Published in final edited form as:

Cell Microbiol. 2014 July ; 16(7): 1105–1118. doi:10.1111/cmi.12264.

The *Bordetella pertussis* Bps Polysaccharide Enhances Lung Colonization by Conferring Protection from Complement-mediated Killing

Tridib Ganguly^{1,*}, John B. Johnson^{1,*}, Nancy D. Kock², Griffith D. Parks¹, and Rajendar Deora^{1,#}

¹Department of Microbiology and Immunology, Wake Forest School of Medicine, Medical Center Blvd., Winston-Salem, NC, 27157

²Department of Pathology/Comparative Medicine, Wake Forest School of Medicine, Medical Center Blvd., Winston-Salem, NC, 27157

Summary

Bordetella pertussis is a human-restricted Gram-negative bacterial pathogen that causes whooping cough or pertussis. Pertussis is the leading vaccine preventable disease that is resurging in the USA and other parts of the developed world. There is an incomplete understanding of the mechanisms by which *B. pertussis* evades killing and clearance by the complement system, a first line of host innate immune defense. The present study examined the role of the Bps polysaccharide to resist complement activity in vitro and in the mouse respiratory tract. The isogenic *bps* mutant strain containing a large non polar in-frame deletion of the *bpsA-D* locus was more sensitive to serum and complement mediated killing than the WT strain. As determined by western blotting, flow cytometry and electron microscopic studies, the heightened sensitivity of the mutant strain was due to enhanced deposition of complement proteins and the formation of membrane attack complex, the end product of complement activation. Bps was sufficient to confer complement resistance as evidenced by a Bps-expressing *E. coli* being protected by serum killing. Additionally, western blotting and flow cytometry assays revealed that Bps inhibited the deposition of complement proteins independent of other *B. pertussis* factors. The *bps* mutant strain colonized the lungs of complement-deficient mice at higher levels than that observed in C57Bl/6 mice. These results reveal a previously unknown interaction between Bps and the complement system in controlling *B. pertussis* colonization of the respiratory tract. These findings also make Bps a potential target for the prevention and therapy of whooping cough.

Introduction

B. pertussis, a Gram-negative pathogen is the etiological agent of whooping cough or pertussis. In the past two decades, this disease has gone from on the verge of eradication (Steele, 2004, Kendrick, 1975) to reemergence in the USA, Europe, Australia and Canada

[#]Correspondent Footnote: Rajendar Deora, Department of Microbiology and Immunology, Wake Forest University School of Medicine, Wake Forest Bio-Tech Place, Room 2N-054, 575 Patterson Ave. (336) 716-1124, (336)-716-9928 (Fax), rdeora@wakehealth.edu.

^{*}These authors contributed equally to this work.

(2012, Mooi, 2010, Celentano *et al.*, 2005, Cherry, 2012). In 2012, 48,277 cases of pertussis were reported in the USA, representing the most number of reported cases since 1955 (www.cdc.gov). Globally in 2008, 16 million cases of pertussis and 195,000 deaths were estimated by the World Health Organization. A better understanding of the interaction of *B. pertussis* with the immune system will lead to more efficacious vaccines.

B. pertussis is an obligate human pathogen. Therefore, in order to survive the hostile host environment and subsequently cause disease, it must have the ability to overcome innate immune defenses. One of the principal components of innate immunity is the complement system. The complement system is a tightly coordinated system comprising of soluble and membrane bound components which interact in a series of reactions to promote the clearance of pathogens by multiple mechanisms including opsonization and direct cell damage due to the formation of the membrane attack complex (MAC). The three major pathways by which complement is activated are the classical, lectin and alternative pathways. Classical pathway (CP) activation occurs when C1 binds to circulating antigen-antibody complexes or directly to activating surfaces, while recognition by mannose binding lectin of the terminal microbial carbohydrate residues leads to activation of the lectin pathway (LP). Lastly, the alternative pathway (AP) is initiated when C3 binds to an appropriate activating surface. Prevention of damage to host cells by complement is avoided by exerting tight control of complement activation. This regulation is mediated by a group of host complement regulatory proteins e.g. Factor H, C1 esterase inhibitor, C4-binding protein and CD55 which target various steps of complement pathways (Walport, 2001b, Walport, 2001a).

B. pertussis has several different mechanisms to resist complement. The protein BrkA mediates resistance to the classical complement pathway (Barnes *et al.*, 2003, Barnes *et al.*, 2001, Fernandez *et al.*, 1994). *B. pertussis* also expresses other proteins that interact with various complement components or its regulators. Filamentous hemagglutinin (FHA) binds C4-binding protein, however the relevance of this binding to serum resistance is unclear since *B. pertussis* mutants deficient in FHA expression exhibit similar levels of serum resistance as the wild type strain. *B. pertussis* autotransporter Vag8 has been shown to bind the C1 esterase inhibitor (C1-inhibitor) and confer serum resistance (Marr *et al.*, 2007, Berggard *et al.*, 1997, Berggard *et al.*, 2001, Marr *et al.*, 2011). *B. pertussis* also recruits factor H, a regulator of AP (Amdahl *et al.*, 2011, Meri *et al.*, 2013).

In addition to proteins, bacterial polysaccharides are associated with avoidance of host innate immunity, including the complement system (Kugelberg *et al.*, 2008, Hyams *et al.*, 2010, Hallstrom *et al.*, 2010). In Gram negative bacteria, the presence of the repetitive O polysaccharide in LPS correlates with enhanced resistance to direct complement mediated lysis (Reeves, 1995). In other *Bordetella* species, *B. bronchiseptica* and *B. parapertussis*, which express the O polysaccharide, deletion of the loci required for its assembly results in enhanced killing by serum complement *in vitro* (Burns *et al.*, 2003). *B. pertussis* differs from these other species in that it naturally lacks the O polysaccharide and has a branched core structure and a trisaccharide with unusual sugars. Mutant strains lacking the terminal trisaccharide do not differ in their ability to be killed by the complement system (Barnes *et*

al., 2003, Caroff *et al.*, 2000). In addition, a recently identified capsular polysaccharide did not protect *B. pertussis* from complement-mediated killing (Neo *et al.*, 2010).

The *B. pertussis* Bps polysaccharide belongs to a large family of β -(1-6)-linked polymeric-*N*-acetylglucosamine (GlcNAc) polysaccharides synthesized by many bacterial pathogens (Franca *et al.*, 2013, Conover *et al.*, 2010, Sloan *et al.*, 2007, Parise *et al.*, 2007). We have previously shown that Bps is essential for efficient early colonization of the mouse respiratory tract in *B. pertussis* (Conover *et al.*, 2010). We further showed that Bps promoted attachment to nasal epithelial cells thereby providing a mechanism for its role in colonization of the nose. In contrast, a role for Bps in attachment to lung epithelial cells was not evident (Conover *et al.*, 2010). Thus, the mechanism by which Bps mediates early colonization of the lungs is not known.

