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Research Article

RESEARCH ARTICLE

Bordetella biofilms: a lifestyle leading to persistent infections

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One sentence summary: Biofilms are emerging as critical for *Bordetella* survival and persistence in animal and human hosts.
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ABSTRACT

Bordetella bronchiseptica and *B. pertussis* are Gram-negative bacteria that cause respiratory diseases in animals and humans. The current incidence of whooping cough or pertussis caused by *B. pertussis* has reached levels not observed since the 1950s. Although pertussis is traditionally known as an acute childhood disease, it has recently resurged in vaccinated adolescents and adults. These individuals often become silent carriers, facilitating bacterial circulation and transmission. Similarly, vaccinated and non-vaccinated animals continue to be carriers of *B. bronchiseptica* and shed bacteria resulting in disease outbreaks. The persistence mechanisms of these bacteria remain poorly characterized. It has been proposed that adoption of a biofilm lifestyle allows persistent colonization of the mammalian respiratory tract. The history of *Bordetella* biofilm research is only a decade long and there is no single review article that has exclusively focused on this area. We systematically discuss the role of *Bordetella* factors in biofilm development *in vitro* and in the mouse respiratory tract. We further outline the implications of biofilms to bacterial persistence and transmission in humans and for the design of new acellular pertussis vaccines.

Keywords: biofilm; *Bordetella*; animal model; transmission; vaccine

INTRODUCTION

Whooping cough or pertussis is increasing steadily in the USA and other developed countries, leading the CDC to classify pertussis as a reemerging disease (CDC 2012; Cherry 2012; Jakinovich and Sood 2014). As a reflection of this resurgence, in 2015, pertussis and its causative organism *Bordetella pertussis* were included in the emerging infectious diseases/pathogens list maintained by the National Institute of Allergy and Infectious Diseases (http://www.niaid.nih.gov/topics/pertussis/Pages/research.aspx). Despite widespread immunization in childhood, 50 million cases and 300 000 deaths due to pertussis are estimated globally each year. Historically, pertussis has been perceived as a disease affecting non- or underimmunized infants. It is classically characterized by a series of short paroxysmal coughs followed by a vigorous inspiratory effort resulting in the whooping sound. In recent years, an increase in the incidence of pertussis has been observed in adolescents and adults with acquired immunity from vaccinations

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or previous infection (Cherry 2012, 2014). These individuals generally display milder symptoms often resembling viral respiratory infections and lack the characteristic 'whoop'. Pertussis in adolescents and adults often results in loss of time from school or work, social isolation, sleep deficiency or anxiety about an undiagnosed condition (McLaughlin *et al.* 2015). These individuals are now recognized as a major source of transmission of bacteria residing mainly in the upper respiratory tract (Cherry 2014).

Bordetella bronchiseptica has a broad host range and causes a spectrum of diseases in animals. It also infects both immunocompromised and healthy humans thereby demonstrating zoonotic transmission (Mattoo and Cherry 2005; Sukumar *et al.* 2014). It is widespread in swine populations and is an important contributor to respiratory disease in pigs (Zhao *et al.* 2011). In dogs, infection with *B. bronchiseptica* and several canine viruses can result in infectious tracheobronchitis or kennel cough (Schulz *et al.* 2014). In cats, *B. bronchiseptica* infection sometimes results in deaths particularly in young kittens when the disease progresses rapidly to bronchopneumonia (Coutts *et al.* 1996). In addition to causing severe diseases, a hallmark of *B. bronchiseptica* infections is long-term to life-long asymptomatic carriage. Carrier animals continue to shed the organism thereby infecting susceptible animals (Bemis, Carmichael and Appel 1977; Coutts *et al.* 1996; Schulz *et al.* 2014). In the laboratory, experimental infection of rats, mice and rabbits results in chronic and asymptomatic colonization of the upper respiratory tract (Goodnow 1980; Akerley, Cotter and Miller 1995; Mattoo, Miller and Cotter 2000; Mattoo and Cherry 2005).

For both *Bordetella* species, while significant insights have been obtained regarding the role of different factors in colonization of the respiratory tract, modulation and evasion of host immune responses and the control of gene expression (Mooi 2010; Hewlett *et al.* 2014), the mechanism by which these bacteria persist in humans and animals is not well characterized. We proposed the hypothesis that the survival and continued persistence of *Bordetella* spp. in mammals is due to the formation of biofilms (Sloan *et al.* 2007; Conover *et al.* 2010; Serra *et al.* 2011). Biofilms are generally defined as multicellular surface-adherent microbial communities often encased in a self-produced or host-derived matrix. This mode of growth confers traits associated with virulence and pathogenesis and resistance to environmental stresses, host defenses and antimicrobial compounds (Hall-Stoodley and Stoodley 2009; Hobley *et al.* 2015). Utilizing several *in vitro* models, the mouse model of respiratory infection and multiple imaging techniques, microscopic and macroscopic multicellular structures of *Bordetella* were observed on abiotic surfaces and in the mouse nose and trachea. The propensity of *Bordetella* to form biofilms raises fundamental questions regarding the (i) existence of unique biofilm-associated phenotypes; (ii) mechanisms by which multicellular structures develop; (iii) factors that contribute to biofilm development; (iv) relationship between biofilms and survival and persistence in humans and animals. In this review, we describe recent advances in the understanding of *B. pertussis* and *B. bronchiseptica* biofilm lifestyle on artificial surfaces and in the mouse respiratory tract. We focus on the developmental and regulatory aspects of biofilm formation as well as key factors involved in this process. Finally, we put forward the proposal that biofilms formed in the human nasopharynx protect bacteria from host clearance, allow transmission by dispersion and can explain the failure of vaccines to break the infectious cycle of *B. pertussis*.

