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1 **_Bordetella bronchiseptica_ diguanylate cyclase BdcA regulates motility**
2 **and is important for the establishment of respiratory infection in mice**
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10 Running Head: Role of c-di-GMP in *B. bronchiseptica* pathogenesis
11
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19 **Abstract**

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

38

39 **Importance**

40 Pathogenesis by *Bordetella* spp., like that of a number of other pathogens, involves biofilm formation.
41 Biofilms increase tolerance to biotic and abiotic factors and are proposed as reservoirs of microbes for
42 transmission to other organs (trachea, lungs) or other hosts. Bis-(3′-5′)-cyclic dimeric GMP (c-di-
43 GMP) is a second messenger that regulate transition between biofilm and planktonic lifestyles. In
44 *Bordetella bronchiseptica,* high c-di-GMP levels inhibit motility and favor biofilm formation. In the
45 present work, we characterized a *B. bronchiseptica* diguanylate cyclase, BdcA, which regulates
46 motility, biofilm formation, and affects the ability of *B. bronchiseptica* to colonize the murine

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47 respiratory tract. These results provide us with a better understanding of how *B. bronchiseptica* can

48 infect a host.

49

50 **Introduction**

51 Cyclic dimeric guanosine monophosphate (c-di-GMP) is a ubiquitous second messenger in bacteria

52 that regulates multiple phenotypes (1,2). In a multitude of bacteria, c-di-GMP regulates transitions

53 between a motile, planktonic lifestyle and a sessile, biofilm mode of growth. Moreover, in several

54 pathogens, c-di-GMP also influences virulence (2). The intracellular concentration of c-di-GMP is

55 regulated by diguanylate cyclases (DGCs) and phosphodiesterases (PDEs). Enzymes with DGC

56 activity share a domain characterized by the presence of a GGDEF (or similar) sequence; these

57 enzymes synthesize c-di-GMP from two molecules of guanosine triphosphate (GTP) (3). The c-di-

58 GMP-degrading PDE activity is associated with enzymes that have either EAL or HD-GYP motifs.

59 The c-di-GMP network is complex and regulates phenotypes through different mechanisms, including

60 gene expression regulation, post-translational regulation, protein conformation modulation, and

61 protein-protein interactions (3). Indeed, c-di-GMP can regulate rapid transitions between lifestyles,

62 thus likely allowing pathogens to adapt quickly to new environments during transitions between hosts.

63 Recently we showed that c-di-GMP regulates motility and biofilm formation in *B. bronchiseptica* (4).

64 As for other bacteria, high c-di-GMP levels in *B. bronchiseptica* correlates with reduced motility and

65 biofilm-forming phenotype, suggesting that c-di-GMP signals could affect interactions with the host.

66

67 *Bordetella bronchiseptica* is a Gram-negative bacterium that causes respiratory tract infections,

68 producing kennel cough in dogs, atrophic rhinitis in pigs, and snuffles in rabbits (5). The ability of

69 this organism to form biofilms has been previously reported; the BvgAS two-component system is

70 involved in control of biofilm formation (6,7). Most known *B. bronchiseptica* virulence factors are

71 also regulated by BvgAS (8). To date, no known signal has been described for the sensor histidine

72 kinase BvgS, but it has been reported that BvgS is typically active, with specific signals inhibiting its

73 function (9). *In vitro* signals such as low temperature, sulfate ions, and nicotinic acid have inhibitory

74 effects on BvgS activity (8).

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

87

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

94

95 **Results**

96 **Expression of *bdcA* (BB3576) increases c-di-GMP levels and reduces motility of *B.***
97 ***bronchiseptica*.** We have shown previously that overexpression of BB3576, a predicted DGC of *B.*
98 *bronchiseptica*, inhibits swimming motility in soft agar (4). We proposed that if BB3576 is a
99 functional DGC, motility inhibition might be due to high c-di-GMP levels produced by this protein.
100 To test this hypothesis, we quantified intracellular c-di-GMP in *B. bronchiseptica* with the BB3576
101 gene expressed from a plasmid under control of a strong promoter (p_{nptII}; Fig. 1A). c-di-GMP levels
102 normalized by dry weight were ~9 times higher when BB3576 was expressed from the plasmid

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103 compared to the vector control (pEmpty) (Fig. 1A). When GGDEF domain was replaced with
104 GGAAF, the measured levels of c-di-GMP were equivalent to levels in wild type (WT) carrying the
105 control vector (Fig. 1A). We tried several approaches to detect the WT and the mutant variant of the
106 BB3576 protein when expressed from a plasmid, including using 6XHis, 3XFlag, and streptavidin
107 epitope tags as well as enriching for the membrane fraction of cell extracts for this predicted inner
108 membrane protein, but we could not detect the WT or mutant protein. Thus, it is not clear if the
109 phenotype of the BB3576-GGAAF mutant protein is due to loss of DGC activity or destabilization of
110 the protein. It is important to note that similar mutations have been made in many DGCs with no
111 apparent loss of protein stability (14,15,16). Moreover, only when intracellular c-di-GMP levels were
112 high was motility reduced in the soft agar assay (Fig. 1A,B). These results suggest that c-di-GMP
113 produced by BB3576 inhibits motility - based on these findings we named BB3576 the *Bordetella*
114 diguanylate cyclase A (*bdcA*).
115
116 We previously showed that overexpression of *bdcA* induced a significant increase in biofilm levels
117 (4), which is consistent with high intracellular c-di-GMP levels observed here for the strain expressing
118 this gene from a plasmid (Fig. 1A). As previously reported, expression of *bdcA* enhanced biofilm
119 formation either modulating bacteria with nicotinic acid or $MgSO_4$ (Fig. 2A and Fig. S1,
120 respectively). The biomass detected by crystal violet (CV) assay includes live and dead cells, lysed
121 cells and other CV-staining matrix components like polysaccharides and eDNA that may lead to the
122 observed increased values. Thus, we also analyzed the biofilm by scanning electron microscopy
123 (SEM). *B. bronchiseptica* expressing *bdcA* from a plasmid showed larger 3D structures than wild type
124 *B. bronchiseptica* in all conditions analyzed by SEM (Fig. 2C). Hence, we confirmed that differences
125 observed are a consequence of more bacteria forming a biofilm rather than simply increased
126 production of matrix components.
127
128 Biofilm enhancement was not observed in bacteria carrying the empty vector or expressing the *bdcA*-
129 GAAF variant, suggesting that c-di-GMP production is required for the enhanced biofilm formation
130 observed when *bdcA* is overexpressed. We also deleted the *bdcA* gene in *B. bronchiseptica Bb*9.73