Complement is present at the mucosal surfaces of the respiratory tract, although at low steady state levels. However, during cellular stress or infection the concentration, activity and profile of complement factors can change dramatically (Bolger *et al.*, 2007). The importance of complement in respiratory tract infections is supported by the fact individuals with complement deficiencies suffer from recurrent respiratory tract infections (Watford *et al.*, 2000). Recently, a complement disorder resulting in low C3 levels has been suggested as a probable cause of severe *B. pertussis* pneumonia in two young infants (Kurvers *et al.*, 2013). Complement-deficient mice infected with some respiratory bacterial pathogens are either likely to die or fail to clear infections (Brown *et al.*, 2002, Gross *et al.*, 1978, Wessels *et al.*, 1995). However, it is not entirely clear how it applies to *B. pertussis* since a previous study failed to reveal a definitive role for complement in the host defense against *B. pertussis* infection of the mouse respiratory tract (Elder *et al.*, 2004).

In the present study, we report that Bps is involved in *B. pertussis* evasion of the complement mediated killing resulting in enhanced colonization of the mouse lungs. To our knowledge, we demonstrate for the first time a direct correlation between *in vitro* complement resistance and *in vivo* bacterial loads in the respiratory tract for *B. pertussis*.

Results

The *bps* mutant of *B. pertussis* is more susceptible to serum killing

To investigate the protective role of Bps in mediating resistance against serum-mediated killing, 1000 CFU of the WT and *bps* strains were incubated for 15 min with either PBS or with various dilutions of normal human serum (Fig. 1A). Serum was used as a surrogate for steady-state levels of complement in tissues such as the respiratory tract (Bolger *et al.*, 2007). Compared to the mutant strain, the WT strain (Bp536) survived better in 1, 2 and 5% serum. At higher serum concentrations (10%), both the WT and the mutant strains were effectively killed, since few if any bacteria grew on BG agar plates (Fig. 1A). We also extended these experiments to a recent *B. pertussis* clinical isolate, GMT1 (Martinez de Tejada *et al.*, 1996). Deletion of the *bps* locus in this strain was generated as described previously (Conover *et al.*, 2010). Lower numbers of the GMT1 strain lacking the *bps* locus (GMT1 *bps*) survived when incubated with 2 and 5% of human serum as compared to the parental strain (Fig. 1B).

Complementation of the mutant strain with a plasmid expressing Bps (*bps*^{Bps}) resulted in higher serum survival as compared to the mutant strain containing the vector plasmid only (*bps*^{Vector}) (Fig. 1C).

We also conducted a time course (5, 10, 15 and 20 mins) of human serum mediated killing. As shown in Fig. 1D, the WT strain exhibited statistically higher rates of survival in 5% human serum. Taken together, these results suggest that Bps promotes resistance of *B. pertussis* to killing mediated by the human serum.

Bps promotes resistance of *B. pertussis* to classical pathway mediated killing

We next determined the complement pathway (Fig. 2A) that was responsible for the enhanced killing of the *bps* mutant strain. The WT and mutant strains showed similar viability in 5% heat-inactivated normal human serum (HI-NHS), which lacks active complement (Fig. 2B). Human serum deplete in the complement component C8 (NHS(-C8)) did not result in any significant killing of either strains, suggesting a role of the membrane attack complex in serum killing of *B. pertussis* (Fig. 2B). Both CP and LP are Ca²⁺ ion dependent and chelation with EGTA in the presence of Mg²⁺ inhibits these pathways. By contrast, the AP is not affected by EGTA treatment (Fig. 2A). Thus, the chelators EGTA and EDTA were used to block CP+LP or all pathways, respectively. Activation of the classical/lectin pathways also requires C4. Serum-killing assays performed with NHS in the presence of either EDTA or EGTA with Mg²⁺ and with C4-depleted NHS resulted in no significant differences in viability compared to that observed during incubation with HI-NHS and PBS. Addition of purified C4 to the C4-depleted serum resulted in considerable reduction in the survival of both the strains (Fig. 2B). Taken together, these results show that a C4-dependent pathway, either CP or LP plays a major role in the serum killing of these strains.

To distinguish between the CP and LP, serum bactericidal assays were conducted with C1q-depleted serum. C1q is required for activation of CP but not for LP. The survival of the WT and the *bps* mutant strain in serum devoid of C1q was similar to that observed during incubation with HI-NHS and PBS. Reconstitution of the C1qdepleted serum with C1q considerably enhanced the serum killing activity (Fig. 2B). Consistent with results obtained using NHS, there was a statistically significant difference in the relative survival of the WT and the *bps* strains in all cases of depleted sera that were reconstituted back to biologically active complement pathways. Taken together, these results suggest that the killing potency of CP is limited by the presence of Bps.

Enhanced deposition of complement proteins C3 and C4 on the *bps* mutant strain

Next, we determined the mechanism by which Bps promotes resistance to serum-mediated killing by *B. pertussis*. Since C3 and C4 are critical effector molecules, anti C3 and anti C4 antibodies were used to determine differences in the deposition of these components on the WT and the mutant strains. Three different assays were employed for this purpose. First, Western blot analyses utilizing whole cell lysates of bacteria after incubation with NHS showed higher levels of deposition of the activated forms of C3 (Fig. 3A) and C4 (Fig. 3B) on the mutant compared to the WT bacteria as evidenced by the higher intensity of the α' and β' fragments. Second, flow cytometry showed that the fluorescence intensity of the WT

cells labeled with antibodies to either C3 or C4 was lower than for the mutant bacteria, indicating more C3 and C4 on the surface of the *bps* strain (Figs. 3D and 3E, respectively and Table 1). Finally, electron microscopy was carried out to verify these findings using immunogold labeling. For both C3 and C4, the mutant bacteria bound considerably more colloidal gold particles compared to the WT strain (Fig. 4).

C3 and C4 deposition studies were also carried out with serum depleted of C8, which unlike NHS did not lead to the killing of *B. pertussis* (Fig. 2B). Western blot analyses showed higher levels of activated forms C3 and C4 on the *bps* strain compared to the WT strain (Fig. 3C). Taken together, these findings indicate that in presence of Bps, there is less C3 and C4 deposition on *B. pertussis* surface consistent with differential cell killing.

