



Bordetella bronchiseptica Diguanylate Cyclase BdcA Regulates Motility and Is Important for the Establishment of Respiratory Infection in Mice

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ABSTRACT Bacteria can be motile and planktonic or, alternatively, sessile and participating in the biofilm mode of growth. The transition between these lifestyles can be regulated by a second messenger, cyclic dimeric GMP (c-di-GMP). High intracellular c-di-GMP concentration correlates with biofilm formation and motility inhibition in most bacteria, including *Bordetella bronchiseptica*, which causes respiratory tract infections in mammals and forms biofilms in infected mice. We previously described the diguanylate cyclase BdcA as involved in c-di-GMP synthesis and motility regulation in *B. bronchiseptica*; here, we further describe the mechanism whereby BdcA is able to regulate motility and biofilm formation. Amino acid replacement of GGDEF with GGAAF in BdcA is consistent with the conclusion that diguanylate cyclase activity is necessary for biofilm formation and motility regulation, although we were unable to confirm the stability of the mutant protein. In the absence of the *bdcA* gene, *B. bronchiseptica* showed enhanced motility, strengthening the hypothesis that BdcA regulates motility in *B. bronchiseptica*. We showed that c-di-GMP-mediated motility inhibition involved regulation of flagellin expression, as high c-di-GMP levels achieved by expressing BdcA significantly reduced the level of flagellin protein. We also demonstrated that protein BB2109 is necessary for BdcA activity, motility inhibition, and biofilm formation. Finally, absence of the *bdcA* gene affected bacterial infection, implicating BdcA-regulated functions as important for bacterium-host interactions. This work supports the role of c-di-GMP in biofilm formation and motility regulation in *B. bronchiseptica*, as well as its impact on pathogenesis.

IMPORTANCE Pathogenesis of *Bordetella* spp., like that of a number of other pathogens, involves biofilm formation. Biofilms increase tolerance to biotic and abiotic factors and are proposed as reservoirs of microbes for transmission to other organs (trachea, lungs) or other hosts. Bis-(3'-5')-cyclic dimeric GMP (c-di-GMP) is a second messenger that regulates transition between biofilm and planktonic lifestyles. In *Bordetella bronchiseptica*, high c-di-GMP levels inhibit motility and favor biofilm formation. In the present work, we characterized a *B. bronchiseptica* diguanylate cyclase, BdcA, which regulates motility and biofilm formation and affects the ability of *B. bronchiseptica* to colonize the murine respiratory tract. These results provide us with a better understanding of how *B. bronchiseptica* can infect a host.

KEYWORDS biofilm, *Bordetella*, motility, c-di-GMP

Cyclic dimeric GMP (c-di-GMP) is a ubiquitous second messenger in bacteria that regulates multiple phenotypes (1, 2). In a multitude of bacteria, c-di-GMP regulates transitions between a motile, planktonic lifestyle and a sessile, biofilm mode of growth.

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Moreover, in several pathogens, c-di-GMP also influences virulence (2). The intracellular concentration of c-di-GMP is regulated by diguanylate cyclases (DGCs) and phosphodiesterases (PDEs). Enzymes with DGC activity share a domain characterized by the presence of a GGDEF (or similar) sequence; these enzymes synthesize c-di-GMP from two molecules of GTP (3). The c-di-GMP-degrading PDE activity is associated with enzymes that have either EAL or HD-GYP motifs. The c-di-GMP network is complex and regulates phenotypes through different mechanisms, including gene expression regulation, posttranslational regulation, protein conformation modulation, and protein-protein interactions (3). Indeed, c-di-GMP can regulate rapid transitions between lifestyles, thus likely allowing pathogens to adapt quickly to new environments during transitions between hosts. Recently, we showed that c-di-GMP regulates motility and biofilm formation in *Bordetella bronchiseptica* (4). As for other bacteria, high c-di-GMP levels in *B. bronchiseptica* correlate with reduced motility and the biofilm-forming phenotype, suggesting that c-di-GMP signals could affect interactions with the host.

Bordetella bronchiseptica is a Gram-negative bacterium that causes respiratory tract infections, producing kennel cough in dogs, atrophic rhinitis in pigs, and snuffles in rabbits (5). The ability of this organism to form biofilms has been previously reported; the BvgAS two-component system is involved in control of biofilm formation (6, 7). Most known *B. bronchiseptica* virulence factors are also regulated by BvgAS (8). To date, no known signal has been described for the sensor histidine kinase BvgS, but it has been reported that BvgS is typically active with specific signals inhibiting its function (9). *In vitro* signals, such as low temperature, sulfate ions, and nicotinic acid (NA), have inhibitory effects on BvgS activity (8).

Inactive BvgAS results in the absence of virulence factor expression, resulting in the so-called “avirulent phase,” thought to be associated with free-living bacteria. When BvgAS is active, virulence factors are expressed and bacteria are considered to be in the “virulent phase,” which can infect a naive host (10). Virulent-phase bacteria are non-motile, and BvgAS is necessary and sufficient to inhibit flagellin expression during the virulent phase (11). It is also possible to observe an intermediate phenotype, presumably involved in transmission between hosts—the “intermediate phase” (12). Although the intermediate phase supports the most robust biofilm formation, *B. bronchiseptica* can form biofilms in any of the three phases (4). Further, transcriptome analysis has shown that genes independent of BvgAS regulation are differentially expressed during biofilm formation compared to those during planktonic culture (13). Thus, further studies are needed to elucidate all factors affecting biofilm formation, motility, and interaction with the host.

We previously showed that a putative DGC from *B. bronchiseptica*, the predicted gene product of the BB3576 gene, enhances biofilm formation and inhibits motility, consistent with its predicted DGC activity (4). Here, we confirm that BB3576 regulates biofilm formation and motility in *B. bronchiseptica*. We further demonstrate that BB3576 impacts flagellar expression and host infection, likely via its DGC activity, which suggests that c-di-GMP-mediated regulation is a key player in the pathogenesis of this organism.

