Cyclic-di-GMP signalling regulates motility and biofilm formation in *Bordetella bronchiseptica*

Federico Sisti,[1]† Dae-Gon Ha,[2] George A. O'Toole,[2] Daniela Hozbor[1] and Julieta Fernández[1]†

[1]Instituto de Biotecnología y Biología Molecular (IBBM)-CCT-CONICET-La Plata, Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, La Plata, Argentina

[2]Department of Microbiology and Immunology, Geisel School of Medicine at Dartmouth, Hanover, New Hampshire 03755

Correspondence
J. Fernández
julieta@biol.unlp.edu.ar.

The signalling molecule bis-(3′–5′)-cyclic-dimeric guanosine monophosphate (c-di-GMP) is a central regulator of diverse cellular functions, including motility, biofilm formation, cell cycle progression and virulence, in bacteria. Multiple diguanylate cyclase and phosphodiesterase-domain-containing proteins (GGDEF and EAL/HD-GYP, respectively) modulate the levels of the second messenger c-di-GMP to transmit signals and obtain such specific cellular responses. In the genus *Bordetella* this c-di-GMP network is poorly studied. In this work, we evaluated the expression of two phenotypes in *Bordetella bronchiseptica* regulated by c-di-GMP, biofilm formation and motility, under the influence of ectopic expression of *Pseudomonas aeruginosa* proteins with EAL or GGDEF domains that regulates the c-di-GMP level. In agreement with previous reports for other bacteria, we observed that *B. bronchiseptica* is able to form biofilm and reduce its motility only when GGDEF domain protein is expressed. Moreover we identify a GGDEF domain protein (BB3576) with diguanylate cyclase activity that participates in motility and biofilm regulation in *B. bronchiseptica*. These results demonstrate for the first time, to our knowledge, the presence of c-di-GMP regulatory signalling in *B. bronchiseptica*.

INTRODUCTION

Bis-(3′–5′)-cyclic-dimeric guanosine monophosphate (c-di-GMP) is a bacterial second messenger known to regulate a variety of cellular processes. c-di-GMP has been found to stimulate biofilm formation, inhibit motility and control the virulence of bacterial pathogens (Jenal & Malone, 2006; Kolter & Greenberg, 2006; Römling & Amikam, 2006; Hengge, 2009).

c-di-GMP is produced from two molecules of GTP by diguanylate cyclases (DGCs) and hydrolysed by a c-di-GMP-specific phosphodiesterases (PDE). DGC activity is conferred by the GGDEF functional domain, whereas PDE activity is performed by unrelated EAL or HD-GYP domains (Römling & Amikam, 2006). Notably, individual bacterial genomes frequently encode numerous GGDEF

and EAL/HD-GYP proteins (Galperin, 2004, 2005), implying that the c-di-GMP network is a highly complex and tightly regulated intracellular signalling system. The observation that most GGDEF and EAL domains are linked directly or through a two-component phosphorylation cascade (Galperin, 2006) to input signal domains (including PAS, blue-light-sensing, Cache, CHASE and MASE domains) implies that numerous environmental and internal signals can be integrated into the c-di-GMP network.

Bordetella bronchiseptica is a Gram-negative bacterium that causes respiratory tract infections in mammals, producing atrophic rhinitis in pigs, kennel cough in dogs and snuffles in rabbits (Goodnow, 1980). *B. bronchiseptica* has a variety of virulence factors and strategies that allows the host infection. Each factor such as pertactin (PRN), filamentous haemagglutinin (FHA), adenylate cyclase (AC), the type three secretory system (TTSS) and LPS are likely to perform specific functions required for successful colonization (Harvill *et al.*, 1999, 2000; Sisti *et al.*, 2002; Skinner *et al.*, 2004; Inatsuka *et al.*, 2010). The BvgAS two-component system plays a central role in the regulation of some of these factors such as PRN, FHA, AC and TTSS (Mattoo & Cherry, 2005). This system comprises a

---

†These authors contributed equally to this work.

histidine kinase sensor protein, BvgS, and a DNA-binding response regulator protein, BvgA. In response to environmental signals, BvgAS undergoes a series of phosphorelay signal transduction events that ultimately lead to differential transcription of target genes (Mattoo & Cherry, 2005). Growth at 37 °C in low concentrations of nicotinic acid (NA) and magnesium sulfate leads to the Bvg^{plus} or virulent phase, in which BvgAS activates expression of most known virulence factors. When the *Bordetellae* are grown at low temperatures (below 26 °C) or in the presence of millimolar concentrations of NA or magnesium sulfate, BvgAS is inactivated, resulting in the Bvg^{minus} or avirulent phase, in which transcription of most virulence factor genes is repressed and the expression of other factors is induced, resulting in the maximal expression of motility loci and genes required for the production of urease (Akerley & Miller, 1993; Akerley *et al.*, 1992; McMillan *et al.*, 1996). Intermediate phase, called Bvg^i (Bvg intermediate), occurs transiently during shifts between the Bvg^{plus} and Bvg^{minus} states and can be induced by the addition of magnesium sulfate or NA at concentrations lower than those needed to fully induce the Bvg^{minus} phase (Cotter and Miller, 1997). The Bvg^i phase is characterized by the expression of a subset of the Bvg^{plus}-phase genes (such as *fhaB* and *prn*) and Bvg^i-specific genes (such as *bipA* and *bcfA*) (Cotter and Miller, 1997; Stockbauer *et al.*, 2001).

The ability of this genus to form biofilms has been reported in previous work, with other authors reporting the ability of *B. bronchiseptica* to form biofilm-like structures on abiotic surfaces regulated by the two-component system BvgAS (Irie *et al.*, 2004; Mishra *et al.*, 2005). Static growth with intermediate NA concentrations represented the best conditions for biofilm formation (Mishra *et al.*, 2005). Like in other biofilms, extracellular DNA and exopolysaccharide are important for biofilm formation in *B. bronchiseptica* (Conover *et al.*, 2011). Sloan and colleagues even observed these structures *in vivo* in the nasal epithelium of mice infected with *B. bronchiseptica*. Those structures present a polysaccharide essential for *in vivo* biofilm development (Sloan *et al.*, 2007). However, the exact mechanism of this process and the bacterial factors involved has not been yet determined.

Although the cyclic-di-GMP signalling system has been characterized in several types of bacteria regulating biofilm formation and motility, the understanding of this important signalling mechanism is limited in *Bordetella* spp. So far there are no reports relating the c-di-GMP network to a specific phenotype in *B. bronchiseptica*. In the published genome of *B. bronchiseptica* RB50 strain there are four hypothetical proteins with EAL domains, ten with GGDEF domains and five with both domains (Amikam & Galperin, 2006). A recent study has identified a GGDEF diguanylate cyclase type protein with ability to synthesize c-di-GMP from the genome sequence of *B. pertussis* Tohama I. Deletion of the gene encoding this enzyme diminished the capacity of *B. pertussis* to adhere to abiotic surfaces (Wan *et al.*, 2009).

In the present work, we demonstrated that c-di-GMP signalling is present in *B. bronchiseptica* by introducing genes coding for proteins with proven PDE or DGC activity. Phenotypes previously linked with the presence of c-di-GMP in other bacteria were analysed, particularly biofilm formation and motility in soft agar. We also describe BB3576, a probable DGC, involved in swimming motility regulation and biofilm formation. The results reported here strongly suggest that c-di-GMP regulates these phenotypes in *B. bronchiseptica*.

