The virulence factors of *Bordetella pertussis*: a matter of control

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Received 3 April 2000; received in revised form 8 November 2000; accepted 19 December 2000

Abstract

*Bordetella pertussis* is the causative agent of whooping cough, a contagious childhood respiratory disease. Increasing public concern over the safety of whole-cell vaccines led to decreased immunisation rates and a subsequent increase in the incidence of the disease. Research into the development of safer, more efficacious, less reactogenic vaccine preparations was concentrated on the production and purification of detoxified *B. pertussis* virulence factors. These virulence factors include adhesins such as filamentous haemagglutinin, fimbriae and pertactin, which allow *B. pertussis* to bind to ciliated epithelial cells in the upper respiratory tract. Once attachment is initiated, toxins produced by the bacterium enable colonisation to proceed by interfering with host clearance mechanisms. *B. pertussis* co-ordinately regulates the expression of virulence factors via the *Bordetella* virulence gene (*bvg*) locus, which encodes a response regulator responsible for signal-mediated activation and repression. This strict regulation mechanism allows the bacterium to express different gene subsets in different environmental niches within the host, according to the stage of disease progression. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Virulence factor expression; Pertussis toxin; Adenylate cyclase; Fimbria; Filamentous haemagglutinin; Pertactin; *Bordetella*; *bvg*

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PII: S0168-6445(01)00056-0
1. Introduction

Pertussis or whooping cough is a highly contagious disease of the human respiratory tract, which is particularly severe in infants. The disease is characterised by bronchopneumonia, paroxysmal coughing and the distinctive 'whooping' intake of air. Pertussis is especially prevalent in developing countries where appropriate medical assistance is often unavailable and disease progression is unimpeded due to the lack of suitable antimicrobials. The readily transmissible nature and the severity of the disease in these countries have seen worldwide deaths from whooping cough increase to approximately 346 000 per year [1]. Whooping cough is caused by the obligate human pathogen, *Bordetella pertussis*, a Gram-negative coccobacillus originally isolated in 1906 by Bordet and Gengou [2].

The normal course of infection begins with the bacterium entering the host via the airways, contained within airborne droplets derived from the cough of an infected individual. The pathogen proceeds down the respiratory tract, adhering to ciliated epithelial cells in the trachea and nasopharynx. Once attachment is initiated, the bacterium begins to replicate and colonises adjacent areas. Toxins secreted by the micro-organism damage the epithelial lining, resulting in the loss of ciliated cells which induces the characteristic coughing. These toxins also allow the bacterium to evade the host immune response by interfering with clearance mechanisms. Halting ciliary function, short-circuiting host G protein signalling apparatus and inhibiting immune cell function by upregulating cAMP levels are all examples of the damage caused by the various toxins of *B. pertussis*.

The incidence of whooping cough has been greatly reduced since the advent of pertussis vaccines in the 1940s. These 'whole-cell' vaccines consist of chemically or heat-killed *B. pertussis* cells. These vaccines, as part of the diphtheria, tetanus, pertussis (DTP) immunisation regime, proved extremely effective at preventing the symptoms of whooping cough. Unfortunately, a loose association with rare neurologic complications and deaths has led to a decline in public confidence, a subsequent reduction in immunisation rates, and an increase in the incidence of the disease. The other constituents of the DTP vaccine are purified diphtheria and tetanus toxoids. These vaccine components have attained a comparatively less controversial reputation, and significant research has been carried out in an attempt to bring the pertussis component into line with the less reactogenic constituents of the vaccine.

2. The genus *Bordetella*

Historically, the bacteria currently classified within this genus have had a very dynamic taxonomic existence, not always being classified together. It was Lopez who proposed that a new group, the genus *Bordetella*, be created to house the then three species, *pertussis, parapertussis* and *bronchiseptica* [3]. More recent molecular evidence, including DNA sequencing analysis, multilocus enzyme electrophoresis and restriction fragment length polymorphism typing, has justified the grouping of these three species into the same genus [4,5]. *B. pertussis* is exquisitely tuned to a single environment and has only been found to infect humans.

*B. parapertussis* causes a milder 'pertussis-like' disease state in humans and has also been found associated with ovine respiratory infections [6]. Most other mammals can be colonised by *B. bronchiseptica* with many commercially important diseases caused by this micro-organism [7], the most notable being an association with atrophic rhinitis in swine which costs the meat industry considerable profits, and kennel cough in dogs [8,9]. The bird pathogen *Bordetella avium*, a later addition to the genus (Table 1) [10], causes turkey coryza and other respiratory diseases of fowl [11].

More recently, a number of new members of the genus have been proposed. *Bordetella hinzii* is the name assigned to a novel species recently isolated from a bacteraemic individual with HIV/AIDS [12]. Associated with human septicaemia, another species, designated *Bordetella holmesii*, has also been identified [13]. The name *Bordetella trematum* has recently been proposed for a novel species isolated from human wounds and ear infections [14]. Although none of these new species were associated with respiratory tract infections, they are similar to other members of the genus based on phylogenetic analyses [12–14]. Since its initial discovery, *B. hinzii* has also been isolated from the respiratory tract of infected poultry [15].

3. Genetic control of virulence

The goal of any pathogenic bacterium, including *B. per-
Table 1

Comparison of the gene products synthesised by the four most studied members of the genus *Bordetella*.

<table>
<thead>
<tr>
<th></th>
<th><em>B. pertussis</em></th>
<th><em>B. parapertussis</em></th>
<th><em>B. bronchiseptica</em></th>
<th><em>B. avium</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Pertussis toxin (PT)</td>
<td>+/−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Adenylate cyclase toxin/haemolysin</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Filamentous haemagglutinin (FHA)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Fimbriae</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Pertactin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tracheal cytotoxin</td>
<td>+++/−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lipopolysaccharide (LPS)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Dermonecrotic toxin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Flagella</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tracheal colonisation factor (Tcf)</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>BkA</td>
<td>+/−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Urease</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
</tbody>
</table>

*aUpdated and modified from Gross et al. [10].

*bAbbreviations: +, gene product expressed; +/−, gene expressed in some strains; −, gene cryptic or absent.

*cExpression of tracheal cytotoxin is not regulated by the bvg locus.*

*tussis*, is to colonise and multiply by exploiting its environment to the fullest extent. This is achieved by the bacterium controlling the production of the specific factors that enable it to infect its host.

### 3.1. Bordetella virulence gene regulation system

It was realised many years ago that *B. pertussis* existed in more than one phase [16]. ‘Phase variation’ occurs when spontaneous mutations during DNA replication give rise to avirulent variants. This process occurs at a frequency of one in a thousand to one in a million cells and produces avirulent bacteria which do not have the ability to invade and colonise the host. However, if, when culturing wild-type *B. pertussis*, the growth conditions are altered, the bacteria undergo a totally reversible conversion to one of principally two states, called X and C modes by Lacey in 1960 [17]. Termed ‘antigenic modulation’, this phenomenon may be important in the survival of this microorganism. This landmark study also recognised a third state (I mode), with growth characteristics intermediate between X and C modes, and “an infinitude of other antigenic states”.

More recent research has dealt almost entirely with the two extreme modes of growth, virulent (X) and avirulent (C). However, studies on the related species *B. bronchiseptica* have provided new insight into the intricacies of antigenic modulation. Due to the expression of a set of genes under modulating growth conditions which are easily monitored, *B. bronchiseptica* may be used as a model for other members of the genus. An intermediate-phase *B. bronchiseptica* mutant similar to Lacey’s I mode has recently been isolated which expresses phenotypes characteristic of both virulent and avirulent states [18].

The members of the genus *Bordetella* share a genetic locus which encodes a biological ‘switch’ enabling these species to oscillate between different phenotypic states depending on their environment. In the human respiratory tract, a subset of *B. pertussis* genes are expressed which allow the bacterium to colonise the host. The genes for a number of adhesins, such as filamentous haemagglutinin (FHA), serotype-specific fimbriae, and a 69-kDa outer membrane protein known as pertactin, are expressed, as are the genes encoding products involved in evasion of the host immune system: pertussis toxin (PT), adenylate cyclase toxin/haemolysin and dermonecrotic toxin.

In the laboratory, the virulence control switch can be manipulated to bring *Bordetella* species into an avirulent state by the addition of sulfate ions or nicotinic acid to the culture medium, or by reducing the culture temperature from 37°C to 25°C [19].

