The *Bordetella pertussis* protein Pertactin:

role in immunity and immune evasion

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Cover: "Clownfish in an anemone". Clownfish are immune to the deadly stings of the anemones tentacles.
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The *Bordetella pertussis* protein Pertactin:
role in immunity and immune evasion

Het *Bordetella pertussis* eiwit pertactine:
rol in immuniteit en het omzeilen daarvan

(met een samenvatting in het Nederlands)

Proefschrift

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Marcel Hijnen

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Voor mijn familie

Review committee:	Prof. Dr. F. Miedema	(Chairman)
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Contents

1

General introduction

Chapter 1

Introduction

1. Pertussis

1.1 History of pertussis

Pertussis or whooping cough is a highly contagious respiratory illness of humans that is caused by the gram-negative bacterium *Bordetella pertussis*. Another member of the *Bordetella* genus, *Bordetella parapertussis*, is able to cause mild pertussis like syndromes. *B. pertussis* is a strictly human pathogen and no known animal or environmental reservoir has been described. *B. pertussis* lacks an ancient written history, which is in contrast to several other severe epidemic infectious diseases of humans. It was suggested that the disease began in France around 1400, where in 1578 the first epidemic was described [1, 2]. The disease was named pertussis by Sydenham in 1679, meaning violent cough, It was not until 1906 that Bordet and Gengou reported the isolation of *B. pertussis* [3]. In view of the severity of the disease, it is not surprising that vaccine development started soon after the isolation and growth of *B. pertussis* in the laboratory. The introduction of effective pertussis vaccines in the 1950s and 60s led to dramatic reductions in morbidity and mortality. Before vaccination, pertussis was a major cause of infant death throughout the world.

1.2 Clinical manifestations

Before widespread introduction of vaccination against pertussis, almost every child contracted pertussis. The disease is most severe in neonates and children under the age of 1. In the 1950s in the Netherlands, the mortality rate due to pertussis was 1,8 deaths per 100.000 [4]. In general, the incubation period of pertussis is 7 to 10 days. The typical pertussis illness most often occurs as a primary infection in un-immunized children [1, 5]. The classical illness lasts 6 to 12 weeks or longer and goes through three distinct stages. In the first, catarrhal stage, there are symptoms of a common cold; rhinnorrhea, malaise, low grade fever and anorexia, symptoms similar to those caused by rhinovirus infections. Over a 7- to 14-day period, the catarrhal stage is followed by the paroxysmal stage, which is characterized by series of repeated coughing with 5 to 10 or more forceful coughs during a single expiration (a paroxysm). The paroxysms are followed by massive inspiratory efforts during which the classic whoops occur. Mucus production is associated with these paroxysms and is partially responsible for the restriction of the airways. After a series of paroxysms, vomiting is common and children are exhausted and may appear apathic. The paroxysmal stage lasts for 2 to 4 weeks. During the gradual transition to the convalescent stage, the frequency of the paroxysms decreases, resulting in a decrease in the severity of the events. The convalescent stage usually lasts several weeks to months. It is very common that secondary infections occur during the convalescent phase, which are mostly of bacterial origin.

1.3 Treatment

Erythromycin has been the basis of treatment of pertussis patients for the last 30 years. When used in the catarrhal stage, erythromycin shortens the duration of symptoms and eliminates

the organism from the upper respiratory tract [6, 7]. Untreated patients with pertussis remain contagious for 2 to 4 weeks. The treatment with erythromycin significantly shortens the period that individuals are contagiousness and helps faster clearance of the bacterium [1]. Since the bacterium is cleared by effector cells from the nasopharynx three weeks after the onset of the disease, antibiotic treatment with erythromycin is only helpful before this period. The remaining symptoms are believed to be caused by the secreted toxins.

1.4 Vaccination and prevention

Due to the morbidity and mortality caused by pertussis, attempts to create vaccines were made as soon as *B. pertussis* was first isolated in the laboratory. In the 1930s, many candidate pertussis vaccines were developed using a large number of methods. The first pertussis vaccines were mono component whole-cell pertussis vaccines. By 1947 these vaccines were combined into the so-called combination vaccines with diphtheria and tetanus toxoids (DTP). Such a whole cell combination vaccine was introduced in the Netherlands in 1952 and children were vaccinated with this vaccine at the age of 3, 4, 5 and 48 months (Table 1). Introduction of mass vaccination reduced the number of pertussis cases decreased significantly and resulted in a 10-fold decrease in pertussis mortality in the Netherlands in the late 1950s [8].

Since the whole cell pertussis vaccines contain strong immunogens like Ptx and LPS, they are rather reactogenic. The reactogenicity of DTP vaccines has been extensively evaluated throughout the entire pertussis vaccine era. There has been concern about the temporally related occurrence of severe neurologic disease and death and DTP vaccination (summarized in: [1]). However in none of the carefully performed studies, there was a clear relation between DTP vaccination and the severe side effects. Although there was no solid scientific evidence, the public distrust against the whole cell vaccine has never completely disappeared.

Unfortunately, this distrust has had a major impact on the effectiveness of many national immunization programs (NIP). To restore the confidence of the general public in the NIP, several changes were made to the pertussis vaccine to make it less reactogenic. The attempts of vaccine manufacturers to make safer variants of their vaccines sometimes resulted in vaccines with a poor efficacy [9, 10].

In the Netherlands in 1975, the potency of the vaccine was reduced to minimize the side effects (Table 1). As a result, in the period between 1975 and 1984, the pertussis incidence increased in the Netherlands. Due to the increase, in 1985 the potency of the Dutch vaccine was restored to its previous level (Table 1). In Sweden there was also concern about "vaccine-induced encephalopathy". These concerns resulted in changes in the Swedish vaccine in 1979, after which it was found to be ineffective and its use was discontinued [11]. In England, the concerns about the presumed serious side effects of the pertussis vaccine led to a decrease of the vaccine coverage to 30%. As a consequence, the pertussis incidence dramatically increased, which in turn led to a recovery of the vaccine coverage [12].

The negative publicity concerning the safety of the WCVs stimulated the development

of acellular pertussis vaccines (ACVs). The first description of the production of acellular pertussis component vaccines was by Sato *et al.* in Japan [13]. Six manufacturers in Japan followed Sato's lead and developed combination vaccines containing acellular pertussis,

Table 1. Overview of changes to the Dutch national immunization program, with the focus on the pertussis vaccine component.

Period	Vaccine combination	Characteristics pertussis component	Vaccination schedule
1952-1959	DTP_w	20 IOU/HD	3,4,5 mo, 4 yr
1959-1962	DTP_w	16 IOU/HD, ≥ 4 IU/HD	3,4,5 mo, 4 yr
1962-1975	DTP_w –IPV	16 IOU/HD, ≥ 4 IU /HD, second strain	3,4,5, 11 mo
1975-1984	DTP_w –IPV	10 IOU/HD, ≥ 4 IU/HD	3,4,5, 11 mo
1985-1992	DTP_w –IPV	16 IOU/HD, ≥ 4 IU/HD	3,4,5, 11 mo
1993-1996	DTP_w -IPV – Hib	16 IOU/HD, ≥ 4 IU/HD	3,4,5, 11 mo
Dec 1997	DTP_w -IPV – Hib	16 IOU/HD, ≥ 7 IU/HD	3,4,5, 11 mo
Jan 1999	DTP_w -IPV – Hib	16 IOU/HD, ≥ 7 IU/HD	2,3,4, 11 mo
Nov 2001	DTP_w -IPV – Hib	16 IOU/HD, ≥ 7 IU/HD	2,3,4, 11 mo, P_A at 4 yr.
Mar 2003	DTP_w -IPV /Hib	16 IOU/HD, ≥ 7 IU/HD	2,3,4, 11 mo, P_A at 4 yr.
Jan 2005	DTP_A-IPV/Hib		2,3,4, 11 mo, P_A at 4 yr.

Abbreviations used: DTP_w, diphtheria toxoid, tetanus toxoid and whole cell pertussis vaccine. IPV, inactivated polio. Hib, Haemophilis influenzae b vaccine. IOU, international opacity units. HD, human dose. IU, international units. P_A, acellular pertussis vaccine. Adapted from de Melker et al., RIVM report, 2004.

diphtheria toxoid and tenanus toxoid (DTaP) [14]. Due to pertussis epidemics in Japan, these vaccines were put into immediate routine use in 1981, without proper efficacy, immunogenicity and reactogenicity studies. However, the epidemical pertussis situation in Japan was significantly controlled by these vaccines [1]. Due to the lack of adequate data about the Japanese vaccines, their use was limited to Japan. However, the success of their DTaP vaccines stimulated extensive studies and trials in Japan, United States, Europe, and Africa, which were carried out over a 15-year period from 1980 to 1995 [1]. A large number of ACVs containing several purified and detoxified *B. pertussis* antigens, including pertussis toxin (Ptx), filamentous hemagglutinin (FHA), Pertactin (Prn) and fimbriae (Fim2 and Fim3) in various combinations were tested for there efficacy, immunogenicity and reactogenicity and were compared to several WCVs. The ACVs were clearly less reactogenic compared to the WCVs and had vaccine efficacies between 59% and 93%, dependent on the vaccine and criteria used (54). In contrast, good WCV's had vaccine efficacies between 89 % and 96%, whereas moderate WCVs had vaccine efficacies between 36 % and 48%.

In the Netherlands a WCV, produced with a process that is essentially similar as in the 1950s, was used until 2004. Several adjustments were made to the vaccine and the vaccination

scheme as described in table 1, including the introduction of a second strain in 1962. In 2001 a booster vaccination with an ACV was introduced for 4-year-old children to reduce the disease burden amongst the 4-9 year olds.
In 2004, there was again a lot of commotion about the reactogenicity of the Dutch WCV. Several parents were claiming that their children experienced neurological damage after vaccination with the Dutch WCV. The Dutch vaccine institute (NVI) had planned to introduce an ACV as part of the NIP in 2007. However, stimulated by the public opinion, it was decided to introduce the DTaP-IPV (Infanrix-IPV, GSK) vaccine in January 2005. This vaccine will be replaced by a DTaP-IPV vaccine produced by the NVI in the near future.

1.5 Epidemiology

Despite a high vaccination coverage is pertussis still endemic in the Netherlands, with epidemic peaks every 2 to 3 years during the last decade (Fig. 1). Also in several other European countries, as well as Canada, The United States and Australia, a re-emergence of pertussis was observed.

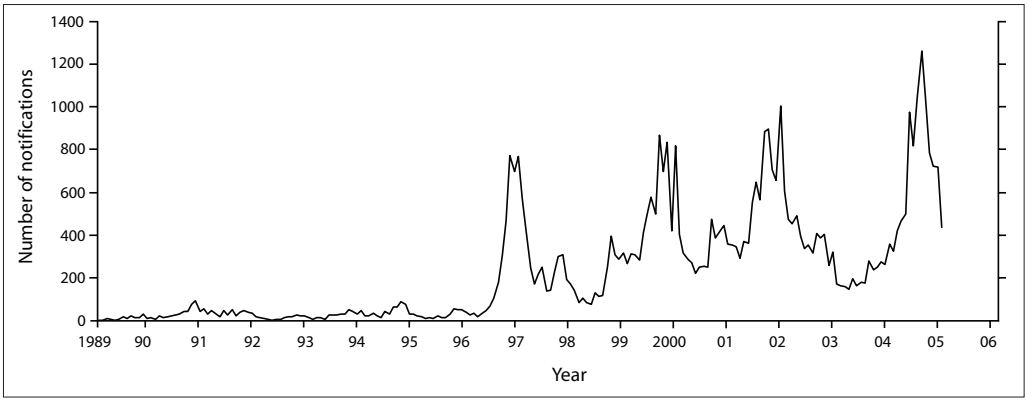

Figure 1. Pertussis notifications in the Netherlands per month in the period 1989-2005.

Insight into the incidence of pertussis is based on different surveillance sources; i.e. notifications, serology data, hospital admissions and registrations of deaths. In 1976 in the Netherlands, pertussis notification was made obligatory by law, which resulted in an increase in the number of notifications. In 1988 the introduction of a stricter case definition, including clinical symptoms and laboratory confirmation, led to a decline in notifications. In 1988 two-point serology and in 1997 one-point serology and positive PCR were officially introduced as diagnostic method [15, 16]. In the period of 1989 to 1995, with a stable case definition for notification, increased numbers of pertussis cases were reported in 1989/1990 and in 1993/1994 despite a vaccine coverage of 96% (Fig. 1). In 1996, a large increase in the number of notifications, positive cultures, positive serology and hospital admissions was observed. Since the outbreak of pertussis in 1996, the Netherlands now has an epidemic

pertussis cycle of 3 years.
Waning immunity in adolescents and adults, increased reporting, improved diagnosis of the disease and the adaptation of the *B. pertussis* population, were all proposed for this sudden increase [17, 18]. Waning immunity is likely to play a role in the epidemiology of pertussis. However, waning immunity alone can not explain the sudden and sharp rise of pertussis cases over a wide age-range in the Netherlands [19].
Analysis of clinical isolates collected in the last 50 years in the Netherlands revealed antigenic divergence between vaccine strains and circulating strains. This antigenic divergence was also observed in isolates collected in several other countries in Europe, the United States, Asia and Australia [20-30]. In the Netherlands, pathogen adaptation has probably played an important role in the resurgence of pertussis [18, 31].

2. The virulence factors of *B. pertussis* and their regulation

2.1 The BvgAS system

The members of the genus *Bordetella* share a genetic locus, designated *bvg* for *Bordetella* virulence genes, which encodes a system that belongs to a large family of two-component signal transduction systems [32]. The *bvg* locus enables these species to oscillate between different phenotypic states depending on their environment [33]. The *bvg* locus comprises a number of genes that enable the bacterium to sense the prevailing environmental conditions and then act accordingly by controlling the expression of specific genes and gene loci [33]. The sensor protein, BvgS, is a trans membrane protein containing a periplasmatic located N-terminus and a cytoplasmatic located C-terminus. The N-terminus senses the environment which, under Bvg^+ conditions results in a phosphorylated C-terminus [34, 35]. The transfer of a phosphoryl group from the cytoplasmic component of BvgS to BvgA, the activator protein, allows the latter to activate the promoters of virulence-activated genes (*vags*) resulting in Bvg^+ phase *Bordetella* [33]. A third protein, BvgR, is regulated under the positive transcriptional control of the BvgAS system and down regulates the expression of virulence repressed genes (*vrgs*) in the Bvg^+ phase. In the laboratory, the addition of sulfate ions, nicotinic acid or a reduction in temperature from 37°C to 25°C results in a-virulent, Bvg^- phase, bordetellae [36]. In the Bvg^- phase, the expression of *vags* is down-regulated and the expression of *vrgs*, through BvgR, is up-regulated [33, 37]. Although the function of the Bvg^+ phase for infection is well defined, the role of the v*rgs* in *B. pertussis* is not clear. It has been suggested that the Bvg^- phase gene products allow *B. bronchiseptica,* which infects a broad range of animal species, to survive outside its host [38]. Since many of these genes are still functional in *B. bronchiseptica* but are pseudogenes in the strict human pathogens *B. pertussis* and *B. parapertussis*, it is plausible that they are an evolutionary remnant. However, recently, a Bvg intermediate phase (Bvg^i) was discovered indicating that the BvgAS-mediated fine tuning of virulence gene expression is still important for the strict human pathogen *B. pertussis*. The Bvg^i phase might be important in the late phases of infection, and is possibly involved in the transmission of bacteria to new hosts. Bacteria in the nasopharynx may express Bvg^i phase factors that could be beneficial for aerosol transmission [33].

2.2 BvgAS regulated virulence factors

The BvgAS two-component system regulates the expression of several virulence factors in a positive or negative manner. During the transition from the Bvg^- to Bvg^+ phase, bordetellae undergo several intermediate phases. The exact role of these intermediate phases is still not fully understood. Early in the Bvg^+ phase, specific adhesins like FHA and fimbriae are expressed ("early" genes) (Fig. 2) [33]. It is believed that these adhesins are important for the initial colonization. Other proteins like Tracheal colonization factor A (TcfA), *Bordetella* resistance to killing A (BrkA), Vag8, Fim, the type III secretion system (TTSS) and P.69 Pertactin, are also expressed during the Bvg^+ phase (Fig. 2). Several of these proteins contribute to adhesion. Later during the colonization by Bvg^+ phase *B. pertussis,* the toxins,

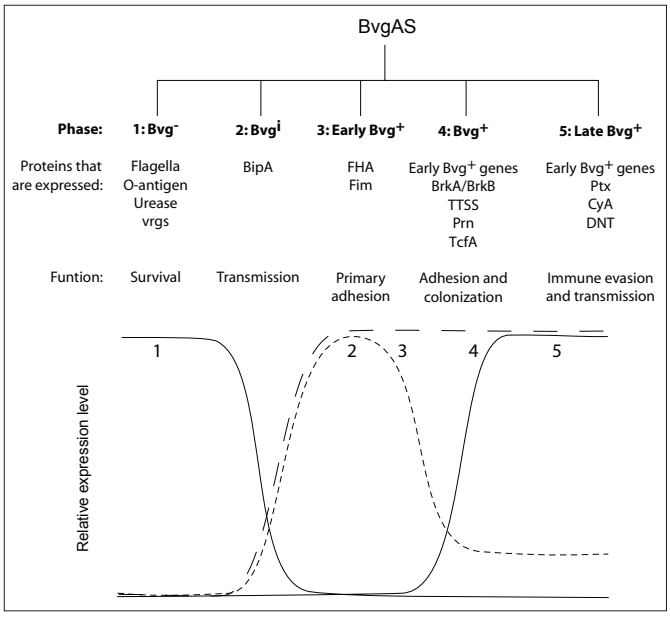

Figure 2. Control of the BvgAS system. The BvgAS system controls the expression of proteins in several phases (1-5). The distinct phases are indicated as well as the gene products that are expressed in that phase including the relative expression level. A possible function of each phase is indicated. Adapted from Cotter and Jones, Trends in Microbiology, 2003.

pertussis toxin (PTX), adenylate cyclase toxin (CYA) and dermonecrotic toxin (DNT) are expressed (the "late" genes) (Fig. 2) [33]. These gene products are assumed to be important for evasion of the host immune system, and transmission to susceptible hosts.

2.3 P.69 Pertactin

P.69 Pertactin, the focus of this thesis, is one of the BvgAS regulated adhesins, and belongs to a class of so-called auto transporter proteins that undergo autoproteolytic processing [39]. P.69 Prn is processed from a 93 kDa large precursor to 69 kDa and 22 kDa proteins. The unprocessed polypeptide is directed via a signal peptide to the secretory machinery in the inner membrane where the signal peptide is cleaved. Subsequently, the polypeptide is directed towards the outer membrane where the 22-kDa protein forms a pore through which the 69-kDa protein is transported. After secretion via the autotransporter domain, proteolytic activities shape the 69-kDa protein to it's final 60.4 or 58.3 kDa form [40]. These final forms (referred to as P. 69 Prn), stay non-covalently bound to the bacterial cell surface and are used in most ACVs [41]. From the first 539 AA of P.69 Pertactin, the X-ray crystal structure has been determined to a resolution of 2.5 Å. From the C-terminal AA 540-677, no structure has been determined. The protein structure consists of a 16-stranded parallel β-helix with a V-shaped cross-section. The structure appears as a helix from which several loops protrude, one of which contains of an Arg-Gly-Asp (RGD) motif implicated in ligand-receptor interactions in eukaryotes. It has been shown that this motif is involved in P.69 Prn-mediated attachment of *B. pertussis* to mammalian cells [42, 43]. P.69 Prn is polymorphic, and 13 variants (P.69 Prn1 - P.69 Prn13) have been identified so far. Variation is mainly limited to two regions, designated region 1 and 2, which are comprised of Gly-Gly-X-

X-Pro (r1 repeat) and Pro-Gln-Pro (r2 repeat) repeats, respectively. In *B. pertussis*, most variation is found in region 1 which is located proximal to the N-terminus and flanks the RGD sequence. Region 2 is located more towards the C-terminus (Fig. 3).
As described in paragraph 1.2, pathogen adaptation has probably played an important role in the resurgence of pertussis in the Netherlands. Analysis of clinical isolates collected in the last 50 years revealed polymorphisms in at least two proteins implicated in protective immunity: P.69 Prn and Ptx [31, 44]. Several observations indicate an important role for P.69 Prn in protective immunity. Antibody levels to P.69 Prn have been shown to correlate with clinical protection [45, 46]. ACV's containing Ptx, FHA and P.69 Prn were more effective compared to ACV's containing Ptx and FHA only [10, 41, 47]. Passive and active immunization studies in mice and pigs have shown that antibodies against P.69 Prn confer protective immunity [48, 49]. Anti-P.69 Prn antibodies, but not anti-Ptx, anti-fimbriae, or anti-FHA antibodies, were found to be crucial for *B. pertussis* phagocytosis [50]. P.69 Prn variants induce type-specific antibodies [16] and, finally, the efficacy of the Dutch whole cell vaccine was also shown to be affected by variation of P.69 Prn in a mouse model [48].

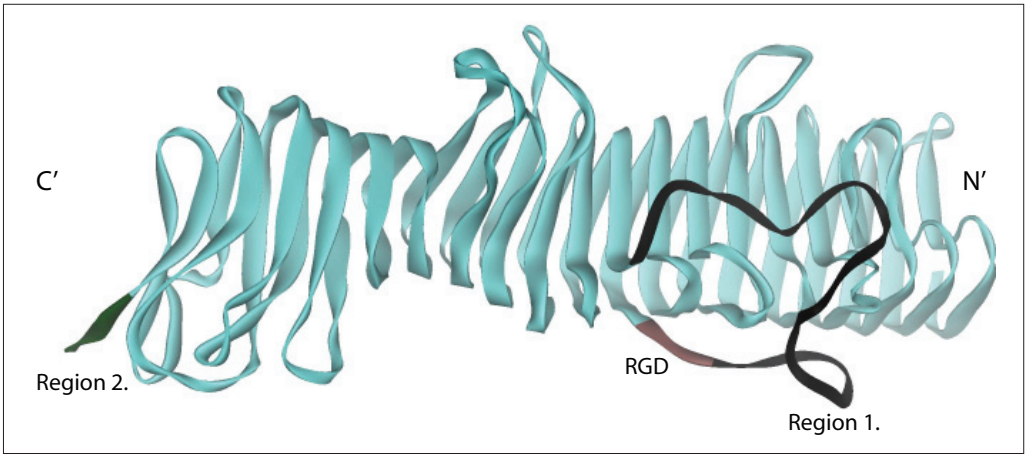

Figure 3. Crystal structure of P.69 Prn (1DAB.pdb). The region 1 loop is indicated in black. The site implicated in attachment, the RGD site, is indicated in brown. Note that the C-terminal amino acid residues 540 to 677, containing region 2, are not part of the structure.

2.4 *Bordetella* bacteriophage BBP-1

The adaptation of parasites to dynamic host characteristics is a common theme in biology [51]. Recently, a unique new mechanism of adaptation was described that governs the interactions between *Bordetella* spp. and a family of bacteriophages [52]. As described in paragraph 2.2, the cell surface of *Bordetella* spp. is highly variable due to the BvgAS mediated gene expression. The control of the BvgAS system results in the presence and absence of certain proteins on the surface of *Bordetella* spp. during their transition through infectious cycles. To maintain their infectivity, the *Bordetella* phages use a novel mechanism to evolve new ligands that

allow the use of alternative surface receptors for host cell entry [51-53]. This mechanism, named tropism switching, is a result of the introduction of single nucleotide substitutions at defined locations in the VR1 (variable region 1) segment of the *mtd* (major tropism determinant) gene, which determines the specificity for receptors on host bacteria. The introduction of the nucleotide substitutions is a template-dependent, reverse transcriptase-mediated mechanism.

The first *Bordetella* phage to be described was, BPP-1 (Bvg Plus tropic Phage–1), which is a temperate bacteriophage initially found in a clinical isolate of *B. bronchiseptica* that displays a marked tropism for Bvg^+ phase *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* [52]. Interestingly, the primary receptor for BPP-1 is Pertactin, which is only expressed in Bvg^+ phase *Bordetella* spp. Deletion of the Pertactin gene eliminated BPP-1 adsorption and decreased phage plaque formation to a level similar as observed with Bvg^- phase bacteria.

At a frequency of approximately 10^{-6}, BPP-1 gives rise to two classes of tropic variants (Fig. 4). One class, designated BMP (Bvg Minus-tropic Phage), has an acquired tropism for Bvg^- phase bacteria. The second class, designated BIP (Bvg indiscriminate phage), can infect both Bvg^+ and Bvg^- phase *B. bronchiseptica* with equal efficiency [51].

The recent discovery of the *Bordetella* phages must change in part our way of thinking about the selective pressure to which Pertactin is exposed. Until recently, only immunological pressure (antibodies) was thought to be the reason for variation in Prn. However, it is likely that Prn has evolved in such a way that it can evade infection by the *Bordetella* phages and binding of antibodies.

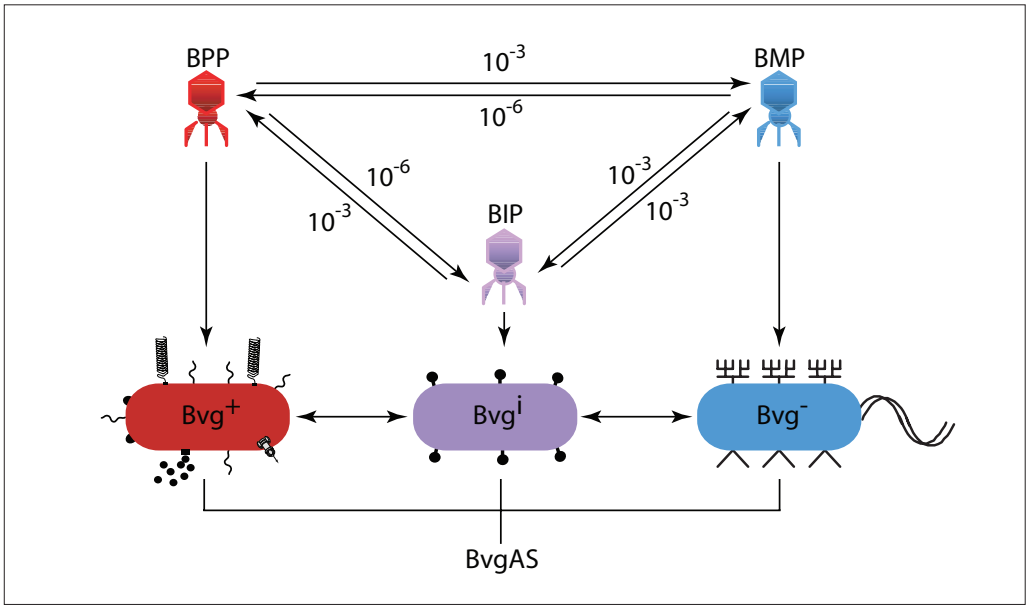

Figure 4. Tropism switching by the *Bordetella* bacteriophages. Specificities and switching frequencies are indicated. Adapted from Doulatov *et al.*, Nature, 2004.

3. Immune evasion

3.1 Introduction

Just as the human immune system has both innate and adaptive mechanisms to defend against bacterial and viral infections, so did both pathogenic and non pathogenic micro-organisms evolve innate and adaptive ways to circumvent these defenses [54]. A large number of defense and evasion strategies are described against both innate and adaptive immune responses, such as down-regulation or suppression of immune responses including complement, seclusion into immune privileged sites, antigenic variation, epitope masking and many other strategies.

The immune evasion strategies employed by Human Immunodeficiency Virus (HIV) are probably the best studied together with several parasitic evasion strategies. In this paragraph, a brief overview of evasion strategies utilized by pathogens that are applicable and relevant to this thesis will be described.

3.2 Immune evasion strategies of *Bordetella*

Although a vast array of evasion strategies are available to micro-organisms to ensure their survival in the "unfriendly" environment of their hosts, relatively little is known about evasion strategies employed by *Bordetella*. Several virulence factors, mainly BvgAS regulated, have been shown contribute to bacterial persistence by modulating or subverting the protective immune response of the host. Several of these are described in this paragraph.

3.2.1 Ptx

Ptx is an exotoxin with a classical AB bipartite structure (A, active domain; B, binding). The B-oligomer binds surface glycoproteins which are present on a variety of mammalian cells, including cilia, macrophages and lymphocytes [55, 56]. The A monomer of Ptx has ADP ribosyltransferase activity. The G_i proteins, which are responsible for transmitting inhibitory signals to the adenylate cyclase complex, are ADP-ribosylated by Ptx which results in elevated levels of intracellular cAMP. These increased cAMP levels affect the signaling pathways in many cells, including cells from the immune system, contributing to impaired immune functions in infected patients [57]. One of the described targets of this mechanism is the innate immune system. It has been shown that, in the airways of the lungs, Ptx interferes with the influx of neutrophils which are able to directly kill bacteria [58]. Ptx has been shown to inhibit macrophage chemotaxis in vivo [59]. Furthermore, the in vitro chemotaxis of neutrophils and lymphocytes has been shown to be inhibited by Ptx [60]. Besides interfering with the innate immune system, also a role of Ptx in interfering with the adaptive immune system has been described. Ptx has been shown to interfere with the delivery of signals that promote the survival of B-cells in vitro, resulting in an accelerated decay of these cells [61]. Furthermore, experimental evidence suggests that Ptx suppresses antibody responses during *B. pertussis* infection [62].

3.2.2 ACT

Unlike Ptx which acts as a freely soluble toxin, ACT is suggested to act as a cell-associated toxin (8). It has been shown that *B. pertussis* can invade and survive within mammalian cells such as macrophages, PMNs and respiratory epithelial cells. The survival in this type of cell has been linked to inhibition of killing mediated by ACT [63, 64, 64-71]. Similar as Ptx, ACT also is a bifunctional toxin, with a haemolysin enzyme domain, responsible for entry into cells, and a adenylate cyclase domain, responsible for elevating the levels of intracellular cAMP [72]. As described for Ptx, elevated levels of intracellular cAMP interfere with cell signaling which in turn can be beneficial for bacterial survival in host cells. Recently, a synergistic role of Ptx and ACT was described. Ptx has been shown to delay the influx of neutrophils, whereas ACT has been suggested to intoxicate the recruited neutrophils. Carbonetti *et al.* therefore suggested that these two toxins provide a one-two punch to neutrophil recruitment and activity, which would be essential for optimal infection and colonization of the respiratory tract [73].

3.2.3 FHA

FHA is one of the major attachment factors of *B. pertussis* to epithelial cells. FHA contains an RGD sequence which is shown to promote binding to leukocyte response integrin ($\alpha_v\beta_3$, CD61), integrin-associated protein (CD47) complex and also stimulates bacterial adherence to the β_2 integrin CR3 (CD11b/CD18, $\alpha_m\beta_2$) which are expressed on macrophages, PMN's and several other cell types [74]. After phagocytosis, due to FHA binding to CR3, intracellular killing is presumably suppressed by the excreted bacterial toxins, which facilitates intracellular survival and persistence of *B. pertussis* in the lungs [63, 65, 75, 76]. It has been shown in a mouse model, that FHA is able to suppress LPS induced IL12 and IFN-γ levels in serum. FHA induces the secretion of IL6 and IL10 by macrophages, with a reciprocal inhibition of IL12 production [75]. The latter could explain the suppressed T-cell response in the lungs of mice during *B. pertussis* infection [77].

3.2.4 BrkA

B. pertussis is relatively resistant to classical complement-dependent killing by human serum. It has been shown that the resistance to complement lysis is mediated by the outer membrane protein BrkA [78, 79]. However, the exact mechanism of action of BrkA is not known.

3.2.5 TCT

Tracheal cytotoxin (TCT) is a disaccharide-tetrapeptide, which is derived from the peptidoglycan of the bacterial cell wall. TCT stimulates the production of IL1 and nitric oxide by respiratory epithelial cells, which are suggested to be responsible for much of the epithelial pathology of pertussis. Is has also been shown that TCT may contribute to *B. pertussis* survival by inhibiting neutrophil and chemotaxis and oxidative metabolism [57, 80].

3.2.6 P.69 Pertactin

The protein of interest of this thesis, P.69 Prn has also been shown to play a role in adhesion to monocytes [69]. There is some evidence that Prn plays a role in subverting the immune response. Like FHA, Prn contains an RGD sequence, and it has been shown that there is a synergistic effect of FHA and Prn on the suppression of IL12 induction by LPS [81]. Furthermore, in this thesis we show that exposed loops on Prn are employed to evade antibody binding by hiding important epitopes from the immune system.

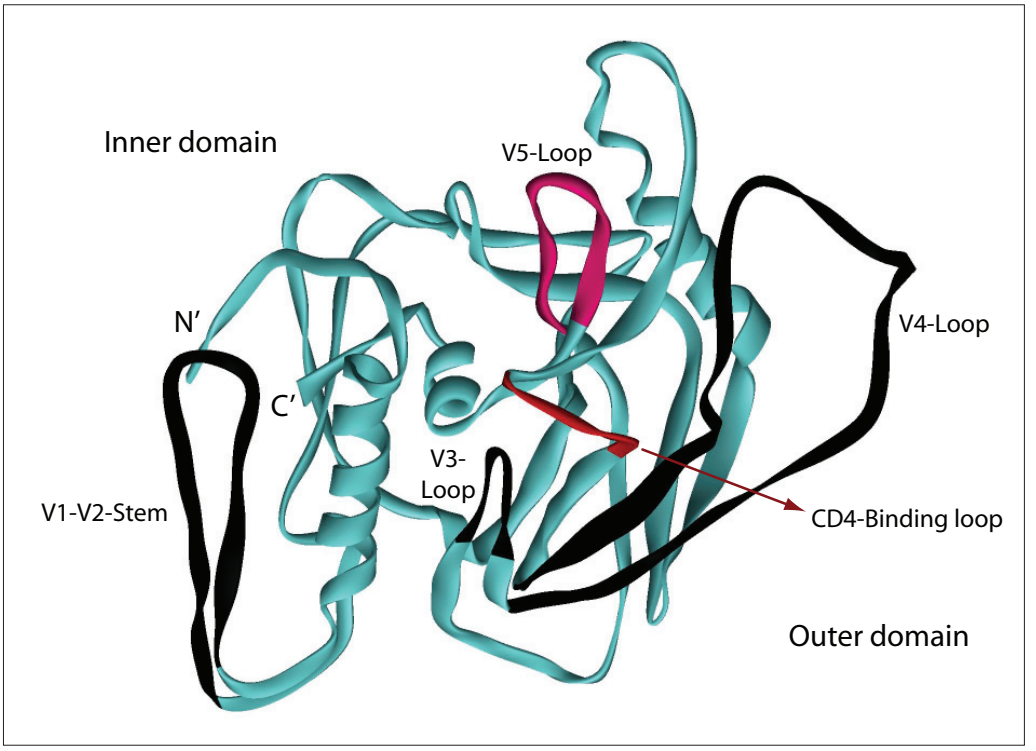

Figure 5. Crystal structure of unliganded gp120 (Chen *et al.*, 2005)(2BF1.pdb). The variable loops V1-V5 are colored to facilitate identification. The CD4-binding loop is indicated in red.

3.3 General mechanisms of evasion of humoral responses

As described in the previous paragraph, a few mechanisms for the evasion of innate immune responses have been reported for *B. pertussis*. The evasion of adaptive immune responses, and in particular evasion of the humoral immune response, has been poorly described for *B. pertussis*. In contrast to *B. pertussis*, quite a number of studies on the evasion of humoral immune responses by viral pathogens have been described. One of these well-studied viruses is HIV, and in particular the HIV glycoprotein gp120. Gp120 serves as an example in this paragraph since it contains all structural features which are also described for other proteins

harboring similar mechanisms to evade humoral immune responses.
The envelope glycoproteins of HIV mediate viral attachment and entry into $CD4^+$ T cells and macrophages through binding interactions with CD4 and chemokine receptors. Since gp120 and gp41 are the only proteins expressed on respectively the surface and membrane of virions and infected cells, these proteins are the only target for antibodies and therefore have evolved to evade antibody responses. Gp120 is heavily glycosilated and contains 5 conserved and 5 variable regions. The first 4 variable regions (V1-V4) are located on loops [82, 83] (Fig. 5). It has been shown that gp120 folds into a globular molecule that is composed of an inner and outer domain which are joined by a bridging sheet [54]. Furthermore gp120 forms oligomers in which the inner domains are predicted to associate with each other, which conceals this domain from antibodies [54, 84]. This feature of gp120 is one of the evasion strategies, since the majority of antibodies are induced to the inner domains of shedded monomeric gp120 which become inaccessible in the oligomeric form [54, 85]. Carbohydrates constitute over 50% of the mass of gp120, and most of the glycosylation is found on the outer domains of gp120 [54, 86]. This limits B-cell responses to the underlying epitopes, which explains the limited number of antibodies to the surface of gp120. The outer domains are therefore regarded as the "immunosilent face" [54, 87]. Another mechanism employed by gp120 is conformational masking. The CD4 binding residues are conserved and therefore potential targets for neutralizing antibodies. However, these conserved residues are conformationally hidden in a deep pocket in gp120 and are exposed only after binding to CD4 [86]. Exposed residues flanking the CD4 binding site, are in turn very tolerant to amino acid substitution and hence antigenic variation, which increases the opportunity for antigenic escape [54]. The CD4 binding site is further protected from antibodies by the position of the variable loops V1 and V2. The V1 and V2 loops are positioned to further conceal the CD4 binding site. These two variable loops are highly tolerant of amino acid substitutions, additions and deletions and therefore induce strain-specific antibodies.
Although gp120 is an extreme example of a protein with humoral immune evasion mechanisms, it clearly indicates the functions certain region can harbor in proteins that are exposed to immunological pressure. Similar mechanisms as employed by gp120 are described for hepadnaviridae family members (Hepatitis B virus) [88, 89], some members of the flavivirus [90] and herpesvirus family [91], Coronaviridae (SARS) [92], influenzaviridae [93], and bacteria like Streptococci (group A and B, *mutans*) [94-96], *Borrelia burgdorferi* [97], Neisseriae [98], bordetellae (this work) and several other pathogens including a large group of parasites.
Since large scale screening methods, such as genome sequencing, micro-arrays and proteomics are becoming more accessible, it is to be expected that immune evasive functions of many other proteins will be identified in the near future. These studies will hopefully also allow a more rational vaccine design and result in effective vaccines to a large number of disastrous diseases.

4. Scopes of this thesis

The aim of this study was to define epitopes on P.69 Prn and to obtain insight in the role that the variable regions play in immunity and immune escape. In **chapter 2** we describe the location of linear epitopes recognized by several mouse monoclonal antibodies (mAbs) and by human serum antibodies. We provide evidence that single mAbs were able to recognize both N- and C-terminal peptides, indicating that the two regions are proximate in the folded molecule. In **chapter 3** we describe a rapid procedure to express large quantities of recombinant P.69 Pertactin in *Escherichia coli*. This procedure was used to isolate and characterize Prn1, 2, and 3 variants as well as several deletion mutants. These deletion mutants facilitated further research on P.69 Pertactin in the subsequent chapters. In **chapter 4** recombinant P.69 Prn mutants were used to map the location of conformational epitopes recognized both by mAbs and human serum antibodies. Furthermore the technique used in chapter 3 was employed to construct P.69 Prn mutants by site-directed mutagenesis. These studies provided additional evidence for the interaction between the N- and C-terminus, as suggested in **chapter 2**. Finally, a role for the interaction of the N- and C-terminus, as well as several loops is discussed. In **chapter 5** we used the data obtained in **chapter 2** to reconstruct a conformational epitope using a novel synthetic peptide scaffold approach. This peptide scaffold was able to induce antibodies that recognize a conformation dependant epitope and provide protection against infection with *B. pertussis*. Furthermore we demonstrated that antibodies against this epitope are normally not induced, possibly since this epitope in Prn is masked from the immune system. In **chapter 6** the role of Pertactin specific antibodies is elucidated. We show that although it is possible to detect Prn specific antibodies, in convalescent sera, the frequency of these antibodies is very low. In contrast, the amount of antibodies directed against the less variable region 2 is relatively high, and we show that immune evasion is likely to occur by interaction of the variable region 1 and 2. In **chapter 7** we tested the effect of antigenic variation in Prn on vaccine efficacy. A large number of Prn variants and mutants which were described in chapter 3 were tested for their protective properties against *B. pertussis*. It became clear that the N-terminus harbors protective epitopes, as mice immunized with a mutant lacking this part were hardly protected. Furthermore, it appeared that Prn1 protected best against Prn1 type strains, and Prn2 against Prn2 strains. Overall Prn2 seemed to provide better protection than Prn1. The role of the variable region 1 seems to be marginal in protection. In **chapter 8** we investigated the role of polymorphisms in P.69 Prn of several *Bordetella* species. It became clear that there is a relation between the location of the polymorphisms and the effect that they have on immune evasion. We provide evidence for a delicate balance between variation and conservation of the P.69 Prn gene. In the discussion in **chapter 9** we summarize the data and discuss the role of variable regions and loops in proteins on immune evasion. Furthermore we discuss the effect of subtle variations in the P.69 Prn gene on vaccine efficacy and protection.

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2 Epitope structure of the *Bordetella pertussis* protein P.69 Pertactin: a major vaccine component and protective antigen

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Abstract

Bordetella pertussis is re-emerging in several countries with a traditional high vaccine uptake. An analysis of clinical isolates revealed antigenic divergence between vaccine strains and circulating strains with respect to P.69 Pertactin. Polymorphisms in P. 69 Pertactin are mainly limited to regions comprised of amino acid repeats, designated region 1 and region 2. Region 1 flanks the RGD motif involved in adherence. Although antibodies against P. 69 Pertactin are implicated in protective immunity, little is known about the structure and location of its epitopes. Here we describe the location of mainly linear epitopes recognized by both human and mouse monoclonal antibodies (mAbs) using Pepscan analysis. A total of 24 epitopes were identified, of these only 2 were recognized by both mAbs and human serum antibodies. A number of immunodominant epitopes were identified, recognized by 78%-93% of the human sera tested. Blocking experiments indicated the presence of high avidity human antibodies against conformational epitopes. Human antibodies against linear epitopes were of much lower avidity as they were unable to block mAbs. Pepscan analyses revealed a number of monoclonal antibodies which bound to both region 1 and region 2. The two regions are separated by 289 amino acids in the primary structure, and we discuss the possibility that they form a single conformational epitope. Thus, both repeat regions may serve to deflect the immune response targeted at the functional domain of P.69 Pertactin. This may explain why variation in P.69 Pertactin is so effective, despite the fact that it is limited to only two small segments of the molecule.

Introduction

In the pre-vaccination era, pertussis was a major cause of infant death throughout the world. Introduction of effective pertussis vaccines 50 years ago led to dramatic reductions in morbidity and mortality. However, there has been a resurgence in the incidence of pertussis in the last ten years in several countries despite high vaccine uptake [1, 2]. Waning immunity in adolescents and adults, increased reporting, improved diagnosis of the disease, and the emergence of *B. pertussis* escape variants are all proposed explanations for the re-emergence of pertussis [3, 4]. In the Netherlands, emergence of escape variants has probably played an important role in the resurgence of pertussis [2, 3]. Analysis of clinical isolates collected in the last 50 years revealed antigenic divergence between vaccine strains and circulating strains. Escape variants showed polymorphism in at least two proteins implicated in protective immunity: P.69 Pertactin (P.69 Prn) and pertussis toxin (Ptx) [2, 5]. The level of antibodies to both P.69 Prn and Ptx have been shown to correlate with clinical protection [6, 7]. Further, acellular vaccines (ACV's) containing Ptx, filamentous hemagglutinin (FHA) and P.69 Prn were more effective compared to ACV's containing Ptx and FHA only, also implicating an important role for P.69 Prn in immunity [8-10]. Finally, variation in P.69 Prn was shown to affect the efficacy of the Dutch whole cell vaccine in a mouse model [11].

P.69 Prn, the focus of this study, belongs to a class of so-called autotransporter proteins that undergo autoproteolytic processing [12]. P.69 Prn is processed from a 93 kDa large precursor to a 69 kDa and 22 kDa protein which are located at the cell-surface and in the outer membrane, respectively [13]. The 69 kDa product (referred to as P.69 Prn) is used in ACV's. P.69 Prn contains an Arg-Gly-Asp (RGD) motif implicated in ligand-receptor interactions in eukaryotes. It has been shown that this motif is involved in P.69 Prn-mediated attachment of *B.pertussis* to mammalian cells [14, 15]. P.69 Prn is polymorphic, and 13 variants (P.69 Prn1 – P.69 Prn13) have been identified so far. Variation is mainly limited to two regions, designated region 1 and two, which are comprised of Gly-Gly-X-X-Pro (r1 repeat) and Pro-Gln-Pro (r2 repeat) repeats, respectively. Most variation is found in region 1 which is located proximal to the N-terminus and flanking the RGD sequence. Region 2 is located more towards the C-terminus. Results presented by He et al., suggest that the r1 repeat induces type-specific antibodies which show little cross-reactivity between P.69 Prn1 and P.69 Prn2 [16].

A number of studies in both animals and humans have indicated that P.69 Prn can elicit protective antibodies [6, 7, 11, 17]. However, information about the location of epitopes to which these antibodies are directed is limited. The aim of this study is to define epitopes on P.69 Prn and to obtain insight in the role that the variable regions play in immunity and immune escape.

Materials and methods

Production of monoclonal antibodies.

MAbs were generated by injection of BALB/c mice subcutaneously three times, either with purified P.69 Prn or a fusion protein comprised of region 1 of P.69 Prn and maltose binding protein (MBP-region 1) [11]. Specol was used as adjuvant (ID-DLO, Lelystad, the Netherlands). Three days before the fusion, mice were boosted intravenously. Spleens cells were fused with mouse SP2/0 myeloma cells using 50% PEG-1500 (Boehringer, Mannheim, Germany). Hybridomas secreting antibody to P.69 Prn were selected by ELISA and cloned twice by limiting dilution [11]. MAbs were purified by protein-G affinity chromatography (Pharmacia, Upsala, Sweden) [18].

Human sera

We selected sera from nine children (median age of 4 years) who had high IgA titers (median of 192U/ml) against *B. pertussis*, which is indicative of a recent infection [19]. In addition, sera from five children were selected who received a booster immunization with acellular vaccine containing 25µg FHA, 25µg PT and 8µg of P.69 Prn1 (Glaxosmithkline, Rixensart, Belgium) at the age of four years in one arm and a regular DT-IPV booster in the other arm. The children were previously vaccinated with four doses of DTP-IPV (containing whole-cell pertussis vaccine, Netherlands Vaccine Institute, Bilthoven, the Netherlands) at the age of 3, 4, 5, and 11 months. Pre-booster sera (n=3) were used as negatives.

Synthesis of peptides derived from region 2

Synthetic penta-decapeptides spanning the r1 and r2 repeat and overlapping by ten amino acids were synthesized. Synthesis of the r2 repeat peptides was performed as described for the r1 peptides [11]. The amino acid sequences of the r2 peptides are listed in Table 1.

R1- and R2-peptide ELISA

Polystyrene 96-well ELISA plates (Immulon II; Dynatech, Chantilly, VA) were coated overnight at 22 °C by adding 100 µl PBS containing 1µg/ml peptide to each well. Plates were washed four times with 200µl PBS supplemented with 0.05% Tween 20 (PBST) per well using the TitertekPlus M96V washer (ICN, Irvine, CA) and subsequently blocked by incubation with PBST supplemented with 0.5% (w/v) bovine serum albumin (BSA; Sigma, St. Louis, MI) for 1½-2hrs at room temperature. Murine mAbs, diluted in 0.5% BSA in PBST to a concentration of 1 µg/ml were added to the wells and were incubated for 2hr at 37 °C followed by four washings as described above. Bound antibodies were detected using HRP-conjugated anti-mouse total IgG (Cappel, Organon Technica, Boxtel, the Netherlands). Extinction (OD_{450}) was measured with a BioTek plate reader (EL312e, BioTek systems, Winooski, VT). To increase the sensitivity of the assay, higher concentrations of coating peptide (5 µg/ml) and mAb (10 µg/ml) were used.

Synthesis of peptides for Pepscan analysis

For Pepscan analysis, 15-mer linear, 15-mer loop and 30-mer linear peptides spanning the entire amino acid sequence of P.69 Prn were synthesized. There was a shift of one amino acid residue, between the subsequent 15- or 30-mer peptides. The synthesis of the 15-mer linear and 15-mer loop peptides was performed on credit-card format mini-PEPSCAN cards (455 peptide format/card) as described previously [20]; Pepscan systems, Lelystad, the Netherlands). The 30-mer peptides were synthesized by linking two 15-mer peptides as follows. The sequence [1-14] was synthesized with an additional cysteine at position 15, whereas the sequence [17-30] was bromoacetylated at its N-terminus. The peptides [1-14]-Cys and BrAc-[17-30] were coupled by formation of a thioether bond. The thioether containing spacer replaces two amino acid residues (i.e. 15 and 16) in the [1-30] sequence.

Pepscan ELISA

The binding of antibodies to peptides was tested in a Pepscan-based ELISA [20], Pepscan systems, Lelystad, the Netherlands). The 455-well creditcard-format polyethylene cards, containing the covalently linked peptides, were incubated (4°C, overnight) with monoclonal antibody (10 µg/ml) diluted in blocking solution which contains 5% horse-serum (v/v) and 5% ovalbumin (w/v) or human serum (diluted 1:100). After washing, the peptides were incubated for 1hr at 25°C with rabbit-anti-mouse IgG peroxidase conjugate (dilution 1:1000) (Dako, Glostrup, Denmark), and subsequently, after washing, with peroxidase substrate, 2,2'-azino-di-3-ethylbenzthiazoline sulfonate (ABTS) and 2 µl/ml 3% H_2O_2. After 1hr the color development was measured at OD_{450}. The color development of the ELISA was quantified with a CCD-camera and an image processing system.

