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Study of CtrA target genes in Brucella abortus

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STUDY OF CTRA TARGET GENES IN *BRUCELLA ABORTUS*

Mémoire présenté pour l'obtention

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Study of CtrA target genes in *Brucella abortus*

PONCIN Katy

Résumé

Brucella abortus est une bactérie pathogène de classe III responsable de la brucellose. La plupart des informations dont nous disposons sur le cycle cellulaire de *B. abortus* ont été déduites à partir de l'étude de l'organisme modèle *Caulobacter crescentus*. Chez cette bactérie, il existe une protéine du nom de CtrA que l'on pourrait qualifier de « master regulator ». En effet, CtrA régule l'activité d'une centaine de gènes impliqués entre autres dans la morphogenèse des pôles, la réplication de l'ADN et la division cellulaire.

Nous avons voulu étudier, à l'aide de systèmes rapporteurs basés sur l'expression d'une *gfp* instable, le rôle éventuel que pourrait jouer CtrA dans la progression du cycle cellulaire de *B. abortus* en culture et en infection. Nous avons choisi de nous concentrer sur quatre promoteurs potentiellement ciblés par CtrA et identifiés par la présence d'une séquence consensus. Ces quatre cibles sont les promoteurs de *ccrM* (une DNA methyltransferase), *repABC* (un opéron impliqué dans la réplication et la ségrégation du petit chromosome), *pleC* (une histidine kinase connue pour réguler CtrA chez *C. crescentus*) et *bmaC* (une adhésine). L'activité du promoteur de *bmaC* n'ayant jamais été détectée par notre système, nous avons rapidement décidé de mettre l'étude de ce promoteur de côté.

Au terme de ce mémoire, nous pouvons émettre les hypothèses suivantes : en culture, CtrA régulerait positivement l'expression de *ccrM* et négativement celle de *repABC*. L'expression de *pleC*, quant à elle, ne semble pas être dépendante de CtrA en culture. A l'opposé, en infection, CtrA ne joue apparemment pas de rôle majeur dans la régulation des promoteurs de *ccrM* et de *repABC*. Or, il apparait que CtrA pourrait avoir un impact sur l'activité du promoteur de *pleC*. De plus, ce promoteur semble être plus actif dans les temps précoces post-infection. Cela suggère une implication importante de PleC lors du processus infectieux.

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Finalement, merci à vous, lecteurs, de prendre le temps de lire ce mémoire. J'espère qu'il vous plaira !

Table of Contents

Abbreviations

Abbreviation	Meaning
bp	Base pairs
ChI	Chromosome I
ChII	Chromosome II
ChIP	Chromatin immunoprecipitation
Cyclic di-GMP	Cyclic diguanylate
DAPI	4',6-diamidino-2-phenylindole
EMSA	Electrophoretic mobility shift assay
FACS	Fluorescence-activated cell sorting
FMN	Flavin mononucleotide
GFP	Green fluorescent protein
IHF	Integration Host Factor
LOV	Light, oxygen, voltage
LPS	Lipopolysaccharide
ORF	Open reading frame
oriI	Replication origin of chromosome I
oriII	Replication origin of chromosome II
PFA	Paraformaldehyde
PI	Post infection
p_x	Promoter of the gene *x*
RACE	Rapid amplification of cDNA end
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
TRSE	Texas Red succinimidyl ester
WT	Wild type
YFP	Yellow fluorescent protein

Introduction

I. The α-proteobacteria class

I.1. General characteristics

I.1.1. An overview of α-proteobacteria lifestyles and morphologies

The Proteobacteria phylum was named after Proteus, a greek god of the sea who could change shape at will (Stackebrandt et al., 1988). Nowadays, the adjective "protean" is used to refer to versatility and adaptability. As their name suggests it, the α-proteobacteria class belongs to the proteobacteria phylum and consists of very diverse bacteria. They are Gram-negative bacteria and this class includes pathogens for animals (*Brucella, Rickettsia*) and plants (*Agrobacterium*), symbionts of arthropods (*Wolbachia*) and plants roots (Rhizobiales which fix nitrogen) as well as free living and opportunistic bacteria like *Caulobacter* and *Ochrobactrum*, respectively (Moreno and Moriyón, 2006). The α-proteobacteria class also seem to represent the most abundant marine cellular organisms (Giovannoni *et al.*, 2005), yet what they are most famous for is the fact that they are considered as the ancestors of mitochondria (Esser et al. 2004).

Without surprise, α-proteobacteria also display very different morphologies. The best known are stalked bacteria. They includes bacteria such as *Caulobacter crescentus*, *Asticaccaulis biprosthecum* and *Hyphomonas neptunium.* These three bacteria are characterized by a dimorphic life cycle resulting from the production of two functionally and morphologically different cells at every cell division: a motile swarmer cell and a sessile stalked cell (**Figure 1**). Stalks are thin tubular extensions of the cell body and seem to be involved in nutrient uptake (Wagner and Brun, 2007). In *Caulobacter crescentus,* the most extensively studied α-proteobacterium, only the stalked cell is competent for replication. The differentiation from a swarmer cell to a stalked cell is controlled by a complex regulatory network which leads to the ejection of the polar flagellum and the formation of a stalk at the same old pole. The new stalked cell can thereafter initiate DNA replication and elongate before dividing and giving rise to a sessile stalked cell and a newborn flagellated cell which can swim away. *Asticcacaulis bisprosthecum* has a similar life cycle but generates two stalks on the side instead of one at the old pole (Porter et al. 1973). The life cycle of *Hyphomonas neptunium* is even more unusual as the flagellated cell arises from a budding of the stalk tip, which implies the transfer of a chromosome through what used to be a stalk (Weiner & Blackman 1973). Even the other α-proteobacteria that appear to have a more classical rod-shaped phenotype can be regarded as a having very different phenotypes. For example, *Ochrobactrum* and *Agrobacterium* possess peritrichous flagellae, while *Brucella* is a non-motile coccobacillus. There also exist spiral-shaped bacteria such as *Rhodospirillum* or the recently discovered mono-flagellated *Kiloniella laminariae* (Wiese et al. 2009).

I.1.2. The asymmetric division and the concept of aging

Despite their obvious heterogeneity, α-proteobacteria seem to share common features. One of them is that they divide asymmetrically, or at least it is the case for *Caulobacter crescentus, Brucella abortus, Sinorhizobium melitoti, Agrobacterium tumefaciens* and *Methylobacterium extorquens* (**Figure 2**) (Bergmiller and Ackermann, 2011; Hallez et al.,

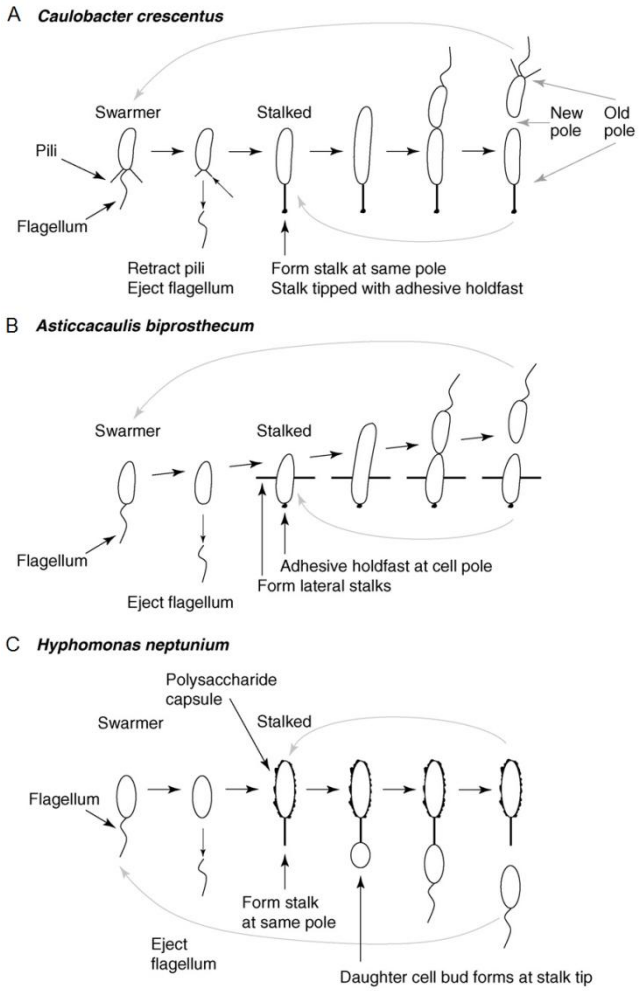

Figure 1: schematic representation of three stalked bacteria cell cycle. (A) *Caulobacter crescentus*, **(B)** *Asticaccaulis biprosthecum* and **(C)** *Hyphomonas neptunium* all undergo a transformation from a flagellated form (swarmer cell) to a stalked form. After the asymmetric division, the newborn swarmer cell must go through a differentiation process before to be able to divide, at the opposite of its sister stalked cell which can directly initiate a new cell division (Lawler and Brun, 2007).

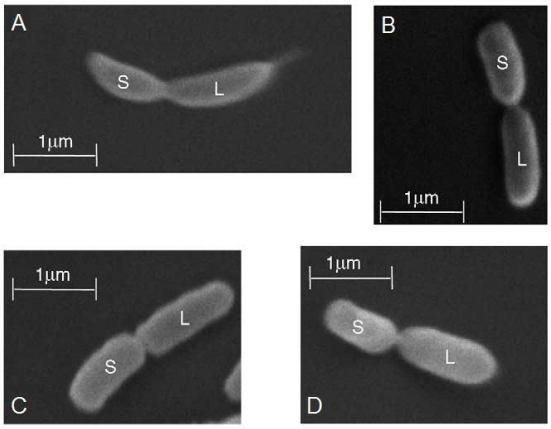

Figure 2: Asymmetric division in α-proteobacteria. This phenomenon has been observed with electron microscopy for **(A)** *Caulobacter crescentus*, **(B)** *Brucella abortus*, **(C)** *Sinorhizobium meliloti* and **(D)** *Agrobacterium tumefaciens*. L and S respectively design the large and small cells (Hallez et al., 2004)

2004). This characteristic is very clear in *C. crescentus* but it is less obvious in the other three bacteria since they do not display polar organelles. However, the two daughter cells have different sizes and an asymmetric subcellular localization of a signal transduction protein called DivK can be observed in *S. melitoti* and *B. abortus*, for example (Hallez et al., 2007; Lam et al., 2003). Recently, it has been shown that a magnetotactic bacterium (i.e. able to orient itself in function of a magnetic field) called *Magnetospirillum gryphiswaldense* had to divide asymmetrically in order to overcome its intrinsic magnetic force (Katzmann et al., 2011).

Dividing asymmetrically can bring an obvious advantage to bacteria in stressful and changing environments. The best example of this is the swarmer cell produced by the asymmetric division of *C. crescentus*. This form is sent away from the stalked cell in order to look for nutrients in a new habitat. Of course, this bet edging strategy (i.e. to produce multiple phenotypes and disperse the offspring across a spatially heterogeneous environment) is not without risks, but it is still an efficient way to generate the long term fitness of the population (Meyers and Bull, 2002). However, in the cases of *B. abortus*, *S. melitoti*, or *A. tumefaciens*, the two daughter cells are not morphologically very different, which suggests there might be another advantage to dividing asymmetrically. Since polar growth is conserved among rhizobiales, its seems logical that the division of the bacteria cited above will eventually lead to the formation of differentially aged cells (Brown et al., 2012). In *C. crescentus*, old stalked cells have been shown to be less fertile than their sister swarmer cells after their have changed into a stalked form (Ackermann et al., 2003). It has been suggested that asymmetry could be a mean to segregate cellular damages in the old bacteria, which would confer a higher fitness to the damage-free daughter (**Figure 3**) (Erjavec et al., 2008; Kysela et al., 2013). The asymmetric division that seems to characterize the α-proteobacteria could thus augur for more important differences between the daughter cells, such as damages segregation.

I.1.3. The repABC-type system

Building a species tree for α-proteobacteria has always been challenging because they also diverge at the genomic level. Phylogenetic trees that are based on rRNA are discordant since 16S and 23S trees do not lead to the same conclusion (Williams et al., 2007). Building the tree from a collection of protein families seems to give better results but a clear consensus has not been reached yet (Brilli et al., 2010; Gupta and Mok, 2007; Williams et al., 2007). Interestingly, α-proteobacteria genomes vary broadly from 1 to 9 Mb. The bacteria associated with animals have undergone gene loss, whereas plant-associated bacteria that are growing on the soil seem to have evolved by expanding their genome (Batut et al., 2004). Such a difference can partly be explained by the presence of dynamic auxiliary replicons (Batut et al., 2004).

Most of the α-proteobacteria plasmids encode at least one *repABC* cassette. *repA*, *repB* and *repC* are organized in an operon in many bacterial species, a list of which can be found in a paper of Cevallos et al. (2008). The roles of these genes are not very clear yet but *repC* is considered to include the origin of replication and to be sufficient for replication, while the two other genes would be required for an efficient partitioning of the daughter plasmids. *repC* has been found exclusively in the α-proteobacteria class, in contrast to *repA* and *repB*, the products of which are similar to other partitioning system proteins that are widely distributed among other bacteria (Pappas et al., 2012). Interestingly, some of the α-proteobacteria plasmids appear to be very similar to the main chromosome. For instance, their sizes and their GC content are alike. Moreover, these plasmids encode at least one essential gene, which is why they are referred to as secondary chromosomes or chromids (Harrison et al., 2010).

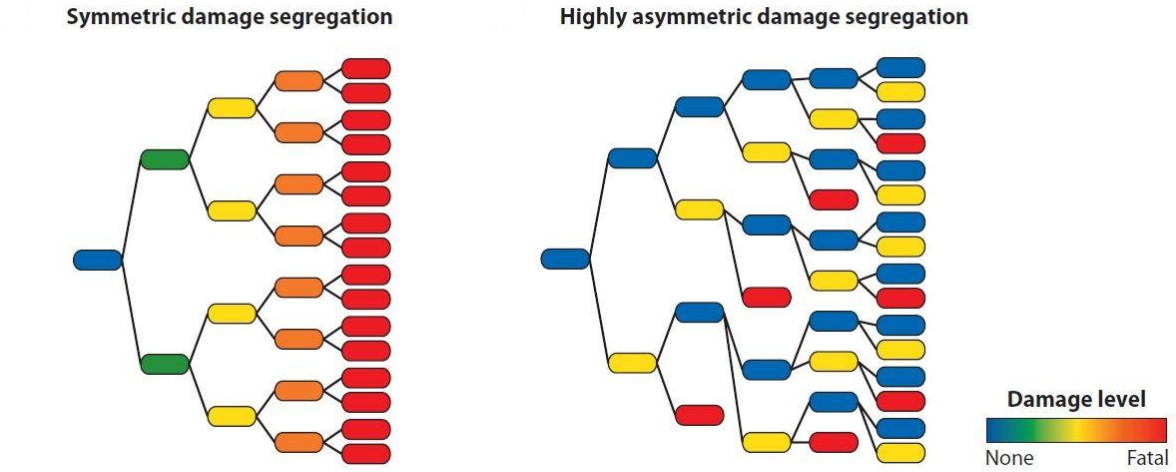

Figure 3: Simplified models of damage segregation. (a) In a symmetric damage segregation model, both daughter cells inherit the same damage levels, whereas in the **(b)** highly asymmetric damage segregation model, all the damages are shunt into one daughter cell (Kysela et al., 2013).

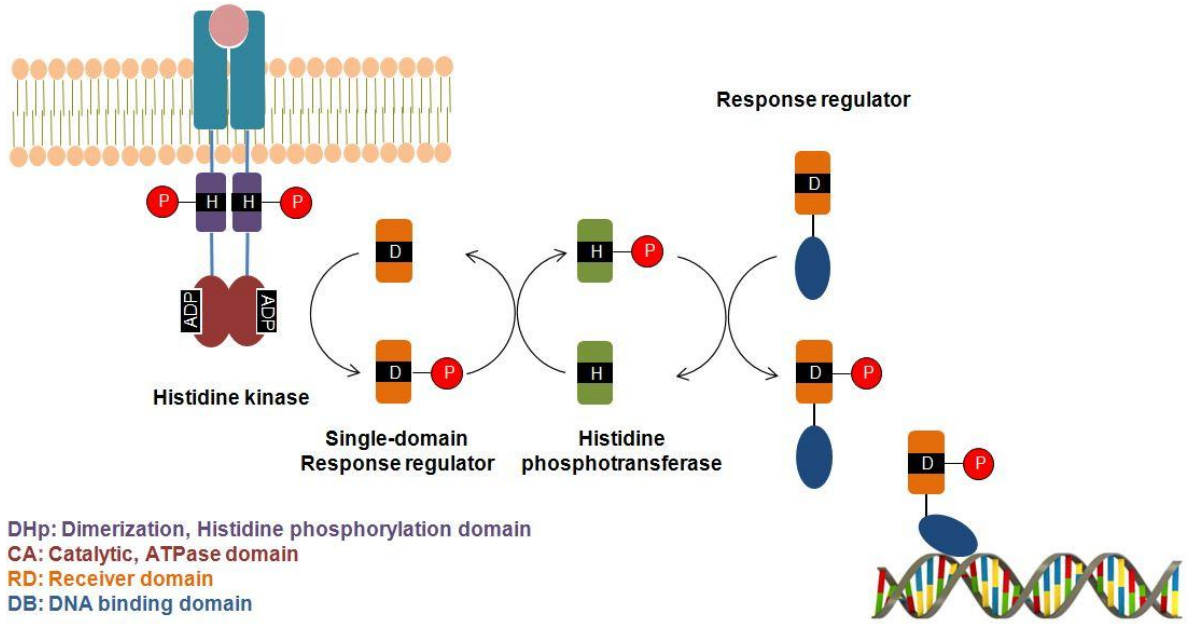

Figure 4: Schematic representation of a phosphorelay signal transduction system. This type of system involves sequential phosphorylations. It begins by the recognition of a signal by a histidine kinase. This leads to its autophosphorylation, which is followed by the transfer of the phosphoryl group on conserved His/Asp residues from protein to protein in the order His -> Asp -> His -> Asp. The final receiver is a response regulator, which is often a transcription factor (N. Francis).

I.1.4. CtrA, a highly conserved gene

A gene that appears to be particularly conserved in the α-proteobacteria class is *ctrA* (Brilli et al., 2010). CtrA, its regulon and its targets have been extensively studied in the model organism *Caulobacter crescentus*, which is why a whole chapter will be dedicated to this question (see point I.2.). In short, in *Caulobacter crescentus*, CtrA is the master regulator of the cell cycle and is responsible for the direct regulation of about 100 genes that are involved in many different processes (Laub et al., 2002). A study based on a BLAST search has revealed that almost all α-proteobacteria have a homologue of the *ctrA* gene. Indeed, over the 65 bacteria that were chosen among the different genus, only 3 did not seem to possess it (Brilli et al., 2010). It has also been shown that several genes that are involved in the regulation of the cell cycle seem to be transcriptionally regulated by CtrA in most of the α-proteobacteria (Brilli et al., 2010; Hallez et al., 2004).