The expression of virulence factors in *Bordetella* species is co-ordinately regulated by what was originally termed the *vir* locus [20] and is now known as the *Bordetella* virulence gene (*bvg*) locus. This gene locus encodes two proteins which enable the bacterium to ‘sense’ the prevailing environmental conditions, and then ‘act’ accordingly by controlling the expression of specific genes and gene loci. The sensor protein is known as BvgS and the activator as BvgA. Together these gene products constitute a two-component signal transduction system and make up part of a signal transduction superfamily [21]. This type of system is utilised to respond to many different environmental stimuli by many prokaryotes, and recently has been described in some eukaryotes [21–24].

The genes encoding BvgA and BvgS are contained within an operon [25] (Fig. 1) [26]. The original sequencing data unfortunately contained mistakes in and downstream of the *bvg* gene, confusing the exact arrangement of the operon and obscuring the presence of the *bvgR* open reading frame (ORF) [25]. The discovery and analysis of a third gene, *bvgR*, has extended both the size and scope of the *bvg* locus [27]. While *bvgA* and *bvgS* are transcriptionally linked, *bvgR* is transcribed separately from the opposing DNA strand (Fig. 1). Transcription of *bvgAS* is controlled by three self-regulated promoters, P₁, P₂,
and P4, and one bvg-independent promoter, P2 (Fig. 1) [28,29]. Transcription from P2 is constitutive, thereby maintaining low-level production of intracellular BvgAS proteins. While P1, P2 and P3 are all involved in the transcription of bvgAS, P4 produces an antisense RNA strand complementary to transcripts expressed from the other three promoters. The exact in vivo function of this promoter remains unclear; however, it may be involved in stabilisation of the sense transcripts via the prevention of secondary structure formation. Hybridisation between mRNA from P1 and P2 may lead to a more efficient interaction with ribosomes [29].

The gene encoding FHA is transcribed from a region just upstream of these four promoters [29] at Pfha (Fig. 1). There has since been discovered a bvg-independent FHA promoter, Pfar, which has been shown to express low levels of the FHA precursor protein, FhaB, in vitro [30,31].

3.1.1. BvgA

The transcriptional activator, BvgA, which can directly and indirectly affect the expression of a number of virulence-controlled genes, is a 23-kDa cytoplasmic protein. The two-domain structure of BvgA (Fig. 2), consisting of a receiver at the N-terminus and a C-terminal helix-turn-helix (HTH) motif, is characteristic of response regulators [25,32]. It is this HTH module which facilitates specific DNA sequence binding at the promoters of bvg-activated genes [33]. Although not part of the HTH domain, the last 20 amino acid residues are also required for binding, probably by stabilising the HTH–DNA binding interaction [33,34].

The binding of DNA by proteins is a common phenomenon within cells, and the C-terminal HTH domain of BvgA shares sequence homology with a number of different protein families. As expected, this domain is homologous to corresponding areas of other response regulators such as FixJ, UhpA and NarL [34]. Other DNA binding proteins such as LuxR from Vibrio Fischeri [35] as well as the C-termini of selected bacterial σ factors [36] also share homology with this evolutionarily conserved domain.

Direct control over the transcription of a number of bvg-activated genes by BvgA is achieved via binding to specific sequences at target promoter regions [37,38]. The heptameric sequence-specific BvgA binding site TTTCCTA first proposed by Roy and Falkow, or homologous sequences, is present as either direct or inverted repeats upstream of various bvg-regulated genes [38–40]. These homologous heptamer repeats are involved in the binding of BvgA to virulence-activated promoters, a point substantiated in a number of virulence-activated promoters by DNase I protection studies [39–42].

3.1.2. BvgS

In a process that is not yet fully understood, a histidine kinase residue in the transmitter domain of BvgS is auto-phosphorylated, a state which can be altered if the environment changes. Following a series of intramolecular phosphorylation events, the phosphoryl group is eventually transferred to BvgA [43]. If there is a change in the environment to modulating conditions, the initial phosphorylation does not occur, preventing the activation of bvgA.

The 135-kDa sensory component of the signal transduction system, BvgS, is often termed an unorthodox sensor protein because of the number of extra functional domains...
it contains. The membrane spanning nature of this protein predicts it possesses both periplasmic and transmembrane domains, and sequence homology studies confirm the absolutely conserved histidine kinase domain [25,32]. However, BvgS also contains a linker section and regions usually reserved for response regulators such as BvgA, a receiver domain and a C-terminal output domain (Fig. 3) [25,32]. The deletion of these ‘extra’ domains has been shown to make BvgS non-functional [25,43,44].

Experiments with BvgS-PhoA fusion proteins have shown that the N-terminal region is located in the periplasm [32]. Mutations in the linker region render the bacterium incapable of sensing the environment providing evidence that this region acts as a molecular relay between periplasmic and C-terminal domains located in the cytoplasm [39,45].

The transmitter region of BvgS is necessary and sufficient for autophosphorylation [43]; however, transfer of this phosphoryl group to the C-terminal (output) domain can only be effected via the receiver domain [46]. Not only does the receiver domain positively regulate the phosphoryl transfer to the C-terminal output domain, it also has the ability to reverse this transfer, thereby rephosphorylating itself and postponing BvgA activation (Fig. 4). It can also totally dephosphorylate BvgS from the receiver itself, thereby completely halting the activation of BvgA (Fig. 4) [46]. It is the output domain (particularly His1172) of the C-terminus which is responsible for the phosphorylation of BvgA [47].

3.1.3. BvgR

While BvgA exercises direct control over the expression of bvg-activated genes by binding to promoter sequences, control over virulence repressed genes (vrgs) is indirect, implicating a Bvg-activated repressor protein. The gene which controls repression of the bvg-repressed genes has been identified and termed bvgR [48].

The DNA sequence of the 5’ end of four of the five bvg-repressed genes (vrg6, vrg18, vrg24, vrg53 and vrg73) has brought to light a conserved consensus sequence element present in the coding region of four of these genes [49,50]. Using a Southwestern analysis with vrg6 DNA, this sequence was recognised by a 34-kDa protein [50]. When analysed, none of the native promoter regions of any of the bvg-repressed genes seem necessary for modulative repression to occur [50].

The bvgR gene lies downstream of and adjacent to bvgAS [48]. Further characterisation led to the identification of the exact location of the bvgR gene (Fig. 1) [27]. Transcribed from its own BvgA-regulated promoter, bvgR lies 43 bp downstream of bvgS and is transcribed in the opposite orientation. Transcriptional analysis has shown that this ORF produces a predicted protein of 32 kDa, close to the molecular mass (34 kDa) obtained by Beattie and associates [27].

It has been shown that B. pertussis mutants lacking bvgR are less efficient colonisers than wild-type strains in a mouse aerosol challenge model, demonstrating that BvgR regulation of the bvg-repressed genes makes a significant contribution to infection in mice [51].

3.2. Transcriptional regulation by BvgA

As the final part of an intricate phosphorelay process, BvgS phosphorylates BvgA. When compared to its unphosphorylated counterpart, this activated ‘BvgA ~ P’ has a far greater affinity for virulence factor promoter BvgA binding sites [30,33,41,42]. Indeed, BvgA must be phosphorylated for bvg-activated promoters to become fully operational in in vitro transcription assays [30,31].
Although BvgA-independent transcription of the fhaB gene has been observed in vitro, expression from this alternative promoter, P_{fla}, only occurs at low levels [30,31].

It was theorised that the positioning of BvgA→P at a promoter region may induce BvgA oligomerisation [29] and raise the affinity of the response regulator for the target DNA [33]. It has been suggested that BvgA→P may bind co-operatively to bvg-activated promoters, forming multimers along the promoter template [31,40–42]. Initially, high-affinity binding occurs far upstream of the transcriptional start site, then weaker sites are progressively bound until the site adjacent to the RNA polymerase recognition sequence is occupied.

3.3. Involvement of RNA polymerase

When bound to the promoter DNA at the correct start site, RNA polymerase catalyses the initiation of transcription. RNA polymerase is composed of four subunits, α, β, β' and σ. The α subunit is generally the portion of RNA polymerase that interacts with transcription factors at positively regulated prokaryotic promoters [52,53]. The σ subunit is involved in promoter recognition, interaction with transcriptional activators, DNA unwinding and setting up the initiation complex [54]. Much research into the important roles played by these two RNA polymerase subunits is currently under way.

The α and major σ factor of B. pertussis have both been cloned and characterised [55,56]. The major σ factor of B. pertussis is larger than the Escherichia coli σ70 and has been designated σ80 [56]. The B. pertussis RNA polymerase σ80 subunit confers enhanced expression of fhaB in E. coli [56]. The authors suggest that the role played by σ80 may be in positioning the RNA polymerase/BvgA→P complex at the correct site along the promoter template. By interacting directly with BvgA→P, the RNA polymerase σ subunit is provided with an interface point through which it can initiate transcription [56].