RESULTS

Expression of *bdcA* (BB3576) increases c-di-GMP levels and reduces motility of *B. bronchiseptica*. We have shown previously that overexpression of BB3576, a predicted DGC of *B. bronchiseptica*, inhibits swimming motility in soft agar (4). We proposed that if BB3576 is a functional DGC, motility inhibition might be due to high c-di-GMP levels produced by this protein. To test this hypothesis, we quantified intracellular c-di-GMP in *B. bronchiseptica* with the BB3576 gene expressed from a plasmid under the control of a strong promoter (p_{nptII}) (Fig. 1A). c-di-GMP levels normalized by dry weight were ~9 times higher when BB3576 was expressed from the plasmid rather than the vector control (pEmpty) (Fig. 1A). When the GGDEF domain was replaced with GGAAF, the measured levels of c-di-GMP were equivalent to levels in the wild type (WT) carrying the control vector (Fig. 1A). We tried several approaches to

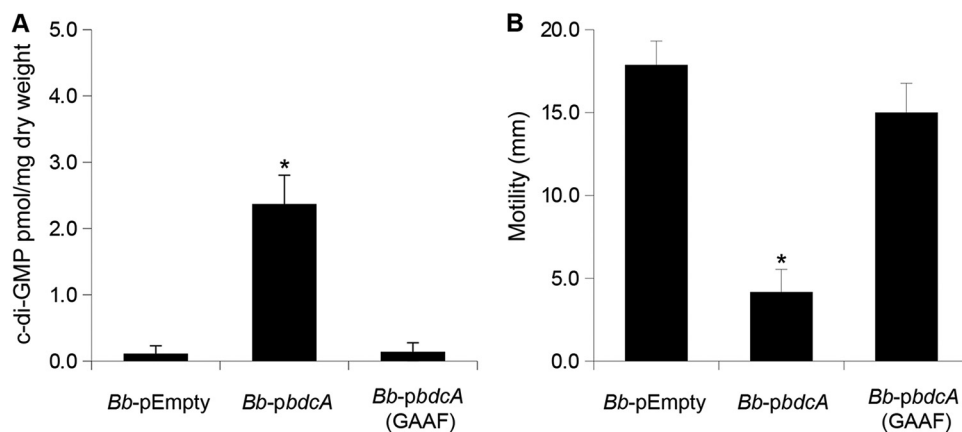


FIG 1 Expression of the *bdcA* (BB3576) gene from a plasmid increases c-di-GMP levels and reduces motility of *B. bronchiseptica*. (A) Quantitative measurements of cellular c-di-GMP concentration from strains carrying the *Bb*-pEmpty, *Bb*-*pbdcA*, and *Bb*-*pbdcA*(GAAF) plasmids. (B) Swimming motility using a soft agar (0.35%) motility assay with the *B. bronchiseptica* strains described for panel A. Diameters greater than 4 mm were indicative of motility. Means with standard errors are shown. Means marked with an asterisk are significantly different from those for *Bb*-pEmpty (Tukey, $P < 0.001$).

detect the WT and the mutant variant of the BB3576 protein when expressed from a plasmid, including using 6×His, 3×Flag, and streptavidin epitope tags as well as enriching for the membrane fraction of cell extracts for this predicted inner membrane protein, but we could not detect the WT or mutant protein. Thus, it is not clear if the phenotype of the BB3576-GGAFF mutant protein is due to loss of DGC activity or destabilization of the protein. It is important to note that similar mutations have been made in many DGCs with no apparent loss of protein stability (14–16). Moreover, only when intracellular c-di-GMP levels were high was motility reduced in the soft agar assay (Fig. 1). These results suggest that c-di-GMP produced by BB3576 inhibits motility; based on these findings, we named BB3576 the *Bordetella* diguanylate cyclase A (*bdcA*).

We previously showed that overexpression of *bdcA* induced a significant increase in biofilm levels (4), which is consistent with the high intracellular c-di-GMP levels observed here for the strain expressing this gene from a plasmid (Fig. 1A). As previously reported, expression of *bdcA* enhanced biofilm formation, modulating bacteria with either nicotinic acid or $MgSO_4$ (Fig. 2A; see also Fig. S1 in the supplemental material). The biomass detected by crystal violet (CV) assay includes live and dead cells, lysed cells, and other CV staining matrix components like polysaccharides and extracellular DNA (eDNA) that may lead to the observed increased values. Thus, we also analyzed the biofilm by scanning electron microscopy (SEM). *B. bronchiseptica* expressing *bdcA* from a plasmid showed larger three-dimensional (3D) structures than wild-type *B. bronchiseptica* in all conditions analyzed by SEM (Fig. 2C). Hence, we confirmed that the observed differences are a consequence of more bacteria forming a biofilm rather than simply increased production of matrix components.

Biofilm enhancement was not observed in bacteria carrying the empty vector or expressing the *bdcA*-GAFF variant, suggesting that c-di-GMP production is required for the enhanced biofilm formation observed when *bdcA* is overexpressed. We also deleted the *bdcA* gene in *B. bronchiseptica* Bb9.73 and evaluated biofilm formation by the *Bb*Δ*bdcA* mutant compared with that of the wild-type strain at virulent, intermediate, and avirulent phases. There was no statistically significant reduction in biofilm formation for any nicotinic acid concentration tested for the *Bb*Δ*bdcA* mutant versus that of the WT control (Fig. 2B).

B. bronchiseptica and *Bordetella pertussis* can form biofilm-like structures on nasal epithelial cells *in vivo* (17). Although the Δ*bdcA* deletion mutant did not affect biofilm formation on the abiotic surface tested here, we evaluated biofilm formation on a biotic

