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REVIEW

# *Bordetella Pertussis* virulence factors in the continuing evolution of whooping cough vaccines for improved performance

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## Abstract

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

**Keywords** *Bordetella pertussis* · Pertussis vaccine · Virulence factors · Immune response · Biofilm-associated antigens

## Introduction

The genus *Bordetella* comprises of Gram-negative bacteria that infect, colonise and cause disease in a wide variety of mammals, humans and birds. Ten species have been identified to-date, including *Bordetella pertussis, B. bronchiseptica, B. parapertussis, B. avium, B. hinzii, B. holmesii, B. ansorpii, B. flabilis, B. trematum*, and *B. petrii* [1, 2]. *B. pertussis, B. parapertussis* and *B. bronchiseptica* are closely related phylogenetically and are often referred to as

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the "classical *Bordetellae*" [3]. *Bordetella bronchiseptica* is a respiratory pathogen infecting a wide range of mammalian hosts generally causing mild, chronic respiratory illness [1]. Two distinct lineages of *B. parapertussis* have been identified—*B. parapertussis*$_{HU}$ in humans and *B. parapertussis*$_{OV}$ in sheep [4]. Phylogenetic analysis indicates that *B. pertussis* and *B. parapertussis*$_{HU}$ evolved independently from *B. bronchiseptica* [5]. *B. pertussis* is an obligate human pathogen, which has undergone significant insertion sequence (IS) mediated gene loss or inactivation. No environmental reservoir for *B. pertussis* has been found until now [6]. This pathogen is the aetiological agent of pertussis (or whooping cough), a chronic and highly contagious respiratory disease particularly severe in infants and children. Infection typically results in paroxysmal cough with a characteristic whooping that may persist for weeks to months and may be followed by post-tussive vomiting. The disease is more severe and pronounced in unimmunised infants and complications of infection with *B. pertussis* may include pulmonary hypertension, pneumonia, febrile seizures, encephalopathy, albeit rare, and brain haemorrhages [7].

Before vaccination programmes were established, whooping cough was the leading cause of death in infants worldwide. However, large-scale vaccination programmes introduced in the 1940s with formalin-killed whole cell (wP) vaccines reduced the incidence and mortality by about 90% in developed countries [7]. While the protection offered by these vaccines were reported to last for 4–12 years [8], they infrequently invoked high fevers with or without febrile seizures, swelling, pain and redness at the site of injection [7]. To reduce the incidence and extent of these side reactions, a second generation of acellular pertussis (aP) vaccines were introduced in the 1990s and administered in combination with diphtheria and tetanus toxoids as "DTaP" vaccines, with incorporation of inactivated polio virus vaccine (IPV) or *Haemophilus influenzae* type b conjugate (Hib) vaccines. A new vaccine formulation, with lower amounts of diphtheria toxoid and pertussis antigens, Tdap, was recently introduced as adult boosters. Although the aP vaccines are less reactogenic than the wP vaccines, the duration of protection offered by aP vaccines has been reported to be less than that offered by wP vaccines with induction of memory B cells being inferior to those induced by wP vaccines [9]. All aP vaccines contain genetically or chemically inactivated pertussis toxin (Ptx), alone or in various combinations with, filamentous haemagglutinin (FHA), pertactin (Prn), serotype 2 fimbriae (Fim2) and serotype 3 fimbriae (Fim3).

Although vaccination has reduced mortality due to *B. pertussis* infection in infants, whooping cough is still a major cause of vaccine-preventable deaths particularly in developing countries [10]. The epidemic cycles occur every 3–5 years and so far vaccination has not changed this incidence significantly [11]. In populations with high vaccine coverage, infection rates can reach as high as 1–7% annually [12]. In 2008, the WHO estimated 16 million pertussis cases worldwide with 95% of them in developing nations and resulted in 195,000 child deaths [7]. Yearly deaths of 285,000–400,000 infants have been reported [6, 13]. The incidence of whooping cough has increased in resource rich countries such as Australia [14], Canada [15], the Netherlands [16], the United Kingdom [17] and the United States [18] despite high levels of immunisation coverage (> 90%). In Australia, whooping cough has re-emerged to become epidemic since 1993 with notifications rising from 1.8/100,000 in 1991 to a peak of 156.9/100,000 in 2010 [10]. Similarly, in England, the year 2012 recorded the highest number of pertussis notification over 12 years. These cases were predominantly among adolescents and adults but with increased deaths of infants under 3 months old, who were too young to complete the recommended vaccination schedule [19]. Figure 1 depicts the incidence of whooping cough in Australia, the United Kingdom and the United States, based on national pertussis notification systems. The recent global rise of pertussis may reflect improved methods of diagnosis, incomplete vaccine coverage, limited duration of vaccine-induced protection and/or pathogen adaptation. A contributing factor may be the inability of the current aP vaccines to induce potent cell-mediated immunity (CMI) after primary immunisation as required for long-term protection against pertussis [20]. Although many factors may contribute to resurgence of pertussis, the limited duration of protection conferred by aP vaccines may facilitate emergence of variant strains capable of evading vaccine-induced protection [21, 22]. Immune pressure may have driven the observed evolution of *B. pertussis*, following replacement of wP vaccines with aP preparations [23]. Isolates have emerged that do not produce Prn, FHA and fimbriae or vary in production of Ptx [24, 25].

The pathogenesis of *Bordetella* species and biofilm formation has been reviewed [26]. This include the potential roles of the surface-associated polysaccharide antigen, poly-β-1,6-*N*-acetyl-D-glucosamine (PNAG) or the *Bordetella* polysaccharides (Bps), and their significance in biofilm formation in vitro and in mice [26]. By functioning as an adhesin, the Bps of *B. pertussis* has been shown to promote colonisation and biofilm formation in the nose of mice [27]. These reports highlight the contributions of biofilms in bacterial persistence and transmission in human hosts. This review describes the potential of virulence factors and novel biofilm antigens to improve pertussis vaccines and prevent the transmission of whooping cough within the community.

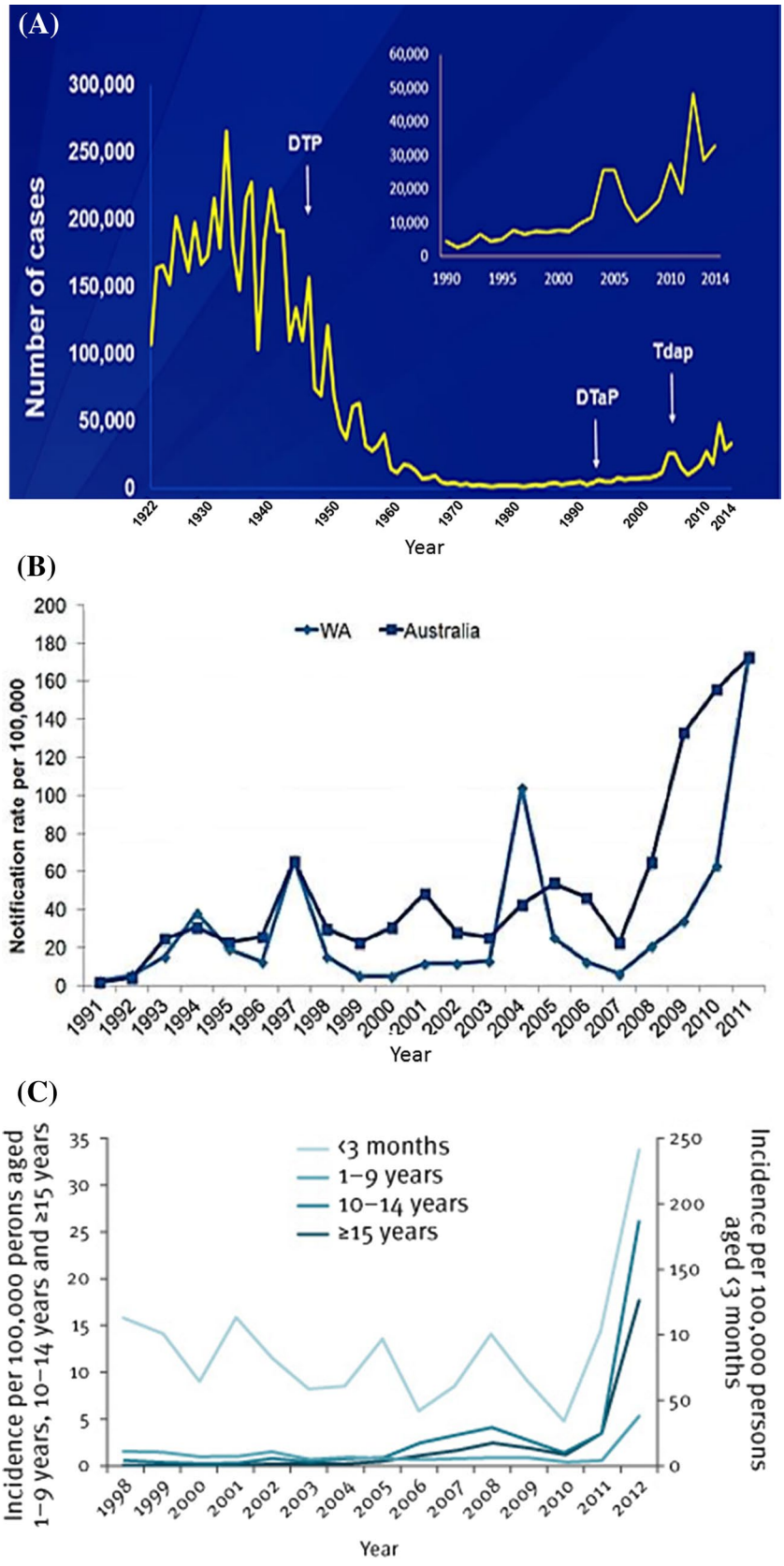


**Fig. 1** Pertussis notification rates from developed countries. Cases from the United States (A)[Ω], Australia (B)[Δ] and United Kingdom (C)[†]. [Ω]Source, Centre for Disease Control and Prevention (http://www.cdc.gov/pertussis/surv-reporting.html), [Δ]Source, Disease Watch, Government of Western Australia, Department of Health (http://www.health.wa.gov.au/diseasewatch/vol16_issue1/all.cfm). Notifications for both national and Western Australia (WA) is shown. [†]Source, Health Protection Agency [17]. Year of report shown in *y*-axis. *DTaP* diptheria, tetanus and pertussis