Studies on response regulator molecules related to BvgA such as LuxR from V. fischeri and UhpA from E. coli have demonstrated that while still able to bind target DNA, transcription from promoters regulated by these proteins is severely hindered by C-terminal deletions [23,57]. Similar experiments carried out on BvgA have shown that a deletion of as few as two C-terminal amino acids resulted in an avirulent phenotype [58]. When this truncated bvgA gene was presented on a plasmid (in trans) in otherwise wild-type B. pertussis strains, a reduced growth rate was observed. Mutations found to remedy the slowed growth rate were traced to the α subunit of RNA polymerase, further suggesting that an interaction between RNA polymerase and the C-terminus of BvgA is required for the transcriptional activation of virulence-associated genes in B. pertussis [58]. These authors postulate that the slowed growth rate was caused by an inappropriate interaction between the truncated BvgA protein and the α subunit of RNA polymerase. This may have led to a significant loss of free RNA polymerase resulting in slowed transcription of normal housekeeping genes, hence a slowed growth rate.

In the absence of BvgA→P, RNA polymerase binds upstream of the in vivo transcription start sites of the P_{fla} promoter leading to low-level expression from the BvgA-independent promoter, P_{fla} [30,31]. However, the addition of BvgA→P allows transcription to be initiated at the normal in vivo site. This is further evidence that BvgA acts upon bvg-activated promoters by correctly positioning RNA polymerase for transcription [30,31].

Recent DNase I protection assays at bvg-activated promoters suggest that the promoter DNA flexes to loop around the BvgA→P/RNA polymerase complex [31,40,41]. This may increase the binding affinity of the BvgA→P/RNA polymerase complex and stabilise the entire transcriptional machinery [31].

3.4. Differential activation of virulence factors

It has been suggested that the expression of bvg-activated genes proceeds in two stages [59,60]. This differential activation of ‘early’ and ‘late’ promoters allows the bacterium to produce adhesin molecules soon after entering the host, whereas the ‘second-wave’ bvg-activated genes are not expressed until attachment is achieved.

The transcription of PT requires the presence of a threshold concentration of BvgA→P [60]. There is actually a 10-fold higher concentration of BvgA→P required to initiate transcription at the P_{ptx} promoter than either P_{fla} or bvg P [40]. Demonstration of the functional difference between early and late expressed genes came when mutations in the C-terminus of BvgA allowed normal levels of FHA expression while abolishing PT expression [61]. This region has been proposed as the region of the BvgA molecule that interacts with RNA polymerase [58,61].

Binding studies at the P_{ptx} promoter have found an initial high-affinity binding site far upstream of the −35 promoter motif [41]. This region includes a 20-bp direct repeat which in turn encompasses heptameric inverted repeats [39,41]. Genetic analysis of this region has demonstrated that it is the heptanucleotide repeats that are crucial for promoter activation [39]. Due to the distance of these repeats from the actual start site, P_{ptx} may require BvgA→P to multimerise along the promoter template with weaker sites progressively bound until the site adjacent to the RNA polymerase recognition sequence is reached.

A similar analysis has been undertaken at the P_{fla} promoter [31] at which the binding of BvgA→P to an extent mirrors that at the P_{ptx} promoter. Again a high-affinity site is bound initially, including an inverted repeat region homologous to that at P_{ptx}, however it lies further downstream than the corresponding element at the P_{ptx} promoter [31,40]. The architecture of the bvg P promoter,
another early promoter, is very similar to that of \( P_{fha} \) and interacts with BvgA \( \sim P \) and RNA polymerase in a similar manner [40]. Since the expression of PT is temporally linked to that of adenylate cyclase toxin/haemolysin it is not surprising that the transcriptional machinery of these two toxins is alike; the \( P_{cya} \) promoter has been shown to behave in a manner which parallels that of \( P_{ptx} \) [42].

Differences in BvgA binding sites at the promoters of the two classes of virulence genes suggest that the specificity of BvgA–RNA polymerase interactions may control differences in transcription at these promoters [31,40–42,62].

3.5. ‘Bvg’

Some of the recent research involving the co-ordinate regulation by the \( bvg \) locus has been performed in the closely related species \( B. bronchiseptica \). While the \( bvg \)-repressed genes of \( B. pertussis \) were seldom studied and little is known about their function, \( B. bronchiseptica \) has an array of important functional \( bvg \)-repressed genes including motility, iron scavenging, urease and phosphatase activity which are at least in part repressed by the \( bvg \) locus [63–67].

Due to this expression of functional genes under modulating growth conditions, \( B. bronchiseptica \) is sometimes used as a model for \( B. pertussis \). An intermediate-phase \( B. bronchiseptica \) mutant has recently been identified which expresses phenotypes characteristic of both \( bvg \)-activated and \( bvg \)-repressed states [18]. Further investigation using polyclonal antisera from animals infected by the intermediate mutant revealed that under growth conditions described as semi-modulating, \( B. bronchiseptica \) expressed elevated levels of a novel class of gene products. The phase-locked ‘\( Bvg \)’ mutant has a C to T transition in the \( bvg \)S gene resulting in the threonine at position 733 of wild-type BvgS being changed to a methionine. The close proximity of this mutation to the putative phosphorylation site (His729) in the transmitter domain is thought to affect kinase activity [18].

3.6. The Ris system

The recent discovery in \( B. bronchiseptica \) of a second two-component regulatory system distinct from \( bvg \) has furthered our knowledge of genetic regulation in \( Bordetella \) spp. Like \( bvg \), this system, designated the \( ris \) locus (regulator of intracellular response), also consists of genes encoding a response regulator (RisA) and a sensor kinase (RisS) [68]. The \( ris \) locus is essential for bacterial resistance to oxidative stress and the production of acid phosphatase, as well as in vivo persistence. DNA sequence analysis of the \( B. pertussis \) and \( B. bronchiseptica \) \( ris \) locus has shown that there is only a single base difference in each of the \( risA \) and \( risS \) genes. However, the function of \( ris \) in \( B. pertussis \) has not yet been determined.

3.7. Bvg accessory factor

The temporally differential regulation employed by BvgA/S to control virulence factor expression in \( B. pertussis \) may require the presence of accessory factors. In \( E. coli \), the activation of either \( fhaB \) or \( bvgAS \) merely requires the presence of \( bvgAS \) in \( trans \) [37,69,70]. It was thought that this was not sufficient however for expression of PT or adenylate cyclase toxin [37,69,71]. When investigating the factors affecting the expression of PT, it was found that the activation of the \( bvg \) locus may depend on the topology of the DNA [72]. These authors also alluded to the possibility that the action of an as yet undiscovered auxiliary protein may be required to activate expression from the \( ptx \) promoter [72]. Stibitz [61] proposed a model suggesting the presence of an accessory protein, which interacts with the C-terminus of BvgA and may be required for \( cya \) and \( ptx \) activation. This protein has been characterised and termed the Bvg accessory factor (Baf). The expression of a \( ptx-lacZ \) fusion in \( E. coli \) requires BvgAS and Baf in \( trans \) [73]. The expression from \( P_{ptx} \) in \( E. coli \) has also been demonstrated without the presence of Baf, thereby contradicting the previous work and questioning the need for Baf [74].

4. Virulence factors of \( B. pertussis \)

The set of genes expressed by \( B. pertussis \) which allow it to invade and persist inside the host are termed virulence factors or virulence determinants. These include adhesins which facilitate attachment to target host cells and toxins which enable the bacterium to evade the host immune system. Mutagenesis studies have shown many of these adhesins and toxins are required for in vivo persistence in animal models [75–78]. At present the consensus with regard to the strategy for developing whooping cough vaccines seems to include a few purified immunogens in an acellular, multicomponent vaccine. Many of the virulence factors of \( B. pertussis \) have been considered for this purpose. Candidate antigens include FHA, serotype-specific fimbriae, pertactin, PT and adenylate cyclase toxin. Other virulence determinants of \( B. pertussis \) which have yet to be considered as vaccine antigens include derrmonecrotic toxin, tracheal cytotoxin, lipopolysaccharide (LPS), tracheal colonisation factor (Tcf) and the serum resistance locus.

