IMMUNE RESPONSES AGAINST THE RECOMBINANT FIMX AND PUTATIVE PEPTIDYL-PROLYL CIS-TRANS ISOMERASE FROM *BORDETELLA PERTUSSIS*

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ABSTRACT

IMMUNE RESPONSES AGAINST THE RECOMBINANT FIMX AND PUTATIVE PEPTIDYL-PROLYL CIS-TRANS ISOMERASE FROM *BORDETELLA PERTUSSIS*

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Whooping cough (pertussis) is a highly contagious respiratory infection caused by *Bordetella pertussis*. It becomes widespread among adolescent and adults as well as infants. Although availability of effective pertussis vaccines seems to decrease the incidence of the disease, *B. pertussis* circulation in population has not been eliminated. It is thought that the antigenic drifts in major protective antigens and continued circulation of *B. pertussis* strains will result in gradual loss of the efficacy of the current pertussis vaccines. Therefore, development of more effective acellular pertussis vaccines with conserved protective proteins is a convenient strategy to provide a better protection against whooping cough.

In this study, immune responses against putative peptidyl-prolyl cis-trans isomerase (PPIase) which was shown to be immunogenic in *B. pertussis* for the first time by our immunoproteome group and FimX whose expression was found higher in our local Saadet strain were determined in mice. The genes encoding FimX and putative

PPIase were amplified by PCR, cloned into pGEM®-T Easy vector and sequenced. The genes were then introduced into pET-28a (+) vector and they were expressed in *Escherichia coli* BL21(DE3) cells. The recombinant proteins were purified by His-tag affinity chromatography and dialyzed. After Western blot analyses, 20 μg and 80 μg recombinant FimX and 80 μg recombinant putative PPIase were used to immunize BALB/c mice (16-18 g) at day 0 and 21. The mice were challenged intranasally with 2.5 x 10^9 live *B. pertussis* Saadet cells. Before second immunization and challenge, the sera were collected to carry out ELISA for measurement of serum-specific IgG levels. According to ELISA results, IgG levels in the mice immunized with 20 μg and 80 μg recombinant FimX were found significantly higher than in control groups at both first and second vaccinations ($p<0.01$). On the other hand, immunization with 160 μg recombinant putative PPIase provided a significant increase in IgG level ($p<0.05$) only at second vaccination. The lungs of the mice were removed at day 2, 5, 8 after challenge and bacterial colonization was determined. No significant decrease in bacterial colonization was observed in the lungs of the mice immunized with 20 μg and 80 μg recombinant FimX and 80 μg recombinant putative PPIase with respect to control groups. After respiratory challenge and second immunization (at day 30) with 20 μg and 80 μg recombinant FimX, the spleens of the mice were removed and a spleen cell culture was obtained. Supernatants were collected after induction of the cells with the recombinant protein and cytokine ELISA was carried out to measure IFN-γ level. No significant difference was observed between control and vaccinated mice in terms of IFN-γ production.

Keywords: Whooping cough, *Bordetella pertussis*, Subunit vaccine, Immune response

ÖZ

BORDETELLA PERTUSSİS'E AİT REKOMBİNANT FİMX VE VARSAYIMSAL PEPTİDİL-PROLİL SİS-TRANS İZOMERAZA KARŞI BAĞIŞIKLIK YANITLARI

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Boğmaca (pertussis), *Bordetella pertussis* bakterisinin neden olduğu oldukça bulaşıcı bir solunum yolu enfeksiyonudur. Hastalık, yeni doğanların yanı sıra, gençler ve erişkinler arasında da yaygınlaşmaya başlamıştır. Etkin pertussis aşılarının kullanımı hastalığın görülme sıklığını azaltmasına rağmen, *B. pertussis* suşlarının populasyondaki dolaşımı ortadan kaldırılamamıştır. Temel koruyucu antijenlerdeki antijenik değişimlerin ve *B. pertussis* suşlarının populasyondaki dolaşımının devam etmesinin şu an kullanılan aşıların zamanla etkinliklerini yitirmesiyle sonuçlanacağı düşünülmektedir. Bu nedenle, koruyuculuğa sahip korunmuş proteinler içeren daha etkin aşıların geliştirilmesi boğmaca hastalığına karşı korunmada uygun bir stratejidir.

Bu çalışmada, grubumuz tarafından *B. pertussis*'de gerçekleştirilen immunoproteom analizinde immunojenik olduğu ilk kez gösterilen varsayımsal peptidil-prolil sis-trans izomeraza proteinine ve yerel bir suj olan Saadet suşunda fazla üretildiği yine

grubumuzca tespit edilen FimX'e karşı farelerdeki bağışık yanıtlar araştırılmıştır. FimX'i ve varsayımsal peptidil-prolil sis-trans izomerazı kodlayan genler PCR ile çoğaltılmış ve pGEM®-T Easy vektörüne klonlanmıştır. Genler daha sonra pET-28a (+) vektörüne yerleştirilmiştir ve *Escherichia coli* BL21(DE3) hücrelerinde gen ifadeleri sağlanmıştır. Rekombinant proteinler His-tag afinite kromatografi yöntemiyle saflaştırılmıştır ve diyaliz edilmişlerdir. Western blot analizlerinden sonra, BALB/c fareleri (16-18 gr), 0 ve 21. günlerde, 20 µg ve 80 µg recombinant FimX ve 80 µg varsayımsal peptidil-prolil sis-trans izomeraz ile aşılanmıştır. Fareler burun içinden 2.5 x 10^9 canlı *B. pertussis* Saadet hücreleri ile enfekte edilmiştir. İkinci aşı uygulamasından ve enfeksiyondan önce, ELISA tekniği ile seruma özel IgG seviyesi ölçülmüştür. ELISA sonuçlarına göre, 20 µg ve 80 µg rekombinant FimX ile aşılanan farelerdeki IgG seviyesi, birinci ve ikinci aşılamalarda, kontrol gruplarındaki IgG seviyesinden önemli ölçüde yüksek bulunmuştur ($p<0.01$). Ancak, 80 µg rekombinant varsayımsal peptidil-prolil sis-trans izomeraz aşılaması sadece ikinci aşı uygulamasında IgG seviyesinde ($p<0.05$) önemli ölçüde artış sağlamıştır. 2., 5. ve 8. günlerde farelerin akciğerleri alınmıştır ve bakteri kolonizasyonu incelenmiştir. 20 µg ve 80 µg rekombinant FimX ve 160 µg rekombinant varsayımsal peptidil-prolil sis-trans izomeraz ile aşılanan hayvanların akciğerlerinde bakteri kolonizasyonunda kontrol gruplarına göre azalma görülmemiştir. Fareler enfekte edildikten ve 20 µg ve 80 µg rekombinant FimX ile ikinci kez aşılandıktan (30. gün) sonra, farelerin dalakları alınarak dalak hücre kültürü oluşturulmuştur. Hücreler rekombinant protein ile uyarıldıktan sonra supernatantlar toplanmıştır ve sitokin ELISA ile IFN-γ seviyesi ölçülmüştür. Kontrol ve aşılı fareler arasında IFN-γ üretimi açısından önemli ölçüde bir farklılık görülmemiştir.

Anahtar kelimeler: Boğmaca, *Bordetella pertussis*, Altbirim aşı, Bağışık yanıt

To my family

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'If there's a will, there's a way'

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bp(s)	: Base pair(s)
B. pertussis	: *Bordetella pertussis*
DC	: Dendritic cell
DTPw	: Diphteria, Tetanus, whole cell Pertussis vaccine
DTPa	: Diphteria, Tetanus, acellular Pertussis vaccine
E. coli	: *Escherichia coli*
FimX	: Fimbrial subunit X
IPTG	: Isopropyl-b D-thiogalactopyranoside
i.p.	: Intraperitoneal
IFN-γ	: Interferon gamma
IL	: Interleukin
IgE	: Immunogobulin E
IgG	: Immunoglobulin G
kDa	: Kilodalton
LPS	: Lipopolysaccharide
NCBI	: National Center for Biotechnology Information
OD	: Optical Density
PPIase	: Peptidyl-prolyl cis-trans isomerase
SDS-PAGE	: Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
Th	: T helper cell
TNF	: Tumor necrosis factor

CHAPTER 1

INTRODUCTION

1.1. Whooping Cough (Pertussis)

Whooping cough (pertussis) is a contagious respiratory tract disease caused by the obligate human pathogen *Bordetella pertussis* which is a small fastidious Gram-negative coccobacillus. In addition, human-adapted *B. parapertussis* causes also a milder pertussis-like disease (Cherry and Heininger, 2004). Complications of the disease include convulsions, encephalopathy, encephalitis, permanent brain damage and death in some cases (Cherry *et al*., 1988; Wortis *et al*., 1999). Although, pertussis is predominant in infants and children aged 1-5 years old, it can also be seen in adolescents and adults (Wood and Mclntyre, 2008). Globally, annual deaths from pertussis were about 295,000 (Bettiol *et al*, 2010). The number of reported cases according to years and regions is given in Table 1.1 (WHO, 2009).

Table 1.1. Regional and global summaries of pertussis incidence (number of reported cases): 2005-2008 (WHO, 2009)

WHO region	**2005**	**2006**	**2007**	**2008**
African Region	22,139	18,399	21,197	19,425
Region of the Americas	34,654	24,516	19,753	26,834
Eastern Mediterranean Region	5,164	10,060	14,213	7,849
European Region	26,425	30,168	28,798	25,278
South-East Asia Region	25,675	27,657	72,981	46,937
Western Pacific Region	21,560	9,164	6,634	25,245
Global	135,617	119,964	163,576	151,568
Number of countries	162	161	164	164

Pertussis has three typical phases, namely catarrhal, paroxismal and convalescent (Table 1.2). Catarrhal stage begins with nonspecific symptoms including rhinorrhoea, sore throat, conjunctivitis and non-productive cough. This stage lasts 1-2 weeks and the bacteria can easily spread. In the second stage, non-productive cough turn into paroxysmal cough with mucous production and episodes of coughing typically end with a deep inspiration (whoop) and vomiting. Paroxysms are more common at night. They occur spontaneously or are precipitated by external stimuli such as noise and cold air. It usually lasts 1-2 weeks or longer. During the third stage, the cough becomes less frequent. If another respiratory infection is acquired, paroxysms increase. This stage can last from 2 weeks to several months (Weiss and Hewlett, 1986; Sinha and Heath, 2005).

Table 1.2. Typical course of pertussis: evolution of symptoms (Tozzi *et al*., 2005).

Variable	Catarrhal phase (1-2 wk)	Paroxismal phase (3-6 wk)	Convalescent phase (>6 wk)
Symptom			
Cough	++	+++	++
Paroxysmal cough	-/+	+++	-/+
Whooping cough	-	+++	-/+
Vomiting	-	+++	-/+
Cyanosis	-	+++	-
apnea	-	+++	-

Note: + present, - absent, -/+ equivocal

All patients may not exhibit typical symptoms of the disease. Age, individual immunity and time elapsed since previous pertussis infection or vaccination can affect the incidence of the symptoms. For example, although severe complications such as pneumonia and apnea are commonly seen in newborns, they may not show coughing spasms. On the other hand, adolescents and adults may present only with prolonged coughing but without other typical findings such as whooping or vomiting (Riffelmann *et al*., 2008).

It is suggested that the transmission of the disease occurs via *B. pertussis* carried with aerosol droplets which are caused by severe coughing or direct contact with respiratory secretions from infected individuals (Tiwari *et al*., 2005). After inhalation of aerosolic bacteria, they colonize the mucosa of the respiratory tract with the help of pertussis toxin and filamentous hemagglutinin (Figure 1.1). The organism interacts with ciliated bronchial epithelial cells and can invade and survive within local immune cells such as macrophages (Maskell and Preston, 2002; Mattoo and Cherry, 2005; Tozzi *et al*., 2005).

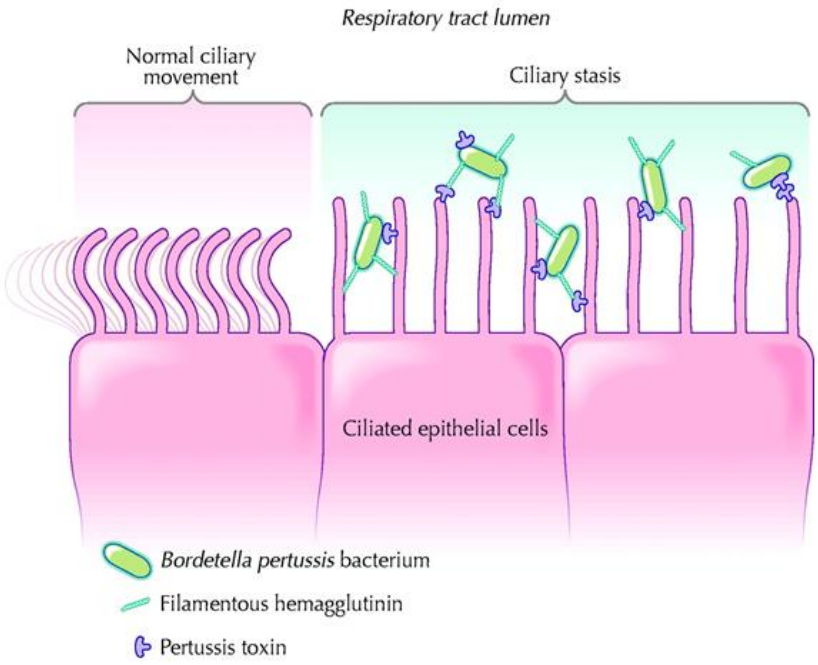


Figure 1.1. Synergy between pertussis toxin and filamentous hemagglutinin in binding to ciliated respiratory epithelial cells (Tozzi *et al*., 2005).

It is thought that the interaction between bacteria and cells results in paralysis of the cilia and death of ciliated cells, possibly due to synergistic effect of pertussis toxin and filamentous hemagglutinin (Tozzi *et al*., 2005). In addition to pertussis toxin,

expression of other toxins cause further damage in respiratory tract and these toxins can interfere with immune responses. At this point, patients start to exhibit characteristic symptoms of pertussis and it is suggested that the primary cause of the prolonged cough is pertussis toxin. Depending on the patient response, the infection can be cleared over time or may proceed to pertussis pneumonia and possibly death (Van Den Berg *et al*., 1999).

For treatment of the disease, erythromycin and newer macrolides such as azithromycin and clarithromycin are used. For clinical effectiveness, antibiotic treatment should be given rapidly (Lebel and Mehr, 2001; Langley *et al*., 2004).

1.2. The Genus *Bordetella* and *Bordetella pertussis*

Bordetella which is a genus that belongs to the phylum proteobacteria encompasses a group of Gram-negative, small coccobacilli. They are obligate aerobes and cannot ferment carbohydrates (Weiss, 2006). The genus currently consists of ten species with the new identified one, *Bordetella ansorpi*; *B. pertussis*, *B. parapertussis* (ovine-adapted), *B. parapertussis* (human-adapted), *B. bronchiseptica*, *B. avium*, *B. hinzii*, *B. trematum*, *B. petrii*, *B. holmesii* (Figure 1.2).

Within these all known species, three members, namely *B. pertussis*, *B. parapertussis*$_{hu}$, and *B. bronchiseptica* have been associated with respiratory infections in humans (Weiss, 2006). Parkhill *et al*. (2003) have sequenced the genomes of them for comparative analysis (Table 1.3) and they indicated that *B. pertussis* and *B. parapertussis* were derived from *B. bronchiseptica*-like ancestors independently. In concordance with this, another study showed that *B. parapertussis* evolved from an animal-associated lineage of *B. bronchiseptica*, while *B. pertussis* evolved from a distinct *B. bronchiseptica* lineage that may already have had a preference for hominids up to 2.5 million years ago (Diavatopoulo *et al*., 2005).

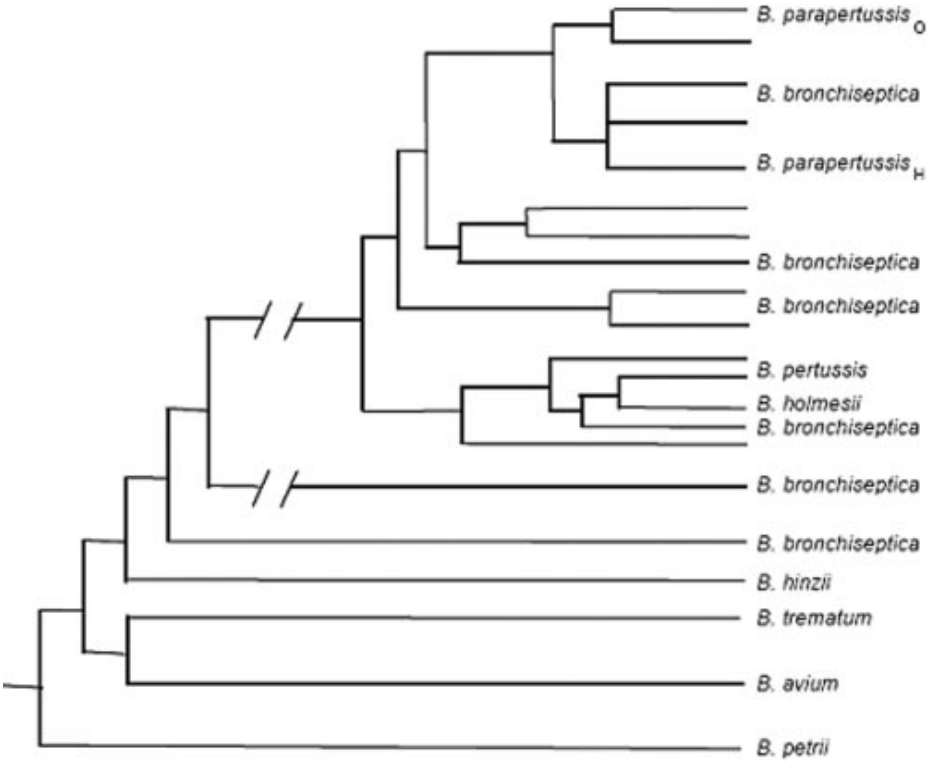


Figure 1.2. Phylogenetic relationships of the nine *Bordetella* species (Mattoo and Cherry, 2005).

Table 1.3. General overview of *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* genomes (Sebaihia *et al*., 2006).

	B. pertussis	***B. parapertussis***	***B. bronchiseptica***
Genome size (bp)	4,086,186	4,773,551	5,339,179
Number of genes	3,816	4,404	5,011

Among the ten species, *Bordetella pertussis* is the causative agent of whooping cough. It is a gram-negative, non-spore forming bacterium that was first isolated by Bordet and Gengou in 1906 (Figure 1.3) (Bordet and Gengou, 1906).

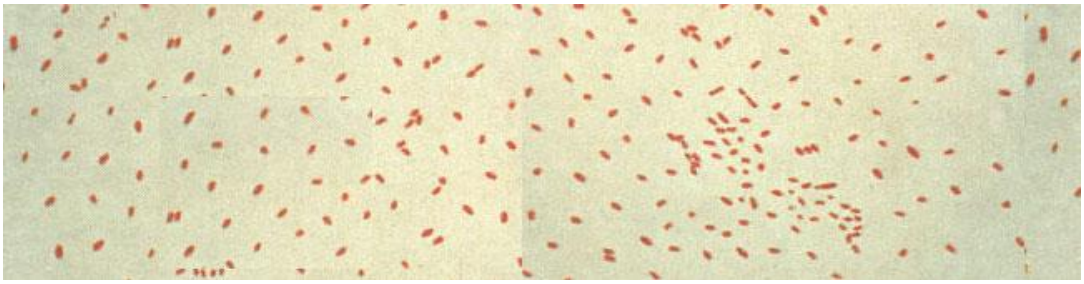


Figure 1.3. Gram stained cells of *B. pertussis* (*http://textbookofbacteriology.net/pertussis.html*).