The *ctrA* gene is essential in *Caulobacter crescentus*. If it has been suggested to also be the case for *Sinorhizobium melitoti* (Barnett et al., 2001), *Agrobacterium tumefaciens* (Kahng and Shapiro, 2001) and *Brucella abortus* (Bellefontaine et al., 2002; Robertson et al., 2000); it does not seem to be systematic in the other α-proteobacteria. As a matter of fact, it is not essential for the viability of *Rhodobacter capsulatus* (Mercer et al., 2010), *Rhodospirillum centenum* (Bird and MacKrell, 2011), *Silicibacter* sp. (Miller and Belas, 2006) and *Magnetospirillum magneticum* (Greene et al., 2012). It is noteworthy that CtrA seems to be involved in the regulation of motility in most α-proteobacteria, which suggests that this function is an ancestral trait (Greene et al., 2012). Besides, CtrA has been linked to the resistance to stress in the obligatory intracellular pathogen *Ehrlichia chaffeensis* (Cheng et al., 2011) and in the free living *C. crescentus* (Bastedo and Marczynski, 2009). In *R. capsulatus*, it is needed to generate virus-like gene transfer agent particles (Lang and Beatty, 2000, 2002) and it is thought to be involved in the symbiotic process of *S. melitoti* (Pini et al., 2013). In *A. tumefaciens*, one of the promoters of the *repABC* operon has been proposed to be regulated by CtrA (Pappas and Winans, 2003; Pappas et al., 2012). Those various roles tend to underline how flexible the CtrA regulatory network can be within the α-proteobacteria class.

I.2. CtrA in the model organism *Caulobacter crescentus*

I.2.1. CtrA regulation

As mentioned before, CtrA has been largely investigated in *C. crescentus*. It is the master regulator that eventually leads to its asymmetric division. Its activation is based on a regulatory network involving sequential phosphorylations. Basically, a signal is received by a histidine kinase. This induces its autophosphorylation, followed by the transfer of the phosphoryl group to an aspartate residue of a single-domain response regulator. The phosphoryl group is then transferred to an intermediate protein called a histidine phosphotransferase before ending up on the receiver domain of a final response regulator which has often a C-terminal DNA binding domain and is thus a transcription factor. This type of system is known as a phosphorelay (**Figure 4**) (Buelow and Raivio, 2010).

CtrA phosphorylation and therefore activation depends of the PleC-DivJ-DivK signaling network (**Figure 5**). DivK is a single-domain response regulator and its phosphorelation state is regulated by the histidine kinases DivJ and PleC. In the swarmer cell,

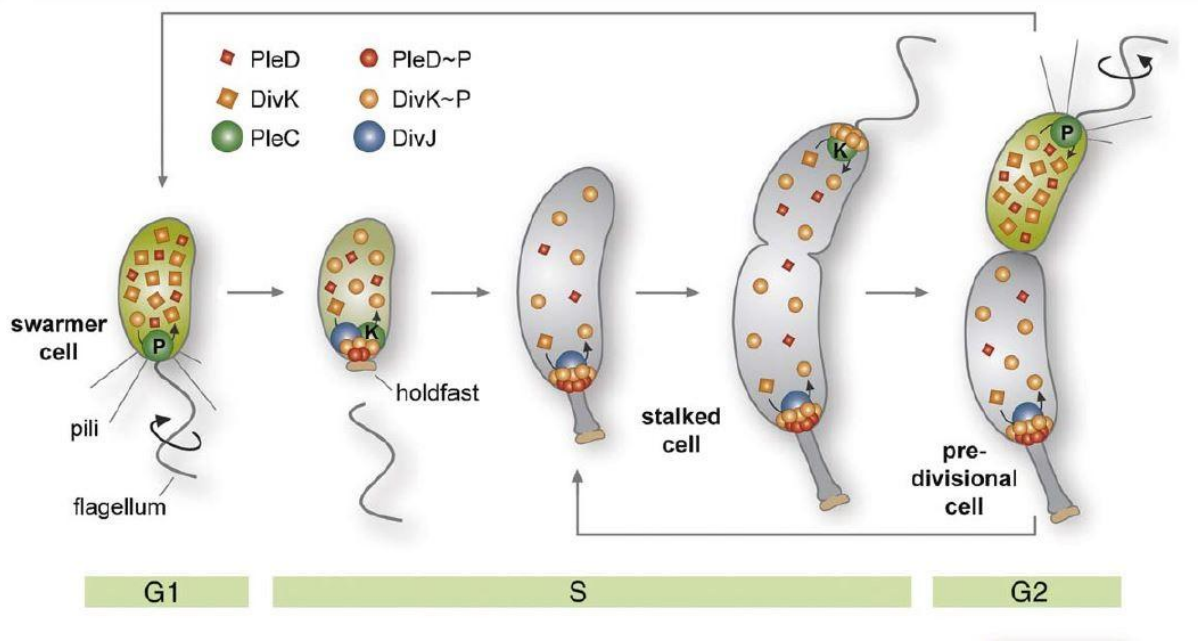

Figure 5: The PleC-DivJ-DivK signaling network. The non-phosphorylated form of the response regulator DivK is evenly distributed within the cell, at the opposite of its phosphorylated form which interacts with the cell poles. Its phosphorylation state is dependant on both pleC, a bifunctionnal enzyme (kinase/phophatase) and DivJ, a histidine kinase. In the swarmer cell, PleC is the main actor present and it works in its phosphatase mode. This leads to an accumulation of non-phosphorylated DivK and, eventually, to CtrA phosphorylation (green background). The differentiation process from a swarmer to a stalked cell occurs, among others, through the phosphorylation of the response regulator PleD by PleC. In the stalked cell, DivJ is present and, in this model, PleC switch of activity from the phosphatase to the kinase mode trigger the phosphorylation of DivK. This induces the elimination of CtrA~P. CtrA stays present in the predivisionnal cell and in the swarmer cell in order to block the inhibition of the chromosomes replication. At the opposite, the stalked cell has to be CtrA-free to directly initiate a new cell division (Thanbichler, 2009).

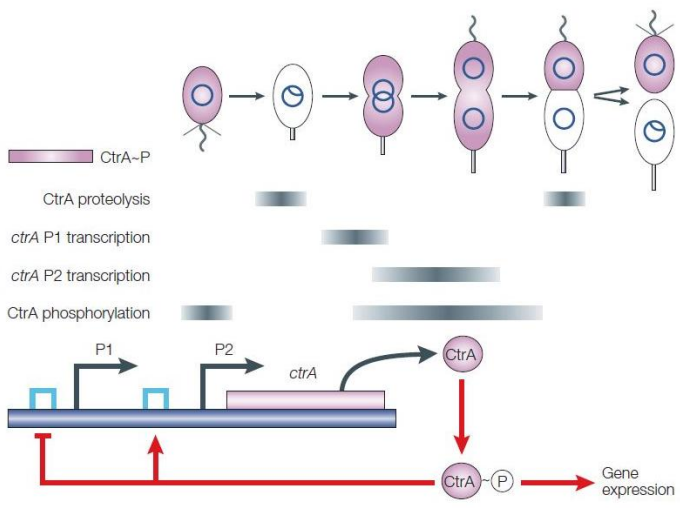

Figure 6:. CtrA regulations. CtrA is regulated at three levels: at the transcriptional level, by phosphorylation and by proteolysis. The first promoter (P1) is activated for a short length of time in the early predivisional cells in order to generate a burst of CtrA, which will be followed by the activation of the second and stronger promoter (P2). Once phosphorylated, CtrA can efficiently bind to its targets, one of which is the origin of replication. Thus, CtrA needs to be degraded in the stalked cell in order to free this binding site and allow the initiation of chromosome duplication to occur (Skerker and Laub, 2004).

DivJ is hardly present and PleC localizes at the swarmer pole where it acts as a phosphatase on DivK. The non-phosphorylated form of DivK is evenly distributed in the cytoplasm of the cell (Lam et al., 2003) and is unable to repress a secondary phosphorelay composed of the hybrid kinase CckA, the histidine phosphotransferase ChpT and the final response regulator CtrA. As a hybrid kinase, CckA possesses both a kinase domain and a receiver domain. Thus it can directly phosphorylate ChpT which will in turn phosphorylate CtrA (Biondi et al., 2006). During the transition from a swarmer cell to a stalked cell, DivJ is produced and has been hypothesized to co-localize with PleC at the old pole. DivJ induces the phosphorylation of DivK and its accumulation at the stalked pole. The phosphorylated form of DivK triggers a switch in PleC activity from the phosphatase to the kinase mode (Paul et al., 2008). After the differentiation event, PleC is found to be delocalized from the stalked pole. Hence, in the stalked cell, phosphorylated DivK is localized to the stalked pole and inhibits the CckA-ChpT phosphorelay, thus preventing CtrA phosphorylation and activation. In the predivisional cell, PleC relocalizes at the swarmer pole. At the same time, DivJ is still present at the stalked pole, where it phosphorylates DivK. In a model suggesting that PleC acts as a phosphatase at the swarmer pole of the predivisional cell, DivK shuttles from one pole to the other (Matroule et al., 2004). The partial dephosphorylation of DivK at the swarmer pole allows again the phosphorylation of CtrA. After cytokinesis, PleC and DivJ are located in physically separated compartments (the future sibling cells), which divides the DivK pool into two: mostly phosphorylated in the (future) stalked cell and predominantly dephosphorylated in the (future) swarmer cell (Matroule et al., 2004). PleC can therefore keep its phosphatase activity in the newborn swarmer cell (Paul et al., 2008), while DivJ ensures DivK phosphorylation in the stalked cell (Lam et al., 2003).

The phosphorylation state of CtrA is not the only level of control exerted on this protein. Actually, CtrA proteolysis has also a major role to play. CtrA is degraded concomitantly to its dephosphorylation during the swarmer-to-stalked cell transition and in the stalked cell after cytokinesis (Domian et al., 1997). The protease responsible for CtrA proteolysis is ClpXP (Jenal and Fuchs, 1998). It is recruited at the stalked pole by the response regulator CpdR when the latter is unphosphorylated (Iniesta and Shapiro, 2008). In addition to leading to CtrA proteolysis, the polar localization of CpdR also enables its own ClpXP-mediated degradation. This triggers ClpXP release from the pole and protects the remaining CtrA from degradation (Jenal, 2009). In predivisional cells, CpdR gets phosphorylated by CckA and CtrA needs to be synthesized again in order to fulfill one of its most crucial roles, the inhibition of the initiation of a second round of DNA replication (Iniesta et al., 2006; Quon et al., 1998). The transcription of *ctrA* is regulated by two promoters known as P1 and P2 (Domian et al., 1999). The weak P1 is activated first in the early predivisional cells to generate a small burst of CtrA synthesis. Its activation requires the action of a protein called GcrA as well as a switch into its hemi-methylated state when the replication fork reaches *ctrA* (Holtzendorff et al., 2004; Reisenauer and Shapiro, 2002). As soon as a sufficient amount of CtrA is present and phosphorylated in the late stalked cell, it can repress P1 activity and activate the strong P2 promoter (**Figure 6**) (Domian et al., 1999). Interestingly, a small protein called SciP can bind to CtrA to prevent it from activating its target genes in swarmer cells. SciP does not seem to hinder CtrA ability to bind DNA, which can explain why CtrA can still efficiently silence the origin of replication. During the G1-S transition, SciP is proteolysed in order to free CtrA and allow it to activate its target genes (Gora et al., 2010).

I.2.2. CtrA targets

It is well known that the initiation of chromosome replication occurs only once every cell cycle and only takes place in the stalked cell of *C. crescentus* (Marczynski, 1999). As explained above, CtrA is responsible for the inhibition of the initiation of replication in the swarmer cell. It does so by binding to five sites situated near the origin of replication (Quon et al., 1998). Those CtrA binding sites are characterized by a 9-mer consensus sequence (TTAA-N_7-TTAAC) (Marczynski and Shapiro, 1992). One of those sites overlaps with a binding site for DnaA (Taylor et al., 2011) as well as with a binding site for an Integration Host Factor (IHF) (Siam et al., 2003). DnaA is in charge of opening the double-stranded DNA helix, whereas IHF is a histone-like protein that is able to bend DNA (Swinger and Rice, 2004). The displacement of CtrA in the stalked cell is thought to be reinforced by the competition arising between those proteins for their common binding site.

CcrM is a DNA methyltransferase known to be tightly regulated by CtrA in *C. crescentus*. The fact that *ccrM* expression requires CtrA implies that the chromosome will be fully methylated only when CtrA is present and represses the initiation of replication. This mechanism thus ensures that there will be only one replication per cell cycle (Curtis and Brun, 2010). This is also supported by the fact that *ccrM* overexpression leads to abnormal chromosome content (Zweiger et al., 1994). Its temporal regulation is also important since it must only be present in late predivisional cells, where it methylates the newly synthesized DNA strands on GANTC sites precisely before cell division occurs (Reisenauer et al., 1999; Stephens et al., 1996). This means that, according to their chromosomal position away from the origin of replication, genes stay hemimethylated for a different amount of time during the cell cycle (Marczynski, 1999). This could have an important impact on gene expression at the whole genome level. As a matter of fact, the transcription of several genes of *C. crescentus* has been observed to change in response to the methylation state of their promoter (Collier and Shapiro, 2007; Gonzalez and Collier, 2013). Remarkably, the promoter of *ctrA* is one of those (Reisenauer and Shapiro, 2002).

As a master regulator, CtrA is also able to regulate, positively or negatively, the transcription of at least 95 genes (Laub et al., 2002). In addition to the classical 9-mer sequence, another consensus sequence has been found to be linked by CtrA in *C. crescentus*. It is the 8-mer sequence TTAACCAT (Laub et al., 2002). The genes that are regulated by CtrA are involved in very diverse processes such as cell division (*ftsZ*), cell wall synthesis, polar morphogenesis (*pilA*, flagellar genes) and proteolysis (*clpP*). Interestingly, it has also been shown that CtrA can modulate its own phosphorylation pathway by regulating *divK* promoter (p_{divK}) in *C. crescentus* (Laub *et al*., 2002).

II. *Brucella*

II.1. Brucellosis

II.1.1. A worldwide zoonosis

Brucellosis has been considered as a major worldwide zoonosis (Pappas et al., 2006). The causative agents of this disease are Gram negative coccobacilli of the genus *Brucella*. They are referred to as facultative intracellular bacteria but it would be more correct to say that they are facultative extracellular intracellular pathogens since they are localized inside

host cells most of the time (Moreno and Moriyon, 2002). They have been divided into six classical species: *B. melitensis, B. abortus, B. suis, B. canis, B. ovis* and *B. neotomae*. Since the improvement of detection methods, several new species have been discovered. For instance, *B. ceti, B. pinnipedialis* and *B. microti*. Even though their genomes share more than 94% of sequence identity, these species have very different host preferences amongst mammals. They infect preferentially goats, cattle, pigs, dogs, sheep, desert wood rats, cetaceans, seals and common voles, respectively. In animals, brucellosis occurs as a chronic infection characterized by orchitis and epididymitis in males or placentitis and abortion in pregnant females (Xavier et al., 2010).

B. melitensis, *B. abortus* and *B. suis* are recognized as pathogens for humans (Moreno and Moriyon, 2006). Human brucellosis is a debilitating disease also known as undulant fever or Malta fever. Clinical manifestations commonly appear within 5 to 60 days after exposure to the bacteria. According to a systematic review of 33 databases, the main symptoms are weakness and fever, followed by joint, muscle, and back pain. Testicular infection concerns one man over ten and severe complications such as endocarditis and neurological cases occur respectively with 1 and 4 cases per 100 patients (Dean et al., 2012). Usually, human infections happen trough the ingestion of contaminated dairy products or by exposure to infected animals. The aerosol route is also a major mean of infection, which is why *Brucella* strains are subjected to strict regulations in laboratories (Yagupsky and Baron, 2005). There are no vaccines available for humans and the usual treatment is the use of antibiotics (Moreno & Moriyon, 2006).

II.1.2. From the infection to the abortion

The mechanism by which *Brucella* manage to invade their host organism is not very clear yet but they seem to cross the mucosal barrier, which implies an interaction with epithelial cells. The role of these cells has not been deciphered yet but epithelial HeLa cells have been effectively used as models for nonprofessional phagocytes (Roop et al., 2009). Since brucellosis can become a chronic illness, the bacteria must also have a persistence niche. The organs from the reticuloendothelial system, such as the spleen and liver, have been proposed as such (Ficht, 2003). *Brucella* is also able to persist and replicate in phagocytic cells like macrophages and dendritic cells (Roop et al., 2009). These cells could therefore be another important reservoir for the bacteria since they provide them with a safe place away from antibodies and the complement. Interestingly, *Brucella* can prevent the apoptosis of the macrophages they have invaded (Gross et al., 2000) and inhibit the maturation of dendritic cells (Billard et al., 2007; Gross et al., 2000; Salcedo et al., 2008).

The placenta is bound to be a relatively suppressed immune zone since it is linking the mother and her genetically different offspring. Its physical and hormonal characteristics thus make it a privileged tissue in which *Brucella* can proliferate in large numbers (Alexander et al., 1981). This particular tropism of the bacteria for the placenta could also be explained by the high concentration of the sugar alcohol erythritol in ruminant placental trophoblasts during the third trimester of pregnancy (Samartino and Enright, 1993; Smith et al., 1962). Indeed, erythritol is one of the favorite carbon sources for *Brucella* (Sperry and Robertson, 1975). The excessive proliferation of the bacteria in the reproductive tract of its host eventually leads to the disruption of the placenta, which can cause abortion or the birth of an infected and weak offspring (Roop et al., 2009). The bacteria can then spread from one animal to another since they are present in high numbers in the aborted foetus, the discharged reproductive tract and milk (Moreno and Moriyon, 2006).