Increased assembly of membrane attack complex (MAC) on the *bps* mutant strain

In addition to the deposition of C4 and C3, the levels of MAC deposition on bacteria can reflect the extent of complement activation and cell killing. Thus, we asked if the enhanced serum-mediated killing of the mutant strain could be correlated with higher levels of MAC assembly on the cell surface. Flow cytometry analysis showed increased levels of C5b-9 association with the mutant bacteria compared to the WT bacteria, since the relative MFI (mean fluorescence intensity) on WT cells was lower than that on the mutant cells (Fig. 3F and Table 1). Electron microscopic analysis further showed abundant colloidal gold labeling of the mutant cells compared to minimal labeling of the WT cells (Fig. 4). These results suggest that presence of Bps leads to reduced deposition of MAC on the surface of *B. pertussis*.

Ectopic expression of Bps in *E. coli* confers serum resistance and interferes with the deposition of complement proteins

To determine if Bps was sufficient for conferring resistance to serum-mediated killing in the absence of other *Bordetella* factors, we expressed Bps in *E. coli*. To avoid potential complications in the interpretation of the results, we utilized a strain that lacks the *E. coli* *pga* locus (Itoh *et al.*, 2008). The protein products encoded by the genes of the *bpsABCD* locus have high amino acid sequence similarity to the enzymes encoded by the *pga* locus (Parise *et al.*, 2007). Moreover, Bps is also antigenically similar to the PNAG polysaccharide of *S. aureus* (Itoh *et al.*, 2008, Parise *et al.*, 2007, Conover *et al.*, 2010). Expression of Bps in the *pga* strain (*pga^{Bps}*) led to a significantly higher level of survival in the presence of human serum compared to that of the strain containing the expression vector alone (*pga^{vector}*) (Fig. 5A). The mechanism of protection of *E. coli* from serum-mediated killing followed a pattern similar to that observed in the case of the WT and the mutant strains of *B. pertussis*, since higher levels of C3 and C4 were associated with the *pga^{vector}* strain compared to the *pga^{Bps}* strain (Fig. 5B). Analysis by flow cytometry further supported the finding that Bps limits C3 and C4 deposition when expressed in *E. coli*, since the *pga^{vector}* strain had a relatively high MFI for both C3 and C4 (Figs. 5C and 5D; and Table 1) compared to that for the *pga^{Bps}* strain. These results confirm that Bps can confer resistance to serum-dependent killing and inhibits the deposition of complement proteins independent of other *B. pertussis* factors.

Comparison of the bps and brkA mutant strains

The *B. pertussis* BrkA protein contributes to complement resistance of *B. pertussis* by inhibiting the deposition of complement proteins C3, C4 and C9 and production of the soluble membrane attack complex (Barnes *et al.*, 2001). Given the overlap in the roles of BrkA and Bps, we compared the serum-dependent killing of the *brkA* and *bps* mutant strains. On incubation with NHS, the *bps* mutant strain survived at slightly higher numbers than the *brkA* mutant strain (% survival for the WT, *bps* and the *brkA* mutant was 57.9 ± 1.42 , 5.87 ± 0.48 and 1.72 ± 0.37 , respectively) (Fig. 6A). By Western blot analyses using a BrkA-specific antibody, we found that there were no significant alteration in the expression of BrkA in the WT and the *bps* mutant strains (Fig. 6B). Furthermore, utilizing an antibody raised against the PNAG polysaccharide of *S. aureus*, we confirmed that the *brkA*-mutant strain produced Bps (Fig. 6C).

Bps confers resistance to complement-mediated clearance of *B. pertussis* in vivo

Previously, we found that the *bps* mutant had an early defect in the colonization of the lungs (Conover *et al.*, 2010). If this colonization defect correlates with the enhanced complement susceptibility observed above, we predicted that the reduced colonization ability of the *bps* mutant strain will be mitigated in the absence of a functional complement system *in vivo*. To test this, we used C3^{-/-} mice, which lack a functional complement system due to the absence of C3.

Before we continued with the animal experiments, we compared the killing ability of the sera from C57Bl/6 mice to that of the sera from C3^{-/-} mice. Anti-*Bordetella* antibodies were not detected in the sera harvested from either C57Bl/6 or C3^{-/-} mice (data not shown). As shown in Fig. 7A, compared to the WT strain, the mutant strain was significantly more sensitive to naïve C57Bl/6 serum after 30 min of incubation. On longer incubation (60 min), both strains were equally susceptible to serum-mediated killing. As expected, C3-deficient mouse serum did not result in killing of either the WT or the *bps* strain. Taken together, these results suggest that the presence of Bps protects *B. pertussis* from being killed by naïve mouse serum and that mouse C3 plays a critical role in serum-mediated killing.

Along with C57Bl/6 mice, C3^{-/-} mice were intranasally inoculated with 5×10^5 CFU of either the WT or the *bps* strains and the bacterial burden in the lungs was determined at the early time point, 3 days post-inoculation. Consistent with previous results (Conover *et al.*, 2010), the lungs of C57Bl/6 mice contained approximately 1.5 log less of the mutant bacteria than that of the WT strain (Conover *et al.*, 2010) (Fig. 7B). There were no significant differences in the bacterial loads of the WT strain contained in the lungs of the C57Bl/6 or the C3^{-/-} mice, a finding consistent with previously reported results (Elder *et al.*, 2004) (Fig. 7B). The lungs of the C3^{-/-} mice had significantly higher load of the mutant bacteria than those found in the lungs of C57Bl/6 mice. Note that while elevated levels of the mutant bacteria were observed in C3^{-/-} mice, these levels did not reach the levels observed for the WT bacteria, suggesting that immune components other than complement proteins are also required for controlling the replication of mutant strain. Collectively, these results suggest that the colonization defect of the *bps* mutant strain in the mouse lungs can be explained by its higher sensitivity to complement. These results further suggest that Bps

functions as a critical pathogenic determinant of *B. pertussis* by resisting the activity of complement in the respiratory tract.

Discussion

We have studied the interaction between the *B. pertussis* Bps polysaccharide and the host complement system. We found that Bps provides resistance to complement-mediated clearance in the mouse respiratory tract. We first established that the expression of Bps by both the prototype laboratory strain and a recent clinical isolate of *B. pertussis* resulted in a significant reduction in *in vitro* killing by serum harvested from human and mouse and from both naïve and immune serum. We present multiple lines of evidence that the classical pathway of the complement system is responsible for the serum-mediated killing of both the WT and the *bps* mutant strain. First, treatment of serum with EGTA, an inhibitor of the classical and MBL pathway resulted in very little killing of the *bps* mutant. Second, serum-mediated killing was dependent on the activity of C4, a complement component necessary for the activation of the classical and MBL pathway and not the alternative pathway. Finally, we show that the killing by serum is dependent on the presence of C1q, a complement component required for the activation of only the classical pathway. Overall, these results suggest that the ability to express Bps by *B. pertussis* is critical for resisting the deleterious actions of complement *in vitro*.