4.1. Filamentous haemagglutinin

The critical step in \( B. pertussis \) infection is attachment of the pathogen to host cells. FHA is consistently referred to as the major adhesin of this bacterium. It is a 220-kDa surface-associated protein secreted to the extracellular environment to facilitate adherence to ciliated respiratory epithelial cells, thereby initiating the pathogenic cycle.
FHA can also work as a bridge adhesin, thereby facilitating the attachment of other micro-organisms [79]. This might in turn explain the superinfections which usually complicate the clinical course of whooping cough. As the name suggests, FHA has a filamentous structure, supported by electron microscopy studies giving the dimensions of the molecule as 2 nm wide and 45–50 nm long [80,81]. An extensive study by Makrov and coworkers [81] culminated in the proposal of a model of FHA: a polypeptide chain folded into a monomeric hairpin. The hairpin comprises head, shaft and tail regions. The model predicts that whilst the head contains the N- and C-termini, and the tail the important RGD (arginine-glycine-aspartic acid) sequence, the shaft is composed of tandem 19-amino acid residue repeat regions R1 (38 cycles) and R2 (13 cycles) which maintain the structural integrity of the molecule. This protein is unusual in a number of respects. Firstly, the gene for FHA, fhaB, has a coding potential of 367 kDa [82,83]. Over one third of this precursor protein is later cleaved in a complex post-translational maturation process to unveil the biologically active polypeptide (Fig. 5) [84]. FHA also possesses a number of possible binding sites and functions. A further interesting point regarding FHA is the level of regulation involved in producing and secreting the polypeptide.

4.1.1. Binding activities

The different domains of FHA encompass various binding capabilities, including integrin-mediated attachment to phagocytes, lectin-like binding to sulfated sugars found in the extracellular matrix (ECM) and on epithelial cells, and a carbohydrate recognition domain which allows attachment to ciliated cells present in the respiratory epithelium. The main target binding site for B. pertussis is the ciliated epithelium of the respiratory tract [85]. Attachment to cells in this region is mediated by the carbohydrate binding domain of FHA [67,86], which has a unique affinity for glycolipids (specifically lactosylceramide residues) and ciliated cells [87]. This carbohydrate recognition domain was narrowed down to amino acid residues 1141–1279 using monoclonal antibodies [87]. Recent studies of this region have enabled high-level, soluble expression in E. coli and further characterisation of this 18-kDa polypeptide, designated fragment A. Anti-fragment A antibodies were shown to inhibit binding of B. pertussis to ciliated rabbit cells in the same fashion as antibodies elicited by whole FHA, raising speculation that with further investigation, the more easily obtained fragment A may replace FHA in component vaccines against whooping cough [88].

The heparin binding activity of FHA may allow B. pertussis to bind to non-ciliated cells as demonstrated using WiDr cells, HeLa cells, and Vero cells [89,90], or perhaps facilitate interactions with the ECM. The lectin-like binding of FHA to heparin and dextran sulfate was characterised when it was shown that binding to epithelial cells could be inhibited by the addition of heparin [91]. Hannah and associates [92] extended this research by revealing that FHA also binds specifically to sulfated, but not uncharged glycolipids and mapped the heparin binding domain to residues 442–863 of the amino-terminal end of FHA. The tail region of FHA includes a binding moiety specific for complement receptor type 3 (CR3, CD11b/CD18) integrins present on the surface of macrophages [93]. The FHA tail region contains an RGD sequence which is present in a number of other bacterial adhesins and is a region of attachment in many eukaryotic integrin binding proteins [94,95]. It has been determined that it is this RGD tripeptide which facilitates binding to CR3 [93]. B. pertussis docks with integrin CR3, which may initiate entry into phagocytes without an oxidative burst. It has since then been shown that the binding process involves the formation of a leukocyte signal transduction complex which upregulates CR3 binding activity, suggesting that FHA could regulate the binding activity of its own receptor. This leukocyte signal transduction complex is composed of a receptor protein called leukocyte response integrin (LRI) and integrin-associated protein (CD47) [96]. These authors suggest that the carbohydrate/glycoconjugate binding capacity of FHA may also be involved in the primary stages of the CR3-LRI-CD47 complex formation. This hypothesis was formulated using the example of leukocyte–endothelial cell recognition [97] whereby a transitory state of attachment is mediated by lectins and this weak connection is strengthened by CR3-dependent binding. Hazenbos and associates [98] found that a macrophage surface fibronectin receptor, very late antigen-5 (VLA-5), may also be involved and proposed that binding and cross-linking of VLA-5 by B. pertussis activates CR3, facilitating attachment to FHA. Further study led to the finding that VLA-5 was acting as a ligand for the minor fimbrial subunit FimD [99]. These authors suggest that fimbriae and FHA may act co-operatively to bind to macrophages, leading to uptake and intracellular survival of B. pertussis.

The advantages gained by B. pertussis in binding to ‘professional phagocytes’ are concerned with evasion of the immune system and may be threefold. Severing cellular communication links to phagocytic cells by obstructing a vital receptor site (CR3, VLA-5 or C4BP) may reduce the impact of the immune system. Secondly, by attaching to CR3, B. pertussis has taken the preliminary step in entry and persistence in phagocytes, and ‘hand-to-hand combat’ using CR3 as a site from which to deliver bacterial toxins at close range may kill macrophages and neutrophils more economically [100,101]. Finally, B. pertussis FHA binds the serum protein C4BP [102]. This protein is known as a regulator of complement activation and acts to inhibit the classical complement pathway, ceasing the formation of the membrane attack complex. How B. pertussis is able to utilise this binding capacity remains unclear, but once
4.1.2. Genetic regulation and biogenesis

Being a virulence determinant of \textit{B. pertussis}, FHA undergoes a high level of genetic regulation. The \textit{bvg} locus controls expression of \textit{fhaB} depending on the prevailing environmental conditions. When \textit{B. pertussis} is in suitable conditions for colonisation, FHA is one of the first proteins produced, detected in a matter of minutes \cite{60}. Production of FHA is also dependent on the expression of at least one accessory protein, FhaC, the gene for which lies downstream of \textit{fhaB} in a cluster of accessory genes involved in the export of both FHA and fimbriae (Fig. 6) \cite{103,104}. FhaC is a pore-forming outer membrane protein that interacts with FhaB allowing for secretion of the adhesin \cite{105-107}.

4.1.3. Post-translational maturation

The DNA sequence of the structural gene \textit{fhaB} has been determined \cite{82,83,86}. The gene consists of approximately 10.1 kb and encodes a protein of 367 kDa termed FhaB. This precursor must undergo a series of modifications which are still only partially understood before mature FHA is secreted to the extracellular environment.

Toward the N-terminus of FhaB lies a domain of 115 residues, bearing homology to regions of the haemolysins of \textit{Serratia marcescens} and \textit{Proteus mirabilis}, and which are required for secretion of these proteins \cite{82}. Secretion of each of these proteins is also dependent on the presence of a homologous accessory protein (FhaC in \textit{B. pertussis}) \cite{107}. Recent studies have shown that the structure of FhaC contains 19 membrane spanning $\beta$-strands that probably allow the molecule to form a $\beta$-barrel in the outer membrane \cite{106,108} (Fig. 5). An FhaC-like outer...
membrane protein, HMWB, has since been found in *Haemophilus influenzae*, where its function is also to aid in the export of an adhesin, HMWA [109]. The precursors of FHA and HMWB share an extra region of homology comprising 22 amino acid residues, followed by a positively charged region and a hydrophobic segment. This acts as an atypical signal peptide and is cleaved from the molecule prior to export [83,105]. Although this is an unusually large signal peptide (71 amino acid residues), it has recently been shown to undergo proteolysis at a leader peptidase (Lep) consensus cleavage site, suggesting that FhaB crosses the inner membrane via the Sec-dependent pathway [110]. The *B. pertussis* *lep* gene has recently been cloned and characterised [111], and future studies may determine whether Lep is an absolute requirement for FHA secretion in *B. pertussis*.

The C-terminal third of the precursor protein is also removed leaving mature FHA comprising the N-terminal 220 kDa of FhaB. Although not part of the mature protein, the C-terminus plays an important role in secretion [112] possibly as a type of intramolecular chaperone to stabilise the protein and prevent incorrect folding, allowing the required secretory interactions to proceed. Thus the current model of FHA maturation and secretion involves the precursor FhaB translocating the inner membrane to the periplasmic space, probably utilising the *B. pertussis* leader peptidase (Lep) and Sec machinery. The 115-residue, haemolysin-homologous domain then interacts with FhaC (Fig. 5). As the N-terminal portion passes through the FhaC-derived β-barrel outer membrane pore, the 150-kDa C-terminal domain is cleaved liberating mature FHA, which then folds into the rigid hairpin structure proposed by Makhov et al. [81].

