ROLE OF THE CAPSULE LOCUS IN THE VIRULENCE OF *BORDETELLA PERTUSSIS*

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DECLARATION

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

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SUMMARY

Our laboratory has recently demonstrated that *Bordetella pertussis*, the etiological agent of whooping cough, produces a surface polysaccharide microcapsule. Pertussis vaccination initiative over the past 60 years has led to significant reduction of incidence rate among young children. However, emergence of adult pertussis cases in recent years suggests that current vaccination fails to provide long-term protection and underscores the need to further study this disease and revisit the pertussis vaccination strategies. Polysaccharide capsules represent an important vaccine and antimicrobial target for many pathogens. The role of the polysaccharide capsule during *B. pertussis* infection has not been investigated. In this work, we have explored the role of the capsule genetic locus in pertussis pathogenesis.

We first constructed *B. pertussis* mutants containing unmarked in-frame deletion in different ORFs within the capsule operon. None of these mutants produced the microcapsule at their surface, similar to KO*caps* mutant deleted for the entire capsule operon. Deletion of the second ORF in the capsule operon, namely *kpsT*, predicted to encode the polysialic acid transport ATP binding protein, led to significant attenuation in colonization of the mouse lungs compared to the parental strain, which recapitulated the virulence defect observed with the KO*caps* mutant. In contrast, mutants deleted for *kspE*, the putative capsule exporter gene and *vipC*, the putative capsule biosynthesis gene displayed modest and no virulence defects respectively. These findings suggested that the polysaccharide capsule exposed at the surface of *B. pertussis* bacteria does not play a role in pertussis pathogenesis. Consistently, the attenuated phenotype observed in *kpsT*-deleted mutant correlated

with the global down-regulation of a variety genes that are either related to bacteria virulence or that encode putative proteins in *B. pertussis*. Key virulence factors FHA, BrkA and PT were slightly down-modulated at both transcriptional and protein levels compared to the parental strain. Since the great majority of the virulence factors in *B. pertussis* is under the control of the two component system BvgA/S, we focused on studying the effect of *kpsT* deletion on the BvgS-mediated signal transduction. Interestingly, we demonstrated that the virulence defect observed with the *kpsT*-deleted mutant was not observed in a *B. pertussis* mutant strain with constitutive activation of its BvgS sensor. This observation thus led us to propose that *kpsT* deletion impaired the function and activity of BvgS sensor. A BvgS pull down approach then revealed that BvgS sensor oligomerizes in parental *B. pertussis* strain, but not in the mutants deleted either for *kpsT* or for the entire capsule operon. This finding demonstrated that KpsT is involved in BvgS oligomerization, presumably BvgS dimerization, which is necessary for the sensor's activity and regulation of *bvg*-regulated genes. Sensitivity tests to antibiotic and chemical treatments supported that membrane associated KpsT protein participate to the plasma membrane integrity and permeability, which is crucial for the conformational integrity and optimal functionality of membrane proteins such as BvgS sensor. Collectively, our data demonstrate an alternative biological function of the capsular transporter KpsT in the central functioning of BvgS-mediated signal transduction in *B. pertussis*.

In addition, we characterized the transcriptional regulation of the capsule locus in different *B. pertussis* strains. Both clinical and laboratory-adapted (BPSM) strains demonstrated increased expression of the capsule locus when the BvgA/S regulatory system is inactive (Bvg^- phase) and vice versa (Bvg^+ phase), supporting that the

capsule locus belong to the class of *vrgs*. We hypothesized that RisA may regulate the transcription of the capsule locus in both BPSM phases; however, over-expression of RisA approaches failed to lend support to this hypothesis. In parallel, *risA* gene deletion could only be obtained in the presence of a wild-type copy of *risA* on a plasmid, thus demonstrating the essentiality of this gene in BPSM. The expression pattern of the capsule locus was also analyzed during *ex vivo* infection (epithelial cells and macrophages) and in the mouse model of pertussis infection. We observed that the capsule locus is highly expressed and dynamically modulated during cellular invasion as well as during the course of *in vivo* infection, reflecting the response of the bacteria to the host microenvironments during infection. These findings prompted us to re-evaluate the genetic regulation of the capsule locus and other *vrgs* during host infection.

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LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
AC	Adenylate cyclase
ADP	Adenosine diphosphate
Amp	Ampicillin
AP	Alkaline phosphatase
ATP	Adenosine triphosphate
B. bronchiseptica	*Bordetella bronchiseptica*
B. holmesii	*B. holmesii*
B. parapertussis	*Bordetella parapertussis*
B. pertussis	*Bordetella pertussis*
BCIP	5-bromo-4-chloro-3'-indolyphosphate p-toluidine
BG	Bordet-Gengou
bp	Base pair
BrkA	Bordetella serum resistance to killing protein A
BSA	Bovine serum albumin
bvg	Bordetella virulence gene
c-di-GMP	Cyclic di-guanine monosphosphate
$CaCl_2$	Calcium chloride
cDNA	Complementary DNA
cm	Centimeter
Cm	Chloramphenicol
CRD	Carbohydrate recognition domain
Ct	Threshold cycle

dCTP	Deoxycytidine triphosphate
DEPC	Diethylpyrocarbonate
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
DNT	Dermonecrotic toxin
dNTP	Deoxyribonucleotide triphosphates
E. coli	*Escherichia coli*
EAL	Glu-Ala-Leu
EDTA	Ethylenedinitrilo tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FHA	Filamentous hemagglutinin
FITC	Fluorescein isothiocyanate
GDP	Guanosine diphosphate
Gm	Gentamicin
GTP	Guanosine triphosphate
h	Hour
H. influenzae	*Haemophilus influenzae*
HCl	Hydrochloric acid
hib	*Haemophilus influenzae* type b
His	Histidine
His-tag	Histidine tag
HK	Histidine kinase

Hpt	Histidine-containing phosphotransfer
HRP	Horseradish peroxidase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
K. pneumoniae	*Klebsiella pneumoniae*
kb	kilobase
LB	Luria-Bertani
mA	Miliampere
µF	Microfarad
µg	Microgram
$MgSO_4$	Magnesium sulphate
min	Minute
µl	Microliter
ml	Milliliter
N	Number
N. meningitides	*Neisseria meningitides*
N. meningitidis	*Neiserria meningitides*
NaCl	Sodium chloride
NaH_2PO_4	Sodium dihydrogen phosphate
NaOH	Sodium hydroxide
NBT	Nitro-blue tetrazolium chloride
Ni-NTA	Nickel-Nitrilotriacetic acid
nm	Nanometer
OD	Optical density
ORF	Open reading frame
P-BvgA	Phosphorylated BvgA

P. aeruginosa	*Pseudomonas aeruginosa*
p.i.	Post-infection
PAGE	Polyacrylamide gel electrophoresis
PAS	Per-Arnt-Sim
PCR	Polymerase chain reaction
PT	Pertussis toxin
PVDF	Polyvinylidene difluoride
RE	Restriction enzyme
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Reverse-transcriptase PCR
s	Second
S. aureus	*Staphylococcus aureus*
S. pneumoniae	*Streptococcus pneumoniae*
S. typhi	*Salmonella typhi*
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
Sm	Streptomicin
sp	Species
SS	Stainer-Scholte
SSC	Saline-sodium citrate
T_a	Annealing temperature
TAE	Tris-acetate-EDTA
TBS	Tris-buffered saline

TCT	Tracheal cytotoxin
TE	Tris-EDTA
Tris-HCl	Tris-hydrochloride
UDP	Uridine diphosphate
UV	Ultra violet
V	Volt
vags	*bvg*-activated genes
VFT	Venus Fly Trap
vrgs	*bvg*-repressed genes

CHAPTER 1 INTRODUCTION

1.1 PATHOGENESIS OF *BORDETELLA PERTUSSIS*

1.1.1 *B. pertussis* Infection and Whooping Cough

Bordetella pertussis is a Gram-negative, obligate aerobe and fastidious coccobacilli that can only be cultivated in an enriched media supplemented with blood. *B. pertussis* is a strict human pathogen and the sole etiological agent for pertussis disease, or commonly known as whooping cough; a respiratory disease that was highly prevalent amongst infants prior to the development of pertussis vaccine in the 1940s. First isolated in 1906 by French microbiologist Bordet and Gengou, *B. pertussis* has since then been widely studied and characterized on its pathogenic and virulence capabilities.

The *Bordetella* genus comprises nine species, with four of them being phylogenetically closely related and all of them being respiratory pathogens of mammalian hosts (Diavatopoulos et al., 2005; Mooi, 2010). The four includes *B. bronchiseptica*, *B. parapertussis*, *B. pertussis* and *B. holmessii*. *B. bronchispetica* causes infectious bronchitis in a variety of mammals and although rarely, can be isolated from humans. The human-associated *B. parapertussis* and *B. pertussis*, which evolve from the former, causes pertussis in humans, while another sub-species of *B. parapertussis* has been reported to cause zoonotic respiratory tract infection in sheep (Diavatopoulos et al., 2005; Mooi, 2010).

B. pertussis is highly contagious with an attack rate of 80% among non-immunized population as it spreads easily via aerosolized droplets when coughed up by an infected host. The infected mammalian host, especially unvaccinated infants will ultimately develop chronic pertussis infection whereas adults will typically display an asymptomatic disease. During the course of infection, *B. pertussis* manifests its pathogenicity through multiple biological activities. The bacteria first establish infection by adhering to the ciliated epithelium linings at the upper respiratory tract by producing a group of virulence factors known as adhesins. Production and secretion of biologically active toxins from *B. pertussis* usually takes place at a later stage of infection, resulting in a more symptomatic and severe illness due to the destruction of mucosal epithelial lining by the toxins (Finger and von Koenig, 1996). Severe, spasmodic coughs with continuous whooping sound and lymphocytosis are hallmarks of pertussis infection in infants (Finger and von Koenig, 1996; Mattoo and Cherry, 2005). Serious complications including bronchopneumonia, seizure and respiratory arrest frequently result in death among infants (Finger and von Koenig, 1996; Mattoo and Cherry, 2005). In addition, following the colonization of the respiratory tract, *B. pertussis* not only adheres to epithelial cells and multiplies extracellularly, it can also persist within epithelial cells and survive within macrophages (Bassinet et al., 2000; Lamberti et al., 2010; Masure, 1992). Such phenomenon indicates that both cellular and humoral mediated immunity are triggered in response to *B. pertussis* infection and elimination (Lamberti et al., 2010).

1.1.2 *B. pertussis* Treatment and Vaccine

Pertussis disease and infectivity can be controlled and treated with common antibiotics including ampicillin, chloramphenicol, azithromycin and erythromycin (Bass et al., 1969; Lambert, 1979). Nevertheless, the development and widespread use of pertussis vaccine has been a primary focus to combat pertussis and has greatly reduced the disease burden among infants. Prior to the widespread use of pertussis vaccine in 1940s, pertussis was one of the most common causes of childhood morbidity and mortality with more than 200,000 cases reported annually in the United States alone according to the World Health Organization. Isolation and characterization of several virulence factors in *B. pertussis* has led to a better understanding of the pathogenesis of pertussis and immunity against the disease, which contributed to the development of acellular pertussis vaccines made of purified *B. pertussis* proteins.

Development of the conventional, inactivated whole-cell pertussis vaccine used in combination with diphtheria and tetanus toxoid has dramatically reduced childhood mortality cases associated with pertussis for the past 60 years (Mattoo and Cherry, 2005). Despite the efficacy of the whole cell vaccine and its routine immunization since the early 1950s to early 1990s, it is no longer as widely used due to the presence of endotoxin component harbored by the bacteria resulting in adverse side effects in children (Cherry, 1996; Cody et al., 1981). The acellular pertussis vaccine was refined in 1990s primarily as a booster for the whole cell vaccine and was subsequently

approved as primary pertussis vaccine due to its effectiveness and significant reduction in reactogenicity as compared to whole cell vaccine (Gustafsson et al., 1996; Olin et al., 1997; Zhang et al., 2011). The current five-component acellular pertussis consists of virulence factors filamentous hemagglutinin (FHA), inactivated pertussis toxin (PT), pertactin, fimbriae 2 and 3 subunits, all of which are major virulence factors that are either cell surface-associated or secreted (Gustafsson et al., 1996). The protective immunity of pertussis vaccine is highly dependent on cell-mediated and humoral immunity, with reports that acellular vaccine specifically drives the Th2 cell-mediated immunity (Mills et al., 1998; Watanabe et al., 2002). Recently, a live-attenuated *B. pertussis* vaccine candidate known as BPZE1 has been developed through targeted genetic manipulation and has reached phase-I human clinical trial (ClinicalTrials.gov NCT01188512)(Skerry et al., 2009). A single nasal administration of live BPZE1 bacteria was shown to confer a long-lasting immunity and strong protection against virulent *B. pertussis* in a murine model of infection, thus promoting a viable and attractive alternative to the current acellular pertussis vaccine (Skerry and Mahon, 2011).

1.1.3 Pertussis Epidemiology: A problem of re-emergence

Despite the widespread use and protective efficacy of acellular pertussis vaccines, pertussis is not completely eradicated unlike many other vaccine-eradicated infectious diseases such as smallpox, polio and rubella. As of 2011, the World Health Organization estimated about 140,000 reported cases of pertussis globally and the estimated number of deaths in 1998 was

close to 200,000. Interestingly, an epidemiological shift of pertussis infection towards adolescents and adults has been increasingly reported in developed countries with high acellular pertussis vaccine coverage (Berbers et al., 2009b; Cherry, 2005; Gilberg et al., 2002; He and Mertsola, 2008; Lin et al., 2007; Mattoo and Cherry, 2005; Pebody et al., 2005). This group of pertussis susceptible hosts, which are often asymptomatic increases the risk of transmission of pertussis to unvaccinated newborn infants, to whom the disease may be life-threatening (Cherry, 2005; Crowcroft and Britto, 2002; He and Mertsola, 2008).

Several hypotheses have been made with regards to factors contributing to the resurgence of pertussis in adolescence and adults. These include waning vaccine-induced immunity for both whole cell and acellular vaccine 10 years after the primary immunization, typically without booster against pertussis over time (Berbers et al., 2009b; Cherry, 2005). Thus, regular immunization booster schedule for pertussis vaccine has been reinforced among the adults and adolescents in developing countries (Berbers et al., 2009b). In contrary to the whole cell pertussis vaccine, the major component of acellular pertussis vaccines was limited to five *B. pertussis* virulence factors; FHA, PT, pertactin, fimbriae 2 and 3 subunit, hence resulting in a narrow, and specific immune response against the bacteria (He and Mertsola, 2008). The relatively specific immune response against the five major virulence factors may drive the emergence of antigenic variants among the circulating *B. pertussis* strains, indicating the adaptative capability of *B.*

pertussis isolates to overcome the current vaccination niche (Berbers et al., 2009b; He and Mertsola, 2008; Mooi et al., 2001).

Antigenic divergence between *B. pertussis* vaccine strains and the circulating *B. pertussis* clinical isolates has been reported in vaccinated populations, with evidences pointing at genetic polymorphisms and allelic variation in the components of current accellular vaccines, mainly the genetic elements encoding PT and pertactin (Berbers et al., 2009a; Cassiday et al., 2000; Gzyl et al., 2001; King et al., 2001; Mooi et al., 1998; Mosiej et al., 2011). In particular, the immunological memory derived from the vaccine strain may not protect against the circulating *B. pertussis* strains that has undergone changes in their genetic elements (Gzyl et al., 2001; King et al., 2001). The resurgence of pertussis in adults has also been attributed to improved disease surveillance and diagnosis methods; from culture to ELISA serology and the widespread use of PCR testing, which resulted in increased detection sensitivity and hence the number of cases being reported (Crowcroft and Pebody, 2006; Wendelboe and Van Rie, 2006). Factors that affect the epidemiological shift of pertussis remain a subject of debate and the current long-term goal focuses on developing a pertussis vaccine that is safe and confers lifelong immunity in children and adults.

1.1.4 Virulence Determinants of *B. pertussis*

The expression of the known virulence factors in *B. pertussis* is essentially governed by the BvgA/S two-component signaling system, which

consists of a sensor protein, BvgS and a cognate response regulator BvgA (Section 1.3). Based on our current understanding, *B. pertussis* BvgA/S regulated virulence determinants can be broadly classified into three groups; namely the toxins, autotransporters and adhesins In this section, we will discuss one major virulence determinant for each class, namely pertussis toxin (PT), the BrkA autotransporter and the filamentous hemagglutinin (FHA). .

Production of toxins by *B. pertussis* typically results in respiratory disease manifestation in infected host through irritation of ciliated epithelial cells and the impairment of ciliary function in the respiratory tract. The surface of *B. pertussis* bacteria is coated with the heat stable lipopolysaccharide endotoxin. In addition, *B. pertussis* secretes several exotoxins, which have been shown to cause a variety of toxic effects. These include PT, an ADP-ribosyl-transferase that interferes with G-protein signaling (Finger and von Koenig, 1996; Katada et al., 1983), the adenylate cyclase (AC) toxin, which increases cAMP levels thereby inhibiting immune effector cell functions (Hanski, 1989), the tracheal cytotoxin (TCT), which causes local damage and extrusion of ciliated epithelia (Wilson et al., 1991) and the dermonecrotic toxin (DNT), which results in modification of GTPases and consequently tissue destruction (Fukui and Horiguchi, 2004). One of the main secreted exotoxins, PT comprises of five different subunits, namely the S2, S3, S4 and S5 subunits each with carbohydrate recognition domains that are capable of binding onto host cell surface receptors (van't Wout et al., 1992; Witvliet et al., 1989). The enzymatically active S1 subunit interferes with cellular GTP and G-protein signaling events (Carbonetti, 2010; Finger and von

Koenig, 1996). PT is transported across the bacterial outer membrane via type IV secretion system encoded by the *ptl* operon, which is located downstream the *ptx* genes (Weiss et al., 1993). The ability for *B. pertussis* to adhere onto host surface is dependent on the production of PT, primarily the S2 and S3 subunits (Tuomanen et al., 1985; van't Wout et al., 1992).

The filamentous hemagglutinin (FHA) is a highly immunogenic, 220 kDa protein, which serves as the dominant adhesin essential for the initial establishment of infection in *Bordetella* sp. Although it is not the sole adhesin in *B. pertussis*, deletion of FHA alone results in dramatic impairment of bacterial colonization in a mouse model of pertussis infection, implying the importance of FHA in colonization. Specifically the carbohydrate recognition domain (CRD) is crucial for the attachment of *B. pertussis* onto the respiratory tract of its infected host (Kimura et al., 1990; Relman et al., 1989). FHA also carries the glycosaminoglycan-binding site, which allows it to bind to sulphated glycolipids and heparin commonly found on the surfaces of various eukaryotic cells (Hannah et al., 1994; Menozzi et al., 1991b). In addition, the Arg-Gly-Asp (RGD) motif promotes bacterial adherence to macrophages and monocytes and possible other leukocytes via the leukocyte integrins (Ishibashi et al., 1994; Saukkonen et al., 1991). Initially, FHA is synthesized as a large 360-kDa FhaB precursor in the cytoplasm and transported into the periplasmic space via the Sec secretion pathway (Chevalier et al., 2004). At the outer membrane, the large FhaB is proteolytically cleaved by SphB protease at the C-terminus end and processed to form the mature 220 kDA FHA adhesin protein (Coutte et al., 2001). Mature FHA is then secreted into the

extracellular milieu or remains associated to the surface of *B. pertussis* through a specialized translocation system at the outer membrane known as FhaC (Guedin et al., 2000; Jacob-Dubuisson et al., 2013; Jacob-Dubuisson et al., 2001).

Proteins that belong to the autotransporter family typically mediate their own export across bacterial cell envelope. As a large protein superfamily, autotransporters comprise of an N-terminal passenger domain and a conserved C-terminal domain, which folds into a beta-barrel channel resulting in the formation of a secretion pore at the outer membrane (Shannon and Fernandez, 1999). Most autotransporters are proteolytically cleaved, resulting in a processed alpha domain that is either secreted into the extracellular milieu via the beta-barrel channel or remain non-covalently associated to bacterial cell surface (Dautin and Bernstein, 2007; Fink et al., 2001; Girard and Mourez, 2006; Oliver et al., 2003; Suhr et al., 1996). BrkA autotransporter, involved in serum resistance, for example, is expressed as a 103 kDa precursor in *B. pertussis*, which is processed to yield a 73 kDa passenger domain and a 30 kDa beta-barrel channel (Dautin and Bernstein, 2007; Shannon and Fernandez, 1999). While BrkA has been implicated in adherence to and invasion of host cells *in vitro*, it also inhibits the classical complement pathway and accumulation of complement C4 proteins, which ultimately protects the bacteria against complement-mediated killing (Barnes and Weiss, 2001).

1.2 BACTERIAL POLYSACCHARIDE CAPSULES

1.2.1 Properties, Structure and Classification

Polysaccharide capsules form a discrete, mesh-liked or slimy layer surrounding the outermost structure of some bacteria species, thereby mediating the initial direct contact between the bacteria and the extracellular environment. First discovered and visualized under the microscope in the early 1900s through various conventional positive and negative staining methods (Gerstley and Morton, 1954; Moller, 1951; Novelli, 1953), the polysaccharide capsule has been described as "a gelatinous ground substance between the micrococci which agglomerates" as illustrated from one of the earliest observations on *Streptococcus pneumoniae* (Austrian, 2011). Increasing evidence of the presence of a gelatinous structure surrounding a microorganism has led to the isolation of the polysaccharide capsules and detailed study of its role in pathogenesis.

Bacterial capsules consist of long polysaccharide chains made of smaller repeating units, whose composition varies largely among bacterial species and among serotypes within the same species. For instance, almost 80 different polysaccharide capsules, also known as K antigens, have been reported and described for *Escherichia coli*, but not all capsulated serotypes lead to the same pathological consequences (Orskov and Orskov, 1992; Roberts, 1996; Whitfield, 2006). The diversity of bacterial polysaccharide macromolecules conveys a diverse virulent potential and is distinguished by the individual polysaccharide chains or monosaccharide units, which are made up either from carbohydrate or non-carbohydrate moieties (Greenfield et al.,

2012; Roberts, 1996; Vann et al., 1981; Whitfield, 1995). In general, polysaccharide capsule polymers are made up of repeating monosaccharide units linked together by glycosidic bond forming either hetero-polymers or homo-polymer as exemplified by the α-(2→8)`-linked sialic acid capsule of *E. coli* K1 strain (Vann et al., 1997). The Vi antigen of *Salmonella typhi* is organized in a linear homo-polymer of α-(1→4)-linked N-acetyl galactosaminuronic acid with a variable O-acetylation at the carbon 3 position (Martin et al., 1967; Yang et al., 2011).

Among different bacterial species, polysaccharide capsules are distinguished by the nature of their branching pattern, chemical linkages and chemical modifications (Bentley et al., 2006; Shu et al., 2009). Although the overall biological and chemical structure of a polysaccharide capsule determines the antibody-mediated immune responses against the bacteria, antigenically similar capsules do not necessarily generate the same response. Chemically identical polysaccharide capsules expressed in different bacteria species such as *S. typhi* Vi antigen and *E. coli* K1 antigen were able to elicit cross-reactive antibody responses (Szewczyk and Taylor, 1983), whereas the structurally identical polysaccharide capsules in *S. pneumoniae* and Group B Streptococcus generated distinct anti-polysaccharide capsule response (Arjunaraja et al., 2012), suggesting the unlimited functional diversity of the polysaccharide capsules in the bacteria kingdom.

The polysaccharide capsules polymer chains are firmly associated onto the bacteria cell surface, whereas those that are loosely connected on the

surface and often secreted into the extracellular milieu are known as exopolysaccharide or extracellular polysaccharide capsules (Figure 1.1) (Cuthbertson et al., 2009). For example, *S. typhi* forms shapeless slimy extracellular layer and releases its polysaccharide content into the extracellular milieu with limited association onto its cell surface (Daniels et al., 1989). In contrast, the *E. coli* surface polysaccharide capsule polymers are packed in matrices and establish into a discrete capsular structure, enveloping the entire outermost surface of the bacteria (Cuthbertson et al., 2009). Surface anchored polysaccharide capsules can also associate or interact with bacterial surface macromolecules or lipids such as phospholipids and lipid-A molecules to form a complex structure known as glycoproteins and glycolipids respectively (Whitfield and Valvano, 1993). In addition, for some Gram-positive bacteria, polysaccharide capsules also form an integral part of the bacterial cell surface through covalent interaction with the outer peptidoglycan layer (Sorensen et al., 1990).

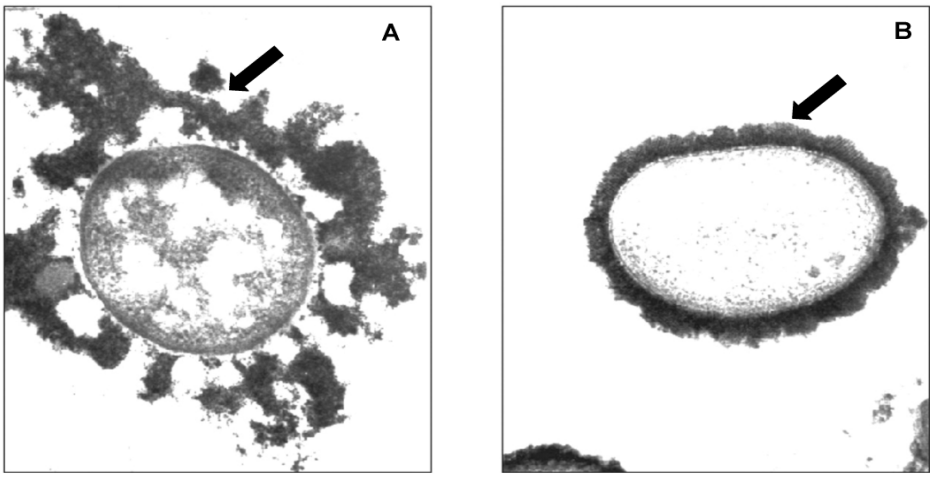


Figure 1.1: Morphology of (A) extracellular polysaccharide capsules in *Klebsiella pneumoniae* serotype K20 and (B) polysaccharide capsules in *E. coli* serotype K30.

The surface capsules for both bacteria are labeled with cationized ferritin. Although both bacteria have identical repeat-unit polysaccharide structures, the capsules of *E. coli* retains most of the polymer in a well-defined structure but the capsules of *K. pneumoniae* has limited association on the bacteria surface as substantial amounts of polymer was dispersed at the extracellular meliue as evident by the black arrows in the above micrographs. Adapted with permission (Cuthbertson et al., 2009).

1.2.2 Biosynthesis and Assembly

Genetic and biochemical evidences have ascertained that the biosynthesis and transport machinery of polysaccharide capsules are broadly similar across different bacteria species. Essentially, three types of polysaccharide biosynthesis and assembly apparatus have been described widely in the literature for both Gram-positive and Gram-negative bacteria; namely the Wzy-dependent system, ATP-binding cassette (ABC) transporter dependent system and the synthase dependent system (Figure 1.2) (Whitfield, 2006; Whitney and Howell, 2013; Yother, 2011). The Wzy and synthase dependent systems are widely characterized in both Gram-positive and Gram negative bacteria, whereas the ABC-transporter dependent system is mainly associated with the transport of capsules in Gram-negative bacteria (Whitfield, 2006; Yother, 2011). In the Wzy-dependent system, the repeating carbohydrates moieties are linked and assembled into polymers at the cytoplasmic face of the inner membrane (Figure 1.2). In contrast, the ABC-dependent pathway is characterized by the ATP-binding cassette transporter system that directs the elongated carbohydrate moieties that were synthesized and assembled independently in the cytoplasm (Figure 1.2).

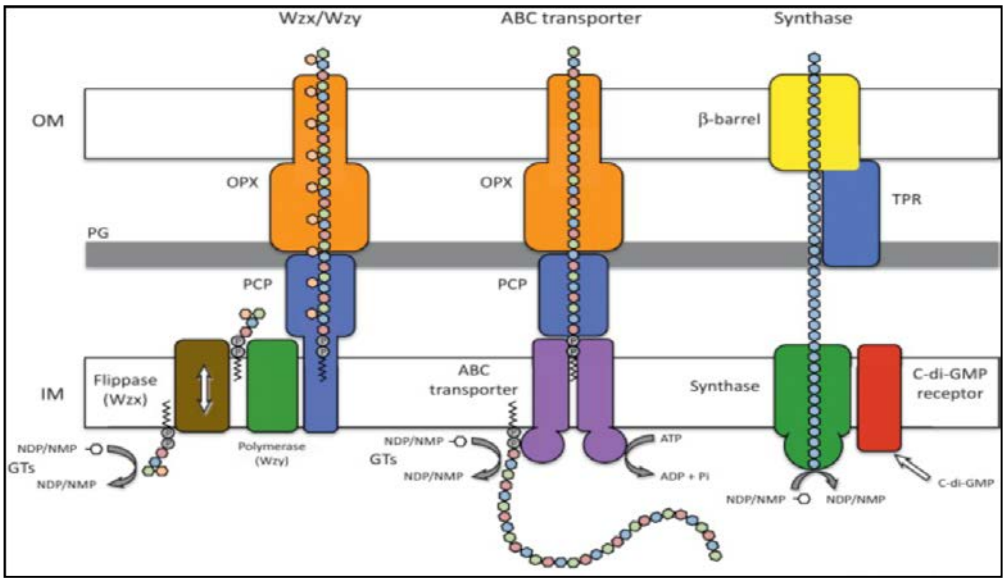


Figure 1.2: Mechanism of polysaccharide biosynthesis and secretion by the Wzy/Wzx, ABC-transporter and synthase dependent pathway.

Abbreviations: OPX, outer membrane polysaccharide export; PCP, polysaccharide copolymerase; TPR, tetratricopeptide repeat proteins; IM, inner membrane; PG, peptidoglycan sacculus; OM, outer membrane; c-di-GMP, bis-(3′-5′)-cyclic dimeric guanosine monophosphate; ABC transporter, ATP-binding cassette transporter. Adapted with permission (Whitney and Howell, 2013).

The prototypical biosynthesis and assembly of polysaccharide capsules in Gram-negative bacteria are mainly based from the biosynthesis and assembly model of group or type 1, 2, 3, and 4 capsules in *E. coli* (Table 1.1) (Whitfield, 2006; Whitfield and Roberts, 1999). More than 80 different serotypes of *E. coli* capsules or K antigens were grouped and classified based on their biochemical composition, structural properties, regulation of expression (as described in Table 1.1) and as well as the sequences of the capsule gene clusters (Whitfield, 2006; Whitfield and Roberts, 1999). In general, the mechanisms of polysaccharide capsule biosynthesis and chain translocation requires multi-protein complexes for co-expression with O-antigen and other carbohydrate moieties (Table 1.1). Biosynthesis usually takes place at the cytoplasm or at the cytoplasmic inner membrane face of the bacteria. Prior to polymer chain elongation, the pool of activated monophospho and/or diphospho-sugar precursors in the cytoplasm first assemble into a nascent polysaccharide at the cytoplasmic inter-face of the inner membrane by biosynthesis enzymes (Whitfield, 2006). Depending on the transporter system (Figure 1.2), the nascent polysaccharide chain grows successively with aid of enzymes for the addition of carbohydrate units or chemical groups at the reducing or non-reducing end of the polymers (Vimr and Steenbergen, 2009). Concurrently, a translocation protein complex spanning the entire cell wall will translocate the elongating mature polymer through the perisplasm and across the outer membrane to the bacterial cell surface, a process typically coupled with ATP hydrolysis for the ABC-transporter system (Figure 1.2) (Whitfield, 2006).

	Group			
Characteristic	**1**	**2**	**3**	**4**
Co-expressed with O serogroups	Limited range	Many	Many	Often O8, O9 but sometimes none
Co-expressed with colanic acid	No	Yes	Yes	Yes
Terminal lipid moiety	Lipid A-core in K_{LPS}; unknown for capsular K antigen	α-Glycero-phosphate	α-Glycero-phosphate ?	Lipid A-core in K_{LPS}; unknown for capsular K antigen
Polymerization system	Wzy-dependent	Processive	Processive ?	Wzy-dependent
Trans-plasma membrane export	Wzx	ABC-2 exporter	ABC-2 exporter?	Wzx
Translocation proteins	Wza, Wzc	KpsD, KpsE (KpsF?)	KpsD, KpsE?	Unknown
Thermo-regulated (not expressed below 20°C)	No	Yes	No	No
Positively regulated by the Rcs system	Yes	No	No	No
Model system	Serotype K30	Serotype K1, K5	Serotype K10, K54	Serotype K40, 0111
Similar to	*Klebsiella*	*Neisseria, Haemophilus*	*Neisseria, Haemophilus*	Many genera

Table 1.1: Classification of *E. coli* capsules

E. coli capsules were classified into type 1, 2, 3 and 4 based on their biochemical properties, polymerization and transport-export system. Table adapted and modified with permission (Whitfield and Roberts, 1999). (?) denotes possible association.

1.2.3 Bacteria Polysaccharide Capsules As Virulence Determinants

Historically, the surface polysaccharide capsule was perceived as a hydrated shield that envelops the bacteria and protects it against external environmental threats, including the harsh effect of desiccation and drastic osmolarity changes (Gibson et al., 2006; Ophir and Gutnick, 1994). Protection against drastic external changes in their natural growth environment is particularly relevant for the survival of pathogens and transmission from one host to another. For many bacteria species, surface polysaccharide capsules also play a significant part in defense against classical host-mediated immunity and promote bacterial adherence onto biotic (host surfaces) and abiotic surfaces, which is often associated with biofilm development (Beloin et al., 2008; Costerton et al., 1981; Reisner et al., 2006).

The later developmental stages of complex biofilm structures from a community of bacteria typically display enhanced resistance to antimicrobial agents and antibiotics treatment (Otto, 2006). Interspecies polysaccharide capsule-associated biofilm matrices reinforce the survival, colonization and invasion of pathogenic bacteria at the site of infection. The variability in carbohydrate and chain modifications are some of the biochemical aspects that drive the functional diversity of the polysaccharide capsules. Whilst certain capsules are associated with adherence as exemplified in *K. pnenumoniae* and *S. pneumoniae* on human respiratory epithelial cells (Favre-Bonte et al., 1999; Hammerschmidt et al., 2005), the surface capsules are also capable of masking surface adhesins, such as fimbriae, crucial for the attachment of the bacteria to

host cells (Favre-Bonte et al., 1999; Schembri et al., 2004). The K1 capsule of the uropathogenic *E. coli* promotes formation of intracellular bacterial communities consisting of polysaccharide-associated biofilm-like matrices within the cytosol of the bladder epithelium (Anderson et al., 2010), resulting in the development of severe and invasive urinary tract infection (Dautin and Bernstein, 2007). The *E. coli* intracellular bacterial communities in turn enhance bacteria proliferation at a distant site of infection, therefore resulting in the recurrence of UTI infection within the gut (Anderson et al., 2010; Goller and Seed, 2010).

The polysaccharide capsules are also capable of subverting host immune recognition, thereby pertinently allowing bacteria to inhibit and evade the host immune defense mechanisms. For instance, the surface capsule of *Neisseria meningitides* protects the bacteria against phagocytosis by inhibiting adherence and uptake into human dendritic cells (Unkmeir et al., 2002). Such anti-phagocytic strategies favor the capsulated bacteria to survive, persist and disseminate within their infected host. This probably explains why capsulated bacteria such as *K. pneumoniae* and Group A Streptococcus, *N. meningitides* and *Staphylococcus aureus* are able to circumvent host defenses and are able to invade host tissues and bloodstream (Barroso et al., 2013; Schrager et al., 1996).

The Vi polysaccharide of Salmonella enterica serotype *Typhi* or commonly known as *Salmonella typhi* is one of the major virulence determinants responsible for typhoid fever. The Vi polysaccharide or Vi

antigen is a linear homopolymer of α-1,4-linked N-acetylgalactosaminuronate (GalNAcA) with variable O-acetylation at the carbon 3 position; a chemical modification that is targeted by majority of the anti-Vi antibody response (Robbins and Robbins, 1984). The surface Vi antigen is one of the major virulent factors for *S. typhi*, which mediates enhanced resistance to innate immune response including phagocytosis and host complement-mediated killing (Robbins and Robbins, 1984; Wilson et al., 2011). It has also been proposed that the capsular Vi antigen prevents host-pathogen recognition event by concealing bacterial LPS recognition by the host pattern recognition receptors (Wilson et al., 2008).

1.2.4 Bacteria Polysaccharide Capsules As Subunit Vaccines

Advances in the knowledge of polysaccharide capsules structure, biochemical properties and host-mediated immune responses ensued the development of vaccines targeting the polysaccharide capsules of pathogenic bacteria. Various polysaccharide capsule-based vaccines have been developed to protect against deadly bacterial diseases including those caused by pneumococci (*S. pneumoniae*), menigococci (*N. menigitidis*), *Haemophilus influenzae* serogroup b (Hib), group B streptococci (*Streptococcus agalactiae*) and *S. typhi*. Immune response, specifically the activation of B-lymphocytes against the large polymeric polysaccharide capsules is largely limited to T-lymphocyte independent mechanisms (Kelly et al., 2004; Weintraub, 2003). The lack of T-lymphocyte memory against capsules is associated with poor immunogenicity, especially in infants and adolescents due to under-developed

B-lymphocytes subpopulation and T-regulatory cells (Weintraub, 2003). To circumvent such immune limitation in children, polysaccharide capsules based vaccines are generally conjugated with protein carriers to enhance immunogenicity and confer stronger protection against encapsulated pathogen.

Whilst the type B polysaccharide capsules of Hib are poorly immunogenic, covalent conjugation with a protein carrier such as diphtheria and tetanus toxoid successfully boosted T-cell immunity and strong antibody responses against Hib polysaccharide capsules (Anderson, 1983; Schneerson et al., 1980). The strong efficacy of Hib polysaccharide capsule-protein conjugated vaccine successfully reduced Hib infection among adolescents for the past 20 years. Similar success was also achieved with the diphtheria toxoid-conjugated meningococcal polysaccharide capsule vaccine used for the prevention of *N. menigitidis* infection among adolescents (Anderson et al., 1994). The currently available diphtheria toxoid-conjugated tetravalent polysaccharide vaccine protects against four different serotypes of *N. menigitidis* (Campbell et al., 2002; Kimmel, 2008). Like many other T-lymphocyte independent polysaccharide capsule based vaccine, the purified *S. typhi* Vi capsular polysaccharide (TYPHIM Vi^{TM}) is only limited for active immunization against typhoid fever in children more than two years of age (Pulickal and Pollard, 2007). To protect children below two years of age, the principle of chemical conjugation between a carrier protein and the capsule Vi antigen was recently developed and subjected to clinical trials. This includes Vi-conjugates with the recombinant, inactivated exoprotein A of *Pseudomonas aeruginosa*, which confers good protection against typhoid

fever in adolescents (Lin et al., 2001; Thiem et al., 2011) and as well Vi-conjugates with diphtheria toxoid (Cui et al., 2010; Rondini et al., 2011).

1.2.5 Genetic Regulation of Bacterial Capsule Expression

The regulatory system that directs the expression of polysaccharide capsule in pathogenic bacteria has been characterized in *E. coli* and *S. typhi*, as they are both regulated by the same Rcs two-component regulatory system. Detailed analysis of effectors and regulators involved in the control of transcriptional activity of the capsule locus and its implication in virulence has been extensively reported for both bacteria.

1.2.5.1 Genetic regulation of extracellular polysaccharide capsule synthesis in *Escherichia coli*

Over 80 chemically distinct capsular polysaccharides or K antigens were reported and characterized in *E. coli* alone, and they are collectively grouped in four different categories. As described in section 1.1.2, the process of biosynthesis, elongation and translocation at the bacterial surface of group 2 and 3 capsules typically requires expenditure of energy. Therefore, production of polysaccharide capsule at the protein and transcriptional levels are tightly regulated and modulated depending on the environmental conditions encountered by the bacteria.

Expression of *E. coli* group 2 capsules is essentially thermo-regulated, with up-regulation of region 1 and 3 at normal physiological temperature of 37°C, but not at temperature below 18°C (Cieslewicz and Vimr, 1996; Whitfield, 2006). Region 1 and 3 denote the conserved group 2 gene clusters involved in the transport and modification of repeating units of the polysaccharide polymers (Roberts, 1996; Whitfield, 2006). Several overlapping regulatory circuits have been shown to mediate the regulation of temperature sensitive capsule promoter in *E. coli*. The transcriptional activator RfaH is necessary for *E. coli* capsule expression at 37°C (Stevens et al., 1994), whereas the H-NS and BipA regulatory proteins play a dual role; both are necessary for activation of the capsule expression at 37°C and repression at 18°C (Rowe et al., 2000). Another transcriptional regulator known as SlyA interacts with H-NS regulator to promote transcription of *E. coli* K5 capsule gene cluster at 37°C.

Different serotypes of encapsulated *E. coli* regulate their capsule differently and involve different molecular events and different regulators. The major exopolysaccharide capsule, also known as colanic acid in *E. coli* K12, is essentially regulated by the Rcs two-component regulatory system. Members of the Rcs signaling pathway consist of RcsC, a trans-membrane sensor kinase, and RcsB, the cystoplasmic response regulator (Figure 1.3) (Gottesman and Stout, 1991; Majdalani et al., 2005). The signal output of RcsS requires RcsD, a phospho-transfer protein formerly known as YojN, to activate the response regulator RcsB via a phospho-transfer reaction (Figure 1.3) (Takeda et al., 2001). Transcriptional regulation of the *cps* gene cluster in

E. coli K12 strain depends on an additional cytoplasmic regulator known as RcsA, which interacts with the response regulator RcsB to activate transcription of *cps* operon (Figure 1.3) (Majdalani et al., 2005). Phosphorylated RcsB either homodimerizes to activate a certain group of genes or heterodimerizes with the accumulating pool of RcsA to activate the capsule *cps* operon (Figure 1.3), resulting in over-expression of colanic acid and mucoid phenotype (Gottesman and Stout, 1991; Majdalani and Gottesman, 2005). RcsF, an alternative sensor kinase for RcsB located at the outer membrane transduces extracellular signals to RcsC in an unknown mechanism (Figure 1.3) (Majdalani et al., 2005). At temperature below 20°C and in the presence of divalent cations, the Rcs signaling system is activated and hence resulting in increased capsule expression (Hagiwara et al., 2003). Intriguingly, a regulatory cross-talk between the Rcs system and PhoP/Q signaling systems has been described for the regulation of extracellular polysaccharide capsule expression in *E. coli* in response to the *in vitro* presence of divalent cations (Hagiwara et al., 2003). The model of Rcs signaling system in regulating the capsule expression illustrates the complexity of signal transduction mechanisms that are responsible for the flexibility and adaptability of *E. coli* in response to its variable host microenvironments.

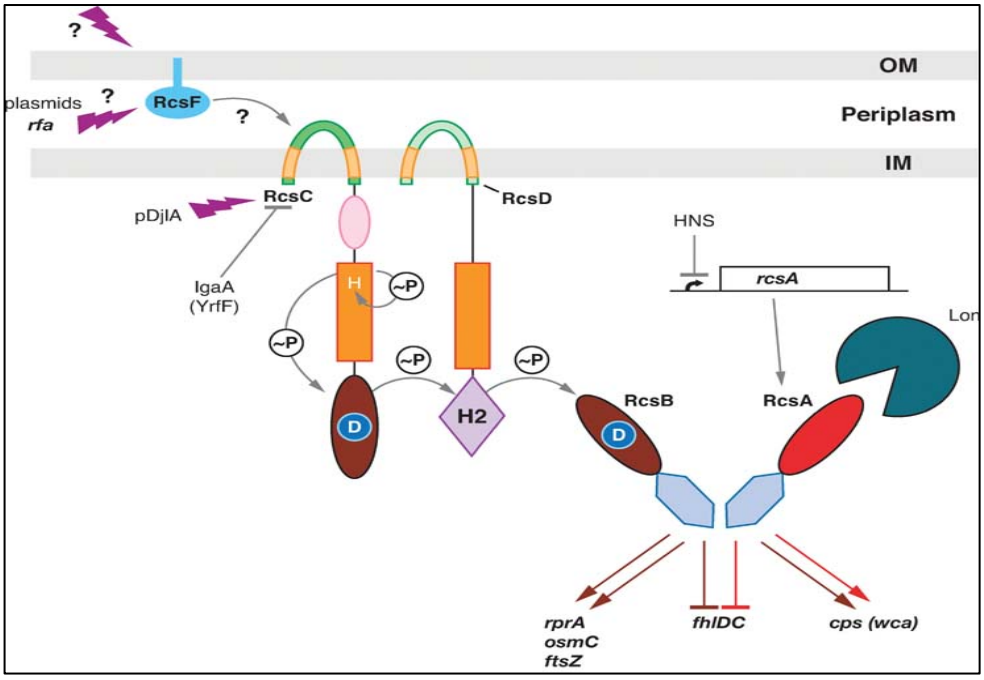


Figure 1.3: Model of Rcs signaling cascade in *E. coli* K12.

The RcsB/C two-component system, including the RcsD phosphotransfer protein, RcsF sensor at the outer membrane and RcsA response regulator encompassed the complexity of the Rcs signaling mechanism in *E. coli*. Transcriptional regulation of the capsule *cps* gene cluster encoding the colanic acid polysaccharide capsule is essentially modulated by the Rcs signaling system. Figure adapted with permission (Majdalani and Gottesman, 2005).

1.2.5.2 Genetic regulation of capsule synthesis in *Salmonella typhi*

S. typhi, the agent of typhoid fever, expresses the Vi polysaccharide capsule antigen or Vi antigen at its surface, which is an important virulent determinant for the bacteria during infection. Sequencing of the *S. typhi* genome revealed a large 134 kb pathogenicity island implicated in virulence, which inlcudes the locus required for biosynthesis and export of Vi antigen (Hashimoto et al., 1993; Seth-Smith, 2008). Expression of Vi antigen in *S. typhi* involves two genetic loci termed as *viaA* and *viaB*. The *viaA* locus, which encodes the TviA protein regulatory protein, is found in many other *Salmonella* serotypes, including in the Vi-negative strains and in other bacteria such as *E. coli*. The *viaB* operon, present only in the Vi-expressing strains, contains ten ORFs necessary for biosynthesis and export of Vi antigen to the cell surface (*tviB* to *vexE*) (Figure 1.4) (Hashimoto et al., 1991; Hashimoto et al., 1993; Kolyva et al., 1992).

Interestingly, it has been shown that a similar RcsB/C regulatory system involved in colanic acid synthesis in *E. coli* (section 1.1.5.1) plays a role in activating the *viaA* locus in *S. typhi* under low osmolarity condition (Figure 1.4) (Houng et al., 1992; Virlogeux et al., 1996). The product of *viaA* locus, TviA positively regulates its own expression and acts as an auxiliary protein by interacting with RcsB, the response regulator of RcsB/C system in *S. typhi*, where both protein cooperatively modulate the expression of Vi antigen at the *viaB* operon, flagella and invasion proteins (Figure 1.4) (Arricau et al., 1998; Houng et al., 1992; Majdalani et al., 2005; Virlogeux et al., 1996).

