INTERNATL MICROBIOL (1999) 2:137–144

REVIEW ARTICLE

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Molecular aspects of *Bordetella pertussis* pathogenesis

Received 3 April 1999
Accepted 10 May 1999

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Summary The molecular mechanisms of *Bordetella* virulence are now well understood, and many virulence factors have been identified and characterized at the molecular level. These virulence factors can be grouped into two major categories: adhesins, such as filamentous hemagglutinin, pertactin and fimbriae, and toxins, such as pertussis toxin, adenylate cyclase, dermonecrotic toxin and tracheal cytotoxin. The production of most virulence factors is coordinately regulated by a two-component signal transduction system composed of the regulator BvgA and the sensor protein BvgS. The adhesins and toxins act in concert to establish infection. Some adhesins exert their effects synergically or are redundant functioning only in the absence of another adhesin, illustrating the importance of adhesion in infection. Most virulence factors are secreted into the culture supernatant or exposed at the surface of the bacterial cell. A notable exception is dermonecrotic toxin, which remains in the cytoplasmic compartment of bacterial cells. Most virulence factors are produced by all of the three major *Bordetella* species, *B. pertussis*, *B. parapertussis* and *B. bronchiseptica*. However, some, such as pertussis toxin and the tracheal colonization factor, are only produced by *B. pertussis*. Our understanding of *Bordetella* virulence at the molecular level has led to the development of new acellular vaccines against whooping cough, and of genetically attenuated *B. pertussis* strains to be used as recombinant live bacterial vaccine vectors for homologous and heterologous protection.

Key words Whooping cough · Adhesins · Toxins · Two-component systems · ADP-ribosyltransferase

Introduction

The genus *Bordetella* contains several pathogenic species generally associated with upper respiratory tract infections in warm-blooded animals. *Bordetella pertussis* is the etiologic agent of whooping cough, a disease that still causes approximately 300,000 deaths worldwide and is currently considered to be reemerging, even in the industrialized world. *Bordetella parapertussis* causes a mild whooping cough-like syndrome, whereas *Bordetella bronchiseptica* is isolated rarely from humans, essentially only from severely immunocompromised individuals. *B. pertussis* and *B. parapertussis* are typical human pathogens, although *B. parapertussis* has also been found in sheep [48]. In contrast, *B. bronchiseptica* is found in many mammalian species, in which it generally remains pathogenically silent. However, it is known to cause kennel cough in dogs and atrophic rhinitis in pigs. These three *Bordetella* species have very similar DNA sequences, although the chromosomes of *B. parapertussis* and *B. bronchiseptica* are approximately 1 Mb larger than that of *B. pertussis* [59]. The more distantly related *Bordetella avium* causes respiratory tract infections in birds. This microorganism appears to be more closely related to two other *Bordetella* species recently isolated from humans, *Bordetella hinzii* [11] and *Bordetella holmesii* [67].

Our understanding of the molecular mechanisms of *Bordetella* pathogenesis is increasing, especially for *B. pertussis*. Many of its virulence factors have been identified, their genes cloned and sequenced and their structure-function relationship, biogenesis and genetic regulation intensively studied in many different laboratories around the world. Since its discovery in 1906 by Bordet and Gengou [5], *B. pertussis* has classically been considered an extracellular pathogen. However, during the past decade it has been realized that it can also survive within a variety of eukaryotic cell types [13], including macrophages and epithelial cells. The physiopathological significance of the intracellular survival of *B. pertussis* has not yet been clearly determined.

The genetic regulation of *B. pertussis* virulence genes

Phenotypic modulation and phase variation *B. pertussis* virulence factors are typically divided into two main categories: adhesins and toxins. Most are proteins, and the expression of their structural genes is coordinately regulated at the transcriptional level in response to environmental signals. The virulence genes are not expressed at low temperature or in the presence of $MgSO_4$ or nicotinic acid, whereas they are expressed at 37°C and in the absence of $MgSO_4$ and nicotinic acid. This reversible physiological adaptation of *B. pertussis* has been termed phenotypic modulation [30]. However, within a population of *Bordetella* cells, irreversible loss of virulence factor production, even at 37°C and in the absence of $MgSO_4$ and nicotinic acid also occasionally occurs, and has been termed phase variation [62].

