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Nat Rev Microbiol. Author manuscript; available in PMC 2014 October 22.

Published in final edited form as:
Nat Rev Microbiol. 2014 April ; 12(4): 274–288. doi:10.1038/nrmicro3235.

Bordetella pertussis pathogenesis: current and future challenges

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Abstract

Pertussis, or whooping cough, has recently reemerged as a major public health threat despite high levels of vaccination against the etiological agent, *Bordetella pertussis*. In this Review, we describe the pathogenesis of this disease, with a focus on recent mechanistic insights into virulence factor function. We also discuss the changing epidemiology of pertussis and the challenges of vaccine development. Despite decades of research, many aspects of *B. pertussis* physiology and pathogenesis remain poorly understood. We highlight knowledge gaps that must be addressed to develop improved vaccines and therapeutic strategies.

Pertussis is a highly contagious respiratory disease that is transmitted directly from human to human[1], most likely via aerosolized respiratory droplets. The primary causative agent, *Bordetella pertussis,* is a Gram-negative bacterium that was first described by Bordet and Gengou in 1906 [2]. The closely related bacterium *Bordetella parapertussis*$_{Hu}$ is responsible for a minority of cases (approximately 14%) and is less capable of causing severe disease[3]. Both *B. pertussis* and *B. parapertussis*$_{Hu}$ are human-specific, and phylogenetic analyses indicate that they evolved from *Bordetella bronchiseptica* or a *B. bronchiseptica*-like ancestor[4, 5] (Box 1). *B. bronchiseptica* infects a broad range of mammals, including humans, and although it can cause overt disease such as kennel cough in dogs and atrophic rhinitis in pigs, it typically colonizes its hosts chronically and asymptomatically[6]. Despite differences in host range and disease-causing propensity, *B. pertussis*, *B. parapertussis*$_{Hu}$ and *B. bronchiseptica* are so closely related that they are now considered subspecies. Together, these organisms provide a paradigm for understanding bacterial adaptation to humans and

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Competing interests statement
The authors declare no competing interests.

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the dichotomy between acute disease and chronic asymptomatic infection[4, 5]. Although other *Bordetella* species have been isolated from humans, they seem to be primarily opportunistic human pathogens.

Box 1

Bordetella phylogenomics

The *Bordetella* genus includes nine species and the "classical" or "mammalian" bordetellae (*B. pertussis*, *B. parapertussis*$_{Hu}$ and *B. bronchiseptica*) are the most important species in the context of mammalian infection. Comparative analyses of their genomes have been informative on at least two levels. The first involves comparisons between subspecies, which has revealed intriguing clues regarding mechanisms of host adaptation and virulence evolution; and the second involves studies of the population dynamics of *B. pertussis* in the context of recent outbreaks and the possibility of vaccine-driven evolution.

Early studies revealed limited genetic diversity between isolates, arguing that the classical bordetellae should be regarded as closely related subspecies and that *B. pertussis* and *B. parapertussis*$_{Hu}$ evolved independently from different *B. bronchiseptica*-like ancestors[172, 173]. This hypothesis suggested that *B. bronchiseptica*, a zoonotic generalist, has the propensity to give rise to host-restricted specialists that cause acute disease. Whole genome sequence comparisons support this idea and show that the transition by *B. pertussis* and *B. parapertussis*$_{Hu}$ to the human-restricted niche was accompanied by large-scale gene loss, an accumulation of pseudogenes and an expansion of IS elements[174, 175]. Based on this framework, a seminal study characterized a set of 132 *Bordetella* isolates from diverse mammalian hosts using a combination of multilocus sequence typing, comparative whole-genome microarray analysis and IS typing[4]. Four distinct complexes comprised of related strains were resolved, representing *B. pertussis* (complex II), *B. parapertussis*$_{Hu}$ (complex III) and two distinct *B. bronchiseptica* complexes (I, IV) (see the figure). Surprisingly, although *B. bronchiseptica* complex I isolates were primarily of animal origin (68%), 80% of *B. bronchiseptica* complex IV strains were from humans that had whooping cough-like symptoms. Notwithstanding the fact that human isolates were overrepresented in the collection, the clustering of *B. bronchiseptica* strains isolated from humans into a genetically related group was intriguing. A follow-up study based on whole-genome sequencing suggested that *B. pertussis* evolved from a *B. bronchiseptica* complex IV-like ancestor[5]. These observations illustrate the evolutionary dynamics of *Bordetella* subspecies and raise the question of whether complex IV *B. bronchiseptica* are on a path towards human adaptation.

At the other end of the evolutionary spectrum, the resurgence of pertussis has prompted numerous efforts to characterize polymorphisms in *B. pertussis* populations in a search for correlations with the introduction of aP vaccines. Although numerous shifts in allelic frequencies have been documented in different countries since the vaccines were introduced[176, 177], a comprehensive analysis of isolates from the United States shows that the majority of these polymorphisms predate the wP to aP transition and are not the result

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of aP-driven selection[178]. Nonetheless, currently circulating strains in the Americas, Asia, Australia and Europe carry a single nucleotide substitution in the PT promoter (the *ptxP3* allele) that confers a slight (ca. 1.6 fold) increase in PT production compared to the previously dominant allele[179]. *ptxP3* strains were present before aP vaccines, but given the roles of PT in immunosuppression it has been suggested that their expansion and apparent fixation reflects a selective advantage for establishing infection in partially immune hosts, regardless of the source of immunity[180]. Cause and effect is unclear, as is the likelihood that such a small difference in PT expression could have such a major effect. One of the difficulties in interpreting these studies is their reliance on allelic variability in small subsets of genes, raising the possibility that "hitchhiker" mutations located elsewhere in the genome are responsible for apparent clonal shifts[177]. It is essential that these analyses continue, but they need to be based on whole genome sequences and appropriate sampling of circulating and reference strains.

In the pre-vaccine era, pertussis was widespread and mainly affected young children (1–9 years old)[7]. The classical manifestation of the disease, which typically occurs in this age group, is characterized by three phases: catarrhal, paroxysmal, and convalescent[8]. Clinical observations combined with results from studies using animal models (Box 2) suggest that classical pertussis is initiated by adherence of bacteria to ciliated respiratory epithelium in the nasopharynx and trachea[9, 10]. Adherent bacteria survive innate host defences, such as mucociliary clearance and the action of antimicrobial peptides, multiply locally and resist elimination by inflammatory cells. Symptoms during this catarrhal phase are similar to those of many upper respiratory infections, including the common cold. After one to two weeks, the disease progresses to the paroxysmal phase, which can persist from one to ten weeks and is characterized by periods of normal airway function interspersed with multiple severe spasmodic coughing fits, followed by characteristic inspiratory whoops and often emesis. The onset of adaptive immunity correlates with bacterial clearance but not with the cessation of symptoms, which typically decline gradually over another month but can persist for much longer (the convalescent phase)[8]. In infants (< 1 year old), pertussis can take a more serious course with bacteria disseminating into the lungs causing necrotizing bronchiolitis, intra-alveolar hemorrhage and fibrinous edema[10]. In severe cases, extreme lymphocytosis occurs, which is positively correlated with intractable pulmonary hypertension, respiratory failure and death[10].

Box 2

Animal models

One of the greatest challenges in studying the pathogenesis of pertussis has been the development of animal models that accurately reflect human disease as the most commonly used laboratory animals do not cough and none of them are natural hosts for *B. pertussis*. Nonetheless, the availability of wild-type, knock-out and transgenic mouse strains, as well as a plethora of murine-specific reagents, have made mice attractive and commonly used model organisms. To establish infection in mice, high numbers of *B. pertussis* must be delivered directly to the lungs, where they multiply for the first week or so post-inoculation and are eventually cleared. Although murine models do not mimic

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classical whooping cough, they have provided insight into the importance of several virulence factors, the roles of various host immune responses in controlling infection and the potential efficacy of vaccines (as outlined in the main text).

