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Étude des gènes essentiels à l'internalisation de Brucella abortus dans les macrophages
une approche Tn-seq

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ESSENTIAL GENES FOR *BRUCELLA ABORTUS* SHORT-TERM INFECTION IN MACROPHAGES : A TN-SEQ APPROACH

Mémoire présenté pour l'obtention

du grade académique de master 120 en biochimie et biologie moléculaire et cellulaire

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Etude des gènes essentiels à l'internalisation de *Brucella abortus* dans les macrophages : une approche Tn-seq

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Résumé

Brucella abortus est un pathogène gram-négatif du groupe des α-proteobactéries et responsable de la brucellose bovine, une anthropo-zoonose mondiale. Ces bactéries intracellulaires facultativement extracellulaires entrainent l'avortement des femelles gestantes ainsi que la stérilité chez les mâles. Récemment, une étude a montré que les bactéries nouvellement dérivées de la division (appelées « *newborns* ») représentent le type cellulaire infectieux prédominant. Afin d'étudier les facteurs des *newborns* pouvant être impliqués dans les étapes précoces de l'infection (adhésion et/ou invasion), une approche « Tn-seq » a été réalisée chez *B. abortus* 2308 à un court temps d'infection. L'analyse statistique des résultats a mis en évidence 77 gènes pouvant conduire à une faible adhésion/invasion/survie des bacteries au sein des cellules hôtes ainsi que 8 gènes montrant un avantage en terme d'internalisation/survie dans les cellules hôtes. Premièrement, *wadB* (glycosyltransférase) et *pgk* (phosphoglycérate kinase) ont été choisis comme contrôles positifs afin de valider les données du Tn-seq. Via la construction de mutants de disruption pour ces gènes, des infections de macrophages RAW 264.7 et des comptages CFU (« *colony-forming units* »), nous avons montré que ces mutants sont atténués à 2 h post-infection (PI). Les mêmes expériences réalisées avec un mutant de disruption pour *gmd* (GDP mannose déhydratase) et une souche délétée pour *wzm* (le composant transmembranaire de l'ABC transporteur de la chaine O du LPS) ont montré une meilleure entrée dans les cellules hôtes pour ces mutants et une forte atténuation pour la souche *Δwzm* à 24 h PI. Grâce à ces résultats, une interprétation possible serait que la chaine O pourrait inhiber la phagocytose des macrophages professionnels et que l'accumulation de celle-ci au sein du cytoplasme serait toxique pour la bactérie. Enfin, l'implication de la boucle RGD (Arginine – Glycine – Aspartate) de la porine Omp2b dans l'adhésion/l'invasion des macrophages RAW 264.7 a été étudiée en la mutant en un motif QGQ (Glutamine – Glycine – Glutamine) chez *B. abortus* 2308 et chez le mutant de disruption *gmd* et la souche *Δwzm*. Les infections réalisées avec ces souches suggèrent que ce motif n'est pas crucial pour les mécanismes d'adhésion et/ou d'invasion de la bactérie.

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Essential genes for *Brucella abortus* short-term infection in macrophages : a Tn-seq approach

GONCALVES de FREITAS Rosa

Abstract

Brucella abortus is a gram-negative pathogen belonging to the α-proteobacteria group and responsible for the bovine brucellosis, a worldwide anthropo-zoonosis. This facultatively extracellular intracellular bacterium causes abortion in pregnant females and sterility in males. Recently, it has been highlighted that during infection, bacteria that are newly derived from the cell division (called newborns) constitute a predominantly infectious bacterial cell type. In order to study *B. abortus* 2308 newborns factors which could be involved early in the infection process (for adhesion to and/or invasion of the host cell), a Tn-seq approach has been performed at short-term post-infection. Statistical analysis of the Tn-seq results brought out 77 genes which could lead to a lower survival, adhesion to and/or invasion of the bacteria in host cells as well as 8 genes showing a strong advantage in term of internalization and/or survival inside the host cells. First, *wadB* and *pgk* (coding for a glycosyltransferase and a phosphoglycerate kinase, respectively) have been chosen as positive controls in order to validate the Tn-seq data. Here we show through the construction of disruption mutants for both genes, infections of RAW 264.7 macrophages and CFU (colony-forming units) countings, that these mutants are attenuated at 2 h post-infection (PI). Then, the same experiments performed for a *gmd* (GDP mannose dehydratase) disruption mutant and a *wzm* (transmembrane part of the O-chain ABC transporter) deletion strain show that both strains enter better in host cells and that *Δwzm* strain CFU number dramatically decrease at 24 h PI. Based on these results a possible interpretation would be that *B. abortus* O-chain could inhibit professional macrophages phagocytosis and that cytoplasmic O-chain accumulation is toxic for the bacteria. Eventually, the implication of porin Omp2b RGD (Arginine – Glycine – Aspartate) motif in the adhesion to and/or invasion of RAW 264.7 macrophages was investigated through its modification into a QGQ (Glutamine – Glycine – Glutamine) motif in *B. abortus* 2308 and in *gmd* disruption and *Δwzm* mutants. Infections performed with these modified strains suggest that Omp2b RGD motif is not crucial for *B. abortus* adhesion and/or invasion mechanisms.

