The "Mystery Phases":
Bordetella Adenylate Cyclase Toxin and Biofilm in the Bvg-Intermediate Phase
&
Bordetella pertussis Motility and Flagella in the Bvg-Minus Phase"

Short Title: *Bordetella* Biofilm and Motility

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Thesis Abstract:

Bordetella pertussis is the Gram-negative bacterial pathogen responsible for the life-threatening disease, whooping cough (pertussis). The bacterium has been of increasing interest in the research community due to the reemergence of this vaccine-preventable disease. In fact, the numbers of pertussis cases reported to the CDC annually are similar to those reported prior to the introduction of the whole cell pertussis vaccine. The increased number of cases coincides with the transition to the acellular vaccine, comprised of just one to five *B. pertussis* antigens (pertussis toxin, filamentous hemagglutinin, pertactin, fimbriae 2 and fimbriae 3). This correlation, while not the only contributing factor to reemergence of the disease, highlights the fact that not enough was known about the biology of *B. pertussis* before the acellular vaccine was created. In fact, it has only recently been shown that *B. pertussis* forms biofilm during infection, and that biofilm may be a virulence trait of *Bordetella*. These biofilms form both *in vitro* on abiotic surfaces and *in vivo* in the mouse trachea and nasopharynx. Biofilms are defined as surface-associated bacterial growth, in which the bacteria are covered in extracellular polymeric substance (EPS or matrix), comprised of polysaccharides, eDNA, and proteins. These microbial communities are often associated with persistent infections and in some cases, asymptomatic infections.

The mechanisms by which biofilm formation occurs and are regulated are not completely understood in the *Bordetella* species, although several pathways and important components of the biofilm have been identified. One of the major observations that inspired this work was that a *B. bronchiseptica* strain from

which the *cyaA* gene, encoding adenylate cyclase toxin (ACT), has been deleted, formed more biofilm than wild type bacteria, suggesting the possibility of an inhibitory effect of ACT. ACT is a host-directed, protein, bacterial toxin, and the toxin's role as an inhibitor of biofilm is unprecedented. It was also discovered by Zaretzky *et al* that ACT binds Filamentous Hemagglutinin (FHA), a surface displayed adhesin required for biofilm formation. Until now, the consequences of this interaction were unknown. We have found that ACT binds to FHA via a direct interaction between the catalytic domain of ACT (AC domain) and the mature c-terminal domain of FHA (MCD). This protein-protein interaction results in the inhibition and disruption of biofilm formation. The AC domain is also able to inhibit biofilm of other bacterial species that express an FHA-like protein.

In fitting these findings into the overall body of knowledge regarding *Bordetellae* biofilm, we found major differences in the regulatory processes controlling *B. bronchiseptica* and *B. pertussis* biofilm. Flagella are essential for initial binding and mature biofilm formation by *B. bronchiseptica*. In contrast, *B. pertussis* forms biofilm despite being classically defined as a non-motile and non-flagellated bacterium. *B. pertussis* encodes all of the required genetic material required for flagella expression, but a stop codon in one of the flagella synthesis gene renders *B. pertussis* unable to express flagella, and therefore non-motile.

Under various conditions, we noted a differential expression of genes required for flagellar synthesis and function in *B. pertussis*. It was because of this apparent regulation of flagellar gene expression that we tested for motility. We found that *B. pertussis* is motile, as demonstrated as outward spreading in the

soft agar motility assay, and that these motile bacteria express flagella. These data challenge the idea that *B. pertussis* is a non-motile organism and also verify that the regulatory mechanisms, involved in *B. bronchiseptica* flagella expression and the motile phenotype, are conserved in *B. pertussis*.

These data raise many questions about what we know and what we do not know about this pathogen, and require further research to discover the meaning of these two phenotypes. The findings described herein add to the general body of knowledge for *B. pertussis* and *B. bronchiseptica,* and alter the manner in which we think about bacterial pathogenesis during the age of next-generation vaccine development.

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Chapter One:

Introduction to *Bordetellae*

Part of this chapter has been adapted from "Review of the neutrophil response to *Bordetella pertussis* infection"

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The *Bordetella* Genus

The *Bordetella* genus is comprised of nine Gram-negative aerobic coccobacilli members (1-3). Three of these are categorized as classical species because of their ability to cause upper-respiratory infections and disease in humans and other mammals. The remaining six non-classical species infect other body sites and cause alternate forms of infections (3, 4). The three classical *Bordetellae* are *Bordetella pertussis, Bordetella bronchiseptica,* and *Bordetella parapertussis* (5). *B. pertussis* is a strict human pathogen, known in the world of microbiological research due to its ability to cause the life-threatening disease, whooping cough (2, 3, 6). Despite widespread vaccine coverage and the lack of an environmental reservoir, pertussis is still frequently reported in the United States and globally (2, 3, 7-10).

B. bronchiseptica is a broad-range mammalian pathogen, which infects animals of a wide variety; pigs, horses, cattle, dogs, cats, rodents, and sometimes humans, both immunocompetent and immuno-compromised (11). *B. bronchiseptica* causes both symptomatic and asymptomatic infections in host organisms, and there is increasing evidence suggesting that *B. pertussis* does the same (2, 3). The final classical *Bordetellae*, *B. parapertussis,* has two distinct isoforms, *B. parapertussis*$_{hu}$, which afflicts humans and causes a whooping cough-like disease, and *B. parapertussis*$_{ov}$, which is restricted to ovine species and causes a respiratory illness in pigs (12, 13).

Currently, the non-classical species, *Bordetella hinzii, Bordetella holmseii*, and *Bordetella avium* are being investigated as their presence and diseases

caused by these species become more prevalent (3). The less well-characterized species include *Bordetella ansorpii*, *Bordetella petrii* and *Bordetella trematum*, all of which have been linked to wound infections and other ailments associated with sites other than the upper respiratory tract (14-16).

DNA sequence analysis shows remarkable similarity between *B. bronchiseptica, B. pertussis* and *B. parapertussis*, so much that some suggest they be classified as subspecies (17). Based upon genome size and insertion sequences, *B. bronchiseptica* is the progenitor from which *B. pertussis* and *B. parapertussis* evolved, through independent and distinct evolutionary events, and by means of genome decay (18). This is most evident in *B. pertussis,* which has lost more than 1 megabase of genetic sequence in comparison to *B. bronchiseptica*. Insertion sequences have resulted in hundreds of pseudogenes present in both *B. pertussis* and *B. parapertussis,* and multiple genome sequence analyses have lead to the hypothesis that the strictly human pathogens minimized the presence of genetic material not required for survival and virulence within the human host (18).

Epidemiology of Whooping Cough

Whooping cough is characterized by a long-lasting paroxysmal cough. The clinical diagnosis of the disease is determined by history (exposure to pathogen) and a positive *B. pertussis* culture, positive PCR, or serology, depending on the time at which a patient is seen after onset of cough (2). The

CDC case definition requires that a patient have an acute cough illness of any duration with isolation of *B. pertussis* from a clinical specimen, or a cough illness lasting ≥ 2 weeks with at lease one of the following symptoms: paroxysms of coughing, an inspiratory “whoop”, post-tussive vomiting, or apnea without cyanosis (for infants ages < 1 year only). The CDC case definition also requires a PCR positive for *B. pertussis* as well (CDC, Pertussis/Whooping Cough Case definition, 2014).

In the 1950’s whole-cell vaccines, comprised of heat-killed *B. pertussis* bacteria, were introduced (19). The whole-cell vaccine markedly decreased the number of cases reported to the CDC each year (Figure 1.1A), but Ddespite widespread vaccination, the organism persisted at extremely low levels (<2,000 reported cases per year) (19). There were several complications, such as fever, febrile convulsions, and fainting, associated with the whole cell vaccine. Due to these complications, the acellular vaccine, comprised of 1-5 antigens was introduced in the 1990’s (Figure 1.1A) (6, 19, 20). In the United States, 2-5 antigens are included in the acellular vaccine: pertussis toxin, pertactin, filamentous hemagglutinin, fimbriae 2 and fimbriae 3 (which will be discussed as individual virulence factors in detail below).

Despite the described efficacy of the acellular vaccine, an increase in the number of cases has been reported since its introduction, reaching rates not seen in the United States since the 1950’s (Figure 1.1B) (21). One of the major issues with the acellular vaccine is that it provides a limited duration of protection. The current vaccines were designed on the concept that antibodies against the

3-4 bacterial adhesins and pertussis toxin would prevent not only disease, but also infection and disease (19). We now know that the acellular vaccine prevents cough and other manifestations of pertussis without preventing infection or subsequent transmission in the baboon model (22). Many hypotheses have been proposed to explain the increasing number of cases of pertussis, such as increased reporting due to knowledge of the disease, refusal of vaccination, the loss of *B. pertussis* genetic material encoding vaccine antigens, and finally a lack of proper protection afforded by the acellular vaccine (6, 20, 23). It is impossible implicate just one of these theories and so it is hypothesized that a combination has lead to the resurgence of whooping cough in the United States and globally. It is clear that more research is required to understand the biology of this bacterium and the disease processes associated with whooping cough.

One of the most notable effects of acellular vaccination has been the shift in incidence of pertussis reported; from infants and unvaccinated children aged 1-9 to vaccinated infants, adolescents, and adults (24). Incomplete immunity in infants, the short-lived immunity that results from vaccination, and a greater awareness of pertussis in adolescents and adults has lead to this striking shift (3, 24, 25). The infection in adolescents and adults manifests in a less severe form, either mild or asymptomatic (25), and so whooping cough is greatly under reported in these age groups. For example, approximately 25% of people who presented a mild cough for two or more weeks were pertussis positive in a case study at UCLA (8, 24, 26, 27). What is more striking is that numerous studies have shown that unknowingly infected individuals, such as those that present

with mild or no symptoms, often transmit the disease to infants and young children (either vaccinated or non-vaccinated). To reinforce that transmission must occur from host to host, there is no environmental reservoir for *B. pertussis,* suggesting again that adults are the main source of transmission. These claims are reinforced by the fact that approximately 60% of all infants who develop pertussis were infected by, or at least in contact with, an infected family member (25). Some form of asymptomatic colonization, or persistence state must exist, during which adults and adolescents are colonized with *B. pertussis,* transmit the disease, and yet display no symptoms (3, 28, 29).

In contrast to the human-specific *B. pertussis,* the closely related species, *B. bronchiseptica,* can infect multiple mammalian species. The bacteria cause infectious tracheobronchitis, or the highly contagious kennel cough, in dogs (3, 30), which can lead to more serious infections if the disease goes untreated (pneumonia) (31). Pigs infected with *B. bronchiseptica* suffer from porcine reproductive and respiratory disease, as well as atrophic rhinitis. In contrast, rodents, cats, and non-human primates suffer from bronchopneumonia (3, 32). A *Bordetella* vaccine exists for canines, but the vaccine provides a limited duration of protection, and so veterinarians suggest that pet owners vaccinate canines once every six months (33). *B. bronchiseptica* has been isolated from asymptomatic animals and humans, further supporting that there is some form of a colonization state without symptoms associated with *Bordetella* infections. Although *B. bronchiseptica* is a mainly animal pathogen, there are cases of *B. bronchiseptica* infections in humans being reported. Humans with HIV and cystic

fibrosis are predisposed to *B. bronchiseptica* infection and are often infected via zoonotic transmission (33-35).

Bordetella Pathogenesis

There are three defined clinical stages of pertussis (28). First is the catarrhal stage, which is much like the common cold, lasting one to two weeks with mild symptoms, such as rhinorrhea. The second stage is the paroxysmal, in which coughing increases in frequency and severity, and patients suffer from coughing fits that include a distinctive whooping sound (massive inspirations after coughing). The paroxysmal stage lasts anywhere from one to ten weeks. Other signs and symptoms that occur during the paroxysmal stage include vomiting, cyanosis, and leukocytosis (28). Diagnosis usually comes during this stage, but by this point the bacterial burden is so low that culture is not suitable for diagnosis. Antibiotics can be prescribed, but are typically not beneficial because the host has already cleared the bacteria, and are often solely prescribed to ensure that the bacteria are not transmitted to new hosts. The third and final clinical stage is the convalescent stage. This is a time of healing and recovery, but can result in weakness, pneumonia, seizures, and encephalopathy, all due to secondary infections.

Pertussis has been classified as a toxin-mediated disease (36), in that symptoms persist after the bacteria are cleared, and are due to actions of toxins. Pertussis is different from other toxin mediated diseases such as *C. difficile*

infection and Tetanus, because administration of the toxins alone does not replicate the disease.

The Host Immune Response to *Bordetella* Infection

The global host immune response to *B. pertussis* infection is outlined in Figure 1.2. The initial production of inflammatory cytokines at the site of infection results in macrophage, dendritic cell, and neutrophil recruitment (37). By day ten, neutrophil levels have peaked and begin to decline as CD4 T cells increase in numbers and IgA levels increase (37). One of the hallmarks of *B. pertussis* infection is the influx of neutrophils, which are one of the major host components that control initial *B. pertussis* bacterial burden (37). The neutrophil response is influenced by several of the *B. pertussis* virulence factors (to be discussed in detail below). In general, after initial inoculation of host airways, the recruitment of neutrophils is signaled by *B. pertussis* lipooligosaccharide (LOS), and then this recruitment can be suppressed by pertussis toxin (38). Over the next few days to weeks, an IL-17 response promotes a massive neutrophil recruitment to the site of infection (peaking at approximately ten to fourteen days after infection) (38). Once at the site of infection, neutrophils are inhibited by the activity of adenylate cyclase toxin and filamentous hemagglutinin (39-42). With the help of antibodies, neutrophils contribute to the clearance of *B. pertussis* between 28 and 25 days in mice (43, 44). The memory response is dependent upon B cells (production of IgA and IgG) and the adaptive CD4 T cell response (37). It is this intricate

interplay between the host and *B. pertussis* in which we see microbial pathogenesis at its fundamental core. The host mechanisms used to combat *B. pertussis* infection are met with an arsenal of tightly regulated bacterial virulence factors.

The BvgAS Two-Component System and *Bordetella* Virulence

The BvgAS two-component system has been classically defined as the major regulator of virulence in *Bordetellae*. Two-component systems are among the most common mechanisms used by bacteria to sense and respond to their environment (45). BvgAS is comprised of a membrane spanning sensor kinase, BvgS, which processes external stimuli through a phosphor-relay cascade to activate a response regulator, BvgA (Figure 1.3) (46, 47). BvgAS is an atypical two-component relay system due to the number of self-phosphorylation events that occur in BvgS (48). The membrane sensor, BvgS, auto-phosphorylates at conserved histidine residue (AA729), this phosphate group is then transferred to an aspartate residue in the receiver domain (AA1023). BvgS is then auto-phosphorylated again at a final histidine residue (AA172) (48). Finally, the BvgA response regulator is phosphorylated by BvgS at an aspartate in the receiving n-terminal domain that alters the DNA-binding capability of the helix-turn-helix c-terminal domain (49-53). Once activated, BvgA upregulates expression of virulence factors as well as its own locus, which includes *bvgA, bvgS,* and *bvgR* (a transcriptional repressor) (49, 53, 54). BvgA regulates virulence-activated

genes (vags) by replacing repressors and/or recruiting RNA polymerase to transcriptional start sites (53), and regulates virulence-repressed genes (vrgs) by turning on a repressor, BvgR. Both activation and repression of genes is vital for *Bordetellae* to be fully virulent; Δ*bvgR* are incapable of causing disease (55-58). Several publications have investigated the Bvg regulon and none are in agreement as to the exact set of genes controlled by BvgAS (54, 59-64). Regardless of these differences, BvgAS is considered the regulator of *Bordetella* virulence and phase variation. The signals that "turn on" BvgAS are unknown, which is due to the fact that at the basal state, BvgS is the "on" conformation, in which auto-phosphorylation occurs (65-68). This is independent of ligand binding to the venus fly trap (VFT2) domain (Figure 1.3). Periplasmic VFT domains associated with two-component systems are typically in the "off" conformation when the ligand is absent (68). In *Bordetellae,* BvgS VFT2 domain binds a ligand (antagonist) and the VFT2 domain switches to the "off" conformation, and BvgS then turns off auto-phosphorylation. The known signals capable of turning off BvgAS are lower temperatures (<30ºC) and certain chemical modulators, which have been used to study the Bvg regulon (69). There are also several genetic manipulations that "lock" *Bordetellae* in a specific Bvg phase, which will be described in detail in the following section.

Phase Variation

In 1931 Leslie and Garner observed that *B. pertussis* change phase to modulate antigen expression based on environmental signals (70). They identified two main phases, the X mode and the C mode, known today as the Bvg(+) and Bvg(-) phases, respectively. They also described a third distinct phase, the I mode, known today as the Bvg(i) phase. These phases are now associated with differential gene expression profiles in both *B. pertussis* and *B. bronchiseptica*. Bvg(+) phase correlates with high BvgA phosphorylation and controls expression of several virulence factors, including adenylate cyclase toxin, filamentous Hemagglutinin, fimbriae, pertussis toxin, pertactin (Figure 1.4) (2, 3). The Bvg(+) phase is the best characterized, as it is the phase is required to establish an infection and cause disease in the mouse model (69). The Bvg(-) phase is considered to be relevant for survival in the environment (71-73), although recent studies have shown that this may not be the only case; Bvg(-) mutants have recently been isolated from patients infected with *B. pertussis* (29, 74, 75). Bvg(-) bacteria are hardier and survive on abiotic surfaces for longer periods of time, and also grow at a faster rate, likely due to major differences in gene expression associated with metabolism and nutrient uptake (Figure 1.4) (54, 64). Bvg(-) phase is also associated with flagella expression and motility in *B. bronchiseptica* (Figure 1.4) (72, 73). Due to a lack of virulence factor expression, Bvg(-) genetically locked *B. bronchiseptica* and *B. pertussis* are cleared more quickly during experimental mouse infections (69).

In 1997, Cotter and Miller confirmed Leslie and Garner's observation of the I mode. They showed that there was a third, genetically distinct, Bvg-phase, Bvg(i), during which *Bordetellae* express Bvg Intermediate Protein, BipA, an adhesin protein with an unknown contribution to *Bordetella* virulence (76, 77). During this phase, there is also a reduction in expression of other Bvg(+) virulence factors, namely toxins (Figure 1.4). This is due to a reduction in phosphorylated BvgA, which binds the promoter regions of virulence associated genes to turn on gene expression. Toxin expression is reduced due to the requirement of more BvgA molecules binding or a lower affinity binding sequence for BvgA. Bvg(i) only proteins are controlled by the promoter sequence structure, in that low levels of BvgA activate gene expression, but additional BvgA molecules alter the promoter structure so that gene transcription is blocked (76, 77). A distinct role for Bvg(i) has not been established, but there is increasing data to support the hypothesis that Bvg(i) is important for transmission and/or for biofilm formation (28). The three separate phases show that the BvgAS two-component system is not a biphasic switch, but rather there is a gradient of BvgA phosphorylation that functions as a rheostat (60, 77).

The standard method(s) used to grow *B. pertussis* in the lab result in the Bvg(+) phase, but researchers can experimentally modulate to the Bvg(-) and Bvg(i) phases using temperature, $MgSO_4$ (sulfate), or nicotinic acid (69). There are also methods to delete *bvgA* to "lock" the bacteria in a Bvg(-) phase or Bvg(i) phase, so that Bvg(+) regulated factors cannot be expressed (46, 78). The overall *in vivo* relevance of the BvgAS two-component system has not yet been

defined, other than studies that have shown that bacteria locked in the Bvg(-) phase do not colonize mice as well as Bvg(+) locked bacteria (69). The Bvg(+) and Bvg(i) bacteria do equally well at establishing infection, likely due to similar levels of adhesin expression, but Bvg(i) bacteria are cleared more rapidly after day 1 post infection (79). The differences in colonization and disease-causing ability are likely due to the lack of, or reduced expression of, virulence factors between the Bvg(i) and Bvg(+) phases. Like many pathogenic bacteria, *Bordetellae* have two classes of virulence factors, adhesins and toxins. There is an intricate and coordinated interplay between the *Bordetella* virulence factors to establish infection and promote disease within the host.

Bordetella Virulence Factors: Adhesins

Filamentous Hemagglutinin

Filamentous Hemagglutinin (FHA) is expressed in both the Bvg(+) and Bvg(i) phases (80), and the 220 kD protein is one of the most immunogenic antigens of *B. bronchiseptica* and *B. pertussis* (81). The protein is located either on the surface of the bacteria, or can be released into the extracellular milieu (82-84). FHA is secreted via the two-partner secretion system, and has been studied as a model for two-partner secretion system proteins. The FHA peptide is translated in the cytoplasm and is transported into the periplasm via a non-specific inner-membrane transporter (85-87). Once in the periplasm, the N-

terminus of the protein is inserted as a hairpin into FhaC (OmpC), the outer membrane transporter (Figure 1.5) (87). The protein begins folding into β-helical sheets until the c-terminal portion reaches the distal tip of the FHA protein. A signal sequence in the periplasm is recognized by an unidentified protease, and the periplasmic portion of the c-terminus is cleaved from FHA. The newly-cleaved, mature c-terminal domain (MCD) folds into its final conformation at the distal tip of FHA (85, 87).

The mature FHA protein has four functional domains that help confer binding to and trigger subsequent signaling within epithelial and immune cells. The RGD motif (arginine-glycine-aspartic acid) aids in binding to complement receptor 3 and leukocyte response integrin/integrin associated protein, found on macrophages, monocytes, and leukocytes (88-90). The RGD motif also interacts with very late antigen 5 (VLA5) on bronchial epithelial cells (91-93). This interaction leads to NF-κβ signaling and leukocyte accumulation. The CRD (carbohydrate recognition domain) of FHA interacts with surface displayed carbohydrates on macrophages and ciliated epithelial cells of the upper respiratory tract to aid in binding (94). The heparin-binding domain promotes haemagglutination, and finally, the CR3 (compliment receptor 3) recognition domain serves an unknown function in disease, but has been implicated in the binding of *B. pertussis* binding to epithelial cells (95).

FHA is required for the inhibition of CD4+ T cell proliferation and induction of apoptosis in these cells, which together decrease the adaptive response to infection with *B. pertussis* (96). FHA also downregulates the innate immune

response by dendritic cells and macrophages, mediated by its induction of IL-10, which suppresses IL-12 production (97, 98). The literature is divided on the importance of FHA *in vivo* during experimental mouse infection, although multiple studies have demonstrated that FHA is required for efficient colonization of the trachea in rodents (not lungs or nares) (99). McGuirk *et al.* demonstrated that FHA was required for colonization of the lungs of mice (100), but Goodwin and Weiss showed that FHA was not needed for mouse lung colonization (101). Major differences that may account for the conflicting results of the FHA studies are the use of different mouse strains, and the use of two genetically distinct *B. pertussis* isogenic backgrounds. To add to the controversial role of FHA during infection, some reports propose that FHA is highly adapted to the targeted host, but it was demonstrated that FHA (*fhaB*) is interchangeable between *B. bronchiseptica* and *B. pertussis.* Exchange of the genes between the two species resulted in no difference in ability of the bacteria to establish infection in the mouse lung or trachea (102).

Fimbriae

Many Gram-negative bacteria express filamentous polymeric proteins, fimbriae (Fim) (103-105). *B. pertussis* expresses Fim2, Fim3, FimX and FimN, but only Fim 2 and Fim3 are expressed during the Bvg(+) phase (106-109). Fimbriae mediate adherence to epithelial cells and monocytes, via an unknown mechanism (110, 111). The specific functions of each fimbriae *in vivo* have not

been unveiled for *Bordetella* species because of the overlapping function with FHA and the other Fim proteins. Fim2 and Fim3 are required *in vivo* for efficient tracheal colonization (112) and downregulate the inflammatory response to *B. pertussis,* as measured by cytokine release from epithelial cells *in vitro* (113). There is an adaptive immune response to Fim2 and Fim3 in the rat model of infection (112), and the inclusion of these two adhesins in the acellular vaccine significantly enhances the protection afforded by the vaccine (114).

Pertactin

An additional adhesin involved in attachment to mammalian epithelial cells is pertactin (PRN). This protein is expressed in the Bvg(+) and Bvg(i) phases (115) and is a 69 kDa protein with an RGD-binding motif in the N-terminal domain (116, 117, 118). PRN is an autotransporter protein; the N-terminal domain, required to elicit effector function, is transported across the bacterial membrane by the C-terminal β-barrel domain (115). When PRN is expressed in *Salmonella,* binding of *Salmonella* to CHO cells is enhanced (118). For *B. pertussis*, PRN imparts binding to CHO cells, but the deletion of PRN (Δ*prn*) from *B. pertussis* has no effect on adherence *in vitro* or *in vivo* (119, 120). Anti-PRN antibodies provide protection during infection (121, 122), but it was discovered that the presence of PRN in the acellular vaccine enhanced antibody response to FHA and pertussis toxin, and that PRN had no protective attributes alone (123). *B. pertussis* Δ*prn* strains are no different from wild type in their ability to colonize

the trachea, nose, or lungs of mice, and are cleared at the same rate as wild type (119, 120). Importantly, these data are the basis for understanding the latest transformation of *B. pertussis* – the loss of PRN expression. The most common method for PRN loss is by the transposable DNA element IS481, which inserts into the target gene and creates a pseudogene (124). Other methods of PRN loss include the deletion of the 5′ signal sequence, premature stop codons, and a large deletion of the promoter and 5′ coding region (124). In recent outbreaks (since 2010), PRN-deficient isolates are reported at a frequency of ≥ 85%, with some areas reporting almost 100% PRN-deficient isolates (124-128). These findings have serious implications for the efficacy of current vaccines, as the loss of vaccine antigens may reduce the ability of hosts to recognize and respond to bacterial infection.

Bordetella Virulence Factors: Toxins

Pertussis Toxin

Pertussis toxin is only expressed by *B. pertussis* within the *Bordetellae* genus. This is due to mutations in the promoter region of the gene that prevent expression in both *B. bronchiseptica* and *B. parapertussis* (129). Pertussis toxin (PT) is an ADP-ribosylating toxin comprised of 6 subunits, S1-S2-S3-S4-S4-S5, encoded by *ptxABCDE* (130-132). PT is an AB toxin, which functions by the B component (S2-S3-S4-S4-S5) forming a ring like structure that binds host membranes and causes the cell to uptake PT via a cytochlasin D-independent

uptake pathway (133-135). ATP binds the B component which causes release of the A component (S1) (136). The A component adds an ADP ribose to GTP binding proteins to disrupt cell signaling and ion channel function (137-139). This results in the inhibition of chemotaxis, a decrease in ROS production, and alteration of lysosome fusion in neutrophils and macrophages (140, 141). PT also serves as an adhesin, which imparts binding to ciliated epithelial cells and macrophages, and is considered the major virulence factor of *B. pertussis* due to its dual function as an adhesin and toxin (142). Strains of *B. pertussis* lacking PT cause a much more robust antibody response in mice; these data implicate PT in the blocking of parts of the adaptive immune response (143). In light of the dual roles PT plays in pathogenesis, the toxin has been included in all currently available acellular vaccines.

Dermonecrotic Toxin

Dermonecrotic Toxin (DNT) was one of the first toxins discovered in *B. pertussis* and it is a heat-liable AB toxin, comprised of a 54 amino acid N-terminal binding domain and a 300 amino acid C-terminal catalytic domain (144). The receptor for DNT is unknown, but uptake of the toxin requires dynamin-dependent endocytosis. Once inside host cells, eukaryotic proteolytic nicking activates DNT. By binding small Rho-GTPases, DNT activates a signaling cascade to alter cytoskeletal rearrangement, DNA replication, and cell movement (144-146). The toxin plays an unknown role in pathogenesis, but has been linked

to necrotic lesion formation in experimentally infected animals, and in the case of *B. bronchiseptica,* turbinate atrophy (bony structures that secrete mucous) in pigs (32, 147).

Tracheal Cytotoxin

Tracheal cytotoxin (TCT) is a disaccharide tetrapeptide monomer of the peptidoglycan cell wall of *Bordetellae*, which unlike many other bacteria, do not recycle peptidoglycan (148, 149). *B. pertussis* is unable to uptake peptidoglycan due to the lack of a functional AmpG, peptidoglycan monomer (N-acetylglucosamine-1,6-anhydro-N-acetylmuramic acid-tetrapeptide) uptake protein, and instead release the monomer into the surrounding environment (149-152). TCT only affects ciliated cells and leaves non-ciliated cells undamaged (153-155); the toxin is involved in cilia destruction, cell blebbling, and the damaging of cell mitochondria. In cell culture, TCT induces production of IL1 alpha and nitric oxide (NO) to destroy cilia in the surrounding area. It is hypothesized that TCT induces IL1 alpha and NO in non-ciliated mucous-secreting cells *in vivo*, which results in destruction of the cilia in the surrounding cells (156, 157).

Lipopolysaccharide and Lipooligosaccharide

Lipopolysaccharide (LPS) in *B. bronchiseptica* and lipooligosaccharide (LOS) in *B. pertussis* are bacterial outermembrane components. LPS is

comprised of Band A and Band B, named for the mobility of the individual bands in sodium dodecyl sulfate polyacrylamide gels. The two bands have similar composition, but the faster migrating band, Band B, has a lipid A molecule which is covalently linked to a branched oligosaccharide core (159, 160). Band A consists of Band B plus one trisaccharide (159, 160). The O-antigen in *B. bronchiseptica* and *B. parapertussis* is made up of repeating sugar molecules linked to Band A and Band B. In contrast, *B. pertussis* does not have an intact *wbm* locus (responsible for synthesis of the O-antigen polymer, its linkage to the trisaccharide or core region, and O-antigen export), and therefore cannot make the enzymes required to construct the final O-antigen, hence it is called LOS (161-163). LPS is a pyrogenic molecule that induces TNF expression in macrophages (158), but plays an unknown role in pathogenesis. Despite its unknown role, a ∆*lps B. bronchiseptica* mutant has a defect in colonization of the mouse respiratory tract (164). It is hypothesized that LPS confers resistance to complement-mediated killing of *B. bronchiseptica* and LOS protects *B. pertussis* from surfactant protein A in mouse lungs (165, 166).

Adenylate Cyclase Toxin

Adenylate Cyclase Toxin (ACT) is a 177 kDa bacterial adenylyl cyclase encoded and expressed by some but not all *Bordetellae* (only *B. pertussis, B. bronchiseptica, B. parapertussis, B. hinzii* and *B. ansorpii* express ACT) (167). The toxin is 1706 amino acids and is comprised of several domains (Figure

1.6A); the type 1 secretion signal, the repeat or RTX domain (42 repeated GGXGXDXLX motifs), the hydrophobic domain, and the catalytic domain (168, 169). This host-directed protein bacterial toxin is secreted by the Type I Secretion System (T1SS) (Figure 1.6B). The secretion apparatus, the toxin, and the Cyclolysin-activating lysine-acyltransferase (CyaC, required for the production of a functional toxin) are encoded in the *cyaABCDE* operon (170, 171).

ACT is ratcheted through the T1SS and efficiency of this process is dependent on physiologic levels (2mM) of calcium (172, 173). In the standard lab culture media, Stainer Scholte Media (SSM) (174), ACT primarily remains surface-associated as an inactive form of the toxin (90%), although some of toxin (10%) is released into the extracellular milieu (175-177). It is now known that this is due to insufficient levels of calcium in SSM. When SSM is supplemented with 2mM calcium, ACT is secreted and released into the extracellular milieu. This released form of ACT is the active form of the toxin, capable of intoxication of host immune cells (177). ACT uses complement receptor 3, the heterodimeric αMβ2 integrin (also known as CD11b/CD18 or Mac-1) as its receptor on myeloid phagocytes (Figure 1.6C) (178, 179). Once the toxin binds its receptor, the catalytic domain of the toxin translocates across the host cell membrane into the cytoplasm where it binds host calmodulin (CaM). This activates the catalytic activity of the toxin, the conversion of ATP to cAMP, and creates supraphysiological levels of cAMP while simultaneously reducing intracellular ATP levels within the host cell (178, 180-188). The toxin activity disrupts cellular signaling and can deplete host cells of energy required for cellular processes.

ACT plays many roles as a toxin and is able to inhibit phagocytosis, chemotaxis and superoxide generation. The toxin is required for the establishment of infection in the mouse model (101, 186, 187, 189-195). ACT also serves as a protective antigen, as demonstrated in several studies, and by the fact that antibodies elicited by infection with *B. pertussis* neutralize toxin activity (189, 193, 196-199). In addition to its role as a potent toxin, capable of altering the host immune response, ACT was shown to interact with FHA (176, 191), the adhesin and virulence factor required for host cell attachment and biofilm formation (200).

Biofilm

The methods used to grow bacteria in the laboratory are far removed from those in which the bacteria grow naturally in the environment and in hosts during infection. Laboratory growth conditions promote planktonic (free-floating) growth, as opposed to natural growth in the environment, during which bacteria are associated with surfaces like metals, plastics, and host tissues (epithelial cells). The presence of what we now call biofilms were described in 1684 by the microscopist, Antonie Van Leeuwenhoek (201). He observed human dental plaque, or “scurf”, and noted the extensive number of bacteria, or as he called them, “animalcules”, contained within the substance. The first documented observations of microbes living as surface-associated communities or in large clusters came from Henrici in 1933 and Zobell in 1943 (201). Henrici noticed that a majority of bacteria grew attached to the surface of his culture system, as

opposed to floating freely in the culture medium. Zobell also noted that marine microorganisms grew better and more frequently on the surface of glass containers, compared to in the seawater itself. In 1969 the first images of biofilm were published; samples were collected from industrial water pipes and the transmission electron micrographs showed bacteria surrounded by what was deemed a “polysaccharide” material (202). And finally, in 1973 the “father of biofilm”, William Costerton, helped open the Center for Biofilm Engineering in Montana. Soon after the center opened, Costerton began explaining the mechanisms by which organisms adhere to living and non-living materials to form biofilm (203, 204).

The use of microscopy and transcriptomics soon showed that the attached bacteria were drastically different that the free-floating bacteria (205). Eventually, these sessile bacteria were described as biofilms, or communities of surface-associated bacteria, encased in a self-produced polymeric matrix, comprised of polysaccharides, extracellular DNA and proteins. Biofilms are organized structures that develop in a step-wise process (Figure 1.7). One free-floating cell or an aggregate of cells comes in contact with a surface, and via some signal attaches in a reversible way (206). This reversible adherence is often mediated by flagella, which in elegant work was shown to signal for irreversible attachment (207, 208). This now permanent attachment is associated with the bacteria or bacterial cluster making a more direct contact with the surface and the subsequent differential gene expression, specifically expression of genes associated with the making and secretion of extracellular polymers (for

example, PGA exopolysaccharide of *E. coli* or PNAG of *S. aureus*) (206, 209). The biofilm continues to grow in one of two ways, either by clonal expansion at the periphery of the biofilm or by clusters of cells migrating together to join the biofilm (210, 211). Typically, cells will grow as a flat field of bacteria, covering as much area as possible. Once the area is seeded with bacteria, the biofilm will expand upwards to create a three-dimensional structure called a microcolony (212). Throughout this process, more matrix material is produced and secreted to encase the biofilm (213, 214). The final stage of biofilm formation is the release of cells into the surrounding environment (dispersal), which is mediated via the expression of enzymes that can degrade the matrix or release attachment factors. These enzymes can include alginate lysase, DNase, and dspB, which cleaves beta 1-6 linked n-acetyl glucosamines (215, 216). The released bacteria gain a new gene expression profile that makes them more similar to the planktonic cells (217). The process of dispersal allows for seeding of bacteria in new locations, so that the biofilm formation can begin again if the conditions are suitable.

Biofilm and Human Infections

Biofilms are associated with an estimated 65-80% of all human infections; some of these infections include otitis media, tonsillitis, dental carries (cavities), wound infections, medical device infections, urinary tract infections, gastrointestinal infections, and chronic lung infections (associated with cystic fibrosis) (218-220). A major issue associated with biofilm is the treatment and

eradication of the bacteria within the biofilm. Many commercially available treatments target metabolically active, free-floating bacteria. These treatments were developed against bacteria grown planktonically in the lab. Treatment of bacteria within biofilm is ineffective, as these biofilm-associated bacteria become metabolically inactive due to changes in gene expression associated with the sessile lifestyle. Research targeting the methods to kill, disrupt, and prevent biofilm is essential to combat the various forms of biofilm-related diseases.

Many long-term infections involve biofilm. One of the best-characterized biofilm-associated infections is associated with Cystic Fibrosis. Immunohistopathological samples of lung tissues from cystic fibrosis patients show that aggregates of *Pseudomonas aeruginosa* with increased matrix material compared to bacteria grown *in vitro.* In addition, many *P. aeruginosa* strains isolated from cystic fibrosis patients are hyper biofilm formers due to mutations in genes associated with polysaccharide production (221, 222). The biofilm state hinders the host response and results in reduced clearance of *P. aeruginosa*. The long-term presence of bacteria in the lung often leads to increased inflammation, reduced in lung function, and eventual respiratory failure (223, 224).

Staphylococcus aureus is known for its ability form biofilm on abiotic materials associated with medical devices, such as catheters, artificial heart valves and prosthetic (225-228). In addition to the biofilm state, antibiotic resistance makes treatment difficult. The abiotic nature of these locations, as well as the restricted ability of the immune system to access these sites, due to

immunosuppressants, hinders both the innate and the adaptive immune response to infection (229). These complications often require replacement of the device, which increases the cost of care associated with these medical devices (226). Other bacterial species associated with medical device infections and biofilm formation in humans include *Streptococcus pneumoniae, Haemophilus influenzae,* and *Escherichia coli* (230-232).

Biofilm formation can result in long-term colonization with mild symptoms, and can often result in infections that go undiagnosed. *B. pertussis* colonization and infection in asymptomatic patients occurs, and the ability of these asymptomatic patients to transmit disease to others has been documented. For these reasons it is hypothesized that *Bordetella* adapts a biofilm lifestyle during infection that leads to an asymptomatic or mild form of disease (3). Importantly, there is a growing body of evidence that supports *B. pertussis* biofilm formation occurs during infection and this contributes to disease and/or transmission. Using high-throughput screens, such as the crystal violet assay to determine biofilm-forming capabilities, and microscopic techniques, including confocal scanning laser microscopy (CLSM) and scanning electron microscopy, researchers have been able to study the structure and composition of biofilm formed by the *Bordetellae* genus. In addition, studies examining the presence of biofilm during infection have provided insight into the *in vivo* structure and function of biofilm.

Bordetella Biofilm

The study of *Bordetella* biofilm is still in its infancy compared to that of *Pseudomonas aeruginosa* and *Staphylococcus aureus*. In the last decade, studies have revealed components necessary for biofilm formation, various forms of regulation, and have demonstrated that biofilm formation occurs not only *in vitro* on abiotic surfaces, but also *in vivo* in the nasal septum and trachea of mice (233). *Bordetella* biofilm are comprised of bacteria cells and matrix, which contains polysaccharide(s), eDNA, and various proteins (233).