Suckling pigs have been used to model infant pertussis and intrapulmonary inoculation with *B. pertussis* results in low-grade fever, mild cough, hypoglycemia, lymphocytosis, weight loss and pneumonia[181]. In this model, passively transferred immunity provides protection for newborn piglets upon challenge[182], supporting the recently instituted policy of maternal immunization during pregnancy[183, 184]. Studies with infant and adult pigs have also revealed that *B. pertussis* is susceptible to host antimicrobial peptides, whereas *B. bronchiseptica* and *B. parapertussis*$_{Hu}$ are resistant [185].

The most recent and exciting advance in modelling human pertussis is the development of a baboon (*Papio anubis*) model[186]. Delivery of *B. pertussis* to the nasopharynx of weanling baboons results in low-grade fever, paroxysmal coughing, lymphocytosis, robust production of anti-PT antibodies and protection from subsequent challenge, all of which are manifestations of pertussis in humans[186]. A unique advantage of this model is the ability to study transmission, which was demonstrated to occur by contact and via aerosols[187]. Importantly, a recent study showed that aP vaccination provided protection against the development of disease symptoms but not against colonization or transmission[165], which suggests that nonsymptomatic individuals might be capable of transmitting the infection to unprotected infants. Use of this model to investigate mechanisms of transmission and disease will be crucial for the development of new vaccines and therapeutics.

Rodents, rabbits, and swine are not natural hosts for *B. pertussis* or *B. parapertussis*$_{Hu}$; however, they are commonly infected with *B. bronchiseptica* in nature. This fact, in combination with the close phylogenetic relationships between these subspecies (Box 1) and the conservation of many known virulence factors among them, has prompted several groups to use *B. bronchiseptica* infection of natural hosts to study features of pathogenesis that are common to *B. pertussis* and *B. parapertussis*$_{Hu}$. Studying *B. bronchiseptica* pathogenesis is also of veterinary and clinical importance, as *B. bronchiseptica* is a common pathogen of domestic animals[188, 189] and occasionally causes disease in humans[190]. These models have revealed roles for several conserved virulence determinants and regulatory factors (see main text), and have also shown that several factors are functionally interchangeable between *B. pertussis* and *B. bronchiseptica*[111, 125, 191], thereby validating the use of *B. bronchiseptica* as model for studying *B. pertussis* virulence and regulatory function. Thus far, the results of these studies suggest that host specificity is determined by differences other than sequence polymorphisms in highly conserved virulence genes.

Introduction of whole-cell pertussis (wP) vaccines in the late 1940s resulted in a rapid reduction in both the incidence of pertussis and death caused by the infection. However, the success of these vaccines was undermined by concerns over their safety (Box 3); thus, they were replaced with acellular pertussis (aP) vaccines in the late 1990s in many developed countries[11]. Since then, pertussis cases have increased and dramatic epidemic cycles have

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returned. In 2012, 48,277 cases of pertussis and 18 deaths were reported to the Centers for Disease Control and Prevention (CDC), which represents the greatest burden of pertussis in the United States in 60 years and similar outbreaks are occurring in other countries[12–14]. However, the epidemiology of contemporary pertussis does not replicate that of the pre-vaccine era. Disease is now more common in infants and older children (ages 9–19) and, strikingly, older children who develop pertussis are often fully vaccinated according to current recommendations[15, 16]. Ominously, studies that have analyzed pertussis incidence among children that were born and vaccinated during the transition to aP vaccines have found that the rate of infection is significantly higher among children vaccinated with only aP vaccines compared to those vaccinated with even a single dose of wP vaccine[17]. To combat the rise of infections in this group, regulatory agencies have called for boosters to be administered earlier[18]. However, the benefit of boosting with aP vaccines is unclear because it is unknown whether the re-emergence of pertussis is due simply to waning immunity or to fundamental differences in the nature of the immune response induced by aP vaccines compared with wP vaccines or with natural infection.

Box 3

Vaccination against pertussis

The introduction of whole cell vaccines (wP) in the 1940s resulted in dramatic decreases in morbidity and mortality caused by pertussis, and by the early 1970s pertussis was nearly eradicated in the developed world[7]. Attention then turned to the side-effects associated with immunization. The National Childhood Encephalopathy Study (NCES), a prospective case-controlled study that was carried out in the U.K. in the late-1970s to evaluate acute neurological illnesses in children aged between 2 and 26 months, concluded that pertussis vaccination was associated with brain damage[192] – although this correlation was later demonstrated to be unfounded. Lack of circulating disease was misinterpreted as being equivalent to a lack of risk in contracting the disease, and the NCES report (among others) led to a precipitous decline in vaccine coverage in many countries worldwide. The reported association with brain damage also effected a change in manufacturing policies; decreased vaccination compliance combined with a rise in legal action associated with previous vaccinations led many pharmaceutical companies to abandon the production of wP vaccines. However, the decrease in vaccine coverage almost immediately sparked the largest pertussis epidemics since the pre-vaccine era in many countries, which resulted in a substantial rise in infant mortality, a much more severe outcome than the alleged dangers caused by the wP vaccine. Eventually, public concern over the increase in pertussis disease and the number of infant deaths rose and vaccine coverage returned to high levels. Efforts to develop improved pertussis vaccines were rekindled, with the subsequent development, testing and deployment of aP vaccines. Although supposedly safer and undoubtedly less reactogenic than the wP vaccine, we now know that aP vaccines are also less effective. The experience with vaccination against pertussis clearly highlights the effect that public perception and misperception can have on the implementation of public vaccination programs.

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The increased incidence of disease among older children and adults is especially worrisome because of the corresponding risk of transmission to non- or incompletely- immunized infants[1]. Compounding the problem, antibiotic treatment has minimal efficacy by the time most diagnoses are made and severe cases can be unresponsive to standard therapies for respiratory distress (such as mechanical ventilation)[10]. Therefore, the re-emergence of pertussis as a global public health problem presents two challenges: first, the development of vaccines that have an acceptable safety profile, provide long-lasting immunity, reduce infection burden and prevent transmission; and second, the development of therapeutic agents and treatment strategies that reduce morbidity and mortality in vulnerable populations. Both goals require a better understanding of the etiological agents of pertussis and the mechanisms by which they cause disease.

In this Review, we discuss our current understanding of the mechanisms used by *Bordetella* spp. to cause respiratory disease, focusing on the roles and functions of virulence factors in pathogenesis. For the interested reader, more specialized recent reviews on pertussis toxin biology[19, 20], virulence gene regulation[21], immunity[22, 23] and vaccines[24] are available, as well as an earlier comprehensive review on *Bordetella* spp. pathogenesis[25].

Bordetella spp. virulence regulation

Several *Bordetella* spp. virulence factors were identified and characterized biochemically before genetic tools became available, including pertussis toxin (PT), adenylate cyclase toxin (ACT), dermonecrotic toxin (DNT), filamentous hemagglutinin (FHA) and fimbriae (Fim). The first transposon mutagenesis screen of *B. pertussis* identified the genes encoding these factors as well as a locus, now known as *bvgAS*, encoding a two-component regulatory system required for their expression[26]. Reasoning that BvgAS also activates expression of genes encoding additional unknown *Bordetella* spp. virulence factors, mutagenesis screens using Tn5*lac* and Tn5*phoA* were conducted[27, 28]. These, together with subsequent genome-wide analyses, revealed that BvgAS controls hundreds of genes in response to changing environmental conditions, including those encoding surface structures and secreted proteins involved in pathogenesis, factors required for survival outside the mammalian host, enzymes involved in cellular metabolism and physiology and additional regulatory systems[29, 30].

