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Investigation of the preferential internalization of newborn Brucella abortus in host cells

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Investigation of the preferential internalization of newborn *Brucella abortus* in host cells

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Etude de l'internalisation préférentielle de la bactérie *Brucella abortus* en phase G1 dans sa cellule hôte

Van der Henst Mathilde

Résumé

Brucella abortus est un pathogène responsable de la brucellose bovine, une anthropozoonose très répandue. Cette bactérie a la capacité de s'internaliser, de survivre et même de se répliquer dans certaines cellules de l'hôte. Mise en contact de cellules en culture, *B. abortus* présente deux phases d'infection bien distinctes. Les premières heures de l'infection sont caractérisées par un nombre constant de bactéries intracellulaires viables. Durant cette première phase, alors appelée « phase non proliférative », on peut observer un blocage du cycle cellulaire du pathogène en phase G1 de son cycle cellulaire (Deghelt *et al.*, 2014), jusqu'à atteindre sa niche réplicative. Une fois que la bactérie a transité jusqu'à cette niche, elle va pouvoir redémarrer de nouveaux cycles cellulaires, ce qui constitue la phase dite « proliférative » de l'infection. Les bactéries se trouvant en phase G1, appelées ici « newborns », constitueraient une forme préférentielle pour l'infection par *B. abortus*. La cause de cette internalisation prédominante des newborns reste cependant inconnue. L'adhésion de la bactérie à sa cellule hôte peut constituer l'une des étapes limitantes de l'infection. Dès lors nous avons suggéré que l'internalisation préférentielle des newborns serait due à la présence de caractéristiques particulières permettant à ce type cellulaire de mieux adhérer à la surface de son hôte et par conséquent serait sélectionné pour l'infection. Récemment il a été montré que la délétion du gène codant pour l'autotransporteur BmaC chez *Brucella suis* entrainait une diminution drastique de l'adhésion et donc de l'internalisation de la bactérie dans les cellules épithéliales HeLa (Posadas *et al.*, 2012). Il a également été montré que BmaC, ainsi que deux autres adhésines de la même superfamille, BtaE et BtaF, sont localisées au niveau du nouveau pôle de *B. suis* (Posadas *et al.*, 2012 ; Ruiz-Ranwez *et al.*, 2013a,b). A partir de ces données, nous avons émis l'hypothèse selon laquelle BmaC serait présente uniquement, ou du moins en quantité plus importante, sur les bactéries newborns, ce qui pourrait expliquer leur plus grande infectiosité. Afin d'investiguer cette hypothèse, nous avons d'une part délété le gène codant pour BmaC chez *B. abortus* afin d'observer un effet possible sur l'internalisation. Nous avons d'autre part surexprimé *bmaC* dans le but d'observer si la souche de surexpression résultante présente un phénotype plus adhérent ou une susceptibilité accrue de se faire internaliser. Les analyses que nous avons effectuées ne sont pas compatibles avec un rôle crucial de BmaC dans l'adhésion de *B. abortus* 2308 aux cellules HeLa ou aux macrophages RAW 264.7. Nous avons donc recherché des structures qui seraient enrichies sur les newborns à l'aide de lectines conjuguées à des sondes fluorescentes. Nous avons identifié une lectine, qui est associée au nouveau pôle de *B. abortus* lorsqu'elle est cultivée jusqu'en phase stationnaire, en milieu riche. Des recherches complémentaires devraient être réalisées afin de déterminer la nature du composé reconnu par cette lectine.

Mémoire de master en biochimie et biologie moléculaire et cellulaire

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Investigation of the preferential internalization of newborn *Brucella abortus* in host cells

Van der Henst Mathilde

Abstract

Brucella abortus is the pathogen responsible for bovine brucellosis, a worldwide anthropozoonosis. This bacteria is able to be internalized, survive and replicate in host cells. *B. abortus* presents two distinct phases in infection. First hours of infection are characterized by a stable number of intracellular viable bacteria. During this first phase of infection, therefore called "non-proliferative phase" we can observe a cell cycle arrest of the pathogen in G1 phase (Deghelt, Mullier *et al.*, 2014), up to reach its replicative niche. Once bacterium has reached this niche it is able to initiate new cell cycles constituting the "proliferative phase" of the infection. In fact bacteria in G1 phase called here "newborns" would constitute the preferential infectious form of *B. abortus*. However the cause of this predominant internalization of newborn bacteria remains unknown. Bacterial adhesion to host cells could constitute a limiting step for the infection. Therefore we have suggested that preferential internalization of newborns could be due to the presence of specific features on these cell types allowing bacteria to be more adherent to host cells and subsequently to be more selected for the infection. Recently it has been shown that deletion of the gene coding for the autotransporter BmaC in *B. suis* led to a drastic decrease of bacterial adhesion and internalization in HeLa cells (Posadas *et al.*, 2012). Moreover it has been reported that BmaC, along with two other proteins from the same superfamily, BtaE and BtaF, are localized at the new pole of *B. suis* (Posadas *et al.*, 2012; Ruiz-Ranwez *et al.*, 2013a,b). From these data we hypothesized that BmaC would only or more present on newborn bacteria that could explain their higher infectiosity. In order to investigate this hypothesis we have first delete the *bmaC* gene in *B. abortus* to observe possible effect on internalization. In another part, we have overexpressed *bmaC* in order to see if this overexpression strain presents an increased adhesion to host cells or is more susceptible to be internalized. Experiments performed have revealed that BmaC has no crucial role in adhesion of *B. abortus* 2308 to HeLa cells and RAW 264.7 macrophages. Therefore we decided to investigate if some structures are enriched on newborn bacteria using lectins conjugated with fluorescent probes. We have identified one lectin which is associated to the new pole of *B. abortus* when cultivated until stationary phase in rich medium. Further investigations should be performed in order to determine the component recognized by this lectin.

Mémoire de master en biochimie et biologie moléculaire et cellulaire

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"The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' but 'That's funny...'"

Isaac Asimov

Table of content

Abbreviations list

AAL, *Aleuria Aurantia* Lectin

AT, Autotransporter

BCV, Brucella Containing Vacuole

bp, base pair

DSL, *Datura Stramonium* Lectin

e.g, exempli gratia

ER, Endoplasmic Reticulum

Fuc, fucose

Gal, galactose

GFP, Green Fluorescent Protein

GlcNAc, N-acetylglucosamine

GNL, *Galanthus Nivalis* Lectin

i.e, ida est

kb, kilobase

kDa, kilos Dalton

LPS, Lipopolysaccharide

MAL, *Maackia Amurensis* Lectin

Man, mannose

MOI, Multiplicity Of Infection

OD, Optic Density

PI, Post-Infection

Sia, sialic acid

SNA, *Sambucus nigra* Lectin

TRSE, Succinimidyl Ester TxRed

TxRed, Texas Red

WGA, Wheat Germ Agglutinin

WT, Wild Type

YFP, Yellow Fluorescent Protein

Introduction

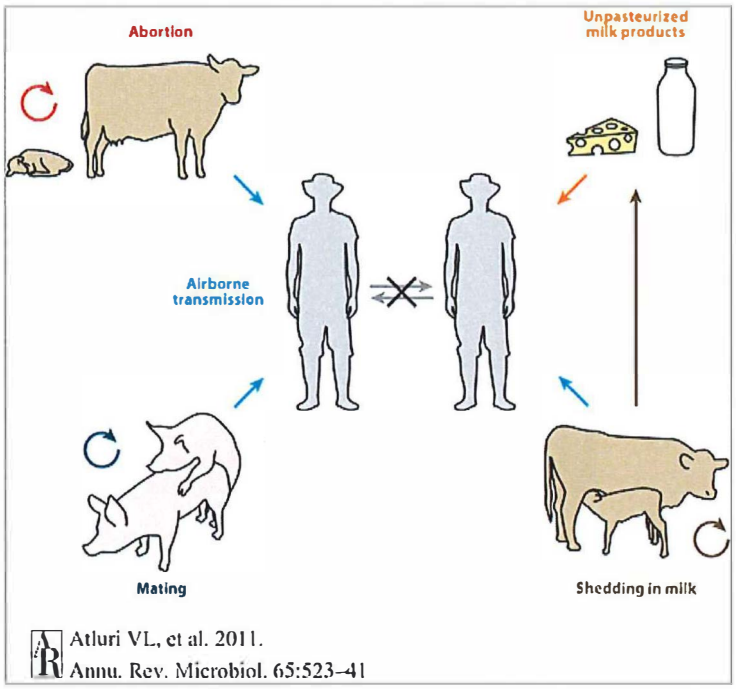

Figure 1: Model describing transmission of *Brucella spp.* The main routes for human contamination by *Brucella* are the comsumption of unpasteurized milk products and inhalation of aerosols containing infectious germs. Regarding infected animals, transmission occurs through close contact or exchange of secretions (Atluri *et al.*, 2011).

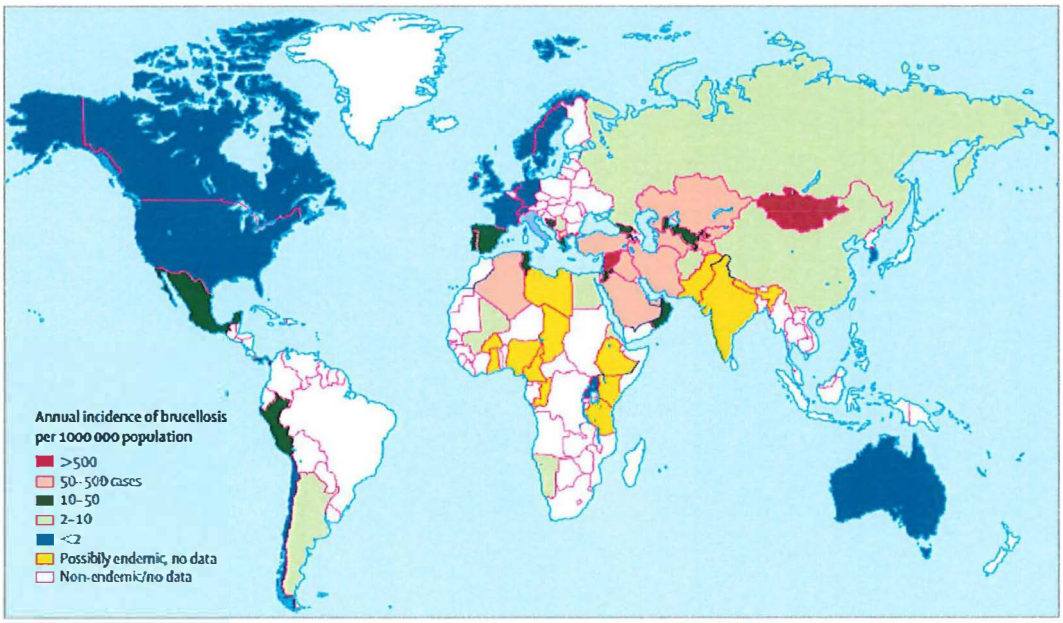

Figure 2: Map of the human brucellosis incidence. (Pappas *et al.*, 2006).

Introduction

Brucellosis

Brucellosis is a worldwide anthropozoonosis affecting many different mammals, including cattle, caprin, sheeps, swines, dogs as well as marine mammals (Figure 1). This infectious disease is also found in human, which is an accidental host, and is often called Malta fever or undulant fever (Moreno and Moriyon, 2006). The name "brucellosis" is derived from David Bruce who isolated bacteria for the first time in the spleen of sick soldiers in Malta during the second part of the 19th century. Symptoms related to the disease are diversified in animals, comprising severe arthritis, infertility in males and abortion in pregnant females (Moreno and Moriyon, 2006). In humans the disease is characterized by different pathological manifestations and evolves by successive impulses of disorder that results in diverse symptoms including headache, weakness and undulant fever. In the long term and without treatment, infection leads to chronicity and more severe symptoms such as arthritis, muscular pain, endocarditis and can have a fatal outcome in several cases (Moreno and Moriyon, 2006). Antibiotic treatment is indicated in the case of human infection, consisting in taking high doses of rifampicin and doxycycline during five weeks (Moreno and Moriyon, 2006).

Infection can be transmitted to humans through several means, a major one being the ingestion of unpasteurized dairy products obtained from infected animals for example, or by inhalation of aerosols containing infectious germs when abortion occurs. However, regarding contagious aspect of the disease, human-to-human transmission remains clearly rare whereas horizontal transmission widely occurs between animals through close contact and exchange of secretions among herds (Moreno and Moriyon, 2006).

After and since brucellosis was discovered, first preventive actions were milk pasteurization and systematic culling of infected flocks when infection was diagnosed in order to eradicate the disease (Moreno, 2002). This implies important economic losses, especially in developing countries where the disease remains endemic (Figure 2).

Up to these days, no effective human vaccine has been found, making bacteria from the genus *Brucella* a class III bioterrorism agent because of their impact on both public health and economy (Moreno, 2002; Moreno, 2014).

The *Brucella* genus

Brucella spp. are facultative intracellular bacteria responsible for Brucellosis. These non-motile pathogens are Gram negative coccobacilli from 0.5 to 1.5 μm long (Moreno and Moriyon, 2006). The genus *Brucella* is commonly divided into different species according to their preferential hosts. Up to now, 10 species have been listed (*B. abortus, B. melitensis, B. suis, B. canis, B. ceti, B. inopinata, B. microti, B. neotomae, B. ovis, and B. pinnipedialis*), among which the first four are known to be pathogenic for human (Moreno and Moriyon, 2006).

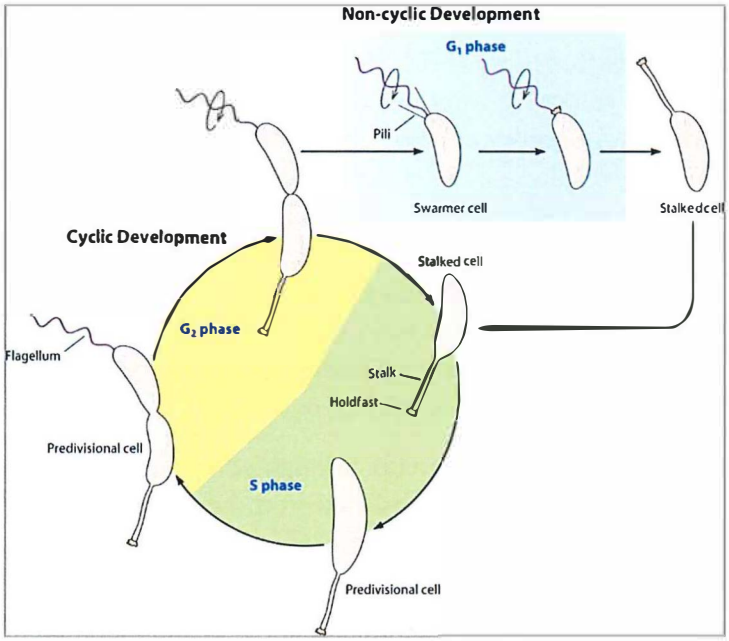

Figure 3: Life cycle of *Caulobacter crescentus*. The stalked cell is able to enter in S phase and replicates its chromosome. When the bacterium is in the predivisional state, a flagellum is synthesized at the opposed pole of the stalk. After cytokinesis, a flagelled cell in G1 phase is generated. This swarmer cell is unable to replicate its chromosome and must differentiate into a stalked cell to initiate replication. (Curtis *et al.*, 2010).

Despite host preference and several phenotypic variations, *Brucella* species are closely related at the genetic level, displaying more than 98 % of sequence identity between strains of different species (Moreno, 2014).

Brucella belongs to the α-proteobacteria class whose members are highly diversified. Indeed this group ranges from soil bacteria like the plant pathogen *Agrobacterium* and plant symbiont *Rhizobium* to the intracellular pathogen *Rickettsia*, or yet the non-pathogenic free-living *Caulobacter crescentus*, *Rhodobacter capsulatus* or *Rhodopseudomonas palustris*, which are aquatic bacteria. Despite these different lifestyles, several α-proteobacteria present common features, showing a unipolar growth (for Rhizobiales) and asymmetric division, sometimes leading to functional asymmetry (Brown *et al.*, 2012). For example, *C. crescentus*, used as a model of bacterial differentiation, presents a morphologically and functional asymmetry since cell division generates two distinct daughter cells having different functional properties. Indeed, each division gives a motile non-replicating flagellated cell in the one hand and secondly a sessile stalked cell displaying an appendage with adherent properties (Curtis *et al.*, 2010) (Figure 3). The flagellated cell named swarmer cell is able to find a suitable niche to differentiate into the stalked replicative-cell and initiate a new cell cycle. This adaptation in lifestyle allows the swarmer bacteria to explore their environment until they reach an appropriate environment for growth and colonization (Skerker and Laub, 2004).

Another classification of the pathogen has emerged as "facultative extracellular, intracellular pathogen" (Moreno and Moriyon, 2006). Indeed, the pathogenic lifestyle of *Brucella* would imply that bacteria need hosts to survive and multiply, however *Brucella* can grow in extracellular environments such as culture medium. Accordingly, the mostly intracellular nature of *B. abortus* means more that host cell environment provides favourable conditions for replication and expansion making it a preferential niche (Moreno and Moriyon, 2006).

Brucella infection and trafficking

Therefore following the new classification, one could imagine that the extracellular environment stage would constitute a stress phase and would be required in order to allow the dissemination of the pathogen from host to another. Founded on this life cycle schema, one plausible interpretation is that *Brucella* would first localize in external environment where it could persist during some days to months, depending on the conditions (Moreno and Moriyon, 2006).

Then bacteria enters in the host through different ways, probably mucosal membranes of the upper digestive tract and the respiratory tract. For example intranasal infection of mice have shown that after bacteria invade epithelium they are mostly found in alveolar professional phagocytes (Archambaud *et al.*, 2010). After internalization by macrophages, bacteria transit to the lymph node where they could establish a systemic infection through transmission to other professional phagocytes that characterize the chronicity of the disease (von Bargen *et al.* 2012). Once bacteria are disseminated throughout the organism the pathogen is able to develop several mechanisms allowing its release into the external environment. One representative example of these mechanisms is the triggering of abortion, to which a very high number of bacteria is found in foetus (containing up to 10^{10} bacteria per cm^3) in order to disseminate, infect, and colonize other hosts (Moreno & Moriyon 2006).

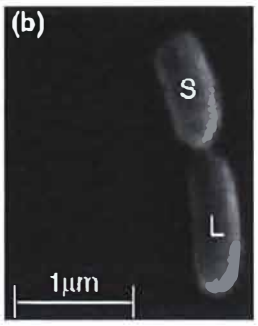

Figure 4: Asymmetric division in *Brucella abortus*. Scanning electron micrograph showing *B. abortus* just before cytokinesis. Small and large cells are represented by S and L respectively. (Hallez *et al*., 2004).

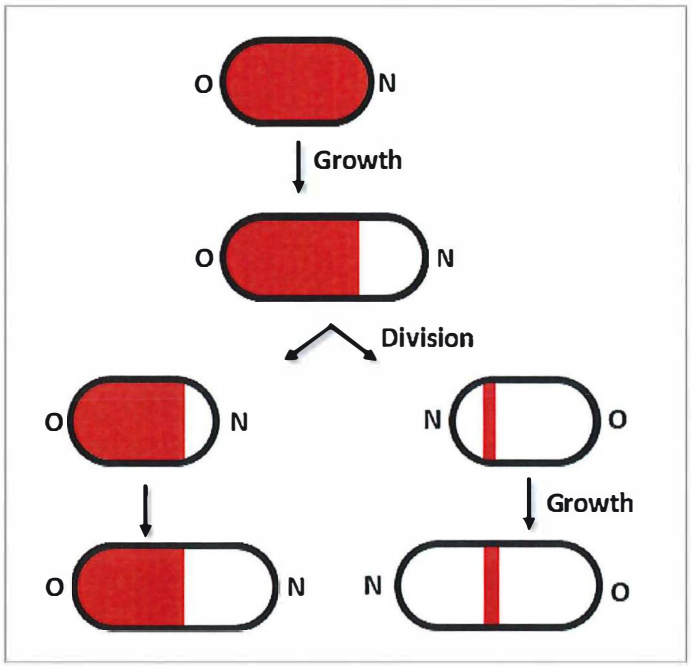

Figure 5: Schematic overview of the unipolar growth of *Brucella abortus* with TRSE labelling. Addition of new non-labelled materiel at the growing pole is revealed by the absence of staining whereas the rest of the bacterium remains stained with TRSE.

It has reported that among the cellular types that can be infected by *Brucella abortus,* epithelial cells and macrophages represent a good model for *in vitro* infection. Infection by *B. abortus* can be divided into two successive steps, a first non-proliferative step during which no increase of the bacterial number is observed, and a second highly proliferative step (Salcedo *et al.*, 2005).