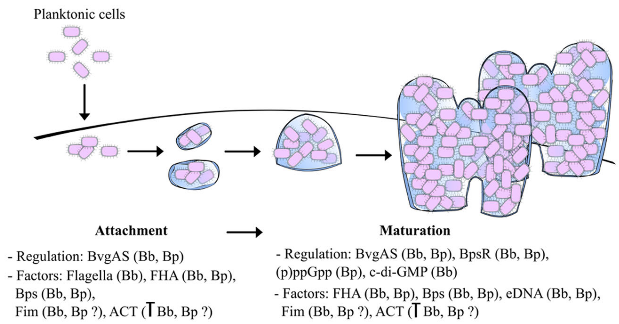

Figure 1. Cartoon of biofilm development and the roles of different factors in various stages of *Bordetella* biofilm formation. Planktonic cells initiate initial surface adhesion followed by formation of a monolayer ultimately giving rise to 3D structures. In one study, the initial attachment of *B. pertussis* (Bp) was Bvg dependent (Bosch *et al.* 2006) whereas in another study (Mishra *et al.* 2005), this step was Bvg independent in both Bp and *B. bronchiseptica* (Bb). Therefore, BvgAS is indicated to play a role in both the attachment and maturation stages of biofilm development. Other regulatory mechanisms ((p)ppGpp, c-di-GMP and BpsR) that control biofilm formation are also indicated. In Bb, flagella and in Bp, FHA promotes initial surface attachment. Bps, eDNA and FHA promote the formation and stability of macrocolonies and 3D structures. The inhibitory effect of ACT in Bb biofilm formation is indicated by bar-headed line. Because the precise mechanism by which Fim and ACT contribute to biofilm development is unknown, these factors are included on both sides of the vertical line. The symbol (question mark) indicates the unknown role of Fim and ACT in biofilm formation of *B. pertussis*.

ROLE OF THE BvgAS SIGNAL TRANSDUCTION SYSTEM IN BIOFILM FORMATION

The BvgAS signal transduction regulates the expression of genes encoding surface, membrane, secreted and regulatory proteins and those involved in bacterial metabolism and physiology. BvgA and BvgS are members of a group of the two-component superfamily of regulatory signal-transducing proteins that communicate by a four-step His-Asp-His-Asp phosphorelay system. In response to changes in the concentrations of certain chemicals, temperature or as a result of specific mutations in the BvgS sensor kinase, BvgAS mediates a transition between multiple phenotypic phases (Bvg^+, Bvg^i and Bvg^-). *In vitro*, BvgAS is active (Bvg^+ phase) when bacteria are grown at 37°C and in the absence of modulators. BvgAS is inactive (Bvg^- phase) at temperatures lower than 26°C or in the presence of high concentrations of modulators. In the Bvg^+ phase, *Bordetellae* are virulent and express several adhesins and toxins, whereas in the Bvg^- phase they are non-virulent. At low or intermediate concentration of modulators, bacteria are in the Bvg^i phase, which is characterized by the expression of specific genes like *bipA* (Deora *et al.* 2001; Cotter and Jones 2003; Deora 2004). Given the importance and the extensive characterization of BvgAS and its regulated gene products in *Bordetella* pathogenesis, it was not surprising that the first two published studies documenting biofilm formation by *Bordetella* showed that BvgAS positively regulated this phenotype in both *B. bronchiseptica* (Irie, Mattoo and Yuk 2004; Mishra *et al.* 2005) and *B. pertussis* (Mishra *et al.* 2005). Comparison of the role of BvgAS in different steps of biofilm development has resulted in different conclusions. While Mishra *et al.* (2005) suggested that biofilm development in both *B. bronchiseptica* and *B. pertussis* was characterized by an initial Bvg-independent attachment stage followed by a Bvg-dependent step that leads to the development of multicellular biofilms, Bosch *et al.* (2006) found both these steps to be Bvg dependent in *B. pertussis* (Fig. 1). Differences in experimental protocols and the nature of the abiotic surfaces utilized to study biofilms may account for the observed discrepancies between the two studies. With respect to the role of different Bvg-regulated phenotypic phases in biofilm formation, utilizing static assays, maximal biofilm formation for *B. bronchiseptica* was reported in the Bvg^{i} phase (Irie, Mattoo and Yuk 2004; Sisti *et al*. 2013). However, another study reported similar levels of biofilm formation when *B. bronchiseptica* was grown under static or flow conditions either in the Bvg^{+} or the Bvg^{i} phase (Mishra *et al*. 2005). The role of the Bvg^{i} phase on *B. pertussis* biofilm formation is unstudied. In summary, although published studies from various groups have reached somewhat different conclusions regarding the role of BvgAS in steps of biofilm development and the role of specific Bvg-regulated phenotypic phases in biofilm formation, it can be concluded that BvgAS plays an important role in regulating biofilm formation in both *B. bronchiseptica* and *B. pertussis*.

BORDETELLA BIOFILM FORMATION ON ABIOTIC SURFACES: A HIGHLY REGULATED DEVELOPMENTAL PROGRAM

In general, bacterial biofilm formation begins with the surface attachment of the planktonic bacteria resulting in the formation of a monolayer followed by the formation of aggregates, clusters and microcolonies. Finally, bacteria develop into differentiated structures in which individual bacteria as well as the entire community are surrounded by an extracellular matrix (Fig. 1) (Hall-Stoodley and Stoodley 2009). Similarly, biofilm formation in both *B. bronchiseptica* and *B. pertussis* can be microscopically visualized as a sequential temporal process that is characterized by initial surface attachment of bacterial cells, followed by the formation of a monolayer covering almost the entire surface area (Fig. 2). At this stage, *Bordetella* biofilms do not display any 3D structural attributes. At later time points, biofilms are characterized by cell clusters separated by individual cells followed by the formation of mature macrocolonies encased in an opaque matrix-like material (in static systems) (Fig. 2), pillar-like structures, water channels and or irregularly shaped microcolonies (in continuous-flow systems) (Parise *et al*. 2007; Serra *et al*. 2008, 2011; Conover *et al*. 2010; Nicholson, Conover and Deora 2012). Thus, based on microscopic analyses, *Bordetella* biofilms are highly differentiated communities compared to their planktonic counterparts and formation of biofilms proceeds in a stage-specific and coordinated manner (Figs 1 and 2).

This model of *Bordetella* biofilm development as a sequential and coordinated process is further supported by gene expression and proteomic analyses of biofilm cells. Transcriptomic analysis of *B. bronchiseptica* biofilms at five different time points representing distinct biofilm stages revealed that greater than 33% of the *B. bronchiseptica* genome undergoes expression changes during biofilm growth. Clustering analysis further revealed a cascade of continuous gene expression patterns with orderly timing of global gene expression lacking sharp transitions. Application of clustering analyses to a specific set of genes annotated to be transcription factors revealed a rigid expression pattern with specific transcription factors maximally expressed at distinct biofilm stages (Nicholson, Conover and Deora 2012). In addition to transcriptomics, independent proteomic analyses in *B. pertussis* revealed that about 8% of the cytosolic subproteome and 10% of the membrane subproteome were found altered in the biofilm condition (Serra *et al*. 2008). These findings along with those from other bacteria (Sauer 2003; Petrova and Sauer 2009; Park *et al*. 2014) strengthen the concept that bacterial biofilms are not simply a mixture of planktonic populations at different growth stages but represent a true microbial developmental process that involves large-scale changes in the expression of biofilm-specific genes and proteins, similar to sporulation by *Bacillus* species (Tan and Ramamurthi 2014) and swarmer-to-stalk cell transition in *Caulobacter crescentus* (Cornejo, Abreu and Komeili 2014).

