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MECHANISMS OF *BORDETELLA* PATHOGENESIS

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1. ABSTRACT

Bordetella are Gram negative bacteria that cause respiratory tract infections in humans and animals. While at least five different species of *Bordetella* are known to exist, this review focuses on *B. pertussis*, *B. bronchiseptica* and *B. parapertussis* subspecies. In their virulent phase, all of these bacteria produce a nearly identical set of virulence factors which include adhesins such as filamentous hemagglutinin (FHA), fimbriae and pertactin, as well as toxins such as a bifunctional adenylate cyclase/hemolysin, dermonecrotic toxin, tracheal cytotoxin, a *B. pertussis* specific pertussis toxin and *B. bronchiseptica* specific type III secreted proteins. Expression of nearly all of these virulence factors is positively regulated by the products of the *bvgAS* locus. BvgA and BvgS comprise a two-component signal transduction system that mediates transition between at least three identifiable phases --- a virulent (Bvg^+) phase, an avirulent (Bvg^-) phase and an intermediate (Bvg^i) phase --- in response to specific environmental signals. *Bordetella* colonize the ciliated respiratory mucosa, a surface designed to eliminate foreign particles, thereby making the adherence and persistence mechanisms of these bacteria crucial. The development of relevant animal models for *B. bronchiseptica* has enabled us to study *Bordetella* pathogenesis in the context of natural host-pathogen interactions. In addition, evolutionary studies across the various *Bordetella* species and detailed analysis of differential regulation of Bvg-activated/repressed genes has greatly enhanced our understanding of the mechanisms of *Bordetella* pathogenesis.

2. INTRODUCTION

Bordetella are small (0.2 micron X 0.7 micron), aerobic, Gram negative coccobacilli that cause respiratory tract infections in humans and animals. Historically, eight species of *Bordetella* have been identified, namely, *B. pertussis*, *B. bronchiseptica*, *B. parapertussis* (human), *B. parapertussis* (ovine), *B. avium*, *B. hinzii*, *B. holmseii*, and *B. trematum*. Very little is known about the virulence mechanisms of *B. avium*, *B. hinzii*, *B. holmseii* and *B. trematum*. In contrast, *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* have been extensively studied.

B. pertussis is the etiological agent for the highly contagious childhood disease called whooping cough (1). It has exclusively adapted to the human host and there is no evidence for the existence of an animal or environmental reservoir. Transmission is thought to occur primarily by respiratory droplets. Infection is characterized by colonization of ciliated respiratory epithelia in the trachea and bronchi. The onset of disease is gradual, with symptoms similar to a mild upper respiratory infection. An acute *B. pertussis* infection is marked by severe, spasmodic coughing episodes during the paroxysmal phase. Leukocytosis with lymphocytosis is also common during this phase of the illness. Dangerous complications are bronchopneumonia and acute encephalopathy. Recovery is associated with immunity to reinfection.

B. parapertussis (human) is also a human adapted pathogen. It causes a pertussis-like syndrome but does not cause lymphocytosis (2, 3). *B. parapertussis* (ovine) are

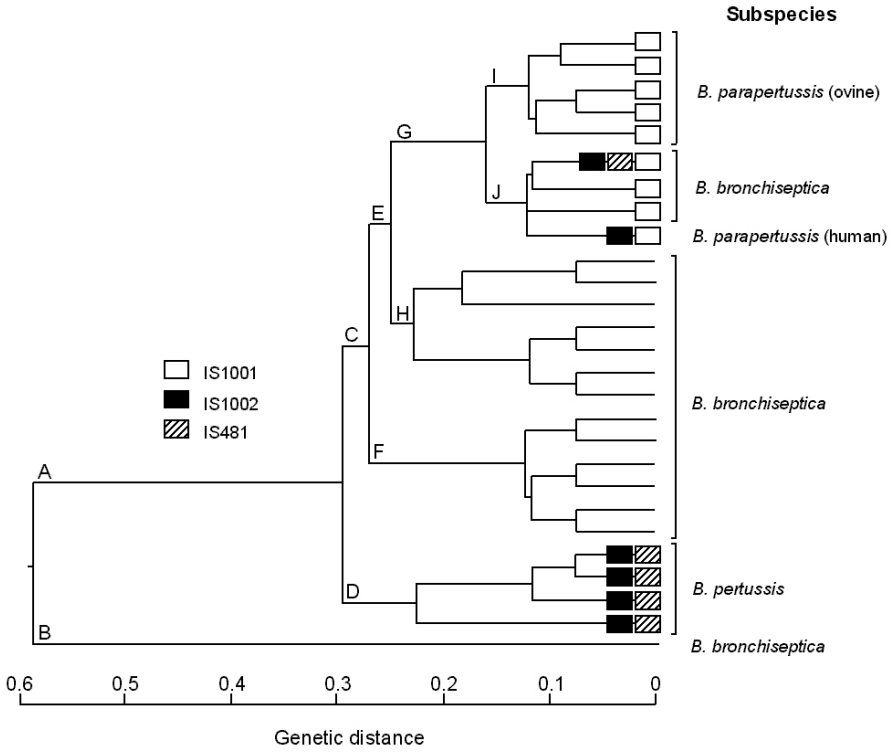

Figure 1. Phylogenetic relationship among *Bordetella* subspecies based on multilocus enzyme electrophoresis and insertion sequence analyses (12). The genetic distance between pairs of electrophoretic types is shown. The presence of IS elements is indicated by boxes. The dendrogram suggests that *B. bronchiseptica* is the likely progenitor for *B. pertussis* and *B. parapertussis*. Based on the host from which each strain was isolated and its electrophoretic type, the *Bordetella* subspecies to which each isolate was assigned is presented.

bordetellae isolated from the respiratory tracts of symptomatic and asymptomatic sheep. *B. parapertussis* (ovine) infection can sometimes predispose animals to pneumonia resulting from secondary infection by other pathogens like *Mannheimia* (*Pasteurella*) *haemolytica* (4).

B. bronchiseptica displays a broad host range which includes mice, rats, guinea pigs, rabbits, cats, dogs, pigs, sheep, horses, and bears (5). Although human infections have been documented, they are usually associated with a severely compromised host (6, 7). *B. bronchiseptica* causes a variety of respiratory diseases such as kennel cough in dogs, atrophic rhinitis in pigs and snuffles in rabbits (4, 8, 9). Infections established by this subspecies are typically chronic, often asymptomatic, and notoriously difficult to clear even with antibiotic therapy (5). *B. bronchiseptica* appears to occupy a position along a continuum with "pathogen" at one end and "commensal" at the other. Its ability to establish long-term asymptomatic infection seems to be an adaptive feature and may represent a balance between immunostimulatory events associated with infection and immunomodulatory events mediated by the bacteria (10). *B. bronchiseptica* infection of laboratory animals provides an excellent model system to understand mechanisms that promote persistent bacterial infections.

Based on rigorous phylogenetic analysis using comparative multilocus enzyme electrophoresis, nucleotide sequence analysis and distribution of insertion sequence elements, *B. pertussis*, *B. parapertussis* (human and ovine) and *B. bronchiseptica* have been reclassified as subspecies, since the overall level of genetic diversity between them is remarkably limited (11-13). The dendrogram in Figure 1 also implies that there have been two independent host range jumps to humans, the earliest by *B. pertussis* and the most recent by *B. parapertussis* (human). In both cases, *B. bronchiseptica* is likely to be the evolutionary progenitor. *B. pertussis* and *B. parapertussis* (human) may, therefore, be considered as human adapted lineages of *B. bronchiseptica*. All four of these bacterial subspecies share a nearly identical virulence control system encoded by the *bvgAS* locus (14-16). They also express a common set of surface associated and secreted molecules involved in colonization and virulence. They differ, however, in a variety of characteristics such as host range specificity, severity of disease, the ability to establish persistent infection and perhaps pathways for transmission. *B. bronchiseptica* also differs from the other subspecies in its ability to survive nutrient limiting conditions, at least *in vitro*, suggesting that in addition to transmission by the aerosol route, this organism may be able to transmit via environmental reservoirs (17, 18). Major phenotypic differences between these subspecies have thus far not been attributed to the presence of pathogenicity islands, plasmids, transposable elements or insertions from bacteriophage genomes. Instead, several Bvg-regulated

loci, such as genes encoding a type III secretion system (19), a motility apparatus (20), and pertussis toxin (21), have been found to be differentially expressed in these species. Thus, differential gene expression and polymorphisms within expressed genes may contribute to complex phenotypic differences. As a result of their extremely high degree of genetic relatedness, a comparative analysis of the similarities and differences in the infectious cycles of *Bordetella* subspecies serves as a guide to understanding fundamental features of bacterial-host interactions.