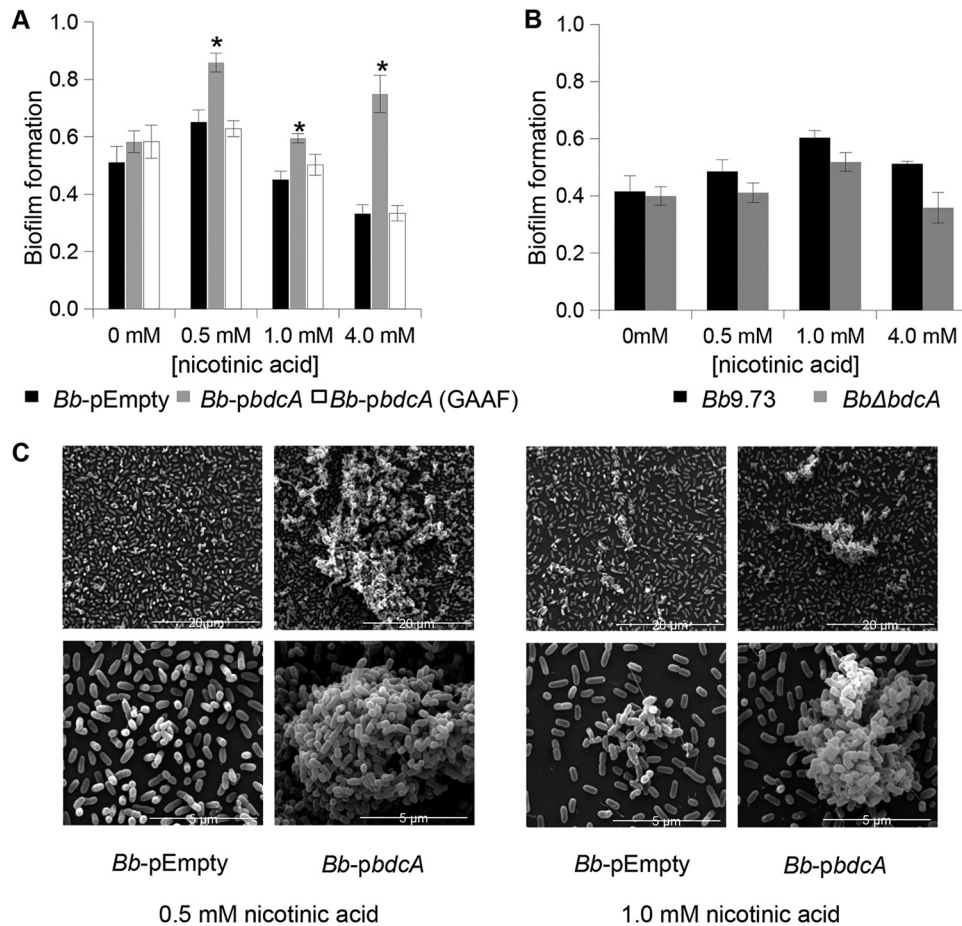


FIG 2 Biofilm formation assay. (A, B) Biofilm formation by the indicated *B. bronchiseptica* strains grown in SS medium either alone or supplemented with nicotinic acid (NA) at the indicated concentration. Biofilm formation was assessed by crystal violet (CV) staining after 24 h in static incubation conditions. Results are based on three biologically independent replicates, and means with standard errors are shown. Means marked with an asterisk are significantly different from those for *Bb-pEmpty* in the same NA concentration (Tukey, $P < 0.01$). (C) Scanning electron microscope images of *B. bronchiseptica* biofilms. *Bb-pEmpty* (left) and *Bb-pbdcA* (right) were grown statically on vertically submerged coverslips at the indicated nicotinic acid concentration. After 24 h of growth, the biofilms formed at the air-liquid interface were visualized. We performed two independent experiments and examined ~20 fields per sample; a representative image is shown.

surface—a cystic fibrosis bronchial epithelial cell line—as described previously (18). In concordance with the abiotic surface experiments, we observed no significant difference in viable biofilm cells recovered for wild-type *B. bronchiseptica* *Bb9.73* or the $\Delta bdcA$ derivative at 6, 10, and 24 h postinoculation (see Fig. S2 in the supplemental material).

c-di-GMP inhibits flagellin production by *B. bronchiseptica*. Motility regulation by c-di-GMP has been described in numerous bacteria (19), and we have observed a similar finding for *B. bronchiseptica* in Fig. 1B. One mechanism of motility control by c-di-GMP is regulation of flagellum production. We performed Western blot analysis to determine if c-di-GMP can inhibit flagellin protein production. In these experiments, we used wild-type *B. bronchiseptica* *Bb9.73*, as well as a *bvgA* mutant strain (*Bb-bvgA* mutant), which is known to regulate expression of the flagellar protein as a control.

Wild-type *B. bronchiseptica* produced the flagellin protein only when grown in the presence of $MgSO_4$ (40 mM), which is known to regulate BvgAS activity (Fig. 3A, first and second panels; see also Fig. S1). However, when *bdcA* was expressed from a plasmid and c-di-GMP levels were high (Fig. 1), flagellin production was repressed (Fig. 3A, second panel). As expected, when the *bvgA* gene was disrupted, flagellin was produced independently of $MgSO_4$ addition to culture medium (Fig. 3A, first lanes of

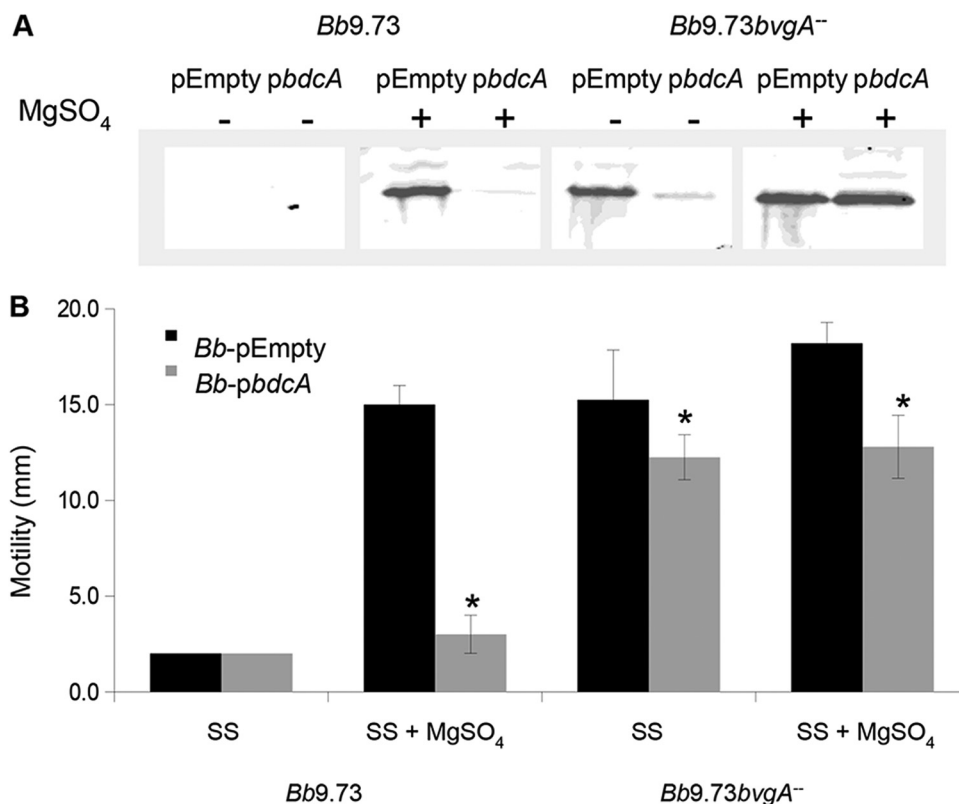


FIG 3 c-di-GMP inhibits flagellin production in *B. bronchiseptica*. (A) Western blot with polyclonal anti-FlaA antibody was used to detect FlaA of *B. bronchiseptica* for each indicated strain grown either in the absence or presence of 40 mM MgSO₄. (B) Motility phenotypes of *B. bronchiseptica* strains used in Western blot analysis in panel A. Diameters greater than 4 mm were indicative of motility. Means with standard errors are shown. Means marked with an asterisk are significantly different from those for the same strain harboring the pEmpty plasmid in the same medium (Tukey, *P* < 0.01).