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« If you want to be interesting, be interested. »

P. Arden

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Abbreviations list

Ala	Alanine
AT	Autotransporter
bp	Base pair
CβG	Cyclic β-glucan
CFU	Colony-forming units
chrI	Chromosome I
chrII	Chromosome II
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
ER	Endoplasmic reticulum
ERES	Endoplasmic reticulum exit sites
Glc*N*ac	*N*-acetylglucosamine
h	Hour
LB	Luria-Bertani
LPS	Lipopolysaccharide
M1P	Mannose-1-phosphate
M6P	Mannose-6-phosphate
MOI	Multiplicity of infection
Mur*N*Ac	*N*-acetylmuramic acid
MW	Molecular weight
OD	Optical density
OMP	Outer membrane protein
ori	Origin of replication
PCR	Polymerase chain reaction
PI	Post infection
QGQ	Glutamine – Glycine – Glutamine
RGD	Arginine – Glycine – Aspartate
RGDS	Arginine – Glycine – Aspartate – Serine
TIS	Transposons insertion site
TTM	Transposons tolerance map
WT	Wild type
YFP	Yellow fluorescent protein

Introduction

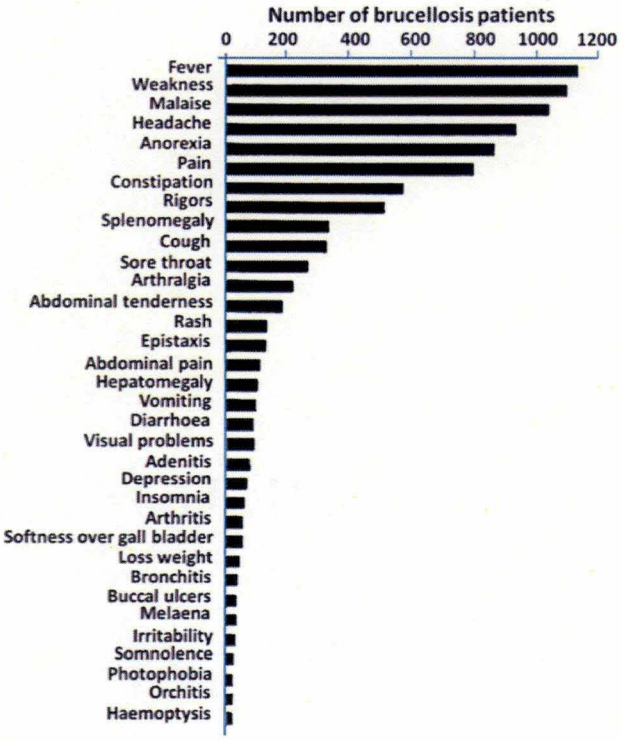

Figure 1 | Brucellosis symptoms. The 34 most frequent symptoms in 1,200 patients suffering from brucellosis (Moreno, 2014).

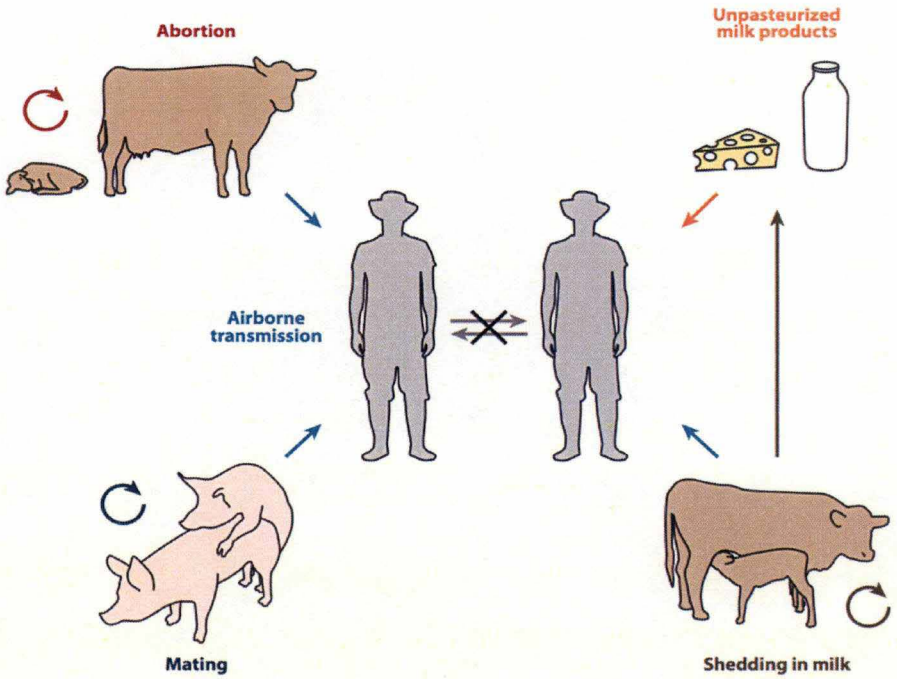

Figure 2 | Brucellosis transmission. Brucellosis transmission can occur within host species through milk or sexual secretions during mating. The generation of aerosols containing a high number of bacteria during abortion can induce the infection of other animals and humans which can also be infected via the consumption of unpasteurized dairy products (Atluri *et al.*, 2011).

Introduction

1. Brucellosis

Brucellosis is an important bacterial anthropo-zoonosis mainly present in third world countries and affecting a broad range of mammals from marine to domestic mammals such as goats, bovines, swines and dogs. Whereas those animals constitute the primary or preferential hosts of brucellosis, this infectious disease can also be contracted by wild animals and human beings who are considered as secondary or incidental hosts (Moreno & Moriyon, 2006). In animals, brucellosis is charachterized as a reticuloendothelial system and reproductive tract disease and is then associated to epididymitis and sterility in males and in colonization of the placenta as well as abortion in females. The manifestation of the disease in humans is more complex as it is usually described as a flu-like illness generating recurrent fevers, muscles and joints pains as well as many other symptoms including chills, weakness, headache, sweating and anorexia (figure 1) (Moreno & Moriyon, 2006). Because of the presence of fever peaks and since the causative agent of human brucellosis was isolated for the first time from the spleen of British soldiers in the Mediterranean island of Malta, this disease is also called « undulant fever » and « Malta fever » (Moreno & Moriyon, 2006).

In the case of human brucellosis a therapy which combines different antibiotics is indicated. Usually doxycycline and rifampicin are recommended and have to be taken in high doses. Unfortunately, even if the treatment is most of the time effective, relapses can occur. If kept untreated, brucellosis may reach a chronical state inducing severe complications like septic arthritis, liver abscess formation, neuronal disorders, endocarditis or even death (Moreno & Moriyon, 2006 ; von Bargen *et al.*, 2012).