### 4.2. Serotype-specific fimbriae

Fimbriae, also known as pili and agglutinogens, are long filamentous protrusions which extend from the bacterial cell surface and facilitate a variety of binding capabilities. The fimbriae of *B. pertussis* incorporate both major and minor subunits. The major subunits form the fimbrial strand, being grouped into pentameric repeat units, each 13 nm in length and comprising two full helical turns [113]. This finding has been supported and extended by more recent research [114] which also obtained an accurate value of 5.7 nm for the diameter of *B. pertussis* fimbriae.

*B. pertussis* produces two distinct types of fimbriae, serotype 2 and serotype 3 (also called serotype 6). These are constructed using major subunits, expressed from the *fim2* and *fim3* genes respectively, and minor fimbrial subunit, encoded by *fimD* [103,104]. Major subunits are stacked to form the long filamentous structure characteristic of fimbriae and the minor subunit is located at the tip [115].

#### 4.2.1. Major subunits

The major fimbrial subunits which form the structural helices, Fim2 and Fim3, are proteins of 22 kDa and 22.5 kDa respectively. The *fim2* and *fim3* genes which encode these proteins have both been cloned and sequenced [116,117] and exhibit considerable homology at both the nucleotide and amino acid level. Two other pseudogenes related to the major fimbrial subunits have also been found in *B. pertussis*, *fimX* and *fimA* [104,118]. The *fimX* gene encodes a protein of approximately 20 kDa which is expressed at very low levels if at all [119]. The other silent fimbrial gene, *fimA*, is located in the cluster of fimbrial and FHA accessory genes (Fig. 6) [104]. This is a region of DNA with sequence homology to *fim2*, *fim3* and *fimX*, however the 5’ end of the gene corresponding to the N-terminal region of FimA is absent in *B. pertussis*. Recently, it was found that *B. bronchiseptica* expresses a fully intact version of *FimA* [120].

The low-level expression of FimA seen in *B. bronchiseptica* is *bvg*-regulated. It has been postulated that the two functional major fimbrial subunit genes and the pseudogene *fimX* may have arisen via gene duplication. The discovery of *fimA*, its chromosomal location within the fimbrial operon and its expression in *B. bronchiseptica* suggest that *fimA* may actually be the original, ancestral major fimbrial subunit from which *fim2* and *fim3* arose [104,120].

Since the discovery of the minor subunit and adhesin FimD, the role of the major subunits in adherence has been somewhat downgraded to almost being regarded as merely the scaffolding on which sits the important adhesin molecule. Recently it has been shown, however, that the major fimbrial subunit has an affinity for sulfated sugars [121], an ability shared by the other principal adhesin molecule, FHA. The association between *B. pertussis* fimbriae and derivatives of heparan sulfate is highly dependent on both the number of sulfate groups present and their placement around the disaccharide molecule [121]. The two Fim2 heparin binding regions (H1 and H2) have since been fully characterised [122]. These regions...
share sequence and structural homology with each other and with similar heparin binding sites of fibronectin, a eukaryotic ECM molecule.

The major antigenic domains present on Fim2 and Fim3 fimbriae have been identified using monoclonal antibodies to screen various synthetic peptides [123]. These epitopes were again confirmed as highly immunogenic when they were exposed to, and recognised serum anti-

bodies to screen various synthetic peptides [123]. These

Fim3 fimbriae have been identified using monoclonal anti-

eukaryotic ECM molecule.

4.2.2. Minor subunit and accessory proteins

The other genes found in the fimbrial cluster (Fig. 6) are designated fimB, fimC and fimD [103,104]. The proteins predicted within this operon are similar to proteins essential for fimbrial biogenesis in both E. coli (Pap) and Klebsiella pneumoniae (Mrk). The genetic organisation of the entire gene cluster is very similar to the mrk operon [125], perhaps suggesting a common origin. Another similar fimbrial gene cluster has also been found in H. influenzae type b [126]. An even greater level of homology to fimB, fimC and fimD is demonstrated by these hif genes. The most interesting aspect of this report was the inference that the entire cluster represents an ancestral mobile genetic element and was originally inserted via a transposition event.

The predicted amino acid sequence of FimB is homolo-
gous to the PapD superfamily of periplasmic chaperones [103,104]. A putative signal peptide sequence directs the newly translated FimB to the periplasm, where the signal sequence is cleaved, leaving a mature polypeptide of approximately 24 kDa [103,104]. Southern hybridisation of B. pertussis, B. parapertussis and B. bronchiseptica DNA disclosed regions homologous to fimB [104] in these closely related species, suggesting that all three may utilise a similar process to escort fimbrial proteins to the cell surface.

FimC is a protein with predicted homology to many outer membrane fimbrial accessory proteins, including PapC, MrkC and HiC, all of which are essential for the biogenesis of fimbriae in their respective organisms. The molecular mass of this predicted protein is approximately 91 kDa after signal peptide cleavage [103,104]. Based on homology data, it was proposed that FimC is probably located in the outer membrane, involved in fimbrial transport and anchorage [103,104].

Much of the more recent research into the fimbriae of B. pertussis has concentrated on the minor fimbrial subunit and primary adhesin, FimD. Lying directly upstream of fimC, the fimD gene encodes a fimbrial adhesin protein with a molecular mass of approximately 40 kDa [127]. Like its genomic neighbours, fimD exhibits homology to genes in the fimbrial clusters of H. influenzae (hi/C), K. pneumoniae (mrkD) and E. coli (fimH) [103,104,126, 128,129]. A fimD homologue is also present in B. parapertussis and B. bronchiseptica [127]. In fact, the corre-
sponding gene in B. bronchiseptica only differs by a single base pair change. There are 20 different amino acids in the FimD polypeptide of B. parapertussis resulting from 34 base pair differences. The differences present in the FimD adhesins of B. pertussis and B. parapertussis may create an evolutionary advantage for both by eliminating binding site competition while sharing the same human host [127].

Binding studies involving fimbrial mutant strains lacking FimD have demonstrated that B. pertussis binds to human monocytes via FimD [99]. Known to interact with B. pertussis fimbriae, VLA-5 has since been confirmed as the FimD receptor on the surface of monocytes [130]. The construction of ‘true’ fimbrial mutants, devoid of both major and minor fimbrial subunits, allowed further investigation [115]. A mouse colonisation model was utilised to show that FimD plays a vital role in colonisation. The Fim2/3−/FimD− mutant colonised the lungs and trachea of mice significantly less efficiently than wild-type, FHA− mutant, and Fim2/3+/FimD− mutant strains [115]. These researchers also demonstrated the binding of FimD to the sulfated sugar heparin, also a possible target molecule for FHA and major fimbrial subunits [91,121, 131].

4.2.3. Genetic regulation

Like that of FHA, fimbrial expression in B. pertussis is regulated at a number of levels. As with other virulence factors employed by B. pertussis to attach to host cells, the expression of fimbriae is controlled by the bvg locus. However, the correct processing and secretion of fimbrial subunits requires the expression of a number of accessory genes, which are located in the fimbrial operon (Fig. 6). B. pertussis produces two distinct fimbriae: serotype 2 and serotype 3. Some strains (serotype 2,3) express both simultaneously [132]. The serotype expressed depends on the level of transcription of fimbrial major subunit genes. Alteration of serotype involves switching promoter activation between the two fimbrial subunit genes in a process termed fimbrial phase variation. The genes encoding the major fimbrial subunits, fim2 and fim3, with which the fimbriae are constructed have been fully characterised [116,117] as have the homologous but transcriptionally inactive fimX and fimA genes [103,104,118]. Sequence data have revealed a high level of homology between all of these genes which extends into the promoter regions, unveiling some interesting features of fimbrial promoter architecture (Fig. 7).

The most noticeable aspect of the fimbrial promoters is the ‘C stretch’, a long run of cytosine residues which has been implicated in fimbrial phase variation [133]. Insertion or deletion of extra cytosine residues in this C stretch is the source of fimbrial phase variation [133]. It is thought that the distance between the putative −10 box and the activator (BvgA) binding site is vital for transcriptional activation, and the alteration in the number of Cs in the
stretch changes this distance [133]. The reason for the lack of transcription from the fimX pseudogene may be the short C stretch in the promoter region.

The expression of the fimbrial accessory genes is also required for the correct delivery and presentation of B. pertussis fimbiae at the cell surface [103,104]. A mutation-al analysis of the fimA/fha gene cluster has provided evidence that fimC, fimD and fhaC are translationally linked. These three genes overlap slightly and there is a putative ribosome binding (Shine-Dalgarno) site directly upstream of the first gene, fimC [127].