To further dissect the relationship between resistance to complement killing and the presence of Bps, we asked if Bps can inhibit the deposition of complement components. We found that deficiency of Bps led to an increased deposition of C3, C4 and the MAC complex. This suggests that without Bps, *B. pertussis* triggers a higher level of complement activation and becomes more susceptible to MAC deposition leading to enhanced killing of the *bps*-mutant strain.

BrkA is the most well-characterized factor of *B. pertussis* that promotes resistance to complement-mediated killing by inhibiting CP (Barnes *et al.*, 2001). We found that the *brkA* and the *bps* mutants displayed approximately similar susceptibility to serum-mediated killing. Our results further show that similar to BrkA, presence of Bps inhibited the deposition of C4, C3, and MAC onto *B. pertussis* surface. It is an open question whether Bps and BrkA promote complement resistance through mechanistically similar or different pathways.

A striking result reported herein was our finding that ectopic expression of Bps in *E. coli* conferred resistance to complement mediated killing and inhibited complement deposition. These results raise two possible roles for Bps in resisting complement activities. First, Bps could serve in a non-specific “masking” role whereby targets of complement activation are physically inaccessible due to the complex matrix. The finding that Bps can protect *E. coli* from complement-mediated killing would indicate that these key complement activating signatures are common to both *E. coli* and *B. pertussis*. Similarly, these common targets must serve as conserved triggers that antibodies recognize, since the *bps* strain activated complement through C1q-dependent classical pathway.

Alternatively, Bps could actively inhibit complement deposition and activity. This could possibly be due to the sequestration of complement factors, competitive inhibition to prevent complex formation and/or recruitment of host regulators. It is unlikely that Bps would do this through mechanisms like the cellular CD46/factor I pathway, since we did not observe increased levels of the cleaved iC3b inactive component when Bps was present. Interestingly, we also did not observe differences in the deposition of C1 inhibitor on the surfaces of either the WT or the *bps* strain (our preliminary data). The elucidation of how Bps confers resistance to complement will be the focus of future work.

It was previously proposed that PIA of *Staphylococcus epidermidis*, a Bps homologue, may promote the lectin pathway of complement activation (Kristian *et al.*, 2008). Mannose binding lectin recognizes mannose on the bacterial surface. In addition both MBL and serum-ficolins have lectin activity for GlcNAc. Among the seven polysaccharides whose chemical structure was found to be similar to that of Bps, mannose has not been found to be a constituent (Izano *et al.*, 2007, Maira-Litran *et al.*, 2002, Choi *et al.*, 2009, Wang *et al.*, 2004, Mack *et al.*, 1996). Our results demonstrate that lectin pathway does not play a major role in recognition of Bps and suggest that this may be true for other polysaccharides of this family.

We then tested the influence of the complement *in vivo*. We previously reported that compared to the WT strain, the *bps* strain colonized the lungs at lower numbers at initial stages of infection (Conover *et al.*, 2010). We reasoned that the reduced ability of the *bps* mutant strain to colonize the mouse lungs is a result of increased *in vivo* susceptibility to complement activity, which in turn is a reflection of the enhanced deposition of complement components on the surface of the mutant strain in the respiratory tract. Based on this line of reasoning, we hypothesized that complement system will be important for controlling the replication of the *bps* mutant strain in the mouse lungs. For this purpose, we utilized C3 knockout mice. In the complete absence of C3, almost all the biological properties associated with the complement system are absent (Walport, 2001b, Walport, 2001a). By quantitating bacterial numbers from the wild-type and C3^{-/-} mice, we found that C3 is required for reduction of the numbers of the mutant strain in the lungs at 3 days post-inoculation. In contrast, there were no significant differences between the numbers of the WT strain harvested from the lungs of wild-type or the C3-deficient mice. This suggests that expression of Bps by *B. pertussis* is important at an early stage of infection for resisting the activity of the complement system in the respiratory tract.

Similar to *B. pertussis*, for two other *Bordetella* species, *B. bronchiseptica* and *B. parapertussis*, no correlation was found between *in vitro* sensitivity to complement-mediated killing and the ability to colonize the mouse lungs (Harvill *et al.*, 2000). Both *B. bronchiseptica* and *B. parapertussis* harbor the genetic capacity to express Bps (Parise *et al.*, 2007) and Bps is critical for respiratory tract colonization of *B. bronchiseptica* (Sloan *et al.*, 2007). Future studies will address if Bps is essential for resisting serum complement mediated killing *in vitro* and in the respiratory tract for these two species.

In summary, by combining the tools of bacterial and mammalian genetics, we were able to assess the importance of a *B. pertussis* virulence factor in resisting innate immune defenses

in the respiratory tract. Our data suggest that the interaction between Bps and the complement system is pivotal in determining the outcome of *B. pertussis* infections.

Experimental Procedures

Bacterial strains, plasmids, culture conditions and reagents

Bacterial strains and plasmids used in this study are listed in Table 2. *B. pertussis* strains were maintained on Bordet–Gengou agar (BG) supplemented with 10% defibrinated sheep blood. Liquid cultures were grown in Stainer–Scholte (SS) broth with heptakis (2,6-di-*O*-methyl- β -cyclodextrin) (Stainer *et al.*, 1970, Conover *et al.*, 2011). *E. coli* strains were grown in Luria–Bertani medium. As necessary, the growth media were supplemented with appropriate antibiotics, chloramphenicol (Cm, 10 or 50 $\mu\text{g ml}^{-1}$), kanamycin (Km, 25 $\mu\text{g ml}^{-1}$) and streptomycin (Sm, 100 $\mu\text{g ml}^{-1}$).

Normal human serum, C4, C8 and C1q depleted human serum and purified proteins C1q and C4 were procured from Complement Technologies (Tyler, TX). Naïve mouse serum from C57Bl/6 mice and C3^{-/-} mouse (C57Bl/6 background) was collected at WFSM, processed and stored as described earlier (Johnson *et al.*, 2008). Heat inactivated human serum was prepared by heating to 56°C for 30 min. A *B. pertussis* specific ELISA showed undetectable levels of anti-*B. pertussis* antibody in mouse sera while the pooled human serum was immunoreactive.

