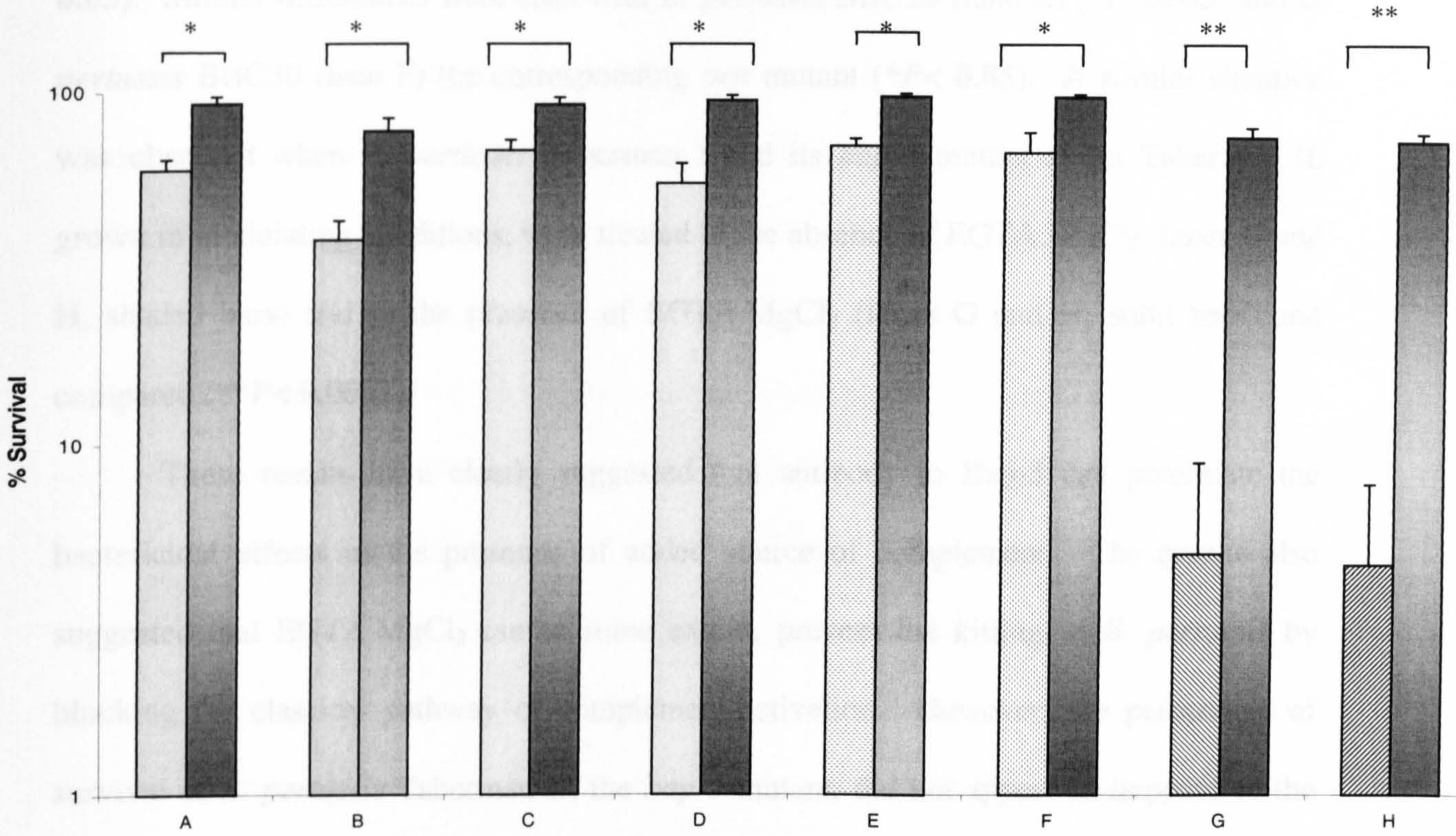
D= *B. pertussis* BP2041, *brkA* mutant

E= *B. pertussis* BBC29

F= *B. pertussis* BBC30, *prn* mutant

G= *B. pertussis* Taberman I, modulated by adding $MgSO_4$ to the growth medium

H= *B. pertussis* Taberman II, modulated by adding $MgSO_4$ to the growth medium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% Survival
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Strains

(solid bar) and a significant difference was seen (**P*< **0.05**). Lane D shows the percentage of survivors of the corresponding *brkA* mutant, BP2041 in the absence (shaded bar) and presence of EGTA.$MgCl_2$ (solid bar), and again a significant difference was observed (**P*< **0.05**). Similar differences were seen with *B. pertussis* BBC29 (lane E) (**P* <**0.05**) and *B. pertussis* BBC30 (lane F) the corresponding *prn* mutant (**P*< **0.05**). A similar situation was observed when *B. pertussis* Taberman I and its *bap-5* mutant strain Taberman II, grown in modulating conditions, were treated in the absence of EGTA.$MgCl_2$ (lanes G and H, shaded bars) and in the presence of EGTA.$MgCl_2$ (lanes G and H, solid bars) and compared (***P*< **0.0001**).

These results have clearly suggested that antibody to Bap-5 can potentiate the bactericidal effects in the presence of added source of complement. The results also suggested that EGTA.$MgCl_2$ can to some extent, prevent the killing of *B. pertussis* by blocking the classical pathway of complement-activation. However, the percentage of survival of *B. pertussis* Taberman II, the *bap-5* mutant, did not appear to improve to the extent as shown by the other *B. pertussis* strains in the presence of EGTA.$MgCl_2$ in complement-mediated bacterial killing assays.

3.2.6 Adhesion studies

The term adhesion describes the relatively stable and irreversible association of bacteria to a surface. The interaction usually occurs between the surface proteins of the bacterium and carbohydrate-containing molecules of the eukaryotic cell membrane or glycocalyx in a ligand-receptor fashion. Adhesion to the host tissues enables *B. pertussis* to avoid host defence mechanisms such as removal by fluid flow, mucociliary clearance and other physical processes. During the colonisation of the respiratory tract, *B. pertussis* can adhere specifically to ciliated epithelium; however, it has also been observed that *B.*

pertussis can adhere to, invade and persist within human macrophage and other cell types (Ewanowich *et al.*, 1989; Masure, 1992; Boschwitz *et al.*, 1997). Adhesion is therefore an essential prerequisite for successful bacterial colonisation of the host and hence can be regarded as a virulence attribute. Among the members of the autotransporter family, PRN is a well-studied, major adhesin of *B. pertussis*. A *prn* mutant of *B. pertussis* was shown to adhere much less well than the wild-type parent strain to Chinese hamster ovary (CHO) cells and a human epithelial laryngeal (HeLa) cell line (Roberts *et al.*, 1991; Leininger *et al.*, 1992). A *brkA* mutant of *B. pertussis* has also been reported to invade and adhere less well to certain cell types (e.g. HeLa cells and human lung fibroblast cell line MRC-5) than its wild-type parent strain (Fernandez and Weiss, 1994).

Due to some structural features (amino acid motifs) shared by Bap-5 protein, with other *B. pertussis* adhesins e.g. the RGD integrin-binding motif, the SGXG glycosaminoglycan-binding motif, it was of interest to explore a potential adhesive function due to such features in Bap-5. Different tissue culture cell lines (Table 2.8) were used to assess the role of Bap-5 as an adhesin *in vitro*. The enzyme-linked immunosorbent assay (ELISA) (section 2.15.4) provides an easy way to manipulate many samples at the same time. A rabbit anti-whole-cell *B. pertussis* antibody was used to detect the adherent bacterium. The antibody concentration was adjusted by measuring the antibody titre in preliminary ELISA experiments (not shown) and a 1 in 10,000 dilution was selected for the ELISA-based adhesion assays. Each well of a tissue culture plate (Costar) was inoculated with 10^5 mammalian target cells and left for 24-48 h to form confluent monolayers. About 10^7 CFU of *B. pertussis* were added to each well (mammalian cell : bacterial cells, 1:100). Some of the wells contained mammalian target cells only. After the ELISA reaction, the absorbance at 492 nm was the measure of adhered bacterial cells (section 2.15.4).

Fig. 3.32 compares the adhesion of different *B. pertussis* wild-type strains and their mutants to HeLa 229 cells. Lanes A and B show the result with *B. pertussis* strain, Taberman I and Taberman II, its derived *bap-5* mutant, respectively. The *bap-5* mutant strain of *B. pertussis* was less able to bind to this cell line compared to its parent, Taberman I strain (***$P < 0.05$**). On the other hand, lanes C and D showed that the adhesion of *B. pertussis* BP338 and its *brkA* mutant BP2041 to HeLa cells were not significantly different from each other ($P > 0.05$). *B. pertussis* BBC29 (lane E) was slightly better in adhering to HeLa cells compared to its *prn* mutant strain BBC30 (lane F) but no significant difference was noted ($P > 0.05$). Another interesting observation was noted when the *B. pertussis* Taberman I (lane G) and Taberman II, the *bap-5* mutant (lane H) were incubated with the pre-absorbed rabbit anti-$_r$Bap-5 serum (pre-absorption was carried out with the cell suspension of *B. pertussis* Taberman II, the *bap-5* mutant strain to minimise any possibility of cross-reaction with other closely-related surface antigen as described in section 2.15.3.3.) at 37°C for one hour prior to the assay. The bacterial cells incubated with the absorbed anti-$_r$Bap-5 serum were then spun down and re-suspended in PBS to standardise their concentration. Thereafter, an equal number of bacterial cells i.e. ~10^7 was added to the wells containing the mammalian cells in a 96-well microtitre plate. A comparison of Lane G (antibody-treated, Taberman I) with lane A (untreated, Taberman I) shows that anti-$_r$Bap-5 antibody seems to interfere with the adherence process of *B. pertussis* Taberman I strain to HeLa cells (****$P<0.0001$**). With Taberman II, the *bap-5* mutant strain, the antibody had no effect on adherence as shown by a comparison of lane H (antibody-treated) and lane B (non-treated) ($P>0.05$). Lane I shows the background level of absorbance in the absence of bacteria which indicates that the anti-whole-cell *B. pertussis* antiserum and the anti-rabbit IgG HRPO-conjugate were adhering to the plastic surface or to the target cells to some extent. Bacterial cells alone showed a high level of

Fig. 3.32: Adhesion study using human epithelial laryngeal cell line (HeLa-229) with different *B. pertussis* strains

An ELISA-based method was used to determine the adhesion ability of different *B. pertussis* strains for HeLa 229 cells. HeLa 229 (~1 x 10^5) cells were infected with ~10^7 CFU of *B. pertussis* at 37°C for 2h. The adherence of bacteria was estimated by measuring the absorbance (492nm) of each strain after adding rabbit anti-whole-cell *B. pertussis* serum followed by the addition of anti-rabbit IgG HRPO conjugate. Each absorbance value shown is the average of 8 independent values and bars represent the standard deviations for each group. ***P*<0.05, ****P*<0.0001**

A= *B. pertussis* Taberman I

B= *B. pertussis* Taberman II, *bap-5* mutant

C= *B. pertussis* BP338

D= *B. pertussis* BP2041, *brkA* mutant

E= *B. pertussis* BBC29

F= *B. pertussis* BBC30, *prn* mutant

G= *B. pertussis* Taberman I pre-incubated with anti-$_r$Bap-5 antibody

H= *B. pertussis* Taberman II pre-incubated with anti-$_r$Bap-5 antibody

I= Background absorbance (antibodies adhering to the plastic surface or target cells in the absence of bacteria)

Fig. 3.33: Adhesion study using a human lung epithelial cell line (A549) with different *B. pertussis* strains

The detailed information of the procedure are same as in legend of Fig. 3.32.

A= *B. pertussis* Taberman I

B= *B. pertussis* Taberman II, *bap-5* mutant

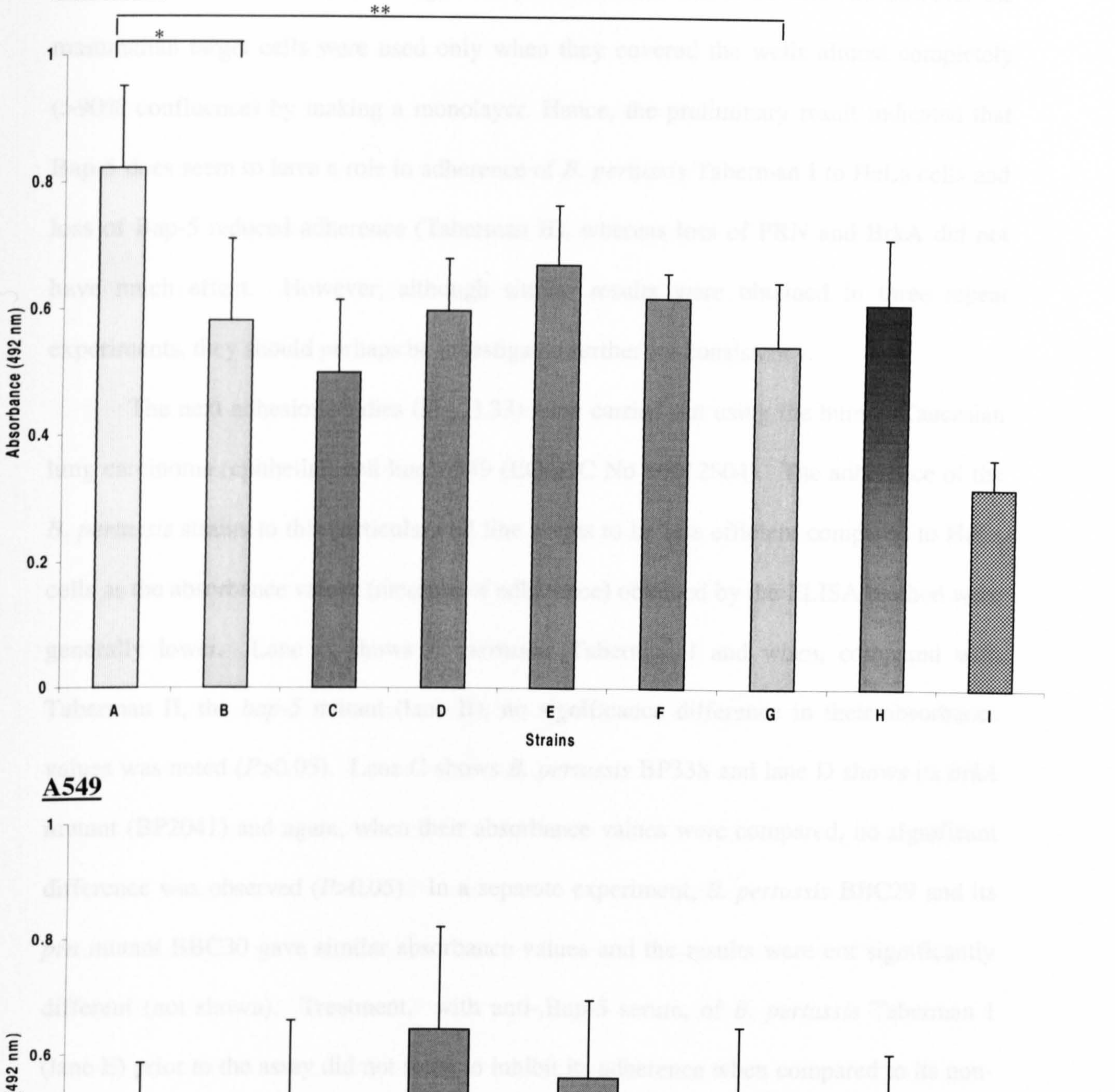
C= *B. pertussis* BP338

D= *B. pertussis* BP2041, *brkA* mutant

E= *B. pertussis* Taberman I pre-incubated with anti-$_r$Bap-5 antibody

F= *B. pertussis* Taberman II pre-incubated with anti-$_r$Bap-5 antibody

G= Background absorbance (as described above)


HeLa 229
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0.2
0
Absorbance (492 nm)
A
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F
G
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Strains
A549
1
0.8
0.6
0.4
0.2
0
Absorbance (492 nm)
A
B
C
D
E
F
G
Strains

background absorbance by adhering to the plastic surface (result not shown), however the mammalian target cells were used only when they covered the wells almost completely (>90% confluence) by making a monolayer. Hence, the preliminary result indicated that Bap-5 does seem to have a role in adherence of *B. pertussis* Taberman I to HeLa cells and loss of Bap-5 reduced adherence (Taberman II), whereas loss of PRN and BrkA did not have much effect. However, although similar results were obtained in three repeat experiments, they should perhaps be investigated further for consistency.

The next adhesion studies (Fig. 3.33) were carried out using the human Caucasian lung carcinoma (epithelial) cell line A549 (ECACC No 86012804). The adherence of the *B. pertussis* strains to this particular cell line seems to be less efficient compared to HeLa cells as the absorbance values (measure of adherence) obtained by the ELISA method were generally lower. Lane A shows *B. pertussis* Taberman I and when, compared with Taberman II, the *bap-5* mutant (lane B), no significance difference in their absorbance values was noted ($P>0.05$). Lane C shows *B. pertussis* BP338 and lane D shows its *brkA* mutant (BP2041) and again, when their absorbance values were compared, no significant difference was observed ($P>0.05$). In a separate experiment, *B. pertussis* BBC29 and its *prn* mutant BBC30 gave similar absorbance values and the results were not significantly different (not shown). Treatment, with anti-$_r$Bap-5 serum, of *B. pertussis* Taberman I (lane E) prior to the assay did not seem to inhibit its adherence when compared to its non-treated counterpart (lane A) ($P>0.05$). Likewise treatment with anti-$_r$Bap-5 of Taberman II, the *bap-5* mutant strain, did not appear to inhibit its adherence to A549 cells compared to its non-treated counterpart (lane B) ($P>0.05$). Lane G shows the background level of absorbance. In conclusion, none of the parent strains showed significantly greater adherence to human lung carcinoma epithelial cell line A549 compared to their corresponding mutant strains.

The next result (Fig. 3.34) shows the adhesion of different wild-type *B. pertussis* strains and their respective mutant strains to the human epithelial cell line (Hep-2). *B. pertussis* filamentous haemagglutinin and fimbriae have been reported to mediate adherence to these cells (section 1.8.1). However PRN did not seem to be involved in adhesion of *B. pertussis* to this epithelial cell line (Mahon *et al.*, 1999), although *E. coli* expressing recombinant PRN has been shown to adhere better than the *E. coli* host alone (Everest *et al.*, 1996). The results showed that *B. pertussis* Taberman I (lane A), seems to be better in adhering to Hep-2 cells compared to Taberman II, the *bap-5* mutant (lane B) (***P< 0.0001**) and compared to all other *B. pertussis* strains included in the study. *B. pertussis* BP338 (lane C) did not show any significant difference in absorbance value to its *brkA* mutant (lane D) ($P > 0.05$), nor was there any differences between *B. pertussis* BBC29 (lane E) and its *prn* mutant BBC30 (lane F) ($P > 0.05$). Lane G shows *B. pertussis* Taberman I strain treated with the absorbed anti-$_r$Bap-5 serum prior to the assay and this treatment seems to inhibit its adherence when compared with its non-treated counterpart (lane A) as shown by their absorbance values (** ***P* <0.0001**). Lane H shows Taberman II, the *bap-5* mutant strain treated with anti-$_r$Bap-5 serum prior to the assay and, when its absorbance value is compared to that of its non-treated counterpart (lane B), no significance difference was noted ($P > 0.05$). Lane I shows background level of absorbance in the absence of bacteria. Thus it appeared from these results that Bap-5 protein does seem to play a role in adherence of *B. pertussis* to Hep-2 cells, although no role for BrkA or PRN was evident.

Fig. 3.35 shows the adhesion of the different wild-type *B. pertussis* strains and mutants to the human colon carcinoma cell line (Caco-2). Lanes A and B show the *B. pertussis*, Taberman I, and its *bap-5* mutant whose absorbance values were not significantly different ($P > 0.05$). Lanes C and D show *B. pertussis* BP338 and its *brkA*-

Fig. 3.34: Adhesion study using human epithelial cell line (Hep-2) with different *B. pertussis* strains

An ELISA-based method (section 2.15.4) was used to determine the adhesion ability of different *B. pertussis* strains for Hep-2 cells. Hep-2 (~1 x 10^5) cells were infected with ~10^7 CFU of *B. pertussis* at 37°C for 2h. The adherence of bacteria was estimated by measuring the absorbance (492nm) of each strain after adding rabbit anti-whole-cell *B. pertussis* serum followed by the addition of anti-rabbit IgG HRPO conjugate. Each absorbance value shown is the average of 8 independent values and bars represent the standard deviations for each group. ***P*<0.05, ****P*<0.0001**

A= *B. pertussis* Taberman I

B= *B. pertussis* Taberman II, *bap-5* mutant

C= *B. pertussis* BP338

D= *B. pertussis* BP2041, *brkA* mutant

E= *B. pertussis* BBC29

F= *B. pertussis* BBC30, *prn* mutant

G= *B. pertussis* Taberman I pre-incubated with anti-$_r$Bap-5 antibody

H= *B. pertussis* Taberman II pre-incubated with anti-$_r$Bap-5 antibody

I= Background absorbance (antibodies adhering to the plastic surface or target cells in the absence of bacteria)

Fig. 3.35: Adhesion study using human colon carcinoma cell line (Caco-2) with different *B. pertussis* strains

The detailed information of the procedure is same as in legend of Fig. 3.34.

A= *B. pertussis* Taberman I

B= *B. pertussis* Taberman II, *bap-5* mutant

C= *B. pertussis* BP338

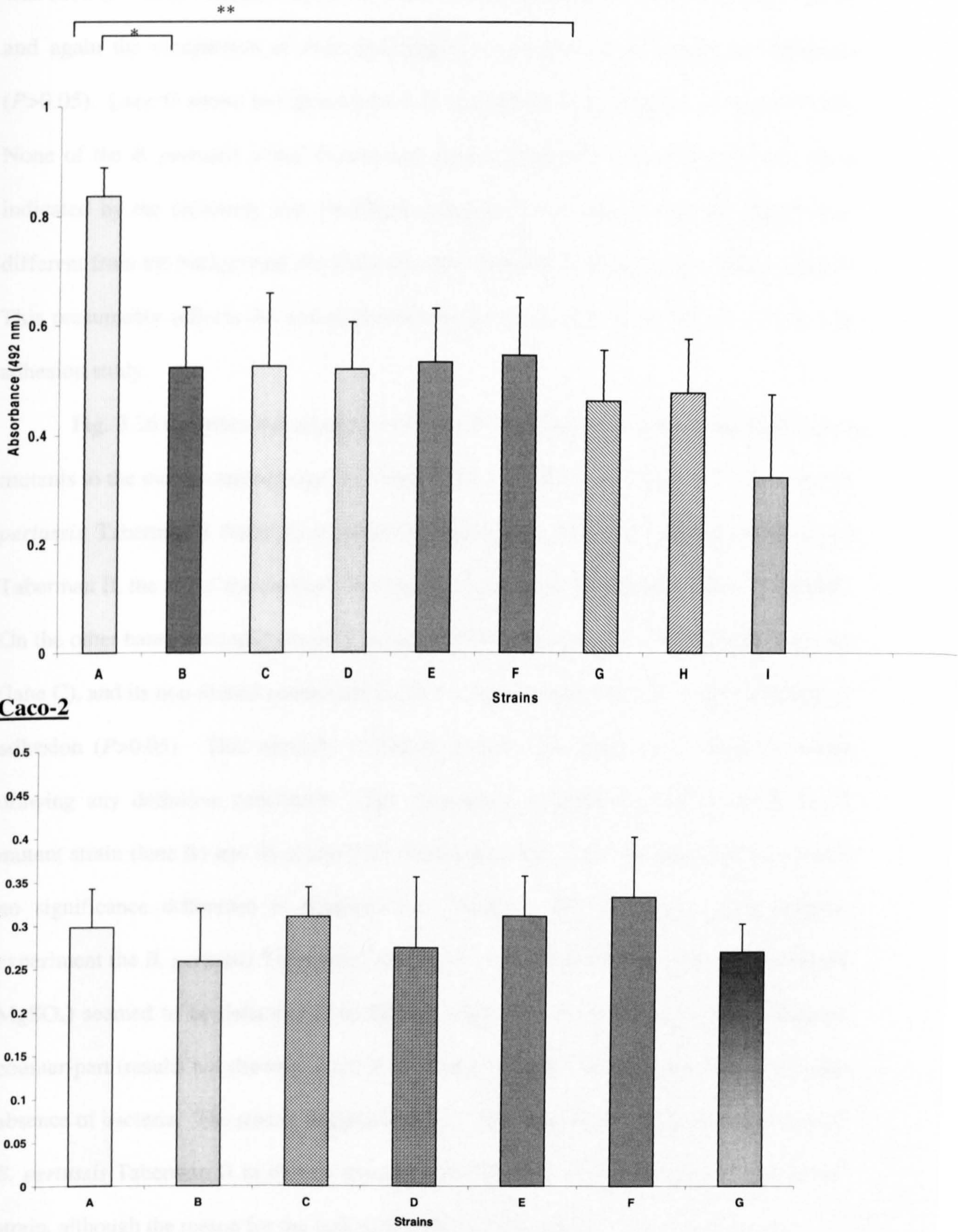
D= *B. pertussis* BP2041, *brkA* mutant

E= *B. pertussis* BBC29

F= *B. pertussis* BBC30, *prn* mutant

G= Background absorbance (as above)

Hep-2


**
*
1
0.8
0.6
0.4
0.2
0
Absorbance (492 nm)
A
B
C
D
E
F
G
H
I
Strains

Caco-2

0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Absorbance (492 nm)
A
B
C
D
E
F
G
Strains

mutant and again, when their absorbance values were compared, no significant difference was seen ($P>0.05$). Lanes E and F show *B. pertussis* BBC29 and its *prn* mutant BBC30 and again the comparison of their absorbance values showed no significant difference ($P>0.05$). Lane G shows background level of absorbance in the absence of bacterial cells. None of the *B. pertussis* strain showed any strong affinity for this particular cell line as indicated by the uniformly low absorbance values. These values were not significantly different from the background absorbance control (lane G) as measured by ELISA method. This presumably reflects the non-respiratory nature of the cell line used (Caco-2) for the adhesion study.

Fig. 3.36 describes the adhesion of different wild-type *B. pertussis* strains and their mutants to the murine macrophage cell line P338D-1 (Table 2.8). The results show that *B. pertussis* Taberman I (lane A) is better in adhering to this cell line compared to the Taberman II, the *bap-5* mutant (lane B) as evident from their absorbance values (***$P<0.05$**). On the other hand, a comparison of *B. pertussis* Taberman I treated with anti-$_r$Bap-5 serum (lane C), and its non-treated counterpart (lane A), did not show any significant inhibition of adhesion ($P>0.05$). This anomaly is hard to explain and needs to be repeated before drawing any definitive conclusion. The comparison between the Taberman II, *bap-5* mutant strain (lane B) and its counterpart treated with anti-$_r$Bap-5 serum (lane D) showed no significance difference in adherence to P338D-1 cells ($P>0.05$). In a separate experiment the *B. pertussis* Taberman I strain grown under modulating conditions (40mM $MgSO_4$) seemed to be defective in its binding capability compared to its non-modulated counter-part (results not shown). Lane E shows the background level of absorbance in the absence of bacteria. The results suggest that the loss of Bap-5 can reduce the adherence of *B. pertussis* Taberman II to murine macrophages P338D-1 compared to wild-type parent strain, although the reason for the lack of inhibition of adherence where bacteria were

Fig. 3.36: Adhesion study using murine macrophage cell line P338D-1 with different *B. pertussis* strains.

An ELISA-based method (2.15.4) was used to determine the adhesion ability of different *B. pertussis* strains for P338D-1cells. P338D-1 (~1 x 10^5) cells were infected with ~10^7 CFU of *B. pertussis* at 37°C for 2h. The other details are the same as described in the legend of the previous Fig 3.34. ***P*<0.05**

A= *B. pertussis* Taberman I

B= *B. pertussis* Taberman II, *bap-5* mutant

C= *B. pertussis* Taberman I pre-incubated with anti-$_r$Bap-5 antibody

D= *B. pertussis* Taberman II pre-incubated with anti-$_r$Bap-5 antibody

E= Background absorbance (antibodies adhering to the plastic surface or target cells in the absence of bacteria)

Fig. 3.37: Adhesion study using murine macrophage cell line (RAW 264.7) with different *B. pertussis* strains.

The detailed information of the procedure is same as in legend of Fig. 3.34. ***P*<0.05**

A= *B. pertussis* Taberman I

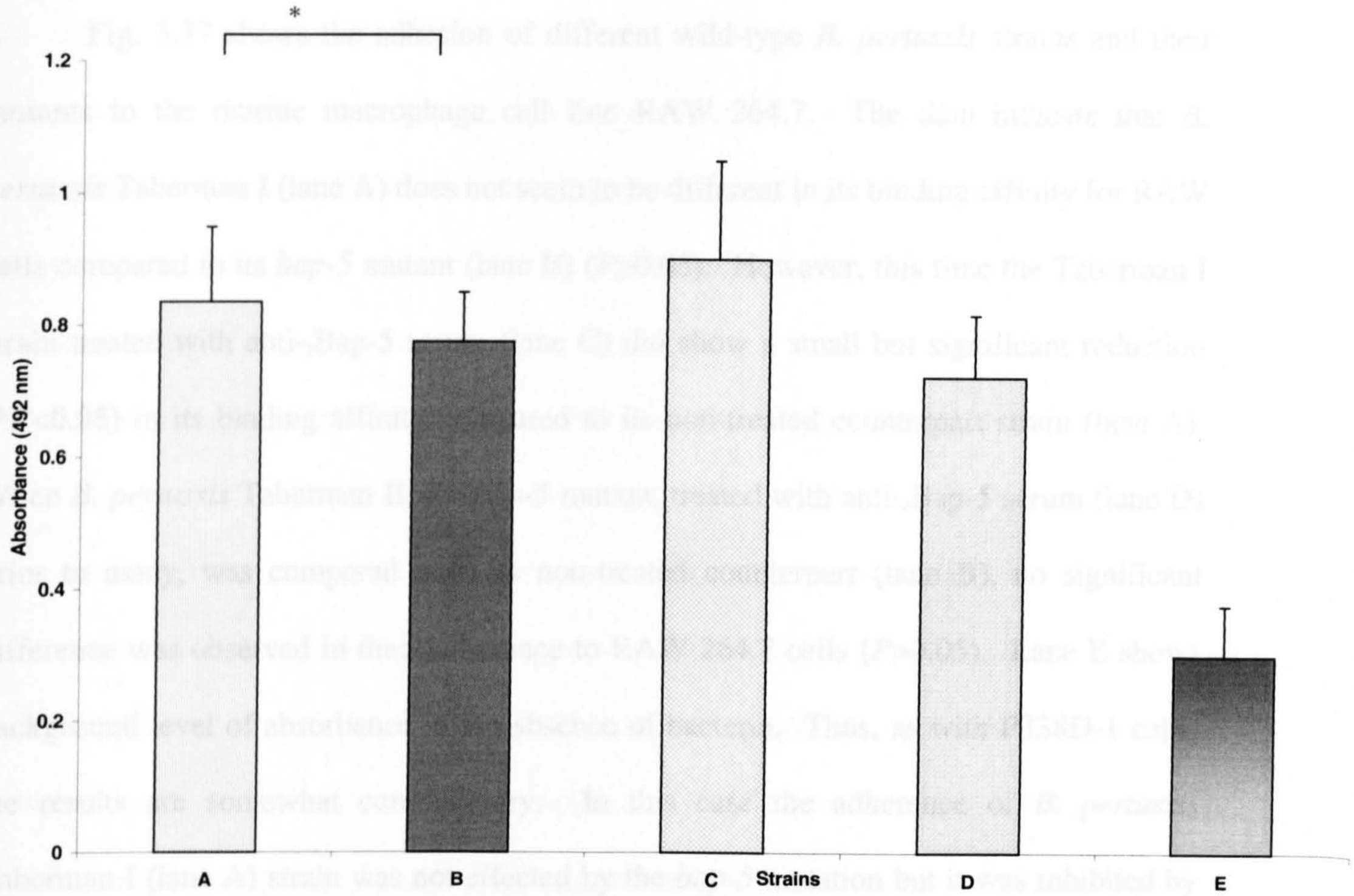
B= *B. pertussis* Taberman II, *bap-5* mutant

C= *B. pertussis* Taberman I pre-incubated with anti-$_r$Bap-5 antibody

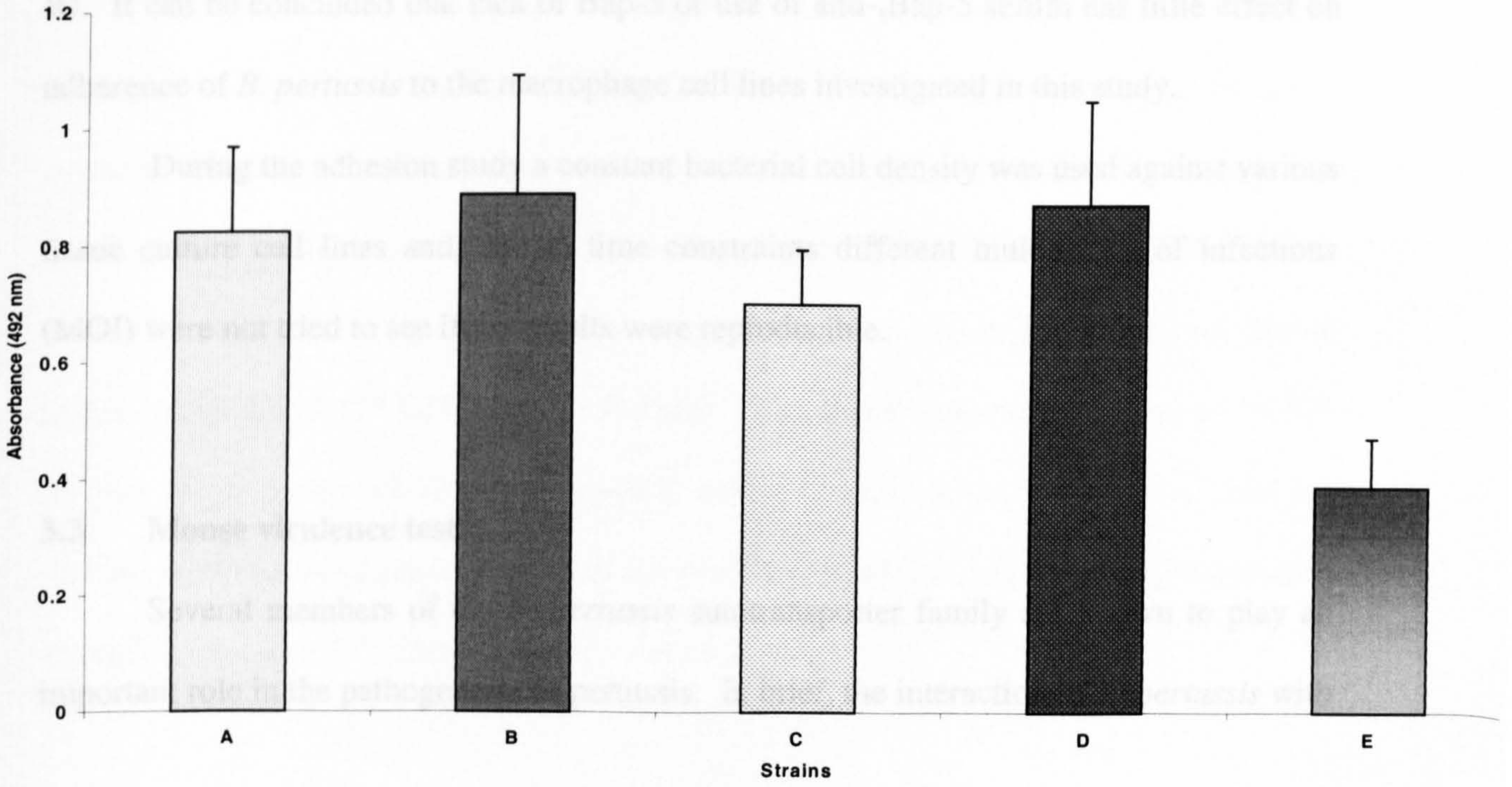
D= *B. pertussis* Taberman II pre-incubated with anti-$_r$Bap-5 antibody

E= Background absorbance (antibodies adhering to the plastic surface or target cells in the absence of bacteria)

P338D-1



RAW264.7



pre-treated with anti-$_r$Bap-5 is unclear.

Fig. 3.37 shows the adhesion of different wild-type *B. pertussis* strains and their mutants to the murine macrophage cell line RAW 264.7. The data indicate that *B. pertussis* Taberman I (lane A) does not seem to be different in its binding affinity for RAW cells compared to its *bap-5* mutant (lane B) ($P>0.05$). However, this time the Taberman I strain treated with anti-$_r$Bap-5 serum (lane C) did show a small but significant reduction (***$P<0.05$**) in its binding affinity compared to its non-treated counterpart strain (lane A). When *B. pertussis* Taberman II, the *bap-5* mutant, treated with anti-$_r$Bap-5 serum (lane D) prior to assay, was compared with its non-treated counterpart (lane B), no significant difference was observed in their adherence to RAW 264.7 cells ($P>0.05$). Lane E shows background level of absorbance in the absence of bacteria. Thus, as with P338D-1 cells, the results are somewhat contradictory. In this case the adherence of *B. pertussis* Taberman I (lane A) strain was not effected by the *bap-5* mutation but it was inhibited by the anti-$_r$Bap-5 serum treatment (lane C). Antibody treatment did not alter the adherence of Taberman II, the *bap-5* mutant (lane D) compared to its non-treated counterpart (lane B). It can be concluded that lack of Bap-5 or use of anti-$_r$Bap-5 serum has little effect on adherence of *B. pertussis* to the macrophage cell lines investigated in this study.

During the adhesion study a constant bacterial cell density was used against various tissue culture cell lines and, due to time constraints different multiplicity of infections (MOI) were not tried to see if the results were reproducible.

3.3 Mouse virulence test

Several members of the *B. pertussis* autotransporter family are known to play an important role in the pathogenesis of pertussis. In brief, the interaction of *B. pertussis* with

ciliated epithelial cells of the respiratory tract is thought to be mediated, at least in part by the autotransporter adhesins PRN, BrkA and TCF. The newly-identified *B. pertussis* autotransporter Bap-5 shares some common features with these well-characterised autotransporter proteins. Therefore, one of the aspects examined in a search for a role for Bap-5 was to determine its virulence potential in a mouse model of *B. pertussis* infection (section 2.13.2).