METHODS

Bacterial strains and growth conditions. Strains and plasmids used in this study are listed in Table S1 available with the online version of this paper. *B. bronchiseptica* strains were grown on Bordet Gengou agar (Britania) supplemented with 10 % (v/v) defibrinated fresh sheep blood (BGA medium) at 37 °C for 48 h and replated in the same medium for 24 h. *Escherichia coli* (DH5-α and S17-1) and *Pseudomonas fluorescens* Pf0-1 strains were routinely cultured with liquid LB medium in a test tube or on solidified LB with 1.5 % (w/v) agar. Proteins with DGC (PA1120) and PDE (PA3947) domains were overexpressed in *B. bronchiseptica* 9.73 by using vectors with the inducible *tac* promoter to assess the consequences of alterations in c-di-GMP levels. Plasmids were introduced into *B. bronchiseptica* or in *Pseudomonas fluorescens* Pf0-1 by electroporation and transformants were selected in the presence of gentamicin (50 μg ml^{-1}).

B. bronchiseptica protein BB3576 was overexpressed in *B. bronchiseptica* by using broad-host-range plasmid pBBR1MCS-5 with a constitutive promoter nptII (Dombrecht *et al.*, 2001) cloned by us in *Sac*I and *Xba*I sites of the pBBR1MCS-5 multiple cloning site. Proof reading Pfx taq polymerase was employed to amplify the BB3576 gene from *B. bronchiseptica* 9.73 with primers BB3576FXhoI (5′ATGC-CTCGAGGACGGGGTCGGATAAGGATA3′) and BB3576RKpnI (5′CTG-AGGTACCTTCGGTCAGGAACGCAGGTC3′). PCR conditions were 94 °C for 4 min (1 cycle), followed by 35 cycles of 94 °C for 20 s, 58 °C for 20 s, and 68 °C for 2 min and a final extension of 68 °C for 5 min. Underlined portions indicated restriction enzyme sites. Underlined portions indicated restriction enzyme sites. The PCR product was digested with *Xho*I and *Kpn*I and cloned into pBB1RMCS-5-nptII (renamed pEmpty) that had been digested with the same enzymes, to generate pBB3576. Constructs were confirmed by sequencing. Plasmids were transferred into *B. bronchiseptica* 9.73 or into *Pseudomonas fluorescens* Pf0-1 by electroporation and transformants were selected in the presence of gentamycin (50 μg ml^{-1}).

Biofilm assays. *B. bronchiseptica* biofilm formation assays using static cultures were performed as described previously (Irie *et al.*, 2004) from overnight cultures into Stainer–Scholte (SS) liquid medium (Stainer & Scholte, 1970). The overnight culture was diluted to OD$_{650}$ 0.1, pipetted into each well of a sterile 96-well U-bottom microtitre plate (both polycarbonate and PVC surfaces) and incubated statically at 37 °C with NA or magnesium sulfate at different concentrations. 1 mM IPTG and gentamicin (50 μg ml^{-1}) were added, when appropriate.

For *P. fluorescens* biofilms an aliquot (1.5 μl) of an overnight culture grown in LB was transferred into 100 μl K10T-1 medium in a 96-well plate (353911; BD Falcon) and grown statically for 6 h at 30 °C. K10T-1 medium, used in prior studies as a biofilm-promoting, phosphate-rich medium, contains 50 mM Tris/HCl (pH 7.4), 0.2 % (w/v) Bacto tryptone, 0.15 % (v/v) glycerol, 0.6 mM MgSO$_4$ and 1 mM K$_2$HPO$_4$ and was prepared as described previously (Newell *et al.*, 2011).

In all assays planktonic bacteria were removed and remnant cells were stained with 0.1 % crystal violet solution (CV). The stain was dissolved by adding 120 µl 33 % acetic acid solution. A 100 µl sample was transferred to a microplate and then quantified by measuring OD_{595}. Each data point results from an average for six wells and each experiment was repeated at least three times with similar results. Figures are representative of one of those replicates.

Measurements of c-di-GMP levels. c-di-GMP levels were analysed via LC-MS. Strains of interest were grown in BGA media for 24 h. Four replicates of each strain was harvested and resuspended in 250 µl extraction buffer [methanol : acetonitrile : water (40 : 40 : 20) plus 0.1 M formic acid at −20 °C] and incubated at −20 °C for 30 min. The cell debris was pelleted for 5 min at 4 °C, and the supernatant containing the nucleotide extract was saved. Samples were immediately adjusted to a pH of approximately 7.5 with 15 % $(NH_4)_2HCO_3$ and stored on dry ice prior to analysis. The resultant extract was analysed via LC-MS using the LC-20AD HPLC system (Shimadzu) coupled to a Finnigan TSQ Quantum Discovery MAX triple-quadrupole mass spectrometer (Thermo Electron) as previously described (Newell *et al.*, 2011).

Motility assays. SS soft-agar motility plate [0.35 % (p/v) agar] supplemented with $MgSO_4$ 40 mM and 1 mM IPTG was used to determine the motility of bacterial strains as previously described (Fernández *et al.*, 2005). The diameter of the migration zone was measured after 18 h of incubation at 37 °C.

Scanning electron microscopy (SEM). *Bordetella* strains were cultured statically on glass coverslips partially submerged vertically in 1.5 ml plastic tubes (Mishra *et al.*, 2005). The glass coverslips were placed such that an air–liquid interface was established. After 48 h the coverslip was removed and washed with sterile PBS (KH_2PO_4 3 mM, Na_2HPO_4 10 mM, NaCl 120 mM), and the bacteria were fixed with 2.5 % glutaraldehyde. Samples were dehydrated in a graded ethanol series (20, 50, 70, 90 and 100 % for 60 min each), dried by critical point using liquid carbon dioxide (EMITECH, K850) and sputter coated with gold. The surface topographies of the biofilm were visualized with a scanning electron microscope (Philips SEM 505), and the images were processed [Image Soft Imaging System ADDA II (SIS)].

RNA extraction and qPCR quantification. RNA preparation from bacteria was performed using the Illustra RNAspin kit (GE). Samples were placed on ice, and quantification of RNA was performed using a ND-1,000 NanoDrop spectrophotometer at 260 nm. Measurements of A_{260}/A_{280} were used to determine the purity of the RNA. DNase (Promega) treatment was performed according to manufacturer's instructions in order to eliminate contaminating DNA. The synthesis of cDNA was performed with a Reverse Transcription System kit (Promega) according to the manufacturer's protocol using random primers. For each sample 1 µg RNA was used. The reaction was incubated at room temperature for 10 min, and reverse transcription was performed in a thermal cycler at 42 °C for 15 min and 95 °C for 5 min. qPCR conditions were 95 °C for 10 min (1 cycle), followed by 40 cycles of 60 °C for 30s, 95 °C for 15 s and a final increasing temperature cycle between 55 and 95 °C for 10 s when melt curve data was obtained. Samples were placed on ice for 5 min to stop the reaction. Samples that had not undergone the reverse transcription process were used as controls for genomic DNA absence in real-time PCR experiments. Only samples with threshold cycle (C_t) values above mock values were used in further experiments. Real-time PCR were performed using the SYBR green master mix 2× (Bio-Rad). Primers employed are summarized in Table S2. All samples, including a negative control using mock samples instead of DNA, were run in triplicates. The data were analysed using C_t calculations using *recA* expression levels as a normalizer. Results were expressed as fold increase over values from bacteria growth in SS media without additional NA.

Statistical analyses. All the results were compared by ANOVA followed by the Tukey test. A value of $P < 0.01$ was considered significant. To identify significant differences in RNA expression, C_t values were analysed using the REST program.