The BvgAS phosphorelay

BvgA is a typical response regulator protein with a receiver domain at its N-terminus and a DNA-binding helix-turn-helix domain at its C-terminus[21] (Fig. 1A). BvgS is a polydomain sensor kinase containing two N-terminal venus flytrap (VFT) domains, which are located in the periplasm[31]. C-terminal to the VFT domains is a membrane-spanning region, followed by a cytoplasmically-located PAS domain, a histidine kinase (HK) domain, a receiver domain and a histidine phosphotransferase (Hpt) domain. During growth in standard medium at 37°C, BvgAS is active and uses ATP to phosphorylate a conserved histidine within the HK domain[32]. The phosphoryl group is subsequently relayed to an aspartate in the receiver domain, then to a histidine in the Hpt domain and finally to an aspartate in the receiver domain of the response regulator BvgA[32]. Phosphorylated BvgA is competent for dimerization and binds to specific DNA sequences to either activate or repress

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transcription[33, 34]. Although the signal(s) to which BvgS responds in nature are unknown, growth at low temperature (~25°C) or in the presence of $MgSO_4$ or nicotinic acid (so-called "chemical modulators" of BvgS) inactivates BvgS; thus, BvgA remains unphosphorylated and is unable to regulate transcription.

BvgAS controls multiple phenotypic phases

The genes regulated by the BvgAS phosphorelay fall into four classes and their differential regulation results in at least three distinct phenotypic phases (Fig. 1B). Class 1 genes include the *ptx-ptl* operon (which encodes PT and its transport system), *cyaA-E* (which encodes ACT) and the *bsc* operon (which encodes a Type III Secretion System (T3SS)). These genes are maximally expressed when BvgAS is fully active (the so-called Bvg^+ phase). Class 2 genes are expressed maximally in both the Bvg-intermediate (Bvgi) and Bvg^+ phase. The Bvg^i phase occurs when bacteria are grown in the presence of low concentrations of chemical modulators or within the first few hours following a switch from Bvg^- phase conditions to Bvg^+ phase conditions. Class 2 genes include *fhaB* (encoding filamentous hemagglutinin (FHA)), *fim* (encoding fimbriae) and *bvgAS* itself; thus *bvgAS* is positively autoregulated. Class 3 genes, of which only one (*bipA*, encoding an outer membrane protein of unknown function) has been characterized so far[35, 36], are expressed maximally in the Bvg^i phase. Class 4 genes, which are also known as *vrg*s (virulence repressed genes), are expressed maximally in the Bvg^- phase and include genes required for flagella synthesis and motility in *B. bronchiseptica*.

Role of BvgAS-mediated gene regulation

The conservation of BvgAS among *Bordetella* spp. and its ability to control multiple phenotypic phases in response to environmental cues suggests that it has an important and conserved role in the infectious cycle. Because *B. pertussis* and *B. parapertussis*$_{Hu}$ strains are unable to survive extended periods of time outside of the human host (unpublished observations from various research groups), it was hypothesized that BvgAS-mediated gene regulation must occur within the mammalian respiratory tract. However, experiments with mutants that were locked in either the Bvg^+ or Bvg^- phase, or that expressed Bvg^- phase factors ectopically in the Bvg^+ phase, showed that the Bvg^+ phase is necessary and sufficient for respiratory infection; that the Bvg^- phase is unable to survive *in vivo*; and that failure to repress Bvg^- phase factors (such as flagella) is detrimental to the development of infection[37–40]. Moreover, recent studies with sensitive reporter systems have provided strong evidence that switching to the Bvg^- phase does not occur *in vivo*[41, 42]. In *B. bronchiseptica*, the Bvg^- phase is required for survival under nutrient-limiting conditions, such as those that might be encountered in an external environment[43]. It has been hypothesized that the Bvg^i phase is important for transmission, and with the development of the baboon model (Box 2), this hypothesis is now testable. Although additional regulatory systems are undoubtedly important during the *Bordetella* spp. infectious cycle, their precise roles have not yet been determined.

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Toxins

Pertussis toxin

One of the first identified and most extensively characterized *B. pertussis* virulence factors, pertussis toxin (PT), sometimes referred to as lymphocytosis-promoting factor for its ability to induce lymphocytosis in mammals[44]. The presumed requirement of PT for the development of infection and the observed positive correlation between PT-specific immunity and bacterial clearance led to the hypothesis that pertussis, like cholera and diphtheria, is a toxin-mediated disease[45]. However, although PT is important for pathogenesis, it is now clear that pertussis results from the coordinated function of many different bacterial factors[46].

PT is an ADP-ribosylating AB_5-type toxin[47] (Fig. 2a). The holotoxin is composed of one catalytic subunit (A) and five membrane-binding/transport subunits (B), which are assembled in the periplasm and then exported by the type IV secretion system encoded by the *ptl* locus[48]. PT holotoxin can bind nearly any sialic acid-containing glycoprotein[49] and thus multiple receptors have been identified and characterized in a broad range of cell types *in vitro*[20]; however, the specific cell types targeted by PT *in vivo* are unknown. After binding, PT enters the host cell by receptor-mediated endocytosis and follows a retrograde transport pathway to the Golgi apparatus and then the endoplasmic reticulum (ER) (Fig. 2b)[50]. The A subunit exits the ER, possibly by hijacking the ER-associated degradation pathway that normally expels misfolded proteins[51]. In the cytoplasm, the A subunit catalyzes the transfer of ADP-ribose from NAD^+ to a cysteine residue near the C-terminus of the alpha subunit of heterotrimeric G-proteins, some of which are inhibitory G-proteins. Amongst other downstream effects, this modification eliminates the ability of these inhibitory G proteins to inhibit adenylate cyclase activity and blocks other G protein regulated enzymes and pathways[20, 52], leading to dysregulation of the immune response.

PT has an extraordinarily broad range of pharmacological effects in cell culture and animal models, which has confounded efforts aimed at identifying its precise role(s) during human infection. PT inhibits the migration of cells that express G-protein coupled chemokine receptors *in vitro*, such as neutrophils, monocytes and lymphocytes[53]. In mouse models, production of PT by *B. pertussis* correlates with decreased proinflammatory chemokine and cytokine production, decreased recruitment of neutrophils to the lungs and increased bacterial burdens early in infection[54, 55]. Experiments in which alveolar macrophages are depleted with clodronate suggest that PT initially targets these cells[56]. PT production at the peak of infection correlates with exacerbated inflammation and pathology in the airways[57]. While these and other observations in animal models suggest that PT contributes to the establishment of infection by suppressing early inflammation and inhibiting the microbicidal action of inflammatory cells, in addition to contributing to inflammatory pathology at the peak of infection, it is unknown whether PT produces these effects during human infection. However, it has been shown that PT production positively correlates with the extreme lymphocytosis that occurs in primary human pertussis cases[58], and antibodies against PT protect against severe disease[59].

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Adenylate cyclase toxin

Adenylate cyclase toxin (ACT, Fig. 2c, d), which is a member of the RTX (repeats in toxin) toxin family, is encoded by *cyaA* and produced by all *Bordetella* subspecies that infect mammals[19]. ACT is secreted by the *cyaBDE*-encoded Type I secretion system and is palmitoylated by the product of *cyaC*[60, 61]. The toxin contains two distinct functional modules: the C-terminal domain, which contains the RTX repeats, mediates binding to target cells and forms cation-selective pores in plasma membranes[62, 63]; and the N-terminal domain is a calmodulin-dependent adenylate cyclase that converts ATP to cyclic AMP (cAMP)[64, 65]. Recent studies indicate that ACT can adopt multiple conformations and that these forms are distinct in their ability to effect pore formation or adenylate cyclase translocation into the host cell[66]. Thus, the observed effects of ACT on different cell types are the result of a combination of ion permeability, increased levels of cAMP (leading to perturbation of downstream signalling events) and possibly the depletion of intracellular ATP.