## The pertussis resurgence

The increasing incidence of whooping cough in several countries is shown in Fig. 1. The resurgence in countries with high vaccination coverage may reflect the short-term protection conferred by the aP vaccines and consequent pathogen evolution. Resurgent and vaccine strains differ in amino acid sequence and the regulation of virulence factors, including those in the current aP vaccines [25, 28]. Resurgent strains are less affected by sulphate-mediated gene suppression and express higher levels of several virulence factors including Ptx, type III secretion toxin (T3SS), Vag8, a protein involved in complement resistance (BrkA) and LpxE, involved in lipid A modification [16, 29]. Whole genome sequencing shed further light on the microevolution and genetic diversity of the p*txP3* strains and differentiated them from the vaccine strain carrying *ptxP1* from which they evolved [30, 31]. Several alleles unique to *ptxP3* strains contribute to its fitness [32]. Diverse genomic events led to Prn and FHA deficiency that also differentiate *ptxP3* strains [30, 33]. *B. pertussis* strains with a novel allele in Ptx promoter, *ptxP3*, were first reported in the Netherlands [25]. It has since been reported in many countries and replaced the indigenous *ptxP1* strains. The *ptxP3* strains display increased production of pertussis toxin and therefore may be more virulent in humans and suppress the host immunity more efficiently [16, 25, 29]. Safarchi et al. [34] found that the *ptxP3* strains colonised the mouse respiratory tract better than the *ptxP1* strain in both vaccinated and unvaccinated mice. Moreover, emerging *B. pertussis* strains can enhance signaling through human pattern recognition receptors (TLR2 and NOD2), and induce secretion of IL-10 by dendritic cells [35]. Increased expression of the regulatory molecule PD-L1 may dampen the vaccine-induced protective response, favouring the survival of this pathogen. The emergence of pertactin-negative ($Prn^-$) isolates in Australia [36], Europe [33], Japan [37] and the USA [38] may reflect a better fitness of the bacterial pathogen, particularly in populations immunised with aP vaccine and carrying anti-Prn antibodies [39]. These findings suggest that *B. pertussis* has adapted to vaccine pressure, so vaccine candidates containing novel antigens from the resurgent strains are warranted.

## Regulation and control of *B. pertussis* virulence factors

### The two-component BvgAS virulence regulatory system in *B. pertussis*

Infection of the host by *B. pertussis* begins with contact with respiratory droplets from an infected individual. At the host's mucosal surfaces, the bacteria produce virulence factors including adhesins and toxins. *B. pertussis* display phase variation in the expression of virulence factors in response to environmental niche [40]. This is achieved by sensory transduction systems, which transduce environmental signals in gene regulation. Transcription of essentially all *B. pertussis* virulence factors is controlled by a locus called the *Bordetella* master virulence regulatory system (BvgAS) [41] and the putative sensor kinase RisAS two-component system (TCS) [42].

The DNA sequence of the BvgAS system revealed one of the first so-called unorthodox TCS, characterised by a composite multi-domain histidine kinase (BvgS) and a four step His-Asp-His-Asp phosphorelay [41]. BvgA and BvgS are 23 and 135 kDa DNA-binding response regulator and a transmembrane sensor kinase, respectively [43], which includes a sensing domain situated in the bacterial periplasm. In response to environmental stimuli, the sensor kinase undergoes a conformational change and transmits signal to the cell resulting in ATP-dependent autophosphorylation of the sensor kinase by its homodimer partner. The phosphorylated BvgS then activates BvgA by transferring its phosphate. The activated BvgA~P, binds to *cis*-acting promoter sequences to activate transcription of virulence-activated genes (*vags*). Simultaneously, a 32 kDa cytoplasmic repressor protein, BvgR, expressed from the BgvAS locus, downregulates the transcription of virulence-repressed genes (*vrgs*) [44]. This phase of *B. pertussis* growth is known as the "non-modulated phase", "$Bvg^+$ phase", or "virulent phase" and is associated with expression of toxins and adhesins required for virulence.

The $Bvg^+$ phase usually manifests when *B. pertussis* is grown at 37 °C in the respiratory tract of a human host [41, 43]. Conversely, when *B. pertussis* is grown in the presence of millimolar amounts of sulphate ions (50 mM $MgSO_4$), 10 mM nicotinic acid or at temperatures around 25 °C, the phosphorylation of BvgA by sensor kinase is suppressed. As a result, BvgA~P is not formed and BvgR is not activated [44]. In this state, BvgAS is not able to activate the transcription of *vags*. The *vrgs* are expressed in the absence of BvgR and the bacteria are in a "modulated, $Bvg^-$ or avirulent phase". During infection, the respiratory environment provides modulating signals that induces the expression of virulence factors [45]. For

example, the $Bvg^-$ phase is characterised by the expression of flagella for motility and genes encoding urease are expressed, aiding its survival in the nutrient-deprived environments [45]. Alternatively, $Bvg^-$ phase may represent an evolutionary remnant that is no longer required in *B. pertussis* [6].

In addition to the $Bvg^+$ and the $Bvg^-$ phases, an "intermediate phase" or "$Bvg^i$ phase" has been identified in *B. pertussis*. The $Bvg^i$ phase is characterised by absence of Bvg-repressed phenotypes owing to the lack of expression of *vrg* genes, with expression of some Bvg-activated virulence factors (eg: FHA), and minimal expression of genes encoding adenylate cyclase toxin (ACT) and pertussis toxin (Ptx) [46]. The $Bvg^i$ phase can be induced in the laboratory by substituting threonine-to-methionine at amino acid residue 733 within the consensus H-box of the transmitter of BvgS of *B. pertussis*, leading to increased resistance to nutrient limitation and reduced virulence [47]. Bvg-intermediate phase A (BipA) protein was one of the first identified $Bvg^i$ phase proteins in *B. pertussis* [48]. It is a 1578 amino acid protein and its N-terminus region shares amino acid sequence with the putative outer membrane localisation domain of intimin (Int) and invasin from *Escherichia coli* and *Yersinia* species, respectively [48]. BipA and the $Bvg^i$ phase may play an important role in the pathogenesis of *Bordetella* species. This may involve aerosol transmission and/or respiratory tract colonisation and survival [48].

Vags have been categorised into three temporal classes; early, intermediate and late genes [49]. Early (or class II) genes include those encoding FHA and Fim and are activated rapidly in response to low concentrations of BvgA~P. Interestingly, the BvgAS system is auto-regulated, and the *BvgAS* locus is categorised as a class II early gene [49]. Auto-phosphorylation of the BvgAS system results in continuous repression and/or expression of downstream genes in *Bordetella* species [50]. The late (or class I) genes include those encoding ACT and Ptx, activated 2–4 h after activation of the BvgAS locus [50]. Genes encoding Prn belong to the class of intermediate genes expressed about 1 h after the activation of the BvgAS locus [50]. This phenomenon of differential gene expression by the BvgAS is due to the architectural differences in the BvgAS regulon promoters. In *B. pertussis*, transcription at the *BvgAS* locus is controlled by four promoters: P1, P2, P3 and P4. P1, P2 and P3 direct expression of the *BvgAS* operon, while synthesis of RNA complementary to the 5′ untranslated region of BvgAS mRNAs is directed by the P4 promoter [50]. Promoters of the late genes require higher concentrations of BvgA~P because of the low-affinity of BvgA binding sites upstream of the transcription start site. Early gene promoters contain high affinity binding sites for BvgA closer to the transcription start site and can be activated by low levels of BvgA~P.

The distinctive $Bvg^+$, $Bvg^-$ and $Bvg^i$ phases have been demonstrated in vitro under stable conditions. The human respiratory tract is a more variable environment, so the BvgAS system is unlikely to function as an "on–off" switch. Rather it facilitates expression of a spectrum of virulence factors transitioning between the $Bvg^+$, $Bvg^-$ and $Bvg^i$ phases [49, 51]. Indeed, the *Bordetella* BvgAS system is often considered a "rheostat" that promotes the infectious cycle of *B. pertussis* by enabling its survival, persistence and dissemination in diverse environmental niches [51].

## The RisAS regulatory system in *Bordetella*

The *Bordetella* regulator of intracellular stress response (ris) system is encoded by the *risAS* locus that encodes a response regulator (RisA) and a sensor kinase (RisS). RisAS is orthologous to the EnvZ–OmpR systems of other Gram-negative bacteria that have been implicated in virulence and shown to reciprocally regulate the expression of the outer membrane proteins, OmpC and OmpF, in response to changes in osmolarity [52]. RisAS is optimally expressed at 37 °C in the absence of magnesium and is important for its intracellular survival independent of the BvgAS regulon [53]. Compared to wild type, a *B. bronchiseptica ris* mutant strain exhibited reduced intracellular survival in mouse macrophages, whereas complementation of *ris* restored its intracellular survival [53]. Moreover, a bacterial acid phosphatase which plays a role in intracellular survival is regulated in response to environmental signals transduced by the RisAS system. The *ris* mutant was susceptible to host intracellular oxidative stress and hence had impaired capacity to persist in the lungs of mice. Complementation of *ris* mutant with the intact *ris* operon restored resistance to oxidative killing in macrophages and survival in the lungs of mice [53].

In *B. pertussis*, the *risAS* allele contains an additional 'C' at position 1848, resulting in a frameshift mutation and leading to a truncated, non-functional form of the RisS sensor protein. The deletion of risA reduces the transcription and expression of *vrgs*, while high levels of *risA* induced strong transcription of *vrgs*, suggesting that *risA* might play an antagonist role to BvgR in the regulation of *vrgs* [52]. RisA may activate *vrgs* by binding directly to the cis-activating sites of the *vrgs*, or indirectly by altering the expression of other key regulatory molecules. The *ris* locus regulates the expression of important factors necessary for intracellular survival of *B. bronchiseptica* but the role of this locus in *B. pertussis* pathogenesis has not been clearly elucidated. A study [42] showed that the expression of almost all *vrgs* is under the control of RisA. Activation of the *ris* locus in *B. pertussis* may require a cross-talk from one or more different, as yet uncharacterised, heterologous regulatory system (s), adding another layer of complexity to regulation of virulence in *B. pertussis* [42].