RESULTS

B. bronchiseptica biofilm formation is enhanced by diguanylate cyclase activity

Because some authors have described that not all proteins with GGDEF domains have DGC activity, we first decided to use a protein with known *in vitro* and *in vivo* DGC activity in our studies (Kulesekara *et al.*, 2006). Protein PA1120 with DGC activity was overexpressed in *B. bronchiseptica* 9.73 (*Bb*-DGC) by using a vector with the inducible *tac* promoter. Clone *Bb*-DGC grown in IPTG-containing media overexpressed PA1120 and this strain was able to grow in a batch culture at the same rate as the parental strain (data not shown). The ability to form biofilm was evaluated by the crystal violet method in 96-well polycarbonate U-bottom and polyvinylchloride (PVC) microtitre plates as described in Methods. Biofilm formation is regulated by the BvgAS two-component system (Irie *et al.*, 2004; Mishra *et al.*, 2005). Hence, we determined the amount of biofilm biomass formed by strains under Bvg-modulated-phase conditions in the presence of NA (0–8.0 mM). This chemical compound serves as an environmental modulator of BvgAS activity (Akerley *et al.*, 1992), at 0 mM NA the BvgAS system should be fully activated or in Bvg^{plus} phase and at 8 mM NA the BvgAS system should be fully inactivated or in Bvg^{minus} phase. In our hands, *B. bronchiseptica* 9.73 (*Bb*WT) strain showed biofilm formation on both polycarbonate and PVC surfaces and the biofilm pellicle was enhanced in intermediate modulation conditions in agreement with results for *B. bronchiseptica* RB50 strain (Irie *et al.*, 2004). In Fig. 1(a) results obtained on polycarbonate surface are shown and are similar to PVC results (not shown). As expected for a high c-di-GMP intracellular concentration, overexpression of PA1120 in *Bb*-DGC significantly enhanced pellicle formation at NA concentrations from 0 to 4.0 mM (Fig. 1a) compared with the parental strain. This difference was observed only when IPTG-supplemented SS was used. Surprisingly, when PA1120 was overexpressed biofilm formation was particularly exacerbated in the Bvg^{minus} phase (4.0 mM NA; Fig. 1a). We observed no biofilm formation in 8.0 mM NA media, but planktonic growth in that condition was significantly slower compared with that for media without additional NA (data not shown). On the other hand, overexpression of PA3947 with PDE activity produced a biologically significant decrease in the biofilm formation as compared with the parental strain only in 1.0 mM NA (Fig. 1a).

The BvgAS system is downregulated by NA and by magnesium sulfate. For that reason, we performed biofilm quantification with different magnesium sulfate concentrations
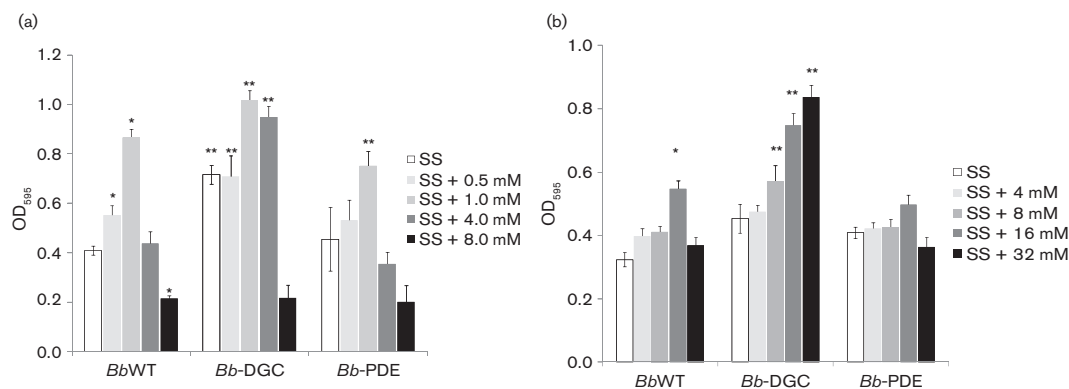
Fig. 1. Effect of DGC or PDE overexpression in *B. bronchiseptica* biofilm formation. Biofilm development by *Bb*WT, *Bb*-DGC and *Bb*-PDE cultured in SS medium either alone or supplemented with NA (a) or magnesium sulfate (b). Biofilm formation was assessed by polycarbonate microtitre plate assays as described in Methods. * $P<0.01$ vs *Bb*WT grown in SS; ** $P<0.01$ vs *Bb*WT at the same modulator concentration.

corresponding to Bvg^{plus}, Bvg^i and Bvg^{minus} phases, as reported previously (Williams & Cotter, 2007). We observed that biofilm formation was also enhanced in *Bb*-DGC at Bvg^i (16 mM) and Bvg^{minus} (32 mM) magnesium sulfate-induced phases compared with the parental strain (Fig. 1b).

In order to confirm that NA concentrations corresponded to established phases in *Bb*WT like in the sequenced *B. bronchiseptica* strain RB50, mRNA extraction and quantification was performed. Bacteria cultures grown in SS with NA at concentrations between 0 and 4.0 mM were subject to mRNA purification. Levels of *cyaA* (AC, Bvg^{plus} phase factor), *bipA* (Bvg^i phase factor) and *flaA* (flagellin, Bvg^{minus} phase factor) mRNA were normalized to *recA* and compared. Results obtained confirmed that with NA in the concentrations assayed all the virulence phases were detected, 0.5 mM NA

and 1 mM NA correspond to Bvg^i phase and 4 mM NA corresponds to Bvg^{minus} phase. (Fig. 2).

Overexpression of a diguanylate cyclase in *B. bronchiseptica* induced increased intracellular c-di-GMP levels

In order to confirm that PA1120 overexpression in *Bb*-DGC correlated with increased DGC activity *in vivo*, intracellular c-di-GMP levels were determined. Cultures with or without IPTG induction were subjected to c-di-GMP extraction and analysis. As shown in Fig. 3, *Bb*-DGC in the presence of IPTG presented significantly higher c-di-GMP levels than the strain grown under non-inducing conditions, supporting the hypothesis that biofilm phenotypes
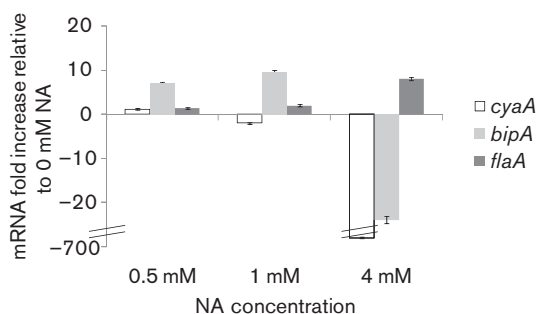
Fig. 2. mRNA expression of *B. bronchiseptica* 9.73 Bvg-regulated factors at different nicotinic acid concentrations. mRNA transcripts levels of *cyaA* (Bvg^{plus} gene), *bipA* (Bvg^i gene) and *flaA* (Bvg^{minus} gene) were quantified by qPCR compared with the constitutive *recA* gene. Values were normalized to levels observed in SS media without additional NA (Bvg^{plus} phase).
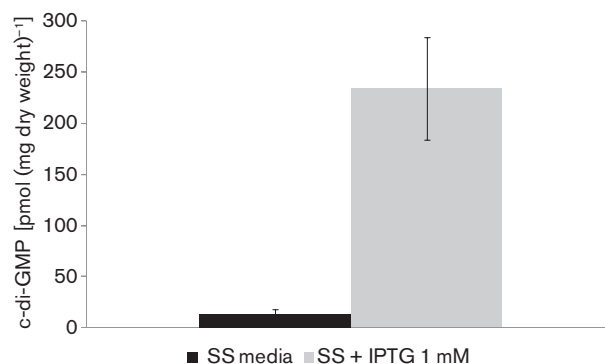
Fig. 3. *In vivo* assessments of DGC activity in *B. bronchiseptica*. Quantitative measurements of cellular c-di-GMP from the *Bb*-DGC strain compared with *Bb*WT. Formic acid-extracted c-di-GMP was measured by LC-MS and normalized to mg dry weight of bacteria after extraction. The difference observed was significant ($P<0.001$).

observed in *Bb*-DGC were a consequence of increased c-di-GMP level.