Interestingly, the osmo- sensitive OmpR/EnvZ two-component system in *S. typhi* has been shown to modulate Vi antigen expression TviA (Figure 1.4) (Pickard et al., 1994). In addition, the alternative sigma factor RpoS, a stress response master regulator required for *S. typhi* survival under unfavorable conditions, has also been described as a repressor for Vi-antigen biosynthesis through the RcsB/C pathway (Figure 1.4) (Santander et al., 2008; Santander et al., 2007). Given the invasive properties of *S. typhi,* it is not surprising that this bacteria encounters a great diversity of microenvironments within the host and that various regulatory systems are involved in the expression of Vi antigen, leading to the complexity of the capsule synthesis regulation.

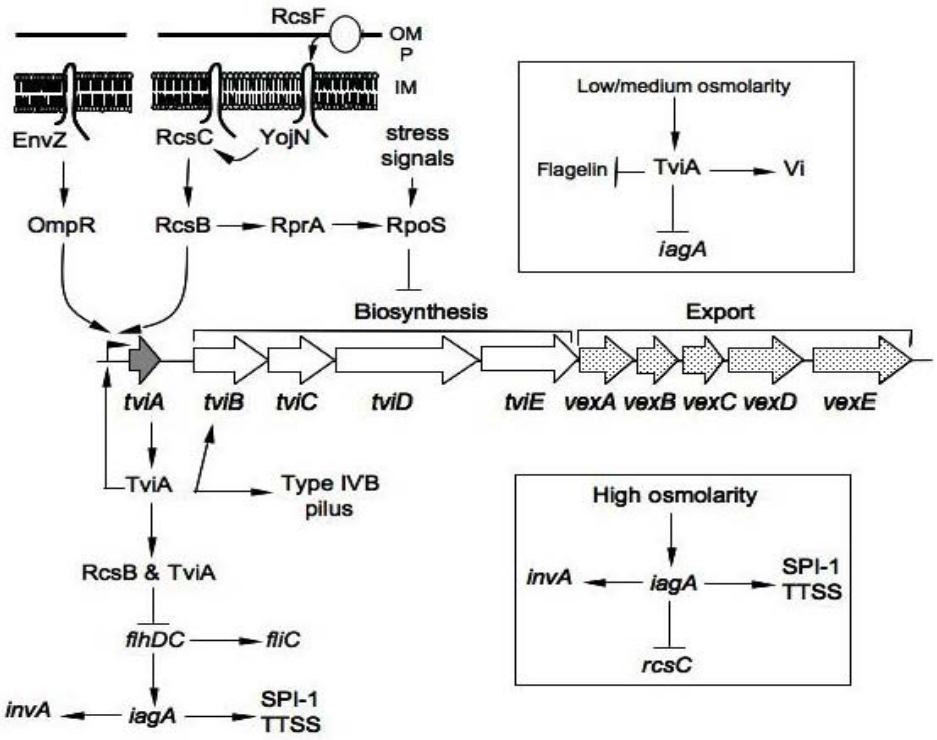


Figure 1.4: Regulatory network of Vi polysaccharide expression by Rcs and Enz/OmpR signaling system.

The Rcs and OmpR/EnvZ two-component regulatory system are involved in the regulation of Vi antigen expression in *S. typhi*. Low osmolarity leads to an increased level of Vi antigen expression via TviA activation, whereas high osmolarity results in a negative regulation of Vi antigen expression via inhibition of *rcsC* expression. OM: Outer membrane; P: Periplasm; IM: Inner membrane. Figure adapted with permission (Santander et al., 2008).

1.2.5.3 Genetic regulation of polysaccharide capsule expression during infection

Precise and temporal regulation of the capsule expression during the course of infection is crucial for bacterial adaptive response within the host environment. Regulation of polysaccharide capsule expression *in vivo* has been a topic of interest as it suggests the flexibility of bacterial regulons in responding to host factors, which in turn promotes bacterial survival and persistence. For instance, expression of *S. typhi* Vi-antigen biosynthesis gene, *tviB* was significantly up-regulated during invasion of intestinal epithelial cells *in vitro* (Tran et al., 2010). Moreover, in an *in vivo* bovine model of *S. typhi* infection, expression of Vi capsular antigen was shown to be induced upon invasion at the ileal mucosa (Tran et al., 2010). Over-expression of Vi antigen renders *S. typhi* less adherent and enhances phagocytic killing whereas, an optimal expression of Vi antigen is necessary for evasion of the host immune response, suggesting that a tightly controlled regulation of Vi antigen expression at different sites and times during infection is crucial for *S. typhi* pathogenesis (Janis et al., 2011; Robbins and Robbins, 1984; Waxin et al., 1993).

For group A *Streptococcus*, the expression of polysaccharide capsule was found immediately up-regulated at early stage of mucosal colonization in mice, which correlates with the bacterial enhanced resistance to opsonization and phagocytic killing by the host immune cells (Dale et al., 1996; Gryllos et al., 2001). In other pathogenic bacteria such as *N. meningitides*, expression of

the polysaccharide capsule was observed to be down-regulated at early stage of infection, upon interaction with epithelial cells to facilitate adherence and invasion of the bacteria (Deghmane et al., 2002; Grifantini et al., 2002). The remarkable flexibility of pathogenic bacteria to dynamically modulate its polysaccharide capsule expression highlights the basis of bacterial adaptability within its infected host environment and the important role played by the polysaccharide capsules for optimal infection.

1.3 POLYSACCHARIDE CAPSULE OF *BORDETELLA PERTUSSIS*

1.3.1 Sequencing and Characterization of The Capsule Operon

Like many other encapsulated bacteria, the capsule locus of *B. pertussis* is organized in a single operon, which comprises of three genetic regions with ten open reading frames (ORF) involved in polysialic acid transport (black arrows), phospholipid modification/export (hashed arrows) and biosynthesis (open arrows) that encode a putative type II polysaccharide capsule (Figure 1.5) (Hot et al., 2003; Parkhill et al., 2003).

The first and second genes of the *B. pertussis* capsule operon, *kpsM* and *kpsT* encode the putative polysialic acid transport and polysialic acid transport ATP-binding proteins respectively (Parkhill et al., 2003). The *kpsM* and *kpsT* genes are organized as a single transcriptional unit with an overlapping *kpsM* stop codon and *kpsT* start codon, implying that the two gene products are likely to be expressed in equal amounts. The polysialic acid

capsules are a subclass of surface capsules that are well characterized for their role in pathogenesis especially in *E. coli* K1 strain (Bliss and Silver, 1996). In *E. coli* K1, the polysialic acid chain translocation across the inner membrane is facilitated by the KpsMT transporter complex at the inner membrane. The KpsMT complex is only functional upon interaction of KpsT with KpsM coupled with the binding of ATP to KpsT (Figure 1.6) (Bliss et al., 1996; Nsahlai and Silver, 2003; Pavelka et al., 1994; Pavelka et al., 1991). Binding of ATP to KpsT leads to a conformational change such that the polysialic acid associated domain on KpsT can be inserted into the KpsM transporter (Bliss and Silver, 1996; Nsahlai and Silver, 2003). This compelling evidence suggests that KpsM/KpsT transporters play a central role to the functioning of the entire polysaccharide capsule biosynthetic, transport and export machinery in *E. coli* (Bliss and Silver, 1996). The presence of motifs conserved in KpsM/T from *B. pertussis*, *E. coli* and *H. influenzae* suggests that KpsM and KpsT produced in *B. pertussis* are likely to form KpsM/T complexes involved in ATP-dependent active transport of a polymers.

Predictive sequence analysis of the third ORF in the capsule operon, *kpsE* reveals a putative polysaccharide capsule export inner membrane protein that resembles the polysaccharide capsule export KpsE protein of *E. coli*, the CtrB protein of *N. meningitides* and BexC of *H. influenzea*. KpsE is located at the inner membrane of *E. coli*, has significant exposure at the periplasmic face and directs the export of polysaccharide capsule across the bacterial inner membrane (Figure 1.6) (Bronner et al., 1993a; Rosenow et al., 1995). Moreover, it has been proposed that KpsE functions in concert with the

specialized ABC transporters KpsM and KpsT for a proper translocation of polymers across the inner membrane to the periplasmic face (Figure 1.6) (Higgins et al., 1990; Rosenow et al., 1995).

The predicted products encoded by the capsular biosynthesis genes (Figure 1.5) are homologous to the *S. typhi* Vi capsular antigen biosynthesis enzymes, in particular WbpT and VipC display 33.15% and 23% homology to *S. typhi* Vi polysaccharide biosynthesis protein TviE and TviD respectively. This implies that the products of the capsule locus may be antigenically similar to the N-acetyl galactosaminuronic acid Vi antigen polymer. Similar to *S. typi* TviE, WbpT harbors a conserved glycosyltransferease domain, which catalyzes the transfer of sugar moieties from activated donor to a specific acceptor ranging from lipid, protein or another carbohydrate compound via glcosidic bonds (Zhang et al., 2006). These proteins usually transfer UDP, ADP, or GDP linked sugars to a variety of substrates and are widely involved in the biosynthesis of polysaccharides (Zhang et al., 2006). Little is known about the role and function of TviD protein except that a functional TviD is required for Vi antigen synthesis in *S. typhi* (Virlogeux et al., 1995).

In *B. pertussis*, the central part of the locus (BP1624-BP1631) is intact, but the 3' region underwent an inversion event and an insertional sequence element-mediated rearrangement, which consists of genes involved in export/modification. Therefore, it has been speculated that the capsule genetic locus is non-functional in *B. pertussis* Tahoma I strain due the

abovementioned mutations end of the locus (black cross) (Figure 1.5) (Parkhill et al., 2003).

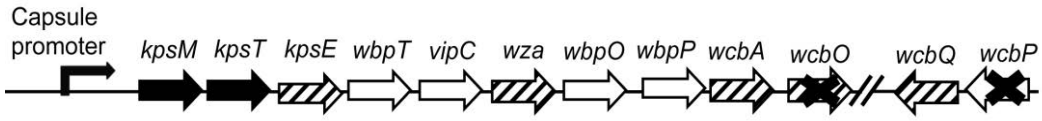


Figure 1.5: The *B. pertussis* capsule operon.

The capsule operon of *B. pertussis* regulated under the capsule promoter is as shown. Black cross represents mutational insertion found at the 3' end of the opeon. Black, hashed and white arrows represent genes involved in polysaccharide capsule transport, polysaccharide modification/translocation and polysaccharide biosynthesis respectively.

Inner membrane

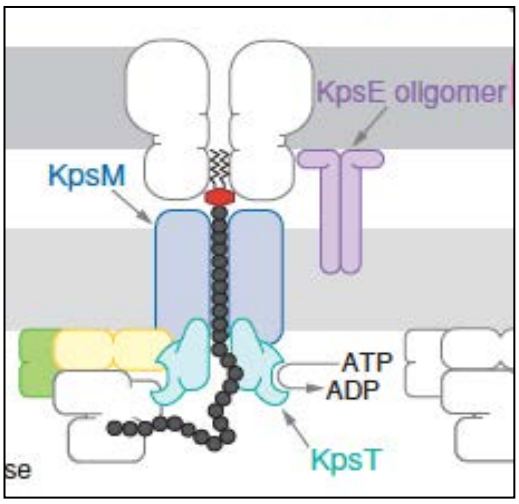


Figure 1.6: A model of biosynthesis and assembly of group II capsules in *E. coli.*

In this model, KpsT associate with polysialic acid polymers and translocate it via the ABC transporter complex; KpsM and KpsT at the inner membrane. The transport requires ATP expenditure. Translocation across the periplasm requires KpsE, possibly function as an adaptor protein. Figure adapted with permission (Whitfield, 2006).

1.3.2 *B. pertussis* Capsule Controversy

Earlier literature has reported that *B. pertussis* is capsulated by direct visualization using modified Wright's staining on virulent and avirulent strains (Lawson, 1939; Ungar et al., 1954). Nevertheless, the authors also noted that the capsular structure observed could not be distinguished from extracellular slime layer, thus raising doubts on the significance of this structure in *B. pertussis* (Ungar et al., 1954). Later on, staining with calcofluor exo-polysaccharide dye revealed that virulent *B. pertussis* may produce a surface capsule that is polysaccharide in nature (Weiss et al., 1989). However, calcofluor stains for all types of polysaccharides and cellulose, and thus they are considered non-specific dyes in detecting capsular polysaccharides. Moreover, conventional capsule stain India ink was unable to conclusively reveal the presence of a capsule in *B. pertussis*. Due to the lack of a capsule-deficient *B. pertussis* mutant in all the staining approaches above mentioned it is not possible to conclusively demonstrate the presence of a polysaccharide capsule produced at the surface of *B. pertussis* bacteria. Since then, no further studies have been reported on the characterization and isolation of the *B. pertussis* polysaccharide capsule.

Transcriptional fusion studies indicated that the *B. pertussis* capsule locus belongs to a new class of *vrgs* (Antoine et al., 2000), with similar modulating profile compared to the group of classical *bvg*-repressed genes *vrg6*, *vrg18*, *vrg24*, *vrg53* (Chapter 1.4.1.3) (Beattie et al., 1990; Beattie et al., 1993). Transcription of the capsule locus was being repressed in the Bvg^+

phase but elevated in the presence of $MgSO_4$ in Bvg^- phase (Chapter 1.4.1.4) (Antoine et al., 2000). Whereas the capsule operon is intact in *B. bronchiseptica*, the genes at the 3' end of the capsule operon that are involved in export/modification of the polysaccharide capsule across the cell wall are interrupted in *B. pertussis* (Parkhill et al., 2003). This observation has led to the assumption that *B. pertussis* is unable to produce an intact polysaccharide capsule which thus appeared to be dispensable for pathogenesis in mammalian hosts. Furthermore, the reduced expression of this locus in Bvg^+ phase virulent *B. pertussis* also supports that the capsule may not be necessary for bacterial virulence in Bvg^+ phase (Antoine et al., 2000). The controversy regarding the presence and functional properties of a polysaccharide capsule in *B. pertussis* has thus remains, which prompted us to undertake a comprehensive study that addressed the production and the role of the polysaccharide capsule in pertussis pathogenesis.

1.3.3 Biofilm Structures on *Bordetella*

The phenomenon of biofilm formation in *Bordetella* genus has been recently reported. Several groups have presented a phenotypic view of the formation of biofilm structures *in vitro*, primarily in the broad host range pathogen, *B. bronchipseptica* and the human pathogen, *B. pertussis* (Irie et al., 2004; Mishra et al., 2005; Serra et al., 2007). These studies indicated that biofilm formation is dominant in Bvg^+ and Bvg^i phase, but not in Bvg^- phase bacteria (Irie et al., 2004; Mishra et al., 2005). Irie and colleagues also suggested that co-existing adhesins FHA and fimbriae are necessary for

maximum biofilm formation in *B. bronchiseptica* and that formation of biofilm is growth phase-dependent, and particularly increased when bacteria reach the stationary phase (Irie et al., 2004; Irie et al., 2006). Although the expression of the biofilm-associated carbohydrate components is Bvg-independent, it appears that the BvgA/S two-component contributes to biofilm formation via its regulation of FHA and fimbriae expression (Irie et al., 2006), suggesting that a network of one or more macromolecules are involved in the formation of *Bordetella* biofilms.

The genetic element responsible for the biofilm formation was first reported in *B. bronchispetica* and it is known as the *Bordetella* polysaccharide locus or *bpsABCD* locus, which displays significant sequence similarities with the *pgaABCD* locus in *E. coli* responsible for the synthesis of poly-β-1,6-N-acetyl-D-glucosamine (Parise et al., 2007). The nature of *B. bronchiseptica* biofilm is antigenically similar to the extracellular poly-β-1,6-N-acetyl-D-glucosamine-like material structure which function as surface polysaccharide adhesin in *E. coli* (Parise et al., 2007). Impairment in biofilm production in both *B. bronchiseptica* and *B. pertussis* due to mutational deletion within the *bps* locus resulted in a reduced bacterial adherence to murine nasal cavity and trachea whereas the colonization of murine lungs was not affected (Conover et al., 2010; Sloan et al., 2007). The *B. bronchiseptica bps* locus was annotated as BB1769 (*bpsA*), BB1768 (*bpsB*) and BB1767 (*bpsC*), which is located way upstream of its putative polysaccharide capsule locus (BB2918-BB2934). Similarly, the *B. pertussis bps* locus (BP1942-BP1944) is physically distinct from the polysaccharide capsule locus (section

1.4.1). It was also reported that expression of the *bps* locus is not under the regulation of the BvgA/S two-component system (Conover et al., 2012; Parise et al., 2007; Parkhill et al., 2003). Therefore, the biofilm structures observed in *B. bronchisptica* and *B. pertussis* are independent from the products of the capsule locus.

1.3.4 Evidence For An Intact Pertussis Capsule

By constructing a *B. pertussis* Tohama I derivative mutant deleted for the entire 10 kb capsule operon (Parkhill et al., 2003), our laboratory has recently demonstrated that *B. pertussis* BPSM strain produces an intact polysaccharide capsule at its bacterial surface using Alcian blue staining visualized by transmission electron microscopy (Figure 1.7) and immuno-detection methods (Neo et al., 2010). The fact that a greater signal was obtained when *B. pertussis* bacteria were grown in Bvg^- phase further supports that the capsule locus belongs to the family of *vrgs* (Neo et al., 2010). We termed the polysaccharide capsule of *B. pertussis* as "microcapsule" as it can only be visualized by TEM upon Acian blue staining, but not by conventional staining and/or light microscopy. Our findings supported earlier studies reporting *B. pertussi*s as a capsulated microorganism (Lawson, 1940; Neo et al., 2010; Weiss et al., 1989).

We also demonstrated that the *B. pertussis* capsular polysaccharide is not involved in classical capsule-mediated defense mechanisms, including adherence to mammalian host cell, complement-mediated killing and

antimicrobial attack (Neo et al., 2010). The *B. pertussis* capsule operon belongs to the *vrg* family with maximal expression in Bvg^- phase (Antoine et al., 2000; Hot et al., 2003). Despite being categorized as a *vrg* family member, we and others have observed that the capsule operon of *B. pertussis* was transcriptionally active with basal expression detected in Bvg^+ phase bacteria (Nakamura et al., 2006; Neo et al., 2010). The fact that the capsule locus is expressed in virulent Bvg^+ phase suggests that this locus may play a role in pertussis pathogenesis.

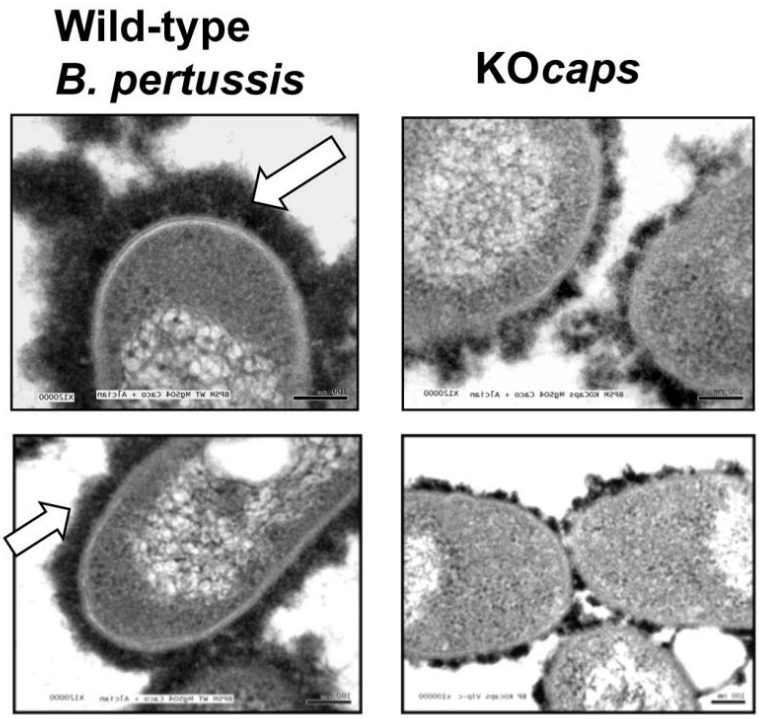


Figure 1.7: Visualization of the *B. pertussis* polysaccharide capsule by transmission electron microscopy.

Wild-type *B. pertussis* BPSM and KO*caps* bacteria grown in Bvg$^-$ phase conditions were fixed, stained with alcian blue, and processed for analysis by transmission electron microscopy. Black scale bar on the bottom right of each figures represent 100 nm. The images were captured at x120,000 magnification and x100,000 (for KO*caps* on the lower right panel). White arrowhead shows the surface microcapsule. Figure adapted with permission from Elsevier (Neo et al., 2010).

1.4 TWO-COMPONENT REGULATORY SYSTEM

1.4.1 The *bvg* Regulon in *B. pertussis*

1.4.1.1 Structure and function of BvgS

BvgS is the 136 kDa virulence sensor protein and a member of the BvgA/S two-component system signaling in *B. pertussis*. The *bvg* locus was first identified as a virulence or *vir* locus crucial for maintaining *B. pertussis* virulent phenotype *in vitro* (Weiss and Falkow, 1984). It was later described in the late 1980s as the single genetic locus responsible for the regulation of the expression of major virulence factors in *Bordetella* sp. such as FHA, PT, AC toxin and DNT (Arico et al., 1989; Stibitz et al., 1989; Stibitz et al., 1988; Stibitz and Yang, 1991). The *bvg* locus encodes the BvgA/S two-component system, which comprises of BvgA protein, a DNA-binding transcriptional regulator and BvgS, the virulence sensor protein (Arico et al., 1989). Predictive sequence analysis suggests that *bvg* locus belongs to the family of environmentally sensitive bacterial regulatory systems involved in signal transduction in response to environmental stimuli (Ronson et al., 1987). Earlier findings have suggested that the BvgA/S system is sensitive to certain *in vitro* stimuli, such as changes in temperature and the presence of salts, for instance $MgSO_4$ and nicotinic acid (Melton and Weiss, 1989; Miller et al., 1989; Stibitz et al., 1988; Weiss and Falkow, 1984). Genetic inactivation on the *bvgS* locus demonstrated that a functional BvgS sensor is absolutely necessary for a full transcriptional expression of virulence factors in *B.*

pertussis thus conferring a virulent phenotype (Arico et al., 1989; Miller et al., 1989; Weiss and Falkow, 1984).

The structural features of BvgS protein comprise of unique multi domains segments corresponding to a histidine kinase sensor (Arico et al., 1989). The BvgS sensor is considered an "unorthodox" histidine sensor kinase, as it has a four-step His-Asp-His-Asp phosphorelay transfer as opposed to a conventional histidine kinase sensor ubiquitously found in many Gram-negative bacteria comprising of two or three-step phosphorelay transfer mechanisms (Figure 1.8) (Arico et al., 1989; Stibitz and Yang, 1991; Stock et al., 2000). The amino acid sequence at the N-terminal end of BvgS contains consensus signal peptide, which harbors the Ala-Gln-Ala signal peptidase cleavage site (Ji et al., 1994; Stibitz and Yang, 1991). Adjacent to the signal peptide is the first trans-membrane domain of BvgS, transversing the inner membrane of *B. pertussis* in a helical manner, thus supporting the sub-cellular localization of BvgS at the inner membrane of the bacteria (Stibitz and Yang, 1991). This is immediately followed by a large periplasmic domain folding into two tandem solute-binding cavities or grooves, presently known as the Venus Fly Trap 1 and 2 (VFT1 and VFT2) domains (Figure 1.8) (Herrou et al., 2009).

The two VFT domains of BvgS have recently been characterized; they share sequence homology with bacterial solute-binding proteins and the periplasmic domain is apparently sensitive to BvgS modulators *in vitro* (Herrou et al., 2010; Martinez de Tejada et al., 1996). The ligand-binding

cavity within the VFT domains confer a hinge-bending motion; whereby it can either adopt an "open" or "close" ligand-binding pocket or cavity conformation, thus restricting the binding of specific solute(s) within the cavity (Herrou et al., 2010; Herrou et al., 2009; Quiocho and Ledvina, 1996). While VFT1 domain is insensitive to BvgS modulators, the VFT2 domain has been shown to display a high binding affinity towards a range of BvgS modulators, including nicotinic acid (Herrou et al., 2010). The flexibility of VFT ligand-binding cavity, and hence as the name "Flytrap" suggests, allows for a rapid response to environmental ligands. Unlike common solute-binding proteins for which binding of extracellular ligands or agonists at the periplasmic receptor results in a close conformational structure (Quiocho and Ledvina, 1996), BvgS VFT2 is by default active and adopts a close conformation even in the absence of ligands or signals (Herrou et al., 2010). It has been proposed that binding of negative signal ligands or antagonists from the milieu into VFT2 clef destabilizes and modifies the entire conformation of BvgS, resulting in an inactive sensor (Herrou et al., 2010). The unusually strong positive electrostatic potential within VFT2 cavity possibly explains for the paradoxical nature of this domain in attracting opposite, negatively charged organic carboxylates or inorganic ions (Herrou et al., 2010).

Mutations within the PAS domain, or formerly known as the linker region located immediately after the second trans-membrane domain (Figure 1.8), renders BvgS insensitive to environmental modulators, leading to the isolation of Bvg-constitutive mutants (Goyard et al., 1994; Manetti et al., 1994; Miller et al., 1992). Although the exact function of PAS domain in *B.*

pertussis is relatively uncharacterized, however by homology, PAS domain in most Gram-negative bacteria responds to oxygen concentrations, redox potential, light and small ligands (Taylor and Zhulin, 1999). Mutations at the BvgS PAS domain (linker region) are dominant over specific mutations at the BvgS periplasmic domain (Goyard et al., 1994; Manetti et al., 1994; Miller et al., 1992), but this does not exclude the importance of the periplasmic domain, including the tandem VFTs domains in stimulus perception. It has been recently shown that the integrity of PAS domain of *B. pertussis* is required for communication of signals from the periplasmic to the downstream kinase domain of BvgS (Dupre et al., 2013).

A signal integration model has been suggested for *B. pertussis* by which the periplasmic VFT domain initially perceive extracellular signals, while the PAS domain transduces the signal recognition events or information to the cytoplasmic histidine-kinase (HK) domain, also known as transmitter domain, thus initiating the autophosphorylation activity at the His residue on the HK domain (Herrou et al., 2010; Herrou et al., 2009; Martinez de Tejada et al., 1996). Moreover, biochemical and structural study in *E. coli* expression system reported that dimerization of BvgS takes place at the HK domain, which is a common dimerization interface domain for a classical two-component system and at the receiver-output domain (Figure 1.8) (Beier et al., 1995). Reconstitution of active phosphotransfer reaction in *trans* between BvgS domains further supports the dimerization capacity of BvgS *in vivo* (Beier et al., 1995; Perraud et al., 2000). Dimerization and higher order oligomerization of signaling complexes in general is important for intrinsic

phospho-transfer activity and activation of downstream regulator proteins (Maeda et al., 2006; Scheu et al., 2010).

The phosphorylation event is successfully transferred downstream towards the C-terminal end of BvgS; from histidine-kinase domain to the Asp residue on the receiver domain, and next to the His residue on the histidine phosphotransfer (Hpt) domain and finally to the Asp residue on the receiver domain of BvgA response regulator (Figure 1.8) (Uhl and Miller, 1994). The primary output of such phosphorelay mechanism ultimately determines the *B. pertussis* virulence genes expression profile and consequently the virulent phenotype of the bacteria.

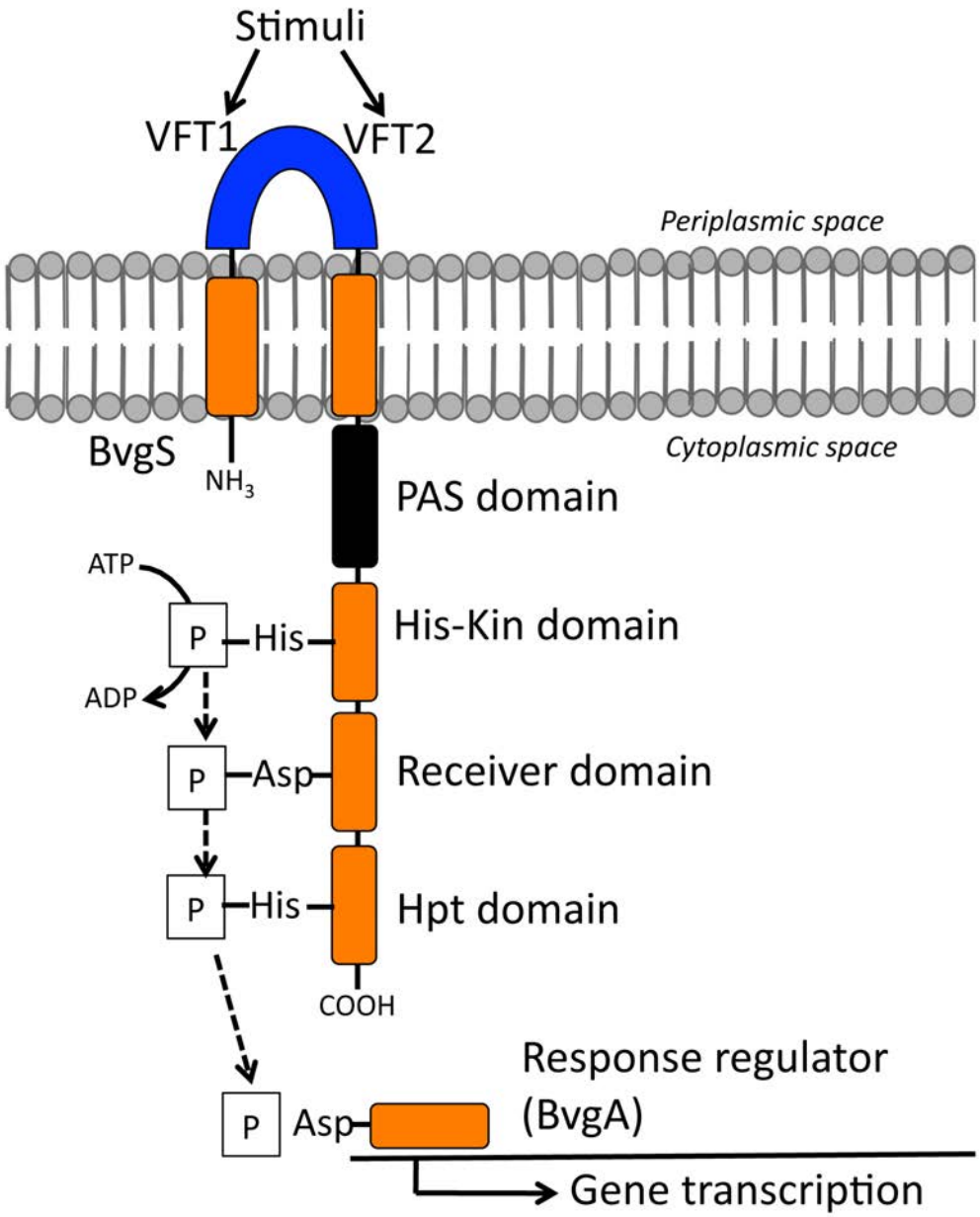


Figure 1.8: Model of an "unorthodox" BvgA/S two-component system in *B. pertussis*.

The BvgA/S two-component system of *B. pertussis*, when in an active form, participates in a four-step phosphorelay event on *B. pertussis* BvgS sensor. The putative signal perception domain comprised of the periplasmic Venus flytrap domains, namely VFT1 and VFT2 domain and the cytoplasmic PAS domain. His-Kin denotes the cytoplasmic histidine-kinase and dimerization domain where autophosphoylation occurs, similar to a classical two-component system. Following the autophosphorylation event at the His residue on His-Kin domain, the phosphate group is transferred to Asp residue on downstream receiver domain and then to the His residue on Hpt domain. Finally the phosphate group is transferred to Asp residue on the receiver domain of BvgA response regulator.

1.4.1.2 Structure and function of BvgA

As the response regulator or the final phosphate receiver for the BvgA/S two-component system, phosphorylation of BvgA would ultimately activate its function thus influencing the overall transcription of a group of genes known as *bvg*-regulated genes in *B. pertussis* (Roy and Falkow, 1991; Uhl and Miller, 1994). Phosphorylated BvgA (P-BvgA) auto-regulates its own transcription at the *bvg* promoter, implying a positive regulatory feedback on the *bvgAS* operon. Analysis of the BvgA amino acid sequence predicts a 23-kDa cytoplasmic protein with an N-terminal phosphate receiver domain and a helical C-terminal DNA-binding domain homologous to bacterial DNA-binding regulatory proteins such as FixJ and UhpA (Arico et al., 1989; Boucher et al., 1994; Stibitz and Yang, 1991).

The presence of negative modulators in the extracellular milieu represses BvgS sensor activation, thus inhibiting the overall phosphorylation of BvgA. P-BvgA protein function as a dimer to activate transcription of *bvg*-regulated genes (Scarlato et al., 1990) and the dimerization capacity of P-BvgA was further supported by several experimental approaches by other groups (Beier et al., 1995; Boucher et al., 1994). The mechanism by which P-BvgA differentially activates the transcription of *bvg*-regulated genes was deciphered from detailed analysis of a few *bvg*-regulated promoters in *B. pertussis*. DNase foot-printing assay on *ptx* and *fhaB* promoters showed variable length of protection, implying that each promoter carries multiple P-

BvgA binding sites, which is consistent with the oligomerization of multiple P-BvgA dimers including the RNA polymerase enzyme at the promoter region to promote mRNA elongation (Boucher et al., 1997; Boucher and Stibitz, 1995). The stability of P-BvgA and RNA polymerase complex interaction is dependent on the promoter architecture. Studies on *fha* promoter revealed that P-BvgA dimer initially binds to high affinity binding site located far upstream of the transcription start site followed by cooperative binding of another set of P-BvgA dimers and RNA polymerase to the next, downstream primary and secondary binding sites (Boucher et al., 2001a; Boucher et al., 2001b; Steffen et al., 1996). The unusually lengthy secondary binding site of *ptx* promoter requires even far more P-BvgA dimers to cooperatively sit on the promoter site as compared to *fha* promoter. Since occupancy of P-BvgA and RNA polymerase at the secondary promoter region is necessary to activate gene transcription, the *ptx* promoter is one of the earliest promoters to have its activity negatively affected when the levels of P-BvgA decreases (Zu et al., 1996).

1.4.1.3 Signal-transduction through BvgA/S two-component system: Regulation of *bvg*-activated and *bvg*-repressed gene

Production and modulation of the great majority of virulence factors in *B. pertusssis* are coordinately regulated by the BvgA/S two-component system. It is essential for *B. pertussis* to rapidly program its cellular responses by modifying its genes expression to adapt to environmental changes (Melton and Weiss, 1989). As described in the above section, BvgA/S activation is

characterized by a sophisticated His-Asp-His-Asp phosphorelay transfer mechanism from the trans-inner membrane sensor; BvgS to the cytoplasmic transcriptional activator; BvgA (Cotter and Jones, 2003; Uhl and Miller, 1994). *In vitro*, BvgA/S system is active when bacteria are grown at 37°C, corresponding to the normal human body temperature, and in the absence of known negative modulators such as $MgSO_4$ or nicotinic acid. Under these conditions, also known as virulent Bvg^+ phase, high intracellular concentrations of P-BvgA are reached thereby allowing the activation of *bvg*-activated promoters, and leading to the up-regulation of a subset of genes important for virulence, referred to as *bvg*-activated genes (*vag*) (Figure 1.9A). The Bvg^+ phase plays a central role in *B. pertussis* pathogenesis (Cotter and Jones, 2003; Cotter and Miller, 1994). In addition, transcription of another set of genes known as *bvg*-repressed genes (*vrg*) is repressed in Bvg^+ phase by the *vag*-encoded transcriptional repressor protein, BvgR (See section 1.4.1.5) (Merkel et al., 2003). In contrast, the BvgA/S system is inactive when bacteria are grown at temperature lower than 26°C or in the presence of milimolar concentrations of $MgSO_4$ or nicotinic acid in the culture media (Figure 1.9A) (Melton and Weiss, 1993). Under these culture conditions, *B. pertussis* bacteria are in avirulent Bvg^- phase, which is characterized by minimal expression of *vags* and maximal expression of *vrgs* as well as outer membrane proteins of unknown function (Vra proteins) (Stenson and Peppler, 1995). However, gene products that are negatively regulated by the BvgA/S two-component in *B. pertussis* such as Vrg6 and Vra proteins and in *B. bronchiseptica* such as flagella, sidephore alcaligin, urease do not appear to be

involved in pertussis pathogenesis (Akerley et al., 1992; Giardina et al., 1995; Knapp and Mekalanos, 1988; McMillan et al., 1996).

Although the activity of BvgA/S two-component system can be easily tweaked under laboratory conditions, the true signals perceived by the BvgS sensor during host infection have yet to be defined. Current understanding of the BvgA/S-signaling pathway suggests that *in vitro B. pertussis* cultures do not require any positive signals to activate the BvgA/S two-component system, active by default. However, recent studies interestingly provided some evidence that BvgS is able to perceive a positive stimulus in the environment. Experiments by Hester and colleagues recently showed that *Bordetella* sp. bacteria are able to modulate the expression of virulence genes in response to experimental *in vivo* levels of carbon dioxide (Hester et al., 2012). By exposing *B. pertussis* and *B. parapertussis* to 5% carbon dioxide, an increase in the expression of virulence gene transcripts encoding FHA, AC toxin, fimbriae, pertactin and type III secretion system was observed (Hester et al., 2012). In another study, BvgS response to potential *in vivo* stimulus, likely via the PAS domain has been directed to redox signals; for instance the kinase activity was abolished when purified truncated form of BvgS is exposed to oxidized ubiquinone (Bock and Gross, 2002).

1.4.1.4 Phenotypic modulation

Rather than an on/off switch, the phenotypic transition between Bvg^+ and Bvg^- phase functions like a rheostat and an intermediate phase, namely

Bvg^i has more recently been described (Figure 1.9) (Cotter and Miller, 1997; Jones et al., 2005; Scarlato et al., 1991; Williams and Cotter, 2007). This distinct intermediate Bvg^i phase between the Bvg^+ and Bvg^- phases can be observed experimentally when *B. pertussis* bacteria are grown *in vitro* at concentration of nicotinic acid and $MgSO_4$ below those that induce the Bvg^- phase (Cotter and Miller, 1997). This Bvg^i phase is characterized by maximal expression of adhesins, surface localized factor, BipA as well as repression of toxins expression (Deora et al., 2001; Stockbauer et al., 2001). Interestingly, the Bvg^i phase-locked mutant of *B. pertussis* was able to persist only at the upper murine respiratory tract, leading to the postulation that Bvg^i phase is probably necessary for the transmission of *B. pertussis* from one host to another (Vergara-Irigaray et al., 2005). The role of the *vrg*-encoded products family in *B. pertussis* virulence has not been clearly established; a Bvg^+ phase-locked *B. pertussis* mutant has been described to colonize and establish infection in mice as efficiently as the wild-type strain, suggesting that the virulent Bvg^+ phase is necessary and sufficient for respiratory infection (Martinez de Tejada et al., 1998). The *bvg*-mediated changes on the transcriptional profile in response to environmental signals are also referred to as phenotypic modulation (Figure 1.9).

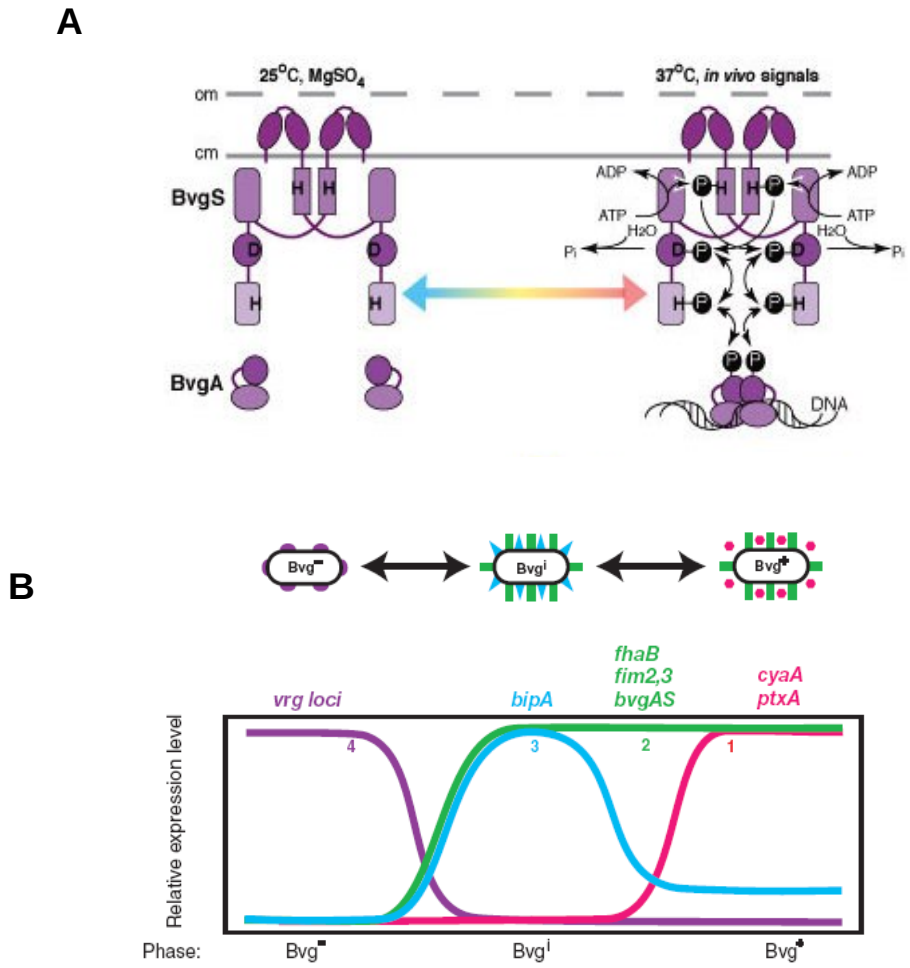


Figure 1.9: Signal transduction through BvgA/S two-component system and regulation of *vags* and *vrgs*.

(A) The BvgA/S two-component signalling event. Left panel: BvgA/S system is inactive under modulating conditions in the presence of $MgSO_4$, nicotinic acid and environmental temperature at 25°C, which is referred to as Bvg^- phase. Right panel: BvgA/S system is active standard growth temperature, which ultimately leads to the transfer of phosphate group to the response regulator BvgA. Phosphorylated BvgA then binds to the promoter region of *vags* and activates its transcription, which is referred Bvg^+ phase. Intermediate phase, referred to Bvg^i phase where concentration of nicotinic acid and $MgSO_4$ is below the concentration used to induced the Bvg^- phase. **(B) Phenotypic transition from Bvg^- to Bvg^i to Bvg^+ phase.** Each of the phases corresponds to differential expression level of various virulence genes. Figure adapted with permission (Jones et al., 2005).

1.4.1.5 BvgR: A repressor for *bvg*-repressed genes

The fact that the group of *vrgs* are repressed in Bvg^+ phase under 37°C, but elevated at a Bvg^- phase temperature below 25°C (presumably the temperature outside of the infected host), suggests that *vrgs* may be necessary for transmission of aerosolized bacteria into the outside environment from one host to another. The *bvgR* locus, located immediately downstream the *bvgAS* locus is directly and positively regulated by the BvgA/S two-component system in Bvg^+ phase (Merkel et al., 1998). The *bvgR* locus encodes the BvgR repressor protein, which in turn mediates the transcriptional repression on *vrgs* promoter (Merkel et al., 1998; Merkel and Stibitz, 1995).

In the presence of negative modulators Bvg^- phase, BvgA/S system is inactive with low intracellular levels of P-BvgA thus resulting in low expression of the BvgR repressor. Repression on *vrgs* promoters is thus relieved; therefore allowing effective transcription of *vrgs* in Bvg^- phase. In addition, a BvgA/S-independent transcriptional factor, RisA was found to promote the transcription of some *vrgs* in Bvg^- phase (Section 1.4.2) (Croinin et al., 2005). However, there has been to date no direct experimental evidence demonstrating how BvgR mediates the repression of *vrgs* in *B. pertussis*. A direct DNA binding of BvgR onto the *vrgs* promoter region was postulated based on sequence homology with a putative transcriptional regulator (Beattie et al., 1993; Galperin et al., 2001). Alternatively BvgR might exert its repressive effect on *vrgs* expression indirectly through the modulation of the transcriptional regulator of *vrgs*, RisA. The protein sequence of BvgR

revealed a unique and conserved EAL domain (Merkel et al., 1998), which can be found in a variety of signaling proteins involved in controlling the levels of bacterial second messenger, c-di-GMP (D'Argenio and Miller, 2004; Galperin et al., 2001). By modulating the intracellular levels of c-di-GMP, the EAL domain has been implicated in regulating a diversity of bacterial phenotypes, including biofilm formation (Cotter and Stibitz, 2007). In *B. pertussis*, it is hypothesized that BvgR acts as a putative c-di-GMP phosphodiesterase, which modulates the activity of RisA through control of the intracellular levels of c-di-GMP (Stibitz, 2001). Further experimental evidence is warranted to elucidate the crosstalk between c-di-GMP signaling with BvgR and RisA in *B. pertussis*.

1.4.2 The *ris* Regulon in *B. pertussis*

1.4.2.1 Discovery of RisA/S two-component system

In addition to the BvgA/S regulatory system, a second two-component regulatory system known as the RisA/S system was identified in all *Bordetella* sp. (Jungnitz et al., 1998). The *risA-risS* locus shares a high degree of homology with the *ompR-envZ* locus in *E. coli*, which is involved in the regulation of genes in response to osmolarity, and to the *S. typhi phoP-phoQ* locus, which is required for bacterial survival within macrophages (Jungnitz et al., 1998; Zimna et al., 2001). A functional RisA/S system is required for *B. bronchiseptica* to resist against oxidative stress and regulating the production of acid phosphatase necessary for intracellular survival (Jungnitz et al., 1998;

Zimna et al., 2001). However, the RisA/S system of *B. bronchiseptica* is considered as an ortholog to *E. coli* OmpR-EnvZ system, as the RisA/S system does not respond to extracellular osmolarity signals (Stenson et al., 2005). A *risAS*-deleted *B. bronchispetica* mutant strain displayed significant attenuation of its ability to colonize the mouse respiratory tract and reduced intracellular survival in macrophages, suggesting that the *ris* locus, presumably via the *ris*-regulated products are necessary for bacterial virulence (Jungnitz et al., 1998; Zimna et al., 2001).