**Table 1** Virulence factors of *Bordetella* and their role in pathogenesis and vaccine development

| Virulence factors/antigens | Molecular character | Class/family | Role in *B. pertussis* pathogenesis | Appearance during infection/Bvg phase | Mechanism of action in disease | Component/references |
|---|---|---|---|---|---|---|
| Toxins | | | | | | |
| Pertussis toxin (Ptx) | 117 kDa hexameric subunit with AB5 configuration | Typical A–B toxin of ADP riboxylating family | Adhesion, immune evasion, local and systemic toxin effects | Expressed as late genes. Acts in synergy with FHA | Coupling of Gαi protein-receptor is inhibited and its signal transduction is blocked | Component of acellular vaccines alone or in combination |
| Adenylate cyclase toxin (ACT) | 177 kDa toxin | Repeats-in-toxin (RTX) family of toxins activated by eukaryotic calmodulin | Evasion of host immune response, local and systemic toxin effects | Expressed as late genes. Acts in synergy with Ptx and FHA | Conversion of intracellular ATP to cyclic cAMP and affects superoxide generation, immune effector cell chemotaxis, phagocytosis and bacterial killing. Pore formation and disrupts cells | Not component of acellular vaccines |
| Tracheal cytotoxin (TCT) | 921 Da | Muramyl peptide family | Evasion of host immune response, local toxin effects | Expressed as early genes | Causes ciliostasis, inhibit DNA synthesis and extrusion of ciliated cells | Not component of acellular vaccines |
| Dermonecrotoxin (DNT) | 160 kDa | A–B family of toxin with polyamination and deamination activity | Local derma necrosis and systemic vasoconstriction toxin effects | NK | Activates host GTP binding protein Rho and results in constitutive expression of GTPase activity | Not component of acellular vaccines |
| T3SS | – | Membrane-embedded nano-injection machinery | Translocates bacterial virulence factors | $Bvg^+$ | Secretion of effectors and translocons | [54, 55] |
| Adhesins | | | | | | |
| Filamentous haemagglutinin (FHA) | 232 kDa | Filamentous protein | Adhesion | Early $Bvg^+$ phase | Binds to ciliated tracheal epithelium, macrophage CR3 receptors and promotes phagocytosis | Component of most acellular vaccines |
| Pertactin (Prn) | 69 kDa | Auto-transporter family of proteins | Adhesion | Expressed as intermediate genes | Binds to ciliated tracheal epithelium, macrophage CR3 due to the presence of RGD motif | Component of three- and five-component acellular vaccines |
| Fimbriae (Fim) | Fim2: 22 kDa<br>Fim3: 21.5 kDa | Filamentous proteins | Adhesion: Fim2 binds to sulphated sugars; Fim3 and FimD to heparin and integrin VLA–5 | Expressed as early genes | Binds to tracheal epithelial cells, predominantly in trachea | Component of acellular vaccines |
| Tracheal colonising factor (Tcf) | 64 kDa | Auto-transporter family | Adhesion | NK | Binds exclusively to tracheal epithelium | Not component of acellular vaccines |

**Table 1** (continued)

| Virulence factors/antigens | Molecular character | Class/family | Role in *B. pertussis* pathogenesis | Appearance during infection/Bvg phase | Mechanism of action in disease | Component/references |
|---|---|---|---|---|---|---|
| Other virulence factors of *Bordetella* used as vaccine antigens | | | | | | |
| Proteoliposome preparation | – | A nanoparticulate vesicular structures that contains proteins, lipids and native LPS | NK | NK | – | [56] |
| PNAG | – | Composition of outer membrane polysaccharide | NK | NK | Major component of bacterial cell wall | Not studied |
| Biofilms | – | Extracellular mesh of sessile bacteria | NK | NK | Protects bacteria from host immune response, antimicrobial peptides and persistence of bacteria inside an extracelluar matrix | [57] |
| Serum resistance factor (BrkA) | 103 kDa | Auto-transporter family of proteins | Adhesion | NK | Serum resistance factor. Confers resistance to bacteria from complement (C) killing | [58, 59] |
| Sph1B | Subtilisin-like serine protease | Auto-transporter family of proteins | Adhesion due to presence of one or more RGD tripeptide motif | NK | Promotes the maturation of FHA adhesion molecules on *Bordetella* | Not component of acellular vaccines |
| Vag8 | C1 esterase inhibitors (C1inh) | Auto-transporter family of proteins | Adhesion due to presence of one or more RGD tripeptide motif | $Bvg^+$ virulent phase | Confer resistance to bacteria from C1 esterase mediated C killing | Not component of acellular vaccines |
| Siderophores (IRP-1 and AfuA) | AfuA: 39 kDa<br>IRP-1: ~ 25 kDa | Iron-binding protein | Overcome host iron restriction | $Bvg^+$ virulent phase | Enable bacteria to thrive in iron limiting niche | [60, 61] |

*NK* not known, *T3SS* type III secretion system

## Virulence-associated factors of *B. pertussis*

The structure and functions of the wide array of virulence factors produced by *B. pertussis* (Table 1) are reviewed below.

### Pertussis toxin

Pertussis toxin (Ptx) is a major virulence factor and a protective antigen produced exclusively by *B. pertussis*. While species such as *B. parapertussis* and *B. bronchiseptica* have genes encoding the toxin, they do not produce functional Ptx. Ptx is an exotoxin that is transported across the bacterial outer membrane by a type IV secretion system. It is a 117 kDa hexameric subunit protein of $AB_5$ configuration consisting of one active subunit (subunit A or S1 subunit), and five binding B oligomers [63, 64]. The A subunit consists of a catalytic ADP-ribosylating domain, and is responsible for the enzymatic activity of Ptx [64]. The B oligomer is composed of S2, S3, two S4 and S5 pentameric-subunits involved in binding of the toxin to extracellular target cell receptors, including the Toll-like receptor 4 (TLR-4) and others glycoconjugate receptors. It enables the translocation of the catalytic A subunit (S1) across the plasma membrane and allows the toxin to enter the cells [64].

The molecular mechanism involved in entry of Ptx into the cell is not well understood. In the cytosol, the A subunit catalyses the transfer of released ADP-ribose from the hydrolysed cellular nicotinamide adenine dinucleotide ($NAD^+$) to a specific cysteine residue at the C-terminus of the α-subunit of the heterotrimeric $G_{i/o}$ family regulatory proteins [65]. This prevents the coupling of G-regulatory proteins to their cognate receptors (GPCRs), resulting in an inactive (GDP-bound) form of the α-subunit. Once inactivated, G-proteins are unable to inhibit adenylate cyclase activity, and thus are unable to halt the conversion of intracellular ATP to cAMP (Table 1). The accumulation of intracellular cAMP disrupts many cellular cascades as shown in Fig. 2.

Diverse effects of Ptx are attributed to the ADP-ribosylation of the $G_{i/o}$ protein family. They include most systemic symptoms of pertussis infection such as leukocytosis, insulinaemia, hypoglycaemia and histamine sensitivity in children [65], and lethality of neonatal mice challenged with virulent *B. pertussis* [66]. Accordingly, *B. pertussis* strain deficient in Ptx isolated from an unvaccinated child with suspected pertussis displayed low virulence [67]. However, most acellular pertussis vaccines contain chemically or genetically detoxified pertussis toxin (PTxoid) in combination with FHA with or without incorporation of Prn and/or fimbriae (Fim2, Fim2/Fim3) (Table 2). Interestingly, Denmark uses hydrogen peroxide inactivated Ptx (40 µg) as a sole pertussis antigen in its immunization schedule [68]. Inactivation of the toxin with hydrogen peroxide instead of the traditional formaldehyde results in higher preservation of antigenic epitopes and therefore more effective immune responses [68]. PTxoid alone as a mono-component antigen tested as an acellular pertussis vaccine yielded an overall efficacy of 71% in vaccinees and reduced *B. pertussis* transmission to household contacts [69, 70]. However, chemical detoxification with formaldehyde or glutaraldehyde, employed by various manufacturers alters the tertiary and quarternary strucures of Ptx, thereby affecting recognition of protective epitopes by antibodies [71].

### Adenylate cyclase toxin

Adenylate cyclase toxin (ACT) is an immunomodulatory toxin produced by *B. pertussis, B. parapertussis* and *B. bronchiseptica* [72]. ACT functions as a haemolysin and cytolysin, and is a member of the repeat-in-toxin (RTX) family that forms pores in bacterial cell membranes [72]. It is a

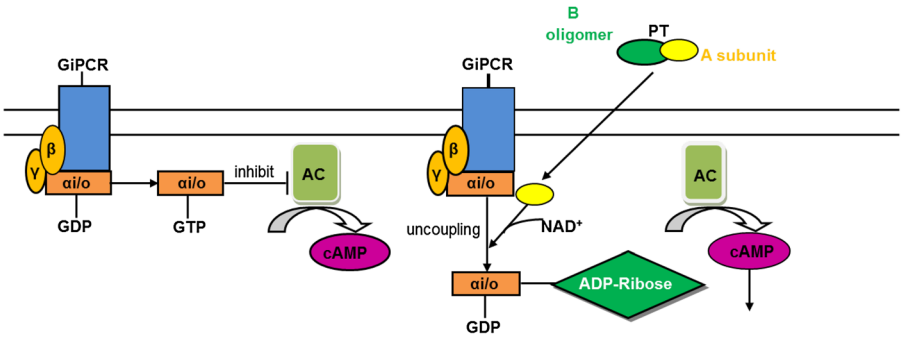


**Fig. 2** Pertussis toxin (Ptx) mediated uncoupling of Gαi/o proteins from GiPCR. Exchange of GTP from GDP results in activation of G-inhibitory proteins (Gαi/o) which inhibit adenylyl cyclase (AC) activity and reduce cAMP levels. The A subunit of Ptx ADP-riboxylates the Gαi/o-inhibitory protein and inactivates Gαi/o resulting in constitutive expression of AC activity, accumulation of cAMP and activation of cAMP-mediated signalling pathways

**Inhibition of $G_i$ proteins results in constitutive expression of AC activity that leads to accumulation of cAMP level and stimulation of cAMP-mediated signalling like protein kinase activity**

