

# Virulence factors of *Bordetella pertussis*

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***Bordetella pertussis* is an aerobic, non-spore-forming Gram-negative coccobacillus that colonizes the respiratory tract of humans, causing whooping cough. It produces a number of virulence factors, which include the pertussis toxin, adenylate cyclase toxin, filamentous haemagglutinin, fimbriae, tracheal cytotoxin, pertactin and dermonecrotic toxin. The *bvg* locus comprising BvgA and BvgS, transcriptionally regulates the expression of these factors and the genes of the Type-III secretory apparatus in response to certain environmental stimuli. Altogether, *B. pertussis* has served as a complete organism exhibiting several disease-causing aspects of other bacterial pathogens and a model system to demonstrate the prowess of the various molecular biology tools used to elucidate its complex mode of pathogenicity.**

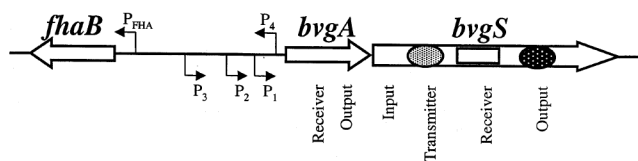
THE genus *Bordetella* in the family *Alcaligenaceae* comprises five species, four of which cause infections of the upper respiratory tract in different host organisms. *Bordetella pertussis* is an obligate human pathogen and is the etiological agent of whooping cough (pertussis), an acute respiratory infection more serious among infants. Annually, more than 50 million cases of pertussis, including 600,000 deaths are reported worldwide<sup>1</sup>. *Bordetella parapertussis* causes a mild pertussis-like disease in humans and is very closely related to *B. pertussis*. An animal pathogen of the *Bordetella* species is *Bordetella bronchiseptica*, a flagellated bacterium that rarely infects humans but can result in pneumonia occasionally accompanied by bacteremia<sup>1,2</sup>. *Bordetella avium* is a pathogen of poultry. *Bordetella hinzii*, which was only recently recognized as a member of the genus *Bordetella*, is frequently associated with poultry, but does not necessarily cause disease<sup>3</sup>.

It is believed that the transmission of *B. pertussis* occurs via aerosol droplets expelled by severe coughing that pass directly from the respiratory tracts of infected individuals to those of susceptible hosts, who inhale the aerosolic bacteria<sup>2</sup>. No animal reservoir for *B. pertussis* has been identified and the bacterium appears unable to survive in the environment for long periods of time<sup>4</sup>. The clinical course of whooping cough is divided into three stages: (i) the initial *catarrhal* stage marked by an

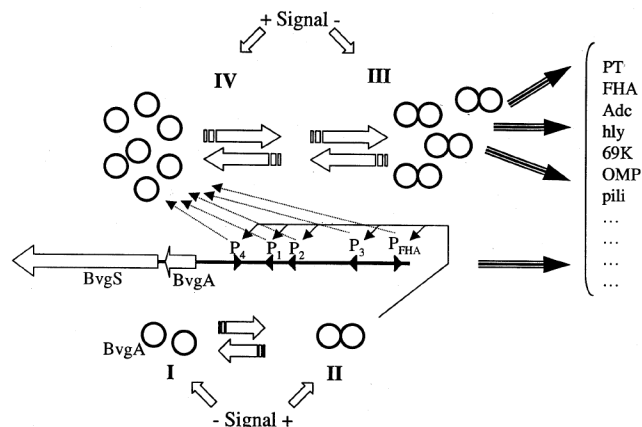
irritating cough and slight fever, lasting 1–2 weeks, (ii) *paroxysmal* cough characterized by an inspiratory ‘whoop’ as air rushes into the lungs against a narrowed glottis, which lasts 1–6 weeks, (iii) a lasting period of convalescence marked by decreased paroxysms, persistent cough and secondary bacterial infections. Severe consequences of this infection include encephalopathy, irreversible brain damage, pneumonia, lung collapse, increased intrathoracic pressure and haemorrhages due to ruptured small and large blood vessels<sup>2</sup>.

During the course of infection, *B. pertussis* adheres to the ciliated epithelium, invades alveolar macrophages, multiplies rapidly on the mucous membrane and expresses an array of virulence factors that help colonize the upper respiratory tract by specific adhesion to the ciliated cells. These factors include cell surface proteins and several extracellular toxins that inhibit host defences and induce damage to host tissues. Like many other bacterial pathogens, the expression of the virulence factors in *B. pertussis* is controlled by growth conditions. Two important phenomena in the regulation of the virulence genes are *phase variation* and *phenotypic modulation*. Phase variation<sup>5</sup> indicates a reversible alteration in the genotype caused by frameshift mutations in which the virulent bacteria simultaneously lose the ability to synthesize toxins and other factors associated with pathogenicity<sup>5</sup>. Erythromycin tolerance has been shown to be a phase marker caused by extensive alterations in the surface properties between the virulent and the avirulent strains, which confer susceptibility to this antibiotic<sup>5</sup>. The natural emergence of phase variants in the later stages of infection implies that phase change could be a defence mechanism to escape immune detection, like *Salmonella* flagellar phase variation where the change of antigenic type helps the bacteria to evade the immune system<sup>6</sup>. The other phenomenon termed phenotypic modulation was first observed by Lacey in 1960 (ref. 7). It implies repression of the expression of virulence factors, except the tracheal cytotoxin at lower temperature (25°C) or in the presence of *in vitro* modulators like SO<sub>4</sub><sup>2-</sup>, ClO<sub>4</sub><sup>-</sup> and nicotinic acid<sup>8</sup>. Using transposon mutagenesis, a single gene locus responsible for both the phenomena was identified<sup>5</sup>. This was termed the *bvg* (*Bordetella* virulence gene) locus. The *bvg* genes share homology with a family of prokaryotic regulatory proteins that respond to environmental stimuli<sup>9</sup>.

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**Figure 1.** The *bvg* locus with *bvgASR* genes and the promoters  $P_1$ ,  $P_2$ ,  $P_3$  and  $P_4$ .



