School of Biomedical Sciences

Novel Key Biofilm-Forming Surface-Associated Polysaccharide and Protein Antigens of *Bordetella pertussis* and the Comparative Immunoprotective Potential

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Declaration

To the best of my knowledge and belief, this thesis contains no material previously published by any other person except where due acknowledgment has been made. This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

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Abstract

Whooping cough is a highly contagious respiratory disease which is most severe in unvaccinated infants. It is caused by *Bordetella pertussis* and infections are becoming more common despite high coverage with whole-cell (wP) and acellular vaccines (aP). Globally, 50 million cases and 300 000 deaths are reported annually. Although the mechanisms for resurgence of pertussis are multifactorial, short-term immunity induced by aP vaccines may contribute.

This project sought an effective vaccine against whooping cough based on the biofilm-forming potential of *B. pertussis*. Twenty-one clinical isolates of *B. pertussis* were recovered during a whooping cough epidemic in Western Australia. The isolates included *ptxP3-prn2-fim3A (*48%), *ptxP3-prn2-fim3B* (28%), not-*ptxP3-prn1-fim3A* (19%) and not-*ptxP3-prn2-fim3A* (5%) genotypes. All clinical isolates formed thicker biofilms compared to vaccine strain *B. pertussis* Tohama I ($P<0.05$). This was confirmed by qualitative scanning electron microscopy that showed a more densely packed multilayered community of cells than was generated by Tohama I. All isolates were susceptible to ampicillin, erythromycin, azithromycin and streptomycin, but resistant to cotrimoxazole and tetracycline. Minimal bactericidal concentrations (MBC) of biofilm-grown *B. pertussis* were 2-256 fold higher than planktonic-grown cells, depending on the antimicrobial agent.

The clinical isolates carried all genes encoding antigens included in current aP vaccines, and genes encoding other accredited virulent antigens including dermonecrotic toxin (*dnt*), tracheal cytotoxin (*tct*), adenylate cyclase toxin (*act*), *Bordetella* serum resistance factor (*brkA*), *Bordetella* intermediate protein (*bipA*), *Bordetella* polysaccharide D (*bpsD*) and the capsular gene (*kpsT*). Phenotypic identification of capsules was achieved in all isolates using a novel Maneval's capsule stain. *Bordetella* polysaccharide A (*bpsA*) was detected in 19 (90%) isolates, and not in the remaining two. There was no correlation between the presence of virulence factor and biofilm formation. Interestingly, nucleotide sequencing of the PAS-associated C-terminal (PAC) domain of virulence sensor gene (*bvgS*), a member of the *Bordetella* two-component master regulatory system, *bvgA*/*bvgS*

phosphorelay, identified a single nucleotide polymorphism in all 21 clinical isolates but not in Tohama I. The A2113G mutation in the *bvgS* gene led to a Lys705Glu substitution in the BvgS protein.

Proteomic characterization of a strong biofilm-forming isolate with *ptxP3* genotype using isobaric tags for relative and absolute quantitation (iTRAQ) found ~5% of proteins were significantly upregulated while 3.5% of proteins were downregulated compared to Tohama I ($P<0.05$). Of the antigens in aP vaccines, serotype 3 fimbrial subunit (Fim3) and chaperone protein FimB/FhaD (FimB) were upregulated in the clinical isolate ($P<0.05$). In biofilm of the clinical isolate, 6% and 7% of proteins were significantly upregulated and downregulated, respectively, relative to planktonic cells ($P<0.05$). Upregulated proteins were enriched for energy metabolism ($P<0.05$).

Mice vaccinated with biofilm produced significantly higher levels ($P<0.05$) of IFN-γ, IL-17a and antigen-specific IgG2a and had reduced bacterial loads in the lungs 7 days after virulent *B. pertussis* challenge compared to mice vaccinated with either planktonic cells, aP or controls. Two membrane proteins upregulated in *B. pertussis* biofilms [outer membrane protein assembly factor (BamB) and lipopolysaccharide assembly protein (LptD)] were investigated as novel vaccine candidates. Vaccination of mice with novel formulation of recombinant BamB, LptD and aP induced unique chemokine profiles and reduced bacterial loads ($P<0.05$) compared to aP-vaccinated mice, suggesting a novel role of biofilm-derived antigens as potential whooping cough vaccines.

In summary, most *B. pertussis* clinical isolates were genotypically divergent from the vaccine strain, indicating an urgent need for aP vaccines containing antigens from circulating variants. The combination of antimicrobial resistance and the capability of forming stronger biofilms than the reference strain offer potential to improve antimicrobial therapeutic regimens. Proteomic characterization identified novel proteins in the biofilm relative to planktonic-grown *B. pertussis*, provides a tool for identification of new antigens that induce responses critical to the control of the bacterial burden.

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Abbreviations

1D-GE	1-dimensional gel electrophoresis
AMP	Ampicillin
aP	Acellular pertussis vaccine
ACT	Adenylate cyclase toxin
BamB	Outer membrane protein assembly factor BamB
BG	Bordet-Gengou
BvgS	Virulence sensor protein,
bvgASR	*Bordetella* master virulence regulatory system
brkA	*S*erum resistance factor A
CFU	Colony forming unit
CHL	Chloramphenicol
CMI	Cell-mediated immunity
CIP	Ciprofloxacin
DAVID	Database for Annotation, Visualisation, and Integrated Discovery
DNT	Dermonecrotic toxin
DTaP	Diphtheria, tetanus and acellular pertussis vaccine
ELISPOT	Enzyme linked immunospot
ERY	Erythromycin
FHA	Filamentous haemagglutinin
Fim	Fimbriae
GPCRs	G-protein coupled receptor
HK	Histidine kinase domain
Hpt	Histidine phosphoryl transfer domain
ID20	*Bordetella pertussis* clinical isolate ID20
IFN-γ	Interferon gamma
IgG1	Immunoglobulin G1
IL-17a	Interleukin-17a
iTRAQ	Isobaric tags for relative and absolute quantitation
LptD	LPS-assembly protein LptD
MBC	Minimal bactericidal concentration

MDDC	Monocyte-derived dendritic cells
NAD^+	Nicotinamide adenine dinucleotide
PAGE	Polyacrylamide gel electrophoresis
PAS	Per/ArnT/Sim
PNAG	Poly-β-1,6-*N*-acetyl-D-glucosamine
(p)ppGpp	5'-triphosphate-guanosine-3'-diphosphate and 5'-diphosphate-guanosine-3'-diphosphate
Prn	Pertactin
Ptx	Pertussis toxin
ptxP3	Pertussis toxin promoter type 3
PTxoid	Detoxified pertussis toxin
PBS	Phosphate buffer saline
SB	Sodium Borate
SEB	*Staphylococcal* enterotoxin B
SEM	Scanning electron microscopy
SFU	Spot forming unit
Sph1B	Subtilisin-like serine protease
SS	Stainer-Scholte
STR	Streptomycin
SXT	Cotrimoxazole
T3SS	Type III secretion system
TCS	Two-component regulatory system
TCF	Tracheal colonising factor
TCT	Tracheal cytotoxin
TET	Tetracycline
Th1	T-helper cell type 1
Th2	T-helper cell type 2
TLR-4	Toll-like receptor 4
VFT1	Venus flytrap domain 1
VFT2	Venus flytrap domain 2
wP	Whole-cell pertussis vaccine

1. Objectives of this research project

The overall goal of this project was to seek an effective vaccine against whooping cough based on the biofilm-forming potential of *B. pertussis*. Specifically, this was achieved through the following objectives:

1. Phenotypically and genotypically characterise clinical isolates of *B. pertussis* isolated during the epidemic period in Western Australia.
2. Determine the biofilm forming potential and identify significant virulence factors associated with biofilm formation.
3. Assess the contribution of biofilm-formation in conferring clinically relevant tolerance to antimicrobial agents.
4. Identify novel antigens that are upregulated in the biofilms using proteomic tools.
5. Determine the immunogenicity and protective potential of biofilm-associated antigens in comparison with currently used acellular pertussis vaccine, using a well-established mouse model system.

2. Outline of thesis

This thesis is presented as a hybrid of accepted publications, submitted and/or prepared manuscripts and traditional thesis chapters. In particular, the materials and methods section and the general discussion are constructed in a traditional format. Each chapter has own reference lists of published papers.

Chapter 1 – Literature Review

Chapter 2 – General Materials and Methods

Materials and methods not described elsewhere in this thesis.

Chapter 3 - Biofilm forming potential and antimicrobial susceptibility of newly emerged Western Australian *Bordetella pertussis* clinical isolates.

The biofilm forming potential of *B. pertussis* recovered during a whooping cough epidemic in Western Australia was investigated using twenty-one clinical isolates. The presence of virulence factors (including *ptx*, *prn*, *bpsAD* and capsular polysaccharide, *kpsT*) was correlated with biofilm formation. Minimal bactericidal

concentration (MBC) of biofilm relative to planktonic forms of *B. pertussis* was also determined. The study showed that the epidemic isolates of *B. pertussis* form significantly ($P<0.05$) thicker biofilm than the vaccine strain *B. pertussis* Tohama I. In addition, most isolates were of different genotype from the vaccine strain. About 5% of proteins were significantly upregulated ($P<0.05$) in the biofilm and antimicrobial resistance of *B. pertussis* was enhanced in the biofilm.

Chapter 4 - Prevalence of virulence factors in resurgent strains of *Bordetella pertussis* clinical isolates: potential relevance to development of an effective pertussis vaccine

The presence of virulence factors in clinical isolates was investigated using PCR and capsules were visualised using Maneval staining and Scanning Electron Microscopy (SEM). The study confirmed the strong biofilm forming potential of the clinical isolates but was not clearly correlated with virulence. Nucleotide sequencing of the PAS-associated C-terminal (PAC) domain of virulence sensor gene (*bvgS*), a member of the *Bordetella* two-component master regulatory system, *bvgA*/*bvgS* phosphorelay, identified a single nucleotide polymorphism in all 21 clinical isolates but not in Tohama I. The A2113G mutation in the *bvgS* gene led to a Lys705Glu substitution in the BvgS protein.

Chapter 5 - Identification of novel antigens of *Bordetella pertussis* by proteomic characterisation of a resurgent strain carrying the *ptxP3* allele and its biofilms

A quantitative isobaric tag for relative and absolute quantitation (iTRAQ)-based proteomic analysis was used to identify proteins differentially expressed in a clinical isolate carrying *ptxP3* allele and Tohama I (carrying *ptxP1* allele). iTRAQ was also used to identify proteins differentially expressed in biofilm and planktonic cells with a goal of identifying novel vaccine candidates.

Chapter 6 - Immunogenicity and protective potential of *Bordetella pertussis* biofilm and its associated antigens in a mouse model system

The immunogenicity and protective potential of biofilm and its associated proteins were investigated. Mice immunised with biofilm cells induced significantly higher

IFN-γ, IL-17a and IgG antibodies compared to a) planktonic cells, b) currently used aP vaccine, Infanrix® hexa (GlaxoSmithKline), and c) Imject (alum) control mice. Furthermore, biofilms provided significantly greater protection compared to all vaccine candidates. Two membrane proteins upregulated in *B. pertussis* biofilms [outer membrane protein assembly factor (BamB) and lipopolysaccharide (LPS) assembly protein (LptD)] were investigated as novel *B. pertussis* vaccine candidates. Immunisation of mice with BamB or LptD significantly reduced ($P \leq 0.05$) bacterial loads as compared to Imject-immunised mice, suggesting a novel role of biofilm-derived antigens as potential whooping cough vaccines.

Chapter 7 – General discussion and conclusions

Chapter 1

Literature Review

Manuscript submitted to the Journal of Medical Microbiology and Immunology

Abstract

Despite high vaccine coverage, whooping cough caused by *Bordetella pertussis* remains one of the most common vaccine-preventable diseases worldwide. Introduction of whole-cell pertussis (wP) vaccines in the 1940s and acellular pertussis (aP) vaccines in 1990s reduced the mortality due to pertussis. Despite induction of both antibody and cell-mediated immune (CMI) responses by aP and wP vaccines, there has been resurgence of pertussis in many countries in recent years. Possible reasons hypothesised for resurgence have ranged from incompliance with the recommended vaccination programmes with the currently used aP vaccine to infection with a resurged clinical isolates characterised by mutations in the virulence factors, resulting in antigenic divergence with vaccine strain, and increased production of pertussis toxin, resulting in dampening of immune responses. While use of these vaccines provide varying degrees of protection against whooping cough, protection against infection and transmission appears to be less effective, warranting continuation of efforts in the development of an improved pertussis vaccine formulations capable of achieving this objective. Major approaches currently under evaluation for the development of an improved pertussis vaccine include identification of novel biofilm-associated antigens for incorporation in current aP vaccine formulations, development of live attenuated vaccines and discovery of novel non-toxic adjuvants capable of inducing both antibody and CMI. In this review, the potential roles of different accredited virulence factors, including novel biofilm-associated antigens, of *B. pertussis* in the evolution, formulation and delivery of improved pertussis vaccines, with potential to block the transmission of whooping cough in the community, are discussed.

1.1 Introduction

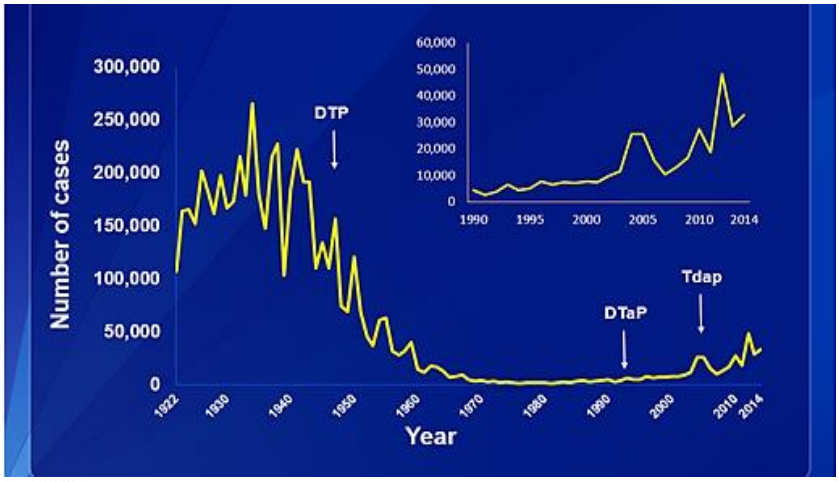
The genus *Bordetella* comprises of Gram-negative bacteria that infect, colonise and cause disease in a wide variety of mammals, humans and birds. Ten species have been identified to-date, including *B. pertussis*, *B. bronchiseptica*, *B. parapertussis*, *B. avium*, *B. hinzii*, *B. holmesii*, *B. ansorpii*, *B. trematum*, *B. flabilis* and *B. petrii* [1,2]. *Bordetella pertussis*, *B. parapertussis* and *B. bronchiseptica* are closely related phylogenetically and are often referred to as the "classical *Bordetellae*" [3]. *Bordetella bronchiseptica* is a respiratory pathogen infecting a wide range of mammalian hosts including generally causing mild, chronic respiratory illness [1,4,5]. The organism is occasionally isolated from humans where it can be an opportunistic pathogen causing severe infection in immune-compromised hosts such as patients with AIDS [6,7], cystic fibrosis [8] or other pre-existing disease conditions [9,10]. Two distinct lineages of *B. parapertussis* have been identified - *B. parapertussis*$_{HU}$ in humans and *B. parapertussis*$_{OV}$ in sheep [11]. Phylogenetic analysis indicates that *B. pertussis* and *B. parapertussis*$_{HU}$ evolved independently from *B. bronchiseptica* [12-14]. *B. pertussis* is an obligate human pathogen, which has undergone significant insertion sequence (IS) mediated gene loss or inactivation. No environmental reservoir for *B. pertussis* has been found until now [12,15-18]. This pathogen is the aetiological agent of pertussis or whooping cough, a chronic and highly contagious respiratory disease particularly severe in infants. Infection typically results in paroxysmal cough with a characteristic whooping that may persist for weeks to months and may be followed by post-tussive vomiting [5,12]. The disease is more severe and pronounced in unimmunised infants and complications of infection with *B. pertussis* may include pulmonary hypertension, pneumonia, febrile seizures, encephalopathy, albeit rare, and brain haemorrhages [12,19-21].

Before vaccination programmes were established, whooping cough was the leading cause of death in infants worldwide. However, large-scale vaccination programmmes introduced in the 1940s with formalin-killed whole cell (wP) vaccines reduced the incidence and mortality by about 90% in developed countries [20]. While the protection offered by these vaccines were reported to last for 4-12 years [22], they infrequently invoked high fevers with or without febrile seizures, swelling, pain and redness at the site of injection [20]. To reduce the incidence and extent of these side

reactions, a second generation of acellular pertussis (aP) vaccines were introduced in the 1990s and administered in combination with diphtheria and tetanus toxoids as "DTaP" vaccines, with incorporation of inactivated polio virus vaccine (IPV) or *Haemophilus influenzae* type b conjugate (Hib) vaccines. A new vaccine formulation, with lower amounts of diphtheria toxoid and pertussis antigens, Tdap, was recently introduced as adult boosters. Although the aP vaccines are less reactogenic than the wP vaccines, the duration of protection offered by aP vaccines has been reported to be less than that offered by wP vaccines with induction of memory B cells being inferior to those induced by wP vaccines [23,24]. All aP vaccines contain genetically or chemically inactivated pertussis toxin (Ptx), alone or in various combinations with, filamentous haemagglutinin (FHA), pertactin (Prn), serotype 2 fimbriae (Fim2) and serotype 3 fimbriae (Fim3).

Although vaccination reduced mortality due to *B. pertussis* infection in infants, whooping cough is still a major cause of vaccine-preventable deaths particularly in developing countries [19,25-27]. The epidemic cycles occur every 3–5 years and so far vaccination has not changed this incidence significantly [28]. In populations with high vaccine coverage, infection rates can reach as high as 1-7% annually [29]. In 2008, the WHO estimated 16 million pertussis cases worldwide with 95% of them in developing nations and resulted in 195,000 child deaths [20]. Yearly deaths of 285,000 to 400,000 infants have been reported [16,30]. The incidence of whooping cough has increased in resource rich countries such as Australia [31], Canada [32], the Netherlands [33], the United Kingdom [34] and the United States [35,36] despite high levels of immunisation coverage. In Australia, whooping cough has re-emerged to become epidemic since 1993 with notifications rising from 1.8/100,000 in 1991 to a peak of 156.9/100,000 in 2010 [26]. Similarly, in England, the year 2012 recorded the highest number of pertussis notification over 12 years. These cases were predominantly among adolescents and adults but with increased deaths of infants under 3 months old, who were too young to complete the recommended vaccination schedule [37]. Figure 1.1 depicts the incidence of whooping cough in Australia, the United Kingdom and the United States, based on national pertussis notification systems. The recent global rise of pertussis may reflect improved methods of diagnosis, incomplete vaccine coverage, limited duration of vaccine-induced

A)



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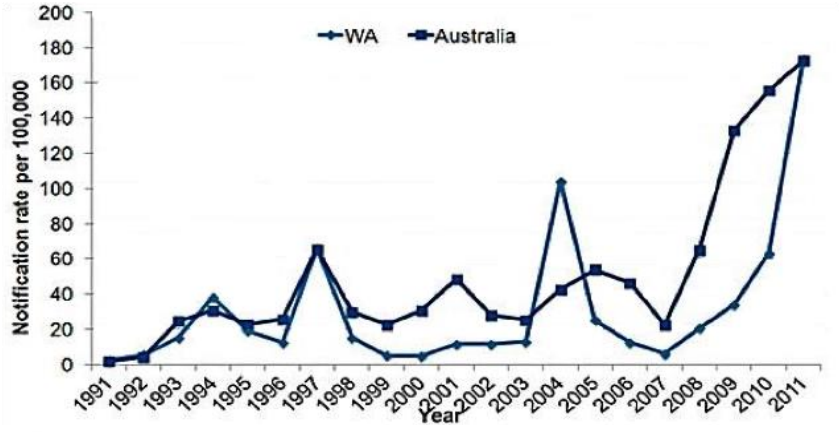

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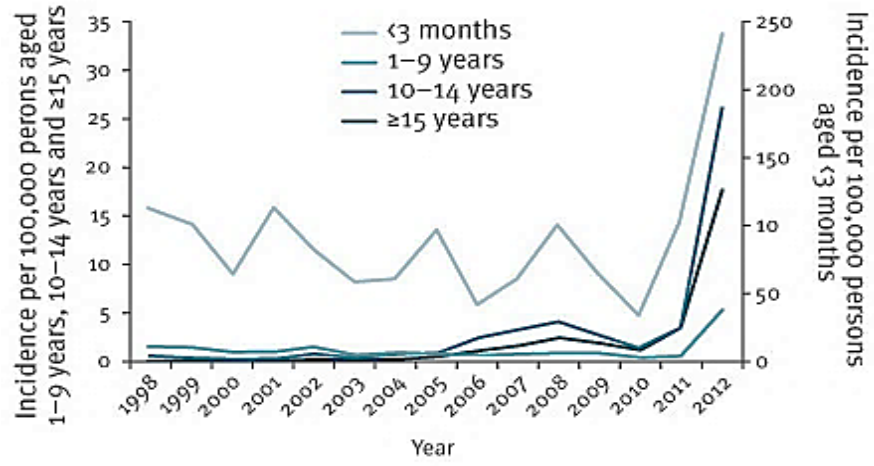

Figure 1.1 Pertussis notification rates from developed countries. Cases from the United States (A)[Ω], Australia (B)[Δ] and United Kingdom (C)[†]. [Ω]Source, Centre for Disease Control and Prevention (www.cdc.gov/pertussis/surv-reporting.html), [Δ]Source, Disease Watch, Government of Western Australia, Department of Health (www.health.wa.gov.au/diseasewatch/vol16_issue1/all.cfm). Notifications for both national and Western Australia (WA) is shown. [†] Source, Health Protection Agency [34]. Year of report shown in y-axis. DTaP; diptheria, tetanus and pertussis.

protection and/or pathogen adaptation. A contributing factor may be the inability of the current aP vaccines to induce potent cell-mediated immunity (CMI) after primary immunisation as required for long-term protection against pertussis [38,39]. Although many factors may contribute to resurgence of pertussis, the limited duration of protection conferred by aP vaccines may facilitate emergence of variant strains capable of evading vaccine-induced protection [40,41]. Immune pressure may have driven the observed evolution of *B. pertussis*, following replacement of wP vaccines with aP preparations [42]. Isolates have emerged that do not produce Prn, vary in production of virulence factors including Ptx [43-46], or are deficient in virulence antigens such as Prn, FHA and fimbriae [47-49].

The pathogenesis of *Bordetella* species, including *B. pertussis* and *B. bronchiseptica* and biofilm formation has been reviewed [50,51]. This include the potential roles of the surface-associated polysaccharide antigen, poly-β-1,6-*N*-acetyl-D-glucosamine (PNAG) or the *Bordetella* polysaccharides (Bps), and their significance in biofilm formation in *vitro* and in mice [51]. By functioning as an adhesin, the Bps of *B. pertussis* has been shown to promote colonisation and biofilm formation in the nose of mice [52]. These reports highlight the contributions of biofilms in bacterial persistence and transmission in human hosts. Here I described the potential of virulence factors and novel biofilm antigens to improve pertussis vaccines and prevent the transmission of whooping cough within the community.

1.2 Regulation and control of *Bordetella pertussis* virulence factors

1.2.1 The two-component BvgAS virulence regulatory system in *Bordetella pertussis*

Infection of the host by *B. pertussis* begins with contact with respiratory droplets from an infected individual. At the host's mucosal surfaces, the bacteria produce virulence factors including adhesins and toxins. *Bordetella pertussis* display phase variation in the expression of virulence factors in response to environmental niche [53]. This is achieved by sensory transduction systems, which transduce environmental signals in gene regulation. Transcription of essentially all *B. pertussis* virulence factors is controlled by a locus called the *Bordetella* master virulence

regulatory system (BvgAS) [54-58] and the putative sensor kinase RisAS two-component system (TCS) [59-61].

The DNA sequence of the BvgAS system revealed one of the first so-called unorthodox TCS, characterised by a composite multi-domain histidine kinase (BvgS) and a four step His-Asp-His-Asp phospohorelay [62]. BvgA and BvgS are 23 and 135 kDa DNA-binding response regulator and a transmembrane sensor kinase, respectively [62], which includes a sensing domain situated in the bacterial periplasm. In response to environmental stimuli, the sensor kinase undergoes a conformational change and transmits signal to the cell resulting in ATP-dependent autophosphorylation of the sensor kinase by its homodimer partner. The phosphorylated BvgS then activates BvgA by transferring its phosphate. The activated BvgA~P, binds to *cis*-acting promoter sequences to activate transcription of virulence-activated genes (*vags*). Simultaneously, a 32 kDa cytoplasmic repressor protein, BvgR, expressed from the BgvAS locus, downregulates the transcription of virulence-repressed genes (*vrgs*) [63,64]. This phase of *B. pertussis* growth is known as the "non-modulated phase", "Bvg^+ phase", or "virulent phase" and is associated with expression of toxins and adhesins required for virulence.

The Bvg^+ phase usually manifests when *B. pertussis* is grown at 37°C in the respiratory tract of a human host [58,62,65]. Conversely, when *B. pertussis* is grown in the presence of millimolar amounts of sulphate ions (50 mM $MgSO_4$), 10 mM nicotinic acid or at temperatures around 25°C, the phosphorylation of BvgA by sensor kinase is suppressed. As a result, BvgA~P is not formed and BvgR is not activated [66]. In this state, BvgAS is not able to activate the transcription of *vags*. The *vrgs* are expressed in the absence of BvgR and the bacteria are in a "modulated, Bvg^- or avirulent phase". During infection, the respiratory environment provides modulating signals that induces the expression of virulence factors [12,67]. For example, the Bvg^- phase in *B. bronchiseptica* is characterised by the expression of flagella for motility and genes encoding urease are expressed, aiding its survival in the nutrient-deprived environments [12,67,68]. Alternatively, Bvg^- phase may represent an evolutionary remnant that is no longer required in *B. pertussis* [16].

In addition to the Bvg^+ and the Bvg^- phases, an "intermediate phase" or "Bvg^i phase" has been identified in *B. pertussis*. The Bvg^i phase is characterised by absence of Bvg-repressed phenotypes owing to the lack of expression of *vrg* genes, with expression of some Bvg-activated virulence factors (eg: FHA), and minimal expression of genes encoding adenylate cyclase toxin (ACT) and pertussis toxin (Ptx) [69]. The Bvg^i phase can be induced in the laboratory by substituting threonine-to-methionine at amino acid residue 733 within the consensus H-box of the transmitter of BvgS of *B. pertussis,* leading to increased resistance to nutrient limitation and reduced virulence [70]. Bvg-intermediate phase A (BipA) protein was one of the first identified Bvg^i phase proteins in *B. pertussis* [69,71]. It is a 1578 amino acid protein and its N-terminus region shares amino acid sequence with the putative outer membrane localisation domain of intimin (Int) and invasin from *Escherichia coli* and *Yersinia* species, respectively [71,72]. BipA was not essential for adherence of *B. bronchiseptica* to rat lung epithelial cells *in vitro*, or for Bvg^i phase-specific persistence and colonisation of the rabbit respiratory tract *in vivo*. However, BipA and the Bvg^i phase may play an important role in the pathogenesis of *Bordetella* species. This may involve aerosol transmission and/or respiratory tract colonisation and survival [71,73,74].

Vags have been categorised into three temporal classes; early, intermediate and late genes [75,76]. Early (or class II) genes include those encoding FHA and Fim and are activated rapidly in response to low concentrations of BvgA~P. Interestingly, the BvgAS system is auto-regulated, and the *BvgAS* locus is categorised as a class II early gene [77]. Auto-phosphorylation of the BvgAS system results in continuous repression and/or expression of downstream genes in *Bordetella* species [78]. The late (or class I) genes include those encoding ACT and Ptx, activated 2-4 hours after activation of the BvgAS locus [79]. Genes encoding Prn belong to the class of intermediate genes expressed about one hour after the activation of the BvgAS locus [79,80]. This phenomenon of differential gene expression by the BvgAS is due to the architectural differences in the BvgAS regulon promoters [12]. In *B. pertussis*, transcription at the *BvgAS* locus is controlled by four promoters: P1, P2, P3 and P4. P1, P2 and P3 direct expression of the *BvgAS* operon, while synthesis of RNA complementary to the 5' untranslated region of BvgAS mRNAs is directed by the P4

promoter [79]. Promoters of the late genes require higher concentrations of BvgA~P because of the low-affinity of BvgA binding sites upstream of the transcription start site. Early gene promoters contain high affinity binding sites for BvgA closer to the transcription start site and can be activated by low levels of BvgA~P [77,78].

The distinctive Bvg^+, Bvg^- and Bvg^i phases have been demonstrated *in vitro* under stable conditions. The human respiratory tract is a more variable environment, so the BvgAS system is unlikely to function as an "on-off" switch. Rather it facilitates expression of a spectrum of virulence factors transitioning between the Bvg^+, Bvg^- and Bvg^i phases [76,81]. Indeed, the *Bordetella* BvgAS system is often considered a "rheostat" that promotes the infectious cycle of *B. pertussis* by enabling its survival, persistence and dissemination in diverse environmental niches [81,82].

1.2.2. The RisAS regulatory system in *Bordetella*

The *Bordetella* regulator of intracellular stress response (ris) system is encoded by the *risAS* locus that encodes a response regulator (RisA) and a sensor kinase (RisS). RisAS is orthologous to the EnvZ-OmpR systems of other Gram-negative bacteria that have been implicated in virulence and shown to reciprocally regulate the expression of the outer membrane proteins, OmpC and OmpF, in response to changes in osmolarity [59]. RisAS is optimally expressed at 37ºC in the absence of magnesium and is important for its intracellular survival independent of the BvgAS regulon [83]. Compared to wild type, a *B. bronchiseptica ris* mutant strain exhibited reduced intracellular survival in mouse macrophages, whereas complementation of *ris* restored its intracellular survival [83]. Moreover, a bacterial acid phosphatase which plays a role in intracellular survival is regulated in response to environmental signals transduced by the RisAS system. The *ris* mutant was susceptible to host intracellular oxidative stress and hence had impaired capacity to persist in the lungs of mice. Complementation of *ris* mutant with the intact *ris* operon restored resistance to oxidative killing in macrophages and survival in the lungs of mice [83,84].

In *B. pertussis*, the *risAS* allele contains an additional 'C' at position 1848, resulting in a frameshift mutation and leading to a truncated, non-functional form of the RisS sensor protein. The deletion of risA reduces the transcription and expression of *vrgs*,

while high levels of *risA* induced strong transcription of *vrgs*, suggesting that *risA* might play an antagonist role to BvgR in the regulation of *vrgs* [59,60]. RisA may activate *vrgs* by binding directly to the cis-activating sites of the *vrgs*, or indirectly by altering the expression of other key regulatory molecules. The *ris* locus regulates the expression of important factors necessary for intracellular survival of *B. bronchiseptica* but the role of this locus in *B. pertussis* pathogenesis has not been clearly elucidated. A study [61] showed that the expression of almost all *vrgs* is under the control of RisA. Activation of the *ris* locus in *B. pertussis* may require a cross-talk from one or more different, as yet uncharacterised, heterologous regulatory system (s), adding another layer of complexity to regulation of virulence in *B. pertussis* [61].

1.3 The pertussis resurgence

The increasing incidence of whooping cough in several countries is shown in Figure 1.1. The resurgence in countries with high vaccination coverage may reflect the short-term protection conferred by the aP vaccines and consequent pathogen evolution. Resurgent and vaccine strains differ in amino acid sequence and the regulation of virulence factors, including those in the current aP vaccines [19,43-46]. Resurgent strains are less affected by sulphate-mediated gene suppression and express higher levels of several virulence factors including Ptx, type III secretion toxin (T3SS), Vag8, a protein involved in complement resistance (BrkA) and LpxE, involved in lipid A modification [33,85]. Whole genome sequencing shed further light on the microevolution and genetic diversity of the p*txP3* strains and differentiated them from the vaccine strain carrying *ptxP1* from which they evolved [86,87]. Several alleles unique to *ptxP3* strains contribute to its fitness [88]. Prn deficiency and FHA deficiency also differentiate *ptxP3* strains [86]. *Bordetella pertussis* strains with a novel allele in Ptx promoter, *ptxP3*, were first reported in the Netherlands [45]. It has since been reported in many countries and replaced the indigenous *ptxP1* strains. The *ptxP3* strains display increased production of pertussis toxin and therefore may be more virulent in humans and suppress the host immunity more efficiently [33,45,85]. Safarchi et al [89] found that the *ptxP3* strains colonised the mouse respiratory tract better than the *ptxP1* strain in both vaccinated and unvaccinated mice. Moreover, emerging *B. pertussis* strains can enhance signaling

through human pattern recognition receptors (TLR2 and NOD2), and induce secretion of IL-10 by dendritic cells [90]. Increased expression of the regulatory molecule PD-L1 may dampen the vaccine-induced protective response, favouring the survival of this pathogen. The emergence of pertactin-negative (Prn^-) isolates in Australia [48], Europe, Japan [91] and the USA [49] may reflect a better fitness of the bacterial pathogen, particularly in populations immunised with aP vaccine and carrying anti-Prn antibodies [92,93]. These findings suggest that *B. pertussis* has adapted to vaccine pressure, so vaccine candidates containing novel antigens from the resurgent strains are warranted.

1.4 Virulence-associated factors of *Bordetella pertussis*

The structure and functions of the wide array of virulence factors produced by *B. pertussis* (Table 1.1) are reviewed below.

1.4.1 Pertussis toxin

Pertussis toxin (Ptx) is a major virulence factor and a protective antigen produced exclusively by *B. pertussis* [94,95]. While species such as *B. parapertussis* and *B. bronchiseptica* have genes encoding the toxin, they do not produce functional Ptx [19]. Ptx is an exotoxin that is transported across the bacterial outer membrane by a type IV secretion system [12,96]. It is a 117 kDa hexameric subunit protein of AB_5 configuration consisting of one active subunit (subunit A or S1 subunit), and five binding B oligomers [12,19,96]. The A subunit consists of a catalytic ADP-ribosylating domain, and is responsible for the enzymatic activity of Ptx [96]. The B oligomer is composed of S2, S3, two S4 and S5 pentameric-subunits involved in binding of the toxin to extracellular target cell receptors, including the Toll-like receptor 4 (TLR-4) and others glycoconjugate receptors. It enables the translocation of the catalytic A subunit (S1) across the plasma membrane and allows the toxin to enter the cells [12,19].

The molecular mechanism involved in entry of Ptx into the cell is not well understood. In the cytosol, the A subunit catalyses the transfer of released ADP-ribose from the hydrolysed cellular nicotinamide adenine dinucleotide (NAD^+) to a specific cysteine residue at the C-terminus of the α-subunit of the heterotrimeric $G_{i/o}$

Table 1.1 Virulence factors of *Bordetella* and their role in pathogenesis and vaccine development

Virulence factors/ Antigens	Molecular Character	Class/ Family	Role in *B. pertussis* pathogenesis	Appearance during infection/Bvg phase	Mechanism of action in Disease	Component/ References
Toxins						
Pertussis Toxin (Ptx)	117 kDa hexameric subunit with AB5 configuration	Typical A-B toxin of ADP riboxylating family	Adhesion, immune evasion, local and systemic toxin effects	Expressed as late genes. Acts in synergy with FHA	Coupling of Gαi protein-receptor is inhibited and its signal transduction is blocked	Component of acellular vaccines
Adenylate cyclase toxin (ACT)	177 kDa toxin	repeats-in-toxin (RTX) family of toxins activated by eukaryotic calmodulin	Evasion of host immune response, local and systemic toxin effects	Expressed as late genes. Acts in synergy with Ptx and FHA	Conversion of intracellular ATP to cyclic cAMP and affects superoxide generation, immune effector cell chemotaxis, phagocytosis and bacterial killing. Pore formation and disrupts cells	Not component of acellular vaccines
Tracheal Cytotoxin (TCT)	921 dalton	Muramyl peptide family	Evasion of host immune response, local toxin effects	Expressed as early genes	Causes ciliostasis, inhibit DNA synthesis and extrusion of ciliated cells	Not component of acellular vaccines
Dermonecrotoxin (DNT)	160 kDA	A-B family of toxin with polyamination and deamination activity	Local derma necrosis and systemic vasoconstriction toxin effects	NK	Activates host GTP binding protein Rho and results in constitutive expression of GTPase activity	Not component of acellular vaccines
T3SS	-	Membrane-embedded nano-injection machinery	translocates bacterial virulence factors,	Bvg+	Secretion of effectors and translocons	[97,98]
Adhesins						
Filamentous Haemaggutinin (FHA)	232 kDa	Filamentous protein	Adhesion	Expressed as both Bvg^+ and Bvg^- phase	Binds to ciliated tracheal epithelium, macrophage CR3 receptors and promotes phagocytosis	Component of most acellular vaccines

Virulence factors/ Antigens	Molecular Character	Class/ Family	Role in *B. pertussis* pathogenesis	Appearance during infection/Bvg phase	Mechanism of action in Disease	Component/ References
Pertactin (Prn)	69 kDa	Auto-transporter family of proteins	Adhesion	Expressed as intermediate genes	Binds to ciliated tracheal epithelium, macrophage CR3 due to the presence of RGD motif	Component of three- and five-component acellular vaccines
Fimbriae (Fim)	Fim2: 22 kDa Fim3: 21.5 kDa	Filamentous proteins	Adhesion: Fim2 binds to sulphated sugars; Fim3 and FimD to heparin and integrin VLA-5	Expressed as early genes	Binds to tracheal epithelial cells, predominantly in trachea	Component of acellular vaccines
Tracheal colonising factor (Tcf)	64kDa	Auto-transporter family	Adhesion	NK	Binds exclusively to tracheal epithelium	Not component of acellular vaccines
Other virulence factors of *Bordetella* used as vaccine antigens						
Proteolisosome preparation	-	A nanoparticulate vesicular structures that contains proteins, lipids and native LPS	NK	NK	-	[99]
PNAG	-	Composition of outer membrane polysaccharide	NK	NK	Major component of bacterial cell wall	Not studied
Biofilms	-	Extracellular mesh of sessile bacteria	NK	NK	Protects bacteria from host immune response, antimicrobial peptides and persistence of bacteria inside an extracelluar matrix	[100]
Serum resistance factor (BrkA)	103 kDa	Auto-transporter family of proteins	Adhesion	NK	Serum resistance factor. Confers resistance to bacteria from complement (C) killing	[101,102]
Sph1B	Subtilisin-like Serine protease	Auto-transporter family of proteins	Adhesion due to presence of one or more RGD tripeptide motif	NK	Promotes the maturation of FHA adhesion molecules on *Bordetella*	Not component of acellular vaccines

Virulence factors/ Antigens	Molecular Character	Class/ Family	Role in *B. pertussis* pathogenesis	Appearance during infection/Bvg phase	Mechanism of action in Disease	Component/ References
Vag8	C1 esterase inhibitors (C1inh)	Auto-transporter family of proteins	Adhesion due to presence of one or more RGD tripeptide motif	Bvg^+ Virulent phase	Confer resistance to bacteria from C1 esterase mediated C killing	Not component of acellular vaccines
Siderophores (IRP-1 and AfuA)	AfuA:39 kDa IRP-1: ~25 kDa	Iron-binding protein	Overcome host iron restriction	Bvg^+ Virulent phase	Enable bacteria to thrive in iron limiting niche	[103,104]

NK; not known, T3SS; type III secretion system

family regulatory proteins [96,105]. This prevents the coupling of G-regulatory proteins to their cognate receptors (GPCRs), resulting in an inactive (GDP-bound) form of the α-subunit [76]. Once inactivated, G-proteins are unable to inhibit adenylate cyclase activity, and thus are unable to halt the conversion of intracellular ATP to cAMP (Table 1). The accumulation of intracellular cAMP disrupts many cellular cascades as shown in Figure 1.2.

Diverse effects of Ptx are attributed to the ADP-ribosylation of the $G_{i/o}$ protein family. They include most systemic symptoms of pertussis infection such as leukocytosis, insulinaemia, hypoglycaemia and histamine sensitivity in children [105], and lethality of neonatal mice challenged with virulent *B. pertussis* [96]. Accordingly, a *B. pertussis* strain deficient in Ptx isolated from an unvaccinated child with suspected pertussis displayed low virulence [106]. However, most acellular pertussis vaccines contain chemically or genetically detoxified pertussis toxin (PTxoid) in combination with FHA with or without incorporation of Prn and/or fimbriae (Fim2, Fim2/Fim3) (Table 2). PTxoid alone as a mono-component antigen tested as an acellular pertussis vaccine yielded an overall efficacy of 71% in vaccinees and reduced *B. pertussis* transmission to household contacts [107,108]. However, chemical detoxification alters the tertiary and quarternary strucures of Ptx, thereby affecting recognition of protective epitopes by antibodies [109].

1.4.2 Adenylate cyclase toxin

Adenylate cyclase toxin (ACT) is an immunomodulatory toxin produced by *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* [110]. ACT functions as a haemolysin and cytolysin, and is a member of the repeat-in-toxin (RTX) family that forms pores in bacterial cell membranes [111,112]. It is a polypeptide of 1,706 amino acids consisting of two domains, a N-terminal adenylate cyclase (AC) domain of approximately 400 amino acid residues and a haemolytic C-terminal domain of 1300 amino acids [113]. The RTX haemolytic (Hly) domain mediates binding of the toxin to target cells and translocation of AC to the cytosol, via cation-selective toxin pores [114]. ACT mediates adherence to host cell by binding to the $\alpha_M\beta_2$ integrin (CD11b/CD18 or CR3 or Mac-1) expressed on myeloid phagocytic and natural killer cells [115,116]. In the cytosol, AC is activated by a Ca^{2+}-binding protein known as

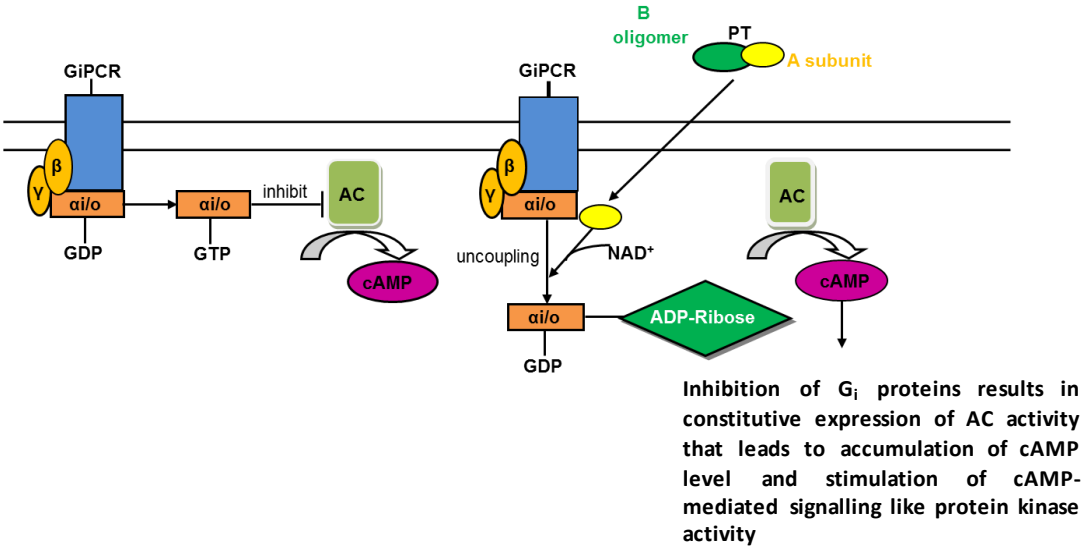

Figure 1.2 Pertussis toxin (Ptx) mediated uncoupling of Gαi/o proteins from GiPCR. Exchange of GTP from GDP results in activation of G-inhibitory proteins ($G\alpha_{i/o}$) which inhibit adenylyl cyclase (AC) activity and reduce cAMP levels. The A subunit of Ptx ADP-riboxylates the $G\alpha_{i/o}$-inhibitory protein and inactivates Gαi/o resulting in constitutive expression of AC activity, accumulation of cAMP and activation of cAMP-mediated signalling pathways.

Table 1.2 Major pertussis vaccines and their antigen components used by different pharmaceutical companies

Vaccine	Trade Name	Manufacturer	Pertussis antigen content (µg)*				Indications/ References
			FHA	Ptx	Prn	Fim 2/3	
DTPa1	Certiva	Baxter	-	40	-	-	3 months to 12 months
DTaP	Daptacel	Sanofi Pasteur	5	10	3	5	6 weeks to 6 years
	Infanrix	GlaxoSmithKline	25	25	8	-	6 weeks to 7 years
	Tripedia	Sanofi Pasteur	23.4	23.4	-	-	6 weeks to 7 years
	ACV-SB	Smithkline Beecham	25	25	8	-	Used in Dutch NIP
	Pentavac[α]	Sanofi Pasteur	25	25	-	-	2, 4 and 6 months
	Acelluvax	Novartis	2.5	5	2.5	-	2, 4 and 6 months
DTaP4	Acel-Immune	Wyeth/Takeda	35	3.5	2	0.8	3, 4.5, 6 and 15 months
DTaP/ Hib	TriHIBit	Sanofi Pasteur	23.4	23.4	-	-	Children 15-18 months
DTaP/ Polio	Kinrix	GlaxoSmithKline	25	25	8	-	Children 4-6 years old
DTaP/ Polio/ Hib	Pediacel[α]/ Pentacel	Sanofi Pasteur	20	20	3	5	Infants and children 6 weeks to 4 years
DTaP/ Polio/ hepatitisB	Pediarix/ Infanrix	GlaxoSmithKline	25	25	8	-	6 weeks to 6 years
Tdap	Adacel	Sanofi Pasteur	5	2.5	3	5	Adolescent and Adults 11-64 years
	Boostrix	GlaxoSmithKline	8	8	2.5	-	10 years and older
	Triaxis	Sanofi Pasteur	5	2.5	3	5	2 months to 4 years
	DTwP-IPV	RIVM, Bilthoven, the Netherlands	2.6	0.16	n.d	n.d	Used in Dutch NIP for 2 months to 4 years [117]

NIP; national immunisation program, DTaP, diphtheria, tetanus and acellular pertussis, Tdap; reduced DTaP, ACV; acellular vaccine, *concentration of pertussis antigen components only, Hib, *haemophilus influenzae* B. [α]Pentavac and Pediacel are DTaP-IPV-Hib vaccine.

calmodulin (CaM) and catalyses the conversion of intracellular ATP to cAMP that inhibits superoxide generation, immune effector cell chemotaxis, phagocytosis and bacterial killing [111,118].

ACT is an important bi-functional virulence factor secreted by *B. pertussis* in the early stage of infection and can elicit a protective immune response [119,120]. Although antibodies against ACT have been found in sera of infants and children infected with *B. pertussis* [121] or immunised with whole cell vaccines [122], it is surprising that ACT has not been included in any currently available aP vaccines. A study reported that immunisation of mice with a purified adenylate cyclase enzyme lacking the toxin fragment protected mice from *B. pertussis* challenge when delivered intranasally, and significantly enhanced the clearance of bacteria from the mouse respiratory tract [123]. It suggested that this protective antigen, if incorporated into aP vaccine formulations, could reduce the asymptomatic human reservoir by limiting bacterial carriage in the respiratory tract. Of interest is the report that protective antibodies induced by ACT were directed against the last correctly folded 800 residues of the C-terminal region of the toxin [124].

Since ACT delivers the N-terminal catalytic domain into the cytosol of eukaryotic cells, including human effector immune cells, it has been exploited as a tool for antigen delivery [96,125]. Fayolle et al [126] chemically linked peptides containing $CD8^+$ T-cell epitopes from several proteins to the catalytic domain of ACT and showed that these recombinant proteins are presented to $CD8^+$ cells inducing cytotoxic T-lymphocytic (CTL) responses in immunised mice. Another study demonstrated that ACT linked to epitopes of antigenic proteins and delivered into CD11b myeloid dendritic cells induced epitope-specific Th1 $CD4^+$ and $CD8^+$ T cell responses [127]. Similarly, immunisation of mice with epitopes of HIV-1 Tat linked to the catalytic domain of ACT (ACT-HIV-1-Tat peptide), elicit a strong Th1-skewed immune response [128,129]. These findings suggest the potential for ACT as a carrier for delivery of a range of therapeutic agents including heterologous vaccines.

1.4.3 Tracheal cytotoxin

Tracheal cytotoxin (TCT) is a peptidoglycan component of bacterial cell wall peptidoglycan [12], a 921 dalton disaccharide-tetrapeptide composed of N-acetylglucosaminyl-1,6-anhydro-N-acetylmuramylalanyl-gamma-glutamyl-diamino-pimelyl-alanine and belongs to the muramyl family of peptides [130,131]. Muramyl peptides are produced by bacteria and are the polymeric components of the cell wall that provide structural rigidity [132]. It has several important biological functions including immunogenicity, somnogenecity and pyrogenicity. During bacterial growth and processing by macrophages, Gram-negative bacteria such as *E. coli* and *Neisseria gonorrhoeae* produce muramyl peptides similar to TCT through the action of transglycosylase enzymes [133]. The muramyl peptides of *N. gonorrhoeae* have been associated with ciliated cell destruction during infections of the fallopian tube [134]. TCT is similar in structure to the sleep promoting factor, FSu, a muramyl peptide found in the human brain that promotes active slow-wave sleep [134]. Of the three major *Bordetella* species, TCT is produced only by *B. pertussis*. In *B. pertussis*, TCT is independent of the BvgAS control system and is constitutively expressed [1]. *Escherichia coli* recycle the peptidoglycan fragment into the cytoplasm for reincorporation into the peptidoglycan biosynthesis pathway through an integral cytoplasmic membrane protein, AmpG [135]. However, *B. pertussis* has no functional AmpG activity so the TCT fragment is released into the external milieu [1] during log phase growth and in the virulent phase [133,136], and damages ciliated cells of the respiratory tract promoting ciliostasis and colonisation. When ciliary movement is retarded, coughing remains the only mechanism to eliminate accumulation of other inflammatory molecules from the respiratory tract, resulting in the eponymous paroxysmal cough. The destruction of ciliary cells by *B. pertussis* TCT also makes the host susceptible to secondary infections, which is often the primary cause of deaths.

Cundell et al [137] showed that TCT inhibits human neutrophil functions including migration, IL-1α production and complement activation by the alternative pathway, and so may increase survival of *B. pertussis* during respiratory infections. Production of IL-1 by the intracellular tracheal epithelial cells drives production of inducible

nitric oxide synthase (iNOS) and hence nitric oxide ($^{\bullet}$NO), which damages tracheal cells [138,139].

1.4.4 Dermonecrotic toxin

Bordetella pertussis dermonecrotic toxin (DNT) is a heat-labile, 1,464 amino acid (160 kDa) protein consisting of a single chain polypeptide with an N-terminal receptor binding domain and C-terminal enzymatic domain [140]. The name dermonecrotic toxin is derived from a characteristic skin lesion induced by the toxin when admintered intradermally into animals. It can catalyse polyamination or deamidation of small Rho family GTPases such as Rho, Rac and Cdc42, through a novel transglutaminase activity [140-142]. Unlike many bacterial toxins, DNT activates Rho family GTPases, which are essential for functions including reorganisation of the actin skeleton, cell motility, focal adhesion, transcription of certain essential genes and cell differentiation [143]. On activation, the inactive GDP-bound forms exchange GDP for GTP. GTP-bound DNT actively transduces downstream signals before reverting back to the inactive GDP-bound form [144]. Polyamination or deamidation of Rho-GDP by DNT at the Gln63 residue of Rho results in formation of Rho-GTP and prevents the reversion of Rho-GTP to its inactive GDP-bound form. The constitutive expression of the active GTP-bound form (Figure 1.3) results in expression of stress fibres, inhibition of cytokinesis, and disturbances of cell differentiation via its association with effector protein, ROCK [145]. DNT can inhibit DNA and protein synthesis, inhibit osteoblastic differentiation, induce organelle formation on membranes and induce formation of caveolae [141,145]. The N-terminal 54 amino acid B (binding) domain is responsible for binding of DNT to target cells, while the C-terminal 288 amino acids A (active) domain is responsible for the enzymatic activity of DNT [146]. Residues 2−30 constitute the binding site within the B domain [147].

Bordetella parapertussis, B. avium and *B. bronchiseptica* DNT molecules are identical [145,148,149]. Despite the reported protective capacity of the DNT-knockout *B. pertussis* vaccine candidate [27], the absence of DNT in a mutant strain did not affect its virulence compared to the wild-type [150] limiting its relevance to the development of a live attenuated vaccine against pertussis.

