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### **RESEARCH ARTICLE**

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# PdeA is required for the rod shape morphology of Brucella abortus

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#### Abstract

Cyclic-di-GMP plays crucial role in the cell cycle regulation of the  $\alpha$ -Proteobacterium Caulobacter crescentus. Here we investigated its role in the  $\alpha$ -Proteobacterium Brucella abortus, a zoonotic intracellular pathogen. Surprisingly, deletion of all predicted cyclic-di-GMP synthesizing or degrading enzymes did not drastically impair the growth of *B. abortus*, nor its ability to grow inside cell lines. As other Rhizobiales, *B. abortus* displays unipolar growth from the new cell pole generated by cell division. We found that the phosphodiesterase PdeA, the ortholog of the essential polar growth factor RgsP of the Rhizobiale Sinorhizobium meliloti, is required for rod shape integrity but is not essential for *B. abortus* growth. Indeed, the radius of the pole is increased by  $31 \pm 1.7\%$  in a  $\Delta pdeA$  mutant, generating a coccoid morphology. A mutation in the cyclic-di-GMP phosphodiesterase catalytic site of PdeA does not generate the coccoid morphology and the  $\Delta pdeA$  mutant kept the ability to recruit markers of new and old poles. However, the presence of PdeA is required in an intra-nasal mouse model of infection. In conclusion, we propose that PdeA contributes to bacterial morphology and virulence in *B. abortus*, but it is not crucial for polarity and asymmetric growth.

#### KEYWORDS

Alpha-Proteobacteria, asymmetric growth, Brucella, cell shape, cyclic-di-GMP, Rhizobiales

### 1 | INTRODUCTION

Brucellaceae are facultative intracellular Gram-negative cocobacilli, belonging to the Rhizobiales order of  $\alpha$ -Proteobacteria family. Members of the Brucella genus are responsible for a worldwide zoonosis known as brucellosis, with important consequences for health and economy. In animal livestock, the disease leads to abortion and sterility. Human brucellosis, mainly caused by *B. melitensis*, *B. suis*, *B. abortus*, and *B. canis*, is characterized by an acute phase with periodic undulant fever. The illness can evolve to a chronic infection, which could also be associated with endocarditis or meningitis (Pappas et al., 2005).

In vitro HeLa and macrophage cell infection by *B. abortus* follows a three-step model in which bacteria are found in three

successive types of vacuoles (Celli, 2019). In Hela cells and RAW264.7 macrophages-like cells, G1 phase bacteria ("new-born") enter the cells, reside in an endosomal *Brucella* containing vacuole (eBCV) but remain blocked in G1 during the first hours after invasion. *B. abortus* resumes growth before the eBCV reaches the replicative niche in the endoplasmic reticulum (rBCV) (Deghelt et al., 2014). After extensive replication in the rBCV, bacteria are captured in autophagosome-like compartments forming an autophagic BCV (aBCV) (Celli, 2019).

One characteristic of *Brucellae* shared with Rhizobiales, is their asymmetric unipolar growth, as it occurs at one pole during elongation and at midcell during the cell division (Brown et al., 2012). Recently, it has been shown that newly synthesized peptidoglycan (PG) and lipopolysaccharide are only inserted at one pole of the bacteria, named the growing pole (Vassen et al., 2019). The growing pole WILEY

is characterized by the presence of the protein AidB, which also localizes at the constriction site in divisional cells (Dotreppe et al., 2011). Division leads to two new growing poles, one in each daughter cell, that will mature into old poles after elongation and the next division event. The maturation of the growing pole into an old pole correlates with the polar accumulation of the histidine kinase PdhS and the fumarase FumC (Hallez et al., 2007; Mignolet et al., 2010). However, the molecular mechanisms regulating cell morphology, cell polarity, and polar growth of *B. abortus*, both in vitro and inside infected cells have not been described so far.

Over the last three decades, extensive studies have been conducted to decipher the role of bis-(3'-5')-cyclic dimeric guanosine monophosphate (cdG) in bacteria. Present in most of the known bacterial species, this second messenger has been shown to be involved in a number of biological processes, such as biofilm formation, transition from acute to chronic infection, motility, and virulence (Jenal et al., 2017). In some bacterial species, cdG also regulates cell cycle, growth, and morphology (Fernandez et al., 2020; Gupta et al., 2016; Kaczmarczyk et al., 2020; Lori et al., 2015). The cdG is synthesized and degraded by diguanylate cyclases (DGC) and phosphodiesterases (PDE) respectively. DGC includes GGDEF domains, while PDE contains EAL or HD-GYP domains (Ryan et al., 2006; Ryjenkov et al., 2005; Schmidt et al., 2005). Recently, the protein RgsP (PDE) has been identified as a key player of the polar growth of Sinorhizobium meliloti (Schaper et al., 2018). Interestingly, the Brucella homologue, BpdA (also named PdeA) has been shown to be required for the virulence of B. melitensis 16M and B. abortus 2308 in a mouse model of infection, but also for the morphology of B. melitensis (Khan et al., 2016; Petersen et al., 2011). Except for these two pioneer studies, nothing is known about the conservation of cdG-metabolizing enzymes among Brucellae species and their role in the pathogen B. abortus. Interestingly, PdeA is a major actor of cell cycle regulation in the  $\alpha$ -Proteobacterium Caulobacter crescentus (Abel et al., 2011).

In this study, we show that cdG family enzymes are not fully conserved among *Brucella* and that this second messenger does not play a crucial role in in vitro conditions in *B. abortus*. We highlighted a role for the phosphodiesterase PdeA in the morphology and in the virulence of *B. abortus* in mice.

### 2 | RESULTS

# 2.1 | *B. abortus* 2308 genome only encodes 7 putatively active cdG metabolizing enzymes

We first performed BLAST analyses on 14 *Brucella* genomes representative of the diversity of this genus (Wattam et al., 2014). We also included the closest known derivative of *Brucella*'s ancestor, *Ochrobactrum anthropi* (Barquero-Calvo et al., 2009; Teyssier et al., 2005). As shown in Figure 1, all *Brucella* strains display 11 genes encoding DGC, PDE, or dual proteins with both domains, with the exception of *B. abortus* 2308 that possess only 10 genes. Twenty genes encoding DGC or PDE have been identified in *O. anthropi*.