**Figure 2.** Proposed model of the regulatory network of the *bvg* locus. **I**, Non-inducing conditions yield a low amount of BvgA, which is inactive. **II**, Under inducing conditions, BvgA is converted to an active form (depicted as phosphorylated) and transactivates  $P_1$ ,  $P_2$ ,  $P_3$ ,  $P_4$ ,  $P_{FHA}$  and all other promoters of the *bvg* regulon. **III**, The active form of BvgA is amplified by this positive transcriptional feedback at the *bvg* locus. **IV**, Changing to non-inducing conditions where BvgA is present in large amounts but in an inactive form (non-phosphorylated). (Adapted from Uhl, M.A. and Miller, J. F.<sup>17</sup>).

## The *bvg* locus

The *bvg* locus is schematically depicted in Figure 1. The *bvg* locus encodes proteins that transmit extracellular signals to the cellular transcription machinery, causing changes in gene expression. With the exception of tracheal cytotoxin, expression of virulence factors is coordinately regulated by the products of the *bvg* locus<sup>10</sup>, which is responsible for both phase variation and phenotypic modulation. This regulation is in turn dependent on the location of the Bvg binding site on the promoter (described individually for the different virulence factors). The *bvg* locus occupies ~ 5 kb on the total genome and codes for BvgA, BvgS and another protein downstream, BvgR<sup>11</sup>. Studies on this locus have revealed that its transcription is controlled by a 350 bp DNA fragment having four promoters<sup>12</sup>. Three of them,  $P_1$ ,  $P_2$  and  $P_3$ , are involved in the transcription of the *bvg* locus, while the fourth,  $P_4$ , is involved in the synthesis of an antisense RNA. Another promoter,  $P_{FHA}$ , located near the *bvg* locus is responsible for the transcription of a virulent adhesion factor (FHA, discussed later). Under non-inducing conditions, the promoter  $P_2$  is active and maintains a low level of the regulatory

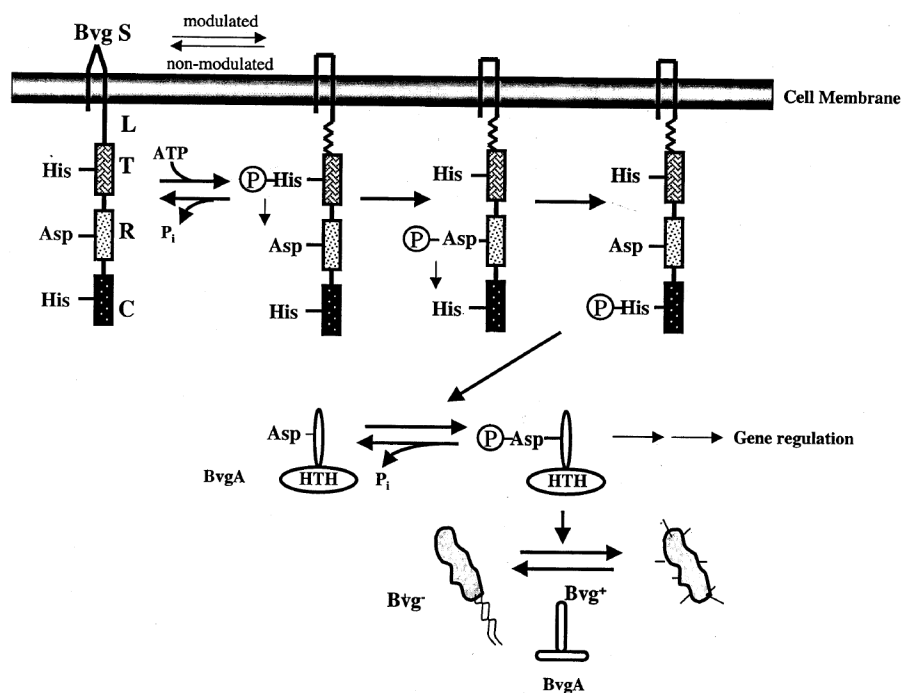
protein BvgA (Figure 2) which is not enough to activate the virulence genes. Under inducing conditions, BvgA is activated by phosphorylation, following which it activates the promoters  $P_1$ ,  $P_2$ ,  $P_3$ ,  $P_4$ ,  $P_{FHA}$  and other promoters of the virulence activated genes (*vag*), a class of loci including genes encoding the adhesins and toxins. This represents the *Bvg*<sup>+</sup> phenotype. The antisense RNA coded from  $P_4$  positively regulates  $P_1$ ,  $P_2$  and  $P_3$  by hybridizing to their 5' untranslated regions. Hybridization inhibits the formation of secondary structure in the mRNAs, thereby favouring their interaction with the ribosome. This causes a 50-fold increase in the rate of translation<sup>12</sup>.

The *Bvg*<sup>-</sup> phase is defined by the absence of *vag*-encoded factors and occurs when the *bvg* locus is deleted or when BvgAS activity is suppressed by modulating conditions such as the presence of sulphate anion, nicotinic acid or reduced temperature. It also involves the expression of a second class of *bvg*-regulated genes during the modulated phase, the virulence repressed genes (*vrg*)<sup>13</sup>, namely *vrg6*, *vrg18*, *vrg24*, *vrg53* and *vrg73*. The first four of the five *vrg* genes mentioned are regulated by *bvg*-activated repressor molecule (BvgR) through a conserved repressor-binding site. While the *bvg*-repressed genes of *B. bronchiseptica*, appear to be involved in survival outside the host<sup>14</sup>, their function in *B. pertussis* is relatively unknown. However it has been shown that BvgR-mediated regulation of gene expression contributes to respiratory infection in mice<sup>15</sup>.

## The BvgAS two-component system

*B. pertussis* employs a two-component signal transduction system comprising BvgS and BvgA, to regulate cellular functions in response to environmental conditions, which are not properly understood so far. BvgS is a 135 kDa periplasmic sensor histidine kinase. It consists of a periplasmic input domain and several cytoplasmic domains, the linker, transmitter, receiver and the C-terminus (Figure 3). The BvgS periplasmic domain senses external stimuli (perhaps via dimerization) and triggers a series of phosphorylation steps starting with autophosphorylation in the transmitter domain at a conserved histidine residue. This is followed by the transfer of the phosphate group to a conserved aspartic acid in the receiver domain, which transfers it to a histidine at the C-terminal domain<sup>1,16-18</sup>.