Results of the mAbs obtained with the three sets of peptides were essentially the same, only in a few cases the intensity of binding of mAbs to the peptides was somewhat different. For the human sera, background correction was performed by subtracting the average extinction of the three negative sera from the positive sera data points. Binding of antibodies was considered to be significant when three successive data points reached values which were 1.5 times higher than the average value of ten flanking data points. Most of the responses found with the human sera were observed in all 3 sets of peptides. When a reaction was observed with only 1 of the 3 peptide series, or with 2 of the 3 series, it was also included in the data set, this however, was observed only occasionally.

Blocking ELISA

The blocking ELISA was performed as described [18]. Briefly, plates were coated with P.69 Prn1 (2 µg/ml) as described above. After washing and blocking, the wells were incubated with 100 µl of serial two-fold dilutions of a pool of human sera with IgG titers of ≥500 IU/ml, diluted in 0.5 % BSA in PBST for 2h at 22 °C. The pool comprised a mix of sera from several recently infected pertussis patients who were fully vaccinated. Subsequently,

plates were washed and incubated with a P.69 Prn-specific murine mAb (2-3µg/ml) for 2h at 22°C. After a final washing step, the amount of mAb was quantified as described above. A mAb was considered to compete with human sera when at least 50% of its binding capacity was blocked by binding of human antibodies.

Table 1. Binding of mAbs to peptides derived from region 2.

Region and peptide	Amino acid sequence[a]	Binding of mAb[b]			
		PeM1	PeM2	PeM19	E4D7
Region 2	WSLVGAKAPPAPKPAPQPGPQPPQPPQPQPEAPAPQPPAGRELSA				
Pep11	WSLVGAKAPPAPKPA				
Pep12	AKAPPAPKPAPQPGP	+	+	-	-
Pep13	APKPAPQPGPQPPQP	+	+	-	-
Pep14	PQPGPQPPQPPQPQP	-	+	-	-
Pep15	QPPQPPQPQPEAPAP	-	-	-	-
Pep16	PQPQPEAPAPQPPAG	-	-	-	-
Pep17	EAPAPQPPAGRELSA	-	-	+	+

[a]The amino acid sequence (aa 530 to 571) for the entire region 2 is given.
[b] +, binding; -, no binding.

Results

Binding of mAbs to peptides derived from region 1 and 2

In view of the fact that polymorphism in P.69 Prn is mainly restricted to the two regions comprised of repeats (region 1 and 2), we initially focused on these regions using a limited set of overlapping peptides. To distinguish these two limited sets of peptides from the more extensive sets used in the Pepscan analysis (see below), we will refer to the two sets of peptides as r1 and r2 peptides, respectively. Previously, we tested 14 mAbs for their ability to bind to synthetic peptides derived from region 1 of P.69 Prn [11]. Of these 14 mAbs, six (PeM3, PeM4, PeM68, PeM71, PeM72 and F6E5) were found to bind r1 repeats, whereas one mAb (PeM70) bound to the sequence ATIRR which forms the N-terminal border of the r1 repeats (Table 2). Due to some discrepancies with the Pepscan analysis (see below), these binding experiments were repeated with higher concentrations of coating peptide and mAbs. Under these conditions, two additional mAbs (PeM7 and PeM85) were identified that bound to r1 peptides. We extended this previous work further by testing the ability of seven additional mAbs, raised against P.69 Prn, to bind to synthetic peptides derived from region 1. Binding was determined also at the high concentrations of coating peptide and mAbs. Of these seven mAbs (PeM6, PeM19, PeM21, PeM29, PeM38 and PeM64), only PeM29 showed binding to the r1 peptides (Table 2).

To further elucidate the epitopes recognized by all the 21 mAbs, a set of seven overlapping peptides corresponding to region 2 was synthesized (Pep 11-17 in Table 1). These r2 peptides were comprised of 15 amino acids with an overlap of 10 residues. Of the 21 mAbs tested, two (PeM1 and PeM2) bound to peptides harboring the PQP repeat, whereas two mAbs (PeM19 and E4D7) bound to the sequence RELSA which forms the C-terminal border of the PQP repeat region (Table 1). Binding of PeM1 to the r2 peptides was found only when high peptide concentrations were used in the assay.

Binding of mAbs to peptides derived from P.69 Prn: Pepscan analysis

A more extensive mapping of mAb epitopes was performed with Pepscan analyses in which the complete primary structure of P.69 Prn was represented. Three sets of peptides were synthesized spanning the entire amino acid sequence of P.69 Prn, the 15-mer linear, the 15-mer loop and the 30-mer linear set (each 670 peptides in total). In case of the 15-mer loop peptides both ends were connected to the plastic as this may mimic loops in the protein and hence, possibly result in an improved representation of the natural epitope [21, 22].

Of the 16 mAbs tested in the Pepscan analyses, 11 showed binding to the peptides (Table 2). Five mAbs (PeM4, PeM7, PeM19, PeM71 and PeM72) recognized a single peptide as illustrated for PeM72 in Figure 1A. In contrast, PeM80, PeM84 and PeM85 recognized multiple peptides (between 5-10) distributed along the whole length of P.69 Prn. However, one peptide was preferentially bound and is indicated in Table 2. Five mAbs (PeM5, PeM6, PeM21, PeM38 and PeM64) failed to show any reactivity with peptides, suggesting they recognize discontinuous or conformational epitopes. Interestingly, three mAbs (PeM1,

PeM2 and PeM29) bound to two or three non-overlapping peptides in the Pepscan as shown for PeM29 in Figure 1B. The binding specificity of these mAbs confirmed the reaction with the r1 and r2 peptides but was extended with additional peptides which were found by Pepscan analysis. There was no obvious similarity in the primary structure of the peptides recognized by either one of the three mAbs.

Some minor discrepancies were observed between the Pepscan analysis and the analyses with the r1 and r2 peptides (Table 2). A number of mAbs (PeM1, PeM2, PeM7, PeM29, PeM84 and PeM85) showed binding to the r1 or r2 repeat in the Pepscan analysis, whereas no, or very weak, binding was observed with the r1 or r2 peptides. This suggested a higher sensitivity of the Pepscan analysis an assumption that was confirmed by the fact that four mAbs (PeM1, PeM7, PeM29 and PeM85) bound to the r1 or r2 peptides when higher concentrations of mAbs and coating peptides were used.

Of the 17 mAbs, raised against native P.69 Prn, 11 were found to bind to r1 or r2 peptides, suggesting these regions are immunodominant in mice (Table 2). The remaining four mAbs were raised against a fusion protein containing region 1 and, as expected, only recognized region 1.

Table 2. Characterization of P.69 Prn mAbs.

MAb	Antigen[a]	Blocking ELISA result[b]	Region 1 peptide	Region 2 peptide	Pepscan analysis peptide(s)
PeM 1	P.69 Prn1	+	-[f]	APKPAPQPGP[d]	TWDDD+GGFGPVLDGW+ PAPQP
PeM 2	P.69 Prn1	-[c]	-[c]	PQPGP	PQP + GGFGPGGFGP
PeM 3	P.69 Prn1	-[c]	GGFGPGGFGP[c]	-	Nd[e]
PeM 4	P.69 Prn1	-[c]	GGFGPGGFGP[c]	-	GGFGPGGFGP
PeM 5	P.69 Prn1	+[c]	-[c]	-	-
PeM 6	P.69 Prn1	+	-	-	-
PeM 7	P.69 Prn1	+[c]	GGFGPGGFGP[c,d]		GGFGPGGFGP
PeM 19	P.69 Prn2	-	-	RELSA	RELSA
PeM 21	P.69 Prn2	+	-	-	-
PeM 29	P.69 Prn2	+	GGFGPGGFGP[d]	-	GERQH+GDTWDDD+GGFGP
PeM 38	P.69 Prn2	+	-	-	-
PeM 64	P.69 Prn2	+	-	-	-
PeM 68	FP	-	GDAPAGGAVP[b]	-	Nd
PeM 70	FP	-	ATIRR[c]	-	Nd
PeM 71	FP	-	GGFGP[c]	-	GGFGPGGFGP
PeM 72	FP	-	GGFGP[c]	-	GGFGPGGFGP
PeM 80	P.69 Prn5	+	-	-	RFAPQ
PeM 84	P.69 Prn5	+	-[c]	-	GGFGPGGFGP
PeM 85	P.69 Prn5	+	GGFGPGGFGP[c,d]	-	GGFGPGGFGP
E4D7	P.69 Prn1	-	-	RELSA	Nd
F6E5	P.69 Prn1	-[c]	GGFGPVLDGW[c]	-	Nd

[a]FP: fusion protein containing region 1. [b]A pool of sera from pertussis patients was able to block at least 50% of binding of the mAb. +, blocking; -, no blocking. [c]Determined previously by King *et al.* [d]Binding observed with high concentration coating peptide and mAb. [e]ND, not done. [f]-, experiment performed but no binding observed.

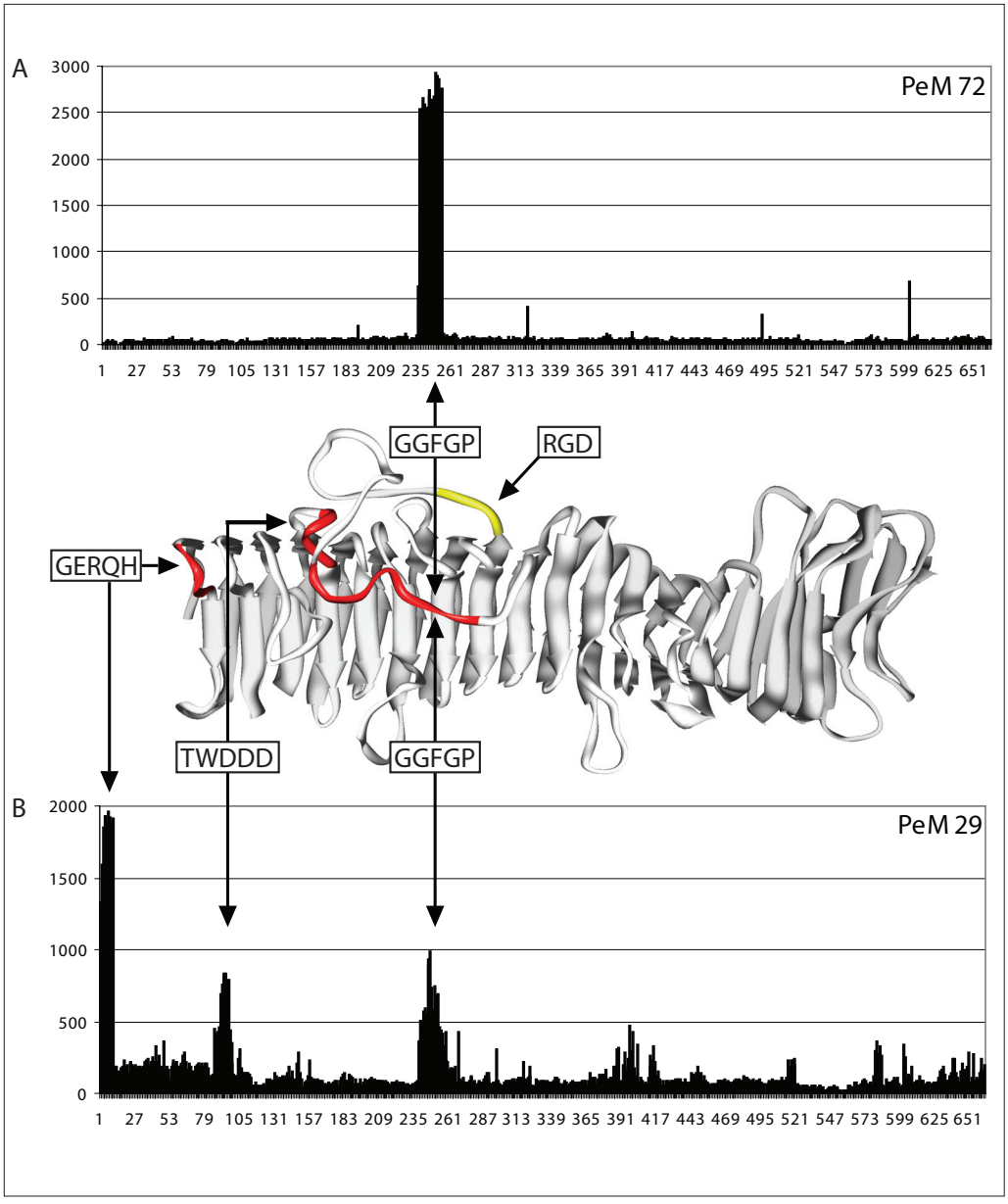


Figure 1. P.69 Prn Pepscan of (A) a mAb (PeM72) that binds to a single linear epitope and (B) a mAb (PeM29) that binds multiple linear epitopes. The peptides recognized by PeM72 and PeM29 are indicated in the P.69 Prn crystal structure. The X-axis shows the residue numbers starting at the N-terminus of the processed protein. The OD450 values are given on the y axis.

Pepscan analysis with human sera

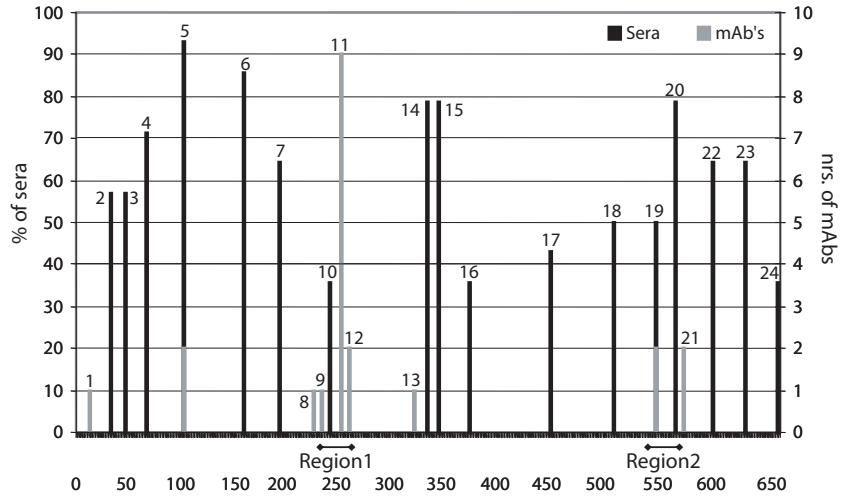
Human sera were also subjected to Pepscan analyses. The sera were obtained from four year old children who received a booster vaccination with a 3-component vaccine comprised of Ptx, FHA and P.69 Prn (n = 5). In addition, sera from children with high titers against *B. pertussis*, which is indicative of a recent infection, were analyzed (n=9). Epitopes unique for antibodies from vaccinated or infected individuals were not observed and the results were combined (Fig. 2). Only epitopes that were recognized by at least five out of 14 sera (36%) are shown in Fig. 2. Epitopes that showed a lower percentage of binding with the human sera were rare. A total of 17 different peptides were recognized by the sera. Three regions with immunodominant linear epitopes were identified in the P.69 Prn. The first region was located in the N-terminal part of P.69 Prn and harbors the peptides TWDDD (aa. 99 to 103) and SLQPED (aa. 156 to 161). The two epitopes are recognized by 93% and 86% of the sera, respectively. The second region was located in the center of P.69 Prn and harbors the peptides SITLQAGAH (aa. 327 to 335) and KALLYR (aa. 339 to 344) both recognized by 78% of the sera. The third region was found in the C-terminal domain of P.69 Prn with the peptide PAPQPP (aa. 562 to 567) as immunodominant epitope which was recognized by 78% of the sera and is located within the r2 repeat region.

Overlap between the 17 epitopes recognized by human antibodies and the nine epitopes of the mouse mAbs was observed in two cases: the epitopes TWDDD and PQP (epitope nrs. 5 and 19 in Fig. 2). In two other cases the epitopes recognized by human antibodies were located in close proximity to epitopes recognized by mouse mAbs. In the r1 region, epitope nr. 10 is flanked by two epitopes recognized by mouse mAbs. The sequence RELSA, recognized by mouse mAbs, forms the C-terminal border of the r2 repeat region and is just a few amino acids removed from epitope nr. 20 which is recognized by human antibodies.

Competition between mAbs and human sera for P.69 Prn

A blocking ELISA was performed with all 21 mAbs to test their ability to compete with a pool of human sera. Six mAbs (PeM2, PeM3, PeM4, PeM5, PeM7 and F5E6) were tested previously [11]. Of the 21 mAbs tested, the binding of ten was not inhibited by human antibodies. Interestingly, seven of these mAbs (PeM3, PeM4, PeM68, PeM70, PeM71, PeM72 and F5E6), were directed against linear epitopes localized in region 1. This indicated that human antibodies bound with low avidity to this region relative to the mAb. One of the ten mAbs whose binding was not affected was PeM2 which recognized the PQP peptide in the r2 repeat. Even when the peptide was used as coat in the assay in stead of purified P.69 Prn and many different human sera were tested (>50) no competition of binding was observed. Since 50% of the human sera contained antibodies against this part of the r2 repeat, lack of inhibition was probably due to low avidity of the human antibodies relative to the mAb. The observation that the remaining two out of ten mAbs (PeM19 and E4D7) that were not blocked by human sera, were directed against the RELSA epitope, that is just a few amino acids apart from the PAPQPP epitope in the r2 repeat which is recognized by

A



B

Nr.	AA's	Epitope	Nr.	AA's	Epitope	Nr.	AA's	Epitope
1	11-15	GERQH	9	227-236	GDALAGGAVP	17	445-450	LAGSGL
2	31-36	SGTIKK	10	237-246	GGAVP	18	503-509	TFTLANK
3	44-49	GILLEN	11	247-251	GGFGP	19	545-547	PQP
4	71-77	GIRRFLL	12	252-261	GGFGPVLDGW	20	562-567	PAPQPP
5	99-103	TWDDD	13	317-321	RFAPQ	21	570-574	RELSA
6	156-161	SLQPED	14	327-335	SITLQAGAH	22	596-603	SNALSKRL
7	190-194	GASEL	15	339-344	KALLYR	23	627-631	DNRAG
8	222-226	ATIRR	16	367-374	TELPSIPG	24	657-664	HLGGLAGY

C

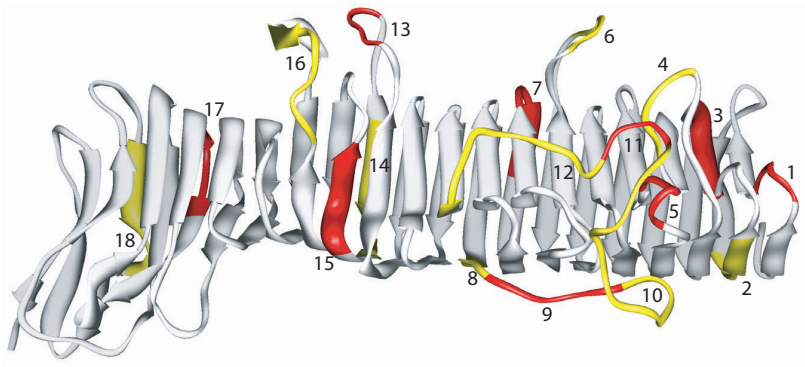


Figure 2. P69 Prn epitopes recognized by mAbs and human sera. (A) The number of mAbs binding to a particular peptide, and the percentage of human sera that contains antibodies binding to a particular peptide, are indicated on the right and left Y-axis, respectively. The numbers on the X-axis refer to the P.69 Prn amino acid residues. Numbering starts with the N-terminus of the processed protein. Epitopes recognized by < 36% of the human sera are not shown. Epitopes are numbered to facilitate identification. (B) A minimal sequence with the corresponding amino acid residues of the epitopes is shown in the table. (C) Visualization of epitopes recognized by human and mouse antibodies. Epitopes are numbered to facilitate identification. Numbering starts with the N-terminus of the processed protein. Epitopes recognized by < 36% of the human sera are not shown. The crystal structure does not include the last 138 C-terminal amino acids of P.69 Prn, which harbors region 2.

78% of the human sera, can be explained in a similar manner.
For 11 mAbs, the binding to P.69 Prn could be blocked by human antibodies. These mAbs included the five (PeM5, PeM6, PeM21, PeM38 and PeM64) that did not bind to peptides and therefore probably recognized conformational or discontinuous epitopes. In fact, the Pepscan indicated that two of this group of 11 mAbs recognized discontinuous, linear, sequences (PeM1 and PeM29). It is conceivable that human antibodies have a stronger affinity for discontinuous epitopes compared to the mouse mAbs. The peptide TWDDD which was recognized by 93% of the sera, forms a part of the epitope recognized by PeM1 and PeM29. The remaining four mAbs (PeM7, PeM80, PeM84 and PeM85) which were blocked by human sera, bound to linear epitopes that were not recognized by human antibodies according to Pepscan analyses. This suggested that human antibodies might recognize these epitopes as part of a larger discontinuous epitope not revealed by Pepscan analyses. The observation that three mAbs directed against the r1 repeat (PeM7, PeM84 and PeM85) were inhibited in binding by human sera indicated the presence of human antibodies directed against this region of P.69 Prn. This was also suggested by the Pepscan analyses.

Discussion

Epitopes on P.69 Prn were mapped with human sera and mAbs using synthetic peptides derived from the repeat regions one and two and with a more extensive set of peptides covering the complete P.69 Prn polypeptide. The interpretation of the results was facilitated by the availability of the crystal structure of a part of P.69 Prn [23, 24].

Human sera

Pepscan analyses did not reveal any differences between epitopes recognized by sera from humans infected with *B.pertussis* or vaccinated with a vaccine containing P.69 Prn. However, Pepscan mainly detects linear epitopes, and it is conceivable that infection and vaccination elicit antibodies that differ with respect to conformational epitopes recognized. The Pepscan analyses revealed 17 linear epitopes recognized by at least 36% of the human sera. The N-terminal region harbored a large cluster of epitopes (nrs. 2-7 in Fig. 2). Located within this cluster was the most immunodominant linear epitope of P.69 Prn (TWDDD) which was recognized by 93% of the sera. The central region of P.69 Prn harbors two main epitopes (nrs. 14 and 15 in Fig. 2), both recognized by 78% of the sera. In the C-terminal region a large number of epitopes was observed with the r2 repeat region being immunodominant (epitopes 19 and 20 in Fig. 2). Most of the identified epitopes (13 out of 18) that could be visualized in the crystal structure of P.69 Prn were located in loops (Fig. 2C).

Comparisons Pepscan of mAbs and human sera

There was little overlap between epitopes recognized by mAbs and human antibodies (Fig. 2). Of the 24 epitopes identified only two were recognized by both human and mouse antibodies. One of these epitopes was TWDDD, emphasizing the immunodominance of this linear epitope. Only 35% of the human sera contained antibodies directed against linear r1 epitopes. In contrast, a high percentage of the human sera (86%) recognized linear r2 repeat epitopes. The percentage of mouse mAbs reacting with these two epitopes showed an opposite trend. Of the 17 mAbs raised against P.69 Prn, 59% was directed against the r1 repeat and only 12% against the r2 repeat or 24% if the RELSA epitope was included in the r2 repeat. Combining the results of Pepscan and blocking ELISA, three different kinds of epitopes recognized by human and mouse antibodies could be identified: 1) strictly linear epitopes localized within region 1 and 2 that were not recognized or only with low avidity by human antibodies compared to mouse mAbs as binding of mAbs to these epitopes was not blocked by human sera, 2) conformational epitopes which could not be localized with linear peptides but were recognized by human antibodies with high avidity compared to mouse mAbs, as the mAbs were blocked by human sera and 3) discontinuous and possible discontinuous epitopes which could be (partly) localized with peptides and were also recognized by human antibodies with high avidity compared to mouse mAbs, as the binding of mAbs was blocked by human sera. Thus, blocking experiments indicated the presence of high avidity human antibodies against conformational epitopes. Interestingly,

human antibodies against linear epitopes were of much lower avidity as they were unable to block mAbs. It should be realized that blocking of binding of mAbs by human sera may be due to competition for identical or overlapping epitopes, thus identifying epitopes recognized by human sera. However, blocking may also result from steric hindrance due to proximity of distinct epitopes recognized by human antibodies and the mAbs tested. At present we cannot distinguish between these possibilities and therefore the identification of discontinuous and possible discontinuous epitopes defined by blocking experiments should be viewed as tentative.

Region 1 and 2 possibly form a single epitope

Interestingly, Pepscan analyses revealed a number of discontinuous epitopes of which region 1 is a part. We found three mAbs (PeM1, PeM2 and PeM29) that bound to two or more non-overlapping peptides. There was no obvious similarity in the primary structure of the peptides recognized by a single mAb, suggesting that peptides are part of a discontinuous epitope. Apparently, the mAbs retain some affinity for the different peptides of which the discontinuous epitope is comprised [25]. This assumption was corroborated for the epitopes of PeM29 and PeM1 by the crystal structure of P.69 Prn. Although the 3 peptides recognized by PeM29 are separated by 85 and 235 amino acids in the primary structure, respectively, they are proximate in the crystal structure (Table 2 and Fig.1B). Similarly, two of the three peptides recognized by PeM1 (TWDDD and GGFGPVLDGW), although separated by 153 amino acids in the primary structure, are in close proximity in the crystal structure (Fig. 2C). Significantly, PeM1 and PeM2 bound peptides derived from both r1 and r2 repeats. The r2 repeats are not part of the published crystal structure of P.69 Prn [23], however, our results suggest that they are in close proximity to the r1 repeats in P.69 Prn.

Immune evasion and repeats

Tandemly repeated sequences, such as the r1 and r2 repeats, are found in the surface antigens of many pathogens and have been implicated in immune escape. This occurs by insertion or deletion of the repeat unit [26]. This type of antigenic variation is unlikely to affect linear epitopes, as deletion or insertion of repeats does not create novel sequences. Thus to be effective, repetitive sequences involved in immune escape should not induce protective levels of antibodies directed against the repeat unit, but should induce antibodies directed against conformational epitopes. There is evidence that this indeed occurs. The protective alpha C protein of group B streptococci harbors a variable number of tandem repeats. It has been shown that deletions in this region are selected for under specific antibody pressure, and appear to lower the organism's susceptibility to killing by antibody specific to the alpha C protein. Importantly, it was shown that antibodies to a repeat harbored by the protective alpha C protein of group B streptococci are recognized predominantly as a conformational epitope [26-28]. This is not to say that antibodies to the repeat are not induced, 36% and 78% of the sera we tested harbored antibodies against region 1 and 2, respectively. However

such antibodies may be less effective than "conformational" antibodies e.g. due to low avidity, concentration, or specific isotype. One reason why the repeats per se may be less protective than when presented as a conformational epitope is that antigens with repeating epitopes may activate B cells without the assistance of T-helper cells. T-cell-independent antibody responses tend to be weaker and are not boosted with repeated immunization. We propose that protective P.69 Prn antibodies induced during infection or vaccination recognize a conformational epitope comprised of r1 and r2 repeats. Antibodies directed against the repeat unit are proposed to be much less effective. This hypothesis is confirmed by the observation that variation in region 1 affected vaccine efficacy in a mouse model [11, 29]. Boursaux-Eude *et al.* (1999) [30] used the mouse model to compare the efficacy of acellular vaccines against isolates producing different Pertactin and pertussis toxin variants. They concluded that a tri-component vaccine was highly effective in promoting lung clearance of all isolates expressing different pertussis toxin and Pertactin variants. The data obtained with different isolates were not compared on a statistical basis, however. Thus relative efficacy against different strains was not determined. The role of Pertactin variation in pertussis epidemiology is still under debate [2]. The extent to which polymorphism affects vaccine efficacy is probably dependent on the potency of the vaccine used. In the Netherlands, in which a relative weak vaccine has been used, pertussis epidemics have been associated with the emergence non-vaccine type strains [2, 31, 32]. Further, the fitness advantage of strains carrying nonvaccine types of Pertactin may be limited to older age categories in which vaccine-induced immunity has waned.

The assumption that region 1 is recognized mainly as a conformational epitope by the human immune system is suggested by the results of He *et al.* [16]. These authors analyzed the type-specificity of the P.69 Prn immune response in humans infected with P.69 Prn2 or P.69 Prn3 strains, or vaccinated with P.69 Prn1. P.69 Prn1 and P.69 Prn3 differ in two amino acid substitutions in region 1, while P.69 Prn2 differs from both, by insertion of a repeat unit comprising five amino acids. Thus of the three P.69 Prn variants, P.69 Prn2 is most structurally distinct while P.69 Prn1 and P.69 Prn3 are structurally more similar. Consistent with this, individuals infected by P.69 Prn2 strains had significantly lower antibodies to P.69 Prn1 than those infected by P.69 Prn3 strains and those who were vaccinated with P.69 Prn1. Moreover, in contrast to vaccinees and subjects infected by P.69 Prn3 strains, individuals infected by P.69 Prn2 strains had hardly any antibodies specific to the variable region of P.69 Prn1.

In summary, our results suggest that the two most variable regions of P.69 Prn may form a single, conformational, epitope. As the RGD motif, involved in adherence to host cells [15], is separated from the first r1 repeat by three amino acids it seems likely that both repeat regions serve to deflect the immune response targeted at the functional domain of P.69 Prn.

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3 The *Bordetella pertussis* virulence factor P.69 Pertactin retains its immunological properties after overproduction in *Escherichia coli*

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Abstract

Bordetella pertussis is re-emerging in several countries with a high vaccine uptake. Analysis of clinical isolates revealed antigenic divergence between vaccine strains and circulating strains with respect to P.69 Pertactin. Polymorphisms in P.69 Pertactin are mainly limited to regions comprised of amino acid repeats, designated region 1 and region 2. Region 1 flanks the RGD motif involved in adherence. Although antibodies against P.69 Pertactin are implicated in protective immunity, little is known about the structure and location of its epitopes. Previously we described the localization of mainly linear epitopes of both human sera and mouse monoclonal antibodies (mAbs). To study the location of conformational epitopes and to investigate the effect of variation in P.69 Pertactin on vaccine efficacy, we cloned, expressed and purified 3 naturally occurring P.69 Pertactin variants, 3 mutants in which the variable regions are missing, 3 N-terminal and 1 C-terminal deletion mutant. Here we describe the procedure to clone, express, and purify up to 0.1 mg of P.69 Pertactin and its derivatives per 1 ml of *Escherichia coli* culture.

Introduction

In several countries pertussis has re-emerged, despite high vaccination coverage[1, 2]. Several explanations have been suggested for the re-emergence of pertussis: waning immunity in adolescents and adults, increased reporting, improved diagnosis of the disease and the emergence of *Bordetella pertussis* escape variants[3]. In the Netherlands, the emergence of escape variants has played an important role in the resurgence of pertussis. Analysis of clinical isolates collected in the last 50 years revealed antigenic divergence between circulating strains and vaccine strains[3, 4]. Escape variants showed polymorphisms in at least two proteins, pertussis toxin and P.69 Pertactin. Polymorphism in P.69 Pertactin is temporally linked to the re-emergence of pertussis. In the pre-vaccination era, Prn1 was the predominant type. After introduction of vaccination the Prn1 strains were gradually replaced by non-vaccine type strains carrying Prn2 and Prn3. Polymorphisms in P.69 Pertactin are essentially limited to 2 regions, designated region 1 and region 2, which are comprised of repeats[5]. Region 1 is located adjacent to an Arg-Gly-Asp motif implicated in adherence[6, 7]. Several studies have shown that P.69 Pertactin is important for immunity against disease. Antibody levels to P.69 Pertactin were shown to correlate with clinical protection[8, 9]. In mice, passive and active vaccination showed that P.69 Pertactin confers protective immunity[8-11]. Variations in Prn also affected the efficacy of the Dutch whole cell vaccine in a mouse model[10]. Furthermore results presented by He *et al.*, suggest that the region 1 repeat can induce type specific antibodies[12].

Pertactin, belongs to the family of autotransporter proteins[13-15] and is processed from a 93-kDa large precursor to 69- and 22-kDa proteins[16]. The unprocessed polypeptide is directed via a signal peptide to the secretory machinery in the inner membrane where the signal peptide is cleaved. Subsequently, the polypeptide is directed towards the outer membrane where the 22-kDa protein is assumed to form a pore through which the 69-kDa protein is transported. After secretion via the autotransporter domain, proteolytic activities shape the 69-kDa protein to it's final 60.37 or 58.34 kDa form[17]. These final forms (referred to as P. 69 Prn) are used in most acellular vaccines (ACV's) [2].

Most studies with Prn have been performed with the P.69 Prn1 variant, which is not representative for the current strains. Our long term aim is to compare the structure, functional and immunological properties of 3 Prn variants. Specifically, we want to determine whether P.69 Prn2 and P.69 Prn3 variants are more effective vaccines against the current strains.

Here we describe a method to express and purify large amounts of Prn variants and Prn mutants in *Escherichia coli*. The recombinant proteins are compared to P.69 Prn produced by *B. pertussis*.

Materials and methods

Construction of expression plasmids

The DNA fragments encoding the extra cellular domain (Fig. 1A) of Prn1, Prn2 and Prn3 were amplified from *B. pertussis* genomic DNA from strains B391 (harboring *prn1*, GenBank accession no. **AJ011091**), B345 (harboring *prn2*, GenBank accession no. **AJ011092**) and B343 (harboring *prn3*, GenBank accession no. **AJ011093**) by polymerase chain reaction (PCR) using primers 1 and 2 (Table 1). Genes were amplified using 1 ng of chromosomal

Table 1. Overview of PCR primers used.

#	Primer name	Sequence (5'- 3')
1	pET-A0-new-L	ACACATATGGACTGGAACAACCAGTCCATCGTCAAGACCGGTG
2	pET-D-new	ATTGGATCCTTAGCCGCCGCCGTCGCCGGTGAA
3	R2-Del-RV1	CGCGGGCTTGGGCGC
4	R2-Del-FW1	CCGGCGGGCAGGGAGTTGTC
5	N-Term-Del1	ACACATATGCAGGGCTCCGACCCGGGCGGCGTACGGAC
6	N-Term-Del2	ACACATATGTCGGGACAGTTGTCCGACGATGGCATCC
7	N-Term-Del3	ACACATATGGGCGAACAGGCCCAGGCCAGCATCGCCGACA
8	R1-Del-FW	GTCCTCGACGGCTGGTATGGCGTGGA
9	R1-Del-RV	GGCAGGCGCGTCCCCGCGCCGT
10	C-term-Del	ATTGGATCCTTACGCGGGCTTGGGCGC

DNA in a 50 µl PCR containing 2.5 U Pfu Ultra HF (Invitrogen, Carlsbad, CA), 0.2 µM primers and 10% dimethyl sulfoxide (DMSO) with the following parameters: 5 min initial denaturation at 95°C; 1 min denaturation at 94°C, 1 min annealing at 63°C, 2 min extension at 72°C (30 cycles) and 7 min final extension at 72°C. To generate mutant proteins lacking the variable regions we used a PCR mutagenesis approach (Fig. 1B). Briefly, two PCR's were performed using primers directed outwards from the variable regions. For the deletion of variable region 1, a PCR was performed with a combination of primers 1 and 9 and primers 8 and 2 (Table 1). PCR products were blunt end ligated with 1U of T4 DNA ligase (Roche, Penzberg, Germany) for 1 hr at room temperature. Subsequently 1 µl of a 100 fold dilution of the ligation mixture was used in the secondary PCR using primers

Table 2. Combination of primers used for construction of recombinant P.69 Prn.

Primer combination	FW primer no.	Forward Primer	RV primer no.	Reversed Primer	Used for cloning of
1	1	pET-A0-new-L	2	pET-D-new	Entire P69 gene
2	1	pET-A0-new-L	3	R2-Del-RV1	Part 1 for R2-K.O.
3	4	R2-Del-FW1	2	pET-D-new	Part 2 for R2-K.O.
4	5	N-Term-Del1	2	pET-D-new	N-terminal deletion mutant
5	6	N-Term-Del2	2	pET-D-new	N-terminal deletion mutant
6	7	N-Term-Del3	2	pET-D-new	N-terminal deletion mutant
7	1	pET-A0-new-L	9	R1-Del-RV	Part 1 for R1-K.O.
8	8	R1-Del-FW	2	pET-D-new	Part 2 for R1-K.O.
9	1	pET-A0-new-L	10	C-term-Del	C-terminus deletion

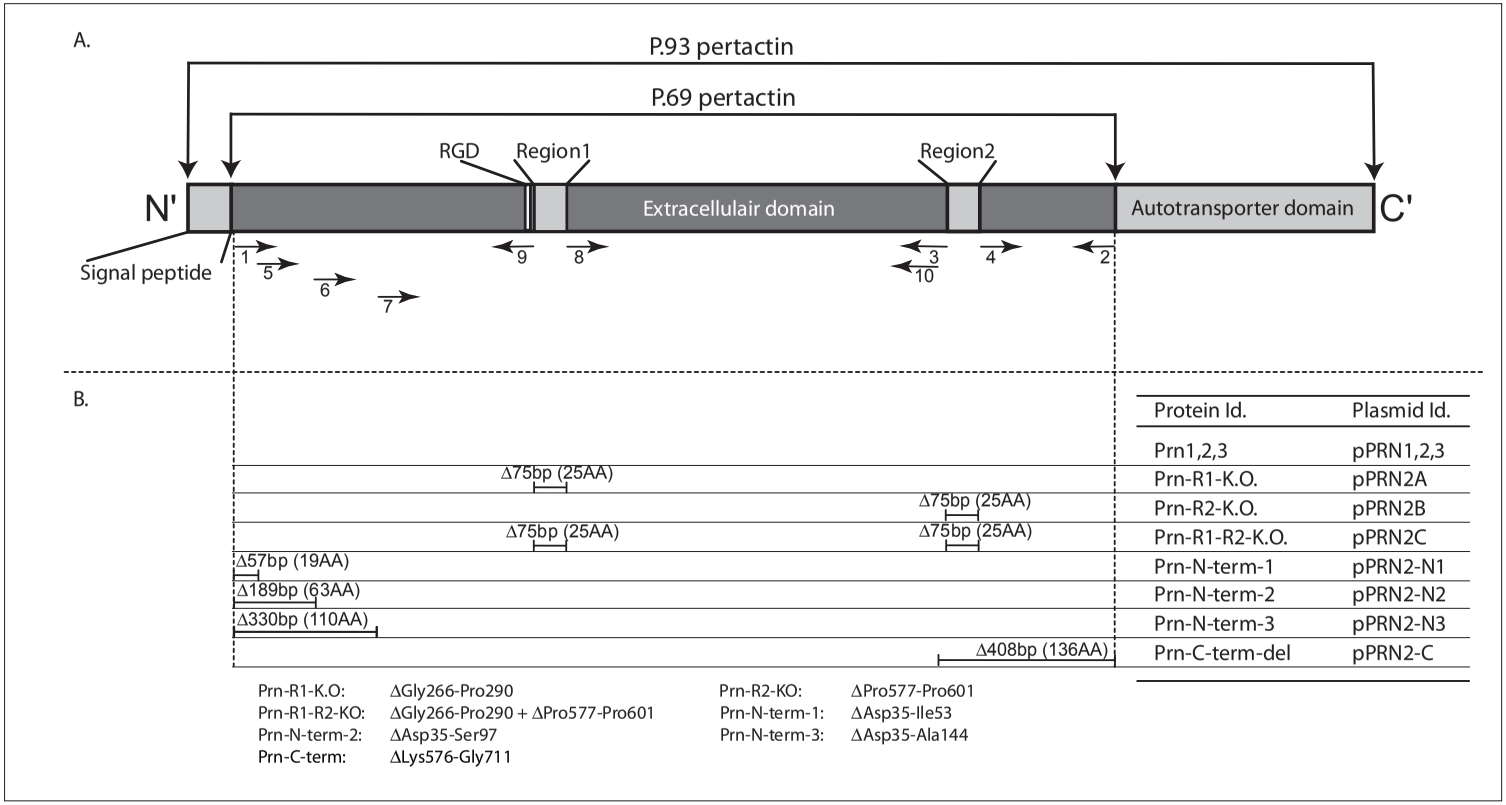

Figure 1. Structure of Prn and constructed deletion derivatives. (A) Numbers indicate the location of primers used for construction of deletions and cloning. (B) Deleted regions, amino acids and the corresponding plasmids have been indicated. AA sequence with GenBank protein accession no. CAA09473 was used as reference.

1 and 2. To generate a mutant lacking both variable regions, 1 ng DNA from the region1 mutant was used as template (Fig. 1B). Two PCR's were performed using a combination of primers 1 and 3 and primers 4 and 2 (Table 1). After blunt end ligation, the secondary PCR using primers 1 and 2 was performed as described above. The secondary PCR products were gel-purified using the QIAquick (Qiagen, Valencia, CA) protocol. Furthermore we also constructed mutants lacking either the C- or N-terminus essentially as described above using primers combinations 4, 5, 6 and 9 (Fig. 1B , Table 2).

PCR fragments containing the mutant genes were digested with endonucleases *Nde*I and *Bam*HI (restriction sites were included in the PCR primers to facilitate cloning) and subsequently ligated into expression vector pET19b (digested with the same enzymes) placing the coding sequences under the control of the inducible T7 promoter (Fig. 1B, Table 3). Plasmids were introduced into TOP10 *E. coli*'s by transformation. Positive clones were subjected to sequence analysis for selection of clones without point mutations.

Table 3. Overview of constructed plasmids (all plasmids are derivatives of pET19b).

Plasmid	Prn type	Characteristics of *prn* insert.
pPRN1	1	-
pPRN2	2	-
pPRN3	3	-
pPRN2A	-	region 1 deleted
pPRN2B	-	region 2 deleted
pPRN2C		region 1 and 2 deleted
pPRN2-N1	-	1st 10 N-terminal AA deleted
pPRN2-N2	-	1st 20 N-terminal AA deleted
pPRN2-N3	-	1st 30 N-terminal AA deleted
pPRN2-C	1	136 C-terminal AA deleted

Expression of recombinant Pertactin

Plasmids were introduced into *E. coli* BL21-Codonplus (DE3)-RP (Stratagene, La Jolla, CA). This strain harbors the lambda DE3 lysogen that carries the T7 RNA polymerase under control of the *lacUV5* promoter. Furthermore these cells contain extra copies of the *argU* and *proL* genes, encoding tRNA's that recognize the arginine codons AGA and AGG and the proline codon CCC, respectively, which are located on a pACYC-based plasmid carrying the chloramphenicol acetyl transferase resistance gene. This strain partially compensates possible codon bias when expressing high GC-content genes in *E. coli*. A single colony of a transformant was used to inoculate 5 ml of LB media containing 100 µg/ml ampicillin and 50 µg/ml chloramphenicol. Bacteria were grown at 37ºC overnight at 250 rpm. From the overnight cultures, 100 µl was added to 1 L fresh LB media containing 100 µg/ml ampicillin and 50 µg/ml chloramphenicol. Cultures were incubated at 37ºC at 250 rpm until the OD_{600} reached 0.6-0.8. Subsequently, cultures were induced with 1 mM isopropyl-ß-D-thiogalactopyranoside (IPTG), and incubated further for 4 hours. Bacteria were harvested by centrifugation at 5000 g for 10 min at 4ºC.

Purification and refolding of inclusion bodies

Inclusion bodies were isolated using the Bug Buster protein extraction reagent (Novagen, Darmstadt, Germany) according to the protocol provided by the manufacturer. Briefly,

induced cells were harvested and subsequently lysed using Bug Buster reagent. The cell lysate was treated with 5,000 U of lysozyme and 125 U of Benzonase nuclease per gram of wet cell paste. Inclusion bodies were collected by centrifugation and were washed three times with 1:10 diluted Bug Buster reagent. The purified inclusion bodies were solubilized in 6 M Guanidine Hydrochloride (GuHCl), 10 mM benzamidine, 1 mM EDTA, 100 mM NaCl and 50 mM Tris/HCl pH 8.8. Refolding of the recombinant Pertactin and variants was initiated by rapid 50-fold dilution into the same buffer without GuHCl. Proteins were allowed to fully refold during overnight dialysis at 4°C against 1 mM EDTA, 100 mM NaCl, 50 mM Tris/HCl pH 8.8. Subsequently, the refolded proteins were dialyzed twice against 50 mM Tris/HCl pH 8.8 using Spectra/Por 7 dialysis membranes with a molecular weight cut off (MWCO) of 50 kDa (Spectrum Laboratories, Rancho Dominguez, CA). The proteins were concentrated on an Amicon Ultra-15 concentrator with a 50 kDa cutoff (Millipore, Billerica, MA). Finally, 2 µg of protease inhibitor (Roche, Penzberg, Germany) was added to 1 mg/ml of the concentrated proteins.

Affinity chromatography of recombinant Pertactin.

The recombinant Pertactin contained an N-terminal His_6-Tag, allowing its purification by affinity chromatography. Both unfolded (in the presence of 6 M GuHCl) and refolded Pertactin was affinity purified under denaturing or native conditions, respectively. After filtration through a 0.22 µm filter, the protein solution was loaded onto a pre-packed His-Trap HP column (Amersham Biosciences, Buckinghamshire, UK) equilibrated with 20 mM $NaPO_4$, 0.5 M NaCl and 40 mM immidazole, pH 7.4. When purifying under denaturing conditions, 6M of GuHCl was included in the buffers. Bound proteins were stepwise eluted with 10 ml of equilibration buffer containing 100 mM of immidazole, 10 ml with 200 mM immidazole and 10 ml with 500 mM immidazole. Eluted fractions were analyzed for the presence of contaminating proteins using SDS-Page.

SDS-PAGE

Proteins and bacterial cells were separated on SDS-Page as described[18]. Samples were mixed with loading buffer and boiled for 5 minutes before loading onto 4-20% Tris-Glycine polyacrylamide gels (Pierce, Rockford, IL). Bands were visualized by Coomassie Brilliant Blue staining.

Western blotting

After SDS-PAGE, proteins were transferred to PVDF membranes (Amersham Biosciences, Buckinghamshire, UK) Membranes were blocked with 0.5% (w/v) Protifar non-fat dry milk, 0.1% BSA (w/v) and 0.1% (v/v) Tween 20 in PBS, and subsequently incubated for 1 h with monoclonal antibodies (mAbs). Membranes were washed three times for 10 min in PBS containing 1% Tween20 and incubated for 1 h with rabbit anti-mouse total IgG conjugated to horseradish peroxidase (HRP) in PBS containing 1% Tween20.

After two 10 min washes in PBS supplemented with 1% Tween20, followed by a 10 min wash in PBS, membranes were incubated with the ECF substrate (Amersham Biosciences, Buckinghamshire, UK) and signals were detected using the FluorImager 595 (Amersham Biosciences, Buckinghamshire, UK).

Circular dichroism spectroscopy

To verify proper refolding of the purified inclusion bodies, refolded recombinant P. 69 Prn1, Prn2 and Prn3 were compared to native P.69 Prn1 by circular dichroism spectroscopy. Circular dichroism (CD) measurements were carried out on an Olis DSM 1000 Spectrophotometer (Olis, Bogart, GA) operating at 25 °C using a 0.02-cm cell. Spectra were signal averaged by adding at least six accumulations. The concentration of protein used was 1 mg/ml in 10 mM $NaPO_4$ pH 8.8.

ELISA

To further verify the proper refolding of the purified inclusion bodies, the binding of several mAbs to the unfolded and refolded proteins was tested in an enzyme-linked immunosorbent assay as described previously[19]. The binding properties of the mAbs to the recombinant proteins were compared with their binding properties to native P.69 Prn1 (kindly provided by Chiron-Biocin, Siena, Italy) and unfolded inclusionbodies. Polystyrene Immulon II 96-well ELISA plates (Dynatech, Chantilly, VA.) were coated overnight at 22°C by adding 100 µl of 0.04 M carbonate buffer pH 9.6 containing 2 µg of protein/ml to each well. The plates were washed four times with 200 µl of PBS supplemented with 0.05% Tween 20 (PBST) per well by using a Titertek Plus M96V washer (ICN, Irvine, CA). Murine mAbs diluted in 0.5% BSA in PBST to a concentration of 2 µg/ml were added to the wells and were incubated for 2 h at 37 °C followed by four washings as described above. Bound antibodies were detected by using horseradish peroxidase-conjugated anti-mouse total IgG (Organon Technica, Boxtel, the Netherlands). The optical density at 450 nm (OD450) was measured with an EL312e plate reader (BioTek Systems, Winooski, VT).

Table 4. Overview of stains used for the comparison of expression levels.

Strain #	Strain	Key features
1	BL21(DE3)pLysS[1]	Standard expression strain
2	Rosetta(DE3)pLysS[1]	Expression of rare tRNA's: AUA, AGG, AGA, CUA, CCC and GGA
3	Rosetta-gami(DE3)pLysS[1]	Features. strain 1 + *trxB/gor* mutant which facilitates cytoplasmic disulfide bond formation.
4	Rosetta-gami B(DE3)pLysS[1]	Features strain 2 + BL21 *lacZY* mutant
5	RosettaBlue(DE3)pLysS[1]	Features strain 1 + *recA*$^-$ and *endA*$^-$ which results in high transformation efficiency
6	Origami(DE3)pLysS[1]	trxB/gor mutant
7	Origami B(DE3)pLysS[1]	Features of strain 5 + BL21 lacZY mutant
8	BL21 Star(DE3)pLysS[2]	rne131$^-$: RNaseE deficient BL21 which results in less mRNA degradation
9	BL21-Codonplus(DE3)-RP[3]	Expression of rare tRNA's: AGA, AGG and CCC

[1]Strains were purchased from Novagen (Darmstadt, Germany). [2]Strains were purchased from Invitrogen (Carlsbad, CA). [3]Strains were purchased from Stratagene (La Jolla, CA).