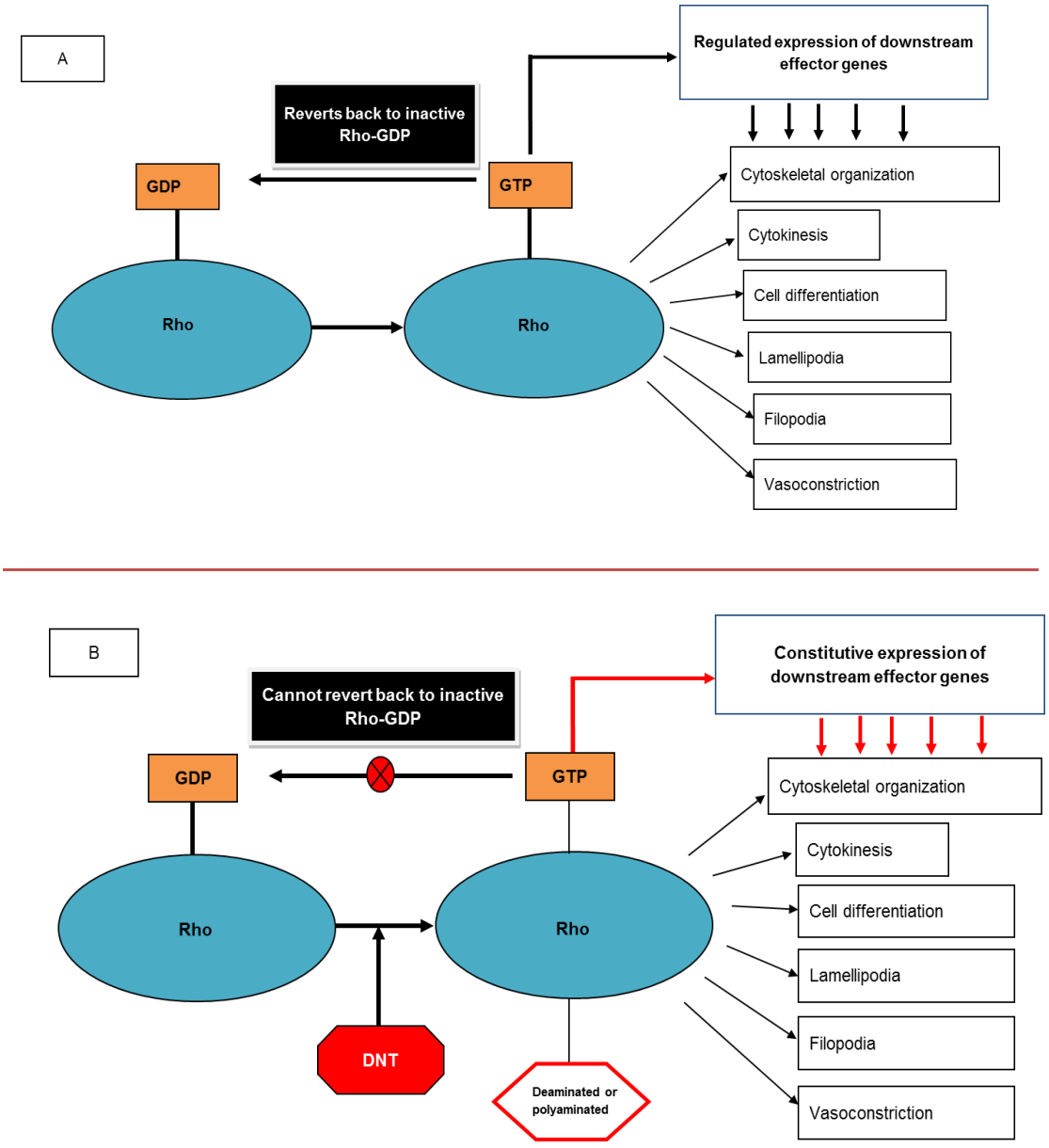

Figure 1.3 Action of DNT on functions of Rho-GTPase. (A) Normal pathways of Rho-GTP activation in the absence of DNT. (B) Deamination or polyamination of Rho-GTP by DNT abrogates the GTP-hydrolysing activity of GTPase and reversion to Rho-GDP is blocked, resulting in constitutive expression of Rho-GTPase activity. Additionally, polyaminated GTPase attains the ability to stimulate downstream effector genes in a GTP-independent manner and induce constitutive expression of GTPase resulting in anomalous cellular events.

1.4.5 Pertactin

Pertactin (Prn) belongs to the family of type V autotransporters virulence factors [110,151]. Mature Prn is a 68 kDa, 69 kDa and a 70 kDa protein in *B. bronchiseptica*, *B. pertussis* and human *B. parapertussis*$_{HU,}$ respectively, and is encoded by the *prn* gene. Prn contains an arginine-glycine-aspartate (RGD) tripeptide motif required for attachment of the *Bordetella* species to integrin binding sites of eukaryotic cells [1]. The nascent Prn polypeptide has a unique central passenger domain flanked by an N-terminal signal sequence and an approximately 30 kDa C-terminal porin domain. The N-terminal signal sequence directs the protein into the periplasm and the C-terminal porin domain forms a channel in the outer membrane that is required for the transport of the passenger domain to the cell surface [151]. On the cell surface, the passenger domain folds into right-handed β-helix comprising 18 three-β-strand repeats connected by turns of differing lengths [152]. Amino acids 260-294 in *B. pertussis* Prn constitute region 1 (R1) and include several Gly-Gly-Xaa-Xaa-Pro repeats and the RGD motif. R1 may be the immunodominant region of *B. pertussis* Prn. Antibodies targeting R1 isolated from a patient with *B. pertussis* conferred protection in mouse respiratory models [153-157]. Residues 563-614 in the C-terminal domain of *B. pertussis* Prn contain Pro-Gln-Pro (PQP) repeats and constitute region 2 (R2). Region R1 is highly polymorphic and has been implicated in vaccine-induced evolution of *B. pertussis* [153,158].

Intriguingly, despite Prn being an important virulence factor, few studies address the function of Prn in *B. pertussis* pathogenesis. The RGD motif in the centre of the passenger domain allows Prn to function as an adhesin. Early studies [159,160] suggested that attachment of *B. pertussis* to Chinese hamster ovary (CHO) or human HeLa cells requires Prn. However, later studies [161,162] failed to demonstrate the essential role of Prn in attachment to mammalian cells. More recently Inatsuka et al [151] implicated *B. bronchiseptica* Prn in resistance to clearance of bacteria from the lungs of mice mediated by neutrophils. A *B. bronchiseptica* Δprn mutant was unable to infect SCID-beige mice (lacking T-cells, B-cells and NK cells) but its ability to cause lethal infection in neutropenic mice was not impaired. The authors inferred that the RGD sequence was not essential for Prn function. Clearly, further studies are necessary to elucidate the role of Prn in the pathogenesis of *B. pertussis*.

Prn is a component of most currently used acellular pertussis vaccines (Table 1.2). *B. pertussis* isolates with variation in Prn and Ptx have been first reported in the Netherlands [44]. After the introduction of whooping cough vaccination, vaccine type variants (PtxA2 and Prn2) were replaced by non-vaccine type variants (PtxA1 and Prn2). Similar shifts are now observed worldwide and PtxA1-Prn2 strains predominate in most vaccinated populations [163]. Since the introduction of the acellular pertussis vaccine, *B. pertussis* strains lacking Prn have been reported in Finland [164], Sweden [165], the United States [166], Japan [91] and Australia [48]. These Prn-negative isolates were fully virulent [167]. In a mouse model, several studies showed that antigenic divergence observed between vaccine strains and resurgent strains reduces the vaccine efficacy [93,153,168,169]. As strains deficient in the expression of Prn have been isolated from infants and children vaccinated with DTaP [49,168], inclusion of Prn in aP vaccines warrants further investigations.

1.4.6 Tracheal colonisation factor

Tracheal colonisation factor (Tcf), first described in 1995 [170], has been found in *B. pertussis* but not in *B. parapertussis* or *B. bronchiseptica*. It is encoded by the *tcfA* gene and expressed in both cell-associated and secreted forms. Tcf functions as an adhesion molecule and contains RGD motifs with high (16.5%) proline content. It is released as a 60 kDa and 30 kDa forms from the N- and C-termini of the 90 kDa precursor protein respectively, where the 30 kDa protein is identical to *Bordetella* pertactin precursor protein. *B. pertussis* strains deficient in Tcf colonised the trachea of mice tenfold less efficiently than the wild type strain, but the establishment and persistence of infection with *B. pertussis* deep in the lungs was unaffected [171].

1.4.7 *Bordetella* resistant to killing (BrkA) protein

BrkA is another autotransporter and a cell surface-associated virulence antigen of *B. pertussis* evading killing via the classical complement pathway [172]. It is a Bvg-regulated protein and contributes to the adherence of *Bordetella* species to the host cells and prevent lysis of the bacteria by select antimicrobial agents [173,174]. BrkA is detectable in *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* but not in *B. avium* [4]. The Brk locus encodes two open reading frames (ORFs), *brkA* and *brkB*, both of which are required for resistance to killing by human serum. The *brkA* ORF

encodes a 103 kDa precursor protein that is processed to yield a 73 kDa N-terminal protein with α-passenger domain possessing functional activity [175]. *brkB* encodes a C-terminal 30 kDa β-domain predicted to be an inner membrane protein [176]. The β-domain facilitates the transport of passenger protein by forming pores in the lipid bilayer, and serves as an intramolecular chaperone facilitating the folding of the passenger domain and translocation across the outer membrane [1,4,177].

While BrkA proteins are not included in any acellular vaccines, a case has been made for their inclusion as a replacement for Prn. Oliver et al [178] showed that rabbit antibodies against BrkA blocked BrkA activity and killed wild type *B. pertussis*. Mice immunised with the purified 73 kDa N-terminal α-domain of BrkA fused to the 60kDa heat shock protein (HSP) of *B. pertussis* produced protective IgG1 and IgG2a antibodies - indicating Th2 and Th1-type immune responses, respectively. When administered together with Diphtheria-Pertussis-Tetanus (DPT), 42% of mice were protected, compared to no protection when the DTP vaccine was used alone [101].

Marr et al [102] showed that immunisation of mice with a three-component vaccine containing Ptx, FHA and BrkA was as efficacious as the commercial Infanrix^{TM} vaccine in protecting mice against colonisation by virulent *B. pertussis* strain Tohama I, but immunisation with BrkA protein alone did not protect against colonisation. Since BrkA is present in all clinical *B. pertussis* isolates, they suggested that the new acellular pertussis vaccine formulation that includes BrkA may confer protection comparable to the currently marketed DTaP vaccines [146].

1.4.8 Other auto-transporter proteins

Other auto-transporter proteins reported in *B. pertussis*, *B. bronchiseptica* and *B. parapertussis*$_{HU}$ species include SphB1 (Section 1.4.9.1) and Vag8. These two proteins demonstrate similarity in amino acid sequence at their C-terminal domains and contain one or more RGD tripeptide motifs, suggesting a role in adherence.

Vag8 is a 95 kDa outer membrane protein encoded by the *vag-8* gene [179]. The C-terminal ends of Vag8 show significant sequence similarity with the C-terminal ends

of Prn, BrkA and Tcf. It was the fourth member of the auto-transporter family of proteins to be identified in *Bordetella* species [179]. The Vag8 protein of *B. pertussis* binds to human C1 esterase inhibitor (C1inh), a major complement regulatory protein in a Bvg-regulated pathway and resists its killing by complement [180]. *Bordetella pertussis* mutants deficient in Vag8 were susceptible to serum killing while wild type *B. pertussis* strains expressing Vag8 were resistant [181]. Furthermore, immunisation of mice with Vag8 induced strong antigen-specific IgG and significantly reduced bacterial load in the lungs of mice challenged with virulent *B. pertussis* [182]. This suggests a novel role of Vag8 in immune evasion, hence Vag8 warrant inclusion in future pertussis vaccines.

An autotransporter protein, BapC, with functions similar to BrkA and Prn was described recently as a virulence antigen of *B. pertussis* [183]. BapC is Bvg-regulated and functions as an adhesin factor responsible for adhesion of *B. pertussis* to various cell lines, while conferring resistance to complement killing [184].

1.4.9 *Bordetella pertussis* adhesins

1.4.9.1 Filamentous haemagglutinin

Filamentous haemagglutinin (FHA) is a cell surface-associated 220 kDa mature protein that is secreted into the extracellular environment [12,19,185,186]. FHA is encoded by the *fhaB* gene in *B. pertussis* and *B. bronchiseptica* [187,188]. It plays a key role in initial attachment of *B. pertussis* to the respiratory mucosal epithelium of host cells, including macrophages [186,189,190]. FHA has three binding sites by which it interacts with host cells: a) an N-terminal glycosaminoglycan binding site, b) an RGD sequence and c) a carbohydrate recognition domain (CRD). FHA has two main immune-dominant regions termed the C-terminal type I and N-terminal type II domains, which are highly immunogenic. The C-terminal domain consists of 456 amino acids and contains most of the reactive epitopes and cell binding sites [186,189]. The N-terminal domain may be responsible for displaying FHA on the surface of *B. pertussis*.

The maturation of FHA from its precursor FhaB, requires a specific protease SphB1. SphB1 is a subtilisin-like serine protease/lipoprotein essential for processing the

precursor of the FHA [191,192]. It was the first reported auto-transporter that is necessary for a maturation of another protein secreted by *B. pertussis*. *Bordetella pertussis* mutants deficient in Sph1B were strongly compromised in their ability to colonise the mouse respiratory tract. However, colonisation ability was restored when purified FHA was instilled nasally or co-infected with an FHA-expressing *B. pertussis* strain [193], highlighting the importance of the SphB1 protease in the maturation and release of FHA.

Despite the importance of FHA in *B. pertussis* pathogenesis and its inclusion in most acellular pertussis vaccines, little is known about the induction of protective immunity [194]. FHA has not been tested as a mono-component acellular vaccine but an 80 kDa protein derived from the N-terminal domain of FHA (Fha44) showed some promise. Intranasal infection of mice with a *B. pertussis* strain producing Fha44 instead of FHA produced a more effective humoral response [189] (See also Table 1.2). However, the 85 kDa protein comprising the 42 kDa maltose-binding domain of *E. coli* combined to 43 kDa type I immune-dominant domain of FHA, performed better as a potential vaccine candidate [194]. However, this work needs re-assessment because of differences in the delivery models used in the above two studies.

1.4.9.2 Fimbriae

Fimbriae (FIM) are filamentous, cell surface-associated polymeric proteins essential for colonisation of the respiratory tract. *Bordetella pertussis* produces contains serologically distinct types of fimbriae (serotypes 2 and 3) composed of major subunits Fim2 and Fim3, respectively [195]. These subunits [also known as agglutinogen2 (AGG2) and agglutinogen3 (AGG3)], are encoded by the scattered and unlinked chromosomal loci, *fim2* and *fim3* genes, respectively. Their expression is regulated by small insertions or deletions within a stretch of a cytosine-rich promoter region (-10 and -35 elements) resulting in fimbrial phase variation in addition to its positive regulation by the BvgAS system [196]. The slip-strand mispairing affects the transcription of the individual fimbrial genes independent of each other. Hence *B. pertussis* may express either Fim2, Fim3 or a combination of fimbriae at any given time [196].

The major fimbrial helices are bundled to form long filaments with FimD on their surface. FimD is encoded by the *fimD* gene cluster involved in fimbrial and FHA biosynthesis. FimD recognises two ligands in the respiratory tract, the integrin very late antigen-5 (VLA-5) and heparin sulphate [197,198]. The binding of FimD to VLA-5 on monocytes activates complement receptor 3 (CR3), which is a ligand for FHA, and enhances the binding of *B. pertussis* to respiratory epithelial cells [198]. The major fimbrial subunits bind to chondroitin sulphate, heparin sulphate and dextran sulphate, which are universally present in the respiratory tract [199].

Early attachment to the host epithelium is often considered critical in bacterial pathogenesis. Although fimbriae are associated with initial attachment, a definitive role of fimbriae as adhesin has not been established for several reasons. The presence of multiple, unlinked and scattered major fimbrial subunit genes complicates the construction of strains completely deficient in fimbriae, the coupling of fimbrial biosynthesis operon with the *fha* operon [1] and the presence of several other putative adhesin molecules with redundant functions masks the detection of clear phenotypes for the Fim-deficient mutants.

It is likely that FimB and FimD are required for fimbrial production but not for FHA biogenesis [200]. Although, *fhaC* was necessary for FHA production, it did not participate in fimbriae biogenesis. On the other hand, *fimD*-mutants had a reduced ability to colonise the nasopharynx, trachea and lungs [201]. A study of the role of fimbriae in adherence of *B. pertussis* to primary human airway epithelial cells from human bronchi and a human bronchial epithelial cell lines suggested that this model was more informative than animal models because the cells used could proliferate *in vitro*, differentiate, and express the same genetic profile as human respiratory cells *in vivo* [202]. Other studies [162,190] showed that both fimbriae and FHA were required for adhesion of *B. pertussis* to the laryngeal epithelial cell line, HEp-2, whereas only FHA was required for adhesion to the bronchial cell line NCI-H_{292}. These authors concluded that fimbriae played an important role in the colonisation of the laryngeal mucosa, whereas FHA was important in colonisation of the entire respiratory tract. However, a mutant of *B. bronchiseptica* that did not express

fimbriae was unable to form biofilm, implying a role of fimbriae in biofilm formation [55]. Whether this holds true for *B. pertussis* requires confirmation.

Fimbrial antigens included in the current five-component acellular vaccines are shown in Table 1.2. Antibodies against fimbriae are protective against *B. pertussis* infection. Sera from mice immunised with fimbriae reduced the adherence of *B. pertussis* to NCI-H_{292} cells *in vitro*, as did the antisera raised against Ptx, FHA, Prn or whole cell pertussis vaccine [203]. Similarly, Rodriguez et al [204] showed that purified antibodies against fimbriae reduced the attachment of *B. pertussis* to respiratory epithelial cells. A study of the five-component vaccine (containing fimbriae) revealed a protective efficiency of 85.25%, while the two-component vaccine (inactivated pertussis toxin and haemagglutinin) and the whole-cell vaccines had protective efficiencies of only 58.9% and 48.3%, respectively [205]. However, in another study the two-component vaccine comprising of Ptx and FHA was as immunogenic and protective as DTwP or DTaP following primary and booster vaccinations [206]. Hallender et al [207] showed that even 71 months after vaccination with the five-component vaccine, 60% of children still had protective levels of anti-fimbriae antibody (IgG anti-Fim2/3 ≥5EU/ml) correlating with decreased risk of *B. pertussis* infection. These authors emphasised the need to include Fim2/3 in future pertussis vaccines.

A new variant of fimbriae, Fim3-2, has reached significant prevalence (up to 80%) in some countries [208-210]. In Finland, the Fim2 type predominated during the early vaccination period while the Fim3 type has predominated since 1999 and represented the major strains responsible for a nation-wide epidemic in 2003 [208]. Intriguingly, about one third of patients infected with Fim2 strains developed antibodies that bind to both Fim2 and Fim3 antigens. These authors suggested that the Fim2 strains could express Fim3 during infection [208]. While a mix of Fim2, Fim3 and Fim2,3 strains circulated before mass vaccination in the UK, Fim3 serotype predominated in later years [46]. Similar shifts were observed in Canada [211], France [212], Russia [213], Serbia [214] and Sweden [165,215].

1.4.10 *Bordetella* Type III secretion system

Bacterial type III secretion system (T3SS) is a membrane-embedded nano-injection structure comprising a hollow extracellular needle and a cylindrical basal body extending beyond bacterial inner and outer membranes [216]. T3SS translocates bacterial virulence factors, termed effectors and translocons, into the host cells to exert their biological functions leading to establishment of infection, persistence and transmission. T3SS is highly conserved in *B. pertussis*, *B. bronchiseptica* and *B. parapertussis* [217]. In *B. bronchiseptica*, T3SS modulates host immune responses [218-220], and contributes to persistent colonisation of the mouse trachea [221]. The *Bordetella* T3SS is transcribed from the *bsc* locus that comprises 30 ORFs and is regulated by the BvgAS virulence regulon [222,223]. In *B. bronchiseptica*, T3SS secreted proteins include the translocons, BopB, BopD, BopN, Bsp22 and the only effector, BopC/BteA.

Despite the conservation of T3SS, the *B. pertussis* T3SS operon is not expressed in the laboratory strain Tohama I. This difference between species may be due to differential regulation of T3SS by BtrA, - a molecule known to reduce T3SS function [224]. Ahuja et al [225] showed that BtrA differentially regulate the expression of BvgAS-regulated genes including *cyaA*, *fha*, *prn*, *ptx* and T3SS-secreted proteins. Deletion of *btrA* in *B. pertussis* enhances the expression of *bopB*, *bopD*, *bopN*, *bsp22*, *bscN* and *bteA*. This finding suggests that T3SS is fully functional in *B. pertussis* but its expression is repressed by the BtrA regulatory node.

Another study [226] attributed the differential expression of T3SS-secreted proteins to the IS481 insertion upstream of T3SS effector protein BteA (a T3SS cytotoxic effector protein described in *B. bronchiseptica*). IS481 insertion reduced BteA expression in common laboratory or vaccine strains but not in the clinical strain. However, Fennelly et al [227] described a functionally active T3SS and its three effector proteins, BopD, BopN and Bsp22, in a low-passage *B. pertussis* clinical isolate. They showed that *B. pertussis* T3SS promotes bacterial adherence, suppresses innate and adaptive immune response and enhances persistence of bacteria in the lungs of mice. Intriguingly, the immunogenicity and protective potential of *Bordetella* T3SS has not been studied so far. Medhekar et al [97] showed

that antisera from mice vaccinated with *B. bronchiseptica* T3SS secreted protein, Bsp22, protected HeLa cells from T3SS-mediated killing and protected mice when challenged with virulent *B. bronchiseptica*. However, neither any detectable antibody response nor protection could be demonstrated in mice immunised with recombinant *B. pertussis* Bsp22 against intranasal challenge with virulent *B. pertussis* [98]. However T3SS functions as an important virulence delivery system and warrants further study.

1.4.11 *Bordetella pertussis* iron regulated proteins as virulence factors

Bordetella pertussis can acquire the essential nutrient iron using a haem uptake system or alcaligin siderophores, with iron-starved *B. pertussis* expressing novel iron uptake systems *in vivo* [228-231]. The iron uptake system is dependent on the Ton system, a Bvg-independent virulence determinant, accompanied by several siderophore receptors including *bfeA*, *bfrB* and *bfrC* [232,233]. Iron is crucial for growth of *B. pertussis* as demonstrated by the inability of the Δ*tonB* mutant of the Tohama I laboratory strain to utilise alcaligin, enterobactin, ferrichrome, desferroxamine B, haemin and haemoglobin, affecting its growth [234]. Two putative iron binding proteins of *B. pertussis* may be potential vaccine candidates. IRP1-3 (Bp1152) and AfuA (BP1605) were identified by comparative proteomics as proteins induced during iron limiting conditions [103]. IRP1-3 is a dimeric membrane protein involved in iron uptake. Its expression was conserved among clinical isolates of *B. pertussis* and enhanced by iron starvation. Immunisation of mice with recombinant IRP1-3 induced antibodies that recognised the native protein on the bacterial surface and promoted bacterial phagocytosis by human neutrophils [235]. Immunisation with IPR1-3 was protective against infection in mice and induced both Th1 and Th2 responses. Mice immunised with recombinant AfuA were also protected compared to control mice immunised with adjuvant alone [103].

Some other iron related proteins may also be promising vaccine candidates. Yilmaz et al [104] showed that recombinant iron superoxide dismutase (rFeSOD) induced substantial IgG1, IgG2a and IFN-γ responses when formulated with the TLR-4 agonist, monophosphoryl lipid A (MPLA), as an adjuvant. The formulation also decreased the bacterial count in the lungs of mice following challenge with a virulent

B. pertussis strain. Banerjee et al [236] showed that a periplasmic protein, $FbpA_{Bp,}$ plays an important role in *B. pertussis* iron uptake system, but this antigen has not been tested as a vaccine. Taken together, these reports support a role for inclusion of putative iron binding proteins in improved pertussis vaccines.

1.4.12 Lipooligosaccharide of *Bordetella pertussis*

There is little doubt on the significance of pertussis toxin to the symptoms of whooping cough [21]. However, it may not be unique because *B. parapertussis* does not produce pertussis toxin but nevertheless causes the characteristic paroxysms of whooping cough [237]. *Bordetella pertussis* lipooligosaccharide (LOS) may be involved in whooping cough syndrome by induction of $^{\bullet}NO$ production by infected tracheal cells, thereby damaging the activity of respiratory ciliated cells [139]. TCT (reviewed in Section 1.4.3) may act synergistically with LPS in the induction of $^{\bullet}NO$, as neither TCT nor LOS alone was able to induce $^{\bullet}NO$ [133].

LOS produced by both *B. pertussis* and *B. parapertussis* are structurally different. *B. pertussis* LOS lacks an O-side chain but has a nonrepeating trisaccharide [238]. *Bordetella parapertussis* O-antigen consists of a homopolymer of 2,3-dideoxy-2,3-di-*N*-acetylgalactosaminuronic acid [238]. In contrast to other *B. pertussis* vaccine antigens, the LOS molecule has escaped any modification in its dodecasaccharide core structure in the post-vaccination era [239]. LOS from both pathogens can modulate dendritic cell responses, with *B. pertussis* LOS inducing higher Th17-polarised immune responses than LOS from *B. parapertussis*. This may influence the severity of pertussis in humans [238]. As such, the LOS of *B. pertussis* is interesting as a potential vaccine target [240].

1.4.13 The *Bordetella pertussis* biofilm lifestyle

Biofilms are multicellular structured communities of bacterial cells that are encased in a self-produced or host-derived polymeric matrix [241]. In the past few decades, biofilms have been shown to affect antibiotic resistance [242,243], susceptibility to host immune responses [244], establish foreign body infections [51,245] and long-term host survival. Studies of *Bordetella* biofilm formation began slowly but several *in vitro* models have illuminated biofilm physiology in mammalian hosts. We and

others have described microscopic and macroscopic multicellular structures of *Bordetella* on several abiotic surfaces [52,246,247]. The *B. pertussis* BvgAS signal transduction system and several Bvg-activated proteins have been shown to be responsible for efficient biofilm formation on abiotic surfaces [55,241]. FHA contributes to efficient biofilm formation by promoting cell-substrate and inter-bacterial adhesions [247]. ACT of *B. pertussis* can inhibit *B. pertussis* biofilm formation by interacting with the mature C terminal domain of FHA [248]. In addition to FHA and ACT, the *Bordetella bpsABCD* locus (required for the synthesis of the Bps polysaccharide) is critical for the stability and maintenance of the complex architecture of biofilms [249,250]. While Bps was not required for initial attachment to artificial surfaces, it was indispensable for the formation of mature biofilms.

Despite the large amount of information on bacterial biofilms formed on artificial surfaces, it is unclear how biofilms develop *in vivo*. Architecturally complex structures of *B. pertussis* have been described on the ciliated epithelium of the mouse nose and the trachea [52,251]. A biofilm matrix was demonstrated by the finding that Bps co-localised with these organ-adherent biofilms and *ex vivo* treatment of biofilms formed on the mouse nose with DNase I resulted in considerable biofilm dissolution [252]. As *Bordetella pertussis* biofilms have been observed in the mouse nose as late as 19 days post-inoculation, biofilms may allow evasion of host immune responses and so promote efficient colonisation of the mouse respiratory tract [252].

1.4.14 Biofilm-associated antigens as potential vaccines

Biofilm formation by *B. pertussis* has significant implications for vaccine design. For this purpose, it is important to identify *Bordetella* genes and factors that are differentially expressed during biofilm formation [12,52,253]. Based on microscopic visualisation of highly differentiated communities of *B. pertussis* on artificial and host surfaces, it appears that biofilms form in a coordinated manner, in contrast to growth under shaking or planktonic conditions. This model has been strengthened by microarray analyses of *B. bronchiseptica* biofilms at five different stages of development [246]. The analyses showed variation in the expression profile of more than 33% of the genome, including several transcriptional regulators. Similarly,

proteomic analyses in *B. pertussis* revealed a large portion of the cytosolic and membrane subproteome to be altered during biofilm formation. Specifically, Serra et al [254,255] and Bosch et al [256] implicated molecules associated with carbohydrate metabolism. BipA is the most abundant surface-associated protein in the biofilm of *B. pertussis*. Immunisation with BipA resulted in significant reduction in colonisation of mouse lungs with a virulent *B. pertussis* challenge strain [100]. This indirectly implicates BipA in colonisation, provoking its inclusion in aP vaccines. Arnal et al [257] reported enhanced biofilm forming capacity of clinical isolates compared to Tohama I, and elevated levels of several proteins compared to planktonic cells [100,257]. This laboratory has demonstrated enhanced biofilm forming capacity in circulating *B. pertussis* clinical isolates from Western Australia ([258]; Chapter 3 of this thesis). The biofilm forming capacity increased tolerance to antimicrobial agents compared to the planktonic state. Furthermore, Isobaric Tags for Relative and Absolute Quantitation (iTRAQ)-based proteomic analyses revealed novel proteins differentially up-regulated in *B. pertussis* biofilms relative to planktonic cells ([258]; Chapter 5 of this thesis). Whether, the novel biofilm-associated proteins prevent colonisation and aerosol transmission of *B. pertussis* remains to be determined.

The development of an improved vaccine for *B. pertussis* requires a better understanding of the factors involved in initial colonisation and survival in the nasopharynx [259]. The current *B. pertussis* vaccines do not prevent the colonisation of the nasopharynx but prevent the development of severe disease [52]. A potential reduction in nasal colonisation would disrupt the transmission cycle of *B. pertussis* and thereby reduce the morbidity and mortality due to whooping cough. To date, only one study by de Gouw et al [100] demonstrated the protective potential of a biofilm-derived proteins, BipA, against *B. pertussis* infection. Therefore, it is important to identify biofilm-associated antigens and establish their potential to eliminate transmission of *B. pertussis* to infants from asymptomatic carriers, vaccinated adults and adolescents [52,260,261].

1.5 Evolution of whooping cough vaccine

The conventional wP whooping cough vaccine consisted of heat-killed or chemically detoxified preparations of bacteria, administered with or without alum as an adjuvant [262]. These first pertussis vaccines conferred protection against whooping cough mainly by induction of humoral antibody as well as Th1-mediated CMI [263,264]. Current aP vaccines are composed of 3 or 5 purified antigens adsorbed to alum and formulated with diphtheria and tetanus toxoids. These vaccines induced mostly Th2-skewed and Th17 cells but weak Th1 cell response in infants [19,39,265] and murine models of infection [266]. Such immune responses are less effective in preventing whooping cough since *B. pertussis* can survive within macrophages and other eukaryotic cells [267,268]. However, CMI is induced following booster doses of vaccines or natural infection [269]. A Th1-mediated response has been reported in adolescents subsequent to primary wP or aP vaccination [270]. In addition, booster vaccination of adult mice with aP vaccine, delivered as DTaP, did not lead to Th1 responses. It is therefore of interest to know whether the reported Th1-skewed immune response obtained after booster vaccination of adolescents with aP was due to silent exposure to infection with wild type *B. pertussis*, as may happen during epidemics of whooping cough. Although the Th2-polarised response appears to be protective in humans, such immune responses are not able to clear *B. pertussis* that has been internalised by macrophages [271]. Thus, improved vaccines that induce protective T-cell responses are required [19].

Since the mouse pertussis model is not considered to be ideal for predicting immune response profiles in humans, it is necessary to validate results using animal models that more closely reflect human infection [272]. Warfel et al [273] compared the immunological profile, and colonisation and transmission potential of pertussis in baboons previously infected with *B. pertussis* or immunised with aP versus wP vaccines. All vaccinated or prior infected animals mounted potent serum antibody responses but with major differences in T-cell mediated immunity. Convalescent or previously infected mice, or wP-vaccinated mice displayed potent *B. pertussis*-specific T helper 17 (Th17) and Th1 memory responses, while vaccination with aP induced a Th1/Th2 response instead as reported previously in humans [263,274]. The mismatch of the immune response induced by aP with that induced by natural

infection may be an explanation for the resurgence of pertussis, warranting the formulation of improved vaccines. Strategies employed in this quest are outlined in the following sections.

1.5.1 Outer membrane vesicle (OMV) as vaccines

The first report suggesting OMVs as a possible candidate for formulation of aP vaccines was based on evidence that they contain a variety of virulence antigens of *B. pertussis*, including ACT among other polypeptides, and uronic-acid containing polysaccharides and LPS [256,275]. Fernandez et al [99] found a proteoliposome (PL) preparation extracted from the outer membrane of *B. pertussis* could protect 90% of mice against lethal infection with *B. pertussis* and achieve total clearance of bacteria after intracerebral and intranasal challenge. Similarly, Raeven et al [276] showed that higher humoral antibody responses (IgG1/IgG2a/IgG2b/IgG3) were elicited by outer membrane vesicles (OMV) than by wP vaccine, aP vaccine or infection. Important *B. pertussis* immunogens including Ptx and FHA are reported in the OMV, in addition to adenylate cyclase and LPS [275]. These studies highlight the importance of *B. pertussis* OMV for future whooping cough vaccines.

1.5.2 *Bordetella pertussis* DNA vaccines

DNA vaccines utilise plasmid DNA encoding critical antigens to transfect host cells *in vivo* [19,30]. Ulmer et al [277] showed that immunisation of mice with DNA encoding influenza A viral proteins induced protective antibody and cytotoxic T-cell responses. Since then, DNA plasmids have been investigated to achieve protective immune responses against parasitic, viral and bacterial infections [30].

Kamachi et al [278] showed that a DNA vaccine expressing the Ptx subunit1 (PtxS1) known as pcDNA/S1 could induce protective IgG antibodies and protect mice from virulent *B. pertussis* challenge. Immunisation with pcDNA/S1 inhibited the leukocytosis-promoting activity induced by *B. pertussis* infection and protected mice from intracerebral challenge with a lethal dose of virulent *B. pertussis*. Three further mutants were constructed from DNA encoding the N-terminal 180-amino-acid fragment of PtxS1: C180-R9K, C180-E129G and C180-R9K/E129G [279]. Immunisation of mice with all three plasmids induced anti-Ptx specific IgG antibody

and inhibited the leukocytosis-promoting activity of Ptx. Furthermore, no toxicity was observed following transfection of CHO cells with C180-R9K and C180-R9K/E129G plasmids. A similar experiment performed using the C-terminal truncated form of Ptx S1 subunit protected mice against infection with lethal dose of virulent *B. pertussis* [280].

Li et al [281] combined three immunodominant antigens from PtxS1, Prn and FHA by cloning these genes into one plasmid (pVAX1/ppf). Immunisation of mice with pVAX1/ppf, elicited more antibodies reactive with all three proteins, induced IL-10 and IFN-γ production, and conferred protection to mice from intracerebral challenge by a lethal dose of *B. pertussis*. Further work on the mechanism of protection imparted by the pVAX1/ppf is clearly warranted, given the induction of IL-10, which has been shown to dampen the CMI responses. Fry et al [30] developed a DNA vaccine that encoded genetically inactivated PtxS1, which when delivered to mice intramuscularly, elicited a T-cell response, with high levels of IFN-γ and IL-2 in stimulated splenocyte with no serum IgG. Whilst lungs of DNA-immunised mice were cleared of *B. pertussis* at a significantly faster rate than mock-immunised mice following aerosol challenge, clearance was faster in DTaP-immunised mice. Similar results have been obtained with the *B. pertussis* FHA and pertactin DNA vaccines [282].

1.5.3 Micro- and nano-particle vaccines

Biodegradable micro- and nano-particle delivery systems hold promise for vaccine development. Mice immunised orally with liposomes coated with FHA produced higher antibody responses than those immunised with FHA and Ptx without liposome as the delivery vehicle [283]. Similarly, parental (intraperitoneal or intramuscular route) immunisation with FHA and Ptx entrapped in micro-particle poly-lactide-co-glycolide (PLG) protected mice against challenge with *B. pertussis* due to induction of potent Th1 and antibody responses [284]. The nanoparticle PLG formulation induced a Th2-skewed immune response [285,286]. Different types of nanoparticle-based vaccines, operating as delivery systems to enhance antigen processing and/or as adjuvants to activate or enhance immunity have been reviewed

[287]. The authors note that a better understanding of *in vivo* bio-distribution and fate will accelerate the rational design of nanoparticle-containing vaccines.

More recently, cell membrane-coated nanoparticles were developed as a new class of biomimetic nanoparticles with strong potential for modulating antibacterial immunity. An in-depth discussion of this topic is beyond the scope of this thesis; however, a recent review discusses the potential of cell membrane-coated nanoparticles sequestering bacterial toxins and mimicking bacterial antigen presentation [288].

1.5.4 Live attenuated vaccines

Roberts et al [289] reported the development of an *aroA B. pertussis* mutant which induced an antibody response and protection, but only in mice given three doses of the vaccine. Induction of CMI by this vaccine was not investigated. Other studies targeted the DNT and Ptx genes by knocking out these genes. The attenuated strains provided protection of animals from virulent *B. pertussis* challenge [290,291]. Mielcarek et al [94] developed a live attenuated *B. pertussis* strain, BPZE1 by genetic detoxification of Ptx, deletion of DNT and replacement of the *B. pertussis ampG* gene by its *E. coli ampG* orthologue. Vaccination of mice with BPZE1 conferred long term protection with the induction of potent pro-inflammatory and regulatory cytokines and stimulation of Th1, Th17 and T-suppressor responses [25,27,292]. Similar potent immune response was also demonstrated in a human preclinical *ex vivo* model using monocyte-derived dendritic cells challenged with BPZE1 [27]. A randomised phase I clinical trial has also been conducted for BPZE1, making it the first live attenuated pertussis vaccine to undergo clinical trials [293]. Whilst BPZE1 was safe for humans, 5 of 12 subjects receiving high dose of BPZE1 (10^7 colony-forming units) were colonised [293]. Moreover, no significant increase in the antigen-specific plasmablast cell or B-cell responses was detected [294].

In an alternative approach, Conford-Nairns et al [26] developed a novel live attenuated *B. pertussis* vaccine candidate, aroQBP, by insertional inactivation of the 3-dehydroquinase (*aroQ*) gene. Immunisation of mice with aroQBP induced antigen-specific IgG1 and IgG2a and stimulated IL-2, IL-12 and IFN-γ responses. Intranasal

immunisation with one dose of aroQBP protected mice against virulent *B. pertussis* infection and cleared the pathogens from lungs seven days post-challenge. Therefore, the aroQBP strain is a promising vaccine candidate that warrants further investigation because it is non-reverting and capable of inducing both systemic and pulmonary antibody as well as CMI responses.

1.6 Novel adjuvants capable of promoting induction of T-cell responses

Several new-generation adjuvants may enhance the immunogenicity of pertussis vaccines. Elahi et al [295] showed that cyclic diguanylate (c-di-GMP; 39, 59-cyclic diguanylate) can be used as a potent immune stimulatory adjuvant that induces strong Th1 response and reduces bacterial loads in the lungs of mice infected with pathogenic *B. pertussis*. Similarly, Dunne et al [296] used a novel TLR2-stimulating lipoprotein from *B. pertussis* as an adjuvant in acellular vaccines (replacing alum) and demonstrated enhanced Th1, Th17 and IgG2a immune responses. Geurtsen et al [297] revealed that a lipopolysaccharide (LPS) analogue, monophosphoryl lipid A (MPLA) and a LPS analogue from *Neisseria meningitides* (Lpxl2) were more effective than alum-formulated acellular vaccines. Recently, a preclinical study showed that an emulsion adjuvant, MF59, and a TLR4 agonist, MPLA, induced stronger IgG immune response against aP vaccines that are skewed towards IgG2a/Th1 isotypes [298]. The authors suggested replacing the aluminium salts with these new adjuvants to enhance the efficacy of the acellular vaccines. Intensive research efforts are needed to establish non-toxic adjuvants that promote antibody and cell-mediated immune responses capable of providing long-term protection against whooping cough [299,300].

1.7 Summary

Although adoption of aP vaccines has improved compliance with the recommended vaccination schedules, the resurgence of pertussis has been attributed to waning of immunity in vaccinated subjects, the emergence non-vaccine type strains and increased or decreased expression of virulence factors [45,85]. Different formulations of pertussis vaccine utilising 2 to 5 antigens (Ptx, Prn, Fim2, Fim3, Fim2 and 3 or FHA) have been compared with the wP using different criteria making direct comparison between studies difficult [205,301]. With no consensus reached so

far, further multicenter clinical trials with defined and uniform outcomes are needed [302]. Meanwhile, circulating *B. pertussis* strains show evidence of genetic selection for vaccine escape mutants. These potentially have increased virulence. The challenges that lie ahead are:

1. Find novel antigens such as BipA, Vag8, TTSS effector proteins
2. Develop non-toxic adjuvants for the reformulation of the currently used aP vaccines
3. Unlock the potential of biofilm as a source of novel antigens
4. Develop novel live attenuated pertussis vaccines [26,293] capable of preventing symptomatic as well as asymptomatic transmission of whooping cough [303].

1.8 References

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Chapter 2

General Materials and Methods

Each results chapter in this thesis (Chapters 3 to 6) contains a separate "Materials and Methods" section in the style of a publication. This chapter details general methods used throughout the work.

2.1 Bacterial strains and reference strains

Twenty one clinical isolates of *B. pertussis* were obtained from the Department of Microbiology, Princess Margaret Hospital, Perth, Western Australia [1]. The vaccine strain *B. pertussis* Tohama I was kindly provided by Associate Professor Ruiting Lan (School of Biotechnology and Biomolecular Sciences, University of New South Wales, Sydney, Australia). *Staphylococcus epidermidis*, used as negative control for capsule stain, was obtained from the Freeze-dry Microbiology biobank of the School of Biomedical Sciences, Curtin University maintained by Mr. Alain Delhaize.

All *B. pertussis* isolates were grown on Bordet-Gengou (BG) agar (Becton Dickenson, Sparks, MD) containing 15% sheep blood or on charcoal agar (Thermo Scientific, Waltham, MA). Following expansion, bacteria were suspended in cryobeads (Medical Wire and Equipment, Corsham, England) followed by storage at -80°C after removal of cryo-preservative fluid.

2.2 Determination of colony forming units (CFU)

Bordetella pertussis suspensions of A_{600}=0.1 were prepared and 100 µL aliquots of serial dilutions from 10^{-2} to 10^{-10} were plated out in triplicate on BG agar plates. The plates were incubated for 4 days at 37°C and colonies were counted on plates containing between 30 and 300 colonies. Bacterial concentrations in the original suspensions are expressed as CFU/mL.

An average of 180±2 (mean±SE) colonies were obtained from a suspension diluted $1x10^4$, so A_{600}=0.1 was calculated to be equivalent to $1.8x10^7$ CFU/mL bacteria.

2.3 Preparation of splenocytes and LN for estimation of IFN-γ and IL-17a

Mice were euthanised using cervical cordotomy in an anaesthetised state, and spleen and lymph nodes (LN) were collected in 1 mL of ice-cold of RPMI-1640. LN from 3 mice were pooled. Organs were ground on a stainless steel tea strainer using a 10 mL syringe piston and cells were collected by rinsing in 3 mL of RPMI-1640.

Splenocytes and LN cells were pelleted at 300 g (10 min, 4°C), pellets were resuspended in 3 mL RPMI-1640 and 500 µL 1.5 M ammonium chloride (Sigma, St. Louis, MO), final concentration 250 mM, was gently added and incubated on ice for 2 min. Cells were washed twice with 5 mL of complete RPMI medium (RPMI-1640/10% foetal calf serum (FCS)/100 µg/mL streptomycin and 100 U penicillin/mL (300 g, 10 min, 4°C) and finally resuspended in 1 mL complete RPMI medium. The viability of splenocytes and LN cells was determined by Trypan blue exclusion (Thermo Scientific).

A concentration of $5x10^6$ cell/mL was prepared and 100 µL transferred to an equivalent volume of medium (containing antigens) to obtain a final splenocyte or LN cell number of $2.5x10^5$ cells/well for enzyme linked immunosorbent spot (ELISPOT) assays (Section 6.2.10).

2.4 Preparation of *Bordetella pertussis* planktonic and biofilm antigens

Antigens of planktonic cells and biofilm were prepared as described previously [2]. Briefly, *B. pertussis* was grown for 5 days on BG agar or charcoal agar. Preparation of planktonic cells and biofilm is described in Chapter 3. Planktonic cells and biofilm were suspended in sterile phosphate-buffered saline (PBS), pH 7.4, washed 3 times in the same buffer and adjusted to A_{600} = 0.1 with PBS. The suspension was heat inactivated (56°C, 1 h) in a pre-heated water bath. The suspension was allowed to cool at room temperature and 100 µL aliquots were plated on to charcoal agar plates to confirm complete inactivation of the bacteria. The suspension was aliquoted into 10 mL aliquots and stored at -80°C for further use.

Based on the growth curve, the final concentrations of inactivated planktonic cells or biofilm were adjusted to 10^7 or 10^9 CFU/mL for vaccination of mice (Section 6.2.6) while $1x10^5$ cells/well was used for stimulation of splenocytes and LN cells in ELISPOT assays (Section 6.2.10).

2.5 Extraction of genomic DNA

Extraction of genomic DNA was performed using Ultraclean® Microbial DNA Isolation kits (MO BIO Laboratories, Carlsbad, CA). *Bordetella pertussis* isolates were grown on BG agar at 37ºC for 3-4 days in moist conditions. Lawns of bacteria

were resuspended in 2 mL Stainer-Scholte (SS) broth and centrifuged (10,000 g, 30 s, room temperature). Pellets were suspended in 300 µL micro-bead solution and transferred to micro-bead tubes, for genomic DNA extractions according to the manufacturer's instructions. DNA was eluted in 50 µL Tris-EDTA (TE) buffer, pH8 and stored at -20°C for further use.

2.6 Extraction of plasmids

Plasmid isolation was performed using an ISOLATE II Plasmid Mini kits (Bioline, Alexandria, Australia). *Escherichia coli* DH5α and *E. coli* BL21-DE3 expression hosts were grown overnight in LB broth [1% tryptone, 0.5% yeast extract (Oxoid, Hampshire, UK) and 1% NaCl with 50 µg/mL kanamycin (Biochemicals, Taren Point, Australia)]. Cells were harvested by centrifugation (30 s, 11000 g). Pellets were resuspended in 500 µL buffer P1. Plasmids were isolated according to manufacturer's instructions and eluted in 50 µL TE buffer, pH8. DNA concentrations were determined using a Nanodrop™ 1000 Spectrophotometer (Thermo Scientific) and stored at -20°C for further use.

2.7 Preparation of *Escherichia coli* competent cells

Competent cells were prepared as described previously [3]. A single colony of *E. coli* DH5α or BL21-DE3 cells were grown in 5 mL LB broth with shaking at 200 rpm. For *E. coli* BL21-DE3, 30 µg/mL chloramphenicol (Sigma) was used in the culture medium. Following overnight incubation, 1.3 mL of each culture was transferred to 100 mL LB broth and allowed to reach A_{600}=0.4. Cultures were cooled on ice for 10 min and centrifuged (2500 g, 10 min, 4°C). Cell pellets were resuspended in 32 mL of sterile ice-cold transformation buffer (10 mM PIPES, 250 mM KCl, 15 mM $CaCl_2$ and 50 mM $MnCl_2$ (Sigma), pH 6.7), incubated for 10 min on ice and centrifuged at 2500 g, 10 min, 4°C. Following removal of the supernatant, pellets were resuspended in 4 mL of ice-cold transformation buffer and 280 µL dimethyl sulphoxide (DMSO; Sigma) was added dropwise while shaking the cultures. The suspension was incubated on ice for 10 min, 50 µL aliquots were prepared in sterile 1.5 mL microcentrifuge tubes on dry ice and stored at -80°C until use.

2.8 Transformation of competent cells with plasmid DNA

Competent cells were thawed on ice and incubated with 200 ng plasmid on ice for 20 min. The mixture was heat-shocked at 42°C for 60 s and immediately cooled on ice for 2 min. LB broth (900 µL) containing 1% glucose (Sigma) was added and the cells incubated at 37°C for 1 h with shaking (200 rpm), following which 100 µL aliquots were evenly spread on to LB agar plates containing 50 µg/mL kanamycin. Plates were incubated overnight at 37°C and observed for transformants.

2.9 Transformation by electroporation

For ligation reactions, cells were transformed by electroporation, following ligation with NEBuilder® HiFi DNA mastermix (New England Biolabs, Ipswich, MA), 2 µL ligation mixture was diluted 1:3 with sterile distilled water and 3 µL was used to transform 50 µL *E. coli* electro-competent cells (Section 6.2.2). The cells and ligation mixture was transferred to a Gene Pulser® electroporation cuvette (Biorad, Gladesville, Australia) with a 0.1 cm electrode gap and pulsed using a Micropulser™ Electroporator (Biorad). The electroporation conditions were set at 25 µF, 2.5 kV and 100 Ω, giving a pulse time of 2.5 ms. Following electroporation, 900 µL LB broth was added and incubated for 1 h (37°C shaken at 200 rpm) and 100 µL aliquots were spread on to LB agar plates containing 50 µg/mL kanamycin or 30 µg/mL chloramphenicol and screened for transformants.

2.10 Restriction digestion of BamB and LptD cloned into the pETM-11 vector

The restriction enzyme of choice was deduced from the restriction map of BamB cloned into pETM-11 expression vector (pETM11-BamB) using SnapGene® Viewer v3.3.4 (GSL Biotech, Chicago, IL). One microgram of plasmid was mixed with 5 µL of 10x CutSmart® buffer and 10 U of SphI-HF® (New England Biolabs) in a total volume of 50 µL. The reaction mixture was incubated at 37°C overnight and electrophoresed on a 1.5% agarose gel (Bioline). The restriction digestion of LptD cloned into pETM-11 (pETM11-LptD) was achieved in a similar way but the restriction enzymes used were XbaI and BsaI-HF® (New England Biolabs).

2.11 Separation of proteins using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were separated by size using both hand-cast 10% gels and pre-cast 4-12% Novex® NuPAGE® Bis-Tris Protein gels (Invitrogen, Carlsbad, CA).

2.11.1 Preparation of hand-cast gels

Hand-cast gels were prepared using 3 mL 4% Bis-acrylmide™ (Biorad) stacking gel and 5 mL of 10% Bis-acrylmide™ resolving gel containing 0.125 and 0.375 M Tris HCl, respectively, 0.1% SDS and ammonium persulphate, pH 8. After mixing, 5 µL *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine (TEMED; Sigma) was added to initiate polymerisation. Gels were subjected to electrophoresis using 1x SDS-PAGE buffer [25 mM Trizma, 190 mM glycine and 0.1% SDS (Sigma), pH 8.8]. Gels were stained and visualised as described in Section 6.2.4

2.12 Ethics statement

All animal experiments used in this study were approved by the Curtin University's Animal Ethics Committee (AEC approval No. AEC_2015_39).

2.13 Statistical analysis

All data comparing across control and test isolates were analysed using Student's T-tests calculated using Microsoft Excel 2010 software.

Two-tailed Mann-Whitney *U* tests were used to compare bacterial loads in the lungs of mice in each vaccinated group (n=7). Unpaired Student's t-tests were used to compare immunological data (n=3). Statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA).

For the proteomic study, *P*-values indicating differential expression were calculated by the ProteinPilot™ 4.5 Software. In all statistical tests, $P \leq 0.05$ was considered statistically significant.

2.14 References

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Chapter 3

Biofilm forming potential and antimicrobial susceptibility of newly emerged Western Australian *Bordetella pertussis* clinical isolates

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An original reprint of this publication is available in the Appendix

Biofilm forming potential and antimicrobial susceptibility of newly emerged Western Australian *Bordetella pertussis* clinical isolates

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Running title: *Bordetella pertussis* biofilm analysis and antibiotic resistance

Key words: *Bordetella pertussis*, biofilm, antimicrobial resistance/tolerance, iTRAQ, proteomic analysis.

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Abstract

Whooping cough caused by *Bordetella pertussis* is increasing in several countries despite high vaccine coverage. One potential reason for resurgence is the emergence of genetic variants of the bacteria. Biofilm formation has recently been associated with the pathogenesis of *B. pertussis*. We investigated biofilm formation by 21 Western Australian *B. pertussis* clinical isolates. All isolates formed stronger biofilms than the reference vaccine strain Tohama I while retaining susceptibility to ampicillin, erythromycin, azithromycin and streptomycin. When two strong biofilm-forming clinical isolates were compared with Tohama I, minimum bactericidal concentrations of antimicrobial agents was increased. Isobaric tags for relative and absolute quantitation (iTRAQ)-based proteomic analysis revealed significant differences in protein expression in *B. pertussis* biofilms providing an opportunity for identification of novel biofilm-associated antigens for incorporation in current pertussis vaccines to improve their protective efficacy. The study also highlights importance of determining antibiograms for biofilms to formulate improved antimicrobial therapeutic regimens.

3.1 Introduction

Bordetella pertussis (*B. pertussis*) is the causative agent of whooping cough or pertussis, a highly contagious respiratory disease. Due to severe systemic complications associated with *B. pertussis* infection, the disease is more severe in infants but adults and adolescents remain susceptible. Severe complications of *B. pertussis* infection include pneumonia, encephalopathy, seizures, otitis media and brain haemorrhages [1,2]. Despite high vaccine coverage amongst infants, whooping cough remains one of the most endemic vaccine-preventable diseases in most developed countries. This includes Australia [3], the Netherlands [4] and the United States [5]. In 2008, the WHO estimated that about 16 million cases of pertussis occurred worldwide (including 95% in developing countries) with 195,000 children dying from the disease [2]. Deaths of 285,000 to 400,000 infants a year has been reported elsewhere [6,7].

The introduction of whole-cell vaccines [DTwP] in the 1940s and acellular pertussis vaccines [DTaP] in the 1990s reduced mortality due to pertussis. However, the recent resurgence of pertussis has been attributed to poor immune responses induced by the acellular pertussis vaccine (aP) (Cherry et al. 2010. These vaccines elicit weak T-cell responses and effective stimulation of T-cell-mediated immunity is a prerequisite for long-term protection [8-10]. Alternatively, another potential reason for resurgence may be evolution of novel genetic variants of *B. pertussis*, which may evade immunity provided by the current aP. Isolates of *B. pertussis* carrying the non-vaccine *prn2* and/or *ptxP3* alleles have been reported from Europe [11], Australia [12,13], Canada [14] and Japan [15]. Some *B. pertussis* isolates from vaccinated populations did not produce pertactin (Prn), an important antigen included in all current aP [16-18], raising questions on the role played by Prn in aP formulations. Another study reported over-production of *ptxP3* type of pertussis toxin (Ptx) [19]. These findings suggest that the emerging strains of *B. pertussis* may have developed selective advantage driven by widespread use of acellular pertussis vaccines, leading to the resurgence of pertussis in vaccinated populations.

Bordetella pertussis forms biofilm in the nasopharyngeal tissue of infected mice, facilitating its persistence and promoting transmission between mammalian hosts

[20,21]. Bacterial biofilms are structured communities of bacterial cells encased in a self-produced polymeric organic matrix that enables the bacteria to become resistant to antimicrobial agents, evade innate host immune defences and create sustained infections [22,23]. Recently, comparative proteomic analysis of Argentinean *B. pertussis* clinical isolates revealed differential expression of ~5.1% proteins in the clinical isolates compared to the reference strain Tohama I, with significant upregulation of proteins associated with energy metabolism [24]. Other studies have linked biofilm formation with virulence factors including extracellular DNA, *Bordetella* polysaccharides or poly-β-1,6-N-acetyl glucosamine (PNAG) encoded by *bpsABCD* operon, filamentous haemagglutinin (FHA) and the signalling molecules, 5'-triphosphate-guanosine-3'-diphosphate and 5'-diphosphate-guanosine-3'-diphosphate, collectively called (p)ppGpp [20,25-27]. The absence of the membrane-associated protein KpsT, involved in the transport of the capsular polymers across the envelope, reduced its colonisation efficacy in mice [28]. Accordingly, *B. per*tussis capsule expressed *in vivo* in murine lungs enhanced the colonisation potential of this pathogen. However, links between the gene encoding capsular polymers and biofilm formation were not investigated.