Sequence analysis of the *risAS* locus in *B. pertussis* Tohama I strain revealed a frameshift mutation within the *risS* locus with a pre-mature stop codon, suggesting that RisS in *B. pertussis* lacks the Enz-like transmitter and autophosphorylation domain (Stenson et al., 2005). However, the *risS* frameshift mutation is absent in *B. bronschisptica* strains. Located immediately downstream the *risS* locus is the *risA* locus, whose amino acid sequences share almost 65% identity with *E. coli* OmpR protein (Stenson et al., 2005), the *risA* locus is co-transcribed with the *risS* locus as a single operon. The *risAS* operon in *B. pertussis* BP536 strain was identified to govern and activate the full expression of *bvg*-repressed surface products VraA and VraB, under modulating conditions, but it does not regulate the expression of *vags*. Furthermore, the *risA* and *risS* locus expression is independent of BvgA/S regulation (Stenson and Peppler, 1995).

1.4.2.2 Regulation of *vrgs* by transcriptional factor RisA and repressor BvgR

Purified transcriptional RisA was found to be able to bind onto the promoter of the *bvg*-repressed genes *vrg6* and *vrg18* in *B. pertussis* BP536 strain (Croinin et al., 2005), suggesting that *vrg6* is directly regulated by RisA. Due to the insolubility of BvgR protein, similar approach was not successful in an attempt to study the promoter binding capacity of the repressor protein (Croinin et al., 2005). However, results obtained with a *bvgR*-deleted mutant implied that BvgR is required for the repression of *vrgs* under Bvg^+ phase, but does not regulate the expression of RisA (Croinin et al., 2005). It was speculated that repressor BvgR acts either by binding to the *vrg6* promoter thus competing with the binding of RisA, or by affecting the function or activity of RisA protein via c-di-GMP signaling (section 1.3.1.5) (Croinin et al., 2005). However, the authors failed to fully demonstrate the conclusive relationship between BvgR and RisA on the regulation of *bvg*-repressed genes. As all *B. pertussis* strains display a frameshift mutation within the *ris*S locus, *risA* activation is believed to be independent of RisS. Phospho-activation of OmpR in an *envZ*-deleted *E. coli* mutant suggests that OmpR can be phosphorylated by another regulatory system besides the EnvZ sensor kinase (Forst et al., 1990). It is unclear how RisA is activated and possible speculation includes activation through another sensor kinase via a molecular crosstalk either with the BvgA/S sytem or with another two-component system in *B. pertussis*. At this stage, it is unclear whether RisA and BvgR regulate the capsule locus expression in *B. pertussis*.

1.5 RATIONALE AND OBJECTIVES

The first and major aim of this thesis is to address the role of the capsule locus in pertussis pathogenesis and characterize the mechanisms involved (Chapter 3). To do so, mutants deleted for ORF involved in the transport, export and biosynthesis of the capsular polysaccharide have been generated and their *in vivo* and *in vitro* phenotypes have been studied. A combination of molecular biology and biochemistry approaches has allowed us to demonstrate a novel and unique mechanism by which the capsule locus is involved in *B. pertussis* virulence.

In the second part of this thesis (Chapter 4), we aimed at characterizing the expression of the capsule locus in various environmental conditions that include *in vitro*, *ex vivo* (macrophage infection) and *in vivo* (mouse model of pertussis) conditions. Furthermore, there has been increasing evidence of some co-regulation between the BvgA/S (via BvgR) and RisA/S two-component systems to modulate the expression of *vrgs* in *B. pertussis* (see section 1.3.2.2). However, the regulation of the capsule locus has not been investigated in details. We thus studied the regulation of the capsule locus expression by BvgR and RisA.

Re-emergence of pertussis cases in adult population has been increasingly reported, suggesting that current vaccination fail to provide long-term protection against pertussis infection. Understanding how the capsule

locus *in B. pertussis* affects bacterial virulence opens up opportunities to exploit it, either by developing new vaccine, drug target or improving current vaccination strategies. Moreover, study on the regulation of a *bvg*-repressed locus will impel an entirely new field of investigation in *B. pertussis* pathogenesis.

CHAPTER 2 MATERIALS AND METHODS

(A) *ESCHERICHIA COLI* WORK

2.1 BACTERIAL STRAINS, PLASMIDS AND GROWTH CONDITIONS

2.1.1 *E. coli* Strains and Plasmids

The *E. coli* strains and plasmids used or generated in this study are listed in the table below.

Strain/ Plasmids	**Description[a]**	**Source/ Reference**
Strains		
One Shot® TOP10	For general purpose cloning, blue/white screening without IPTG	Invitrogen
Backbone plasmids		
pCR® 2.1-TOPO®	Amp^r, Kan^r; high copy number TA cloning vector with single 3'thymidine overhangs and covalently-bound Topoisomerase I, *LacZα* gene for blue/white screening	Invitrogen
pBR322	Amp^r, Tet^r; medium to low copy number plasmid, used as an intermediate plasmid	(Bolivar et al., 1977)
pJQ200mpl8-rpsl	Gm^r, Sm^s; low copy number suicide vector, wild type rpsL thus is streptomycin sensitive	(Quandt and Hynes, 1993)
pBBR1MCS	Cm^r; broad-host-range cloning vector	(Elzer et al., 1995)
pUC19	Amp^r; High copy number cloning vector; *LacZα* gene for blue/white screening	(Yanisch-Perron et al., 1985)
pUC57	Amp^r; high copy number *E. coli* cloning vector derived from pUC19	Genescript
Plasmids for Δ*kpsT* cloning		
TOPO-T1	Amp^r, Kan^r; TOPO® derivative containing the PCR1 (*kpsM*) insert	This study

	flanked by *BamH*I and *Nhe*I restriction sites	
TOPO-T2	Amp^r, Kan^r; TOPO® derivative containing the PCR2 (*kpsE*) insert flanked by *Nhe*I and *Hind*III restriction sites	This study
pBR-T1	Amp^r, Tet^r; pBR322 derivative containing the PCR1 insert flanked by *BamH*I and *Nhe*I restriction sites	This study
pBRT1-2	Amp^r, Tet^r; pBR322 derivative containing the PCR1 + PCR2 fragment flanked by *BamH*I and *Hind*III restriction sites	This study
pJQT1-2	Gm^r, Sm^s; pJQ200mp18-rpsl derivative containing the PCR1 + PCR2 flanked by *BamH*I and *Hind*III restriction sites, used for the construction of Δ*kpsT*	This study
Plasmids for Δ*kpsE* cloning		
TOPO-E1	Amp^r, Kan^r; TOPO® derivative containing the PCR1 (*kpsT*) insert flanked by *EcoR*I and *Hind*III restriction sites	This study
TOPO-E2	Amp^r, Kan^r; TOPO® derivative containing the PCR2 (*wbpT*) insert flanked by *Hind*III and *BamH*I restriction sites	This study
pBR-E1	Amp^r, Tet^r; pBR322 derivative containing the PCR1 insert flanked by *EcoR*I and *Hind*III restriction sites	This study
pBRE1-2	Amp^r, Tet^r; pBR322 derivative containing the PCR1 + PCR2 fragment flanked by *EcoR*I and *BamH*I restriction sites	This study
pJQE1-2	Gm^r, Sm^s; pJQ200mp18-rpsl derivative containing the PCR1 + PCR2 flanked by *EcoR*I and *BamH*I restriction sites, used for the construction of Δ*kpsE*	This study
Plasmids for Δ*vipC* cloning		
pJQV1-2	Gm^r, Sm^s; pJQ200mp18-rpsl derivative containing the PCR1 + PCR2 flanked by *EcoR*I and *BamH*I restriction sites, used for the construction of Δ*vipC*	This lab (Neo Y.L)
Plasmids for complementation of Δ*kpsT* mutant		
pUC57-Pcaps	Amp^r; pUC57 derivative containing 866 bp of native *B. pertussis* capsule promoter flanked by *Xba*I and *BamH*I restriction sites	Genescript
pBBR-*kpsT*	Cm^r; pBBR1MCS derivative containing *B. pertussis kpsT* ORF flanked by *BamH*I	This study

	and *Hind*III restriction sites	
pBBR::Pcaps*k psT*	Cm^r; pBBR1MCS derivative containing native *B. pertussis* capsule promoter and *kspT* ORF flanked by *Xba*I and *Hind*III restriction sites	This study
Plasmids for KO*caps* mutant expressing the *kpsT* and *kpsMT* ORFs		
TOPO-Pcaps2	Amp^r, Kan^r; TOPO® derivative containing 858 bp of native *B. pertussis* capsule promoter flanked by *Xba*I and *BamH*I restriction sites (using pUC57-Pcaps as template)	This study
pBBR::Pcaps*k psT*	Cm^r; pBBR1MCS derivative containing native *B. pertussis* capsule promoter and *kspT* ORF flanked by *Xba*I and *Hind*III restriction sites	This study
pUC57-*kpsMT*	Amp^r; pUC57 derivative containing 1529 bp *B. pertussis kpsM* and *kpsT* ORFs flanked by *BamH*I and *Hind*III restriction sites	This study
pBBR-*kpsMT*	Cm^r; pBBR1MCS derivative containing 1529 bp *B. pertussis kpsM* and *kpsT* ORFs flanked by *BamH*I and *Hind*III restriction sites	This study
pBBR::Pcaps*k psMT*	Cm^r; pBBR1MCS derivative containing native *B. pertussis* capsule promoter and *kspM + kpsT* ORFs flanked by *Xba*I and *Hind*III restriction sites	This study
Plasmids for His_6-BvgS cloning		
TOPO-BvgAS-PCR1	Amp^r, Kan^r; TOPO® derivative containing the PCR1 (*bvgA* and *bvgS*) insert flanked by *BamH*I and *Xba*I restriction sites	This study
TOPO-BvgSHis_6PCR 2	Amp^r, Kan^r; TOPO® derivative containing the PCR2 insert flanked by *Xba*I and *Hind*III restriction sites	This study
pJQ-BvgSHis_6PCR 1+2	Gm^r, Sm^s; pJQ200mp18-rpsl derivative containing the PCR1 + PCR2 flanked by *BamH*I and *Hind*I restriction sites	This study
pJQSY4	Gm^r, Sm^s; pJQ200mp18-rpsl derivative containing the PCR1 + PCR2 flanked by *Sal*I and *EcoR*I restriction sites, used for the construction of KO*caps* mutant	(Neo et al., 2010)
Plasmid for over-expression of *risA*		
TOPO-P*fha*	Amp^r, Kan^r; TOPO® derivative containing *fha* promoter insert flanked by *Kpn*I and *BamH*I restriction sites	This study
TOPO-P*recA*	Amp^r, Kan^r; TOPO® derivative containing *recA* promoter insert flanked by *Hind*III and *BamH*I restriction sites	This study

TOPO-*risA*	Amp^r, Kan^r; TOPO® derivative containing risA insert flanked by *BamH*I and *Xba*I restriction sites	This study
pBBR::P*fha*-*risA*	Cm^r; pBBR1MCS derivative containing native *fha* promoter insert and *risA* ORF flanked by *Kpn*I and *Xba*I restriction sites	This study
pBBR::P*recA*-*risA*	Cm^r; pBBR1MCS derivative containing native *recA* promoter insert and *risA* ORF flanked by *Hind*III and *Xba*I restriction sites	This study
Plasmid for cloning of Δ*risAS*		
TOPO-RisAS-PCR1	Amp^r, Kan^r; TOPO® derivative containing the PCR1 (*risA*) insert flanked by *BamH*I and *Nhe*I restriction sites	This study
TOPO-RisAS-PCR2	Amp^r, Kan^r; TOPO® derivative containing the PCR2 (*ahpC*) insert flanked by *Nhe*I and *Hind*III restriction sites	This study
pJQ*risAS*1+2	Gm^r, Sm^s; pJQ200mp18-rpsl derivative containing the PCR1 + PCR2 flanked by *BamH*I and *Hind*III restriction sites, used for the construction of Δ*risAS* mutant	This study

Table 2.1: *E. coli* strain and plasmid

2.1.2 Growth Conditions

All *E. coli* strains were grown at 37°C overnight in 3 ml of fresh Luria-Bertani broth (Difco, Detroit, Mich.) with shaking at 220 rpm or on LB agar (Difco) plates. When appropriate, 100 μg/ml ampicillin, 50 μg/ml kanamycin, 10 μg/ml gentamicin and 30 μg/ml chloramphenicol were added to select for antibiotic-resistant strains.

2.2 MOLECULAR BIOLOGY

2.2.1 List of Primers

The list of primers that were used for cloning, screening and/ or sequencing in *E. coli* is as shown in Table 2.2. When necessary, primers used to amplify inserts had appropriate restriction sites added at their 5' ends (shown in bold and underlined) to facilitate subsequent cloning work. Annealing temperature (T_a) was calculated according to the following equation: $[(4 \times N_{G/C}) + (2 \times N_{A/T})] - 5$, where $N_{G/C}$ is the number of G and C bases and $N_{A/T}$ is the number of A and T bases.

Oligo name	**Sequence (5' to 3')**	**Description**
Primers for Δ*kpsT* cloning		
kpsM1F	tt**ggatcc**tgtccaccaccatctacgtggtgt	Forward and reverse primers to amplify PCR1 (*kpsM*) fragment
kpsM2R	tt**gctag**ccagctccatgccgcagatca	
kpsE1F	tt**gctag**ccttggacgaaaccatcgcgc	Forward and reverse primers to amplify PCR2 (*kpsE*) fragment
kpsE2R	tt**aagct**tgccagctgcagattggcctc	
Primers for Δ*kpsE* cloning		
kpsT1F	tt**gaattc**cgcatgatctgcggcatcga	Forward and reverse primers to amplify PCR1 (*kpsT*) fragment
kpsT2R	tt**aagctt**gacatactggtcggacgcaat	
wbpT7F	tt**aagctt**gaggccaatctgcagctggc	Forward and reverse primers to amplify PCR2 (*wbpT*) fragment
wbpT6R	tt**ggatcc**tatgcccgcggcgcggctt	
Primers for Δ*kpsT* mutant complementation cloning/screening		
kpsTcomF	tt**ggatcc**cgttgatggagacggccatg	Forward and reverse primers to amplify *B. pertussis kpsT* ORF
kpsTcomR	tt**aagctt**tcaggattgctcagcgtcgac	
Primers for KO*caps* mutant expressing the *kpsT* and *kpsMT* ORFs		
Pcaps*Xba*I-F	tt**tctaga**cgcgaatctgtcagtagctgc	Forward and reverse primers to amplify *B. pertussis* capsule promoter (Pcaps2) using pUC57-Pcaps as template
Pcap2.2*BamH*I-R	tt**ggatcc**acctgggctcccgatgcctgcaa	

Primers for BvgS-His$_6$ cloning		
BvgA-*BamH*I-F	tt**ggatcc**tgtactgagattcgccgtc	Forward and reverse primers to amplify PCR1 from 3' end of *bvgA* ORF to 5' end of *bvgS* signal peptide ORF
BvgS-*Xba*I-R	tt**tctaga**gcttgcctgcgcgggc	
BvgS-*Xba*I-His$_6$-F	tt**tctaga**catcatcaccatcaccaccaggag ctgaccctg	Forward and reverse primers to amplify PCR2 downstream of *bvgS* signal peptide sequence; forward primer carries nucleotides encoding for six histidines
BvgS-*Hind*III-R	tt**aagctt**ggcgactacgcgaacgtcattgaa	
Primers for *risA* over-expression		
P*fha*-F	tt**ggtacc**tttgagtttcgtggcgag	Forward and reverse primers to amplify *B. pertussis fha* promoter
P*fha*-R	tt**ggatcc**catattccgaccagcgaagtgaag	
P*recA*-F	tt**ggatcc**gtaaagtcctgtattgaag	Forward and reverse primers to amplify *B. pertussis recA* promoter
P*recA*-R	tt**aagctt**gcctgcgcagcacctcca	
*BamH*I-RisA-F	tt**ggatcc**atgaacacgcaaaaacacc	Forward and reverse primers to amplify *B. pertussis risA* ORF
*Xba*I-RisA-R	tt**tctaga**tcaactgccgccatccg	
Primers for Δ*risAS* cloning		
*risA*1.1BamHI-F	tt**ggatcc**caccgcctcatgcgacac	Forward and reverse primers to amplify PCR1 (*risA*) fragment
risA-NheI-R	tt**gctagc**agatcatcgacaggccatcctc	
RisS-NheI-F	tt**gctagc**gtagaatttagggcttgag	Forward and reverse primers to amplify PCR2 (*ahpC*) fragment
AhpC-HindIII-R	tt**aagctt**ttacagcgtggcgccgcc	
Prmers for sequencing		
M13-F	gtaaaacgacggccag	Forward and reverse primers to sequence TOPO® construct (Invitrogen)
M13-R	caggaaacagctatgac	

Table 2.2: Primers used for *E. coli* work

2.2.2 Polymerase Chain Reaction

2.2.2.1 Polymerase Chain Reaction

All PCR reactions were carried out using HotStarTaq® PCR kit (Qiagen, Hilden, Germany) or GoTaq® DNA polymerase (Promega, MA, Wisconsin) with suitable primer pairs (Table 2.2). A 50 µl reaction mix was set up for each of the PCR amplification according to the manufacturer's instruction. Amplification was conducted in the GeneAmp® PCR system 2400 thermal cycler (PE Applied Biosystem, CA, USA) with initial denaturation step of 95°C for 15 min or 2 min, followed by 25-35 cycles of DNA denaturation at 95°C for 45 s, primer annealing at suitable T_a for 50 sec -1 min, and DNA elongation at 72°C for 1 min-2 min depending on the size of the product to be amplified (approx. 1 min / kb of DNA template). A final extension step of 5-10 min at 72°C was also included, after which the reaction was maintained at 4°C in the machine.

2.2.2.2 Colony PCR screening

A loopful of bacteria was dissolved in 30 µl of ultrapure water, heated at 95°C for 10 min and centrifuged at 13,000 rpm for 5 min. PCR was performed as described in Section 2.2.2.1 for 35 cycles, using 2 µl of the supernatant as template.

2.2.3 Restriction Enzyme Digestion

DNA digestion was performed with appropriate restriction enzymes (RE) with their corresponding buffers (New England Biolabs, Ipswich, MA) following the manufacturer's protocol. A one-step digestion of 1-2 h at 37°C was performed in case of single RE or when two REs share the same buffer. Sequential digestion was carried out when two or more REs have incompatible buffers. In this scenario, the first RE was heat-inactivated according to manufacturer's instructions, followed by membrane dialysis (Millipore, Billerica, MA) against deionized water for 10 min prior to setting the digestion reaction with the second RE. The restriction profile was analyzed by DNA gel electrophoresis (Section 2.2.4).

2.2.4 Agarose Gel Electrophoresis

2.2.4.1 Gel migration

DNA/RNA electrophoresis was performed using 0.8% to 1.5% agarose in 1x Tris-Acetate (TAE) running buffer (0.04 M Tris-Acetate and 0.0001 M ethylenedinitrilo tetraacetic acid (EDTA), pH 7.8). Each DNA/RNA sample to be analyzed was mixed with 1/5 volume of blue/orange 6x loading dye (Promega) containing 0.4% orange G, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 15% Ficoll® 400, 10 mM Tris-HCl (pH 7.5) and 50 mM EDTA (pH 8.0) prior to loading into gel wells. 6 µl of 1 kb or 100 bp DNA

ladder (Promega) were used as markers to estimate bad sizes. Electrophoresis was carried out at 100 V for 45 min, after which the gel was stained in 0.5 μg/ml of ethidium bromide (Invitrogen, Carlsbald, CA) for 15 min before visualization under a ultra-violet (UV) transilluminator (ChemiGenius, Syngene, UK).

2.2.4.2 Gel extraction

After agarose DNA electrophoresis, DNA fragments of interest were excised from agarose gel under low UV light (366 nm) to prevent DNA damage. The DNA fragments were purified directly using the QIAquick Gel Extraction and PCR Purification Kit (Qiagen) as described in the manufacturer's protocol.

2.2.5 Plasmid Extraction

Plasmid DNA was isolated and purified using the QIAprep Spin Miniprep Kit (Qiagen) or GeneJET Plasmid Miniprep Kit (Thermo, Waltham, MA) according to the manufacturer's instructions. High and low copy plasmids were eluted in 50 and 20 μl of elution buffer respectively.

2.2.6 DNA Cloning

Digestion with appropriate RE(s) was performed on plasmids to open up the cloning vectors and to generate DNA inserts. Both digested vectors and inserts were electrophoresed on agarose gel and the DNA bands of interest were excised and purified from the gel as described in Section 2.2.4.2. Cloning of inserts into the TOPO® vector was performed using the TOPO® TA Cloning Kit (Invitrogen), while DNA inserts were ligated into pBR322, pBBR1MCS and pJQ vectors using the Fast-Link DNA Ligation Kit (Epicentre Technologies, Madison, WI) or Takara DNA Ligation Kit (Otsu, Shiga, Japan) according to the manufacturer's protocol.

2.2.7 Transformation of Chemically Competent *E. coli*

Chemically competent One Shot® TOP10 *E. coli* (Invitrogen) were transformed with slight modifications from the manufacturer's protocol. Half of the ligation mix (section 2.2.6) was added into one vial of TOP10 cells and mixed gently. The vial was incubated on ice for 30 min, followed by heat shock at 42ºC for 45 s. The tube was immediately transferred on ice and 500 µl of LB medium without antibiotics was added to the mixture. After incubation at 37ºC for 1 h, 100 µl of the transformation mixture was plated on LB agar supplemented with appropriate antibiotics or on imMedia™ Amp Blue ready-mix agar (Invitrogen) for blue-white screening selection. The plates were incubated at 37ºC overnight.

2.2.8 DNA sequencing

Plasmids from the recombinant clones were sent for DNA sequencing (AIT Biotech) with suitable primers (Table 2.2). Alignment of the obtained sequences with the relevant nucleotide databases was performed using the NCBI BLAST programme (http://blast.ncbi.nlm.nih.gov/) and the sequence alignment programme ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

(B) *BORDETELLA PERTUSSIS* WORK

2.3 BACTERIAL STRAINS AND GROWTH CONDITIONS

2.3.1 *B. pertussis* Strains

The *B. pertussis* strains used in this study are listed in the table below.

Strain	**Description**	**Source/ Reference**
BPSM	TohamaI derivative, mutant rpsL; Sm resistance	Pasteur Institute of Lille
Tohama-I	Clinical isolate from 1954, Japan	Dr. Frits Mooi, Centre for Infectious Diseases Control, The Netherlands
18323	Clinical isolate from 1946, USA	Dr. Frits Mooi, Centre for Infectious Diseases Control, The Netherlands
KO*caps*	BPSM carrying an in-frame	(Neo et al., 2010)

	deletion from *kpsM* to *wcbO* ORFs	
Δ*kpsT*	BPSM carrying an in-frame deletion in *kpsT* ORF	This study
Δ*kspE*	BPSM carrying an in-frame deletion in *kpsE* ORF	This study
Δ*vipC*	BPSM carrying an in-frame deletion in *vipC* ORF	This lab (Neo Y.L.)
Δ*kpsT*com	BPSM carrying an in-frame deletion in *kpsT* ORF containing vector pBBR::Pcaps-*kpsT*	This study
BvgS-VFT2	BPSM carrying amino acid substitution at F375E and Q461E at the periplasmic VFT2 domain	(Herrou et al., 2010)
BvgS-VFT2-Δ*kpsT*	BvgS-VFT2 carrying an in-frame deletion in *kpsT* ORF	This study
KO*caps*:*kpsT*	KO*caps* containing vector pBBR::Pcaps-*kpsT*	This study
KO*caps*:*kpsMT*	KO*caps* containing vector pBBR::Pcaps-*kpsMT*	This study
BPSH	BPSM derivative expressing his-tagged BvgS at the N-terminal	This study
BPSH-KO*caps*	BPSH cotaining an in-frame deletion from *kpsM* to *wcbO* ORFs	This study
BPSH-Δ*kpsT*	BPSH carrying an in-frame deletion in *kpsT* ORF	This study
BPSH-Δ*kpsT*com	BPSH carrying an in-frame deletion in *kpsT* ORF containing vector pBBR::Pcaps-*kpsT*	This study
BPSM-P*fha-risA*	BPSM containing vector pBBR::P*fha-risA*	This study
BPSM-P*recA-risA*	BPSM containing vector pBBR::P*recA-risA*	This study
BPSM-pbbr1mcs	BPSM containing vector pBBR1MCS	This study
Δ*bvgAS*	BPSM carrying an in-frame deletion in *bvgAS* ORF	This lab (Ho S.Y.)
Δ*bvgAS*-pbbr1mcs	Δ*bvgAS* containing vector pBBR1MCS	This study
Δ*bvgAS*-P*recA-risA*	Δ*bvgAS* containing vector pBBR::P*recA-risA*	This study
Δ*risAS*	BPSM carrying an in-frame deletion in *risAS* ORF	This study

Table 2.3: *B. pertussis* strains

2.3.2 Growth Conditions

All *B. pertussis* strains were grown at 37ºC on pre-warmed sterile Bordet-Gengou (BG) agar (Difco) supplemented with 1% glycerol and 10% defibrinated sheep blood or in pre-warmed sterile modified Stainer-Scholte (SS) medium containing 1 g/L 2,6-*O*-dimethyl-ß-cyclodextrin (Sigma) as described previously (Menozzi et al., 1991a). When appropriate, 10 µg/ml gentamicin, 100 µg/ml streptomycin, 30 µg/ml chloramphenicol or 0.05 µg/ml of erythromycin (Sigma) were added to select for antibiotic-resistant strains. For EDTA treatment assay, *B. pertussis* strains grown on BG agar for 24 h were adjusted to initial OD_{600nm} of 0.02. The bacterial suspension (50 µl) was incubated with 1 or 2 mg/ml of EDTA for 2 h at 37°C. Treated and untreated controls were serially diluted and plated onto BG agar. The number of CFU (colony forming units) was determined after 3-4 days incubation at 37°C.

2.4 MOLECULAR BIOLOGY

2.4.1 List of primers

The list of primers that were used for screening of *B. pertussis* strain and Southern blot analysis is as shown in Table 2.4.

Primer name	**Sequence (5' to 3')**	**Description**
Primers for PCR screening of capsule-deficient mutants and complemented strains		
kpsTKO1F	atcgagccgatcctgcacgt	Forward and reverse primers to amplify upstream of PCR1 (*kpsM*) for screening
kpsTKO1R	attgctcagcgtcgaccgtg	

kpsTKO2F	attctcgatgacgtgtcgttcga	Forward and reverse primers to amplify downstream of PCR2 (*kpsE*) for screening
kpsTKO2R	atggtcattgaggtccttgagct	
kpsEKO1F	attctcgatgacgtgtcgttcg	Forward and reverse primers to amplify upstream of PCR1 (*kpsT*) for screening
kpsEKO1R	atcaccgtgtacagcacctgg	
kpsEKO2F	ggagaaaacctatacaggcc	Forward and reverse primers to amplify upstream of PCR2 (*wbpT*) for screening
kpsEKO2R	ctcgagcaggtcgagaatcgt	
kpsTF	attctcgatgacgtgtcgttcga	Forward and reverse primers to amplify the middle portion of *kpsT* ORF
kpsTR	ggcgaacacctcgagacacattg	
mid-PcapsF	gacggatgcgcggcattg	Forward and reverse primers to amplify upstream of 5' *kpsM*
mid-kpsMR	gatgtccatgttggtgatcg	
Primers for PCR screening of BPSH strain		
bvgShis6F	tctcagaacatcatcaccatcacacc	Forward primer binds to the six histidine coding nucleotides and reverse primer bind downstream of His_6 insertion
bvgShis6R	gggcgactacgcgaacgta	
bvgAhisSeqF	ataacggcattgacgggctc	Forward and reverse primers flanking the six histidine coding nucleotides used for sequencing
bvgShisSeqR	gcatcgccgatgaatacgtc	
Primers for PCR screening of *B. pertussis* strain over-expressing *risA*		
P*fha*-F	acttcacttcgctggtcggaa	Forward and reverse primers to screen for pBBR::P*fha-risA* plasmid in BPSM
risA-R	cgggttgaagggcttggaca	
p*recAriA*-F	ggatacgcatgcgtgcaacat	Forward and reverse primers to screen for pBBR::P*recA-risA* plasmid in BPSM and Δ*bvgAS*
p*recAriA*-R	cgggttgaagggcttggaca	
Primers for PCR screening of Δ*risA*S mutant strain		
Pfha-Fp	acttcacttcgctggtcggaa	Forward and reverse primers flanking to screen for pBBR::P*fha-risA* plasmid
risAKO-R	ttggatccgtcgaacgcctcgtattcgc	
AhpD-Fp	acgtacggataccaggtgtt	Forward and reverse primers flanking the *risAS* deleted region
risA-Rp	ctgctggttctcgacctgatg	
Primers for Southern blot probe synthesis		
kpsTKO1F	atcgagccgatcctgcacgt	Forward and reverse primers to amplify probe for screening of Δ*kpsT*, BvgS-VFT2-Δ*kpsT* and BPSH-Δ*kpsT*
kpsT3R	cgaacgacagctcatcgagaat	
wbpT7-F	ttaagcttgaggccaatctgcagctggc	Forward and reverse primers to amplify probe for screening of

kpsEKO2-R	ctcgagcaggtcgagaatcgt	Δ*kpsE* mutant
P1-F	tgctcgccgttcttcgatcg	Forward and reverse primers to amplify probe for screening of BPSH-KO*caps* mutant
P2-R	tcctggacctcgcgcatatc	

Table 2.4: Primers used for *B. pertussis* PCR screening work and Southern blot

2.4.2 Transformation of *B. pertussis*

2.4.2.1 Preparation of electrocompetent cells

10 ml of bacteria exponentially grown up to OD_{600} 3 to 4 in SS medium were centrifuged at 7,000 rpm for 10 min at room temperature. The bacteria pellet was washed 3 times with 10 ml of sterile 10% glycerol. After the final wash, the pellet was re-suspended in 1 ml of 10% glycerol and the cells were immediately used for electroporation.

2.4.2.2 Electroporation of plasmid DNA into *B. pertussis*

About 1 µg of plasmid DNA was mixed with 200 µl of electrocompetent *B. pertussis* cells in an electroporation cuvette (0.2 cm) (Biorad) and incubated on ice for 5 min. An electrical pulse of 2.5 kV, 800 Ω resistance and 25 µF capacitance was administered. 1 ml of pre-warmed SS medium without antibiotics was added to recover the bacteria cells, which were then incubated for 5 h at 37°C with shaking. 200 µl of bacteria cells were

plated onto pre-warmed BG agar plates containing appropriate antibiotics and incubated for 5-7 days at 37°C.

2.4.3 Selection of Transformants

Gentamicin-resistant (Gm^r) colonies obtained upon electroporation were patched first onto streptomycin then on gentamicin plates. Streptomycin sensitive (Sm^s) and Gm^r clones were observed within 24 h and were identified as transformants that have successfully undergone first event of recombination. These intermediate transformants were then streaked onto a Sm plate to select for the second event of recombination. After incubation 5-7 days at 37°C, isolated Sm^r colonies were then patched first onto Gm then onto Sm plates. Sm^r and Gm^s clones were observed within 24 h and were identified as transformants with completed second event of recombination.

2.4.4 Analysis of True Recombinants

Colony PCR screening was performed on Sm^s and Gm^r intermediate clones and Sm^r and Gm^s clones to distinguish between true recombinants from revertants at the genomic level. DNA template was harvested as described in section 2.2.2.2 except that *B. pertussis* bacteria were heated for 30 min instead of 10 min. PCR was carried out as described in section 2.2.2.1 for 35 cycles using appropriate primers (Table 2.4) followed by agarose gel electrophoresis (Section 2.2.4.1).

2.4.5 Chromosomal DNA Extraction

10 ml of *B. pertussis* liquid culture grown in SS medium was centrifuged at 8,000 rpm for 10 min and DNA extraction was performed using Genomic-tip 100/G Anion-Exchange Resin and Genomic DNA Buffer Set (Qiagen) according to the manufacturer's instructions. DNA was finally precipitated in 100% isopropanol and dissolved in 0.5-1 ml of ultrapure water at 4°C overnight. Alternatively, *B. pertussis* chromosomal DNA was harvested by phenol-chloroform extraction method. 2 ml of *B. pertussis* culture grown to mid-exponential phase in SS medium was centrifuged at 8,000 rpm for 10 min, re-suspended in 600 µl of lysis buffer (3% SDS, 1 mM $CaCl_2$, 10 mM Tris-HCl, 100 mM NaCl, pH 8.0) and heat-inactivated at 95°C for 30 min. Cell debris were pelleted by centrifugation at maximum speed for 10 min and the supernatant was mixed with EGTA to a final concentration of 2mM. The supernatant was transferred to a MaXtract™ High Density phase-locked Eppendorf tube (Qiagen). Equal volume of phenol/chloroform/isoamylalcohol (49:49:2, vol/vol/vol) (Invitrogen) was added to supernatant in the phased locked tube. The liquid mixture in the phase-locked tubes was mixed vigorously for 1 min to evenly mix the liquid phases. The phase-locked tubes were centrifuged at maximum speed for 5 min to separate the aqueous phase, inter phase containing MaXtract™ gel and the organic phase. The aqueous phase was transferred to a clean tube and 0.8 volume of 100% isopropanol and 0.1 volume of 3 M sodium acetate were added for precipitation of chromosomal DNA. The mixture was centrifuged at maximum speed for 15 min and the chromosomal DNA pellet was washed once with 70% ethanol,

air-dried at room temperature and dissolved in 1 ml TE buffer (10 mM Tris pH 8.0, 0.2 mM EDTA). The extracted chromosomal DNA was quantified by NanoDrop™ ND-1000 spectrophotometer (Thermo) and was visualized by DNA gel electrophoresis (Section 2.2.4.1).

2.4.6 Southern Blot Analysis

2.4.6.1 Synthesis of DIG-labeled probe

Probes for Southern blot analysis were PCR synthesized and labeled with digoxigenin (DIG) using the PCR DIG Probe Synthesis Kit (Roche) according to the manufacturer's instructions. To estimate probe-labeling efficiency, an unlabeled control reaction without DIG-labeled dNTPs was included. Amplification of DIG-labeled probe was conducted in GeneAmp® PCR system 2400 thermal cycler (Biorad) with initial denaturation at 95°C for 2 min, followed by 35 cycles of DNA denaturation at 95°C for 30 s, primer annealing at appropriate annealing temperature for 40 s and DNA elongation at 72°C for 1 min. An additional 7 min of DNA extension at 72°C was included, after which the reaction was maintain at 4°C. The synthesized probes, both labeled and unlabeled, were analyzed and quantified by gel electrophoresis (Section 2.2.4.1).

2.4.6.2 Southern blot

Approximately 1 µg of *B. pertussis* chromosomal DNA was digested

for 4 h with appropriate RE(s) as described in Section 2.2.3 and the digested chromosomal DNA was subjected to 0.8% agarose gel electrophoresis. The agarose gel containing the digested DNA was treated twice in depurination solution (0.25 M HCl) for 10 min with gentle agitation, rinsed in deionised water, soaked twice in denaturation solution (5% of 10 N NaOH and 8.75 % NaCl) for 15 min and finally soaked twice in neutralization solution (7.7 % ammonium acetate, 0.2 % 10 N NaOH solution) for 30 min. The DNA fragments were transferred for 2-4 h onto a nitrocellulose membrane (Milipore). Before the assembly as illustrated in Figure 2.1, the nitrocellulose membrane was pre-wet in neutralization solution. After transfer, the membrane was UV-fixed for 1 min and equilibrated with 10 ml of pre-heated DIG Easy Hyb solution (Roche) at 65°C for 20 min, with gentle agitation. For hybridization, about 5-25 ng/ml of heat-denatured DIG-labeled DNA probe in DIG Easy Hyb solution was incubated with the membrane overnight at 65°C. After hybridization the membrane was washed twice in 2xSSC (0.15 M NaCl and 0.015 M sodium citrate) containing 0.1% SDS for 5 min at room temperature, followed by 2 wash steps in 0.1x SSC containing 0.1% SDS for 15 min at 65°C. Blocking and washing were done using the DIG Wash and Block Buffer Set (Roche) according to the manufacturer's instructions. Detection was performed by incubating the membrane with alkaline phosphatase (AP)-conjugated anti-DIG antibody (Roche) at a dilution of 1:5,000 for 1 h at room temperature. The membrane was developed using NBT/BCIP AP substrate (Chemicon) and reaction was stopped by washing the developed membrane in deionised water.

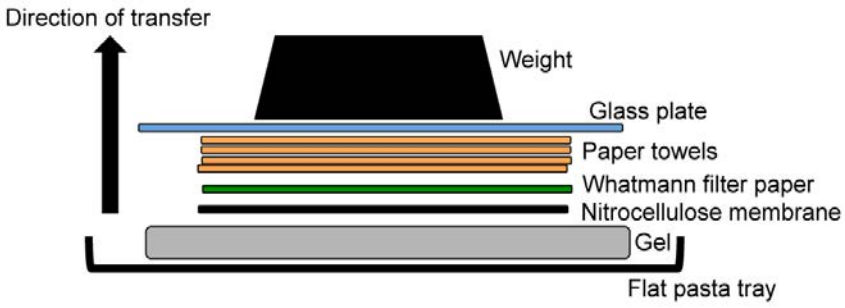


Figure 2.1: Semi-dry transfer of nucleic acids onto nitrocellulose membrane.

2.4.7 RNA Extraction

2.4.7.1 RNA extraction from *in vitro B. pertussis* culture

500 µl of mid-exponential bacterial culture was harvested into 1 ml of RNAprotect™ Bacteria Reagent (Qiagen). The mixture was immediately vortexed and incubated at room temperature for 15 min. The bacterial cells were centrifuged for 10 min at 8,000 rpm, and the supernatant was discarded. The bacterial pellet was first re-suspended in 100 µl 1x TE buffer (Tris-EDTA at pH 8) containing 20 mg/ml of lysozyme (Sigma) and was incubated at room temperature for 10 min, vortexed for 10 s for every 2 min of incubation. RNA extraction was carried out using the RNeasy Mini Kit (Qiagen) according to the manufacture's protocol. The RNeasy clean up with an on-column DNase treatment was employed using the DNase-I set (Qiagen) as recommended by the manufacturer's protocol. Finally, total RNA was eluted using 30-50 µl of DEPC-RNase free water (Invitrogen).

2.4.7.2 RNA extraction from *B. pertussis* infected eukaryotic cells

10 ml of RNAprotect™ Bacteria Reagent (Qiagen) were added into the tissue culture flasks of *B. pertussis* infected mammalian cells. The cell monolayers were then mechanically scraped, pooled and centrifuged at maximal speed. The cell pellet obtained was then re-suspended in 2 ml of DPEC water, vortexed vigorously and incubated at 37ºC for 20 min for lysis of mammalian cells. Free bacteria and cell debris were then pelleted at 10,000 rpm for 10 min, followed by re-suspension of the pellet in 300 µl of of 1 x TE buffer containing 50 µl of 20 mg/ml lysozyme. The RNA extraction procedure was similar to that described in section 2.4.7.1 except that the amount of RLT buffer and ethanol used per collecting tube was scaled up to 700 µl and 500 µl respectively. In addition, in-solution DNA digestion with DNase-I was performed according to the manufacturer's protocol to further eliminate genomic DNA contaminants. The essentially cleaned RNA was then eluted in 20-30 µl of DPEC water.

2.4.7.3 RNA extraction from *B. pertussis* infected mice lungs

B. pertussis-infected BALB/c mice (CARE) were euthanized and their lungs were aseptically removed (as described in section 2.12) and immediately immersed in 3 ml of RNAprotect™ Bacteria Reagent (Qiagen) for 1 h in 4ºC. The stabilized lungs were homogenized using the High Shear homogenizer (Omni International, Reasearch Biolabs). The lung homogenates from

individual lungs were filtered through a cell strainer. The filtered suspension was centrifuged at 1,500 rpm for 7 min to pellet the remaining cell debris. The supernatant containing free bacteria was centrifuged at 10,000 rpm for 10 min. The bacterial pellet was again stabilized in 1 ml of RNAprotect Bacteria Reagent for 5 min at room temperature. Bacterial RNA was extracted using lysozyme and RNeasy Mini Kit buffer as described in section 2.4.7.1. In-solution DNA digestion was performed with DNase-I, and finally total RNA was eluted in 20-30 µl of DPEC water.

2.4.7.4 Quantification of total RNA

The concentration of total RNA extracted was quantified by UV spectroscopy using the NanoDrop™ Spectrophotmeter (Thermo). Quantification was done in an absorbance range from 220 nm to 750 nm. 2 µl of the total RNA extracted was placed onto the measurement pedestal to estimate its concentration in ng/µl and the ratio of absorbance measured at 260 nm and 280 nm (A260/A280) was determined to assess RNA purity. RNA preparations with an absorbance ratio within the range of 1.8 to 2 are considered of satisfactory purity. The quality of the total RNA extracted was checked visually upon agarose gel electrophoresis as described in section 2.2.4.1.

2.4.8 Reverse-transcription Polymerase Chain Reaction (RT-PCR)

Total bacterial RNA extracted from *in vitro* culture (10 ng) from the infected cell lines (1 µg) and from infected mice lungs (1 µg) was subjected to RT-PCR to synthesize complementary DNA (cDNA) strands using the iScript™ cDNA synthesis kit (BioRad) (Table 2.5). For each RNA sample, a reaction with (+RT) or without (-RT) reverse transcriptase was set up. Amplification was conducted in the iCycler™ Thermal Cycler system (BioRad) with a pre-incubation at 25ºC for 5 min and cDNA amplification at 42ºC for 40 min. Finally, the RT was inactivated at 85ºC for 5 min and reaction was kept at 4ºC.

Component	Volume per reaction (µl)
5x iScript reaction mix	4
iScript reverse transcriptase	1
Nuclease-free water	10
RNA template (10 ng-1 µg)	10
Total volume	20

Table 2.5: Reaction components for RT-PCR amplification per sample tube for RNA input less than 1µg. The reaction was scaled up to a final volume of 40 µl when using more than 1 µg of RNA.

2.4.9 Real-Time Polymerase Chain Reaction

2.4.9.1 Reaction setup

iTaq™ SYBR Green Supermix with ROX cocktail (BioRad) was used in all real-time quantitative PCR (qPCR) reaction. A 50 µl singleplex PCR reaction mix was prepared according to the manufacturer's instructions as shown in Table 2.6. 2-4 µl of cDNA template was added in triplicates into

each of the assigned wells in the MicroAmp® Optical 96-well Reaction Plate (Applied Biosystem). A master mix consisting of the iTaq™ cocktail, forward-reverse primers and DEPC water was initially prepared before adding 48 µl of this master mix into each of the assigned optical wells containing either the cDNA sample, the no-template control as well as the –RT control. For no-template control, 2-4 µl of RNase-DNase free DEPC water was added into the optical well instead of the cDNA template, which is used to estimate the formation of primer-dimer and to ensure the PCR reaction proceeds in a contamination-free environment. The -RT control was used to estimates the amount of genomic DNA contamination present in the target samples. Passive reference ROX dye in the iTaq™ cocktail provides an internal fluorescence reference to which the reporter SYBR dye signal was normalized during data analysis.

Component	Volume per reaction (µl)	Final concentration
iTaq™ SYBR Green Supermix with ROX	25	1x
Forward primer	0.5	500nM
Reverse primer	0.5	500nM
DEPC water	20-22	
cDNA template	2-4	
Total volume	50	

Table 2.6: Reaction components for Real-time PCR amplification per sample tube.

Both forward and reverse primers were designed with the Applied Biosystem Primer Express® and OligoTech software. The list of primers is as shown in Table 2.7. Annealing temperature (T_a) was calculated according to

the following equation: $(4 \times n_{G/C}) + (2 \times n_{A/T}) - 5$ where $n_{G/C}$ and $n_{A/T}$ represent the number of G & C, and A & T bases respectively.

bvgA	TCCTCATCATTGACGATCACCC	CGATGACTTCCAGCCCGTCCA
bvgR	AACAGCTGCTGGCGCAGGTT	GCCGCAGGCTATGCAGGCTT
brkA	GTATCTCGATAGATTCCGTCAAT	CGTGTTGTCCCGTGGTCG
fhaB	TGTCCGCCATGGAGTATTTCAA	CCCAAATGTACTCGTAGCGATTC
kpsT	ATTCTCGATGACGTGTCGTTCGA	GGCGAACACCTCGAGACATTTG
ptx	GCGTTGCACTCGGGCAATTC	CAGATGGTCGAGCACATTGTC
recA	GACGACAAAACCAGCAAGGCC	CGTAGACCTCGATCACGCGG
risA	CTGCTGGTTCTCGACCTGATG	CGGGTTGAAGGGCTTGGACA
sphB1	TGCTGCAGGACAACCTGTATTC	TCAGGCCGGCCGAGACTTCG
vrg6	AAGTGGTTCGTTGCTGCCGG	TACACCACCTGCGGGCGC
BP3838	GCGAGTTCGACCTGGTAATG	AATCGCGCACGTGCGACGT
BP3818	CCATCGGGTTGCGCTACC	AACAGATAGCCCGCGACCG

Table 2.7: List of primers used for Real-time PCR.

Amplification and relative quantification for gene expression was conducted using the Applied Biosystem Standard 7500 sequence detector. Both forward and reverse primers for selected targets and endogenous control is as listed in Table 2.7. The amplification begins with an initial denaturation step at 95ºC for 3 min, followed by PCR cycling for 45 cycles consisting of DNA denaturation at 95ºC for 15 s and primer annealing and extension steps at suitable T_a of various primers for 45 s. A dissociation stage was added for

melting curve analysis at 95°C for 15 s, 60°C for 15 s, and a slow ramp to 95°C for 15 s.

2.4.9.2 Configuring data analysis setting in real-time PCR

The real-time data on gene expression quantitation or RQ was automatically collected by the Applied Biosystem Standard 7500 system throughout the PCR cycling process. At the end of the run, data analysis was done either on a single plate document or multi-plates document. SYBR green fluorescence intensity was depicted by the amplification curve generated from the system. Baseline value was set at initial stage of cycling where minimal change in SYBR green fluorescent signal was detected. Significant amplification was characterized by the point in time during cycling when amplification of a target is first detected above the set threshold value, which was set above the baseline and within the exponential part of the amplification curve. The threshold cycle (Ct) value was determined at which the amplification curve intersects with the set threshold value. The determination of Ct values across the samples is essential for subsequent relative quantitation assay. To quantify the relative expression of each gene, the average Ct values calculated from the triplicate samples was normalized against the endogenous reference gene, *recA* (equation 1 and 2). Normalization step is necessary to correct for fluorescence fluctuations caused by variations in template concentration or in volume from one well to another. The selected endogenous control *rec*A was expected to be constitutively expressed in all the experimental samples. The ΔCt value obtained was then compared with a

calibrator, using the ΔΔCt method (equation 3). From the ΔΔCt values, the relative gene expression of target sample was calculated (equation 4).