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131 and evaluated biofilm formation by the *Bb*Δ*bdcA* mutant compared with the wild-type strain at
132 virulent, intermediate and avirulent phases. There was no statistically significant reduction in biofilm
133 formation for any nicotinic acid concentration tested for the *Bb*Δ*bdcA* mutant versus the WT control
134 (Fig. 2B).
135
136 *B. bronchiseptica* and *B. pertussis* can form biofilm-like structures on nasal epithelial cells *in vivo*
137 (17). Although the Δ*bdcA* deletion mutant did not affect biofilm formation on the abiotic surface
138 tested here, we evaluated biofilm formation on a biotic surface - a cystic fibrosis bronchial epithelial
139 cell line—as described previously (18). In concordance with the abiotic surface experiments, we
140 observed no significant difference in viable biofilm cells recovered for wild-type *B. bronchiseptica*
141 *Bb*9.73 or the Δ*bdcA* derivative at 6, 10, and 24 hours post-inoculation (Fig. S2).
142
143 **c-di-GMP inhibits flagellin production by *B. bronchiseptica*.** Motility regulation by c-di-GMP has
144 been described in numerous bacteria (19), and we have observed a similar finding for *B.*
145 *bronchiseptica* in Fig. 1B. One mechanism of motility control by c-di-GMP is regulation of flagellum
146 production. We performed Western blot analysis to determinate if c-di-GMP can inhibit flagellin
147 protein production. In these experiments we used wild-type *B. bronchiseptica* 9.73, as well as a *bvgA*
148 mutant strain (*Bb-bvgA*$^{--}$), which is known to regulate expression of the flagellar protein as a control.
149
150 Wild-type *B. bronchiseptica* produced the flagellin protein only when grown in the presence of
151 $MgSO_4$ (40 mM), which is known to regulate BvgAS activity (Fig. 3A, first and second panels and
152 Fig. S1). However, when *bdcA* was expressed from a plasmid and c-di-GMP levels were high (see
153 Fig. 1), flagellin production was repressed (Fig. 3A, second panel). As expected, when the *bvgA* gene
154 was disrupted, flagellin was produced independently of $MgSO_4$ addition to culture media (Fig. 3A,
155 first lanes of third and fourth panels). Interestingly, the presence of flagellin in the *bvgA* mutant
156 background was drastically diminished when *bdcA* was expressed from plasmid, but only in the
157 absence of $MgSO_4$ (Fig. 3A, third panel). We repeated the Western blot with a *bvgS* deletion mutant
158 (RB54); the flagellin expression pattern phenocopied the *bvgA* mutant (Fig. S3A). Together, these

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159 data suggest that $MgSO_4$ may be enhancing flagellar gene expression independently of the BvgAS
160 system and/or c-di-GMP. These data are also consistent with the model that increased c-di-GMP
161 reduces motility at least in part via reduction in the level of flagella.
162
163 Finally, motility in soft agar assay was observed when flagellin production was detected (Fig. 3B). In
164 the *Bb-bvgA* mutant grown in $MgSO_4$ 40 mM, despite relatively high levels of flagellin, the strain
165 showed lower motility when BdcA was overexpressed (Fig. 3B, far right column). This was also
166 observed in *B. bronchiseptica* RB54 background (Fig. S3B). These data are consistent with findings
167 from other organisms that c-di-GMP-mediated regulation can impact both flagellar gene expression
168 and flagellar function (19).
169
170 To assess whether the change in flagellar protein levels might be due to changes in *flaA* gene
171 expression, we transformed *B. bronchiseptica* with a *flaA* promoter-*gfp* transcriptional fusion
172 introduced onto the genome. The fluorescence of the wild-type strain was significantly higher when
173 bacteria were grown with $MgSO_4$ (40 mM) compared to medium only (Fig. S3). In agreement with
174 motility and Western blot results, expression of the *bdcA* gene from a plasmid resulted in a modest,
175 but significant reduction in fluorescence from the *flaA*-*gfp* transcriptional fusion (Fig. S4).
176
177 **BdcA reduces motility in the soft agar assay.** Given that c-di-GMP apparently regulates motility,
178 we wondered if BdcA has a physiological role in generating c-di-GMP. We hypothesized that if BdcA
179 inhibits motility, deletion of *bdcA* gene would trigger an increased motility phenotype. As predicted,
180 the strain with a *bdcA* deletion showed significantly higher zone of motility (increased by 31%) in the
181 soft agar assay (Fig. 4A). Our results support the idea that BdcA is involved in producing c-di-GMP
182 levels contributing to the inhibition of motility by *B. bronchiseptica* in the avirulent phase (i.e., in the
183 presence of $MgSO_4$). Accordingly, intracellular c-di-GMP concentration in the *BbΔbdcA* mutants was
184 significantly lower than wild-type levels of this second messenger (Fig. 4B). Thus, while loss of the
185 *bdcA* gene did not significantly reduce biofilm formation, it did show enhanced motility.
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187 **The YcgR homolog of *B. bronchiseptica* plays no apparent role in motility regulation in our**
188 **assay conditions.** c-di-GMP binding proteins are responsible for signal-transduction pathways
189 responsive to c-di-GMP. Given that c-di-GMP regulates biofilm formation and motility in *B.*
190 *bronchiseptica*, we predicted that one or more c-di-GMP binding proteins participate in this
191 regulation. One class of c-di-GMP receptor in bacteria is proteins with PilZ domains. The *B.*
192 *bronchiseptica* genome harbors only one gene encoding a protein with a PilZ domain, *ycgR.* YcgR of
193 *B. bronchiseptica* is a predicted homologue of the YcgR protein of *E. coli*, a reported PilZ domain
194 protein important for regulation of flagellar function (20,21,22); YcgR of *B. bronchiseptica* and YcgR
195 of *E. coli* share 22% sequence identity and 42% similarity at the amino-acid level. We generated a
196 clean deletion of the *ycgR* gene and expressed *bdcA* from a plasmid in this mutant background to
197 establish if c-di-GMP produced by BdcA is sensed by YcgR. The *ycgR* mutant showed no change in
198 motility in the soft agar assay compared to the WT in these experimental conditions (Fig. 5A, first
199 columns). If YcgR is necessary to inhibit motility when *bdcA* is expressed from a plasmid, absence of
200 YcgR would abolish the BdcA-mediated inhibition of motility. As shown in Fig. 5A, motility in soft
201 agar was inhibited by BdcA even in absence of YcgR.
202
203 **C-di-GMP motility inhibition and biofilm regulation is not dependent on the LapD homolog of**
204 ***B. bronchiseptica*.** We previously described a LapD homolog of *B. bronchiseptica* (23). LapD is a c-
205 di-GMP receptor protein that controls the cell surface localization of BrtA, a large adhesin required
206 for biofilm formation (23). We speculated that LapD may sense c-di-GMP produced by BdcA to
207 regulate motility. The *B. bronchiseptica* Δ*lapD* mutant had no significant effect on motility in the soft
208 agar assay (Fig. 5A). We also expressed *bdcA* in the *B. bronchiseptica* Δ*lapD* mutant background and
209 performed motility experiments. As shown in Fig. 5A, expression of the *bdcA* gene from a plasmid
210 could inhibit motility even in the absence of the LapD homolog. Together, these data indicate no role
211 for the LapD homolog of *B. bronchiseptica* in motility as assessed in the soft agar assay.
212
213 **C-di-GMP motility inhibition and biofilm regulation are dependent on BB2109.** Dahlstrom and
214 coworkers postulated that some DGCs interact with EAL domains through particular protein surfaces

215 they called "bar codes and readers" (24). These investigators were able to predict interaction of
216 proteins from *P. aeruginosa* by comparing modeled structures of DGCs and EALs domains. We
217 modeled BdcA and every EAL domain-containing protein encoded by the *B. bronchiseptica* RB50
218 genome with Phyre2 software (25) and searched for bar-code/reader matching pairs for the BdcA
219 protein. We found that the predicted α2 helix of the EAL domain of BB2109, a membrane protein
220 that also contains GGDEF domain, is a plausible match with the α5 helix of the DGC domain of
221 BdcA based on complementary charge-charge interactions (Fig. 5B). The BB2109 protein also
222 harbors another predicted domain (BaeS) usually present in histidine kinases. Individual alignment of
223 either EAL or GDDEF domains suggested absence of PDE or DGC activity for BB2109 based on a
224 lack of key residues in the predicted active sites (Fig. 5C and supplemental material).
225
226 To test whether BB2109 impacts motility, we constructed a deletion of the BB2109 gene. The
227 ΔBB2109 mutant resulted in small, but not significant, reduction in motility (Fig. 5A, rightmost black
228 column). To evaluate whether the BB2109 protein is needed for BdcA-mediated motility inhibition in
229 *B. bronchiseptica*, we assessed this phenotype both when *bdcA* is expressed from a plasmid and in a
230 *Bb*ΔBB2109 mutant background. In the mutant, BdcA was unable to inhibit motility, indicating that
231 this protein is needed for motility inhibition under these conditions (Fig. 5A). To determine if the
232 combined role of BB2109 and BdcA in motility inhibition was specific, we analyzed another
233 predicted *B. bronchiseptica* DGC, BdcB. Expression of *bdcB* from a plasmid inhibited motility either
234 in the presence or absence of BB2109 (Fig. 5A, rightmost white column), indicating that BB2109 is
235 involved specifically in BdcA-mediated motility inhibition.
236
237 We also checked whether the protein BB2109 was necessary for biofilm formation when *bdcA* was
238 expressed from a plasmid. As shown in Fig. 6, biofilm formation in the strain carrying the *bdcA*-
239 expressing plasmid only was observed in presence of BB2109. Dependence on the presence of
240 BB2109 was observed in all nicotinic acid (Fig. 6) or $MgSO_4$ concentrations (not shown) where BdcA