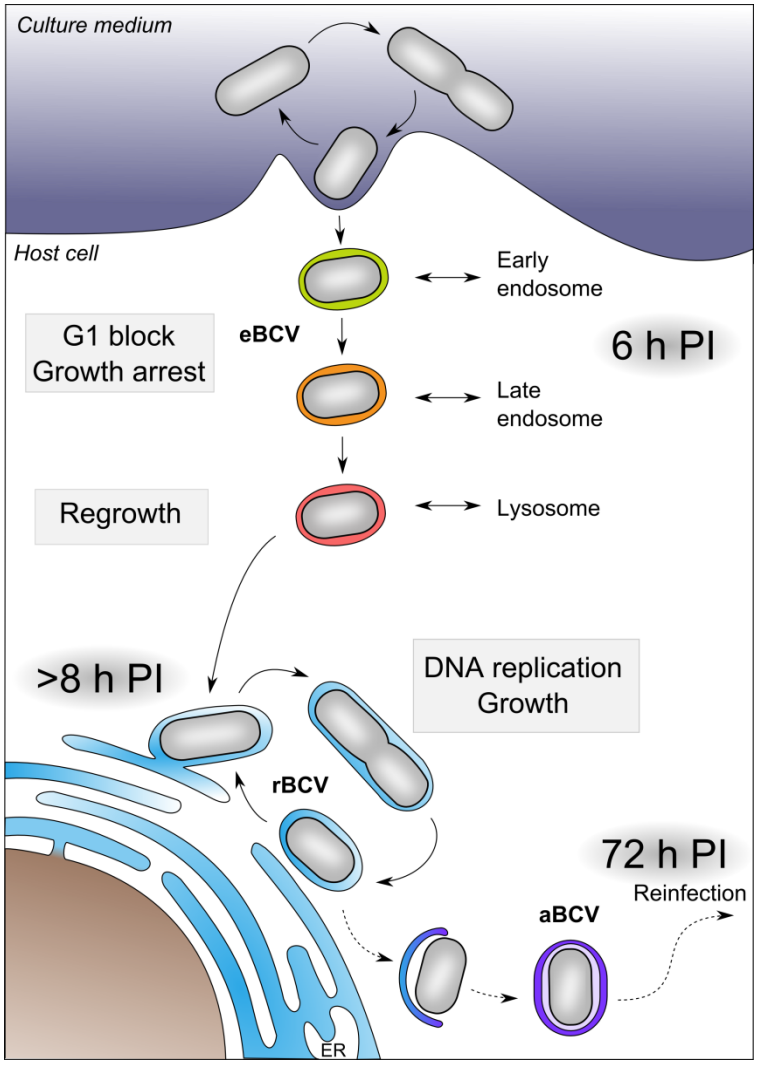

Figure 7: *Brucella abortus* trafficking. Once inside its host cell, *B. abortus* extensively interacts with the endocytic pathway. The compartment in which it resides at that stage can be referred to as the endocytic *Brucella*-containing vacuole (**eBCV**). During this first step of the infection, the bacterium is blocked in G1 and its growth is arrested. After a transient interaction with the lysosomes, the bacterium reaches its replicative niche (**rBCV**), which is an endoplasmic reticulum-like compartment. Later on, the bacteria are thought to be able to reinfect neighbor cells through an autophagy-dependant vacuole (**aBCV**).

II.2. The intracellular life of *Brucella*

II.2.1. Brucella's trafficking

Adhesion to the host cell is often a limiting step of bacterial infection (Pizarro-Cerdá and Cossart, 2006). However, in the case of *Brucella*, it has not been scrutinized much yet. Non-opsonized *Brucella* uptake into murine macrophages, human monocytes and human dendritic cells is at least partially mediated by lipid rafts (Billard et al., 2005; Naroeni and Porte, 2002; Watarai et al., 2002). Remarkably, it appears that all the epithelial cells of a monolayer are not evenly infected by the bacteria since only a few of them are associated with bacteria (Gorvel and Moreno, 2002). The internalization of *Brucella* in HeLa cells seems to occur through a discrete recruitment of actin filaments and sometimes in a phagocytosis-like manner (Gorvel and Moreno, 2002). The activation of Rho GTPases as well as the MAPK kinase pathway and the PIP3 kinase also seems to be involved in this process (Guzmán-Verri et al., 2001). *B. suis* adhesion to HeLa cells apparently requires the binding of a protein called BmaC, a predicted monomeric autotransporter of 340 KDa (Posadas et al., 2012). This adhesin is able to bind fibronectin, a component of the extracellular matrix, which has been proposed to be a key player in *B. abortus* internalization (Campbell et al., 1994). Interestingly, the *bmaC* gene seems to be conserved in *B. abortus* and the presence of possibly two CtrA binding boxes in its putative promoter were found *in silico* using RSA-tools (van Helden, 2003). Recently, two other adhesins called BtaE and BtaF have been found and, like BmaC, they are associated to the bacterial surface of the new cell pole (Ruiz-Ranwez et al., 2013a, 2013b). BtaE is involved in the binding of *B. suis* to hyaluranic acid (Ruiz-Ranwez et al., 2013a), whereas BtaF can bind to several components of the extracellular matrix and to an abiotic surface (Ruiz-Ranwez et al., 2013b).

The entry of *Brucella* into epithelial cells occurs within minutes after inoculation and it happens for one to two bacteria per cell (Pizarro-Cerdá et al., 1998), and only for a small fraction of the cellular population (typically 1 to 10% in HeLa cells). Once internalized, the bacterium stays in a *Brucella*-containing vacuole (BCV) that interacts with the endocytic pathway (Pizarro-Cerdá et al., 1998). It manages to alter the pathway in order to only undergo transient interactions with the late endosomes and the lysosomes (Starr et al., 2008). This induces the BCV acidification that is necessary for the bacterium to survive and reach its replicative niche (Boschiroli et al., 2002; Porte et al., 1999; Starr et al., 2008). The acidic pH of the BCV has been linked with the capacity of *Brucella* to induce the expression of specific genes such as the *virB* operon. This operon encodes a type IV secretion system (T4SS) that is essential for the bacteria to traffic until their proliferation niche (Boschiroli et al., 2002). The bacteria replicative niche is an endoplasmic reticulum-like (ER) compartment in both HeLa cells and macrophages (**Figure 7**) (Celli et al., 2003; Pizarro-Cerdá et al., 1998). However, it is interesting to note that opsonized *B. abortus* strains do not seem to interact intensively with the ER when they proliferate in the human monocytic cell line THP-1 (Bellaire et al., 2005).

Until recently, the way Brucella reinfectes another cell after its replication in the ER-like compartment was not very clear. A destruction of the host cell had been observed when the bacteria number reached an excessive amount but besides this, no other mean of spreading from one cell to its neighbors was known (Moreno and Moriyon, 2006). The formation of a compartment with macroautophagic features could be the key to this missing step since autophagy-deficient *Brucella* are not able to perform cell-to-cell spreading, when cellular infections are prolonged for long periods, typically 72 h (Starr et al., 2012). Macroautophagy,

here referred to as autophagy, is a cellular process in charge of the lysosomal degradation and recycling of long-lived cytoplasmic proteins and organelles (Ferraro and Cecconi, 2007). It begins with the formation of a phagophore (or isolation membrane), a double membrane structure that grows at both ends before closing and engulfing cytoplasm and organelles to form an autophagosome, which will eventually fuse with a lysosome (Ferraro and Cecconi, 2007). Interestingly, only the initiation complex seems to be needed by *Brucella* to promote reinfection (Starr et al., 2012). Indeed, markers of the elongation phase of autophagy such as ATG5 and LC3 were not found within the autophagic-like BCV (Starr et al., 2012). It should be noted that autophagy is particularly important at birth. At that time, the transplacental nutrient supply is no more available, which suggests that autophagy is strongly activated in the neonate in order to adapt to the early neonatal starvation period (Kuma et al., 2004). The use of this process by the bacteria could therefore be relevant for the spreading of the bacteria inside new-born calves.

II.2.2. Brucella cell cycle

Without taking the initial killing occurring in macrophages and the newly discovered autophagic-like BCV into account, the infection of eukaryotic cells by *B. abortus* can be considered as biphasic. Indeed, it begins by a non-proliferative stage in which the number of colony forming unit (CFU) is stable. This step corresponds to the transit of the bacteria within endocytic vacuoles. The second phase occurs when the Brucellae reach the ER-like compartment. At that time, the number of CFU increases, which suggests the relaunch of the active growth and cell division of the bacteria (Celli et al., 2003; Pizarro-Cerdá et al., 1998; Starr et al., 2008). Since *B. abortus* growth is unipolar, it is possible to follow its state by labeling the bacteria with Texas-Red conjugated to succinimidyl ester (TRSE) (Brown et al., 2012). This technique confirmed the assumption that the bacteria are arrested for growth during the first stage of their trafficking (Mullier, unpublished data; Deghelt et al., article in revision).

Contrarily to *C. crescentus*, *B. abortus* has got two distinct chromosomes (Chain et al., 2005). On the one hand, the large chromosome (ChI) of 2.1 Mb possesses a *parAB* segregation system with three centromere-like *parS* sites. On the other hand, the small chromosome (ChII) of 1.2 Mb is a chromid, the replication of which is controlled by a *repABC* system. This operon also contains two centromere-like sequences called *repS* (Livny et al., 2007; Deghelt et al., article in revision). Both chromosomes are oriented along the cell length, with a privileged association of the ChI origin and terminator with the poles, on the contrary of the origin and terminator of ChII which are commonly found closer to the midcell (Deghelt et al., article in revision). The chromosomal replication status of *B. abortus* can be followed with fluorescent reporters of the segregation markers ParB and RepB, as well as reporters allowing localization of the replication origins and the terminators. Those markers made it possible to observe that during the non-proliferative stage of the trafficking, the bacteria are blocked in G1, similarly to what happens in the carbon-starved swarmer cells of *C. crescentus* (Lesley and Shapiro, 2008; Deghelt et al., article in revision). Moreover, the bacteria that are found within the BCV at early times after infection are predominantly newborn cell types. This term indicates bacteria that just underwent cell division and that have not initiated their chromosomes replication yet (Mullier, unpublished data; Deghelt et al., article in revision).

TRSE labeling showed that bacterial growth restarts between 6 and 8 hours post infection in HeLa cells, when they are not supposed to be in the ER-like compartment yet

(Mullier, unpublished data). Contrarily to the first stage of the infection when most bacteria are blocked in G1, the proportion of proliferating bacteria at 18 hours post infection reaches the level obtained in rich culture medium. This indicates that they are in an adequate environment for optimal proliferation. It is also interesting to note that *oriII* is systematically duplicated after *oriI*. This suggests that there is a mechanism coordinating the two chromosome replication systems (Deghelt et al., article in revision).

Objectives

In *B. abortus*, not much is known about CtrA regulation and target genes. In 2002, several putative target promoters (*ctrA*, *ccrM*, *pleC*, *minC*, *ftsE*, *rpoD*) of CtrA have been identified by their binding by a recombinant His_6-CtrA in DNAse I footprinting experiments (Bellefontaine et al., 2002). All these promoters possess the 9-mer TTAA-N7-TTAAC motif in their promoter, which suggests that this consensus sequence is conserved in other α-proteobacteria. It should also be noted that, in these experiments, His_6-CtrA could not bind to *divK* and *ftsZ* promoters or the origin of replication, which are all known to be CtrA targets in *C. crescentus* (Bellefontaine et al., 2002). Nevertheless, the six identified targets in *B. abortus* are involved in similar cellular processes than those controlled by CtrA in *C. crescentus* (Bellefontaine et al., 2002). In addition to this study, we were able to predict other putative targets of CtrA using RSA-tools (van Helden, 2003) to search *in silico* for the presence of 8-mer and 9-mer CtrA binding boxes in all the intergenic regions of *B. abortus* genome. Most of the identified genes found by this method were involved in cell wall biogenesis, but a few others drew our attention. For instance, we found that *bmaC* and the *repABC* operon both had CtrA binding boxes consensus sequences in their promoters. As a reminder, the *bmaC* gene supposedly codes for an adhesin able to bind fibronectin (Posadas et al., 2012), and the *repABC* operon is predicted to control ChrII initiation of replication and the segregation of the replicated *oriII* (Deghelt et al., article in revision).

The aim of this master thesis was to investigate the regulation of several potential CtrA target promoters during either cell cycle or the course of a cellular infection. Indeed, CtrA is proposed to be involved in the control of cell cycle progression, and we were interested to investigate the possible role of CtrA in the cell cycle arrest detected inside host cells. The four putative CtrA-dependent targets we chose to look at are the promoters of *bmaC* (p_{bmaC}), *repABC* (p_{repABC}), *ccrM* (p_{ccrM}) which is a DNA methyltransferase and *pleC* (p_{pleC}) that is probably involved in *ctrA* regulation. They have not been well characterized yet in *B. abortus*, which is why the first part of this master thesis focused on trying to follow the activity of each promoter in *B. abortus* grown in rich medium (2YT) and in HeLa cell infection model. To monitor the activity of the promoter, we decided to use a reporter system based on the expression of a gene coding for an unstable GFP. In the second part, we checked if CtrA had an active role in the regulation of the expression of the four chosen genes. To do so, we mutated the 9-mer consensus sequences of their promoters in the hope that it would prevent the binding of CtrA. We constructed similar reporter systems than for the wild type promoters and compared their activity in 2YT medium and in HeLa cells.

Results

I. The overall strategy

Since the limits of the promoters of *bmaC*, *repABC*, *ccrM* and *pleC* are not clearly defined, we decided to consider the whole intergenic regions separating the coding sequences of interest from the upstream open reading frames as "promoters". To study the activity of those four promoters, we constructed reporter systems. They are based on the fusion between the promoter of each chosen gene and a sequence coding for an unstable GFP. This protein is indeed susceptible to endogenous housekeeping proteases. It has been reported that the last three residues of the C-terminal end of the GFP protein is determinant for its stability (Andersen et al., 1998). We thus constructed strains coding for either the gfp_{asv} or the gfp_{laa} version. In *Escherichia coli*, GFP_{laa} is known to be degraded faster than GFP_{asv}. In *Pseudomonas putida*, however, they have approximately the same half-life (Andersen et al., 1998). The degradation rate of each GFP being unknown in *B. abortus*, we wondered if one of them could be a better reporter of the activity of the studied promoters. We tested in parallel the two *gfp* versions under the control of p_{repABC}. It appeared that, even though they do not reach their peak intensity at the same time, they still displayed a similar expression profile (data not shown). We thus chose to keep on working with GFP_{asv}.

The reporter systems were cloned in the medium copy number plasmid pBBRMCS1 and in the low copy number plasmid pMR10. Having obtained numerous data for those two systems, we realized that the fluorescence intensities were reproducible with the pBBRMCS1-borne reporter systems but not with the pMR10 (data not shown). This lack of reproducibility could be due to a variation in plasmid copy-number from clone to clone. All the results presented bellow are therefore based on the expression of the gfp_{asv} allele on a pBBRMCS1 plasmid.

For the experiments done in a rich culture medium, the mean GFP fluorescence intensity and the cell length of each bacterium were measured by using MicrobeTracker, an extension of the software Matlab (Garner, 2011). The variability of the fluorescence intensity values was very high. This is why, in order to have an idea of the underlying trend, we decided to consider the data obtained by plotting the average values for groups of 300 values. Given the fact that, up to now, there is no way of synchronizing a culture of *B. abortus*, we have to keep in mind that bacteria of the same length are not all at the exact same stage of their cell cycle. Our observations were done several times with one to three different clones per strain.

As for the experiments performed in the HeLa cell infection model, only the mean GFP fluorescence intensity was measured with MicrobeTracker. Indeed, it was risky to determine the cell length since we could not see in which axis the bacteria were photographed. Data were grouped in function of the time post infection (PI) at which the bacteria had been fixed. Each dot on the graph represents the mean GFP fluorescence intensity for one bacterium. The experiments were done several times with one clone per strain. Statistics tests (Mann-Whitney) were performed to see if there was a significant difference in the intensity values between the populations at different times PI.

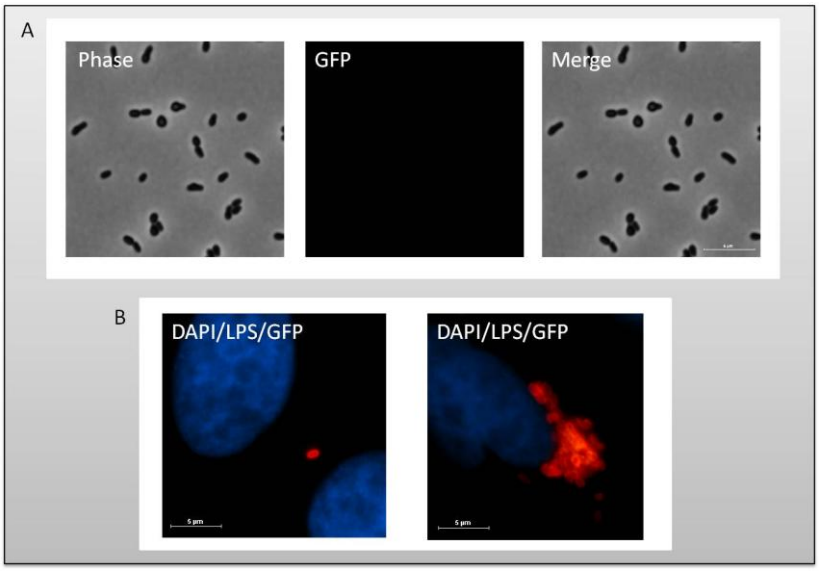

Figure 8: Results for the *B. abortus* strain carrying the p_{bmaC}-gfp_{asv} reporter system. GFP fluorescence could be detected in neither the culture medium **(A)** nor in the HeLa cell infection model **(B).** The lower left picture was taken at 30 min PI, whereas the lower right picture was taken at 24 h PI. Scale bars represent 5 µm.

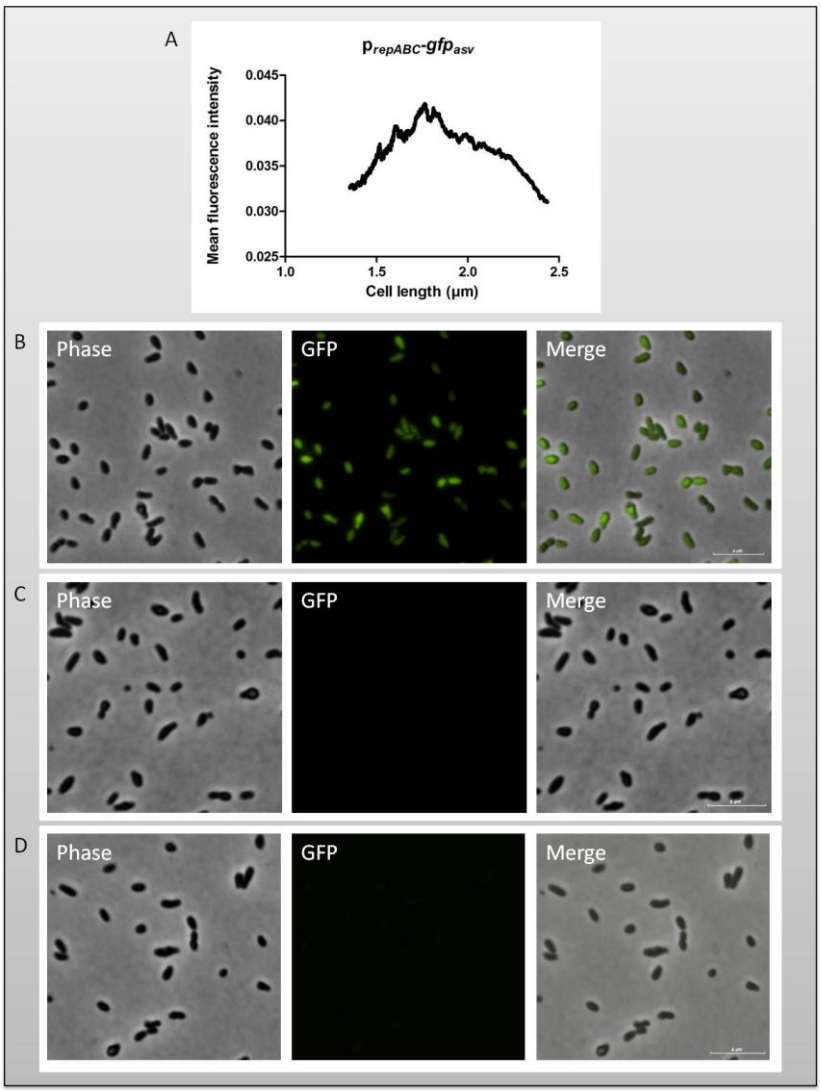

Figure 9: Results for *B. abortus* strains carrying different versions of the p_{repABC}-gfp_{asv} reporter system in a 2YT culture medium. (A) Plot of the mean GFP fluorescence intensities per bacterial cell over the cell length for the WT reporter system carrying strain. The mean intensities and cell lengths were computed and monitored with MicrobeTracker (Garner, 2011). Bacteria were sorted according to cell length and averaged for a window of 300 bacteria of similar size. Pictures were taken for **(B)** the WT reporter system carrying strain, **(C)** the mutated version of the reporter system carrying strain and **(D)** the p_{lac}-*ctrA*-p_{repABC}-gfp_{asv} carrying strain. Scale bars represent 5 µm.

