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The *Brucella* pathogens are polarized bacteria

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Abstract

Brucella pathogens are responsible for brucellosis, a worldwide zoonosis. They are facultative intracellular pathogens characterized by their asymmetric division and their unipolar growth. This growth modality generates poles with specialized functions (through polar recruitment of polar adhesins or of cell cycle regulators) and progeny cells with potentially different fates.

Keywords: Polarity; *Brucella*; Growth; Adhesion; Cell cycle; Host–pathogen interaction

1. Introduction

Bacteria belonging to the alphaproteobacteria group display highly diversified ecological niches. Their lifestyle can be closely related to a host organism, being pathogenic or symbiotic for plants, insects or mammals, either extracellular or intracellular, facultative or obligate [3]. Free-living alphaproteobacteria can also be found in water and soils. Their genome size and organization also differ [53], and following the rule of genome reduction process during the course of evolution [47], obligate intracellular pathogens like *Rickettsia prowazekii* display a small genome (1.1 Mb) compared to the free living bacterium *Bradyrhizobium japonicum* (9.1 Mb), highlighting the loss of genes coding for metabolic pathways that become no more necessary. Despite those differences, several alphaproteobacteria display common features such as (i) asymmetric division [23], (ii) unipolar growth [7], (iii) the presence of a highly and specifically conserved master regulator of the cell cycle called CtrA and its associated control network [6] and (iv) polar functions such as signal transduction systems and adhesion [23,28,44,54]. The intracellular pathogen *Brucella* belongs also to the alphaproteobacteria class and does not make exception to these shared features. In this review we report on the recent data suggesting that the polarity and the asymmetry of *Brucella* have a functional role that could impact the infectious cycle.

2. *Brucella* spp.

Brucella spp. are Gram-negative bacteria belonging to the alpha-2 subclass of proteobacteria [33] and harboring a 3.2 Mb genome divided in two chromosomes [34]. They are described as small coccobacilli (0.6–0.8 μm) (Fig. 1), non-sporulating and non-motile. Until now, *Brucella* genus comprises 10 species according to their host specificities [58]. *Brucellae* are responsible for a worldwide zoonosis called brucellosis. Some *Brucella* species are very infectious for humans and cause an undulant fever called Malta fever [4,30,48]. The disease can become chronic and debilitating if left untreated. Infections can occur by ingestion of contaminated and unpasteurized milk or by working closely with infected animals. There is few if any human to human transmission. No vaccine is available for humans. The cardinal clinical signs of brucellosis in

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animals are abortion in pregnant females and sterility in males. The chronicity of the disease is thought to be linked to the intracellular location of the pathogen and to its ability to deal both with the stressful conditions encountered as well as with the innate and adaptive immune defense. *Brucella* enters the host mainly through the mucosal membrane of respiratory, digestive and genital tracts [16]. Mice intranasal infections with *Brucella abortus* showed that the bacterium is mainly found in alveolar macrophages and occasionally in pulmonary dendritic cells [2]. After internalization inside a host macrophage, *Brucella* is found in a membrane-bound compartment called BCV (for *Brucella*-containing vacuole). The BCV will sequentially acquire markers of early endosomes and late endosomal/lysosomal pathway [36,51]. Early acidification of the BCV is required for successful trafficking [38], probably because this environment activates transcription of virulence factors such as the type IV secretion system [5]. The BCV will finally interact with endoplasmic reticulum (ER) exit sites to reach an ER-like compartment where bacteria will actively proliferate [36]. Very late in the infection of cultured cells, BCV will acquire autophagic features, which will allow them to complete an infectious cycle by cell-to-cell spreading [50]. It is important to note that this intracellular lifestyle can also be divided into two steps: a non-proliferative phase during the traffic of *Brucella* (about 12 h after internalization) is followed by a proliferative phase once inside the replication niche [36]. Interestingly, *Brucella* proliferation is also observed in the chorionic trophoblasts of experimentally infected goats [1] and in both immortalized and primary human trophoblasts [46].