Within the preferential host species, the illness is usually transmitted through milk or sexual secretions during mating. Moreover, the presence of high numbers of bacteria in aborted fœtus can induce brucellosis transmission in susceptible animals due to the generation of aerosols during abortion or through some common animal behaviors such as aborted products licking and smelling (Atluri *et al.*, 2011 ; Moreno & Moriyon, 2006). In humans, the disease often occurs after the consumption of unpasteurized dairy products. But in many countries the disease is more considered as an occupational disease which can be contracted by farmers, veterinarians, slaughterhouse staff and laboratory workers (figure 2) (Moreno & Moriyon, 2006).

Nowadays, preventive actions including dairy products pasteurization, diagnosis and culling of infected herds are important in developed countries to contain and limit the spreading of the disease among animals as well as humans. Unfortunately, those actions are difficult to implement in low-income countries since livestock slaughter leads to big economic losses (Moreno, 2014 ; Moreno & Moriyon, 2006).

2. The genus *Brucella*

Bacteria from the genus *Brucella* are non-motile Gram-negative coccobacilli responsible for brucellosis. Those pathogens belong to the diversified α-proteobacteria group which includes *Rhizobium* (symbiont), *Agrobacterium* (plant pathogen), *Bartonella* and

Rickettsia (intracellular parasite) as well as *Caulobacter* (free living bacterium) (Moreno & Moriyon, 2006).

Currently, ten *Brucella* species have been identified and named according to their preferential hosts : *B. melitensis* (caprines and camelids), *B. abortus* (bovines), *B. suis* (swines), *B. canis* (dogs), *B. ovis* (sheeps and rams), *B. neotomae* (desert wood rats), *B. pinnipediae* (seals), *B. ceti* (dolphins, porpoises and whales), *B. microti* (voles and foxes) and *B. inopinata* (isolated from breast implant of a sick patient). Among those species, only the first three are of high pathogenicity for humans (von Bargen *et al.*, 2012).

Because of its high infectivity through aerosols and the absence of effective vaccines for humans, *Brucella* is considered as a category B biological warfare agent and placed in the microorganisms risk group 3 which means that those pathogens have to be carefully manipulated in a biosafety level 3 laboratory (von Bargen *et al.*, 2012 ; Moreno & Moriyon, 2006).

For a long time, *Brucella spp.* were considered as facultative intracellular pathogens because of their ability to grow in culture media and to replicate in cells. But currently, they are seen more as facultatively extracellular, intracellular pathogens since their aim is to avoid extracellular environments which could be hostile and to invade their preferential niche (especially phagocytic cells and trophoblasts) in order to survive, replicate and disseminate inside the host or from a host to one another (Moreno & Moriyon, 2006).

3. *Brucella* infection and trafficking

Although the epithelium of the mucous membrane present in the digestive and respiratory tracts constitutes the classical route of bacteria invasion, bacteria entrance can also occurs through the conjunctiva and the membranes of the reproductive organs (vagina and prepuce) in natural hosts. Once engulfed by resident phagocytic cells (usually macrophages and dendritic cells), bacteria can reach the regional lymph nodes following the lymphatics and subsequently induce a systemic dissemination by infecting new cells and reaching first the blood and then the reticuloendothelial system, the reproductive tract and the mammary glands in primary hosts and any organs in secondary hosts (von Bargen *et al.*, 2012 ; Moreno & Moriyon, 2006). This final spread of the bacteria leads to the chronic state of the disease.

In vitro, *Brucella* poorly but consistently infects epithelial cells and macrophages. Usually, infection of those cells is composed of two phases : a non-proliferative phase, characterized by a proliferation arrest of the bacterium, and a proliferative phase, showing a resumption of the bacterium cell cycle between 6 and 8 hours after their entry in the host cell (Deghelt & Mulier *et al.*, 2014).

The non-proliferative step of infection takes place once the bacterium enters the host cell. Straight after internalization by a macrophage or invasion of a non-phagocytic cell, *Brucella* is localised in a compartment named « *Brucella*-containing vacuole » or BCV (Celli, 2015). The presence of early endocytic markers, including Rab5 (a GTP-binding protein) and its effector EEA1 (Early Endosomal Antigen 1), followed by the appearance of late endocytic markers, like LAMP1 (Lysosome-associated membrane protein 1) and Rab7, seems to