$(\Delta Ct_{target} = Ct_{target} - Ct_{recA})$ ----------(equation 1)

$(\Delta Ct_{calibrator} = Ct_{calibrator} - Ct_{recA})$-------(equation 2)

$(\Delta\Delta Ct = \Delta Ct_{target} - \Delta Ct_{calibrator})$---------(equation 3)

$(RQ = 2^{-\Delta\Delta Ct})$----------(equation 4)

The calibrator sample was used as the basis for comparative gene expression in which its RQ value was set as 1. Consequently, an increase in gene expression would have an RQ greater than 1 whereas a decrease in gene expression would be reflected by an RQ < 1. Relative fold change was then calculated from the RQ values obtained.

2.4.10 Microarray Analysis

8 ml of mid-exponential bacterial culture was harvested at OD_{600nm} 2 and mixed with 2 ml of phenol-ethanol mixture containing 5% of UltraPure™ Buffer-Saturated Phenol (Invitrogen) and 95% Ethanol. The bacteria-phenol-ethanol mixture was pelleted at 8,000 rpm for 10 min. The pellet was frozen and shipped to the Institute Pasteur Lille, France for whole genome transcriptomics analysis. In brief, long oligonucleotide probes were designed from the sequences of the 3554 open reading frames (all coding CDS except transposases of IS elements) of *B. pertussis* Tohama I genome using OligoArray v2.1 (Rouillard et al., 2003). Oligonucleotides were synthesized

by Sigma-Aldrich and spotted on Nexterion AL slides (Schott Nexterion) in 1x SciSpot-AM buffer (Scienion) using a Q-Array II spotter (Genetix). For each sample, 15 μg of total RNA was reverse transcribed with 400 units of SuperScript III (Invitrogen) in presence of 100μM Cy3-dCTP or Cy5-dCTP (GE) and 300 mM of random hexanucleotide (Roche). The labelled cDNA was then NaOH treated to degrade RNA and purified on Qiaquick PCR purification kit (Qiagen). Hybridization was performed in 40% formamide, 5x Denhardt's solution, 0.1% SDS, 1 mM sodium pyrophosphate and 5x SSC for 14-16 h at 52°C under agitation. Slides were then washed sequentially in 2x SSC with 0.2% SDS for 5 min, 0.5x SSC for 10 min, 0.05x SSC for 5 min and 0.01x SSC for 1 min before drying. Hybridized slides were scanned using an Innoscan 700 (Innopsys) microarray scanner and analyzed with Mapix v3.1 (Innopsys). Normalisation and differential expression were carried out using the LIMMA package (Linear Models for Microarray Data) (Smyth et al., 2003) running under the statistical language R v2.11.1. Identification of statistically significant regulation was performed using moderated t-statistic with empirical Bayes shrinkage of the standard errors (Lonnstedt and Speed, 2002). Because of multiple testing, obtained *P* values were corrected using Benjamini & Hochberg method to controls the false discovery rate (Benjamini and Hochberg, 1995).

2.5 PROTEIN EXPRESSION STUDIES

2.5.1 Preparation of *B. pertussis* Samples for Protein Expression Studies

2.5.1.1 Supernatant

10 ml of *B. pertussis* culture grown in SS medium were harvested in mid-exponential phase at OD_{600nm} and centrifuged at 8,000 rpm for 10 min at room temperature. The supernatant was collected and subjected to further spinning for 10 min at maximum speed to remove any remaining cell debris. Equal volume of commercial protein loading buffer; the Laemmli Blue sample buffer (Biorad) mixed with 5% β-mercaptoethanol (Sigma) was added to 500 µl of the clarified supernatant and the mixture was heated at 95°C for 15 min. When necessary the supernatant was concentrated up to 10 times using Ultra-4 Centrifugal Filter Device (Amicon) according to the manufacturer's instructions prior to adding the Laemmli Blue sample buffer and 5% β-mercaptoethanol.

2.5.1.2 Whole cell extract

1 ml of *B. pertussis* culture grown in SS medium was harvested in mid-exponential phase at OD_{600nm} and centrifuged at 8,000 rpm for 10 min at room temperature. The pellet was re-suspended in 500 µl of RNases-free water, and an equal volume of Laemmli Blue sample buffer and 5% β-mercaptoethanol was added before heating the mixture at 95°C for 15 min. Chomosomal DNA was sheared by passing the suspension through a 27G needle followed by heating at 95°C for 15 min.

2.5.2 Preparation of *B. pertussis* Samples for Protein Purification Studies

2.5.2.1 Growth of bacteria

A 10 ml pre-culture of *B. pertussis* culture grown in SS medium was used to inoculate 50 ml of SS medium to an initial OD_{600nm} of 0.1. The culture was incubated overnight until OD_{600nm} 2-2.5 was reached.

2.5.2.2 Clarified whole cell extract

50 ml of *B. pertussis* culture were centrifuged at 8,000 rpm for 10 min and washed twice with 25 ml of 1x PBS. The washed bacteria pellet was re-suspended in 5 ml of lysis buffer A (containing 20 mM Tris-HCl pH7.9, 10 mg/ml lysozyme, 50 µM KCL, 10% glycerol and 1xprotease inhibitor) and incubated at 37°C with rocking for 1 h. The bacterial cells were sonicated in a bioruptor closed system (Diagenode) for 15 min with 15 s ON/OFF interval. Cellular contents and debris were removed upon centrifugation at 8,000 rpm for 10 min. The bacteria pellet was re-suspended again in 5ml lysis buffer A, with an addition of 1% Triton-X-100. The bacterial cells were incubated at 37°C with rocking for 1 h, prior to centrifugation at 10,000 rpm for 10 min. Finally, the supernatant was discarded and the bacteria pellet was solubilized in 5 ml of solubilization buffer (containing 20mM Tris-HCl, 50 µM KCL, 10% glycerol and 6 M guanidine hydrochloride, pH 8) with rocking for 1 h at

4°C. The cellular lysate was clarified by centrifugation at maximum speed for 15 min to pellet unsolubilized cells and debris.

2.5.3 Protein Quantification Using Bicinchoninic Acid (BCA) Assay

200 µl of BCA working reagent diluted 1:50 (Thermo) were added to 10 µl of the sample cellular lysate and to a range of bovine serum albumin standards at 2 mg/ml, 1 mg/ml, 0.5mg/ml, 0.25 mg/ml and 0.125 mg/ml. The mixture was incubated at 37°C for 30 min and absorbance was measured at 562 nm with Infinite200 Pro (Tecan, Switzerland) 96-well plate reader.

2.5.4 Protein Purification Using Ni-NTA Column Chromatography

400 µl of Ni-NTA slurry (Qiagen) were centrifuged at 5,000 rpm for 5 min to remove slurry solution. The beads were pre-equilibrated with 400 µl of solubilization buffer containing 20 µM imidazole. Pre-equilibration of the Ni-NTA beads was performed on a rocker for 10 min at room temperature. Approximately 5 mg of clarified lysate were mixed with imidazole to a final concentration of 20 µM. If obvious viscosity was observed, 20 units of TURBO DNase-I (Invitrogen) was added to the cell lysate. The cell lysate was then mixed with the pre-equilibrated Ni-NTA beads at 4°C overnight with rocking. The mixed lysate and Ni-NTA beads were loaded onto an empty Poly-Prep^{TM} chromatography column (Biorad). The Ni-NTA beads were allowed to settle at the bottom of the tube before the bottom cap was opened for gravity flow purification. The Ni-NTA beads were washed with 5 column

volumes of wash buffer (containing 6 M urea, 100 mM NaH_2PO_4, 10mM Tris-HCl and 20 µM of imidazole. pH6.3). Proteins bound to the Ni-NTA beads were batch eluted four times in elution buffer (containing 6 M urea, 100 mM NaH_2PO_4, 10mM Tris-HCl and 200 µM of imidazole. pH 4.5). Eluted proteins were aliquoted in batch and stored at -80°C. Prior to SDS-PAGE, defrost purified proteins were mixed with Laemmli Blue sample buffer alone and unheated or with β-mercaptoethanol and heated at 95°C for 15 min.

2.5.5 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein separation was conducted under denaturing conditions via SDS-PAGE using a vertical slab gel unit (Hoefer, USA) according to the manufacturer's instructions. The slab gel consisted of a 5% polyacrylamide stacking gel and a 8%, 10% or 12% polyacrylamide resolving gel, both containing 10% SDS. Cell extract, supernatant samples or purified proteins were heated at 95ºC (or not for purified proteins) for 5-15 min immediately before loading onto the SDS-gels. 5 µl of BenchMark™ Pre-Stained protein ladder (Invitrogen) or Spectra Multicolor Broad Range protein ladder (Thermo) were loaded as well as the molecular weight standard. Electrophoresis was conducted in 1xTris-Glycine SDS running buffer at constant 80 V for the first 20 min and increased to 100 V for protein separation in resolving gel for 2 h.

2.5.6 Coomassie Blue Staining

To visualize the protein bands after electrophoresis, the PAGE gel was soaked in Coomassie Staining InstantBlue™ solution (Expedeon, Cambridge, UK) for 1-2 h with constant shaking until bands appeared. The PAGE gel was then destained in deionized water with constant shaking until the protein bands could be visualized as sharp blue bands against a clear background. The gel was placed on clear cellophane and scanned for record and analysis.

2.5.7 Western Blot

After SDS-PAGE, the separated proteins were electro-transferred to a methanol activated PVDF membrane (Bio-Rad) using either a semi-dry transfer system (Vann et al.) at 65 mA for 2-4 h or a wet transfer system (Biorad) at 30 V overnight. Prior to assembly for semi-dry transfer, the SDS-PAGE gel, PVDF membrane and filter papers were soaked in transfer buffer containing 1xTowbin buffer made up of Tris-Glycine and methanol (containing 25 mM Tris, 192 mM glycine and 20% (v/v) methanol) for 15 min. Similarly for wet transfer, the PVDF membrane and filter papers were soaked in 1xTowbin buffer containing 40% (v/v) methanol instead of 20% methanol. PVDF membranes from completed semi-dry or wet transfer were blocked in 5% skim milk (Bio-Rad) diluted in 1xTBS with 0.1% Tween20 at room temperature for 1 h. The membranes were then incubated with the appropriate primary antibody (Table 2.8) diluted in the same blocking buffer for 1 h at room temperature or at 4ºC overnight, followed by 3-5 washing

steps in an excess of washing buffer (containing 1xTBS, 0.1% Tween20) at 15 min intervals. Thereafter, the membrane was incubated for 1 h with gentle shaking at room temperature, with the appropriate AP-conjugated or HRP-conjugated secondary antibody (Table 2.8) diluted in blocking buffer, followed by washing as described above. Finally, the AP-conjugated antibody-reactive bands were revealed by chromogenic detection upon addition of NBT/BCIP alkaline phosphatase substrate (Chemicon, Temecula, CA). For chemiluminescent detection, the membrane with HRP-conjugated secondary antibody was incubated with Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare, Waukesha, WI) and reactive bands were developed on CL-XPosure X-ray Film (Thermo).

Primary antibody	Dilution	Secondary Antibody	Dilution
Rabbit anti-BrkA polyclonal antibody (New England Peptide, Gardner,MA)	1:30,000	Goat anti-rabbit IgG AP conjugate (H+L) (Bio-rad)	1:3000
Mouse anti-FHA monoclonal antibody (National Institute for Biologica Standards and Control, UK)	1:5000	Goat anti-mouse IgG AP conjugate (H+L) (Bio-rad)	1:3000
Mouse anti-PTX monoclonal antibody (National Institute for Biologica Standards and Control, UK)	1:1500		
Rat anti-BvgS polycolonal antibody (Kind gift from Dr. F. Jacob-Dubuisson, Institute Pastuer Lille)	1:3000	Goat anti-rat IgG (H+L) HRP conjugate (Abcam, Cambridge, UK)	1:5000
Mouse anti-penta Histidine-HRP conjugated monoclonal antibody (Qiagen)	1:10,000	NA	

Table 2.8: Antibodies used in Western blot.

Figure 2.2: Western blot setup for semi-dry transfer.

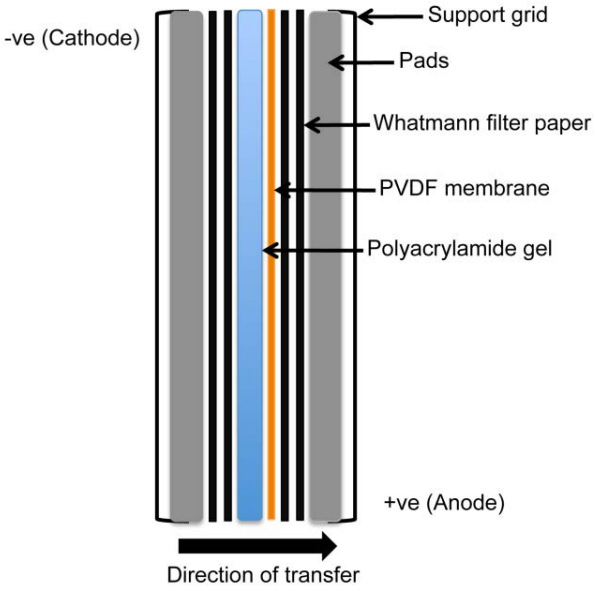


Figure 2.3: Western blot setup for wet transfer.

2.6 FLUORESCENCE ACTIVATED CELL SORTING (FACS)

2.6.1 Preparation of *B. pertussis* Samples for FACS

To detect surface polysaccharide capsule, 5 ml of *B. pertussis* strains grown in SS medium containing 50 mM $MgSO_4$ were harvested at mid-exponential phase and centrifuged at 8,000 rpm for 10 min. The bacteria pellet was washed twice with 2 ml of sterile HEPESG buffer (1xHEPES, 5% glycerol; 0.05% Tween80). Bacteria concentration was adjusted to 10^8 CFU/ml in 2 ml of HEPESG. 500 µl of the bacteria were incubated with mouse anti-Vi antiserum (see section 2.10) diluted 1:50 for 2 h at 4°C with constant agitation. The bacteria-antibody complexes were centrifuged at 8,000 rpm for 10 min and gently washed twice with 2 ml of HEPEG. The bacteria-anti-Vi antibody complexes were then gently re-suspended in 500 µl of HEPESG with 1:100 dilution of FITC-conjugated goat anti-mouse IgG (Chemicon) for 1 h at room temperature with constant agitation. The suspension was centrifuged at 8,000 rpm for 10 min, washed twice with 2 ml of HEPEG and finally re-suspended in 100 µl of 1xHEPES with 4% paraformaldehyde to fix the bacteria cells.

To evaluate bacteria membrane integrity with propidium iodide (PI) staining assay, *B. pertussis* strains grown on BG agar for 24 h were washed once with PBS and adjusted to initial OD_{600nm} of 0.7 with SS media. 500 µl of bacterial suspension was incubated with or without 0.02% SDS for 2 h at 37°C. 50 ng of PI (BD Pharmigen) was then added to the culture and

incubated for 15 min at room temperature. Bacteria cells were washed twice with 1xPBS prior to fixing with 4% paraformaldehyde for 30 min and analyzed with LSRFortessa cell analyzer (BD Pharmigen).

2.6.2 FACS Analysis

100 µl of each sample were diluted with 700 µl of BD FACSFlow^{TM} sheath fluid (Becton-Dickinson, Heidelberg, Germany) prior to FACS profiling and acquisition. A Coulter Epics machine (Beckman Coulter, Palo Alto, CA) was used for the flow cytometric study. Samples were analyzed with laser excitation at 488 nm, and data acquisition was performed using EXPO Version 2.0 software (Applied Cytometry Systems, Sheffield, U.K.) and analyzed with WinMDI-2.8 software.

2.7 CELL BIOLOGY

2.7.1 Cell Line and Culture Conditions

J774.A1 murine macrophages (ATCC, TIB 67) were cultured at 37ºC and 5% CO_2 atmosphere, and were maintained in Dulbecco's modified essential medium (DMEM) medium supplemented with 10% heat-inactivated fetal calf serum (FCS) (Gibco, Invitrogen), 2% of 200 mM GlutaMAX-I (Gibco, Invitrogen) and 1% of 100mM sodium pyruvate (Gibco, Invitrogen). A549 human lung epithelial cells (ATCC, CCL-185) cells were cultivated in RPMI-1640 medium containing L-glutamine and 25 mM HEPES buffers

(Gibco, Invitrogen), supplemented with 10% heat-inactivated FCS (Gibco, Invitrogen), 2% of 200mM Glutamax-I (Gibco, Invitrogen) and 1x penicillin-streptomycin (100U/ml of penicillin and 100 μg/ml of streptomycin) antibiotics (Gibco, Invitrogen). Both J774.A1 and A549 cells were cultivated in 25 cm^2 and 75 cm^2 tissue culture flasks. Upon 80% confluency, usually within 2-3 days, the spent medium was discarded and the cell monolayers were rinsed thrice with 10 ml sterile 1xPBS to remove dead cells. Cells were detached from the tissue culture flask surface by mechanical scraping. J774.A1and A549 cells were passage in a ratio of 1:3 in 75 cm^2 tissue culture flasks.

2.7.2 Trypan Blue Assay

Single cell suspensions were mixed with equal volume Trypan blue dye (Sigma) and transferred to a cover slip chamber on a hemocytometer. Under the inverted light microscope, the viable and non-viable cells can be distinguished, whereby viable cells exclude the trypan blue dye whereas dead cells take up the blue dye. The number of viable (unstained) cells located in the 25 squares of the hemocytometer was counted and the cell concentration per ml was determined using the following calculation:

Cells per ml = the average count in 25 squares x dilution factor x 10^4

2.7.3 Cell Culture Infection Assay

J774.A1 and A549 cells were grown as described in section 2.7.1. Two days before the infection assay, *B. pertussis* strains were plated on BG agar as mentioned in section 2.3.2. The bacterial lawns were harvested and washed once in 5 ml sterile 1x PBS, followed by centrifugation at 8,000 rpm for 10 min at room temperature to remove traces of blood agar. Bacteria pellet was re-suspended in 1 ml of incomplete cell culture medium (without FCS) for OD measurement at 600 nm. The final bacterial concentration was adjusted with appropriate incomplete cell culture medium to allow a multiplicity of infection (M.O.I) of 100 for Real-time PCR analysis of bacterial gene transcripts (Section 2.4.9). In addition, 200 ul of the final bacterial suspension were serially diluted and plated on BG agar for quantification of the inoculums.

Prior to infection assay, J774.A1 and A549 cell monolayers were washed with 10 ml of 1xPBS and 10 ml of bacterial suspension in incomplete medium were added into the 75 cm^2 tissue culture flasks. Similarly, 10 ml of incomplete DMEM medium were added into the non-infected flask. The tissue culture flasks were incubated in 37ºC at 5 % CO_2 atmosphere for 1.5 h. After 1.5 h, bacterial suspension was removed from all flasks and the monolayers were rinsed thrice with 1xPBS. The monolayers were either lysed (time point 1.5 h p.i.) or further incubated with medium containing 100 µg/ml of gentamicin for 2 h at 37ºC at 5 % CO_2 atmosphere to kill extracellular bacteria. After 2 h, the flasks were washed thrice with sterile 1x PBS. Except for those cells, which are to be lysed immediately (time point 3.5 h p.i.), cell culture medium containing 20 µg/ml of gentamicin was added into the flasks. For each time point, prior to cell lysis, the flasks were washed thrice with 1x

PBS and the monolayers were immediately processed for bacterial RNA extraction as described in section 2.4.7.2.

(C) ANIMAL WORK

2.8 Ethics Statement

All the animal experiments were approved by NUS IACUC under the protocol number 089/09. The animal experiments were carried out under the guidelines of the National Advisory Committee for Laboratory Animal Research (NACLAR) in the AAALAC-accredited NUS animal facilities. All efforts were done to minimize suffering of the animals and all non-terminal procedures were performed under anesthesia.

2.9 Mouse Strain

6-8 weeks-old pathogen-specific free (SPF) female BALB/c mice were purchased from Centre for Animal Resources (CARE), NUS Singapore. The mice were housed in individual ventilated cages (IVCs) in a ABSL2 facility.

2.10 Generating Polyclonal Anti-Vi Antisera

BALB/c mice were immunized intraperitoneally (ip.) with 5 µg (100 µl) of Typhoid Vi polysaccharide vaccine of *S. typhi* Ty2 strain (TYPHIM Vi^{TM},

Aventis Pasteur SA, France) mixed with equal volume of incomplete Freund's adjuvant (Calbiochem, San Diego, USA). Immunization was performed once and blood was collected from the sedated immunized mice by retro-orbital plexus puncture after 10 days and 17 days post-immunization. The blood was allowed to clot at 37°C for 30 min and 4°C for 1 h, followed by centrifugation at 3,500 rpm for 10 min at 4°C. The serum was collected and stored at -20°C until used.

2.11 Intranasal Infection

B. pertussis strains grown on BG agar at 37ºC for 3 days were re-suspended in sterile PBST (containing 1x PBS, 0.05% Tween80) and adjusted to a final concentration of approximately $2.5x10^7$ CFU/ml (for colonization assay) or $2.5x10^9$ CFU/ml (for bacterial RNA extraction). Prior to infection, mice were sedated upon ip. administration of 150 µl per mouse of an anaesthetic cocktail (6% valium, 10% atropine, 20% ketamine, 64% 1x sterile PBS) . Infection was performed through the intranasal route with 20 µl of the bacterial suspension slowly deposited onto the nostrils of the animals.

2.12 Murine Lung Colonization Study

Four mice per time point were sacrificed at the indicated time points after intranasal infection with various *B. pertussis* strains. The lungs from each individual mouse were aseptically removed and homogenized in 2 ml or 5 ml

sterile PBST using High Shear homogenizer (Omni International, Reaserch Biolabs). Serially diluted lung homegenates from individual mice were then plated onto BG agar supplemented with 100 µg/ml Sm and the total CFU per lung was counted after 4-5 days incubation at 37ºC.

2.13 Statistical Analysis

Statistical analysis of all results was performed using the unpaired Student *t*-test. Differences were considered significant at a *P* value of < 0.05.

CHAPTER 3 ROLE OF THE CAPSULE OPERON IN PERTUSSIS PATHOGENESIS

(A) CHARATERIZATION OF *B. PERTUSSIS* MUTANTS CARRYING A SINGLE GENE DELETION WITHIN THE CAPSULE OPERON

3.1 RESULTS

3.1.1 Construction of *B. pertussis kpsT, kpsE* and *vipC*-deleted Mutants

A non-polar single gene deletion was constructed for *kpsT*, *kpsE* and *vipC* ORFs via double homologous recombination at the chromosomal locus of wild-type BPSM. Approximately, 600-800 bp of genes, termed as PCR1, flanking from the 5' internal region and PCR2, flanking from the 3' internal region of the respective ORFs to be deleted, were PCR amplified from BPSM chromosomal DNA using the primers listed in Table 2.2 (Figure 3.1). The PCR1 and PCR2 fragments were cloned into TOPO vector for sequencing and then into pBR322 intermediate vector. PCR1+2 were eventually cloned into the *Bordetella* suicide vector pJQ200mp18rpsL, yielding pJQT1-2, pJQE1-2 and pJQV1-2 respectively. Electrocompetent *B. pertussis* BPSM strain was electroporated with pJQT1-2, pJQE1-2 and pJQV1-2 plasmids as described in section 2.4.2. *B. pertussis* clones deleted for *kpsT*, *kpsE* and *vipC* genes, respectively, were selected as described in section 2.4.3 and colony PCR screening was performed using primers as listed in Table 2.4.

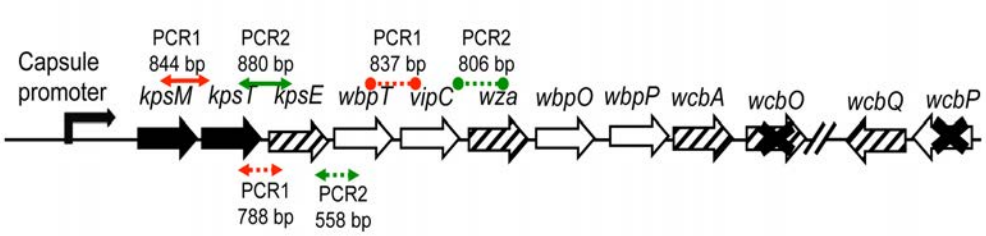


Figure 3.1: Schematic organization of the ORFs for *B. pertussis* capsule operon.

The capsule operon of *B. pertussis* regulated under the capsule promoter is as shown. Black cross represents mutational insertion found in the locus. Black, hashed and white arrows represent genes involved in polysaccharide capsule transport, polysaccharide modification/translocation and polysaccharide biosynthesis respectively. The homologous PCR1 and PCR2 fragments indicated by the filled red and green arrowheads (Δ*kpsT*), dotted red and green arrowheads (Δ*kpsE*) and filled red and green rounded arrowheads (Δ*vipC*) were used for the construction of the respective mutants.

3.1.2 Obtaining The Δ*kpsT*, Δ*kpsE* and Δ*vipC* Mutants

3.1.2.1 Southern blot analysis

The BPSM-derivative knockout (KO) mutant strains designated as Δ*kpsT*, Δ*kpsE* and Δ*vipC* were further analyzed by Southern blot analysis. Chromosomal DNA from wild-type BPSM, Δ*kpsT*, Δ*kpsE* and Δ*vipC* strain was extracted and restriction digested as described in section 2.4.5 and 2.2.3. Southern blot strategy for Δ*kpsT*, Δ*kpsE* and Δ*vipC* mutant and the hybridization of DIG-labeled probes are as shown in Figure 3.2 A. The band sizes obtained for Δ*kpsT* (~2.7 kb), Δ*kpsE* (~3.9 kb) and Δ*vipC* (~3.4 kb) versus the BPSM counterpart confirms that *kpsT*, *kpsE* and *vipC* is deleted at the correct locus (Figure 3.2 B).

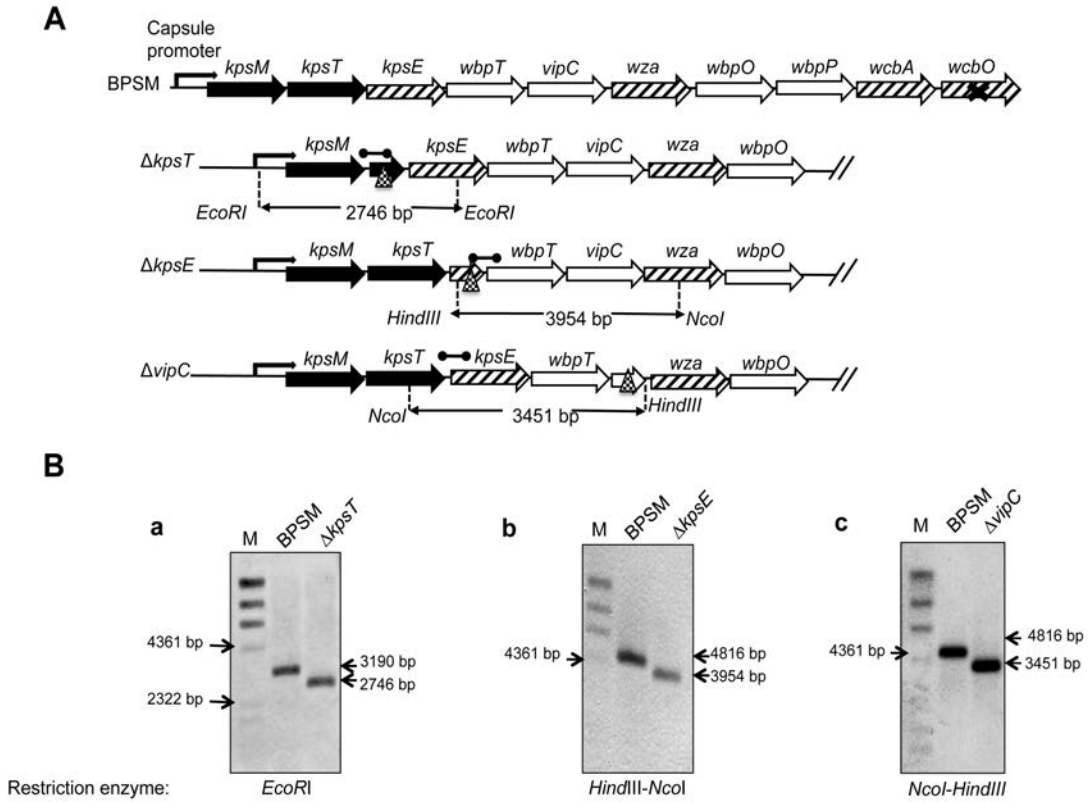


Figure 3.2: Southern blot analysis of Δ*kpsT*, Δ*kpsE* and Δ*vipC* chromosomal DNA.

(A) Strategy for Southern blot analysis for Δ*kpsT*, Δ*kpsE* and Δ*vipC* mutant.
Dotted triangle in panel indicate site of deletion that render each mutant non-capsulated. The DIG-labeled probe binding region (black rounded arrow), restriction sites and size of restriction-digested chromosomal DNA for Southern blot analysis are as shown.
(B) Southern blot analysis of *B. pertussis* chromosomal DNA.
Chromosomal DNA of Δ*kpsT* was digested with *Eco*RI, whereas chromosomal DNA of Δ*kpsE* and Δ*vipC* was digested with *Hind*III and *Nco*I. Chromosomal DNA of BPSM was used as control and digested with the same RE used for each of the respective mutants. Restriction-digested chromosomal DNA from BPSM, Δ*kpsT*, Δ*kpsE* and Δ*vipC* were electrophoresed, transferred onto a nitrocellulose membrane and hybridized with the DIG-labeled probe (Figure 3.2 A showed probe binding site). Panel **a**, *Eco*RI-restricted BPSM and Δ*kpsT* DNA yielded 2.7-kb and 3.2-kb respectively. Panel **b**, *Hind*III-*Nco*I restricted BPSM and Δ*kpsE* DNA yielded 4.8-kb and 3.9-kb respectively. Panel **c**, *HindIII-NcoI* restricted BPSM and Δ*vipC* DNA yielded 4.8-kb and 3.4-kb respectively. M, DIG-labeled DNA ladder.

3.1.3 Construction of *B. pertussis* Δ*kpsT*-Complement Strain

An 866 bp DNA fragment corresponding to the native capsule promoter in BPSM was synthesized and cloned into *Xba*I and *BamH*I digested pUC57 plasmid (GenScript, Piscataway, NJ), yielding pUC57-Pcaps. A 764 bp DNA fragment corresponding to the *kpsT* ORF was PCR amplified from purified BPSM chromosomal DNA using primers listed in Table 2.2. The *kpsT* ORF was cloned into pBBR1MCS to form pBBR-*kpsT*. The 866 bp capsule promoter fragment from pUC57-Pcaps was cloned upstream *kpsT* ORF in pBBR-*kpsT*, yielding pBBR::Pcaps*kpsT*.

3.1.4 Obtaining the Δ*kpsT*-Complemented Strain

Electrocompetent *B. pertussis* Δ*kpsT* bacteria were electroporated with pBBR::Pcaps*kpsT* replicative plasmid. Complemented Δ*kpsT* clones were selected on Cm–containing BG plates. Using primers as listed in Table 2.4, *B. pertussis* Δ*kpsT*com (complemented) strains were further confirmed by PCR screening based on the presence of full-length *kpsT* ORF (Appendix 1).

3.1.5 Transcriptional Analysis of Downstream Genes in Δ*kpsT*, Δ*kpsE* and Δ*vipC* Mutants

To confirm that the in-frame deletion of these individual ORFs does not terminate the transcription efficacy of the downstream ORFs within the capsule operon, reverse transcription-PCR was performed on total RNA purified from Δ*kpsT*, Δ*kpsE* and Δ*vipC* strains grown in Bvg$^-$ phase using primers mapping in downstream ORFs of the respective deleted regions. The KO*caps* mutant strain (Neo et al., 2010) for which the entire capsule operon has been deleted was used as negative control. Specific PCR products were obtained for both parental BPSM and Δ*kpsT*, Δ*kpsE* and Δ*vipC* strains (Figure 3.3). Taking into account the variation in loading levels for *risA* in Figure 3.3B and Figure 3.3C, these variations probably lead to the observed changes in the intensity level observed for the PCR products amplified from *wbpT* and *wbpO* region (Figure 3.3 B and C). This study suggests that deletion of the respective *kpsT*, *kpsE* and *vipC* ORF does not affect transcription efficacy of the downstream ORFs within the capsule operon.

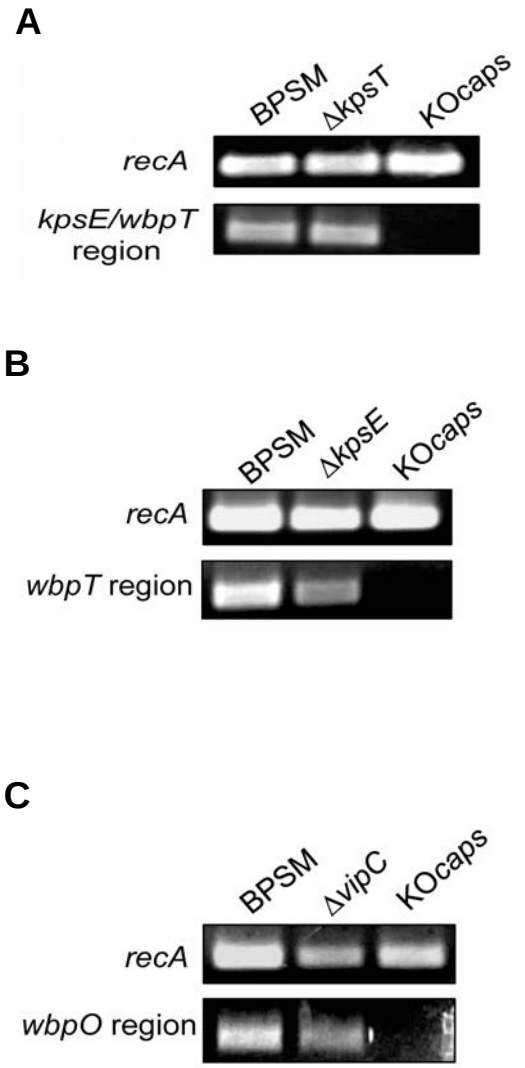


Figure 3.3: Reverse transcription-PCR on downstream gene.

Total RNA extracted from exponential SS liquid cultures of BPSM, KO*caps* and **(A)** Δ*kpsT*, **(B)** Δ*kpsE* and **(C)** Δ*vipC* was reverse-transcribed followed by PCR amplification using primers specific to the endogenous control gene *recA* and primers mapping to the respective downstream region of the deleted ORFs. The KO*caps* strain which was deleted for the entire capsule locus was used as a negative control.

3.1.6 *In vitro* Fitness of Δ*kpsT*, Δ*kpsE* and Δ*vipC* Mutants

3.1.6.1 Growth kinetics

To determine the *in vitro* fitness of *B. pertussis* Δ*kpsT*, Δ*kpsE* and Δ*vipC* mutants, their growth kinetic was determined and compared to the wild-type BPSM in virulent (Bvg^+) culture conditions. All the mutant strains displayed similar growth profiles to BPSM, except Δ*vipC*, which showed a delay in growth at the mid-logarithmic growth phase. Nevertheless, all of mutant strains were able to multiple up to a maximum OD_{600nm} of more than 5 at the late growth phase, indicating that the in-frame single gene deletion of the *kpsT*, *kpsE* and *vipC* ORF does not affect the *in vitro* growth abilities (Figure 3.4).

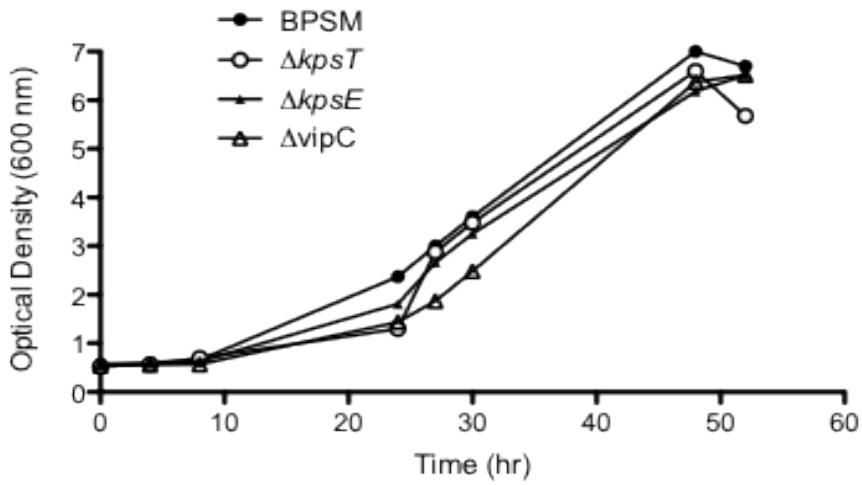


Figure 3.4: Growth kinetics for BPSM, Δ*kpsT*, Δ*kpsE* and Δ*vipC* mutant.

SS liquid medium was inoculated with BPSM (closed circles), Δ*kpsT* (open circles), Δ*kpsE* (closed triangles) and Δ*vipC* (open triangles) at initial OD_{600nm} of 0.5 at time-point 0 h. OD_{600nm} was monitored throughout 52 h of incubation at 37ºC. The growth kinetics assay was performed twice independently for each strain and each culture conditions. The data shown is a representative of two independent experiments.

3.1.7 Expression of Surface Polysaccharide Capsule

3.1.7.1 FACS analysis

Based on homology mapping, KpsT and KpsE are membrane proteins predicted to be involved in the transport-export of the polysaccharide capsule accross the bacterial cell wall, whereas VipC is cytoplasmic and involved in capsule biosynthesis. Therefore, the absence of KpsT, KpsE and VipC proteins in Δ*kpsT*, Δ*kpsE* and Δ*vipC* strains, respectively, is expected to result in the absence of the capsule at the bacterial surface, due to the lack of effective capsule polymer transport and biosynthesis, respectively (Pavelka et al., 1994). To confirm this hypothesis, FACS analysis was performed on non-permeabilized bacterial cells using the cross-reactive anti-Vi antigen immune sera as previously described by our laboratory (Neo et al., 2010) and as described in Chapter 2.10. The parental BPSM and capsule-deleted mutant KO*caps* strains were used as positive and negative controls, respectively (Figure 3.5 A and B). All the strains were grown in Bvg$^-$ phase culture conditions to allow optimal expression of the capsule operon, which is known as a *vrg* (Hot et al., 2003). Whereas approx. 20% of the parental BPSM cells exhibited a substantial shift in fluorescent signal compared to isotype control; KO*caps*, Δ*kpsT*, Δ*kpsE* and Δ*vipC* cells displayed background levels of fluorescent shift, indicating the absence of polysaccharide capsule at the surface of these mutant bacteria (Figure 3.5 C, D and E). Δ*kpsT*com displayed approx. 18% shift in the detection of surface capsule, similar to that of parental level (Figure 3.5 F).

Altogether, these results indicate that deletion of the single ORF *kpsT*, *kpsE* or *vipC* within the capsule locus is sufficient to prevent the production of the polysaccharide capsule at the bacterial surface.

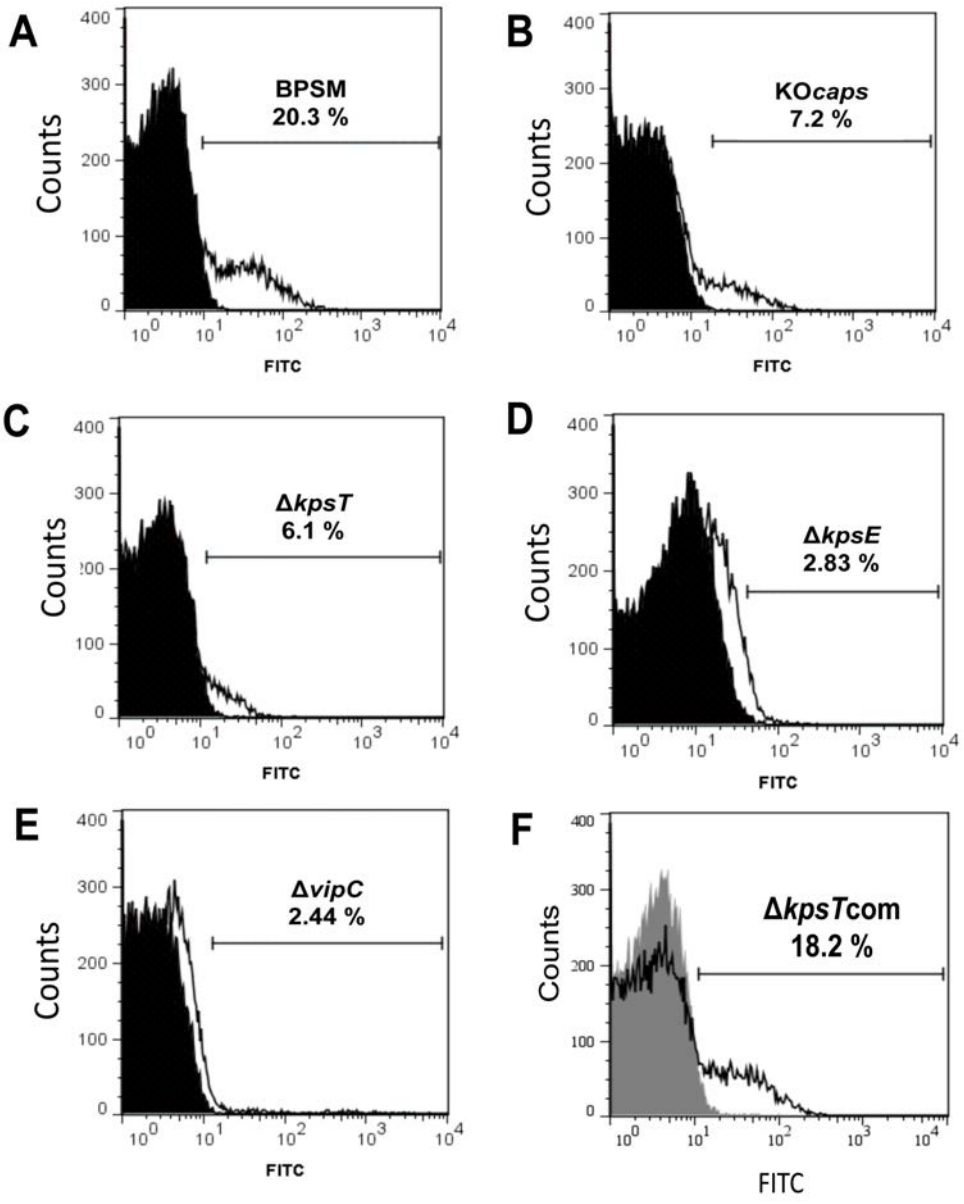


Figure 3.5: Detection of the polysaccharide capsule at the surface of *B. pertussis* strains.

Mouse polyclonal anti-*Salmonella typhi* Vi antigen immune serum was co-incubated with non-permeabilized **(A)** BPSM, **(B)** KO*caps*, **(C)** Δ*kpsT*, **(D)** Δ*kpsE*, **(E)** Δ*vipC* and **(F)** Δ*kpsT*com bacteria strains grown in avirulent (Bvg$^-$) phase, followed by anti-mouse FITC-conjugated secondary antibody. Isotype-matched controls are incubated with an anti-mouse antibody as shown in black/grey. The fluorescent cells were detected by flow cytometry, with 20,000 events counted for each sample. A representative experiment is shown, with percentage of fluorescent cells indicated in each panel.

3.1.8 Lung Colonization Profile of Δ*kpsT*, Δ*kpsE* and Δ*vipC* Mutants

To investigate whether the *B. pertussis* polysaccharide capsule plays a role during infection, Balb/c mice were nasally infected with wild type BPSM, KO*caps*, Δ*kpsT*, Δ*kpsE* and Δ*vipC* strains, and the bacterial loads in the lungs were monitored over time. The parental BPSM showed typical lung colonization profile with a multiplication peak at 7 days post-infection (p.i.) followed by a progressive reduction in bacterial load over the next 3 weeks p.i. (Figure 3.6 A). In contrast, mice infected with KO*caps* bacteria displayed no peak of multiplication, and instead a rapid decrease in the bacterial load was observed as early as 3 days p.i. with complete clearance within 17 days (Figure 3.6 A), indicating that deletion of the 10-kb capsule operon in *B. pertussis* greatly impaired its colonization efficiency, thus supporting that the polysaccharide capsule plays a role during pertussis pathogenesis.

Since the Δ*kpsT*, Δ*kpsE* and Δ*vipC* mutants do not produce any surface polysaccharide capsule; a similar attenuated phenotype was expected to be observed in the mouse model. Consistently, the Δ*kpsT* mutant displayed a colonization profile comparable to that observed with KO*caps* (Figure 3.6 B). The complemented strain Δ*kpsT*com displayed a colonization profile similar to that of BPSM (Figure 3.6 B), demonstrating that *kpsT* deletion is responsible for the attenuated phenotype observed with the Δ*kpsT* mutant. Interestingly, since *kpsT* complementation was achieved using the pBBR1MCS replicative plasmid (Elzer et al., 1995; Kovach et al., 1994), substantial loss of the plasmid occurred during *in vivo* bacterial replication as evidenced by the

increasingly reduced percentage of colonies recovered over time from the infected mouse lungs that have retained the plasmid construct (Appendix 2). Nevertheless parental colonization efficacy was observed with the Δ*kpsT*com strain at all the time points analysed, suggesting a bystander or paracrine effect whereby bacteria that have retained the plasmid are able to support the colonization of those that have lost the plasmid, likely through the secretion of critical virulence factors such as PT and FHA as reported before (Alonso et al., 2001). The lung colonization profile observed in mice nasally infected with Δ*kpsE* bacteria was also significantly attenuated throughout the course of infection compared to the parental BPSM strain (Figure 3.6 C), albeit to a much lesser extent than the KO*caps* and Δ*kpsT* strains. In contrast, the colonization efficiency of the Δ*vipC* mutant was comparable to the parental BPSM strain, with the exception of a lower bacterial load at day 17 p.i. (Figure 3.6 D).

Taken together, it appears that although the capsule was absent from the cell surface of all the mutant strains, their colonization profile substantially differed ranging from drastic (KO*caps* and Δ*kpsT*) to moderate (Δ*kpsE*) or no (Δ*vipC*) attenuation. This observation thus suggests that the presence of the polysaccharide capsule at the bacterial surface does not play a critical role in pertussis pathogenesis. Instead, we propose that the membrane-associated polysaccharide transport proteins KpsT and to a lesser extent KpsE, are specifically involved in *B. pertussis* ability to colonize the mouse respiratory tract.