Scanning electron microscopy of *B. bronchiseptica* cells adhered to glass coverslips

To further investigate the consequences of DGC activity in biofilm development, biofilms formed at the air–liquid interface in biphasic cultures on glass coverslips were observed by SEM. As expected for Bvg^i conditions, at 48 h all strains grown in 1.0 mM NA presented thick multi-layered stack of cells alternated with stacked cells aggregated in clusters similar to those observed by other authors (Fig. 4) (Mishra *et al.*, 2005). Wild-type cells grown in Bvg^{plus} phase (0 mM NA) formed a thin layer with small aggregates, whereas cells grown in Bvg^{minus} phase (4.0 mM NA) appear as diffuse, interspersed bacteria (Fig. 4). Interestingly, SEM analysis of *Bb*-DGC biofilms revealed stacked bacteria more frequently and with bigger dimensions, resulting in an architecture that appeared to encase the bacterial microcolonies. These structures were observed in all virulence phases tested. In the case of *Bb*-PDE, cells grown at Bvg^{plus} (0 mM NA) or Bvg^{minus} (4.0 mM NA) phase were spread around the disks with large regions remaining uncolonized. Altogether these results further corroborate those from CV quantification for *Bb*-DGC, with the exception that no differences were observed at 1.0 mM NA between *Bb*-PDE and *Bb*WT in this analysis.

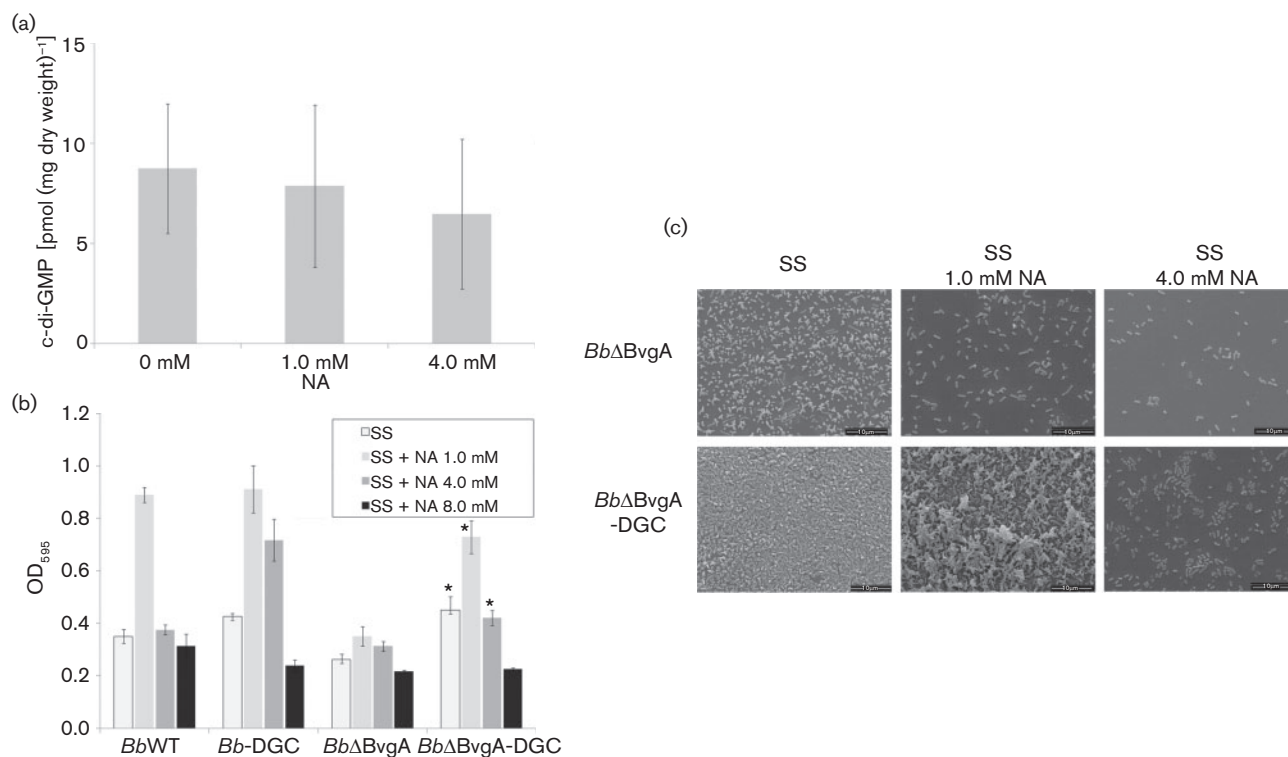## BvgA is not necessary for diguanylate-cyclase-dependent biofilm formation

Bacterial proteins that mediate c-di-GMP turnover and signal transduction are often composed of multiple domains, allowing for a variety of regulatory inputs (Galperin *et al.*, 2001). In particular, interplay between c-di-GMP network and two-component systems has been observed in many bacteria (Lai *et al.*, 2009; Mikkelsen *et al.*, 2009). *B. bronchiseptica* biofilm formation was dependent on Bvg phase conditions and c-di-GMP signalling as described above, indicating a probable connection between the two regulatory systems.

As described above, biofilm formation was dependent on Bvg phase condition even if high c-di-GMP intracellular levels were induced. A hypothesis to explain this observation is that the BvgAS system controls c-di-GMP levels. However, quantification of intracellular c-di-GMP levels under different Bvg-modulated conditions shows no significant differences between c-di-GMP concentrations (Fig. 5a), thus we must reject this hypothesis.

Although c-di-GMP concentration was apparently independent of direct BvgAS regulation, we assessed the effect of a *bvgA* mutant in a strain overexpressing a DGC. We
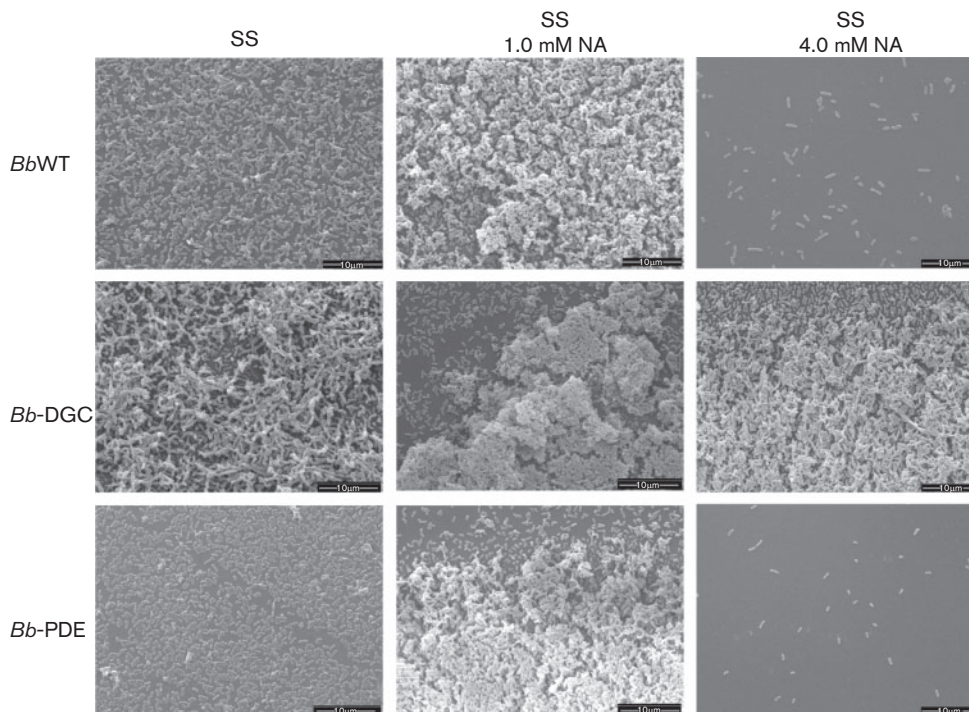
Fig. 4. SEM images of *B. bronchiseptica* biofilms. *Bb*WT (upper row of panels), *Bb*-DGC (middle row of panels) and *Bb*-PDE (bottom row of panels) were grown statically on vertically submerged coverslips in SS medium alone (left column of panels), SS supplemented with 1.0 mM NA (middle column of panels) or SS supplemented with 4.0 mM NA (right column of panels). After 48 h of growth, the biofilms formed at the air–liquid interface were visualized by SEM.