BvgA, the response regulator, is a 23 kDa cytoplasmic protein comprising an N-terminal receiver and a C-terminal output domain, which contains a helix–turn–helix (HTH) DNA binding motif. BvgA is activated by the transfer of the phosphate group from the C-terminal of BvgS to a conserved aspartic acid in the BvgA N-terminal receiver domain. Upon phosphorylation, BvgA



**Figure 3.** Model for the two-component signal transduction system by the BvgAS proteins. L, Linker; T, Transmitter; R, Receiver and HTH, Helix-turn-helix. Conserved histidine (H) and aspartic acid (D) residues to which phosphotransfer occurs are shown.

**Table 1.** Putative type-III secretion proteins identified in *B. pertussis* (Adapted from Kerr *et al.*<sup>77</sup>)

Putative <i>B. pertussis</i> protein	No. of amino acids	% homology to <i>Yersinia</i>	Predicted location and properties
BscC	600	YscC (33)	Outer membrane
BscU	349	YscU (38)	Inner membrane
BscT	266	YscT (33)	Inner membrane
BscS	88	YscS (50)	Inner membrane
BscR	223	YscR (53)	Inner membrane
BscQ	242	YscQ (24)	Secreted
BscO	169	YscO (22)	Mobile core protein
BscN	444	YscN (65)	ATPase
BscL	212	YscL (34)	Unknown
BscK	220	YscK (17)	Unknown
BscJ	274	YscJ (36)	Lipoprotein
BscI	135	YscI (21)	Unknown
LcrH2	231	LcrH/sycD (16)	Chaperone
BopB	400	YopB (24)	Translocation
BopD	313	YopD (17)	Translocation
LcrH1	161	LcrH/sycD (25)	Chaperone
LcrE	363	LcrE/YopN (19)	Secretion regulation
LcrD	699	LcrD (57)	Inner membrane
BscD	298	YscD (21)	Inner membrane
BscF	97	YscF (23)	Cytosol

positively regulates the *bvgAS* and other virulence factor promoters by binding to the target sequence TTTCCTA<sup>19</sup>. It has also been shown that BvgA is able to dimerize in solution as a possible means to bring about its DNA-binding property<sup>12</sup>.

Several lines of evidence suggest that the mechanism of regulation by the *bvg* locus is different for different virulence loci. Although BvgA can directly activate transcription from the *fha* and *bvg* promoters, the *bvg* locus is not sufficient for the activation of the *ptx* and

**Table 2.** Similarity in pathogenic potential between *B. pertussis* and other organisms

Virulence factor in <i>B. pertussis</i>	Corresponding virulence factor in other pathogens		
	Organism	Virulence factor	Nature of similarity
BvgAS	<i>Vibrio cholerae</i> <sup>78</sup>	ToxRS	Two-component signal transduction system
Pertussis toxin	<i>Enterotoxigenic E. coli</i> <sup>79</sup> <i>V. cholerae</i> <sup>78</sup> <i>Shigella dysenteriae</i> <sup>80</sup>	Labile toxin I Cholera toxin Shiga toxin	AB <sub>5</sub> class of toxin
FHA	<i>V. cholerae</i> <sup>78</sup>	Mannose–Fucose–Resistant Haemagglutinin and Mannose-sensitive Haemagglutinin	Surface molecules with haemagglutinin activity
Adenylate cyclase toxin	<i>Bacillus anthracis</i> <sup>81</sup> <i>Pseudomonas aeruginosa</i> <sup>82</sup>	Edema Factor Eyo Y	Adenylate cyclase activity
Adenylate cyclase toxin	<i>Enterohaemorrhagic E. coli</i> <sup>83</sup>	Hemolysin	Hemolytic activity
Adenylate cyclase toxin	<i>Shigella flexneri</i> <sup>84</sup>	IpaB	Ability to trigger apoptosis in macrophages
Tracheal cytotoxin	<i>Neisseria gonorrhoeae</i> <sup>85</sup>	Tracheal cytotoxin	Ciliostasis
Type-III secretion proteins	<i>Yersinia</i> sp. <sup>86</sup> <i>Pseudomonas</i> sp. <sup>86</sup> <i>Salmonella</i> sp. <sup>86</sup>	Type-III secretion proteins	Type-III secretion apparatus

*cya* promoters (detailed later). This can be interpreted in terms of the lower binding affinity or higher levels of activated BvgA required to stimulate transcription at the *ptx* compared to the *fha* promoter<sup>20</sup>. Recent work has also implicated the requirement of additional accessory factors such as the recently identified Baf protein<sup>21</sup> or the differential effects of DNA topology<sup>22</sup> for the selective activation of some promoters. Given the varied aspects of BvgA-mediated transcriptional activation, the *bvg* regulon has proved to be an excellent system for the study of differential and timely regulation of target promoters to optimize the infection process.

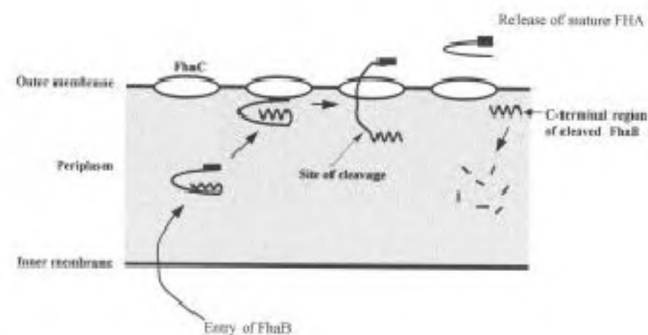
The various virulence factors associated with pertussis, from adhesion factors to extracellular toxins are discussed below.