Sequence alignments revealed that several events of pseudogenization occurred in the evolution of the Brucella genus (Figure 1; empty symbol), likely leading to the inactivation of the encoded protein. Strikingly, we found out that BpdB (Bab1\_0512), Bab2\_0622, Bab2\_0859, and Bab2\_0228 (according to B. abortus 2308 coding sequences annotations) appear to be specific to Brucellae. This suggests that those four genes might have been acquired through horizontal transfer (or duplication and strong divergence) after the separation of Brucella and Ochrobactrum genus. Interestingly, three of them are located on the second chromosome, which is thought to be a stabilized megaplasmid having a lower density of essential genes compared to chromosome 1 (Deghelt et al., 2014; Paulsen et al., 2002; Sternon et al., 2018). Moreover, only pleD and bab2\_0228 seem to remain intact among the Brucella genus analyzed here. Overall, it seems that these families of enzymes were subjected to intense rearrangements along the evolution within Brucellae, which contrasts with the high sequence identity shared by the different species of the Brucella genus (Verger et al., 1985).

B. abortus 2308 exhibits most of the gene decay we could observe in the strains we examined here. Three proteins are either inactivated due to a nucleotide deletion resulting in a frameshift (bab2\_0255 and bab2\_0622, Figure 1 and Figure S1) or absent (Bmei 0929). Our in silico analyses revealed 8 genes encoding putative cdG-metabolizing enzyme in B. abortus 2308. Four of them contain a GGDEF domain (DGC), Bab1\_0441 (or CsgB), Bab2\_0228, Bab2 0630 (or PleD), and Bab2 0966 (Figure S2). Two proteins have an EAL domain (PDE), Bab1\_0512 (or BpdB), and Bab2\_0859. Two proteins contain both GGDEF and EAL domain: Bab1\_0220 and Bab1\_0507 (or PdeA). We did not find any proteins containing a HD-GYP domain. Detailed investigation of the protein sequences showed that Bab2\_0966 has a truncated GGDEF domain, suggesting that this protein is no longer an active DGC. PdeA presents several mutations in the DGC active site residues, with SSDQF instead of the GG(D/E)EF. That is a conserved feature among Brucellae and in O. anthropi. Bab1\_0220 has a mutation in the active site residues EAL (EAF), which seems to be specific to B. abortus species (to the exception of B. inopinata in which the homolog of bab1\_0220 seems to be a pseudogene). Those results suggest that both PdeA and Bab1\_0220 could actually be monofunctional in B. abortus 2308.

This in silico analysis suggests that *B. abortus* 2308 only encodes 4 DGC and 3 PDE, which contrasts with the 11 enzymes identified in *B. melitensis* 16M (Petersen et al., 2011). These genes and predicted encoded proteins were also identified in *B. abortus* 544 with 100% identity (data not shown).

# 2.2 | cdG does not regulate *B. abortus* in vitro growth

To determine which role cdG might play in *B. abortus*, we generated single deletion mutants of each of the seven genes previously identified in *B. abortus* 544 wild-type strain (WT). We assessed their ability to grow in rich (TSB) or defined medium (Plommet Erythritol, PE) and



1451





**FIGURE 2** PdeA is required for the rod-shaped morphology of *Brucella abortus*. (a) Colony morphologies on TSB agar plates. Strains were plated on TSB agar plates and imaged after 4 days of incubation at 37°C. Deletion of *pdeA* leads to small colony formation. Pictures are from one experiment representative of at least 3 independent experiments. Scale bar, 0.5 cm. (b) Phase-contrast micrographs of *B. abortus* 544 pMR10 (WT),  $\Delta pdeA$  pMR10 ( $\Delta pdeA$ ) mutant and complemented strain ( $\Delta pdeA$  pMR10::*pdeA*) grown until exponential phase in TSB at 37°C. Scale bar, 2 µm. (c-e) Violin plot of width to length ratio (w/l) (c), lengths (d), and widths (e). The  $\Delta pdeA$  mutant is significantly rounder, shorter, and wider than the WT strain, respectively (\*\*\*\*, *p* < .0001, in one-way analysis of variance). For each violin plot graphs, the totality of measured bacteria from three independent experiments is shown. Statistics were calculated on the three independent medians. Medians (hatched bars) and quartile (dot lines) are indicated

in RAW264.7 macrophages-like cells. None of the mutants displayed a phenotype (Figure S3). As redundancy or compensatory activities of cdG metabolizing enzymes might explain the absence of strong phenotypes with single deletion mutants, we generated a quadruple mutant of the DGCs ( $\Delta csgB \ \Delta pleD \ \Delta bab1_0220 \ \Delta bab2_0228$ , here referred as  $\Delta 4$ DGC), and a triple mutant of the PDEs ( $\Delta pdeA \ \Delta bpdB \ \Delta bab2_0859$ , referred as  $\Delta 3$ PDE). While the  $\Delta 4$ DGC does not have a notable phenotype, the  $\Delta 3$ PDE mutant showed a minor growth defect in TSB (Figure S4, \*\*, p < .05, in a Mann and Whitney test).

We noticed that the  $\Delta pdeA$  and  $\Delta 3PDE$  mutant strains formed small colonies, compared to the WT strain, after 4 days on TSB agar plates (Figure S5). Time course analyses revealed that these small colonies reach the same size as the WT strain colonies (Figure S5). In order to complement the phenotype, empty pMR10 was introduced in both the WT strain and the  $\Delta pdeA$  mutant. We observed that the pMR10 vector slightly slows down the growth of the  $\Delta pdeA$ mutant, both in vitro and in RAW264.7 cells but these defects were fully complemented when pdeA is inserted in the pMR10 (Figure S6). Complementation of the mutant strain with the pMR10 bearing pdeA restored bacterial growth to the WT level on plates (Figure 2a and Figure S5).

Taken all together, those results suggest that cdG does not play a crucial role in in vitro growth, regardless of the medium, or in intracellular growth of *B. abortus* in a simple cellular infection model.