It should be noted that graphs cannot be compared between each other, except if the bacterial strains were observed on the same day. Indeed, fluorescence intensities can vary from one day to another. The general profile is thus more relevant than the exact intensity values.

II. The promoter of *bmaC*

II.1. In the 2YT culture medium

Two CtrA binding boxes had been found *in silico* with RSA-tools (van Helden, 2003) in the region regarded as being the promoter of the *bmaC* gene of *B. abortus*. To characterize p_{bmaC} activity, we first looked at the GFP expression profile of a *B. abortus* strain carrying the p_{bmaC}-gfp_{asv} fusion in a 2YT culture medium. No GFP signal could be detected this way, suggesting a very low activity of this promoter in such conditions (**Figure 8A**).

II.2. In the HeLa cell infection model

The *bmaC* gene being known for its role in *B. suis* adhesion to host cells, we also investigated the potential activity of our reporter system in a HeLa cell infection model. We fixed the cells with paraformaldhehyde (PFA) 2% at 30 min, 1 h, 6 h and 24 h PI and looked for the presence of fluorescent bacteria. In the same way than for the experiments done in the rich culture medium, no GFP fluorescence could be observed at any time (**Figure 8B**). We were expecting to observe fluorescence at 30 min PI because one hour after the initiation of the contact between the bacteria and the HeLa cells, they are treated with Gentamycin in order to kill the non-internalized bacteria. This means that, at 30 min PI, there should still be bacteria that are trying to enter the HeLa cells.

Being unable to detect GFP in neither the culture medium nor the infection model, we decided not to look further into the activity of p_{bmaC}.

III. The promoter of *repABC*

III.1. In the 2YT culture medium

The use of RSA-tools (van Helden, 2003) also allowed us to consider the p_{repABC} as possessing a putative CtrA binding box. Here, too, we first tried to characterize the *B. abortus* strain carrying the pBBRMCS1-p_{repABC}-gfp_{asv} reporter system in culture. The graph representing the mean GFP intensity over the mean cell length suggests that the activity of the promoter increases until it reaches a peak, and then decreases (**Figure 9A**). The bacteria with an intermediate cell length harbor the highest mean GFP fluorescence intensities. The RepA, RepB and RepC proteins being needed for the ChII initiation of replication and segregation, it seems logical that the promoter regulating their expression would be active in growing bacteria. The average cell size at which the peak of GFP intensity is detected could also be meaningful as RepA, RepB and RepC production needs to be tightly regulated. Indeed, an overexpression of those genes in large bacteria would be toxic as it could lead to a second replication initation of ChII and thus an aberrant cell cycle.

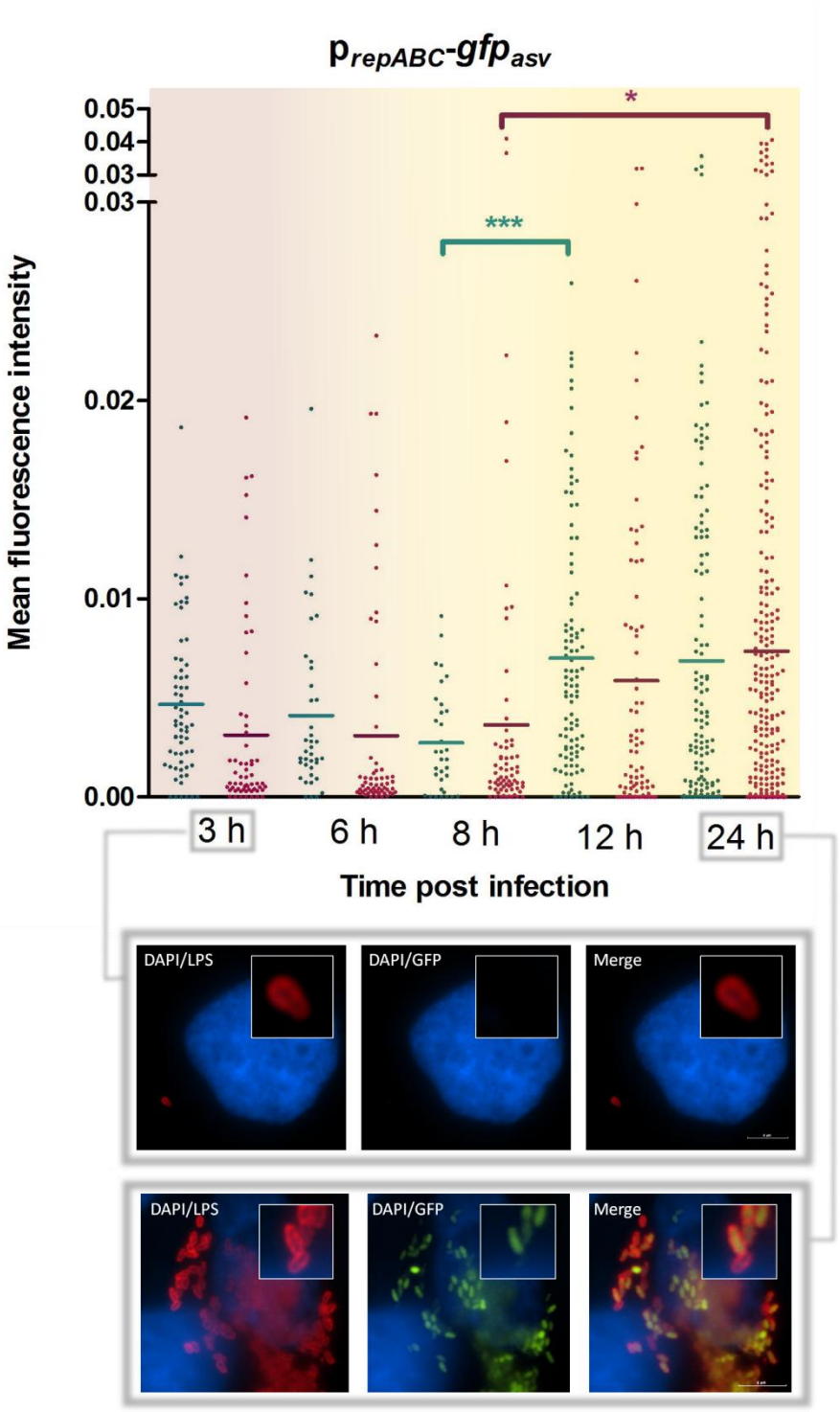

Figure 10: Results for the *B. abortus* strain carrying the p_{repABC}-gfp_{asv} reporter system in the HeLa cell infection model. The graph represents the mean GFP fluorescence intensities at different times PI for two separate experiments (in blue and in magenta). Each dot corresponds to the mean fluorescence intensity value of one bacterium quantified with MicrobeTracker (Garner, 2011). Intensity values were measured for 64, 60, 39, 60, 33, 68, 105, 80, 130 and 286 bacteria, respectively. The horizontal bar represents the average intensity value. Mann-Whitney U tests were performed to determine if there is a difference between the populations at different times post infection. The p values are shown by one star (*) for 0.05 and three stars (***) for 0.001. The colored background represents the transition from the non-proliferative phase (in purple) to the proliferative phase (in orange) of the infection (see Introduction point II.2.2. for more information). Pictures shown here correspond to 3 h PI and 24 h PI. Scale bars represent 5 µm.

The same experiment was performed with the mutated version of the reporter system (pBBRMCS1-p_{repABC}mut-*gfp*$_{asv}$), in which the CtrA binding box was altered (see Discussion and perspectives, point I.2. for more details). The GFP fluorescence intensity drastically decreased to the point that we could not visually discriminate it from the background fluorescence (**Figure 9C**). This could eventually suggest a strong implication of CtrA in the regulation of p_{repABC} activity.

Since p_{repABC} appeared to be potentially dependent on CtrA in the 2YT rich culture medium an since deleting ctrA is probably impossible (Bellefontaine et al., 2002), we decided to look at the GFP expression profile in a *ctrA* overexpressing strain. This way, we should have an idea about CtrA regulation on p_{repABC} without having to mutate its sequence. We thus constructed a pBBRMCS1 plasmid carrying both the p_{lac}-*ctrA* fusion and the p_{repABC}-*gfp*$_{asv}$ reporter system. This plasmid was incorporated into a wild-type (WT) *B. abortus* 544 strain. Unexpectedly, the observation of this strain in a 2YT culture medium showed that, similarly to what happens with the pBBRMCS1-p_{repABC}mut-*gfp*$_{asv}$ carrying strain, no GFP fluorescence could be observed (**Figure 9D**). This observation should be approached with caution as it has only been done once. Moreover, the bacterial population seemed to have growth issues.

III.2. In the HeLa cell infection model

In the HeLa cell infection model, the pBBRMCS1-p_{repABC}-*gfp*$_{asv}$ reporter system *B. abortus* carrying strain displayed a clear difference of activity between the non-proliferative phase and the proliferative phase of the infection. We chose to look at closer ranges of time PI to have a better idea of when the promoter was switched on and off. This strain was thus observed at the following times: 3, 6, 8, 12 and 24 h PI. During the first phase (until approximately 8 h PI), the bacteria are blocked in G1, which should dispense them from needing to express the *repABC* operon. As a matter of fact, at this time, the *B. abortus* strain carrying the reporter system apparently expressed a lower level of GFP than in the second phase (after 8 h PI), when the bacteria have reached their replicative niche. The variations of the mean GFP fluorescence intensity among the same stage of the infection vary slightly but the overall difference between the two stages is conserved from one experiment to another (**Figure 10**).

In the interest of saving time and because we could not observe GFP fluorescence in culture with the pBBRMCS1-p_{repABC}mut-*gfp*$_{asv}$ carrying strain, we chose not to look at it in the infection model.

IV. The promoter of *ccrM*

IV.1. In the 2YT culture medium

A DNAse I footprinting experiment showed that a recombinant His_6-CtrA could bind to p_{ccrM} *in vitro* (Bellefontaine et al., 2002). In addition, a *B. abortus* pBBRMCS1-p_{ccrM}-*gfp*$_{asv}$ carrying strain had already been observed in a 2YT culture medium (C. Van de Henst, unpublished data). Predivisional bacteria appeared to be the most fluorescent (data not shown). This had allowed us to extrapolate a model for p_{ccrM} activation. It suggests that *ccrM* is only expressed at the end of DNA replication, before cell division. This model is a simple transposition to *B. abortus* of what is known about *ccrM* in *Caulobacter crescentus*: p_{ccrM} activation also seems transient and cell cycle-dependent. Indeed, in *C. crescentus* p_{ccrM}

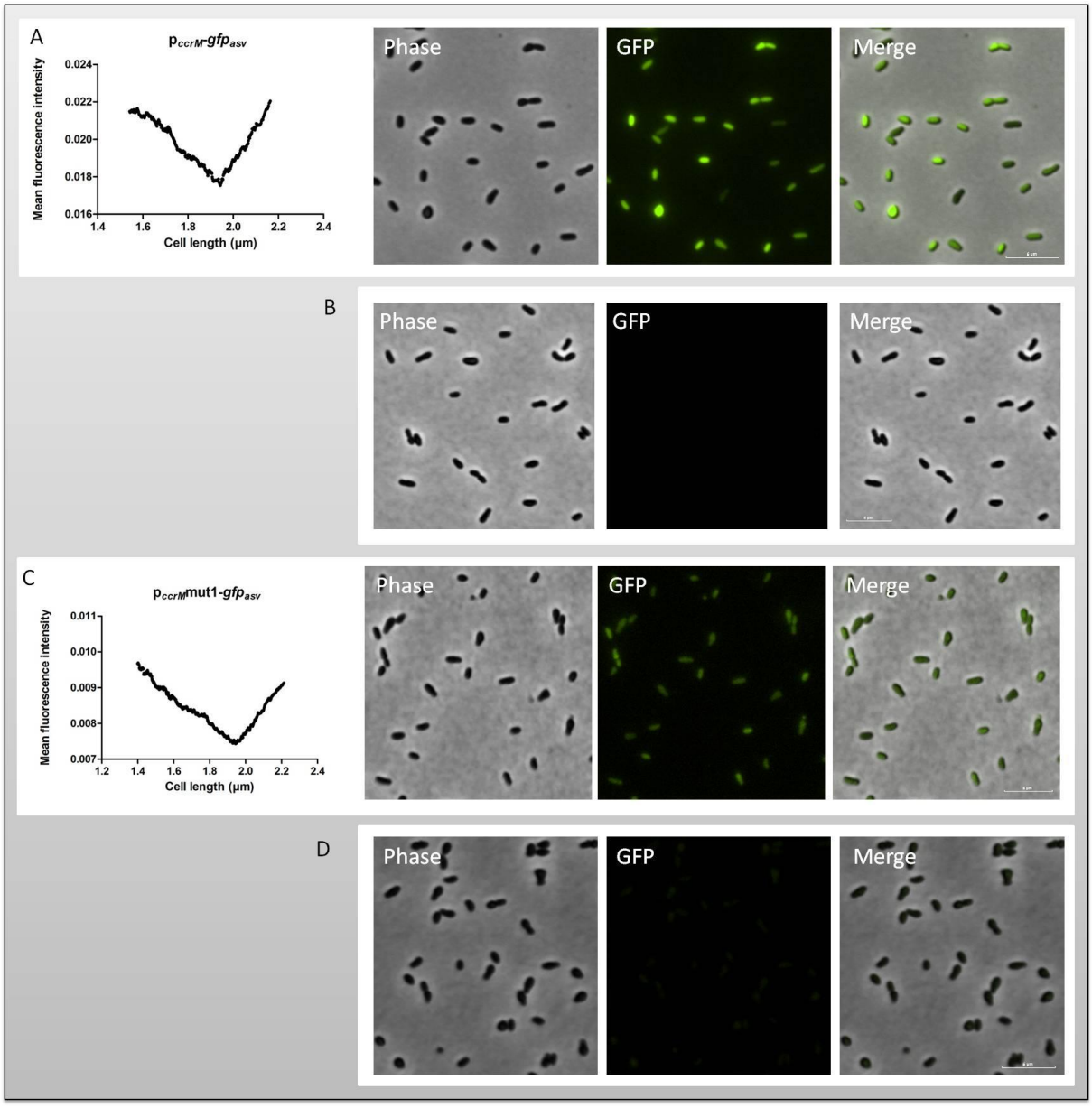

Figure 11: Results for *B. abortus* strains carrying different versions of the p_{ccrM}-gfp_{asv} reporter system in a 2YT culture medium. (A and C) Plots of the mean GFP fluorescence intensities per bacterial cell over the cell length for the strain carrying the WT reporter system and for the strain carrying the reporter system mutated for the first CtrA binding box (p_{ccrM}mut1-gfp_{asv}), respectively. The mean intensities and cell lengths were computed and monitored with MicrobeTracker (Garner, 2011). Bacteria were sorted according to the cell length and averaged for a window of 300 bacteria of similar size. Pictures were taken for **(A)** the WT reporter system carrying strain, **(B)** the double mutant reporter system carrying strain, **(C)** the p_{ccrM}mut1-gfp_{asv} carrying strain and **(D)** the strain carrying the reporter system mutated for the second CtrA binding box. Scale bars represent 5 µm.

activation occurs only at the end of the S phase when CcrM is needed to methylate the newly synthesized DNA strands (Reisenauer et al., 1999; Stephens et al., 1996). When this *B. abortus* strain was observed, however, we did not have the technical tools to measure the mean GFP fluorescence intensity at a single cell scale.

We therefore repeated the experiment and analyzed, for each bacterium, the mean GFP fluorescence intensity and the cell length by using MicrobeTracker. The graph depicting the mean GFP intensity per bacterial cell over the mean cell length clearly shows that p_{ccrM} is mostly active in large bacteria (**Figure 11A**). This matches the model previously mentioned. The high fluorescence intensity observed in the smallest bacteria is probably due to the GFP produced in predivisional cells and not yet degraded. It could also be explained by the fact that p_{ccrM} might take some time to be shut down. In bacteria of medium size, the GFP fluorescence decreases and reaches a minimum, suggesting that p_{ccrM} activity is repressed.

p_{ccrM} actually possesses two potential CtrA binding boxes. We thus constructed three reporter systems on pBBRMCS1 medium plasmids: a double mutant (p_{ccrM}mut1+2-*gfp*$_{asv}$), and two single mutants (p_{ccrM}mut1-*gfp*$_{asv}$ and p_{ccrM}mut2-*gfp*$_{asv}$) for the first and the second CtrA binding box, respectively. The *B. abortus* double mutant carrying strain displayed no visible GFP fluorescence (**Figure 11B**). This loss of activity seems to be mainly due to the mutations of the second CtrA binding box. Indeed, the strain that carries the pBBRMCS1-p_{ccrM}mut2-*gfp*$_{asv}$ plasmid presents almost no GFP fluorescence in a 2YT culture medium (**Figure 11D**). At the opposite, a bacterial population of the other strain, which carries the p_{ccrM}mut1-*gfp*$_{asv}$ reporter system, clearly expressed GFP. The general profile of the graph representing the mean GFP fluorescence intensity over the mean cell length is also conserved between this bacterial strain and the one carrying the WT reporter system. However, its intensity looks weaker than in the WT reporter system (**Figure 11C**).