The master regulator of virulence, BvgAS (Figure 1.3), also regulates *Bordetella* biofilm formation. Two publications show similar, but not identical results, in that biofilm formation occurs in the Bvg(+) and Bvg(i) phases and no biofilm formation occurs in the Bvg(-) phase. In 2004, Irie *et al*. showed that *B. bronchiseptica* formed more biofilm in the Bvg(i) phase compared to the Bvg(+) phase (234), while Mishra *et al.* in 2005 showed that *B. pertussis* biofilm formation was equal in both the Bvg(+) and Bvg(i) phases (235). The differences between these two studies (between *B. pertussis* and *B. bronchiseptica*) have not been explored, but these data raise several questions about the differences between the regulatory aspects of *B. pertussis* and *B. bronchiseptica* biofilm, and bring to light other differences between Bvg(+) and Bvg(-) phases. The BvgAS regulon controls only one of the biofilm components that have been identified thus far, filamentous hemagglutinin (FHA) (54, 63, 64). Other major components of biofilm are not directly linked to the Bvg regulon, including the polysaccharide (*Bps*), lipids, and extracellular DNA (200, 236-238).

One of the best-characterized biofilm proteins, FHA, is required for biofilm formation both *in vitro* and *in vivo;* FHA-FHA interactions and FHA-surface interactions promote biofilm formation and maintain biofilm structure (200). Several other matrix proteins, such as BtrA (*Bordetella* RTX-family adhesin) and BipA (*Bordetella* intermediate-phase protein A), were identified in a proteomic analysis of *in vitro* biofilm samples (239). BipA enhances biofilm formation by increasing attachment to surfaces (240), and BrtA promotes Ca^{2+}-dependent bacteria-substrate adherence (240). Interestingly, FHA is a Bvg(+) factor, BipA is expressed only in the Bvg(-) phase, and BrtA is a Bvg(-) expressed factor, and all three of these adhesins are important for biofilm formation. This data demonstrate that suggest a stepwise transition between the Bvg phases may be important for biofilm formation, or that a mixture of Bvg phase bacteria are involved in biofilm.

In addition to these protein components, several other factors have been implicated in regulation of *Bordetella* biofilm. These factors and signals have not been integrated to develop an all-encompassing model of biofilm regulation for *Bordetella* species. Figure 1.8 was generated to demonstrate the various factors that have been identified as biofilm components, as either regulatory factors or matrix components. As of now, there has not been a master regulator of biofilm identified, and all identified regulatory mechanisms seem to act independently.

Non-Protein Matrix Components and *Bordetella* Biofilm Regulation

Bordetella biofilm matrix is similar to that of other bacterial species and includes polysaccharides, LPS or LOS, proteins, and eDNA (200, 236, 238). The polysaccharide component is comprised of xylose (*B. bronchiseptica* only), Bps (*Bordetella* polysaccharide) (*B. pertussis* and *B. bronchiseptica*), and uronic acids (*B. pertussis* only) (237, 238, 241). These and other sugars have been detected within *Bordetella* matrix, but only Bps is required for stability and maintenance of biofilm structures. The machinery required for production of Bps is encoded in the *bpsABCD* operon (241). These genes are highly conserved amongst the *Bordetellae* species and the operon is homologous to the *pgaABCD* locus of *Escherichia coli* and the *icaADBC* loci of Gram-positive bacteria (i.e. *Staphylococcus aureus*) (241). Based upon various biochemical and immunological tests, Bps has a similar backbone to poly-β-(1,6)-N-acetyl-D-glucosamine polysaccharides and the two polysaccharides have similar antibody reactivity (238, 241). The exact biochemical composition remains to be determined. Dispersin B, an enzyme that specifically hydrolyzes linear polymers of N-acetyl-D-glucosamines, is able to prevent and disrupt *Bordetella* biofilm by degrading the Bps portion of the matrix (237). There is a lack in biofilm formation in the nasal cavities and tracheas of mice infected with the Δ*bps B. pertussis* compared to the wild type strain, as determined by immuno-histochemical analyses of excised tissue from these sites (237). The lack of biofilm in the nasal septum and trachea is not due to a decreased ability of Δ*bps B. pertussis* to colonize these sites. In contrast, Δ*bps B. pertussis* are deficient at establishing

an infection in the lung, where CFUs recovered are similar between the wild type and the Δ*bps* strain (237). In addition, it was shown that Δ*bps* does not bind to lung epithelial cells (A549) in cell culture, which may be responsible for the colonization defect in the lung (237). The expression of Bps is regulated at the transcriptional level by a repressor of Bps expression, BpsR (242). The transcription factor was found to repress *bpsABCD* expression by occupying the promoter regions of the genes (Figure 1.8) (242). The signals required to relieve BpsR repression have not been discovere, but the BvgAS locus has no control over Bps production or BpsR repression (242).

Extracellular DNA (eDNA) is another major component of the Bordetella matrix. When *B. bronchiseptica* and *B. pertussis* are grown in the presence of DNase, no biofilm forms (236), and when preformed biofilm is treated with DNase, biofilm is degraded (236). The mechanisms involved in regulation of eDNA secretion and release are unknown, but in other bacterial species, factors such as pH and Ca^{2+}, and bacterial encoded DNA specific transporters, and enzymes, increase and decrease the total eDNA mass and the association of eDNA with bacterial membrane (243, 244).

Bacterial Signaling Molecules and *Bordetella* Biofilm Regulation

Two sets of signaling molecules have been directly linked to biofilm formation in several species of bacteria. Intracellular levels of Bis-(3'–5')-cyclic-dimeric guanosine monophosphate, c-di-GMP, directs bacteria to either a

planktonic (free-floating) or biofilm lifestyle (245-247). c-di-GMP is produced by digaunylate cyclase enzymes encoding a GGDEF domain, which catalyze the linkage of two GTP molecules by phosphodiester bonds. The signaling molecule is degraded by phosphodiesterase enzymes with an EAL or HY-GYP domain (248). In many bacteria, high levels of c-di-GMP decrease biofilm by changing gene expression and increasing bacterial motility and low levels of c-di-GMP result in the opposite, increased biofilm formation and decreased bacterial motility (245-247). *B. bronchiseptica* express at least one functional phosphodiesterase (PDE), but encode several predicted PDEs in the genome (249). Over expression of the PDE results in increased biofilm formation and decreased motility. Deletion of the PDE results in the opposite (decreased biofilm, increased motility) (249). No studies have been conducted to elucidate the role(s) of c-di-GMP in *B. pertussis*, but several predicted PDEs and DGCs have been identified within the *B. pertussis* genome.

c-di-GMP is considered to be the key signaling molecule for biofilm, although (p)ppGpp (guanosine pentaphosphate, or tetraphosphate) has also been implicated in *B. pertussis* biofilm regulation (250). (p)ppGpp is a bacterial alarmone involved in the stringent response to amino acid starvation (251, 252). Upon accumulation of high levels of the alarmone, RNA synthesis is halted (251). *B. pertussis* has two proteins involved in (p)ppGpp synthesis, degradation, and balance. RelA is required for (p)ppGpp synthesis and SpoT is required for degradation and balance of the signaling molecule (both synthesis and degredation) (250). Nutrient limitation (specifically glutamate) and oxidative

stress influence RelA and SpoT expression and activity within the bacterial cell; high levels of (p)ppGpp lead to increased biofilm formation (250). When RelA is deleted, the alarmone response does not occur and *B. pertussis* does not form biofilm (250).

Major Differences Between *B. pertussis* and *B. bronchiseptica* Biofilm

Flagella are required for the early stages of *B. bronchiseptica* biofilm, specifically the initial binding step, and for subsequent mature biofilm formation (Figure 1.8) (253). From elegant studies in other bacterial species, we know that flagella are the initial adhesin that binds to surfaces. Through the subsequent signaling event, an increase in gene expression associated with adhesins and matrix production promotes biofilm (210, 217, 220, 254). *B. bronchiseptica* flagella expression must eventually decrease, so that mature biofilm structures can form (253). If *B. bronchiseptica* are engineered to constitutively express flagella, mature biofilm formation never occurs, but a thin, single layer of bacteria adhere to surfaces. In contrast, despite being classified as a non-flagellated and non-motile organism, *B. pertussis* forms mature biofilm and the bacteria are not hindered by a lack of flagella.

***Bordetella* Motility and Flagella**

B. bronchiseptica has been historically classified as a motile organism, whereas the closely-related species, *B. pertussis,* has been classified as a non-

motile organism that lacks flagella (7, 72, 73, 255, 256). A comparative analysis of *B. bronchiseptica* and *B. pertussis* genomes revealed that all of the genetic material required for flagella assembly and function are encoded in the *B. pertussis* genome, but a stop codon in *flhA*, one of the necessary flagella apparatus assembly proteins renders *flhA* a pseudogene (257). The predicted *B. pertussis* flagella structure, based on the genetic material encoded in the genome is described in Figure 1.9. *B. pertussis* motility was never explored to the best of our knowledge.

Several studies have been published about *B. bronchiseptica* motility and flagella expression, but the overall relevance of the motile phenotype and flagella expression are unknown in the context of pathogenesis. *B. bronchiseptica* motility and flagella expression are regulated by the BvgAS two component system; Bvg(-) phase *B. bronchiseptica* are motile, Bvg(i) and Bvg(+) phase bacteria are not (73). The studies linking mature biofilm formation and flagella expression illustrate that there are major gaps in our knowledge with regards to *B. pertussis* and *B. bronchiseptica* biofilm formation, and with *Bordetellae* biology in general.

Project Rationale

The goal of this body of research was to better understand biofilm formation by *B. pertussis*, and to relate this complex and highly coordinated process to pathogenesis. The hypothesis for this project was that the differential

regulation of virulence factors, specifically adenylate cyclase toxin and filamentous hemagglutinin, results in varying amounts of biofilm formation. Strong preliminary evidence showed that by not expressing ACT, *B. bronchiseptica* was able to form more biofilm, supporting our hypothesis that ACT plays an inhibitory role in biofilm formation. We then explored the mechanism by which this occurs, building upon previous studies that showed ACT and FHA interact on the surface of *B. pertussis*. We further characterized this protein-protein interaction and provide evidence that shows ACT must bind FHA in order to inhibit biofilm formation.

To determine if this biofilm inhibitory molecule had inhibitory effects on other bacterial species, ACT and the much smaller AC domain were tested as biofilm inhibitors for other biofilm-formation bacterial species. Specifically species that express FHA-like proteins (both structure and sequence homology) were tested. In fact, ACT and the AC domain demonstrated moderate to efficient inhibitory effects on biofilm of *Pseudomonas aeruginosa*, *Escherichia coli*, and *Salmonella typhimurium.*

While fitting the novel regulatory data of ACT into the pre-existing body of knowledge for *Bordetella* biofilm, we found a discrepancy between *B. pertussis* and *B. bronchiseptica* biofilm. *B. bronchiseptica* require flagella in order to initially adhere to a surface and form mature biofilm. *B. pertussis* has long been defined as a non-flagellated organism, and yet it is able to form mature biofilm. Based on these and other observations, namely that *B. pertussis* encodes all the genetic material to assemble a functional flagellum, and that differential regulation of the

flagella operon occurs in *B. pertussis*, we hypothesized that *B. pertussis* is able to express flagella and is motile. Upon testing for the motile phenotype, we found that *B. pertussis* is motile and express flagella. The findings herein prompt several questions that warrant further investigation, and these studies will lay the foundation for a novel perspective to emerge in *B. pertussis* research. Importantly, these data alter the way we think about *B. pertussis* biology and the host-pathogen interaction.

FIGURES:

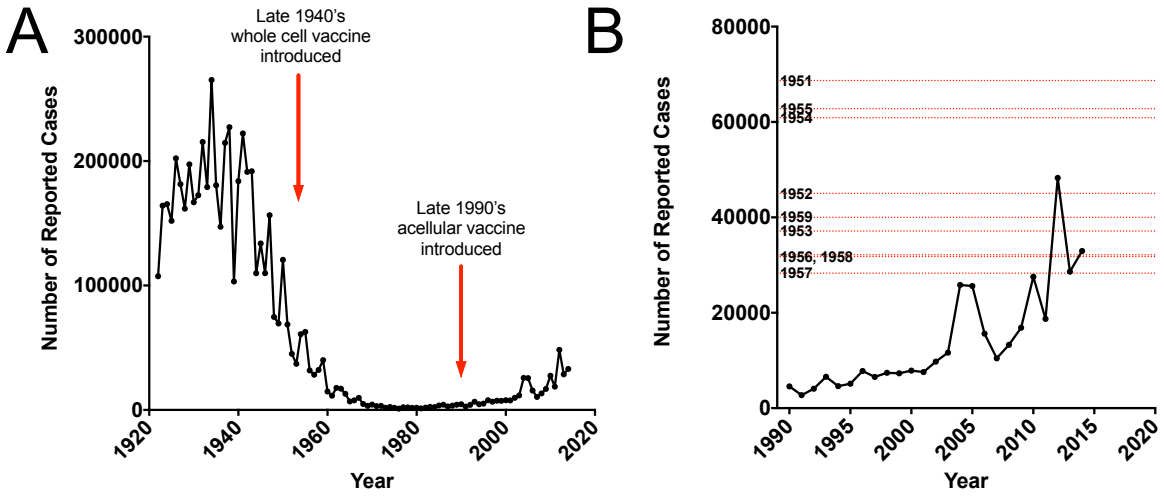

Figure 1.1. Number of pertussis cases reported to the CDC in the United States. (A) The number of cases reported to the CDC from 1922 through 2014. The introduction of the whole cell and acellular vaccines are denoted as arrows on the graph. **(B)** A closer look at the number of cases reported to the CDC since 1990. The number of cases reported during the 1950's are denoted on the graph as red dotted lines for reference.

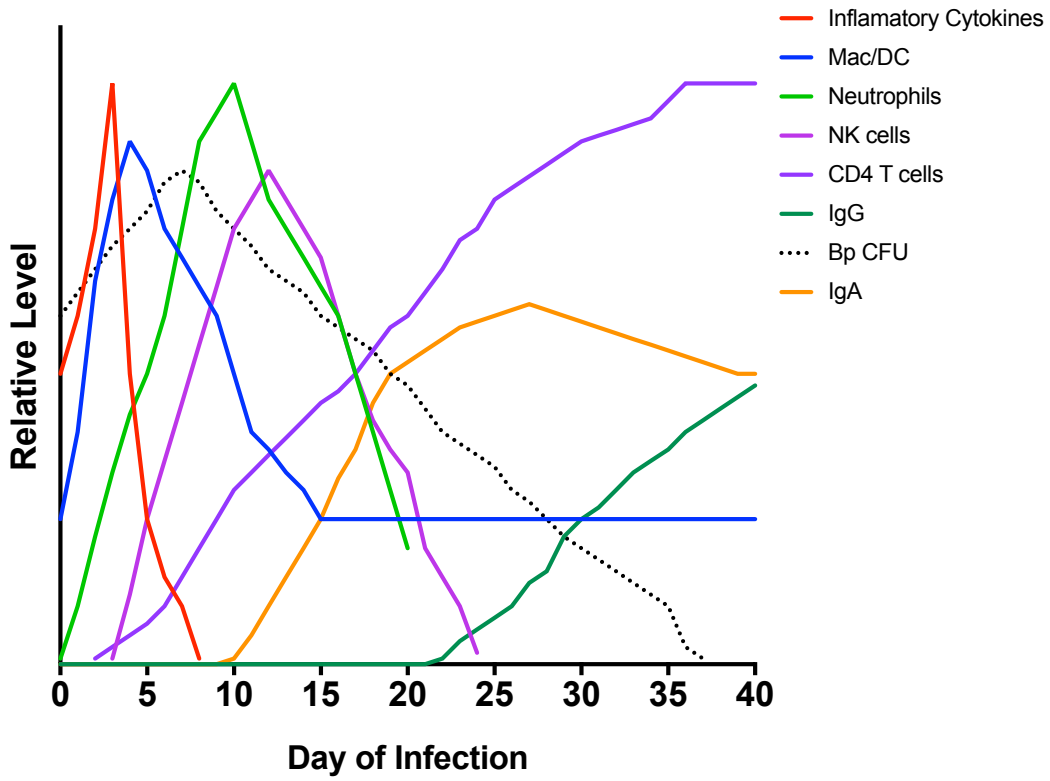

Figure 1.2. The global host response to *B. pertussis* infection. Data presented here have been condensed into one infographic (modeled off of Higgs, *et al* diagram (37)) and are based upon years of human, rabbit, mouse, and baboon research in the *B. pertussis* field. Upon infection with *B. pertussis,* there is an early inflammatory cytokine response, which recruits macrophages (Mac), dendritic cells (DC), and neutrophils to the site of infection. At peak *B. pertussis* CFUs (Bp CFU), NK cell populations increase and soon after the CD4 T cell response begins (Th17 cells during natural infection). IgA levels increase before IgG levels. Bacteria are cleared naturally by the host by day 35-40.

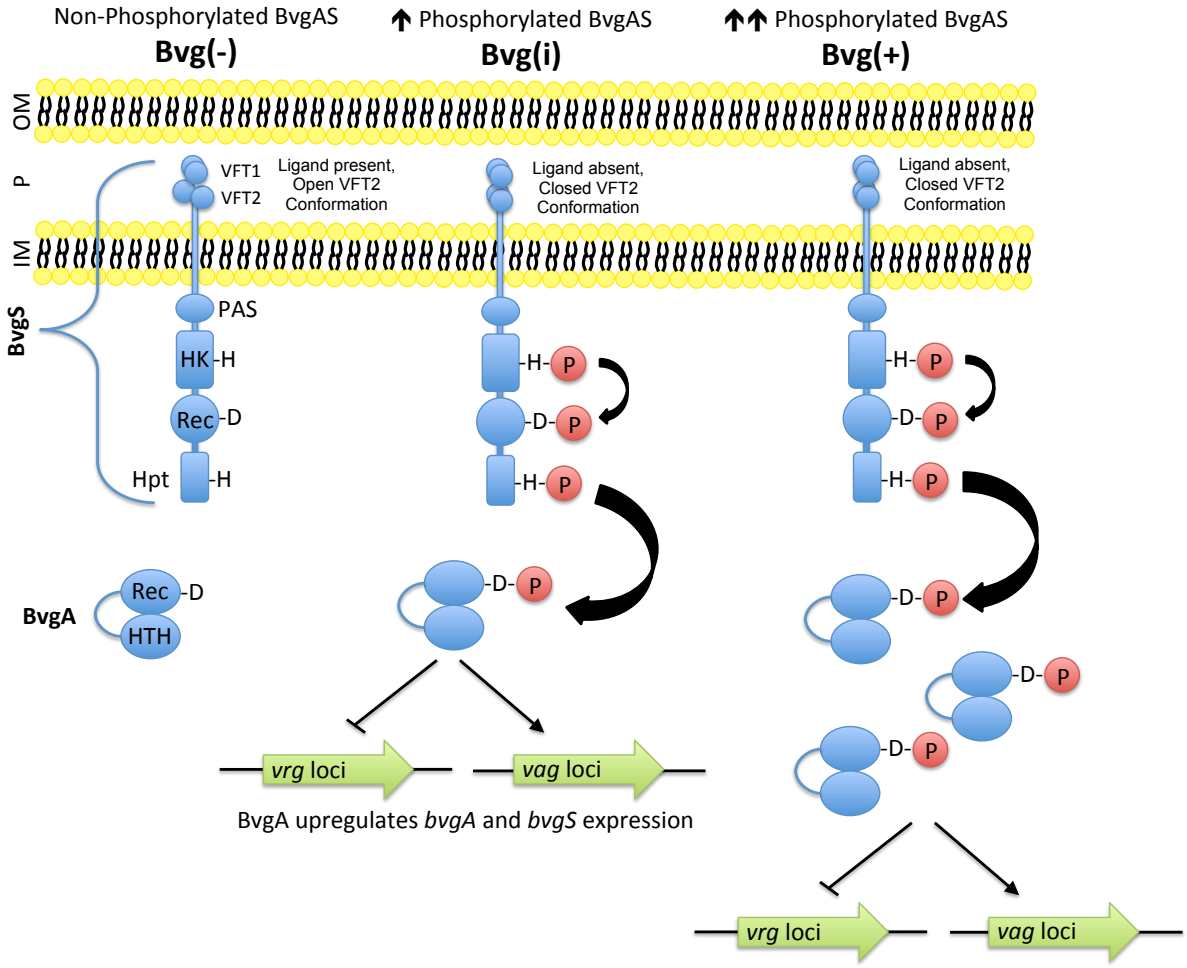

Figure 1.3. The master regulator of virulence, the BvgAS two-component system. The BvgAS two component system, comprised of the BvgS transmembrane (inner membrane, IM) has VFT1 and VFT2 (venus fly trap domain). Under basal conditions, VFT2 is closed, which typically hinders self-phosphorylation activity of the kinase, but in *Bordetella* the closed conformation of VFT2 renders BvgS active for self-phosphorylation. Under modulating conditions, VFT2 binds an agonist and opens, which turns off BvgS (68). When BvgS is active, the protein auto-phosphorylates the Histidine Kinase (HK) domain,

which in turn phosphorylates the receiver (Rec) domain. BvgS then auto-phosphorylates at the final HK domain, where it is finally transferred to the Rec domain of BvgA, a transcription factor. Upon phosphorylation, BvgA alters its helix-turn-helix (HTH) domain to more efficiently bind DNA and either turn on expression of virulence activated genes (vag) or turn off virulence repressed genes (vrg).

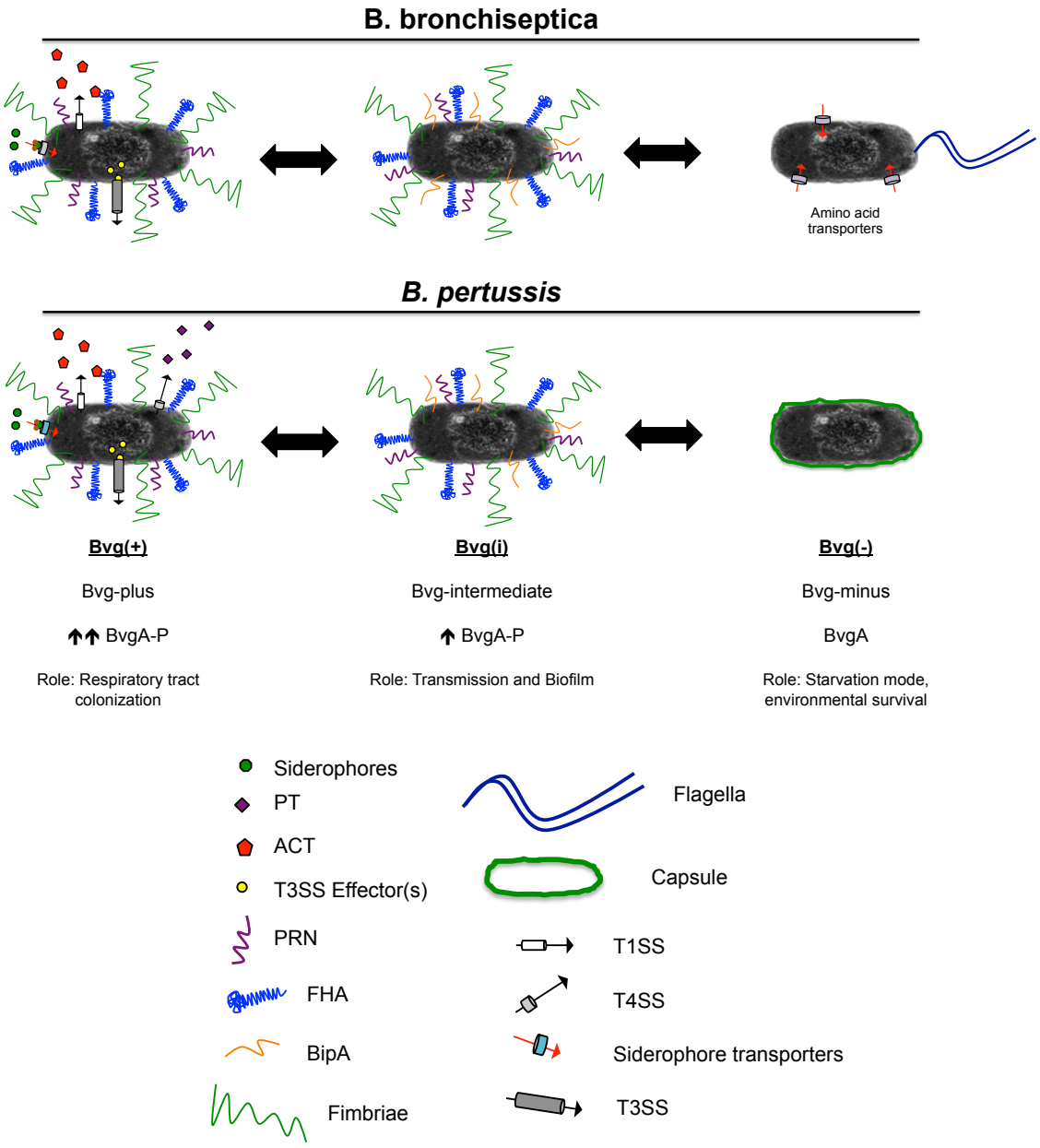

Figure 1.4. *Bordetella* Phase Variation BvgAS controls three distinct phenotypic phases in response to various environmental stimuli. The Bvg(+) phase (historically the X mode) is necessary and sufficient for respiratory tract colonization and is associated with the expression of virulence factors (adhesins and toxins). The Bvg(i) phase (historically the I mode) is hypothesized to be

important for respiratory transmission and is characterized by the expression of only a subset of Bvg+ phase-specific factors, namely adhesins, as well as factors expressed only in the Bvg(i) phase, *Bordetella* Intermediate Protein A (BipA). *B. pertussis* and *B. bronchiseptica* express a significantly different set of genes in the Bvg(−) phase (historically the C mode). The Bvg(-) phase has an unknown function in the context of infection, but has been shown to be necessary for growth in nutrient-limiting conditions and plays a role in survival in the environment. Both *B. pertussis* and *B. bronchiseptica* grow at increased rates in the Bvg(-) phase (adapted from Matto and Cherry, 2005 (3)).

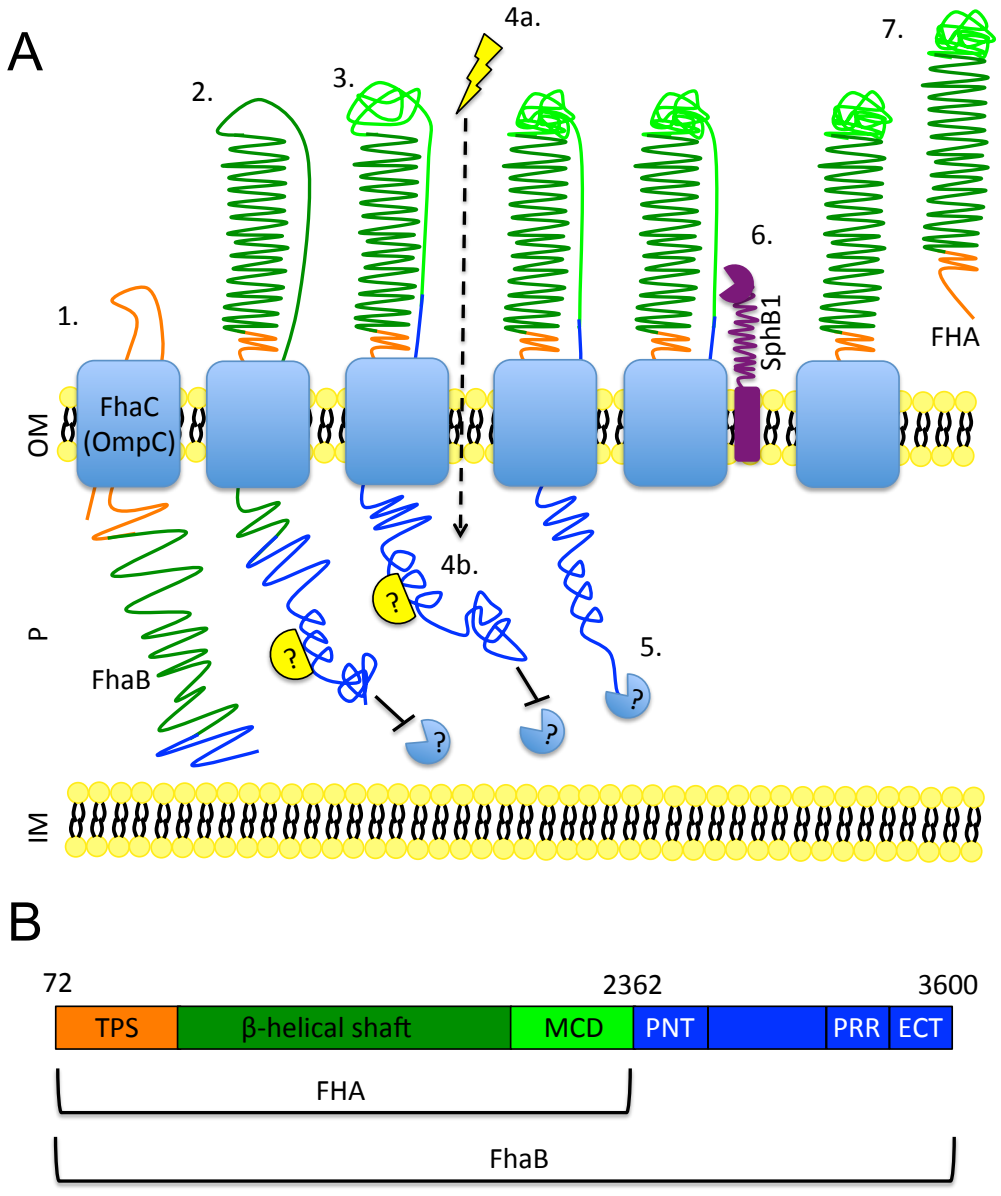

Figure 1.5. Filamentous hemagglutinin protein processing. (A) FhaB secretion and FHA release is a multistep process, involving several proteases for the final FHA product, which is displayed as a mature protein on the surface, or released into the extracellular milieu. 1. Once inside the periplasm, FhaB (**(B)** peptide map of FhaB and FHA) is inserted into the FhaC outermembrane protein

in a hairpin shape. 2. The peptide begins folding into layers of β-helical sheets, to form the shaft of the protein. 3. The portion of FHA deemed the mature c-terminal domain (MCD) begins folding non-specifically at the distal tip of FhaB, and 4a. upon some unknown signal, possibly binding to target cells, 4b. an unknown chaperone is released from the proline rich region (PRR), and 5. a periplasmic protease begins degrading FhaB from the extreme c-terminal towards the prodomain N-terminal (PNT). This degradation results in the MCD folding into its final conformation at the distal tip of FHA. 6. SphB1 is an extracellular protease that cleaves the remainder of the prodomain and a portion of the MCD peptide that is not folded. This results in the final FHA structure, which can then be released from the cell surface via an unknown signal. This model has been adapted from Noel *et al.* (87).

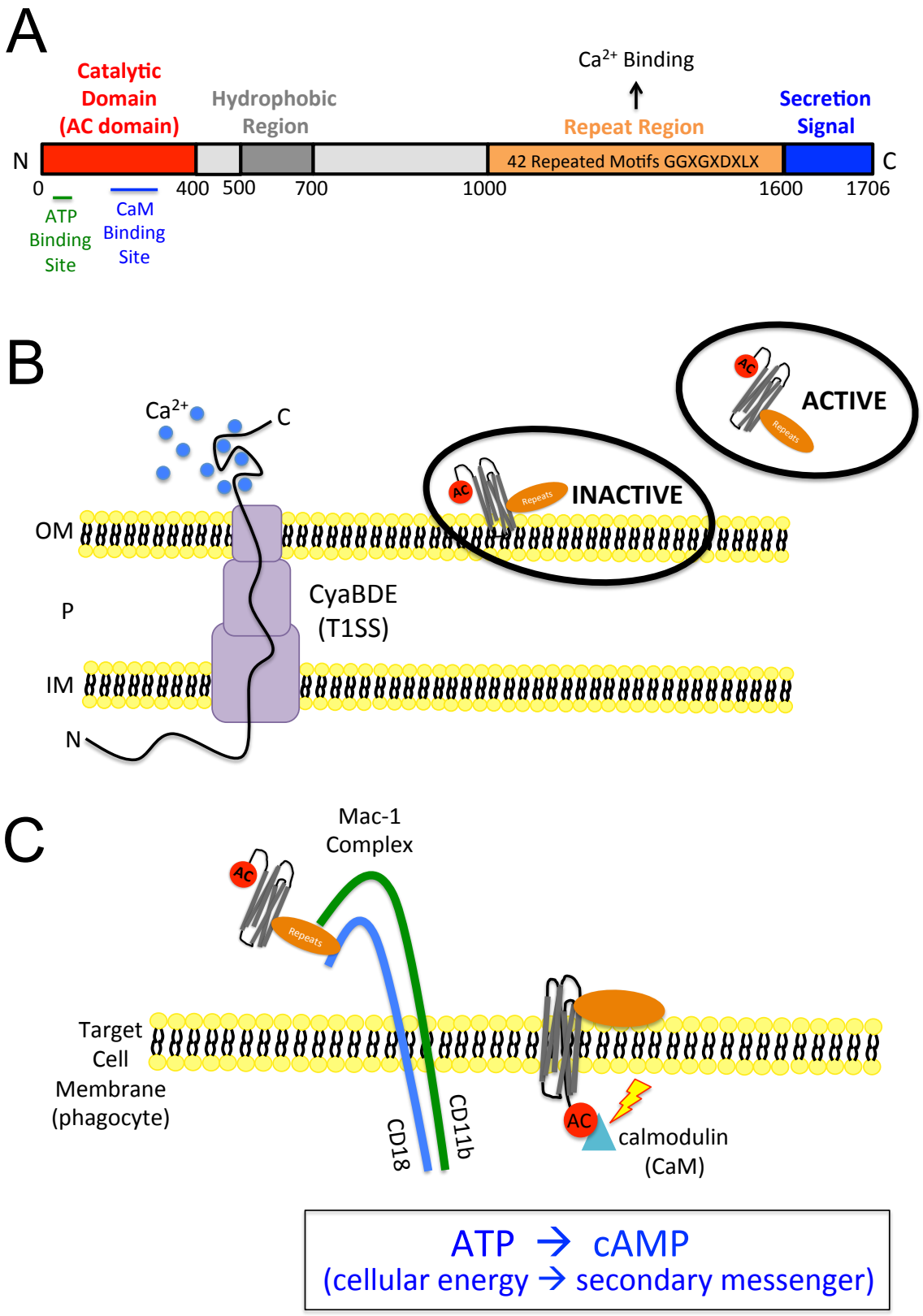
A
Catalytic Domain (AC domain)
Hydrophobic Region
Ca2+ Binding
Repeat Region
Secretion Signal
N
42 Repeated Motifs GGXGXDXLX
C
0
400
500
700
1000
1600
1706
ATP Binding Site
CaM Binding Site
B
Ca2+
C
ACTIVE
INACTIVE
AC
Repeats
OM
P
IM
N
CyaBDE (T1SS)
C
Mac-1 Complex
AC
Repeats
Target Cell Membrane (phagocyte)
CD18
CD11b
AC
calmodulin (CaM)
ATP → cAMP
(cellular energy → secondary messenger)

Figure 1.6. Adenylate cyclase toxin structure, secretion and release, and interactions with host cells. (A) Adenylate cyclase toxin is a 1706 AA peptide with 4 specific domains that have been identified and well characterized. The Catalytic domain is approximately 400 AA and contains the ATP binding site and the calmodulin (CaM) binding sites. Upon CaM binding, the toxin becomes catalytically active. The hydrophobic region is involved in inserting the toxin into host membranes, the repeat region binds ~40 calcium ions. And finally, the type 1 secretion system (T1SS) signal directs the peptide to the T1SS (CyaBDE). **(B)** ACT is transcribed from the c-terminal catalytic domain to the N terminal T1SS signal. The peptide is directed to the T1SS CyaBDE and is inserted into the channel. With proper calcium (Ca^{2+}) concentrations, the peptide begins binding Ca^{2+} ions to ratchet the toxin out of the pore. After secretion and folding, the toxin either remains surface-associated, or becomes released into the intracellular milieu. **(C)** The released toxin, but not the surface associated toxin, can then act upon host cells. ACT binds the CD11B/CD18 complex via the repeat domain. This binding triggers the recruitment of cholesterol rich lipid rafts, so that the hydrophobic domain of ACT can insert into the host cell membrane. The catalytic domain (AC) is translocated through the membrane to the cytoplasm, where there is a hypothesized cleavage event to release AC from the holotoxin. AC then binds CaM to activate enzyme activity inside the cell, the conversion of ATP to cAMP.

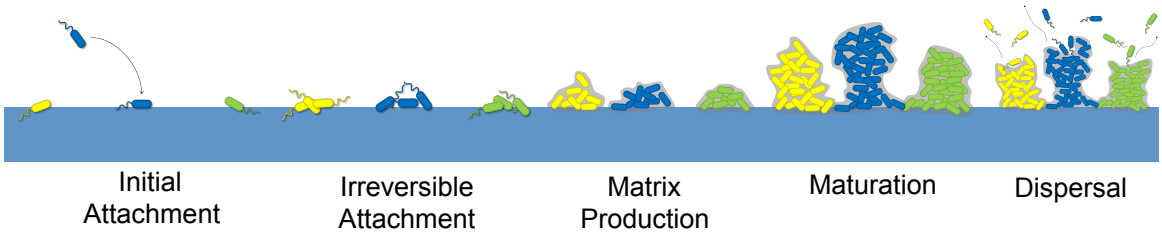

Figure 1.7. Steps in the formation of mature biofilm. Planktonic bacteria are "signaled" to attach to a biotic or abiotic surface (blue bar) via weak attachment forces. Usually, via a signaling event facilitated by flagella binding, the bacteria enter the irreversible attachment phase. The interaction with the surface is now mediated by pilus, fimbriae, flagella, and other proteins, and involves stronger forces (hydrogen, dipole, ionic, hydrophobic). The bacteria then begin secreting exopolymeric substance (polysaccharides, eDNA, and proteins) to produce a matrix that encases the bacteria. The biofilm continues to mature which results in resistance to shear forces and the formation of channels within the biofilm structure. Finally, upon reaching its carrying capacity, the biofilm disperses bacteria to seed a biofilm in a new location.