# 2.3 | The phosphodiesterase PdeA modulates rod shape morphology of *B. abortus*

We next assessed if the disruption of each of these genes could impair the morphology of B. abortus. Only the pdeA mutant ( $\Delta pdeA$ ) displayed significant size modifications, with smaller and wider shape, adopting a coccoid almost spherical morphology (Figure 2b and Figure S7, Table S1a). Lengths and widths of the WT and  $\Delta pdeA$  mutant, both bearing the empty pMR10 vector were measured to obtain the width to length (w/l) ratios. This analysis revealed that the  $\Delta p de A$ mutant was significantly rounder than the WT strain, with mean w/l ratio of 0.888 and 0.536, respectively (Figure 2c, p < .0001, in oneway analysis of variance). Deeper investigations of bacterial length and width showed that the  $\Delta pdeA$  mutant was significantly shorter but also wider than the WT strain (Figure 2d,e and Figure S8a,b, Table S1b). The  $\Delta pdeA$  mutant displays an average length of 1.227  $\mu$ m and an average width of 1.058  $\mu$ m compared to 1.521 and 0.80  $\mu$ m for the WT strain, respectively (p < .0001 in a one-way ANOVA). Complementation of the mutant strain with the pMR10 bearing pdeA restored the wild-type shape (length 1.513 µm; width 0.806 µm), showing that this alteration is indeed due to the loss of PdeA and not to polar effects or ectopic mutations. This morphology alteration was also observed in constricting bacteria (Figure S8c-d, p < .0001in a one-way ANOVA). The wider shape of the bacteria implies that the size of the pole is strongly impaired in the  $\Delta p deA$  mutant. Indeed, measurements of the pole radius showed that the  $\Delta pdeA$  mutant pole is  $31 \pm 1.7\%$  (mean  $\pm$  SD) larger than the WT strain.

Deletion of the two other PDEs in the  $\Delta pdeA$  strain did not worsen the morphological aspect of the strain (Figure S9, Table S1c). The  $\Delta$ 4DGC mutant was morphologically identical to the WT strain. These results suggest that PdeA is required to maintain the integrity of the rod shape morphology.

# 2.4 | PdeA does not rely on its phosphodiesterase activity to impact bacterial morphology

From our in silico analyses, we showed that PdeA is likely a phosphodiesterase for cdG. Hence, the absence of this protein could potentially lead to an accumulation of cdG in bacterial cells. It has been previously shown that high levels of intracellular cdG might lead to small colony variant or alter the cell morphology (Gupta et al., 2016; Starkey et al., 2009). Therefore, we wanted to assess if the alterations of the  $\Delta pdeA$  mutant colony size and morphology were due to an accumulation of cdG. We introduced a point mutation in the catalytic site of the EAL domain (PdeA<sup>E742A</sup>) to abrogate its phosphodiesterase activity (Rao et al., 2008). This mutation did not lead to a colony size defect (Figure 3a and Figure S5). Interestingly, this mutation did not generate coccoid morphology either (Figure 3b-d, Table S1d). These data suggest that the alteration of morphology in the  $\Delta pdeA$  mutant is not due to the lack of phosphodiesterase activity of PdeA.

As the deletion of *pdeA* leads to a strong morphological defect independent of the phosphodiesterase activity, we wanted to reevaluate the role of the other phosphodiesterases on cell morphology. Hence, we also introduced the PdeA<sup>E742A</sup> point mutation in a  $\Delta bpdB \ \Delta bab2_0859$  double mutant strain, leading to a catalytically dead triple mutant (referred as  $\Delta B59 \ PdeA^{E742A}$ ). This mutant did not display any colony size defect (data not shown) nor morphological defect (Figure 3e–g, Table S1d). It is thus likely that morphological defects of the  $\Delta pdeA$  mutant are not due to the absence of a cdG phosphodiesterase activity in this strain.

Lastly, evaluation of in vitro growth and in macrophages-like cells showed no difference between the WT strain and the two catalytically dead mutants (Figure S10). Overall, these results confirm that putative changes in cdG concentration do not seem to play a crucial role in the in vitro growth conditions tested here.

# 2.5 | Asymmetric growth is maintained in the *pdeA* mutant

Considering the coccoid shape of the  $\Delta pdeA$  mutant, we hypothesized that this loss of rod-shape morphology might be correlated with a perturbation of the polar (thus asymmetric) growth. To address this hypothesis, bacteria were labeled with the eFluor<sup>670</sup> dye (later named eFluor). eFluor binds to the accessible reactive amines on the bacterial surface. As the cells grow in absence of the dye, newly synthesized material that is incorporated at the growing pole of the cell are unlabeled, and since the outer membrane has a poor



FIGURE 3 The role of PdeA in morphology is not related to its phosphodiesterase activity. (a) Colony morphologies on TSB agar plates. Strains were plated on TSB agar plates and imaged after 4 days of incubation at 37°C. PdeA carrying a point mutation in its catalytic site grows as the WT strain. Pictures are from one experiment representative of at least three independent experiments. Scale bar: 0.5 cm. (b, e) Phase-contrast micrographs of *Brucella abortus* WT and the catalytic mutants grown until exponential phase growth in TSB at 37°C. Scale bar, 2 μm. (c-d; f-g) Violin plot of bacterial lengths (c and f) and widths (d and g). Bacteria from 3 independent experiments are plotted. Numbers of measured bacteria, average length, width, and w/l ratio are provided in Table S1d. Medians (hatched bars) and quartile (dot lines) are indicated

apparent fluidity, the unlabeled zone remains at the pole or at the division site (Vassen et al., 2019). Hence, eFluor labeling allows to discriminate the labeled old pole from the unlabeled growing pole (Vassen et al., 2019). As for the WT strain (Figure 4a eFluor and b, upper panels), an eFluor unlabeled region was observed at one pole of the bacterium in the  $\Delta pdeA$  mutant (Figure 4a eFluor and b, bottom panels), revealing that despite its almost round morphology, the  $\Delta pdeA$  mutant still exhibits asymmetric growth. As it can be tricky to correctly identify poles in almost spherical bacteria, we took advantage of the eFluor labeling. This analysis allowed us to confirm that the  $\Delta pdeA$  mutant is indeed shorter and wider than the WT strain (p < .0001 in an unpaired t-test, data not shown).