A critical aspect of any such evaluation is to ensure that results seen *in vitro* mimic events occurring *in vivo*. As such, an animal model where naturally occurring host-pathogen interactions can be studied becomes important. *B. bronchiseptica* has a major advantage over *B. pertussis* and *B. parapertussis* in this respect. Its broad host range allows the use of natural hosts for experimental infection. Further, its high degree of similarity with *B. pertussis* and *B. parapertussis* and the ability to construct chimeric strains (22) justifies the *B. bronchiseptica* model as a foundation for comparative studies of pathogenesis.

Animal models for *B. bronchiseptica* have been developed that reflect both the natural course of infection as well as those that are specifically skewed toward disease. A *B. bronchiseptica* strain, RB50, has been isolated from the nose of a naturally infected New Zealand White rabbit (23). Specific pathogen free rabbits inoculated with RB50 become colonized in the nasal cavity, larynx, trachea and lungs. The ID_{50} is less than 200 cfu delivered in a 5 microliter droplet to the nares. Colonization of the nasal cavity, larynx and trachea persists indefinitely. Despite the presence of *Bordetella* in the upper and often lower respiratory tract, clinical signs of respiratory disease are not observed. Larynx, trachea and lung specimens show no gross pathology and histological examination of tissue sections rarely show indications of inflammation or abnormal tissue structure. Rat and mouse models have also been developed. For RB50, the intranasal ID_{50} is less than 25 cfu in Wistar rats (24) and less than 5 cfu in BALB/c and C57BL/6 mice (25). Rats become persistently colonized in the nasal cavity, larynx and trachea. In mice, tracheal colonization lasts for 4-5 weeks and nasal colonization persist indefinitely.

Infection in all of the above models is characterized by efficient establishment, life long persistence and the absence of disease. This accurately reflects the epidemiology of naturally occurring infection. It also reflects a type of interaction that, from the standpoint of the bacterium, would appear to be adaptive. The availability of mice with knock out mutations in genes required for immune effector functions allows investigation of interactions between *Bordetella* virulence factors and the host defense. In immunocompromised animals, the balance is tipped towards disease (25). These models are appropriate for probing mechanisms of colonization and signal transduction, which are expected to be shared to a significant degree by all the *Bordetella* subspecies. They also provide an excellent opportunity to address how bacteria establish persistent infections and simultaneously avoid damaging their hosts.

3. VIRULENCE GENE REGULATION

Bordetella-host interactions occur predominantly at respiratory surfaces. Many surface and secreted molecules involved in mediating these interactions have been identified (Figure 2). Those proven or predicted to function in adherence include filamentous hemagglutinin (FHA) (24, 26), fimbriae (27), pertactin (28), and lipopolysaccharides (LPS) (29). *Bordetella* species also produce toxins such as tracheal cytotoxin (TCT) (30), a bifunctional adenylate cyclase toxin/hemolysin (ACT/Hly) (31), dermonecrotic toxin (32, 33), a *B. pertussis* specific pertussis toxin (34) and a *B. bronchiseptica* specific toxin(s) which is secreted by a recently identified type III secretion system (19). The genes and operons of all of the above virulence factors, except TCT, are positively regulated by the BvgAS master locus (16). BvgA and BvgS are members of a subfamily of two component signal transduction systems that uses phosphorylation reactions to regulate gene expression pathways. BvgAS uses a four-step His-Asp-His-Asp phosphotransfer signaling mechanism (35). BvgS is a 135kDa integral cytoplasmic membrane sensor kinase and contains the first three phosphotransfer domains of the BvgAS phosphorelay (36). BvgA is a typical cytoplasmic response regulator and contains the fourth phosphotransfer domain. Mutational studies have shown that His-729 of the transmitter domain, Asp-1023 of the receiver domain, His-1172 of the histidine phosphotransfer domain (HPD), and Asp-54 of the BvgA response regulator are essential for the phosphorylation cascade and virulence gene activation (35). Using a biochemical approach in which the BvgS signaling domains, expressed and purified alone and in combination, were analyzed in *in vitro* phosphorylation assays with purified BvgA, Uhl *et al.* deciphered the phosphorelay modeled in Figure 3 (37). In this model, signal inputs detected by the periplasmic domain are relayed through the membrane to the transmitter which autophosphorylates at His-729 by a reaction that is reversible *in vitro*. His-729 then donates the phosphoryl group to Asp-1023 of the receiver domain. Asp-1023 can donate the phosphoryl group to His-1172 of the HPD or to water to form inorganic phosphate. The HPD can then transfer the phosphate back to BvgS or alternatively, it can phosphorylate (and thus activate) BvgA.

Under growth conditions of 37^{o}C in the relative absence of $MgSO_4$ or nicotinic acid, the BvgAS phosphorelay is active and bordetellae are referred to be growing under Bvg^+ phase conditions. Under Bvg^+ phase conditions, transcription of genes encoding various virulence factors is triggered via the interaction of the BvgA carboxyl terminus and the alpha subunit of RNA polymerase (38). Promoters of Bvg-activated genes vary in their affinity for BvgA. Genes encoding adhesins, such as *fhaB*, have high affinity BvgA binding sites while genes encoding toxins, such as *cyaA*, have lower affinity BvgA binding sites (39, 40). In addition, there is another set of genes that are repressed under Bvg^+ phase conditions.