Randomised groups of 5 mice were inoculated intransally with approximately $1x10^5$ (in experiment 1) and $1x10^6$ (in experiment 2) colony forming units /mouse. The mice were sacrificed on day 7 and their tracheas and lungs were removed aseptically and nasal washes were also collected, to study the colonisation of *B. pertussis* at different sites in the mouse respiratory tract. The virulence of *B. pertussis* Taberman I strain was compared with Taberman II, the *bap-5* mutant strain. The standard mouse virulent strain 18-323 was used as a positive control (in experiment 1). In the experiment 2, *B. pertussis* BBC29 and its *prn* mutant BBC30 were included. The raw viable count data are given in appendices IV, V and VI. The number of colony forming units (CFU) of *B. pertussis* strains recovered from the lungs of different mouse groups in these two separate experiments is presented in Figs. 3.38a and 3.38b. The recovered lung count for the *B. pertussis* 18-323 group (mean log count= 6.893 CFU/lung) was significantly higher than for *B. pertussis* Taberman I strain (mean log count= 5.741 CFU/lung) (**$P<0.0001$). However, this latter strain colonised the lungs to a greater extent than its *bap-5* mutant (mean log count= 4.979 CFU/lung) (* $P<0.05$). The higher colonisation ability of 18-323 reflects the fact that 18-323 is a standard mouse-virulent *B. pertussis* strain used for vaccine tests and this particular batch of the challenge strain had previously been passaged through the mouse (R. Parton, personal communication).

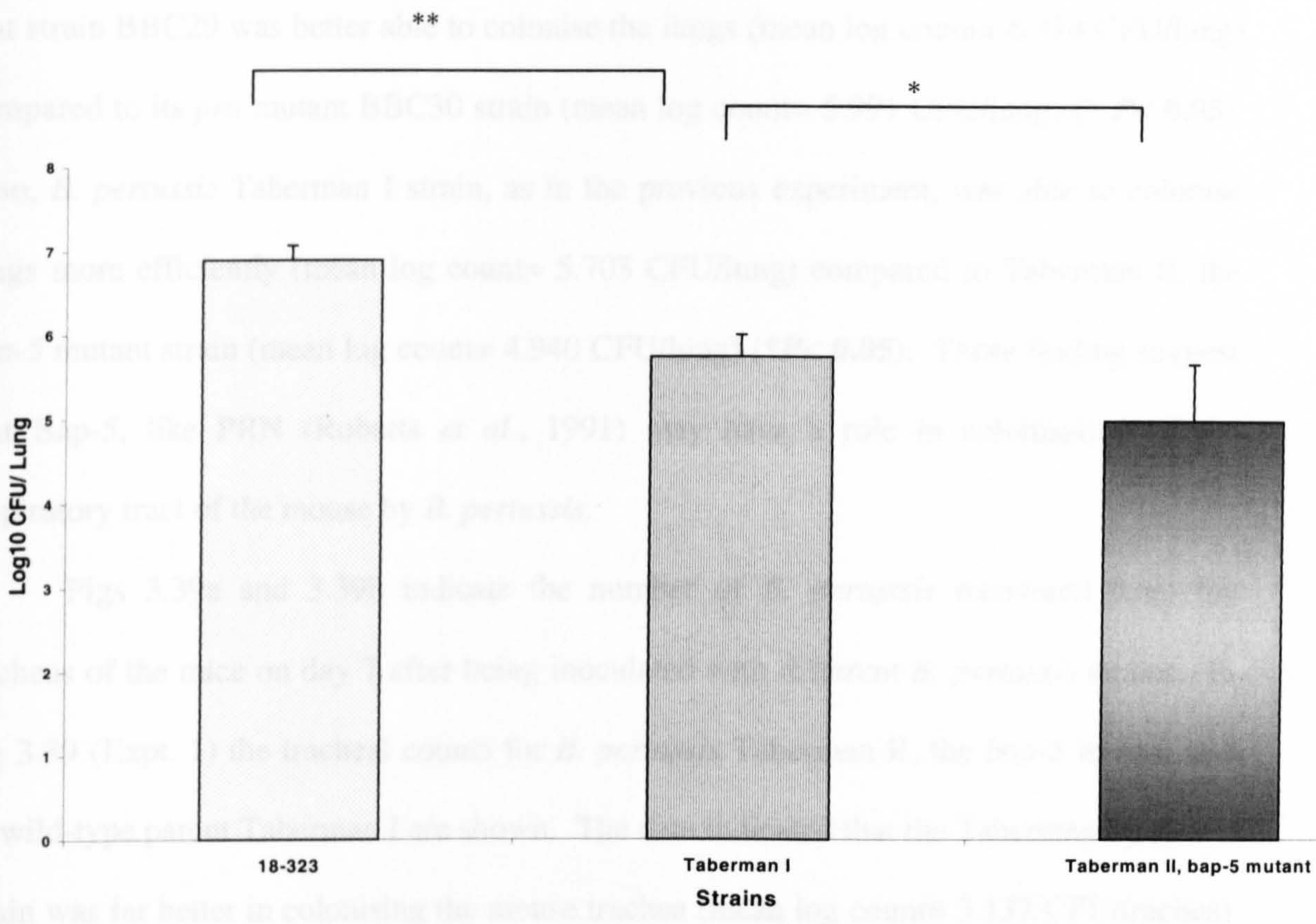
Fig. 3.38a: Mouse virulence data showing numbers of *B. pertussis* wild type and mutant strains recovered from the mouse lungs after intranasal challenge

Mice (CD1 strain, female ~3 weeks of age) in groups of five were challenged intranasally either with *B. pertussis* 18-323, *B. pertussis* Taberman I or its *bap-5* mutant Taberman II strain (~1 x 10^5 CFU/ mouse), under mild halothane anaesthesia (section 2.13.2). On day 7 after challenge, mice were killed with an overdose of CO_2 and the lungs were removed aseptically and viable count data were obtained by plating different dilutions of the lung homogenate on the BG-agar plates. The data presented are the means and standard deviations of the Log_{10} CFU/lung. Raw data of lung counts from individual mice are presented in appendix IV. Student's *t*-test was applied to compare the groups. ***P*<0.05, ****P*<0.0001

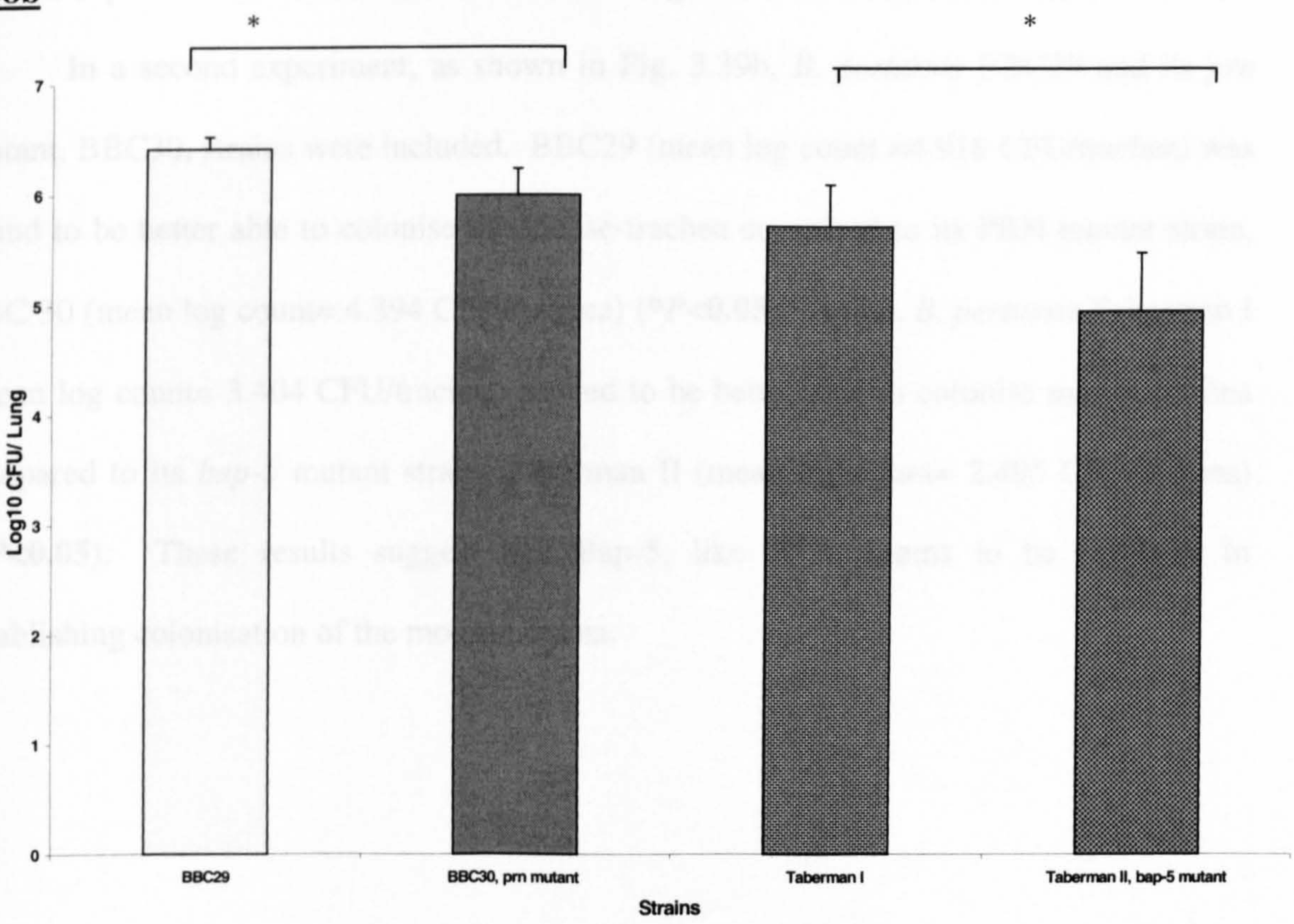
Fig. 3.38b: Mouse virulence data showing number of *B. pertussis* wild type and mutant strains recovered from the mouse lungs after intranasal challenge

Mice (CD1 strain, female ~3 weeks of age) in groups of five were challenged intranasally either with *B. pertussis* BBC29, BBC30 (*prn* mutant), *B. pertussis* Taberman I or Taberman II (*bap-5* mutant) strain (~1 x 10^6 CFU/ mouse) and lung count data were obtained as described above.

3.38a


**
*
Log10 CFU/ Lung
8
7
6
5
4
3
2
1
0
18-323
Taberman I
Taberman II, bap-5 mutant
Strains

3.38b


*
*
Log10 CFU/ Lung
7
6
5
4
3
2
1
0
BBC29
BBC30, prn mutant
Taberman I
Taberman II, bap-5 mutant
Strains

The results obtained in the second experiment (experiment 2), (Fig. 3.38b) showed that strain BBC29 was better able to colonise the lungs (mean log count= 6.414 CFU/lung) compared to its *prn* mutant BBC30 strain (mean log count= 5.991 CFU/lung) (* ***P*< 0.05**). Also, *B. pertussis* Taberman I strain, as in the previous experiment, was able to colonise lungs more efficiently (mean log count= 5.703 CFU/lung) compared to Taberman II, the *bap-5* mutant strain (mean log count= 4.940 CFU/lung) (**P*< **0.05**). These finding suggest that Bap-5, like PRN (Roberts *et al.*, 1991) may have a role in colonisation of the respiratory tract of the mouse by *B. pertussis.*

Figs 3.39a and 3.39b indicate the number of *B. pertussis* recovered from the tracheas of the mice on day 7 after being inoculated with different *B. pertussis* strains. In Fig 3.39 (Expt. 1) the tracheal counts for *B. pertussis* Taberman II, the *bap-5* mutant and its wild-type parent Taberman I are shown. The data indicated that the Taberman I parent strain was far better in colonising the mouse trachea (mean log count= 3.137 CFU/trachea) than its *bap-5* mutant Taberman II strain (mean log count=1.449 CFU/trachea) (**P*=**0.05**).

In a second experiment, as shown in Fig. 3.39b, *B. pertussis* BBC29 and its *prn* mutant, BBC30, strains were included. BBC29 (mean log count =4.918 CFU/trachea) was found to be better able to colonise the mouse-trachea compared to its PRN mutant strain, BBC 30 (mean log count= 4.394 CFU/trachea) (**P*<**0.05**). Again, *B. pertussis* Taberman I (mean log count= 3.404 CFU/trachea) proved to be better able to colonise mouse trachea compared to its *bap-5* mutant strain, Taberman II (mean log count= 2.495 CFU/trachea) (**P*<**0.05**). These results suggest that Bap-5, like PRN, seems to be involved in establishing colonisation of the mouse trachea.

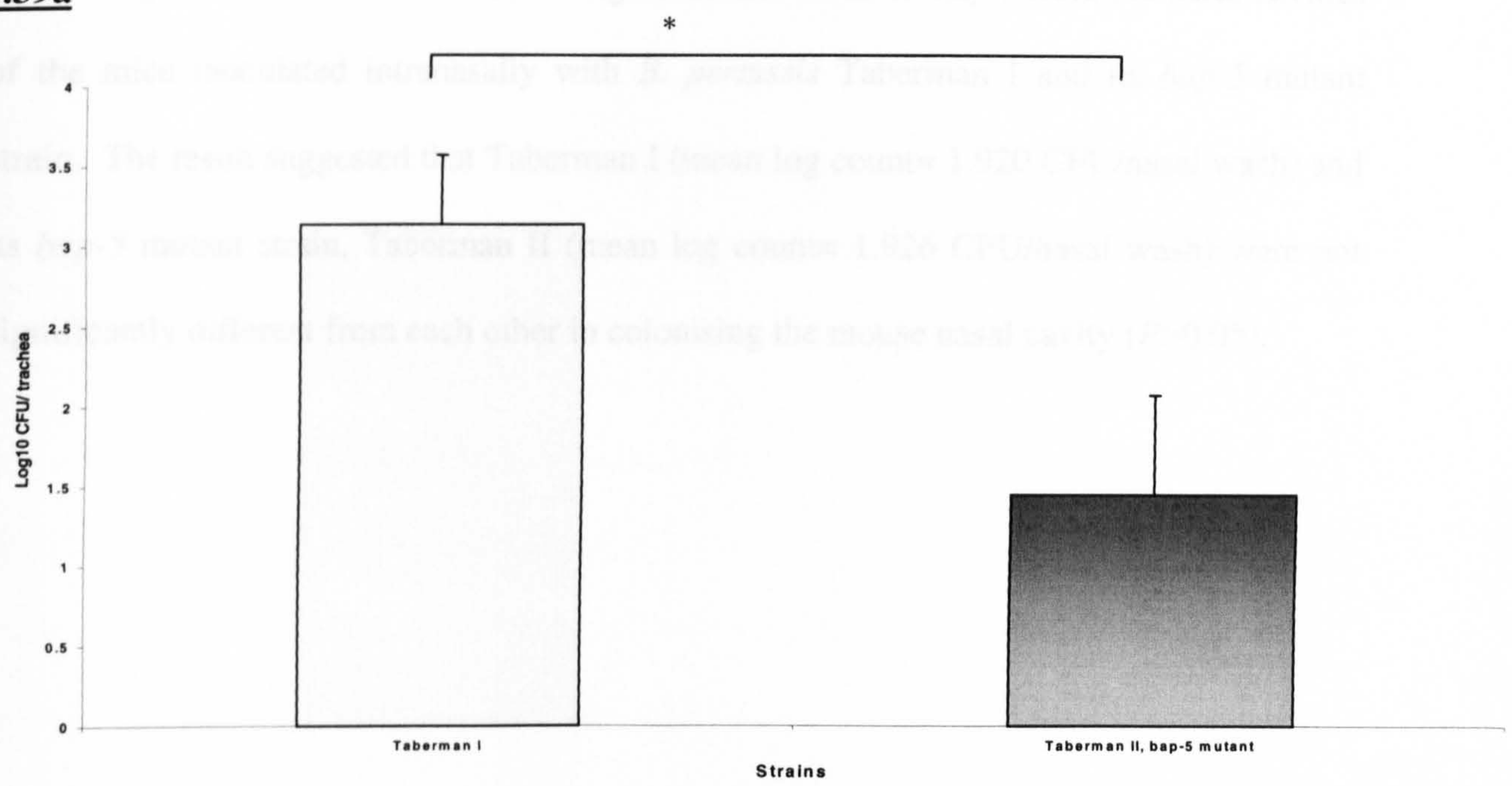
Fig. 3.39a: Mouse virulence data showing number of *B. pertussis* wild type and mutant strains recovered from the mouse tracheas after intranasal challenge

Mice (CD1 strain, female ~3 weeks of age) in groups of five were challenged intranasally either with *B. pertussis* 18-323, *B. pertussis* Taberman I or its *bap-5* mutant Taberman II strain (~1 x 10^5 CFU/ mouse), under mild halothane anaesthesia (section 2.13.2). On day 7 after challenge, mice were killed with an overdose of CO_2 and the tracheas were removed aseptically and viable count data were obtained by plating different dilutions of the tracheal homogenate on the BG-agar plates. The data presented are the means and standard deviations of the Log_{10} CFU/trachea. Raw data of tracheal counts from individual mice are presented in appendix V. Student's *t*-test was applied to compare the groups. ***P*<0.05, ****P*=0.0001**

Fig. 3.39b: Mouse virulence data showing number of *B. pertussis* wild type and mutant strains recovered from the mouse tracheas after intranasal challenge

Mice (CD1 strain, female ~3 weeks of age) in groups of five were challenged intranasally either with *B. pertussis* BBC29, BBC30 (*prn* mutant), *B. pertussis* Taberman I or Taberman II (*bap-5* mutant) strain (~1 x 10^6 CFU/ mouse) and tracheal count data were obtained as described above. Other details are same as above. ***P*<0.05.**

3.39a


*
Log10 CFU/ trachea
4
3.5
3
2.5
2
1.5
1
0.5
0
Taberman I
Taberman II, bap-5 mutant
Strains

3.39b

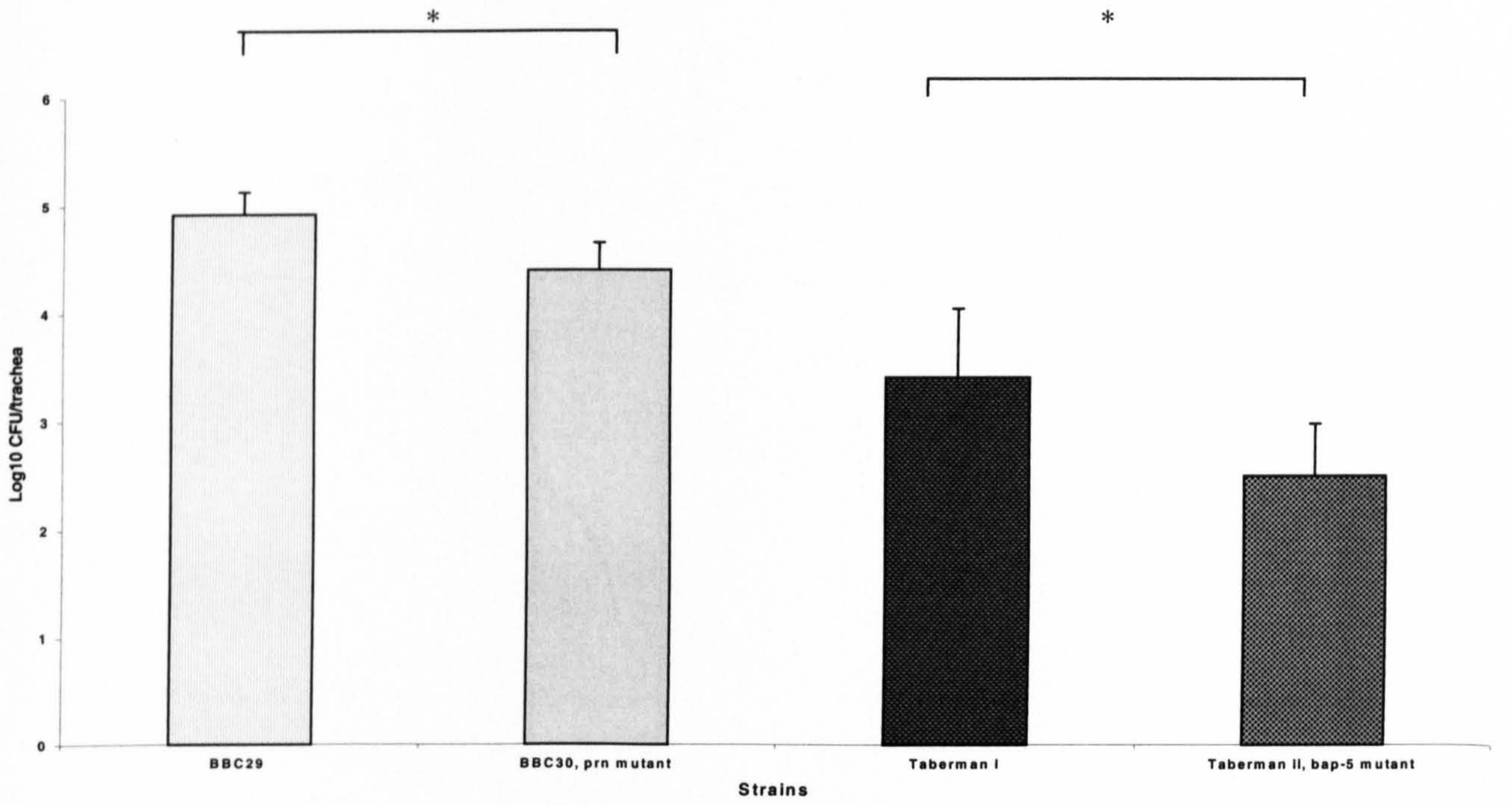
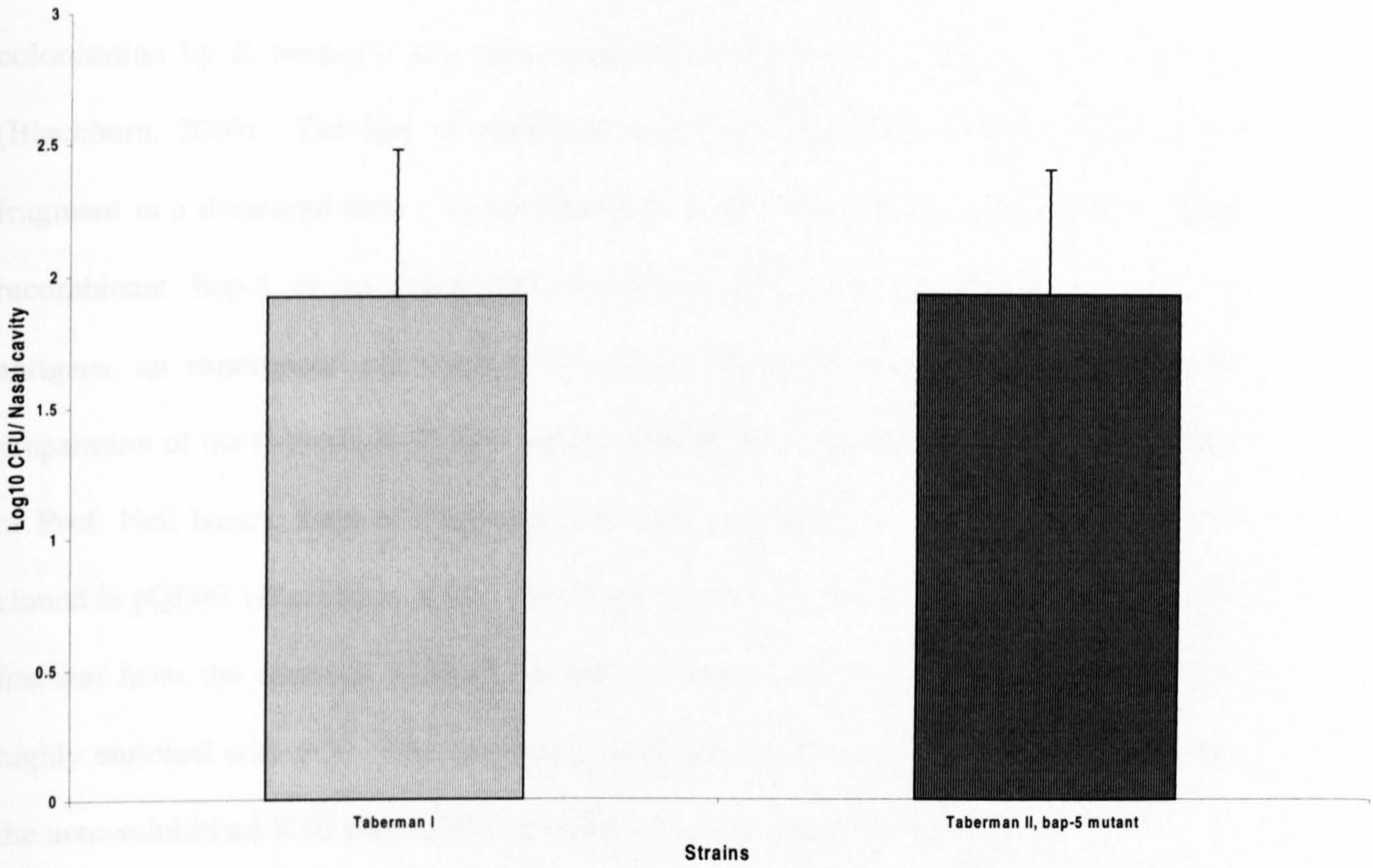

*
*
Log10 CFU/trachea
6
5
4
3
2
1
0
BBC29
BBC30, prn mutant
Taberman I
Taberman II, bap-5 mutant
Strains

Fig. 3.40 shows the number of organism recovered on day 7 from the nasal cavities of the mice inoculated intranasally with *B. pertussis* Taberman I and its *bap-5* mutant strain. The result suggested that Taberman I (mean log count= 1.920 CFU/nasal wash) and its *bap-5* mutant strain, Taberman II (mean log count= 1.926 CFU/nasal wash) were not significantly different from each other in colonising the mouse nasal cavity ($P>0.05$).

Fig. 3.40: Mouse virulence data showing number of *B. pertussis* wild type and mutant strains recovered from the mouse nasal washes after intranasal challenge

Mice (CD1 strain, female ~3 weeks of age) in groups of five were challenged intranasally either with *B. pertussis* 18-323, *B. pertussis* Taberman I or its *bap-5* mutant Taberman II strain (~1 x 10^5 CFU/ mouse), under mild halothane anaesthesia (section 2.13.2). On day 7 after challenge, mice were killed with an overdose of CO_2 and the nasal washes were collected (in 1 ml volume of casamino acids) aseptically and viable count data were obtained by plating neat and dilutions of the nasal washes on BG-agar plates. The data presented are the means and standard deviations of the Log_{10} CFU/nasal wash. Raw data of nasal wash counts from individual mice are presented in appendix VI. Student's *t*-test was applied to compare the groups.


3
2.5
2
1.5
1
0.5
0
Log10 CFU/ Nasal cavity
Taberman I
Taberman II, bap-5 mutant
Strains

3.4 Mouse protection test using re-natured P.30, the C-terminal domain of pertactin

In a previous mouse protection study by Blackburn (2000), mice were vaccinated twice (subcutaneously, at a three week interval) with 10 µg of P.30 /injection. Two weeks after the 2nd injection they were given an intranasal challenge with a sub-lethal dose (~1x10^5 CFU/mouse) of *B. pertussis* 18-323. No protection against respiratory tract colonisation by *B. pertussis* was seen compared to the control, non-vaccinated animals (Blackburn, 2000). The lack of protection may have been due to the use of the P.30 fragment in a denatured state. As a preliminary to determine any protective effect using recombinant Bap-5 or its component N-terminal or C-terminal domains as vaccine antigens, an experiment was done to investigate the protective ability of a re-natured preparation of the C-terminal 30 kDa moiety of PRN (P.30) kindly provided by the group of Prof. Neil Isaacs, Dept of Chemistry, University of Glasgow. The recombinant P.30 cloned in pQE60, (Blackburn, 2000), was expressed in *E. coli* strain M 15). A urea-soluble fraction from the resultant inclusion bodies (section, 2.10.3), was obtained which was highly enriched with P.30. The re-natured preparation of P.30 were prepared by diluting the urea-solubilised P.30 with refolding buffer (Personal communication).

A preparation of native purified PRN (P.69) (kindly provided by Prof. M. Roberts) (section 2.13.1) being a known protective antigen in mice against respiratory tract colonisation, acted as a positive control. Alhydrogel alone (diluent) was included as a negative control. The same route(s) of injection, timing and challenge dose of the antigens were used as in the experiment by Blackburn (2000). Mouse lungs and tracheas were removed and nasal washes collected on post challenge days 0, 3, 9 and 14. Viable counts from individual mice were obtained and the raw data of the experiment are given in appendices VII, VIII and IX and are summarised in appendix X.

Fig. 3.41 shows the number of colony forming units (CFU) of *B. pertussis* recovered from the nasal cavities of mice in the different groups at intervals after challenge with *B. pertussis* 18-323. At day 0, i.e. immediately after the challenge, approximately 2.32 CFU/nasal cavity (mean log. count) of *B. pertussis* could be recovered. However, by day 3, the mouse group immunised with P.69 showed some protection (mean log count= 1.345) against colonisation compared to the sham group (mean log count= 2.932) (***P*<0.05**). The group vaccinated with P.30 also showed a lower count than the sham group but the difference was not significant (*P*>0.5). The counts for all groups had increased by day 9 and had declined by day 14. Although the counts for the P.30 group and especially the P.69 group were lower than those of the sham group on these days, no significant difference was observed (*P*>0.05). The results also indicated that the mice treated with P.30 appeared to show no significant protection against nasal colonisation compared to the sham group (*P*>0.05) throughout the experiment, whereas P.69 gave some protection at day 3.

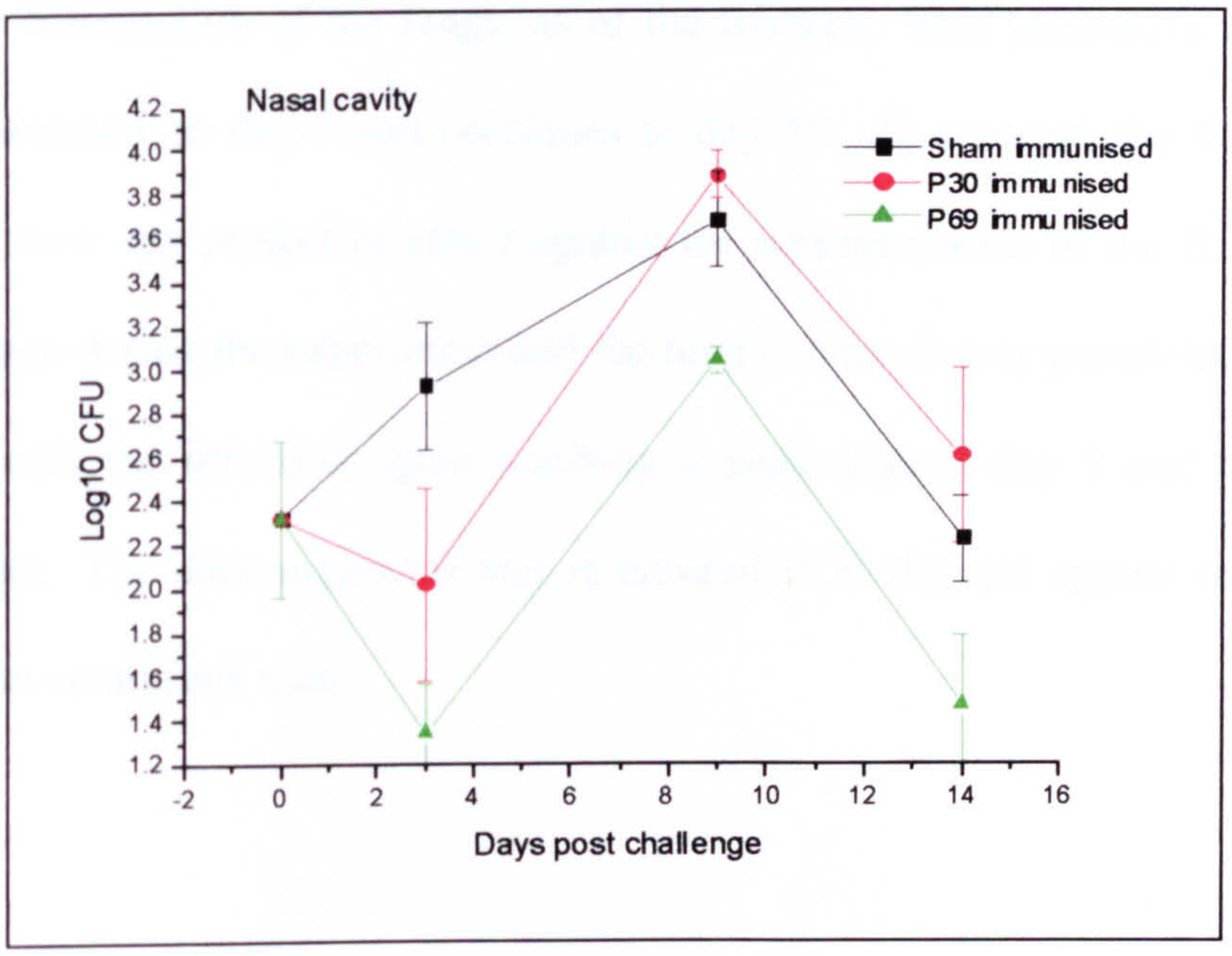
Fig. 3.42 shows that P.69 appeared to provide strong protection against tracheal colonisation in mice compared to the sham group and the protection was evident throughout the duration of the experiment (***P*<0.05**). There was little difference in *B. pertussis* colonisation of the trachea in the P.30 and sham group (*P*>0.05), where the infection reached a peak at day 9 and then showed some clearance by day 14, as was seen in the nasal cavity (Fig. 3.41).

Fig. 3.41: Mouse protection data showing the CFU recovered from the nasal cavities of the mice after intranasal challenge with *B. pertussis* 18-323.

Mice vaccinated twice subcutaneously with P.69, re-natured P.30 or with the diluent (alhydrogel) only were challenged intranasally with a sub-lethal dose of the standard mouse-virulent *B. pertussis* strain 18-323 (~$1x10^5$ CFU/mouse) and then sacrificed at intervals. One-way analysis of variance (ANOVA) test was applied to determine the statistical difference between the groups. The *P* value <0.0001 is considered extremely significant whereas the *P* value <0.05, is significant. Each point represents the mean of the Log_{10} CFU recovered from the nasal cavities of four individual mice and the error bars the S.E.M. Raw data of nasal wash counts from individual mice are given in appendix VII.

Fig. 3.42: Mouse protection data showing the CFU recovered from the tracheas of the mice after intranasal challenge with *B. pertussis* 18-323

Each point represents the mean of the Log_{10} CFU recovered from the tracheas of four individual mice and the error bars the S.E.M. Other details are same as described in the legend to Fig. 3.41. Raw data of tracheal counts from individual mice are given in appendix VIII.