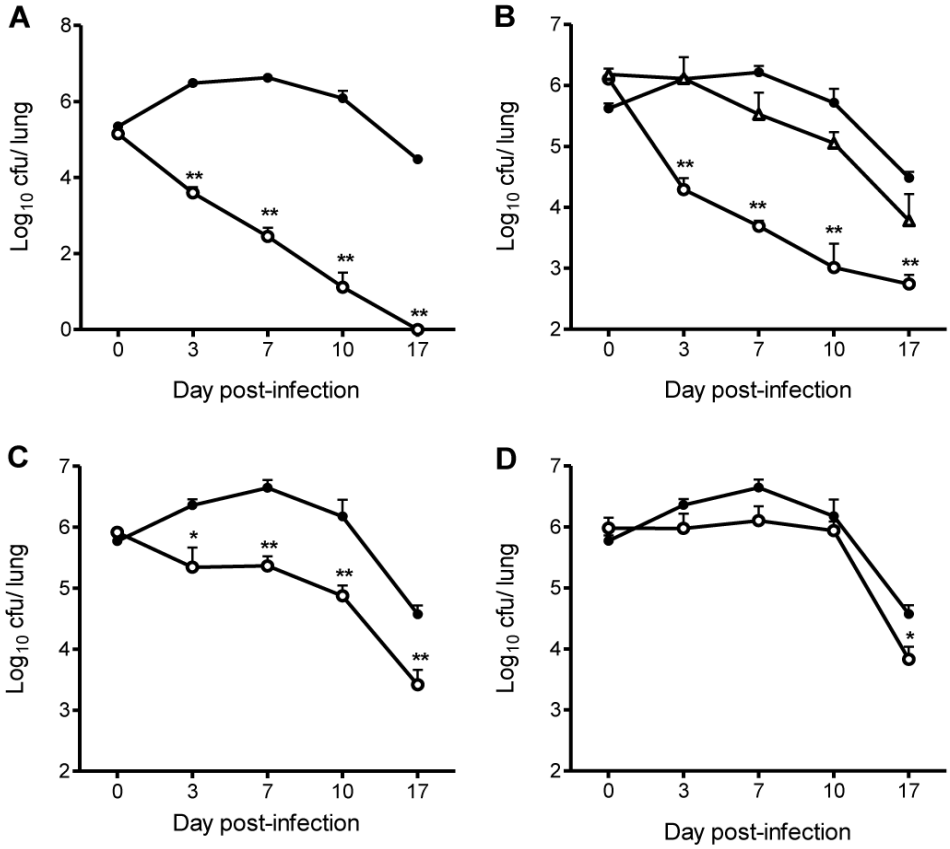


Figure 3.6: Lung colonization profile by *B. pertussis* BPSM, Δ*kpsT*, Δ*kpsE* and Δ*vipC* strains.

Balb/C mice were infected intranasally with $5x10^5$ CFU of *B. pertussis* BPSM (solid circles) and **(A)** KO*caps* (open circles), **(B)** Δ*kpsT* (open circles) and Δ*kpsT*com (open triangles), **(C)** Δ*kpsE* (open circles) and **(D)** Δ*vipC* (open circles). At the indicated time points, four infected mice per group were euthanized and their lungs were harvested, homogenized and plated on blood agar to determine the total number of CFU per lung. The results are expressed as the mean ± SEM of four mice per group. ** *p* value < 0.01 and * *p* value < 0.05 relative to BPSM. Results are representative of two independent experiments.

3.1.9 Expression of Virulence Factors in Δ*kpsT*, Δ*kpsE* and Δ*vipC* Mutants

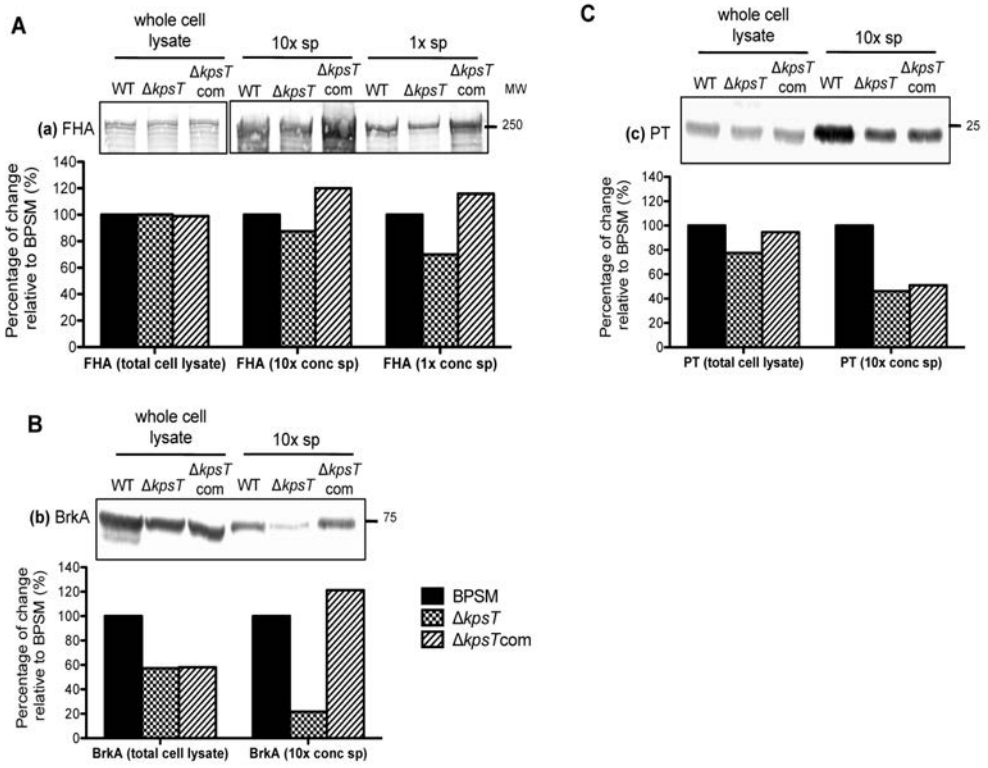
3.1.9.1 Western blot analysis

To further investigate the mechanism(s) involved in the capsule-mediated virulence in *B. pertussis*, we examined the production of several major virulence factors responsible for bacteria colonization including the adhesin filamentous hemagglutinin; FHA, the serum resistance protein; BrkA, and pertussis toxin; PT in Δ*kpsT,* Δ*kpsE* and Δ*vipC* mutants compared to wild type BPSM strain all grown in virulent (Bvg^+) phase in order to mimic the growth conditions *in vivo*. All bacteria cultures were harvested at OD_{600nm} 3. Compared to BPSM, production in the Δ*kpsT* mutant for FHA, BrkA and PT in the whole cell lysate or in the concentrated and non-concentrated culture supernatant were markedly reduced (Figure 3.7 I A, B and C). However, taking into account the slight variation in Δ*kpsT*com whole cell lysate loading control analyzed by Coomassie-stained SDS-PAGE (Figure 3.8), production/secretion level of FHA, PT and BrkA was concluded to be partially restored to parental level in Δ*kpsT*com strain (Figure 3.7 I A, B and C), implying that a negative feedback event occurs in the Δ*kpsT*com.

A 40% reduction in the production BrkA and secretion of PT in the Δ*kpsE* mutant (Figure 3.7 II B and C) was observed compare to wild type BPSM. Δ*vipC* mutant also displayed similar fold reduction in BrkA expression, while expression of FHA and PT remains comparable to wild type

BPSM (Figure 3.7 II B). From the protein expression analysis between the wild-type and capsule-deleted mutant strains, we propose that the absence of the membrane-associated KpsT protein and to a lesser extent KpsE, results in reduction of the production and/or secretion of key virulence factors in *B. pertussis.* Nevertheless, it is unlikely that such mild reduction observed in these three virulence factors in Δ*kpsT* and Δ*kpsE* could compromise the overall bacteria virulence and lung colonization ability observed *in vivo*.

I


A
whole cell lysate
10x sp
1x sp
WT ΔkpsT ΔkpsT com WT ΔkpsT ΔkpsT com WT ΔkpsT ΔkpsT com MW
(a) FHA
250
Percentage of change relative to BPSM (%)
FHA (total cell lysate)
FHA (10x conc sp)
FHA (1x conc sp)
C
whole cell lysate
10x sp
WT ΔkpsT ΔkpsT com WT ΔkpsT ΔkpsT com
(c) PT
25
PT (total cell lysate)
PT (10x conc sp)
B
whole cell lysate
10x sp
WT ΔkpsT ΔkpsT com WT ΔkpsT ΔkpsT com
(b) BrkA
75
BrkA (total cell lysate)
BrkA (10x conc sp)
BPSM
ΔkpsT
ΔkpsTcom

II

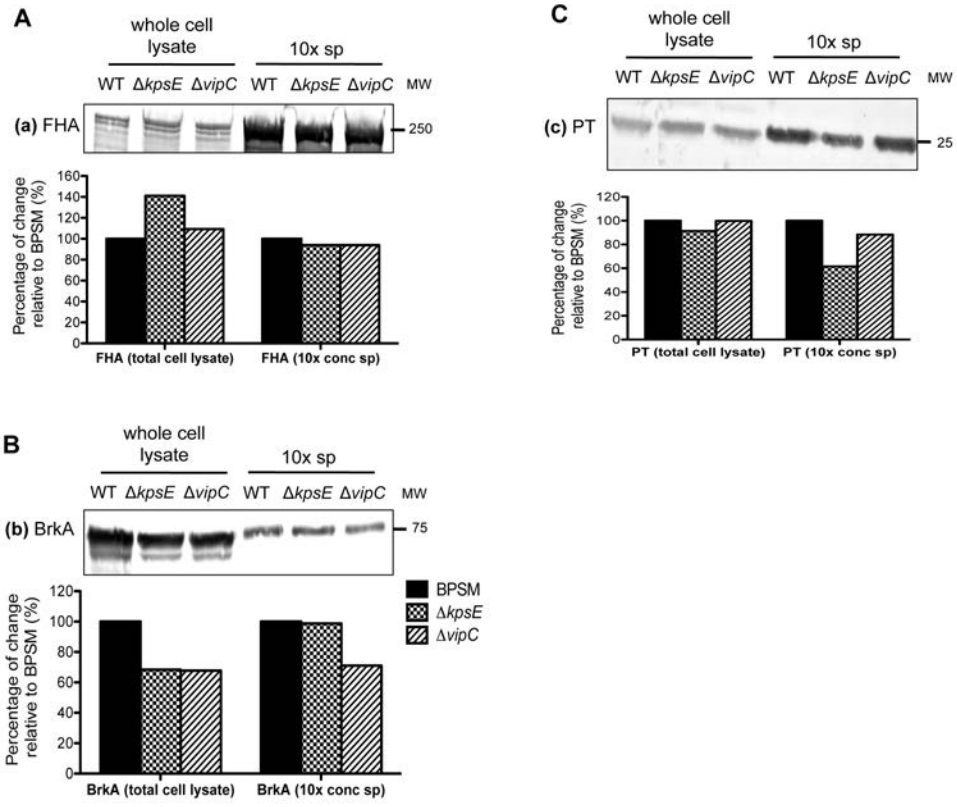

A
whole cell lysate
10x sp
WT ΔkpsE ΔvipC WT ΔkpsE ΔvipC MW
(a) FHA
250
Percentage of change relative to BPSM (%)
FHA (total cell lysate)
FHA (10x conc sp)
C
whole cell lysate
10x sp
WT ΔkpsE ΔvipC WT ΔkpsE ΔvipC MW
(c) PT
25
PT (total cell lysate)
PT (10x conc sp)
B
whole cell lysate
10x sp
WT ΔkpsE ΔvipC WT ΔkpsE ΔvipC MW
(b) BrkA
75
BrkA (total cell lysate)
BrkA (10x conc sp)
BPSM
ΔkpsE
ΔvipC

Figure 3.7: Production of *bvg*-regulated virulence proteins in capsule-deficient mutants.
(I) BPSM, Δ*kpsT* and Δ*kpsT*com strains and **(II)** BPSM, Δ*kpsE* and Δ*vipC* were exponentially grown in virulent (Bvg^+) phase. Western blot analysis was performed on 10x concentrated or non-concentrated culture supernatants, and whole cell lysates using **(A)** anti-FHA, **(B)** anti-BrkA or **(C)** anti-PT primary antibodies. The results are representative of three independent experiments. Molecular weights are indicated on the right side. Densitometry plot corresponding to the each of the blots is plotted as percentage of fold change relative to wild type BPSM.

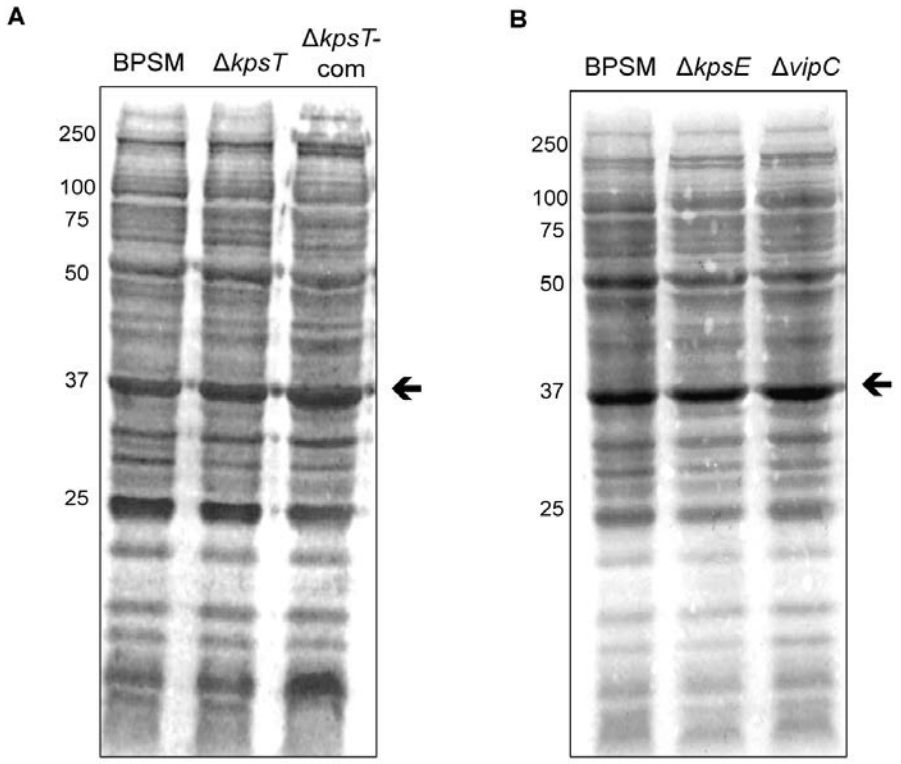


Figure 3.8: Coomassie blue-stained 12% SDS-PAGE of whole cell lysates.

(A) BPSM, Δ*kpsT* and Δ*kpsT*com strains and **(B)** BPSM, Δ*kpsE* and Δ*vipC* strains were exponentially grown in virulent (Bvg^+) phase. Equal amount of protein from whole cell lysate was loaded in each well. SDS-PAGE and Coomassie blue staining was performed to estimate equal loading of protein content, as indicated by the band intensity as shown by the black arrowhead. Molecular weights are indicated on the left side.

3.1.10 Transcriptional Analysis of Virulence Genes Expression

3.1.10.1 Real-time PCR analysis

To gain further insights in the mechanisms responsible for the lower production and/or secretion of key virulence factors observed with the Δ*kpsT* mutant, the relative expression of the corresponding genes in Δ*kpsT* namely *brkA*, *ptx*, and *fhaB* was measured by real-time PCR. Relative quantification of these transcripts in Δ*kpsT* was compared to that obtained with wild type BPSM grown in virulent (Bvg^+) phase at early logarithmic phase. Consistent with the Western blot analysis (Figure 3.7), the transcriptional activity of *brkA*, *ptx* and *sphB1* was significantly down-regulated about 10-fold, 4-fold and 2.5-fold, respectively in Δ*kpsT* compared to BPSM (Figure 3.9). The clear transcriptional down-regulation of *brkA* and *ptx* does not seem to correlate with the modest down-modulation of the corresponding protein levels (Figure 3.7 I), as the translation efficiency in Δ*kpsT* appears almost as efficient as in wild-type BPSM. However, it is to be noted that the sensitivity of the methods employed to monitor the transcriptional and translational activities respectively is significantly different. It is thus not surprising that a 2-3 fold difference observed by real-time PCR is not reflected by a 2-3 fold difference by Western blot analysis. The expression level of *brkA*, *ptx* and *sphB1* genes in the complemented strain Δ*kpsT*com was partially restored up 2-fold for *brkA*, 2-fold for *ptx* and 1.25-fold for *sphB1* compared to BPSM (Figure 3.9). Consistently, the corresponding protein expression level observed in Δ*kpsT*com was close to the parental level (Figure 3.7 I).

Instead, expression of the *fhaB* gene in Δ*kpsT* was not significantly different from that measured in the parental BPSM and Δ*kpsT*com strains, supporting the expression of FHA detected by Western blot analysis in the whole cell lysate of BPSM, Δ*kpsT* and Δ*kpsT*com (Figure 3.7 I A). The lower levels of FHA detected by Western blot in the culture supernatant of Δ*kpsT* but not in the total cell lysate (Figure 3.7 I A) could imply that the secretion of FHA but not its production may be impaired in Δ*kpsT*. Alternatively, *fhaB* translational efficiency and/or post-translational modification aberration in Δ*kpsT* may also result in FHA degradation and secretion impairment. Since, the FHA, BrkA and PT encoding genes are regulated by the two-component system BvgA/S, we also investigated the transcriptional activity in the Δ*kpsT* mutant of the *bvgAS* locus (Antoine et al., 2000; Roy and Falkow, 1991) and *bvgR* (Merkel et al., 2003). Comparable transcriptional activities of *bvgAS* and *bvgR* were obtained in Δ*kpsT,* Δ*kpsT*com and BPSM strains (Figure 3.9), suggesting that the lower expression of *ptx* and *brkA* in Δ*kpsT* is not directly correlated to a lower expression of the *bvgAS* locus.

Together, these data indicate that deletion of the *kpsT* ORF in the capsule locus altered the expression of at least two key *bvg*-regulated genes (*brkA*, *ptx* and *sphB1*) at the transcriptional level, as well as the secretion of FHA.

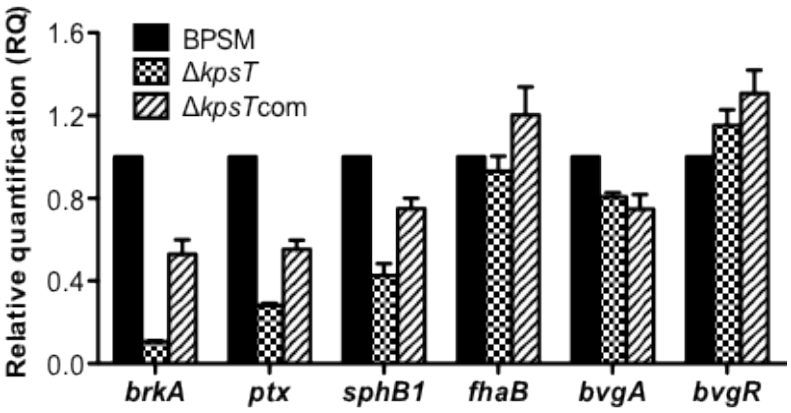


Figure 3.9: Relative transcriptional activity of *vags* in BPSM, Δ*kpsT* and Δ*kpsT*com in virulent phase.

Total RNA was extracted from BPSM (solid bars), Δ*kpsT* (dotted bars) and Δ*kpsT*com (stripped bars) strains grown in virulent Bvg^+ phase. Real-time PCR analysis was performed using primers mapping in the *brkA*, *ptx*, *fhaB*, *bvgA* and *bvgR* genes. *recA* gene was used as the endogenous control. Results are expressed as average relative quantification (RQ) vs wild type BPSM (RQ=1). Results are expressed as the average relative quantification RQ ± SD of triplicate versus BPSM. Results are representative of 3 independent experiments.

3.1.10.2 Microarray analysis

To further explore the effect of *kpsT* deletion on the expression of *bvg*-regulated genes, the global transcriptional profile in the Δ*kpsT* mutant was determined and compared to its parental counterpart BPSM using DNA microarray technology which screens for a total of 3554 *B. pertussis* ORFs. Mid-exponential Bvg^+ phase BPSM and Δ*kpsT* strains (OD_{600nm} 2) were harvested and processed for RNA extraction and microarray hybridization as described in section 2.4.10.

The global transcriptional profiling revealed a large number of genes that were significantly (adjusted *P* value < 0.01) down-regulated in the Δ*kpsT* mutant (Appendix 3). The down-regulated transcripts included genes coding for autotransporters (*vag8, brkA*), serine protease (*sphB1*), putative RNA polymerase sigma factor (*brpL*), components and effector of the type 3 secretion system T3SS (*bcrD*, *bscD*, *bopD*, *bopN*, *bsp22*), pertussis toxin accessory genes (*ptxABDE*), tracheal colonization factor A (*tcfA*), outer membrane porin (*ompQ*) and components for iron acquisition (*hemC*, *bfrD*) (Figure 3.10). Furthermore, consistent with our Real-time PCR analysis, expression of the *bvgAS* locus and *fhaB* was not found to be down-regulated in the Δ*kpsT* mutant (Appendix 3). Notably, expression of the loci BP0454 and BP0455, which encode for the hypothetical tripartite ATP-independent periplasmic transporters (TRAP) was strongly down-modulated in Δ*kpsT* mutant (Appendix 3). The energy dependent TRAP is ubiquitous in gram-negative bacteria and plays a crucial role in bacteria physiology and virulence

by driving carboxylate sugar and sialic acid uptake into bacteria cell across the inner membrane (Mulligan et al., 2011).

The transcriptomic analysis also revealed a gene cluster (BP3812-BP3838), which exhibited an increased amount of transcripts in a range from 1.7 to 4 fold in Δ*kpsT* compared to parental BPSM (Appendix 3). A search from GeneDB database (Sanger Institute) revealed that this particular gene cluster is flanked by short insertional sequence element (ISE), also known as transposable element that encodes transposase. To address whether up-regulation of this gene cluster is due to *kpsT* deletion, Real-time PCR analysis was performed on BPSM, Δ*kpsT* and Δ*kpsT*com strains with two different sets of primers mapping within the BP3812-BP3838 cluster. Consistent with the microarray data, increased transcript levels compared to BPSM were observed for Δ*kpsT* (Figure 3.11). However, comparable expression levels were obtained for both Δ*kpsT* and Δ*kpsT*com strains, thus indicating that the increased expression of the BP3812-BP3838 gene cluster in the Δ*kpsT* strain is independent of *kpsT* deletion (Figure 3.11). It is possible that this cluster has undergone some genetic rearrangement in the Δ*kpsT* mutant.

In addition to support our Real-time PCR analysis, the microarray data revealed that the absence of KpsT affects negatively the expression of a large number of *bvg*-regulated genes. Such overall down-regulation is likely to be responsible for the attenuated phenotype observed with the Δ*kpsT* mutant in mice. These observations led us to draw the hypothesis that the BvgA/S-mediated gene regulation is affected in the Δ*kpsT* mutant.

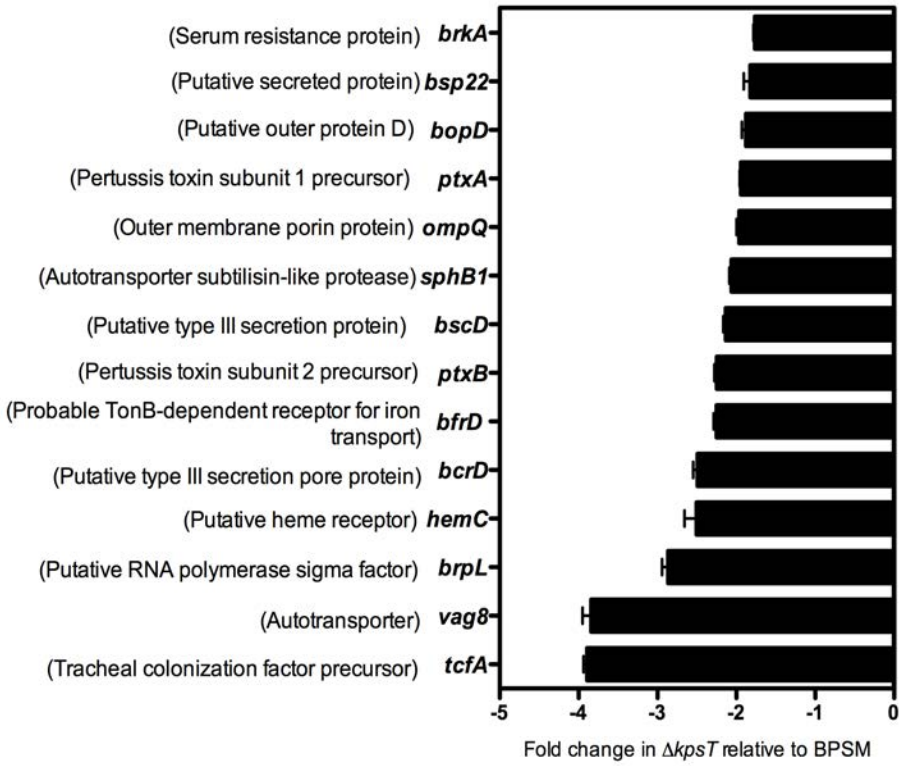


Figure 3.10: Microarray analysis of relative expression levels of selected genes that was down-modulated in Δ*kpsT* mutant.

Total RNA was extracted from BPSM and Δ*kpsT* strains grown in virulent Bvg^+ phase. Microarray gene expression values were selected based on $\log_2$ fold change < -0.8, with adjusted *P* value <0.01. Results are expressed as average fold change Δ*kpsT* compared to BPSM, negative value indicates gene repression. Solid bars represent fold change ± SD of 2 independent experiments.

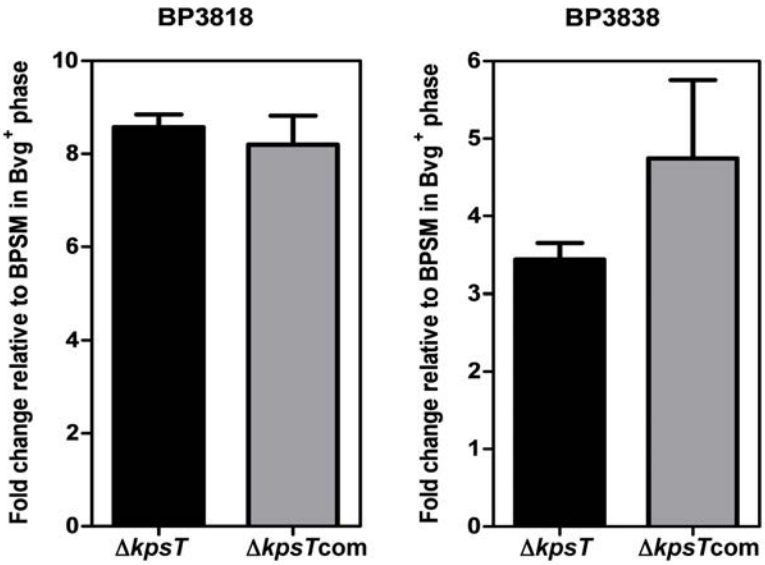


Figure 3.11: Relative transcriptional activity of *BP3818* and *BP3838* ORFs in BPSM, Δ*kpsT* and Δ*kpsT*com in virulent phase.

Total RNA was extracted from BPSM, Δ*kpsT* (black bar) and Δ*kpsT*com (grey bar) strains grown in virulent phase. Real-time PCR analysis was performed using primers mapping in the *BP3818* ORF and *BP3838* ORF. *recA* gene was used as the endogenous control. Results are expressed for each target gene as average fold change ± SD of triplicate Ct values obtained Δ*kpsT* and Δ*kpsT*com versus the Ct value obtained with BPSM strain. The results are representative of 3 independent experiments.

(B) ROLE OF KPST AND THE POLYSACCHARIDE CAPSULE TRANSPORT-EXPORT COMPLEX IN THE VIRULENCE OF *B. PERTUSSIS*

3.2 RESULTS

3.2.1 Construction of The *B. pertussis* KO*caps* Strains Expressing *kpsT* and *kpsMT* Under The Control of Native Capsule Promoter

Since deletion of the *kpsT* ORF did not affect the expression of the upstream and downstream ORFs in the capsule operon, it is expected that the corresponding proteins involved in polysaccharide capsule transport-export and biosynthesis are still being produced in the Δ*kpsT* mutant. We postulate that the role of KpsT on the modulation of *bvg*-regulated virulence factors may require the presence of some other capsule locus-encoded proteins, in particular those located in the bacterial envelope, thus susceptible to affect the function of BvgS sensor located within the inner membrane. To test this hypothesis, *kpsT* ORF was expressed in KO*caps* mutant (deleted for the entire capsule operon) under the control of native capsule promoter. Plasmid pBBR::Pcaps*kpsT* was electroporated into KO*caps* strain and selected based on Cm resistance and PCR screening.

In addition, in *E. coli*, KpsT is a peripheral inner membrane protein that binds ATP for active transport of capsule polymers from the cytoplasm to the periplasmic face through the integral inner membrane KpsM, forming the

KpsMT transporter (Bliss et al., 1996; Pigeon and Silver, 1994). Thus, we reasoned that similarly in *B. pertussis*, KpsM and KpsT may form the KpsMT transporter and instead of KpsT alone, KpsMT could interact with the BvgA/S signaling pathway. To address this possibility, *kpsMT* was expressed into the KO*caps* mutant under the control of native capsule promoter. This strain was obtained upon electroporation of the KO*caps* mutant with the pBBR::Pcaps*kpsMT* plasmid construct.

3.2.2 Lung Colonization Profile

To evaluate whether KpsT and/or KpsMT alone is sufficient to restore bacterial virulence when expressed in KOcaps, the lung colonization profile of KO*caps*:*kpsT* and KO*caps*:*kpsMT* strains was determined in mice and compared with wild type BPSM and KO*caps* mutant. The KO*caps*, KO*caps*:*kpsT* and KO*caps*:*kpsMT* strains displayed a significant reduction in CFU counts at 3 days and 7 days p.i. compared to BSPM (Figure 3.12). However, significantly higher CFU counts were obtained with KO*caps*:*kpsT* and KO*caps*:*kpsMT* at 3 days p.i. compared to KO*caps* mutant but far lower than those observed with BPSM (Figure 3.12). The results here support that neither KpsT alone nor the KpsMT complex is sufficient to restore a parental colonization efficacy of the KO*caps* mutant, suggesting that the role of KpsT on the modulation of *bvg*-regulated factors and the overall virulence requires the entire polysaccharide capsule transport-export machinery. Moreover, it is plausible that absence of KpsT at the inner membrane may compromise structurally the overall capsule transport-export complex across the cell wall

and consequently affect the *bvg*-mediated virulence, as single gene deletion of *kpsT* leads to bacterial attenuation *in vivo* and decreased transcriptional activity of *bvg*-regulated genes.

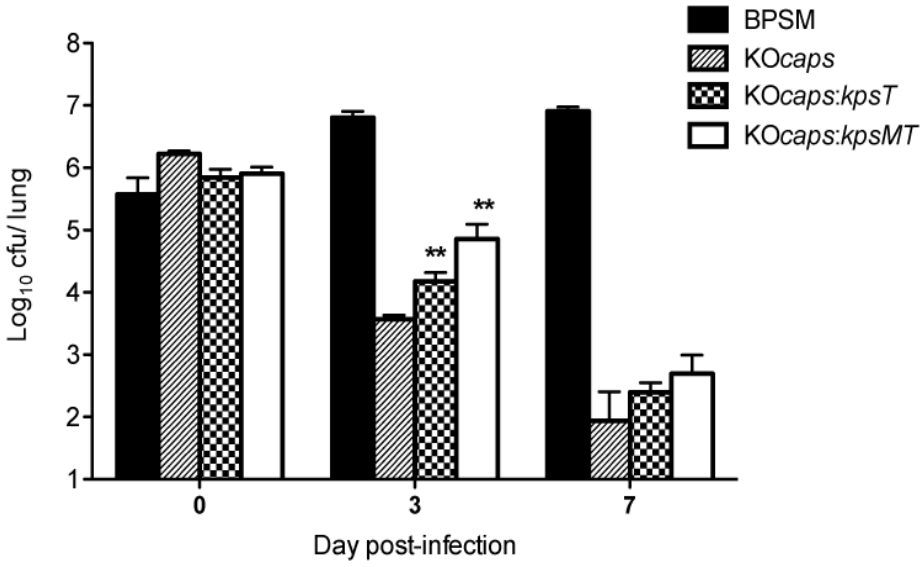


Figure 3.12: Lung colonization profile by *B. pertussis* BPSM, KO*caps*, KO*caps*:*kpsT* and KO*caps*:*kpsMT* strains.

Balb/C mice were infected intranasally with $5x10^5$ CFU of *B. pertussis* BPSM (solid bars), KO*caps* (striped bars), KO*caps*:*kpsT* (dotted bars) and KO*caps*:*kpsMT* (open bars). At the indicated time points, four infected mice per group were euthanized and their lungs were harvested, homogenized and plated on blood agar to determine the total number of CFU per lung. The results are expressed as the mean ± SEM of four mice per group. ** *p* value < 0.01 relative to KO*caps*. Results are representative of two independent experiments.

(C) STUDY OF THE ROLE OF THE CAPSULE LOCUS IN BVG-MEDIATED SIGNAL TRANSDUCTION

3.3 RESULTS

3.3.1 Effects of *kpsT* Deletion In a Bvg-Constitutive Background

3.3.1.1 Construction of the *B. pertussis kpsT*-deleted mutant in a Bvg-constitutive active strain, BvgS-VFT2

Our data so far demonstrate that bvg-regulated gene expression is altered in the absence of KpsT. Given the predicted localization of KpsT at the inner membrane, we hypothesized that KpsT may directly or indirectly exert its effect on the BvgS sensor, an integral plasma membrane protein, thus affecting the overall BvgS-mediated phosphorelay and signal transduction. To test this hypothesis, we introduced the *kpsT* deletion in a BPSM-derivative Bvg^+ phase-locked mutant (Herrou et al., 2009). Such mutant, termed as BvgS-VFT2, contains amino acid substitutions at the periplasmic solute-binding Venus Fly Trap 2 (VFT2) domain of the BvgS sensor, which becomes insensitive to environmental modulator $MgSO_4$, thereby resulting in the constitutive expression of *vags* in both modulating and non-modulating conditions (Herrou et al., 2010; Herrou et al., 2009). The BPSM derivative BvgS-VFT2 strain was kindly provided by Dr. F. J. Dubuisson from Institute Pasteur Lille, France.

Electrocompetent B. pertussis BvgS-VFT2 strain was electroporated with pJQT1-2 plasmid used for the construction Δ*kpsT* as described in section 3.1.1. The recombinant pJQT1-2 construct was integrated into BvgS-VFT2 genome via double homologous recombination, leading to an in-frame deletion of the *kpsT* ORF. Using primers as listed in Table 2.4, BvgS-VFT2 clones deleted for *kpsT* were selected by colony PCR screening based on the integration into the correct locus. Positive clones were propagated and subjected to Southern blot analysis using the same probing strategy as described in Figure 3.2 A for the construction of Δ*kpsT* strain. Southern blot reveals a larger fragment size for BvgS-VFT2 and a smaller fragment size for BvgS-VFT2-Δ*kpsT* mutant. The size obtained for BvgS-VFT2-Δ*kpsT* (~2.7 kb) versus BvgS-VFT2 (~3.2 kb) counterpart confirms *kpsT* deletion in the capsule operon (Figure 3.13).

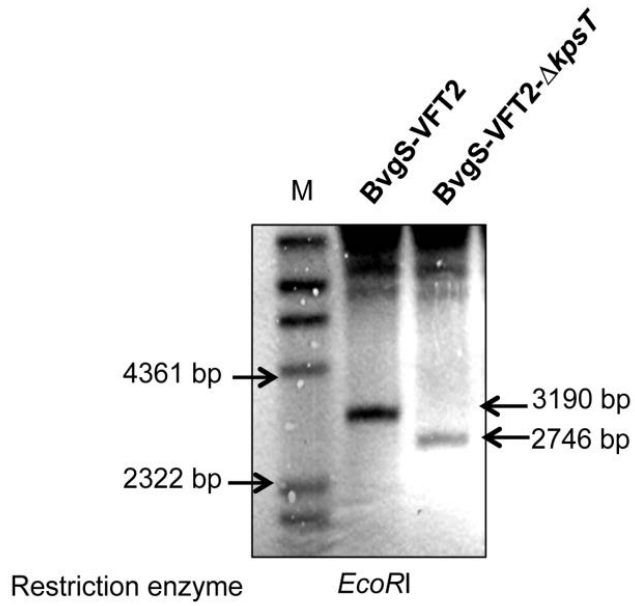


Figure 3.13: Southern blot analysis of BvgS-VFT2-Δ*kpsT* chromosomal DNA.

Restriction-digested chromosomal DNA from BvgS-VFT2 and BvgS-VFT2-Δ*kpsT* were electrophoresed, transferred onto a nitrocellulose membrane and hybridized with the DIG-labeled probe (Refer to Figure 3.8 A showed probe binding site). *EcoRI*-restricted BvgS-VFT2 and BvgS-VFT2-Δ*kpsT* chromosomal DNA yielded 2.7-kb and 3.2-kb respectively. M, DIG-labeled DNA ladder.

3.3.1.2 Production and expression of virulence factors

The production of the three major virulence factors FHA, BrkA and PT in BvgS-VFT2-Δ*kpsT* was compared to that observed with the single mutants, BvgS-VFT2 and Δ*kpsT*, as well as wild type BPSM strains grown in both virulent (Bvg^+) and avirulent (Bvg^-) culture conditions. As previously reported (Herrou et al., 2010; Herrou et al., 2009), the BvgS-VFT2 Bvg^+ phase-locked mutant displayed a constitutive production of the three virulence factors in both virulent and avirulent phases (Figure 3.14 A). Expectedly, production of the virulence factors BrkA and PT was clearly down-modulated in avirulent phase for BPSM and Δ*kpsT* compared to the virulent phase (Figure 3.14 A). Interestingly, higher amounts of BrkA, PT and to a lesser extent FHA were detected with the BvgS-VFT2-Δ*kpsT* double mutant compared to Δ*kpsT* single mutant in virulent phase, with band signal intensities comparable to those observed for wild type BPSM (Figure 3.14 A). This observation thus suggests that deletion of *kpsT* alone in a BvgS-constitutive mutant does not affect the production of virulence factors.

Furthermore, real-time PCR analysis was conducted and showed that down-regulation of the *brkA, ptx* and *sphB1* genes observed with the Δ*kpsT* single mutant was not observed in the BvgS-VFT2-Δ*kpsT* double mutant (Figure 3.14 B). The western blot and real-time PCR analyses thus indicated that deletion of *kpsT* in a Bvg^+-phase locked mutant does not affect *vag* expression and that VFT2 mutation in the BvgS sensor is dominant over the *kpsT* deletion.

A

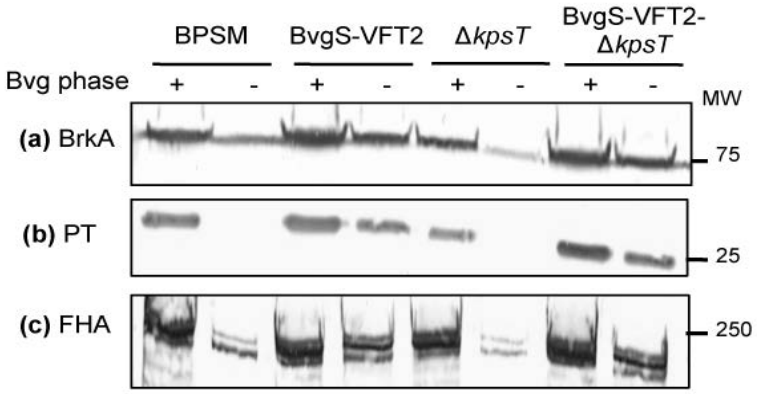


Figure 3.14: Production and expression of virulence factors in BvgS-VFT2-Δ*kpsT* mutant.

(A) Production of *bvg*-regulated virulence proteins in BvgS-VFT2-Δ*kpsT* mutant.
BPSM, BvgS-VFT2, Δ*kpsT* and BvgS-VFT2-Δ*kpsT* strains were exponentially grown in virulent (Bvg^+) and avirulent (Bvg^-) phase. Western blot analysis was performed on whole cell extract (panel **a**) and 10x concentrated (panel **b**) or non-concentrated (panel **c**) culture supernatants using **(a)** anti-BrkA, **(b)** anti-PT or **(c)** anti-FHA primary antibodies. The results are representative of three independent experiments. Molecular weights are indicated on the right side.

B

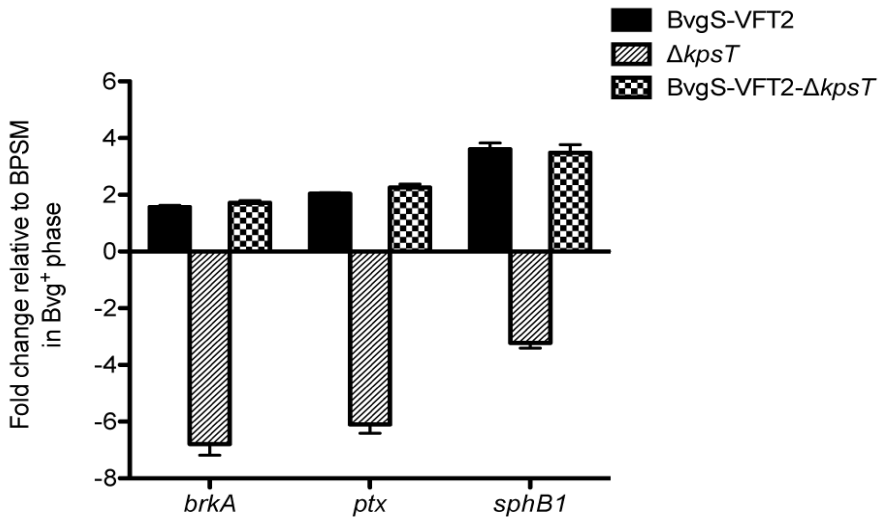


Figure 3.14: (B) Relative transcriptional activity of *vags* in BvgS-VFT2, Δ*kpsT* and BvgS-VFT2-Δ*kpsT* versus BPSM in virulent phase.

Total RNA was extracted from BPSM, BvgS-VFT2 (solid bars), Δ*kpsT* (striped bars) and BvgS-VFT2-Δ*kpsT* (dotted bars) strains grown in virulent phase. Real-time PCR analysis was performed using primers mapping in the *brkA*, *ptx*, and *sphB1* genes. *recA* gene was used as the endogenous control. Results are expressed for each target gene as average fold change ± SD of triplicate Ct values obtained with BvgS-VFT2, Δ*kpsT* and BvgS-VFT2-Δ*kpsT* versus the Ct value obtained with BPSM strain. The results are representative of two independent experiments.

3.3.1.3 Lung colonization profile

To assess whether the restoration of the production of virulence factors in the BvgS-VFT2-Δ*kpsT* double mutant (Figure 3.15) is able to re-establish bacterial virulence, the lung colonization profile was determined in mice and compared to that of BvgS-VFT2 and Δ*kpsT* single mutants. BvgS-VFT2 strain was able to persist and multiply in the mouse respiratory tract up to 7 days p.i. at least, in contrast to the Δ*kpsT* mutant, which displayed an attenuated lung colonization profile as early as day 3 p.i. (Figure 3.15). The BvgS-VFT2-Δ*kpsT* double mutant displayed a colonization profile similar to that observed with BvgS-VFT2 (Figure 3.15), demonstrating that the *kpsT* deletion-associated attenuation phenotype is lost in a BvgS-VFT2 constitutive mutant. Altogether, the results indicate that a *bvgS* constitutive mutation is able to restore the production and expression of *bvg*-regulated virulence factors in Δ*kpsT*, and hence restoring the virulence of the bacteria *in vivo*. The results here suggest for a potential crosstalk between the membrane-associated KpsT protein and the BvgA/S signaling pathway.

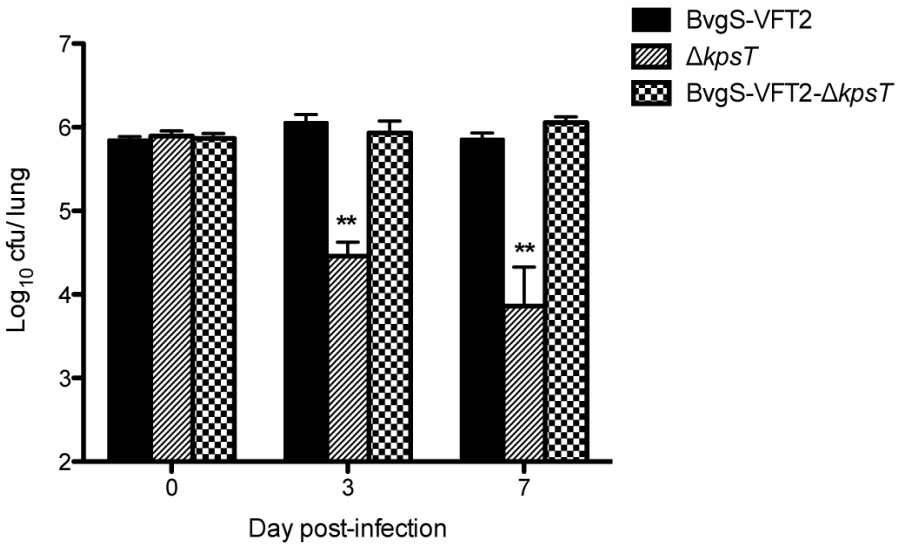


Figure 3.15: Lung colonization profile by *B. pertussis* BvgS-VFT2, Δ*kpsT* and BvgS-VFT2-Δ*kpsT* strains.

Balb/C mice were infected intranasally with $5x10^5$ CFU of *B. pertussis* BvgS-VFT2 (solid bars), Δ*kpsT* (striped bars) and BvgS-VFT2-Δ*kpsT* (dotted bars). At the indicated time points, four infected mice per group were euthanized and their lungs were harvested, homogenized and plated on blood agar to determine the total number of CFU per lung. The results are expressed as the mean ± SEM of four mice per group. ** *p* value < 0.01 relative to BPSM. Results are representative of two independent experiments.