Serum sensitivity assays

Bacteria were grown to mid-log phase, washed and diluted in PBS with Mg^{2+} and Ca^{2+} to a final concentration of 10^4 CFU ml^{-1} . Approximately 1000 CFU of bacteria were treated with varying concentrations of serum for fixed time or set serum concentration for different times as indicated and incubated at 37°C. In order to inhibit the CP, 10 mM EGTA was added to the serum, while 10 mM EDTA was used to block all complement pathways. The assays were stopped by transferring the samples to ice. Colonies were enumerated by plating on BG agar. Percent survival was calculated from the PBS controls. Resistance to CP was specifically addressed using C1q and C4 depleted human serum or by reconstituting the respective depleted serum with either C1q (180 $\mu\text{g ml}^{-1}$) or C4 (640 $\mu\text{g ml}^{-1}$) and assayed as described above.

Western blot analysis

Bacteria were grown to mid-log phase. 1×10^9 cells of WT or *bps* strains were treated either with 5% NHS, 5% NHS depleted of C8 (NHS(-C8), or PBS for 10 min at 37°C. Cells were pelleted by centrifugation, washed twice with PBS followed by resuspension in PBS. The cells were boiled for 5 min in the presence of 1X SDS-PAGE gel loading buffer containing β -mercaptoethanol. Following boiling, the suspension was sonicated, centrifuged and the supernatants were loaded on SDS polyacrylamide gel. Following SDS-PAGE, western blotting was carried out by transferring the samples onto nitrocellulose membranes. Blots were blocked overnight in 5% milk, followed by probing with appropriate primary antibodies. Goat anti-human C3 or C4 (Complement Technology) was used at a dilution of 1:2000 followed by HRP-labeled anti goat antibody (1:5000, BioRad).

For detection of BrkA, strains were grown as above and 1×10^9 bacteria after washing were directly lysed in 1X SDS-PAGE gel loading buffer containing β -mercaptoethanol. The membrane was probed with rabbit anti-BrkA antibody (1:50,000) (kindly provided by Dr. Rachel Fernandez). Following primary antibody incubation, membrane was probed with appropriate HRP-labeled secondary antibodies. The chemiluminescence substrate ECL plus (GE Healthcare) kit was used for detection.

For detection of Bps, approximately 1×10^{10} cells of stationary phase cultures of different strains were collected by centrifugation, resuspended in 100 μ l of 0.5 M EDTA pH 8.0 and boiled for 5 min at 100°C. Cells were removed by centrifugation and the supernatant was treated with proteinase K (1 mg/ml) for 3 h at 65°C followed by incubation for 15 min at 85°C. After extracting twice with Phenol: Chloroform (1:1), 10 μ l aliquot of the extracts was spotted onto a nitrocellulose membrane and allowed to air dry overnight. The blot was blocked with 5% nonfat milk and then probed with a 1:2,000 dilution of goat anti-PNAG antibody raised against *S. aureus* dPNAG followed by HRP-labeled anti goat antibody (1:5000, BioRad) as described earlier. The chemiluminescence substrate ECL plus (GE Healthcare) kit was used for detection.

Flow cytometry analysis

Mid-log phase bacteria (1×10^9) were washed with PBS, treated with 5% NHS at 37°C for 10 min, followed by addition of EDTA to a final concentration of 10 mM and incubation on ice to stop the reaction. Bacteria were pelleted down and washed with PBS, the deposited C3, C4 or C5b-9 was probed using goat anti-human C3 or C4, (1:750 dilution, MyBiosource) or a mouse monoclonal antibody against a neo-epitope of C5b-9 (10 μ g ml⁻¹, Quidel). The bound antibody was detected using an Alexa Fluor 633 conjugated anti goat or anti-mouse (Molecular Probes) secondary antibody and the samples were analyzed on a FACS Calibur instrument (BD Biosciences) post fixation with 2% paraformaldehyde. Relevant controls included unstained samples or serum exposed bacteria probed with goat polyclonal antibody (Jackson Immunoresearch) or mouse IgG1 κ isotype (Biolegend).

Electron microscopy

Electron microscopic analysis of C3, C4 deposition and C5b-9 formation on bacterial surface was carried out by adsorbing 1×10^3 bacteria onto carbon coated gold grids (Electron microscopy sciences, PA) in a humidified chamber for 1 h. The bacteria on the grids were exposed to 5% NHS for 10 min at 37°C, blocked with 2% bovine serum albumin (BSA) in PBS, then probed with anti-C3 and anti-C4 polyclonal antibodies at a dilution of 1:10 or 50 μ g ml⁻¹ of anti-sC5b-9 in the blocking buffer. The bound antibodies were detected with 12 nm gold labeled anti-goat or anti-mouse antibody (Jackson ImmunoResearch Laboratories, PA). Finally the bacteria were subjected to negative staining with 2% phosphotungstic acid (pH 6.6) and analyzed with a Technai transmission electron microscope.

Animal experiments

C3^{-/-} and C57Bl/6 mice were procured from Jackson Laboratories and maintained in *Bordetella*-free environment under aseptic conditions on irradiated chow and autoclaved water. Mice were intranasally inoculated with 5×10^5 CFU of the strains in 50 μ l of sterile

PBS. Prior to inoculation, mice were anesthetized by isoflurane, delivered using a SurgiVet® Vaporstick small animal anesthesia machine equipped with a Classic T3™ isoflurane vaporizer (Smith Medical, Dublin, OH). Mice were exposed to 2% isoflurane delivered in O₂ (1.5 l min⁻¹). Intranasal administration was performed by pipetting either PBS or bacterial suspension onto the outer edge of each nostril. Animals were sacrificed 3 days post-infection and the lungs were harvested. Three right lung lobes were homogenized in sterile PBS, plated on BG agar containing Sm followed by enumeration of CFU. All animal experiments were approved by the WFSM Animal Care and Use Committee (IACUC).

Acknowledgments

We thank Drs. Rachel Fernandez, Allison Weiss, Dr. Gerald B. Pier and Tony Romeo for providing strains and sera. Research in the laboratory of RD is supported by funds from the NIH (grant no. 1R01AI075081).