Results and discussion

Expression of recombinant Pertactin

The gene for P.69 Prn was initially cloned into a pET-expression vector without an affinity tag and expressed in BL21 cells. Expression levels were low, and P.69 Prn was only detectable using western blots. Comparison of codon usage of *E. coli* and *B. pertussis* suggested differences in codon usage bias underlie the poor expression levels. In P.69 Prn, both the proline and glycine codon are five times more abundant then in *E. coli.* In an attempt to overcome codon usage bias and increase the expression levels, we compared the expression efficiencies of various *E. coli* strains (Table 4). An increase in expression levels was observed using BL21-Codonplus(DE3)-RP cells compared to BL21(DE3)pLysS. BL21-Codonplus(DE3)-RP contains extra copies of the *argU* and *proL* genes, which encode tRNA's that recognize the arginine codons AGA and AGG and the proline codon CCC, respectively, some of which are abundant in *B. pertussis*, but are rare in *E. coli*. Although expression was increased in BL21-Codonplus(DE3)-RP, P.69 Prn was only detectable using western blots (data not shown). Analyses of the cloned DNA upstream of the initiation codon of the pET vector revealed that the first 17 nucleotides of the cloned gene contain inverted repeats adjacent to the ribosomal binding site (RBS)[20]. When transcribed into RNA such regions may form stem and loop structures which inhibit translation[21]. To prevent effects of the secondary structure on the availability of the RBS we re-cloned the gene for Prn to an expression vector with an N-terminal HIS_6-tag (pET19b). Addition of an N-terminal HIS_6-tag led to very high expression levels, presumably due to improved accessibility of the ATG-site. Again we compared the expression efficiencies of these new constructs using the various expression hosts described above. Differences in expression levels were observed between the different *E. coli* hosts (Fig. 2). The BL21-Codonplus(DE3)-RP strain was chosen as expression host due to its high growth rate combined with high expression levels. Therefore, all recombinant proteins were expressed only in this host.

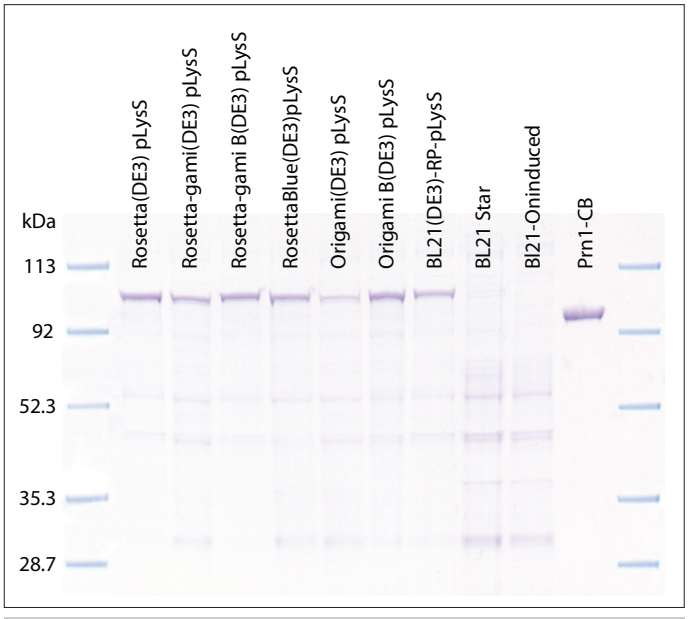

Figure 2. Coomassie stained SDS-PAGE gel (4-20%) for the comparison of expression levels of P.69 Prn1 in various *E. coli* stains. $2*10^4$ CFU were loaded in each lane. 2µg of purified P.69 Prn1 was loaded as a control.

Inclusion body purification and refolding

Overexpression of Prn in *E. coli* resulted in the formation of Prn inclusion bodies. Approximately 10 mg of inclusion bodies proteins could be isolated from 100 ml of IPTG induced culture. Since the protein was N-terminally fused to a His_6-tag we tried to further purify the recombinant P.69 Prn via affinity chromatography. Both unfolded inclusion bodies and fully refolded proteins were subjected to purification via Ni-His affinity chromatography. However, this did not result in further removal of impurities (not shown). Also, affinity chromatography in the presence of Tween20, high NaCl concentrations (>1 M) and imidazole did not result in an improved purification. Since most protein impurities had molecular masses smaller than 50 kDa we attempted to remove impurities by dialysis of the refolded proteins using a membrane with a 50 kDa cut-off. After 3 subsequent dialyses, no impurities were visible when 2 µg of purified proteins were loaded onto a SDS-page gel (Fig. 3).

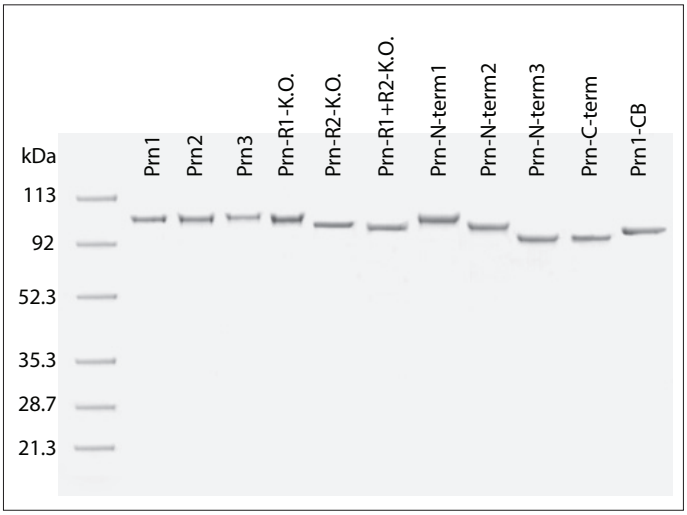

Figure 3. Coomassie stained SDS-PAGE gel (4-20%) with purified Prn recombinants. Due to it's high proline content22, on SDS-PAGE, PRN migrates slower then the marker proteins. Approximately 2µg of purified PRN was loaded in each lane.

Analysis of refolded Pertactin.

Refolded proteins were tested in an ELISA to verify proper refolding. We tested several mAbs, of which a number recognize conformational epitopes (Table 5)[10, 19]. No difference was observed between binding of mAbs to the refolded proteins compared to the binding to P.69 Prn purified from *B. pertussis* (Table 5), indicating that the protein has refolded into its native conformation. Furthermore none of the mAbs that recognize a conformational epitope showed reactivity with the inclusionbodies. The mAbs directed against a linear epitope however, display the same binding characteristics with inclusionbodies as they do on fully refolded and native material.

The secondary structure of the refolded proteins was compared to the structure of native P.69 Prn isolated from *B. pertussis* by circular dichroism spectroscopy (CD). Within experimental error, the CD spectra for native and recombinant Pertactin are identical, confirming the proper refolding of the purified proteins (Fig. 4).

Table 5. Analysis of refolded Pertactin.

	Rec-P.69 Prn1	Rec-P.69 Prn2	Rec-P.69 Prn3	P.69 Prn1	Unfolded Rec-Prn1	Putative epitope structure[1]
Pem 1	+	+	+	+	-	Conformational
Pem 2	++	++	++	++	-	Conformational
Pem 3	++	++	++	++	+	Linear
Pem 4	++	++	++	++	+	Linear
Pem 5	+	+	+	+	-	Conformational
Pem 6	+	+	+	+	-	Conformational
Pem 7	+	+	+	+	+	Linear
Pem 19	++	++	++	++	+	Linear
Pem 21	+	+	+	+	-	Conformational
Pem 29	+	+	+	+	-	Conformational
Pem 38	+	+	+	+	-	Conformational
Pem 64	+	+	+	+	-	Conformational
Pem 68	++	++	++	++	+	Linear
Pem 70	+	+	+	+	+	Linear
Pem 71	++	++	++	++	+	Linear
Pem 72	++	++	++	++	+	Linear
Pem 80	+	+	+	+	+	Linear
Pem 84	+	+	+	+	+	Linear
Pem 85	+	+	+	+	+	Linear
MUM-84	-	-	-	-	-	Not known

Microtiter plates were coated with P.69 Prn recombinants produced by *E. coli* (Rec-P.69 Prn1-3 and Unfolded Prn1 inclusionbodies) or P.69 Prn isolated from *B. pertussis* (P.69 Prn1). Subsequently, the binding of mAbs to the coated proteins was investigated. Mab MUM84 was used as a negative control and was raised against the mumps virus. A + indicates an OD450 >1.0, a ++ indicates an OD450 >2.0. [1]Determined previously by Hijnen *et al.*, 2004 [19].

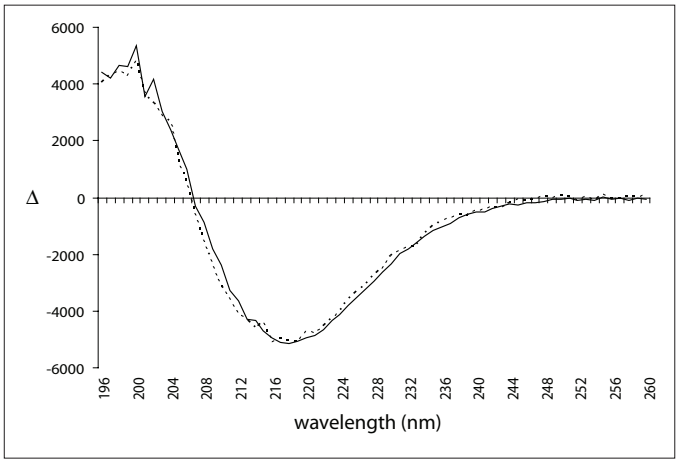

Figure 4. CD-spectrum of purified recombinant (dashed line) and native (solid line) Prn1. The data were collected at 25 °C on a Olis DSM 1000 Spectrophotometer. The protein concentrations were 1 mg/mL in 10 mM phosphate, pH 8.8. CD-spectra are presented as Δε values versus wavelength (nm).

In conclusion, we have developed a method to obtain large amounts of P.69 Prn which is indistinguishable from P.69 Pertactin isolated from *B. pertussis* as determined with mAbs and CD spectroscopy. The availability of large amounts of P.69 Pertactin variants and deletion mutants will facilitate functional and immunological studies.

Acknowledgements

We thank Muriel van Schilfgaarde for encouraging ideas and stimulating discussions concerning the expression of Prn, Chiron-Biocin for providing us with native P.69 Prn 1 and Bernard Metz for help with the CD measurements.

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4

The role of peptide loops of the *Bordetella pertussis* protein P.69 Pertactin in immune evasion.

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Abstract

Bordetella pertussis, the ethiological agent of whooping cough, is re-emerging in several countries with a traditionally high vaccine uptake. In *B. pertussis* strains associated with the re-emergence, polymorphisms were found in several proteins, including P.69 Pertactin (P.69 Prn). P.69 Pertactin, an adhesin, contains two variable regions comprised of repeats, one of which flanks the receptor binding site. Although P.69 Prn is one of the components of acellular pertussis vaccines, and is shown to induce protective antibodies, little is known about the structure and location of epitopes to which these protective antibodies are directed. In this report we describe the location of several conformational epitopes that are recognized by human antibodies. Furthermore we provide evidence that the two variable repeat regions of P.69 Prn, although separated by 289 amino acids in the primary structure, form a single polymorphic site which surrounds the functional domain of P.69 Prn. We propose that the repeat regions have evolved to allow rapid antigenic variation to deflect the immune response from the functional domain of P.69 Prn. We also provide evidence for an indirect role of loops in immune evasion by masking of epitopes. The results presented here provide a deeper understanding of the structure and function of variable loops and their role in the persistence of pathogens in immunologically primed populations.

Introduction

Bordetella pertussis, a small gram-negative bacterium, is the causative agent of whooping cough or pertussis. Although vaccination against pertussis has been highly successful in reducing morbidity and mortality, it has remained one of the 10 most common causes of death from infectious diseases worldwide [1]. During the past 10 years there has been a resurgence of pertussis in countries with a high vaccine uptake [1-6]. Several explanations have been suggested for the re-emergence of pertussis in these countries: increased reporting, improved diagnosis of the disease, waning immunity in both adolescents and adults and the adaptation of the *B. pertussis* population [2]. Pathogen adaptation has probably played an important role in the resurgence of pertussis in the Netherlands [2,7].

Analysis of clinical isolates collected in the last 50 years revealed antigenic divergence between vaccine strains and circulating strains in both Europe, the United States, Japan and Australia [4,8-17]. Polymorphisms were found in at least two proteins implicated in protective immunity: P.69 Pertactin (P.69 Prn) and pertussis toxin (Ptx) [2,18]. Several observations indicate an important role of P.69 Prn in protective immunity. Antibody levels to P.69 Prn have been shown to correlate with clinical protection [19,20]. Acellular vaccines (ACV's) containing Ptx, filamentous hemagglutinin (FHA) and P.69 Prn were more effective compared to ACV's containing Ptx and FHA only [21-23]. Passive and active immunization studies in mice and pigs have shown that antibodies against P.69 Prn confer protective immunity [24,25]. Anti-pertactin antibodies, but not anti-pertussis toxin, anti-fimbriae, or anti-filamentous hemagglutinin antibodies, were found to be crucial for *B. pertussis* phagocytosis [26]. P.69 Prn variants induce type-specific antibodies [27] and, finally, the efficacy of the Dutch whole cell vaccine was also shown to be affected by variation of P.69 Prn in a mouse model [24].

P.69 Prn, belongs to the family of so-called autotransporter proteins [28-30] which undergo autoproteolytic processing [29]. P.69 Prn is processed from a 93 kDa large precursor to a 69 kDa and 22 kDa protein [31]. The unprocessed polypeptide is directed via a signal peptide to the secretory machinery in the inner membrane where the signal peptide is cleaved. Subsequently, the polypeptide is directed towards the outer membrane where the 22-kDa protein forms a pore through which the 69-kDa protein is transported. After secretion via the autotransporter domain, proteolytic activities shape the 69-kDa protein to it's final 60.37 or 58.34 kDa form [32]. These final forms (referred to as P. 69 Prn), stay non-covalently bound to the bacterial cell surface and are used in most ACVs [22].

The X-ray crystal structure of P.69 Pertactin has been determined to a resolution of 2.5 Å. The protein fold consists of a 16-stranded parallel β-helix with a V-shaped cross-section. The structure appears as a helix from which several loops protrude, one of which contains the sequence motif associated with the biological activity of the protein; adherence to host tissues [33].

P.69 Prn is polymorphic, and 13 variants (P.69 Prn1 – P.69 Prn13) have been identified so far [34]. Variation is mainly limited to two regions, designated region 1 and 2, which are comprised of Gly-Gly-X-X-Pro (r1 repeat) and Pro-Gln-Pro (r2 repeat) repeats, respectively.

Most variation is found in region 1 which is located proximal to the N-terminus and flanks the Arg-Gly-Asp (RGD) motif, implicated in ligand-receptor interactions in eukaryotes. It has been shown that the RGD motif is involved in P.69 Prn-mediated attachment of *B. pertussis* to mammalian cells [35,36]. Region 2 is located at the C-terminus.

Although a number of studies in both animals and humans have indicated that P.69 Prn can elicit protective antibodies [19,20,24,37], information about the location of epitopes to which these antibodies are directed is limited. Previously, we described the location of several linear epitopes on P.69 Prn of both human serum antibodies and mouse mAbs. The aim of this study is to define the location of conformational epitopes on P.69 Prn, recognized by human antibodies, and to gain insight in the role that both the variable and non variable regions play in immunity and immune evasion.

Materials and Methods

Production of monoclonal antibodies

Details on the production of mAbs used in this study are previously described [24].

Biacore

The competition between anti-P.69 Prn mAbs in binding to pertactin was investigated by Biosensor technology using the Biacore. The Biacore (Pharmacia, Uppsala, Sweden) uses Surface Plasmon Resonance (SPR) technology to study macromolecular interaction [38]. In the Biacore one of the macromolecules, the ligand, is immobilized on a sensor chip. The sensor chip is composed of a carboxymethylated dextran coating on a thin gold film covering a glass plate. Other macromolecules, the analytes, flow continuously over the sensor chip surface and are allowed to interact with immobilized ligand [39]. The amount of material bound to the sensor chip surface in any experiment was expressed in arbitrary Resonance Units (RU). One RU represents approximately 1 pg of protein per mm^2 of the sensor chip surface [40].

In all experiments the Biacore buffer HBS-EP from Pharmacia was used at a flow rate of 5µl/min and a thermostatic temperature of 25ºC. To immobilize affinity purified anti-mouse-Fc (RAM-Fc, Pharmacia) on the sensor, the dextran layer of the CM5 sensor chip was activated by injection of 35 µl of a mixture consisting of 0.2M N-ethyl-N'-(dimethylaminopropyl) carbodiimide (EDC; Pharmacia) and 0.05 M N-hydroxysuccinimide (NHS; Pharmacia). Subsequently, 5000RU of Rabbit anti Mouse IgG (RAM-Fc)(diluted in 200mM sodium acetate pH 4.0) was immobilized. Next, the remaining uncoupled activated dextran of the sensor chip was saturated by injection of 50 µl 1M ethanolamine-HCl pH 8.5 (Pharmacia). Finally, the sensor chip surface was regenerated by injection of 15 µl 100mM Glycin-HCl solution pH 1.5 (Pharmacia).

To determine whether anti-P.69 Prn mAbs compete in binding to P.69 P.69 Prn, 15 affinity purified mAbs were tested pair wise in the Biacore system. The concentration of the individual MAbs was set for an optimal response in the Biacore. (PeM 68, 70 and 71 were not measured as their binding to P.69 Prn in the Biacore was low) After immobilization of 5000RU RAM IgG-F_c, a primary mAb in its optimal concentration (1-100µg/ml) was injected and allowed to bind to RAM-Fc. Next, the unbound RAM-Fc was saturated by injection of 35µl of an irrelevant mAb. Subsequently, 25µl of P.69 Prn 1 (50µg/ml) was injected and allowed to bind to the primary anti-P.69 Prn mAb. Then a secondary mAb was injected (1-100µg/ml) and allowed to bind to the P.69 Prn presented by the primary anti- P.69 Prn mAb. Finally, the sensor chip was regenerated with a Glycin-HCl solution (Pharmacia). For every secondary mAb the percentage of binding to P.69 Prn was determined relative to the maximal amount of binding to P.69 Prn in the absence of the first mAb. To correct for non-specific binding, the response obtained with a similar Biacore run in which P.69 Prn was replaced with buffer was subtracted from the response obtained with P.69 Prn as analyte. A reduction in binding with >90%, between 50% and 90%, and with

>50% compared maximal binding was considered as complete inhibition, partial inhibition and no inhibition, respectively [39].

P.69 Prn deletion mutants

The construction of plasmids, pPRN1, 2, 3, pPRN2A, B, C, pPRN2-N-1, 2, 3 and pPRN2-C1 which code for both wild-type and truncated P.69 Prn polypeptides has been described [41]. The plasmids pPRN2-C2, 3, 4 and 5 were constructed in a similar way. P.69 Prn recombinant proteins were over expressed in *E. coli* and purified as described [41].

Site directed mutagenesis

To localize the epitopes recognized by mAbs, exposed loops on P.69 Prn were mutated using site directed mutagenesis. On every exposed loop we mutated 2 adjacent amino acids (AA) (Fig. 1, Table1). Briefly, two PCR's were performed with the desired mutations in the reversed and forward primer of primer set 1 and 2 respectively. Purified plasmid DNA containing the native P.69 Prn1 sequence was used as template. Subsequently 25ng of a mixture of PCR-product 1 and 2 was used as a template for a secondary PCR using the forward and reversed primer of primer set 1 and 2 respectively. PCR fragments containing the mutant genes were digested with endonucleases *Nde*I and *Bam*HI (restriction sites were included in the PCR primers to facilitate cloning) and subsequently ligated into expression vector pET19b (digested with the same enzymes) placing the coding sequences under the control of the inducible T7 promoter. Plasmids were introduced into TOP10 *E.coli* by transformation for propagation and storage. All constructs were sequenced to confirm the presence of the desired point mutations and the absence of undesired mutations.

Table 1. Mutations introduced by SDM. Numbers correspond to the loops indicated in Figure 1. Amino acids that were mutated are indicated in bold.

Name	Original Site	Mutated Site	Name	Original Site	Mutated Site
SDM1	TG**ER**QH	TG**DH**QH	SDM10	SL**QP**ED	SL**TM**ED
SDM2	QGS**DP**GGV	QGS**EM**GGV	SDM11	VP**AS**GAP	VP**IN**GAP
SDM3	VS**GR**QAQ	VS**AK**QAQ	SDM12	**GA**	**IL**
SDM4	SDD**GI**RR	SDD**AL**RR	SDM13	**RGD**	**KIE**
SDM5	VGDTW**DD**DG	VGDT**FE**DG	SDM14	GARR**FAP**	GARR**WIM**
SDM6	G**EQ**	G**DN**	SDM15	V**LP**EP	V**IM**EP
SDM7	G**AG**	G**LA**	SDM16	AP**EL**	AP**DI**
SDM8	**RG**A	**KA**A	SDM17	ATEL**PS**I	ATEL**MN**I
SDM9	V**DG**G	V**EI**G	SDM18	QQPAE**AG**R	QQPAE**IL**R
SDM9a	V**DG**G	**AN**GG	SDM19	TP**LG**SAA	TP**IA**SAA
SDM9b	V**DG**G	V**NA**G	SDM20	AN**GN**G	AN**IT**G

Expression of Recombinant Pertactin

Plasmids containing constructs for the over expression of P.69 Prn recombinant proteins, were introduced into BL21 Codonplus (DE3)-RP (Stratagene, La Jolla, CA). P.69 Prn recombinant proteins were expressed and purified essentially as described [41]. A single colony of a transformant was used to inoculate 50 ml of auto induction Overnight Express (OE) medium (Novagen, Darmstadt, Germany) containing 100µg/ml ampicillin and 50µg/ml chloramphenicol. Bacteria were grown at 37°C overnight at 250 rpm. The next morning, bacteria from the induced cultures were harvested by centrifugation at 5000 g for 10 min at 4°C.

Purification and refolding of inclusion bodies

Inclusion bodies were isolated and refolded as described, except that 200mM of L-Arginine was added to the first refolding buffer (1 mM EDTA, 100 mM NaCl, 50 mM Tris/HCL pH 8.8) resulting in higher refolding efficiencies [41].

ELISA

An enzyme-linked immunosorbent assay (ELISA) was used to investigate the effect of mutations or deletions introduced in P.69 Prn on the binding of mAbs. Details about the procedure have been described previously [42]. The binding properties of the mAbs to the recombinant proteins were compared with their binding properties to native P.69 Prn1 (kindly provided by Chiron-Biocin). Polystyrene 96-well ELISA plates (Immulon II; Dynatech, Chantilly, Va.) were coated overnight at 22°C by adding 100 µl of 0.04 M carbonate buffer pH 9.6 containing 2 µg of protein/ml to each well. The plates were washed four times with 200 µl of PBS supplemented with 0.05% Tween 20 (PBST) per well by using a Titertek Plus M96V washer (ICN, Irvine, Calif.). Murine mAbs, diluted in PBST, were added to the wells and were incubated for 2 h at 37 °C followed by four washings as described above. Bound antibodies were detected by using horseradish peroxidase-conjugated anti-mouse total IgG (Cappel, Organon Technica, Boxtel, the Netherlands). The optical density at 450 nm (OD450) was measured with a plate reader (EL312e; BioTek Systems, Winooski, Vt.).

Binding of mAbs to mutant P.69 Prn was categorized as follows (Fig. 2): complete loss of binding of the mAb (binding not above background, “black square”), partial loss of binding (a 1 to 4 fold reduction compared to binding to native P.69 Prn, “grey square”), no loss of binding (binding equal to native P.69 Prn, “no square”) and increase of binding (at least a twofold increase of binding compared to native P.69 Prn, “striped square”).

Affinity ELISA

To measure the affinity of the mAbs for P.69 Prn, an affinity ELISA was performed. The affinity ELISA was performed as described above with the following adjustments. After

the coated P.69 Prn was incubated with a mAb for 2 h, plates were washed 4 times as described above. Subsequently plates were incubated for 15 minutes at RT with increasing concentrations of NaSCN. Plates were washed for 4 times after which the remaining bound antibodies were detected. The concentration of NaSCN (M) needed for fully disrupting the binding of mAb to P.69 Prn was used as the avidity index (Fig. 2).

In silico analysis of mutations

To locate variation between the three major *Bordetella* spp., *Bordetella pertussis* (Tohama I), *parapertussis* (12822) *and bronchiseptica* (RB50) an alignment of the P.69 Prn amino acid sequences was performed using the program Kodon (Applied Maths, Belgium).

To investigate the effect that the introduced amino acid substitution could have on the folding of the protein or accessibility of possible epitopes, several in silico analyses were performed. The coordinates of pertactin were downloaded from the Protein Data Bank [43], under code 1DAB.pdb. First, the effect of the substitution of the resident amino acid with the other 19 amino acids at every given position was evaluated with the WhatIf commando MUTQUA[44]. MUTQUA will find a `likely' side chain conformation for every mutant, and calculate the quality value for the mutant in that position. The quality value is a weighted total of several terms (W-Q); how well does backbone of database fragment fit (fit-Q), how well does this rotamer look like the most frequently observed rotamer (Rot Q), how is the side chain packing quality (Pack Q) and the bumps made by this rotamer (Bumps). A low quality value for an amino acid indicates that this mutation is unlikely to occur. A high quality value indicates that this mutation is preferred above the resident amino acid.

Secondly, some of the least conserved mutations were checked for energetically feasible sidechain rotamers using the programme DeepView. Thirdly, the solvent accessible surface area was computed per residue with a 1.4 Å probe, using the programme Getarea 1.1 [45].

Results

In a previous study, a Pepscan of P.69 Prn was used to identify epitopes recognized by mAbs raised against the native protein [42]. This approach mainly reveals linear epitopes. Indeed a number of the mAbs did not react with synthetic peptides, suggesting they recognized discontinuous or conformational epitopes [42]. Here we used three different approaches to further identify epitopes recognized by our set of mAbs; site-directed mutagenesis (SDM), deletion mapping and competition experiments. It was hoped that these studies would allow us to recognize not only linear, but also conformational epitopes. The crystal structure of P.69 Prn [33] was used to identify regions for SDM.

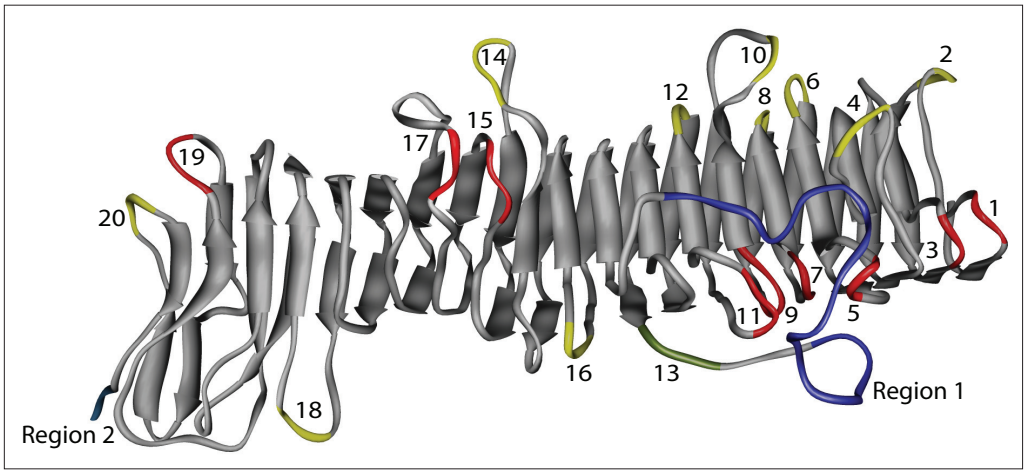

Figure 1. Crystal structure of P.69 Prn 33. Loops that were selected for site-directed mutagenesis are numbered. Note that the C-terminal amino acid residues 540 to 677, containing region 2, are not part of the structure.

The crystal structure appears as a β-helix from which several loops protrude. Unlike the rigid core, the loops of P.69 Prn are well exposed and therefore likely to be more immunogenic. SDM was performed on all loops located between residues 1 and 539 (crystal structure 1DAB.pdb was used as a reference) (Fig. 1).

In the second approach, defined deletions were introduced in P.69 Prn. Region 1 and 2 were deleted, respectively, by removing all 5 GGXXP repeats and peptide PQPGP~PAPQP, comprising 25 residues (Fig. 2A). It should be noted that region 1 is part of loop 13 which was also modified by SDM. Three N-terminal deletion derivatives (designated N1-3) were constructed in which 19, 63 and 110 residues were deleted, respectively (Fig. 2A). The following loops were removed by the N-terminal deletions: N1, loop 1, N2 loops 1, 2, 3 and N3 loops 1, 2, 3, 4, 5. The four C-terminal mutants (designated C1-4) contained deletions of 136, 154, 229 and 247 residues, respectively (Fig. 2A). All C-terminal deletions removed region 2. The deletions in C3 and C4 extended into a junction region, important for folding of BrkA [46], a protein with a large degree of sequence identity with P.69 Prn, and we anticipated that these deletions would have a significant effect on the structure of P.69

Prn.

We observed that mutations could both decrease and increase binding of the mAbs (see below). These effects could be caused in a direct way by modifying the epitope. However, indirect effects of the modifications were also possible. E.g. a modification outside the epitope, could affect its three dimensional structure. Further, an indirect effect can be caused by a modification which affects accessibility of the epitope for the mAb. E.g. modification of a loop can result in increased or decreased steric hindrance of a proximate epitope. Since a number of loops (loops 3, 5, 7, 9, 11, and 15) are not exposed to solvent in the X-ray conformation, several observations were attributed to indirect effects.

For modification by SDM, a total of 20 loops were selected (Fig. 1), but we were unable to introduce mutations in 5 loops (loops 7, 8, 11, 12 and 16). Consequently, a total of 15 loops were modified and the modification of 4 loops (loops 3, 10, 17 and 20) had no effect on the binding of any of the mAbs tested. This suggested that the residues modified are not part of an epitope recognized by these mAbs or that their contribution to binding is negligible. Modification of loop 9 by replacement of Gly147 by Ile, resulting in loop 9a, abolished binding of 6 mAbs that recognize N-terminal peptides, and decreased binding of 1 mAb. To validate these results, 2 more loop 9 mutants (9b and c) were constructed containing more conserved mutations. These modifications did not have an effect on the binding of any of the mAbs tested, suggesting that the first modification resulting in loop 9a had an indirect effect on the tertiary structure of the N-terminus of P.69 Prn. Solvent accessibility calculations with the GetArea programme pointed out that loops 3, 5, 7, 9, 11, and 15 are not exposed to solvent and any effect of SDM in these loops could be indirect.

MAbs PeM3, PeM4, PeM68, PeM70, PeM71 and PeM72 showed similar binding characteristics with respect to P.69 Prn mutant-derivatives. With the exception of Pem68, modification of loops did not affect binding of these mAbs. Modification of loop 13 abolished binding of PeM68. Binding of all mAbs was abolished by deletion of region 1, while deletion of region 2 had no effect. N- and C-terminal deletions did not affect binding of any of these mAbs. Since the C3 and C4-deletions were expected to have a significant

Figure 2. (A) Primary structure of P.69 Prn. The location and size of N-terminal, C-terminal and central deletions are indicated. Positions of loops 1 to 20 are shown below the bar. r1, r2 and RGD indicate the variable regions 1, 2 and the RGD receptor binding motif, respectively. Abbreviations: del, deletion. AA, amino acid. Bp, base pair.
(B) Binding properties of mAbs. Left panel: The ability of mAb to block binding of human antibodies, their avidity and their identification numbers are shown on the left. + and - indicate blocking and no blocking, respectively. In the Avid column, the numbers indicate the concentration of NaSCN that was needed to fully disrupt antibody binding. Right panel: N-terminal, C-terminal deletion mutants and loops are indicated by N1-3, C1-4 and lp1-20, respectively. The region 1 and 2 deletion mutants are indicated by r1-del and r2-del, respectively. Thickset squares indicate regions which have been shown to bind mAbs by Pepscan analyses [42]. Black and grey boxes indicate loss and 1-4 fold decrease in binding, respectively. Striped boxes indicate a 1-4 fold increase in binding.Bottom panel: Polymorphisms in loops is indicated and is based on a comparison of P.69 Prn orthologues from *B. pertussis, B. parapertussis* and *B. bronchiseptica*. "Flank" refers to polymorphisms in the flanking regions of the loops. Surface exposure of the loops is indicated and was determined using the program Getarea 1.1. Abbreviations. Block, blocking. Avid, avidity. Lp, loop. PS, Pepscan. *Determined previously by Hijnen *et al*., 2004 [42].

A.
Δ57bp (19AA)
Δ189bp (63AA)
Δ330bp (110AA)
Δ408bp (136AA)
Δ462bp (154AA)
Δ687bp (229AA)
Δ741bp (247AA)
N1
N2
N3
C1
C2
C3
C4
N'
C'
RGD
r1-del:
Δ75bp (25AA)
r1
cfd
r2
r2-del:
Δ75bp (25AA)
1 2 3 4 5 6 9 10 13 14 15 17 18 19 20
B.
Block.* Avid. PeM N1 N2 N3 Ip1 Ip2 Ip3 Ip4 Ip5 Ip6 Ip9a Ip9b Ip9c Ip10 Ip13 PS r1-pep r1-del Ip14 Ip15 Ip17 Ip18 Ip19 Ip20 PS r2-pep r2-del C4 C3 C2 C1
Polymorphic loop: Yes Yes No Yes Flank No No No No No No No Yes Yes No Yes No Yes
Surface exposed loop: Yes Yes No Yes No Yes No No No No No Yes Yes No Yes Yes Yes Yes
= Loss of binding
= 1 to 4 fold decrease in binding
= 1 to 4 fold increase in binding
= mAb binding previously determined by Pepscan analysis

influence on the structure of P.69 Prn, as described above, these results suggest that the mAbs recognize linear epitopes. These data are consistent with our previous studies which showed that the mAbs bind to peptides derived from region 1 [42]. The negative effect on binding of modification of loop 13 fits into this picture, as region 1 is part of loop 13. In conclusion, all available data indicate that these mAbs recognize a linear epitope in region 1. MAbs PeM2 and PeM19 also showed similar binding characteristics with respect to P.69 Prn mutant derivatives. Binding of the 2 mAbs was not affected by modification of the loops or deletion of region 1. However, deletion of region 2 abolished binding. N-terminal deletions did not affect binding, while all C-terminal deletions abolished binding. The latter was expected as these deletions also removed region 2. These results suggested that the mAbs bound to an epitope which located in region 2. Consistent with this assumption, previous studies showed that both mAbs bound to peptides derived from region 2. Interestingly, previous data indicated that PeM2 also bound to a region 1 peptide. The fact that region 1 can be deleted without affecting binding of PeM2 to P.69 Prn indicates that the region 2 comprises the major binding site of PeM2. In conclusion, PeM2 and 19 recognize an epitope located in region 2. Region 1 may also be part of the binding site of PeM2.
Binding of PeM84 and PeM85 to P.69 Prn was affected by modification of the loops 9 (to 9a), 13, 18 and 19. PeM85 was also affected by modification of loop 5, which resulted in a 2-fold increase in binding. Modification of loop 9 to 9a abolished binding of both mAbs, while modification of loops 13, 18 en 19 decreased binding 2-fold. Since modification of loop 9 to 9a (Gly to Ile) was likely to have an effect on the local tertiary structure of the N-terminus of P.69 Prn, the observed effect with the loop 9a mutant could be caused by an indirect effect. This was corroborated by the results with the loop 9b and c mutants, to which mAbs PeM84 and 85 were still able to bind. Deletion of region 1 or region 2 did not affect binding of the 2 mAbs. However, N-terminal deletion N1 decreased binding of PeM84 3-fold, while deletions N2 and N3 abolished binding of PeM84. The binding of PeM85 was decreased 2-fold by deletions N1, N2 and N3. The deletion N3 removed loop 5, modification of which affected binding of PeM85. C-terminal deletion C2 reduced binding of both mAbs 2-fold, while C3 and C4 abolished binding. Since C3 and C4 deletions do not remove any of the loops implicated in binding of the mAbs, but affect the overall structure of P.69 Prn, the effect of these deletions suggests that the mAbs recognize a conformational epitope. Previous work showed that both mAbs bound to peptides derived from region 1. This is consistent with the observation that modification of loop 13 reduced binding, as region 1 is part of this loop. The fact that removal of region 1 still allows binding of both mAbs, indicates that residues outside region 1 contribute significantly to binding. These regions may comprise loop 5, which is in close proximity to loop 13 in the crystal structure. There is likely a contribution of other loops in the N-terminus, since modification of loop 9a abolished binding indirectly presumably through modification of the tertiary structure of the N-terminus. Since PeM84 was unable to bind to N-terminal deletion 2 and 3, but showed some binding with N-terminal deletion 1, and bound to loop

2 and 3 mutants, it is conceivable that peptides other then loops 2 or 3, located in between deletions N-term1 and 2, contribute to binding. In conclusion, the data suggest that the 2 mAbs recognize an N-terminal conformational epitope, which is formed by peptides located in between loops 2 or 3 and loop 13 in the case of PeM84, and loops 5 and 13 in the case of PeM85. It is unclear how modification of the loops18 and 19 affect binding of PeM84 and PeM85. Parts of discontinuous conformational epitopes generally are within 6-8 Ångstrom from each other and the distance between loop 13, 18 and 19 is much larger [47]. Thus modification of loop 18 and 19 may affect antibody binding indirectly through conformational changes of P.69 Prn.

Binding of PeM29 was affected by modification of the loops 1, 2, 9 (to 9a), 13 and 19. Modification of loop 1 abolished binding consistent with the fact that PeM29 recognized a peptide derived from this loop [42]. Modification of loop 2 and 9a resulted in a 2-fold decrease in binding and loss of binding, respectively. As described for PeM84 and 85, the modification of loop 9a abolished the binding of PeM29 indirectly. Modification of loop 13 decreased binding 2-fold which is in line with data showing that PeM29 bound to peptides derived from region 1 [42]. In previous work we found that PeM29 also bound to a peptide derived from loop 5. Modification of loop 5 did not affect binding, however, indicating that the modified residues were not part of the antibody binding site. In view of the proximity of loops 1, 2, 5, and 13 it is conceivable that they are part of the PeM29 epitope. Modification of loop 19 reduced binding 2-fold. The distance between loop 19 and the other loops implicated in binding is probably too large for loop 19 to contribute directly to binding. Modification of loop 19 may affect binding indirectly through the conformation of P.69 Prn. Unexpectedly deletion of region 1 did not affect binding. This suggests that while region 1 is involved in binding, it is not absolutely required. The binding affinity is probably mainly determined by loop 1 and 2. The essential contribution of loop 1 is in line with the observation that modification of loop1 or the N1 N-terminal deletion, which removes loop 1 but no other loops, abolishes binding. Deletion of region 2 decreased binding 2.5-fold, consistent with our hypothesis that region 1 and 2 can be proximate in conformations other than the X-ray structure, and may form a single conformational epitope [42]. The C-terminal deletion C1 and C2 decreased binding 2-fold, while C3 and C4 abolished binding. Deletions C3 and C4 probably affect the overall structure of P.69 Prn. In summary, these data suggest that loops 1, 2, 13 and region 2 comprise the PeM29 epitope.

PeM5 was previously shown not to bind to any synthetic peptides, suggesting it binds to an unknown conformational epitope. Similar as PeM29, the binding of PeM5 was affected by modification of the loops 1, 2, 4, 5, 9a, 13 and 19. Modification of loops 5 and 9 (to 9a) abolished binding of PeM5. Similar as for mAb PeM84, 85 and 29, the modification of loop 9 (to 9a) abolished the binding of PeM5 indirectly. Modification of loops 1, 2, 4, 13 and 19 resulted in a 2-fold decrease in binding. In view of the proximity of loops 1, 2, 4, 5, and 13 it is conceivable that they are part of the PeM5 epitope. Similarly as described for

PeM29, the distance between loop 19 and the other loops implicated in binding is probably too large for loop 19 to contribute directly to binding. Modification of loop 19 may affect binding indirectly through the conformation of P.69 Prn. Deletion of region 1 did not affect binding. N-terminal deletion N1, in which only loop 1 is deleted, abolished binding of PeM5, implicating that this region is an essential part of the PeM5 epitope. Larger N-terminal deletions, N2 and N3, also abolished binding of PeM5. Deletion of region 2 decreased binding of PeM5 by 2-fold. Similar as described for PeM29, C-terminal deletions C1 and C2 decreased binding 2-fold, while C3 and C4 abolished binding. Summarizing, these data suggest that the epitope of PeM5 comprises loops 1, 2, 4, 5, 13 and region 2.
Binding of PeM1 was affected by modification of loops 1, 9 (to 9a), 13, 18 and 19. Binding was decreased 2-fold and abolished by modification of loops 1 and 9 (to 9a), respectively. As described for mAbs PeM84, 85, 29 and 5, the modification of loop 9 (to 9a) abolished the binding of PeM1 indirectly. Modification of loops 13, 18 and 19 increased binding 2-fold. As was observed for PeM29, modification of loop 5 did not affect binding, although a peptide derived from this loop was implicated in binding of the mAb. Thus the residues modified in loop 5 are apparently not essential for binding. PeM1 was shown to bind to peptides derived from region 1. However, no effect was observed when region 1 was deleted but modification of loop 13 increased binding. Since Pepscan analyses did not identify the residues in loop 13 that were modified as part of the antibody binding site, it is possible that the increase in binding observed is due to indirect effect on the accessibility of the epitope of PeM1. PeM1 was also shown to bind to peptides derived from region 2, which is consistent with the observation that deletion of region 2 reduced binding 2.5-fold. N-terminal deletions N1, N2, N3 and the C-terminal deletions C1 and C2 reduced binding 3-fold. The latter deletions also remove region 2. The C-terminal deletion C3 and C4 abolished binding. Based on similar arguments as was used for loop 19 and PeM29, modification of loops 18 and 19 may affect binding of PeM1 indirectly. Together, these data suggest that PeM1 binds to an epitope comprised of loop 1, 5, 13, and region 2.
Binding of PeM6 was affected by modification of loops 1, 4, 5, 6, 9 (to 9a), 13, 18 and 19. Binding was decreased 2-fold by modification of loops 1 and 9. Modification of loops 4, 5, 6, 13, 18 and 19 increased binding 2-fold. Deletion of region 2 reduced binding of PeM6 by 4-fold. N-terminal deletions and the C-terminal deletions C1 and C2 reduced binding of PeM6 by 3-fold. The C-terminal deletions C3 and C4 abolished binding. Since the binding of PeM6 was affected by modification of many N-terminal loops, it is likely that its epitope is comprised of several of these loops. Since binding was decreased by modification of loop 1, it is likely that it is part of the epitope of PeM6. Since loops 4, 5, 6 and 13 are all in close proximity, it is possible that they are also part of the epitope of PeM6. Binding of PeM6 was affected by deletion of region 2 and parts of both the C- and N-terminus, therefore, similar arguments can be used as for several other mAbs described above which suggest an epitope comprising both the N- and C-terminus. In summary, these data suggest two possibilities for the epitope of PeM6, an epitope comprised of N-terminal loops, or an

epitope comprising both the N- and C-terminus.
Modification of loops 2 and 9 (to 9a) resulted in a loss of binding of PeM7. Based on similar arguments as was used for loop 9 and PeM84, 85, 29, 5, 1 and 6, modification of loop 9 (to 9a) may affect binding of PeM7 indirectly. Mutation of loops 1, 13 and 19 decreased binding of PeM7 2-fold. In contrast, modification of loops 14 and 18 resulted in a 2-fold increase in binding. PeM7 was previously found to bind to region 1 peptides and this is consistent with the negative effect of modification of loop 13. However, deletion of region 1 did not affect binding suggesting that the high affinity binding sites of PeM7 lie outside region 1. Deletion of region 2 resulted in a 3-fold reduction of binding. N-terminal deletions abrogated binding, consistent with the presence of loop 2 in this region, which was shown to be essential for binding. C-terminal deletions C1 and C2 resulted in a 3-fold reduction in binding, whereas deletions C3 and C4 abolished binding. In conclusion, we suggest the following composition for the PeM7 epitope: loops 1, 2, 13 and region 2.
Binding of PeM21 and PeM64 to P.69 Prn was affected identically by modification of the loops 15 and 18, which decreased binding 3-fold and increased binding 2-fold, respectively. PeM64 was also affected by modification of loop 4, which resulted in a 2-fold increase in binding. Deletion of region 2 reduced binding of PeM21 and 64 by 3 and 4-fold respectively. N-terminal deletions and the C-terminal deletion C1 reduced binding for both mAbs by 3-fold. The latter deletions also remove region 2. The C-terminal deletions C2, C3 and C4 abolished binding. Together, these data suggest several possibilities for the epitope recognized by PeM21 and 64. Since loops 15 and 18 are located on opposite sides of the protein, it is unlikely that they comprise the epitope of PeM21 and 64. Since loop 15 is located in the center of P.69 Prn, it is unlikely that the epitope consists of loop 15 and the N- or C-terminus. The two remaining possibilities are an epitope that is comprised of loop 18 and the C-terminus, or an epitope in which region 2 folds towards region 1, resulting in a close proximity of loops 15, 18 and the N-terminus.
PeM38 was unable to bind synthetic peptides in previous studies, suggesting it recognizes an unknown conformational epitope. Modification of loop 18 resulted in a 2-fold increase in binding for PeM38. Deletions of the N-terminus resulted in a 3-fold decrease in binding of PeM38. Deletion of region 2 also resulted in a 3-fold reduction of binding. The C-terminal deletion C1 decreased binding 3-fold, while C2 abolished binding. We previously hypothesized that region 1 and 2 are proximate, which would also result in a close proximity of the N- and C-terminus of which region 1 and 2 are part of, respectively. Based on the location of loop 18 close to region 2, we speculate that its modification may result in an increased interaction between regions 1 and 2, implicating an indirect effect. Deletion of region 2 decreased binding 3-fold, consistent with our hypothesis that region 1 and 2 are proximate and may form a single conformational epitope [42]. In summary, these data suggest that the N- and C-terminus, including region 2, comprise the PeM38 epitope.
PeM80 was affected by modification of the loops 5, 6, 13, 14, 15 and 19. Modification of loops 5, 6, 13 and 19 increased binding 2-fold. Modification of loop 14 and 15 abolished

and reduced binding 2-fold, respectively. The effect of modification of loop 14 is consistent with previous data which showed that PeM80 binds to RFAPQ which is part of loop 14. In view of its proximity to loop 14 (Fig. 1) it is conceivable that loop 15 is also part of the PeM80 epitope. N-terminal deletions N1 and N2 resulted in a 2-fold decrease in binding, whereas deletion N3 had no effect. In view of the distance between the N-terminus and loop14 it is unlikely that the effect of the N-terminal deletion can be explained by a reduction in steric hindrance. More likely, these deletions may affect binding of PeM80 indirectly by changes in the tertiary structure of P.69 Prn. The C-terminal deletion C2 decreased binding of PeM80 2-fold, while C3 and C4 abolished binding. Together, these data suggest that PeM80 binds to an epitope comprised of loop 14 and possibly 15.

Competition between mAbs for binding to P.69 Prn

To validate the results obtained with both SDM and P.69 Prn deletion mutants and to identify the few remaining epitopes, the ability of mAbs to compete with each other for binding to P.69 Prn was tested. For the competition assay, P.69 Prn was allowed to bind to a primary mAb (PA) immobilized to the sensor chip of the BIACore. Subsequently, the ability of a second antibody (SA) to bind to the immobilized P.69 Prn was determined. In this way, 15 mAbs were assayed for mutual competition resulting in the identification of 7 competition groups, defined as a group of mAbs that showed competition with one or more mAbs within that group (Fig. 3). We will present the inhibition results from the perspective of the primary mAb.

The mAbs PeM4 and 72 comprise the first group, and show only a mutual inhibition. Both mAbs were shown by SDM, P.69 Prn deletion mutants, and Pepscan to strictly recognize region 1 peptides.

The second group consists of PeM2 and 19. Although no mutual inhibition was observed, both mAbs inhibited subsequent binding of PeM4. P.69 Prn deletion mutant data showed that both mAbs bind to region 2 whereas mAb PeM4 was shown to strictly bind region 1. PeM2 partially inhibited the subsequent binding of PeM6, which was shown to recognize a conformational epitope located in the N-terminus. Finally, PeM19 partially inhibited the subsequent binding of PeM72 which recognizes an epitope located in region 1. The observed inhibition is consistent with a close proximity of region 1 and region 2.

PeM84 and 85 comprise a third group and show no competition with any of the mAbs from the other groups. Both mAbs have almost identical SDM and P.69 Prn deletion mutant binding patterns, explaining their mutual inhibition.