We investigated the biofilm-forming potential and antimicrobial susceptibility profile of genetic variants of clinical *B. pertussis* isolated from Western Australia. Having confirmed the strong biofilm-forming potential of the clinical isolates, the potential association of genes encoding antigens incorporated in the currently used aPs with biofilm formation was investigated. Finally, using 2 of the strong biofilm-forming clinical isolates as prototypes, antimicrobial tolerance and proteome profiles were investigated in comparison with the reference strain Tohama I.

3.2 Materials and methods

3.2.1 Ethics statement

Samples were obtained from patients after approval of the Human Ethics Committee of Princess Margaret Hospital (PMH), affiliated with the University of Western Australia and Curtin University, Western Australia.

3.2.2 Bacterial strains, growth conditions and DNA extraction

Twenty-one clinical isolates, recovered from 2008 to 2010 during a whooping cough epidemic [12], were obtained from the Department of Microbiology, Princess Margaret Hospital, Perth, Western Australia. Bacterial isolates were inoculated on to BG agar (Beckton Dickenson) supplemented with 15% sheep blood and cultured at 37°C for 5 days. For biofilm experiments, the isolates were grown on Stainer-Scholte (SS) broth supplemented with 0.2 mg/mL Heptakis (2,6-di-*O*-methyl)-β-cyclodextrin (Sigma) to enhance growth. The reference strain, *Bordetella pertussis* Tohama I, was prepared in parallel. DNA was extracted using Ultraclean® Microbial DNA Isolation Kits (MO BIO Laboratories).

3.2.3. Genotypic detection of virulence factors

The primers used in this investigation are shown in Table 3.1. Genes encoding pertussis toxin (*ptx*) and pertactin (*prn*) were detected by PCR as described previously [29]. For *prn* PCR, amplification was carried out in 1xfinal concentration of PCR master mix (Thermo Scientifica) using 3% DMSO (Sigma). Genes encoding *Bordetella* polysaccharides, *bpsA* and *bpsD*, were detected by PCR as described previously [30] in 1xfinal concentration of PCR master mix (Thermo Scientific) using GP1F and GP1R primers (Table 3.1). For *bpsD*, the reaction mixture contained 3% DMSO (Sigma, Australia). The *kpsT* capsular gene was amplified as described previously [31]. All PCR products were separated on 1.5% agarose gel using 1xSodium Borate (SB) buffer and stained with Midori green (Nippon Genetics, Duren, Germany).

3.2.4 Antimicrobial susceptibility testing of clinical isolates

Antimicrobial susceptibility was determined using the Calibrated Dichotomous Sensitivity (CDS) method [32]. Antibiotics discs, ampicillin (5 µg), erythromycin (5 µg), tetracycline (10 µg), streptomycin (10 µg), ciprofloxacin (2.5 µg), nalidixic acid (30 µg) and co-trimoxazole (25 µg), were purchased from Oxoid. Briefly, clinical isolates were inoculated on BG agar supplemented with 15% sheep blood and cultured at 37°C for 5 days [33]. About 3-5 small colonies were stabbed with a nichrome wire, suspended in 2 mL of SS broth and poured on to pre-warmed charcoal agar plates (Thermo Scientific). Excess fluid was drained and discs placed.

Table 3.1 List of primers used in this study

Target gene	Primer Name	Primer Sequence	Expected product size (bp)	References
prn	AF	GCCAATGTCACGGTCCAA	568	[29,34]
	AR	GCAAGGTGATCGACAGGG		
ptx	S1-F2	CCCCCTGCCATGGTGTGATC	930	[29,34]
	S1-R2	AGAGCGTCTTGCGGTCGATC		
*bps*A	GP1F	CTAGTCTAGAGGCGAAATTATACCGCGTT	575	[21,30]
	GP1R	CCCAAGCTTCCCCGCCACCAGCAGCCGAGT		
*bps*D	GP3F	CCCAAGCTTCAGCGGCAACCCGACGGACGCAT	563	[21,30]
	GP3R	CGGGGTACCGGGCGCGGCTGCTGCTGCAGG		
kpsT	CAPF	ATTCTCGATGACGTGTCGTTCGA	450	[31]
	CAPR	GCGAACACCTCGAGACATTTG		

3.2.5 Quantitation of *Bordetella pertussis* biofilm formation

Biofilm formation was assessed in triplicate in microtiter plates [21,26,30] inoculated with 200 µL suspension of *B. pertussis* cells at A_{600nm} = 0.1 and allowed to form biofilm for 96 h at 37°C under static conditions. After 96 h the plates were gently shaken (60 rpm) for 20 minutes. The supernatant was removed and the wells were washed with 200 µL of sterile distilled water. Adherent cells were stained with 200 µL 0.1% crystal violet (CV), washed, solubilised in 200 µL of 95% ethanol and quantitated at A_{540nm}. Non-biofilm producing strains were defined as $A_{540nm} \leq 0.301$ (mean±3SD of blank) [35]. Arbitrary cut-offs of $A_{540nm} \leq 0.602$, $A_{540nm} \leq 1.204$ and $A_{540nm} > 1.204$ was used to define weak, intermediate and strong biofilm formers, respectively, according to classification system for biofilm quantification [36].

3.2.6 Biofilm antimicrobial resistance assay

Biofilm antimicrobial resistance assays for the selected *B. pertussis* isolates ID20, ID14 and the reference strain Tohama I were performed as described previously [37]. Ampicillin, kanamycin and streptomycin powders were obtained from Astral Bioscience (Astral Scientific, Australia). Azithromycin, ciprofloxacin, clarithromycin, erythromycin, nalidixic acid and co-trimoxazole as trimethoprim and sulphamethoxazole powders were obtained from Sigma.

For planktonic cells, *B. pertussis* isolates were grown for 5 days on BG agar plates and suspensions at A_{600nm} = 0.1 were prepared in SS broth. Bacterial suspensions (200 µL) were inoculated into 96-well microtitre plates containing antimicrobial agent(s) ranging in concentrations from 0.25 µg/mL to 64 µg/mL. After 96 h at 37°C, 100 µL of medium containing antimicrobial agent and bacteria was plated on to fresh BG agar plates. The minimal bactericidal concentration for planktonic (MBC-P) cultures was defined as the lowest concentration of an antimicrobial agent at which no visible growth of *B. pertussis* was seen on BG agar plates.

To investigate antimicrobial resistance of biofilm state, 96-well microtitre plates were inoculated with *B. pertussis* isolates at A_{600nm} = 0.1 and incubated for 96 h at 37°C in static conditions to allow biofilm formation. The medium was removed and replaced with fresh SS broth containing different concentrations of each

antimicrobial agent and re-incubated for additional 96 h. Medium (100 µL) containing bacteria was plated on to fresh BG agar plates. Minimal bactericidal concentration of biofilm (MBC-B) cultures was defined as the lowest concentration of an antimicrobial agent at which no visible growth of *B. pertussis* was seen on BG agar plates.

3.2.7 Proteomic analysis using isobaric tags for relative and absolute quantitation (iTRAQ)

Samples for proteomic analysis were prepared as described previously [38,39]. Briefly, bacteria were cultured for 18 h in SS broth at 37°C with shaking at 180 rpm. Planktonic cultures were adjusted to A_{600} = 0.1 and harvested by centrifugation (8000 g, 20 min), washed 3 times with PBS and frozen at -80°C overnight. Approximately 200 mL of the planktonic cultures were inoculated into a tissue culture flask and allowed to form biofilm at 37°C for 96 h. The attached biofilms were collected by centrifugation (10000g, 10 min), washed and frozen at -80°C overnight, and solubilised in 1 mL of lysis buffer (7 M Urea, 2 M Thiourea, 30 mM Tris pH 8.8, 1 mM EDTA and 1.5% Triton X-100). The suspensions were further separated by sonication (Misonix, USA) at amplitude of 30% for 2 minutes with a pulse "On" and "Off" of 10 s each. The clear suspension was then centrifuged at 14000 g, 30 min and the supernatant stored at -80°C. Protein concentration was determined using a Direct Detect™ spectrometer (Millipore, Australia). Differential protein expression analysis was performed by Proteomics International, Perth, Australia using iTRAQ. Peptide fractions were analysed by electrospray ionisation mass spectrometry using a 1260 Infinity HPLC system (Agilent) coupled to an Agilent 1260 Chipcube Nanospray interface on an Agilent 6540 mass spectrometer (Agilent). Spectral data were analysed against the SwissProt Bacteria database using ProteinPilot™ 4.5 Software (AB Sciex). Functional classification of proteins was determined using TIGRFAMs database (ftp://ftp.jcvi.org/pub/data/TIGRFAMs/). Fisher's exact test was used to determine the significance of functional classification of biofilm-associated protein relative to planktonic cultures. The subcellular localisation of proteins was determined using PSORTb version 3.0.2 [40].

3.2.8 Statistical analysis

Means were compared using Students' two-tailed paired t-test with $P \leq 0.05$ considered as statistically significant.

3.3 Results

3.3.1 Characteristics of *Bordetella pertussis* clinical isolates

All 21 *B. pertussis* clinical isolates were obtained in Western Australia (WA) during a period from 2008 to 2010 when Australia experienced epidemics of whooping cough [12]. The characteristics of the isolates are summarised in Table 3.2. All isolates and Tohama I demonstrated a clear zone of haemolysis on BG-blood agar. Seventeen (81%) and 4 (19%) isolates carried the *prn2* and *prn1* alleles, respectively. Sixteen (76%) isolates carried the *fim3A* allele while 5 (24%) isolates carried the *fim3B* allele (Table 3.2). Similarly, 16 (76%) isolates carried the *ptxp3* allele while 5 (24%) isolates carried not-*ptxP3* allele. Overall, the 21 clinical isolates represented four genotypes comprising 10 (48%), 6 (28%), 4 (19%) and 1 (5%) isolates with *ptxP3-prn2-fim3A*, *ptxP3-prn2-fim3B*, not-*ptxP3-prn1-fim3A* and not-*ptxP3-prn2-fim3A* genotypes, respectively. Based on single nucleotide polymorphism (SNP) typing [12,41], 16 (76%) isolates in this study belong to cluster I comprising SP13 (10 isolates or 48%), SP14 (4 isolates or 19%) and SP16 (2 isolates or 9%), while one isolate each belonged to SP1, SP6, SP7, SP11 and SP18 (Table 3.2).

3.3.2 Antibiotic susceptibility of *Bordetella pertussis* clinical isolates

Antimicrobial susceptibility of *B. pertussis* clinical isolates to 7 antimicrobial agents was determined using the disc diffusion method on charcoal dextrose agar and interpreted according to the CDS method [32]. All 21 clinical isolates were susceptible to streptomycin, ampicillin and erythromycin (Table 3.3). Thirteen (62%) isolates that were susceptible to ciprofloxacin were also susceptible to nalidixic acid while 8 (38%) isolates that were resistant to ciprofloxacin were susceptible to nalidixic acid. All isolates were resistant to co-trimoxazole and tetracycline.

3.3.3 Genotypic detection of *Bordetella pertussis* virulence factors

Genes encoding virulence factors including pertussis toxin, pertactin, gene encoding membrane-associated protein involved in the transport of capsule polymers across

Table 3.2 Characteristics of clinical isolates and biofilm formation assessed at 96 h

ID	SNP type	RBC lysis	Biofilm formation Mean Absorbance (A_{540}) ± SE	P^{γ} value	Genotype*			Virulence factors PCR				
					ptxP type	*prn* type	*fim type*	*ptx*	*prn*	*bpsA*	*bpsD*	*kpsT*
Toh	-	+	2.34±0.03	-	*ptxP1*	*prn1*	*fim2*	+	+	+	+	+
1	SP13	+	3.18±0.07	<0.05	*ptxP3*	*prn2*	*fim3A*	+	+	+	+	+
2	SP6	+	3.10±0.12	<0.05	*not ptxP3*	*prn1*	*fim3A*	+	+	+	+	+
3	SP13	+	3.23±0.11	<0.05	*ptxP3*	*prn2*	*fim3A*	+	+	+	+	+
4	SP13	+	3.23±0.06	<0.05	*ptxP3*	*prn2*	*fim3A*	+	+	+	+	+
5	SP13	+	3.07±0.01	<0.05	*ptxP3*	*prn2*	*fim3A*	+	+	+	+	+
6	SP1	+	3.20±0.05	<0.05	*not ptxP3*	*prn1*	*fim3A*	+	+	+	+	+
7	SP14	+	3.26±0.03	<0.05	*ptxP3*	*prn2*	*fim3B*	+	+	+	+	+
8	SP13	+	3.07±0.07	<0.05	*ptxP3*	*prn2*	*fim3A*	+	+	+	+	+
9	SP7	+	3.17±0.03	<0.05	*not ptxP3*	*prn1*	*fim3A*	+	+	+	+	+
10	SP11	+	3.06±0.05	<0.05	*not ptxP3*	*prn2*	*fim3A*	+	+	-	+	+
11	SP18	+	3.26±0.08	<0.05	*not ptxP3*	*prn1*	*fim3A*	+	+	+	+	+
12	SP13	+	3.31±0.05	<0.05	*ptxP3*	*prn2*	*fim3A*	+	+	+	+	+
13	SP13	+	3.23±0.08	<0.05	*ptxP3*	*prn2*	*fim3A*	+	+	+	+	+
14	SP16	+	2.99±0.01	<0.05	*ptxP3*	*prn2*	*fim3B*	+	+	+	+	+
15	SP13	+	2.86±0.02	<0.05	*ptxP3*	*prn2*	*fim3A*	+	+	+	+	+
16	SP14	+	2.92±0.08	<0.05	*ptxP3*	*prn2*	*fim3B*	+	+	-	+	+
17	SP16	+	2.84±0.03	<0.05	*ptxP3*	*prn2*	*fim3B*	+	+	+	+	+
18	SP14	+	3.22±0.06	<0.05	*ptxP3*	*prn2*	*fim3B*	+	+	+	+	+
19	SP14	+	3.02±0.09	<0.05	*ptxP3*	*prn2*	*fim3B*	+	+	+	+	+
20	SP13	+	3.37±0.08	<0.05	*ptxP3*	*prn2*	*fim3A*	+	+	+	+	+
21	SP13	+	2.77±0.02	<0.05	*ptxP3*	*prn2*	*fim3A*	+	+	+	+	+

+; positive, -; negative, SNP; single nucleotide polymorphism. ID; isolate number, Toh; Tohama I

**prn*, *fim* and *ptxP* typing for the WA isolates, used in this investigation was previously carried out by A/Professor Ruiting Lan's group at the University of New South Wales, Sydney, Australia.

$^{\gamma}$values indicate the absorbance of clinical isolates in comparison to Tohama I

Table 3.3 Susceptibility of *B. pertussis* clinical isolates to antimicrobial agents

Strain ID	Antimicrobial susceptibility testing							Genotype		
	NAL (30 µg)	CIP (2.5 µg)	SXT (25 µg)	AMP (5 µg)	ERY (5 µg)	TET (10 µg)	STR (100 µg)	*ptxP* type	*prn* type	*fim* type
Toh	S	S	R	S	S	R	S	*ptxP1*	*prn1*	*fim2*
1	S	R	R	S	S	R	S	*ptxP3*	*prn2*	*fim3A*
2	S	R	R	S	S	R	S	*not ptxP3*	*prn1*	*fim3A*
3	S	R	R	S	S	R	S	*ptxP3*	*prn2*	*fim3A*
4	S	S	R	S	S	R	S	*ptxP3*	*prn2*	*fim3A*
5	S	R	R	S	S	R	S	*ptxP3*	*prn2*	*fim3A*
6	S	S	R	S	S	R	S	*not ptxP3*	*prn1*	*fim3A*
7	S	R	R	S	S	R	S	*ptxP3*	*prn2*	*fim3A*
8	S	S	R	S	S	R	S	*ptxP3*	*prn2*	*fim3B*
9	S	R	R	S	S	R	S	*not ptxP3*	*prn1*	*fim3A*
10	S	R	R	S	S	R	S	*not ptxP3*	*prn2*	*fim3A*
11	S	S	R	S	S	R	S	*not ptxP3*	*prn1*	*fim3A*
12	S	S	R	S	S	R	S	*ptxP3*	*prn2*	*fim3A*
13	S	S	R	S	S	R	S	*ptxP3*	*prn2*	*fim3A*
14	S	S	R	S	S	R	S	*ptxP3*	*prn2*	*fim3B*
15	S	S	R	S	S	R	S	*ptxP3*	*prn2*	*fim3A*
16	S	S	R	S	S	R	S	*ptxP3*	*prn2*	*fim3B*
17	S	R	R	S	S	R	S	*ptxP3*	*prn2*	*fim3A*
18	S	S	R	S	S	R	S	*ptxP3*	*prn2*	*fim3B*
19	S	S	R	S	S	R	S	*ptxP3*	*prn2*	*fim3B*
20	S	S	R	S	S	R	S	*ptxP3*	*prn2*	*fim3A*
21	S	S	R	S	S	R	S	*ptxP3*	*prn2*	*fim3A*

NAL; nalidixic acid, CIP; ciprofloxacin, SXT; cotrimaxazole, AMP; ampicillin, ERY; erythromycin, TET; tetracycline, STR, streptomycin.

R, resistant; S, susceptible; Toh; Tohama I.

envelope, *kpsT*, and *Bordetella* polysaccharide A (*bpsA*) and *Bordetella* polysaccharide D (*bpsD*) were investigated using traditional PCR. Pertussis toxin (*ptx*) was detected in all 21 (100%) clinical isolates including the Tohama I reference strain (Table 3.2). The *bpsA* gene was detected in 19 (90%) isolates including Tohama I strain while 2 (10%) isolates were negative for *bpsA*. The *bpsD* and *prn* genes were detected in all the 21 clinical isolates including Tohama I reference strain. The presence of the gene, *kpsT*, the second open reading frame in the *B. pertussis* capsule operon that is responsible for transport of capsular polymers from the periplasmic space to the bacterial cell surface was also detected in all the 21 clinical isolates and Tohama I reference strain (Table 3.2).

3.3.4. *Bordetella pertussis* clinical isolates form strong biofilms

Previous studies reported the biofilm production by the laboratory strains of *B. pertussis* and/or *B. bronchiseptica* [20,25,26]. The present study investigated the ability of clinical isolates of *B. pertussis* to form biofilms *in vitro*. Using 96-well microtitre plate assays, all 21 clinical isolates formed strong biofilms (Table 3.2) with A_{540nm} >1.20. Furthermore, the mean absorbance of all 21 clinical isolates was higher than Tohama I (P<0.05).

3.3.5 Biofilm grown *Bordetella pertussis* clinical isolates confer increased tolerance to antimicrobial agents

MBC-P versus MBC-B was determined in two, randomly selected, strong biofilm-producing clinical isolates, ID20 and ID14, and compared with Tohama I, which is of a different genotype. The genotype of ID20 was *ptxP3, prn2* and *fim3A*, which belongs to the SP13 profile and harboured the *kpsT, bpsA* and *bpsD* genes, whereas that of ID14 was *ptxP3, prn2* and *fim3B*. Tohama I have the genotype *ptxP1, prn1*and *fim2* and was positive for *kpsT, bpsA* and *bpsD*. Among the nine different antimicrobial agents tested, there was a notable increase in the antimicrobial resistance of the clinical isolates of *B. pertussis* grown in biofilm cultures compared to planktonic cultures, particularly with respect to ampicillin and clarithromycin (Table 3.4). The fold increase in antimicrobial resistance to different antimicrobial agents, particularly streptomycin, clarithromycin and kanamycin was lower with strain ID14 as compared to clinical isolate ID20, the strongest biofilm producer in

Table 3.4 Minimal bactericidal concentrations (MBC) of planktonic and biofilm state of selected *B. pertussis* isolates and Tohama I

Antimicrobial agent	Bacterial strains tested								
	ID20			Tohama I			ID14		
	MBC-P[a] (µg/mL)[c]	MBC-B[b] (µg/mL)[c]	MBC-B/MIC-P (Fold change)	MBC-P[a] (µg/mL)[c]	MBC-B[b] (µg/mL)[c]	MBC-B/MIC-P (Fold change)	MBC-P[a] (µg/mL)[c]	MBC-B[b] (µg/mL)[c]	MBC-B/MIC-P (Fold change)
Ampicillin	8	64	8	8	16	2	8	32	4
Azithromycin	1	1	1	0.25	1	4	0.25	1	4
Ciprofloxacin	1	2	2	1	2	2	1	2	2
Clarithromycin	0.25	64	256	0.25	16	64	0.25	16	64
Co-trimaxazole	8	8	1	8	32	4	4	16	4
Erythromycin	1	2	2	0.25	2	8	1	4	4
Kanamycin	2	32	16	2	16	8	0.5	2	4
Nalidixic acid	64	64	1	64	64	1	64	64	1
Streptomycin	2	64	32	8	8	1	0.5	1	2

[a]MBC-P, MBC of planktonic *B. pertussis*; [b]MBC-B, MBC of biofilm grown *B. pertussis.* [c]Lowest concentration of antimicrobial agents at which no visible growth was observed on BG agar.

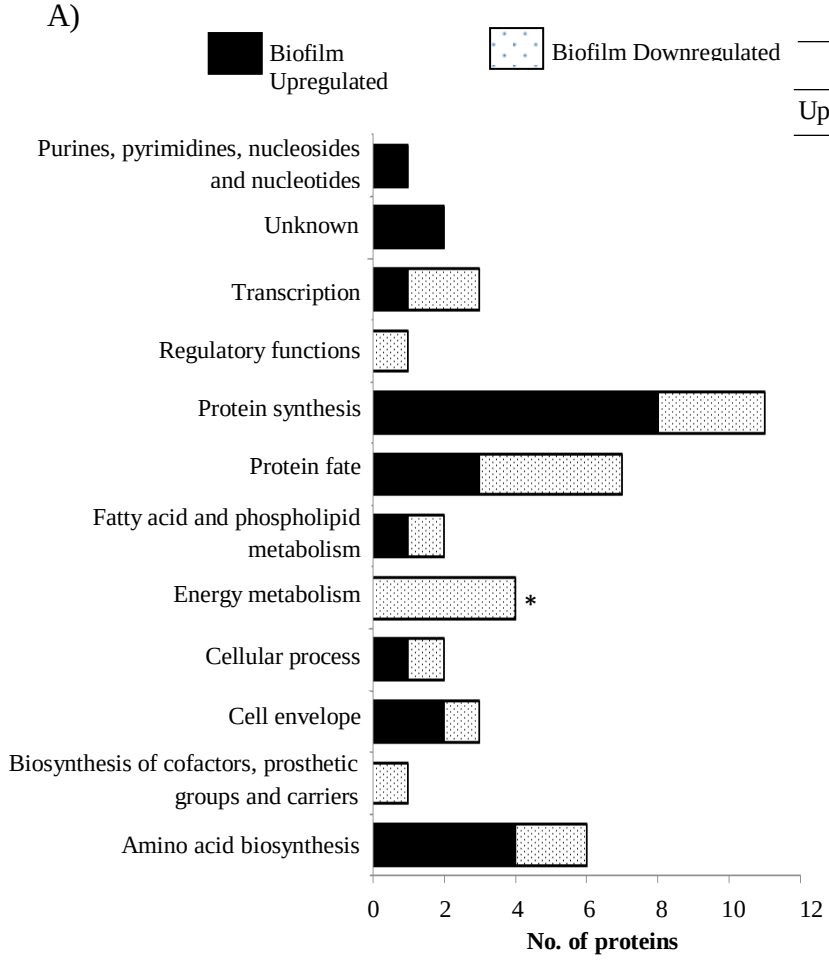
[a]MBC-P, MBC of planktonic *B. pertussis*; [b]MBC-B, MBC of biofilm grown *B. pertussis.* [c]Lowest concentration of antimicrobial agents at which no visible growth was observed on BG agar.

this study. Antimicrobial resistance of the biofilm grown *B. pertussis* clinical isolates were between 2 and 256 fold, 1 and 64 fold, and 2 and 64 fold higher than planktonically grown isolates for ID20, ID14 and Tohama I, respectively, depending on the antimicrobial agents tested (Table 3.4).

3.3.6 Proteomic of *Bordetella pertussis* biofilm and planktonic cells

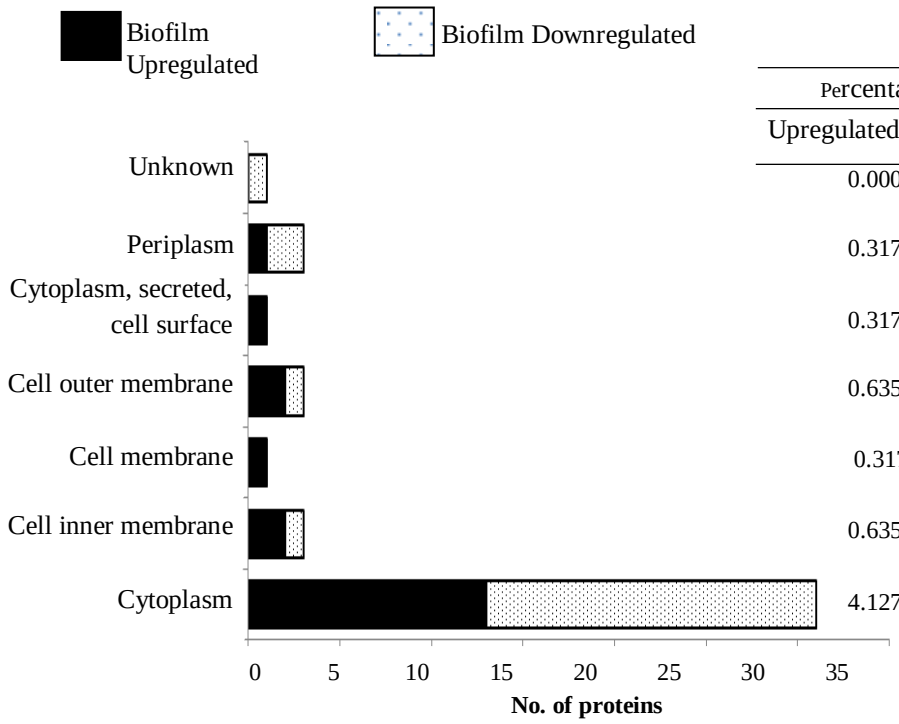
To identify novel proteins associated with *B. pertussis*, biofilms of the reference strain Tohama I and the clinical isolate ID20 were compared with their planktonic cells using iTRAQ. A total of 315 proteins were identified from the soluble fractions of both biofilm and planktonic-grown *B. pertussis*. The mean ratios of proteins in biofilm-grown cells relative to planktonic-grown cells were calculated. Protein ratios that were significantly higher or lower ($P<0.05$) compared to planktonic cells were defined as biofilm upregulated or biofilm downregulated, respectively. A total of 43 (13.5%) proteins were differentially expressed in biofilm cells of *B. pertussis* clinical isolate ID20 compared to planktonic cells with 20 (6%) and 23 (7%) proteins being significantly upregulated or downregulated in biofilm, respectively (Appendix 3.1 and 3.2). Clustering of proteins based on function showed that the proteins involved in energy metabolism were significantly upregulated in biofilm compared to the planktonic state (Figure 3.1). On the other hand, 41 (13%) proteins were differentially expressed in biofilms of *B. pertussis* Tohama I reference strain, with 18 (5.7%) and 23 (7.3%) proteins being significantly upregulated or downregulated, respectively (Appendix 3.1 and 3.2). However, no significant enrichment for specific functional categories of proteins was observed in *B. pertussis* Tohama I biofilms compared to planktonic cells. Interestingly, of the 38 proteins that were upregulated in either strain, seven proteins, polyribonucleotide nucleotidyltransferase (PnP), aspartate-tRNA(Asp/Asn) ligase (AspS), ATP synthase subunit alpha (AtpA), membrane protein insertase (YidC), outer membrane protein A (OmpA), RNA-binding protein (Hfq) and elongation factor Tu (TuF), were upregulated in biofilms of both strains. Among 46 proteins that were downregulated in planktonic cells of either strains, 5 proteins, chaperone protein (ClpB), trigger factor (Tig), probable parvulin-type peptidyl-prolyl cis-trans isomerase (BB3803), UDP-N-acetylmuramate-L-alanine ligase (MurC) and chaperone protein FimB/FhaD, precursor of FimB, were downregulated in both the strains.

A)



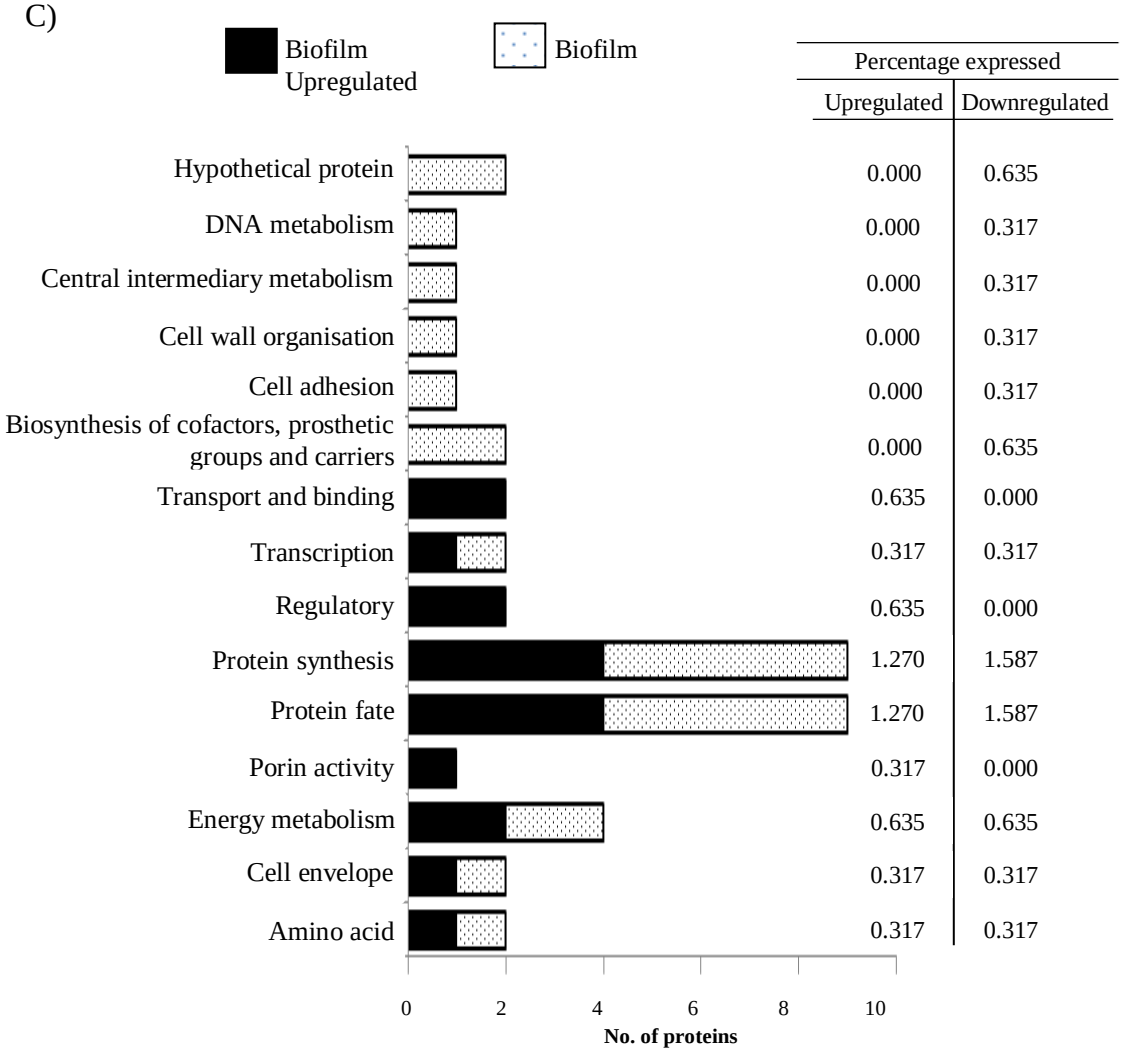
	Percentage expressed	
	Upregulated	Downregulated
Purines, pyrimidines, nucleosides and nucleotides	0.000	0.317
Unknown	0.000	0.635
Transcription	0.635	0.317
Regulatory functions	0.317	0.000
Protein synthesis	0.952	2.540
Protein fate	1.270	0.952
Fatty acid and phospholipid metabolism	0.317	0.317
Energy metabolism *	1.270	0.000
Cellular process	0.317	0.317
Cell envelope	0.317	0.635
Biosynthesis of cofactors, prosthetic groups and carriers	0.317	0.000
Amino acid biosynthesis	0.635	1.270

B)



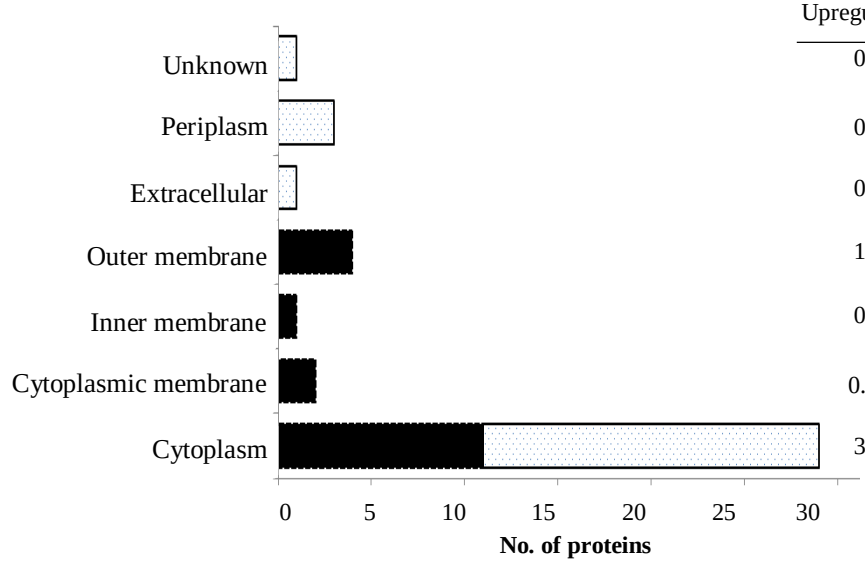
	Percentage expressed	
	Upregulated	Downregulated
Unknown	0.000	0.317
Periplasm	0.317	0.635
Cytoplasm, secreted, cell surface	0.317	0.000
Cell outer membrane	0.635	0.317
Cell membrane	0.317	0.000
Cell inner membrane	0.635	0.317
Cytoplasm	4.127	5.714

C)



	Percentage expressed	
	Upregulated	Downregulated
Hypothetical protein	0.000	0.635
DNA metabolism	0.000	0.317
Central intermediary metabolism	0.000	0.317
Cell wall organisation	0.000	0.317
Cell adhesion	0.000	0.317
Biosynthesis of cofactors, prosthetic groups and carriers	0.000	0.635
Transport and binding	0.635	0.000
Transcription	0.317	0.317
Regulatory	0.635	0.000
Protein synthesis	1.270	1.587
Protein fate	1.270	1.587
Porin activity	0.317	0.000
Energy metabolism	0.635	0.635
Cell envelope	0.317	0.317
Amino acid	0.317	0.317

D)



	Percentage expressed	
	Upregulated	Downregulated
Unknown	0.000	0.317
Periplasm	0.000	0.952
Extracellular	0.000	0.317
Outer membrane	1.270	0.000
Inner membrane	0.317	0.000
Cytoplasmic membrane	0.635	0.000
Cytoplasm	3.492	5.714

Figure 3.1 Functional classification of differentially expressed biofilm associated proteins. Proteins of *B. pertussis* ID20 clinical isolate (A) and *B. pertussis* Tohama I strain (C) classified using TIGRFAMs database**.** Subcellular localisation of differentially expressed proteins in biofilms of *B. pertussis* ID20 (B) and *B. pertussis* Tohama I (D) predicted by PSORTb. The relative percentage of proteins upregulated or downregulated in biofilms compared to total number of protein identified is shown on the right as percentage expressed. Asterisks shows the significant expression of biofilm associated protein in respective function categories as determined by Fisher's exact test ($P<0.005$).

3.4 Discussion

The re-emergence of *B. pertussis* infection associated with new genotypic variants represents an interesting and a serious concern, as the currently used aP may not offer protection against these new variants. This study supports the view that the new variants, of genotype *ptxP3-prn2-fim3A*, comprising 48% of the isolates in the present study, may be able to evade immune responses generated by the acellular pertussis vaccine expressing *ptxP1* allele [42,43]. In this study, all *B. pertussis* isolates were susceptible to erythromycin, the current drug of choice for the treatment of whooping cough [44]. Azithromycin was the most effective antimicrobial agent tested against the strong biofilm-forming clinical isolate. The mechanism of antimicrobial action of azithromycin clearly differs from that of clarithromycin or erythromycin [45]. Azithromycin inhibits bacterial protein synthesis by interfering with the 50S large ribosomal subunit assembly while clarithromycin and erythromycin inhibits cytochrome P450 enzyme systems [45,46]. Its efficacy in this study coupled with its reported lesser side effects as compared to erythromycin, warrants further study for its use in the clinical management of whooping cough, particularly in infants under 1 month of age. The minimum bactericidal concentration of streptomycin, an aminoglycoside that acts by inhibiting ribosomal protein synthesis was also raised in this study for the strong biofilm forming isolate ID20. The reduced efficacy of streptomycin observed in this study may be as a result of increased *de novo* synthesis of proteins in biofilm state rendering it ineffective.

This study demonstrated that strong biofilm formation by clinical isolates of *B. pertussis* may be one reason underpinning their greater resistance to antimicrobial agents compared to planktonic cultures. It supports a previous report that *B. bronchiseptica* biofilms had higher tolerance to antimicrobial agents compared to their planktonic counterpart [37]. Given the previous demonstration of the presence of *B. pertussis* in the nasopharyngeal epithelium of infected mice [20,22,23] and strong biofilm-forming potential of the clinical isolates, it is likely that biofilms allow the bacteria to persist in upper respiratory epithelium of the human adult and adolescent carriers [47]. Since the antibiotic tolerance of the biofilm cells of clinical *B. pertussis* isolates are increased, determination of antibiogram for clinical *B.*

pertussis isolates *in vit*ro in the planktonic as well as in biofilm cultures may provide better therapeutic outcomes. It is also desirable to obtain evidence for role of biofilms in evasion of protection offered by the currently used aP. de Gouw et al [38] showed that biofilm derived protein, BipA, a Bvg^{i} or Bvg-intermediate phase protein, protected mice against infection with virulent *B. pertussis* and elicited protective antibodies. Further studies are warranted to demonstrate the contribution of biofilm formation in the evasion of immunity induced by the aP. Future studies are also needed to investigate the protective potential of novel biofilm-associated antigens of *B. pertussis* that may be of interest for the formulation of potent pertussis vaccines.

Given the observed genetic diversity in the virulence-associated genes encoding pertussis toxin, pertactin and fimbrial antigens [19,42,48,49], it is necessary to evaluate whether currently used aP induces protection against the newly emerged *B. pertussis* strains. This is highly relevant given the recent report of severe whooping cough cases with pertactin-negative (Prn^{-}) isolates in vaccinated populations in Australia [50], Europe [18], Japan [51] and the USA [16,17]. Pertactin, like other auto-transporter proteins, contains an RGD-rich motif that mediates attachment of *B. pertussis* to many eukaryotic cells and is associated with multiple virulence functions including adhesion, toxicity, invasion and aggregation and biofilm formation [52]. It is difficult to hypothesise the mechanisms underpinning the greater virulence of pertactin-negative isolates since loss of pertactin should ideally reduce the pathogenesis of whooping cough, given its adhesion and anti-phagocytic properties [53]. Fimbriae and FHA of *B. pertussis* may directly or indirectly contribute to biofilm formation [25,27]. However, downregulation of FhaB in clinical isolate ID20 and FimB in biofilms state of both the clinical isolate ID20 and Tohama I reference strain observed in this study warrants further investigation.

The *Bordetella bpsABCD* locus has been reported to be homologous to other bacterial loci implicated in the synthesis of PNAG [21]. Previous studies have shown that *bps* is critical to promote colonisation and subsequent biofilm formation by *B. pertussis* [21,30,54]. Since *bpsA* and *bpsD* were detected in all except two clinical isolates, there may be an apparent association of strong biofilm formation with the *bps* operon. The *kpsT* gene was found in all 21 circulating *B. pertussis* isolates but

there was no association between *kpsT* and biofilm formation. Further studies are necessary to elucidate the role *kpsT* in biofilm formation by *B. pertussis* and the protective potential capsular of the antigen against infection caused by the pathogen.

This study also investigated the differential expression of *B. pertussis* proteins associated with biofilm cells relative to the planktonic cells using iTRAQ analysis of soluble proteins. It identified novel biofilm upregulated proteins in a newly emerged clinical isolate compared with the Tohama I reference strain. Interestingly, functional classification of proteins using TIGRFAMs with *B. pertussis* database searches showed that proteins involved in energy metabolism are significantly upregulated in biofilm cells of the newly emerged clinical isolates ($P<0.03$) in comparison to planktonic cells. Laboratory strain Tohama I biofilms did not show any significant enrichment for any particular functional class of proteins compared to its planktonic cells. This is consistent with a recent study in which higher expression of energy metabolism enzymes in clinical isolates was observed [24]. However, another study showed that proteins involved in protein biosynthesis such as ribosomal subunit proteins and elongation factors are significantly enriched in *B. pertussis* biofilms [38].

This study did not identify any of the proteins included in the formulation of the current aP to be upregulated in biofilms of either *B. pertussis* ID20 or Tohama I strain. However, filamentous haemagglutinin, FhaB, was found to be downregulated in biofilm cells of clinical isolate *B. pertussis* ID20, while chaperone protein FimB/FhaD, precursor of FimB, was found to be downregulated in biofilm cells of both the clinical isolate and the reference strain Tohama I (Appendix 3.1 and 3.2). This is consistent with earlier reports of de Gouw et al [38] and Serra et al [55] who did not report any significant upregulation of filamentous haemagglutinin and fimbrial proteins in *B. pertussis* biofilm proteome suggesting that these two antigens may not be protective antigen against biofilm forms of *B. pertussis*. Therefore, it is important to continue studies on the identification of novel biofilm-associated antigens, including surface-associated proteins and polysaccharides, and determine their protective potential with a view to formulating improved pertussis vaccines capable of providing protection against whooping cough associated with resurgent

strains of *B. pertussis* as well as preventing the persistence and transmission of *B. pertussis* among susceptible populations.

3.5 Acknowledgements

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3.6 Conflict of interest

No potential conflict of interest was reported by the author(s).

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Chapter 4

Prevalence of virulence factors in resurgent strains of *Bordetella pertussis* clinical isolates*:* potential relevance to development of an effective pertussis vaccine

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Part of this Chapter has been prepared as manuscript entitled "**Genome sequence of *Bordetella pertussis* strain ID20 with *ptxP3* allele isolated during a whooping cough epidemic from Western Australia**", for submission to the journal, Genome Announcements.

Key words: *Bordetella pertussis*, whole genome sequencing, genetic analysis, resurgence, whooping cough, pertussis resurgence

4.1 Introduction

Bordetella pertussis typically expresses a wide range of virulence factors responsible for bacterial adhesion, invasion and modulation of host immune systems [1]. These includes the toxins, pertussis toxin (*ptx*), tracheal cytotoxin (*tct*), dermonecrotic toxin (*dnt*), adenylate cyclase toxin (*cya*); the adhesins, filamentous haemagglutinin (*fha*), fimbriae (*fim*) and pertactin (*prn*), and others like lipopolysaccharides (LPS) and serum resistance factor A (*brkA*). Acting in concert, these factors alter the local environment and prevent rapid clearance of the bacteria by the host [2]. This enables bacterial replication and dissemination to the lower respiratory tract, leading to typical symptoms of whooping cough [2]. Whooping cough is traditionally a childhood disease, but an increasing number of cases are being seen in adolescents and adults [3,4]. This shift represents a significant public health concerns, as infected adults act as a reservoir for transmission to susceptible infants. Carriage of virulence factors may explain why *B. pertussis* has reemerged in countries with high vaccine coverage causing 200,000 to 400,000 pertussis-related deaths, annually [5].

Bordetella pertussis display variations in the expression of virulence factors in response to its environmental stimuli [6]. This variation is attributed to the *Bordetella* master virulence regulatory system (*bvg-ASR*) locus [7,8], and a second, more recently identified, two-component regulatory system (TCS) called the RisAS system [9-11]. BvgS is a polydomain histidine sensor kinase that contains two periplasmically located venus flytrap domains (VFT1 and VFT2), a transmembrane domain, a cytoplasmic Per/ArnT/Sim (PAS domain), a histidine kinase domain (HK), a receiver domain (Rec) and a histidine phosphoryl transfer domain (Hpt) [6,12]. BvgA is a response regulator protein with an N-terminal Rec domain and a C-terminal helix–turn–helix domain (HTH) [6]. The BvgAS system is active (Bvg^+) at 37°C and in laboratory growth conditions, resulting in activation of virulence-activated genes (*fim2*, *fim3*, *fhaB*, *ptx* and *cyaA*) necessary for infections [6,13], while it is inactive (Bvg^-) at 25°C or in the presence of high concentrations of $MgSO_4$ or nicotinic acid. The Bvg^- phase represents bacteria in nutrient-limiting conditions (usually *ex vivo*) while the Bvg^+ phase is necessary for *in vivo* respiratory infections.

Bordetella pertussis virulence factors associated with adhesion of the bacteria to host airways are FHA, fimbriae and Prn [1,14,15] (See Chapter 1 for review). FHA is a 220 kDa protein that binds to host cells using three different binding domains– the carbohydrate binding, the Arg-Gly-Asp (RGD) domain and the heparin binding domain [1,14]. The RGD domain is involved in invasion of respiratory epithelial cells. In mice, FHA modulates the immune system by inducing apoptosis of phagocytes, stimulating IL-10 responses and skewing immune responses to Th17 responses [1,14]. In humans, FHA mediates immune suppression by time-dependent suppression of the NF-κB pathway [16]. In addition, *B. pertussis* FHA is implicated in biofilm formation in the mouse respiratory tract [17]. Prn is a 69 kDa protein containing an RGD-motif and proline- and leucine-rich repeat motifs associated with binding to eukaryotic cells. However, *B. pertussis* strains lacking Prn invaded human monocyte-derived dendritic cells (MDDC) more efficiently than the wild type [18]. Intriguingly, most resurgent strains of *B. pertussis* are Prn-deficient [19]. Fimbriae, also known as pili or agglutinogens consist of one of the major fimbrial subunit (Fim2 or Fim3) depending on *B. pertussis* serotype. FHA, Prn and Fim are the principle components of currently licensed aP [20].

Toxins that play a role in whooping cough pathogenesis include Ptx, CyaA, TCF and TCT (See Chapter 1 for review). These factors also support the adhesion of *B. pertussis* to the human respiratory tract. Ptx is a secreted, hexameric AB_5 configuration toxin consisting of one active subunit (subunit A or S1 subunit), and five binding B-oligomers (S2 to S5 with two S4 subunits) [1,21,22]. Ptx targets the α-subunit of heterotrimeric Gi/o proteins, thereby disrupting a wide range of downstream signalling pathways [21,23]. It modulates host immune responses by inhibiting the recruitment of immune cells, disrupting innate immune cells and suppressing phagocytic activity of monocytes [14]. Ptx induces lymphocytosis (a hallmark of pertussis in children), hyperinsulinaemia, hypoglycaemia and sensitisation to histamine [14]. Inactivated Ptx is another major component of currently licensed aP vaccines [20].

Bifunctional haemolysin/adenylate cyclase (ACT) encoded by *cyaA* is another toxin that plays an important role in *B. pertussis* pathogenesis. It is a 200 kDa protein and functions as a haemolysin and cytolysin by catalyzing the conversion of cellular ATP

to cAMP. Together, ACT and Ptx play a role in the pathogenesis of *B. pertussis* with Ptx responsible for early establishment of infection and ACT in the maintenance of the later part of infection [24]. Other important toxins include TCT, TCF and DNT but and like ACT, they are not included in any aP (see Chapter 1 for review). Other virulence factors including BrkA play a role in *B. pertussis* infection. BrkA inhibits the classical pathways of complement-mediated killing [25]. Marr et al [26] showed that vaccination of mice with 3 proteins, Ptx, FHA and BrkA, was effective in protecting mice against colonisation by *B. pertussis*.

Biofilm formation is a complex process in which bacteria transit through different stages of development depending on their surrounding environment [27]. The BvgAS signal transduction system of *B. pertussis* and several Bvg-activated proteins are responsible for efficient biofilm formation on abiotic surfaces [28,29]. Various studies have described the microscopic and macroscopic multicellular structures of *B. pertussis* biofilms [17,30,31]. In addition to FHA [17] and ACT [32], the *Bordetella bpsABCD* locus (required for the synthesis of the *B. pertussis* polysaccharide) is critical for the stability and maintenance of the complex architecture of biofilms [33,34]. As biofilm formation may affect vaccine design, it is important to identify biofilm-associated antigens and establish their potential to block transmission of *B. pertussis* to infants from asymptomatic carriers, vaccinated adults and adolescents [31,35,36].

In this study, major *B. pertussis* virulence factors were investigated in 21 clinical isolates. ID20 was chosen as representative of the clinical isolates due to its ability to form strong biofilm. Whole-genome sequencing of ID20 was employed to compare variations in major virulence factors included in the aP vaccine relative to Tohama I.

4.2 Materials and methods

4.2.1 *Bordetella pertussis* isolates

Twenty-one clinical isolates of *B. pertussis*, described in Chapter 3 were used. For Scanning Electron Microscopy (SEM), biofilm of the clinical isolate ID20 was compared with *B. pertussis* Tohama I biofilms. ID20 was used for whole-genome sequencing. The vaccine strain, *Bordetella pertussis* Tohama I (ATCC BAA-589), which carries the *ptxP1* allele, was used as a positive control and for comparison

with isolates carrying the *ptxP3* allele. Bacterial isolates were cultured and DNA extraction carried out as described in Chapter 3.

4.2.2 Scanning electron microscopy of *Bordetella pertussis* biofilms

A suspension of *B. pertussis* equivalent to 10^7 CFU/mL was prepared in SS broth. Sterile coverslips were placed in each well of 24-well plates and 40 µL of the bacterial suspension was added to 360 µL of SS broth to obtain 10^6 CFU/mL. The suspensions were incubated without shaking for 6, 24, 48 or 96 hours. Following incubation, the supernatant was gently removed and the coverslips gently loaded on to aluminium stubs coated with carbon tape. The stubs were washed three times with sterile PBS by gently pipetting over inverted surfaces. The cells were chemically fixed with 2.5% (v/v) glutaraldehyde at room temperature for 10 min and then fixed in 3% glutaraldehyde overnight. The stubs were washed with PBS and then dehydrated by transferring to a tube containing 50% ethanol for 5 min. This step was repeated with 80% and 90% ethanol and then repeated twice with 100% ethanol for 10 min each. Stubs were then transferred to a ScanVac Coolsafe freeze dryer (Labogene, Lynge, Denmark) and freeze dried at -50°C overnight. The stubs were then coated with 3nm of platinum and viewed using a Zeiss Neon scanning electron microscope and images taken at 1000x, 1500x and 3000x magnifications.

4.2.3 Detection of antigens present in aP vaccine

Detection of *ptx* and *prn* in 21 *B. pertussis* clinical isolates was described in Chapter 3. Detection of *fim2* and *fim3* was carried out using primers shown in Table 4.1 as described previously [37]. For *fim2*, 1 µL of DNA was added to 1xMyTaq buffer™ (Bioline) with 5% DMSO (Sigma). The amplification protocol was 95°C for 3 min, 30 cycles of 95°C for 45 s, annealing temperature (Ta) 69°C for 40 s and 72°C for 45 s with a final extension of 72°C for 7 min. Amplification conditions for *fim*3 and *fha* were the same as for *fim2* except that the annealing temperature was 52°C and 57°C and DMSO was not used.

4.2.4 Detection of other virulence factors of *Bordetella pertussis*

PCR was carried out to detect known virulence and biofilm-associated genes of *B. pertussis* from clinical isolates, using primers shown in Table 4.1. Briefly, the amplification protocols for *brkA*, *cyaA*, *dnt* and *tcf* were 95°C for 3 min, 30 cycles of

Table 4.1 Primers* used to detect virulence factors in *B. pertussis* isolates

Virulence factor		Primer Sequence (5' – 3')	Expected product size (bp)	Reference
fim2	Forward	GCGCCGGGCCCTGCATGCAC	848	[37]
	Reverse	GGGGGGTTGGCGATTTCCAGTTTCTC		
fim3	Forward	GACCTGATATTCTGATGCCG	714	[37]
	Reverse	CGCAAGGCTTGCCGGTTTTTTTTGG		
tcf	Forward	TAGAATTCCTTAAGCTCCCGTCGCTG	1076	[38]
	Reverse	CGTCTAGACTAGCGCTTGCTCAACGCG TT		
brkA	Forward	TCAGTCCATGGCGCAGGAAGGAGAGTT CGAC	1900	[39]
	Reverse	CAGTGCAAGCTTCTGCAAGCTCC AGACATG		
dnt	Forward	GTTCGCCTACGACGAATTGG	2400	[40]
	Reverse	CTCCTGCAGGTATCGATATG		
bvgS	Forward	ACCGCAACGAGATCTACCTG	723	[41]
	Reverse	TATCTAGAATGTCGCCTATCAGC		
fha	Forward	GCCACGATTTCACG GTGCA	248	[42]
	Reverse	5'CAGCGTCGCGTC ATGCT		

* The primer sequences of *ptx*, *prn*, *kpsT* and *bpsA* and *bpsD* primers are shown in Table 3.1

95°C for 40 s, Ta (60°C, 46°C, 48°C or 56°C, respectively) for 40 s and 72°C for 45 s with a final extension of 72°C for 7 min.

All PCR products were analysed by 1.5% agarose (Bioline, Australia) gel electrophoresis, stained with 0.8 µL/100 mL of Midori Green DNA Stain (Nippon Genetics) using 1×sodium borate (SB) buffer (10mM NaOH, 40mM Boric Acid, pH 8). GeneRuler 100 bp DNA ladders (100–1000 bp, Fermentas) were used to size the amplicons following visualizing on a UV transilluminator.