3. Asymmetric division and unipolar growth

Asymmetric division is a mechanism that can generate two functionally differentiated but clonally identical sibling cells. This mechanism is well studied in eukaryotes. Symmetric transversal binary fission is thought to be the main dividing mode for bacteria. Nevertheless, alternatives to the binary fission exist [28] and cases of asymmetric cytokinesis are also well known in both Gram-positive and Gram-negative bacteria such as the sporulation of *Bacillus subtilis* [17] and the cell cycle of *Caulobacter crescentus* [22], respectively. Few years ago, it was reported that several alphaproteobacteria including *Brucellae* divide asymmetrically, giving after division a large cell and a small cell [23].

In addition to the asymmetric localization of their septal plane [23], several alphaproteobacteria undergo a new type of cellular growth that is strictly unipolar [7]. First identified for *Agrobacterium tumefaciens* using Texas red-X succinimidyl ester (TRSE) and D-Cys cell surface labelings [7], this polar growth was also shown to be conserved in other alphaproteobacteria belonging to the order Rhizobiale like *B. abortus*, *Ochrobactrum anthropi* and *Sinorhizobium meliloti*. As depicted in Fig. 2B, the unipolar growth of *B. abortus* allows to generate two different cells after cell division: a "mother" cell inheriting the old envelope material, and a "daughter" cell having a newly synthesized envelope.

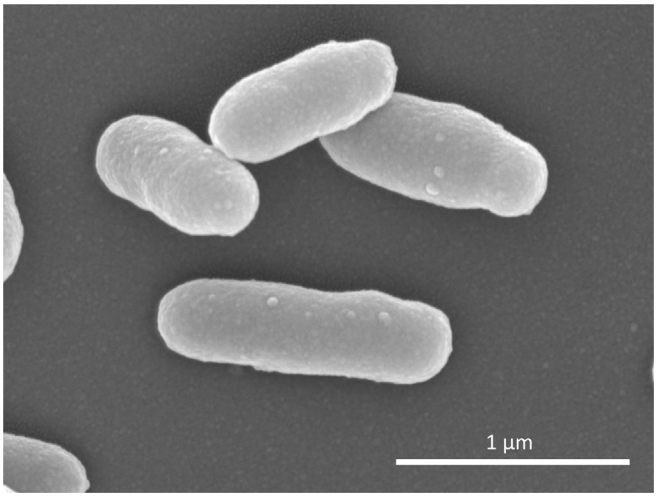

Fig. 1. Scanning electron micrography of *Brucella abortus* grown in rich defined medium.

4. Cell cycle regulation and life cycle

Signal transduction systems allow the sensing of environmental and intracellular conditions in order to engage an appropriate cellular response. Among the most widespread bacterial response systems are the two-component systems that are classically composed of a sensory histidine kinase that autophosphorylates on a conserved histidine in the presence of specific signal. The response regulator catalyzes the transfer of the phosphoryl group from this histidine of the histidine kinase to a conserved aspartate, resulting in response regulator activation [25]. Two-component systems are also used as regulatory networks involved in the control of the bacterial cell cycle regulation [40]. CtrA is a master and global regulator of the cell cycle [41], conserved and specifically found only in alphaproteobacteria. A complex regulation network allows the