During the non-proliferative phase of infection, internalized bacteria traffic within a vacuole called BCV for Brucella Containing Vacuole. It has been reported that *Brucella* can manipulate the intracellular trafficking to prevent fusion of the BCV with lysosomes (Pizarro-Cerda *et al.*, 1998). BCV interacts first with endosomal pathway and subsequently acquires markers of early endosomes such as Rab5 and EEA1 (Pizarro-Cerda *et al.*, 1998). This vacuole maturates and successively loses markers of early endosomes to acquire the late endosomal/lysosomal marker LAMP-1 (for Lysosomal Associated-Membrane Protein-1) (von Bargen *et al.*, 2012). It should be noted that BCV acidification is necessary for *Brucella* trafficking and survival within host cell. Indeed inhibition of pH acidification with bafilomycin in BCVs results in absence of replication niche and a non-proliferation of the bacteria (Boschiroli *et al.*, 2002). In fact pH variation in the bacterial environment is an important step since it triggers expression of virulence genes such as the *virB* operon coding for type IV secretion system (T4SS) shown to be essential to reach the replication niche (Boschiroli *et al.*, 2002). It should be noted that during the first steps of infection, an important killing of intracellular bacteria has been reported in professional phagocytes suggesting that most BCVs fuse with lysosome to become a phagolysosomal compartment leading to the degradation of bacteria. However this killing is less intense in non-professional phagocyte (Celli *et al.*, 2003).

Finally BCVs reach a compartment derived from endoplasmic reticulum to establish a proliferative niche corresponding to the proliferative phase. These ER-like compartments are characterized by the presence of ER markers and the absence of the LAMP-1 marker (Celli and Gorvel, 2004). It has been shown that this fusion event requires the presence of intact ERES (for Endoplasmic Reticulum Exit Site) since disruption of these elements prevents BCV-ER fusion and thereby prevents active proliferation of bacteria (Celli *et al.*, 2005). During this proliferative step, host cell integrity seems to be preserved and up to now few is known about the exit of bacteria from host cells (von Bargen *et al.*, 2012). Recent findings suggest the implication of autophagy in the dissemination of *Brucella* from host cells. Indeed, it has been reported that after bacterial replication the BCV is converted into a vacuole displaying autophagic features named aBCV (for autophagic BCV) and that this vacuole modification is necessary for cell-to-cell spreading of *Brucella* (Starr *et al.*, 2012).

Unipolar growth and asymmetric division markers

As in *C. crescentus*, we can observe asymmetric division during the cell cycle of other α-proteobacteria such as *Sinorhizobium meliloti*, *Agrobacterium tumefaciens* and especially in *Brucella abortus*. In this later both sibling cells resulting from bacterial division present different sizes and thus are named "small cell" and "large cell" (Hallez *et al.* 2004) (Figure 4).

It has been reported that several α-proteobacteria also present a unipolar growth (Brown *et al.*, 2012). Using TRSE (for Texas Red®-X Succinimidyl Ester) staining (Figure 5), this polar phenomenon was first discovered in *Agrobacterium tumefaciens* (Brown *et al.*, 2012).

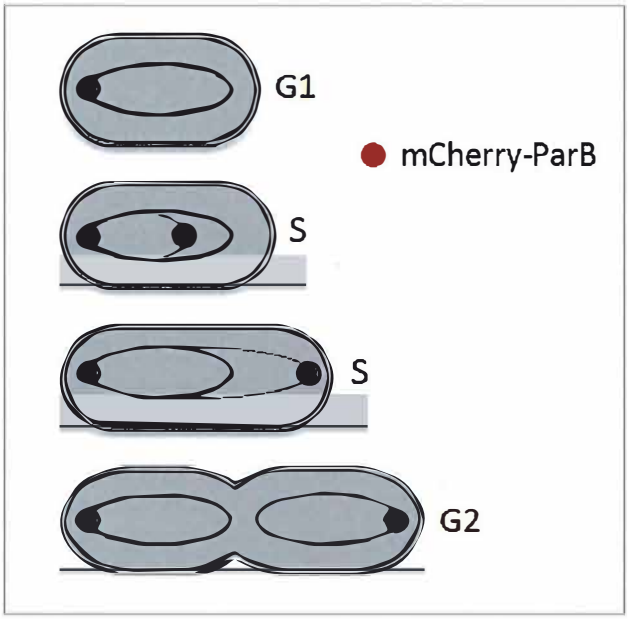

Figure 6: Monitoring of *B. abortus* cell cycle. In bacteria in G1 phase only one red focus is shown, corresponding to the segregation protein ParB fused to mCherry fluorescent protein (red) which binds replication origin of the chromosome I. When bacteria initiate a new cell cycle, chromosome replication occurs and bacteria enter in S phase. Thus two red foci indicate that replication has been initiated.

In fact it has been observed that bacteria grew unidirectionaly from the new pole generated after cell division. Interestingly using TRSE labelling we can observe the same global pattern of growth in *B. abortus*. This unipolar growth leads to emergence of a polarization in the bacterium, and subsequently different sibling cells after division (Brown *et al.*, 2012).

Different fluorescent polar markers have been characterized to detect the new pole from the old in addition to distinguish small cells from the large cells. PdhS (for PleC DivJ Homologue Sensor) is a histidine kinase displaying a polar localization in the bacterial cell. After cell division PdhS is found at the old pole of the mother cell and the other cell needs to acquire PdhS at its old pole in turn before division occurs. Therefore PdhS could be used as a tool to identify the old pole in *B. abortus* (Hallez *et al.*, 2007). IfoP (for In Front Of PdhS), shows also polar localization and allows to highlight the pole opposite to PdhS, *i.e* the new pole (Hallez *et al.*, 2007). Another characterized new pole marker is AidB, a homologue of acyl-CoA dehydrogenase protein involved in the resistance to alkylating compounds. In addition to highlight the new cell pole, this marker was also shown to be present at the constriction site in predivisional cells (Dotreppe *et al.*, 2011).

Cell cycle monitoring

To monitor cell cycle progress we have taken advantage of the partitioning system of chromosomes segregation used as a reporter of the DNA replication status. The *parABS* system has been reported to drive the bacterial chromosome I segregation in different species such as *B. subtilis, V. cholerae* or *C. crescentus* (Toro *et al.*, 2010). In *C. crescentus* this system consists in a centromere-like sequence named *parS,* neighbouring the origin of replication *oriC,* that is recognized and bound by the protein ParB. This later is then recognized by ParA, a filamentous ATPase protein localized close to the new pole of the bacteria. The depolymerisation of ParA leads to force generation and when bound to ParB resulting in movement of *oriC* toward cell pole allowing the correct segregation of one replicon relative to the other (Mohl, Gober *et al.*, 1997). Recently a homologous *parABS* system was characterized in *B. abortus* where ParB showed the same pattern of localization than in *C. crescentus* (Deghelt, Mullier *et al.*, 2014).

As the *parABS* partitioning system drives segregation of the chromosome I, the chromosome II segregation is also driven by a *repABC* system of partition. RepA and RepB, functional homologues of ParA and ParB respectively, allow the plasmid segregation through the binding of RepB to a *repS* sequence near the replication origin (Deghelt, Mullier *et al.*, 2014).

Fusion proteins have been constructed to highlight the position and the initiation of replication of both chromosomes of *B. abortus*. ParB, to which the fluorescent protein mCherry has been fused (mCherry-ParB), and YFP-RepB fusion can be detected by fluorescence microscopy displaying specific patterns of foci distribution depending on the chromosomes replication status (Figure 6) (Deghelt, Mullier *et al.*, 2014). Subsequently, initiation of chromosome replication can be detected by following the number of foci corresponding to *ori*I or *ori*II.

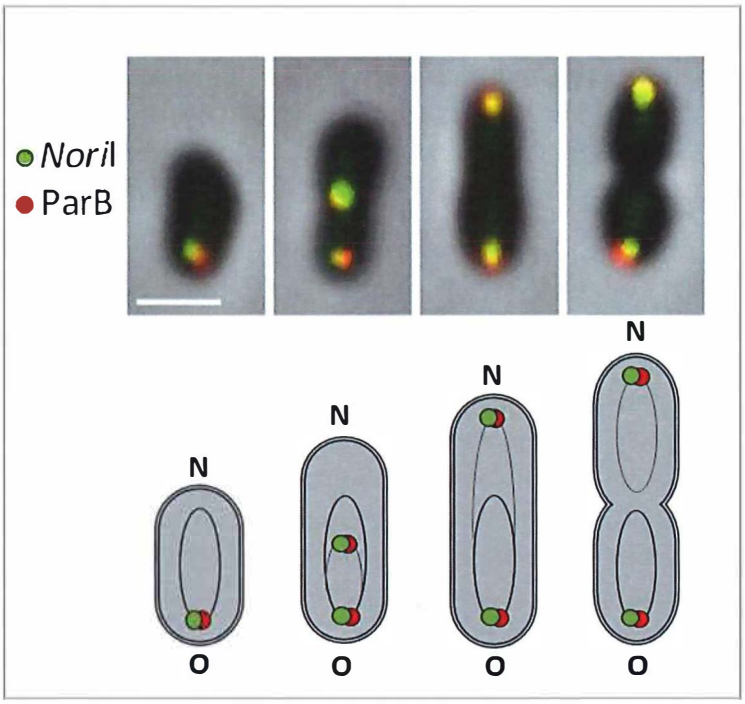

Figure 7: Colocalization of *Noril* (green) and mCherry-ParB (red). mCherry-ParB fusion protein recognizes the endogenous *parS* sequence highlighting the replication origin of the chromosome I (*oril*). The $parS_{pMT1}$ sequence, recognized by YFP-$ParB_{pMT1}$, was inserted near the replication origin of the chromosome I (*Noril*). Modified from Deghelt, Mullier *et al.*, 2014.

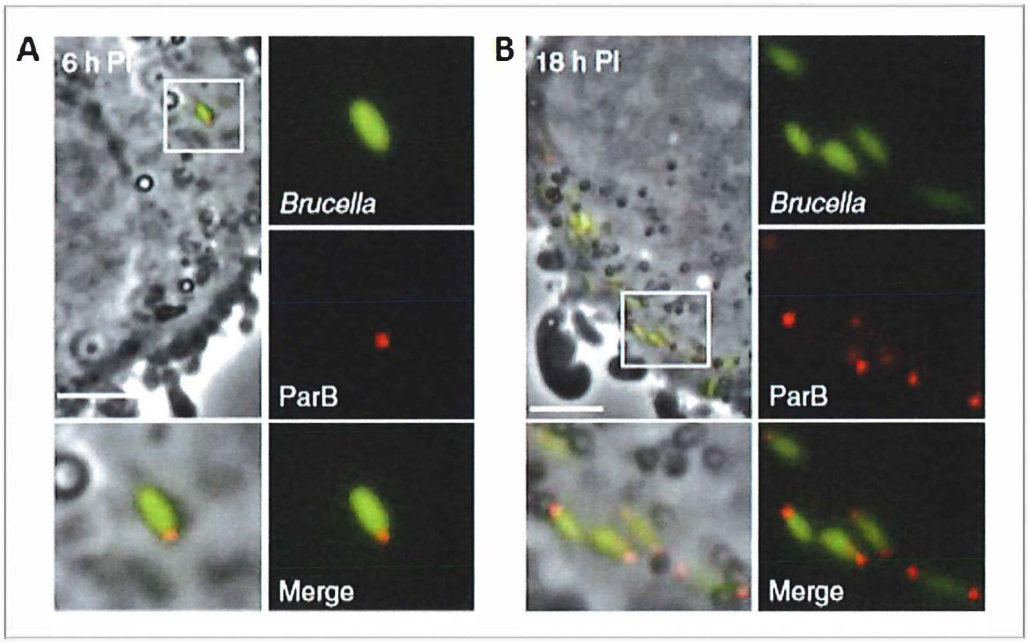

Figure 8: HeLa cells infection with *B. abortus* strain expressing mCherry-ParB (red) and diffuse GFP (green). HeLa cells were infected with *B. abortus* and were observed by fluorescence microscopy. **(A)** At 6 h PI 79 % of intracellular bacteria presented only one focus compared to 25% in *in vitro* culture. **(B)** At 18h PI bacteria showed one or two red foci. (Deghelt, Mullier, *et al.*, 2014). Scale bars represent 5 µm.

In order to confirm that *parS* is recognized by ParB in *B. abortus*, the same strategy was followed using an exogenous system to check the localization of mCherry-ParB in *B. abortus,* named $parS_{pMT1}$/$ParB_{pMT1}$ derived from the *Yersinia pestis* plasmid pMT1. The *parS* sequence was inserted in a region called *Nori*I (for Neighbouring *oriI*). This sequence is recognized and bound by YFP-$ParB_{pMT1}$ which colocalized in most cases with mCherry-ParB validating this latter reporter for the detection of chromosome replication status (Figure 7). Moreover in cells displaying only one focus, the signal colocalized with the old pole marker PdhS-mCherry as well as with mCherry-ParB (Deghelt, Mullier *et al.*, 2014).

It should be noted that using mCherry-ParB/YFP-RepB reporters, chromosome I replication was observed to be initiated before the chromosome II. Therefore the mCherry-ParB system or the $parS_{pMT1}$/$ParB_{pMT1}$ system allow us to distinguish first bacteria in G1 phase, called here **newborn** cells, which have not yet replicated any of their two chromosomes (only one focus detected for each fusion), secondly bacteria in intermediary stage characterized by one or two chromosome(s) replication(s) initiated which correspond to S and G2 phase, and finally predivisional bacteria displaying a constriction site in addition to the replication of both chromosomes (Figure 6) (Deghelt, Mullier *et al.*, 2014).

Cell cycle and infection

Given that the first phase of infection is characterized by a constant number of bacteria it was decided to investigate growth and cell cycle progress of *B. abortus* in an infection model. In order to assess the DNA replication status during the non-proliferative phase of HeLa cells infection, and therefore a possible cell cycle block, we have taken advantage of reporter strains described above and investigated chromosome replication during infection of host cells. Cell cycle of bacteria was monitored at 6 hours post-infection, *i.e.* before the beginning of intracellular proliferation (Figure 8). About 79 % of intracellular bacteria were found in G1 phase (*e.g.* no DNA replication was initiated) whereas this newborn subpopulation only accounts for 26 % of the bacterial culture when grown in rich medium. More importantly, at 15 min post-infection, about 73% of the internalized bacteria displayed only one mCherry-ParB focus, thus corresponding to newborn bacteria (Deghelt, Mullier *et al.*, 2014). These results indicated that most of the intracellular bacteria display a G1 phenotype from 15 min post-infection to 6 h post-infection, indicating that the newborn *Brucella* undergo a G1 block during the first non-proliferative phase of HeLa cells infection. Moreover, this suggests that these specific bacteria could be the predominant infectious bacterial cell type. It was also shown that the cell cycle progress is recovered during the proliferative phase (observation at 18 hours PI). It should be noted that similar results have been obtained for the infection of RAW 264.7 macrophages, another host cell model for *B. abortus* infection, where cell cycle was shown to restart earlier (Deghelt, Mullier *et al.*, 2014).

TRSE labelling was used to characterize the growth status of *B. abortus* in HeLa cells. Bacteria showing no growth, illustrated by totally labelled bacteria by TRSE (Figure 5), represented 93% of total intracellular population at 2 hours p.i. (during the non-proliferative phase) and this arrest is present up to 6 hours PI (Deghelt, Mullier *et al.*, 2014). However, the number of growing bacteria characterized by partially labelled or non-labelled bacteria increased after 8 hours PI, *i.e* during proliferative phase of infection.

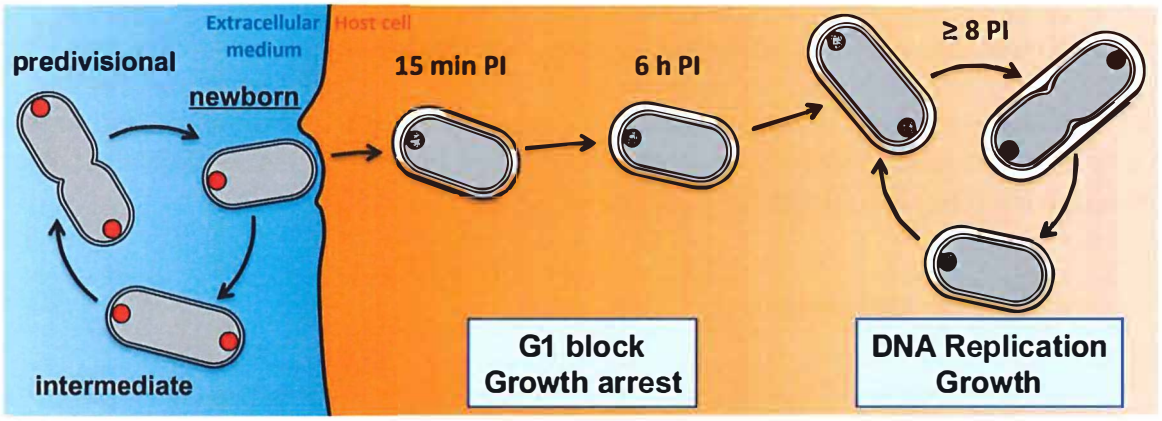

Figure 9: Characterization of *B. abortus* cell cycle in infection of HeLa cells. In *in vitro* culture, bacteria are able to enter in S phase and initiation of DNA replication can occur. Thus two red foci are shown, corresponding to the segregation protein ParB fused to mCherry fluorescent protein which bind replication origin of the chromosome, indicate that replication has been initiated. Bacteria found in early stage of infection (15 minutes to 6 h post-infection) are mainly blocked in G1 phase, which resulted in the presence of only one red focus per bacterium. At 6 to 8 hours post-infection, bacterial growth and replication start again (two red foci) and *B. abortus* are able to proliferate in its host cell. Modified from Deghelt, Mullier *et al.*, 2014

This suggests that bacterial growth is resumed between 6 and 8 hours post infection in HeLa cells. The growth was also analysed in RAW 264.7 macrophages showing a global arrest during first phase of infection (up to 4 hours p.i.) and shown to be resumed between 4 and 6 hours p.i. (Deghelt, Mullier *et al.*, 2014). These observations matched with previous investigation about *B. abortus* cell cycle in infection model, *i.e* consisting in a G1 block and growth arrest of bacteria in first hours post-infection.

It should be noted that bacterial cell cycle and growth are resumed when bacteria are still in LAMP-1 positive compartment, *i.e.* before reaching ER-like compartments, suggesting that cell cycle progression could already resume within endosomal compartment. Nevertheless, division starts at 4 to 6 hours post-infection according to the infected cell type, RAW 264.7 macrophages and HeLa cells respectively (Deghelt, Mullier *et al.* 2014).

Newborn selection

The fact that the majority of observed intracellular bacteria are in G1 phase at 15 minutes post-infection suggests that bacteria entering the host are already in G1 phase (Deghelt, Mullier *et al.*, 2014). In fact, 15 minutes is not enough time for *Brucella* to generate newborns out of another bacterial cell type. Therefore data presented above demonstrate that newborn bacteria are the predominant form of invasion in both professional and non-professional phagocytes (Figure 9). However the mechanisms underlying the preferential internalization of bacteria in G1 phase remain unclear and we are therefore interested in investigating this phenomenon. Since the bias directed toward newborn selection is observed at 15 minutes post-infection that is just after internalization, one plausible hypothesis is that the preferential internalization of bacteria in G1 phase depends on a preferential adhesion and/or invasion of these bacteria.

Host-pathogen interactions are closely related to adhesion and adhesion is often required for a successful infection establishment. Indeed attachment plays a key role in the virulence of intracellular bacteria since it allows pathogens to bind and adhere to host surface molecules before invading the cell. Some bacterial adhesion structures have been described in the literature, ranging from simple polysaccharides or proteins to macromolecular complexes such as pili machinery (Pizarro-Cerda and Cossart, 2006). In *Brucella,* it has been reported that adherence to extracellular matrix (ECM) components in addition to host cell receptors is a crucial step for invasion. Especially, adhesion mediated by sialic acid residues and fibronectin were identified as being implicated in interaction of brucellae with different host cells (Rocha-Gracia *et al.*, 2002; Castaneda-Roldan *et al.*, 2004).

Recently, a gene cluster conserved in all *Brucella* species has been identified. This locus showed a G+C content significantly reduced compared to other chromosomal regions indicating a putative horizontally genomic transfer (Czibener *et al.*, 2011). Horizontal gene transfer, which constitutes an important source of genetic variation, is generally associated with an improved adaptability to the environment. For pathogenic organisms, these adaptations could be characterized by increased virulence factors in order to promote invasion and survival in the host(s). Deletion of the entire gene cluster in *B. abortus* resulted in a decrease of bacterial attachment to professional and non-professional phagocytes *in vitro* as well as in mouse infection model where the mutant showed an attenuation when administered through oral route but not in peritoneal injection (Czibener *et al.*, 2011).