B. pertussis is fastidious in terms of nutrition and it usually grows on rich media containing blood. It is also possible to cultivate the bacteria on synthetic medium supplemented by nicotinamide for growth, amino acids for energy, charcoal for absorption of fatty acids and other noxious substances (Long and Edwards, 2003). The growth of the organism is slow and colonies start to be seen after 3-6 days, even on blood agar (Todar, 2008).

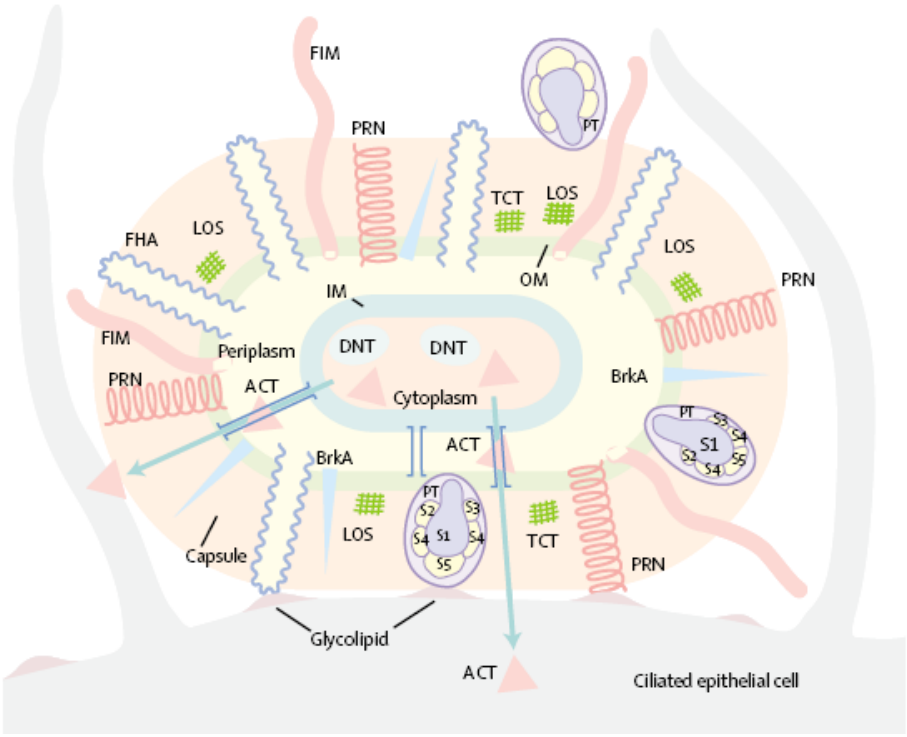
During the disease, *B. pertussis*-host interactions which occur predominantly at respiratory surfaces are mediated by virulence factors of the organism (Mattoo *et al*., 2001).

1.3. Virulence Factors of *Bordetella pertussis*

Virulence factors of *B. pertussis* can be divided in two categories: adhesins and toxins. Adhesins contain filamentous hemagglutinin, pertactin, fimbriae, tracheal colonization factor and serum-resistance protein. Toxins include pertussis toxin, adenylate cyclase toxin, dermonecrotic toxin, tracheal cytotoxin and lipopolysaccharide (Mattoo and Cherry, 2005).

Like many other bacterial pathogens, growth conditions affect the expression of these virulence factors (Figure 1.4). Two important phenomena; phase variation and

phenotypic modulation are related to the regulation of the virulence genes. In phase variation, the genotype of the bacteria is irreversibly altered by frameshift mutations. It results in loss of the ability to synthesize toxins and other pathogenicity factors. In phenotypic modulation, expression of the virulence factors is repressed at low temperature or in the presence of nicotinic acid or $MgSO_4$. The responsible locus for both phenomena is called *bvg* (*Bordetella v*irulence *g*ene) which encodes two proteins, BvgS and BvgA (Lacey, 1960; Locht, 1999; Babu *et al*., 2002).



FIM	Fimbriae	DNT	Dermonecrotic toxin
FHA	Filamentous haemagglutinin	TCT	Tracheal cytotoxin
PRN	Pertactin	BrkA	Serum resistance protein
PT	Pertussis toxin	ACT	Adenylate cyclase toxin
LOS	Lipooligosaccharide		

Figure 1.4. Virulence factors associated with the pathogenesis of *Bordetella pertussis* infection (Crowcroft and Pedoby, 2006).

1.3.1. BVG System

Like all organisms, bacteria sense their environment and control their behavior appropriately to survive. The two-component regulatory system is the most common mechanism for this purpose (Ninfa and Magasanik, 1986; Nixon *et al*., 1986; Hess *et al*., 1988). The mechanism in *B. pertussis* is termed BVG system which is encoded by *bvgAS* locus. The system is composed of two proteins, BvgS as a transmembrane, polydomain integral membrane sensor kinase and BvgA as a DNA binding response regulator (Uhl and Miller, 1995). The *bvgAS* locus regulates expression of the virulence genes both positively and negatively. Response to environmental conditions causes three distinct phenotypic phases that are controlled by BVG system (Mattoo and Cherry, 2005).

BvgAS phosphorelay (Figure 1.5) is activated under the growth conditions at 37°C and in the absence of $MgSO_4$ or nicotinic acid. Under bvg^+ phase condition, BvgS autophosphorylation takes place leading to activation of BvgA by phosphorylation. Active BvgA interacts with alpha subunit of RNA polymerase and the transcription of vir-activated genes (*vag*) is triggered while an additional class of genes, *vrg* (*vir*-repressed genes), is repressed (Boucher *et al*., 1997; Mattoo and Cherry, 2005). The repression of some of these genes is controlled by the expression of an intermediate regulatory gene, *bvgR*, under the transcriptional control of BvgA. So, in the virulent phase, active BvgAS system is involved in the expression of both *vags* and *bvgR* which in turn represses some of the genes in *vrg* class (Locht, 1999).

Under "modulating" conditions, such as at 25°C in the presence of ≥10 mM nicotinic acid or ≥40 mM $MgSO_4$, the BvgAS phosphorelay can be inactivated (Melton and Weiss, 1993). So, under bvg^- phase condition, *vag*s cannot be activated by BvgAS and *vrg*s are expressed (Deora *et al*., 2001). In bvg^i phase, Bvg-repressed phenotypes are absent and some of bvg-activated virulence genes such as *fhaB* are expressed

(Deora *et al*., 2001). It is thought that this phase is involved in transmission of the bacteria between hosts (Vergara-Irigaray *et al*., 2004).

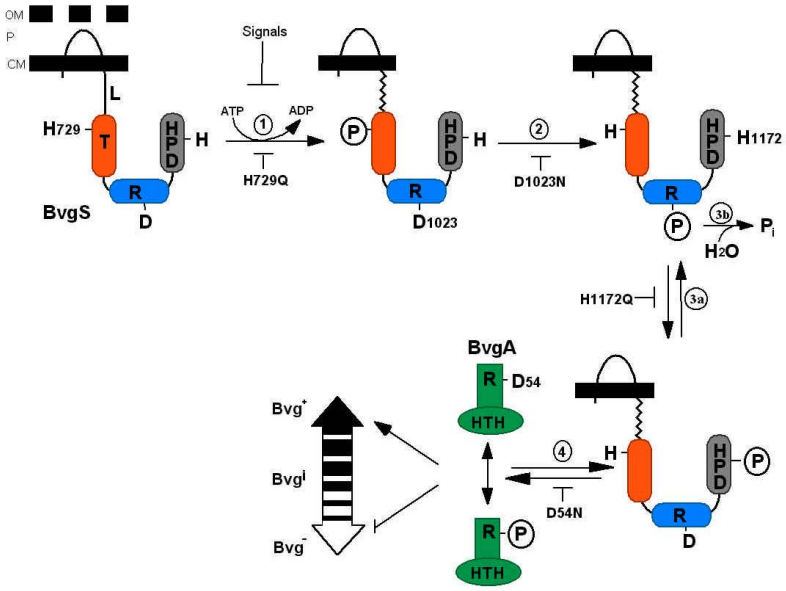


Figure 1.5. The BvgAS phosphorelay (Mattoo *et al*., 2001).

Characteristics of these three phases imply the presence of four classes of Bvg-regulated genes (Figure 1.6). Class 1 contains the genes that are expressed maximally only in the Bvg^+ phase such as *cyaA-E* coding adenylate cyclase toxin and *ptxA-E* coding pertussis toxin (Gross and Rappuoli, 1988; Steffen *et al*., 1996; Julio and Cotter, 2005). Class 2 genes such as *fhaB* coding for filamentous hemagglutinin are expressed maximally in both Bvg^+ and Bvg^i phases (Relman *et al*., 1989; Domenighini *et al*., 1990; Jacob-Dubuisson *et al*., 2000). Class 3 contains *bipA* gene expressed exclusively in the Bvg^i phase. It encodes BipA (Bvg-intermediate phase protein A) which shares amino acid sequence similarity with intimin of enteropathogenic *Escherichia coli* and invasin of *Yersinia* spp. (Stockbauer *et al*., 2001). Class 4 contains the genes that are expressed maximally only in the Bvg^- phase (Mattoo and Cherry, 2005).

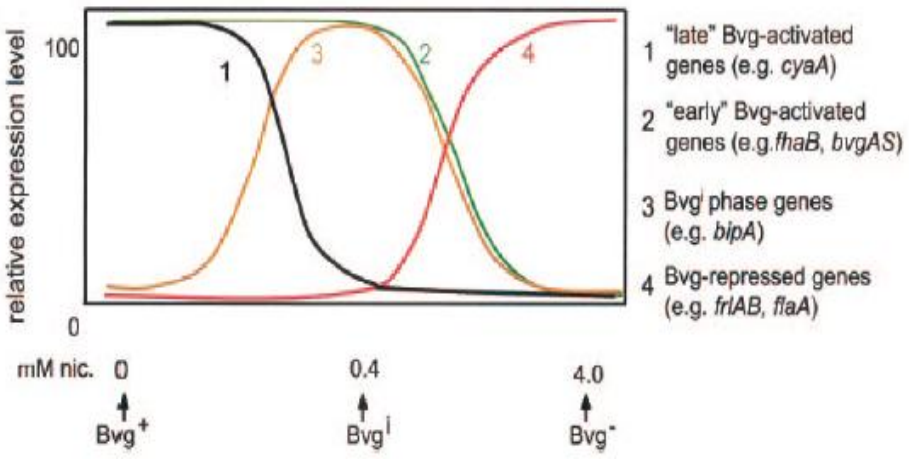


Figure 1.6. BvgAS controls expression of at least four classes of genes (Mattoo and Cherry, 2005).

1.3.2. Adhesins

1.3.2.1. Filamentous Hemagglutinin (FHA)

Filamentous hemagglutinin is a large, highly immunogenic, mainly cell-associated protein which is included in acellular pertussis vaccines. Identification of several binding domains suggested that FHA functions for adhesion (Inatsuka, 2005). FHA structural gene, *fhaB*, encodes a 367 kDa precursor, referred to as FhaB and exported into the periplasm (Hodak *et al*., 2006). After modification of N terminus and cleavage of C terminus of FhaB, the mature protein FHA (220 kDa) represents 60% N-terminal portion of FhaB (Renauld-Mongenie *et al*., 1996). FHA is mainly found on cell surface, but can be released in substantial amounts by the protease SphB1.

The full-length FHA contains three distinct binding domains: (1) an N-terminal glycosaminoglycan-binding site, (2) two arg–gly-asp (RGD) sequences which interact with leukocyte response integrin/integrin-associated protein complex on macrophages and (3) a carbohydrate recognition domain (CRD) which has role in

adherence to respiratory epithelial cells and macrophages (Prasad *et al*., 1993; Ishibashi *et al*., 1994).

The protein contains two main immunodominant regions, identified as type I and II domains (Piatti *et al*., 1999). Type I domain is located at C-terminus and seems to be more immunodominant than type II located at N-terminus. While purified protein alone is not sufficient to induce a protective immune response, the antigen with adjuvant is efficient in terms of protectivity (Knight *et al*., 2006). *In vitro* and *in vivo* studies show that FHA mediates induction of FHA-specific T regulatory cells in a respiratory model and secretion of inflammatory and anti-inflammatory cytokines by macrophages (Abramson *et al*., 2001; Mcguirk *et al*., 2002).

1.3.2.2. Fimbriae (FIM)

B. pertussis produces filamentous, polymeric protein cell surface structures called fimbriae which belong to the same family as the type 1 fimbriae of enterobacteria (Willems *et al*., 1994). The *B. pertussis* fimbriae contain major and minor subunits. The major subunits form the two predominant serotypes, Fim2 (22.5 kDa) and Fim3 (22 kDa) encoded by *fim2* and *fim3*, respectively (Mattoo *et al*., 2001). In a recent study, SDS-PAGE and Western blot analysis using anti-Fim3 monoclonal antibodies showed that Fim3 could not be detected in Tahoma I strain, unlike in Saadet (Atakan and Ozcengiz, 2007). Different studies showed the immunogenicity of Fim2 and Fim3 serotypes in different strains (Zhang *et al*., 1985; Atakan and Ozcengiz, 2007; Xu *et al*., 2009). The minor subunit named FimD (40 kDa) functions in binding to the integrin Vla-5 and heparin (Smith *et al*., 2001). Another fimbrial subunit gene called *fimX* is also found in the bacteria and it is suggested that this gene is silent or expressed at undetectable level (Pedroni *et al*., 1989; Willems *et al*., 1990; Riboli *et al*., 1991). The length of the C-stretch in *fim2* and *fim3* promoter has effect on transcriptional activity and the C stretch in the *fimX* promoter is much shorter than in the promoter of *fim2* and *fim3* (Willems *et al*., 1990; Gogol *et al.,* 2007). So, it is

suggested that *fimX* promoter is active but less strong than the other two (Riboli *et al*., 1991).

For the assembly of fimbriae, two proteins are required, FimB and FimC which are periplasmic chaperone and outer-membrane usher protein, respectively. While the *fim2, fim3* and *fimX* genes are dispersed along the *B. pertussis* chromosome, the *fimBCD* genes are found together with the *fha* and *bvg* genes (Willems *et al*., 1994). It is thought that *fim2, fim3* and *fimX* genes may have been evolved after initial duplication from *fimA* gene which is a pseudogene located at upstream of *fimB*. Interestingly, *fimA* is truncated in *B. pertussis*, whereas it is found as complete in *B. bronchiseptica* and *B. parapertussis* (Boschwitz *et al*., 1997). Expression of the fimbrial subunit genes is under positive control of Bvg system. In addition to this regulation, the *fim* genes are subject to fimbrial phase variation which occurs by small insertions or deletions of C-residues in promoter regions (Mattoo *et al*., 2001). These mutations have effects on the transcription of the *fim* genes. Thus, while some strains produce both types of fimbriae (serotype 2, 3), some contain only one, or none at all (Locht, 1999).

Fimbriae of *B. pertussis* have a role in colonization of mouse trachea and induction of nitric oxide production in murine macrophages. *In vivo* studies have demonstrated that *B. pertussis* strains lacking fimbriae lose the ability to multiply in the nasopharynx and trachea of mice (Mooi *et al*., 1992; Geuijen *et al*., 1997; Xing *et al*., 2000). Additional protection of Fim 2 and 3 in multicomponent pertussis vaccines were demonstrated in humans. So, they are included in five-component acellular pertussis vaccines (Marwick, 1995; Parton, 1999).

1.3.2.3. Pertactin (PRN)

Pertactin encoded by *prn* gene is an outer-membrane protein with an apparent molecular weight of 69,000 Da (Locht, 1999). It is produced as a 93,478 Da

precursor called P.93 which undergoes two proteolytic cleavages. One of them is the cleavage of its 34 amino acid signal peptide and the other one is the cleavage of its 30 kDa C-terminal domain (P.30) (Figure 1.7) (Charles *et al*., 1994; Junker *et al*., 2006). The C-terminal region (P.30) remains anchored in the outer membrane and is essential for the correct localization of pertactin on the cell surface and its secretion. P.69 pertactin is transported into the periplasm by a classical signal-peptide dependent secretion mechanism (Locht, 1999). Pertactin is polymorphic between the various *Bordetella* species, and even between different strains within the same species. The *B. pertussis*, *B. bronchiseptica* and *B. parapertussis* pertactins differ primarily in the number of proline-rich regions they contain (Li *et al*., 1992; Mooi, *et al*., 1998).

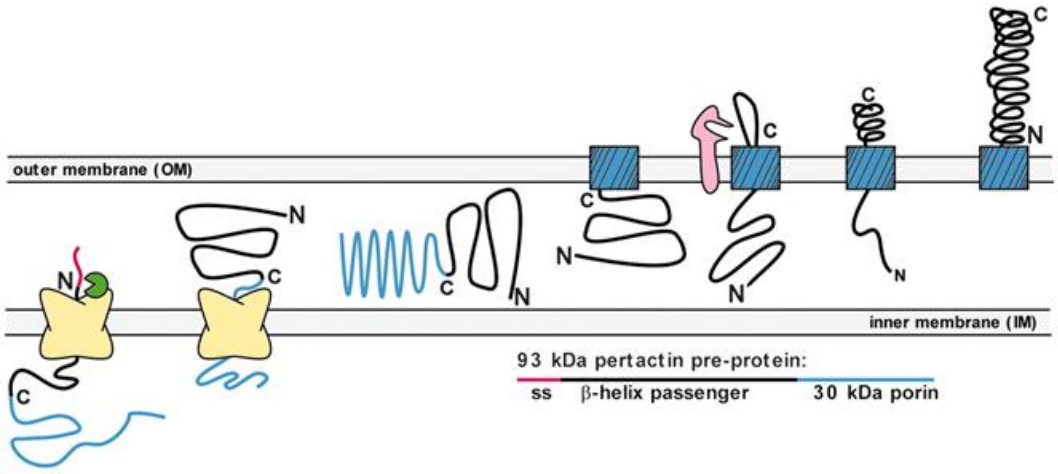


Figure 1.7. Pertactin biogenesis (Junker *et al*., 2006).

The studies showed that mutants deficient in PRN are able to colonize the mouse respiratory tract efficiently. However, mutants lacking both PRN and FHA are cleared more rapidly than the wild-type parents. This suggests that pertactin is involved in bacterial adhesion. Due to its role in adhesion and its protective activity

in animal models, PRN is included in some of the new acellular vaccines (Gustafsson *et al*., 1996).

1.3.2.4. Serum Resistance Protein (BrkA)

The *brk* (*Bordetella* resistance to killing) locus which is also found in *Bordetella parapertussis* and *Bordetella bronchiseptica* encodes two proteins, BrkA and BrkB. BrkA produced as a 103-kDa precursor is proteolytically processed to a 73-kDa N-terminal passenger domain and a 30-kDa C-terminal domain. It shares homology with pertactin and provides resistance to classical-pathway, complement-dependent killing by human serum. BrkA is also involved in adherence and invasion. BrkB has similar structure to various transporters and it is thought that the protein is found at cytoplasmic membrane (Fernandez and Weiss, 1994; Marr *et al*., 2008).