PeM29 and 5 form a fourth group. Both mAbs show a mutual inhibition. Pepscan, SDM and P.69 Prn deletion mutant data indicated that these two mAbs bind several N-terminal loops. MAb PeM5 shows a non-reciprocal inhibition of PeM4 and 2. MAbs PeM5 and 2 may compete for an epitope in region 2, since the mAbs showed a decreased and no binding to a P.69 Prn mutant in which region 2 was deleted, respectively. Finally, mAbs PeM5 and 4 may compete for an epitope located in, or near region 1, since PeM4 was shown to strictly

bind region 1, whereas PeM5 showed a decreased binding to the loop 13 mutant, which is part of region 1.
The mAbs PeM1, 6, 7, 21 and 64 comprised the fifth competition group. The binding of one of these mAbs was inhibited by almost all other mAbs within this group. These results confirm the data generated with SDM, P.69 Prn deletion mutants and Pepscan. In all these experiments these five mAbs tend to bind to N-terminal loops. Although PeM64

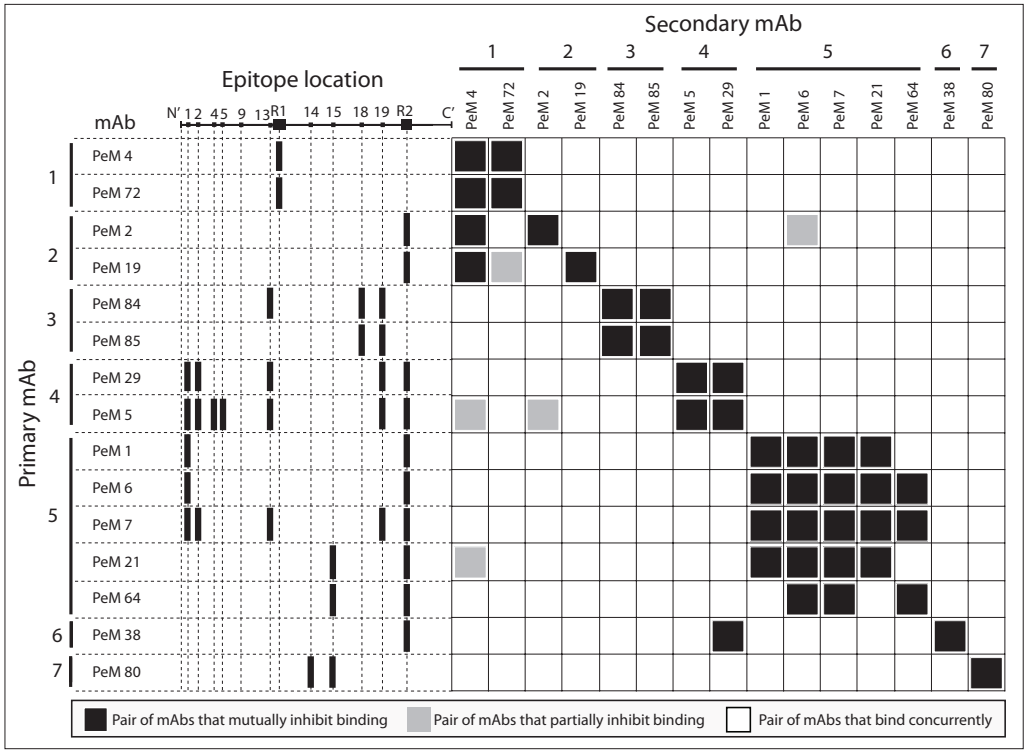

Figure 3. Competition matrix visualizing the binding ability of pairs of mAbs to native Prn1. The results of the SDM and deletion mutant mapping is indicated on the left. Only the decreases in binding observed by SDM and deletion mutant mapping are indicated.

and 21 show similar binding patterns with the SDM and P.69 Prn deletion mutants, they do not compete for binding. PeM64 and PeM1 also do not compete for binding, which is consistent with the observation that they show different binding to SDM and P.69 Prn deletion mutants. Although mAbs PeM21 and 4 show different binding patterns with mutant P.69 Prn derivatives, PeM21 partially inhibits the subsequent binding of PeM4. PeM38, comprising group six, did not compete with any other mAb except for a non-reciprocal inhibition of PeM29. PeM38 bound all SDM and P.69 Prn deletion mutants, but showed a slight decrease in binding with both the N- and C-terminal deletion mutants. These results could explain the inhibition of binding of PeM29, which was shown to bind

to several N-terminal loops and region 2.
PeM80, comprising group 7, showed no competition with any of the other mAbs. PeM80 was the only mAb of which the binding was negatively affected by modification of loop14.

Affinity ELISA
To measure the affinity of the mAbs for P.69 Prn, an affinity ELISA was performed (Fig. 2B). There was no clear relation between the affinity and the type of epitope, linear or conformational, that was recognized by the respective mAb. The affinity of mAbs recognizing a linear epitope, varied between 1.5 and 4.5M. The affinity of mAbs recognizing conformational epitopes, varied between 2 and 3.5M. In a previous study we observed that although many human Abs bind to region 2, no blocking of PeM2 and 19, also recognizing region 2, was observed by these Abs. We suggested that this could be due to the high affinity of these two mAbs, which was corroborated by the affinity ELISA data described here.

In silico analysis of mutations
To determine if natural variation occurred in the Prn loops, we compared the Prn orthologues from *B. pertussis*, *B. parapertussis* and *B. bronchiseptica*. Residues were assigned to loops based on the crystal structure [33]. Residues flanking loops were considered part of the loop. Polymorphism was observed in loops 1, 2, 4, 5, 12, 13, 14, 16, 17 and 19. With the exception of loop 17, modifications of these loops affected mAb binding, generally resulting in a decreased binding. Loops 3, 6, 9, 10, 15, 18, 20 were conserved. Modification of four of these loops (6, 9, 15, 18) affected binding of mAbs,
Modification of four loops (3, 10, 17 and 20) was not found to affect binding of mAbs. Three of the four loops (3, 10 and 20) were conserved. Modification of 11 loops (loops 1, 2, 4, 5, 6, 9, 13, 14, 15, 18, 19) resulted in a decrease or increase in mAb binding. Seven of these loops were polymorphic (loops 1, 2, 4, 5, 13, 14, 19). These results suggest that loops that are not important for immune recognition do not display polymorphism, while loops that are involved in antibody binding are polymorphic.
Loops that after mutation showed only a decrease in binding with mAbs (loops 1 and 2) had polymorphisms at the same location as the SDM mutations. Although after mutation of loop 15 the binding of 3 mAbs decreased, no polymorphisms were observed in this loop. In contrast, after mutation of loop 6, the binding of 2 mAbs increased, but no polymorphisms were found. Several loops that were polymorphic showed both an increase and decrease in binding with mAbs (loops 4, 5, 14 and 19).
To investigate, and find possible explanations for the observed in- and decrease of antibody binding to mutant derivatives of P.69 Prn, we first employed the WhatIf MUTQUA command to investigate the effect that the substitution of a resident amino acid with another amino acid has. If a different amino acid then the resident one fits a certain location better, MUTQUA will propose this. MUTQUA did not propose any amino acid changes in the native P.69 Prn sequence except for residue Pro58. This amino acid could easily be

changed to a Glu, Thr or Val, without interfering with the tertiary structure. We mutated this Pro into a Met, to be certain to change a possible conformational epitope and this substitution was found to affect mAb binding. When introducing very subtle changes, it could theoretically be possible that mAbs still retain part of their affinity. The fact that no mutations were considered viable a priori by MUTQUA, suggested that SDM were likely to lead to in- or decreased affinity. We then focused on the SDM mutants that showed either an in- or decrease in binding with at least two mAbs. When loop 9 was mutated, 6 mAbs (PeM's 1, 5, 7, 29, 84 and 85) were affected in their ability to bind to this mutant. Either this site is very immunogenic or the introduction of a large aliphatic side chain (mutation from Gly to Ile) caused loops 11 and 13 to move side wards. The latter explanation is most likely, since loop 9 is not solvent exposed in the X-ray conformation and we were unable to find energetically favorable side chain positions during mutation to Ile with the modeling program DeepView. It is possible that these 6 mAbs do not recognize loop 9 as part of their epitope, but recognize one of the adjacent loops, of which the accessibility or location is affected by mutation of loop 9. The latter was confirmed by loop9 mutants 9b and c. Mutants 9b and c contain more conserved mutations, which should not affect the tertiary structure. When in loop 13, Gly261 was mutated to Ile, a positive effect on the binding of several mAbs was observed. It is likely that the exposed hydrophobic Ile drives loop 13 to desolvate, possibly by binding to a hydrophobic antibody, or by folding inwards the protein. This could explain the increased binding of several mAbs to this mutant P.69 Prn. Similar results were obtained when Ile was introduced in loops 14, 18 and 19. In contrast, when Gly565 in loop 20 was mutated to an Ile, no effect on the binding of mAbs was observed. Another, non-conservative mutation did lead to an increase of binding of mAbs. When the negatively charged Asp135 in loop 5 was mutated to a large hydrophobic aromatic Phe, the binding of 3 mAbs increased. The Phenylalanine will try to avoid being solvated, or avoid polar binding partners of Asp135. Therefore it will interact with the adjacent Trp134, fold inwards or bind with an antibody. The first 2 options will lead to a conformational change that possibly changes the location of loop 13, thereby exposing loop 5.

Discussion

Until recently, little was known about the location of epitopes on P.69 Prn that are recognized by human antibodies. Previously, we identified several linear epitopes recognized by human serum antibodies and mAbs. The location of antibodies recognizing conformational epitopes remained unclear. Here we describe the location of several conformational epitopes recognized by mAbs that compete with human serum antibodies (mAbs PeM1, 5, 6, 7, 21, 29, 38, 64, 80, 84, 85). Thus these epitopes are also recognized by human antibodies or located proximate to epitopes to which human antibodies bind.

A three pronged approach was used to identify conformational epitopes involving site-directed mutagenesis, deletion mapping and competition assays. Site-directed mutagenesis was focused on regions of P.69 predicted to form loops according to the crystal structure [33]. We observed that modification of most loops (11 out of 15) resulted in a loss or decrease in binding of mAbs. These results are consistent with in silico analyses with MUTQUA which predicted that most substitutions in the loops would affect their conformation. We identified a region of P.69 Prn important for its overall conformation. Deletion of this region abolished binding of all antibodies, except those binding to non-conformational epitopes. This segment of P.69 Prn is similar in amino acid sequence to a region found in other autotransporter proteins that has been shown to be important in protein folding [46].

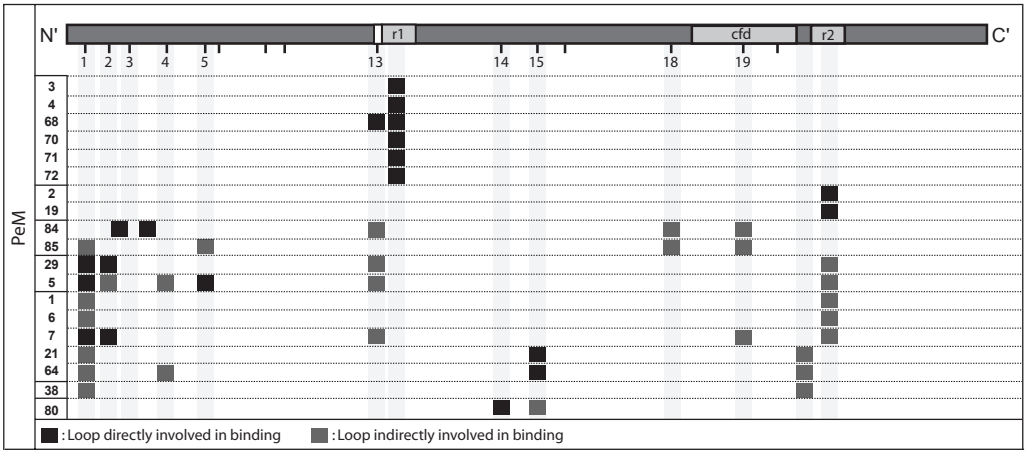

Figure 4. Location of epitopes recognized by the mAbs described in this report. Based on Pepscan, SDM, deletion mutant, and competition data, we suggest these epitopes for the mAbs.

In Fig. 4, the interpretation of the results of the three approaches is indicated. Linear epitopes were mapped previously using peptides and these results were confirmed and refined in this study. All of the mAbs recognizing linear epitopes, bound to region 1 (mAbs PeM3, 4, 68, 70, 71 and 72) or to region 2 (mAbs PeM 2 and 19). In addition, our approach allowed us to identify regions of P.69 Prn which are part of conformational epitopes. MAb PeM84 bound to peptides located in between loops 2 and 4, region 1 and the C-terminus. PeM85 showed a similar binding pattern, suggesting it recognizes a similar epitope. PeM29

clearly bound to loop1, which was already shown by Pepscan analysis. Furthermore, our results suggested that the epitope of PeM29 also compromised loop 2, 13 and region 2 (Fig. 5). MAb PeM5 bound a similar epitope as PeM29, comprising loop 5 and possibly loops 1, 2, 4, 13 and region 2 (Fig. 5). A large group of mAbs, PeM1, 6, 7, 21 and 64 recognized loops located in the N-and C-terminal region of P.69 Prn (Fig. 5). MAb PeM38 possibly recognized a conformational epitope comprising both C-and N-terminal peptides. MAb PeM80 recognized a conformational epitope comprised of loop 14 and possibly also loop15 (Fig. 5). Our data indicated an important role of the N-terminus in immune recognition. Deletion of 19 residues from the N-terminus affected binding of all 11 mAbs that recognized a conformational epitope. Further, the epitopes of 4 of these mAbs were shown to comprise residues located in the N-terminus.

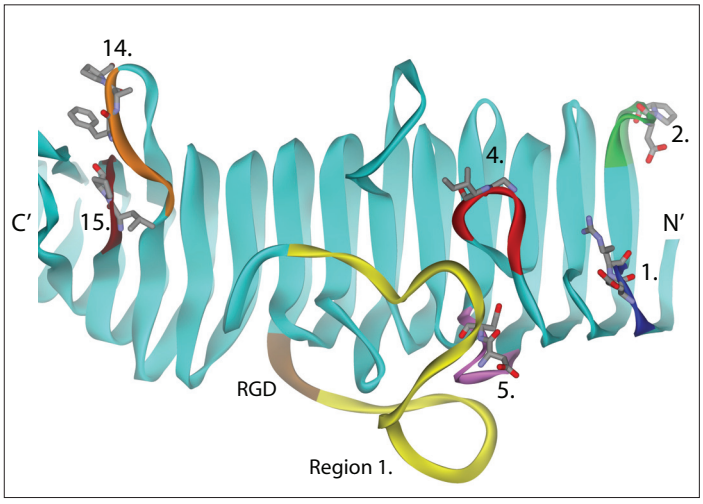

Figure 5. Part of the crystal structure of P.69 Prn 33. Loops that are recognized by mAbs are indicated. Loops 1 and 2: PeM29, loops 2 and 1: PeM7, loops 1, 4 and 5: PeM5, loops 14 and 15: PeM80.

Interestingly, in several cases, the modification of loops resulted in an increase in binding of mAbs compared to P.69 Prn. It is conceivable that the introduction of mutations in P.69 Prn loops resulted in an increased exposition of epitopes that are normally masked by adjacent loops. The fact that mutations in the non-exposed loops 5, 9, and 15 changed the binding profiles, strongly supports this line of reasoning (Fig. 6). An analogous mechanism for the HIV envelope protein gp120 was described by Koch *et al.* and Kang *et al.* They recently reported that after mutation of glycosylation sites in variable loops of the HIV envelope proteins, several neutralizing antibodies showed an enhanced binding [48,49]. They suggested that, as a result of these mutations or deletions, neutralizing epitopes were more exposed. These mutations removed glycoslylation sites and hence their results are probably due to the absence of sugar chains which mask epitopes. We cannot exclude that a similar mechanism occurs in *B. pertussis*, although glycosylation is not a general phenomenon in bacteria. More likely, the mutations introduced in P.69 Prn affected the conformation of the loop, thereby exposing neighboring epitopes that were previously hidden. The observed increase in binding of mAbs could also be an effect of an improved lock-key fit, resulting in an increased affinity of the mAb for its epitope. However, the affinities of mAbs for native and SDM P.69 Prn were compared, and no differences were observed (data not shown).

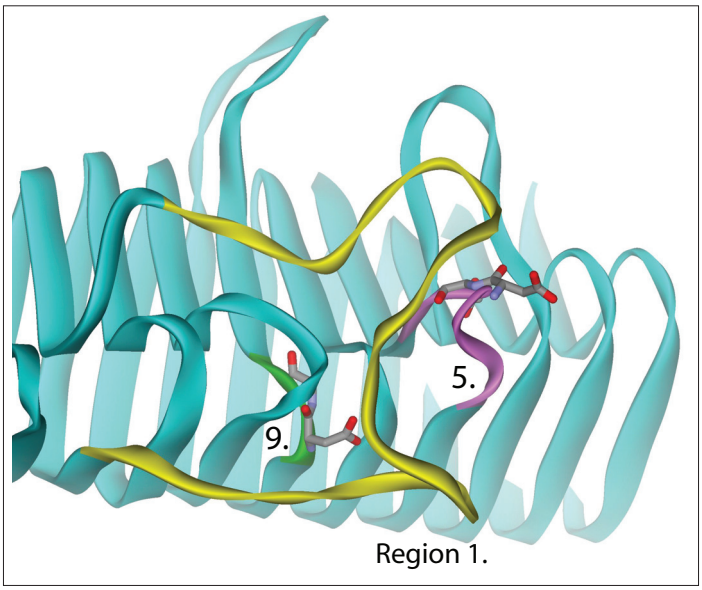

Figure 6. Location of non-exposed loops 5 and 9 and the exposed region 1 loop.

To investigate the role of variation in loops on immune evasion, an alignment of the P.69 Prn amino acid sequences of *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* was performed to locate polymorphic sites. Of the loops that, after mutation by SDM, showed mainly a decrease in binding with mAbs, 67% (4 out of 6 loops) was polymorphic. In contrast, 75% (3 out of 4 loops) of the loops that mainly showed an increase in binding after mutation by SDM were conserved. We suggest that there is a delicate balance between variation and conservation to evade antibody binding. Good examples for this hypothesis are loops 18 and 19. Although both are exposed, loop 18 is conserved while loop 19 is polymorphic. After mutation of the conserved loop 18, the binding of 2 mAbs decreased but the binding of 6 mAbs increased. The net effect of this mutation would result in more antibody binding, possibly explaining why this loop is conserved. Conversely, when the polymorphic loop 19 was mutated, the binding of 2 mAbs increased, whereas the binding of 5 mAbs decreased, thus the net effect is that fewer antibodies can bind. Another example is mutation of loop 13. This mutation resulted in a decrease and increase of binding of 6 and 3 mAbs respectively. Mutation of this site would be beneficial as it decreases the number of antibodies binding to P.69 Prn. However this mutation alters the RGD site, implicated in binding of Prn to host cells. Mutation of this site would result in a loss of function of Prn, thus there is a strong selective pressure to conserve this sequence. While the RGD sequence is conserved, the adjoining region 1 is hyper variable. Deletion of region 1 decreased the binding of 5 mAbs. Together the modifications of loop 13 (comprised of the RGD sequence and region 1) account for a decrease in binding of 53% (10 out of 19) of the mAbs. Deletion of region 2 also decreased the binding of 10 out of 19 mAbs. Taken together, the modifications of loop 13 and region 2 affected the binding of 90% (17 out of 19) of the mAbs tested. In this and previous work, we provided evidence that region 1 and 2, are proximate in the folded protein comprising a single epitope. Variation of this epitope results in a highly effective evasion of antibody binding.

In previous studies in mice, it was shown that *B. pertussis* Prn does not protect against infection with *B. parapertussis* [37,50]. An alignment of *B. pertussis* and *B. parapertussis* P.69 Prn revealed that the loops comprised 79% of the accessible residues and that 78% of all polymorphisms are located in loops. Several of these polymorphisms were found in loops that were shown in this work to be recognized by mAbs. This indicates that the loops play an important role in immune evasion.

To confirm the data obtained with site-directed mutagenesis and the deletion mapping, we studied the ability of the mAbs to compete for binding to P.69 Prn with the Biacore. Due to the relatively low affinity of several mAbs (PeM68, 70 and 71) no consistent binding to P.69 Prn was detected in the Biacore. Therefore these mAbs were excluded from competition experiments. As expected, inhibition was always observed between identical mAbs. In several cases we observed non-reciprocal inhibition between mAbs that were shown by SDM and P.69 Prn deletion mutant analysis to bind to different regions on P.69 Prn. In a number of cases, competition between mAbs binding to either the N-terminus (containing region 1) with the C-terminus (containing region 2) was observed. These data, together with the SDM and deletion mapping results, indicate a possible interaction of loops in the N- and C-terminus. Recently, Seifert *et al.*, Rhodin *et al.* and Dolleweerd *et al.* reported that several mAbs directed to the *Streptococcus mutans* adhesin P1, recognize a discontinuous epitope comprising an N-terminal alanine-rich region (A-region) and a C-terminal proline-rich repeat region (P-region) [51-53]. Similar to our findings with P.69 Prn, they observed that the binding of several anti P1 mAbs depends on the simultaneous presence of both the A and P region, thus suggesting that these domains comprise a single epitope. Since one of the mAbs directed against the A- and P-region of P1 , designated Guy's 13, provides protection against infection, Dolleweerd *et al.* suggested that this mAb holds the P1 protein in a conformation which prevents the adhesin activity of P1 [51]. This suggests that the interaction of the A- and P-region serves as an immune deflector to keep the active site, hidden from the immune system until it bind to its receptor.

We propose a similar hypothesis for P.69 Prn. Both region 1 and 2 are comprised of repeats. Such regions are known to create polymorphisms at a much higher rate than can be achieved by point mutations [54] and have been shown to be important for immune escape [55]. Our results suggest that that region 1 and 2 are proximate and may comprise a single conformational epitope. We propose that this domain has evolved as an immunodominant, polymorphic, site that surrounds the functional domain of P.69 Prn, the RGD site, and is able to create polymorphisms at high frequency allowing the bacteria to escape from immunity. Although variation in repeat units may not affect the binding of antibodies that recognize linear epitopes, such antibodies may be less effective due to a lower affinity. Consistent with this assumption, we observed that the affinity of antibodies binding to linear epitopes in the variable region 1 (average affinity of 2.3 M) was somewhat lower (P=0.096) then antibodies binding to conformational epitopes (average affinity of 2.7 M), which supports this hypothesis. Our results also provide evidence for an indirect role of

loops in immune escape through the masking of important epitopes.
To our knowledge this is the one of the first reports that describes a complex role of loops of a bacterial adhesin in evading immune responses. These results facilitate the further improvements for bacterial vaccine design by directing the immune response away from the "decoy" epitopes towards the protective conformational epitopes.

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5 Mimicry of a discontinuous epitope of *Bordetella pertussis* P.69 Pertactin using a selectively addressable synthetic scaffold

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Abstract

Bordetella pertussis, the etiological agent of whooping cough, is re-emerging in several countries with a traditionally high vaccine uptake. In *B. pertussis* strains associated with re-emergence, polymorphisms were found in several proteins, including P.69 Pertactin (P.69 Prn). In a previous study we identified a discontinuous conformational epitope on P.69 Prn that is recognized by a mouse monoclonal antibody (mAb) PeM29. Human antibodies recognized a similar epitope, suggesting it may be a potential vaccine target. In this report we employed a selectively addressable synthetic TAC-scaffold, for the mimicry of the P.69 discontinuous conformational epitope by confined presentation of several different peptide arms. The X-crystal structure of P.69 Prn was used in relatively straightforward modeling to position the peptides on the TAC-scaffold in order to mimic the epitope recognized by PeM29. The design, synthesis, immunogenicity and protective properties of the resulting peptide containing TAC (PEPTAC) constructs are described. We show that this approach yielded protective antibodies that were not induced during immunization with native P.69 Prn. This approach could be employed to design new synthetic vaccines that induce functional antibodies which are not induced during natural infection.

Introduction

In the pre-vaccination era, pertussis was a major cause of infant death throughout the world. Introduction of effective pertussis vaccines 50 years ago led to dramatic reductions in morbidity and mortality. However, there has been a resurgence in the incidence of pertussis in the last ten years in several countries despite high vaccine uptake [1,2]. Waning immunity in adolescents and adults, increased reporting, improved diagnosis of the disease, and *B. pertussis* strain adaptation are all proposed explanations for the re-emergence of pertussis [2]. In the Netherlands, strain adaptation probably played an important role in the resurgence of pertussis [2-4]. Analysis of clinical isolates collected in the last 50 years revealed antigenic divergence between vaccine strains and circulating strains. The *B. pertussis* variants showed polymorphism in at least two proteins implicated in protective immunity: P.69 Pertactin (P.69 Prn) and pertussis toxin (Ptx) [2,3,5]. Several lines of evidence indicate an important role for P.69 Prn in immunity. A number of studies in animals have indicated that P.69 Prn can elicit protective antibodies (Abs) [6-9]. The level of Abs to both P.69 Prn and pertussis toxin (Ptx) have been shown to correlate with clinical protection [6,7]. Further, acellular vaccines (ACV's) containing Ptx, filamentous hemagglutinin (FHA) and P.69 Prn were more effective compared to ACV's containing Ptx and FHA only, also implicating an important role for P.69 Prn in immunity [10-12]. Finally, variation in P.69 Prn was shown to affect the efficacy of the Dutch whole cell vaccine in a mouse model [8].

P.69 Prn, the focus of this study, belongs to a class of so-called autotransporter proteins that undergo autoproteolytic processing [13]. P.69 Prn is processed from a 93 kDa large precursor to a 69 kDa and 22 kDa protein which are located at the cell-surface and in the outer membrane, respectively [14]. The 69 kDa product (referred to as P.69 Prn) is used in ACV's. P.69 Prn contains an Arg-Gly-Asp (RGD) motif implicated in ligand-receptor interactions in eukaryotes. It has been shown that this motif is involved in P.69 Prn-mediated attachment of *B.pertussis* to mammalian cells [15,16]. P.69 Prn is polymorphic, and 13 variants (P.69 Prn1 – P.69 Prn13) have been identified so far. Variation is mainly limited to two regions, designated region 1 and 2, which are comprised of Gly-Gly-X-X-Pro (r1 repeat) and Pro-Gln-Pro (r2 repeat) repeats, respectively. Most variation is found in region 1 which is located proximal to the N-terminus and flanking the RGD sequence. Region 2 is located more towards the C-terminus. Results presented by He et al., suggest that the r1 repeat induces type-specific Abs which show little cross-reactivity between P.69 Prn1 and P.69 Prn2 [17].

Acceptance of pertussis vaccination has been hampered by the reactogenicity of whole cell vaccines. For this reason, many countries have switched to the less reactogenic ACV's. The pertussis ACV's contain 3 to 5 purified proteins and have fewer side effects, but provide less protection against the second causative agent of pertussis, *Bordetella parapertussis*. Current ACV's contain P.69 Prn1, whereas most clinical isolates produce P.69 Prn2 or P.69 Prn3.

Ideally, a vaccine in general should provide a broad protection against strain variants, induce no, or few, side effects and provide long term immunity. The efficacy of vaccines against bacterial respiratory pathogens depends on the induction of broadly protective, high

affinity, neutralizing antibody responses and the induction of T-cell responses [18]. Due to the tertiary and quaternary structure of a protein, Abs are often induced against discontinuous epitopes. This has clearly been shown for Abs directed against the HIV-1 glycoproteins gp120 and gp41 [19-26]. In several studies, Abs that were protective or had neutralizing or blocking properties, were shown to be directed against conformation dependant epitopes, and the avidity of antibody binding has been shown to correlate with their neutralizing capacity [27-32].

Synthetic peptides represent convenient chemical entities that can be rapidly synthesized in clinical grade for therapeutic applications, and due to their purity, induce no or limited side effects. Mimicry of discontinuous epitopes is the major obstacle in the design of synthetic peptide vaccines. To our knowledge, the design of assemblies of peptides that mimic the native three-dimensional structure of discontinuous conformational B-cell epitopes, against which protective Abs are often induced, has not been realized so far.

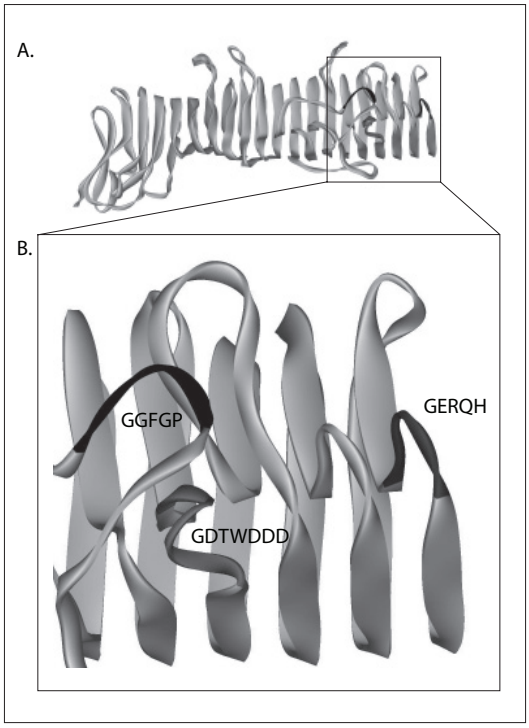


Figure 1. (A) Crystal structure of P.69 Prn1. (B) Enlargement of the crystal structure of P.69 Prn. The three peptides implicated in binding of PeM29 are indicated.

In a previous study we employed a Pepscan to identify the location of epitopes recognized by anti-Prn mouse monoclonal antibodies (mAbs) [33]. One of the mAbs, PeM29, showed moderate binding to several linear peptides, suggesting that they were part of a discontinuous conformational epitope. PeM29 bound the N-terminal peptides; GERQH (AA 11-15), GDTWDDD (AA 97-103) and GGFGP (AA 247-251) (Fig. 1). Analysis of the crystal structure of P.69 Prn [34] revealed that although these peptides are far apart in the primary structure, they are rather close in the folded protein. Peptides GGFGP/GDTWDDD and GDTWDDD/GERQH are located approximately 3.5Å and 12Å apart, respectively. However, all 3 peptides are located in an 8Å radius of each other. We previously showed that human Abs recognized a similar epitope as PeM29, thus this epitope may represent a potential vaccine target [33]. In this report we employed a selectively addressable synthetic TriAzaCyclophane-scaffold (TAC-scaffold), for the mimicry of a discontinuous conformational epitope by confined presentation of several different peptide arms. The design, synthesis and immunogenicity of the resulting peptide containing TAC constructs

(PEPTAC's) are described. Furthermore, the protective properties of antibodies induced by immunization with this peptide containing TAC-scaffold that mimics a discontinuous conformational epitope of P.69 Prn is described and discussed.

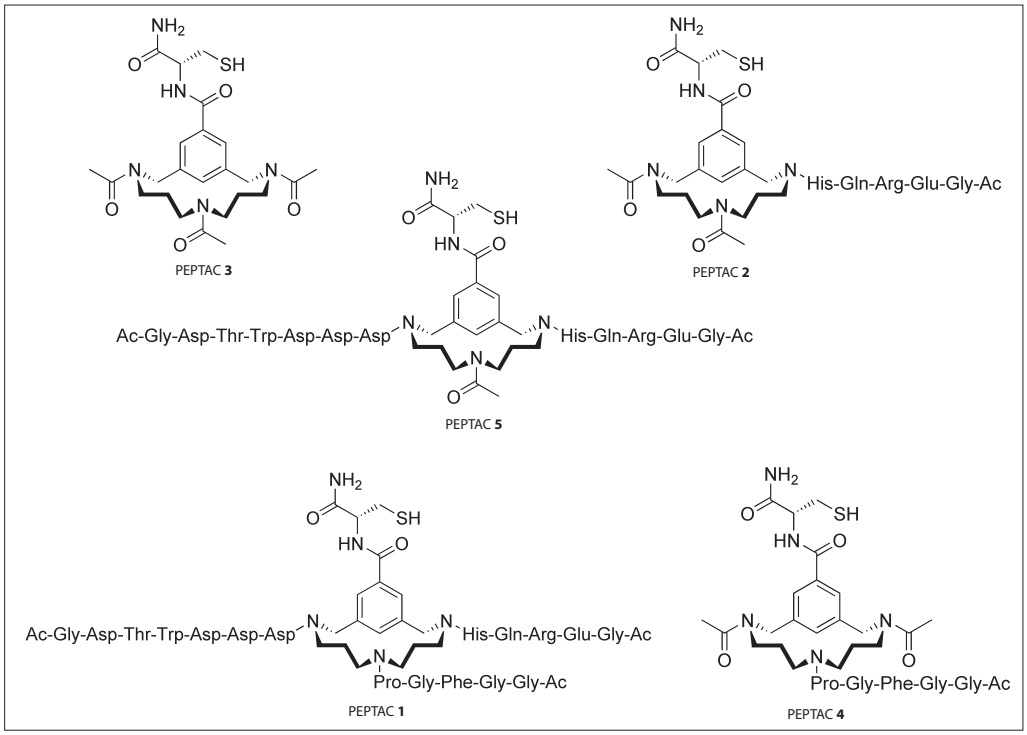


Figure 2. Schematic representation of PEPTAC's used in this study.

Table 1. Overview of the peptides present on the PEPTAC's and their respective locations on the PEPTAC.

	Peptide present on position:		
Name	Fmoc	o-NBS	Aloc
PEPTAC 1	GERQH	GDTWDDD	GGFGP
PEPTAC 2	GERQH	-	-
PEPTAC 3	-	-	-
PEPTAC 4	-	-	GGFGP
PEPTAC 5	GERQH	GDTWDDD	-

Materials and Methods

General

Synthesis of the PEPTAC molecular constructs was carried out manually in syringes containing a frit. Analytical HPLC runs were performed on a Shimadzu HPLC system (detection at 220 nm). Preparative HPLC runs (detection at 220 nm) were performed on a Gilson workstation (Middleton, Wisconsin, USA). For analysis and purification Adsorbosphere XL C8, 90 Å columns were used and appropriate gradients of water to water/acetonitrile buffers containing 0.1% TFA. ESI-MS was carried out on a Shimadzu QP-8000 LCMS spectrometer. MALDI-TOF spectra were recorded on a Kratos CFR spectrometer where CHCA was used as a matrix and ACTH 18-39 as internal standard. Modelling studies were performed on a SGI O2 workstation with MacroModel version 7 [35]. Solid phase resin, chemicals and solvents were obtained commercially and used without further purification.

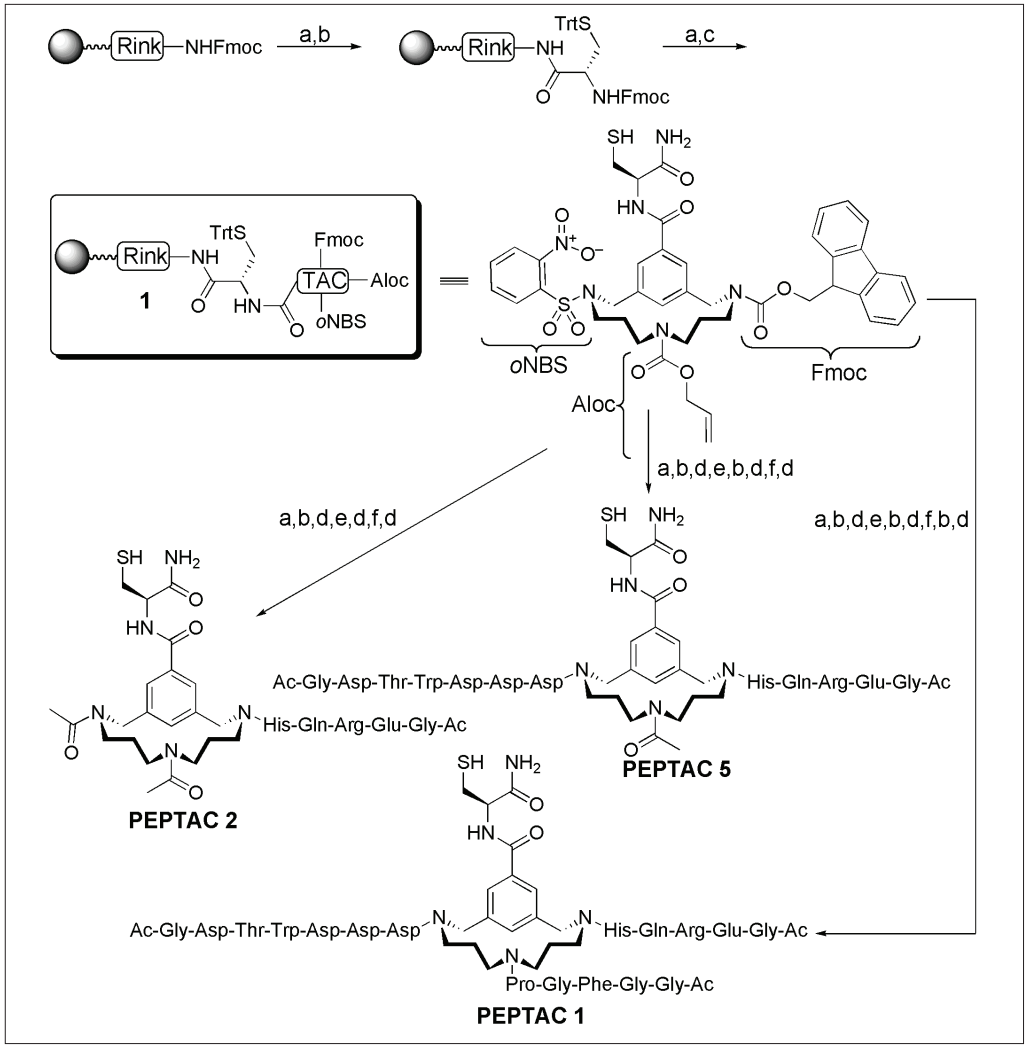
Synthesis of PEPTAC's 1 - 5

The TAC-scaffold was prepared as described earlier [36,37]. Selective and subsequent deprotection of the semi-orthogonal protecting groups was carried out as described earlier [36-38]. Fmoc cleavage was achieved with 20% piperidine in NMP. *o*-NBS cleavage was carried out using a mixture of DBU and mercaptoethanol in DMF. Removal of the Aloc group was effected with Tetrakis-(triphenylphosphine) palladium and anilinium p-toluenesulfinate in NMP. The molecular constructs containing a cystein residue necessary for conjugation to tetanus toxoid (i.e. PEPTAC 1 - 5) were prepared by coupling of FmocCys(Trt)-OH to Fmoc-deprotected ArgoGel™ Rink resin followed by attachment of the orthogonally protected TAC-scaffold (Scheme 1).

PEPTAC 1

Starting from solid phase attached cysteine-TAC, the required peptide arms were introduced onto the TAC-scaffold using appropriate Fmoc amino acids by BOP-coupling.

The peptide arms were assembled subsequently, essentially as was the case for the syntheses of dipeptides on the TAC-scaffold as was described earlier [38]. The first peptide arm was assembled after cleavage of the Fmoc-group on the TAC-scaffold (see above) and upon completion capped with an acetyl group. After removal of the *o*-NBS group, the second peptide arm was assembled concluded by capping. Assembly of the third peptide followed Aloc-removal and its solid phase synthesis was ended by capping. Crude PEPTAC 1 was obtained after cleavage and simultaneous deprotection using a TFA/TIS/H_2O/EDT mixture followed by precipitation (twice) from MBTA/n-hexane. Then, the precipitate was lyophilized from water. Pure PEPTAC 1 was obtained after preparative HPLC. The fractions were analyzed by analytical HPLC and fractions containing a pure product which had the correct masses were pooled and lyophilized to yield PEPTAC 1 (40 mg, 27%). MALDI-TOF: $[M]^+$ found 2333.92, calculated 2333.41.



Scheme 1. Synthesis of PEPTAC 1, 2 and 5. a). 20% Piperdine, NMP; b). FMOC-AA-OH, BOP, DiPEA, NMP; c). TAC-scaffold, BOP, DiPEA; d). Ac2O, DiPEA, HOBt; e). HSCH2CH2OH, DBU, DMF; f). Pd(PPh3)4, Phenylsilane, NMP; g). TFA/TIS/H2O/EDT (94.5/1/2.5/2.5); Fmoc = 9H-fluoren-9-ylmethyloxycarbonyl; oNBS = nitrobenzene sulfonamide; Aloc = Allyloxycarbonyl; Ac = Acetyl; BOP = (benzotriazol-1-yloxy)-tris(dimethyl-amino)-phosphonium hexafluorophospate); TFA = trifluoroacetic acid; TIS = triisopropylsilane; EDT = ethanedithiol.

PEPTAC 2

According to the above procedure for PEPTAC 1, only the first peptide arm was assembled on the solid phase attached cysteine-TAC-scaffold. Instead of a peptide arm on the second and third position, the positions were acetylated. Simultaneous cleavage and deprotection gave after preparative HPLC pure PEPTAC 2 (30 mg, 73%). MALDI-TOF: $[M]^+$ found 1113.78, calculated 1113.25.

PEPTAC 3

For the preparation of PEPTAC 3 solid phase attached cysteine-TAC(tri-*o*-NBS)$_3$ was used [39]. All three *o*-NBS protecting groups were removed by DBU mercaptoethanol followed by acetylation. After cleavage from the resin, precipitation, lyophilizing and purification, PEPTAC 3 (24 mg, 97%) was obtained. ESI-MS: $[M+H]^+$ found 506.50, calculated 506.63. The purity was verified by analytical HPLC.

PEPTAC 4

According to the above procedure for PEPTAC 1, the Fmoc and *o*-NBS protecting groups were removed subsequently and the thus liberated secondary amines were acetylated. Palladium effected removal of the Aloc group was followed by introduction of the third peptide arm as is present in PEPTAC 1. Then, the synthesis of resin bound PEPTAC 4 was completed by acetylation. TFA/TIS/H_2O/EDT induced cleavage and deprotection led, after purification, to PEPTAC 4 (38 mg, 83%). ESI-MS: $[M+H]^+$ found 921.65, calculated 921.07, $[M+Na]^+$ found 944.50 calculated 944.07.

PEPTAC 5

The synthesis of this molecular construct was carried out analogous to the synthesis of PEPTAC 1, except for assembly of the third arm. At this position and acetyl group was introduced. Cleavage, deprotection, precipitation and purification by preparative HPLC gave PEPTAC 5 (29 mg, 43%). MALDI-TOF: $[M]^+$ found 1918.46, calculated 1917.97.

Coupling of PEPTAC's to tetanus toxoid

The cysteinyl-containing PEPTAC's (3-5 mg) were dissolved in a freshly prepared solution of bromoacetylated tetanus toxoid [40] (1.2 – 2.0 ml, protein content 2.1 mg/ml) in 0.1 M sodium phosphate + 5 mM EDTA, pH 6.0, and left to stand at room temperature for 16 h. Each mixture was transferred into an Amicon Ultra-15 centrifugal filter device (15 ml, 10 K MWCO), diluted with 13 ml PBS, pH 7.2, and centrifuged at room temperature for 30 min. at 3000 g. The concentrate (≈ 250 µl) was diluted with 15 ml PBS and centrifuged again. The final concentrate was removed from the filter device, diluted with PBS to a protein content of ≈ 1 mg/ml, and stored at 4°C until further use

Mouse experiments

To investigate the protective properties of the PEPTAC's, and to raise Abs, mice were immunized and subsequently challenged with 2 *B. pertussis* strains. To determine if there was a difference in protection after immunization against Prn1 and Prn2 strains, mice were challenged with a mixture of 2 isogenic Prn1 and Prn2 strains.

Immunization scheme
Mice were immunized 3 times with either unconjugated or TT-conjugated PEPTAC's with a 14 day interval. Per immunization, a total of 50µg of PEPTAC or purified P.69 Prn1 mixed with 20µg of the adjuvant QuilA (Spikoside)(Iscotec AB, Stockholm, Sweden) was administered subcutaneously.

In vivo challenge
Fourteen days after the last immunization, mice were intranasally challenged with a mixture of 4.0 X 10^7 colony forming units (CFU's) containing two isogenic *B. pertussis* strains harboring *prn1* and *prn2*, respectively. To be able to discriminate between the 2 strains, a kanamycin and gentamycin resistance gene was inserted into the *fim3* gene of *B. pertussis* Prn1 and *B. pertussis* Prn2, respectively. Three days after the challenge, mice were sacrificed and the blood, trachea and lungs were collected. The number of CFU's were counted on plates with kanamycin or streptomycin as described previously [8].

Calculations
The Log protection values were calculated by subtracting the Log10 CFU of immunized mice from the mean Log10 CFU of non-immunized mice. The statistical significance of differences between two groups was assessed by the Student t test (2-sided). *P* values below 0.05 were regarded as statistically significant.

Antibody depletion
To determine the specificity of the humoral response, mouse sera were depleted using synthetic peptides with identical sequences as peptides on the scaffold. Linear peptides used for depletion comprised the sequences GERQH (pep1), GDTWDDD (pep2) and GGFGP (pep3). Sera were also depleted with the PEPTAC's 1-5. Sera were mixed in a 1:1 ratio with PBS containing 1mg/ml of peptide or PEPTAC's. Sera were depleted overnight at 4ºC after which the samples were centrifuged at 4ºC for 15 minutes. Supernatants were used for ELISA and Biacore experiments.

Analysis of antibody binding using ELISA
Binding of mAbs and mouse sera to the PEPTAC's or purified P.69 Prn1, was measured by ELISA. Polystyrene 96-well ELISA plates (Immulon II; Dynatech, Chantilly, Va.) were coated overnight at 22°C by adding 100 µl of PBS containing 2 µg/ml of peptide or protein to each well. When the conjugated PEPTAC 1 was used as a coating antigen to determine the antibody titer, the response doubled, indicating that also a large amount of Abs was induced against TT (data not shown). To prevent interference of the anti-TT Abs, unconjugated PEPTAC's were used in all assays.
The plates were washed four times with 200 µl of PBS supplemented with 0.05% Tween 20 (PBST) per well by using a Titertek Plus M96V washer (ICN, Irvine, Calif.). Murine

mAbs and sera, diluted in PBST, were added to the wells and were incubated for 2 h at 37 °C followed by four washings as described above. Bound Abs were detected by using horseradish peroxidase-conjugated anti-mouse total IgG (Cappel, Organon Technica, Boxtel, the Netherlands). The optical density at 450 nm (OD450) was measured with a plate reader (EL312e; BioTek Systems, Winooski, Vt.). The dilution at half of the maximum OD_{450} was used as the ELISA titer

Analysis of antibody binding to PEPTAC's using surface plasmon resonance

Binding of mAbs and mouse sera to the PEPTAC's was also studied on a Biacore 1000 instrument. PEPTAC's were coupled to a carboxymethyl dextran sensor chip CM5 using N-ethyl-N'-(dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) chemistry with the Thiol coupling kit (Pharmacia Biacore) to activate the CM5 dextran. After activation, 20µl of PDEA was injected and subsequently, 35µl of the cysteine containing PEPTAC 1-5, 0.5mg/ml in 0.1M NaAc, 1M NaCl, pH4.0, were injected during 7 minutes. Unreacted groups were blocked by injection of 20µl of L-cysteine during 4 minutes. The binding assays were performed at a constant flow rate of 5µl/min at 25°C. MAbs and sera were diluted 1:100 in HBS-EP buffer (10 mM HEPES (pH 7.4) containing 150 mM NaCl, 3 mM EDTA and 0.005 % surfactant P20). Antibodies were allowed to interact with the immobilized PEPTAC's for two minutes after which the dissociation was followed for another minute. Residual bound antibody was removed by washing the chip for three minutes with 10mM glycine-HCl (pH 1.5) or 20mM NaOH + 1M NaCl solution.

Results

In a previous study we used a Pepscan analysis to identify epitopes on P.69 Prn that were recognized by mAbs raised against the native protein [33]. One of mAbs tested, PeM29, showed binding to 3 N-terminally located peptides, suggesting these are part of a discontinuous conformational epitope. The peptides recognized by PeM29 are located on 2 small and 1 large loop that protrude from the β-helix backbone. Two of these loops (GGFGP and GDTWDDD) are in close proximity (±3.5Å), whereas the third loop (GERQH) is located further towards the N-terminus. All of the peptides recognized by PeM29 are in located in an 8Å radius of each other. Using molecular modeling we attempted to reconstruct the epitope recognized by PeM29.

Design and Synthesis of PEPTAC's

Our recently developed TAC-scaffold enables the selective, sequential introduction of three peptide sequences using semi-orthogonal protecting groups (Scheme 1, 1) [36,38]. In principle even four positions are available, but in this work the fourth position was reserved for the introduction of a cysteine residue, thereby enabling the selective attachment of tetanus toxin. Six different peptide containing TAC-scaffold isomeric molecular constructs were possible upon attachment of three peptides. These corresponded to the three peptides that were part of a discontinuous epitope of P.69 Prn. The PEPTAC isomers where constructed in the molecular modeling package MacroModel using the X-ray structure of P.69 Prn (1DAB.pdb)[34,41]. Pertinent epitopes where dissected in MacroModel and attached to the TAC-scaffold leading the six isomeric PEPTAC's followed by energy minimization (PRG gradient, MMFF force field in water). The resulting minimized PEPTAC's were superimposed on the crystal structure of P.69 Prn. From this, it was concluded that a promising fit with the parent Prn was obtained with a PEPTAC containing peptide GERQH attached to position protected with an Fmoc-group, peptide GDTWDDD on the position occupied by the *o*-NBS group and peptide GGFGP on the Aloc protected site.