Prior to microscopic observations, bacteria were short pulsed with the fluorescent p-amino acid HCC-amino-p-alanine (HADA) to follow the sites of synthesis and recycling of PG (Kuru et al., 2012). In agreement with the previously described insertion sites (Vassen et al., 2019), HADA was inserted at the new pole and at the constriction site in 84.5% and 11% of the WT bacteria, respectively (Figure 4a,c, upper panels, 4d). Interestingly, PG was also incorporated at the opposite pole of the eFluor labeling in the  $\Delta pdeA$  mutant (Figure 4a,c, bottom panels), consistent with the preservation of asymmetric growth in the absence of PdeA. However, analysis of the intensity of fluorescence indicated that the proportion of bacteria with a polar incorporation of HADA was significantly reduced to 57% in the  $\Delta pdeA$  cells but HADA incorporation is found at midcell (no visible sign of constriction) in 19% of the bacteria (2% for the WT strain) (Figure 4d, \*\*\*\*, *p* < .0001, \*\*, *p* < .01 in a two-way ANOVA). HADA incorporation was found at the constriction site in 19% of the

population of  $\Delta p deA$  mutant. These data show that the localization of HADA-labeled PG is perturbed in the absence of PdeA since the sharp transition from polar to midcell signal clearly visible in the WT is replaced by a smooth transition and an extended polar localization to midcell in the  $\Delta p deA$  mutant. Moreover, the proportion of the cell surface labeled with HADA seems to be much more extended in the  $\Delta pdeA$  mutant compared to the WT strain. Indeed, it appears from the microscopic observations and the demographic representation that HADA labeled the sidewalls of the growing zone of the cells in the mutant strain, while HADA labeling is concentrated at the tip of the pole in the WT strain (Figure 4c). In order to quantify this difference in the incorporation zone, we measured the HADA labeled area (quantified with ImageJ plug-in MicrobeJ [Ducret et al., 2016]) in the WT and the mutant strains. There is no significant difference in the bacterial area between the WT and the mutant strains (Figure 4e). Hence, we calculated the percentage of HADA labeled area per bacterium. As this difference is mostly observed when HADA incorporation is polar, we only considered polarly labeled bacteria, regardless of the eFluor staining. In the WT strain, HADA-PG covers  $11.5 \pm 2\%$ (mean  $\pm$  SD) of the area of the cells, versus 23  $\pm$  3% for the mutant strain (Figure 4f, p < .01 in an unpaired *t*-test). Altogether, these results suggest that PdeA is not required for asymmetric growth but might be needed to focus the insertion of newly synthesized material at the growth tip. Moreover, this clearly raises the hypothesis that asymmetric growth might not be driven by the cell morphology or the size of the pole.

Considering the round shape of the *pdeA* mutant strain and the higher number of constricting bacterial that we observed with the



FIGURE 4 Asymmetric growth is maintained in the  $\Delta pdeA$  mutant. (a) Microscopic observations of WT strain (upper panels) and ΔpdeA mutant (bottom panels). Scale bar, 1 μm. (b) Demographic representation of eFluor labeling from (a). Bacteria were sorted by their length and oriented with eFluor signal (representing the old pole) at the top of the demograph. (c) Demographic representation of HADA labeled bacteria from (a). Bacteria were sorted by their length and oriented with eFluor signal (representing the old pole) at the top of the demograph. The intensity of purple and cyan corresponds to the normalized fluorescence intensity (NFI). Demograph were aligned on the center of the bacteria. (d) Quantification of the PG insertion sites from (a). The graph shows the mean of three individual experiments,  $n_{\rm WT}$  = 1,115 (black bars) bacteria and  $n_{\rm mutant}$  = 939 (white bars). GP, Growing Pole; M, Midcell; C, Constriction site (\*\*\*\*, p < .0001, \*\*, p < .01, in an two-way ANOVA). Only statistically significant differences are highlighted. (e) Bacterial area. Shown is the total number of considered bacteria from three independent experiments. Statistical analysis was performed on the three individual medians. (f) Percentage of HADA labeled area per bacteria. Main graph is the distribution of all measured bacteria from three independent experiments,  $n_{WT} = 2,162$  bacteria, n<sub>mutant</sub> = 784. Insert represents the individual measured medians and the mean of the three medians (bar) (\*\*, p < .01, in an unpaired t-test)

HADA labeling, we performed time-lapse experiment to measure the doubling time of bacteria. Even though there is a tendency of the mutant to grow slightly slower than the WT strain (Table S2, Movies S1-S3), this difference is not statistically significant.

## 2.6 | PdeA does not trigger the localization of polar markers

As the pole size of  $\Delta p de A$  is disturbed, we wondered if this defect might impair the localization of the pole-associated proteins PdhS, FumC, and AidB. We first investigated if PdeA plays a role in the maturation of the growing pole into an old pole or in the localization of

the old pole markers, through the analysis of PdhS and FumC. We labeled bacteria with eFluor in order to identify the old pole. PdhS-YFP and FumC-YFP localized at the eFluor labeled pole, both in the WT and the  $\Delta p deA$  strain (Figure 5a,b and Figure S11). In rare cases, we detected PdhS-YFP or FumC-YFP at the growing pole or at midcell in the  $\Delta p deA$  mutant. However, these phenotypes were also observed in a few WT bacteria. Although there are statistically different recruitment patterns between the WT strain and the  $\Delta p deA$  mutant, the amplitude of these differences is small. This supports the hypothesis that, despite a strongly affected morphology, there is no major alteration of the old pole or of its maturation in the absence of PdeA.

We then analyzed the localization of AidB-YFP. In most exponentially growing cells, AidB-YFP localized at the growing pole and at the constriction site in elongated cells, regardless of the strain (Figure 5c and Figure S11). In a few bacteria, we did observe AidB at the same pole as the eFluor labeling, what was never observed with the WT strain. AidB-YFP was found more often at the midcell and at the constriction site in the  $\Delta p deA$  mutant (Figure 5c, right panel), which is

consistent with the higher number of constricting bacteria we observed with the PG labeling. However, we should be careful regarding the localization of AidB-YFP at the midcell. Indeed, the coccoid morphology might lead to a particular orientation on the pad and to a misinterpretation in the localization of our protein of interest.