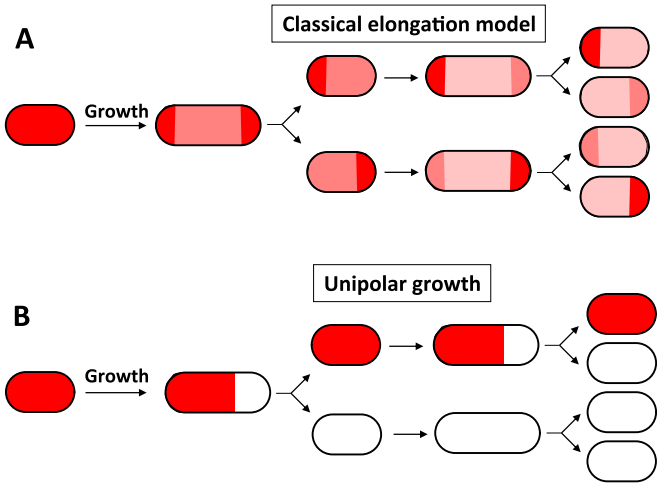

Fig. 2. Comparison between non-polar and unipolar growth in bacteria. (A) In the case of classical elongation, the newly incorporated envelope is diluting the previously synthesized envelope. (B) The cellular growth of *Brucella abortus* and other alphaproteobacteria belonging to the order Rhizobiales is unipolar. Dark red, previous envelope material; white, newly incorporated envelope material and light red, diluted envelope material.

fine-tuning of CtrA abundance and activity through control of proteolysis, phosphorylation and *ctrA* transcription [49]. Actors of the CtrA regulation network are also conserved among alphaproteobacteria [23] and some will be described in the next sections.

4.1. *The* C. crescentus *model and the* Ehrlichia chaffeensis *pathogen*

The processes of cell division and differentiation are well documented for the free-living bacterium *C. crescentus* in which the morphological asymmetry is obvious. Each division gives rise to a large sessile stalked cell and a motile flagellated cell. Several advantages can explain why *C. crescentus* represents a well-studied and useful prokaryotic model: (i) an identifiable polar structure (the stalk) is easily observed by optical microscopy and allows to distinguish between the two different progeny cells, (ii) a simple synchronization protocol allows to enrich a population of small flagellated cells [18], which permits to study cell cycle process at the molecular level, e.g. using large scale approaches, (iii) a rather short generation time (e.g. 90 min, depending on the bacteriological medium), (iv) this is a non-pathogenic bacterium compared to other alphaproteobacteria and (v) on the contrary to *Escherichia coli*, there is one round of chromosome replication per cell cycle, which makes easier to follow the bacterial cell cycle. Interestingly, alphaproteobacterial pathogens have conserved genes involved in the asymmetric cell cycle control, identified and characterized in *C. crescentus*. Even in small genomes of obligate intracellular pathogens like *E. chaffeensis*, *Anaplasma phagocytophilum* [42] and *R. prowazekii* [3], a *ctrA* homolog is present while other actors of the regulatory pathway are lost. Interestingly, in *E. chaffeensis*, only three pairs of two-component systems are predicted and two of them involve homologs that regulate CtrA activity in *C. crescentus*. Rikihisa and colleagues showed that (i) histidine kinase activity was required for preventing lysosomal fusion of *E. chaffeensis* [27] and (ii) CtrA regulated genes products were involved in bacterial stress resistance for the passage between two host cells [11]. In these pathogens, the possible roles of CtrA homologs in the control of cell cycle and the localization of CtrA or its regulators are currently unknown. The knowledge of the molecular details of cell cycle control and polarized structures in *C. crescentus* is exceeding by far the knowledge we have on many alphaproteobacterial pathogens. The example of *E. chaffeensis* suggests that cell cycle regulation could be linked to virulence in other pathogenic alphaproteobacteria. In recent years, some progresses have been made on the description of polarized structures and functions in the *Brucella* genus.

4.2. *PdhS, an essential and polarly localized histidine kinase in* Brucella

As part of the CtrA regulatory central network first identified in *C. crescentus*, two histidine kinases, called PleC and DivJ, were shown to participate in the regulation of DivK phosphorylation [29]. In a predivisional cell, PleC and DivJ display antagonist functions and opposite localizations. PleC acts as a phosphatase on DivK∼P and is localized at the flagellated pole while DivJ, acting as a kinase of DivK, is localized at the stalked pole. Phosphorylated and unphosphorylated DivK can thus freely diffuse from one pole to the other [29]. This mechanism implies that after septum formation, the flagellated cell will be depleted of phosphorylated DivK while the stalked cell will accumulate DivK in its phosphorylated form. The fact that some signaling proteins are localized in clusters could be a way to (i) segregate their function between the two sibling cells after cytokinesis completion if localized at one pole and (ii) allow increasing the efficiency of a function at a specific location. Thus, the two sibling cells will inherit a different developmental program.