This PEPTAC (1) was assembled by solid phase synthesis starting from Rink amide resin. A cysteine residue was introduced first followed by attachment of the TAC-Scaffold. The Fmoc on the TAC-scaffold was removed by piperidine followed by synthesis of peptide arm GERQH using appropriately side chain protected Fmoc-amino acids. Upon completion of the sequence the peptide was capped leading to the N-acylated sequence. Then, selective removal of the *o*-NBS group by thiolysis was followed by synthesis of the GDTWDDD sequence and after capping to the N-acetylated peptide, the resin bound molecular construct was ready for deprotection of the third position by palladium(0). Peptide GGFGP was now synthesized on this position and after capping PEPTAC 1 was cleaved and deprotected simultaneously.

In addition, several biomolecular constructs were prepared to evaluate the influence of the scaffold in the mimicry of the discontinuous epitopes as well as contribution of each of the three peptides attached the TAC-scaffold. Furthermore, the immunological responses to

free, non scaffolded, peptides was investigated.
Acetylated TAC-scaffold was prepared by deprotection of resin bound Cys-TAC(*o*-NBS)$_3$ followed by acetylation leading to Cys-TAC(Ac)$_3$. This construct was used to determine the immune response caused solely by the TAC-scaffold.

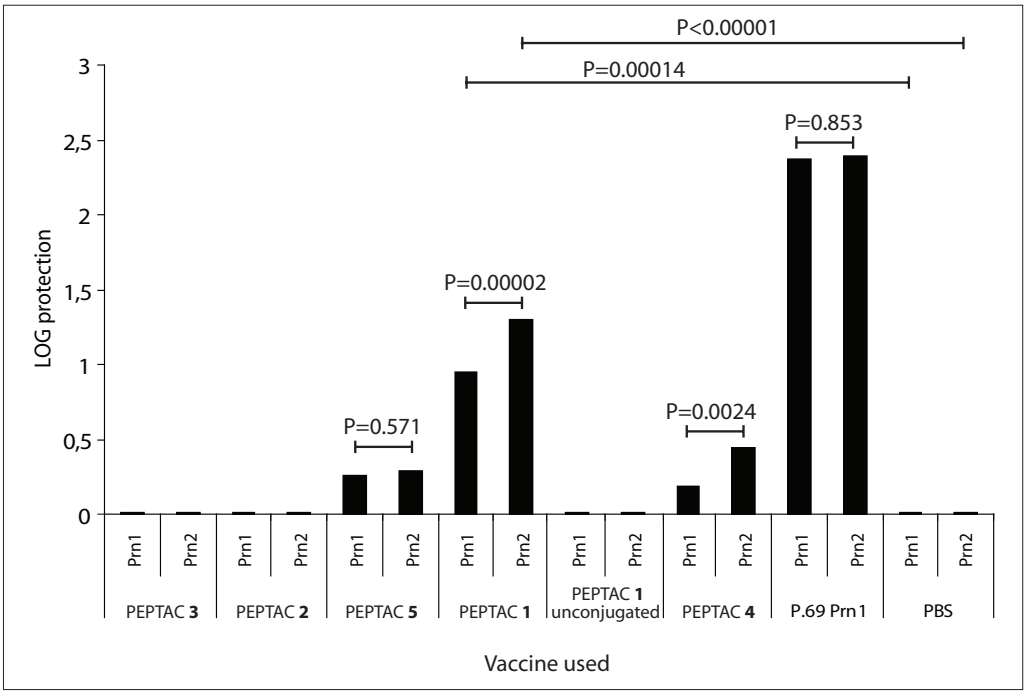


Figure 3. Log protection of mice challenged with a mixture of 2 B. pertussis strains. Mice were vaccinated with PBS, P.69 Prn1 or PEPTAC constructs and challenged with a mixture of two isogenic strains producing P.69 Prn1 and P.69 Prn2, respectively. The amount of protection against strains was calculated by subtracting the Log10 CFU of each unvaccinated mice by the average of Log10 CFU of vaccinated mice. Statistical significance of differences observed between isogenic strains was calculated using a paired T-Test. Statistical significance of differences between vaccines was assessed by the unpaired T-Test. The protection was compared between P.69 Prn1 and P.69 Prn2 strains from the vaccinated and unvaccinated groups. Mice vaccinated with PEPTAC 5 were not significantly protected against P.69 Prn1 or 2 strains compared to the PBS group (P=0.096 and P=0.088 respectively). Mice vaccinated with PEPTAC 1 were significantly protected against P.69 Prn1 or 2 strains compared to the PBS group (P=0.00014 and P<0.00001 respectively). Mice vaccinated with PEPTAC 4 were significantly protected against P.69 Prn1 or 2 strains compared to the PBS group (P=0.001 and P=0.001 respectively). Mice vaccinated with P.69 Prn1 were significantly protected against P.69 Prn1 or 2 strains compared to the PBS group (P=1.5E-06 and P=1.4E-06 respectively). Abbreviations used: PEPTAC 3: Cys-TAC-3Ac, PEPTAC 2: Cys-TAC-HQREG-2Ac, PEPTAC 5: Cys-TAC-HQREG-DDDWTDG-Ac, PEPTAC 1: Cys-TAC-HQREG-DDDWTDG-PGFGG, PEPTAC 4: Cys-TAC-PGFGG-2Ac, Prn1: Strain producing P.69 Prn1, Prn2: strain producing P.69 Prn2.

Molecular constructs PEPTAC 2 and 4, each possessing one peptide arm were prepared analogously to PEPTAC 1. However, instead of introducing all three arms, for PEPTAC 2, assembly of the first peptide onto the TAC-scaffold was followed by deprotection and acetylation steps of the *o*-NBS and Aloc-position leading to Cys-TAC-(Ac)$_2$-GERQH. For PEPTAC 4 deprotection and acetylation of the Fmoc and *o*-NBS protecting group,

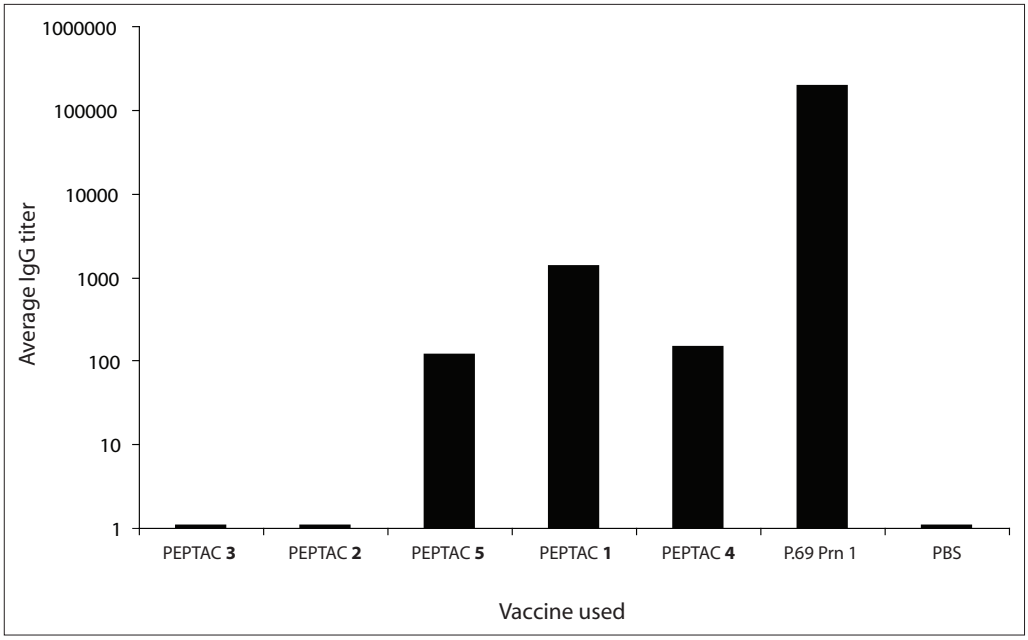


Figure 4. Serum titer of anti-P.69 antibodies in mice vaccinated with PEPTAC constructs. Abbreviations used: Abbreviatons used: PEPTAC 3: Cys-TAC-3Ac, PEPTAC 2: Cys-TAC-HQREG-2Ac, PEPTAC 5: Cys-TAC-HQREG-DDDWTDG-Ac, PEPTAC 1: Cys-TAC-HQREG-DDDWTDG-PGFGG, PEPTAC 4: Cys-TAC-PGFGG-2Ac.

followed by deprotection of the Aloc position and peptide GGFGP was constructed yielding Cys-TAC-$(Ac)_2$GGFGP. The two peptide arm construct PEPTAC 5 was also synthesized analogously to PEPTAC 1, now halting the introduction of the third peptide and acetylating this position and Cys-TAC-(Ac)-GERQH-GDTWDDD was obtained. PEPTAC 3 was obtained after deprotection of the three *o*-NBS groups followed by acetylation.
Finally, the "free" non scaffolded peptides GERQH, GDTWDDD and GGFGP were synthesized to pertinently demonstrate the necessity of scaffolding discontinuous peptide epitopes as opposed to their mere combined solution.

PeM29 does not recognize PEPTAC 1

A total of 5 PEPTAC's were synthesized (Table 1). PEPTAC 1 contained the 3 peptides that were recognized by PeM29 in a Pepscan analysis [33]. The binding of PeM29 to the 5 PEPTAC's was tested in ELISA and on a Biacore1000 instrument, but no binding was observed. MAbs PeM71 and 72, both recognizing GGFGP peptides showed a high affinity for the GGFGP containing PEPTAC's (PEPTAC 1 and 4), indicating that the GGFGP peptides are accessible on the scaffold.

Protective properties and antibody responses induced by the PEPTAC's

To investigate whether it was possible to mimic a discontinuous epitope using a synthetic scaffold, we raised Abs against the PEPTAC's and tested their protective properties in a

mouse model. Since small peptides are poor immunogens and induce low amounts of Abs in mice, we coupled the scaffold constructs to a tetanus toxoid (TT). The immune response of mice to unconjugated PEPTAC's, was also tested.

Groups of eight mice were immunized with PEPTAC's 1-5 either conjugated to TT or unconjugated. After three immunizations with peptides, mice were challenged with a mixture containing 4 x 10^7 CFUs of two isogenic *B. pertussis* strains which produced P.69 Prn1 and P.69 Prn2, respectively. The strains could be distinguished on the basis of their antibiotic susceptibility. This allowed us to calculate a possible differential protection against strains carrying distinct P.69 Prn variants. Three days after the challenge, the number of CFUs in the lungs and trachea were determined. Since the data obtained from the trachea and lungs were similar, we will only discuss the data from the lungs.

In a first experiment, the protective property of unconjugated PEPTAC 1 was compared with the TT-conjugated PEPTAC 1. Mice immunized with unconjugated PEPTAC's were not protected against infection whereas mice immunized with the conjugated PEPTAC's were protected ($P > 0.00014$ compared to the PBS control, Fig. 3). Interestingly, a higher protection was observed against the P.69 Prn2 strain compared to the P.69 Prn1 strain ($P > 0.00002$). Mice vaccinated with the conjugated PEPTAC 1 showed high titers against P.69 Prn1, consistent with the observed protection (Fig. 4).

In mice immunized with unconjugated PEPTAC 1, Abs against PEPTAC 1 were not detected (data not shown), whereas in mice that received the conjugated PEPTAC 1, high anti-PEPTAC 1 titers were observed (Fig. 5). Since the unconjugated PEPTAC did not induce protection, TT-conjugated PEPTAC's were used in the mouse model in subsequent experiments, unless stated differently.

Several control experiments were performed. First, to rule out a protective effect of anti-TT Abs, or anti-PEPTAC Abs, an "empty" PEPTAC, which did not contain peptides (PEPTAC 3) (Fig. 2), was coupled to TT. Secondly, the protective properties of the PEPTAC intermediates (PEPTAC 2 and PEPTAC 5 containing peptides GERQH, and GERQH+DDDWTDG, respectively) (Fig. 2) were tested in the mouse model. In previous studies, linear synthetic peptides containing a GGFGP sequence were shown to induce Abs that confer protection [8] . To investigate the protective role of the GGFGP peptide on PEPTAC 1, a TAC containing only the GGFGP peptide (PEPTAC 4) (Fig. 2) was synthesized and subsequently coupled to TT. The degree of protection induced in mice with conjugated PEPTAC 1- 5 was compared to P.69 Prn1 [42] or PBS (Fig. 3).

When mice were immunized with the "empty" PEPTAC 3, no protection against infection was observed (Fig. 3), which is consistent with the observation that the Abs from mice immunized with PEPTAC 3 did not bind to purified P.69 Prn1 in an ELISA (Fig. 4). Similarly, PEPTAC 2 did not induce protection and antibodies against P.69 Prn1. (Fig. 4). Mice receiving PEPTAC 5 showed a slight insignificant protection of 0.25 Log10 CFU ($P = 0.088$ compared to the PBS control, Fig. 3). In addition, a small amount of Abs to P.69 Prn1 was detected in ELISA (Fig. 4).

Mice vaccinated with the conjugated PEPTAC 1 showed high titers against P.69 Prn1, consistent with the observed protection (Fig. 4).
To rule out that the observed protection was induced by the GGFGP peptide only, mice were immunized with the PEPTAC containing only the GGFGP peptide (PEPTAC 4). PEPTAC 4 immunized mice were less well protected against infection compared to PEPTAC 1 immunized mice (Fig 3, P > 0.001). Similar as observed with PEPTAC 1, PEPTAC 4 immunized mice were better protected against P.69 Prn2 strains compared to P.69 Prn1 strains (P = 0.0024). A decrease of respectively 0.2 and 0.4 Log10 in the number of Prn1 and Prn2 CFU's, was observed in the lungs of PEPTAC 4 immunized mice. PEPTAC 4 induced a similar level of protections as PEPTAC 5, which is consistent with the similar titers against P.69 Prn1 produced by both vaccines (Fig. 4).
Mice receiving purified P.69 Prn1 showed the highest protection, a 2.4-Log10 decrease in the number of CFU/ml was observed (Fig 3) (P > 1.5E-6 compared to the PBS control, Fig. 3). Furthermore, P.69 Prn1 induced higher antibody titers against P.69 Prn1 compared to the PEPTAC constructs (Fig. 4). As expected, mice that were immunized with PBS were not protected against infection (Fig. 3) and did not react with P.69 Prn1 in an ELISA (Fig. 4)

Analysis of anti-PEPTAC 1 antibodies
We investigated the specificity of Abs, induced after immunization with the TT-conjugated scaffolds, in several ways. First, we measured the response of induced Abs against the five PEPTAC variants in the Biacore (Fig. 5). Secondly, we depleted the sera with several linear peptides, PEPTAC's and a combination of both, after which we measured the response of the remaining Abs (Fig. 6). Finally, we tested the response of the Abs with several Prn deletion mutants lacking parts of P.69 Prn1 (Fig. 7). The results of these experiments will be discussed in this order.

Reaction of sera with PEPTAC 1-5
To investigate the response of the anti-PEPTAC sera to the five PEPTAC constructs, all five were coupled to a CM5 sensor chip, after which individual sera were allowed to interact with the PEPTAC's in the Biacore (Fig. 5).
Antibodies induced by PEPTAC 3 were very reactive with chip bound PEPTAC 3, 2 and 4. Almost no reaction was observed with PEPTAC 5 and 1 (Fig. 5). PEPTAC 2 and 4 both contain only one peptide arm whereas PEPTAC 5 and 1 contain two and three peptide arms, respectively. It is possible that when two or more peptides are present on the TAC scaffold, binding of Abs to the TAC-scaffold structure is blocked. This may explain that Abs against PEPTAC 3 show little reactivity with PEPTAC 5 and 1.
PEPTAC 2 induced lower levels of Abs directed against the 5 PEPTAC's compared to PEPTAC 3. The highest responses of anti-PEPTAC 2 Abs were observed with chip-bound PEPTAC 3 and 2. Similar as described for anti-PEPTAC 3 Abs, anti-PEPTAC 2 Abs were

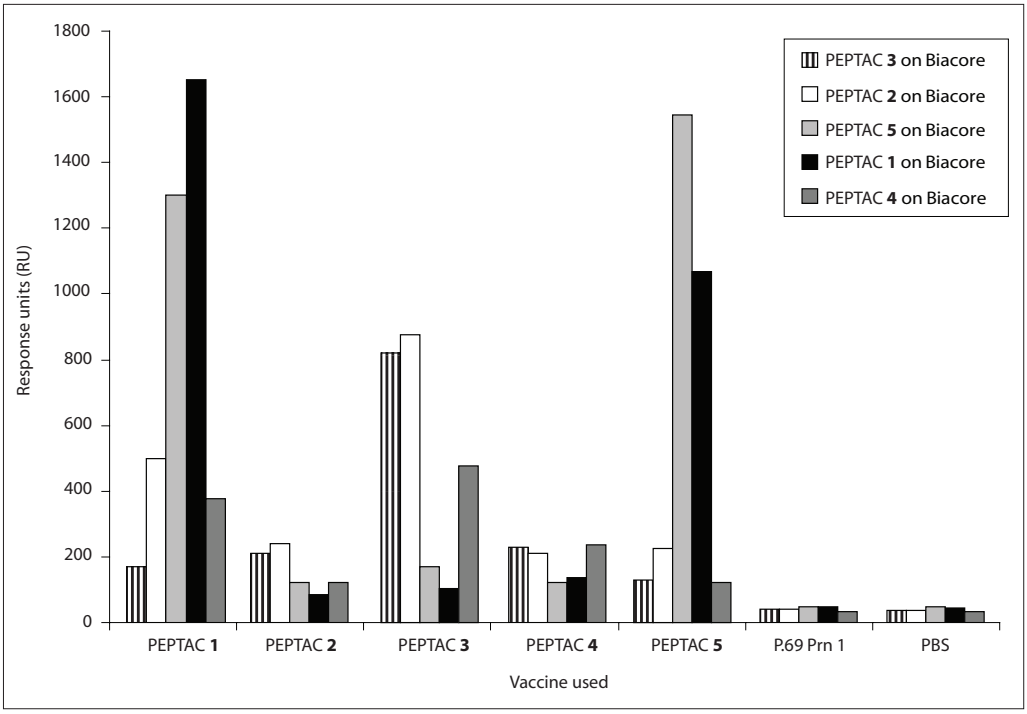


Figure 5. Mice were vaccinated with PEPTAC 1-5 after which the response of the serum Abs was tested to PEPTAC 1-5 in the Biacore. The average response of all sera per group is displayed. Equal amounts of PEPTAC's were coupled to a CM5 sensor chip. Abbreviations used: PEPTAC 3: Cys-TAC-3Ac, PEPTAC 2: Cys-TAC-HQREG-2Ac, PEPTAC 5: Cys-TAC-HQREG-DDDWTDG-Ac, PEPTAC 1: Cys-TAC-HQREG-DDDWTDG-PGFGG, PEPTAC 4: Cys-TAC-PGFGG-2Ac.

almost unable to react with PEPTAC 5 and 1. In contrast to anti-PEPTAC 3 Abs, anti-PEPTAC 2 Abs were not very reactive with PEPTAC 4.

Antibodies from PEPTAC 5 immunized mice showed almost no reaction with PEPTAC 3 and 4, which can be explained by the absence of peptides (PEPTAC 3) or a peptide arm other than present in PEPTAC 5. Furthermore, this suggests that when two or more peptides are present on the TAC scaffold, they block the binding or induction of Abs to the TAC-scaffold structure. It is interesting, however, that PEPTAC 5 antisera strongly reacted with PEPTAC 5 and 1, but only a small reaction was observed with PEPTAC 2. This could indicate that almost no Abs are induced against the peptide perse present on PEPTAC 2 (GERQH), but that most Abs recognized GDTWDDD only, or an epitope that comprised both peptides present on PEPTAC 5 (GERQH and GDTWDDD).

Sera from mice immunized with PEPTAC 1 showed only a minor reaction with PEPTAC 3. Antibodies induced against PEPTAC 1 very effectively bound to PEPTAC 5. The highest response was measured with PEPTAC 1. Anti-PEPTAC 1 Abs were also able to bind to PEPTAC 4 with a similar response as to PEPTAC 2. Both PEPTAC 2 and 4 contain only one peptide arm. When anti-PEPTAC 4 Abs were tested, a reaction was observed with PEPTAC 3, 2 and 4. A lower reaction was observed with PEPTAC 5 and 1, a result similar

to that obtained with anti-PEPTAC 3 or 2 antibodies. This result can be explained in a similar manner as for anti-PEPTAC 3 and 2 antibodies and indicated that the majority of Abs induced by PEPTAC 4 were directed against the TAC-scaffold structure.
Surprisingly, sera from mice immunized with P.69 Prn1 were unable to bind to either one of the five PEPTAC's. This result was unexpected since anti-PEPTAC 1 Abs were able to recognize purified P.69 Prn1 (shown above). As expected, no reaction of antibodies from mice immunized with PBS was observed.

Reaction of depleted sera with PEPTAC's

The observation that antibodies against PEPTAC 5 hardly bind to PEPTAC 2, and only small amounts of anti-PEPTAC 1 Abs bind to PEPTAC 2 and PEPTAC 4, suggested that PEPTAC 5 and 1 induced Abs against a discontinuous epitope. To confirm the assumption that Abs induced by PEPTAC 1 were directed against discontinuous epitope, anti-PEPTAC 1 sera were depleted with linear peptides, the five PEPTAC's and a mix of both. Since we were only interested in the discontinuous epitope mimicked by PEPTAC 1, only anti-PEPTAC 1 sera were depleted. After depletion, the binding of sera to PEPTAC's 1-5 was measured. However, we will only present data generated with PEPTAC 1 since these are the most informative. The results are summarized in Fig. 6.
The amount of response units (RU) obtained with PBS depleted serum was set to 100% (Fig. 6). Anti-PEPTAC 1 sera were first depleted with PEPTAC 3, and only a small decrease

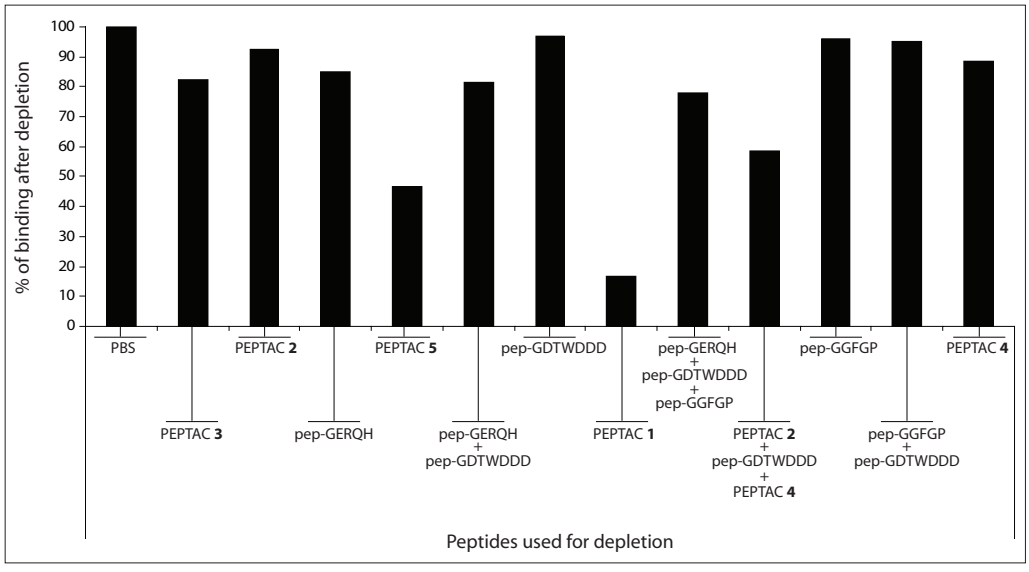


Figure 6. Average antibody responses of mice that were vaccinated with peptide PEPTAC 4 after depletion with several peptides (indicated below the X-axis). The relative response is indicated, using PBS as a reference (set at 100%). Abbreviations used: Abbreviations used: PEPTAC 3: Cys-TAC-3Ac, PEPTAC 2: Cys-TAC-HQREG-2Ac, PEPTAC 5: Cys-TAC-HQREG-DDDWTDG-Ac, PEPTAC 1: Cys-TAC-HQREG-DDDWTDG-PGFGG, PEPTAC 4: Cys-TAC-PGFGG-2Ac, Pep = linear peptide.

of 20% in the amount of binding was observed (Fig. 6). This result is consistent with results described earlier showing that a small amount of Abs were directed against the TAC-scaffold structure of the PEPTAC's. When the anti-PEPTAC 1 sera were depleted with PEPTAC 2, only a small decrease of 10% in binding was observed (Fig. 6). Similarly, depletion of anti-PEPTAC 1 sera with the peptide GERQH resulted in a 15% decrease in Ab binding. Both results are consistent with the results presented in Fig. 5 showing that PEPTAC 1 hardly induced Abs to GERQH. When anti-PEPTAC 1 sera were depleted with PEPTAC 5, a decrease of 50% in Ab binding was observed. This result was consistent with the observation that a large amount of anti-PEPTAC 1 Abs bound to PEPTAC 5 (Fig. 5).
To test whether the Abs that were depleted with PEPTAC 5 were directed against a discontinuous epitope, a depletion with either one or a mix of two linear peptides, both present on PEPTAC 5, was performed. A decrease of 20% and 5% in Ab binding was observed when the serum was depleted with a mix of both peptides or with a single GDTWDDD peptide, respectively (Fig. 6). In contrast, when the two peptides were scaffolded (PEPTAC 5) a decrease of 50% in Ab binding was realized. When anti-PEPTAC 1 sera were depleted with PEPTAC 1, the amount of binding decreased with 80% (Fig. 6). In contrast, when the anti-PEPTAC 1 serum was depleted with a mix of the three linear peptides present on PEPTAC 1, a 20 % decrease in antibody binding was observed. Apparently, the mix of three peptides was not able to mimic the epitopes recognized by the majority of PEPTAC 1 antibodies. To investigate whether confined, scaffolded, peptides were better able to mimic these epitopes, a mix containing PEPTAC 2, 4 and a linear peptide with the sequence GDTWDDD was used adsorb PEPTAC 1 serum. The GDTWDDD peptide was used since a TAC-scaffold containing only the peptide GDTWDDD was not synthesized. This depletion resulted in a decrease of 40 % of antibody binding. This suggested that although the individual peptides were scaffolded, (except for GDTWDDD) they need to presented in a distinct spatial position in order to be recognized by an Ab. Anti-PEPTAC 1 sera were also depleted with a GGFGP peptide and with a mix of GGFGP and GDTWDDD peptides. Both depletions did not result in a decrease of more then 5 % of antibody binding, however. This result indicated that almost no antibodies (or only antibodies with low avidity) to linear GGFGP or GDTWDDD epitopes were induced by PEPTAC 1. Finally when anti-PEPTAC 1 sera were depleted with PEPTAC 4, a decrease of 10% of antibody binding was observed.
Taken together, these results indicated that anti-PEPTAC 1 Abs recognize a discontinuous epitope which requires the simultaneous presence of all peptides on distinct spatial positions with respect to each other. These spatial positions are not present when linear peptides are free in solution, or presented as separate peptides on the TAC-scaffold.

Analysis of antibody binding to Prn deletion mutants
The observation that PEPTAC 1 induced mainly antibodies against a discontinuous epitope, was further confirmed using recombinant P.69 Prn1 mutants. The binding of anti-PEPTAC 1, 4 and 5 Abs to P.69 Prn1 mutants lacking parts of the N-terminus or the variable region

1 was tested and compared to the binding to native P.69 Prn1 (Fig. 7).
The binding of anti-PEPTAC 5 sera were first tested to N-terminal P.69 Prn1 mutants 1 and 2, in which the peptides GERQH and GERQH+GDTWDDD are missing respectively. These deletions resulted in a 5-fold 12-fold decrease in binding, respectively. Deletion of region 1 did not affect the binding of anti-PEPTAC 5 Abs, which as expected since PEPTAC 5 does not contain peptides which are present in region 1 (Fig. 7).

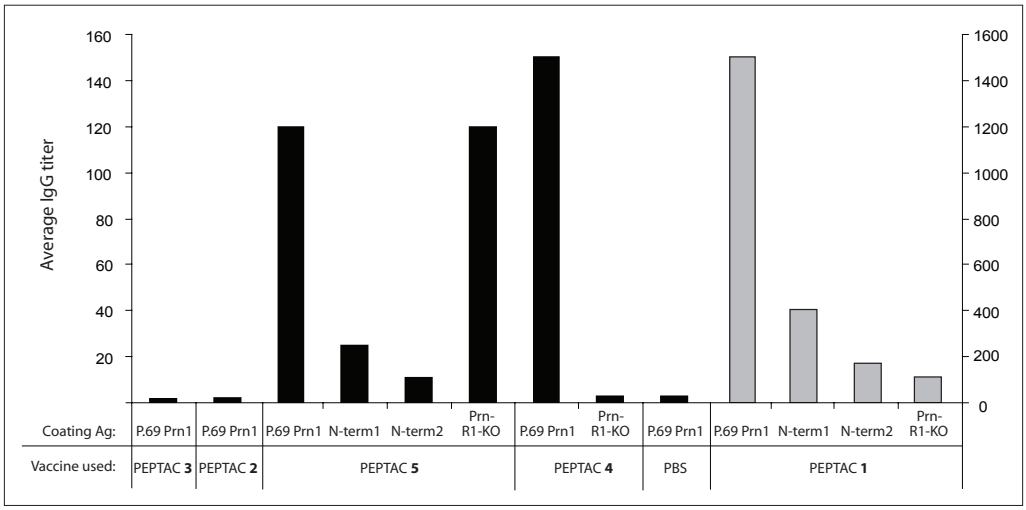


Figure 7. Analysis of antibody responses of groups of sera to native Prn1 and several Prn mutants. The values of the black bars and grey bars are plotted on the left and right Y-axes, respectively. Abbreviations used: PEPTAC 3: Cys-TAC-3Ac, PEPTAC 2: Cys-TAC-HQREG-2Ac, PEPTAC 5: Cys-TAC-HQREG-DDDWTDG-Ac, PEPTAC 1: Cys-TAC-HQREG-DDDWTDG-PGFGG, PEPTAC 4: Cys-TAC-PGFGG-2Ac, Ag: antigen.

When the binding of anti-PEPTAC 1 sera were tested to Prn mutants lacking parts of the N-terminus, a dramatic decrease in the amount of antibody binding was observed. The binding of PEPTAC 1 antisera to N-terminal mutants 1 and 2 decreased 4- and 7- fold, respectively. Deletion of region 1 resulted in a 14 fold decrease in antibody binding (Fig. 7). The binding of anti-PEPTAC 4 sera was abolished when region 1 was deleted from Prn. This result was as expected since region 1 contains the peptide that is present on PEPTAC 4.
Since PEPTAC 2 and 3 did not induce Abs against P.69 Prn1 in the mouse, they were not tested on the Prn mutants. The observation that anti-PEPTAC 1 Abs were not reactive with PEPTAC 4 and PEPTAC 2 (containing GGFGP and GERQH respectively), but that binding to P.69 Prn was reduced 4 and 16 fold when the corresponding peptides were deleted, indicated that most of the induced anti-PEPTAC 1 Abs were directed against a molecular construct containing a discontinuous epitope of Prn. Furthermore, the observation that anti-P.69 Prn titers induced by the PEPTAC 1 vaccine were a 10-fold higher than those induced by PEPTAC 4 and 5 supports this line of reasoning.

Discussion

In a previous attempt to identify antigenic sites on P.69 Prn1, we employed a Pepscan to map the location of binding sites of mAbs and human serum Abs. One of the mAbs, PeM29, recognized a discontinuous epitope, that comprised three loops which are located in the N-terminus of P.69 Prn. PeM29 also competed with human serum Abs for binding to Prn, suggesting it binds to a similar epitope as human serum Abs. Since the epitope recognized by mAb PeM29 comprises two conserved loops, it could potentially be a suitable vaccine target. In addition, Abs directed against conformation dependant epitopes often harbor protective or neutralizing properties [27-32].

In an attempt to mimic the discontinuous epitope recognized by PeM29 and test its potential as a vaccine candidate, we employed a novel synthetic scaffold. Synthetic peptides are attractive vaccine candidates since they are easy and inexpensive to produce. Large quantities of peptides can be obtained relatively easy without complicated purification procedures [43-45]. Furthermore, peptide vaccines have the advantage that they can be designed to include specific T- or B-cell epitopes that stimulate the immune response against the peptides [44]. Including T-cell epitopes could possibly also stimulate immunological memory [18].

The TAC-scaffold used in this study contains 4 flexible sites (arms) to which peptides can be coupled. In our approach, peptides were synthesized to 3 arms. The 4th arm was used to couple the TAC-scaffold via via the free SH-group of a cysteine residue to tetanus toxoid, which is shown to contain several T-cell epitopes [46], and to the dextran layer of a CM5 sensor chip for presentation in the Biacore.

Using molecular modeling, the location of three the loops in the crystal structure of P.69 Prn recognized by PeM29, was compared with the spatial position that the synthetic peptides could display when synthesized on the TAC-scaffold. The PEPTAC that resembled the native form of the three loops closest, was synthesized. When subsequently the binding of PeM29 was tested to PEPTAC 1, no reaction was observed indicating that the epitope presented by PEPTAC 1 did not exactly mimic the epitope that is recognized by this mAb.

A four-pronged approach was used to investigate the protective properties and the conformational nature of the antibody response of mice to the TAC-scaffolds. This involved immunization and protection studies in mice, Biacore analysis, deletion mapping and depletion ELISA analysis.

A total of 5 PEPTAC's were synthesized, of which PEPTAC 1 contained the peptides recognized by PeM29 (Fig. 2). When mice were immunized with PEPTAC 5 or 4, a slight protection was observed whereas PEPTAC 1 immunized mice were well protected against infection. Mice immunized with PEPTAC 1 and 4, were better protected against *B. pertussis* P.69 Prn2 strains then to P.69 Prn1 strains. P.69 Prn2 strains contain four GGFGP repeats in the variable region 1 of P.69 Prn whereas Prn1 strains only have two GGFGP repeats. It is possible that anti GGFGP Abs are capable of a better multivalent binding to P.69 Prn2 as compared to P.69 Prn1 proteins. Although the GGFGP repeats are located adjacent to each other in the variable loop of P.69 Prn, the tertiary structure of this large loop may allow the

subsequent binding of two Abs without steric hindrance. It is also possible that the P.69 Prn2-region 1, due to the increased size, is more exposed, and therefore Abs are able to bind better to P.69 Prn2 compared to P.69 Prn1.

When the binding of the serum Abs was tested to the 5 individual PEPTAC's, it became clear that the peptides that were presented on the scaffold adopted a tertiary structure as predicted by molecular modeling. Anti-PEPTAC 2, 3 and 4 Abs were unable to bind to PEPTAC 1 and 5 which contain 2 and 3 peptides respectively. When 2 or more peptides are presented on the scaffold, they adopt a tertiary structure which blocks the binding, or prevents the induction of antibodies directed against the TAC-scaffold structure. This result is very favorable for the future use of TAC-scaffolds as vaccine candidates, since no additional, potentially cross-reactive, Abs to the TAC-scaffolds are induced.

The discontinuous nature of the induced anti-PEPTAC 1 and 5 Abs, was investigated by Biacore analysis, depletion ELISAs and deletion mutant mapping (Fig. 5-7). Using deletion mutant mapping and depletion ELISAs it became clear that anti-PEPTAC 1 and 5 Abs recognized a discontinuous epitope that comprised 3 or 2 peptides respectively. Depletions with linear peptides were much less efficient in adsorbing the anti-PEPTAC Abs compared depletions using to scaffolded peptides. In contrast depletions with scaffolded peptides (PEPTAC's) were very efficient in adsorbing the sera from Abs compared to depletions with linear peptides. Anti PEPTAC 1 Abs were not very reactive with PEPTAC 4 (GGFGP) but bound to P.69 Prn1. However, deletion of region 1 (containing GGFGP peptides) from P.69 Prn1 reduced binding of anti-PEPTAC 1 Abs 16 fold, indicating that the GGFGP peptide is part of a discontinuous epitope presented by PEPTAC 1. Although anti-PEPTAC 1 Abs were not reactive with PEPTAC 2, deletion of parts of the N-terminus of P.69 Prn1 resulted in a 4 to 8 fold decrease in Ab binding. Together these results clearly indicated that Abs were directed against a discontinuous epitope.

The majority of the induced antibodies were directed against a discontinuous epitope comprising the peptides GDTWDDD and GGFGP. It is probable that the distance between peptides GERQH and GDTWDDD (>12Å in the crystal structure) is too large to for an antibody to bind to. Since peptides GDTWDDD and GGFP are only 3.5Å apart, it is possible that they are recognized more easily as a single discontinuous epitope. Another possibility remains that the GERQH peptide is not immunogenic and that therefore almost no Abs were induced against this peptide.

When the Ab response of immunized mice was tested with P.69 Prn1, only sera from mice immunized with PEPTAC 1, 4 and 5 were able to bind. Sera from mice immunized with purified P.69 Prn1 however, were unable to bind to any of the PEPTAC's. This could indicate that P.69 Prn1 does not induce antibodies against the epitope presented on PEPTAC 1. Interestingly, mice immunized with PEPTAC 1 were protected against infection with *B. pertussis*, and anti-PEPTAC 1 Abs bound to P.69 Prn. Therefore it seems likely that PEPTAC 1 induces antibodies to an epitope that is hidden in native P.69 Prn. Several explanations are possible for this observation. It has been described for the HIV glycoprotein gp120 that

epitopes are hidden until the protein binds to the receptor [47]. In contrast to anti-PEPTAC 1 Abs, anti-gp120 Abs are unable to bind to native protein. It is also possible that the epitope mimicked by PEPTAC 1 is not immunogenic on P.69 Prn1, but becomes immunogenic when presented on a PEPTAC. A third possibility is that the immune response is directed to immunodominant regions of P.69 Prn1. Therefore no or only a small number of Abs are induced against this epitope. A final possibility remains.

It has been described that Abs binding to residues located in close proximity of a hidden epitope, can result in a conformational change of the protein, thereby exposing the hidden epitope [48]. It is possible that anti-PEPTAC 1 Abs display a similar mode of action. The X-ray crystal structure of P.69 Prn appears as a helix from which several loops protrude. The largest loop, containing the variable region 1, folds towards the N-terminus of P.69 Prn and is thereby likely to cover at least one or two of the loops present on PEPTAC 1. Due to this loop, the anti PEPTAC 1 Abs may not be able to directly bind to all 3 loops, and this binding may require a 2-step process. The following scenario can be envisaged. Region 1 that contains the GGFGP peptides is well exposed, and a low affinity binding to this region may take place first. This binding results in a conformational change that exposes the underlying loops. Subsequently the Ab will be able to bind all 3 peptides, which results in a high affinity binding. In a previous report, Hijnen *et al.*, submitted we presented evidence that loops on P.69 Prn may play an indirect role in immune evasion by masking of loops containing important epitopes. The epitope displayed by PEPTAC 1 is consistent with this hypothesis.

Our work shows it is possible to induce Abs to epitopes that are hidden in the native protein using a synthetic scaffold. As shown for gp120, epitopes that are hidden in the native conformation of a protein are often epitopes to which an immune response can be highly effective. However, since these epitopes are often buried in the native protein, B-cell response against such epitopes are limited [47].

To our knowledge this is the first example that describes the synthesis of a novel selectively addressable synthetic scaffold that is capable of spatially presenting peptides, thus displaying a set of discontinuous epitopes. This approach did not use screening of large phage display libraries, but rather used a crystal structure in combination with straightforward molecular modeling to construct a mimotope of a discontinuous epitope. Using this synthetic TAC-scaffold, we have constructed a mimotope of a discontinuous conformational epitope of P.69 Prn that induces functional antibodies in mice against a conformational epitope which are not induced when using native purified protein. This approach could be employed to design new synthetic vaccines that induce functional antibodies which are not induced during natural infection, and which cannot be induced by single peptides and mixture thereof.

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6

Antibody responses to the *Bordetella pertussis* virulence factor P.69 Pertactin

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Chapter 6

Abstract

Bordetella pertussis, the causative agent of whooping cough, is re-emerging in several countries with a traditional high vaccine uptake. Analysis of clinical isolates revealed antigenic divergence between vaccine strains and circulating strains with respect to the *B. pertussis* virulence factor P.69 Pertactin. Polymorphisms in P. 69 Pertactin are mainly limited to regions comprised of amino acid repeats, designated region 1 and region 2. Region 1 flanks the RGD motif involved in adherence. Although antibodies against P. 69 Pertactin are implicated in protective immunity, and monoclonal antibodies against the variable regions have been shown to be protective, until recently, little was known about antibody specificity against Pertactin. Using P. 69 Pertactin variants and deletion derivates, we investigated the immune response against the native protein, and against deletion derivatives. The N-terminus of P. 69 Pertactin was found to be immunodominant in both rabbits and humans. P. 69 Pertactin type specific antibodies were detected in rabbits, but not in humans. Furthermore, we found evidence for a role of the N- and C-termini and the variable region 1 in the evasion of antibody responses by conformational masking of epitopes. These results shed new light about the role of the variable regions and the N- and C-termini in the evasion of antibody responses.

Introduction

Despite high vaccine coverage, the causative agent of whooping cough (pertussis) has re-emerged in several countries [1-5]. Several explanations have been suggested for the re-emergence of pertussis including antigenic divergence between vaccine and circulating strains [6-15]. Antigenic divergence of P.69 Pertactin (P.69 Prn) has been suggested to be one of the causes for the return of pertussis in the Netherlands [2, 16].

The role of P.69 Prn in immunity to pertussis has been well documented. Antibody levels to P.69 Prn have been shown to correlate with clinical protection [17, 18]. Passive and active immunization studies in mice and pigs have shown that antibodies (Abs) against P.69 Prn confer protective immunity [19, 20]. Anti- P.69 Prn Abs, but not anti-Ptx, anti-fimbriae, or anti-FHA Abs, were found to be crucial for *B. pertussis* phagocytosis [21]. Recently it was described that P.69 Prn induces type-specific Abs in humans [22]. Furthermore, the efficacy of the Dutch whole cell vaccine was affected by variation of P.69 Prn in a mouse model [19]. Finally, acellular vaccines (ACV's) containing Ptx, FHA and P.69 Prn were more effective compared to ACV's containing Ptx and FHA only [23-26].

P.69 Prn, an autotransporter protein, is located at the cell surface of *B. pertussis*. P.69 Prn is polymorphic but variation is mainly limited to two regions, designated region 1 and 2, which are comprised of Gly-Gly-X-X-Pro and Pro-Gln-Pro repeats, respectively. A total of 13 P.69 Prn types have been isolated. In the Netherlands and Finland [22], the 3 most common *B. pertussis* P. 69 Prn types are P.69 Prn1, P.69 Prn2 and P.69 Prn3. P.69 Prn1 and P.69 Prn3 only differ in 2 aminoacids (AA) in 1 of the repeats in region 1, whereas P.69 Prn2 contains an additional GGFGP repeat (Fig. 1A). The variable regions 2 of P.69 Prn1, 2 and 3 are identical (Fig.1A). P.69 Prn contains an N-terminally located Arg-Gly-Asp (RGD) motif, implicated in ligand-receptor interactions in eukaryotes, and most variation is found adjacent to this site [27, 28]. The second variable region, region 2, is located at the C-terminus of P.69 Prn (Fig. 1A).

In a previous study, we showed that P.69 Prn induces type-specific Abs in humans [22]. However, since purified P.69 Prn2 and P.69 Prn3 were not available at that time, an indirect approach was used to identify P.69 Prn-type-specific antibodies, involving synthetic peptides and a blocking ELISA. Recently we described the cloning, expression and purification of P.69 Prn1, 2 and 3 and several P.69 Prn mutants that lack parts of the protein [29]. These proteins were used in this study where we investigated the presence or absence of P.69 Prn type-specific Abs in humans and rabbits using a direct approach. In this direct approach we isolated the Abs of interest and tested their specificity. Based on the results described in this study, we propose an adjusted hypothesis about the effects of variation in Prn on the antibody response to Prn.

Materials and Methods

Recombinant Pertactin

P.69 Pertactin variants and Prn deletion mutants were expressed and refolded as described, except that 200mM of L-Arginine was added to the first refolding buffer (1 mM EDTA, 100 mM NaCl, 50 mM Tris/HCL pH 8.8) resulting in higher refolding efficiencies [29].

Rabbit polyclonal anti-sera

Polyclonal rabbit anti-P.69 Prn1, 2 and 3 sera were raised at the Eurogentec facility (Eurogentec, Seraing, Belgium). New Zealand white Rabbits (n=3) were immunized 3 times with 50 µg of purified P.69 Prn per immunization using Freund's complete adjuvant for the first immunization, followed by two boosts using Freund's incomplete adjuvant.

Human sera

A set of paired sera from Finnish individuals were selected which included vaccine recipients and patients [22]. Sera from 11 patients infected by Prn2 *B. pertussis* strains, 5 sera from patients infected with Prn3 *B. pertussis* strains, 10 sera from patients infected with *B. parapertussis*, and 10 sera from adolescents after booster vaccination with the GSK ACV (Boostrix) were selected. The antibody response of these sera to purified P.69 Prn1, 2, 3 and the Prn-R1-KO (lacking the variable region 1) was tested in ELISA as decribed previously [22], and a subset was used in the depletion assay.

Furthermore, a set of 21 sera from Dutch patients infected with *B. pertussis* was selected. Although, in the Netherlands, the P.69 Prn type of the strain causing an infection is not routinely characterized, surveillance studies have shown that over 90% of the strains are of the P.69 Prn2 type. The children were vaccinated at the age of 3, 4, 5, and 11 months with DTP-IPV which at that time contained the whole-cell pertussis vaccine from the Netherlands Vaccine Institute (NVI, Bilthoven, the Netherlands). The whole cell vaccine is derived from strains which produce P.69 Prn1 [30]. The children received a booster immunization with acellular vaccine containing 25µg FHA, 25µg PT and 8µg of P.69 Prn1 (Infanrix, Glaxosmithkline, Rixensart, Belgium) at the age of four years. The response of these sera to several P.69 Prn variants and deletion mutants was tested in ELISA and in a depletion assay.

Depletion of Sera

Human and rabbit sera were diluted 1:40 and 1:100 with PBS, respectively. Purified His-tagged recombinant P.69 Prn or PBS was added to a final concentration of 25µg/ml. Sera were incubated overnight at 22°C under constant rocking. After incubation, possibly formed P.69 Prn-Ab complexes were removed by centrifugation for 15 min at 15.000 g. Additional purified recombinant His-tagged P.69 Prn was added to the supernatant to a concentration of 25 µg/ml, after which the sample was incubated for 1 hr at 22 °C while rocking. Complexes were removed by an additional centrifugation step at 15.000g for 15

min. Since not all of the Prn-Ab complexes were removed by centrifugation, a 1:20 volume of washed magnetic Ni^{2+} beads (His-Mag-Beads; Novagen) was added to the supernatant to remove the final Prn-Ab complexes via the His-tag of the recombinant Prn. After incubation at 22°C for 15 min, the beads were removed and the supernatant was used in ELISA.

ELISA

The binding of Abs to the recombinant proteins was tested in an ELISA as described previously [31]. Polystyrene 96-well ELISA plates (Immulon II; Dynatech, Chantilly, Va.) were coated overnight at 22°C by adding 100 µl of 0.04 M carbonate buffer pH 9.6 containing 2 µg of protein/ml to each well. The plates were washed four times with 200 µl of PBS supplemented with 0.05% Tween 20 (PBST) per well by using a Titertek Plus M96V washer (ICN, Irvine, CA.). Sera were added to the wells and were incubated for 2 h at 37 °C followed by four washings as described above. Bound rabbit Abs were detected with alkaline phosphatase conjugated goat anti-rabbit total IgG (DakoCytomation, Glostrup, Denmark). Bound human Abs were detected with alkaline phosphatase conjugated goat anti-human total IgG (Sigma, Saint Louis, MO). The optical density at 405 nm (OD405) was measured with a plate reader (EL312e; BioTek Systems, Winooski, Vt.). To verify that the equal amounts of protein were coated in the ELISA assays, in all of the ELISA experiments, mAb PeM72 was used as a control. MAb PeM72 is directed against a linear epitope that is located in R1, and therefore does not react with mutants in which this region is deleted (Prn-R1-KO and Prn-R1+R2-KO).

Structure prediction

The published crystal structure of P.69 Prn1 lacks the C-terminal AA residues 540-677. The tertiary structure of the passenger domain of P.69 Prn1 (AA 1-677) (CAA09473) was determined using the Robetta server (http://robetta.bakerlab.org) with the published crystal structure (1DAB.pdb) as a reference [32-35].

Analysis

To calculate the remaining amount of antibody after depletion, a curve comparison was performed between the PBS depleted samples (un-depleted) and the P.69 Prn depleted samples. The OD405-reading of three subsequent data points in the linear range of a graph were multiplied with the corresponding dilution factor. The PBS-curve was set to a 100%. The statistical significance of the observed differences in binding of human serum Abs to P.69 Prn variants and its deletion derivatives was determined using a two-tailed T-test.

Results

From here on, we will use the following nomenclature: P.69 Prn1, 2 or 3 are termed Prn1, Prn2 or Prn3 respectively. The Prn variable regions 1 and 2 will be referred to as R1 and R2, respectively. The Prn region 1, 2 and 1+2 knockouts will be termed Prn-R1-KO, Prn-R2-KO and Prn-R1-R2-KO, respectively. The Prn mutants lacking parts of the C- or N-terminus will be referred to as C- and N-terminal Prn mutants, respectively. The rabbit anti-P.69 Prn1, 2 or 3 sera will be referred to as P1, P2 or P3 serum, respectively. Furthermore, Abs that bind to all three Prn variants are referred to as cross-reactive Abs. Antibodies that are specific for only one of the P.69 Prn types will be referred to as type-specific Abs. All of the data is summarized in Figure 1B.