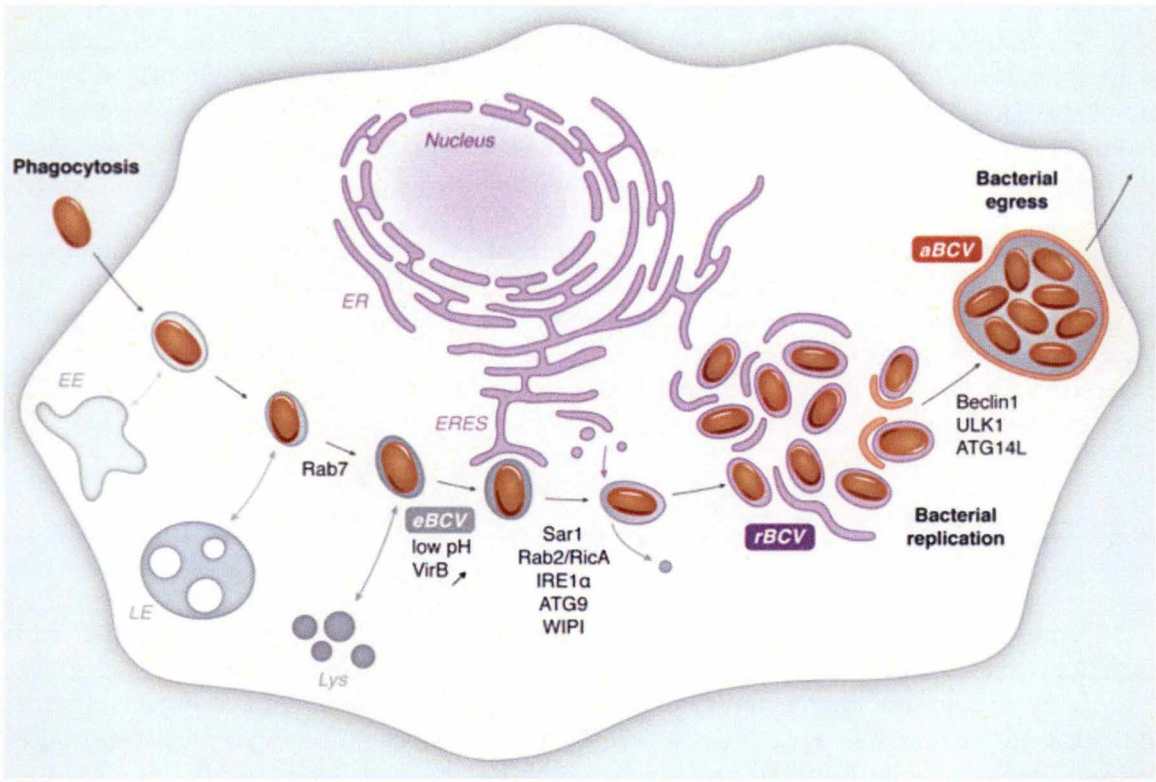

Figure 3 | *Brucella* trafficking inside mammalian host cells. After internalization, *Brucella* is found in a BCV (*Brucella*-containing vacuole) which interacts with the endosomal pathway (*e*BCV). After several hours (between 6 and 8 hours depending the host cell) the BCV interacts with the secretory pathway and becomes a favourable niche for *Brucella* replication (*r*BCV). After replication, bacteria are surrounded by a double-membrane vacuole derived from autophagosomes (*a*BCV) before being released in the extracellular environment (Celli ; 2015). **Abbreviations : EE**, early endosomes, **LE**, late endosomes, **Lys**, lysosomes, **ER**, endoplasmic reticulum, **ERES**, endoplasmic reticulum exit sites.

indicate that the BCV first interacts with the endosomal pathway (figure 3) (Pizarro-Cerda *et al.*, 1998 ; Celli, 2015). During a long time, the difficulty to highlight the presence of the lysosomal hydrolase cathepsin D inside the BCV (Pizarro-Cerda *et al.*, 1998 ; Celli *et al.*, 2003) suggested that *Brucella*, in order to survive, was able to avoid the fusion of its BCV with lysosomes. But later, the emergence of live cell imaging experiments showed that the fusion between the BCV and the lysosomes could take place, although in a limited manner, which is consistent with the pH decrease observed in the BCV once the late endocytic markers are acquired (Starr *et al.*, 2008). It has been shown that this acidification is an essential step for *Brucella* trafficking since it allows the activation of the type IV secretion system encoded by the *virB* operon (Boschiroli *et al.*, 2002). Thanks to the diverse protein effectors released by this important virulence factor, the bacterium is able to reach a beneficial niche in term of proliferation (figure 3) (von Bargen *et al.*, 2012 ; Celli, 2015).

The proliferative step arises when the replication niche is established, namely once the endosomal derived BCV interacts with the endoplasmic reticulum (ER). This late BCV derived from the ER loses its late endocytic markers and eventually displays early secretory pathways markers including calnexin and calreticulin (lectin chaperones) (figure 3) (von Bargen *et al.*, 2012). Even if the molecular mechanisms hidden behind this interaction are not totally identified, it is known that BCV interacts with the small GTPase Sar1, present at ER exit sites (ERES). Indeed, an inhibtion of the Sar1 activity impedes the endosomal derived BCV transformation into a favorable proliferative niche (Celli *et al.*, 2005).

In order to begin another infection cycle, *Brucella* has to be released and spread to other host cells. Recently, it has been proposed that after replication one or several bacteria are surrounded by double-membrane vacuole which is derived from a maturing autophagosome. This newly created autophagic BCV ends the bacterium intracellular cycle and allows its export from the initial host cell (figure 3) (Starr *et al.*, 2012).

4. Intracellular aggressions

To fight intracellular parasites present in phagolysosomes, phagocytic host cells have developped different systems. Indeed, macrophages are able to kill intracellular bacteria through bactericidal mechanisms involving reactive oxygen and nitrogen species as well as several enzymes and cationic peptides (Moreno & Moriyon, 2006). Cationic peptides are antibacterial peptides able to bind to negatively charged elements exposed on the bacterial outer membrane. Once linked to anionic compounds, cationic peptides are able to permeabilize the bacterium envelope in order to sensitize it to lytic enzymes and to interrupt bacterial functions which need an intact outer membrane (Martinez de Tejada *et al.*, 1995). However, it has been shown that after internalization, *B. abortus* does not seems to induce high respiratory burst (transforming oxygen into oxygen reactive species) and appears to resist against antibacterial cationic peptides and lysosomal proteins (Moreno & Moriyon, 2006). Several experiments performed those last years highlighted that bacterial envelope is a crucial factor for intracellular parasites to resist against host cells aggressions and that cell cycle and infection seems to be coordinated (Moreno & Moriyon, 2006).

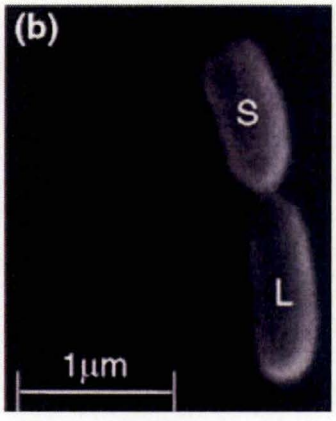

Figure 4 | *Brucella abortus* asymmetric division. Scanning electron micrograph showing the small (S) and the large (L) cells generated during *B. abortus* asymmetric division (Hallez *et al.*, 2004).