Abbreviations

CP	classical pathway
LP	lectin pathway
AP	alternative pathway
MAC	membrane attack complex
NHS	normal human serum

References

1. Pertussis epidemic--Washington, 2012. MMWR Morb Mortal Wkly Rep. 2012; 61:517–522. [PubMed: 22810264]
2. Amdahl H, Jarva H, Haanpera M, Mertsola J, He Q, Jokiranta TS, Meri S. Interactions between Bordetella pertussis and the complement inhibitor factor H. Mol Immunol. 2011; 48:697–705. [PubMed: 21167605]
3. Barnes MG, Weiss AA. BrkA protein of Bordetella pertussis inhibits the classical pathway of complement after C1 deposition. Infect Immun. 2001; 69:3067–3072. [PubMed: 11292725]
4. Barnes MG, Weiss AA. Activation of the complement cascade by Bordetella pertussis. FEMS Microbiol Lett. 2003; 220:271–275. [PubMed: 12670691]
5. Berggard K, Johnsson E, Mooi FR, Lindahl G. Bordetella pertussis binds the human complement regulator C4BP: role of filamentous hemagglutinin. Infect Immun. 1997; 65:3638–3643. [PubMed: 9284130]
6. Berggard K, Lindahl G, Dahlback B, Blom AM. Bordetella pertussis binds to human C4b-binding protein (C4BP) at a site similar to that used by the natural ligand C4b. Eur J Immunol. 2001; 31:2771–2780. [PubMed: 11536176]
7. Bolger MS, Ross DS, Jiang H, Frank MM, Ghio AJ, Schwartz DA, Wright JR. Complement levels and activity in the normal and LPS-injured lung. Am J Physiol Lung Cell Mol Physiol. 2007; 292:L748–759. [PubMed: 17071722]
8. Brown JS, Hussell T, Gilliland SM, Holden DW, Paton JC, Ehrenstein MR, et al. The classical pathway is the dominant complement pathway required for innate immunity to Streptococcus pneumoniae infection in mice. Proc Natl Acad Sci U S A. 2002; 99:16969–16974. [PubMed: 12477926]
9. Burns VC, Pishko EJ, Preston A, Maskell DJ, Harvill ET. Role of Bordetella O antigen in respiratory tract infection. Infect Immun. 2003; 71:86–94. [PubMed: 12496152]

10. Caroff M, Brisson J, Martin A, Karibian D. Structure of the *Bordetella pertussis* 1414 endotoxin. *FEBS Lett.* 2000; 477:8–14. [PubMed: 10899302]
11. Celentano LP, Massari M, Paramatti D, Salmaso S, Tozzi AE. Resurgence of pertussis in Europe. *Pediatr Infect Dis J.* 2005; 24:761–765. [PubMed: 16148840]
12. Cherry JD. Epidemic pertussis in 2012--the resurgence of a vaccine-preventable disease. *N Engl J Med.* 2012; 367:785–787. [PubMed: 22894554]
13. Choi AH, Slamti L, Avci FY, Pier GB, Maira-Litran T. The *pgaABCD* locus of *Acinetobacter baumannii* encodes the production of poly-beta-1-6-N-acetylglucosamine, which is critical for biofilm formation. *J Bacteriol.* 2009; 191:5953–5963. [PubMed: 19633088]
14. Conover MS, Mishra M, Deora R. Extracellular DNA is essential for maintaining *Bordetella* biofilm integrity on abiotic surfaces and in the upper respiratory tract of mice. *PLoS One.* 2011; 6:e16861. [PubMed: 21347299]
15. Conover MS, Sloan GP, Love CF, Sukumar N, Deora R. The Bps polysaccharide of *Bordetella pertussis* promotes colonization and biofilm formation in the nose by functioning as an adhesin. *Mol Microbiol.* 2010; 77:1439–1455. [PubMed: 20633227]
16. Elder KD, Harvill ET. Strain-dependent role of BrkA during *Bordetella pertussis* infection of the murine respiratory tract. *Infect Immun.* 2004; 72:5919–5924. [PubMed: 15385494]
17. Fernandez RC, Weiss AA. Cloning and sequencing of a *Bordetella pertussis* serum resistance locus. *Infect Immun.* 1994; 62:4727–4738. [PubMed: 7927748]
18. Fernandez RC, Weiss AA. Serum resistance in *bvg*-regulated mutants of *Bordetella pertussis*. *FEMS Microbiol Lett.* 1998; 163:57–63. [PubMed: 9631546]
19. Franca A, Vilanova M, Cerca N, Pier GB. Monoclonal antibody raised against PNAG has variable effects on static *S. epidermidis* biofilm accumulation in vitro. *Int J Biol Sci.* 2013; 9:518–520. [PubMed: 23781145]
20. Gross GN, Rehm SR, Pierce AK. The effect of complement depletion on lung clearance of bacteria. *J Clin Invest.* 1978; 62:373–378. [PubMed: 27534]
21. Hallstrom T, Riesbeck K. *Haemophilus influenzae* and the complement system. *Trends Microbiol.* 2010; 18:258–265. [PubMed: 20399102]
22. Harvill ET, Preston A, Cotter PA, Allen AG, Maskell DJ, Miller JF. Multiple roles for *Bordetella* lipopolysaccharide molecules during respiratory tract infection. *Infect Immun.* 2000; 68:6720–6728. [PubMed: 11083787]
23. Hyams C, Camberlein E, Cohen JM, Bax K, Brown JS. The *Streptococcus pneumoniae* capsule inhibits complement activity and neutrophil phagocytosis by multiple mechanisms. *Infect Immun.* 2010; 78:704–715. [PubMed: 19948837]
24. Itoh Y, Rice JD, Goller C, Pannuri A, Taylor J, Meisner J, et al. Roles of *pgaABCD* genes in synthesis, modification, and export of the *Escherichia coli* biofilm adhesin poly-beta-1,6-N-acetyl-D-glucosamine. *J Bacteriol.* 2008; 190:3670–3680. [PubMed: 18359807]
25. Izano EA, Sadovskaya I, Vinogradov E, Mulks MH, Velliyagounder K, Ragunath C, et al. Poly-N-acetylglucosamine mediates biofilm formation and antibiotic resistance in *Actinobacillus pleuropneumoniae*. *Microb Pathog.* 2007; 43:1–9. [PubMed: 17412552]
26. Johnson JB, Capraro GA, Parks GD. Differential mechanisms of complement-mediated neutralization of the closely related paramyxoviruses simian virus 5 and mumps virus. *Virology.* 2008; 376:112–123. [PubMed: 18440578]
27. Kendrick PL. Can whooping cough be eradicated? *J Infect Dis.* 1975; 132:707–712. [PubMed: 1202113]
28. Kovach ME, Phillips RW, Elzer PH, Roop RM 2nd, Peterson KM. pBBR1MCS: a broad-host-range cloning vector. *Biotechniques.* 1994; 16:800–802. [PubMed: 8068328]
29. Kristian SA, Birkenstock TA, Sauder U, Mack D, Gotz F, Landmann R. Biofilm formation induces C3a release and protects *Staphylococcus epidermidis* from IgG and complement deposition and from neutrophil-dependent killing. *J Infect Dis.* 2008; 197:1028–1035. [PubMed: 18419540]
30. Kugelberg E, Gollan B, Tang CM. Mechanisms in *Neisseria meningitidis* for resistance against complement-mediated killing. *Vaccine.* 2008; 26(Suppl 8):I34–39. [PubMed: 19388162]