IV.2. In the HeLa cell infection model

As usual, we tried to characterize our *B. abortus* WT reporter system carrying strain in the HeLa cell infection model. The first time we did this experiment, we could observe a slight increase of the mean fluorescence intensity with time. Higher intensity values seem to break away from the others at 24 h PI but this increase of signal is not statistically relevant (**Figure 12**). This strain was observed a second time by Luca Rappez and the results he had were quite different from the first time: the increase of mean fluorescence intensity happened at 8 h PI to slightly drop at 24 h PI (data not shown). Still, the variations of signal were not statistically significant. This strongly suggests that p_{ccrM} is not as strictly regulated in infection as it is in culture. It is also possible that our system is not sensitive enough to detect the variations of fluorescence intensities with this strain.

The mutated reporter systems carrying strains have not been observed in the infectious context yet.

V. The promoter of *pleC*

V.1. In the 2YT culture medium

His_6-CtrA has also been shown to bind to p_{pleC} *in vitro* (Bellefontaine et al., 2002). The analysis of the *B. abortus* strain carrying the p_{pleC}-*gfp*$_{asv}$ construct allowed us to propose that

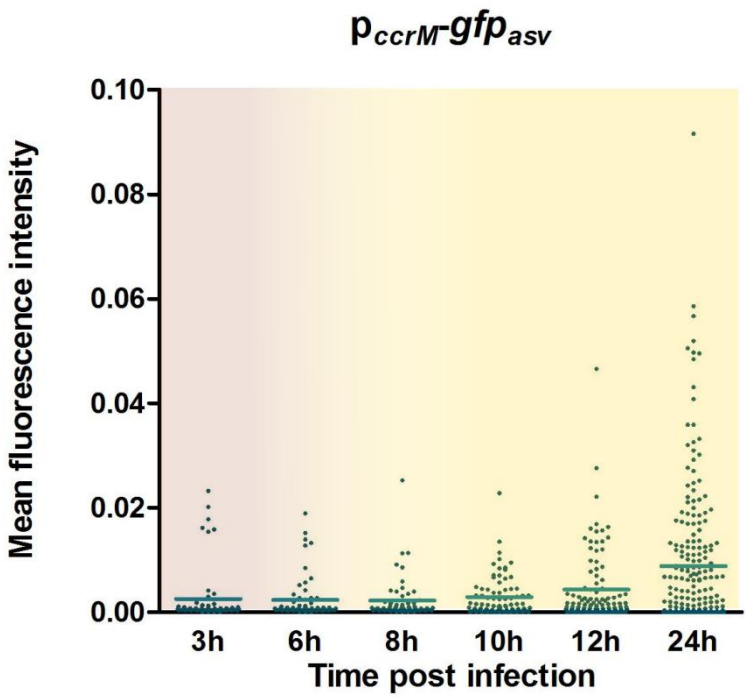

Figure 12: Results for the *B. abortus* strains carrying the p_{ccrM}-gfp_{asv} reporter in the HeLa cell infection model. The graphs represent the mean GFP fluorescence intensities at different times PI. Each dot corresponds to the mean fluorescence intensity value of one bacterium quantified with MicrobeTracker (Garner, 2011). From left to right, intensity values were measured for 59, 62, 58, 79, 102 and 217 bacteria. The horizontal bar represents the average intensity value. Mann-Whitney U tests were performed to determine if there is a difference between the populations at different times post infection and it appears there is not. The colored background represents the transition from the non-proliferative phase (in purple) to the proliferative phase (in orange) of the infection (see Introduction point II.2.2. for more information).

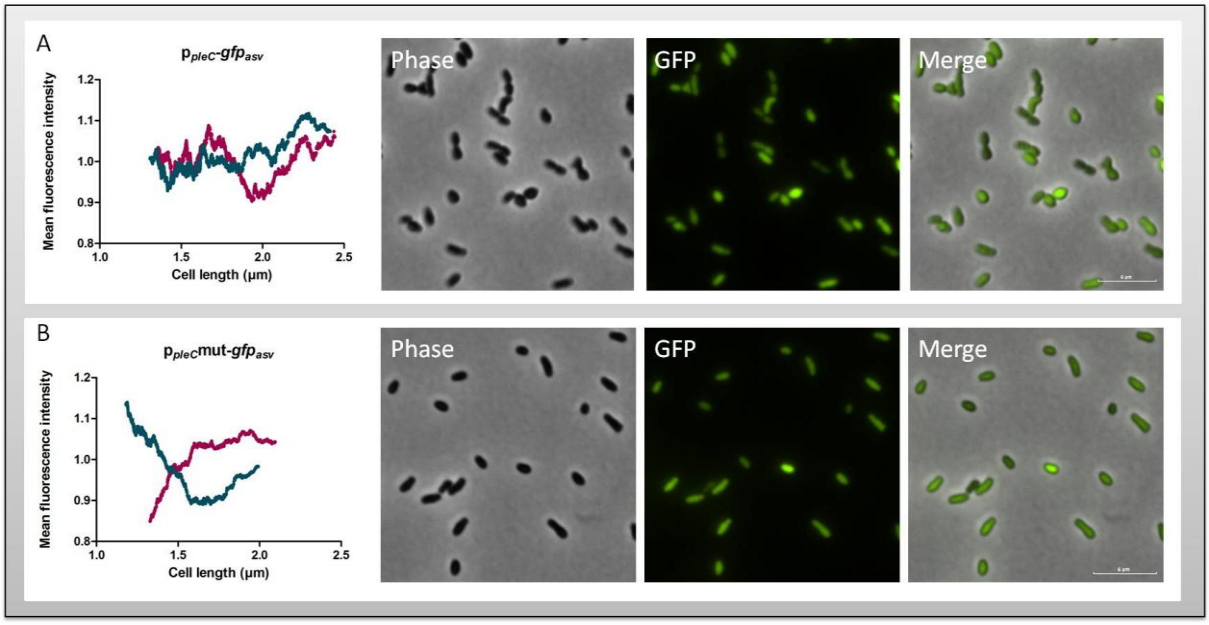

Figure 13: Results for *B. abortus* strains carrying (A) the p_{pleC}-gfp_{asv} reporter system or (B) its mutated version in a 2YT culture medium. The plots represent the normalized mean GFP fluorescence intensities per bacterial cell over the cell length for two experiments (in blue and in magenta). The mean intensities and cell lengths were computed and monitored with MicrobeTracker (Garner, 2011). Normalizations were made by dividing each intensity value by the mean intensity value of the population. Bacteria were sorted according to their cell length and averaged for a window of 300 bacteria of similar size. Pictures were taken for **(A)** the WT reporter system carrying strain, **(B)** the mutated version of the reporter system (p_{pleC}mut-gfp_{asv}) carrying strain. Scale bars represent 5 µm.

the activity of p_{pleC} is not rigorously regulated throughout the cell cycle. Indeed, the fluctuations that can be seen on the graph depicting the mean GFP fluorescence intensities over the bacteria cell lengths were not reproducible from one test to another (**Figure 13A**). In conclusion, the p_{pleC} activity does not seem to be cell cycle dependent in culture.

The mutated version of the reporter system carrying strain displayed a similar phenotype than the WT version. Here also, the mean GFP fluorescence intensities fluctuate from one experiment to another (**Figure 13B**).

V.2. In the HeLa cell infection model

A proteomic analysis conducted by Lamontagne et al. in 2009 showed that, unlike most of the other proteins, PleC is present at higher levels in *B. abortus* during the early stage of macrophages infection, compared with bacteria grown in culture medium. The p_{pleC} activity could thus be tightly regulated during infection, even though it does not seem cell cycle dependent in culture. The proteomic data match with our results showing that *B. abortus* carrying the p_{pleC}-gfp_{asv} construct display the highest intensity at 3 h PI (**Figure 14,** left graph). In fact, Lamontagne et al. (2009) only assessed PleC abundance at 3, 20 and 44 h PI. It has been reported to drop slowly to reach pre-infection levels only at 44 h PI. Our study brings new insight as to what could happen during the gap between 3 and 20 h PI at the transcriptional level. If we take the two extremes times, namely 3 h PI and 24 h PI, we can see that there is a clear drop of fluorescence intensity with time. As for the transition from a high to a low fluorescence intensity, it seems to occur between 6 and 8 h PI. Interestingly, even if there is no significant change from 12 to 24 h PI, it seems that several bacteria have an increased p_{pleC} activity at the later time (**Figure 14,** left graph). Additional data are needed to test whether this tendency will keep on going with time and if it will rejoin pre-infection levels like it did in the macrophage infection model (Lamontagne et al., 2009). Actually, the culture that had been used to infect the HeLa cells have also been observed to make sure that the fixation with PFA did not impact on the GFP fluorescence profile (data not shown). However, we can not compare the intensity values in culture and in infection because the bacteria were not excited for the same amount of time in both experiments. The times of exposition were chosen in order not the photobleach our fluorescent markers, which are not as strongly visible in the HeLa cells as they are in culture.

The first time the pBBRMCS1-p_{pleC}mut-gfp_{asv} carrying strain has been observed, we could see that the GFP fluorescence intensities were low and relatively constant from 6 h PI to 24 h PI (**Figure 14,** right graph). Unfortunately, we had no result for the time 3 h PI. We therefore still had to confirm the stillness of the mutated p_{pleC} activity throughout the infection.

Intriguingly, we could observe a high variability of the GFP fluorescence intensities for the bacteria carrying the WT reporter system that were photographed at the same time PI (**Figure 14**). This tendency was even clearer at early times PI. We thus thought that this variability of GFP intensities could be due to the fact that we included extracellular bacteria into our analysis. Therefore, when we reproduced the experiment with the WT reporter system and its mutated version, we performed differential staining for extracellular and intracellular bacteria (see material and methods, point I.2.3.). Analyses were thereafter made on the basis of bacteria that were without doubt marked as intracellular. As it is shown in **Figure 15**, the p_{pleC}mut-gfp_{asv} fluorescence intensity profile is conserved. As for the p_{pleC}-

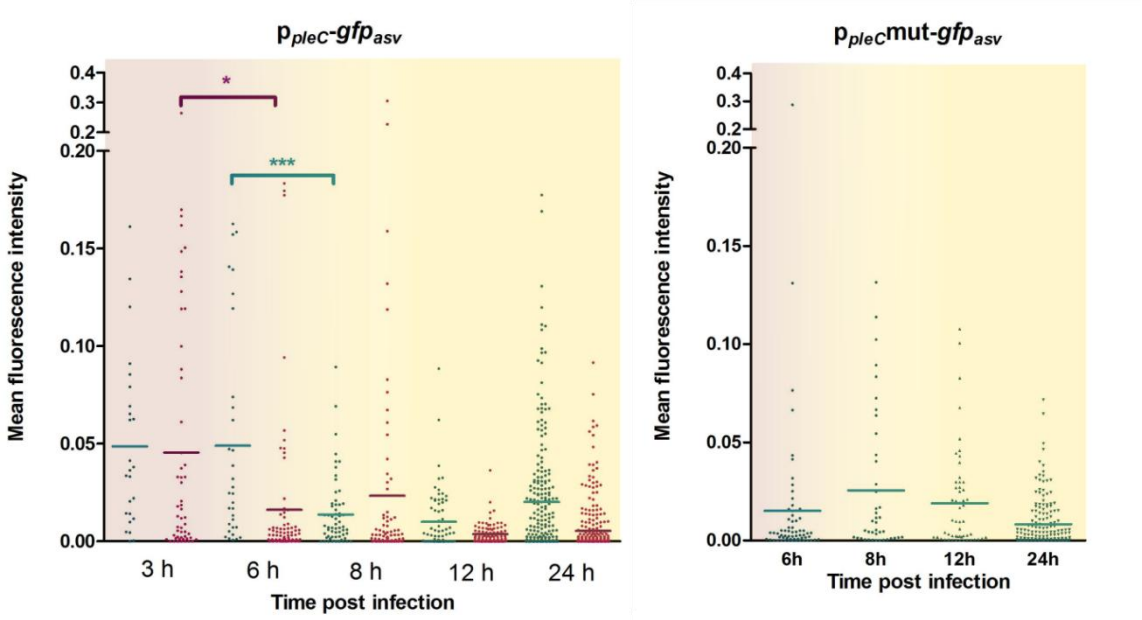

Figure 14: Results for the *B. abortus* strains carrying the p_{pleC}-gfp_{asv} reporter system or its mutated version in the HeLa cell infection model. The graphs represent the mean GFP fluorescence intensities at different times PI. Two separate experiments (in blue and in magenta) for the WT version of the reporter system are represented on the left plot. The plot on the right represents the results obtained for the first experiment performed with the mutated version of the reporter system. Each dot corresponds to the mean fluorescence intensity value of one bacterium quantified with MicrobeTracker (Garner, 2011). From left to right, intensity values were measured for 25, 54, 32, 73, 62, 70, 71, 101, 233, 305, 65, 42, 54 and 159 bacteria. The horizontal bar represents the average intensity value. Mann-Whitney U tests were performed to determine if there is a difference between the populations at different times post infection. The p values are shown by two stars (**) for 0.01 and three stars (***) for 0.001. The colored background represents the transition from the non-proliferative phase (in purple) to the proliferative phase (in orange) of the infection (see Introduction point II.2.2. for more information).

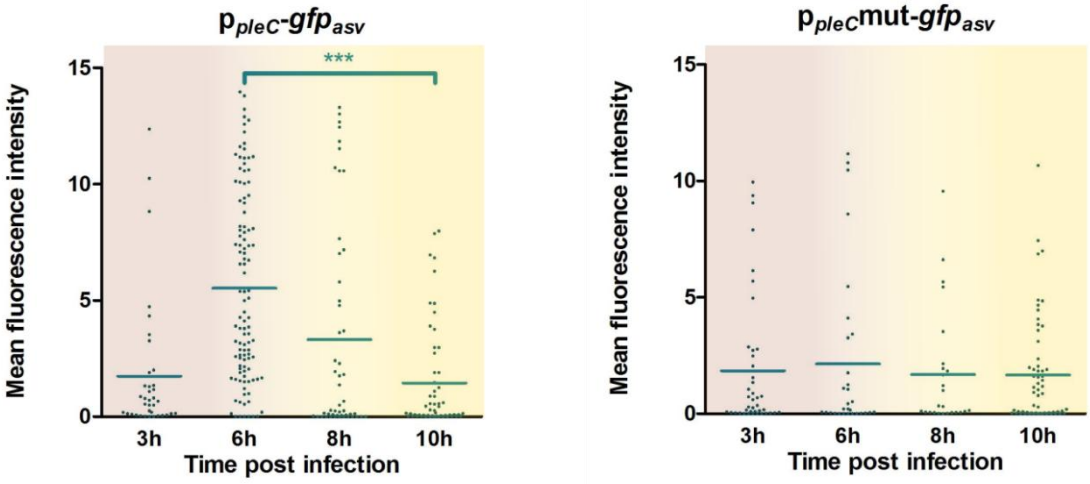

Figure 15: Results for the intracellular-marked bacteria from *B. abortus* strains carrying the p_{pleC}-gfp_{asv} reporter system or its mutated version in the HeLa cell infection model. The graphs represent the mean GFP fluorescence intensities at different times PI for bacteria that were marked as being intracellular. Each dot corresponds to the mean fluorescence intensity value of one bacterium quantified with MicrobeTracker (Garner, 2011). From left to right, intensity values were measured for 37, 109, 50, 56, 41, 30, 32 and 66 bacteria. The horizontal bar represents the average intensity value. The colored background represents the transition from the non-proliferative phase (in purple) to the proliferative phase (in orange) of the infection (see Introduction point II.2.2. for more information). Mann-Whitney U tests were performed to determine if there is a difference between the populations at different times post infection. The p value is shown by three stars (***) for 0.001.

gfp$_{asv}$ fusion carrying strain, it appears to have a similar profile than with previous results, except for the time 3 h PI. This could be explained by the relatively low number of bacteria that were analyzed. Indeed, in such a plot, extreme values influence greatly the mean value. What we can also observe on these plots is that the variability among the bacteria observed at the same time is still present. This suggests that the variations of activity of p_{pleC} are intrinsic to the bacteria and that the selection criteria we used until now, i.e. to only consider the fluorescence intensities from bacteria that were perfectly focused, is relevant.

Discussion and perspectives

I. About the method

I.1. The reporter system

I.1.1. The use of different gfps *as reporter genes*

GFP has already been used as a reporter system to identify genes that are differentially expressed by *B. abortus* and *B. suis* during macrophage infections (Eskra et al., 2001; Köhler et al., 1999). The aim of these studies was to start without any *a priori*. They created constructs based on the fusion between a random fragment of genomic DNA and a *gfp* allele. Fluorescent bacteria were therefore the ones who had inherited a plasmid carrying a *gfp* downstream to a sequence possessing a promoter activity. These studies therefore suggested that *gfp*-based reporter systems are efficient tools to study promoters activity in *Brucella* spp.

What we wanted to do here is a bit different, though. We not only wanted to see the *gfp* expression switching on and off, but also to follow it through time and as close as possible to reality. We thus chose to follow the fluorescence of an unstable version of GFP. As previously mentioned, we first tested in parallel the p_{repABC}-gfp_{asv} and the p_{repABC}-gfp_{laa} constructs. We could not see a striking difference in their expression profiles, which is why we arbitrarily chose to keep on working with the gfp_{asv} version. Andersen et al. (1998) showed that the GFP_{asv} and the GFP_{laa} proteins seem to have approximately the same half life in *Pseudomonas putida* but not in *E. coli*. Actually, they also studied two other versions of unstable GFP: GFP_{aav} and GFP_{lva}. GFP_{lva} turned out to be the fastest degraded in both *E. coli* and *P. putida*. This form could then be a more reliable reporter of the promoters activity than the ones we used, if it allows a sufficient sensibility. Nevertheless, it is a fact that proteins are degraded differently in each bacterial species.

A way to determine which *gfp* allele is the most suitable in *B. abortus* would be to determine their half-life. We could do so by putting a *gfp* gene under the control of an exogenous promoter on a replicative plasmid. We would incubate the *B. abortus* strain carrying this plasmid with an inhibitor of translation such as chloramphenicol or puromycin. The fluorescence profile we would obtain from such an experiment should be directly dependent of the GFP proteolysis and not of its production. Note that another way to strongly decrease translation would be to shift the bacterial population from a rich culture medium to a minimal medium. This phenomenon happens because the bacteria have to undergo an adaptation phase before to be able to metabolize the new source of nutrients. This latter method was the one chosen by Andersen et al. (1998) to determine the approximate half-life of the studied unstable GFPs.

Another aspect of the GFP should be looked at. Indeed, the fluorescence profile arising from the *gfp* expression is influenced by the protein proteolysis, but also by its maturation. A mean to check if the different GFP versions maturate at the same speed could be to express them under the control of an inducible promoter such as an IPTG-dependent promoter. The kinetics at which the fluorescence increases above the background for each strain would give us an indication about the rate at which the maturation of the GFPs occurs. In *E. coli*, the four

unstable GFP versions and the WT version appear to have the same maturation speed (Andersen et al., 1998).