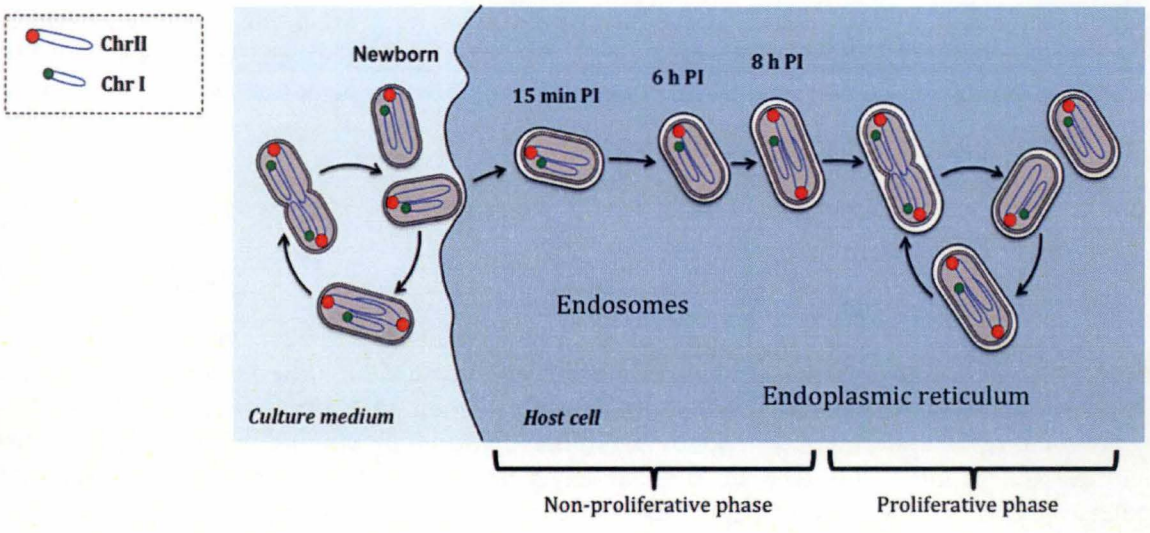

Figure 5 | *Brucella abortus* infection cycle. In the early stage of infection, newborns predominantly enter in the host cells. Between 15 minutes and 6 hours post-infection, those cells stay blocked in G1, during the non-proliferative stage, before reactivation of their cell cycle between 6 and 8 hours post-infection just before the bacterium reach its proliferative niche (proliferative stage). **Abbreviations : ChrI**, chromosome I ; **ChrII**, chromosome II ; **PI**, post-infection.

5. *Brucella* cell cycle

As the cell cycle model *Caulobacter crescentus*, *Brucella* presents an asymmetric division. This conserved trait among several α-proteobacteria leads to the production of two daughter cells of different size, a small one and a large one (figure 4) (Hallez *et al.*, 2004). It is important to underline that cell division generates two new poles, the old pole of each daughter cell being the pre-existing to cell division (Deghelt & Mulier *et al.*, 2014). Furthermore, it has been shown that during *Brucella* unipolar growth, the addition of new enveloppe takes place at the new pole and at the division site (Brown *et al.*, 2012).

In order to efficiently perform cell cycle, replication and chromosomal segregation have to take place. It is well known that *B. abortus* contains two chromosomes : a large chromosome (chrI) and a small one (chrII), displaying their own origin of replication (*oriI* and *oriII*, respectively) (Deghelt & Mulier *et al.*, 2014). In *Brucella*, thanks to the presence of an homologous system of the *C. crescentus* ParABS system, which allows chromosome segregation, the monitoring of the *oriI* replication and segregation is possible (Deghelt *et al.*, 2014). Briefly, the ParABS system is composed of three major elements : a centromeric DNA sequence localized near *oriI* (*parS*), an effector protein able to bind *parS* (ParB) and an ATPase (ParA) (Ptacin *et al.*, 2010). The replacement of the *parB* sequence by a fusion of *parB* with *mcherry* allows to follow the replication and the segregation of the first chromosome when a rescue copy of *parB* is added. Indeed, mCherry-ParB is able to bind to *parS* and generates a fluorescent signal observable by microscopy (Deghelt *et al.*, 2014). ChrII replication and segregation can be followed in an identical manner, but by using the *repABC* operon instead of the ParABS system. The system encoded by this operon is composed of two proteins named RepA and RepB involved in chrII segregation, a centromeric sequence named *repS* recognized by RepB and the sequence *repC* initiating the chrII replication. As for *parB*, *repB* can be replaced by an *YFP-repB* (YFP standing for yellow fluorescent protein) sequence whose protein is still able to bind *repS* and thus allowing the monitoring of the chrII replication and segregation by fluorescence microscopy (Deghelt & Mullier *et al.*, 2014). Those monitoring systems allow to follow the duplication of each focus, namely if the replication/segregation of chrI or chrII have begun or not. In a whole bacteria population containing these monitoring systems, it was underlined that the number of loci (red or yellow regarding the monitoring system) can be different from one bacterium to another (Deghelt & Mullier *et al.*, 2014). Furthermore, it has been shown that when the bacteria display one or two red foci (mCherry-ParB) those are localised at the old pole and following chrI segregation, one focus is found at the new pole and the other at the old pole (Deghelt & Mullier *et al.*, 2014). On the contrary, it has been highlighted that yellow foci (YFP-RepB) are found in different regions during chrII replication, before they each reach a pole after segregation (Deghelt & Mullier *et al.*, 2014). Eventually, those experiments showed that chrI replication is generated before replication of the chrII (Deghelt & Mullier *et al.*, 2014).