**Table 2** Major pertussis vaccines and their antigen components used by different pharmaceutical companies

| Vaccine | Trade name | Manufacturer | Pertussis antigen content (μg)[a] | | | | Indications/references |
|---|---|---|---|---|---|---|---|
| | | | FHA | Ptx | Prn | Fim2/3 | |
| DTPa1 | Certiva | Baxter | – | 40 | – | – | 3–12 months |
| DTaP | Daptacel | Sanofi Pasteur | 5 | 10 | 3 | 5 | 6 weeks to 6 years |
| | Infanrix | GlaxoSmithKline | 25 | 25 | 8 | – | 6 weeks to 7 years |
| | Tripedia | Sanofi Pasteur | 23.4 | 23.4 | – | – | 6 weeks to 7 years |
| | ACV-SB | Smithkline Beecham | 25 | 25 | 8 | – | Used in Dutch NIP |
| | Pentavac[b] | Sanofi Pasteur | 25 | 25 | – | – | 2, 4 and 6 months |
| | Acelluvax | Novartis | 2.5 | 5 | 2.5 | – | 2, 4 and 6 months |
| DTaP4 | Acel-Immune | Wyeth/Takeda | 35 | 3.5 | 2 | 0.8 | 3, 4.5, 6 and 15 months |
| DTaP/Hib | TriHIBit | Sanofi Pasteur | 23.4 | 23.4 | – | – | Children 15–18 months |
| DTaP/Polio | Kinrix | GlaxoSmithKline | 25 | 25 | 8 | – | Children 4–6 years old |
| DTaP/Polio/Hib | Pediacel[b]/Pentacel | Sanofi Pasteur | 20 | 20 | 3 | 5 | Infants and children 6 weeks to 4 years |
| DTaP/Polio/hepatitisB | Pediarix/Infanrix | GlaxoSmithKline | 25 | 25 | 8 | – | 6 weeks to 6 years |
| Tdap | Adacel | Sanofi Pasteur | 5 | 2.5 | 3 | 5 | Adolescent and Adults 11–64 years |
| | Boostrix | GlaxoSmithKline | 8 | 8 | 2.5 | - | 10 years and older |
| | Triaxis | Sanofi Pasteur | 5 | 2.5 | 3 | 5 | 2 months to 4 years |
| | DTwP–IPV | RIVM, Bilthoven, The Netherlands | 2.6 | 0.16 | n.d | n.d | Used in Dutch NIP for 2 months to 4 years [62] |

*NIP* national immunisation program, *DTaP* diphtheria, tetanus and acellular pertussis, *Tdap* reduced DTaP, *ACV* acellular vaccine, *Hib haemophilus influenzae* B

[a]Concentration of pertussis antigen components only

[b]Pentavac and pediacel are DTaP–IPV–Hib vaccine

polypeptide of 1706 amino acids consisting of two domains, a N-terminal adenylate cyclase (AC) domain of approximately 400 amino acid residues and a haemolytic C-terminal domain of 1300 amino acids [73]. The RTX haemolytic (Hly) domain mediates binding of the toxin to target cells and translocation of AC to the cytosol, via cation-selective toxin pores. ACT mediates adherence to host cell by binding to the $\alpha_M\beta_2$ integrin (CD11b/CD18 or CR3 or Mac-1) expressed on myeloid phagocytic and natural killer cells [74]. In the cytosol, AC is activated by a $Ca^{2+}$-binding protein known as calmodulin (CaM) and catalyses the conversion of intracellular ATP to cAMP that inhibits superoxide generation, immune effector cell chemotaxis, phagocytosis and bacterial killing [75].

ACT is an important bi-functional virulence factor secreted by *B. pertussis* in the early stage of infection and can elicit a protective immune response. Although antibodies against ACT have been found in sera of infants and children infected with *B. pertussis* [76] or immunised with whole cell vaccines [77], it is surprising that ACT has not been included in any currently available aP vaccines. A study reported that immunisation of mice with a purified adenylate cyclase enzyme lacking the toxin fragment protected mice from *B. pertussis* challenge when delivered intranasally, and significantly enhanced the clearance of bacteria from the mouse respiratory tract [78]. It suggested that this protective antigen, if incorporated into aP vaccine formulations, could reduce the asymptomatic human reservoir by limiting bacterial carriage in the respiratory tract. Of interest is the report that protective antibodies induced by ACT were directed against the last correctly folded 800 residues of the C-terminal region of the toxin [79]. It is thus surprising that the option of using ACT in the acellular pertussis vaccine has not been appropriate attention [80].

Since ACT delivers the N-terminal catalytic domain into the cytosol of eukaryotic cells, including human effector immune cells, it has been exploited as a tool for antigen delivery [66, 81]. Fayolle et al. [82] chemically linked peptides containing $CD8^+$ T-cell epitopes from several proteins to the catalytic domain of ACT and showed that these recombinant proteins are presented to $CD8^+$ cells inducing cytotoxic T-lymphocytic (CTL) responses in immunised mice. Another study demonstrated that ACT linked to epitopes of antigenic proteins and delivered into CD11b myeloid dendritic cells induced epitope-specific Th1 $CD4^+$ and $CD8^+$ T cell responses [83]. Similarly, immunisation of mice with epitopes of HIV-1 Tat linked to the catalytic domain of ACT (ACT-HIV-1-Tat peptide), elicit a strong Th1-skewed immune response [84]. These findings suggest

the potential for ACT as a carrier for delivery of a range of therapeutic agents including heterologous vaccines.

## Tracheal cytotoxin

Tracheal cytotoxin (TCT) is a peptidoglycan component of bacterial cell wall peptidoglycan, a 921 Da disaccharide-tetrapeptide composed of *N*-acetylglucosaminyl-1,6-anhydro-*N*-acetylmuramylalanyl-gamma-glutamyl-diaminopimelyl-alanine and belongs to the muramyl family of peptides [85]. Muramyl peptides are produced by bacteria and are the polymeric components of the cell wall that provide structural rigidity [85]. It has several important biological functions including immunogenicity, somnogenicity and pyrogenicity. During bacterial growth and processing by macrophages, Gram-negative bacteria such as *E. coli* and *Neisseria gonorrhoeae* produce muramyl peptides similar to TCT through the action of transglycosylase enzymes. The muramyl peptides of *N. gonorrhoeae* have been associated with ciliated cell destruction during infections of the fallopian tube [86]. TCT is similar in structure to the sleep promoting factor, FSu, a muramyl peptide found in the human brain that promotes active slow-wave sleep [86]. Of the three major *Bordetella* species, TCT is produced only by *B. pertussis*. In *B. pertussis*, TCT is independent of the BvgAS control system and is constitutively expressed [1]. *E. coli* recycle the peptidoglycan fragment into the cytoplasm for reincorporation into the peptidoglycan biosynthesis pathway through an integral cytoplasmic membrane protein, AmpG [87]. However, *B. pertussis* has no functional AmpG activity so the TCT fragment is released into the external milieu [1] during log phase growth and in the virulent phase [88], and damages ciliated cells of the respiratory tract promoting ciliostasis and colonisation. When ciliary movement is retarded, coughing remains the only mechanism to eliminate accumulation of other inflammatory molecules from the respiratory tract, resulting in the eponymous paroxysmal cough. The destruction of ciliary cells by *B. pertussis* TCT also makes the host susceptible to secondary infections, which is often the primary cause of deaths.

Cundell et al. [89] showed that TCT inhibits human neutrophil functions including migration, IL-1α production and complement activation by the alternative pathway, and so may increase survival of *B. pertussis* during respiratory infections. Production of IL-1 by the intracellular tracheal epithelial cells drives production of inducible nitric oxide synthase (iNOS) and hence nitric oxide (·NO), which damages tracheal cells.

## Dermonecrotic toxin

*Bordetella pertussis* dermonecrotic toxin (DNT) is a heat-labile, 1464 amino acid (160 kDa) protein consisting of a single chain polypeptide with an N-terminal receptor binding domain and C-terminal enzymatic domain [90]. The name dermonecrotic toxin is derived from a characteristic skin lesion induced by the toxin when administered intradermally into animals. It can catalyse polyamination or deamidation of small Rho family GTPases such as Rho, Rac and Cdc42, through a novel transglutaminase activity [91]. Unlike many bacterial toxins, DNT activates Rho family GTPases, which are essential for functions including reorganisation of the actin skeleton, cell motility, focal adhesion, transcription of certain essential genes and cell differentiation [92]. On activation, the inactive GDP-bound forms exchange GDP for GTP. GTP-bound DNT actively transduces downstream signals before reverting back to the inactive GDP-bound form [92]. Polyamination or deamidation of Rho-GDP by DNT at the Gln63 residue of Rho results in formation of Rho-GTP and prevents the reversion of Rho-GTP to its inactive GDP-bound form. The constitutive expression of the active GTP-bound form (Fig. 3) results in expression of stress fibres, inhibition of cytokinesis, and disturbances of cell differentiation via its association with effector protein, ROCK [93]. DNT can inhibit DNA and protein synthesis, inhibit osteoblastic differentiation, induce organelle formation on membranes and induce formation of caveolae [93]. The N-terminal 54 amino acid B (binding) domain is responsible for binding of DNT to target cells, while the C-terminal 288 amino acids A (active) domain is responsible for the enzymatic activity of DNT and residues 2–30 constitute the binding site within the B domain [94].

*Bordetella parapertussis, B. avium* and *B. bronchiseptica* DNT molecules are identical [93]. Despite the reported protective capacity of the DNT-knockout *B. pertussis* vaccine candidate, the absence of DNT in a mutant strain did not affect its virulence compared to the wild-type [95] limiting its relevance to the development of a live attenuated vaccine against pertussis.