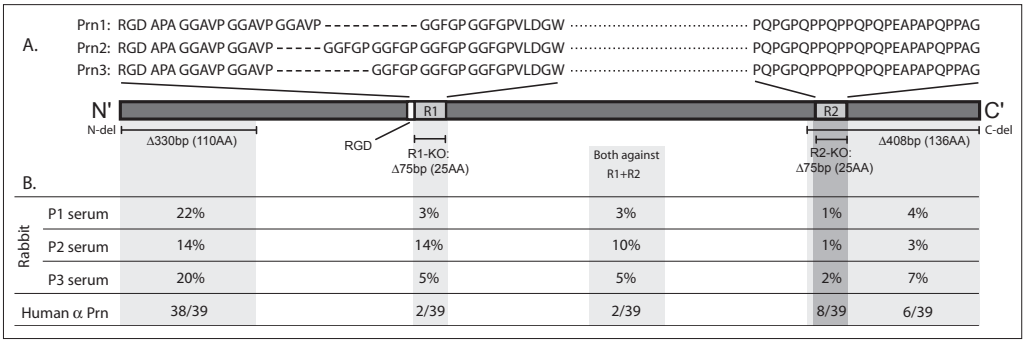

Figure 1. (A) Structure of Prn variants and deletion derivatives. Amino acid sequence of the variable region R1 and R2 are indicated. Prn1 and Prn2 differ in 2 amino acids whereas Prn2 contains an additional repeat. The location and size of N-terminal, C-terminal and central deletions are indicated. R1, R2 and RGD indicate the variable regions 1, 2 and the RGD receptor binding motif, respectively. Abbreviations: del, deletion. AA, amino acid. Bp, base pair. (B) The residual activity of anti-Prn1, 2 and 3 rabbit Abs directed against the respective parts of P.69 Prn are indicated in gray panels. The fraction of human sera that contain Abs directed against the respective part of P.69 Prn are also indicated in the gray panels. Only 2 of the 39 human sera had Abs whose binding was dependent on de presence of both R1 and R2.

Depletion of rabbit sera

In human sera, antibody levels against Prn are often low, and only small amounts of serum are available whereas in rabbits it is possible to generate Ab titers that are over a 1,000 times higher than those found in human serum. Therefore we decided to immunize rabbits with various Prn types and investigated their immune responses. After immunization, sera were tested for the presence of type specific Abs using a depletion ELISA. By adding purified Prn to the sera, we aimed to remove all of the cross reactive Abs while preserving the type specific Abs. Since His-tagged recombinant Prn was used, it was possible to remove Prn-Ab complexes by adding magnetic Ni^{2+} beads.

The 3 most common Prn types produced by current *B. pertussis* strains are Prn1, 2 and 3, we therefore focussed on the Ab responses against these 3 types. The presence of region- or type-specific Abs was investigated by depleting sera with Prn1, 2, 3 and with three deletion mutants that lack either R1, R2 or both variable regions. Furthermore, the Ab responses

to the N- and C-termini were characterized by depleting sera with N- and C-terminal Prn mutants (Fig. 1A). When the rabbit sera were depleted with Prn mutants, in many cases the reaction of the remaining Abs in a single sample was different to Prn1, 2 and 3 (Table 1 & Fig. 2). For a more straightforward presentation of the results, the range of the reaction to Prn1, 2 and 3 variants will be described in the first paragraphs. In the last paragraph, the difference in binding of depleted sera to Prn1, 2 and 3 will be discussed in more detail. To verify each depletion, the reaction of the depleted serum was tested on a homologous protein used as coating antigen in ELISA (e.g. depletion of serum with Prn1 and ELISA plates were coated with Prn1). These controls were always negative, indicating that the sera were fully depleted (indicated with "0" in Table 1). Furthermore, as a second control, the reaction of every depleted serum was tested on all of the Prn variants and mutants. All data is summarized in Table 1. No differences were observed in the anti Prn titers of the rabbit sera (n=3), excluding the possibility of artefacts due to non-responder rabbits.

Table 1. Percentage of binding activity after the depletion of rabbit sera

		Coating Antigen (Ag)[c]							
Serum[a]	Depletion Ag[b]	Prn1	Prn2	Prn3	R1-KO	R2-KO	R1-R2-KO	N-term	C-term
P1	PBS	100	100	100	100	100	100	100	100
P1	Prn1	0	0	0	0	0	0	0	0
P1	Prn2	0	0	0	0	0	0	0	0
P1	Prn3	0	0	0	0	0	0	0	0
P1	Prn-R1-KO	3	3	2	0	4	0	2	2
P1	Prn-R2-KO	1	1	0	1	0	0	1	0
P1	Prn-R1-R2-KO	3	3	2	0	3	0	2	1
P1	Prn-N-term	18	25	22	25	21	28	0	20
P1	Prn-C-term	5	3	3	4	2	3	5	0
P2	PBS	100	100	100	100	100	100	100	100
P2	Prn1	0	0	0	0	0	0	0	0
P2	Prn2	0	0	0	0	0	0	0	0
P2	Prn3	0	0	0	0	0	0	0	0
P2	Prn-R1-KO	18	8	15	0	16	0	10	9
P2	Prn-R2-KO	2	1	1	2	0	0	2	0
P2	Prn-R1-R2-KO	14	7	11	2	12	0	8	8
P2	Prn-N-term	17	10	14	18	17	17	0	10
P2	Prn-C-term	4	2	2	3	1	1	4	1
P3	PBS	100	100	100	100	100	100	100	100
P3	Prn1	0	3	2	0	0	0	0	0
P3	Prn2	0	0	0	0	0	0	0	0
P3	Prn3	0	0	0	0	0	0	0	0
P3	Prn-R1-KO	3	6	7	0	4	0	3	3
P3	Prn-R2-KO	1	3	3	3	0	0	3	0
P3	Prn-R1-R2-KO	2	7	6	2	3	0	5	2
P3	Prn-N-term	14	25	21	22	30	28	0	26
P3	Prn-C-term	3	11	10	9	5	4	10	1

[a]Rabbit sera that were depleted. [b]Antigens that were used for depletion of rabbit sera. PBS was used as the undepleted control and the response was set to a 100%. [c]Response of depleted sera to the 8 different coating antigens.

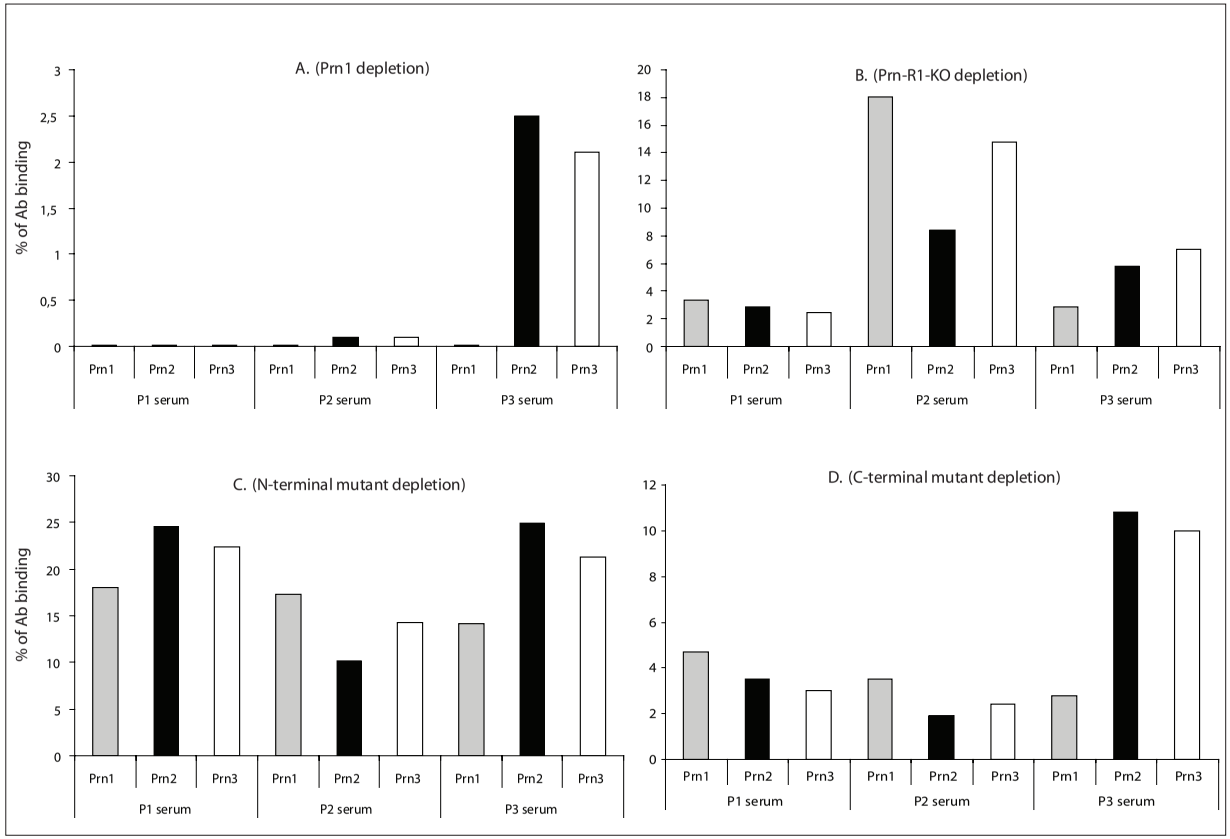

Figure 2. Percentage of antibody binding to Prn variants after depletion. Antibodies raised against Prn variants were depleted with Prn variants and its deletion derivatives. Subsequently, the percentage of remaining activity was determined. Response of Rabbit P1, P2 and P3 Abs to Prn1, Prn2 and Prn3 after depletion with; (A) PrnR1-KO, (B) N-terminal Prn mutant, (C) C-terminal Prn mutant, (D) Prn1. The amount of remaining activity after depletion is indicated in a % relative to the total pool of Abs. On the X-axes, the coating antigen (Prn1, Prn2 or Prn3) is indicated together with the type of antiserum (P1, P2 or P3). Grey, black and white bars indicate the remaining activity after depletion to Prn1, Prn2 and Prn3, respectively.

Only Prn3 induces type specific Abs in rabbits

To identify type specific Abs, rabbit P1, P2 and P3 sera were depleted with Prn1, Prn2 and Prn3. After depleting rabbit P1 and P2 sera with either Prn1, Prn2 or Prn3, no Ab response was detected against the three variants, indicating that the sera were fully depleted (Fig. 1A & Table 1). This indicates that the Abs directed to Prn1 or Prn2 are able to bind to all 3 Prn types. However when rabbit P3 serum was depleted with Prn1, a relative large Ab reactivity against Prn2 and Prn3 (>2%) was still detected (Table 1 & Fig. 2A). These are Prn3 type-specific Abs. When the rabbit P3 serum was depleted with Prn2, a much smaller Abs response (0.2%) was detected (not shown in Table 1 due to round off to integers). As expected, depletion of the rabbit P3 serum with Prn3 fully depleted the serum of anti Prn Abs. None of the Prn3- type-specific Abs bound to the Prn-R2-KO, R1-R2-KO, N- or C-terminal mutant. Surprisingly, a response of 0.3% of the Prn3-type-specific Abs was detected with the Prn-R1-KO (not shown in Table 1 due to round off to integers). This latter result was unexpected since Prn1 and 3 vary only in 2 AA in 1 repeat in region 1. Variation in region 1 apparently also affects the binding of Abs directed to other regions of Prn. Although the percentages seem very low, due to the high Ab titers of the rabbits, the presence of the Prn-type and region specific Abs could accurately be determined.

The immunogenicity of region 1 varies between the 3 main Prn types

Rabbit sera were depleted with the Prn-R1-KO to determine the amount of Abs directed against the variable region 1 of Prn. When rabbit antiserum, raised against Prn1, was depleted with the Prn-R1-KO, only a response of 2-3% was detected against the Prn variants (Table 1 & Fig.2B). Rabbit serum raised against Prn3 also contained only a small amount of Abs specific for region 1. After depletion, only a response of 3-7% was observed against the Prn variants (Table 1 & Fig. 2B). In contrast, when serum raised against Prn2 was depleted with the Prn-R1-KO a response of 8-18% was detected, indicating that Prn2 induces more Abs, or Abs with a higher affinity against the variable region 1 compared to Prn1 and Prn3 (Table 1 & Fig. 2B).

Region 2 has a low immunogenicity in rabbits

To investigate the amount of Abs directed against R2, rabbit P1, P2 and P3 sera were depleted with the Prn-R2-KO. Subsequently, the ability of remaining Abs to bind to purified Prn and the Prn-deletion mutants was tested. Depletion of rabbit P1 sera revealed that only 0-1% of the Abs response was directed against R2. In P2 rabbit sera, only 1-2% of the Abs response was directed against R2. In the P3 rabbit sera, 1-3% of the Abs response was directed against R2 (Table 1).

The N-terminus is highly immunogenic in rabbits

The rabbit P1, P2, and P3 sera were depleted with a Prn mutant that lacks a large part of the N-terminus. A total of 18-25% and 14-25% of the Abs raised against respectively, Prn1

and Prn3 were directed against epitopes that are dependent on the presence of the first 110 AA (Table 1 & Fig. 1C). Prn2 induced less Abs directed against the N-terminus, 10-17% of the Abs were directed against this region (Table 1 & Fig. 1C).

The C-terminus of Prn3 is more immunogenic in rabbits than that of Prn1 and Prn2

When rabbit P3 serum was depleted with the C-terminal Prn mutant, a total of 3-11% of Abs remained, indicating they are directed against the C-terminus (Fig. 2D). In the rabbit P1 and P2 sera, 3-5% and 2-4% of the Abs were directed against the C-terminus, respectively (Fig. 2D). This could indicate that the C-terminus of Prn3 is more immunogenic.

Differences in binding of serum Abs to Prn variants

After depletion of the rabbit sera with the different Prn variants, the binding of the remaining Abs to Prn1, Prn2 and Prn3 was tested. One would expect the binding response of Abs to be the highest for the antigen they were raised against. In 4 out of 5 cases, the response of rabbit P1 serum Abs to Prn1, 2 or 3 was either equal to all 3 proteins, or higher to Prn1. However, the reaction of rabbit P2 serum Abs was always higher to Prn1 and Prn3 than to Prn2. Furthermore, in 3 out of 5 cases the reaction of rabbit P3 serum Abs was higher to Prn2 than to Prn3. A good example is the result of the depletion of rabbit P2 serum with the Prn-R1-KO. When the reaction of the remaining Abs was tested to Prn1, Prn2 and Prn3, the highest binding response was observed with Prn1 and 3, rather than with Prn2 (Table 1 & Fig. 2B). Another example is the depletion of rabbit P3 serum with the C-terminal Prn mutant. After depletion, the reaction of the remaining Abs to Prn1 was 3-4 times lower than to Prn2 and Prn3 (Table 1 & Fig. 2D). This result was rather unexpected since the C-termini of Prn1, Prn2, and Prn3 are identical (Fig. 1A).

ELISA with human sera and Prn variants

Anti-Prn Abs in the serum from Dutch children

We determined the titers of 21 sera from Dutch children to Prn variants and its deletion derivatives. When the response of a human serum sample to a Prn variants was at least 1.5 times higher or lower compared to the response to Prn1, it was statistically significant ($P<0.05$).

No significant differences in the response of any of the 21 sera to Prn1, Prn2 or Prn3 were observed. Interestingly, the response of 16 out of 21 sera was significantly higher to the C-terminal Prn mutant compared to the response with Prn1 ($P=0.004$) (Fig. 3A). Furthermore, the response of 10 out of 21 sera was significantly higher to the N-terminal Prn mutant compared to the response to Prn1 ($P=0.0001$) (Fig. 3A). From these 10 sera, the reaction of 9 sera was also significantly higher to the C-terminal mutant. Although the response of 3 out of 21 sera showed a decrease in the reaction with the Prn-R1-KO compared to Prn1, none of these decreases was significant ($P=0.419$). Although the response of 6 out of 21 of the sera to the Prn-R2-KO was lower than to Prn1, none of these responses was significant

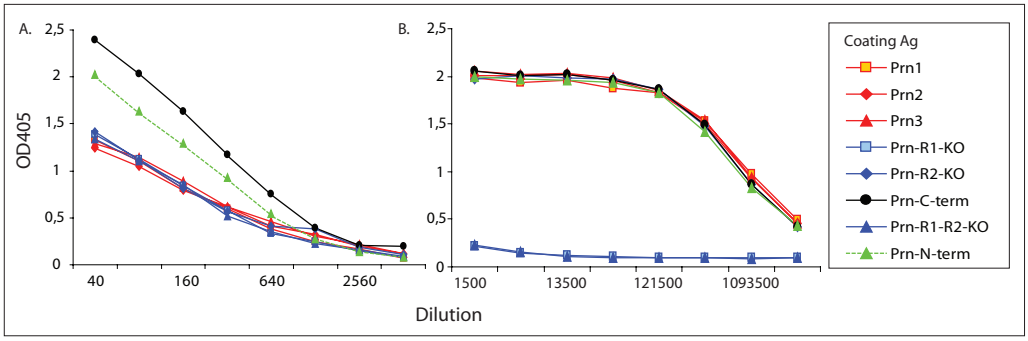

Figure 3. Response of antibodies to Prn variants and deletion derivatives. Reaction of (A) Randomly selected human serum and (B) mAb Pem72 to Prn variants and deletion derivatives. The OD405 is indicated on the Y-axes, and the Ab dilution on the X-axes.

(P=0.143). No differences were observed in binding of mAb PeM72 to Prn1, Prn2, Prn3, Prn-R2-KO, and the N- and C-terminal mutants. This indicated that the amount of protein coated in ELISA was similar for all proteins (Fig. 3B).
Since the amount of information about the sera was limited, no correlations between Ab responses and e.g. the type of strain causing the infection could be established.

Anti-Prn Abs response of Finnish sera

The response of 36 Finnish sera from patients that were vaccinated with Prn1 or infected with *B. pertussis* Prn2, Prn3 or *B. parapertussis* strains was tested to Prn1, Prn2, Prn3 and the Prn-R1-KO. No significant differences in the Ab responses to these variants were found. From 8 of these sera the response was also tested to the Prn-R2-KO, Prn-R1-R2-KO, and N- and C-terminal Prn mutants. Similar as described for the response of the Dutch sera, the response of 7 out of 8 sera to the N-terminal Prn mutant was significantly higher then the response to Prn1. All of the 8 sera showed a significant increase to the C-terminal Prn mutant. Interestingly, 4 of the 8 sera showed a significant decrease in binding to the Prn-R1-R2-KO mutant, but not to the Prn-R1-KO, indicating that Abs directed to region 2, but not region 1 were present. From these 4 sera, 3 were from children that had just been vaccinated (data not shown) indicating that vaccination with Prn1 induces a relatively large amount of Abs directed against region 2. No further correlations were found between the Prn type of the strain causing the infection and the direction of the Ab response.

Depletion of human sera

A set of 39 samples, comprising Dutch and Finnish human sera, were depleted in a similar manner as the rabbit sera. The titers of the human sera varied between 15 and 3,798 EU/ml. Unless stated otherwise, no differences in the reaction of the depleted sera were observed with the different Prn-coating antigens. In 38 out of 39 depletions, the response of the human sera depleted with the N-terminal Prn mutant was similar as the response of the

undepleted (PBS) serum (Fig. 4A+B). This could indicate that a very large amount of Abs is directed against the N-terminus of Prn. Only when the sera that were depleted with the N-terminal Prn mutant were tested with the N-terminal Prn mutant as coating antigen in an ELISA, the response was abolished, indicating that the depletions were successful. The only serum sample that after depletion with the N-terminal mutant had no remaining Abs, had the lowest anti-Prn IgG titer (15 EU) of all the sera tested.
In 6 out of 39 sera, a residual activity of approximately 15% was observed after depletion with mutants Prn-R1+R2-KO and the C-terminal Prn mutant. This response was abolished

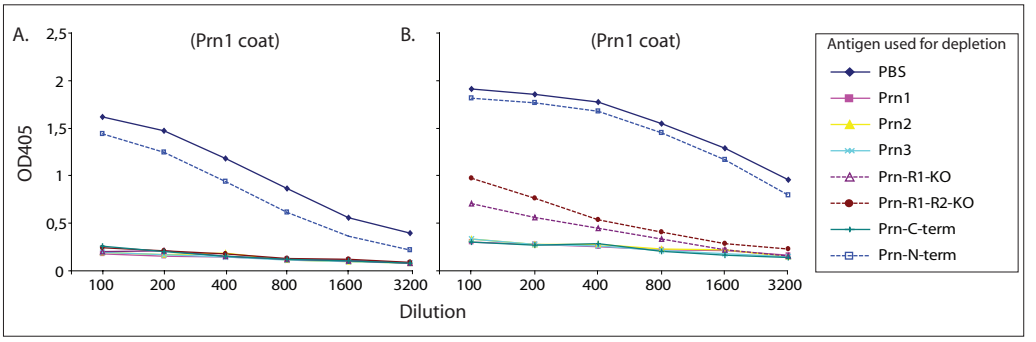

Figure 4. Reaction of 2 human sera to Prn1 after depletion with several Prn variants and deletion derivatives. The OD405 is indicated on the Y-axes, and the Ab dilution on the X-axes.

when either the Prn-R2-KO or the C-terminal mutant was used as coating antigen, indicating that the Abs were directed against R2. Of these 6 sera, 3 were from children that had just been vaccinated. Furthermore, the IgG titers of these 3 sera were among the highest. In 2 of the 39 sera, a response was observed after depletion with the Prn-R1-KO or the Prn-R1-R2-KO (Fig. 4B). When the Prn-R1-KO or the Prn-R2-KO was used as coat in an ELISA, the response was halved. Only when the Prn-R1-R2-KO was used as a coating antigen, the response was abolished. This indicated that this serum contained Abs directed against both variable regions. In contrast to the results obtained with the rabbit sera, depletions of human sera with Prn1, Prn2 or Prn3, fully depleted the sera from Abs (Fig. 4A+B). Besides the observation that vaccination induces a large amount of Abs (15%) against region 2, no correlations were found between the Prn type of the strain causing the infection and the specificity of the Ab response.

Discussion

There has been a resurgence of pertussis in several countries, including the Netherlands. One of the factors which may have contributed to this re-emergence is the variation in Prn, a protein that has been shown to elicit protective immunity in both animals and humans [19, 20]. Most variation in Prn is found in R1 which is comprised of GGXXP repeats and is located proximal to the N-terminus. Of the 3 most prevalent Prn types, Prn2 is the most distinct since it contains 1 additional repeat. Variation between Prn1 and 3 is limited to 2 AA. In this study we investigated the antibody response of rabbits and humans, immunized or infected with different Prn variants. The results are summarized in figure 1A.

In a first attempt to identify Prn specific Abs, we tested the response of rabbits vaccinated with Prn1, 2 or 3. Using a depletion assay we were able to detect the Abs of interest. Depletion of rabbit P1, P2 and P3 sera, revealed that P2 serum contained 3-4 times more R1 specific Abs compared to P1 and P3 serum (Fig.2B). It is possible that the larger number of repeats in R1 of Prn2 caused this region to become more exposed and therefore more immunogenic compared to the R1 of Prn1 and Prn3. In contrast, rabbit P1 and P3 sera contained almost 2 times more Abs directed against the N-terminus of Prn than anti Prn2 sera (Fig. 2C). We previously hypothesized that the variable R1 acts as a shield that hides important epitopes located in the N-terminus from the immune system Hijnen *et al.* Submitted. Region 1 appears as a loop that protrudes from the backbone of Prn, and folds towards the N-terminus [34]. The R1 of Prn1 covers at least parts of 4 loops in the N-terminus (Fig. 5). It is likely that the increased size of the Prn2-R1 covers an even larger part of the N-terminus than the R1 of Prn1 and 3 (Fig. 5). The observation that Prn2 induces more Abs directed against R1, but less to the N-terminus (compared to Prn1 and Prn3), supports this line of reasoning. When rabbit P2 serum was depleted with the Prn-R1-KO, and subsequently the reaction of the remaining Abs was tested to Prn1, Prn2, and Prn3, the lowest response was observed with Prn2 (Fig. 2B). Apparently Prn2 evolved a way to circumvent the increased immunogenicity of its R1. The amount of binding energy needed for an interaction between a highly flexible loop and an Ab is likely to be larger than the amount of energy needed for an interaction between a conformationally stable defined epitope and a mAb. It is possible

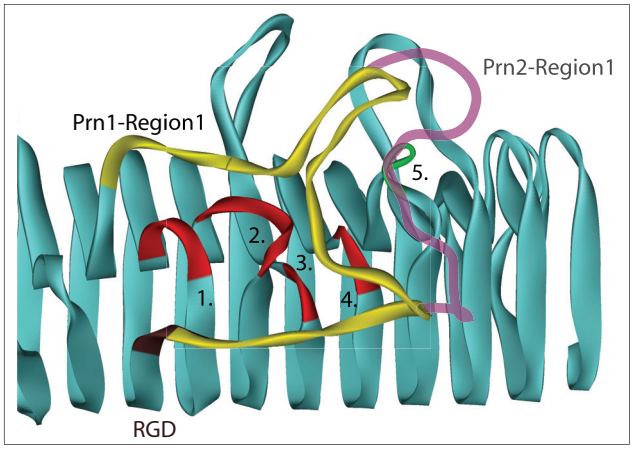

Figure 5. The Crystal structure of Prn. The R1 of Prn1 is indicated. A hypothetical R1 of Prn2 is indicated in transparent pink. The loops that are covered by the R1 of Prn1 are numbered 1-4 . The extra loop that possibly will be covered by the R1 of Prn1 is numbered 5.

that R1, due to its increased size is more flexible and that therefore Abs pay a significant entropy penalty to bind to this flexible region. This could explain the observation that the reaction of P2-R1 Abs is higher to the R1 of Prn1 and Prn3 then that of Prn2, since the amount of energy needed for this interaction is possibly lower.

Pertactin type specific Abs were only observed when rabbit P3 serum was depleted with Prn1. Interestingly, these Abs were unable to bind to any of the Prn deletion mutants. This was rather unexpected since Prn1, Prn2 and Prn3 only vary in R1. This observation however, is in line with our previously described hypothesis that the two variable regions interact [31,(Hijnen *et al.* Submitted)]. However, due to structural limitations this cannot be a direct interaction. It is more likely that the N- and C-termini are interacting and the variation in R1 and R2 indirectly affects this interaction.

Due to the close proximity of R1 (located in the N-terminus) and the C-terminus (Fig. 6), it is possible that Abs are induced that recognize a conformational, type-specific, epitope that is comprised of both R1 and the C-terminus. It is conceivable that when either R1, R2, the N- or C-terminus are deleted, the conformational epitope is destroyed, explaining that Prn3-specific Abs are not able to bind to either one of these deletion mutants.

To test whether similar antibody responses were induced in humans after infection or vaccination, identical experiments were performed with human sera. When the binding of Abs from human sera were tested to Prn1, Prn2 and Prn3 in a regular ELISA, no differences were measured. In contrast to the rabbit sera, depletion of human serum with Prn1, Prn2 or Prn3 did not reveal Prn type specific Abs. Possibly, Prn specific Abs are not induced in humans, or the amount of these Abs is so small that they are not detectable. Even if Prn-type-specific Abs are induced in humans, the role of the Abs is questionable. We were only able to detect Prn-specific Abs in Prn3 immunized rabbits whereas humans are vaccinated with Prn1, which did not induce Prn-specific Abs in rabbits. Furthermore, infections with Prn3 strains are very rare.

When we tested the response of human sera to N- and C-terminal mutants, an unexpected result was found. From the 30 sera tested, 24 sera reacted significantly better to the C-terminal mutant, and 17 of the 30 sera reacted significantly better to the N-terminal mutant compared to their reaction to Prn1 (Fig. 2A). This result seems to contrast with the results obtained from the human serum depletion assay. In 38 out of the 39 cases when a human serum was depleted with the N-terminal mutant, almost all of the Abs remained present. In contrast, only in 6 of the 39 depletions with the C-terminal mutant, Abs remained present. These results may indicate that deletion of the N-terminus results in the exposition of additional epitopes allowing an increased Ab binding. Apparently, some regions of Prn are immunogenic, but not very well accessible to antibodies in native Prn. The second result however, suggests that a large amount of Abs is directed against the N-terminus since depletion with this mutant did not result in a decrease of Ab binding. The latter result is consistent with one of our previous studies where 6 of the 17 mapped epitopes recognized by human sera were located in the N-terminus of Prn [31]. Although these results

seem contradictory, they could be explained as follows. The N-terminus harbors important discontinuous conformational epitopes to which Abs with a high affinity are induced [31,(Hijnen *et al.* Submitted)]. Abs binding to this region block the subsequent binding of other Abs. When in an ELISA the response of sera is tested to the N-terminal mutant, the binding of Abs to other parts of Prn is not blocked by Abs directed against the N-terminus, resulting in a higher response. When the N-terminal mutant is used in a depletion assay, the Abs directed against the discontinuous conformational epitopes in the N-terminus remain present in the sample. When subsequently the depleted sample is tested to Prn variants, still a large response is measured.

It is possible that a similar mechanism as just described for the N-terminus is applicable for the C-terminus. However, in contrast to the N-terminus, only a small amount of human sera contained Abs directed against the C-terminus. Although the tertiary structure of the C-terminus has not been determined, structure prediction programs predicted it to be a flexible linear coiled region. It is conceivable that the C-terminus folds into the direction of the N-terminus, thereby covering a large part of the molecule. This hypothesis was confirmed when the tertiary structure of P.69 Prn1 including the C-terminus was predicted by the Robetta server (Fig. 6) [32-35]. We previously showed that the center and the N-terminus of

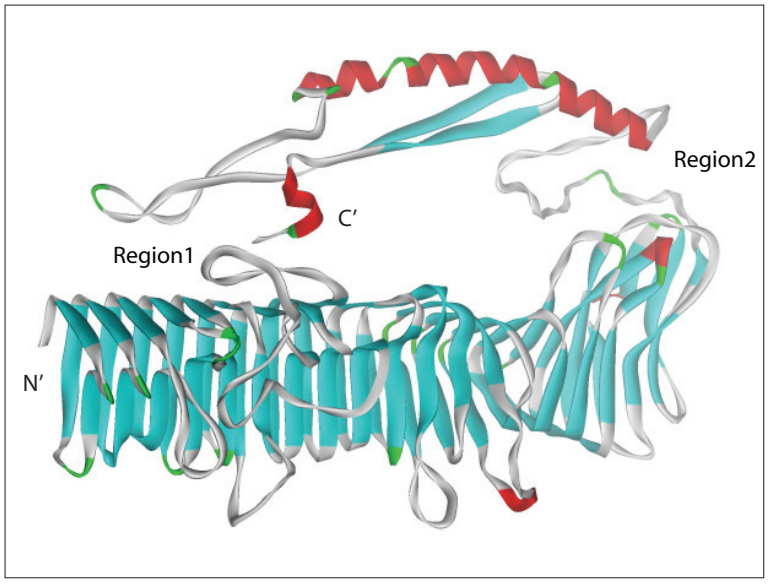

Figure 6. The predicted tertiary structure of Prn including the C-terminus. The tertiary structure of the entire passenger domain of P.69 Prn1 was determined by the Robetta server with the published crystal structure as a reference.

Prn harbor several conformational epitopes to which Abs with a high affinity are induced. It is possible that the C-terminus blocks the binding of these Abs. When the C-terminus is deleted, the binding of Abs directed against the remainder of the protein will not be blocked, resulting in a higher response then obtained with the entire protein.

A second explanation remains for the results obtained with the N-terminal Prn mutant. It is possible that the tertiary structure of the N-terminal mutant is affected by the deletion

of the 110 N-terminal AAs. This could result in the inability of Abs directed against conformational epitopes to bind. A depletion assay with this mutant could result in the depletion of Abs directed to linear epitopes. When subsequently the binding of this depleted serum was tested to Prn1, Abs directed against conformational epitopes would be able to bind. This would indicate that antibodies directed against linear epitopes are induced in large numbers, which block the binding of antibodies directed against conformational epitopes. The latter are often considered to be more important. However, it was possible to partly deplete rabbit sera with this mutant, which speaks against this hypothesis.

Together, these and previously described results, explain the effects of variation in region 1 on the antibody response. The variation does not only affect the Ab response directed against region 1 as suggested by us previously, but rather affects Ab binding to the N-terminus of Prn. As previously described, Prn2 infected patients do not develop Abs that block the binding of mAbs PeM1 and PeM5 to Prn [22]. Recently, PeM5 was found to recognize an N-terminal loop as its primary binding site and not a strictly R1 dependant epitope [Hijnen *et al.* Submitted]. The binding of PeM1 was found to be affected by deletion of parts of the N- and C-terminus, but not by deletion of region 1. However mutation of the RGD site led to an increase and decrease in binding of PeM1 and 5 respectively, suggesting the epitopes of these mAbs are located in the vicinity of region 1. Since the Prn2-region 1 is larger than that of Prn1 and 3, no, or less Abs are induced against the epitopes recognized by mAbs PeM1 and 5, which could explain that no blocking was observed due to improved conformational masking. Depletion of rabbit sera showed that, compared to Prn1 and 3, Prn2 induces more Abs against region 1, and less Abs directed to the N-terminus. These results support our hypothesis that variation in region 1 affects the Ab response directed against the N-terminus.

It is feasible that Prn1 induces antibodies to epitopes that are present in Prn2 but are not exposed due to the conformational masking by the larger region 1 of Prn2. Therefore, in theory, immunization with Prn2 would be more efficient, as this would not induce Abs to epitopes that are hidden in Prn1 or Prn2. These results could facilitate the development of new pertussis vaccines that direct the Ab response to epitopes that are exposed in all Prn variants.

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7 Differential protection of the *Bordetella pertussis* protein Pertactin studied in two mouse models

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Abstract

A resurgence of *Bordetella pertussis*, the causative agent of whooping cough, has been observed in several countries with a traditional high vaccine uptake. Variation in P.69 Pertactin has been proposed to be partially responsible for this re-emergence. In this study we investigated if there is a differential protection against Prn1 and Prn2 strains after immunization with several Prn variants and Prn deletion derivatives. When low doses of Prn were used to immunize mice, a clear difference in the protection against these 2 Prn variants was observed. Furthermore, the role of the variable region 1 and the N- and C-termini in the evasion of humoral immune responses was investigated. Our results suggest that when immunity wanes, variation in Prn could lead to an increased risk of infection.

Introduction

Before widespread introduction of vaccination against pertussis, almost every child contracted this disease. Although vaccination against pertussis has been highly successful in reducing morbidity and mortality, it has remained one of the 10 most common causes of death from infectious diseases worldwide [1].

In many countries the traditional whole cell vaccines (WCV's) are gradually being replaced by the less reactogenic acellular vaccines (ACV's). The ACV's are composed of different amounts and combinations of pertussis toxoid, P.69 Pertactin 1 (P.69 Prn1), filamentous hemagglutinin (FHA) and Fimbriae (Fim2 and 3).

Despite high vaccination coverage, the incidence of pertussis increased significantly during the last 10 years in many countries including the Netherlands [2-12]. A number of explanations were proposed for the resurgence of pertussis, including increased reporting, improved diagnosis of the disease, waning immunity and the adaptation of the *B. pertussis* population to vaccination [13, 14]. Pathogen adaptation has probably played an important role in the resurgence of pertussis in the Netherlands [14, 15]. When clinical isolates were analysed that were collected during the last 50 years, polymorphisms were observed in at least two proteins implicated in protective immunity: P.69 Prn and pertussis toxin (Ptx) [15, 16]. Both proteins play an important role in protective immunity. Antibody levels to P.69 Prn and Ptx have been shown to correlate with clinical protection [17-19]. Passive and active immunization studies in mice and pigs have shown that antibodies against P.69 Prn confer protective immunity [20, 21]. Anti- P.69 Prn antibodies, but not anti-Ptx, anti-fimbriae, or anti-FHA antibodies, were found to be crucial for *B. pertussis* phagocytosis [22]. Furthermore, ACV's containing Ptx, FHA and P.69 Prn were more effective compared to ACV's containing Ptx and FHA only. Finally, the efficacy of the Dutch whole cell vaccine was shown to be affected by variation of P.69 Prn in a mouse model [20]. These results underline a particular important role for Prn in the induction of protective immunity [23-26].

P.69 Prn, an autotransporter protein, is located at the cell surface of *B. pertussis*. P.69 Prn is polymorphic but variation is mainly limited to two regions, designated region 1 and 2, which are comprised of Gly-Gly-X-X-Pro and Pro-Gln-Pro repeats, respectively (Fig. 1). A total of 13 P.69 Prn types have been isolated. In the Netherlands, the 3 most common *B. pertussis* P. 69 Prn types are P.69

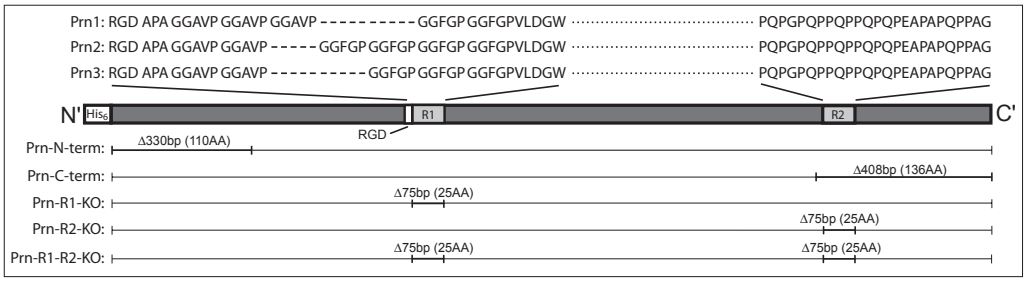

Figure 1. Structure of 3 Prn variants and deletion derivatives. Amino acid sequence of the variable region R1 and R2 are indicated. Prn1 and Prn2 differ in 2 amino acids whereas Prn2 contains an additional repeat. The location and size of N-terminal, C-terminal and central deletions are indicated. R1, R2 and RGD indicate the variable regions 1, 2 and the RGD receptor binding motif, respectively. Abbreviations: AA, amino acid. Bp, base pair, His_6, extension of the N-terminus with 6 His residues.

Prn1, P.69 Prn2 and P.69 Prn3. P.69 Prn1 and P.69 Prn3 only differ in 2 amino acids (AA's) in 1 of the repeats in region 1, whereas P.69 Prn2, the predominant type during the last 10 years, contains an additional GGFGP repeat (Fig. 1). The C-terminally located variable region 2 is identical in P.69 Prn1, 2 and 3. It has been shown that Prn functions as an adhesin, a function which is mediated, at least in part, by an N-terminally located RGD site [27, 28] (Fig. 1). Most of the variation in *B. pertussis* Prn found adjacent to this site.

A number of studies have investigated the role of variation in P.69 Prn on vaccine efficacy in a mouse model with conflicting results [6, 20, 26]. Several reasons may underlie these discrepancies. First, in general extremely large doses of vaccines were used in the mouse model. Further, after vaccination mice were challenged with wild type strains which may differ at many (unknown) loci. Another drawback of these studies is that only the P.69 Prn1 type was used. In this study the naturally occurring Prn variants P.69 Prn1, P.69 Prn2 and P.69 Prn3 were used to immunize mice. Further, we investigated the effect of different doses of antibodies and antigens on differential protection against Prn1 and Prn2 strains. Finally, mice were challenged with isogenic strains which differed only at the Prn locus

Materials and Methods

Recombinant Pertactin

Recombinant His-tagged P.69 Prn 1, 2 and 3 and Prn deletion mutants were expressed and purified as previously described [29].

Rabbit polyclonal antisera

Polyclonal rabbit anti- P.69 Prn1, 2, 3 and Prn-R1-KO sera were raised at the Eurogentec facility (Eurogentec, Seraing, Belgium). New Zealand white Rabbits were immunized 3 times with 50µg of purified P.69 Prn per immunization using Freund's complete adjuvant for the first immunization, followed by two booster immunizations in Freund's incomplete adjuvant. The rabbit anti sera were affinity purified using the same antigen that was used for immunization.

Active immunization

Determination of the optimal dose for immunization

To determine the optimal dose of P.69 Prn for use in the mouse model, a dose-response experiment was performed. Groups of 8 BALB/c mice (Harlan, Horst, the Netherlands) were immunized 3 times subcutaneously with various concentrations of purified recombinant P.69 Prn1 mixed with 20µg of the adjuvant QuilA (Spikoside) (Iscotec AB, Stockholm, Sweden). Groups 1-7 received respectively; 40µg, 1µg, 0.2µg, 0.02µg, 0.004µg, 0.002µg and 0µg of P.69 Prn1. These amounts corresponded to, respectively, 5, 1/8, 1/40, 1/400, 1/2000 and 1/4000th human dose (HD) based on the acellular vaccine produced by GSK which contains 8µg of P.69 Prn1 per dose.

Immunization of mice with P.69 Prn variants and deletion derivatives

Mice were immunized three times subcutaneously with 0.02 µg P.69 Prn1, P.69 Prn2, P.69 Prn3 or Prn deletion derivatives (Fig. 1). The Prn preparations were adjuvated with 20µg QuilA. Two weeks after the final immunization, mice were challenged as described below.

Intranasal infection of mice

For the infection of mice, 2 isogenic *B.pertussis* Tohama strains were used harboring *prn1* (B1584) and *prn2* (B1586), respectively (manuscript in preparation). To be able to discriminate between the 2 strains, a kanamycin (*kan*) and gentamycin (*gen*) resistance gene was inserted into the *fim3* gene of *B. pertussis* Prn1 and *B. pertussis* Prn2, respectively.

To increase the reproducibility, mice were infected with a single batch of suspensions containing B1584 and B1586. These suspensions were prepared as follows. Bacteria were cultured on Bordet Gengou agar plates, supplemented with 1% glycerol, 15% sheep blood. For culturing B1854 (Prn1, Kan) and B1586 (Prn2, Gen) strains, 50µg/ml kanamycin and 10µg/ml gentamycin was added to the plates respectively. Plates were incubated at 35°C for 24 hrs, after which a number of colonies were transferred to a new plate without antibiotics. The BG plates were incubated at 35°C for 24hrs, after which bacteria were suspended in Verwey medium and the optical density at 600nm (OD) was adjusted to approximately $1.0*10^{10}$ bacteria/ml. Aliquots of the bacterial suspension were mixed with

15% glycerol and were flash-frozen in a mixture of ethanol and dry ice. The aliquots were stored at -80°C. Prior to infection, the viability of the frozen bacteria was determined and used to coinfect mice intranasally with $1.0*10^7$ CFU's of each strain. Three days after the challenge, mice were sacrificed and the blood and lungs were collected. The number of CFU's were counted as described previously [20] on plates containing either kanamycin or gentamycin.

Passive immunization

Determination of the optimal dose for passive immunization

Similar as described for the active immunizations, we first determined the optimal dose for passive immunization with rabbit Abs directed against P.69 Prn1. A total of 8 groups (group1-8) of 10 mice were immunized. Groups 1-6 received respectively; 400µg, 100µg, 50µg, 5µg, 1µg or 0.1µg of anti-Prn1 Abs. As a control, mice received either PBS, or 100µg of Abs from a non-immunized rabbit. One day after immunization, mice were challenged with a mixture of the 2 isogenic strains as described above. Three and 7 days after the challenge, mice were sacrificed and the number of CFUs in the lungs was determined.

Passive immunization with antibodies directed against P.69 Prn variants and Prn deletion derivatives

Mice were immunized with purified antibodies directed against P.69-Prn1, 2, 3 and a Prn region 1 deletion derivative (Fig. 1). The results were compared with PBS vaccinated mice. To normalize the amount of antibodies against the different Prn molecules, titers were determined with an ELISA. The titers of the purified Abs to P.69 Prn1 coated plates were determined. Dilutions that yielded similar titers in the ELISA were used to pre-dilute the sera before passive vaccination.

Aerosol challenge protocol

Bacterial suspensions (*B. pertussis* B1584, B1586), stored at -70°C in 5% glycerol, were cultured as described above. Bacteria were harvested and resuspended in 0.9% saline containing 1% casein. The OD623 of the bacterial suspension was adjusted to an OD of 0.2 and kept on ice until it was used in the aerosol challenge. Aerosol challenge was performed on groups of 10 BALB/c mice (Harlan, Horst, the Netherlands). Mice were exposed to the mixture of the 2 *B. pertussis* strains during 5 min by use of a custom-made aerosol apparatus [30]. Three and 7 days after the challenge, mice were sacrificed and the blood and lungs were collected. CFU counts from the lungs were made as described previously [31].

ELISA

The binding of serum antibodies from immunized mice was tested in an enzyme-linked immunosorbent assay as described previously [32]. Polystyrene 96-well ELISA plates (Immulon II; Dynatech, Chantilly, Va.) were coated overnight at 22°C by adding 100 µl of 0.04 M carbonate buffer pH 9.6 containing 2 µg of Prn1/ml to each well. The plates were washed four times with 200 µl of PBS supplemented with 0.05% Tween 20 (PBST) per well by using a Titertek Plus M96V washer (ICN, Irvine, CA.). Sera were added to the wells and were incubated for 2 h at 37 °C followed by four washings as described above. Bound antibodies were detected with HPO-conjugated rabbit anti-mouse total IgG (DakoCytomation, Glostrup, Denmark). The optical density at 450 nm (OD450) was measured

with a plate reader (EL312e; BioTek Systems, Winooski, Vt.). The dilution at OD450=1.5 was used as the ELISA titer.

Statistical analysis

Mice were infected with a mixture of P.69 Prn1 and P.69 Prn2 CFU. To correct for a difference in the amount of Prn1 or Prn2 CFU in the inoculum, the ratio of Prn1/Prn2 CFU in the lungs of PBS vaccinated mice was used to correct the data set. To this purpose, CFUs of the Prn2 infected mice were multiplied by the Prn1/Prn2 ratio of the control mice. The means and SEMs were calculated from the Log10 CFU. The Log protection values were calculated by subtracting the Log10 CFU of individual immunized mice from the mean Log10 CFU of non-immunized mice.

Since mice were infected with a mixture of 2 isogenic strains, it was possible to accurately calculate a differential protection against Prn1 and Prn2 strains per mice. The P1-P2 value was calculated by subtracting the Prn2 Log10 CFU counts from the Prn1 Log10 CFU counts. If mice were equally well protected against Prn1 and Prn2 strains, the P1-P2 value would equal to "0". If mice were better protected against Prn1 compared to Prn2 strains, the P1-P2 value would be <0. If mice were better protected against Prn2 compared to Prn1 strains, the P1-P2 value would be >0.

Differences in the P1-P2 ratio and, differences in the Log10 CFU between groups were assessed by an unpaired two-sample unequal variance T-test. *P* values below 0.05 were regarded as statistically significant.

Results

From here on, we will use the following nomenclature: P.69 Prn1, 2 or 3 will be designated Prn1, Prn2 or Prn3 respectively. The Prn region 1, 2 and 1+2 deletion mutants will be termed Prn-R1-KO, Prn-R2-KO and Prn-R1-R2-KO, respectively. The Prn mutants lacking parts of the C- or N-terminus will be referred to as Prn-C-term and Prn-N-term, respectively. The rabbit anti-P.69 Prn1, 2, 3 or R1-KO Abs will be referred to as P1, P2, P3 or P-R1-KO Abs, respectively. Actively immunized mice were intranasally challenged with the Prn1 and Prn2 strains. Mice that were passively vaccinated were challenged with the Prn1 and Prn2 strain using the aerosol model.

Active immunization

Dose response active immunization

Previous work showed that differences in protection against Prn1 and Prn2 were only detected at relative low doses of a WCV [20]. Therefore, we first established the dose of Prn1 which showed the largest differential protection against Prn1 and Prn2 strains. To this purpose, mice were vaccinated with a dose of Prn1 ranging from 5 to 1/4000th HD and subsequently challenged with a suspension containing a mixture of isogenic Prn1 and Prn2 strain. PBS vaccinated mice served as control.

All doses, except the lowest (0.004µg), conferred significant protection against both Prn1 and Prn2 strains compared tot the PBS control mice (Fig. 2). As expected, lower doses of Prn1 resulted in less protection against both strains.