B. abortus cell cycle was also monitored inside host cells, especially in HeLa cells as well as in RAW macrophages. This experiment revealed that during infection, bacteria that are newly derived from the cell division, termed newborns here, are the predominantly infectious bacterial cell type (figure 5) (Deghelt & Mullier *et al.*, 2014). Indeed, it has been shown that newborn bacteria are enriched in infection since their relative proportion goes from around 25% in *in vitro* culture, to around 80% in infection (Deghelt & Mullier *et al.*, 2014). Moreover, it has been shown that after entering the host cell, newborns are blocked in G1 and are able to survive inside host cell during the first hours of infection prior to reactivate

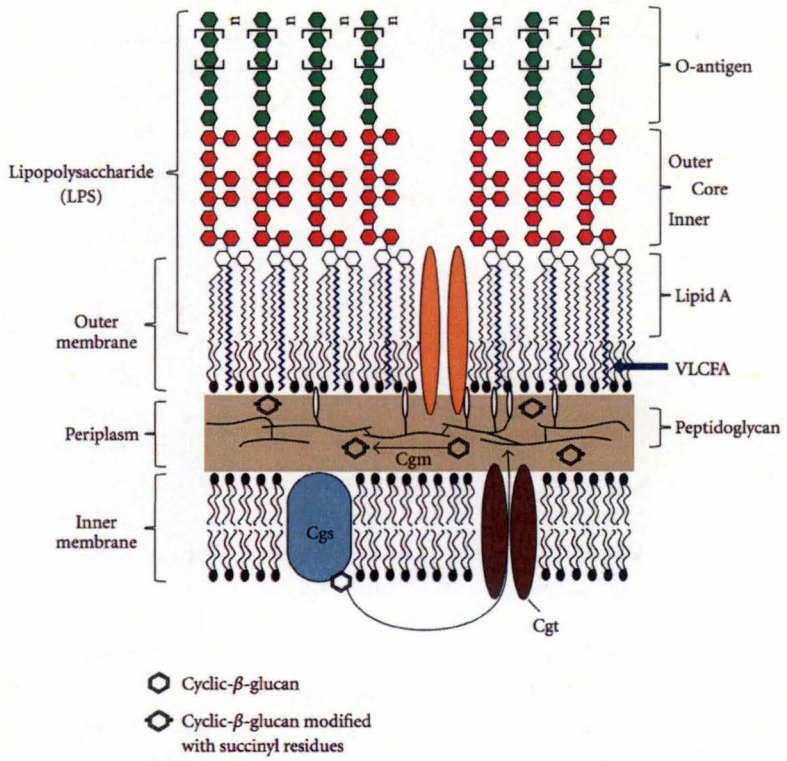

Figure 6 | *Brucella* cell envelope structure. *Brucella* cell envelope is composed of a periplasmic space surrounded by an inner- and an outer- membrane. Peptidoglycan and cyclic β-glucan (CβG) are the major consitutive elements of the periplasm. CβG is produced by a unique cyclic glucan synthase named Cgs and transported into the periplasm thanks to the Cgt ABC transporter. Eventually, once in the periplasm CβG is transformed with two succinyl residues by the cyclic glucan modifier protein (Cgm). The LPS is the most important element of the outer-membrane. It is constituted of a lipid A (modified with very long-chain fatty acid, VLCFA), a sugar core and a O-antigen (Haag *et al.*, 2010).

their replication between 6 and 8 hours after entrance, just before reaching their proliferative niche (Deghelt & Mullier *et al.*, 2014). This means that there is a link between *Brucella* cell cycle progression and infection (De Bolle *et al.*, 2015). Furthermore, since it is known that newborns selection is very quickly performed (as early as 15 minutes PI), it is postulated that they are more adhesive and/or invasive that other bacterial cell types.

6. *Brucella* envelope

Since *Brucella spp.* are Gram negative bacteria they exhibit a cell envelope composed of a periplasm delimited by two membranes : the inner- and the outer membranes (figure 6) (Moreno & Moriyon, 2006).

The periplasm is a space where resides soluble components as well as the peptidoglycan. This one, also called murein, represents an important structural part of the bacterial envelope. Peptidoglycan has two major functions : assuring the bacteria cell shape and maintaining the cell integrity towards the osmotic conditions (Vollmer *et al.*, 2008). Briefly, murein structure is constituted of linear glycan molecules reticulated with short peptides. Glycan molecules are composed of two alternating monosaccharide residues, *N*-acetylglucosamine (Glc*N*Ac) and *N*-acetylmuramic acid (Mur*N*Ac), linked together by β-1,4 connections. During the synthesis of the glycan chain, Mur*N*ac molecule displays a peptide branch generally constituted of L-Alanine (Ala), D-Glutamate, 2,6-diaminopimelic acid (or L-Lysine), D-Ala and D-Ala. It is important to note that in the fully mature glycan chain, the last D-Ala residue of the peptide branch is deleted (Vollmer *et al.*, 2008). Even if no rich analysis of *Brucella* peptidoglycan has not been performed yet, this one appears to be similar to the one of other Gram negative bacteria (Moreno & Moriyon, 2006).

Among the soluble elements which can be found in the periplasmic space, cyclic β-glucans (CβG) are important (Moreno & Moriyon, 2006). These molecules are composed of cyclic glucose molecules (from 17 to 24 monomers) which are linked through β-1,2 bounds. CβG synthesis is divided in 4 enzymatic reactions (initiation, elongation, phosphorolysis and cyclization) catalyzed by a unique cyclic glucan synthase named Cgs. After their biosynthesis, CβG are brought in the periplasm thanks to an ABC-transporter containing a protein displaying characteristics of ATP-binding proteins and called Cgt. Eventually, each CβG molecule present in the periplasm are transformed with two succinyl residues by a cyclic glucan modifier protein (Cgm) (figure 6) (Haag *et al.*, 2010).
Since CβG biosynthesis in *Sinorhizobium meliloti* and in *Agrobacterium tumefaciens* is activated in low osmotic conditions and its cellular accumulation is inhibited in hyper-osmotic conditions, it was hypothesized that CβG biosynthesis could be regulated by the osmotic conditions in *Brucella* too (Zorreguieta *et al.*, 1990). But experiments lead on *B. abortus* and *B. ovis* underlined that CβG production is not osmoregulated (Briones *et al.*, 1997). Furthermore, since β-glucans mutants show a modified intracellular trafficking as well as difficulties to infect host cells, these molecules appear to be important for *Brucella* virulence (Inon de Iannino *et al.*, 1998).

The other soluble components found in the perisplam are mainly enzymes involved in the resistance to oxydative stress including Cu^{++} and Zn^{++} superoxide dismutase (SodC) as well as a catalase (Moreno & Moriyon, 2006).

