Pertussis: a matter of immune modulation

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Abstract

Pertussis, or whooping cough, is a highly contagious, acute respiratory disease of humans that is caused by the Gram-negative bacterial pathogen Bordetella pertussis. In the face of extensive global vaccination, this extremely monomorphic pathogen has persisted and re-emerged, causing approximately 300 000 deaths each year. In this review, we discuss the interaction of B. pertussis with the host mucosal epithelium and immune system. Using a large number of virulence factors, B. pertussis is able to create a niche for colonization in the human respiratory tract. The successful persistence of this pathogen is mainly due to its ability to interfere with almost every aspect of the immune system, from the inhibition of complement- and phagocyte-mediated killing to the suppression of T- and B-cell responses. Based on these insights, we delineate ideas for the rational design of improved vaccines that can target the ‘weak spots’ in the pathogenesis of this highly successful pathogen.

Introduction

Whooping cough or pertussis is a highly contagious, acute, respiratory disease of humans caused by several Gram-negative bacterial species of the genus Bordetella. Four Bordetella species have been mainly associated with respiratory disease in humans: i.e. Bordetella pertussis, Bordetella parapertussis, Bordetella bronchiseptica, and Bordetella holmesii (Mooi et al., 2007). The first three species are closely related and are often referred to as the classical Bordetellae, while B. holmesii forms a distinct genetic lineage (Diavatopoulos et al., 2005, 2006). Bordetella bronchiseptica infects a large range of mammalian hosts in which it generally causes mild and chronic disease (Goodnow, 1980). Infrequently, B. bronchiseptica is also isolated from the respiratory tract of humans, mostly from immunocompromised individuals (Woolfrey & Moody, 1991). Two B. parapertussis lineages with distinct host specificities for humans and sheep have been identified, designated B. parapertussisHU and B. parapertussisOAS respectively (Porter et al., 1996; van der Zee et al., 1997; Parkhill et al., 2003; Diavatopoulos et al., 2005). Interestingly, this switch to humans was accompanied by a change from chronic disease to acute. Although there will undoubtedly be many parallels that can be drawn to other Bordetella species that cause human respiratory disease, this review will focus on B. pertussis as it causes the highest morbidity and mortality in humans.

The hallmark symptoms of pertussis are paroxysmal coughing with whooping and post-tussive vomiting. Persistent coughing may last for weeks to months with a gradual decrease in frequency and severity. However, it should be noted that B. pertussis infections, particularly in hosts with partial immunity to the bacterium, may also follow a much milder or subclinical course (Bordet & Gengou, 1906; Cherry & Heininger, 2004). Complications that are frequently found in other mammalian species, B. pertussis, B. parapertussisHU, and B. holmesii have been isolated only from humans. Phylogenetic analysis has indicated that both B. pertussis and B. parapertussisHU evolved from B. bronchiseptica separately, implying that adaptation to humans occurred as two independent events (van der Zee et al., 1997; Parkhill et al., 2003; Diavatopoulos et al., 2005). Pertussis, or whooping cough, is a highly contagious, acute respiratory disease of humans that is caused by the Gram-negative bacterial pathogen Bordetella pertussis. In the face of extensive global vaccination, this extremely monomorphic pathogen has persisted and re-emerged, causing approximately 300 000 deaths each year. In this review, we discuss the interaction of B. pertussis with the host mucosal epithelium and immune system. Using a large number of virulence factors, B. pertussis is able to create a niche for colonization in the human respiratory tract. The successful persistence of this pathogen is mainly due to its ability to interfere with almost every aspect of the immune system, from the inhibition of complement- and phagocyte-mediated killing to the suppression of T- and B-cell responses. Based on these insights, we delineate ideas for the rational design of improved vaccines that can target the ‘weak spots’ in the pathogenesis of this highly successful pathogen.

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The persistence and resurgence of pertussis in countries with highly vaccinated populations has been attributed to various factors including increased awareness, improved diagnostics, suboptimal vaccines, waning immunity, and pathogen adaptation (He & Mertsola, 2008; Berbers et al., 2009; Mooi, 2010). Of significant concern is that most currently used acellular pertussis vaccines (ACV) protect against infections only for a period of 6–8 years, while protection against subclinical infections declines even faster (De Serres & Duval, 2005; Hallander et al., 2005; Gustafsson et al., 2006).

Several recent reviews have focused on the resurgence of pertussis and the evolution of Bordetella (He & Mertsola, 2008; Berbers et al., 2009; Elomaa et al., 2009; Mooi, 2010). Knowledge about the molecular mechanisms by which B. pertussis infects the host and evades clearance by the immune system will provide insight into its survival strategies and may facilitate the rational design of novel therapeutic and/or preventive strategies that target the ‘weak’ spots of this versatile pathogen. Thus, in this review, we focus on the molecular interplay between host and pathogen and provide an overview of how this bacterium is able to persist and resurge in the face of intensive vaccination.

Regulation of virulence genes

Infection of the host by B. pertussis is initiated through contact with respiratory secretions from an infected individual. Following the inhalation of these particles, bacteria enter the upper respiratory tract and adhere to (ciliated) epithelial cells in the nasopharynx and trachea. Once attached to the mucosal surface, B. pertussis produces a myriad of virulence factors, including adhesins, immunomodulating factors, and toxins. The concerted expression of these factors prevents rapid clearance of the bacteria and enables replication and dissemination to the lower areas of the respiratory tract, causing pneumonia. Since the recognition of B. pertussis as the causative agent of whooping cough in humans (Bordet & Gengou, 1906), research has focused on understanding its pathogenic lifestyle. In particular, the identification of bacterial factors that contribute to the development of disease has been comprehensively investigated. One of the initial, intriguing observations was that B. pertussis displayed variation in the expression of surface antigens (designated antigenic modulation at that time) in response to environmental signals (Leslie & Gardner, 1931; Lacey, 1960). We now know that these phenomena can be explained by the existence of a master regulator that controls transcription of nearly all known virulence genes: the Bordetella master virulence regulatory system (bvgaSR) locus, as well as a second, more recently identified, two-component regulatory system (TCS) called the RisAS system. Although there are a number of other sensory systems present in the Bordetella genome (Parkhill et al., 2003), the BvgASR and RisAS systems have been the most extensively studied and will be discussed here.

The bvgaSR virulence regulon

The transcription of many of the B. pertussis genes known to be involved in virulence is controlled by the activity of BvgS and BvgA, originally designated the vir locus by Weiss & Falkow (1984; Stibitz, 2007). These two proteins form a typical TCS that allows dynamic variation of gene expression in response to changes in extracellular signals from the surrounding environment. BvgA is a 23-kDa DNA-binding response regulator (Boucher & Stibitz, 1995) and BvgS is a 135-kDa transmembrane sensor kinase. BvgS contains a periplasmic domain, a linker region, a transmitter, a receiver, and a histidine phosphotransfer domain (Stibitz & Yang, 1991). BvgAS is responsive to several known environmental signals that can modulate its expression, at least under laboratory conditions. For instance, growing B. pertussis at 37°C induces the expression of BvgAS, while the presence of millimolar amounts of sulfate or nicotinic acid, or growth at or below 25°C, suppresses BvgAS production (Melton & Weiss, 1989, 1993). Bordetella pertussis strains cultured under so-called ‘nonmodulating’, virulent conditions are...
referred to as Bvg+ phase bacteria. Of all currently known inducers of BvgAS activity, temperature seems to be most relevant for sensing changes in the in vivo environment. Currently, the in vivo contributions of sulfate and nicotinic acid remain unclear.

During the virulent Bvg+ phase, the periplasmic domain of BvgS relays environmental signals through the membrane to the transmitter domain, which then autophosphorylates. Phosphorylated BvgS subsequently transfers its phosphate group to BvgA (BvgA-P), which then becomes activated and binds to specific cis-acting promoter sequences, thus inducing the transcription of Bvg+ phase-specific genes, commonly designated vags (for ‘vir-activated genes’) (Uhl & Miller, 1994, 1996b). Simultaneously, the transcription of vrgs (for ‘vir-repressed genes’) is repressed by a third protein that is expressed from the bvgASR locus, the 32-kDa cytoplasmic repressor protein BvgR (Akerley et al., 1992; Merkel & Stibitz, 1995). Based on in vitro growth experiments, vags have been classified into three different temporal classes: early, intermediate, and late genes (Scarlat et al., 1991; Veal-Carr & Stibitz, 2005; Cummings et al., 2006).

Early genes, encoding the adhesins filamentous hemagglutinin (FHA) and fimbriae (Fim) respond rapidly to activation and require a lower concentration of BvgA-P for induction (Scarlat et al., 1991). Late genes, which include those encoding adenylate cyclase toxin (ACT) and pertussis toxin (Ptx), show relatively slow induction kinetics and require higher concentrations of BvgA-P for expression (Scarlat et al., 1991). The gene encoding pertactin (Prn) has been suggested to belong to the class of intermediate genes (Kinnear et al., 1999). The BvgASR locus is autogenously regulated and belongs to the early genes, providing an additional level of regulation for Bvg-induced class-switching (Roy et al., 1990). This phenomenon of differential gene expression has been attributed to architectural promoter differences, with the promoters of the late genes requiring higher levels of BvgA-P compared with the early promoter sequences (Zu et al., 1996). A similar expression pattern has been observed in vivo (Veal-Carr & Stibitz, 2005), which presumably enables the bacterium to adapt its virulence factor expression to different stages of infection or to niches containing different signals (e.g. temperature changes). The molecular mechanisms by which these virulence factors interact with the host will be discussed later in this review.

When B. pertussis is grown under ‘modulating conditions,’ the BvgAS phosphorelay is inactivated (Melton & Weiss, 1993). Under these conditions, BvgAS is unable to activate transcription of vags, whereas in turn, vrgs are expressed due to the absence of the repressor BvgR, resulting in an avirulent phenotype referred to as Bvg− phase. It is currently unknown whether this avirulent phase is still relevant and functional in B. pertussis. In contrast to the host-restricted B. pertussis, B. bronchiseptica has the capacity to survive for weeks in water, suggesting the ex vivo environment may play a role in its transmission cycle (Porter et al., 1991). In B. bronchiseptica, motility (flagella) and urease-encoding genes are expressed specifically in the Bvg− phase, and it has been shown that this phase is essential for survival under nutrient-limiting conditions (Cotter & Miller, 1994, 1997). In comparison with B. bronchiseptica, B. pertussis has undergone significant loss of genes during its host adaptation, presumably because of the lack of a need for the environmental phase to enhance transmission rates (Parkhill et al., 2003). It is therefore not unlikely that the Bvg− phase in B. pertussis represents an evolutionary remnant.

In addition to the Bvg− and Bvg+ phenotypes that were originally observed in vitro, a third phase has since been identified, designated the intermediate or the Bvgi phase. This phase can be induced under semi-modulating conditions or by substituting one basepair in the bvgS gene (Cotter & Miller, 1997). The Bvgi+ phase is typically characterized by the absence of vrg expression, the expression of some vags (e.g. FHA) but not others (e.g. Ptx, ACT) (Deora et al., 2001), and a third class of genes (class III genes) that is expressed exclusively in this phase (Cummings et al., 2006). The main representative of the Bvgi+ phase is the Bvg-intermediate phase A (BipA) protein, which has been suggested to be important for early colonization of the airways (Stockbauer et al., 2001; Sukumar et al., 2007). A potential explanation for the existence of this phase is that it may play a role in the transmission and early respiratory tract colonization of B. pertussis (Stockbauer et al., 2001). Mouse respiratory tract infection experiments with Bvgi-locked and BipA deletion mutants have strengthened this hypothesis. The Bvgi+locked mutant was found to colonize the upper respiratory tract efficiently during early phases of the infection, but was unable to persist during later stages of the infection (Vergara-Irigaray et al., 2005). Interestingly, using a Bvgi+locked BipA deletion mutant, this successful early colonization of the Bvgi+locked mutant was shown to be dependent on BipA (Vergara-Irigaray et al., 2005).