A clear correlation was observed with between the anti-Prn titers induced by immunization and the amount of protection against infection (Fig. 2). In mice immunized with 40, 1 or 0.2µg Prn1, high Ab titers against Prn1 were measured. No significant differences in the Ab titers were observed between these 3 groups (Fig. 2). In mice immunized with 0.02µg Prn1, the anti-Prn1 titers were 10 times lower compared to the mice immunized with a 0.2µg Prn1 or higher (Fig. 2). Interestingly, in

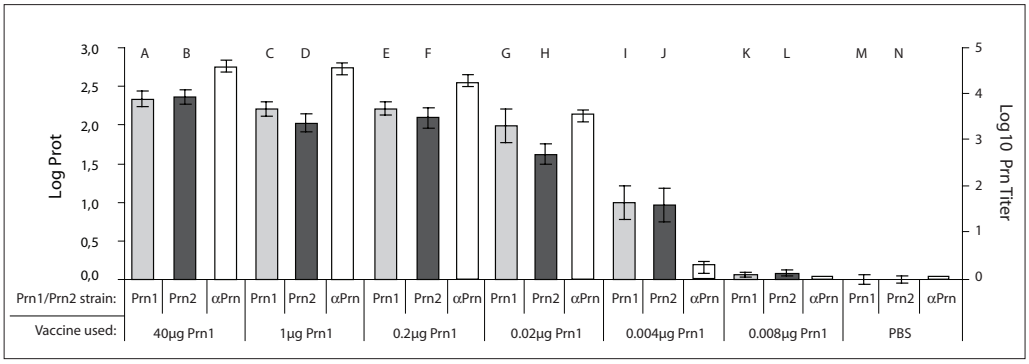

Figure 2. Log Protection levels after active immunization with different doses of Prn1.
Mice were immunized with different doses of Prn1 and challenged with a mixture of two isogenic strains producing Prn1 and Prn2, respectively. The Log protection values were calculated by subtracting the Log10 CFU of individual immunized mice from the mean Log10 CFU of non-immunized mice. The titers of anti-Prn antibodies are indicted by the open columns. P values were as calculated for the Prn1 and Prn2 columns A-L, relative to the non-immunized mice (columns M and N). A:1.1*10^{-9}, B: 8*10^{-10}, C: 2.3*10^{-07}, D: 5.1*10^{-06}, E: 1.4*10^{-10}, F: 7*10^{-07}, G: 5.9*10^{-05}, H: 3.7*10^{-06}, I: 0.289, J:0.327, K:0.385, L:0.203. Titers indicate the ELISA dilution at OD405 = 1.5. Abbreviation, αPrn, anti-Prn1 antibody titer

mice immunized with 0.004µg Prn1, although no Ab response against Prn1 was detected, a decrease of 1.0 Log10 in the number of CFU was observed compared to unvaccinated mice suggesting that even at his low dose some immunity was induced. In mice immunized with a 0.008µg Prn1 or PBS, no Ab titers against Prn1 were detected, which is consistent with the lack of protection in these 2 groups (Fig. 2).

Differential protection was observed when 1, 0.2 and 0.02µg of Prn1 was used to vaccinate mice (Fig. 3). In all cases vaccination with Prn1 protected better against the Prn1 strain compared to the Prn2 strain. However, only when 0.02µg of Prn1 was used to vaccinate mice, a significant difference in differential protection was observed with the PBS control (Fig. 3). Based on these results, the subsequent experiments were performed with 0.02µg of purified Prn types and its deletion derivatives.

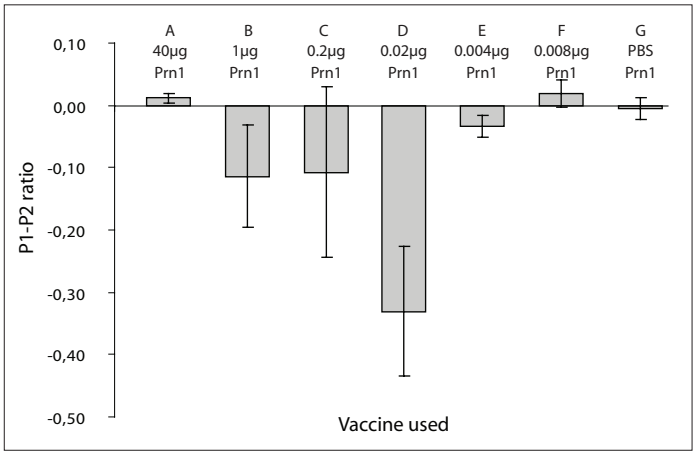

Figure 3. Differential protection against Prn1 and Prn2 strains in mice immunized with Prn1. Mice were immunized with different doses of Prn1 and challenged with a mixture of two isogenic strains producing Prn1 and Prn2, respectively. The P1-P2 ratio was determined by subtracting the Log10 Prn2 CFU from the Log10 Prn1 CFU. Statistically significant P values are displayed as column X versus column Y (XY:). AD: 0.008, DE: 0.013, DF: 0.006, DG: 0.008.

Active immunization with Prn variants

Three experiments were performed in which mice were immunized with different Prn types and mutant derivatives. The data from these three experiments were pooled. To compare the efficacy of the three natural Prn variants (Prn1, Prn2 and Prn3) against Prn1 and Prn2 strains, mice were vaccinated with 0.02µg of the Prn variant. Further, to identify domains of Prn which affected immunity, mice were also vaccinated with deletions mutants derived from Prn. After immunization, mice were challenged with a suspension containing the isogenic Prn1 and Prn2 strains. The experimental set up allowed us address several questions. First, the level of protection induced by the Prn variants and its deletion derivatives against, respectively, Prn1 and Prn2 strains. Second, the differential protection conferred by these vaccines against Prn1 and Prn2 strains.

Protection induced by the Prn variants and their deletion derivatives against Prn1

All three natural Prn variants conferred significant protection against the Prn1 strain compared to the PBS control (P from Prn1, Prn2 and Prn3, $1.4*10^{-19}$, $4.2*10^{-28}$ and $5.2*10^{-17}$, respectively) (Fig. 4). The degree of protection conferred by the three natural Prn variants, Prn1, Prn2 and Prn3, against the Prn1 strain was not significantly different. All of the Prn deletion derivatives induced

significant protection against Prn1 strains compared to the PBS control (Fig. 4). The P values for the Prn-R1-KO, Prn-R2-KO, Prn-R1-R2-KO, Prn-C-term and Prn-N-term were $6.7*10^{-13}$, $1.3*10^{-18}$, $9.3*10^{-18}$, $3.0*10^{-09}$ and $3.3*10^{-06}$, respectively. The degree of protection decreased in the order Prn-R2-KO>Prn-R1-R2-KO>Prn-R1-KO>Prn-C-term>Prn-N-term. However, there was only a significant difference in the level of protection induced by the Prn-R1-KO, Prn-R2-KO and Prn-R1-R2-KO compared to Prn-N-term (P = 0.033, 0.006 and 0.017, respectively) (Fig. 4).
When protection induced by the natural Prn variants was compared with the deletion derivatives, the Prn-R2-KO was found to induce a significantly higher level of protection against the Prn1 strain compared to the level of protection induced by Prn1 (P=0.027). Furthermore, Prn2 and Prn3 were found to induce a significantly higher level of protection against the Prn1 strain compared to Prn-N-term (P=0.018 and 0.023, respectively) (Fig. 4). The differences between the other Prn variants and deletion derivatives were not significant, however.

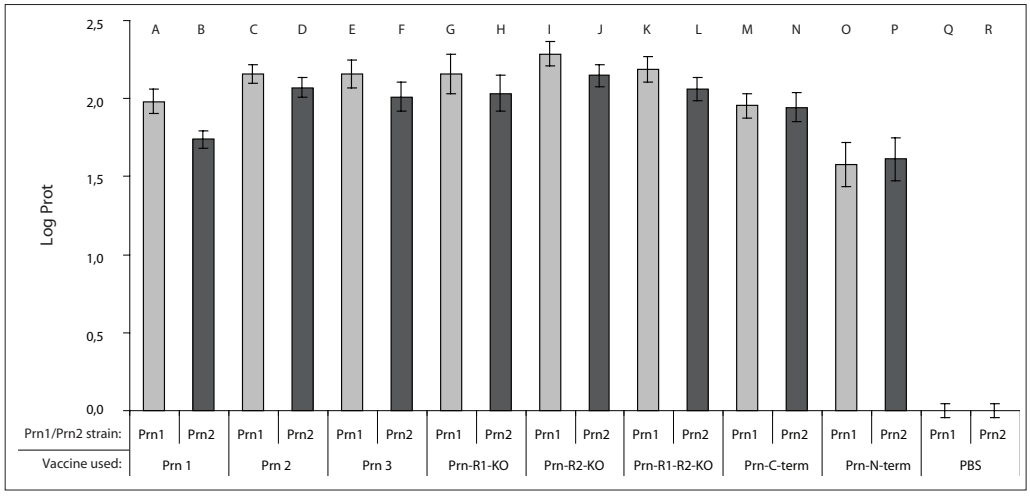

Figure 4. Log Protection levels of mice immunized with Prn variants and deletion derivatives. Mice were immunized with different Prn variants and Prn deletion derivatives and were challenged with a mixture of two isogenic strains producing Prn1 and Prn2, respectively. The Log protection values were calculated by subtracting the Log10 CFU of individual immunized mice from the mean Log10 CFU of non-immunized mice. The results are the pooled data from 3 independent experiments, except for Prn-C-term and Prn-N-term which were used in two experiments only. Statistically significant P values are displayed as column X versus column Y (XY:). AQ: $1.4*10^{-19}$, BR: $2.3*10^{-23}$, CQ: $4.2*10^{-28}$, DR: $5.9*10^{-26}$, EQ: $5.2*10^{-17}$, FR: $7.3*10^{-16}$, GQ: $6.7*10^{-13}$, HR: $4.6*10^{-13}$, IQ: $1.3*10^{-18}$, JR: $1.8*10^{-20}$, KQ: $9.3*10^{-18}$, LR: $1.8*10^{-18}$, MQ: $3.0*10^{-09}$, NR: $3.3*10^{-08}$, OQ: $3.3*10^{-06}$, PR: $1.7*10^{-06}$. AI: 0.027, BD: 0.001, BF: 0.044, BJ: 0.001, BL: 0.008. CO: 0.018, DP: 0.049, EO: 0.023, GO: 0.033, IO: 0.006, JR: 0.026, KO: 0.017.

Protection induced by the Prn variants and their deletion derivatives against Prn2

All three natural Prn variants conferred significant protection against the Prn2 strain compared to the PBS control (P for Prn1, Prn2 and Prn3, $2.3*10^{-23}$, $5.9*10^{-26}$ and $7.3*10^{-16}$, respectively) (Fig. 4). The level of protection against the Prn2 strain induced by Prn1 was significantly lower compared to the level of protection induced by Prn2 or Prn3 (P=0.001 and 0.044, respectively). The degree of protection conferred by the two natural Prn variants, Prn2 and Prn3, against the Prn2 strain was not significantly different.

All of the Prn deletion derivatives induced significant protection against the Prn2 strain compared to the PBS control (Fig. 4). The P values for the Prn-R1-KO, Prn-R2-KO, Prn-R1-R2-KO, Prn-C-term and Prn-N-term were $4.6*10^{-13}$, $1.8*10^{-20}$, $1.8*10^{-18}$, $3.3*10^{-08}$ and $1.7*10^{-06}$, respectively.
Similar as observed for the Prn1 strain, the degree of protection decreased in the order Prn-R2-KO>Prn-R1-R2-KO>Prn-R1-KO>Prn-C-term>Prn-N-term. However, there was only a significant difference in the level of protection induced by the Prn-R2-KO compared to Prn-N-term (P = 0.026) (Fig. 4). When protection induced by the natural Prn variants was compared with the deletion derivatives, the Prn-R2-KO and Prn-R1-R2-KO were found to induce a significantly higher level of protection against the Prn2 strain compared to the level of protection induced by Prn1 (P=0.001 and 0.008, respectively). Furthermore, Prn2 was found to induce a significantly higher level of protection against Prn2 strains compared to Prn-N-term (P=0.049) (Fig. 4). The differences in protection conferred by the other Prn variants and deletion derivatives were not significant, however.

Differential protection conferred by Prn variants and their deletion derivatives against Prn1 and Prn2 strains

In mice vaccinated with the natural Prn variants, the P1-P2 ratio was significantly lower compared tot the PBS control (P for Prn1, Prn2 and Prn3 vaccinated mice, respectively, $2.9*10^{-04}$, $6.7*10^{-03}$, and $6.7*10^{-05}$) (Fig. 5). This implicates that all Prn variants protected better against the Prn1 strain compared to the Prn2 strain. The largest and smallest difference in differential protection was observed between the Prn1 and Prn2 vaccinated mice, respectively. The differences in differential protection between the three Prn variants were not significant however.

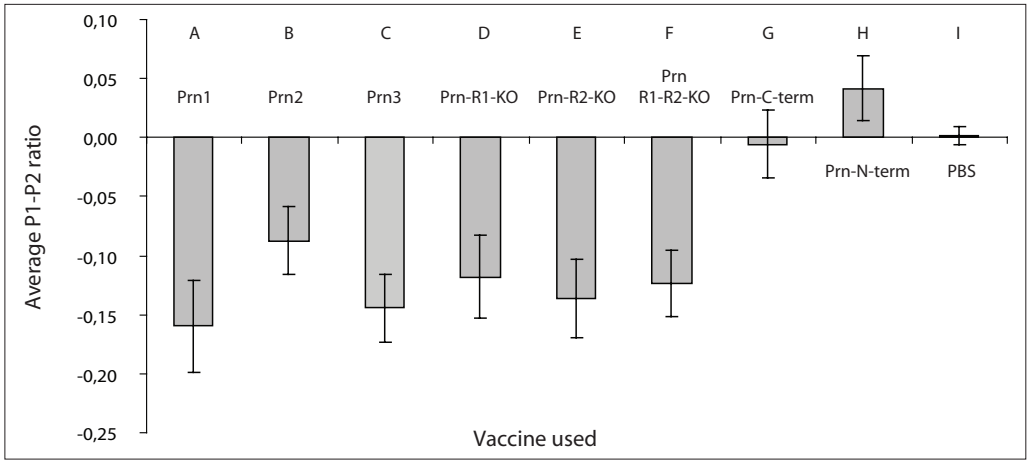

Figure 5. P1-P2 ratios of mice immunized with Prn variants and deletion derivatives. Mice were immunized with different Prn variants and Prn deletion derivatives and were challenged with a mixture of two isogenic strains producing Prn1 and Prn2, respectively. The P1-P2 ratio was determined by subtracting the Log10 Prn2 CFU from the Log10 Prn1 CFU. The results are the pooled data from 3 independent experiments, except for Prn-C-term and Prn-N-term who were used in two experiments only. Statistically significant P values are displayed as column X versus column Y (XY:). AI: $2.9*10^{-04}$, BI: $6.0*10^{-03}$, CI: $1.9*10^{-04}$, DI: $2.1*10^{-03}$, EI: $5.7*10^{-04}$, FI: $6.5*10^{-04}$. AH: $2.9*10^{-04}$, BH: $6.7*10^{-03}$, CH: $6.7*10^{-05}$, DH: $2.9*10^{-03}$, EH: $5.3*10^{-04}$, FH: $3.2*10^{-04}$. AG: 0.008, CG: 0.009, DG: 0.040, EG: 0.017, FG: 0.023.

In mice vaccinated with the deletion derivatives Prn-R1-KO, Prn-R2-KO or the Prn-R1-R2-KO the P1-P2 ratio was significantly lower compared to the PBS control (P = $2.9*10^{-03}$, $5.3*10^{-4}$ and $3.2*10^{-04}$, respectively) (Fig. 5). Furthermore, the P1-P2 ratio was significantly lower compared to the C-terminal mutant (P= 0.040, 0.017 and 0.023, respectively) and the N-terminal mutant (P= 0.002, 0.001 and 0.001, respectively) (Fig. 5). Thus these mutant derivatives all protected better against Prn1 compared to Prn2 strains. Although differences in the P1-P2 ratios were observed between the mice vaccinated with the Prn-R1-KO, Prn-R2-KO or the Prn-R1-R2-KO Prn deletion derivatives, they were not significant. The deletion derivatives Prn-C-term and Prn-N-term protected equally well against both Prn variants (Fig. 5). When the mice vaccinated with the Prn variants were compared to those vaccinated with the deletion derivatives significant differences in the P1-P2 ratio were only observed between Prn1 and 3 vaccinated mice compared to the C-terminal mutant (P=0.008 and 0.009, respectively). Furthermore, a significant difference in the P1-P2 ratio was found between Prn1, 2 and 3 vaccinated mice compared to the N-terminal mutant (P=$2.9*10^{-04}$, $6.0*10^{-03}$ and $1.9*10^{-04}$, respectively) (Fig. 5).

Passive immunization with anti-Prn antibodies

Dose response passive immunization with anti-Prn1 Antibodies

Similar as described for the active immunization, a dose response experiment was performed with rabbit P1 Abs for the passive immunizations. The numbers of CFUs in the lungs were determined at day 3 and 7. Since no significant differences were observed in the levels of protection between the vaccinated groups at day3, only the data from day 7 will be presented.

The P1 Ab doses of 400, 100, 50 and 5µg conferred significant protection against both Prn1 and Prn2 strains compared tot the PBS control mice (Fig. 6). Mice immunized with 1µg of P1 Abs were

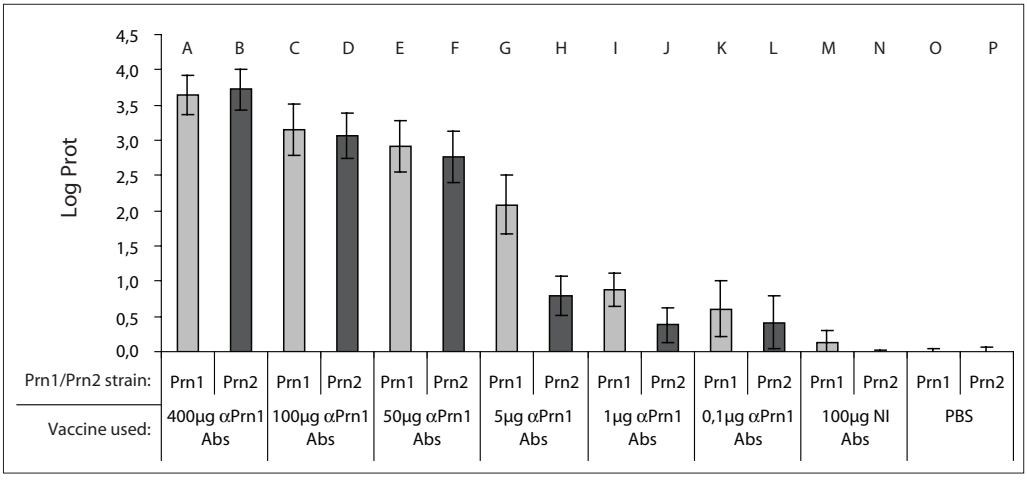

Figure 6. Log protection levels after passive immunization with Prn1 antibodies. Mice were passively immunized with different doses of Abs raised against Prn1 and were challenged with a mixture of two isogenic strains producing Prn1 and Prn2, respectively. The Log protection values were calculated by subtracting the Log10 CFU of individual immunized mice from the mean Log10 CFU of non-immunized mice. The results are the pooled data from 2 independent experiments. Statistically significant P values are displayed as X versus Y (XY:). AO: $1.4*10^{-04}$, BP: $1.5*10^{-04}$, CO: $8.6*10^{-04}$, DP: $5.6*10^{-04}$, EO: $1.2*10^{-03}$, FP: $1.3*10^{-03}$, GO: $7.4*10^{-03}$, HP: $4.3*10^{-02}$, IO: $1.8*10^{-02}$.

significantly protected against Prn1, but not against Prn2 strains (P=0.017 and 0.195, respectively). The doses 0.1µg and 100µg of P1 Abs from non-immunized rabbits did not confer protection. As expected, lower doses of P1 Abs resulted in less protection against both strains (Fig. 6).
The largest degree of differential protection was observed at a dose of 5 µg of P1 Abs. The difference with the PBS control was marginally significant (P=0.052) (Fig. 7). Based on these results, the subsequent experiments were performed with 5µg of Abs.

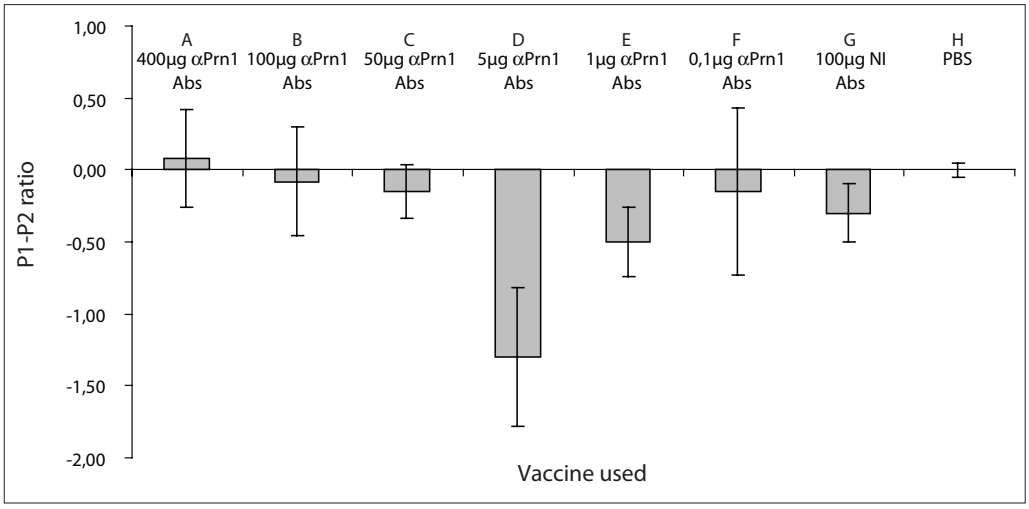

Figure 7. P1-P2 ratio of mice passively immunized with different doses of anti-Prn1 antibodies. Mice were passively immunized with different doses of Abs raised against Prn1 and were challenged with a mixture of two isogenic strains producing Prn1 and Prn2, respectively. The P1-P2 ratio was determined by subtracting the number of Log10 Prn2 CFU from the number of Log10 Prn1 CFU. Statistically significant or interesting P values are displayed as X versus Y (XY:). AD: 0.052, DH: 0.054.

Passive immunization with antibodies directed against Prn

Two experiments were performed in which mice were passively immunized with purified Abs that were raised against Prn1, Prn2, Prn3 and Prn-R1-KO. The data of both experiments were pooled.

Protection induced by the Prn antibodies against Prn1 strains

P1, P2, P3 and P-R1-KO Abs conferred a significant protection against the Prn1 strain compared to the PBS control (P= $5.0*10^{-04}$, $1.9*10^{-03}$, $4.7*10^{-02}$ and $4.0*10^{-03}$, respectively) (Fig. 8). The highest degree of protection was conferred by the P2 Abs, followed by the Abs against Prn-R1-KO, Prn1 and Prn3. These differences were not significant however.

Protection induced by the Prn antibodies against Prn2 strains

P1, P2, and P-R1-KO Abs conferred a significant protection against the Prn2 strain compared to the PBS control (P= $1.4*10^{-03}$, $6.6*10^{-06}$, and $4.6*10^{-03}$, respectively) (Fig. 8). Mice immunized with the P3 Abs were not significantly protected against the Prn2 strain compared to the PBS control (P=0.121) (Fig. 8). Furthermore, mice immunized with the P2 Abs were protected significantly

better against the Prn2 strain compared to P1 or P3 Abs (P= $2.4*10^{-04}$ and $8.2*10^{-03}$, respectively). The highest degree of protection was conferred by the P2 Abs, followed by the P-R1-KO Abs, P1 and P3 Abs (Fig. 8). However, the differences between the levels of protection conferred by P1, P3 and P-R1-KO Abs were not significant.

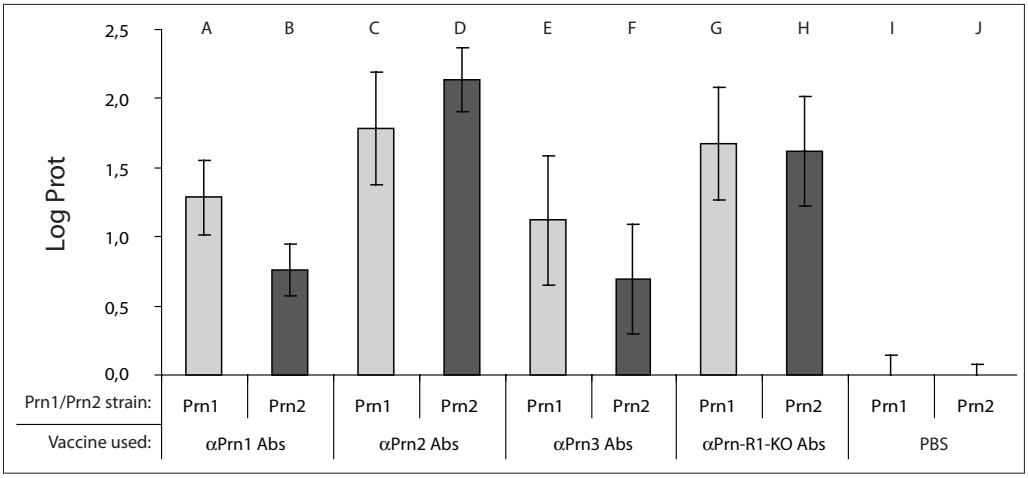

Figure 8. Log protection levels in mice passively immunized with antibodies raised against Prn variants and Prn deletion derivatives. Mice were passively immunized with Abs that was raised against Prn1, 2, 3 and Prn-R1-KO and were challenged with a mixture of two isogenic strains producing Prn1 and Prn2, respectively. The Log protection values were calculated by subtracting the Log10 CFU of individual immunized mice from the mean Log10 CFU of non-immunized mice. Statistically significant P values are displayed as X versus Y (XY:). AI: $5.0*10^{-04}$, BJ: $1.4*10^{-03}$, CI: $1.9*10^{-03}$, DJ: $6.6*10^{-06}$, EI: $4.7*10^{-02}$, GI: $4*10^{-03}$, HJ: $4.6*10^{-03}$. BD: $2.4*10^{-04}$, BF: 0.008.

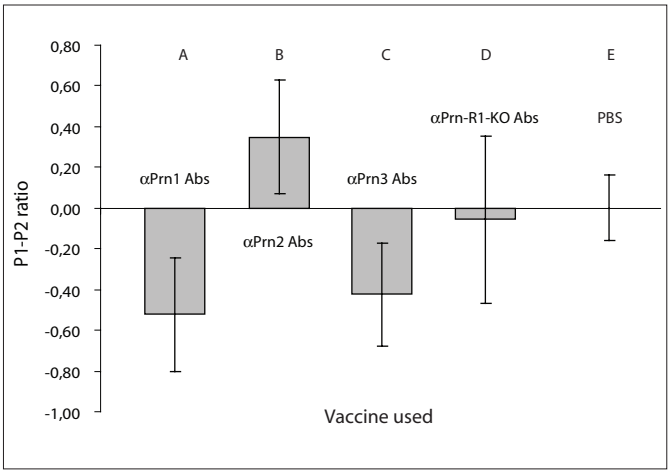

Figure 9. P1-P2 ratio of mice passively immunized with antibodies raised against Prn variants and Prn deletion derivatives. Mice were passively immunized with Abs raised against Prn1, 2, 3 and Prn-R1-KO and were challenged with a mixture of two isogenic strains producing Prn1 and Prn2, respectively. The P1-P2 ratio was determined by subtracting the number of Log10 Prn2 CFU from the number of Log10 Prn1 CFU. Statistically significant or interesting P values are displayed as X versus Y (XY:). AB: 0.039, BC: 0.056.

Differential protection conferred by Prn antibodies against Prn1 and Prn2 strains

Mice passively immunized with P1, P2 P3 and P-R1-KO Abs did not show a P1-P2 ratio that was significantly different from the PBS control mice (Fig. 9). However, P1 and P3 Abs conferred a higher degree of protection against the Prn1 strain compared to the Prn2 strain. The reverse was observed with the P2 Abs. Only the difference between the mice treated with the P1 and P2 Abs was significant (P= 0.039). The difference between mice treated with P2 and P3 Abs was just above the significance level (P= 0.056) (Fig. 9).

Discussion

In this study we investigated the effect of variation in Prn on the efficacy of vaccine-induced immunity by 3 naturally occurring Prn variants and several Prn deletion derivatives. We studied the protective properties of these Prn proteins using two isogenic *B. pertussis* strains that produce Prn1 and Prn2, respectively. Mice were co-infected with these strains and both absolute CFU's per strain and the ratio's in CFU's between the Prn1 and Prn2 strains were determined. This allowed us to accurately calculate a possible difference in protection against Prn1 and Prn2 strains per mouse.

When mice were challenged with Prn1 and Prn2 strains after immunization with different doses of Prn1, it became evident that when high vaccine doses were used, no difference in protection against Prn1 and Prn2 strains was observed. A similar phenomenon was observed when mice were passively vaccinated with high doses of rabbit anti-Prn1 Abs. Again, mice that received high doses of Abs were equally well protected against Prn1 and Prn2 strains. These results are consistent with field data which show that recently vaccinated humans are well protected against pertussis, irrespective of strain characteristics. Our hypothesis was that the small differences observed between Prn variants will only confer a selective advantage in individuals with low (or waning) immunity. Consistent with this hypothesis, only when mice were vaccinated with 1/400th human dose of Prn1 (0.02µg) or with 5µg of purified anti-Prn1 Abs, they were less well protected against Prn2 compared to Prn1 strains. In a recent report, the protective properties of 3 pertussis ACVs were tested against 6 *B. pertussis* strain that are currently circulating in Sweden [26]. Of the 3 ACVs tested, 2 contained 8µg and 3µg of Prn1, respectively. In that study no differences in protection against Prn1, 2 or 3 strains were observed after immunization with these ACVs. This is consistent with our results, that immunization with high doses of Prn or Prn antibodies did not result in differential protection against strain variants. It should also be noted that in the published study wild type strains were used which may differ at loci other than the gene for Prn, while we used well defined isogenic strains.

In absolute terms Prn1, the Prn type currently used in vaccines, was less effective against Prn2 strains compared to Prn2 and Prn3 (P=0.01 and P=0.044, respectively). Interestingly, deletion of R1, R2 or both regions did not significantly affect efficacy. Indeed, the most effective vaccine was comprised of the Prn-R2-KO (Prn1 vs. Prn-R2-KO; P=0.027 (Prn1 strain) and P=0.001 (Prn2 strain). This was unexpected as previous work showed that both regions are highly immunogenic in rabbits [33]. Furthermore, in a previous study we showed that 86% of human sera contained Abs directed against epitopes in region 2 [32].

Thus our results suggest that antibodies against these regions are not very effective. R1 and R2 could serve to deflect the immune response from regions which would induce protective antibodies. The effectiveness of the R1 and R2 KO molecules could also be due to unmasking of such protective epitopes. The C- and N-term Prn mutants were the least effective vaccines, although they still conferred significant protection. The deletion in the N-term resulted in the largest decrease in efficacy. In previous work we provided evidence that the N-terminus was highly immunogenic as over 50% of the Abs in human sera were directed towards N-terminal epitopes Hijnen *et al.*, submitted.

This work shows that N-terminal epitopes (or epitopes that are dependent on the presence of the N-terminus) induce protective antibodies. As suggested previously, R1 and R2 may serve to deflect the immune response form the N-term, either by masking or by providing an immunodominant (and variable) region. Deletion of the C-terminus also decreased the vaccine efficacy when compared to vaccination with Prn2, but the effect was less or non-existent compared to vaccination with the

N-terminal mutant or Prn1, respectively. The C-terminal mutant contained a Prn1-region 1, thus these results suggest that the C-terminus does not contain protective epitopes as the vaccine efficacy did not decrease compared to Prn1.

Consistent with active vaccination, passive vaccination showed that P2 Abs were more effective than P1 Abs against Prn2 strains ($P=2.4*10^{-4}$). However, in contrast to active vaccination where Prn3 was found to be as effective as Prn2, P3 Abs were less effective compared to P2 Abs to Prn2 strains ($P=0.08$). Both P1, P2 and P3 Abs were equally effective to Prn1 strains. Consistent with the active vaccination, Abs against the Prn-R1-KO were highly effective, suggesting that R1 Abs are not crucial for immunity.

Mice immunized with a low dose of Prn1, 2, 3, Prn-R1-KO, Prn-R2-KO or the Prn-R1-R2-KO, were less well protected against the Prn2 strain compared to the Prn1 strain. A similar phenomenon was observed using P1 and P3 Abs. In contrast, P2 Abs were found to protect better against Prn2 strains compared to Prn1 strains. The latter differential protection was not significant however. In mice immunized with Prn2, the difference in protection against Prn1 and 2 strains was less than in mice immunized with other Prn variants or the region 1 and 2 deletion derivatives. Further, mice immunized with a Prn mutant lacking a part of the N- or C-terminus, were equally well protected against Prn1 and Prn2 strains and slightly better protected against the Prn1 strain, respectively. The latter differential protection was not significant however. The results with the Prn R1 and R2 KO mutants were unexpected. Since the variable regions are removed from these Prn derivatives, the differential protection indicates that antibodies directed against conserved epitopes in Prn are more effective against Prn1 than Prn2 strains. It is possible that R1 is better able to block binding of antibodies in Prn2, or that binding of antibodies to Prn2 have less effect on the receptor-ligand interaction. Clearly this aspect requires further study.

Consistent with previous studies [6, 20], the results described in this work clearly indicate that variation in Prn has an effect on the immunity in the mouse model. Several studies have shown that findings in the mouse model are relevant to immunity against pertussis in humans [30, 34-37]. Further, our results may have important implications as they suggest that replacement of Prn1 by Prn2 may improve the efficacy of pertussis vaccines, not only against the predominant Prn2 strains, but also against Prn1. As mentioned above we presume that such an improvement may be visible mainly in individuals with low vaccine-induced immunity.

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8 Evolution of the *Bordetella* autotransporter Pertactin: identifications of regions subject to positive selection

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Chapter 8

Abstract

The virulence factor Pertactin is expressed by the closely related mammalian pathogens *Bordetella pertussis, Bordetella parapertussis*$_{ov}$, *Bordetella parapertussis*$_{hu}$ and *Bordetella bronchiseptica. B. pertussis* and *B. parapertussis*$_{hu}$ are obligate human pathogens, and cause whooping cough. *B. bronchiseptica* is usually an animal pathogen, but recently it was shown that a human-associated lineage also exists. Extensive variation has been observed in the Pertactin repeat regions 1 and 2, as well as in other regions of the protein. This variation is not only inter-specific, but also occurs between isolates from the same species. Currently, Pertactin is an important component of many acellular pertussis vaccines. Knowledge about codons that are under positive selection could possibly facilitate the development of more broadly protective vaccines. In this study, a large set of Pertactin genes from *B. bronchiseptica*, *B. parapertussis*$_{hu}$, *B. parapertussis*$_{ov}$ and *B. pertussis* were compared using different nucleotide substitutions models, and positively selected codons were identified using an empirical Bayesian approach. This approach yielded 15 codons subject to diversifying selection pressure. The location of these codons was compared to the locations of epitopes.

Introduction

The very closely related pathogens *Bordetella pertussis, Bordetella parapertussis* and *Bordetella bronchiseptica* (referred to as the mammalian bordetellae) express a similar array of virulence factors, including Pertactin. *B. pertussis* is a strictly human pathogen that causes pertussis or whooping cough. *B. parapertussis* comprises two distinct lineages, found in humans and sheep, designated *B. parapertussis*$_{hu}$ and *B. parapertussis*$_{ov}$, respectively. *B. bronchiseptica* has been isolated from a large number of mammalian host species, and recently it was shown that also a human-associated lineage exists (Diavatopoulos *et al.*, PLOS pathogens, in press). *B. pertussis* and *B. parapertussis*$_{hu}$ have evolved from distinct branches of a *B. bronchiseptica*-like ancestor [1, 2, (Diavatopoulos *et al.*, submitted)].

Pertactin (Prn) belongs to the type V autotransporter protein family [3-5], and these proteins are characterized by the ability to catalyze their own transport through the outer membrane. After secretion, autoproteolytic activities reduce the 69-kDa protein to its final 60.37 or 58.34 kDa forms [6], which remain non-covalently bound to the bacterial cell surface [7]. The X-ray crystal structure indicates that Prn consists of a 16-stranded parallel β-helix with a V-shaped cross-section [8]. From this helix, several loops protrude, one of which contains the Arg-Gly-Asp (RGD) motif that is associated with adherence to host tissues [8-11]. The protein further contains two hyper variable regions, designated region 1 (R1) and region 2 (R2), which are comprised of amino acid (AA) repeats (Gly-Gly-X-X-Pro and Pro-Gln-Pro, respectively). Region 1 is located proximal to the N-terminus and directly adjacent to the RGD motif, and R2 is located at the C-terminus.

One of the known biological functions of Prn is that it serves as an adhesin to the epithelium [12]. The exact host receptor to which Prn binds is unknown. Pertactin elicits high antibody titers and anti-Prn antibodies (Abs) have been shown to confer protective immunity [13, Hijnen *et al.*, submitted]. Furthermore, anti-Prn Abs, but not anti-Ptx, anti-fimbriae, or anti-FHA antibodies, were found to be crucial for *B. pertussis* phagocytosis [14], indicating an important role of Prn in immunity to pertussis.

Especially polymorphism in regions 1 and 2 has been suggested to be important for evasion of antibody responses [13, 15]. Mice vaccinated with *B. pertussis* Prn1 were protected less against *B. pertussis* Prn2 strains then to *B. pertussis* Prn1 strains [13]. Prn2 differs only from Prn1 by the presence of an additional repeat unit in region 1. Further, vaccination of mice with *B. pertussis* Prn1 did not protect against infection with *B. parapertussis* [16, 17], suggesting a lack of Prn cross-reactivity between these species. These observations indicate that anti-Prn Abs significantly affect transmission of the bordetellae, at least for *B. pertussis*.

A new light has been cast on variation in Prn with the recent identification of a *Bordetella* phage (BPP-1, Bvg Plus tropic Phage–1) [18]. The mammalian bordetellae can switch between Bvg-phases (*Bordetella* virulence gene), depending on environmental stimuli. Phase switching results in a different expression of surface-associated molecules, including Prn which is specifically expressed in the Bvg$^+$ phase [19]. BPP-1 showed a marked tropism for the Bvg$^+$ phase of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* [18], and its primary

receptor for BPP-1 was shown to be Prn. Phase switching of the bacteria would expectedly result in the loss of the receptor for BPP-1. However, BPP-1 has been shown to specifically generate polymorphism in its ligand-binding domain, resulting in phages with increased binding capacities to alternative surface receptors for host cell entry [18, 20, 21].

Extensive variation has been observed in the Prn repeat regions 1 and 2, as well as in other regions of the protein, both between species but also within species. Prn is an important component of many current acellular pertussis vaccines. Therefore, knowledge about codons that are under positive selection could possibly facilitate the development of more broadly protective vaccines. Our aim was to identify regions that are subject to positive selection within the Prn gene and to compare AAs under diversifying selective pressure to the locations of epitopes. Pertactin genes of different species were compared using different models of nucleotide substitutions, and positively selected codons were identified using an empirical Bayesian approach. The location of positively selected sites was visualized in the crystal structure of *B. pertussis* Prn and compared to the location of epitopes.

Materials and Methods

Sequence data and alignment

In this study, the nucleotide sequence encoding the extracellular domain of Prn was used, represented by AAs 1-Asp to 677-Gly in the *B. pertussis* Tohama sequence. Regions 1 (232-Gly to 256-Pro) and 2 (545-Pro to 566-Pro), which contain repeats, were excluded from the positive selection analysis, which resulted in 1908 nucleotides in total (or 636 codons). Nucleotide sequences were obtained from a previous study [Diavatopoulos *et al.*, PLOS pathogens, in press], and additionally the Genbank database was searched for Prn nucleotide sequences that included the region encoding the extracellular domain. This search yielded 147 *prn* sequences, of which 25 were unique (Fig. 1). Nucleotide sequences were aligned using Kodon 2.5 (Applied Maths, Sint-Martens-Latem, Belgium) and alignment gaps were omitted.

Detection of selection

Detection of selective pressures acting on individual codons within genes is generally estimated from varying ratios (ω) of non-synonymous (d_N) to synonymous mutations (d_S). In the case of positive selection, ω is expected to be >1. However, positive selection often occurs only at a limited number of codons and therefore, the ω for the complete gene may be <1, although the ω for individual codons can still be >1. A maximum-likelihood approach can be used to estimate varying ω-values across sequences using different models of codon evolution [22]. These models assume a certain statistical distribution of ω and estimate the likelihood for the model, thereby also accounting for the phylogenetic relationships of the sequences. The following models are commonly compared to estimate the distribution of ω: model M1A to M2A and model M7 to M8. The first nested model pair consists of the "nearly neutral model" M1A and the positive selection model M2A [23]. In M1A, codons are assigned to two classes of ω of which the value is between 0 and 1, thus always assuming essentially neutral evolution. This model is compared to the "positive selection" model M2A, which has exactly the same codon classes as the M1A model, but in this model an additional class of codons is allowed with ω free to assume a value >1. The second nested pair consists of models M7 and M8. M7 assumes eight codon classes that are distributed in a β-shaped manner with 0<ω<1. Model M8 is similar to model M7, but differs in the existence of an additional codon class with ω>1. A likelihood ratio test respectively compares each nested model pair, and this gives an estimation of the extent of positive selection acting on the sequences under investigation. If M1A is rejected in favor of M2A, positive selection may be concluded. Similarly, positive selection can also be concluded if M7 is rejected in favor of M8. In the case of positive selection, individual positively selected codons can be identified using a Bayes empirical Bayes approach [24]. Likelihood ratio tests were performed using the CODEML program from the PAML software package [25]. Statistical significance for the fit of the models to the actual data was obtained using a χ^2-test. Twice the difference in log-likelihood ($2\Delta l=2(l_1-l_0)$ with l the log-likelihood of a model was calculated, and this was

compared to a χ^2 distribution with two degrees of freedom and a 95% confidence interval. Maximum likelihood trees were reconstructed from the aligned 25 *prn* sequences using Treefinder (Jobb, G. 2005 Treefinder version of June 2005, Munich, Germany, http://www.treefinder.de) under the general-time-reversible (GTR) model of nucleotide substitution, with 1000 bootstrap replicates.

Epitopes in Prn

In previous studies, a Pepscan was used to map the location of linear epitopes recognized by mAbs [26], and site-directed mutagenesis (SDM) was used to identify the location of conformational epitopes recognized by monoclonal antibodies (mAbs) [Hijnen *et al*, submitted]. In addition to these experimentally identified epitopes, the location of additional, putative discontinuous epitopes was determined using the Conformational Epitope Prediction (CEP) server (http://bioinfo.ernet.in/cep.htm) [27]. CEP predicts the location of conformational epitopes, based on the exposure of stretches of AAs that are located in a 6Å proximity of each other. For this analysis, the crystal structure of *B. pertussis* Prn (1DAB.pdb) was used [8, 28].

Solvent exposure and secondary structure

The solvent accessibility of AAs is a measure for their surface exposure; AAs with very high solvent accessibility are thus more likely targets for e.g. the immune system while AAs with low solvent accessibility will not likely come into contact with antibodies. The solvent accessibility was determined for each AA in Prn 1DAB.pdb with the program Getarea1.1 [29]. Since Getarea1.1 was only able to determine the exposure of the AAs present in the crystal structure, we also used the PredictProtein server to determine the solvent accessibility for the remainder of Prn [30]. The secondary structure of the entire Pertactin protein was determined with the PredictProtein server as well [30].

Visualization of sites

The coordinates of Prn were downloaded from the Protein Data Bank, under code 1DAB.pdb [8, 28]. The location of epitopes identified by the above described methods and the location of positively selected sites were visualized in Chimera [31].

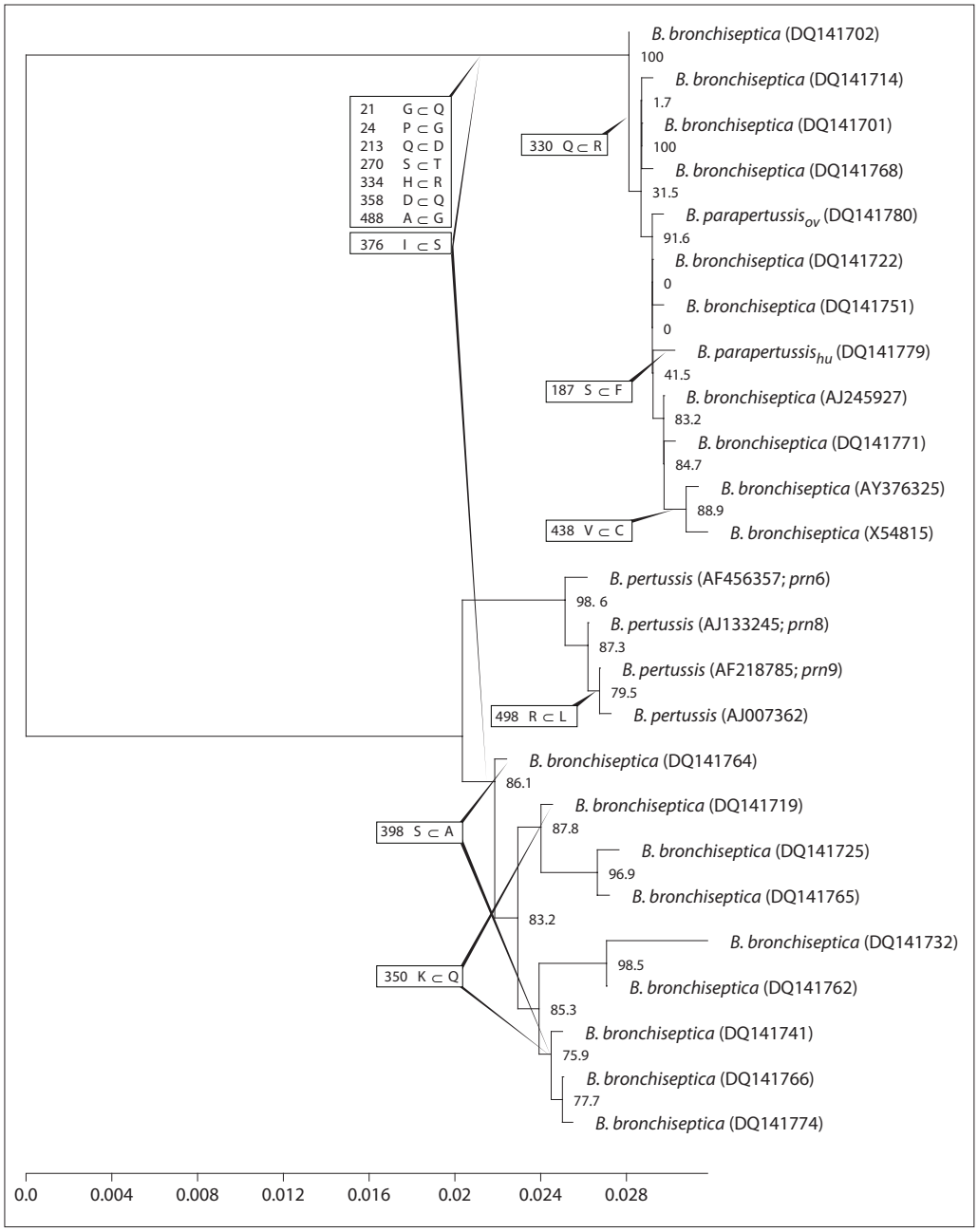

Figure 1. Maximum likelihood tree of 25 unique prn sequences, encoding the exposed domain of Prn, with the exclusion of regions 1 and 2 and alignment gaps. Accession numbers are indicated between parentheses. Positively selected amino acids and their substitutions are indicated in boxes. Numbers near the branches indicate the bootstrap values, based on 1,000 bootstrap replicates. The scale indicates the evolutionary distance in substitutions per site.

Results

A Genbank search yielded 25 unique *prn* sequences out of a total of 147 that included the nucleotide region encoding the surface-exposed domain, representing 10 *B. bronchiseptica* complex I, 9 *B. bronchiseptica* complex IV, 4 *B. pertussis*, 1 *B. parapertussis*$_{ov}$ and 1 *B. parapertussis*$_{hu}$ sequences. A maximum-likelihood tree of these sequences is shown in Figure 1. *B. bronchiseptica* comprises two distinct complexes, complexes I and IV, isolated predominantly from animals and humans, respectively [Diavatopoulos *et al.*, PLoS Pathogens, in press]. The mammalian bordetellae comprise a genetically closely related group of pathogens. Based on housekeeping gene sequence data, comparative genomic hybridization and Pertactin sequence data, it was concluded that *B. pertussis* forms a separate branch with *B. bronchiseptica* complex IV, and *B. parapertussis* clusters with *B. bronchiseptica* complex I [Diavatopoulos *et al.*, PLoS Pathogens, in press]. Interestingly, *B. bronchiseptica* complex I strains and *B. parapertussis*$_{hu}$ cannot be discriminated based on their *prn* genes, while the housekeeping gene tree does allow distinction between the two complexes.

Sequences coding for Prn are subject to positive selection

Positive selection can be estimated from the ratio (ω) of non-synonymous substitutions (d_N) to synonymous substitutions (d_S). For genes and codons evolving under positive selection pressure, ω is expected to be larger than one, indicating that mutations in those codons resulting in AA changes are selected for. We used a likelihood ratio test (LRT) to determine if Prn was evolving under positive selection pressure. In the LRT, the likelihood of a model assuming positive selection is compared to a model that assumes a different (non-positive) selection. The LRT indicated that for Prn, both models M2A and M8 had a significantly better likelihood (p>0.95) than models M1A and M7, respectively. The average ω for Prn was 0.29, but the ω-values for the additional codon class in M2A and M8 were well above 1, indicating that positive selection is a driving force of evolution for some codons in Prn. An empirical Bayesian approach identified 11 codons in model M2A and 15 codons in model M8 to be under positive selection. The 15 codons identified in model M8 also contained the 11 codons that were identified under the M2A model (Table 1). Of the 15 positively selected codons in Prn, all but one (codon 22) resulted in an AA change. Interestingly, although the first two nucleotides of codon 22 had been substituted, this did not result in an AA change.