third and fourth panels). Interestingly, the presence of flagellin in the *bvgA* mutant background was drastically diminished when *bdcA* was expressed from plasmid but only in the absence of MgSO₄ (Fig. 3A, third panel). We repeated the Western blot with a *bvgS* deletion mutant (RB54); the flagellin expression pattern phenocopied the *bvgA* mutant (see Fig. S3A in the supplemental material). Together, these data suggest that MgSO₄ may be enhancing flagellar gene expression independently of the BvgAS system and/or c-di-GMP. These data are also consistent with the model that increased c-di-GMP reduces motility at least in part via reduction in the level of flagella.

Finally, motility in soft agar assay was observed when flagellin production was detected (Fig. 3B). In the *Bb*-*bvgA* mutant grown in 40 mM MgSO₄, despite relatively high levels of flagellin, the strain showed lower motility when BdcA was overexpressed (Fig. 3B, far right bars). This was also observed in the *B. bronchiseptica* RB54 background (Fig. S3B). These data are consistent with findings from other organisms that c-di-GMP-mediated regulation can impact both flagellar gene expression and flagellar function (19).

To assess whether the change in flagellar protein levels might be due to changes in *flaA* gene expression, we transformed *B. bronchiseptica* with an *flaA* promoter-*gfp* transcriptional fusion introduced onto the genome. The fluorescence of the wild-type strain was significantly higher when bacteria were grown with MgSO₄ (40 mM) compared to medium only (Fig. S4). In agreement with motility and Western blot results, expression of the *bdcA* gene from a plasmid resulted in a modest but significant reduction in fluorescence from the *flaA-gfp* transcriptional fusion (see Fig. S4 in the supplemental material).

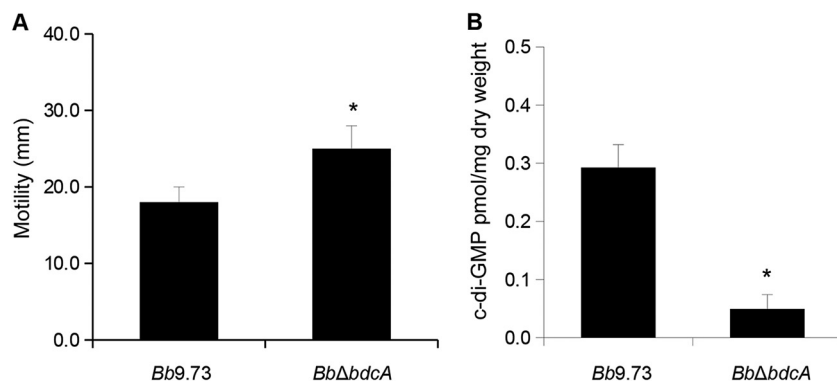


FIG 4 BdcA regulates motility by modifying c-di-GMP levels. (A) Motility phenotype of *B. bronchiseptica* 9.73 and the *BbΔbdcA* strains in SS motility agar, as described in Materials and Methods. Means with standard errors are shown. The mean marked with an asterisk is significantly different from that for wild-type *B. bronchiseptica* Bb9.73 (Tukey, $P < 0.01$). (B) Quantitative measurement of cellular c-di-GMP level of *B. bronchiseptica* Bb9.73 and the *BbΔbdcA* mutant. Formic acid-extracted c-di-GMP was measured by liquid chromatography-mass spectrometry and normalized to milligrams dry weight of bacteria after extraction. Means with standard errors are shown. The mean marked with an asterisk is significantly different from that for *Bb-pEmpty* (Tukey, $P < 0.001$).

BdcA reduces motility in the soft agar assay. Given that c-di-GMP apparently regulates motility, we wondered if BdcA has a physiological role in generating c-di-GMP. We hypothesized that if BdcA inhibits motility, deletion of the *bdcA* gene would trigger an increased motility phenotype. As predicted, the strain with a *bdcA* deletion showed a significantly higher zone of motility (increased by 31%) in the soft agar assay (Fig. 4A). Our results support the idea that BdcA is involved in producing c-di-GMP levels contributing to the inhibition of motility by *B. bronchiseptica* in the avirulent phase (i.e., in the presence of $MgSO_4$). Accordingly, intracellular c-di-GMP concentration in the *BbΔbdcA* mutants was significantly lower than wild-type levels of this second messenger (Fig. 4B). Thus, while loss of the *bdcA* gene did not significantly reduce biofilm formation, it did show enhanced motility.

The YcgR homolog of *B. bronchiseptica* plays no apparent role in motility regulation in our assay conditions. The c-di-GMP binding proteins are responsible for signal transduction pathways responsive to c-di-GMP. Given that c-di-GMP regulates biofilm formation and motility in *B. bronchiseptica*, we predicted that one or more c-di-GMP binding proteins participate in this regulation. One class of c-di-GMP receptor in bacteria is proteins with PilZ domains. The *B. bronchiseptica* genome harbors only one gene encoding a protein with a PilZ domain, *ycgR*. YcgR of *B. bronchiseptica* is a predicted homologue of the YcgR protein of *Escherichia coli*, a reported PilZ domain protein important for regulation of flagellar function (20–22); YcgR of *B. bronchiseptica* and YcgR of *E. coli* share 22% sequence identity and 42% similarity at the amino acid level. We generated a clean deletion of the *ycgR* gene and expressed *bdcA* from a plasmid in this mutant background to establish if c-di-GMP produced by BdcA is sensed by YcgR. The *ycgR* mutant showed no change in motility in the soft agar assay compared to the WT in these experimental conditions (Fig. 5A, first columns). If YcgR is necessary to inhibit motility when *bdcA* is expressed from a plasmid, absence of YcgR would abolish the BdcA-mediated inhibition of motility. As shown in Fig. 5A, motility in soft agar was inhibited by BdcA even in the absence of YcgR.