Although the Bvg+, Bvgi+, and Bvg− phases are highly distinctive in that each phase shows specific expression of certain genes, it is important to note that these findings apply to B. pertussis cultured under relatively static in vitro conditions. In contrast, the human respiratory tract is a highly variable environment, and under these conditions, it is unlikely that the BvgAS system functions as an ‘on–off’ switch, but rather that it facilitates a spectrum of expression levels transitioning between the Bvg+, Bvgi+, and Bvg− phases in response to local changes in environmental conditions (Cummings et al., 2006). Due to its flexibility, the BvgASR system is nowadays considered a ‘rheostat’ (Cotter & Jones, 2003).
The RisAS system

In addition to the BvgASR system, B. pertussis contains a second TCS called the regulator of intracellular response (Ris) system. This system is encoded by the risAS locus, originally identified in B. bronchiseptica. The RisAS system encodes a response regulator (RisA) and a sensor kinase (RisS) (Jungnitz et al., 1998). In B. bronchiseptica, this system functions independently of BvgAS, and contributes to persistence in the host by regulating the expression of multiple factors involved inintracellular survival (Jungnitz et al., 1998; Zimna et al., 2001). The ability and mechanisms by which B. pertussis may survive in host cells will be discussed later in this review. In B. pertussis, the risA gene contains a conserved frameshift, and consequently encodes a truncated nonfunctional form of the RisS sensor (Stenson et al., 2005). In contrast, RisA is still expressed and functional in B. pertussis. Indeed, the observation that deletion of risA results in a strong reduction of transcription of vrgs suggests that RisA plays an antagonistic role to BvgR in the regulation of vrg gene expression (Croinin et al., 2005; Stenson et al., 2005). Possibly, BvgR inhibits vrg gene expression by preventing binding of RisA to the vrg promoters, either by direct competition for promoter sequences or indirectly by binding to RisA. Alternatively, BvgR may act as a regulator of RisA expression analogous to its repression of vrg gene expression in the BvgA phase. However, a direct link between BvgR and RisA expression could not be established as no changes in risA transcription were observed when the bvgR gene was deleted (Croinin et al., 2005). Another unresolved issue is the question as to whether or not RisA, similar to BvgA, requires phosphorylation in order to be active, and if so, which factor mediates this reaction. Further research is needed to elucidate the exact interactions between RisA and BvgR and to characterize the contribution of RisA to the in vivo survival of B. pertussis.

Interaction with the mucosal epithelium

The human respiratory tract poses a significant challenge to airway pathogens such as B. pertussis. The ciliated epithelium that lines the respiratory tracts ensures that mucus secretions containing particles (e.g. pathogenic microorganisms or pollen) are mechanically cleared from the nasal cavities, trachea, and bronchial tubes. Colonizing B. pertussis also faces direct competition with the resident flora for nutrients and space, and is exposed to a range of host defense mechanisms that have evolved to limit or prevent microbial colonization, such as iron sequestration and the innate immune system. Successful infection of the host by B. pertussis is therefore critically dependent on its ability to resist these antimicrobial defense mechanisms. To this end, B. pertussis produces a number of adhesins and toxins that can alter the local environment to promote adhesion, invasion, and biofilm formation on the host respiratory mucosa.

Iron acquisition

Bordetella pertussis, like all other bacteria, requires iron for a number of essential cellular processes. However, the free iron concentration in the human host is tightly regulated, with free [Fe3+] in the order of 10^-24 M on mucosal surfaces, while bacterial growth is only supported above 10^-7 M (Ratledge & Dover, 2000). The human glycoprotein lactoferrin, as well as other iron-binding proteins, play an important role in sequestering extracellular iron (Aisen & Listowsky, 1980). To enable infection of the host, B. pertussis has evolved multiple iron acquisition pathways (reviewed in Brickman & Armstrong, 2009). Additionally, B. pertussis is also able to use iron bound by exogenous siderophores. So far, three endogenous high-affinity transport systems have been genetically characterized in B. pertussis. These include the alcaligin (Moore et al., 1995), the enterobactin (Beall & Sanden, 1995a), and the heme utilization systems (Vanderpool & Armstrong, 2001). Under iron-replete conditions, global expression of these systems is inhibited by the iron-dependent transcriptional repressor Fur (Beall & Sanden, 1995b). Conversely, under iron-limiting conditions, Fur-regulated genes become derepressed and transcription of these genes occurs. Importantly, the expression of each of these systems can be regulated independently by specific transcriptional activators that are responsive to their cognate iron source (Brickman et al., 2008). Upon early colonization of the host mucosal surface, lactoferrin, which is abundantly present in the mucin-rich airway surface fluid, is most likely the predominant iron source taken up by the alcaligin siderophore system (Brickman & Armstrong, 2007). This is supported by the observation that a B. pertussis strain with an inactive alcaligin transport system is outcompeted by its isogenic counterpart during early phases of the infection (Brickman & Armstrong, 2007). During the progression of infection, both the host inflammatory response to B. pertussis and the pathogenic effects of secreted toxins compromise the integrity of the host epithelial barrier. Ultimately, when significant host cell lysis occurs, additional intracellular iron sources such as transferrin and heme proteins become available, which can also be utilized by B. pertussis. The importance of these iron sources during the later stages of infection has also been recognized in vivo, as inactivation of the heme system rendered B. pertussis less infectious (Brickman et al., 2006).

Although the B. pertussis iron transport systems have been well-studied, little is known about the role of other important trace metals such as magnesium, manganese, copper, cobalt, lithium, zinc, etc., which have been shown
to play essential roles in other pathogenic bacteria (Agranoff & Krishna, 2004; Papp-Wallace & Maguire, 2006). Analysis of the *B. pertussis* genome has indicated the existence of genes coding for predicted trace metal transporters such as a putative permease protein for cobalt transport, a CorC protein for magnesium and cobalt efflux, and two homologs of GufA, a member of the ZIP zinc transporter family (Parkhill et al., 2003; Armstrong & Gross, 2007).

In conclusion, the existence of at least three distinct iron uptake mechanisms in the genome of *B. pertussis* clearly emphasizes the importance of iron during infection of the host. Using multiple iron acquisition pathways, *B. pertussis* is able to adapt rapidly to changes in the available iron sources during infection. It is likely that other, less-studied trace metals may be of similar importance and further research into the utilization mechanisms for these metals is warranted to elucidate their exact contribution to colonization of the host.

**Adherence and cytotoxicity**

Many bacterial pathogens that occupy the mucosal surfaces of the human host produce adhesins and toxins that facilitate adhesion and transmission. The main *B. pertussis* virulence factors that have been associated with direct adhesion to host airways are FHA, fimbriae, and Prn. Furthermore, three major protein toxins have been identified thus far, Ptx, ACT, and BteA, an effector of the type III secretion system (T3SS). Additionally, tracheal cytotoxin (TCT) is released from the cell wall of colonizing *B. pertussis*. These factors jointly facilitate the adhesion of *B. pertussis* to the human respiratory tract (see Fig. 1 and Table 1). Before discussing the molecular mechanisms in detail, the modular structure of the adhesins and toxins will be briefly reviewed.

**The major adherence factors**

FHA is a 232-kDa protein, present both in the secreted and the surface-associated form (Renauld-Mongenie et al., 1996). The translocation and secretion of FHA is mediated by a two-partner secretion system. An important step in the FHA secretion pathway is the proteolytic removal of roughly one-third of the C-terminus of FHA by SphB1, a specific protease of the subtilisin superfamily (Siezen & Leunissen, 1997; Coutte et al., 2001). Following cleavage, mature FHA remains noncovalently bound to the cell surface, probably via interactions between its N-terminal domain and a translocation-mediating protein called FhaC (Mazar & Cotter, 2006). Because adhesins typically remain associated with the bacterial surface to promote maximal attachment,
the observation that FHA is weakly surface-associated and even released in copious amounts seems counterintuitive (Coutte et al., 2001). However, colonizing bacteria can also benefit from a weaker interaction between bacterial adhesins and their host cell-ligands, as this may facilitate bacterial dissemination to other regions of the respiratory tract. The significance of this phenomenon has been demonstrated with an SphB1-deletion mutant that produces FHA exclusively in an immature, strongly membrane-associated form. Despite the enhanced ability of the mutant to bind pulmonary epithelial cells in vitro, it is significantly attenuated in the lungs of mice (Coutte et al., 2003). Binding of FHA to cell-surface receptors on respiratory epithelial cells is mediated by the action of multiple binding domains including an Arg-Gly-Asp (RGD) domain, a carbohydrate recognition domain (CRD), a binding domain for heparin and other sulfated carbohydrates [heparin-binding domain (HBD)] present on host cell surfaces, a potential integrin-binding domain, and its mature C-terminal domain (MCD), located distally from the cell surface (Mazar & Cotter, 2006).

Fimbriae are long, thin adhesive structures that extend from the outer bacterial membrane. Bordetella pertussis fimbriae consist of one of the major fimbrial subunits (Fim2 or Fim3) and a minor subunit called FimD (Blom et al., 1983; Locht et al., 1992; Willems et al., 1992, 1993; Geuijen et al., 1997). FimD and two additional fimbrial chaperone proteins, designated FimB and FimC, mediate correct formation of the fimbrial structure and outer membrane translocation (Willems et al., 1992, 1994). The Fim2 and, most likely, also the Fim3 subunits contain two regions with heparin-binding activity (Geuijen et al., 1998), which may be involved in binding to the extracellular matrix of respiratory epithelial cells.

Prn is a member of the autotransporter family and is characterized by its ability to direct its own secretion across the outer membrane using the Sec machinery (Leininger et al., 1991; Henderson & Nataro, 2001). Mature Prn (also known as p.69) is produced from a precursor protein of 93 kDa (p.93), following the removal of the 34-aa N-terminal signal peptide and a 30-kDa C-terminal part (p.30) (Charles et al., 1988; Makoff et al., 1990). Prn contains two proline-rich regions consisting of tandem repeats and an RGD motif that has been implicated in adhesion (Emsley et al., 1996).

Toxins

Ptx is a secreted protein toxin and a key virulence factor produced exclusively by B. pertussis (for a recent review, see Carbonetti, 2010). Ptx is a hexameric AB5 protein toxin with a total mass of 117 kDa, consisting of five dissimilar subunits (PtxA–E or S1–S5, respectively). It is transported across the
bacterial outer membrane by the Ptl type IV secretion system, which is cotranscribed with the Ptx genes (Verma & Burns, 2007; Verma et al., 2008). The A part consists of the PtxA subunit that contains the catalytic (ADP-ribosylating) domain. The B5 oligomer contains the PtxB–PtxE subunits (with two copies of PtxD) that are involved in receptor binding. The N-terminus of the PtxB and PtxC subunits contain lectin-like CRDs that have been implicated in binding to cell-surface receptors (Heerze et al., 1992; Saukkonen et al., 1992). For Ptx activity, binding of NAD is critical as it donates its ADP moiety during the ADP-ribose transfer reaction.

Another secreted toxin with a key role in B. pertussis pathogenesis is ACT (also reviewed in Carbonetti, 2010), which is a 200-kDa polypeptide that is secreted by a type I secretion system. It contains two major domains: a catalytic domain that includes the active site as well as a calmodulin-binding site and a hemolytic binding domain that is composed of a hydrophobic channel domain and calcium-binding RTX repeats (Vojtova et al., 2006). The functional activity of ACT is critically dependent on the toxin having undergone post-translational palmitoylation of the K983 residue, which is catalyzed by CyaC during protein maturation (Hackett et al., 1995). Additionally, post-translational acylation is important for the apoptotic and cytotoxic effects of ACT (Boyd et al., 2005). Although the majority of ACT remains associated with the bacterium in an inactive form, a small amount of active toxin is secreted into the environment (Zaretzky et al., 2002; Gray et al., 2004).

The T3SS represents a specialized secretory apparatus used by a wide variety of gram-negative bacteria to interfere with host cell functioning. These systems enable translocation of effector proteins directly into the host cell cytosol. The majority of effector proteins are enzymes that modify cytoplasmic targets involved in important cellular events, including actin cytoskeleton remodeling, intracellular signaling, and apoptosis (Cossart & Sansonetti, 2004). T3SS have been implicated as important mediators of virulence in a range of bacterial pathogens including Escherichia coli, Salmonella spp., and Yersinia spp. (Hueck, 1998; Yuk et al., 2000). A BvgASR-regulated T3SS and its effector proteins, such as BteA, have also been identified in Bordetella species, initially in B. bronchiseptica (Yuk et al., 1998, 2000) but later also in B. pertussis (Fennelly et al., 2008). BteA is a 69-kDa T3SS effector protein with an N-terminal lipid raft-targeting domain and a C-terminal domain implicated in cytotoxicity (Kuwae et al., 2006; French et al., 2009).

Another toxin contributing to respiratory epithelial adhesion by B. pertussis is TCT (Goldman et al., 1982). TCT is a 921-Da disaccharide–tetrapeptide belonging to the mumamyl peptide family (Goldman & Herwaldt, 1985). In contrast to the other toxins described above, TCT is not a protein toxin, but is released from B. pertussis cell wall peptidoglycans at relatively high levels (Rosenthal et al., 1987).