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241 stimulated biofilm formation. These results indicated that BB2109 and BdcA may work together to
242 generate c-di-GMP, a hypothesis we test below.
243
244 **BdcA-mediated c-di-GMP production is BB2109-dependent.** Motility inhibition and enhancement
245 of biofilm formation by BdcA was BB2109-dependent. The BB2109 protein may be either a receptor
246 for c-di-GMP synthesized by BdcA or necessary for proper function of BdcA as a DGC. In the first
247 proposed mechanism, we would expect to observe high c-di-GMP levels when BdcA was expressed
248 from a plasmid independently of a functional BB2109 protein. In contrast, the increased c-di-GMP
249 level would be dependent on BB2109 if this protein were necessary for BdcA function. To distinguish
250 between these possibilities, we quantified c-di-GMP level when *bdcA* was expressed from a plasmid
251 in the wild-type and ΔBB2109 mutant backgrounds. We observed that enhanced c-di-GMP levels in a
252 strain expressing the *bdcA* gene depended on the presence of the BB2109 protein (Fig. 7), supporting
253 the hypothesis that BB2109 may stimulate BdcA activity.
254
255 **BdcA is necessary to effectively establish a respiratory tract infection in the murine model.** In
256 many pathogens, c-di-GMP network has been linked to the ability of bacteria to efficiently colonize a
257 host (26). The deletion of *bdcA* resulted in modified intracellular c-di-GMP levels, suggesting that
258 Bb*ΔbdcA* may less effectively colonize the murine respiratory tract. To test this hypothesis, two
259 groups of mice were intranasally challenged with 5 μL of PBS containing a variable number (between
260 6 and 100 CFU) of wild-type or mutant bacteria. Our results show that the wild-type bacteria could
261 efficiently colonize nasal cavity, trachea, and lungs with a low inoculum of 12-25 CFU. However, the
262 mutant strain required higher numbers of CFU (50-100 CFU) to colonize mice, and those mice had
263 lower overall bacterial numbers recovered from their respiratory tracts seven days later (Fig. 8). These
264 differences were observed despite the *bdcA* mutant having no apparent defect on growth *in vitro* (not
265 shown). Overall, these results indicate that *BbΔbdcA* strain colonizes mice less efficiently than wild
266 type bacteria.
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269 **Discussion**

270 In this work we examined the role of BdcA, a diguanylate cyclase of *B. bronchiseptica*. BdcA is a
271 predicted inner membrane protein of 540 amino acids with two domains, an N-terminal CACHE
272 (calcium channels and chemotaxis receptor) domain and a C-terminal GGDEF. This CACHE domain
273 has been described as a sensor domain that may bind small ligands (27). In previous work we showed
274 that BdcA inhibits motility and enhances biofilm formation (4); here we supported the idea that BdcA
275 is an active DGC by showing that intracellular levels of c-di-GMP were enhanced by *bdcA*
276 expression. While we assume that the GGDEF domain of BdcA is responsible for c-di-GMP
277 synthesis, the inability to detect the mutant protein by Western blot limits our ability to make a solid
278 conclusion.
279
280 The physiological role of BdcA was evaluated by deleting this gene in *B. bronchiseptica*. Deletion of
281 *bdcA* resulted in lower c-di-GMP levels in *B. bronchiseptica*, supporting the idea that BdcA is an
282 active DGC; however, we observed no difference in biofilm formation between WT and the *bdcA*
283 mutant strain on an abiotic surface or on airway cells. Although it is well established that c-di-GMP
284 synthesized by DGCs enhances biofilm formation, absence of a single DGC is usually not enough to
285 impact formation of biofilms. For example, in *P. fluorescens*, deletion of four DGCs was needed to
286 abolish biofilm formation in standard laboratory assay conditions (28). Alternatively, deletion of a
287 single DGC can be important for biofilm formation depending on carbon source used (29), indicating
288 that BdcA might participate in biofilm formation in other environments.
289
290 The finding that loss of the *bdcA* gene increased motility supports the hypothesis that *bdcA* regulates
291 motility in *B. bronchiseptica*, but the deletion of *bdcA* only increases motility by 31%, indicating that
292 there may be other DGCs involved in motility regulation in this microbe. In previous work we
293 described that *bdcA* is predominantly transcribed in the avirulent phase, when this organism is motile
294 (4). It is intriguing that *B. bronchiseptica* triggers this apparent inhibitory mechanism during its motile
295 phase. One report has indicated transient expression of flagellin in virulent phase (13), and
296 transcriptome analysis of *B. pertussis* has also showed discrepancies between virulent repressed genes

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297 and flagellar gene regulation (30). Expression of a negative regulator of motility during the motile
298 phase of this organism may explain these disparate observations.
299
300 Motility, and particularly flagellin expression, is repressed by the active two-component system
301 BvgAS. Here we determined that overexpression of BdcA can inhibit motility even when functional
302 BvgA or BvgS are absent. These data suggest that high c-di-GMP levels can reduce motility even
303 when the organism is in a stage wherein BvgAS is inactive. However, when $MgSO_4$ was added to
304 medium, c-di-GMP inhibition of flagellin production was dependent on BvgA or BvgS, suggesting a
305 complex interaction among c-di-GMP signaling, the BvgAS system and the regulatory effects of
306 $MgSO_4$. Importantly, there are conditions wherein flagellin is produced but the cells are less motile,
307 indicating that c-di-GMP can inhibit motility by one or more other mechanisms.
308
309 We demonstrated that homologs of a PilZ domain protein and of LapD, known c-di-GMP receptors,
310 did not participate in the BdcA-dependent regulation of motility. Instead, using an approach reported
311 by Dahlstrom and coworkers (24), we were able to predict a possible interacting partner to BdcA –
312 BB2109, which appears to lack functional DGC or PDE domains due to the absence of critical
313 catalytic residues. Some such nonfunctional proteins have been described as c-di-GMP binding
314 proteins (31). Overexpression of *bdcA* in the WT background results in almost complete loss of
315 motility and increased c-di-GMP production, this loss of motility and enhanced c-di-GMP production
316 are BB2109-dependent. These data suggest that the DGC BdcA and the putative c-di-GMP receptor
317 BB2109 likely participate in a common pathway to regulate biofilm formation and motility.
318
319 Finally, we noted a connection between c-di-GMP and virulence, illuminating for the first time that an
320 airborne pathogen like *B. bronchiseptica* requires a functional c-di-GMP network to efficiently infect
321 a natural host like the mouse. Interestingly, dosages of above 50 CFU are necessary for the mutant
322 strain to be able to colonize the murine respiratory tract, suggesting that high c-di-GMP levels might
323 be required in the initial steps of infection. Thus, it is plausible that c-di-GMP may participate in

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324 regulating adaptation to hosts or to tissues of the same host. Unwrapping the c-di-GMP network in
325 *Bordetella* may give us a better understanding of how this organism infects hosts.
326
327