3.3.2 Study of The Interaction Between the Capsule Locus Members and BvgS

3.3.2.1 Construction of the *B. pertussis* BPSH strain expressing histidine-tagged BvgS

Our findings so far indicate that KpsT is required for proper expression of *bvg*-regulated virulence factors and the entire polysaccharide capsule translocon spanning the cell envelope is crucial for *B. pertussis* virulence. Based on these findings, we proposed that the polysaccharide capsule transport-export complex, including KpsT, directly or indirectly influences the activity of BvgS sensor. To date, interaction between a large translocon complex with a two-component sensor within the inner membrane of a bacteria has never been described. To decipher whether the BvgS sensor protein physically interacts with the polysaccharide transport-export machinery and/or KpsT at the inner membrane, we constructed a *B. pertussis* recombinant strain expressing the histidine-tagged BvgS as “bait” protein for *in vivo* affinity purification studies. While the conventional strategy of detecting interacting partners involves “bait” protein over-expression, isolation and purification from *E. coli* system followed by *in vitro* binding assay with *B. pertussis* total cell lysate, it was not particularly relevant in our study as we aim to pull down potential interacting partners within the membrane envelope of *B. pertussis*. Moreover, due to its high molecular weight (137 kDa) and hydrophobic nature, over-expression of full-length BvgS membrane protein in *E. coli* may not be feasible and has been reported

to be toxic to the bacteria cell (Wagner et al., 2006). To construct the *B. pertussis* strain expressing a histidine tag at the N-terminal end of BvgS, six histidines encoding sequences were inserted downstream of *bvgS* signal peptide (Figure 3.16). The final plasmid constructed using primers listed in Table 2.2, termed as pJQ-BvgSHis$_6$PCR1+2 was electroporated into electrocompetent wild-type BPSM. The His$_6$PCR1+2 sequence was integrated into the *bvgS* chromosomal locus via allelic exchange, leading to in-frame insertion of histidines coding sequence downstream of the signal peptide sequences at the N-terminal end. This is to ensure that the membrane insertion of BvgS directed by the signal peptide will not be interfered. The rationale of cloning the histidines coding sequences nearer to the N-terminal is to avoid interfering with the phopho-transfer reaction that occurs at the C-terminal end of BvgS where the phosphate receiver and output domain are located (Figure 3.16). The resulting strain was named BPSH, where His-tag is fused and expressed at the N-terminal end of BvgS.

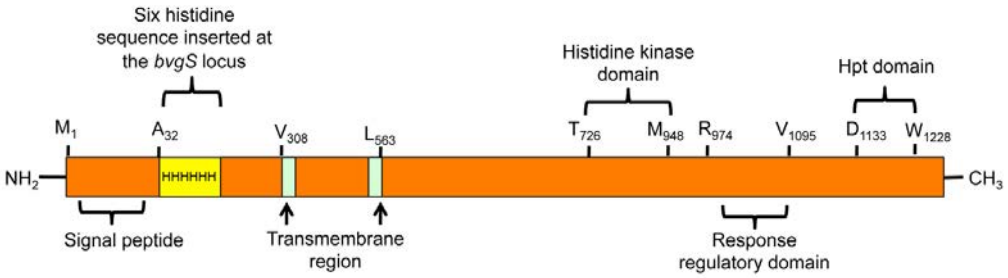


Figure 3.16: Schematic diagram of His-BvgS chimera.

The amino acid residue numbers are specific for *B. pertussis* Tohama I BvgS, figure adapted and modified from UniProt database P16573 (BVGS_BORPE). Yellow region represents the position of His-tag insertion in BvgS.

To ensure that insertion of six His at the N-terminal end of BvgS sensor does not impair its function and activity, expression of several *bvg*-regulated virulence genes such as *brkA*, *ptx*, *fhaB*, *bvgR* and as well as the capsule operon, was accessed in BPSH strain by real-time PCR of the expression level of these genes was found comparable to that of parental BPSM, suggesting that the presence of six His at the BvgS N-terminal end does not interfere with the overall function of BvgS (Figure 3.17).

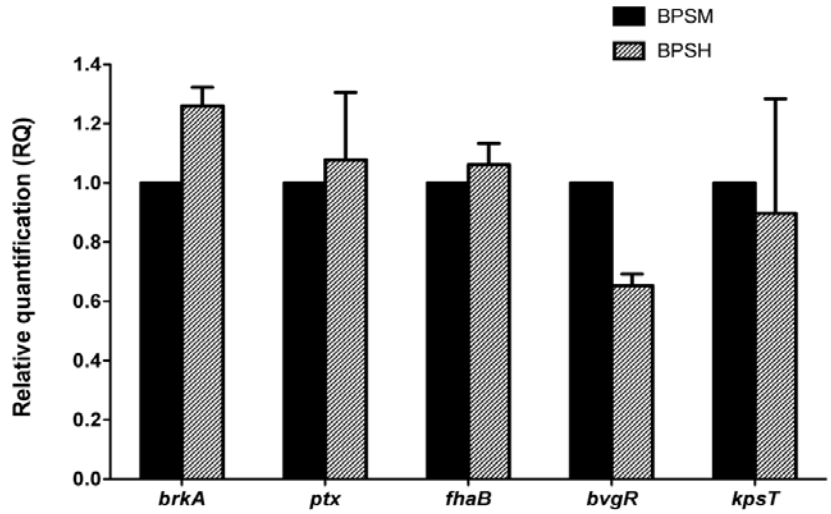


Figure 3.17: Relative transcriptional activity of *vags* and *kpsT* in BPSM and BPSH in virulent phase.

Total RNA was extracted from BPSM (solid bars) and BPSH (stripped bars) strains grown in virulent Bvg^+ phase. Real-time PCR analysis was performed using primers mapping in the *brkA, ptx, fhaB, bvgR* and *kpsT* genes. *recA* gene was used as the endogenous control. Results are expressed as the average relative quantification (RQ) ± SD of triplicate vs Bvg^+ phase BPSM. Results are representative of 2 independent experiments.

3.3.2.2 Optimization of His-BvgS solubilization

Bioinformatics tools predict that BvgS protein consists of two trans-membrane helical domain nearer to the N-terminal end, suggesting that the sensor is membrane-associated (Figure 3.16). Prior to elucidate whether the BvgS sensor physically interacts with the polysaccharide transport-export machinery and/or KpsT at the inner membrane, we first optimized the cell extracts preparation enriched in BvgS protein in *B. pertussis*. According to the protocol described by Zaretzky and co-workers, *B. pertussis* outer and inner membrane fraction can be separated by ultracentrifugation and differential solubilization in presence of 2% Triton-X-100 (Zaretzky et al., 2002). The purpose of separating the membrane fraction from total cellular lysates is to enrich the fraction in His-BvgS proteins and to reduce contamination with other proteins from the cytoplasmic extract prior to Ni-NTA pull down assay.

Total cell extract was harvested from mid-exponential virulent phase bacteria grown in 50 ml of SSAB medium as described in section 2.5.2. BPSH cells were sonicated in lysis buffer containing 10mg/ml of lysozyme, followed by centrifugation at low speed; 4000 x *g* for 10 min to remove unbroken cell debris. The supernate was subjected to ultra-centrifugation at 100, 000 x *g* for 1 h to pellet the total membrane fraction containing both inner and outer membrane. The total membrane fraction was dissolved in 2% Triton-X-100 on a rotating shaker overnight at 4°C. A small aliquot of each fraction obtained from low speed centrifugation (cell pellet and crude supernatant in lysis buffer) and ultra-centrifugation (insoluble membrane pellet and solubilized

membrane proteins in the supernatant) was mixed with Laemelli blue SDS loading buffer containing β-mercaptoethanol. Each of these fractions was heated at 95°C and analyzed by Western blot using anti-His and anti-BvgS antibody for the detection of His-tagged BvgS protein (Figure 3.18).

Despite a thorough enzymatic and mechanical lysis of *B. pertussis*, BvgS was not released into neither the supernatant nor the membrane-enriched fraction obtained by ultra-centrifugation and solubilization with 2% Triton-X-100 (Figure 3.18). Instead, the 137 kDA BvgS was mainly detected in the cell pellet fraction consisting of cellular debris and insoluble proteins after sonication and low-speed centrifugation, indicating that BvgS is highly insoluble and hydrophobic in nature (Figure 3.18). Nevertheless, the detection here confirms that the His-BvgS fusion is successfully expressed in BPSH strain, and that the level of expression is comparable to the parental BPSM (Figure 3.18). The findings here also suggest that insertion of His-tag at the N-terminal end of BvgS does not affect its expression at the protein level in *B. pertussis*.

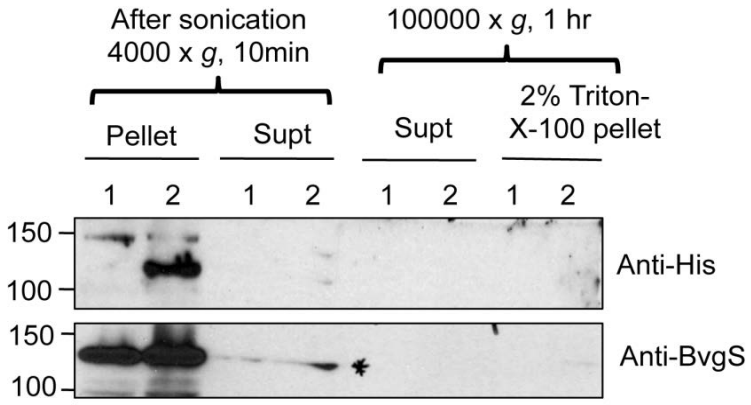


Figure 3.18: Western blot analysis for the detection of His-tagged BvgS.

50 ml of BPSH grown in SS medium was harvested in mid-exponential phase. Cells were lysed by sonication using a bioruptor, followed by a brief low-speed centrifugation (4000 x *g*) for 10 min and ultracentrifugation (100, 000 x *g*) for 1 h. Each of the fractions was heated to 95 °C for 15 min and analyzed under reducing 10% SDS-PAGE. Lanes: 1; BPSM, 2; BPSH. MW markers are indicated on the left.

Due to the insolubility of BvgS under mild lysis treatment, His-BvgS from BPSH was solubilized under denaturing conditions. The washed bacterial pellet was lysed by mechanical disruption and incubation with lysozyme, Triton-X-100 detergent and finally in 6 M urea as described in section 2.5.2.2. Total protein content in the solubilized lysate was measured by BCA assay. A total of 5 mg of protein from the solubilized lysate was mixed with charged Ni-NTA agarose beads and a final concentration of 20 µM of imidazole prior loading to a column chromatography. Purification scheme was first optimized with lysate from BPSH bacteria as described in section 2.5.4. After 5 rounds of stringent washes through the chromatography column with wash buffer containing 20 µM imidazole at pH 6.3 to remove unbound proteins, His-BvgS was released in batch from the Ni-NTA beads with the urea elution buffer containing 250 µM imidazole at pH 4.5.

A small aliquot of the solublized "input" lysate, column flow-through and eluted fractions were mixed with equal volume of Laemelli blue SDS loading buffer containing β-mercaptoethanol and heated at 95°C for 15 min. Each of these fractions was analyzed by Coomassie blue staining (Figure 3.19 A) and Western blot (Figure 3.19 B). Untag parental control, BPSM was harvested and purified concurrently with BPSH. His-BvgS was successfully purified from the solubilized cellular extract of BPSH under denaturing conditions on a Ni-NTA chromatography column, with a majority of the His-BvgS protein detected in the second (E2) and third elution (E3) fractions as evidenced by the detection of a band of an apparent molecular weight (MW) of 140 kDa which corresponds to monomeric His-BvgS (predicted size of 137

kDa) on a Coomassie blue stained SDS-PAGE denaturing gel (Figure 3.19 A). Western blot analysis using anti-His and anti-BvgS antibodies further confirmed the identity of the 140 kDa eluted protein as His-BvgS monomers in E2 and E3 from BPSH but not from BPSM extracts (Figure 3.19 B). A band at 50 kDa MW was also observed in E3 and E4 from both BPSM and BPSH, suggesting that this unknown protein bound to the Ni-NTA agarose beads may correspond to a histidine-rich protein (Figure 3.19 A).

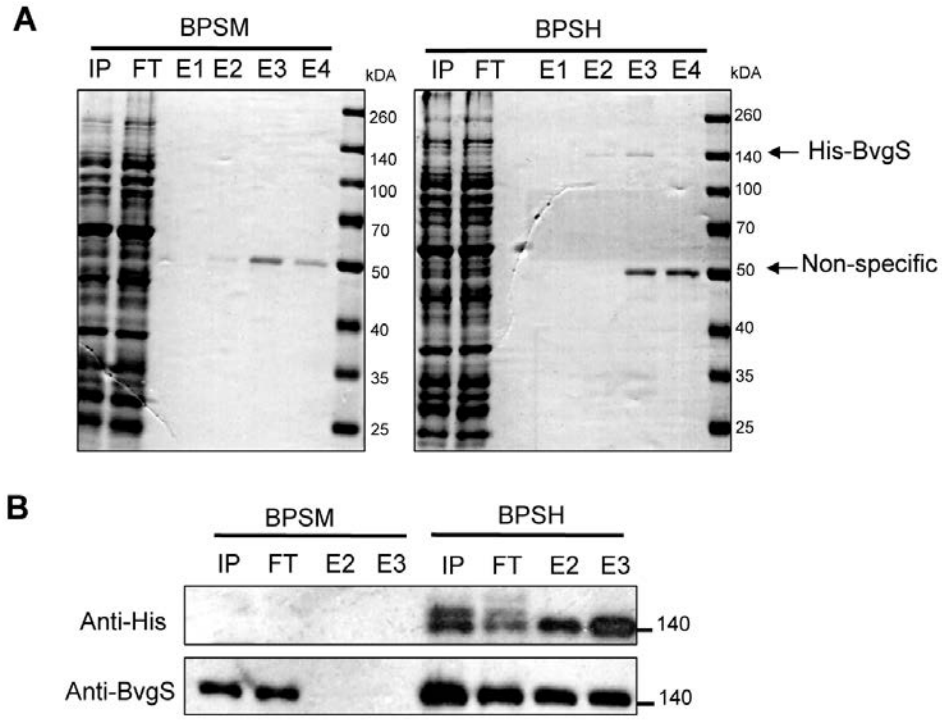


Figure 3.19: Expression and purification of His-BvgS by Ni-NTA chromatography.

5 mg of solubilize cell lysate harvested from BPSM (untag control) and BPSH was mixed with Ni-NTA agarose beads prior to loading onto a chromatography column. Solubilized lysate input, flow-through and batch eluted fractions were heated to 95 °C for 15 min and analyzed under reducing 10% SDS-PAGE.
(A) Coomassie blue staining, Lane IP; Input, FT; Flow through, E1; Eluted fraction 1, E2; Eluted fraction 2, E3; Eluted fraction 3, E4; Eluted fraction 4. Molecular weights are indicated on the right side.
(B) Western blot analysis with anti-His and anti-BvgS antibodies. Lane IP; Input, FT; Flow through, E2; Eluted fraction 2, E3; Eluted fraction 3. Molecular weights are indicated on the right side.

3.3.2.3 Detection of purified His-BvgS under reducing and non-reducing conditions

A comparative analysis for the formation of protein complexes from purified His-BvgS was analyzed by denaturing SDS-PAGE and Western blot in the presence and absence of reducing agent β-mercaptoethanol. Western blot analysis revealed the presence of a high MW protein complex (greater than 260 kDa) which reacts with both anti-His and anti-BvgS antibodies and disappears under reducing conditions and/or upon heat treatment (Figure 3.20 A). Concomitantly, a stronger signal intensity of the 140 kDa band was observed under reducing conditions (Figure 3.20 A). The observation here thus strongly suggests that BvgS is able to form high MW complexes that dissociate upon heat treatment and/or addition of β-mercaptoethanol. The denaturing mechanism of urea and guanidine hydrochloride mainly targets the intramolecular hydrogen bonds, thus weakening the overall hydrophobic structure of a protein (England and Haran, 2011). Given the denaturing conditions used for purification of BvgS, it unexpected that such high molecular complexes were still detected upon Ni-NTA column elution in the absence of heat and reducing agent. This observation may be explained by the presence of several functionally important amino acid residues within the hydrophobic core of BvgS involved in inter- and intramolecular interactions for example covalent, peptide and disulphide bonds that may be involved in maintaining the conformational tension of the sensor kinase.

To further investigate the nature of the high MW complexes that are resistant to guanidine hydrchloride treatment, the corresponding band gel was excised from a Coomassie-blue stained non-reducing SDS-PAGE gel and subjected to Triple-TOF mass spectrometry analysis (Figure 3.20 B). MS analysis further confirmed with high confidence that majority of the protein complex consists of BvgS protein with no detection of any of the membrane proteins from the capsule transport machinery (Table 3.1). Nevertheless, the possibility that KpsT or any other membrane proteins involved in the capsule transport machinery interacting physically and directly with BvgS should not be disregarded based on this pull-down assay, as potential interacting partner for BvgS may be lost under denaturing conditions. The high MW complexes captured from His-BvgS purification likely consist of BvgS multimers, confirming previous reports on the possible homodimerization of truncated domains of BvgS in *E. coli* (Beier et al., 1995; Perraud et al., 2000).

Due to the solubility of BvgS under denaturing condition which may result in the loss of association between proteins, we have attempted another alternative pull-down scheme, whereby both BPSM control and BPSH strain were treated with cross-linking agent to link potential interacting partners with His-BvgS. The solubilized bacteria lysate was subjected to Ni-NTA column chromatography as described in Figure 3.19. However, pull-down of His-BvgS from cross-linked BPSH samples could not be distinguished from BPSM untag control due to high non-specific binding to the Ni-NTA column (Data not shown). Chemical cross-linkers are known to link protein(s) that are in close proximity with the "bait" protein, leading to high background

consisting of non-specific binding partners (Kaake et al., 2010; Klockenbusch and Kast, 2010). Thus, a number of membrane proteins spanning the cell envelope of *B. pertussis* were likely cross-linked to His-BvgS and hence co-purfied with BvgS despite having no true interaction with BvgS. More stringent washes conditions before elution from the column could potentially help reduce aspecific binding and enrich for true interacting partners with BgvS.

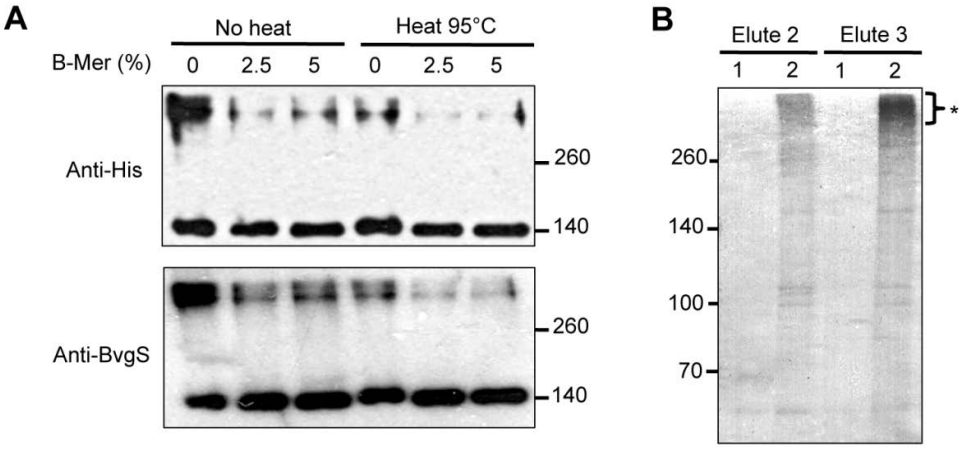


Figure 3.20: Detection of purified BvgS by Western blotting and SDS-PAGE.

(A) Detection of BvgS associated oligomers and BvgS monomer by Western blotting.
Purified His-BvgS from BPSH cells were mixed with equal volume of Laemmli Blue sample buffer containing either no reducing agent (0% β-mercaptoethanol) or increasing concentrations (2.5% and 5% β-mercaptoethanol). The proteins samples were then subjected to either no heat or heat denaturation at 95°C for 15 min prior to SDS-PAGE analysis and Western blotted with anti-His or anti-BvgS antibody. Molecular weights are indicated on the right side.
(B) Non-reducing SDS-PAGE analysis of purified His-BvgS.
Purified His-BvgS batch eluted from fraction 2 and 3 are subjected non-reducing SDS-PAGE and Coomassie blue staining. BPSM was used as untag control. The asterisk labelled regions of above 260 kDA corresponding to the Western blot signals was gel excised for Triple-TOF MS analysis. Lane 1; BPSM, Lane 2; BPSH. Molecular weights are indicated left side.

Elution fraction	Unused score	Total score	% Coverage	Pubmed accession number	Name	Peptides (95%)
Elute 2	57.56	57.56	29.2	gi| 34978356	Virulence sensor protein BvgS	36
Elute 3	76.01	76.01	34.6	gi| 34978356	Virulence sensor protein BvgS	54

Table 3.1: Protein summary report generated by ProteinPilot software.

Based on Figure 3.20 B, high molecular weight complex observed on the Coomassie blue stained SDS-PAGE gel from elution fraction 2 and 3 of His-BvgS purified from BPSH were gel excised and sent for Triple-TOF mass spectrometry analysis, a service provided by the Protein and Proteomics Centre, Department of Biological Science, NUS. Identities and scoring were analyzed using ProteinPilot^{TM} software.

3.3.2.4 Construction of the BPSH strain deleted for *kpsT* or the entire capsule operon

In the context of the bacterial cell wall, the structural stability of membrane proteins forming oligomers is fundamental for their function, and in this case the stability of BvgS oligomers in signal integration and transduction from the extracellular environment. To extend our understanding whether the products of the capsule locus affect BvgS oligomerization, deletion of the entire capsule operon and *kpsT* was introduced into the BPSH strain.

To construct *B. pertussis* BPSH strain deleted for the entire capsule operon and *kpsT*, suicide vector pJQSY4 (Neo et al., 2010) and pJQT1-2 (Section 3.1.1) were used for allelic exchange in BPSH. pJQSY4 and pJQT1-2 were separately integrated into the *B. pertussis* genome via double homologous recombination, leading to in-frame deletion of the capsule operon and *kpsT* gene, respectively. Positive clones were expanded and subjected to Southern blot analysis using the DIG-labeled probing strategy as depicted in Figure 3.21 A for the confirmation of BPSH-KO*caps* strain, and Figure 3.2 A under section 3.1.2 for the confirmation of BPSH-Δ*kpsT* mutant.

Southern blot analysis revealed the expected sizes for both BPSH-KO*caps* (~1 kb) and BPSH-Δ*kpsT* (~2.7 kb) relative their wild-type counterpart (Figure 3.21 B), thus confirming the deletion of the 10 kb capsule operon and *kpsT* at the correct region. To complement the BPSH-Δ*kpsT*

mutant, pBBR::Pcaps*kpsT* plasmid (section 3.1.3) was electroporated into the electerocompetent BPSH-Δ*kpsT*, yielding the chloramphenicol resistant strain designated as BPSH-Δ*kpsT*com.

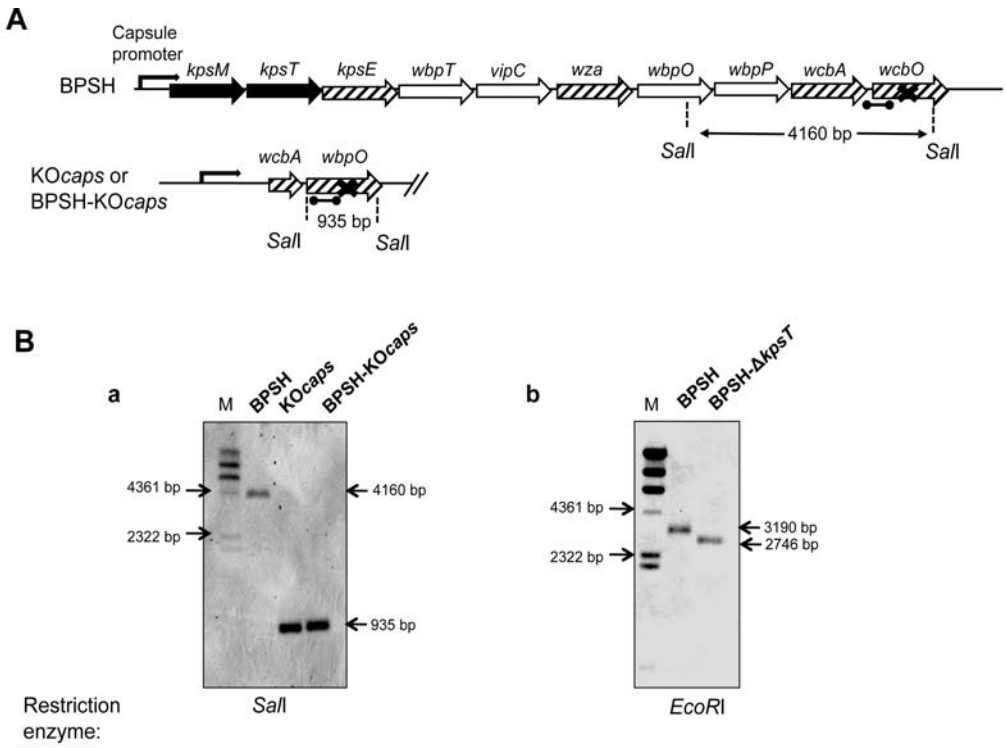


Figure 3.21: Southern blot analysis of BPSH-KO*caps* and BPSH-Δ*kpsT* chromosomal DNA.

(A) Strategy for Southern blot analysis for BPSH-KO*caps* mutant.
The DIG-labeled probe binding region (black rounded arrow), restriction sites and size of restriction-digested chromosomal DNA for Southern blot analysis are as shown.
(B) Southern blot analysis of *B. pertussis* chromosomal DNA.
Restriction-digested chromosomal DNA from BPSH, KO*caps* and BPSH-KO*caps* or BPSH-Δ*kpsT* were electrophoresed, transferred onto a nitrocellulose membrane and hybridized with the DIG-labeled probe (Refer to Figure 3.32 A showed probe binding site for BPSH-Δ*kpsT*). Panel **a**, *Sal*I-restricted BPSH, KO*caps* and BPSH-KO*caps* DNA yielded 4.1-kb and 935 bp respectively. Panel **b**, *Eco*RI restricted BPSH and BPSH-Δ*kpsT* DNA yielded 2.7-kb and 3.2-kb respectively. M, DIG-labeled DNA ladder.

3.3.2.5 Purification of His-BvgS from BPSH, BPSH-KO*caps* and BPSH-Δ*kpsT* strains

Affinity purification of His-BvgS from BPSH, BPSH-KO*caps* and BPSH-Δ*kpsT* strains was performed as described in section 3.3.2.3. Purified His-BvgS fractions from all of the constructs were subjected to Western blot analysis under reducing and non-reducing conditions. Strikingly, Western blot analysis revealed that under non-reducing conditions, the signal intensity of the high MW band >260 kDa was much lower for the BPSH-KO*caps* and BPSH-Δ*kpsT* compared to BPSH (Figure 3.22 A, B). Instead, a higher signal intensity of the 140 kDa band was observed for both mutants compared to BPSH under non-reducing condition. The complemented BPSH-Δ*kpsT*com displayed a partial parental BPSH phenotype in non-reducing condition whereby a greater signal intensity of the high MW band was observed with BPSH-Δ*kpsT*com compared to BPSH-Δ*kpsT* (Figure 3.22 B). Under reducing conditions, expectedly, the high MW band disappeared and comparable signal intensities of the 140 kDa band were observed for all the strains (Figure 3.22 A, B), suggesting that the capsule locus, including KpsT, is fundamental for BvgS oligomerization. Taken together, these pull-down approaches further support a link between the capsule locus (KpsT in particular) and BvgS oligomerization in *B. pertussis*.

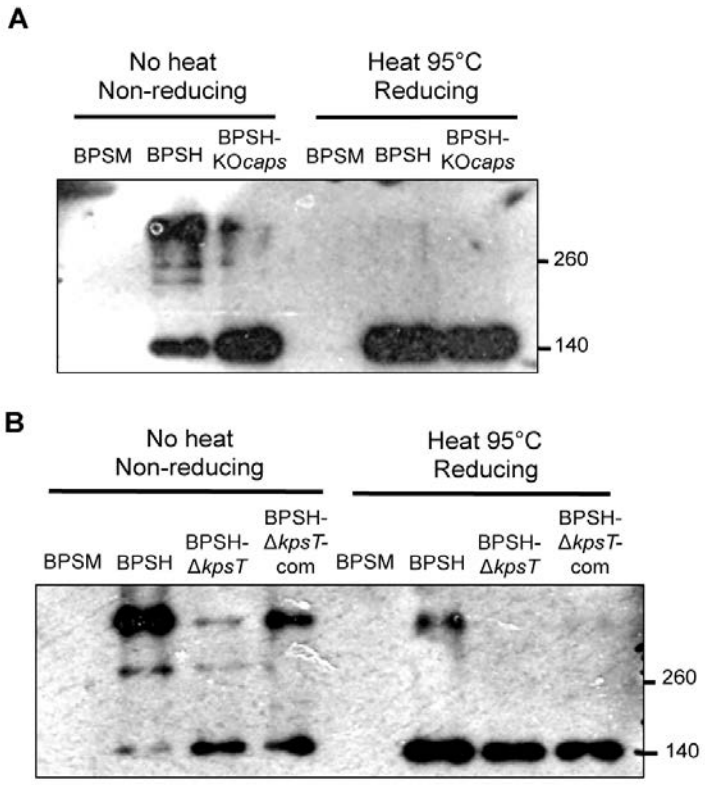


Figure 3.22: Detection of BvgS associated oligomers and BvgS monomer.

(A) Detection of BvgS associated oligomers and BvgS monomer in BPSH and BPSH-KO*caps*.
Equal amount of purified His-BvgS from BPSH and BPSH-KO*caps* cells were mixed with Laemmli blue sample buffer containing either no reducing agent or with 5% β-mercaptoethanol. The proteins samples were then subjected to either no heat or heat denaturation at 95°C for 15 min. Equal amount of protein were loaded for each well for SDS-PAGE analysis and Western blotted with anti-BvgS antibody. Molecular weights are indicated on the right side.
(B) Detection of BvgS associated oligomers and BvgS monomer in BPSH, BPSH-Δ*kpsT* and BPSH-Δ*kpsT*com by Western blotting.
Equal amount of purified His-BvgS from BPSH, BPSH-Δ*kpsT* and BPSH-Δ*kpsT*com cells were mixed with Laemmli Blue sample buffer containing either no reducing agent or with 5% β-mercaptoethanol. The proteins samples were then subjected to either no heat or heat denaturation at 95°C for 15 min. Equal amount of protein were loaded for each well for SDS-PAGE analysis and Western blotted with anti-BvgS antibody. Molecular weights are indicated on the right side.

3.3.3 Assessment of Membrane Integrity In *kpsT*-Deleted Mutant

We postulated that absence of KpsT and the polysaccharide capsule transport-export machinery may alter the overall cell envelope structure and/or the cellular membrane integrity, thus affecting the ability of BvgS to sense and integrate extracellular signals, thereby altering the dimerization of BvgS and/or signal transduction to downstream regulator protein BvgA. The putative KpsM and KpsT protein sequences of *B. pertussis* are highly similar to an ABC transporter protein family, with KpsM harboring the predictive trans-membrane domain and KpsT the nucleotide ATP-binding domain. Several studies have suggested that alteration of the ABC transporter structure or function and absence of the ATP-binding protein cognate partner induces membrane stress and damage (Attia et al., 2010; Zhong et al., 1996). Therefore, it is conceivable here that the absence of KpsT in *B. pertussis* may alter the inner membrane environment and/or integrity thereby affecting the function of other membrane proteins such as BvgS sensor.

To test this hypothesis, we compared the sensitivity of BPSM, Δ*kpsT* and Δ*kpsT*com to erythromycin, a large hydrophobic macrolide, and one of the most commonly used antibiotics to treat pertussis (Bergquist et al., 1987; Trollfors, 1978; Zackrisson et al., 1983). To reach its cytoplasmic target the 50s ribosomal subunit, erythromycin must cross the bacterial envelope and inner membrane. We reasoned that the absence of KpsT protein may modify the diffusion rate of erythromycin across the plasma membrane thereby leading to a different sensitivity of Δ*kpsT* mutant to this antibiotic. While the

in vitro growth profiles of BPSM, Δ*kpsT* and Δ*kpsT*com in the absence of erythromycin were comparable (Figure 3.23, left panel), growth of Δ*kpsT* was completely inhibited in the presence of sub-lethal concentration of erythromycin 0.05 μg/ml (Zackrisson et al., 1983), as opposed to parental BPSM and Δ*kpsT*com which kept multiplying over time (Figure 3.23, right panel). The increased susceptibility of Δ*kpsT* mutant to erythromycin may therefore reflect some structural changes and perturbed integrity of the bacterial plasma membrane.

To further investigate this possibility, Δ*kpsT* was subjected to other chemical treatments known to perturb the bacterial membrane integrity (Attia et al., 2010; Baud et al., 2009; Plesa et al., 2006). BPSM, Δ*kpsT* and Δ*kpsT*com were incubated with 0.02% SDS for 2 h to induce membrane permeabilization and analyzed by flow cytometry for the uptake of propidium iodide (PI), a membrane impermeant and DNA specific dye. The results indicated a significantly higher percentage of PI^+ Δ*kpsT* bacteria compared to the parental and complemented strains (Figure 3.24 A). The increased sensitivity to SDS seen with Δ*kpsT* thus provides further support to the idea that in this mutant the membrane integrity and permeability are perturbed.

Cationic chelating agent EDTA induces membrane permeabilization, irreversible destabilization and release of LPS from Gram-negative bacteria (Leive, 1965; Vaara, 1992). BPSM, Δ*kpsT* and Δ*kpsT*com bacteria were incubated with sub-lethal concentrations of EDTA for 2 h and bacteria viability was assayed by CFU counting. At both EDTA concentrations of

2mg/ml and 1mg/ml, it was found that parental BPSM survived significantly better than Δ*kpsT* mutant (Figure 3.24 B). Parental susceptibility to EDTA-mediated killing was observed with Δ*kpsT*com strain (Figure 3.24 B). It must be noted however, that although Δ*kpsT* mutant is more susceptible to killing and permeabilization by these agents, our data do not rule out the possibility that absence of the surface capsule polysaccharide may also be a factor that may be involved in the increased sensitivity to erythromycin, SDS and EDTA. Taken together, these findings support that the membrane associated KpsT protein could possibly contribute to *B. pertussis* plasma membrane integrity.

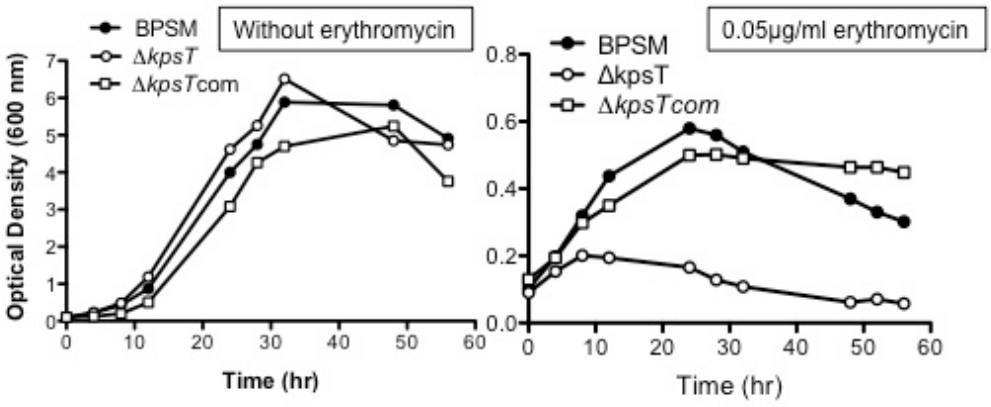


Figure 3.23: Growth kinetics of BPSM, Δ*kpsT* and Δ*kpsT*com in the presence of erythromycin.

SS liquid medium was inoculated with BPSM (closed circles), Δ*kpsT* (open circles) and Δ*kpsT*com (open squares) at initial $OD600_{nm}$ of 0.1 at time-point 0 h without (left panel) and with 0.05µg/ml erythromycin (right panel). $OD600_{nm}$ was monitored throughout incubation at 37ºC. A representative of 3 independent experiments is shown.

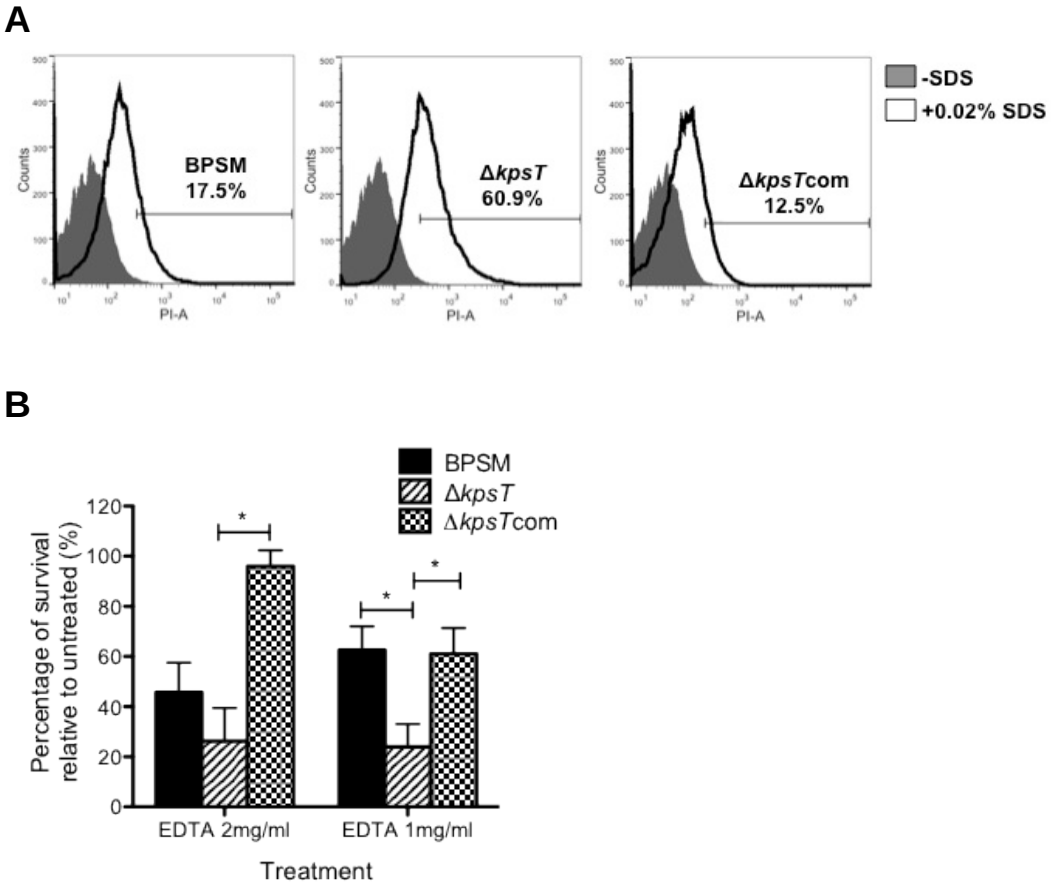


Figure 3.24: Effect of SDS and EDTA on BPSM, Δ*kpsT* and Δ*kpsT*com strain.

(A) Effect of SDS on BPSM, Δ*kpsT* and Δ*kpsT*com strain.

BPSM, Δ*kpsT* and Δ*kpsT*com strain were incubated with 0.02% SDS (white histogram) and without SDS (grey histogram). Both SDS-treated and untreated bacteria were stained with propidium iodide (PI). The fluorescent cells were detected by flow cytometry, with 20,000 events counted for each sample. A representative experiment is shown from 3 independent experiments, with percentage of fluorescent cells indicated in each panel with respect to the untreated control for each sample.

(B) Effect of EDTA treatment on BPSM, Δ*kpsT* and Δ*kpsT*com viability. BPSM, Δ*kpsT* and Δ*kpsT*com strains were incubated with 2mg/ml and 1mg/ml of EDTA for 2 h. Viable bacteria were enumerated on BG agar after 3 days of incubation. Data is expressed as the mean ± SEM from 3 independent experiments. * p value < 0.05 relative to the indicated experimental group.

3.4 DISCUSSION

3.4.1 Construction of *B. pertussis* Capsule Deficient Mutants

The biological role of the polysaccharide capsule, a complex structure often associated with microbial virulence and an important vaccine target for many pathogens, remains uncharacterized in *B. pertussis*. In a previous work, our lab showed that *B. pertussis* bacteria produces an intact and distinct polysaccharide capsule at the bacteria surface, and demonstrated that this structure does not participate in the classical capsule-defense mechanisms including phagocytosis, complement-mediated killing and antimicrobial peptides attack (Neo et al., 2010). Although classified as a *bvg*-repressed gene (*vrg*) with maximal expression in Bvg^- growth phase (Antoine et al., 2000; Hot et al., 2003), substantial transcriptional activity of the capsule operon was detected when bacteria are grown in virulent Bvg^+ phase (Nakamura et al., 2006; Neo et al., 2010), implying that the capsule operon is expressed during the virulent phase and may play a role in pertussis pathogenesis.

The role of *B. pertussis* polysaccharide capsule was investigated by constructing an unmarked in-frame deletion of *kpsT*, *kpsE* and *vipC* ORFs within the capsule operon of *B. pertussis* Tohama I derivative strain, BPSM. The predicted amino acid sequence of *B. pertussis* KpsT protein exhibits significant degree of homology with several other proteins responsible for active transport of capsular polysialic acid polymers, including KpsT from *E. coli* (40% identity) and HexA from *Pasteurella multocida* (44% identity),

supporting that *B. pertussis* KpsT performs a similar function (Parkhill et al., 2003). *B. pertussis* KpsE has been proposed to function as the capsular polysaccharide exporter across the periplasmic space, a function similar to that of KpsE from *E. coli* (27% identity) and CtrB of *N. meningitides* (30% identity). Using HMMTOP 2.0 trans-membrane topology prediction software (Tusnady and Simon, 2001), KpsT was found to adopt six transmembrane spanning domains whereas KpsE has two trans-membrane domains. The *B. pertussis* VipC protein instead does not harbor any trans-membrane domain and is homologous to the Vi polysaccharide biosynthesis protein TviD of *S. typhi* (23% identity), thus suggesting a role of VipC in the polysaccharide biosynthesis.

Immuno-detection method using anti-Vi antigen antibodies as previously described (Neo et al., 2010), suggested that deletion of either ORF namely *kpsT*, *kpsE* and *vipC* is sufficient to prevent the detection of a capsular structure at the bacterial surface, a similar phenotype observed in Δ*kpsT* or Δ*kpsE* of *E. coli* mutant strains (Bronner et al., 1993b; Pavelka et al., 1994). It is conceivable that the polysaccharide capsule transport-export process might be defective in *B. pertussis kpsT* and *kpsE* deleted mutants, thus resulting in the accumulation of polysaccharide polymers within the cytoplasm. It has been reported that deletion of *kpsT* in *E. coli* lead to accumulation of polysaccharide polymers at the inner cell periphery due to defect in the transport process (Bliss et al., 1996; Pavelka et al., 1994). Similarly, intracellular polysaccharide accumulation may also occur in the *B. pertussis* Δ*kpsT* and Δ*kpsE* strains, which may affect the cell viability and overall

fitness. However, no *in vitro* growth defect was noticed for these mutants. In addition, the *B. pertussis* KO*caps* mutant deleted for the entire capsule locus and for which polysaccharide accumulation does not occur, displayed an attenuation profile *in vivo* comparable to that seen with the Δ*kpsT* mutant. These observations therefore do not support the hypothesis that intracellular accumulation of capsule polysaccharide polymers occurs in the Δ*kpsT* and Δ*kpsE* strains and is responsible for the *in vivo* attenuated phenotype observed with these mutants, which is further discussed in the next section 3.4.2.

3.4.2 Attenuation of *B. pertussis* Capsule Deficient Mutants

Mice lung colonization profile revealed that the membrane-associated KpsT and to a lesser extent KpsE are required for optimal and efficient *B. pertussis* colonization whereas VipC is dispensable. This is a key observation that indicates that rather than the surface-exposed capsule itself, the membrane-associated protein KpsT and to a lesser extent KpsE, is important for pertussis pathogenesis. In addition, Western blot and Real-time PCR approaches have shown that the production and/or secretion of key virulence factors such as BrkA, PT and FHA essential for bacterial colonization were slightly impaired in Δ*kpsT,* and to a lesser degree for BrkA and PT in Δ*kpsE*, whereas the Δ*vipC* mutant displayed parental levels of PT and FHA production. While the transcriptional activity of *ptx* and *brkA* was significantly reduced in the Δ*kpsT* mutant, transcription of *bvgA/S*, *bvgR* and *fhaB* was not affected. Importantly, complementation of Δ*kpsT* was not fully restored to a parental level expression at the transcript and protein level. The reason for the

partial restoration of these genes expression in Δ*kpsTcom* is unclear whereas complete restoration of the *in vivo* phenotype was observed. It is possible that expression of *kpsT* ORF from a multicopy replicative plasmid (pBBR1MCS) (Elzer et al., 1995) may alter the KpsM/ KpsT protein ratio to form an ABC transporter complex, which may in turn result in sub-optimal transcriptional and/or translational feedback onto these genes.

The differential down-regulation of these *bvg*-regulated genes in Δ*kpsT* likely reflects the different affinities of each promoter for phosphorylated BvgA (P-BvgA), the transcriptional regulator of the BvgA/S two component system (Zu et al., 1996). As such, a decrease in P-BvgA levels will first affect the bvg-regulated promoters with low affinities for P-BvgA. Consistently, the *ptx* promoter was shown to require higher concentrations of P-BvgA than the *fhaB* and *bvgA* promoters to activate their transcription (Steffen et al., 1996). The affinity of the *brkA* promoter for BvgA-P has not been studied in details, but it appears from our study that it might be rather low as evidenced by the 10-fold reduction of *brkA* transcriptional activity in the Δ*kpsT* mutant.

Nevertheless, the mild attenuation in the production of FHA, BrkA and PT is unlikely to account for the *in vivo* attenuation observed. It is instead more plausible that a general effect may occur in the Δ*kpsT* mutant leading to the significant attenuated *in vivo* phenotype. Indeed, genome wide microarray analysis conducted on Δ*kpsT* mutant further revealed significantly reduced transcriptional activity of a number of *bvg*-regulated genes, in particular those

that encode virulence factors associated with bacterial colonization. In addition to *fhaB, brkA* and *ptx,* the *tcfA* gene coding for the tracheal colonization precursor factor A shown to play a role in bacterial adherence to the mouse trachea (Finn and Stevens, 1995), was found down-regulated. *Vag8* encoding the autotransporter Vag8 involved in *in vivo* colonization (Elder and Harvill, 2004; Finn and Amsbaugh, 1998) was also down-regulated in the Δ*kpsT* mutant. Interestingly, also down-regulated was *sphB1* encoding the autotransporter subtilisin-like serine protease SphB responsible for the proteolytic cleavage of FHA at the bacterial surface (Coutte et al., 2003; Coutte et al., 2001). SphB down-regulation likely explains the lower amounts of FHA detected in the culture supernatant but not in the bacterial lysates of Δ*kpsT*, supporting that FHA secretion, but not production is impaired in this mutant. Altogether, the microarray data thus support that the down-regulation in Δ*kpsT* of a number of virulence genes involved in the colonization efficacy is likely responsible for the attenuated phenotype observed in mice. Moreover, previous work has shown that, whereas the absence of a single virulence factor in *B. pertussis* resulted only in mild or no attenuation, multiple deletions in genes encoding adhesins and toxins significantly impaired the ability to colonize the mice lungs, supporting some degree of functional redundancy among the different virulence factors (Alonso et al., 2001; Carbonetti et al., 2005).