### Filamentous haemagglutinin

*B. pertussis* confers infection by adhering to host tissues of the upper respiratory tract implicating several adhesins in the process, especially the filamentous haemagglutinin or FHA. It is a filamentous structure about 2 nm wide and 50 nm long, folded into a monomeric rigid rod based on a 19-residue repeat motif, rich in beta strands and turns<sup>23</sup>. The adherence of *B. pertussis* to ciliated cells and macrophages is critical to colonization and infection of the upper respiratory tract, requiring the recognition of eukaryotic carbohydrates or integrins by FHA. Three distinct domains of the mature FHA are responsible for the differential recognition of receptors on these host cells: (1) the first spanning residues 1 to 400 are responsible for binding to sulphated sugars on

mucus-secreting epithelial cells<sup>24</sup>, (2) a region comprising residues 1097 to 1099, the RGD sequence which binds to CR3 integrins on macrophages and ciliary cells,<sup>25</sup> (3) the carbohydrate recognition domain (CRD) in the region 1141 to 1279, responsible for binding to lactosylceramides on macrophages and ciliated cells<sup>26</sup>.

The 220 kDa mature form of FHA represents the N-terminal 60% of the 367 kDa precursor gene product of the structural gene *fhaB*<sup>27</sup>. The FHA operon constitutes the *fhaB* gene and several downstream open-reading frames required for the biogenesis and the secretion of mature FHA and its expression requires the absolute presence of phosphorylated BvgA. Mutations that overcome the suppression of FHA expression conferred by short, C-terminal BvgA deletions have been mapped to the gene encoding the  $\alpha$ -subunit of RNA polymerase (RNAP)<sup>28</sup>. These results show that BvgA interacts directly with the  $\alpha$ -subunit of RNAP in activating FHA genes without necessitating the requirement of any other accessory factor. Despite its size FHA is efficiently secreted into the extracellular environment and has been found to require at least one outer-membrane accessory protein FhaC, whose gene locus is located immediately downstream of the structural *fhaB* gene<sup>29</sup>. The N-terminal 100 residues of FHA share homology with calcium-dependent hemolysins of *S. marcescens* and *P. mirabilis*<sup>30</sup> and interact with the membrane-bound FhaC to lead to the secretion of mature FHA into the extracellular milieu. It was also found that the C-terminal domain of the FHA precursor, FhaB, acts as an intramolecular chaperone to prevent the premature formation of the rigid hairpin rod, which can potentially impede the transmembrane movement of FHA<sup>31</sup>.



**Figure 4.** Depiction of potential roles of FhaC and the C-terminal domain of FhaB. The N-terminal domain of FhaB and the mature FHA are shown as a black box. FhaC is shown embedded in the outer membrane. The cleavage site of FhaB to yield mature FHA is shown. FhaB is translocated through the inner membrane and its C-terminal domain covers the C-terminal half of the mature FHA, leaving its N-terminal domain to interact with FhaC. Upon interaction with FhaC, the N-terminal domain crosses the outer domain first, followed by the C-terminal domain of the mature portion. The C-terminal domain of the precursor is then cleaved off, allowing the mature FHA to fold into its hairpin structure outside the cell. The C-terminal domain of FhaB stays in the cell and is eventually degraded. (Adapted from Mongenie, G. R. *et al.*<sup>31</sup>).

Figure 4 schematically explains the roles played by both the C- and N-termini of the FHA precursor in the processing and secretion of mature FHA.

### Fimbriae

*B. pertussis* fimbriae are submicroscopic proteinaceous appendages that protrude from the cell surface. They are comprised of a major and a minor subunit and are believed to cause persistency of infection. The minor subunit, named FimD binds to the integrin V $\alpha$ -5, located on monocytes<sup>32</sup>. The major subunit binds to sulphated sugars like heparan sulphate, chondroitin sulphate and dextran sulphate which are ubiquitous in the respiratory tract<sup>33</sup>. Studies have shown that both the degree and arrangement of sulphate groups determine the affinity of the binding of fimbriae<sup>34</sup>. It is interesting to note that these binding activities are also found in fibronectin, a host protein found in the extracellular matrix and that the major subunit shares considerable homology with fibronectin within regions implicated with heparin binding<sup>34</sup>. Thus *B. pertussis* fimbriae mimic natural host ligand-receptor interactions to further their infection process. Sulphated sugars on respiratory tract cells are also recognized by FHA; hence the presence of fimbriae could possibly help to differentiate between different host cells and tissues exhibiting many different configurations of heparan sulphate<sup>35</sup>. Binding of fimbriae to monocytes results in activation of CR3 (complement receptor type-3)<sup>32</sup>, the monocyte receptor of FHA, reflecting their synergistic action during infection.

### Pertussis toxin

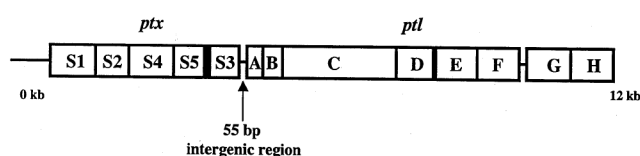
Pertussis Toxin (PTX) is a major colonizing factor and an exotoxin, which remains cell-bound as well as released into the extracellular environment. PTX is a 105 kDa hexamer with typical AB<sub>5</sub> architecture, consisting of A subunit S1 (MW 26,026) and a complex pentamer B comprising S2 (MW 21,925), S3 (MW 21,873), S4 (MW 12,059) and S5 (MW 11,013) in a respective molar ratio of 1 : 1 : 2 : 1 (ref. 36). The crystal structure (Figure 5) of PTX has been determined at 2.9 Å resolution<sup>37</sup>. The A and B subunits share significant structural homology with other bacterial toxins like the cholera toxin of *Vibrio cholerae*, shiga toxin of *Shigella dysenteriae* and the labile toxin of *E. coli*, which have the same AB<sub>5</sub> pattern.