328 Material and Methods

329 **Bacterial strains and growth conditions.** Strains and plasmids used in this study are listed in Table
330 S1. Wild type *B. bronchiseptica* 9.73H$^+$ was isolated from a rabbit and described previously (32). *B.*
331 *bronchiseptica* wild type and mutants were grown on Bordet Gengou agar (Difco) supplemented with
332 15% (v/v) defibrinated fresh sheep blood (BGA medium) at 37 °C for 48 h and replated in the same
333 medium for 24 h. Stainer-Scholte liquid medium (SS) was used to grow *B. bronchiseptica* in broth
334 cultures (33). When appropriate, BGA or SS was supplemented with streptomycin (200 μg ml^{-1}),
335 kanamycin (80 μg ml^{-1}) and/or gentamycin (50 μg ml^{-1}). *Escherichia coli* (DH5-α, BTH101 and S17-
336 1) strains were cultured with lysogeny broth (LB) (34) in a test tube or on solidified LB with 1.5%
337 agar. When appropriate, antibiotics were added to the medium at the following concentrations: 10 μg
338 ml^{-1} gentamycin and 50 μg ml^{-1} kanamycin.
339

340 **Plasmid and strain construction.** Plasmids and strains were constructed using standard molecular
341 biology techniques; detailed descriptions of construction procedures are in the supplemental material.
342 Oligonucleotides used in this study are listed in Table S2.
343

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

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353 **Scanning electron microscopy.** *B. bronchiseptica* biofilm assays for scanning electron microscopy
354 were performed as previously described by our group (23). Briefly, glass coverslips were vertically
355 submerged in plastic tubes containing bacteria adjusted to $OD_{650} = 0.1$. After 24 h, the coverslip was
356 removed and treated to perform a critical-point procedure using liquid CO_2 (EMITECH, K850) and
357 sputter-coated with gold. Samples were visualized with a scanning electron microscope (Philips SEM
358 505) and the images were processed using an Image Soft Imaging System ADDA II.

359

360 **SDS PAGE and Western blot analysis**. *B. bronchiseptica* strains were grown at 37 ^{o}C for 16 h in SS
361 medium plus gentamycin, with or without $MgSO_4$ (40 mM) as indicated. Cells were collected,
362 normalized to an equal OD_{650nm} value, harvested by boiling samples in 1× Laemmli buffer for 10 min,
363 and subjected to SDS polyacrylamide gel electrophoresis followed by transfer of the contents of the
364 gel to a polyvinylidine fluoride (PVDF) filter. After transfer, the PVDF membrane was blocked with
365 5% non-fat milk powder in tris-buffered saline (TBS) for 1 h followed by incubation with polyclonal
366 anti-FlaA sera diluted 1:2,000 in TBS containing 5% non-fat milk powder at 4°C overnight (35). The
367 filter was then incubated with anti-mouse IgG-HRP conjugated (1:5,000) (Invitrogen) in TBS
368 containing 5% non-fat milk powder at room temperature for 2.5 h. Horseradish peroxidase conjugated
369 anti-mouse antibody (BioRad, USA) was used as secondary antibody. Chemi-luminescent reagent was
370 used for developing according to manufacturer's instructions. Samples from each strain and growth
371 condition combination were prepared and analyzed independently three times.

372

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

378

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379 **Motility assays.** SS soft-agar motility plate (0.35% agar) supplemented with 40 mM $MgSO_4$ and 1.0
380 mM $CaCl_2$ was used to determine the motility of bacterial strains as previously described (35). We
381 found that adding 1.0 mM $CaCl_2$ to motility medium allowed better visualization of the migration
382 zone border. The diameter of the migration zone was measured after 18 h of incubation at 37 °C.
383 Experiments were repeated at least three times, with at least three technical replicates.
384
385 **ID_{50}**. Wild-type C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME). All
386 experiments were carried out in accordance with institutional guidelines (Bordetella Host Interactions
387 AUP: A2016 02-010-Y2-A6). Power-G was used to calculate the number of mice needed to obtain
388 statistical significance (assuming an effect size of 0.60, power level of 0.80, and a probability level for
389 statistical significance of 0.05). Briefly, following anesthesia with 5% isoflurane, groups of 7 mice
390 were intranasally inoculated with 5 μL of PBS containing 6-100 CFU of wild-type or mutant *B.*
391 *bronchiseptica*. Seven days post-inoculation, mice were euthanized with CO_2 followed by cervical
392 dislocation and organs were collected in 1 mL of PBS and homogenized using a bead tissue disruptor
393 (Bead Mill 24, Fisher Scientific). Bacterial load was enumerated by dilution plating; the limit of
394 detection was 10 CFU. Results were graphed in GraphPad Prism (v8) and statistical significance was
395 calculated using one-way ANOVA.
396
397 **Ethics Statement**. This study was carried out in strict accordance with the recommendations in the
398 Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, USA. The
399 protocols used on this study have been approved by the Institutional Animal Care and Use Committee
400 at the University of Georgia, Athens, USA (A2016 02-010-Y2-A3 *Bordetella*-Host). Animals were
401 handled following institutional guidelines, in keeping with full accreditation from the Association for
402 Assessment and Accreditation of Laboratory Animal Care International.
403
404 **Statistical analyses.** Means were analyzed for significance using a one-way ANOVA with a Tukey's
405 multiple comparisons test to compare differences among groups. Shapiro-Wilks test was performed to
406 confirm normal distribution of data. Significance level is stated in each figure legend.

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408 **GenBank accession number.** *ycgR*: Gene ID 2663780, locus tag BB_RS07780; *bdcA* Gene ID
409 2661253, locus tag BB_RS17940; BB2109 Gene ID 2659958, locus tag BB_RS10615; BcdB Gene ID
410 2661408, locus tag BB_RS19575; LapD Gene ID 2662323, locus tag BB_RS05885.

411

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510 **Figure legends.**

511

512 **Fig. 1. Expression of the *bdcA* (BB3576) gene from a plasmid increases c-di-GMP levels and**
513 **reduces motility of *B. bronchiseptica*. A**. Quantitative measurements of cellular c-di-GMP
514 concentration from strains carrying the *Bb*-pEmpty, *Bb*-p*bdcA* and *Bb*-p*bdcA*(GAAF) plasmids. **B**.
515 Swimming motility using a soft agar (0.35%) motility assay with the *B. bronchiseptica* strains
516 described for panel A. Diameters greater than 4 mm were indicative of motility. Means with standard
517 errors are shown. Means marked with an asterisk are significantly different from *Bb*-pEmpty (Tukey,
518 $p < 0.001$).

519

520 **Fig. 2. Biofilm formation assay. A, B.** Biofilm formation by the indicated *B. bronchiseptica* strains
521 grown in SS medium either alone or supplemented with nicotinic acid (NA) at the indicated
522 concentration. Biofilm formation was assessed by crystal violet (CV) staining after 24 h in static
523 incubation conditions. Results are based on three biologically independent replicates and means with
524 standard errors are shown. Means marked with an asterisk are significantly different from *Bb*-pEmpty
525 in same NA concentration (Tukey, * $p < 0.01$, ** $p < 0.001$). **C.** Scanning electron microscope images
526 of *B. bronchiseptica* biofilms. *Bb*-pEmpty (left) and *Bb*-p*bdcA* (right) were grown statically on
527 vertically submerged coverslips at the indicated nicotinic acid concentration. After 24 h of growth, the
528 biofilms formed at the air–liquid interface were visualized. We performed two independent
529 experiments and examined ~20 fields per sample; a representative image is shown.