4.3. Pertactin

Also known by the aliases p.69 and OMP 69 because of its electrophoretic mobility in SDS-PAGE, pertactin is actually a 60-kDa outer membrane protein involved in bacterial adherence [134,135]. Similar molecules are produced by other members of the genus, p.70 in B. parapertussis [136] and p.68 in B. bronchiseptica [137].

The crystal structure of pertactin has been elucidated, revealing a 16-stranded parallel β-helix with a V-shaped cross-section [138]. The secondary structure of pertactin contains two immunodominant direct repeat regions. The first, (Gly.Gly.Xaa.Xaa.Pro)₅, is located directly after the important RGD tripeptide and the other, (Pro.Gln.Pro)₅, thought to be the major immunoprotective epitope, lies toward the C-terminal end [139,140].

Pertactin is transcribed from the prn gene, which encodes a 93.5-kDa polypeptide comprising 910 amino acids [139]. This precursor, termed p.93, later undergoes the removal of a 34-amino acid N-terminal signal peptide [135] and cleavage of a 30-kDa polypeptide (p.30) from the C-terminus. Although the precise role of p.30 is unclear, it is detected in outer membrane fractions and is likely to be involved in the export of pertactin to the outer membrane [141]. In a comparison of the prn gene sequences of B. pertussis, B. parapertussis and B. bronchiseptica, the precursors of the pertactins were found to be extremely homologous and interestingly, the most highly conserved region is the C-terminus, suggesting the functional relevance of p.30 to these organisms [142].

The mechanism by which pertactin promotes adherence to eukaryotic cells is unknown and no receptor has been found. The amino acid sequence revealed an RGD motif (amino acids 225–227), a known integrin binding moiety present in many bacterial adhesins such as FHA and eukaryotic ECM proteins including fibronectin and vitronectin [93,143]. It was assumed that this region facilitates binding to mammalian cells. Mutants deficient in pertactin expression adhered 30–40% less well to CHO (Chinese hamster ovary) cells and HeLa cells [134]. Synthetic peptides containing RGD derived from pertactin were found to inhibit pertactin binding to epithelial cells and reduce B. pertussis entry into HeLa cells [134,144]. However, a recent study has found no role for the pertactin RGD sequence as a mediator of eukaryote cell invasion [145]. These researchers used site-directed mutagenesis (aspartic acid to glutamic acid) to obtain a pertactin molecule with an RGE site rather than RGD. Strains expressing the mutation displayed no difference in their ability to promote adhesion to HEp-2 or CHO cells. It would be useful to determine the role of pertactin in wild-type B. pertussis infection, which would lead to a better understanding of whooping cough and the vaccines which prevent it.

It has been demonstrated by a number of groups that pertactin is an immunoprotective antigen, being used in subunit and live oral vaccines to protect mice from respiratory challenge with virulent B. pertussis [146–150]. Many human vaccine trials suggest that the inclusion of pertactin in the formulations is imperative. Although the role of pertactin in vaccine-based immunoprotection is unknown, preparations including pertactin have attained promising vaccine efficacy results [151,152].
4.4. Pertussis toxin

A member of the A-B bacterial toxin superfamily, PT is a 106-kDa, hexameric protein comprising five distinct subunits [153]. PT is an ADP ribosyltransferase [154] with the ability to cause a plethora of effects on host cells. It is the A protomer or S1 subunit which possesses enzymatic capabilities, catalysing the transfer of the ADP-ribose moiety of NAD\(^+\) to a family of G proteins, which are involved in signal transduction pathways within the host cell. This short-circuits the cell signalling machinery of epithelial and immune system cells by breaking down G protein interactions [154]. The B oligomer comprises the remaining subunits; S2, S3, S4 and S5 make up the B oligomer in the ratio 1:1:2:1 [153]. This region of the protein facilitates attachment of the toxin to host cells, delivering the toxic action of the S1 subunit [153].

The genes encoding the PT subunits are clustered together in an operon typical of many other bacterial toxins (Fig. 8). Genetic analysis has revealed that each subunit is translated separately with an amino-terminal signal sequence which is cleaved during transport to the periplasm where the holotoxin is then assembled and secreted [155,156].

A major hurdle yet to be overcome in the preparation of acellular vaccines is the inability to obtain purified antigens in large amounts. The incompatibility of PT gene expression signals with respect to E. coli makes the production of PT in this organism difficult to achieve [157,158]. There are also problems associated with obtaining high yields of purified PT from virulent B. pertussis. These include the slow growth rate, fastidious nutritional requirements and the inherent low PT production levels of the bacterium. There is also the risk that the PT operon is positively regulated by the bvg locus, are major reasons why pertussis holotoxin is yet to be expressed in E. coli. Expression of PT subunits has been achieved in E. coli and Bacillus subtilis [158,160,161]. Unfortunately, expression of PT subunits in E. coli caused the majority of the protein to congregate in inclusion bodies, and not be secreted into the periplasm [158]. The other major problem with expression of subunits for vaccine purposes is their inability to assemble into holotoxin in vitro. The individual PT subunits have been shown to induce antibody responses in immunised mice. However, these mice were not protected when challenged intracerebrally with B. pertussis [158]. Simultaneous expression of all five PT subunits has been achieved in E. coli and Salmonella typhimurium aroA [162,163]. However, varying levels of expression and post-translational processing, together with a lack of protection in orally immunised mice, left these strains unsuitable for vaccine production. In a significant recent study the PT S1 subunit expressed in Mycobacterium bovis BCG has been shown to induce protection against live B. pertussis in the mouse intracerebral challenge model [164]. This reinforces that the system of delivery and the induction of an appropriate immune response are very important elements of vaccine design.

Efficient expression of pertussis holotoxin has to date only been achieved in Bordetella species. The major concern here is that PT production takes place only in virulent strains of B. pertussis. Co-purification of other toxins and thus contamination of vaccine preparations may therefore occur. Lee and associates [165] have used recombinant plasmids to express PT in B. parapertussis and B. bronchiseptica. Yields were comparable to those of B. pertussis and although the majority of the PT produced was localised in the periplasm, PT was detected in the culture supernatant. B. parapertussis and B. bronchiseptica have also been used to produce genetically altered detoxified PT [166,167]. Bacterial strains engineered to produce a safe pertussis ‘toxoid’ are very good candidates for the production of acellular vaccines. Overexpression and secretion of genetically detoxified PT has been achieved in B. pertussis [168] by using allelic exchange techniques to introduce multiple copies of a genetically altered PT operon into the chromosome. Fully inducible production of PT in B. bronchiseptica has resulted in improved growth rates and PT yields when compared to B. pertussis [169]. Expression of PT was achieved in a permanently avirulent strain of

\[
\text{P}_\text{et} \quad S1 \quad S2 \quad S4 \quad S5 \quad S3 \quad A \quad B \quad C \quad D \quad I \quad E \quad F \quad G \quad H
\]

Fig. 8. Schematic representation of the ptx/pl operon. PT subunit genes (open boxes; S1–S5) and PT liberation genes (shaded boxes; A-I) are indicated. The ptx/pl promoter (filled arrow; P\text{et}) is also depicted. Modified from Smith and Walker [171]; Farizo et al. [173].

\[\text{E. coli} \text{ consensus ribosome binding site. This, and the fact that the PT operon is positively regulated by the bvg locus, are major reasons why pertussis holotoxin is yet to be expressed in E. coli. Expression of PT subunits has been achieved in E. coli and Bacillus subtilis [158,160,161]. Unfortunately, expression of PT subunits in E. coli caused the majority of the protein to congregate in inclusion bodies, and not be secreted into the periplasm [158]. The other major problem with expression of subunits for vaccine purposes is their inability to assemble into holotoxin in vitro. The individual PT subunits have been shown to induce antibody responses in immunised mice. However, these mice were not protected when challenged intracerebrally with B. pertussis [158]. Simultaneous expression of all five PT subunits has been achieved in E. coli and Salmonella typhimurium aroA [162,163]. However, varying levels of expression and post-translational processing, together with a lack of protection in orally immunised mice, left these strains unsuitable for vaccine production. In a significant recent study the PT S1 subunit expressed in Mycobacterium bovis BCG has been shown to induce protection against live B. pertussis in the mouse intracerebral challenge model [164]. This reinforces that the system of delivery and the induction of an appropriate immune response are very important elements of vaccine design.