It was demonstrated that recombinant BrkA significantly increased the efficacy of two-pertussis-component DTPa vaccine against *B. pertussis* in the sublethal intranasal murine respiratory challenge model of *B. pertussis* infection. This suggests that it is a promising candidate antigen to improve existing acellular pertussis vaccines for use in humans (Marr *et al*., 2008).

1.3.2.5. Tracheal Colonization Factor (TCF)

Tcf encoded by the *tcfA* gene is expressed by *B. pertussis,* but not by *B. parapertussis* and *B. bronchiseptica*. Tcf has an important role in colonization of the trachea and the amino terminal of the protein shows 50% identity with the pertactin precursor (Finn and Stevens, 1995; Kerr and Matthews, 2000). There are several proline-rich regions in its sequence and, like FHA, pertactin and Brk, Tcf also contains an Arg-Gly-Asp sequence. The C-terminal end of Tcf is an autotransporter domain and it has high degree of sequence similarity to those of pertactin and Brk. Tcf can be found in two forms, either as cell-associated form, or as secreted form

(Locht, 1999). The amino terminal of the protein shows 50% identity with the pertactin precursor (Kerr and Matthews, 2000).

It was shown that the strain lacking Tcf displayed ten-fold decrease in the number of bacteria from the trachea of infected mice when compared with the parent strain (Finn and Stevens, 1995).

1.3.3. Toxins

There are five toxins of *B. pertussis*. Most of them are protein toxins, except for lipopolysaccharide, also called endotoxin, and the tracheal cytotoxin, which is a fragment of the *Bordetella* peptidoglycan. The three major protein toxins are the pertussis toxin, the adenylate cyclase toxin and the dermonecrotic toxin (Locht, 1999).

1.3.3.1. Pertussis Toxin (PT)

Pertussis toxin is secreted only by *B. pertussis*. In non-pertussis species, *B. parapertussis* and *B. bronchiseptica*, expression of the gene does not occur due to inactivation in the promoter region (Arico and Rappuoli, 1987).

PT is an AB type toxin composed of six polypeptides, S1, S2, S3, S4 (2) and S5, which are encoded by the *ptxA* to *ptxE* genes, respectively. A subunit contains S1 polypeptide and B subunit contains S2, S3, S4 and S5 with a ratio 1:1:2:1 (Figure 1.8) (Mattoo and Cherry, 2005). While B subunit of the toxin has a role for binding to receptors of target cells, A subunit which is the toxin itself has an ADP-ribosyl transferase activity. A subunit causes an increase in cAMP in bronchial mucosal cells. The toxin leads to gain resistance to phagocytosis by deregulating macrophages (Higashi *et al*., 2009). PT targets a large number of G proteins. Its ADP-ribosylation activity causes activation of adenylate cyclase by inhibiting hydrolysis of GTP to

GDP. This causes an increase in cAMP levels which leads to disorder in cellular functions and decrease in phagocytic function of phagocytes (Babu *et al*., 2002).

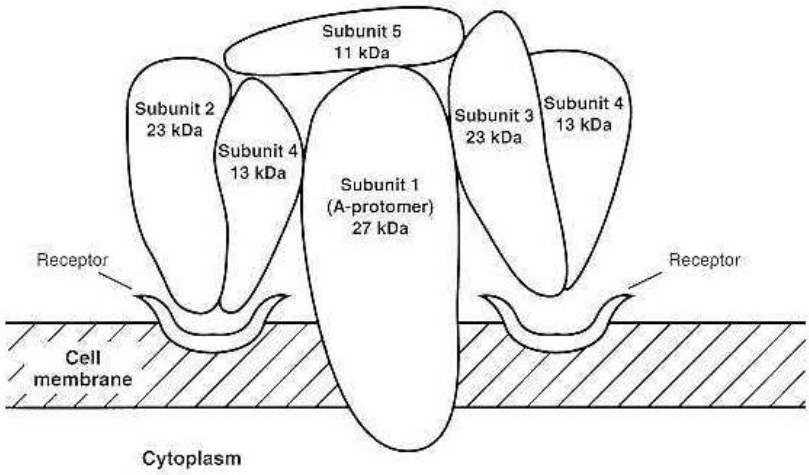


Figure 1.8. Binding of pertussis toxin to a target cell *(http://www.freewebs.com/ekelley13/index.htm).*

The studies have shown that PT has protective activity in mice. It induces high level of antibody production and protects mice which were challenged intracerebrally with live *B. pertussis* (Sato *et al*., 1984; Munoz *et al*., 1989). Also, it is believed that antibody to PT has a critical role in protection in humans (Pittman, 1984). For these reasons, PT is one of the major components of new acellular vaccines (Locht, 1999).

It has been demonstrated that the S1 subunit of PT is polymorphic and that the frequency of its variant shows shifts in time (Mooi *et al*., 1998). Also, in recent studies, *B. pertussis* strains having increased pertussis toxin production and polymorphism in the promoter region of the gene were observed which were associated with the increase in pertussis incident in the Netherlands and the United States (Cassiday *et al*., 2000; Mooi *et al*., 2009).

1.3.3.2. Adenylate Cyclase Toxin (ACT or CyaA)

Adenylate cyclase is a biofunctional calmodulin-sensitive toxin with adenylate cyclase function and hemolytic activity. CyaA toxin expressed maximally in the Bvg+ phase has a critical role in the early stages of bacterial colonization (Mattoo and Cherry, 2005). It has hemolytic and catalytic (CA) domains. While hemolytic domain plays a role in binding and endocytosis of the toxin into target cells, AC domain is activated by calmodulin and exhibits catalytic activity (Ladant and Ullman, 1999). After entry into the cell, AC leads to increase in intracellular cAMP level which causes paralysis of the functions of phagocytic and immune effector cells (Kerr and Matthews, 2000).

1.3.3.3. Dermonecrotic Toxin (DNT)

Dermonecrotic toxin is a 102 kDa heat-labile toxin which is also called lethal toxin and found in all species of *B. pertussis*. It has two domains, a 30 kDa binding domain at N-terminal and a 24 kDa active domain at C-terminal (Kashimoto *et al.*, 1999). DNT is in the family of cytotoxic necrotizing factor (CNF 1 and 2) which has effects on regulation of cell division or growth. It induces inflammation and causes dermonecrotic lesions where the bacteria colonize. DNT decreases the expression of type I collagen in an osteoblast-like cell line and causes polynucleation by stimulating DNA and protein synthesis without cell division. It also leads to the assembly of actin stress fibres and tyrosine phosphorylation of focal adhesion kinase. It is believed that these effects are the result of glutamine 63 deamidation of the small GTP-binding protein called RhoA caused by DNT (Babu *et al*., 2002).

A study showed that intradermal injection of the toxin led to necrotic lesions in mice and intravenous administration was lethal for mice at low doses (Mattoo and Cherry, 2005).

1.3.3.4. Tracheal Cytotoxin (TCT)

Tracheal cytotoxin is a fragment of peptidoglycan which is a disaccharide-tetrapeptide monomer (Cookson *et al*., 1989). Although this fragment is found in all Gram-negative bacteria during breaking down and rebuilding of cell wall, the studies showed that it was released into the environment only by *Bordetella* spp. and *Neisseria gonorrhoeae* (Sinha *et al*., 1980; Rosenthal *et al*., 1987).

Besides *B. pertussis*, other *Bordetella* species also produce TCT and its production is not under the control of the Bvg system (Locht, 1999). It seems that the toxin causes specific destruction of ciliated epithelial cells in respiratory tract and prevents DNA synthesis in vitro (Goldman, 1986; Cookson *et. al*., 1989).

1.3.3.5. Lipopolysaccharide (LPS)

Lipopolysaccharide is found in all *Bordetella* species. However, its structure shows differences between species. There are two different lipids, A and X, and two different oligosaccharides, I and II, in *B. pertussis.* The ratio of LPS I to LPS II may show variety between strains. The intact LPS has endotoxin-like effects and causes hypersensitivity to histamine (Munoz *et al*., 1978; Locht, 1999). In addition, it activates macrophages and stimulates tumor necrosis factor production (Watanabe *et al*. 1990).

1.4. Vaccine Development

The history of vaccines starts with the first vaccine against rabies developed by Louis Pasteur in 1881. For over a century, Pasteur's method including the isolation, inactivation and injection of the causative agent of an infectious disease, were used as a guide to produce vaccines. Many different types of vaccines have been developed with application of Pasteur's principles (Table 1.4) (Serruto and Rappuoli, 2006).

Traditional vaccines such as inactivated vaccines and live, attenuated vaccines are produced from microorganism itself. Microorganisms causing diseases are killed with chemical or physical treatments to produce inactivated vaccines. These vaccines are safe and pathogens cannot revert to disease-causing form. Live, attenuated vaccines include pathogens which have lost virulence properties. They induce both cell-mediated and antibody-mediated immunity (NIH, 1998). In the last decade, genomic and immunoproteomic studies have provided new approaches for vaccine development. Conjugate vaccines, subunit vaccines, recombinant vector vaccines and DNA vaccines are produced by using these new approaches such as recombinant DNA technology. In conjugate vaccines, an outer coat component of the pathogen is linked to a protein carrier. Subunit vaccines include antigenic fragments from pathogen that induce immune response. Recombinant vector vaccines are produced from weakened virus or bacterium which is used to carry pathogen DNA. DNA vaccines contain plasmid DNA that encodes antigenic proteins and they elicit both humoral and cellular immunity. Besides recombinant genetic engineering, reverse vaccinology also provides genome-based way for vaccine development (Figure 1.9) (Donnely *et al*., 1997; NIH, 1998; Serruto and Rappuoli, 2006).

With the development of technology, new strategies will be available to produce effective vaccines, especially against the pathogens that cannot be cultivated or for which the applications of Pasteur's principles have failed.

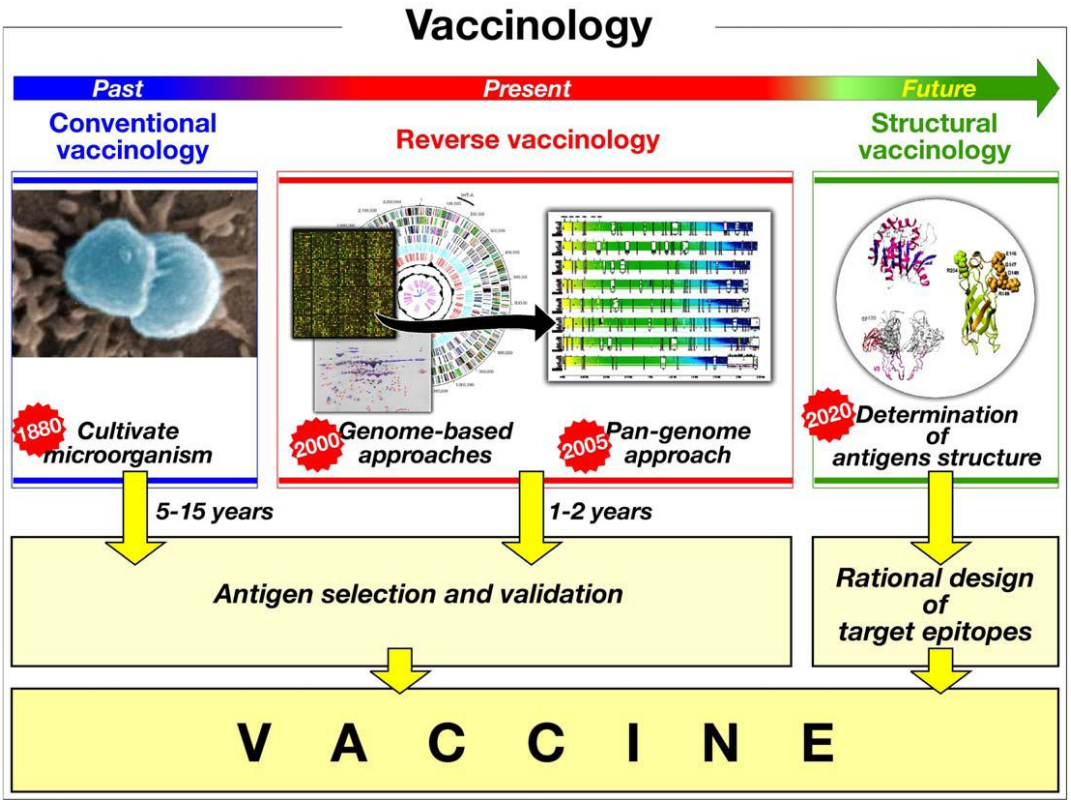


Figure 1.9. Different approaches for vaccine development (Serruto and Rappuoli, 2006).

Table 1.4. Different types of vaccines (Serruto and Rappuoli, 2006).

Type	Description	Advantages	Drawbacks	Examples
Killed microorganisms	The causative agent is inactivated by chemical or physical treatments	Efficacious	*Some pathogens are difficult or almost impossible to cultivate in a scalable setting *Regulatory authorities require high safety and quality standards for all new vaccine formulations; obtaining approval might be difficult	Polio virus vaccine (Salk) Influenza vaccine Rabies vaccine Oral cholera vaccine
Live attenuated microorganism	The causative agent is live but it has lost the ability to cause the disease	Efficacious, induce a protective immune response	As above	Polio virus (Sabin) Intranasal influenza vaccine (Cold adapted) Measles, mumps and rubella (MMR)
Subunit	Vaccines contain purified portions of the causative agents	*There is no risk that vaccines can provoke the disease *If recombinant form of the selected components are utilized, the pathogen need not be cultivated	The identification of the few protective components from the pool of molecules present in the pathogen is usually complex and time consuming	Diphtheria toxoid Tetanus toxoid Pertussis toxoid Hepatitis B vaccine
Subunit-conjugated	A polysaccharide component of the causative agent is chemically linked to a protein carrier	The conjugated polysaccharide that is poorly immunogenic on its own becomes immunogenic	*Need to grow the pathogen in vitro to obtain the capsule polysaccharide *Capsule not always immunogenic *Too many capsule types	*Haemophlius influenza* Meningococcus A, C, Y, W135 Pneumococcus

1.5. Current Vaccines against Whooping Cough (Pertussis)

Following the identification of pertussis, many vaccines were developed against the disease. The first pertussis vaccines were whole-cell vaccines developed in the 1940s. The first test of whole-cell vaccine containing dead *B. pertussis* organisms was done with children in Denmark and Tunisia (Howson *et al*., 1991).

Many different methods were used to prepare whole cell vaccines. While some vaccines included culture media, some of them contained other bacteria found in respiratory tract as well as *B. pertussis* (Mattoo and Cherry, 2005; Crowcroft *et al*., 2006). They have been used worldwide until replacing them with acellular pertussis vaccines. At first, acellular pertussis vaccines were monocomponent. Since 1947, combined pertussis vaccines with diphtheria and tetanus toxoids (DTP) have been used to protect against the diseases caused by the three bacteria (Mattoo and Cherry, 2005). The preparation of DTP vaccines differ between manufacturers due to the different *B. pertussis* strains used in vaccine development (Edwards *et al*., 2004).

It has been shown that while whole cell pertussis vaccines (DTwP) induce Th1 cells and production of IgG2a antibodies predominantly, acellular pertussis vaccines (DTaP) induce Th2 cells and high levels of IgG1 in mice (Figure 1.10). In addition, it seems that immunization with DTwP provides protection by cellular immunity, whereas humoral immunity mediates protection in DTaP immunization (Mills, 2001).

Although whole-cell vaccines were successful in significantly reducing morbidity and mortality worldwide, its reactogenicity and association with mild to severe neurological complications such as encephalopathy and convulsions led to the development of acellular pertussis vaccines (Church, 1979; Miller *et al*., 1981; Blumberg *et al*., 1993). The risks of encephalopathy and convulsions differ according to the vaccine origin and strains used in vaccine preparation. According to the study,

the estimated risk for encephalopathy is 3/106, the risk for fever convulsions is 1/19,496 and it is 1/76,133 for convulsion without fever (Edwards *et al.*, 2004).

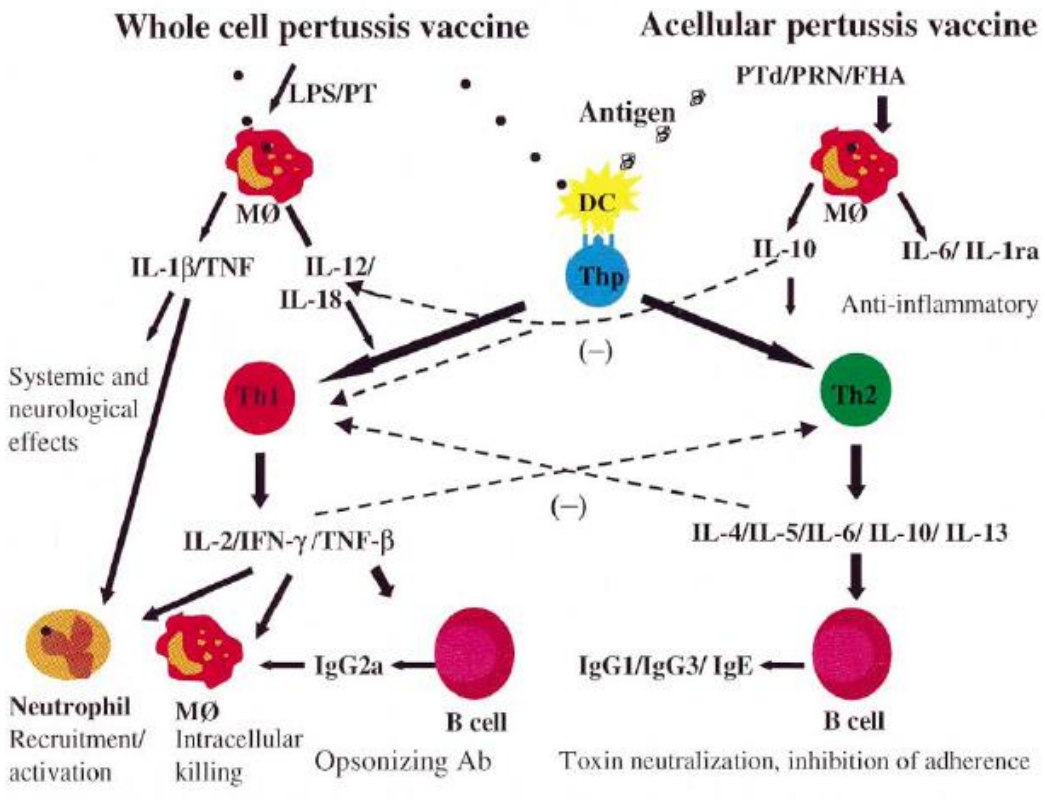


Figure 1.10. Distinct mechanisms of immunity induced with whole cell and acellular vaccines (Mills, 2001).

Decrease in vaccine coverage due to the fear of whole-cell pertussis vaccine toxicity led to increase in incidence of pertussis and to overcome this, the studies have been started to produce acellular pertussis vaccines having less or no adverse effects in 1970s. First acellular pertussis vaccine was developed in Japan by Sato *et al* (1984). The vaccine was composed of two major virulence factor of the bacteria, filamentous hemagglutinin (FHA) and pertussis toxin (PT). The study showed that these two components are enough to obtain protection against whooping cough (Sato *et al*.

