Bordetella biofilms: a lifestyle leading to persistent infections

Natalia Cattelan¹, Purnima Dubey², Laura Arnal¹, Osvaldo M. Yantorno¹ and Rajendar Deora³,*

¹Microbial Biofilm Laboratory, CINDEFI-CONICET-CCT La Plata, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, La Plata (1900), Argentina, ²Department of Pathology, Wake Forest School of Medicine, Medical Center Blvd., Winston-Salem, NC 27157, USA and ³Department of Microbiology and Immunology, Wake Forest School of Medicine, Medical Center Blvd., Winston-Salem, NC 27157, USA

*Corresponding author: Department of Microbiology and Immunology, Wake Forest School of Medicine, 575 N. Patterson Ave., North Carolina, 27104, USA. Tel: +(336)716-1124; E-mail: rdeora@wakehealth.edu

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

Editor: Nicholas Carbonetti

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 resurfaced 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...
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 (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_, cDNA 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 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_ 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._Bvg_A_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_ 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_ phase, which is characterized by the expression of specific genes like _hpB_ (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 Yue 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-dependent 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
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 Kormeli 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*-regulated 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, Matteo and Yuk 2004). The role of FHA in *B. pertussis* biofilm...
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 cyA 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 bpsABC 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 icaA/B/C 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 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 O2 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.
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) biofilm 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 (ΔfhaB and ΔbpsABCD) 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 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 $5 \times 10^7$ CFUs of either the WT or ΔfhaB 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).
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.

ACKNOWLEDGEMENT

RD is supported by Federal funds from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services, under Contract No. HHSN272201200073005C. OY is supported by grants from the Ministerio de Ciencia, Tecnología e Innovación Productiva, Argentina (ANPCyT-FICT 2012–2514 and MINCyT-Dirección de Relaciones Internacionales). NC is a fellow of CONICET-Argentina.

Conflict of interest. None declared.

REFERENCES