The A subunit has the ADP-ribosyltransferase activity, whereas the B oligomer recognizes and binds the intact toxin to the surface of the host target cells. Following binding, the A subunit cleaves off and inserts through the membrane into the cytoplasm. It then transfers an ADP-ribose from NAD to cysteine352 of the  $\alpha$  subunit of the membrane-bound inhibitory trimeric GTP-binding protein (G<sub>i</sub> protein), which normally inhibits the adenylate cyclase<sup>38</sup>. The ADP-ribosylation prevents the G<sub>i</sub> complex from hydrolysing GTP linked to the G<sub>s</sub> subunit to GDP, thus constitutively activating adenylate cyclase. This increases intracellular levels of cAMP leading to disruption of cellular functions, decrease in phagocytic function of phagocytes such as chemotaxis, engulfment, oxidative burst and bactericidal killing. The systemic effects of the toxin include lymphocytosis and alteration of hormonal activities that are regulated by cAMP, such as increased insulin and histamine production<sup>4</sup>. The B oligomer of pertussis toxin interacts with the glycoproteins and glycolipids on many types of eukaryotic cells serving several distinct and independent functions, namely mitogenicity for T lymphocytes, adherence of the bacteria to eukaryotic cells and the delivery of the toxic S1 subunit to its target. The differential recognition of cilia and macrophages has been localized respectively to subunits S2 and S3 of the B oligomer despite >80% homology<sup>36</sup>. In this study, ciliary lactosylceramide was found to bind exclusively to S2 and leukocytic gangliosides only to S3. Moreover, mutational exchange of residues 37–52 between S2 and S3 showed interchange in their carbohydrate and target cell specificities.

In order to be exported out into the extracellular environment, the toxin must traverse both the inner and outer membranes of *B. pertussis*. While it has not been possible so far to elucidate the exact mechanism of this membrane transport, it is known that PTX requires the products of the *ptl* (pertussis toxin liberation) operon encoding 8 genes (*ptlA* through *ptlH*)<sup>39</sup>. This operon



**Figure 5.** The enormous complexity of the pertussis toxin is shown. Notice the dissimilarity in structure among the B subunits of PT. The A subunit S1 contacts the B oligomer through its C-terminus involving residues 219 to 235 and its enzymatic activity resides within residues 195 to 204 (Krueger *et al.*<sup>78</sup>). The B oligomer recognizes the glycolipid receptors on the surface of the host respiratory tract cells.



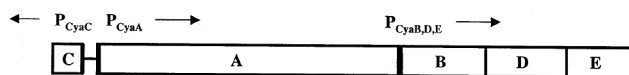
**Figure 6.** Arrangement of genes in the *ptx* and the *ptl* operons. The position of the 55-bp intergenic region acting as a weak termination point, having a stem-loop structure is also demarcated.

covers 9.5 kb located 55 bp downstream of the *ptx* operon. (Figure 6). The PTL proteins share a high degree of homology with the VirB proteins of *Agrobacterium tumefaciens*, which are involved in the transport of single-stranded DNA across bacterial membranes<sup>40</sup>. PtlC is specifically required for the export of assembled holotoxin from the periplasmic space. Mutation in PtlC results in a 20-fold reduction in the amount of PTX present in the culture supernatant, but has no effect upon the assembly or the steady-state level of holotoxin present in the periplasmic space<sup>41</sup>. The *ptx* and the *ptl* operons form the same transcriptional unit controlled by the *ptx* promoter ( $P_{TOX}$ ). This is evident by the non-expression of the downstream *ptl* genes by insertion of a transposon within the coding region of S3 and that only the first gene of the PT operon encoding S1 subunit contains a Shine–Dalgarno sequence<sup>42</sup>. The intervening 55 bp region between S3 and *ptlA* genes is believed to form a stem loop structure and its deletion results in at least 30% higher accumulation of *ptl* transcripts compared to the wild type strain grown at 35°C (ref. 43). Thus the 55 bp region could potentially be a weak rho-independent transcription termination signal of the PTX operon whose read-through is responsible for maintaining equilibrium amounts of PTX and PTL for efficient secretion.

The phosphorylation of BvgA is an absolute necessity for the activation of *ptx* and *cya* operons. Gel shift assays and DNaseI protection studies<sup>44</sup> show that phosphorylated BvgA binds synergistically with *E. coli* RNA polymerase (RNAP) via its C-terminal domain to a region upstream of the PTX promoter that encompasses a 20 bp directly repeated sequence (positions –157 to –117). However, unlike the sole requirement of the BvgAS proteins for the expression of *fhaB* gene, the transcription of *ptx* and *cya* (encoding adenylate cyclase toxin) operons also requires a trans-acting 28 kD accessory factor, Baf<sup>45</sup>. This factor carries no significant homology to any known bacterial transcriptional regulator and its G + C content of 74% is the highest among all *B. pertussis* genes.

### Adenylate cyclase toxin

Adenylate cyclase (CyaA) is an important invasive toxin secreted by *B. pertussis*. It has both adenylate cyclase and hemolytic activities. The two activities are on different domains of the protein, with the hemolytic domain enabling the entry of the enzymatic domain into the cell. Following entry into the cell, the toxin has direct adenylate cyclase activity, which is activated in eukaryotic cells, only in the presence of calmodulin. It causes nearly a thousand-fold increase in the intracellular cAMP levels<sup>46</sup>. This may be important in the inhibition of phagocytic cell activity. Edema Factor (EF), released by *Bacillus anthracis* is another calmodulin-dependent adenylate cyclase. Recently, another adenylate cyclase, ExoY, secreted by *Pseudomonas aeruginosa*, whose activity is not dependent on activation by calmodulin, has also been identified<sup>47</sup>.



**Figure 7.** The *cya* operon. *CyaA*, the structural gene encoding the toxin is under the control of a single promoter. Another promoter between *cyaA* and *cyaB* controls the expression of *cyaB*, *D* and *E* whose products are essential for the secretion of *CyaA*. The gene *cyaC* is transcribed in the opposite direction to that of the other genes in the *cya* operon. The gene product *CyaC* is required for the palmitoylation of *CyaA* at Lys 983 in order to convert it to the active form.