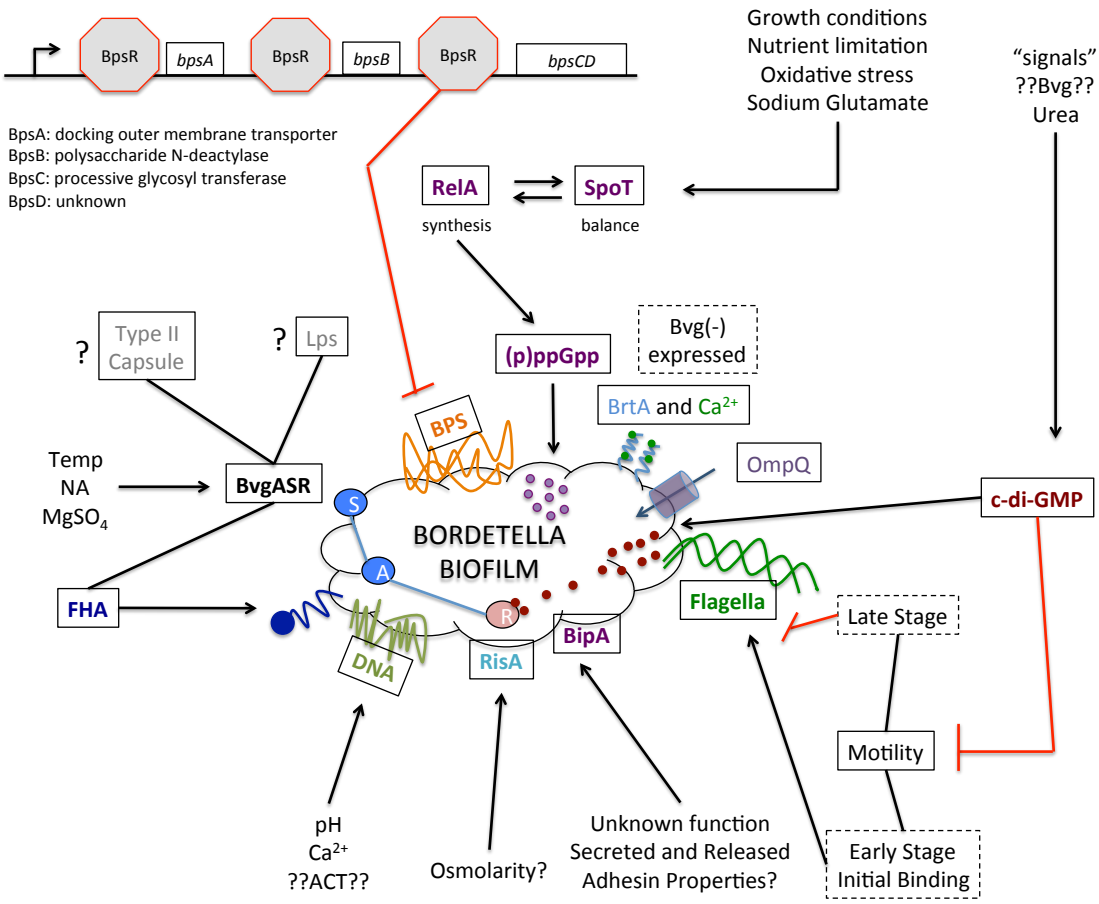

Figure 1.8. What we know about *Bordetella* biofilm regulation and components. The studies discussed in the biofilm portion of the introduction have been integrated to create a diagram of all of the inputs that have an effect on biofilm formation. *Bordetella* biofilm includes bacteria, and the matrix, which is comprised of eDNA, BPS, and various other proteins (FHA, BipA, BrtA, and in the early stages, flagella). Regulatory molecules such as (p)ppGpp and c-di-GMP have been implicated in signaling to alter biofilm formation and the enzymes that control the levels of these have been implicated in biofilm as well. The changes that occur that directly enhance or hinder biofilm are unknown, but

future studies will hopefully elucidate changes in gene expression or protein expression that result in more or less biofilm. The master regulator of biofilm has been implicated in biofilm formation control as well, but its links to other systems of biofilm regulation are unknown. BvgAS must be on in order for biofilm to form, likely because it controls the expression of the major biofilm adhesin, FHA. Several other factors have been hypothesized to play a role in *Bordetella* biofilm, but have not yet been tested (RisA, osmolarity, capsule formation, LPS/LOS, etc.) The goal is to one day integrate all of these signals to determine which matter the most, and if there is truly a master regulator of biofilm.

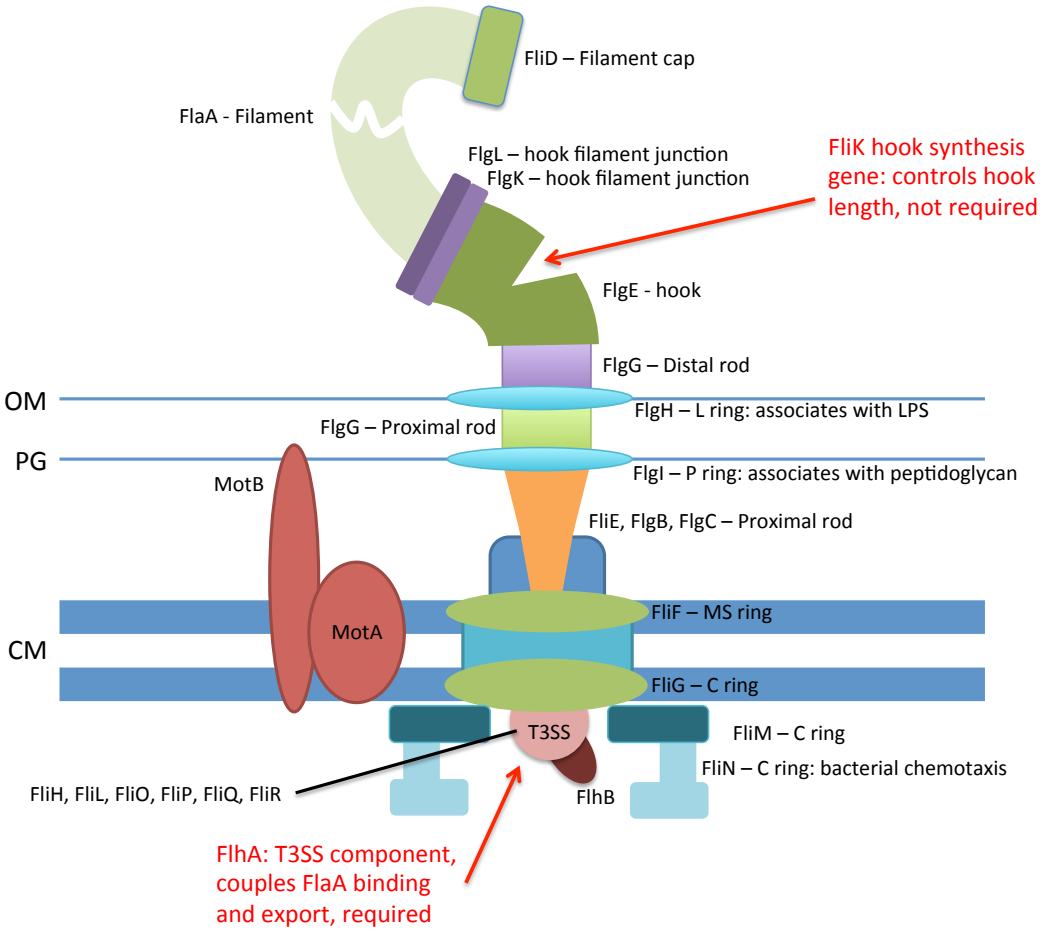

Figure 1.9. Predicted *B. pertussis* flagella structure. This schema was made using the KEGG PATHWAY modeling software. The flagella structure was predicted based on the genes present in the *B. pertussis* Tohama I genome. Red text and arrows denote genes that are not present, or pseudogenes that are present in the genome.

Chapter Two:

Bordetella Adenylate Cyclase Toxin Interacts with Filamentous Hemagglutinin to Inhibit Biofilm Formation *in vitro*

Adapted from "*Bordetella* Adenylate Cyclase Toxin Interacts with Filamentous Hemagglutinin to Inhibit Biofilm Formation *in vitro*"

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SUMMARY:

Bordetella pertussis, the causative agent of whooping cough, secretes and releases adenylate cyclase toxin (ACT), which is a protein bacterial toxin that targets host cells and disarms immune defenses. ACT binds filamentous hemagglutinin (FHA), a surface-displayed adhesin, and until now, the consequences of this interaction were unknown. A *B. bronchiseptica* mutant lacking ACT produced more biofilm than the parental strain, leading Irie *et al*. to propose the ACT-FHA interaction could be responsible for biofilm inhibition. Here we characterize the physical interaction of ACT with FHA and provide evidence linking that interaction to inhibition of biofilm *in vitro*. Exogenous ACT inhibits biofilm formation in a concentration-dependent manner and the N-terminal catalytic domain of ACT (AC domain) is necessary and sufficient for this inhibitory effect. AC Domain interacts with the C-terminal segment of FHA with ~650 nM affinity. ACT does not inhibit biofilm formation by *Bordetella* lacking the mature C-terminal domain (MCD), supporting that the direct interaction between AC domain and the MCD is required for the inhibitory effect. Additionally, AC domain disrupts preformed biofilm on abiotic surfaces. The demonstrated inhibition of biofilm formation by a host-directed protein bacterial toxin represents a novel regulatory mechanism and identifies an unprecedented role for ACT.

INTRODUCTION:

Bordetella pertussis is the causative agent of whooping cough (pertussis) and a reemerging health threat in the United States and globally, as illustrated by the increasing number of cases reported each year. Despite high vaccination rates of children and adolescents, there were approximately 33,000 cases in the United States reported to the CDC in 2014. The most striking shift in the age-specific incidence of pertussis has been in patients aged 15 and older (24), who are now more frequently infected with *B. pertussis*. In contrast to the potential fatality of pertussis in infants and young children, adolescents and adults develop a persistent cough with fewer systemic manifestations of the disease (258, 259), and often serve as sources of pertussis transmission (260-263).

B. pertussis has been shown to form biofilm *in vitro* on abiotic surfaces and *in vivo*, primarily on nasal septum and the trachea of infected mice (200, 237, 264, 265). Ongoing studies support the concept that *B. pertussis* forms biofilm during infection; recent clinical isolates form more biofilm compared to a lab-passaged isolate, BP338, and de Gouw *et al.* showed that biofilm-derived antigens protect mice from *B. pertussis* infection (239, 266). The closely related animal pathogen, *B. bronchiseptica* forms biofilm *in vitro* on abiotic surfaces and biofilm formation contributes to its chronic infection of dogs and other mammals (255). Although the specific role of biofilm in human infections with *B. pertussis* has not yet been established, it is clear that these organisms produce biofilm both *in vitro* and *in vivo* and thus the regulation of this process warrants further investigation.

Biofilms are complex structures controlled by a variety of bacterial signaling systems. They are comprised of aggregated bacteria surrounded by a matrix of polysaccharides, proteins and extracellular DNA (eDNA). *Bordetella* biofilm has been shown to require eDNA (236), Bps (*Bordetella* polysaccharide) (237, 238), which resembles *S. aureus* poly-N-acetyl-beta-(1-6)-glucosamine, and multiple proteins. Of significance to this study is the observation that filamentous hemagglutinin (FHA) is an important component of biofilm in *B. pertussis* and *B. bronchiseptica*. This surface-displayed adhesin promotes the formation and maintenance of biofilm by mediating bacteria-substrate as well as bacteria-bacteria interactions. Serra *et al.* showed that anti-FHA antibodies blocked biofilm formation by *B. pertussis,* and a strain lacking FHA (Δ*fhaB* BPGR4) made less biofilm *in vitro* and *in vivo* on mouse trachea and nasal septum compared to WT BPSM (200).

Although less is known about the regulation of biofilm in *Bordetellae* compared to other medically relevant, biofilm-forming bacterial species, several modes of regulation have been implicated. Nutrient limitation and oxidative stress activate (p)ppGpp signaling to enhance biofilm formation in *B. pertussis* (250), and c-di-GMP signaling regulates motility and biofilm formation in *B. bronchiseptica* (249). The machinery for synthesis of the Bps matrix component is encoded by the *bpsABCD* operon and is under control of the BpsR repressor, but the factor, process, or signal that relieves BpsR repression is unknown (242). Multiple signals are integrated to control *Bordetella* biofilm. One of these signals is from the “master regulator” of virulence, *Bordetella* virulence gene two-

component regulatory system, BvgAS. Biofilm formation occurs in the Bvg(+) phase and Bvg(i) phase, but is not observed in the Bvg(-) phase. Irie *et al*. showed biofilm formation was maximal in the Bvg(i) phase in *B. bronchiseptica* (234). In contrast, Mishra *et al.* found biofilm formation was equal in the Bvg(+) and Bvg(i) phases for *B. pertussis* (235). Irie *et al*. also showed that a *B. bronchiseptica* strain lacking adenylate cyclase toxin (ACT) (Δ*cyaA* RB58) made more biofilm than the parental WT RB50 strain and in light of the earlier observation demonstrating a direct interaction between ACT and FHA (176), suggested that this protein-protein interaction could function to regulate biofilm production in *Bordetella*.

ACT is an important virulence factor of both *B. pertussis* and *B. bronchiseptica.* The 177 kDa protein toxin is secreted by a type I secretion system and remains surface-associated or is released as a function of free-calcium concentration in the medium (172). ACT that has been released is the active form of the toxin, which affects target cells (177). ACT uses complement receptor 3, the heterodimeric $\alpha_M\beta_2$ integrin (CD11b/CD18 or Mac-1), as its receptor (178, 179), but can also intoxicate cells that lack this integrin heterodimer (267). Following binding to the host cell, the catalytic domain of the toxin is translocated across the plasma membrane and into the host cytoplasm, where calmodulin (CaM) binds the enzymatic (catalytic) domain, activating it to convert ATP → cAMP (178, 180-185). This in turn leads to supraphysiological levels of cAMP and can cause a reduction in intracellular ATP (184, 186-188). Through these mechanisms, ACT inhibits phagocytosis, chemotaxis, and

superoxide generation by neutrophils, and is required for the establishment of infection in the mouse model. Human infections with the attenuated strain, BPZE1, reveal that ACT is required for establishment of infection in humans (190, 268). And finally, ACT serves as a protective antigen (113, 186, 187, 189, 192-195, 269, 270). In previous studies, the secretion, release, binding to host cells, interaction with host cells, functional effects of ACT on host cells, and its role in establishing an infection have been characterized. This host-directed protein bacterial toxin has not, however, been studied for effects on the bacterium itself, and here we describe a novel function for ACT.

In the present work we have added purified ACT to cultures of *B. pertussis* and *B. bronchiseptica in vitro* to directly tested the hypothesis that the ACT-FHA interaction inhibits biofilm. Indeed, exogenous ACT inhibits biofilm formation, adding to the effect of endogenously produced and secreted ACT. This effect of added ACT occurs through binding of the catalytic AC domain, independently of its enzyme activity, to the mature C-terminal domain (MCD) of FHA, which must be properly folded for the inhibitory effect of ACT to occur. The possible relationship of this novel regulatory role for a bacterial toxin to the hypothetical “life cycle” of *B. pertussis*, controlled by BvgAS, is discussed.

RESULTS:

Exogenous ACT inhibits biofilm in a concentration-dependent manner.

In light of the observation by Irie *et al*., which showed that *B. bronchiseptica* Δ*cyaA* (RB58) produced more biofilm than WT, we tested the hypothesis that ACT has an inhibitory effect on *B. pertussis* biofilm as well. The *B. pertussis* mutant, BP348, lacking ACT by virtue of a transposon insertion in *cyaA*, and the parental WT strain, BP338, were compared for their abilities to form biofilm. BP338, BP348 and BP347 were grown in 96 well plates and biofilm formation was measured by bacterial accumulation on wells using the crystal violet assay. As shown in figure 2.1A, BP348 made more biofilm than BP338. Bvg(-) BP347, a negative control, formed no biofilm; the low, but measurable, OD_{595} reflects basal levels of bacterial adherence, and has been previously observed for Bvg(-) *B. pertussis* (235). These differences in biofilm formation are not due to differences in bacteria growth, in 10 mL SSM shaking cultures all strains grew at the same rate, when cultured under 100μL static culture conditions in 96 well plates, BP338 and BP348 grew at similar rates. BP347, which does not make biofilm, reached a higher OD_{600} more quickly (Figure 2.2), excluding impaired bacterial growth as the cause of differences in biofilm formation.

We next tested exogenous, purified, recombinant ACT for its ability to inhibit biofilm formation by *B. pertussis* and *B. bronchiseptica*. Exogenous ACT was added at concentrations of 10, 100 or 1000 ng mL^{-1} (56 pM, 565 pM, 5650 pM respectively) to BP338 cultures. The concentration-dependent inhibition of

biofilm is demonstrated in Figure 2.1B (IC_{50} 17.32 ng ml^{-1} to reduce biofilm to crystal violet OD_{595} equal to negative control OD_{595}). *B. pertussis* grown in the presence of added ACT or urea, a major component of the solution in which ACT is stored, grew at the same rate as in media alone, further showing that differences in growth rate do not account for reduced biofilm formation. (Figure 2.3). The same concentration-dependent inhibition of biofilm by ACT occurred with *B. bronchiseptica* (Figure 2.4A). Importantly, the concentrations of ACT (approximately 100 ng mL^{-1}) used in this study are comparable to calculated concentrations in nasopharyngeal washes from baboons and infants infected with *B. pertussis* (175).

The AC domain is necessary and sufficient for inhibition of biofilm.

ACT is a 177 kDa, multi-domain protein, and to determine which part(s) of the ACT molecule are involved in the inhibitory effect, we tested truncated ACT variants and an ACT protein mutant lacking AC enzymatic activity (iACT) for their abilities to inhibit biofilm formation (Figure 2.1C is a schematic representation of modifications). These variants, which are partial deletions from full-length *cyaA* (271, 272), were previously used to characterize monoclonal antibodies (273), and have been used previously to study ACT functions (39, 274-276).

All ACT variants, with one exception, inhibited biofilm formation to the same extent as full-length ACT (Figure 2.1C). $ACT_{\Delta AC}$, ACT lacking the catalytic domain, was without an inhibitory effect at 100 ng mL^{-1}, and the 43 kDa AC domain was comparable to native ACT in inhibiting biofilm formation at 10 ng mL^{-}

^{1} (comparable molar concentrations), and did so in a concentration-dependent manner (Figure 2.5). iACT, the enzymatically inactive form of the toxin (1000-fold reduction in enzymatic activity in comparison to ACT holotoxin), inhibited biofilm comparably to ACT at 100 ng mL^{-1} (Figure 2.1D), establishing that the enzymatic activity of the toxin is not required for the inhibitory effect. Thus the AC domain is both necessary and sufficient for biofilm inhibition, yet its catalytic activity is not required.

The AC domain is the most conserved portion of ACT among *Bordetella* species that encode the toxin (5), indicating the possibility of a comparable inhibitory role for the AC domain in biofilm regulation among the *Bordetellae*. The AC domain is also necessary and sufficient to inhibit *B. bronchiseptica* RB50 biofilm (Figure 2.4B). To confirm the role of endogenous ACT and its catalytic domain, we constructed BP338 ΔAC. The portion of *cyaA* encoding the AC domain (Δ1-373) was deleted and the resultant strain, BP338 ΔAC, was tested for biofilm formation in *in vitro* assays described above. A western blot shows that the BP338 ΔAC mutant expresses truncated ACT peptide (Figure 2.6). As expected, BP338 ΔAC made more biofilm than the parental WT BP338 (Figure 2.1E), confirming the necessity for and specificity of the AC domain to inhibit biofilm in *B. pertussis*.

In that the crystal violet assay is an indirect quantification of biofilm, we used Scanning Electron Microscopy to visualize the effects of AC domain on BP338 biofilm, using BP347 as a biofilm-negative control. We allowed *B. pertussis* to form biofilm on glass coverslips in the absence and presence of

exogenous AC domain to complement data from the crystal violet assay. Samples obtained under these conditions were imaged using a Zeiss Sigma VP HD Field Emission Scanning Electron Microscope (SEM). Figure 2.7 illustrates the dramatic effects of AC domain under these conditions. WT BP338 (Figure 2.7A) and Bvg(-) BP347 (Figure 2.7B) grown in SSM were compared to BP338 grown in SSM + 10 ng mL^{-1} AC domain (Figure 2.7C). The exogenous AC domain precluded biofilm accumulation on glass coverslips, such that BP338 plus AC domain was equivalent to the negative control, BP347. In the images of BP347 and BP338 plus AC domain, there are few bacteria adherent to the coverslip. The lack of bacterial accumulation under these conditions suggests a defect in the initial binding of bacteria, which then impairs subsequent biofilm development. Thus, the initial step of binding to the abiotic surface is a determinant of the ability of *B. pertussis* to produce robust biofilm.

The AC domain inhibits bacterial aggregation and disrupts preformed *B. pertussis* biofilm.

To address the underlying mechanisms by ACT inhibits biofilms, we tested other steps in the biofilm life cycle for susceptibility to ACT inhibition. Bacterial aggregates form in shaking culture and positively correlate with biofilm formation in many bacterial species (277, 278). Exogenous AC domain was added to growing cultures of *B. pertussis* and an aggregation index was determined at 24 hours, as previously described for *B. pertussis* (266). Exogenous AC domain decreased bacteria aggregation by 75% (Figure 2.8A).

The final stages of the biofilm lifecycle involve dispersal of the bacteria from the biofilm structure. In order to investigate further the regulatory effect of ACT on biofilm, we tested the effect of AC domain when added to existing biofilm. Figure 2.8B shows the time course of biofilm formation in the presence (red solid line) and absence of AC domain (black solid line). AC domain was added to BP338 biofilm at 72 hours post inoculation. Biofilm measured 24 hours later (96 hours) was reduced 76% (blue dotted line) compared to BP338 alone at 96 hours, resulting in quantities comparable to biofilm formed in the continuous presence of AC domain (red line). This disruption of existing biofilm did not occur when full-length ACT was added at 72 hours (data not shown). Although we do not know the mechanism of disruption, we hypothesize that the lack of effect of ACT was due to limitations in the ability of the large hydrophobic protein to access the necessary site(s) within the biofilm.

The inhibition of biofilm formation by ACT is specific.

To characterize the inhibitory effects of exogenous ACT and AC domain on *B. pertussis* biofilm formation, we tested molecules that interact with ACT for their ability to affect ACT-mediated inhibition. Calmodulin (CaM) binds the AC domain of ACT with high affinity (K_d ~2 nM) and activates its enzymatic activity (279). It has been previously demonstrated that addition of CaM to ACT, prior to incubation with cells, blocks translocation of the AC domain into the cell cytosol, thereby precluding cAMP production (280, 281). In the present studies, purified ACT or AC domain and CaM were combined before addition to bacteria. Under

these conditions, a molar excess of CaM prevented the inhibitory effect of ACT or AC domain on biofilm formation (Figure 2.9). Similarly, an antibody directed against the catalytic domain of ACT blocked the inhibitory effects of ACT and AC domain on biofilm (Figure 2.9). The fact that CaM or an antibody blocks the inhibitory effect of ACT suggests the possibility that CaM prevents a physical interaction between ACT and another bacterial factor, such as filamentous hemagglutinin (FHA), which is involved in biofilm formation.

The AC domain interacts with FHA.

In a *B. pertussis* mutant lacking FHA, ACT is present in the media as opposed to remaining surface-associated (191), and ACT interacts with FHA on the surface of bacteria (176). These data led us to the hypothesis that ACT directly interacts with FHA to inhibit biofilm formation. In light of this collection of observations and the fact that AC domain is necessary and sufficient for inhibition of biofilm, we explored the interaction of the AC domain with FHA by surface plasmon resonance (SPR).

The FHA (~220 kDa) protein, purified from *B. pertussis* culture supernatant (Figure 2.10), was immobilized on GLC sensor chip and real-time kinetics of the interaction of the recombinant AC domain with FHA was analyzed by parallel injection of diluted AC protein over the sensor chip surface at a constant flow rate of 30 µl/min (Figure 2.11A). Interaction of the AC domain with FHA was specific, since negligible binding of the AC domain was observed to the chip coated with FHA44, a truncated FHA protein corresponding to residues 72-

862 of FHA, which does not contain the c-terminal domain (Figure 2.11B). Kinetic parameters of the AC-FHA interaction were calculated from global fitting of concentration-dependent binding curves. As shown in Figure 2.11A, the binding curves fit well to a Langmuir-type binding model indicating a simple 1:1 interaction between the AC domain and FHA with equilibrium dissociation constant (K_D) of approximately 650 nM. These data demonstrate that the AC domain only interacts with FHA when c-terminal segment is present.

To complement the functional data which shows CaM blocks the inhibitory effects of AC domain on biofilm formation, AC domain was mixed with CaM in molar ratios of 10:1, 1:1 or 1:10 and the capacity of AC domain-CaM complex to interact with FHA was probed by SPR. As shown in Figure 2.11C, binding of the AC domain to FHA in the presence of CaM (10:10) was reduced approximately 75% as compared to AC domain alone, suggesting that CaM and FHA compete for the same site on AC domain, or that CaM binding alters conformation of the AC domain thereby interfering with the ACT-FHA interaction. The data presented in Figure 2.9 show that an excess of CaM blocks AC domain inhibition of biofilm and the data in Figure 2.11C show that equal molar ratios of AC domain:CaM reduce binding to FHA by 75%. Based on these data we hypothesized that the molar excess of CaM used in the biofilm assay blocks the inhibitory effects of ACT on biofilm formation by blocking the physical interaction between ACT and FHA.

In that the c-terminal portion of FHA is required for AC domain binding to FHA (Figure 2.11B, FHA44), we hypothesized that ACT and the AC domain

would block antibody interactions with specific epitopes on FHA. To test this hypothesis, we developed an ELISA-based assay to characterize the interaction between ACT and the c-terminal segment of FHA. Plates were coated with full length FHA and incubated with buffer, ACT, or AC domain over a range of concentrations (0.1-10 μg mL^{-1}), or $ACT_{\Delta AC}$ at 10 μg mL^{-1}. Monoclonal antibodies directed against the mature C-terminal domain (MCD) of FHA (residues 1870-2362) were used to determine the accessibility of the c-terminal segment of FHA. This anti-MCD antibody has been used previously to detect FHA and study FHA processing (86, 87), and was used in this study because it recognizes a large portion of FHA that was deleted from the truncated FHA44 mutant protein.

The presence of ACT or AC domain blocked anti-MCD antibodies from binding to FHA (Figure 2.12). Both ACT and the AC domain produced a concentration-dependent inhibition of anti-MCD antibody binding to FHA, but, in accordance with the earlier functional data on inhibition of biofilm, $ACT_{\Delta AC}$ had no effect (Figure 2.12). Furthermore, incubation of ACT or the AC domain with CaM prior to addition to FHA-coated plates precluded them from blocking the binding of MCD antibody to FHA (striped bars, Figure 2.12). Because the data regarding ACT inhibition of biofilm correlate with the physical binding of the AC domain and FHA, the consequences of their physical interaction were investigated to better understand the molecular mechanisms involved in biofilm inhibition.

The MCD of FHA is required for ACT inhibition of biofilm.

FHA is delivered to the bacterial surface via a two-partner secretion pathway. This process involves translocation of FhaB, the FHA precursor, through FhaC, an FhaB-specific outer-membrane transporter (282). FhaB enters the FhaC channel as a hairpin and then begins folding in an N-to-C-terminal manner on the cell surface, creating a β-helical shaft (85, 86). After the region distal to the β-helical shaft reaches the cell surface, the C-terminal prodomain is proteolyzed in the periplasm, creating the "mature C-terminal domain" (MCD), which is located on the distal portion of FHA (87). To better understand the functional domains involved in ACT binding and inhibition of biofilm, we studied *B. pertussis* and *B. bronchiseptica* mutants with altered secretion and processing of FHA.

Since the MCD of FHA is required for AC domain binding (Figure 2.11B), we hypothesized that the MCD must be present in order for ACT inhibition to occur. A *B. pertussis* mutant lacking the MCD was assessed for biofilm formation in the presence and absence of ACT to determine the role of the MCD in the inhibitory process. BPSM JS20, a derivative of parental strain BPSM, produces a truncated FHA by virtue of deletion of the MCD and C-terminal prodomain and, is therefore, composed only of the β-helical shaft. BPSM and BPSM JS20 were grown in the presence and absence of ACT and biofilm formation was measured at 96 hours. WT BPSM formed biofilm that was susceptible to inhibition by ACT. BPSM JS20 formed equivalent amounts of biofilm in the presence and absence of exogenous ACT (Figure 2.14A) and made more biofilm than the parental

BPSM strain in the absence of exogenous ACT; this may be due to the inability of endogenous ACT to have an effect on BPSM JS20 biofilm. Importantly, the BPSM isogenic strain and the BP338 isogenic strain, both of which are Tohama I derivatives, were compared for biofilm formation, ACT expression and FHA expression. No significant differences were observed in biofilm formation between the parental WT strains (Figure 2.13A). FHA protein expression was similar between the two strains, although there was slightly more ACT protein expression in BPSM. (Figure 2.13B). The equivalent BPSM JS20 mutant strain in *B bronchiseptica,* which is derived from RBX11 and lacks the MCD and C-terminal prodomain, produced biofilm that is not inhibited by ACT (Figure 2.15). Although the MCD is not required for biofilm formation, it appears to be necessary for ACT-mediated inhibition of biofilm to occur. These data are consistent with the SPR results showing ACT does not bind FHA44, which lacks the MCD (Figure 2.11B).

To validate the role of the MCD in ACT inhibition of biofilm, we tested a mutant in which the MCD is improperly folded. BPSM T-N, also derived from BPSM parental WT strain, contains a mutation in *fhaB* such that a stop codon is introduced in the region encoding the N-terminus of the prodomain. As a result, the MCD is present and located distally from the cell surface, but is not folded in its native conformation (86, 87). BPSM T-N formed similar amounts of biofilm compared to BPSM, yet like BPSM JS20, ACT did not inhibit biofilm formation of this strain (Figure 2.14A). The same was true for RBX11 T-N, the equivalent *B. bronchiseptica* strain with a misfolded MCD (Figure 2.15). Although the MCD

itself is not required for biofilm formation, the MCD of FHA must be present and in the proper conformation for the inhibition of biofilm by ACT to occur. These data directly link the ACT-FHA interaction to inhibition of biofilm by ACT in *B. pertussis* and *B. bronchiseptica*.

In light of the inhibitory effects of ACT and the fact that ACT and anti-MCD antibody both bind to the MCD, the ability of the anti-MCD antibody to block biofilm was tested. Indeed, when BP338 was grown in the presence of anti-MCD antibodies, there was a reduction in biofilm (Figure 2.14B). These data suggest that the anti-MCD antibody may block biofilm formation in a similar manner to ACT, support the competition between the ACT and the anti-MCD antibodies for FHA binding, and corroborate previous studies showing that polyclonal antibodies directed against FHA block biofilm formation (200). It is clear that the MCD of FHA plays a part in inhibition of biofilm, although its exact role is unclear. When binding partners, either anti-MCD antibodies or the AC domain, are present, biofilm formation is inhibited. Further investigation of the MCD and its role in regulating biofilm will be important to understand *Bordetella* biofilm and the effects of ACT.

DISCUSSION:

The data presented here link the ACT-FHA interaction to inhibition of biofilm formation by *Bordetella pertussis* and *Bordetella bronchiseptica in vitro*. The AC domain is necessary and sufficient, yet the catalytic activity of the toxin is not required for this inhibitory phenomenon. These effects of ACT can be blocked by CaM or by a catalytic domain-specific antibody. We have identified the AC domain as a sufficient binding partner for FHA, and the MCD as necessary for this binding and the inhibitory effect on biofilm to occur. The inhibitory effect may result from the AC domain – MCD interaction simply blocking the FHA molecule in its yet-to-be-identified role in *Bordetella* biofilm production, or by inducing a conformational change in FHA that has this and other effects. Our working model for the inhibition of *B. pertussis* biofilm by ACT is diagrammed in Figure 2.16; the AC domain of ACT binds the MCD of FHA to interfere with inter-bacterial FHA-FHA interactions, which have been previously described as important for biofilm formation. The inhibition by ACT may start in the early steps of biofilm formation, by ACT blocking initial bacteria-substrate, as well as bacteria-bacteria interactions and thus limiting subsequent biofilm accumulation. We have, however, been unable to relate our data to the observations by Vidakovics *et al.* showing that the absence of ACT reduces *B. pertussis* binding to alveolar epithelial cells (283).

Our data illustrating inhibition of *Bordetella* biofilm by ACT through its interaction with FHA raise the important question of how this phenomenon fits with the current concepts of *Bordetella* pathogenesis and biofilm production.

Others have shown that multiple factors, ranging from (p)ppGpp and c-di-GMP to transcriptional regulators of Bps polysaccharide production, affect biofilm production by *Bordetellae* (242, 249, 250). Specifically, Irie *et al*. and Mishra *et al*. demonstrated that BvgAS modulates the formation of biofilm and that there is an increase in *B. bronchiseptica* biofilm under Bvg(i) conditions (234). This scenario can now be explained, at least in part, by a reduction in the amount of inhibitory ACT in the presence of a constant level of FHA in the Bvg(i) phase (3, 76, 79). Thus, during infection in which conditions are optimal for virulence factor production by the bacteria, ACT is produced for its inhibitory effects on the host immune response and biofilm production is suppressed (Figure 2.16). Under less favorable conditions, during which a defensive posture might be beneficial, a reduction in ACT production could be one of several mechanisms by which production of biofilm is initiated.

Given the active production of ACT during the Bvg(+) phase, it is appropriate to ask why there is any biofilm produced during these *in vitro* assays. We now know that the quantity and distribution of ACT is different than what occurs *in vivo*. Previously, we have demonstrated that in *ex vivo* samples obtained during active infection, concentrations of ACT can reach approximately 100 ng mL^{-1} and all of the ACT is in the supernatant fraction, as opposed to being surface associated (175). This is in contrast to *B. pertussis* cultured *in vitro* in SSM, in which >90% of the ACT remains surface-associated and concentrations are rarely as high as those measured in the *ex vivo* samples (175). We have also shown that the functional form of the toxin is that which is

released into the media (177), whereas the surface-associated toxin is likely an improperly folded, inert pool.

Finally, this is not the first report of a single protein having multiple, seemingly unrelated biochemical functions. Dr. Constance Jeffery has described and catalogued (www.moonlightingproteins.org) a number of dual function protein molecules, in which a single protein performs multiple physiologically relevant biochemical or biophysical roles (284-288). On the basis of recognizing additional functions for known proteins, these fascinating molecules, which are from both prokaryotic and eukaryotic sources, have been designated “moonlighting proteins”. Their study has enabled identification of novel biochemical pathways and protein functions, and allowed systems biologists to better understand cellular processes. Prior to this study, ACT has been studied and characterized solely a host-directed protein bacterial toxin that modulates function and is cytotoxic for some target cells by increasing cAMP levels and, depending on concentration, depleting ATP levels. ACT is also a hemolysin and member of the RTX family of pore-forming toxins, which includes *E. coli* hemolysin, HlyA (289). The pore-forming function, which for ACT is involved in delivery of its catalytic domain to the target cell interior, has an additional effect of compromising membrane integrity and polarization and contributes to cytotoxicity. The additional role for this protein bacterial toxin, contained within its catalytic domain, namely interaction with a surface adhesion to impair formation of biofilm makes it unlike any other moonlighting protein that has been described in the Moonprot database (290). This information can now be used to study

Bordetella biofilm and to hypothesize when formation may occur *in vivo*. The ability of ACT to inhibit biofilm needs to be investigated further to understand the therapeutic and prophylactic implications. Additionally this information may provide a building block for future studies that elucidate similar novel functions in other bacterial protein toxins.

EXPERIMENTAL PROCEDURES:

Bacterial Strains and Growth Conditions: *B. pertussis* WT BP338 (Tohama I); Bvg(-) BP347 (TN5::*bvgS* mutant derived from BP338); WT BPSM (Tohama I); BPSM JS20 (*fhaB* ΔMCD and C-terminal prodomain derivative of BPSM); and BPSM T-N (*fhaB* with transposon insertion in C-terminal prodomain derivative of BPSM) were grown on Bordet-Gengou (BG) agar (Gibco) supplemented with 15% defibrinated sheep blood (Cocalico) for 48 hours at 37ºC. The same growth conditions were used for *B. bronchiseptica* strains, WT RB50; Bvg(-) RB54; RBX11-JS20 (*fhaB* ΔMCD and C-terminal prodomain); and RBX11 T-N (*fhaB* with transposon insertion in C-terminal domain). These *B. pertussis* strains have been previously reported (86, 191), as have *B. bronchiseptica* (86). Bacteria were then transferred to liquid culture in modified synthetic Stainer-Scholte liquid medium (SSM) (174), and grown for 24 hours at 37ºC, shaking at 150 RPM. Bacteria were pelleted and washed in SSM, and then resuspended to an OD_{600} of 0.1 for biofilm experiments.

Strain Construction (ΔAC domain): Two DNA fragments (517 and 513 bps), corresponding to the 5 and the 3 flanking region of the in-frame deletion, were amplified from B. pertussis genomic DNA as a template by PCR using two pairs of primers

F1: 5-TTTACTAGTGGGATTGAGGAGGGAGGGC-3;

R1: 5-TTTATGCATGTGGATCTGTCGATAAGTAGTC -3

F2: 5-TTTATGCATAAGTTCTCGCCGGATGTACTG -3;

R2: 5-TTTGAATTCGCCGCCTCCCAGCGCCAT -3

The primers were cut with the SpeI/NsiI and NsiI/EcoRI, respectively, and ligated with the SpeI/EcoRI-cleaved pSS4245 vector (291). Bacteria were mated following the previously described methods for *B. pertussis* . Briefly, *B. pertussis* BP338 was passaged 2-3 days on BG and grown over night in SSM. The OD_{600} was between 0.7 and 0.8. RHO3 *E. coli* were grown in antibiotic selection media, LB + 150 µg mL^{-1} ampicillin (AMP) supplemented with 400 µg mL^{-1} DAP to mid-log phase. A 2:1 donor to recipient ratio was used. After washing, the bacteria were resuspended in 100 µL SSM and plated on BG + $MgSO_4$ + DAP plates. After an overnight incubation (approximately 15 hours), the plates were swabbed, the material was washed to remove residual DAP and resuspended in 100 µL SSM. Bacteria were plated on BG + $MgSO_4$ + AMP. Colonies were isolated and confirmed to lack the AC domain via western blot and PCR.

ACT and ACT Mutant Protein Purification: As previously described, calmodulin columns were used to isolate holotoxin ACT from whole-cell urea extracts of XL1-Blue *E. coli* transformed with plasmid *pT7cACT1*. Similar methods were used to purify the ACT deletion mutants expressed from the previously described plasmid constructs with similar backbone, whole-cell urea extracts of XL1-Blue *E. coli* transformed with the following plasmids and urea-extracted material was used in assays described below; $ACT_{\Delta AC}$ (pCACT Δ-373), $ACT_{\Delta HR}$ (pCACT Δ385-1489), $ACT_{\Delta H}$ (pCACT Δ385-1006), $ACT_{\Delta R}$ (pCACT ΔC217), and iACT (pGW44/188) (273). The AC domain was purified from the *E. coli* BL21 (λDE3) strain expressing the AC-int-CBD fusion protein (272). Briefly, bacteria were grown at 30°C in MDO medium supplemented with 150 mg mL^{-1} of

ampicillin. Cultures were induced with IPTG (1 mM), the cells were washed and resuspended in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl (TN buffer) containing 1 mM EDTA (EDTA buffer), and disrupted by sonication. For protein purification by chitin affinity chromatography, the cell extract was cleared at 20,000 × g and loaded on a chitin bead column. After washing, EDTA buffer containing 50 mM dithiothreitol was loaded on the column to promote self-excision of the intein-CBD from the AC-intein-CBD fusion protein during overnight incubation at 4°C. The AC domain was then eluted with EDTA buffer and dithiothreitol was removed by dialysis against TN buffer. Purified proteins were stored at -70ºC until use.