Although ACT can intoxicate many cell types, it binds with high affinity to CR3 (CD11b/CD18, Mac-1), which is present on neutrophils, macrophages and dendritic cells[67], and early work correlated ACT-dependent cAMP production in human neutrophils with inhibition of phagocytosis and oxidative burst[68]. More recent studies have shown that ACT blocks complement-dependent phagocytosis by macrophages[69]. In addition, this toxin also suppresses activation and chemotaxis of T-cells[70]. The significance of these *in vitro* observations is unclear; however, a recent study using the baboon model and clinical samples from humans showed that the concentrations of ACT in *B. pertussis*-infected respiratory tissues are significantly lower than the amount of purified protein used in most *in vitro* studies[71]. In mouse models, ACT-deficient bacteria are cleared faster than wild-type bacteria, and studies with immunodeficient and neutropenic mice suggest that ACT has a crucial role in enabling bacteria to resist neutrophil-mediated clearance[72, 73]. These data, in addition to the fact that ACT is one of the few virulence factors that is conserved and produced by all pathogenic *Bordetella* species[5], suggest that ACT has the potential to be an effective antigen in future vaccine formulations[74].

Type III Secretion

For reasons of experimental tractability, the *Bordetella* spp. Bsc type III secretion system (T3SS) is most extensively studied in *B. bronchiseptica* and induces caspase-independent necrotic death in a diverse array of cell types *in vitro*[75]. Mutations that eliminate T3SS activity decrease bacterial persistence in the lower respiratory tract following intranasal inoculation of rats and mice[76, 77]. Infection of mice with T3SS-defective *B. bronchiseptica* mutants also results in a more robust antibody response and re-stimulated splenocytes from animals infected with these mutants show increased production of pro-inflammatory IFN-γ and decreased production of anti-inflammatory IL-10[78]. Consistently, IFN-γ facilitates clearance of *B. bronchiseptica* from the lower respiratory tract, whereas IL-10 delays it [78]. Together, these observations suggest that the Bsc T3SS has an immunomodulatory role that promotes persistence in the lower respiratory tract but the mechanistic basis of this phenomenon remains to be determined.

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Remarkably, and despite concerted efforts by several research teams, only a single effector protein, BteA, has been definitively identified as a translocated substrate of the Bsc T3SS[79, 80]. BopN, a homolog of YopN (which regulates type III secretion in pathogenic *Yersinia* spp.) has been proposed as a second effector[81] but thus far, evidence that BopN is translocated by the Bsc system is lacking. BteA is both necessary and sufficient for cytotoxicity *in vitro*, and mutations in *bteA* recapitulate the phenotypes associated with eliminating T3SS activity *in vitro* and *in vivo*[77, 79]. Following translocation into host cells, the N-terminal targeting domain results in BteA localization to ezrin-rich lipid rafts that underlie sites of bacterial attachment[82]. However, the mechanisms responsible for the potent cytotoxicity of BteA remain unclear.

Type III secretion is tightly regulated in *Bordetella* spp. The *bteA* and *bsc* genes are transcriptionally activated by the alternative sigma factor BtrS, which is activated by BvgAS[83]. Expression of the *bcs* genes is also up-regulated by iron starvation[84]. In addition to these regulatory mechanisms, the partner-switching proteins BtrU, BtrV and BtrW mediate a cycle of serine phosphorylation and dephosphorylation events that regulate secretion activity[83, 85].

Perhaps the most pressing question regarding the Bsc T3SS relates to its potential role during human infection. A requirement of T3SS activity for *B. pertussis* cytotoxicity has not been documented, despite the fact that T3SS genes are intact, highly conserved, transcribed and regulated, in addition to the observation that *bteA* alleles are functionally interchangeable between subspecies[82, 83]. Fortunately, recent studies are beginning to shed light on this paradox. Although Bsc activity is not generally observed with laboratory-adapted *B. pertussis* strains, the tip complex of the T3SS, Bsp22, is secreted by clinical isolates *in vitro*, and mutations in the ATPase gene, *bscN*, result in elevated production of pro-inflammatory cytokines and accelerated clearance of *B. pertussis* from the lungs of aerosol-infected mice[86]. Furthermore, T3SS activity seems to be lost following laboratory passage of *B. pertussis* and regained after passage in mice[86, 87].

Tracheal cytotoxin

Tracheal cytotoxin (TCT) is a disaccharide-tetrapeptide monomer of peptidoglycan that is produced during cell wall remodelling[88]. Although most Gram-negative bacteria recycle this molecule[89, 90], *B. pertussis* does so inefficiently and releases a large amount of TCT into the extracellular environment. TCT is the only known *B. pertussis* virulence factor that is not regulated by BvgAS. In hamster tracheal rings, TCT functions synergistically with lipooligosaccharide to stimulate the production of pro-inflammatory cytokines (TNF-α, IL-1α, IL-1β, and IL-6) and iNOS, resulting in destruction and extrusion of ciliated cells from the epithelial surface[91, 92]. The biological activity of TCT depends on NOD1, a cytosolic pattern recognition receptor that senses bacterial peptidoglycan and induces the production of pro-inflammatory mediators[93]. NOD1-dependent detection of TCT seems to be host specific, as human NOD1 poorly detects TCT whereas mouse NOD1 does so efficiently[93]. Although it has been postulated that TCT-mediated cytopathology contributes to the characteristic cough in pertussis, the lack of relevant animal models has prevented

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testing of this hypothesis. Thus, the contribution of TCT to pertussis pathogenesis in humans remains unclear.

Dermonecrotic toxin

Subcutaneous injection of *B. pertussis* or *B. bronchiseptica* cells into mice results in the formation of necrotic lesions due to the activity of dermonecrotic toxin (DNT)[94]. Consistent with a role in infection, DNT production is positively regulated by BvgAS [26, 29] and there is evidence that DNT contributes to the ability of *B. bronchiseptica* to induce turbinate atrophy and lung pathology in swine[95]. DNT has transglutaminase activity, can activate Rho GTPases[96, 97] and inhibits osteogenic cell differentiation *in vitro*, suggesting that the toxin acts directly on host cells[98, 99]. However, as DNT lacks a signal sequence for export and is not secreted from bacterial cells grown in culture[94, 100], it may actually function within the bacterial cytoplasm during infection, possibly by facilitating bacterial survival within a specific host niche and hence functioning indirectly in pathogenesis.

Surface Structures

Filamentous hemagglutinin

Filamentous hemagglutinin (FHA, Fig. 3a) is a large rod-shaped protein and, together with FhaC, serves as a prototypical member of the Two-Partner Secretion (TPS) pathway [101]. It is initially synthesized as an ~370 kDa preproprotein (FhaB) that undergoes processing to produce the mature ~250 kDa FHA as it is translocated across the cytoplasmic membrane by the Sec translocation system and across the outer membrane by FhaC[102]. The N-terminal signal peptide is likely removed by leader peptidase and the C-terminal prodomain is processed by SphB1 and other as yet unidentified factors[103, 104]. Mature FHA is oriented with its mature C-terminus (the MCD) distal to the bacterial surface, and a substantial amount of FHA is also released into culture supernatants when the bacteria are grown *in vitro*[104].

FHA is both necessary and sufficient to mediate bacterial adherence to several eukaryotic cell types *in vitro*[105, 106]. However, FHA is only one of several factors contributing to bacterial adherence to tracheal explants[9, 107], suggesting that additional adhesins are important for adherence *in vivo*. Studies using cultured, non-ciliated cells have reported that FHA binds to CR3, Very Late Antigen V (VLA-5) and Leukocyte Response Integrin/ Integrin Associated Protein (LRI/IAP) complexes, and an RGD motif located in the centre of the FHA molecule is implicated in this process[108–110]. More recent studies that have examined *B. bronchiseptica* infection of animal and cell culture models have shown that the FHA molecules produced by *B. pertussis* and *B. bronchiseptica* are functionally interchangeable. These studies have also demonstrated that production of an FHA protein containing an RAE motif instead of RGD results in no observable differences and that the MCD is required for function[111]. Whether FHA interacts with CR3, VLA-5, LRI/IAP or other mammalian receptors during infection has yet to be determined.