31. Kurvers RA, Westra D, van Heijst AF, Walk TL, Warris A, van de Kar NC. Severe infantile *Bordetella pertussis* pneumonia in monozygotic twins with a congenital C3 deficiency. *Eur J Pediatr*. 2013
32. Mack D, Fischer W, Krokotsch A, Leopold K, Hartmann R, Egge H, Laufs R. The intercellular adhesin involved in biofilm accumulation of *Staphylococcus epidermidis* is a linear beta-1,6-linked glucosaminoglycan: purification and structural analysis. *J Bacteriol*. 1996; 178:175–183. [PubMed: 8550413]
33. Maira-Litran T, Kropec A, Abeygunawardana C, Joyce J, Mark G 3rd, Goldmann DA, Pier GB. Immunochemical properties of the staphylococcal poly-N-acetylglucosamine surface polysaccharide. *Infect Immun*. 2002; 70:4433–4440. [PubMed: 12117954]
34. Marr N, Luu RA, Fernandez RC. *Bordetella pertussis* binds human C1 esterase inhibitor during the virulent phase, to evade complement-mediated killing. *J Infect Dis*. 2007; 195:585–588. [PubMed: 17230419]
35. Marr N, Shah NR, Lee R, Kim EJ, Fernandez RC. *Bordetella pertussis* autotransporter Vag8 binds human C1 esterase inhibitor and confers serum resistance. *PLoS One*. 2011; 6:e20585. [PubMed: 21695123]
36. Martinez de Tejada G, Miller JF, Cotter PA. Comparative analysis of the virulence control systems of *Bordetella pertussis* and *Bordetella bronchiseptica*. *Mol Microbiol*. 1996; 22:895–908. [PubMed: 8971711]
37. Meri T, Amdahl H, Lehtinen MJ, Hyvarinen S, McDowell JV, Bhattacharjee A, et al. Microbes bind complement inhibitor factor H via a common site. *PLoS Pathog*. 2013; 9:e1003308. [PubMed: 23637600]
38. Mooi FR. *Bordetella pertussis* and vaccination: the persistence of a genetically monomorphic pathogen. *Infect Genet Evol*. 2010; 10:36–49. [PubMed: 19879977]
39. Neo Y, Li R, Howe J, Hoo R, Pant A, Ho S, Alonso S. Evidence for an intact polysaccharide capsule in *Bordetella pertussis*. *Microbes Infect*. 2010; 12:238–245. [PubMed: 20005302]
40. Parise G, Mishra M, Itoh Y, Romeo T, Deora R. Role of a putative polysaccharide locus in *Bordetella* biofilm development. *J Bacteriol*. 2007; 189:750–760. [PubMed: 17114249]
41. Reeves P. Role of O-antigen variation in the immune response. *Trends Microbiol*. 1995; 3:381–386. [PubMed: 8564356]
42. Sloan GP, Love CF, Sukumar N, Mishra M, Deora R. The *Bordetella* Bps Polysaccharide is Critical for Biofilm Development in the Mouse Respiratory Tract. *J Bacteriol*. 2007
43. Stainer DW, Scholte MJ. A simple chemically defined medium for the production of phase I *Bordetella pertussis*. *J Gen Microbiol*. 1970; 63:211–220. [PubMed: 4324651]
44. Steele RW. Pertussis is eradication achievable? *Pediatr Ann*. 2004; 33:525–534. [PubMed: 15354604]
45. Walport MJ. Complement. First of two parts. *N Engl J Med*. 2001a; 344:1058–1066. [PubMed: 11287977]
46. Walport MJ. Complement. Second of two parts. *N Engl J Med*. 2001b; 344:1140–1144. [PubMed: 11297706]
47. Wang X, Preston JF 3rd, Romeo T. The *pgaABCD* locus of *Escherichia coli* promotes the synthesis of a polysaccharide adhesin required for biofilm formation. *J Bacteriol*. 2004; 186:2724–2734. [PubMed: 15090514]
48. Watford WT, Ghio AJ, Wright JR. Complement-mediated host defense in the lung. *Am J Physiol Lung Cell Mol Physiol*. 2000; 279:L790–798. [PubMed: 11053012]
49. Wessels MR, Butko P, Ma M, Warren HB, Lage AL, Carroll MC. Studies of group B streptococcal infection in mice deficient in complement component C3 or C4 demonstrate an essential role for complement in both innate and acquired immunity. *Proc Natl Acad Sci U S A*. 1995; 92:11490–11494. [PubMed: 8524789]

Fig. 1.

Presence of Bps promotes resistance of *B. pertussis* to serum-mediated killing. *B. pertussis* strains were incubated with different concentrations of normal human serum (NHS) followed by enumerating the surviving bacteria by plating on BG agar. Percent survival was calculated by dividing the CFU obtained from control reactions incubated with PBS from the CFU obtained from the test reactions. Data are representative of one of three experiments performed in triplicates. **, $p < 0.01$ and ***, $p < 0.001$. The different strains were incubated with indicated concentrations of NHS (A and B), with 2% NHS (C) for 15 min or with 5% NHS for indicated time points (D). Strains used were: the WT strain Bp536 and its isogenic derivative the Δbps strain (A and D); the clinical isolate GMT1 and its isogenic derivative GMT1 Δbps (B); the Δbps strain in the Bp536 background containing the vector plasmid pBBR1MCS (Δbps^{Vector}) or the vector plasmid containing the cloned *bpsA-D* locus (Δbps^{BPS}) (C).

Fig. 2.

The WT and *bps* strains differ in susceptibility to killing by C4 and C1q-dependent pathways. A. The complement cascade. The classical, lectin and alternative pathways of complement activation are represented without substantial details. In the classical pathway, antigen-antibody forms an immune complex thereby activating C1. The lectin pathway after activation proceeds through the action of C4 and C2 to produce activated complement proteins further down the cascade. Alternative pathway activates with spontaneous hydrolysis of C3 on microbial surface. All three pathways converge on the cleavage of C3 via formation of C3b. Activated C3 triggers a proteolytic cascade leading to the assembly of C5b-9 complex or the membrane attack complex (MAC) on microbial surface.

B. *B. pertussis* WT strain or the *bps* mutant were treated for 15 min with 5% NHS, heat inactivated NHS (HI-NHS), C8 depleted NHS (NHS(-C8)), NHS with addition of either EDTA (10mM) or EGTA (10mM) with MgCl₂ (2mM), C1q depleted or C1q reconstituted NHS and C4 depleted or C4 reconstituted NHS. Cells were plated on BG agar to enumerate the CFU recovered. Percent survival was calculated by dividing the CFU obtained from control reactions incubated with PBS from the CFU obtained from the test reactions. Data are representative of one of three experiments performed in triplicates. Error bars denote standard error. *, p < 0.05; **, p < 0.01.