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\text{P}_\text{et} \quad S1 \quad S2 \quad S4 \quad S5 \quad S3 \quad A \quad B \quad C \quad D \quad I \quad E \quad F \quad G \quad H
\]

Fig. 8. Schematic representation of the ptx/pl operon. PT subunit genes (open boxes; S1–S5) and PT liberation genes (shaded boxes; A-I) are indicated. The ptx/pl promoter (filled arrow; P\text{et}) is also depicted. Modified from Smith and Walker [171]; Farizo et al. [173].
4.5. Adenylate cyclase toxin/haemolysin homologous recombination results in the expression and Pasteurella haemolytica leukotoxins of various pathogenic Gram-negative bacteria. Calmodulin is, however, crucial for the enzyme once inside the cell, where the formation of ultraphysiological levels of cyclic AMP is catalysed [186,187].

The entire *B. pertussis* *ptl* operon, the products of which direct the secretion of PT to the culture supernatant, has been cloned and sequenced [172]. There were originally thought to be eight ORFs present in the operon, designated *ptlA*–*ptlH* [172]. Evidence for the presence of a ninth gene, *ptlI*, has since been found (Fig. 8) [173].

Partial *ptl* sequence data of *B. parapertussis* and *B. bronchiseptica* can be compared to the equivalent genomic region of *B. pertussis* [155,156,172]. Fusion proteins expressed in *E. coli* have been used to raise polyclonal antibodies against the predicted *PtlA*, *B*, *C*, *E*, *F*, *G* and *H* proteins [174]. Immunoreactive bands in Western blots of *B. pertussis* whole-cell extracts were only detected using polyclonal antibodies against *PtlE*, *F* and *G*. Replacement of the *B. bronchiseptica* *Ppx* promoter with the *B. pertussis* *Ppx* promoter results in detectable expression of *Ptf* in Western blots in *B. bronchiseptica* [175]. These results suggested that *Ppx* actually drives the transcription of the complete *ptx*/*ptl* region. This was confirmed when the 11-kb mRNA for the entire *ptx*/*ptl* operon was detected in *B. pertussis* [176]. The introduction of the *B. pertussis* *Ppx* promoter together with a portion of the PT S1 subunit gene into *B. bronchiseptica* and *B. parapertussis* via homologous recombination results in the expression and secretion of this heterologous toxin [177].

4.5. Adenylate cyclase toxin/haemolysin

The adenylate cyclase-haemolysin of *B. pertussis* is a bifunctional protein belonging to the RTX (repeat in toxin) family. This assemblage also includes the much studied α-haemolysin of *E. coli*, together with haemolysins and leukotoxins of various pathogenic Gram-negative bacteria including *Pasteurella haemolytica*, and *Actinobacillus species* [178]. Both the adenylate cyclase and the haemolytic activities have been shown to be essential for the initiation of *B. pertussis* infection [179]. Adenylate cyclase-haemolysin is a secreted polypeptide chain consisting of 1706 amino acid residues [180], with a molecular mass of between 175 and 220 kDa [181–183].

The adenylate cyclase activity of this protein is located within the first 400 amino acids at the amino-terminal end of the protein and is activated by calmodulin [180,184]. Although the high affinity of adenylate cyclase toxin/haemolysin for calmodulin was initially thought to be involved in the translocation of the toxin into host cells, recent evidence has proven this to be incorrect [185]. The calmodulin is, however, crucial for the enzyme once inside the cell, where the formation of ultraphysiological levels of cyclic AMP is catalysed [186,187].

The remaining 1300 residues contain an independently functional haemolysin [180,188] among other structurally and functionally important domains. A repeat domain of adenylate cyclase toxin/haemolysin contains 41 repeats of the glycine- and aspartic acid-rich regions indicative of the RTX family. Homology with other RTX members suggests that this region is involved in host cell recognition and calcium binding [189,190]. In fact, each repeat motif binds a single calcium ion, leading to a major conformational change and possibly the translocation of the catalytic portion of the molecule into host cells [191].

The gene encoding the adenylate cyclase toxin/haemolysin (*cyaA*) is contained within an operon, together with a number of other factors involved in toxin activation and secretion [180] (Fig. 9) [192]. Toxin secretion from *B. pertussis* will only proceed with the expression of *cyaB*, *cyaD* and *cyaE* [193]. The final member of the *cya* operon, *cyaC*, is required to activate adenylate cyclase toxin/haemolysin, and is involved in post-translational modification of the toxin [194] (Fig. 10). This protein causes the acylation of adenylate cyclase toxin/haemolysin resulting in the addition of a palmitoyl fatty acid residue to Lys983 of the polypeptide chain [195] (Fig. 10).

Host cell invasion by adenylate cyclase toxin/haemolysin is not effected via a receptor-mediated endocytotic pathway [196], and instead uses a specialised calcium- and temperature-dependent entry system which is not yet fully understood [197].

The use of a detoxified form of adenylate cyclase toxin/haemolysin in acellular vaccine preparations has been advocated by a number of groups [198–200]. Immunisation with adenylate cyclase toxin/haemolysin has been shown to be protective against bacterial colonisation in animal models [199,201,202]. Humans infected with *B. pertussis* are known to produce antibodies against adenylate cyclase toxin/haemolysin to fight the infection [198]. The interaction between adenylate cyclase toxin/haemolysin and aspects of the human immune system has been the focus of many research groups. Invasion and survival of *B. pertussis* into many different immune cells is now well documented [100,203,204]. The inhibition of antigen-dependent T-cell proliferation by *B. pertussis* has recently been dem-
onstrated and necessitates the presence of adenylate cyclase toxin/haemolysin [120]. The potential of recombinant adenylate cyclase toxoids as vaccine carrier proteins has recently been highlighted due to their ability to deliver heterologous epitopes fused to their N-terminal domain into the cytosol of antigen presenting cells [205]. This system is still being refined, however it has been used to deliver CD8+ and CD4+ T-cell epitopes to antigen presenting cells to induce antiviral responses [205,206]. This may be particularly useful for the presentation of vaccine antigens when a polarised Th1 immune response is required for protection.

The role which adenylate cyclase toxin/haemolysin plays in apoptosis has been demonstrated in vitro and in vivo in a number of different cell types [207–209]. It has been proposed that this may be a method by which B. pertussis might escape the antibacterial effects of macrophages [207]. Alternatively, it could be an action facilitated by the immune system to remove the bacterium from their possibly safer intracellular niche and expose it to the rigours of the extracellular environment, patrolled by killer cells and antibodies.

4.6. Dermonecrotic toxin

Although known for many years [210], this toxin has not been as extensively studied as other Bordetella virulence determinants. This may be due, in part, to difficulty in the purification process. Terminated heat-labile toxin because it becomes inactive at 56°C, it was given its current name due to its ability to cause necrotic skin lesions when injected subcutaneously into mice [211,212]. This injection is lethal when given intravenously. Dermonecrotic toxins are produced by Bordetella species and are regulated by the bvg locus [210,213–215]. The toxin is not secreted to the extracellular environment and has been localised to the cytoplasm [216]. This and its very low expression levels are probably the major purification problems. Later studies agree on a molecular mass of 140 kDa and, contrary to previous reports, a single polypeptide chain [217,218].

The gene encoding the B. pertussis dermonecrotic toxin was sectionally cloned and sequenced [219]. The toxin can be placed by homology and functionality into a family of toxins including the cytotoxic necrotising factors of E. coli, botulinum C3 toxin and Pasteurella multocida toxin [220–223]. All members of this family induce dormant cells to begin DNA synthesis, leading to either increased cell division or multinuclear cells, as is the case with dermonecrotic toxin. The mechanism of action has recently been determined. The C-terminal part of dermonecrotic toxin deamidates a glutamine residue (Glu-63) of Rho protein [224]. Although dermonecrotic toxin is considered a virulence factor, mutants deficient in dermonecrotic toxin do not exhibit any differences in virulence in mouse studies.
4.7. Tracheal cytotoxin

The major biological activity endowed by this toxin is the ability to damage ciliated respiratory epithelial cells [225]. Tracheal cytotoxin is a disaccharide-tetrapeptide derivative of the cell wall peptidoglycan layer. Both structurally and functionally, tracheal cytotoxin falls into the muramy l peptide family. Gram-negative bacteria produce a layer of peptidoglycan, however only Bordetella and Neisseria release fragments extracellularly, in the form of muramy l peptides, normally during exponential growth [226,227].