Experiments in which *B. bronchiseptica* is delivered in a small volume to the nasal cavities of rats and pigs have revealed FHA is essential for progression of the infection from the upper to the lower respiratory tract[111, 112]. In mouse models, in which large numbers of

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bacteria are delivered directly into the lungs, FHA-deficient *B. bronchiseptica* strains induce a more robust inflammatory response than wild-type bacteria[73, 111]. This response is characterized by increased production of proinflammatory cytokines and chemokines in lung tissue (such as TNF-α, KC, MCP-1 and IL-17) and increased recruitment of neutrophils to the lungs during the first four days post-inoculation[73]. Animals that do not succumb to inflammation-mediated pulmonary damage clear the FHA-deficient bacteria from their lungs much faster than animals inoculated with wild-type bacteria[73, 111]. These data suggest that FHA enables *B. bronchiseptica* to modulate inflammation during the establishment of infection, thereby facilitating bacterial persistence. It is currently unknown whether FHA exerts these effects by binding directly to host receptors while attached to the bacterial cell surface or after release from the bacterial cell. Furthermore, it has been suggested that FHA serves as a scaffold to direct the delivery of other virulence factors (such as ACT[113]); however, the *in vivo* relevance of this activity has not been determined.

Fimbriae

Bordetella spp. produce type 1 pili, which are also known as fimbriae (Fig. 3b). The putative chaperone (FimB), usher (FimC) and tip adhesin (FimD) proteins are encoded by the *fimBCD* operon, which is located between the *fhaB* and *fhaC* genes[114]. The genes encoding the two primary major fimbrial subunits, *fim2* and *fim3*, are located elsewhere on the chromosome and can undergo phase variation[115]. Alternative major fimbrial subunit genes (*fimA*, *fimN* and *fimX*) have also been identified[116–118]. Although *in vitro* adherence assays using cultured cells have yielded variable results[119, 120], studies with tracheal explants indicate a role for fimbriae in mediating adherence to ciliated respiratory epithelium[9, 107]. Studies with both *B. pertussis* and *B. bronchiseptica* have demonstrated a requirement for fimbriae during colonization of the lower respiratory tract in rodents[120, 121], and mice inoculated with Fim-deficient *B. pertussis* display a more robust inflammatory response than mice inoculated with wild-type bacteria[122]. Similarly to FHA, fimbriae seem to be involved in adherence and/or suppression of the initial inflammatory response to infection, potentially contributing to persistence.

Pertactin

Pertactin (PRN) is a member of the classical autotransporter family of outer membrane proteins (Fig. 3c)[123]. The surface-localized ‘passenger’ domain forms a β-helix with β-strands connected by short turns or, in a few cases, large extrahelical loops[124]. Similarly to fimbriae, studies using non-ciliated mammalian cells to investigate a role for PRN in adherence or invasion have yielded equivocal results[112, 125]. Studies using ciliated rabbit tracheal explant cultures suggest that PRN contributes to *B. pertussis* adherence to ciliated respiratory epithelium[9], although experiments with mice failed to identify a role for PRN *in vivo*[126]. However, in the case of *B. bronchiseptica*, studies indicate that PRN is involved in mediating resistance to neutrophil-mediated clearance and promoting persistence in the lower respiratory tract[112, 125]. In recent years, *B. pertussis* strains that do not produce PRN have been isolated from pertussis patients[127], raising the concern that such strains have been selected due to the presence of anti-PRN antibodies generated in response to immunization with PRN-containing aP vaccines. Whether vaccine driven evolution of *B. pertussis* strains

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is actually occurring is currently under investigation, as it has decisive implications for the development of new and improved vaccines.

Lipopolysaccharide

B. pertussis, *B. parapertussis*$_{Hu}$ and *B. bronchiseptica* produce different forms of lipopolysaccharide (LPS). *B. pertussis* produces a penta-acylated lipid A linked to a complex core trisaccharide, *B. bronchiseptica* produces hexa-acylated lipid A linked to a similar, if not identical, complex core trisaccharide and O-antigen repeats and *B. parapertussis*$_{Hu}$ produces a hexa-acylated lipid A linked to an altered core structure and O-antigen repeats[128–130]. Because it lacks O-antigen, *B. pertussis* LPS is often referred to as lipooligosaccharide (LOS)[131]. The genes required for synthesis of O-antigen in *B. bronchiseptica* and *B. parapertussis*$_{Hu}$ are repressed by BvgAS[132]; however, some O-antigen is produced under Bvg$^+$ phase conditions and mutants unable to produce O-antigen display defective virulence in mouse models[132, 133].

In mice, *B. bronchiseptica* LPS is sensed by TLR4, resulting in an early TNF-α response and recruitment of neutrophils to the lungs[134, 135]. Although *B. parapertussis*$_{Hu}$ LPS and *B. pertussis* LOS can stimulate murine TLR4, they do so less efficiently and TLR4$^{-/-}$ mice are only modestly impaired in their ability to control infection by these organisms[136–138]. In addition, it has been reported that *B. pertussis* LOS stimulation of murine dendritic cells results in the development of anti-inflammatory regulatory T cells[136]. On the basis of these observations it has been suggested that *B. pertussis* and *B. parapertussis*^{Hu} have evolved to be less inflammatory than *B. bronchiseptica* and that diminished inflammation might facilitate persistence during human infection[136, 137]. However, subsequent studies have demonstrated that human and murine TLR4-MD-2-CD14 complexes differ in their ability to recognize different forms of lipid A. Although murine TLR4-MD-2-CD14 responds similarly to both penta- and hexa-acylated lipid A, human TLR4-MD-2-CD14 responds robustly to hexa-acylated lipid A but only weakly to penta-acylated lipid A[139]. Furthermore, as opposed to murine TLR4-MD-2-CD14, which responds to *B. pertussis* lipid A regardless of whether the phosphate groups are modified or not, human TLR4-MD-2-CD14 responds more robustly to lipid A containing glucosamine (GlcN)-modified phosphate groups than to lipid A with unmodified phosphates[140]. Although it seems that the majority of *B. pertussis* LOS contains GlcN-modified phosphate groups[141], the fact that it is penta-acylated suggests that its ability to stimulate TLR4 in humans is even weaker than its ability to stimulate TLR4 in mice. These data provide additional support for the hypothesis that *B. pertussis* and *B. parapertussis*$_{Hu}$ strains have evolved to be relatively non-inflammatory in humans. However, they also raise concerns about extrapolating conclusions drawn from murine studies to humans, as the TLR4-MD-2-CD14-dependent immune responses clearly differ in these hosts.

Additional surface proteins

Many additional BvgAS-activated genes encode known or predicted surface-localized or secreted proteins and are suspected to have roles in pathogenesis[29, 142]. BrkA, TcfA, BapC, BatB, Vag8, SphB1 and Phg are BvgAS-activated classical autotransporter proteins, and their putative roles in pathogenesis include mediating adherence, serum resistance, evasion

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of antibody-mediated clearance and proteolytic processing of other surface proteins[103, 143–148]. BipA and BcfA are BvgAS-regulated members of the intimin/invasin family, and although their roles in pathogenesis are unknown[35, 149], immunization of mice with BcfA can accelerate clearance of *B. bronchiseptica* following intranasal challenge[150], suggesting that these poorly characterized surface molecules should be considered for the development of new vaccines containing different or additional antigens.

Metabolic proteins

Many BvgAS-regulated genes encode proteins that are probably involved in metabolism, respiration and other physiological processes[29, 142], presumably reflecting the diversity of environmental conditions encountered by *Bordetella* spp. as they travel within and outside the mammalian respiratory tract. Among these factors, those involved in the acquisition and use of iron have been the focus of most studies. In addition to producing and using the siderophore alcaligin[151], *B. pertussis* and *B. bronchiseptica* can use a variety of xenosiderophores (including enterobactin[152]) and haem iron sources such as hemoglobin[153]. Most, if not all, of these iron acquisition mechanisms are required during murine respiratory infection[154, 155], demonstrating the necessity of iron for bacterial survival, the variety of mechanisms used by the host to sequester iron and the reciprocal array of mechanisms used by the bacteria to acquire this essential element.