4.2.5 Detection of capsule

Bordetella pertussis was visualised using modified Maneval's staining to stain its outer capsule as described previously [43]. Briefly, *B. pertussis* isolates were grown at 37°C. A few colonies of each isolate were scraped with a sterile loop and suspended in 1% glucose and centrifuged at 2500 x g for 2 min. The supernatant was discarded and pellets resuspended in 1 mL 5% glucose solution, incubated at 4°C for 5 min and centrifuged at 2500 x g. Pellets were resuspended in a drop of 1% carbol fuchsin and allowed to mix gently. A smear was prepared on to a clean and grease-free glass slide and allowed to air dry. The smears were stained with Maneval's solution (Carolina Biologicals, Burlington, NC) for 5 min and gently washed with running tap water. After air drying, the smears were observed using oil immersion (100x) microscopy and images were captured using Olympus DP70 camera (Olympus, Japan).

4.2.6 Typing the *bvgS* gene

4.2.6.1 bvgS PCR

bvgS was amplified from all clinical isolates using primers listed in Table 4.1. The amplification protocol was 95°C for 3 min, 30 cycles of 95°C for 45 s, 51.5°C for 30 s and 72°C for 45 s with a final extension of 72°C for 7 min. The reaction mixture contained 1cQ5® Hi-Fidelity DNA polymerase (New England Biolabs), 1XQ5® enhancer (New England Biolabs) and 200 µM of dNTP (New England Biolabs). PCR products were visualised as described in Section 4.2.4.

4.2.6.2 Sequencing of bvgS

bvgS amplicons were purified using Ultraclean® PCR Clean-up kits (MO BIO Laboratories, USA), according to the manufacturer's instructions and quantitated using a Nanodrop™ 1000 Spectrophotometer (Thermo Scientific), with PCR products separated on 1.5% agarose gel to confirm purity. Approximately 30 ng DNA templates with 10 pmol primers were sent for sequencing at the Australian Genome Research Facility (AGRF), Western Australia.

Sequencing data was cleaned manually and aligned with the *bvgS* gene, GenBank ID: 2667057 of *B. pertussis* Tohama I genome (NC_002929.2) using ClustalW Multiple alignment Editor, BioEdit v7.2.0 [44]. The quality score of the sequencing results were evaluated using Q20 base scores (http://www.agrf.org.au/docs/sanger-sequencing-interpreting-batch-summary-reports.pdf). A Q20 score greater than 85% of the total fragment length was considered acceptable.

4.2.7 Genome sequencing and virulence tracking

The clinical isolate ID20 was chosen for whole-genome sequencing as it was characterised as the strongest biofilm producer (Chapter 3). DNA was extracted as described in Chapter 3. Sequencing was performed using Illumina MiSeq (925112 pair-ended 300 bp reads). The reads were trimmed using Cutadapt v1.8.1 (http://journal.embnet.org/index.php/embnetjournal/article/view/200) and TrimGalore v0.4.0 (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore) and re-ordered to the start codon of glucose-inhibited cell division protein A (*gidA*), similar to available genome sequences of *Bordetella pertussis* Tohama I (NC_002929.2). Variants were called using Burrows-Wheeler Aligner (BWA) [45] and Freebayes [46] and the variants were annotated using SnpEff [47].

4.3 Results

4.3.1 Prevalence of *Bordetella pertussis* virulence factors

The prevalence of *ptx*, *prn*, *kpsT* and *bpsA* and *bpsD* in all 21 clinical isolates and their potential to form biofilm was reported in Chapter 3. Here, PCR amplification showed that virulence antigens *fha*, *fim2* and *fim3*, included in the aP vaccines, were detectable in all the clinical isolates investigated (Figure 4.1).

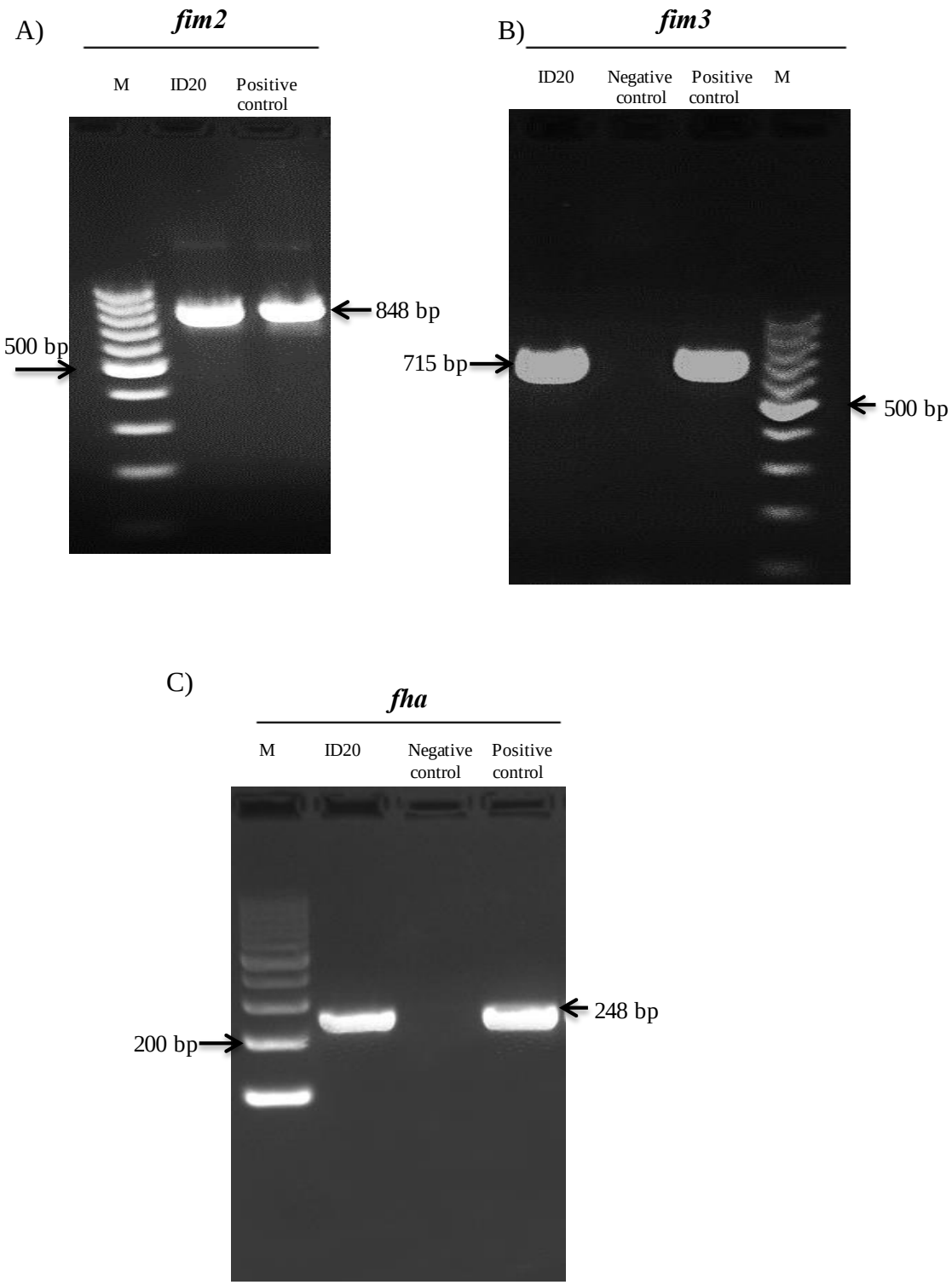

Figure 4.1 Detection of *B. pertussis* virulence factors included in the current aP vaccines. Approximately 848, 715 and 248 bp PCR products represent *fim2* (A), *fim3* (B) and *fha* (C), respectively, in ID20 (shown) and all other clinical isolates. Lanes with positive and negative controls are shown. M; 100 bp DNA marker.

4.3.2 Prevalence of other known *Bordetella pertussis* virulence factors

In addition, *cya*A, *dnt*, *tcf* and *brkA* were detected in all the clinical isolates (Figure 4.2). However, no correlation was established between the presence of virulence factors and biofilm formation as all the studied virulence factors were demonstrated in clinical isolates, irrespective of their genetic background or biofilm forming potential.

4.3.3 SEM confirms the stronger biofilm forming potential of clinical isolate ID20

Qualitative SEM comparing the biofilm-forming potential of *B. pertussis* ID20 and vaccine strain Tohama I showed that ID20 formed a more densely packed multilayered community of cells than Tohama I at all time points observed (Figure 4.3). These results confirm the quantitative finding reported in Table 3.3 that clinical isolate ID20 forms a stronger biofilm compared to the vaccine strain, Tohama I.

4.3.4 Detection of capsules in *Bordetella pertussis* clinical isolates

All isolates carried the gene *kpsT* (Table 3.2, Chapter 3), encoding the second open reading frame in the *B. pertussis* capsule operon responsible for transport of capsular polymers from the periplasmic space to the bacterial cell surface. In addition, *B. pertussis* capsules were demonstrated by modified Manvel's staining in all the isolates (Figure 4.4).

4.3.5 *Bordetella pertussis* clinical isolates carry a single nucleotide polymorphism (SNP) in the *bvgS* gene

Typing of *bvgS*, a member of the *Bordetella* two-component master regulatory system, *bvgAS* phosphorelay, was performed by sequencing the PAS-associated C-terminal (PAC) domain of *bvgS*. Quality of sequencing results were evaluated as described in Section 4.2.6. A Q20 base score of >85% was achieved for all the amplicons. Alignment of *bvgS* nucleotide sequences of all the 21 clinical isolates with Tohama I showed a SNP in all 21 clinical isolates not present in Tohama I *bvgS* (Figure 4.5). The A2113G non-synonymous mutation in the *bvgS* gene led to a Lysine705Glutamic acid substitution (K705E) in the BvgS protein of all the clinical isolates.

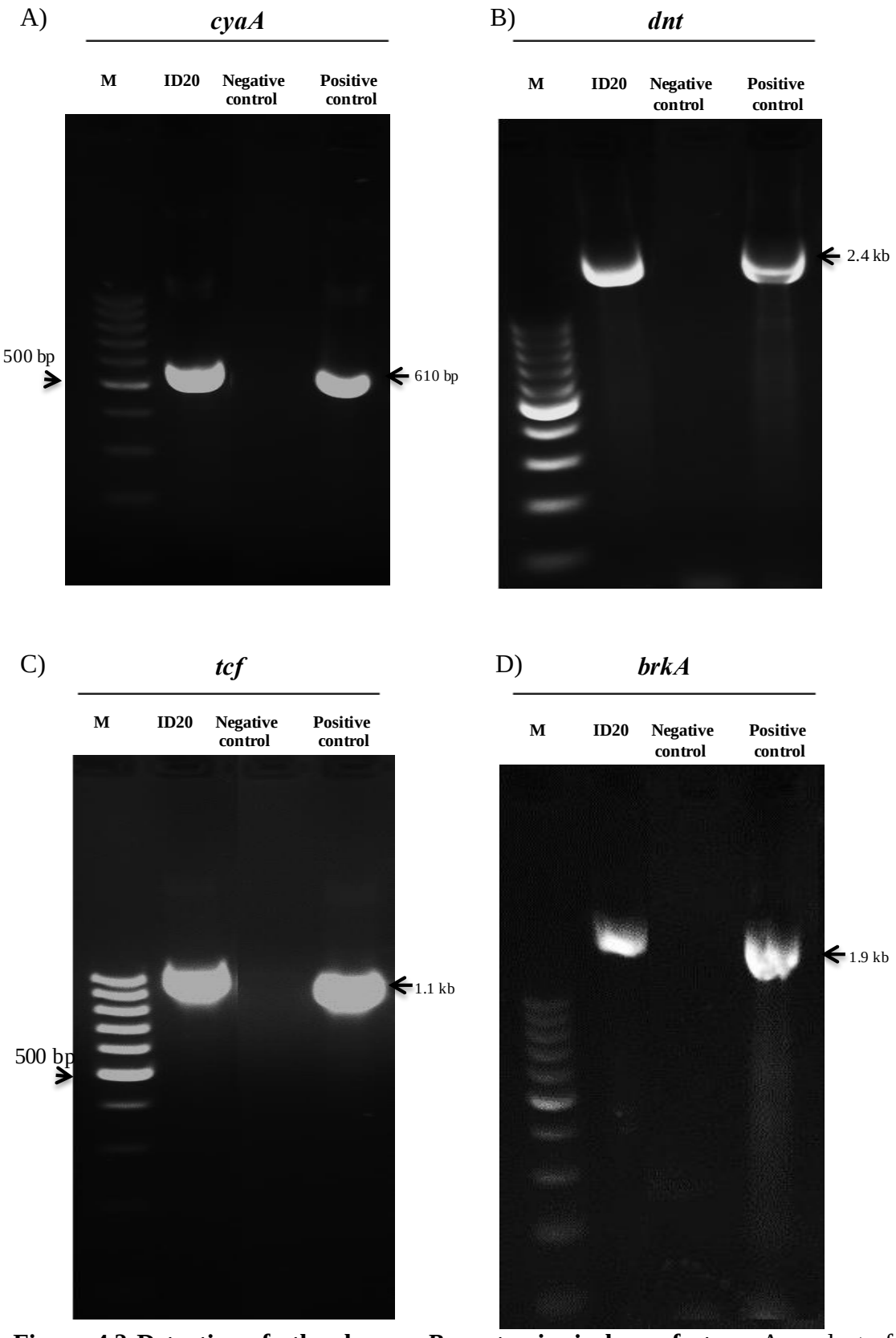

Figure 4.2 Detection of other known *B. pertussis* virulence factors. A product of 0.5, 2.4, 1.5 and 1.9 kb PCR products mark carriage of *cyaA*, *dnt*, *tcf* and *brkA* (respectively) in ID20 (shown) and all the clinical isolates. Positive and negative controls are included. M; 100 bp DNA marker.

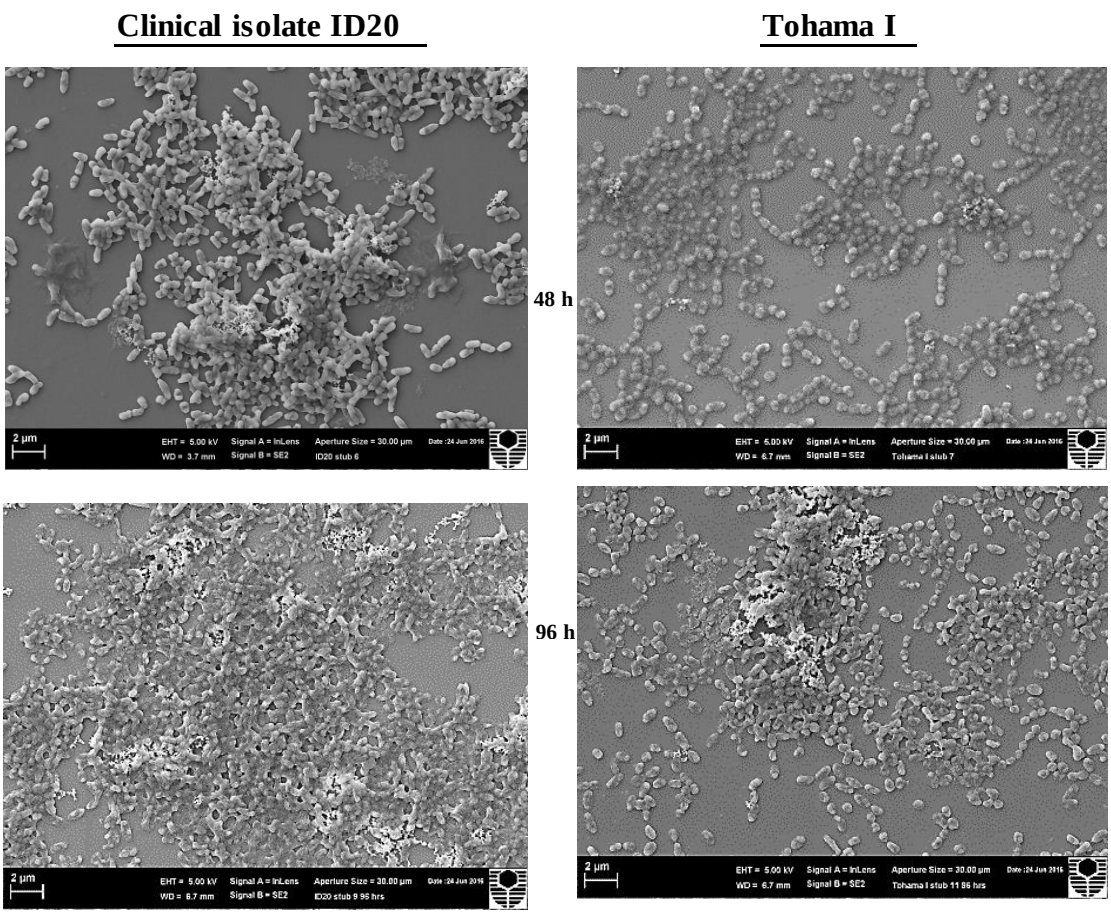

Figure 4.3 Scanning electron micrograph of *B. pertussis* biofilm formed on glass coverslips. Clinical isolate ID20 (left panels) and Tohama I (right panels) were grown for 48 h and 96 h. Scale bar is shown on left bottom corner.

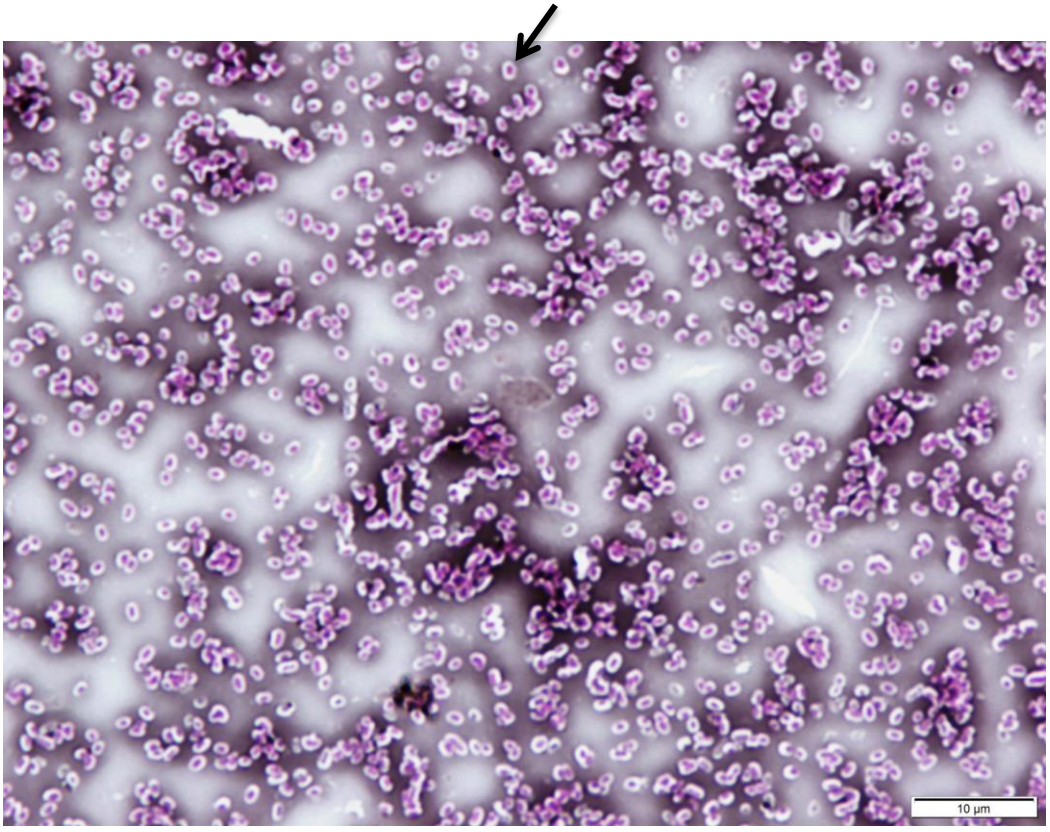

Figure 4.4 Demonstration of *B. pertussis* capsules using Maneval's stain. *B. pertussis* isolates were stained as described in Section 4.3.4. Bacteria stained bright red while the capsules are seen as a clear halo around the bacterial cells. Images were obtained with light microscopy using oil immersion. An arrow indicates an example of a red bacterial cell with a clear halo capsule around it.

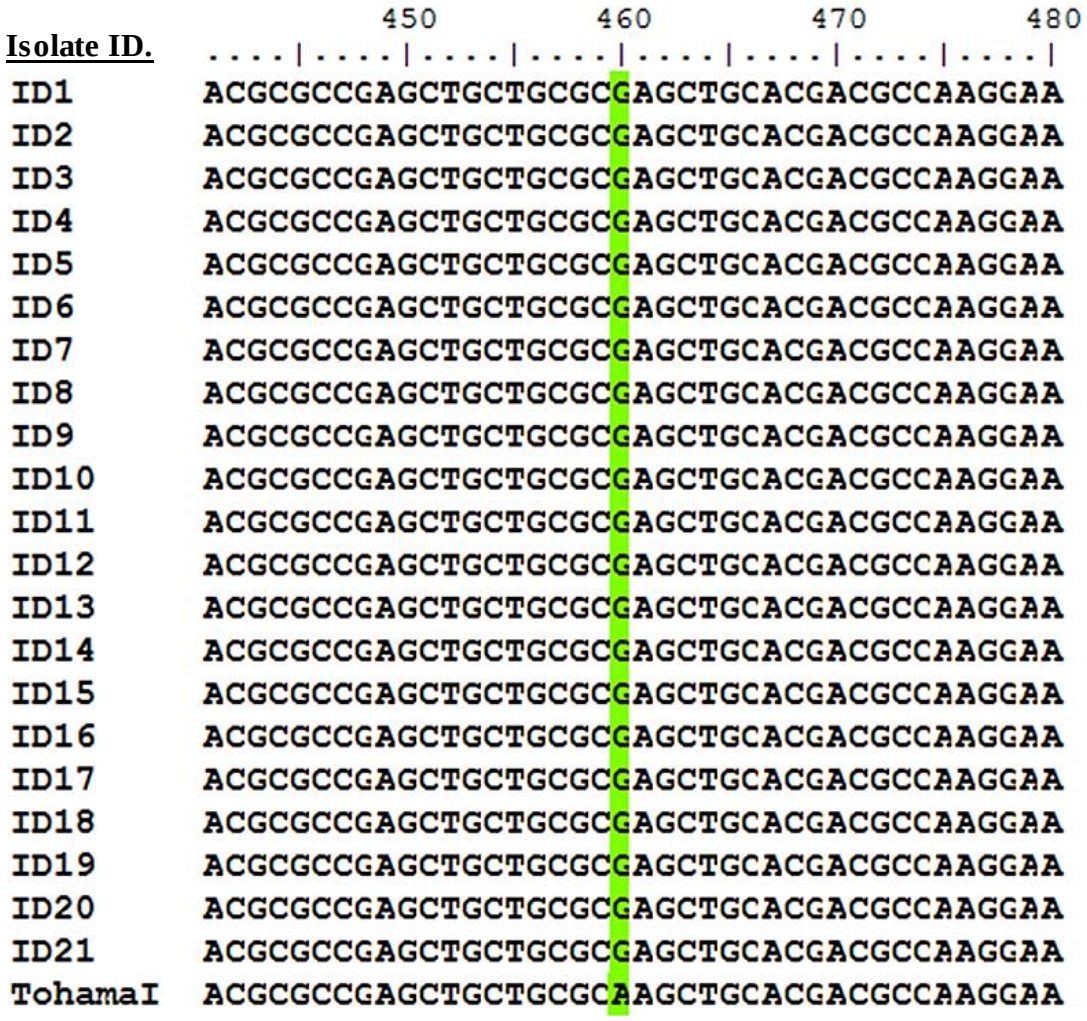

Figure 4.5 ClustalW multiple sequence alignment of *bvgS* gene of 21 *B. pertussis* clinical isolates and Tohama I. A substitution A to G at position 460 of alignment is highlighted in green colour. The ID of clinical isolates is shown at the left. Numbers on the top indicate the nucleotide position in the sequenced *bvgS* gene.

4.3.6 Whole-genome sequencing of *Bordetella pertussis* clinical isolate ID20 reveals SNPs in major virulence factors

To dissect the genetic signatures of the resurgent strains used in this study, the whole-genome sequencing of ID20 - the strongest biofilm producer among the 21 clinical isolates investigated (Chapter 3) was performed. The sequencing generated 1,051,279 paired 300 bp read pairs. The reads were trimmed and variants analysed using Burrows-Wheeler Aligner (BWA) [45] and Freebayes [46]. The assembled genome had 275 contigs with 99% coverage. The estimated genome size was 4.1 Mb with a GC content of 67.7%. The whole-genome assembly has been deposited at GenBank BioProject under the accession number **PRJNA390469 (**https://www.ncbi.nlm.nih.gov/bioproject/PRJNA390469).

The genome was mapped to previously sequenced genome [48-50] available in the database using BWA [45] and Freebayes [46]. Based on the highest number of properly paired reads, the genome mapped closest to clinical isolate ID20 was the Swedish isolate with Accession no CP011444 [50]. Compared to CP011444, the Freebays variant analysis detected 18 SNPs, 3 insertions and 3 deletions. The SNPeff variant analysis detected 48 variants, 7 (15%) of which were located in the intergenic region.

In sharp contrast, 344 SNPs, and 19 insertions and 11 deletions were detected when ID20 was compared to Tohama I. Three high impact variants were detected in the *prn* gene of clinical isolate ID20 when compared to Tohama I. This includes a 6 base missense substitution at position 828, a 1 base frameshift insertion at position 836 and a 16 bp insertion sequence at position 872 (attributed to *prn2/prn1* variations). The 16 bp insertion in *prn* was found in 19 Australian isolates studied by Safarchi et al [51]. Clinical isolate ID20 had a SNP (substitution G to A) in the promoter region of *ptx*, consistent with the *ptxP3* allele, as well as a SNP in the *ptxA*, *ptxB*, *ptxC* subunits. In addition, *fim*2 had one upstream and downstream intergenic variant, while *fimC* and *fimD* had one downstream variant and one missense variant, respectively. Interestingly, the *fimC* variant was not found in 31 *B. pertussis* isolates from US [52] or in the Swedish isolate CP011444 [50]. However, 8 out of 27 isolates studied in Australia [51] demonstrated the variant in the upstream region of *fim2*.

Analysis of the genome for other accredited *B. pertussis* virulence factors showed a SNP in *sphB1* and *sphB2*, while no polymorphisms were observed in *fimA*, *fimB*, *cyaA*, *dnt*, *bipA*, *brkA* or *vag8*. The missense variant in *sph1B* was reported in all the epidemic *B. pertussis* isolates while it was absent in the pre-epidemic isolates and Tohama I [51].

4.4 Discussion

Bordetella pertussis is well known to contain a diverse array of virulence factors which modulate responses of the host immune system [14]. Further exquisite gene control is mediated by the global transcription regulator, *bvg-AS* system, in response to environmental cues [8]. The *bvg-AS* system enables *B. pertussis* to switch between virulent (Bvg^+) and non-virulent (Bvg^-) phases depending on the local host environment. The *bvg-AS* system thus controls the expression of virulence factors required only at particular environmental conditions.

4.4.1 *Bordetella pertussis* clinical isolates carry other known virulence factors investigated in this study

Virulence factors were assessed in 21 *B. pertussis* clinical isolates from Western Australia. PCR amplification demonstrated carriage of all virulence factor genes including those encoding toxins- *ptx*, *dnt*, *cya* and *tcf* - and adhesins- *fim2*, *fim3* and *prn* in all 21 clinical isolates. In addition, *brkA* was also detected in all clinical isolates. These results suggest that *B. pertussis* is largely monomorphic [53] and highly clonal in spread [14,54]. Furthermore, these results may support the hypothesis that instead of complete gene loss or acquisition of new genes, evolution of *B. pertussis* may be attributed to large genome rearrangements primarily mediated by insertion sequences (IS), *IS481*, or accumulation of mutations, particularly SNPs, over the last 60 years of vaccination [55-58]. The gene rearrangements or SNPs alter the orientation of protein-encoding genes or mediate change in gene expression [50,59]. This is evidenced by the recent emergence of *B. pertussis* isolates that do not produce *prn* [60] or produce elevated levels of *ptx* [50], despite the presence of conserved structural genes. To elucidate the expression of these virulence factors at the protein level in the clinical isolates, quantification of these virulence factors employing Western-blot or quantitative-PCR are warranted as a next phase of the study.

4.4.2 The clinical isolate ID20 forms stronger biofilm than the vaccine strain

A correlation between biofilm formation and presence of virulence factors could not be established as all isolates carried virulence factors. The SEM results support the findings in Chapter 3 that ID20 was able to form stronger biofilm than vaccine strain Tohama I, supporting evidence [42] that biofilm formation may be a strategy used by resurgent strains to enhance survival and spread in vaccinated populations. Since biofilm formation has been increasingly associated with the pathogenesis of *B. pertussis* [27,31], vaccines prepared from inactivated biofilm represent a novel strategy for development of an effective whooping cough vaccine.

4.4.3 *Bordetella pertussis* capsules were present in all the clinical isolates

This study demonstrated the phenotypic presence of capsules. The presence of *kpsT*, a capsular gene responsible for transport of capsular polysaccharides from the bacterial periplasmic space to the bacterial cell surface was reported in Chapter 3. Earlier studies reported *B. pertussis* as encapsulated though conventional methods of capsule detection such as negative staining using India ink and presumptive colony morphology on agar plates could not confirm the presence of capsule [61]. Neo et al [61] used transmission electron microscopy and immunostaining to demonstrate intact capsules in the virulent and avirulent phases of *B. pertussis* replication. Cherry et al [62] reviewed evidence that the as-yet unidentified "cough toxin" in pertussis could be the polysaccharide capsule of *B. pertussis*. Conjugated capsular polysaccharide antigens have been successfully used as vaccines in the control of highly invasive bacterial disease including *Neisseria meningitidis*, *Haemophilus influenzae* type b (Hib) and *Streptococcus pneumoniae* and are now part of the routine vaccination schedule in most countries [63]. Taken together, these results indicate the potential of exploring *B. pertussis* conjugated polysaccharide capsular vaccines as an alternative formulation strategy to control mortality and morbidity associated with whooping cough.

4.4.4. The *Bordetella pertussis* master regulatory system, *bvgS*, carries a SNP in all the clinical isolates compared to vaccine strain

Sequence analysis of the *bvgS* gene showed a A2113G transition resulting in a non-conservative K705E change in the resultant protein for all the 21 clinical isolates (Figure 4.5) compared to Tohama I. A similar *bvgS* sequence was also revealed by

the whole-genome sequencing of clinical isolate ID20. Two previous studies [41,42] reported similar mutations in the *bvgS* gene and attributed it to enhanced response to $MgSO_4$ or nicotinic acid-mediated modulation. Furthermore, Arnal et al [42] showed that the modulated *B. pertussis* clinical isolate and mutant carrying K705E demonstrated significantly faster adherence and biomass forming capability compared to Tohama I or a wild type strain without K705E. The *bvgS* typing results thus support evidence described here and in Chapter 3 that all clinical isolates produced stronger biofilm than Tohama I. If *bvgS* is indeed a master regulator of *B. pertussis* virulence factors, the mutation in *bvgS* affect all the virulence factors of *B. pertussis*. Designing a vaccine targeting the *bvgAS* virulence regulatory system may achieve subversion of *B. pertussis* epidemics.

4.4.5 Whole-genome sequencing identified variants in major *Bordetella pertussis* virulence factors

Clinical isolate ID20 was selected to represent resurgent strains with enhanced biofilm formation. Whole-genome sequencing revealed important information regarding polymorphisms relevant to antigens included in the aP and other well characterised virulence factors. When the whole-genome *de novo* assembled reads of ID20 was compared to Tohama I, 424 variants were identified, with 344, 19 and 11 SNPs, insertions and deletions, respectively. The isolate had an intergenic SNP located in the promoter region of *ptx*, indicating a *ptxP3* allele, consistent with the genotype results reported in Chapter 3. Interestingly, the clinical isolate ID20 exhibited variants in *ptxA-C* subunits, *prn*, *fim2*, *fimC* and *fimD*. Therefore, ID20 had variations in all the virulence antigens included in the aP. Previous reports show that the *ptxP3* strains colonise the mouse better than *ptxP1* strains [19,64,65]. The genetic background of ID20, representing the resurgent strains, may be responsible for the enhanced fitness of these isolates against current aP vaccination.

The variant in *fimC* identified in this study was not found in a study of 31 clinical isolates from US [52] or in the Swedish isolate CP011444 [50], while 8 of 27 *B. pertussis* isolates studied in Australia [51] demonstrated the variant in the upstream region of *fim2*. Furthermore, the missense variant in *sphB1*, required for maturation of *fha* [66], was detected in all the 21 epidemic isolates studied but not in the pre-epidemic isolates from Australia [51]. Combined, these results suggest that variants

in *fimC* and *fim2* or *sph1B* may represent the genetic signature of *B. pertussis* epidemic strains unique to Australia.

In summary, the results from this study suggest that the representative resurgent strains of *B. pertussis* ID20 has different genetic makeup compared to the vaccine strain. Studies on whole genome sequence of other resurgent strains from Western Australia require a larger study and are beyond the scope of this thesis. The escape from vaccine-induced immunity in the resurgent *B. pertussis* population may be attributed to these variations. Therefore, novel *B. pertussis* antigens, preferably prepared from the resurgent strains, are clearly warranted. Conjugated-polysaccharide capsule vaccines and biofilm vaccine formulations represent novel whooping cough vaccines for future testing.

4.5 References

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Chapter 5

Identification of novel antigens of *Bordetella pertussis* by proteomic characterisation of a resurgent strain carrying the *ptxP3* allele and its biofilm

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Key words: *Bordetella pertussis*, biofilms, proteomics, iTRAQ, pathways analysis, whooping cough vaccines.

5.1 Introduction

Conventional whole cell (wP) whooping cough vaccines consist of heat-killed or chemically detoxified preparations of *B. pertussis* administered with or without alum as an adjuvant [1]. These first generation vaccines conferred protection against whooping cough mainly by induction of Th1-dependent cell-mediated immunity (CMI) [2,3]. On the other hand, the current acellular vaccines (aP) are composed of 3- or 5-component antigens adsorbed to alum and formulated with diphtheria and tetanus toxoids and, more recently, with hepatitis B, polio and influenza B antigens (GlaxoSmithKline) [4]. These vaccines elicit mostly Th2-skewed humoral antibody responses in infants [5,6] and murine models of infection [7]. These immune responses are sub-optimal in preventing whooping cough since *B. pertussis* is an intracellular pathogen shown to survive within macrophages and other eukaryotic cells [8,9]. Though several factors, acting concomitantly, are likely contribute to pertussis resurgence in countries with high vaccination coverage, the short term protection conferred by aP and pathogen evolution may be important [10].

Other studies [6,11-14] of *B. pertussis* evolution and results from this project (Chapter 4) of *B. pertussis* evolution have identified differences between the resurgent strains and the vaccine strains in genetic contents and production of toxins and antigens that are incorporated into the aP. Resurgent strains of *B. pertussis* lacking pertactin (Prn; a major component of the aP vaccine) have been reported from developed countries including the USA [15], Australia [16] and Europe [17]. Resurgent strains are unaffected by sulphate-mediated gene suppression [18] and over-produce the vaccine antigens Prn and Ptx, and other virulence antigens like Vag8 [a type III secretion toxin (T3SS)], BrkA (a protein involved in complement resistance), and LpxE (involved in lipid A modification) [18]. These differences can affect virulence, survival, vaccine escape and resurgence.

Recently, biofilm formation has gained acceptance as an important factor in the pathogenesis of *B. pertussis*. In the human nasopharynx, biofilm formation allows bacteria to evade immune defences even after vaccination and serves as a reservoir for transmission to susceptible infants and children [19,20]. A recent study [21] identified 11 proteins expressed more than three-fold higher in biofilms compared to planktonic cultures. Subsequent vaccination of mice with the most abundant biofilm-

associated protein reduced colonisation of lungs with virulent *B. pertussis* infection [21]. Therefore, antigens expressed in biofilms of *B. pertussis* may represent an important therapeutic target for effective whooping cough vaccines.

In addition to conventional genomic tools to study differences in emerging *B. pertussis* populations, proteomic-based technology can reveal differences in protein expression of resurgent strains [22-24]. Isobaric tags for relative and absolute quantitation (iTRAQ) is a powerful gel-free technique for quantitative comparison of global proteomic studies in microbial research [25]. Previously, iTRAQ was used to identify a novel T3SS effector protein in *B. bronchiseptica* wild-type by comparing to its isogenic T3SS-deficient mutant strain [26]. In this chapter, semi-quantitative iTRAQ-based proteomic analysis has been used to identify proteins differentially expressed in the clinical isolate ID20 carrying the *ptxP3* allele associated with recent epidemics in WA and compared to the vaccine strain, *B. pertussis* Tohama I, which carry *ptxP1* allele. In addition, iTRAQ was used to identify proteins that were differentially expressed in biofilm and planktonic cells of *B. pertussis*.

5.2 Materials and methods

5.2.1 Bacterial strains and growth conditions

Proteomic characterisation was performed on two *B. pertussis* strains, Tohama I and a clinical isolate, designated *B. pertussis* ID20 [27]. Tohama I was first isolated in Japan in 1954 and is a well-characterised strain that has been used for preparing vaccines in many countries for several years [28]. The genome of Tohama I is completely sequenced. It possesses the pertactin 1 (*prnA1*) and pertussis toxin (*ptxS1B*) genotype typical of pre-vaccine-era isolates. Strain *B. pertussis* ID20 was isolated from patients during an epidemic in Western Australia between 2008 and 2010. Its genotype was *ptxP3*, *prn2*, typical of currently circulating isolates. All *B. pertussis* isolates were plated on to Bordet-Gengou (BG) agar supplemented with 15% sheep blood, or charcoal agar, and incubated at 37°C for 4-5 days. Lawns of bacteria were inoculated into beads and stored at −80°C until required. Before use, single beads were sub-cultured on to pre-warmed BG agar plates. For broth cultures, bacteria were subsequently sub-cultured in Stainer-Scholte (SS) broth at 37°C with shaking at 120 rpm.

5.2.2 Preparation of samples for proteomic analysis

Preparation of samples for proteomic analysis was described in Chapter 3 or [27].

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

Following protein quantification, protein lysates were loaded on to one-dimensional SDS-PAGE (1D-GE) for separation of proteins as described in Section 2.11. Gels were stained with SimplyBlue™ SafeStain (Invitrogen) and visualised using a ChemiDoc™ MP imager system (Biorad).

5.2.4 Preparation of gel bands for proteomic analysis

Bands of interest were cut from SDS-PAGE gels with a clean scalpel blade. Bands were stored at 4°C in sterile 1.5 mL tubes before analysis.

5.2.5 iTRAQ analysis

Differential protein expression analyses were performed at Proteomics International, Perth, Australia, using iTRAQ. The protein samples were diafiltrated, reduced, alkylated and trypsin digested according to the standard iTRAQ protocols as described previously [29]. The samples were labelled using 4-plex iTRAQ reagents. Peptide fractions were analysed by electrospray ionisation mass spectrometry using a 1260 Infinity HPLC system (Agilent Technologies, Australia) coupled to an Agilent 1260 Chipcube Nanospray interface on an Agilent 6540 mass spectrometer. Spectral data were analysed against the SwissProt Bacteria database using ProteinPilot™ 4.5 Software (AB Sciex, Australia). For quantification, each test sample was analysed separately. Average protein ratios and *P*-values indicating differential expression were calculated by the ProteinPilot™ 4.5 Software. $P \leq 0.05$ was considered as statistically significant. Quantitative ratios of identified proteins are colour coded to indicate differential expression. Red indicates up-regulation and blue indicates down-regulation. The intensity of the colouring indicates the certainty of the differential expression.

5.2.6 Prediction of subcellular localisation and functions

The subcellular localisation of proteins was determined using PSORTb version 3.0.2 [30]. Functional classification of proteins was determined using TIGRFAMs

database (ftp://ftp.jcvi.org/pub/data/TIGRFAMs/). Fisher's exact test was used to determine the significance of functional classification of biofilm-associated protein relative to planktonic cultures.

5.2.7 Pathway enrichment and protein interaction analysis

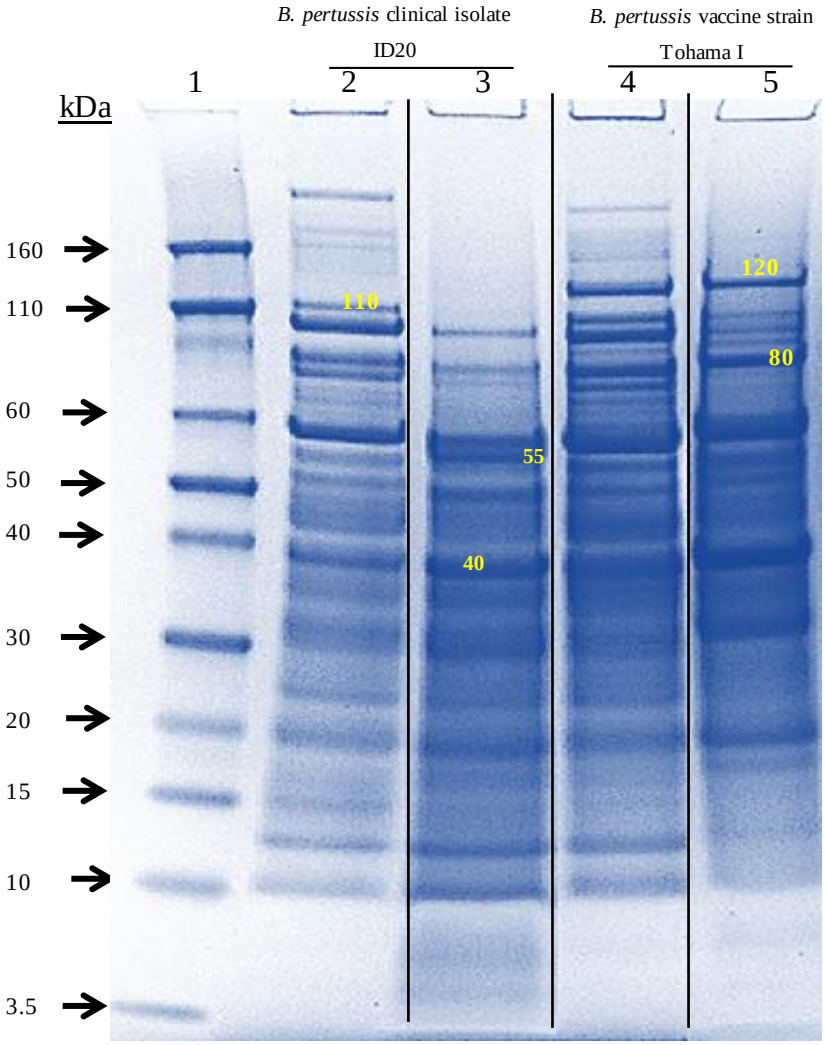
Functional annotation of genes was carried out using the Database for Annotation, Visualisation, and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov/) [31,32]. Pathway enrichment analyses were performed using Gene Ontology Consortium (GO, http://geneontology.org/) and KEGG mapper (http://www.kegg.jp/kegg/), to derive associated functions along with their enrichment scores and *P*-values. *P*-values ≤ 0.05 were considered statistically significant. Protein-protein interaction analysis was performed using STRING v10 (http://string-db.org) [33].

5.3 Results

5.3.1 1D-GE of *Bordetella pertussis* soluble proteins

The soluble protein fraction was initially separated by 1D-GE, using three replicates per sample to ensure consistency in protein banding patterns. *Bordetella pertussis* vaccine strain Tohama I and clinical isolate ID20 were included, as well as the biofilms of both strains. Overall, different protein banding profiles among the strains were observed, with 1D-GE revealing major differences with unique protein banding patterns. There were also differences in band intensities between strains (Figure 5.1A). To identify proteins that were differentially expressed, bands with higher intensity or bands that were present in one but missing from the other preparations were cut out and processed for protein identification with mass spectroscopy. Forty (40) and 55 kDa bands from ID20 biofilm of were identified as outer membrane porin protein (BP0840) and an uncharacterised protein (V8ZBL3) of *B. pertussis* strain Tohama I and I176, respectively (Figure 5.1B). Similarly, the 80 and 120 kDa proteins from biofilm of Tohama I were identified as an outer membrane porin protein (BP0840) and a putative N-acetyltransferase (YedL), respectively (Figure 5.1B). Both proteins may be present in the gel as dimers since their calculated monomeric molecular weights were approximately 41 kDa.

A)



B)

Protein Mass (kDa)	Protein description	Protein ID by mass spectrometer
110	ID20 planktonic	Middle cell wall protein (WP_026557553.1)
40	ID20 biofilm	Outer membrane porin protein (BP0840)
55	ID20 biofilm	Uncharacterised protein (V8ZBL3)
80	Tohama I biofilm	Outer membrane porin protein (BP0840)
120	Tohama I biofilm	N-acetyltransferase (YedL)

Figure 5.1 Comparative 1-DE SDS gel images of B. pertussis planktonic and biofilm cells. Lane 1: Novex® sharp pre-stained protein standard. Lane 2 and 4 are planktonic cells of clinical isolate B. pertussis ID20 and Tohama I, respectively. Lanes 3 and 5 are biofilm of the same isolates. Approximate band sizes (kDa) identified by mass-spectrometry are indicated in yellow.

5.3.2 *Bordetella pertussis* proteome profiling by iTRAQ

A 4-plex iTRAQ was performed on trypsin digested peptides of the soluble fractions of planktonic and biofilm of *B. pertussis* Tohama I and clinical isolate ID20. In total, 315 proteins were identified (Appendix 5.1). The quantitation of 16 proteins could not be determined. Based on PSORTb prediction, more than 83% of the proteins identified were localised to the cytoplasm, while 12% constitute periplasmic, cytoplasmic membrane, outer membrane, multi-pass proteins, extracellular or cell membrane proteins, with the remaining 5% being undetermined (Appendix 5.1). Functional classification of the identified proteins showed that the majority (81%) are related to cellular processes such as protein synthesis, cell biosynthesis, cellular metabolism, transcription, translation, and protein fate while approximately 4% are related to pathogenesis, adhesion and regulatory functions. About 15% of the proteins could not be classified to any functional categories. For each strain, protein expression in the biofilm was compared to that in the planktonic state. Protein expressions between planktonic cells of the two strains were also compared. No significant change in expression in any of the comparisons was observed in 227 (72%) proteins.

5.3.3 Differences in the proteome of *Bordetella pertussis* ID20 and Tohama I

To identify proteins that are characteristic of the *ptxP3* lineage of the currently circulating Western Australian isolates, protein expression was investigated in planktonic cells of clinical isolate ID20 and the vaccine strain *B. pertussis* Tohama I (Table 5.1). iTRAQ analysis identified 27 (8.5%) proteins exhibiting significant (P<0.05) differential expression; 16 (5%) were significantly upregulated while 11 (3.5%) were significantly downregulated in ID20 compared to Tohama I (Figure 5.2). Among the upregulated proteins, 5 of 16 (31%) were related to protein fate (Figure 5.3A). Four of 11 downregulated proteins (36%) were associated with energy metabolism (Figure 5.3A). Interestingly, functional classification showed that proteins localised in the periplasmic space were significantly upregulated (P<0.03) in ID20 compared to Tohama I (Figure 5.3B). Of the antigens included into the aP, serotype 3 fimbrial subunit (Fim3), and chaperone protein FimB/FhaD (FimB) were significantly upregulated in the clinical isolate compared to Tohama I (P<0.001 and

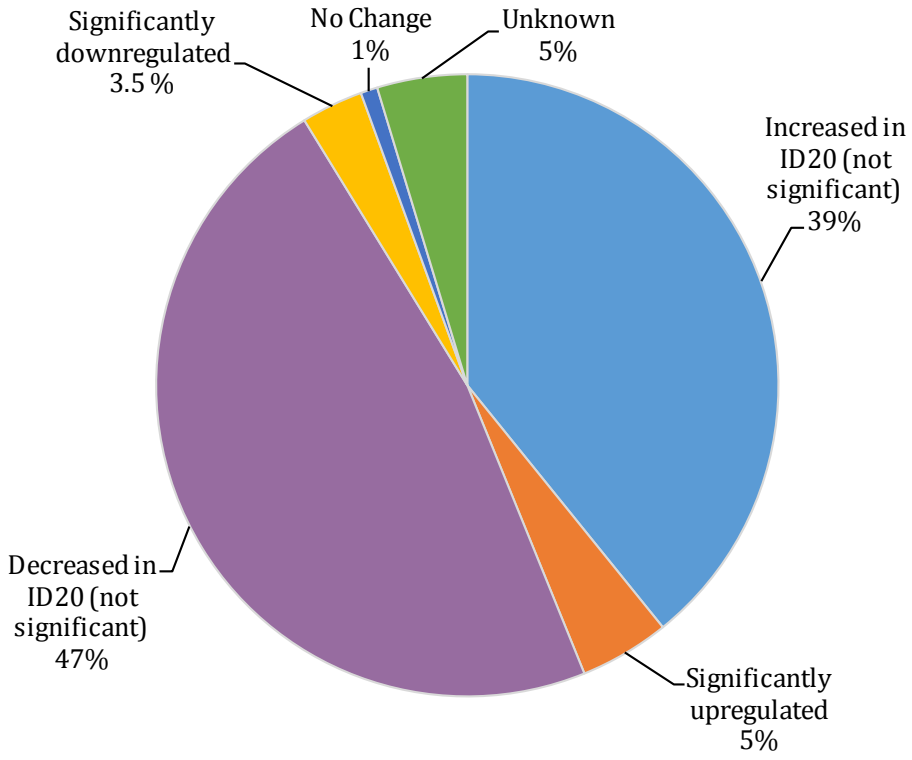


Figure 5.2 Distribution of proteins differentially expressed in *B. pertussis* ID20 and Tohama I.

Table 5.1 Differential expression of proteins in planktonic cells of clinical ID20 compared to planktonic cells of vaccine strain Tohama I.

Protein name, gene	Cellular localisation	Function	Ratio (ID20:Tohama I)	*P*-value[‡]
60 kDa chaperonin, *groL*	Cytoplasm	Protein fate	0.031	0.002
Succinyl-CoA ligase [ADP-forming] subunit beta, *sucC*	Cytoplasm	Energy metabolism	0.056	0.002
Aminomethyltransferase, *gcvT*	Cytoplasm	Energy metabolism	0.157	0.004
ATP synthase subunit alpha, *atpA*	Cytoplasm	Energy metabolism	0.083	0.015
DNA-directed RNA polymerase subunit alpha, *rpoA*	Cytoplasm	Transcription	0.305	0.016
ATP synthase subunit alpha, atpA	cytoplasm	Energy metabolism	0.180	0.019
DNA-directed RNA polymerase subunit beta, *rpoB*	Cytoplasm	Transcription	0.328	0.023
Acetylglutamate kinase, *argB*	Cytoplasm	Amino acid biosynthesis	0.288	0.030
N-acetyl-gamma-glutamyl-phosphate reductase, *argC*	Cytoplasm	Amino acid biosynthesis	0.219	0.031
Elongation factor Tu, *tuf*	Cytoplasm	Protein synthesis	0.011	0.041
Surface layer protein P38538	Unknown	Unknown	0.020	0.044
Chaperone protein, *clpB*	cytoplasm	Protein fate	1.096	0
Threonine--tRNA ligase, *thrS*	Cytoplasm	Protein synthesis	1.107	0.001
Serotype 3 fimbrial subunit, *fim3*	Extracellular	Cell adhesion	23.768	0.001
Outer membrane protein A, *ompA*	outer membrane	Protein fate	2.399	0.002
Arginine biosynthesis bifunctional protein, *argJ*	cytoplasm	Amino acid biosynthesis	3.191	0.002
Azurin BPP3406	Periplasm	Energy metabolism	35.645	0.003
3-isopropylmalate dehydratase small subunit 1, *leuD1*	Cytoplasm	Amino acid biosynthesis	6.368	0.003
10 kDa chaperonin, *groS*	Cytoplasmic	Protein fate	2.965	0.004
Chaperone protein FimB/FhaD, *fimB*	Periplasm	Cell wall organisation	2.399	0.004
Phosphoglucosamine mutase, *glmM*	Cytoplasm	Cell envelope	1.486	0.006
Protein TolB, *tolB*	Periplasm	Transport and binding proteins	1.853	0.001
Cytochrome c oxidase subunit 1, *ctaD*	Cytoplasmic Membrane	Energy metabolism	87.902	0.018
Probable parvulin-type peptidyl-prolyl cis-trans isomerase BB3803	Periplasm	Hypothetical protein	1.148	0.022
60 kDa chaperonin, *groL*	Cytoplasm	Protein fate	2.051	0.035
LPS-assembly protein LptD, *lptD*	Outer membrane	Cell envelope	3.373	0.038
Membrane protein insertase, *yidC*	Cytoplasmic membrane	Protein fate	8.551	0.042

[‡]Red indicates up-regulation and blue indicates down-regulation. The intensity of the colour indicates the certainty of the differential expression based on *P*-value as described in Materials and Method.