In addition to the known *vags* that have been well studied, other *bvg*-regulated genes that might be involved in pathogenesis of *B. pertussis* were also found negatively regulated in the Δ*kpsT* mutant. Previous studies have

suggested that *bfrD* encoding the TonB-dependent receptor for iron transport and *hemC* encoding the putative iron scavenger, both regulated by the BvgA/S system, were up-regulated in wild-type *B. pertussis* under iron starvation condition (Brickman et al., 2011). Down-modulation of these iron-scavenging genes in Δ*kpsT* may suggest that this mutant is impaired in its iron acquisition ability, which may likely affect its survival within the host. The T3SS locus consisting of *bsp22*, *bopA*, *bscD* and *bcrD* genes was also found down-regulated in Δ*kpsT*. This locus has been reported in several *B. pertussis* clinical isolates and *B. bronchiseptica* to be necessary for optimal bacterial colonization and persistence, and to be involved in host immunomodulation (Fennelly et al., 2008; Skinner et al., 2005). However, although transcriptionally active under the control of *bvg* regulation, the T3SS locus in the laboratory-adapted *B. pertussis* Tohama I strain was not translated, as opposed to that of *B. pertussis* clinical isolates, with reports suggesting that long-term laboratory passage, minimal contact with host cells and large-scale genomic re-arrangements have led to post-transcriptional silencing of the T3SS locus (Fennelly et al., 2008; Mattoo et al., 2004). Therefore, the attenuated colonization profile displayed by the BPSM Tohama I derivative Δ*kpsT* mutant cannot be attributed to the down-modulation of the T3SS locus.

3.4.3 Molecular Cross-talk Between the *B. pertussis* Capsule Locus and *bvg*-Mediated Signal Transduction

The predominant molecular events that were observed in Δ*kpsT* mutant suggest that *bvg*-mediated mechanism(s) may be compromised in this mutant.

Therefore, this prompted us to elucidate whether the *bvg*-mediated mechanism is dependent or independent of KpsT. Deletion of *kpsT* in a Bvg^{+} phase-locked background did not lead to reduced *vag* expression and impairment of *in vivo* virulence as observed with the parental Δ*kpsT* mutant, thus suggesting a regulatory link between KpsT and the BvgA/S-mediated signal transduction pathway. Moreover, failure to restore parental colonization of the KO*caps* mutant by expressing KpsT alone or the KpsMT complex suggested that other protein members from the capsule operon are necessary.

The model of a two-component system comprises of a sensor kinase and response regulator, which are often thought to organize in a linear mode of action, from the perception of stimulus to downstream phosphorelay activation and transcriptional responses. Many other studies, however, have shown that the complexity of a bacterial two-component system was generally overlooked. Direct and/or indirect cross-talk between two-component systems and its non-cognate partners have been widely reported as a new paradigm in bacterial signal transduction (Casino et al., 2010; Eguchi and Utsumi, 2005; Fink et al., 2012; Jung et al., 2012; Krell et al., 2010; Mitrophanov and Groisman, 2008). They affect the downstream phosphorylation activity of the senor and response regulators, thus modulating the overall output of the two-component system (Mitrophanov and Groisman, 2008). Furthermore, and more relevant to our own observations, reports of inner membrane proteins interacting physically and influencing a two-component sensor kinase activity have been recently described in different pathogens (Eguchi et al., 2007; Jung et al., 2012; Lippa and Goulian, 2009).

We initially predicted that within the context of cell envelope, the polysaccharide capsule transporter-exporter complexes in *B. pertussis* might have a functional or interactional effect on the trans-membrane BvgS sensor. In this study, we purified BvgS directly from *B. pertussis* strains grown in Bvg^+ phase. Prior to purification, a series of conditions were optimized to ensure that BvgS is fully solubilized and released from *B. pertussis* cellular membranes. We noted that full length BvgS was insoluble under native lysis buffer even with strong mechanical disruption, unlike the truncated BvgS domain previously expressed in *E. coli* (Beier et al., 1995; Dupre et al., 2013). Denaturation of BvgS by guanidine hydrochloride ultimately solubilized His-BvgS proteins and could be purified via Ni-NTA column. Under such harsh denaturing conditions, BvgS is expected to be in unfolded state due to the breakage of hydrogen bonds and hydrophobic interactions upon guanidine hydrochloride treatment. Nevertheless, we still observed by SDS-PAGE the presence of BvgS-associated oligomers, which dissociated into BvgS monomers upon heat treatment or upon addition of a reducing agent. While it is surprising that such high molecular weight structure is resistant to strong denaturant such as guanidine hydrochloride, it has been reported that large hydrophobic proteins, in particular membrane proteins or proteins that contain proline rich homeodomain are resistant to denaturation by urea or guanidine hydrochloride (Gokhale et al., 1996; Makino et al., 1981; Shukla et al., 2012). BvgS sensor contains two separate alanine-proline rich regions within the cytoplasmic histidine kinase and receiver domains (Miller et al., 1992; Uhl and Miller, 1996), and it has been reported in eukaryotic system that proline

rich regions mediate protein dimerization and oligomerization (Shukla et al., 2012; Soufi et al., 2006). Whether these features are responsible for maintaining the conformational tension between BvgS oligomers in *B. pertussis* remain to be investigated. In the context of chemical bonds within BvgS oligomers interface, it is also plausible that strong covalent bonds and disulphide bonds exist within the macromolecular structure associated with BvgS oligomers.

Our data demonstrate that the entire polysaccharide capsule translocon machinery and KpsT is essential for the oligomerization, presumably homodimerization, of the BvgS sensor. Biochemical and structural evidences have indeed confirmed that BvgS homodimerizes at two domains within the C-terminal cytoplasmic region, namely the transmitter and receiver-output domains (Beier et al., 1995). Moreover, active phosphotransfer could be reconstituted in *trans* between BvgS domains, thus further supporting the dimerization capacity of BvgS *in vivo* (Beier et al., 1995; Perraud et al., 2000). Dimerization and higher order oligomerization of signalling complexes in general and BvgA/S in particular are believed to be important for intrinsic phospho-transfer activity and activation of downstream regulator proteins (Maeda et al., 2006; Scheu et al., 2010). Our work is the first experimental demonstration of the existence of BvgS oligomers in the *B. pertussis* bacteria since all the previous studies were performed in *E. coli* with truncated BvgS proteins (Beier et al., 1995; Perraud et al., 2000).

It is conceivable here that the absence of KpsT and the polysaccharide capsule transport-export machinery may alter the inner membrane environment and integrity such that it affects its ability to sense and integrate extracellular signals, thereby altering the dimerization of BvgS and/or signal transduction to downstream regulator protein BvgA, which in turn fine tune transcriptional regulation of genes involved in pathogenesis. Fine tuning of virulence gene expression during *B. pertussis in vivo* infection is crucial for bacterial virulence during different stages of infection within the host environment (Beier and Gross, 2006; Strauss, 1995; Veal-Carr and Stibitz, 2005). From a different perspective, some unknown post-translational modification(s) that are involved in the synthesis and dissociation of chemical bonds such as covalent bonds, peptide bonds and disulphide bridges, may eventually lead to the variation in BvgS oligomerization observed between the wild-type bacteria and Δ*kpsT* mutant.

The precise mechanisms underlying the role of KpsT in BvgS oligomerization are intriguing and we reasoned that it might have an indirect effect via its role on the overall membrane integrity. Our further observations on the sensitivity of Δ*kpsT* towards erythromycin, SDS and EDTA, compared to the parental BPSM strain, seem to land support to the above hypothesis. However, the absence of the surface polysaccharide capsule in Δ*kpsT* mutant may also be responsible for these latest phenotypes observed. To distinguish between these two possibilities, the Δ*vipC* mutant should be included in in this assay. Indeed, Δ*vipC* mutant was shown to be capsule-free but did not display a drastic *in vivo* phenotype in the mouse model of pertussis. Should Δ*vipC*

display sensitivity to erythromycin, SDS and EDTA that is similar to that observed with Δ*kpsT,* it will indicate that the PS capsule at the bacterial surface is primarily responsible for these phenotypes. Instead, should Δ*vipC* display a parental phenotype, it will support the idea that KpsT is specifically involved in the bacterial resistance to these treatments and may therefore contribute to the plasma membrane integrity. Absence of KpsT, the ATPase cognate partner of the integral membrane KpsM, may indeed eventually lead to a complete disorganization of the entire capsule transport machinery within the cell envelope which may affect the overall membrane integrity and permeability. This is further supported by the observation that *in vivo* attenuation was seen with a Δ*kpsE* mutant, and that re-introduction of KpsT or KpsMT into the KO*caps* mutant was not sufficient to restore a parental phenotype. Collectively, these findings support that absence of KpsT in the Δ*kpsT* mutant may actually affect the trans-envelope complex formed by the PS capsule transport proteins. Further structural analysis would be necessary to strengthen this hypothesis.

3.4.4 Role of The Capsule Locus, a *bvg*-Repressed Factor in Pertussis Pathogenesis

The pathogenesis of *B. pertussis* has been extensively studied and reviewed for the past 25 years, including the characterization of numerous bacterial factors responsible for virulence. As an obligate human pathogen, *B. pertussis* has evolved favorably by expressing a wide variety of virulence factors that promote its life cycle, transmission, colonization and defense

against host immune responses in the upper respiratory tract. It has been well established that the Bvg^+ phase-locked *B. pertussis* and *B. bronchiseptica*, a close relative of *B. pertusiss*, were able to colonize the respiratory tract as efficiently as the wild type strains, indicating that the *bvg*-activated genes, namely the *vags* are sufficient for *B. pertussis* virulence (Cotter and Miller, 1994; Martinez de Tejada et al., 1998). In contrast, the avirulent *Bordetella* variants such as the Bvg^- phase-locked mutant, characterized by high expression levels of *vrgs* and low expression levels of *vags*, displayed an attenuated phenotype *in vivo* (Cotter and Miller, 1994; Martinez de Tejada et al., 1998). However, it is necessary to note that transcription and expression levels of the capsule locus, albeit reduced, are not totally inhibited in virulent bacteria.

Although *vrg*6 was first reported to play a role in *B. pertussis* virulence (Beattie et al., 1992), later reports disputed that the attenuated phenotype observed in mice was actually due to a secondary mutation (Martinez de Tejada et al., 1998). Thus, there had been no firm evidence of a possible role of a *vrg* during pertussis pathogenesis. Our work here demonstrates for the first time and unambiguously that a *vrg* locus (the capsule locus) plays a critical role in pertussis pathogenesis. Rather than playing a direct and conventional role in pertussis pathogenesis such as the adhesins and toxins, the determinants involved in polysaccharide capsule transport and export affects *Bordetella* virulence in an indirect manner, via the BvgA/S two-component regulatory system.

3.5 CONCLUSIONS AND FUTURE DIRECTIONS

Our work here provides the evidence that the *B. pertussis* capsule locus is instrumental for optimal expression of the pathogen's virulence factors and represents the first report of a *vrg* locus that is clearly involved in pertussis pathogenesis. Rather than the surface polysaccharide capsule, our findings led to a novel concept that KpsT and the capsular transporter-exporter system in *B. pertussis* are necessary for bacterial virulence. Specifically, the products of the capsule locus, in particularly KpsT, play a role in the regulation of Bvg-mediated genes in *B. pertussis*. Mechanistically, KpsT and the capsular transporter-exporter system influence the dimeric and oligomeric state of BvgS necessary for optimal signal sensing and/or transduction. Our findings led us to contemplate that KpsT and the capsular transporter-exporter complexes may be responsible for maintaining the plasma membrane integrity and permeability, which are crucial for the conformational integrity and optimal functionality of membrane proteins such as BvgS sensor.

Therefore, future studies could progress towards elucidating the BvgA/S signaling output in Δ*kpsT* and KO*caps* mutant. Perhaps the most pertinent strategy is to analyze the phosphorylation state of the response regulator BvgA in these mutants compared to wild-type *B. pertussis*. We hypothesized that the pool of phosphorylated BvgA may be compromised in Δ*kpsT* and KO*caps* mutant, which correlates with the changes in the *bvg*-regulated gene expression. Our preliminary analysis on the pool of P-BvgA dimer in *B. pertussis* cell lysate by SDS-PAGE and Western blot was

unsuccessful due to poor specificity of the polyclonal anti-BvgA antibodies. However, a group recently reported that by employing the latest Phos-tag™ technology, they were able to distinguish the pool of BvgA monomer from its phosphorylated counterpart (P-BvgA) in *B. pertussis* total cell lysates by Western blot analysis (Boulanger et al., 2013). Hence, such alternative method of detection/quantification of P-BvgA proteins in *B. pertussis* would provide further support, that the sensing and/or transduction of extracellular signals are impaired in Δ*kpsT* and KO*caps* mutant.

Moreover, additional controls should be included in several of the above-mentioned experiments to refine the overall relationship between BvgS and the products of the capsule locus. Given the lack of complete understanding on the bonds that maintain BvgS homodimers, the oligomerization capacity of BvgS observed *in situ* in *B. pertussis* could be further confirmed by targeted site-directed mutagenesis on BvgS cysteine residues or the alanine-proline residues whose substitutions may abolish BvgS dimerization in BPSH strain. Such experimental concepts will provide further support on the existence of chemical bonds that are responsible for the oligomerization of BvgS and its possible interaction with capsular transporter-exporter proteins.

It is also worthwhile to construct the recombinant His-BvgS tag fusion in the BvgS-VFT2 background strain to leverage on the observation of BvgS oligomerization in BPSM and its capsule-deleted counterpart. As the BvgS-VFT2 mutant contains constitutive active BvgS sensor and hence constitutive

dimerization, it is nevertheless a necessary control to address the structural interactions associated with BvgS and to support our hypothesis that BvgS oligomerizes in *B. pertussis.* Finally, inclusion of Δ*vipC* mutant in the erythromycin, SDS and EDTA sensitivity assays is necessary to support the claim that the cellular membrane integrity affects the conformational changes of BvgS sensor.

CHAPTER 4 GENETIC REGULATION OF THE CAPSULE OPERON IN *B. PERTUSSIS*

The genetic organization of the putative type II polysaccharide capsule operon in *B. pertussis* Tohama I (ATCC BAA-589) strain has been fully sequenced by the Sanger Institute, UK (Parkhill et al., 2003). In Chapter 3, we have shown that the products of the capsule locus, in particularly KpsT, influence the oligomeric state of BvgS necessary for optimal signal sensing and/or transduction. However, the genetic regulation of the capsule locus has remained largely unexplored. Thus far, the capsule locus in *B. pertussis* is only known as a *bvg*-repressed gene or *vrg*, i.e its expression is optimal in Bvg^- phase. We have also previously reported that the capsule locus is expressed at a basal detectable level in Bvg^+ phase (Neo et al., 2010). In this chapter, we provide some additional information on the regulation of the expression of the *B. pertussis* capsule locus *in vitro*, *ex vivo* and *in vivo*.

(A) ANALYSIS OF THE TRANSCRIPTIONAL REGULATION OF THE CAPSULE LOCUS IN *IN VITRO B. PERTUSSIS* CULTURE

4.1 RESULTS

4.1.1 Transcriptional Analysis of The Capsule Locus in *B. pertussis* Clinical Isolates

To gain better insight into the regulation of the capsule expression in *B. pertussis*, we systematically analyzed the transcriptional pattern of the capsule locus (represented by *kpsT*) and other transcripts including *vrg6*, *risA* and *bvgR* in several *B. pertussis* clinical isolates grown in *in vitro* culture of Bvg^+ and Bvg^- phase. For comparison, the laboratory-adapted strain BPSM was also included in the study. As previously reported, expression of the capsule locus in BPSM, represented by *kpsT* gene, and another *bvg*-repressed gene *vrg6* was significantly elevated in the presence of modulators $MgSO_4$ which switch the bacteria to Bvg^- phase, while basal expression was observed in the absence of $MgSO_4$ (Bvg^+ phase) (Figure 4.1, black and green bars) (Croinin et al., 2005; Neo et al., 2010; Stenson et al., 2005). Unlike *bvgR* whose transcripts were strongly down-regulated in Bvg^- phase, expression of *risA* was not significantly modulated between the two Bvg phases (Figure 4.1), confirming that transcription of *risA* is independent of BvgA/S modulation (Stenson and Peppler, 1995).

Two different clinical strains isolated from pre-vaccination era were assessed for the levels of expression of their capsule locus; Tohama I, a Japan isolate from year 1954 and strain 18323, a USA isolate from year 1946. Tohama-I displayed a similar trend of expression for *risA*, *vrg6* and greater expression of the capsule locus in both phases when compared to BPSM (Figure 4.1, grey and red bars). However, the levels of *bvgR* in Bvg^- phase Tohama-I is not completely repressed unlike in BPSM strain, suggesting a BvgA/S-independent basal level of expression of *bvgR* in Bvg^- phase in Tohama I. Alternatively, a differential sensitivity of the BvgS sensor to

$MgSO_4$ modulator between Tohama I and BPSM may exist. However, despite the presence of some *bvgR* expression in Bvg^- Tohama I, significant up-regulation of *vrgs* (*vrg6* and *kpsT*) can be observed and to the same extent as what is observed with BPSM. This observation suggests the existence of a potential transcriptional activator of *vrg6* and *kpsT* that outcompetes BvgR.

Strikingly, expression of *risA* in strain 18323 was strongly down-regulated in both Bvg^+ and Bvg^- phase compared to BPSM and Tohama I strains (Figure 4.1, stripped grey and stripped red bars). Consistently, the *vrg6* and *kpsT* transcripts were significantly reduced in strain 18323, particularly in the Bvg^- phase compared to BPSM (Figure 4.1). In fact, the *vrg6* and capsule locus seem to be constitutively expressed in strain 18323 regardless of the presence of modulators (Figure 4.1). However, *bvgR* was modestly enhanced to 3-fold in Bvg^+ phase, and completely repressed in Bvg^- phase in strain 18323 compared to BPSM (Figure 4.1).

The results here suggest that the reduced levels of *risA* may account for the reduced *vrgs* expression (*kpsT* and *vrg6*) in strain 18323 in both Bvg^+ and Bvg^- phase, as compared to BPSM and Tohama I strains. In addition, the variability of *vrgs* expression, in particular the capsule locus, observed between laboratory-adapted and clinical isolates highlight the possibility of selective genetic regulation between distinct *B. pertussis* strains.

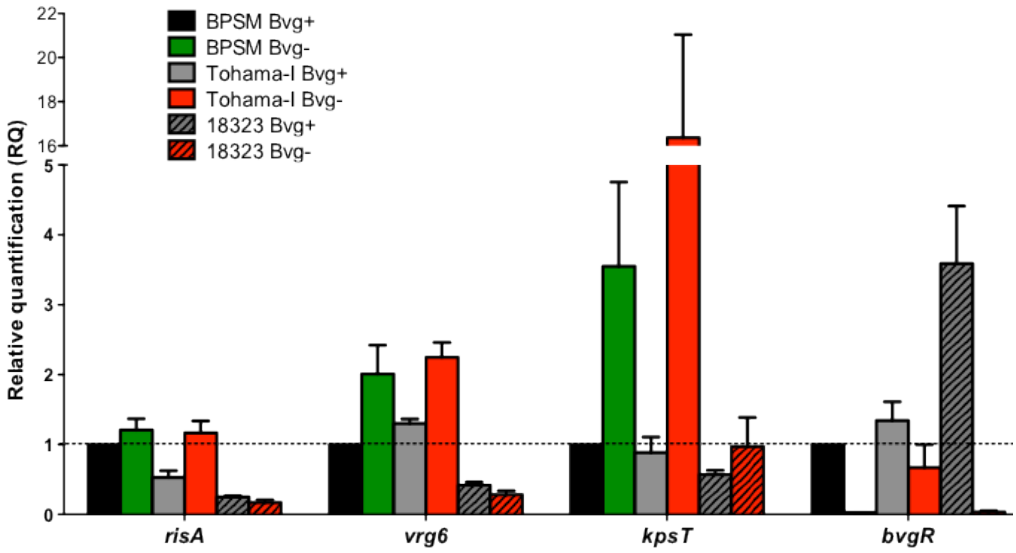


Figure 4.1: Relative transcriptional activity of *risA*, *vrg6*, *bvgR* and the capsule locus in BPSM, Tohama I and 18323 strain in virulent and avirulent phase.

Total RNA was extracted from BPSM, Tohama-I and 18323 strains grown in virulent (Bvg^+) and avirulent (Bvg^-, in the presence of 50mM $MgSO_4$) phase, as indicated on the figure legend on the top left of the graph. Real-time PCR analysis was performed using primers mapping in the *risA*, *vrg6*, *kpsT* and *bvgR* genes. *recA* gene was used as the endogenous control. Results are expressed as the average relative quantification RQ ± SD of triplicate versus Bvg^+ phase BPSM. Results are representative of 2 independent experiments. Dotted line represents RQ equal to 1 in relative to Bvg^+ phase BPSM.

4.1.2 Transcriptional Analysis of The Capsule Locus in Δ*bvgAS* Mutant

It was previously shown that deletion of *bvgR* in strain 18323 led to a constitutive expression of *bvg*-repressed gene *vrg6* and surface proteins VraA/B in both Bvg^+ and Bvg^- phase (Croinin et al., 2005; Merkel et al., 1998), demonstrating the role of BvgR as a repressor for *vrgs* expression in *B. pertussis*. We initially took a similar approach to study the expression level of the capsule locus. However, after several attempts we were unable to obtain a *bvgR*-deleted mutant in BPSM strain, suggesting that in this strain, *bvgR* is an essential gene. Assuming that in a Δ*bvgAS* mutant expression of *bvgR* will be constitutively repressed, we thus performed a transcriptional profile analysis on the capsule locus and a few selected genes including *risA*, *vrg6* and *bvgR* in a Δ*bvgAS* BPSM derivative mutant grown in both Bvg^+ and Bvg^- phase. The parental strain BPSM grown in Bvg^+ phase was used as the calibrator for gene expression analysis.

We first monitored the expression of *risA* in wild-type BPSM and Δ*bvgAS* mutant. Expression of *risA* was not significantly modulated in Δ*bvgAS* and in the presence of modulators, supporting that regulation of *risA* is BvgA/S-independent (Figure 4.2 A) (Jungnitz et al., 1998; Stenson et al., 2005). As expected, BPSM strongly repressed the expression of *bvgR* in Bvg^- phase, but the magnitude of repression was lesser than in the Δ*bvgAS* mutant (Figure 4.2 B). This is a surprising observation, as deletion of the *bvgAS* locus is known to lock the bacteria into a Bvg^- phase phenotype and that expression of *bvgR*, a *bvg*-regulated factor, should be completely repressed (Martinez de

Tejada et al., 1998). The basal level of *bvgR* expression in Δ*bvgAS* mutant was consistently observed in a few independent experiments, suggesting that a basal BvgA/S-independent expression of *bvgR* exists in BPSM. As expected expression of *vrg6* and the capsule locus were up-regulated in Bvg^- phase BPSM (Figure 4.2 C, D). While the expression level of *vrg6* was similar in both Bvg^+ and Bvg^- phase in Δ*bvgAS* (Figure 4.2 C), expression of the capsule locus remained distinctly modulated, where the expression of the capsule locus is significantly higher in Bvg^- phase compare to Bvg^+ phase Δ*bvgAS* (Figure 4.2 D).

The results of this analysis indicate that the lower level of *bvgR* expression in Δ*bvgAS* mutant (Figure 4.2 B) leads to up-regulation of the capsule locus in Bvg^+ phase (Figure 4.2 D, BPSM vs Δ*bvgAS*), but not to the level observed in Bvg^- phase (Figure 4.2 D, Δ*bvgAS* Bvg^+ vs Bvg^- phase). This implies the existence of a transcriptional activator present in the Bvg^- phase that up-regulates the expression of the capsule locus. Additionally, another factor repressed in the Δ*bvgAS* mutant would account for the repression of the capsule locus in Bvg^+ phase BPSM. It has been formally demonstrated that RisA binds to the promoter of *vrg6* and activates its transcription in BP536 *B. pertussis* strain (Croinin et al., 2005). Similarly, we postulate that RisA might act on the capsule locus promoter. Whether RisA directly modulates the expression of the capsule locus in BPSM remains to be investigated.

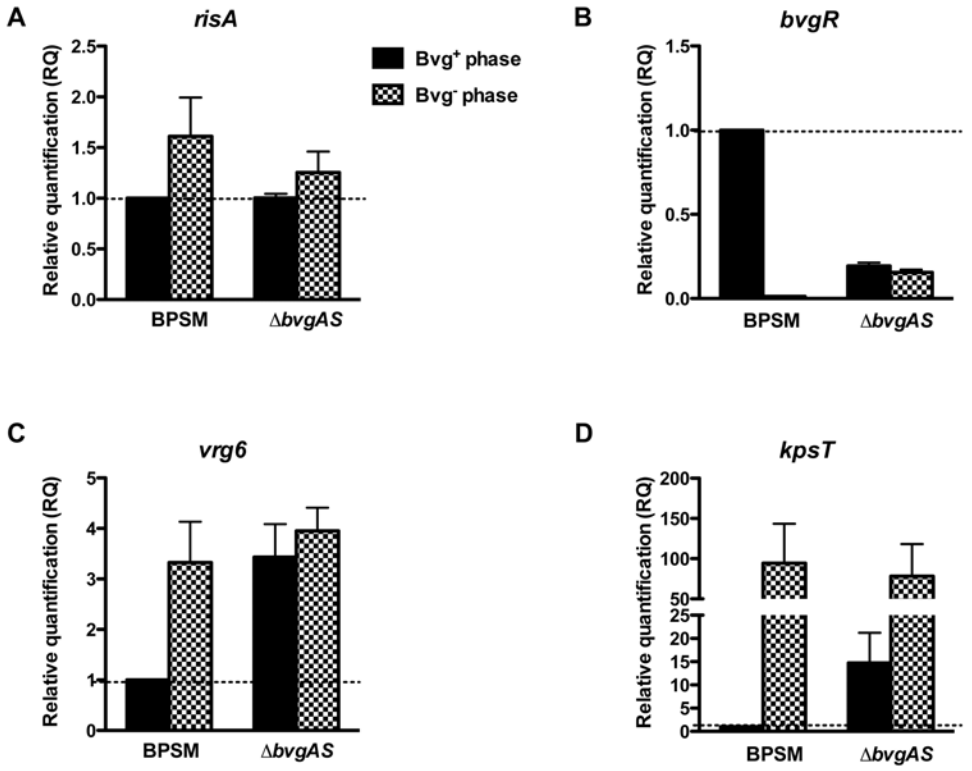


Figure 4.2: Relative transcriptional activity of *risA*, *bvgR*, *vrg6* and the capsule locus in BPSM and Δ*bvgAS* strain in virulent and avirulent phase.

Total RNA was extracted from BPSM (black bars) and Δ*bvgAS* (dotted bars) strains grown in virulent Bvg^+ phase and avirulent Bvg^- phase. Real-time PCR analysis was performed using primers mapping in the *risA*, *vrg6*, *kpsT* and *bvgR* genes. *recA* gene was used as the endogenous control. Results are expressed as the average relative quantification RQ ± SD of triplicate versus Bvg^+ phase BPSM. Results are representative of 2 independent experiments. Dotted line represents RQ equal to 1 in relative to Bvg^+ phase BPSM.

4.1.3 Transcriptional Analysis of The Capsule Locus by The *Ris*-Regulon

4.1.3.1 Construction of a *ris*-deleted mutant in BPSM background strain

Loss of a functional RisA in *B. pertussis* would be expected to enhance the expression of *vrgs*, in particular the capsule locus expression as previously observed for *B. pertussis* BP563 strain (Croinin et al., 2005; Stenson et al., 2005). To investigate the modulation of capsule locus expression by the *risA* locus in the laboratory adapted BPSM strain, we proposed to construct a *risA*-deleted BPSM mutant strain. However, despite several attempts, deletion of *risA* by double homologous recombination was unsuccessful; whereby all the colonies screened reverted to parental genotype suggesting that *risA* may be an essential gene for BPSM strain. Polar effects are unlikely as construction of *risA* deletion was in-frame and the downstream gene *BP3555* is transcribed in the opposite direction (Figure 4.3 A).

In contrast, previous studies reported the construction of a *risA*-deleted mutant in the *B. pertussis* BP536 strain (Croinin et al., 2005; Stenson et al., 2005). The expression of *vrg6, vrg18, vrg24* and *vrg73* was reduced to basal level in Bvg$^-$ phase Δ*risA*-BP536 mutant, but no complementation study was performed to confirm the phenotype described in the Δ*risA*-BP536 mutant (Croinin et al., 2005). In a separate study, Stenson et al. found that expression of Vra surface proteins and transcription of *vrgs* in Δ*risA*-BP536 mutant was reduced to basal level and that expression of these *vrgs* was restored by *risA*

complementation on a low-copy-number plasmid, thus supporting the phenotype observed (Stenson et al., 2005). However, the authors offered no explanation on the impairment of *in vitro* growth profiles and fitness of the Δ*risA*-BP536 mutant. It is possible that the laboratory adapted BPSM strain may have undergone a different selective pressure in terms of genetic regulation and fitness compared to BP536 strain (Gaillard et al., 2011; Hot et al., 2003; Park et al., 2012), which may result in different physiology and fitness status when *risA* is deleted. As a transcriptional factor, RisA may regulate a variety of essential factors in BPSM, thus the absence of RisA and/or the Ris-regulated factors may contribute to an overall pleiotropic effect, as previously described for *B. bronchiseptica* (Jungnitz et al., 1998).

Essentiality of a bacterial gene can be demonstrated by successful deletion of the target gene only in the presence of an extra copy of that gene (Parish and Stoker, 2000; Reyrat et al., 1998). Therefore, to confirm the essentiality of *risA*, we attempted to delete the chromosomal *risAS* locus in BPSM in the presence of a wild-type copy of *risA*, which was expressed under the control of *fha* promoter in the pBBR1MCS replicative plasmid. Because RisS is truncated in *B. pertussis* and has been shown to be dispensable for the expression of *vrgs* (Stenson et al., 2005), the entire *risAS* chromosomal locus was deleted in this study. We attempted to construct *risAS*-deletion in BPSM-P*fha-risA* strain via double homologous recombination. Positive Δ*risAS* clones were obtained in the presence of a wild-type copy of *risA* expressed on a plasmid (Figure 4.3 B), thus demonstrating the essentiality of this gene in BPSM strain.

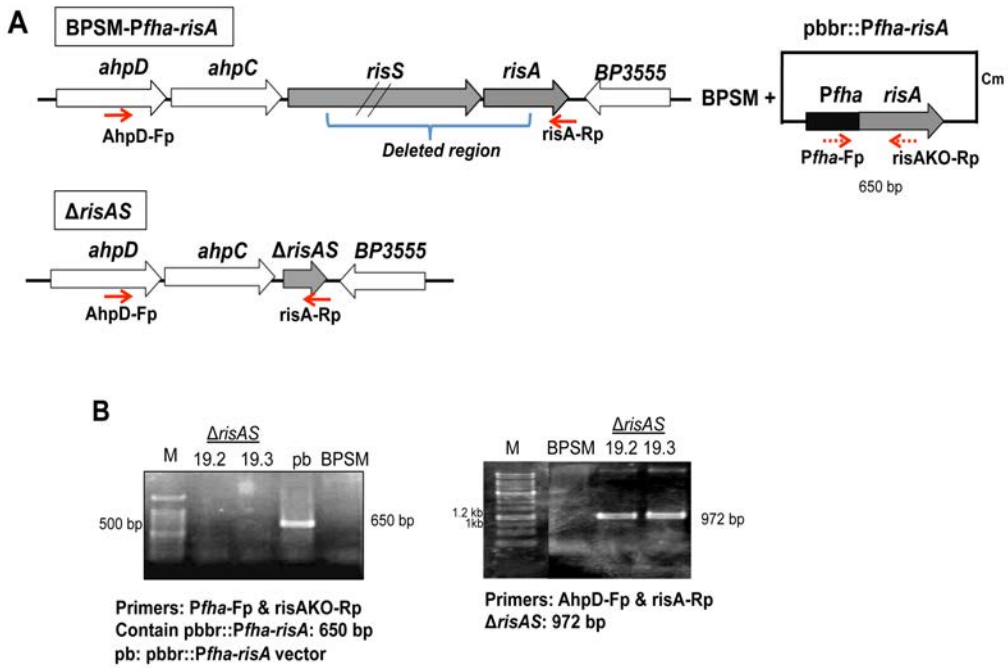


Figure 4.3: Construction of *ris*-deleted mutants in BPSM background strain.

(A) Genomic organization of the *risA* and *risS* locus in *B. pertussis* BPSM-P*fha-risA* and Δ*risAS* strain, pBBR::P*fha-risA* vector over-expressing *risA* is shown on the right. Red arrows below each locus/gene indicates the primer used to screen for PCR screening.
(B) PCR screening of Sm resistant Δ*risAS* chromosomal DNA with primer pairs and expected size as indicated on the bottom of each gel picture. The Δ*risAS* has been propagated in the absence of Cm antibiotics, thus allowing the mutant to loose the pbbR::P*fha-risA* plasmid and retain the mutant genotype. Lanes; M, DNA ladder (5 µl); 19.2 and 19.3, Δ*risAS* clones; pb, pBBR::P*fha-risA* vactor (10 µl); BPSM chromosomal DNA (10 µl).

4.1.3.2 Transcriptional analysis of the capsule locus in BPSM and Δ*bvgAS* strains over-expressing *risA*

Since RisA appears to be essential for BPSM survival, an alternative approach to study the role of RisA in capsule regulation is to over-express RisA in wild-type BPSM strain. We thus constructed a BPSM strain over-expressing *risA* under the control of the strong *fhaB* promoter, giving rise to BPSM-P*fha-risA* strain. The capsule locus expression as well as expression of other genes (*risA*, *vrg6*, *kpsT* and *bvgR*) in both parental BPSM and BPSM-P*fha-risA* were monitored in Bvg^+ phase by Real-time PCR. As shown in Figure 4.4, over-expression of *risA* in Bvg^+ phase BPSM-P*fha-risA* led to a 5-fold increase in *risA* transcripts compared to BPSM. However, the increased *risA* expression did not significantly enhance expression of neither the *vrg6* locus nor the capsule locus (Figure 4.4). Expression of all the genes analyzed were not affected in BPSM carrying empty vector control; BPSM-pbbr1mcs (Figure 4.4). From this data, we reasoned that BvgR repressor, which is expressed in BPSM-P*fha-risA* strain, acts as a strong negative regulator and competes with RisA in modulating *vrg6* and capsule locus expression in Bvg^+ phase.

Given the low expression level of *bvgR* in Δ*bvgAS* mutant (Figure 4.2 B), we thus decided to over-express *risA* in Δ*bvgAS* using the constitutive *recA* promoter, which is independent of the BvgA/S regulatory system, unlike *fhaB* promoter used in the previous construct (Hot et al., 2003). The resulting

strain, known as Δ*bvgAS*-P*recA*-*risA* was analyzed for its gene expression profile in Bvg^+ phase. Over-expression of *risA* under *recA* promoter in Δ*bvgAS* mutant led to a modest 2-fold increase in *risA* transcripts compared to Bvg^+ phase BPSM (Figure 4.5), reflecting that *recA* promoter appears weaker than *fhaB* promoter. Therefore, expectedly, neither the expression of *vrg6* nor the capsule locus was found up-regulated in Δ*bvgAS*-P*recA*-*risA* strain (Figure 4.5). The modest over-expression of *risA* also did not result in modulation of the expression of *bvgR*, which remained basal in the Δ*bvgAS*-P*recA*-*risA* strain in both Bvg^+ and Bvg^- phase (Figure 4.5). Despite the low levels of *bvgR* expression in Δ*bvgAS*-P*recA*-*risA* strain, we postulate that it is sufficient to negatively repress the capsule locus and *vrg6*, thus overcoming the over-expression of RisA in the overall modulation.

In conclusion, our *risA* over-expression approaches failed to provide any further information on the possible modulation of the expression of the capsule locus by RisA.

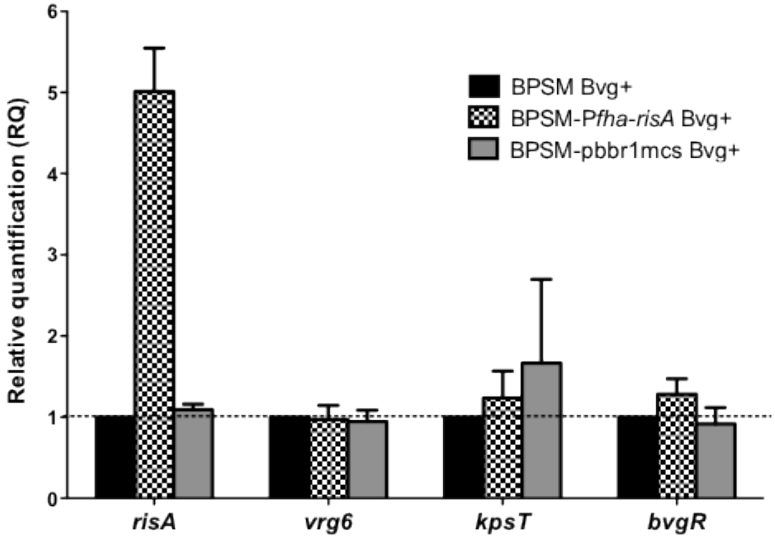


Figure 4.4: Relative transcriptional activity of *risA, vrg6, bvgR* and the capsule locus in BPSM and BPSM-P*fha-risA* strain in virulent phase.

Total RNA was extracted from BPSM (black bars), BPSM-P*fha-risA* (dotted bars) and BPSM-pbbr1mcs (grey bars) grown in virulent Bvg^+ phase. Real-time PCR analysis was performed using primers mapping in the *risA, vrg6, kpsT* and *bvgR* genes. *recA* gene was used as the endogenous control. Results are expressed as the average relative quantification RQ± SD of triplicate versus Bvg^+ phase BPSM. Results are representative of 2 independent experiments. Dotted line represents RQ equal to 1 in relative to Bvg^+ phase BPSM.

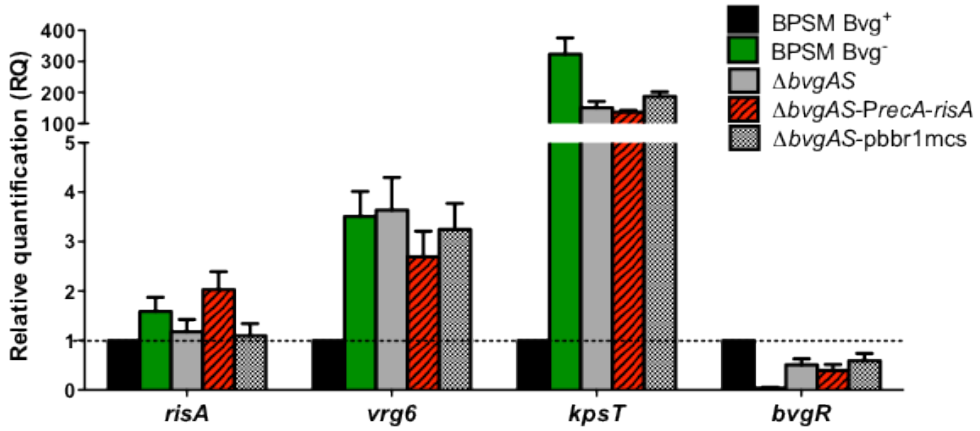


Figure 4.5: Relative transcriptional activity of *risA, vrg6, bvgR* and the capsule locus in BPSM, Δ*bvgAS*, Δ*bvgAS*-P*recA-risA* and Δ*bvgAS*-pbbr1mcs empty vector control strain in virulent and avirulent phase.

Total RNA was extracted from BPSM Bvg$^+$ phase (black bars), BPSM Bvg$^-$ phase (green barr), Δ*bvgAS* Bvg$^+$ phase (grey bars), Δ*bvgAS*-PrecA-*risA* Bvg$^+$ phase (red stripped bar) and Δ*bvgAS*-pbbr1mcs Bvg$^+$ phase (stripped bars). Real-time PCR analysis was performed using primers mapping in the *risA*, *vrg6*, *kpsT* and *bvgR* genes. *recA* gene was used as the endogenous control. Results are expressed as the average relative quantification RQ ± SD of triplicate versus Bvg$^+$ phase BPSM. Results are representative of 2 independent experiments. Dotted line represents RQ equal to 1 in relative to Bvg$^+$ phase BPSM.

(B) ANALYSIS OF THE TRANSCRIPTIONAL REGULATION OF THE CAPSULE LOCUS IN *B. PERTUSSIS* DURING *EX VIVO* AND *IN VIVO* INFECTION

In this section, we sought to further investigate whether the capsule locus is being expressed and modulated during the course of infection in human pulmonary epithelial A549 cells and in mouse macrophages J774.A1 as well as in the mouse respiratory tract.

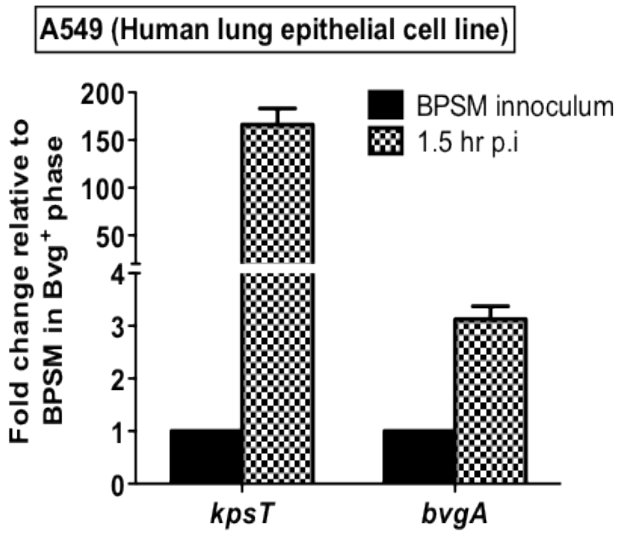
4.2 RESULTS

4.2.1 Transcriptional Analysis of The Capsule Locus in *B. pertussis* During Infection of Lung Epithelial Cells

The human lung epithelial cells represent the major cell type *B. pertussis* interacts with upon infection (Ishibashi et al., 1994; Wilson et al., 1991). Although *B. pertussis* has been recognized as extracellular pathogen that adheres to pulmonary and tracheal epithelial cells *in vitro* (Alonso et al., 2001; Coutte et al., 2003; van den Berg et al., 1999), studies have shown that it can also invade the A549 pulmonary epithelial cell line and survive intracellularly up to 4 h p.i. (Ishibashi *et al*., 2001). In this study, expression of the capsule locus in BPSM was quantified by real time PCR using primers mapping in the *kpsT* ORF upon invasion in A549 cells. For invasion assay, the co-incubation was performed for 1.5 h at 37°C. *In vitro* BPSM culture applied

for the cellular infection was used as comparison for the expression of the capsule locus.

As shown in Figure 4.6, expression of the capsule locus in BPSM was significantly up-regulated upon during invasion in A549 cells compared to the level of expression measured during *in vitro* Bvg^+ culture condition. Interestingly, expression of *bvgA,* an indicator for *bvg*-regulated response, showed a 3-fold increase upon uptake within A549 cells (Figure 4.6), suggesting that uptake of *B. pertussis* into mammalian epithelial cells induce a rapid and transient change in the bacteria gene expression.



Gene/ Average Ct values	Uninfected	No template control
kpsT	36.087	36.215
bvgA	39.789	38.612
recA (ENDO)	34.367	35.754

Figure 4.6: Relative transcriptional activity of *kpsT* and *bvgA* in BPSM recovered from A549 versus *in vitro* BPSM grown in virulent phase.

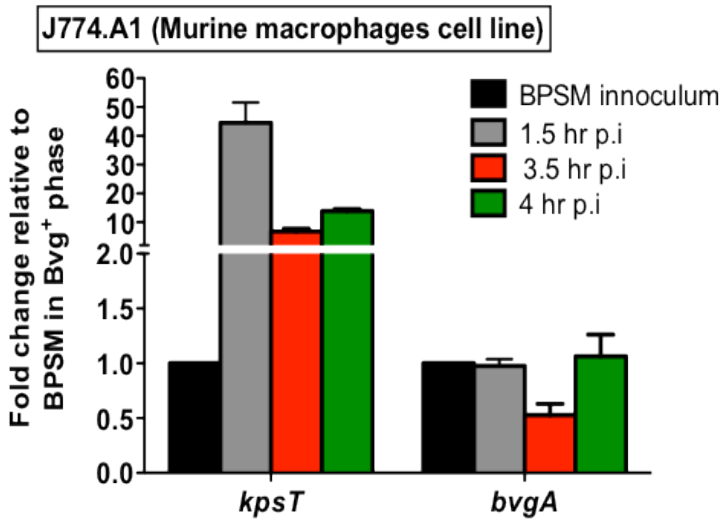
Invasion of A549 cells were done by incubating the cells and BPSM at MOI of 100 at 37ºC for 1.5 h. Unbound bacteria were washed away prior to total RNA extraction from BPSM recovered from infected A549 cells. Real-time PCR analysis was performed using primers mapping in the *kpsT* and *bvgA*. *recA* gene was used as the endogenous control. Results are expressed as the average RQ ± SD of triplicate versus BPSM innoculum. Results are representative of 2 independent experiments. Dotted line represents RQ equal to 1 in relative to BPSM innoculum. The bottom table shows average Ct values for each gene obtained from the mock-infected A549 (as negative control) and the no template control.

4.2.2 Transcriptional Analysis of the Capsule Locus in *B. pertussis* During Infection of Macrophages

As *B. pertussis* can invade and persist in human macrophages at MOI of 100 up to 48 h p.i. without affecting the viability of the macrophages (Friedman et al., 1992b), we further evaluated the response of the capsule locus expression during murine macrophages J774.A1 invasion, from which BPSM RNA was extracted at different time intervals p.i.

Similar to A549 cell infection assay, expression of the capsule locus was significantly up-regulated upon during invasion in J774.A1 when compared to *in vitro* BPSM culture (Figure 4.7). Transcriptional activity of the capsule locus was significantly increased within 1.5 h p.i. upon uptake into the macrophages compared to inoculum (Figure 4.7). At 3.5 h and 4 h p.i, expression of the capsule locus remained elevated when compared to *in vitro* culture, but appeared to be down-regulated with reference to the 1.5 h time point after uptake (Figure 4.7). Unlike the capsule locus, levels of *bvgA* expression in BPSM were moderately regulated during the course of infection in J774.A macrophages (Figure 4.7).