The *cya* operon of *B. pertussis* codes for the adenylate cyclase toxin and comprises five genes: *cya A*, *B*, *C*, *D* and *E* (Figure 7) is under the coordinate regulation of the *bvg* locus. *cyaA*, the structural gene encoding the toxin, is controlled by a single promoter which binds BvgA phosphate to an extensive sequence covering positions –137 to –51 on the promoter. Another promoter, situated between *cyaA* and *cyaB* controls the expression of *cya B*, *D* and *E*. All these gene products are required for the secretion of a functional *CyaA*<sup>48</sup>. *cyaC* is separated from the polycistron by a 260 base pair intergenic region and is transcribed in the opposite orientation<sup>49</sup>. *cyaC* converts the inactive precursor of the toxin to its active form by palmitoylation<sup>50</sup>. The *CyaA* toxin is a 200 kDa, 1706 residue, bifunctional protein with a hemolytic and a catalytic domain<sup>51</sup>. It belongs to the AB toxin family like the diphtheria toxin. The C-terminal 1306 residue hemolytic domain facilitates the entry of the N-terminal 400 residue catalytic domain into the host cell<sup>52</sup>.

The catalytic domain can be further divided into two subdomains, a 18 kDa region –T18 and a 25 kDa region –T25, which interact with calmodulin to form the active complex<sup>53</sup>. The T25 region (1–224 amino acids) is responsible for cAMP increase and the T18 region (224–399 amino acids) is involved in calmodulin binding<sup>54</sup>. The C terminus of this protein (residues 1007 and 1706) has about 45 repeats of the sequence GGXGXDXLX which is responsible for low-affinity calcium binding. Calcium binding to this region causes a conformational change (increase in helicity) in the protein which effects the internalization process<sup>55</sup> (Figure 8). Other characteristics of the hemolytic region include (1) four hydrophobic segments which forms the pore (residues 500–700) (b) a site for *CyaC*-mediated palmitoylation on the  $\epsilon$  amino group of Lys 983 (ref. 50) and (c) an unprocessed carboxy terminal secretion signal<sup>55</sup>.

The entry of the *CyaA* toxin into the eukaryotic cells occurs in the following manner: (i) Binding of the toxin to the cell surface, which is dependent on the palmitoylation of Lys 983 and (ii) pore formation due to the hemolytic activity of the toxin. Monomer oligomerization is thought to be a prerequisite for this process<sup>56</sup>. The catalytic domain of the toxin is translocated through the pore created on the cell membrane by the hemolytic do-

main. The presence of an amphipathic helix containing Glu 509 and Glu 516 has been reported to be crucial for the translocation<sup>57</sup>. Other factors affecting the translocation are (a) calcium binding properties of the toxin<sup>55</sup>; (b) the electrostatic charge of the stretch of amino acids, 224–242 in the catalytic domain<sup>58</sup>; and (c) the potential of the membrane across which the translocation occurs<sup>59</sup>.

*B. pertussis* has been found to trigger apoptosis in macrophages. From mutation studies, the expression of the adenylate cyclase – hemolysin has been found essential for the induction of programmed cell death in macrophages. This property of the bacteria may be useful for the initiation of infection, evasion of host immune system and its survival in the host<sup>60,61</sup>. Recent experiments report that phagocytosed bacteria are killed by human neutrophils and so cannot survive in them<sup>62</sup>.

This bifunctional property of the adenylate cyclase toxin has been exploited advantageously by manipulations in the catalytic domain. Recombinant toxins obtained by the insertion of CD8<sup>+</sup> T-cell epitopes into the catalytic domain of the toxin have been used to elicit cytotoxic T-cell responses through MHC class I molecules<sup>63</sup>. Suitable vaccines to confer cell-mediated immunity on the host may be developed using this strategy. The complete dependence of the toxin on calmodulin has been exploited to use *CyaA* as a reporter for protein targeting.

### Pertactin

Pertactin, a 69 kDa nonfimbrial outer membrane protein, under the control of the *bvg* locus, is partly responsible for the adhesion of the bacteria to the host cells. It also functions as a protective antigen in animal models<sup>64,65</sup>. The pertactin crystal structure having a  $\beta$ -helical conformation has been solved to 2.5 Å resolution<sup>66</sup> (Figure 9).

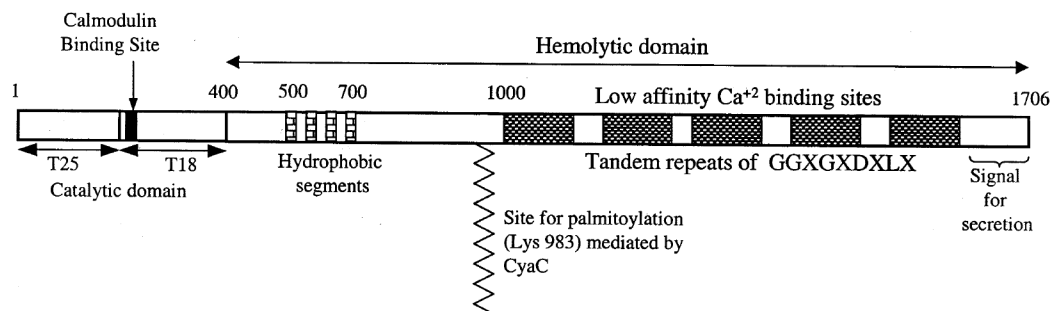
The mature protein has two arginine–glycine–aspartic acid (RGD) sequences at 225–227 and 665–667 (Figure 10). These sequences are also present on other mammalian adhesion proteins like fibronectin, vitronectin and fibrinogen and bind specifically to the integrin family of proteins, located on the mammalian cell surface<sup>64</sup>.

*In vitro* cell adhesion studies on Chinese hamster ovary (CHO) cells using purified pertactin in the presence of synthetic peptides containing RGD showed that these three residues were critical for cell adhesion and any substitution resulted in marked decrease in their ability to adhere to cell surfaces. Mutant strains, which do not express pertactin, were 30–40% less adhesive to HeLa and CHO cells than the wild type<sup>64</sup>.