### Binding to the respiratory epithelium

Direct physical binding of B. pertussis to epithelial cells of the respiratory tract has been shown to be mediated predominately by the two major adhesins FHA and Fim described above (Relman et al., 1989; van den Berg et al., 1999; Rodriguez et al., 2006). A number of in vitro adhesion studies have shown that deletion of FHA from B. pertussis almost completely abrogates adherence to a wide variety of ciliated and nonciliated epithelial cells, emphasizing the importance of FHA in this process. Tuomanen et al. (1985) first established a crucial role for FHA in a human tracheal epithelial cell model, followed by the identification of its host-ligand, i.e. galactose–glucose-containing glycolipids (Tuomanen et al., 1988). Subsequent studies implicated the CRD and HBD domains in the binding of sulfated carbohydrates and glycolipids present on the surface of ciliated and nonciliated epithelial cells, respectively (Prasad et al., 1993; Hannah et al., 1994; Menozzi et al., 1994). Interestingly, using blocking antibodies that specifically bind to each of the major FHA domains implicated in binding, it was shown recently that besides the HBD, the MCD is also involved in bacterial adhesion to nonciliated cells (Julio et al., 2009). The binding potential of FHA is to some extent determined by its interaction with ACT (Zaretzky et al., 2002). Membrane-bound ACT physically associates with FHA, and it has been postulated that this interaction may enhance the adhesive capacity of FHA as it changes the orientation of its binding domains (Perez Vidakovics et al., 2006). Furthermore, FHA-mediated binding of B. pertussis to respiratory epithelial cells is dependent on the presence of lipid rafts in the host cell membrane (Lamberti et al., 2009). Lipid rafts are clusters of proteins and lipids in the membrane bilayer that are held together by cholesterol (Simons & Ehehalt, 2002). When cholesterol was depleted, bacteria were significantly less able to bind to host cells, suggesting that cholesterol-containing lipid rafts are important for binding of FHA (Lamberti et al., 2009). This lipid raft dependency is not exclusive to B. pertussis, as lipid rafts are a prominent target for attaching bacterial pathogens by providing a platform with clustered signaling proteins (e.g. receptors) and lipid cofactors (reviewed in Lafont et al., 2004).

In both ciliated and nonciliated tracheal and laryngeal adhesion models, deletion of the major and/or minor subunit genes of fimbriae significantly reduced adherence by > 50%, emphasizing the importance of this virulence factor to cell attachment (Funnell & Robinson, 1993; van den Berg et al., 1999). Although a specific receptor for fimbriae has not been identified on host tissues yet, sulfated sugar-containing molecules may be involved in fimbrial
binding to the respiratory epithelium (Guéijen et al., 1996, 1998).

Despite initial reports that describe the effects of Prn deletion on adherence to Chinese hamster ovary cells and human nonrespiratory epithelial HeLa cells (Leininger et al., 1991; Everest et al., 1996), the exact contribution of Prn to adherence remains enigmatic. When Prn was first described by Leininger et al. (1991), they showed a significant reduction in adherence when the Prn gene was deleted. Furthermore, binding inhibition studies with synthetic Prn-derived RGD-containing peptides suggested that the RGD domain is important for adherence. However, later studies were unable to show a role for Prn in adherence to human laryngeal and bronchial epithelial cells (Roberts et al., 1991; van den Berg et al., 1999). Moreover, as Prn host ligands have as yet not been identified, the question remains whether Prn primarily contributes to adhesion or to other aspects of pathogenesis.

Early studies suggested a role for Ptx in bacterial binding to ciliated cells. A Ptx-deficient strain was shown to be attenuated in its ability to adhere to ciliated cells in vitro, which could be restored when exogenous Ptx was provided (Tuomanen et al., 1985). In support of this observation, it was reported that Ptx-mediated binding occurred in a carbohydrate-dependent manner, by means of the lectin-like binding properties of the PtxB (S2) and PtxC (S3) subunits (Saukkonen et al., 1991, 1992). Although purified Ptx has been shown to bind to nonciliated human bronchial and laryngeal cells in a dose-dependent fashion, adherence of a Ptx deletion mutant strain to these cells was not attenuated (van den Berg et al., 1999). Based on these and other recent observations, the current hypothesis is that Ptx is not an adhesin, but rather facilitates adhesin-driven binding by modulating host immune responses (Carbonetti et al., 2003) (discussed later in this review).

* Bordetella pertussis * adhesion to the respiratory epithelium is further supported by toxin-induced cytotoxicity, as this leads to the exposure of cryptic receptors on the basement membrane. ACT for instance is able to penetrate the cytoplasmic site of lipid rafts in the host cell membrane via an N-terminal lipid raft-targeting domain (French et al., 2009). Interestingly, BteA was shown to associate with the cytoplasmic side of lipid rafts, which are known to function as Bordetella attachment sites (French et al., 2009). However, BteA is not directly involved in adhesion, as a T3SS-inactivated mutant was found to adhere to lipid rafts in a manner similar to its parental wild-type strain (French et al., 2009). Instead, BteA acts indirectly by inducing a rapid, nonapoptotic death, as shown in HeLa, rat lung epithelium (L2), and human renal epithelial (293T) cell models (Stockbauer et al., 2003; Panina et al., 2005; Shrivastava & Miller, 2009). Thus far, the mechanisms underlying this potent cytotoxicity have not been established. In contrast, the molecular mechanisms of TCT-induced toxicity have been the subject of study for decades and are very well-understood. In vitro studies performed in the 1980s already showed that TCT was able to inhibit DNA synthesis in tracheal epithelial cells and that it caused progressive cytotoxic alterations in ciliated cells of primary hamster tracheal organ cultures (Goldman et al., 1982). These cytotoxic effects are very similar to the TCT-induced effects on human-derived tissue (Wilson et al., 1991). Further research showed that TCT-like muramyl peptides were able to stimulate the production of interleukin-1 (IL-1) by phagocytes (Dinarello & Krueger, 1986) and hamster tracheal epithelial cells (Heiss et al., 1993). In the latter study, IL-1 production was found to induce cellular cytotoxicity. As the induction of IL-1 in and of itself may also be associated with a protective response against infection with * B. pertussis *, the specific downstream effects of IL-1 induction were investigated in a follow-up study by Heiss et al. (1994). They showed that the TCT-mediated expression of IL-1 induced type II nitric oxide synthases (inducible NOS), leading to the synthesis of nitric oxide (NO). Under normal circumstances, the synthesis of NO is tightly regulated, as too much free NO may lead to the production of reactive oxygen or nitrogen species (ROS or RNS, respectively). On the one hand, these free radicals play an important protective role in the immunological response, as they are released by phagocytic cells in a respiratory burst, thus contributing to the control of invading microbial pathogens. On the other hand, the TCT-mediated induction of IL-1 may also lead to the uncontrolled formation of high levels of free radicals, which may subsequently inhibit vital cellular processes. It is currently believed that the high levels of NO are responsible for the observed respiratory cytotoxicity of TCT (Heiss et al., 1994). A later study showed that the
production of NO is not induced by TCT alone, but requires the synergistic action of TCT and lipo-oligosaccharide, the endotoxin produced by *B. pertussis* (Flak et al., 2000). Although NO primarily affects ciliated epithelial cells, a recent study showed that the source of NO is in fact not the ciliated epithelium itself, but adjacent nonciliated mucus-producing goblet cells. These goblet cells themselves remain undamaged during this process (Flak & Goldman, 1999), and NO may be released through diffusion, thus contributing to remote cytopathologic effects on the ciliated epithelium.

In conclusion, current evidence indicated that FHA and Fim are the major adhesins used by *B. pertussis* to bind receptors on the host cell epithelium, while Ptx, ACT, BteA, and TCT (in synergy with lipo-oligosaccharides) indirectly contribute to bacterial attachment at different levels. First, cytotoxic activity establishes a niche devoid of ciliary epithelium, thus preventing mechanical clearance of attaching bacteria. Secondly, induced cellular damage results in the exposure of cryptic receptors on the basement membrane, thus allowing the pathogen to attach more efficiently. The inability of the host to remove mucus from the respiratory tract due to lack of ciliary action has been linked to the persistent coughing observed in individuals suffering from pertussis. As coughing is likely to contribute to spread of *B. pertussis* throughout the population, these virulence factors may play a pivotal role in the ecology of *B. pertussis* not only by enabling adherence, but also by contributing to transmission.

**Importance of adhesins and toxins for colonization in animal models**

The contribution of FHA, fimbriae, and Prn to infection in the host has also been studied in various animal models. The flaB and fim genes belong to the early genes, while prn is optimally expressed in the intermediate phase. This may suggest that FHA and Fim are important at an earlier phase of infection compared with Prn. Roberts et al. (1991) tested a Prn deletion mutant in a mouse pulmonary infection model and found no significant colonization defect in either the trachea or the lungs. This would suggest that Prn is not essential for successful colonization of the lower respiratory tract, at least in mice. In contrast, we have recently found that deletion of the Prn gene does affect replication in the mouse (unpublished data). Furthermore, studies with *B. bronchiseptica* have shown that although inactivation of the Prn gene did not affect adherence to epithelial and macrophage-like cells or respiratory infection in rats (Inatsuka et al., 2010), the mutant was cleared much faster than wild-type bacteria in a mouse model. This was predominantly caused by the effect of Prn on the inhibition of neutrophil-mediated clearance. It was also shown that the RGD motif, in contrast to previous reports, did not contribute to Prn function in the mouse model (Inatsuka et al., 2010). *Bordetella bronchiseptica* was also used to study the role of FHA and Prn in its natural host, the swine (Nicholson et al., 2009). Colonization by the FHA mutant was strongly reduced compared with the wild type. In comparison, while the Prn mutant caused similar disease severity as the wild type, it was significantly attenuated in colonization during the early and late infection. These results emphasize the multifactorial contribution of Prn to infection, and suggest that the primary role of Prn may not be adhesion but that it rather has toxin-like activities.

Using a mouse model of infection, FHA and Fim were both shown to be important for bacterial persistence in the trachea, whereas only FHA contributed to nasopharyngeal colonization (Mooi et al., 1992). In this study, neither FHA nor Fim mutants were attenuated in the lungs (Mooi et al., 1992). This study also used fimbrial mutants lacking the major fimbrial subunit genes. It was later found that such mutants still expressed the minor fimbrial subunit FimD on their surface (Geuijen et al., 1997), confounding the interpretation of previous findings. A comparison of an FHA mutant to a strain lacking both major and minor fimbrial subunits showed that the Fim mutant had the maximum defect, as it was significantly less able to colonize nasopharynx, trachea, and lungs (Geuijen et al., 1997). Interestingly, a later study showed that the complete Fim mutant induced a more severe lung inflammation compared with an FHA mutant and the wild-type strain, suggesting that Fim may also act as an immunosuppressant (Vandebriel et al., 2003). Kimura et al. (1990) found that FHA was important only for initial colonization of the mouse trachea, but not for colonization of the lungs. In summary, these animal studies suggest an especially important role for fimbriae in colonization, but also imply FHA and Prn in this process.

Similar to the adhesins, the roles of the toxins have also been studied in various animal models. Using mixed infection experiments in mice, Carbonetti et al. (2003) established markedly distinct roles for Ptx and ACT in respiratory tract colonization. A Ptx-deficient strain was shown to be severely attenuated in colonization. This became apparent within 1–2 days postinfection, but showed the strongest defect 7 days postinfection. Co-administration of soluble Ptx with the Ptx-deficient strain restored colonization to wild-type levels. Although an ACT deletion mutant was also attenuated in colonization, the phenotype was significantly different from the Ptx mutant. The ACT mutant was able to colonize as efficiently as the wild-type parental strain during the early phase of infection, but was unable to persist beyond the first 4 days postinfection. In contrast to Ptx, providing soluble ACT together with the deletion mutant did not have any significant effect (Carbonetti et al., 2005). However, because ACT is an integral membrane protein that...
is functional only upon direct contact with its target cell (Basler et al., 2007), it cannot be excluded that the protein production and purification procedure may have rendered ACT inactive. Thus, while Ptx already contributes during the initial phases of infection, the effects of ACT do not become apparent until later during infection. This suggests that these two toxins serve complementary functions in the pathogenesis of infection, by contributing firstly to the initial establishment of infection and secondly to persistence in the respiratory tract.

The in vivo contribution of the T3SS in B. pertussis has been studied by Fennelly and colleagues. Similar to studies with Salmonella and Yersinia (Eichelberg et al., 1994; Woeslyn et al., 1994), they deleted the BscN gene in B. pertussis, which resulted in an abolished secretion of T3SS effector proteins (Fennelly et al., 2008). The BscN deletion mutant colonized the lungs significantly less efficiently during the complete course of infection, and it was cleared earlier than the wild-type strain (Fennelly et al., 2008).