Therefore these results suggest an important role for this pathogenic island in the process of adherence. The pathogenicity island contained a gene coding for an adhesin named IlgA. This protein harbours an immunoglobulin-like domain also found in other bacterial adhesins such as the intimin from enterohemorragic *E. coli* which mediates bacterial adhesion to intestinal epithelial cells (Czibener *et al.*, 2011; Batchelor *et al.*, 2000). By gene deletion and overexpression, *ilgA* was shown to be implicated in the binding of *Brucella* to epithelial cells (Czibener and Ugalde, unpublished).

Particular surface proteins (SPs) of *Brucella* have been characterized to be associated with adhesion and invasion process of epithelial cells (Castaneda-Roldan *et al.*, 2006). Especially SP41 was shown to be implicated in bacteria binding to HeLa cells in a dose-dependent manner (Castaneda-Roldan *et al.*, 2006). The gene encoding SP41 known as *ugpB* showed homology with the periplasmic component of an ATP-binding cassette transport system also found in other bacteria. The use of an *ugpB* deletion strain or anti-SP41 antibodies in binding assay on HeLa cells leads to drastic reduction of *B. suis* adherence to the host. Adherence of SP41 to host cells appeared to be mediated by sialic acid residues since HeLa cells treated with neuraminidase prevents SP41 binding to epithelial cells (Castaneda-Roldan *et al.*, 2006).

Other proteins indirectly involved in the binding of *Brucella* to host cells have already been described in the literature. For example, BMEI0216 gene from *B. melitensis*, conserved in all *Brucella* species, codes for a hypothetical transglycosylase-associated protein and has been shown to participate to invasion process in epithelial cells but not in macrophages (Hernandez-Castro *et al.*, 2008). A deletion mutant showed a strong decrease in the internalization in HeLa cells suggesting that this gene is implicated in the *Brucella* internalization process (Hernandez-Castro *et al.*, 2008). Transglycosylation proteins are involved in peptidoglycan lysis and maintenance of cell wall integrity. Thus, loss of this integrity could lead to modification of several membrane components or envelope structures which could explain the decreased ability of the mutant to invade host cells (Hernandez-Castro *et al.*, 2008). Therefore it should be noted that rise of bacterial virulence could also result from indirect modifications of membrane components.

Recent investigations about adhesins from the autotransporters family

The autotransporters (AT) protein superfamily is widely spread in Gram-negative bacteria and is often associated with pathogenicity (Henderson and Nataro, 2001). These proteins are composed of three principal domains; a signal peptide, a N-terminal passenger domain and a C-terminal β-barrel domain. The β-barrel domain is anchored in the outer membrane of the (Gram negative) bacteria and allows the translocation of the passenger domain across the membrane that leads to presentation of this peptide at the surface (Henderson *et al.*, 1998). Generally, functions of autotransporters are dependent on the N-terminal passenger domain located in the extracellular environment (remaining bound or being released from the outer membrane of the bacteria), which could be decorated by different sugar motifs (Benz and Schmidt, 2001). Autotransporters have been associated with a variety of functions such as toxicity, aggregation, biofilm formation and especially adhesion.

For example a *Neisseria gonorrhoeae* autotransporter has been reported to release an IgA protease to alter the immune system of the host (Koomey *et al.*, 1982). Pertactin, an AT protein from *Bordetella pertussis* also used as a vaccine due to a high immunogenic feature, is well characterized as an adhesion protein since it binds to the lung epithelium (Diavatopoulos *et al.*, 2006).

In *Brucella suis*, adhesion functions for autotransporters have recently been reported. Indeed, three autotransporters have been characterized in this species, called BmaC (Posadas *et al.*, 2012), BtaE (Ruiz-Ranwez et al., 2013a) and BtaF (Ruiz-Ranwez *et al.*, 2013b). BmaC monomeric AT was first discovered to bind fibronectin, a glycoprotein present in the extracellular matrix and known to have a role in cell adherence. Following this, two other proteins BtaE and BtaF have been characterized as predicted trimeric AT using *in silico* analysis (Ruiz-Ranwez *et al.*, 2013a, b).

It has been shown that BtaE displays an adhesion function and especially binding to epithelial cells using as a ligand hyaluronic acid, a major component of the ECM (Ruiz-Ranwez *et al.*, 2013a). BtaE adhesion phenotype has been observed using a deletion mutant for *btaE* (*ΔbtaE*) displaying a decrease in its ability to bind to HeLa cells, A549 epithelial cells and hyaluronic acid *in vitro*. This deletion strain was also outcompeted by the wild-type strain in competition experiment to assess binding to host cells. These results have been confirmed *in vivo* using mouse infection model where BtaE have also shown to be required for full virulence in mice (Ruiz-Ranwez *et al.*, 2013a).

Similar to BtaE, BtaF was proposed to play a role in the adhesion to HeLa cells and A549 epithelial cells (Ruiz-Ranwez *et al.*, 2013b). Moreover, in addition to hyaluronic acid, *ΔbtaF* strain displayed a strong decrease in binding to collagen and fetuin (a sialic acid rich protein) compared to the wild-type *B. suis* strain (Ruiz-Ranwez *et al.*, 2013b). BtaF has also reported to be important in *in vivo* model of infection since it is also required for full virulence in mice (Ruiz-Ranwez *et al.*, 2013b).

BmaC was discovered using a phage display library of peptides from *B. suis* genomic DNA fragments, and selected with fibronectin (Posadas *et al.*, 2012). The fibronectin-binding domain isolated by this process was 113-amino-acids-long and predicted to belong to the passenger domain of BmaC. To test the function of BmaC, a predicted monomeric AT of 3420 amino acids, a *ΔbmaC* deletion mutant of *B. suis* has been constructed and tested in infection or binding assay. Results have shown a decrease of 45-fold in the binding of the mutant strain compared to the wild-type strain and the complemented strain showed a restauration of the WT phenotype (Posadas *et al.*, 2012). *ΔbmaC* strain was outcompeted by wild-type strain in a co-infection assay with HeLa cells and A549 cells. Moreover in presence of phage displaying fibronectin-binding peptide from BmaC, *B. suis* was outcompeted in the binding to HeLa cells and fibronectin. Finally bacteria attachment to HeLa cells was decreased in the presence of anti-fibronectin and anti-BmaC antibodies (Posadas *et al.*, 2012). Taken together these observations highlight the role of BmaC autotransporter in the initial process of invasion of *B. suis*.

Interestingly a specific feature was that in the reference strain *B. suis* 1330 BmaC, BtaE and BtaF all colocalized only at the new pole as AidB-YFP localization (Posadas *et al.*, 2012, Ruiz-Ranwez *et al.*, 2013). Although the percentage of labelled bacteria was very low, these observations could suggest that the new pole is differentiated as an adhesion pole in *Brucella suis*. Moreover, it has been reported that Brucella interact with host cell membrane through one pole, the same than BmaC was observed (Pizarro-Cerdá *et al.*, 2000, Ruiz-Ranwez *et al.*, 2013).

Exopolysaccharides-mediated adherence

It is not excluded that *Brucella* adhesion to host cells is mediated, or partly mediated, by exopolysaccharides exposed on the surface of the bacterium. Because of its highly virulent characteristics, lipopolysaccharide (LPS) of bacterial pathogens is well documented in literature so far. LPS constitutes the most abundant component of the outer membrane of Gram negative bacteria. *Brucella* smooth LPS (S-LPS) is constituted of three principal domains; the lipid A, the core oligosaccharide and the O polysaccharide chain also called O antigen due to its high immunogenic feature. In fact, lipid A layer represents the external leaflet of the outer membrane bilayer (Godfroid *et al.*, 1998). It is composed of multiple fatty acids linked to a phosphorylated glucosamine disaccharide. The core domain, directly linked to the lipid A, is mainly composed of oligosaccharides and is also bound to the O side chain. This later, only present in S-LPS, contained polymer of 4,6-dideoxy-4-formamido-α-D-mannopyranosyl in *Brucella* (Bundel *et al.*, 1987). Due to its external localization on the surface, the O chain is characterized as a highly immunogenic molecule but could also play an important role in host pathogen interactions. A perosamine synthetase gene deletion in *B. melitensis* leads to a "rough" mutant, named B3B2, where the O antigen is not produced. The rough mutant showed a strong attenuation in mouse model of infection but not in bovine macrophages (Godfroid *et al.*, 1998).

It has been shown that in several cases the entry of bacteria could be partially mediated by lipid rafts, a process depending of TLR4 and PI3-kinase (Guzman-Verri *et al.*, 2001). This mode of internalization seems to be important for the intracellular survival of certain *Brucella* species since a rough mutant which mediates its entry independently of lipid rafts fails to decrease the macrophage activation leading to more bacterial killing (von Bargen *et al.*, 2012). However structure modification of bacterial surface components, including LPS, could induce several pleiotropic effects turning out to be difficult to link a specific function with a phenotype observed (von Bargen *et al.*, 2012).

Exopolysaccharides have been reported to be essential for adhesion process and biofilm formation in several bacterial species. For example surface glucosaminoglycans have been reported to promote bacterial colonisation and virulence in *Bordetella bronchiseptica* and transmission of *Yersinia pestis* from flea to mammals (Hinnebusch *et al.*, 2008; Sloan *et al.*, 2007). In the α-proteobacterium *Caulobacter crescentus*, the holdfast, a polar adhesive polysaccharide composed in part of N-acetylglucosamine, is produced on the tip of the stalk (Bodenmiller *et al.*, 2003; Li *et al.*, 2005). This structure allows the bacteria to adhere and colonize diverse surfaces leading to resistance of various stresses such as fluid flow for example (Merker *et al.*, 1988). Recent studies on *Agrobacterium tumefaciens*, another α-proteobacterium, have highlighted a polar adhesive structure called UPP for "unipolar polysaccharide". This UPP is produced at one pole of the bacterial cell when it comes into contact with a surface (Xu *et al.*, 2013).

As in *Caulobacter crescentus* this phenomenon leads to a transition from a free swimming state to a stably adherent state. Holdfast and UPP were shown to be abundantly stained with the specific lectin WGA (Wheat Germ Agglutinin) which specifically recognizes β1-4 linked N-acetyl-D-glucosamines and sialic acid residues (Xu *et al.*, 2013).

It has been reported that in many bacteria transition from motile stage to adherent stage could be regulated by the intracellular signalling molecule c-di-GMP (cyclic diguanosine monophosphate) functioning as a second messenger in bacteria (Ryan *et al.*, 2006).

Recently, the cellular pool of c-di-GMP has been shown to control flagellar motility and adherent polysaccharide synthesis especially by transcription regulation or allosteric modifications of several target processes (Hengge, 2009). Intracellular levels of c-di-GMP are controlled by diguanylate cyclases (DGCs) and phosphodiesterases (PDEs). The c-di-GMP is produced from two GTP by DCGs containing a conserved GGDEF domain essential for enzymatic activity. The degradation of c-di-GMP is mediated by specific PDEs, containing a conserved EAL or HD-GYP domain, releasing two molecules of GMP (Hengge, 2009).

Holdfast from *C. crescentus* and UPP from *A. tumefaciens* have been described to be controlled by c-di-GMP level. In *Caulobacter* DGC proteins are localized close to the stalked pole leading to local c-di-GMP increase in concentration. This increase seems to promote stalk morphogenesis and entry into the replication DNA S phase (Abel *et al.*, 2011). It has been observed that an increase of c-di-GMP in *A. tumefaciens* leads to production of UPP independently of surface contact and also promotes synthesis of the exopolysaccharide cellulose (Xu *et al.*, 2013). Moreover using transpositional mutagenesis, regulators have been detected to inhibit UPP and cellulose production, null mutation of these genes leading to a hyperadherent phenotype. Among these negative regulators, genes involved in flagellar motility have been detected. One of them, *visR*, has been found to inhibit three diguanylate cyclases (Xu *et al.*, 2013). These results confirm that motile-to-sessile transition could be regulated by the pool of c-di-GMP which can be modified in turn by different effectors.

Objectives

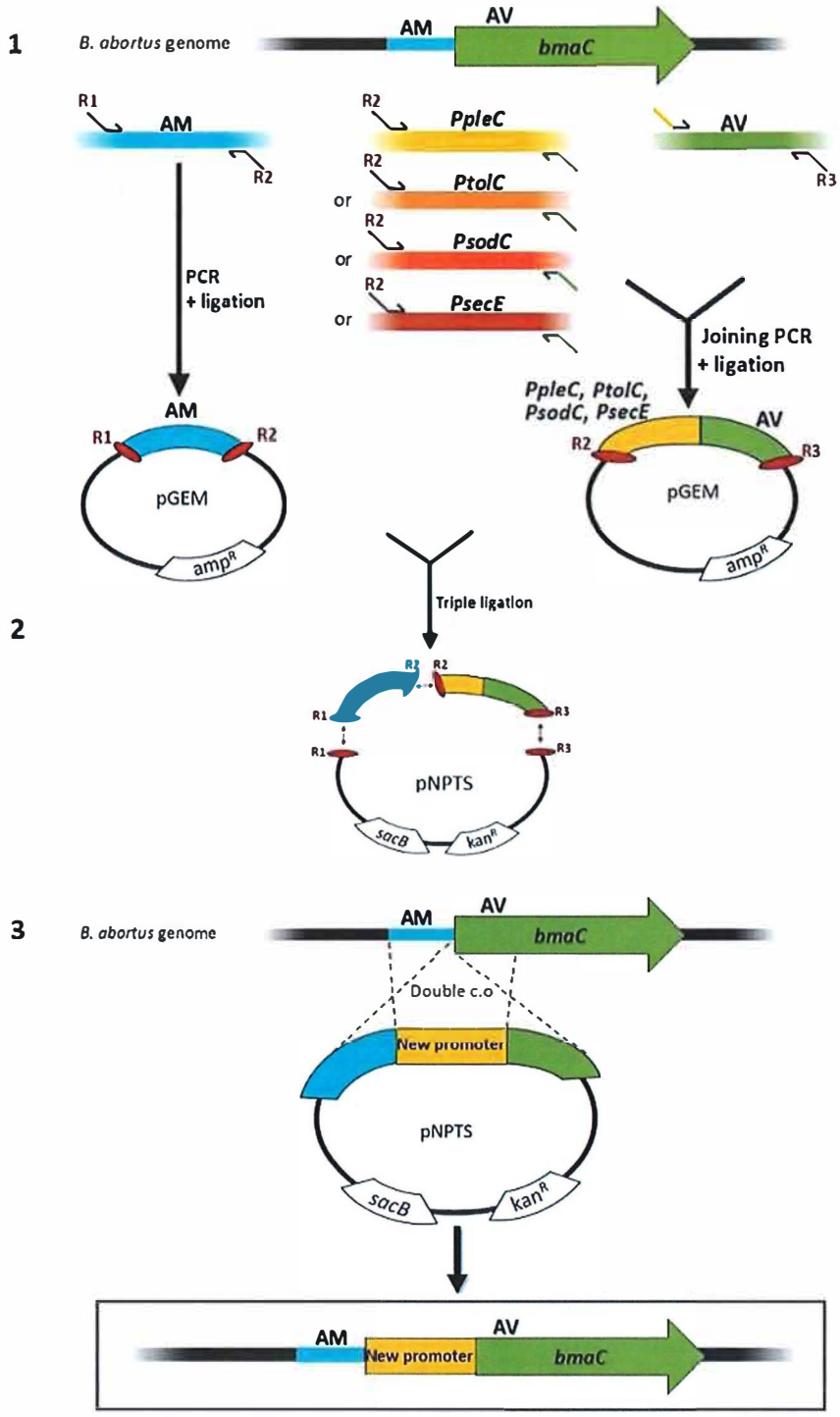

Figure 10. Molecular strategy to construct *bmaC* overexpression strain (*bmaC*$^{++}$). 1. The region upstream of the *bmaC* coding sequence (AM) is amplified by PCR. Four endogenous promoters (*PpleC*, *PtolC*, *PsodC* and *PsecE*) of *B. abortus* are also amplified. The region corresponding to the beginning of the *bmaC* coding sequence (AV) is also amplified. The reverse primer corresponding to the promoter amplification contains a part of the AV sequence (green) and vice-versa forward primer corresponding to the AV amplification contains a part of the end sequence of the promoter amplified (yellow). Thus, the ends of these two PCR products are complementary and allow to perform a joining PCR to ligate these two fragments. Note that restriction sites (R1, R2 and R3) are included in primers that permits to restrict AM and Promoter+AV fragments and to ligate them in the correct orientation into a pNPTS plasmid. Once the recombinant pNPTS is obtained, it is conjugated in *B. abortus* and used in an allelic replacement strategy.

Objectives

Investigation on BmaC

As mentioned in the introduction, newborn bacteria are the predominantly infectious bacterial cell types in both phagocytic and non-phagocytic host cells. However, the molecular mechanism involved in this preferential internalization of newborn bacteria is not understood. One plausible hypothesis could be that this increase in internalization of newborn bacteria depends on the preferential adhesion of these bacteria to host cells. In fact we hypothesized that adhesion molecules would be present and/or active mostly on newborns cells, thus enabling these bacteria to be internalized preferentially. Therefore, we decided to investigate BmaC and its potential role in newborn adherence to host cells in *B. abortus* due to its strong deletion phenotype in adherence reported in *B. suis* (Posadas *et al.*, 2012).

To test the BmaC function in adhesion, we want on the one hand to delete *bmaC* gene from the *B. abortus* 2308 strain and on the other hand to overproduce BmaC in order to observe variations regarding adherence and internalization of the bacteria. Indeed, BmaC overproduction could lead to the internalization of all cell types of the cell cycle, and not only newborns. This would be an interesting tool to compare the intracellular survival and trafficking of newborn bacteria compared to the other cell types. Considering the fact that the sequence encoding BmaC is 10,269 nucleotides long, cloning it in an overexpression vector is likely to be difficult to perform. For this reason we decided to attempt *bmaC* overexpression by adding a new promoter in the upstream region of the *bmaC* coding sequence. To the best of our knowledge, this "promoter replacement method" has never been used in *B. abortus.* We based our choice for candidate promoters on previous microarray data obtained in *Brucella melitensis* 16M grown until the late exponential phase in rich (2YT) medium (Rossetti *et al.*, 2009). For this ***a priori*** strategy, we chose four endogenous promoters, the *pleC* (P*pleC*), *tolC* (P*tolC*), *sodC* (P*sodC*), and *secE* (P*secE*) promoters. Both P*sodC* and P*secE* are strong promoters, and P*secE* (also called P*sojA*) has already been used to constitutively produce GFP or mCherry in *B. abortus* 2308 (Köhler et al., 1999). P*pleC* and P*tolC* have an intermediary level of transcripts according to microarray data. PleC is a histidine kinase suspected to function as a phosphatase on its cognate response regulator DivK (Matroule *et al.*, 2004; Hallez *et al.*, 2007), conserved in α-proteobacteria and putatively involved in cell cycle regulation. TolC is an outer membrane protein which is implicated in the transport accross the enveloppe of Gram-negative bacteria, and more particularly serves as a multidrug efflux pump to expel unwanted molecules from the cytoplasm (Posadas *et al.*, 2007). SodC is a superoxide dismutase which plays a role in oxidative stress resistance (Gee *et al.*, 2005). Finally, SecE is a member of a translocase complex (SecYEG) localized in the inner membrane of the bacteria and is probably involved in the formation of an inner membrane channel for protein export in the periplasm. The molecular strategy used to add a new strong promoter upstream the *bmaC* coding sequence is detailed in Figure 10.

Potential adhesion polysaccharides detection

To study surface exposed polysaccharides which are poorly documented in *B. abortus* we decided to use several lectins conjugates recognizing specific sugar motifs as staining tools.

Lectin name	Lectin symbol	Ligand motif
Wheat Germ Agglutinin	WGA	(β-1,4)GlcNAc, Sia
Maackia amurensis leukoagglutinin	MAL I	Sia(α2-3)Gal(β1-4)GlcNAc
Galanthus nivalis lectin	GNL	(α-1,3)Man
Aleuria aurantia lectin	AAL	Fuc(α1-2)Gal(β1-4)Gal(β1-4)GlcNAc
		R2-GlcNAc(β1-4)(Fucα1-6)GlcNAc-R1
Sambucus nigra lectin	SNA	Sia(α2-6)Gal(NAc)-R
Datura stramonium lectin	DSL	(β-1,4)GlcNAc oligomer

Table 1: Describing of lectins used for *B. abortus* staining and their ligand motif binding.