As expected and consistent with the *bvg*-dependent control of biofilm formation, transcriptomic analyses also suggested that in *B. bronchiseptica*, expression of many of the *bvg*-activated genes varied in a temporal manner with the progression of biofilm formation. Surprisingly, at the initial time points of biofilm formation, many of the classical *bvg*-activated genes were repressed whereas expression of *bvg*-repressed genes (those involved in motility and synthesis of flagella) was induced. This suggested that a Bvg^{-} phase phenotype was preferred during early stages of biofilm formation and suggests a role for flagella in initial surface contact (Nicholson, Conover and Deora 2012). The role of flagella in biofilm formation is discussed later.

ROLE OF *BvgAS*-REGULATED PROTEIN FACTORS IN BIOFILM DEVELOPMENT

BvgAS-activated proteins

Bordetella pertussis and *B. bronchiseptica* produce several Bvg-activated adhesins namely filamentous hemagglutinin (FHA), pertactin, fimbriae and BrkA and toxins like adenylate cyclase toxin (ACT) and pertussis toxin (produced only by *B. pertussis*). FHA is a rod-like structure that is both surface-associated and secreted (Villarino Romero, Osicka and Sebo 2014). Microtiter plate assays under static conditions showed that both FHA and fimbriae contribute to biofilm formation in *B. bronchiseptica* (Irie, Mattoo and Yuk 2004). The role of FHA in *B. pertussis* biofilm

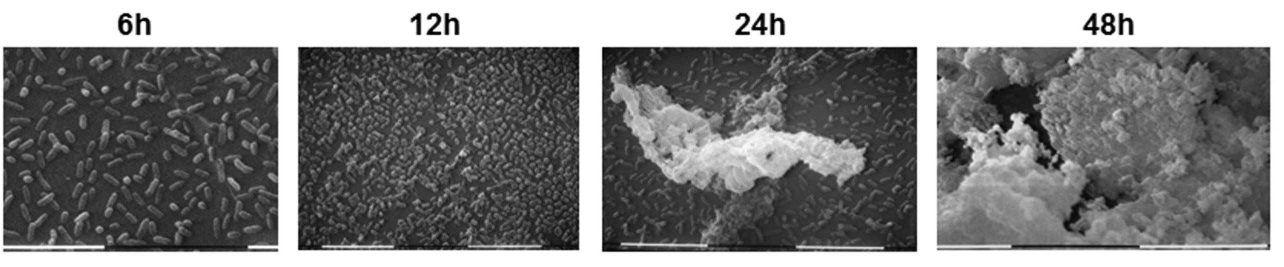

Figure 2. Different stages of *B. bronchiseptica* biofilm development examined by scanning electron microscopy (SEM). At 6 h, initial attachment is evident as individual cells dispersed in the surface. At 12 h, more cells adhere to the surface showing cell–cell interaction, but without any structural organization. At 24 h, cell clusters are apparent, evolving in mature macrocolonies enclosed in a self-produced extracellular matrix by 48 h of culture. The figure was taken from Nicholson, Conover and Deora (2012).

formation and the mechanisms by which FHA promotes biofilm formation were later pursued by our groups (Serra *et al.* 2011). A mutant strain of *B. pertussis* lacking FHA exhibited reduced surface attachment, decreased biofilm biomass and did not form microcolonies. Absence of FHA from *B. pertussis*, antibody-mediated blockade of surface-associated FHA and addition of exogenous FHA inhibited the attachment of bacteria to the pre-existing biofilms (Serra *et al.* 2011). Thus, FHA promotes the structural integrity of *B. pertussis* biofilm by mediating cell substrate and interbacterial adhesions. The mechanisms by which Fimbriae promote biofilm formation remain to be determined for both *B. bronchiseptica* and *B. pertussis*.

ACT is a protein toxin produced by both *B. pertussis* and *B. bronchiseptica* (Vojtova, Kamanova and Sebo 2006). It is secreted by the type I secretion system and remains both surface associated and released. A *B. bronchiseptica* strain lacking the *cyaA* gene formed higher levels of biofilms than the wild-type strain. It was proposed that ACT was inhibiting biofilm formation by interacting primarily with FHA (Irie, Mattoo and Yuk 2004). The role of ACT in *B. pertussis* biofilm formation and the precise mechanism of biofilm inhibition remain to be tested.

Role of flagella, a Bvg-repressed surface structure in biofilm formation

Gene expression profiling of *B. bronchiseptica* biofilm cells revealed that the expression of genes encoding flagella and motility, classical Bvg$^-$ phase phenotypes occurred early and was under tight regulatory control (Nicholson, Conover and Deora 2012). By utilizing strains lacking either *flaA* (encoding the flagellin monomer) or other genes that regulate the production of flagella, it was shown that flagella were critical for initial surface attachment but were not required for biofilm maturation (Nicholson, Conover and Deora 2012). While work in other bacterial systems suggested that repression of flagellar expression after the initial attachment is a necessary step for formation of mature bacterial biofilms (Moorthy and Watnick 2004; Lemon, Higgins and Kolter 2007), direct experimental evidence for such a requirement was lacking. By utilizing mutant strains that harbored alterations in the regulatory hierarchy of flagella production, it was shown that constitutive production of flagella by *B. bronchiseptica* results in immature and unstructured biofilms (Nicholson, Conover and Deora 2012).

The *Bordetella* biofilm matrix and role of matrix components in biofilm formation

The bacterial biofilm matrix is composed of several extracellular polymeric substances (EPS) whose composition varies based on the species. EPS is mainly composed of polysaccharides, proteins, metabolites and extracellular DNA (eDNA) (Hall-Stoodley and Stoodley 2009). *Bordetella* biofilm matrix also contains polysaccharides, LPS, eDNA and proteins. The sugar content of the biofilm matrix is composed of xylose (*B. bronchiseptica*), poly-β-1-6-N-acetyl-D-glucosamine (*B. bronchiseptica* and *B. pertussis*) and uronic acids (*B. pertussis*) (Irie, Preston and Yuk 2006; Parise *et al.* 2007; Serra *et al.* 2008).