We should also make sure that the *gfp* expression is only dependant on the chosen promoter located upstream. To do so, we should make a reporter system with the *gfp* being under the control of a constitutive promoter. As a matter of fact, Luca Rappez constructed a pBBRMCS1-p_{lac}-gfp_{asv} reporter system in the context of his Master thesis. It should then be useful as a control of "no variation". However, the mean GFP fluorescence profile of the *B. abortus* strain carrying this fusion was not as flat as expected. This could be explained by the fact that, even though the *lac* operon coming from *E. coli* does not exist in *B. abortus*, the sigma factor that triggers the initiation of its transcription seems to be conserved in both bacteria. Moreover, in *B. abortus*, the gene coding for this sigma factor is suspected to be regulated by CtrA (Bellefontaine et al., 2002). This could explain why the fluorescence intensity profile of this strain is varying with the cell length.

I.1.2. An alternative to gfp*?*

There exists a problem with the use of GFP family members as reporters for a promoter activity. Indeed, it has been shown that the YFP chromophore undergoes a reversible inhibition of its maturation under oxygen-limited conditions (Drepper et al., 2010). Fluctuation of oxygen availability in culture has been demonstrated and makes it so that YFP cannot be used as an accurate reporter for quantitative analysis (Drepper et al., 2010). Of course, this should also be true in an infection model. Other environmental parameters such as the pH can also alter the GFP signal intensity (Bizzarri et al., 2009). Nevertheless, our observations can still be considered as valid since what really interest us are not the intensity values but the differences between the global profiles obtained for our reporter system *B. abortus* carrying strains. In addition, we do not look at the fluorescence intensity through time but through the cell length at a given time for the experiments done culture and at fixed times PI for the experiments done with the infection model.

There are alternatives to GFP family members. One of them is the flavin-dependent fluorescent protein family. Its members are all derived from the LOV (light, oxygen, voltage) photoreceptors proteins. Their preferred chromophore is FMN (flavin mononucleotide) and their main characteristic is that their maturation is not dependant of oxygen. They display a cyan-green fluorescence (λ_{max}= 495 nm) after being excited with blue light (λ_{max}= 450 nm) (Drepper et al., 2013). Using a LOV-derived reporter could be a mean to solve the problem we had with the fluctuation of oxygen levels in our models. However, an intrinsic LOV-Histidine kinase has been discovered in *B. abortus*. It seems to be a very important aspect of the bacteria virulence. Indeed, the replication rate in mouse macrophages of the *B. abortus* WT strain exposed to visible light is 10-fold higher than its dark control (Rinaldi et al., 2012). The excitation of a LOV-derived reporter system could thus have an impact on the global kinetics of the infection. This is why the use of such a protein in *B. abortus* should be considered with caution.

I.2. The choice of mutations

In order to determine what part CtrA plays in the regulation of the four putative targets, we mutated the CtrA binding boxes that we found in their promoter. We first looked in the literature to see if there was a consensus as to where and what bases to mute. Since there was apparently none (**Table 1**), we chose to limit the number of mutations to four. We distributed

Sequence changes below WT	Source
TTAAGATCTTT**CTAA**CATATA**TTAA**T |→ -14 bp ←|	Siam & Marczynski, 2000
ACACC**TTAA**TGAATTC**TTAA**GTCCT ACAGG	Siam & Marczynski, 2000
AGTGG**TTAA**GCAACCG**TTAA**CGGAT CGCT	Siam & Marczynski, 2000
GGG**TTAA**CGCTCTG**TTAA**TCA ATCC	Siam et al., 2003
GGG**TTAA**CGCTCTG**TTAA**TCA GAAT	Siam et al., 2003
GGG**TTAA**CGCTCTG**TTAA**TCA AATT	Spencer et al., 2009
CTAACATATA**TTAA**TAAGAAA**TTAA**G AATT	Bastedo and Marczynski, 2009
TTA**TTAA**CAATTGG**TAAA**CGC AGCTT	Shaheen et al., 2009
TTA**TTAA**CAATTGG**TAAA**CGC AATTC	Shaheen et al., 2009

Table 1: Systematic review of the mutations made in CtrA binding boxes in *C. crescentus*. The two TTAA half boxes are highlighted in bold.

Sequence changes below WT	Promoter
GATA**TTAA**GAAAAGA**TTTAC**GA CG CC	p_{ccrM} (CtrA binding box 1)
GGCG**TTAA**CGGCATA**TTTAC**CC CG CC	p_{ccrM} (CtrA binding box 2)
GATT**TTTA**CGCCTCG**TTAAC**GA CC CG	p_{pleC}
CATC**TTAA**CAAAGAG**TTAGC**GG CG CG	p_{repABC}

Table 2: WT and mutated versions of the CtrA binding boxes studied with the reporters systems. The two TTAA half boxes are highlighted in bold. The -35 element is in green; its consensus sequence is TTGACA in *E. coli*.

them on both TTAA half-sites and, to avoid any unnecessary disturbance, we replaced purines by purines and pyrimidines by pyrimidines (transitions only) (**Table 2**).

We also had to be careful not to modify the -10 and -35 boxes. The only promoter for which we had the precise -10 and -35 elements position is p_{ccrM}. Its -35 sequence actually overlaps with the second CtrA binding box (Robertson et al., 2000). Fortunately, the -35 element consensus sequence was still as conserved as it used to be (two substitutions in both cases) after mutating it as described above (**Table 2**). As for the other promoters, we do not know where their +1 start site is positioned so we can not be sure not to have disrupted the -10 and -35 boxes. Inserting other point mutations in the reporter systems could allow us to confirm the obtained results.

By preventing CtrA to bind to its boxes and by repeating the same experiments than the ones we did with the wild type promoters, we aimed at better understanding how CtrA regulates *B. abortus* cell cycle in culture and in the HeLa cell infection model, if CtrA is indeed the major regulator of these promoters. Another way to look at it is to overproduce CtrA in *B. abortus* strains that are already carrying the p_{target}-gfp_{asv} constructs. This has been done with the p_{repABC}-gfp_{asv} reporter system carrying *B. abortus* but it still needs to be done with the other strains. We could also overexpress a non-phosphorylable version of *ctrA* as a control.

I.3. Other techniques

We could also follow the GFP fluorescence intensities resulting from our reporter systems in parallel with the amount of DNA of the bacteria by using FACS (fluorescence-activated cell sorting). This would give us an indication about the link between the cell cycle and the putative CtrA-dependent promoters activity.

Reporter systems are not the only way to monitor gene expression. We could also perform RT-qPCR (reverse transcription quantitative polymerase chain reaction) on WT and *ctrA* overexpressing strains. This would allow us to directly follow the transcription levels of the putative CtrA-dependent genes, without having to rely on mutations and plasmids.

An EMSA (electrophoretic mobility shift assay) could also be performed. Basically, this method consists in the comparison of the migration profiles of a protein-DNA mixture versus an unbound DNA fragment. If CtrA is indeed bound to the putative CtrA binding box of our promoter of interest, their complex should migrate more slowly than the unbound control.

An even more powerful technique is the ChIP (chromatin immunoprecipitation). It is also based on the isolation of protein-DNA complexes from cell lysates. DNA is cleaved into ~500 bp fragments by sonication and selected by immunoprecipitation against the protein of interest (CtrA in our case). The DNA fragments are purified, amplified and labeled to be distinguished from an "unbound DNA" control. They are finally sequenced and identified.

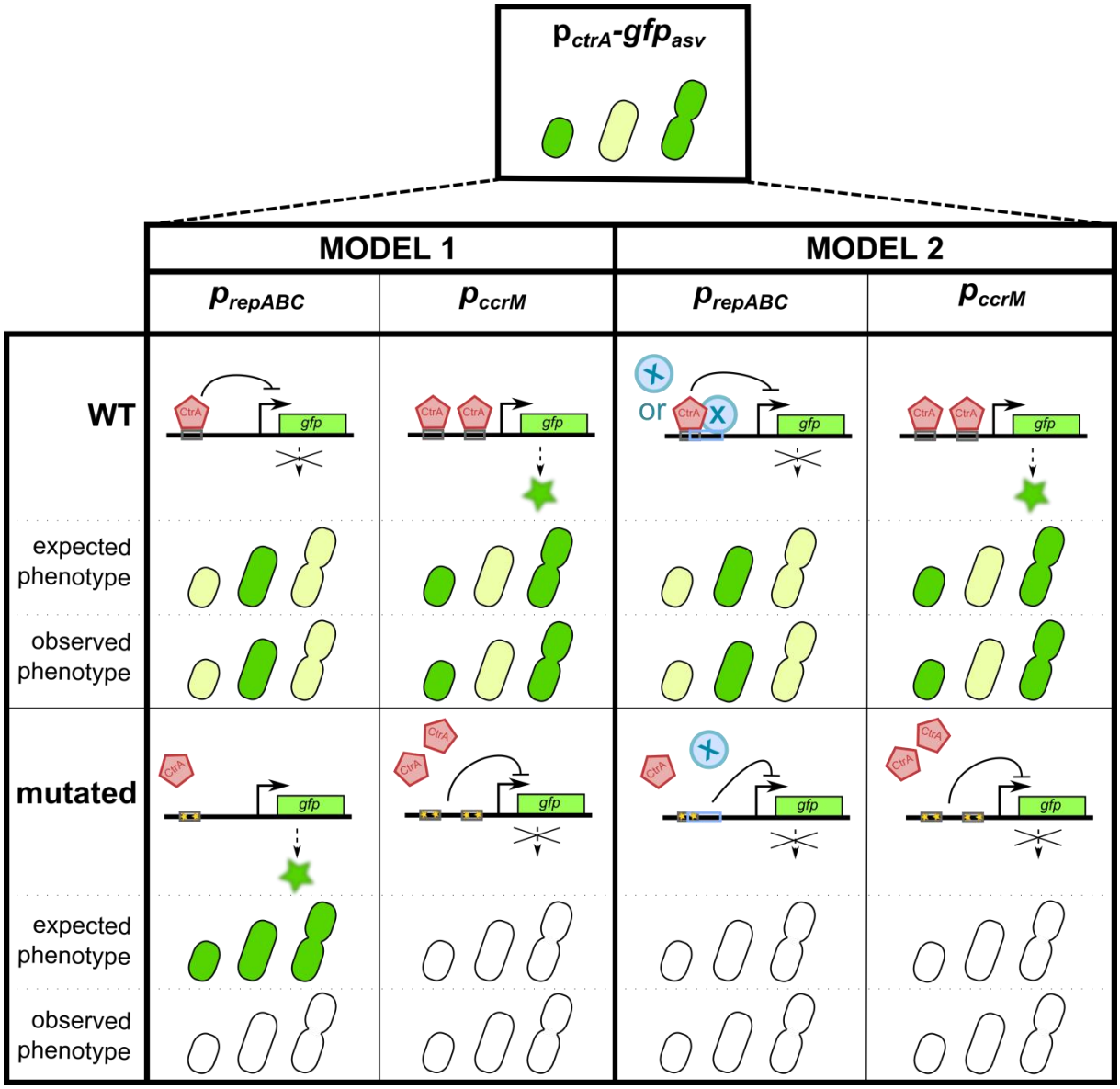

Figure 16: Two models representing the role of CtrA in the regulation of p_{repABC} and p_{ccrM}. These models are inferred from the fluorescence intensity profile obtained with the p_{ctrA}-*gfp*$_{asv}$ *B. abortus* carrying strain (Luca Rappez's master thesis). X represents an unknown factor. The grey and blue boxes represent CtrA binding boxes and X binding boxes, respectively. The small yellow stars represent mutations. The green stars represent GFP production. Green color intensities represent the GFP fluorescence intensities.

II. "If you please… draw me a *model* !"

II.1. In the culture medium

II.1.1. What we can deduct from p_{ccrM}-gfp_{asv} and p_{repABC}-gfp_{asv} profiles

Since drawing a box and telling the readers to imagine what is inside would probably not be welcomed, here is are a few suggestions as to how the results can be explained. As a reminder, the aim of this master thesis was to investigate the potential regulation of four promoters by CtrA in *B. abortus*. The inversed GFP fluorescence profiles of p_{ccrM}-gfp_{asv} and p_{repABC}-gfp_{asv} in culture could therefore be consistent with CtrA activation of one promoter and CtrA repression of the other one.

The limited information we have about *ctrA* expression in *B. abortus* comes from Luca Rappez's master thesis. He showed that, in a 2YT culture medium, a p_{ctrA}-gfp_{asv} reporter system displays a similar profile than the one we obtained with the p_{ccrM}-gfp_{asv} fusion: the highest fluorescence intensity values are reached in the smallest and largest bacteria, while the bacteria of medium size show a low fluorescence. Of course, this does not give us any information about CtrA abundance or phosphorylation state, which is important for it to efficiently bind to its targets (Bellefontaine et al., 2002). Nevertheless, in *C. crescentus*, *ctrA* transcription and its product phosphorylation are coordinated. We could then extrapolate, with some reserves of course, that the p_{ctrA}-gfp_{asv} fluorescence profile obtained somehow reflects CtrA distribution in *B. abortus*. Based on this, p_{ccrM} would be the one that is activated by CtrA, at the opposite of p_{repABC} that would be inhibited. If this hypothesis is right, the mutated versions of the reporter systems should display opposite profiles as well. The mutations should not allow CtrA to bind to the promoters at any time of the cell cycle. This implies that the expected phenotypes should be the same for the whole bacterial population. We should therefore observe high fluorescence intensities for all cell lengths with the *B. abortus* strain carrying the p_{repABC}mut-gfp_{asv} version and no detectable fluorescence for all cell lengths with the p_{ccrM}mut-gfp_{asv} version. However, it is not what we observed. Indeed, if the results we got with the p_{ccrM}mut-gfp_{asv} reporter system match with this model, it is not the case for the p_{repABC}mut-gfp_{asv} fusion (**Figure 16, model 1)**.

Nevertheless, CtrA implication in the regulation of p_{ccrM} and p_{repABC} should not be discarded immediately since our reporter systems definitely have flaws. We do know that the second CtrA binding box of p_{ccrM} overlaps with the -35 element consensus sequence (Robertson et al., 2000) but we made it so that this consensus would be relatively conserved (**Table 2**). This can not be said for p_{repABC}, though. If the -10 or -35 element sequences have been altered in p_{repABC}, the binding of sigma factor would be hindered and we would not be able to observe the GFP fluorescence with the p_{repABC}mut-gfp_{asv} fusion. As suggested in point I.2., doing the same reporter systems with other mutations should help us to understand if we are in such a situation or not.

Actually, it is also possible that the mutations affect another consensus sequence which would have no link with the sigma factor but would be necessary for an important transcription factor. A way to discern it from the sigma factor would be to determine the exact positions of the -10 and -35 boxes. If they do not overlap with the CtrA binding box of p_{repABC}, the implication of the sigma factor can be overlooked. A 5' RACE (rapid amplification of cDNA end) could be used to determine the +1 transcription site. It consists in doing a reverse transcription on the mRNA of the gene of interest. The cDNA is then

Figure 17: DNase I protection of p_{ccrM}. $Fp1_{ccrM}$ and $Fp2_{ccrM}$ represent the first CtrA binding box and the second CtrA binding box, respectively. Vertical bars indicate the sites that are protected in the experiment (adapted from Bellefontaine et al., 2002)**.**

circularized or it is used to form a concatemer, i.e. a DNA sequence containing the same sequence repeated in tandem. This serves as a template for a PCR with the primers chosen to be complementary of the known regions. The sequencing of the PCR products would finally tell us where the transcription had started.

The **model 2** in **Figure 16** is a summary of some of the possible interactions between CtrA and an unknown other factor (designed as X) which could explain the fluorescence profiles of our reporter systems. There are several possibilities:

- CtrA and X both bind to the WT p_{repABC}. X is required for the gene expression and CtrA is an inhibitor of the promoter activity. The mutations on the CtrA binding box should release CtrA and activate the promoter. However, if the mutations overlap both CtrA and X consensus binding sequences, none of them could bind to p_{repABC} which would stay inactive. X could be a sigma factor, for example.
- CtrA and X both bind to the WT p_{repABC}. If X is required for the gene expression and if CtrA is an inhibitor of X, the global effect would be an inhibition of the gene expression in the presence of CtrA. The mutations on the CtrA binding box should release CtrA and stop its inhibition on X. The resulting phenotype would be the activation of the promoter. However, if the mutations overlap both CtrA and X consensus binding sequences, none of them could bind to p_{repABC} which would stay inactive.
- CtrA and X compete for the binding to the WT p_{repABC}. X is required for the gene expression. In the presence of CtrA, X is not able to bind to the promoter, which stays inactive. The mutations on the CtrA binding box should release CtrA and allow the binding of X. However, if the mutations are overlapping their consensus binding sequences, none of them can bind to p_{repABC} which would stay inactive for all cell lengths. For instance, it is the case in *C. crescentus* with DnaA and CtrA (Taylor et al., 2011).

Note that it is also possible that CtrA is not involved in p_{repABC} regulation and that we modified X binding sequence by chance.

Concerning the respective role of each CtrA binding box in p_{ccrM}, things are not very clear. Bellefontaine et al. (2002) showed that, *in vitro*, the first CtrA binding box of p_{ccrM} is bound more strongly by His_6-CtrA than the second box (**Figure 17**). In our experiments, however, it appears that the mutations on the second CtrA binding box have a more drastic effect on the *gfp* expression than the mutations on the first CtrA binding box. As previously mentioned, the second box overlaps with the -35 element binding sequence. Since the WT -35 sequence is diverging by two bases from the consensus sequence known for *E. coli* (TT**A**AC**G** instead of TT**G**AC**A**) and that it is also the case for our mutated version (T**C**GAC**G** instead of T**T**GAC**A)**, we considered it would have no impact on the reporter gene expression. However, an in-depth study of the literature revealed that all substitutions are not equally significant in the -35 element binding sequence, though. The -33 (Gardella T, Moyle H, 1989; Keener and Nomura, 1993) et -31 positions (Gregory et al., 2005; Siegele et al., 1989) have been reported to be more relaxed than the others. The substitution in the WT sequence (at position -33) would then be much less meaningful than the one we introduced in the mutated version (at position -32). This should be taken into account if we have to design other mutated versions of the reporter systems.