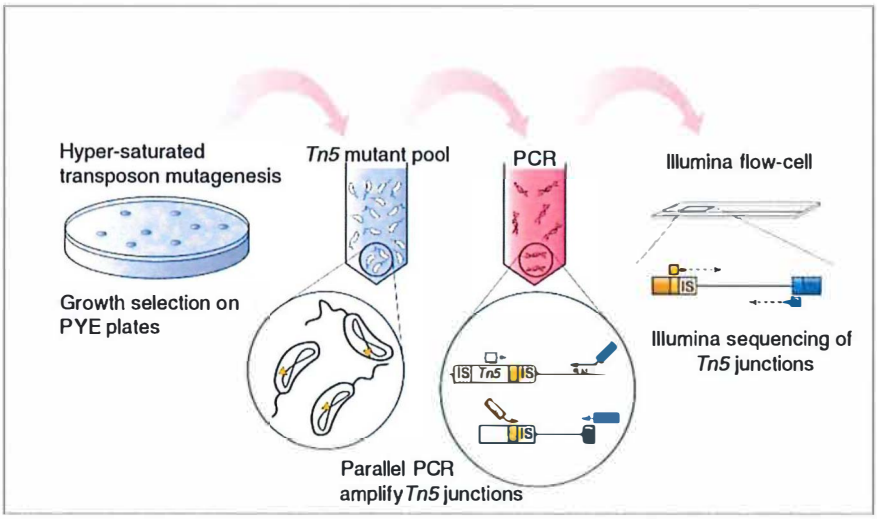

Figure 11: Large scale approach to determine essential genes of *Caulobacter crescentus*. Transposon mutagenesis was first performed on *C. crescentus* using random insertion of the Tn*5* transposon. Viable mutants able to grow on PYE plate were harvested and transposon junctions DNA were amplified by PCR using terminal adapters compatible to Illumina sequencing. (From Christen *et al.*, 2011).

To detect polysaccharides putatively involved in adherence of *B. abortus* to host cells we have used wheat germ agglutinin (WGA) conjugated with Alexa Fluor 488, as a fluorescent lectin conjugate. WGA recognizes β-1,4 linked N-acetylglucosamine, known to be present in the holdfast of *C. crescentus* and UPP of *A. tumefaciens* (as mentioned in the introduction), as well as sialic acid residues. We also tested other lectins, to increase range of polysaccharidic structures detection, such as *Maackia amurensis* leukoagglutinin (MAL I) recognizing sialic acid linked to galactose residue linked to N-acetylglucosamine, *Galanthus nivalis* lectin (GNL) which binds preferentially mannose residues. *Aleuria aurantia* lectin (AAL) was also tested, allowing detection of fucose residues linked to N-acetylglucosamine, *Sambucus nigra* lectin (SNA) recognizing sialic acid residues linked to galactose and *Datura stramonium* lectin (DSL) binding to N-acetylglucosamine oligomers. All lectins specific liaisons are described in more detail in Table 1.

Without *a priori* approach

In order to identify genomic region potentially involved in adhesion and/or invasion of *B. abortus* in host cells, we have also used a large-scale method to screen a high number of transposition mutants simultaneously.

Recently, the essential part of *Caulobacter* genome (meaning all genes required for colony formation on standard PYE culture plates) has been identified using transposon mutagenesis combined with high-throughput sequencing (Christen *et al.*, 2011). First, a large library of 8.10^5 random transposition mutants was created, statistically resulting in a single transposon insertion every 8 bp. Next, for all mutants that were able to grow on plates, the genomic region adjacent to the transposon insertion site was amplified by arbitrary PCR and sequenced by the Illumina technology (Figure 11). This screen resulted in a map representing all regions of the genome of *C. crescentus* where transposon insertion was tolerated for the colony forming ability of the bacterium, meaning that all gaps between these tolerant regions are composed of essential genomic regions. Thereby coding and non-coding sequences, regulatory sequences and intergenic regions were discovered to be a part of the essential genome of *Caulobacter crescentus* (Christen *et al.*, 2011). We decided to conduct similar experiments in order determine DNA elements essential for growth and for infection by *B. abortus*.

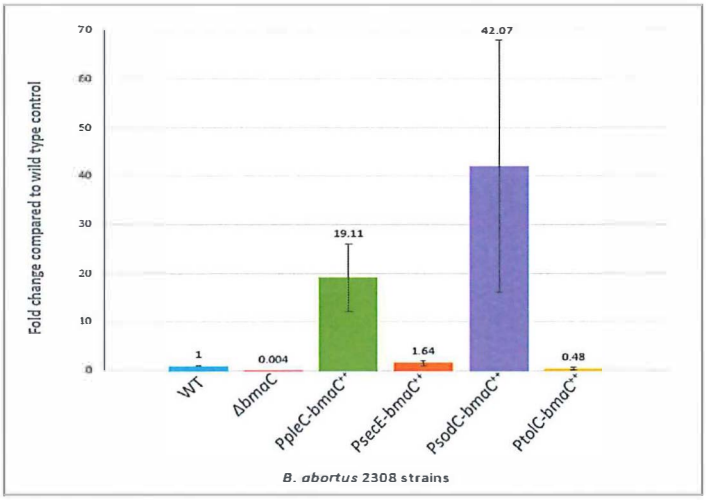

Figure 12: The *bmaC* transcription level in deletion and overexpression strains evaluated by qRT-PCR. WT, *ΔbmaC*, *PpleC-bmaC*$^{++}$, *PsecE-bmaC*$^{++}$, *PsodC-bmaC*$^{++}$ *and PtolC-bmaC*$^{++}$ bacterial strains were grown in rich medium (2YT) up to reach an OD comprised between 0.4 and 0.8. The *bmaC* transcription level was set to 1 by default for the WT strain. The housekeeping gene used to normalize mRNA quantity was 16S ribosomal RNA. Negative controls were performed for each strains and consisted in preparation that were not retrotranscribed into cDNA.

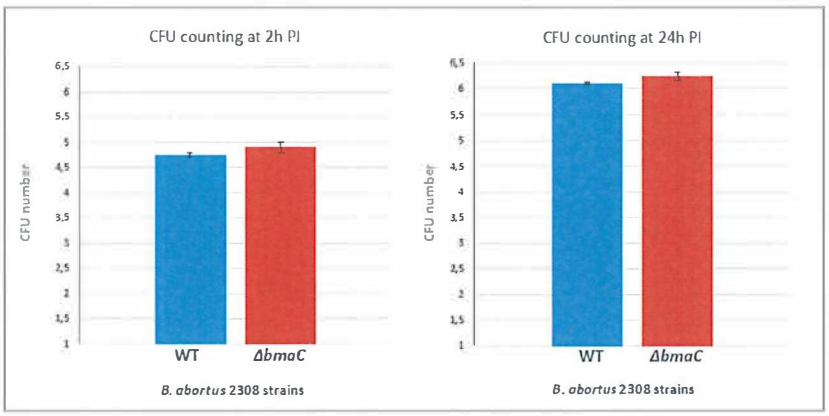

Figure 13: Comparison of WT and *ΔbmaC* strains in RAW 264.7 infection assay at 2 hours and 24 hours PI. RAW 264.7 macrophages were infected with WT or Δ*bmaC* strain, with a MOI of 50. Internalized bacteria were harvested at 2 hours and 24 hours post-infection, plated on rich medium and CFUs were counted (expressed in $\log_{10}$) some days later.

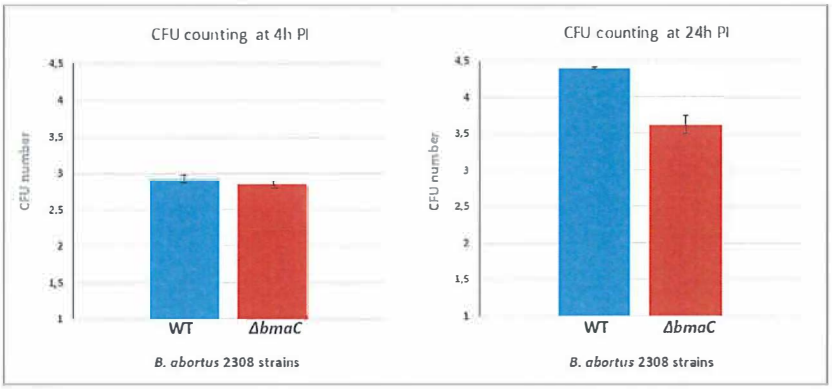

Figure 14: Comparison of WT and *ΔbmaC* strains in HeLa cells infection assay at 4 hours and 24 hours PI. Similarly to the experience reported in Fig. 4, HeLa cells were infected with WT or *ΔbmaC* deletion strain, with a MOI of 300. Internalized bacteria were harvested at 4 hours and 24 hours post-infection and CFUs were counted (expressed in $\log_{10}$).

Results

Results

Investigation of BmaC protein

First, a *B. abortus bmaC* deletion mutant named *ΔbmaC* was generated by allelic replacement in the *B. abortus* 2308 in order to investigate the role of *bmaC* in this strain.

To obtain *bmaC* overexpression strains, we have constructed allelic replacement plasmids in which four endogenous promoters have been separately fused in between the *bmaC* promoter and the beginning of BmaC coding sequence while keeping the ribosome binding site (RBS) predicted for this coding sequence (Figure 10). These vectors were then mated in *Brucella* and allelic replacement was performed, thus leading to the integration of a new promoter upstream of *bmaC* (see experimental procedure for allelic replacement). All four constructions have been obtained in *Brucella abortus* resulting in four *bmaC* overexpression strains corresponding to the four promoters, and named *PpleC-bmaC*$^{++}$, *PsecE-bmaC*$^{++}$, *PsodC-bmaC*$^{++}$ *and PtolC-bmaC*$^{++}$overexpression strain.

Transcription level determination

We have first compared the transcription level of *bmaC* in the four overexpression mutants with the wild type (WT) strain. In order to evaluate this mRNA level a qRT-PCR was performed including the *ΔbmaC* mutant strain, as a negative control due to the absence of *bmaC* transcript. Transcription level was evaluated in culture condition (2YT, rich medium, in mid to late exponential phase) with an optical density at 600 nm (OD) between 0.4 and 0.8. Results obtained are presented in Figure 12. As expected *ΔbmaC* showed no *bmaC* transcript expression. *PpleC-bmaC*$^{++}$ and *PsodC-bmaC*$^{++}$ revealed the strongest *bmaC* (and more variable) expression level with respectively an average of 19 and 42 times more transcripts than the WT strain. The transcription level for *PsecE-bmaC*$^{++}$ and *PtolC-bmaC*$^{++}$ was respectively 1.6 and 0.5 times compared to WT strain.

Infection assay with *bmaC* deletion strain

The ability of the *ΔbmaC* strain to adhere and invade host cells was first evaluated in infection assay on RAW 264.7 macrophages. These professional phagocytic cells were infected with both WT and *ΔbmaC* bacteria with a multiplicity of infection (MOI, average number of bacteria per host cell) of 50. Bacteria were harvested at either 2 hours or 24 hours PI and colony forming units (CFUs) were counted (Figure 13). No significant difference in CFU number was observed at 2 hours PI between the WT and the *ΔbmaC* strains. In the same way, CFU counting at 24 hours PI revealed no significant differences between both strains.

We also tested the effect of *bmaC* deletion on *B. abortus* infection of non-professional phagocytic cells (HeLa cells) which in fact constitute another cellular infection model for *B. abortus*. HeLa cells were infected with WT or *ΔbmaC* strain with a MOI of 300. Bacteria were harvested and CFUs were counted at 4 hours and 24 hours PI (Figure 14). As in RAW 264.7 macrophages infection, no significant decrease was observed for *ΔbmaC* strain compared to WT strain at 4 hours PI. However a highly significant ($p < 0.01$) decrease of CFUs number was observed at 24 hours PI according to the Student t test.

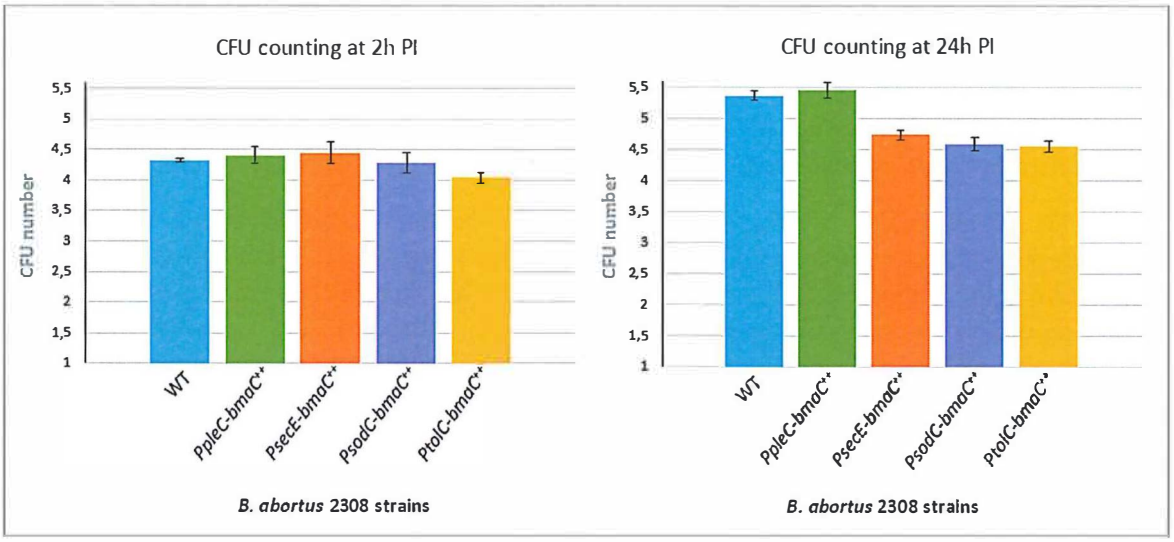

Figure 15: Comparison between WT and *bmaC* overexpression strains in RAW 264.7 infection assay at 2 hours and 24 hours PI. Macrophages were infected by WT, *PpleC-bmaC*$^{++}$, *PsecE-bmaC*$^{++}$, *PsodC-bmaC*$^{++}$ and *PtolC-bmaC*$^{++}$ bacterial strains with a MOI of 50. Internalized bacteria were harvested at 2 hours and 24 hours post-infection and CFUs were counted (expressed in $\log_{10}$).

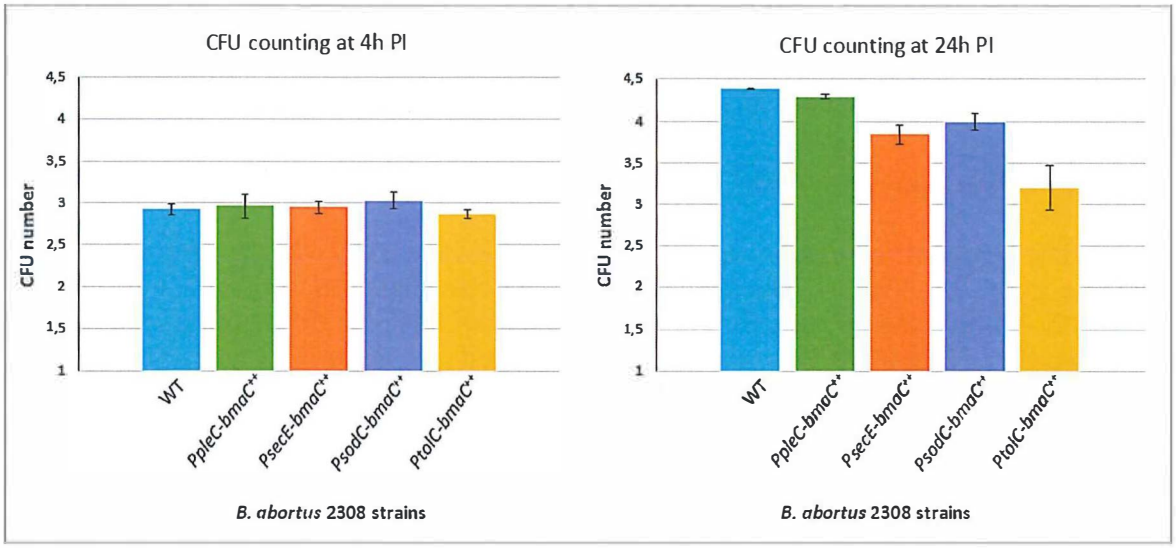

Figure 16: Comparison between WT and *bmaC* overexpression strains in HeLa cells infection assay at 4 hours and 24 hours PI. HeLa cells were infected by WT, *PpleC-bmaC*$^{++}$, *PsecE-bmaC*$^{++}$, *PsodC-bmaC*$^{++}$ *and PtolC-bmaC*$^{++}$ bacterial strains with a MOI of 300. Internalized bacteria were harvested at 4 hours and 24 hours post-infection and CFUs were counted (expressed in $\log_{10}$).

These results suggest that *bmaC* deletion strain was not compromised in the binding and/or internalization in host cells but can influence bacterial intracellular trafficking or growth given that *Brucella* has already reached its proliferative niche at 24 hours PI.

Infection assay with *bmaC* overexpression strains

The previous results obtained concerning the ability of *ΔbmaC* to be internalized as well as the WT strain suggested that BmaC protein is not involved in this process. However other adhesins (or adherence components) could be implicated in adhesion process offsetting *bmaC* deletion. Nevertheless, we were interested in overproducing BmaC in order to detect possible variations regarding adherence and internalization into host cells.

To test the ability of *bmaC* overexpression strains to be more internalized than the WT strain, we performed infection on RAW 264.7 macrophages. Host cells were infected by *PpleC-bmaC*$^{++}$, *PsecE-bmaC*$^{++}$, *PsodC-bmaC*$^{++}$, *PtolC-bmaC*$^{++}$ or WT strains with a MOI of 50 and bacteria were harvested at 2 hours and 24 hours PI (Figure 15). The number of CFUs obtained at 2 hours PI was not significantly different between WT strain and the different *bmaC* overexpression strains. We also observed no significant increase in bacteria number at 24 hours PI for strains overexpressing *bmaC*. However, *PsecE-bmaC*$^{++}$, *PsodC-bmaC*$^{++}$ and *PtolC-bmaC*$^{++}$ strains presented a significant decrease in CFU number at 24 hours PI.

The same experiment was repeated in HeLa cells with a MOI of 300 and CFUs were counted after 4 hours and 24 hours of infection (Figure 16). No significant difference between WT and *bmaC* overexpressing strains was shown at 4 hours PI but we observed again a decrease for *PsecE-bmaC*$^{++}$, *PsodC-bmaC*$^{++}$ and *PtolC-bmaC*$^{++}$ at 24 hours PI as previously observed in RAW 264.7 macrophages infection.

Growth test of *bmaC* deletion and overexpression strains

The decrease of bacterial number at 24 hours post-infection for *PsecE-bmaC*$^{++}$, *PsodC-bmaC*$^{++}$ and *PtolC-bmaC*$^{++}$ strains suggested an affected intracellular trafficking or survival, but could also be due to a general growth defect. To investigate this possibility we performed a bioscreen (automated culture system recording OD along time of culture) in order to characterize bacterial growth of the different strains. We analysed the growth of *B. abortus* WT, *ΔbmaC, PpleC-bmaC*$^{++}$, *PsecE-bmaC*$^{++}$, *PsodC-bmaC*$^{++}$ *and PtolC-bmaC*$^{++}$ strains in rich medium (2YT) and obtained growth curves for each strain (Figure 17). Growth curves revealed a general growth delay for *PsecE-bmaC*$^{++}$, *PsodC-bmaC*$^{++}$ *and PtolC-bmaC*$^{++}$ strains.compared to the WT. Therefore the most likely explanation for the decrease in the number of CFU at 24 hours PI for these three strains is that bacteria present a general growth defect also detectable in culture.

Binding assay on fibronectin

As it has been previously shown that BmaC from *B. suis* is able to bind fibronectin we decided to test if *B. abortus ΔbmaC* as well as *bmaC* overexpression strains were also modified in their binding to this extracellular matrix component. We thus performed a binding assay with *B. abortus* WT, *ΔbmaC,* and *PpleC-bmaC*$^{++}$ against fibronectin, since the three other overexpression strains seem to present growth problems. Bacteria were first incubated during 2 hours with fibronectin. Bacteria not bound to fibronectin were removed by washing.