Cells sorted by length



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# 2.7 | PdeA localizes at the growing pole and at the division site

We next aimed at localizing PdeA in the bacterial cell. To that end, we replaced the native *pdeA* with *pdeA-mcherry* at the chromosomal locus. This strain did not show a growth defect on plates, like the *ΔpdeA* strain, suggesting that the PdeA-mCherry fusion is at least partially functional. To determine its subcellular localization, we coexpressed PdeA-mCherry with the old pole marker PdhS-YFP (Hallez et al., 2007). In most of the cells, PdeA-mCherry is either mainly found at the growing pole of the bacteria, opposite to PdhS-YFP, or at the constriction site (Figure 6a-c and Figure S12). Quantitative analyses of the fluorescence intensity showed that PdeA-mCherry localized at the growing pole in 80% of the cells and at the constriction site in 8% (Figure 6g).

As PdeA seemed to dynamically localize either at the growing pole or at the division site, we next assessed its localization compared to the growing pole-associated protein AidB. In 77% of the cells, PdeA-mCherry was observed at the same pole as AidB-YFP (Figure 6d–f,h and Figure S12). It has also been observed at the constriction site together with AidB-YFP (7%). In elongated cells where AidB-YFP is already relocated to the midcell or the constriction site, PdeA-mCherry was mainly found at the pole of the bacteria (7.6%). These results strongly suggest that PdeA co-localizes at the growing pole with AidB and is then later redirected to the constriction site.

# 2.8 | PdeA regulates the virulence of *B. abortus* in a mice model of infection

The homologs of PdeA in *B. melitensis* 16M and *B. abortus* 2308 have been previously shown to be required in a mouse model of infection through the intraperitoneal route (Petersen et al., 2011). Here we assessed the role of PdeA in the virulence of *B. abortus* 544 in an intranasal model of infection in C57BL/6 mice that mimics the natural route of infection (Hanot Mambres et al., 2016). We evaluated the capacity of the  $\Delta pdeA$  mutant strain to colonize lung and spleen at 48 hr, 5 days (d), 12 d, and 28 d post-infection. The  $\Delta pdeA$ mutant failed to colonize the lungs as efficiently as the WT strain from the early times of infection (as soon as 48 hr post-infection), while the complemented strain is fully capable of multiplying in the lungs (Figure 7a and Figure S13a). Deletion of *pdeA* also leads to a strong attenuation of *B. abortus* colonization of the spleen (Figure 7b and Figure S13b).

### 3 | DISCUSSION

Bacterial genomes are in continuous evolution, sometimes allowing the emergence of pathogenicity. As demonstrated by Wattam et al., *Brucellae* are no exception (Wattam et al., 2014). In this study, we only identified seven (six in the case of *B. abortus*) cdG metabolizing enzymes in common with its closest known derivative ancestor, O. anthropi (that possess twenty putative enzymes, data not shown). We also identified four enzymes that are specific to Brucellae. Differences in the distribution of cdG signaling proteins in close relatives have been reported and likely relies on the adaptation of the organisms to its lifestyle (Galperin, 2005). As such, free-living organisms possess more DGC/PDE proteins, among others signaling pathways, than do host-adapted organisms or obligate intracellular bacteria. For instance, several cdG encoding proteins were lost or inactivated during the evolution of Yersinia pestis from Y. pseudotuberculosis, which probably contributed to the adaptation of Y. pestis to the flea vector (Bobrov et al., 2011; Hinnebusch & Erickson, 2008). Despite a very strong genetic homogeneity between Brucellae species, we spotted several pseudogenization events, even in the four genes that are specific to Brucellae. This actually is not so unexpected as small genomic variations such as pseudogenes occurrences have been shown to be associated with host preference (Suarez-Esquive) et al., 2017, 2020; Tsolis et al., 2009; Wattam et al., 2009). As a hostassociated bacterium, Brucellae species might have evolved and selected mutations to ensure a tighter regulation of intracellular cdG concentration.

Our systematic approach to evaluate the role of PDE and DGC identified major differences regarding the role of cdG in Brucellae compared to what is known in other bacteria. We did not find any defect in growth or in host adaptation in vitro, with the exception of the putative encoded PDE PdeA. BpdA, the homologue of PdeA has been described as an active phosphodiesterase in B. melitensis (Khan et al., 2016; Petersen et al., 2011). Deletion of pdeA in both strains leads to common phenotypes, such as the alteration of the bacterial shape. Thus, we reasoned that PdeA is also an active phosphodiesterase in B. abortus, its deletion leading to an accumulation of cdG. The phenotypes we observed with the pdeA mutant strain correlate with previously described phenotypes associated with increased level of cdG. For instance, the mutant forms small colony on plates similar to Pseudomonas aeruginosa (Malone et al., 2010; Starkey et al., 2009). Alteration of the shape morphology is also a common characteristic of a high concentration of cdG (Fernandez et al., 2020). As an example, in Mycobacterium smegmatis, which also exhibits polar growth but from both poles of the cells, high concentration of cdG reduces cell length (Gupta et al., 2016). However, we clearly established here that none of the phenotype displayed by *B. abortus*  $\Delta pdeA$  relies on its catalytic activity as catalytically inactive mutant exhibited normal growth and morphology. Surprisingly, strains lacking all predicted DGC or PDE were also not affected in any of the phenotypes we investigated here. Yet, we cannot exclude that B. abortus possesses an undiscovered class of cdG metabolizing enzymes or that one of the genes we excluded in this study as a pseudogene actually encodes a functional DGC or PDE. Still, our results are in accordance with the findings reported for S. meliloti, in which a strain devoid of cdG does not show drastic growth or morphological defects (Schaper et al., 2016). In contrast, in the free-living bacterium C. crescentus, the phosphodiesterase PdeA, and the diguanylate cyclases PleD and DcgB control cell fate, asymmetric division and pole morphogenesis (Abel et al., 2011, 2013; Aldridge et al., 2003; Duerig et al., 2009;



**FIGURE 6** PdeA localizes at the growing zones. (a, d) Fluorescence microscopy images of *Brucella abortus* 544 expressing *pdeA-mcherry pdhS-yfp* (a) or expressing *pdeA-mcherry aidB-yfp* (d). Scale bar, 1  $\mu$ m. (b, c, e, f) Bacteria were sorted by their lengths and oriented with PdhS at the top of the demographs (b, c, *n* = 546 bacteria) or AidB at the bottom of the demographs (f, g, *n* = 141 bacteria). A representative experiment out of three individual biological replicates is shown. Replicates are shown in Figure S12. (g, h) Quantification of fluorescence intensity of *B. abortus* 544 expressing *pdeA-mcherry pdhS-yfp* (g) or expressing *pdeA-mcherry aidB-yfp* (h). Graphs display the mean percentages from three individual experiments

Kaczmarczyk et al., 2020; Lori et al., 2015; Paul et al., 2004, 2008). One might hypothesize that in the process of evolution and adaptation to a more restricted niche, cdG lost its role as regulator of cell cycle in favor of its role in virulence. This hypothesis might explain or be explained by the recent evolutional modifications of cdG metabolizing enzymes. Alternatively, it is also possible that the role of cdG as a cell cycle regulator is not ancestral in the  $\alpha$ -Proteobacteria and could be specific to Caulobacteraceae.