If we compare the cell length at which p_{repABC}-gfp_{asv} reaches its maximum mean fluorescence intensity with the cell length at which p_{ccrM}-gfp_{asv} reaches its minimum mean fluorescence intensity, we can observe that they are not synchronized. Interestingly, p_{ccrM}-gfp_{asv} reaches its minimum GFP intensity at a cell length of about 1.95 µm. This is also the case for the p_{ctrA}-gfp_{asv} reporter system of Luca Rappez. This could suggest that p_{ccrM} activity

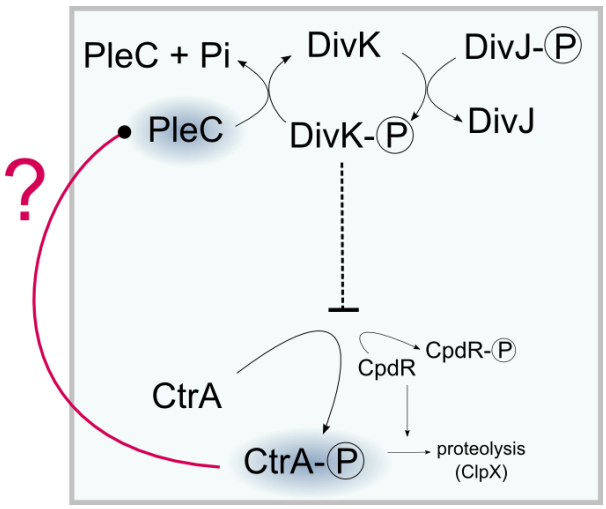

Figure 18: Simplified representation of the pathway regulating CtrA phosphorylation in *C. crescentus*. The phosphorylation of the histidine kinase PleC eventually leads to CtrA activation by phosphorylation. The red curve represents a possible interaction between CtrA and p_{pleC} in *B. abortus* if this pathway is conserved.

is tightly linked to p_{ctrA} activity. As for the p_{repABC}-*gfp*$_{asv}$ reporter system, it reaches its maximum fluorescence intensity at an average cell length of 1.7 µm. In *C. crescentus*, it has been observed that some promoters are more sensitive to CtrA concentration changes than others (Curtis et al., 2013). A hasty conclusion would be to say that, in *B. abortus*, p_{repABC} is very sensitive to CtrA concentration. This way, as soon as CtrA level decreases, p_{repABC} would become active.

These hypotheses have to be considered with great caution because the profiles obtained are the reflections of the GFP synthesis but also of its maturation and degradation. Nothing tells us that it is proteolysed with the same kinetics than CtrA, CcrM, RepA, RepB or RepC. Moreover, the bacterial populations were not synchronized, which means that bacteria with the same cell length can be at different stages of their cell cycle. Furthermore, vertical variations (i.e. due to the individual variability) are bound to occur in a population. This highlights how important it is to confirm the obtained results with other methods, such as those proposed in point I.3.

II.1.2. What about p_{bmaC} and p_{pleC} ?

p_{pleC} appeared to not be cell cycle regulated in culture. The mutations in its promoter did not seem to change anything in the fluorescence profile. *A priori*, this would mean that CtrA does not regulate its activity. This is contrasting with the results obtained by Bellefontaine et al. (2002) who showed that His_6-CtrA was able to bind to p_{pleC}. This experiment was done *in vitro* and not with a WT CtrA version, though. A ChIP assay against CtrA would be particularly interesting to do in p_{pleC} case. The overexpression of *ctrA* in a *B. abortus* strain carrying the p_{pleC}-*gfp*$_{asv}$ reporter system would also give us information about the implication, or not, of CtrA in p_{pleC} regulation. In fact, this strain has already been constructed, but it has not been observed yet. We also constructed a pBBRMCS1-p_{lac}-*pleC* and pMR10-p_{pleC}-*gfp*$_{asv}$ carrying strain. If CtrA is involved in *pleC* expression and if the pathway leading to CtrA activation is conserved between *B. abortus* and *C. crescentus*, the overexpression of *pleC* should, through CtrA, have an impact on its own promoter activation (**Figure 18**). This strain has not been observed yet either.

No fluorescence could be detected with the p_{bmaC}-*gfp*$_{asv}$ reporter system. This could suggest that p_{bmaC} might be activated only in particular conditions in *B. abortus*. In *B. suis*, BmaC is involved in the bacterial adhesion to host cells (Posadas et al., 2012). Adhesins of *C. crescentus*, *A. biprosthecum* and *A. tumefaciens* are apparently produced just-in-time as a result of surface contact (Li et al., 2012). If it is the case with *B. abortus* too, it could explain why we did not detect GFP in culture.

As to its regulation by CtrA, it still is a hypothesis. Indeed, the two putative CtrA binding boxes (one 8-mer box and one 9-mer box) that were found in the sequence regarded as p_{bmaC} were situated quite far away from the ATG start codon of *bmaC*. The ORF upstream on *bmaC* being oriented in the opposite sense, it is possible that the consensus binding sequences actually regulate this gene rather than *bmaC*.

II.2. In the HeLa cell infection model

We could not detect any fluorescence with the p_{bmaC}-*gfp*$_{asv}$ reporter system in the infection model either. Since only a small proportion of newborn *B. abortus* are able to enter host cells (Mullier, unpublished data; Deghelt et al., manuscript in revision), we can

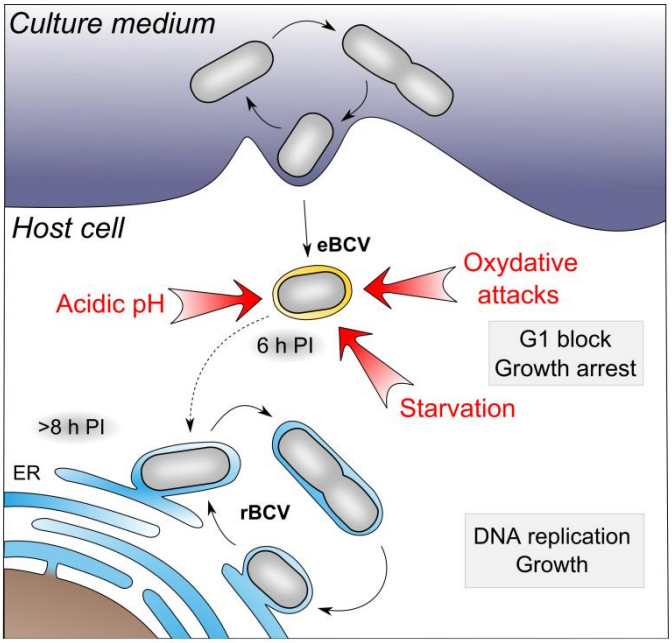

Figure 19: Schematic representation of Brucella trafficking in its host cell. During the first stage of the infection, when the bacterium is in the eBCV compartment, its cell cycle is blocked in G1 and its growth is arrested. The bacterium has to face several stresses, such as acidic pH, starvation and oxidative attacks. Once in the rBCV, the stresses decrease. Moreover, DNA replication and growth are resumed.

hypothesize that a small proportion of the bacterial population needs BmaC. If its production follows the just-in-time regulation mentioned in the preceding point, we would have to be lucky to look at it at the right time. Even if it is not the case, we can not be sure that p_{bmaC} is active during the internalization process. Actually, it may be required for the bacterium adhesion in a later phase of the infection. Until now, we have no mean to synchronize a *B. abortus* population. If a technique could be set up to do it, we would at least be able to follow bacteria that are at the same stage of their cell cycle.

Even though the bacterial populations were not synchronized, we can still clearly observe a striking difference in the fluorescence intensities of the p_{pleC}-gfp_{asv} and the p_{repABC}-gfp_{asv} *B. abortus* carrying strains between the proliferative and the non-proliferative stages of the infection. At the opposite, this observation could not be done with the p_{ccrM}-gfp_{asv} reporter system carrying strain. The constant and low level of fluorescence displayed by the p_{ccrM}-gfp_{asv} fusion is puzzling. We do know that the bacteria are blocked in G1 during the first stage of the infection and that growth begins between 6 and 8 h PI (Mullier, unpublished data; Deghelt et al., manuscript in revision). According to our model, CcrM is needed in predivisional cells to fully methylate DNA just before the end of cell division. Therefore, we were expecting to observe much stronger fluorescence intensities when the bacteria resume their cell cycle. However, we have to keep in mind that proliferating bacteria are not all at the same stage of their cell cycle. This could "dilute" the mean intensity value. Unfortunately, our method does not allow us to clearly discriminate predivisional bacteria from others in infection. In culture, predivisional bacteria but also small bacteria have a high mean fluorescence intensity value. The GFP observed in the smaller bacteria could be due to the remaining GFP after its production in the predivisional bacteria. It is also possible that p_{ccrM} is still active in those bacteria. Whatever the reason, this should also be the case in infection. This means that the "dilution" theory can probably not explain the low intensity values by itself.

The mean GFP fluorescence values of the p_{repABC}-gfp_{asv} *B. abortus* carrying strain in the HeLa cell infection model are less surprising. As expected p_{repABC} activity gets stronger when the bacteria are in their proliferative phase. That is logical because RepA, RepB and RepC are not supposed to be needed as long as the bacterium is not replicating its second chromosome. It should be noted that the replication of ChI begins before the replication of ChII and that the bacterial growth stars between 6 and 8 h PI (Mullier, unpublished data; Deghelt et al., manuscript in revision). The fact that p_{repABC} activity seems to shift systematically after 8 h PI could thus be meaningful. A tight regulation of this operon expression could be necessary to insure that the bacterium stays blocked in G1 until the very last moment.

If our model is right in culture, both p_{ccrM} and p_{repABC} should be regulated by CtrA. However, L. Rappez has observed relatively constant fluorescence intensities with the p_{ctrA}-gfp_{asv} *B. abortus* carrying strain during infection (L. Rappez's Master thesis). If CtrA is not the factor that is inducing the difference of activity in p_{repABC} during the two stages of the infection, then what is it? Bacterial virulence gene expression is often dependent on physical or chemical factors such as reactive oxygen species, pH, temperature, nutrient availability and inorganic ion concentrations (Guiney, 1997). In *B. abortus*, what mainly distinguishes the proliferative and the non-proliferative stages is the habitat. During the first stage of the infection, the bacterium has to face multiple stresses, such as starvation, oxidative attacks and acidic pH (Roop et al., 2004). At the opposite, once the bacterium has reached its replicative niche, those stresses lessen (**Figure 19**). Several *B. abortus* genes that are involved in the adaptation to early intracellular conditions have been found to be active at 4 h PI in

macrophages. These genes are needed for detoxification, osmotic protection and DNA repair (Eskra et al., 2001). A proteomic analysis also showed that *B. abortus* 2308 upregulates the production of proteins involved in DNA mismatches repair and DNA relaxation at 3 h PI, to let them drop at pre-infection levels afterwards (Lamontagne et al., 2009). The same study proposed that the bacterium tends to shift to alternative energy sources early after infection. Several proteins associated with the central carbon metabolism were downregulated at 3 h PI, whereas enzymes required for amino acids catabolism were upregulated (Lamontagne et al., 2009). This suggests that the bacterium undergoes an adaptation period under stressful conditions. One, or several, of those stresses could regulate the activity of p_{repABC} and p_{pleC}.

Unexpectedly, the use of the mutated version of the p_{pleC}-*gfp*$_{asv}$ reporter system in parallel with a WT version has allowed us to propose that, during infection, p_{pleC} regulation could be dependent on CtrA. Indeed, the mean fluorescence intensity values that appear to be higher during the early hours PI with the WT strain drop to the level usually reached at later times in the mutant strain. Interestingly, no striking difference could be observed between the two strains in culture. This suggests that, in the particular context of infection, p_{pleC} activity needs to be tightly regulated. Even though CtrA does not seem to be deeply involved in p_{repABC} regulation during infection, two promoters can be differentially sensitive to the same stimulus. Moreover, we should not forget that we know nothing about CtrA post-translational and post-transcriptional regulation in the infectious context.

The roles of the RepA, RepB and RepC in the infectious context are relatively obvious but that is another story with PleC. In *C. crescentus*, it is part of the phosphorelay that leads to CtrA activation. It can also, through PleD phosphorylation and cyclic di-GMP (a second messager) production, lead to the polar development of the stalk. It is characterized by the flagellar ejection and the stalk biogenesis (Aldridge and Jenal, 1999). Another notable information to know about PleC is that it has been proposed to be involved in the regulation of bacterial density during cell differentiation (Aldridge et al., 2003). PleD is conserved in *B. abortus* and it is thus conceivable that PleC could not only control the DivK-CtrA pathway, but also the production of cyclic di-GMP. The function of PleC could be very interesting to study, first by checking its abundance, the effect(s) of its absence (i.e. analysing growth and replication of a ΔpleC mutant inside host cells) and its role in the (de)phoshorylation of DivK and PleD. To conclude, PleC might actually play the role of an important virulence factor for *B. abortus*, which should definitely not be overlooked.

Conclusion

The main goal of this study was to investigate the role of CtrA in the regulation of several of its putative target genes in *B. abortus*. The four targets we chose to look at are the *ccrM*, *repABC*, *pleC* and *bmaC* promoters. The first part of this master thesis focused on characterizing the activity of those reporters in a rich culture medium (2YT) and in the HeLa cells infection model. To do so, we set up reporter systems. We fused each of those promoters to a *gfp* allele in order to monitor the fluorescence intensities at a single-cell level. In the second part, we constructed similar reporter systems but with a mutated version of the CtrA binding boxes that we had found in the four promoters. By doing so, we aimed at preventing the binding of CtrA on its putative targets in order to check if it had an active role in the regulation of their expression. Like for the wild type reporter systems, we compared their activity in 2YT medium and in HeLa cells. The results we obtained require further studies to be confirmed and clarified. Still, these experiments were rich in information.

No fluorescence could be detected with the p_{bmaC}-gfp_{asv} reporter system. We thus decided to put it aside and to focus on the other promoters. As expected, p_{repABC} seems to be required in the bacterial populations that are proliferating to initiate chromosome replication. Indeed, we found high fluorescence intensity values with the p_{repABC}-gfp_{asv} construct in bacteria of medium size in culture and, more generally, in the bacteria having reached their replicative niche in infection. The role of CtrA in the regulation of p_{repABC} has been neither rejected nor confirmed. It is possible that the mutations we made in its CtrA binding box might have touched the binding sequence of another important transcription factor. As long as this hypothesis has not been cleared off, we can not conclude. At the opposite, it seems likely that p_{ccrM} is regulated by CtrA. Based on the little information we have about p_{ctrA} activity in culture (from L. Rappez's Master thesis), we can propose a model in which CtrA directly activates *ccrM* transcription. If CcrM plays the same role in *B. abortus* than it does in *C. crescentus* (i.e. methylate DNA at the very end of chromosomes segregation), it can explain why the highest fluorescence intensity values were reached in predivisional bacteria in culture. In infection, p_{ccrM} regulation is not that clear and further data are needed to confirm our model. Contrarily to p_{ccrM} and p_{repABC}, p_{pleC} activity does not seem to be rigorously controlled in culture, yet, surprisingly, it looks like it is the case in infection. In addition, even though CtrA does not seem to be deeply involved in p_{ccrM} and p_{repABC} regulation during infection, it appears to play a role in *pleC* expression in this context. In agreement with a paper from Lamontagne et al. (2009), the study of p_{pleC} in the HeLa cell infection model has led us to consider PleC as being an important actor during the non-proliferative stage of the infection. This exciting hypothesis should definitely be tested.

Materials and methods

I. Bacterial strains, growth conditions and plasmids

I.1. Bacterial strains cultures

Escherichia coli strains (DH10B or S17-1) were cultivated on Solid Luria-Bertani (LB) or liquid LB medium at 37°C. *Brucella abortus* 544 strains were cultivated on solid or liquid 2YT medium at 37°C. Antibiotics were used at the following final concentrations:

Antibiotics	Concentration
Ampicilline	100 µg/ml
Kanamycine	50 µg/ml
Chloramphenicol	20 µg/ml
Nalidixic acid	25 µg/ml

The plasmids used in this study were derived from:

Plasmid	Characteristics
pGEMT	High copy number plasmid; resistant to Ampicilline
pBBRMCS1	Medium copy number plasmid; resistant to Chloramphenicol
pMR10	Low copy number plasmid; resistant to Kanamycine

The bacterial strains used in this study were:

Strain	Plasmid carried	Strain number
E. coli DH10B	pMR10-p_{repABC}-*gfp*$_{laa}$	128
	pMR10-p_{repABC}-*gfp*$_{asv}$	132
	pMR10-p_{pleC}-*gfp*$_{laa}$	138
	pMR10-p_{pleC}-*gfp*$_{asv}$	130
	pMR10-p_{bmaC}-*gfp*$_{laa}$	146
	pMR10-p_{bmaC}-*gfp*$_{asv}$	137
	pGEMT-SacI-p_{repABC}-*gfp*$_{asv}$-XbaI	153
	pGEMT-p_{repABC}mut-*gfp*$_{asv}$	152
	pGEMT-p_{repABC}-*gfp*$_{laa}$	122
	pGEMT-p_{repABC}-*gfp*$_{asv}$	124
	pGEMT-p_{pleC}mut-*gfp*$_{asv}$	157
	pGEMT-p_{pleC}-*gfp*$_{laa}$	119
	pGEMT-p_{pleC}-*gfp*$_{asv}$	121
	pGEMT-p_{pleC}-*gfp*$_{asv}$	125
	pGEMT-p_{ccrM}-WT-*gfp*$_{asv}$	159
	pGEMT-p_{ccrM}mut2-*gfp*$_{asv}$	184
	pGEMT-p_{ccrM}mut1-*gfp*$_{asv}$	158
	pGEMT-p_{ccrM}mut1+2-*gfp*$_{asv}$	185
	pGEMT-p_{bmaC}-*gfp*$_{laa}$	120
	pGEMT-p_{bmaC}-*gfp*$_{asv}$	123