c-di-GMP motility inhibition and biofilm regulation are not dependent on the LapD homolog of *B. bronchiseptica*. We previously described a LapD homolog of *B. bronchiseptica* (23). LapD is a c-di-GMP receptor protein that controls the cell surface localization of BrtA, a large adhesin required for biofilm formation (23). We speculated that LapD may sense c-di-GMP produced by BdcA to regulate motility. The *B. bronchiseptica* $\Delta lapD$ mutant had no significant effect on motility in the soft agar assay (Fig. 5A). We also expressed *bdcA* in the *B. bronchiseptica* $\Delta lapD$ mutant background and

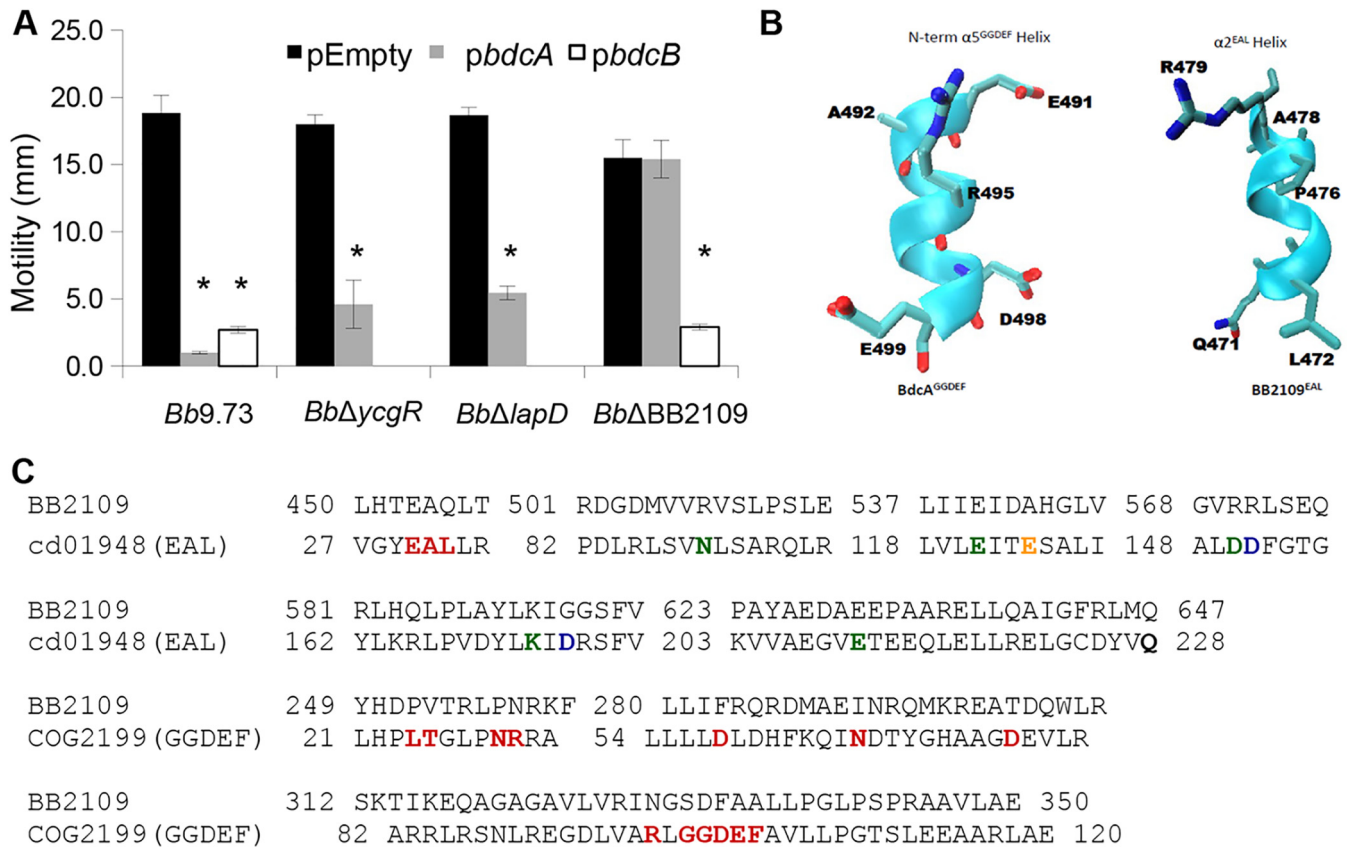


FIG 5 BB2109 is required for c-di-GMP-mediated motility inhibition. (A) Motility phenotypes of *B. bronchiseptica* *Bb9.73* and the *BbΔycgR*, *BbΔlapD*, and *BbΔBB2109* mutants carrying pEmpty, *pbdcA*, or *pbdcB* plasmids on SS motility agar as described in Materials and Methods. Means with standard errors are shown. Means marked with an asterisk are significantly different from those for the same strain harboring the pEmpty plasmid (Tukey, $P < 0.001$). (B) The regions of BdcA and BB2109 that may interact with each other are shown in this ribbon diagram. Five surface residues that are possible points of contact are shown in $\alpha 5$ BdcA and $\alpha 2$ BB2109 helices. (C) Conservation of residues in EAL and GGDEF domains of BB2109. The residues that form the enzyme active sites and are required for diguanylate cyclase activity are shown in red. In the EAL domain, the catalytic EAL domain is shown in red. Green indicates amino acids involved in Mg^{2+} binding; blue indicates amino acids involved in substrate binding; orange indicates glutamate-stabilizing loop 6. Colors are as used by Römmling et al. (31). For more details, see the supplemental material.

performed motility experiments. As shown in Fig. 5A, expression of the *bdcA* gene from a plasmid could inhibit motility even in the absence of the LapD homolog. Together, these data indicate no role for the LapD homolog of *B. bronchiseptica* in motility as assessed in the soft agar assay.

c-di-GMP motility inhibition and biofilm regulation are dependent on BB2109.

Dahlstrom and coworkers postulated that some DGCs interact with EAL domains through particular protein surfaces they called “bar codes and readers” (24). These investigators were able to predict the interaction of proteins from *Pseudomonas aeruginosa* by comparing modeled structures of DGCs and EAL domains. We modeled BdcA and every EAL domain-containing protein encoded by the *B. bronchiseptica* RB50 genome with Phyre2 software (25) and searched for bar code/reader matching pairs for the BdcA protein. We found that the predicted $\alpha 2$ helix of the EAL domain of BB2109, a membrane protein that also contains the GGDEF domain, is a plausible match with the $\alpha 5$ helix of the DGC domain of BdcA based on complementary charge-charge interactions (Fig. 5B). The BB2109 protein also harbors another predicted domain (BaeS) usually present in histidine kinases. Individual alignment of either EAL or GGDEF domains suggested the absence of PDE or DGC activity for BB2109 based on a lack of key residues in the predicted active sites (Fig. 5C; see also the supplemental material).