In addition, accumulating evidence suggests that biofilm production by pathogenic *Bordetella* spp. *in vitro* and during infection may contribute to colonization of the respiratory tract. This process is regulated by a complex program of both Bvg-dependent and Bvg-independent gene expression[156–159], with genes that promote biofilm formation being maximally expressed in the Bvg^i phase[156]. Bvg-independent production of an exopolysaccharide via expression of the *bps* locus and the presence of extracellular DNA are also required for biofilm production[157, 160, 161]. Recent evidence suggests that the second messenger cyclic-di-GMP is also crucial for the regulation of biofilm formation[162].

Current and future challenges

Despite high rates of immunization with aP vaccines, epidemics of pertussis have recently occurred in the US, Europe, Australia and Japan (CDC, Australian Government Department of Health and Aging, Japanese National Institute of Infectious Diseases)[163, 164] and similar outbreaks seem imminent in developed countries throughout the world. Moreover, irrespective of socioeconomic status, the highest rates of mortality are in infants, who are also the most difficult population to treat and protect. In considering these challenges and looking ahead, we suggest three priorities for future studies.

The first priority is to improve the robustness and duration of protection conferred by vaccination, which will require further study of the immunological responses to infection and vaccination (Box 4). The deficiencies of current aP vaccines are well documented, including the striking observation that aP vaccination of baboons only protects against disease symptoms but not colonization or transmission[165]. Numerous efforts are in progress to overcome these deficiencies[24], such as the inclusion of additional antigens in aP vaccines, reformulation with adjuvants that favour Th1/Th17 responses as opposed to the Th2-type

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immunity generated by alum-adjuvanted vaccines as well as the development of live, attenuated *B. pertussis* vaccines[166]. The latter approach has significant advantages, including the ability to generate mucosal immunity, but the issue of public acceptance looms large. In a similar vein, it is interesting to note that outside of North America, Europe and parts of Asia, wP vaccines remain in widespread use and approaches to decrease their reactogenicity while retaining immunogenicity should be considered[167]. The known efficacy of these vaccines combined with the cost effectiveness of this approach might be of more benefit to people than the development of improved but more costly vaccines composed of purified proteins. It is important to remember that the development and approval of novel vaccines will be a prolonged process. In light of recent findings concerning the lack of protection against colonization or transmission by aP vaccination[165], maximizing the efficacy of current vaccines through prenatal vaccination, additional boosting and additional strategies is imperative.

Box 4

Immunity to pertussis

Clinical studies suggest that both humoral and cell-mediated immunity are important for controlling human pertussis. Measurements of *B. pertussis*-specific T cell proliferation, cytokine production and titers of different IgG subclasses from human samples suggest that naive infection causes primarily a Th1 response, resulting in pro-inflammatory cytokine and opsonizing antibody production, combined with the stimulation of antigen presenting cells[193, 194]. wP vaccines also stimulate a Th1 response[195, 196], whereas aP vaccines seem to produce a mixed Th1/Th2 response[196–198]. Many studies have demonstrated that vaccination with aP vaccines produces antibody responses that are equal to or exceed those produced by vaccination with wP vaccines[194, 196, 198], yet the immunity induced by aP vaccines is inefficient, which underscores the importance of investigating and evaluating cell-mediated immune responses induced by vaccination and infection. Studies that have evaluated human infection or vaccination have not comprehensively addressed the role of Th17 responses, which result in pro-inflammatory cytokine production and stimulation of professional phagocytes. However, studies with the baboon infection model show that infection causes a mixed Th1/Th17 response that is long-lived and protective against colonization following subsequent challenge[186, 199]. Immunological data from animal models mostly agree with these findings[23, 165, 199]. Regardless of the natural immune response to infection, efficacious vaccines need to be long lasting, prevent transmission and reduce disease burden. As *B. pertussis* is primarily an extracellular respiratory pathogen, it is likely that an effective immune response will require the induction of a mixed Th1/Th17 response that stimulates the production of opsonizing, toxin-neutralizing and mucosal antibodies, along with memory T cells that produce cytokines to recruit and activate professional phagocytes at the site of infection. As such, there is a pressing need to re-evaluate antigens, adjuvants and immunization routes to achieve these goals.

A second priority is to mitigate infant mortality. Nearly 90% of all deaths due to pertussis occur in infants that are less than four months of age[168], and the most frequent cause is

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intractable pulmonary hypertension associated with marked lymphocytosis and bronchopneumonia. Currently, the only efficacious therapy for severe cases is rapid leukodepletion, which is only available at advanced critical care centres[169, 170]. Respiratory samples obtained during autopsies show luminal aggregates of leukocytes occluding small pulmonary arteries, along with an abundance of *B. pertussis*[10, 171]. The pathology of fatal pertussis pneumonia appears to be largely caused by pertussis toxin. Thus, in addition to protecting susceptible infants by maternal vaccination or by vaccination at birth, it is also imperative to pursue approaches for limiting PT activity during infection. Potential therapeutic modalities include humanized monoclonal antibodies and small molecules to target PT interactions with host cell receptors or the enzymatic activity of PT, as well as regulatory factors such as the BvgAS system.

Finally, although animal models have proven useful, we need to enhance our understanding of human disease. Decades of research on *B. pertussis* virulence determinants have primarily been based on tissue culture models and murine infections. These studies have shown what adhesins, toxins and other virulence factors *can do* under laboratory conditions, but very little, if anything, is known about what they *really do* during human disease. Specificity is the rule for human adapted pathogens and it can manifest at several levels including gene expression, virulence factor delivery, binding specificity and activity. Perhaps the most vivid illustration of our lack of understanding of *B. pertussis* is that we still don't know why infection makes people cough!

Acknowledgments

The authors thank N. Carbonetti, P. Sebo, and R. Fernandez for their comments on the manuscript and their help preparing figures. Due strict limitations on citations, we apologize to our colleagues whose work we were unable to cite. This work was supported by the National Institute of Allergy and Infectious Disease of the National Institutes of Health under Award Numbers T32 AI007151 (J.A.M.) and R01 AI094991 (P.A.C.).

Glossary

paroxysmal coughing	Multiple, rapid coughing attacks that are characteristic of pertussis
***B. parapertussis*$_{Hu}$**	Two clades of bacteria have been classified as *B. parapertussis*. Those isolated from humans are designated *B. parapertussis*$_{Hu}$ and those isolated from sheep are designated *B. parapertussis*$_{Ov}$
Lymphocytosis	An increase in the number of lymphocytes (T-cells, B-cells and NK cells) in the bloodstream
type IV secretion system	A protein complex that is homologous to the conjugative machinery that spans the cell envelope. Type IV secretion systems (T4SS) are capable of both secretion and uptake of DNA and proteins
clodronate	bisphosphonate clodronate is a chemical that, when delivered to macrophages via phagocytosis of clodronate containing liposomes, causes apoptotic cell death

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type I secretion system	A protein complex that spans the inner and outer membrane of Gram-negative bacteria, the Type I secretion machinery consists of an ABC transporter protein, a membrane fusion protein and an outer membrane protein and is responsible for the secretion of various molecules
oxidative burst	(Respiratory burst)The rapid production of reactive oxygen species (such as hydrogen peroxide and superoxide radicals), typically by neutrophils and monocytes, which assist in combating bacterial and fungal infections
Rho GTPases	A family of small signalling GTPases within the Ras superfamily. Rho GTPases are found in eukaryotic cells and are involved in a wide range of cellular activities
Pattern recognition receptors	Proteins of the innate immune system that recognize pathogen-associated molecular patterns (PAMPs); for example the toll-like receptor 4 (TLR4), which recognizes lipopolysaccharide
Two-Partner Secretion (TPS) pathway	A secretion pathway consisting of two proteins, a large exoprotein (TspA) and an outer membrane pore-forming β-barrel protein (TspB)
Sec translocation system	A protein complex that transports molecules across the cytoplasmic membrane
phase variation	A mechanism by which bacteria vary the expression of genes encoding extracellular structures, such as flagella and fimbriae, typically as a means to avoid host immunity. This is frequently accomplished by DNA rearrangement or slip-strand mispairing
autotransporter	Family of proteins found in Gram-negative bacteria that consist of an N-terminal passenger domain and a C-terminal β-barrel. The β-barrel is responsible for transporting the passenger domain into the extracellular space and anchoring the protein to the outer membrane
intimin/invasin family	Virulence factors that mediate invasion and adherence
alternative sigma factor	Protein factors that associate with RNA polymerase and are important for promoter recognition and transcription initiation. They are typically induced under different environmental conditions. Alternative sigma factors differ from primary sigma factors, which are required for transcription of essential genes
Siderophore	Iron chelating compounds secreted by microorganisms to scavenge iron for cellular metabolism. Xenosiderophores refer to siderophores that can be utilized across different species

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IS (insertion sequence) element	A short, transposable DNA sequence
multilocus sequence typing	A molecular method that is used to characterize microorganisms by comparing the sequences of housekeeping genes

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Biographies

Jeffrey A. Melvin received his Ph.D. in Biochemistry from Duke University in 2012 and is currently a post-doctoral fellow in the laboratory of Dr. Peggy Cotter where his research focuses on the mechanism of secretion and the physiological function of *Bordetella* spp. filamentous hemagglutinin.