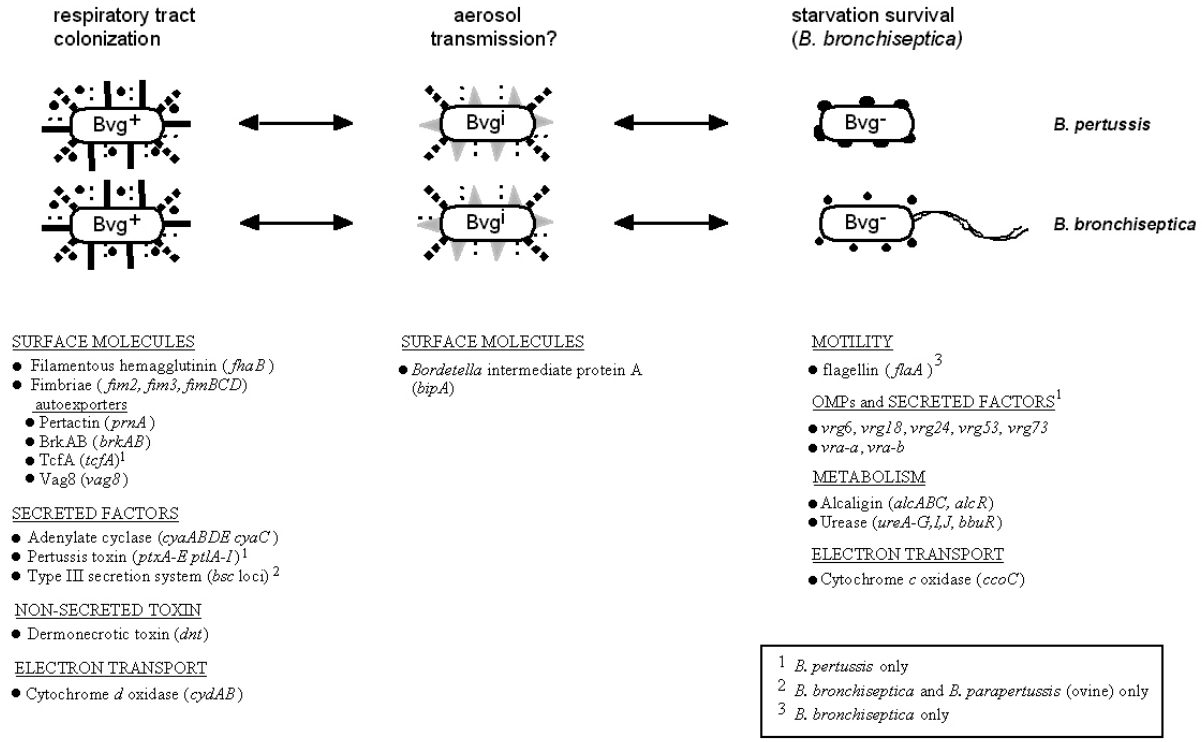

Figure 2. The three phases of *Bordetella* and their associated phenotypes. BvgAS controls at least three distinct phenotypic phases in response to environmental conditions. Under Bvg^+ phase conditions, *B. pertussis* and *B. bronchiseptica* produce a nearly identical set of secreted (solid circles), surface associated (solid and dashed lines) and metabolic factors. The Bvg^+ phase has been shown to be necessary and sufficient for respiratory tract colonization. The Bvg^i phase is characterized by the expression of a subset of Bvg-activated factors (dashed lines) as well as factors expressed maximally in this phase (shaded triangles). The Bvg^i phase is hypothesized to be involved in aerosol transmission. Under Bvg^- phase conditions, *B. pertussis* and *B. bronchiseptica* express different sets of proteins. *B. pertussis* expresses *vrg*s/*vra*s (solid ovals), while *B. bronchiseptica* expresses flagella (curved lines) and genes required for motility, as well as other coregulated factors such as urease (solid circles). The Bvg^- phase of *B. bronchiseptica* has been shown to be necessary and sufficient for survival under nutrient limiting conditions.

These include genes required for motility (20), siderophore production and synthesis of various metabolic, biosynthetic, and respiratory enzymes in *B. bronchiseptica* (41-43), a similar but not identical set of genes in *B. parapertussis* (human and ovine), and genes (vrg's) encoding outer membrane and secreted proteins in *B. pertussis* (44) (Figure 2). In *B. pertussis*, BvgA mediates repression of *vrg*'s indirectly through a repressor called BvgR. The gene encoding BvgR is located immediately downstream of the *bvgAS* locus and is activated by BvgA (45, 46). The Bvg^- phase is expressed when bordetellae are grown under "modulating" conditions (i.e. at room temperature or in the presence of $\geq$10mM nicotinic acid or $\geq$40mM $MgSO_4$) or when *bvgAS* is inactivated by a mutation. It was recently discovered that wild type *Bordetella* grown in the presence of submodulating concentrations of $MgSO_4$ or nicotinic acid (e.g. between 0.4 and 2.0mM nicotinic acid for *B. bronchiseptica*) expresses a phenotypic phase distinct from those described above (47). This phase is characterized by the absence of the Bvg-repressed phenotypes, the presence of some (but not all) Bvg-activated virulence factors, and the presence of a newly discovered set of phenotypes that are unique to this phase. Since the gene expression profile of this new phase falls in between those of the Bvg^+ and Bvg^- phases, this new phase has been designated as the Bvg-intermediate (Bvg^i) phase (47-49). Characteristics of the Bvg^+, Bvg^i and Bvg^- phases indicate the existence of four classes of Bvg-regulated genes: (a) those that are expressed maximally only in the Bvg^+ phase, (b) those that are expressed maximally in both the Bvg^+ and Bvg^i phases, (c) those that are expressed maximally only in the Bvg^i phase, and (d) those that are expressed maximally only in the Bvg^- phase (Figure 4). The complexity of the BvgAS phosphorelay may reflect its ability to respond to signal intensity rather than signal diversity, such that rather than functioning as a switch that is responsive to many different signals, BvgAS may function as a rheostat that is adjusted in response to variations in intensity of a limited number of signals.

4. VIRULENCE GENE FUNCTION

Based on *in vitro* attachment assays and *in vivo* infection experiments, several surface exposed and secreted

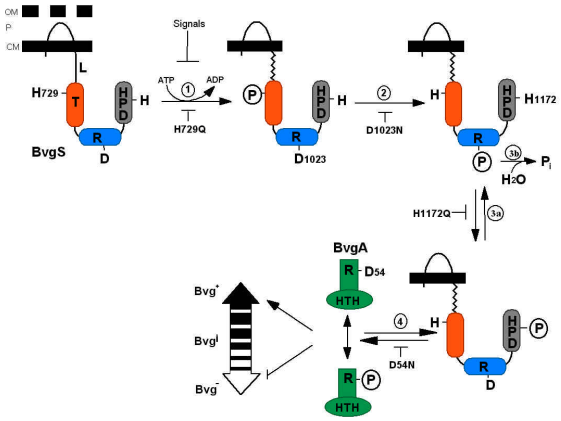

Figure 3. The BvgAS phosphorelay. BvgS is a transmembrane sensor protein containing a periplasmic domain, a linker region (L), a transmitter (T), a receiver (R) and a histidine phosphotransfer domain (HPD). BvgA contains a receiver (R) and a helix-turn-helix (HTH) motif. Sequential steps in the phosphorelay and the amino acid residues involved are shown. In this model, signal inputs detected by the periplasmic domain are relayed through the membrane to the transmitter which autophosphorylates at His-729 by a reaction that is reversible *in vitro*. His-729 then donates the phosphoryl group to Asp-1023 of the receiver domain. Asp-1023 can donate the phosphoryl group to His-1172 of the HPD or to water to form inorganic phosphate. The HPD can then transfer the phosphate back to BvgS or alternatively, it can phosphorylate (and thus activate) BvgA. Abbreviations used: OM (outer membrane), P (periplasm), IM (inner membrane).

molecules have been proposed to play a role in *Bordetella* pathogenesis (Figure 2).

4.1. Filamentous hemagglutinin (FHA)

FHA is a large, highly immunogenic, hairpin shaped molecule which has been included as a primary component in acellular pertussis vaccines. It is synthesized as a 367 kDa precursor, FhaB, which is modified at its N-terminus (50) and cleaved at its C-terminus (51) to form the mature 220 kDa FHA protein. Efficient secretion of FHA requires an outer membrane associated accessory protein, FhaC (52, 53). In the absence of FhaC, FHA is no longer secreted and gets degraded. FHA and FhaC have been classified as members of the two-partner secretion (TPS) system which includes several secretory/accessory protein secretion systems from Gram negative bacteria (51). Although efficiently secreted via this process, a significant amount of FHA remains associated with the cell surface by an unknown mechanism (51). *In vitro* studies using a variety of mammalian cell types suggest FHA possesses at least four distinct attachment activities and four separate FHA binding domains have been proposed (28, 54-57). The Arg-Gly-Asp (RGD) triplet, situated in the middle of FHA and localized to one end of the proposed hairpin structure, stimulates adherence to monocyte/macrophages and possibly other leukocytes via the leukocyte response integrin/integrin associated protein (LRI/IAP) complex and complement receptor type 3 (CR3) (58-60). The CR3 recognition domain in FHA has yet to be identified. FHA also possesses a carbohydrate recognition domain (CRD) which mediates attachment to ciliated respiratory epithelial cells as well as to macrophages *in vitro* (56). Finally, a lectin-like activity for heparin and other sulfated carbohydrates, which can mediate adherence to non-ciliated epithelial cell lines, has been identified. This heparin binding site is distinct from the CRD and RGD sites and is required for FHA mediated hemagglutination (61).