## Pertactin

Pertactin (Prn) belongs to the family of type V auto-transporters virulence factors [72]. Mature Prn is a 68, 69 and a 70 kDa protein in *B. bronchiseptica, B. pertussis* and human *B. parapertussis*$_{HU,}$ respectively, and is encoded by the *prn* gene. Prn contains an arginine-glycine-aspartate (RGD) tripeptide motif required for attachment of the *Bordetella* species to integrin binding sites of eukaryotic cells [1]. The nascent Prn polypeptide has a unique central passenger domain flanked by an N-terminal signal sequence and an approximately 30 kDa C-terminal porin domain. The N-terminal signal sequence directs the protein into the periplasm and the C-terminal porin domain forms a channel in the outer membrane that is required for the transport of the passenger domain to the cell surface [96]. On the

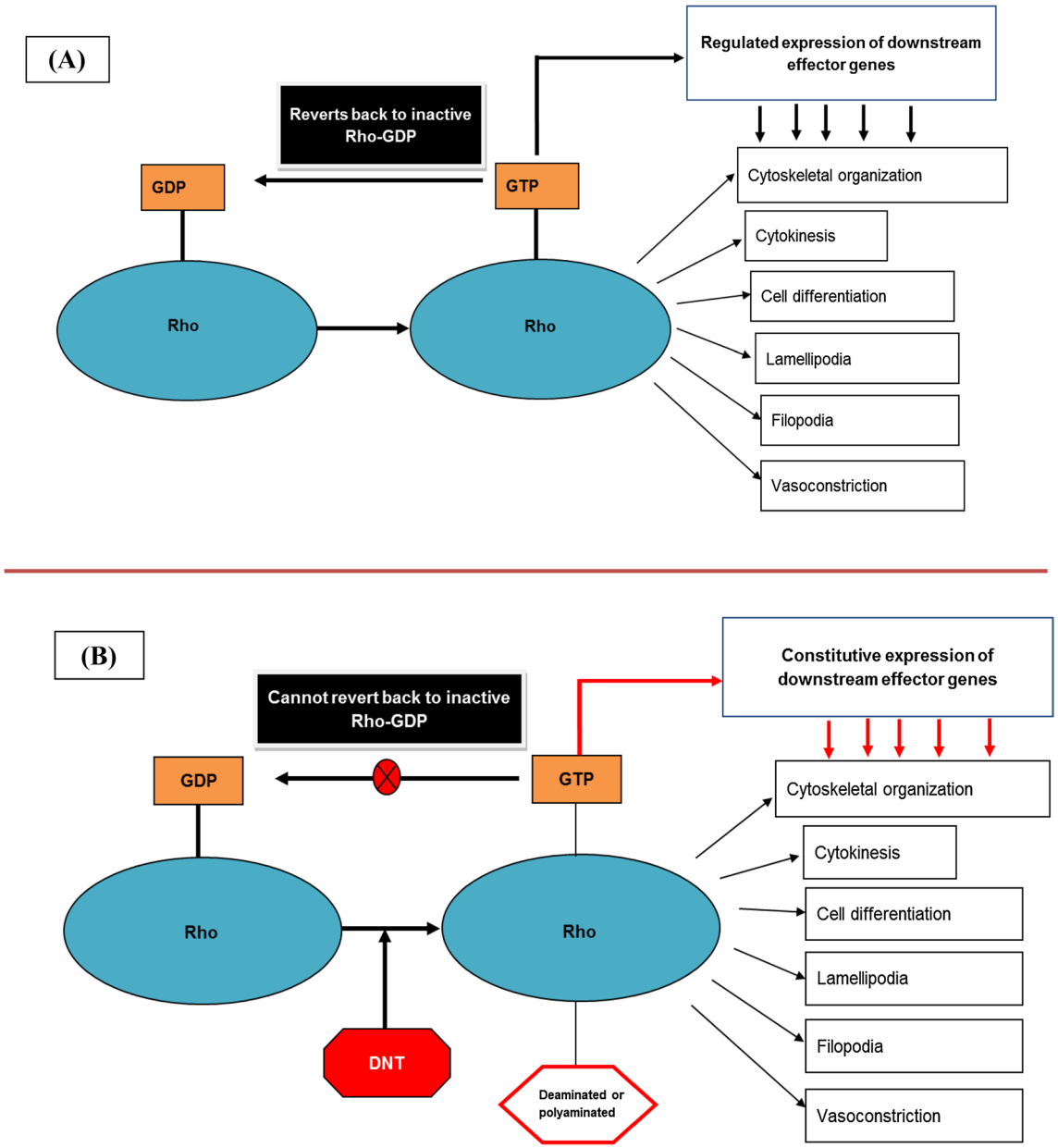


**Fig. 3** Action of DNT on functions of Rho-GTPase. **a** Normal pathways of Rho-GTP activation in the absence of DNT. **b** Deamination or polyamination of Rho-GTP by DNT abrogates the GTP-hydrolysing activity of GTPase and reversion to Rho-GDP is blocked, resulting in constitutive expression of Rho-GTPase activity. Additionally, polyaminated GTPase attains the ability to stimulate downstream effector genes in a GTP-independent manner and induce constitutive expression of GTPase resulting in anomalous cellular events

cell surface, the passenger domain folds into right-handed β-helix comprising 18 three-β-strand repeats connected by turns of differing lengths. Amino acids 260–294 in *B. pertussis* Prn constitute region 1 (R1) and include several Gly-Gly-Xaa-Xaa-Pro repeats and the RGD motif. R1 may be the immunodominant region of *B. pertussis* Prn. Antibodies targeting R1 isolated from a patient with *B. pertussis* conferred protection in mouse respiratory models [97]. Residues 563–614 in the C-terminal domain of *B. pertussis* Prn contain Pro-Gln-Pro (PQP) repeats and constitute region 2 (R2). Region R1 is highly polymorphic and has been implicated in vaccine-induced evolution of *B. pertussis*.

Intriguingly, despite Prn being an important virulence factor, few studies address the function of Prn in *B. pertussis* pathogenesis. The RGD motif in the centre of the passenger domain allows Prn to function as an adhesin. Early studies [98, 99] suggested that attachment of *B. pertussis* to Chinese hamster ovary (CHO) or human HeLa cells requires Prn. However, later studies [100, 101] failed to demonstrate the essential role of Prn in attachment to mammalian cells. More

recently Inatsuka et al. [96] implicated *B. bronchiseptica* Prn in resistance to clearance of bacteria from the lungs of mice mediated by neutrophils. A *B. bronchiseptica* Δprn mutant was unable to infect SCID-beige mice (lacking T-cells, B-cells and NK cells) but its ability to cause lethal infection in neutropenic mice was not impaired. The authors inferred that the RGD sequence was not essential for Prn function. Clearly, further studies are necessary to elucidate the role of Prn in the pathogenesis of *B. pertussis*.

Prn is a component of most currently used acellular pertussis vaccines (Table 2). *B. pertussis* isolates with variation in Prn and Ptx have been first reported in the Netherlands [102]. After the introduction of whooping cough vaccination, vaccine type variants (PtxA2 and Prn2) were replaced by non-vaccine type variants (PtxA1 and Prn2). Similar shifts are now observed worldwide and PtxA1–Prn2 strains predominate in most vaccinated populations. Since the introduction of the acellular pertussis vaccine, *B. pertussis* strains lacking Prn have been reported in Finland [103], Sweden [104], Europe [33] the United States [105], Japan [37] and Australia [36]. These Prn-negative isolates were fully virulent. In a mouse model, several studies showed that antigenic divergence observed between vaccine strains and resurgent strains reduces the vaccine efficacy [39, 106]. As strains deficient in the expression of Prn have been isolated from infants and children vaccinated with DTaP [38, 107], inclusion of Prn in aP vaccines warrants further investigations.

## Tracheal colonisation factor

Tracheal colonisation factor (Tcf), first described in 1995 [108], has been found in *B. pertussis* but not in *B. parapertussis* or *B. bronchiseptica*. It is encoded by the *tcfA* gene and expressed in both cell-associated and secreted forms. Tcf functions as an adhesion molecule and contains RGD motifs with high (16.5%) proline content. It is released as a 60 and 30 kDa forms from the N- and C-termini of the 90 kDa precursor protein respectively, where the 30 kDa protein is identical to *Bordetella* pertactin precursor protein. *B. pertussis* strains deficient in Tcf colonised the trachea of mice tenfold less efficiently than the wild type strain, but the establishment and persistence of infection with *B. pertussis* deep in the lungs was unaffected [109].

## *Bordetella* resistant to killing (BrkA) protein

BrkA is another auto-transporter and a cell surface-associated virulence antigen of *B. pertussis* evading killing via the classical complement pathway [110]. It is a Bvg-regulated protein and contributes to the adherence of *Bordetella* species to the host cells and prevent lysis of the bacteria by select antimicrobial agents [110]. BrkA is detectable in *B. pertussis, B. parapertussis* and *B. bronchiseptica* but not in *B. avium* [64]. The Brk locus encodes two open reading frames (ORFs), *brkA* and *brkB*, both of which are required for resistance to killing by human serum. The *brkA* ORF encodes a 103 kDa precursor protein that is processed to yield a 73 kDa N-terminal protein with α-passenger domain possessing functional activity [111]. *brkB* encodes a C-terminal 30 kDa β-domain predicted to be an inner membrane protein. The β-domain facilitates the transport of passenger protein by forming pores in the lipid bilayer, and serves as an intramolecular chaperone facilitating the folding of the passenger domain and translocation across the outer membrane.

While BrkA proteins are not included in any acellular vaccines, a case has been made for their inclusion as a replacement for Prn. Oliver et al. [112] showed that rabbit antibodies against BrkA blocked BrkA activity and killed wild type *B. pertussis*. Mice immunised with the purified 73 kDa N-terminal α-domain of BrkA fused to the 60 kDa heat shock protein (HSP) of *B. pertussis* produced protective IgG1 and IgG2a antibodies—indicating Th2 and Th1-type immune responses, respectively. When administered together with diphtheria–pertussis–tetanus (DPT), 42% of mice were protected, compared to no protection when the DTP vaccine was used alone [58].

Marr et al. [59] showed that immunisation of mice with a three-component vaccine containing Ptx, FHA and BrkA was as efficacious as the commercial Infanrix™ vaccine in protecting mice against colonisation by virulent *B. pertussis* strain Tohama I, but immunisation with BrkA protein alone did not protect against colonisation. Since BrkA is present in all clinical *B. pertussis* isolates, the new acellular pertussis vaccine formulation that includes BrkA may confer protection comparable to the currently marketed DTaP vaccines.