530

531 **Fig. 3. c-di-GMP inhibits flagellin production in *B. bronchiseptica*. A.** Western blot with
532 polyclonal anti-FlaA antibody was used to detect FlaA of *B. bronchiseptica* for each indicated strain
533 grown either in the absence or presence of 40 mM $MgSO_4$. **B.** Motility phenotypes of *B.*
534 *bronchiseptica* strains used in Western blot analysis in Panel A. Diameters greater than 4 mm were
535 indicative of motility. Means with standard errors are shown. Means marked with an asterisk are
536 significantly different from same strain harboring pEmpty plasmid in same media (Tukey, * $p <$
537 0.001, * $p < 0.01$).

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539 **Fig. 4. BdcA regulates motility by modifying c-di-GMP levels. A.** Motility phenotype of *B.*

540 *bronchiseptica 9.73* and the BbΔ*bdcA* strains in SS motility agar, as described in Materials and

541 Methods. Means with standard errors are shown. Means marked with an asterisk are significantly

542 different from wild-type *B. bronchiseptica Bb*9.73 (Tukey, p <0,01). **B.** Quantitative measurement of

543 cellular c-di-GMP level of *B. bronchiseptica Bb9.73* and the *Bb*Δ*bdcA* mutant. Formic acid-extracted

544 c-di-GMP was measured by liquid chromatography–mass spectrometry and normalized to mg dry

545 weight of bacteria after extraction. Means with standard errors are shown. Means marked with an

546 asterisk are significantly different from *Bb*-pEmpty (Tukey, p <0,001).

547

548 **Figure 5. BB2109 is required for c-di-GMP-mediated motility inhibition. A.** Motility phenotype

549 of *B. bronchiseptica Bb9.73, Bb*Δ*ycgR, Bb*Δ*lapD,* or *Bb*ΔBB2109 mutants carrying the pEmpty,

550 p*bdcA,* or p*bdcB* plasmids on SS motility agar as described in Materials and Methods. Means with

551 standard errors are shown. Means marked with an asterisk are significantly different from the same

552 strain harboring pEmpty plasmid (Tukey, p <0,001). **B.** The regions of BdcA and BB2109 that may

553 interact with each other are shown in this ribbon diagram. Five surface residues that are possible

554 points of contact are shown in α5 BdcA and α2 BB2109 helixes. **C.** Conservation of residues in EAL

555 and GGDEF domains of BB2109. The residues that form the enzyme active sites and are required for

556 diguanylate cyclase activity are shown in bold red. In the EAL domain, the catalytic EAL domain is

557 shown in bold red. Green, amino acids involved in Mg^{+2} binding; blue, amino acids involved in

558 substrate binding; orange, glutamate-stabilizing loop 6. Colors adapted from Römling et al., 2016

559 (29). For more details see the supplemental material.

560

561 **Figure 6. BB2109 is required for c-di-GMP-mediated biofilm formation.** Biofilm formation by *B.*

562 *bronchiseptica* strains grown in SS medium either alone or supplemented with nicotinic acid (NA) at

563 the indicated concentration. Biofilm formation was assessed by crystal violet (CV) staining after 24 h

564 in static incubation conditions. Means with standard errors are shown. Means marked with an asterisk

565 are significantly different (Tukey, * p < 0.01; ** p < 0.001).

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567 **Figure 7. BdcA activity is BB2109 dependent.** Quantitative measurement of cellular c-di-GMP level

568 of *B. bronchiseptica Bb9.73* and the *Bb*ΔBB2109 mutant carrying the pEmpty or p*bdcA* plasmids, as

569 indicated. Means with standard errors are shown. Means marked with an asterisk are significantly

570 different from *Bb*-pEmpty (Tukey, p <0,001).

571

572 **Figure 8. BdcA is required to effectively establish a respiratory tract infection in a mouse model.**

573 Five-week old mice were intranasally inoculated with 5 µL of PBS containing bacteria at different

574 bacterial load (6, 12, 25, 50, 100 CFU). Seven days post-inoculation, bacterial load in the organs of

575 the respiratory tract was enumerated. Each symbol represents a single animal, with the mean

576 colonization depicted as short horizontal bars. Data are pooled from 2 separate experiments conducted

577 independently. Means marked with an asterisk are significantly different from (Tukey, p <0,05). Note:

578 some points were allowed to be superimposed when plotted to increase the clarity of the presentation

579 but all data were included in the statistical analysis.

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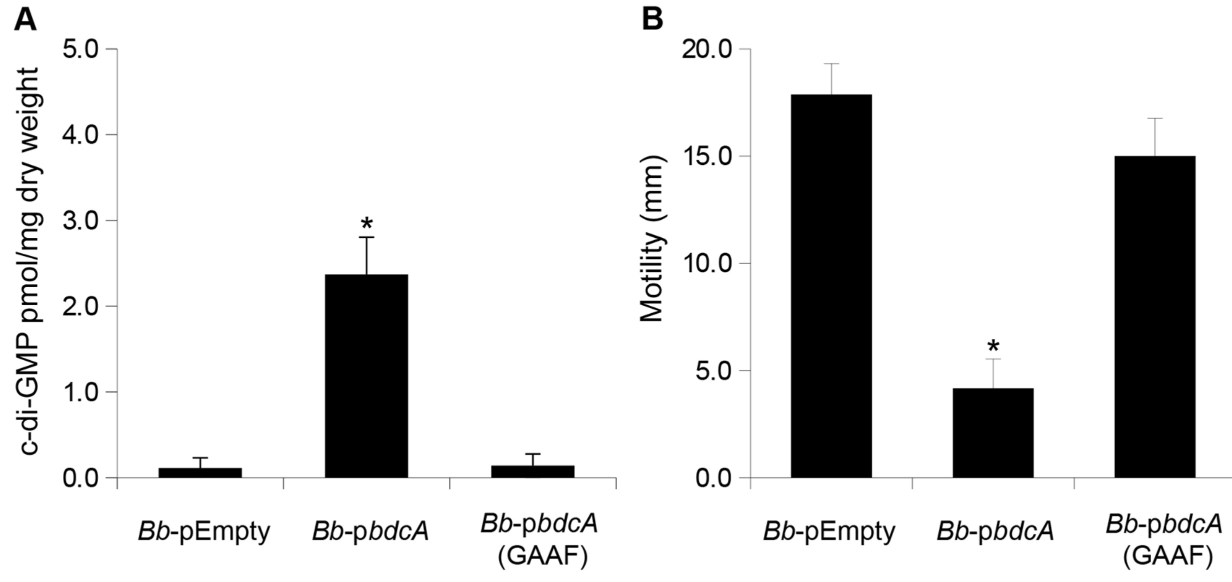