Characterization of positively selected codons in Prn

In Figure 2, the positively selected codons are indicated on the primary structure of Prn. Of the 15 sites predicted to be positively selected for, the majority was located in the N-terminus or in the center (87%). Only two (13%) positively selected sites could be identified in the C-terminus of Prn, and these were only detected using the M8 model, which has been described to be less conserved than the M2A model [23, 24].

Amino acids may be part of a putative conformational or discontinuous epitope if they

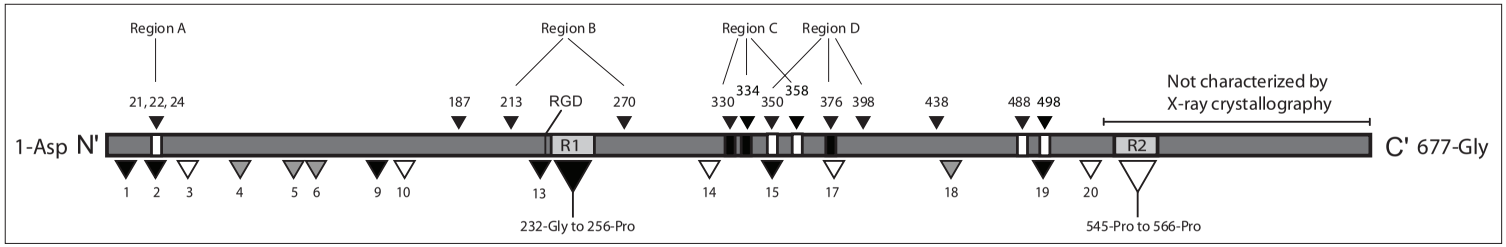

Figure 2. Location of positively selected codons and regions on the primary structure of Tohama Prn. Positively selected codons are indicated by black triangles on top of the primary structure. AA-residues with a maximal distance of 6Å and a minimum solvent accessibility of 25% have been designated as regions, indicated by the connecting lines. Numbering starts with the first amino acid of the mature protein. White boxes indicate the location of conformational epitopes co-localized with positively selected codons. Black boxes indicate the co-localization of both conformational and linear epitopes with positively selected codons. Black, white and gray triangles below the primary structure indicate loops that after mutation by SDM showed respectively a decrease, no effect or increase in binding with mAbs.

Table 1. Characteristics of positively selected sites in the different *Bordetella* complexes

	21G	22S	24P	187S[a]	213Q	270S	330Q	334H	350K	358D	376I	398S	438V[a]	488A[a]	498R[a]
BP (4; 60)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	L
BB-4 (9; 9)	-	-	-	-	-	-	-	-	Q	-	S	A	-	-	-
BPP-hu (1; 10)	Q	-	G	F	D	T	-	R	-	Q	S	-	-	G	-
BPP-ov (1; 3)	Q	-	G	-	D	T	-	R	-	Q	S	-	-	G	-
BB-1 (10)	Q	-	G	-	D	T	R	R	-	Q	S	-	C	G	-
Solvent exposure (%)	65,8	87,2	88,2	0,1	59,9	26,2	36,2	41,1	44,4	39,3	25,2	35,5	29,2	64,5	84,7
Located in	C	C	C	β	C	β	β-C	β-C	β-C	β-C	C	β	β-C	C	C
CEP	Yes	Yes	Yes	No	No	No	Yes	Yes	No	Yes	Yes	No	No	Yes	Yes
SDM/PEPSCAN	S+P	S+P	S+P	No	No	No	P	P	S	No	f-P	No	No	No	S

[a] Positively selected codons identified only by model M8

Abbreviations: BP, *B. pertussis*; BB-4, *B. bronchiseptica* complex IV; BPP-hu *B. parapertussis*-hu; BPP-ov *B. parapertussis*-ov; BB-1; *B. bronchiseptica* complex I, C, loop or coil; β, β-sheet; β-C, β-sheet adjacent to coil; CEP, conformational epitope prediction; SDM, site-directed mutagenesis; S+P, ; f-P, flanks pepscan epitope

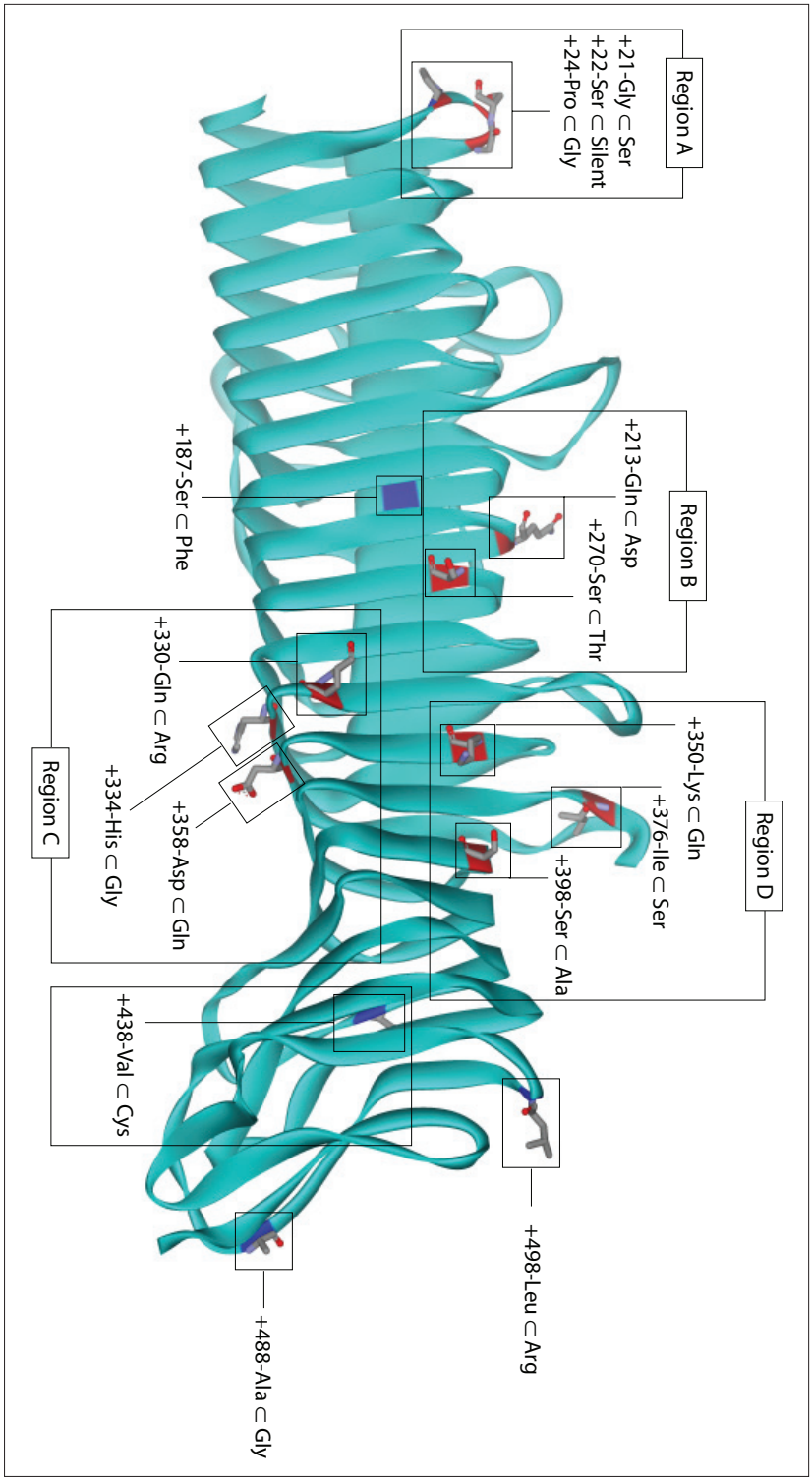

Figure 3. Projection of the positively selected codons and regions on the crystal structure of *B. pertussis* Prn1 (1DAB.pdb) (Emsley, 1996). Numbers indicate the positively selected codons. Codons that are part of a region are indicated by black rectangles.

are within a 6Å proximity, and their solvent accessibility is more than 25% [27]. Positively selected codons that correspond to these criteria may be recognized by a single antibody species and were therefore designated regions. In total, we identified four regions (A-D), representing 11 codons. Four codons could not be assigned to a region under these criteria (Fig. 2, 3 and Table 1).

Positively selected regions in Prn

Region A, located in the N-terminus of Prn, consisted of three positively selected codons that were in very close proximity (AAs 21, 22 and 24) and located in an exposed loop of Prn, designated loop 2 (Fig. 3). In a previous report we described that the N-terminus contained a number of conformational epitopes [Hijnen *et al.*, submitted], and one of these N-terminal conformational epitopes was found to co-localize with Region A. The highly variable loop 2 contains 6 codons (20-Gln to 25-Gly), and 12 of the respective 18 nucleotides were found to be polymorphic between *B. pertussis* and *B. bronchiseptica* complex IV on the one hand and *B. parapertussis* and *B. bronchiseptica* complex I on the other hand. The solvent accessibility of the AAs in loop two was also very high, suggesting they are all well exposed (Table 1).

Region B comprised two codons (213-Gln and 270-Ser), located partially in the N-terminus and partially in the center of Prn. Although separated by 57 AAs in the primary sequence, they are within a 4Å radius of each other in the crystal structure. Of these two AAs, 213-Gln is well exposed (59.9%), but 270-Ser is only 26.2% exposed to solvent. In the β-sheet where 270-Ser is located (AAs 261-274), only three AAs (including 270-Ser) are exposed to solvent. Both codons were not part of previously identified epitopes [26, Hijnen *et al.*, submitted] or epitopes predicted by CEP [27].

Three positively selected codons comprise region C (330-Gln, 334-His and 358-Asp), all of which are within a 6Å proximity. The solvent accessibility of these AAs indicated that they are all well exposed to the environment. Further, these three AAs were predicted to co-localize with a putative conformational epitope, as predicted by CEP [27].

In the center of Prn, three closely located positively selected codons were identified (Region D; 350-Lys, 376-Ile and 398-Ser). These codons were all located in a 6Å radius of each other. Although 350-Lys and 398-Ser are well exposed, 376-Ile is only 25.2% exposed. In contrast, the α-helix adjacent to 376-Ile (373-Gly to 375-Ser) is well exposed. It is likely that the mutation of the hydrophobic 376-Ile to a hydrophilic serine may have an effect on the tertiary structure, or the adjacent α-helix.

Positively selected codons in Prn not assigned to a region

Located in the N-terminus of Prn, 187-Ser was predicted to be under positive selection. Although 187-Ser was in a 6Å radius of Region B (see above), the β-sheet in which 187-Ser is located is inaccessible to solvent, suggesting that this β-sheet does not constitute an epitope. The mutation of the hydrophilic 187-Ser to a bulky aromatic phenylalanine,

as observed in a number of Prn variants, will likely affect the local tertiary structure of the protein, possibly indirectly affecting the exposure of adjacent epitopes or leading to a change in receptor binding. This could possibly suggest an indirect role of this loop in antigenic variation.

Residue 438-Val was mutated into a cysteine in two *B. bronchiseptica* Prn sequences. This mutation was very unusual, as cysteine residues are not normally present in Prn. Residue 488-Ala, located in the beginning of the C-terminus, was also predicted to be under positive selection. The loop in which this AA resides was predicted to be part of five distinct putative conformational epitopes, suggesting this loop is well exposed and possibly very immunogenic. Two of these predicted epitopes also contained the loop that is comprised of AAs 428-436. This loop is flanked by residue 438-Val (see above) which was also found to be under positive selection. It is likely that these two mutations affect the structure and location of several of these conformational epitopes.

The last C-terminally located positively selected site was the well exposed residue 498-Leu, which was predicted to be part of two conformational epitopes. In a previous study, we also identified this residue as part of a conformational epitope recognized by both human and mouse Abs Hijnen *et al.*, submitted.

Analysis of repeat regions 1 and 2

The repeat regions 1 and 2 are located in the N-terminus and in the C-terminus, respectively. Region 1 is comprised of repeats of 5 AA in length, which may also vary in composition (GXXXP), and is located adjacent to the RGD-site. The RGD site has been implicated in adherence of the bacterium to host cells [9], but it is likely that other, uncharacterized domains may also be involved. Region 1 has been shown to induce Prn-specific Abs and variation in this region affected the efficacy of the Dutch whole cell vaccine [13, 32]. This suggests that variation in region 1 is important for evasion of host immunity. Diversity in regions 1 and 2 was also observed in *prn* sequences which were otherwise conserved (Table 2). The length of region 1 was found to be statistically significantly associated with the length of region 2. Longer region 1 sequences were associated with shorter region 2 sequences, and vice versa (Pearson correlation $P<10e^{-16}$). Further, the ratio of region 1: region 2 length was found to be phylogenetically associated. Long region 1 sequences and short region 2 sequences were found almost exclusively in the human-associated *B. pertussis* and *B. bronchiseptica* complex IV branch. In contrast, short region 1 sequences combined with long region 2 sequences were observed predominantly in the *B. parapertussis* and *B. bronchiseptica* complex I branch (Table 2).

Table 2. Characteristics of regions 1 and 2.

	Length in AA	
Complex	Region 1	Region 2
1	16.7 (+/-3.1)[a]	25.5 (+/-2.9)
2	26.5 (+/-3.3)	14.6 (+/-1)
3	20 (+/-0)	31.4 (+/-1.3)
4	25 (+/-6.6)	22.2 (+/-2.4)

[a]Numbers between parentheses indicate the standard deviation

Discussion

In this study, we provide evidence for the presence of positively selected codons in the autotransporter protein Pertactin. The majority of these codons were well exposed to solvent and located in linear or conformational epitopes, suggesting that adaptive changes in these codons may lead to immune escape, decreased phage-recognition or a better fit with the host receptor. Further, the length of repeat region 1 (R1) was found to be significantly associated to that of region 2 (R2), and possible explanations are put forward for this association.

Characterization of positively selected codons

An analysis of Prn from which the hyper variable regions 1 and 2 were excluded resulted in 25 unique Prn sequences. These sequences were analyzed for positive selection using a combination of a likelihood ratio test and empirical Bayes estimates. This approach identified 15 codons that were subject to positive selection. Out of these 15 codons, 14 were exposed for more than 25% to solvent, indicating they are surface exposed and therefore likely to be affected by the immune system or phage binding. The majority of the positively selected codons was located in (n=6) or directly adjacent to (n=5) a loop. In contrast, only 3 positively codons were located in a β-sheet, including the only non-exposed codon (Table 1). These data indicate that in Prn, amino acids located in or near loops are more amenable to diversifying selection than those in β-sheets. The backbone of Prn is comprised of mainly β-sheets and variation in the composition of these sheets may result in structural changes and thus possibly loss of biological function. Variation in the exposed loops however, is not likely to affect the overall structure and function of the protein, and thus these loops may be important for immune evasion. The majority of positively selected codons (n=10) co-localized with linear and conformational epitopes that were predicted by CEP or experimentally identified previously [26, 27, Hijnen *et al.*, submitted]. A total of six codons were located in linear epitopes recognized by human Abs in *B. pertussis* Prn (Table 1) [26]. Further, we recently modified exposed loops of *B. pertussis* Prn by site-directed mutagenesis (SDM), after which the binding of well-characterized mAbs to these Prn variants was investigated [Hijnen *et al.*, submitted]. Several of these Prn variants showed a decreased affinity for a number of mAbs, indicating that mutations in these loops may be important for immune evasion. Out of the 15 positively selected codons, 5 were located in loops of which modification by SDM resulted in decreased affinity to at least 3 mAbs. In the same study, several loops in Prn were identified that upon SDM showed an increase in binding with mAbs [Hijnen *et al.*, submitted]. We hypothesized that these mutations affected the conformation of the loop, thereby unmasking epitopes. Since these loops could be important for masking of epitopes, they are possibly under purifying selection pressure. Consistent with this hypothesis, none of the codons that we identified to be under positive selection in this work were located in these loops. The majority of the positively selected codons were found to be variable between *B. bronchiseptica* complex I and *B. parapertussis* on the one hand and *B. bronchiseptica* complex IV and *B. pertussis* on the other hand. Previously, it was shown that while vaccination with *B. pertussis* Prn1 protected at least partially against *B. pertussis* strains, including those with different

Prn sequences [13, 33], it did not protect against *B. parapertussis* [16, 17]. The latter observation is consistent with an important role of the variable codons in immune evasion.
We previously provided evidence that *B. pertussis* and *B. bronchiseptica* complex IV strains were subject to immune competition resulting in antigenic divergence between these two species [Diavatopoulos *et al.*, PLoS Pathogens, in press]. This analysis was based on the presence or absence of genes coding for dermonecrotic toxin, pertussis toxin and LPS. Here we looked for more subtle changes due amino acid substitutions in Prn. Two substitutions, in the codons 350 and 398, were found which may have been caused by immune competition between *B. pertussis* and *B. bronchiseptica* complex IV strains. In *B. pertussis* en *B. bronchiseptica* complex I strains, these codons code for Lys and Ser, respectively. In contrast, in *B .bronchiseptica* complex IV strains, the residues Gln and Ala are found at these positions, respectively. Similarly, the polymorphism in codon 187 may be due to immune competition between *B. pertussis* and *B. parapertussis*$_{hu}$. All *Bordetella* species code for Ser at this position, except for *B. parapertussis*$_{hu}$ in which Phe is found at this position.
Although data about the location of epitopes was available, functional data concerning receptor specificity and residues possibly involved in this interaction were unavailable. We therefore compared the location of the positively selected AA residues present in human adapted strains with the animal adapted strains to locate residues possibly involved in host receptor specificity. This approach yielded two residues, 213 and 270 that could possibly play a role in host receptor specificity. Both residues are identical in *B. pertussis* and *B. bronchiseptica* complex IV strains but different from *B. parapertussis* and *B. bronchiseptica* complex I strains. Furthermore, both residues were not mapped or predicted to be part of an epitope. Both residues are located closely together in between two large loops (Fig. 3). This creates a groove that could be a potential receptor binding site. The subtle variations observed for these residues (Q>D and S>T) which are located on the bottom of the groove could potentially enhance the affinity or the fit to the human receptor.

Polymorphism in Repeat Regions 1 and 2

Comparison of R1 and R2 sequences between the 147 isolates revealed a striking correlation between the length of R1 and R2. Long R1 sequences were found to be accompanied with short R2 sequences, and vice versa (Pearson correlation $P<10e^{-16}$). This association was correlated to the phylogenetic tree, high R1:R2 ratios were found almost exclusively in the human-associated *B. pertussis* and *B. bronchiseptica* complex IV isolates; low R1:R2 ratios were observed mainly in the *B. bronchiseptica* complex I and *B. parapertussis* isolates. We previously provided evidence that one of the roles of R1 was masking of epitopes [Hijnen *et al.*, submitted]. Further, we observed that R1 and R2 may be part of single discontinuous epitope implicating close proximity of these regions. In the light of these observations it is plausible that variation in the length of R1 is compensated by variation in the length of R2 to maintain the close proximity of the variable epitope, or to maintain masking of underlying epitopes.

In this study we have identified codons of Prn that are under diversifying selection. The results we obtained are largely consistent with immunological and structural data. Our analyses may facilitate the development of more effective vaccines against pertussis by identifying regions which induce an effective immune response and are not subject to diversifying selection. It should be noted that variation in Prn may not only be driven by the interaction with the host. Recently, phage BBP-1 was described that infects Bvg^+ bordetellae via Prn as its main receptor. It is likely that this phage has had a diversifying effect on Prn.

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9

Summarizing discussion

Chapter 9

Pertussis is a highly contagious infectious disease of the respiratory tract which is caused by *Bordetella pertussis*. Before widespread introduction of vaccination against pertussis, almost every child contracted pertussis. The disease is most severe in neonates and children under the age of 1. Most of the hospitalized cases are children that were too young to be vaccinated. Pertussis is now being recognized not only as a childhood disease but also an important infectious disease in vaccinated adults. Possibly due to waning immunity, this group of individuals now represents an important reservoir for transmission of the bacterium to susceptible (un-immunized) individuals.
Introduction of mass vaccination reduced the number of pertussis cases significantly and resulted in a 10-fold decrease in pertussis mortality in the Netherlands in the late 1950s [1]. Despite vaccination, pertussis remained endemic. A major epidemic occurred in the Netherlands in 1996 [2]. Since the 1996 outbreak, the Netherlands now has an epidemic pertussis cycle with peaks every 2 to 3 years during the last decade. Also in several other European countries, as well as Canada, The United States and Australia, a re-emergence of pertussis was observed.
Several explanations were proposed for the sudden increase of pertussis in the Netherlands including waning immunity in adolescents and adults, increased reporting, improved diagnosis of the disease and the adaptation of the *B. pertussis* population. In the Netherlands, pathogen adaptation has probably played an important role in the resurgence of pertussis [3, 4]. Vaccine adapted strains showed polymorphisms in two proteins implicated in protective immunity: P.69 Prn and Ptx [3, 5]. The role of P.69 Prn in protective immunity has been well documented in both humans and in animal models [6-11, 11-13]. Furthermore, P.69 Prn is present in several of the currently most used ACVs.

Until recently, relatively little was known about the antibody response to P.69 Prn, the location of epitopes on P.69 Prn and the role of variation in Prn on immune evasion. Variation in the variable region 1 was described to affect the Ab response to this region [14], but no direct evidence or a feasible mechanism for immune evasion was described. The goal of this thesis was to identify the location of (protective) epitopes to which human Abs are directed, and to investigate the role of variation in P.69 Prn and the implications for the anti-P.69 Prn Ab response.

The observed variation in *B. pertussis* Prn is mainly limited to two regions that are comprised of repeats. These two regions were thought to be immunodominant and therefore it was assumed that a large Ab response would be directed to these regions. In **chapter 2** we tested the Ab response of human sera from infected and vaccinated individuals in a Pepscan. In contrast to the general belief, only a small percentage of Abs were found to recognize the variable region 1. Only 35% of the sera tested contained Abs directed against linear epitopes in region 1. In contrast, 86% of the sera contained Abs directed against linear epitopes in region 2. Our results suggested that no Abs with a high avidity were induced

against region 1 and 2 as the binding of mAbs to these epitopes was not blocked by human sera. However, the binding of mAbs that were found to recognize conformational epitopes was blocked by human sera, indicating that in humans Abs with a high avidity are induced against conformational epitopes. This result was rather unexpected as Abs directed against region 1 were shown to be, at least in part, responsible for protection against infection [11]. A total of 24 epitopes were identified, of which 12 were located in the N-terminus of P.69 Prn, suggesting an important role of the N-terminus in immunity to pertussis.

Three mAbs bound to two or three non overlapping peptides in a Pepscan analysis. Although these peptides were located far apart in the primary structure, they were rather close in the folded protein, suggesting they were part of a discontinuous epitope. Furthermore, two of these mAbs bound to peptides derived from region 1 and the C-terminus. We speculated that perhaps region 1 and 2 are interacting. However, this seems not likely due to structural limitations in monomeric P.69 Prn (**chapter 6**). It now seems more likely that the N- and C-termini are interacting and the variation in the region 1 and 2 indirectly affects this interaction. A similar interaction has been described for the Streptococcus mutants adhesin P1. Here the polymorphic regions interacted to hide the receptor binding site from the immune system until the protein binds to its receptor [15]. Hidden epitopes are also present in the HIV glycoprotein gp120. Here, the CD4 binding site is hidden from the immune system until receptor binding [16]. It is conceivable that the interactions of the N- and C-termini of P.69 Prn mask the RGD site, implicated in adherence to host cells, from the immune system.

To study the interaction of the N- and C-terminus in further detail, to map the location of epitopes to which the high avidity human Abs are directed and to identify region and Prn type specific Abs, we cloned and expressed several P.69 Prn variants and deletion mutants (**chapter 3**). It was possible to express large quantities of P.69 Prn in E. coli which was indistinguishable from P.69 Prn isolated from *B. pertussis* as determined by CD-spectroscopy and by comparing the binding of mAbs to recombinant and native Prn. The technique for expressing P.69 Prn was combined with site directed mutagenesis (SDM) to construct Prn mutants with mutations in sites that were exposed, or believed to be part of an epitope (**chapter 4**). With these SDM mutants, Prn deletion mutants and a competition assay we mapped the location of several conformational epitopes. Interestingly, the binding of several mAbs was affected by deletion of parts of the N- and C-terminus. This indicated that these mAbs recognized an epitope that comprised parts of both the N- and C-termini, as suggested in **chapter 2**. This result was further substantiated when the mAbs were tested for their ability to compete with each other in the Biacore. In several cases, mAbs that were shown by SDM and deletion mutant mapping to bind to either N- or C-terminal loops were found to compete with each other. This competition could be explained by a close proximity of the N- and C-termini of P.69 Prn.

Interestingly in several cases the binding of mAbs to SDM mutants increased (**chapter 4**). This increase in binding was not due to an improved lock-key fit, since the affinity

of the mAbs for the SDM-Prn mutants did not increase. It is more likely that due to the introduction of non-neutral mutations in certain loops, epitopes in adjacent loops became more exposed. This hypothesis was confirmed by the mutation of 3 non-exposed loops (loops 5, 9 and 15) (for the location of loops see Fig. 1 in **chapter 4**) which changed the binding profile of 10 mAbs. Furthermore, of the 7 loops that after mutation showed an increase in binding with mAbs, 4 loops (loops 4, 14, 18 and 19) were large enough to cover several adjacent epitopes. It is conceivable that mutation of these loops influenced their tertiary structure and thus possibly their location, resulting in the exposure of adjacent epitopes. These results indicate that loops other than the region 1 loop are also involved in epitope masking.

Unexpectedly, the role of peptide loops in immune evasion become more evident when we reconstructed one of the discontinuous conformational epitopes of P.69 Prn (**chapter 5**). When we mimicked the epitope recognized by mAb PeM29 using a synthetic peptide scaffold (PEPTAC), we were able to induce Abs in mice that were not induced during natural infection or vaccination with P.69 Prn1. However the anti-PEPTAC Abs were protective, suggesting that Abs were induced against an epitope that is normally hidden in P.69 Prn1. As suggested in **chapter 4**, several epitopes are hidden in P.69 Prn, since Ab responses directed to these sites are protective. This hypothesis was further confirmed when we tested the response of human serum Abs to the Prn deletion mutants (**chapter 6**). No differences were observed when serum from infected or vaccinated individuals was tested to P.69 Prn1, 2, 3 or a mutant that lacks the variable region 1. However, when we tested the reaction of human sera to the N- and C- terminal mutants, an increase in binding was observed, indicating that certain epitopes became available or more exposed. We previously provided evidence that the N- and C-terminus could be interacting (**chapters 2** and **4**). Unfortunately, the crystal structure of P.69 Prn1 lacks the 133 C-terminal AA residues [17]. However, we were able to predict the tertiary structure of the C-terminus. In these structure predictions, the C-terminus of P.69 Prn1 appeared as a flexible linear region that folds towards the N-terminus, thereby covering large parts of the molecule (**chapter 6**) (Fig. 1). The avidity of Abs directed against linear epitopes is generally somewhat lower then those directed towards conformational epitopes [18-25]. It is conceivable that when the C-terminus is deleted, high affinity Abs directed to conformational epitopes located in parts of the molecule that are partly covered by the C-terminus are able to bind, resulting in a higher Ab reactivity with the molecule. It is likely that the flexible C-terminus acts as a decoy region, similar as the loops in the N-terminus as described in **chapter 4**.

A contrasting result was found when human sera were depleted with the N-terminal mutant. In 96% of the cases when a human serum was depleted with a mutant lacking a large part of the N-terminus, no decrease in the amount of Ab binding to P.69 Prn was observed, indicating that many Abs are directed towards the N-terminus. However, when the binding of human sera was tested to the N-terminal Prn mutant in a regular ELISA, a similar binding and sometimes even an increase in binding was observed compared to the

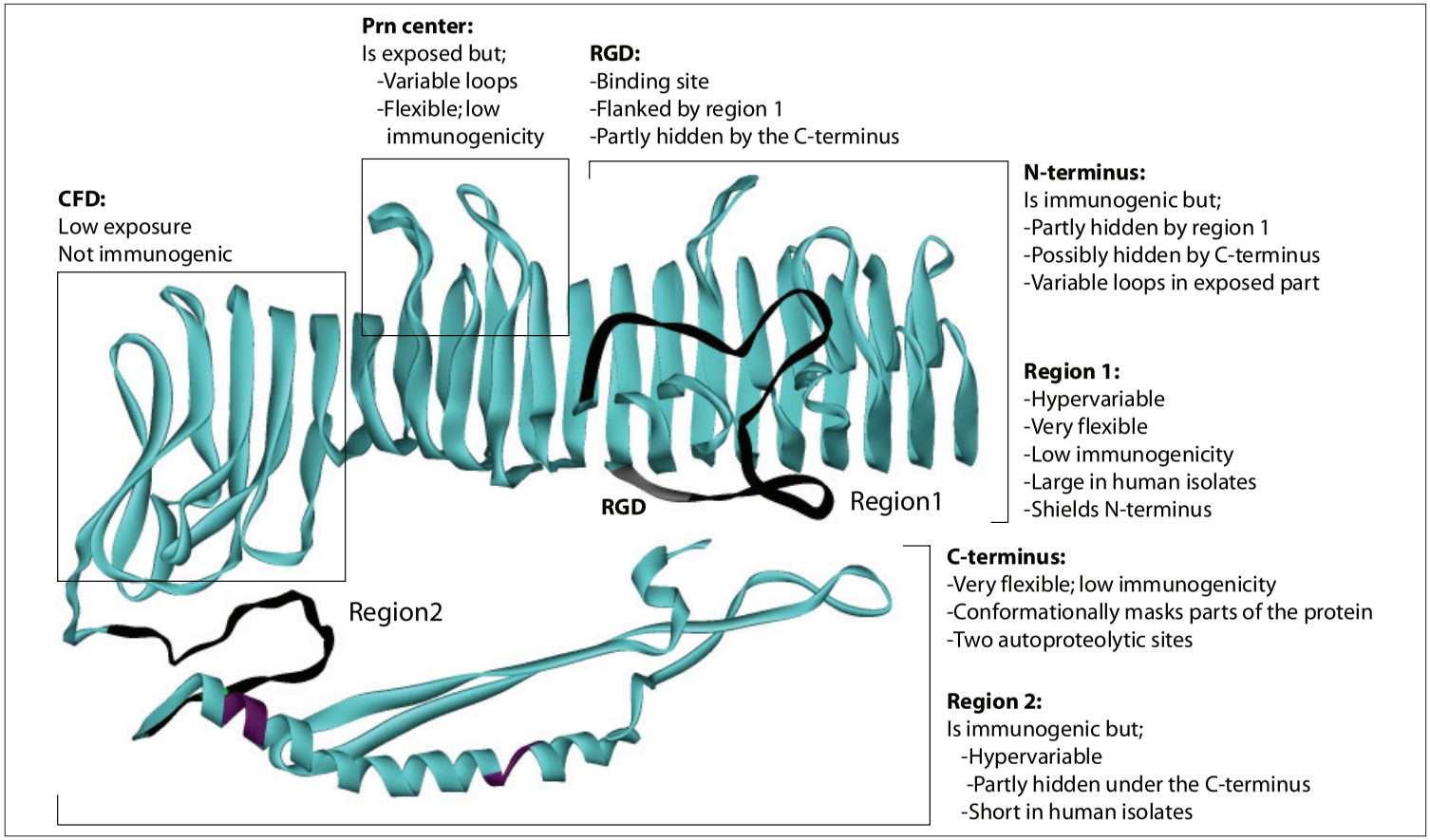

Figure 1. Summary of the immune evasion strategies employed by Pertactin. Abbreviations. CFD, conserved folding domain.

binding to P.69 Prn. This result seemed to indicate that the contribution of the N-terminus in Ab binding is marginal. However, we have shown that human Abs with a high affinity are directed against the N-terminus, as they were able to block the binding of several mAbs that were shown in **chapter 4** to bind to the N-terminus. It is possible that when these Abs bind to P.69 Prn, they block the binding of Abs directed to other parts of the protein. When the N-terminus is deleted and sera are adsorbed with this protein, the high affinity Abs do not block the binding of Abs to other parts of the protein. When the response of the adsorbed sera is subsequently tested to P.69 Prn1, still a large reaction is measured. These results indicate that a large Ab response is directed towards the N-terminus, and therefore underline the importance of this region once more.

An interesting relation between Ab affinity and peptide loops was found when rabbit anti-P.69 Prn1, 2 and 3 sera (P1, P2 and P3 sera) were depleted with a Prn mutant lacking region 1 (**chapter 6**). Anti-P2 serum contained the most Abs directed against region 1 (compared to P1 and P3 sera), however, the lowest response of this serum was measured to P.69 Prn2. Apparently, the increased immunogenicity of the larger region 1 of P.69 Prn2 is compensated by a higher flexibility of region 1 resulting in a lower affinity of the induced anti-region 1 Abs.

In **chapter 6**, we identified an additional role for region 1 in immune evasion. Recently, it was described that P.69 Prn induces type specific Abs in humans [14]. It was shown that patients infected or vaccinated with P.69 Prn1 strains were able to block the binding of two mAbs (PeM1 and 5) one of which (PeM5) was shown in **chapter 4** to recognize N-terminal loops as its main binding site. However, sera from patients infected with *B. pertussis* P.69 Prn2 strains did not contain Abs that were able to block the binding of these 2 mAbs. Since the Prn2-region 1 is larger than that of Prn1 and 3, no, or less Abs are induced against the N-terminal epitopes recognized by mAbs PeM1 and 5, which could explain that no blocking was observed due to improved conformational masking. Depletion of rabbit sera showed that, compared to Prn1 and 3, Prn2 induces more Abs against region 1, and less Abs directed to the N-terminus. These results support our hypothesis that variation in region 1 affects the Ab response directed against the N-terminus.

In **chapter 8** we used a large number of Prn sequences from *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* to identify codons in Prn that are under positive or negative selection pressure. We identified a total of 15 codons that were predicted to be under positive selection pressure, and 9 out of 15 codons were located in predicted conformational epitopes. Furthermore, the majority (n=8) of these 15 positively selected codons co-localized with both linear as well as conformational epitopes that were mapped in **chapter 2** and **4** by Pepscan or SDM, respectively. Furthermore, these results could explain the described lack of cross reactivity of Abs to *B. pertussis* Prn to *B. parapertussis* as many of the observed mutations are found in *B. parapertussis* strains.

Besides immunological pressure by Abs, T-cells and other immune-factors, Prn is also exposed to selective pressure by the Bordetella phages (BPP-1) (**chapter 1**). Although

the receptor for BPP-1 has not yet been determined, it is possible that it recognizes one of 15 sites under positively selection pressure. In **chapter 8** we identified a loop (loop 2) in the N-terminus that contained 3 sites under positive selection. Furthermore, this loop was flanked by polymorphic residues. These polymorphisms were mainly found in *B. bronchiseptica* strains, from which the BPP-1 phage was first isolated. This site could be a potential receptor for the BPP-1 phage since it is fully exposed. Since this site is so fully exposed, and cannot rely on the masking properties of the region 1 loop or the N- and C-terminal interaction, it is possible that it has become so extremely polymorphic in Prn to evade phage or Ab binding.

The acceptance of pertussis vaccination has been hampered by the reactogenicity of whole cell vaccines (WCVs). For this reason, many countries have switched to the less reactogenic ACVs. The pertussis ACVs contain 1 to 5 purified proteins and cause fewer side effects, but provide less protection against the second causative agent of pertussis, *B. parapertussis* compared to the WCVs. Although it was shown in a previous study that variation in P.69 Prn affected the efficacy of the Dutch whole cell vaccine, the current ACVs still contain P.69 Prn1, whereas most clinical isolates produce P.69 Prn2 and some P.69 Prn3.

In **chapter 7** we investigated the role of variation in P.69 Prn on the level of protection against *B. pertussis* Prn1 and Prn2 strains. Mice were vaccinated with Abs against Prn, with purified P.69 Prn and with Prn deletion derivatives. Mice immunized with Prn1, 2, 3, Prn-R1-KO, Prn-R2-KO or Prn-R1-R2-KO, were less protected against infection with Prn2 strains compared to Prn1 strains. However in Prn2 vaccinated mice this difference was less compared to the other groups. These results are in line with the hypothesis described in **chapter 4** and **6** that region 1 shields epitopes located in the N-terminus, thereby blocking the binding, or preventing the induction of Abs directed to N-terminally located epitopes. This hypothesis is also supported by the correlation between the length of region 1 and region 2 in human adapted strains. In **chapter 8** we described that human adapted Bordetellae have a significantly larger region 1 compared to the animal adapted strains. In the animal adapted strains an inverse correlation was found. Animal adapted strains had a significantly shorter region 1 and a longer region 2. This coincided with the observation described in **chapter 2** and **6** that in human sera, more Abs are directed towards region 2 compared to region 1. This would favor a longer region 1 and shorter region 2 for effective evasion of immune responses in the human host. It is therefore not surprising that vaccination with a Prn mutant lacking region 1 did not result in a lower level of protection.
Furthermore, it became clear that the N-terminus is essential for protection against infection. As described in **chapters 2**, **4** and **6**, a large Abs response is directed towards the N-terminus indicating that this region is immunogenic. Mice vaccinated with a Prn mutant that lacks a large part of the N-terminus (110 AA), were the least protected against infection compared to the level of protection after vaccination with P.69 Prn2 or 3. This result in line

with our hypothesis that region 1 blocks the binding of Abs to the N-terminus. In contrast to the N-terminal mutant, mice that were immunized with the C-terminal mutant were equally well protected compared to mice immunized with P.69 Prn2 or 3 or any of the deletion derivatives. This result supports our hypothesis described in **chapter 6** that the role of the C-terminus is protecting the remainder of the protein from Ab binding.
Similar as observed for region 1, deletion of region 2 did not result in a lower level of protection against infection (compared to P.69 Prn1). The overall trend was that P.69 Prn2 vaccinated mice were best protected against P.69 Prn1 and 2 strains. These results indicate a complex role of the variable regions in the evasion of Ab responses. The approach of deleting the variable regions to skew the Ab response towards conserved regions of Prn does not automatically improve the immune response against Prn. In fact, by doing this, it is possible that the immune response becomes less effective, as Abs are induced to regions that in the native protein might be masked by either region 1, region 2 or by the interaction of both variable regions.
The approach that we describe in **chapter 5** seems to be more promising. Using a synthetic scaffold (PEPTAC), we were able to construct a synthetic discontinuous epitope that induces Abs that are not induced during natural infection or immunization with P.69 Prn1. Since these Abs are not induced during natural infection, the chance of vaccine escape mutants induced by this PEPTAC vaccine is lower than that induced by immunizing with 1 type of antigens or whole bacteria. However, the disadvantage of this observation is that natural boosting won't occur or to a lesser extent.
Using a single epitope we induced a protection level that was about half of that when purified P.69 Prn1 was used. Since we only presented a single epitope, the level of protection can possibly be improved by including multiple conformational epitopes presented on different PEPTAC's. Since the PEPTAC vaccine that was used consisted purely of synthetic peptides conjugated to a well defined adjuvant, high purities can be guarantied. This is likely to minimize the undesirable side effects that are inextricably linked to the pertussis vaccine.
A final point of consideration should be taken into account when evaluating vaccines in mice. As previously reported [11, 26] and described in **chapter 7**, when high vaccine doses are used in the mouse model no differences in protection are observed between any of the Prn variants and mutants. In the human situation, pertussis is not a problem in fully vaccinated individuals, but becomes a problem when immunity wanes [2]. It is difficult to study waning immunity in our mouse model. However, it would be very interesting to investigate the protective properties of the Prn variants and mutants and that of the PEPTAC's in a model with waning immunity.

To summarize, our data clearly indicate that Prn has evolved several ways to escape antibody and possibly phage binding. These results are summarized in Figure 1. In **chapters 2**, **4**, **5**, **6**, **7** and **8** we showed that the N-terminus of P.69 Prn harbors important epitopes. Our data suggests that the variable region 1 has evolved to hide those important epitopes from

immune recognition and possibly phage binding. Furthermore Prn has several other flexible (and variable) loops that are employed to hide adjacent epitopes from the immune system (epitope masking). In addition, the N- and C-termini of P.69 Prn interact physically, which results in the masking of a part of Prn. Since the C-terminus has a linear structure that folds towards the N-terminus, it is likely that the Ab response to this region is less efficient then an Ab response directed to the conformational epitopes located in parts of the molecule that are covered by the C-terminus (conformational masking). The loops that are exposed, e.g. region 1, region 2, and several other loops, are shown in **chapter 8** to be highly variable (antigenic variation). In **chapter 7** we showed that deletion of the variable regions does not improve protection against infection. Apparently deletion of the decoy regions does not automatically skew the Ab response towards the conserved protective epitopes. However, in **chapter 5** we showed that it is possible to employ synthetic peptides to induce a protective response towards conserved and protective epitopes.

The results described in this thesis have shed new light on the mechanisms employed by bacteria, and *B. pertussis* in particular, to evade immune recognition. These results will facilitate the development of new, possibly synthetic, but mainly more effective vaccines.

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Nederlandse samenvatting

Kinkhoest is een ernstige en zeer besmettelijke ziekte van de bovenste luchtwegen welke wordt veroorzaakt door de bacterie *Bordetella pertussis*. In de jaren vóór de introductie van het kinkhoest vaccin, kwam bijna elk kind in aanraking met deze bacterie. De introductie van grootschalige en systematische vaccinatie in de jaren 50 had een enorme reductie van het aantal kinkhoest gevallen tot gevolg. Ondanks de introductie van vaccinatie bleef de kinkhoest bacterie endemisch aanwezig in de populatie maar van uitbraken was geen sprake. De vaccins die geïntroduceerd werden in de jaren 50 bestonden uit geïnactiveerde bacteriën. Dit type vaccin bleek erg effectief in het bestrijden van kinkhoest. Echter was er veel negatieve publiciteit over vermeende ernstige bijwerkingen van deze totaal-cel vaccins, wat de ontwikkeling van nieuwe vaccins stimuleerde. Eind jaren 70 begon de ontwikkeling van nieuwe acellulaire vaccins, welke bestonden uit gezuiverde delen van de bacterie. Doordat er slechts enkele gezuiverde en goed gedefinieerde delen van de bacterie in deze vaccins aanwezig zijn, geven deze vaccins minder bijwerkingen. Veel landen zijn overgestapt op acellulaire vaccins, waaronder Nederland in 2005.
Ondanks de hoge vaccinatiegraad in Nederland was er in 1996 een plotselinge uitbraak van kinkhoest. Sinds deze uitbraak heeft Nederland nu een epidemische kinkhoest cyclus, met uitbraken iedere 2 tot 3 jaar (Fig. 1, pagina 14). Ook in andere Europese landen, de VS, Canada en Australië is een vergelijkbare kinkhoest situatie ontstaan. De plotselinge terugkeer van kinkhoest was een raadsel. Er werd een aantal oorzaken geopperd voor de terugkeer van kinkhoest, waaronder veranderingen in de surveillance en diagnostiek van kinkhoest, een daling van de vaccinatiegraad, veranderingen in de vaccinproductie en het langzaam wegebben van de immuniteit tegen kinkhoest. Echter geen van deze mogelijkheden kon de plotselinge terugkeer verklaren. Een laatste mogelijke oorzaak lijkt meer voor de hand te liggen, namelijk aanpassing van de bacterie aan het vaccin. Deze oorzaak lijkt in ieder geval deels verantwoordelijk te zijn geweest voor de terugkeer van kinkhoest in Nederland. In verschillende voorgaande studies bleken veranderingen in de *B. pertussis* populatie te zijn ontstaan na de introductie van vaccinatie tegen kinkhoest. Een van deze veranderingen werd gevonden in pertactine, een eiwit van *B. pertussis*. Uit analyse van de stammen die werden gevonden voor en na de introductie van vaccinatie werd duidelijk dat het oorspronkelijke pertactine type, dat in de gebruikte vaccins zit, niet meer voorkomt na de introductie van vaccinatie. Pertactine is een eiwit dat op de buitenmembraan van *B. pertussis* wordt gevonden. In een aantal studies werd een belangrijke rol voor pertactine beschreven in het opwekken van immuniteit tegen kinkhoest. Zo werd duidelijk dat de hoeveelheid antilichamen tegen pertactine correleert met het niveau van bescherming tegen kinkhoest.

Een van de doelen van de studie beschreven in dit proefschrift was het onderzoeken van het effect van variaties in de aminozuur sequentie van pertactine op de immuun respons tegen het eiwit en de mogelijke consequenties voor vaccinatie met de huidige vaccins. De meeste acellulaire vaccins bevatten 3 tot 5 gezuiverde eiwitten van de kinkhoest bacterie,

waaronder pertactine. Het type pertactine in de huidige vaccins is nog steeds hetzelfde type dat tot expressie werd gebracht door kinkhoest bacterien uit de jaren 50 en komt nu niet meer voor.

In **hoofdstuk 2** hebben we onderzocht tegen welke delen van het eiwit pertactine antilichamen worden opgewekt (epitopen). Dit werd gedaan om te kunnen voorspellen of variatie in het eiwit een effect heeft op de effectiviteit van de antilichamen gericht tegen pertactine. Met behulp van humane sera hebben we de locatie van diverse epitopen kunnen identificeren. Echter bonden er bijna geen antilichamen aan de variabele regio's, wat impliceert dat de variatie in pertactine geen direct effect op antilichaam binding blijkt te hebben.

Om het effect van variatie verder te bestuderen hebben we het eiwit door de *E.coli* bacterie laten produceren, waardoor we de beschikking kregen over grote hoeveelheden pertactine (**hoofdstuk 3**). Tevens konden we zo variaties in het eiwit aanbrengen waarbij o.a. de variabele delen verwijderd waren.

Met behulp van de eiwitten uit **hoofdstuk 3,** hebben we in **hoofdstuk 4** een nieuwe rol voor de variabele regio's en een aantal andere delen van pertactine ontdekt. We vonden dat verschillende geëxposeerde delen van pertactine betrokken waren bij het afschermen van andere, mogelijk belangrijkere, epitopen. In **hoofdstuk 6** werd gekeken naar de aanwezigheid van antilichamen tegen bepaalde delen van pertactine. Hierdoor werd bevestigd dat de geëxposeerde delen van pertactine een belangrijke rol spelen in het afschermen van epitopen.

In **hoofdstuk 8** hebben we een groot aantal DNA sequenties vergeleken die coderen voor pertactine. We ontdekten een correlatie tussen de lengte van de variabele regio's van stammen die alleen voorkomen bij mensen of bij dieren. Hierdoor worden de resultaten, beschreven in **hoofdstuk 4,** bevestigd dat de rol van één van de variabele regio's, het afschermen van epitopen, effectiever gaat als deze regio groter is.

In **hoofdstuk 5** hebben we de kennis uit **hoofdstuk 2** en **4** aangewend om een nieuw synthetisch vaccin te maken. Dit vaccin bootst een belangrijke epitoop van pertactine na. In een muismodel gaf dit vaccin een goede bescherming tegen de kinkhoest bacterie. Omdat het een synthetisch vaccin betreft, kan het simpel en heel zuiver worden gemaakt, punten die heel belangrijk zijn bij mogelijke toepassing als nieuw vaccin.

In **hoofdstuk 7** hebben we met behulp van het muismodel de invloed van pertactine varianten getest op bescherming tegen verschillende kinkhoest bacteriën. Verder hebben we gekeken of varianten van pertactine betere vaccinkandidaten zijn dan de variant die in het huidige vaccin gebruikt wordt. Pertactine varianten waarin de variabele regio's verwijderd

waren bleken een vergelijkbare of betere bescherming te geven dan normaal pertactine. Dit resultaat bevestigd wederom de rol van de variabele regio's in het afschermen van epitopen. De in dit hoofdstuk beschreven resultaten ondersteunen de studies uit **hoofdstuk 2, 4** en **6**, waarin werd beschreven dat de N-terminus van pertactine heel belangrijk is, maar de C-terminus geen belangrijke rol speelt bij antilichaam herkenning. Tevens laten we in dit hoofdstuk zien dat pertactine type 2 een betere bescherming geeft dan pertactine type 1 dat in de huidige vaccins wordt gebruikt.

De resultaten beschreven in dit proefschrift geven meer inzicht in locaties van belangrijke epitopen op pertactine en meer inzicht in de rol die pertactine speelt bij het omzeilen van vaccin geïnduceerde immuniteit door de kinkhoest bacterie. We hebben deze kennis deels toegepast om een verbeterd kinkhoest vaccin te maken. Mogelijk zullen deze resultaten bijdragen aan de ontwikkeling en productie van nieuwe en betere kinkhoest vaccins die de incidentie en circulatie van kinkhoest kunnen verminderen of zelfs kunnen eradiceren.

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Op naar de volgende uitdaging, Australia here we come!

Marcel

Curriculum vitae

The author of this thesis was born on October 16, 1978 in Beek en Donk. He attended high school at the Dr. Knippenberg College in Helmond and completed his final exam in 1995. He studied Microbiology at the Fontys Hogeschool in Eindhoven and graduated in 2001. During his final project he studied the opportunistic yeast, *Candida albicans*. This research was performed at the Eijkman-Winkler Institute at the University Medical Center Utrecht under the supervision of Dr. A. Fluit and Dr. A. Borst. After his graduation he worked for nine months at the Immunology department of the Eemland Hospital in Amersfoort. In the same year he started working on his PhD project at the Laboratory for Vaccine Preventable Diseases at the National Institute of Public Health and the Environment in Bilthoven under the supervision of Prof. Dr. F.R. Mooi and Dr. G.A.M. Berbers. The work described in this thesis is the result of the research performed during this period. In March 2006 he will start working as a post-doc at the Macfarlane Burnet Institute in Melbourne, Australia.