Nasal cavity
Sham immunised
P30 immunised
P69 immunised
Log10 CFU
4.2
4.0
3.8
3.6
3.4
3.2
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
-2
0
2
4
6
8
10
12
14
16
Days post challenge

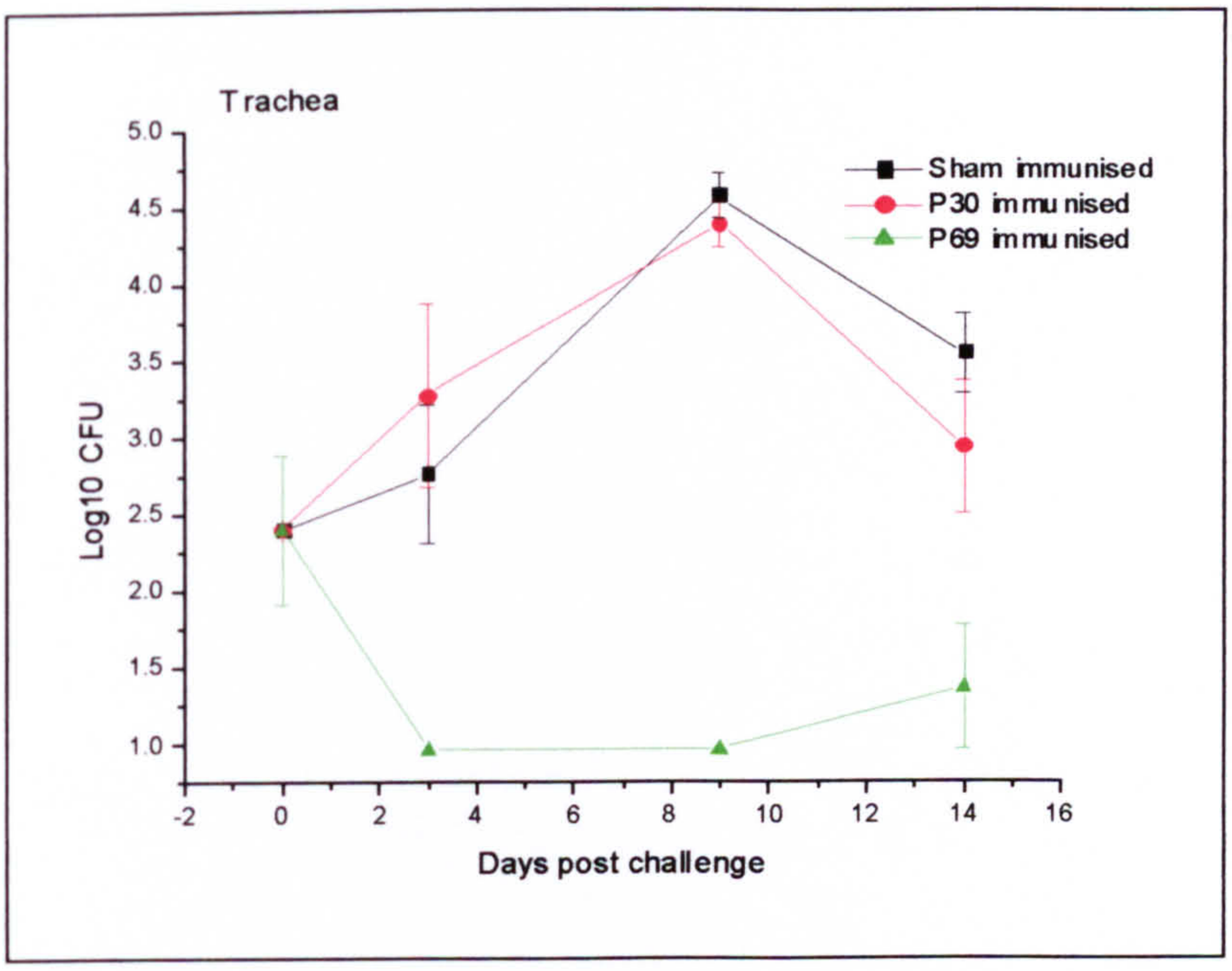
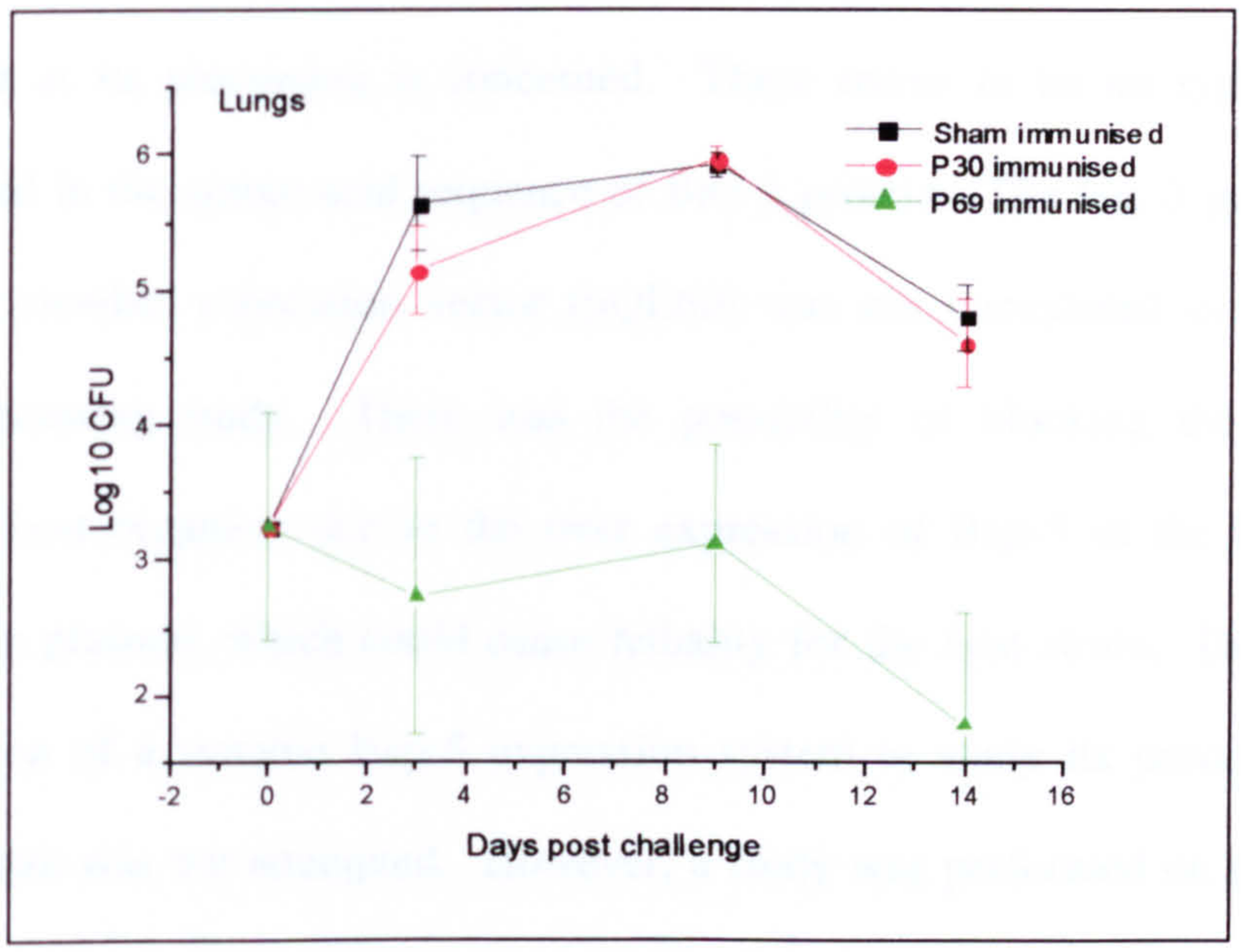

Trachea
Sham immunised
P30 immunised
P69 immunised
Log10 CFU
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
-2
0
2
4
6
8
10
12
14
16
Days post challenge

Fig. 3.43 shows the lung counts obtained from the different groups of mice during the course of experiment. It is apparent that immunisation with P.69 provided clear protection against colonisation of the lungs, as of the trachea. This protection is evident from the results obtained on day 3 and continues to day 14. In contrast, the P.30 group does not seem to show any protective effect against the natural course of the *B. pertussis* infection at any stage during the experiment and the lung counts closely paralleled those in the sham group, with the infection again reaching a peak around day 9 and thereafter beginning to decline. The data suggested that re-natured P.30 did not appear to provide protection under the conditions used.

Fig. 3.43: Mouse protection data showing the CFU recovered from the lungs of the mice after intranasal challenge with *B. pertussis* 18-323

Each point represents the mean of the Log_{10} CFU recovered from the lungs of four individual mice and the error bars the S.E.M. Other details are same as described in the legend to Fig. 3.41. Raw data of nasal wash counts from individual mice are given in appendix IX.


Lungs
Sham immunised
P30 immunised
P69 immunised
Log10 CFU
6
5
4
3
2
-2
0
2
4
6
8
10
12
14
16
Days post challenge

3.5 Secretion mechanism of a *Bordetella pertussis* autotransporter protein in protease-deficient strains of *E. coli*

Many of the virulence determinants of *B. pertussis* are proteinacious in nature and are usually either exported to the bacterial cell surface or secreted into the external environment. Bap-5, being the newest member of the autotransporter family, is the least understood as far as its processing is concerned. There seems to be no typical signal sequence identified in the amino acid sequence of Bap-5 protein. The *bap-5* gene cloned in the high copy number expression vector (pQE60) was not considered as a suitable system for a processing study. There was the possibility of blocking the secretion apparatus of the host organism due to the over expression of Bap-5 in the high copy number expression plasmid, which could cause lethality for the host strain. Due to time constraints, creation of a suitable Bap-5 expression system to study its processing and secretion mechanism was not attempted. However, a study was performed on another *B. pertussis* autotransporter protein, PRN (section 1.8.1). This particular protein was selected as the *prn* gene (2770 bp) was available in a form, ready to use, cloned into a broad host range, medium copy number vector (7.9 Kb plasmid pMMB66EH) kindly provided by Prof. M. Roberts, Department of Veterinary Pathology, University of Glasgow. As with Bap-5, the mechanism of processing of the precursor PRN (P.93) to mature PRN (P.69), is not clear. The release of passenger domain from the pro-protein may occur either by auto-proteolytic cleavage as in the case of the IgA protease of *Neisseria gonorrhoeae*, or by the help of some of the outer membrane-localised proteases e.g. serine protease (OmpT) (Table 2.2).

A set of protease-deficient *E. coli* strains (Table 2.2) was kindly provided by Prof. G. Georgiou, Department of Chemical Engineering, University of Austin, U.S.A. As these strains were isogenic, any differences in the protein profiles of the various cell fractions of

the *E. coli* expressing *prn* should correlate with the presence or absence of particular protease and hence the accumulation of the different forms of PRN (processed or unprocessed, secreted or non secreted). Thus by comparing the protein profiles in different compartments of the cell it may be possible to determine the cell envelope proteases that may have a role in the processing of the precursor protein into a mature protein at the different stages of the secretion process.

The export of the passenger domain (P.69) was investigated in *E. coli* strains lacking various combinations of envelope-associated proteases i.e. DegP, Esp, Ptr and OmpT (Table 2.2; section 2.1.2). The pertactin gene (*prn*) (2.7 Kb) cloned into the broad host range vector pMMB66EH (7.9 Kb) was under the control of an IPTG-inducible *tac* promoter to give p41869 (10.5 Kb) (Fig. 3.44) and this plasmid was transformed by electroporation into the *E. coli* protease deficient strains. Fig. 3.45 shows the plasmid preparations from these *E. coli* transformants. All the *E. coli* strains carrying pMMB66EH (control) or p41869 (expressing *prn*) were grown in modified Luria Broth medium supplemented with glucose 0.2% w/v. The expression of the recombinant PRN was induced by adding 1mM IPTG to the culture during the late log phase (OD_{600nm} ~0.9) with incubation at 37°C continued for ~3 h.

Figs. 3.46a and 3.46b show the protein profiles, obtained by SDS-PAGE, of the whole-cell fractions of the various protease-deficient strains of *E. coli,* and the corresponding immunoblot. The protein profiles of the parent strain carrying only the backbone vector pMMB66EH (lane A) or expressing *prn* (p41869) (lane B) and all the protease-deficient strains expressing *prn* (p41869) (lanes C-I), were complex and indistinguishable by SDS-PAGE. However there were some differences evident in the corresponding immunoblot (Fig. 3.46b). All the lanes with the exception of lane A (parent strain, pMMB66EH) showed the ~93 kDa band which is the expected size of the full

Fig. 3.44: A restriction map showing the *prn* gene cloned into a broad host range vector pMMB66EH to create p41869.

The map shows the important features of the plasmid 41869. The expression of the full length *prn* gene is regulated by the *tac* promoter which is controlled by the *lacI*^{q} repressor. It also contains an ampicillin resistance encoding gene (*bla*).

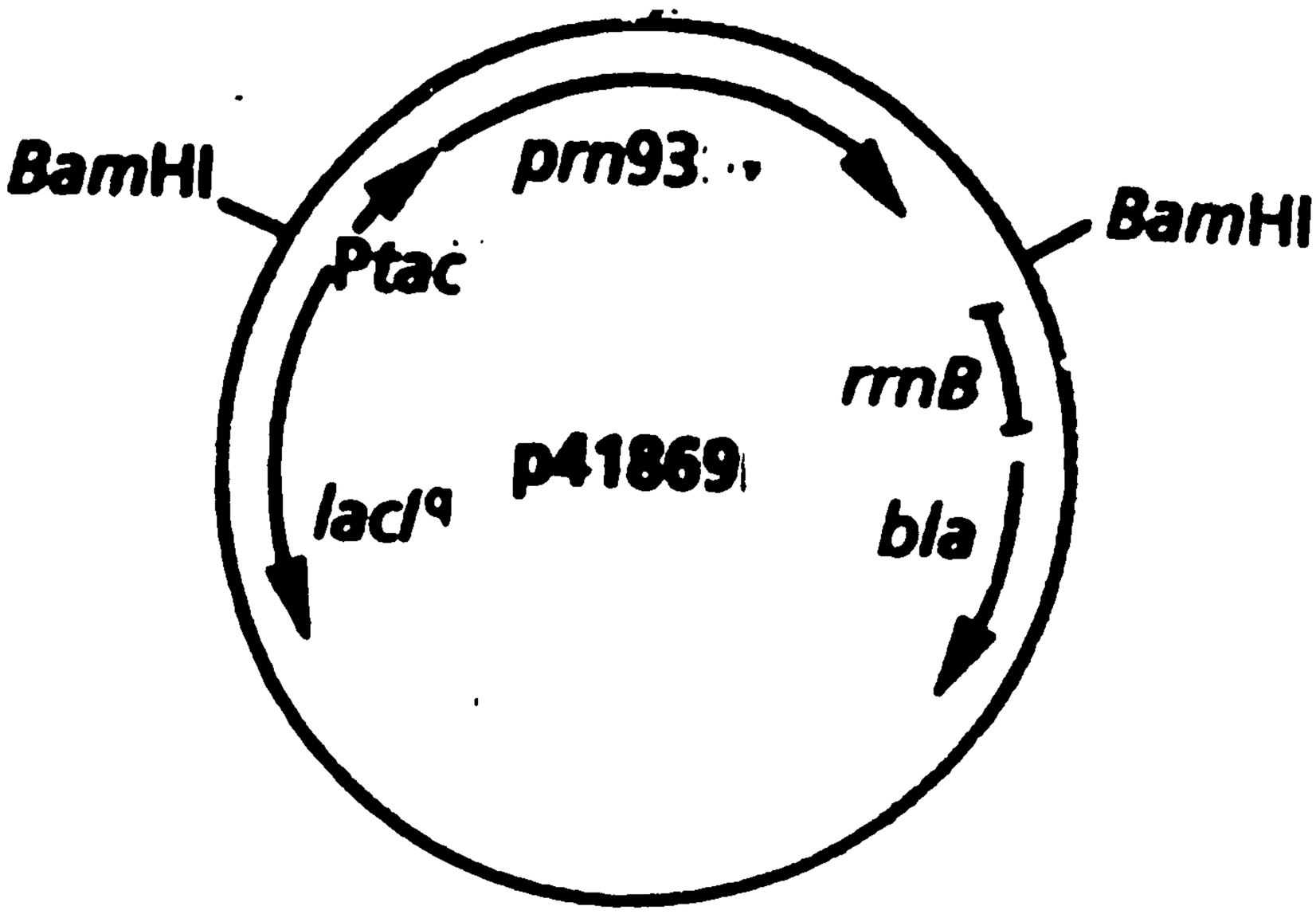

BamHI
prn93
BamHI
Ptac
rrnB
p41869
lacI^q
bla

Fig. 3.45: Agarose gel (0.7 %) showing the broad host range vector, pMMB66EH (7.9 Kb) and plasmid p41869 (10.5 Kb), containing the gene encoding full length pertactin (P.93) isolated from the range of protease-deficient *E. coli* strains

A = *E. coli* KS272	parent	containing	pMMB66EH
B = *E. coli* KS272	//	//	p41869
C = *E. coli* SF110	*ompT, degp*	//	p41869
D = *E. coli* SF120	*ompT, degp, ptr*	//	p41869
E = *E. coli* HM101	*tsp, eda*	//	p41869
F = *E. coli* HM111	*ompT, tsp, eda*	//	p41869
G = *E. coli* HM112	*ptr, tsp, eda*	//	p41869
H = *E. coli* HM119	*degp, ompT, tsp, eda*	//	p41869
I = *E. coli* HM130	*degp, ompT, tsp, eda, ptr*	//	p41869

1 = Super coiled DNA marker

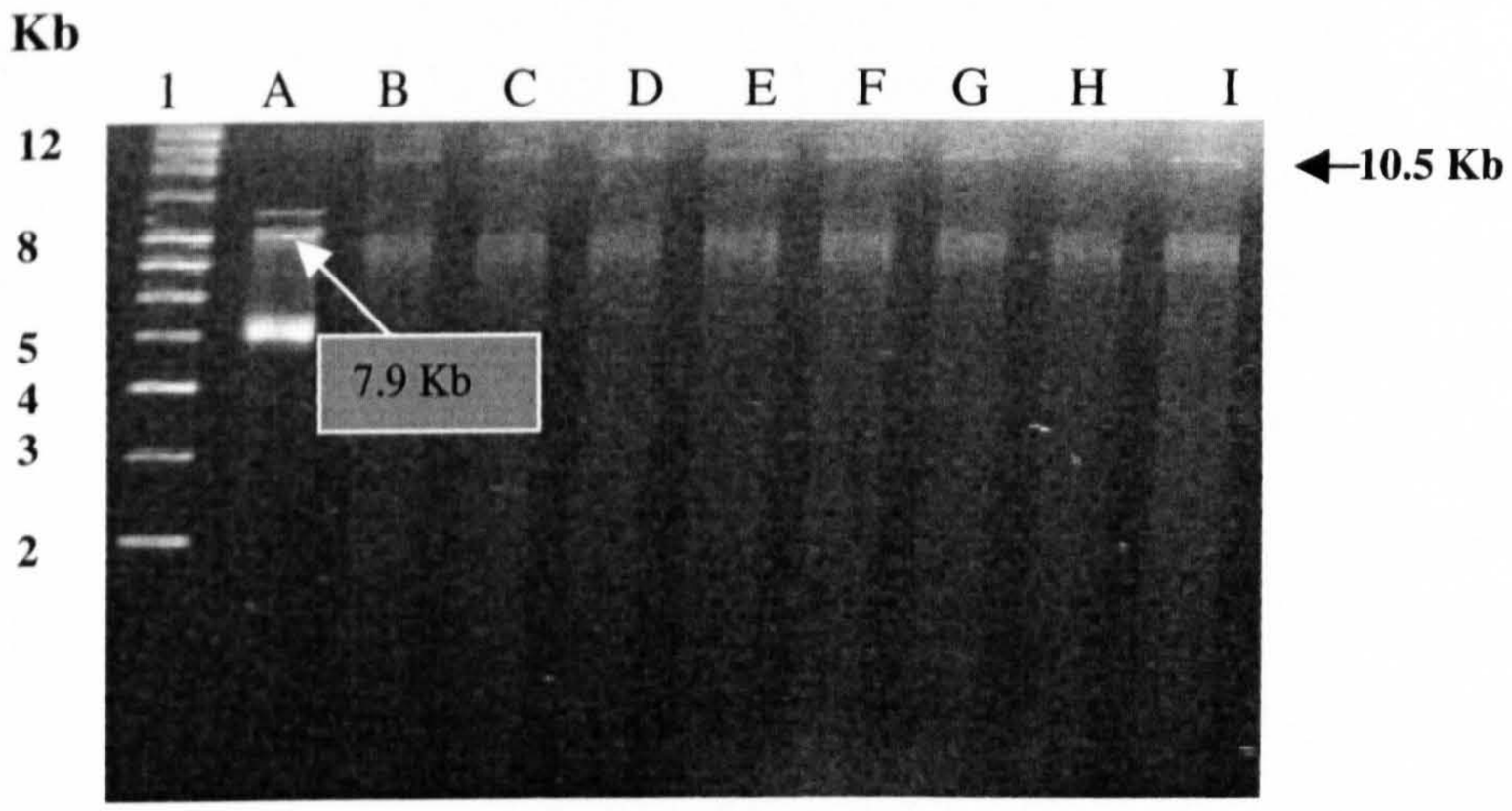

Kb
1 A B C D E F G H I
12
8
5
4
3
2
7.9 Kb
10.5 Kb

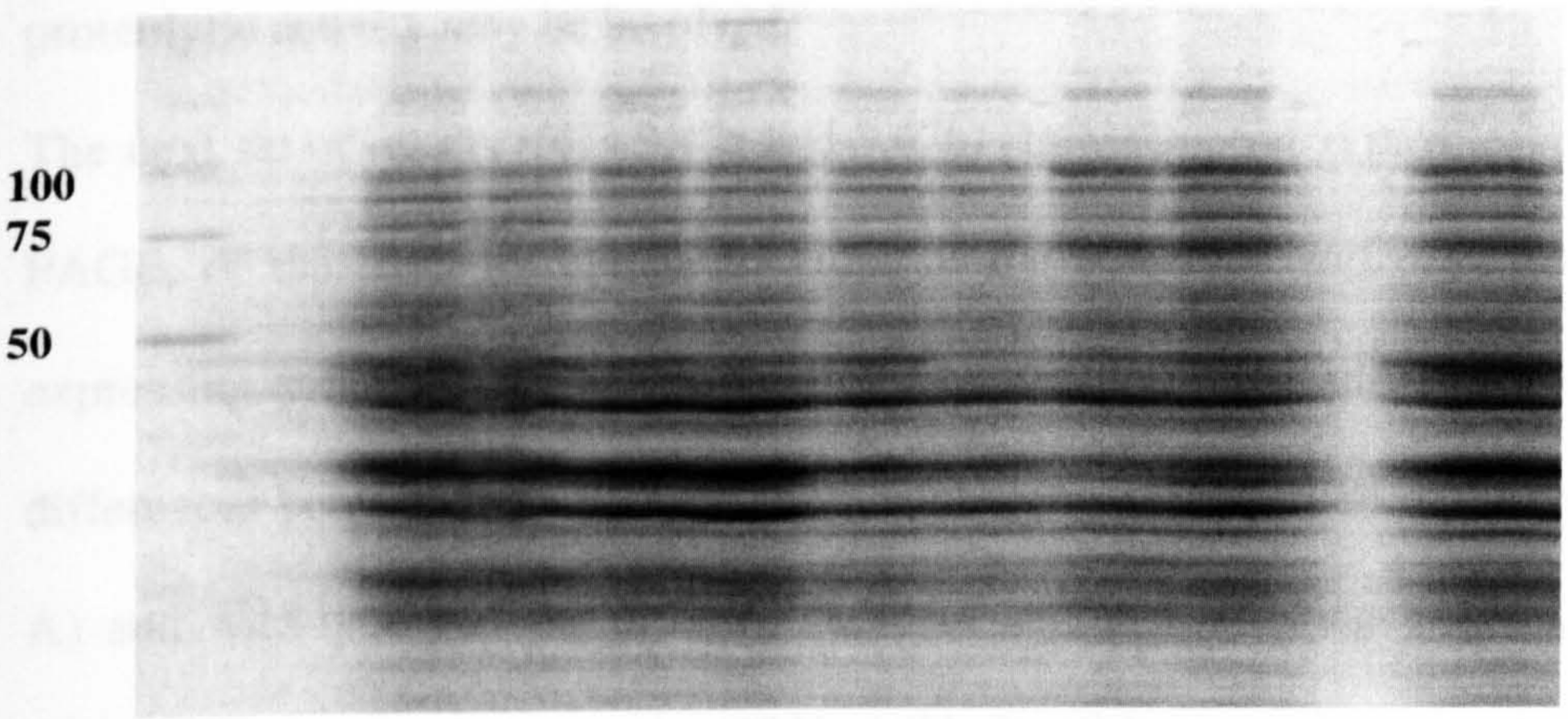
Fig. 3.46: Protein profiles and corresponding immunoblot showing the whole-cell fractions of various protease-deficient strains expressing full length pertactin

The figure shows the whole-cell protein profiles by the SDS-PAGE (12%) of various *E. coli* strains grown in LB medium. Fig. 3.46b shows an immunoblot of Fig. 3.46a, obtained with rabbit anti-P.69 antiserum (Table 2.7).

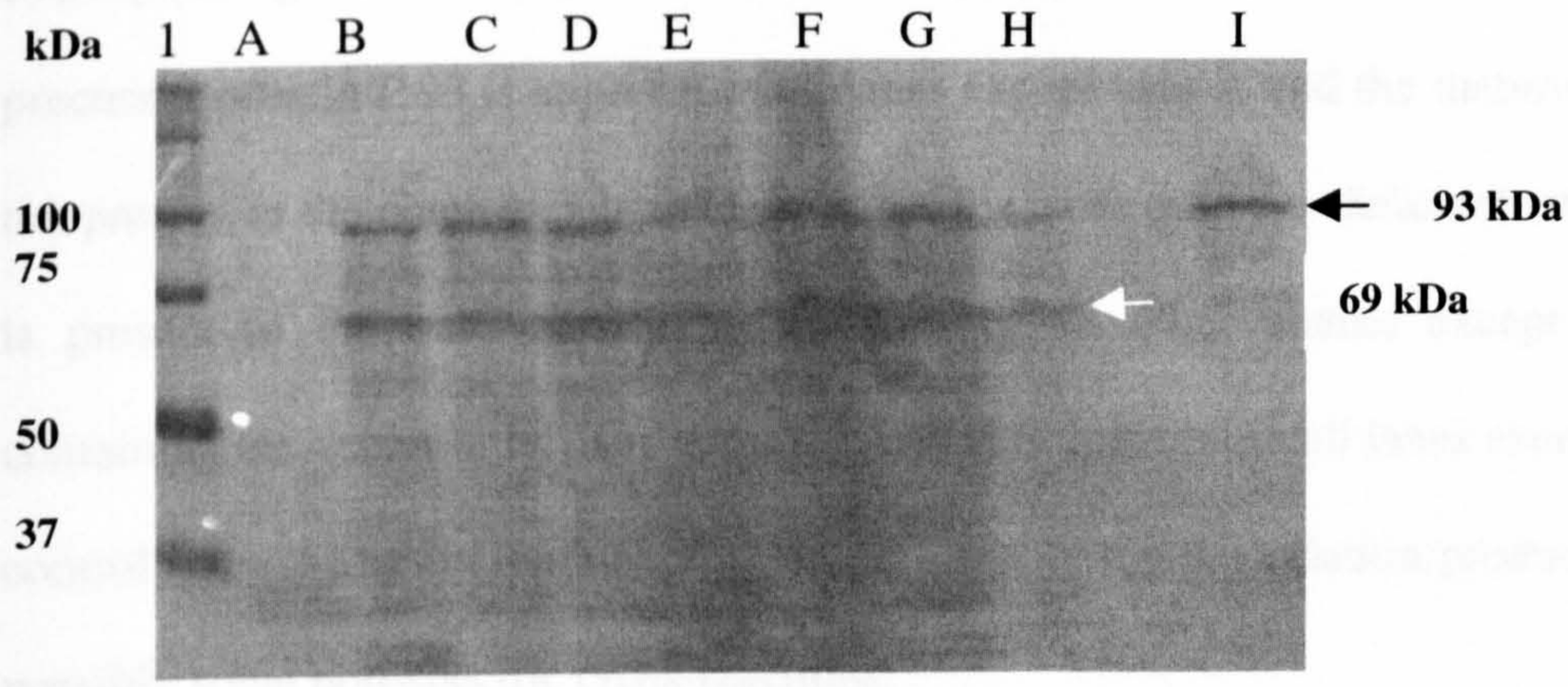
1 = Protein standard with molecular weights shown in the left

A = *E. coli* KS272	parent	containing	pMMB66EH
B = *E. coli* KS272	//	//	p41869
C = *E. coli* SF110	*ompT, degp*	//	p41869
D = *E. coli* SF120	*ompT, degp, ptr*	//	p41869
E = *E. coli* HM101	*tsp, eda*	//	p41869
F = *E. coli* HM111	*ompT, tsp, eda*	//	p41869
G = *E. coli* HM112	*ptr, tsp, eda*	//	p41869
H = *E. coli* HM119	*degp, ompT, tsp, eda*	//	p41869
I = *E. coli* HM130	*degp, ompT, tsp, eda, ptr*	//	p41869

3.46a


kDa
1
A
B
C
D
E
F
G
H
I
100
75
50

3.46b


kDa
1
A
B
C
D
E
F
G
H
I
100
75
50
37
93 kDa
69 kDa

length of PRN. They also showed a band at 69 kDa which is the expected size of the mature form of PRN, except in lane I (HM130, strain deficient in most of the proteases) which did not seem to produce the mature PRN (P.69). The result suggested that several protease-activities had to be absent to prevent processing, indicating that a non-specific proteolytic activity may be involved.

The next set of results (Figs 3.47a and 3.47b) show the protein profiles, obtained by SDS-PAGE, of the outer-membrane fractions of the wild-type and protease-deficient strains expressing *prn* and their corresponding immunoblot. The protein profiles show some differences between the wild-type strain containing pMMB66EH, not expressing *prn* (lane A) and wild-type and the protease-deficient strains containing p41869, expressing *prn*. There seems to be the precursor PRN visible in all lanes at the correct predicted size (93 kDa) with the exception of lane A (wild-type, carrying the vector pMMB66EH only). The mature PRN (P.69) seems to be visible at the predicted size (69 kDa) in the outer-membrane fractions of all the strains expressing *prn*, with the exception of the most deficient strain HM130 (lane I). Confirmation of the SDS-PAGE results is provided by the corresponding immunoblot, obtained with the polyclonal anti P.69 serum (Fig. 3.47b). The precursor protein P.93 is apparent in all lanes except lane A and the mature PRN (P.69) is not present in the outer-membrane fraction of the most protease-deficient strain (lane I) but is present in the outer-membrane fractions of all other strains except lane A again containing the vector only. The other minor bands apparent in all lanes except the negative control (lane A) in the immunoblot, are presumably the degradation products of PRN and possibly some non-specific cross-reactions.

To determine whether PRN, after being exported to the outer-membrane, is released or not, concentrated (50 times) culture supernate fractions from the wild-type and protease-deficient strains were collected (section 2.10.1).

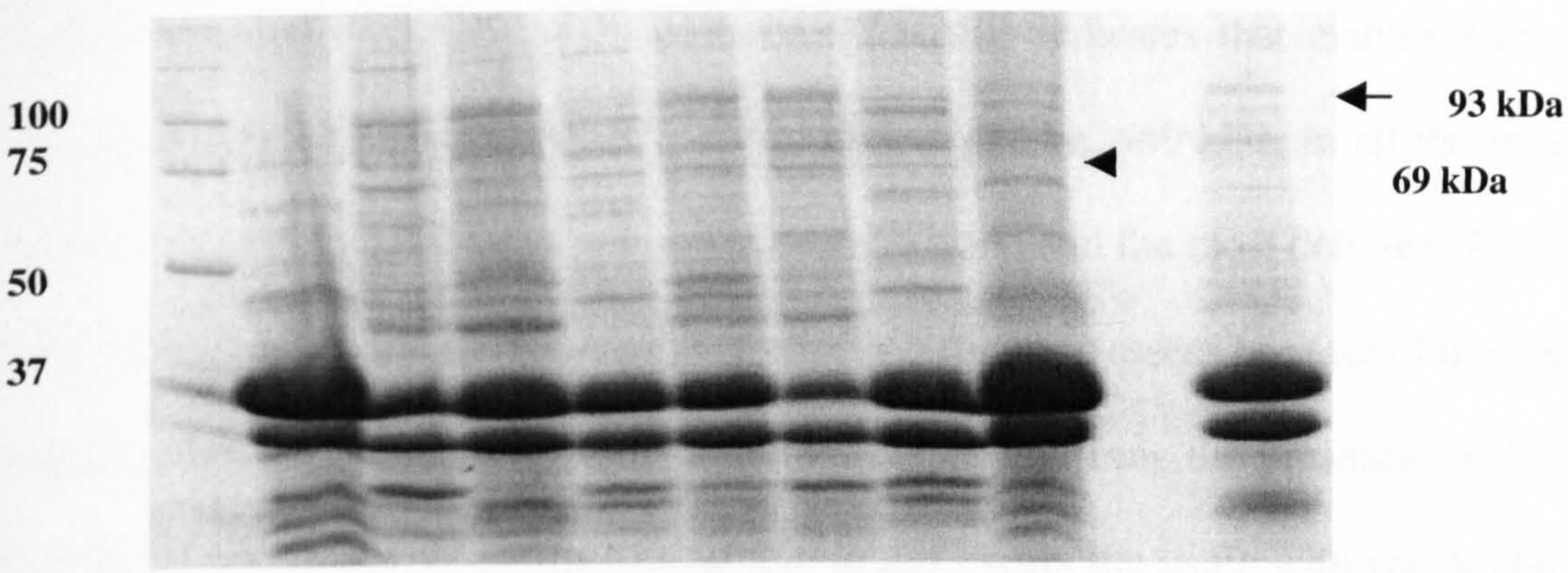
Fig. 3.47: Protein profile and corresponding immunoblot showing outer-membrane fractions of various protease-deficient strains and their parent expressing full length pertactin.

The figure shows the outer-membrane preparations by the SDS-PAGE (12%) of various *E. coli* strains grown in LB medium. profiles (loaded with same amount of protein in each well). Fig. 3.47b shows an immunoblot of Fig. 3.47a, obtained with rabbit anti-P.69 antiserum (Table 2.7).

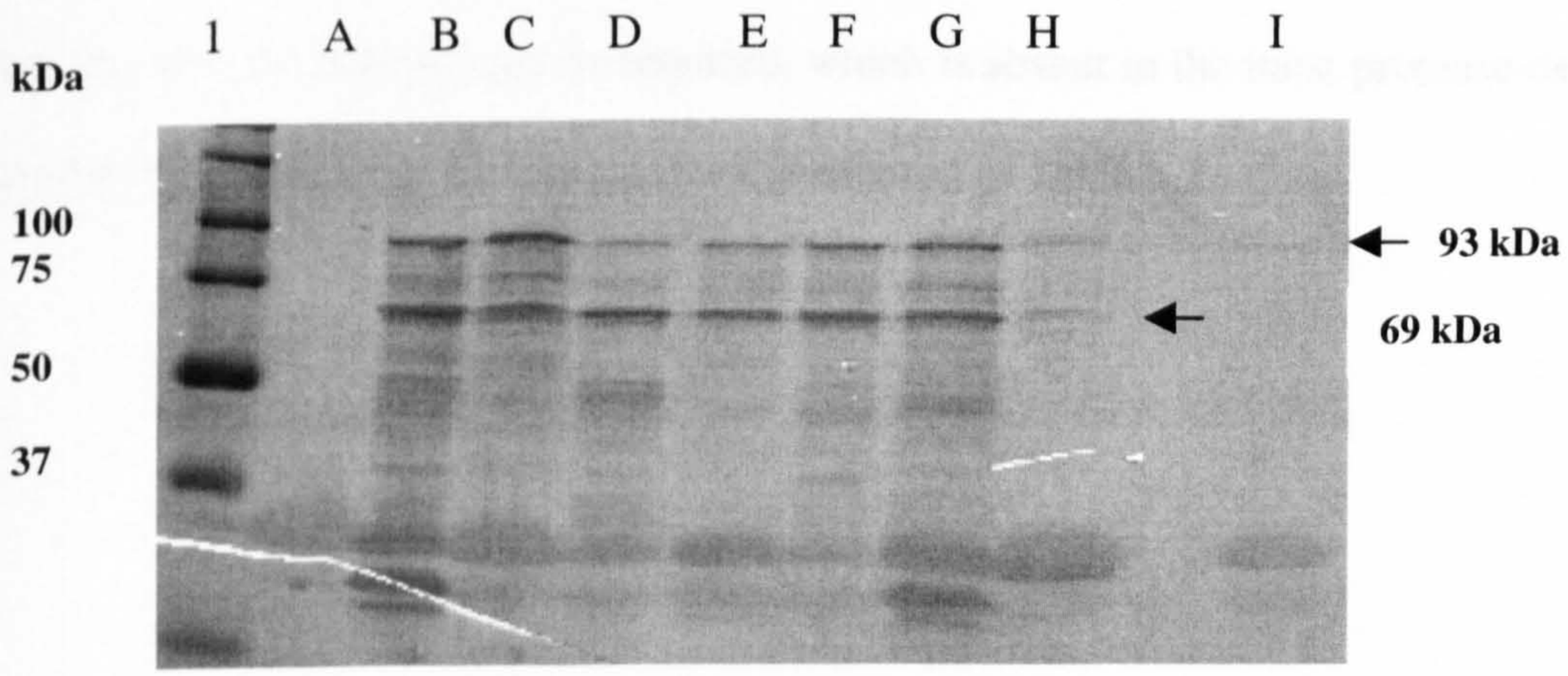
1 = Protein standard with molecular weights shown in the left

A = *E. coli* KS272	parent	containing	pMMB66EH
B = *E. coli* KS272	//	//	p41869
C = *E. coli* SF110	*ompT, degp*	//	p41869
D = *E. coli* SF120	*ompT, degp, ptr*	//	p41869
E = *E. coli* HM101	*tsp, eda*	//	p41869
F = *E. coli* HM111	*ompT, tsp, eda*	//	p41869
G = *E. coli* HM112	*ptr, tsp, eda*	//	p41869
H = *E. coli* HM119	*degp, ompT, tsp, eda*	//	p41869
I = *E. coli* HM130	*degp, ompT, tsp, eda, ptr*	//	p41869

3.47a


kDa
1
A
B
C
D
E
F
G
H
I
100
75
50
37
93 kDa
69 kDa

3.47b


1
A
B
C
D
E
F
G
H
I
kDa
100
75
50
37
93 kDa
69 kDa

Figs. 3.48a and 3.48b shows the protein profile obtained by SDS-PAGE of these supernate fractions and their corresponding immunoblot screened with rabbit anti-P.69 serum. There was a clear difference between the supernate fraction of the wild-type strain not expressing *prn* (lane A) and wild-type and protease-deficient strains expressing *prn*. The strains expressing *prn* seem to be producing a range of degradation products in their culture supernate fractions. The SDS-PAGE profile indicates that mature PRN may be present in the supernate fractions at the predicted size i.e. ~69 kDa, in all the strains with the exception of wild-type not expressing *prn* (lane A) and the most deficient *E. coli* strain HM 130 expressing *prn* (lane I) although this is difficult to assess because of the number of polypeptides visible. The immunoblot (Fig. 3.48b) confirms the presence of the mature P.69 form in supernatant fractions of all the strains except the strain with vector alone (lane A) and the most deficient-protease strain (lane I).

The overall results indicate that the processing of the precursor PRN protein P.93 into its passenger domain P.69 and the P.30 domain (β-barrel forming domain) was not dependent on any specific protease investigated in this study, but suggests that a non-specific proteolytic activity may be required, which is absent in the most protease-deficient strain HM130. A summary of the results is presented in Table 3.2.