1984). The findings about the components of *B. pertussis* have provided development of many different acellular pertussis vaccines containing one or more antigens and the studies demonstrated that these vaccines had lower reactogenicity (Table 1.5) (Edwards *et al*., 1986; Lewis *et al*., 1986; Edwards, 1993; Higashi *et al*., 2009). Since 1991, Food and Drug Administration (FDA) have given licenses for five different acellular vaccines (DTaP) which are ACEL-IMUNE, Tripedia®, Infanrix™, DAPTACEL® and Certiva™ in the United States. These vaccines have been used for the children aged 6 weeks-6 years (Mattoo and Cherry, 2005). Although the disease is predominant in infants, its incidence also increases among the adolescents and adults. For adolescents and adults, there are two licensed vaccines (Tdap), ADACEL® and Boostrix™. Boostrix™ and ADACEL® are used for people aged 10-18 and 11-64, respectively (Bamberger *et al*., 2008).

Table 1.5. Adverse reactions to whole cell and acelullar pertussis vaccines (Edwards, 1993).

	Frequency (%)	
Adverse reaction	**Whole cell**	**acellular**
Local redness	40	8-15
Local tenderness	52	5-7
Local swelling	38	5
Fever 37.7-38.7°C	46	3-5
Fever ≥ 38.8°C	4	0.5
Irritability	34	10

Although whole-cell vaccines have been used in most nations, most developed countries started to use acellular vaccines (Crowcroft *et al*., 2006). Vaccination schedules vary between countries but none of them starts earlier than 6 weeks of age (Wood *et al*., 2008) (Table 1.6).

Table 1.6. Pertussis vaccination schedules in selected countries (WHO, 1996).

Country/ Organization	Type of vaccine	Primary immunization schedule	Adolescent dTp booster recommended
WHO	DTPw	6,10,14 months	No
Australia	DTPa	2,4,6 months	Yes
Brazil	DTPw	2,4,6 months	No
Canada	DTPa	2,4,6 months	Yes
Egypt	DTPw	2,4,6 months	No
France	DTPa	2,3,4 months	Yes
Germany	DTPa	3,4,5 months	Yes
Italy	DTPa	3,5,11 months	No
South Africa	DTPw	6,10,14 months	No
Sweden	DTPa	3,5,12 months	Yes
UK	DTPa	2,3,4 months	No
USA	DTPa	2,4,6 months	Yes

DTPw: Diphtheria and tetanus toxoid with whole cell pertussis vaccine
DTPa: Diphtheria and tetanus toxoid with acellular pertussis vaccine

1.5.1. Vaccines against *B. pertussis* in Turkey

In Turkey, pertussis vaccine coverage reached a level of about 80% among children younger than six years of age following the National Vaccination Campaign of 1985, and it was maintained over the following years with improvement in vaccination procedures by the Ministry of Health (Aksakal *et al*., 2007) (Table 1.7). This led to decrease in incidence of pertussis from 21 cases per 100.000 in 1970 to 0.55 per 100.000 in 2004 (Figure 1.11).

Table 1.7. Vaccination coverage rates and pertussis incidence by regions, Turkey, 2005 (Dilli *et al*., 2008).

Regions	Coverage (%)	Pertussis cases (*n*)	Population (n)	Incidence per 100,000
1-Marmara	93	29	19,348,429	0,15
2-Egean	93	42	9,427,675	0,44
3-Mediterranean	93	12	9,884,437	0,12
4-Middle Anatolia	90	47	12,288,126	0,38
5-Black Sea	87	13	8,123,939	0,16
6-East Anatolia	84	114	6,404,473	1,78
7-Southeast Anatolia	85	15	7,492,222	0,20
Turkey (general)	~90	272	72,969,301	0,38

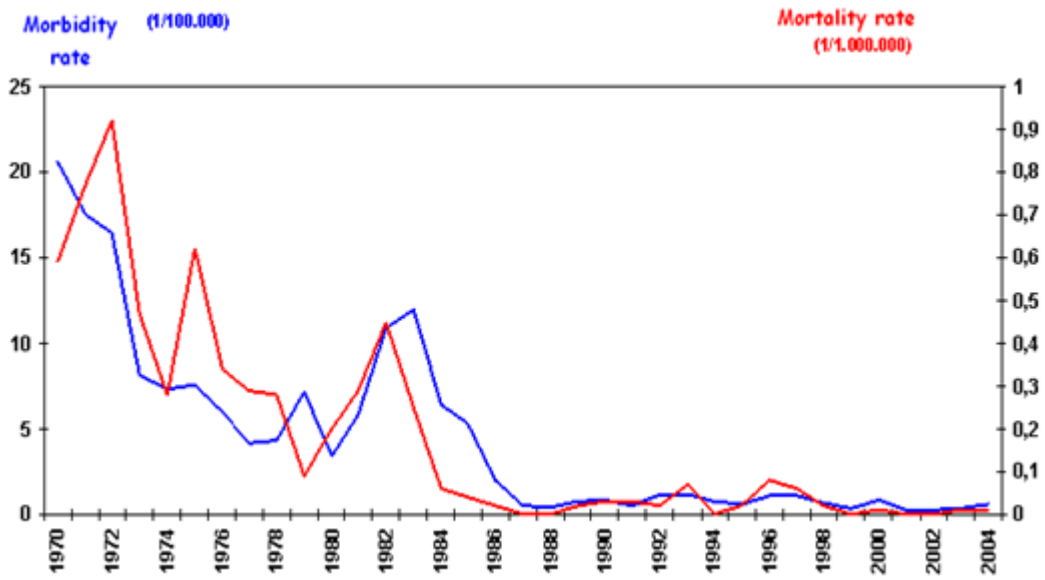


Figure 1.11. Pertussis morbidity and mortality rates in Turkey between 1970 and 2004 (*http://www.saglik.gov.tr/extras/istatistikler/temel2004/tablo-39.htm*).

Since 1968, the vaccination schedule was not changed in Turkey. Whole-cell vaccines were used in 2nd, 3rd and 4th months of age, with a booster dose administered between 16th and 24th months. If there was a delay in the booster, it could be administered into children younger than 6 years old. In 2008, whole-cell vaccines were replaced by acellular vaccines. After the alteration of vaccines, the schedule was changed into 2nd, 4th and 6th months and the date for booster became narrower between 18th and 24th months (Table 1.8) (Özmert *et al.* 2008).

Until 1960s, vaccines were prepared as Diphteria+Pertussis (DB). After 1968, vaccines containing Diphteria+Pertussis+Tetanus (DBT) were manufactured in Turkey till 1996. Then, according to the "Expanded Program on Immunization" (EPI) plan; combined DBT containing *Haemophilus influenza* B. (Hib) and inactive polio (IPA) vaccines (DaBT-IPA-Hib) have been used (Özmert *et al.* 2008).

Table 1.8. Current vaccination program in Turkey (as of January 2008) (Özmert *et al.*, 2008)

		End of month				Month		Grade	
	Birth	1	2	4	6	12	18-24	1.	8.
BCG			I						
HepB	I	II			III				
DTaP-IPV-Hib			I	II	III		B*		
OPV					I		II		
MMR						I		B	
Td									B

BCG; Tuberculosis, HepB; Hepatitis B, DTaP-IPV-Hib; Diptheria, Acellular pertussis, Tetanus, Inactive Polio, *Haemophilus influenza* B, OPV; Oral Polio, MMR; Measles, Mumps and Rubella, Td; Diptheria-Tetanus for adults.
*Booster dose

Although, the vaccines are used according to the schedule, the circulation of *B. pertussis* still exists and whooping cough is still seen in all age groups in Turkey. It occurs endemically with 2-to-5- year cycles of increased disease incidence (Vatansever *et al*., 2005; Dilli *et al*., 2008)

1.6. Re-emergence of Pertussis

Although high vaccination coverage seems to decrease the incidence of pertussis over time, the disease has reemerged in some countries having highly vaccinated populations such as Australia, The Netherlands, Israel and Canada (DeSerres *et al*., 1995; Andrews *et al*., 1997; De Melker *et al.*, 1997; Moerman *et al.*, 2006). The resurgence of the disease is frequent among the adolescents and adults (de Greeff *et al.*, 2008). Various factors such as increased awareness, improved diagnostics, decreased vaccination coverage, suboptimal vaccines, waning vaccine-induced immunity, and pathogen adaptation are thought to be main reasons for the resurgence (Mooi *et al*., 2001; Mooi *et al*., 2007). Some studies provided supports for pathogen adaptation. It has been shown that antigenic divergence is present between vaccine strains and clinical isolates with respect to protective proteins: pertussis toxin, fimbriae, filamentous hemagglutinin and pertactin. Polymorphism has been found in all these proteins (Mooi *et al*., 1998; Mooi *et al*., 2007; Berbers *et al*., 2009). In addition, different procedures of manufacturers for vaccine preparation in different countries may lead to production of suboptimal vaccines and this may play a role in resurgence of the disease. There is a consensus about the fact that the immunity against pertussis gained by vaccination is not life-long. Therefore, adolescents and adults can infect infants easily and cause increase in circulation of *B. pertussis* (Berbers *et al*., 2009).

To prevent the pertussis resurgence, some strategies are suggested. The important one is improvement of pertussis vaccines. For whole-cellular vaccines, reactogenicity of them should be reduced. Modification of the *B. pertussis* LPS may provide this

reduction (Geurtsen *et al*., 2007). The vaccines can also be improved by containing the strains whose virulence gene transcription is enhanced. For acellular pertussis vaccines, protein variants from circulating strains can be used for vaccine production. In addition, besides main virulence factors, conserved protective antigens can be included in new generation acellular pertussis vaccines (Berbers *et al*., 2009).

1.7. The Present Study

Despite availability of effective vaccines and high vaccine coverage, re-emergence of whooping cough has been observed in recent years. Although pertussis is mainly a childhood disease, its incidence increases among adolescents and adults, due to continued circulation of *B. pertussis* strains. There are reports about antigenic divergence between clinical isolated and vaccine strains and polymorphisms have been found in filamentous hemagglutinin, pertactin, fimbriae and pertussis toxin which are the main components of acellular pertussis vaccines (Mooi *et al*., 1998; Mooi *et al*., 2007; Berbers *et al*., 2009). These adaptations cause decrease in vaccine efficiency. For better protection against the disease, a strategy might be to produce more effective acellular vaccines with conserved protective proteins. Immunoproteomic studies can supply expanded knowledge of components of pathogens. Immunoproteomic analysis of *B. pertussis* carried out by Altindis *et al*., (2009) provided to identify 25 new immunogenic proteins 21 of which were shown to be the novel antigens for *B. pertussis*.

Putative PPIase is one of the newly identified immunogenic proteins in *B. pertussis* and functions in acceleration of cis-trans conformational changes at the Xaa-Pro peptide bonds during protein folding (Fischer *et al*., 1984, Altindis *et al*., 2009; Tefon *et al*., 2011). PPIases of *Legionella pneumophila*, *Neisseria gonorrhoeae* and *Salmonella enterica* has been defined as virulence factors (Fischer *et al*., 1992; Humphreys *et al*., 2003; Leuzzi *et al*., 2005). FimX is one of fimbrial proteins of the bacteria and it has been suggested that the gene is silent or expressed at very low level

that cannot be detected (Willems *et al*., 1990; Riboli *et al*., 1991). A recent study showed that FimX has been found in surfaceosome of *B. pertussis* Saadet strain which is a local strain found in Turkey, but not in commonly used *B. pertussis* Tohama I in LC-MS/MS analysis (Tefon *et al*., 2011).

The aim of this study is to determine possible cellular and humoral responses against the recombinant FimX and putative PPIase from *B. pertussis* in mice. These kinds of studies provide perspective in development of new generation acellular pertussis vaccines.

CHAPTER 2

MATERIALS AND METHODS

2.1. Bacterial Strains and Plasmids

Tohama I and Saadet strains of *B. pertussis* were used in this study (Table 2.1). While Tohama is a standard strain of *B. pertussis* used worldwide for both vaccine preparation and research, Saadet is a local *B. pertussis* strain which was isolated in 1948 from a baby girl named Saadet. *Escherichia coli* DH5α and *E. coli* BL21(DE3) strains were used as hosts for sub-cloning and expression studies, respectively (Table 2.1). pGEM®-T Easy (Promega) and pET-28a (+) vectors (Novagen) were used for cloning and expression of the recombinant proteins in *E. coli* BL21(DE3), respectively (Figures 2.1, 2.2 and 2.3).

Table 2.1. Strains used in the study.

Strain	Characteristics	Source and Reference
B. pertussis Tohama I and Saadet	Tohama I: standard strain Saadet: local strain	Kindly provided by Dr. Erkan Özcengiz (VBR Vaccine Res. Co., Ankara).
E. coli DH5α	F' ϕd*lac*ZΔ(*lac*ZY A-argF)U169 *sup*E44λ$^-$ *thi*-1 *gyr*A *rec*A1 *rel*A1 *end*A1 *hsd*R17	American Type Culture Collection; Hanahan (1983)
E. coli BL21(DE3)	F$^-$ *ompT gal dcm lon* *hsd*S_B(r_B^- m_B^-) λ(DE3 [*lac*I *lac*UV5-T7 gene 1 ind1 sam7 nin5])	Novagen, Merck (Germany)

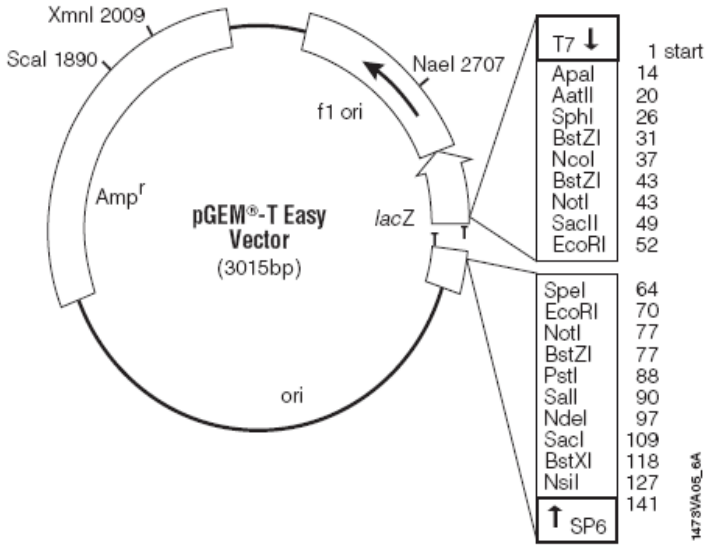


Figure 2.1. Restriction map of the pGEM-T vector.

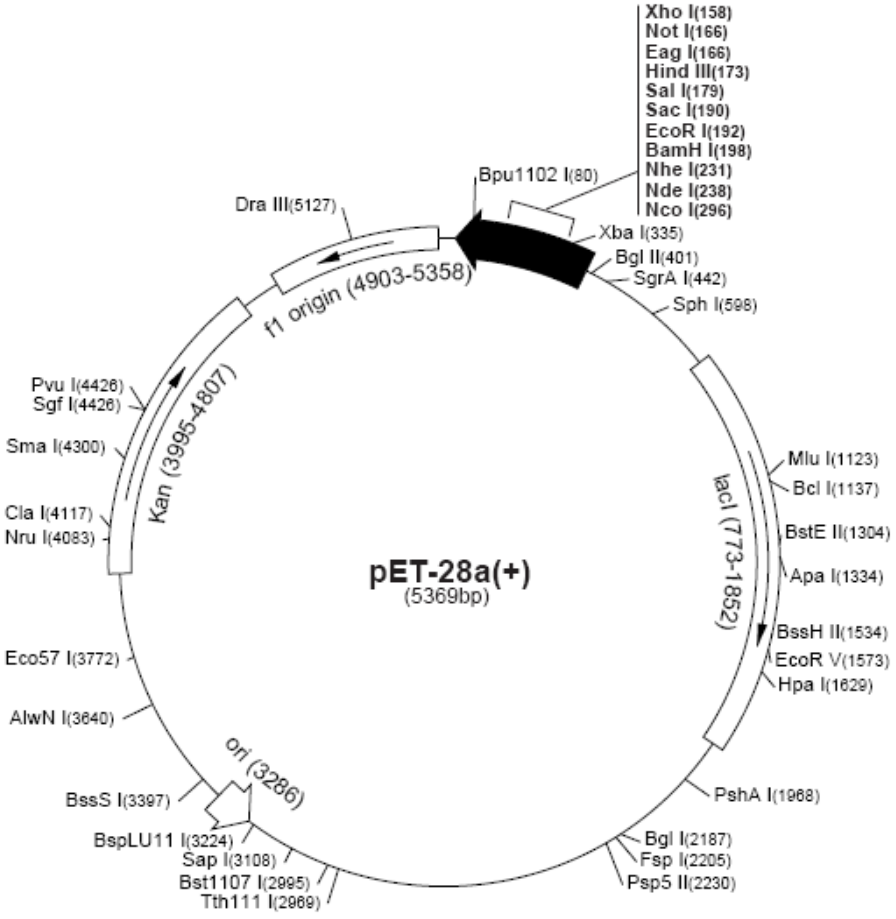


Figure 2.2. Restriction map of the pET-28a (+) vector.

2.2. Culture Media

Composition and preparation of culture media are given in the Appendix A.

2.3. Buffers and Solutions

Composition and preparation of buffers and solutions are given in the Appendix B.

2.4. Chemicals and Enzymes

The chemicals and enzymes used in the study are given in the Appendix C.

2.5. Maintenance of Bacterial Strains

B. pertussis Tohama I and Saadet strains were grown on Cohen-Wheeler agar medium (Appendix A) for 48 h at 37^0C, stored at 4^0C (Sato *et al*., 1972). *E. coli* DH5α strain were grown in Luria-Bertani (LB) liquid medium (Appendix A) containing 100µg/mL ampicillin and kept on LB agar plates (Appendix A) supplemented with ampicillin. *E. coli* BL21(DE3) strains were grown in LB liquid medium containing 30µg/mL kanamycin and kept on LB agar plates supplemented with kanamycin. 20% glycerol stock was prepared for each bacterial strain and kept at -80^{o}C.