## Other auto-transporter proteins

Other auto-transporter proteins reported in *B. pertussis, B. bronchiseptica* and *B. parapertussis*$_{HU}$ species include sphB1 ("*Bordetella pertussis* adhesins") and Vag8. These two proteins demonstrate similarity in amino acid sequence at their C-terminal domains and contain one or more RGD tripeptide motifs, suggesting a role in adherence.

Vag8 is a 95 kDa outer membrane protein encoded by the *vag-8* gene [113]. The C-terminal ends of Vag8 show significant sequence similarity with the C-terminal ends of Prn, BrkA and Tcf. It was the fourth member of the auto-transporter family of proteins to be identified in *Bordetella* species. The Vag8 protein of *B. pertussis* binds to human C1 esterase inhibitor (C1inh), a major complement regulatory protein in a Bvg-regulated pathway and resists its killing by complement [114]. *B. pertussis* mutants deficient in Vag8 were susceptible to serum killing while wild type *B. pertussis* strains expressing Vag8 were resistant [114]. Furthermore, immunisation of mice with Vag8 induced strong

antigen-specific IgG and significantly reduced bacterial load in the lungs of mice challenged with virulent *B. pertussis* [115]. This suggests a novel role of Vag8 in immune evasion, hence Vag8 warrants inclusion in future pertussis vaccines.

An auto-transporter protein, BapC, with functions similar to BrkA and Prn was described recently as a virulence antigen of *B. pertussis* [116]. BapC is Bvg-regulated and functions as an adhesin factor responsible for adhesion of *B. pertussis* to various cell lines, while conferring resistance to complement killing [116].

## *Bordetella pertussis* adhesins

### Filamentous haemagglutinin

Filamentous haemagglutinin (FHA) is a cell surface-associated 220 kDa mature protein that is secreted into the extracellular environment [117, 118]. FHA is encoded by the *fhaB* gene in *B. pertussis* and *B. bronchiseptica*. It plays a key role in initial attachment of *B. pertussis* to the respiratory mucosal epithelium of host cells, including macrophages [119]. FHA has three binding sites by which it interacts with host cells: (a) an N-terminal glycosaminoglycan binding site, (b) an RGD sequence and (c) a carbohydrate recognition domain (CRD). FHA has two main immune-dominant regions termed the C-terminal type I and N-terminal type II domains, which are highly immunogenic. The C-terminal domain consists of 456 amino acids and contains most of the reactive epitopes and cell binding sites. The N-terminal domain may be responsible for displaying FHA on the surface of *B. pertussis*.

The maturation of FHA from its precursor FhaB, requires a specific protease sphB1. sphB1 is a subtilisin-like serine protease/lipoprotein essential for processing the precursor of the FHA [120]. It was the first reported auto-transporter that is necessary for a maturation of another protein secreted by *B. pertussis. B. pertussis* mutants deficient in Sph1B were strongly compromised in their ability to colonise the mouse respiratory tract. However, colonisation ability was restored when purified FHA was instilled nasally or co-infected with an FHA-expressing *B. pertussis* strain [121], highlighting the importance of the sphB1 protease in the maturation and release of FHA.

Despite the importance of FHA in *B. pertussis* pathogenesis and its inclusion in most acellular pertussis vaccines, little is known about the induction of protective immunity [122]. FHA has not been tested as a mono-component acellular vaccine but an 80 kDa protein derived from the N-terminal domain of FHA (Fha44) showed some promise. Intranasal infection of mice with a *B. pertussis* strain producing Fha44 instead of FHA produced a more effective humoral response [123] (see also Table 2). However, the 85 kDa protein comprising the 42 kDa maltose-binding domain of *E. coli* combined to 43 kDa type I immune-dominant domain of FHA, performed better as a potential vaccine candidate [122]. However, this work needs re-assessment because of differences in the delivery models used in the above two studies.

### Fimbriae

Fimbriae (FIM) are filamentous, cell surface-associated polymeric proteins essential for colonisation of the respiratory tract. *B. pertussis* produces contains serologically distinct types of fimbriae (serotypes 2 and 3) composed of major subunits Fim2 and Fim3, respectively [124]. These subunits [also known as agglutinogen2 (AGG2) and agglutinogen3 (AGG3)], are encoded by the scattered and unlinked chromosomal loci, *fim2* and *fim3* genes, respectively. Their expression is regulated by small insertions or deletions within a stretch of a cytosine-rich promoter region (− 10 and − 35 elements) resulting in fimbrial phase variation in addition to its positive regulation by the BvgAS system [125]. The slip-strand mispairing affects the transcription of the individual fimbrial genes independent of each other. Hence *B. pertussis* may express either Fim2, Fim3 or a combination of fimbriae [125].

The major fimbrial helices are bundled to form long filaments with FimD on their surface. FimD is encoded by the *fimD* gene cluster involved in fimbrial and FHA biosynthesis. FimD recognises two ligands in the respiratory tract, the integrin very late antigen-5 (VLA-5) and heparin sulphate [126]. The binding of FimD to VLA-5 on monocytes activates complement receptor 3 (CR3), which is a ligand for FHA, and enhances the binding of *B. pertussis* to respiratory epithelial cells [126]. The major fimbrial subunits bind to chondroitin sulphate, heparin sulphate and dextran sulphate, which are universally present in the respiratory tract [126].

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

It is likely that FimB and FimD are required for fimbrial production but not for FHA biogenesis. Although, *fhaC* was necessary for FHA production, it did not participate in fimbriae biogenesis. On the other hand, *fimD*-mutants had a reduced ability to colonise the nasopharynx, trachea and lungs [127]. A study of the role of fimbriae in adherence of *B. pertussis* to primary human airway epithelial

cells from human bronchi and a human bronchial epithelial cell lines suggested that this model was more informative than animal models because the cells used could proliferate in vitro, differentiate, and express the same genetic profile as human respiratory cells in vivo [128]. Other studies [101, 119] showed that both fimbriae and FHA were required for adhesion of *B. pertussis* to the laryngeal epithelial cell line, HEp-2, whereas only FHA was required for adhesion to the bronchial cell line NCI-$H_{292}$. These authors concluded that fimbriae played an important role in the colonisation of the laryngeal mucosa, whereas FHA was important in colonisation of the entire respiratory tract. However, a FHA mutant that did not express fimbriae was unable to form biofilm, implying a role of fimbriae in biofilm formation [129]. Whether this holds true for *B. pertussis* requires confirmation.

Fimbrial antigens included in the current five-component acellular vaccines are shown in Table 2. Antibodies against fimbriae are protective against *B. pertussis* infection. Sera from mice immunised with fimbriae reduced the adherence of *B. pertussis* to NCI-$H_{292}$ cells in vitro, as did the antisera raised against Ptx, FHA, Prn or whole cell pertussis vaccine [130]. Similarly, Rodriguez et al. [131] showed that purified antibodies against fimbriae reduced the attachment of *B. pertussis* to respiratory epithelial cells. A study of the five-component vaccine (containing fimbriae) revealed a protective efficiency of 85.25%, while the two-component vaccine (inactivated pertussis toxin and haemagglutinin) and the whole-cell vaccines had protective efficiencies of only 58.9 and 48.3%, respectively [132]. However, in another study the two-component vaccine comprising of Ptx and FHA was as immunogenic and protective as DTwP or DTaP following primary and booster vaccinations [133]. Hallander et al. [134] showed that even 71 months of vaccination with the five-component vaccine, 60% of children still had protective levels of anti-fimbriae antibody (IgG anti-Fim2/3 $\geq$ 5 EU/ml) correlating with decreased risk of *B. pertussis* infection. These authors emphasised the need to include Fim2/3 in future pertussis vaccines.

A new variant of fimbriae, Fim3-2, has reached significant prevalence (up to 80%) in some countries [135, 136]. In Finland, the Fim2 type predominated during the early vaccination period while the Fim3 type has predominated since 1999 and represented the major strains responsible for a nation-wide epidemic in 2003 [137]. Intriguingly, about one-third of patients infected with Fim2 strains developed antibodies that bind to both Fim2 and Fim3 antigens. These authors suggested that the Fim2 strains could express Fim3 during infection [137]. While a mix of Fim2, Fim3 and Fim2,3 strains circulated before mass vaccination in the UK, Fim3 serotype predominated in later years [28]. Similar shifts were observed in Canada [138], France [139], Finland [139], Russia [140], Serbia [141] and Sweden [142].

## *Bordetella* type III secretion system

Bacterial type III secretion system (T3SS) is a membrane-embedded nano-injection structure comprising a hollow extracellular needle and a cylindrical basal body extending beyond bacterial inner and outer membranes [143]. T3SS translocates bacterial virulence factors, termed effectors and translocons, into the host cells to exert their biological functions leading to establishment of infection, persistence and transmission. T3SS is highly conserved in *B. pertussis, B. bronchiseptica* and *B. parapertussis*. The *Bordetella* T3SS is transcribed from the *bsc* locus that comprises 30 ORFs and is regulated by the BvgAS virulence regulon [144]. T3SS secreted proteins include the translocons, BopB, BopD, BopN, Bsp22 and the only effector, BopC/BteA.

Despite the conservation of T3SS, the *B. pertussis* T3SS operon is not expressed in the laboratory strain Tohama I. This difference between species may be due to differential regulation of T3SS by BtrA—a molecule known to reduce T3SS function [145]. Ahuja et al. [146] showed that BtrA differentially regulate the expression of BvgAS-regulated genes including *cyaA, fha, prn, ptx* and T3SS-secreted proteins. Deletion of *btrA* in *B. pertussis* enhances the expression of *bopB, bopD, bopN, bsp22, bscN* and *bteA*. This finding suggests that T3SS is fully functional in *B. pertussis* but its expression is repressed by the BtrA regulatory node.