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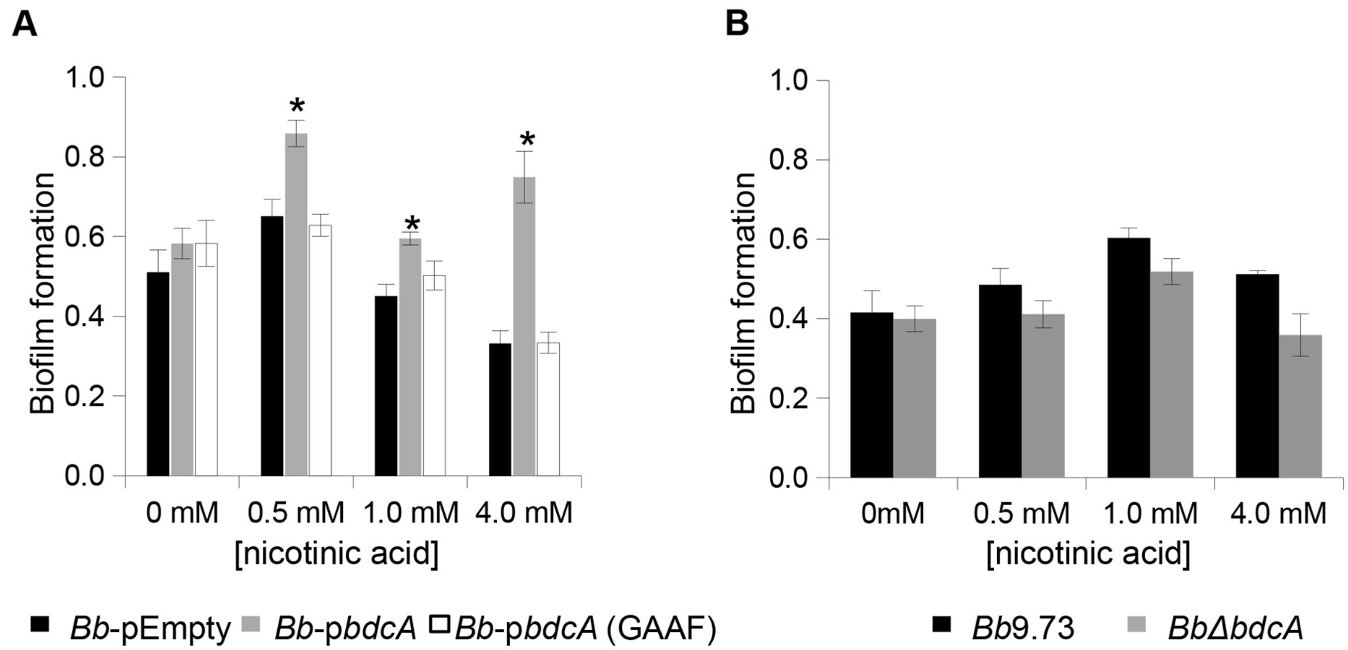

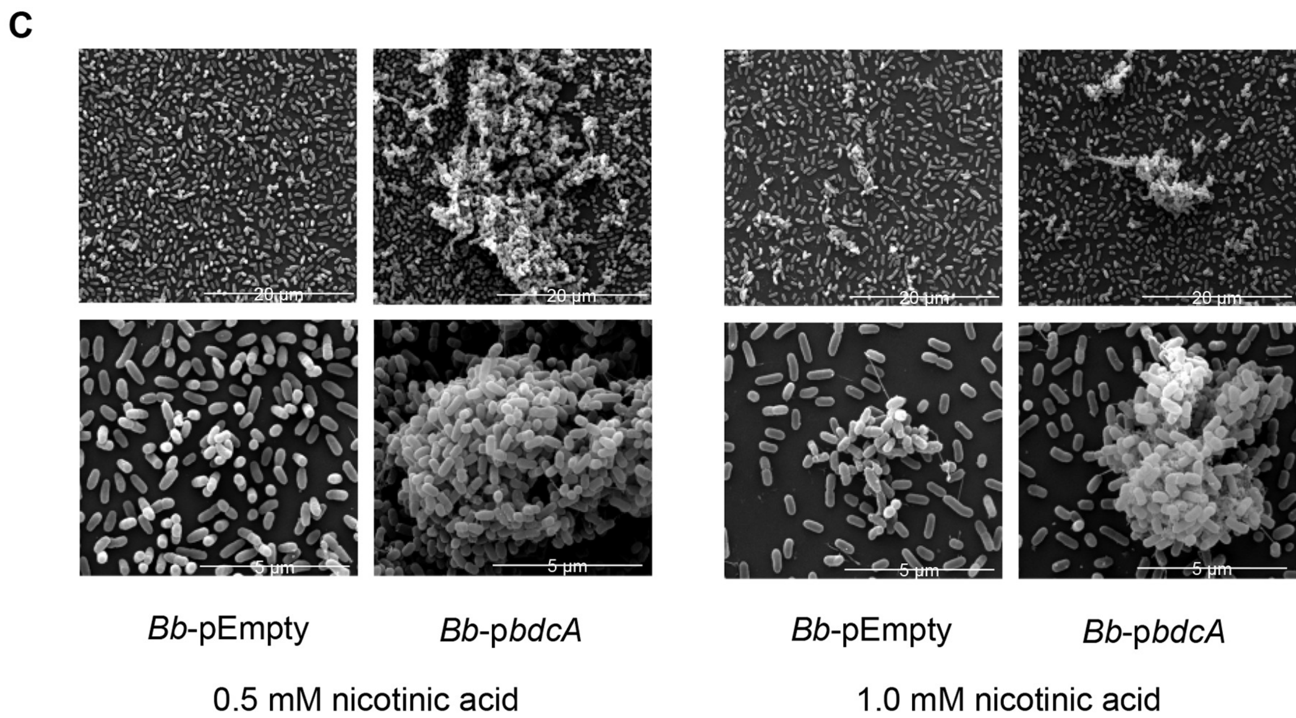

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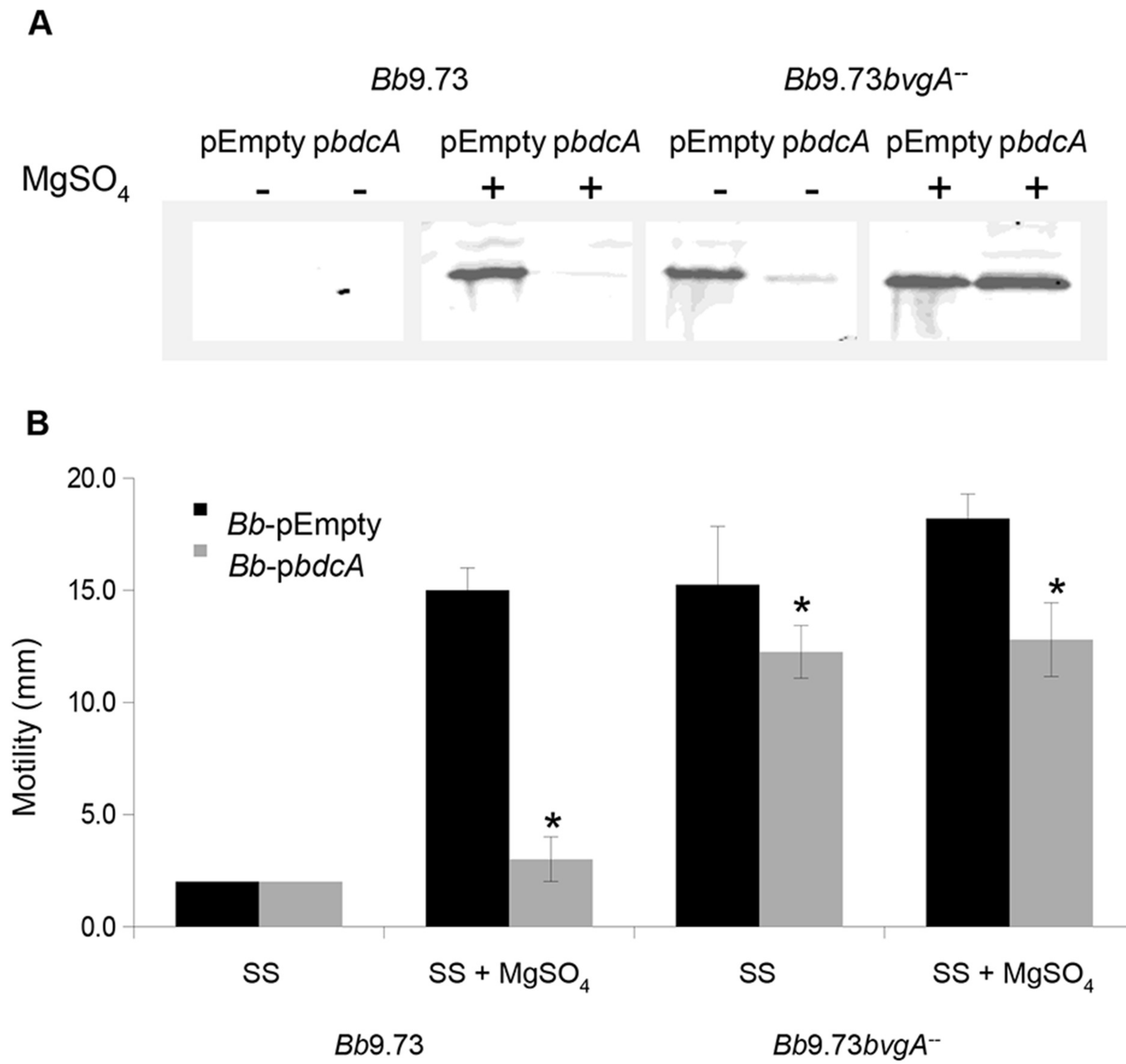