In addition to the virulence factors discussed above, B. pertussis produces several other factors that are important for colonization. One of these factors is a 34-kDa protein designated tracheal colonization factor (TcfA) (Finn et al., 1991; Finn & Stevens, 1995). Finn & Stevens (1995) showed that a TcfA-deficient mutant strain colonized the trachea of mice at 10-fold lower levels, but found similar colonization rates in the lungs. In spite of this interesting observation, the mechanisms through which TcfA contributes to B. pertussis pathogenesis have not yet been elucidated.

The effects observed in these animal studies are not easily associated with a single aspect of disease, as the majority of virulence factors are implicated in multiple virulence-related processes. For instance, FHA is an important adhesin, but it has also been linked to the apoptotic induction of host epithelial and phagocytic cells (Abramson et al., 2001). Furthermore, both Ptx and ACT facilitate adhesin-mediated adherence to host cells, but are primarily involved in modulating the host immune response (discussed later in this review). Nonetheless, the virulence factors discussed above are all important for the ability of B. pertussis to colonize the human respiratory epithelium.

**Epithelial cell invasion and survival**

Many pathogens invade host cells to hide from the immune system, to intoxicate effector cells, or to gain access to scarce (metabolic) resources (Knodler et al., 2001; Cossart & Sansonetti, 2004). A number of studies have provided evidence that B. pertussis may also be capable of cell invasion, using both primary cell cultures and cell lines. Thus far, several virulence factors that are involved in this process have been identified. However, it should be noted that these studies often presented conflicting roles for particular virulence factors. For example, a B. pertussis Hela cell invasion model identified both FHA and Ptx as positive mediators of invasion (Ewanowich et al., 1989). In contrast, two other studies were unable to reproduce these findings, although a similar positive trend was observed for FHA and Ptx (Lee et al., 1990; Bassinet et al., 2000). A more convincing role for FHA in cellular invasion of human respiratory epithelial cells has been established subsequently using three other cell invasion models of both human cell lines and primary cultures (Bassinet et al., 2000; Ishibashi et al., 2001).

Ishibashi et al. (2001) investigated which molecular domains of FHA are involved in this process. By substituting one amino acid in the RGD domain of FHA, they were able to show that this domain is essential for bacterial entry into host epithelial cells. Follow-up studies implicated the intercellular adhesion molecule 1 (ICAM-1, or CD54) and the very late antigen 5 (VLA-5, also known as ITGAV5 or CD49e) integrins in FHA-mediated cellular entry (Ishibashi et al., 2001; Ishibashi & Nishikawa, 2002). ICAM-1 expression by epithelial cells was found to be upregulated in response to B. pertussis in a nuclear factor κB (NF-κB)-dependent manner, and this upregulation required participation of both FHA (with its RGD domain) and VLA-5 (Ishibashi & Nishikawa, 2002, 2003). Although invasion by B. pertussis is associated with increased ICAM-1 expression through the early expressed FHA, induction of ICAM-1 has also been shown to be associated with increased leukocyte recruitment and clearance of bacterial pathogens (O’Brien et al., 1999). Interestingly, Ptx, which is expressed maximally later during infection, has been shown to inhibit the expression of ICAM-1 (Ishibashi & Nishikawa, 2002). This may point to a concerted modulation of the host by B. pertussis, by which the bacterium first enables adhesion and cellular entry through FHA-mediated ICAM-1 upregulation. Later during infection, when the ability to spread to other niches and hosts outweighs the need for adherence, inhibition of ICAM-1 by Ptx would become important. Although VLA-5 integrin has been shown to be involved in invasion, a direct interaction with FHA appears unlikely, as VLA-5 has been found to interact specifically with FimD (Hazenzos et al., 1995b). To study the contribution of fimbriae to invasion, Fim2 and Fim3 deletion mutants were tested for their ability to invade epithelial cells. No difference in uptake levels compared with wild type was seen for either mutant, suggesting that they are not required for invasion (Bassinet et al., 2000). However, given that these mutants still express FimD on their surface, a role for fimbriae in invasion cannot be completely ruled out yet.

Studies showed conflicting results with regard to involvement of Prn in invasion. Initially, it was suggested that Prn was not involved in bacterial uptake, as a Prn deletion mutant invaded Hela cells to the same extent as its parental wild-type strain (Roberts et al., 1991). In contrast, the same...
The role of Prn in invasion was further supported by the observation that this process was inhibited in the presence of peptides derived from Prn with the RGD motif (Leininger et al., 1992). Based on the last two studies, it seems plausible that Prn contributes to the ability of B. pertussis to bind and invade respiratory epithelial cells, but the underlying molecular mechanism has not yet been resolved.

Similar to FHA, Fim, and Prn, the involvement of ACT in epithelial cell invasion remains an enigma. An identical ACT deletion mutant (Weiss et al., 1983) has been used in three different studies, all of which used a Hela cell invasion model. Although one study could not identify a role for ACT in invasion (Lee et al., 1990), the other two studies showed that inactivation of ACT enhanced the uptake of B. pertussis by Hela cells (Ewanich et al., 1999; Bassinet et al., 2000). Bassinet et al. (2000) also showed that the ACT mutant was more rapidly taken up by tracheal epithelial cells, suggesting that ACT may be able to inhibit epithelial cell invasion. Given the indirect effect of ACT on FHA-mediated binding (Zaretzky et al., 2002), and the role of FHA in epithelial cell invasion, one may expect that ACT inactivation would reduce uptake. However, as experimental evidence points to the contrary, further research is required to unravel the specific mechanism by which ACT contributes to epithelial cell invasion.

The first in vivo evidence of intracellular survival of B. pertussis in an animal host dates from 1969, when Cheers & Gray challenged mice intranasally with B. pertussis. Following infection, bacteria were detected inside alveolar macrophages (AMs) (Cheers & Gray, 1969). This intracellular phenotype has later been confirmed in two other animal studies that described the presence of viable, intracellular bacteria in the bronchoalveolar lavage 19 days after infection (Hellwig et al., 1999; Vandebriel et al., 2003). Although these findings suggest that B. pertussis can survive inside cells, interpretation of these results is complex as the studies do not discriminate between bacteria that have only just entered cells and bacteria that have survived intracellularly for a longer time. All of these findings bring us to an important question: when and where during infection does cellular invasion contribute to the survival of B. pertussis? For instance, if invasion reflects an immune evasion strategy to survive in the host, one would expect B. pertussis to also be able to survive for an extended period of time inside these cells. However, there are strong data that suggest otherwise. For example, despite the fact that B. pertussis was highly proficient in invading human colonic epithelial (Caco-2) cells, survival of intracellular bacteria was impaired (Schipper et al., 1994). Similarly, B. pertussis was also unable to survive in human tracheal epithelial cells (Bassinet et al., 2000).

To conclude, despite a significant amount of data in support of the invasion of epithelial cells, its role in the life cycle of B. pertussis remains enigmatic. Importantly, the exact molecular mechanisms underlying this process remain currently unknown, and many of the experimental observations from invasion studies may in fact be more incidental as a result of tight adherence, rather than represent a specific invasion mechanism. It may be possible that epithelial cell invasion does not play a role in the ecology of B. pertussis at all, but represents an evolutionary deadend road, similar to the invasion of meninges caused by Neisseria meningitidis (Join-Lambert et al., 2010). Further research is clearly warranted to establish the importance of these mechanisms for bacterial infection and persistence in the host.

Biofilm formation

Many pathogenic bacteria are capable of forming well-structured communities on various substrates or cells, commonly referred to as biofilms. Biofilm formation is an essential process for many mucosal pathogens, as it facilitates successful colonization and persistence in the host (Costerton et al., 1999), mainly by providing resistance to antibiotics and host immune defenses (Lewis, 2001). In the last few years, it has been recognized that B. pertussis forms biofilms in vitro and in vivo (Mishra et al., 2005; Parise et al., 2007). Here, we will discuss the role and consequences of this phenotype during B. pertussis infection.

The development of a biofilm is a very complex process, in which bacteria transit through distinct stages of multicellular organization in response to various environmental stimuli (Monds & O’Toole, 2009). Initially, planktonic bacteria irreversibly attach to a surface, for instance the respiratory tract mucosa. This is followed by the formation of discrete cell clusters called microcolonies. This can either be due to clonal expansion of attached bacteria or via the recruitment of additional planktonic cells. These microcolonies then grow in size and coalesce to form mature macrocolonies, in which cells are embedded in an amorphous slimy material, primarily consisting of exopolysaccharides, proteins, and DNA (Karatan & Watnick, 2009). Finally, macrocolonies can dissolve, leading to the release and dissemination of bacteria to other surface areas. Several models are available for studying biofilm formation in vitro, ranging from simple methodologies such as growth on agar plates and plastic microtiter dishes to more advanced flow cell methods (reviewed in Branda et al., 2005). It seems likely that these methods mimic biofilm formation in the host only to varying degrees.

Biofilm formation by pathogenic bacteria is generally considered to be a highly regulated process, tightly coupled to the regulation of virulence (Gotter & Stibitz, 2007). Studies from the last 20 years revealed that bis-(3′-5′)-cyclic
dimeric GMP (c-di-GMP) is a conserved key regulatory molecule controlling the transition between motile planktonic and sedentary biofilm-associated lifestyles of many bacterial species. It does this by regulating genes involved in motility, cell–cell communication (or quorum sensing), and the biosynthesis of adhesins and exopolysaccharide matrix substances (for recent reviews, see Cotter & Stibitz, 2007; Hengge, 2009; Schirmer & Jenal, 2009). Thus far, the role of c-di-GMP in the regulation of B. pertussis biofilm formation remains largely speculative, as most of our assumptions are based on in silico analyses that have not been confirmed in the laboratory yet. Analysis of the B. pertussis genome identified the presence of five putative diguanylate cyclase (DGC)-encoding genes (Wan et al., 2009). These enzymes are characterized by the presence of a GGDEF domain and are capable of catalyzing the formation of c-di-GMP (Galperin et al., 2001). Wan et al. (2009) showed that a mutant strain deficient in one of the DGCs (BpeGReg, B. pertussis globin-coupled regulator) has a reduced ability to form biofilms on plastic, suggesting a role for c-di-GMP signaling in biofilm formation by B. pertussis. Additionally, the B. pertussis genome contains four genes encoding putative phosphodiesterases. Phosphodiesterases contain an EAL domain, associated with cyclic di-GMP phosphodiesterase activity, and are able to hydrolyze c-di-GMP (Galperin et al., 2001). Interestingly, one of the B. pertussis proteins that contain an EAL domain is BvgR (Merkel et al., 1998). It has been postulated that the increased expression of BvgR in the virulent phase may result in a reduction of the amount of cellular c-di-GMP, thereby inhibiting biofilm formation. However, whether BvgR actually displays phosphodiesterase activity has not yet been confirmed. Furthermore, in vitro experiments with wild-type B. pertussis and a Bvg− phase-locked derivative showed that biofilm formation occurred exclusively under Bvg− conditions, suggesting that BvgR may not play a crucial role in this process (Mishra et al., 2005; Serra et al., 2007). Although in vitro experiments with B. bronchiseptica indicated maximal biofilm formation under Bvg− phase conditions (Irie et al., 2004), B. pertussis has so far not been studied under similar conditions. Further research is warranted to elucidate the role of c-di-GMP signaling and the BvgASR system in B. pertussis biofilm formation.

For a better understanding of the significance of biofilm formation in pertussis pathogenesis, identification of the molecular factors involved in this process is of utmost importance. The observation that Bvg− phase-locked B. pertussis are unable to form biofilms (Mishra et al., 2005) may suggest that vags are important for biofilm formation. As biofilm formation is initiated by the attachment of planktonic cells (Monds & O’Toole, 2009), adhesins are hypothesized to play an essential role in this process. In vitro experiments with B. bronchiseptica suggested that this is indeed the case, as FHA and Fim deletion mutants were shown to be highly attenuated in their ability to form biofilms on plastic (Irie et al., 2004). The observed attenuation was Bvg-phase dependent, as the Fim deletion mutant was affected in biofilm formation under Bvg+ conditions, whereas the FHA deletion mutant showed a defect under Bvg− conditions (Irie et al., 2004). Furthermore, this study also showed that deletion of ACT resulted in an increased biofilm formation (Irie et al., 2004), which suggests that ACT may directly or indirectly inhibit biofilm formation in B. bronchiseptica. Given that ACT is expressed maximally in the Bvg+ phase while FHA is expressed under both Bvg+ and Bvg− conditions (Cotter & Miller, 1997), it has been speculated that the weaker biofilm phenotype of Bvg+− phase B. bronchiseptica is at least partially mediated by the interaction of ACT with FHA (Zaretzky et al., 2002). Interestingly, B. pertussis shows a similar phase-dependent expression pattern of FHA and ACT (Cummins et al., 2006); further research is required to elucidate the molecular mechanisms by which these factors contribute to biofilm formation.