FHA and FHA44 purification: FHA was produced using *B. pertussis* Tohama I, and *B. pertussis* Fha44-stop was used to produce the ~80 kDa N-terminal fragment of FHA that comprises residues 72 to 862 of FhaB (the fhaB allele having a stop codon inserted at position 863 was constructed according to Renaud-Mongenie). Bacteria were grown in 1 L shaking liquid cultures in SSM supplemented with 1 g L^{-1} of (2,6-O-dimethyl)-β-cyclodextrin (Sigma). At OD600nm ≥ 4.0, culture supernatants were collected by centrifugation at 10,000 x g for 20 minutes at 4°C and were sterilized by passage through filters with 0.22 µm pore diameter before loading onto 5 ml Cellufine sulphate columns (JNC corporation) equilibrated with 10 mM sodium phosphate buffer at pH 7.6 (buffer A). After sample application, both matrices containing adsorbed FHA or FHA44 were washed with 80 column volumes (cv) of buffer A, after which a second wash of 20 cv was performed with buffer A containing 300 mM NaCl. The purified FHA and FHA44 proteins were eluted with 700 mM NaCl in buffer A and were

stored frozen at – 70°C until use. The entire purification procedure on chromatography column was performed at 4°C.

Microtiter Crystal Violet Assay: *Bordetella* biofilm was measured using the microtiter plate assay, coupled with crystal violet staining, as previously described (292). Briefly, *Bordetella* bacteria were grown in a total volume of 100 µL of Stainer-Scholte medium at 37°C in 96-well polyvinylchloride (PVC), round-bottom, non-tissue-culture treated microtiter plates (NuncBrand). *B. pertussis* biofilm was measured at 96 hours and *B. bronchiseptica* biofilm was measured at 72 hours. Wells were washed at the final time point to remove planktonic bacteria. Bacterial cells that remained attached to the wells were stained with a 0.1% solution of crystal violet (CV) and were incubated at room temperature for 30 min. The washing process was repeated, and the CV stain was solubilized from bacterial cells with 200 µl of 95% ethanol. Biofilm formation was quantitated by measuring OD_{595}.

Scanning Electron Microscopy: Biofilms were grown statically in 24-well non-tissue culture treated polypropylene plates (0.5 mL cultures per well), and a circular microscope cover glass (12CIR.-1.5) was inserted into each well. Bacteria successfully adhered to the coverslip, as initially confirmed by Gram stain, and matrix was present as determined by Calcofluor White staining. Coverslips were placed in 4% paraformaldehyde to fix samples. *B. pertussis* biofilm was observed on the surfaces of the coverslips at 96 hours under various conditions.

Aggregation Assay: Bacterial aggregation was measured using previously described methods for *B. pertussis (*266). Briefly, BP338 was grown in the presence and absence of AC domain for 24 hours at 37°C shaking. At 24 hours, six 1 mL samples were collected. Three were homogenized by vigorous vortexing and three were not homogenized before centrifugation at 650 x g for 2 min. The OD_{600} was then measured from the supernatant, and the aggregation index (AI) was calculated based on the following equation: $(OD_{homogenized} - OD_{nonhomogenized})/OD_{homogenized}$. Three independent experiments were performed in triplicate.

Surface Plasmon Resonance: SPR measurements were performed at 25°C using a ProteOn XPR36 Protein Interaction Array System (Bio-Rad, Hercules, CA, USA). FHA and FHA44 were diluted to a final concentration of 1 μg mL^{-1} in PBS containing 0.005 % Tween-20 and immobilized as ligands to a GLC sensor chip (Bio-Rad) at flow rate of 30 μl/min. SPR measurements were carried out in the running buffer containing PBS supplemented with 0.005% Tween-20 at the flow rate of 30 μL min^{-1} for association and dissociation phase of sensograms. The purified AC domain was diluted in the running buffer to the indicated concentrations and injected in parallel (“one-shot kinetics”) over the chip surface. The sensograms were corrected for sensor background by interspot referencing (the sites within the 6 × 6 array which are not exposed to ligand immobilization but are exposed to analyte flow), and double referenced by subtraction of analyte (channels 1–5) using a “blank” injection (channel 6). The data were analyzed globally by fitting both the association and the dissociation phases

simultaneously for five different AC domain concentrations using a 1:1 Langmuir-type binding model. An apparent equilibrium dissociation constant (K_D) was determined as $K_D=k_d/k_a$.

ELISA-based Binding Assay: ELISA-specific MaxiSorp 96-well immunoplates (Thermo Scientific) were coated with 0.5 μg mL^{-1} FHA overnight in 100μL bicarbonate solution. Before beginning the assay, the wells were washed and then blocked in 5% milk 1X PBS 0.05% Tween for 1 hour. Wells were washed and control (no protein), ACT, AC domain or $ACT_{\Delta AC}$ (50 μL) was added for 30 minutes. Anti-MCD antibodies described previously (87) (50 μL of 1:100,000 dilution) were added to wells for 30 minutes after the addition of the first 50 μL solution. Wells were washed and a secondary anti-rabbit-HRP linked antibody was added to wells for 1 hour. Wells were washed again and the detection solution (SureBlue TMB Microwell Peroxidase Substrate) was added for fifteen minutes. HCl 1N was added to stop the detection solution reaction and the absorbance at OD_{450} was read using an uQuant Bio-Tek ELISA reader.

Statistics: Statistical analysis was performed using student's unpaired t test with Welch's correction, assuming Gaussian distribution (parametric test), these tests were performed on data sets to compare conditions within experiments.

FIGURES:

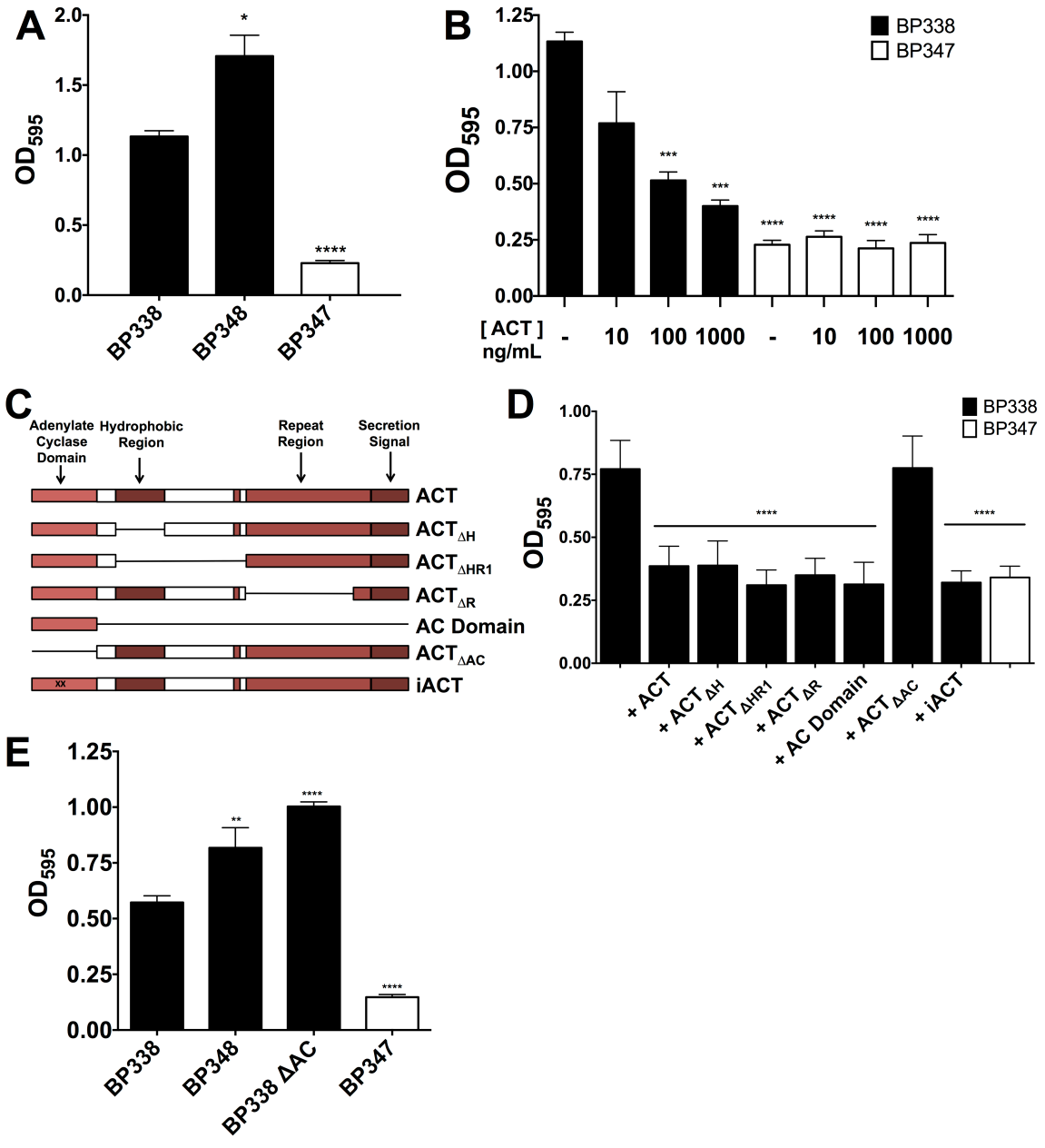

Figure 2.1. The AC Domain of ACT is necessary and sufficient for *B. pertussis* biofilm inhibition. (A) BP348, a *B. pertussis* strain lacking ACT,

makes more biofilm than WT BP338 *B. pertussis*. Strains were grown in 96 well microtiter plates and biofilm formation was assessed using the crystal violet assay at 96 hours. Bvg(-) BP347 serves as a negative control. Data expressed as the mean ± standard deviations, compiled from 3 experiments run in triplicate. * = $p < 0.05$ and **** = $p < 0.0001$ compared to WT BP338. **(B) ACT inhibits biofilm in a concentration-dependent manner.** WT BP338 biofilm formation in the presence of increasing concentrations of recombinant purified ACT (ng mL^{-1}) was assessed at 96 hours. Biofilm formation was measured by crystal violet assay. Bvg(-) BP347 serves as negative control. Data expressed as the mean ± standard deviations, compiled from 5 experiments run in triplicate. *** = $p < 0.001$, **** = $p < 0.0001$ compared to WT BP338 without ACT. **(C)** Schema of ACT truncated and enzymatically inactive mutant proteins (273). **(D) The AC domain is necessary and sufficient for biofilm inhibition, although the catalytic activity of ACT is not required.** ACT, iACT or other ACT mutant proteins were added to WT BP338 and biofilm formation was measured by crystal violet assay at 96 hours. AC domain was added at 10 ng mL^{-1} and additional ACT proteins including ΔH, ΔHR1, ΔR, and ΔAC were all added to a final concentration of 100 ng mL^{-1}. Data expressed as the mean ± standard deviations, compiled from 3 experiments run in triplicate. *** = $p < 0.0005$ compared to WT BP338 without ACT. **(E) BP338 lacking the AC domain (BP338 ΔAC) makes more biofilm than the parental WT strain.** Strains were grown in 96 well microtiter plates and biofilm formation was assessed using the crystal violet assay at 96 hours. Mean values represented by bars, error bars represent standard deviations. Bvg(-)

strain serves as a negative control. Data expressed as the mean ± standard deviations, compiled from 3 experiments run in triplicate. ** = $p < 0.01$ and **** = $p < 0.0001$ compared to WT.

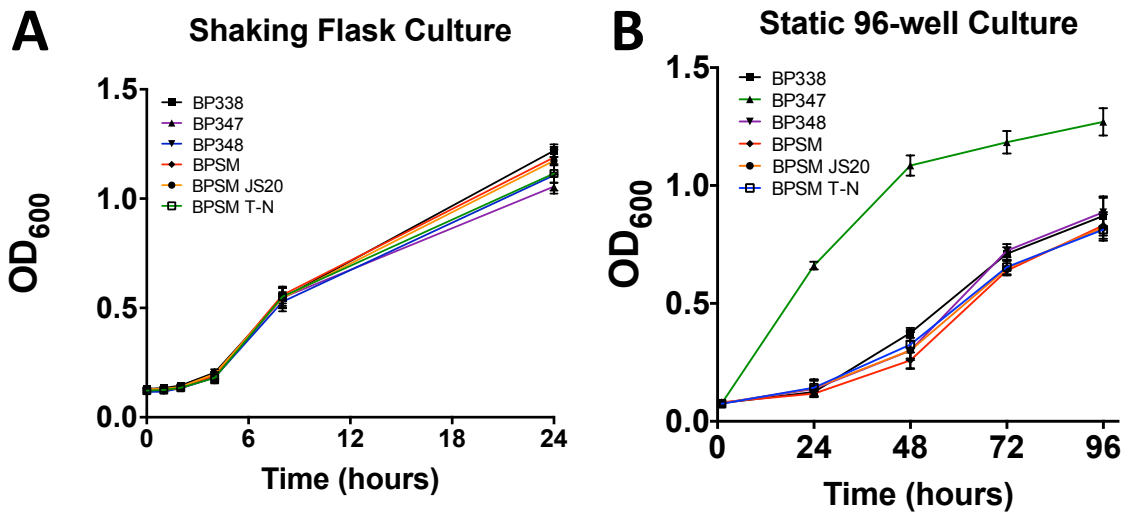

Figure 2.2. Growth curves of bacterial strains used in the study grown in SSM **(A)** shaking 10 mL culture **(B)** static 100µL culture in 96 well plates. OD_{600} measurements recorded over 24 hours for shaking cultures and 96 hours for static 100 µL cultures.

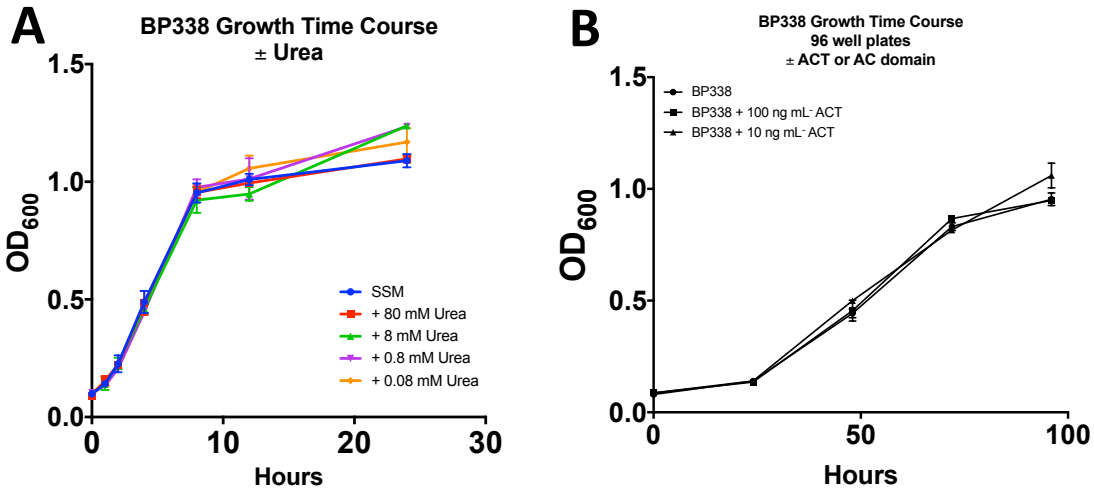

Figure 2.3. Urea has no effect on bacterial growth. (A) *B. pertussis* strains were grown in 5 mL cultures and increasing concentrations of urea were added to ensure urea had no effect on bacterial growth. Data expressed as the mean ± standard deviations, compiled from 3 experiments run in triplicate. **(B)** Static 100µL cultures were grown in the presence and absence of ACT or AC domain in 96 well plates. OD_{600} measurements were recorded every 24 hours.

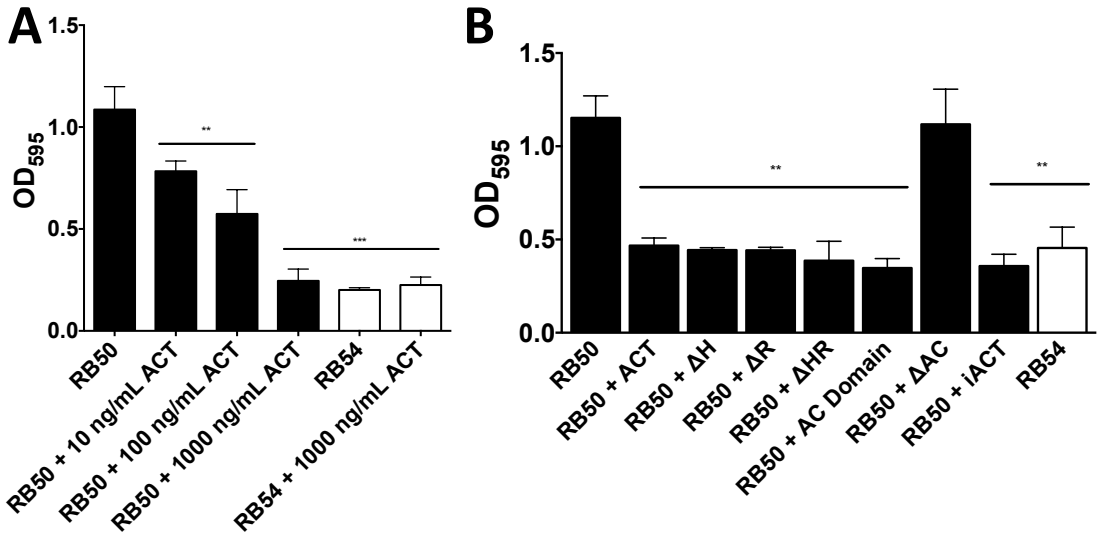

Figure 2.4. The AC domain of ACT is necessary and sufficient for *B. bronchiseptica* biofilm inhibition. (A) ACT inhibits *B. bronchiseptica* biofilm in a concentration-dependent manner. WT RB50 biofilm formation in the presence of increasing concentrations of recombinant purified ACT (10, 100, or 1000 ng mL^{-1}) for 96 hours. Biofilm formation was measured by crystal violet assay. Bvg(-) RB54 strain serves as negative control. Data expressed as the mean ± standard deviations, compiled from 3 experiments run in triplicate. ** = $p < 0.01$ and *** = $p < 0.001$ compared to WT without ACT. **(B) The AC domain is necessary and sufficient for *B. bronchiseptica* biofilm inhibition, although the catalytic activity of ACT is not required.** ACT, iACT or other ACT truncated mutant proteins were added to RB50 and biofilm formation was measured by crystal violet assay at 96 hours. AC domain was added at 10 ng mL^{-1} and additional ACT proteins including ΔH, ΔHR1, ΔR, and ΔAC were all

added to a final concentration of 100 ng mL^{-1}. Bvg(-) RB54 strain serves as negative control. Data expressed as the mean ± standard deviations, compiled from 3 experiments run in triplicate. ** = $p < 0.01$ compared to WT RB50

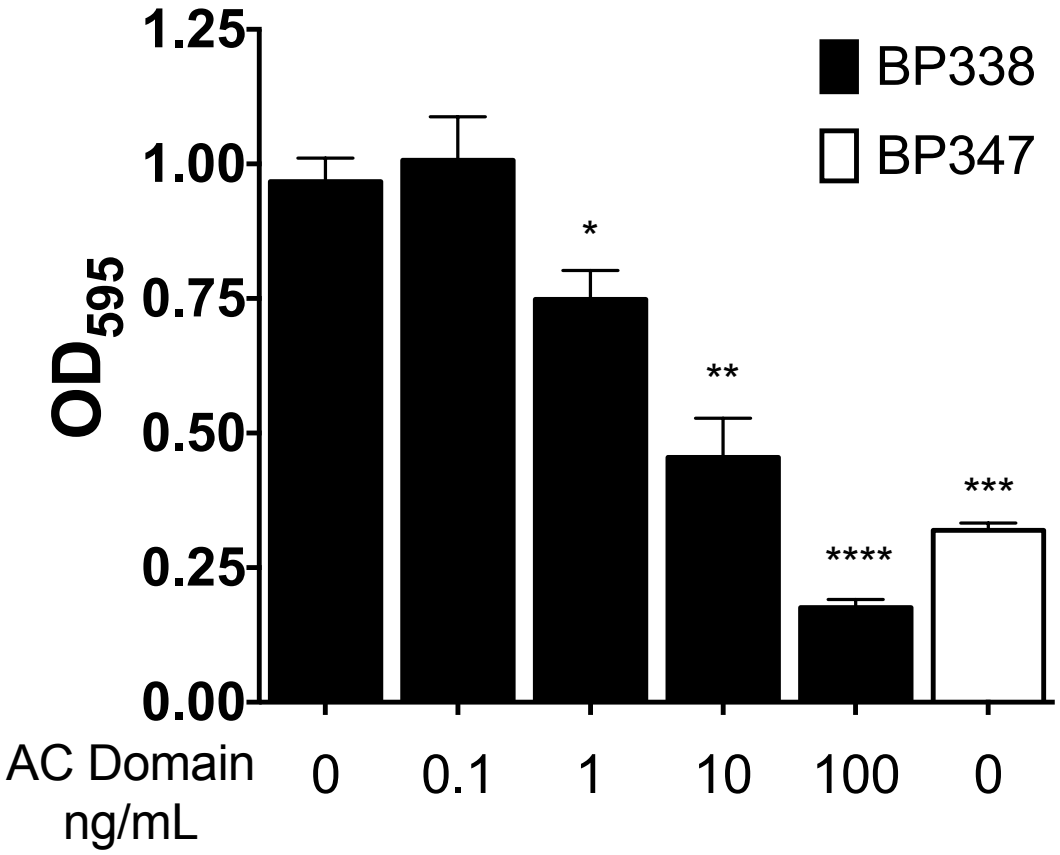

Figure 2.5. AC domain inhibits biofilm in a concentration-dependent manner. BP338 biofilm formation in the presence of increasing concentrations of recombinant purified ACT (10, 100, or 1000 ng mL^{-1}) for 96 hours. Biofilm formation was measured by crystal violet assay. Bvg(-) strain serves as negative control. Data expressed as the mean ± standard deviations, compiled from 3 experiments run in triplicate. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$ compared to WT without ACT.

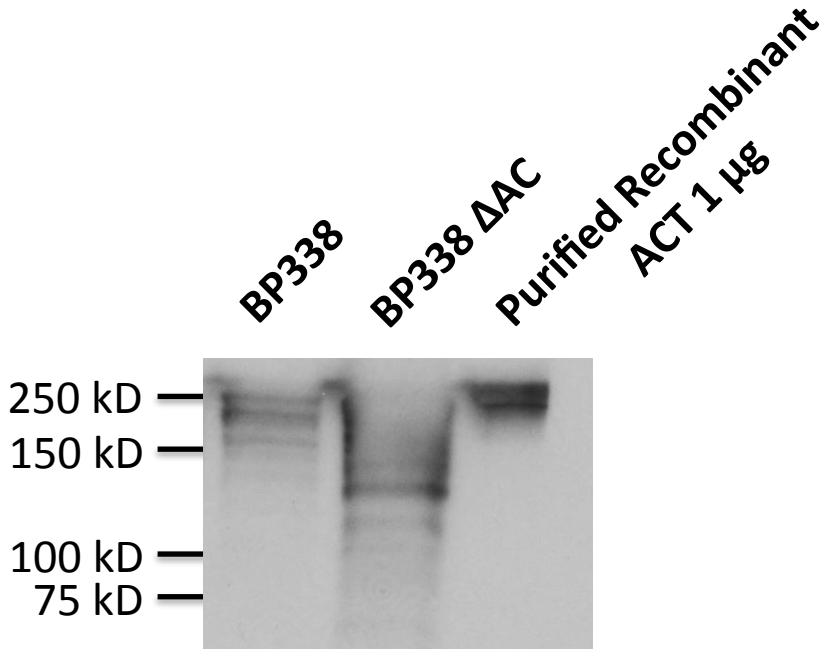

Figure 2.6. Western blot of BP338 ∆AC, confirmation of deletion of AC domain. Bacterial strains were grown 48 hours on BG plates at 37ºC, transferred to 10 mL shaking SSM cultures, and grown for 24 hours. At 24 hours samples were taken and the OD_{600} of each sample was matched. Samples were boiled 5 minutes and 30 µL of the sample was loaded per well. 1 µg of ACT was loaded. A western blot was performed using a polyclonal ACT antibody (273).

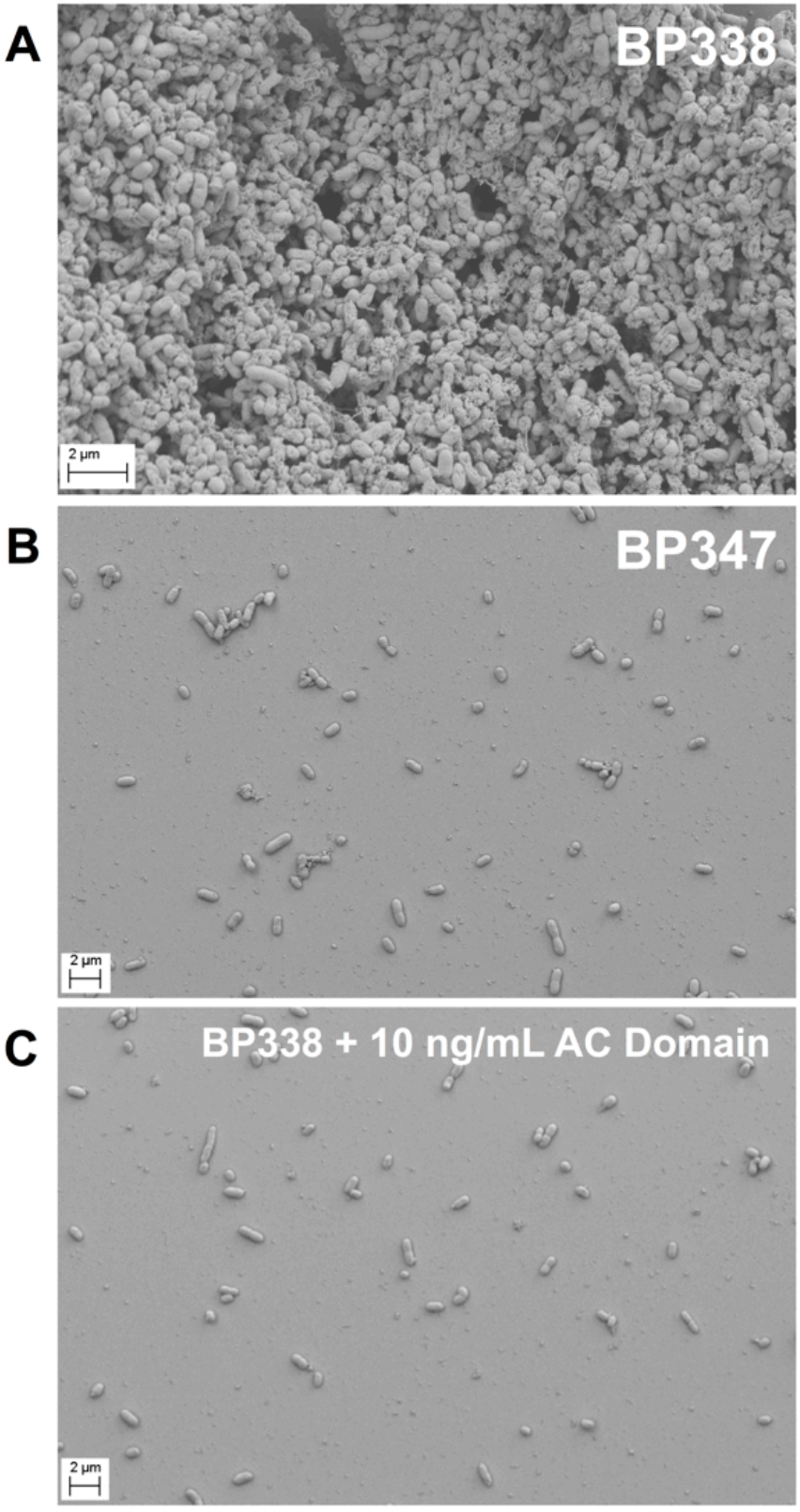
A
BP338
2 µm
B
BP347
2 µm
C
BP338 + 10 ng/mL AC Domain
2 µm

Figure 2.7. SEM images show that AC domain inhibits biofilm formation on glass coverslips. *B. pertussis* was grown in 24 well plates with inverted glass coverslips so that biofilm formation could occur at the air liquid interface. At 96 hours, the coverslips were fixed in 4% paraformaldehyde and prepared for SEM imaging using a Zeiss Sigma VP HD field emission Scanning Electron Microscope at the University of Virginia Microscopy Core. Representative images were chosen from four experimental replicates. **(A)** WT BP338 (15000 X). **(B)** Bvg(-) BP347 *B. pertussis* (5000 X). **(C)** WT BP338 + 10 ng mL^{-1} AC domain (5000 X).

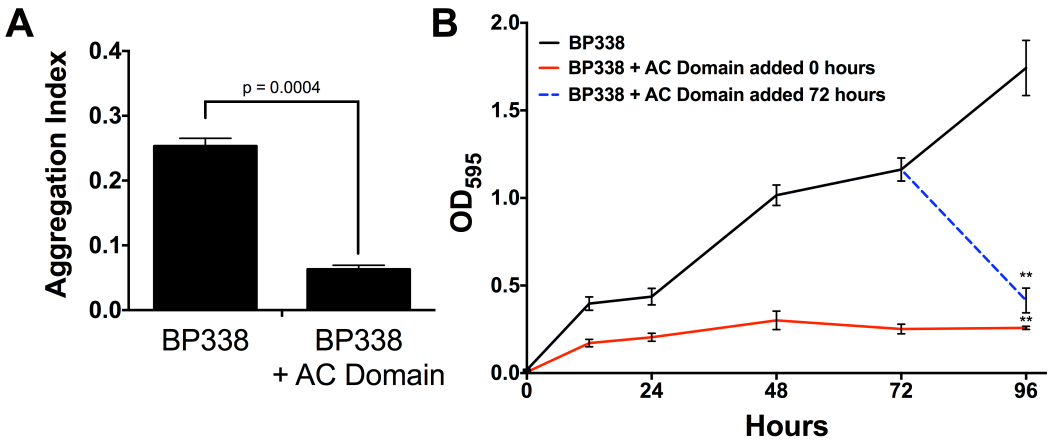

Figure 2.8. (A) Exogenous AC domain inhibits bacterial aggregation. *B. pertussis* strains were grown as 5 mL shaking cultures, in the presence or absence of 100 ng/mL AC domain. At 24 hours, samples were removed from the culture and the Aggregation Index was determined. Mean values are represented by bars, error bars represent standard deviations. Data expressed as the mean ± standard deviations, compiled from 3 experiments run in triplicate. **(B) Exogenous AC domain disrupts preformed biofilm.** BP338 biofilm formation (circles, black line) was measured every 24 hours via the crystal violet assay. AC domain was added at time zero (squares, red line) or was added at 72 hours (open blue circles, dashed line) and biofilm was measured every 24 hours. Mean values represented by lines and error bars represent standard deviations. Data compiled from 5 experiments run in triplicate. ** = $p < 0.001$ compared to BP338.

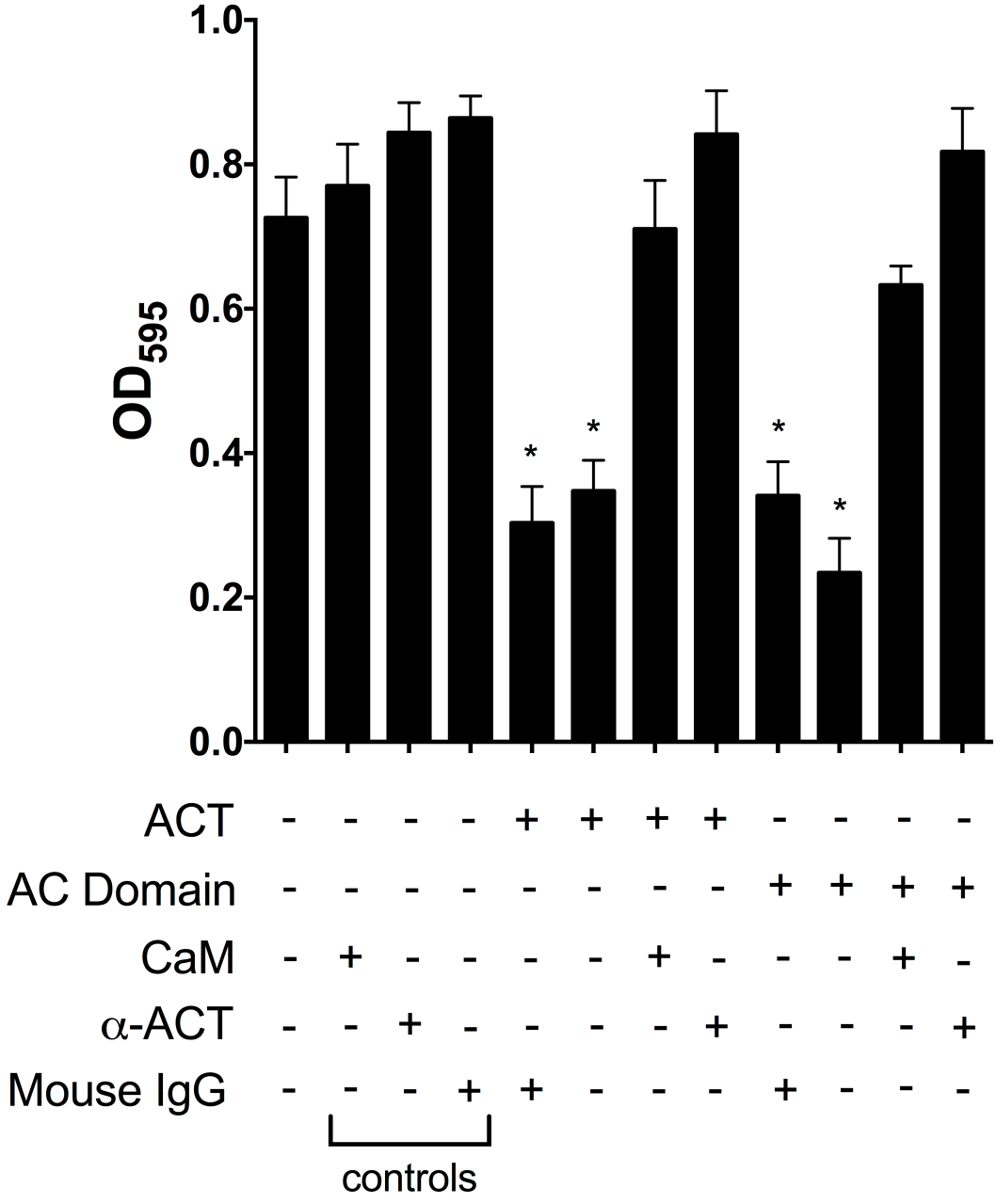

Figure 2.9. CaM and anti-ACT antibodies block ACT inhibitory effects. Calmodulin (1 μM) was incubated with ACT (100 ng mL^{-1}, 565 pM) or AC domain (10 ng mL^{-1}, 0.233 pM) for 15 minutes before adding the combination to BP338 cultures. ACT and AC domain alone were also incubated for 15 minutes prior to

addition to bacterial cultures. Monoclonal antibody 7CE4B1 directed against the AC domain (273), produced by Hewlett Lab, (2.4 μg mL^{-1}) was incubated with ACT or AC domain for 15 minutes before adding the combination to cultures in 96 well microtiter plates. Mixtures as indicated were added to bacterial cultures and biofilm formation was measured at 96 hours by crystal violet assay. Data expressed as the mean ± standard deviations, compiled from 3 experiments run in triplicate. * = $p < 0.05$ compared to WT.

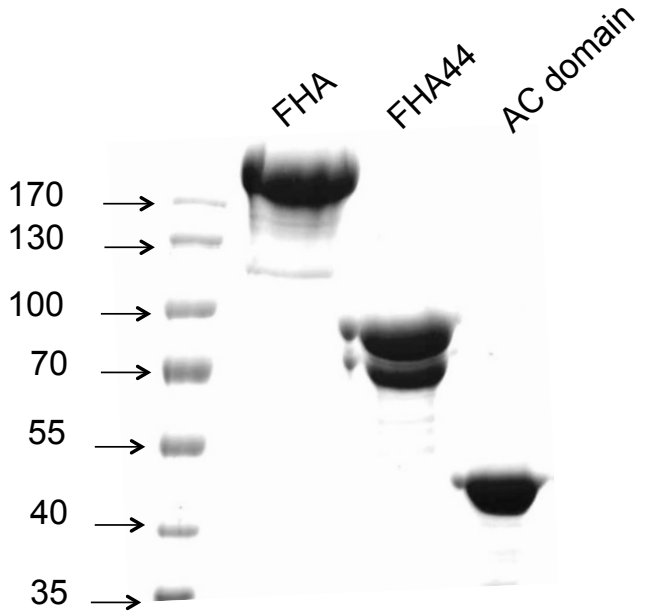

Figure 2.10. Purified proteins used in SPR experiments. 10 µg of each protein was loaded into wells of a 7.5% SDS-PAGE gel. Coomassie staining shows purity of proteins used in SPR experiments.