Evidence for a role for FHA *in vivo* has been more difficult to obtain mainly due to the lack of a natural animal host (other than humans) for *B. pertussis*, as well as the complexity of this molecule and its associated biological activities. Fewer FHA mutants than wild type *B. pertussis* were recovered from the lungs of rabbits at 24 hours after intra-tracheal inoculation (60). A comparison of *in vivo* results with *in vitro* binding characteristics of the various mutant strains used in the above study suggested that wild type *B. bronchiseptica* were capable of adhering to both ciliated epithelial cells and macrophages. Further, competition experiments with lactose and anti-CR3 antibody suggested both CRD- and RGD-dependent binding was involved (60). Using mouse models, however, others have found FHA mutants to be indistinguishable from wild type *B. pertussis* in their ability to persist in the lungs, but defective for tracheal colonization (62, 63). Still others, also using mouse models, have observed no difference between FHA mutants and wild type *B. pertussis* (27, 64-67).

We have recently explored the role of FHA in pathogenesis by constructing two types of FHA mutant derivatives of *B. bronchiseptica*, one containing an in-frame deletion in the structural gene, *fhaB*, and one in which FHA is expressed ectopically in the Bvg^- phase, in the absence of the array of Bvg^+ phase virulence factors with which it is normally expressed (24, 68). Comparison of these mutants with wild type *B. bronchiseptica* showed that FHA is both necessary and sufficient to mediate adherence to rat lung epithelial cells *in vitro*. Using a rat model of respiratory infection, we showed that FHA is absolutely required, but not sufficient, for tracheal colonization in healthy, unanesthetized animals (24). FHA was not required for initial tracheal colonization in anesthetized animals, however, suggesting its role in establishment may be dedicated to overcoming the clearance activity of the mucociliary escalator (24).

B. pertussis has recently been shown to inhibit T cell proliferation to exogenous antigens *in vitro* in an FHA dependent manner (69). Further, McGuirk and Mills have demonstrated that interaction of FHA with receptors on macrophages results in suppression of the proinflammatory cytokine, IL-12, via an IL-10 dependent mechanism (70). These data reveal a role for FHA in facilitating persistence by curbing protective Th1 immune responses. In contrast, a recent study suggests that FHA can elicit proinflammatory and proapoptotic responses in human monocyte-like cells and bronchial epithelial cells (71). FHA specific antibodies generated by rats infected with *B. bronchiseptica* have also been found to be necessary to

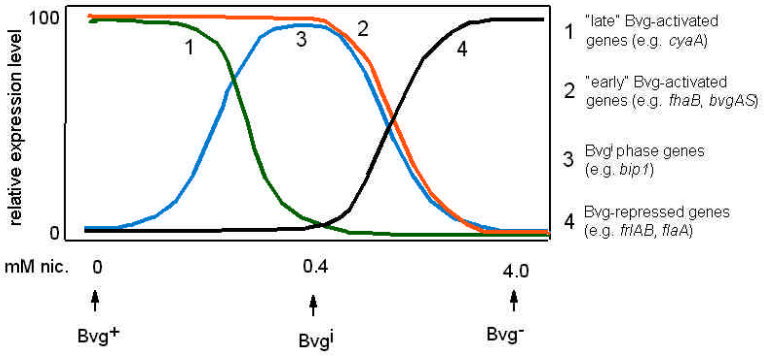

Figure 4. BvgAS controls expression of at least four classes of genes. Those that are expressed maximally only under Bvg^+ phase (such as *cyaA*) are referred to as "late" Bvg-activated genes (curve 1, shown in green). Those that are expressed maximally under both the Bvg^+ and Bvg^i phases (such as *fhaB*) are referred to as "early" Bvg-actiavted genes (curve 2, shown in red). Those that are expressed maximally only under Bvg^i phase conditions (such as *bipA*) are represented by curve 3 (shown in blue). Finally, those that are repressed by BvgAS and expressed maximally only under Bvg^- phase conditions (such as genes involved in motility) are represented by curve 4 (shown in black).

protect against further infection (superinfection) by *B. bronchiseptica* in the rat model (Mattoo *et al.*, manuscript in preparation). Taken together, these data suggest FHA performs several immunomodulatory functions *in vivo*.

4.2. Fimbriae

Like most Gram-negative pathogenic bacteria, *Bordetellae* express filamentous, polymeric protein cell surface structures called fimbriae. The major fimbrial subunits that form the two predominant *Bordetella* fimbrial serotypes, Fim2 and Fim3, are encoded by unlinked chromosomal loci *fim2* and *fim3*, respectively (72, 73). A third unlinked locus, *fimX*, is expressed only at very low levels if at all (74), and recently, a fourth fimbrial locus, *fimN*, was identified in *B. bronchiseptica* (75). In addition to positive regulation by BvgAS, the *fim* genes are subject to fimbrial phase variation by slip-strand mispairing within a stretch of cytosine residues located between the -10 and -35 elements of the *fim2*, *fim3*, *fimX*, and *fimN* promoters (75, 76). In *B. pertussis*, a truncated major fimbrial subunit gene, *fimA*, is located at the 5' end of the *fimBCD* gene cluster (77). It was recently shown that in *B. bronchiseptica* and *B. parapertussis*, *fimA* is intact and capable of encoding a fourth fimbrial subunit type, FimA (78). The putative promoter region of *fimA* does not contain a "C stretch" and therefore probably does not undergo phase variation. Since slip-strand mispairing affects transcription of the individual fimbrial genes independently of each other, bacteria may express Fim2, Fim3, FimX, FimN or any combination at any given time. However, all fimbrial serotypes share a common minor fimbrial subunit, FimD, that forms the tip adhesin (79). The *fimD* gene is located within the fimbrial biogenesis operon downstream of *fimB* and *fimC* (77, 80). Interestingly, this operon is positioned between *fhaB* and *fhaC*, genes required for synthesis and processing of FHA. Based on the predicted amino acid sequence similarity to the *E. coli* PapD and PapC proteins, FimB and FimC have been proposed to function as a chaperone and usher, respectively (77, 80). Mutation of any one of the genes in the *fimBCD* locus results in a complete lack of fimbrial structures on the bacterial cell surface, suggesting *fimBCD* is the only functional fimbrial biogenesis locus on the *Bordetella* chromosome (81).

As a critical, early step in bacterial pathogenesis, attachment to host epithelia is often mediated by fimbriae. Establishment of a definitive role for *Bordetella* fimbriae as adhesins has been difficult for several reasons. First, the multiple, unlinked major fimbrial subunit genes, as well as the transcriptional and translational coupling of the fimbrial biogenesis operon with the *fha* operon has impeded the ability to construct strains completely devoid of fimbriae. Second, the presence of several other putative adhesins with potentially redundant functions has, in many cases, obscured detection of clear phenotypes for Fim- mutants. Finally, since the interactions between bacterial adhesins and host receptor molecules are expected to be highly specific, the use of heterologous hosts for studies with *B. pertussis* has limited the ability to detect *in vivo* roles for putative adhesins. Nonetheless, several studies suggest fimbriae may mediate binding of *Bordetella* to respiratory epithelium via the major fimbrial subunits and to monocytes via FimD (63, 82, 83). Geuijen *et al.* have shown that purified *B. pertussis* fimbriae, with or without FimD, were able to bind to heparan sulfate, chondroitin sulfate and dextran sulfate, sugars that are ubiquitously present in the mammalian respiratory tract (84). Heparin binding domains within the Fim2 subunit were identified and found to be similar to those of the eukaryotic extracellular matrix protein, fibronectin. Studies by Hazenbos *et al.* suggest that FimD mediates binding of non-opsonized *B. pertussis* to the very late antigen 5 (VLA-5) on the surface of monocytes, which then causes activation of CR3 which enhances its ability to bind FHA (82, 83).