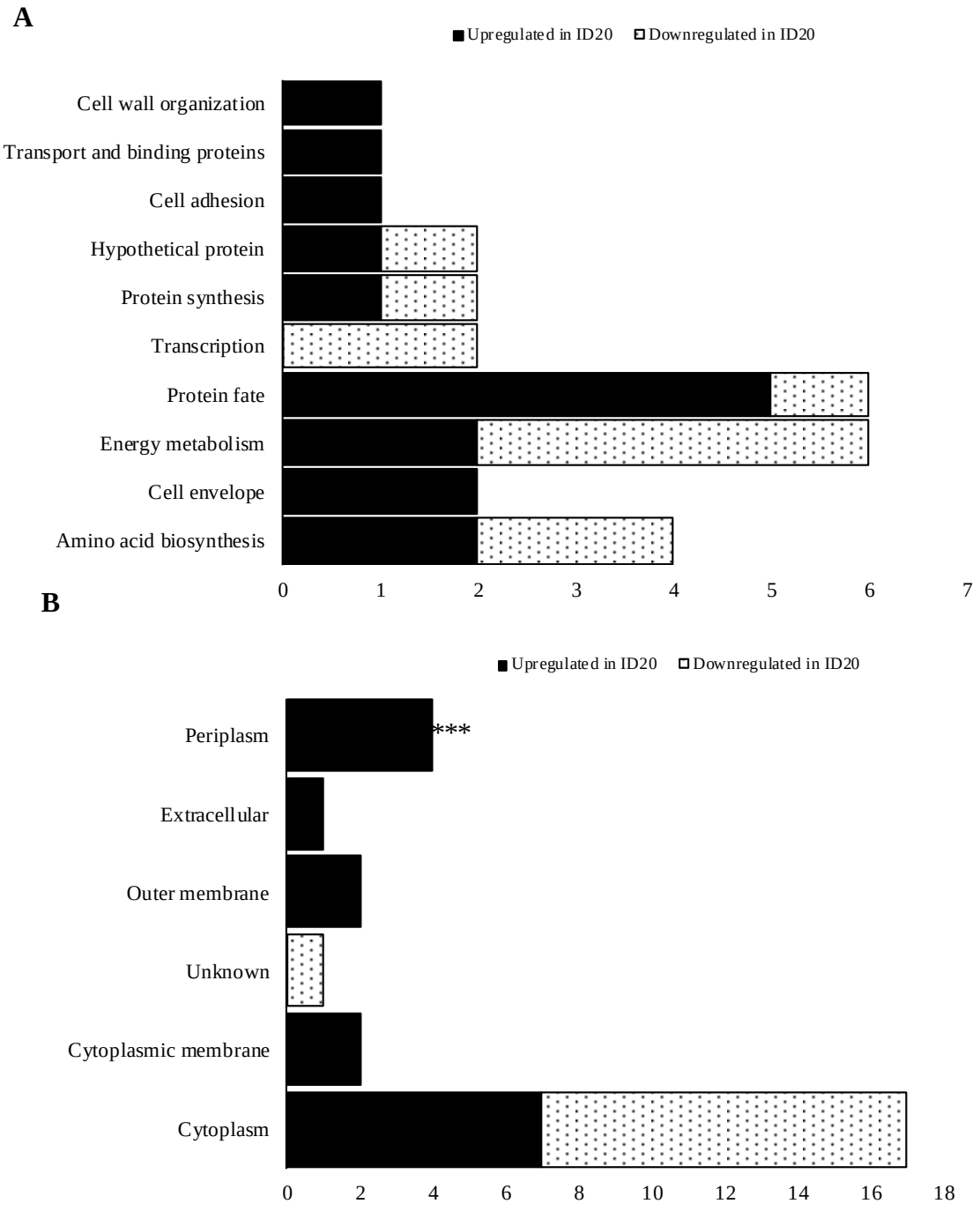


Figure 5.3 Classifications of differentially expressed proteins in *B. pertussis* ID20 and *B. pertussis* Tohama I strain using TIGRFAMS database. Functional classification (A) and Subcellular localisation (B) of differentially expressed proteins predicted by PSORTb. ***Biofilm proteins localised to periplasm were enriched in ID20 compared to Tohama I (Fisher's exact test, $P<0.05$).

P<0.005, respectively) (Table 5.2). The increases in other aP antigens, including filamentous haemagglutinin (FhaB), serotype 2 fimbrial subunit (Fim2), pertussis toxin subunit 1 and 4, and pertactin (Prn) were not significant (*P*>0.05) (Table 5.2). Three membrane proteins, outer membrane protein A (OmpA), membrane protein insertase (YidC) and LPS-assembly protein (LptD), were significantly upregulated in the clinical isolates, while no membrane proteins were found to be upregulated in Tohama I.

5.3.4 Differences in the proteome of planktonic and biofilm of *Bordetella pertussis* ID20 and Tohama I

The proteomes of biofilm and planktonic cells of the Tohama I (with *ptxP1*) and ID20 (with *ptxP3*) allele were compared in Chapter 3 [27]. None of the proteins included in the current aP were upregulated in the biofilm of either the clinical isolate ID20 or *B. pertussis* Tohama I. However, FHA was significantly downregulated in biofilm of clinical isolate ID20 and Fim3 was downregulated in biofilm of *B. pertussis* Tohama I strain. The chaperone protein, FimB/FhaD, was downregulated in biofilm of both strains. Interestingly, virulence factors putative positive transcription regulator, BvgA, and probable TonB-dependent receptor, BfrD, were upregulated in Tohama I biofilm compared to its planktonic counterpart. Proteins localised to the outer membrane were significantly enriched [(*P*<0.05) in *B. pertussis* Tohama I biofilms (see Figure 3.1A and B, Chapter 3).

5.3.5 Pathway enrichment analysis of significantly expressed proteins in planktonic cells of ID20 relative to Tohama I

To identify proteins in common pathways that were upregulated or downregulated, pathway enrichment analysis was performed using DAVID as described in Section 5.2.7. Protein-protein interactions were determined using STRING as described in Section 5.2.7. The summary of results of pathway analysis is shown in Table 5.3. Pathway enrichment analysis of 16 significantly upregulated proteins of *B. pertussis* clinical isolate ID20 did not show any significant enrichment for any particular pathway. However, among 11 proteins that were significantly downregulated in *B. pertussis* ID20 compared to Tohama I, three pathways [biosynthesis of RNA polymerase, metabolic pathway (glycan biosynthesis and metabolism) and antibiotic (streptomycin) biosynthesis] were significantly enriched (*P*<0.05, Table 5.3).

Table 5.2 Comparative ratio profile of major *Bordetella pertussis* proteins in ID20 compared to Tohama I

***Bordetella pertussis* proteins**	**Ratio (ID20:Tohama I)**	***P*-value**
***Bordetella pertussis* acellular vaccine components**		
Filamentous haemagglutinin , FhaB	0.99	0.20
Serotype 3 fimbrial subunit, Fim3	23.77	0.001
Chaperone protein FimB/FhaD, FimB	2.40	0.005
Serotype 2 fimbrial subunit, Fim2	0.02	0.07
Pertussis toxin subunit 1, PtxA	1.46	0.54
Pertussis toxin subunit 4, PtxD	2.47	0.19
Pertactin autotransporter , Prn	1.15	0.498
Other accredited virulence factors of *Bordetella pertussis*		
Tracheal cytotoxin, Tct	Not detected	-
Tracheal colonising factor, Tcf	Not detected	-
Dermonecrotoxin, Dnt	Not detected	-
Bifunctional haemolysin/adenylate cyclase, CyaA	1.14	0.11
BrkA autotransporter, BrkA	1.84	0.08
Virulence factors putative positive transcription regulator, BvgA	0.85	0.88
Virulence sensor protein, BvgS	1.31	0.14
Probable TonB-dependent receptor, BfrD	0.76	0.48
RNA-binding protein, Hfq [34]	0.97	0.178

Table 5.3 Pathway enrichment of differentially expressed proteins

Bacterial strain	Gene Ontology Term	Pathway description	Count	*P*-value
***B. pertussis* ID20 Biofilms**				
Upregulated				
	00034	RNA degradation	4	0.03
Downregulated				
	00970	Aminoacyl tRNA biosynthesis	5	0.03
	03010	Ribosome	4	0.03
***B. pertussis* Tohama I Biofilms**				
Upregulated	-	-	-	-
Downregulated	-	-	-	-
***B. pertussis* ID20 Vs Tohama I**				
B. pertussis ID20 upregulated	-	-	-	-
B. pertussis ID20 downregulated				
	03020	RNA polymerase	2	0.004
	01100	Metabolic pathways (glycan biosynthesis and metabolism)	7	0.005
	01130	Biosynthesis of antibiotics (streptomycin)	4	0.007

5.3.6 Pathway enrichment analysis of proteins upregulated in biofilm

Pathway analysis of 20 proteins upregulated in the biofilm of ID20 relative to its planktonic cells showed that the RNA degradation pathway was significantly enriched (P<0.03) (Table 5.3; Figure 5.4). This pathway comprises four significantly upregulated proteins: polyribonucleotide nucleotidyltransferase (Pnp), 60 kDa chaperonin (GroEL), enolase (Eno) and RNA-binding protein (Hfq). Aminoacyl tRNA (P<0.03) and ribosomal (P<0.03) biosynthesis pathways were significantly enriched amongst 23 biofilm downregulated proteins, suggesting tight control of protein biosynthesis in biofilm of the clinical isolate. The aminoacyl tRNA pathway comprised 4 proteins: 1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino) methylideneamino] imidazole-4-carboxamide isomerase (hisA), 3-isopropylmalate dehydratase small subunit (leuD), ATP phosphoribosyltransferase (hisG) and tryptophan synthase subunit alpha (trpA). The ribosomal biosynthesis pathway comprised the 30S ribosomal protein S10 (rpsJ), 50S ribosomal protein L1 (rplA), 50S ribosomal protein L17 (rplQ) and 50S ribosomal protein L22 (rplV).

In contrast, there was no significant enrichment for any pathways amongst 18 and 23 upregulated or downregulated proteins, respectively, when Tohama I biofilms were compared to planktonic cells.

5.3.7 Protein-protein functional association of differentially expressed *Bordetella pertussis* proteins

STRING database analyses were used to establish the functional protein-protein association of differentially expressed proteins in biofilm as well as in planktonic cells of ID20 and Tohama I. This showed significant interactions (P<0.001) amongst the differentially upregulated or downregulated by biofilm formation in ID20 (Figure 5.5) as well as among proteins differentially expressed in clinical isolate ID20 relative to Tohama I (Figure 5.6). This result suggests that the differentially expressed proteins identified in this study have more interactions among themselves than expected for a random set of proteins of similar size drawn from the genome. Such enrichment indicates that the proteins are biologically connected as a group. As shown in Figure 5.5A, the interaction between in BamB (a quinoprotein that is involved in assembly and insertion of beta-barrel proteins into the outer membrane) and Hfq (RNA-binding protein or chaperone that binds small regulatory RNA

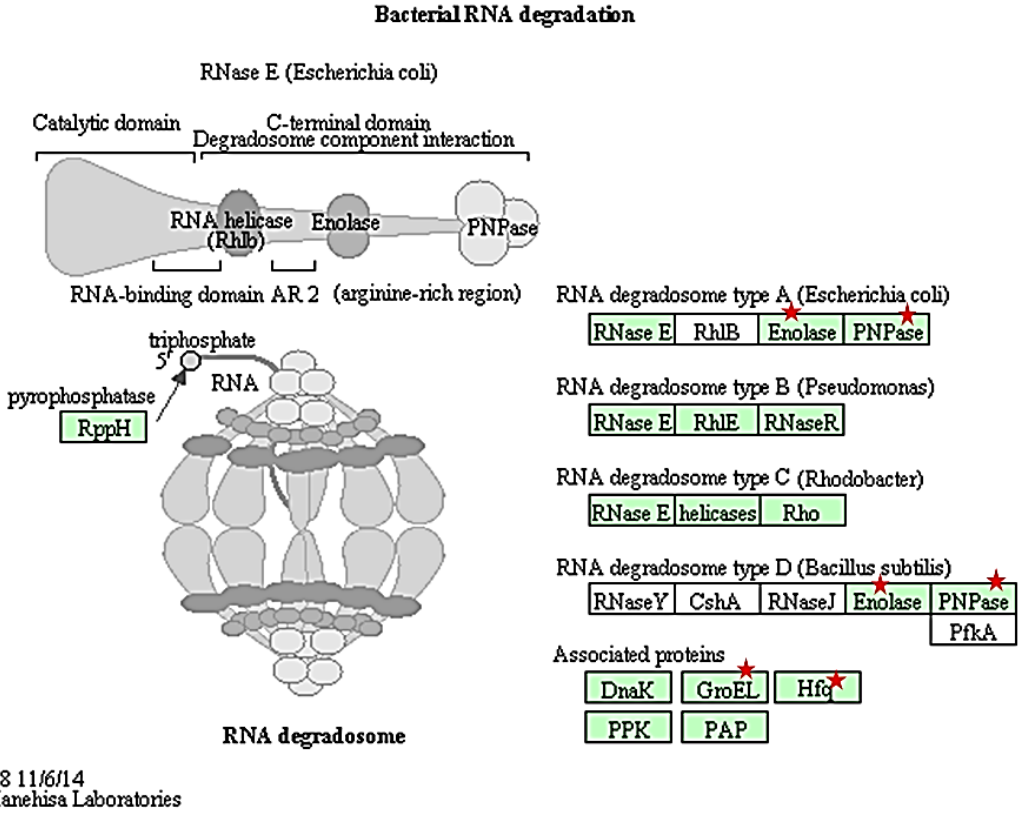


Figure 5.4 RNA degradation pathway. Locations of proteins significantly upregulated in *B. pertussis* ID20 biofilm that participate in the pathway are shown with red asterisks. http://www.genome.jp/kegg-bin/show_pathway?bpe03018 (accessed 17.2.2017).

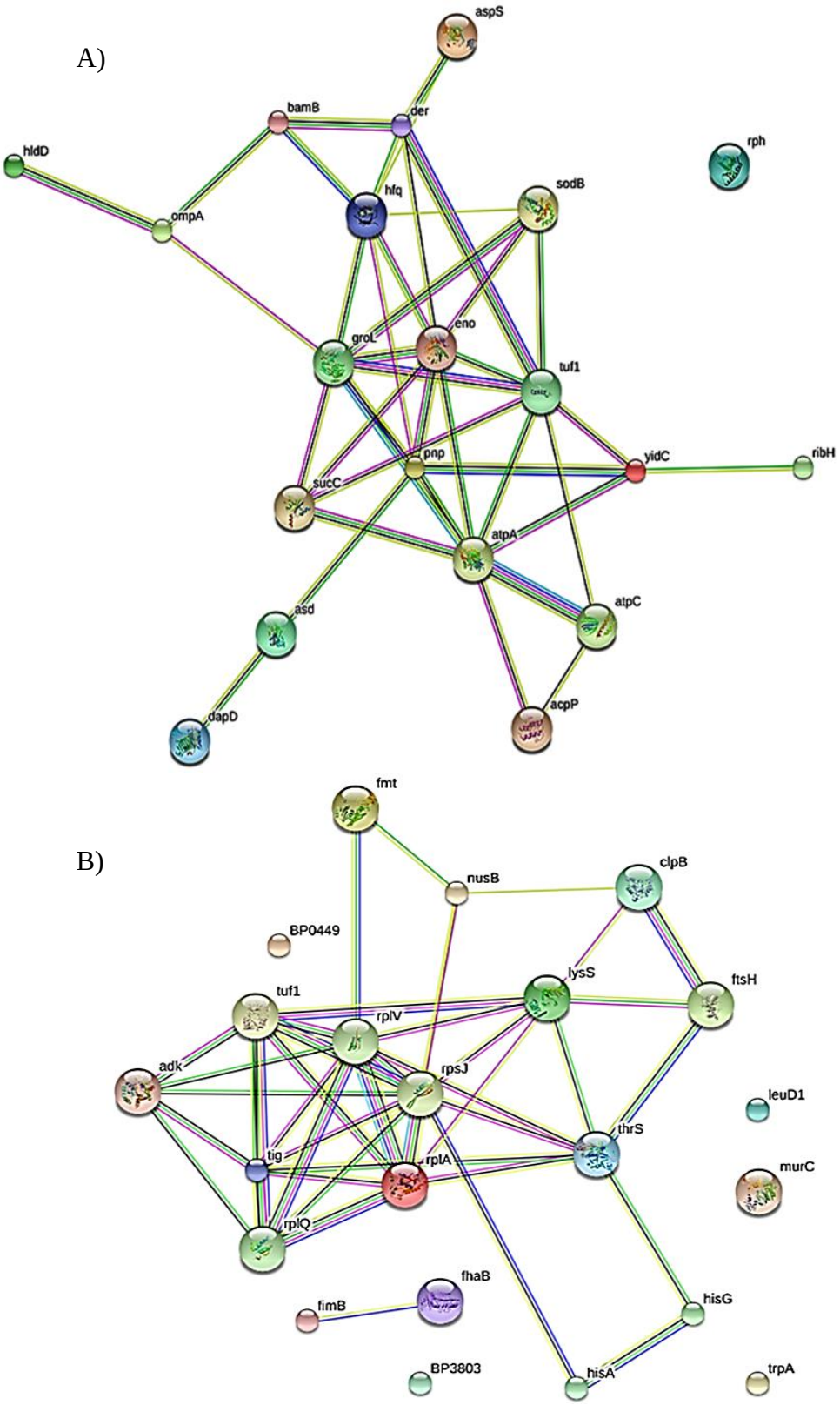


Figure 5.5 STRING analysis differentially expressed protein in biofilms. Protein-protein interaction of significantly upregulated (A) and downregulated (B) in biofilm proteins of ID20 relative to planktonic cells is shown. Detailed figure legends are presented in Figure 5.6.

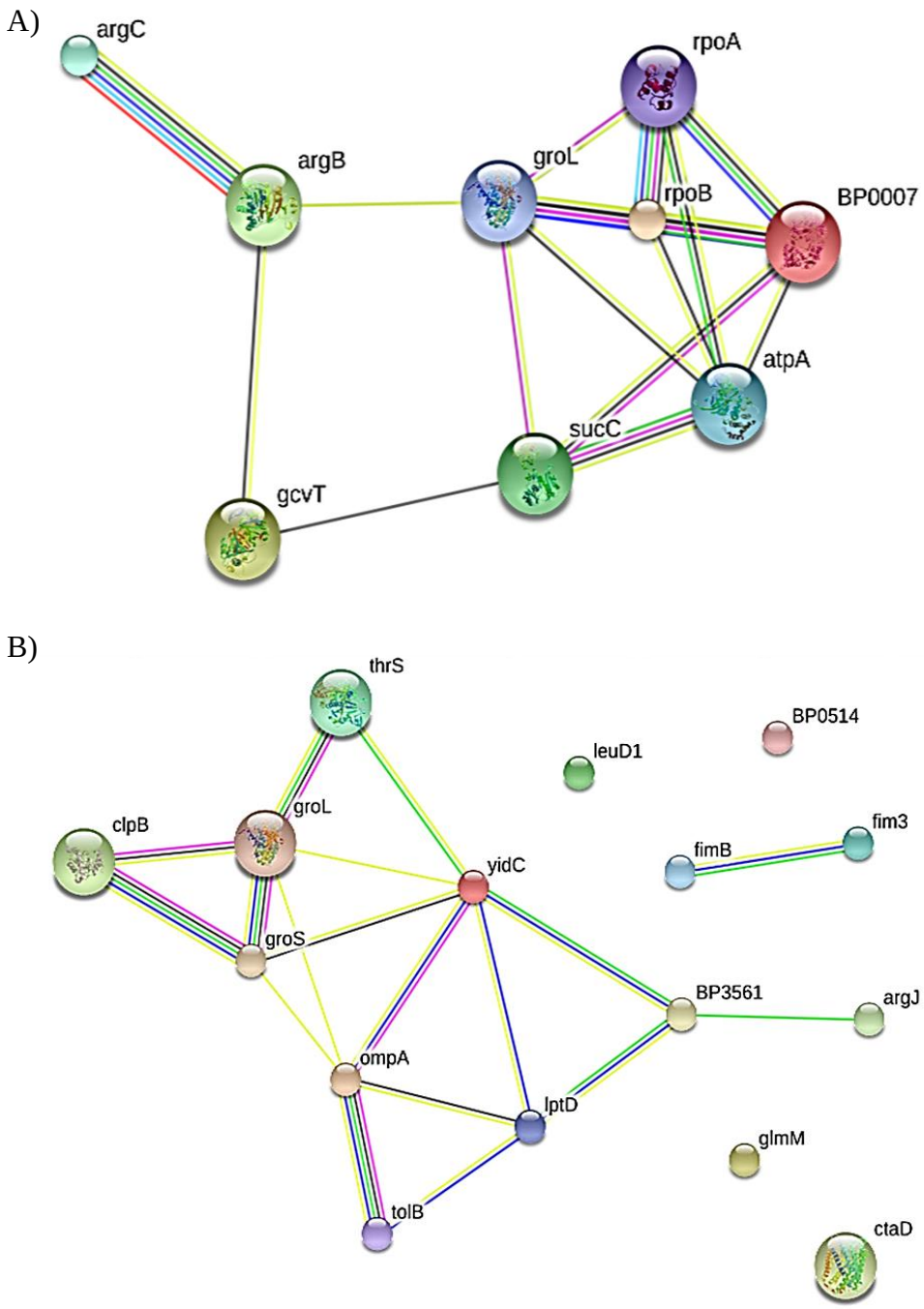


Figure 5.6 STRING analyses of differentially expressed protein in ID20 relative to Tohama I. Protein-protein interactions between upregulated (A) and downregulated (B) proteins of ID20 compared to Tohama I. Each node represents one protein and edges (connecting lines) represent protein-protein interactions. Known interactions are shown in light blue (database) or purple (experimentally determined) while predicted interactions are shown in green (gene neighbourhood), red (gene fusion), light green (text-mining) or dark blue (gene co-occurrence). Small blank nodes and large nodes represent protein with unknown and known 3D structures, respectively.

(sRNAs) and mRNAs to facilitate mRNA translational regulation in response to envelope stress, environmental stress and changes in metabolite concentrations), is evidenced by predicted gene neighbourhood (dark green line), co-occurrence (blue) and putative homologues in the text literature (light green) (Figure 5.5A).

5.4 Discussion

Proteomics has emerged as a powerful tool to study large subsets of proteins expressed in an organism. For many years, proteomic analyses were based on one-dimensional (1D) or two-dimensional (2D) gel electrophoresis based on a proteins characteristic molecular mass and isoelectric point (pI). However, these technologies are labour intensive and may not completely resolve large proteins or protein complexes into discernable subunits, while proteins expressed in low quantities may escape detection [35]. More recently, researchers have developed several gel-free proteomics techniques to overcome these challenges [36]. Here I have used both gel-based and gel-free (iTRAQ) proteomics to investigate differential expression in *B. pertussis*.

5.4.1 iTRAQ identified differentially expressed *Bordetella pertussis* proteins

The proteomic profile of a new variant of *B. pertussis*, isolated from a clinical sample and carrying the *ptxP3* allele was compared with the vaccine strain Tohama I to identify proteins that could contribute to pathogen adaptation and the fitness of the resurgent strains. The proteomic profiles of *B. pertussis* biofilms relative to planktonic cells were compared with the goal of identifying novel antigens with potential for inclusion in the formulation of effective whooping cough vaccines. A literature search indicated that this is the first study employing iTRAQ to elucidate the differential proteomic profile of a *B. pertussis* clinical isolate relative to the vaccine strain. The differential expression of ID20 proteins, either in planktonic forms or in biofilm cultures, provide insight regarding the survival strategies and enhanced fitness of the resurgent *B. pertussis* strains to persist in vaccinated populations. A previous study [37] identified 25 immunoreactive proteins using 2D-gel electrophoresis of soluble proteins derived from Tohama I and a local *B. pertussis* strain, Saadet. Interestingly, 3 proteins [60 kDa chaperonin (GroL), DNA-directed RNA polymerase subunit alpha (rpoA) and elongation factor (Tu)] and 2 proteins, [probable parvulin-type peptidyl-prolyl cis-trans isomerase (BB3803) and

10 kDa chaperonin (groS)], which were downregulated and upregulated, respectively, in this study were found to be immunoreactive in their study. In another study, Williamson et al [22] identified a total of 1363 whole cell proteins among six *B. pertussis* outbreak isolates from California, using nanoflow Liquid Chromatography-Electrospray Ionisation-Mass Spectrometry (nLC-MS/MS). They reported that 2% of proteins identified were specifically associated with the outbreak isolates.

5.4.2 aP antigens are upregulated in ID20 relative to Tohama I

Fim3 and FimB/FhaD (FimB), both components of current acellular whooping cough vaccines, were significantly upregulated in the clinical isolate. FimB (required for biogenesis of haemagglutinin and fimbriae) is an outer membrane protein important for binding of *B. pertussis* to host cells while Fim3 is an extracellular protein necessary for attachment to host cell, and both of these proteins play important roles in the initial steps of *B. pertussis* infection. This is consistent with the previously mentioned study from the USA [22] in which clinical isolates demonstrated higher expression of these proteins compared to the vaccine strain. That study reported relatively higher amount of Fim3 and FHA in the whole cell fraction of *B. pertussis* Tohama I while the culture supernatants of clinical isolates showed 3- to 6- fold higher concentrations of FHA together with sphB1, a subtilisin-like protease required for FHA maturation and release. In contrast to Fim3, expression of Fim2 trended downwards ($P<0.07$) in the clinical isolate compared to the vaccine strain. This finding suggests that the resurgent strains of *B. pertussis* may have adapted towards the expression of Fim3 in response to vaccine pressure, as seen in the UK [14], Sweden [38,39], France [40] and Russia [41].

5.4.3 Ptx production is not enhanced in ID20

Ptx is a major virulence factor and a protective antigen produced exclusively by *B. pertussis* [42,43]. It is a 117 kDa hexameric protein of AB_5 configuration consisting of one active subunit (subunit A or S1), and five binding (B) oligomers (subunit B, C and D, or S2, S3 and S4) [6,44,45]. The *ptxP3* allele has been associated with increased Ptx production [13,22]. The results from this study showed no differences in Ptx production between ID20 and Tohama I. Previous immunoproteomic [37] and surfaceomes [46] studies could not detect Ptx in their study while the study by

Willianson et al [22] demonstrated higher Ptx production in *B. pertussis* outbreak isolates. Similarly, there was no difference in the expression of Prn, a 69kDa autotransporter protein and a major component of the aP, between ID20 and Tohama I. Most resurgent strains reported deficient Prn production [15,16]. However, early studies reported enhanced expression of vaccine antigens including Ptx and Prn by the *ptxP3* lineage [18]. The relative expression of Prn in clinical isolates warrants further investigation against the backdrop of increasing Prn-deficient isolates in Australia [16].

5.4.4 Novel outer membrane proteins were upregulated in ID20

The current study identified three membrane proteins upregulated in the clinical isolate: outer membrane protein A (OmpA, $P<0.01$), LPS-assembly protein (LptD, $P<0.04$) and membrane protein insertase (YidC, $P=0.04$). OmpA was described as being "immunoreactive" using antigen-antibody affinity capture with specific mouse antisera [46]. However, immunisation of mice with recombinant OmpA did not confer protection against lethal *B. pertussis* challenge [47]. This may have been due to incorrect folding of the recombinant protein resulting in failure to induce anti-bacterial antibodies [47]. Further studies using optimally purified recombinant OmpA are required. YidC and LptD are other outer membrane proteins identified here in resurgent strains. LPS-assembly protein (LptD) is also upregulated in Tohama I biofilm relative to its planktonic cells. It is an outer membrane protein responsible for assembly of lipopolysaccharide at the surface of the bacterial membrane in *E. coli* and other Gram-negative bacteria [48]. BlastP search of LptD showed that besides *Bordetella* species, the closest match was *Achromobacter* species with 77% homology. Protein sequence alignment using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) showed only 10% similarity when aligned with clinically relevant bacterial pathogens *Escherichia coli*, *Neisseria meningitidis* and *Haemophilus influenzae*, suggesting LptD is unique to *Bordetella* species. The immunogenicity and protective efficacy of LptD was reported in detail in Chapter 6. In addition, YidC may have potential for inclusion in aP, if their immunogenicity and protective potentials can be proven. A periplasmic translocation protein B (TolB), involved in TonB-independent uptake of proteins, is also significantly upregulated in ID20. This protein has been shown to be essential for growth, pathogenicity and antimicrobial resistance of *Pseudomonas aeruginosa* [49].

5.4.5 Other novel proteins were upregulated in ID20 carrying *ptxP3* allele

Other proteins upregulated in ID20 include the heat shock proteins, 60 kDa chaperonin (groL), 10 kDa chaperonin (groS), chaperone protein (clpB) and peptidyl-prolyl isomerase (PPIase or BP3561). All these proteins were found to be immunogenic in a previous study [37]. The stress response protein, clpB, is involved in the recovery of cells from heat-induced damage and contributes to stress tolerance and virulence in *Staphylococcus aureus* [50] and pathogenic *Leptospira* species [51]. The significant upregulation of these proteins in the clinical isolate may indicate their adaptation to enhanced survival and virulence. Though there is no enrichment of any particular pathway among these upregulated proteins, the protein STRING network showed significant (P<0.01) interactions between them in ID20 (Figure 5.5). It is likely that disruption of one protein in the network may affect the interaction of all other proteins in the network.

5.4.6 iTRAQ identified proteins upregulated in *Bordetella pertussis* biofilm

The study next compared protein expression profiles of biofilm and planktonic ID20 with Tohama I. These profiles were discussed in Chapter 3. Of note, Hfq is upregulated (P<0.03) in the ID20 biofilm. Hfq is an RNA chaperone protein that binds small regulatory RNA (sRNA) and mRNA to facilitate post-translational regulation in response to environmental stress [52]. The KEGG pathway mapping implicated Hfq in quorum sensing, RNA degradation and biofilm formation in *Vibrio cholerae*. DAVID analysis confirmed that Hfq participate in the RNA degradation pathway together with enolase, PNPase and GroEL (Figure 5.4). In *Bordetella* species, Hfq is required for expression of various virulence factors including Type III secretion system (T3SS), pertussis toxin, adenylate cyclase, tracheal colonising factor (Tcf) and Vag8 [34,53]. A Δ*hfq* strain had reduced ability to infect the mouse respiratory tract compared to wild type [34]. Considering its importance in virulence and pathogenicity, Hfq represents a novel candidate for targeting *B. pertussis* biofilm associated persistence and infections in the respiratory tract.

In summary, this study identified novel proteins that are upregulated in planktonic cells of clinical isolate ID20 relative to the vaccine strain. It also identified novel proteins upregulated in the biofilm of ID20 and Tahoma 1 relative to their planktonic forms. The functions of some of these proteins suggest they may have potential in a

vaccine effective against the resurgent strains as well as against the biofilm forms of *B. pertussis*. The data from this study also confirm that gel-free based proteomics is an efficient tool for identification of differentially-expressed novel antigens and virulence factors at different stages of bacterial pathogenesis.

5.5 References

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Chapter 6

Immunogenicity and protective potential of *Bordetella pertussis* biofilm and its associated antigens in a murine model system

6.1 Introduction

An ideal whooping cough vaccine would induce both Th1 and Th17 immune cells, with concomitant induction of specific antibody responses [1]. The first-generation wP vaccines did this but were associated with adverse reactions [2] leading to acute neurological disorders in children. This led to the introduction of safer second generation aP, which induce potent IgG1 antibody responses in children, but marking a predominantly Th2-cell response. Thus, the duration of protection is short, with immunity waning after 5 years [3]. The induction of memory B cells by aP is inferior to that induced by a wP [4,5]. T-cell responses do not increase with aP boosters in aP-primed children while in wP-primed children, these responses are enhanced by aP booster or natural infections [6,7]. Suboptimal immune responses to aP vaccination may create selective pressure [8] favouring the emergence of genetically distinct variants of *B. pertussis,* as seen from results shown here in Chapter 4. *Bordetella pertussis* strains with antigenic divergence in genes encoding Prn and Ptx were initially described in 1998 [9], but they are common amongst epidemic strains in most countries. Alarmingly, this new "P3" lineage is antigenically distinct from vaccine strains and produces higher levels of Ptx [10,11]. P3 strains that do not produce Prn are now found in most developed countries [12-14] including Australia [15]. Clearly, there is an urgent need for improved whooping cough vaccines that are able to stimulate potent CMI response and confer long lasting immunity to circumvent the emerging genetic variants of this pathogen.

Results from Chapter 3 and other studies [16,17] indicate that the new variants of circulating *B. pertussis* isolates demonstrate enhanced ability to form biofilms compared to the common vaccine strain *B. pertussis* Tohama I. Biofilm formation may enhance the survival and continued persistence of *B. pertussis* in human nasopharynx, facilitating transmission to susceptible infant hosts [18,19]. Thus far, all pertussis vaccines have been formulated with antigens derived from planktonic bacterial cells. *Bordetella* intermediate protein A (BipA) was the only *B. pertussis* biofilm-derived membrane protein identified that protected mice against virulent *B. pertussis* infection [20].

Chemokines may play a role in protective immunity induced by vaccination. For example; Ptx can delay early neutrophil recruitment in lungs by inhibiting the

production of CXCL1 (KC), CXCL5 (LIX) and CXCL2 (MIP1) chemokines [21]. Studies suggest that Ptx and ACT modulate the neutrophil chemotaxis and affect their function [21,22]. A study reported no role for neutrophils in initial infection of naïve mice but a significant role in previously immunised mice was observed [23]. Chemokines may also affect adaptive immune responses. Expression of CXCL13 (B-cell chemoattractant) was detected after only 7 days in unprotected mice while it was detected before 2 days in protected mice [24]. T-cell activation was also accelerated in immune mice.

In this study, the biofilm lifestyle of this versatile pathogen was explored to identify potential whooping cough vaccine antigens. The proteomic profiles of *B. pertussis* biofilms relative to planktonic cells were presented in Chapters 3 and 5. Novel *B. pertussis* biofilm-associated surface antigens were identified. Here, mice will be vaccinated with *B. pertussis* biofilm and concentrations of IFN-γ and IL-17a cytokines and IgG1 and IgG2a antibodies compared to vaccination with a) planktonic cells, b) currently used aP (Infanrix® hexa; GlaxoSmithKline, Rixensart, Belgium) or DTaP and c) control mice. Furthermore, the protective potential of biofilm vaccination will be compared to other vaccine candidates. Having confirmed the protective potential of biofilm, the immunogenicity and protective potential of two selected biofilm upregulated proteins [outer membrane protein assembly factor (BamB) and lipopolysaccharide assembly protein (LptD)], identified in Chapter 5 will be explored. Finally, the ability of these two proteins to protect mice against infection with a new variant of *B. pertussis* of recent lineage and the probable chemokine signature associated with vaccination will be investigated.

6.2 Materials and methods

6.2.1 Animal ethics statement

In accordance with the Australian Animal Welfare Act 2002, all animal experiments were approved by the Curtin University's Animal Ethics Committee according to approval No. AEC_2015_39.

6.2.2 Bacterial strains and growth conditions

All *B. pertussis* strains were grown on Bordet-Gengou (BG) agar (Becton Dickenson) supplemented with 15% sheep blood or on Charcoal agar (Thermo

Scientific). Broth cultures were carried out in SS-broth containing 0.2% cyclodextrin (Sigma). The common vaccine strain, *B. pertussis* Tohama I, was used in the initial challenge experiment. In the second challenge experiment, a clinical *B. pertussis* isolate ID20, the strongest biofilm producer in this study (Chapter 3), was used to challenge mice vaccinated with *B. pertussis* Tohama I biofilm or planktonic cells. This strain also represents currently circulating isolates with a *ptxP3* allele and Prn variant (Chapter 4).

Escherichia coli strain DH5α (New England Biolabs) was transformed with recombinant plasmids. *Escherichia coli* BL21-DE3, electro-competent *E. coli* and pETM-11 vector for expression of recombinant proteins were obtained from Dr. Joshua Ramsay, School of Biomedical Sciences, Curtin University. All *E. coli* strains were grown on Luria Bertani (LB) agar containing 1% tryptone, 0.5% yeast extract (Oxoid), 1% NaCl (Biochemicals) and 1.5% bacteriological agar or LB broth containing 1% tryptone, 0.5% yeast extract (Oxoid) and 1% NaCl with 50 μg/mL kanamycin (Biochemicals) and 100 μg/mL chloramphenicol (Sigma), where appropriate.

6.2.3 Design and cloning of recombinant BamB (rBamB) and LptD (rLptD)

The DNA sequences of BamB and LptD excluding the N-terminal signal sequence was amplified using primers specific for BamB and LptD. The primers were designed with 15-22 nucleotide overlaps (Figure 6.1) of the vector sequence at the 5' end of the gene-specific primer sequence using NEBuilder® Assembly tool v1.12.15 (New England Biolabs). Fragments with high predictive immunogenicity scores and outer membrane localisation were chosen for amplification. PCR amplification was carried out using Q5® Hi-Fidelity DNA polymerase (New England Biolabs). The amplified PCR products were sliced from gels and purified using a gel/PCR purification kit (Bioline) as per the manufacturer's instructions. The purified PCR product was cloned between the BamHI and NcoI sites of pETM-11 vector using NEBuilder® HiFi DNA Assembly cloning kit (New England Biolabs), to generate N-terminal His_6-tag proteins, rBamB and rLptD, respectively. Briefly, 1:3 molar ratios of vector:insert were mixed with 10 μl of NEBuilder® HiFi DNA mastermix and incubated in a thermocycler for 20 min at 56°C. Following assembly, 2 μl of the

assembled product was transformed into *E. coli* competent cells (Section 2.5, General methods). The clones were verified by colony PCR, DNA restriction analyses, DNA sequencing of the purified insert containing plasmids. Recombinant proteins were separated using 1D-PAGE and the identification of the proteins confirmed by MS analyses at Proteomics International, Perth, Western Australia.

6.2.4 Expression and purification of rBamB and rLptD

Expression of recombinant proteins was induced in *E. coli* BL21-DE3 cells cultured at 18°C overnight with 0.4mM isopropyl- β-D-1-thiogalactopyranoside (IPTG; Sigma) with shaking (200 rpm). Induced cells were harvested by centrifugation (8000 g, 30 min, 4°C). Pellets were resuspended in wash buffer containing 20 mM sodium phosphate (Sigma), 500 mM NaCl (Biochemical) and 20 mM imidazole with 1 mM PMSF (Sigma). The cells were disrupted using a high pressure cell disruptor at 30kPa. Cell lysates were centrifuged at 30,000 g, 30 min, 4°C and the supernatant was used for protein purification using Ni-NTA sepharose® (Qiagen, Nutley, NJ). His_6-tagged rBamB and rLpTD were further purified using size exclusion chromatography Sepharose, 200 on an AKTApurified™ 10 FPLC system (GE Healthcare, Uppsala, Sweden). The presence of protein was determined by visualisation at A_{280} and fractions containing recombinant proteins were pooled. Protein concentrations were determined using a Nanodrop™ 1000 Spectrophotometer (Thermo Scientific). Fractions of interest were stored at -80°C for further use. Protein purity was determined by 1D-PAGE using a gradient 4-12% novex® NuPAGE® Bis-Tris Protein gels (Invitrogen, Carlsbad, CA), stained using SimplyBlue™ SafeStain (Invitrogen), according to manufacturer's instructions. Gels were imaged using Chemidoc (Biorad) and bands were excised and subjected to MS to confirm the identity of proteins.

6.2.5 Preparation of vaccines and antigens

Planktonic and biofilm cells were harvested as detailed in Chapter 3 and the A_{600} was adjusted to 0.1. The planktonic and biofilms suspensions were inactivated at 56°C for 1 h (Section 2.4). All vaccines and antigens were prepared in one batch to ensure consistency, and stored at -80°C in aliquots for further use.

6.2.6 Animal experiments

Animal experiments in this study comprised four phases with specific objectives as described below. Phase I was carried out to optimise sampling times and analytical procedures. Eighteen naïve, 8-10 week old BALB/c mice (Animal Research Centre, Murdoch University, Western Australia) were divided into two groups and immunised subcutaneously on days 0 and 14 with 10^7 and 10^9 CFU/mL, respectively, of inactivated *B. pertussis* planktonic or biofilm vaccines in 0.1 mL volume [consisting of 0.05 mL of vaccine antigent mixed 1:1 in alum adjuvant or Imject (Thermo Scientific)]. On days 9, 19 and 24, 3 mice from each group were sacrificed and sampled for blood, spleen and lymph nodes (LN). Splenocytes and LN cell preparations were stimulated with *Staphylococcal* enterotoxin B [(SEB); Sigma)], biofilm, planktonic cells, DTaP, rBamB or rLptD.

Phase II investigated the immunogenicity of the *B. pertussis* vaccine candidates. Three groups of 6 mice each were immunised similarly with inactivated planktonic cells, biofilm or Imject. Another group was immunised with $1/50^{th}$ of the human dose of the commercially available hexavalent acellular vaccine Infanrix® hexa (GlaxoSmithKline) or DTaP, as previously described [20]. On days 24 and 35, three mice from each group were sampled for blood, spleen and LN for quantitation of antigen-specific IgG1 and IgG2a antibodies in serum, and IFN-γ and IL-17a production by stimulated splenocytes and LN cells, as described in Section 6.2.9 below.

In Phase III, the protective potential of *B. pertussis* vaccine candidates against challenge with *B. pertussis* Tohama I was determined. Four groups of 7 mice each were immunised with planktonic, biofilm, DTaP or Imject within two week interval. On day 24, mice were lightly sedated with Isoflurane and challenged with virulent *B. pertussis* Tohama I by carefully instilling their nostrils with 40 µl of bacterial suspension containing $2x10^7$ CFU [20]. The mice were weighed daily and observed twice daily for morbidity and mortality. Seven days after the intranasal challenge, lungs were harvested, collected in 2 mL of sterile SS broth Ten-fold serial dilutions of lung homogenates were inoculated onto charcoal agar plates and incubated at 37°C, following which bacterial loads were determined [20].

Following results from phase III, phase IV determined the protective potential of *B. pertussis* Tohama I- and biofilm-derived vaccine candidates against the resurgent strains of *B. pertussis* carrying *ptxP3* allele. Seven groups of 7 mice each were immunised subcutaneously with planktonic or biofilm-derived *B. pertussis* Tohama I, 1/250th dose of DTaP (calculated based on ratio of average weight (5.5 kg) of 3 months old child and 9 weeks old mice (0.022 kg) or Imject within a two-week interval as described above. Another 3 groups of 7 mice were immunised subcutaneously with 2 μg rBamB, rLptD or combined rBamB-rLptD-DTaP within a 2 week interval. On day 24, mice were challenged with a clinical isolate, *B. pertussis* ID20. The mice were weighed daily and observed twice daily for morbidity. Seven days after the intranasal challenge, lungs were harvested and bacterial load was determined. Blood was also collected post-challenge and serum antibody levels and chemokine levels were determined as described in Section 6.2.9 below.

6.2.7 Clinical observation and monitoring of vaccinated mice

All mice were monitored following vaccination and challenge. The clinical features noted include coat quality (smooth/ruffled), activity, body weight, swelling or infection of injection site and signs of dehydration. These were scored as 0 - no adverse changes, + low grade adverse changes, ++ medium grade adverse changes and +++ severe adverse changes.

6.2.8 Whole cell ELISA for detection of antigen-specific IgG1 and IgG2a in mouse serum

IgG1 and IgG2a reactive with whole bacterial cells were assessed using an enzyme-linked immunosorbent assay (ELISA) method adapted from Raymonds et al [25]. Briefly, 96-well half-volume plates were coated with A_{600}=0.1 heat-inactivated *B. pertussis* suspended in phosphate-carbonate buffer overnight at room temperature. The plates were washed with PBS containing 0.05% Tween-20 (BDH Prolabo, Fontenay-sous-Bois, France) and blocked with 5% bovine serum albumin (BSA; Bovogen Biologicals, Keilor, Australia), before incubation with 4 serial dilutions (3-fold dilutions starting at 1:1000 for IgG1 and 1:4 for IgG2a) of mouse sera for 2 h at room temperature, followed by horseradish peroxidase (HRP)-conjugated Goat Anti-Mouse IgG1 (1:10,000 dilution) or HRP-conjugated Goat Anti-Mouse IgG2a (1:10,000 dilution; Abcam, Melbourne, Australia) for 1 h at room temperature. After

washing, 3,3',5,5'–tetramethylbenzidine (TMB; Sigma) added with 1 µL concentrated H_2O_2 (Sigma) was added to each well and incubated at room temperature for 15 min or 30 min for IgG1 and IgG2a, respectively. The reactions were stopped by adding 25 µL 1M H_2SO_4 (Sigma) to each well. The absorbance was measured at 450 nm with an Envision multilabel reader (PerkinElmer, Turku, Finland). Standards and quality control (QC) samples were prepared by pooling samples known to contain high concentrations of antibodies. Seven 3-fold dilutions of standard and four 3-fold dilution of QC were run on each plate. Intra-assay variation was considered acceptable if within-run standard deviation (SD) of QC samples were ≤8%. Antibody concentrations were determined from standard curves calculated using least-squares curve-fitting. IgG1 and IgG2a levels were reported as arbitrary units per (AU/mL).

6.2.9 DTaP-antigen ELISA

To assess IgG1 and IgG2a reactive with DTaP antigens, plates were coated with 5 µg/mL Infanrix® hexa (GlaxoSmithKline) or DTaP vaccine and incubated overnight at 4°C. The plates were washed, blocked and reacted as described in Section 6.2.8. Antibody isotype concentrations were determined using standard curves and reported as arbitrary units per mL (AU/mL), as described in Section 6.2.8.

6.2.10 Quantification of IFN-γ and IL-17a in antigen stimulated splenocytes and lymph nodes.

Production of IFN-γ and IL-17a was assessed using Mouse IFN-γ and IL-17a ELISPOT Ready-SET-GO!® kits (eBioscience, San Diego, CA), according to manufacturer's instructions. Briefly, 96-well ELISPOT Plates (Millipore, Billerica, MA) were coated with anti-mouse capture antibodies overnight at 4°C, washed and blocked with RPMI-1640/10% fetal calf serum (FCS) for 1 h. After washing, 100 µL/well of antigens were added. These comprised $1x10^5$ cells of planktonic cells or biofilm, 5 µg/mL of rBamB, rLptD or DTaP or 1 µg/mL of SEB in RPMI-1640/10% FCS. These were followed by 100 µL of medium containing $2.5x10^5$ spleen or LN cells prepared as described in Section 2.3. The cells were incubated at 37°C in a 5% CO_2/95% air humidified incubator for 24 or 48 h for IFN-γ and IL-17a, respectively. The plates were washed and incubated with biotinylated anti-mouse detection

antibody for 2 h at room temperature. Plates were washed again, incubated with Avidin-HRP at room temperature for 45 min and re-washed. One hundred microliters of freshly prepared 3-amino-9-ethyl carbazole (AEC) substrate solution (Becton Dickenson) was added and allowed to develop at room temperature for 30 min. The reaction was stopped by washing with distilled water. Spots were counted using an automated ELISPOT plate reader (Autoimmun Diagnostica, Strassberg, Germany) and data analysed using EliSpot2.9 software (Autoimmun Diagnostica). Images were edited manually to check the spot morphology and results are reported as spot-forming units (SFU) per $2x10^5$ cells.

6.2.11 Determination of chemokines

Concentrations of chemokines in mouse serum were determined using a LEGENDPLEX™ Mouse Proinflammatory Chemokine Panel (Biolegend, San Diego, CA) according to the manufacturer's instructions. Mouse sera were diluted 4-fold and 40 µL aliquots were added to 20 µL of mixed beads in wells of a V-bottom plate. After 2 h with shaking at 200 rpm, the plate was centrifuged at 250 g for 5 min using a swing bucket rotor with a microplate adaptor. The supernatant was carefully removed, the wells were washed and 20 µL mouse proinflammatory chemokine detection antibody was added to each well. After 1 h shaking at room temperature, 20 µL streptavidin-phycoerythrin (SA-PE) was added to each well. After a further 30 min, the plate was centrifuged, supernatants were discarded, beads were resuspended in 100 µL wash buffer and analysed in a BD FACS LSRFortessa™ (Becton Dickenson) flow cytometer at Curtin Health Innovation and Research Institute (CHIRI) Facility, Curtin University supervised by Dr. Jeanne Le Masurier. The excitation was achieved using red (640nm) and blue (488nm) lasers with emission of 670/14 and 575/26 bandpass. The data was acquired using BD FACSDiva™ Software v8.0.1 (Beckton Dickenson) and analysed by FlowJo™ v10.1r7 (FlowJo, Ashland, OR). The concentrations of chemokines were interpolated from the standard curve and reported as pg/mL.

6.2.12 Statistical analyses

Two-tailed Mann-Whitney *U* tests were used to compare bacterial loads in the lungs of mice in each vaccinated group (n=7). Unpaired Student's t-tests were used to compare immunological data (n=3). All statistical analyses were performed using

GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA). $P<0.05$ was considered statistically significant.

6.3 Results

6.3.1 Vaccine candidate selection

BamB and LptD were selected for evaluation as novel whooping cough vaccine antigens. BamB was significantly ($P<0.05$) upregulated in the biofilm of the clinical isolate ID20, while LptD was upregulated ($P<0.05$) in the biofilm of Tohama I as well in the clinical isolate ID20 relative to planktonic cells of Tohama I. These proteins represent antigens specifically expressed in biofilms and PSORTb prediction suggested localisation in the cell outer membrane. Detailed proteomic characterisation of *B. pertussis* biofilms was reported in Chapter 3 or [16] and in Chapter 5.

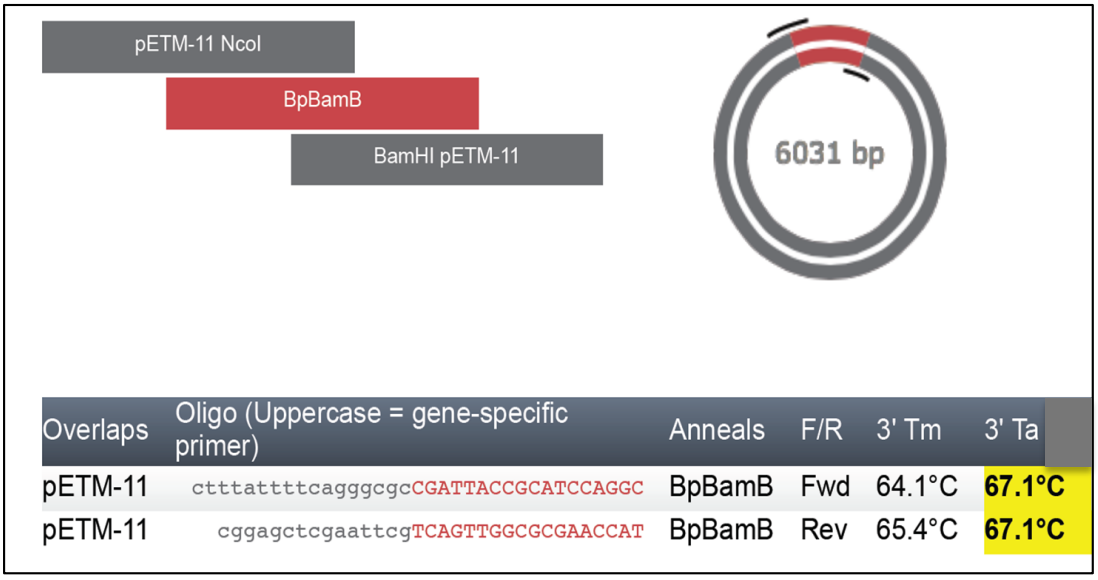
6.3.2 Production of recombinant rBamB and rLptD

PCR products of 685 bp and 2166 bp for BamB and LptD, respectively, were obtained using primers shown in Figure 6.1. The PCR products were cloned into the BamHI and NcoI sites of the pETM-11 to obtain 6031 bp (pETM11-BamB) and 7531 bp (pETM11-LptD) products, as vector plus insert (Figure 6.2). *Escherichia coli* DH5α were transformed to prepare stocks of the plasmid constructs (Section 2.9). For protein expression, *E. coli* DE3-BL21 expression hosts were transformed with purified plasmids. All plasmids were verified by DNA restriction, colony PCR and DNA sequencing (Figures 6.3 and 6.4). Digestion of pETM11-BamB with SphI resulted in ~400 bp and ~5.6 kb fragments (Figure 6.3A) while XbaI and BsaI digestion of pETM11-LptD obtained a ~1.5 and 6 kb fragments (Figure 6.4A). PCR using pETM-11 primers (flanking the cloning sites) obtained expected 1086 and 2568 bp bands for BamB and LptD, respectively. The identity of purified recombinant proteins was confirmed using mass spectrometry (MS) (Figure 6.3C and 6.4C).

6.3.3 Optimisation of vaccine schedules (see Section 6.2.6)

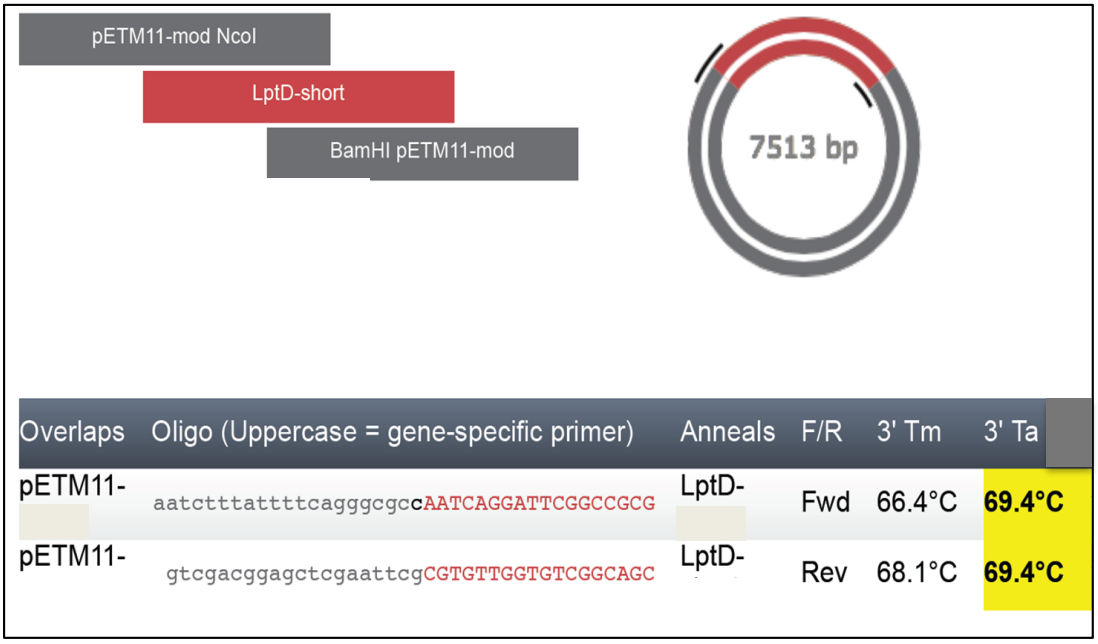
Animal experiments carried out in this study are outlined in Table 6.1. The optimisation trial showed that the levels of IgG1 reactive with bacterial whole cell

A)



Overlaps	Oligo (Uppercase = gene-specific primer)	Anneals	F/R	3' Tm	3' Ta
pETM-11	ctttattttcagggcgcCGATTACCGCATCCAGGC	BpBamB	Fwd	64.1°C	67.1°C
pETM-11	cggagctcgaattcgTCAGTTGGCGCGAACCAT	BpBamB	Rev	65.4°C	67.1°C

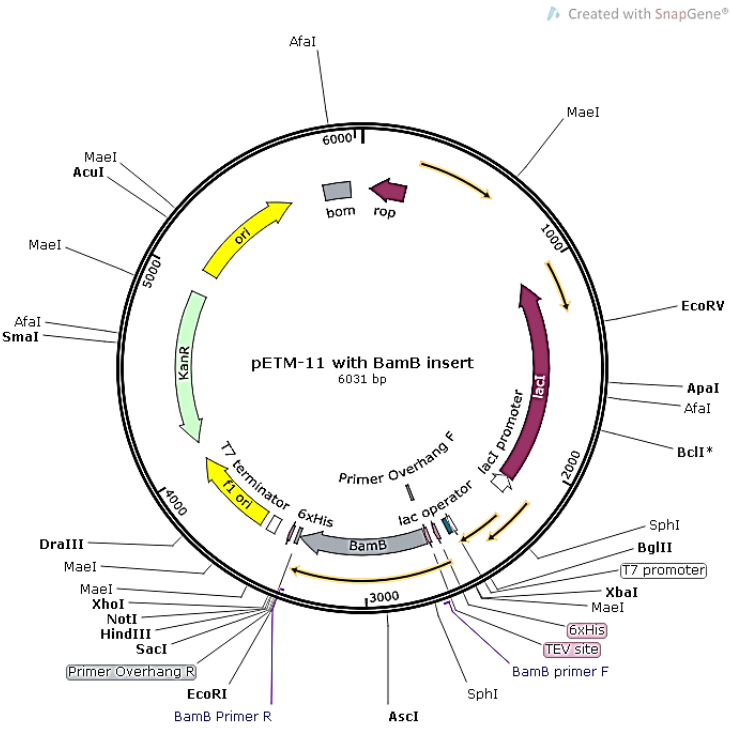
B)



Overlaps	Oligo (Uppercase = gene-specific primer)	Anneals	F/R	3' Tm	3' Ta
pETM11-	aatctttattttcagggcgccAATCAGGATTCGGCCGCG	LptD-	Fwd	66.4°C	69.4°C
pETM11-	gtcgacggagctcgaattcgCGTGTTGGTGTCGGCAGC	LptD-	Rev	68.1°C	69.4°C

Figure 6.1 Primer design scheme for rBamB and rLptD using NEB Hi-Fi DNA Assembly system™. The nucleotide sequences in lower case represent the vector sequence overhang while gene specific primer sequences are shown in uppercase red. The annealing temperature (Ta) used for amplification is highlighted in yellow. The total size of plasmid after insertion of the gene of interest is shown.