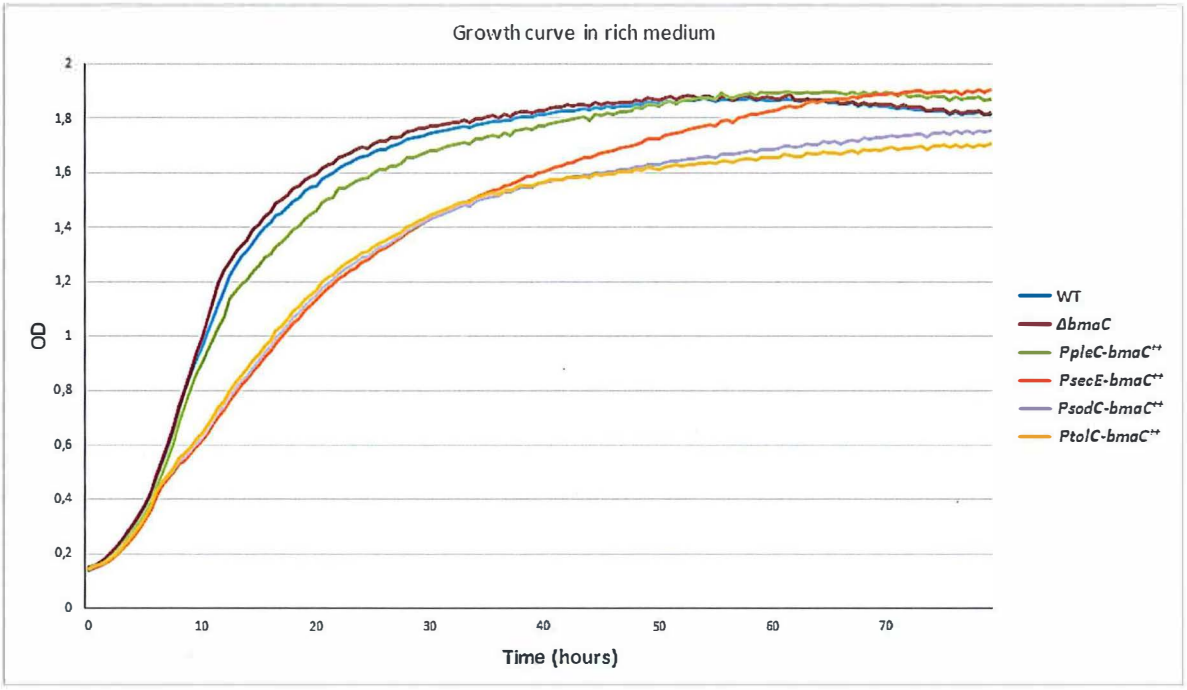

Figure 17: Growth curves of WT, *ΔbmaC*, *PpleC-bmaC*$^{++}$, *PsecE-bmaC*$^{++}$, *PsodC-bmaC*$^{++}$ *and PtolC-bmaC*$^{++}$ bacterial strains. The different bacterial strains were grown in rich medium (2YT) overnight. Cultures were diluted at OD of 0.1 and OD was measured every 30 minutes.

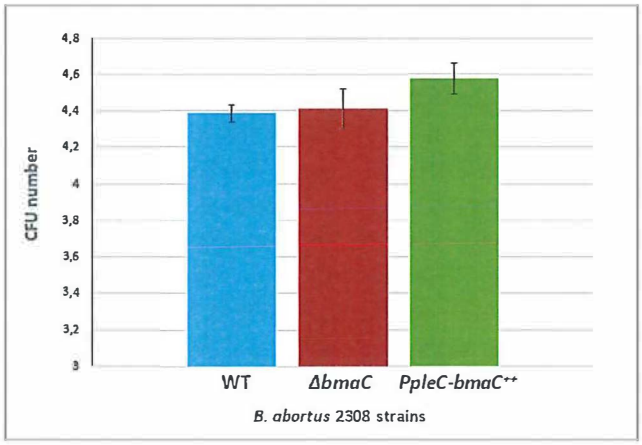

Figure 18: Adherence of *ΔbmaC* and *PpleC-bmaC*$^{++}$ strains tested by binding assay on fibronectin. The different bacterial strains were grown in rich medium (2YT) overnight. The OD was measured and dilutions were performed to normalize the number of bacteria for each strain. Bound bacteria were harvested by adding Tryspin-EDTA solution and serial dilutions were performed before CFU counting.

Bacteria able to bind fibronectin were harvested by addition of Trypsin-EDTA. CFUs were then counted for the different strains (Figure 18). Controls were performed to confirm viability of bacteria when they are put in contact to both Trypsin and EDTA. The *ΔbmaC* strain showed no difference in its ability to bind fibronectin compared to the WT strain. However a slight but yet significant ($p < 0.05$) increase was observed for the strain overexpressing *bmaC* with the *PpleC* promoter, of according to a Student t test.

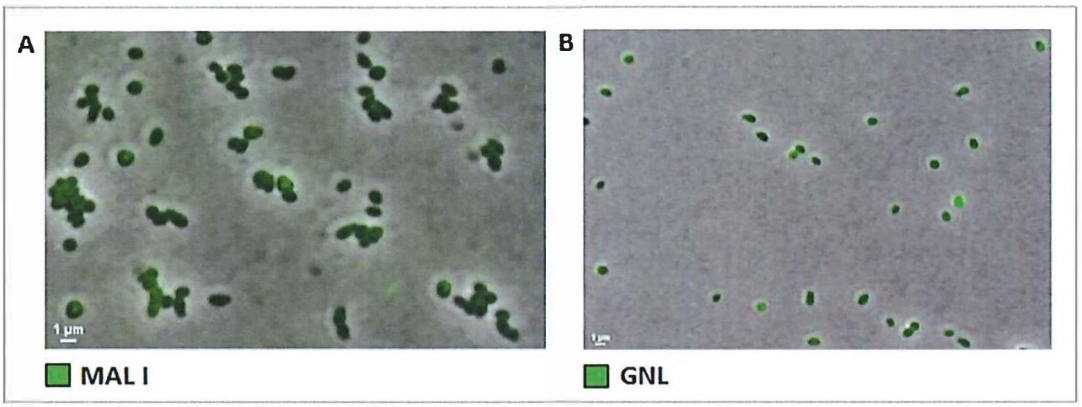

Figure 19: Staining of *B. abortus* 2308 with MAL I (A) or GNL (B). For both labelling bacteria were cultivated in 2YT medium and labelled with MAL I-Alexa Fluor 488 or GNL-Alexa Fluor 488 in stationary phase. Bacteria were observed by fluorescence microscopy on PBS-agarose pad. Scale bars represent 1 µm.

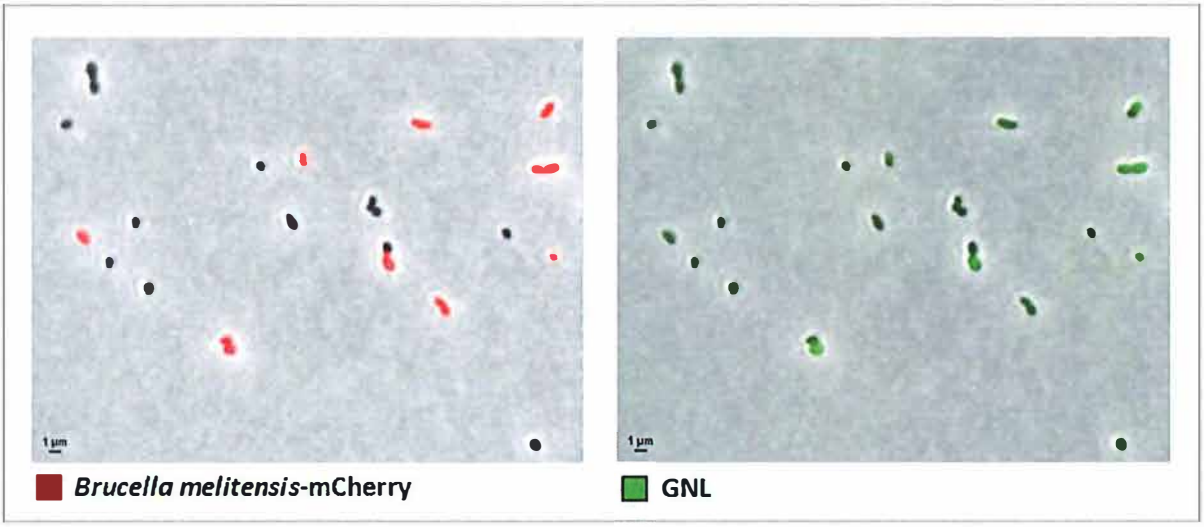

Figure 20: *Brucella melitensis* 16M mCherry and *Brucella abortus* 2308 stained with GNL. *B. melitensis* mCherry (red signal) and *B. abortus* (unlabelled) were mixed together and stained with GNL. Bacteria were observed with fluorescence microscopy on PBS-agarose pad. Scale bars represent 1 µm.

Surface-exposed exopolysaccharides investigation

Two other α-proteobacteria, *C. crescentus* and *A. tumefaciens*, were shown to produce polar and surface-exposed polysaccharides mediating adhesion (see introduction). Several bacterial pathogens are known to adhere to their host cells through mechanisms involving carbohydrate-peptide interactions. We thus investigate the possibility that *B. abortus* could produce surface-exposed polysaccharides other than lipopolysaccharide (LPS), maybe polar. We explored this possibility by incubating *B. abortus* with different lectins labelled with fluorescent molecule (Alexa 488) to detect potential polysaccharides on the surface of the bacteria, and investigated them localizations.

We tested *Aleuria aurantia* lectin (AAL, recognizing fucose residues linked to N-acetylglucosamine), *Sambucus nigra* lectin (SNA, also named Elderberry lectin, binding to sialic acid residues linked to galactose), *Maackia amurensis* leukoagglutinin (MAL I, recognizing N-acetylglucosamine linked to galactose and could also tolerates sialic acid at the third position on galactose), *Galanthus nivalis* lectin (GNL, recognizing mannose, binding being preferentially directed toward mannose residues), *Datura Stramonium* lectin (DSL, binding to *N*-acetylglucosamine oligomers) and the Wheat Germ Agglutinin (WGA recognizing N-acetylglucosamine and sialic residues) (specific liaisons detailed in Table 1 in objectives part).

B. abortus is labelled with different lectins

We first performed a staining of *B. abortus* grown in rich liquid medium until stationary phase with each lectin conjugate described above and observed the presence or absence of labelling by fluorescence microscopy. Staining assay with AAL, SNA and DSL revealed no bacterial labelling above the background (data not shown). MAL I and GNL bacterial staining showed a fluorescent signal distributed throughout the bacteria surface indicating the presence of N-acetylglucosamine-galactose and mannose residues (Figure 19 A, B). It should be noted that the lipopolysaccharide (LPS) composition of *Brucella* varies with a species-specific manner. In fact, the LPS of *B. melitensis* is constituted by a higher amount of α-1,3 linked N-formylperosamine, derived from mannose, compared to the LPS of *B. abortus*. Because GNL recognizes mannose residues we supposed that it recognizes LPS containing mannose derivatives. In order to investigate the nature of the structure detected with GNL we simultaneously performed a staining on *B. abortus* and *B. melitensis* with this lectin (Figure 20). We observed a stronger GNL signal for *B. melitensis* than for *B. abortus*. These first data suggest that GNL could also recognize N-formyl perosamine, and not only mannose.

A WGA polar signal is detected in a small part of the population

Interestingly, when labelled with WGA after being grown in 2YT until stationary phase, a part of the bacterial population (15.6 %) clearly displayed a fluorescent signal. Moreover, in a high proportion of this subpopulation, bacteria displayed a unipolar fluorescent signal (Figure 21 A). To test if this labelling was dependent of the physiological state of bacteria, we examined bacteria grown until the exponential phase. Surprisingly, a very small number of bacteria were then labelled with WGA (Figure 21 B). Given that WGA recognizes and binds N-acetylglucosamine which is a major component of the bacterial peptidoglycan we cannot exclude that WGA binds to the peptidoglycan in damaged bacteria which would appear in the stationary phase of culture.

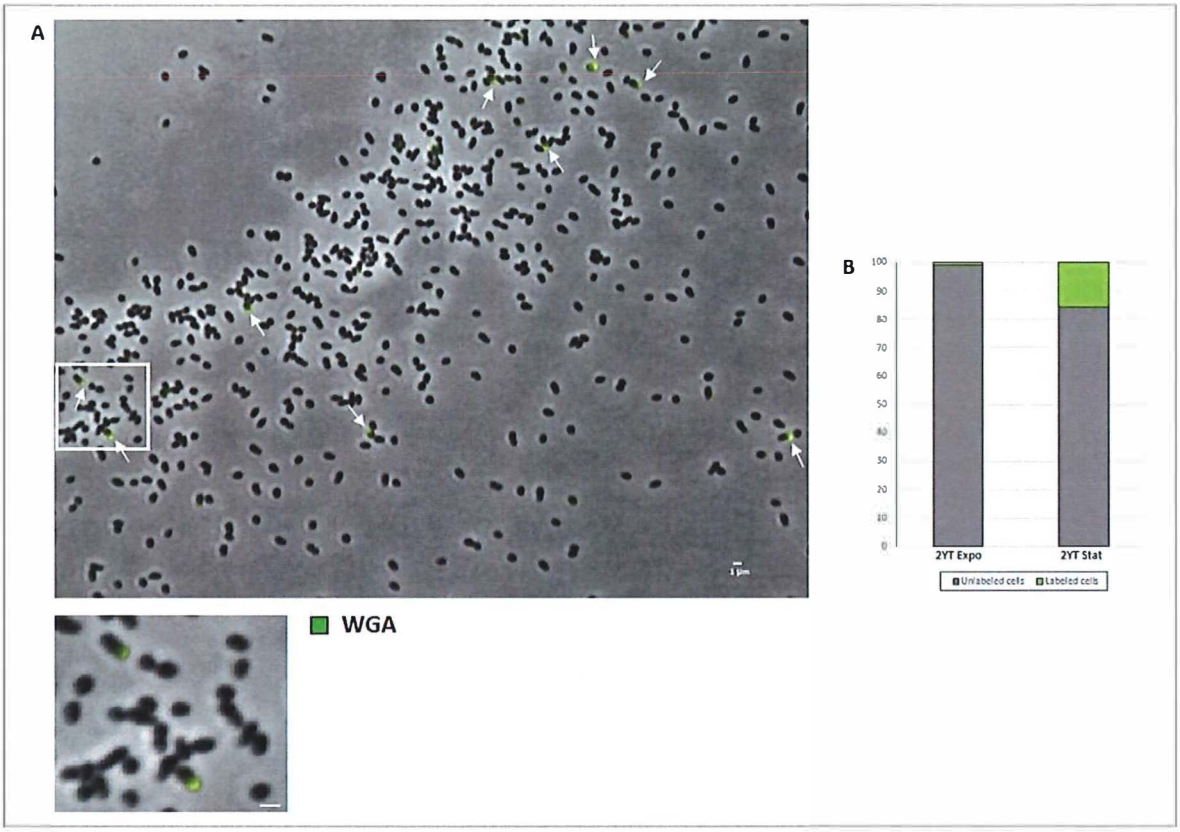

Figure 21: ***Brucella abortus*** **2308 labelling with WGA. (A)** Bacteria were cultivated in 2YT medium until stationary phase and labelled with WGA-Alexa Fluor 488. Scale bars represent 1 µm. **(B)** Proportion of bacteria labelled with WGA (n=558) in green, depending on the conditions, *i.e.* culture phase (exponential or stationary).

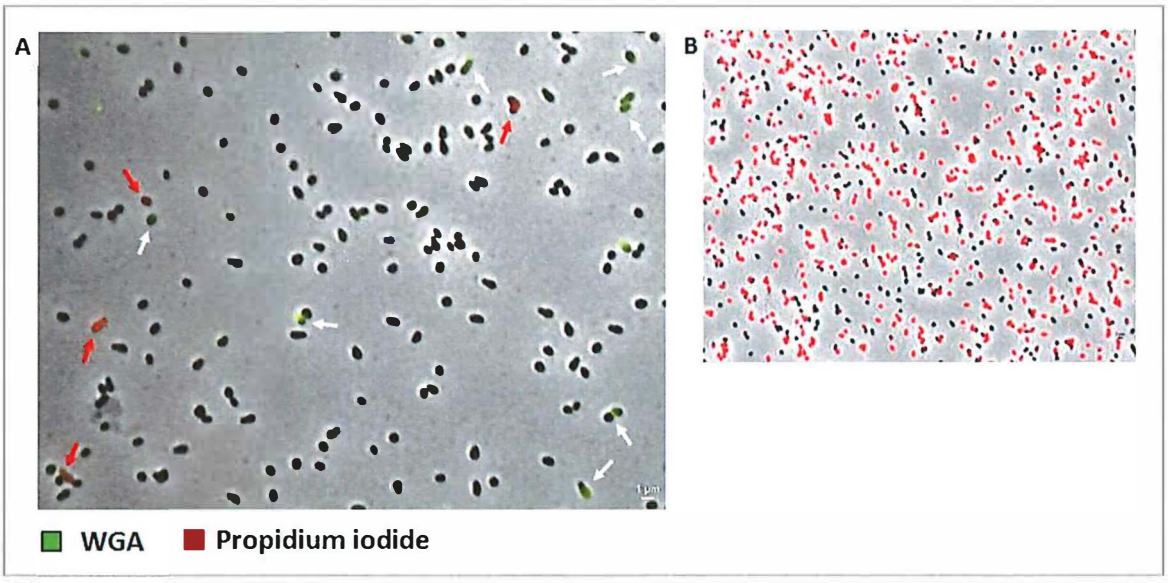

Figure 22: Live dead test on ***B. abortus*** **labelled with WGA. (A)** Bacteria were first labelled with WGA and then incubated during 15 minutes with propidium iodide. Bacteria were observed with fluorescence microscopy. Red arrow = bacteria positives for propidium iodide staining indicating bacterial death. White arrow = bacteria stained with WGA. **(B)** Positive control for the live dead test. Bacteria were resuspended and incubated in ethanol 70 % during 10 minutes at room temperature. Staining with propidium iodide was then performed during 15 minutes and bacteria were observed by fluorescence microscopy. Scale bars represent 1 µm.

Thus, a "live dead" viability assay, consisting in testing the membrane integrity using specific fluorescent dye (propidium iodide) able to enter only in permeabilized cells, recorded here as dead bacteria, has been performed to ensure that bacteria labelled with WGA are not dead and subsequently that WGA did not label peptidoglycan instead of a surface exposed polysaccharidic structures (Figure 22 A). As a positive control for the live dead test, bacteria were treated with ethanol and then labelled with propidium iodide, suggesting that this compound is indeed able to label dead bacteria (Figure 22 B).

To test if a component of the rich medium (2YT, an undefined and rich culture medium) was implicated in this staining we labelled bacteria grown in the defined Plommet medium supplemented by erythritol as a carbon source. In this condition, no fluorescent signal was detected in either exponential or stationary phase (data not shown). Absence of labelling on bacteria grown in this defined medium suggests that the signal detected in 2YT could be due to one or more component from the rich media. One alternative hypothesis could be that such a structure is not synthesized in this defined medium. For example the presence of erythritol could indeed repress the production of a polar exopolysaccharide in the Plommet medium since it has been reported that this molecule presents signalling functions in *B. abortus* (Rodriguez *et al.*, 2012). Therefore to test this later hypothesis we performed a WGA staining on bacteria grown in Plommet supplemented by xylose (another carbon source instead of erythritol) and no labelling was observed (data not shown). In the same way to confirm that erythritol does not interfere with WGA positive labelling we repeated the staining on bacteria grown in rich medium supplemented by erythritol. Bacteria still presented fluorescent signal indicating that erythritol has no effect on WGA labelling (data not shown).

The fact that only a small part of the population was stained with WGA led us to investigate if the staining was biased toward bacteria in a specific cell cycle phase. We also addressed whether the polar signal was randomly distributed between the old and the new bacterial pole or if it is specific for a given pole. To test this, we have taken advantage of the mCherry-ParB fusion highlighting *oriI* and thus informing us about the cell cycle phase of the bacterium as well as the location of the new and the old pole in newborn cells. Results showed a WGA labelling on bacteria displaying only one focus (newborn bacteria) as well as on bacteria with two distinct foci (S or G2 phase). However an interesting observation was that a high number of newborn bacteria positive for the WGA staining displayed a strongly fluorescent signal focused at the new pole of the cell whereas bacteria in S or G2 phase presented a signal more diffuse around the bacteria (Figure 23 A). Another interesting data was that almost all predivisional cells were strongly labelled only at the constriction site (Figure 23 B). A bacterial counting was performed to evaluate proportion of newborn (G1) bacteria presenting strong polar signal or G1 bacteria presenting a diffuse signal, S/G2 presented in all cases a more diffuse signal and predivisional cells presented signal at the constriction site only in all cases (Figure 23 C).

Because the three adhesins BmaC, BtaE and BtaF have been reported to be present only in a small part of the bacterial population and were localized at the new pole we tested WGA lectin staining on triple deletion strain for these adhesins. However we were still able to observe a labelling on this mutant (data not shown), indicating that BmaC, BtaE and BtaF have no impact on WGA labelling.