Inner- and outer membranes are both composed of phospholipids and triglycerides but whereas the inner-membrane displays several proteins playing a role in components transport

or in other metabolic pathways, the outer-membrane is composed of different outer membrane proteins (OMPs) and the lipopolysaccharide (or LPS) (Moreno & Moriyon, 2006). It is important to underline that the outer-membrane is an essential factor since it is the bacterium region which is in direct contact with the host cells (Haag *et al.*, 2010).

The major OMPs of *Brucella spp.* are divided in three groups (1, 2 and 3) depending on their molecular weight (MW) (Moreno & Moriyon, 2006). Group 1 encompasses OMPs with a MW of 88 or 94 kDa and whose functions are still unknown. Group 2 is constituted of two porins named *omp2a* and *omp2b* which have a MW included between 36 and 38 kDa. Finally, group 3 displays OMPs which are not related, including *omp25* whose MW ranges from 25 to 27 kDa (Moreno & Moriyon, 2006).

Different studies concerning *omp25* sequence have revealed that this gene is well conserved between the different species and strains of *Brucella*. The function of this OMP is not clearly known but some experiments in mice have revealed that deletion of *omp25* in *B. abortus*, *B. melitensis* and *B. ovis* lead to an attenuation in infection (Edmonds *et al.*, 2002). Furthermore, it has been shown that the expression of *omp25* is transcriptionnally regulated by the the two-component regulatory system BvrR/BvrS (standing for *Brucella* virulence related regulatory and sensory protein, respectively) which is involved in *B. abortus* cell invasion and virulence (Sola-Landa *et al.*, 1998). Indeed this OMP is not detected in *bvrR* and *bvrS* mutants (Guzman-Verri *et al.*, 2002).

The *omp2a* and *omp2b* present at the *omp2* locus were the first OMPs genes discribed. Those genes, which are separated by 900 bp, exhibit more than 85% of DNA sequence homology. In *B. abortus*, *omp2a* shows a deletion of 108 pb compared to *omp2b* and only this last one is expressed and essential (Cloeckaert *et al.*, 1996). In other *Brucella* species, Omp2a seems to form a larger pore than Omp2b which is interesting, especially during infections when the bacterium suffers of starvation (Cloeckaert *et al.*, 1996).

LPS is localized at the outer leaflet of the outer-membrane and is constituted of 3 major elements : the lipid A, an hydrophobic component which is inside the outer-membrane, the core, linked to the lipid A, and the O-antigen bound to the core (figure 6) (Haag *et al.*, 2010). For many pathogenic bacteria, LPS plays a major role in infection since it is able to activate the innate immune system and thus to induce an immune response in the host (Haag *et al.*, 2010). However, it has been highlighted that *Brucella* LPS is less immunogenic than those of other bacteria and then allows the bacteria to evade the immune system of the infected host and promote bacteria survival (Haag *et al.*, 2010).

Lipid A of *Brucella* LPS is constituted of 2,3-diamino-2,3-dideoxy-D-glucose combined with saturated fatty acids and hydroxylated fatty acids (Haag *et al.*, 2010). As for *Sinorhizobium meliloti*, modified lipid A with very long-chain fatty acids (VLCFA ; figure 6) appears to be important for the interaction of *Brucella* with its hosts. Indeed, it could stabilize the bacterium membrane within host environment as well as protect the bacterium against the antibacterial stress present in host cells (Ferguson *et al.*, 2004). Several genes which are involved in lipid A biosynthesis have been identified in *B. melitensis* 16M and *B. suis* but their precise role have not been characterized yet (annex 1).

The LPS core is constituted of glucose, mannose, quinovosamine, glucosamine, 3-deoxy-D-manno-2octulosonic acid as well as other sugar molecules which have not been identified yet, whereas the O-chain polysaccharide, also composed of sugar, consists of a linear homopolymer constituted of around 96 to 100 subunits of D-mannopyranosyl residues protruding across the extracellular medium (figure 6) (Haag *et al.*, 2010).

In *Brucella spp.*, two types of strains are distinguished according to the presence or the absence of the O-chain and are called smooth- and rough- strains, respectively. *B. abortus*, *B.*