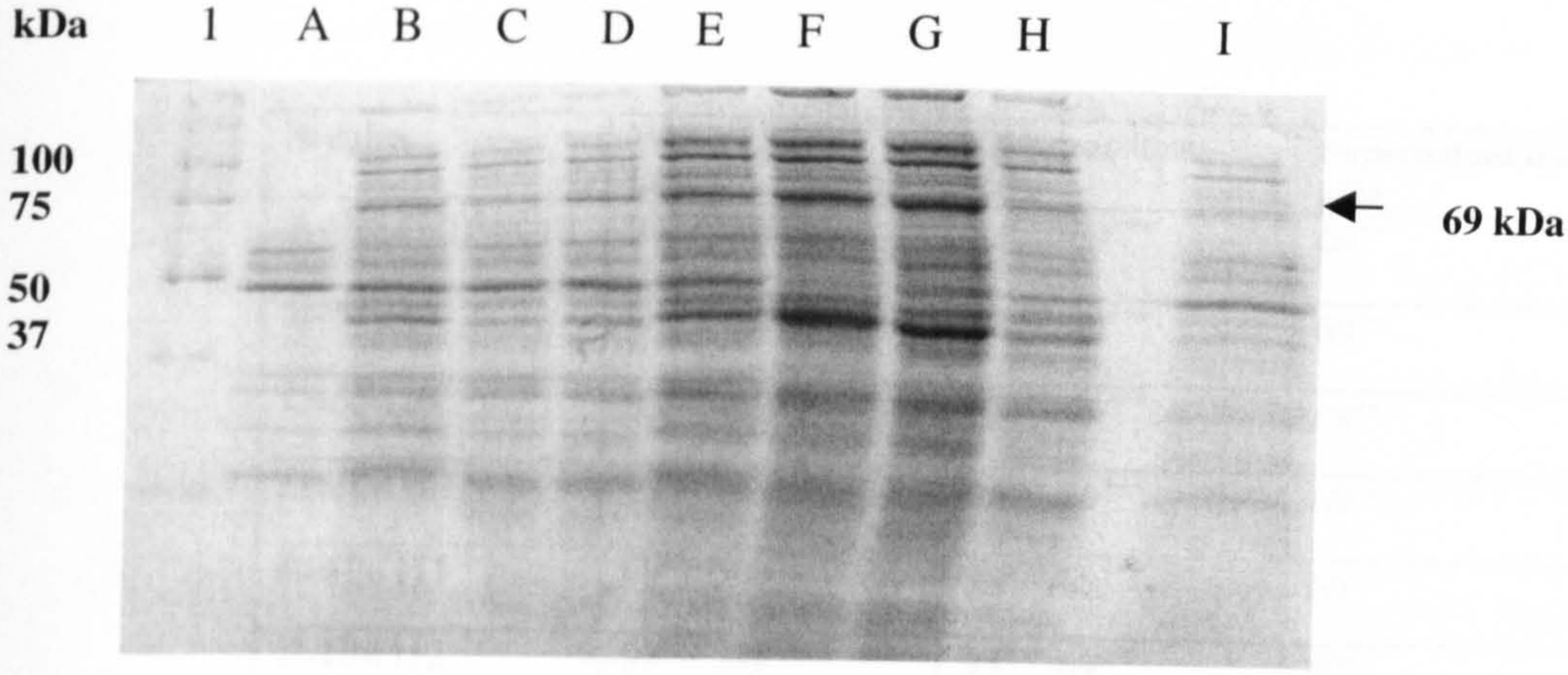
Fig. 3.48: Protein profiles and corresponding immunoblot showing supernatant fractions of various protease-deficient strains and their parent expressing full length pertactin.

The figure shows the concentrated supernate fractions by the SDS-PAGE (12%) of various *E. coli* strain grown in LB medium and their supernates were concentrated to same degree (section 2.10.1). Fig. 3.48b shows an immunoblot of Fig. 3.48a, obtained with rabbit anti-P.69 antiserum (Table 2.7).

1 = Protein standard with molecular weights shown in the left

A = *E. coli* KS272	parent	containing	pMMB66EH
B = *E. coli* KS272	//	//	p41869
C = *E. coli* SF110	*ompT, degp*	//	p41869
D = *E. coli* SF120	*ompT, degp, ptr*	//	p41869
E = *E. coli* HM101	*tsp, eda*	//	p41869
F = *E. coli* HM111	*ompT, tsp, eda*	//	p41869
G = *E. coli* HM112	*ptr, tsp, eda*	//	p41869
H = *E. coli* HM119	*degp, ompT, tsp, eda*	//	p41869
I = *E. coli* HM130	*degp, ompT, tsp, eda, ptr*	//	p41869

3.48a


kDa
1
A
B
C
D
E
F
G
H
I
100
75
50
37
69 kDa

3.48b

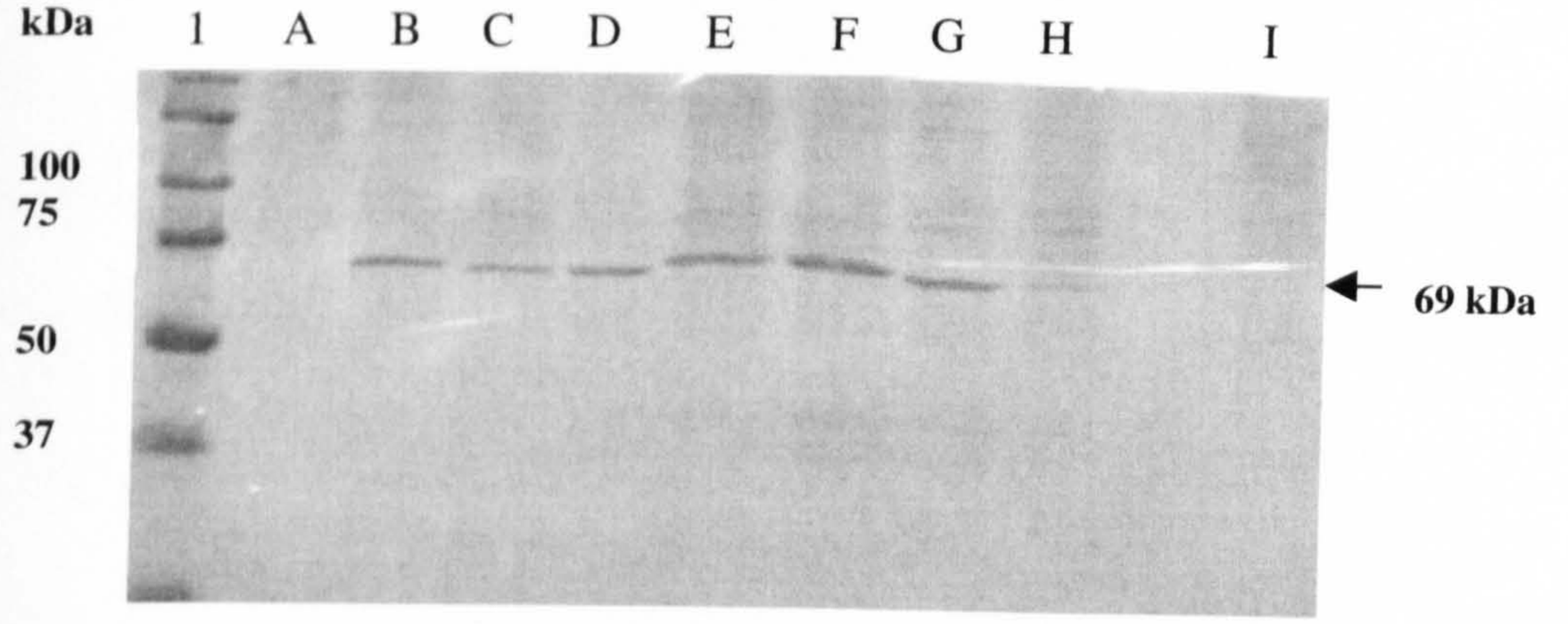

kDa
1
A
B
C
D
E
F
G
H
I
100
75
50
37
69 kDa

Table 3.2 Presence of precursor pertactin (P.93) and mature pertactin (P.69) in various protease-deficient strains in different cellular compartments.

Strains	Whole Cells kDa	OM Fractions kDa	Supernatant fractions kDa
A=KS272(parent)	$93^{++++}/69^{++++}$	$93^{+++}/69^{+++}$	69^{++}
B=SF110	$93^{++++}/69^{++++}$	$93^{+++}/69^{+++}$	69^{++}
C=SF120	$93^{++++}/69^{++++}$	$93^{+++}/69^{+++}$	69^{++}
D=HM101	$93^{++++}/69^{++++}$	$93^{+++}/69^{+++}$	69^{++}
E=HM111	$93^{++++}/69^{++++}$	$93^{+++}/69^{+++}$	69^{++}
F=HM112	$93^{++++}/69^{++++}$	$93^{+++}/69^{+++}$	69^{++}
G=HM119	$93^{+++}/69^{+++}$	$93^{++}/69^{++}$	69^{+}
H=HM130	$93^{++}/69^{-}$	$93^{+}/69^{-}$	69^{-}

3.5.1 Secretion and processing of C-terminal and linker region of pertactin in *E. coli* strains

The C- terminal domains of the autotransporter proteins are postulated to have a β-barrel structure consisting of several amphipathic anti-parallel β-strands. The next experiment was carried out to see whether the C-terminal (30 kDa) portion and the linker region (15 kDa; involving PQP repeat region; Fig 1.4) of the PRN, located between the beginning of the C-terminus and the end region of the N-terminus, are processed like the full length PRN (p41869), which is processed into P.69 and P.30 domains in the parent *E. coli* strain KS272 and others but appeared not to get processed and surface exposed in the most protease deficient strain HM130 of *E. coli*. A *Sac*I and *Eco*RI fragment (1.1 Kb insert) encoding P.30 and linker (15 kDa) was cloned into the 4.1 Kb vector pBAD/gIII (Invitrogen) (Fig. 3.49) which is provided with a signal sequence (Blackburn, 2000). This plasmid was transformed by electroporation into the parent strain (KS272) and into the most protease-deficient *E. coli* strain (HM130). Expression of the P.30 and its linker domains is tightly regulated by an arabinose promoter (*araC*). *E. coli* was grown in modified Luria broth medium in the presence of ampicillin (60μg/ml) and expression of the recombinant protein was induced by 0.2% L-arabinose (Sigma) when an OD_{600nm} ~0.9 was obtained.

Ideally, the recombinant protein should be expressed in a host which lacks the ability to metabolise arabinose. In a previous study (Blackburn, 2000), P.30 and linker region was expressed in *E. coli* strain LMG194 (*ara*$^-$). The outer-membrane profile of the *E. coli* LMG194 expressing the recombinant protein showed two prominent bands of about 14 and 16 kDa size and it was the bigger band which reacted with the monoclonal anti-PRN linker antibody (BBO5) (Table 2.8) These two polypeptides may be due to processing of the precursor (C-terminal + linker region) at two different sites and the

additional 2 kDa protein in the ~16 kDa represents the immunodominant region of the molecule, which actually reacted with the monoclonal antibody (BBO5) (Blackburn, 2000). The *E. coli* strains used in this study i.e. KS272 parent and HM130, the most protease-deficient strain, showed some arabinose utilising activity as determined by the API20E kit, but the arabinose concentration (0.2%) used gave reasonable expression of the recombinant proteins in these strains.

Results in Fig. 3.50a show the protein profiles of whole-cell fractions of the wild-type and most protease-deficient strains expressing linker region and P.30 domain of *prn*, obtained by SDS-PAGE. Lane A represents the non-induced wild-type *E. coli* strain (KS272), whereas the lanes B and C show the induced parent and the most protease-deficient *E. coli* strain (HM130). There is a clear difference seen between the lane A and lanes B and C at 30 kDa which is the predicted size of the PRN C-terminal domain. There is a minor difference at ~45 kDa which is presumably the un-processed precursor protein (linker + C-terminal). The corresponding immunoblot (Fig. 3.50b) was developed with a monoclonal antibody (BBO5) raised to the PRN-linker region and kindly provided by Prof. M. Roberts, Department of Veterinary Pathology, University of Glasgow. The immunoblot shows reactions at ~45 kDa and ~15 kDa. As the antibody can detect the linker region only, it has reacted with the intact ~45 kDa PRN molecule in both the lanes B (parent) and C (most protease-deficient strain). There is another reaction seen only in lanes B and C at ~15 kDa and that is reproducible and is clearer in the outer-membrane preparation of these strains (Fig. 3.51). There appears to be three bands reacting with the monoclonal anti-PRN linker antibody presumably due to some abnormal processing or protein degradation. These results indicated that cleavage of the expressed protein (45 kDa) had occurred in both parent and most protease-deficient *E. coli* strains.

Fig. 3.49: Shows the restriction map of the plasmid pBAD/gIII (linker region of *prn* and its C-terminal insert (1.1 Kb) cloned into the vector (4.1 Kb)

The expression of the pertactin C-terminal and linker region is under the tight the tight regulation of the arabinose promoter (*araC*). It possesses a ColE1 origin of replication and ampicillin resistance marker. The vector is provided with a signal sequence (gIII) which is capable of transporting proteins via the Sec-dependent pathway into the periplasm. The insert was ligated into it as *Sac*I and *EcoR*I fragment.

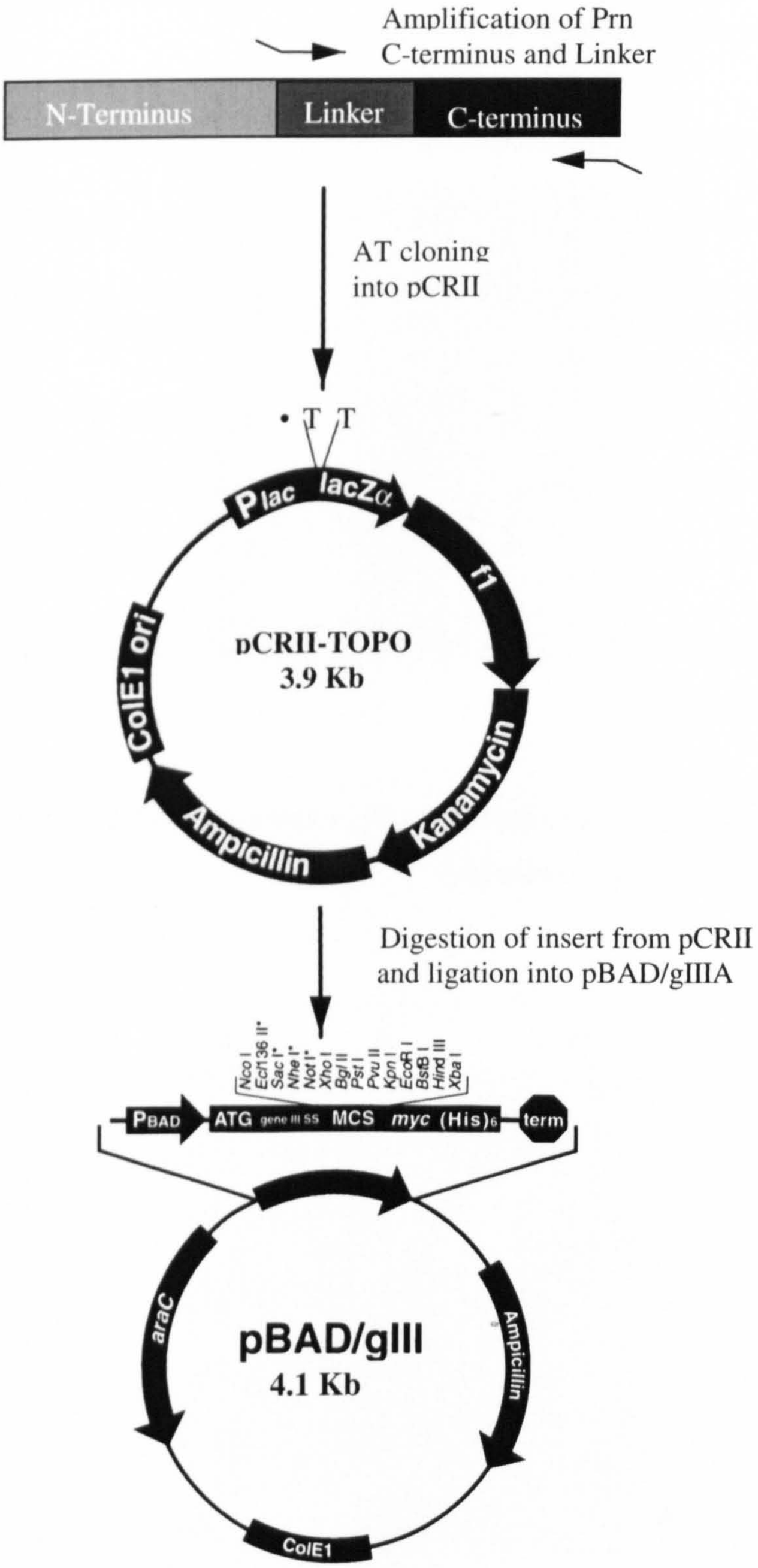

Amplification of Prn
C-terminus and Linker
N-Terminus
Linker
C-terminus
AT cloning
into pCRII
T T
Plac
lacZα
f1
pCRII-TOPO
3.9 Kb
ColE1 ori
Ampicillin
Kanamycin
Digestion of insert from pCRII
and ligation into pBAD/gIIIA
Nco I
Ech136 II*
Sac I*
Nhe I*
Not I*
Xho I
Bgl II
Pst I
Pvu II
Kpn I
EcoR I
BstB I
Hind III
Xba I
PBAD
ATG gene III SS MCS myc (His)6 term
araC
pBAD/gIII
4.1 Kb
Ampicillin
ColE1

Fig. 3.50: Protein profile and its corresponding immunoblot showing the whole cell fractions of *E. coli* parent and most protease-deficient strains expressing the pertactin C-terminal and linker region

The figure shows the whole-cell profile by the SDS-PAGE (12%) of different *E. coli* strains grown in LB medium with and without arabinose induction. Fig. 3.50b shows an immunoblot of the Fig 3.50a obtained with monoclonal anti-PRN-linker antibody (BBO5) (Table 2.7).

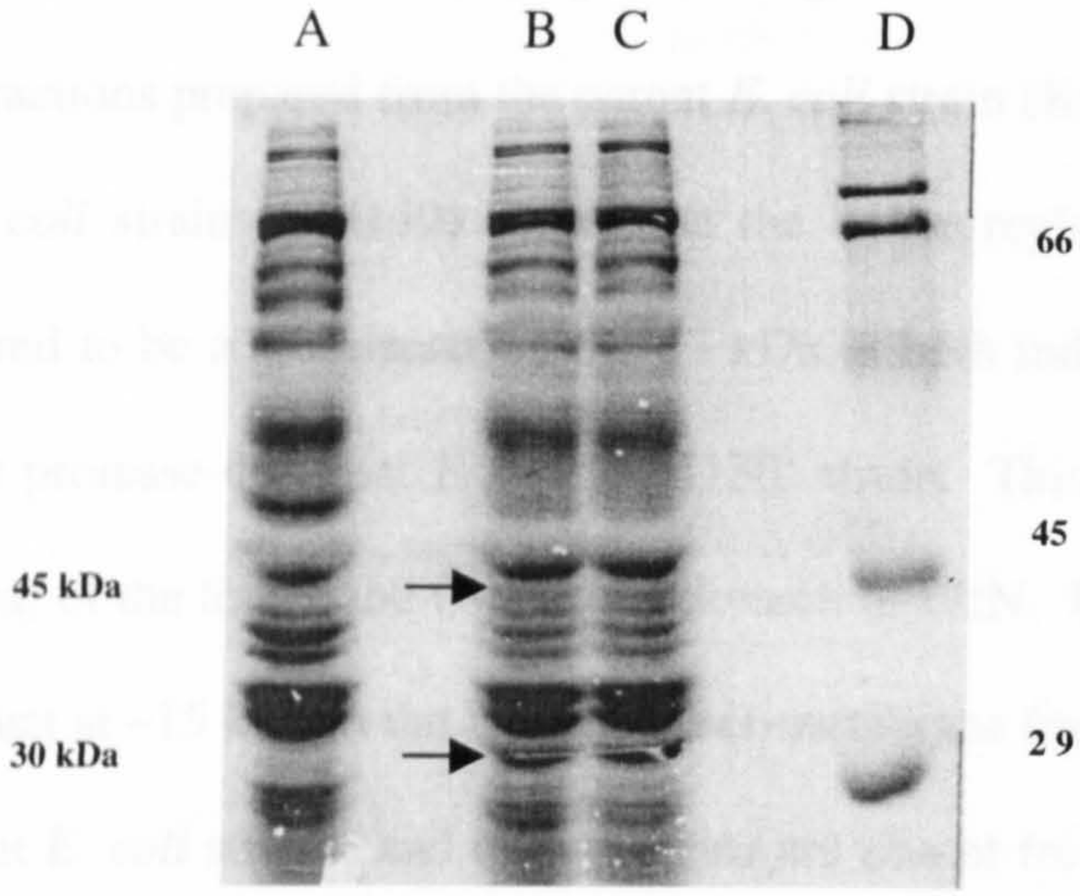
A = *E. coli,* parent strain (KS272), expressing PRN C-terminus and linker region (non-induced)

B = *E. coli,* parent strain (KS272), expressing PRN C-terminus and linker region (induced with L-arabinose)

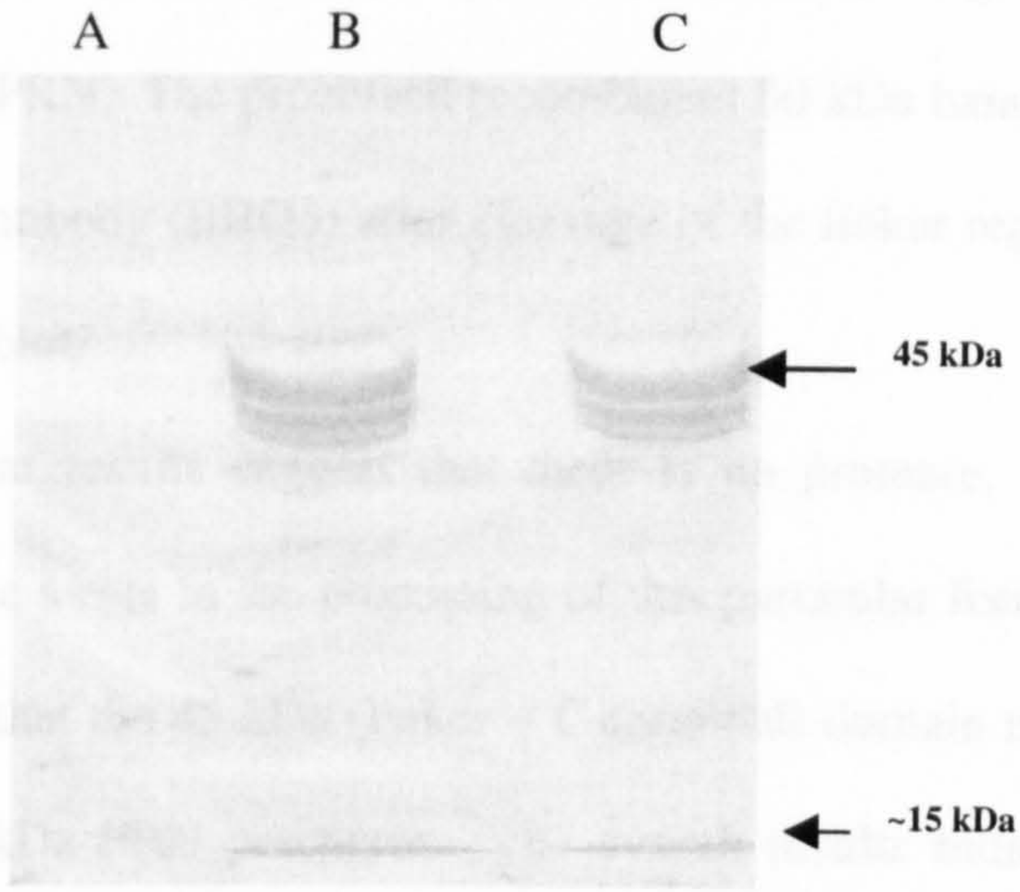
C = *E. coli,* most protease-deficient strain (HM130), expressing PRN C-terminus and linker region (induced with L-arabinose)

D = Protein ladder

3.50a



3.50b



To further elucidate the processing of the PRN C-terminus and its linker region into ~30 kDa C-terminal domain and the ~15 kDa linker polypeptide, the outer-membrane preparations of both strains were prepared. Fig. 3.51 shows an immunoblot of outer-membrane fractions prepared from the parent *E. coli* strain (KS272) and the most protease-deficient *E. coli* strain (HM130) expressing the linker region and P.30 domain of *prn*. There appeared to be a cross-reaction at ~37 kDa in both induced *E. coli* parent (KS272) and the most protease-deficient *E. coli* (HM130) strain. This was not quite the predicted size (~45 kDa) of the linker and C-terminal domain of PRN. However, there seemed to be another product at ~15 kDa in the induced outer-membrane fractions of both the parent and most deficient *E. coli* strains, and both proteins are absent from the non-induced fractions of both the strains. This result was reproducible and the ~15 kDa band was presumably the processed linker region that, after being cleaved from the C-terminal domain seemed to be associated with the outer-membrane of both *E. coli* strains. The 15 kDa band was associated with the outer-membrane surface after cleavage, although it may behave differently in this respect than the mature ~69 kDa PRN molecule generated by cleavage of the 93 kDa precursor PRN. The processed recombinant 30 kDa band would not react with the anti-PRN linker antibody (BBO5) after cleavage of the linker region and would not be visible in the immunoblot.

These particular results suggest that there is no protease, amongst those under investigation, that have a role in the processing of this particular form of PRN. However, there is the provision that the 45 kDa (linker + C-terminal) domain may not behave in the same way as the 93 kDa PRN precursor. The overall results indicate that none of the proteases lacking in the most protease-deficient strain HM130 were involved in processing of PRN and they do not support the conclusions of the work presented in the previous section.

Fig. 3.51: Immunoblot of the OMP fractions of the *E. coli* parent and most protease-deficient strain expressing the linker+C-terminal region (~43kDa) of pertactin

The outer membrane-enriched fractions were prepared from the parent and most protease-deficient strain grown under inducing and non-inducing conditions, separated on 10% SDS-PAGE and screened using monoclonal anti-PRN-linker antibody (BBO5, Table 2.7).

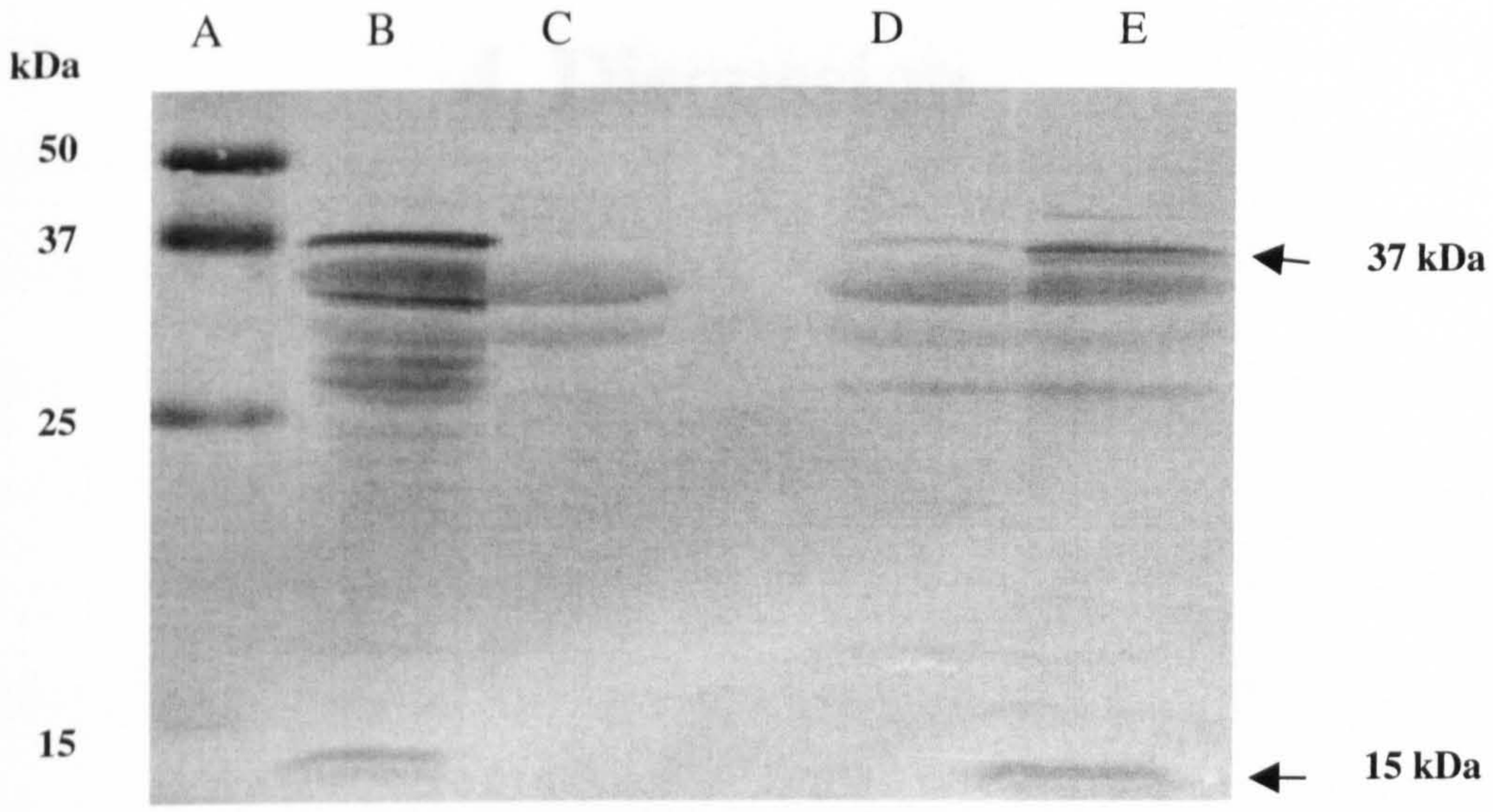
A = Pre-stained Protein ladder with molecular weights shown on the left

B = Outer membrane fraction prepared from *E. coli* parent strain (KS272) expressing PRN C-terminus and linker region (induced with L-arabinose)

C = Outer membrane fraction prepared from *E. coli* parent strain (KS272) expressing PRN C-terminus and linker region (non-induced)

D = Outer membrane fraction prepared from *E. coli* most protease-deficient strain (HM130) expressing PRN C-terminus and linker region (non-induced)

E = Outer membrane fraction prepared from E. coli most protease-deficient strain (HM130) expressing PRN C-terminus and linker region (induced with L-arabinose)


A
B
C
D
E
kDa
50
37
25
15
37 kDa
15 kDa

4. Discussion

4.1: Overview

The main objective of any research on *B. pertussis* in recent years has been to identify the nature of its interaction with its host during infection. This can be achieved by identifying the bacterial surface components and the host tropism and the host response towards the pathogen during the disease process. The candidate genes thought to be involved in pathogenesis, once identified, can then be mutated to generate a *B. pertussis* strain deficient in expression of the putative virulence-associated factor. The mutated strain can then be tested for virulence by comparison with its wild-type *B. pertussis* strain.

Bordetella autotransporter-five gene (*bap-5*; Appendix II) was identified initially due to a misprimed PCR reaction designed for amplification of the *prn* gene from the *B. pertussis* genome (section 1.8.1). A search of the databases has shown that homologues to the *bap-5* gene also exist in other *Bordetella* species i.e. *B. bronchiseptica*, *B. parapertussis* and *B. avium* (Blackburn, 2000). In this study, the *bap-5* gene has been shown by RT-PCR as well as by immunoblotting with a rabbit anti-$_r$Bap-5 serum, to be expressed in *B. pertussis*. RT-PCR results have also suggested that *bap-5* expression is regulated by the two component regulatory system *bvgAS*. *B. pertussis bap-5* mutants of Taberman and Tohama wild-type strains (Table 1.4) were constructed successfully by insertion of a kanamycin-resistance cassette. *B. pertussis* Taberman I and its derived Taberman II *bap-5* defective mutant were selected for further characterisation studies of *bap-5*. SDS-PAGE profiles, immunoblots and RT-PCR results confirmed the absence of expression of *bap-5* in *B. pertussis* Taberman II, the *bap-5* mutant.

The structural similarities between Bap-5 (the product of *bap-5*) and other already well-characterised *B. pertussis* autotransporter proteins i.e. PRN, TCF and BrkA (Fig 1.3) suggested that the Bap-5 protein could perhaps function as an adhesin or a serum resistance factor. The results suggested that Bap-5 behaves like BrkA (serum resistance factor) in *B.*

pertussis (Taberman I) i.e. it confers resistance on *B. pertussis* to killing by human serum mainly by the classical pathway. The mouse virulence tests (section 3.3) suggested that Bap-5 aids *B. pertussis* in colonising the lower respiratory tract of the animal. The results of *in vitro* adhesion studies were less clear but there was some suggestive evidence that Bap-5 contributes to *B. pertussis* adherence to certain cell types.

A mouse protection study (section 3.4) was carried out with the re-natured PRN autotransporter domain (P.30), as a previous result with the denatured P.30 showed that it had no protective capacity in a murine model (Blackburn, 2000). The re-natured P.30 used in a mouse protection study did not prove to be a protective antigen against respiratory tract colonisation of *B. pertussis*. It would have been of interest to test the processed 30 kDa C-terminal and mature forms of Bap-5 as a protective antigen but there was insufficient time to clone the required gene sequences in an expression vector in order to obtain enough protein for immunisation studies.

There is little information in the literature regarding the processing of the autotransporter proteins of *B. pertussis*. A study was carried out to investigate this using the well-characterised *B. pertussis* autotransporter, PRN. The PRN precursor (P.93) is processed into a mature passenger domain (P.69) and a C-terminal pore forming domain (P.30). There appears to be no information as to whether PRN has an inherent autoproteolytic activity to produce the above mentioned two final processed products or whether some unknown envelope-associated protease activity is required. The processing of PRN was studied in a set of *E. coli* strains (section 3.5) which were deficient in known envelope-associated or periplasmic proteases.

4.2: *B. pertussis* genome sequence and *bap-5*

Recently, the sequencing of the *B. pertussis* Tohama I genome has been completed, although the published annotated version is still awaited, whereas genome sequencing of *B. bronchseptica* and *B. parapertussis* is close to completion. A comparative study between these three closely-related species of the genus *Bordetella* may reveal why such closely related organisms exhibit different host ranges and produce different disease symptoms.

The presence of *bap-5* (1.8.5; Appendix II) in the genomes of *B. pertussis* and *B. bronchiseptica* was confirmed from the genome sequences. *B. bronchiseptica bap-5* gene shows close homology to that of its *B. pertussis* counterpart. The sequence in the *B. pertussis* Tohama I strain sequenced by the Sanger centre has 100% nucleotide identity to the *bap-5* nucleotide sequence obtained for the Taberman strain of *B. pertussis* (Blackburn, 2000) using the BLAST search engine at http://www.Sanger.ac.uk (BLAST search results). The sequence analysis of the *bap-5* region has shown that it does not possess a typical Shine-Delgarno sequence (Shine and Delgarno, 1975) in the 5' region of its proposed open reading frame, like other known autotransporter genes. However, the genome sequence search during this study showed the existence of two potential BvgA dimer binding sites TTTC(A/G)TA (section 1.5) upstream from the predicted translational start site of *bap-5* (ATG 1) (Appendix III), which are similar to a characteristic heptameric sequence TTTC(C/T)TA (Marques and Carbonetti, 1997; Kinnear *et al.*, 1999). Similar repeats are located upstream of the Bvg-regulated *prn, fha, ptx, cya* and *bvg* genes of *B. pertussis*. The BvgA dimer is required for regulating the transcription of these genes through its interaction with the RNA polymerase. Three classes of *bvg*-activated promoters have been reported in *B. pertussis* which are categorised on the basis of their temporal activation after an inducing signal. The early class is represented by *fha* and *bvg* and the late class is represented by *ptx* and *cya*, whereas *prn* promoter activation is thought to occur at the time

between *fha* and *ptx* and hence it constitutes an intermediate class of *bvg*-activated promoter (Kinnear *et al.*, 1999). The distance of the putative primary BvgA binding site upstream from the promoter regions perhaps determines the affinity of BvgA interaction with RNA polymerase for the primary binding sites and hence the kinetics of these promoters (Kinnear *et al.*, 1999). The promoter region of *bap-5* could be analysed to determine which class it belongs to by determining at what stage it begins to be transcribed compared with the other *vag* genes. This could be done by an RT-PCR method similar to that described earlier by preparing RNA samples at intervals after switching the culture from Bvg^- to Bvg^+ phase. Alternatively, RNA preparations could be separated by electrophoresis in a formaldehyde agarose gel followed by transfer of the RNA to a nylon membrane and detecting the relevant RNA by hybridisation with a labelled probe. These probes could be prepared by using Dig-labelled dNTPs in PCR reaction or by a random Dig-labelling method. The preliminary work of designing the primer sets for the housekeeping gene i.e. s*odB* as a control and other *bvg*-regulated genes like *fha* (early *bvg*-class) and *cyaA* (late *bvg*-class) was done but, due to insufficient time, this work could not be continued. Alternatively, fusion of the promoter region of *bap-5* to a reporter function such as GFP or Lux would allow the time of promoter-activation to be determined.

There appears to be a *rho*-independent terminator sequence downstream of the *bap-5* gene (Appendix II, shown in green) as with *prn* (Charles *et al.*, 1989), with the exception that it is further from the translational stop codon and smaller than that of *prn*. The secondary structure formed by this *rho*-independent terminator adopts the shape of a hairpin or loop and presumably is responsible for RNA polymerase pausing and thereafter preventing mRNA synthesis beyond the point of palindrome formation. There are some common features shared by the *bap-5* gene product with other *B. pertussis* autotransporter members (section 1.8) such as an integrin-binding motif (RGD), a glycosaminoglycan-

binding site (SGXG), a proteolytic processing site (ALSKRLGEL) and an outer membrane localisation signal which ends at a cyclic amino-acid like other autotransporters in Gram-negative bacteria (FHLGYRYTW) (Appendix II, shown in yellow).

Some other novel *B. pertussis* putative autotransporters, Bap-6 and Bap-9 (Blackburn, 2000) and Phg (Antoine *et al.*, 2000), have been identified in the genome sequence which need to be characterised. For example, the latter is deposited in Genbank as a cold shock protein of *B. pertussis*. There are also some recent reports about other possible autotransporters in *B. pertussis*, which are mentioned in section 1.8.5.

4.3: Expression of *bap-5* and construction of *bap-5*-defective mutant

The expression of *bap-5* was confirmed by RT-PCR results which produced the products of predicted size (section 3.1). The results also suggested the existence of some other smaller transcript in the Taberman I (wild-type) which could be the product of non-specific primer-binding or there could be another promoter to regulate the production of this smaller transcript. There is also the possibility of the involvement of some other *bap-5* homologue in *B. pertussis*. The RT-PCR and Western blotting results suggested that the expression of Bap-5 is under *bvg*-regulation like other *B. pertussis* autotransporter proteins such as PRN and BrkA.