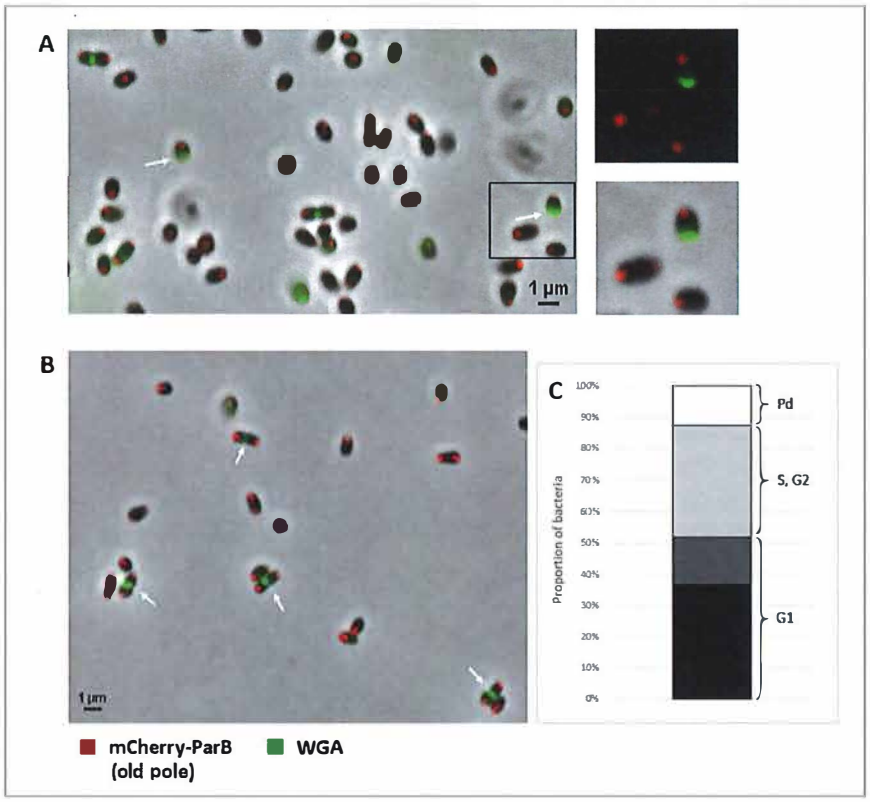

Figure 23: ***Brucella abortus*** **mCherry-ParB labelled with WGA. (A,B)** Bacteria were cultivated in 2YT medium and labelled with WGA-AlexaFluor488 in stationary phase. **(C)** Bacteria positives for WGA staining were counted (representing 100 %). Black = proportion of G1 bacteria labelled with a polar signal (new pole). Dark grey = proportion of G1 bacteria labelled with a diffuse signal. Light grey = proportion of bacteria labelled which are in S or G2 phase (diffuse signal). White = proportion of bacteria labelled presenting a constriction site. (n=440) Pd = predivisional cells. Scale bars represent 1 µm.

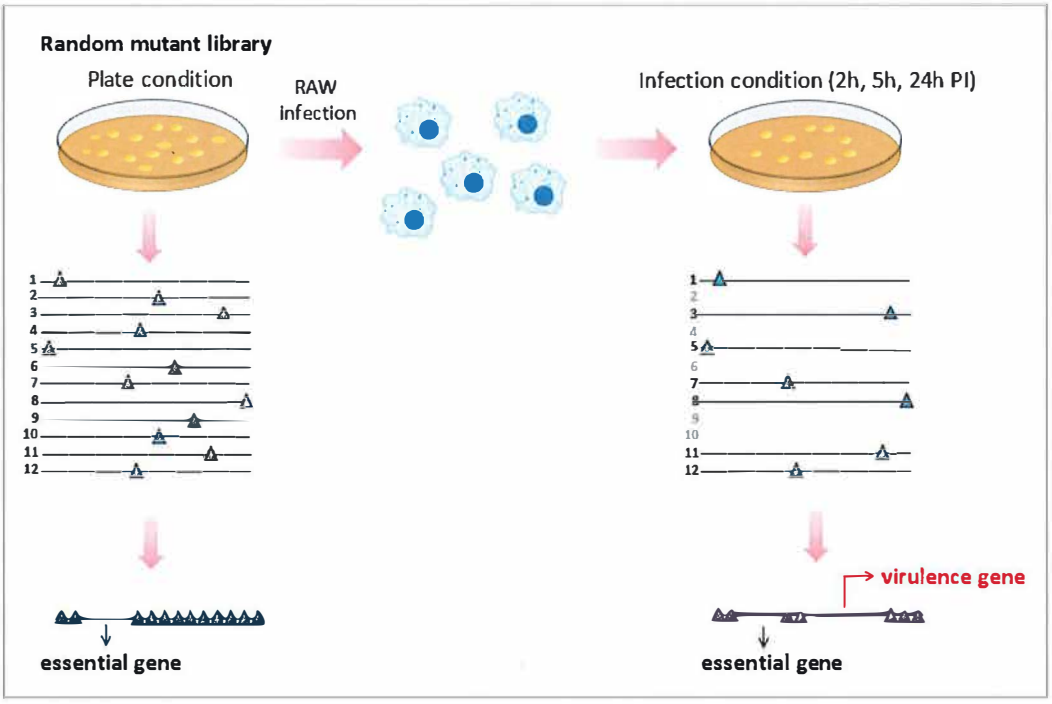

Figure 24: Tn-seq method to identify *Brucella abortus* essential genes involved in infection. Random mutant library in *B. abortus* was obtained by Tn*5* transposon insertion. Essential genes are first identified from mutants able to grow on rich medium (plate condition) by PCR amplification and sequencing of Tn*5* flanking regions. Library mutants was then used for infection of RAW 264.7 macrophages. Bacteria able to survive were harvested at 2h, 5h and 24h PI. All insertion sites will also be determined by sequencing and essential genes involved in infection ("virulence gene") will be established by comparison with essential genes obtained with the plate condition.

Without *a priori* approach

A transposon mutagenesis was performed to generate a large random mutant library in *B. abortus* followed by high-throughput sequencing to determine the position of transposon insertion sites in bacteria able to form colonies. Subsequently, essential genes on the one hand, and virulence genes involved in infection on the other hand were established by this process, since colonies were also collected at three times post-infection (Figure 24). The hyperactive transposon Tn*5* (Christen *et al.*, 2011) containing an antibiotic resistance cassette was used to perform a first mutant library. The random insertion mutant library was constructed to obtain approximately one transposon insertion site every base pair which represent 3.10^6 mutants. A first round of selection of resistant mutants was performed on culture plate containing kanamycin to select transpositional mutants. Thus, this condition was called "plate condition". At this stage only clones which have the transposon carrying the resistance cassette inserted in their genome are selected. From these clones, transposon insertion sites were amplified by arbitrary PCR and are currently analysed by high-throughput Illumina sequencing. By this process, essential genes for growth on standard 2YT culture plates could be identified because they will correspond to gaps between regions where transposons are tolerated (Figure 24). In order to identify genes crucial for adhesion and/or invasion, infections were performed using the previous mutant library. RAW 264.7 macrophages were put in 6-wells plates for cell culture (16x3 plates for 3 different times post-infection, *i.e* 96 wells for each time). Macrophages were infected and mutants being able to invade host cells were harvested at 2 h, 5 h, and 24h post-infection. Bacteria harvested from each well were plated on 2YT agar and CFUs were recovered. Transposon insertion sites of viable mutants (from three conditions corresponding to each time post-infection) were also amplified in order to be sequenced thereafter. This step will allow us to find new gaps between tolerant insertion sites by comparison with previous gaps defined as essential genomic regions (Figure 24). Data obtained from these new gaps will point to essential genes for attachment, internalization, survival or replication of bacteria in host cells. Up to now we have obtained DNA samples for each condition tested and the two first samples (control on plate and 2 h PI time point) are being sequenced.

Discussion and perspectives

Discussion and perspectives

During host cell colonization, many bacterial pathogens must first adhere to the cell surface through mechanisms involving adhesion molecules. Bacteria have evolved several classes of ligands/receptors to promote interaction with the host surface or with the extracellular matrix (ECM) to facilitate their internalization. The BmaC protein of *B. suis* has been found to interact with fibronectin, a component of ECM, and the *ΔbmaC* mutant showed a strong decrease in the binding to HeLa cells indicating that BmaC mediates the attachment of *B. suis* to host cells (Posadas *et al.*, 2012). Given that the *bmaC* coding sequence of *B. abortus* shares 99.6% of homology with *bmaC* of *B. suis* and that this later is reported to have a specific localization at the new pole of the bacterium (Ruiz-Ranwez *et al.*, 2013), we wondered if this protein could be involved in the attachment of *B. abortus* and more specifically in the attachment of the more infectious newborn bacteria to host cells. Indeed one particularity of newborn cells is the presence of a newly generated pole, produced by cell division (Figure 5).

To test BmaC adhesion function in *B. abortus* we first generated a deletion mutant strain for this adhesin. Surprinsingly, the CFU counting after 2 h and 4 h of RAW 264.7 macrophages and HeLa cells infection, respectively, did not generate a significant difference between the wild type strain and the *ΔbmaC* strain. These early times post-infection were interesting time points since they can inform us in a quantitative way about bacterial internalization, itself being mediated in part by the adhesion process. Therefore results obtained seem to be not in favour of BmaC as an adhesive protein as opposed to what has been published in *B. suis*. Regarding later times post infection non-significant difference was observed between both strains in RAW 264.7 macrophages. However in HeLa cells infection model, *ΔbmaC* presented an attenuation compared to the wild type strain, suggesting either that *bmaC* deletion could affect intracellular trafficking of bacteria or that this strain displays a slower growth once the replication niche has been reached. Data obtained from a growth analysis in rich culture medium performed on *ΔbmaC* and wild type strains indicate that the Δ*bmaC* strain does not suffer from a generalized growth defect in rich medium, like several *bmaC* overexpression strains (Figure 17). These data would reveal also that RAW 264.7 and HeLa cells are indeed distinct cellular infection models for the study of *B. abortus in vitro*.

The fact that *ΔbmaC* is not impaired in its ability to adhere and to be internalized in host cells suggests either that BmaC has no adherence function in *B. abortus* or that its deletion is compensated by other rescue mechanisms. Therefore an overexpression of *bmaC* could help reveal a specific phenotype possibly linked to adhesion on host cells.

The four overexpression strains showed different *bmaC* expression profiles according to the promoter used to control transcriptional expression. The promoter of *pleC* and *sodC* showed the strongest expression phenotype compared to the wild type strain or strains overexpressing *bmaC* via *secE* or *tolC* promoters (Figure 12). However this increase in *bmaC* transcripts cannot confirm that the protein is overproduced and more abundant at the surface of bacteria. According to *B. melitensis* microarray data, *PsodC* and *PsecE* should display stronger expression compared to *PpleC* and *PtolC*. However microarray data used for the selection of the four promoters do not inform us about a possible regulation of these promoters. Therefore the particular conditions in which the qRT-PCR was performed (culture medium, culture phase,...) could influence on one hand the expression level of these promoters.

Another plausible explanation is that insertion of the promoter sequence in another chromosomal region could interfere with a possible regulation of this promoter, *i.e.* steric hindrance for transcription factors for example. However, the *PtolC* promoter does not induce overexpression of *bmaC* at the mRNA level (Figure 12), suggesting a limited overexpression of *bmaC* in this strain, which is nevertheless strongly impaired for growth (Figure 17).

Infection with the four overexpression strains did not generate a significant difference between the wild type strain and the *bmaC*$^{++}$ strains at early times post-infection in macrophages as well as in HeLa cells, suggesting that BmaC has no adherence function *in infection* in *B. abortus* in the tested conditions. The CFU decrease observed at 24 hours post-infection for *PsecE-bmaC*$^{++}$, *PsodC-bmaC*$^{++}$ and *PtolC-bmaC*$^{++}$ suggested either a general bacterial growth deficiency, an affected intracellular trafficking or a loss of bacterial survival, to cite some of the possible hypotheses. To test the possibility of a general growth problem, growth curves have been generated for all the overexpression strains. The resulting data suggested that the intracellular decrease of CFU could be due to a general growth defect for these bacterial strains, since the three strains with a intracellular growth defect also have a growth problem in culture in rich medium (Figure 17). Several reasons could explain these bacterial generalized growth defects. Among these, overexpression of *bmaC* could induce abnormalities in the outer membrane of *Brucella*, similarly to the effect of the *bvrRS* mutations, which leads to perturbation of the outer membrane and to a high sensitivity to cationic compounds (Guzman-Verri *et al.*, 2001). Another hypothesis could be that promoter addition upstream of the *bmaC* coding sequence could generate some perturbations at this chromosomal locus leading to gene expression modifications for example.

We chose the *PpleC-bmaC*$^{++}$ strain for a binding assay on fibronectin, excluding *PsecE-bmaC*$^{++}$, *PtolC-bmaC*$^{++}$, *PsodC-bmaC*$^{++}$ strains because they present growth defects (moreover *PsecE-bmaC*$^{++}$ and *PtolC-bmaC*$^{++}$ strains seem to express *bmaC* at a very low level). We observed no difference between the wild type and Δ*bmaC* strains in the fibronectin binding assay, however a slight but significative increase for *PpleC-bmaC*$^{++}$ binding to fibronectin was detected, indicating nonetheless a potential adhesion function to fibronectin for BmaC (Figure 6), as observed in *B. suis* (Posadas *et al.*, 2012).

The data summarized above highlight a rather ambiguous situation regarding BmaC function in *B. abortus*. In fact the adhesion function of *B. suis* BmaC is not conserved in *B. abortus*, thus explaining why little or no phenotype is observed in infection when deleting the *bmaC* gene, eventhough this phenotype seems to be host cell type dependent. Moreover, BmaC overexpression leads to a more efficient fibronectin binding whereas the absence of BmaC does not influence fibronectin binding. Therefore, one could imagine that when *bmaC* is deleted, other components would mimic the BmaC adhesion function, or that BmaC is produced at a very low level in the *B. abortus* WT strain and thus it does not contribute to fibronectin binding.

Figure 25: Putative model describing recognition by WGA in rich medium. Representation of a newborn bacterium. Following the model, bacterium would produce first an unknown component on the surface of the new pole. This bacterial component would recognize and bind a component of the rich medium which is then recognized by the WGA lectin. Red dot represent mCherry-ParB focus.

Although they display varied ecological niches and lifestyles, members of α-proteobacteria group share several common features. *Brucella,* along with other members such as *Caulobacter* or *Agrobacterium* are characterized by asymmetric division and different polar functions or share highly conserved master regulators. In particular, *Caulobacter crescentus* and *Agrobacterium tumefaciens* present a polysaccharidic adhesive structure polarly localized. This phenomenon suggested us to explore the possibility of having surface-exposed (possibly polar) polysaccharides in *Brucella abortus*. Therefore we took advantage of the recognition of sugar motifs by lectins to identify polysaccharides exposed on the surface of the bacteria.

We used different lectins in order to detect polysaccharides potentially involved in adherence of *B. abortus* and especially of newborn bacteria which constitute the preferential infectious form of *B. abortus* (Deghelt, Mullier *et al.*, 2014). The staining with MAL I and GNL revealed a positive signal distributed throughout the bacterial surface suggesting the presence of galactose linked to N-acetylglucosamine and mannose residues at this location. Since the lipopolysaccharide (LPS) O chain of *Brucella* is known to contain sugar units derived from mannose (N-formylperosamine) we supposed that GNL revealed in fact LPS component. The results obtained regarding mixed *B. abortus* and *B. melitensis* co-labelling with GNL indicated that this lectin probably recognizes LPS from *B. abortus* suggesting that this lectin is also able to recognize mannose derives such as N-formylperosamine. The labelling of *Brucella* rough strains (*i.e.* without O chain at their LPS) could also provide a simple answer to this hypothesis.

More interestingly the staining with wheat germ agglutinin (WGA) showed a polar signal that prompted us to more deeply investigate this phenomenon, especially because UPP from *A. tumefaciens* and holdfast from *C. crescentus* are also recognized by this specific lectin. Given that the bacterial peptidoglycan is in part composed of N-acetylglucosamine residues, we wanted to ensure that bacteria labelled with WGA were not dead and subsequently that WGA did not label peptidoglycan instead of an exposed polysaccharidic structures. To do so, we performed a live dead test. Results showed that membrane integrity of bacteria labelled with WGA was not altered indicating that the detected component does probably not correspond to peptidoglycan.

Interestingly we have observed that WGA signal is only detected under certain conditions *i.e.* when bacteria were cultivated in rich medium (2YT) and until stationary phase of culture. Absence of labelling on bacteria grown in defined medium (called Plommet) supplemented with erythritol could be due to specific physiological state of bacteria in this condition. One hypothesis could be that the presence of a particular condition, *e.g.* the presence of erythritol, could repress the production of a polar exopolysaccharide in the Plommet medium as it is known that erythritol has additional properties than being a nutrient source for *Brucella* (Thibault Barbier, personal communication). In fact it has been reported that erythritol, in addition to be a carbon source for *Brucella*, could also influence gene expression and virulence factors regulation (Rodriguez *et al.*, 2012). We therefore investigate the possible role of this component in the production of the structure detected by WGA. However no labelling was observed on bacteria when grown in Plommet medium supplemented by another carbon source, *i.e.* xylose. Moreover labelling of bacteria grown in the rich medium 2YT supplemented with erythritol displayed the standard staining phenotype. From these results we can conclude that erythritol does not affect the WGA staining. One hypothesis could be that such a structure is not synthesized in defined medium involving that bacterial physiology status could have an effect on this polar component production. Alternatively another explanation could be the WGA labelling is due to one or more component from the rich medium as suggested by the absence of labelling on bacteria when grown in defined media.

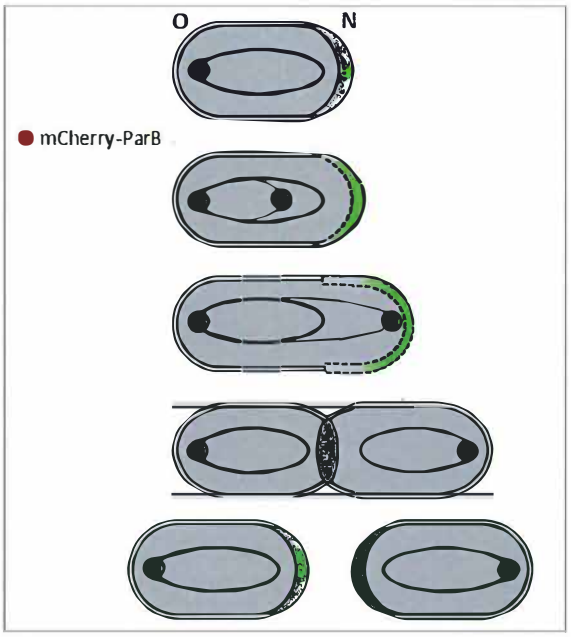

Figure 26: Model describing polar production of a bacterial component. Bacteria in predivisional phase accumulate the labelling at the septum of division. After division both daughter cells present the labelling at their new poles. The unipolar growth which occurs at the new pole would then lead to diffusion of the component throughout the surface of the cell. O = old pole. N = new pole.

However, the staining pattern proved to be reproducible and highly specific to given bacterial sublocations (Figure 23), leading us to think that such signal would highlight the presence of (an) unknown bacterial component(s). We hypothesize a model in which one or several components of the 2YT medium could possibly adhere to the surface of *B. abortus* and then be recognized by WGA. In fact, such interactions would form a "sandwich" complex involving three parts; an adhesive molecule localized on the outer membrane of the bacteria, a medium component bound by this adhesive structure and the wheat germ agglutinin recognizing the bound component (Figure 25). This unknown medium component could therefore contain N-acetylglucosamine or sialylated residues according to what WGA is able to recognize. Chitin, a long polymer of N-acetylglucosamine contained in yeast wall, would be a candidate for this medium component since the 2YT rich medium contains yeast extract. We could therefore repeat staining with WGA on bacteria grown in defined medium supplemented by chitin or N-acetylglucosamine and see if bacteria present fluorescent signal. It would be also interesting to test if eukaryotic cell culture medium (DMEM + BSA) has an effect on the WGA staining given that bacteria are first resuspended in this medium before infecting host cells. In fact, one could imagine that such a binding of medium-borne molecules onto the bacterial surface could affect and either lower or increase infection efficiency.

In stationary phase only a part of the bacterial population displayed WGA signal (15.6 %) suggesting that the production of the bacterial component occurs with a selection of individuals in this phase. To investigate if production of this component is linked to a specific cell cycle phase we used a *B. abortus* strain producing mCherry-ParB. Observation of both fluorescent signals by microscopy showed that newborns as well as S or G2 bacteria were labelled with WGA indicating that production or binding of the polysaccharidic component is not specific to one phase of the cell cycle. However newborn bacteria were labelled at the new pole indicating that such a bacterial component is not randomly produced to both poles, seems not very mobile and is probably induced at the time of bacterial division. Indeed, all predivisional bacteria displayed a strong fluorescent signal at the constriction site which constitutes the future new pole of both sibling cells. Moreover a particularity of the more infectious newborn bacteria is the presence of a pole newly generated by cell division implicating addition of new and possibly specific material. With these correlations we could hypothesize that the structure detected by WGA, which is preferentially found at the new pole, could also be involved in adherence. Therefore we suggest a model where, in late exponential/early stationary phase, bacteria in predivisional phase accumulate WGA signal at the division septum, resulting after cell division in both daughter cells displaying WGA signal only on their new poles. Then unipolar bacterial growth from the new pole consisting in addition of new material in order to extend cell wall lead to a progressive diffusion of the WGA signal throughout bacterial surface (Figure 26).