A)



B)

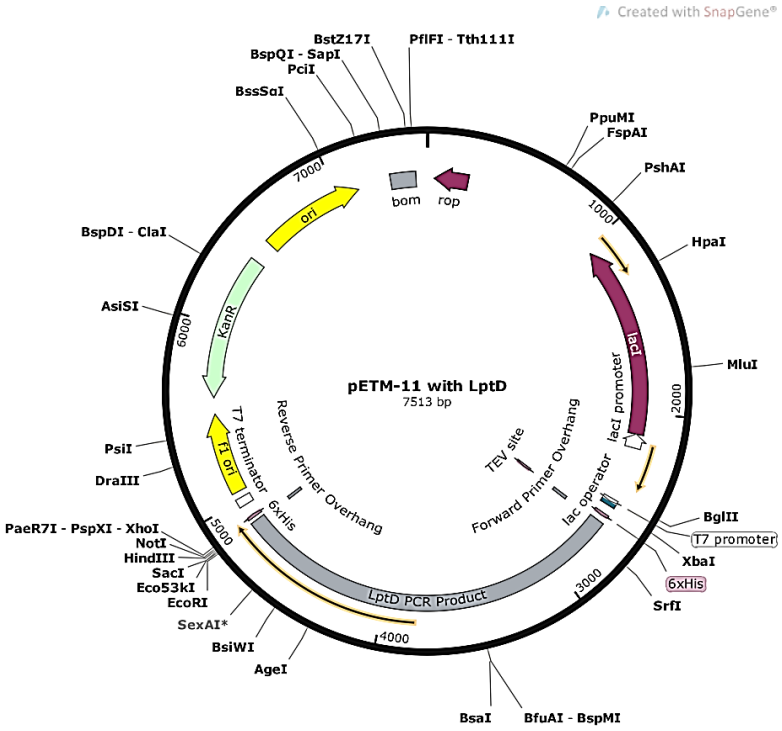
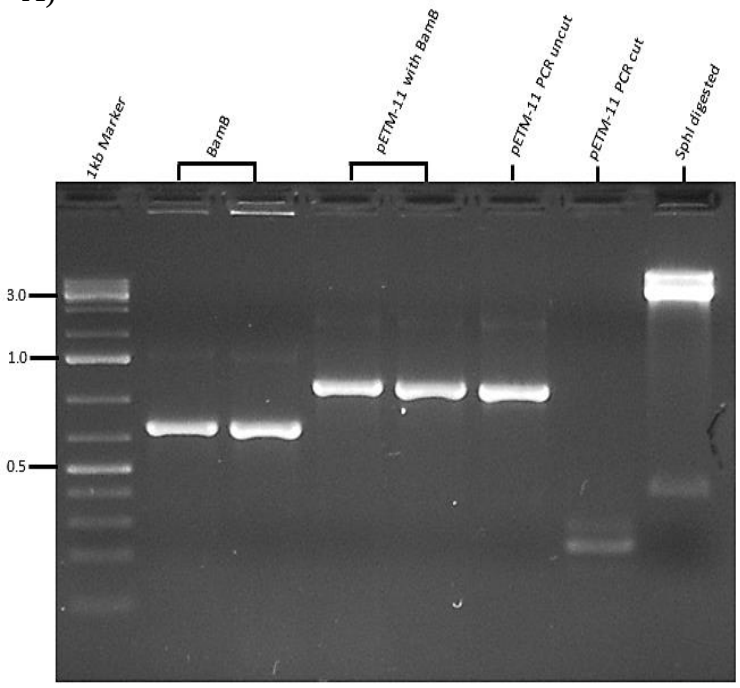
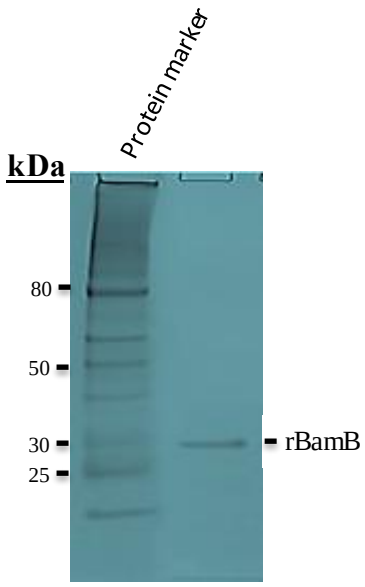


Figure 6.2 Plasmid map of *bamB* and *lptD* genes cloned into the pETM-11 vector. A 685 bp and 2166 bp fragments of *bamB* (A) and *lptD* (B) were cloned into the pETM-11 vector. The orientation of the gene with respect to the N-terminal His_6-tag and TEV-site is shown. The plasmid map is generated using SnapGene Viewer™ version 3.3.4. Origin of replication, kanamycin resistance gene (*kanR*) and *lacI* gene is shown in yellow, light green and purple, respectively.

A)



B)



C)

MASCOT Search Results

User :
E-mail :
MS data file : C:\Documents and Settings\Proteomics\Desktop\3945\160715 3945A.mgf
Database : MSPnr100 (75,925,788 sequences; 27,045,014,025 residues)
Taxonomy : Bacteria (Eubacteria) (51,492,816 sequences)
Timestamp : 20 Jul 2016 at 09:19:47 GMT

Not what you expected? Try the peptide summary.

▸Search parameters
▸Score distribution
▸Legend

Protein Family Summary

Significance threshold p< 0.05 Max. number of families AUTO
Ions score or expect cut-off 0 Dendrograms cut at 0
Preferred taxonomy All entries

A0A058YGG7 101 tr|A0A058YGG7|bamB A0A058YGG7_BORBO Outer membrane protein assembly factor BamB n=1 Tax_Id=1331202...

	Score	Mass	Matches	Sequences	emPAI
A0A058YGG7	101	39935	18 (3)	4 (2)	0.17

tr|A0A058YGG7|bamB A0A058YGG7_BORBO Outer membrane protein assembly factor BamB n=1 Tax_Id=1331202 [Bordetella bronchiseptica 99-R-0433]

▾11 samesets of A0A058YGG7

A0A0E1QT54	101	40066	18 (3)	4 (2)	

tr|A0A0E1QT54|bamB A0A0E1QT54_BORBO Outer membrane protein assembly factor BamB n=3 Tax_Id=1208657 [Bordetella bronchiseptica 1289]

A0A0E7UU36	101	39935	18 (3)	4 (2)	

tr|A0A0E7UU36|yfgL A0A0E7UU36_BORPT Quinoprotein n=2 Tax_Id=520 [Bordetella pertussis]

A0A0E8DJ16	101	39935	18 (3)	4 (2)	

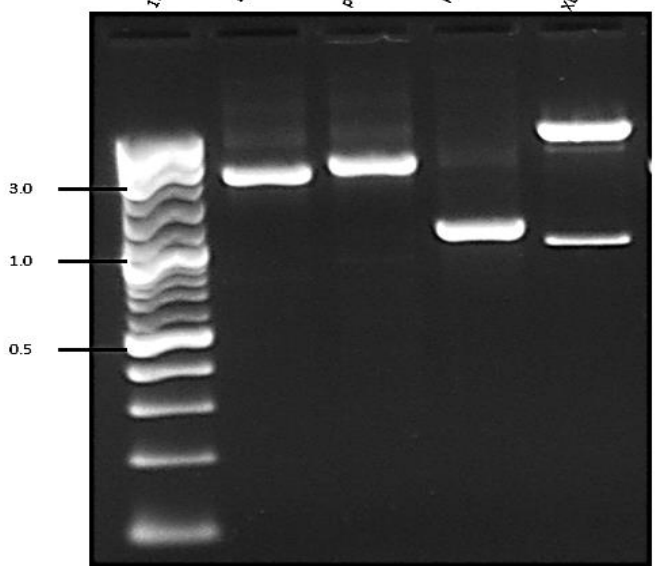
tr|A0A0E8DJ16|yfgL A0A0E8DJ16_BORPT Quinoprotein n=1 Tax_Id=520 [Bordetella pertussis]

Q7VWL3	101	40066	18 (3)	4 (2)	

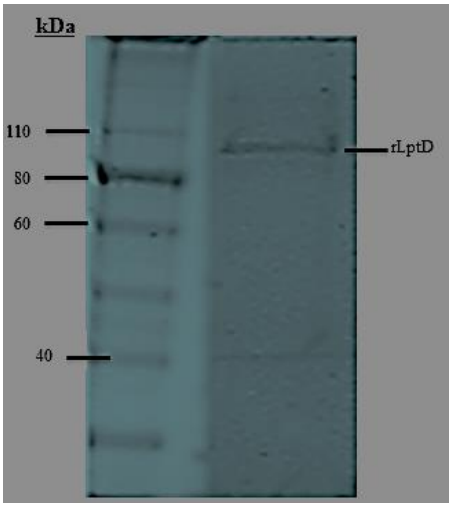
sp|Q7VWL3|bamB BAMB_BORPE Outer membrane protein assembly factor BamB n=4 Tax_Id=257313 [Bordetella pertussis (strain Tohama I / ATCC BAA-589 / NCTC ...

Figure 6.3 Cloning and production of rBamB. The *bamB* gene was cloned into the pETM-11 vector and confirmed by PCR using *bamB* or pETM-11 primers in DE3-BL21 *E. coli* cells (A). AKTA-purified rBamB separated on SDS-gel (B) and MS identification of rBamB performed using MSPnr100 database search (C).

A)



B)



C)

MASCOT Search Results

Protein View: Q7VU13

sp|Q7VU13|lptD LPTD_BORPE LPS-assembly protein LptD n=6 Tax_Id=257313 [Bordetella pertussis (strain Tohama I / ATCC BAA-589 / NCTC 13251)]

Database:	MSPnr100
Score:	1538
Nominal mass (M_r):	88533
Calculated pI:	8.32
Taxonomy:	**Unknown species**

Sequence similarity is available as **an NCBI BLAST search of Q7VU13 against nr**.

Search parameters

MS data file:	C:\Documents and Settings\Proteomics\Desktop\3975B-20160927T020048Z\3975B\160923 3975B.mgf
Enzyme:	Trypsin: cuts C-term side of KR unless next residue is P.
Variable modifications:	**Oxidation (M)**

Protein sequence coverage: 40%

Matched peptides shown in ***bold red***.

```
  1 MRMLRWLILS AFSVAGAVQA QGNQDSAAAS APSASIGAPV LRTSPGLRVH
 51 RLPDEKIPAF MEADQISGDP DSEVTLTGNA QVRRVDGIIK GDRINYRRDT
101 GDVDVQGSAR MLRDGTLITG PSARLNVDTY SGEIQEPNFW IGASGGTAQA
151 RHADIFSKSQ MRLSQVTYSG CPCPKPSWYI KADTVDLDFD ENEGVARNGV
201 LYFKDVPILA SPYLTFPVKK ERKSGFLMPT YGTTSNSGFD ISLPYYFNLA
251 PNYDLTLVPR YLSKRGAQLG GEFRYLGSGY RGVAIGTYLP DDNETGRDRW
301 MYRTYHRQLL GNGFYTDWDI AGASDDNYFR DISELGLNTA STTYLPRRGR
351 VGWSSTYWQT YAQVYKYQTL QDPDAPLAPP YDKVPELWLK GARYDWGGFD
401 AEWVSTAVRF QRSLLNGRRL GPDGDRLQTY PTVSYPIGRP GWFLVPKVGV
451 HYTQYRTDWY NRDWNRIGLS NYKRTESRTV PIMSLDAGMI FERDASLFGK
501 AATQTLEPRL YYLRVPYRDQ SALPVYDTTL ADFSFDQAFQ ENIYTGGWDR
551 IANANQLTAA LTTRWLDANT GFERLSLSAA QRIYFQDQEV TLPAEQPRKN
601 VRSDFLVGAT AALTDTLITD VAAQYNPYDN KWSRGMVSAR WSPQRLTTVA
651 VAYRYQRDPL PGISYQPQGQ NQVSLAVQWP IHRRWYGVGR VDYSLRSEPA
701 TAAAAEQSPR VTQAIAGLEY KGDCCWVGRV VYQRYAVSAA DTNIALFFQL
751 ELTGLGALGT DPISLLNRSI PGYQSVVPPT PTGTTFERYE
```

Figure 6.4 Cloning and production of rLptD. The *lptD* gene was cloned into the pETM-11 vector and confirmed by PCR using *lptD* or pETM-11 specific PCR in DE3-BL21 *E. coli* cells (A). AKTA-purified rLptD separated on SDS-gel (B) and MS identification of rLptD performed using MSPnr100 database search (C).

Table 6.1 Experimental design for determination of immunogenicity and protective potential of *B. pertussis* biofilm and associated vaccines (n=119)

Phase	Experiment	Vaccination Group	Mice in each group
I	Optimisation	Biofilms	9
		Planktonic bacteria	9
II	Immunogenicity trial	Biofilms	6
		Planktonic bacteria	6
		DTaP	6
		Imject	6
III	Protective potential against Tohama I	Biofilms	7
		Planktonic bacteria	7
		DTaP	7
		Imject	7
IV	Protective potential against clinical isolate ID20	Biofilms	7
		Planktonic bacteria	7
		DTaP	7
		Imject	7
		rBamB	7
		rLptD	7
		rBamB-rLptD-DTaP	7
Total mice used in the study			**119**

antigen increased from day 9 to day 19 and day 24, whilst levels of IgG2a were low and variable, peaking at day 19. This study did not obtain satisfactory stimulation of spleen and LN cells on day 9 or 19, but IFN-γ and IL-17a were measurable on day 24. Hence, day 24 post-vaccination was used to compare responses to our novel vaccines. Stimulation of splenocytes and LN cells with heat-inactivated biofilms and planktonic cells (Section 2.4) achieved IFN-γ and IL-17a responses similar to SEB, so SEB was omitted as a positive control in further studies.

6.3.4 Immunogenicity of *Bordetella pertussis* biofilm and associated proteins

6.3.4.1 Bordetella pertussis biofilm and associated antigens stimulate potent IFN-γ responses in splenocytes of vaccinated mice

In Phase II (Table 6.1), groups of 6 mice were vaccinated with inactivated *B. pertussis* planktonic bacteria, biofilm or DTaP on days 0 and 14. Control mice were vaccinated with Imject as described in Section 6.2.6 and responses to vaccines were considered positive when significantly greater than in these mice. Spleen and LN cells were harvested from 3 mice from each group on day 24 and day 35. LN from each group were pooled to obtain adequate cells. Splenocytes and LN cell preparations were stimulated with planktonic cells, biofilm, DTaP, rBamB or rLptD antigens (see Section 6.2.6) and cytokine profiles were determined using ELISPOT assays (see Section 6.2.10). Spots for unstimulated controls were subtracted and values less than unstimulated controls were considered as zero for analysis.

IFN-γ responses were evident in cultures of splenocytes stimulated with planktonic cells or biofilm bacteria (Figure 6.5). These responses subsided but remained strong on day 35. When cultures were stimulated with *B. pertussis* planktonic cells, splenocytes of mice vaccinated with biofilm produced higher (P<0.001) IFN-γ responses (median SFU 394 per $2x10^5$cells) than mice vaccinated with planktonic bacteria, DTaP or Imject [median SFU 337, 197 or 167 per $2x10^5$cells, respectively (Figure 6.5A)]. Moreover, stimulation of splenocyte cultures with biofilm generated higher IFN-γ responses than stimulation with planktonic cells (Figure 6.5B) following priming mice with biofilm vaccine. Similarly, when cultures were stimulated with DTaP, only the splenocytes of mice vaccinated with biofilm produced higher (P<0.05) IFN-γ responses than Imject control mice (Figure 6.5C).

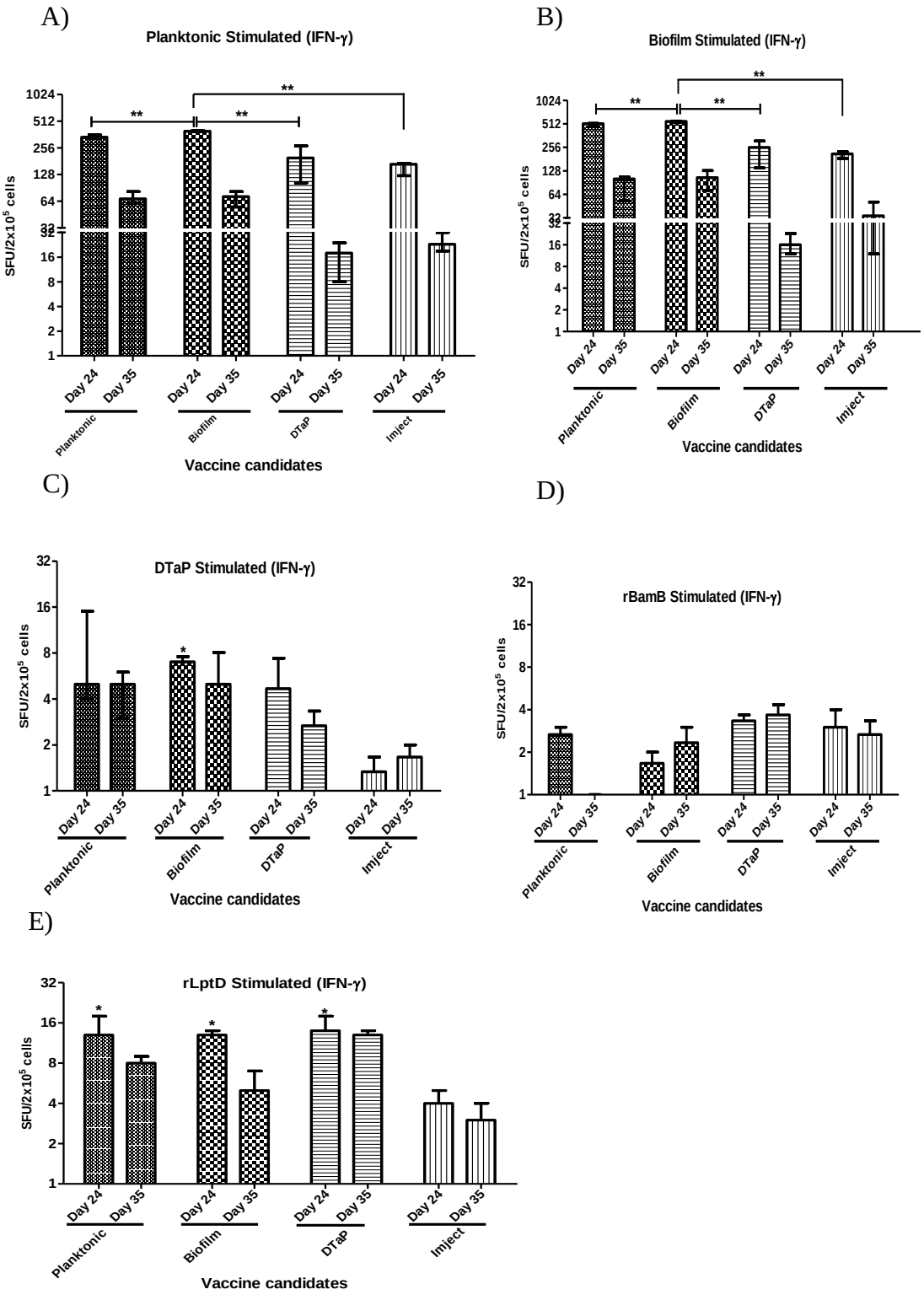


Figure 6.5 IFN-γ levels in mice vaccinated with *B. pertussis* vaccine candidates. Splenocytes stimulated with planktonic (A), biofilm (B), DTaP (C), rBamB (D) or rLptD (E). **P*<0.05 relative to Imject-vaccinated mice at the same time point. Each graph represents n=3 mice. Error bars represent range.

No mice displayed significant production of IFN-γ when their splenocytes were stimulated with rBamB (Figure 6.5D). However, rLptD induced IFN-γ responses in splenocytes of mice vaccinated with planktonic bacteria, biofilm or DTaP above that seen in Imject controls (P<0.05) (Figure 6.5). This result suggests that biofilm and associated protein, rLptD, induce potent production of IFN-γ either through vaccination, or after *in vitro* stimulation of splenocytes from vaccinated mice, representing a booster vaccination.

6.3.4.2 Bordetella pertussis biofilm and associated antigens stimulate potent IL-17a responses in splenocytes of vaccinated mice

The IL-17a responses subsided at day 35 but remained above Imject controls. Stimulation of splenocytes with planktonic or biofilm antigens produced significantly higher IL-17a responses (P<0.05) in biofilm-vaccinated mice compared to planktonic-, DTaP- or Imject-vaccinated mice (Figures 6.6A and B). The IL-17a response was highest in splenocytes of biofilm-vaccinated mice stimulated with biofilm antigens (P<0.05).

When stimulated with DTaP, biofilm-vaccinated mice also produced stronger IL-17a responses but the differences were not significant (Figure 6.6C). Similarly, stimulation of splenocytes with rBamB did not induce IL-17a in any groups (Figure 6.6D). When cultures were stimulated with rLptD, higher IL-17a responses were induced in biofilm-vaccinated mice compared to planktonic-, DTaP- or Imject-vaccinated mice (P<0.05) (Figure 6.6E).

6.3.4.3 Bordetella pertussis biofilm and associated antigens stimulate IFN-γ and IL-17a production in LN cells of vaccinated mice

IFN-γ and IL-17a production by LN cells stimulated *in vitro* was assessed in mice vaccinated as described above (Table 6.2 and 6.3). LN cells from 3 mice were pooled from each group to ensure an adequate number of cells, so there is no measure of individual variation. LN cells of biofilm-vaccinated mice produced higher IFN-γ responses to all stimulants when compared to mice immunised with planktonic bacteria, DTaP- or Imject (Table 6.2). Similarly, stimulation of LN cells with biofilm

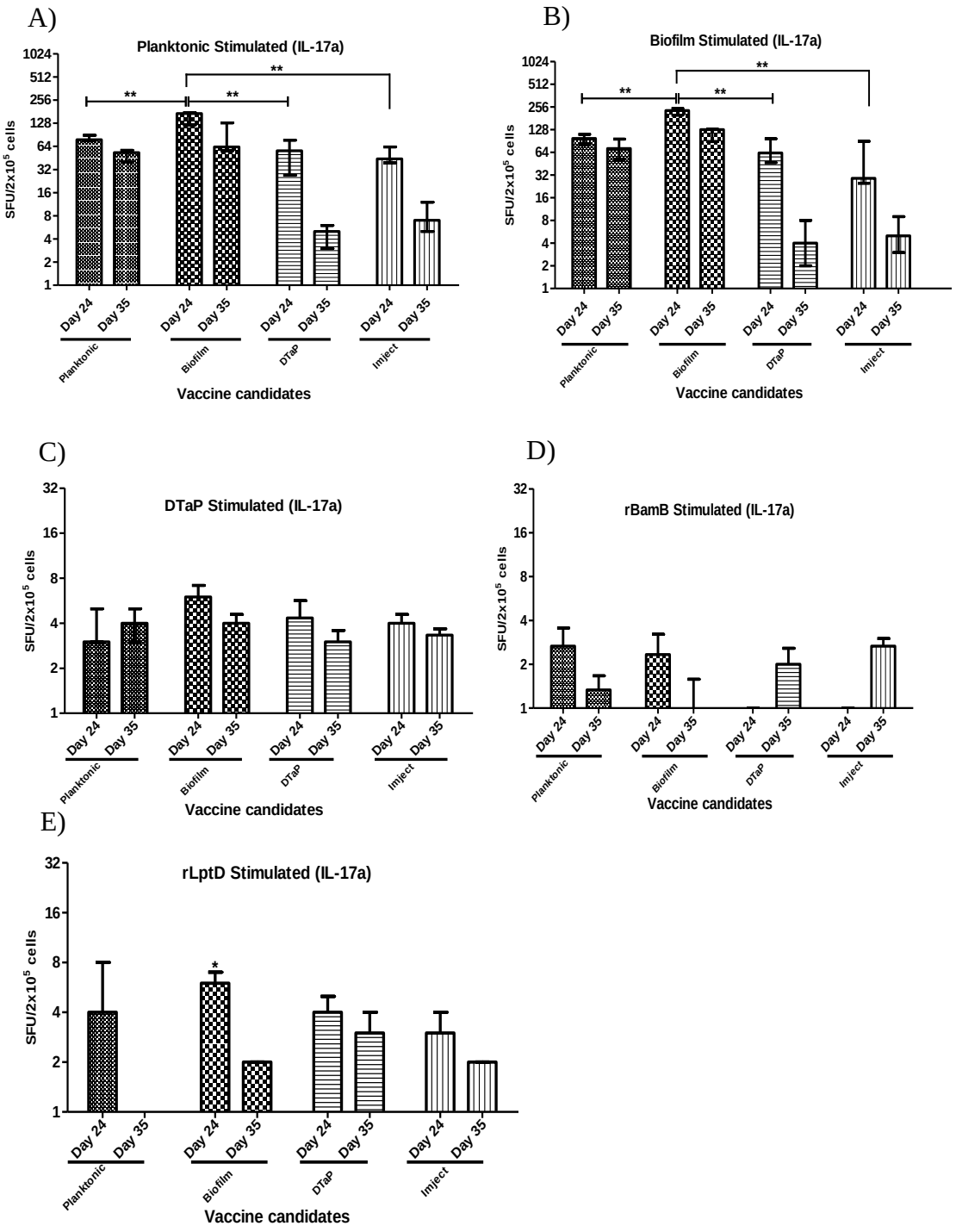


Figure 6.6 IL-17a levels in mice vaccinated with *B. pertussis* vaccine candidates. Splenocytes stimulated with planktonic (A), biofilm (B), DTaP (C), rBamB (D) or rLptD (E). **P*<0.05 relative to DTaP-vaccinated mice at the same time point. SFU, spot forming unit. Each graph represents median for n=3 mice. Error bars represent range.

Table 6.2 IFN-γ levels in LN cells of vaccinated mice stimulated with planktonic, biofilm, rBamB, DTaP, rBamB or rLptD.

Stimulants	Days	IFN-γ levels in vaccine groups (SFU/2x10^5 cells)			
		Planktonic	Biofilm	DTaP	Imject
Planktonic	24	390	446	270	33
	35	38	212	25	21
Biofilm	24	471	506	368	56
	35	70	177	17	40
DTaP	24	0	0	2	0
	35	0	0	3	0
rBamB	24	7	16	5	4
	35	4	0	10	7
rLptD	24	25	26	4	15
	35	5	2	4	6

Table 6.3 IL-17a levels in LN cells of vaccinated mice stimulated with planktonic, biofilm, rBamB, DTaP, rBamB or rLptD.

Stimulants	Days	IL-17a levels in vaccine groups (SFU/2x10^5 cells)			
		Planktonic	Biofilm	DTaP	Imject
Planktonic	24	138	159	16	1
	35	23	87	0	3
Biofilm	24	225	263	26	8
	35	29	143	7	12
DTaP	24	0	1	2	1
	35	0	0	0	0
rBamB	24	0	0	2	3
	35	9	5	4	6
rLptD	24	8	6	20	15
	35	6	3	8	2

SFU; spot forming unit

induced clearer IFN-γ responses in all vaccinated mice compared to *in vitro* stimulation with planktonic, DTaP, rBamB or rLptD antigens. Responses to rLptD were greater than responses to rBamB, which in turn were greater than responses to DTaP, irrespective of the vaccination.

The LN cells of all groups of vaccinated mice generated IL-17a response in the same pattern as was seen with IFN-γ (Table 6.3).

6.3.4.4 Vaccination of mice with Bordetella pertussis biofilm stimulates antigen-specific IgG1 and IgG2a antibodies

Mice vaccinated with heat-inactivated planktonic and biofilm cells produced higher (P<0.05) IgG1 levels at day 24 compared to mice vaccinated with DTaP or Imject controls, with a similar pattern at day 35 (Figure 6.7A and B). Responses to planktonic and biofilm bacteria were similar. Biofilm-vaccinated mice produced significantly higher levels of IgG2a compared to planktonic-, DTaP- or Imject-vaccinated mice at both the days studied (Figure 6.7B and C). This links biofilm vaccination with an efficient Th1 response.

6.3.4.5 DTaP vaccination induces predominantly IgG1 type antibody response

DTaP-ELISA was used to investigate antibody responses in mice vaccinated with planktonic bacteria, biofilm, DTaP or Imject (see Section 6.2.9). Mice vaccinated with DTaP produced higher levels of IgG1 antibody compared to planktonic- or biofilm-vaccinated mice when DTaP was used as coating antigen (Figure 6.8). IgG2a was not detectable in any mice, confirming the link between DTaP and Th2 responses.

6.3.5 *Bordetella pertussis* biofilm and associated proteins protects mice against infection with virulent *Bordetella pertussis* strains

Given the strong Th1 and Th17 responses to *B. pertussis* biofilms, protection against virulent bacterial challenge was assessed. The first study used *B. pertussis* Tohama I vaccine strain, while the second used the clinical isolate ID20, which had the highest biofilm-forming potential of the isolates studied (see Chapter 3). In Phase III (Table 6.1), mice were vaccinated twice over 2 weeks with biofilm, planktonic bacteria, DTaP or Imject and challenged with virulent *B. pertussis* Tohama I by intranasal

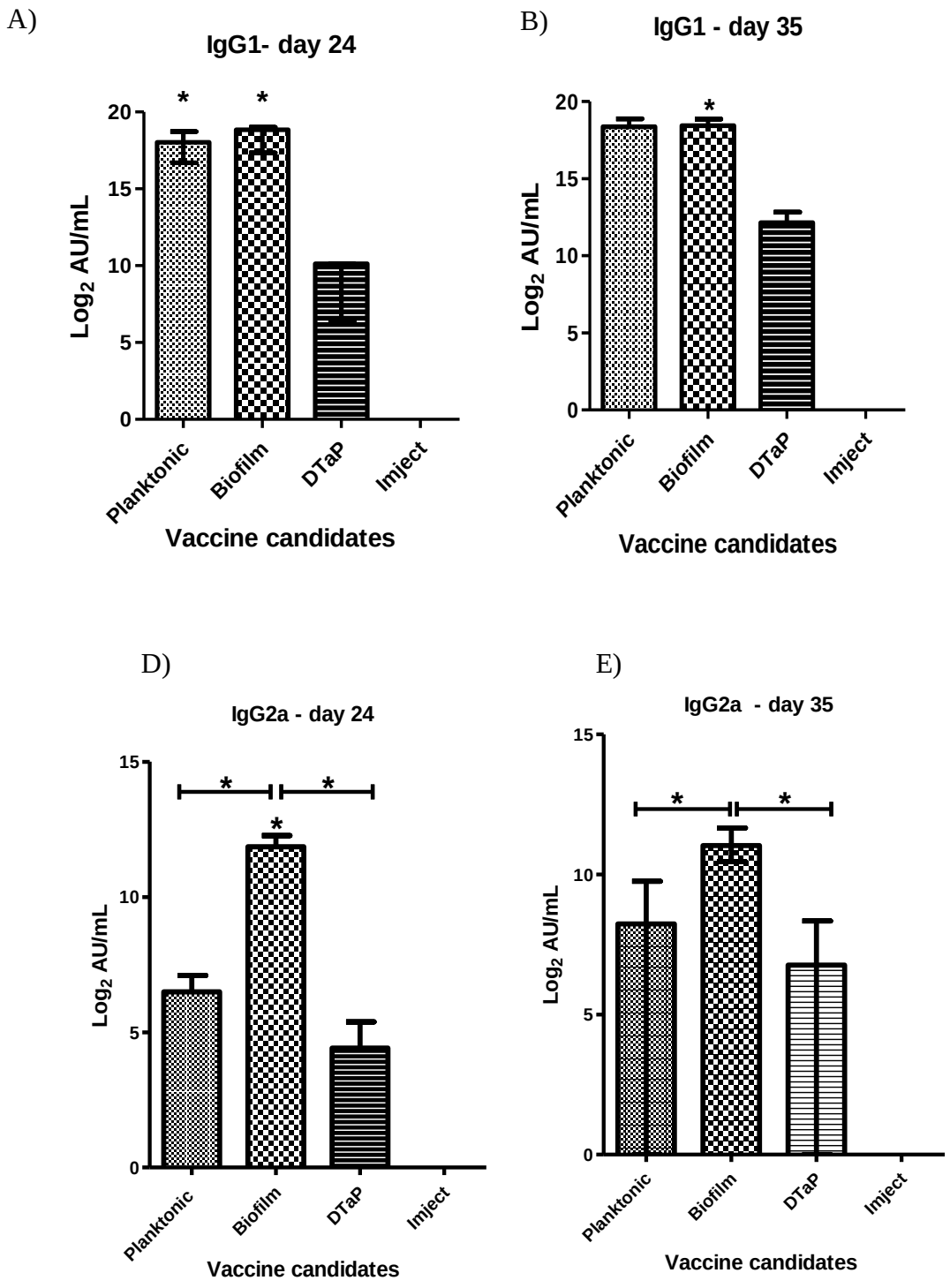


Figure 6.7 Levels of antigen-specific IgG1 and IgG2a antibody in sera of mice vaccinated with planktonic bacteria, biofilm, DTaP or Imject. Levels of IgG1 antibody at day 24 (A) and 35 (B) was shown, while IgG2a levels are shown in (C) and (D), respectively. Columns represent median levels from 3 mice and error bars represent range. *P<0.05 relative to DTaP-vaccinated mice at the same time point.

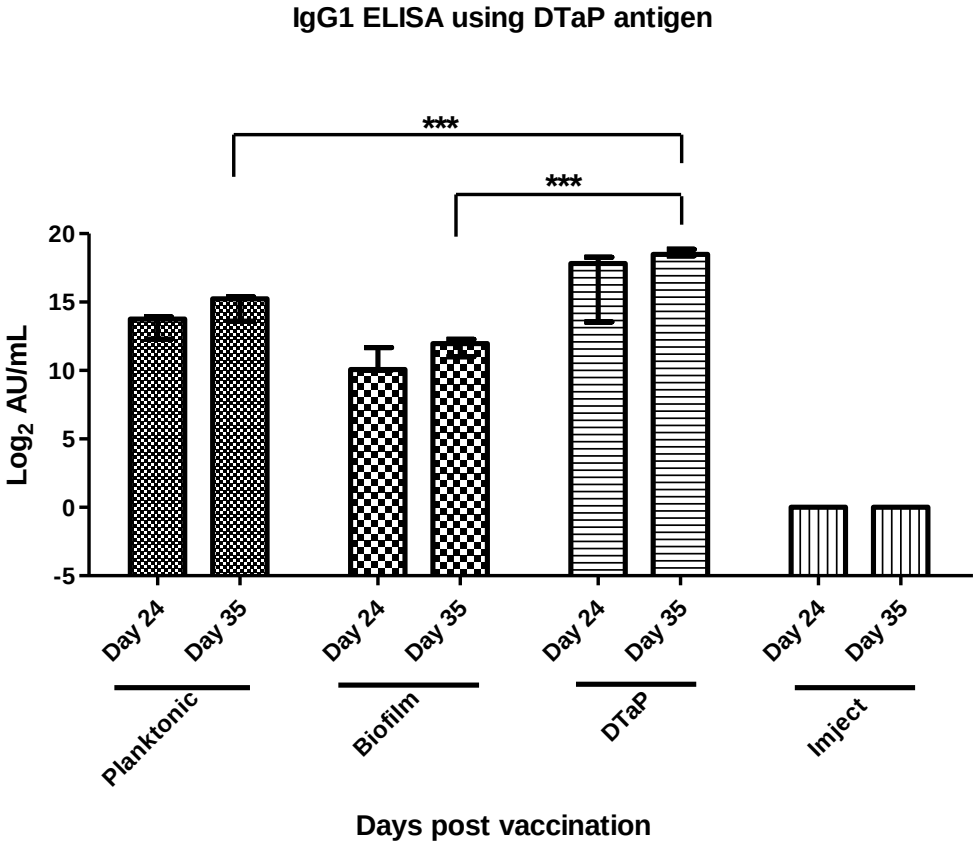


Figure 6.8 Levels of DTaP-specific IgG1 antibody in mice vaccinated with planktonic bacteria, biofilm, DTaP or Imject. Columns represent median values from 3 mice and error bars show the range.

route at day 24. After 7 days, mice were sacrificed and bacterial loads in the lung homogenates were analysed. Mice vaccinated with biofilm cells had lower bacterial loads in their lungs compared to mice vaccinated with DTaP (P<0.02) or Imject (P<0.002) (Figure 6.9). There was no significant difference (P=0.6) between biofilm-vaccinated and planktonic-vaccinated mice. Overall it appears that *B. pertussis* biofilm is protective.

6.3.6 The resurgent *Bordetella pertussis* strain ID20 generates higher bacterial loads than Tohama I

In Phase IV, mice were vaccinated with planktonic cells or biofilm-derived from Tohama I, DTaP and novel *B. pertussis* vaccine candidates, rBamB and rLptD alone or in combination with DTaP and challenged with virulent ID20 by intranasal route (see Section 6.2.6). The experiment sought: a) the protective potential of the *B. pertussis* biofilm-associated proteins and b) the protective potential of *B. pertussis* Tohama I-derived antigens against a resurgent strain carrying the *ptxP3* allele and Prn variant (see Chapter 4). Vaccination with planktonic and biofilm bacteria reduced (P<0.001) bacterial loads in the lungs compared to DTaP- or Imject-vaccinated mice (Figure 6.9) following challenge with ID20. Moreover, mice challenged with ID20 had higher bacterial loads than those challenged with Tahoma I, irrespective of the vaccine candidates (Figure 6.10). This suggests the better fitness of the resurgent strain to survive in current vaccinated populations.

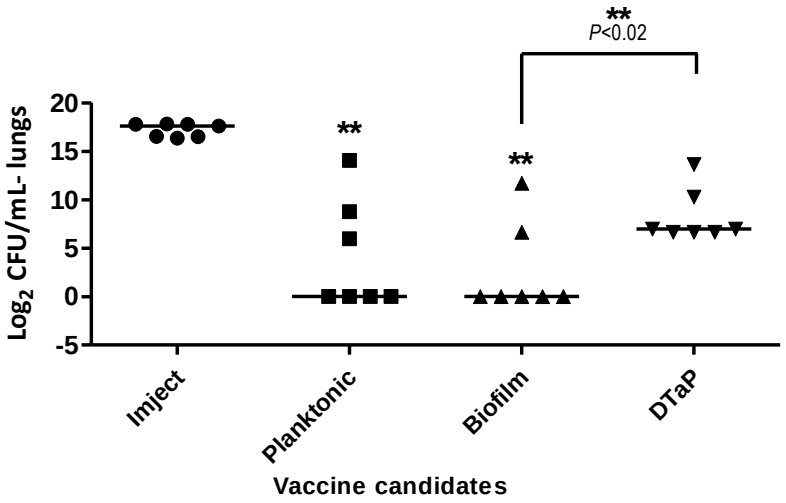
6.3.7 Vaccination with biofilm-associated proteins, rBamB or rLptD, protect mice against resurgent *Bordetella pertussis* infection

Vaccination with rBamB or rLptD as stand-alone vaccines reduced (P<0.003 and P<0.001, respectively) bacterial loads in lungs compared to Imject control mice (Figure 6.9B). Combined rBamB-rLptD-DTaP was the most potent vaccine against ID20, with lower bacterial loads than achieved with Imject or DTaP (P<0.02). Hence, rBamB and rLptD may enhance the protective efficacy of DTaP against resurgent *B. pertussis*.

6.3.8 Clinical features in mice after challenge in vaccinated mice

No mice given any vaccine candidate showed adverse features. Following challenge of vaccinated mice with clinical isolate ID20, the Imject-, rBamB- and rLptD-

A)



B

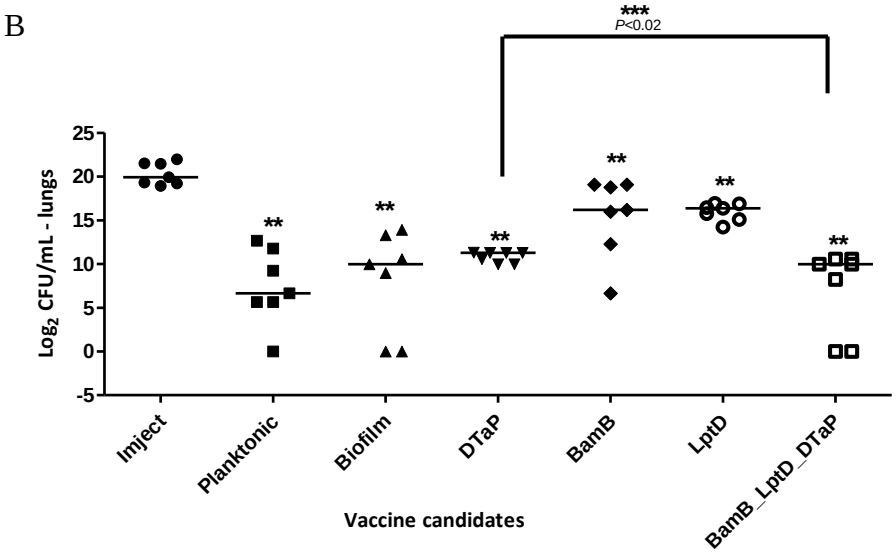
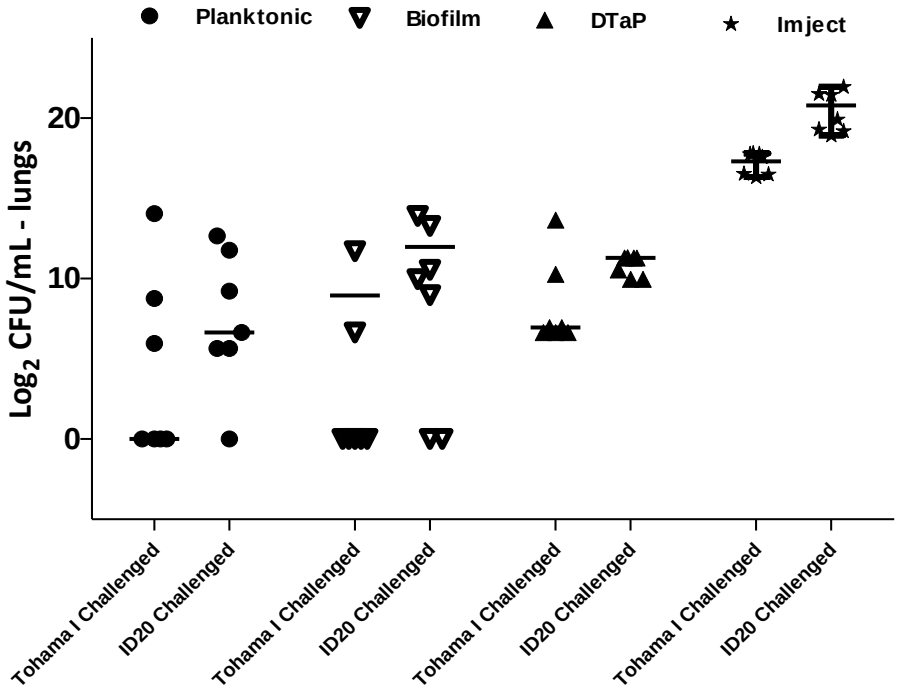


Figure 6.9 Protective potential of *Bordetella pertussis* biofilm and associated proteins. Naïve BALB/c mice were subcutaneously immunised on days 0 and 14 with vaccine candidates as described in Section 6.2.6 and challenged with Tohama I (A) or clinical isolate ID20 (B) on day 24. Bacterial loads were determined from lung homogenates collected 7 days post-challenge. Negative control consists of adjuvant only (Imject) in PBS while DTaP was used as positive control. Each symbol represents bacterial loads in one mouse. Horizontal lines represent the median CFU/mL. ***P*<0.05 relative to control mice and ****P*=0.02 relative to DTaP-vaccinated mice. Two-tailed Mann-Whitney *U* tests were used.

A)



B)

	Vaccine candidates			
	Planktonic	**Biofilm**	**DTaP**	**Imject**
Median CFU x 10^6/mL (Tohama I challenged)	1	1	125	197500
Median CFU x 10^6/mL (ID 20 challenged)	100	1000	2500	1000000

Figure 6.10 Comparative protective potential of biofilm vaccine against Tohama I and ID20 challenge. Bacterial loads in lung homogenates of mice following challenge with Tohama I or ID20 (A). Comparative median bacterial loads (B).

vaccinated mice showed low to medium grade illness persisting at day 7 post-challenge. DTaP-vaccinated mice showed low grade illness. The features include observed rough coat, low activity and low movement (Table 6.4). The planktonic-, biofilm- and rBamB-rLptD-DTaP-vaccinated mice did not show any adverse clinical features at day 7 post-challenge (see Table 6.4, Figure 6.11A and B).

6.3.9 Chemokine signatures in vaccinated and post-challenged mice

In a pilot study, the proinflammatory chemokine signatures were compared in protected mice (termed "clearers" or "C") and non-protected mice (termed "non-clearers" or "NC") using sera from Phase IV (see Section 6.2.6), collected on day 7 post-challenge. Levels of eight chemokines are shown in Table 6.5. Pools from 3 or 4 mice based on bacterial loads were used so there is no measure of variability.

Concentrations of CCL11, CXCL10 and CXCL13 were generally higher in non-clearers, so they appear to be induced by the challenge. These are discussed below in relation to the generation of IgG1 or IgG2a as these are associated with induction of predominantly Th2 or Th1 immune responses, respectively [26].

Clearance of bacteria in planktonic-, biofilm- or LptD-vaccinated mice was associated with higher IgG2a levels compared to non-clearers, consistent with a Th1 response and IFN-γ production (Figure 6.12). However this was not accompanied by higher CXCL10 concentrations – a chemokine induced by IFN-γ.

Considering this in more detail - amongst planktonic- and rLptD-vaccinated mice, clearers had lower concentrations of CXCL10 compared to non-clearers. This suggests induction of the chemokine by the challenge dose. However, clearers did not show lower concentrations of CXCL10 in mice given the biofilm vaccine. This paralleled >10-fold higher IgG2a production and a low bacterial count, which is consistent with protective Th1 responses in biofilm-vaccinated mice.

DTaP-vaccinated mice had low CXCL10 concentrations and produced IgG1 with no IgG2a responses (Table 6.5 and Figure 6.8). Accordingly, non-clearers displayed over >1.5-fold higher CCL11 and CXCL13 responses. The clearers' low CCL11 and

Table 6.4 Clinical features observed in different groups of mice immunised with different *Bordetella pertussis* vaccines.

Clinical signs* observed in mice								
Sl. no	**Vaccine Group**	**Days post-vaccination**						
		Day1	**Day2**	**Day3**	**Day4**	**Day 5**	**Day 6**	**Day 7**
1	Planktonic	++	+++	+++	++	+	0	0
2	Biofilm	++	+++	+++	++	+	0	0
3	Imject control	+++	+++	+++	+++	+++	++	++
4	DTaP	+++	+++	+++	+++	++	++	+
5	rBamB	+++	+++	+++	+++	++	++	++
6	rLptD	+++	+++	+++	++	+	+	+
7	rBamB-rLptD-DTaP	++	+++	+++	++	+	0	0

*Clinical features scored based on monitoring of mouse coat, activity and movement post-challenge. Scores compare observed features to the most severe changes: 0 - no macroscopic changes, + low grade, ++ medium grade, +++ severe grade in mouse health. Observations made up to 7 days post challenge. Sl. No; serial no.

Figure 6.11A Clinical features of mice vaccinated with rBamB-rLptD-DTaP at day 2 post-challenge with ID20. Macroscopic ruffled coats and reduced activity were scored as a "severe grade" outcome (+++).

Figure 6.11B Clinical features in mice vaccinated with rBamB-rLptD-DTaP at day 7 post-challenge with ID20. Mice recovered by day 7 and were scored as “no macroscopic changes” (0).

Vaccine Group	Concentration of chemokines (pg/mL) in sera from vaccinated challenged with ID20								Median CFU (CFU/mL) in Lungs	Median IgG1 (AU/mL)	Median IgG2a (AU/mL)	IgG1:IgG2 ratio
	RANTES (CCL5)	MIP-3a (CCL20)	EOTAXIN (CCL11)	IP-10 (CXCL10)	MIP-Ia (CCL3)	MIP-1b (CCL4)	BLC (CXCL13)	MDC (CCL22)				
Imject C	125	247	7012	1286	27	272	17095	2861	583333	1	1	77
Imject NC	138	547	24307	4616	20	280	132401	1824	2700000	235	136	1
Planktonic C	127	253	10566	443	17	248	25206	5140	67	815875	7087	9
Planktonic NC	133	255	16573	1500	24	265	20094	4992	3533	900580	4065	158
Biofilm C	129	264	14422	1413	14	255	30223	3655	167	1448800	86185	16
Biofilm NC	135	271	16731	1308	25	262	32088	3936	12750	1480705	8566	173
DTaP C	126	244	8479	578	17	251	7498	3136	150	5081	1	5705
DTaP NC	128	239	14912	491	18	254	12016	3488	2417	3624	1	66
rBamB C	129	246	13302	983	19	261	8135	3428	26700	1	1	1
rBamB NC	131	256	20431	2242	24	269	19432	2341	516667	1	50	1
rLptD C	135	316	7743	1086	44	329	11793	4692	19000	2561	647	4
rLptD NC	149	294	10286	3377	59	327	19434	4285	111667	671	275	1
rBamB-rLptD-DTaP C	133	257	5885	2212	39	278	11923	2830	267	3941	109	1
rBamB-rLptD-DTaP NC	131	252	5454	1570	32	275	7910	2672	1250	3623	433	9

Table 6.5 Heat-map depicting levels of eight chemokines in vaccinated challenged with ID20. Median CFU is bacterial load from 3 mice in a group, 7 days post-challenge. IgG1 and IgG2a levels are median value of 3 mice from each group, 7 days post-challenge. Dark green boxes indicate chemokine levels >2-fold higher among non-clearers compared to clearers. Light green marks >1.5 fold higher in non-clearers. Light blue indicate >1.5 fold higher in clearers. White box marks <1.5 fold differences. C = clearer, NC= non-clearer.