In vivo studies have shown that Fim^- *B. pertussis* strains are defective in their ability to multiply in the nasopharynx and trachea of mice (63, 79). Using a *B. bronchiseptica* strain devoid of fimbriae but unaltered in its expression of FHA and other putative adhesins, we have recently shown that fimbriae contribute to the efficiency of establishment of tracheal colonization and are absolutely

required for persistence in the trachea using both rat and mouse models (27). Moreover, the serum antibody profiles of animals infected with Fim- bacteria differ qualitatively and quantitatively from those of animals infected with wild type *B. bronchiseptica* (27). Lastly, challenge experiments suggest that the presence of fimbriae is important to elicit an immune response that is protective against superinfection (Mattoo *et al.*, manuscript in preparation). Taken together, these results suggest Fim-mediated interactions with epithelial cells and/or monocyte/macrophages may play important roles, not only in adherence, but also in the nature and magnitude of the host immune response to *Bordetella* infection.

4.3. Pertactin and other auto-exporters

Bordetella express a number of related surface-associated proteins that appear to direct their own export to the outer membrane where they undergo autoproteolytic processing of their C-termini. This mechanism is similar to that used by the IgA proteases of *Neisseria gonorrhoeae* and *Haemophilus influenzae* and elastase of *Pseudomonas aeruginosa* (85-87).

The first and best characterized member of this family to be identified in *Bordetella* is pertactin. Mature pertactin is a 68 kDa protein in *B. bronchiseptica* (88), a 69 kDa protein in *B. pertussis* (89) and a 70 kDa protein in *B. parapertussis* (human) (90). Pertactin has been proposed to play a role in attachment as all three pertactin proteins contain an Arg-Gly-Asp (RGD) tripeptide motif as well as several proline-rich regions and leucine-rich repeats, motifs commonly present in molecules that form protein-protein interactions involved in eukaryotic cell binding (91). The *B. pertussis*, *B. bronchiseptica* and *B. parapertussis* pertactins differ primarily in the number of proline-rich regions they contain (92). The X-ray crystal structure of *B. pertussis* pertactin suggests it consists of a 16-stranded parallel beta-helix with a V-shaped cross-section and is the largest beta-helix known to date (93). Other *Bordetella* proteins with predicted autoexport ability include TcfA (94), BrkA (95), and Vag8 (96). All of these proteins show significant amino acid sequence similarity in their C-termini and contain one or more RGD tripeptide motifs. Based on predicted amino acid sequence similarity with all of these proteins, the *B. pertussis* genome appears to encode at least three additional members of this autoexporter family. It is hypothesized that the C-termini of the autoexporting precursor proteins form a pore in the outer membrane through which the N-terminal portions are threaded then cleaved but which remain cell-associated by an uncharacterized mechanism. In support of this model, Charles *et al.* have shown that deletion of the 3' region of *prn*Bp prevents surface exposure of the molecule (97). In the case of Vag8, however, cleavage may not occur since the predicted size of the entire protein encoded by *vag8* corresponds to the size seen by SDS-PAGE (96).

The ability of pertactin and the other auto-exporters to function as adhesins has been tested both *in vitro* and *in vivo*. *In vitro* studies demonstrated that purified pertactin could promote binding of CHO cells to tissue culture wells and that expression of *prn* in *Salmonella* or *E. coli* could increase the adherence and/or invasiveness of these bacteria to various mammalian cell lines (98). In contrast, a Prn$^-$ strain of *B. pertussis* did not differ from its wild type parent in its ability to adhere to or invade HEp2 cells *in vitro* or to colonize the respiratory tracts of mice in *vivo* (99). Similarly, we have constructed a *B. bronchiseptica* strain with an in-frame deletion mutation in *prn* and found it to be indistinguishable from wild type *B. bronchiseptica* in its ability to establish a persistent respiratory tract infection in rats (our unpublished data). Thus, although pertactin appears to be a strong and potentially protective immunogen (88, 89, 100), its role in pathogenesis remains unknown. Potential adhesive functions for TcfA, BrkA and Vag8 have not been investigated directly although TcfA$^-$ *B. pertussis* show a decreased ability to colonize the murine trachea compared to wild type *B. pertussis* (94). BrkA has been proposed to play a role in serum resistance (95, 101).

4.4. Lipopolysaccharides (LPS)

Like endotoxins from other Gram-negative bacteria, the LPS of *Bordetella* species, are pyrogenic, mitogenic, toxic, and can activate and induce tumor necrosis factor production in macrophages (102-104). *Bordetella* LPS molecules differ in chemical structure from the well-known smooth-type LPS expressed by *Enterobacteriaceae.* Specifically, *B. pertussis* LPS lacks a repetitive O-antigenic structure and is therefore more similar to rough-type LPS. It resolves as two distinct bands (A and B) on silver-stained sodium dodecyl sulfate-polyacrylamide gels (105). The faster migrating moiety, band B, consists of a lipid A molecule linked via a single ketodeoxyoctulosonic acid (KDO) residue to a branched oligosaccharide core structure containing heptose, glucose, glucuronic acid, glucosamine and galactosaminuronic acid (GalNAcA) (106-108). The charged sugars, GalNAcA, glucuronic acid and glucosamine, are not commonly found as core constituents in other LPS molecules. The slower migrating moiety (band A) consists of band B plus a trisaccharide consisting of N-acetyl-N-methyl-fucosamine (FucNAcMe), 2,3-deoxy-di-N-acetylmannosaminuronic acid (2,3-diNAcManA), and N-acetylglucosamine (GlcNAc) (106-108). *B. bronchiseptica* LPS is composed of band A and band B plus an O-antigen structure consisting of a single sugar polymer of 2,3-dideoxy-di-N-acetyl-galactosaminuronic acid (109). *B. parapertussis* (human) isolates contain LPS that lacks band A, has a truncated band B, and contains an O-antigen that, like *B. bronchiseptica*, consists of 2,3-dideoxy-di-N-acetyl-galactosaminuronic acid. *B. parapertussis* (ovine) isolates lack O-antigen and contain band A- and B-like moieties that appear to be distinct from those of the other *Bordetella* species (110).

Although a distinct role(s) for LPS in *Bordetella* pathogenesis has not yet been demonstrated, its importance is suggested by the observation that changes in LPS structure in *B. bronchiseptica* are controlled by the BvgAS virulence regulatory system (110). Allen and Maskell have recently identified, cloned, and sequenced genetic loci required for LPS biosynthesis in *B. pertussis*, *B. parapertussis* (human) and *B. bronchiseptica* and have constructed strains with various mutant LPS phenotypes (111-113). Compared with their wild type parental strains,

B. pertussis, *B. parapertussis* (human) and *B. bronchiseptica* strains which synthesize only band B LPS show decreased colonization in a mouse model of respiratory infection (29). For *B. bronchiseptica* and *B. parapertussis* (human), this difference may be attributed to differences in sensitivity to antibody-dependent serum killing (29). Further characterization of these and other mutants with defined mutations affecting LPS structure will greatly facilitate deciphering the precise role(s) of LPS in *Bordetella* pathogenesis.

4.5. Tracheal Cytotoxin (TCT)

Of the various virulence factors synthesized by *Bordetellae*, only tracheal cytotoxin TCT has been shown to reproduce the specific epithelial cytopathology characteristic of pertussis. TCT corresponds to a disaccharide-tetrapeptide monomer of peptidoglycan. Its structure is N-acetylglucosaminyl-1,6-anhydro-N-acetylmuramyl-(L)-alanyl-g-(D)-glutamyl-mesodiaminopimelyl-(D)-alanine (114). Although this peptidoglycan fragment is produced by all Gram-negative bacteria as they break down and rebuild their cell wall during growth, only *Bordetella* spp. (115) and *Neisseria gonorrhoeae* (116) have been shown to release it into the environment. Other bacteria, such as *E. coli*, recycle this peptidoglycan fragment by transporting it back into the cytoplasm via an integral cytoplasmic membrane protein called AmpG (117, 118). It appears that *Bordetella* do not express a functional AmpG, although sequences resembling *ampG* can be identified in the recently released *B. pertussis* genome database (Lyon and Goldman, personal communication). Expression of *E. coli ampG* in *B. pertussis* results in a substantial decrease in the amount of TCT produced (Lyon and Goldman, personal communication).