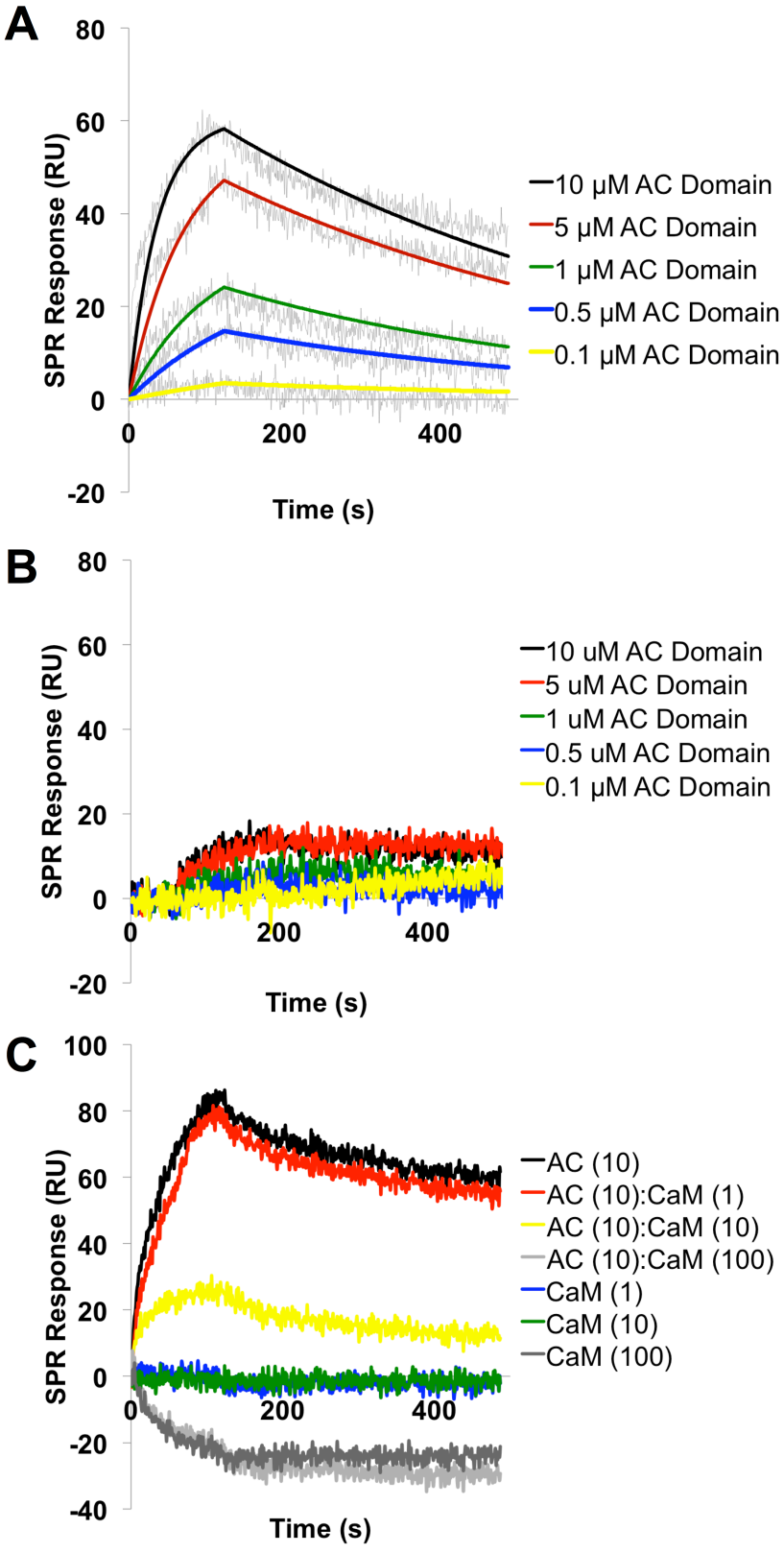
A
80
60
40
20
0
-20
SPR Response (RU)
0
200
400
Time (s)
10 μM AC Domain
5 μM AC Domain
1 μM AC Domain
0.5 μM AC Domain
0.1 μM AC Domain
B
80
60
40
20
0
-20
SPR Response (RU)
0
200
400
Time (s)
10 uM AC Domain
5 uM AC Domain
1 uM AC Domain
0.5 uM AC Domain
0.1 μM AC Domain
C
100
80
60
40
20
0
-20
-40
SPR Response (RU)
0
200
400
Time (s)
AC (10)
AC (10):CaM (1)
AC (10):CaM (10)
AC (10):CaM (100)
CaM (1)
CaM (10)
CaM (100)

Figure 2.11. SPR kinetic binding analysis of the interaction between FHA and the AC domain of ACT. The recombinant AC domain at indicated concentrations was injected in parallel ("one-shot kinetics") over the sensor chip coated with purified **(A) FHA** or **(B) FHA44** proteins at a flow rate of 30 µL/min for both association and dissociation phases of the sensogram. The kinetic data were fitted globally by using a 1:1 Langmuir model (see Materials and Methods) to obtain association [k_a=2.9±0.4 (×10^3) $M^{-1}s^{-1}$] and dissociation [k_d=1.9±0.2 (×10^{-2}) s^{-1}] rate constants of the interaction. The equilibrium dissociation constant, K_D, was determined as k_d/k_a and found to be 650 nM between AC domain and FHA. No binding was observed between AC domain and FHA44. The fitted curves are superimposed as colored lines on top of the binding curves. Representative data from a single experiment shown here. **(C) CaM blocks the AC domain – FHA interaction.** The AC domain (10 µM) and the freshly-prepared complexes of the AC domain with CaM mixed in molar ratios of 10:1, 1:1 and 1:10 AC domain:CaM were injected in parallel over the SPR sensor chip coated with FHA at flow rate of 30 µL/min. Inhibition of binding of the AC/CaM 1:10 complex to FHA is represented by a decrease of SPR signal response. No binding of CaM alone was observed to FHA. Results are representative data from three independent experiments.

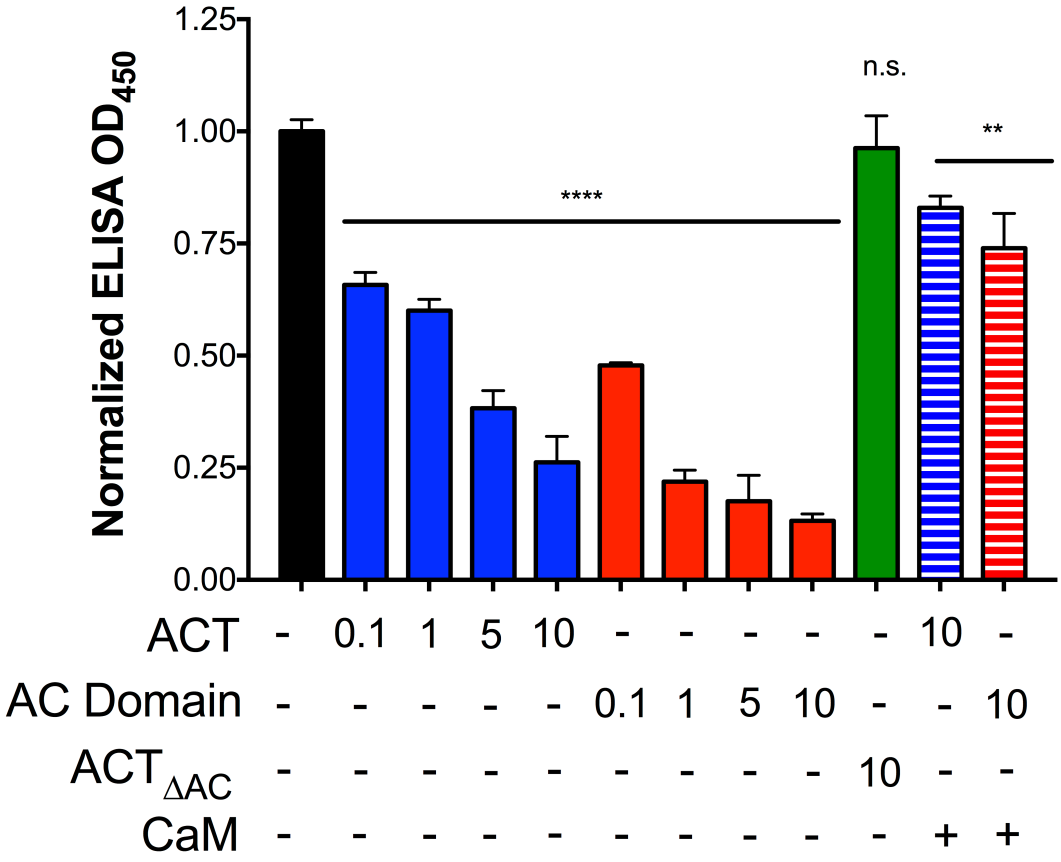

Figure 2.12. The AC domain and ACT inhibit MCD antibody binding to FHA. In an ELISA binding assay, plates were coated with FHA and an anti-MCD antibody was used as a detection reagent. ACT, AC domain and $ACT_{\Delta AC}$, added at a range of μg mL^{-1} concentrations, were added prior to the addition of the anti-MCD antibody to determine if AC domain binding interfered with MCD antibody detection. In addition to these conditions, 10 μM CaM was incubated with the various concentrations of ACT or AC domain for 15 minutes prior to addition of ACT to the wells. All values were normalized to the control (OD_{450} 0.284) using GraphPad Prism6 software. Data expressed as the mean ± standard deviations,

compiled from 3 experiments run in triplicate ** = p <0.01 and **** = p<0.0001 compared to control.

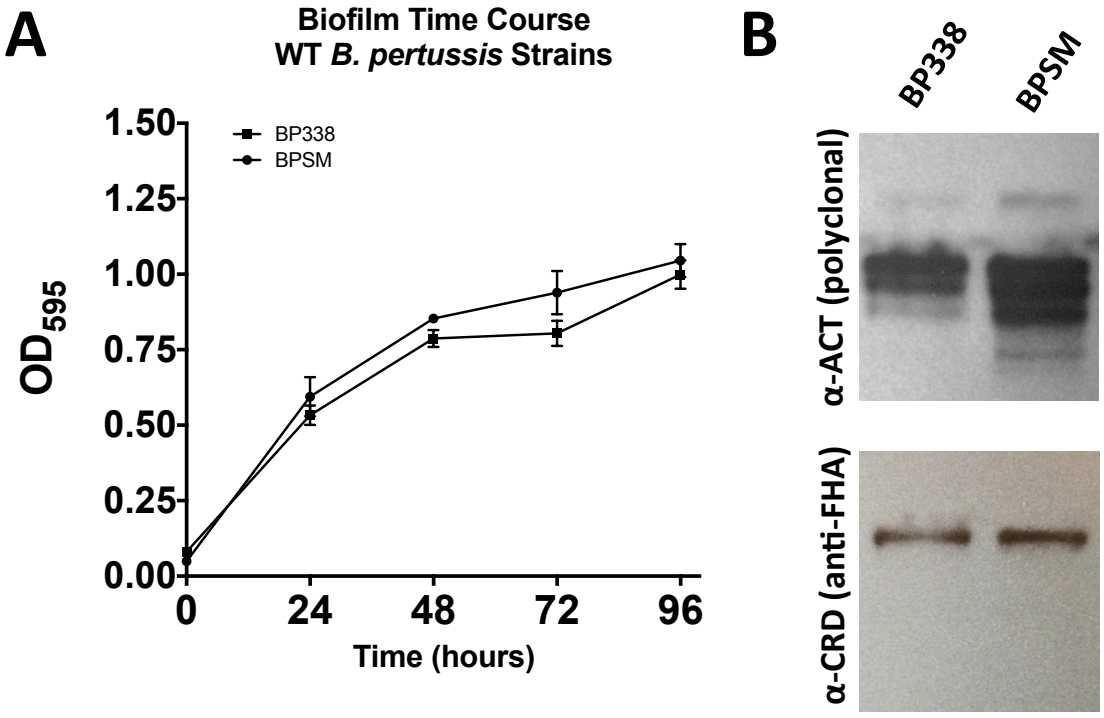

Figure 2.13. Comparison of parental *B. pertussis* WT strains used in this study (A) Biofilm time course of WT *B. pertussis* strains used in the study, BP338 and BPSM **(B)** ACT and FHA protein expression of BP338 and BPSM at 24 hours in shaking culture. 20 µL of OD_{600} normalized bacteria were run on SDS-PAGE gel and protein expression was determined by western blot analysis; polyclonal anti-ACT antibody was used to detect ACT and monoclonal anti-CRD antibody was used to detect FHA (87).

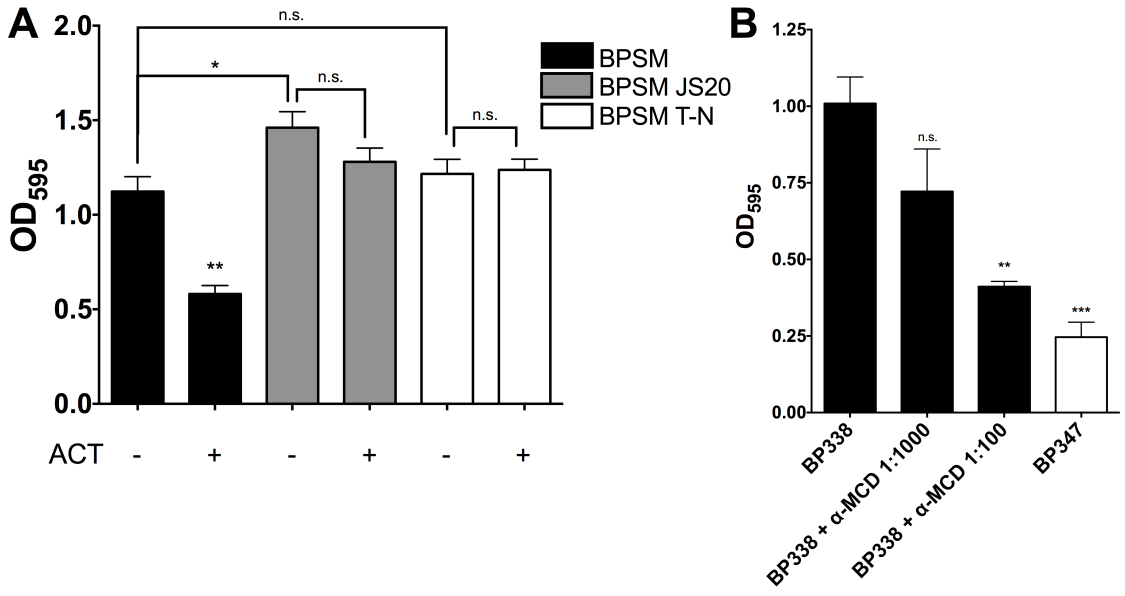

Figure 2.14. (A) The MCD of FHA must be present and properly folded for ACT inhibition of *B. pertussis* biofilm. FHA mutant proteins were generated in the WT *B. pertussis* BPSM parent strain. BPSM JS20 (ΔMCD) has the entire MCD sequence deleted. BPSM T-N has a transposon inserted into the prodomain sequence, precluding prodomain cleavage and processing of the MCD, leaving the MCD unfolded in the final FHA molecule. *B. pertussis* strains were allowed to form biofilm for 96 hours in the presence or absence of 100 ng mL^{-1} ACT. Biofilm was measured by crystal violet assay. Data expressed as the mean ± standard deviations, compiled from 3 experiments run in triplicate. * = $p < 0.05$ and ** = $p < 0.01$ compared to WT (BPSM). **(B) Anti-MCD antibodies block biofilm.** Anti-MCD antibodies were added at 1:1000 and 1:100 dilutions to WT *B. pertussis* (BP338) cultures in 96 well microtiter plates to observe their effects on biofilm formation. Biofilm formation was measured at 96 hours using

the crystal violet assay. Data expressed as the mean ± standard deviations, compiled from 3 experiments run in triplicate ** = $p < 0.01$ compared to WT alone.

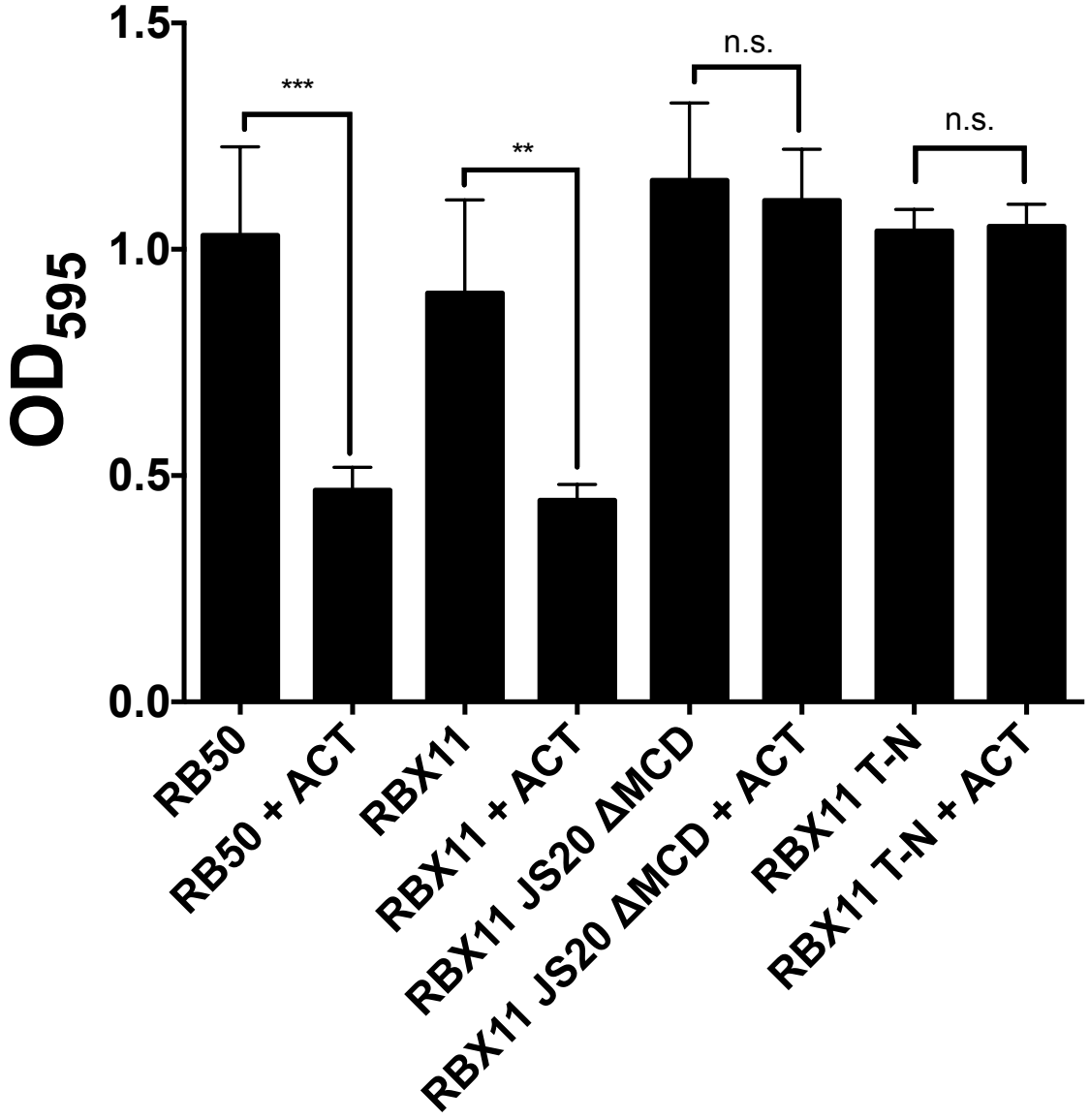

Figure 2.15. The MCD of FHA must be present and properly folded for ACT inhibition of *B. bronchiseptica* biofilm. FHA mutant proteins were generated in the WT *B. bronchiseptica* RBX11 parent strain. JS20 (ΔMCD) has the entire MCD sequence deleted. RBX11 T-N has a transposon inserted into the prodomain sequence, precluding prodomain cleavage and processing of the

MCD, leaving the MCD unfolded in the final FHA molecule. *B. bronchiseptica* strains were allowed to form biofilm for 96 hours in the presence or absence of ACT. Biofilm was measured by crystal violet assay. Data expressed as the mean ± standard deviations, compiled from 3 experiments run in triplicate.** = $p < 0.01$ and *** = $p < 0.001$ compared to WT (RB50).

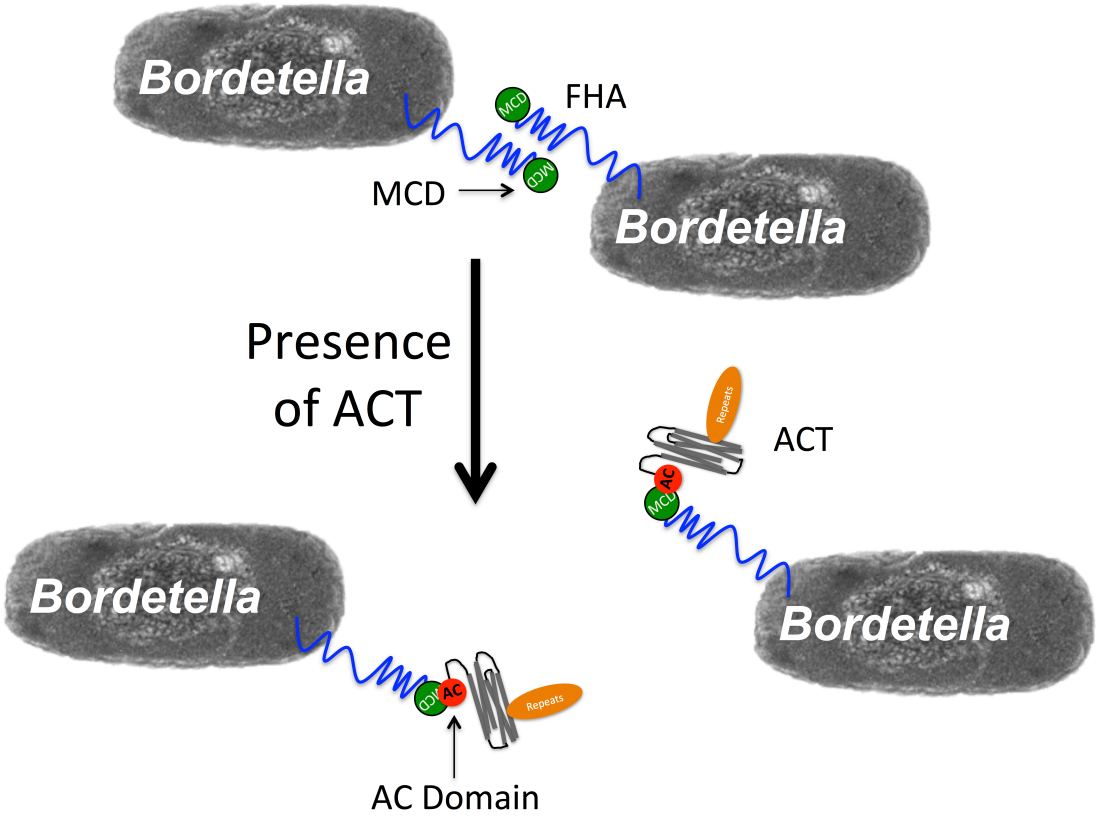

Figure 2.16. Working model of biofilm inhibition by ACT. The AC domain of ACT binds FHA via the MCD at the distal tip of the FHA molecule. This binding blocks FHA function in biofilm, either through FHA-FHA interactions within biofilm or FHA-surface interactions or possibly through some signaling event due to conformation changes in the FHA protein.

Chapter Three:

B. pertussis Adenylate Cyclase Toxin and Derived Peptides as Broad-Spectrum Biofilm Inhibitors

SUMMARY:

Biofilms are communities of surface-associated bacteria, encased in a matrix of polysaccharides, DNA, and proteins. In natural environments, bacteria are frequently encased in biofilm structures. This is in comparison to lab settings, in which bacteria are isolated as individual, free-floating organisms. Biofilms lead to serious medical problems in humans and it is estimated that more than two thirds of all infections of bacterial origin are associated with biofilm. In addition to its role in human infections, biofilm can cause substantial environmental and industrial problems by clogging water pipelines and by disrupting processes in sewage treatment plants, recycling plants, paper-pulping plants, and even in oil pipelines. The removal or prevention of biofilm is a major goal in many fields, and although treatments are available to remove biofilm, including abrasive mechanical disruption coupled with harsh chemical treatments. Broad-spectrum biofilm inhibitors are needed for the prevention of biofilm.

The 177 kD Adenylate Cyclase Toxin (ACT) of *Bordetella* and the derived 40 kD derived catalytic domain (AC domain) have been identified as potent biofilm inhibitors for *B. pertussis* and *B. bronchiseptica*. The inhibitor works by directly binding the distal tip of the surface-displayed biofilm adhesin, Filamentous Hemagglutinin (FHA). Many bacterial species express FHA-like proteins that display high structural or sequence homology to *Bordetella* FHA, and some of these proteins are involved in biofilm formation. The ability of ACT, the AC domain, and peptides derived from the AC domain were tested for their ability to inhibit biofilm of *Bordetellae, Pseudomonas aeruginosa, Escherichia coli,*

and *Salmonella typhimurium in vitro*. The AC domain demonstrated an inhibitory effect on biofilm of most, but not all bacteria strains tested. Using a *Pseudomonas aeruginosa* transposon mutant that lacks the FHA-like protein, CdrA, we have demonstrated that the AC domain likely inhibits biofilm of other bacteria via a similar mechanism. This mechanism would involve the AC domain binding FHA-like proteins to prevent the adhesin's role in biofilm. These data, taken together, show that the AC domain and derived peptides serve as general biofilm inhibitors, specifically for Gram-negative bacteria that encode FHA-like proteins.

INTRODUCTION:

Bacterial biofilms are surface-associated communities of bacteria embedded in a self-produced matrix of polysaccharides, eDNA, and proteins. These communities are the most widely distributed and successful modes of life on earth, found in humans, plants, animals, and on surfaces in the environment. While some biofilms drive important bio-geochemical processes, such as organic matter decomposition, nitrogen fixation, nitrification, denitrification, iron reduction, and sulfate reduction, some harmful types of biofilms can be associated with persistent infections in humans, plants, and animals, including infection of medical devices and implants (356). In addition to these biofilms which form on surfaces associated with living organisms, biofilms also occur on man-made structures in the environment, such as water and sewage pipelines, bathroom drains and faucets, water holding tanks, etc (201, 203, 204, 225, 226, 356). These biofilms can lead to complications with the efficiency of processes that involving these structures and materials. One of the main goals of biofilm research is to eradicate these unwanted communities of bacteria, either by disrupting preformed biofilm or preventing biofilm from occurring in the first place (365). Virtually all surfaces, both man-made and naturally occurring, are susceptible to bacterial deposition and subsequent biofilm formation; thus prevention has been difficult. Disruption of biofilm typically consists of abrasive mechanical treatment and the application of harsh chemicals, which kills all living cells (366-371). While this is an option for abiotic sites such as sewage pipelines and sinks, these types of treatments are not suited for use in humans, animals,

or plants. In comparison, treatment for biofilm in humans and animals requires antibiotics, but bacteria in biofilms are frequently resistant to antibiotics due to changes in metabolism and the acquisition of antibiotic resistance genes/plasmids, rendering antibiotic treatment ineffective (365, 368, 372, 373).

A recent study has shown that the 170 kD *Bordetella* adenylate cyclase toxin (ACT) and the derived 40 kD peptide, which comprises the catalytic domain of the toxin (AC domain), are both able to inhibit biofilm formation (299). ACT is a bacterial adenylate cyclase expressed by most *Bordetella* species (*B. pertussis*, *B. bronchiseptica, B. parapertussis, B. hinzii,* and *B. ansorpii).* The holotoxin is a major virulence factor of *Bordetellae,* which kills macrophages, blocks neutrophil function, and helps *B. pertussis* and *B. bronchiseptica* establish infection in hosts (mice and humans) (167, 189, 374). The study which identified ACT as a potent inhibitor of *Bordetellae* biofilm demonstrated that the inhibitory effect on biofilm was mediated by a direct binding event between ACT and the 220 kD surface displayed adhesin, Filamentous Hemagglutinin (FHA) (299). *Bordetella* FHA is involved in a variety of processes, such as binding to epithelial cells in the nares, trachea and lungs, and altering the host immune response, but is also described as one of the major protein components of *Bordetella* biofilm (84, 200). *B. pertussis* and *B. bronchiseptica* lacking FHA do not form biofilm compared to their parental wild type strains *in vitro* or *in vivo* (200, 234). The ACT-FHA interaction occurs between the 40kD catalytic domain of ACT (AA 1-398) (AC domain) and the distal tip of FHA, the mature C-terminal domain (MCD AA 1870-2362). The AC domain is sufficient for FHA-binding, and necessary and sufficient

for biofilm inhibition. The c-terminal portion of FHA is required for AC domain-FHA binding, and the MCD must be present and properly folded for biofilm inhibition (299). We hypothesize that the ACT-FHA interaction results in some hindrance of FHA for biofilm formation, either through a conformational change of FHA or through spatial hindrance of FHA, as opposed to sending a signal via the binding event. In addition to inhibitory properties of the AC domain, the peptide is able to disrupt preformed *B. pertussis* biofilm (299). The full-length holotoxin lacks this biofilm disruptive activity, which may be due to the size difference between ACT and AC domain and inability of full-length ACT to access FHA within mature biofilm structures (299).

The MCD is just one of many domains within the FHA protein. Several of the proteins domains confer binding to substrates, such as epithelial cells, macrophages, leukocytes, and monocytes (84, 94). These domains and the overall structure of FHA are highly conserved amongst *Bordetellae* (*B. ansorpii* do not express FHA) and bacterial species outside of the *Bordetella* genus (15). Many of these FHA-like proteins act as adhesins, similar to FHA of *B. pertussis*, and some have been implicated in biofilm and aggregative growth, a precursor to biofilm (300, 357-364).

In other bacterial species, specifically *Pseudomonas fluorescens* and *Pseudomonas aeruginosa*, the FHA-like protein, CdrA, is a major component of biofilm matrices (375, 376). CdrA directly binds the polysaccharide component of *Pseudomonas* matrix, Psl, to reinforce biofilm structure. Strains that lack CdrA form less complex biofilm structures than the parental wild type strains. Biofilm of

the Δ*cdrA* strain accumulates as a thin field, as opposed to growing into complex three-dimensional structures (375, 376). The CdrA deficient mutant accumulates biofilm at low levels, likely because *Pseudomonads* have other mechanisms to partially compensate for this defect.

P. aeruginosa CdrA shows high structural similarity to *Bordetella* FHA, although the homology between the two genes encoding the proteins is low. Figure 3.1 shows that both the predicted structures for FHA and CdrA contain β-helical shafts, a globular c-terminal domain located at the distal tip of the protein, and CRD binding domains. The peptides are both inserted into a specific transporter as hairpin structures, begin folding into β-helical sheets, and via proteolytic processing, the protein reaches its final structure and is displayed on the surface of the bacterial outer membrane. FHA is secreted via the two-partner secretion system (87), while CdrA is an RTX toxin secreted via the Type 1 Secretion System (375).

Both FHA and CdrA can be released into the extracellular milieu, albeit via different mechanisms. CdrA is cleaved by the LapG protease, located in the periplasm, and is dependent on c-di-GMP levels within the cell. The mechanism by which FHA is released into the media is unknown (375), but does not require a proteolytic cleavage event (85, 86). Because of the similarities between FHA and CdrA, we hypothesized that *Pseudomonads*, and possibly other bacteria that express FHA-like proteins, may be susceptible to biofilm inhibition by AC domain.

RESULTS:

ACT and the AC domain Inhibit Biofilm Formation of *Pseudomonas aeruginosa.*

Although there are multiple bacterial species that express FHA-like proteins, *P. aeruginosa* is one of the best-studied biofilm forming bacteria due to its prevalence in cystic fibrosis and wound infections (218, 221, 377, 378). We chose to focus primarily on *P. aeruginosa* biofilm, as multiple publications have shown that the FHA-like protein, CdrA, is involved in biofilm formation, and because of the striking structural homology between *P. aeruginosa* CdrA and *Bordetella* FHA (Figure 3.1).

First, we tested the ability of full-length holotoxin ACT to inhibit biofilm formation of *P. aeruginosa*. Two lab-adapted parental strains were used, PA14 and PA01. Bacteria were grown over night in shaking cultures, and using procedures described for measuring biofilm in the microtiter assay for *P. aeruginosa,* bacteria were diluted to an OD_{600} of 0.05 before inoculating 100 µL Luria Broth (LB) cultures in 96 well plates. Recombinant purified *B. pertussis* ACT was added exogenously to *P. aeruginosa* cultures at concentrations of 0.1, 1, and 10 µg ml^{-1}. Biofilm formation, or bacterial accumulation in wells, was determined at 12 hours by crystal violet assay. A concentration-dependent inhibition of biofilm by ACT was observed in both PA01 and PA14 (Figure 3.2A and A.2B blue bars). We next tested the ability of AC domain to inhibit biofilm, as the AC domain is necessary and sufficient for biofilm inhibition in *Bordetellae*. AC domain was added at 0.1, 1, and 10 µg ml^{-1} to PA01 and PA14 cultures in 96 well

plates and biofilm formation was assessed at 12 hours using the crystal violet assay (Figure 3.2A and 3.2B red bars). The AC domain inhibited biofilm formation of both *P. aeruginosa* strains in a concentration-dependent manner. There were no observed differences in growth between PA14 or PA01 grown in the presence or absence of the highest concentration of ACT or AC domain (10 μg mL^{-1}), as measured by OD_{600}. Therefore ACT and the AC domain do not inhibit biofilm by altering growth rates under these conditions (Figure 3.2C).

In order to test our major hypothesis, that ACT and AC domain inhibit biofilm formation by bacteria that express FHA-like proteins via a conserved mechanism, we obtained a transposon mutant from the PA14 transposon library, PA14 32575 (tn::*cdrA*, transposon insertion at 249 codons from start) which lacks CdrA, the *Pseudomonas* FHA-like protein (379-382). Biofilm formation of PA14 and the CdrA transposon mutant was determined over a 12-hour time course in the presence and absence of 10 μg mL^{-1} AC domain (Figure 3.3). PA14 32575 (blue solid line) made less biofilm than the parental wild type strain PA14 (black solid line), which is consistent with previous observation that a Δ*cdrA* mutant made less biofilm than the parental wild type strain. The AC domain inhibited PA14 biofilm throughout the experiment (black dotted line), but did not significantly decrease biofilm formation of PA14 32575 (blue dotted line) at anytime point during the experiment (Figure 3.3). These data suggest that the AC domain inhibits *P. aeruginosa* biofilm by targeting (and possibly binding) the FHA-like protein, CdrA. Future studies will elucidate if AC domain binds CdrA. If the two proteins bind, the binding affinity of the two proteins will be determined. *P.*

aeruginosa mutants will be created to determine if the c-terminal globular distal tip of the protein is required to be present for biofilm inhibition by AC domain.

In that the AC domain was able to disrupt preformed *Bordetella* biofilm, the AC domain was tested for its ability to disrupt preformed *P. aeruginosa* biofilm. PA14 formed biofilm for 6, 8, and 10 hours before the AC domain was added to cultures. Biofilm formation was measured at 12 hours by the crystal violet assay. No differences in biofilm formation were observed; the AC domain does not disrupt biofilm of *Pseudomonas* (data not shown). The AC domain may be too large to access the correct sites once biofilm formation has already occurred in *P. aeruginosa,* as the matrix material is denser than that of *B. pertussis,* or because once mature biofilm has formed, *P. aeruginosa* has other matrix components and proteins that reinforce the biofilm independent of CdrA.

Anti-MCD Antibodies Inhibit *P. aeruginosa* biofilm.

As demonstrated in *B. pertussis*, anti-FHA antibodies prevent biofilm formation (200) and more specifically, anti-MCD monoclonal antibodies prevent biofilm formation (299). Because the AC domain and anti-MCD antibodies both bind the MCD, the two binding events may prevent biofilm in a similar manner. The anti-MCD antibody was tested for its ability to inhibit *P. aeruginosa* PA14 biofilm, and was added at dilutions of 1:100 and 1:1000 to PA14 cultures in 96 well plates, similar to concentrations used in *B. pertussis* biofilm experiments. Biofilm formation was assessed at 12 hours using the crystal violet assay (Figure 3.4). Anti-MCD antibodies inhibited PA14 biofilm formation in a concentration-

dependent manner, suggesting that a direct binding event to the distal globular tip of CdrA, or some other MCD-like epitope, prevent biofilm formation.

The AC domain Inhibits Biofilm Formation of Other Bacterial Species.

In addition to testing the inhibitory ability of the AC domain on *P. aeruginosa*, the ability of AC domain to decrease or inhibit biofilm was tested on other bacterial species, including *Escherichia coli* and *Salmonella typhimurium*. Species were selected based upon availability and presence of FHA-like proteins found in their genomes. *Escherichia coli* expresses Filamentous Hemagglutinin (hypothetical protein, member of the ShlA/HecA/Fha exoprotein family) that shares sequence and structural homology to *Bordetella* FHA (including the portion of the protein which maps to the MCD of *Bordetella* FHA). *E. coli* also expressed amyloid proteins that are involved in biofilm formation. These amyloid proteins react with congo red dye; FHA also reacts with congo red dye (unpublished data) (383). *Salmonella enterica* serovars express BapA (biofilm associated protein), which shares structural homology to *Bordetella* FHA and is involved in biofilm (359).

Biofilm formation of *E. coli* and *S. typhimurium* was assessed in the presence and absences of 10 μg mL^{-1} AC domain using the crystal violet assay. Bacteria were grown overnight in liquid LB culture and diluted to an OD_{600} of 0.05 in the morning. 100 μL cultures of bacteria were added to wells in 96 well plates and biofilm was measured at 12 hours for both *E. coli* and *S. typhimurium.* Two strains of *E. coli* were tested, *E. coli* MC4100, which has been studied in the

context of curli proteins and biofilm (384-388), and *E. coli* 87-23, which is a shigatoxin negative 0157:H7 *E. coli* strain (389). The AC domain was able to inhibit biofilm of one strain of *E. coli,* 87-23 (Figure 3.5A), but AC domain was ineffective against biofilm formation of the other *E. coli* strain tested, MC4100 (Figure 3.5B). Currently, we are unsure as to why AC domain only inhibited one of the *E. coli* strains tested, but in some bacteria, such as *Salmonella*, curli proteins can compensate for biofilm defects (300). Future studies will determine the presence of FHA-like proteins in both of the *E. coli* strains using polyclonal anti-*B. pertussis* FHA antibodies (and the anti-MCD antibody). The requirement of these predicted FHA-like proteins in biofilm formation and inhibition by AC domain will be determined as well. And finally, the AC domain inhibited biofilm formation of *S. typhimurium* at 10 μg mL^{-1} at 12 hours, as measured by the crystal violet assay (Figure 3.5C).

DISCUSSION:

These data establish that the AC domain can act as a non-specific biofilm inhibitor for bacterial species that express FHA-like proteins. Future studies will assess the ability of the AC domain to inhibit other medically-relevant biofilm forming bacteria that express FHA homologs, such as other *Pseudomonads, Salmonella typhi, Acetinobacter baumanni, Burkholderia mallei, Burkholderia pseudomallei* and *Avibacterium paragallinarum.* Because the AC domain is only able to inhibit *P. aeruginosa* biofilm when the FHA-like protein, CdrA, is expressed, there may be a conserved mechanism of inhibition of biofilm.

The AC domain does not fully inhibit to “zero levels” of biofilm in any species described herein, although proper biofilm negative controls are required to determine the efficacy (EC_{50} values) of the AC domain for each species described. In order to develop the AC domain as a tool for broad-spectrum biofilm inhibition several things must be considered and assessed. The long-term effects of the AC domain on biofilm formation for each of these species, beyond 12 hours, will need to be determined. In order to fully understand the inhibitory properties of the AC domain, different abiotic surfaces (other plastics and metals) and biotic surfaces (plants, human tissue cells, etc.) should be used as substrates in biofilm assays. At this point, only polyvinylchloride (PVC) has been tested. And finally, to better understand the ability of the AC domain to inhibit biofilm mixed bacterial-species biofilm should be assessed for AC domain inhibition. Single species biofilms are not frequently found in settings outside of the laboratory.