	pGEMT-p_{bmaC}-*gfp*$_{asv}$	129
	pBBRMCS1-p_{repABC}-mut-*gfp*$_{asv}$	155
	pBBRMCS1-p_{repABC}-*gfp*$_{laa}$	126
	pBBRMCS1-p_{repABC}-*gfp*$_{asv}$	127
	pBBRMCS1-p_{pleC}-mut-*gfp*$_{asv}$	173
	pBBRMCS1-p_{pleC}-*gfp*$_{laa}$	150
	pBBRMCS1-p_{pleC}-*gfp*$_{asv}$	139
	pBBRMCS1-p_{lac}-ctrA-p_{repABC}-*gfp*$_{asv}$	154
E. coli S17-1	pMR10-p_{repABC}-*gfp*$_{laa}$	131
	pMR10-p_{pleC}-*gfp*$_{laa}$	141
	pMR10-p_{pleC}-*gfp*$_{asv}$	142
	pMR10-p_{bmaC}-*gfp*$_{laa}$	149
	pMR10-p_{bmaC}-*gfp*$_{asv}$	147
	pBBRMCS1-p_{repABC}mut-*gfp*$_{asv}$	156
	pBBRMCS1-p_{repABC}-*gfp*$_{laa}$	133
	pBBRMCS1-p_{repABC}-*gfp*$_{asv}$	134
	pBBRMCS1-p_{pleC}mut-*gfp*$_{asv}$	176
	pBBRMCS1-p_{pleC}-*gfp*$_{laa}$ clone 5	136
	pBBRMCS1-p_{pleC}-*gfp*$_{laa}$ clone 2	135
	pBBRMCS1-p_{pleC}-*gfp*$_{laa}$	151
	pBBRMCS1-p_{pleC}-*gfp*$_{asv}$	143
	pBBRMCS1-p_{lac}-ctrA-p_{repABC}-*gfp*$_{asv}$	160
	pBBRMCS1-p_{ccrM}-*gfp*$_{asv}$	178
	pBBRMCS1-p_{ccrM}mut2-*gfp*$_{asv}$	192
	pBBRMCS1-p_{ccrM}mut1-*gfp*$_{asv}$	177
	pBBRMCS1-p_{ccrM}mut1+2-*gfp*$_{asv}$	196
	pBBRMCS1-p_{bmaC}-*gfp*$_{laa}$	148
	pBBRMCS1-p_{bmaC}-*gfp*$_{asv}$	144
B. abortus 544	pMR10-p_{repABC}-*gfp*$_{laa}$ clone 2	37
	pMR10-p_{repABC}-*gfp*$_{laa}$ clone 1	36
	pMR10-p_{repABC}-*gfp*$_{asv}$ clone 2	35
	pMR10-p_{repABC}-*gfp*$_{asv}$ clone 1	34
	pMR10-p_{pleC}-*gfp*$_{asv}$ pBBRMCS1-p*lac-pleC* clone 2	77
	pMR10-p_{pleC}-*gfp*$_{asv}$ pBBRMCS1-p*lac-pleC* clone 1	76
	pMR10-p_{pleC}-*gfp*$_{asv}$ pBBRMCS1-p*lac-ctrA* clone 2	75
	pMR10-p_{pleC}-*gfp*$_{asv}$ pBBRMCS1-p*lac-ctrA* clone 1	74
	pMR10-p_{pleC}-*gfp*$_{asv}$ 3	53
	pMR10-p_{pleC}-*gfp*$_{asv}$ 2	52
	pMR10-p_{pleC}-*gfp*$_{asv}$ 1	51
	pBBRMCS1-p_{repABC}mut-*gfp*$_{asv}$ clone 3	44
	pBBRMCS1-p_{repABC}mut-*gfp*$_{asv}$ clone 2	43
	pBBRMCS1-p_{repABC}mut-*gfp*$_{asv}$ clone 1	42
	pBBRMCS1-p_{repABC}-*gfp*$_{laa}$ clone 2	33
	pBBRMCS1-p_{repABC}-*gfp*$_{laa}$ clone 1	32
	pBBRMCS1-p_{repABC}-*gfp*$_{asv}$ clone 2	31
	pBBRMCS1-p_{repABC}-*gfp*$_{asv}$ clone 1	30
	pBBRMCS1-p_{pleC}mut-*gfp*$_{asv}$ clone 3	58
	pBBRMCS1-p_{pleC}mut-*gfp*$_{asv}$ clone 2	57
	pBBRMCS1-p_{pleC}mut-*gfp*$_{asv}$ clone 1	56
	pBBRMCS1-p_{pleC}-*gfp*$_{asv}$ clone 2	29

	pBBRMCS1-p_{pleC}-*gfp*$_{asv}$ clone 1	28
	pBBRMCS1-p_{lac}-*ctrA*-p_{repABC}-*gfp*$_{asv}$ clone 2	55
	pBBRMCS1-p_{lac}-*ctrA*-p_{repABC}-*gfp*$_{asv}$ clone 1	54
	pBBRMCS1-p_{ccrM}-*gfp*$_{asv}$ clone 3	61
	pBBRMCS1-p_{ccrM}-*gfp*$_{asv}$ clone 2	60
	pBBRMCS1-p_{ccrM}-*gfp*$_{asv}$ clone 1	59
	pBBRMCS1-p_{ccrM}mut2-*gfp*$_{asv}$ clone 3	67
	pBBRMCS1-p_{ccrM}mut2-*gfp*$_{asv}$ clone 2	66
	pBBRMCS1-p_{ccrM}mut2-*gfp*$_{asv}$ clone 1	65
	pBBRMCS1-p_{ccrM}mut1-*gfp*$_{asv}$ clone 3	64
	pBBRMCS1-p_{ccrM}mut1-*gfp*$_{asv}$ clone 2	63
	pBBRMCS1-p_{ccrM}mut1-*gfp*$_{asv}$ clone 1	62
	pBBRMCS1-p_{ccrM}mut1+2-*gfp*$_{asv}$ clone 3	70
	pBBRMCS1-p_{ccrM}mut1+2-*gfp*$_{asv}$ clone 2	69
	pBBRMCS1-p_{ccrM}mut1+2-*gfp*$_{asv}$ clone 1	68
	pBBRMCS1-p_{bmaC}-*gfp*$_{laa}$ clone 2	41
	pBBRMCS1-p_{bmaC}-*gfp*$_{laa}$ clone 1	40
	pBBRMCS1-p_{bmaC}-*gfp*$_{asv}$ clone 2	39
	pBBRMCS1-p_{bmaC}-*gfp*$_{asv}$ clone 1	38

I.2. Plasmids construction and mating

I.2.1. For the WT reporter systems

Three consecutive PCR were done in order to create the reporter system sequence. The PCR mixes (for a final volume of 50 µl) were composed of dNTPs (4 µl each), 5x Phusion buffer (10 µl), primers (0.5 µl each), Phusion DNA polymerase (0.5 µl), template DNA (1µl of genomic DNA or about 1 µl of minipreparation diluted 10 times) and nuclease free water (to 50 µl). The thermocycling conditions were programmed as:

(1) a denaturation step at 98°C for 30 s,

(2) 25 amplification cycles composed of:

- a first denaturation step (10 s at 98°C),
- a hybridization step (30 s at a suitable temperature based on the primers predicted melting temperatures),
- an elongation step at 72°C (30 s/kb of the expected amplification product).

(3) a final elongation step (10 min at 72°C).

An electrophoretic migration was used to check the absence of aspecific amplification products and to see if the products had approximately the expected size. The gel was composed of 1% agarose supplemented with ethidium bromide to visualize DNA under UV light. The fragment size marker used was the GeneRulerTM 1kb DNA Ladder (Fermentas).

The first PCR, made on genomic DNA, served to amplify the intergenic region situated upstream of the CtrA putative target gene and the region considered as "promoter". The Forward primer (F1) that we used contained a non-complementary XbaI restriction site at its 3' end. The Reverse primer (R1) possessed the beginning of the *gfp* sequence at its 5' end. The second PCR was made on a plasmid-borne copy of a *gfp* gene coding for an unstable GFP. The Forward primer (F2) directly began at the ATG codon of the *gfp* gene. The Reverse primer (R2) began by a non-complementary XhoI restriction site. The two PCR products were

then joined by their common "*gfp* ends" during the first 5 cycles of the third PCR (done in absence of primers). The next 25 cycles of the third PCR served to amplify the resulting hybrid product with F1 and R2. Note that the p_{ccrM}-gfp_{asv} fusion was bounded by SacI and XbaI restriction sites instead of XhoI and XbaI and that it contained a BglII restriction site just after the ATG codon. The final PCR product purification was made on column using the MSB SpinPCRapace (Invitek, Berlin, Germany) and following the manufacturer's protocol. It was then inserted in a linearized (EcoRV) pGEMT plasmid. The ligation mix was composed of the 5x ligase buffer (1x), the restricted plasmid and the insert sequence (volumes variable but always with a large excess of the insert sequence) and the T4-ligase (Fermentas). The final mix was incubated overnight at 18°C.

A $CaCl_2$-competent DH10B *E. coli* strain was transformed with the pGEMT containing the reporter system. The transformation protocol is based on a thermic shock. 50 µl of competent bacterial cells were defrosted on ice and 5 µl of DNA (1-2 µl for the final construct) were added. The mix was left on ice for 30 min before a 2 min thermic shock at 42°C. The bacteria were resuspended with 700 µl liquid LB and incubated at 37°C for 45 min. The culture was centrifuged 3 min at 5000 rpm. The pellet was resuspended in 100 µl of supernatant, then spread onto solid LB-agar medium supplemented with ampicilin for overnight culture.

Five clones were cultivated overnight at 37°C in liquid LB. The plasmids were then extracted. The cultures were centrifuged 2 min at 13000 rpm and the pellet was resuspended in 300 µl of P1 solution (RNAase A 100 µg/ml, Tris HCl 50 mM, EDTA 80 mM, pH 8 and at 4°C). 300 µl of P2 lysis solution (NaOH 200 mM and SDS 1%) were then added. The tubes were turned over and after 5 min, P2 was neutralized by 300 µl of P3 solution (KAc 3 M, pH 5.5). After 5 min, the lysates were centrifuged for 15 min at 13000 rpm. The supernatant was transferred to a new tube and 700 µl of isopropanol were added. The tubes were turned over, then centrifuged for 15 min at 13000 rpm. The supernatant was discarded and the pellet was washed in 300 µl of cold 70% ethanol. After being centrifuged 5 min at 13000 rpm, the supernatant was discarded and the pellet was dried before to be resuspended in ddH_2O (milliQ purification system, Millipore).

The plasmids were checked by XbaI and XhoI diagnostic restriction before to be sent for sequencing (Beckman Coulter Genomics, UK). The reporter systems were then extracted from the pGEMT with XbaI and XhoI restriction enzymes and inserted into linearized (XbaI/XhoI) pBBRMCS1 or pMR10 plasmids. The enzymatic restriction protocol consists in incubating DNA with the appropriate restriction enzyme(s) (10 U/µl) (Roche®) and with the 10x appropriate buffer (1x). The time of incubation depends on the amount of DNA to be restricted (1h to 1h30).

The pBBRMCS1 or pMR10 plasmids containing the reporter systems were used to transform a DH10B *E. coli* strain. Several clones were selected and checked by XbaI and XhoI diagnostic restriction. A conjugative S17-1 *E. coli* strain was then transformed with the purified pBBRMCS1/pMR10-p_{target}-*gfp* plasmids. The newly generated S17-1 *E. coli* strains were conjugated with a wild type *B. abortus* strain. To do so, we mixed 1 ml of *B. abortus* 544 WT strain, which was cultivated in 2YT medium, with 50 µl of the S17-1 *E. coli* strain that was cultivated in LB medium and the appropriate antibiotic. The sample was centrifuged for 2.5 min at 7000 rpm, then the pellet was resuspended in 1 ml of 2YT medium. After being centrifuged again for 2.5 min at 7000 rpm, the pellet was resuspended back in 50 µl of 2YT medium and transferred on a Petri dish with solid 2YT-agar medium. The Petri dish was

incubated 4h at 37°C. The culture was then streaked on a solid 2YT-agar medium supplemented with nalidixic acid (50 µg/ml) and the antibiotic corresponding to the resistance cassette harbored by the plasmid. The Petri dish was incubated 3-4 days at 37°C before to isolate 2-3 clones and to grow them on 2YT-agar medium supplemented with the above-mentioned antibiotics but devoid of nalidixic acid. It was incubated for 2-3 days at 37°C. The obtained colonies were cultivated on liquid 2YT and antibiotics medium.

I.2.2. For the mutated reporter systems

The mutated version of the reporter systems were basically done in the same way than the WT versions. The templates for PCR1 and PCR2 were the plasmids encoding the WT reporter systems. The R1 and F2 primers were designed to be partially complementary of the CtrA binding box. Their "floating" ends contained the chosen mutations. In the case of the p_{ccrM}-*gfp*$_{asv}$ mutated versions, we worked by step by step: we first constructed the p_{ccrM}mut1-*gfp*$_{asv}$ version, then the p_{ccrM}mut1+2-*gfp*$_{asv}$ with the preceding version serving as template. The p_{ccrM}mut2-*gfp*$_{asv}$ version was constructed in parallel.

I.2.3. For the ctrA *overexpressing strains*

The plasmids containing the WT reporter systems were used as template for a PCR. The F and R primers contained floating XhoI and SacI restriction sites, respectively. The PCR products were inserted into a linearized (EcoRV) pGEMT plasmid and sequenced (Beckman Coulter Genomics, UK). This plasmid and a pBBRMCS1-p_{lac}-*ctrA* plasmid (from laboratory stock) were then digested in parallel with XbaI and SacI. The p_{target}-*gfp*$_{asv}$ sequences were ligated into the linearized pBBRMCS1-p_{lac}-*ctrA* plasmid.

The *B. abortus* strains carrying both the pBBRMCS1-p_{lac}-*ctrA* (or pBBRMCS1-p_{lac}-*pleC*) and the pMR10-p_{pleC}-*gfp*$_{asv}$ plasmids were created by doing a mating between a pBBRMCS1-p_{lac}-*ctrA* (or pBBRMCS1-p_{lac}-*pleC*) *E. coli* S17-1 carrying strain and a pMR10-p_{pleC}-*gfp*$_{asv}$ *B. abortus* 544 carrying strain.

II. Microscopy

II.1. HeLa cells infection

The *B. abortus* strains carrying the plasmids of interest were cultivated in liquid 2YT and antibiotics medium at 37°C overnight. HeLa cells were placed in a 24-well plate with a concentration of 6. 10^4 cells/ml. *B. abortus* cultures were diluted in DMEM (Dulbecco's Modified Eagle Medium) to reach a MOI (multiplicity of infection) of 300. The HeLa cells culture medium was discarded and replaced by 500 µl of bacteria-containing DMEM before to be centrifuged 10 minutes at 1200 rpm. After 1 h of incubation at 37°C, the culture medium was discarded and replaced by DMEM and gentamycin (50 µg/ml). Hela cells were stored at 37°C until the end of the experiment. They were washed twice with PBS (Phosphate Buffered Saline), fixed with paraformaldehyde (2%) for 20 min at room temperature, and finally washed with PBS once more.

II.2. Immunostaining

II.2.1. Classical protocol

HeLa cells were permeabilized with Triton X-100 (0.1%), then stained for 30-40 min with the primary antibody (mouse anti-LPS 12G12). Coverslips were washed twice in PBS before to begin the staining with the secondary antibody (anti-IgG mouse TxRed) and DAPI. After 30 min, the coverslips were washed 3 times in PBS, once in water and eventually mounted in mowiol on a microscope slide. It was conserved in the dark.

II.2.2. Intracellular vs extracellular staining protocol

HeLa cells were stained for 30-45 min with a first primary antibody (rabbit anti-Brucella 1/2000 completed with BSA3%), then the coverslips were washed twice with PBS. Next, cells were permeabilized with Triton X100 (0.1%) + BSA (3%), treated with a second primary antibody (mouse anti-LPS 12G12) and washed twice with PBS. After 10 min, HeLa cells were stained for 30-45 min with the secondary antibodies (anti-IgG rabbit PacificBlue 1/500 and anti-IgG mouse TxRed 1/500 + BSA 3% + Triton X100 and completed with PBS). After 30 min, the coverslips were washed 3 times in PBS, once in water and eventually mounted in mowiol on a microscope slide. It was conserved in the dark.

II.2. B. abortus observation in culture and during infection

The *B. abortus* strains carrying the plasmids of interest were cultivated in liquid 2YT and antibiotics medium at 37°C overnight, then diluted approximately five times the next morning. 2 µl of each culture were spotted on a PBS-agarose pad for observations. The Phase100 and FITC channels were used to observe the bacteria and GFP fluorescence, respectively. The microscope which has been used is a Nikon Eclipse E1000 (objective 100X, plan Apo) connected to a Hamamatsu ORCA-ER camera. We also used DF type immersion oil (Nikon oil) with a refraction indice of 1.5150 +/- 0.0002.

Microscope slides with infected HeLa cells were observed in the same way but with a filter to protect fluorochromes from bleaching. The Phase100, FITC, TxRed and UV channels were used to observe HeLa cells, GFP fluorescence, bacteria LPS and DNA, repectively.

Pictures were encoded with NIS-element software and analyzed with MicrobeTracker (Garner, 2011) for bacteria detection and GFP fluorescence intensities quantification. For the culture condition, bacteria were detected automatically on the base on LPS presence. The automatic selections were then checked manually. In the infection condition, the bacteria had to be selected manually. We chose to only consider the bacteria which were focused with the LPS being only clearly visible at the borders of the bacteria.

The parameters used were:

In culture		**In infection**	
algorithm = 2	gradSmoothArea = 0.5	algorithm = 4	erodeNum = 1
scalefactor = 1	thresFactorM = 0.95		
	thresFactorF = 0.95	% Pixel-based parameters	% Image force parameters
% Pixel-based parameters	attrCoeff = 0	areaMin = 150	fitqualitymax = 0.5
areaMin = 150	repCoeff = 0.2	areaMax = 1000	forceWeights = 2/3 1/3 0

```
areaMax = 630
thresFactorM = 1
thresFactorF = 1
splitregions = 1
edgemode = 1
edgeSigmaL = 1
erodeNum = 1

% PDM parameters
scaleFactor = 1
trainingFile = PDMdataEcoli
Nkeep = 11
rigidity = 0.000001
fitqualitymax = 2
fsmooth = 16
fitDisplay1 = 0
fitConvLevel1 = 0.01
fitMaxIter1 = 200
fitStep1 = 1
fitDisplay = 0
fitConvLevel = 0.0001
fitMaxIter = 125
fitStep = 0.4

% Image force parameters
forceWeights = 2/3 1/3 0
dmapThres = 2
dmapPower = 2
attrCoeff1 = 0
repCoeff1 = 0
attrRegion = 4
repArea = 0.9
attrPower = 12
neighRep = 0.5

% Mesh creation parameters
roiBorder = 20.5
noCellBorder = 5
maxmesh = 1000
maxCellNumber = 2000
maxRegNumber = 10000
meshStep = 1
meshWidth = 11
meshTolerance = 0.01

% Joining and splitting
splitThreshold = 0.35
joindist = 5
joinangle = 0.8
joinWhenReuse = 0
split1 = 0

% Other
bgrErodeNum = 5
sgnResize = 1
aligndepth = 1
```

```
thresFactorM = 1
thresFactorF = 1
splitregions = 1
edgemode = logvalley
edgeSigmaL = 1
edveSigmaV = 1
valleythresh1 = 0
valleythresh2 = 1
erodeNum = 1
opennum = 0
threshminlevel = 0.02

% Constraint parameters
fmeshstep = 1
cellwidth = 13
fsmooth = 18
imageforce = 4
wspringconst = 0.3
rigidityRange = 2.5
rigidity = 1
rigidityRangeB = 8
rigidityB = 5
attrCoeff = 0.1
repCoeff = 0.3
attrRegion = 4
horalign = 0.2
eqaldist = 2.5

areaMin = 150
areaMax = 630
thresFactorM = 1
thresFactorF = 1
splitregions = 1
edgemode = 1
edgeSigmaL = 1
dmapThres = 2
dmapPower = 2
gradSmoothArea = 0.5
repArea = 0.9
attrPower = 12
neighRep = 0.5

% Mesh creation parameters
roiBorder = 20.5
noCellBorder = 5
maxmesh = 1000
maxCellNumber = 2000
maxRegNumber = 10000
meshStep = 1
meshTolerance = 0.01

% Fitting parameters
fitConvLevel = 0.0001
fitMaxIter = 500
moveall = 0.1
fitStep = 0.2
fitStepM = 0.6

% Joining and splitting
splitThreshold = 0.35
joindist = 5
joinangle = 0.8
joinWhenReuse = 0
split1 = 0

% Other
bgrErodeNum = 5
sgnResize = 1
aligndepth = 1
```

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