Erich V. Scheller received a B.A from Harvard University. He is currently a Ph.D. candidate in the Department of Microbiology and Immunology at the University of North Carolina at Chapel Hill, working in the laboratory of Dr. Peggy Cotter. His current research focuses on the contribution of *Bordetella* spp. fimbriae to adherence and virulence.

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Jeff F. Miller earned his Ph.D. in Molecular Biology and Microbiology from Tufts University School of Medicine in 1986. His interests in *Bordetella* spp. stem from postdoctoral studies with Dr. Stanley Falkow at Stanford and have continued at UCLA where he is Professor and Chair of Microbiology, Immunology and Molecular Genetics. In addition to *Bordetella* spp. pathogenesis, interests of the Miller lab include diversity-generating retroelements in phage, pathogens and the human microbiota and plasma membrane fusion by intracellular *Burkholderia* spp. Dr. Miller is currently President of the American Society for Microbiology.

Peggy A. Cotter earned her Ph.D. in Microbiology and Molecular Genetics at UCLA in 1992. She began studying the mechanisms of *Bordetella* spp. pathogenesis as a post-doctoral fellow with Jeff F. Miller at UCLA and continued those studies at UC Santa Barbara as an assistant, associate and full professor, before moving to the University of North Carolina, Chapel Hill in 2009. The Cotter lab also studies Contact-Dependent Growth Inhibition (CDI) in *Burkholderia* spp. Dr. Cotter was elected to the American Academy of Microbiology in 2013.

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Online summary

- *Bordetella pertussis*, the causative agent of whooping cough, has reemerged as a public health threat despite broad vaccine coverage.
- Reemergence of this disease correlates with a transition from the use of whole-cell pertussis vaccines to acellular component vaccines and the epidemiology of pertussis has shifted while the overall number of cases of pertussis has increased.
- *Bordetella* spp. serve as paradigms for studying global virulence control systems, bacterial toxins, type V secretion proteins and the evolution of pathogens.
- *Bordetella* spp. produce many virulence factors that contribute to pathogenesis, including toxins, adhesion factors, iron acquisition systems and surface structures.
- Considering the fact that *Bordetella pertussis* is a strict human pathogen, the development of appropriate animal models has been challenging. However, a novel baboon infection and transmission model provides a promising new research avenue.
- Future goals include improving the efficacy of vaccines, protecting unvaccinated infants from infection and developing better treatment strategies for infants who do become infected with *B. pertussis*. Reaching these goals requires a more thorough understanding of the mechanisms used by *B. pertussis* to establish infection and cause disease.

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Fig. 1A

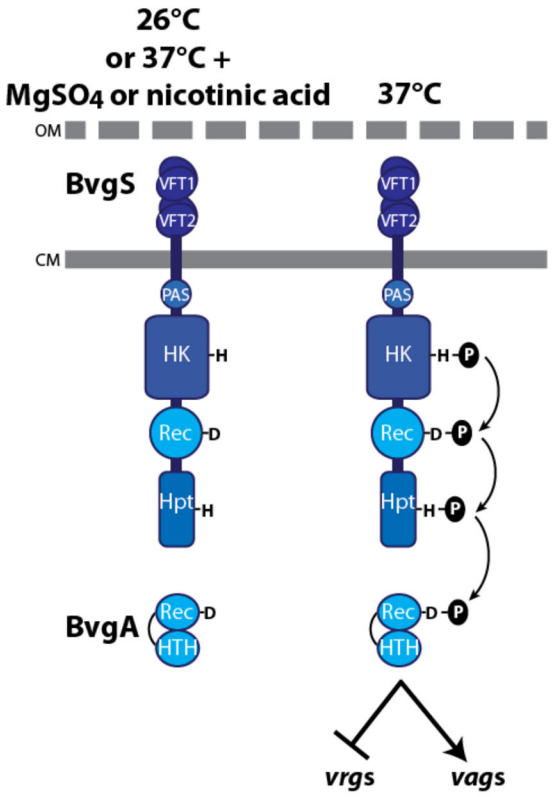

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Fig. 1B

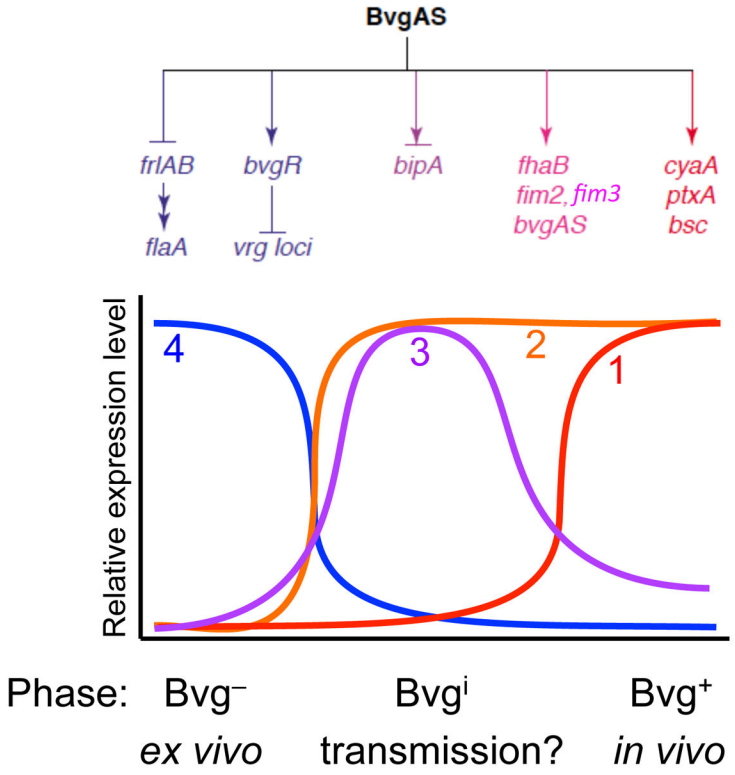

Figure 1. The BvgAS master regulatory system

(a) BvgS is a polydomain histidine sensor kinase containing (from the N- to the C-terminus) two periplasmically-located venus flytrap domains (VFT1 & VFT2), a transmembrane domain, a PAS domain (PAS), a histidine kinase domain (HK), a receiver domain (Rec) and a histidine phosphoryl transfer domain (Hpt). BvgA is a response regulator protein with an N-terminal receiver domain (Rec) and a C-terminal helix-turn-helix domain (HTH). BvgS is activate at 37°C, autophosphorylates at a conserved histidine in the HK domain, and transfers the phosphoryl group to the Rec, the Hpt and then to the Rec domain of BvgA. Phosphorylated BvgA (BvgA-P) activates expression of virulence-associated genes (*vag*s; which are subdivided into class 1 and 2 genes) and represses expression of virulence-repressed genes (*vrg*s; known as class 4 genes). BvgS is inactive and remains unphosphorylated when bacteria are grown at a low temperature (~25°C) or at 37°C in the presence of chemical modulators (such as $MgSO_4$ or nicotinic acid). (OM-outer membrane, CM-cytoplasmic membrane) **(b)** BvgAS controls four classes of genes and three distinct phenotypic phases. The Bvg^+ phase occurs when BvgAS is fully active and is characterized by maximal expression of genes encoding adhesins (class 2 genes, such as *fhaB, fim2* and *fim3,* expression levels indicated by an orange line) and toxins (class 1 genes, such as *cyaA-E, ptx-ptl* and *bsc* genes, expression levels indicated by a red line), and minimal expression of class 3 and class 4 genes (expression levels indicated by purple and blue lines, respectively). The Bvg^+ phase is necessary and sufficient to cause respiratory infection (i.e.,