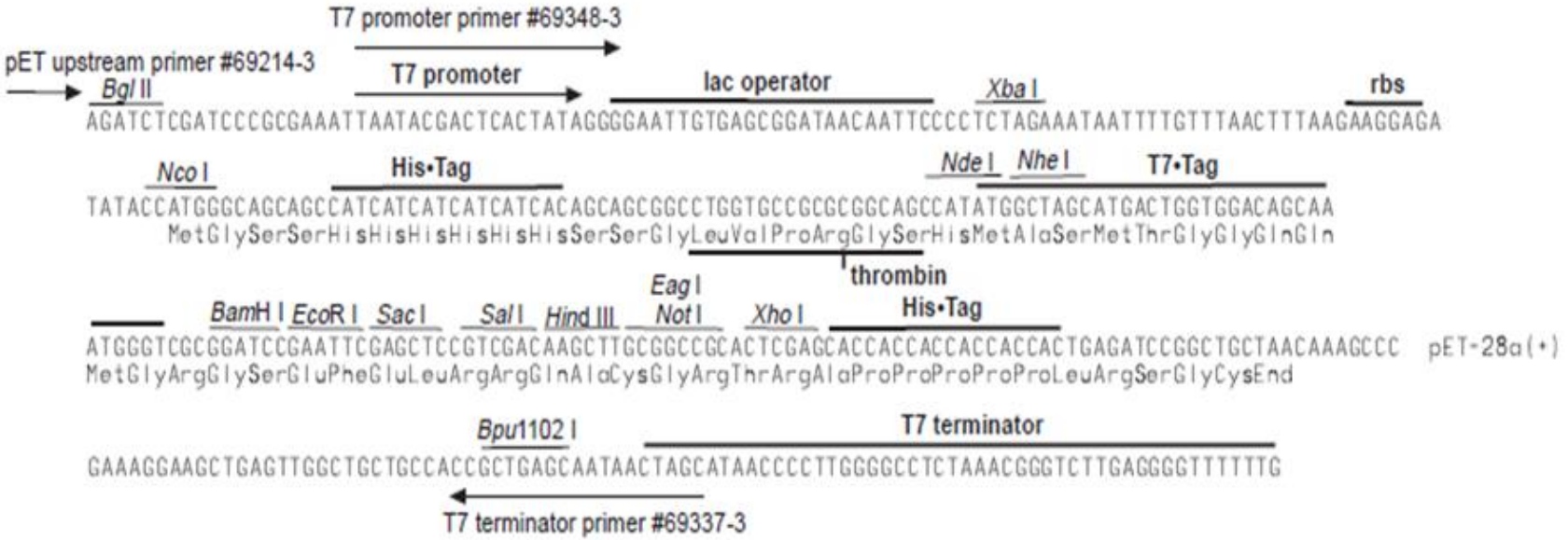


Figure 2.3. pET-28a (+) cloning/expression region

2.6. Genomic DNA Isolation

Chromosomal DNA of *B. pertussis* was isolated according to slightly modified procedure described by Storm *et al*. (2006). *B. pertussis* strains were grown on Cohen-Wheeler agar medium for 3 days at 37^{o}C. Colonies were scraped from the agar plate and resuspended in 200 µl TEN buffer (Appendix B) and incubated at 100°C for 10 min. Sample was centrifuged for 2 min at 13,000 rpm. Supernatant was stored at 4°C until use in PCR.

2.7. Primer Design

Primers to amplify *fimX* and BP3561 (putative peptidyl-prolyl cis-trans isomerase) genes from *B. pertussis* Tohama I were designed according to the sequences of *B. pertussis fimX* and BP3561 genes (NCBI gene IDs: 2665538 and 2665184, respectively) (Table 2.1).

Table 2.2. Primers used in PCR amplification. Restriction enzyme sites were underlined.

Gene name	Primer	Oligonucleotide Sequence	Size of the PCR product
fimX	FF	5'- ggatccatgcaagccaaaacgttc - 3'	606 bp
fimX	FR	5'- agatcttcaggggtagacgaccg - 3'	
BP3561	PF	5'- ggatccatgaaacgcatcgccat - 3'	777 bp
BP3561	PR	5'- agatctttactggatcttggcctgt - 3'	

2.8. Polymerase Chain Reaction (PCR)

The oligonucleotide primers (Table 2.1) were purchased from IONTEK, Co. (Istanbul, Turkey). PCR mixture contained 5 µl 10X PCR buffer, 5 µl 25 mM

$MgCl_2$, 2 µl of each 10 µM primers, 1 µl of 10 mM dNTP, 0.5 µg genomic DNA, 1 µl *Taq* polymerase and sterile dH_2O to complete the volume to 50 µl. PCR condition for both genes is given in Table 2.3. Reaction mixtures were run in 1% agarose gel.

Table 2.3. PCR condition used for the amplification

	Temperature	**Time**	
Initial denaturation	94 °C	3 min	
Denaturation	94 °C	1 min	40 cycles
Annealing	58 °C	1 min	
Extension	72 °C	1 min	
Final extension	72 °C	10 min	

2.9. Agarose Gel Electrophoresis

Electrophoresis was carried out on a horizontal apparatus. 1% agarose gel was prepared in 1X TAE buffer (Appendix B). Gel loading dye (6X) was mixed with samples and electrophoresis was run at 90 Volts for 50-60 min. After run, the gel was dyed with ethidium bromide (0.5 µg/ml). The DNA bands were visualized and photographed by using Vilber Lourmat Gel Imaging System (Vilber Lourmat, Marne-la-Valle´e, France). Lambda DNA/*Pst*I Marker and GeneRuler™ 100 bp DNA Ladder were used to determine the size of the DNA bands. Qiagen Gel Extraction Kit (Qiagen Inc., Valencia, CA) was used to extract the desired bands from the gel.

2.10. Sequencing reactions

DNA sequencing was carried out in RefGen Inc. (Ankara, Turkey). Deduced nucleotide sequences were compared with the National Center for Biotechnology

Information (NCBI) database using the BLAST search at the website (http://www.ncbi.nlm.nih.gov/BLAST).

2.11. Ligation Reactions

The mixture for ligation of PCR products to pGEM®-T subcloning vector contained 5 µl 2X ligase buffer, 1 µl pGEM®-T vector, 500 ng DNA (insert), 1 µl T4 ligase and sterile dH_2O to complete the volume to 10 µl. Ligation of inserts to pET-28a(+) expression vector was performed as follows: 1 µl 10X ligase buffer, 2 µl pET-28a(+) vector, 500 ng DNA (insert), 1 µl T4 ligase were mixed and volume was completed to 10 µl with sterile dH_2O. Ligation was carried out at 4°C for 16 h.

2.12. Transformation

2.12.1. Preparation of *E. coli* DH5α and BL21(DE3) CaCl2 Competent Cells

The competent cells were prepared according to the protocol described by Sambrook *et al*. (1989) with slight modifications. In a 100 ml flask, 50 ml of LB broth was inoculated with *E. coli* from a fresh LB agar plate and incubated overnight with shaking at 37°C. 300 µl of seed culture was inoculated into a fresh flask containing 50 ml LB broth. The culture was incubated for 2–2.5 h at 37°C with shaking at 300 rpm until OD_{600} reaches to 0.6. Then the culture was split into two sterile pre-chilled 40 ml screwcap centrifuge tubes aseptically and stored on ice for 10 min. After centrifuging at 4,000 rpm for 10 min at 4°C, each pellet was resuspended in 5 ml of ice-cold 10 mM $CaCl_2$ by vortexing. The cells were centrifuged at 3,000 rpm for 10 min at 4°C. Finally, each pellet was resuspended gently in 1 mL ice-cold 75 mM $CaCl_2$. The competent cells were stored at - 80°C as 100 µl aliquots.

2.12.2. Transformation of Bacteria

The competent *E. coli* cells were kept on ice for 10 min. 10 µl of ligation products was added to the cells and mixed gently. The mixture was incubated on ice for 20 min. After a heat shock at 42°C for 60 - 90 sec, it was incubated on ice for 5 min. 900 µl of LB was added to the mixture and incubated at 37^{o}C for 80 min by gentle agitation (100 rpm). The cells were centrifuged at 3000 rpm for 10 min and the pellets were resuspended in 100 µl LB. Transformed cells were plated on selective medium containing appropriate antibiotics. For blue-white colony selection, the cells were plated on LB agar media containing 20 mg/mL X-gal, 100 mg/ml IPTG and appropriate antibiotic.

2.13. Plasmid DNA Isolation

Qiagen Plasmid Purification Mini and Midi Kits (Qiagen Inc., Valencia, CA) were used for isolation of plasmids from *E.coli* strains according to the procedure described by the manufacturer.

For manual plasmid isolation, the method described by Hopwood *et al*. (1985) was used with slight modifications. Each *E.coli* strain was grown on LB agar containing appropriate antibiotics for overnight. A few colonies were resuspended in 100 µl STE solution containing 2 mg/ml lysozyme (Appendix B). Samples were mixed by vortexing. They were incubated at room temperature for 10 min to lyse the cells and then at 70°C for 10 min to denature genomic DNA. After cooling, an equal amount of phenol-chloroform (water saturated, Appendix B) was added. The samples were centrifuged at 13,000 rpm for 5 min to separate phases. 15 µl of supernatant was run on agarose gel electrophoresis.

2.14. Restriction Enzyme Digestion

Restriction enzymes were added in a suitable buffer to the DNA to introduce 2 Units per µg of DNA. The mixture was incubated at 37°C for 3-5 h. The samples were stored at -20^{o}C when needed.

2.15. Alkaline Phosphatase Treatment

Alkaline Phosphatase treatment was carried out according to protocol of rAPid Alkaline Phosphatase Kit (Roche). For treatment, 1 µg vector DNA, 2 µl 10X rAPid Alkaline Phosphatase buffer and rAPid Alkaline Phosphatase was mixed and the volume was completed to 20 µl with sterile deionized water. The mixture was incubated at 37^{o}C for 30 min and then it was incubated at 75^{o}C for 2 min to inactivate the rAPid Alkaline Phosphatase. The sample was stored at -20 ^{o}C until use.

2.16. Protein Overexpression and Purification

Overexpression and purification of the proteins were carried out according to the protocols described by Ayalew *et al.* (2008) and Protino® Ni-TED 2000 protein purification system manufacturer (Macherey-Nagel) with slight modifications. A single colony of *E. coli* BL21(DE3) carrying the genes was inoculated into 10 ml of LB with kanamycin and grown overnight. 1.5 ml of culture was inoculated into 150 ml LB with kanamycin and incubated at 37°C 200 rpm until OD_{600} reaches 0.6. One of the cultures was induced with IPTG to a final concentration of 1 mM and the other culture was left as uninduced control. All the flasks were incubated for 5 h after induction. 150 ml of the cultures were centrifuged and the pellets were resuspended in 5 ml of denaturing solubilization buffer (DSB) (Appendix B). After 2 cycles of freeze-thaw, the mixtures were sonicated 5x10 sec with 5 sec incubations on ice. The mixtures were centrifuged at 15 000 rpm for 15 min. The supernatants were taken and their optical densities were measured at 280 nm.

For purification, the His-tag columns containing immobilized nickel ions were used. The columns were equilibrated with 4 ml DSB and the supernatants were loaded. The polyhistidine parts of his-tagged proteins bind strongly to nickel ions and all untagged proteins passed through the column. Then, the columns were washed for 3 times with DSB. The proteins were eluted with 3 ml denaturing elution buffer (DEB) (Appendix B) and the optical densities of the eluted proteins were read at 280 nm. The samples were run on SDS-PAGE gel.

2.17. Dialysis

One liter of dialysis buffer was prepared (Appendix B). Dialysis membrane was wetted by distilled water and protein solution was loaded. The sample was incubated in the buffer at 4°C for overnight. The sample was passed through 0.2 μm filter and the concentration of the protein was measured with Bradford assay.

2.18. Determination of Protein Concentration

To determine total protein concentration, the modified Bradford assay described by Ramagli and Rodrigez (1985) was used with slight modifications. 5X Bradford reagent (containing 500 mg Coomassie Brillant Blue G-250, 250 ml of 96% ethanol and 500 ml of 85% ortho-phosphoric acid; completed to 1 liter with dH_2O) was diluted 1:5 with dH_2O and filtered at least three times using Whatman No. 1 filter paper. For determination of the protein concentration, to 20 μl of the supernatant, 1580 μl dH_2O and 400 μl of 1:5 diluted 5X Bradford reagent were added. The mixture was incubated at room temperature for 10 min at dark and absorbance was measured at 595 nm. Bovine Serum Albumin (BSA) (Table 2.4) was used as a standard for the construction of calibration curve (Figure 2.4).

Table 2.4. Standards for preparation of protein calibration curve.

Protein concentration	Mixture*	dH_2O	Bradford reagent
2 µg/ml	8 µl	1592 µl	400 µl
3 µg/ml	12 µl	1588 µl	400 µl
5 µg/ml	20 µl	1580 µl	400 µl
7,5 µg/ml	30 µl	1570 µl	400 µl
10 µg/ml	40 µl	1560 µl	400 µl

*Mixture= 100 µl DEB containing 100 µl BSA

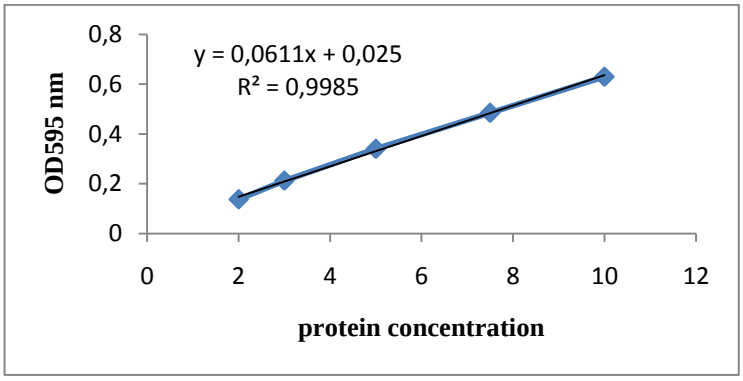


Figure 2.4. Calibration curve for quantification of protein concentrations.

2.19. SDS-PAGE and Coomassie Brilliant Blue Staining

SDS-PAGE of the proteins was carried out by using a 4% stacking gel (pH 6.8) and a 12% separating gel (pH 8.8) with a Bio-Rad Cell system (Bio-Rad, USA). The gels were prepared according to Bio-Rad Cell system manufacturer's protocol (Table 2.5). The electrophoresis was performed with a constant current of 16 mA per gel in 1X running buffer (Appendix B). After electrophoresis, the gels were treated with fixation buffer (Appendix B) for 1 h. To visualize, the gels were stained with Coomassie Brilliant Blue R-250 (Neuhoff *et al.*, 1988) (Appendix B).

Table 2.5. SDS-PAGE gel preparation

	Separating Gel (12%)	**Stacking Gel (4%)**
Acrylamide/bis	40.0 ml	1.3 ml
Distilled water	33.5 ml	6.1 ml
1.5 M Tris-HCl pH 8.8	25.0 ml	-
0.5 M Tris-HCl pH 6.8	-	2.5 ml
10 % (w/v) SDS	1.0 ml	100 µl
10 % Ammonium persulfate	500 µl	50 µl
TEMED	50 µl	10 µl
Total	100 ml	10 ml

2.20. Preparation of Antisera against *B. pertussis*

B. pertussis Tohama I and Saadet strains were grown on Cohen-Wheeler agar medium for 3 days. The cells were suspended in 0.85% saline solution to contain ca. 4×10^{10} bacteria/ml. The suspension was inactivated at 56°C for 30 min and mice were immunized with inactivated cells to obtain anti-*B. pertussis* polyclonal antibodies. For each strain, 10 mice were injected subcutaneously (sc) with 0.5 ml per animal at two week intervals for twice. After 14 days from second injections, the Th and Sa antisera were collected and stored at -20°C until use.

2.21. Western Blotting

The modified method described by Towbin *et al.* (1979) was used for Western analysis. Proteins were transferred to a nitrocellulose membrane for 1.5 h at $1.5mA/cm^2$ in transfer buffer (Appendix B) using a semi-dry blotting system (Cleaver Scientific Ltd, UK)**.** After the transfer, the membrane was blocked for 2 h with 10% skim milk in TBS (Appendix B) at 37^{o}C. After rinsing for 10 min with 0.5% Tween-20 in TBS, the membrane was incubated with the serum taken from

vaccinated mice or raised against whole cell *B. pertussis* at a dilution of 1:300 in TBS containing 2.5% skim milk for 1 h at room temperature. The membrane was rinsed for 10 min and incubated with anti-mouse IgG-alkaline phosphatase in TBS containing 2.5% skim milk for 1 h. The membrane was then washed with TBS and developed with substrate (AP Conjugate Substrate Kit, Bio-Rad, USA).

2.22. Dot Blotting

For dot blotting, the proteins were placed onto nitrocellulose membrane directly and the method used for Western analysis was carried out.

2.23. Mice Experiments

BALB/c female and male mice (3 weeks old) weighing between 18 and 20 g from Ankara University, Faculty of Medicine (Ankara, Turkey) were used in this study. Experiments for 80 μg FimX and putative PPIase vaccination were carried out with 18 mice and 14 mice were used for 20 μg FimX vaccination experiments.

2.23.1. Vaccination

In each experiment set for 80 μg FimX and putative PPIase vaccination, 9 of 18 mice were injected intraperitoneally for FimX and putative PPIase with 0.5 ml of vaccines containing the proteins absorbed to $AlOH_3$ (Alhydrogel) adjuvant on day 0 and 21. 9 mice were injected with PBS for negative control. For 20 μg FimX vaccination, 7 mice were immunized and 7 mice were injected with PBS.

2.23.2. Challenge and Determination of Bacterial Colonization

After 10 days from second vaccination, mice were challenged with live *B. pertussis* Saadet strain. *B. pertussis* was grown on Cohen-Wheeler agar medium. The bacteria

were collected and diluted in 0.85% NaCl solution (pH 7.0) containing 1% casamino acid. After light anesthesia with ether, 50 µl of the inoculum containing 2.5 x 10^9 CFU (colony forming unit) of bacteria was administered into each nostril and allowed to be inhaled by the mice. For the determination of bacterial colonization, the lungs of mice were removed aseptically on day 2, 5 and 8 into 0.85% NaCl solution containing 1% casamino acid. The lungs were homogenized and serial dilution was carried out before inoculation. 100 µl of each sample was inoculated on two plates of Cohen-Wheeler agar medium. The plates were incubated at 37^{o}C for 3-4 days and the colony counting was carried out.

2.23.3. Spleen Cell Culture Conditions to Assess Cytokine Profile and Cytokine Assay

Before challenge, spleens of one of the mice from control and vaccinated groups were removed aseptically and suspended in 5 ml RPMI containing 10% FBS, 3% NEA and 1% pen/strep. Spleens of the remaining mice were taken after challenge. The spleens were placed in cell strainers (BD Falcon) and ground by adding RPMI. 1x10^7 cells were added to each well of 6-well plate and medium was completed to 2.5 ml with RPMI. The plate was incubated in CO_2 incubator at 37°C. After one day, medium was changed and 4 µg of Fimx protein was added for induction. Culture supernatant was removed after 72 h to measure IFN-γ secretion.

Determination of IFN-γ secretion was carried out with Mouse IFN- γ Minikit (Thermo Scientific, Rockford, USA) according to the manufacturer's protocol. 35 µl of coating antibody was mixed with 11 ml coating buffer (Appendix B) and 100 µl of the solution was added to each well of 96-well plate. The plate was incubated at room temperature for overnight. Coating antibody solution was aspirated and 300 µl of blocking buffer (Appendix B) was added to each well. The plate was incubated at room temperature for 1 hr and then blocking buffer was aspirated. Samples were prepared with 1:4, 1:8, 1:16 and 1:32 dilutions. 100 µl of each sample was added to

wells in duplicate and incubated at room temperature for overnight. The plate was aspirated and washed three times with 300 µl/well washing buffer (Appendix B). 35 µl of detection antibody was combined with 11 ml assay buffer (Appendix B) and 100 µl of the solution was added to each well. The plate was covered and incubated at room temperature for 1 hr. It was aspirated and washed three times with 300 µl/well washing buffer. Streptavidin-HRP was diluted in assay buffer with 1/10000 dilution. 100 µl of the solution was added to each well and the plate was incubated at room temperature for 30 min. The plate was aspirated and washed three times with 300 µl/well washing buffer. 100 µl of TBM substrate solution was added to each well and the plate was incubated in dark for 30 min at room temperature. The reaction was stopped by adding 100 µl of stop solution (Appendix B) to each well and the plate was read at 450 nm.