eDNA and extracellular polysaccharides also play critical roles in different aspects of bacterial biofilm formation (Das, Sehar and Manefield 2013; Payne and Boles 2015). DNase I treatment of *B. pertussis* and *B. bronchiseptica* biofilms inhibited biofilm growth and disrupted established mature biofilms formed under both static and continuous flow conditions, suggesting that eDNA is involved in maintaining biofilm structure and stability (Conover, Mishra and Deora 2011). While the detection of several sugars in the biofilm matrix suggests a role for many polysaccharides with distinct biochemical composition in biofilm formation, Bps remains the only polysaccharide that has been experimentally shown to be required for biofilm formation (Parise *et al.* 2007; Conover *et al.* 2010). The *bpsABCD* operon, which encodes the machinery for Bps synthesis, is highly conserved in *Bordetella* species and is homologous to the *pgaABCD* locus of *Escherichia coli* (Wang, Preston and Romeo 2004) and the *icaADBC* loci of Gram-positive bacteria (O'Gara 2007). While the exact biochemical composition of Bps remains to be determined, based on immune reactivity and enzymatic susceptibility to dispersin B, it is similar in composition to poly-β-(1,6)-*N*-acetyl-D-glucosamine type of polysaccharides (Parise *et al.* 2007; Conover *et al.* 2010; Little *et al.* 2015). In both *B. bronchiseptica* and *B. pertussis*, Bps is dispensable for initial attachment to abiotic surfaces. Instead, Bps contributes to the stability and maintenance of the complex architecture of biofilms (Parise *et al.* 2007; Conover *et al.* 2010).

Additional mechanisms of biofilm regulation

In *B. bronchiseptica*, expression of the *bpsA-D* locus was elevated in biofilms (Conover *et al.* 2012). While, the inducing signals and control mechanisms of Bps synthesis have not yet been completely defined, the expression of the *bpsA-D* locus in *B. bronchiseptica* was not regulated by BvgAS (Conover *et al.* 2012). A DNA-binding repressor protein BpsR that negatively regulated Bps expression and synthesis was identified in *B. bronchiseptica*. The absence of BpsR from *B. bronchiseptica* increased expression and production of Bps, enhanced biofilm formation and produced more structured biofilms (Conover *et al.* 2012). The function of BpsR is not known in *B. pertussis*. Continued research on the role of Bps and BpsR in biofilm formation and regulatory mechanisms will further elucidate biofilm developmental processes.

The signaling molecule bis-(3′–5′)-cyclic-dimeric guanosine monophosphate c-di-GMP plays a key role in the decision between planktonic or biofilm growth, where low intracellular levels of c-di-GMP lead to a planktonic phenotype and high concentrations lead to a biofilm phenotype. c-di-GMP is produced from two GTP molecules by enzymes that contain GGDEF domains, and is degraded by enzymes with EAL or HD-GYP domains (Romling, Galperin and Gomelsky 2013). Overexpression of *Pseudomonas aeruginosa* genes that encode enzymes involved in either the production or degradation of c-di-GMP led to modest but statistically significant enhancement or reduction, respectively in biofilm levels of *B. bronchiseptica* (Sisti *et al.* 2013). Plasmid-mediated expression of a *B. bronchiseptica* gene encoding a potential diguanylate cyclase increased biofilm formation in *B. bronchiseptica* and complemented the biofilm defective phenotype of a *P. fluorescens* strain lacking the genes encoding four diguanylate cyclase proteins (Sisti *et al.* 2013). Similar to *B. bronchiseptica*, a mutant strain lacking the gene encoding for a diguanylate cyclase displayed reduced biofilm formation in *B. pertussis* (Wan *et al.* 2009). It has been proposed that this protein synthesizes c-di-GMP and therefore may influence biofilm formation by sensing O_2 tension (Wan *et al.* 2009). *Bordetella bronchiseptica* encodes four hypothetical proteins with EAL domains, ten with GGDEF domain and five with both domains (Sisti *et al.* 2013). Similarly, *B. pertussis* encodes five proteins with GGDEF domain and four with EAL domains (Wan *et al.* 2009). The presence of several genes encoding proteins with either GGDEF or EAL domains in the genomes of *B. bronchiseptica* and *B. pertussis* suggests the existence of multigene control on the levels of c-di-GMP and biofilm formation.

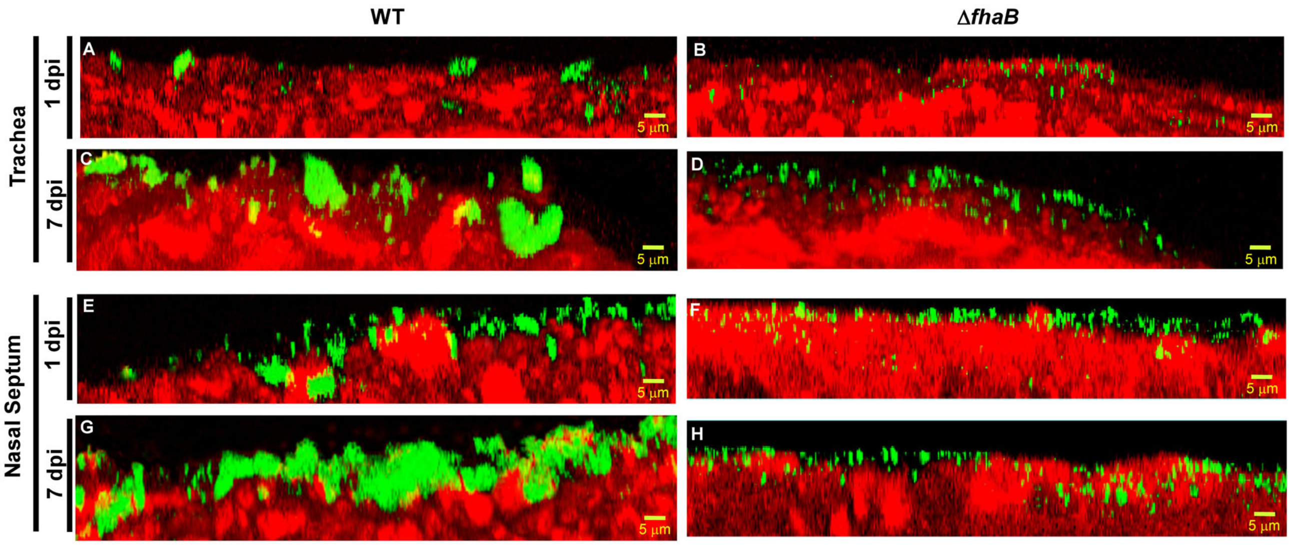

Figure 3. *Bordetella pertussis* biofilms and the role of FHA in biofilm formation in the mouse respiratory tract. Groups of 6-week-old C57BL/6 mice were intranasally inoculated with 5×10^5 CFUs of either the WT or Δ*fhaB* strain. Sections of trachea and nasal septum were harvested at 1 or 7 days post-infection, immediately fixed, and probed with rat anti-*Bordetella* serum followed by a donkey anti-rat secondary antibody conjugated to Alexa Flour 488 (stains bacteria green). Respiratory epithelium was visualized by staining for F-actin using phalloidin conjugated to Alexa Fluor 633 (red staining). For detailed figure legend and the experimental procedure, please see Serra *et al.* (2011).