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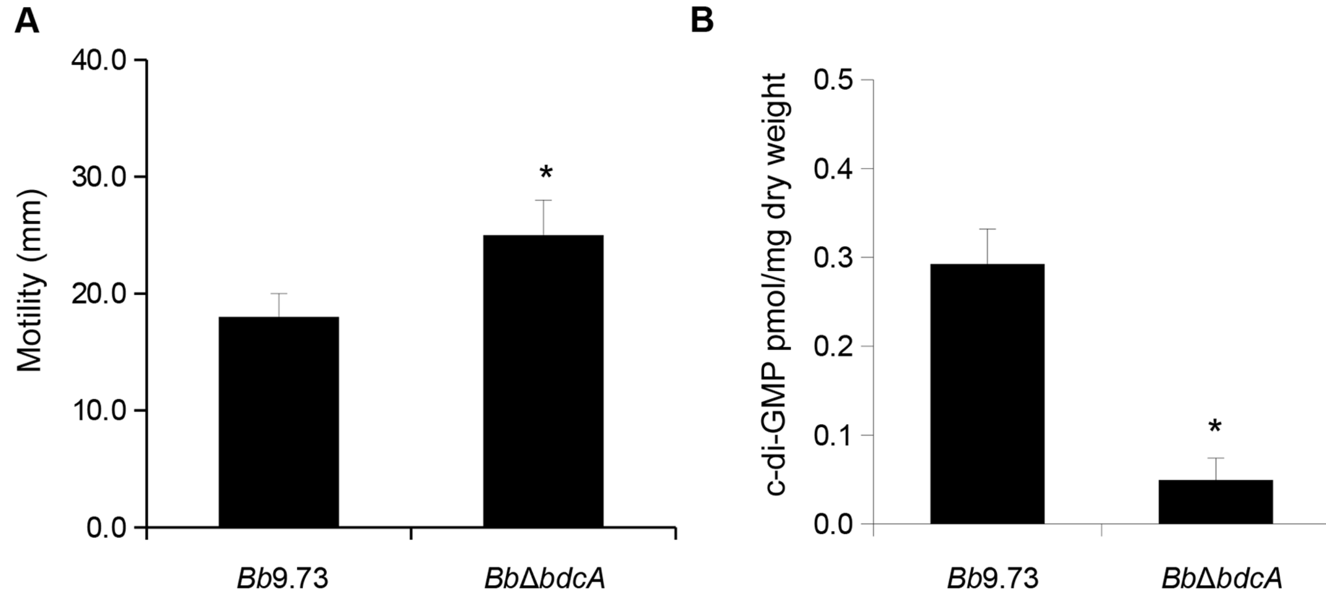

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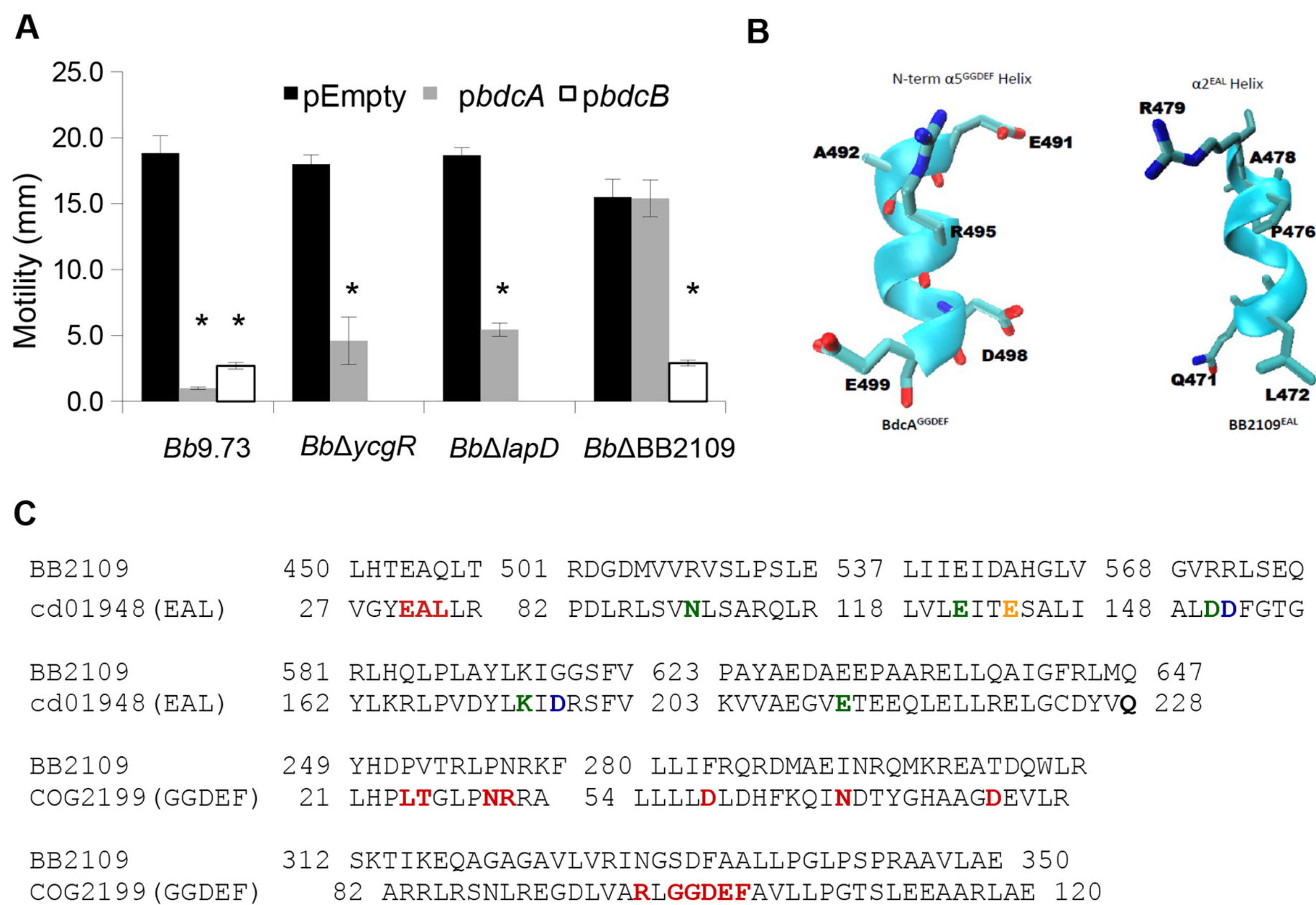