To test whether BB2109 impacts motility, we constructed a deletion of the BB2109 gene. The Δ BB2109 mutant resulted in a small, but not significant, reduction in motility (Fig. 5A, rightmost black column). To evaluate whether the BB2109 protein is needed

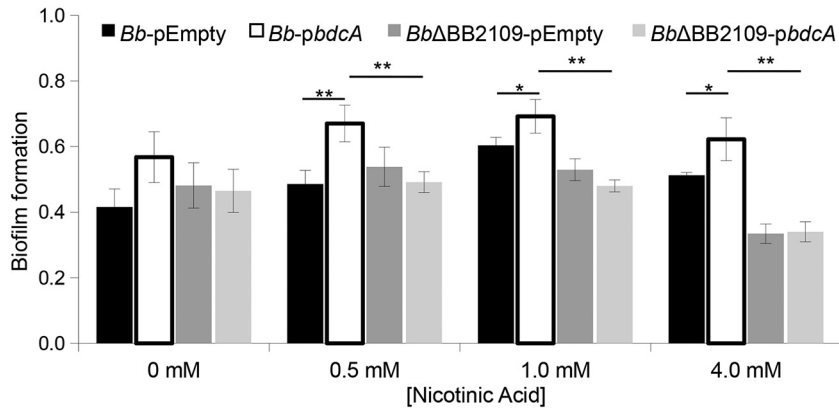


FIG 6 BB2109 is required for c-di-GMP-mediated biofilm formation. Biofilm formation by *B. bronchiseptica* strains grown in SS medium either alone or supplemented with nicotinic acid (NA) at the indicated concentration. Biofilm formation was assessed by crystal violet (CV) staining after 24 h in static incubation conditions. Means with standard errors are shown. Means marked with asterisks are significantly different (Tukey; *, $P < 0.01$; **, $P < 0.001$).

for BdcA-mediated motility inhibition in *B. bronchiseptica*, we assessed this phenotype both when *bdcA* is expressed from a plasmid and in a *Bb*Δ*BB2109* mutant background. In the mutant, BdcA was unable to inhibit motility, indicating that this protein is needed for motility inhibition under these conditions (Fig. 5A). To determine if the combined role of BB2109 and BdcA in motility inhibition was specific, we analyzed another predicted *B. bronchiseptica* DGC, BdcB. Expression of *bdcB* from a plasmid inhibited motility either in the presence or absence of BB2109 (Fig. 5A, rightmost white bar), indicating that BB2109 is involved specifically in BdcA-mediated motility inhibition.

We also checked whether the protein BB2109 was necessary for biofilm formation when *bdcA* was expressed from a plasmid. As shown in Fig. 6, biofilm formation in the strain carrying the *bdcA*-expressing plasmid was only observed in the presence of BB2109. Dependence on the presence of BB2109 was observed in all nicotinic acid (Fig. 6) or $MgSO_4$ concentrations (not shown) where BdcA stimulated biofilm formation. These results indicated that BB2109 and BdcA may work together to generate c-di-GMP, a hypothesis we test below.

BdcA-mediated c-di-GMP production is BB2109 dependent. Motility inhibition and enhancement of biofilm formation by BdcA were BB2109 dependent. The BB2109 protein may be either a receptor for c-di-GMP synthesized by BdcA or necessary for proper function of BdcA as a DGC. In the first proposed mechanism, we would expect to observe high c-di-GMP levels when BdcA was expressed from a plasmid independently of a functional BB2109 protein. In contrast, the increased c-di-GMP level would be dependent on BB2109 if this protein were necessary for BdcA function. To distinguish between these possibilities, we quantified c-di-GMP level when *bdcA* was expressed from a plasmid in the wild-type and Δ*BB2109* mutant backgrounds. We observed that enhanced c-di-GMP levels in a strain expressing the *bdcA* gene depended on the presence of the BB2109 protein (Fig. 7), supporting the hypothesis that BB2109 may stimulate BdcA activity.

BdcA is necessary to effectively establish a respiratory tract infection in the murine model. In many pathogens, the c-di-GMP network has been linked to the ability of bacteria to efficiently colonize a host (26). The deletion of *bdcA* resulted in modified intracellular c-di-GMP levels, suggesting that *Bb*Δ*bdcA* may less effectively colonize the murine respiratory tract. To test this hypothesis, two groups of mice were intranasally challenged with 5 μ l of phosphate-buffered saline (PBS) containing a variable number (between 6 and 100 CFU) of wild-type or mutant bacteria. Our results show that the wild-type bacteria could efficiently colonize nasal cavity, trachea, and lungs with a low inoculum of 12 to 25 CFU. However, the mutant strain required higher numbers of CFU (50 to 100 CFU) to colonize mice, and those mice had lower overall

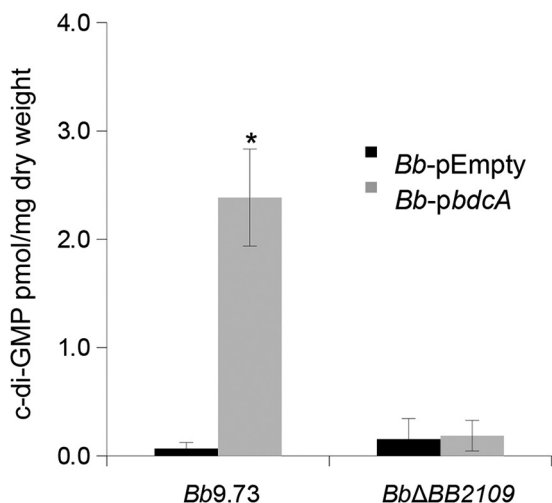


FIG 7 BdcA activity is BB2109 dependent. Quantitative measurement of cellular c-di-GMP level of *B. bronchiseptica* *Bb9.73* and the *BbΔBB2109* mutant carrying the pEmpty or *pbdcA* plasmids, as indicated. Means with standard errors are shown. The mean marked with an asterisk is significantly different from that for *Bb-pEmpty* (Tukey, $P < 0.001$).

bacterial numbers recovered from their respiratory tracts 7 days later (Fig. 8). These differences were observed despite the *bdcA* mutant having no apparent defect on growth *in vitro* (not shown). Overall, these results indicate that the *BbΔbdcA* strain colonizes mice less efficiently than wild-type bacteria.