Fig. 3.

Complement factors C3, C4 and MAC are deposited to a greater extent on the *bps* strain. 1×10^9 cells of WT or *bps* bacteria were treated either with 5% NHS (A, B, D, E and F) or with 5% NHS depleted of C8 (C) for 10 min at 37°C.

Top panels; Western blot analyses for deposition of C3b (A and left panel of C), C4b (B and right panel of C). Purified C3b (panel A, extreme left lane) and C4b (panel B, extreme right lane) were used as markers while bacteria without serum treatment (control) served as the negative control. Following detection of C3b and C4b, the respective membranes were stripped of antibodies and immunoblotting for detection of BrkA as a loading control (bottom panels in figures A, B and C) was carried out.

Bottom panels, Flow cytometry analyses of C3 (D), C4 (E) and C5b-9 (F) deposition on WT and *bps* strains. For detection, anti-C3 (D), anti-C4 (E) and anti- C5b-9 neo-epitope specific (F) primary antibodies followed by Alexa Fluor 633 conjugated anti-goat antibody (D and E) and Alexa fluor 633 conjugated anti-mouse antibody (F) were used. Solid lines represent isotype control, dotted lines represent WT bacteria and the dashed lines represent *bps* bacteria. M1 and M2 mark the negative and positive populations, respectively as determined by the isotype control.

Fig. 4. Complement factors C3, C4 and MAC are deposited to a greater extent on the *bps* strain. Electron micrograph of C3 (top panels), C4 (middle panels) and MAC (lower panels) deposition on WT (left panels) and *bps* (right panels) surface. Each arrow indicates one or a group of gold beads on the bacterial surface. Bar = 0.1 micron.

Fig. 5.

Bps polysaccharide confers resistance to *E. coli* from complement mediated killing. A. The *pga* strain of *E. coli* transformed either with a plasmid that allows expression of Bps (*pga*^{BPS}) or the vector plasmid only (*pga*^{vector}) was incubated with different concentrations of NHS followed by enumerating the surviving bacteria by plating on LB agar. Percent survival was calculated by dividing the CFU obtained from control reactions incubated with PBS from the CFU obtained from the test reactions. Data are representative of one of three experiments performed in triplicates. **, $p < 0.01$. B. Western blot analysis of C4 (top panel) and C3 (bottom panel) deposition on the *pga*^{BPS} or the *pga*^{vector} strains. Purified proteins C4b and C3b were used as markers (extreme left lanes) while bacteria treated without serum were used as control. The blots were probed with anti-C4 or anti-C3 antibodies. C and D. Flow cytometry analysis of C3 and C4 deposition on the *pga*^{BPS} and *pga*^{vector} strains. Anti-C3 and anti-C4 antibodies followed by anti-goat Alexa fluor 633 conjugated secondary antibody were used. Solid line represents an isotype control, dotted line represents *pga*^{BPS} bacteria and the dashed line represents *pga*^{vector} bacteria. M1 and M2 mark the negative and positive populations, respectively as determined by the isotype control.

Fig. 6. Comparison of WT, *bps* and *brkA*-deficient strains

A. Comparative serum survival of WT, *bps* and *brkA* (RFBP2152) mutant. Different strains were treated for 15 min with 2% NHS. Percent survival was calculated as described in Fig. 1. Data are representative of one of two experiments performed in triplicates *, $p < 0.05$; ***, $p < 0.001$.

B. Expression of BrkA is not altered in the *bps* strain. Equal numbers of RFBP2152 (*brkA* mutant) (Fernandez *et al.*, 1998), WT or the *bps* strain were lysed in SDS-PAGE loading buffer and whole cell lysates were analyzed by Western blotting for BrkA levels with anti-BrkA antibody. The positions of the protein markers (in kDa) are indicated to the left of the gel.

C. The *brkA* mutant expresses Bps. Exopolysaccharides were extracted from stationary phase cultures of different strains as described in the experimental procedures. Ten microliters of boiled EDTA extracts treated with proteinase K were spotted onto a nitrocellulose membrane and probed with a 1:2,000 dilution of goat antiserum raised against *S. aureus* dPNAG polysaccharide followed by mouse anti-goat IgG conjugated to HRP.

Fig. 7.

Bps polysaccharide promotes resistance of *B. pertussis* to killing by mouse serum and to complement in the respiratory tract. A. WT or *bps* strains were incubated with 10% naïve mouse serum harvested from C57Bl/6 mice or C3 knockout mice (C3^{-/-}) for the indicated time points followed by enumerating the surviving bacteria by plating on BG agar. B. WT or *bps* strains were inoculated through the intranasal route in either C57Bl/6 or C3^{-/-} mice. Three days post inoculation the residing bacteria in the lungs were enumerated by plating out the lung homogenates on BG agar. Bars represent the average of each group. **, p 0.01.

Table 1

Mean fluorescence intensity of strains for deposition of C3, C4 and MAC after incubation in 5% human serum.

Strains	C3	C4	MAC
WT	30.5 ± 4.5	28.67 ± 3.00	7.66 ± 0.14
<i>bps</i>	186.33 ± 17.92	91.83 ± 12.19	90.2 ± 14.34
<i>pga</i> ^{BPS}	61.7 ± 9.10	22.03 ± 0.96	n.d
<i>pga</i> ^{vector}	219.33 ± 25.96	141.33 ± 15.53	n.d

n.d.=not determined

Table 2

Bacterial strains and plasmids

Strains and plasmids	Characteristics	Reference/source
<u>Strains</u>		
<u><i>B. pertussis</i></u>		
Bp536	Wild-type reference strain	Laboratory stock
<i>bps</i>	Bp536 derivative containing an in-frame deletion in the <i>bpsABCD</i> locus	(Conover <i>et al.</i> , 2010)
GMT1	Clinical strain of <i>B. pertussis</i>	(Martinez de Tejada <i>et al.</i> , 1996)
GMT1 <i>bps</i>	GMT1 derivative containing an in-frame deletion in the <i>bpsABCD</i> locus	This study
RFBP2152	<i>brkA</i> mutant strain	(Fernandez <i>et al.</i> , 1998)
<u><i>E. coli</i></u>		
<i>pga</i>	<i>E. coli</i> strain TRXWMG1655 containing a deletion in <i>pga</i> locus	(Itoh <i>et al.</i> , 2008)
<u>Plasmids</u>		
pBBR1MCS	Broad-host-range plasmid; Cm ^R	(Kovach <i>et al.</i> , 1994)
pMM11	<i>bpsABCD</i> locus cloned into pBBR1MCS.	(Parise <i>et al.</i> , 2007)