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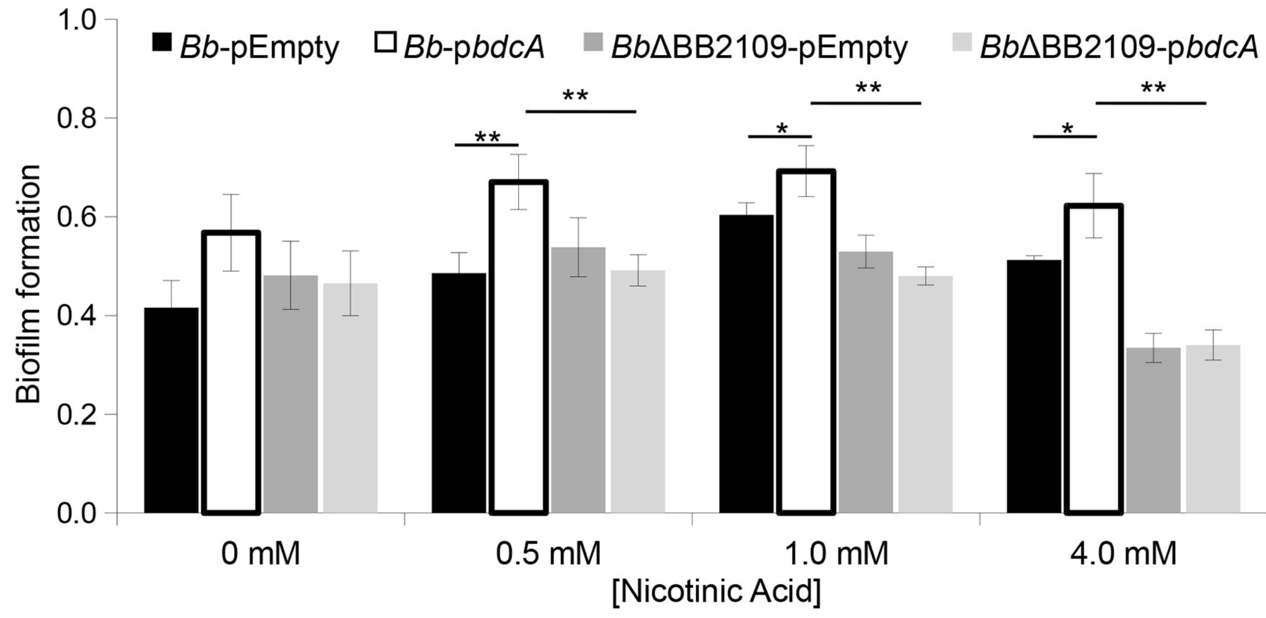

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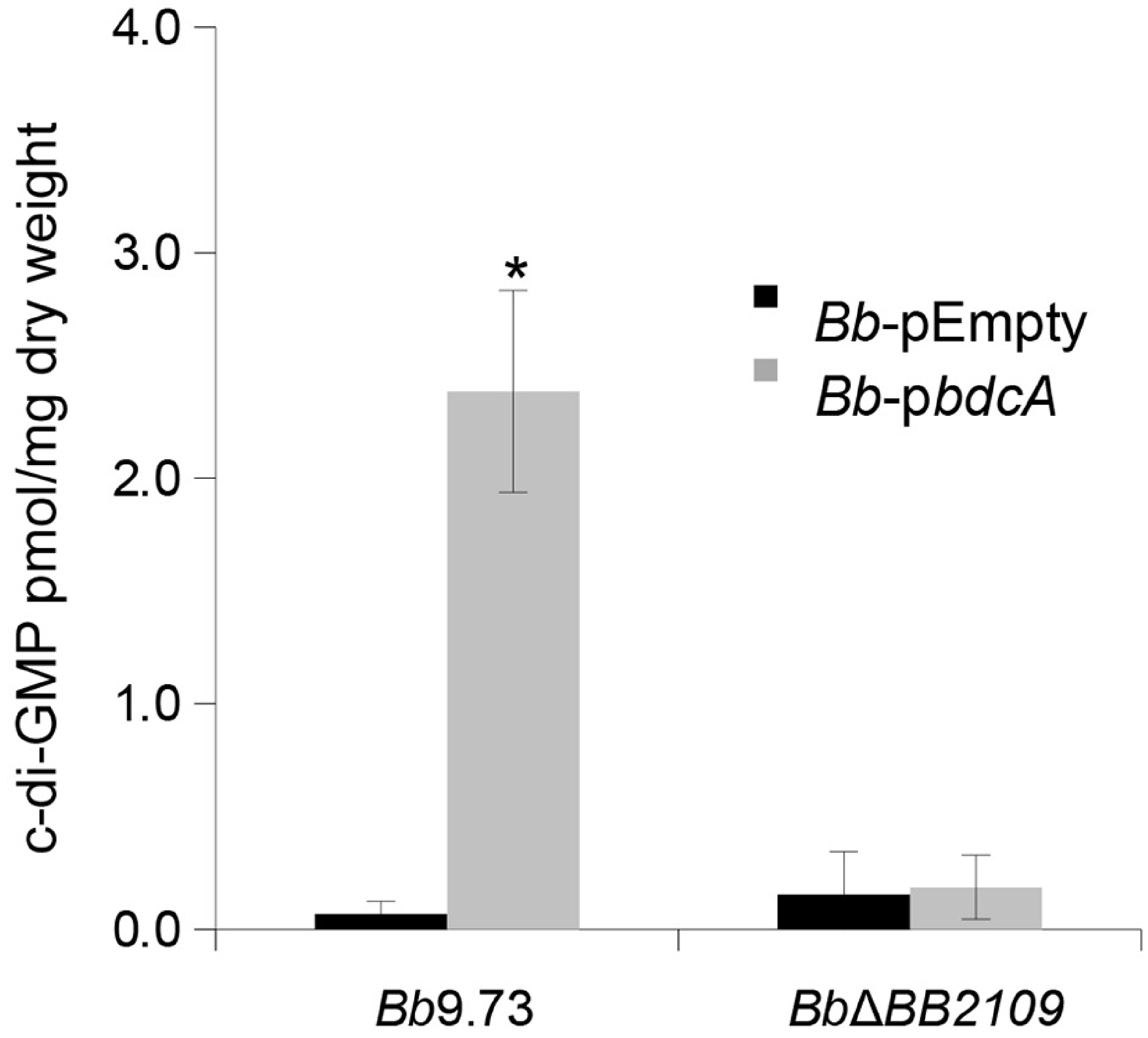

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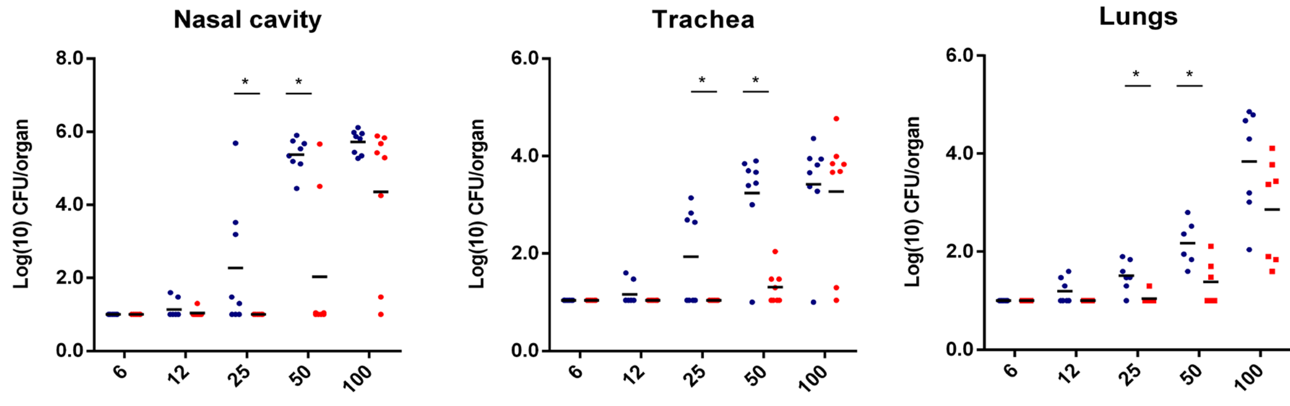

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