The BvgA/S two-component system The genetic locus responsible for both phenotypic modulation and phase variation is the *bvg* locus, consisting of at least two closely linked genes, *bvgA* and *bvgS* [62]. These genes encode the constituents of a two-component regulatory system. BvgA is a typical transcriptional activator, containing an N-terminal receiver domain and a C-terminal output domain with a helix-turn-helix motif capable of binding its DNA target sequences. BvgS is a sensor protein located in the inner membrane of the organism. It contains a large periplasmic domain, which is connected to a cytoplasmic domain by a membrane-spanning and a linker region. Within the large family of two-component sensor proteins, BvgS is unusual in having a particularly large cytoplasmic region containing several distinguishable subdomains. In addition to the usual transmitter module with a conserved histidine residue and an ATP-binding site, it contains a receiver module and a C-terminal subdomain with a second conserved histidine residue of transmitter domains and a second ATP-binding site.

Signal transduction by the BvgA/S system occurs via a succession of phosphotransfer reactions. At high temperature and in the absence of $MgSO_4$ and nicotinic acid, BvgS autophosphorylates at the first conserved histidine residue within the transmitter domain, using ATP as a phosphodonor. The phosphate is then transferred from this histidine to an aspartate from the receiver module within the cytoplasmic domain of BvgS, and finally from this aspartate to the second conserved histidine in the C-terminal subdomain of BvgS. From this C-terminal subdomain, the phosphate group is then finally transferred to the N-terminal receiver domain of BvgA. Phosphorylation of BvgA increases the affinity of its output domain for the target DNA sequences located within the promoter regions of the virulence genes [8], which activates the transcription of these genes. Interestingly, as found for many other two-component systems, amplification of the signal occurs via transcriptional auto-activation of the *bvgA*/*S* genes by phosphorylated BvgA itself.

The *vag* and *vrg* genes The BvgA/S system not only activates a set of virulence genes, called *vags* (for virulence activated genes) but may also repress the expression of another set of genes, the *vrgs* (for virulence repressed genes). The repression of at least some of these genes requires the expression of an intermediate regulatory gene, *bvgR*, located directly downstream from the *bvgA* and *bvgS* genes [42]. The expression of *bvgR* is under the transcriptional control of BvgA. In the virulent phase, when the BvgA/S system is activated, both *vags* and *bvgR* are expressed, which in turn represses the *vrgs*. In modulating conditions, (i.e. at 25°C or in the presence of $MgSO_4$ or nicotinic acid), the BvgA/S system is inactivated, *vags* are not expressed and *vrgs* are derepressed. The functional role of the *vrgs* in *B. pertussis* is unknown. However, ectopic expression of these genes results in a defect in tracheal colonization, at least in laboratory animal models [1].

Adhesins

Of the various virulence factors *B. pertussis* produces under the control of the BvgA/S system, a number of adhesins are found at the surface of the organism, several of which have been studied in some detail (Table 1). These include filamentous hemagglutinin, fimbriae, also called agglutinogens, pertactin, the serum-resistance protein Brk and the attachment factor Tcf.

Filamentous hemagglutinin The best studied *B. pertussis* adhesin is filamentous hemagglutinin (FHA). It is produced as a 370 kDa precursor encoded by the *fhaB* gene, which is located directly upstream from the *bvg* genes [39]. The FhaB precursor undergoes N-terminal and C-terminal proteolytic processing, resulting in the 220 kDa mature FHA [54]. The structural model of mature FHA, based on high-resolution electron microscopy and circular dichroism measurements, as well as secondary structure predictions, is a filamentous monomeric molecule approximately 50 nm long and folded like a hairpin [40]. It has a rod-like shape with a diameter of 4 nm, and contains at one extremity a globular structure formed by the N-terminal and C-terminal ends of the molecule. It has been suggested that 19-residue repeated motifs form an elongated amphiphilic β-sheet structure, joining the N-terminal and C-terminal moieties of the molecule by their hydrophobic interfaces to form the rod-like structure.