It should be noted that *Brucella*'s new pole has been already suggested to be specialized for adhesion, since the three *B. suis* adhesins BmaC, BtaE and BtaF have been reported to be localized at this specific pole (Posadas *et al.*, 2012; Ruiz-Ranwez *et al.*, 2013). It has been also reported that bacterial cells interact with host cell membrane through one pole, the same than BmaC was observed (Pizarro-Cerdá *et al.*, 1999; Ruiz-Ranwez *et al.*, 2013).

Labelling of bacteria in rich medium in stationary phase revealed a strong polar signal that is not present when bacteria are grown until exponential phase. The fact that little to no labelling occurs in exponential phase in rich medium support the hypothesis that the polar structure is produced only under specific physiological state. It has been reported that *B. melitensis* presents differences in infection efficiency depending on its culture phase when harvested for infection (Rossetti *et al.*, 2009). Indeed bacteria in early stationary phase have been shown to be significantly more infectious than bacteria grown until mid-exponential phase, and were highly significantly more infectious than bacteria in late stationary phase (Rossetti *et al.*, 2009). *Brucella abortus* is a facultatively extracellular intracellular pathogen, which means that bacteria are able to survive in extracellular environments but prefer to establish their niche in host cells where conditions are more suitable to grow and proliferate. During exponential phase, bacteria are presumably not under stress conditions (nutrients, space, oxygen,...). We could imagine that when culture reaches stationary phase the bacterial population encounters different stresses triggering adaptive responses. Among these, virulence factors expression could be more expressed in order to increase infection efficiency and find more suitable conditions in the host. Following this idea, if the polysaccharidic structure detected by WGA is involved in host cells adhesion and *in extenso* to colonize a new host or host cell, its production is not necessary for bacteria growing in exponential phase.

To conclude, further investigations must be performed in order to identify the component(s) directly recognized by WGA and/or the bacterial structure that is recognized. Moreover it would be interesting to test if this specific bacterial component is involved in adherence and *in extenso* contributes to the success of *Brucella* infection process. However, if these experiments fail to generate interesting data regarding the study of the mechanisms regarding *B. abortus* infectiosity, the large scale and without *a priori* Tn-seq approach performed in *B. abortus* could highlight novel perspectives regarding genes implicated in the infection of host cells. In fact, the "2YT culture" control condition and 2 h PI condition are currently being sequenced, and comparing the two resulting transposon insertion maps should bring new insights about genes involved in adherence and internalization of *B. abortus* in RAW 264.7 macrophages.

Experimental procedures

Experimental procedures

Strains and growth conditions

The reference strain *B. abortus* 2308 was used for the overexpression and polysaccharides detection experiments and was grown on solid or in liquid 2YT medium (LB 32 g/L Invitrogen, Yeast Extract 5g/L, BD and Peptone 6 g/L, BD) at 37°C. *E. coli* strain DH10B was used for plasmid constructions and the conjugative strain *E. coli* S17-1 was used for mating in *B. abortus*. Both strains were cultivated in LB medium (Luria Bertani, Casein Hydrolysate 10g/L, NaCl 5g/L, Yeast Extract 5g/L) at 37°C overnight. Depending on the plasmid used, different selection markers were added to the culture medium: Ampicillin (100 µg/mL); Kanamycin (50 µg/mL for plasmid borne resistance cassettes, and 10 µg/mL for chromosome-encoded resistance cassettes); Nalidixic acid (25µL/mL). Plasmids used in this study were pGEM5-Zf(+) (Promega®, Madison, USA) and pNPTS138.

PCR and sequencing

Two types of PCR were performed, preparative PCR used for subsequent constructions and diagnostic PCR to check for the presence of a given DNA fragment.

Preparative PCR

The PCR mix contains Phusion polymerase (0.02 U/µL, Finnzymes), 5X Phusion buffer (1X, BioLabs), primers (20 µM each), dNTPs (5 mM each), template DNA (about 60 ng), and milliQ water (milliQ purification system, Millipore). Preparative PCR programs were composed of a first DNA denaturation step of 98°C for 30 seconds followed by 30 amplification cycles. Each cycle was composed of a DNA denaturation step (98°C for 10 seconds), a primer hybridization step (temperature set according to primers tm for 30 seconds), and an elongation step at 72°C (duration depending on the length of the PCR product to be amplified, typically one 30 seconds per kilobase). Eventually, after the 30 amplification cycles, a final elongation step was performed at 72°C during 5 minute. In the particular case of joining PCR, allowing to ligate two PCR products together through sequence complementarity, a classical PCR program (without primers added) of 5 cycles was performed to join both products, then, primers were added into the mix to amplify the entire product (composed of the joined initial products) for 25 cycles.

Diagnostic PCR

The mix was composed of Taq polymerase (Promega®, Madison, USA), 5X GoTaq buffer (1X, PROMEGA), primers (20 µM each), dNTPs (5 mM each), template DNA (60 ng) or immersion of a tip which touched one bacterial colony, and milliQ H_2O. The PCR programme was composed of a first DNA denaturation step (94°C during 4 minutes), followed by 35 cycles containing an initial denaturation step (94°C during 30 seconds), followed by a hybridization step (Tm according to primers during 30 seconds), and eventually an elongation step (72°C for a time dependent on the length of the PCR product, typically 1 minute per kilobase). The programme was then ended by a final elongation step of 72°C for 5 minutes.

Purification of PCR products

Each PCR was checked by electrophoretic migration using agarose gel containing ethidium bromide to control the quantity and size the amplification product by correspondence to a given DNA Ladder (Gene Ruler 0.1 µg/µL Thermo Scientific). The purification of PCR products on column was made using the MSB SpinPCRapace (Invitek, Berlin, Germany) following the manufacturer's protocol. DNA gel extraction was performed with QIAquick® Gel Extraction Kit (QUIAGEN). Sequencing was performed by Beckman Coulter Genomics to check the sequence of the different constructions.

Plasmidic DNA extraction

A bacterial culture grown overnight was centrifuged at 13000 rpm during 1 minute and the supernatant was removed (this step can be repeated to increase the amount of input material). The pellet was resuspended using 300 µL of P1 buffer (RNAase A 100 µg/ml, Tris HCl 50 mM, EDTA 80 mM, pH 8, stored at 4°C), then 300 µL of P2 lysis solution (NaOH 100 mM, SDS 1%) were added and the mix was incubated during 5 minutes at room temperature. Then, 300 µL of P3 buffer (KAc 3M, pH 5.5 stored at 4°C) were added, followed by centrifugation of the lysate at 13000 rpm during 10 minutes. The supernatant was then transferred into a new tube to which 700 µL of isopropanol were added. The mix was centrifuged at 13000 rpm for 10 minutes. Supernatant was removed and 400 µL of ethanol 70 % stored at -20°C were added on the pellet and centrifuged again at 13000 rpm during 5 minutes. Then supernatant was discarded and the tube was left to dry in a 65°C incubator for 15 to 20 minutes in order to remove all traces of ethanol. After that, pellet was resuspended with 20 µL of miliQ water.

Enzymatic restriction

Restriction of DNA sequence was performed with the appropriate restriction enzyme (10 U/µL, Roche®) during 45 minutes at the appropriate temperature. The quantity of DNA used in mix differed with the restriction type; about 300 ng is used for preparative restriction and 120 ng for diagnostic restriction. DNA was incubated with (1X) and 10X appropriate buffer (1X).

Ligation protocol

Ligation of inserts into different plasmids was performed with T4-ligase (Fermentas), 5X ligase buffer (1X, Invitrogen), and the quantity of plasmid and insert used was determined to have 1/5 ratio respectively. The final mix was incubated overnight at 18°C.

Transformation in competent strains

The competent strains used were *E. coli* DH10B and *E. coli* S17-1 stored at -80°C. The quantity of DNA added in bacterial culture was 120 ng for a DNA purification product or 300 ng for ligation mix. Bacteria were incubated on ice during 20 minutes and then heat shocked at 42°C for 2 minutes. 700 µL of LB medium were added in the mix before being put at 37°C with agitation during 45 minutes. After this incubation time, bacteria were centrifuged at 5000 rpm for 3 minutes. A part of supernatant was removed and the bacterial pellet was resuspended with the rest of supernatant (approximately 100 µL).

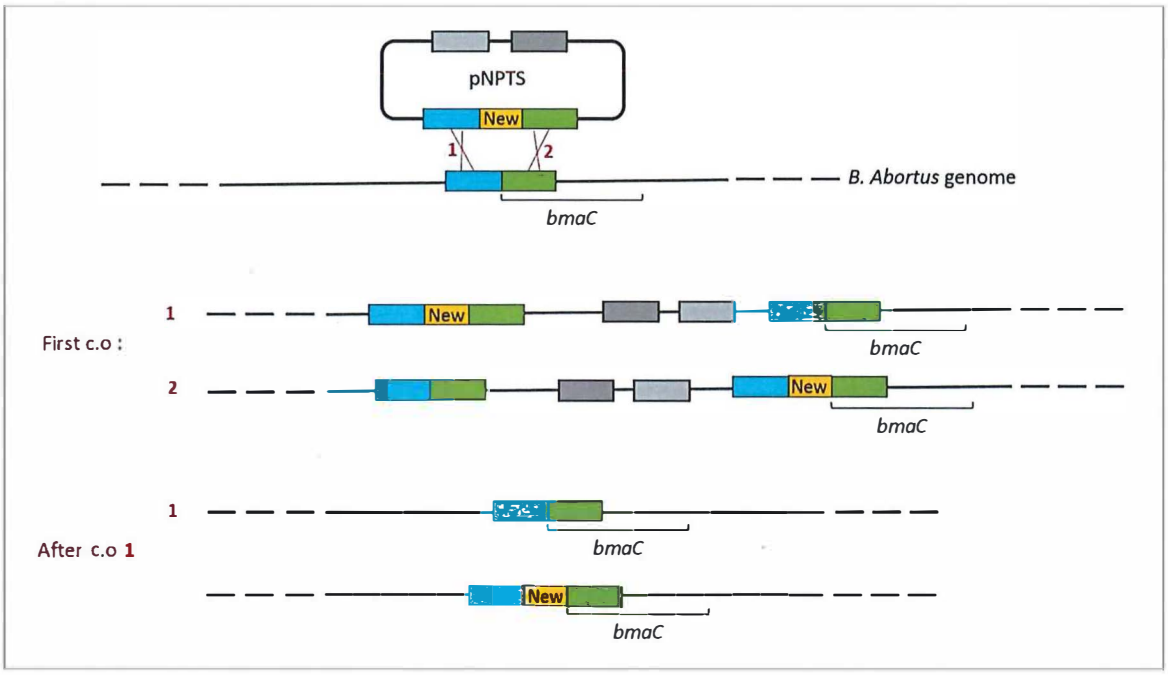

Figure 27: Allellic replacement using the pNPTS plasmid. After a first crossing over (c.o) two plasmid integration possibilities are observed, corresponding the upstream c.o (1) or downstream c.o (2). Then a second homologue recombination was performed again with two possibilities, upstream or downstream. If the first c.o (1) occurs, the second c.o must occur in downstream region to obtain the new sequence insertion. If two c.o occur in the same region, a WT genotype is regenerated.

Bacteria were then plated on petri dish containing LB agar with the appropriate antibiotic as well as X-gal (0.004 %) and IPTG (1 mM) if white blue screen was performed. Petri dish are placed in incubator at 37°C overnight.

Bacterial conjugation

The conjugative strain *E. coli* S17 containing the plasmid of interest was used for mating with *B. abortus* 2308. 50 µL of an overnight culture of *E. coli* were added to 1 mL of *B. abortus* overnight culture and centrifuged at 7000 rpm during 2 minutes. The supernatant was removed and the pellet was resuspended in 100 µL of 2YT medium. This suspension was centrifuged again at 7000 rpm for 2 minutes, then most of the supernatant was removed and the pellet was resuspended with the residual supernatant (approximately 100 µL). The bacterial suspension was spotted on 2YT agar without being spread (this drop configuration increases the conjugation likelihood). The next day a part of the drop was resuspended in 100 µL of 2YT medium, spread on 2YT agar containing nalidixic acid (1 µL/mL) and kanamycin (10 µg/mL) and was placed at 37°C during approximately 4 days (time for colonies grow and appear). Colonies obtain were streaked on 2YT agar with only kanamycin (10 µg/mL) and incubated at 37°C. A bacterial culture was performed from a streak in liquid 2YT and was incubated at 37°C overnight (this culture without antibiotics allows the second crossing over *i.e.* the loss of the integrated plasmid, Figure 27). Then 100 µL of this culture were spread on 2YT agar containing sucrose (5%) (to select bacteria which had excised their plasmid and thus lost counter-selection marker) and incubated at 37°C for approximately 5 days. The colonies obtain were picked and spread on two plates containing respectively kanamycin and sucrose. Only bacteria which had grown on sucrose but not on kanamycin were selected to check for the presence of insert after being inactivated in sterile PBS at 80°C during 1 hour.

Polysaccharide staining

Polysaccharides staining was performed using different lectins conjugated to Alexa Fluor 488 (Invitrogen). First, *B. abortus* was grown in rich medium (2YT) overnight at 37°C. 1 mL of the bacterial culture was centrifuged at 5000 rpm during 2 minutes and pelleted bacteria were resuspended in 100 µL sterile PBS at neutral pH. This washing was performed a second time and bacteria were then diluted 10X. 1 µL of labelled lectin (1 mg/mL) was added in the suspension and the mix was incubated during 20 minutes away from light. Then bacteria were washed with sterile PBS and 2 µL of bacteria suspension were placed on a PBS-agarose pad. A coverslip was applied and sealed with hot VALAP (mix of equal amounts of paraffin, lanoline and vaseline). Samples were observed by fluorescence microscopy.

Live dead test

Propidium iodide was used to stain and detect dead bacteria by microscopy. Bacteria stained previously or not with lectins were incubated with propidium iodide during 15 minutes away from light at room temperature and were washed with PBS. Bacteria pre-treated with ethanol (70 %) and then labelled with propidium iodide was used as positive control to confirm the dead test.

Microscopy

The microscope which has been used is a Nikon 80i (objective 100X, plan Apo) connected to a Hamamatsu ORCA-ER camera. We also used DF type immersion oil (Nikon oil) with refraction indice of 1.5150 +/- 0.0002.

Agarose pad construction

Agarose was added in PBS solution to have a final concentration of 1 %. The mix was heated until dissolution of agarose and stored at 55°C. 350 µl of the hot agarose solution were injected between two slightly and uniformly spaced parallel microscopy slides and wait until the agarose solution solidifies in between the slides. Then, the top microscopy slide was removed in order to conserve the pad on a single slide. To prevent pads from drying, they were stored in a petri dish containing a piece of water-soaked paper towel.

CFU (Colony Forming Unit)

RAW 264.7

B. abortus 2308 was grown in 2YT at 37°C overnight. RAW macrophages were put in wells in DMEM medium (with decomplemented bovine serum, glucose, glutamine, and no pyruvate, Gibco®) to have 10^5 cells/mL. The next day OD of the bacterial culture was measured and dilutions were performed to have MOI equal to 50 (50 times more bacteria than macrophages). Cells medium was removed to add the appropriate bacterial dilution. The mix was centrifuged 10 minutes at 1200 rpm (4°C) and incubated at 37°C (this time point is set as time zero). After one hour of incubation, medium was removed and replaced by medium containing gentamycin (50 µg/ml) in order to kill extracellular bacteria. In this study, at either 2 hours or 24 hours post infection, cells were first washed twice with sterile PBS and were then incubated in PBS + triton 0.1% at 37°C during 10 minutes in order to lyse the cells while keeping bacteria alive. After that, cells were flushed and lysates were harvested. Serial dilutions were performed and each dilution was spotted on 2YT agar plates. Eventually, 3 to 5 days after plating CFU were counted.

HeLa cells

B. abortus 2308 was grown in 2YT at 37°C overnight. HeLa cells were put in wells in DMEM medium (with sodium pyruvate, non-essential amino acid, glucose, glutamine, and no pyruvate, Gibco®) to have $4x10^4$ cells/mL. The next day OD of the bacterial culture was measured and dilutions were performed to have MOI equal to 300. Cells medium was removed to add the appropriate bacteria dilution. The mix was centrifuged 10 minutes at 1200 rpm (4°C) and incubated at 37°C (this time point is set as time zero). After one hour of incubation, medium was removed and replaced by medium containing gentamycin (50 µg/ml) in order to kill extracellular bacteria. In this study, at either 4 hours or 24 hours post infection, cells were first washed twice with sterile PBS and were then incubated in PBS + triton 0.1% at 37°C during 10 minutes. After that, cells were flushed and lysates were harvested. Serial dilutions were performed and each dilution was spotted on 2YT agar plates. Eventually, 3 to 5 days after plating CFU were counted.

Bioscreen

The different *B. abortus* strains were grown in 2YT at 37°C overnight. The next day OD of the bacterial culture was measured and dilutions were performed in 2YT to have an OD equal to 0.1. 200 µL of each bacterial cultures were put in wells (Honeycomb 2 plate) and measurements of OD were done every 30 minutes (Bioscreen C MBR).

Reverse transcription polymerase chain reaction (RT-qPCR)

Nucleic acid extraction

The wild-type strain of *Brucella abortus* 2308 was used to compare transcript level between the different strains tested. Bacterial strains were grown in 2YT medium at 37°C overnight as classic bacterial cultures. The next day OD of the cultures were measured and dilutions were performed to obtain a final volume of 50 mL with an OD equal to 0.1 in Erlenmeyer flasks. Cultures were placed at 37°C by the time the OD reaches between 0.4 and 0.8 (approximately 15 hours). When adequate OD of cultures were reached RNA extraction was performed (from this step RNAse-free work area is required). Cultures were centrifuged at 7000 rpm during 2 minutes. Supernatants were removed and pellets were resuspended with 100 µL of SDS 10% and 20 µL of proteinase K (stocked at 4°C) and the mix were incubated at 37°C during one hour. 5 mL of Trizol (Tripure Isolation Reagent, Roche®) were added, mixed and incubated at 65°C during 10 minutes and 1 mL of chloroform were then added and incubated at room temperature during 10 minutes. All mixes were aliquoted in eppendorfs, centrifuged at 14000 rpm during 15 minutes at 4°C and each supernatant containing nucleic acids were recovered and pooled depending on conditions. One equal volume of ethanol 100% was added in these supernatants and mix was stocked at -20°C overnight.

Nucleic acid treatment

The next day 2 mL of each conditions were centrifuged at 14000 rpm during 30 minutes at 4°C, 200 µL of ethanol 70% were then added and mixes were centrifuged at 8500 rpm during 5 minutes at 4°C. Supernatants were removed and the DNA/RNA pellets were dried during 15 minutes at room temperature before being resuspended in 50 µL of DEPC H_2O, water treated to inactivate RNase enzymes. Mixes were incubated at 55°C during 10 minutes.

DNAse treatment

The next step was to remove DNA for each conditions. Each mixes containing DNAse was made with 5 µg of RNA, DNAse buffer (10X, Thermo Scientific), DNAseI (1 U/µL used 1 µg of RNA, Thermo Scientific), and DEPC H_2O. These mixes were incubated at 37°C during 30 minutes and 2 µL of EDTA (50 mM, Thermo Scientific) were added and incubated 10 minutes at 65°C.

Reverse transcription

For each conditions, twice 9 µL of the previous DNAse mix were put separately in 2 tubes, one used as negative control. 2 µL of Oligo dT Random Primer (500 ng/µL, Invitrogen) were added and incubated 10 minutes at 70°C and placed then directly on ice for 5 minutes. Then, dNTP Mix (20 mM, Eurogentec), DTT (0.1 M, Invitrogen), RT buffer (5X, Roche), RNasin Ribonuclease Inhibitor (40 U/µL, 18X, Promega), and DEPC H_2O were added in all tubes and incubated 5 minutes at RT. 1 µL of SuperScript II (200 U/µL, Invitrogen) was added in each sample except negative controls and all tubes were incubated first 1H30 at 42°C and 15 minutes at 70°C then. After that 1 µL Ribonuclease H (2 U/µL, Roche) was added in each mixes incubated 20 minutes at 37°C and frozen at -20°C directly. To be sure there was enough cDNA in samples before starting RT-qPCR a first amplification by PCR was be performed using protocol corresponding to classic PCR (see above) and using primer dedicated to RT-qPCR.