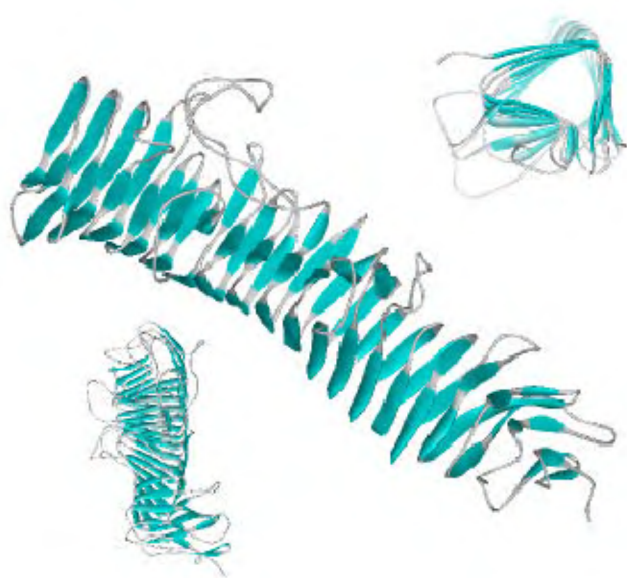
### Tracheal cytotoxin

The characteristic whooping cough (pertussis) caused by *B. pertussis* can be attributed to the elaboration of

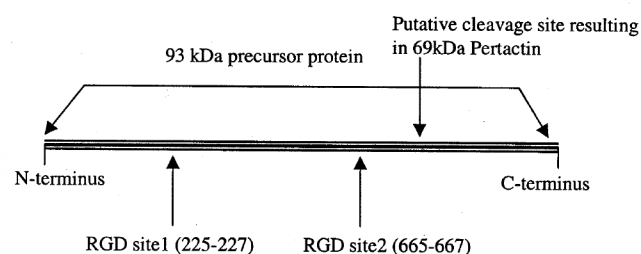




**Figure 8.** Structural organization of the CyaA toxin. The catalytic domain which extends from 1 to 400 residues is divided into the T25 and T18 subdomains. The calmodulin-binding site in the T18 subdomain is shown. The hemolytic domain containing Lys 983 (required for activation by palmitoylation mediated by CyaC) and low-affinity calcium binding repeat consensus sequence GGXGXDXLX is also shown. (Adapted from Ladant, D. *et al.*<sup>63</sup>).



**Figure 9.** Pertactin crystal structure at 2.5 Å resolution, having the  $\beta$ -helical structure is shown here. (Emsley, *et al.*<sup>66</sup>).



**Figure 10.** Mature form of pertactin, 69 kDa, is obtained from its 93 kDa precursor. The putative point of cleavage is shown. The RGD sequence (225–227) has been found to facilitate the adherence of bacteria to the cell surface.

the tracheal cytotoxin (TCT). This is a 921 Da disaccharide tetrapeptide bearing resemblance to peptidoglycan and cannot be classified as a classical exotoxin though it is elaborated in the extracellular fluid by the bacteria<sup>4,49</sup>. The structure of TCT has been determined to be GlcNAc-1,6-anhydro-MurNAc-L-Ala- $\gamma$ -D-Glu-*meso*-A<sub>2</sub>pm-D-Ala, where MurNAc is N-acetylmuramic acid and A<sub>2</sub>pm is diaminopimelic acid<sup>67</sup> (Figure 11). It has been associated with the respiratory cytopathology observed in the disease and it targets the ciliated epithelial cells in the bronchial tract. It has the ability to cause ciliostasis (restriction of the free movement of cilia) and effects the killing and extrusion of the ciliated epithelial cells from the mucosa. It has also been found to stimulate release of IL-1, which causes increase in body temperature<sup>49</sup>.

Studies on structure–activity relationships using analogues for TCT on hamster trachea epithelial (HTE) cell cultures have been conducted. Analogues containing modified sugars but with the same peptide had no change in their toxicity on HTE cells. Analogues with the same sugar group, but with truncated peptides were at least five hundred times less toxic than TCT. Analogues with complete deletion of A<sub>2</sub>pm or truncation of the side chain amine or carboxyl group of A<sub>2</sub>pm, or replacement of A<sub>2</sub>pm (by  $\alpha$ -aminopimelic acid) have been found to be a thousand times less active than TCT. Use of other TCT analogues have led to conclusions that the C-terminal dipeptide has a crucial role in determining its toxicity. Results from all the tests conducted have revealed that the peptide portion of the toxin dictates its toxicity and the disaccharide moiety has no evident role in its contribution to the toxicity for respiratory epithelium. One can hypothesize that the disaccharide moiety may be involved in recognition of specific sites on target cells. It has also been suggested that  $\gamma$ -D-Glu-*meso*-A<sub>2</sub>pm may be the smallest fragment for TCT toxicity<sup>67</sup>.

*N. gonorrhoeae* has also been found to damage human fallopian tubes due to loss of ciliated cells from the mucosa by the secretion of TCT. Both the bacteria seem to have evolved similar mechanisms for colonization of



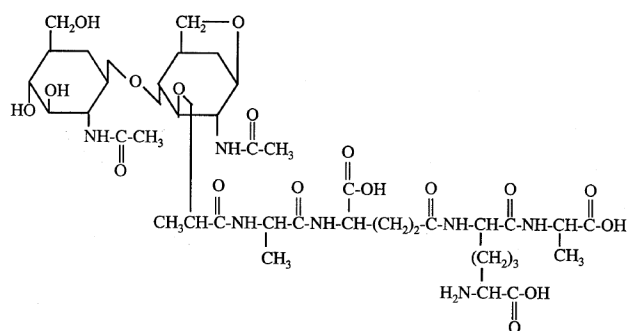


Figure 11. Disaccharide tetrapeptide structure of TCT.

their respective ciliated epithelia and the toxins may act through related pathways<sup>68,69</sup>.