The N-terminal region of the precursor molecule contains an unorthodox signal peptide [27], which is unusual in that it is 71 residues long and consists of two parts. The first part, the N-terminal extension, is homologous to the N-terminal extremities of several other adhesin precursors from Gram-

Table 1 *Bordetella* virulence factors

	Produced by		
	B. pertussis	*B. parapertussis*	*B. bronchiseptica*
Adhesins			
Filamentous hemagglutinin	+	+	+
Pertactin	+ (69 kDa)	+ (70 kDa)	+ (68 kDa)
Fimbriae[a]	+	+	+
Tracheal colonization factor	+	–	–
Serum-resistance protein (Brk)[b]	+	?	?
Toxins			
Pertussis toxin	+	–	–
Adenylate cyclase toxin	+	+	+
Dermonecrotic toxin	+	+	+
Tracheal cytotoxin	+	+	+

[a]In addition to the major Fim2 and Fim3 subunits, and the minor subunit FimD, *B. parapertussis* and *B. bronchiseptica* produce an additional fimbrial subunit, FimA.
[b]The *brk* genes are present in *B. parapertussis* and *B. bronchiseptica*, but it is not known whether they are expressed in these two species.

negative organisms. Its function is unknown. It is followed by a sequence with the features of a typical signal peptide, suggesting that the FHA precursor is transported through the cytoplasmic membrane by a classical Sec system. The N-terminal residue of mature FHA is pyroglutamate, a modified glutamine residue, that has undergone posttranslational cyclization by an unknown mechanism [32].

The N-proximal region of mature FHA is homologous to a domain found in various hemolysins and other adhesins from Gram-negative pathogens. This approximately 120 residue domain is essential for the translocation of FHA through the outer membrane. It contains very well conserved AN(P/S)NL and NPNGI motifs, in which the asparagines play a particularly important, though not yet defined role in FHA export [28].

The secretion of FHA requires an accessory protein located in the outer membrane. This protein, FhaC, is homologous to the accessory proteins involved in the secretion of the hemolysins and adhesins of other microorganisms containing an N-proximal domain homologous to FHA. It is likely that the conserved domains of these proteins interact with the FhaC-like accessory proteins during the course of transport through the outer membrane. The *fhaC* gene lies downstream from the *fhaB* gene, and the two genes are separated by three genes involved in *B. pertussis* fimbrial biogenesis [69], indicating that the production of FHA is closely coregulated with that of the fimbriae.

The secretion domain of FHA is followed by several repeats. These repeats may be involved in the binding of FHA to sulfated glycosaminoglycans (ligands for FHA) via which *B. pertussis* may bind to epithelial cells [22]. In addition, an Arg-Gly-Asp motif is located downstream from these repeats and has been shown to be involved in the interaction of FHA with the CR3 integrin on the surface of macrophages [53]. The hairpin structure model suggest that the Arg-Gly-Asp sequence should be located at one of the extremities of the rod. Finally, a third region has been identified in FHA, downstream from the Arg-Gly-Asp sequence. This third region may be responsible for the adhesion of FHA to galactose-containing glyco-conjugates [50]. This region is also the immunodominant part of the molecule [34].

The C-terminal portion of the FHA precursor is removed during processing. Its role has not been clearly identified, but it appears to be important for the secretion of full-length FHA, but not for truncated FHA molecules, provided they contain the secretion domain. It probably does not itself contain secretion determinants, but has been suggested to act as an intramolecular chaperone to prevent the premature folding of FHA in the periplasm, which would hamper its transport through the outer membrane [54].

FHA has long been considered to be the major *B. pertussis* adhesin. However, it functions in conjunction with other adhesins, such as the fimbriae, pertactin and even pertussis toxin. Some of these adhesins may even substitute for FHA or may be redundant, especially in strains lacking FHA. During infection, in both humans and animal models, FHA induces a strong antibody response, both systemically and mucosally [61], and vaccination with purified FHA confers protection against respiratory challenge in mice. For this reason, FHA has been included in new, second-generation vaccines against whooping cough [56]. FHA also has adjuvant activity for antigens delivered with it by the nasal route [49]. These properties can be exploited to develop systems for presenting heterologous antigens to the respiratory mucosa [55].

The fimbriae *B. pertussis* produces fimbriae of two serotypes, serotype 2 and serotype 3, composed of the major fimbrial subunits Fim2 (22.5 kDa) and Fim3 (22 kDa) respectively. The *B. pertussis* genome also harbors a homologous *fimX* gene, the product of which has not been identified. In addition to the major subunits, the fimbriae also contain the minor 40 kDa subunit FimD, which serves as the adhesin [68]. Expression of

the *fim2* and *fim3* genes is regulated by mutations in their promoter regions, which result in serotype switching [70]. In particular, a C-rich region may undergo relatively frequent deletions and insertions. These changes strongly affect the transcription of the fimbrial genes. As a result, some strains produce both types of fimbria (serotype 2, 3), some only one (serotype 2 or 3), or none at all.