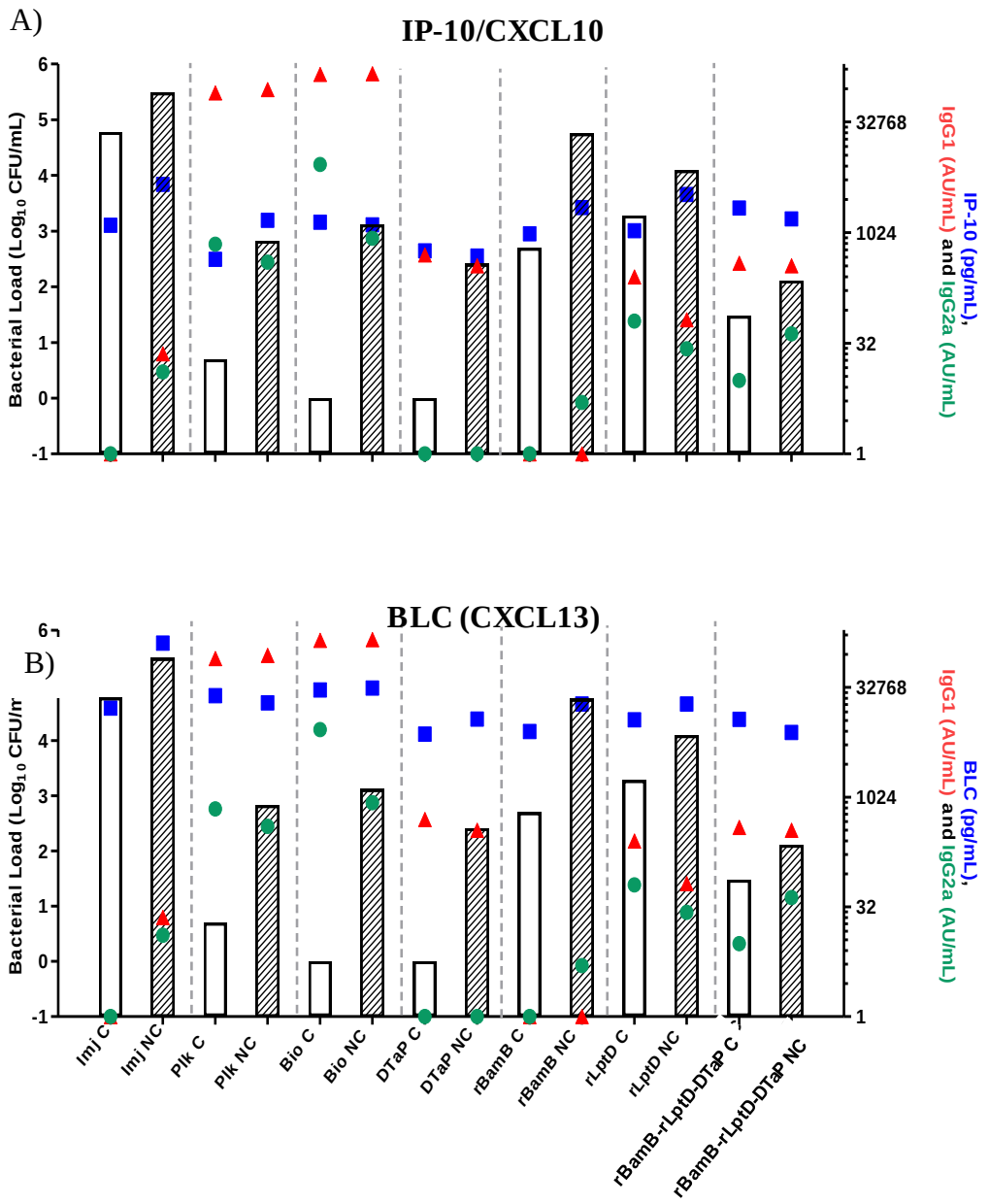


Figure 6.12 Chemokines concentrations (pg/mL) in sera from vaccinated mice challenged with ID20. The serum concentrations of chemokines (blue squares) were analysed on day 31 after vaccinating mice on day 0 and day 14 with Imject (Imj), planktonic (Plk), biofilm (Bio), DTaP, rBamB, rLptD or rBamB-rLptD-DTaP combination, followed by challenge with ID20 on day 24. Two to three mice from each group were pooled. Letters 'C' and 'NC' denote clearers and non-clearers, respectively, from each group and indicated by bacterial loads (hatched and open bars, respectively). The median IgG1 and IgG2a levels are shown in red triangles and green circles, respectively. Each vaccination group is separated by a dotted line.

CXCL13 responses but higher IgG1 responses was consistent with Th2 responses seen with DTaP vaccination.

The rBamB-rLptD-DTaP-vaccinated mice exhibit similar levels of chemokines in clearers and non-clearers, except CXCL13 which was >1.5-fold higher while CXCL10 was >1.4-fold higher in clearers (Figure 6.12B). In these mice, antibody may not be protecting from infection as IgG2a was low. The higher CXCL10 and CXCL13 level may indicate a combined Th1- and Th2-mediated clearance. Since this is a preliminary study, the role of chemokines warrants further investigation with this regime.

6.4 Discussion

All current whooping cough vaccines are formulated with antigens expressed in planktonic grown bacteria. In addition, the resurgent strains predominantly carry the *ptxP3* allele while aP vaccines in routine use are prepared from *B. pertussis* Tohama I carrying the *ptxP1* allele. In this study, the ability of *B. pertussis* to form biofilm was explored to identify novel antigens that may protect against resurgent *B. pertussis* strains. Moreover protection against the biofilm form of *B. pertussis* may interrupt its persistence and spread [18]. I am aware of only one *B. pertussis* biofilm vaccine trial [20], so immunogenicity and comparative protective potential need further work.

Mice were vaccinated on day 0 and day 14 with inactivated *B. pertussis* planktonic or biofilm cells and immune responses assessed on days 10, 19 and 24. Day 24 was chosen as an optimum time-point for quantification of immune responses. The results suggest that a more detailed time-point study would be informative.

6.4.1 Selection of novel vaccine candidate for investigation

BamB and LptD were selected as novel vaccine candidate antigens as they were significantly (P<0.05) upregulated in *B. pertussis* biofilm and the resurgent strain ID20 (see Chapter 5). So far only one *B. pertussis* biofilm-associated protein, BipA, has been tested for protection against *B. pertussis* infection [20]. Using iTRAQ, this study identified 5% and 3.5% proteins upregulated and downregulated (respectively) in biofilm cultures of clinical isolate ID20. In addition to BamB and LptD, other

differentially expressed biofilm proteins may have potential in the design of a novel whooping cough vaccine.

6.4.2 Vaccination with *Bordetella pertussis* biofilm induced significantly higher Th1 and IL-17a responses

In this study, *B. pertussis* biofilm was more immunogenic than planktonic or DTaP vaccine candidates. Vaccination of mice with *B. pertussis* biofilm induced higher (P<0.005) IgG1 levels than was seen in DTaP- or Imject-vaccinated mice. Importantly, mice vaccinated with biofilm produced 50-fold higher median (P<0.05) levels of IgG2a compared to vaccination with any other candidate vaccines (Figure 6.7C). Accordingly in splenocytes and LN cells of mice vaccinated with planktonic cells, biofilms, DTaP or Imject, stimulation with biofilm induced stronger IFN-γ and IL-17a responses compared to stimulation with planktonic bacteria, DTaP, rBamB or rLptD. Biofilm-vaccinated mice produced more IFN-γ than planktonic-, DTaP- or Imject-vaccinated mice, when splenocytes were stimulated with biofilm. Moreover, in mice immunised with biofilm and challenged with live bacteria, serum levels of CXCL10 were high and similar to mice who failed to clear the challenge dose. This suggests that the CXCL10 was induced by the vaccination not the challenge (Figure 6.12). As CXCL10 is induced by IFN-γ, the finding supports the conclusion from the ELISpot data that biofilm vaccination induces Th1 and IL-17a responses, an advantage for novel whooping cough vaccines.

Interestingly, compared to stimulation with DTaP, rLptD induced more IFN-γ production in all vaccinated groups (Figure 6.5E). Similarly, in Imject vaccinated mice, rBamB stimulated higher levels of IFN-γ responses than DTaP (Figure 6.5C and D). Recombinant BamB and rLptD also induced marginally more IL-17a than were induced by DTaP in the LN cells. Taken together, these data indicate the superior immunogenicity of *B. pertussis* biofilm associated antigens (rBamB and rLptD) over DTaP vaccines. Further studies are warranted to support this finding.

6.4.3 DTaP vaccination induces predominantly IgG1 antibody responses

The DTaP-antigen ELISA confirmed that mice vaccinated with DTaP produce IgG1 but not IgG2a antibody (Figure 6.8). This is in consistent with other study [1] that vaccination of mice with aP induce predominantly IgG1 antibody responses.

Combined with IFN-γ and whole-cell IgG1 and IgG2a antibody levels (Figure 6.6 and 6.7), this fits with evidence from mice [1] and humans [27] showing that currently used aP vaccines induce predominantly Th2 immune response with weak IL-17 and Th1 immune response. This highlights a need for replacement of current aP vaccines with novel pertussis vaccine able to stimulate Th1/IL-17 immune responses.

6.4.4 rBamB and rLptD formulations protect mice from virulent *Bordetella pertussis* challenge

Having demonstrated the immunogenic potential of the biofilm and its associated proteins, the study assessed the protective potential of these antigens against virulent *B. pertussis* challenge. Groups of 7 mice were vaccinated with planktonic cells, biofilms or DTaP derived from the *B. pertussis* Tohama I strain. On day 24 post-vaccination, mice were challenged with $2x10^7$ CFU of virulent Tohama I. Lungs were harvested after 7 days and bacterial loads determined in lung homogenates. Biofilm vaccination reduced ($P<0.02$) bacterial loads compared to DTaP vaccinated mice (Figure 6.9). In the next phase, mice were vaccinated with the vaccine candidates described above, or with rBamB, rLptD or a combined rBamB-rLptD-DTaP formulation. These mice were then challenged with virulent *B. pertussis* ID20 which carries the *ptxP3* allele, Prn variant (Chapter 4) and was most able to create biofilm when assessed with 21 clinical isolates (Chapter 3). Mice vaccinated with rBamB or rLptD had lower ($P<0.05$) bacterial loads than Imject control mice. Moreover, the rBamB-rLptD-DTaP formulation significantly enhanced ($P<0.02$) the protective efficacy of DTaP alone vaccination and had an acceptable safety profile (see Figure 6.9B and Table 6.4). This is a novel and important finding. de-Gouw et al [20] showed that vaccination of mice with biofilm protein, BipA, significantly reduced colonisation of the lungs following virulent *B. pertussis* challenge, but the effect of BipA on the protective potential of DTaP was not studied. This result suggests that BamB and LptD can be safely formulated with DTaP to enhance its protective efficacy against the resurgent *B. pertussis* strains.

6.4.5 Vaccines prepared from Tohama I provide lower protection against resurgent strains

Vaccination with Tohama I derived antigens provided lower protection against challenge with ID20 carrying *ptxP3* allele (Figure 6.10). This result is in accordance with other reports [28-30] that demonstrated enhanced fitness and spread of resurgent strains in the aP vaccinated population. Clearly, there is an urgent need for pertussis vaccines that are effective against resurgent strains.

In summary, a vaccine comprising inactivated *B. pertussis* biofilm may induce protective immune response predominantly via the induction of IFN-γ, IL-17a, CXCL10 and IgG2a, although IgG1 is also seen. The planktonic cell vaccine produced immune responses inferior to biofilm vaccine with significantly lower IgG2a antibody responses. The current aP vaccine, DTaP, induces predominantly IgG1 antibody response with little or no IgG2a, IFN-γ or IL-17a. Alarmingly, the current results suggest that antigens derived from *B. pertussis* Tohama I are not optimally effective in protecting against infection with resurgent *B. pertussis* strains. Formulation of DTaP with BamB and LptD may enhance the protective potential of DTaP against resurgent strains, pointing towards BamB- and LptD-based formulations as potential novel pertussis vaccines.

6.5 References

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Chapter 7

General discussion and conclusions

The resurgence of whooping cough has called for an integrated approach to control and prevention of the disease. Pertussis epidemiology in developing countries, genomic analysis of *B. pertussis*, analyses of virulence factor expression and the need for a novel whooping cough vaccines are recognised as problems that require immediate attention [1]. In this project, I investigated the genetic variants of epidemic strains, identified variations in virulence factors and identified novel antigens for the formulation of effective whooping cough vaccines. In the last two decades since the resurgence of whooping cough, very little progress has been made to identify an alternative vaccine or vaccine antigens [2]. An important aim of this project was to identify a novel vaccine candidate against whooping cough using the mouse as a model system. This study used the biofilm-forming potential of *B. pertussis* to determine the immunogenicity and protective potential of proteins upregulated in biofilm as novel vaccine antigens for the control and prevention of whooping cough.

7.1 The resurgent strains of *Bordetella pertussis* comprise of new variants

Twenty-one clinical isolates of *B. pertussis* representing the resurgent strains were studied. Many isolates (48%) carried the *ptxP3-prn2-fim3A* genotype. This genotype differs from that of the Tohama I strain used to prepare vaccine. Hence, the resurgent strains may be able to evade immune responses generated by the current aP vaccine, as Tohama I expresses the *ptxP1* allele rather than the *ptxP3* allele [3]. Tohama I-derived antigens offered lower protection when challenged with the *ptxP3*-carrying clinical isolate ID20 (Figure 6.10). Similarly, other reports [4-6] have shown that resurgent *B. pertussis* strains demonstrate enhanced fitness and have spread in aP vaccinated populations. Clearly, there is an urgent need for an alternative whooping cough vaccine that is effective against the resurgent strains.

7.2 Clinical isolate ID20 exhibits variants in the antigens included in the aP vaccines

All the clinical isolates carried all genes encoding antigens included in current aP vaccines, as well as genes encoding other accredited virulent antigens including dermonecrotic toxin (*dnt*), tracheal cytotoxin (*tct*), adenylate cyclase toxin (*act*), *Bordetella* serum resistance factor (*brkA*), *Bordetella* intermediate protein (*bipA*),

Bordetella polysaccharide D (*bpsD*) and the capsular gene (*kpsT*). Furthermore, draft whole genome sequencing of the representative clinical isolate, ID20 identified 344 SNPs along with 19 insertions and 11 deletions, when aligned with the Tohama I genome. For example, the *prn* gene with a 1 bp frameshift mutation may affect the expression of functional *prn*, an important component of all aP vaccines. The pertussis toxin carried a polymorphism (G to A transition) in its promoter region, resulting in the *ptxP3* allele. The *ptxP3* alleles are characteristic of epidemic resurgent strains and are associated with increased Ptx production [7]. There are also SNPs in the *ptxA*, *ptxB*, *ptxC* subunits and *fimD* genes. Given the observed genetic diversity in the virulence-associated genes encoding pertussis toxin, pertactin and fimbrial antigens [3,7-9], it is necessary to evaluate whether the currently used aP vaccine induces protection against newly emerged *B. pertussis* strains. Severe whooping cough cases with pertactin-negative (*prn*$^-$) isolates have been reported in vaccinated populations in Australia [10], Europe [11], Japan [12] and the USA [13,14]. Like other auto-transporter proteins, pertactin contains an Arg-Gly-Asp (RGD)-repeat motif that mediates attachment of *B. pertussis* to eukaryotic cells. It is associated with multiple virulence functions including adhesion, toxicity, invasion and aggregation, and biofilm formation [15]. *In vitro* and *in vivo* studies showed that *B. pertussis* strains lacking Ptx, Prn and FHA retained cytotoxicity and caused lethal infections in mice [16]. Ptx, Prn, Fim and FHA may be minor virulence factors affecting the cytotoxicity and lethality of resurgent *B. pertussis* strains. Therefore, the inclusion of Ptx and Prn in aP, and identification of other novel virulence factors against resurgent *B. pertussis* strains, warrants immediate consideration.

7.3 *Bordetella pertussis* clinical isolates form stronger biofilm compared to the vaccine strain

Biofilm formation allows *B. pertussis* to persist in the upper respiratory epithelium of human adult and adolescent carriers [17] and act as a reservoir of infection for unvaccinated infants. This is underlined by the greater ability of all clinical isolates investigated in the present study to form stronger biofilms *in vitro* ($P<0.05$) compared to Tohama I. A study [18] from Argentina similarly showed clinical isolates were stronger biofilm producers than the reference strain. Furthermore, biofilm formation may play a role in the evasion of protection conferred by the currently used aP. Since biofilm of clinical *B. pertussis* isolates displayed increased

antibiotic resistance, *in vitro* determination of antibiograms for clinical *B. pertussis* biofilm may provide information that will lead to better therapeutic interventions. Further studies to elucidate the contribution of biofilm formation to the evasion of immunity induced by aP vaccines are warranted.

7.4 Proteomic profile identified large subsets of proteins for inclusion in novel whooping cough vaccines

Proteomic characterisation of a strong biofilm-forming isolate, ID20, with *ptxP3* allel, identified 5% proteins were significantly upregulated, while 3.5% of proteins were downregulated compared to Tohama I ($P<0.05$) (Chapters 3 and 5). Fim3 and FimB were significantly upregulated in the clinical isolated ID20 compared to vaccine strain. Fim3 is *B. pertussis* fimbriae (subunit 3) necessary for attachment to epithelial cells and persistence of infection [19]. It is one of the important components of aP. FimB is required for biogenesis of fimbriae and FHA. Both fimbriae and FHA are *B. pertussis* adhesins and contribute to biofilm formation in *B. bronchiseptica* [20]. The higher expression of Fim3 and FimB therefore support the stronger biofilm forming potential of the clinical isolate. Subsequently, differential protein expressions in the biofilm of clinical isolate ID20 relative to its planktonic cells showed 6% and 7% of proteins were significantly upregulated or downregulated in biofilm, respectively. Interestingly, functional classification of upregulated proteins showed that proteins involved in energy metabolism were significantly upregulated in biofilm of the newly emerged clinical isolate ID20 ($P<0.03$) compared to its planktonic cells. Four proteins associated with energy metabolism were upregulated in the clinical isolate ID20. Among them, two ATP-biosynthesis enzymes, AtpA and AtpC, are responsible for synthesis of ATP that serves as an energy currency for cells [21]. The upregulation of proteins associated with energy metabolism was attributed to cellular need to produce higher cell biomass [18]. The result from this study suggests that clinical isolate ID20 has different energy requirements in the biofilm compared to planktonic cells. Results from other studies [22-24] showed that ATP is important for bacterial biofilm formation. Therefore, inhibition of ATP-biosynthesis enzymes could be a novel strategy to prevent *B. pertussis* biofilm-associated infections.

7.5 Novel antigens for formulation of improved whooping cough vaccines identified in this study

Differentially expressed proteins, whether they are upregulated or downregulated, may have an important role in the biology and evolution of *B. pertussis* resurgent strains. Furthermore, any differentially expressed biofilm proteins may be useful as antigens in a novel whooping cough vaccine. To identify novel whooping cough vaccine candidates, this study investigated the immunogenicity and protective potential of biofilm and associated proteins. Two proteins (BamB and LptD) that were significantly upregulated ($P<0.05$) in *B. pertussis* biofilm were constructed and expressed as recombinant proteins. These proteins were then tested in combination with biofilm and DTaP, a currently used aP vaccine.

In accordance with other reports in mice [25] and in humans [26], the DTaP-vaccinated mice produced high IgG1 but not IgG2a antibody responses (Figure 6.8), suggesting Th2-skewed immune response. This highlights an urgent need for replacement of current aP vaccines with novel pertussis vaccines capable of additionally stimulating Th1/IL-17 immune responses. Vaccination of mice with *B. pertussis* biofilm alone induced higher ($P<0.005$) IgG1 responses than were seen in DTaP- and Imject- vaccinated mice. Importantly, IgG2a responses were >50-fold higher ($P<0.05$) than levels induced by vaccination with planktonic cells, DTaP or control (Figure 6.7C). In addition, *ex vivo* stimulation of splenocytes and LN cell cultures of vaccinated mice with biofilm induced stronger IFN-γ and IL-17a responses compared to stimulation with planktonic bacteria, DTaP, BamB or LptD. Interestingly, LptD induced IFN-γ responses above that seen with Imject-vaccinated control ($P<0.05$), with the highest IFN-γ response elicited in splenocyte and LN cell cultures of biofilm-vaccinated mice. These results suggest that biofilm vaccination induces Th1 and IL-17a responses, an advantage for formulating novel whooping cough vaccines.

Furthermore, vaccination of mice with biofilm achieved lower ($P<0.02$) bacterial loads compared to DTaP- or control-vaccinated mice 7 days after virulent intranasal *B. pertussis* challenge. Preliminary chemokine data showed that the mice clearing the bacteria had high levels of serum CXCL10 - a chemokine induced by IFN-γ (Figure 6.12), indicating Th1-mediated clearance of bacteria. Interestingly, mice vaccinated

with BamB or LptD had lower (P<0.05) bacterial loads than Imject-vaccinated mice, while the BamB-LptD-DTaP formulation significantly enhanced (P<0.02) the protective efficacy of DTaP-alone vaccination. This approach was different from a previous study [27] in that the protective potential of biofilm-associated proteins was studied in combination with DTaP vaccine. I believe this is the first study to demonstrate the enhanced protective potential of *B. pertussis* biofilm-associated proteins. However, the responses of vaccination with whole cell whooping cough vaccines was not compared to biofilm vaccination, as whole cell vaccines were no longer used in developed countries where the resurgent strains were mostly reported.

The major findings of this study were that vaccination of mice with inactivated *B. pertussis* biofilm induced significant production of Th1-associated cell-mediated immune response and conferred protection of mice against *B. pertussis* infection. Vaccination with the recombinant proteins, BamB and LptD, resulted in significant reduction of bacterial loads compared to control mice and when used in combination with DTaP, significantly enhanced the protective efficacy of DTaP-alone vaccination. Hence, biofilm, BamB and LptD hold potential as a novel antigens to improve the efficacy of the current whooping cough vaccine.

7.6 Future directions

The findings from this study lay a foundation for future research into the development of novel whooping cough vaccine. Given the majority of the clinical isolates investigated in this study belongs to a new variant of *B. pertussis* with epidemic potential, formulation of whooping cough vaccines with antigens derived from these newly emerged variants need urgent consideration. This was demonstrated convincingly in this study by the protective efficacy experiments that showed *B. pertussis* Tohama I-derived antigens provided lower protection against challenge with clinical isolates carrying *ptxP3* genotype.

Since the polysaccharide capsule of *B. pertussis* was demonstrated phenotypically in all clinical isolates used (Chapter 4), conjugated polysaccharide-capsular vaccines may be explored as an alternative strategy to control mortality and morbidity associated with whooping cough. To the best of my knowledge, conjugated polysaccharide-capsular vaccines for whooping cough have not been formulated,

despite their successful use in routine vaccination programs for the control of highly invasive bacterial diseases like *Neisseria meningitidis*, *Haemophilus influenzae* type b (Hib) and *Streptococcus pneumoniae* [28].

To counter the continuously evolving biology of *B. pertussis*, whole genome sequencing of clinical isolates may be used to dissect the genetic signatures of the resurgent strains and effective whooping cough vaccine formulated accordingly.

Alternatively, the peptide library of the resurgent *B. pertussis* genome may be screened to identify the most immunogenic peptides and subsequently the *in vitro* and *in vivo* immunogenicity and protective potential determined. This approach will identify the most predominant virulence factors expressed during the pathogenesis of *B. pertussis*. Vaccines targeting these predominant virulence factors would be expected to prevent adhesion, colonisation, persistence and, therefore, infection with *B. pertussis*.

The results presented in this study suggest that refining the *B. pertussis* biofilm proteins BamB and LptD as novel antigens for inclusion in the current whooping cough vaccine would be a novel strategy to target emergent strains that produce biofilm. Targeting the biofilm has the advantage of reducing its protective effect, potentially also preventing its formation, and increasing susceptibility of the bacteria to the immune response induced by other components of the vaccine. Ideally, clinical trials of novel vaccine formulations would include Th1-enhancing adjuvants.

7.7 References

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Appendix A

A.1 Buffers, solutions and media

Stainer-Scholte (S-S) broth

Ingredients	grams
$CaCl_2$	0.02
KCl	0.20
KH_2PO_4	0.50
$MgCl_2.6H_2O$	0.10
NaCl	2.50
L-Glutamic Acid	0.67
L-Proline	0.24

The mixture was dissolved in 970 mL of distilled water, pH adjusted to 7.2 to 7.3 with 2 M NaOH and autoclaved at 121°C for 15 min. After cooling, 10 mL of filter sterilised Stock Solution and 20 mL of 10 mg/mL cyclodextrin was added.

Stock Solution

Ingredients	grams
Ascorbic Acid	0.020
Nicotinic Acid	0.004
Glutathione	0.100
$FeSO_4.7H_2O$	0.010
L-Cysteine	0.040

The mixture was dissolved in 10 mL of distilled water, filter-sterilised using 0.2 µM syringe filter and stored at -20^{O}C up to 6 months.

NaCl (0.9%)

9 g of NaCl was dissolved in 1 L of distilled water and autoclaved at 121°C for 15 min.

Cyclodextrin (10mg/mL)

Ingredients	
Cyclodextrin	10 mg

Dissolved in 10 mL of distilled water, filter-sterilised using 0.2 µm filter and stored at -20^{O}C up to 6 months.

1xPBS

Ingredients	grams
NaCl	8.0
KCl	0.2
Na_2HPO_4	1.44
KH_2PO_4	0.24

The mixture was dissolved in 800 mL of distilled water and pH adjusted to 7.4 with 1 M NaOH. The volume was adjusted to 1 L and autoclaved at 121°C for 15 min.

0.5 M EDTA

93.5 g of disodium ethylene diamine tetra-acetate, 2H20 was added to 400 mL of distilled water, volume adjusted to 500 mL and autoclaved at 121°C for 15 min.

Sodium borate (SB) buffer (20X)

Ingredients	grams
NaOH	8.0
Boric acid	47.0

The mixture was dissolved in 900 mL of distilled water, pH adjusted to 8 and final volume adjusted to 1 L.

Agar rose gel (1.5%)

1.5g Agarose

100 mL 1xSB buffer

Bordet-Gengou agar

Ingredients	grams
BG agar	30.0

Dissolved in 975 mL of distilled water and 10 mL glycerol was added. The mixture was autoclaved at 121°C for 15 min. The mixture was cooled to 50°C and 150 mL of sterile sheep blood was aseptically added, mixed and poured into sterile 90 mm petri plates.

ELISA reagents

Carbonate/bicarbonate buffer

Ingredients	gram
Na_2CO_3	1.59
$NaHCO_3$	2.55

The mixture was dissolve in 1 L of distilled water, pH adjusted to 9.5, autoclaved at 121°C for 15 min and stored at 4°C.

PBS/Tween

Two hundred fifty microliter of Tween-20 was added to 500 mL of sterile PBS

TMB substrate

TMB	1 tablet
Phosphate/citrate Buffer	10 mL
H_2O_2 (30%w/w)	1 µL

Dissolved tablet in buffer and added H_2O_2 immediately prior to use.

Na_2HPO_4 (0.2M)

Ingredients	gram
Na_2HPO_4	14.2g

Dissolved in 500 mL of distilled water and stored at room temperature.

Citric acid (0.1M)

Ingredients	gram
Citric Acid	10.5

Dissolved in 500 mL of distilled water and stored at room temperature.

Phosphate/citrate Buffer

Na_2HPO_4 (0.2M)	128.5mL
Citric acid (0.1M)	21.5mL
MilliQ Water	250mL

Adjusted pH to 5.0 and stored at 4°C.

Blocking buffer

Ingredients	gram
Bovine Serum Albumin	25

Dissolved in 500 mL of sterile PBS and stored at 4°C.

Appendix B

Supplementary materials

B.1 Chapter 3

Appendix 3.1 Proteins significantly upregulated in biofilm of *B. pertussis* clinical isolate ID20 and reference strain Tohama I

Protein upregulated in biofilm cells of clinical isolate ID20			Protein upregulated in biofilm cells of reference strain Tohama I		
Name	**Localisation**	**Function**	**Protein description**	**Localization**	**Function**
*Polyribonucleotide nucleotidyltransferase, PnP	Cytoplasm	Transcription	Polyribonucleotide nucleotidyltransferase, Pnp	Cytoplasm	Transcription
*Aspartate--tRNA(Asp/Asn) ligase, AspS	Cytoplasm	Protein synthesis	Aspartate--tRNA(Asp/Asn) ligase, AspS	Cytoplasm	Protein synthesis
*ATP synthase subunit, AtpA	Inner membrane	Energy metabolism	ATP synthase subunit alpha, AtpA	Cytoplasm	Energy metabolism
*Outer membrane protein A, OmpA	Outer membrane	Protein fate	Outer membrane protein A, OmpA	Outer membrane	Protein fate
*Membrane protein insertase, YidC	Cell membrane	Protein fate	Membrane protein insertase, YidC	Cell membrane	Protein fate
*Elongation factor Tu, TuF	Cytoplasm	Protein synthesis	Elongation factor, TuF	Cytoplasm	Protein synthesis
*RNA-binding protein, Hfq	Cytoplasm	Regulatory functions	RNA-binding protein, Hfq	Cytoplasm	Regulatory functions
Aspartate-semialdehyde dehydrogenase, AsD	Cytoplasm	Amino acid biosynthesis	Outer membrane porin protein, BP0840	Outer membrane	Porin activity
Superoxide dismutase [Fe], SodB	Periplasm	Cellular process	ATP synthase subunit beta, AtpD	Inner membrane	Energy metabolism
6,7-dimethyl-8-ribityllumazine synthase, RibH	Cytoplasm	Biosynthesis of cofactors, prosthetic groups, and carriers	Probable TonB-dependent receptor, BfrD	Outer membrane	Transport and binding proteins
ATP synthase epsilon chain, AtpC	Inner membrane	Energy metabolism	Probable cytosol aminopeptidase, PepA	Cytoplasm	Protein fate
2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-succinyltransferase, DapD	Cytoplasm	Amino acid biosynthesis	ATP-dependent Clp protease ATP-binding subunit, ClpX	Cytoplasm	Protein fate
ADP-L-glycero-D-manno-heptose-6-epimerase, HldD	Cytoplasm	Cell envelope	ATP phosphoribosyltransferase regulatory subunit, HisZ	Cytoplasm	Protein synthesis
60 kDa chaperonin, GroL	Cytoplasm	Protein fate	Putative positive transcription regulator, BvgA	Cytoplasm	Regulatory
Acyl carrier protein, AcpP	Cytoplasm	Fatty acid and phospholipid metabolism	Threonine--tRNA ligase, ThrS	Cytoplasm	Protein synthesis
Enolase, EnO	Cytoplasm, secreted, cell surface	Energy metabolism	ATP phosphoribosyltransferase, HisG	Cytoplasm	Amino acid biosynthesis
Ribonuclease PH, RpH	Cytoplasm	Transcription	LPS-assembly protein, LptD	Outer membrane	Cell envelope
GTPase Der, DeR	Cytoplasm	Protein synthesis	Phosphate-binding protein, PstS	Cytoplasmic membrane	Transport and binding proteins
Outer membrane protein assembly factor, BamB	Outer membrane	Protein fate			
Succinyl-CoA ligase [ADP-forming] subunit beta, SucC	Cytoplasm	Energy metabolism			

*proteins upregulated in both clinical isolate ID20 and Tohama I

Appendix 3.2 Proteins significantly downregulated in biofilm of *B. pertussis* clinical isolate ID20 and reference strain Tohama I

Protein downregulated in biofilm cells of clinical isolate ID20			**Protein downregulated in biofilm cells of reference strain Tohama I**		
Name	**Localization**	**Function**	**Protein description**	**Localization**	**Function**
‡Chaperone protein, ClpB	Cytoplasm	Protein fate	Chaperone protein, ClpB	Cytoplasm	Protein fate
‡Trigger factor, Tig	Cytoplasm	Protein fate	Trigger factor, Tig	Cytoplasm	Protein fate
‡Probable parvulin-type peptidyl-prolyl cis-trans isomerase, BB3803	Periplasm	Hypothetical protein	Probable parvulin-type peptidyl-prolyl cis-trans isomerase, BB3803	Periplasm	Hypothetical protein
‡Chaperone protein, FimB/FhaD	Periplasm	cell wall organization	Chaperone protein, FimB/FhaD	Periplasm	cell wall organization
‡UDP-N-acetylmuramate--L-alanine ligase, MurC	Cytoplasm	Cell envelope	UDP-N-acetylmuramate--L-alanine ligase, MurC	Cytoplasm	Cell envelope
Filamentous hemagglutinin, FhaB	Outer membrane	Protein fate	Elongation factor, Tu	Cytoplasm	Protein synthesis
Tryptophan synthase alpha chain, TrpA	Cytoplasm	Amino acid biosynthesis	Putative amino-acid ABC transporter-binding protein, YhdW	Periplasm	Hypothetical protein
1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino) methylideneamino] imidazole-4-carboxamide isomerase, HisA	Cytoplasm	Amino acid biosynthesis	Elongation factor, Ts	Cytoplasm	Protein synthesis
3-isopropylmalate dehydratase small subunit 1, LeuD1	Cytoplasm	Amino acid biosynthesis	Chaperone protein, DnaK	Cytoplasm	Protein fate
50S ribosomal protein L1, RplA	Cytoplasm	Protein synthesis	Aminomethyltransferase, GcvT	Cytoplasm	Energy metabolism
N utilization substance protein B homolog, NusB	Unknown	Transcription	Chaperone protein, HtpG	Cytoplasm	Protein fate
Nucleotide-binding protein, BB4511	Cytoplasm	General	DNA-directed RNA polymerase subunit alpha, RpoA	Cytoplasm	Transcription
Adenylate kinase, Adk	Cytoplasm	Purines, pyrimidines, nucleosides, and nucleotides	Single-stranded DNA-binding protein, Ssb	Cytoplasm	DNA metabolism
ATP-dependent zinc metalloprotease, FtsH	Inner membrane	Cellular processes	Phosphopantetheine adenylyltransferase, CoaD	Cytoplasm	Biosynthesis of cofactors, prosthetic groups, and carriers
Threonine--tRNA ligase, ThrS	Cytoplasm	Protein synthesis	S-adenosylmethionine synthase, MetK	Cytoplasm	Central intermediary metabolism
30S ribosomal protein S10, RpsJ	Cytoplasm	Protein synthesis	Serotype 3 fimbrial subunit, (Fim3)	Extracellular	cell adhesion
ATP phosphoribosyltransferase, HisG	Cytoplasm	Amino acid biosynthesis	Protein, CyaY	Unknown	Biosynthesis of cofactors, prosthetic groups, and carriers
Lysine--tRNA ligase, LysS	Cytoplasm	Protein synthesis	Imidazole glycerol phosphate synthase subunit, HisH	Cytoplasm	Amino acid biosynthesis
50S ribosomal protein L17, RplQ	Cytoplasm	Protein synthesis	50S ribosomal protein, RplP	Cytoplasm	Protein synthesis

Protein downregulated in biofilm cells of clinical isolate ID20			Protein downregulated in biofilm cells of reference strain Tohama I		
Name	**Localization**	**Function**	**Protein description**	**Localization**	**Function**
Methionyl-tRNA, Fmt	Cytoplasm	Protein synthesis	50S ribosomal protein L24, RplX	Cytoplasm	Protein synthesis
50S ribosomal protein L22, RplV	Cytoplasmic	Protein synthesis	10 kDa chaperonin, GroS	Cytoplasm	Protein fate
Elongation factor, TuF1	Cytoplasm	Protein synthesis	30S ribosomal protein S18, RpsR	Cytoplasm	Protein synthesis
Acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha, AccA	Cytoplasm	Fatty acid and phospholipid metabolism	Succinyl-CoA ligase [ADP-forming] subunit beta, SucC	Cytoplasm	Energy metabolism

‡proteins downregulated in both clinical isolate ID20 and Tohama I

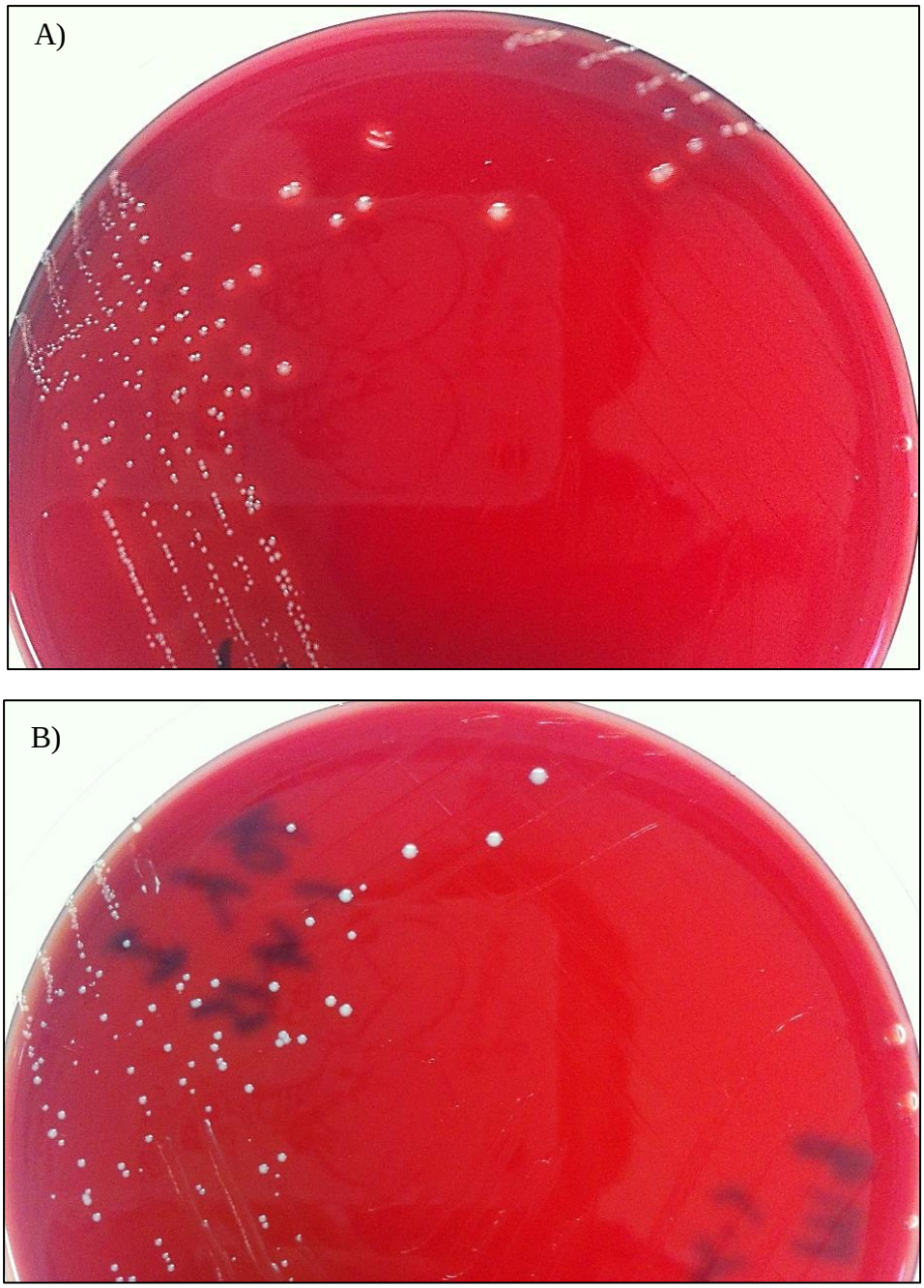
B. 2 Chapter 4

Appendix 4.1 Quality control report for samples submitted for *bvgS* sequencing.

SANGER SERVICE: Batch summary

ample Name	Q20 Bases	Intensity	Total bases	Q20 score (%)
S1_A01	677	1239	699	97
S2_A02	674	896	700	96
S3_A03	677	1175	699	97
S4_A04	674	697	698	97
S5_A05	674	657	700	96
S6_A06	674	1667	701	96
S7_A07	672	1643	700	96
S8_A08	672	1722	716	94
S9_A09	673	1583	700	96
S10_A10	673	781	700	96
S11_A11	667	2271	698	96
S12_A12	675	626	702	96
S13_B01	674	1975	716	94
S14_B02	772	1868	836	92
S15_B03	676	1360	701	96
S16_B04	674	513	700	96
S17_B05	672	1678	700	96
S18_B06	677	1404	701	97
S19_B07	676	845	700	97
S20_B08	682	1576	720	95
S21_B09	668	1906	699	96
T1_B10	679	1308	700	97

Sample name; name of the sample (S1 to S21 represent 21 clinical isolates and T1 represent Tohama I, A and B indicate well position of micro-titre plate), Q20 Bases: the number of bases in the sample sequence that give a Q20 score (>99% confidence) for each base call. Intensity: The average raw signal intensity of the sample. Q20 score for all the samples were shown.



Appendix 4.2. *B. pertussis* demonstrates haemolytic activity during virulent phase. A clear zone of haemolysis is seen on BG agar (A) when *B. pertussis* Tohama I (shown) was grown under common laboratory conditions while the presence of 50 mM $MgSO_4$ inhibits the haemolytic activity (B).

B.3 Chapter 5

Appendix 5.1 List of proteins identified from soluble fractions of *B. pertussis* biofilm and planktonic cells using iTRAQ. The table is ordered based on the total protein score. Cov; coverage, Bio; biofilm, Plk; planktonic cells, ID20; clinical isolate ID20, Toh; Tohama I. Significantly upregulated or downregulated proteins were indicated by *P*-value and coloured as described in Materials and Method section.

No.	Total	% Cov	Protein name	Peptides (95%)	Localisation	Function	Signal Peptide	Tohama I		ID20:Toh	*P* val ID20:Toh	ID20	
								Bio:Plk	*P* val (Bio:Plk)			Bio:Plk	*P* val (Bio:Plk)
1	161.91	77	60 kDa chaperonin, GroL	189	Cytoplasm	Protein fate	No	1.2134	0.1157	2.0512	0.0354	1	0.8799
2	117.31	36.6	Filamentous haemagglutinin, FhaB	95	Outer membrane	Adhesion	No	1.0471	0.3787	0.9908	0.2001	0.863	0.0126
3	83.33	60.5	Chaperone protein ClpB	55	cytoplasm	Protein fate	No	0.8091	0	1.0965	0	0.9376	0.0249
4	64.19	55.9	Chaperone protein DnaK	51	cytoplasm	Protein fate	No	0.912	0.001	1.0093	0.7336	0.9638	0.5465
5	46.13	72.7	Elongation factor Tu	50	cytoplasm	Protein synthesis	No	0.1854	0.0034	0.6607	0.1791	0.1644	0.16
6	42.28	66.5	Trigger factor Tig	32	cytoplasm	Protein fate	No	0.0308	0.0002	0.9727	0.335	0.1368	0
7	38.66	85.7	Virulence factors putative positive transcription regulator BvgA	40	Cytoplasm	Regulatory functions	No	2.2284	0.0015	0.8472	0.8731	0.2704	0.0563
8	37.37	39.9	Polyribonucleotide nucleotidyltransferase PNP	27	cytoplasm	Transcription	No	1.5136	0.0226	0.6918	0.557	4.0179	0.0102
9	36.42	58.4	ATP synthase subunit beta, AtpD	24	cytoplasm	Energy metabolism	No						
10	35.55	34.9	DNA-directed RNA polymerase subunit beta, RpoC	18	Cytoplasm	Transcription	No	1.2589	0.1524	0.8551	0.6449	0.7447	0.1613
11	32.44	57.6	Elongation factor G, FusA	23	Cytoplasm	Protein synthesis	No	0.5598	0.7529	0.6427	0.796	0.5012	0.271
12	32.34	34.6	BrkA autotransporter, BrkA	32	Outer membrane	Protein fate	Yes	0.5105	0.1619	1.8365	0.0794	0.2377	0.3565
13	29.88	58.6	Succinyl-CoA ligase [ADP-forming] subunit beta, sucC	20	Cytoplasm	Energy metabolism	No	0.1019	0.0425	1.8707	0.1407	0.3404	0.0635
14	28.31	62.3	Elongation factor Ts OS=Bordetella bronchiseptica, tsf	18	cytoplasm	Protein synthesis	No	0.078	0.0251	1.0471	0.2886	0.6194	0.9155
15	28.2	66.3	Malate dehydrogenase OS=Bordetella bronchiseptica, mdh	24	cytoplasm	Energy metabolism	No	0.3664	0.0917	0.6855	0.7135	0.8872	0.8291
16	27.03	36.6	Aspartate--tRNA(Asp/Asn) ligase, aspS	15	cytoplasm	Protein synthesis	No	2.208	0.026	2.355	0.1534	3.3729	0.0043

No.	Total	% Cov	Protein name	Peptides (95%)	Localisation	Function	Signal Peptide	Tohama I		ID20:Toh	*P* val ID20:Toh	ID20	
								Bio:Plk	*P* val (Bio:Plk)			Bio:Plk	*P* val (Bio:Plk)
17	26.26	36.4	Pertactin autotransporter, prn	17	Outer membrane	Protein fate	Yes	1.406	0.4628	1.1482	0.4918	0.2606	0.7493
18	25.75	52.6	Protein TolB, tolB	20	Periplasm	Transport and binding proteins	Yes	0.492	0.3656	1.8535	0.0096	0.871	0.6552
19	25.54	43.2	Chaperone protein HtpG, htpG	13	cytoplasm	Protein fate	No	0.133	0.0018	1.1376	0.7407	0.5152	0.6711
20	25.11	65.6	Aminomethyltransferase, gcvT	18	Cytoplasm	Energy metabolism	No	0.3467	0.0236	0.157	0.0039	0.1871	0.1442
21	24.47	31.7	Protein translocase subunit, *secA*	13	Cytoplasm	Protein fate	No	1.5417	0.4382	0.6607	0.1241	1.3932	0.6782
22	24.42	31	DNA-directed RNA polymerase subunit beta, *rpoB*	12	Cytoplasm	Transcription	No	0.3837	0.2209	0.3281	0.0228	2.6303	0.2537
23	24.13	58.2	N-acetyl-gamma-glutamyl-phosphate reductase, argC	16	Cytoplasm	Amino acid biosynthesis	No	0.6194	0.9702	0.2188	0.0309	3.6644	0.1415
24	24.02	50.3	Arginine biosynthesis bifunctional protein, ArgJ	15	cytoplasm	Amino acid biosynthesis	No	0.3532	0.8869	3.1915	0.0019	0.879	0.8669
25	24	38.7	Outer membrane porin protein, BP0840	18	Outer membrane	Porin activity	Yes	5.1051	0.0368	0.4613	0.2031	2.0893	0.8013
26	21.96	39.6	ATP synthase subunit alpha, atpA	13	cytoplasm	Energy metabolism	No	0.955	0.2728	0.1803	0.0188	8.5507	0.0291
27	21.04	63.5	ATP synthase subunit beta, atpD	17	Cytoplasmic membrane	Energy metabolism	No	14.8594	0	0.1096	0.0732	0.955	0.7202
28	20.73	32	Probable TonB-dependent receptor, BfrD	13	Outer membrane	Transport and binding proteins	Yes	7.2444	0	0.7586	0.4784	4.6989	0.074
29	20.14	47.7	Probable parvulin-type peptidyl-prolyl cis-trans isomerase, BB3803	19	Periplasm	Hypothetical protein	Yes	0.6486	0.0039	1.1482	0.0223	0.8551	0.0424
30	20.08	29	Proline--tRNA ligase, proS	10	Cytoplasm	Protein synthesis	No	1.0093	0.5662	0.3631	0.1199	11.695	0.4672
31	20	50	Ketol-acid reductoisomerase, ilvC	13	cytoplasm	Amino acid biosynthesis	No	0.4325	0.4016	0.8472	0.9996	0.7178	0.8486
32	19.77	30.9	Translation initiation factor IF-2, infB	10	cytoplasm	Protein synthesis	No	0.871	0.483	0.5649	0.2744	2.0324	0.0598
33	18.98	61.4	Acetylornithine aminotransferase 2, argD	11	cytoplasm	Amino acid biosynthesis	No	1.2134	0.2527	0.3373	0.2087	4.2073	0.1654
34	18	48.4	Ribose-phosphate pyrophosphokinase, prs	10	Cytoplasm	Purines, pyrimidines, nucleosides, and nucleotides	No	0.9817	0.6068	0.8166	0.2151	1.0765	0.8885
35	18	58.7	Azurin, BPP3406	29	Periplasm	Energy metabolism	Yes	0.3733	0.2042	35.6451	0.0028	0.3436	0.2054

No.	Total	% Cov	Protein name	Peptides (95%)	Localisation	Function	Signal Peptide	Tohama I		ID20:Toh	*P* val ID20:Toh	ID20	
								Bio:Plk	*P* val (Bio:Plk)			Bio:Plk	*P* val (Bio:Plk)
36	16.84	42.8	Tryptophan synthase alpha chain, trpA	10	Cytoplasm	Amino acid biosynthesis	No	0.2655	0.2002	1.6144	0.2468	0.2489	0.0298
37	16.8	39.5	Enolase, eno	13	Cytoplasm	Energy metabolism	No	1.5704	0.6464	2.0324	0.3434	6.1944	0.4117
38	16.65	46.7	Phosphoglucosamine mutase, glmM	9	Cytoplasm	Cell envelope	No	0.6252	0.9739	1.4859	0.0064	0.597	0.735
39	16.53	52.7	Aspartate-semialdehyde dehydrogenase, asd	8	Cytoplasm	Amino acid biosynthesis	No	0.912	0.5319	0.3373	0.1483	2.3988	0.0444
40	16.23	42.2	UDP-N-acetylmuramoylalanine--D-glutamate ligase, murD	10	Cytoplasm	Cell envelope	No	0.4742	0.1689	1.1376	0.1801	0.3802	0.2658
41	16.06	60.6	1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino)methylideneamino] imidazole-4-carboxamide isomerase,hisA	11	Cytoplasm	Amino acid biosynthesis	No	0.1259	0.099	1.4723	0.2623	0.092	0.0212
42	15.84	34.5	DNA-directed RNA polymerase subunit alpha, rpoA	11	Cytoplasm	Transcription	No	0.1306	0.0054	0.3048	0.016	1.4997	0.34
43	15.69	42.2	Malate synthase G, glcB	13	Cytoplasm	Energy metabolism	No	0.7379	0.176	0.5649	0.3112	3.767	0.2818
44	14.21	35.3	Probable cytosol aminopeptidase, pepA	10	cytoplasm	Protein fate	No	8.7902	0.0004	0.2128	0.1798	5.445	0.9562
45	14	27	Chaperone SurA, surA	7	Periplasm	Protein synthesis	Yes	0.1127	0.5845	2.2699	0.4383	0.0637	0.0916
46	13.82	55.5	4-hydroxy-tetrahydrodipicolinate synthase, dapA	7	Cytoplasmic	Amino acid biosynthesis	No	0.5012	0.2679	0.5702	0.1664	0.2884	0.2955
47	13.8	40.2	Adenylosuccinate synthetase, purA	8	Cytoplasm	Purines, pyrimidines, nucleosides, and nucleotides	No	2.5823	0.0636	0.2911	0.3371	9.0365	0.1586
48	13.26	39.5	Phosphoribosylformylglycinamidine cyclo-ligase, purM	9	Cytoplasm	Purines, pyrimidines, nucleosides, and nucleotides	No	0.9908	0.8283	0.8472	0.1505	1	0.2198
49	13.18	50	Superoxide dismutase [Fe], sodB	13	Periplasm	Unknown	No	0.1393	0.7269	0.955	0.3991	4.1305	0.0023
50	13.11	70.5	10 kDa chaperonin, groS	11	Cytoplasmic	Protein fate	No	0.0655	0.0924	2.9648	0.0038	2.3768	0.3221
51	12.04	50.8	ATP synthase subunit delta, atpH	7	Cytoplasm	Energy metabolism	No	0.4831	0.4346	0.6982	0.0588	0.1854	0.1028
52	12.03	35.5	Acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha, accA	6	cytoplasm	Fatty acid and phospholipid metabolism	No	0.7447	0.4429	1.3305	0.8871	0.0938	0.0191

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								Bio:Plk	*P* val (Bio:Plk)			Bio:Plk	*P* val (Bio:Plk)
53	12.01	72.6	6,7-dimethyl-8-ribityllumazine synthase, ribH	13	cytoplasm	Biosynthesis of cofactors, prosthetic groups, and carriers	No	1.1695	0.8302	0.3436	0.0999	7.3114	0.0308
54	12	34.5	Phosphoglycerate kinase, pgk	6	Cytoplasm	Unknown	No	0.3133	0.5499	1.3932	0.079	0.597	0.5864
55	12	46.7	Imidazoleglycerol-phosphate dehydratase, hisB	9	Cytoplasm	Protein synthesis	No	0.5297	0.4191	0.5916	0.5339	9.7275	0.2265
56	11.68	30.9	Indole-3-glycerol phosphate synthase, trpC	8	Cytoplasm	Protein synthesis	No	0.9204	0.5595	0.8017	0.4516	1.0186	0.9074
57	11.64	26	ATP-dependent Clp protease ATP-binding subunit, ClpX	7	Cytoplasm	Protein fate	No	3.9084	0.0265	4.1305	0.0879	0.631	0.4037
58	10.9	45.5	Orotate phosphoribosyltransferase, pyrE	8	Cytoplasm	Purines, pyrimidines, nucleosides, and nucleotides	No	0.9727	0.2579	0.9462	0.4504	0.7112	0.089
59	10.85	10.3	Bifunctional haemolysin/adenylate cyclase, cya	5	Extracellular	Regulatory functions	No	0.912	0.6248	14.8594	0.1013	0.0625	0.8506
60	10.78	47.8	50S ribosomal protein L4, rplD	7	Cytoplasm	Protein synthesis	No						
61	10.48	19.8	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase, metE	6	Cytoplasm	Amino acid biosynthesis	No	0.7178	0.1575	0.7047	0.2362	0.9727	0.9769
62	10.42	25.7	ATP phosphoribosyltransferase regulatory subunit, hisZ	5	Cytoplasm	Amino acid biosynthesis	No	1.5276	0.0416	0.4571	0.5408	10.5682	0.1055
63	10.38	35.3	Argininosuccinate synthase, argG	6	Cytoplasm	Amino acid biosynthesis	No	0.9376	0.3969	1.0471	0.9923	1.0965	0.6335
64	10.24	42.4	D-alanine--D-alanine ligase, ddl	6	Cytoplasm	Cell envelope	No	1.9055	0.9006	0.5546	0.5312	4.0926	0.6949
65	10.23	30.3	Adenosylhomocysteinase, ahcY	6	Cytoplasm	Energy metabolism	No	2.2699	0.7972	3.0479	0.8856	1.0375	0.7003
66	10.14	22.2	GTPase Der, der	5	Cytoplasm	Protein synthesis	No	2.1878	0.4497	1.0471	0.879	4.4055	0.0062
67	10.09	33.4	Outer membrane protein assembly factor, BamB	6	Outer membrane	Protein fate	No	2.9376	0.5722	2.704	0.3894	2.5119	0.009
68	10.07	25.1	Fructose-1,6-bisphosphatase class 1, fbp	6	Cytoplasm	Energy metabolism	No	0.955	0.8007	1.1066	0.2905	1.0093	0.8856
69	10.05	58.2	50S ribosomal protein L6, rplF	5	Cytoplasm	Protein synthesis	No	0.4613	0.529	1.5996	0.1228	0.2655	0.0629