For the formation of a mature biofilm, or macrocolony, bacteria present in microcolonies need to produce and secrete matrix components such as proteins, lipids, DNA, and, most importantly, polysaccharides (Vu et al., 2009). Parise et al. (2007) recently identified a locus required for the production of cell surface-associated poly-β-1,6-N-acetyl-d-glucosamine (poly-β-1,6-GlcNAc, also called Bps polysaccharide), which was designated the Bordetella BpsABCD locus. This study showed that biofilm formation was inhibited both in the presence of dispersin B, a Bps polysaccharide-degrading enzyme, or in a BpsABCD-deficient background (Parise et al., 2007). Furthermore, a follow-up study showed that the BpsABCD deletion mutant was unable to form a robust biofilm in the mouse respiratory tract, validating previous in vitro data (Sloan et al., 2007). Moreover, a recent study showed that the role of the Bps polysaccharide is limited to early colonization and biofilm formation in the nose and trachea but not in the lungs (Conover et al., 2010). Interestingly, the Bps polysaccharide was found to function as an adhesin, as it specifically promoted adhesion to human nasal epithelial cells and not to human lung epithelial cells (Conover et al., 2010). This suggests that the cryptic receptor for Bps is specifically expressed in the upper respiratory tract, and that biofilm formation may be especially important for colonization, and possibly also for transmission.

In order to gain a deeper insight into the macromolecular composition of B. pertussis biofilms, Serra et al. (2008) used a combined approach of proteomics and Fourier transform infrared spectroscopy to identify molecules involved in biofilm formation. Their analysis showed that in vitro biofilm formation has a distinctive and specific impact on
the bacterial physiology compared with planktonic growth. The proteome profile revealed that about 8–10% of the proteins are differentially regulated in biofilms, including an increased expression of Prn and BipA (Serra et al., 2008). Significantly, the most outstanding and distinctive physiological feature of Bordetella pertussis biofilms was linked to carbohydrate metabolism. Bordetella pertussis biofilms displayed an increase in carbohydrate production, which coincided with the de novo expression and overexpression of three proteins involved in polysaccharide biosynthesis, modification, and transport (Serra et al., 2008). Further characterization showed that B. pertussis specifically produces an acidic-type polysaccharide. However, they did not identify the Bps polysaccharide discussed earlier (Parise et al., 2007), suggesting that the polysaccharide composition of Bordetella pertussis biofilms is rather complex.

In summary, biofilm formation by B. pertussis is a novel and largely unexplored field of research, with potentially significant implications for the design of therapeutics. Further experiments using specific gene-deficient strains are required for the clarification of the factors involved in the complex process of biofilm formation by B. pertussis. Importantly, the mechanisms by which the BvgASR system controls biofilm formation, and under which conditions and in which niche biofilm formation occurs in the host, should be further elucidated.

Modulation of the host immune response

Infection with B. pertussis leads to the induction of an innate response and the subsequent development of specific immunity (Mills, 2001). The respiratory epithelium, together with resident antigen-presenting cells (APCs) such as alveolar macrophages (AMs) and DCs are the primary host innate immune cells that sense and shape initial local immune responses towards B. pertussis. As the infection progresses, B. pertussis is challenged by an additional host defense mechanism: the adaptive immune system. The main effectors of this system are the T and B lymphocytes, which eventually mediate clearance through the development of protective immunity against the pathogen. However, B. pertussis has evolved a number of strategies to prevent or modulate specific aspects of this response, thus delaying clearance and improving survival chances and transmission to the next host (see Fig. 2 and Table 2). In this section, we will discuss how B. pertussis interacts with specific components of the immune system to modulate the host immune response to facilitate survival and replication in the respiratory tract.

Innate recognition

The initial recognition by resident immune cells and respiratory epithelial cells represents an important step in the successful induction of specific immunity. DCs and macrophages are specialized inflammatory cells that are strategically localized throughout the body near potential pathogen entry portals, including the respiratory tract. These cells act as sentinels for the immune system by sensing the presence of potential pathogens through PRRs. Their main aim is to recognize and process antigens and present these to the adaptive immune system. PRRs recognize distinct evolutionary conserved microbial components called pathogen-associated molecular patterns (PAMPs) such as lipids, lipoproteins, proteins, and nucleic acids, derived from a wide range of bacteria, viruses, parasites, and fungi (reviewed by Kawai & Akira, 2009, 2010). There are many different classes of PRRs, including surface-expressed Toll-like receptors (TLRs), cytosolic nucleotide-binding oligomerization domain (NOD)-like receptors, C-type lectin receptors, and retinoic acid-inducible gene-I-like receptors. Prevention of an effective immune response, either by directly preventing recognition or by modulating the downstream pathways, is an effective survival mechanism for bacteria. The biological mechanisms by which B. pertussis achieves this will be discussed here.

Most efforts aimed at elucidating the interaction between B. pertussis and innate immune cells have looked at human monocytic-derived DCs (MDDCs), as these cells are relatively easy to culture in vitro. Immature MDDCs express a repertoire of PRRs, such as TLR2, TLR4, TLR5, but not TLR9, as well as a range of intracellular PRRs, which are able to recognize several bacterial PAMPs.

TLR4 is involved in the recognition of lipopolysaccharide (Poltorak et al., 1998; Kawai & Akira, 2009). Typically, lipopolysaccharide structures are comprised of three distinct structural domains: the lipid A, the core oligosaccharide, and O-antigenic repeats. The lipopolysaccharide is bound by a lipopolysaccharide-binding protein, which forms a complex with CD14, followed by the transfer of the lipopolysaccharide to the surface-exposed TLR4–MD-2 complex. Following recruitment of MyD88 and TRIF to the cytoplasmic domain of TLR4, NF-κB and interferon regulatory factor 3 (IRF3) signaling pathways are induced, leading to the production of proinflammatory cytokines including type I interferons and IL-6 (Kawai & Akira, 2010).

In contrast to most Gram-negative bacteria, B. pertussis produces a lipo-oligosaccharide that has a branched core structure with a nonrepetitive trisaccharide, rather than a long repeating O-side chain (Caroff et al., 2000). The lipid A moiety of the B. pertussis lipo-oligosaccharide also activates TLR4 signaling pathways in MDDCs, albeit significantly less efficiently than the typical lipid A domain present on the lipopolysaccharide structure of enteric Gram-negative pathogens (Fedele et al., 2007, 2008).

Activation of TLR4 signaling pathways by lipo-oligosaccharide is dependent on recognition of the relatively
conserved lipid A structure moiety (Kawai & Akira, 2010). In recent years, it has become apparent that variation in bacterial lipopolysaccharide is not restricted to the O-antigen repeats but also occurs in the lipid A region, leading to differential inflammatory responses (Dixon & Darveau, 2005). Initially, Preston et al. (2003) showed that the lipid A moiety of *B. bronchiseptica* lipopolysaccharide is modified through the palmitoyl transferase activity of the BvgS phase-specific PagP enzyme. By expressing *B. bronchiseptica* PagP and its antagonist PagL in *B. pertussis*, Geurtsen et al.

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**Fig. 2.** *Bordetella pertussis* virulence factors modulating the host immune response. The most relevant aspects of host immune manipulation by *B. pertussis* in the respiratory tract are schematically illustrated. A, The lipo-oligosaccharide (LOS) molecule of *B. pertussis* is not recognized and bound by host surfactant protein A (SP-A) due to its atypical structure, and thereby protects the pathogen from SP-A-mediated clearance. B, *Bordetella pertussis* inhibits complement-mediated killing (classical pathway) through the action of *Bordetella* resistance to killing A (BrkA), and by the recruitment of host C1 esterase inhibitor (C1INH) and C4b-binding protein (C4BP). C, Pertussis toxin (Ptx) is able to intoxicate alveolar macrophages (AMs) by ADP ribosylation of its G-proteins. D, Ptx inhibits the recruitment of immune cells including AMs, neutrophils (N), and T cells (not shown) by inducing the release of chemokines (IL-8 and CXCL5) from epithelial cells. E, Adenylate cyclase toxin (ACT) and filamentous hemagglutinin (FHA) inhibit macrophage effector functioning by inducing apoptosis in a complement receptor 3 (CR3) dependent manner. F, Cellular immunity is suppressed by the concomitant action of LOS and Ptx on Toll-like receptor 4 (TLR4), FHA, and ACT on CR3, and the type III secretion system (T3SS) secreted protein BopN, on dendritic cells (DCs), which mediate the suppression of IL-12 and the induction of IL-10. G, ACT is able to subvert cyclic AMP (cAMP) signaling in phagocytes and thereby affects their bactericidal activity by inhibiting chemotaxis, the production of superoxides (and hence the respiratory burst), and killing. H, Ptx is able to enter the circulation and suppress antibody responses to *B. pertussis* antigens.
occurs in a BvgAS-dependent manner under BvgA's lipo-oligosaccharide with glucosamine, which also has been found to substitute the lipid A phosphate groups of B. pertussis is Bvg-regulated (van den Akker, 1998). In fact, it can modulate its lipo-oligosaccharide in response to changing B. pertussis that other enzymes with similar activity may be expressed in served by the authors (Geurtsen et al.). They are both pseudogenes and therefore cannot explain the significance of this observation has not yet been determined, although the genome of B. pertussis contains both genes, which are BvgA-regulated (van den Akker, 1998). This implies that other enzymes with similar activity may be expressed in B. pertussis, such as the outer membrane phospholipase A. It is tempting to speculate that B. pertussis is able to modulate its lipo-oligosaccharide in response to changing conditions within the host, as for example has been observed in B. bronchiseptica in which lipo-polysaccharide biosynthesis is Bvg-regulated (van den Akker, 1998). In fact, B. pertussis has been found to substitute the lipid A phosphate groups of its lipo-oligosaccharide with glucosamine, which also occurs in a BvgAS-dependent manner under BvgA conditions (Marr et al., 2008; Geurtsen et al., 2009). Importantly, glucosamine substituents have been shown to be more potent inducers of the release of proinflammatory cytokines by macrophages (Marr et al., 2010). Although the in vivo significance of this observation has not yet been determined, it could be hypothesized that glucosamine modifications of lipo-oligosaccharide are important for skewing the host immune response, as they induce a different cytokine response. The mucosal surfaces of the upper respiratory tract are colonized with a plethora of other species, and consequently, the innate immune system is stimulated with a complex mixture of PAMPs, including B. pertussis lipo-oligosaccharide. In contrast, B. pertussis lipo-oligosaccharide may be able to interact in a more direct manner with the innate immune system in the lungs, which are normally sterile. It would be interesting to investigate whether glucosamine modification of lipo-oligosaccharide is dependent on the location and differs between the upper and lower respiratory tract. One interesting alternative hypothesis for the biological effect of the modified lipo-oligosaccharide is that it may also play a role in altering the outcome of bacterial competition by directing the innate immune response to competing flora. There is precedent for this, as Lysenko et al. (2005) have shown that species-specific stimulation of the innate immune response can be an effective strategy to outcompete nasal flora. The lipid A and the oligosaccharide core domain of lipopolysaccharide can also be recognized by the surfactant proteins A and D (SP-A and SP-D, respectively), hydrophilic lipid-binding lectins that are ubiquitously expressed in the lower respiratory tract of humans (reviewed in Chaby et al., 2005). Binding of SP-A to lipopolysaccharide induces agglutination, destabilizes the bacterial membrane, and facilitates phagocytosis (reviewed in McCormack & Whitsett, 2002). Further, SP-A-mediated recognition of lipopolysaccharide can also modify LBP–CD14 complexes, thus contributing to an altered recognition of lipopolysaccharides by TLR4 (Stamme et al., 2002). Interestingly, wild-type B. pertussis lipo-oligosaccharide is not recognized and bound by SP-A or SP-D (Schaeffer et al., 2004a, b). However, after removal of one or more sugars from the terminal trisaccharide, the Table 2. Virulence factors that modulate the host immune system* Bacterial factor(s) Innate immune system Adaptive immune system Comment(s) FHA Drives phagocytes into apoptosis, Stimulates IL-10 expression in DCs; Implicated in C4BP binding and recruitment Skews T cell differentiation towards Th17 subtype Induces CR3 upregulation in PMN; suppresses IL-12 and induces IL-10 and IL-6 in macrophages PAMP for TLR2/4, Induces CR3 upregulation in PMN Ptx Inhibits immune cell recruitment; Intoxicates AMs through ADP ribosylation of their G-proteins; Suppresses phagocytic activity of monocytes Suppresses antibody responses; Skews T cell differentiation towards Th17 subtype TCT Inhibits the deposition of soluble complement factors on the bacterial membrane PAMP for murine Nod1 LOS Penetrates DCs and induces IL-10 expression Skews T cell differentiation towards Th17 subtype PAMP for TLR4, not recognized by SP-A BrkA Inhibits immune cell recruitment; Synergizes LOS–PAMP for TLR4, not recognized by SP-A* See text for references. AMs, alveolar macrophages; C4BP, C4-binding protein; CR3, complement receptor 3; DCs, dendritic cells; IL, interleukin; LOS, lipo-oligosaccharide; Nod1, nucleotide-binding oligomerization domain-like receptor 1; PAMP, pathogen-associated molecular patterns; PMN, polymorphonuclear neutrophils; SP-A, surfactant protein A; TLR, Toll-like receptor.