The *B. pertussis* fimbriae belong to the same family as the type 1 fimbriae of enterobacteria. Their assembly requires a periplasmic chaperone, FimB and an outer-membrane usher protein, FimC. The *fim2*, *fim3* and *fimX* genes are scattered along the *B. pertussis* chromosome, whereas the *fimBCD* genes are clustered together with the *fha* and *bvg* genes and constitute a polycistronic operon with *fhaC* [69]. A pseudogene, *fimA*, is located immediately upstream from *fimB* and may be an ancestral major structural fimbrial gene from which *fim2*, *fim3* and *fimX* may have been derived after initial duplication. Interestingly, *fimA* is truncated in *B. pertussis*, whereas it is complete in *B. bronchiseptica* and *B. parapertussis* [7].

Like FHA, the major fimbrial subunit can also bind sulfated glycoconjugates present on the surface of epithelial cells [16]. The minor subunit FimD binds to the VLA-5 integrin of macrophages. The binding of *B. pertussis* to VLA-5 upregulates CR3 integrin expression, thereby increasing the binding of the organism via FHA to the macrophages [23]. Both the fimbriae and FHA may thus cooperate in *B. pertussis* adhesion to, and perhaps invasion of, alveolar macrophages. Studies in animal models with defined *B. pertussis* mutants also suggest that FHA and fimbriae cooperate in the colonization of the trachea, which provides some biological significance for the tightly coordinated expression of these two adhesins.

Many years ago, it was observed that anti-fimbrial antibodies in the sera of infected or vaccinated children agglutinate the bacteria. Therefore, the fimbriae were initially called agglutinogens. In addition, a correlation was found between the presence of agglutinating antibodies and protection [51], which has led to the inclusion of Fim2 and Fim3 as antigens in some of the new acellular pertussis vaccines.

Pertactin Pertactin is an outer-membrane protein with an apparent molecular weight of 69,000. The structural gene, *prn*, encodes a protein of 94 kDa which is transported into the periplasm by a classical signal-peptide dependant secretion mechanism [9]. It then undergoes proteolytic processing resulting in the removal of an approximately 30 kDa C-terminal region. This C-terminal region remains anchored in the outer membrane and is essential for the secretion of pertactin. It resembles the C-terminal domains of the precursors of several secreted proteins from Gram-negative bacteria that are known to be autotransporters. The best studied example of this family of proteins is the *Neisseria* IgA protease [25]. The C-terminal domains of such proteins are predicted to contain 14 amphipathic β-strands, which may form a β-barrel within the outer membrane through which the N-terminal moieties of the proteins may then extrude. The secretion of these proteins does not therefore require additional specific accessory proteins. In the case of the IgA protease, the C-terminal domain of the precursor is cleaved by the autocatalytic activity of the IgA protease at a precise site resembling the proteolytic cleavage site within the IgA substrate. However, there is no evidence that pertactin has protease activity, which suggests that the processing of pertactin involves other proteases or that pertactin is not cleaved in physiological conditions.

The crystal structure of pertactin has been determined at 2.5 Å resolution [12]. The protein is a monomer and is composed of 16 parallel strands that form a β-helix with a V-shaped cross-section. Several coils protrude from the β-helix core. These coils have been associated with the biological activities of pertactin. An Arg-Gly-Asp sequence involved in the binding of pertactin to host cells, in particular to integrins on the surface of epithelial cells, is located adjacent to one of these coils containing proline-rich repeats $(GGXXP)_5$. The C-terminal region of pertactin contains other proline-rich repeats $(PQP)_5$, such repeats being major protective epitopes of the protein.

Pertactin is polymorphic between the various *Bordetella* species, and even between different strains within the same species [44]. *B. parapertussis* and *B. bronchiseptica* produce pertactins with slightly different molecular weights, 70 and 68 kDa, respectively [35]. These differences are mainly attributed to differences in the copy numbers of the proline-rich repeats.

Pertactin was initially discovered during the development of a veterinary vaccine against *B. bronchiseptica* [46] and has been shown to provide protection in animal models. Mutants deficient in pertactin appear to colonize the mouse respiratory tract as efficiently as the wild-type parent strains. However, mutants deficient in both pertactin and FHA are cleared much more rapidly than the wild-type parents, supporting the notion that pertactin is involved in bacterial adhesion. Due to its involvement in adhesion and its protective effect in animal models, pertactin is also one of the antigens included in some of the new acellular vaccines [20].