We cannot exclude a more specific role of those enzymes in conditions we have not tested in this study. This is first supported by the differences between in vitro cell infection and mice infection we observed for the *pdeA* mutant, and secondly by the requirement of several enzymes to survive in bone marrow-derived macrophages (Khan et al., 2016). In many bacteria, cdG has been shown to be involved in the adaptation to stress conditions, that is, in *S. meliloti* for acid stress response or in *Legionella pneumophila* 

to survive in poor-nutrients environments (Hughes et al., 2019; Schaper et al., 2016). In Escherichia coli, the phosphodiesterase PdeC is maintained in its inactive form through the formation of a disulfide bond in its periplasmic loop, through the system DsbA/DsbB (Herbst et al., 2018). In conditions of low oxygen supply, such as the deeper layers of a biofilm, a shift from oxidized PdeC to thiol-free state increases the phosphodiesterase activity of PdeC. Although Bab2\_0859 only shares 32% of identity with PdeC, it is interesting to note that, additionally to the conserved EAL domain and the periplasmic loop of about 200 amino acids flanked by two transmembrane domains, both the cysteines required for the formation of the disulfide bond are also conserved. As a human intracellular pathogen, Brucellae could face a low level of oxygen in the phagocytes and in deeper organs such as the spleen, liver, and brain in some cases (Ariza et al., 2001; Colmenero et al., 2002; Colmenero Jde et al., 2002; Sohn et al., 2003).

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2 ⊽ ⊽ 1 48h 5d 12d 28d Time post infection (hours and days)

V

**∀**⊽ **₹** 

V

FIGURE 7 PdeA is required for optimal organs colonization. Time course colonization of lung (a) and spleen (b) at 48 hr, 5, 12, and 28 d following intranasal infection of C57BL/6 mice with 2.10<sup>4</sup> WT strain (black circles),  $\Delta p deA$  strain (white triangles) and the complemented strain (gray triangles). A representative experiment out of two is shown. Replicate is shown in Figure S13. Detection threshold is 10 CFU. Red bars represent median values (\*, p < .05, \*\*, *p* < .01, \*\*\*, *p* < .001, \*\*\*\*, *p* < .0001, in a two-way ANOVA with Bonferroni post-test). Data were obtained using 7 or 8 mice per group

As mentioned above, we provided evidence that PdeA is required to maintain a proper rod-shape of the bacteria but is strikingly less required than its ortholog in S. meliloti which is essential (Schaper et al., 2018; Sternon et al., 2018). This suggests that B. abortus could possess alternate mechanisms to ensure its polar growth which is surprising for a pathogen with a reduced genome. A decreased conjugation efficiency in the *pdeA* mutant prevents Tn-seq analyses to identify such a redundant molecular mechanism. Analyses of the B. abortus mutant also reveal that PdeA is required for the pole size and that recycling or incorporation of immature PG is slightly affected in the absence of PdeA. This is consistent with a role of PdeA in the recruitment of PG synthesis enzymes at the growing pole, as reported for RgsP in S. meliloti. Indeed, alteration in the composition and/or the localization of the PG synthesis and/or modification enzymes in the pdeA mutant could explain its aberrant morphology. As for the implication of RgsP in S. meliloti polar growth, PdeA displays the same localization as RgsP along the cell cycle, and all domains of S. meliloti RgsP are conserved in B. abortus PdeA. Similar to RgsP, the PDE function of PdeA is not

required for pole size in B. abortus. The GGDEF domain of S. meliloti is required for polar growth (Schaper et al., 2018). However, several mutations in this domain abrogate its ability to bind or synthesize cdG (Schaper et al., 2018). This domain might also be important for the role of PdeA in B. abortus. Interestingly, degenerated GGDEF domain is conserved throughout the Rhizobiales homologs but this domain is intact in Caulobacteraceae. This could be an important feature in the evolution of micro-organisms and the discrepancies regarding the role of PdeA in C. crescentus, S. meliloti, and B. abortus. RgsP control of the cell growth involves the proteins RgsM, a metallopeptidase and RgsA, an transmembrane protein but yet functionally and structurally uncharacterized (Krol et al., 2020). While a putative ortholog for RgsM is present in B. abortus (Bab1\_1866, 52% identity), we did not find such ortholog for RgsA. All these data suggest that PdeA could fulfill a similar role as RgsP, but in a different context, generating a difference in the phenotype of the corresponding mutants.

We showed here, that despite a coccoid morphology and an enlarged pole size, asymmetric growth is kept in the pdeA mutant. HADA is incorporated via enzymes such as the LD-transpeptidases and this incorporation is not exclusively representative of the incorporation of new PG in the envelope (Kuru et al., 2019; Radkov et al., 2018; Williams et al., 2021). The extended HADA labeling that we observed in the absence of PdeA might also be due to cell wall remodeling.

We showed that the definition of the poles is conserved in the absence of PdeA. AidB recruitment to the growing pole is not affected, and further maturation in an old pole through the acquisition of PdhS and FumC still occurs like in WT strain. This suggests that the pole size is not a crucial determining factor in the fate of the bacterial pole. It is thus possible that polarity and asymmetric growth, which probably are intimately linked and evolutionary ancient, are resistant to genetic perturbations that impair pole morphology.