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								Bio:Plk	*P* val (Bio:Plk)			Bio:Plk	*P* val (Bio:Plk)
70	10.03	31.2	ATP synthase gamma chain, atpG	5	Cytoplasm	Energy metabolism	No	1.0093	0.4027	0.1138	0.1037	27.0396	0.1599
71	10	7.8	Surface layer protein	7	Unknown	Unknown	No	0.9376	0.2436	0.0196	0.044	2.3121	0.2091
72	10	27.1	Anthranilate phosphoribosyltransferase, trpD	5	Cytoplasm	Amino acid biosynthesis	No	0.631	0.2685	0.0759	0.071	0.2858	0.243
73	10	33.6	Uridylate kinase, pyrH	10	Cytoplasm	Purines, pyrimidines, nucleosides, and nucleotides	No	1.1272	0.4025	1.2474	0.3449	1.028	0.6295
74	9.78	40.6	Chaperone protein FimB/FhaD, fimB	6	Periplasm	Cell wall organisation	No	0.2312	0.0082	2.3988	0.0045	0.0832	0.0012
75	9.73	64.6	50S ribosomal protein L7/L12, rplL	13	Unknown	Protein synthesis	No	0.4613	0.4178	0.7798	0.4679	0.871	0.5373
76	9.68	68.5	Protein GrpE	9	cytoplasm	Protein fate	No	0.0887	0.264	0.4018	0.6035	0.4169	0.1761
77	11.73	27.3	Elongation factor Tu, tuf	16	cytoplasm	Protein synthesis		8.2414	0.0043	0.0366	0.0869	99.0832	0.2171
78	9.46	37.8	Bifunctional protein, FolD	8	Cytoplasm	Protein synthesis	No	0.4446	0.0876	1.9055	0.4666	0.492	0.1966
79	9.33	18.9	GMP synthase [glutamine-hydrolyzing], guaA	5	Cytoplasm	Purines, pyrimidines, nucleosides, and nucleotides	No	1.6293	0.7296	0.631	0.1953	0.2032	0.5076
80	9.09	44.3	Serine hydroxymethyltransferase 2,glyA2	7	Cytoplasm	Protein synthesis	No	0.1028	0.1912	0.5346	0.5339	0.7178	0.4956
81	8.68	57.2	Single-stranded DNA-binding protein,ssb	4	Cytoplasm	DNA metabolism	No	0.1871	0.0208	0.879	0.9756	0.138	0.1676
82	8.66	31.5	3-isopropylmalate dehydratase small subunit 1, leuD1	5	Cytoplasm	Amino acid biosynthesis	No	1.977	0.6045	6.368	0.0032	0.1837	0.028
83	8.65	38.7	Acetylglutamate kinase , argB	4	Cytoplasm	Amino acid biosynthesis	No	0.4966	0.1335	0.2884	0.0303	0.3532	0.2553
84	8.55	49.3	Uracil phosphoribosyltransferase , upp	6	Cytoplasm	Purines, pyrimidines, nucleosides, and nucleotides	No	0.3767	0.1957	1.3062	0.404	0.3908	0.3651
85	8.33	45.5	50S ribosomal protein L11, rplK	7	Cytoplasm	Protein synthesis	No	1	0.2793	1.2359	0.9553	0.1318	0.0848
86	8.29	27.6	50S ribosomal protein L1, rplA	6	Cytoplasm	Protein synthesis	No	0.5152	0.5298	1.5996	0.2856	0.0752	0.0256

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								Bio:Plk	*P* val (Bio:Plk)			Bio:Plk	*P* val (Bio:Plk)
87	8.21	48.4	Orotidine 5'-phosphate decarboxylase, pyrF	5	Cytoplasm	Purines, pyrimidines, nucleosides, and nucleotides	No	0.2032	0.1624	0.631	0.7995	1.8535	0.5519
88	8.21	30.9	Protein RecA, recA	4	Cytoplasm	DNA metabolism	No	1.1272	0.4335	1.4588	0.3677	0.7516	0.8238
89	8.15	38.2	Peptide deformylase 1, def1	4	Cytoplasm	Protein fate	No	0.1923	0.1498	0.7943	0.5231	2.729	0.1015
90	8.14	55.6	UPF0234 protein, BB1300	4	Cytoplasm	Unknown	No	0.492	0.2083	0.4966	0.4553	0.1923	0.126
91	8.11	37.3	Porphobilinogen deaminase, hemC	4	Cytoplasm	Biosynthesis of cofactors, prosthetic groups, and carriers	No	0.2938	0.5102	1.9588	0.6245	0.2228	0.7633
92	8.1	46	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase, gpmA	4	Cytoplasm	Energy metabolism	No	1.1272	0.9751	0.8017	0.3661	2.2909	0.0606
93	8.1	27.4	Triosephosphate isomerase, tpiA	4	Cytoplasm	Energy metabolism	No	0.2535	0.5828	0.8872	0.8258	0.9908	0.7773
94	8.06	21.5	4-hydroxy-3-methylbut-2-enyl diphosphate reductase, ispH	4	Cytoplasm	Biosynthesis of cofactors, prosthetic groups, and carriers	No	0.8472	0.5728	0.9462	0.6301	0.863	0.9191
95	8.02	70.8	N utilisation substance protein B homolog, nusB	5	Unknown	Transcription	No	0.5754	0.5918	1.1376	0.6342	0.1202	0.0297
96	8.01	69.3	ATP synthase epsilon chain, atpC	7	Cytoplasm	Energy metabolism	No	0.6138	0.6685	0.8551	0.7982	1.6444	0.0094
97	8	23.2	Bifunctional protein GlmU, glmU	5	Cytoplasm	Cell envelope	No	0.2399	0.9883	0.8551	0.8945	0.8241	0.8955
98	8	16.4	Catalase, katA	6	Periplasm	Energy metabolism	No	0.7112	0.1802	1.9055	0.2723	1.1588	0.3066
99	8	26.5	Serotype 3 fimbrial subunit, fim3	5	Extracellular	Adhesion	Yes	0.492	0.0063	23.7684	0.0013	1.8535	0.1708
100	8	31.8	50S ribosomal protein L19, rplS	5	Cytoplasm	Protein synthesis	No	0.9036	0.8305	0.955	0.5702	1.028	0.4007
101	7.94	48.3	50S ribosomal protein L10, rplJ	6	Cytoplasm	Protein synthesis	No	1.9953	0.1613	1.0471	0.6101	0.1159	0.1521
102	7.87	29.8	Putative phosphoenolpyruvate synthase regulatory protein, BB2621	4	Cytoplasm	Unknown	No	1.2706	0.352	1.0864	0.9633	0.787	0.8917

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								Bio:Plk	*P* val (Bio:Plk)			Bio:Plk	*P* val (Bio:Plk)
103	7.79	34.1	3-isopropylmalate dehydrogenase 2, leuB2	5	Cytoplasm	Amino acid biosynthesis	No	0.6486	0.9697	2.7542	0.0675	0.4285	0.7633
104	7.67	32.1	Deoxycytidine triphosphate deaminase, dcd	4	Cytoplasm	Purines, pyrimidines, nucleosides, and nucleotides	No	1.0568	0.7076	1.3183	0.0836	0.8954	0.5038
105	7.46	30.1	Tyrosine--tRNA ligase, tyrS	4	Cytoplasm	Protein synthesis	No	0.2109	0.4713	0.7112	0.941	2.8314	0.3223
106	7.45	13.4	Glycine dehydrogenase (decarboxylating), gcvP	5	Cytoplasm	Energy metabolism	No	1.1272	0.907	0.8241	0.4493	0.9817	0.5164
107	7.4	40.8	50S ribosomal protein L5, rplE	4	Cytoplasm	Protein synthesis	No	0.8318	0.4711	0.6486	0.3473	0.3076	0.6677
108	7.32	53.2	30S ribosomal protein S7, rpsG	4	Cytoplasm	Protein synthesis	No	0.4487	0.4038	1.1588	0.5249	0.1905	0.4156
109	7.21	33.6	Pantothenate synthetase, panC	4	Cytoplasm	Biosynthesis of cofactors, prosthetic groups, and carriers	No	1.1169	0.9191	0.9817	0.5377	1.1912	0.4191
110	9.35	33.4	Elongation factor Tu, tuf	11	Cytoplasm	Protein synthesis	No	22.08	0.1372	0.3311	0.0502	0.787	0.2445
111	6.97	22.7	S-adenosylmethionine synthase, metK	4	Cytoplasm	Central intermediary metabolism	No	0.0879	0.0343	1	0.6592	0.5754	0.4458
112	6.95	35.5	2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-succinyltransferase, dapD	4	Cytoplasm	Amino acid biosynthesis	No	0.7516	0.7006	0.9817	0.9947	2.5823	0.029
113	6.95	27.2	3-isopropylmalate dehydratase large subunit, leuC	4	Cytoplasm	Amino acid biosynthesis	No	1.1912	0.4189	1.9588	0.0963	0.5808	0.369
114	6.6	51.8	Cell division topological specificity factor, minE	5	Cytoplasm	Cellular processes	No	0.3597	0.6383	0.863	0.4621	0.0483	0.2765
115	8.57	15.3	ATP synthase subunit alpha , atpA	9	Cytoplasm	Energy metabolism	No	22.2843	0.0011	0.0832	0.0154	1.5996	0.2018
116	6.43	34.6	Imidazole glycerol phosphate synthase subunit HisF, hisF	3	Cytoplasm	Amino acid biosynthesis	No	0.1629	0.1805	1.0471	0.8456	0.4966	0.8251
117	6.42	33.4	ADP-L-glycero-D-manno-heptose-6-epimerase , hldD	3	Cytoplasm	Cell envelope	No	3.8726	0.2581	1.6444	0.5816	6.7298	0.0206
118	6.19	28.5	50S ribosomal protein L9, rplI	5	Cytoplasm	Protein synthesis	No	0.1148	0.1185	1.6596	0.575	0.0586	0.1857

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								Bio:Plk	*P* val (Bio:Plk)			Bio:Plk	*P* val (Bio:Plk)
119	6.14	16	Alanine--tRNA ligase, alaS	3	Cytoplasm	Protein synthesis	No	2.884	0.3774	2.1281	0.4318	0.4613	0.2745
120	6.11	34.3	Phosphopantetheine adenylyltransferase, coaD	3	Cytoplasm	Biosynthesis of cofactors, prosthetic groups, and carriers	No	0.1202	0.0171	0.3105	0.0605	0.1871	0.5645
121	6.08	34.3	Pyridoxine/pyridoxamine 5'-phosphate oxidase, pdxH	3	Cytoplasm	Biosynthesis of cofactors, prosthetic groups, and carriers	No	0.1282	0.2817	0.6081	0.5194	0.1514	0.069
122	6.07	22.3	Argininosuccinate lyase, argH	3	Cytoplasm	Amino acid biosynthesis	No	1.3552	0.1787	1.0093	0.8499	1.4322	0.3647
123	6.02	15.8	Virulence sensor protein BvgS, bvgS	3	Cytoplasm	Regulatory functions	No	0.9727	0.7316	1.3062	0.1372	0.9036	0.3868
124	6	16.2	CTP synthase, pyrG	3	Cytoplasm	Purines, pyrimidines, nucleosides, and nucleotides	No	0.6607	0.5665	0.863	0.7951	0.2014	0.3296
125	6	27.6	60 kDa chaperonin , groL	4	Cytoplasm	Protein fate	No	0.7112	0.8901	0.0308	0.0023	5.0119	0.0007
126	6	23.3	Glutathione synthetase, gshB	3	Cytoplasm	Biosynthesis of cofactors, prosthetic groups, and carriers	No						
127	6	29.8	Guanylate kinase, gmk	3	Cytoplasm	Purines, pyrimidines, nucleosides, and nucleotides	No	0.9727	0.5293	0.7244	0.752	0.7943	0.4162
128	6	31	Nucleotide-binding protein, BB4511	3	Cytoplasm	Protein synthesis	No	1.9588	0.6386	1.2706	0.2153	0.5445	0.0073
129	6	34	2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase, ispF	3	Cytoplasm	Biosynthesis of cofactors, prosthetic groups, and carriers	No	0.6918	0.2706	1.0864	0.5424	0.8472	0.4658
130	6	32.9	Pertussis toxin subunit 4, ptxD	4	Multiple	Protein fate	No	1.0375	0.6588	2.466	0.1898	0.4487	0.4563

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								Bio:Plk	*P* val (Bio:Plk)			Bio:Plk	*P* val (Bio:Plk)
131	6	30.9	50S ribosomal protein L25, rplY	3	Cytoplasmic	Protein synthesis	No	1.2823	0.3875	1.0471	0.8677	0.9638	0.6125
132	6	36.2	Deoxyuridine 5'-triphosphate nucleotidohydrolase, dut	3	Unknown	Purines, pyrimidines, nucleosides, and nucleotides	No	0.169	0.1257	0.8395	0.6047	0.3342	0.954
133	6	28	N-(5'-phosphoribosyl)anthranilate isomerase, trpF	3	Unknown	Amino acid biosynthesis	No	0.631	0.7043	0.3767	0.3504	0.3251	0.8542
134	6	15.5	Outer membrane protein A, ompA	5	Outer membrane	Protein fate	Yes	2.7797	0.0018	2.3988	0.0018	4.529	0.031
135	6	55.2	Cold shock-like protein, cspA	5	Cytoplasm	Protein synthesis	No	0.0325	0.1862	3.02	0.2456	0.1556	0.1752
136	5.95	33	Protein CyaY , cyaY	4	Unknown	Biosynthesis of cofactors, prosthetic groups, and carriers	No	0.4487	0.0096	1.1376	0.0569	0.8395	0.9347
137	5.65	33.2	Thiazole synthase, thiG	5	Cytoplasm	Energy metabolism	No	0.1786	0.767	2.6546	0.3829	0.1738	0.4355
138	9.18	11.8	60 kDa chaperonin, groL	5	Cytoplasm	Protein fate	No	1.1482	0.6986	0.0377	0.0811	15.1356	0.1118
139	5.62	39.2	Acyl carrier protein, acpP	8	Cytoplasm	Fatty acid and phospholipid metabolism	No	0.3945	0.243	0.863	0.776	7.4473	0.0227
140	5.44	21.6	Elongation factor P, efp	5	Cytoplasm	Protein synthesis	No	0.8954	0.8715	1.1695	0.6864	0.1941	0.977
141	5.41	37	Glutamate-1-semialdehyde 2,1-aminomutase, hemL	3	Cytoplasm	Biosynthesis of cofactors, prosthetic groups, and carriers	No	1.3428	0.7745	0.9908	0.6363	7.5858	0.5169
142	5.35	19.8	Phosphoribosylaminoimidazole-succinocarboxamide synthase, purC	3	Cytoplasm	Purines, pyrimidines, nucleosides, and nucleotides	No	0.3281	0.3891	0.1803	0.0906	6.4863	0.0682
143	5.31	17	Dihydroxy-acid dehydratase 1, ilvD	3	Cytoplasm	Amino acid biosynthesis	No	1.0186	0.8957	0.9817	0.9183	1.3428	0.1442
144	5.3	32.3	30S ribosomal protein S3, rpsC	5	Cytoplasm	Protein synthesis	No	0.4018	0.4182	0.2443	0.2586	0.1432	0.2931

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								Bio:Plk	*P* val (Bio:Plk)			Bio:Plk	*P* val (Bio:Plk)
145	5.28	13.6	Glutamine--fructose-6-phosphate aminotransferase [isomerizing], glmS	3	Cytoplasm	Cell envelope	No	1.2589	0.267	1.0864	0.893	1.0765	0.5606
146	5.25	48.1	30S ribosomal protein S8, rpsH	3	Cytoplasm	Protein synthesis	No	0.3945	0.312	1.1376	0.9481	0.2443	0.1416
147	5.14	37.4	30S ribosomal protein S2, rpsB	3	Cytoplasm	Protein synthesis	No	0.5445	0.5361	0.955	0.7709	0.2188	0.2762
148	5.03	22.8	Tryptophan synthase beta chain, trpB	3	Cytoplasm	Amino acid biosynthesis	No	1.4588	0.7276	1.3552	0.2048	0.7244	0.4466
149	4.99	19.9	Enolase, eno	3	Cytoplasm	Energy metabolism	No	0.7447	0.6309	0.0213	0.0515	2.6792	0.0338
150	4.98	24.3	Adenylate kinase, adk	3	Cytoplasm	Purines, pyrimidines, nucleosides, and nucleotides	No	0.6026	0.3552	1.2942	0.2266	0.0938	0.0317
151	4.36	15.9	Threonine--tRNA ligase, thrS	3	Cytoplasm	Protein synthesis	No	1.4322	0.0009	1.1066	0.0009	0.4656	0.004
152	4.36	46.3	Putative septation protein, spoVG	3	Unknown	Unknown	No	0.2559	0.369	0.0366	0.1796	8.1658	0.4457
153	4.25	31.1	30S ribosomal protein S10 , rpsJ	2	Cytoplasm	Protein synthesis	No	0.2535	0.0915	1.2823	0.4626	0.0718	0.0234
154	4.17	33.3	Imidazole glycerol phosphate synthase subunit, hisH	2	Cytoplasm	Amino acid biosynthesis	No	0.6427	0.0091	1.028	0.8677	0.9638	0.1543
155	4.17	6.5	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, sucB	4	Cytoplasm	Energy metabolism	No	3.0479	0.3308	5.1523	0.1551	0.1472	0.1418
156	4.14	19.1	Glutamate--tRNA ligase, gltX	3	Cytoplasm	Protein synthesis	No	0.0809	0.2539	1.028	0.7828	0.227	0.625
157	4.08	33.2	ATP phosphoribosyltransferase, hisG	2	Cytoplasm	Amino acid biosynthesis	No	1.4191	0.0177	1.0568	0.1096	0.9817	0.0307
158	4.07	42.3	Transcriptional regulator MraZ, mraZ	3	Cytoplasm	Unknown	No	0.9727	0.741	1.0765	0.7957	0.7586	0.7567
159	4.05	15.5	30S ribosomal protein S4, rpsD	2	Cytoplasm	Protein synthesis	No	0.52	0.3354	0.4742	0.4575	0.2089	0.2244
160	4.03	63.3	50S ribosomal protein L7/L12, rplL	6	Cytoplasm	Protein synthesis	No	0.2109	0.2337	0.0146	0.0903	4.0551	0.2267
161	4.02	21.8	Transcription termination factor, rho	4	Cytoplasm	Transcription	No	1.1066	0.9843	1.3677	0.588	0.9376	0.9805
162	4.02	13.6	Lysine--tRNA ligase, lysS	2	Cytoplasm	Protein synthesis	No	0.6918	0.4169	1.1272	0.3096	0.52	0.0073

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								Bio:Plk	*P* val (Bio:Plk)			Bio:Plk	*P* val (Bio:Plk)
163	4.02	29.8	50S ribosomal protein L17, rplQ	3	Cytoplasm	Protein synthesis	No	0.3597	0.3652	0.8091	0.9431	0.0445	0.006
164	4.01	37.6	Ribosome-recycling factor, frr	3	Cytoplasm	Protein synthesis	No	0.0787	0.0509	1.3804	0.3618	0.2109	0.1815
165	4.01	46.4	Thiol:disulfide interchange protein, dsbA	3	Cytoplasm	Protein fate	Yes	0.1432	0.287	11.3763	0.128	0.035	0.0992
166	4.01	25.6	ATP synthase subunit b, atpF	3	Cytoplasm	Energy metabolism	No	3.1333	0.4008	0.3597	0.5547	2.6792	0.5188
167	8	14.9	ATP synthase subunit beta 2, atpD2	8	Cytoplasm	Energy metabolism	No	30.761	0.0873	0.0483	0.1906	99.0832	0.2568
168	6	7.7	Chaperone protein ClpB 2, clpB2	3	Cytoplasm	Protein fate	No	0.7379	0.5487	1.0864	0.6502	0.1225	0.1828
169	4	14	RNA polymerase sigma factor, rpoD	3	Cytoplasm	Transcription	No	4.4463	0.3157	0.7943	0.7043	8.9536	0.2391
170	4	18.2	Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit B, gatB	2	Multiple	Protein synthesis	No	1.1803	0.4638	1.1169	0.8211	1.0666	0.3651
171	4	6.2	Aconitate hydratase B, acnB	3	Cytoplasm	Energy metabolism	No	2.5823	0.2286	7.9433	0.1912	0.3311	0.5527
172	4	36.3	50S ribosomal protein L15, rplO	2	Cytoplasm	Protein synthesis	No	0.6982	0.1891	1.1803	0.7682	0.6427	0.8421
173	4	13	Arginine--tRNA ligase, argS	2	Cytoplasm	Protein synthesis	No	0.7727	0.7233	0.1959	0.6572	1.2134	0.9563
174	4	9.6	ATP-dependent zinc metalloprotease, ftsH	3	Cytoplasm	Cellular processes	No	1.0375	0.2882	0.2884	0.6269	0.2128	0.0406
175	4	10	UPF0365 protein OB1959	2	Cell Membrane	Unknown	Yes	15.5597	0.1313	0.4018	0.8156	5.6494	0.621
176	4	26.9	Protein-export protein, secB	6	Cytoplasm	Protein fate	No	0.4786	0.7308	1.3932	0.6798	0.6138	0.8417
177	4	12.8	Chorismate synthase, aroC	2	Cytoplasm	Amino acid biosynthesis	No	0.5649	0.9349	0.4529	0.3501	2.8054	0.1653
178	4	41.8	DNA-directed RNA polymerase subunit omega, rpoZ	2	Multipass	Transcription	No	0.3565	0.3234	0.6368	0.3202	0.0787	0.32
179	4	9.9	Inosine-5'-monophosphate dehydrogenase, guaB	2	Cytoplasm	Purines, pyrimidines, nucleosides, and nucleotides	No	0.4446	0.5656	0.3105	0.5276	4.3251	0.348
180	4	37.1	UPF0434 protein BB2007	2	Unknown	Unknown	No	0.0752	0.0519	0.8954	0.5569	0.2655	0.1029
181	4	17.7	Translation initiation factor IF-3, infC	2	Cytoplasm	Protein synthesis	No	0.0497	0.0521	0.8472	0.8554	0.078	0.1798

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								Bio:Plk	*P* val (Bio:Plk)			Bio:Plk	*P* val (Bio:Plk)
182	4	18.1	Transcriptional repressor, nrdR	2	Cytoplasm	Regulatory functions	No	1.3428	0.3029	0.9727	0.9272	1.5849	0.2923
183	4	12	50S ribosomal protein L13, rplM	2	Cytoplasm	Protein fate	No	0.4742	0.1711	1.1376	0.8266	0.3981	0.2597
184	4	14.2	Thiopurine S-methyltransferase, tpm	2	Cytoplasm	Unknown	No	13.1826	0.2902	1.7061	0.27	2.1281	0.3447
185	4	12	NADH-quinone oxidoreductase subunit C, nuoC	2	Cytoplasm	Energy metabolism	No	1.4997	0.4646	1.7539	0.3666	0.912	0.945
186	4	9.6	Alpha-keto acid-binding periplasmic protein, takP	3	Periplasm	Unknown	Yes	2.1281	0.595	0.138	0.3925	9.5499	0.2432
187	4	2.3	Bifunctional proteinA, putA	2	Cytoplasm	Energy metabolism	No	7.8705	0.2621	4.6989	0.5182	0.1393	0.3203
188	4	21.4	50S ribosomal protein L23, rplW	2	Cytoplasm	Unknown	No	1.1376	0.6459	1.0375	0.6956	1.1272	0.9256
189	4	14.4	30S ribosomal protein S2, rpsB	2	Cytoplasm	Protein synthesis	No	7.2444	0.128	0.0236	0.0972	0.879	0.9132
190	3.88	14.5	Chaperone protein, dnaJ	2	Cytoplasm	Unknown	No	0.8954	0.5243	0.8091	0.7335	1.2823	0.5739
191	3.8	6.3	LPS-assembly protein, lptD	2	Outer membrane	Cell envelope	Yes	17.5388	0.0291	3.3729	0.0385	2.729	0.273
192	3.77	3.1	Lon protease, lon	2	Cytoplasm	Protein fate	No	1.2942	0.3931	0.2489	0.704	0.871	0.5232
193	3.74	22.9	UDP-N-acetylmuramate--L-alanine, murC	3	Cytoplasm	Cell envelope	No	0.8472	0.0294	1.1803	0.1889	0.7727	0.0047
194	3.73	4.5	Bifunctional purine biosynthesis protein, purH	2	Cytoplasm	Purines, pyrimidines, nucleosides, and nucleotides	No	0.7178	0.3907	1.1803	0.5469	0.8551	0.588
195	3.65	22.6	Thymidylate synthase, thyA	2	Cytoplasm	Purines, pyrimidines, nucleosides, and nucleotides	No	0.7311	0.0958	1.3552	0.187	1.0186	0.7443
196	3.62	11.7	Putative amino-acid ABC transporter-binding protein, yhdW	2	Periplasm	Hypothetical protein	Yes	0.1202	0.0431	0.955	0.3925	0.6026	0.1975
197	3.46	37.3	ADP-dependent (S)-NAD(P)H-hydrate dehydratase, nnrD	3	Cytoplasm	Unknown	No	1.4191	0.9612	0.5297	0.1657	4.6559	0.1045
198	3.38	47.3	Ribosomal RNA small subunit methyltransferase H, rsmH	2	Cytoplasm	Cell envelope	No	0.6546	0.5692	1.9055	0.4709	3.6644	0.231
199	3.34	16.6	Ribosomal RNA small subunit methyltransferase A, rsmA	2	Cytoplasm	Protein synthesis	No	1.4859	0.5512	1.0965	0.8291	0.8017	0.1376

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								Bio:Plk	*P* val (Bio:Plk)			Bio:Plk	*P* val (Bio:Plk)
200	3.33	9.8	Lipid A export ATP-binding/permease protein, msbA	2	Cytoplasmic Membrane	Cell envelope	No	0.9204	0.4799	1.1803	0.5996	1.7219	0.3188
201	3.03	22.6	UPF0246 protein BB3890	2	Cytoplasm	Unknown	No	1.4454	0.2877	6.0813	0.1712	0.0655	0.2291
202	2.96	12.4	Uncharacterised protein	2	Cytoplasm	Unknown	No	0.1959	0.2849	2.0701	0.1641	0.2168	0.2614
203	2.83	26.6	Methionyl-tRNA formyltransferase , fmt	2	Cytoplasm	Protein synthesis	No	0.9376	0.3002	0.0946	0.0693	0.5152	0.0496
204	4.92	7	DNA-directed RNA polymerase subunit beta', rpoC	3	Cytoplasm	Transcription	No	3.4995	0.1918	0.4875	0.3223	3.02	0.2149
205	2.66	9.6	Phosphoribosylglycinamide formyltransferase 2, purT	2	Cytoplasmic membrane	Purines, pyrimidines, nucleosides, and nucleotides	No	0.7178	0.3946	0.863	0.1521	1.0965	0.0792
206	2.59	6.8	2-oxoglutarate dehydrogenase E1 component, odhA	2	Cytoplasm	Energy metabolism	No	1.1272	0.4501	5.3456	0.2874	2.6303	0.3338
207	2.59	11.5	Holliday junction ATP-dependent DNA helicase, ruvB	2	Cytoplasm	DNA metabolism	No	1.0965	0.8498	0.9817	0.9342	1.2942	0.4456
208	2.54	15.5	Ribosome maturation factor, rimP	2	Cytoplasm	Unknown	No	0.1585	0.1589	0.302	0.3854	1.9055	0.8817
209	2.49	35.7	30S ribosomal protein S6, rpsF	2	Cytoplasm	Protein synthesis	No	0.7656	0.4208	1.0375	0.7707	0.2884	0.396
210	2.4	65.4	RNA-binding protein, hfq	2	Cytoplasm	Regulatory functions	No	1.803	0.0288	0.9638	0.171	2.0893	0.0265
211	2.33	24.6	50S ribosomal protein L14, rplN	1	Cytoplasm	Protein synthesis	No	0.9727	0.8779	0.7112	0.5471	1.5276	0.4365
212	2.28	17.1	Membrane protein insertase, yidC	1	Cytoplasmic membrane	Protein fate	No	20.1372	0.0271	8.5507	0.0425	1.5996	0.0324
213	2.27	15.9	Adenine phosphoribosyltransferase, apt	2	Cytoplasm	Purines, pyrimidines, nucleosides, and nucleotides	No	1.9055	0.2187	0.2965	0.2128	2.8314	0.1348
214	2.26	41.5	30S ribosomal protein S9, rpsI	2	Cytoplasm	Translation	No	0.2032	0.4431	1.2359	0.6713	0.0991	0.2073
215	2.2	52.2	DNA-binding protein HU, hupA	1	Multiple	Unknown	Yes	0.0291	0.0699	0.3311	0.445	13.9316	0.1168
216	2.16	21.2	Probable thiol:disulfide interchange protein, dsbC	1	Periplasm	Unknown	Yes	0.3837	0.2243	2.3988	0.2849	0.4487	0.5488
217	2.1	21.7	UDP-2,3-diacylglucosamine hydrolase, lpxH	1	Cytoplasm	Cell envelope	No	1.0093	0.447	1.5136	0.1726	0.6918	0.3335

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								Bio:Plk	*P* val (Bio:Plk)			Bio:Plk	*P* val (Bio:Plk)
218	2.07	19.8	Putative anti-sigma factor antagonist, btrV	1	Unknown	Regulatory functions	No	1.2246	0.4771	3.4041	0.2347	0.4571	0.3188
219	2.06	45	50S ribosomal protein L22, rplV	1	Cytoplasm	Protein synthesis	No	1.1912	0.159	0.8091	0.9163	0.0545	0.0023
220	2.06	27.3	30S ribosomal protein S13, rpsM	1	Cytoplasm	Protein synthesis	No	0.912	0.8747	1	0.9596	0.673	0.4784
221	2.04	36.2	Ribonuclease PH, rph	2	Cytoplasm	Transcription	No	0.6792	0.4182	0.4571	0.1016	1.9953	0.0345
222	2.04	36.9	Aspartate 1-decarboxylase , panD	1	Cytoplasm	Biosynthesis of cofactors, prosthetic groups, and carriers	No	0.8318	0.859	7.5858	0.3107	2.466	0.3477
223	2.03	20.4	PKHD-type hydroxylase BPP2533	1	Cytoplasm	Unknown	No	0.6792	0.6818	0.6982	0.588	1.0568	0.9659
224	2.02	47.8	50S ribosomal protein L16, rplP	1	Cytoplasm	Protein synthesis	No	0.2965	0.0496	1.1169	0.7213	0.631	0.1769
225	2.02	43.4	50S ribosomal protein L24, rplX	1	Cytoplasm	Protein synthesis	No	0.4487	0.0176	3.4041	0.1126	0.1202	0.1311
226	2.02	25.5	30S ribosomal protein S3, rpsC	1	Cytoplasm	Protein synthesis	No	0.6427	0.0816	0.8318	0.1669	0.9908	0.2077
227	2.01	51.8	Nucleoside diphosphate kinase, ndk	1	Cytoplasm	Unknown	No	0.3873	0.0876	0.4246	0.1111	2.1086	0.0699
228	2.01	11.5	UPF0307 protein, BP2965	1	Cytoplasm	Unknown	No	0.0238	0.0574	1.0765	0.8305	0.0461	0.1514
229	2.01	6.6	GTP-sensing transcriptional pleiotropic repressor , codY	1	Cytoplasm	Regulatory functions	No	1.1169	0.8261	0.0692	0.0979	0.1236	0.2125
230	75.35	51.1	60 kDa chaperonin, groL	116	Cytoplasm	Protein fate	No						
231	36.42	58.4	ATP synthase subunit beta, atpD	24	Multiple	Energy metabolism	No						
232	21.63	23.3	60 kDa chaperonin, groL	32	Cytoplasm	Protein fate	No						
233	17.55	29.2	60 kDa chaperonin, groL	34	Cytoplasm	Protein fate	No						
234	10.78	47.8	50S ribosomal protein L4, rplD	8	Cytoplasm	Unknown	No	1.3932	0.7149	1.4322	0.4955	0.9376	0.4193
235	10.01	30.3	Elongation factor Tu, tuf1	13	Cytoplasm	Protein synthesis	No	0.6918	0.1203	1.3183	0.5199	0.047	0.0487
236	6	23.3	Glutathione synthetase, gshB	3	Cytoplasm	Biosynthesis of cofactors, prosthetic	No						

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								Bio:Plk	*P* val (Bio:Plk)			Bio:Plk	*P* val (Bio:Plk)
						groups, and carriers							
237	4	14.9	Elongation factor Tu, tuf	7	Cytoplasm	Protein synthesis	No	0.1406	0.1262	0.0111	0.0414	87.9023	0.0167
238	2.07	17.2	Uracil phosphoribosyltransferase, upp	1	Cytoplasm	Purines, pyrimidines, nucleosides, and nucleotides	No	1.9055	0.3416	0.1343	0.1231	3.6644	0.1857
239	2	9	Chromosome partition protein, smc	1	Cytoplasm	Cellular processes	No	1.406	0.5322	1.8535	0.3551	0.5808	0.4018
240	2	16.4	Cysteine--tRNA ligase, cysS	1	Cytoplasm	Protein synthesis	No						
241	2	24.2	Fumarate hydratase class II, fumC	1	Cytoplasm	Energy metabolism	No	0.6368	0.454	0.7727	0.6394	1.977	0.3314
242	2	22.3	CCA-adding enzyme , cca	1	Cytoplasm	Unknown	No	0.2992	0.2718	0.0247	0.1246	99.0832	0.1172
243	2	10.7	Probable aldehyde dehydrogenase SCO1174	1	Unknown	Energy metabolism	Yes	17.0608	0.1414	0.2089	0.476	1.9231	0.5467
244	2	7.9	Modification methylase XhoI	1	Cytoplasm	Unknown	No						
245	2	7.2	Methyl-accepting chemotaxis protein, mcpS	1	Cytoplasmic Membrane	Unknown	Yes	3.3113	0.1988	0.0391	0.1028	2.9923	0.1941
246	2	15.3	ATP-dependent protease ATPase subunit, hslU	1	Cytoplasm	Protein fate	No	0.912	0.9109	0.9817	0.6608	0.9376	0.5007
247	2	17.2	Dihydroorotate dehydrogenase (quinone), pyrD	1	Cytoplasm	Purines, pyrimidines, nucleosides, and nucleotides	No	0.4325	0.2753	0.6918	0.5126	0.7656	0.5885
248	2	5.5	Elongation factor G, fusA	1	Cytoplasm	Protein synthesis	No						
249	2	10.4	Putative glutamate--cysteine ligase 2, BB0289	1	Cytoplasm	Unknown	No	1.3062	0.5635	6.0256	0.2127	0.3221	0.1826
250	2	18	GTPase, obg	1	Unknown	Protein synthesis	No	0.1076	0.1478	0.5445	0.358	0.0955	0.192
251	2	7.8	Methionine--tRNA ligase , metG	1	Cytoplasm	Protein synthesis	No	0.4966	0.5222	0.7311	0.7751	0.0461	0.2173
252	2	57.8	Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit C,	1	Cytoplasm	Protein synthesis	No	0.5058	0.8561	2.5823	0.337	0.1096	0.3036
253	2	7.2	Vegetative catalase, katA	1	Periplasm	Unknown	No	1.4191	0.4985	0.3908	0.5984	26.7917	0.1103

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								Bio:Plk	*P* val (Bio:Plk)			Bio:Plk	*P* val (Bio:Plk)
254	2	8.3	Phospho-2-dehydro-3-deoxyheptonate aldolase, Phe-sensitive, aroG	1	Cytoplasm	Amino acid biosynthesis	No	0.1472	0.3162	0.3076	0.491	0.2249	0.3757
255	2	6.1	ATP-dependent zinc metalloprotease, ftsH	1	Cytoplasmic membrane	Cellular processes	No						
256	2	9.2	S-adenosylmethionine synthase, metK	2	Cytoplasm	Central intermediary metabolism	No	8.2414	0.1928	1.3932	0.7032	2.421	0.4377
257	2	6.5	2-isopropylmalate synthase, leuA	1	Cytoplasm	Amino acid biosynthesis	No	0.9462	0.6782	0.5649	0.32	1.7701	0.2245
258	2	13.5	30S ribosomal protein S8, rpsH	1	Cytoplasm	Protein synthesis	No	4.0551	0.1786	0.0118	0.0562	9.8175	0.0854
259	2	21.9	50S ribosomal protein L3, rplC	1	Cytoplasm	Protein synthesis	No	0.1138	0.6801	0.0991	0.1825	15.8489	0.1155
260	2	12.5	50S ribosomal protein L18, rplR	1	Cytoplasm	Protein synthesis	No	0.4406	0.5103	0.6252	0.978	0.2399	0.2974
261	2	12.8	D-beta-D-heptose 7-phosphate kinase, rfaE	1	Cytoplasm	Cell envelope	No	1.3428	0.7704	0.0871	0.625	0.9908	0.3843
262	2	11.6	3-oxoacyl-[acyl-carrier-protein] synthase 3, fabH	1	Multiple	Fatty acid and phospholipid metabolism	No	0.9204	0.91	0.8472	0.6891	1.1803	0.5493
263	2	11.1	Phosphate acyltransferase, plsX	1	Cytoplasm	Fatty acid and phospholipid metabolism	No	0.4169	0.2198	0.5598	0.1687	0.3133	0.9908
264	2	27	30S ribosomal protein S15, rpsO	1	Cytoplasm	Protein synthesis	No	0.2729	0.3064	0.631	0.9605	0.1107	0.1859
265	2	9.2	tRNA (guanine-N(7)-)-methyltransferase, trmB	1	Cytoplasm	Protein synthesis	No	0.9036	0.9506	0.597	0.9286	0.5395	0.2555
266	2	9.9	Electron transfer flavoprotein subunit alpha, etfA	2	Unknown	Unknown	No	1.7539	0.7993	3.5318	0.3795	0.8017	0.7732
267	2	19.6	Nitrogen regulatory protein P-II, glnB	2	Cytoplasmic membrane	Unknown	No	0.0236	0.1744	1.1376	0.8191	0.7311	0.6786
268	2	6.8	Alcohol dehydrogenase, adh	1	Cytoplasm	Unknown	No	1.6444	0.4665	0.0249	0.1369	7.5162	0.2386
269	2	13	Serotype 2 fimbrial subunit, fim2	1	Extracellular	Unknown	Yes	2.9648	0.2285	0.0227	0.0726	3.4356	0.3285
270	2	18.9	Cytochrome c	2	Cytoplasmic Membrane	Unknown	No	2.9107	0.3883	1.1803	0.6133	0.1614	0.2702
271	2	7	Taurine--pyruvate aminotransferase, tpa	1	Cytoplasm	Unknown	No	0.8017	0.689	0.5702	0.3932	3.3729	0.1974

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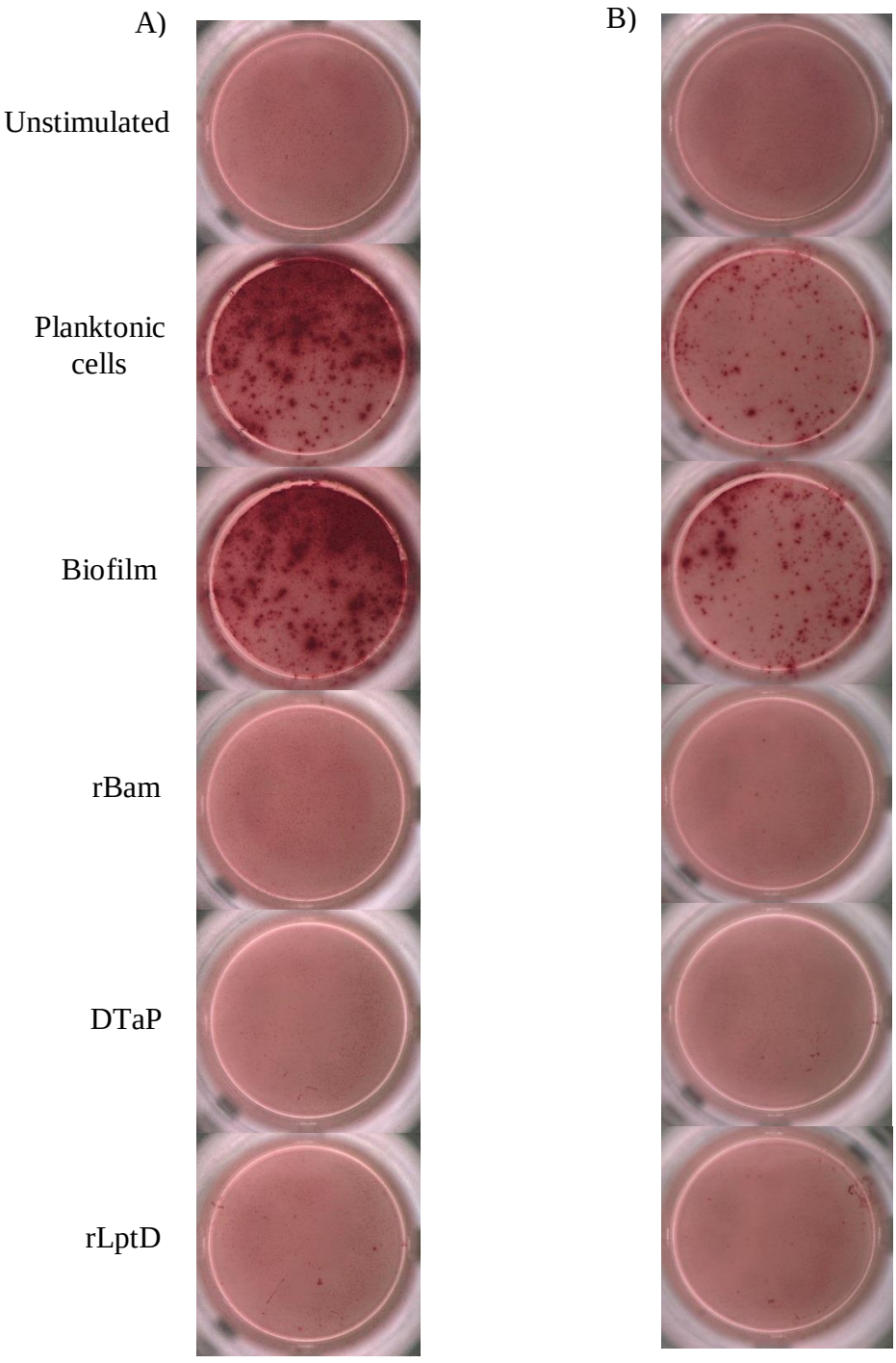
								Bio:Plk	*P* val (Bio:Plk)			Bio:Plk	*P* val (Bio:Plk)
272	2	40.4	10 kDa chaperonin, groS	1	Cytoplasm	Protein fate	No	0.0111	0.0335	0.0497	0.0829	6.0256	0.136
273	2	22.8	50S ribosomal protein L13, rplM	1	Cytoplasm	Protein synthesis	No	0.5754	0.4069	0.0273	0.0756	7.1121	0.4004
274	2	5.1	Chaperone protein, dnaK	1	Cytoplasm	Protein fate	No	0.787	0.659	0.2109	0.1573	1.4454	0.5151
275	2	7	50S ribosomal protein L10, rplJ	1	Cytoplasm	Unknown	No	0.4207	0.4521	0.0328	0.1418	2.3768	0.5221
276	2	15.1	Dual-specificity RNA methyltransferase, rlmN	1	Cytoplasm	Unknown	No						
277	2	9.7	50S ribosomal protein L16, rplP	1	Cytoplasm	Protein synthesis	No	3.767	0.2044	0.0256	0.1106	6.1944	0.5482
278	2	13.3	30S ribosomal protein S18, rpsR	1	Cytoplasm	Protein synthesis	No	0.0111	0.0351	0.8872	0.8303	0.2208	0.1626
279	2	7.4	NAD kinase, nadK	1	Cytoplasm	Unknown	No	6.4269	0.1314	0.4875	0.3211	0.0113	0.0627
280	2	11.4	Putative iron-sulfur cluster insertion protein, erpA	1	Cytoplasm	Biosynthesis of cofactors, prosthetic groups, and carriers	No	0.0809	0.1811	1.4191	0.8234	1.3428	0.6616
281	2	4.1	Pertussis toxin subunit 1 homolog, ptxA	1	Extracellular	Pathogenesis	Yes	0.0847	0.2092	1.4588	0.5442	0.2559	0.2649
282	2	3.2	Chromosomal replication initiator protein, dnaA	1	Cytoplasm	DNA metabolism	No	0.4699	0.4548	0.5105	0.367	0.7798	0.7955
283	2	11.2	Phosphoheptose isomerase, gmhA	1	Cytoplasm	Unknown	No	1.0375	0.5381	1.2359	0.6163	1.0568	0.7629
284	2	12.3	UPF0145 protein DSY2697	1	Unknown	Unknown	No	4.529	0.1982	0.0525	0.121	1.4859	0.358
285	2	24.3	Primosomal replication protein, priB	1	Unknown	Unknown	No	0.4093	0.8459	0.5248	0.5852	2.2284	0.4908
286	2	20.9	Stage V sporulation protein S, spoVS	1	Unknown	Unknown	No	1.5417	0.4598	0.8318	0.7357	0.2355	0.1691
287	2	4.2	Glyceraldehyde-3-phosphate dehydrogenase, gap	1	Cytoplasm	Energy metabolism	No	0.6855	0.493	1.2823	0.4205	0.8166	0.4679
288	2	10.4	Peptidyl-prolyl cis-trans isomerase B, ppiB	5	Cytoplasm	Unknown	No						
289	2	1.5	Cytochrome c oxidase subunit 1, ctaD	1	Cytoplasmic Membrane	Energy metabolism	No	55.4626	0.0774	2.1086	0.5586	5.445	0.3915
290	2	6.8	Uncharacterised oxidoreductase, ytbE	1	Cytoplasm	Unknown	No	1.6904	0.4038	0.7798	0.6453	2.421	0.2631
291	1.83	39.8	50S ribosomal protein L21, rplU	1	Cytoplasm	Protein	No	12.5892	0.4412	13.5519	0.5631	1.2474	0.6086

No.	Total	% Cov	Protein name	Peptides (95%)	Localisation	Function	Signal Peptide	Tohama I		ID20:Toh	*P* val ID20:Toh	ID20	
								Bio:Plk	*P* val (Bio:Plk)			Bio:Plk	*P* val (Bio:Plk)
						synthesis							
292	1.82	6.9	Argininosuccinate synthase, argG	1	Cytoplasm	Amino acid biosynthesis	No	1.2246	0.7638	0.1445	0.1855	1.1588	0.1152
293	1.82	17.7	50S ribosomal protein L19, rplS	1	Cytoplasm	Protein synthesis	No	0.7244	0.5618	2.3988	0.2687	0.4656	0.3092
294	1.81	8.5	Isoleucine--tRNA ligase, ileS	2	Cytoplasm	Protein synthesis	No	3.767	0.1809	1.5276	0.4706	0.7379	0.5938
295	1.8	5.5	Succinyl-CoA ligase [ADP-forming] subunit alpha, sucD	1	Cytoplasm	Energy metabolism	No	1.4191	0.7314	0.0649	0.1571	0.6026	0.6417
296	4	4.9	Transcription termination factor Rho, rho	2	Cytoplasm	Transcription	No	0.3532	0.6259	1.1912	0.7767	1.4859	0.8085
297	1.77	7.4	Histidinol dehydrogenase, hisD	1	Cytoplasm	Amino acid biosynthesis	No	0.227	0.2904	0.8872	0.4867	1.3428	0.4422
298	1.77	8.3	Thioredoxin, trxA	1	Cytoplasm	Energy metabolism	No	0.0718	0.2392	1.1588	0.8931	0.52	0.556
299	1.77	9.8	Nitrogen regulatory protein P-II, glnB	1	Cytoplasm	Unknown	No	0.1047	0.3863	0.9638	0.8829	1.1066	0.9646
300	1.77	13.8	Oligoribonuclease, orn	1	Cytoplasm	Unknown	No	1.3804	0.5097	0.2109	0.4121	1.4997	0.1485
301	1.77	4.3	Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta, accD	1	Cytoplasm	Fatty acid and phospholipid metabolism	No	2.2284	0.9633	6.9183	0.3396	0.4699	0.3789
302	1.72	5.8	Lon protease 1, lon1	1	Cytoplasm	Protein fate	No	1.0864	0.9025	0.3467	0.3977	4.0926	0.2545
303	1.68	7.1	30S ribosomal protein S7, rpsG	1	Cytoplasm	Protein synthesis	No	3.6644	0.2244	0.0394	0.0926	5.5463	0.1653
304	1.66	14	Phosphate-binding protein, pstS	2	Cytoplasmic membrane	Transport and binding proteins		87.9023	0.0172	87.9023	0.0179	9.5499	0.1089
305	1.57	4.9	GTP cyclohydrolase, folE2	1	Cytoplasmic	Unknown	No	0.2992	0.2011	1.2589	0.6454	0.4656	0.3034
306	3.66	13.3	Enolase, eno	2	Cytoplasm	Energy metabolism	No						
307	1.49	1.8	Elongation factor 4, lepA	1	Cytoplasm	Unknown	No	0.7311	0.5842	1	0.9886	1.0666	0.8774
308	2.85	14.5	Elongation factor G, fusA	4	Cytoplasm	Protein synthesis	No						
309	1.45	14.8	Succinyl-CoA ligase [ADP-forming] subunit beta, sucC	2	Cytoplasm	Energy metabolism	No	4.4875	0.1136	0.0555	0.0024	10.5682	0.0007
310	1.41	22.1	6,7-dimethyl-8-ribityllumazine synthase, ribH	1	Cytoplasm	Biosynthesis of cofactors,	No	1.2134	0.6933	1.3305	0.5889	0.8954	0.8379

No.	Total	% Cov	Protein name	Peptides (95%)	Localisation	Function	Signal Peptide	Tohama I		ID20:Toh	*P* val ID20:Toh	ID20	
								Bio:Plk	*P* val (Bio:Plk)			Bio:Plk	*P* val (Bio:Plk)
						prosthetic groups, and carriers							
311	1.41	2.1	1-deoxy-D-xylulose-5-phosphate synthase, dxs	1	Cytoplasm	Biosynthesis of cofactors, prosthetic groups, and carriers	No	0.9817	0.8478	0.1977	0.4716	3.5975	0.8977
312	1.39	27.6	Phosphoribosyl-AMP cyclohydrolase, hisI	2	Cytoplasm	Unknown	No	0.5495	0.3709	1.4588	0.5059	1.4588	0.503
313	1.39	1.2	UvrABC system protein C, uvrC	1	Cytoplasm	DNA metabolism	No	0.0247	0.1138	1.5996	0.481	0.0196	0.0985
314	1.38	4.4	RNA polymerase sigma factor, rpoH	1	Cytoplasm	Cellular processes	No	0.3802	0.2463	3.9084	0.1768	0.1644	0.1363
315	1.35	11.7	Pyridoxine 5'-phosphate synthase, pdxJ	1	Cytoplasm	Biosynthesis of cofactors, prosthetic groups, and carriers	No	1.3428	0.6479	0.4169	0.2672	10.5682	0.1063

B.4 Chapter 6



Appendix 6.1 Representative images of an ELISPOT plate. IFN-γ (A) and IL-17a (A) production by splenocytes of mice vaccinated with biofilm (shown) and stimulated with planktonic cells, biofilm, rBamB, DTaP or rLptD as described in section 6.2.10. Unstimulated control is shown.

Appendix C

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Biofilm forming potential and antimicrobial susceptibility of newly emerged Western Australian *Bordetella pertussis* clinical isolates

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ABSTRACT

Whooping cough caused by *Bordetella pertussis* is increasing in several countries despite high vaccine coverage. One potential reason for the resurgence is the emergence of genetic variants of the bacterium. Biofilm formation has recently been associated with the pathogenesis of *B. pertussis*. Biofilm formation of 21 Western Australian *B. pertussis* clinical isolates was investigated. All isolates formed thicker biofilms than the reference vaccine strain Tohama I while retaining susceptibility to ampicillin, erythromycin, azithromycin and streptomycin. When two biofilm-forming clinical isolates were compared with Tohama I, minimum bactericidal concentrations of antimicrobial agents increased. Isobaric tags for relative and absolute quantitation (iTRAQ)-based proteomic analysis revealed significant differences in protein expression in *B. pertussis* biofilms, providing an opportunity for identification of novel biofilm-associated antigens for incorporation in current pertussis vaccines to improve their protective efficacy. The study also highlights the importance of determining antibiograms for biofilms to formulate improved antimicrobial therapeutic regimens.

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KEYWORDS
Bordetella pertussis; biofilm; antimicrobial resistance/tolerance; iTRAQ; proteomic analysis

Introduction

Bordetella pertussis is the causative agent of whooping cough or pertussis, a highly contagious respiratory disease. Due to severe systemic complications associated with *B. pertussis* infection, the disease is more severe in infants but adults and adolescents remain susceptible. Severe complications of *B. pertussis* infection include pneumonia, encephalopathy, seizures, otitis media and brain haemorrhages (WHO 2010; CDC 2014). Despite high vaccine coverage among infants, whooping cough remains one of the most endemic vaccine-preventable diseases in most developed countries. This includes Australia (NNDSS 2013), The Netherlands (King et al. 2013) and the USA (CDC 2014). In 2008, the WHO estimated that about 16 million cases of pertussis occurred worldwide (including 95% in developing countries) with 195,000 children dying from the disease (WHO 2010). Deaths of 285,000 to 400,000 infants a year have been reported elsewhere (Parkhill et al. 2003; Fry et al. 2008).

The introduction of whole-cell vaccines [DTwP] in the 1940s and acellular pertussis vaccines [DTaP] in the 1990s reduced mortality due to pertussis. However, the recent resurgence of pertussis has been attributed to poor immune responses induced by the acellular pertussis vaccine(s) (APV) (Cherry et al. 2010). These vaccines elicit weak T-cell responses and effective stimulation of T-cell-mediated immunity is a prerequisite for long-term protection (Mills 2001; Rowe et al. 2005; Fadugba et al. 2014). Alternatively, another potential reason for resurgence may be evolution of novel genetic variants of *B. pertussis*, which may evade immunity provided by the current APVs. Isolates of *B. pertussis* carrying the non-vaccine *prn2* and/or *ptxP3* alleles have been reported from Europe (Kallonen et al. 2011), Australia (Octavia et al. 2012; Lam et al. 2013), Canada (Shuel et al. 2013) and Japan (Miyaji et al. 2013). Some *B. pertussis* isolates from vaccinated populations did not produce pertactin (PRN), an important antigen included in all current APVs (Martin et al. 2014; Pawloski et al. 2014; Zeddeman et al. 2014), raising questions on the role played by PRN in APV formulations. Another study reported over-production of *ptxP3* type of pertussis toxin (PTX) (Mooi et al. 2009). These findings suggest that the emerging strains of *B. pertussis* may have developed selective advantage driven

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To whom it may concern

I, Dorji Dorji, as a first author declare myself as the primary contributor to the experimental design, interpretation and writing to the paper/publication entitled **"Biofilm forming potential and antimicrobial susceptibility of newly emerged Western Australian *Bordetella pertussis* clinical isolates"**.

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I, as co-author, endorse that this level of contribution by the candidate indicated above is appropriate.

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