Homologous proteins of the *C. crescentus* PleC, DivJ and DivK are present in *Brucella*. Interestingly, a third histidine kinase, homologous to both PleC and DivJ is also found in *Brucella*. This protein called PdhS (PleC/DivJ homologous sensor) was demonstrated to be polar in *B. abortus* [24]. PdhS is only detected at the old pole after division (Fig. 3), and the sibling cell that did not inherit PdhS has to acquire it at its old pole prior to divide, suggesting a differentiation event. Several observations suggest that PdhS function is involved in DivK control in *Brucella*. First, PdhS is interacting with DivK in a yeast two-hybrid assay [24], and in a bacterial recruitment assay [56]. Second, PdhS and DivK are co-localized at the same pole [24]. Third, DivK polar localization is decreased in a *pdhS* thermosensitive mutant at restrictive temperature [55]. PdhS is probably required for an appropriate control of cell cycle progression since the thermosensitive mutant does not grow at restrictive temperature, and several overexpression mutants (e.g. non-phosphorylatable and truncated proteins) generate abnormal cellular morphologies in *Brucella* that are typical of cell division defects [55]. PdhS homologous protein

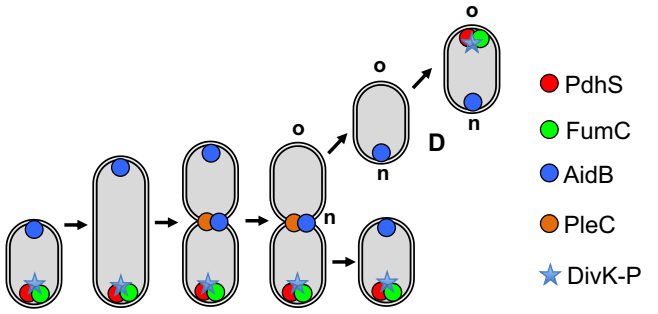

Fig. 3. Summary of pole-anchored proteins in *Brucella abortus*. The histidine kinase PdhS, the fumarase FumC and the phosphorylated response regulator DivK are localized at the old pole (o), while the alkylation response protein AidB is localized at the new pole (n), and possibly at the constriction site. The histidine kinase PleC is also localized at the constriction site or the very new poles. The DivK protein is only partially localized to the old pole, since this polar foci are only seen in a fraction of the population, and moreover a signal is also detectable in the cytosol (light grey). A non-phosphorylated mutant of DivK (DivK-D53A) is not detectable to the old pole, suggesting that only phosphorylated DivK is able to localize at the old pole. The differentiation event (D) is detected by the maturation of the old pole, that acquires specific markers (PdhS, FumC and DivK).

in *S. meliloti* called CbrA was also recently shown to play a role in the cell cycle regulation [45].

4.3. A histidine kinase interacting with a citric cycle enzyme

Mignolet and colleagues performed a *Brucella* ORFeome-based [15] yeast two-hybrid screen to identify potential PdhS interactors [32]. Surprisingly, an enzyme of the Krebs cycle called FumC was shown to specifically interact with PdhS. FumC is a fumarate hydratase (also called fumarase) that reversibly converts fumarate into L-malate [59]. PdhS–FumC interaction was further confirmed by colocalization assay in *Brucella* [32] and in *E. coli* [56]. An additional gene coding for another fumarase (called FumA) is present in the *Brucella* genome. FumA does not display a polar localization but rather a diffuse cytoplasmic pattern. This shows that polar localization is not a general feature of fumarases and raises the question of the meaning of FumC localization at the old pole of *Brucella*. One major difference between FumC and FumA is the presence of an iron-sulfur cluster in FumA that renders this enzyme sensitive to reactive oxygen species (ROS) [59]. Thus, the presence of FumC at one pole can allow a pool of ROS resistant enzyme ready to continue the Krebs cycle under stress conditions. Whether the origin of this putative oxidative stress is endogenous to *Brucella* or linked to its intracellular location inside host cell [26] has to be determined. Histidine kinases usually interact with themselves or with their cognate response regulator. So, the interaction between PdhS and FumC still remains intriguing and could represent a link between cell cycle progression and the metabolic state of this bacterium. Additional data are required to sustain this hypothesis.