Such strong morphological defects could have been predicted to generate pleiotropic modification, leading to virulence attenuation. Surprisingly, the pdeA mutant is not affected in its ability to grow in vitro in RAW264.7 macrophages-like cell line. However, our results showed that PdeA is required to efficiently colonize lungs and spleens in a mouse intranasal infection model, which is obviously much more complex than cells in culture and subjects Brucella to innate and adaptive immune defenses. This is in agreement with the results of Khan et al. and Petersen et al. who showed the involvement of the phosphodiesterase in the virulence of B. melitensis and B. abortus 2308 in a intraperitoneal infection model (Khan et al., 2016; Petersen et al., 2011). cdG has been involved in the regulation of virulence for several bacteria (reviewed in Hall & Lee [2018]), such as Klebsiella pneumoniae in which it attenuates virulence in lungs or P. aeruginosa where it controls the transition from acute to chronic infection and vice versa, a feature shared by Brucellae (Galinska & Zagorski, 2013; Moscoso et al., 2011; Rosen et al., 2018). However, further characterizations are needed to confirm the role of the phosphodiesterase activity of PdeA in mice.

### 4 | EXPERIMENTAL PROCEDURES

#### 4.1 | Bioinformatics

Brucella assemblies (ASM18242v1, ASM15877v1, ASM74021v1, ASM5400v1, ASM1852v1, ASM18272v1, ASM712v1, ASM2274v1, ASM74225v1, ASM1684v1, ASM22100v1, ASM750v1, ASM 15775v1, and ASM1890v1) and O. anthropi assembly (ASM74295v1) were manually retrieved from NCBI. As the genome of B. abortus 544, our laboratory strain for biovar 1 of B. abortus, is not available in the NCBI data bank, we used the genome of B. abortus 2308 (also biovar 1), as a template. Cyclic di-GMP metabolizing enzymes were retrieved via annotation and BLAST-style alignment that queried for C. crescentus PleD (CCNA 02546; GGDEF domain), P. aeruginosa PA2567 (EAL domain) and Xanthomonas campestris RpfG (XAACFBP6369 RS13410; HD-GYP domain) (Hecht & Newton, 1995; Kulasakara et al., 2006; Ryan et al., 2006). We validated our screen by comparing published B. melitensis 16M c-di-GMP enzymes to our list (Petersen et al., 2011). Orthology relations were assessed by reciprocal best blast hit. Domains were aligned using MUSCLE (Edgar, 2004). Sequence polymorphism was manually screened. Domain identifications were performed with SMART (Schultz et al., 1998).

# 4.2 | Bacterial strains, plasmids, and growth conditions

All strains, plasmids and sets of primers used in this study are listed in Table S3. *E. coli* DH10B (Invitrogen) and S17.1 (Simon et al., 1983) were grown in Luria-Bertani (LB) medium at 37°C. *B. abortus* 544 (nalidixic acid-resistant strain, referred as the WT strain) and its derivatives were grown in tryptic soy liquid broth (TSB) at 37°C with shaking (160 rpm). When required, antibiotics were added to the medium at the following concentrations, nalidixic acid, 25  $\mu$ g/ml; kanamycin, 10 (integrative plasmid) or 50 (replicative plasmid)  $\mu$ g/ml; gentamycin, 10 or 50  $\mu$ g/ml.

Deletion strains were constructed by double homologous recombination using the pNPTS138 vector and checked with polymerase chain reaction (PCR), as previously described (Deghelt et al., 2014). Briefly, upstream and downstream regions of about 500 base pairs (bp) were PCR amplified for each gene of interest using the sets of primers listed in Table S3. PCR products were joined by PCR overlap and cloned into the *EcoRV* site of the pNPTS138 vector. The resulting plasmids were introduced into *B. abortus* by conjugation. Merodiploids were selected on kanamycin-nalidixic acid supplemented TSB agar plates, while excision of the plasmid was counterselected on 5% sucrose TSB agar plates. The  $\Delta pdeA$  mutant was complemented with the pMR10 vector containing the operon *bab1\_0508-bab1\_0507* (amplified with the primers set F-*Pst1-pdeA* and R-*Xhol-pdeA*, listed in Table S3) under the control of its own promoter (pMR10::*pdeA*).

Point mutation in the EAL active site of PdeA (E742A) was obtained by overlap PCR carrying the mutation with the primers listed in Table S3 (catalytic mutant) and cloned into the *EcoRV* site of the pNPTS138. Double homologous recombination was applied to exchange the WT allele with the mutated one, as previously published (Deghelt et al., 2014). Allelic replacement was checked by sequencing with primers that hybridize out of the homologous recombination zone.

When needed, the old pole markers PdhS-YFP (Hallez et al., 2007), FumC-YFP (Mignolet et al., 2010), and the growing pole marker AidB-YFP (Dotreppe et al., 2011) (on pSK-integrative plasmid, Kan<sup>R</sup>) were integrated into the genome through a single homologous recombination.

The analyses of colony size were assessed by awakening strains from frozen stocks (-80°C) onto TSB agar plates (with Kanamycin when needed). Plates were incubated at 37°C and images after 3, 4, and 7 days. Assessment of growth was performed in TSB medium or in the chemically modified Plommet medium with erythritol as the sole carbon source (PE) (Barbier et al., 2014). Overnight (O/N) culture were diluted to an optical density (OD<sub>600</sub>) of 0.05 (TSB) or 0.1 (PE), 200 µl were subsequently inoculated in flat bottom 96 well plates (Eppendorf) and grown for 48 hr at 37°C. OD<sub>600</sub> was measured every 30 min (with shaking prior to each measure) using an Epoch2 (Bioscreen).

#### 4.3 | Cell culture, infection and intracellular survival

RAW264.7 mouse macrophage-like cells were cultured at 37°C with 5% CO<sub>2</sub> in DMEM Glutamax<sup>TM</sup> (Thermofisher Scientific) supplemented with 10% fetal bovine serum (Gibco).  $5 \times 10^4$  cells were seeded into 24-well plates (Greiner® Bio-One) and infected at a multiplicity of infection of 50. When needed, antibiotic was added to the bacterial suspension prior to infection and kept during the infection. Infected cells were centrifuged for 10 min at 200 g at RT. After 1 hr of contact at 37°C with 5% CO<sub>2</sub>, medium was replaced with DMEM Glutamax<sup>TM</sup> supplemented with 50  $\mu$ g/ml of gentamycin to kill the remaining extracellular bacteria. Medium was changed after 1 hr to DMEM Glutamax<sup>TM</sup> containing 10  $\mu$ g/ml of gentamycin. At different times post-infection, the medium was removed; cells were washed two times with PBS and lysed with Triton X-100 (0.1%) in PBS for 10 min at RT. Intracellular survival and growth were assessed by colony forming unit (CFU) counting on TSB agar plates.