*See text for references. AMs, alveolar macrophages; C4BP, C4-binding protein; CR3, complement receptor 3; DCs, dendritic cells; IL, interleukin; LOS, lipo-oligosaccharide; Nod1, nucleotide-binding oligomerization domain-like receptor 1; PAMP, pathogen-associated molecular patterns; PMN, polymorphonuclear neutrophils; SP-A, surfactant protein A; TLR, Toll-like receptor.
lipo-oligosaccharide is recognized by SP-A and SP-D, leading to effective bacterial opsonization and phagocytosis (Schaeffer et al., 2004a). Thus, it appears that the terminal trisaccharide of \textit{B. pertussis} lipo-oligosaccharide prevents access of SP-A and SP-D to the lipid A domain through steric hindrance, and thereby protects the bacteria from surfactant-mediated clearance.

Because lipopolysaccharide is such a characteristic and dominant feature of Gram-negative bacteria, the host has evolved multiple recognition receptors to sense the presence of this molecule, for example through surfactants. By expressing the lipo-oligosaccharide, \textit{B. pertussis} is able to effectively prevent surfactant-mediated recognition and clearance. Furthermore, by changing the composition of the lipid A moiety, it is able to modulate the lipo-oligosaccharide-mediated immune response. It remains to be investigated whether or not the stronger inflammatory charide-mediated immune response.

Recruitment of immune cells

Colonization of the mucosal surfaces of the respiratory tract by \textit{B. pertussis} initiates a local chemokine response, resulting in the recruitment of immune cells to the site of infection. Usually, neutrophils are the first immune cells to arrive, followed by a second wave of NK cells, macrophages, DCs, and lymphocytes (Khelef et al., 1994; McWilliam et al., 1996; McGuirk et al., 1998; Vandebriel et al., 2003; Byrne et al., 2004).

It has been well-established that the toxins Ptx and TCT are able to inhibit immune cell trafficking to the respiratory tract. Meade et al. (1985) already showed that systemic administration of Ptx resulted in reduced recruitment of macrophages to the site of infection. Additionally, in vitro experiments have shown that Ptx is able to inhibit chemotaxis of lymphocytes and neutrophils (Spangrude et al., 1985). This effect on neutrophil trafficking has also been observed in vivo (Thomazzi et al., 1995; Kirimanjeswara et al., 2005), and the underlying molecular mechanisms are well-understood. Ptx has been shown to affect chemotaxis indirectly by suppressing the release of chemokines from resident airway cells. Using a mouse model of infection, it was shown that a mutant \textit{B. pertussis} strain lacking Ptx induced significantly higher levels of keratinocyte-derived chemokine and macrophage inflammatory protein 2, i.e. the murine functional equivalents of human IL-8, as compared with the wild-type strain. Further, lipopolysaccharide-induced CXCL chemokine, the murine homolog to human CXCL5/ENA-78, was also inhibited by Ptx (Andreasen & Carbonetti, 2008). It is likely that the Ptx-mediated decrease in chemokine release also contributed to the reduced early infiltration of leukocytes observed in earlier studies (Meade et al., 1985; Spangrude et al., 1985). In addition to Ptx, TCT has also been shown to inhibit neutrophil chemotaxis, at least in vitro (Cundell et al., 1994), which may also partially contribute to this effect.

In conclusion, by producing Ptx and TCT, \textit{B. pertussis} is able to delay phagocytic clearance by inhibiting the recruitment of phagocytes.

Phagocytosis

The efficiency of bacterial phagocytosis differs between phagocytic cell types, and distinct innate immune cells may process internalized bacteria differently. An important question is which are the bacterial and host factors that are predominantly involved in the interaction of \textit{B. pertussis} with these phagocytic cells? The current model is that phagocytosis of \textit{B. pertussis} occurs either via direct recognition of surface-exposed molecules on the bacterial membrane or via antibody-mediated recognition of surface-bound opsonins (antibodies and complement system components). Both mechanisms will be discussed here, as well as...
the strategies used by B. pertussis to interfere with phagocytosis and subsequent cellular killing mechanisms.

**The role of antibodies in phagocytosis**

In recent years, the importance of antibody opsonization in the phagocytosis of B. pertussis has been studied extensively. Based on *in vitro* experiments with fluorescein isothiocyanate (FITC)-labeled B. pertussis, it was initially suggested that opsonization enhances the uptake of the pathogen by human monocytes and neutrophils (Steed et al., 1991; Hazenbos et al., 1994). However, the FITC labeling procedure was later shown to have intrinsic effects on protein function, including ACT, thereby confounding previous results (Weingart et al., 1999). Subsequent experiments using a genetically modified B. pertussis strain stably expressing green fluorescent protein actually showed an opposite effect, whereby B. pertussis failed to be internalized efficiently (Weingart et al., 1999). A subsequent study suggested that incubating bacteria with heat-inactivated serum containing antibodies to B. pertussis lipo-oligosaccharide as well as to several other surface-localized protein virulence factors (Weiss et al., 1999) inhibited both attachment and uptake by human neutrophils (Weigart & Weiss, 2000). In these experiments, the majority of surface-exposed bacterial antigens were bound by antibodies, and hence were shielded from phagocyte binding. Based on these studies, it was suggested that phagocytosis of B. pertussis occurs independent of opsonization, but requires a specific, direct interaction with factors present on the bacterial membrane.

Although antibody-independent phagocytosis plays an important role (discussed below), there is also substantial clinical and epidemiological evidence, including passive immunization trials, that antibodies are essential for protection against B. pertussis. Miller et al. (1943) already observed that individuals with high antibody titers were significantly less susceptible to infection with B. pertussis. Since then, multiple studies established a strong correlation between clinical protection and the presence of specific immunoglobulin G (IgG) antibodies against fimbriae, Ptx, and most importantly Prn (Cherry et al., 1998; Storsaeter et al., 1998, 2003; Hellwig et al., 2003). It is therefore not surprising that recent studies established a role for antibody-mediated phagocytosis in the clearance of B. pertussis from the host.

IgA and IgG antibodies against B. pertussis were both found to mediate binding, phagocytosis, and subsequent killing of the pathogen by polymorphonuclear neutrophils (PMN) *in vitro* (Hellwig et al., 2001a, b). IgA-dependent uptake by PMN was shown to be mediated by the human myeloid IgA receptor FcαRI (CD89), whereas the IgG-dependent uptake was shown to be mediated by the synergistic action of the IgG receptors FcγRIa (CD32) and FcγRIIb (CD16) (Hellwig et al., 2001b; Rodriguez et al., 2001). Although CR3 (discussed below) and Fc receptors both play an important role in phagocytosis, the contribution of each pathway to bacterial clearance remained unknown. Therefore, the CR3- vs. FcR-mediated uptake of B. pertussis was compared in an *in vivo* model. Importantly, only FcR-mediated uptake was shown to facilitate clearance of B. pertussis from the lungs (Hellwig et al., 2001a). These findings support a model whereby internalization of bacteria through the CR3-dependent pathway is advantageous to B. pertussis, whereas FcR-mediated uptake is associated with protection against infection. Further proof of the crucial role of antibodies in protection comes from a recent study from Andreasen & Carbonetti (2009), where they showed that neutrophil depletion before inoculation of naïve mice did not have any effect on bacterial colonization. In contrast, immunized neutropenic mice showed a significant reduction in clearance compared with immunized wild-type mice.

Taken together, the presence of B. pertussis antibodies facilitates Fc-receptor-mediated phagocytosis by phagocytic cells, eventually leading to clearance of the pathogen.

**Antibody-independent phagocytosis**

The construction of B. pertussis deletion mutants has made it possible to study the involvement of specific bacterial molecules and host receptors in the recognition of B. pertussis by phagocytes. In particular, the interaction of B. pertussis with monocytes and monocyte-derived macrophages has been well-characterized. Although there are a number of dissimilarities between these two cell types, they also share many surface receptors and signaling pathways. In *in vitro* adhesion assays using FHA, FimD, or Prn deletion mutants suggested that they are all involved in the recognition and binding of B. pertussis by monocytes (Hazenbos et al., 1994). Although the involvement of FHA and FimD in this process is undisputed, the extent to which Prn contributes to this process is currently not well-understood. Relman et al. (1990) initially found that the RGD domain of FHA binds to the CR3 integrin on macrophages, a receptor known to be involved in the phagocytosis of C3-mediated complement-opsonized bacteria. Later work showed that FHA, in addition to the RGD domain, contains three regions with sequence and functional similarity to the binding loops of factor X of the coagulation cascade, a factor known to bind CR3 with high affinity (Rozdzinski et al., 1995). Thus, both the RGD domain and the factor X-like domains may facilitate FHA-mediated binding of B. pertussis to CR3. However, CR3 has been shown to recognize multiple other ligands with varying affinity, including lipopolysaccharides, iC3b, ICAM-1, and fibrinogen, and binding of CR3 may activate different signaling pathways, related to extravasation, chemotaxis, and activation of
phagocytic cells (reviewed by Gramonte-Hevia et al., 2002). It is currently not well understood which signaling pathways are induced by the binding of FHA to CR3. Another study showed that FHA-mediated binding to monocytes can be inhibited by a monoclonal antibody specific for the fibronectin receptor VLA-5 (Hazenbos et al., 1993). Based on these data, the authors suggested that the recognition of FHA by CR3 is dependent on VLA-5. Subsequent efforts to identify the precise mechanisms by which these factors facilitate bacterial uptake showed that the bacterial ligand for VLA-5 is FimD. Further, binding of FimD to VLA-5 facilitated the subsequent interaction between FHA and CR3 in a phosphorylation-dependent manner (Hazenbos et al., 1995a, b). This model complies with previous work that showed that binding and activation of VLA-5 allowed the CR3 receptor to mediate phagocytosis (Pommier et al., 1983).

Monocytes and macrophages both express CR3 and VLA-5, suggesting that monocytes may be able to phagocytose B. pertussis in a similar FHA-dependent manner. However, Schaeffer & Weiss (2001) could not reproduce previous findings with FHA in the context of monocytes. A possible explanation for this observation could be that monocytes are significantly less able to internalize bacteria compared with more differentiated macrophages, potentially caused by differences in signaling pathways. Furthermore, the mechanism by which CR3 facilitates bacterial uptake may be dependent on other, unknown factors. For instance, CR3 is known to interact with the surface-expressed low-affinity immunoglobulin receptor CD16, and this interaction is lost during the differentiation to DCs (Preynat-Seauve et al., 2004). It is conceivable that differentiation-stage-dependent factors, such as CD16, may also contribute to the CR3-mediated uptake of B. pertussis.

The ability of neutrophils to internalize and kill B. pertussis has also been studied. Work by Hazenbos et al. (1993) initially showed that human neutrophils were unable to bind B. pertussis. At that time, the authors suggested that this may be due to the 10-fold lower expression level of VLA-5 on the cell surface of neutrophils, compared with monocytes or macrophages (Hazenbos et al., 1993). In contrast, a later study suggested that murine neutrophils were able to recognize and bind B. pertussis efficiently (Weingart & Weiss, 2000). These differences might be explained by the host-specific recognition of B. pertussis by VLA-5, similar to the observed differences in the recognition of TCT by Nod1 described above. Furthermore, Weingart & Weiss (2000) showed that differences in experimental design between the studies could also have contributed to these differences. To determine the effect of FHA binding to CR3, human neutrophil adhesion assays were performed with either an FHA-deletion mutant or in the presence of a CR3-specific blocking antibody. Results from these experiments indicated that the interaction between B. pertussis and human neutrophils, similar to previous findings with macrophages, was in fact also mediated by CR3 and FHA (Weingart & Weiss, 2000; Mobberley-Schuman & Weiss, 2005). The importance of this interaction is further supported by the observation that an SphB1-deletion mutant, with immature FHA strongly bound to the bacterial cell surface, was more efficiently bound and phagocytosed by neutrophils (Mobberley-Schuman & Weiss, 2005). It was mentioned before that lipid rafts containing cholesterol are crucial for the ability of B. pertussis to bind to epithelial cells in an FHA-mediated manner (Lamberti et al., 2009). A similar mechanism might apply to the recognition of B. pertussis by neutrophils, as cholesterol depletion of neutrophils drastically diminished their ability to bind and internalize nonopsonized B. pertussis (Lamberti et al., 2008). The findings that lipid rafts contribute to this complex process may also explain the observation that monocytes were unable to bind FHA, as receptor clustering within these rafts plays an important role in determining the molecular interaction between receptor and ligand (van Zanten et al., 2009).