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in vivo). The Bvg^{-} phase occurs when BvgAS is inactive and is characterized by maximal expression of class 4 genes and minimal expression of class 1, 2, and 3 genes. (Note that regulation of some *vrg*s is indirect; when BvgAS is inactive, it does not repress *frlAB,* a positive regulator at the top of the motility regulon, and it does not activate *bvgR*, a negative regulator of *vrg* loci.) The Bvg^{-} phase is required for growth under nutrient limiting conditions, such as may be encountered in the environment (i.e., *ex vivo*). The Bvg^{i} phase occurs when BvgAS is partially active and is characterized by maximal expression of class 3 genes and minimal expression of class 1, 2, and 4 genes. The only class 3 gene characterized so far is *bipA*, which is activated by BvgA under Bvg^{i} phase conditions and repressed by BvgA under Bvg^{+} phase conditions. The Bvg^{i} phase may be important for transmission between hosts, but this has not been fully elucidated.

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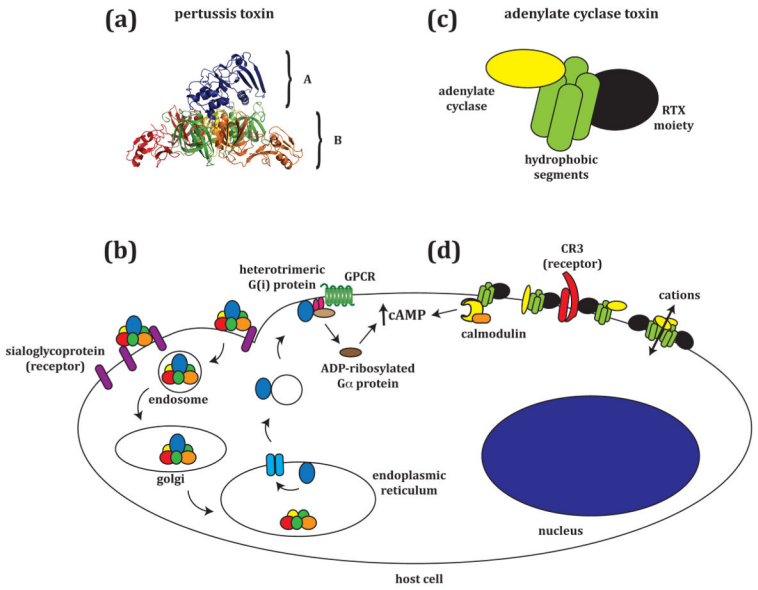

Figure 2. Toxin-mediated virulence of *Bordetella* spp

(a) Pertussis toxin (PT, PDB ID 1PRT), is an AB_5-type toxin composed of one catalytic subunit (A subunit) and five membrane-binding/transport subunits (B subunits)[47]. PT is assembled in the bacterial periplasm and exported by a type IV secretion system. **(b)** On binding to a sialoglycoprotein host cell receptor, PT is endocytosed and trafficked through the Golgi to the endoplasmic reticulum. In the endoplasmic reticulum, the B_5 complex binds to ATP and dissociates from the A subunit. The A subunit is then transported into the cytoplasm and traffics on exosomes to the cytoplasmic membrane, where it ADP-ribosylates the α subunit of heterotrimeric G proteins. This modification alters the ability of G proteins to regulate multiple enzymes and pathways, including their ability to inhibit cyclic AMP (cAMP) formation. The overall result of these modifications is an initial suppression of inflammatory cytokine production and inhibition of immune cell recruitment to the site of infection. **(c)** *Bordetella* spp. adenylate cyclase toxin (ACT) is composed of two primary domains, a calmodulin-responsive adenylate cyclase enzymatic domain (yellow) and an RTX domain (black), which are connected by hydrophobic segments (green). **(d)** The RTX domain of ACT interacts with CR3 receptors that are expressed on host cell membranes from a wide range of cell types. The hydrophobic segments of the linker region (green) form pores in the membrane that enable the passage of ions and translocation of the adenylate cyclase domain into the cytoplasm. Adenylate cyclase activity is stimulated by binding to calmodulin in the host cell. The combined effects of ACT intoxication and pore formation result in inhibition of complement-dependent phagocytosis, induction of anti-inflammatory cytokines, suppression of pro-inflammatory cytokines and inhibition of immune cell recruitment.

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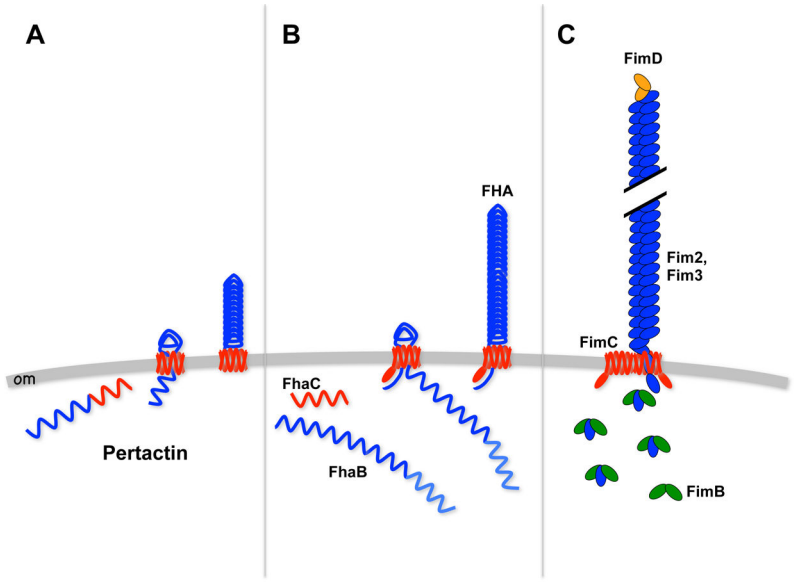

Figure 3. Presentation of filamentous hemagglutinin, fimbriae and pertactin on the *Bordetella* cell surface

(a) Filamentous hemagglutinin (FHA) is a TpsA exoprotein (blue) that is translocated across the outer membrane through its cognate TpsB pore protein (red), FhaC. This translocation occurs via the two-partner secretion pathway. Processing during translocation removes the C-terminal prodomain (yellow) from the full-length FhaB protein to produce the mature ~250 kDa FHA protein. FHA is required for adherence to ciliated epithelial cells and for persistence during infection, possibly by directly or indirectly modulating the host immune system. **(b)** *Bordetella* spp. fimbriae are type 1 pili. FimB is similar to chaperone proteins that traffic major fimbrial subunits (Fim2 and Fim3, in this case) to the membrane usher FimC. FimB and FimC are necessary for fimbrial secretion and FimD (the tip subunit) is necessary for fimbrial assembly. Fimbriae are required for persistence during infection, possibly by functioning similarly to FHA by directly or indirectly modulating the immune system. Furthermore, studies have suggested that fimbriae are necessary for adherence to ciliated epithelial cells. **(c)** Pertactin is a classical autotransporter. The C-terminal ~30 kDa region (red) forms a channel in the outer membrane (om) that is required for translocation of the ~70 kDa β-helical passenger domain (blue) to the cell surface. Although the precise role of pertactin is unclear, data suggests that pertactin may contribute to virulence by resisting neutrophil-mediated clearance.