Taken together, the observations here suggest that uptake of *B. pertussis* into mammalian cells induced a rapid, transient and strong increase in the capsule locus expression, supporting that the capsule locus may play an important role in the early stages of mammalian cell invasion.



Gene/ Average Ct values	Uninfected	No template control
kpsT	35.279	34.902
bvgA	34.717	31.664
recA (ENDO)	27.093	26.583

Figure 4.7: Relative transcriptional activity of *kpsT* and *bvgA* in BPSM recovered from J774.A1 macrophages versus *in vitro* BPSM grown in virulent phase.

Invasion of J774.A1 cells were done by incubating the cells and BPSM at MOI of 100 at 37ºC for 1.5 h, 3.5 h and 4 h. Unbound bacteria were washed away prior to total RNA extraction from BPSM recovered from infected J774.A1 cells. Real-time PCR analysis was performed using primers mapping in the *kpsT* and *bvgA*. *recA* gene was used as the endogenous control. Results are expressed as the average RQ ± SD of triplicate versus BPSM innoculum. Dotted line represents RQ equal to 1 in relative to BPSM innoculum. The bottom table shows average Ct values for each gene obtained from the mock-infected J774.A1 (as negative control) and the no template control.

4.2.3 Transcriptional Analysis of the Capsule Locus in *B. pertussis* During Infection of The Mouse Respiratory Tract

We have shown that deletion of the membrane-associated capsule locus-encoded products impaired mice colonization efficiency as early as 3-days p.i., suggesting that the capsule locus plays an important role in the establishment of pertussis infection (Chapter 3). Our *ex vivo* data also suggest that the capsule locus is transcriptionally active at early stage of cellular infection (Section 4.2.1 and 4.2.2). Based on these findings, we moved on to monitor the capsule expression profile of *B. pertussis* during the course of infection *in vivo*.

Mice were nasally infected with BPSM bacteria and at different time points p.i., the animals were sacrificed and their lungs harvested for bacterial RNA extraction and purification. To minimize the changes in bacterial transcription during the processing of the infected lungs, infected mice lungs were soaked in RNAprotect Bacteria Reagent (Qiagen) for 1 h to immediately stabilize *B. pertussis* RNA prior to RNA isolation procedures. We observed that expression of the capsule locus was significantly up-regulated throughout the course of infection, with a peak of expression at day 3 p.i. compared to BPSM inoculum grown on BG agar plate (Figure 4.8), implying that the capsule locus is actively transcribed at early phase of pertussis infection. Interestingly, a similar expression profile was observed for another *bvg*-repressed factor, *vrg6* (Figure 4.8), indicating that BPSM harvested from the lungs environment induces the expression of *vrgs*, including the capsule locus,

as compared to BPSM harvested from BG agar plates in the absence of modulators.

In contrast to the *vrgs*, expression of *vags* including *bvgA, bvgR, fhaB* and *ptx* was elevated in a step-wise manner from day 0 (3 h post-nasal administration) to day 3 and day 7 p.i., likely explaining the peak of bacterial multiplication in the lungs at day 7 (Figure 4.8). Expression of the *fhaB* transcript was more greatly induced than the *ptx* transcript at 3 h p.i.; consistent with the fact that *fhaB* is an "early" *bvg*-activated gene necessary for the initial colonization of *B. pertussis* (Veal-Carr and Stibitz, 2005). At day 3 and 7 p.i. when *B. pertusiss* begins to multiply in the lungs, *ptx* was greatly up-regulated compared to *fhaB*; a stage where pertussis toxin may be highly produced by the bacteria. Surprisingly, elevated expression of *bvgR* contradicts the capsule locus and *vrg6* expression during the course of infection, indicating an altered *vrgs* modulation in *in vivo* versus *in vitro* bacteria. Our findings also support a previous study reporting on the differential regulation of *vags* assessed in a small time frame (between 5 h to 36 h post-inoculation) by the RIVET system during *in vivo* infection (Veal-Carr and Stibitz, 2005).

Despite the fact that the capsule locus has been classified as a *bvg*-repressed locus based on *in vitro* modulating and non-modulating conditions, the results here revealed for the first time that laboratory adapted BPSM strain activates the capsule locus expression, together with other *vrgs* during *in vivo* infection. Whether the *in vivo* lung microenvironment mimics the Bvg^+ phase

in vitro culture conditions remain to be determined. Moreover, the observation here indicates that host-mediated transcriptional changes on the capsule locus expression likely reflect a response of the bacteria towards the presence of unknown *in vivo* signals throughout the course of pertussis infection.

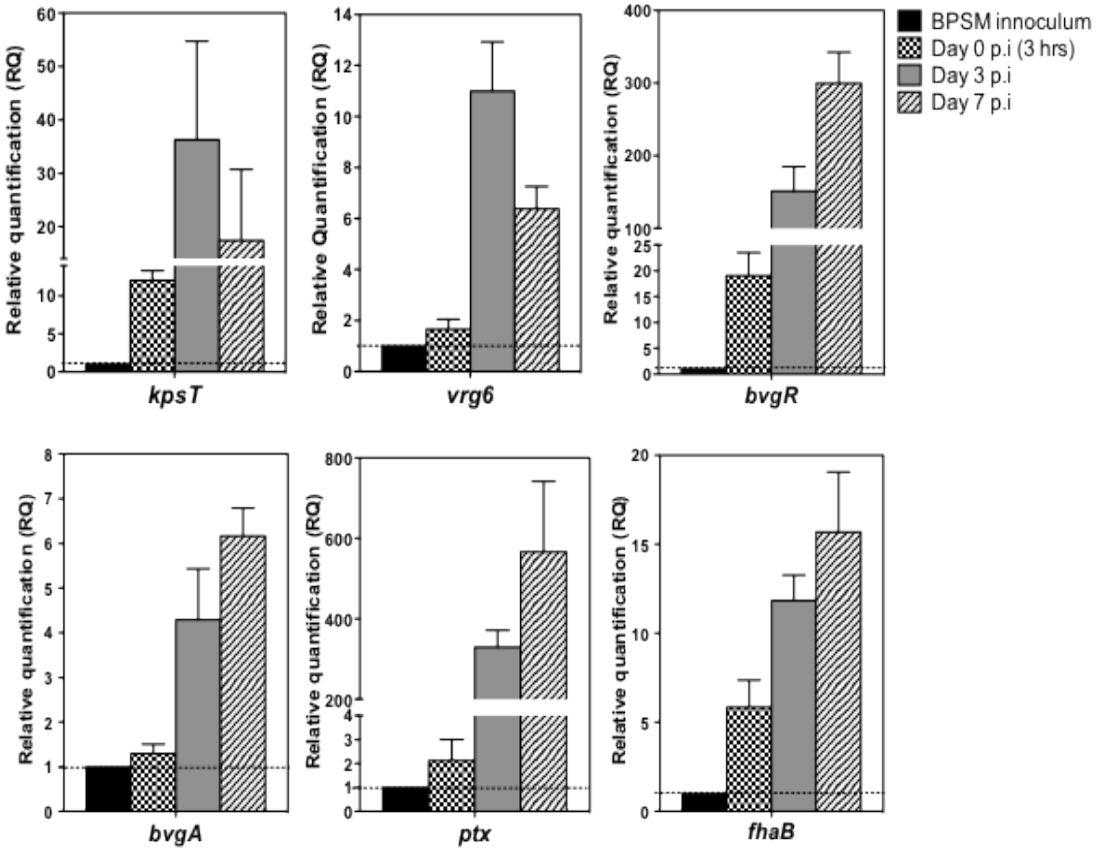


Figure 4.8: Relative transcriptional activity of *vrgs* and *vags* in BPSM recovered from mice lungs versus *in vitro* BPSM grown in virulent phase.

Mice were infected intranasally with approx. $5x10^7$ CFU of BPSM and the bacteria were recovered from the mice lungs at different time points (day 0/3 hours, day 3 and day 7 p.i.) through differential centrifugation. Bacterial RNA extracted and purified from group of 4 mice were pooled and subjected to Real-time PCR analysis using primers mapping in the *kpsT*, *vrg6*, *bvgR*, *bvgA*, *fhaB* and *ptx* genes. *recA* gene was used as the endogenous control. Results are expressed as the average RQ ± SD of triplicate versus BPSM innoculum. Dotted line represents RQ equal to 1 in relative to BPSM innoculum. Mock-infected mice were used as negative control.

4.3 DISCUSSION

4.3.1 Genetic Regulation of The Capsule Locus by The Ris System

Expression of the capsule locus in *B. pertussis* is highly elevated under *in vitro* modulating conditions that inactivates BvgA/S system or in a Δ*bvgAS* mutant. This supports that BvgA/S signaling system is involved in the regulation of the capsule locus in *B. pertussis*, presumably through the activity of the known *vrg* repressor, BvgR (Merkel et al., 1998; Merkel et al., 2003). Interestingly, the clinical isolate *B. pertussis* strain 18323 expresses low levels of the capsule transcripts even under modulating Bvg^- phase conditions when compared with the clinical isolate Tohama I strain and its laboratory adapted derivative strain BPSM. Concurrently, expression of *risA*, a proposed transcriptional regulator of *vrgs*, was greatly reduced in strain 18323. Whether the repression of the capsule locus under modulating Bvg^- phase in strain 18323 is a result of reduced *risA* expression and/or reduced binding affinity of RisA to the promoter of the capsule locus remains to be investigated.

However, attempts in determining the regulatory role of RisA on the capsule locus have not been successful due to the failure of obtaining a *risA*-deleted mutant in BPSM background strain. We demonstrated the essentiality of the *risA* gene in BPSM, thus somehow likely contributing to the overall bacteria *in vitro* growth and survival. Consistently, in *B. bronchiseptica*, deletion of the *ris* locus resulted in a general pleiotropic effect on the expression of a large numbers of unknown proteins (Jungnitz et al., 1998).

Deletion of *risA* in a Bvg^+ phase-locked BP536 strain was also reported previously and was found to reduce bacteria invasion when compared to a Bvg^+ phase-locked mutant in an *ex vivo* model of infection (Stenson et al., 2005). These observations land further support that Ris proteins are involved in some important biosynthesis or metabolic molecular pathways in *Bordetella* sp.

Over-expression of *risA* transcripts (upon expression under the strong *fhaB* promoter) did not significantly alter the expression of the capsule locus as well as *vrg6* in the Bvg^+ phase. It is likely that over-expression of RisA (5 fold) may not be sufficient to overcome the repressive effect of BvgR on the expression of these *vrgs*. Alternatively, RisA may not be involved or may not be the main activator for the capsule locus expression in BPSM strain. As exemplified by the Rcs signaling system in section 1.1.5.1, the transcriptional control of *E. coli* group 2 gene cluster is regulated by more than one regulator involving several overlapping regulatory circuits (Majdalani and Gottesman, 2005). As RisS is a pseudogene in *B. pertussis*, it is speculated that phospho-activation of RisA may be driven by another kinase (Stenson et al., 2005), drawing the possibility of molecular cross-talk between two regulatory systems in *B. pertussis*. Since *risA* expression is BvgAS-independent, it is unlikely that a cross-talk exists between RisA and the BvgA/S system.

4.3.2 Genetic Regulation of The Capsule Locus During Mammalian Cells Invasion

Analysis on the *B. pertussis* capsule locus expression pattern during *ex vivo* infection supports that the capsule locus is expressed during invasion into mammalian cells. We have previously shown that capsule-deleted mutant and wild-type *B. pertussis* equally adheres and invades the human pulmonary epithelial cells and murine macrophages (Neo et al., 2010). *B. pertussis* Bvg^+ phase-locked mutant with constitutive activation of *vags* and repression of *vrgs* (including the capsule locus) are able to adhere and survive in mammalian cells as well as the wild-type bacteria (Stenson et al., 2005). Therefore, the increased capsule locus expression during A549 and J774 macrophages infection is not associated with *B. pertussis* adherence and invasion properties into mammalian cell. The ability for *B. pertussis* to mediate the capsule locus expression in a temporal manner following invasion suggests that cellular "signals" may initiate a response governing the modulation of capsule locus and other genes expression in *B. pertussis*. Instead of being repressed under a presumably Bvg^+ virulent phase (as evidenced by the sustained high levels of *bvgA* transcripts) during mammalian cells infection, expression of the capsule locus as a *vrg* is dynamically modulated over the course invasion into epithelial cells and macrophages. The findings here argue against the fact that *bvg*-repressed factors are not expressed during infection.

Moreover, exposure of *B. pertussis* to different host cell microenvironments, such as extracellular and intracellular compartments may also influence signals perceived by the bacteria, and hence the overall expression of the capsule locus, *vrg6* and other *vags*. For instance within the cellular context of macrophages, *B. pertussis* was shown to confine into early phagosomal compartment and subsequently progress into late phagolysosome (Schneider et al., 2000). During the transition from phagosome to phagolysosome, the bacteria are exposed to an increasingly hostile environment including the presence of bactericidal cationic peptides, hydrolytic enzymes and acidic pH (Schneider et al., 2000). It is likely that the different environmental stress signals from these compartments play a role in activating the regulatory mechanism involved in the modulation of the capsule locus expression in *B. pertussis* during macrophage infection.

Consistently, the modulation of *vags* was observed for AC toxin during entry and invasion into human macrophages; a significant increase in AC activity was observed 30 min p.i. followed by a progressive down-modulation at 2 h and 4 h p.i. (Masure, 1992). This supports the transition-modulation of *B. pertussis* from *in vitro* to a host microenvironment, presumably as a adaptation strategy for the bacteria to express the appropriate levels of *vags* such as adhesin and toxin necessary for bacterial adherence and invasion in mammalian cells (Alonso et al., 2001; Friedman et al., 1992a; Ishibashi and Nishikawa, 2002; van den Berg et al., 1999; Vojtova et al., 2006).

4.3.3 Genetic Regulation of The Capsule Locus During *in vivo* Infection

The striking modulation of the capsule locus during mammalian cell infection prompted us to further investigate the expression pattern of the capsule locus in *B.pertussis* during colonization in the mouse respiratory tract. Immediately after infection, similar to the *ex vivo* epithelial and macrophage infection model, expression of the capsule locus was up-regulated, reflecting the response of *B. pertussis* towards the host environment changes upon infection. Expression of the capsule locus peaked at day 3 p.i. and lowered by day 7 p.i. A similar pattern of expression was observed for *vrg6* as well. These observations demonstrate for the first time that *vrgs* are expressed during infection. However, a previous study indicated that only the Bvg^+ phase is necessary and sufficient for a successful pertussis infection in mice (Martinez de Tejada *et al*., 1998), implying that the products of *vrgs* may not play a critical role during infection as they are expected to be down-modulated during *in vivo* infection. Here, we show that unlike the common belief, *vrgs* are readily expressed and modulated during infection which supports the idea that the *in vivo* microenvironment(s) encountered by the bacteria during infection are likely to be different from the artificial *in vitro* Bvg^+ growth condition, implying that the global transcriptional patterns in both *in vivo* and *in vitro* Bvg^+ conditions are likely to be very different.

Expression pattern of several *vags* was also monitored; in contrast to the *vrgs*, expression of *vags* (*bvgR*, *bvgA*, *ptx* and *fhaB*) was increased over the course of infection, with highest expression levels observed at day 7 p.i.

Consistently, a previous study has shown that in contrast to *in vitro B. pertussis* culture grown on BG agar, the environment in a mammalian respiratory tract is more highly inducing towards *vags* (*prn*, *fhaB*, *cyaA*) expression (Veal-Carr and Stibitz, 2005). The authors concluded that the environment in the mouse lungs is a Bvg^+ phase environment; however, they did not investigate the expression of *vrgs* following intranasal infection in mice (Veal-Carr and Stibitz, 2005).

It is interesting to note that whilst the *vags* are dynamically modulated during the course of early respiratory infection, the *vrgs* including the capsule locus are differentially modulated as well. Dynamic modulation of *vags* and *vrgs* at different days p.i is an important strategy for *B. pertussis* to disseminate from the site of infection to the upper and lower respiratory tract. Whereas constitutive expression of *vags* does not affect bacteria virulence, it was reported that ectopic expression of *vags* (*ptx* and *fhaB*) is detrimental to *B. pertussis in vivo* virulence (Kinnear et al., 2001). Furthermore, constitutive expression of *vrgs* in a *bvgR*-deleted *B. pertussis* mutant interferes with the ability of the bacteria to cause disease (Merkel et al., 1998). Therefore, the flexibility of *B. pertussis* regulon to appropriately express the right amount of *vags* and *vrgs* at distinct sites and times of infection plays an important part for the establishment of pertussis infection.

Modulation of bacterial gene expression in response to host microenvironment and host immune responses during infection has been well documented in other pathogen, implying the ability of a pathogen to sense and

respond to its host microenvironment (Heithoff et al., 1997; Howden et al., 2008; Talaat et al., 2004). Migration of *B. pertussis* from the mice nasal cavity to the lungs likely results in exposure of the bacteria to different mucosal surfaces (nasal, trachea and lungs) and immune cells at different time-points p.i (3 h, day 3 and day 7). Infiltrated immune cells such as neutrophils and macrophages into the lungs encountered by the bacteria at day 3 and 7 p.i. may also contribute to the differential modulation of *vags* and the capsule locus expression in *B. pertussis*. Besides the major Bvg^+ phase virulence factors, convalescence immune sera from *B. pertussis* infected individuals were able to recognize several *B. pertussis bvg*-intermediate phase factors, suggesting that during natural infection the bacteria are able to concurrently express different surface factors that were categorized as Bvg^+ vs Bvg^i factors under *in vitro* phase culture (Martinez de Tejada et al., 1998). Therefore, despite evidence that Bvg^+ phase is sufficient for respiratory infection in animal models (Cotter and Miller, 1994; Martinez de Tejada et al., 1998), our observations here further support the possibility that *B. pertussis* is able to modulate both the *vags* and *vrgs* (the capsule locus) during natural infection.

With multiple putative transcription factors deduced from the full *B. pertussis* genome sequence analysis (Parkhill et al., 2003), regulation of the capsule locus in *Bordetella* sp is likely to be more complex than expected. The ability of *B. pertussis* to sense its environment and dynamically modulate its genes expression may confer a selective advantage to the bacteria in promoting optimal survival within its host.

4.4 CONCLUSIONS AND FUTURE WORK

4.4.1 Transcriptional Regulation of The Capsule Locus in *B. pertussis*

Genetic regulation of the polysaccharide capsule locus in *B. pertussis* remains unclear, although the Ris system has been described to positively regulate other *bvg*-repressed factors including *vrg6* and surface Vra antigen expression in *B. bronchiseptica* and BP536 strain. Our results show that RisA is essential for BSPM, therefore we were not able to isolate a *risA*-deficient mutant strain. Hence, several aspects regarding the function of Ris system towards the capsule regulation remain to be determined. Perhaps the most pertinent is to delete *risA* in a *B. pertussis* strain where RisA is non-essential. In addition, further study on the role of BvgR repressor by mutational analysis will aid in a better understanding on the capsule locus regulation in *B. pertussis*.

Further work should also be directed at elucidating the binding capability of RisA onto the capsule locus promoter, as previously described for the *vrg6* promoter (Croinin et al., 2005). *In vitro* DNaseI footprinting and/or electro-mobility gel shift assay (EMSA) using purified RisA protein (over-expressed in *E. coli*) and the putative capsule promoter sequence could be performed to confirm direct binding of RisA to the capsule promoter and to delineate the promoter region to which RisA binds. RisA is speculated to be phosphorylated prior to its binding to *vrg6* promoter (Croinin et. al., 2005), therefore it is likely that binding of RisA to its target DNA motifs may require

it to be phosphorylated. Hence, *in vitro* phosphorylation of RisA should be performed prior to EMSA analysis. In addition to RisA, other proteins may interact with the capsule promoter. To test this hypothesis, an *in vitro* DNA-protein pull-down assay can be performed. The putative capsule promoter can first be synthesized and labeled with a high affinity tag (i.e biotin) and incubated with enriched cytoplasmic extract of wild-type *B. pertussis* prior to purification using streptavidin agarose or magnetic beads. Such *in vitro* DNA pull-down assay would allow the identification of possible regulatory protein(s) (hypothetically RisA, BvgR and perhaps other proteins) that bind(s) onto the capsule locus promoter. This approach would eventually help characterize the molecular mechanisms involved in the genetic regulation of the capsule locus in *B. pertussis*

4.4.2 Genetic Modulation of The Capsule Locus of *B. pertussis* During *in vivo* Infection

The results show that the capsule locus of *B. pertussis* is highly expressed and dynamically modulated during cellular invasion, as well as during the course of *in vivo* infection, reflecting the response of the bacteria to the host microenvironments during infection. The fact that the capsule locus, classified as a *vrg* is actually readily expressed and differentially modulated during *in vivo* infection, forces to re-evaluate the role of *vrgs* during *in vivo* infection. Furthermore, our observations suggest that *in vitro* culture conditions with or without modulators which so far have helped define the Bvg^- and Bvg^+ phases in *B. pertussis* may be far from the environmental

conditions actually encountered by the bacteria *in vivo* and may not be predictive of the potential role and importance of genes during pertussis pathogenesis. The current findings add to the growing body of literature on the modulation of *Bordetella* genes during infection. Thus far, there is limited knowledge on the genetic regulation of *vrgs* during infection; this is the first report that *vrgs* including the capsule locus is expressed and modulated during infection. A microarray/deep sequencing approach to map the expression of *vrgs* during infection is awaited and would greatly enhance our knowledge regarding the role of *vrgs* during infection.

Similar to the temporal modulation of *vags* expression during infection, the modulation of the expression of the capsule locus during infection may imply that the temporal expression of this locus is important for optimal virulence of *B. pertussis*. These findings provide the following insights for future work; a further study on the capsule locus and other *vrgs* expression in different laboratory-adapted and clinically isolated *B. pertussis* strains during *in vivo* infection would provide supporting evidence on the genetic regulation of the capsule locus during pertussis pathogenesis. In addition, it would be very informative to study the virulence phenotype of *B. pertussis* bacteria in which the chromosomal capsule locus is ectopically expressed under the control of a strong *vag* promoter such as the *fhaB* promoter. Such approach would certainly help apprehend the role and importance of the capsule locus during infection.

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APPENDICES

APPENDIX 1: Obtaining the Δ*kpsT*-Complemented Strain

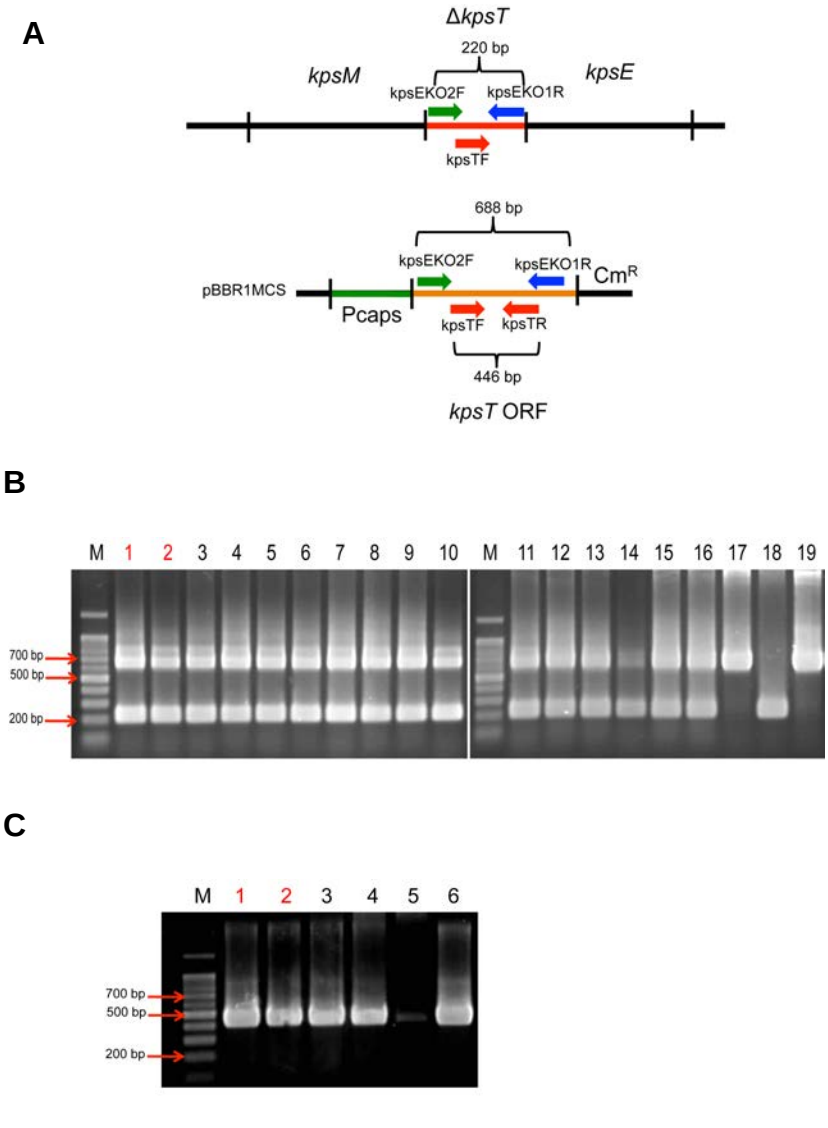

Figure 3.7: Colony PCR screening for *B. pertussis* Δ*kpsT*com strains.
(A) PCR screening strategy for Δ*kpsT* complemented strain using the primer pairs as indicated. pBBR1MCS represents the complement replicative plasmid in a Δ*kpsT* mutant.
(B) *B. pertussis* Δ*kpsT*com clones screened with kpsTKO2F and kpsEKO1R primers. Lanes; M, 100 bp ladder (5 µl); 1 to 17, *B. pertussis* Δ*kpsT*com clone 1 to 17 (10 µl); 18, Δ*kpsT* chromosomal DNA; 19, BPSM chromosomal DNA (10 µl). Colony PCR screening of Δ*kpsT*com strains are expected to show both 220 bp and 688 bp fragment, while Δ*kpsT* should have only a 220 bp fragment. BPSM should have longer fragment of 688 bp.
(C) *B. pertussis* Δ*kpsT*com clones screened with kpsTF and kpsTR primers. Lanes; M, 100 bp ladder (5 µl); 1 to 4, *B. pertussis* Δ*kpsT*com clone 1 to 4 (10

µl); 5, Δ*kpsT* chromosomal DNA; 6, BPSM chromosomal DNA (10 µl).
Colony PCR screening of Δ*kpsT*com strains are expected to show 446 bp fragment, while Δ*kpsT* should not have any band. BPSM should have a 446 bp fragment.
Positive clones are indicated in red.

APPENDIX 2: Estimated percentage of Δ*kpsT*com Cm-resistant colonies recovered from the lungs of infected mice at the indicated time-points.

Twenty random CFUs obtained from plated lung homogenate on BG agar at indicated time-points were subjected PCR using primers mapping in *kpsT*-deleted region and primers flanking *kpsT*-deleted region as described in appendix 1.

Day p.i	**0**	**3**	**7**	**10**	**17**
Estimate percentage of Cm-resistant colonies recovered	90%	70%	45%	20%	10%

APPENDIX 3: DNA microarray analysis of statistically significant differentially modulated transcripts in Δ*kpsT* mutant compared to BPSM.

DNA microarray analysis was performed to measure relative transcript levels in Δ*kpsT* compared to the transcript levels present in wild-type BPSM. Differences in transcript levels are listed as mean $\log_2$ fold change (FC) from two biological replicates filtered with adjusted *p* value < 0.01 and $\log_2$ FC > 0.8 or < -0.8. . Down-regulated transcripts are represented by negative values of $\log_2$ FC and up-regulated transcripts are represented by positive values of $\log_2$ FC.

BP ORF	Gene Symbol	Product	NCBI_GeneID	$\log_2$FC	*p* value	Adjusted *p* value	Average $\log_2$ FC
BP0454	_	putative exported protein	ncbi-geneid:2664448	-4.80	1.05E-14	4.40E-11	-4.77
BP0454	_	putative exported protein	ncbi-geneid:2664448	-4.75	5.57E-15	4.40E-11	
BP0455	_	putative membrane protein	ncbi-geneid:2664449	-4.17	1.67E-13	4.66E-10	-4.15
BP0455	_	putative membrane protein	ncbi-geneid:2664449	-4.12	1.56E-12	3.27E-09	
BP1201	tcfA	tracheal colonization factor precursor	ncbi-geneid:2666888	-1.97	7.05E-10	7.56E-08	-1.96
BP1201	tcfA	tracheal colonization factor precursor	ncbi-geneid:2666888	-1.95	4.39E-10	5.59E-08	
BP2315	vag8	autotransporter	ncbi-geneid:2666501	-1.97	1.31E-10	3.53E-08	-1.94
BP2315	vag8	autotransporter	ncbi-geneid:2666501	-1.91	6.23E-10	6.94E-08	
BP2925	_	conserved hypothetical protein	ncbi-geneid:2667044	-1.70	3.19E-10	5.38E-08	-1.69
BP2925	_	conserved hypothetical protein	ncbi-geneid:2667044	-1.68	9.06E-12	1.05E-08	
BP2926	_	conserved hypothetical protein	ncbi-geneid:2667045	-1.61	2.21E-10	5.38E-08	-1.61
BP2926	_	conserved hypothetical protein	ncbi-geneid:2667045	-1.60	5.48E-11	2.12E-08	

BP2234	brpL	putative R_ polymerase sigma factor	ncbi-geneid:2667457	-1.55	4.09E-10	5.43E-08	-1.52
BP2234	brpL	putative R_ polymerase sigma factor	ncbi-geneid:2667457	-1.49	1.11E-09	1.01E-07	
BP3694	_	conserved hypothetical protein (pseudogene)	ncbi-geneid:2664938	-1.47	1.81E-11	1.05E-08	-1.46
BP3694	_	conserved hypothetical protein (pseudogene)	ncbi-geneid:2664938	-1.46	4.26E-11	1.86E-08	
BP2924	_	putative exported protein	ncbi-geneid:2667043	-1.44	1.87E-11	1.05E-08	-1.44
BP2924	_	putative exported protein	ncbi-geneid:2667043	-1.44	1.76E-11	1.05E-08	
BP0499	_	hypothetical protein	ncbi-geneid:2664691	-1.40	2.20E-07	4.63E-06	-1.36
BP0499	_	hypothetical protein	ncbi-geneid:2664691	-1.32	2.93E-10	5.38E-08	
BP0456	hemC	putative heme receptor	ncbi-geneid:2664098	-1.39	3.41E-09	2.30E-07	-1.32
BP0456	hemC	putative heme receptor	ncbi-geneid:2664098	-1.26	3.27E-10	5.38E-08	
BP3696	_	putative exported protein	ncbi-geneid:2664940	-1.35	3.77E-10	5.38E-08	-1.34
BP3696	_	putative exported protein	ncbi-geneid:2664940	-1.33	3.20E-10	5.38E-08	
BP2261	bcrD	putative type III secretion pore protein	ncbi-geneid:2665956	-1.34	8.79E-08	2.44E-06	-1.32
BP2261	bcrD	putative type III secretion pore protein	ncbi-geneid:2665956	-1.30	2.48E-09	1.90E-07	
BP3011	_	hypothetical protein	ncbi-geneid:2665904	-1.25	3.43E-09	2.30E-07	-1.24
BP3011	_	hypothetical protein	ncbi-geneid:2665904	-1.24	9.49E-10	9.13E-08	
BP1363	_	putative amino-acid ABC transporter, permeaseprotein	ncbi-geneid:2665277	-1.23	2.13E-10	5.38E-08	-1.19
BP1363	_	putative amino-acid ABC	ncbi-geneid:2665277	-1.16	1.70E-09	1.43E-07	

		transporter, permeaseprotein					
BP1364	_	putative amino-acid ABC transporter, periplasmicamino acid-binding protein	ncbi-geneid:2665278	-1.23	5.36E-09	3.21E-07	-1.21
BP1364	_	putative amino-acid ABC transporter, periplasmicamino acid-binding protein	ncbi-geneid:2665278	-1.19	1.43E-08	6.40E-07	
BP3695	_	putative hydroxymethylglutaryl-CoA lyase	ncbi-geneid:2664939	-1.16	4.97E-10	6.03E-08	-1.19
BP3695	_	putative hydroxymethylglutaryl-CoA lyase	ncbi-geneid:2664939	-1.22	1.07E-10	3.21E-08	
BP0856	bfrD	probable TonB-dependent receptor for iron transport	ncbi-geneid:2664308	-1.19	2.27E-10	5.38E-08	-1.17
BP0856	bfrD	probable TonB-dependent receptor for iron transport	ncbi-geneid:2664308	-1.15	2.80E-10	5.38E-08	
BP3784	ptxB	pertussis toxin subunit 2 precursor	ncbi-geneid:2665069	-1.18	1.24E-08	5.84E-07	-1.17
BP3784	ptxB	pertussis toxin subunit 2 precursor	ncbi-geneid:2665069	-1.16	1.81E-10	4.73E-08	
BP0500	_	hypothetical protein	ncbi-geneid:2664714	-1.18	7.97E-10	7.94E-08	-1.14
BP0500	_	hypothetical protein	ncbi-geneid:2664714	-1.10	1.18E-09	1.06E-07	
BP1198	clpB, htpM	ATP-dependent protease, ATPase subunit	ncbi-geneid:2666478	-1.14	9.00E-07	1.23E-05	-1.11
BP1198	clpB, htpM	ATP-dependent protease, ATPase subunit	ncbi-geneid:2666478	-1.08	1.62E-06	1.85E-05	
BP2499	d_K	molecular chaperone	ncbi-geneid:2666522	-1.13	4.17E-08	1.40E-06	-1.12
BP2499	d_K	molecular chaperone	ncbi-geneid:2666522	-1.10	4.08E-10	5.43E-08	

BP1203	_	conserved hypothetical protein	ncbi-geneid:2666845	-1.11	1.77E-09	1.45E-07	-1.10
BP1203	_	conserved hypothetical protein	ncbi-geneid:2666845	-1.09	2.93E-10	5.38E-08	
BP2262	bscD	putative type III secretion protein	ncbi-geneid:2665957	-1.11	8.93E-10	8.79E-08	-1.09
BP2262	bscD	putative type III secretion protein	ncbi-geneid:2665957	-1.08	2.33E-09	1.82E-07	
BP0074	htpG	heat shock protein	ncbi-geneid:2666131	-1.09	3.26E-09	2.23E-07	-1.06
BP0074	htpG	heat shock protein	ncbi-geneid:2666131	-1.03	1.04E-09	9.71E-08	
BP1204	_	conserved hypothetical protein	ncbi-geneid:2666846	-1.09	3.69E-10	5.38E-08	-1.07
BP1204	_	conserved hypothetical protein	ncbi-geneid:2666846	-1.06	1.18E-10	3.39E-08	
BP3575	_	putative exported protein	ncbi-geneid:2665198	-1.08	3.08E-09	2.17E-07	-1.07
BP3575	_	putative exported protein	ncbi-geneid:2665198	-1.06	3.86E-10	5.38E-08	
BP0216	sphB1	autotransporter subtilisin-like protease	ncbi-geneid:2664729	-1.06	5.35E-10	6.13E-08	-1.04
BP0216	sphB1	autotransporter subtilisin-like protease	ncbi-geneid:2664729	-1.03	2.85E-10	5.38E-08	
BP0822	hyuA	hydantoin utilization protein A	ncbi-geneid:2664341	-1.05	5.09E-10	6.08E-08	-1.05
BP0822	hyuA	hydantoin utilization protein A	ncbi-geneid:2664341	-1.05	2.33E-09	1.82E-07	
BP3785	ptxD	pertussis toxin subunit 4 precursor	ncbi-geneid:2665406	-1.04	2.42E-10	5.38E-08	-1.03
BP3785	ptxD	pertussis toxin subunit 4 precursor	ncbi-geneid:2665406	-1.02	3.77E-10	5.38E-08	
BP2263	bscE	hypothetical protein	ncbi-geneid:2665958	-1.03	2.97E-07	5.73E-06	-1.00
BP2263	bscE	hypothetical protein	ncbi-geneid:2665958	-0.96	1.25E-09	1.10E-07	
BP3432	cysI	putative sulfite reductase	ncbi-geneid:2666024	-1.02	1.27E-09	1.11E-07	-1.00
BP3432	cysI	putative sulfite reductase	ncbi-geneid:2666024	-0.98	2.19E-08	8.76E-07	
BP3455	_	putative taurine dioxyge_se	ncbi-geneid:2666952	-1.00	8.45E-09	4.47E-07	-0.98
BP3455	_	putative taurine dioxyge_se	ncbi-geneid:2666952	-0.95	5.77E-09	3.35E-07	

BP2183	_	conserved hypothetical protein	ncbi-geneid:2666908	-0.99	2.08E-07	4.48E-06	-0.96
BP2183	_	conserved hypothetical protein	ncbi-geneid:2666908	-0.93	1.04E-07	2.73E-06	
BP3405	ompQ	outer membrane porin protein OmpQ	ncbi-geneid:2667075	-0.99	9.25E-10	8.99E-08	-0.97
BP3405	ompQ	outer membrane porin protein OmpQ	ncbi-geneid:2667075	-0.96	7.51E-10	7.75E-08	
BP2141	_	putative exported protein	ncbi-geneid:2666978	-0.99	1.36E-08	6.21E-07	-0.98
BP2141	_	putative exported protein	ncbi-geneid:2666978	-0.97	8.51E-09	4.48E-07	
BP3871	_	putative cold shock-like protein	ncbi-geneid:2665120	-0.99	1.09E-08	5.33E-07	-0.99
BP3871	_	putative cold shock-like protein	ncbi-geneid:2665120	-0.98	2.08E-07	4.48E-06	
BP2662	_	putative aldolase	ncbi-geneid:2665526	-0.98	3.46E-10	5.38E-08	-0.96
BP2662	_	putative aldolase	ncbi-geneid:2665526	-0.95	2.06E-09	1.66E-07	
BP3783	ptxA	pertussis toxin subunit 1 precursor	ncbi-geneid:2665068	-0.97	1.01E-08	5.12E-07	-0.96
BP3783	ptxA	pertussis toxin subunit 1 precursor	ncbi-geneid:2665068	-0.95	5.02E-09	3.07E-07	
BP1200	bapB	autotransporter (pseudogene)	ncbi-geneid:2666887	-0.96	3.25E-08	1.17E-06	-0.92
BP1200	bapB	autotransporter (pseudogene)	ncbi-geneid:2666887	-0.88	4.27E-08	1.42E-06	
BP2233	_	hypothetical protein	ncbi-geneid:2667456	-0.96	8.19E-09	4.37E-07	-0.95
BP2233	_	hypothetical protein	ncbi-geneid:2667456	-0.94	1.38E-08	6.29E-07	
BP2253	bopD	putative outer protein D	ncbi-geneid:2667094	-0.94	5.91E-09	3.41E-07	-0.91
BP2253	bopD	putative outer protein D	ncbi-geneid:2667094	-0.88	6.09E-09	3.49E-07	
BP0162	_	putative membrane protein	ncbi-geneid:2664287	-0.91	4.73E-09	2.93E-07	-0.91
BP0162	_	putative membrane protein	ncbi-geneid:2664287	-0.90	1.11E-09	1.01E-07	
BP2256	bsp22	putative secreted protein	ncbi-geneid:2665951	-0.90	2.07E-08	8.49E-07	-0.86
BP2256	bsp22	putative secreted protein	ncbi-geneid:2665951	-0.82	6.26E-08	1.89E-06	

BP3574	_	putative branched-chain amino acid transportpermease	ncbi-geneid:2665197	-0.90	9.40E-09	4.89E-07	-0.90
BP3574	_	putative branched-chain amino acid transportpermease	ncbi-geneid:2665197	-0.90	5.72E-09	3.34E-07	
BP1362	_	putative amino-acid ABC transporter, ATP-bindingprotein	ncbi-geneid:2665276	-0.89	6.18E-09	3.51E-07	-0.85
BP1362	_	putative amino-acid ABC transporter, ATP-bindingprotein	ncbi-geneid:2665276	-0.81	2.30E-08	9.02E-07	
BP2683	paaB	phenylacetic acid degradation protein	ncbi-geneid:2665547	-0.88	7.04E-10	7.56E-08	-0.86
BP2683	paaB	phenylacetic acid degradation protein	ncbi-geneid:2665547	-0.85	3.22E-08	1.17E-06	
BP0723	_	probable ABC transporter, ATP-binding protein	ncbi-geneid:2666778	-0.86	2.77E-09	2.02E-07	-0.84
BP0723	_	probable ABC transporter, ATP-binding protein	ncbi-geneid:2666778	-0.82	9.89E-09	5.06E-07	
BP3786	ptxE	pertussis toxin subunit 5 precursor	ncbi-geneid:2665407	-0.86	5.90E-10	6.66E-08	-0.86
BP3786	ptxE	pertussis toxin subunit 5 precursor	ncbi-geneid:2665407	-0.86	3.70E-07	6.77E-06	
BP2255	_	hypothetical protein	ncbi-geneid:2665950	-0.86	6.18E-08	1.88E-06	-0.83
BP2255	_	hypothetical protein	ncbi-geneid:2665950	-0.80	1.10E-08	5.33E-07	
BP0120	_	inner membrane component of binding-protein-dependent transport system	ncbi-geneid:2664359	-0.84	1.60E-07	3.74E-06	-0.83
BP0120	_	inner membrane component of binding-protein-dependent transport system	ncbi-geneid:2664359	-0.82	7.68E-08	2.17E-06	

BP3086	hslV, htpO	ATP-dependent protease heat shock protein	ncbi-geneid:2667055	-0.83	4.20E-08	1.40E-06	-0.83
BP3086	hslV, htpO	ATP-dependent protease heat shock protein	ncbi-geneid:2667055	-0.83	5.24E-08	1.65E-06	
BP3494	brkA	serum resistance protein	ncbi-geneid:2664892	-0.83	1.01E-06	1.33E-05	-0.82
BP3494	brkA	serum resistance protein	ncbi-geneid:2664892	-0.81	4.78E-08	1.54E-06	
BP3812	_	putative outer membrane efflux proteinbpe	ncbi-geneid:2665087	0.804	5.09E-09	3.08E-07	0.80
BP3812	_	putative outer membrane efflux proteinbpe	ncbi-geneid:2665087	0.805	2.05E-08	8.45E-07	
BP3838	ubiE	bpe:BP3838	ncbi-geneid:2664850	1.99	1.79E-11	1.05E-08	2.00
BP3838	ubiE	bpe:BP3838	ncbi-geneid:2664850	2.02	7.15E-12	1.05E-08	

APPENDIX 4: Reagents for gel electrophoresis

4.1 DNA Electrophoresis

4.1.1 50x Tris-Acetate-EDTA (TAE) Buffer

	Per 1000 ml
Tris base	242 g
Glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8)	100 ml
Final pH adjusted to 7.8	

4.1.2 Agarose Gel

	1%	1.5%
Agarose	0.5 g	0.75g
1x TAE	50 ml	50 ml

1x TAE was prepared by diluting 20 ml of 50x TAE with 980 ml of ddH_2O

4.1.3 6x DNA Loading Dye

Bromophenol blue	0.25%
Xyelene cyanol	0.24%
Ficoll (type 400) in ddH_2O	25%

4.2 Protein Electrophoresis

4.2.1 SDS-PAGE

4.2.1.1 5x SDS/Glycine Electrophoresis Buffer

	Per 1000 ml
Tris base	15.1 g
Glycine	72 g
SDS	5 g

4.2.1.2 SDS-PAGE Seperating/Resolving Gel

	Per 10 ml		
	8%	10%	12%
30% Acrylamide-bisacrylamide (29:1)	2.65ml	3.33ml	4ml
1.5 Tris-HCl (pH 8.8)	2.5ml	2.5ml	2.5ml
10% SDS	0.1ml	0.1ml	0.1ml
10% Ammonium persulfate	0.1ml	0.1ml	0.1ml
TEMED	0.004ml	0.004ml	0.004ml
ddH_2O	4.65ml	3.97ml	3.30ml

4.2.1.2 SDS-PAGE Stacking Gel

	Per 10 ml
	5%
30% Acrylamide-bisacrylamide (29:1)	1.65ml
1 M Tris-HCl (pH 6.8)	2.5ml
10% SDS	0.1ml
10% Ammonium persulfate	0.1ml
TEMED	0.004ml
ddH_2O	5.65ml

APPENDIX 5: Reagents for growth media

5.1 *E. coli* Culture Media

5.1.1 Luria-Bertani (LB) Agar

	Per 1000 ml
Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Agar	15 g

To ensure sterility, medium was autoclaved at 121°C for 15 min.

5.1.2 Luria-Bertani (LB) Broth

	Per 1000 ml
Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Final pH adjusted to 7.0	

To ensure sterility, medium was autoclaved at 121°C for 15min.

5.2 *B. pertusssis* culture media

5.2.1 Stainer-Scholte (SS) Medium

Fraction A:	Per 950ml
Na-L-Glutamate	11.84 g
L-Proline	0.24 g
NaCl	2.5 g
KH_2PO_4	0.5 g
KCl	0.2 g
$MgCl_2.6H_2O$	0.1 g
$CaCl_2.2H_2O$	0.02 g
Tris	1.5 g
Casamino acids	10 g

Dimethyl-β-Cyclodextrine	1 g

Fraction B:	Per 50 ml
L-Cysteine	0.04 g
$FeSO_4.7H_2O$	0.01 g
Nicotinic acid	0.04 g
Ascorbic acid	0.4 g
Glutathione	0.15 g

Dissolve Fraction A and B completely prior to mixing Fraction A and B to a final volume of 1000 ml.
Medium was filter sterilized using 0.2 µm filter unit.

5.2.2 Bordet-Gengou (BG) Agar

	Per 1000 ml
Potato infusion from 125 g	4.5g
NaCL	5.5g
Agar	20 g
Glycerol	10 g

To ensure sterility, medium was autoclaved at 121°C for 15 min.
10% sterile, defibrinated sheep blood was added at 45°C-50°C.

5.3 Tissue culture media

5.3.1 Dulbecco's modified essential medium (DMEM)

	Per 1000 ml
DMEM	900ml
FCS	100ml
200mM Glutamax-I	0.02ml
100mM Sodium pyruvate	0.01ml

Medium was filter sterilized using 0.2 µm filter unit.

5.3.2 RPM1-1640 medium modified

	Per 1000 ml
RPMI-1640	900ml
FCS	100ml
200mM Glutamax-I	0.02ml
Penicillin-streptomycin	10ml

Medium was filter sterilized using 0.2 µm filter unit.

APPENDIX 6: Reagents for animal work

6.1 Anaesthetic cocktail for nasal administration

Valium	6%
Atropine	10%
Ketamine	20%
1x PBS	64%

Cocktail must be prepared under sterile conditions. 120 µl cocktail is injected intraperitoneally for a mouse of approximately 17 g of body weight.