The other regulatory molecule involved in *B. pertussis* biofilm formation is the alarmone (p)ppGpp. This molecule regulates stringent responses and processes important for bacterial growth, stress survival and virulence (Gaca, Colomer-Winter and Lemos 2015). A mutant strain of *B. pertussis* deficient in the production of (p)ppGpp was impaired in autoaggregation and biofilm formation. It was proposed that the effect of (p)ppGpp on biofilms was mediated by changes in filamentous structures, since compared to the wild-type strain, the mutant strain resulted predominantly in short filaments (Sugisaki *et al.* 2013). Thus, although the intricacies of the various controls on *Bordetella* biofilm formation remain unknown, it is clear that *Bordetella* utilizes multiple regulatory mechanisms to maintain a sessile lifestyle.

BORDETELLA BIOFILM LIFESTYLE IN THE MAMMALIAN RESPIRATORY TRACT

Despite the wealth of data on the mechanisms by which bacteria form biofilms on abiotic surfaces, limited information is available on factors and mechanisms that contribute to biofilm formation *in vivo*. Parsek and Singh proposed several criteria to define biofilm infections, which were later revised by Hall-Stoodley and Stoodley. In essence, these criteria are that (i) infecting bacteria should be adherent or attached to the surface, (ii) bacterial microcolonies or aggregates encased in an extracellular matrix either of bacterial or host origin should be directly observed; (iii) infection should be localized to a particular anatomical site; (iv) bacteria should be recalcitrant to antibiotic treatment compared to planktonic counterparts, or bacterial clusters/macrocolonies should be localized in host tissues as evidence of ineffective host clearance (Parsek and Singh 2003; Hall-Stoodley and Stoodley 2009). Utilizing well-established intranasal mouse models of *B. bronchiseptica* and *B. pertussis* infections (Harvill, Cotter and Miller 1999; Carbonetti *et al.* 2005; Sukumar *et al.* 2010), a biofilm mode of existence for these bacteria was demonstrated in the mouse nose and trachea (Sloan *et al.* 2007; Conover *et al.* 2010; Serra *et al.* 2011). In both of these models, distinct architectural features (in the form of mats, towers or pillars separated by void spaces for *B. bronchiseptica* and clusters and macrocolonies for *B. pertussis*; Fig. 3) adherent to ciliated epithelium of the nose and trachea were observed. These surface-adherent biofilms colocalized with Bps (Sloan *et al.* 2007; Conover *et al.* 2010). *Ex vivo* treatment with DNase I considerably dissolved both *B. pertussis* and *B. bronchiseptica* biofilms formed on the nasal septum, suggesting that eDNA is an additional biofilm matrix component and contributes to the structural stability of respiratory tract biofilms (Conover, Mishra and Deora 2011). *Bordetella bronchiseptica* biofilms formed *in vitro* are as much as 1000-fold more resistant to antibiotics compared to their planktonic counterparts (Mishra *et al.* 2005). Respiratory tract biofilms of *B. bronchiseptica* have been visualized as long as 38 days post-inoculation (Sloan *et al.* 2007) suggesting that biofilm formation supports bacterial persistence in the respiratory tract. It has not yet been experimentally demonstrated that *B. pertussis* biofilms enhance antimicrobial resistance. However, respiratory tract biofilms of *B. pertussis* have been observed 19 days post-inoculation of bacteria (Conover *et al.* 2010) and mutants of *B. pertussis* (Δ*fhaB* and Δ*bpsABCD*) defective in attachment to epithelial cells, cell–cell interactions and development of mature biofilms *in vitro* and *in vivo* are cleared faster from the respiratory tract (Fig. 3) (Conover *et al.* 2010; Serra *et al.* 2011). Thus, these results link *B. pertussis* biofilms to increased respiratory tract survival.

In conclusion, the mouse models of *Bordetella* biofilm infection satisfy the overall criteria for true biofilm infections. These models have tremendous potential to enhance the understanding of host–pathogen interactions in the context of biofilm infections.

IS THERE A ROLE FOR BIOFILMS IN HUMAN INFECTIONS OF *B. PERTUSSIS*?

Although *B. pertussis* forms biofilms in the mouse respiratory tract, the existence of *B. pertussis* biofilms and its role in *B. pertussis* life cycle in humans remains controversial. In 1912, the

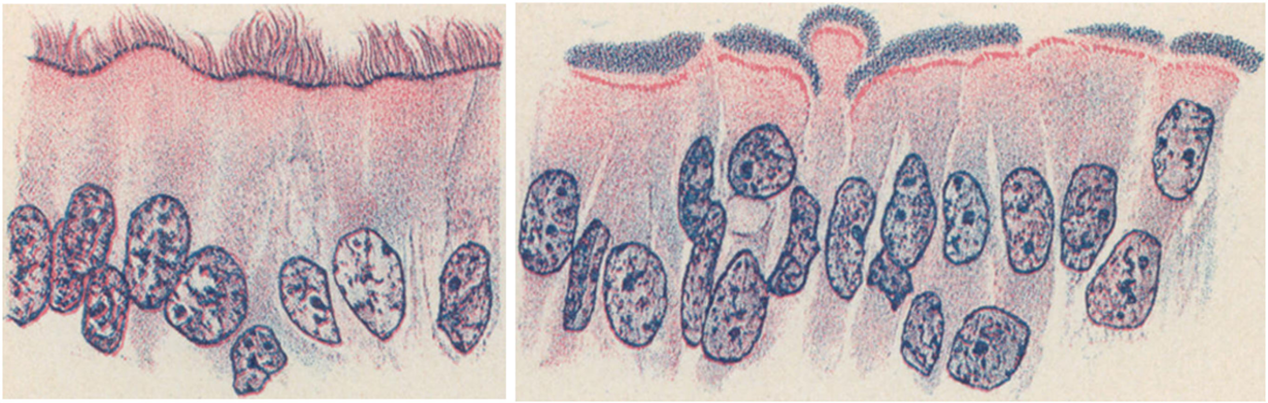

Figure 4. Drawing of normal and infected ciliated epithelia lining the trachea. This drawing was reported in 1912, showing the appearance of infected epithelium of a child dying in acute phase of pertussis. Picture taken from Mallory and Hornor (1912).