Another study [147] attributed the differential expression of T3SS-secreted proteins to the IS481 insertion upstream of T3SS effector protein BteA (a T3SS cytotoxic effector protein described in *B. bronchiseptica*). IS481 insertion reduced BteA expression in common laboratory or vaccine strains but not in the clinical strain. However, Fennelly et al. [148] described a functionally active T3SS and its three effector proteins, BopD, BopN and Bsp22, in a low-passage *B. pertussis* clinical isolate. They showed that *B. pertussis* T3SS promotes bacterial adherence, suppresses innate and adaptive immune response and enhances persistence of bacteria in the lungs of mice. Intriguingly, the immunogenicity and protective potential of *Bordetella* T3SS has not been studied so far. Medhekar et al. [54] showed that antisera from mice vaccinated with *Bordetella* T3SS secreted protein, Bsp22, protected HeLa cells from T3SS-mediated killing and protected mice when challenged with virulent bacteria. However, neither any detectable antibody response nor protection could be demonstrated in mice immunised with recombinant *B. pertussis* Bsp22 against intranasal challenge with virulent *B. pertussis* [55]. However T3SS functions as an important virulence delivery system and warrants further study.

## *Bordetella pertussis* iron regulated proteins as virulence factors

*Bordetella pertussis* can acquire the essential nutrient iron using a haeme uptake system or alcaligin siderophores, with iron-starved *B. pertussis* expressing novel iron uptake systems in vivo [149]. The iron uptake system is dependent on the Ton system, a Bvg-independent virulence determinant, accompanied by several siderophore receptors including *bfeA, bfrB* and *bfrC*. Iron is crucial for growth of *B. pertussis* as demonstrated by the inability of the Δ*tonB* mutant of the Tohama I laboratory strain to utilise alcaligin, enterobactin, ferrichrome, desferroxamine B, haemin and haemoglobin, affecting its growth. Two putative iron binding proteins of *B. pertussis* may be potential vaccine candidates. IRP1-3 (Bp1152) and AfuA (BP1605) were identified by comparative proteomics as proteins induced during iron limiting conditions [60]. IRP1-3 is a dimeric membrane protein involved in iron uptake. Its expression was conserved among clinical isolates of *B. pertussis* and enhanced by iron starvation. Immunisation of mice with recombinant IRP1-3 induced antibodies that recognised the native protein on the bacterial surface and promoted bacterial phagocytosis by human neutrophils [150]. Immunisation with IPR1-3 was protective against infection in mice and induced both Th1 and Th2 responses. Mice immunised with recombinant AfuA were also protected compared to control mice immunised with adjuvant alone [60].

Other iron related proteins may also be promising vaccine candidates. Yilmaz et al. [61] showed that recombinant iron superoxide dismutase (rFeSOD) induced substantial IgG1, IgG2a and IFN-γ responses when formulated with the TLR-4 agonist, monophosphoryl lipid A (MPLA), as an adjuvant. The formulation also decreased the bacterial count in the lungs of mice following challenge with a virulent *B. pertussis* strain. Banerjee et al. [151] showed that a periplasmic protein, $FbpA_{Bp,}$ plays an important role in *B. pertussis* iron uptake system, but this antigen has not been tested as a vaccine. Taken together, these reports support a role for inclusion of putative iron binding proteins in improved pertussis vaccines.

## Lipooligosaccharide of *B. pertussis*

There is little doubt on the significance of pertussis toxin to the symptoms of whooping cough. However, it may not be unique because *B. parapertussis* does not produce pertussis toxin but nevertheless causes the characteristic paroxysms of whooping cough. *B. pertussis* lipooligosaccharide (LOS) may be involved in whooping cough syndrome by induction of ·NO production by infected tracheal cells, thereby damaging the activity of respiratory ciliated cells [152]. TCT (reviewed in "Tracheal cytotoxin") may act synergistically with LPS in the induction of ·NO, as neither TCT nor LOS alone was able to induce ·NO [88].

LOS produced by both *B. pertussis* and *B. parapertussis* are structurally different. *B. pertussis* LOS lacks an O-side chain but has a nonrepeating trisaccharide [153]. *B. parapertussis* O-antigen consists of a homopolymer of 2,3-dideoxy-2,3-di-*N*-acetylgalactosaminuronic acid [153]. In contrast to other *B. pertussis* vaccine antigens, the LOS molecule has escaped any modification in its dodecasaccharide core structure in the post-vaccination era [154]. LOS from both pathogens can modulate dendritic cell responses, with *B. pertussis* LOS inducing higher Th17-polarised immune responses than LOS from *B. parapertussis*. This may influence the severity of pertussis in humans [153]. As such, the LOS of *B. pertussis* is interesting as a potential vaccine target.

## *Bordetella pertussis* biofilm lifestyle

Biofilms are multicellular structured communities of bacterial cells that are encased in a self-produced or host-derived polymeric matrix [155]. In the past few decades, biofilms have been shown to affect antibiotic resistance [156, 157], susceptibility to host immune responses [158], establish foreign body infections [26, 159] and long-term host survival. Studies of *Bordetella* biofilm formation began slowly but several in vitro models have illuminated biofilm physiology in mammalian hosts. We and others have described microscopic and macroscopic multicellular structures of *Bordetella* on several abiotic surfaces [27, 160, 161]. *B. pertussis* BvgAS signal transduction system and several Bvg-activated proteins have been shown to be responsible for efficient biofilm formation on abiotic surfaces [129, 155]. FHA contributes to efficient biofilm formation by promoting cell-substrate and inter-bacterial adhesions [161]. ACT of *B. pertussis* can inhibit *B. pertussis* biofilm formation by interacting with the mature C terminal domain of FHA [162]. In addition to FHA and ACT, the *Bordetella bpsABCD* locus (required for the synthesis of the Bps polysaccharide) is critical for the stability and maintenance of the complex architecture of biofilms [163, 164]. While Bps was not required for initial attachment to artificial surfaces, it was indispensable for the formation of mature biofilms.

Despite the large amount of information on bacterial biofilms formed on artificial surfaces, it is unclear how biofilms develop in vivo. Architecturally complex structures of *B. pertussis* have been described on the ciliated epithelium of the mouse nose and the trachea [27, 165]. A biofilm matrix was demonstrated by the finding that Bps co-localised with these organ-adherent biofilms and ex vivo treatment of biofilms formed on the mouse nose with DNase I resulted in considerable biofilm dissolution [166]. As *B. pertussis* biofilms have been observed in the mouse nose as late as 19 days post-inoculation, biofilms may allow evasion of host

immune responses and so promote efficient colonisation of the mouse respiratory tract [166].

### Biofilm formation as an important virulence factors

Biofilm formation by *B. pertussis* has significant implications for vaccine design. Interestingly, the circulating isolates of *B. pertussis* were associated with increased biofilm forming potential [167, 168]. Recent study implicated *B. pertussis* biofilm formation to enhanced virulence in the mouse nose and trachea [169]. For this purpose, it is important to identify *Bordetella* genes and factors that are differentially expressed during biofilm formation [27, 117, 170]. Based on microscopic visualisation of highly differentiated communities of *B. pertussis* on artificial and host surfaces, it appears that biofilms form in a coordinated manner, in contrast to growth under shaking or planktonic conditions. This model has been strengthened by microarray analyses of biofilms at five different stages of development [160]. The analyses showed variation in the expression profile of more than 33% of the genome, including several transcriptional regulators. Similarly, proteomic analyses in *B. pertussis* revealed a large portion of the cytosolic and membrane subproteome to be altered during biofilm formation. Specifically, Serra et al. [171, 172] and Bosch et al. [173] implicated molecules associated with carbohydrate metabolism. BipA is the most abundant surface-associated protein in the biofilm of *B. pertussis*. Immunisation with BipA resulted in significant reduction in colonisation of mouse lungs with a virulent *B. pertussis* challenge strain [57]. This indirectly implicates BipA in colonisation, provoking its inclusion in aP vaccines.

Arnal et al. [168] reported enhanced biofilm forming capacity of clinical isolates compared to Tohama I, and elevated levels of several proteins compared to planktonic cells [57, 168]. This laboratory has demonstrated enhanced biofilm forming capacity in circulating *B. pertussis* clinical isolates from Western Australia [167]. The biofilm forming capacity increased tolerance to antimicrobial agents compared to the planktonic state. Furthermore, Isobaric Tags for Relative and Absolute Quantitation (iTRAQ)-based proteomic analyses revealed novel proteins differentially up-regulated in *B. pertussis* biofilms relative to planktonic cells [167]. Whether, the novel biofilm-associated proteins prevent colonisation and aerosol transmission of *B. pertussis* remains to be determined.

## Evolution of whooping cough vaccine

The conventional wP whooping cough vaccine consisted of heat-killed or chemically detoxified preparations of bacteria, administered with or without alum as an adjuvant. These first pertussis vaccines conferred protection against whooping cough mainly by induction of humoral antibody as well as Th1-mediated CMI [174]. Current aP vaccines are composed of three or five purified antigens adsorbed to alum and formulated with diphtheria and tetanus toxoids. These vaccines induced mostly Th2-skewed and Th17 cells but weak Th1 cell response in infants [20] and murine models of infection [175]. Such immune responses are less effective in preventing whooping cough since *B. pertussis* can survive within macrophages and other eukaryotic cells. However, CMI is induced following booster doses of vaccines or natural infection. A Th1-mediated response has been reported in adolescents subsequent to primary wP or aP vaccination [176]. In addition, booster vaccination of adult mice with aP vaccine, delivered as DTaP, did not lead to Th1 responses. It is therefore of interest to know whether the reported Th1-skewed immune response obtained after booster vaccination of adolescents with aP was due to silent exposure to infection with wild type *B. pertussis*, as may happen during epidemics of whooping cough. Although the Th2-polarised response appears to be protective in humans, such immune responses are not able to clear *B. pertussis* that has been internalised by macrophages [177]. Thus, improved vaccines that induce protective T-cell responses are required [63].

Since the mouse pertussis model is not considered to be ideal for predicting immune response profiles in humans, it is necessary to validate results using animal models that more closely reflect human infection [178]. Warfel et al. [179] compared the immunological profile, and colonisation and transmission potential of pertussis in baboons previously infected with *B. pertussis* or immunised with aP versus wP vaccines. All vaccinated or prior infected animals mounted potent serum antibody responses but with major differences in T-cell mediated immunity. Convalescent or previously infected mice or wP-vaccinated mice displayed potent *B. pertussis*-specific T helper 17 (Th17) and Th1 memory responses, while vaccination with aP induced a Th1/Th2 response instead as reported previously in humans [174]. The mismatch of the immune response induced by aP with that induced by natural infection may be an explanation for the resurgence of pertussis, warranting the formulation of improved vaccines. Strategies employed in this quest are outlined in the following sections.