The activities of TCT have been studied *in vitro* using hamster tracheal organ culture and cultured hamster tracheal epithelial (HTE) cells (30, 119). TCT causes mitochondrial bloating, disruption of tight junctions and extrusion of ciliated cells, with little or no damage to non-ciliated cells, in hamster tracheal ring cultures and a dose-dependent inhibition of DNA synthesis in HTE cells. TCT has also been shown to cause loss of ciliated cells, cell blebbing and mitochondrial damage in human nasal epithelial biopsies (120). There is strong evidence that this cytopathology is due to a TCT-dependent increase in nitric oxide (NO). Interestingly, a synergistic increase in NO production is seen when HTE cells are treated with both TCT and LPS. Inducible nitric oxide synthase (iNOS) expression is positively controlled by interleukin-1alpha (IL-1alpha), and TCT has been shown to trigger IL-1alpha production in HTE cells (121, 122). Both TCT and IL-1alpha result in increased NO production when added to HTE cells (123). It is hypothesized that TCT stimulates IL-1alpha production in non-ciliated mucus-secreting cells, which activates expression of iNOS leading to high levels of NO production. NO then diffuses to neighboring ciliated cells which are much more susceptible to its damaging effects (124). The ability to construct TCT deficient mutants by expressing a heterologous *ampG* gene in *Bordetella* will allow this hypothesis to be tested using *in vivo* models.

4.6. Dermonecrotic toxin (Dnt)

Although initially misidentified as endotoxin, dermonecrotic toxin (Dnt) was one of the first *B. pertussis* virulence factors to be described (125). This heat labile toxin induces localized necrotic lesions in mice and other laboratory animals when injected intradermally, and is lethal for mice at low doses when administered intravenously (72, 125-127). The Dnt's of *B. pertussis* and *B. bronchiseptica* are cytoplasmic, single polypeptide chains of about 140 kDa (32, 33, 128, 129).

In vitro studies have shown that purified Dnt from *B. bronchiseptica* induces dramatic morphological changes, stimulates DNA replication, and impairs differentiation and proliferation in osteoblastic clone MC3T3 cells (130, 131). Recent evidence indicates that these effects are due to Dnt-mediated activation of the small GTP binding protein rho (132) which results in tyrosine phosphorylation of focal adhesion kinase (p125fak) and paxillin (133). P125fak and paxillin are involved in embryonic development and cell locomotion (134) and their activation leads to profound alterations in the actin cytoskeleton and the assembly of focal adhesions (135-138). Lacerda *et al*. also showed that Dnt stimulates DNA synthesis without activation of p42mapk and p44mapk, providing evidence for a novel p21rho-dependent signaling pathway that leads to entry into the S phase of the cell cycle in Swiss 3T3 cells (133). If and how these effects of Dnt contribute to *Bordetella* pathogenesis is not known. Although *B. bronchiseptica* strains with decreased dermonecrotic toxin activity have been associated with decreased turbinate atrophy in infected pigs (9, 139), transposon mutants of *B. pertussis* lacking dermonecrotic toxin are no less virulent than wild type bacteria in mice (67).

4.7. Adenylate Cyclase Toxin (ACT or CyaA)

All of the *Bordetella* species that infect mammals secrete ACT, a bifunctional calmodulin-sensitive adenylate cyclase/hemolysin. ACT is synthesized as a protoxin monomer of 1706 amino acids. Its adenylate cyclase catalytic activity is located within the N-terminal 400 amino acids (140, 141). The 1300 amino acid long C-terminal domain mediates delivery of the catalytic domain into the cytoplasm of eukaryotic cells and possesses low but detectable hemolytic activity for sheep red blood cells (141-143). Amino acid sequence similarity between the C-terminal domain of ACT, the hemolysins of *E. coli* (HlyA) and *Actinobacillus pleuropneumoniae* (HppA), and the leukotoxins of Mannheimia (*Pasteurella) haemolytica* (LktA) and *Actinobacillus actinmycetemcomitans* (AaLtA), places ACT within a family of calcium-dependent, pore-forming cytotoxins known as RTX (repeats in toxin) toxins (144). Each of these toxins contains a tandem array of a nine amino acid repeat (L-X-G-G-X-G-(N/D)-D-X) that is thought to be involved in calcium binding (144). Before the ACT protoxin can intoxicate host cells, it must be activated by the product of the *cyaC* gene, which is located adjacent to, and transcribed divergently from, the *cyaABDE* operon (145). CyaC activates the ACT protoxin by catalyzing the palmitoylation of an internal lysine residue (Lys-983) (146, 147). The *E. coli* HlyA protoxin is also

activated by fatty acyl group modification (148-150).

ACT can enter a variety of eukaryotic cell types (151). Once inside, ACT is activated by calmodulin (152) and catalyzes the production of supraphysiologic amounts of adenosine 3',5'-monophosphate (cAMP) from (adenosine triphosphate (ATP) (153-156). Purified ACT inhibits chemiluminescence, chemotaxis and superoxide anion generation by peripheral blood monocytes and PMNs *in vitro* (157). ACT has also been shown to induce apoptosis in cultured murine macrophages (65) and inhibit phagocytosis of *B. pertussis* by human neutrophils (158, 159). *In vivo* studies have shown that, compared to wild type *B. pertussis*, ACT deficient mutants are defective in their ability to cause lethal infections in infant mice (67, 160) and to grow in the lungs of older mice (64, 160). Moreover, significantly fewer inflammatory cells, particularly PMNs, are recruited to the lungs in response to murine respiratory infection with ACT deficient mutants compared with wild type *B. pertussis* (161). Taken together, these results suggest ACT functions primarily as an anti-inflammatory and anti-phagocytic factor during infection.

The importance of ACT in resisting constitutive host defense mechanisms was further demonstrated using mice that lack the ability to mount an adaptive immune response. SCID, SCID-beige and Rag-1$^{-/-}$ mice, which are deficient in T and B cells and NK cell activities, are dependent on constitutive, innate defense mechanisms for protection against microbial pathogens. When these mice were inoculated with wild type *B. bronchiseptica* they died within 50 days, while those inoculated with the ACT deficient strain remained healthy (25). Conversely, neutropenic mice, made so by treatment with cyclophosphamide or by a homozygous null mutation in the granulocyte-colony stimulating factor (G-CSF) gene, were killed by both wild type and ACT deficient strains of *B. bronchiseptica*, indicating that in the absence of neutrophils ACT is not required to cause a lethal infection (25). This data indicates that T and B cells are required to prevent killing by wild type *B. bronchiseptica*, but innate defenses alone are adequate to control infection by an ACT deficient mutant. It also suggests that phagocytic cells, particularly PMNs, are a primary *in vivo* target of the adenylate cyclase toxin.

4.8. Pertussis toxin (Ptx)

Of the *Bordetella* species identified so far, only *B. pertussis* synthesizes and secretes an ADP-ribosylating toxin called pertussis toxin (Ptx). Ptx is composed of six polypeptides, designated S1 - S5, which are present in a 1:1:1:2:1 ratio. Each subunit is synthesized with an N-terminal signal sequence, suggesting that transport into the periplasmic space occurs via a general export pathway analogous to the *sec* system of *E. coli*. Secretion across the outer membrane requires a specialized transport apparatus composed of nine Ptl (Pertussis toxin liberation) proteins (162, 163). The *ptl* locus bears extensive similarity to the *Agrobacterium tumefaciens virB* operon, which encodes a secretion system involved in exporting single-stranded "T-DNA", suggesting that these systems function by a common mechanism (164-166). Both appear to be involved in transporting large protein complexes (167). Furthermore, there is evidence that only the fully assembled Ptx holotoxin is efficiently secreted (168, 169). The *ptl* genes are located directly 3' to, and within the same transcriptional unit as, the *ptxA-E* genes which encode the Ptx subunits (163, 170). While the chromosomes of *B. parapertussis* and *B. bronchiseptica* also contain *ptx-ptl* loci which encode functional polypeptides, these genes are transcriptionally silent due to nucleotide differences in the promoter regions (21, 171-173). In both *B. parapertussis* and *B. bronchiseptica*, replacement of native *ptx-ptl* promoter sequences with those of *B. pertussis* results in the secretion of biologically active Ptx (174). The biological relevance of differential Ptx expression amongst *Bordetellae* is not known.