The *bap-5*-defective mutant of *B. pertussis* was created using the suicide vector pSS11229 (Stibitz, 1989) for gene replacement. This vector was previously used for creating *fhaB*-defective and *bvgA*-defective mutants in *B. pertussis*. The ligation of the mutated (with kanamycin-resistance cassette) *bap-5* gene into the pSS11229 vector proved to be a difficult task perhaps due to the high GC-content of the *bap-5* gene. The Nal and Sm spontaneous antibiotic resistant strain of *B. pertussis* proved to be valuable selection markers for selecting the mutant strains of *B. pertussis* and also for inhibiting the growth of

the undesired DNA-mobilising *E. coli* strain SM10.λ_{PIR}. PCR and Southern blotting results showed the successful incorporation of the mutated *bap-5* into the chromosome of *B. pertussis* strains of Taberman and Tohama. However, only Taberman I (wild-type) strain and its *bap-5*-defective mutant were selected for the characterisation studies because the *bap-5* mutant of Tohama seemed to have lost its virulence properties, as suggested by its non-haemolytic nature and growth kinetics. However, it would be worth trying to create *bap-5* mutants of other *B. pertussis* strains and of other *Bordetella* species.

4.4 Characterisation of Bap-5

Bap-5, the product of the *bap-5* gene (Appendix II), is predicted to be a protein of ~79.9 kDa. However, it appears to run anomalously on SDS-PAGE, at ~90 kDa, and thus shows some similarity to PRN (predicted size 91 kDa, apparent size 93 kDa). Like BrkA, but unlike other *B. pertussis* autotransporter proteins such as PRN, Bap-5 does not possess a typical amino-terminal signal sequence upstream from its putative translational start site. It can be speculated that another protein, perhaps BrkB (section 1.8.3) or an analogous protein, may be involved in aiding Bap-5 translocation across the inner membrane. However, there are many membrane proteins that lack discrete signal peptides but their first transmembrane segments can act as targeting signals (Friedlander and Blobel, 1985).

The processing of Bap-5 at the putative cleavage site (ALSKRLGEL) would lead to a mature product of 49.9 kDa and a 30 kDa C-terminal domain in the outer membrane. Unfortunately, although a rabbit antiserum was raised against the whole precursor Bap-5 molecule cloned and expressed in *E. coli*, it did not seem to react with its C-terminal moiety. The putative ~30 kDa, β barrel-forming domain of Bap-5 would be expected to be present in the outer membrane, but it was not possible to detect it either in whole-cells or in outer membrane-enriched fractions using the rabbit anti-$_r$Bap-5 serum. It is possible that

this domain did not have immunodominant epitopes and hence was not recognised by the anti-$_r$Bap-5 serum. It can also be speculated that antiserum was raised against SDS-denatured Bap-5 and so this could have reduced its immunogenicity. However, the anti-$_r$Bap-5 serum reacted with the precursor Bap-5 whose expected size is 79.9 kDa from the predicted translational site but ran at ~90 kDa in whole cell fractions and in outer membrane-enriched fractions of *B. pertussis* Taberman I. It also reacted with a band at ~65 kDa which is presumably the mature Bap-5 that again, runs at a higher molecular weight than the expected size (49.9 kDa). This anomaly again can be compared with the anomalous mobility on SDS-PAGE of PRN, whose processed passenger domain is actually 61 kDa, but runs at 69 kDa on SDS-PAGE. The difference between the predicted and apparent size of the mature Bap-5 i.e. ~16 kDa, appears to be much higher than in the case of PRN (~8 kDa). A possible explanation for this size discrepancy may be that Bap-5 is translated further from the first ATG site towards the 3', at one of two other putative start sites to produce a ~65 kDa precursor protein (Appendix II), as the true translational start site of *bap-5* is yet to be confirmed.

The outer membrane preparations (Fig. 3.20, 3.21) showed a very clear and significant difference between *B. pertussis* Taberman I and its derived Taberman II the *bap-5* mutant i.e. the presence of a ~16 kDa band in the parent Taberman I strain of *B. pertussis,* in addition to the above described differences. There seems to be a precedent in the literature with another outer membrane associated protein Ail encoded by the chromosomal gene *ail* in *Yersinia enterocolitica,* which is 17 kDa protein (Miller *et al.*, 1990). Ail is predicted to form eight membrane-spanning amphipthic β-strands and four extracellular loops (Miller *et al.*, 1990) and, coincidentally it is known to play a role in serum resistance (like Bap-5) and invasion (Miller *at al.*, 2001). Recombinant Ail has been shown to confer a high level of serum resistance to *E. coli* (Bilska and Falkow, 1992;

Pierson and Falkow, 1993). Sequencing of the ~16 kDa band present in the outer-membrane-enriched fraction of *B. pertussis* Taberman I could generate further information about the processing of Bap-5 (~79.9 kDa) but there is the possibility of some other, smaller transcript within the larger *bap-5* ORF, which encodes the ~16 kDa outer membrane protein. Alternatively it may be cleaved further away from the putative predicted cleavage side towards the C-terminal to produce a ~16 kDa C-terminal domain and a mature ~65.5 kDa passenger domain.

The rabbit anti-$_r$Bap-5 serum also cross-reacted to some extent with the BrkA protein (~73 kDa) in the outer membrane-enriched fractions of *B. pertussis.* This may be due to the existence of common immunodominant epitopes. The work also indicated that precursor BrkA (103 kDa) seemed to be processed efficiently from its C-terminal domain (30 kDa) into a mature 73 kDa form that remained surface bound. This surface localisation is perhaps due to some non-covalent interaction with its C-terminal domain or with other surface components of *B. pertussis,* as seen in the case of the AIDA-I autotransporter protein of *E. coli* (Benz and Schmidt, 1989).

4.4.1 Bap-5 as a serum-resistance factor

B. pertussis appears to thrive well in its host by employing a range of surface associated or released virulence factors to overcome, delay or evade the immune effector mechanisms deployed against it. Although *B. pertussis* is a mucosal pathogen, it can still be exposed to levels of antibody and complement which in the respiratory tract are about 20% of what is present in the normal human serum due to plasma exudation from the underlying capillaries. These levels may be further increased during inflammation (Persson, 1991; Barnes and Weiss, 2001). Like *Vibrio cholerae,* another mucosal pathogen, *B. pertussis* appears to have mechanisms in place to resist complement (Weiss *et*

al., 1999). It was seen in the case of *V. cholerae* that immunity to cholera did not correlate with the immune responses to cholera toxin. The vibriocidal assay, which reflects the ability of the serum to kill the *V. cholerae* by antibody-mediated complement fixation, has been correlated with immunity to cholera as well as immunity from asymptomatic colonisation (Glass *et al.*, 1985).

The complement cascade can be activated by either carbohydrates on bacterial surfaces (such as lipopolysaccharide, LPS), that can activate the alternate pathway (Taylor, 1992; Moffit and Frank, 1994) or by the deposition of antibody on the bacterial surface which can activate the classical or primarily antibody-dependent pathway of the complement system (section 1.9, Fig 1.6). Thus Complement is part of the innate immune defences, and provides a defence against pathogens that have not previously infected the host by recognising repeating structures such as lipopolysaccharide (LPS) and other components found on the surface of bacteria. It is also a part of the inducible defences activated by antibodies.

This study has suggested a role for Bap-5 in *B. pertussis* Taberman I in resisting complement-mediated killing. Bap-5 was shown to confer a considerable resistance on *B. pertussis* Taberman I to killing by normal human serum compared to its Bap-5-deficient mutant (***P*<0.05**) (section 3.2.4). Moreover, it was observed that Bap-5 protects *B. pertussis* mainly from the classical (antibody-dependent) pathway of complement activation.

However, some preliminary data also suggested that Bap-5 may have a role in providing some resistance to *B. pertussis* Taberman I against the alternative (antibody-independent) pathway of complement activation (section 3.2.5, Fig 3.29). The result presented in Fig 3.29 showed that the *bap-5* mutant is sensitive to killing by complement alone (<60 % survival) compared to the wild-type Taberman I parent strain (91 %

survival). However, the results with modulated (Phase IV) strains (lanes G and H) showed that some other functions may also contribute to resistance to complement-mediated killing because these strains were hugely sensitive to killing (<10 % survival) by guinea pig serum (source of complement). The *brkA* mutant BP2041 strain (87% survival) was not sensitive to complement alone like its wild-type strain BP338 (92% survival). There was some discrepancy in this result as the Fig. 30 showed that the sensitivity to complement alone can be inhibited by the addition of EGTA.$MgCl_2$ as shown in lanes B, G and H. This suggests that complement killing is Ca^+ dependent and therefore due to the classical pathway or MBL pathway. Even with the addition of EGTA.$MgCl_2$ to guinea pig serum with *bap-5* mutant there was still some residual killing effect (~70 % survival). The modulated strains were slightly better survivors (~77%), which was reproducible but unexpected result. However, further investigation would be required to determine which arm of the complement pathways Bap-5 mediates protection against. Further investigation, perhaps by blocking or inhibiting the key components of these various pathways (section 1.9) might give a clearer picture of the actual mechanisms involved.

The presence of BrkA (serum resistance factor) inhibits deposition of C4, C3 and C9 and production of soluble membrane attack complex (section 1.9, Fig 1.6). In contrast, BrkA does not affect the deposition of C1, suggesting that BrkA possibly acts before C4 (second component of the classical pathway to be activated) deposition. It is proposed that BrkA inhibits the activation or promotes degradation of C4 after its deposition on the bacterial surface (Barnes and Weiss, 2001). The actual molecular mechanism by which BrkA inhibits complement has not been determined but in theory it could adopt one of the following routes as suggested by Barnes and Weiss, (2001): BrkA could prevent complement functions by acting as a protease; It could recruit complement-inhibitory soluble C4 binding protein or membrane-bound cofactors CD46 and CR1; It could recruit

host protectin (CD59), another complement inhibitor, which is usually considered to be bound by the host membrane, as seen in the case of *E. coli* (Rautemmaa *et al.*, 1998).

The mechanism of action of Bap-5 was not examined, but there is a possibility that Bap-5 may affect/prevent the deposition of C1 in the first instance, a role which BrkA has not been suggested to play (Barnes and Weiss, 2001). In this way it could possibly prevent killing of *B. pertussis* by the classical-pathway of complement-mediated killing and also by the alternative pathway of complement-mediated killing, as spontaneous deposition of C1 on the bacterial surface can initiate the formation of membrane-linked terminal lytic complex C5b-9 on the bacterial surface.

As this study has suggested that Bap-5 played a role in protecting *B. pertussis* from killing by the complement source (guinea pig serum), there is a possibility that it could recruit some host protein, such as vitronectin or S protein (section 1.9, Fig 1.6), which, after linking with the C5b-9 complex forms the fluid phase SC5b-9 complex. It has been proposed that vitronectin combines with fluid-phase terminal lytic complex at the stage of C5b-7 and this can lead to the formation of less destructive SC5b-9 with regard to its membrane insertion into bacteria (Mills *et al.*, 1993).

One possible line of investigation would be to restore the ability of the Bap-5-deficient mutant to resist killing by serum or complement by allowing the spontaneous deposition of purified recombinant Bap-5 protein on its surface and then carrying out serum killing assays on this strain along with parent and untreated *bap-5* mutant strain. A similar experiment was done using a purified preparation of BrkA with a *brkA* mutant and the outcome of the experiment confirmed the role of BrkA in serum resistance (Oliver and Fernandez, 2002).

It was interesting to note that in both the BrkA-deficient and Bap-5-deficient mutants, there remained a considerable amount of residual serum resistance whereas

almost greater sensitivity was exhibited by the Bvg-modulated parent strain of Bap-5 Taberman I (section 3.2.4). The interpretation of this result could be that both BrkA and Bap-5 contribute to serum resistance and, when neither is expressed, under modulating conditions, the bacterium is highly serum sensitive. This hypothesis could be tested by the creation of a BrkA and Bap-5 double mutant construct, which should show full serum sensitivity. PCR results with chromosomal DNA prepared from BP338 and its *brkA* mutant strain BP2041 confirmed the presence of the *bap-5* gene and a streptomycin spontaneous resistant mutant was created (Sm^R) for BP2041. An attempt was made to make a *bap-5, brkA* double mutant of BP338 in the *brkA* mutant BP2041 background but, unfortunately, the first few conjugations between *E. coli* SM10.λ_{PIR} carrying the mutated *bap-5* gene cloned in the suicide vector pBap5-Kana and *B. pertussis* BP2041 did not yield transconjugants. Due to time constraints, this line of investigation was not continued.

The restoration of resistance to serum killing by putting back the wild-type copy of the *bap-5* gene on a plasmid into the *bap-5* mutant Taberman II strain of *B. pertussis* would also be a step forward in order to confirm the phenotype conferred by *bap-5*. Another investigation would be to study serum resistance in *E. coli* by expressing recombinant Bap-5. The agglutination test with anti-$_r$Bap-5 serum using *E. coli* M15 expressing Bap-5 (pQE60) suggested that it is surface exposed in this organism and its serum-resistance potential of this *E. coli* should be compared with that of the *E. coli* containing vector alone.

A previous study has suggested that BrkA is not involved in conferring resistance to *B. bronchiseptica* against serum killing (Rambow *et al.*, 1998), so it would be interesting to determine the role, if any, of Bap-5 in *B. bronchiseptica* and other bordetellae in this regard. The presence of the smooth LPS in *B. bronchiseptica* has been correlated with the resistance to killing *in vitro* by antibody and complement (Byrd *et al.*, 1991) and anti-

microbial peptides (Baneman *et al.*, 1998). The LPS of *B. pertussis* was reported to have a simple structure consisting of lipid A, core polysaccharide and a single O-chain trisaccharide (Lebbar *et al.*, 1994; Allen *et al.*, 1998). *B. pertussis* lacking the oligosaccharide side chains was found to be more sensitive to these agents (Preston and Maskell, 2002). However many new genes and some unexpected genes (e.g. the capsule biosynthesis locus) have been identified with the completion of the genome sequence of *B. pertussis,* which may lead to a new outlook on the organism and the manner in which it interacts with the innate immune system (Preston and Maskell, 2002).

Another area to look for a complement resistance mechanism is to explore the possible involvement of the antibody-independent route of the classical pathway i.e. the lectin pathway, in *B. pertussis* killing. There is evidence that a deficiency of mannan-binding lectin (MBL) is associated with an increased susceptibility to bacterial infection in mice as it reduces the efficiency of the lectin-pathway of complement-mediated killing mechanism (section 1.9; Fig 1.6) (Turner, 1996). MBL binds to carbohydrate surface structures (Ikeda *et al.*, 1987) on bacteria, yeast, parasitic protozoa and viruses (Turner, 1996) and is structurally similar to C1q and is able to activate complement via associated serine proteases (MASP-1 and MASP-2) (Matsushita and Fujita, 1992). Killing is mediated by the terminal lytic complement pathway or possibly by increased phagocytosis. Thus complement and MBL have an important role in innate immunity through both opsonisation and alternative complement pathway activation as is the case with *N. meningitidis.* Similar mechanisms may be operative in *B. pertussis* via Bap-5 as it may be involved in preventing the initial deposition of MBL protein on the surface of *B. pertussis.*

In the light of the outcome of this study, the most probable strategies employed by *B. pertussis* to evade host defences can be updated, with the inclusion of defence strategies

against the classical pathway of complement-mediated killing (BrkA and Bap-5) and possibly the alternative pathway of complement-mediated killing (Bap-5) (Table 4.1).

4.4.2 Anti-Bap-5 serum and its bactericidal effect on *B. pertussis*

Understanding the role of antibody-mediated immunity against intracellular and extracellular pathogens is important for fundamental immunology, vaccine design and development of immunotherapy.

Surface-located components of bacteria could either mediate serum resistance by blocking complement killing or act as targets for bactericidal antibodies. Antibodies to surface antigens play a role in protection against *B. pertussis*. Antibodies may act either by neutralising bacterial toxins, inhibiting the extracellular bacterial binding to the cells in mucosal tracts or by facilitating bacterial uptake and destruction by macrophages and neutrophils. It is presumed that anti-LPS antibodies are the major antibodies responsible for bactericidal activity against *B. pertussis*, as they can activate the classical pathway of complement and they have been detected in humans and mice (Weiss *et al.*, 1999). *B. pertussis* is known to express two forms of LPS: band A, consisting of lipid A, core and O-chain (LOS) and band B, a partial structure consisting of lipid A and core without O-chain. In other words, unlike the highly polymerised LPS of the enteric bacteria, the LPS of *B. pertussis* does not seem to protect it from complement-mediated killing.

B. pertussis infection results in humoral and cell-mediated immunity that is largely influenced by the polarised Th1 response (Ryan *et al.*, 1997; Mills *et al.*, 1998). Th1 responses (via cytokines, especially IFN-γ) can also stimulate the production of opsonising antibody and complement-fixing IgG antibody subtypes e.g. IgG3. Th1 responses generated either by *B. pertussis* infection or by vaccination with the whole-cell pertussis vaccine have been shown to correlate with increased clearance of *B. pertussis* (Mills *et al.*,

Table 4.1: Probable evasion strategies employed by *B. pertussis*

Immune effector mechanisms	**Probable evasion strategy**	**References**
Muco-ciliary clearance	Attachment colonization factors (FHA, PRN,TCF, BrkA, Bap-5), destruction of ciliated cells by tracheal cytotoxin	Tuomanen and Weiss, 1985. Luker *et al.*, 1993. This study
Complement (classical, possibly alternative)	BrkA , BrkB and Bap-5	Fernandez and Weiss, 1994. Fernandez and Weiss,1998. This study
Phagocyte function	Avoidance of respiratory burst, neutrophil intoxication FHA, PTX, ACT.	Cundell *et al.*, 1994. Pearson *et al.*, 1987. Meade *et al.*, 1985. McGuirk *et al.*, 1998.
B cells, antibody	Intracellular niche, PT mediated-immunomodulation, switching from more severe to less protective arm of the immune response	Saukonen *et al.*, 1991. Masure, 1992.
Cell-mediated immunity	Altered co-stimulation, migration and antigen presentation FHA, PTX	McGuirk *et al.*, 1998. Spangrude *et al.*, 1985.

Data has been taken from Mahon and Mills (1999) and modified

1998). Bactericidal activity of the serum from adults exposed to *B. pertussis* or immunised with Pa (acellular pertussis vaccine) correlated with the presence of IgG3 antibodies to LPS, the IgG subclass which is most efficient at fixing complement. Natural infection or immunisation with Pw (whole-cell pertussis vaccine) induces a significant protection in mice via Th1 cells and mostly IgG2a antibodies. The Pa, which is not as protective as Pw, induces Th2 cells and low levels of IgG2a antibodies (Mahon *et al.*, 1996; Mills *et al.*, 1998). However, Pa induces high levels of IgG1 in the mouse and this subclass may be important in neutralising toxins and inhibiting bacterial adherence to the ciliated epithelial cells in the respiratory tract (Mills *et al.*, 1998). Among the antibody classes, IgM is known to activate complement very well, whereas IgA is not effective. In humans, IgG3 activates complement better than IgG1, which in turn is better than IgG2. IgG4 does not activate complement.

A previous study showed that neither the antibodies to pertussis toxin or FHA nor immunisation with vaccines containing these antigens were correlated with bactericidal activity (Cherry *et al.*, 1998; Storsaeter *et al.*, 1998). This is most likely due to the secreted nature of pertussis toxin or the loosely surface-bound FHA antigen, which make them inappropriate targets for bactericidal antibodies. Surface-association is a property that is required for directing the membrane attack complex to the bacterial membrane. It was reported that the monoclonal antibodies to band A LPS (LOS) have a bactericidal effect and that passive immunisation with anti-LPS antibodies from the sera of individuals convalescing from natural infection (Shahin *et al.*, 1994; Weiss *et al.*, 1999), or with monoclonal antibodies to LOS, resulted in increased clearance of *B. pertussis* from the mouse lungs following an aerosol challenge (Mountzouras *et al.*, 1992).

The immunisation of mice with PRN (P.69) resulted in reduced colonisation of the lungs after subsequent challenge and the antibodies produced in response to P.69 were

shown to be bactericidal (Gotto *et al.*, 1993). More recently it has been shown that anti-BrkA serum can augment the killing of *B. pertussis* when added to human serum (Oliver and Fernandez, 2002).

The present study has suggested that antiserum raised against the Bap-5 precursor can not only recognise the surface-exposed antigen but like anti-BrkA antibodies (Oliver and Fernandez, 2002) can potentiate the killing of *B. pertussis* in the presence of complement (section 3.3.5). However, the killing ability of the antiserum seems to vary for each *B. pertussis* strain e.g. BP338 and BBC29 strains were less sensitive to complement-mediated killing via the classical pathway than *B. pertussis* Taberman. This difference can presumably be attributed to the level of production, processing and surface exposure of Bap-5 or possibly other cross-reacting factors in different strains of *B. pertussis*. It is also likely that antiserum raised against a preparation of the Bap-5 protein in native form would be more effective in recognising and binding to the surface-exposed epitope of Bap-5, which may include conformational epitopes not present in denatured preparations of the protein. As mentioned before (section 3.2.3.2.3), the rabbit anti-$_r$Bap-5 serum cross-reacted to some extent with another outer membrane protein, possibly BrkA, hence all the bactericidal assays were carried out using the anti-Bap-5 serum absorbed with the outer membrane-enriched fractions extracted from the *B. pertussis bap-5* mutant of the Taberman I strain. Due to time constraints, it could not be evaluated whether anti-$_r$Bap-5 serum has a potentiating effect on the killing ability of the human serum. This study also strongly suggests that Bap-5, like BrkA, appears to be mainly surface located rather than secreted.

4.4.3 *In vitro* adhesion studies

B. pertussis colonises the respiratory tract and is thought to preferentially adhere to ciliated epithelial cells (van den Berg *et al.*, 1999) and also possibly to alveolar macrophages (Relman *et al.*, 1990). There is a diverse range of virulence factors, including FHA, PRN, FIM, PTX and TCF that are considered to play a role in adherence of *B. pertussis* to epithelial cells (Locht *et al.*, 1993; Funnell and Robinson, 1993: Everest *et al.*, 1996; Finn and Stevens, 1995). *B. pertussis* FHA has been suggested to mediate adherence to macrophages and possibly other leukocytes *in vitro* (Ishibashi *et al.*, 1994), a function which may have immunomodulatory effects, either by altering the host cell signal transduction systems or by facilitating the delivery of secreted toxins, such as ACT/HLY (Boschwitz *et al.*, 1997). Moreover FHA has also been shown to be involved in adhesion to laryngeal, bronchial and human lung epithelial cells, whereas fimbriae are involved in adhesion to laryngeal epithelial cells (Mahon *et al.*, 1991; Ishibashi *et al.*, 2001). Tracheal colonisation factor (TCF) is thought to be involved in *B. pertussis* colonisation of the trachea of mice but not the lungs (Finn and Stevens, 1995).

A *B. pertussis* strain deficient in PRN autotransporter protein, another well-characterised adhesin has been shown to have reduced adherence to human epithelial cell lines of non-respiratory origin such as HeLa 229, Chinese hamster ovary (CHO) cells and human laryngeal epithelial (Hep-2) cells *in vitro* compared to the PRN parent strain of *B. pertussis* (Ewanowich *et al.*, 1989; Everest *et al.*, 1996). There are some contradictory reports regarding the role of PRN in adhesion of *B. pertussis* to Hep-2 cells. The study from Roberts *et al.* (1991) using *B. pertussis* BP536, a Sm^R derivative of Tohama and its derived BB52 PRN mutant strain, suggested no significant difference in their ability to adhere to and invade Hep-2 cell lines. On the other hand Leininger *et al.* (1991) suggested that PRN seemed to play a role in *B. pertussis* adherence to Hep-2 cells. However,

recombinant PRN expressed on the surface of *E. coli* (HB101) was shown to impart better adhesive properties for Hep-2 and CHO cells (Everest *et al.*, 1996). So far, no receptor for PRN-mediated adhesion has been identified. An understanding of the precise roles of the individual bacterial virulence factors (adhesins) involved in adhesion to specific primary cells and tissue culture cell lines will be difficult, because of the overlapping functions of different factors during the course of infection.

An ELISA-based adhesion study like that used by Everest *et al* (1996), using different *B. pertussis* strains with different tissue culture cell lines was performed (section 3.2.6). The Taberman strain of *B. pertussis* was selected as its Bap-5-deficient mutant was available. This adhesion study suggested that Bap-5 may play a role in adherence of *B. pertussis* to non-respiratory, HeLa 229 cells, human respiratory epithelial laryngeal (Hep-2) cells and to murine macrophages P338D-1 as demonstrated by the better adherence of the wild type Taberman I strain compared to its Bap-5-deficient mutant Taberman II strain. However there were some inconsistencies in the results such as the adhesion study with Hep-2 cells which showed that it was only the *B. pertussis* Taberman I wild-type strain which was more adherent compared to other wild type *B. pertussis* strains. The adhesion study also showed that Bap-5 wild-type strain of *B. pertussis* was better able to adhere to HeLa-229 cells, Hep-2 cells as well as P338D-1 macrophages compared to its Taberman II *bap-5* mutant. The other wild-type *B. pertussis* strains were generally only slightly better than their mutants i.e. *prn* mutant (BBC30) and *brkA* mutant (BP2041) in adhering to different cell lines used in this study and no significant differences were observed. However, It would be worth trying to use different *B. pertussis* strains other than those used in this study and the *prn* and *brkA* mutants derived from them as well as different concentrations of the bacterial cells in the adhesion study to confirm the results.

In many adhesins that have been studied it is the RGD peptide which is implicated as the key player in catalysing the adhesion of their carrier organism to the host cells. The cell lines like human enterocyte like cell line (Caco-2) are known to have receptors for binding proteins carrying the RGD peptide and there are certain cells, like CHO cells, which lack such receptors. These can be useful tools for assessing the adhesion activity of RGD-containing proteins. However, none of the *B. pertussis* strains showed strong affinity towards Caco-2 cells in this study. In addition, Everest *et al* (1996) presented evidence that the RGD sequence on PRN was not involved in adhesion.

There is also evidence that most of the adhesins if not all, in *B. pertussis* are regulated by the two component Bvg system. In the present study this was shown by the reduction of adherence of *B. pertussis* Taberman to Hep-2 cells when the organism was modulated with $MgSO_4$. However, it would be worth mentioning, that due to repeated sub-culturing, there is a strong possibility that these cell lines, particularly human respiratory epithelial cells, undergo morphological variation and hence have altered surface characteristics. This could have affected the adhesion study. It would be an important consideration that any such study should be done with the primary cell lines as they produce a homogenous cell population or perhaps with engineered cell lines which show little variation in their surface properties (Dowling and Wilson, 1998). Moreover it will be extremely difficult to manipulate adhesion studies *in vitro* by replicating the exact environment of respiratory mucosa where the cells are bathed with the mucus and ciliary beating is operative. However, these studies can give some lines of direction to investigate the role of any functionally unknown gene.

The ability of antibodies to the surface antigens of *B. pertussis* to inhibit adherence to cells in culture should in theory provide a useful functional assay for assessing their ability to prevent infection in the respiratory tract. It was shown that antisera from children

immunised with Pw (pertussis whole-cell vaccine) or convalescing from pertussis inhibit adherence of *B. pertussis* to the human respiratory cells *in vitro* (Tuomanen *et al.*, 1984). Antibodies to FIM have been shown to block the adherence of *B. pertussis* to Vero cells (Ashworth *et al.*, 1988) and HeLa cells. Also, the antibodies to FHA, PRN, PTX, LPS and a 40 kDa outer membrane protein were also shown to inhibit adherence to human bronchial epithelial cells (Tuomanen *et al.*, 1984; Van Den *et al.*, 1999). Another study has suggested that monoclonal antibodies raised against either *B. pertussis* FHA, fimbriae, PRN or PTX inhibited adherence of *B. pertussis* but not *B. parapertussis* to human bronchial epithelial cells (Mahon *et al.*, 1999).

An anti-$_r$Bap-5 serum was also used in this study to demonstrate a role for Bap-5 in adhesion of *B. pertussis* to different mammalian cell lines. The anti-$_r$Bap-5 serum was absorbed with outer membrane-enriched fractions from the Bap-5-deficient mutant prior to use in the study to reduce any cross-reaction against other surface components such as BrkA. There were also some anomalies in these adhesion studies, for example the anti-Bap-5 serum appeared to reduce adherence of *B. pertussis* Taberman to some cells i.e. HeLa 229, Hep-2 and murine macrophages RAW 264.7 cells but did not have an effect with other cells i.e. A549 (human lung epithelial cells), Caco-2 (human carcinoma intestinal cell line) and to P338D-1 macrophages even though, in some cases, the bacteria adhered equally well.

These findings suggest that interaction between the bacteria and mammalian cells is complex and possibly depends on many different adhesins and receptors. In those cases where the *bap-5* mutant adhered less well than the wild-type strain or the anti-$_r$Bap-5 serum has significant effect in reducing adherence, the interaction of Bap-5 and its unspecified receptors may have a significant role to play in adhesion process.

4.5 Mouse virulence tests

B. pertussis infection begins by attachment to the respiratory tract of its human host with the help of a variety of surface-exposed virulence-related proteins. The successful establishment of colonisation depends on the ability of the organism to avoid clearance by the mucociliary escalator, either by developing strong attachments to the ciliated epithelial cells or by paralysing the cilia, and resisting the host immune defences that are operative in this site. However, bacterial colonisation and infection of the airways may also result from pre-existing damage to local defence mechanisms, rather than to bacterial virulence factors (Dowling and Wilson, 1998). There is a range of *B. pertussis* virulence factors , including toxins and adhesins such as FHA, PRN, TCF, BrkA and fimbriae, that are co-ordinately regulated by the *bvg* locus. Strains with mutations in the *bvg* locus are unable to produce these factors and are avirulent in animal models (Weiss and Goodwin, 1989). It is likely that the expression of the members of the *bvg* regulon is altered or tuned, dependent on the different sites or environmental niches encountered in the respiratory tract. Most of the autotransporter proteins studied to date have been found in pathogenic bacteria and are often associated with virulence e.g. PRN acts as an adhesin, BrkA acts as a serum resistance factor.

The present study (section 3.3) has suggested that a *bap-5* mutant of the Taberman I strain of *B. pertussis* was less able to persist and colonise the trachea and the lungs of mice compared to its isogenic parent strain, Taberman I. However, no significant difference was observed in their colonisation of the nasal cavities of mice as was the case with a *prn* mutant of *B. bronchiseptica* which colonised the nasal cavity as well as the parent strain (Prof. M. Roberts, unpublished). There may be some other unknown factors involved in colonising this particular site. The results with other strains also produced some useful information. *B. pertussis* BBC30, a *prn* mutant, was also found to be less able

to persist and colonise the trachea and the lungs of mice compared to its isogenic parent strain, BBC29. These results suggested that Bap-5, like PRN, appears to participate in establishing *B. pertussis* infection in the trachea and lungs of mouse. However, such findings should be treated with caution as they may not be representative of the natural *B. pertussis* infection in man and because relatively large challenge doses are required to initiate the infectious process.

A previous study suggested that a *B. pertussis* PRN mutant was cleared more quickly from the lungs of intranasally-infected mice than its wild-type strain (Khelef *et al.*, 1994). A *B. bronchiseptica* PRN mutant was reduced in its ability to colonise the lower respiratory tract but not the nasal cavity of mice (Prof. M. Roberts, unpublished). A *B. pertussis* TCF mutant was found to have reduced ability to colonise the tracheas of mice and intranasal immunisation of mice with an attenuated *Vibrio cholerae* strain expressing TCF reduced the ability of *B. pertussis* to subsequently colonise the trachea (Chen *et al.*, 1998). A *B. pertussis brkA* mutant was found to be 10-fold less virulent in colonising the respiratory tract (lungs) in intranasally challenged infant mice than the parent strain (Fernandez and Weiss, 1994). However, a *B. pertussis vag-8* mutant colonised the trachea and lungs of mice as efficiently as the isogenic parent (Finn and Amsbaugh, 1998).

These animal studies illustrate the important role that most of the *B. pertussis* autotransporter proteins play in the infection process.

4.6 Mouse protection test

Previous mouse protection studies using the purified C-terminal domains of *B. pertussis* autotransporter proteins of BrkA, TCF, PRN and Bap-5 suggested that they were not immunodominant, protective regions of the autotransporter proteins (Blackburn, 2000). These C-terminal domains however, were presented to the mice in denatured form after

expression in *E. coli* and solubilisation of inclusion bodies in urea, which could be the reason for the lack of provision of protection by the protective epitopes against *B. pertussis*. In the present study, to circumvent the doubt regarding the denatured form of P.30, a re-natured preparation of the recombinant C-terminal domain was used. In parallel, a purified preparation of P.69, which is a known immunogen, was used in its native form.

This study (section 3.4) suggested that no protection was afforded by the re-natured P.30 against *B. pertussis* colonisation of the respiratory tract of the mice, whereas P.69 (at the same dose) afforded a clear protection against tracheal and lung colonisation and to some extent colonisation of the nasal cavity. There is a possibility that P.30 may not have an immunodominant epitope which can generate an effective immune response and hence provide protection against *B. pertussis* or, even if it does, there is a possibility that anti-P.30 antibodies may not have access to this antigen because of its presumed embedded nature in the outer membrane (Charles *et al.*, 1994). Unfortunately, blood serum samples were not collected from the pre-immunised and post-immunised mice, which would have given useful information regarding the presence of P.30-specific antibodies in the mouse model. Similar protection studies could be devised for the Bap-5 protein to see if its re-natured mature and C-terminal domains have any role in providing protection against *B. pertussis*. It has been reported that a good immune response occurs when some *B. pertussis* antigens are presented in their correct form, for example as outer membrane complexes (Hamstra *et al.*, 1995; Shahin *et al.*, 1995). It has been reported that the TCF C-terminus (~33 kDa), protein when used in the form of outer membrane complexes was protective in the intracerebral mouse protection test (Hamstra *et al.*, 1995). However, the vaccine preparations, challenge doses and routes of administration of the vaccines and adjuvant effects of different formulations are all potential areas to be explored in the field of pertussis vaccinology. Moreover, the complete genome sequence data is available and it

will give information on all the surface exposed and secreted proteins of an organism. This can lead to the identification of potential novel antigens from the genome sequence for possible inclusion in the next generation of acellular vaccines, a strategy known as reverse vaccinology (Preston and Maskell, 2002).

Previous studies using the mouse model of infection have suggested that immunisation with purified *B. pertussis* FHA, PTX, Fim or PRN protects against an intranasal or aerosol challenge with *B. pertussis* (Mills *et al.*, 1993, 1998; Mahon *et al.*, 1999; Guiso *et al.*, 1999). This is consistent with the presence of antibodies against *B. pertussis* FHA and fimbriae in humans which protect against *B. pertussis* by interfering with its adherence (Mahon *et al.*, 1999). The antibodies against the other surface structures i.e. LPS (Shahin et al., 1994) and other outer-membrane associated proteins can also reduce the adherence of *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* as evident by the protection provided by these immunogens (Fernandez and Weiss, 1998).

The protection against *B. pertussis* provided by the members of its autotransporter family is another area to be exploited by researchers, to enhance the efficacy of the acellular vaccines. Natural and recombinant preparations of purified pertactin/P.69 have been shown to induce a protective immune response against an intranasl or aerosol challenge of *B. pertussis* in experimental models (Shahin *et al.*, 1990; Roberts *et al.*, 1992). The protective capacities of purified TCF, BrkA and Vag-8 have not been reported so far. Vag-8 (92 kDa protein) had been reported to be protective only in the presence of non-protective levels of pertussis toxin (Hamstra *et al.*, 1995). The autotransporter PRN is known to be an important protective component of many current acellular vaccines. However, there is increasing evidence of sequence variations of at least two important constituents of current acellular vaccines e.g. PRN and PTX in *B. pertussis* strains circulating in the population (Gerlach *et al.*, 2001). This has been suggested to be due to

the selective pressure imposed by current vaccines (Mooi *et al.*, 1998) and this situation has prompted the search for additional protective antigens that will be useful in enhancing the protective efficacy of the new generation of acellular pertussis vaccines.

4.7 Secretion mechanism of a *B. pertussis* autotransporter protein in protease-deficient strains of *E. coli*

B. pertussis autotransporters, i.e. PRN, TCF, BrkA, Vag-8 and Bap-5 are associated with bacterial virulence. There are many examples of such virulence associated autotransporter proteins in other Gram negative bacteria, e.g. IgA protease (*N. gonorrhoeae*), adhesins like AIDA-1 (*E. coli*) (Benz and Schmidt, 1989) and invasins such as Hap (*H. influenzae*) (St Geme *et al.*, 1994), proteases such as Esp (*E. coli*) Mecsas and Strauss, 1996) and SepA (*S. flexneri*) (Benjelbun-Touimi *et al.*, 1995) and toxins such as VacA (*H. pylori*) (Cover and Blaser, 1992) and Pet (Henderson *et al.*, 1999) (Table 1.1). Some of these proteins e.g. IgA1 and Hap proteases are members of the serine protease family whose outer membrane translocation is dependent on the activity of the trypsin-like active site (GDGSP, where S is the catalytic residue). Most of the members of this family, after being exposed on the cell surface, are processed and released into the medium. There is another class within the autotransporter family of Gram-negative bacteria which are known as non-protease autotransporters e.g. Ag43 and AIDA-1. They are grouped on the basis of amino acid homology and the presence of repetitive amino acid motifs (Henderson et al., 1999). In this way, they appear to resemble PRN of *Bordetella* spp which also contains repetitive sequences e.g. $(GGXXP)_5$ and $(PQP)_5$. The members of the non-protease autotransporter family, upon cleavage, appear to remain bound to the outer membrane of the bacteria due to noncovalent interactions with their respective C-termini which form β-domains in the outer membrane (Benz and Schmidt, 1992). There appears to

be no information regarding the exact mechanism of their processing into the surface-exposed passenger domains and C- terminal, β-domains. It is possible either that they are processed by membrane-associated proteases or they may have an inherent autoproteolytic ability which brings about their cleavage into the passenger domain and C-terminal domain. The autoproteolytic ability of IgA-1 protease is well known as it can bring about its own processing in the absence of the outer membrane serine protease (*ompT*) in recombinant *E. coli* (Klauser *et al.*, 1992). *B. pertussis* PRN (P.93) is known to produce two fragments, a N-terminal passenger domain P.69 and a C-terminal domain P.30, after processing (Roberts *et al.*, 1991). The possibility of any involvement of any protease(s) in the processing of the autotransporters was investigated in a range of protease-deficient *E. coli* strains (section 3.6).