RT-qPCR

The mix was composed of cDNA obtained from reverse transcription (5 ng/µL), SYBER Green (FastStart Universal 2X, Roche), primers forward and reverse (10 µM, 20X each), and water. Samples were put in a 96-well plate (AppliedSystem Micro Amp Fast Optical 96-well) and sealed with a protective film. The plate was centrifuged at 4°C during 2 minutes at 2000 rpm. RT-qPCR was then performed using StepOnePlus, Real Time PCR Sustem (Applied Biosystems).

Tn-Seq

Generation of mutant library

Brucella abortus 2308 was grown on 2YT solid medium and Escherichia coli S17-1 containing the hyperactive Tn5 transposon GentaR modified in KanR on LB solid medium with ampicillin (100 µg/mL) and kanamycin (50 µg/mL). Five bacterial conjugations were performed between these strains (see above). Drops containing *B. abortus* and *E. coli* were harvested and resuspended in 300 µL of 2YT. The mix were then diluted 40 times and plated on 96 solid 2YT medium plates containing kanamycin (10 µg/mL) and nalidixic acid (1 µL/mL). After drying, petri dishes were put in incubator at 37°C during 3 days (time for colonies to grow and appear). Colonies were resuspended in 2 mL of 2YT liquid medium added directly on petri dishes and bacterial suspensions were harvested. A determined fraction of each suspension was set aside to infect RAW macrophages. The rest was centrifuged during 3 minutes at 7000 rpm and pellets were resuspended in 150 µL of SDS 2% and inactivated at 80°C during 2 hours.

RAW infection

The day before infections, RAW macrophages were put in wells to a concentration of 10^5 cells/mL. OD of bacterial suspensions obtained previously were measured and dilutions were performed in RAW culture medium in order to have a MOI of 50. Cells medium was removed and bacterial dilutions were added. The mix was centrifuged 10 minutes at 1200 rpm (4°C) and incubated at 37°C (this time point is set as time zero for infection) during one hour. Then medium was removed to add fresh medium containing gentamycin (50 µg/mL).

Mutants harvesting after infection

At 2 hours, 5 hours and 24 hours post-infection, cell medium was removed and cells were washed twice with sterile PBS. Then PBS + triton 0.1% was added in wells and was incubated for 10 min. After incubation, wells were flushed leading to cell lysis while keeping bacteria alive. After that, the bacteria-containing lysate were harvested, diluted (3X for time 2 hours and time 5 hours post-infection, and 30X for time 24 hours post-infection) in sterile PBS and plated on 2YT agar containing kanamycin (10 µg/mL). Petri dishes were dried and placed at 37°C during 3 days (time for colonies to grow and appear). Bacteria were then harvested by resuspension in 2YT liquid medium and mixes were centrifuged at 7000 rpm during 3 minutes. Pellets obtained were resuspended in 150 µL of SDS 2% and placed in 80°C during 2 hours to inactivate bacteria.

DNA treatment

Inactivated bacteria were treated with proteinase K overnight and genomic DNA was purified by ethanol precipitation before sequencing.

Fibronectin binding assay

B. abortus 2308 strains were grown in rich medium 2YT overnight at 37°C. Fibronectin was coated in plate wells (96 Well Culture Plate, Cellstar) (100 µL/well, 100 µg/mL) and was incubated overnight at 4°C. The next day wells containing fibronectin were washed three times with PBS to remove excess ligand. 100 µL of PBS-BSA (1 %) were added in wells and incubated during 2 hours at room temperature (negative controls were performed consisting in empty wells and wells containing only PBS-BSA). Bacteria were washed once with a PBS-BSA solution (1 %) and OD were measured. Bacteria were diluted to obtain an OD of 0.3 representing 1.10^9 bacteria/mL. 50 µL of bacterial mixes were added in wells and plate was put at 37°C during 2 hours. After that bacteria were removed and wells were washed three times with PBS in order to eliminate bacteria not bound. Bound bacteria were harvested by adding 100 µL of Trypsine-EDTA (Trypsin 0.05 %, EDTA 0.5%) incubated at 37°C during 10 minutes. Bacteria were therefore harvested and serial dilutions were performed before plated on 2YT agar. Viability control was performed to test effect of Trypsin-EDTA on bacteria. Bacteria were resuspended and incubated with EDTA or Trypsin-EDTA during 10 minutes at 37°C and CFUs were counted.

Data were analized using Student t Test for independent samples.

Annex

Primers table

bmaC AM	bmaC-over-AM-F	ATAAGCTTgttgatattagcgacgatcg
	bmaC-over-AM-R	TAATCGATgcctctgtcatttaccacat
PpleC over	PpleC-over-F	ATATCGATttcgcaaaatccgaaggtc
	PpleC-over-R	CAAATTAGGCATctctcggcccctcttgaatc
PsecE over	PsecE-over-F	TATTCGAAttttgaaagcgggtgtctgtag
	PsecE-over-R	CAAATTAGGCATtcagcaaaagcgaatcaaatgt
PsodC over	PsodC-over-F	TATTCGAAgagcaaggcccgatgc
	PsodC-over-R	AGGCATcacttctcctgaatatagttagaacagttcc
PtolC over	PtolC-over-F	ATATCGATttcgggaattggtacaattctgc
	PtolC-over-R	TAGGCATcagaacaaacgaatccatcatcg
bmaC AV	bmaC-pleCover-ORF-F	CGAGAGatgcctaatttggccaatcag
	bmaC-secEover-ORF-F	TGCTGAatgcctaatttggccaatcag
	bmaC-sodCover-ORF-F	ATTCAGGAGAAGTGatgcctaatttggccaatcag
	bmaC-tolCover-ORF-F	TCGTTTGTTCTGAGatgcctaatttggccaatcag
	bmaC-over-ORF-R	ATGGATCCcagaggcactagcagagcttcctac
B. ab chrm integr. Check	PpleC-bmaC-check-AM-F	cttgacagtgatcttgtcaacc
	PpleC-bmaC-check-AM-R	ctcgaccttcggattttgc
	PpleC-bmaC-check-AV-F	gatttcagttcatacagttctgc
	PpleC-bmaC-check-AV-R	tcgtatgattgttgaagatatttcc
bmaC qRT-PCR	bmaC-qRT-PCR-F	caatccgtttcgatgacttcaag
	bmaC-qRT-PCR-R	Cgataccgtagatgtgcatg

Bacterial strains table

E. coli DH10B	pGEM5-Zf(+) over-bmaC-AM	ampR
	pGEM5-Zf(+) over-bmaC-AV	ampR
	pGEM5-Zf(+) over-bmaC-PpleC-AV	ampR
	pGEM5-Zf(+) over-bmaC-PsecE-AV	ampR
	pGEM5-Zf(+) over-bmaC-PsodC-AV	ampR
	pGEM5-Zf(+) over-bmaC-PtolC-AV	ampR
	pNPTS138 over-bmaC-AM-PpleC-AV	kanR/sucS
	pNPTS138 over-bmaC-AM-PsecE-AV	kanR/sucS
	pNPTS138 over-bmaC-AM-PsodC-AV	kanR/sucS
	pNPTS138 over-bmaC-AM-PtolC-AV	kanR/sucS
E. coli S17-1	pNPTS138 over-bmaC-AM-PpleC-AV	kanR/sucS
	pNPTS138 over-bmaC-AM-PsecE-AV	kanR/sucS
	pNPTS138 over-bmaC-AM-PsodC-AV	kanR/sucS
	pNPTS138 over-bmaC-AM-PtolC-AV	kanR/sucS
B. abortus 2308	pNPTS138 bmaC-over-PpleC	
	pNPTS138 bmaC-over-PsecE	
	pNPTS138 bmaC-over-PsodC	
	pNPTS138 bmaC-over-PtolC	

References

Abel S, Chien P, Wassmann P, Schirmer T, Kaever V, Laub MT, Baker TA, Jenal U. (2011) Regulatory cohesion of cell cycle and cell differentiation through interlinked phosphorylation and second messenger networks. Mol Cell. 43(4):550-60

Archambaud C, Salcedo SP, Lelouard H, Devilard E, de Bovis B, Van Rooijen N, Gorvel JP, and Malissen B. (2010) Contrasting roles of macrophages and dendritic cells in controlling initial pulmonary *Brucella* infection. Eur. J. Immunol. 40:3458-3471.

Batchelor M, Prasannan S, Daniell S, Reece S, Connerton I, Bloomberg G, Dougan G, Frankel G, Matthews S. (2000) Structural basis for recognition of the translocated intimin receptor (Tir) by intimin from enteropathogenic *Escherichia coli*. EMBO J. 19(11):2452-64.

Benz I, Schmidt MA. (2001) Glycosylation with heptose residues mediated by the aah gene product is essential for adherence of the AIDA-I adhesin. Mol Microbiol. 40(6):1403-13.

Bodenmiller D, Toh E, Brun YV. (2004) Development of surface adhesion in *Caulobacter crescentus*. J Bacteriol. 186(5):1438-47.

Boschiroli ML, Ouahrani-Bettache S, Foulongne V, Michaux-Charachon S, Bourg G, Allardet-Servent A, Cazevieille C, Lavigne JP, Liautard JP, Ramuz M, O'Callaghan D. (2002) Type IV secretion and *Brucella* virulence. Vet Microbiol. 20;90(1-4):341-8.

Boschiroli ML, Ouahrani-Bettache S, Foulongne V, Michaux-Charachon S, Bourg G, Allardet-Servent A, Cazevieille C, Lavigne JP, Liautard JP, Ramuz M, O'Callaghan D (2002) Type IV secretion and *Brucella* virulence. Vet Microbiol. 90(1-4):341-8.

Brown PJ, de Pedro MA, Kysela DT, Van der Henst C, Kim J, De Bolle X, Fuqua C, Brun YV. (2012) Polar growth in the Alphaproteobacterial order Rhizobiales. Proc Natl Acad Sci U S A. 109(5): 1697-701.

Bundel DR, Cherwonogrodzky JW, Carrof M, Perry MB. (1987) The lipopolysaccharides of *Brucella abortus* and *B. melitensis*. Ann Inst Pasteur Microbiol. 138(1):92-8.

Bundle DR, Cherwonogrodzky JW, Perry MB. (1987) The structure of the lipopolysaccharide O-chain (M antigen) and polysaccharide B produced by *Brucella melitensis* 16M. FEBS Lett. 216(2):261-4.

Castañeda-Roldán EI, Avelino-Flores F, Dall'Agnol M, Freer E, Cedillo L, Dornand J, Girón JA. (2004) Adherence of *Brucella* to human epithelial cells and macrophages is mediated by sialic acid residues. Cell Microbiol. 6(5):435-45.

Castañeda-Roldán EI, Ouahrani-Bettache S, Saldaña Z, Avelino F, Rendón MA, Dornand J, Girón JA. (2006) Characterization of SP41, a surface protein of *Brucella* associated with adherence and invasion of host epithelial cells. Cell Microbiol. 8(12):1877-87

Celli J and Gorvel JP (2004) Organelle robbery: *Brucella* interactions with the endoplasmic reticulum. Curr Opin Microbiol. 7(1):93-7.

Celli J, de Chastellier C, Franchini DM, Pizarro-Cerda J, Moreno E, Gorvel JP. (2003) *Brucella* evades macrophage killing via VirB-dependent sustained interactions with the endoplasmic reticulum. J Exp Med. 198(4):545-56.

Celli J, Salcedo SP, Gorvel JP. (2005) *Brucella* coopts the small GTPase Sarl for intracellular replication. Proc Natl Acad Sci U S A. 102(5):1673-8.

Christen B, Abeliuk E, Collier JM, Kalogeraki VS, Passarelli B, Coller JA, Fero MJ, McAdams HH, Shapiro L. (2011) The essential genome of a bacterium. Mol Syst Biol. 30;7:528.

Curtis Patrick D. (2010) Getting in the loop: Regulation of development in *Caulobacter crescentus.* Microbiol. Mol. Biol. Rev. 74(1):13.

Czibener C. and Ugalde JE. (2011) Identification of a unique gene cluster of *Brucella spp.* that mediates adhesion to host cells. Microbes Infect. (1):79-85.

Deghelt M, Mullier C, Sternon JF, Francis N, Laloux G, Dotreppe D, Van der Henst C, Jacobs-Wagner C, Letesson JJ, De Bolle X. (2014) G1-arrested newborn cells are the predominant infectious form of the pathogen *Brucella abortus*. Nat Commun. 9;5:4366.

Diavatopoulos DA, Hijnen M, Mooi FR. (2006) Adaptive evolution of the *Bordetella* autotransporter pertactin. J Evol Biol. 19(6):1931-8

Dotreppe D, Mullier C, Letesson JJ, De Bolle X. (2011) The alkylation response protein AidB is localized at the new poles and constriction sites in *Brucella abortus*. BMC Microbiol. 11:257

Figge RM, Easter J, Gober JW. (2003) Productive interaction between the chromosome partitioning proteins, ParA and ParB, is required for the progression of the cell cycle in *Caulobacter crescentus*. Mol Microbiol. 47(5): 1225-37.

Gee JM, Valderas MW, Kovach ME, Grippe VK, Robertson GT, Ng WL, Richardson JM, Winkler ME, Roop RM 2nd. (2005) The *Brucella abortus* Cu,Zn superoxide dismutase is required for optimal resistance to oxidative killing by murine macrophages and wild-type virulence in experimentally infected mice. Infect Immun. 73(5):2873-80.

Godfroid F, Taminiau B, Danese I, Denoel P, Tibor A, Weynants V, Cloeckaert A, Godfroid J, Letesson JJ. Identification of the perosamine synthetase gene of *Brucella melitensis* 16M and involvement of lipopolysaccharide O side chain in *Brucella* survival in mice and in macrophages. (1998) Infect Immun. 66(11):5485-93.

Guzmán-Verri C, Chaves-Olarte E, von Eichel-Streiber C, López-Goñi I, Thelestam M, Arvidson S, Gorvel JP, Moreno E. (2001) GTPases of the Rho subfamily are required for *Brucella abortus* internalization in nonprofessional phagocytes: direct activation of Cdc42. J Biol Chem. 276(48):44435-43

Hallez R, Bellefontaine AF, Letesson JJ, De Bolle X. (2004) Morphological and functional asymmetry in alpha-proteobacteria. Trends Microbiol. 12(8):361-5.

Hallez R, Mignolet J, Van Mullem V, Wery M, Vandenhaute J, Letesson JJ, Jacobs-Wagner C, De Bolle X. (2007) The asymmetric distribution of the essential histidine kinase PdhS indicates a differentiation event in *Brucella abortus*. EMBO J. 26(5): 1444-55

Henderson IR, Nataro JP. (2001) Virulence functions of autotransporter proteins. Infect Immun. 69(3):1231-43

Hengge R. (2009) Principles of c-di-GMP signalling in bacteria. Nat Rev Microbiol. 7(4):263-73.

Hernández-Castro R, Verdugo-Rodríguez A, Puente JL, Suárez-Güemes F. (2008) The BMEI0216 gene of *Brucella melitensis* is required for internalization in HeLa cells. Microb Pathog. 44(1):28-33.

Hinnebusch BJ. (2008) The evolution of flea-borne transmission in *Yersinia pestis*. Curr Issues Mol Biol. (2):197-212.

Köhler S, Ouahrani-Bettache S, Layssac M, Teyssier J, Liautard JP. (1999) Constitutive and inducible expression of green fluorescent protein in *Brucella suis*. Infect Immun. 67(12):6695-7.

Koomey JM, Gill RE, Falkow S. (1982) Genetic and biochemical analysis of gonococcal IgA1 protease: cloning in *Escherichia coli* and construction of mutants of gonococci that fail to produce the activity. Proc Natl Acad Sci U S A. 79(24):7881-5

Li G, Smith CS, Brun YV, Tang JX. (2005) The elastic properties of the *Caulobacter crescentus* adhesive holdfast are dependent on oligomers of N-acetylglucosamine. J Bacteriol. 187(1):257-65.

Matroule JY, Lam H, Burnette DT, Jacobs-Wagner C. (2004) Cytokinesis monitoring during development; rapid pole-to-pole shuttling of a signaling protein by localized kinase and phosphatase in *Caulobacter*. Cell. 118(5):579-90.

Merker RI, Smit J. (1988) Characterization of the adhesive holdfast of marine and freshwater *Caulobacters*. Appl Environ Microbiol. 54(8):2078-85

Mohl DA and Gober JW. (1997) Cell Cycle–Dependent Polar Localization of Chromosome Partitioning Proteins in *Caulobacter crescentus*. Cell. 88(5):675-84.

Moreno E. (2014) Retrospective and prospective perspectives on zoonotic brucellosis. Front. Microbiol. 13;5:213.

Moreno E. and Moriyo I. (2006) The genus *Brucella*. Prokaryotes. 5:315–456.

Pizarro-Cerdá J, Cossart P. (2006) Bacterial adhesion and entry into host cells. Cell. 124(4):715-27

Pizarro-Cerdá J, Moreno E, Gorvel JP. (2000) Invasion and intracellular trafficking of *Brucella abortus* in nonphagocytic cells. Microbes Infect. 2(7):829-35.

Pizarro-Cerdá J, Moreno E, Sanguedolce V, Mege JL, Gorvel JP. (1998) Virulent *Brucella abortus* prevents lysosome fusion and is distributed within autophagosome-like compartments. Infect Immun. 66(5):2387-92.

Posadas DM, Martín FA, Sabio y García JV, Spera JM, Delpino MV, Baldi P, Campos E, Cravero SL, Zorreguieta A. (2007) The TolC homologue of *Brucella suis* is involved in resistance to antimicrobial compounds and virulence. Infect Immun. 75(1):379-89.

Posadas DM, Ruiz-Ranwez V, Bonomi HR, Martín FA, Angeles Zorreguieta. (2012) BmaC, a novel autotransporter of *Brucella suis*, is involved in bacterial adhesion to host cells. Cell Microbiol. 14(6):965-82.

Rocha-Gracia del C, Castañeda-Roldán EI, Giono-Cerezo S, Girón JA. (2002) *Brucella sp.* bind to sialic acid residues on human and animal red blood cells. FEMS Microbiol Lett. 213(2):219-24.

Rodríguez MC, Viadas C, Seoane A, Sangari FJ, López-Goñi I, García-Lobo JM. (2012) Evaluation of the effects of erythritol on gene expression in *Brucella abortus*. PLoS One. 7(12):e50876

Rossetti CA, Galindo CL, Lawhon SD, Garner HR, Adams LG. (2009) *Brucella melitensis* global gene expression study provides novel information on growth phase-specific gene regulation with potential insights for understanding *Brucella*:host initial interactions. BMC Microbiol. 9:81

Ruiz-Ranwez V, M. Posadas DM, Van der Henst C, Estein SM, Arocena GM, Abdian PL, Martin FA, Sieira R, De Bolle X, Zorreguieta A. (2013, a) BtaE, an Adhesin That Belongs to the Trimeric Autotransporter Family, Is Required for Full Virulence and Defines a Specific Adhesive Pole of *Brucella suis*. Infect Immun. 81(3):996-1007.

Ruiz-Ranwez V, Posadas DM, Estein SM, Abdian PL, Martin FA, Zorreguieta A. (2013, b) The BtaF Trimeric Autotransporter of *Brucella suis* Is Involved in Attachment to Various Surfaces, Resistance to Serum and Virulence. PLoS ONE 8(11): e79770.

Ryan RP. (2006) Cyclic di-GMP signalling and the regulation of bacterial virulence. Microbiology. 159(Pt 7):1286-97

Salcedo SP, Holden DW. Bacterial interactions with the eukaryotic secretory pathway. 2005. Curr Opin Microbiol. 8(1):92-8.

Skerker, J.M. and Laub, M.T. (2004) Cell-cycle progression and the generation of asymmetry in *Caulobacter crescentus*. Nat Rev Microbiol. 2, 325-337.

Sloan GP, Love CF, Sukumar N, Mishra M, Deora R. (2007) The *Bordetella* Bps polysaccharide is critical for biofilm development in the mouse respiratory tract. J Bacteriol. 189(22):8270-6.

Starr T, Child R, Wehrly TD, Hansen B, Hwang S, López-Otin C, Virgin HW, Celli J. (2012) Selective subversion of autophagy complexes facilitates completion of the *Brucella* intracellular cycle. Cell Host Microbe. 11(1):33-45

Toro E, Shapiro L. (2010) Bacterial chromosome organization and segregation. Cold Spring Harb Perspect Biol. 2(2):a000349.

von Bargen K, Gorvel J.P and Salcedo S.P. (2012) Internal affairs: investigating the *Brucella* intracellular lifestyle. FEMS Microbiol Rev. 36(3):533-62

Xu J, Kim J, Koestler BJ, Choi JH, Waters CM, Fuqua C. (2013) Genetic analysis of *Agrobacterium tumefaciens* unipolar polysaccharide production reveals complex integrated control of the motile-to-sessile switch. Mol Microbiol. 89(5):929-48.