The Ptx subunits are held together by non-covalent interactions and arranged in an A-B architecture typical of many bacterial toxins. The A protomer, consisting of the enzymatically active S1 subunit, sits atop the B oligomer, a ring like structure formed by the remaining S2-S5 subunits (175-177). The B oligomer binds to eukaryotic cell membranes and dramatically increases the efficiency with which the S1 subunit gains entry into host cells (177). Unlike diphtheria toxin, Ptx does not require an acidic environment for entry into eukaryotic cells (178). It has, therefore, been suggested that Ptx may traverse the membrane directly without the need for endocytosis. Within the host cell cytosol, binding of ATP to the B oligomer which has intercalated into the cytoplasmic membrane causes release of S1 subunit which then becomes active upon reduction of its disulfide bond (179).

The biochemical properties and biological effects of Ptx have been extensively characterized *in vitro*. In its reduced form, the S1 subunit catalyzes the transfer of ADP-ribose from NAD to the alpha subunit of guanine nucleotide binding proteins (G proteins) in eukaryotic cells (175, 177, 180). The G proteins that Ptx has been shown to inactivate by ADP-ribosylation are G_i, G_t (transducin), and G_o. When active, G_i inhibits adenylyl cyclase and activates K^+ channels, G_t activates cyclic GMP phosphodiesterase in specific photoreceptors, and G_o activates K^+ channels, inactivates $Ca2^+$ channels, and activates phospholipase C-beta (181). Biological effects attributed to the disruption of these signaling pathways include histamine sensitization, enhancement of insulin secretion in response to regulatory signals, and both suppressive and stimulatory immunologic effects (182, 183). Ptx has been shown to inhibit chemotaxis, oxidative responses, and lysosomal enzyme release in neutrophils and macrophages (180, 184-191). Using mouse and rat models, Ptx has been shown to inhibit chemotaxis and migration of neutrophils, monocyte/macrophages and lymphocytes (192-194). Ptx has also been suggested to function as an adhesin involved in adherence of *B. pertussis* to human macrophages and ciliated respiratory epithelial cells (59, 195).

Ptx is commonly cited as the major virulence factor expressed by *B. pertussis* and pertussis has been proposed to be a toxin-mediated disease with Ptx being

responsible for many, if not all, of the disease's typical symptoms (183). However, despite a plethora of experimental evidence demonstrating what Ptx can do *in vitro* and in animal models, clear evidence for an *in vivo* role for Ptx in human disease is lacking. One approach has been to compare symptomatology in children infected with either *B. pertussis* or *B. parapertussis* (human) since these organisms differ primarily in the absence of Ptx expression by *B. parapertussis* (human). Such studies have indicated that the only significant difference between the two is increased leukocytosis in *B. pertussis*-infected children (196, 197). These observations suggest Ptx may not play a decisive role in causing the paroxysmal coughing, whooping, and vomiting characteristic of pertussis. The exact role of Ptx in establishment of infection, disease and/or transmission of pertussis is currently unknown.

4.9. The Type III secretion system (TTSS)

A functional type III secretion system has recently been discovered in *B. bronchiseptica* (19). Type III, or contact-dependent, secretion is found in many Gram negative bacteria including the human pathogens *Yersinia*, *Shigella*, *Salmonella* and *Enteropathogenic E. coli*, as well as plant pathogens *Pseudomonas syringae* and *Erwinia* (for review see (198, 199). Type III secretion systems allow bacteria to translocate bacterial effector proteins directly into the plasma membrane or cytoplasm of eukaryotic cells through a needle-like injection apparatus (200). These effector proteins then alter normal host cell signaling cascades and other processes (201). The genes encoding the secretion apparatus are relatively conserved among different genera, but the effector proteins are quite diverse.

While the type III effector proteins secreted by the *B. bronchiseptica* TTSS have not yet been identified, *B. bronchiseptica* causes a variety of *in vitro* phenotypes that are dependent on an intact and functional TTSS. The TTSS of *B. bronchiseptica* induces cytotoxicity in several cultured cell lines (19, 202), dephosphorylation of specific host cell proteins (19), and activation of the MAP kinases, ERK1 and ERK2 (10). Additionally, the TTSS causes aberrant localization of the transcription factor NF-kappaB into aggregates within the host cell cytoplasm (10); the NF-kappaB of cells infected with wild type *B. bronchiseptica* does not translocate to the nucleus even upon stimulation of the cells with TNFalpha (10). *B. bronchiseptica* also causes very rapid apoptosis in macrophage and epithelial cell lines (10).

In vivo, the *B. bronchiseptica* TTSS contributes to persistent colonization of the trachea in both rat and mouse models of respiratory infection (10, 19). The inflammatory cells that infiltrate the lungs during infection undergo apoptosis in mice infected with a wild type strain but not with a mutant strain deficient in type III secretion (10). Additionally, mice infected with the type III secretion deficient strain elicit higher titers of anti-*Bordetella* antibodies (specifically serum IgA) than animals infected with wild type *Bordetella* (10). Consistent with this, animals infected with the type III deficient strain are completely protected against superinfection with wild type *B. bronchiseptica* (Mattoo *et al.*, manuscript in preparation). Taken together, these data suggest the *B. bronchiseptica* TTSS may be involved in modulating the host immune response and could contribute to the typically chronic nature of *B. bronchiseptica* infections.

Interestingly, while the chromosomes of all *Bordetella* subspecies shown in Figure 1 contain *bsc* (Bordetella secretion) loci, only *B. parapertussis* (ovine) isolates, an atypical *B. pertussis* strain (18323), and *B. bronchiseptica* strains express these genes *in vitro* (19). Although it is possible that the requirements for the induction of the TTSS for *B. parapertussis* (human) and most *B. pertussis* strains may be more stringent *in vivo*, immunoblot analysis using sera from children recovering from pertussis suggest that the TTSS of these strains is not expressed *in vivo* (Yuk and Miller, unpublished data). We have recently identified a regulatory locus just downstream of the *Bordetella bsc* locus. This locus contains a sigma factor, homologous to the HrpL protein which activates type III secretion in *P. syringae* (Mattoo *et al.*, manuscript in preparation). Analysis of this locus in *B. bronchiseptica* reveals an intricate mechanism of regulation of type III secretion, which falls downstream of the role of BvgA. Analysis of this locus in other *Bordetella* subspecies is currently under way. Additional phylogenetic analyses of this locus and other type III related loci will help clarify the relationship between expression of the *bsc* locus, host range and the course of disease.

Recently, in an in silico scan of the partially completed genome sequence of *B. pertussis*, Antoine *et al.* have identified additional potential virulence factors which include a putative siderophore receptor, adhesins, and an autotransporter protein (203).

5. PERSPECTIVE

Bordetella species interact with their mammalian hosts primarily, and perhaps exclusively, at respiratory surfaces. Several scanning electron micrographic studies have demonstrated that *Bordetella* bind specifically to the cilia of respiratory epithelia (204-207). In the nasal cavity, requirements for colonization appear to be few; *B. bronchiseptica* strains multiply deficient in the expression of FHA, Fim, Prn, and ACT are capable of persisting in the nasal cavities of rats for at least 60 days, albeit at levels lower than wild type (Mattoo *et al.*, manuscript in preparation). Establishment of infection in the trachea, however, requires that bacteria be able to resist or overcome the clearance action of the mucociliary escalator as well as the killing effects of defensins, complement and other antimicrobial factors. FHA, in its secreted as well as surface associated form, serves as a strong adhesin and appears to be essential for overcoming mucociliary clearance (24). LPS may be important for resistance to complement (29). TCT, released by *Bordetella* growing among the cilia, and bacterial LPS is proposed to stimulate NO production causing several cytopathological changes along the mucosal surface. Damage and loss of tracheal epithelial cells containing adherent bacteria probably contributes to respiratory disease symptoms and possibly also to transmission by the aerosol route.