The study suggested that lack of any of the proteases alone or certain combinations appeared to have no effect in preventing the processing of P.93 in *E. coli* strains as P.69 was found to be outer-membrane associated in these strains as well as released into the supernate. Unfortunately, the anti-P.30 serum available was unable to detect P.30 in the outer membrane fractions of protease-deficient strains expressing full length PRN (P.93) containing plasmid p41869. However, by comparing the SDS-PAGE profiles of the outer membrane proteins of the protease-deficient strains of *E. coli* expressing P.93 (p41869) with the *E. coli* parent strain containing the vector only (pMMBE66H), a 30 kDa C-terminal region of pertactin, P.30, and the mature passenger domain, P.69 could be visualised in the former case. However, the most deficient *E. coli* strain (HM130) did not appear to process the surface exposed P.93 into P.69 and P.30. This observation can perhaps be explained by the fact that due to many protease-deficiencies in HM130 strain, it was not active in processing the P.93 at the outer membrane. Alternatively, these deficiencies could have caused blockage of the secretion channels due to the accumulation

of undegraded proteins in the periplasm. P.93 which reached the outer membrane may not have been comparable to that found in the other less protease-deficient strains in terms of its quantity. Moreover, there is the possibility that P.93 would not be able to gain a correct conformation in the most protease-deficient strain which may have hindered its processing and release. This possibility can be supported by the successful processing of the linker region from its C-terminal domain (section 7.2) as seen in the outer membrane fraction of the most protease-deficient strain of *E. coli* expressing linker region and C-terminal of pertactin (pBADpct/linker). The linker region (15 kDa) appeared to be detached from the P.30 domain and found equally in the outer-membrane fractions of parent *E. coli* strain and its most protease-deficient strain HM130. It would be worth checking the culture supernate fraction of the latter strain for the presence or absence of linker region. However, it would be more appropriate if various domains of interest of PRN and other autotransporters could be investigated in the *B. pertussis* native system. Moreover, with the recent availability of the complete *B. pertussis* genome information, the hunt for any envelope-associated proteases of interest will become easier and simplify the investigation of their role in the processing of *B. pertussis* autotransporter proteins. In this regard, a series of protease-deficient strains of *B. pertussis* could be created (similar to those of *E. coli*) and their ability to export and release the various *B. pertussis* autotransporter proteins in the native system could be examined.

The host-pathogen interaction can also play a crucial role in determining the fate of the surface-located protein. For example the autotransporter proteins Hap (adhesin) and IgA protease of *Haemophilus influenzae* has been reported to be cleaved by lactoferrin (milk protein) without interrupting bacterial cell growth (Plaut *et al.*, 2002). This opens up new possibilities for the processing and release of the auotransporter proteins of *B. pertussis* by host proteins with proteolytic activity. The benefit of this sophisticated action

for the host may be to make initial colonisation by the bacteria of the respiratory tract less effective and for the bacteria it may result in the conversion of the more severe host immune response into a comparatively less severe response.

In conclusion, the study showed that Bap-5 is expressed and regulated by the Bvg system in *B. pertussis*. It also showed that Bap-5 plays a role in conferring resistance to *B. pertussis* against complement-mediated (classical and possibly alternative) killing and it possibly aids *B. pertussis* in adhering better to certain tissue culture cell types *in vitro*. Antibody to Bap-5 can potentiate killing of *B. pertussis* in the presence of a source of complement and it also can inhibit *B. pertussis* adhesion to certain tissue culture cell lines *in vitro*. Moreover, the mouse virulence tests have shown that Bap-5 aids *B. pertussis* in colonising the lower respiratory tract of mice. Characterisation of the other autotransporter protein PRN showed that its C-terminal protein does not possess the ability to provide protection in a mouse model. The study also suggested that PRN (P.93) appeared to be processed into mature P.69 as well as the 15 kDa linker plus the C-terminal domain (30 kDa) of PRN into its components without requiring any specific accessory protein in various protease-deficient *E. coli* strains.

5. References

Ackers, J. P. and Dolby, J. M. **(1972).** The antigen of *Bordetella pertussis* that induces bactericidal antibody and its relationship to protection of mice. *J. Gen. Microbiol.* **70**, 371-382.

Akerley, B. J., Cotter, P. A. and Miller, J. F. (1995). Ectopic expression of the flagellar regulon alters development of the *Bordetella*-host interaction. *Cell.* 80, 611-620.

Allaoui, A., Schulte, R. and Corneils, G. R. **(1995).** Mutational analysis of the *Yersinia enterocolitica virC* operon: Characterization of YscE, F. G. I. J. K. required for Yop secretion and yscH encoding YopR. *Mol. Microbiol.* **18**, 343-355.

Allaoui, A., Woestyn, S., Sluiters, C. and Corneils, G. R. **(1994).** YscU, a *Yersinia enterocolitica* inner membrane protein involved in Yop secretion. *J. Bacteriol.* **176**, 4534-4542.

Allen, A. G., Thomas, R. M., Caddisch, J. T. and Maskell, D. J. **(1998).** Molecular and functional analysis of the lipopolysacchariode biosynthesis locus *wbl from Bordetella pertussis, Bordetella parapertussis* and *Bordetella bronchiseptica. Mol. Microbiol.* **29**, 27-38.

Amsbaugh, D. F., Li, Z. M. and Shahin, R. D. **(1993).** Long lived respiratory immune response to filamentous haemagglutinin following *Bordetella pertussis* infection. *Infect. Immun.* **61**, 1447-1452.

Anderson, R., Dougan, G. and Roberts, M. **(1996).** Delivery of the pertactin/P.69 polypeptide of *Bordetella pertussis* using an attenuated *Salmonella typhimurium* vaccine strain: expression levels and immune response. *Vaccine*, **14**(14), 1384-1390.

Antoine, R., Alonso, S., Raze, D., *et al.* **(2000).** New virulence-activated and virulence-repressed genes identified by systematic gene inactivation and generation of transcriptional fusions in *Bordetella pertussis. J. Bacteriol.* **182**(20), 5902-5905.

Antoine, R., Raze, D. and Locht, C. **(2000)**. Genomics of *B. pertussis* toxins. *Int. J. Med. Microbiol.* **290**, 301-305.

Arico, B., Miller, J. F., Roy, C., *et al.* **(1989).** Sequences required for expression of *Bordetella pertussis* virulence factors share homology with prokaryotic signal transduction proteins. *Proc. Natl Acad. Sci. USA.* **86**, 6671-6675.

Arico, B., Nuti, S., Scarlato, V and Rappuoli, R. **(1993).** Adhesion of *Bordetella pertussis* to eukaryotic cells requires a time dependent export and maturation of filamentous haemagglutinin. *Proc. Natl Acad. Sci. USA.* **90**, 9204-9208.

Armstrong, S. K. and Parker, C. D. **(1986)**. Heat-modifiable envelope proteins of *Bordetella pertussis. Infect. Immun.* **54**(1), 109-117.

Armstrong, G. D., Clark, C. G. and Heerze, L. D. **(1994).** The 70 kilo-dalton pertussis toxin-binding protein in Jurkat cells. *Infect. Immun.* **62,** 2236-2243.

Ashworth, L. A., Robinson, A., Irons, L. I. *et al.* **(1982)**. Rabbit nasopharyngeal colonization by *Bordetella pertussis:* the effects of immunization on clearance and on serum and nasal antibody levels. *J. Hyg (Lond).* **88**, 475-486.

Ashworth, L. A., Dowsett, A. B., Irons, L. I. and Robinson, A. **(1985)**. The location of the surface antigens on the surface of *Bordetella pertussis* by immuno-electron microscopy. *Dev. Biol. Stand.* **61**, 143-151.

Ashworth, L. A., Robinson, A., Irons, L. I. *et al.* **(1986).** Fimbriae of *Bordetella pertussis*: A chapter from Protein-Carbohydrate interactions in Biological systems by Academic press Inc. (London) Ltd. 291-293.

Ashworth, L. A., Robinson, A., Funnell, S. *et al.*, **(1988)**. Agglutinogens and fimbriae of *Bordetella pertussis. Tok. J. Expt. Clin. Med.* **13** suppl:203-10.

Babu, M. M., Bhargavi, J., Saund, R. S. and Singh, S. K. **(2001)**. Virulence factors of *Bordetella pertussis*. *Curr. Sci.* **80** (12), 1512-1522.

Baneyx, F. and Georgiou, G. **(1990)**. *In vivo* degradation of secreted fusion proteins by the *Escherichia coli* outer membrane protease OmpT. *J. Bacteriol.* **172**(1), 491-494.

Banemann, A., Deppish, H. and Gross, R. **(1998)**. The lipopolysaccharide of *Bordetella bronchiseptica* acts as a protective shield against antimicrobial peptides. *Infect. Immun.* **66**, 5607-5612.

Baneyx, F. and Georgiou, G. **(1991)**. Construction and characterization of *Escherichia coli* strains deficient in multiple secreted protesaes: Protease III degrades high- molecular-weight substrates *in vivo*. *J. Bacteriol.* **173**(8), 2696-2703.

Barnard, A., Mahon, B. P., Watkins, J., et al. (1996). Th1/Th2 cell dichotomy in acquired immunity to *Bordetella pertussis*: variables in the invivo priming and in vitro cytokine detection techniques affect the classification of T- cell subsets as Th1, Th2 or Th0. *Immunol.* 87, 372-380.

Barnes, M. G. and Weiss, A. **(2001)**. BrkA protein of *Bordetella pertussis* inhibits the classical pathway of complement after C1 deposition. *Infect. Immun.* **69**, (5), 3067-3072.

Bassinet, L., Gueirard, P., Maitre, B., *et al.* **(2000)**. Role of adhesins and toxins in invasion of human tracheal epithelial cells by *Bordetella pertussis*. *Infect. Immun.* **68**(4), 1934-1941.

Beattie, D. T., Mahan, M. J. and Mekalanos, J. J. **(1993)**. Repressor binding to a regulatory site in the DNA coding sequence is sufficient to confer transcriptional regulation of the *vir*-repressed genes (*vrg* genes) in *Bordetella pertussis*. *J. Bacteriol.* **175**(2), 519-527.

Bechler, C. E., Drenkow, J., Kehoe, B., *et al.* **(2000)**. The transcriptional responses of respiratory epithelial cells to *Bordetella pertussis* reveal host defensive and pathogen counter-defensive strategies. *Proc. Natl Acad. Sci. USA.* **97**, 13847-13852.

Beier, D. Deppisch, H. and Gross, R. **(1996)**. Conserved sequence motifs in the unorthodox BvgS two-component sensor protein of *Bordetella pertussis*. *Mol. Gen. Genet*. **252**, 168-176.

Benjelloun-Touimi, Z., Sansonetti, P. J. and Parssot, C. **(1995)**. SepA, the major extracellular protein of Shigella flexneri: autonomous secretion and involvement in tissue invasion. *Mol. Microbiol*. **17**(1), 123-135.

Benz, I. and Schmidt, M. A **(1989)**. Cloning and expression of an adhesin (AIDA-I) involved in diffuse adherence of enterpathogenic *Escherichia coli*. *Infect and Immun*. **57**(5), 1506-11.

Benz, I., and Schmidt, M. A. **(1992)**. AIDA-1, the adhesin involved in diffuse adherence of the diarrhoeagenic *Esclierichia coli* strain 2787 (O126:H27), is synthesised via a precursor molecule. *Mol. Microbiol*. **6**(11), 1539-1546.

Bergman, T., Erickson, K., Galvoy, E., *et al*. **(1994)**. The *crB* (yscN/U) gene cluster of *Yersinia pseudotuberculosis* is involved in Yop secretion and shows high homology to the *spa* gene clusters of *Shigella flexneri* and *Salmonella typhimurium*. *J. Bacteriol*. **176**, 2619-2626.

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

Beuzon, C. R., Banks, G., Deiwick, J., *et al*. **(1999)**. PH-dependent secretion of SseB, a product of the SPI-2 type II secretion system of *Salmonella typhimurium*. *Mol. Microbiol*. **33**(4), 806-816.

Binet, R., Letoffe, S., Ghigo, J. M., *et al*. **(1992)**. Protein secretion by Gram-negative bacterial ABC exporters-a review. *Gene*, **192**, 7-11.

Black, S. **(1997)**. Epidemiology of pertussis. *Pediatr. Infect. Dis. J*. **16**, S85-S89.

Blackburn, P. E. **(2000).** Characterisation of the virulence related outer-membrane proteins of *Bordetella pertussis*. Ph.D. thesis. University of Glasgow.

Bliska, J. B. and Falkow, S. **(1992).** Bacterial resistance to complement killing mediated by Ail protein of *Yersinia entercolitica*. *Proc. Natl. Acad. Sci. USA*. **89**(8). 3561-3565

Blobel, G. and Dobberstein, B. **(1975).** Transfer to proteins across membranes. II. Reconstitution of functional rough microsomes from heterologous componenets. *J. Cell. Bio*. **67**(3), 852-862.

Bock, S. C., Skriver, K., Nielsen, E., *et al*. **(1986).** Human C1 inhibitor: Primary structure cDNA cloning, and chromosomal localisation. *Biochemistry*. **25**, 4292-4301.

Bordet, J. and Gengou, O. **(1906)**. Le microbe de la coqueluche. *Ann Inst Pasteur*, **23**, 415-419.

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

Boursaux-Eude, C., Thiberge, S., Carletti, G. and Guiso, N. **(1999).** Intranasal murine model of *B. pertussis* infection: II. Sequence variation and protection induced by a tricomponenet acelluar vaccine. *Vaccine*. **17**(20-21), 2651-2660.

Brennan, M. J., David, J. L., Kenimer, J. G. and Manclark, C. R. **(1988)**. Lectin-like binding of pertussis toxin to 165-kilodalton Chinese hamster ovary cell glycoprotein. *J. Biol. Chem*. **263,** 4895-4899.

Brennan, M. J., Hannah, J. H. and Leininger, E. **(1991)**. Adhesion of *Bordetella pertussis* to sulfatides and to the GalNAc-beta4-Gal sequence found in glycosphingolipids. *J. Biol. Chem*. **266**, 18827-18831.

Brezin, C., Guiso, N., Ladant, D., *et al.* **(1987)**. Protective effects of anti-*Bordetella pertussis* adenylate cyclase antibodies against lethal respiratory infection of the mouse. *FEMS Microbiol. Lett.* **42**, 75-80.

Brockmeier, S. L. **(1999)**. Early colonization of the rat upper respiratory tract by temperature modulated *Bordetella bronchiseptica*. *FEMS Microbiol Lett.* **74**(2), 225-229.

Brockmeier, S. L., Register K. B. and Kunkle R. A. (2000). Evaluation of the virulence of a dermonecrotic toxin mutant of *Bordetella bronchiseptica* in swine. *16th Internatl. Pig. Vet. Soc. Cong. Melbourne, Australia.* pp. 480-480

Brunder, W., Schmidt, H. and Karch, H. **(1997)**. EspP, a novel extracellular serine protease of enterohaemorrhagic *Escherichia coli* O157:H7 cleaves human coagulation factor V. *Mol. Microbiol.* **24**, 767-778.

Burns, D. L. **(1999)**. Biochemistry of type IV secretion. *Curr. Opin. Microbiol.* **2**, 25-29.

Burns, S. M and Hull, S. I. **(1998)**. Comparison of loss of serum resistance by defined lipopolysaccharide mutants and an acapsular mutant of uropathogenic *Escherichia coli* O75:K5. *Infect. Immun.* **66**, 4244-4253.

Busse, H. J. and Augling, G. **(1992)**. The genera *Alcaligenes* and *Achromobacter*, In : Balows, A., Truper, H. G *et al.* (Eds). *The prokaryotes, Springer, Heidelberg*. pp. 2544-2555.

Buttner, D. and Bonas, U. **(2002)**. Port of entry-the type III secretion translocons. *Trends Microbiol.* **10** (4), 186-192.

Byrd, D. W., Roop, R. M., Veit, H. P. and Schurig, G. G. **(1991)**. Serum sensitivity and lipopolysaccharide characteristics in *Bordetella bronchiseptica*, *Bordetella pertussis* and *Bordetella parapertussis*. *J. Med. Microbiol.* **34**(3), 159-165.

Capiau, C., Carr, S. A., Hemling, M. E., *et al.* **(1990)**. Purification, characterisation, and immunological evaluation of the 69-kDa outer membrane protein of *Bordetella pertussis*. In: C. R. Manclark (Ed.), *Proceedings of the Sixth International Symposium on Pertussis*, (pp. 75-85). DHSS Publication Number (FDA) 90-164.

Caroff, M., Brisson, J. R., Martin, A. and Karibian, D. **(2000)**. Structure of *B. pertussis* 1414 endotoxin. *FEBS letters*. **477**, 8-14.

Castro, M. G., McNamara, U. and Carbonetti, N. H. **(2001)**. Expression, activity and cytotoxicity of pertussis toxin S1 subunit in transfected mammalian cells. *Cellular Microbiol.* **3**(1), 45-54.

Chaby, R. and Caroff, M. **(1988)**. Lipopolysaccharide of *Bordetella pertussis* endotoxin. In: *Pathogenesis and Immunity in Pertussis,* edited by Wardlaw, A. C and Parton, R. Chichester: John Wiley and Sons, pp. 247-271.

Charles, I. G., Dougan, G., Pickard, D., *et al.* **(1989)**. Molecular cloning and characterisation of protective outer membrane protein P.69 from *Bordetella pertussis*. *Proc. Natl Acad. Sci. USA*. **86**, 3554-3558.

Charles, I. G., Li, J. L., Roberts, M., *et al.* **(1991)**. Identification and characterisation of a protective immunodominant B-cell epitope of pertactin (P.69) from *Bordetella pertussis*. *Eur. J. Immunol.* **21**, 1147-1153.

Charles, I., Fairweather, N., *et al.* **(1994)**. Expression of the *Bordetella pertussis* P.69 pertactin adhesin in *Escherichia coli*: fate of the carboxy terminal domain. *Microbiology*. **140**, 3301-3308.

Chen, I., Finn, T. M., Yanqing, L., Guoming, Q., *et al.* **(1998)**. A recombinant live attenuated strain of *Vibrio cholera* induces immunity against tetanus toxin and *Bordetella pertussis* tracheal colonisation factor. *Infect. Immun.*, **66**(4), 1648-1653.

Cherry, J. D., Brunnel, P. A., Golden, G. S. and Karzon, D. T. (**1988**). Report on the task force on pertussis and pertussis immunization. *Paediatrics*, **81**, 939.

Cherry, J. D., Hewlett, E. L., Mortimer, E. A. Jr. (**1990**). Protective antibodies in pertussis. *J. Paediat.* **117**, 347-348.

Cherry, J. D., Gombein, J., Heininger, U. and Stehr, K. (**1998**). A search for serologic correlates of immunity to *Bordetella pertussis* cough illnesses. *Vaccine.* **16**, 1901-1906.

Cherry, J. D. (**1999**) Epidemiological, clinical, laboratory aspects of pertussis in adults. *Clin. Infect. Dis.* **28** (Suppl.2): S112-S117.

Cobb, B. D. and Clarkson, J. M. (1994). A simple procedure for optimising the polymerase chain reaction (PCR) using modified Taguchi methods. N. A. Res. 22(18), 3801-3805.

Cooper, N. R. (**1991**). Complement evasion strategies of microorganisms. *Immunol. Today.* **12**, 327-331.

Coote, J. G. (**1991**). Antigenic switching and pathogenicity: environmental effects on virulence gene expression in *Bordetella pertussis*. *J. Gen. Microbiol.* **137**, 2493-2503.

Corneils, G. R. and Gijisegem, F. V. (**2000**). Assembly and function of type III secretory systems. *Annu. Rev. Microbiol.* **54**, 735-774.

Cotter, P. A., Akerley, B. J. and Miller, J, F. (**1995**). BvgAS dependent phenotypic modulation of *Bordetella* species. In: *Signal transduction and bacterial virulence,* edited by Rappuoli, R., Scarlato, V., Arico, B. Austin, Texas. R.G. Landes Company. pp21-42.

Cotter, P. A., Akerley, B. J., Miller, J, F., *et al.* (**1998**). Filamentous Hemagglutinin of *Bordetella bronchiseptica* is required for efficient establishment of tracheal colonization. *Infect. Immun.* **66**(12), 5921-5929.

Cotter, P. A. and Miller, J. F. **(1994)**. BvgAS-mediated signal transduction: analysis of phase-locked regulatory mutants of *Bordetella bronchiseptica* in a rabbit model. *Infect. Immun.* **62**, 3381-3390.

Cover, T. L. and Blaser, M. J. **(1992).** *Helicobater pylori* and gastroduodenal disease. *Ann. Rev. Med.* **43**, 135-145.

Cover, T. L., Cao, P., *et al.* **(1994).** Divergence of genetic sequences for the vacuolating cytotoxin among *Helicobacter pylori* strains. *J. Biol. Chem.* **269**, 105666-10573.

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

Cookson, B. T., Vandamme, P., Carlson, L. C., *et al.* **(1994)**. Bacteraemia caused by a novel *Bordetella* species, "*Bordetella hinzii*". *J. Clin. Microbiol.* **32**(10), 2569-2571.

Cooper, N. R. **(1991).** Complement evasion strategies of microorganisms. *Immunol. Today.* **12**, 327-331.

Craig, F. F., Lackie, J. M., Parton, R. and Freer, J. H. **(1988)**. Interaction of *Bordetella pertussis* virulence components with neutrophils: effect on chemiluminescence induced by a chemotactic peptide and by intact bacteria. *J. Gen. Microbiol.* **134**, 2201-2211.

Craig-Mylius, K. A. and Weiss, A. A. **(1999)**. Mutants in the ptlA-H genes of *Bordetella pertussis* are deficient for pertussis toxin secretion. *FEMS Microbiol lett.* **179**(2), 479-484.

Craig-Mylius, K. A., Stenson, T. H. and Weiss, A. A. **(2000)**. Mutations in the S1 subunit of pertussis toxin that affect secretion. *Infect. Immun.* **68**, 1274-1281.

Cundell, D.R., Kanthakumar, K., Taylor, G. W. and Goldman, W. E. **(1994)**. The effect of tracheal cytotoxin from *Bordetella pertussis* on human neutrophils function *in vitro*. *Infect. Immun.* **62**, 639-643.

d'Enfert, C. (**1993**). Yet another chaperone? *Trends. Microbiol.* **1**(5), 161-162.

De Ley, J., Segers, P., Kersters, K., *et al.* (**1986**). Intra- and intergenic similarities of the *Bordetella* ribosomal ribonucleic acid cistrons: Proposal for a new family, *Alcaligenaceae. Intl. J. Syst. Bacteriol.* **36**(3), 405-414.

Diano, M., Bivic, A. L and Hirn., M. (**1987**). A method for the production of highly specific polyclonal antibodies: *Analy. Biochem.* **166,** 224-229.

Dinh, T., Paulsen, I. T. and Saier, M. H. J. (**1994**). A family of extracytoplasmic proteins that allow transport of large molecules across the outer membranes of Gram-negative bacteria. *J. Bacteriol.* **176**(13), 3825-3831.

Domenighini, M., Relman, D., Capiau, C., *et al.* (**1990**). Genetic characterisation of *Bordetella pertussis* filamentous haemagglutinin: a protein processed from an unusually large precursor. *Mol. Microbiol.* **4**(5), 787-800.

Dowling, R.B and Wilson, R. (**1998**). Bacterial toxins which perturb ciliary function and respiratory epithelium. *J. Appl. Microb.* **85**, 138S-148S.

Economou, A. (**1999**). Following the leader: bacterial protein export through the Sec pathway. *Trends. Microbiol.* **7**, 315-319.

Egile, C., d'Hauteville, H., *et al.* (**1997**). SopA, the outer membrane protease responsible for polar localization of IcsA in *Shigella flexneri. Mol. Microbiol.* **23**, 1063-1073.

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

Eslava, C., Navarro-Garcia, F., Czeczulin, J. R., *et al.* (**1998**). Pet, an autotransporter enterotoxin from enteroaggregative *Escherichia coli. Infect. Immun.* **66**(7), 3155-3163.

Everest, P., Li, J., Douce, G., *et al.* (**1996**). Role of the *Bordetella pertussis* P.69/pertactin protein and the P.69/pertactin motif in the adherence to and invasion of mammalian cells. *Microbiology.* **142**, 3261-3268.

Ewanowich, C. A., Melton, A. R., Weiss, A. A., *et al.* (**1989**). Invasion of HeLa 229 cells by virulent *Bordetella pertussis*. *Infect. Immun.*. **57**, 2698-2704.

Fairweather, N. F., Makoff, A. J., Oxer, M. D., *et al.* (**1990**). P.69 Pertactin: high-level expression and purification from *Eschericlzia coli* and protective properties. In: C. R. Manclark (Ed.), *Proceedings of the Sixth International Symposium on Pertussis*, (pp. 275-280). DHSS Publication Number (FDA) 90-164.

Farizo, K. M., Huang, T. and Burns, D. L. (**2000**). Importance of holotoxin assembly in Ptl-mediated secretion of pertussis toxin from *Bordetella pertussis*. *Infect. Immun.* **68**, 4049-4054.

Fekkes, P. and Driessen, A. J. M. (**1999**). Protein targeting to the bacterial cytoplasmic membrane. *Microbiol. Mol. Biol. Rev.* **63**(1), 161-173.

Femlee, T., Pellet, S. and Welch, R. A. (**1985**). *Escherichia coli* hemolysin is released extracellularly without cleavage of a signal peptide. *J. Bacteriol.* **163,** 88-93.

Ferry, N. S. (**1910**). A preliminary report of the bacterial findings in canine distemper. *Am. Vet. Rev.* **37**(499-504).

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

Fernandez, R. C. and Weiss, A. A. (**1996**). Susceptibilities of *Bordetella pertussis* strains to antimicrobial peptides. *Antimicrobial Agents and Chemotherapy*. **40**(4), 1041-1043.

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

Filip, C., Fletcher G., Wulf, J. L. and Earhart, C. F. **(1973)**. Solubilisation of the cytoplasmic membrane of *Eschericlhia coli* by the ionic detergent sodium-lauryl sarcosinate. *J. Bacteriol.* **115**(3), 717-722.

Filloux, A. and Hardie, K. R. **(1998)**. A systematic approach to the study of protein secretion in gram-negative bacteria. *Meth. Microbiol.* **27**, 301-314.

Finlay, B. B. and Falkow, S. **(1997)**. Common themes in microbial pathogenicity revisited. *Microbiol. Mol. Bio. Rev.* **61**(2), 136-169.

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

Finn, T. M. and Stevens, L. A. **(1995)**. Tracheal colonization factor: a *Bordetella pertussis* secreted virulence determinant. *Mol. Microbiol.* **16**, 625-634.

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

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

Forde, C. B., Parton, R. and Coote, J. C. **(1998)**. Bioluminescence as a reporter of intracellular survival of *Bordetella bronchiseptica* in murine phagocytes. *Infect. Immun.* **66**(7), 3198-3207.

Forde, C. B., Shi, X., Li, J. and Roberts, M. **(1999)**. *Bordetella bronchiseptica*-mediated cytotoxicity to macrophages is dependent on *bvg*-regulated factors, including pertactin. *Infect. Immun.* **67**, 5972-5978.

Frank, M. M. **(1992)**. The mechanism by which micro-organisms avoid complement attack. *Curr. Opin. Immunol.* **4**, 14-19.

Friedlander, M. and Blobel, G. **(1985)**. Bovine opsin has more than one signal sequence. *Nature*. **318**(6044), 338-343.

Funke, G., Hess, t., von Gravenitz, A., Vandamme, P., *et al.* **(1996)**. Charecteristics of *Bordetella hinzii* strains isolated from a cystic fibrosis patient over a 3 year period. *J. Clin. Microbiol.* **34**, 966-999.

Funnell, S. G., Robinson A. **(1993)**. A novel adherence assay for *Bordetella pertussis* using tracheal organ cultures. *FEMS Microbiol. Lett.* **110**, 197-203.

Galan, J. E., Ginocchio, C., Costeas, P. **(1992)**. Molecular and functional charactetrisation of the Salmonella invasion gene *invA*: homology of InvA to memebers of a new protein family. *J. Bacteriol.* **174**(13), 4338-4349.

Gerlach, G., Von-Wintzingerode, F., Middendorf, B. and Gross, R. **(2001)**. Evolutionary trends in the genus *Bordetella. Microb. Infect.* **3**, 61-72.

Geuijen, C. A. W., Willems, R. J. L., Bongaerts, M. Top, J., *et al.* **(1997)**. Role of the *Bordetella pertussis* minor fimbrial subunit, FimD in colonisation of the mouse respiratory tract. *Infect. Immun.* **64,** 4222-4228.

Geuijen, C. A. W., Willems, R. J. L. and Mooi, F. R. **(1996)**. The major fimbrial subunit of *B. pertussis* binds to sulfated sugars. *Infect. Immun.* **64**(7), 2657-2665.

Glass, R. I.., Svennerholm, A. M., Khan, M. R., *et al.* **(1985)**. Seroepidemiological studies of E1 Tor cholera in Bangladesh: association of serum antibody levels with protection. *J. Inf. Dis.* **151**(2), 236-242.

Goldman, W. E. **(1988)**. Tracheal cytotoxin of *Bordetella pertussis*. In A. C. Wardlaw and R. Parton (Eds.), *Pathogenesis and Immunity in Pertussis* (pp. 231-246). John Wiley and Sons Ltd.

Gotto, J. W., Eckhardt, T., Reilly, P. A., *et al.* **(1993)**. Biochemical and immunological properties of two forms of pertactin, the 69,000-molecular-weight outer-membrane protein of *Bordetella pertussis*. *Infect. Immun.* **61,** 2211-2215.

Graeff, W. H., Deppish, H. and Gross, R. **(1995).** Global regulatory mechanisms affect virulence gene expressions in *Bordetella pertussis*. *Mol. Gen Genet*. **247**, 86-94.

Granowitz, E. V. and Keenholtz, S. L. **(1998)**. Apsuedoepidemic of Alcaligens xylosoxidants attributable to contaminated saline. *Am. J. Infect. Control.* **26**, 146-148.

Griffith, A. H. **(1989).** Permanent brain damage and pertussis vaccination: is the end of the saga insight. *Vaccine*. **7,** 199-210.

Gross, R. and Rappuoli, R. **(1998)**. Positive regulation of pertussis toxin exporesion. Proc. *Natl. Acad. Sci*. **85**, 3913-3917.

Gueirard, P. and Guiso, N. **(1993)**. Virulence of *Bordetella bronchiseptica*: role of adenylate cyclase-hemolysin. *Infect. Immun*. **61**, 4072-4078.

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

Guiso, N., Capiau, C., Carletti, G., Poolman, J. and Hauser, P. **(1999)**. Intranasal murine model of *Bordetella pertussis* infection: Prediction of protection in human infants by acellular vaccines. *Vaccine*. **17**, 2366-2376.

Guiso, N., Szatanik, M. and Rocancourt, M. **(1991)**. Protective activity of *Bordetella* adenylate cyclase-haemolysin against bacterial colonisation. *Microb. Patho*. **11**, 423-431.

Gustafsson, L., Hallander, H. O., Olin, P. Reizenstein, E. and Storsaeter, J. **(1996)**. A controlled trial of a two component acellular, a five-component acellular, a whole cell pertussis vaccine. *N. Engl. J. Med*. **334**, 349-355.

Hallander, H, O. **(1999)**. Microbiological and serological diagnosis of pertussis. *Clin. Infec. Dis.* **28** (Suppl.2): S99-S106.

Hall, E., Parton, R. and Wardlaw, A. C. **(1994)**. Cough production, leucocytosis and serology of rats infected intrabronchially with *Bordetella pertussis*. *J. Med. Microbiol.* **40.** 204-213.

Hamstra, H., Kuipers, B., Schijf-Evers, D., *et al.* **(1995)**. The purification and protective capacity of *Bordetella pertussis* outer membrane proteins. *Vaccine*. **13**(8), 747-752.

Hancock, R. E. W. **(1991).** Bacterial outer membranes: evolving concepts. *ASM News*, **57**(4), 175-182.

Hannah, J. H., Menozzi, F. D., Renauld, G., Locht, C. and Brennan, M. J. **(1994)**. Sulfated glycoconjugate receptors for the *Bordetella pertussis* adhesin filamentous haemagglutinin (FHA) and mapping of the heparin-binding domain on FHA. *Infect. Immun.* **62** (11), 5010-5019.

Hardy, S. J. S. **(1989)**. Biochemical investigation of protein export in *Escherichia coli. J. Cell. Sci. Supp.* **11**, 29-43.

Harvill, E. T., Preston, A., Cotter, P. A., *et al.* In press **(2000).** Multiple roles for *Bordetella* LPS molecules during respiratory tract infection. *Infect. Immun.*

Henderson, B., Poole, S. and Wilson, M. **(1996).** Bacterial modulins: a novel class of virulence factors which cause host tissue pathology by inducing cytokine synthesis. *Microbiol. Rev.* **60**(2), 316-341.

Henderson, I. R., Navarro-Garcia, F. and Nataro, J. P. **(1998).** The great escape: structure and function of the autotransporter proteins. *Trends. Microbiol.* **6**(9), 370-378.

Henderson, I. R. and Nataro, J. P. **(2001)**. Virulence functions of "Autotransporter Proteins". *Infect. Immun.* **69** (3), 1231-1243.

Henderson, I. R. and Owen, P. **(1999)**. The major phase-variable outer membrane protein of *Escherichia coli* structurally resembles the immunoglobulin A1 class of exported protein and is regulated by a novel mechanism involving Dam and OxyR. *J. Bacteriol.* **181**, 21-32.

Herrington, D. A., Hall, R. H., Losonsky, G., Mekalanos, J. J., Taylor, R. K. and Levine, M. M. **(1988)**. Toxin, toxin-coregulated pili, and the toxR regulon are essential for *Vibrio cholerae* pathogenesis in humans. *J. Exp.Med.* **168.** 1487-1492.

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

Hewlett, E. L. **(1995)**. *Bordetella* species In: Mandell, G. L. *et al.* (eds). Principles and practice of infectious diseases, 3rd eds. New York: Churchill Livingstone. pp 2708-2784.

Hewlett, E. L. **(2000)**. *Bordetella* species In: Mandell, G. L. et al. Eds. Principles and practice of infectious diseases, Philadelphia: Churchill Livingstone. pp 2414-2442.

Hirst, T. R. and Welch, R. A. **(1988)**. Mechanisms for secretion of extracellular proteins by Gram-negative bacteria. *Trends. Bio. Sci.* **13,** 263-269.

Hodder, S. L. and Mortimer, E. A. **(1992)**. Epidemiology of pertussis and reactions to pertussis vaccine. *Epidemiol. Rev.* **14**, 243-268.

Holland, B. **(1998)**. Autotransporters: protein contortionists whose carboxy termini translocate their own amino terminal domains. *Trends. Microbiol.* **6**(10), 388-389.

Hoppe, J. E. **(1992)**. Comparison of erythromycin estolate and erythromycin ethylsuccinate for treatment of pertussis. The erythromycin study group. *Pediatr. Infec. Dis. J.* **11,** 189-193.

Hong, Y. Q. and Ghebrehiwet, B. **(1992).** Effect of *Pseudomonas aeuroginosa* elastase and alkaline phosphatase on serum complement and isolated components C1q and C3. *Clin. Immunol. Immunopatho.* **62**, 133-138.

Hong, K., T. Kinoshita., Kozono, H., Pramoonjago, Y., Kim, Y. U and Inoue, K. **(1990).** Inhibition of the alternative C3 convertase and classical C5 convertase of complement by group A streptococcal M protein. *Infect. Immun.* **58**, 2535-2541.

Hopkins, B. A., Skeeles, J. K., Houghten, G. E. and Story, J. D. **(1988)**. Development of an enzyme-linked immunosorbent assay for *Bordetella avium*. *Avian Dis.* **32**(2):353-61.

Horiguchi, Y., Sugimoto, N. and Mastuda, M. **(1994).** *Bordetella bronchiseptica* dermonecrotizing toxin stimulates protein synthesis in an osteoblastic clone, MC3T3-E1 cells. *FEMS Microbiol. Lett.* **120**, 1-2.

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

Hornibrook, J. W. **(1940)**. Nicotinic acid as a growth factor for *Haemophilus pertussis*. *Proc. Soc. Exp. Biol. Med.* **45**, 598-599.

Horstmann, R. D., Sievertsen, H. J., Knobloch. J and Fischetti, V. A. **(1988)**. Antiphagocytic activity of streptococcal M protein: selective binding of complement control protein factor H. *Proc. Natl Acad. Sci. USA*. **85**, 1657-1661.

Hueck, C. J. **(1998)**. Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiol. MolBiol. Rev.* **62**(2), 379-433.

Ikeda, K., Sannoh, T., kawasaki, N., *et al.* **(1987)**. Serum lectin with known structure activates complement through the classical pathway. *J. Biol. Chem.* **262**, 7451.

Ishibashi, Y., Relman, D. A. and Nishikawa, A. **(2001)**. Invasion of human respiratory epithelial cells by *Bordetella pertussis*: a possible role for a filamentous hemagglutinin Arg-Gly-Asp sequence and $\alpha_5\beta_1$ integrin. *Microb. Patho.* **30**, 279-288.

Ishibashi, Y., Claus, S. and Relman, D. A. (**1994**). *Bordetella pertussis* filamentous hemagglutinin interacts with a leukocyte signal transduction complex and stimulates bacterial adherence to monocyte CR3 (CD11b/CD18). *J. Exp. Med.* **180**, 1225-1233.

Jacob-Dubuisson, F., Buisine, C., Mielcarek, N., *et al.* (**1996**). Amino terminal maturation of the *Bordetella pertussis* filamentous hemagglutinin. *Mol. Microbiol.* **19**, 65-78.

Jacob-Dubuisson, F., Hamel, C, E., Saint, N., *et al.* (**1999**). Channel formation by FhaC, the outer-membrane protein is involved in the secretion of the *Bordetella pertussis* filamentous hemagglutinin. *J. Biol. Chem.* **274**, 37731-37735.

Jacob-Dubuisson, F., Kehoe, B., Willery, E., *et al.* **(2000).** Molecular characterization of *Bordetella bronchiseptica* filamentous hemagglutinin and its secretion machinery. *Microbiology*. **146**, 1211-1221.