The *Bordetella* serum-resistance protein Brk Brk (for *Bordetella* resistance to killing) is responsible for the resistance of *Bordetella* to killing by serum [14]. The resistance mechanism is unknown. However, Brk presents several features that suggest it is involved in adhesion to host cells. It contains two Arg-Gly-Asp motifs and two potential binding sites for sulfated glycoconjugates. Mutant strains that lack Brk are impaired in binding to eukaryotic cells in vitro and, in murine models, are less virulent than wild-type parent strains.

Analyses of the primary structure of the protein suggest that Brk is derived from a larger, 103 kDa, precursor. Like pertactin, the Brk precursor contains an approximately 300 residue C-terminal autotranslocator domain with outer-membrane localization signals. This C-terminal putative translocator domain has 54.5% of residues identical to those

in pertactin. It is thus likely that Brk and pertactin use a similar secretion mechanism.

The tracheal colonization factor A third protein with a structure similar to pertactin is the tracheal colonization factor (Tcf). This protein is produced by *B. pertussis*, but not by *B. parapertussis* and *B. bronchiseptica*, and appears to be specifically involved in the colonization of the trachea [15]. Its sequence shows several proline-rich regions and, like FHA, pertactin and Brk, Tcf also contains an Arg-Gly-Asp sequence. Also, like pertactin and Brk, the C-terminal end of Tcf is probably an autotransporter domain with a high degree of sequence similarity to those of pertactin and Brk, again suggesting a similar secretion mechanism. Tcf is encountered in two forms, either as a large, cell-associated form, or as a small, secreted form.

Toxins

Most *B. pertussis* toxins are protein toxins (see Table 1), except for lipopolysaccharide, also called endotoxin, and the tracheal cytotoxin, which is a fragment of the *Bordetella* peptidoglycan. The three major protein toxins are the pertussis toxin, the adenylate cyclase toxin and the dermonecrotic toxin.

Pertussis toxin Pertussis toxin (PTX) consists of five dissimilar subunits, named S1 through S5, according to their decreasing molecular weights, and are arranged in a 1S1:1S2:1S3:2S4:1S5 stoichiometry [60]. The S2 to S5 subunits form the B moiety of the toxin, which is responsible for the interaction of the toxin with the target cell receptors. The S1 subunit, also called the A protomer, has enzymatic activity that is responsible for most, if not all, of the biological activities. PTX is therefore a member of the A-B bacterial protein toxin group.

S1 has ADP-ribosyltransferase activity using NAD as ADP-ribosyl donor and signal transduction, G proteins as ADP-ribosyl acceptors. The molecular action of the toxin can be divided into three major steps, (i) binding of the toxin to target cell receptors via the B oligomer, (ii) membrane translocation of the S1 subunit into the cytosol, and (iii) ADP-ribosyltransferase activity by the translocated S1 subunit. The ADP-ribosylation of the acceptor substrate G proteins, essentially of the G_i and G_o families, results in their inactivation, and signals that normally regulate intracellular processes, such as the formation of cAMP, can no longer exert their regulatory actions. As a consequence, PTX poisoning results in dysregulation of major metabolic pathways, although the toxin does not usually kill the target cells. Depending on the cell type, the toxin may have a variety of physiological effects, such as histamine sensitization, activation of insulin secretion, or lymphocytosis, one of the hallmarks of the whooping cough syndrome [45]. However, this toxin is probably not directly involved in the typical cough.

The enzymatic mechanism of PTX has been extensively studied and the catalytic residues have been identified. Glu-129 [3] is a key catalytic residue strictly conserved among all ADP-ribosylating toxins and enzymes. The carboxyl group of this residue interacts with the 2'-OH group of the ribose on NAD [57], which may increase the ionization potential of the diol, thereby weakening the *N*-glycoside bond between the ribose and nicotinamide [37]. This bond can then be cleaved by the attacking nucleophile, a cysteine in the G protein. A second catalytic residue, His-35 is thought to activate the cysteine to increase its nucleophilicity [2]. This residue is less well conserved among ADP-ribosyltransferases, consistent with the heterogeneity of acceptor substrates used by the various ADP-ribosylating toxins.