5. A pole specialized for adhesion?

Adhesion to host cell surface is a crucial step during cellular infection process of intracellular pathogens as it is required before internalization. Moreover, this first contact determines the subsequent invasion steps and the intracellular fate of the bacterium [35,58]. Several adhesive structures have been identified in *Brucella*. SP41 is a surface protein that interacts with host eukaryotic receptors containing sialic acid residues [9]. The deletion of a genomic island (BAB1_2009–2012 for *B. abortus*, BMEI0060-0063 for *Brucella melitensis*) leads to a cellular adhesion defect, decreasing the number of bacteria at 4 h post infection in HeLa cells and in J774 macrophages compared to the wild type control [13].

Recently, panning of a *Brucella suis* phage-display library against fibronectin was used to identify a new bacterial adhesin [39]. Fibronectin was chosen as a target because it can be bound, in a dose-dependent manner, by *Brucella* [8] and it is present inside the BCV after internalization [21]. This allowed the identification of BmaC (*Brucella* monomeric autotransporter), a large protein of 340 kDa predicted to belong to the type I autotransporter family [39]. Accordingly to the proposed adhesin function, a *bmaC* deletion mutant (i) was impaired in the attachment to immobilized fibronectin and to the surface of HeLa and A549 epithelial cells, (ii) was outcompeted by the wild type strain in co-infection experiments and (iii) anti-BmaC and anti-fibronectin antibodies significantly inhibited the binding of *B. suis* to HeLa cells. Interestingly, immuno-fluorescence (IF) labeling experiments using anti-BmaC antibodies showed that BmaC is localized at one bacterial pole in *B. suis*, but only in a small proportion of the population growing in rich defined medium. To note, it seems that single *Brucella* initially binds through one of its poles with the cell surface [37,39] and when the IF was performed on adherent bacteria during a cellular infection, BmaC was detected at the pole in contact with the host cell surface.

More recently, a comprehensive bioinformatic search in the *B. suis* genome led to the identification of a novel adhesin BtaE (*Brucella* trimeric autotransporter) from the type II autotransporter family. As shown for BmaC, the deletant strain for *btaE* (i) displays a decreased ability to adhere to HeLa and A549 epithelial cells and (ii) was outcompeted by the wild type *B. suis* strain in binding assay to the host cells. Moreover, it was shown that BtaE is required not only for cellular infection *in vitro* but also for full virulence during mice infection. It was also shown that the heterologous production of this protein in a "non-adherent" *E. coli* strain increases the interaction to immobilized hyaluronic acid and fibronectin. Immunodetection using anti-BtaE antibodies revealed that BtaE is surfaced localized at one pole of *B. suis* [44].

Interestingly, BmaC and BtaE are both localized at one pole, raising the hypothesis of a common adhesive pole in *Brucella*. To show this, both BmaC and BtaE were co-localized with specific markers of old and new pole in *Brucella* (Fig. 3) [44]. Although the number of bacteria positively labeled with BtaE and BmaC was low, both adhesins were found to localize at the same pole as AidB-YFP (a new pole marker [14]) at the opposite to PdhS-GFP labeling (old pole marker). These observations identify the newly formed pole as functionally differentiated pole for adhesion.

Virulence of *Brucella* relies on its ability to modulate the host endocytic pathway to reach the endoplasmic reticulum of the host cell. The compartment containing *Brucella* interacts with the host endoplasmic reticulum exit sites (ERES) to reach the bacterial proliferative niche [10]. Confocal microscopy analysis of infected macrophages revealed that *B. abortus* containing vacuoles are often polarly apposed to Sec31, a protein involved in COPII complex localized at the ERES [10]. This polar apposition suggests that asymmetry is also exploited outside the bacterium during the intracellular trafficking, at a crucial step of the cellular infection.