Jacob-Dubuisson, F., Buisine, C., Willery, E., *et al.* **(1997).** Lack of functional complementation between *Bordetella pertussis* filamentous hemagglutinin and *Proteus mirabilis* HpmA hemolysin secretion machineries. *J. Bacteriol.* **179**(3), 775-83, 1997.

Jarvis, G. A. and Verdos, N. A. **(1987).** Sialic acid of group B *Neisseria meningitidis* regulates alternative complement pathway activation. *Infect. Immun.* **55**, 174-180.

Joiner, K. A. (**1988**). Complement evasion by bacteria and parasites. *Annu. Rev. Microbiol.* **42**, 201-230.

Jones. C. H., Danese, P. N., Pinker, J. S., *et al.*, **(1997).** The chaperone-assisted membrane release and folding pathway is sensed by two signal transduction system. EMBO Journal. **16**(21), 6394-6406.

Jose, J., F, Jahnig and Meyer, T. F. **(1995)**. Common structural features of IgA1 protease-like outer membrane protein autotransporters. *Mol. Microbiol.* **18**, 377-382.

Jose. J., J. Kramer, T. Klauser., *et al.* **(1996).** Absence of periplasmic DsbA oxidoreductase facilitates export of cysteine-containing passenger proteins to the *Escherichia coli* cell surface via the IgA-β autotransporter pathway. *Gene.* **178**:107-110.

Kattar, M. M., Chavez, J. F., Limaye, A. M., *et al.* **(2000)**. Application of 16S rRNA gene sequencing to identify *Bordetella hinzii* as the causative agent of fatal septicaemia. *J. Clin. Microbiol.* **38**, 789-794.

Kaslow, H. R. and Burns, D. L. **(1992)**. Pertussis toxins and target eukaryotic cells: binding, entry, activation. *FASEB J.* **6**, 2684-2690.

Karamanou, S., Vrontou, E., Sianidis, G., *et al.* **(1999)**. A molecular switch in SecA protein couples ATP hydrolysis to protein translocation. *Mol. Bio.* **34**(5), 1133-1145.

Karimova, G. and Ullmann, A. **(1997)**. Characterisation of DNA binding sites for the BvgA protein of *Bordetella pertussis*. *J. Bacteriol.* **179**(11), 3790-3792.

Kathleen A., Craig-Mylius. and A. A. Weiss. **(1999)**. Mutants in the ptlA-H genes of *Bordetella pertussis* are deficient for pertussis toxin secretion. *FEMS.* **179:**479-484.

Kawasaki, N., Kawasaki, T. and Yamashina, I. **(1989)**. A serum lectin (mannan-binding protein) has complement-dependent bacterial activity. *J. Biochem.* **106**, 483.

Kerr, J. R. **(1999)**. Type III (contact-dependent) secretion in Gram-negative bacteria. *Rev. Med. Microbiol.* **10**(3), 155-164.

Kerr, J. R. **(1999)**. Cell adhesion molecules in the pathogenesis of and host defence against microbial infection. *J. Clin. Path; Mol. Pathology*, **52**, 220-230.

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

Kerr, J. R., Rigg, G. P., Mathews, R. C. and Burnie, J. P. **(1999)**. The *BpeI* locus encodes type III secretion machinery in *Bordetella pertussis*. *Microb. Patho.* **27**, 349-367.

Khelef, N., Danve, B., N. guiso et al. **(1993)**. *Bordetella pertussis* and *Bordetella parapertussis*: two immunologically distinct species. *Infect. Immun.* **61**, 486-490.

Khelef, N., Bachelet, C. M., Vargaftig, B. B. and Guiso, N. **(1994)**. Characterization of murine lung inflammation after infection with parental *Bordetella pertussis* and mutants deficient in adhesions or toxins. *Infect. Immun.* **62**, 2893-2900.

Keital, W. A. and Edwards, K. M. **(1995)**. Pertussis in adolescents and adults: time to reimmunize? *Semin. Respir. Infect.* **10**, 51-57.

Keitel, W. A. **(1999)**. Cellular and acellular vaccines in adults. *Clin. Infec. Dis.* **28**, S118-123.

Kinnear, S. M., Boucher, P. E., Stabitz, S. and Carbonetti, N. H. **(1999)**. Analysis of BvgA activation of the pertactin gene promoter in *Bordetella pertussis*. *J. Bacteriol.* **181**(17), 5234-5241.

Kinnear, S. M., Marques, R. R. and Carbonetti, N. H. **(2001)**. Differential regulation of Bvg-activated virulence factors plays a role in *Bordetella pertussis* pathogenicity. *Infect. Immun.* **69** (4), 1983-1992.

Klauser, T., Pohlner, J. and Meyer, T. F. **(1993)**. The secretion pathway of IgA protease-type proteins in gram-negative bacteria. *Bioessays*. **15**, 799-805.

Klauser, T., Otzelberger, K., Pohlner, J. and Meyer, T. F. (1993). Characterization of the *Neisseria* IgA-β core. The essential unit for outer membrane targeting and extracellular protein secretion. *J. Mol. Bio.* 234, 579-593.

Koebnik, R. (1999). Structural and functional roles of the surface-exposed loops of the β-barrel membrane protein OmpA from *Escherichia coli*. *J. Bacteriol.* 181(12), 3688-3694.

Kolb, W. P. and Muller-Eberhard, H. J. (1975). The membrane attack mechanism of complement. Isolation and subunit composition of the C5b-9 complex. *J. Exp. Med.* 141, 724-735

Kreuger, K. M. and Barbieri, J. T. (1995). The family of bacterial ADP-ribosylating exotoxins. *Clin. Microbiol. Rev.* 8, 34-47.

Kurzchalia, T. V., Wiedmann, M., Girshovich, A. S. *et al.* (1986). The signal sequence of nascent preprolactin interacts with the 54K polypeptide of the signal recognition particle. *Nature.* 320(6063), 634-636.

Lacey, B. W. (1960). Antigenic modulation in *Bordetella pertussis*. *J. Hygiene.* 58, 57-93.

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

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227, 680-685

Lattemann, C. L., Maurer, J., Gerland, E. and Meyer, T. F. (2000). Autodisplay: functional display of active β-lactamase on the surface of *Escherichia coli* by the AIDA-1 autotransporter. *J. Bacteriol.* 182(13), 3276-3733.

Lebbar, S., Caroff, M., Szabo, L., Merienne, C. and Szilogyi, L. (1994). Structure of a hexasaccharide proximal to the hydrophobic region of lipopolysaccharides present in *Bordetella pertussis* endotoxin preparations. *Carbohydrate. Res.* 259(2), 257-275.

Le Blay, K., Caroff, M., Richards, J. C., Perry, M. B. and Chaby, R. (1994). Specific and cross-reacting monoclonal antibodies to *Bordetella parapertussis* and *Bordetella bronchiseptica* lipopolysaccharides. *Microbiology.* 140, 2459-2465.

Leininger, E., Ewanowich, C. A., Bhargava, A., *et al.* (1992). Comparative roles of the Arg-Gly-Asp sequence present in the *Bordetella pertussis* adhesins pertactin and filamentous haemagglutinin. *Infect. Immun.* 60(6), 2380-2385.

Leininger, E., Roberts, M., Kenimer, J. G., *et al.* (1991). Pertactin, an Arg-Gly-Asp-containing *Bordetella pertussis* surface protein that promotes adherence of mammalian cells. *Proc. Natl Acad. Sci.USA.* 88, 345-349.

Lewthwaite, J., Skinner, A. Henderson, B. (1998). Are molecular chaperones microbial virulence factors?. *Trends. Microbiol.* 6(11), 426-428.

Li, L. J., Dougan, G., Novotny, P., and Charles, I. G. (1991). P.70 pertactin, an outer-membrane protein from *Bordetella parapertussis*: cloning, nucleotide sequence and surface expression in *Escherichia coli. Mol. Microbiol.* 5(2), 409-417.

Locht, C. and Keith, J. M. (1986). Pertussis toxin gene: nucleotide sequence and genetic organization. *Science.* 6, 232(4755): 1258-64.

Locht, C., Bertin, P., Menozzi, F. D. and Renauld, G. (1993). The filamentous haemagglutinin, a multifaceted adhesin produced by virulent *Bordetella* spp. *Mol. Microbiol.* 9(4), 653-660.

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

Lory, S. (1998). Secretion of the proteins and assembly of bacterial surface organelles:shared pathways of extracellular protein targeting. *Curr. Opin. Microbiol.* 1, 27-35.

Loveless, B. J. and Saier M. H. (1997). A novel family of channel-forming, autotransporting, bacterial virulence factors. *Mol. Membr. Bio.* 14, 113-123.

Luker, K. E., Collier, J. L., Kolodziej, E. W., *et al.* (1993). *Bordetella pertussis* tracheal cytotoxin and other muramyl peptides: distinct structure-activity relationships for respiratory epithelial cytopathology. *Proc. Natl Acad. Sci. USA*. 90(6), 2365-2369.

Mahon, B. P., Ryan, M. S., Griffin, F. and Mills, K. H. G. (1996). Interleukin-12 is produced by macrophages in response to live or killed *Bordetella pertussis* and enhances the efficacy of an acellular pertussis vaccine by promoting induction of Th1 cells. *Infect. Immun.* 64(12). 5295-5301.

Mahon, B, P. and Mills, K. H. G. (1999). Interferon-γ mediated immune effector mechanisms against *Bordetella pertussis. Immunol. Lett.* 66, 213-217

Makoff, A. J., Oxer, M. D., Ballantine, S. P., *et al.* (1990). Protective surface antigen P69 of *Bordetella pertussis*: Its characterisation and very high level expression in *Escherichia coli. Biotechnol.* 8, 1030-1033.

Martin, D., Peppler, M. S. and Brodeur, B. R. (1992). Immunological characterization of the lipoligosaccharide B band of *Bordetella pertussis. Infect. Immun.* 60, 2718-2725.

Martinez de Tejada, G., Cotter, P. A., Heininger, U. *et al.* (1998). Neither the Bvg⁻ phase nor the vrg6 locus of the *Bordetella pertussis* is required for respiratory tract infection in mice. *Infect. Immun.* 66, 2762-2768.

Matoglio B. and B. Dobberstein. (1998). Signal sequences: more than just greasy peptides. *Trends. Cell. Bio.* 8, 410-415.

Matsushita, M. and Fujita, T. (1992). Activation of the classical complement pathway by mannose-binding protein in association with a novel C1s-like serine protease. *J. Expt. Med.* 176, 1497-1502.

Mattoo, S., Miller, J. F. and Cotter, P. A. (2000). Role of *Bordetella bronchiseptica* fimbriae in tracheal colonization and development of a humoral immune response. *Infect. Immun.* 68(4), 2024-2033.

Maurer, J., Jose, J. and Meyer, T. F. (1997). Autodisplay: One-component system for efficient surface display and release of soluble recombinant proteins from *Escherichia coli*. *J. Bacteriol.* 179(3), 794-804.

Maurer, J., Jose, J. and Meyer, T. F. (1999). Characterization of the essential transport function of the AIDA-I autotransporter and evidence supporting structural predictions. *J. Bacteriol.* 181(22), 7014-7020.

Mazengia, E., Silva, E. A., Peppe, J. A., *et al.* (2000). Recovery of *Bordetella holmesii* from patients with pertussis-like symptoms: Use of pulse-field gel electrophoresis to characterize circulating strains. *J. Clin. Microbiol.* 38(6), 2330-2333.

McMillan, D. J., Shojaei, M., Chhatwal, G. S., *et al.* (1996). Molecular analysis of the bvg-repressed urease of the *Bordetella bronchiseptica*. *Microb. Patho.* 21, 379-394.

McGillivary, D. M., Coote, J. G. and Parton, R. (1989). Cloning of the virulence regulatory (*vir*) locus of *Bordetella pertussis* and its expression in *Bordetella bronchiseptica*. *FEMS. Microbiol. Lett.* 65, 333-338.

McGuirk, P. and Mills, K. H. (2000). A regulatory role of interleukin 4 in differential inflammatory responses in the lungs following infection of mice primed Th1- or Th2-inducing pertussis vaccines. *Infect. Immun.* 68, 1383-1390.

Mecsas, J. J. and Strauss, E. J. (1996). Molecular mechanisms of bacterial virulence type III secretion and pathogenicity islands. *Emerg. Infect. Dis.* 2, 270-288.

Meerman, H. J. and Georgiou, G. (1994). Construction and characterisation of a set of *Escherichia coli* strains deficient in all known loci affecting the proteolytic stability of secreted recombinant proteins. *Bio/Technology*. 12, 1107-1110.

Merkel, T. J., Stibitz, S., Keith, M. L. and Shahin, R. (1998). Contribution of regulation by the *bvg* locus to respiratory infection of mice by *B. pertussis*. *Infect. Immun.* 66, 4367-4373.

Mertsola, J. (1985). Mixed outbreak of *Bordetela pertussis* and *Bordetella parapertussis* infection in Finland. *Eur. J. Clin. Microbiol.* 4, 123-128.

Menozzi, F. D., Boucher, P.E., Riveau, G., *et al.* (1994). Surface- associated filamentous haemagglutinin induces autoagglutination of *Bordetella pertussis*. *Infect. Immun.* 62, 769-778.

Michiels, T., Wattiau, P., Brasseur, R., *et al.* (1990). Secretion of Yop proteins by *Yersinia*. *Infect. Immun.* 58, 2840-2849.

Mills, L., Morris, C. A., Sheehan, C. *et al.*, (1993). Vitronectin-mediated inhibition of complement: evidence for different binding sites for C5b-7 and C9. *Clin. Exp. Immunol.* 92, 114-119.

Miller, V. L., Bliska, J. B. and Falkow, S. (1990). Nucleotide sequence of the *Yersinia enterocolitica ail* gene and characterization of the Ail protein product. *J. Bacteriol.* 172(2), 1062-1069.

Miller, J. F., S. A. Johnson, W. J. Black., *et al.* (1992). Isolation and analysis of constitutive sensory transduction mutations in the *Bordetella pertussis bvgS* gene. *J. Bacteriol.* 174, 970-979.

Miller, E., Vurdien, J. E. and White, J. M. (1992). The epidemiology of pertussis in England and Wales. *Commun Dis Rep CDR Rev.* 2(13):R152-4

Miller, V. L., Beer, K. B., Heusipp, G., *et al.* (2001). Identification of regions of Ail required for invasion and serum resistance phenotypes. *Mol. Microbiol.* 4(5), 1053-1062.

Mills, K. H., Ryan, M., Ryan, E. and Mahon, B. P. (1998). A murine model in which protection correlates with pertussis vaccine efficacy in children complementary role for humoral and cell-mediated immunity in protection against *Bordetella pertussis*. *Infect. Immun.* 66, 594-602.

Mills, K.H. (2001). Immunity to *Bordetella pertussis*. *Microb. Infect.* 3, 655-677.

Millis, L., Morris, C. A., Sheehan, M. C., *et al.* (1993). Vitronectin-mediated inhibition of complement: evidence for different binding sites for C5b-7 and C9. *Clin. Expt. Immunol.* 92, 114-119

Moffitt, M. C. and Frank, M. M. (1994). Complement resistance in microbes. Springer semin. *Immunopatohl.* 15, 327-344.

Monji, N., Stebbins, M. R., McCoy, D. W. and Kuo, J. S.-C. (1986). Isolation of the outer membrane components of *Bordetella pertussis* which enhance the immunogenicity of *Haemophilus influenzae* type b capsular polysaccharide polyrybosyl ribotol phosphate. *Infect. Immun.* 51(3), 865-871.

Mooi, F. R., van Oirschot, H., Heuvelman, K. and van der Heide, H. G. J. (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(2), 670-675.

Mountzouros, K. T., Kimura, A. and Cowell, J. L. (1992). A bactericidal monoclonal antibody specific for lipoligopolysaccharide of *Bordetella pertussis* reduces colonization of the respiratory tract of mice after aerosol infection with *Bordetella pertussis*. *Infect. Immun.* 60, 5316-5318.

Muller, F. M., Hoppe, J. and Wirsing, von Koenig, C. (1997). Laboratory diagnosis of pertussis: State of the art in 1997. *J. Clin. Microbiol.* 35, 2435-2443.

Nikai, T., Swata, A. and Kume, K. (1985). Intracellular locations of dermonecrotic toxins in *Pasteurella multocida* and in *Bordetella bronchiseptica*. *Am. J. Vet. Res.* 46, 870-874.

Nunn, D. (1999). Bacterial type II protein export and pilus biogenesis: more than just homologies? *Trends. Cell Bio.* 9, 402-408.

Oliver, D. C. and Fernandez, R. C. (2002). Antibodies to BrkA augment killing of *Bordetella pertussis*. *Vaccine*. 20, 235-241.

Ohnishi, Y. M.,Nishiyama, M., Horinouchi. S. and Beppu, T. (1994). Involvement of the COOH-terminal pro-sequence of *Serratia marcescens* serine protease in the folding of the mature enzyme. *J. Biol. Chem.* 269, 32800-32806.

O'Toole, P. W., Austin, J. W. and Trust, T. J. (1994). Identification and molecular characterization of a major ring-forming surface protein from the gastric pathogen *Helicobacter mustelae*. *Mol. Microbiol.* 11, 349-361.

Parton, R. (1998). Chapter 9 *Bordetella*. Topley and Wilson's Microbiology and Microbial Infections, Volume 2, 901-918.

Parton, R. (1999). Review of the biology of *Bordetella pertussis*. *Biologicals*. 27, 71-76.

Parton, R., Hall, E. and Wardlaw, A. C. (1994). Responses to *Bordetella pertussis* mutant strains and to vaccination in the coughing rat model of pertussis. *J. Med. Microbiol.* 40, 307-312.

Passerini de Rossi, B., Friedman, L., Luis Gonzalez Flecha, F., *et al.* (1999). Identification of *Bordetella pertussis* virulence-associated outer membrane proteins. *FEMS Microbiol. Lett.* 172, 9-13.

Perlman, D. and Halvorson, H. O. (1983). A putative signal peptidase recognition site and sequence in eukaryotic and prokaryotic signal peptides. *J. Mol. Bio.* 167, 391-409.

Persson, C. G. (1991). Plasma exudation in the airways: mechanisms and function. *Eur. Res. J.* 4(10), 1268-74.

Pierson, D. E. and Falkow, S. (1993). The ail gene of *Yersinia enterocolitica* has a role in the ability of the organism to survive serum killing. *Infect. Immun.* 61(5), 1846-1852.

Pilz, D., Vocke, T., Heesemann, J. and Brade, V. (1992). Mechanism of YadA-mediated serum resistance of *Yersinia enterocolitica* serotype O3. *Infect. Immun.* 60, 189-195.

Plaut, A. G., Qiu, J., Fine, D. H., *et al.* (2002). *Proceedings of the International Pasteurellaceae Society Conference*, Banff National Park, Canada. p15.

Pohlner, J., Halter, R., Beyreuther, K. and Meyer, T. F. (1987). Gene structure and extracellular secretion of *Neisseria gonorrhoeae* IgA protease. *Nature*, 325, 458-462.

Pollard, A. J. and Frasch, A. (2001). Development of natural immunity to *Neisseria meningitidis*. *Vaccine*. 19, 1327-1346.

Poulsen, K., Brandt, J., *et al.*, (1989). Cloning and sequencing of the immunoglobulin A1 protease gene (*iga*) of *Haemophilius influenzae* serotype b. *Infect. Immun.* 57, 3097-3015.

Preston, A., Mandrell, R. E., Gibson, B. W and Apicella, M. A. (1996). The lipooligosaccharides of pathogenic gram-negative bacteria. *Crit Rev Microbiol.* 22(3), 139-80.

Preston, A and Maskell, D. J. (2002). A new era of research into *B. pertussis* pathogenesis. *J. Infect.* 44, 13-16.

Provence, D. L. and Curtis III, R. (1995). Isolation and characterisation of a gene involved in haemagglutination by an avian pathogenic *Escherichia coli* strain. *Infect. Immun.* 62, 1369-1380.

Pugsley, A. P. **(1993)**. The complete general secretory pathway in gram-negative bacteria. *Microbiol. Rev.* **57**(1), 50-108.

Pugsley, A. P., Francetic, O., Possot, O. M., *et al.* **(1997)**. Recent progress and future directions in studies of the main terminal branch of the general secretory pathway in Gram-negative bacteria- a review. *Gene.* **192**, 13-19.

Pullinger, G. D., Adams, T. E., Mullan, P. B., *et al.* **(1996).** Cloning, expression and molecular characterization of the dermonecrotic toxins gene of *Bordetella* spp. *Infect. Immun.* **64**, 4163-4171.

Rappuoli, R., Pizza, M., Covacci, A., *et al.* **(1992a)**. Recombinant acellular pertussis vaccine from the laboratory to the clinic: improving the quality of the immune response. *FEMS. Microbiol. Immunol.* **5**, 161-170

Rappuoli, R., Pizza, M., Magistris, M. T., *et al.* **(1992b)**. Development and clinical testing of an acellular pertussis vaccines containing genetically detoxified pertussis toxin. *Immunobiol.*. **184**, 230-239.

Rappuoli, R. **(1999).** The vaccine containing recombinant pertussis toxin induces early and long-lasting protection. *Biologicals*. **27**(2): 99-102.

Rambow, A. A., Fernandez, R. C. and Weiss, A. A. **(1998)**. Characterisation of BrkA expression in *Bordetella bronchiseptica*. *Infect. Immun.* **66**(8), 3978-3980.

Rautemaa, R., Jarvis, G. A., Marnila, P. and Meri, S. **(1998).** Acquired resistance of *Escherichia coli* to complement lysis by binding of glycophosphoinositol-anchored protectin (CD59). *Infect. Immun.* **66**, 1928-1933.

Relman, D., Domenighini, M., Tuomanen, E., *et al.* **(1989)**. Filamentous haemagglutinin of *Bordetella pertussis*: Nucleotide sequence and crucial role in adherence. *Proc. Natl Acad. Sci. USA*. **86**, 2637-2641.

Relman, D., Tuomanen E., Falkow, S., *et al.* **(1990)**. Recognition of a bacterial adhesion by an integrin: macrophage CR3 (alpha M beta 2, CD11b) binds filamentous hemagglutinin of *Bordetella pertussis*. *Cell.* **61**(7), 1375-1382.

Renauld-Mongenie, G., Cornette, J., Mielcarek, N. *et al.* **(1996)**. Distinct roles of the N-terminal and C-terminal precursor domains in the biogenesis of the *Bordetella pertussis* filamentous haemagglutinin. *J. Bacteriol.* **178**, 1053-1060.

Ricci, S., Rappuoli, R. and Scarlato, V. **(1996)**. The pertussis toxin liberation genes of *Bordetella pertussis* are transcriptionally linked to the pertussis toxin operon. *Infect. Immun.* **64**(4), 1458-1460.

Rinder, C. S., Rinder, H. M., Smith, M. J., *et al.* **(1999)**. Selective blockade of membrane attack complex formation during simulated extracorporeal circulation inhibits platelet but not leukocyte activation. *J. Thorac. Cardiovasc. Sur.* **118**, 460-466.

Roberts, M., Fairweather, N. F., Leininger, E., *et al.* **(1991)**. Construction and characterisation of *Bordetella pertussis* mutants lacking the *vir*-regulated P.69 outer-membrane protein. *Mol. Microbiol.* **5**(6), 1393-1404.

Roberts, M., Tite, J. P., Fairweather, N. F., *et al.* **(1992)**. Recombinant P.69/pertactin: immunogenicity and protection of mice against *Bordetella pertussis* infection. *Vaccine*, **10**(1), 43-48.

Roberts, M., Bacon, A., Rappuoli. R., *et al.* **(1995)**. A mutant pertussis toxin molecule that lacks ADP-ribosyltransferase activity, PT-9K/129G, is an effective mucosal adjuvant for an intranasally delivered proteins. *Infect. Immun.* **63**, 2100-2108.

Robinson, A. and Funell, S. G. **(1992)**. Potency testing of acellular pertussis vaccines. *Vaccine.* **10**. 139-141.

Robinson, A., Duggleby, C. J., Gorringe, A. R. and Livey, I. **(1986)**. Antigenic variation in *Bordetella pertussis*. In: *Antigenic variation in infectious diseases,* edited by Birkbeck, T. H., and Penn, C. W. (eds). Oxford: IRL press. Vol. **19**, pp 147-161.

Robinson, A., Gorringe, A. R. Funnel, S. G. and Fernandez, M. **(1989)**. Serospecific protection of mice against intranasal infection with *Bordetella pertussis*. *Vaccine*. **7**, 321-324.

Rostrand, K. S. and Esko, J. D. **(1997)**. Microbial adherence through peptidoglycans. *Infect. Immun.* **65**(1), 1-8.

Roy, C. R., Miller, J. F and Falkow, S. **(1989)**. The *bvgA* gene of *Bordetella pertussis* encodes a transcriptional activator required for coordinate regulation of several virulence genes. *J. Bacteriol.* **171**, 6338-6344.

Ryan, M. S., Griffin, F., Mahon, B. and Mills, K. H. **(1997)**. The role of S-1 and B oligomer components of pertussis toxin in its adjuvant properties for Th1 and Th2 cells. *Biochem. Soc. Trans.* **25**, 126s.

Salmond, G. P. C. and Reeves, P. J. **(1993)**. Membrane traffic wardens and protein secretion in Gram-negative bacteria. *Trends. Bio. Sci.* **18**, 7-12.

Sandkvist, M. **(2001)**. Type II secretion and pathogenesis. *Infect. Immun.* **69**, 3523-3535.

Sandkvist, M. and Bagdasarian, M **(1996)**. Secretion of recombinant proteins by Gram-negative bacteria. *Curr. Opin. Biotech.* **7**, 505-511.

Sandros, J. and Tuomanen, E. **(1993)**. Attachment factors of *Bordetella pertussis*: mimicry of eukaryotic cell recognition molecules. *Trends. Microbiol.* **1**(5), 192-195.

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

Sato, Y and Sato, H. **(1988)**. Animal models of pertussis. In: Wardlaw, A. C. and Parton, R. (Edi). *Pathogenicity and Immunity in Pertussis*. Cheichester: John Wiley and Sons pp. 309-325.

Sato, H and Sato, Y. **(1988).** Protective activities in mice of monoclonal antibodies against pertussis toxin. *Infect. Immun.* **58**, 3369-3374.

Saukkonen, K., Burnette, W. N., Mar, V. L., *et al.* **(1992)**. Pertussis toxin has eukaryotic-like carbohydrate recognition domains. *Proc. Natl Acad. Sci. USA.* **89,** 118-122.

Scarlato, V., Prugnola, A., Arico, B. and Rappuoli, R. **(1990)**. Positive transcriptional feedback at the *bvg* locus controls expression of virulence factors in *Bordetella pertussis. Proc. Natl Acad. Sci.USA*, **87**, 6753-6757.

Scarlato, V., Arico, B and Rappuoli, R. **(1993)**. DNA topology affects transcriptional regulation of the pertussis toxin gene of *Bordetella pertussis* in *Escherichia coli* and *in vitro. J. Bacteriol.* **175**, 4764-4771.

Schmidt, G., Goehring, U. M., Schirmer, J., *et al.* **(1999)**. Identification of the C-terminal part of *Bordetella* dermonecrotic toxin as a transglutaminase for rho GTPases. *J. Biol. Chem.* **274**, 31875-31881.

Schweinle, J. E., Ezekowitz, R. A. B., Tenner, A. J., *et al.* **(1989)**. Human mannose binding protein activates the alternative complement pathway and serum bactericidal activity on a mannose-rich isolate of *Salmonella. J. Clin. Invest.* **84**, 1821.

Segal, E., Cha, J., Lo, J., Falkow, S. and Tompkins, L. **(1999)**. Altered states: Involvement of the CagA in the induction of host cellular growth changes by *Helicobacter pylori. Proc. Natl Acad. Sci. USA.* **96**, 14559-14564.

Settles, M. and Martienssen, R. (1998). Old and new pathways of protein export in chloroplasts and bacteria. *Trends. Cell. Bio.* **8**, 494-500.

Shareck, F. and Cameron, J. **(1984)**. Cloning of Bordetella pertussis outer membrane proteins in *Escherichia coli. J. Bacteriol.* **159**(2), 780-782.

Shahin, R. D., Brenan, M. J., Li, Z. M. Meade. B. D and Manclark, C. R. **(1990)**. Characterisation of the protective capacity and immunogenicity of the 69-kDa outer membrane protein of *Bordetella pertussis*. *J. Expt. Med.* **171**, 63-73.

Shahin, R. D., Hammel, J., Leef, M. F. and Brodeur, B. R. **(1994)**. Analysis of protective and non-protective monoclonal antibodies specific for *Bordetella pertussis* lipoligosaccharide. *Infect. Immun.* **62**, 722-725.

Shahin, R., Leef, M., Eldridge, J., *et al.* **(1995)**. Adjuvanticity and protective immunity elicited by *Bordetella pertussis* antigens encapsulated in poly (DL-lactide-co-glycolide) microspheres. *Infect. Immun.* **63**(4), 1195-2000.

Shanon, J. L and Fernandez, R. C. **(1999)**. The C-terminal domain of the *Bordetella pertussis* autotransporter BrkA forms a pore in lipid bilayer membranes. *J. Bacteriol.* **181**, 5838-5842.

Shea, J. E., Hensel, M., Gleeson; C. and Holden, D. W. **(1996)**. Identification of a virulence locus encoding a second type III secretion system in *Salmonella typhimurium*. *Proc. Natl. Acad. Sci. USA*. **93**(6), 2593-2597.

Silber, K. R., Keiler, K. C. and Sauer, R. T. **(1992)**. Tsp: A tail-specific protease that selectively degrades protein with non polar C termini. *Proc. Natl Acad. Sci. USA*. **89**, 295-299.

Sindt, K. A., Hewlett, E. L., Redpath, G. T., *et al.* **(1994).** Pertussis toxin activates platelets through an interaction with platelet glycoprotein Ib. *Infect Immun*. **62**(8):3108-14.

Skeeles, J. K., Arp L. H. **(1988)**. *In: Clanck, B. W., Barnes , H. J. et al (Eds).* Diseases of poultry, lowa state university press, Ames. 275-288.

Soane, M. C., Jackson, A., Maskell, D., *et al.* **(2000)**. Interaction of *Bordetella pertussis* with human respiratory mucosa *in vitro*. *Respir. Med.* **94**, 791-799.

Spangrude, G. J., Araneo, B. A. and Daynes, R. A. (**1985**. Site-selective homing of antigen-primed lymphocyte populations can play a crucial role in the efferent limb of cell-mediated immune responses *in vivo*. *J. Immunol*. **134**, 2900-2907.

Stainer, D. W and Scholte, M. J. (**1971**). A simple chemically defined medium for the production of phase I *Bordetella pertussis*. *J. Gen. Microbiol*. **63**, 211-220.

St. Geme III, J. W., Morena, M. L. d. l. and Falkow, S. (**1994**). A *Haemophilus influenzae* IgA protease-like protein promotes intimate interaction with human epithelial cells. *Mol. Microbiol*. **14**(2), 217-233.

Stein, P. E., Boodhoo, A., Armstrong, G. D., Cockle, S. A., *et al*. (**1994**). The crystal structure of the pertussis toxin. *Structure*. **2**, 45-57.

Stein, M., Rappuoli, R and Covacci, A. (**2000**). Tyrosine phosphorylation of the *Helicobacter pylori* CagA antigen after Cag-driven host cell translocation. *Proc. Natl Acad. Sci. USA*. **97**, 1263-1268.

Stenson, T. H. and Weiss. A. A. (**2002**). DsbA and DsbC are required for secretion of pertussis toxin by *B. pertussis*. *Infect. Immun*. **70**(5), 2297-2303.

Stibitz, S. (**1994**). Use of conditionally counterselectable suicide vectors for allelic exchange. *Meth. Enzymol*. **235**, 458-465.

Stibitz, S. (**1994**). Mutations in the bvgA gene of *B. pertussis* that differentially affect regulation of virulence determinants. *J. Bacteriol*. **176** (18), 5615-5621.

Stibitz, S., Black, W. Falkow, S. (**1986**). The construction of a cloning vector designed for gene replacement in *Bordetella pertussis*. *Gene*. **50**(1-3), 133-140.

Stibtz, S., Weiss, A. A and Falkow, S. (**1988**). Genetic analysis of a region of *Bordetella pertussis* encoding filamentous hemagglutinin and the pleiotropic regulatory locus *vir*. *J. Bacteriol*. **170**, 2904-2913.

Stibitz, S., Weiss, A. A. and Falkow, S. (**1988**). Genetic analysis of a region of the *Bordetella pertussis* chromosome encoding filamentous haemagglutinin and the pleiotropic regulatory locus *vir*. *J. Bacteriol*. **170**(7), 2904-2913.

Stibitz, S and Yang, M. S. (**1991**). Subcellular localization and immunological detection of proteins encoded by the *vir* locus of *Bordetella pertussis*. *J. Bacteriol*. **173**, 4288-4296.

Stibitz, S and Garletts, T. L. (**1992**). Derivation of the physical map of the *Bordetella pertussis* Tohama I. *J. Bacteriol*. **174,** 7770-7777.

Stockbauer, K. E., Fuchslocher, B., Miller, J. F. and Cotter, P. A. (**2001**). Identification and characterization of BipA, a Bordetella Bvg-intermediate phase protein. *Mol. Microbiol.* **39** (1), 65-78

Storsaeter, J., Hallander, HO., Gustafsson, L. and Olin, P. (**1998**). Levels of anti-pertussis antibodies related to protection after house hold exposure to *Bordetella pertussis*. *Vaccine*. **16**, 1907-1916.

Strauss, E. (**1999**). New clues to whooping cough pathology. *Science*. **285**, 811-812.

Strugnell, R., Dougan, G., Chatfield, S., Charles, I., *et al*. (**1992**). Characterisation of a *Salmonella typhimurium aro* vaccine strain expressing the P.69 antigen of *Bordetella pertussis*. *Infect. Immun.* **60**(10), 3994-4002.

Suhr, M., Benz, I. And Schmidt, M. A. (**1996**). Processing of the AIDA-1 precursor; removal of AIDA-1 and evidence for the outer membrane anchoring as a β-barrel structure. *Mol. Bio.* **22**, 31-42.

Suzuki, T., Lett, M. and Sasakawa, C. (**1995**). Extracellular transport of VirG protein in *Shigella*. *J. Bio. Chem*. **270**, 30874-30880.

Taylor, P. W. (**1992**). Complement-mediated killing of susceptible Gram-negative bacteria: an elusive mechanism. *Exp. Clin. Immunogenet*. **9**, 48-56.

Temple, L. M., Weiss, A. A., Walker, K. E., *et al.* **(1998)**. *Bordetella avium* virulence measured *in vivo* and *in vitro*. *Infect. Immun.* **66**, 5244-5251.

Touimi, B. Z., Sansonetti, P. J. and Parsot, C. **(1995)**. SepA, the major extracellular protein of *Shigella flexneri*: autonomous secretion an involvement in tissue invasion. *Mol. Microbiol.* **17**, 123-135.

Towbin, H. Staehelin, T. and Gordon, J. **(1979)**. Electrophoretic transfer of proteins from nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA*. **76**(9), 4350-4354.

Trollfors, B., Taranger, J., Lagergard, T., *et al.* **(1995)**. A placebo controlled trial of a pertussis toxoid vaccine. *N. Engl. J. Med.* **333,** 1045-1050.

Trun, N. J. and Silhavy, T. J. **(1989)**. The genetics of protein targeting in *Escherichia coli* K12. *J. Cell Sci. (Suppl).* **11**, 13-28.

Tuomanen, E. I., Zapiain, L. A., Galvan, P. and Hewlett, E. L. **(1984)**. Characterization of antibody inhibiting adherence of *Bordetella pertussis* to human respiratory epithelial cells. *J. Clin. Microbiol.* **20**(2), 167-170.

Tuomanen E. S. J. **(1988)**. *Bordetella pertussis* adhesins. *In A. C. Wardlaw & R. Parton (Eds.), Pathogenesis and Immunity in Pertussis* (chapter 4). John Wiley and Sons.

Tuomanen, E. S. J. **(1993)**. Attachment factors of *Bordetella pertussis*: mimicry of eukaryotic cell recognition molecules. *Trends. Microbiol.* **1**(5), 192-196.

Tuommassen, J., Bosch, D., De Cock, H. *et al.*, **(1989).** Involvement of membrane lipids in protein export in *Escherichia coli. J. Cell Sci.* **11**, 73-83.

Turner, M. W. **(1996).** Mannose-binding lectin: the pluripotent molecule of the innate immune system. *Immunol. Today*. **17,** 532-540.

Tyrrell, G. J., Peppler, M. S., Bonnah, R. A., *et al.* **(1989)**. Lectin-like properties of pertussis toxin. *Infect. Immun.* **57**, 1854-1857.

Ui, M. **(1988)**. The multiple biological activities of pertussis toxin. In: Wardlaw, A. C and Parton, R (eds). Pathogenesis and Immunity in Pertussis. Chichester: John Wiley and Sons, pp. 121-172.

Uhl, M. A. and Miller, J. F. **(1995)**. *Bordetella pertussis* BvgAS. Virulence control system. *In T. Silhavy and J. A. Hoch. (Eds), Signal transducing genetic switches ASM. press Washington, D. C* 333-349.

Vandamme, P., Hommez, J., Vancanneyet, M., *et al.* **(1995)**. *Bordetella hinzii* sp. nov. isolated from poultry and humans. *Intl. J. Syst. Bacteriol.* **45**, 37-45.

Vandamme, P., Heyndrickx, M., Vancanneyt, M., *et al.* **(1996)**. *Bordetella trematum* sp. nov., isolated from wounds and ear infections in humans, and reassessment of *Alcaligenes denitrificans* Ruger and Tan 1993. *Int. J. Syst. Bacteriol.* **46**(4), 849-858.

Van den Akker, W. M. R. (1998). Lipopolysaccharide expression within the genus *Bordetella*: influence of temperature and phase variation. *Microbiology.* **144,** 1527-1535.

Van Den Berg, B. M., Beekhuizen, H., Willems, R. j. L., *et al.* **(1999)**. Role of *Bordetella pertussis* virulence factors in adherence to epithelial cell lines derived from the human respiratory tract. *Infect. Immun.* **67**(3), 1056-1062.

Van Gijsegem, F., Genin, S. and Boucher, C. **(1993)**. Conservation of secretion pathways for pathogenicity determinants of plant and animal bacteria. *Trends. Microbiol.* **1**(5), 175-180.