Fig. 5. *BvgA* is not necessary for diguanylate-cyclase-dependent biofilm formation. (a). Quantitative measurements of cellular c-di-GMP from *B. bronchiseptica* grown planktonically in BGA supplemented with different NA concentrations. Formic-acid-extracted c-di-GMP was measured by LC-MS and normalized to mg dry weight of bacteria after extraction. (b) Biofilm formation by *Bb*WT, *Bb*-DGC, *Bb*ΔBvgA and *Bb*ΔBvgA-DGC cultured in SS medium either alone or supplemented with NA was analysed by CV staining after 24 h in static conditions. (c) SEM of 48 h air–liquid interface submerged coverslips in SS medium alone (left panels), SS supplemented with 1.0 mM NA (middle panels) or SS supplemented with 4.0 mM NA (right panels). * $P<0.01$ vs *Bb*ΔBvgA grown at the same NA concentration.

overexpressed PA1120 with DGC activity in *B. bronchiseptica* ΔBvgA (*Bb*ΔBvgA), a mutant locked in the BvgA^{minus} phase and previously constructed by our group (Fernández *et al.*, 2005). All strains were assayed for biofilm formation in 96-well polycarbonate U bottom plates. As expected, *Bb*ΔBvgA showed poor biofilm formation in all Bvg modulatory conditions, similar to the BvgA^{minus} phase (Fig. 5b). However, when PA1120 in *Bb*ΔBvgA-DGC was overexpressed, significantly higher biofilm formation was detected at NA concentrations lower than 8.0 mM.

Biofilms produced by DGC-expressing bacteria in a ΔBvgA background were analysed by SEM. As shown in Fig. 5(c), *Bb*ΔBvgA-DGC in 1.0 mM NA was the only strain and condition that formed a uniform multilayered stack of cells, but the volume of the structure was smaller than that for *Bb*-DGC, corroborating the CV determinations (Fig. 4). As expected, *Bb*ΔBvgA showed no distinct region of biofilm formation and cells were spread evenly across the coverslips (Fig. 5c).

c-di-GMP regulates swimming motility in *B. bronchiseptica*

In several bacteria c-di-GMP suppresses swimming, swarming and twitching motility (Jenal & Malone, 2006;

Römling & Amikam, 2006). Flagellar expression and motility in *B. bronchiseptica* is regulated by the BvgAS two-component system and is maximal in the BvgA^{minus} phase (Akerley *et al.*, 1992). Therefore, we evaluated strain motility in the presence of modulating concentrations of magnesium sulfate. As expected, all strains showed motility in soft agar in modulated conditions. However, when DGC overexpression was induced by IPTG in WT reduced motility was observed (Fig. 6). This phenotype was observed also in a ΔBvgA background. *Bb*-PDE showed no significant differences from the wild-type strain with regard to motility.

BB3576: a hypothetical GGDEF protein regulating swimming motility and biofilm formation in *B. bronchiseptica*

The *B. bronchiseptica* genome contains 15 proteins with GGDEF domains. As observed above, DGC overexpression in *B. bronchiseptica* inhibits swimming motility in soft agar. In *E. coli*, swimming is regulated by c-di-GMP through diverse DGCs and PDEs. Deletion of genes encoding GGDEF proteins YeaI, YedQ, YfiN, YeaJ and YneF in *E. coli* increased swimming motility (Sanchez-Torres *et al.*, 2011).
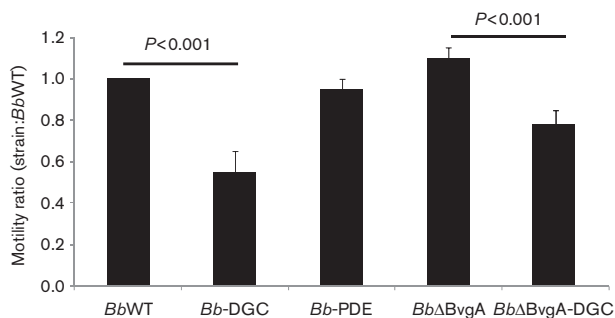
Fig. 6. Motility phenotypes of *B. bronchiseptica* strains. The diameters of migration zones of the wild-type, overexpression strains and mutants were measured and expressed relative to wild-type diameter after 18 h of incubation at 37 °C on SS motility plates supplemented with 32 mM magnesium sulfate. One hundred per cent motility in *Bb*WT was 12 mm. The results are based on three replicates.

Also, YeaJ, YedQ and YfiN overexpression negatively regulate motility without impairing it completely (Pesavento *et al.*, 2008). These results suggested that some proteins with DGC activity are involved in fine-tuning regulation of flagellar activity. Among these proteins, YeaJ also has a Cache domain similar to the domain found in the BB3576 protein of *B. bronchiseptica*. Swimming motility in *B. bronchiseptica* is regulated mainly by the two-component system BvgAS and is fully expressed in Bvg^{minus} phase. Hence we hypothesized that if BB3576 is involved in motility regulation it should be expressed in the Bvg^{minus} phase. Bacteria grown in the presence of NA were subjected to RNA extraction and subsequent cDNA synthesis. qPCR relative quantification of BB3576 mRNA demonstrated that BB3576 is expressed in all phases tested and 36 times higher in the Bvg^{minus} than in the Bvg^{plus} phase, suggesting that BB3576 protein may be present when flagellar apparatus is functionally active (Fig. 7a).

As was observed for *Bb*-DGC, overexpression of BB3576 should inhibit swimming motility in Bvg^{minus} phase. The BB3576 gene was cloned into a broad-host-range plasmid pBB1RMCS-5 under control of the *nptII* constitutive promoter and transferred to *Bb*-DGC to obtain *Bb*-BB3576. These bacteria showed reduced swimming motility in soft agar compared with wild-type *B. bronchiseptica* transformed with the empty vector (Fig. 7b). This behaviour was also observed when *Bb*ΔBvgA transformed with the empty vector was compared with *Bb*ΔBvgA–BB3576 (data not shown).

As described above, DGC overexpression correlates with impaired motility and enhanced biofilm formation in *B. bronchiseptica*. *Bb*-BB3576 showed impaired swimming in soft agar, hence we decided to evaluate if biofilm formation was different from that of the wild-type strain. As expected for a strain with high c-di-GMP levels, biofilm formation

of *Bb*-BB3576 was enhanced in Bvg^{i} phase (1.0 mM NA) compared with *B. bronchiseptica* transformed with the empty vector (Fig. 7c). Furthermore, biofilm formation for *Bb*ΔBvgA–BB3576 in Bvg^{i} phase was enhanced compared with *Bb*ΔBvgA transformed with the empty vector (Fig. 7c).