The crystal structure of pertussis toxin, defined at a 3.5 Å resolution, shows that the catalytic Glu-129 and His-35 are located next to each other, close to the active site into which NAD can be modeled [58]. The holotoxin is a pyramid, with its triangular base formed by the B oligomer. The S1 subunit is located at the top of the pyramid and inserts its C-terminal end into the central hole formed by the juxtaposition of the B oligomer subunits. The triangular base is formed by one S2-S4 dimer, one S3-S4 dimer and one connecting S5 subunit. S2 and S3 share approximately 70% identical residues [38], but have different receptor-binding specificities [36].

All the subunits are exported individually through the inner membrane, using classical signal peptides. After the removal of these signal peptides, the subunits assemble into the holotoxin structure within the periplasm, and the fully assembled toxin is secreted into the culture medium of *B. pertussis*. This final step in toxin secretion requires 9 accessory proteins encoded by the *ptl* genes. These proteins are homologous to the *Agrobacterium tumefaciens* plasmid transport machinery encoded by the *virB* genes [66]. The *Bordetella ptl* genes are located immediately downstream from the five structural *ptx* genes. They are cotranscribed with the *ptx* genes, in a single polycistronic operon. The full set of *ptx* and *ptl* genes is also present in *B. bronchiseptica* and *B. parapertussis* [41]. However, the genes are not expressed in these two species due to an accumulation of inactivating point mutations, mostly in the promoter region.

PTX induces high levels of antibody after infection or vaccination with classical whole cell vaccine [61], and the toxin provides full protection against challenge in mouse models. For this reason, inactivated PTX is the major protective antigen in all the new acellular vaccines available today.

Adenylate cyclase/hemolysin toxin Adenylate cyclase/hemolysin is a bifunctional protein composed of 1,706 amino acid residues [18]. This toxin is produced by *B. pertussis*, *B. parapertussis* and *B. bronchiseptica*. Via its hemolytic domain, the toxin delivers its catalytic cyclase domain into

the cytosol of the target cell. The catalytic domain then expresses adenylate cyclase activity upon activation by calmodulin [31]. The catalytic domain is located in the N-terminal half of the toxin. The C-terminal half contains multiple GGXGXDXLX repeats making this toxin a member of the RTX (for repeats in toxin) toxin family. These repeats may be the site of binding to calcium, an ion essential for toxin activity [18].

A variety of biochemical and molecular biological studies have identified several residues, including Trp-242, involved in the interaction with calmodulin. In addition, Lys-58 and Lys-65 have been implicated in the catalytic activity of the enzyme [17].

Unlike the other *Bordetella* virulence factors, adenylate cyclase is secreted without undergoing proteolytic processing. Its secretion depends upon three accessory proteins, CyaB, CyaD and CyaE, the genes of which are located immediately downstream from the cyclase structural gene [19]. These three proteins are homologous to the accessory proteins essential for the secretion of other RTX toxins, such as the alpha-hemolysin of *Escherichia coli*. Together, the three proteins form the secretion apparatus, which belongs to the class of ABC transporters (for ATP-Binding Cassettes) because ATP binding is required for secretion. The secretion determinant of the RTX toxins usually lies within the C-terminal end of the protein and is not removed upon secretion.

The adenylate cyclase structural gene is preceded by the *cyaC* gene, which is transcribed in the opposite direction from the structural gene [4]. The *cyaC* gene product is a protein necessary for activation of the toxin. It catalyzes the transfer of a palmitoyl group onto the ε nitrogen of lysine 983 [21]. This palmitoylation is essential for the toxin and hemolytic activities of adenylate cyclase/hemolysin.

B. pertussis mutants that do not produce adenylate cyclase colonize the respiratory tract of infected mice much less than do the wild-type strains [65]. The toxin has been shown to be responsible for cell death by apoptosis of the alveolar macrophages in contact with *B. pertussis*, which may be a major way that the organism has evolved to escape the immune system of the infected host. This may enable it to colonize the host efficiently. Both enzyme and the hemolytic activities are required for the induction of apoptosis [29].