Polar localization could be an elegant way of increasing the adhesive power, by concentrating the adhesive proteins and thus providing avidity. Moreover, host invasion by a bacterial pole *per se* can facilitate entry because of the bacterial shape. There are other examples of alphaproteobacteria that show polar adherence to host cell or a substrate. The extracellular plant pathogen *A. tumefaciens* binds to plant and abiotic surfaces through one of its poles [54] and *C. crescentus* adheres to

a solid surface by the holdfast at the tip of the polar stalk [31]. Therefore, polar adherence to surfaces can be proposed as a conserved mechanism shared by several alphaproteobacteria.

6. The flagellum of *Brucella*

While *Brucellae* are described as non-motile, a polar gained-flagellar structure was reported in a small proportion of *B. melitensis* population at the start of the exponential phase of growth [20]. As the flagellum is potentially an adhesive structure, it would be interesting to observe if this appendage is localized at the new or the old pole in *Brucella*. One of the most surprising observations with regard to the *Brucella* flagellar genes is the absence of classical genes encoding the membrane chemoreceptors, proteins of the signal transduction pathway and the flagellar motor [12]. Moreover, the deletant strains for *ftcR*, *fliF*, *flgE* and *fliC* flagellar genes still produce an empty sheated structure [19]. Interestingly, *Brucella* flagellar genes are required for the establishment of infection in mice and goats [20,60] and flagellin monomer was recently shown to modulate *Brucella* innate immunity [52]. However, the function played by the flagellum and the conditions in which it is produced in the natural host or infections models remain to be investigated.

7. AidB, a polar DNA repair enzyme

In *E. coli*, AidB is described as a DNA binding protein [43] proposed to play a role in the protection against alkylating agents [57]. The homologous protein of *B. abortus* (AidB) was found to be recruited at the new pole and the constriction site in *B. abortus* [14] (Fig. 3). The *B. abortus aidB* mutant is more sensitive to methanesulfonic acid ethyl ester (EMS), suggesting that AidB is also playing a role in the response to alkylating agents. Interestingly, overexpression of *aidB*, but not of two other paralogs (acyl-CoA dehydrogenases) generates strong morphological aberrations. While the exact molecular function of *B. abortus* AidB is unknown, the branched, swollen and elongated morphologies observed with the *aidB* overexpression strain suggest that AidB could play a role in a checkpoint at cell division.

8. Conclusion and future directions

Various cellular mechanisms such as the asymmetric division, the polar growth and polar functions can generate different cell types. Those cell types could be functionally differentiated, helping the intracellular bacteria such as *Brucella* to cope with the different environments encountered during its life cycle. All these data also show that clonal bacterial populations are more heterogeneous than previously foreseen and this has to be taken into account to better understand how bacteria interact with their environment, and particularly those associated to a host organism. In that view, working at the single cell level becomes an attractive option and microfluidic devices combined with automated microscopy will allow recording the behavior of a large number of individual bacteria in an infection context. This opens new perspectives for antibiotics design that can target specific mechanisms during the appropriated step of the infectious cycle of the pathogen. In addition, drug design can aim to disturb the specific localization of some proteins complexes, decreasing the success rate of the pathogen. For example, polar adhesins seem to be an interesting target since they are surfaced exposed and thus accessible to drugs that could interfere with adhesion, a crucial step in host–pathogen interaction.

Questions box

- What are the physiological differences between the sibling cells generated by asymmetric division?
- Why are adhesins at new poles only?
- Is there a subpopulation of bacteria expressing adhesins that would be more infectious?
- Are there other polar proteins or appendage?
- What is the selective advantage of unipolar growth?
- What is the role of the DivK-CtrA pathway in *B. abortus*?
- What is the selective advantage of the polar localization of enzymes such as FumC and AidB?

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