presence of 'masses of minute bacilli packed between the cilia of the lining epithelium' and 'in the secretion in the trachea and bronchi' in the lungs patients who died of whooping cough was reported (Mallory and Hornor 1912). The drawings presented (Fig. 4) clearly showed large numbers of bacteria in the form of aggregates or clusters. Approximately, a hundred years later, Paddock *et al.* (2008) observed clusters and tangles of *B. pertussis* on the cilia of columnar epithelial cells lining the trachea and bronchioles in human infants who succumbed to pertussis. *Bordetella pertussis* was also found adherent to ciliated cells of human nasal explants in the form of microcolonies (Soane *et al.* 2000). While these authors did not specifically classify the various bacterial forms as biofilms, the structures observed resemble biofilms described by us on abiotic surfaces and in the mouse nose and trachea (Conover *et al.* 2010; Serra *et al.* 2011). Moreover, Bps polysaccharide, which is required for biofilm formation, expressed at higher levels during *in vitro* biofilm growth and colocalizes with biofilms formed on mouse tissues, is expressed during human infections as evidenced by the presence of anti-Bps antibodies in pertussis-positive patients (Conover *et al.* 2010). Colocalization of either Bps and/or eDNA with the bacterial structures formed on human respiratory tissues will allow classification of the observed structures on human tissues as biofilms.

BIOFILMS, A POTENTIAL EXPLANATION FOR PERTUSSIS VACCINE FAILURE AND REEMERGENCE

Bordetella pertussis infection or immunization with whole cell vaccines (wPV) confers long-term immunity against *B. pertussis* reinfection. However, wPVs are highly reactogenic, and the safety and compliance concerns associated with wPV were the impetus for implementation of acellular pertussis vaccines (aPV), for pediatric immunizations and adult boosters. Although the mechanisms that lead to reemergence of pertussis are likely multifactorial, lower vaccine efficacy and poor short-lived vaccine-induced immunity are some explanations (Guiso 2009; Mooi 2010; Higgs *et al.* 2012). In recent years, a rapid drop in protection and waning protective immunity following aPV vaccination has been observed suggesting that aPVs do not generate long-term immunity (Klein *et al.* 2012; Witt *et al.* 2013). All commercial aPVs are formulated with alum, an adjuvant that induces Th2-type immune responses (Marrack, McKee and Munks 2009), while wPVs and prior infection induce Th1/Th17-type responses (Higgs *et al.* 2012; Ross *et al.* 2013). Accumulated evidence suggests that this difference in polarization is at least partly responsible for the incomplete protection mediated by aPVs (Higgs *et al.* 2012; Ross *et al.* 2013). Thus, the majority of recent therapeutic efforts are focused on the identification of novel adjuvant and antigen combinations that will remodel the aPV response towards a response similar to that induced by wPV.

Another viable hypothesis for vaccine failure is that current vaccines do not protect against the bacterial biofilm state. The choice of the current aPV components is based on functional studies conducted under planktonic growth conditions. In general, *B. pertussis* vaccination studies do not evaluate colonization of the mouse nose or trachea, organs where biofilms are observed. Inclusion of biofilm-promoting factors or antigens expressed at higher levels during biofilm development may result in enhanced protection against biofilms thereby facilitating the clearance of the infection. Recent work addressed the ability of biofilm-derived proteins to enhance *B. pertussis* clearance (de Gouw *et al.* 2014). In this study, while vaccination with biofilm-derived proteins reduced the number of bacteria in the lungs of mice, the numbers of bacteria harvested were still higher than those harvested from lungs of aPV-vaccinated mice. Surprisingly, vaccination with biofilm-derived proteins or aPV did not reduce bacterial numbers in the mouse nose. One factor that contributes significantly to colonization of *B. pertussis* in the mouse nose and trachea is Bps (Conover *et al.* 2010). Bps is also (i) required for biofilm formation; (ii) colocalizes with biofilms; (iii) functions as a nasal adhesin and (iv) resists complement (Conover *et al.* 2010; Ganguly *et al.* 2014). Thus, development of a Bps conjugate vaccine either alone or in combination with aPV may prevent bacterial colonization and establishment of biofilms in the nose and the trachea. In this context, it is quite encouraging that vaccination of mice with a protein-conjugated PNAG (a Bps homolog) vaccine reduced *Acinetobacter baumannii* bacterial burden in the blood (Bentancor *et al.* 2012). Additionally, adoptive transfer of anti-PNAG antibodies reduced *Staphylococcus aureus* bacterial burden (Kelly-Quintos *et al.* 2006).

CONCLUSIONS

Less than a decade after the first description, biofilms in *B. bronchiseptica* and *B. pertussis* are beginning to be recognized as important contributors to the pathogenesis of these organisms. This emerging view is probably best reflected by the increased pace of research on *Bordetella* biofilms. Several important discoveries and concepts applicable not only to *Bordetella* spp. but also in general to bacterial biofilms have resulted from these studies: (i) bacterial biofilm formation is a developmental program that proceeds by a number of complex and highly ordered regulatory steps involving extensive and stage-specific changes in gene expression, (ii) eDNA plays a critical role in the stability of biofilms formed on host organs, (iii) repression of flagella expression subsequent to initial attachment stage is critical for the maturation of abiotic biofilms and (iv) establishment of an animal model of biofilms that complies well with the criteria established for bacterial biofilm infections. The cellular processes of biofilm formation and maintenance, and the various biofilm matrix components have the potential to serve as targets for novel antimicrobials and more efficient vaccines that will better control the entire infectious cycle including colonization, persistence, disease presentation and transmission.

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REFERENCES

Akerley BJ, Cotter PA, Miller JF. Ectopic expression of the flagellar regulon alters development of the Bordetella-host interaction. *Cell* 1995;**80**:611–20.

Bemis DA, Carmichael LE, Appel MJ. Naturally occurring respiratory disease in a kennel caused by *Bordetella bronchiseptica*. *Cornell Vet* 1977;**67**:282–93.

Bentancor LV, O'Malley JM, Bozkurt-Guzel C, *et al.* Poly-N-acetyl-beta-(1-6)-glucosamine is a target for protective immunity against *Acinetobacter baumannii* infections. *Infect Immun* 2012;**80**:651–6.

Bosch A, Serra D, Prieto C, *et al.* Characterization of *Bordetella pertussis* growing as biofilm by chemical analysis and FT-IR spectroscopy. *Appl Microbiol Biot* 2006;**71**:736–47.