BB3576 complements a diguanylate cyclase mutant phenotype

The BB3576 protein expressed in *B. bronchiseptica* resulted in phenotypes consistent with this protein being an active DGC. To confirm that BB3576 has DGC activity, we transformed the *P. fluorescens* Δ4DGC (*Ps*Δ4) strain with the pBB3576 plasmid. Strain *Ps*Δ4 has the genes coding for four DGC proteins deleted from its genome and is not able to produce biofilm under static growth conditions (Newell *et al.*, 2011). A previous study showed that complementation with one DGC protein was sufficient to restore biofilm formation to this strain (Newell *et al.*, 2011). Biofilm formation for the *P. fluorescens* strains was evaluated in a 96-well plate grown statically for 6 h at 30 °C in K10T-1 medium (Fig. 8). When *Ps*Δ4 was complemented with a DGC-encoding-gene *gcbB*, biofilm formation was restored as previously described (Newell *et al.*, 2011). When a plasmid carrying BB3576 was introduced, biofilm formation was also observed, suggesting a DGC activity for this protein in concordance with the results described above (Fig. 8).

DISCUSSION

The signalling molecule c-di-GMP has been reported to regulate phenotypes like biofilm formation, motility and virulence. It is generated from two GTP molecules by GGDEF-domain-containing DGC enzymes, and degraded by PDE enzymes containing either EAL or HD-GYP protein domains. Despite the frequent occurrence of these protein domains encoded in bacteria genomes, information on mechanisms and physiological roles regarding their function and regulation is sparse. Many of these proteins have also other domains, suggesting that c-di-GMP activity is regulated by these additional domain functions. So we can imagine that cell c-di-GMP turnover is regulated through the consensus of various external and internal factors. The balance of these signals ultimately regulates biofilm formation, motility and virulence.

In the present work we showed that c-di-GMP signalling is involved in *B. bronchiseptica* biology. We overexpressed proteins with demonstrated *in vitro* and *in vivo* activity in order to establish if c-di-GMP regulation was present in this microbe. We overexpressed PA1120 or PA3947 with known DGC and PDE activities, respectively, in wild-type *B. bronchiseptica*. As shown in Fig. 1, PA1120 with a GGDEF functional domain overexpression leads to an enhanced biofilm phenotype compared with the wild-type strain under all conditions tested. Biofilm formation was
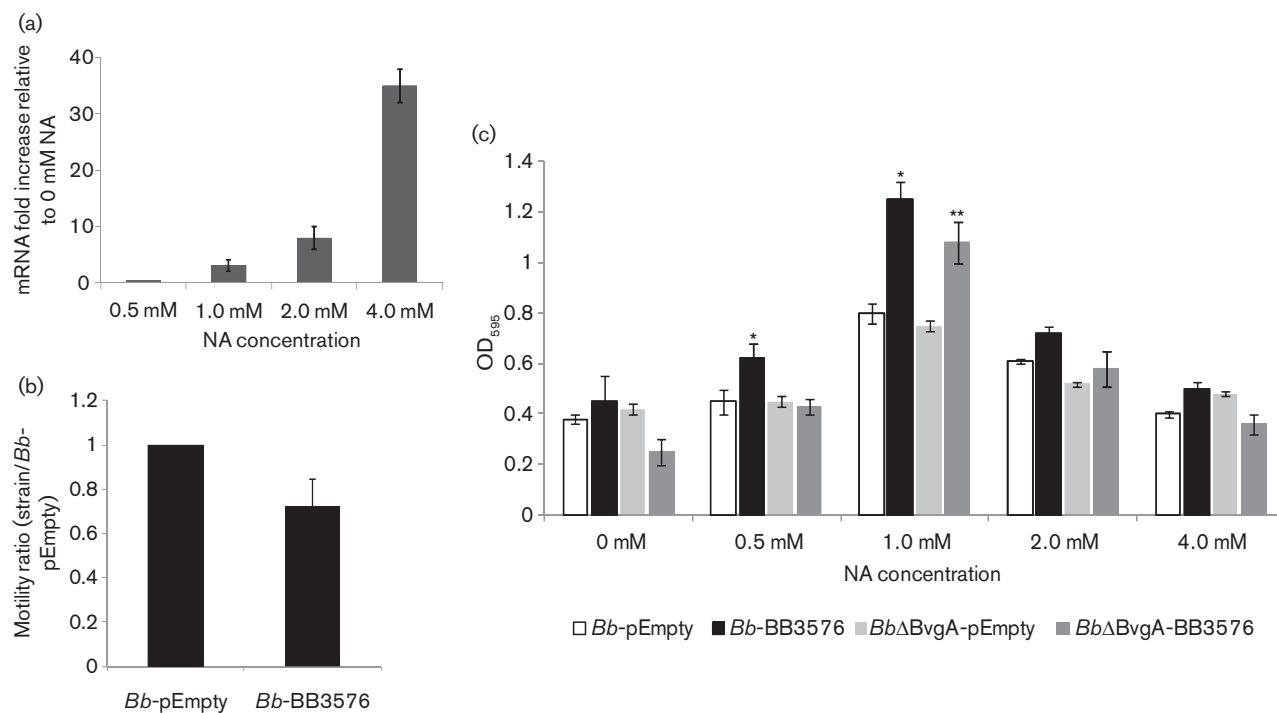
Fig. 7. Motility and biofilm phenotypes of *B. bronchiseptica* overexpressing BB3576. (a) mRNA transcripts levels of BB3576 were quantified by qPCR compared with the constitutive *recA* gene. Values were normalized to levels observed in SS media without additional NA. (b) The diameters of migration zones of the wild-type strain with pBBR1MCS-5 plasmid alone (*Bb*-pEmpty) or with the BB3576 gene (*Bb*-BB3576) were measured and expressed relative to *Bb*-pEmpty vector diameter after 18 h of incubation at 37 °C on SS motility plates supplemented with 32 mM magnesium sulfate. One hundred per cent motility for *Bb*-pEmpty was 12 mm. The results are based on three replicates. * $P < 0.01$ vs *Bb*-pEmpty. (c) Biofilm development by *Bb*-BB3576 compared with *Bb*-pEmpty was assessed by microtitre plate assays as described in Methods. Biofilm level was determined in a ΔBvgA background (*Bb*ΔBvgA-BB3576 and *Bb*ΔBvgA-pEmpty). * $P < 0.01$ vs *Bb*-pEmpty grown at the same modulator concentration, ** $P < 0.01$ vs *Bb*ΔBvgA-pEmpty grown at the same modulator concentration.

quantified by the CV method and confirmed by SEM. These findings suggest that an increase in the levels of this DGC and in intracellular c-di-GMP levels causes an enhanced production of factors that promote bacterial binding to abiotic surfaces and/or inter-bacterial adherence.

As mentioned above, biofilm formation is regulated by BvgAS in *B. bronchiseptica*. Nicotinic acid or magnesium sulfate can modulate BvgAS activity *in vitro* through the three phases, virulent (Bvg^{plus}), intermediate (Bvg^i) and avirulent (Bvg^{minus}). Interestingly, although for all modulator conditions *Bb*-DGC showed more biofilm formation than the parental strain, the difference is bigger in Bvg^{minus} phase (4.0 mM NA). Irie *et al.* (2004) suggested that while AC activity inhibits biofilm formation, FHA is necessary for full biofilm development. Both proteins are present in Bvg^{plus} phase but only FHA is present in Bvg^i phase and both are absent in Bvg^{minus} phase (Mattoo & Cherry, 2005). This is in agreement with results showing that Bvg^i phase is the condition where major biofilm formation is observed in *B. bronchiseptica*. Nevertheless, it is noteworthy that with overexpression of a non-physiological DGC, and in the absence of FHA during growth in the Bvg^{minus} phase,

a biofilm is still established by the *Bb*-DGC strain. The group of factors involved in biofilm regulation and presumably activated by c-di-GMP might be sufficient for establishment of this phenotype regardless of virulence factors like FHA.