DISCUSSION

In this work, we examined the role of BdcA, a diguanylate cyclase of *B. bronchiseptica*. BdcA is a predicted inner membrane protein of 540 amino acids with two domains, an N-terminal CACHE (calcium channels and chemotaxis receptor) domain and a C-terminal GGDEF. This CACHE domain has been described as a sensor domain that may bind small ligands (27). In previous work, we showed that BdcA inhibits motility and enhances biofilm formation (4); here, we supported the idea that BdcA is an active DGC by showing that intracellular levels of c-di-GMP were enhanced by *bdcA* expression. While we assume that the GGDEF domain of BdcA is responsible for c-di-GMP synthesis, the inability to detect the mutant protein by Western blotting limits our ability to make a solid conclusion.

The physiological role of BdcA was evaluated by deleting this gene in *B. bronchiseptica*.

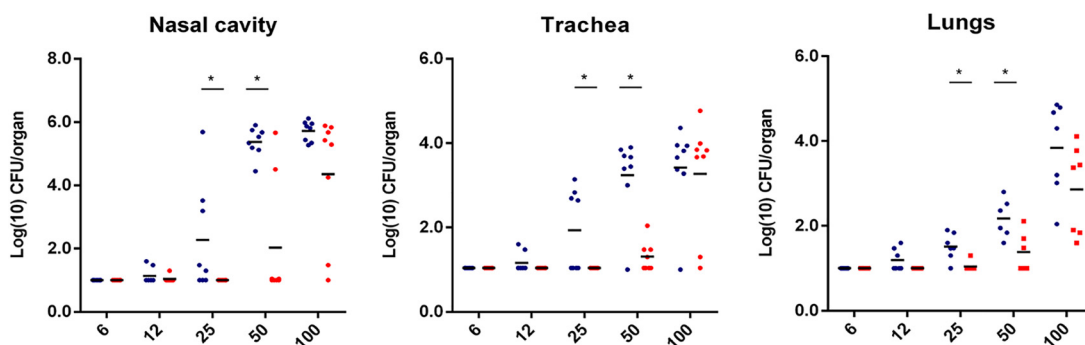


FIG 8 BdcA is required to effectively establish a respiratory tract infection in a mouse model. Five-week-old mice were intranasally inoculated with 5 μ l of PBS containing bacteria at different bacterial load (6, 12, 25, 50, 100 CFU). Seven days postinoculation, bacterial loads in the organs of the respiratory tract were enumerated. Each symbol represents a single animal, with the mean colonization depicted as short horizontal bars. Data are pooled from 2 separate experiments conducted independently. Means marked with an asterisk are significantly different (Tukey, $P < 0.05$). Note that some points were allowed to be superimposed when plotted to increase the clarity of the presentation, but all data were included in the statistical analysis.

tica. Deletion of *bdcA* resulted in lower *c*-di-GMP levels in *B. bronchiseptica*, supporting the idea that BdcA is an active DGC; however, we observed no difference in biofilm formation between WT and the *bdcA* mutant strain on an abiotic surface or on airway cells. Although it is well established that *c*-di-GMP synthesized by DGCs enhances biofilm formation, absence of a single DGC is usually not enough to impact formation of biofilms. For example, in *Pseudomonas fluorescens*, deletion of four DGCs was needed to abolish biofilm formation in standard laboratory assay conditions (28). Alternatively, deletion of a single DGC can be important for biofilm formation depending on the carbon source used (29), indicating that BdcA might participate in biofilm formation in other environments.

The finding that the loss of the *bdcA* gene increased motility supports the hypothesis that *bdcA* regulates motility in *B. bronchiseptica*, but the deletion of *bdcA* increases motility only by 31%, indicating that there may be other DGCs involved in motility regulation in this microbe. In previous work, we described that *bdcA* is predominantly transcribed in the avirulent phase, when this organism is motile (4). It is intriguing that *B. bronchiseptica* triggers this apparent inhibitory mechanism during its motile phase. One report has indicated transient expression of flagellin in the virulent phase (13), and transcriptome analysis of *B. pertussis* has also shown discrepancies between virulent repressed genes and flagellar gene regulation (30). Expression of a negative regulator of motility during the motile phase of this organism may explain these disparate observations.

Motility, and particularly flagellin expression, is repressed by the active two-component system BvgAS. Here, we determined that overexpression of BdcA can inhibit motility even when functional BvgA or BvgS is absent. These data suggest that high *c*-di-GMP levels can reduce motility even when the organism is in a stage wherein BvgAS is inactive. However, when $MgSO_4$ was added to medium, *c*-di-GMP inhibition of flagellin production was dependent on BvgA or BvgS, suggesting a complex interaction among *c*-di-GMP signaling, the BvgAS system, and the regulatory effects of $MgSO_4$. Importantly, there are conditions wherein flagellin is produced but the cells are less motile, indicating that *c*-di-GMP can inhibit motility by one or more other mechanisms.

We demonstrated that homologs of a PilZ domain protein and of LapD, known *c*-di-GMP receptors, did not participate in the BdcA-dependent regulation of motility. Instead, using an approach reported by Dahlstrom and coworkers (24), we were able to predict a possible interacting partner to BdcA-BB2109, which appears to lack functional DGC or PDE domains due to the absence of critical catalytic residues. Some such nonfunctional proteins have been described as *c*-di-GMP binding proteins (31). Overexpression of *bdcA* in the WT background results in almost complete loss of motility and increased *c*-di-GMP production; this loss of motility and enhanced *c*-di-GMP production are BB2109 dependent. These data suggest that the DGC BdcA and the putative *c*-di-GMP receptor BB2109 likely participate in a common pathway to regulate biofilm formation and motility.