### Dermonecrotic toxin

Dermonecrotic toxin (DNT), also called lethal toxin, is a 102 kDa heat-stable toxin found in all species of *B. pertussis*. It induces inflammation, vasoconstriction and dermonecrotic lesions around the areas which *B. pertussis* colonize in the respiratory tract. DNT belongs to the family of the newly discovered cytotoxic necrotizing factor (CNF1 and 2) which affects regulation of cell growth or division<sup>70</sup>. Progress in determining its exact mode of action or its role in disease has so far been relatively slow. However it has been reported that DNT inhibits the elevation of alkaline phosphatase activity and reduces the expression of type-I collagen in an osteoblast-like cell line<sup>71</sup>. DNT has also been found to stimulate DNA and protein synthesis in these cells without cell division, leading to polynucleation. It also induces the assembly of actin stress fibres and tyrosine phosphorylation of focal adhesion kinase<sup>72</sup>. DNT is believed to cause these effects by glutamine 63 deamidation of the small GTP-binding protein, RhoA, involving residues 1136 to 1451 of DNT<sup>73</sup>. Glutamine 63 is essential for GTP hydrolysis by Rho. Deamidation of glutamine by DNT inhibits the GTPase activity of Rho and renders the Rho protein constitutively active. Rho GTPases are regulators of actin cytoskeleton and act as molecular switches to trigger several intracellular signalling pathways. Thus DNT is a complicated toxin having significant, but as yet unclear roles to play in the alteration of the host physiological reactions in response to *B. pertussis* infection.

### Type-III secretion system

Type-III secretion systems, found in various Gram-negative bacteria are responsible for transporting virulence factors or effector proteins directly from the cytoplasm to the external surface of the bacterium, where they interact with or are delivered into the host cell.

These virulence factors interfere with normal host cell metabolism, enabling the pathogen to survive and replicate effectively under unfavourable host environmental conditions. The genes encoding the type-III secretion system reside in pathogenicity islands, which are regions on the bacterial chromosome that confer a variety of virulence traits to the pathogen. It has been reported that the ability of the pathogen to obtain complex virulence traits is a single genetic event from a foreign source, rather than by undergoing natural selection for many years<sup>74,75</sup>. This can be substantiated by the fact that many of the proteins in the type-III secretion apparatus share a considerable degree of homology among themselves.

Recently, using *B. bronchiseptica* in laboratory animals, it was demonstrated that a particular locus was differentially expressed in the Bvg<sup>+</sup> phase. This locus was then found to code for a protein named BscN, whose predicted sequence is similar to the *Yersinia* YscN protein, an ATPase that provides the drive for the export of the *Yersinia* secretory protein by the type-III apparatus. Analysis of the flanking regions revealed other ORFs, which were similar to the members of the *Yersinia* and other bacterial pathogenic type-III secretion apparatus. This was the first report on the existence of the type-III secretory system in the genus *Bordetella*. The same study revealed loci encoding the type-III secretion system homologues in strains of *B. pertussis*<sup>76</sup>.

Kerr *et al.*<sup>77</sup> examined a 35.6 kb DNA sequence amplified from the virulent *B. pertussis* Tohoma I, for homology with known type-III secretion genes of the bacterial pathogens. Upon investigation, a total of 20 ORFs were determined and putative type-III secretion proteins were annotated according to their homology with type-III secretory proteins in *B. bronchiseptica*, *Yersinia* and *Pseudomonas*. This region was designated as the BpeI locus (Figure 12). The % G + C content of this locus was around 66.16, which is similar to the % G + C content of the *B. pertussis* chromosome<sup>76,77</sup>.

The ORFs were found to be arranged in two operons. The first operon spans nucleotide 23385 through 7888 and codes for putative proteins LcrH1, BopD, BopB, LcrH2, BscI, BscJ, Bcs, K, BscL, BscN, BscQ, BscR, BscS, BscT, BscU and BscC. The second operon spans nucleotide 23580 through 29863 and codes for putative proteins LcrE, LcrD, BscD and BscF. The homology of these proteins with the type-III secretory proteins was

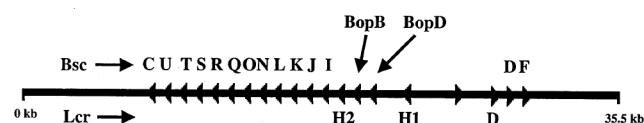


Figure 12. The BpeI locus encoding the type-III secretory machinery in *B. pertussis*. Putative functions and details about the protein length are summarized in Table 1 (Adapted from Kerr, *et al.*<sup>77</sup>).

around 73–99% with *B. bronchiseptica*, 17–65% with *Yersinia* and 18–64% with *Pseudomonas*<sup>77</sup>.

## Conclusion

This review has dealt primarily with the effects of the various virulence factors elaborated during pertussis infection. The present level of understanding of the role of these factors in pathogenesis comes from the past four decades of intense genetic and biochemical analysis utilizing all contemporarily available molecular biology tools. *B. pertussis* has proved to be a demanding organism in terms of its growth requirements and fragility to transformation by conventional methods. However, plasmid conjugation with *E. coli* as an alternative to direct transformation, followed by transposon mutagenesis allows easy characterization of the mutated gene loci using antibiotic resistance and probe hybridization techniques. DNA footprinting studies as well as specific mutational analysis have made clear the role of DNA-binding regulatory proteins and their synergistic interaction with RNA polymerase demonstrating a host-assisted virulence control mechanism. The various methods to detect *B. pertussis* include PCR, using specific primers for different gene loci and ELISA, involving detection of specific antibodies IgG, IgM, IgA and IgE produced against both the whole cell and the released virulence determinants. The antibiotics commonly used for control following diagnosis are erythromycin, tetracycline and chloramphenicol. However their limited efficacy in completely subverting the disease has prompted the use of preventive measures as more appealing and effective.

Whooping cough is a prime example of a once highly contagious and dangerous disease, eventually tamed by effective, widespread vaccination. Still, several pockets exist around the world, mostly in developing nations, where the mortality rate remains high due to non-availability of proper vaccination. Earlier attempts at vaccination were based on chemically killed whole cell suspensions (as part of the DPT vaccine), which were subsequently discontinued due to local and systemic side reactions caused by the vaccine components. Present-day vaccines focus on combinations of inactivated PTX, FHA, pertactin and fimbriae, termed 'acellular vaccines'. These are more effective, have fewer side effects and moderate dose requirement compared to whole cell vaccines.

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