### 4.4 | Fluorescence microscopy

Bacteria were observed on PBS-1% agarose pad (if not otherwise stated) with an inverted microscope Nikon Eclipse Ti2 (oil objective 100x, CFI plan Apo Lambda DM 100XH 1.45/0.13 mm) with the filter sets: Cy5.5B (eFluor, ex 655/40, DM 685, BA716/40); CFP-2432C (HADA, ex 438/24, DM 458, BA483/32); YFP-2427B (ex 500/24, DM 520, BA542/27); TxRed-4040C (mCherry, ex 562/40, DM 593, BA624/40). Pictures were acquired with an Orca Flash 4.0

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V3 (Hamamatsu). All images were analyzed with ImageJ the plug-in MicrobeJ (Ducret et al., 2016; Schneider et al., 2012). Detailed procedures are provided in Supplementary Methods.

For microscopic observations and morphology quantification. bacteria were grown O/N in TSB, diluted into fresh TSB to an OD<sub>600</sub> of 0.1 and grown until early exponential phase was reached ( $OD_{600}$ between 0.3 and 0.5). Two  $\mu$ l of culture were mounted on TSB agar pads and imaged. At least 300 bacteria from minimum three different fields were analyzed per pad. Only individual bacteria have been considered, divisional cells with a visible septum were manually selected and analyzed separately. Length and width of bacteria were determined and measured with the ImageJ plug-in MicrobeJ. For the statistical analyses, we calculated the median length and width for each experiment and we performed statistics on the mean of these three medians. For the pole radius measurement, we divided individual bacteria mean width by two to get the radius, calculated the median for each of the three replicates and the percentage of augmentation on the medians with the formula: ((Radius mutant - Radius WT)/Radius WT) \* 100. To measure the doubling time (i.e., the time period between two consecutive divisions), exponentially grown bacteria were labeled with eFluor and mounted on TSB agar pad. Bacteria were imaged every 20 min to produce time-lapse movies. At least 6 bacteria were considered per experiment.

# 4.5 | eFluor staining, peptidoglycan labeling, and localization of polar proteins

To assess unipolar growth, O/N cultures were diluted to an  $OD_{600}$  of 0.2, stained with eBioscience<sup>™</sup> Cell Proliferation Dye eFluor<sup>™</sup> 670 (eFluor, Invitrogen) to a final concentration of 5 µM in phosphatebuffered saline (PBS) for 15 min at RT, washed twice in PBS and incubated at 37°C with shaking in TSB (Vassen et al., 2019). Bacteria were short-pulsed with HADA for 5 min at room temperature to a final concentration of 500 µM in TSB (Kuru et al., 2012). Cells were fixed in ethanol for 15 min in 70% ice-cold ethanol on ice. Bacteria were imaged at the beginning of the experiment and in exponential phase (5-6 hr of growth). eFluor fully labeled bacteria were manually excluded as they cannot be oriented. To assess the localization of the PG incorporation or the polar proteins, only bacteria with a polar signal for eFluor have been taken into consideration in order to properly orient the bacteria. To quantify the HADA labeled area, we used the ImageJ plug-in MicrobeJ to detect the HADA area. Bacteria were manually checked and false maxima were excluded.

### 4.6 | Localization of PdeA-mCherry

To localize PdeA in live bacterial cells, we replace the *pdeA* wild-type gene with a *pdeA-mcherry* gene fusion, by double homologous recombination. Around 500 bp of the 3' part of the gene (excluding the last 3 codons) and around 500 bp of the 3' portion of the gene

(including the last 13 codons, bearing a putative proteolysis tag) were amplified. *mcherry* gene was amplified from the pUC19::*mcherry* (F. Renzi, UNamur). All three fragments were joined by overlap PCR and cloned into the *EcoRV* site of the pNPTS138. Double homologous recombination was applied to exchange the WT allele with the mCherry fusion one. Allelic replacement was checked by PCR. O/N cultures were diluted to an OD<sub>600</sub> of 0.2 and imaged in exponential phase of growth. Bacteria were manually selected in the smallest cell aggregated when possible. Only bacteria with one signal for PdhS-YFP or 1 or 2 signal(s) for AidB-YFP and at least one signal for PdeA-mCherry have been considered.

### 4.7 | Mice infection

C57BL/6 mice were acquired from Harlan (Envigo, Bicester, UK). Eight 10-weeks female mice per group were infected intranasally with 30  $\mu$ l of a 6.6  $\times$  10<sup>5</sup> CFU/ml (infectious dose: 2  $\times$  10<sup>4</sup> CFU) in PBS. Prior to infection, mice were anesthetized with a mix of Xylazine/Ketamin (9 mg/kg and 36 mg/kg, respectively). The doses of infection were checked by plating serial dilutions on TSB agar plates. At desired time point, mice were sacrificed by cervical dislocation. Spleen and lungs were immediately recovered and crushed in PBS 0.1% Triton X-100 (Sigma Aldrich). We performed the experiment with and without (data not shown) the empty vector pMR10 in the WT and the  $\Delta pdeA$  strains. Bacterial burden was evaluated by plating serial dilutions on TSB agar plates containing nalidixic acid or kanamycin (only for the experiment in which strains bear the pMR10). All experiments were handled in a Biosafety level 3 facility.

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#### AUTHOR CONTRIBUTIONS

AR and XDB designed the work. AR and FXS performed the in silico analyses. AR, EC, PTAO, and AG performed the in vitro experiments. AR, EB, and AD did the in vivo experiments. EM provided tools and skills for the in vivo experiments. AR and XDB wrote the manuscript.

#### ETHIC STATEMENT

The procedure used in this study and the handling of mice are compliant with current European Legislation (Directive 86/609/ EEC). The Animal Welfare Committee of the University of Namur (UNamur, Belgium) reviewed and approved the complete protocol for *B. abortus* infection (Permit Number: UN GIN 20/344).

### DATA AVAILABILITY STATEMENT

All data supporting this study results are provided within the article and its supplementary information files or from the corresponding author upon request.

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1462 | WILEY

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