In conclusion, in the absence of opsonizing antibodies, B. pertussis can be bound and phagocytosed by monocytes, macrophages, and neutrophils, promoting bacterial survival. This process is dependent on the interactions between FHA-CR3 and FimD-VLA-5. The specific interactions between these factors and the effects on the activation of signaling pathways remain largely unknown, although it is known that CR3- and FcγR-dependent phagocytosis is mediated through different signal transduction pathways (Caron & Hall, 1998). Interestingly, CR3-mediated phagocytosis seems to occur in the absence of an oxidative burst (Berton et al., 1992), providing further evidence that CR3 is exploited by B. pertussis for intracellular survival. Both FHA and Ptx were found to induce CR3 expression by PMNs (Mobberley-Schuman & Weiss, 2005). In addition, in vivo respiratory colonization experiments in mice revealed that Ptx is able to suppress serum antibody responses to various B. pertussis antigens (Mielcarek et al., 1998; Carbonetti et al., 2004). Together, these effects may shift the balance towards the nonkilling CR3-mediated phagocytic route, allowing the pathogen to escape from clearance by the more efficient Fc receptor-mediated pathway.

**Foiling phagocytosis**

Phagocytic uptake of bacteria is generally followed by fusion of the phagosome with the lysosome, generating a new compartment called the phagolysosome (Cohn & Fedorko, 1969). Bacteria present in this compartment are then exposed to an acidified environment as well as a number of...
antibacterial molecules, including ROS and proteolytic enzymes that break down the bacterium. It is thought that once *B. pertussis* is taken up into the phagolysosome of phagocytes, it is killed rapidly because the acidic environment of this compartment is detrimental for the growth and survival rate of *B. pertussis* (Schneider et al., 2000). For instance, phagocytic killing assays by adherent monocytes and neutrophils showed virtually no bacterial survival after only 2 h (Lenz et al., 2000; Schaeffer & Weiss, 2001). In contrast, other studies suggest that internalization by macrophages does not automatically lead to killing of the pathogen. This was already observed by Friedman and colleagues in the early 1990s, who showed that *B. pertussis* was able to survive for at least 3 days following internalization (Friedman et al., 1992). Recently, Lamberti et al. (2010) established that the route of intracellular trafficking determines the fate of internalized *B. pertussis*. They observed that while the majority of bacteria taken up by macrophages end up in an acidic compartment and are killed rapidly, the residual bacteria were able to survive and replicate inside a nonacidic early endosome-like compartment. The mechanisms that underlie this nonkilling route of intracellular trafficking have not yet been established.

Besides survival through entering the endosomal compartment, *B. pertussis* uses several other strategies to suppress phagocytic activity, which will be discussed here. Within minutes to hours of infection, *B. pertussis* encounters AMs. The importance of these cells in the host defense against *B. pertussis* has been elegantly demonstrated by Carbonetti et al. (2007). They showed that depletion of AMs from the lungs of mice before inoculation with wild-type *B. pertussis* resulted in enhanced respiratory infection. Interestingly, a similar phenotype was observed after challenge with a Ptx-deficient strain, suggesting that Ptx is able to suppress AMs. Indeed, Ptx was shown to intoxicate AMs by ADP ribosylation of its G-proteins, thereby contributing to the initial establishment of infection (Carbonetti et al., 2007). The specific effects of Ptx on G-coupled proteins have been well-studied and have been reviewed elsewhere recently (Carbonetti, 2010). As infection progresses, other phagocytic cell types such as monocytes and neutrophils migrate to the site of infection. Importantly, Ptx is able to suppress the phagocytic activity of monocytes (Schaeffer & Weiss, 2001), although the effect of Ptx on neutrophils has not been studied yet.

*Bordetella pertussis* also evades phagocytosis by suppressing the bactericidal activity of phagocytes (Weingart et al., 2000). As mentioned before, ACT rapidly induces cellular cAMP levels in CR3⁺ phagocytes, including PMNs (Guermonprez et al., 2001). The rapid elevation of cAMP signals causes a transient and selective inactivation of RhoA, a member of the Rho family of GTPases, which are key regulators of actin cytoskeletal dynamics (Kamanova et al., 2008). This inactivation of RhoA results in the dephosphorylation of the actin filament-severing protein coflin, and causes massive actin cytoskeletal rearrangements that coincide with phagocytic ruffling and the loss of macropinocytic fluid-phase uptake (Kamanova et al., 2008). cAMP-induced pathways were shown to affect the *in vitro* bactericidal activity of monocytes and neutrophils by inhibiting chemotaxis and the production of superoxides, thereby hampering their ability to elicit a respiratory (or oxidative) burst of ROS (Friedman et al., 1987; Pearson et al., 1987).

*Bordetella pertussis* also evades phagocytic killing by driving phagocytes into apoptosis. Both *in vitro* (Khelef et al., 1993) and *in vivo* (Gueirard et al., 1998) experiments have shown that ACT is the major virulence factor involved in host cell apoptosis. In contrast to the anti-phagocytic effects of ACT reviewed above, the induction of apoptosis occurs independently of its enzymatic activity and the concurrent supraphysiological rise in cAMP levels (Boyd et al., 2005). For ACT to induce apoptosis, it must be post-translationally acylated (Boyd et al., 2005), internalized by means of CR3-mediated vesicular transport (Khelef et al., 2001) and conformationally changed (Cheung et al., 2009). The importance of the correct delivery of ACT into cells is reflected by the observation that ACT-mediated apoptotic effects are significantly reduced upon inhibition of macropinocytosis, depletion of cholesterol, or disruption of the Golgi network (Khelef et al., 2001). Once ACT has entered the cell via the vesicular transport pathway, apoptotic signals reduce mitochondrial functioning. For instance, when monocytes were treated with purified ACT, their mitochondrial membrane potential was found to be disrupted (Bachelet et al., 2002). Furthermore, ACT induces the activity of the effector caspases 3 and 7 in macrophages, which are the key factors triggering apoptosis (Cheung et al., 2009). Programmed cell death is further promoted by the ability of ACT to form cation-selective pores in the host cell membranes (Benz et al., 1994), which has been attributed to its tendency to form multimers (Szabo et al., 1994). Another *B. pertussis* virulence factor that affects apoptosis is FHA. Abramson et al. (2001) showed that purified FHA induced dose-dependent apoptosis in human phagocytic and epithelial cells. Using a FHA deletion mutant, they showed that strains lacking FHA induced significantly less apoptosis compared with wild-type strains (Abramson et al., 2001). However, because an earlier study did not identify FHA as an inducer of apoptosis (Khelef & Guiso, 1995), the specific role of FHA in the induction of apoptosis requires further investigation.

To summarize, by producing Ptx, ACT, and FHA, *B. pertussis* delays the efficient removal by phagocytes by (1) entering the phagocytic endosomal compartment, (2) intoxicating resident AMs, (3) suppressing the bactericidal activity of monocytes and macrophages, and (4) inducing apoptosis. Thus, the concerted action of these three virulence factors allows the bacterium to persist longer in the respiratory tract.
The complement system

The complement system plays an important role in host defense and can be activated via three different pathways, i.e. the classical, antibody-dependent pathway, the lectin pathway, and the alternative pathway. Activation of these pathways can initiate a number of antibacterial mechanisms, such as agglutination, opsonization and phagocytosis, the recruitment of inflammatory cells through chemotaxis, and finally direct killing of bacteria through the formation of the membrane attack complex. As the complement system can have damaging effects on the host itself if kept unchecked, regulation of complement activation occurs through complement-inhibitory proteins. In the past few years, it has become clear that many bacterial pathogens have evolved mechanisms to evade complement activation, either by recruitment of these inhibitory proteins or by direct inhibition of complement components (for recent reviews, see Lambris et al., 2008; Blom et al., 2009; Laarman et al., 2010). Similarly, B. pertussis uses a number of strategies to overcome complement-mediated removal from the respiratory tract, which will be reviewed here.

Bordetella pertussis inhibits complement-induced phagocytosis through Bordetella resistance to killing A (BrkA), a virulence factor that interferes specifically with the classical pathway of complement activation (Fernandez & Weiss, 1994). In this pathway, opsonizing antibody complexes on the surface of a bacterium provide a docking site for the C1 complex. Binding of C1 to the bacterial surface initiates an enzymatic cascade of proteolytic cleavages, resulting in the deposition of other complement components including C4b, C2a, C3b, and C5b. Membrane-bound C3b functions as an opsonin for phagocytosis and is recognized by the CR1 and CR3 receptors expressed on phagocytes (Newman et al., 1984). Work by Barnes & Weiss (2001) showed that BrkA interferes with the deposition of C4b, C2a, C3b, and C5b onto the C1 complex, thereby preventing subsequent phagocytosis and killing by neutrophils. It is currently unknown whether BrkA exerts its effect via direct inhibition of complement function or alternatively through the recruitment of a complement-inhibitory protein.

More recently, two BrkA-independent mechanisms of complement resistance have been identified. Bordetella pertussis has been found to bind and recruit the C4b-binding protein (C4BP) as well as the human C1 esterase inhibitor (C1INH), both major inhibitors of the classical complement pathway (Berggard et al., 1997; Martz et al., 2007). C4BP inhibits killing of host cells by binding C4b, which renders C4b more susceptible to degradation by factor I (C3b inactivator) (Fujita et al., 1978; Gigli et al., 1979). Conversely, C1INH interferes with the formation of the C1 complex by inhibiting the action of the C1s and C1r proteases (Caliezi et al., 2000). Importantly, both mechanisms of complement resistance were shown to be regulated by the BvgASR system, emphasizing their importance during colonization of the host. FHA has been implicated in the binding and recruitment of C4BP by B. pertussis (Berggard et al., 1997). In contrast, the specific bacterial factor that interacts with C1INH has yet to be determined. The absence of FHA does not render B. pertussis completely susceptible to complement killing (Fernandez & Weiss, 1998), suggesting that other complement resistance mechanisms provide sufficient protection in the absence of C4BP recruitment.

In summary, the recruitment and binding of host C1INH and C4BP act in concert with the expression of BrkA to ensure inhibition of complement activation at a very early stage. Experiments with antibody-depleted serum suggested that the lectin and alternative pathways may be less relevant for B. pertussis compared with the classical pathway (Barnes & Weiss, 2001). This is supported by the identification of three distinct mechanisms to prevent activation of the classical pathway, which further highlights the important role of antibodies in the protection against B. pertussis.

Cellular immunity

During infection with B. pertussis, multiple PRRs engage bacterial molecules, leading to the induction of innate signaling pathways. Consequently, immune cells are recruited through the release of chemokines, and an adaptive response is initiated through cytokine production and the upregulation of costimulatory molecules. APCs, such as DCs and macrophages, are pivotal to this process. In response to PRR activation, these cells initiate the presentation of B. pertussis-derived antigens to cells of the adaptive immune system, with the aim to induce specific immunity. Insight into the development of T-cell immunity during infection with B. pertussis is of critical importance for the rational design of novel vaccines. In this section, we will focus on how infection with B. pertussis affects the differentiation of naive lymphocytes to specific T-cell subtypes with antibacterial effector functions. Furthermore, B. pertussis virulence factors that are known to modulate this process, in particular regarding the production of cytokines, will also be discussed.

T-cell responses to B. pertussis

Cellular immunity against B. pertussis is predominantly mediated by CD4+ T lymphocytes, whereas CD8+ T lymphocytes are mostly dispensable (Mills et al., 1993; Leef et al., 2000). Traditionally, CD4+ cells have been subdivided as T helper type 1 (Th1) or Th2 cells, based on their cytokine expression profile (Mosmann & Coffman, 1989; Liew, 2002). Th1 cells typically express interferon-γ (IFN-γ) and IL-2, and these cells play an important role in cell-mediated inflammatory responses (Mosmann et al., 1986). Conversely, Th2 cells are more important for the development of...
antibody responses and are characterized by the secretion of high levels of IL-4, IL-5, and IL-13 (Mosmann et al., 1986). More recently, a distinct subset of T helper cells has been discovered, designated Th17 cells (Park et al., 2005). Th17 cells typically express IL-17, IL-21, and IL-22, and have been implicated in host inflammatory responses against various bacterial pathogens (Ouyang et al., 2008).