If as effective and broadly applicable, the AC domain could be developed as a commercial peptide to inhibit and disrupt biofilm formation. The AC domain itself is a harmless peptide, that will not enter or disrupt cellular processes within living hosts, and because the inhibitory effects of the AC domain do not require enzymatic activity, the peptide could be “detoxified” via a point mutation in order to remove greater that 99.9% of the toxin activity, making it even safer for administration. The AC domain could serve as a safe alternative to harsh chemical and mechanical treatments currently in use to prevent and disrupt biofilm.

EXPERIMENTAL PROCEDURES:

Bacterial Strains and Growth Conditions: *P. aeruginosa* PA01, PA14 and PA14 32575 were grown over night on Luria Broth Agar plates and then transferred to liquid culture in Luria Broth and grown overnight at 37ºC, shaking at 180 RPM. Bacteria were pelleted and washed in LB, and then resuspended to an OD_{600} of 0.05. *Salmonella typhimurium* S1344 (390, 391), *E. coli* MC4100 (387, 388), and *E. coli* 87-23 (389) were grown and passaged in the same manner before biofilm experiments.

ACT and ACT Mutant Protein Purification: As previously described (392, 393), calmodulin columns were used to isolate holotoxin ACT from whole-cell urea extracts of XL1-Blue *E. coli* transformed with plasmid *pT7cACT1 (*273). The AC domain was purified from the *E. coli* BL21 (λDE3) strain expressing the AC-int-CBD fusion protein (272). Briefly, bacteria were grown at 30°C in MDO medium supplemented with 150 mg mL^{-1} of ampicillin. Cultures were induced with IPTG (1 mM), the cells were washed and resuspended in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl (TN buffer) containing 1 mM EDTA (EDTA buffer), and disrupted by sonication. For protein purification by chitin affinity chromatography, the cell extract was cleared at 20,000 × g and loaded on a chitin bead column. After washing, EDTA buffer containing 50 mM dithiothreitol was loaded on the column to promote self-excision of the intein-CBD from the AC-intein-CBD fusion protein during overnight incubation at 4°C. The AC domain was then eluted with EDTA buffer and dithiothreitol was removed by dialysis against TN buffer. Purified proteins were stored at -80ºC until use.

Microtiter Crystal Violet Assay: Biofilm was measured using the microtiter plate assay, coupled with crystal violet staining, as previously described (292). Briefly, bacteria were grown in a total volume of 100 µL of LB medium at 37°C in 96-well polyvinylchloride (PVC), round-bottom, non-tissue-culture treated microtiter plates (NuncBrand). Biofilm was measured at 12 hours, unless otherwise stated. Wells were washed at indicated time points to remove planktonic bacteria. Bacterial cells that remained attached to the wells were stained with a 0.1% solution of crystal violet (CV) and were incubated at room temperature for 30 min. The washing process was repeated, and the CV stain was solubilized from bacterial cells with 200 µl of 95% ethanol. Biofilm formation was quantitated by measuring OD_{595}.

Statistics: Statistical analysis was performed using student's unpaired t test with Welch's correction, assuming Gaussian distribution (parametric test), these tests were performed on data sets to compare conditions within experiments.

FIGURES:

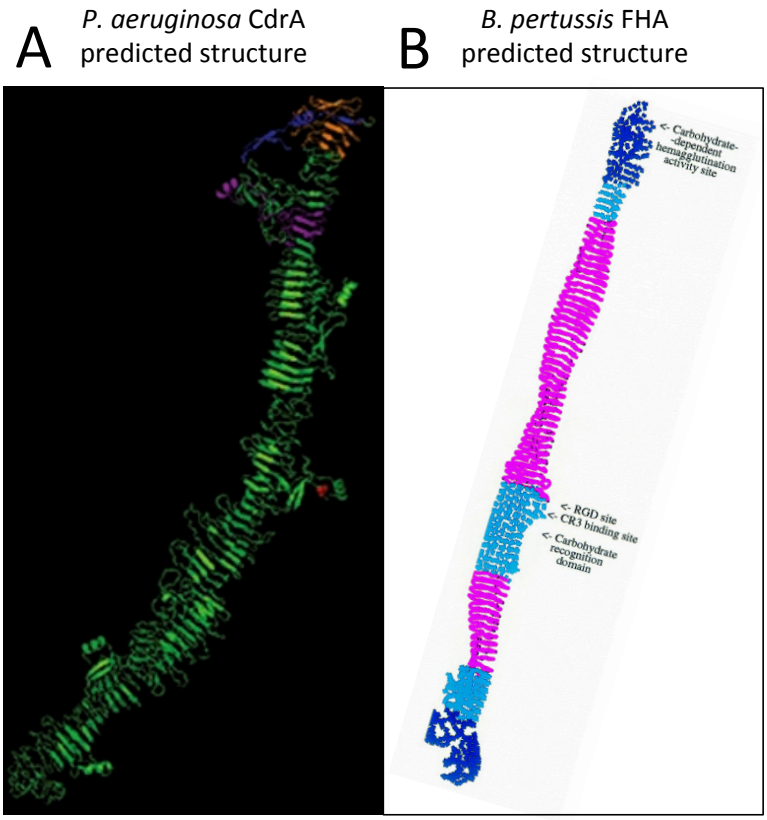

Figure 3.1. The CdrA and FHA protein structures are similar. Predicted structures for **(A)** CdrA of *P. aeruginosa* (courtesy of Wiley Online Publishing, copyright permission of replication, license 4039600160922) (375), and **(B)** FHA of *B. pertussis* (courtesy of Wiley Online Publishing, copyright permission of replication, license 4039600162316) (394). The arrow in A points to the RGD binding motif of CdrA, which is also highlighted for FHA in figure B. The proteins are oriented in N terminal at the top of the figure to C terminal at the bottom of the figure. The globular, unstructured C-terminal domains are at the bottom of the figure.

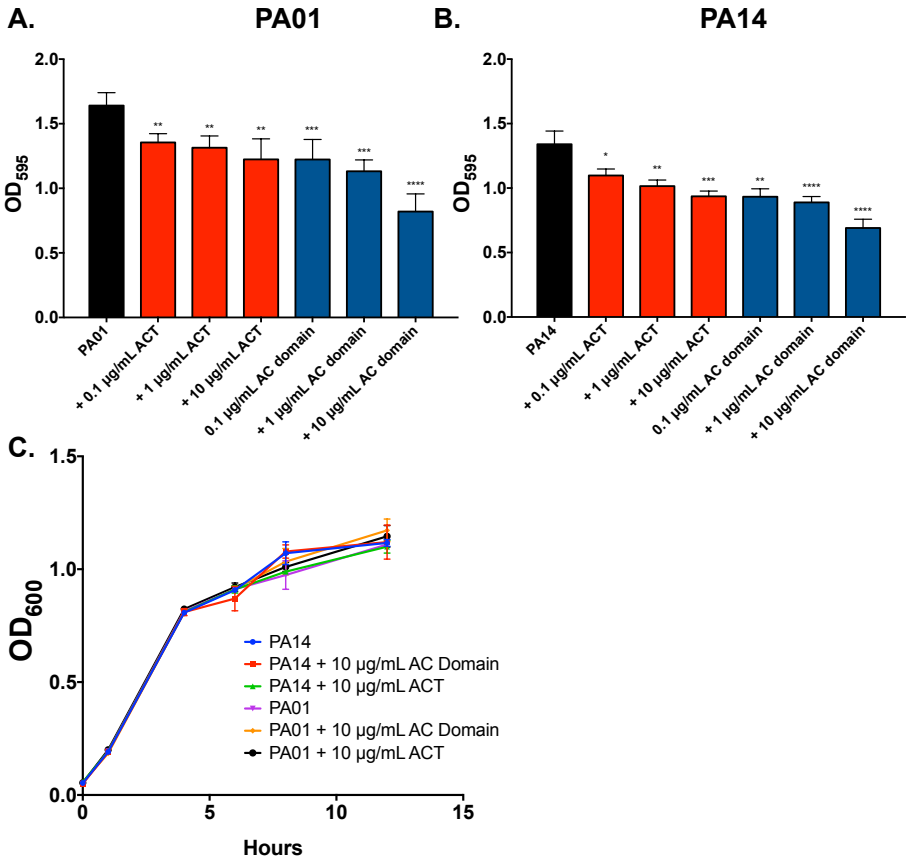

Figure 3.2. ACT and AC Domain inhibit *P. aeruginosa* biofilm in a concentration-dependent manner. (A) WT *P. aeruginosa* PA01 and **(B)** WT *P. aeruginosa* PA14 biofilm formation in the presence of increasing concentrations of recombinant purified ACT (0.1, 1 and 10 µg mL^{-1}, red bars) and AC domain ACT (0.1, 1 and 10 µg mL^{-1}, blue bars) was assessed at 12 hours. Biofilm formation was measured by crystal violet assay. Data expressed as the mean ± standard deviations, compiled from 4 experiments run in triplicate. * = p <0.05, ** = p <0.01, *** = p <0.001, **** = p <0.0001 compared to WT *P. aeruginosa,* (A) PA01 and (B) PA14 without ACT or AC domain added

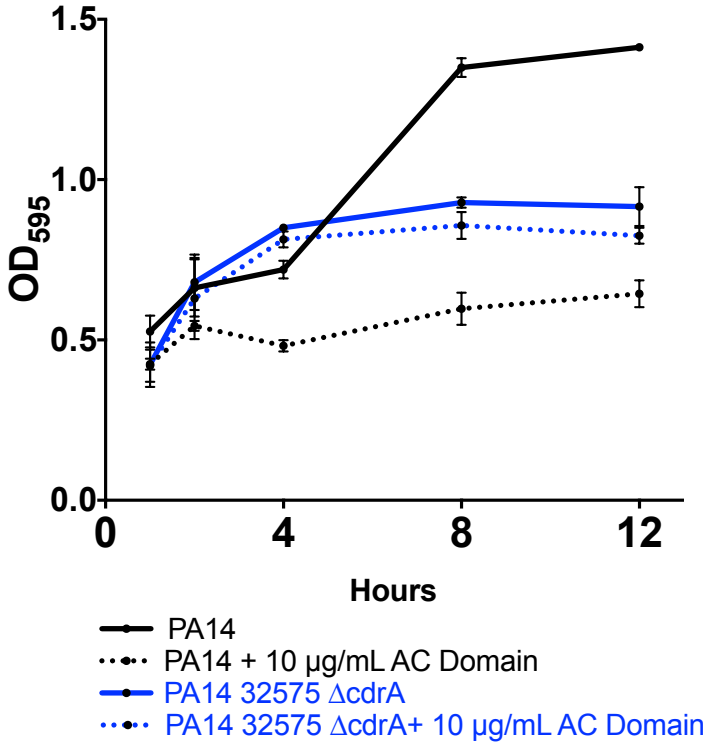

Figure 3.3. Exogenous AC domain inhibits *P. aeruginosa* PA14 biofilm, but not biofilm of the CdrA transposon mutant, PA14 32575 (tn::*cdrA*). Time course of biofilm formation of the WT PA14 (solid black line) and CdrA transposon mutant, PA14 32575 (solid blue line) was measured over a 12 hour time course via the crystal violet assay. Concurrently, 10 µg mL ˉ1 AC domain was added at time zero to PA14 (dotted black line) and the PA14 32575 mutant (dotted blue line) and biofilm was measure at the same time points. Mean values represented by circles and error bars represent standard deviations. Data compiled from 3 experiments run in triplicate. Statistics not included on graph for clarity: At 12 hours, PA14 vs. PA14 + AC domain $p < 0.0001$, PA14 vs. PA14 32575 $p < 0.001$, and PA14 32575 vs. PA14 32575 + AC domain not significant.

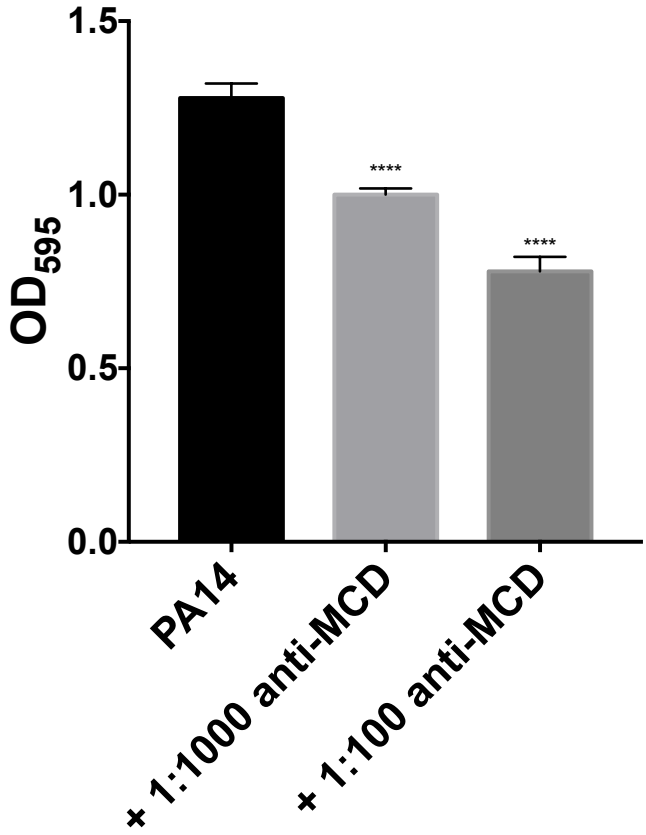

Figure 3.4. Anti-MCD antibodies inhibit *P. aeruginosa* PA14 biofilm. Anti-MCD antibodies were added at 1:1000 and 1:100 dilutions to WT *P. aeruginosa* PA14 cultures in 96 well microtiter plates to observe their effects on biofilm formation. Biofilm formation was measured at 12 hours using the crystal violet assay. Data expressed as the mean ± standard deviations, compiled from 3 experiments run in triplicate **** = $p < 0.0001$ compared to WT PA14 alone.

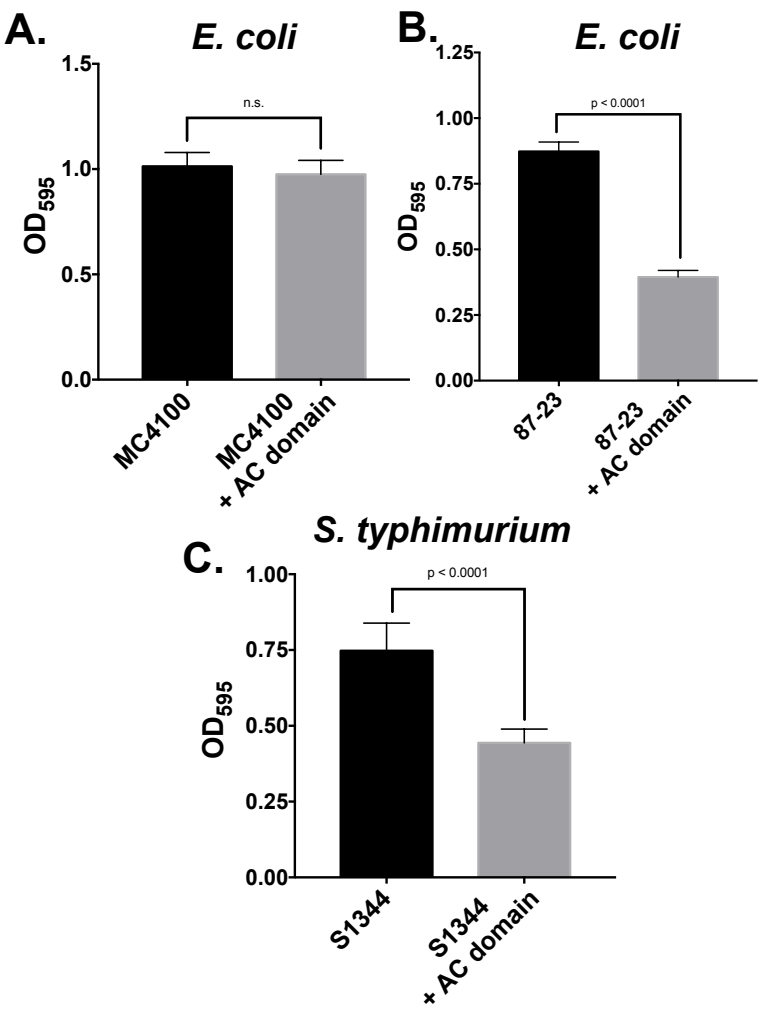

Figure 3.5. AC Domain inhibits *E. coli and S. typhimurium* biofilm (A) WT *E. coli* MC4100, **(B)** WT *E. coli* 87-23 and **(C)** WT *S. typhimurium* S1344 biofilm formation ± 10 μg mL^{-1} (+ AC domain, gray bars) was assessed at 12 hours. Biofilm formation was measured by crystal violet assay. Data expressed as the mean ± standard deviations, compiled from 4 experiments run in triplicate. P values indicated on graphs.

Chapter Four:

Bordetella pertussis are Motile and Express Flagella in the Bvg(-) Phase

Part of this chapter has been adapted from "*Bordetella pertussis* are Motile and Express Flagella in the Bvg(-) Phase"

Casandra L. Hoffman, Laura A. Gonyar, Mary Gray, Federico Sisti, Julieta Fernandez, Ting Wong, F. Heath Damron, and Erik L. Hewlett.

In preparation

SUMMARY:

Bordetella bronchiseptica encodes and expresses a flagellar apparatus required for multiple processes, including swimming motility and mature biofilm formation. The role of flagella during *B. bronchiseptica* infection is unknown, but the primary component of flagella, flagellin, is recognized by the innate immune system, which can signal for the recruitment of innate immune effectors to the site of infection.

In contrast, *Bordetella pertussis*, the causative agent of whooping cough, has historically been described as a non-motile and non-flagellated organism, and to our knowledge, there are no published reports of *B. pertussis* flagella expression or motility being evaluated. The idea that *B. pertussis* is non-motile and non-flagellated appeared consistent with the stop codon found in the flagellar biosynthesis gene, *flhA*, in the *B. pertussis* Tohama I genome. This same stop codon is present in all other *B. pertussis* isolates that have been sequenced since the original report. Interestingly, the *B. pertussis* genome encodes all of the genetic material required for flagellar synthesis and function, and these flagellar genes were differentially regulated in several *B. pertussis* growth conditions, as determined by RNAseq. In light of the RNAseq data and the ability of *B. pertussis* to form mature biofilm, which in *B. bronchiseptica* requires flagella, we tested for motility in *B. pertussis* and found that multiple strains, including laboratory-adapted strains and several clinical isolates, were motile in the soft agar motility assay. Upon isolation of the motile bacteria, we discovered that *B. pertussis* also express flagella, as reflected by negative-stain transmission electron microscopy

and by detection of flagellin protein by western blot with anti-FliC antibody. In addition, we demonstrate that *B. pertussis* motility and flagella expression occurs predominantly in the Bvg(-) phase, the avirulent phase. The Bvg-regulation in *B. pertussis* is consistent with the regulation of motility and flagella expression observed in *B. bronchiseptica*. These findings lay the groundwork for additional studies to assess *B. pertussis* motility and flagella expression. The regulatory mechanisms that control flagella expression and motility, such as c-di-GMP and chemotaxis, can be explored. And importantly, the immune response to the strong inflammatory mediator and the role of flagella and motility in pathogenesis will be assessed.

INTRODUCTION:

B. pertussis was first identified as the causative agent of whooping cough by Jules Bordet in 1900 (295, 296), and by the 1950's whole cell pertussis vaccines were used to combat the life-threatening disease (3, 28, 126, 261). Whole-cell vaccines were subsequently replaced in many countries by acellular pertussis vaccines (APVs) because of concerns about reactogenicity (261). Following adoption of APVs in the 1990's, the number of reported cases of whooping cough has increased steadily, recently reaching levels not seen since the 1950's. The current resurgence of pertussis has revealed that there are major gaps in our knowledge about the biology of *B. pertussis* and the mechanisms by which it causes disease (297, 298). For these reasons, the investigation of these mechanisms and the general biology of *B. pertussis* is required to better understand pathogenesis and develop the next generation of vaccines.

For example, in recent years it has been shown that *B. pertussis* is able to form biofilm *in vitro* and *in vivo (*200, 233, 234, 236-238, 240, 242, 250, 253, 265, 266, 297, 299). Based on these reports, researchers hypothesize that biofilm plays a role in *B. pertussis* pathogenesis in both asymptomatic infections and transmission from host to host (3, 233). These hypotheses are supported by the fact that *B. pertussis* evolved from the closely related organism, *B. bronchiseptica*, for which biofilm contributes to long-term infections in dogs and pigs (30, 31, 35).

The two bacterial species, *B. pertussis* and *B. bronchiseptica*, share many of the same mechanisms in regulation of biofilm as described in Chapter 1. As

noted, one of the major differences in biofilm formation between these two species is the initial step of the bacteria binding to surfaces. Like many other bacterial species, *B. bronchiseptica* rely on a flagellar structure that acts as an adhesin to initiate contact with surfaces before biofilm formation begins (253). Elegant studies have elucidated mechanisms in other bacterial species that show flagella binding signals for changes in biofilm gene expression, specifically for an increase in polysaccharide production and adhesin expression, and a decrease in virulence factor expression (207, 210, 217, 253, 254, 300).

B. pertussis is described as a non-motile, non-flagellated bacterium (1, 20, 256), and despite this, *B. pertussis* forms mature biofilm. There are additional adhesins involved in biofilm formation, but those do not compensate in the initial steps of biofilm formation by *B. bronchiseptica* (253); the flagella are required for initial attachment. How, then, does *B. pertussis* make initial contact for biofilm formation? Interestingly, *B. pertussis* encodes all of the genetic material required to assemble a flagellar apparatus, and based on the *B. pertussis* genome, we predicted the flagellar structure for *B. pertussis* (Figure 4.1A), which was modeled from the *E. coli* flagella structure (Figure 4.1B).

The bacterial flagellum consists of six major parts: a basal body (the MS ring, the P ring, and the L ring), a motor, a switch, a hook, a filament, and an export apparatus (Figure 4.1B) (301, 302). Based on the genomes of flagellated bacteria across multiple taxa, a core set of flagellar proteins was defined. This set consists of 24 proteins that comprise the flagellar structure, motor proteins, regulatory elements, and assembly machinery (301). The core proteins include:

FliC (FlaA in *Bordetella*, filament protein that polymerizes to form the flagellar tail); FlgL and FlgK (hook-filament junction); FlgE (oligermerizes to form the hook); FlgB, FlgC, FlgG and FlgF (rod); FliF (MS ring); FliG, FliM and FliN (C ring); MotA and MotB (motor proteins); and FlhA, FlhB, FliI, FliP, FliR, and FliQ (export apparatus) (301). The genes for all of these core proteins are encoded in the *B. pertussis* genome, although one mutation has occurred.

Parkhill *et al.* sequenced the *B. pertussis* Tohama I genome and discovered that a stop codon mutation was located 1313 codons into the flagellar biosynthesis gene *flhA* (total gene 2119 codons) (5, 18, 257). The stop codon in *flhA* is present in all sequenced *B. pertussis* strains tested in this study, and is is presumed to render the gene non-functional. FlhA is a transmembrane type III secretion protein that serves as the docking site for the Fli(X) ATPases and the FliC filaments (303). FlhA couples FliC to the transport apparatus, which results in export of FliC filaments across the bacterial membrane for assembly of the flagellar tail (303, 304). FlhA is the only core protein missing in *B. pertussis*, but the gene is still present.

The *B. pertussis* genome analysis from Parkhill *et al.,* 2003 supported the concept that *B. pertussis* was non-flagellated (257), but the idea that *B. pertussis* was a non-motile organism began much earlier. A personal correspondence with Jeffrey Miller, a prominent researcher in the *Bordetellae* field, provided insight into some of the original motility experiments (305). Dr. Miller went to the basement at University of California, Berkley, where Bruce Alberts, who studied *Salmonella* motility, maintained his storeroom. Dr. Miller obtained standard

clinical motility agar, comprised of pancreatic digest of gelatin, sodium chloride, beef extract, and agar (306), and used this media to conduct the first experiments to determine if *B. pertussis* and *B. bronchiseptica* were motile organisms. Single colonies of each species were stabbed into motility agar plates, and motility was measured as outward spreading from the point of inoculation. *B. pertussis* did not grow in the agar, and studies concerning *B. pertussis* motility were not continued. It was later discovered that gelatin greatly hinders growth of *B. pertussis*, and because of this lack of growth, for over 25 years, the idea that *B. pertussis* was non-motile persisted. Since the first publication describing motility and flagella expression in *B. bronchiseptica,* there have only been a total of eight published reports about flagella and motility, only some of which explore their relationship to *Bordetella* pathogenesis.

Dr. Miller and his group completed the initial characterizations of motility and regulation of flagella expression in *B. bronchiseptica*. In all publications that investigated *B. bronchiseptica* motility, the clinical motility agar was replaced by the standard *Bordetella in vitro* growth medium. Stainer-Scholte media (SSM), a synthetic media comprised of amino acids and salts (174) was used in combination with 0.4% agar (73). *B. bronchiseptica* is motile in 0.4% agar and these motile bacteria, when isolated from the motility agar, display flagellar structures on their surfaces as determined by negative stain Transmission Electron Microscopy (TEM) (73). The flagellar structures were purified and it was found that the flagellins from *B. bronchiseptica* corresponded to 35kD and 42kD proteins that react with an anti-FliC antibody (monoclonal antibody 15D8 against

Salmonella typhimurium flagellin, which recognizes a conserved epitope of flagellins in the *Enterobacteriaceae* family) as determined by western blot (73). Akerley *et al.* determined there are two flagellin isoforms, and that *B. bronchiseptica* isolates only express one of these two isoforms, never both. Flagellin was also purified from *B. avium* and all isolates tested expressed a 42kD flagellin (73). The amino acid sequence of the *B. bronchiseptica* flagellins were analyzed and the N-terminal sequence of both flagellins were 100% identical, and both showed 80% homology to *Salmonella typhimurium* (73).

Akerley *et al*. also demonstrated that *B. bronchiseptica* motility and flagella expression are controlled by the BvgAS two-component system (72, 73). BvgAS acts as a rheostat to modulate the bacteria between the virulent Bvg(+) phase, intermediate Bvg(i) phase, and the avirulent Bvg(-) phase, based on environmental signals (47, 307, 308). The motile phenotype and flagella expression (gene and protein) occur only in the Bvg(-) phase, during which virulence factor production is decreased and the bacteria are described as more environmentally hardy (73, 79, 309). This is true for both Bvg(-) modulated *B. bronchiseptica* (grown in the presence of $MgSO_4$ or Nicotinic Acid), and for genetically locked Bvg(-) (Δ*bvgA*) *B. bronchiseptica* mutants. Multiple *B. bronchiseptica* and *Bordetella avium* isolates, lab-adapted and clinical, were tested. All strains became motile under Bvg(-) modulated conditions (73). These findings lead to questions about the relevance of motility and flagella expression in the context of infection, if these phenotypes only occur in the Bvg(-) phase.

West *et al.* identified non-motile mutants in a transposon screen; these mutants were both flagellated and non-flagellated (e.g. the flagellated non-motile mutants had defects in flagella function, but were still able to express the flagellar structures on the bacterial surface). All non-motile mutants were defective for HeLa cell invasion in comparison to the parental wild type strains (310). These data suggest that motility, independent of flagella expression, is an important factor in cell invasion. Although intracellular *Bordetella* were initially thought to be an artifact of *in vitro* cell culture conditions, recent reports demonstrate that *B. pertussis* enter alveolar macrophages, and that approximately 25% of the bacteria that enter survive and replicate in non-acidic compartments (312). Once inside these compartments, *B. pertussis* recruit transferrin and other nutrients via the host cell recycling pathway (312). This phenomenon may be an important aspect of pathogenesis, which requires motility for cell invasion, as bacteria have been isolated from macrophages in infected mice and humans (311-313).

In regards to the role of flagella during infection, Akerley *et al*. showed that ectopic expression of flagella in *B. bronchiseptica* resulted in defects in tracheal colonization, but not colonization of the nares or the lungs (314). In fact, *B. bronchiseptica* mutants that ectopically expressed flagella were detected more frequently in the lungs from infected mice (a positive or negative detection read out was used, no bacterial burden data were provided). In contrast, a Bvg(+) *B. bronchiseptica* mutant, which cannot express flagella, has no defect in colonization compared to the wild type, and colonizes the nares better than the *B. bronchiseptica* mutant which ectopically expresses flagella (314). The study

comes to the conclusion that the ectopic expression of flagella alters the overall development of the *Bordetella*-host interaction, but does not determine if flagella play a role during mouse infection (314).

For many bacteria, motility and flagella expression are important virulence phenotypes during infection. Hosts have developed methods to respond to these pathogen-associated molecular patterns (PAMPs), and the monomeric component of flagella is a potent proinflammatory factor (315-317). Flagellin of most bacterial species is recognized by TLR5 (318), however some bacteria have altered flagellin structures that are no longer recognized by TLR5. These altered flagellin structures can instead be recognized by the inflammasome complex (319-325). In either of these two cases, flagellin is recognized by the host innate immune system to induce chemokine, cytokine, and host defense gene expression in epithelial cells. *B. bronchiseptica* flagellin induces production of MIP3α (CCL20), which participates in the recruitment and activation of immature dendritic cells, and IL-8, which is involved in recruitment of neutrophils (326, 327). This was demonstrated in both A549 lung epithelial cells and human bronchial epithelial cells. A Bvg(+) *B. bronchiseptica* mutant and a Δ*flaA* (deletion of the gene that encodes flagellin subunit) *B. bronchiseptica* mutant were both unable to induce secretion of MIP3α and IL-8 in A549 lung epithelial cells. Additionally, *B. bronchiseptica* flagellin signals through TLR5, as demonstrated in HEK293 cells expressing either human or mouse TLR5 (326). The same was observed in RAW 264.7 cells (326).

Despite the demonstrated innate immune response to *B. bronchiseptica* flagellin, the controversial role of flagella in pathogenesis has not been investigated for many years, and because these structures are only expressed in the Bvg(-) phase, they are not thought to contribute to virulence. These ideas may soon change, as recent reports have isolated Bvg(-) phase *B. pertussis*, both mutated genetically and modulated temporarily, from patients infected with *B. pertussis*. These data have began to prompt research on the Bvg(-) phase (29, 74), and raise the question, do Bvg(-) phase *Bordetella* and flagella expression play a role during infection?

Finally, multiple RNAseq and microarray experiments have shown under various conditions, that there is differential regulation of the genes associated with flagella assembly and function in *B. pertussis* (294, unpublished data). One of these RNAseq data sets compared a spontaneous Bvg(-) *B. pertussis* mutant to its parental wild type strain. The genes involved in flagella assembly were more highly expressed in the Bvg(-) mutant (unpublished data). In light of these RNAseq data, the fact that *B. pertussis* forms mature biofilm, and that *B. pertussis* encodes all of the genetic material to assemble functional flagella, we tested for motility in *B. pertussis*. The overall hypothesis for this work is that *B. pertussis* express a functional flagella that allows the bacteria reach its preferred niche within the host, and that the host has evolved mechanisms to detect and respond to the bacterial flagellin to initiate the innate immune response. In the present work, we demonstrate that *B. pertussis* is motile and able to express

flagella, and begin characterizing some of the conserved mechanisms of regulation involved in these processes

RESULTS:

***Bordetella pertussis* is motile in the Bvg(-) phase.**

Preliminary studies from two separate RNAseq datasets demonstrated that there is differential regulation of *B. pertussis* genes associated with flagellar assembly and function. One RNAseq data set compared a *B. pertussis* wild type strain to a *B. pertussis* RpoE mutant. RpoE is a sigma factor, which in other bacteria, responds to oxidative, heat, and other environmental stress (UT25 Δ*rseA* – negative regulator of RpoE). Flagellar gene expression was increased in the RpoE mutant between 1.5 fold and 22 fold (294). In comparison, another RNAseq data set compared a wild type strain and a spontaneous Bvg(-) mutant. As suspected based on findings from *B. bronchiseptica,* the flagella operons were significantly upregulated 1.5 to 400 fold in the Bvg(-) mutant (unpublished data). Based on these observations, we hypothesized that flagella expression occurs and thus that *B. pertussis* could be motile.

We first tested *B. pertussis* motility using the soft agar motility assay described for *B. bronchiseptica* (73). As a control we also tested *B. bronchiseptica* WT RB50, a Bvg(+) RB53, and a Bvg(-) RB54 mutant, which have been previously characterized for motility (73). In these assays, as previously described for *B. bronchiseptica,* WT RB50 is motile, as it can modulates between the Bvg(+) and Bvg(-) phases, Bvg(+) RB53 is non-motile, and Bvg(-) RB54 is motile (Figure 4.2A). *B. bronchiseptica* motility was observed in these assays at 24 hours, much earlier than *B. pertussis* motility was detected due to differences in growth rate (72 hours).

B. pertussis WT BP338 and a Tn5 transposon Bvg(-) mutant (Tn5::*cyaA*) BP347 were grown over night in shaking culture. The over night cultures were diluted to an OD_{600} of 0.800 to match bacterial number between the two strains. Two microliters of these OD_{600}-matched cultures were stabbed into 0.4% *Bordetella* motility agar that had been prepared fresh for the experiment (Stainer-Scholte Media with proline and supplement, 0.4% agar). The *B. pertussis* Bvg(-) mutant BP347 was chosen because of previous studies that show Bvg(-) locked *B. bronchiseptica* are motile and express flagella, and because the Bvg(-) locked *B. pertussis* mutant had higher flagellar gene expression than the WT. In support of our hypothesis, the *B. pertussis* Bvg(-) mutant was motile at 72 hours, as demonstrated in Figure 4.2B. The Bvg(-) mutant BP347 was more motile than the WT strain in multiple experiments, measured by a larger zone of spreading (radius, millimeters) from the initial point of inoculation (quantitated based on multiple experiments in Figure 4.2B). In some experiments we observed that *B. pertussis* WT BP338 is motile, but this does not occur regularly. We postulate that when *B. pertussis* WT strains become motile, it is due to either phase variation to the Bvg(-) phase or a genetic mutation that results in Bvg(-) mutants that dominate and spread. We plan to isolate these bacteria and screen for phase variation (based on colony size and hemolysis on blood agar plates) and sequence for mutations that render the bacteria Bvg(-). Importantly, the bacterial spreading we observed was inside the agar layer, not on top or below. This is indicative of swimming motility (328), which is flagella-dependent.

To confirm Bvg-regulation of *B. pertussis* motility, plates were supplemented with 40 mM $MgSO_4$, which is used to chemically modulate *Bordetella* to the Bvg(-) phase. Agar concentration remained at 0.4% in the plates ± 40 mM $MgSO_4$. *B. pertussis* WT BP338 was grown over night and 2 µL of an OD_{600} 0.800 solution was stabbed into the motility agar plates. After 72 hours at 37ºC, the radius of the motile zone was recorded and representative images from six experiments show that $MgSO_4$ increases *B. pertussis* BP338 motility (Figure 4.3A and 4.3B). Data from these six experiments were combined (Figure 4.3C), and show the radius of the *B. pertussis* BP338 motile zone ± 40 mM $MgSO_4$. Importantly, these observations that *B. pertussis* is motile and that motility increases in the presence of 40 mM $MgSO_4$, were confirmed by collaborators, Federico Sisti and Julieta Fernandez, at the Universidad de La Plata in Argentina, and are not resultant of a contaminant present in our lab. Federico and Julieta study *B. bronchiseptica* motility, specifically c-di-GMP signaling and its control over motility and biofilm.

To determine if this is a generalizable phenomenon, we tested several other lab-adapted and clinical isolates and found that some, but not all were motile in our hands. Table 4.1 describes all strains tested and their motility phenotype (+, motile or −, non-motile) in the presence of 40 mM $MgSO_4$, and Figure 4.4 provides examples of some of these strains demonstrating motility in the assay when modulated to Bvg(-) conditions with 40 mM $MgSO_4$. As noted in Table 4.1, we also tested recent clinical isolates; some, but not all, of these clinical isolates were motile. The lack of motility observed in some strains may be

due to decreased rates of growth, as overnight cultures of clinical isolates frequently did not reach OD_{600}'s of greater than 0.7 compared to our lab-adapted *B. pertussis* WT strains, which typically reach an OD_{600} of > 1.0.

***Bordetella pertussis* express flagella.**

B. bronchiseptica motility is mediated by flagella, as demonstrated by the fact that *B. bronchiseptica* Δ*flaA* and Bvg(+) phase locked mutants, both of which cannot express flagella, are non-motile in the soft agar motility assay (72). Because *B. pertussis* is motile, we tested for the presence of flagella on motile bacteria isolated from the agar plates. Motile bacteria were isolated from the outer edges of the spreading zones in motility agar plates with a polystyrene swab, and resuspended in filter-sterilized deionized water. Samples were prepared for negative stain transmission electron microscopy (TEM) using methods adapted from those used in *B. bronchiseptica* flagella studies (73). Bacteria were fixed, added to Formvar grids, and stained with 2% phophsotungstic acid. Bacteria were imaged using a JEOL 1230 Transmission Electron Microscope. Three independent experiments were conducted for *B. bronchiseptica* RB50, RB54, and RB53, and for *B. pertussis* BP338 and BP347. Two independent experiments were conducted for *B. pertussis* UT25 and clinical isolate V015. ≥ 500 bacteria per sample were imaged. Both flagellated and non-flagellated bacteria were observed for all strains, representative images of flagellated bacteria were chosen (Figure 4.5). Motile *B. bronchiseptica* strains

were flagellated (RB50, Figure 4.5A and RB54, Figure 4.5B) as has been previously described (73).

When comparing *B. pertussis* BP338 WT and the BP347 Bvg(-) mutant isolated from motility agar + 40 mM $MgSO_4$, there were no observable differences in the number or frequency of flagellated bacteria, which we presume is due to the fact that only motile bacteria were isolated and imaged using this method. Not all individual bacteria isolated from the motility agar were flagellated. Representative images of flagellated BP338 and BP347 are shown in Figure 4.6A and Figure 4.6B. Another lab-adapted motile strain, *B. pertussis* UT25, was grown in the presence of 40 mM $MgSO_4$ and examined for expression of flagellar structures. UT25 also displays flagellar structures (Figure 4.6C). And finally, the *B. pertussis* clinical isolate, V015, which was the most reproducibly motile clinical isolate, displayed flagellar structures on its surface when grown in motility agar supplemented with $MgSO_4$ (Figure 4.6D).

We then tested for reactogenicity of *B. pertussis* isolated from motility agar with anti-flagellin antibodies from a variety of organisms. The motility and flagellar studies for *B. bronchiseptica* and *B. avium* used the monoclonal antibody, 15D8, which recognizes a conserved epitope of FliC in *Enterobacteriaceae*, such as *Salmonella* and *Escherichia* (73). *B. bronchiseptica* expressed a 42 kD and 35 kD proteins that were both recognized by monoclonal antibody 15D8. This antibody is now commercially available as the anti-FliC antibody (BioLegend Cat # 629701), which recognizes a conserved epitope of both *Salmonella* and *E. coli* flagellins. Using this anti-FliC antibody, we tested for flagellin protein expression

in *B. pertussis*. Bacteria were isolated from the motility agar for western blot analysis using similar methods described for the TEM imaging; bacteria were swabbed from the outer edges of the spreading zone and resuspended in filter sterilized PBS. The OD_{600} of the solutions were matched, and sample buffer was added before the samples were boiled. Proteins were separated on an SDS-PAGE gel, transferred to nitrocellulose and then the membrane was probed with a variety of anti-flagella antibodies. Figure 4.7A shows that all motile strains react with the monoclonal Fli-C antibody and that non-motile strains do not react (Bvg(+) RB53, BP338 in non-modulated conditions, and non-motile clinical isolates).