Carbonetti NH, Artamonova GV, Andreasen C, *et al.* Pertussis toxin and adenylate cyclase toxin provide a one-two punch for establishment of *Bordetella pertussis* infection of the respiratory tract. *Infect Immun* 2005;**73**:2698–703.

CDC. Pertussis epidemic–Washington, 2012. *MMWR Morb Mortal Wkly Rep* 2012;**61**:517–22.

Cherry JD. Epidemic pertussis in 2012–the resurgence of a vaccine-preventable disease. *N Engl J Med* 2012;**367**:785–7.

Cherry JD. Adult pertussis in the pre- and post-vaccine eras: lifelong vaccine-induced immunity? *Expert Rev Vaccines* 2014;**13**:1073–80.

Conover MS, Mishra M, Deora R. Extracellular DNA is essential for maintaining *Bordetella* biofilm integrity on abiotic surfaces and in the upper respiratory tract of mice. *PLoS One* 2011;**6**:e16861.

Conover MS, Redfern CJ, Ganguly T, *et al.* BpsR modulates *Bordetella* biofilm formation by negatively regulating the expression of the Bps polysaccharide. *J Bacteriol* 2012;**194**:233–42.

Conover MS, Sloan GP, Love CF, *et al.* The Bps polysaccharide of *Bordetella pertussis* promotes colonization and biofilm formation in the nose by functioning as an adhesin. *Mol Microbiol* 2010;**77**:1439–55.

Cornejo E, Abreu N, Komeili A. Compartmentalization and organelle formation in bacteria. *Curr Opin Cell Biol* 2014;**26**: 132–8.

Cotter PA, Jones AM. Phosphorelay control of virulence gene expression in Bordetella. *Trends Microbiol* 2003;**11**:367–73.

Coutts AJ, Dawson S, Binns S, *et al.* Studies on natural transmission of *Bordetella bronchiseptica* in cats. *Vet Microbiol* 1996;**48**:19–27.

Das T, Sehar S, Manefield M. The roles of extracellular DNA in the structural integrity of extracellular polymeric substance and bacterial biofilm development. *Environ Microbiol Rep* 2013;**5**:778–86.

de Gouw D, Serra DO, de Jonge MI, *et al.* The vaccine potential of *Bordetella pertussis* biofilm-derived membrane proteins. *Emerg Microbes Infect* 2014;**3**:e58.

Deora R. Multiple mechanisms of bipA gene regulation by the Bordetella BvgAS phosphorelay system. *Trends Microbiol* 2004;**12**:63–5.

Deora R, Bootsma HJ, Miller JF, *et al.* Diversity in the Bordetella virulence regulon: transcriptional control of a Bvg-intermediate phase gene. *Mol Microbiol* 2001;**40**:669–83.

Gaca AO, Colomer-Winter C, Lemos JA. Many means to a common end: the intricacies of (p)ppGpp metabolism and its control of bacterial homeostasis. *J Bacteriol* 2015;**197**:1146–56.

Ganguly T, Johnson JB, Kock ND, *et al.* The *Bordetella pertussis* Bps polysaccharide enhances lung colonization by conferring protection from complement-mediated killing. *Cell Microbiol* 2014;**16**:1105–18.

Goodnow RA. Biology of *Bordetella bronchiseptica*. *Microbiol Rev* 1980;**44**:722–38.

Guiso N. *Bordetella pertussis* and pertussis vaccines. *Clin Infect Dis* 2009;**49**:1565–9.

Hall-Stoodley L, Stoodley P. Evolving concepts in biofilm infections. *Cell Microbiol* 2009;**11**:1034–43.

Harvill ET, Cotter PA, Miller JF. Pregenomic comparative analysis between *Bordetella bronchiseptica* RB50 and *Bordetella pertussis* tohama I in murine models of respiratory tract infection. *Infect Immun* 1999;**67**:6109–18.

Hewlett EL, Burns DL, Cotter PA, *et al.* Pertussis pathogenesis—what we know and what we don't know. *J Infect Dis* 2014;**209**:982–5.

Higgs R, Higgins SC, Ross PJ, *et al.* Immunity to the respiratory pathogen *Bordetella pertussis*. *Mucosal Immunol* 2012;**5**: 485–500.

Hobley L, Harkins C, MacPhee CE, *et al.* Giving structure to the biofilm matrix: an overview of individual strategies and emerging common themes. *FEMS Microbiol Rev* 2015;**39**: 649–69.

Irie Y, Mattoo S, Yuk MH. The Bvg virulence control system regulates biofilm formation in *Bordetella bronchiseptica*. *J Bacteriol* 2004;**186**:5692–8.

Irie Y, Preston A, Yuk MH. Expression of the primary carbohydrate component of the *Bordetella bronchiseptica* biofilm matrix is dependent on growth phase but independent of Bvg regulation. *J Bacteriol* 2006;**188**:6680–7.

Jakinovich A, Sood SK. Pertussis: still a cause of death, seven decades into vaccination. *Curr Opin Pediatr* 2014;**26**: 597–604.

Kelly-Quintos C, Cavacini LA, Posner MR, *et al*. Characterization of the opsonic and protective activity against *Staphylococcus aureus* of fully human monoclonal antibodies specific for the bacterial surface polysaccharide poly-N-acetylglucosamine. *Infect Immun* 2006;**74**:2742–50.

Klein NP, Bartlett J, Rowhani-Rahbar A, *et al*. Waning protection after fifth dose of acellular pertussis vaccine in children. *N Engl J Med* 2012;**367**:1012–9.

Lemon KP, Higgins DE, Kolter R. Flagellar motility is critical for *Listeria monocytogenes* biofilm formation. *J Bacteriol* 2007;**189**:4418–24.

Little DJ, Milek S, Bamford NC, *et al*. BpsB is a poly-beta-1,6-N-acetyl-D-glucosamine deacetylase required for biofilm formation in *Bordetella bronchiseptica*. *J Biol Chem* 2015;**290**: 22827–40.

McLaughlin JM, McGinnis JJ, Tan L, *et al*. Estimated human and economic burden of four major adult vaccine-preventable diseases in the United States, 2013. *J Prim Prev* 2015;**36**: 259–73.

Mallory FB, Hornor AA. Pertussis: the histological lesion in the respiratory tract. *J Med Res* 1912;**27**:115–24, 113.

Marrack P, McKee AS, Munks MW. Towards an understanding of the adjuvant action of aluminium. *Nat Rev Immunol* 2009;**9**:287–93.