The destruction of cilia and ciliated epithelial cells by tracheal cytotoxin causes cell lysis and forces the infected individual to cough relentlessly in order to remove mucus, a task ordinarily performed by ciliated cells. It has been demonstrated in hamster trachea epithelial cells that the lactyl tetrapeptide portion of the molecule is responsible for its full toxic activity [228]. The toxicity conferred by tracheal cytotoxin is indirect, being caused by the induction of host cells to produce interleukin-1 [229]. This activates host cell nitric oxide synthase leading to high levels of nitric oxide radicals [230]. It is still not certain whether it is the tracheal cytotoxin or the interleukin-1 which stimulates nitric oxide synthase. The nitric oxide acts by destroying iron-dependent enzymes, eventually inhibiting mitochondrial function and DNA synthesis in nearby host cells [229]. Since B. pertussis is attached to the ciliated epithelial cells, it is these which undergo the most damage. Tracheal cytotoxin also has a toxic effect on other cells, impairing neutrophil function at low concentrations and conferring toxic activity in larger quantities [231]. Evolutionarily, the production of tracheal cytotoxin by B. pertussis may merely represent a breakdown by-product of peptidoglycan manufacture. However, it is considered an integral component in the pathogenesis of B. pertussis infection.

4.8. Lipopolysaccharide

The LPS molecule of B. pertussis is smaller than many other bacterial LPS structures and is therefore often referred to as a lipooligosaccharide. The role which LPS plays in the pathogenesis of B. pertussis infection is unclear; however, biological activities exhibited by B. pertussis LPS include antigenic and immunomodulating properties [232,233]. The most common action associated with LPS is the potent endotoxin activity [234].

Present in most Gram-negative bacteria, LPS generally comprises lipid A, core oligosaccharide and a long polysaccharide O antigen. The structure of B. pertussis LPS differs in that it lacks the O antigen. B. pertussis actually produces two types of LPS, designated A and B [235]. These molecules possess differing electrophoretic mobilities due to the presence of an extra trisaccharide moiety on LPS-A [236]. All of the species of Bordetella express different LPS molecules, which may be a factor in the high level of species specificity demonstrated within this genus [237]. B. parapertussis strains isolated from humans and sheep display distinct LPS profiles [237] adding to the host-specific feature of this molecule [238,239]. The LPS produced by B. bronchiseptica is similar to that of B. parapertussis, as they both express temperature-dependent O antigen [237,240]. The charged O antigen of B. bronchiseptica is thought to be the element which confers protection against antimicrobial peptides since B. pertussis, which lacks O antigen, is not protected [241]. B. bronchiseptica also produces host-specific LPS molecules, with isolates from dogs having more heterogeneous LPS structures than isolates from pigs [237]. A human isolate of B. bronchiseptica displays a different LPS profile than that of a rabbit isolate [207,242]. Subsequent isolation of B. bronchiseptica from the same human patient over 2 years has shown a variation in LPS profiles during the course of infection [207].

Transposon mutagenesis has allowed the identification of a gene cluster encoding LPS biosynthesis in B. pertussis [243]. Many of these genes are similar to polysaccharide and LPS synthesis genes present in other bacteria, however the B. pertussis LPS operon is arranged in a unique manner [243].

Unfortunately, our knowledge of the B. pertussis LPS remains limited and further research is necessary before we are able to define the involvement of this molecule in both the pathogenesis of disease caused by this bacterium, and the immunogenicity and adjuvanticity of vaccines targeted against whooping cough.

4.9. Tracheal colonisation factor

Originally discovered with the aid of a transposon-mutated strain which was deficient in four outer membrane proteins [244], Tcf is a bvg-regulated protein, exclusively produced among the Bordetellae by B. pertussis. The gene encoding Tcf, tcfA, was first mapped using a TnphoA insertion mutation and was termed virulence-activated gene #34 (vag34) [244]. The original TnphoA mutants were 10-fold less able to colonise and persist in the trachea of mice, but were not significantly disadvantaged in the lungs [245]. The tcfA gene has now been cloned and sequenced, with the derived amino acid sequence predicting a 68.6-kDa precursor, the first 39 amino acids of which comprise a likely signal peptide, leaving a 64.4-kDa protein [245]. The C-terminal half of Tcf exhibits over 50% homology to the C-terminus of the precursors of the pertactins of Bordetella species, including the Lys-Arg puta-
5. Elicitation of protective immune responses against B. pertussis

The development of whooping cough vaccines has concentrated on the elicitation of high antibody titres and protective immunity, and on the reduction of side-effects. Recently, a number of issues have been brought to light which also bear serious consideration in the preparation of whooping cough vaccines.

5.1. Cell-mediated immunity

One of the major challenges in developing superior vaccines is our limited understanding of the mechanisms involved in the development of immunity against pertussis following either natural infection or vaccination. Which components of the human immune system become primed to fight B. pertussis infection after vaccination? Defence against intracellular pathogens is usually dependent on the activation of a strong Th1 helper response. B. pertussis has been shown to invade an increasing number of cell types [100,249,250] and is now considered by some a facultative intracellular pathogen. Intracellular bacteria may be protected from host clearance mechanisms resulting in the infection persisting. It is therefore reasonable to assume that cell-mediated immunity (CMI) may play a key role in eliminating B. pertussis infection, at the very least by facilitating the release of bacteria surviving within phagocytes. Studies have demonstrated the importance of CMI in natural and vaccine-based immunity [251,252]. Immunisation of mice with whole-cell vaccine or recovery from natural B. pertussis infection induces a Th1 response, whereas immunisation with an acellular vaccine elicits a Th2 response [252]. Production of interleukin-12 is induced by native infection or whole-cell vaccination, but not by acellular vaccination [253].

Recent studies have shown that protection after respiratory challenge in the murine model is an excellent correlate for efficacy in humans of acellular and whole-cell vaccines, and that humoral and cell-mediated immunity play complementary roles in vaccine-elicited protection [254]. Using different knockout mice, Mills and associates [255] found that both humoral and cell-mediated responses are required for the stimulation of protective immunity following vaccination. The initial response may be antibody-mediated and is involved in restricting the extent of infection and minimising the damage to epithelial and immune cells. To achieve complete bacterial clearance however, the induction of a CMI response is also required. The Th2 response elicited after immunisation with an acellular vaccine results in delayed bacterial clearance in the murine respiratory challenge model. In contrast, natural infection or immunisation with killed whole cells, inducing a Th1 response, was associated with rapid clearance [255]. However, analyses of the CMI responses of children in recent vaccine efficacy trials revealed that acellular vaccines induce Th1 responses [256,257] equivalent to responses exhibited after natural infection [258].

5.2. Development of vaccine resistance

Despite the time spent on research into mechanisms of immunity, efficacy and reactogenicity of vaccines against
B. pertussis, the bacterium itself has been fighting back. Recent data from The Netherlands reported a dramatic increase in the incidence of pertussis in that country in 1996, despite high levels of immunisation [259]. An extensive study has traced the problem to the changing population structure of B. pertussis itself [260]. The antigenic variation of the protective antigens pertactin and the S1 subunit of PT was analysed using circulating clinical isolates collected over a long period. Three different variants of each pertactin and S1 were discovered and the presence of these variants showed temporal shifts. Polymorphism in pertactin was found to occur in the midst of a repeat region (Gly.Gly.Xaa.Xaa.Pro)₃ termed region I. The mutations in the S1 subunit were similarly confined to a small region of the polypeptide. The mutations in S1 and pertactin are concentrated in regions defined as T-cell and B-cell epitopes respectively [140,261]. This highlights the importance of maintaining a constant global monitoring of circulating isolates and the necessity to adapt present formulations according to temporal antigenic shifts.

6. Concluding remarks

B. pertussis is exquisitely fine-tuned to inhabit the human respiratory tract. This has been achieved via an evolutionary process. The twentieth century had caught up with this pathogen somewhat, with the advent of antimicrobials and immunisation, and it was on the way out. Now it is again evolving and re-emerging as a dangerous pathogen. Together with the attention to vaccine reactogenicity, the induction of appropriate immune response mechanisms and raising the protective efficacy of future anti-pertussis vaccines, we must also consider the rapid evolution of B. pertussis in the development of future whooping cough vaccines.

An exciting development in B. pertussis research is the ongoing genome sequencing project of this bacterium by the Sanger Centre, details of which are available on their website [262]. This will undoubtedly facilitate the development of better vaccines against whooping cough.

References


terization of a Bordetella pertussis fimbrial gene cluster which is located directly downstream of the filamentous haemagglutinin gene. Mol. Microbiol. 6, 2661–2671.


[120] Charles, I.G., Dougan, G., Pickard, D., Chatfield, S., Smith, M.,