Damage to respiratory epithelia also results in the release of inflammatory cytokines. Inflammatory cells, predominantly neutrophils, are recruited into the lungs of mice within three days following intranasal inoculation with either *B. pertussis* or *B. bronchiseptica* (25, 161, 208). This inflammatory response is significantly decreased in animals infected with *ptx* or *cyaA* mutants (25, 161, 208). Both Ptx and ACT have been shown to inhibit the microbicidal activities of neutrophils and macrophages *in vitro* (158, 159, 187, 189, 190). Thus, these toxins may serve as anti-defense mechanisms, allowing *Bordetella* to resist the killing action of phagocytic cells. Without ACT and Ptx, *Bordetella* mutants are efficiently eliminated by fewer numbers of neutrophils and macrophages and hence less inflammation occurs. Experiments with mice have shown that ACT, by targeting neutrophils and macrophages, is an important factor in resisting constitutive host defense mechanisms.

Experiments using immunodeficient mice also revealed the importance of adaptive immunity in controlling *Bordetella* infections. *B. bronchiseptica* is confined to the upper respiratory tract in immunocompetent hosts but causes a lethal systemic infection in SCID or SCID/Beige mice (25). For *B. pertussis*, the ability of mice to mount an adaptive immune response determines whether the infection persists indefinitely in the lungs or is cleared entirely (209). While these observations demonstrate a crucial role for adaptive immunity, they do not reveal the nature of the immune response that is involved. It has been assumed that *B. pertussis*, as a non-invasive respiratory pathogen, is controlled primarily by a humoral immune response. The generation of anti-*Bordetella* antibodies in response to *Bordetella* infection is not only well-documented but is considered diagnostic for pertussis in the absence of positive nasopharyngeal swab cultures. Using mice which do not express the interferon-γ receptor, interleukin-4, or immunoglobulin heavy chain genes, Mills *et al.* have recently demonstrated an absolute requirement for B cells, or their products, in clearance of *B. pertussis* infection in mice (210) and we have recently demonstrated that serum containing anti-*Bordetella* antibodies can rescue SCID/Beige mice from lethal infection by *B. bronchiseptica* (209). Challenge of rats infected with *B. bronchiseptica* for 30 days with a larger dose of a marked wild type strain demonstrated that anti-FHA antibody titers play an important role in preventing superinfection (Mattoo, *et al.*, manuscript in preparation). Superinfection experiments also suggested a role for fimbriae in modulating a protective immune response (Mattoo, *et al.*, manuscript in preparation). Recently, a role for IgA in mediating protection against *B. pertussis* infection in mice has been suggested (211). Recovery from pertussis in humans corresponds to the development of long-lasting protection against subsequent disease. It has been assumed that this immunity is antibody mediated and vaccination strategies have been focused on the induction of anti-*Bordetella* antibodies. Recent evidence suggests, however, that while vaccination against *B. pertussis* in humans, and *B. bronchiseptica* in lower animals, confers protection against disease, it is less effective at preventing colonization (196, 212). The ability to detect *Bordetella*-specific CD4+ T cell clones in humans recovering from pertussis suggests the possibility that while antibody may suffice to neutralize the effects of secreted toxins, activation of phagocytic cells may be required for eliminating bacteria from the respiratory tract. There is increasing evidence that cell mediated immunity also plays a significant role in controlling *Bordetella* infections. $CD4^+$ T cell clones which proliferate when stimulated with Ptx or FHA have been identified in peripheral blood mononuclear cells from children and adults following recovery from pertussis (210, 212-216). Secretion of interferon-gamma by these cells suggests they are Th-1 T cells (217), which are crucial for activation of phagocytic cells such as macrophages. Spleens of mice recovering from respiratory infection with *B. pertussis* were also shown to contain T cells that proliferate in response to heat killed *B. pertussis* and secrete cytokines indicative of a Th-1 response (212, 218). These spleen cells, upon adoptive transfer, were capable of inducing clearance of *B. pertussis* from the lungs of *nu/nu* mice which lack functional T cells (219). It therefore appears that both humoral and cell mediated immune responses are important for the control and/or clearance of *Bordetella* infections. The relative contributions of each, and the mechanisms involved, await further investigation. Using high-density DNA microarrays, a recent analysis of the host transcriptional profile generated in response to *B. pertussis* infection of a human bronchial epithelial cell line (BEAS-2B) sheds light on the complex interactions occurring between the host and the pathogen (220).

At the bacterial level, the multiple events occurring during *Bordetella*-host interactions all seem to be controlled by BvgAS. The different patterns of gene expression required to produce the various phenotypic phases occur in response to variations in phosphorylated BvgA levels, which are in turn regulated by the ability of BvgS to sense its environment and mediate subsequent phosphorylation/dephosphorylation events (36). So, what is the role of BvgAS mediated signal transduction in the *Bordetella* life cycle? The functional conservation of BvgAS within and across *Bordetella* subspecies suggests it mediates a common and important adaptive response in these organisms.

It is clear that the Bvg^+ phase, which is nearly identical among the *Bordetella* that infect mammals, is required for virulence. A Bvg^+ phase-locked mutant was found to be indistinguishable from wild type *B. bronchiseptica* in its ability to efficiently colonize the respiratory tracts of rabbits and rats, suggesting that the Bvg^+ phase is both necessary and sufficient for establishment of respiratory infection and that the Bvg^- phase is not required *in vivo* (23). Further, antibodies against Bvg^- phase factors could not be detected in sera from any animal, suggesting wild type *B. bronchiseptica* do not switch to the Bvg^- phase during infection. Ectopic expression of Bvg^- phase factors, such as flagella, in the Bvg^+ phase severely compromised the ability of *B. bronchiseptica* to establish tracheal colonization in rats (68). This result demonstrates that inappropriate expression of Bvg^- phase factors can be detrimental to the infectious process and underscores the importance of Bvg-mediated repression of gene expression *in vivo*. Further, Kinnear *et al.* have demonstrated that altering the kinetic pattern of expression

of Bvg-activated genes can significantly reduce the ability of *B. pertussis* to colonize the respiratory tracts of mice, thereby suggesting a role for differential regulation of Bvg-activated genes in *Bordetella* pathogenesis (221). Comparison of wild type *B. bronchiseptica* and Bvg phase-locked mutants indicated that only Bvg^- phase bacteria, induced either by mutation or by the presence of modulating signals, could survive and multiply in PBS or in other nutrient-deplete media (47), demonstrating a clear advantage for the Bvg^- phase under nutrient limiting conditions. Together with the apparent lack of an *in vivo* role for the Bvg^- phase, these data suggest that the Bvg^- phase may allow *B. bronchiseptica* to survive in an environmental reservoir, if such a reservoir exists. The Bvg^i phase shows phenotypic characteristics intermediate between the Bvg^+ and Bvg^- phases. Using a recently developed rabbit model of aerosol transmission, transmission by wild type *B. bronchiseptica* but not Bvg^+ phase-locked mutants was observed (Cotter *et al.*, unpublished). This result suggests phenotypic modulation, to some extent, is required for transmission and the Bvg^i phase may be involved in transmission by the aerosol route. Since all *Bordetella* subspecies can be transmitted by the aerosol route, there should be strong selection to maintain Bvg^i phase phenotypes. This is in fact the case as both *B. pertussis* and *B. bronchiseptica* express cross-reactive Bvg^i phase antigens, under semi-modulating conditions (Martinez de Tejada *et al*., unpublished data and 48).

Clearly, BvgAS controls expression of a spectrum of phenotypic phases in response to subtle yet distinct quantitative differences in environmental cues. It must be noted that only the signals to which BvgAS responds in the laboratory have so far been identified and characterized. The true signals that are sensed in nature remain unknown, and await further study.

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