We also characterized a panel of antibodies that were raised against flagellins from individual bacterial species; *Salmonella typhi* (Figure 4.7B) (329), *Shigella sonnei* (Figure 4.7C) (330), *Vibrio cholera* (Figure 4.7D) (329), and several monoclonal antibodies for *E. coli* (331). The antibodies raised against *E. coli* flagella were non-specific and did not confirm the presence or absence of flagella in *B. pertussis* or *B. bronchiseptica* (data not shown). The remaining antibodies all recognized an approximately ~35kD band in all motile strains, providing evidence that *B. pertussis* express flagellin protein (Figure 4.7). These data, taken with the negative stain TEM images, confirm that *B. pertussis* express flagella, which are similar not only to *B. bronchiseptica* flagella, but also other *Enterobacteriaceae*.

DISCUSSION:

Despite the statement that, "*B. pertussis* is a non-motile organism", which is widespread in *Bordetella* literature, we demonstrated that *B. pertussis* are motile and express flagella. Several lab-adapted strains and recent clinical isolates are motile, and collaborators confirmed these findings. *B. pertussis* becomes motile in the Bvg(-) phase, either by genetic mutation or by chemical modulation to the Bvg(-) phase with $MgSO_4$. Finally, all motile *B. pertussis* expressed flagella. These data prompt the question, "how has this feature not been observed previously?" After speaking with the group of researchers who characterized *B. bronchiseptica* motility, we now know that the early experiments were conducted with media that inhibited the growth of *B. pertussis*, and because of the lack of *B. pertussis* growth, and therefore lack of motility, it was presumed that *B. pertussis* was a non-motile organism.

In 2003, genome sequencing showed that the *B. pertussis* genome contained a stop codon in one of the core genes required for flagella assembly (257). This reinforced the earlier observations that presumed *B. pertussis* was non-motile. Until now, there were no reports of *B. pertussis* motility or flagella being evaluated, but after repeatedly observing differential regulation of flagellar gene expression in RNAseq data sets, the idea could no longer be accepted without testing for motility and flagella in *B. pertussis*.

The data contained within this chapter are to be taken as an initial observation, which will lay the groundwork for future studies. Flagella will be purified as described for *B. bronchiseptica* and antibodies against the structure

will be produced. This will provide a tool to detect flagella during various forms of bacterial growth, such as conditions in which *B. pertussis* is in biofilm or attached to epithelial cells. In addition, we will determine if the motility observed in the soft agar motility assay is flagella-mediated swimming. *B. pertussis* mutants will be created that are unable to assemble the flagellar filament (Δ*flaA*), and these strains will be tested for motility in the soft agar assay.

The ability of *B. pertussis* to express flagella, despite the presence of a stop codon in the flagellar synthesis gene, *flhA*, raises another important question. How does *B. pertussis* over come the lack of FlhA? Is FlhA protein expressed? In other bacteria, there is evidence of “anti-termination”, or the ability to bypass an apparent termination codon (332). This would allow for *flhA* mRNA and subsequent FlhA protein expression. Transcripts that map to the portion of *flhA* after the stop codon (between codons 1313 and 2119) were detected in both RNAseq datasets. Alternatively, *BP2261* (*bcrD*, putative type III secretion apparatus protein) is encoded in the *B. pertussis* genome; this gene has sequence homology to FlhA of *B. pertussis* (69% homologous) and FlhA of *P. aeruginosa* (68% homologous). It is possible that BcrD substitutes for FlhA in the flagella machinery. In order to test these two hypotheses, Δ*flhA,* Δ*bcrD*, and a double mutant will be created in *B. pertussis,* and motility and flagella expression will be tested in each strain. If anti-termination of *flhA* is occurring, the Δ*flhA* strain will be non-motile if BcrD does not substitute for FlhA. If BcrD is substituting for FlhA, Δ*bcrD* will be non-motile if FlhA is not expressed. To confirm either of these outcomes the double mutant will be assessed as well.

Beyond further characterization of the flagella structures and motility in *B. pertussis*, it will also be important to understand the role that flagella play in host infection with *B. pertussis* and pathogenesis of pertussis. Flagellin of *B. bronchiseptica* is a potent inflammatory mediator, which signals through TLR5 to activate MIP3α (CCL20) and IL-8 production (326). If *B. pertussis* expresses flagella, TLR5 could be activated *in vivo* during infection and would alter the way in which we think about the host immune response to *B. pertussis*.

The role of *B. bronchiseptica* flagella in host infection has not been resolved. While ectopic expression of flagella in *B. bronchiseptica* increased the rate of bacterial clearance from mice lungs and nares (314), experiments that examined a strain lacking flagella did not conclusively determine if flagella were required or not required during infection (314). These studies provide the conclusion that the lack of flagella, or ectopic expression of flagella, alters the development of the host-pathogen interaction. Future studies that address the role of *B. pertussis* flagella will resolve some of these issues.

Finally, because flagella expression is linked to the Bvg(-) phase, it supports the idea that flagella are not important for infection. Experimental mouse studies with both *B. bronchiseptica* and *B. pertussis* have shown that Bvg(-) locked bacteria are defective for colonization and are cleared more rapidly than WT and Bvg(+) locked strains (46, 76, 77, 79, 333, 334). Though this may be the case for initial colonization, a set of recent publications by Karataev *et al*. and Medkova *et al.* have shown that Bvg(-) mutants have been isolated from the upper respiratory tracts of acutely and chronically infected humans and mice (29,

74, 75). The ability of these Bvg(-) mutants to persist and be isolated from the host provides evidence that the Bvg(-) phase occurs during infection. Flagella genes are only expressed in the Bvg(-) phase; the Bvg(-) mutants from patients will be an important tool for understanding flagella expression during infection. The novel observation that *B. pertussis* are motile and express flagellum warrants further investigation and provides the initial foundations for future studies which will explore the role of *B. pertussis* flagella expression and motility, and determine how these phenomena relate to pathogenesis.

EXPERIMENTAL PROCEDURES:

Bacterial Strains and Growth Conditions: *B. pertussis* WT (wild type) BP338 (Tohama I) (191); Bvg(-) BP347 (TN5::*bvgS* mutant derived from BP338) (95, 191); WT BPSM (Tohama I) (86); WT UT25 (294, 335); WT Bpe60 (336); WT BP536 (337); clinical isolate V235 (originated in Hewlett Lab, isolated from either University of Virginia patient); clinical isolates UVA009, UVA 010, UVA015, UVA018, UVA052, UVA062, UVA145, UVA150, UVA190, UVA194, UVA198 (originated from Hewlett Lab, provided by from Virginia State Labs) were grown on Bordet-Gengou (BG) agar (Gibco) supplemented with 15% defibrinated sheep blood (Cocalico) for 48 hours at 37ºC. The same growth media were used for *B. bronchiseptica* strains, WT RB50 (333); Bvg(-) RB54 (333); and Bvg(+) RB53 (333). *B. bronchiseptica* were grown at 37ºC for 24 hours. After growth on BG plates, bacteria were transferred to 10 mL liquid culture in modified synthetic Stainer-Scholte liquid medium (SSM) with proline and supplement, and grown for 20 hours at 37ºC, shaking at 150 RPM.

Motility Assay: The motility assay was modeled after the assay described for *B. bronchiseptica* (73). Motility agar plates were made fresh before each assay from SSM with proline and supplement, and the final concentration of agar was 0.4% (Hoefer, Inc. GR140500). Each motility agar plate had 15 mL of media added, plates were allowed to set for 2 hours before bacteria were stabbed into the agar. This reduced evaporation that could lead to an increase in the agar concentration. *B. pertussis* were grown on BG, and passaged once in SSM for 20 hours. Bacteria were then diluted to an OD_{600} of 0.800 and 2 µL of the suspension was

stabbed into SSM motility agar plates. *B. pertussis* were grown for 72 hours at 37ºC under the various conditions for the motility assay, *B. bronchiseptica* were grown for 24 hours at 37ºC under the various conditions.

Negative Stain TEM: Motile (from outer edges of the spreading zones) *B. pertussis* and *B. bronchiseptica* were isolated from motility agar plates using a Fisherbrand Polyester-Tipped Applicator (Cat No. 23-400-122), and swabbed into 1mL filter sterilized, autoclaved, deionized water. Most of the agar went into solution; any remaining chunks were removed by slow speed 2,000-RPM centrifugation. Bacteria solution was centrifuged at 10,000-RPM for 10 minutes to pellet bacteria, which were resuspended in 2.5% gluteraldehyde and fixed for one hour. Bacteria were added to 200 mesh Formvar, copper Transmission Electron Microscopy grids (Ted Pella, Inc. 01700-F), excess liquid was dabbed away. Grids were stained with 2% phophsotungstic acid (pH 7.0) (*79690 SIGMA-ALDRICH*) and excess liquid was dabbed away. Negative-stained *B. bronchiseptica* and *B. pertussis* were imaged with the JEOL 1230 Transmission Electron Microscope with real-time digital imaging (2Kx2K) and ultra high resolution digital imaging (4Kx4K).

Western blot analysis for flagellin in bacterial cultures: Motile (from outer edges of the spreading zones) *B. pertussis* and *B. bronchiseptica* were isolated from motility agar plates using a Fisherbrand Polyester-Tipped Applicator (Cat No. 23-400-122), and swabbed into 0.5 mL filter sterilized, autoclaved, deionized water. Most of the agar went into solution; any remaining chunks were removed by slow speed 2,000-RPM centrifugation. In each experiment, OD_{600} was

matched to standardize bacterial number. Sample buffer was added and samples were boiled 5 minutes before loading 35 µL to each well of a 12 well, SDS PAGE 10% gel (Criterion TGX,10% 12+2 well, item no. 5671033). Proteins were transferred at 4ºC to nitrocellulose (MILLIPORE Immobilon-FL, cat no. IPFL00010) overnight at 20V. Blots were probed with several flagellin antibodies as described in results and figure legends.

TABLES:

Strain Name	Motile Phenotype + 40 mM $MgSO_4$
WT BP338 (Tohama I)	+
Bvg(-) BP347	+
WT Bpe60 (Tohama I)	+
WT BP536 (Tohama I)	−
WT UT25	+
WT BPSM (Tohama I)	+
WT GMT1	+
V235*	−
UVA009*	−
UVA010*	+
UVA015*	+
UVA018*	−
UVA052*	+
UVA062*	−
UVA145*	−
UVA150*	+
UVA175*	+
UVA190*	+
UVA194*	−
UVA198*	−

Table 4.1. *B. pertussis* Motility in the presence of Bvg(-)-modulating conditions (40 mM $MgSO_4$). Strains from different isogenic backgrounds and clinical isolates were grown as described for the motility agar assay. Motility, measured by outward spreading from point of inoculation in the agar, was determined to be "+" if the strain was consistently (≥80%) motile. If the strain was not consistently motile, the motility phenotype was determined to be "−". Experiments were repeated 5 times, the strains were tested in duplicate each experiment. An asterisk denotes clinical isolates.

FIGURES:

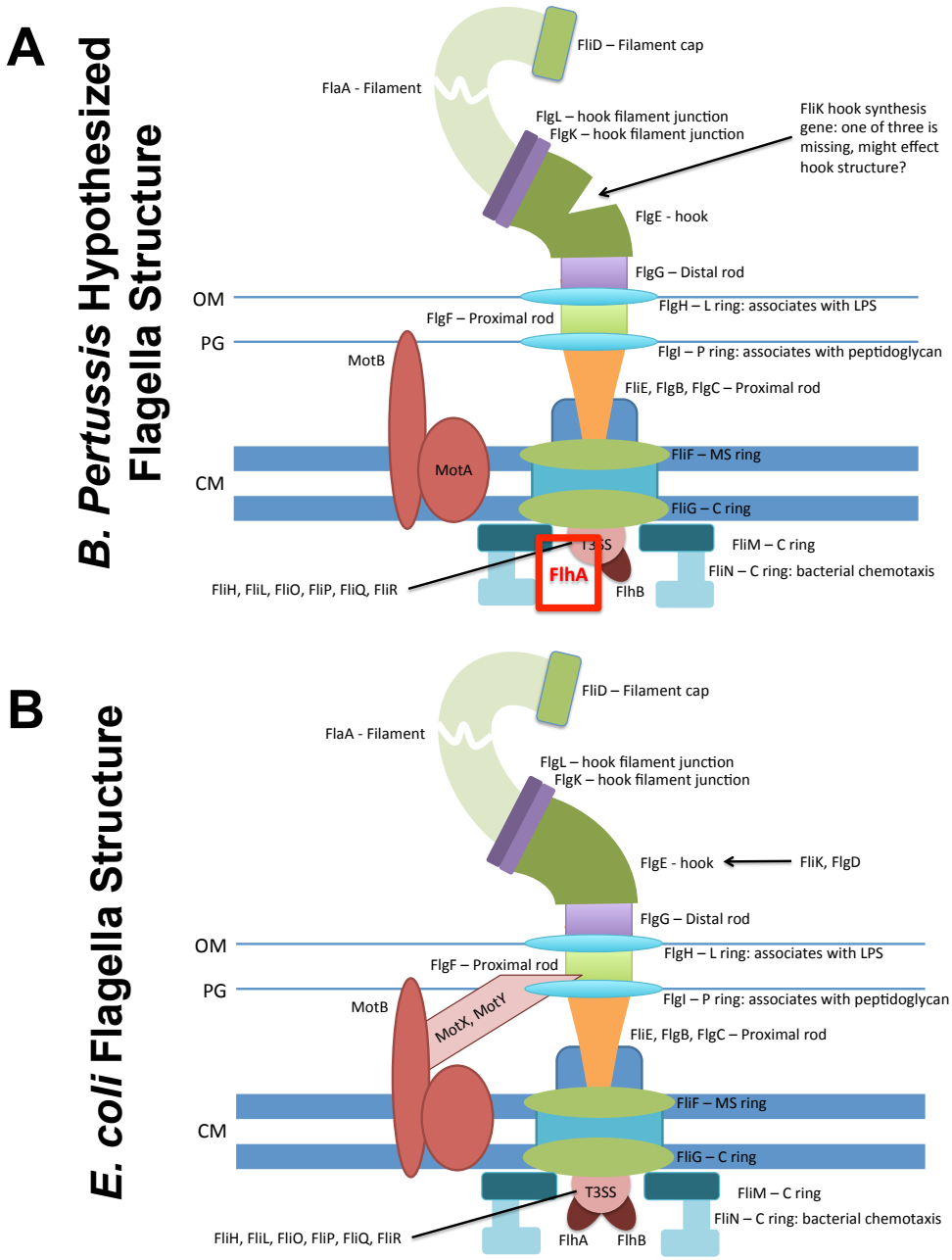

Figure 4.1. Comparison of Flagella Structures for **(A)** the predicted *B. pertussis* Tohama I flagella, based on the genes present in the genome and **(B)** the *E. coli* flagella, based off of the genes and proteins present in the genome.

The *B. pertussis* genome lacks FliK (non-essential) and there is a stop codon in the FlhA biosynthesis gene (required); for these reasons *B. pertussis* has been described as non-flagellated. *E. coli* also has additional Mot proteins (MotX/Y, involved in flagella rotation). Structures predicted by the KEGG PATHWAY modeling software.

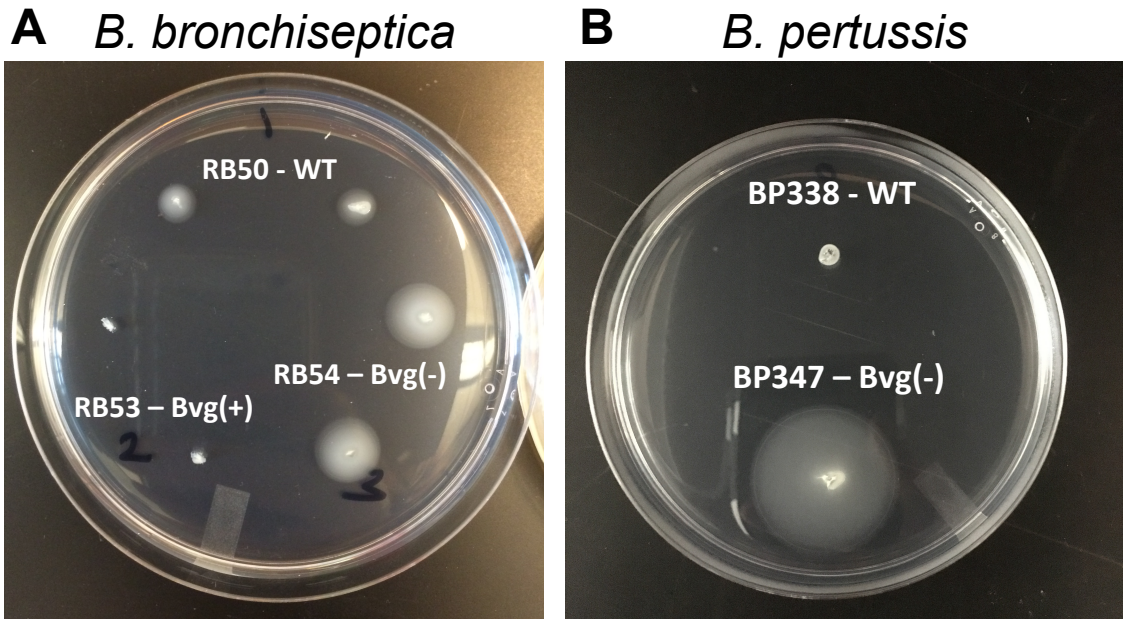

Figure 4.2. *B. bronchiseptica* and *B. pertussis* are motile in the Bvg(-) phase. Bacteria were grown over night as shaking cultures in SSM and diluted to an OD_{600} of 0.800. 2 µL of diluted cultures were stabbed into 0.4% SSM motility agar plates. *B. bronchiseptica* strains were grown for 24 hours at 37ºC at ambient CO_2 levels, *B. pertussis* were grown for 72 hours in the same conditions. **(A)** *B. bronchiseptica* WT RB50, Bvg(-) RB54, and Bvg(+) RB53 were tested for motility. **(B)** *B. pertussis* WT BP338 and Bvg(-) BP347 were tested for motility.

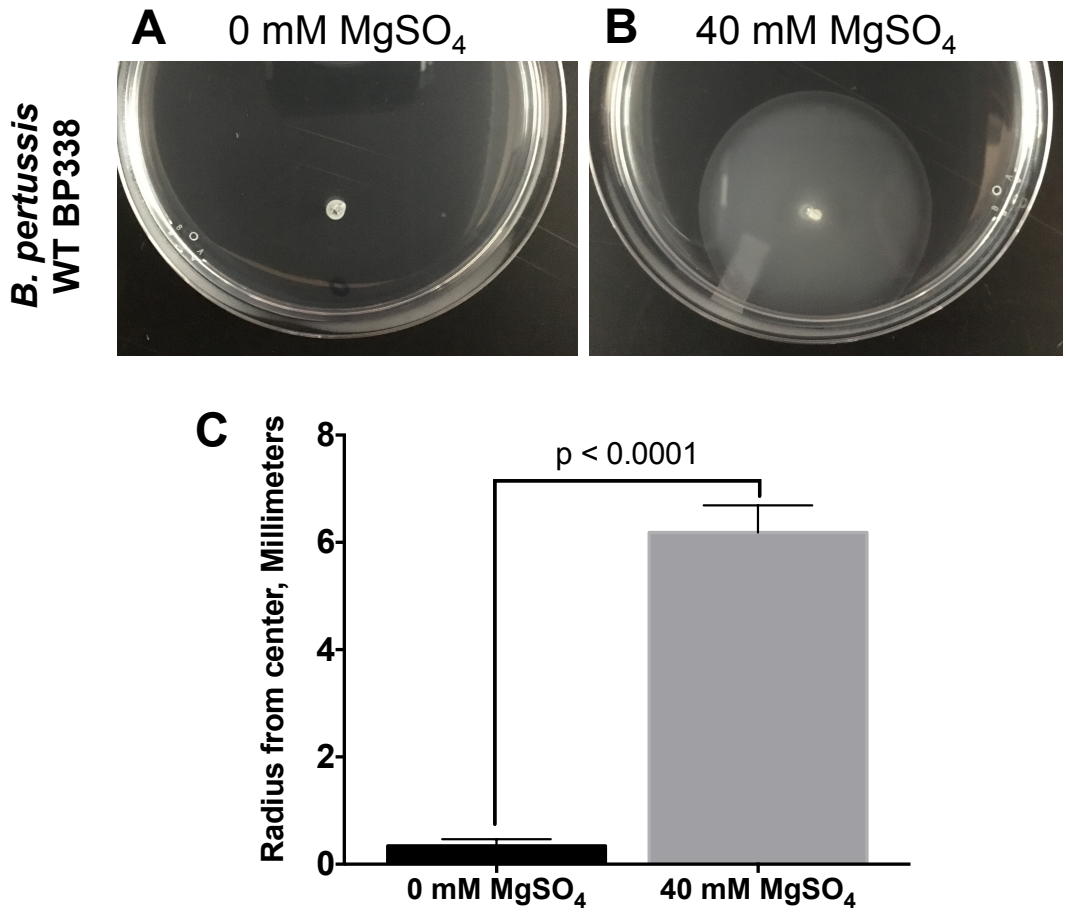

Figure 4.3. *B. pertussis* motility increases when the bacteria are modulated to the Bvg(-) phase with 40 mM $MgSO_4$. *B. pertussis* WT BP338 were grown over night as shaking cultures in SSM and diluted to an OD_{600} of 0.800. 2 µL of diluted cultures were stabbed into 0.4% SSM motility agar plates. *B. pertussis* strains were grown for 72 hours at 37ºC at ambient CO_2 levels. Representative images of BP338 grown **(A)** without and **(B)** with 40 mM $MgSO_4$, which modulates the bacteria to Bvg(-). Experiment was repeated 6 times and the radius was quantitated each time. **(C)** The mean radius with standard deviation was graphed for each condition (± 40 mM $MgSO_4$). p value < 0.0001.

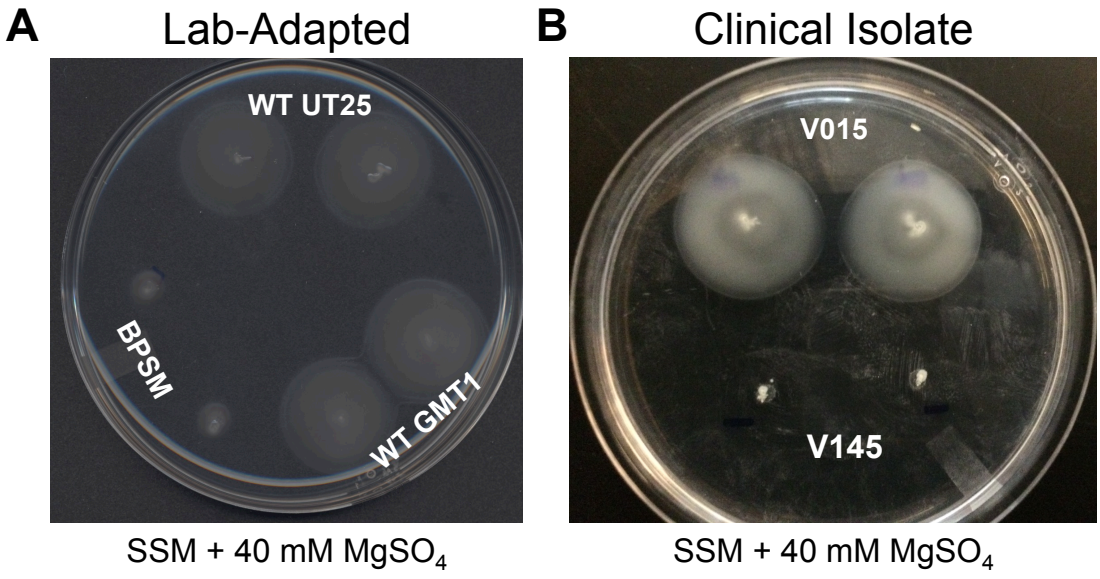

Figure 4.4. Lab-adapted and clinical isolates demonstrate a motile phenotype under Bvg(-) modulated conditions. Bacteria were grown over night as shaking cultures in SSM and diluted to an OD_{600} of 0.800. 2 µL of diluted cultures were stabbed into 0.4% SSM + 40 mM $MgSO_4$ motility agar plates. *B. pertussis* strains were grown for 72 hours at 37ºC at ambient CO_2 levels. **(A)** WT UT25, WT BPSM, and GMT1 **(B)** Clinical Isolate V015 and V145

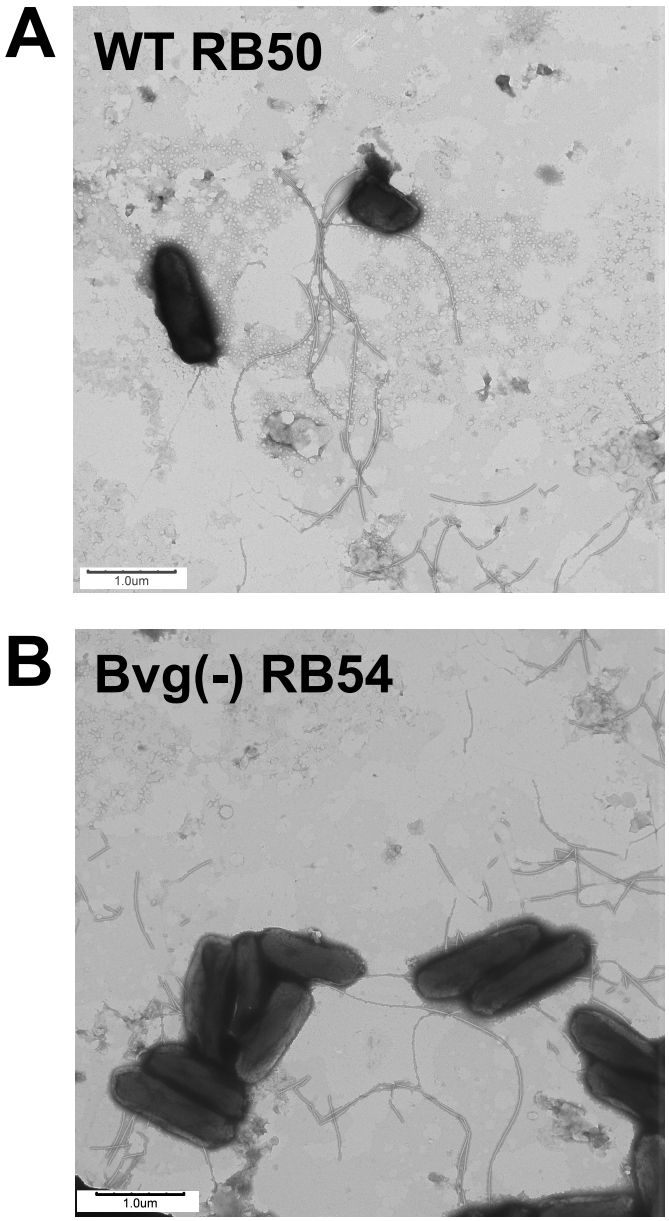

Figure 4.5. Negative stain TEM of *B. bronchiseptica* shows flagella structures on bacterial surface. Motile *B. bronchiseptica* were isolated from the outer edges of the spreading zones in SSM 0.4% agar plates. **(A)** WT RB50 and **(B)** Bvg(-) RB54 were isolated for negative stain TEM as described in the methods and imaged with JEOL 1230 Transmission Electron Microscope.

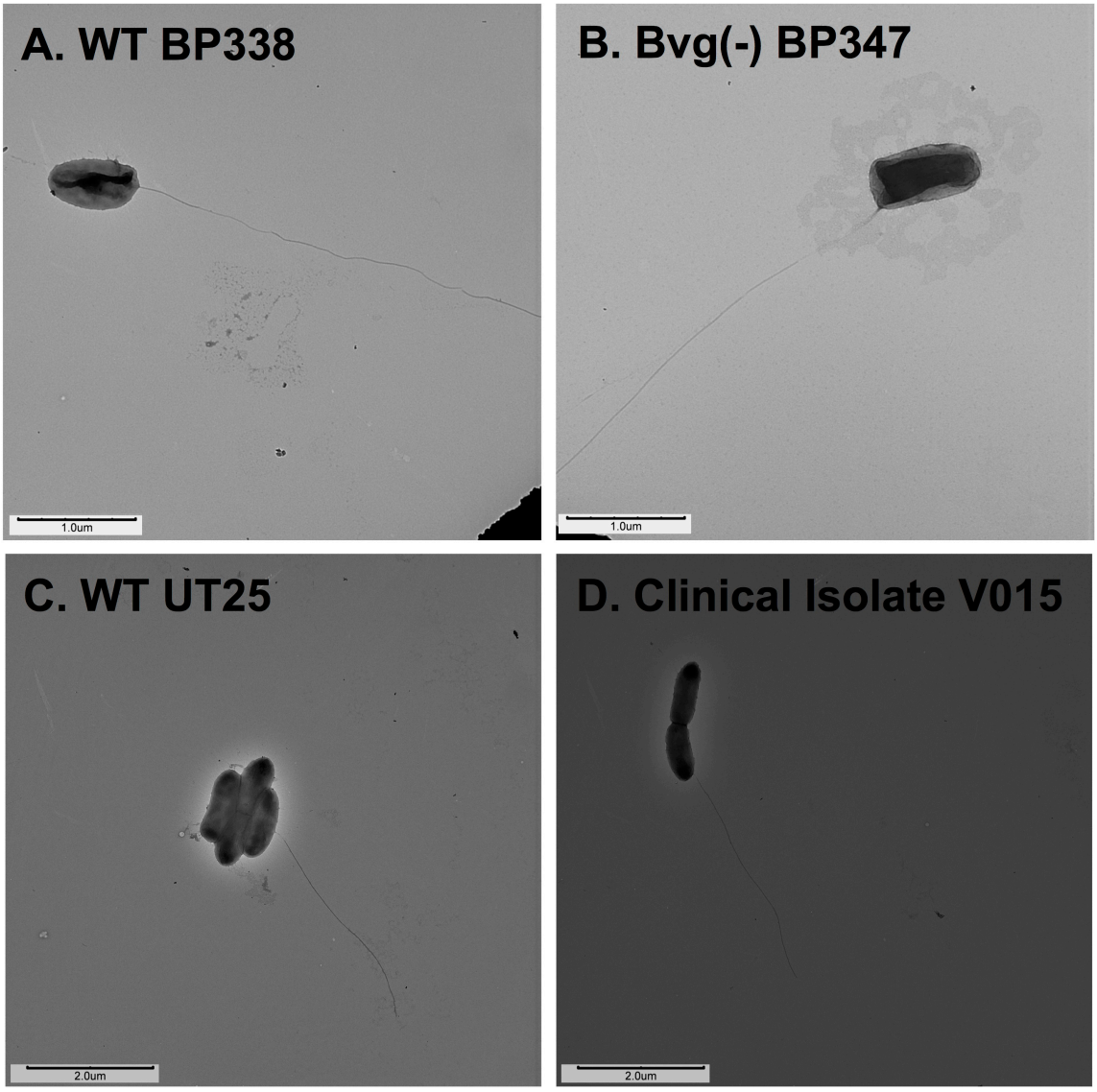

Figure 4.6. Negative stain TEM of *B. pertussis* shows flagella structures on bacterial surface. Motile *B. pertussis* were isolated from the outer edges of the spreading zones in SSM + 40 mM $MgSO_4$ 0.4% agar plates. **(A)** Lab adapted WT BP338, **(B)** the BP338-derived Bvg(-) BP347, **(C)** lab-adapted WT UT25, and **(D)** recent clinical isolate V015 were isolated for negative stain TEM as described in the methods and imaged with JEOL 1230 Transmission Electron Microscope.

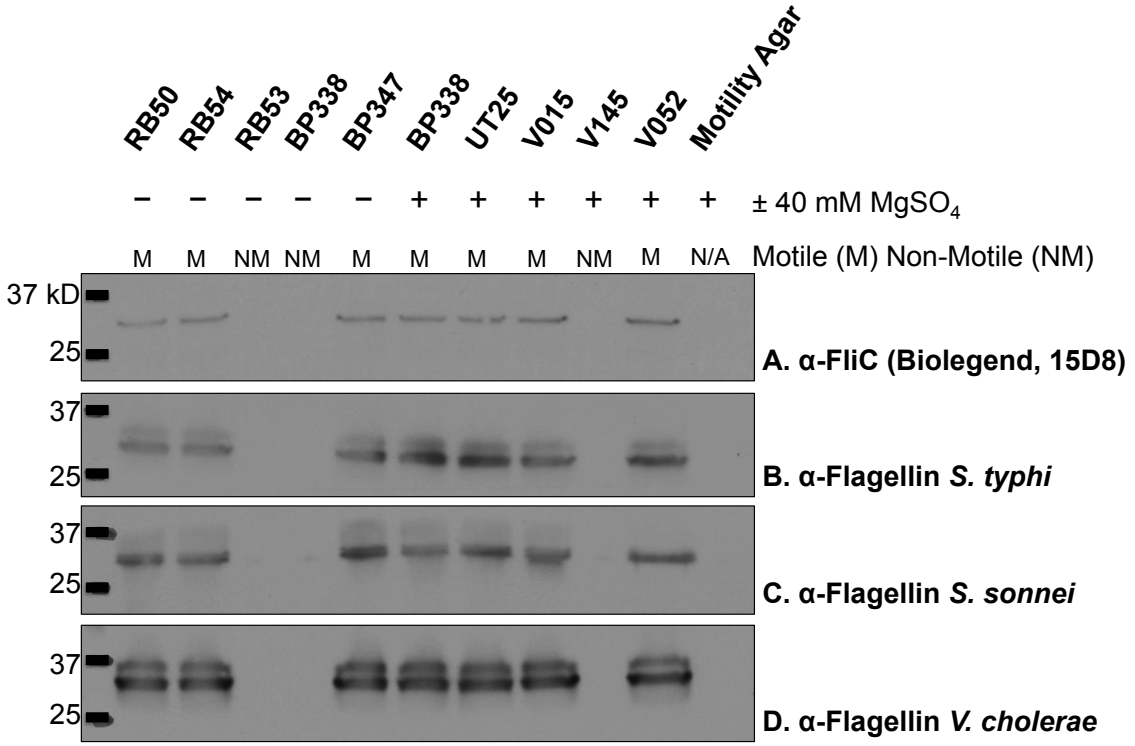

Figure 4.7. Western blot analysis of flagellin protein expression of motile *B. bronchiseptica* and *B. pertussis* strains. Motile *B. pertussis* were isolated from the outer edges of the spreading zones in SSM + 40 mM $MgSO_4$ 0.4% agar plates. Samples were prepared as described in the methods. Nitrocellulose membranes were probed with a variety of flagellin antibodies (diluted 1:300 in 5% skim milk). **(A)** BioLegend monoclonal anti-FliC antibody **(B)** anti-*Salmonella typhi* flagellin antibody **(C)** anti-*Shigella sonnei* flagellin antibody **(D)** anti-*Vibrio cholera* flagellin antibody. Non-commercial antibodies were obtained from Jorge Giron, and used previously to characterize *Shigella* flagella (329).

Chapter Five:

Conclusions and Future Directions

SUMMARY: Adenylate Cyclase Toxin-Filamentous Hemagglutinin Interaction and Inhibition of *Bordetella* Biofilm

When describing bacterial toxins, we think of proteins or molecules that bind to host cells and become internalized or injected into the host cytoplasm. Once inside, these toxins are able to disrupt cellular signaling and processes. Bacterial toxins are stereotypically thought of as weapons employed by the bacteria to fight or take advantage of the host. The novel regulatory role of Adenylate Cyclase Toxin (ACT) in the life style switch between planktonic (free-floating) and biofilm (surface-associated) is unprecedented (299), as ACT has been characterized primarily for its effects on host immune cells (macrophages, neutrophils, and dendritic cells) (189).

In a 2002 study, ACT was shown to bind the surface displayed adhesin, Filamentous Hemagglutinin (FHA) (176), and up until now, this interaction served an unknown function. Initial hypotheses suggested that the ACT-FHA interaction was a method for ACT delivery to host cells, but it was soon demonstrated that the interaction was not necessary for delivery of ACT to or subsequent intoxication of macrophages (J774). It is the non-surface associated and non-FHA associated ACT that is the active form of the toxin (177). We have since discovered a function for the ACT-FHA interaction – the inhibition of biofilm formation of *B. pertussis* and *B. bronchiseptica.* This discovery was prompted by the findings of Irie *et al*., who first showed that a *B. bronchiseptica* mutant lacking ACT formed more biofilm than the parental wild type strain (234). We have since shown that ACT inhibits biofilm, and that it is the catalytic domain of ACT (AC

domain) that binds the distal mature C-terminal domain of FHA (MCD) for this inhibitory phenomena to occur. This inhibition of biofilm occurs independently of ACT catalytic activity contained in the AC domain.

The MCD, albeit required for inhibition of biofilm by ACT, is not required for biofilm formation. The binding of ACT to the MCD may inhibit biofilm in one of several ways: 1) ACT blocks the portion of FHA required for biofilm, 2) ACT binding alters the conformation of FHA so that the adhesin's role in biofilm is altered, or 3) ACT binding signals through FHA, via a short periplasmic tail, to turn off biofilm (i.e. genetic changes, altering the levels of signaling molecules c-di-GMP or (p)ppGpp). Our data demonstrate that anti-MCD antibodies block biofilm formation, and suggest that ACT binds and alters FHA availability for biofilm, as opposed to this interaction causing a signaling event. There is evidence that antibodies can serve as agonists for signaling molecules, but we do not think this is the case. The AC domain also disrupts preformed biofilm, providing evidence for a role of endogenous toxin in dissolution of biofilm. Exogenously added full-length toxin does not disrupt preformed biofilm, which may be due to access issues of the 170kD holotoxin in comparison to the much smaller, 40kD AC domain. During biofilm formation ACT is produced within the biofilm and secreted at the surface of the bacteria, which would allow for direct access to FHA, also located on the surface.

IMPACT: Adenylate Cyclase Toxin-Filamentous Hemagglutinin Interaction and Inhibition of *Bordetella* Biofilm

The ability of a host-directed protein bacterial toxin to inhibit biofilm is unprecedented. Other bacterial toxins have been shown to inhibit biofilm, however, these are much different than the role of *Bordetella* ACT. Several bacteria encode Toxin-Antitoxin (TA) systems that consist of a stable toxin and its labile antitoxin that inhibits toxin action (338). These TA systems control growth, defend against phages, induce programmed cell death, and regulate biofilm formation, bacterial persistence, and general stress responses (338-340). In contrast, ACT is a host-directed protein bacterial toxin that inhibits parts of the host immune response. Herein we have described a novel role for the toxin as a regulator for the switch between the biofilm and planktonic lifestyles of *Bordetella*.