The fate of T-cell differentiation is to a large extent determined by the local cytokine environment during priming, together with the antigen dose, antigen affinity, major histocompatibility complex haplotypes, and the costimulatory molecules present on DCs (reviewed in Liew, 2002).

High levels of IFN-γ and IL-12 typically promote expansion of Th1 cells, while simultaneously inhibiting Th2 cell proliferation. Conversely, high levels of IL-4 induce Th2 cell proliferation and suppress Th1 subtype expansion (Fernandez-Botran et al., 1988; Gajewski & Fitch, 1988). IL-1β, IL-6, and IL-23 have been implicated as mediators for the expansion of the Th17 subtype (Veldhoen et al., 2006; Acosta Rodriguez et al., 2007; Wilson et al., 2007). Another important cytokine in this respect is the anti-inflammatory cytokine IL-10. The induction of this cytokine has been shown to suppress the host immune response to certain pathogens, such as S. pneumoniae and other species (Saraiva & O’Garra, 2010).

Thus far, several studies have demonstrated a dominant role for antigen-specific IFN-γ secreting Th1 cells in the protection against B. pertussis (Peppoloni et al., 1991; Barbic et al., 1997; Mahon et al., 1997). Additionally, the generation of antigen-specific Th17 cells also correlates with protection, further supported by the finding that IL-17-deficient mice showed increased susceptibility to B. pertussis infections (Higgins et al., 2006; Dunne et al., 2010). Taken together, these findings suggest that the induction of a combined Th1 and Th17 response is essential to induce protective immunity against pertussis. Furthermore, although antibodies have also been shown to correlate with protection, it is currently unknown how Th2 cells contribute to the induction of these antibodies.

Suppression of T-cell responses

Several bacterial factors have been shown to induce cytokine production in APCs that are known to induce Th17 T cells. For instance, exposing MDDCs to Ptx has been shown to induce the production of the Th17 expansion-mediator cytokines IL-1β, IL-6, and IL-23 in a TLR2/4-dependent manner (Nasso et al., 2009). Other bacterial factors that drive Th17 induction are FHA and ACT (McGuirk et al., 2002; Spensieri et al., 2006), both of which have been shown to suppress a key inhibitor of Th17 expansion, IL-12 (Hoeve et al., 2006). FHA inhibits the secretion of IL-12 by lipopolysaccharide (and lipo-oligosaccharide)-stimulated macrophages through the upregulation of IL-6 and IL-10 (McGuirk & Mills, 2000). Furthermore, FHA may also inhibit IL-12 production by APCs through its cognate interaction with CR3, as it was shown that monoclonal antibody-mediated activation of CR3 also reduced IL-12 secretion (Marth & Kelsall, 1997). The mechanism by which ACT affects IL-12 production has also been characterized. Dependent on the adenylate cyclase activity, ACT inhibits the induction of IRF-1, IRF-8, and IFN-β, which are master regulators of IL-12 p35, one of the two subunits of IL-12 (Spensieri et al., 2006; Hickey et al., 2008). In addition, ACT potently induces the inflammasome of DCs (Dunne et al., 2010), a multiprotein complex involved in the initiation of the inflammatory response. Activation of the inflammasome results in the secretion of proinflammatory cytokines, including IL-1β (Sutterwala et al., 2006). Similarly, the pore-forming capacity of ACT induces a decrease in intracellular K⁺ levels, and this has been shown to activate caspase-1 and NALP3, resulting in an increased release of IL-1β and a Th17 response (Dunne et al., 2010).

Because the induction of a Th17 response has also been shown to be associated with protection, the bacterium uses another strategy to enable survival on the respiratory mucosal surface. Importantly, clinical studies have shown that high levels of IL-10 are clinically associated with an increased susceptibility to infections with B. pertussis, which is due to the IL-10-mediated suppression of IFN-γ (Dirix et al., 2009). Through the action of multiple virulence factors, B. pertussis is able to induce an IL-10 phenotype.

As discussed before, activation of TLR4 by lipo-oligosaccharide is essential for efficient recognition by DCs. As such, lipo-oligosaccharide represents one of the most important immunostimulating factors of B. pertussis (Fedele et al., 2007, 2008). In vitro stimulation of MDDCs with purified lipo-oligosaccharides resulted in a low expression of Th1 cytokines IFN-γ and IL-12, together with high amounts of the Th2 cytokines IL-5 and IL-10 and the Th17 cytokines IL-1β, IL-6, and IL-23 (Fedele et al., 2007, 2008). This suggests that the lipo-oligosaccharide, through its aberrant structure and lipid A modifications, may skew the cytokine production of DCs to a Th2/Th17 phenotype. Furthermore, ACT has been found to act synergistically with lipo-oligosaccharides in the induction of IL-6 and IL-10 (Ross et al., 2004). Importantly, FHA is also able to stimulate IL-10 expression by DCs, resulting in the induction of local IL-10-producing T cells (McGuirk et al., 2002).

In addition to these well-known virulence factors, the T3SS has also been implicated in suppression of both cell-mediated and humoral immune responses. Infection of mice with a mutant B. pertussis strain lacking a functional T3SS resulted in an increased proinflammatory cytokine response, as well as an increased IL-17, IFN-γ, and IgG.
response (Fennelly et al., 2008). BopN has recently been characterized as the T3SS secreted molecule that is responsible for these effects. In an MDDC infection model, it was shown that BopN induces the expression of IL-10 through the inhibition of both extracellular signal-regulated kinase and NF-κB pathways (Nagamatsu et al., 2009).

Thus, recognition of B. pertussis by APCs results in the induction of Th1/Th17-associated cytokines, which are protective. However, through the modulating effect of several virulence factors, IL-10 is also produced, reducing the effectiveness of the T-cell response. Considering the importance of costimulatory molecules in the presentation of antigens to T- and B-cells, it will be interesting to investigate the effects of the above-mentioned virulence factors on costimulatory molecule expression.

Conclusions and future perspectives

Despite the fact that pertussis morbidity and mortality have decreased drastically after vaccine introduction, pertussis remains one of the least well-controlled vaccine-preventable childhood diseases in the world. Bordetella pertussis is a highly monomorphic pathogen, suggesting that its gene repertoire is well-adapted to its present niche and that only fine-tuning is required for its persistence (Mooi, 2010). With this review, we aimed to clarify the mechanisms by which B. pertussis is able to persist in the face of vaccination. In particular, we discussed the interaction of B. pertussis with the host mucosal epithelium and immune system (Figs 1 and 2), and how this knowledge may be used to improve pertussis vaccines.

Recently vaccinated children are well-protected, indicating that B. pertussis cannot cope with an activated adaptive immune response. We propose that B. pertussis has evolved to delay the adaptive immune response, in particular the induction of antibodies, until transmission occurs. The implication is that immunity against B. pertussis can be maintained if sufficiently high levels of antibodies are present in a large part of the population. There is evidence of pertussis immunity in the absence of (high levels of) antibodies (Cassone et al., 2000; Mahon et al., 2000). This suggests that if memory is still robust, antibody induction can outpace immune suppression. Choice of antigens and ways to prevent decay of memory are crucial to the solution of the B. pertussis problem.

Adaptations may reveal weak spots in the bacterial defense, and B. pertussis has used different strategies to adapt to vaccinated populations, including antigenic divergence with vaccine strains and upregulation of Ptx production (He & Mertola, 2008; Mooi, 2010). Antigenic divergence has been observed in particular with respect to Prn and Ptx, and pertussis vaccines may be improved by including protein variants found in current B. pertussis populations. This may improve both the effectiveness of antibodies and memory. Increased Ptx production confers an advantage in populations where transmission of B. pertussis is mainly dependent on primed (vaccinated) hosts (Mooi et al., 2009) and this adaptation can be neutralized by ‘adapting’ vaccines so that they induce Ptx-neutralizing antibodies that persist longer. One way this can be accomplished is by replacing chemically detoxified Ptx with the genetically detoxified variant, which has been shown to be more immunogenic (Peppoloni et al., 1995; Robbins et al., 2005). Higher initial titers of Ptx antibodies may increase the period in which protective antibody levels are maintained. Additional reasons to replace chemically detoxified Ptx are that formaldehyde treatment of Ptx has been shown to constrain antigen presentation to T cells, and that chemical detoxification has a significant effect on protein structure and may destroy protective epitopes (Di Tommaso et al., 1994). Both may affect immunological memory.

Significantly, Ptx is the only known B. pertussis virulence factor showing polymorphisms in both structure and regulation, underlining the importance of this protein in the ecology of B. pertussis. Ptx-induced leukocytosis has been associated with mortality (Pierce et al., 2000; Paddock et al., 2008) and it is difficult to envisage a pertussis vaccine without this toxin. As more genome sequences become available, comparative genomics can be exploited to identify genetic changes that are associated with the introduction of vaccination. This might reveal novel genes that are important for the suppression of host immune responses, and the associated gene products may represent potential vaccine candidates.

A number of toxins shown to be crucial for infection are promising vaccine candidates. Antibodies against these toxins may target the pathogen directly, through effector functions, and indirectly, through toxin neutralization. Promising toxin vaccine candidates include ACT, lipooligosaccharide, TCT, and T3SS toxins. ACT functions as an adjuvant, enhancing both Th1 and Th2 responses as well as protective antibody responses (Cheung et al., 2006). Lipooligosaccharide also possesses powerful adjuvant activity, but requires detoxification before being used as a vaccine component (Geurtsen et al., 2008). TCT could be made immunogenic by conjugation. The T3SS and its effector proteins have been tested in animal models as vaccines (reviewed in Abe et al., 2008). It is doubtful whether antibodies against effector molecules will be very effective, as these toxins are directly injected into host cells. However, structural components of the T3SS have been shown to induce protective immunity against Pseudomonas aeruginosa in a mouse infection model (Sawa et al., 1999). Medhekar et al. (2009) recently showed that the B. pertussis T3SS protein, Bsp22, which forms a filamentous T3SS tip complex...
on the cell surface, is highly immunogenic in mice. Furthermore, Bsp22 antibodies were found to be protective against *B. pertussis* infection.

Antibodies against antigens expressed early in infection may be particularly effective, especially if they prevent manipulation of the (subsequent) host immune response. Such proteins can be identified by applying currently available genome-wide approaches such as transcriptional profiling, proteomics, or elegant *in vivo* technologies (Veal-Carr & Stibitz, 2005). These techniques can also be used to identify proteins important for biofilm formation. Although this field of research is largely unexplored for *B. pertussis*, the approach of targeting specific bacterial biofilm factors with vaccines has already found proof of principle for other human pathogens including staphylococci, *Escherichia coli*, and the fungus *Cryptococcus neoformans* (Lewis, 2001; Martinez & Casadevall, 2005; Cerca et al., 2007; Visal et al., 2007). The effectiveness of such vaccines may rely on their ability to suppress the activity of biofilm-stimulating factors and thereby prevent this process at the early stages of infection. *B. pertussis* also forms biofilms in the nose, trachea, and lungs (Conover et al., 2010), and it is known that biofilms are inherently more resistant towards host immune defenses (reviewed in Lewis, 2001). This suggests that the inclusion of biofilm antigens in novel vaccines, likely in combination with vaccine antigens that target essential processes of the pathogenesis of *B. pertussis*, may provide an additional level of protection compared with currently used vaccines.

Finally, a promising approach to induce protective immunity in infants is to mimic natural infection, using a live attenuated *B. pertussis* strain. Vaccination with an attenuated strain was shown to provide superior protection compared with an ACV in a mouse model. Interestingly, the nasal route of vaccination was also shown to protect against *B. parapertussis*, which is another highly desired feature of pertussis vaccines (Mielcarek et al., 2006).

There clearly are plenty of scientifically valid rationales to improve current pertussis vaccines. However, the major hurdle to this is not a scientific one, but an economic one. Most commercial vaccine producers have little incentive to improve pertussis vaccines, as they have made significant investments in the new generation of ACVs. It can therefore be expected that vaccine producers promote the strategy of repetitive boostering. The development and testing of the next-generation pertussis vaccines may require incentives from both commercial vaccine producers and governments.

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**References**


Cherry JD & Heininger U (2004) Pertussis and other *Bordetella* infections. *Textbook of Pediatric Infectious Diseases* (Feigin RD,
Modulation of the host immune response by *B. pertussis*


Eichelberg K, Ginocchio CC & Galan JE (1994) Molecular and functional characterization of the *Salmonella typhimurium*...


McWilliam AS, Napoli S, Marsh AM, Pemper FL, Nelso DJ, Pimm CL, Stumbles PA, Wells TN & Holt PG (1996) Dendritic cells are recruited into the airway epithelium during the


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