In concordance with this finding, when PA1120 was overexpressed in a ΔBvgA background, biofilm formation was observed. However, the biofilm magnitude was significantly weaker than in wild-type background and SEM analysis showed only biofilm architecture in 1.0 mM NA. These results are surprising, since neither *Bb*ΔBvgA nor *Bb*ΔBvgA-DGC is expected to respond to different concentrations of known BvgAS modulators, thus the interplay between NA and the c-di-GMP network may be present in *B. bronchiseptica* through mechanisms not yet explored. We can speculate that BvgAS regulates the c-di-GMP network regulating protein expression, particularly those proteins directly involved in c-di-GMP signalling. Interestingly, the BvgR regulatory protein, which represses the expression of virulence genes in Bvg^{plus} phase in *Bordetella*, contains an EAL domain (Merkel *et al.*, 1998). The expression of this protein is stimulated by the BvgAS
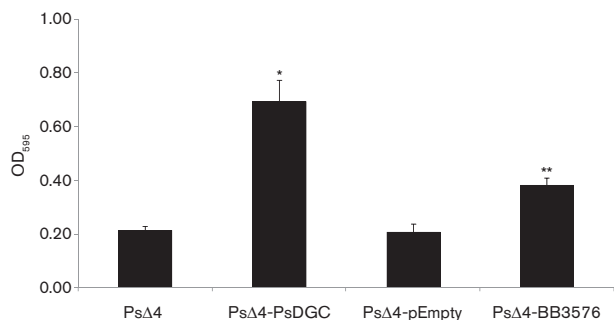
Fig. 8. BB3576 complements biofilm phenotype in a *P. fluorescens* diguanylate cyclase mutant. Biofilm development by the *P. fluorescens*Δ4DGC strain and *P. fluorescens*Δ4DGC complemented with BB3576-expressing vector (PsΔ4-BB3576) was determined by CV staining after 6 h growth in static conditions in K10T-1 medium. *P. fluorescens* with expression vector without insertion (PsΔ4-pEmpty) was used as negative control and *P. fluorescens* complemented with vector expressing a *P. fluorescens* diguanylate cyclase *gcbB* (PsΔ4-PsDGC) as a positive control. *gcbB* expression was induced by presence of arabinose 0.2 % (p/v). * $P<0.001$ vs PsΔ4. ** $P<0.001$ vs PsΔ4-pEmpty.

system, strongly suggesting a relationship between the BvgAS system and c-di-GMP that requires further investigation.

In other bacteria where c-di-GMP function has been determined, low c-di-GMP levels are frequently associated in planktonic phenotypes with flagellar expression. As shown above, DGC overexpression in *B. bronchiseptica* enhanced biofilm formation, probably as a consequence of high c-di-GMP levels, which may also repress motility in *Bb*-DGC when a flagellar system is present. It is known that *B. bronchiseptica* shows motility in Bvg[minus]-modulated conditions like high-sulfate concentrations. Motility assays in soft agar plates showed that *Bb*-DGC exhibited significantly diminished motility. This result is in concordance with behaviour of other bacteria and with a c-di-GMP network that regulates the transition between sessile and motile lifestyles of bacteria. Moreover, we described for the first time, to our knowledge, in *B. bronchiseptica* a protein with a GGDEF domain that probably had diguanylate cyclase activity *in vivo*. Real-time PCR results clearly demonstrated that BB3576 expression was higher in Bvg[minus] phase compared with Bvg[plus] conditions. However, NA regulation appears to be dominant over BB3576 activity for biofilm formation, even in a ΔBvgA background, since differences in biofilm quantification of strains overexpressing BB3576 were only significant at NA concentrations inducing the Bvg[i] phase. One possible explanation is that conformational changes in the expressed BB3576 protein may be required for optimal DGC function. BB3576 has two predicted domains, a DGC domain and an extracellular sensory Cache domain (Ca[+2] channels, chemotaxis receptors). In general, Cache

domains sense stimuli present in the periplasm and transmit signals to an output domain such as GGDEF, resulting in a specific adaptive response (Anantharaman & Aravind 2000; Galperin 2006). We speculate that BB3576 regulates biofilm and motility when a ligand binds to the Cache domain. The nature of this ligand remains unknown at the moment. Diguanylate cyclase activity was demonstrated *in vivo* for BB3576 as shown in Fig. 8. Overexpression of BB3576 in a *P. fluorescens* strain with a low-DGC-activity background restored biofilm formation. The partial complementation achieved by BB3576 may be explained by the absence from the growth media of the unknown ligand.

Recently, Amarasinghe and co-workers described YeaJ in *Salmonella enterica* serovar Typhimurium as an active DGC membrane protein involved in motility regulation and exopolysaccharide production (Amarasinghe *et al.*, 2013). This protein, like BB3576, has a GGDEF domain with demonstrated DGC activity and a putative Cache domain. They proposed that the association of a protective monoclonal IgA antibody with the O-antigen induces outer membrane stress triggering a c-di-GMP signalling pathway that effectively promotes a less motile and non-invasive biofilm state, thereby rendering the bacterium unable to invade intestinal epithelial cells. The particular expression profile of BB3576 in Bvg[plus], Bvg[i] and Bvg[minus] phases is in concordance with putative motility regulation of the bacteria during Bvg[minus] phase. Flagellar synthesis and function is regulated in bacteria at different levels by c-di-GMP (Wolfe & Visick, 2008). This newly described protein BB3576 might fine tune flagellar motor function using c-di-GMP as a second messenger in response to chemosensory activity of the Cache domain, as reported for *E. coli* (Pesavento *et al.*, 2008).

Although further research is needed to elucidate c-di-GMP regulation and signalling in *B. bronchiseptica*, we demonstrated here for the first time, to our knowledge, its role regarding the control of *B. bronchiseptica* biofilm formation and motility. More over we would now argue that c-di-GMP is involved in motility regulation in response to as yet unknown ligands that may be sensed by BB3576.

REFERENCES

Akerley, B. J. & Miller, J. F. (1993). Flagellin gene transcription in *Bordetella bronchiseptica* is regulated by the BvgAS virulence control system. *J Bacteriol* **175**, 3468–3479.

Akerley, B. J., Monack, D. M., Falkow, S. & Miller, J. F. (1992). The *bvgAS* locus negatively controls motility and synthesis of flagella in *Bordetella bronchiseptica*. *J Bacteriol* **174**, 980–990.

Amarasinghe, J. J., D'Hondt, R. E., Waters, C. M. & Mantis, N. J. (2013). Exposure of *Salmonella enterica* Serovar Typhimurium to a protective monoclonal IgA triggers exopolysaccharide production via a diguanylate cyclase-dependent pathway. *Infect Immun* **81**, 653–664.

Amikam, D. & Galperin, M. Y. (2006). PilZ domain is part of the bacterial c-di-GMP binding protein. *Bioinformatics* **22**, 3–6.

Anantharaman, V. & Aravind, L. (2000). Cache – a signaling domain common to animal Ca^{2+}-channel subunits and a class of prokaryotic chemotaxis receptors. *Trends Biochem Sci* **25**, 535–537.

Conover, M. S., Mishra, M. & Deora, R. (2011). Extracellular DNA is essential for maintaining *Bordetella* biofilm integrity on abiotic surfaces and in the upper respiratory tract of mice. *PLoS ONE* **6**, e16861.

Cotter, P. A. & Miller, J. F. (1997). A mutation in the *Bordetella bronchiseptica bvgS* gene results in reduced virulence and increased resistance to starvation, and identifies a new class of Bvg-regulated antigens. *Mol Microbiol* **24**, 671–685.

Dombrecht, B., Vanderleyden, J. & Michiels, J. (2001). Stable RK2-derived cloning vectors for the analysis of gene expression and gene function in Gram-negative bacteria. *Mol Plant Microbe Interact* **14**, 426–430.