Mattoo S, Cherry JD. Molecular pathogenesis, epidemiology, and clinical manifestations of respiratory infections due to *Bordetella pertussis* and other Bordetella subspecies. *Clin Microbiol Rev* 2005;**18**:326–82.

Mattoo S, Miller JF, Cotter PA. Role of *Bordetella bronchiseptica* fimbriae in tracheal colonization and development of a humoral immune response. *Infect Immun* 2000;**68**:2024–33.

Mishra M, Parise G, Jackson KD, *et al*. The BvgAS signal transduction system regulates biofilm development in Bordetella. *J Bacteriol* 2005;**187**:1474–84.

Mooi FR. Bordetella pertussis and vaccination: the persistence of a genetically monomorphic pathogen. *Infect Genet Evol* 2010;**10**:36–49.

Moorthy S, Watnick PI. Genetic evidence that the *Vibrio cholerae* monolayer is a distinct stage in biofilm development. *Mol Microbiol* 2004;**52**:573–87.

Nicholson TL, Conover MS, Deora R. Transcriptome profiling reveals stage-specific production and requirement of flagella during biofilm development in *Bordetella bronchiseptica*. *PLoS One* 2012;**7**:e49166.

O'Gara JP. ica and beyond: biofilm mechanisms and regulation in *Staphylococcus epidermidis* and *Staphylococcus aureus*. *FEMS Microbiol Lett* 2007;**270**:179–88.

Paddock CD, Sanden GN, Cherry JD, *et al*. Pathology and pathogenesis of fatal *Bordetella pertussis* infection in infants. *Clin Infect Dis* 2008;**47**:328–38.

Parise G, Mishra M, Itoh Y, *et al*. Role of a putative polysaccharide locus in *Bordetella* biofilm development. *J Bacteriol* 2007;**189**:750–60.

Park AJ, Murphy K, Krieger JR, *et al*. A temporal examination of the planktonic and biofilm proteome of whole cell *Pseudomonas aeruginosa* PAO1 using quantitative mass spectrometry. *Mol Cell Proteomics* 2014;**13**:1095–105.

Parsek MR, Singh PK. Bacterial biofilms: an emerging link to disease pathogenesis. *Annu Rev Microbiol* 2003;**57**:677–701.

Payne DE, Boles BR. Emerging interactions between matrix components during biofilm development. *Curr Genet* 2015.

Petrova OE, Sauer K. A novel signaling network essential for regulating *Pseudomonas aeruginosa* biofilm development. *PLoS Pathog* 2009;**5**:e1000668.

Romling U, Galperin MY, Gomelsky M. Cyclic di-GMP: the first 25 years of a universal bacterial second messenger. *Microbiol Mol Biol R* 2013;**77**:1–52.

Ross PJ, Sutton CE, Higgins S, *et al*. Relative contribution of Th1 and Th17 cells in adaptive immunity to *Bordetella pertussis*: towards the rational design of an improved acellular pertussis vaccine. *PLoS Pathog* 2013;**9**:e1003264.

Sauer K. The genomics and proteomics of biofilm formation. *Genome Biol* 2003;**4**:219.

Schulz BS, Kurz S, Weber K, *et al*. Detection of respiratory viruses and *Bordetella bronchiseptica* in dogs with acute respiratory tract infections. *Vet J* 2014;**201**:365–9.

Serra DO, Conover MS, Arnal L, *et al*. FHA-mediated cell-substrate and cell-cell adhesions are critical for *Bordetella pertussis* biofilm formation on abiotic surfaces and in the mouse nose and the trachea. *PLoS One* 2011;**6**:e28811.

Serra DO, Lucking G, Weiland F, *et al*. Proteome approaches combined with Fourier transform infrared spectroscopy revealed a distinctive biofilm physiology in *Bordetella pertussis*. *Proteomics* 2008;**8**:4995–5010.

Sisti F, Ha DG, O'Toole GA, *et al*. Cyclic-di-GMP signalling regulates motility and biofilm formation in *Bordetella bronchiseptica*. *Microbiology* 2013;**159**:869–79.

Sloan GP, Love CF, Sukumar N, *et al*. The Bordetella Bps polysaccharide is critical for biofilm development in the mouse respiratory tract. *J Bacteriol* 2007;**189**:8270–6.

Soane MC, Jackson A, Maskell D, *et al*. Interaction of *Bordetella pertussis* with human respiratory mucosa *in vitro*. *Respir Med* 2000;**94**:791–9.

Sugisaki K, Hanawa T, Yonezawa H, *et al*. Role of (p)ppGpp in biofilm formation and expression of filamentous structures in *Bordetella pertussis*. *Microbiology* 2013;**159**: 1379–89.

Sukumar N, Nicholson TL, Conover MS, *et al*. Comparative analyses of a cystic fibrosis isolate of *Bordetella bronchiseptica* reveal differences in important pathogenic phenotypes. *Infect Immun* 2014;**82**:1627–37.

Sukumar N, Sloan GP, Conover MS, *et al*. Cross-species protection mediated by a *Bordetella bronchiseptica* strain lacking antigenic homologs present in acellular pertussis vaccines. *Infect Immun* 2010;**78**:2008–16.

Tan IS, Ramamurthi KS. Spore formation in *Bacillus subtilis*. *Environ Microbiol Rep* 2014;**6**:212–25.

Villarino Romero R, Osicka R, Sebo P. Filamentous hemagglutinin of *Bordetella pertussis*: a key adhesin with immunomodulatory properties? *Future Microbiol* 2014;**9**:1339–60.

Vojtova J, Kamanova J, Sebo P. Bordetella adenylate cyclase toxin: a swift saboteur of host defense. *Curr Opin Microbiol* 2006;**9**:69–75.

Wan X, Tuckerman JR, Saito JA, *et al*. Globins synthesize the second messenger bis-(3′-5′)-cyclic diguanosine monophosphate in bacteria. *J Mol Biol* 2009;**388**:262–70.

Wang X, Preston JF, 3rd, Romeo T. The pgaABCD locus of *Escherichia coli* promotes the synthesis of a polysaccharide adhesin required for biofilm formation. *J Bacteriol* 2004;**186**:2724–34.

Witt MA, Arias L, Katz PH, *et al*. Reduced risk of pertussis among persons ever vaccinated with whole cell pertussis vaccine compared to recipients of acellular pertussis vaccines in a large US cohort. *Clin Infect Dis* 2013;**56**: 1248–54.

Zhao Z, Wang C, Xue Y, *et al*. The occurrence of *Bordetella bronchiseptica* in pigs with clinical respiratory disease. *Vet J* 2011;**188**:337–40.