B. pertussis enters the upper respiratory tract of hosts, binds to epithelial cells by use of adhesins, and begins secreting toxins to combat the host immune system (3, 28). These toxins disrupt the host response to infection and protect the bacteria from cell-mediated killing. ACT is expressed during infection, and is estimated to reach concentrations of ~100 ng mL^{-1} in the respiratory tract of infected humans and baboons during peak bacterial CFUs (175). As *B. pertussis* is cleared and CFUs decrease, ACT concentrations decrease as well (there is a direct correlation between bacterial burden and ACT concentration) (175). The decrease in ACT concentration at later stages during infection could allow biofilm to begin forming in the upper respiratory tract. In this scenario, the bacteria do not actively secrete ACT to inhibit biofilm, but rather its absence allows the

bacteria to form biofilm within the host. This suggests that *B. pertussis* at some point switches from an aggressive “attack” phase within the host to become more defensive (Figure 5.1A). This defensive phase, if it were to include biofilm formation, would leave the bacteria protected from the immune system independent of its arsenal of toxins (Figure 5.1A). For example, antibodies and host immune cells cannot access bacteria within biofilms because the bacteria are protected by the matrix encasement (341). In addition, during the later phases of disease, the host immune response switches from an innate to a more adaptive response. ACT functions primarily to alter the innate immune response by inhibiting the function of macrophages, neutrophils, and dendritic cells. It is possible that the bacteria do not require ACT during later stages of disease, specifically in this defensive phase in which *B. pertussis* forms biofilm.

The hypothesized defensive phase above is similar to what has been described in the literature as the Bvg(i) phase. *Bordetella* transition among the virulent Bvg(+), intermediate Bvg(i), and avirulent Bvg(-) phases by virtue of activation (phosphorylation) and inactivation of the BvgAS-two component system. The system functions as a rheostat to allow differential expression of factors based primarily on the promoter structure of Bvg-regulated genes. During the Bvg(i) phase, adhesin expression remains high, while toxin expression is reduced (76); specifically *fhaB* (encodes FHA) expression remains high and *cyaA* (encodes ACT) expression is decreased (Figure 5.1B). Previous studies have shown that *B. bronchiseptica* biofilm formation is maximal in the Bvg(i) phase (234), and we have confirmed these observations in *B. pertussis*. We now think

that this increase in biofilm in the Bvg(i) phase is due at least in part to decreased expression of ACT (Figure 5.1B).

Unfortunately, the Bvg(i) phase, like biofilm, currently has an unknown role in *Bordetella* pathogenesis (3, 6, 7, 79). These studies raise questions of the importance of studying the Bvg phases during all aspect of Whooping Cough: initial colonization, establishment of infectious, persistence, and transmission. When a phase-locked *Bordetella* mutant is created, multiple aspects of *Bordetella* virulence are altered. In regards to the Bvg(i) phase, studies have shown that Bvg(i) locked bacteria are cleared more rapidly than their respective parental wild type strains from mice (79). These data are important in understanding general features of infection (i.e. initial adherence, colonization, and rate of clearance) and how BvgAS regulates the toxins and adhesins involved in these processes, but the data do not explain the full spectrum of a pertussis infection. *B. pertussis* enters hosts, adheres to ciliated epithelial cells in the upper respiratory tract, and establishes an infection by disrupting the host immune response (6). The final stages of disease are often ignored in the mouse model; the bacteria must be able to transmit in order to remain in circulation within the population, as there is no environmental reservoir. For these reasons, mouse models do not fully replicate the entire infectious cycle of *B. pertussis*.

It is hypothesized that the Bvg(i) phase is involved in transmission (3, 6), which has only been explored in the baboon model (22, 298, 342, 343). Despite the ability of the baboon model to explore transmission, the current tools (e.g. Bvg(i) locked mutants) used study the link between BvgAS and transmission do

not address initial establishment of infection, which requires the Bvg(+) phase and the toxins expressed only in Bvg(+) phase. Transmission studies using Bvg(i) mutants would result in an incomplete understanding of transmission.

The importance of each of the Bvg phases in the context of pathogenesis has been difficult to study, as has biofilm formation and its role in *Bordetella* virulence and pathogenesis. Collectively, others and we have shown that surface-associated growth of *Bordetella* meets all of the requirements to qualify as biofilm *in vitro*. A matrix comprised of polysaccharides, eDNA, and proteins encase the bacteria, and in the biofilm mode of growth there is increased resistance to antibiotics (200, 235-237). Additionally, biofilm-associated bacteria have a different gene expression profile compared to those in planktonic growth (253). Just four years ago, *Bordetella* biofilm was considered irrelevant to many outside of the group of *Bordetella* biofilm researchers, but great strides have been made to establish the fact that *Bordetella* does form biofilm and that biofilm formation occurs *in vivo*. *B. pertussis* and *B. bronchiseptica* form biofilm on the trachea and the nasal septum during experimental mouse infections, and these biofilms contain all of the same matrix components found in *in vitro* biofilms formed by these bacteria (200, 236, 237). Linking biofilm formation to human disease has been a difficult task, but recent studies have begun to show that biofilm components are expressed during human infection. For example, convalescent serum from pertussis patients recognizes the polysaccharide component of *B. pertussis* biofilm (237). Additionally, BipA, which was identified in a proteomics screen of biofilm matrix proteins, serves as a protective antigen

in the mouse model; immunization with BipA (with alum adjuvant) results in decreased colonization compared to non-immunized controls (239).

The difficulties associated with determining the role of biofilm in pathogenesis are similar to those listed above for understanding the role of Bvg(i). *B. pertussis* strains defective for biofilm formation *in vitro,* such as those that lack the *bps* polysaccharide locus (237, 241, 242), or *fhaB*, a major adhesin for biofilm formation (200), are also incapable of biofilm formation *in vivo*. And although we can determine which factors are important for biofilm formation, the inability of *Bordetella* to form biofilm has not been linked to a reduction in CFUs or increased rates of clearance. Teasing apart the reasons for a reduction in CFUs has been challenging as the mutants developed for studying biofilm also have defects in binding to cells in the upper and lower respiratory tract (237), or lack major virulence factors required for infection (84, 344).

The development of ACT as a tool to study biofilm inhibition *in vivo* may provide a method to assess the role of biofilm independent of the loss of required adhesins, toxins, and other factors required for infection. The AC domain, capable of biofilm inhibition, is a small peptide that has minimal to no effects on host tissues because it lacks the domain required for entry into host cells, and therefore does not exhibit toxin activity. Additionally, because the catalytic activity of ACT is not required for inhibitory properties, a safer peptide, in which the AC domain is mutated to remove catalytic activity, could be created for *in vivo* studies. The peptide would be similar to that of the catalytically inactive ACT_i protein which has a >1,000 fold reduction in catalytic activity and would be non-

toxic to host cells (described in Chapter 2). Administration of the AC domain would result in biofilm inhibition without deleting any of the factors required for adherence to host cells or factors required to fight off the host immune response. This would allow for the assessment of *B. pertussis* infection independent of biofilm formation and would bring us a step closer to understanding the role of biofilm in disease.

Evaluating the presence of biofilm in the context of human disease will be instrumental in assessing the relevance of *B. pertussis* biofilm and future studies concerning this phenomenon. In considering how to determine if biofilm is present in human patients, we were in contact with the pertussis group at the CDC. We proposed a change in the protocol for tissue handling upon patient death from pertussis. Samples would be saved and sent to either us or to a clinical lab to evaluate biofilm in the lungs, trachea, and nasopharynx, using methods similar to those described for mouse tissue samples (200, 236, 237). While the pertussis group at the CDC was eager to determine the possible presence of biofilm in human samples, the broader CDC community was not enthusiastic about the request, as the logistics would be hard to implement. Very few people die from pertussis on an annual basis and cases occur across the country. A nation-wide change in the protocol would be difficult to implement and enforce. This is why we believe it is imperative to first understand the role of biofilm in disease in animal models that are available to us; we must determine the contribution of biofilm to virulence, long-term colonization, asymptomatic colonization, and transmission.

In addition to understanding the regulatory mechanisms of biofilm formation *in vitro* and *in vivo*, these findings raise important questions about the inclusion of ACT in next-generation acellular pertussis vaccines. One of the prime candidates for inclusion in the vaccine is ACT (189). Antibody neutralization of the toxin activity of ACT will be useful in the prevention of pertussis (197, 345), but an antibody response that impairs this ACT-mediated inhibition of biofilm may have unanticipated consequences. It is important to note, however, that FHA antibodies inhibit biofilm formation *in vitro* (200, 299), and so the anti-FHA antibody response would theoretically safeguard from a major increase in biofilm in vaccinated populations if ACT were added to the aP vaccine. Since many of the assumptions made in the design of current aPs are now known to be incorrect, the fundamental concepts about *B. pertussis* and the pathophysiology of pertussis need to be carefully examined before the next generation of vaccines is released (298).

The basic mechanism of ACT inhibition of biofilm, the concept of an interaction between ACT and FHA, was first suggested in 1983 when a Δ*fhaB* mutant was shown to release all ACT into the extracellular milieu, as opposed to the ACT remaining associated with the bacterial surface (hypothesized to bind via FHA interactions) (191). In 2002 Zaretzky *et al.* confirmed the ACT-FHA interaction occured and we have since shown the specific domains required for this interaction and the specific consequences of this interaction (176). These studies will provide the basis for studying this interaction in other *Bordetellae*, and even other bacterial species that express FHA-like proteins. The AC domain

is the most conserved portion of ACT amongst *Bordetellae* that encode *cyaA (*5, 257), and this protein-protein interaction may be conserved amongst all *Bordetellae* that express the two proteins. See Appendix One: Adenylate Cyclase Toxin Inhibits *Bordetella parapertussis* Biofilm.

FUTURE DIRECTIONS: Adenylate Cyclase Toxin-Filamentous Hemagglutinin Interaction and Inhibition of *Bordetella* Biofilm

In order to better understand this inhibitory phenomenon, it is imperative to begin *in vivo* studies and test the ability of ACT and derived peptides to inhibit biofilm. Although *B. pertussis* and *B. bronchiseptica* ΔACT strains make more biofilm *in vitro*, it would be impossible to study the effects of endogenous ACT *in vivo* because ACT is required by *Bordetella* to establish infection. We propose that administration of exogenous ACT or AC domain will inhibit biofilm independently of altering virulence factor expression. Alternatively, a bacterial strain can be constructed to express the AC domain under a highly active, constitutive promoter (*ompA* or *groEL* promoters drive high expression of genes *in vivo* based on preliminary RNAseq analysis). The constructed strain would need to be characterized for AC domain secretion before being used *in vivo*, but could provide a tool for direct delivery of AC domain to the site where biofilm formation occurs. It is important to remember that the growth conditions in the host are extremely different that those in which we culture *B. pertussis* in the laboratory, and could alter ACT-FHA interactions within the host.

Finally, FHA has homologs in various other bacterial species. Chapter Three explores the ability of ACT and AC domain to inhibit biofilm of other bacteria that express an FHA-like protein. The characterization of the AC domain as an inhibitor of multiple bacterial species could provide a broad-spectrum biofilm inhibitor.

SUMMARY: *Bordetella pertussis* are Motile and Express Flagella

The longstanding belief in the pertussis field, and even the broader microbiology community, has been that *B. pertussis* is a non-motile organism. This idea has been perpetuated in textbooks for years, and though we have shown that the opposite it true, there are certainly reasons why the motile phenotype of *B. pertussis* had not been observed before. The idea may have stemmed from clinical labs that conduct characterization of patient samples to determine the pathogen responsible for disease. The nutrient-rich media used for the clinical motility assay should theoretically allow for most pathogens to grow (346, 347), but the gelatin in the media is inhibitory for *B. pertussis* growth. Based upon the readout of this clinical assay, *B. pertussis* is a “non-motile” organism. The idea persisted when researchers began studying *B. bronchiseptica* motility; the same clinical motility agar was used and *B. pertussis* was deemed non-motile again, simply due to the fact that the bacteria did not grow under the assay conditions (305). To add to the data in support of a non-motile *B. pertussis*, a stop codon was found in one of the core flagellar genes during genome sequence analysis of the *B. pertussis* Tohama I strain. The gene

encoding FlhA, a protein required for flagellin export to the bacterial surface, was rendered a pseudo gene due a frame shift mutation that created a stop codon (257). To our knowledge, this study marked the end of assessment of *B. pertussis* motility and flagellar expression.

Since then, several key observations prompted our investigation of *B. pertussis* motility: 1.) *B. bronchiseptica* require flagella for mature biofilm formation, but *B. pertussis* forms mature biofilm despite being non-flagellated (253); 2.) *B. pertussis* encodes all of the genetic material required to assemble a functional flagella; 3.) Multiple RNAseq data sets that show differential regulation of the flagellar operon in *B. pertussis*. 3.) And finally, the massive increase in flagella gene expression in a *B. pertussis* Bvg(-) mutant compared to the parental WT strain.

We have demonstrated that *B. pertussis* is motile, and that motility is induced in the Bvg(-) phase. This pattern of regulation by BvgAS is also found in *B. bronchiseptica* and *B. avium* (73), two of the *Bordetella* species previously shown to be motile. These data demonstrate that some of the regulatory mechanisms for motility in *Bordetella* are conserved. To confirm our findings, collaborators at the Universidad de le Plata in Argentina have replicated these observations. In addition to the motile phenotype observation, we have also shown the presence of flagellar structures on the surface of *B. pertussis*, both lab-adapted strains and clinical isolates, and have characterized a panel of flagellin antibodies that react specifically with *B. pertussis* flagellin (the major component of the flagella “tail”). These findings need to be further explored to

confirm that the motility is mediated by the flagella expressed by the bacteria. If the motility is not flagella-mediated, we will pursue the study of other forms of bacterial motility. We will also continue to investigate the role of flagella expression, regardless the outcome of the type of motility used by *B. pertussis,* because flagella are virulence factors for many bacterial pathogens.

IMPACT: *Bordetella pertussis* are Motile and Express Flagella

Bacterial motility does not occur by a single mechanism; rather there are several types, which are mediated by a collection of complex molecular mechanisms and bacterial structures (254, 302, 328, 348, 349). Bacterial motility is used for a variety of purposes, among which is the ability to reach and maintain a preferred niche for colonization and survival (349). Several types of bacterial motility have been detected, described, defined, and classified as various forms of spreading during bacterial growth on or in semi-solid media (302, 328). Some of the types of bacterial motility and the appendages/structures associated with each form are illustrated in Figure 5.2. Because we have identified flagella-like structures in motile *B. pertussis*, and none of the required genes for type IV pili assembly (involved in twitching motility) are present in the *B. pertussis* genome*,* we hypothesize that motility is dependent on flagella. Determining the type of motility will better characterize the phenomenon and help us understand the basic biology of *B. pertussis*. This will lay the groundwork to eventually understand the role of motility and flagella expression in the context of disease.

Flagella are highly immunogenic structures recognized by the host immune system. *B. bronchiseptica* flagellin (*flaA*) is a proinflammatory determinant that signals through TLR5 on epithelial cells and promotes IL-8, GM-CSF, and MIP3α production in Calu-3 human airway epithelial cells (326). Purified *B. bronchiseptica* flagellin produces an IL-8 response in epithelial cells comparable to the purified flagellins of *P. aeruginosa* and *S. enterica* (326). If it is determined that *B. pertussis* flagella are recognized by TLR5, this would alter the way in which we think about the innate immune response to *B. pertussis*, and allow for exploration of this phenomenon both *in vitro* and *in vivo*.

Alternatively, some bacteria alter flagellar structure to avoid recognition by TLR5. These altered structures are instead recognized by the cytoplasmic NLRC4 (also known as Ipaf) receptor, a part of the inflammasome complex (319-324). Upon activation, the inflammasome stimulates production of IL-1β and IL-18 (319). If *B. pertussis* expresses flagella that are recognized by the inflammasome complex as opposed to TLR5, this would suggest that the bacteria have evolved flagella sequence and/or structure to avoid detection by TLR5 and provide an argument for the fact that flagellar expression is an important aspect of *B. pertussis* pathogenesis.

In the context of disease, *B. bronchiseptica* that cannot express flagella have no defect in colonization of mouse lungs and are cleared from the host lungs at the same rate as the parental wild type strain (314). Ectopic expression of flagella in *B. bronchiseptica,* however, results in defects in tracheal colonization, but not colonization of the nares or the lungs (measured by positive

culture, no CFUs provided) (314). The differences of bacterial burden between these sites within the host raise important questions about the overall requirement of flagella, and studies should be repeated to better understand these differences. Importantly, now that we are aware that *B. pertussis* express flagella, these studies should be conducted with both organisms.

FUTURE DIRECTIONS: *Bordetella* pertussis are Motile and Express Flagella

The discovery of flagella expression and motility prompts many questions. Flagella mediate movement of the bacteria from one location to another, and this movement can often be driven by chemotaxis. Once *B. pertussis* enters the host, how does it reach its niche? Do the flagella aid in this movement from the initial inhalation location to the bacteria's niche on ciliated epithelial cells? How do *B. pertussis* enter the lungs of infected mice and humans? Are there chemical gradients that the bacteria follow?

First, the involvement of flagella in the motile phenotype must be assessed; Δ*flaA* mutants will be tested for motility in the soft agar assay. Secondly, *B. pertussis* flagellin will be tested as a proinflammatory mediator in relevant cell culture models (Calu-3 airway epithelial cells, HBE bronchial epithelial cells, Nasal Epithelial cells, etc). IL-8, GM-CSF, and MIP3α will be measured in the presence and absence of flagellin (WT and ΔflaA). The ability of purified *B. pertussis* flagellin to bind and signal through mouse and human TLR5 will be evaluated using HEK-blue TLR5 cells (which will compliment the above experiments).

In order to understand the role of flagella during of infection, the Δ*flaA* mutant will be tested in the mouse model to determine if there are defects in colonization in the lungs, trachea, and nares. If so, the defect could arise from a decrease in binding (confirmed by defects in binding to cells in cell culture models). The defect could also arise from a decrease in cell invasion, as reported for *B. bronchiseptica* (confirmed by HeLa cell invasion assays). Alternatively, due to a lack of motility (i.e. these bacteria cannot travel to the specific sites within the host) the bacteria may have a defect in colonization. Finally, to finish this work with biofilm-flagella studies, biofilm formation of the Δ*flaA* will be assessed *in vitro* and *in vivo* to determine if a lack of flagella alters the ability of *B. pertussis* to form a mature biofilm.

CONCLUSIONS AND IMPLICATIONS OF THIS STUDY TO THE FIELD:

Although the two studies contained herein seem separate and independent, the underlying theme between them is the connection to the BvgAS two-component system. Since the discovery of BvgAS, the longstanding view in the field has been that Bvg(+) is all that is needed for the bacteria to cause infection, but what studies have missed are other aspects of the disease: transmission, long-term infection, and asymptomatic infection. Studies need to be conducted in order to understand these other aspects of *B. pertussis* pathogenesis, and experiments need to be designed to assess the role of the other Bvg phases in the context of transmission, long-term infection and asymptomatic infection. The current literature compares a Bvg(i) locked or Bvg(-)

locked strain to a wild type strain *in vivo*. What is forgotten is that the wild type strain can modulate freely among the three Bvg-phases to occupy whatever niche is necessary.

Bvg(i) and Bvg(-) bacteria have been isolated from mice during experimental infection and Bvg(-) phase *B. pertussis* have been recently isolated from humans (29, 74, 75). These non-Bvg(+) phase bacteria arise through two different mechanisms; slip-stream pairing, which transiently modulates the bacteria to a Bvg(-) or Bvg(i) phase, or genetic mutations which render the bacteria locked in either Bvg(-) or Bvg(i) phase (52, 291, 350-353). Regardless of the mechanism behind the change, these bacteria enter the experimentally infected host (mouse or baboon) as Bvg(+) phase, and can modulate themselves and survive for extended periods of time in the host without being cleared. The roles that these individual Bvg(i) and Bvg(-) populations play is currently unknown, and so little attention is paid to their isolation from both symptomatic and asymptomatic and vaccinated and unvaccinated patients (29, 75)

If the Bvg(i) and Bvg(-) phases occur within hosts, their associated phenotypes may play a role for these specific populations of bacteria. Biofilm occurs maximally in the Bvg(i) phase and motility is demonstrated in the Bvg(-) phase. It is possible that small subsets of the total *B. pertussis* population form biofilm or become motile in the host, and that the subsets of bacteria have connections to persistence and long-term infections, asymptomatic infections, and transmission. Better models for understanding these Bvg phases and their associated phenotypes are important for developing an all-encompassing model

of *B. pertussis* pathogenesis, because as of now, all we know is that the Bvg(+) phase is what is required to establish an infection. What comes after is unknown.

COMING FULL CIRCLE: My First Presentation as a MIC Student to My Last

On March 22nd, 2013, approximately one month after I joined Erik Hewlett's lab and began studying *Bordetella* biofilm, I gave a presentation for the MIC Colloquium Course. The manuscript I presented was entitled "Transcriptome Profiling Reveals Stage-Specific Production & Requirement of Flagella during Biofilm Development in *Bordetella bronchiseptica*". I remember having issues with the manuscript; they all seemed to revolve around the fact that *B. pertussis* had no defects in biofilm formation despite being classified as a non-flagellated bacterium. I had not revisited the manuscript for its take away message, that flagella were required for the initial binding step in biofilm formation, until approximately three years after I presented the manuscript to my MIC Colloquium classmates. This was a key piece of literature that would link the biofilm studies to the unanticipated discovery that *B. pertussis* is motile. From this experience, I realized how important it is to fully understand what a manuscript is saying, and to not simply toss aside the findings if they do not fit your model. These pieces of information are often more important than we initially think and highlight the importance of revisiting old literature to think about the findings in the context of what we know now.

FIGURES:

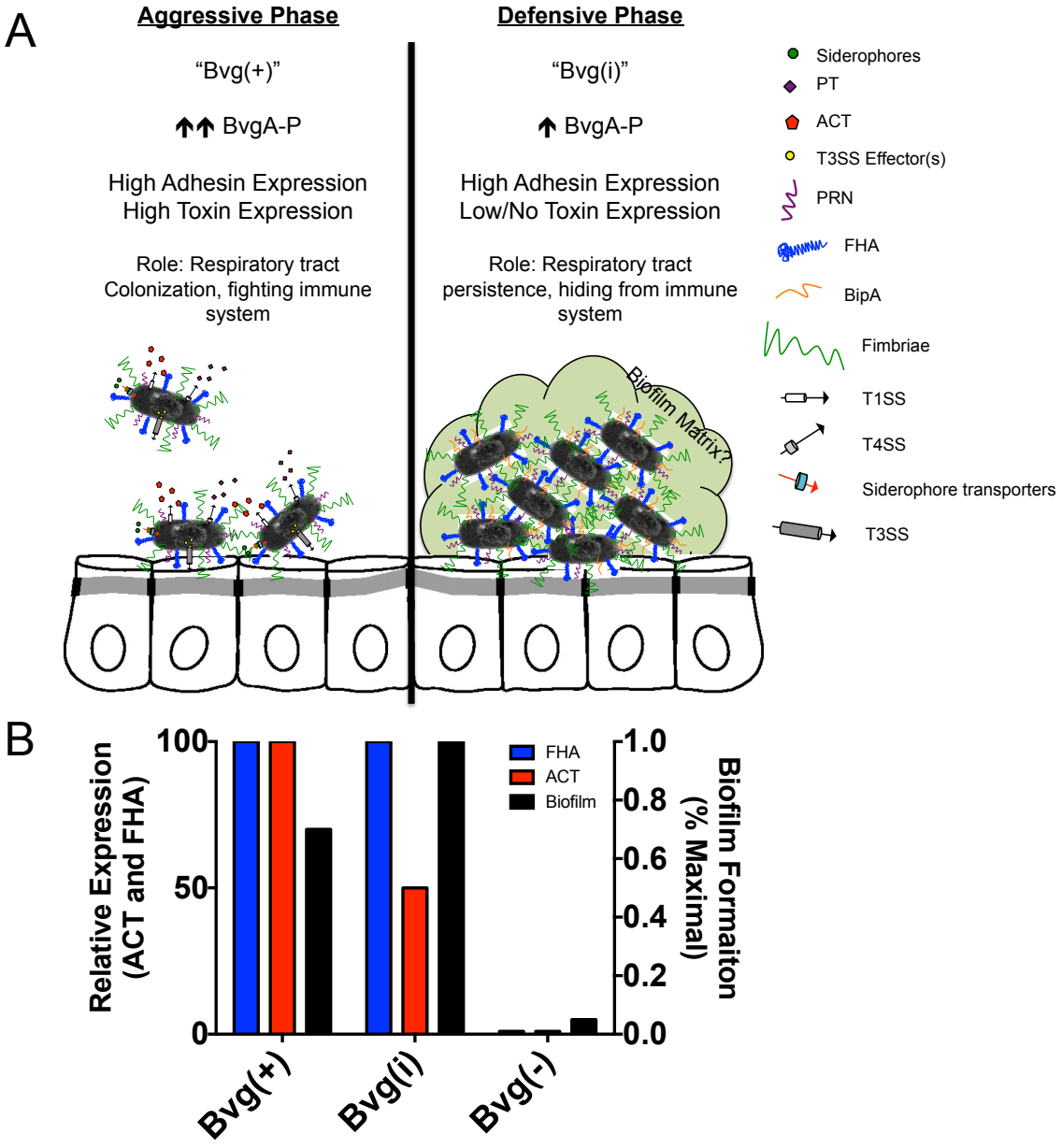

Figure 5.1. (A) Hypothesized Aggressive and Defensive Phases During Infection. During *B. pertussis* infection, bacteria enter the host, adhere to cells, and begin secreting toxins. This initial "Aggressive Phase" correlates with the Bvg(+) phase in which toxin and adhesin expression is high. During infection it is

unknown if toxin expression remains high throughout, but data has shown that as *B. pertussis* CFUs decrease, adenylate cyclase toxin decreases (175). This decrease in toxin expression would correlate with the Bvg(i) phase, in which adhesin expression remains high. This is the hypothesized “Defensive Phase” during which the bacteria stick to surfaces and possibly begin secreting biofilm matrix material to protect themselves from the host immune response. The defensive phase could play a role in persistence. **(B) Diagrammatic Characterization of the Bvg Phases.** The relative expression levels for ACT and FHA have previously been determined via qPCR for mRNA levels and protein expression has also been measured (76). Based on these data, this diagram shows that ACT and FHA expression is high in the Bvg(+) phase (left y axis). FHA expression remains high in the Bvg(i) phase, but ACT expression is significantly reduced. FHA and ACT expression is minimal to none in the Bvg(-) phase. Biofilm formation has been measured in all three of the Bvg phases as well. Based on these data, biofilm formation was described for each of the phases. Biofilm is maximal in the Bvg(i) phase, while it is significantly reduced in the Bvg(+) phase. No biofilm occurs in the Bvg(-) phase.

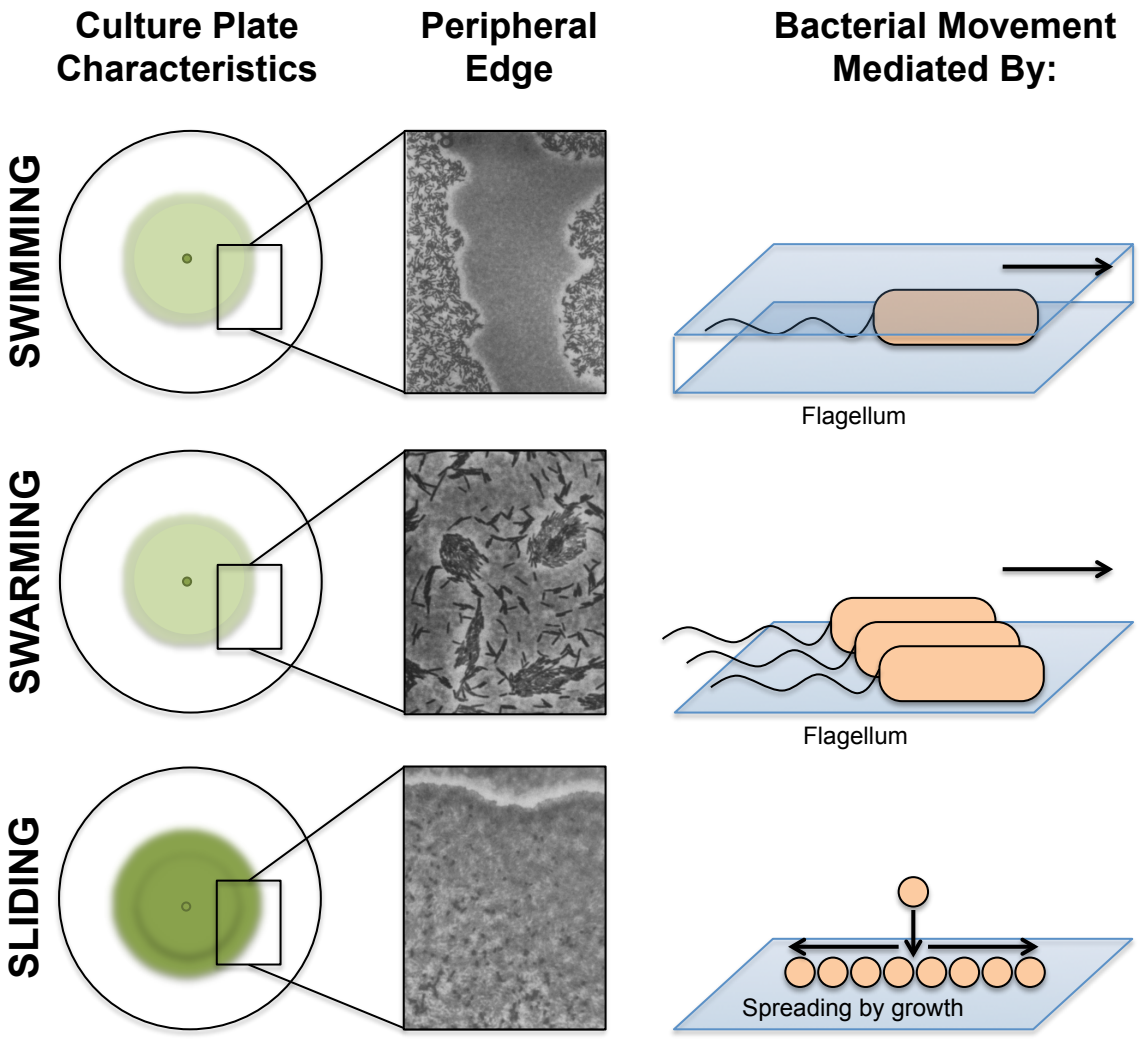

Figure 5.2. Possible Forms of Bacterial Motility Utilized by *B. pertussis*. Diagram of the various forms of bacterial motility as observed in the soft agar motility assay, and example images of the peripheral edges. Black arrows describe the direction in which the bacteria are moving. Red arrow describes pilus retraction direction. Adapted (302, 328).

Appendix One:

Adenylate Cyclase Toxin Inhibits *Bordetella parapertussis* Biofilm

RESULTS:

Bordetella parapertussis is grouped into the classical *Bordetella* species with both *Bordetella bronchiseptica* and *Bordetella pertussis*. *B. parapertussis* encodes and expresses Adenylate Cyclase Toxin and Filamentous Hemagglutinin. In addition, *B. parapertussis* was shown to form biofilm in multiple studies and biofilm formation of this species of *Bordetella* is also regulated by the BvgAS two-component system (235). It was demonstrated that the host-directed protein bacterial toxin, Adenylate Cyclase Toxin (ACT), and the derived catalytic domain (AC domain) are able to inhibit biofilm of both *B. pertussis* and *B. bronchiseptica.* We have also shown that ACT and the AC domain inhibit biofilm of *B. parapertussis*. Figure A.1 shows that both 100 ng mL^{-1} ACT and 10 ng mL^{-1} AC domain inhibits biofilm formation of *B. parapertussis* to the same extent as the two peptides inhibit *B. pertussis* and *B. bronchiseptica* biofilm.

EXPERIMENTAL PROCEDURES:

Bacterial Strains and Growth Conditions: *B. pertussis* WT BP338 (Tohama I); Bvg(-) BP347 (TN5::*bvgS* mutant derived from BP338); and *B. parapertussis* WT CN8234 (1983) were grown on Bordet-Gengou (BG) agar (Gibco) supplemented with 15% defibrinated sheep blood (Cocalico) for 48 hours at 37ºC. The same growth conditions were used for *B. bronchiseptica* strains, WT RB50; Bvg(-) RB54; RBX11-JS20 (*fhaB* ΔMCD and C-terminal prodomain); and RBX11 T-N (*fhaB* with transposon insertion in C-terminal domain). These *B. pertussis* strains have been previously reported (86, 191), as have *B. bronchiseptica* (86) and *B. parapertussis* (395). Bacteria were then transferred to liquid culture in modified synthetic Stainer-Scholte liquid medium (SSM) (174), and grown for 24 hours at 37ºC, shaking at 150 RPM. Bacteria were pelleted and washed in SSM, and then resuspended to an OD_{600} of 0.1 for biofilm experiments.

Microtiter Crystal Violet Assay: Biofilm was measured using the microtiter plate assay, coupled with crystal violet staining, as previously described (292). Briefly, bacteria were grown in a total volume of 100 µL of LB medium at 37°C in 96-well polyvinylchloride (PVC), round-bottom, non-tissue-culture treated microtiter plates (NuncBrand). Biofilm was measured at 12 hours, unless otherwise stated. Wells were washed at indicated time points to remove planktonic bacteria. Bacterial cells that remained attached to the wells were stained with a 0.1% solution of crystal violet (CV) and were incubated at room temperature for 30 min. The washing process was repeated, and the CV stain

was solubilized from bacterial cells with 200 µl of 95% ethanol. Biofilm formation was quantitated by measuring OD_{595}.

Statistics: Statistical analysis was performed using student's unpaired t test with Welch's correction, assuming Gaussian distribution (parametric test), these tests were performed on data sets to compare conditions within experiments.

FIGURES:

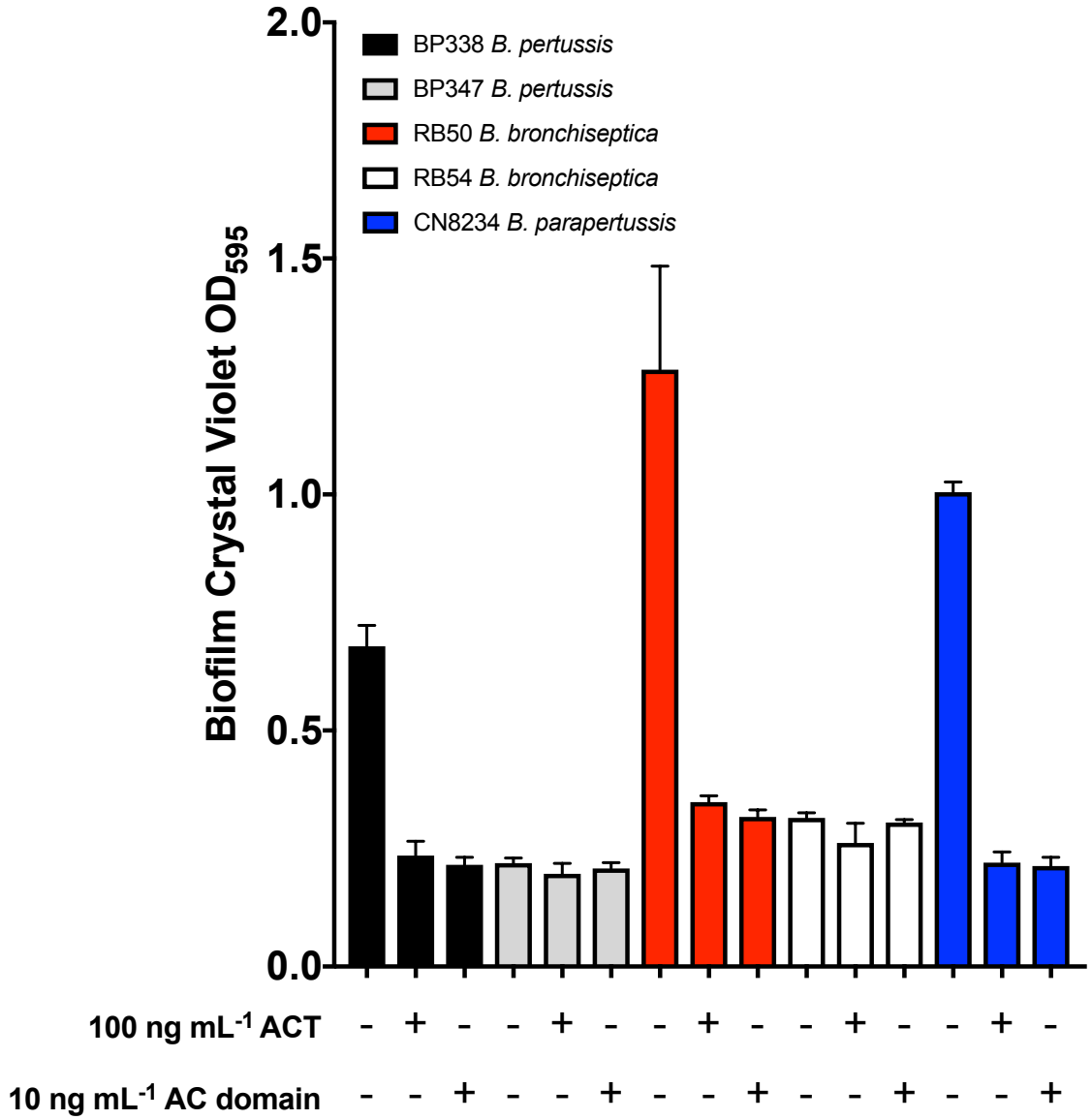

Figure A.1. ACT and the AC domain inhibit *Bordetellae* biofilm. *B. pertussis* WT BP338 biofilm formation in the presence of increasing concentrations of recombinant purified ACT (ng mL^{-1}) was assessed at 96 hours (black bars). Biofilm formation was measured by crystal violet assay. *B. pertussis* Bvg(-) BP347 serves as negative control (gray bars). In comparison, *B. bronchiseptica*

WT RB50 (red bars) and *B. bronchiseptica* Bvg(-) RB54 (white bars) biofilm formation was assessed at 72 hours in the presence and absence of ACT and the AC domain. WT *B. parapertussis* CN8234 biofilm formation in the presence and absence of 100 ng mL^{-1} recombinant purified ACT and 10 ng mL^{-1} recombinant purified AC domain was assessed at 72 hours (blue bars). Data expressed as the mean ± standard deviations, compiled from 5 experiments run in triplicate. *** = $p < 0.001$, **** = $p < 0.0001$ compared to WT BP338 without ACT.

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