### Outer membrane vesicle (OMV) as vaccines

The first report suggesting OMVs as a possible candidate for formulation of aP vaccines was based on evidence that they contain a variety of virulence antigens of *B. pertussis*, including ACT, Ptx and FHA, and uronic-acid containing polysaccharides and LPS [173, 180]. Fernandez et al. [56] found a proteoliposome (PL) preparation extracted from the outer membrane of *B. pertussis* could protect 90% of

mice against lethal infection with *B. pertussis* and achieve total clearance of bacteria after intracerebral and intranasal challenge. Similarly, Raeven et al. [181] showed that higher humoral antibody responses (IgG1/IgG2a/IgG2b/IgG3) were elicited by outer membrane vesicles (OMV) than by wP vaccine, aP vaccine or infection. These studies highlight the importance of *B. pertussis* OMV for future whooping cough vaccines.

### *Bordetella pertussis* DNA vaccines

DNA vaccines utilise plasmid DNA encoding critical antigens to transfect host cells in vivo [13, 63]. Ulmer et al. [182] showed that immunisation of mice with DNA encoding influenza A viral proteins induced protective antibody and cytotoxic T-cell responses. Since then, DNA plasmids have been investigated to achieve protective immune responses against parasitic, viral and bacterial infections [13].

Kamachi et al. [183] showed that a DNA vaccine expressing the Ptx subunit1 (PtxS1) known as pcDNA/S1 could induce protective IgG antibodies and protect mice from virulent *B. pertussis* challenge. Immunisation with pcDNA/S1 inhibited the leukocytosis-promoting activity induced by *B. pertussis* infection and protected mice from intracerebral challenge with a lethal dose of virulent *B. pertussis*. Three further mutants were constructed from DNA encoding the N-terminal 180-amino-acid fragment of PtxS1: C180-R9K, C180-E129G and C180-R9K/E129G [184]. Immunisation of mice with all three plasmids induced anti-Ptx specific IgG antibody and inhibited the leukocytosis-promoting activity of Ptx. Furthermore, no toxicity was observed following transfection of CHO cells with C180-R9K and C180-R9K/E129G plasmids. A similar experiment performed using the C-terminal truncated form of Ptx S1 subunit protected mice against infection with lethal dose of virulent *B. pertussis* [185].

Li et al. [186] combined three immunodominant antigens from PtxS1, Prn and FHA by cloning these genes into one plasmid (pVAX1/ppf). Immunisation of mice with pVAX1/ppf, elicited more antibodies reactive with all three proteins, induced IL-10 and IFN-γ production, and conferred protection to mice from intracerebral challenge by a lethal dose of *B. pertussis*. Further work on the mechanism of protection imparted by the pVAX1/ppf is clearly warranted, given the induction of IL-10, which has been shown to dampen the CMI responses. Fry et al. [13] developed a DNA vaccine that encoded genetically inactivated PtxS1, which when delivered to mice intramuscularly, elicited a T-cell response, with high levels of IFN-γ and IL-2 in stimulated splenocyte with no serum IgG. Whilst lungs of DNA-immunised mice were cleared of *B. pertussis* at a significantly faster rate than mock-immunised mice following aerosol challenge, clearance was faster in DTaP-immunised mice. Similar results have been obtained with the *B. pertussis* FHA and pertactin DNA vaccines [187].

### Micro- and nano-particle vaccines

Biodegradable micro- and nano-particle delivery systems hold promise for vaccine development. Mice immunised orally with liposomes coated with FHA produced higher antibody responses than those immunised with FHA and Ptx without liposome as the delivery vehicle [188]. Similarly, parental (intraperitoneal or intramuscular route) immunisation with FHA and Ptx entrapped in micro-particle polylactide-*co*-glycolide (PLG) protected mice against challenge with *B. pertussis* due to induction of potent Th1 and antibody responses [189]. The nanoparticle PLG formulation induced a Th2-skewed immune response. Different types of nanoparticle-based vaccines, operating as delivery systems to enhance antigen processing and/or as adjuvants to activate or enhance immunity have been reviewed [190]. The authors note that a better understanding of in vivo bio-distribution and fate will accelerate the rational design of nanoparticle-containing vaccines.

More recently, cell membrane-coated nanoparticles were developed as a new class of biomimetic nanoparticles with strong potential for modulating antibacterial immunity. An in-depth discussion of this topic is beyond the scope of this review; however, a recent review discusses the potential of cell membrane-coated nanoparticles sequestering bacterial toxins and mimicking bacterial antigen presentation [191].

### Live attenuated vaccines

Roberts et al. [192] reported the development of an *aroA B. pertussis* mutant which induced an antibody response and protection, but only in mice given three doses of the vaccine. Induction of CMI by this vaccine was not investigated. Other studies targeted the DNT and Ptx genes by knocking out these genes. The attenuated strains provided protection of animals from virulent *B. pertussis* challenge [193]. Mielcarek et al. [194] developed a live attenuated *B. pertussis* strain, BPZE1 by genetic detoxification of Ptx, deletion of DNT and replacement of the *B. pertussis ampG* gene by its *E. coli ampG* orthologue. Vaccination of mice with BPZE1 conferred long term protection with the induction of potent pro-inflammatory and regulatory cytokines and stimulation of Th1, Th17 and T-suppressor responses [195]. Similar potent immune response was also demonstrated in a human preclinical ex vivo model using monocyte-derived dendritic cells challenged with BPZE1 [196]. A randomised phase I clinical trial has also been conducted for BPZE1, making it the first live attenuated pertussis vaccine to undergo clinical trials [197]. Whilst BPZE1 was safe for humans, 5 of 12 subjects receiving high dose of BPZE1 ($10^7$

colony-forming units) were colonised [197]. Moreover, no significant increase in the antigen-specific plasmablast cell or B-cell responses was detected [198].

In an alternative approach, Cornford-Nairns et al. [10] developed a novel live attenuated *B. pertussis* vaccine candidate, aroQBP, by insertional inactivation of the 3-dehydroquinase (*aroQ*) gene. Immunisation of mice with aroQBP induced antigen-specific IgG1 and IgG2a and stimulated IL-2, IL-12 and IFN-γ responses. Intranasal immunisation with one dose of aroQBP protected mice against virulent *B. pertussis* infection and cleared the pathogens from lungs 7 days post-challenge. Therefore, the aroQBP strain is a promising vaccine candidate that warrants further investigation because it is non-reverting and capable of inducing both systemic and pulmonary antibody as well as CMI responses.

### Biofilm-associated antigens as potential whooping cough vaccine

The development of an improved vaccine for *B. pertussis* requires a better understanding of the factors involved in initial colonisation and survival in the nasopharynx [199]. The current *B. pertussis* vaccines do not prevent the colonisation of the nasopharynx but prevent the development of severe disease [27]. A potential reduction in nasal colonisation would disrupt the transmission cycle of *B. pertussis* and thereby reduce the morbidity and mortality due to whooping cough. To date, only one study by de Gouw et al. [57] demonstrated the protective potential of a biofilm-derived protein, BipA, against *B. pertussis* infection. Therefore, it is important to identify biofilm-associated antigens and establish their potential to eliminate transmission of *B. pertussis* to infants from asymptomatic carriers, vaccinated adults and adolescents.

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

Several new-generation adjuvants may enhance the immunogenicity of pertussis vaccines. Elahi et al. [200] showed that cyclic diguanylate (c-di-GMP; 39, 59-cyclic diguanylate) can be used as a potent immune stimulatory adjuvant that induces strong Th1 response and reduces bacterial loads in the lungs of mice infected with pathogenic *B. pertussis*. Similarly, Dunne et al. [201] used a novel TLR2-stimulating lipoprotein from *B. pertussis* as an adjuvant in acellular vaccines (replacing alum) and demonstrated enhanced Th1, Th17 and IgG2a immune responses. Geurtsen et al. [202] revealed that a lipopolysaccharide (LPS) analogue, monophosphoryl lipid A (MPLA) and a LPS analogue from *Neisseria meningitides* (Lpxl2) were more effective than alum-formulated acellular vaccines. Recently, a preclinical study showed that an emulsion adjuvant, MF59, and a TLR4 agonist, MPLA, induced stronger IgG immune response against aP vaccines that are skewed towards IgG2a/Th1 isotypes [203]. The authors suggested replacing the aluminium salts with these new adjuvants to enhance the efficacy of the acellular vaccines. Intensive research efforts are needed to establish non-toxic adjuvants that promote antibody and cell-mediated immune responses capable of providing long-term protection against whooping cough.

## Conclusions and future directions

Although adoption of aP vaccines has improved compliance with the recommended vaccination schedules, the resurgence of pertussis has been attributed to waning of immunity in vaccinated subjects, the emergence non-vaccine type strains and increased or decreased expression of virulence factors [25, 29]. Different formulations of pertussis vaccine utilising 2–5 antigens (Ptx, Prn, Fim2, Fim3, Fim2,3 or FHA) have been compared with the wP vaccines using different criteria making direct comparison between studies difficult [132, 204]. With no consensus reached so far, further multicenter clinical trials with defined and uniform outcomes are needed [205]. Meanwhile, many aspects of basic *B. pertussis* biology remains to be further elucidated. Circulating *B. pertussis* strains show evidence of genetic selection for vaccine escape mutants. These potentially have increased virulence [34, 39]. The challenges that lie ahead are:

a. Explore novel antigens such as BipA, Vag8, TCT and TTSS effector proteins as potential whooping cough vaccine
b. Develop non-toxic and Th1-stimulating adjuvants for the reformulation of the currently used aP vaccines
c. Unlock the potential of biofilm as a source of novel whooping cough vaccine
d. Develop novel live attenuated pertussis vaccines capable of preventing symptomatic as well as asymptomatic transmission of whooping cough.

The resurgence of whooping cough has called for an integrated approach to control and prevention of the disease. Pertussis epidemiology in developing countries, genomic analysis of *B. pertussis* circulating isolates, analyses of virulence factor expression and the need for a novel whooping cough vaccine that stimulate concomitant Th1 and Th2 immune responses are recognised as problems that require immediate attention [206].

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest. The authorship of this article complies with the Australian code for the responsible conduct of research (https://nhmrc.gov.au/guidelines-publications/R39).

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