2.23.4. IgG Enzyme-linked Immunosorbent Assay (ELISA)

96-well plates were coated with 4 µg of the protein per well in 200 µl of carbonate/bicarbonate buffer (Appendix B). The plates were covered with parafilm and incubated at 4^{o}C for overnight. They were washed three times with washing solution (Appendix B). The wells were blocked with 50 µl of blocking solution (Appendix B) and incubated at 37^{o}C for 1 h. 100 µl of test sera was added into each well at a 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400, 1:12800, 1:25600 dilutions in blocking solution and the plates were incubated at 37^{o}C for 1 h. The plates were washed four times with washing solution. 100 µl of alkaline phosphotase conjugated mouse IgG at dilution of 1:1000 in blocking solution was added and the plates were incubated at 37^{o}C for 1 h. They were washed four times with washing solution and 100 µl of substrate (AP Conjugate Substrate Kit, Bio-Rad, USA) was added into each well. They were incubated at room temperature for 30 min at a dark place. 50 µl of 1M NaOH was added to stop the reaction and the plate was read at 490 nm.

2.24. Statistical Analysis

Statistical significance in ELISA and cytokine assay was determined using one-way analysis of variance (ANOVA). Tukey's range test was used for comparisons of data sets.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Cloning of Genes Encoding Fimbrial Subunit X (*fimX*) and Putative Peptidyl-Prolyl Cis-Trans Isomerase (BP3561) from *B. pertussis* in *E. coli*

3.1.1. PCR Amplification and Cloning into pGEM®-T Easy Vector

Genomic DNA of *B. pertussis* Tohama I was isolated and used for the amplification of *fimx* and BP3561 genes (Figures 3.1 and 3.2)

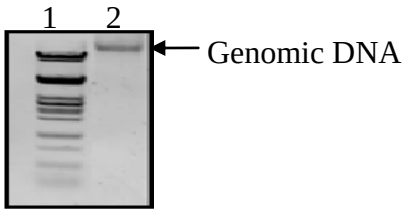


Figure 3.1. Genomic DNA isolation. Lane 1: Marker (Lambda DNA/*Pst*I, 24), Lane 2: The genomic DNA of *B. pertussis.*

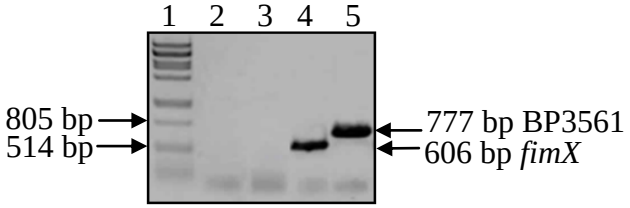


Figure 3.2. Amplification of *fimX* and BP3561 genes from the genomic DNA of *B. pertussis.* Lane 1: Marker (Lambda DNA/*Pst*I, 24), Lane 2: Negative control for *fimX* (no template), Lane 3: Negative control for BP3561 (no template), Lane 4: PCR product for *fimX*, Lane 5: PCR product for BP3561.

The PCR products were extracted from the gel and cloned into pGEM®-T subcloning vector (Figure 2.1.).

3.1.2. Transformation of *E. coli* DH5α with Recombinant Plasmids and Verification of Cloning into *E. coli* DH5α

The ligation products of *fimX* and BP3561 were transformed into *E. coli* DH5α competent cells. The cells were inoculated into LA plates containing X-gal, IPTG and ampicillin to select the recombinants. Plasmids from the white colonies were isolated (Figure 3.3) and digested with restriction enzymes to verify the cloning (Figure 3.4). For restriction enzyme digestion, *BamH*I and *Bgl*II enzymes were used.

Recombinantion was also verified by sequence analysis by comparing known sequences in NCBI database. The amplified products showed significant homology with the genes of *B. pertussis*. Figure 3.5 and 3.6 show the nucleotide sequences of *fimx* and BP3561 genes in FASTA format, respectively.

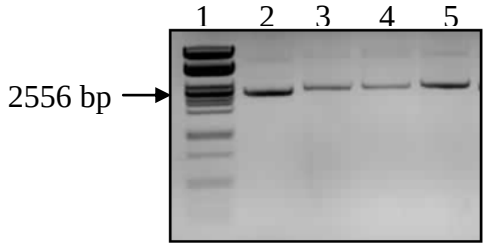


Figure 3.3. Plasmid isolation from the white colonies. Lane 1: Marker (Lambda DNA/*Pst*I, 24), Lane 2: pQE60 vector as control, Lane 3-4: pGEM®-T carrying *fimX*, Lane 5: pGEM®-T carrying BP3561.

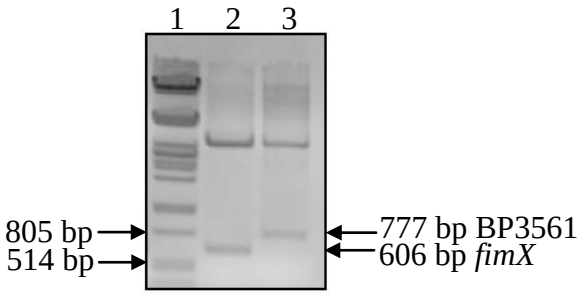


Figure 3.4. Verification of cloning of *fimX* and BP3561 into pGEM®-T vector via restriction enzyme digestion. Lane 1: Marker (Lambda DNA/*Pst*I, 24), Lane 2: *BamH*I and *Bgl*II digested pGEM®-T carrying *fimX*, Lane 3: *BamH*I and *Bgl*II digested pGEM®-T containing BP3561.

>gi|33591275:2839779-2840384 Bordetella pertussis Tohama I, complete genome

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ATGCAAGCCAAAACGTTCCTCCTGGGCGCGGCGCTCGCCGGCGTCGCGCTCGCCGCCCATGCCGAAGA
CGGCACCATTGTCATTACCGGCACGATCACCGACCAGACCTGCACGATCGAGGACCCGAGCCCCGGTT
ACATCAAGGTCGTGCACCTGCCCACGATCTCCAAGAGCGCGCTGAAGAACGCCGGCGACGTGGCGGGG
CGCACTCGCTTCGATATCAAGCTGAAGGACTGCCCGACCACCGTCAACACTCTCAAGCTGTACTTCGA
GCCCGGCCCCACCACGGATTACGGCACCAAGGATCTGAAAGCCTATAAGCAGGCTTGGTACGTCGACG
CCGCAACGCTGCTCAAATCGCCGCCCAGTGTGACCGAAGCCAAGGGGGTGCAGATCCGGCTGATGAAC
CTGAACGGCAAGCAGATTCCCATGGGCGAGACCGAGCCCAACCAGCATGCCGCGGCATTTTCCGGCAC
CATGCAAGCCGGCCAGGCGAAGAAATCGTTCACCTTGCACTACCTGGCCGGCTACGTGAAGAAGGCCA
GTGGAGAGGTCGAGGCGACCATGCTGACCACCTACGTGGGCTTTTCGGTCGTCTACCCCTGA
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Figure 3.5. The nucleotide sequence of *fimx* amplicon (*http://www.ncbi.nlm.nih.gov*).

>gi|33591275:c3777424-3776648 Bordetella pertussis Tohama I, complete genome

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ATGAAACGCATCGCCATGCTGGCTGCTGCCTGCGTCATTGCCGTGCCCGCTTTCGCCCAGAACGTGGC
GACCGTGAACGGCAAGCCCATTACTCAGAAGAGCCTGGATGAGTTCGTCAAGCTGGTCGTCAGCCAGG
GCGCTACCGATTCGCCCCAGCTGCGTGAGCAGATCAAGCAGGAAATGATCAACCGCCAGGTGTTCGTG
CAGGCGGCCGAGAAGGACGGCGTCGCCAAGCAGGCCGACGTGCAGACTGAGATCGAGCTGGCCCGCCA
GGGCATCCTGGTGCGCGCCCTGATGGCCGACTACCTGCAAAAACACCCCGTCACCGACGCCCAGGTCA
AGGCCGAATACGAAAAGATCAAGAAAGAACAGGCCGGCAAGATGGAATACAAGGTCCGTCACATCCTG
GTCGAGGACGAAAAGACGGCCAACGACCTGCTGGCCCAGGTCAAGAGCAACAAGAACAAGTTCGACGA
TCTGGCCAAGAAGAACTCCAAGGACCCCGGCAGCGCCGAGCGCGGCGGCGACCTGGGTTGGGCGCCTG
CCACCAACTACGTCCAGCCGTTTGCCGAGGCCGTGACCAAGCTGAAGAAGGGCCAACTGGTCGACAAG
CCGGTGCAGACCCAGTTCGGCTGGCACGTGATCCAGGTCGACGATACCCGTCCGGTCGAATTCCCCGC
CATGGACCAGGTGCGCCCGCAACTGGAAGAAATGCTGCGCCAGCAAACCCTGGCCAACTACCAGAAGC
AATTGCGCGAACAGGCCAAGATCCAGTAA
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Figure 3.6. The nucleotide sequence of BP3561 amplicon (*http://www.ncbi.nlm.nih.gov*).

3.1.3. Subcloning of *fimX* and BP3561 Genes into pET-28a (+) Expression Vector

pET-28a (+) expression vector carries His-tag sequences at C and N terminal and the expression of the genes are under the control of T7 promoter. For subcloning, the vector was digested with *BamH*I enzyme and treated with alkaline phosphatase. The genes digested from the pGEM®-T vector were ligated into pET-28a vector (Figure 2.2).

3.1.4. Transformation of *E. coli* BL21(DE3) with Recombinant Plasmids and Verification of Cloning in *E. coli* BL21(DE3)

The ligation products in pET-28a (+) were transformed into *E. coli* BL21(DE3) competent cells. The cells were inoculated into LB agar plates containing kanamycin. Plasmids from recombinant colonies were isolated (Figure 3.7) and digested with restriction enzymes to verify the cloning. *BamH*I and *EcoR*I enzymes were used for pET-28a (+) that contains *fimX* and pET-28a (+) carrying BP3561 was digested with *Nco*I (Figures 3.8 and 3.9).

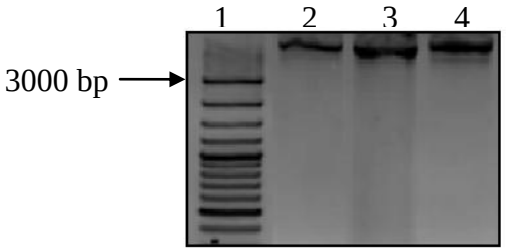

Figure 3.7. Plasmid isolation from putative recombinant colonies. Lane 1: Marker (O'GeneRuler™ 100 bp Plus DNA Ladder), Lane 2: pET-28a (+) carrying *fimX*, Lane 3: pET-28 a (+) as control, Lane 3: pET-28a (+) carrying BP3561.

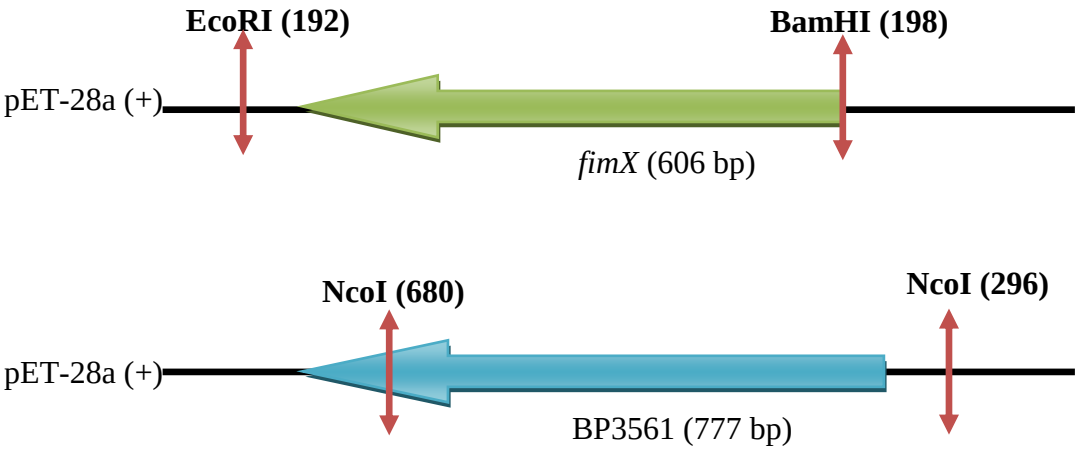

Figure 3.8. Restriction digestion profile of *fimX* and BP3561 genes in pET-28a (+).

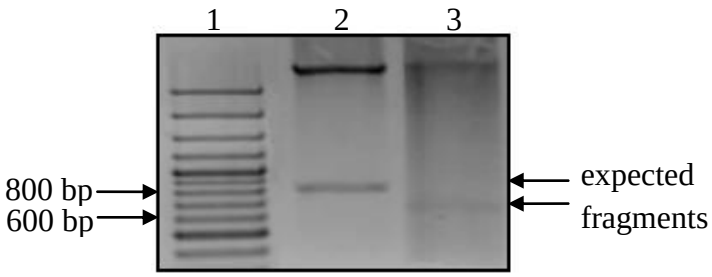

Figure 3.9. Verification of cloning of *fimX* and BP3561 into pET-28a (+) vector via restriction enzyme digestion. Lane 1: Marker (O'GeneRuler™ 100 bp Plus DNA Ladder), Lane 2: *Nco*I digested pET-28a (+) containing BP3561, Lane 3: *BamH*I and *EcoR*I digested pET-28a (+) carrying *fimX*.

3.2. Expression of Recombinant *fimX* and BP3561 Genes in *E. coli* BL21(DE3)

For each sample, two flasks of LB were inoculated with *E.coli* BL21(DE3) cells which were transformed with pET-28a (+) carrying the gene of interest. IPTG was added into one of the cultures to induce the expression of recombinant His-tagged FimX (21.4 kDa) and putative PPIase protein (28 kDa) and the other culture was used as uninduced control (Figures 3.10 and 3.11).

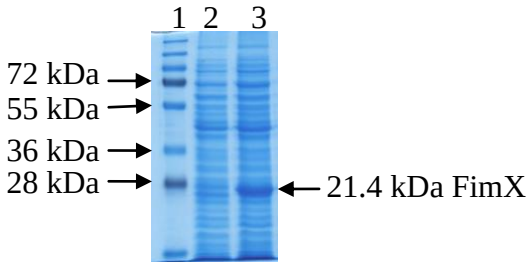

Figure 3.10. Coomassie blue-stained SDS polyacrylamide gel of expressed recombinant FimX. Lane 1: Marker (Pageruler^{Tm} Plus Prestained Protein Ladder, #SM1811), Lane 2: Control (uninduced sample), Lane 3: Expressed FimX (IPTG-induced sample).

The molecular mass of recombinant FimX protein was larger than the expected size. It was previously reported that protein might undergo modification or tertiary structure of protein may affect SDS polyacrylamide gel migration and cause low mobility (Rainbow *et al*., 1997; Yu *et al*., 1997; Benz and Schmidt, 2002; Mudd *et al*., 2008; Rath *et al*., 2008). In adddition, his tag parts of the protein can lead to a band with a size larger than the expected on SDS-PAGE (Alger and Williams, 2002).

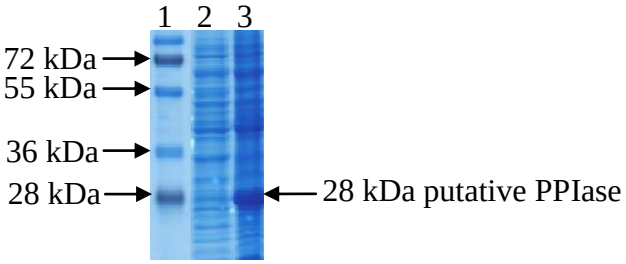

Figure 3.11. Coomassie blue-stained SDS polyacrylamide gel of expressed recombinant putative PPIase. Lane 1: Marker (Pageruler^{Tm} Plus Prestained Protein Ladder, #SM1811), Lane 2: Control (uninduced sample), Lane 3: Expressed putative PPIase (IPTG-induced sample).

3.3. Purification of Recombinant FimX and Putative PPIase Proteins by His-tag Affinity Chromatography

Recombinant His-tagged FimX and putative PPIase were purified with Protino® Ni-TED 2000 protein purification system. After purification, the proteins were dialyzed against dialysis buffer (Appendix B) to decrease urea and NaCl concentrations and were passed through 0.2 μm filters (Figures 3.12 and 3.13). The His-tag parts of the recombinant proteins were not removed due to small size, weak immunogenicity and less potential to interfere with protein folding (Constans, 2002).

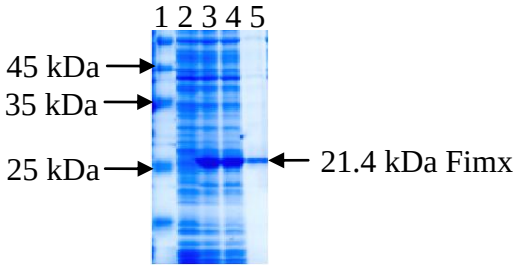

Figure 3.12. Coomassie blue-stained SDS polyacrylamide gel of purified His-tagged FimX. Lane 1: Marker (Pageruler^{Tm} Plus Unstained Protein Ladder, #SM0431), Lane 2: Control (uninduced sample), Lane 3: Expressed FimX (IPTG-induced sample), Lane 4: Flowthrough, Lane 5: Purified His-tagged FimX.

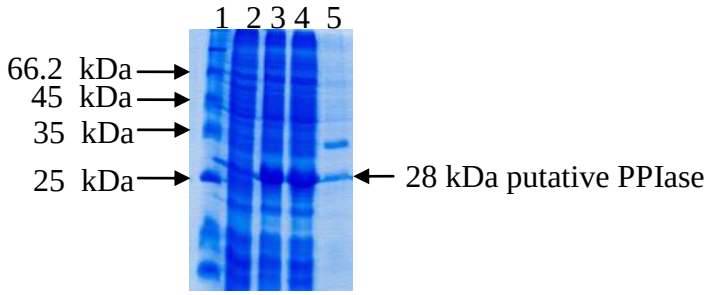

Figure 3.13. Coomassie blue-stained SDS polyacrylamide gel of purified His-tagged putative PPIase. Lane 1: Marker (Pageruler^{Tm} Plus Unstained Protein Ladder, #SM0431), Lane 2: Control (uninduced sample), Lane 3: Expressed putative PPIase (IPTG-induced sample), Lane 4: Flowthrough, Lane 5: Purified His-tagged putative PPIase.

In the SDS polyacrylamide gel analysis of purified recombinant putative PPIase, two bands were detected with different molecular masses although the larger band was not seen clearly in expression (Figure 3.11). The 28 kDa protein should correspond to putative PPIase while the other band might be modified product of the protein or aggregated protein (Gal *et al*. 1998; Brurberg *et al*. 2000). Overproduction of recombinant proteins might have resulted in partial or complete deposition into aggregates due to interactions between the hydrophobic regions of the proteins, as reported earlier in Villaverde and Carrio, 2003 and Baneyx and Mujacic, 2004.

3.4. Western Blot Analyses of Recombinant FimX and Putative PPIase Proteins

Sera taken from the mice injected subcutaneously with *B. pertussis* Tohama I and Saadet strains were used for Western blotting of the recombinant FimX (Figures 3.14 and 3.15) and putative PPIase (Figures 3.16 and 3.17). Each protein was analyzed with both Th and Sa antisera.