Dermonecrotic toxin The dermonecrotic toxin (DNT) was one of the first *B. pertussis* toxins to be identified and studied, but much less is known about this toxin than about the others. This may be due to the absence of a dramatic effect on pathogenesis in experimental animal models [63]. However, the toxin is lethal if injected intravenously into mice, even at low doses. If injected subcutaneously, it induces skin lesions. The absence of dramatic effects of the toxin in infection experiments, comparing isogenic *dnt*$^{+/-}$ strains, may be due to the intracellular location of the toxin. Unlike the other *Bordetella* toxins, DNT is not secreted and was therefore initially called endotoxin [6]. Its action in vivo, if any, may require the lysis of bacterial cells. Alternatively, the toxin may be secreted at very specific anatomic sites, in a tightly regulated manner by a mechanism that has not been identified yet.

The toxin has a molecular weight of approximately 140,000 and is produced by all three major *Bordetella* species. Its molecular action has recently been elucidated. The toxin deaminates the glutamine 63 residue of the RhoA protein and changes this glutamine into glutamate. This modification alters the electrophoretic mobility of the RhoA protein and decreases its GTPase activity by a factor of 10. This decrease in GTPase activity blocks RhoA in a permanently active position, thereby inducing the formation of actin stress fibers, which are readily detected if the host cells are incubated with DNT [26].

Tracheal cytotoxin The primary structure of tracheal cytotoxin (TCT) has been determined by fast atom bombardment mass spectrometry as a 921 Da *N*-acetyl-glucosaminyl-1,6-anhydro-*N*-acetylmuramylalanyl-gamma-glutamyldiaminopimelylalanine, identical to the *Neisseria gonorrhoeae* ciliostatic anhydropeptidoglycan and the slow-wave sleep-promoting factor [10]. It destroys ciliated epithelial cells of the respiratory tract, causes an increase in the number of cells with sparse ciliation and the extrusion of cells from the epithelial surface. In vitro, TCT inhibits DNA synthesis in tracheal epithelial cells and induces the production of intracellular interleukin-1 and of nitric oxide, the most likely triggers of TCT-mediated cytopathy [24]. The alanyl-gamma-glutamyldiaminopimelate moiety appears to be the smallest derivative of TCT with biological activity. However, the alanyl group may be replaced by other amino acids or blocking groups. TCT is also produced by the other *Bordetella* species, suggesting that it may be of general importance in bordetellosis. TCT production is not under the control of the BvgA/S regulatory system.

Lipopolysaccharide The *B. pertussis* lipopolysaccharide (LPS) may act in synergy with other toxins, such as TCT or pertussis toxin. By itself, it has endotoxin activities, similar to those of LPS from enteric bacteria. It is lethal in galactosamine-sensitized mice, and is pyrogenic and mitogenic in spleen cell cultures. It activates macrophages and induces the production of tumor necrosis factor [64]. Although all *Bordetella* species produce LPS, its structure differs between species, possibly reflecting differences in the strength of biological activity between *Bordetella* species. *B. pertussis* contains two LPS classes, LPSI and LPSII [33], which differ in their polysaccharide moieties. The ratio of LPSI to LPSII may differ between variants of the same strain [52]. However, the precise role in pathogenesis of these structural differences is not clear.

Concluding remarks

Our understanding of the molecular mechanisms of *B. pertussis* pathogenesis has increased over the past 15 years, since the cloning of its first virulence gene [38]. This has led to major improvements in whooping cough vaccines. The original whole cell vaccines may now be replaced by much less reactogenic acellular vaccines. These are based on the use of very well defined and properly inactivated virulence determinants from *B. pertussis*. These virulence factors are also the major protective antigens. PTX inactivation has been achieved by chemical treatment of the protein with formaldehyde or gluteraldehyde [56], and more elegantly, by genetic inactivation using site-directed alterations of key amino acid residues in the active site of the enzymatic subunit S1 [47] and/or of residues of the B oligomer involved in receptor binding [36].

In addition, attenuated *B. pertussis* has been proposed for use as a live vaccine that can be administered by the intranasal route [43]. Interestingly, and in contrast to most other live bacterial vaccines, genetic attenuation by alteration of the *ptx* genes does not decrease the immunogenicity of the organism, but in fact appears to increase immunogenicity over that of the wild-type strain. This makes attenuated *B. pertussis* particularly well suited to intranasal administration as a single dose mucosal vaccine to protect against whooping cough. In addition, taking advantage of what is known about the molecular mechanism of FHA secretion, attenuated *B. pertussis* can be engineered such that it produces and secretes heterologous protective antigens and can therefore be used to protect simultaneously against pertussis and other infectious diseases.

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