Finally, we noted a connection between *c*-di-GMP and virulence, illuminating for the first time that an airborne pathogen like *B. bronchiseptica* requires a functional *c*-di-GMP network to efficiently infect a natural host like the mouse. Interestingly, dosages of above 50 CFU are necessary for the mutant strain to be able to colonize the murine respiratory tract, suggesting that high *c*-di-GMP levels might be required in the initial steps of infection. Thus, it is plausible that *c*-di-GMP participates in regulating adaptation to hosts or to tissues of the same host. Unwrapping the *c*-di-GMP network in *Bordetella* may give us a better understanding of how this organism infects hosts.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains and plasmids used in this study are listed in Table S1 in the supplemental material. Wild-type *B. bronchiseptica* 9.73H⁺ was isolated from a rabbit and described previously (32). *B. bronchiseptica* wild-type isolates and mutants were grown on Bordet-Gengou agar (BGA) (Difco) supplemented with 15% (vol/vol) defibrinated fresh sheep blood (BGA medium) at 37°C for 48 h and replated in the same medium for 24 h. Stainer-Scholte (SS) liquid medium

was used to grow *B. bronchiseptica* in broth cultures (33). When appropriate, BGA or SS was supplemented with streptomycin ($200 \mu\text{g ml}^{-1}$), kanamycin ($80 \mu\text{g ml}^{-1}$), and/or gentamicin ($50 \mu\text{g ml}^{-1}$). *Escherichia coli* (DH5 α , BTH101, and S17-1) strains were cultured with lysogeny broth (LB) (34) in a test tube or on solidified LB with 1.5% agar. When appropriate, antibiotics were added to the medium at the following concentrations: $10 \mu\text{g ml}^{-1}$ gentamicin and $50 \mu\text{g ml}^{-1}$ kanamycin.

Plasmid and strain construction. Plasmids and strains were constructed using standard molecular biology techniques; detailed descriptions of construction procedures are in the supplemental material. Oligonucleotides used in this study are listed in Table S2 in the supplemental material.

Biofilm formation assays. *B. bronchiseptica* biofilm assays were performed as previously described by our group (23) from colonies grown in SS semisolid medium (1.5% agar) supplemented with 15% (vol/vol) defibrinated fresh sheep blood. Bacteria were resuspended in SS medium and pipetted into wells of a sterile 96-well U-bottom microtiter plate (polyvinylchloride [PVC]). Nicotinic acid or MgSO_4 were added at the indicated concentrations when appropriate. After 24 h, attached cells were stained with crystal violet (CV) solution. The stain was dissolved by adding 33% (vol/vol) acetic acid solution and then quantified by measuring optical density (OD) at 595 nm. Experiments were repeated at least three times with at least six technical replicates.

Scanning electron microscopy. *B. bronchiseptica* biofilm assays for scanning electron microscopy were performed as previously described by our group (23). Briefly, glass coverslips were vertically submerged in plastic tubes containing bacteria adjusted to an optical density at 650 nm (OD_{650}) of 0.1. After 24 h, the coverslip was removed and treated to perform a critical-point procedure using liquid CO_2 (EmiTech K850) and sputter coated with gold. Samples were visualized with a scanning electron microscope (Philips SEM 505), and the images were processed using an image soft imaging system ADDA II.

SDS-PAGE and Western blot analysis. *B. bronchiseptica* strains were grown at 37°C for 16 h in SS medium plus gentamicin, with or without MgSO_4 (40 mM) as indicated. Cells were collected, normalized to an equal OD_{650} value, harvested by boiling samples in $1\times$ Laemmli buffer for 10 min, and subjected to SDS-polyacrylamide gel electrophoresis followed by transfer of the contents of the gel to a polyvinylidene fluoride (PVDF) filter. After transfer, the PVDF membrane was blocked with 5% nonfat milk powder in Tris-buffered saline (TBS) for 1 h followed by incubation with polyclonal anti-FlaA serum diluted 1:2,000 in TBS containing 5% nonfat milk powder at 4°C overnight (35). The filter was then incubated with anti-mouse IgG conjugated to horseradish peroxidase (HRP) (1:5,000) (Invitrogen) in TBS containing 5% nonfat milk powder at room temperature for 2.5 h. Horseradish peroxidase-conjugated anti-mouse antibody (Bio-Rad, USA) was used as the secondary antibody. Chemiluminescent reagent was used for developing according to manufacturer's instructions. Samples from each strain and growth condition combination were prepared and analyzed independently three times.

Measurement of c-di-GMP levels. The c-di-GMP levels were analyzed via liquid chromatography-mass spectrometry (LC-MS) as previously described (4). Strains were grown in SS liquid medium for 14 h. Four replicates of each strain were analyzed via LC-MS using the LC-20AD high-performance LC system (Shimadzu, Columbia, MD) coupled to a Finnigan TSQ Quantum Discovery Max triple-quadrupole mass spectrometer (Thermo Electron Corp., San Jose, CA). Results are means of three independent experiments.

Motility assays. SS soft agar motility plate (0.35% agar) supplemented with 40 mM MgSO_4 and 1.0 mM CaCl_2 was used to determine the motility of bacterial strains as previously described (35). We found that adding 1.0 mM CaCl_2 to motility medium allowed better visualization of the migration zone border. The diameter of the migration zone was measured after 18 h of incubation at 37°C . Experiments were repeated at least three times with at least three technical replicates.

Fifty percent infective dose. Wild-type C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME). Power-G was used to calculate the number of mice needed to obtain statistical significance (assuming an effect size of 0.60, a power level of 0.80, and a probability level for statistical significance of 0.05). Briefly, following anesthesia with 5% isoflurane, groups of 7 mice were intranasally inoculated with $5 \mu\text{l}$ of PBS containing 6 to 100 CFU of wild-type or mutant *B. bronchiseptica*. Seven days postinoculation, mice were euthanized with CO_2 followed by cervical dislocation, and organs were collected in 1 ml of PBS and homogenized using a bead tissue disruptor (Bead Mill 24; Fisher Scientific). Bacterial load was enumerated by dilution plating; the limit of detection was 10 CFU. Results were graphed in GraphPad Prism (v8), and statistical significance was calculated using one-way analysis of variance (ANOVA).

Ethics statement. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, USA. The protocols used in this study have been approved by the Institutional Animal Care and Use Committee at the University of Georgia, Athens, GA (animal usage protocol:A2016 02-010-Y2-A3 *Bordetella*-Host). Animals were handled following institutional guidelines, in keeping with full accreditation from the Association for Assessment and Accreditation of Laboratory Animal Care International.

Statistical analyses. Means were analyzed for significance using a one-way ANOVA with a Tukey's multiple-comparison test to compare differences among groups. Shapiro-Wilks test was performed to confirm normal distribution of data. Significance level is stated in each figure legend.

GenBank accession number. The GenBank accession number for the *B. bronchiseptica* RB50 genome is [NC_002927.3](https://www.ncbi.nlm.nih.gov/nuclink/NC_002927.3). Gene identification numbers and locus tags, respectively, for all genes mentioned are as follows: *ycgR*, 2663780 and BB_RS07780; *bdcA*, 2661253 and BB_RS17940; BB2109, 2659958 and BB_RS10615; *bdcB*, 2661408 and BB_RS19575; *lapD*, 2662323 and BB_RS05885.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00011-19>.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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