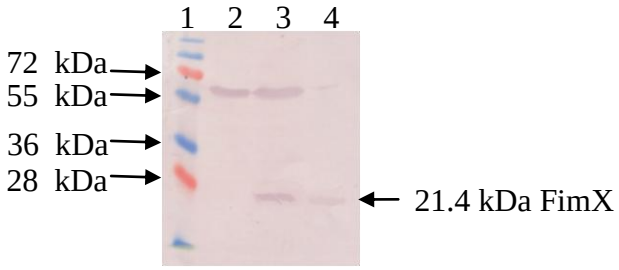

Figure 3.14. Western blot analysis of purified recombinant FimX against Th antiserum. Lane 1: Marker (Pageruler^{Tm} Plus Prestained Protein Ladder, #SM1811), Lane 2: Control (uninduced sample), Lane 3: Expressed His-tagged FimX (IPTG-induced), Lane 4: Purified His-tagged FimX.

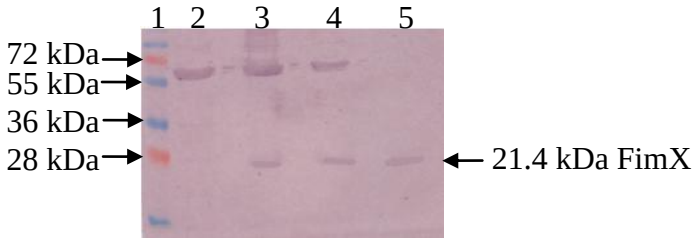

Figure 3.15. Western blot analysis of purified recombinant FimX against Sa antiserum. Lane 1: Marker (Pageruler^{Tm} Plus Prestained Protein Ladder, #SM1811), Lane 2: Control (uninduced sample), Lane 3: Expressed His-tagged FimX (IPTG-induced), Lane 4: Flowthrough, Lane 5: Purified His-tagged FimX.

The *fim* promoters have homology with the pertussis toxin promoter in terms of C-stretch at approximately same position. When four of six Cs were deleted in the promoter of pertussis toxin, it was inactivated, suggesting a similar role for the C stretch in *fim* promoters (Gross and Rappuoli, 1989; Willems *et al*., 1990). The C stretch in *fimx* promoter is shorter than that in the *fim2* and *fim3* promoter and it has been thought that FimX is a silent gene or expressed at an undetectable level due to weak promoter of the gene (Willems *et al*., 1990). In 1991, Riboli *et al*. cloned *fimX* from *B. pertussis* strain BPSA1 to *B. bronchiseptica* and showed that *fimX* was expressed at a very low level. In 2003, the transcript of *fimX* in *B. pertussis* BPSM was detected by microarrays and RT-PCR (Hot *et al*., 2003). In a recent study, Tefon *et al*. (2011) clearly showed in geLC-MS/MS analysis that FimX was found only in surface proteome of Saadet strain of *B. pertussis*, but not in Tohama I strain. In this study, FimX was recognized in Western blot analysis using both Th and Sa antisera.

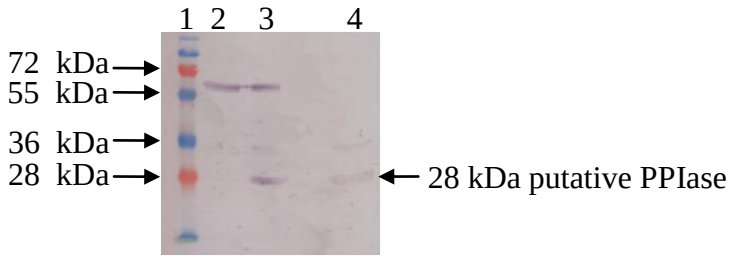

Figure 3.16. Western blot analysis of purified recombinant putative PPIase against Th antiserum. Lane 1: Marker (Pageruler^{Tm} Plus Prestained Protein Ladder, #SM1811), Lane 2: Control (uninduced sample), Lane 3: Expressed His-tagged putative PPIase (IPTG-induced), Lane 4: Purified His-tagged putative PPIase.

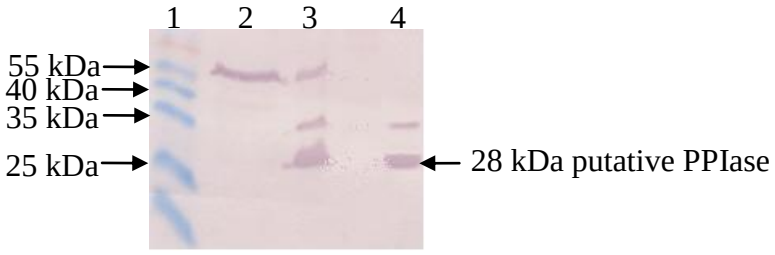

Figure 3.17. Western blot analysis of purified recombinant putative PPIase against Sa antiserum. Lane 1: Marker (Pageruler^{Tm} Plus Prestained Protein Ladder, #SM0671), Lane 2: Control (uninduced sample), Lane 3: Expressed putative PPIase (IPTG-induced), Lane 4: Purified His-tagged putative PPIase.

It was characterized that PPIases have been defined as virulence factors in *Salmonella enterica*, *Neisseria gonorrhoeae* and *Legionella pneumophila* (Fischer *et al*., 1992; Humphreys *et al*., 2003; Leuzzi *et al*., 2005). In addition, Cj0596, a periplasmic PPIase, of *Campylobacter jejuni* was found as an immunogenic protein and had involved in invasion and bacterial colonization (Rathbun *et al*., 2009). In *B. pertussis*, there are several different PPIases and another putative PPIase was first identified as an immunogenic protein in total soluble proteome of Tohama I and Saadet strains of the organism (Altindis *et al*., 2009). In a recent study, putative PPIase of *B. pertussis* was also found in surface proteome as immunogenic protein

(Tefon *et al*., 2011). The results of Western blot analyses with Th and Sa antisera were consistent with these findings.

3.5. Western and Dot Blot Analyses of FimX, Fim2 and Fim3 from *B. pertussis*

As explained earlier, there are some other fimbrial proteins in *B. pertussis* in addition to FimX. Major fimbrial proteins are Fim2 and Fim3 which are included in some acellular pertussis vaccines (Figures 3.18 and 3.19). The purified Fim2 and Fim3 were kindly provided by Dr. Erkan Özcengiz. The homology among the deduced amino acid sequences of the proteins are 60.7%, 60.3% and 57.7% between Fim3 and Fim2, Fim2 and FimX and Fim3 and FimX, respectively (Cuzzoni *et al*., 1990). Figure 3.20 shows ClustalW2 result of the amino acid sequence alignment between FimX, Fim2 and Fim3. Although amino acid sequences of Fim2 and Fim3 show over 57 - 69 % of homology, only weak cross-reactivity was observed with antibodies raised against the purified proteins or induced during infection (Preston, 1985; Robinson *et al.*, 1989; Williamson and Matthews, 1995). To observe cross-reactivity between FimX and other fimbrial proteins of *B. pertussis*, Western and dot blot analyses were carried out with the serum taken from the mice vaccinated with recombinant FimX protein (Figures 3.21 and 3.22).

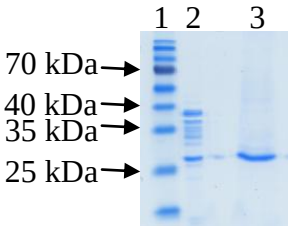

Figure 3.18. Coomassie blue stained SDS polyacrylamide gel of purified Fim2, Fim3 and purified recombinant FimX. Lane 1: Marker (Pageruler^{Tm} Plus Prestained Protein Ladder, #SM0671), Lane 2: Purified Fim2 and Fim3 from *B. pertussis* Saadet strain, Lane 3: Purified recombinant FimX.

In SDS polyacrylamide gel, more bands were present in Lane 2 (Figure 3.18) possibly due to aggregation of Fim2 and Fim3 proteins.

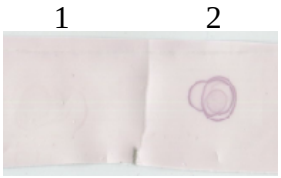

Figure 3.19. Dot blot analysis of purified Fim2 and Fim3 with whole Sa antiserum. Lane 1: Control (PBS) Lane 2: Purified Fim2 and Fim3.

```
FimX -----MQAKTFLLGAALAGVAL---AAHAEDGTIVITGTITDQTCTIEDPS-PGYIKVVH 51
Fim 2 MQIPFQRALRLCLRAALAAIAS---AAHADDGTIVITGTITDTTCVIEDPSGPNHTKVVQ 57
Fim 3 ----MSKFSYPALRAALILAASPVLPALANDGTIVITGSISDQTCVIEEPSTLNHIKVVQ 56
          :     * ***   *     .* *:********:*:* **.**:**  .: ***:

FimX LPTISKSALKNAGDVAGRTRFDIKLKDCPTTV-NTLKLYFEPGPTTDYGTKDLKAYKQAW 110
Fim 2 LPKISKNALKANGDQAGRTPFIIKLKDCPSSLGNGVKAYFEPGPTTDYSTGDLRAYKMVY 117
Fim 3 LPKISKNALRNDGDTAGATPFDIKLKECPQAL-GALKLYFEPGITTNYDTGDLIAYKQTY 115
      **.***.**:  ** ** * * ****:** :: . :* ***** **:*.* ** *** .:

FimX YVDAATLLKSPPSVTEAKGVQIRLMNLNGKQIPMGETEPNQHAAAFSGTMQAGQAKKSFT 170
Fim 2 ATNPQTQLSNITAATEAQGVQVRISNLNDSKITMGANEATQQAAGFDPEVQTGGTSRTVT 177
Fim 3 NASGNGNLSTVSSATKAKGVEFRLANLNGQHIRMGTDKTTQAAQTFTGKVTNG--SKSYT 173
       ..    *.. .:.*:*:**:.*: ***..:* **  :..* *  *   :  *  .:: *

FimX LHYLAGYVKKASGEVEATMLTTYVGFSVVYP 201
Fim 2 MRYLASYVKKN-GDVEASAITTYVGFSVVYP 207
Fim 3 LRYLASYVKKPKEDVDAAQITSYVGFSVVYP 204
      ::***.****   :*:*: :*:*********
```

Figure 3.20. ClustalW2 result of amino acid sequence alignment between FimX, Fim2 and Fim3 (*http://www.ebi.ac.uk/Tools/msa/clustalw2/*).

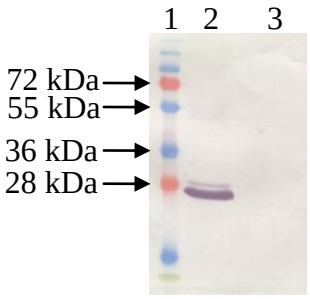

Figure 3.21. Western blot analysis of purified recombinant FimX, Fim2 and Fim3 with serum from the mice vaccinated with recombinant FimX. Lane 1: Marker (Pageruler^{Tm} Plus Prestained Protein Ladder, #SM1811), Lane 2: Purified recombinant FimX, Lane 3: Purified Fim2 and Fim3 from *B. pertussis* Saadet strain.

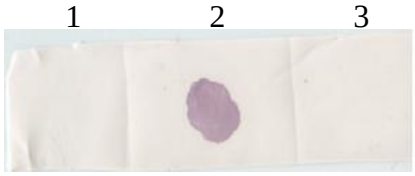

Figure 3.22. Dot blot analysis of purified recombinant FimX , Fim2 and Fim3 with serum from the mice vaccinated with recombinant FimX. Lane 1: Control (PBS) Lane 2: Purified recombinant FimX, Lane 3: Purified Fim2 and Fim3 from *B. pertussis* Saadet strain.

As seen in Figures 3.21 and 3.22, Fim2 and Fim3 did not give any reaction with antibody raised against recombinant FimX. Although there is homology between the proteins with respect to the amino acid sequences, no cross-reactivity was seen between FimX and the other two fimbrial proteins, Fim2 and Fim3.

3.6. Mice Challenge Experiments

BALB/c mice were immunized with 20 µg and 80 µg recombinant FimX or 80 µg putative PPIase proteins at day 0 and day 21. The mice were challenged with 2.5 x 10^9 CFU of *B. pertussis* Saadet cells intranasally. Before second injection and

challenge, their sera were collected. After intranasal challenge, lungs of the mice were removed at day 2, 5, 8 for determination of bacterial colonization. After challenge and second immunization (at day 30) with 20 μg and 80 μg recombinant FimX, the spleens of the mice were removed and spleen cell culture was obtained to measure IFN-γ level.

Respiratory challenge by intranasal administration of the bacteria instead of intracerebral model was considered during the infection of the mice with *B. pertussis* due to the similarities between human infection and respiratory infection of mice. Younger mice are more susceptible to *B. pertussis* and postinfection symptoms such as histamine sensitization and lymphocytosis can be seen in intranasal model (Guiso *et al*., 1999).

3.6.1. Colonization of *B. pertussis* on the Lungs of the Mice Immunized with FimX and Putative PPIase

Some earlier studies carried out for protective activity determination of major antigens of *B. pertussis* demonstrated effectively bacterial clearance from lungs of mice immunized with those antigens. Filamentous hemagglutinin (FHA) immunization, for instance, caused reduction in bacterial colonization in lungs of adult mouse model which were challenged with aerosol *B. pertussis* Tohama I (Kimura *et al*., 1990). It was observed that although DTPa vaccine containing FHA and pertussis toxin induced limited bacterial clearance, addition of BrkA to the vaccine provided more efficient removal of the bacteria (Marr *et al*., 2008). In addition, the antibodies against pertussis toxin, the most important protective antigen of *B. pertussis*, lowered bacterial growth in lungs significantly (Sato and Sato, 1990). Although the studies showed decrease in bacterial colonization after immunization with the antigens, a correlation between bacterial clearance and protective activity of antigens is not always present. For instance, Guiso *et al*. (1999) tested bicomponent (PT-FHA), tricomponent (PT-FHA-PRN) and pertactin DTPa vaccines in sublethal

aerosol and lethal and sublethal intranasal *B. pertussis* respiratory challenge models. They recorded numbers of dead mice daily and lungs of mice were removed to observe bacterial clearance besides measurement of antibody levels. In lethal intranasal challenge, while all nonimmunized mice died within 4 days, only 1 of the 30 immunized mice died. Although all three combinations of acellular vaccine components provided protective immunity against lethal challenge, bacterial clearance were not detected in mice immunized with monocomponent (pertactin) or bicomponent vaccines.

The results of bacterial clearance tests obtained in the present study are presented in Tables 3.1, 3.2 and 3.3.

Table 3.1. Number of CFU/ml recovered from the lungs of the mice immunized with 20 μg of FimX after intranasal challenge with live *B. pertussis* Saadet cells.

Group	**Day 2**	**Day 5**	**Day 8**
Control	0	$8.3x10^5$	0
Immunized	0	$1.7x10^6$	0

Table 3.2. Number of CFU/ml recovered from lungs of mice immunized with 80 μg of FimX after intranasal challenge with live *B. pertussis* Saadet cells.

Group	**Day 2**	**Day 5**	**Day 8**
Control	$1.4x10^3$	$2\ x10^3$	$1.3x10^3$
Immunized	$9.6x10^2$	$1.2x10^3$	$1\ x10^4$

It was seen that 20 μg FimX immunization did not have effect on bacterial clearance (Table 3.1). No colony was observed at day 2 and 8 from either the control and

immunized mice and there was no decrease in bacterial colonization in the lungs of the immunized mice at day 5. In addition, 80 μg FimX immunization did not provide significant bacterial clearance although there was little amount decrease in colony number in immunized mice in day 5 but not in day 2 and 8 (Table 3.2).

Table 3.3. Number of CFU/ml recovered from the lungs of the mice immunized with 80 μg of putative PPIase after intranasal challenge with live *B. pertussis* Saadet cells.

Group	**Day 2**	**Day 5**	**Day 8**
Control	0	0	$4.1x10^{1}$
Immunized	0	$7\ x10^{4}$	$6\ x10^{5}$

No decrease in bacterial colonization was detected in the immunized mice with 80 μg putative PPIase as compared to the control groups (Table 3.3).

3.6.2. Serum Antibody Levels Against Recombinant FimX and Putative PPIase Proteins

To determine serum-specific IgG levels to the recombinant proteins, sera of immunized and control mice were collected before second immunization and challenge and they were pooled. ELISA was carried out to measure antibody levels (Figures 3.23 and 3.24). For comparison of data sets of control and vaccinated groups, one way analysis of variance (ANOVA) with Tukey's test was used and absorbance at cutoff values was compared.

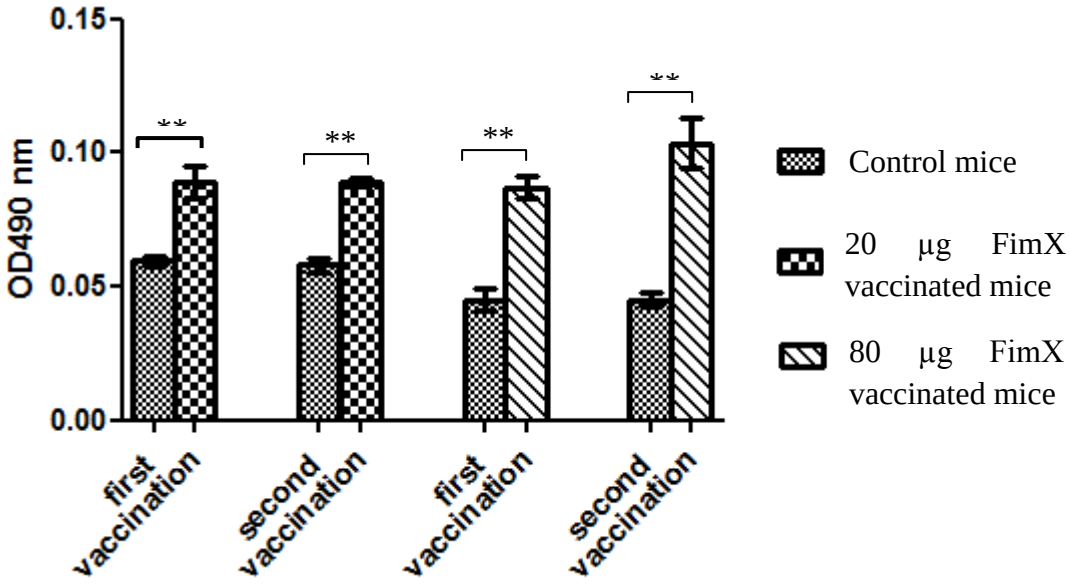

Figure 3.23. Antibody levels in mice immunized with 20 µg and 80 µg of recombinant FimX protein (titer: 1/12800) (**p<0.01 between immunized and control groups).

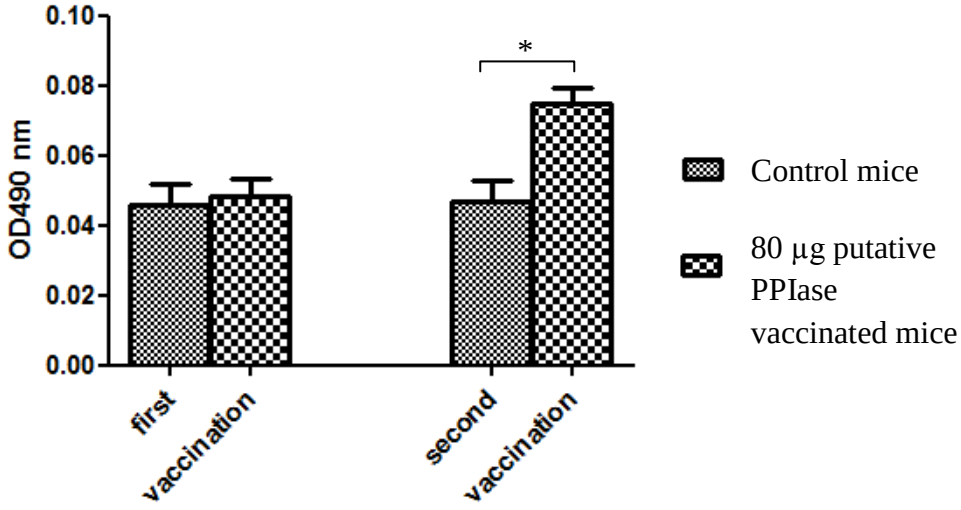

Figure 3.24. Antibody levels in mice immunized with 80 µg of recombinant putative PPIase protein (titer: 1/6400) (*p<0.05 between immunized and control groups).