Veiga, E., de Lorenzo, V. and Fernandez, L. A. **(1999)**. Probing secretion and translocation of a β-autotransporter using a reporter single chain antibody Fv as a cognate passenger domain. *Mol. Microbiol.* **33**(6), 1232-1243.

Von Henge, G. **(1986)**. A new method for predicting signal sequence cleavage sites. *Nucleic Acids Res.* **14**(11), 4683-4690.

Von Wintzingerode, F., Schattke, A., Siddiqui, R. et al. (2001 in press). *Bordetella petrii* sp. Nov., isolated from an anaerobic bioreactor and emended description of the genus Bordetella. *Int. Jour. Syst. Evol. Microbiol.*

Waittiau, P., Woestyn, S. and Corneils, G. R. **(1996)**. Customized secretion chaperones in pathogenic bacteria. *Mol. Microbiol.* **20**, 255-262.

Wandersman, C. **(1992)**. Secretion across the bacterial outer membrane. *Trends. Gen.* **8**(9), 317-332.

Wardlaw, A. C. **(1988)**. Virulence factors and species specificity in *Bordetella*. In: Donachie, W., Griffiths, E and Stephen, J (eds). *Bacterial Infections of the Respiratory tract and Gastrointenstinal Tract*. Oxford: IRL press, pp. 41-56.

Wardlaw. A. C. and Parton, R, (Eds). **(1988)**. The host-parasite relationship in pertussis, *Pathogenesis and Immunity in pertussis*, John Wiley & Sons, Chichester. 327-352

Walker, K. E. and Weiss, A. A. **(1994)**. Characterisation of the dermonecrotic toxin in members of the genus *Bordetella*. *Infect. Immun.*, **63**(9), 3817-3828.

Wardlaw, A. C. and Parton, R. (1988). Pathogenesis and immunity in *Pertussis*. *Chichester*: John Wiley.

Weingart, C. L. and Weiss, A. A. **(2000)**. *Bordetella pertussis* virulence factors affect phagocytosis by human neutrophils. *Infect. Immun.* **68**, 1735-1739.

Weiss, A. A and Falkow, S. **(1984)**. Genetic analysis of phase change in *Bordetella pertussis*. *Infec. Immun.* **43**, 263-269.

Weiss, A. A., Hewlett, E. L., *et al.* **(1983).** Tn5-induced mutations affecting virulence factors of *Bordetella pertussis*. *Infect. Immun.* **42**, 33-41.

Weiss, A. A., Hewlett, E. L., Myers, G. A and Falkow, S. **(1984)**. Pertussis toxin and extracytoplasmic adenylate cyclase as a virulence factor of *Bordetella pertussis*. *J. Infect. Dis*. **150**, 219-222.

Weiss, A. A., Hewlett, E. L., Myers, G. A and Falkow, S. **(1985)**. Genetic studies of the molecular basis of whooping cough. *Dev Biol Stand.* **61**:11-9.

Weiss, A. A. and Goodwin, M.S.M. **(1989)**. Lethal infection by *Bordetella pertussis* mutants in the infant mouse model. *Infect. Immun.* **42**, 33-41.

Weiss, A. A., Johnson, F. D and Burns, D. L. **(1993)**. Molecular characterization of an operon required for pertussis toxin secretion. *Proc. Natl Acad. Sci. USA*. **90**, 2970-2974.

Weiss, A. A. **(1994)**. Chapter22: Unorthodox secretion by Gram-Negative bacteria. In: V. L. Miller, J. B., Kaper, D. A., Portnoy, R.R. Isberg (eds), Molecular Genetics of Bacterial Pathogenesis (pp.341-349). Washington: *ASM.*

Weiss, A. A., Mobberley, P. S., Fernandez, R. C. and Mink, C. M. **(1999)**. Characterization of human bactericidal antibodies to *Bordetella pertussis*. *Infect. Immun.* **67**(3), 1424-1431.

Weyant, R. S., D.G., H., Weaver, R. G., Amin, M. F., P., *et al.* **(1995)**. *Bordetella holmesii* spp. nov., a new gram-negative species associated with septicaemia. *J. Clin. Microbiol.* **33**, 1-7.

Willems, R. J. L., Kamerbeek, J., Geuijen, C. A. W., *et al.* **(1998)**. The efficacy of a whole cell vaccine and fimbriae against *Bordetella pertussis* and *Bordetella parapertussis* in a respiratory mouse model. *Vaccine*, **16**(4), 410-416.

Willems, R. J. L., Geuijen, C., van der Heide, H. G. J., *et al.* **(1993)**. Isolation of a putative fimbrial adhesion from *Bordetella pertussis* and the identification of its gene. *Mol. Microbiol.* **9**, 623-634.

Winstanley, C., Hales, B. A., Sibanda, L. M., Dawson, S. *et al.* **(2000)**. Detection of type III secretion system genes in animal isolates of *Bordetella bronchiseptica. Vet. Microbiol.* **72**(3-4), 329-337.

Winstanley, C. and Hart, C. A. **(2001)**. Type III secretion systems and pathogenicity islands. *J. Med. Microbiol.* **50**, 116-126.

Woolfrey, B. F and Moody, J. A. (1991). Human infections associated with *Bordetella bronchiseptica. Clin. Microbiol. Rev.* **4**, 243-255.

Yanagida, N., Uozumi, T. and Beppu, T. **(1986)**. Specific excretion of *Serratia marcescens* protease through the outer membrane of *Escherichia coli. J. Bacteriol.* **166**(3), 937-944.

Yuk, M. H., Cotter, P. A. and Miller, J. F. **(1996)**. Genetic regulation of airway colonisation by *Bordetella* species. *Am. J. Respir. Crit. Care. Med.* **154**, S150-S154.

Yuk, M. H., Harvill, E. T. and Miller, J. F. **(1999)**. The BvgAS virulence control system regulates type III secretion in *Bordetella bronchiseptica. Mol. Microbiol.* **28**, 945-959

Yuk, M. H., Harvill, E. T., Cotter, A. and Miller, J. F. **(2000)**. Modulation of host immune responses, induction of apoptosis and inhibition of NF-kB activation by the *Bordetella* type III secretion system. *Mol. Microbiol.* **35**(5), 991-1004.

Zhang, J. M., Cowell, J. L. Steven, A. C. *et al.* **(1985)**. Purification and characterization of fimbriae isolated from *Bordetella pertussis. Infect. Immun.* **48**(2), 422-7.

Appendix I

Media composition

Luria Bertani broth (LB)

1 litre

Tryptone	10g
Yeast extract	5g
Sodium chloride	10g

Add 1.2% agar for solid media

Cyclodextrin Liquid (CL) media

1 litre

Sodium-L-glutamate	10.7g
L-proline	0.24g
Sodium chloride	2.5g
Sodium di-hydrogen orthophosphate	0.5g
Magnesium chloride ($6H_2O$)	0.1g
Calcium chloride	0.02g
Tris	6.1g
Casamino acids	10g
Methyl-ß-cyclodextrin	0.25g
Potassium chloride	0.2g

pH to 7.6 and add vitamin solution to 0.5%

Vitamin Solution/CL-supplements

75 ml

L-cysteine	0.04g
Iron sulphate ($7H_20$)	0.01g
Nicotinic acid	0.004g
Glutathione	0.15g
Ascorbic acid	0.4g

Stainer-Scholte medium

L-Glutamic acid (mono-sodium salt)	63.4mM
Proline	2.1mM
NaCl	43.0mM
KH_2PO_4	3.7mM
KCl	2.7mM
Tris.HCl	20.1mM
Tris	48.5mM
$MgCl_2.6H_2O$	0.5mM
$CaCl_2.2H_2O$	0.135mM

Phosphate-buffered saline (PBS)

NaCl	128mM
KCl	2.7mM
KH_2PO_4	1.5mM
K_2HPO_4	5.0mM

Casamino acid (CAA) solution

1 Litre

Casein hydrolysate	10g
Magnesium chloride ($6H_2O$)	0.1g
Calcium chloride	0.016g
Sodium chloride	5g

pH to 7.1; supplement with 20% glycerol for storage of *B. pertussis* at -80°C

SOC Medium

1 Litre

Tryptone	20g
Yeast extract	5g
Salts I (250 mM KCl, 1M NaCl)	10ml

After autoclaving, add 10ml of sterile 2M glucose and 10ml of Salts II (1M $MgCl_2.6H_2O$, 1M $MgSO_4.7H_2O$)

Molecular biology solutions

Stop Solution

SDS	0.5% w/v
Tris	50mM (pH= 7.5)
EDTA	0.4M

Add 1 mg/ml proteinase K immediately before use.

Tris-Borate-EDTA (TBE) buffer. 5x stock solution

Tris	54g
Boric acid	27.5g
EDTA (0.5M stock)	20ml

DNA loading buffer. 6x stock solution

Tris	60mM
EDTA	6mM
Sucrose	40%
Bromophenol blue	0.25%

Southern blot solutions

Denaturing solution

Sodium hydroxide	0.5M
Sodium chloride	1.5M

Neutralising solution

Sodium chloride	3M
Tris.HCl	0.5M, pH= 8.0

SSC. 20x stock solution

Sodium chloride	3M
Sodium citrate	0.3M, pH= 7.0

Maleic acid buffer

Maleic acid	0.1M
NaCl	0.15M

pH adjusted to 7.5

Blocking reagent

Blocking stock solution (Boehringer Mannheim) (10% w/v)	20ml
Maleic acid buffer	180ml

Dissolved with heating and shaking

Prehybridisation solution

In a 5 x SSC (prepared from 20 x SSC stock) was added

10 ml N-laurylsarcosine (from 10% w/v stock solution)
2ml SDS (from 10% w/v stock solution)
100 ml blocking reagent (from 10% stock solution)

Added 888ml of dH_2O to make 1 litre prehybridisation slution.

Washing solution 1

2x SSC
0.1% SDS

Washing solution 2

0.1x SSC
0.1% SDS

Detection

Equilibrating solution

Maleic acid	100mM

NaCl	150mM
pH adjusted to 7.5 and added	
Tween-20	0.3%

Antibody blocking solution/ Blocking buffer

Maleic acid	100mM
NaCl	150mM
pH adjusted to 7.5 and added	
Blocking reagent	1.0%

Detection buffer

Tris.HCL	100mM
NaCl	100mM
pH adjusted to 9.5.	

<u>Protein analysis solutions</u>

Protein sample buffer

Glycerol	5ml
20% SDS	2.5ml
2-mercaptoethanol	0.5ml
Tris (0.5ml, pH 6.8)	2.5ml
Bromophenol blue	0.25%

Stacking gel (6%)

Acrylamide/Bis solution	4ml
dH_2O	10.69ml
Tris-HCl (0.5M, pH 6.8)	5ml
20% SDS	100μl
TEMED	10μl
10% Ammonium persulfate (APS)	200μl

Envelope buffer

NaH_2PO_4	20mM
Na_2HPO_4	20mM

28 ml of first is mixed with 72 ml of second and then added 100 ml of dH_2O to produce a 10x envelope buffer

Separating gel (12%, adjust volume of Acrylamide/Bis solution and dH_2O to change percentage)

Acrylamide/Bis solution	4ml
dH_2O	12.6ml
Tris (1.5M, pH 8.8)	10ml
10% SDS	0.4ml
TEMED	30μl
10% APS	0.3ml

PAGE running buffer (10x)/Electrode buffer

1 Litre

Tris	30g
Glycine	144g
SDS	10g

pH 8.3

Coomassie gel stain

Coomassie blue	0.5g
Methanol	500ml
Acetic acid	100ml
dH_2O	400ml

For destain preparation, coomassie blue was omitted from the Coomassie gel stain recipe.

Blocking buffer

In PBS

3% Blotto powder (dried milk)
0.2% Tween 20

RNA analysis

10x DNase buffer

Tris.HCl (pH=8.5)	500mM
MgCl2	50mM
DTT	10mM

RNA running buffer/MOPS (5 X)

0.1 M MOPS (pH 7.0)
40 mM Sodium acetate
5 mM EDTA (pH 8.0)

Dissolve 20.6g of 3-(N-morpholino) propane-sulfonic acid (MOPS) in 800 ml 0.1% DEPC-treated (overnight) 50 mM sodium acetate. Adjust pH to 7.0 with 2 N NaOH and then add 10 ml DEPC-treated 0.5 M EDTA (pH 8.0) and adjusted final volume to 1 litre with DEPC-treated water and covered the solution with tin foil to protect from light.

Appendix II

```
          10         20         30         40         50         60         70
          |          |          |          |          |          |          |
TCCCGATTATTACGGTGCCGCGGCTGTCTACGCCGGTACGCTGAATATCGAGAATTCCACGGTTCACCATAACTATGC
  P  I  I  T  V  P  R  L  S  T  P  V  R  •  I  S  R  I  P  R  F  T  I  T  M  R

 80         90         100        110        120        130        140        150
 |          |          |          |          |          |          |          |
GGCCCAGCCGTTCGAAGACGCGGTAGGAGTCGGGGTAACCTCGCTCGGGGATAAGGCCATACTCAACGTTACCGACAG
  P  S  R  S  K  K  T  R  •  E  S  G  •  P  R  S  G  I  R  P  Y  S  T  L  P  T  A

   160        170        180        190        200        210        220        230
   |          |          |          |          |          |          |          |
CGAGGTATCGGGTGCGAGGGGCGCGGTCATCGGTTGGGGGGGGGGGCGAAGCGACATTTACCGATTCGGTCCTGCGTG
  R  Y  R  V  R  G  A  R  S  S  V  G  G  G  G  E  A  T  F  T  D  S  V  L  R  G
                                                  bap-5 forward

    240        250        260        270        280        290        300        310
    |          |          |          |          |          |          |          |
GTTCGGCCTTCGGGCTGTACGCCGAAATGTGCGACACCTGCAGAGATGATGATGGCACCTCGCCTTCGATTCGCGTCC
  S  A  F  G  L  Y  A  E  M  C  D  T  C  R  D  D  D  G  T  S  P  S  I  R  V  Q

       320        330        340        350        360        370        380
                             NTS-Forward
                                                                        390
       |          |          |          |          |          |          |          |
AAGGCGGGGTTGTTCAGGGCGGCATGGGTGCAAATAACGTCGCTGTGGTGGCAACAGGGTCTGGAAAGGTCGCGATCG
  G  G  V  V  Q  G  G  M  G  A  N  N  V  A  V  V  A  T  G  S  G  K  V  A  I  E

     400        410        420        430        440        450        460
     |          |          |          |          |          |          |
AGAATGCGGAACTGCTCGGAGCCAGCGGCATGTACGCCACGTTCGGCGCGCAGGTCGATATGAAAGGCGGGCGCATTC
  N  A  E  L  L  G  A  S  G  M  Y  A  T  F  G  A  Q  V  D  M  K  G  G  R  I  L

 470        480        490        500        510        520        530        540
 |          |          |          |          |          |          |          |
TGGCGCACAACACCAATATCCTGGGAAGCCAGGGTTACGCCGATGGTCCCTATGGCGGCGTGGTCGTGACAGAGGACG
  A  H  N  T  N  I  L  G  S  Q  G  Y  A  D  G  P  Y  G  G  V  V  V  T  E  D  G

   550        560        570        580        590        600        610        620
   |          |          |          |          |          |          |          |
GTCAAGTCAACCTGGAGGGCGCCAAGGTCAGTGCAACTGGCCTGGGGGCCGCCGGCTTGTGGTTGCTGGGCGACAAGG
  Q  V  N  L  E  G  A  K  V  S  A  T  G  L  G  A  A  G  L  W  L  L  G  D  K  D

     630        640        650        660        670        680        690        700
     |          |          |          |          |          |          |          |
ACACCAGCCCGCGAGCCAGCCTGCGCAACACCGACGTCCACGGAGAGGTCGCCGCCATTGCGCTGGGGTTCAATGGCG
  T  S  P  R  A  S  L  R  N  T  D  V  H  G  E  V  A  A  I  A  L  G  F  N  G  E

       710        720        730        740        750        760        770
                                                                        780
       |          |          |          |          |          |          |          |
AGGCGAACATCTCGGGCGGCAGCTTGAGCGTAGAGGATGGGGCCGTGCTCACCACCCTGACGCCCGATGCAGTCGAGT
  A  N  I  S  G  G  S  L  S  V  E  D  G  A  V  L  T  T  L  T  P  D  A  V  E  Y

       790        800        810        820        830        840        850
                  NTS-Reverse  NcoI
       |          |          |          |          |          |          |
ATTACTACGACTACGCCTTGTCCATGGAGCATCTGCCAGCTGATGCGCCGTTGACGCCGGTCCGCGTCACGCTGTCCG
  Y  Y  D  Y  A  L  S  M  E  H  L  P  A  D  A  P  L  T  P  V  R  V  T  L  S  D
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860        870        880        890        900        910        920        930
|          |          |          |          |          |          |          |
ATGGCGCGCGCGCCAGCGGAGAAACGTTGATCGCGCATGGCGGGTTGTTGCCCATGACGCTGCGCTTGAGCAGCGGGG
  G  A  R  A  S  G  E  T  L  I  A  H  G  G  L  L  P  M  T  L  R  L  S  S  G  V

   940        950        960        970        980        990        1000       1010
   |          |          |          |          |          |          |          |
TCGACGCCCGCGGCGACATCGTCACGCTGCCGCCTTCCGCGCCGCCCGATTCCGCGGAGCAACCGGATGCCGAGCCGG
  D  A  R  G  D  I  V  T  L  P  P  S  A  P  P  D  S  A  E  Q  P  D  A  E  P  E

    1020       1030       1040       1050       1060       1070       1080       1090
    |          |          |          |          |          |          |          |
AACCGGATGCCGAGCTGGAACCGGACGCCGCGGCGCAGTCGGACGCCAAGGCGAATGCGCGGGTCATGGCGCAGGTAG
  P  D  A  E  L  E  P  D  A  A  A  Q  S  D  A  K  A  N  A  R  V  M  A  Q  V  D

      1100       1110       1120       1130       1140       1150       1160       1170
      |          |          |          |          |          |          |          |
ATGGCGGGGAACCTGTTGCCGTGCCGATCCCGGCCCCTTCGCATCCCGATGCCCCGATCGACGTGTTCATCGACAGCG
  G  G  E  P  V  A  V  P  I  P  A  P  S  H  P  D  A  P  I  D  V  F  I  D  S  G

         1180       1190       1200       1210       1220       1230       1240
         |          |          |          |          |          |          |
GTGCCCAATGGCGGGGCATGACCAAGACCGTCAATGCGTTGCGCATCGAGGACGGCACCTGGACCGTCACCGGGTCGT
  A  Q  W  R  G  M  T  K  T  V  N  A  L  R  I  E  D  G  T  W  T  V  T  G  S  S

1250       1260       1270       1280       1290       1300       1310       1320
|          |          |          |          |          |          |          |
CCACGGTGAACAGCCTGCACCTGCAGGCAGGCAAGGTGGCGTACGCAACGCCTGCCGAAAGCGACGGAGAATTCAAAC
  T  V  N  S  L  H  L  Q  A  G  K  V  A  Y  A  T  P  A  E  S  D  G  E  F  K  H

   1330       1340       1350       1360       1370       1380       1390       1400
   |          |          |          |          |          |          |          |
ACCTGCGGGTCAAGACCCTCTCGGGAAGCGGCCTGTTCGAGATGAACGCCAGCGCCGACCTGAGCGATGGCGACCTGC
  L  R  V  K  T  L  S  G  S  G  L  F  E  M  N  A  S  A  D  L  S  D  G  D  L  L

      1410       1420       1430       1440       1450       1460       1470       1480
      |          |          |          |          |          |          |          |
TGGTCGTGTCCGACGAGGCCAGCGGGCAGCACAAGGTGCTGGTGCGAGGAGCCGGCACGGAACCCACCGGTGTGGAAA
  V  V  S  D  E  A  S  G  Q  H  K  V  L  V  R  G  A  G  T  E  P  T  G  V  E  S

         1490       1500       1510       1520       1530       1540       1550       1560
         |          |          |          |          |          |          |          |
GCCTGACGCTGGTCGAGCTGCCCGAGGGCAGCCAGACGAAGTTCACGCTTGCCAACCGGGGCGGGGTGGTCGACGCCG
  L  T  L  V  E  L  P  E  G  S  Q  T  K  F  T  L  A  N  R  G  G  V  V  D  A  G

   1570       1580       1590       1600       1610       1620       1630
   |          |          |          |          |          |          |
GCGCGTTCCGCTATCGCCTGACGCCGGACAACGGTGTCTGGGGCCTGGAACGGACCAGCCAGCTTTCGGCCGTCGCCA
  A  F  R  Y  R  L  T  P  D  N  G  V  W  G  L  E  R  T  S  Q  L  S  A  V  A  N

1640       1650       1660       1670       1680       1690       1700       1710
|          |          |          |          |          |          |          |
ACGCGGCCTTGAATACCGGGGGCGTGGGCGCGGCCAGCAGCATCTGGTATGCGGAAGGCAATGCGCTCTCCAAGCGCC
  A  A  L  N  T  G  G  V  G  A  A  S  S  I  W  Y  A  E  G  N  A  L  S  K  R  L

   1720       1730       1740       1750       1760       1770       1780       1790
   |          |          |          |          |          |          |          |
TGGGCGAGTTGCGGCTCGATCCCGGCGCGGGCGGCTTCTGGGGGCGCACGTTCGCCCAGAAGCAGCAGCTCGACAACA
  G  E  L  R  L  D  P  G  A  G  G  F  W  G  R  T  F  A  Q  K  Q  Q  L  D  N  K

      1800       1810       1820       1830       1840       1850       1860       1870
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     |         |         |         |         |         |         |         |
AGGCTGGCCGACGCTTCGACCAGAAGGTGTACGGTTTCGAGCTGGGGGCCGACCATGCCATCGCAGGACAGCAAGGGC
  A   G   R   R   F   D   Q   K   V   Y   G   F   E   L   G   A   D   H   A   I   A   G   Q   Q   G   R

          1880      1890      1900      1910      1920      1930      1940      1950
          |         |         |         |         |         |         |         |
GCTGGCACGTGGGCGGCCTGCTGGGCTATACCCGCGCAAGGCGCAGCTTCATCGATGACGGCGCCGGGCATACCGACA
  W   H   V   G   G   L   L   G   Y   T   R   A   R   R   S   F   I   D   D   G   A   G   H   T   D   S

              1960      1970      1980      1990      2000      2010      2020
              |         |         |         |         |         |         |
GCGCGCATATCGGGGCCTACGCGGCGTACGTGGCGGACAACGGCTTCTATTTCGATTCGACCCTGCGCGCCAGCCGCT
  A   H   I   G   A   Y   A   A   Y   V   A   D   N   G   F   Y   F   D   S   T   L   R   A   S   R   F

      2030      2040      2050      2060      2070      2080      2090      2100
      |         |         |         |         |         |         |         |
TCGAGAACGACTTCACGGTAACGGCCACCGACGCCGTTTCCGTACGGGGCAAGTACCGGGCCAATGGGGTAGGCGCCA
  E   N   D   F   T   V   T   A   T   D   A   V   S   V   R   G   K   Y   R   A   N   G   V   G   A   T

          2110      2120      2130      2140      2150      2160      2170      2180
          |         |         |         |         |         |         |         |
CCTTGGAGGCCGGCAAACGTTTCACGTTGCACGACGGCTGGTTCGTCGAACCTCAGTCCGAGGTGTCGCTGTTCCATG
  L   E   A   G   K   R   F   T   L   H   D   G   W   F   V   E   P   Q   S   E   V   S   L   F   H   A

              2190      2200      2210      2220      2230      2240      2250      2260
              |         |         |         |         |         |         |         |
CCAGCGGCGGAACCTACCGTGCCGCGAACAACCTGTCGGTCAAGGACGAAGGCGGCACCTCCGCCGTGCTGCGCCTGG
  S   G   G   T   Y   R   A   A   N   N   L   S   V   K   D   E   G   G   T   S   A   V   L   R   L   G

          2270      2280      2290      2300      2310      2320      2330      2340
          |         |         |         |         |         |         |         |
GCTTGGCGGCCGGGCGACGCATCGACCTGGGCAAGGACCGCGTGATCCAGCCCTATGCCACCCTGAGCTGGCTGCAGG
  L   A   A   G   R   R   I   D   L   G   K   D   R   V   I   Q   P   Y   A   T   L   S   W   L   Q   E

      2350      2360      2370      2380      2390      2400      2410
      |         |         |         |         |         |         |
AATTCAAAGGCGTCACGACCGTTCGCACCAACGGGTACGGGCTGCGCACCGACCTGAGCGGTGGCCGGGCTGAATTGG
  F   K   G   V   T   T   V   R   T   N   G   Y   G   L   R   T   D   L   S   G   G   R   A   E   L   A

  2420      2430      2440      2450      2460      2470      2480      2490
  |         |         |         |         |         |         |         |
CGCTGGGCCTGGCCGCCGCGTTGGGGCGCGGCCACCAGCTCTACACTTCGTACGAGTACGCCAAGGGCAACAAGCTGA

  L   G   L   A   A   A   L   G   R   G   H   Q   L   Y   T   S   Y   E   Y   A   K   G   N   K   L   T
                                  bap-5 Reverse
      2500      2510                  2530      2540      2550      2560      2570
      |         |         |         |         |         |         |         |
CCTTGCCTTGGACGTTCCACCTGGGCTATCGCTACACCTGGTAGCGGCGCATCGCAACAACGGGGTCATCGCGACCCC
  L   P   W   T   F   H   L   G   Y   R   Y   T   W   •   R   R   I   A   T   T   G   S   S   R   P   R

          2580      2590      2600      2610      2620      2630      2640      2650
          |         |         |         |         |         |         |         |
GTTGTTGCATTCCCGGACCGCGCTGCGGTCAGACCAGCCCGGGCCGTCGACCACGCGTGCCCTATAGTAAGGGCGAAT
  C   C   I   P   G   P   R   C   G   Q   T   S   P   G   R   R   P   R   V   P   Y   S   K   G   E   F

      2660      2670      2680      2690      2700      2710      2720
      |         |         |         |         |         |         |
TCTGCAGATATCCATCACACTGGCGGCCGCTCGAGCATGCATCTAGAGGGCCCAATTCGCCCTATAGTGATAG
  C   R   Y   P   S   H   W   R   P   L   E   H   A   S   R   G   P   N   S   P   Y   S   D
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Appendix III

CCGTCGCCTGATCGATATGGCGCGCCGCATGGGCGCGCGCGAGCCCGAAGTGCTCGGCGAC

GGCTTGATGCTGCTCATCGAGGGCGCTTTCATTTCGGGCCAGCTATTTCACGGCGACGGGC

CGGCGCGTCACGTCGCGCGATTGGCCGACAGATTGATCGAAGCCAGTCTGTAGCGATTCCG

TTTCATATTCGTTCGATTTTCAAGCCGCCCCTCGGGGCGGTTTTTTTTGGTGGGGGAGGGC

GATGTGCCGCGTGTAAGTCCGCCATTTTCGTACGTGTTCAGGTGCCGATTGCCGGTATCGG

GATATATGGGCTTATTTGAAACTAGAACAGCTTTTTCACCGGGTGCCAGGCACCTGTTCGC

AGCAGTTTTTTCACGACTGTTTATTGGCCTCCTTTACCGATTTGAATATGAATGACAGAAA

ATCCAATAGCATGTCGCCAGCGGCGAGATACTTGCGTTTCGGCGTTGTCGCGGTAGCGGGT

GTGGCGGCGGGCGCGCTGCCTTCGTCGGATGTCGATGCCCAGGCCGCGCCGGCCGCCGCCG

AGGTAGCCAAGATCGAGGCTCTGTCGGATGCGGACATTTACAGCGACTACGAGCACGAGCA

TGGCATCGTGATGACGCCCGATGGCAAGGACGACTACATCAGTTACAGGTCCGCCGAGAGC

GGTCGTCCGAAGCCCCCCCCCCCCCCTTTCAAACTTCAAACCATTGGGTAATGACGTCGTA

GCGGAGCGGATACGGGTAGAAGTGCACGGTGACGAAACCCTCGGCGTGTATGTCGACTCGG

AGCACCGTTCGCTCACCGTGCGTGACAGCACGATAGATGCATACGGCAAGCCGCCTTCCGT

CGACTCTCCCGATTATTACGGTGCCGCGGCTGTCTACGCCGGTACGCTGAATATCGAGAAT

TCCACGGTTCACCATAACTATGCGGCCCAGCCGTTCGAAGACGCGGTAGGAGTCGGGGTAA

CCTCGCTCGGGGATAAGGCCATACTCAACGTTACCGACAGCGAGGTATCGGGTGCGAGGGG

CGCGGTCATCGGTTGGGGGGGGGGGCGAAGCGACATTTACCGATTCGGTCCTGCGTGGTTC

GGCCTTCGGGCTGTACGCCGAAATGTGCGACACCTGCAGAGATGATGATGGCACCTCGCCT

TCGATTCGCGTCCAAGGCGGGGTTGTTCAGGGCGGCATGGGTGCAAATAACGTCGCTGTGG

TGGCAACAGGGTCTGGAAAGGTCGCGATCGAGAATGCGGAACTGCTCGGAGCCAGCGGCAT

GTACGCCACGTTCGGCGCGCAGGTCGATAT

Appendix IV (mouse-virulence tests)

Mouse lung counts obtained at day 7, after intranasal challenge with *B. pertussis* strains.

Experiment	*B. pertussis* challenge strains	Total lung counts from individual mice (CFU)
I	Taberman I	$2.8x10^5$, $1.19x10^6$, $9.1x10^5$, $4.1x10^5$, $4.1x10^5$
	Taberman II, *bap-5* mutant	$1.7x10^5$, $1.0x10^4$, $7x10^5$, $1.1x10^5$, $6.0x10^4$
	18-323	$1.0x10^7$, $8.7x10^6$, $4.0x10^6$, $1.0x10^7$, $7.4x10^6$
	Control (diluent only)	$<10^2$, $<10^2$, $<10^2$, $<10^2$, $<10^2$
II	Taberman I	$9.7x10^5$, $7.3x10^5$, $1.3x10^3$, $1.0x10^6$, $3.5x10^5$
	Taberman II, *bap-5* mutant	$2.7x10^5$, $3.9x10^5$, $3.0x10^4$, $4.0x10^4$, $4.0x10^4$
	BBC29	$1.82x10^6$, $2.94x10^6$, $2.22x10^6$, $3.12x10^6$, $3.2x10^6$
	BBC30, *prn* mutant	$1.19x10^6$, $4.0x10^5$, $8.9x10^5$, $1.53x10^6$, $1.4x10^6$

*Limit of detection is $=10^2$ CFU/lung

Appendix V (mouse-virulence tests)

Mouse tracheal counts obtained at day 7, after intranasal challenge with *B. pertussis* strains.

Experiment	*B. pertussis* challenge strains	Total tracheal counts from individual mice (CFU)
I	Taberman I	600, $1.5x10^3$, $6.0x10^3$ 500, $1.8x10^3$
	Taberman II, *bap-5* mutant	$<10^2$, $<10^2$, 800, 200, $<10^2$,
	18-323	$1.0x10^5$
	Control (diluent only)	$<10^2$, $<10^2$, $<10^2$, $<10^2$, $<10^2$,
II	Taberman I	$1.6x10^3$, $4.6x10^3$, $2.9x10^3$, $1.64x10^4$, 300
	Taberman II, *bap-5* mutant	500, 500, $1.2x10^3$, 100,100
	BBC29	$9.6x10^4$, $1.17x10^5$, $1.09x10^5$, $3.6x10^4$, $8.9x10^4$
	BBC30, *prn* mutant	$3.9x10^4$, $3.1x10^4$, $4.3x10^4$, $1.5x10^4$, $1.2x10^4$

*Limit of detection is $=10^2$ CFU/trachea

Appendix VI (mouse-virulence tests)

Mouse nasal counts obtained at day 7, after intranasal challenge with *B. pertussis* strains.

Experiment	*B. pertussis* challenge strains	Total nasal counts from individual mice (CFU)
I	Taberman I	80, 100, 200, 250,10
	Taberman II, *bap-5* mutant	60, 180, 60, 200, 330
	Control (diluent only)	<10, <10, <10, <10, <10

*Limit of detection is = 10 CFU/nasal cavity

Appendix VII (mouse-protection tests)

Mouse nasal counts obtained at different days after intranasal challenge with *B. pertussis* strain 18-323, the standard mouse-virulent challenge strain.

Day	Vaccine Group	Total counts from nasal washes of individual mice (CFU/ml)
0	Sham	20, 270, 360, 1000
3	Sham	120, 1040, 1800, 2380
3	P.30 (Renatured)	9, 60, 200, 1150
3	P.69	9, 9, 9,9
9	Sham	$1.6x10^2$, $2.4x10^2$, $1.12x10^3$, $1.15x10^3$
9	P.30 (Renatured)	$4.4x10^3$, $6.4x10^3$, $8.48x10^3$, $1.42x10^4$
9	P.69	780, 1000, 1120, 1600
14	Sham	60, 100, 320, 410
14	P.30 (Renatured)	40, 240, 1030, 2710
14	P.69	9, 9, 30, 240

The limit of detection is 10 and for statistical purposes, these were assigned a nominal values of 9 CFU.

Appendix VIII (mouse-protection tests)

Mouse lung counts obtained at different days after intranasal challenge with *B. pertussis* strain 18-323, the standard mouse-virulent challenge strain.

Day	Vaccine Group	Total counts from lungs of individual mice (CFU/ml)
0	Sham	<100, $1.0x10^3$, $2.9x10^4$, $3.6x10^4$
3	Sham	$4.0x10^4$, $8.64x10^5$, $9.6x10^5$, $1.06x10^6$
3	P.30 (Renatured)	$4.0x10^4$, $4.6x10^4$, $1.96x10^5$, $1.0x10^6$
3	P.69	<100, <100, $2.6x10^4$, $3.7x10^4$
9	Sham	$4.7x10^5$, $9.6x10^5$, $1.04x10^6$, $1.1x10^6$
9	P.30 (Renatured)	$5.0x10^5$, $1.05x10^6$, $1.1x10^6$, $1.2x10^6$
9	P.69	<100, $3.0x10^3$, $8.0x10^3$, $1.4x10^4$
14	Sham	$1.2x10^4$, $7.8x10^4$, $9.7x10^4$, $1.65x10^5$
14	P.30 (Renatured)	$4.0x10^3$, $7.4x10^4$, $7.8x10^4$, $1.08x10^5$
14	P.69	<100, <100, <100, $1.7x10^3$

The limit of detection is $>10^2$ CFU/lung

Appendix IX (mouse-protection tests)

Mouse tracheal counts obtained at different days after intranasal challenge with *B. pertussis* strain 18-323, the standard mouse-virulent challenge strain.

Day	Group	Total counts from trachea of individual mice (CFU/ml)
0	Sham	<100, 400, 700, 1500
3	Sham	100, 200, 500, $1.09x10^4$
3	P.30 (Renatured)	100, 400, $7.1x10^3$, $4.07x10^4$
3	P.69	<100, <100,<100, <100
9	Sham	$1.5x10^4$, $3.37x10^4$, $4.88x10^4$, $7.2x10^4$
9	P.30 (Renatured)	$1.12x10^4$, $2.03x10^4$, $2.47x10^4$, $6.0x10^4$
9	P.69	<100, <100,<100, <100
14	Sham	700, $3.3x10^3$, $7.0x10^3$, $9.7x10^3$
14	P.30 (Renatured)	100, 600, 700, $1.29x10^4$
14	P.69	<100, <100,<100, $4.0x10^3$

The limit of detection is $>10^2$ CFU/trachea

Appendix X (mouse-protection tests)

Statistical analysis of respiratory tract colonisation (nasal cavities) of the different mouse vaccine groups after intranasal challenge with *B. pertussis* strain 18-323.

The P value is <0.0001 is extremely significant (ANOVA) and if the value of q is greater than 4.764 (Tukey-Kramer multiple comparison test) then the P value is less than 0.05 (significant)

Group comparison on day number	Mean difference	q	*P*
Sham-3 vs. P 69-3	1.587	5.696	<0.05
Sham-9 vs. P 69-9	0.6425	2.306	>0.05
Sham-14 vs. P 69-14	0.7597	2.727	>0.05
Sham-3 vs. P 30-3	0.9080	3.261	>0.05
Sham-9 vs. P 30-9	-0.2037	0.7312	>0.05
Sham-14 vs. P 30-14	-0.3830	1.375	>0.05
P 30-3 vs. P 69-3	0.6785	2.435	>0.05
P 30-9 vs. P 69-9	0.8462	3.037	>0.05
P 30-14 vs. P 69-14	1.143	4.101	>0.05

Statistical analysis of respiratory tract colonisation (tracheas) of the different mouse vaccine groups after intranasal challenge with *B. pertussis* strain 18-323.

Group comparison on day number	Mean difference	q	P
Sham-3 vs. P 69-3	1.805	5.361	<0.05
Sham-9 vs. P 69-9	3.608	10.716	<0.001
Sham-14 vs. P 69-14	2.183	6.483	<0.01
Sham-3 vs. P 30-3	-0.5064	1.504	>0.05
Sham-9 vs. P 30-9	0.1805	0.5360	>0.05
Sham-14 vs. P 30-14	0.6154	1.828	>0.05
P 30-3 vs. P 69-3	2.311	6.865	<0.01
P 30-9 vs. P 69-9	3.428	10.18	<0.001
P 30-14 vs. P 69-14	1.567	4.655	>0.05

Statistical analysis of respiratory tract colonisation (lungs) of the different mouse vaccine groups after intranasal challenge with *B. pertussis* strain 18-323.

Group comparison on day number	Mean difference	q	P
Sham-3 vs. P 69-3	2.914	5.350	<0.05
Sham-9 vs. P 69-9	2.808	5.156	<0.05
Sham-14 vs. P 69-14	3.021	5.547	<0.05
Sham-3 vs. P 30-3	0.4973	0.9131	>0.05
Sham-9 vs. P 30-9	-0.0319	0.0587	>0.05
Sham-14 vs. P 30-14	0.1947	0.3575	>0.05
P 30-3 vs. P 69-3	2.416	4.437	>0.05
P 30-9 vs. P 69-9	2.84	5.215	<0.05
P 30-14 vs. P 69-14	2.826	5.189	<0.05