Fernández, J., Sisti, F., Bottero, D., Gaillard, M. E. & Hozbor, D. (2005). Constitutive expression of *bvgR*-repressed factors is not detrimental to the *Bordetella bronchiseptica*–host interaction. *Res Microbiol* **156**, 843–850.

Galperin, M. Y. (2004). Bacterial signal transduction network in a genomic perspective. *Environ Microbiol* **6**, 552–567.

Galperin, M. Y. (2005). A census of membrane-bound and intracellular signal transduction proteins in bacteria: bacterial IQ, extroverts and introverts. *BMC Microbiol* **5**, 35.

Galperin, M. Y. (2006). Structural classification of bacterial response regulators: diversity of output domains and domain combinations. *J Bacteriol* **188**, 4169–4182.

Galperin, M. Y., Nikolskaya, A. N. & Koonin, E. V. (2001). Novel domains of the prokaryotic two-component signal transduction systems. *FEMS Microbiol Lett* **203**, 11–21.

Goodnow, R. A. (1980). Biology of *Bordetella bronchiseptica*. *Microbiol Rev* **44**, 722–738.

Harvill, E. T., Cotter, P. A., Yuk, M. H. & Miller, J. F. (1999). Probing the function of *Bordetella bronchiseptica* adenylate cyclase toxin by manipulating host immunity. *Infect Immun* **67**, 1493–1500.

Harvill, E. T., Preston, A., Cotter, P. A., Allen, A. G., Maskell, D. J. & Miller, J. F. (2000). Multiple roles for *Bordetella* lipopolysaccharide molecules during respiratory tract infection. *Infect Immun* **68**, 6720–6728.

Hengge, R. (2009). Principles of c-di-GMP signalling in bacteria. *Nat Rev Microbiol* **7**, 263–273.

Inatsuka, C. S., Xu, Q., Vujkovic-Cvijin, I., Wong, S., Stibitz, S., Miller, J. F. & Cotter, P. A. (2010). Pertactin is required for *Bordetella* species to resist neutrophil-mediated clearance. *Infect Immun* **78**, 2901–2909.

Irie, Y., Mattoo, S. & Yuk, M. H. (2004). The Bvg virulence control system regulates biofilm formation in *Bordetella bronchiseptica*. *J Bacteriol* **186**, 5692–5698.

Jenal, U. & Malone, J. (2006). Mechanisms of cyclic-di-GMP signaling in bacteria. *Annu Rev Genet* **40**, 385–407.

Kolter, R. & Greenberg, E. P. (2006). Microbial sciences: the superficial life of microbes. *Nature* **441**, 300–302.

Kovach, M. E., Elzer, P. H., Hill, D. S., Robertson, G. T., Farris, M. A., Roop, R. M., II & Peterson, K. M. (1995). Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* **166**, 175–176.

Kulesekara, H., Lee, V., Brencic, A., Liberati, N., Urbach, J., Miyata, S., Lee, D. G., Neely, A. N., Hyodo, M. & other authors (2006). Analysis of *Pseudomonas aeruginosa* diguanylate cyclases and phosphodiesterases reveals a role for bis-(3′–5′)-cyclic-GMP in virulence. *Proc Natl Acad Sci U S A* **103**, 2839–2844.

Lai, T. H., Kumagai, Y., Hyodo, M., Hayakawa, Y. & Rikihisa, Y. (2009). The *Anaplasma phagocytophilum* PleC histidine kinase and PleD diguanylate cyclase two-component system and role of cyclic di-GMP in host cell infection. *J Bacteriol* **191**, 693–700.

Mattoo, S. & Cherry, J. D. (2005). Molecular pathogenesis, epidemiology, and clinical manifestations of respiratory infections due to *Bordetella pertussis* and other *Bordetella* subspecies. *Clin Microbiol Rev* **18**, 326–382.

McMillan, D. J., Shojaei, M., Chhatwal, G. S., Guzmán, C. A. & Walker, M. J. (1996). Molecular analysis of the *bvg*-repressed urease of *Bordetella bronchiseptica*. *Microb Pathog* **21**, 379–394.

Merkel, T. J., Barros, C. & Stibitz, S. (1998). Characterization of the *bvgR* locus of *Bordetella pertussis*. *J Bacteriol* **180**, 1682–1690.

Mikkelsen, H., Ball, G., Giraud, C. & Filloux, A. (2009). Expression of *Pseudomonas aeruginosa* CupD fimbrial genes is antagonistically controlled by RcsB and the EAL-containing PvrR response regulators. *PLoS ONE* **4**, e6018.

Mishra, M., Parise, G., Jackson, K. D., Wozniak, D. J. & Deora, R. (2005). The BvgAS signal transduction system regulates biofilm development in *Bordetella*. *J Bacteriol* **187**, 1474–1484.

Newell, P. D., Yoshioka, S., Hvorecny, K. L., Monds, R. D. & O'Toole, G. A. (2011). Systematic analysis of diguanylate cyclases that promote biofilm formation by *Pseudomonas fluorescens* Pf0-1. *J Bacteriol* **193**, 4685–4698.

Pesavento, C., Becker, G., Sommerfeldt, N., Possling, A., Tschowri, N., Mehlis, A. & Hengge, R. (2008). Inverse regulatory coordination of motility and curli-mediated adhesion in *Escherichia coli*. *Genes Dev* **22**, 2434–2446.

Römling, U. & Amikam, D. (2006). Cyclic di-GMP as a second messenger. *Curr Opin Microbiol* **9**, 218–228.

Sanchez-Torres, V., Hu, H. & Wood, T. K. (2011). GGDEF proteins YeaI, YedQ, and YfiN reduce early biofilm formation and swimming motility in *Escherichia coli*. *Appl Microbiol Biotechnol* **90**, 651–658.

Sisti, F., Fernández, J., Rodríguez, M. E., Lagares, A., Guiso, N. & Hozbor, D. F. (2002). *In vitro* and *in vivo* characterization of a *Bordetella bronchiseptica* mutant strain with a deep rough lipopolysaccharide structure. *Infect Immun* **70**, 1791–1798.

Skinner, J. A., Reissinger, A., Shen, H. & Yuk, M. H. (2004). *Bordetella* type III secretion and adenylate cyclase toxin synergize to drive dendritic cells into a semimature state. *J Immunol* **173**, 1934–1940.

Sloan, G. P., Love, C. F., Sukumar, N., Mishra, M. & Deora, R. (2007). The *Bordetella* Bps polysaccharide is critical for biofilm development in the mouse respiratory tract. *J Bacteriol* **189**, 8270–8276.

Stainer, D. W. & Scholte, M. J. (1970). A simple chemically defined medium for the production of phase I *Bordetella pertussis*. *J Gen Microbiol* **63**, 211–220.

Stockbauer, K. E., Fuchslocher, B., Miller, J. F. & Cotter, P. A. (2001). Identification and characterization of BipA, a *Bordetella* Bvg-intermediate phase protein. *Mol Microbiol* **39**, 65–78.

Wan, X., Tuckerman, J. R., Saito, J. A., Freitas, T. A., Newhouse, J. S., Denery, J. R., Galperin, M. Y., Gonzalez, G., Gilles-Gonzalez, M. A. &

Alam, M. (2009). Globins synthesize the second messenger bis-(3′–5′)-cyclic diguanosine monophosphate in bacteria. *J Mol Biol* **388**, 262–270.

Williams, C. L. & Cotter, P. A. (2007). Autoregulation is essential for precise temporal and steady-state regulation by the *Bordetella* BvgAS phosphorelay. *J Bacteriol* **189**, 1974–1982.

Wolfe, A. J. & Visick, K. L. (2008). Get the message out: cyclic-di-GMP regulates multiple levels of flagellum-based motility. *J Bacteriol* **190**, 463–475.

Edited by: M. Whiteley