It was shown that there was statistically significant elevation in the antibody level in mice immunized with 20 μg and 80 μg FimX protein at first and second vaccinations (Figure 3.23). The increase in the second vaccination of 80 μg FimX immunization was more noticeable than in that of 20 μg FimX immunization, probably due to increased protein concentration. While there was no significant difference between control and immunized mice with 80 μg putative PPIase in terms of antibody level at first vaccination, a statistically significant ($p<0.05$) increase in antibody titer was observed at second vaccination (Figure 3.24).

Measurement of total IgG levels gives little information what is actually happening in immune system and there are studies that show protective activities with low or undetectable antibody responses (Roberts *et al*., 1990; Shahin *et al*., 1990; Strugnell *et al*., 1992; Mills *et al*., 1993). In this study, even though antibody levels in immunized mice were significantly higher than in control groups, they were not sufficient to clear the bacterial colonization.

3.6.3. IFN-γ Levels in Mice Vaccinated with FimX Protein

Cellular immune responses are more consistent than antibody response and they can be characterized as Th1- or Th2-type responses. While Th2-type responses are composed of high level of IL-4, IL-10 and IgG1, Th1-type responses involve increased IFN-γ, IgG2a levels and activation of infected macrophages to induce antibacterial mechanisms. IFN-γ can activate macrophages whose phagocytosis ability plays role in bacterial clearance (Fisher *et al*., 1988). When *B. pertussis* enters the respiratory tract, the cells of innate immune system such as macrophages, dendritic cells and natural killer cells, recognize the bacteria. Dendritic cells function in presentation of the bacterial antigens to T cells and it results in polarization of T cells to the Th1 subtype. IFN-γ secreted by Th1 cells later in infection provides recruitment and activation of neutrophils and macrophages for intracellular bacterial killing by NO or reactive oxygen intermediates. In addition, IFN-γ stimulates B cell

to produce opsonizing and complement-fixing antibody (IgG2a in the mouse) (Mills, 2001). It was shown that higher IFN-γ production correlated with the resolution of *Listeria monocytogenes*, *Burkholderia pseudomallei*, *Chlamydia trachomatis* and *Bordetella bronchiseptica* infections (Buchmeier and Schreiber, 1985; Perry *et al*, 1997; Santanirand *et al*., 1999; Pilione and Harvill, 2006). Barbic *et al*. (1997) observed increased IFN-γ mRNA levels in the lungs of the mice infected with *B. pertussis* and they showed that mice which lack mature T lymphocytes could not clear the *B. pertussis* infection and died after the aerosol challenge. In addition, immunized mice having low levels of antigen-specific IFN-γ production following DTP (whole cell) vaccination had low survival rate (Barnard *et al*., 1996). Therefore, the studies demonstrate that INF-γ has a key role in controlling *B. pertussis* infection.

To determine IFN-γ levels, supernatants from spleen cell culture were removed, pooled and analyzed with Mouse IFN- γ Minikit before and after challenge. The results of cytokine ELISA are presented in Figure 3.25.

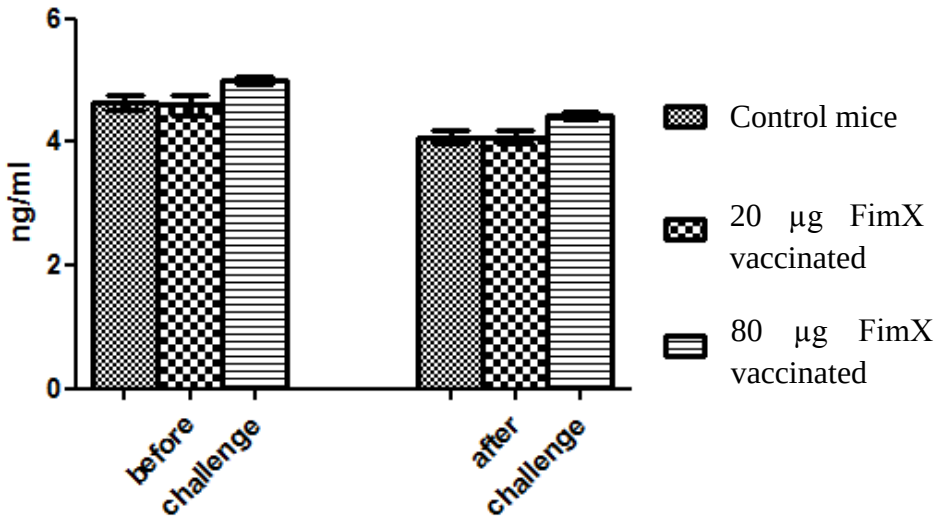

Figure 3.25. IFN-γ levels in control and vaccinated mice with 20 µg and 80 µg of FimX protein before and after challenge.

According to one-way ANOVA and Tukey's test, there was no significant difference between control and vaccinated groups upon immunization with 20 μg and 80 μg of FimX protein before and after challenge. This indicated that sufficient INF-γ was not produced by T lymphocytes in spleens of the immunized mice. Although significant amount of antibody production was detected in the mice sera, no bacterial clearance was observed at the lungs. The reason could be that Th2-type response was induced instead of Th1-type during immunizations. While IgG1 was produced and the majority of measured IgG in ELISA was probably IgG1 from Th2-type response, significant INF-γ level was not present due to lack of Th1-type response.

CHAPTER 4

CONCLUSION

- In addition to FimX, *B. pertussis* contains some other fimbrial proteins which are Fim2 and Fim3 as the major ones. Fim2, Fim3 and FimX share homology in terms of amino acid sequences. In spite of homology in amino acid sequences of Fim2 and Fim3, only weak cross-reactivity was observed with antibodies raised against the proteins or induced during infection. In this study, the Western and dot blot analyses using the serum taken from the mice immunized with recombinant FimX protein did not result in cross-reactivity between FimX and the other two fimbrial proteins despite homology of amino acid sequences to some extent.

- Previously, it was suggested that FimX was a silent gene or expressed at undetectable level. In 1991, *fimX* from *B. pertussis* strain BPSA1 was cloned into *B. bronchiseptica* and low level expression was observed. Then, the transcript of *fimx* in *B. pertussis* strain BPSM was detected by microarrays and RT-PCR. A recent study carried out by our immunoproteomics group reported detection of FimX only in surfaceome of *B. pertussis* Saadet, not in Tohama I strain in LC-MS/MS analysis. In this study, FimX was recognized by both Th and Sa antisera in the Western blot analysis.

- In ELISA, serum-specific IgG levels were measured due to its importance in second immune response. Significant increase in IgG levels ($p<0.01$) in the mice immunized with 20 μg and 80 μg recombinant FimX protein was

detected at first and second vaccinations. In 80 μg recombinant putative PPIase immunization, IgG level was significant only at second vaccination ($p<0.05$). Overall, it seems that FimX and PPIase proteins increase antibody response.

- Although serum-specific IgG levels were significantly high, no bacterial clearance was observed in the lungs of the mice at 20 μg and 80 μg recombinant FimX and 80 μg recombinant putative PPIase immunizations. It was suggested that increase in antibody level was not sufficient to decrease bacterial colonization itself in the lungs. Besides antibody response, cellular immune responses, specifically Th1-type responses which involve increased IgG2a and IFN-γ levels play critical role in the clearance.

- Cytokine ELISA showed that there was no difference between control and immunized groups in terms of IFN-γ production at 20 μg and 80 μg recombinant FimX vaccination. The result can be possibly the reason for no decrease in bacterial colonization in the lungs. Th2-type response involving high level of IL-4, IL-10 and IgG1 was probably induced instead of Th1-type response during vaccinations. Therefore, in spite of high level of IgG response against FimX, lack of a clearance in bacterial colonization can be explained with low levels of IFN-γ.

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APPENDIX A

COMPOTIONS AND PREPARATION OF CULTURE MEDIA

Modified Cohen-Wheeler Medium for *Bordetella pertussis* (pH 7.2 - 7.5) (1000ml)

Bacto-agar	20 g
NaCl	2.5 g
KH_2PO_4	0.5 g
$MgCl_2.6H_2O$	0.4 g
Soluble starch	1.5 g
Yeast extract	2 g
Casamino acid	10 g
$CaCl_2$ (1%)	1 cc
$FeSO_4.7H_2O$ (0.5%)	2 cc
L-cystein	2.5 cc
$CuSO_4.5H_2O$	1 cc
Active coal	4 g
NaOH (26%)	for pH adjustment

- Mix all of them and autoclave

Luria Bertani (LB) Medium (1000ml)

Tryptone	10 g
Yeast Extract	5 g
NaCl	5 g

- Complete to 1000ml with distilled H_2O and autoclave

Luria Bertani (LB) Agar Medium (1000 ml)

Tryptone	10 g
Yeast Extract	5 g
$NaCl_2$	5 g
Agar	15 g

- Complete to 1000ml with distilled H_2O and autoclave

APPENDIX B

COMPOSITIONS OF BUFFERS AND SOLUTIONS

TAE Buffer (50X)

Tris base	242 g
Glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml

Loading Buffer (6X)

0,25% Bromophenol blue

0,25% Xylene cyanol FF

40% (w/v) Sucrose in water

TEN Buffer

40 mM Tris

1 mM EDTA

150 mM NaCl

IPTG (Isopropyl-β-D-thiogalactoside)

IPTG	100 mg
Distilled water	1 ml

- Filtered and stored at at -20° C

TBS (1X) (1000ml)

Tris	2.42 g
NaCl	29,2 g

STE Buffer

10,3% Sucrose

25 mM EDTA pH:8.0

25 mM Tris-HCl pH:8.0

2mg/ml Lyzozyme

Transfer Buffer (1X) (1000ml)

20% Methanol	200 ml
25 mM Tris	3,63 g
192 mM Glycine	14,4 g
0,037% SDS	0,37 g

PBS (1X) (1000ml)

NaCl	8 g
KH_2PO_4	0,2 g
Na_2HPO_4. $7H_2O$	2,17 g
KCl	0,2 g

Denaturing Solubilization Buffer (pH:8.0)

50 mM NaH_2PO_4

1 M NaCl

8 M Urea

Denaturing Elution Buffer (pH:8.0)

50 mM NaH_2PO_4

1 M NaCl

8 M Urea

250 mM Imidazole

Dialysis Buffer (pH:8.0)

50 mM NaH2PO4

500 mM NaCl

4 M Urea

Carbonate/Bicarbonate Buffer (1000ml, 0,05M, pH:9.6)

Na_2CO_3	1,59 g
$NaHCO_3$	3,88 g

Washing Solution (pH:7.2)

1X PBS

0,1% Tween 20

Running Buffer (5X) (1000ml, stored at 4°C)

Tris base	15 g
Glycine	72 g
SDS	5 g

Fixation Buffer

40 % Ethanol

10 % Acetic Acid

50 % dH2O

Coating Buffer (pH:7.4, 0.2 µm filtered)

0.014 M NaCl

0.002 M KH_2PO_4

0.008 M Na_2HPO_4. $7H_2O$

0.01 M KCl

Blocking Buffer

4 % Bovine Serum Albumin, 5 % Sucrose in PBS

Assay Buffer (pH:7.2-7.4)

2 % Bovine Serum Albumin in PBS

Wash Buffer (pH:7.0-7.5, prepared fresh)

50 mM Tris, 0.2 % Tween-20 in PBS

Stop Solution

0.18 M H_2SO_4

Coomassie Blue R-250 Staning

Coomassie blue R-250	0,25 g
Methanol	125 g
Glacial acetic acid	25 ml
dH_2O	100 ml

Blocking Solution

2% BSA in washing solution

Phenol-Chloroform Solution (water-saturated)

Phenol 500 g

Chloroform 500 mL

Distilled water 400 mL

- The solution was stored at room temperature, protected from light.

APPENDIX C

CHEMICALS AND THEIR SUPPLIERS

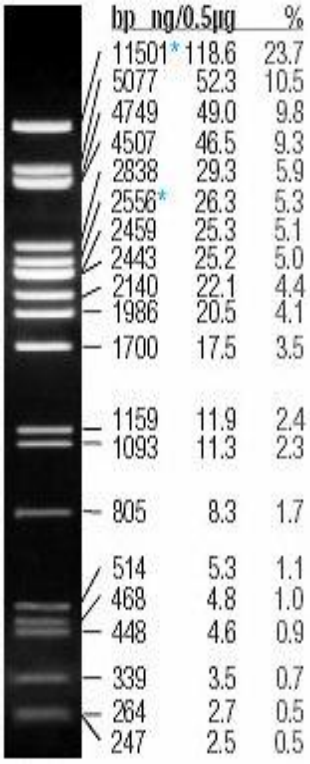
Chemicals	**Supplier**
Agar Bacteriological	Merck
Agarose	Prona
Ampicillin	Sigma
Bovine Serum Albumin	Sigma
Bromophenol Blue	Sigma
$CaCl_2.2H_2O$	Merck
Casamino Acid	Sigma
Coomassie Brilliant Blue G-250	Merck
Coomassie Brilliant Blue R-250	Sigma
dNTPs	MBI Fermentas
Dimethylformamide	Merck
EDTA	AppliChem
Ethanol	Botafarma
Ethidium Bromide	Sigma
Glacial Acetic Acid	Merck
Glycerol	Merck
Glycine	Merck
HCl	Merck
Imidazole	Merck
IPTG	Sigma
Kanamycin	Sigma

KH_2PO_4	Merck
KCl	Merck
L-cystein	Sigma
Ligase Buffer (2X)	MBI Fermentas
Ligase Buffer (10X)	MBI Fermentas
Luria Broth	Q-Biogene
Methanol	Merck
$MgCl_2.6H_2O$	Merck
NaCl	Merck
Na_2CO_3	Merck
$NaHCO_3$	Merck
NaH_2PO_4	Merck
NaOH	Merck
Phenol	Merck
SDS	Merck
Skim Milk	Fluka
Sucrose	Merck
Tris-base	Merck
Urea	Fluka
X-gal	MBI Fermentas
Yeast Extract	Difco

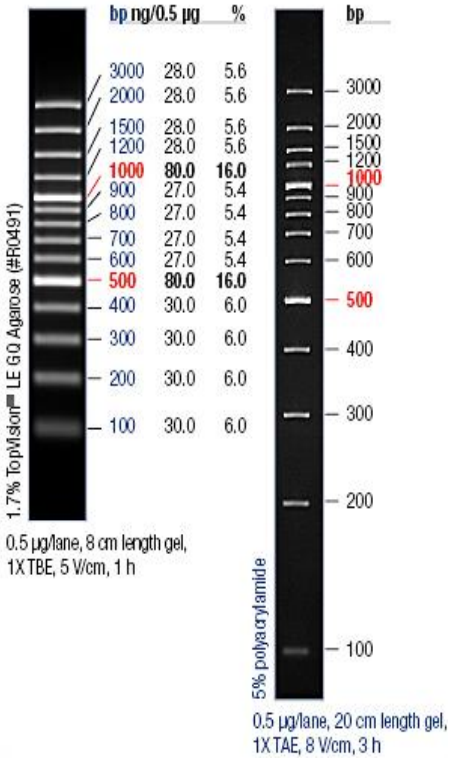
Enzymes	**Supplier**
*BamH*I	MBI Fermentas
*Bgl*II	MBI Fermentas
*EcoR*I	MBI Fermentas
Lysozyme	AppliChem
*Nco*I	MBI Fermentas
T4 DNA Ligase	MBI Fermentas
Tag DNA Polymerase	MBI Fermentas

APPENDIX D

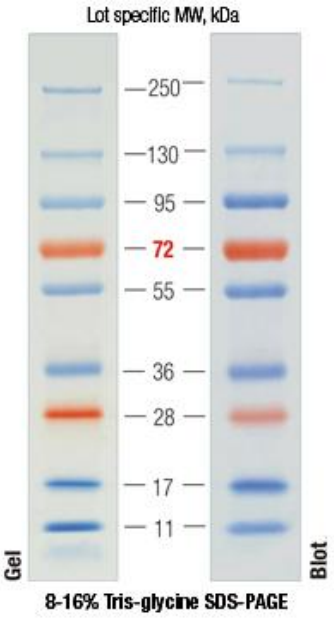
MARKERS



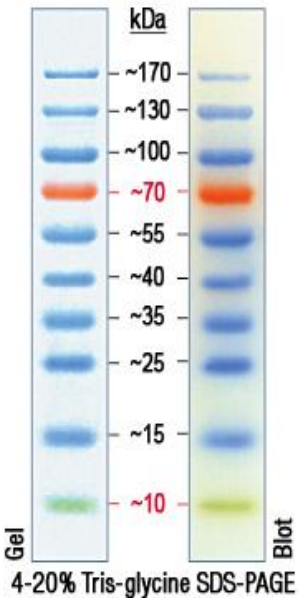
Fermentas Lambda DNA/PstI, 24 Ladder



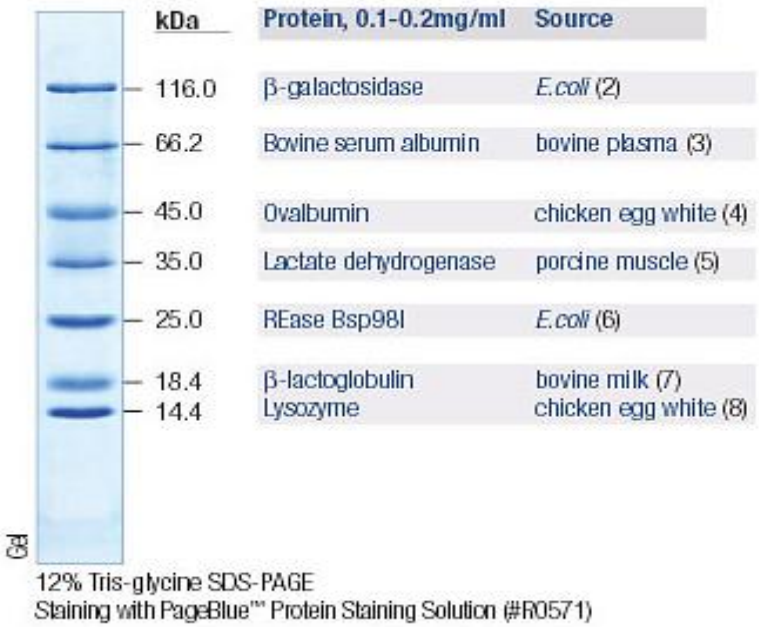
O'GeneRuler™ 100 bp Plus DNA ready-to-use, 100-3000bp



PageRuler™ Plus Prestained Protein Ladder, #SM1811, Fermentas



PageRuler™ Prestained Protein Ladder, #SM0671, Fermentas



Unstained Protein Molecular Weight Marker, #SM0431, Fermentas