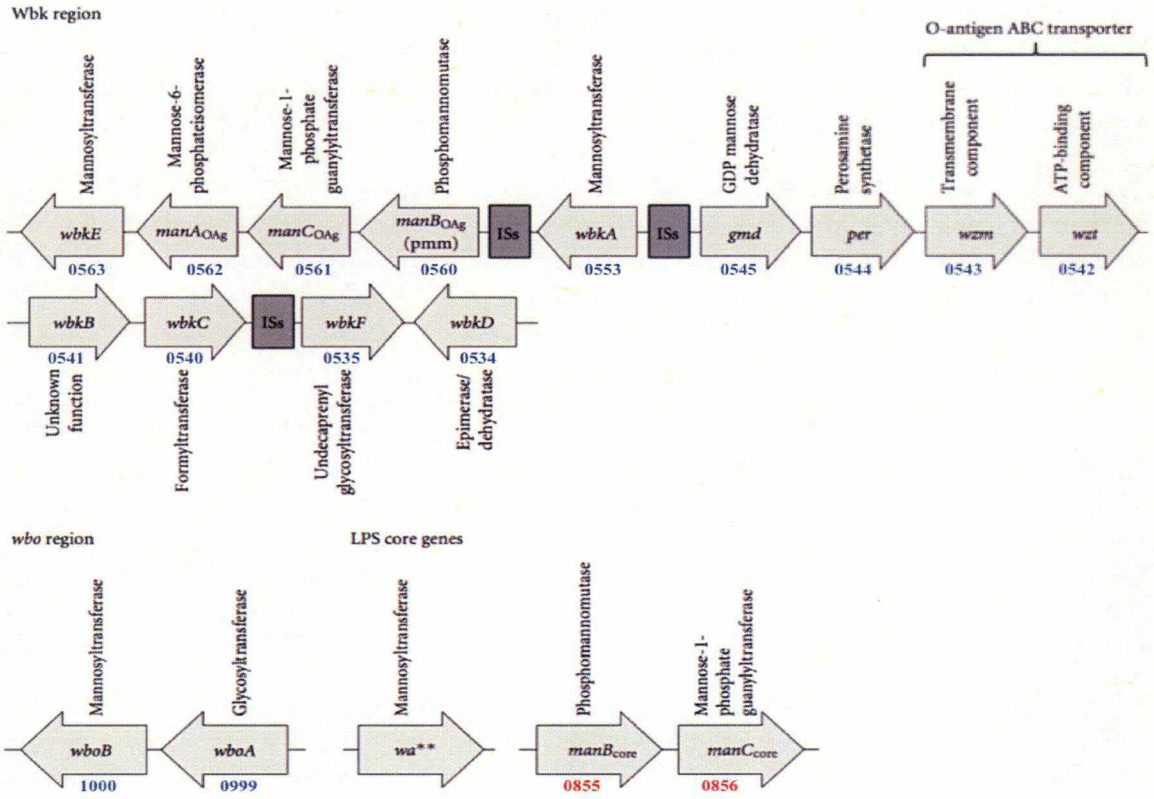

Figure 7 | Genomic regions involved in LPS O-chain and core synthesis. Four different loci dispersed on both chromosomes are predicted to be involved in the biosynthesis of the O-chain and the core of the LPS. Genes involved in the O-chain biosynthesis are located on chromosome I in the *wbk* and *wbo* regions. Genes involved in the LPS core biosynthesis are located on chromosome I (*wa*** gene) and on chromosome II (*$manB_{core}$ and $manC_{core}$*) (Haag *et al.*, 2010). Blue numbers stand for the locus tag for genes belonging to chromosome I (all the number preceded by « BAB1_ ») and red numbers stand for chromosome II genes locus tag (all the number preceded by « BAB2_ »).

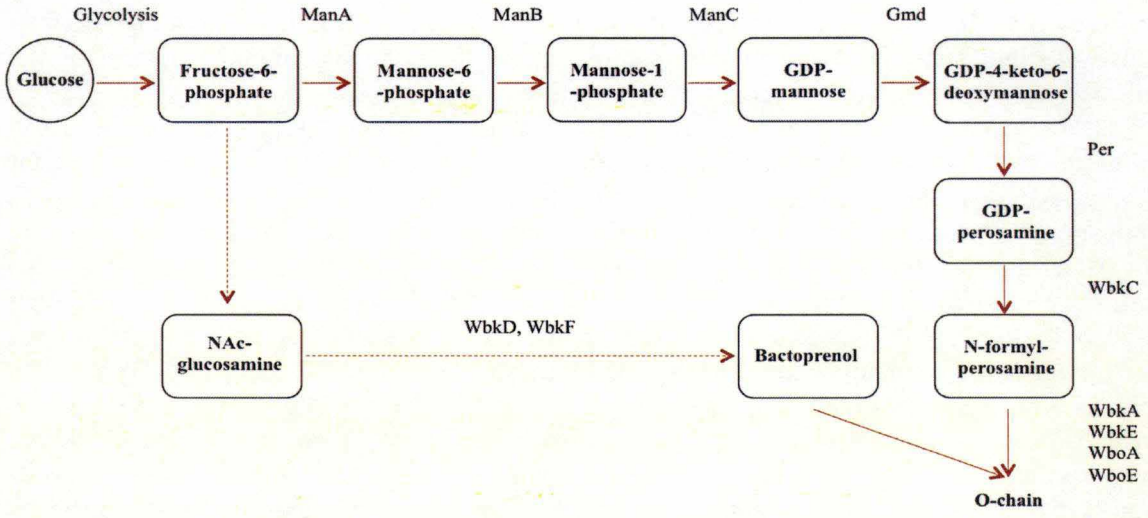

Figure 8 | LPS O-chain biosynthesis. The biosynthesis of the LPS O-chain involved 10 genes of the *wbk* locus (*manA*, *manB*, *manC*, *Gmd*, *per*, *wbkC*, *wbkD*, *wbkF*, *wbkA* and *wbkE*) as well as 2 genes of the *wbo* locus (*wboA* and *wboB*) (Adapted from Haag *et al.*, 2010).

suis as well as *B. melitensis* are smooth strains whereas other species such as *B. canis* and *B. ovis* are natural rough strains (Haag *et al.*, 2010). It has been often reported that this absence of O-antigen led to rough strains less virulent than the smooth ones as well as less resistant against bactericidal molecules generated inside the host cells (Jimenez de Bagues *et al.*, 2005). Furthermore, it has been shown that the O-chain is also essential for the bacterium to reach its replicative niche since *B. abortus* mutants with no O-chain are more phagocyted by the macrophages and destroyed in the lysosomes (Jimenez de Bagues *et al.*, 2005).

It has been shown in *B. melitensis* that genes involved in the O-antigen biosynthesis belong to the locus *wbk* present on the chr I whereas those of the core biosynthesis are dispersed on both chromosomes (figure 7) (Haag *et al.*, 2010). The *wbk* region exhibits several genes involved in the LPS O-chain biosynthesis such as *manA* (mannose-6-phosphateisomerase), *manB* (phosphomannomutase), *gmd* (GDP mannose dehydratase), *per* (perosamine synthetase), *wbkC* (formyltransferase), as well as *wzm* and *wzt* (ABC transporter of the O-chain to cross the inner membrane) (figure 7) (Gomes Cardoso *et al.*, 2006 ; Haag *et al.*, 2010).
For O-antigen synthesis (figure 8), perosamine is required. Perosamine synthesis needs fructose-6-phosphate (derived from glucose) which is successsively transfromed into four intermediate products : mannose-6-phosphate (M6P), mannose-1-phosphate (M1P), GDP-mannose and GDP-4-keto-6-deoxymannose. The perosamine synthetic pathway involved different genes. The *manA* product transforms fructose-6-phosphate into M6P. The phosphomannomutase, encoded by the gene *pmm* in *B. abortus* and by *manB* in *B. melitensis*, is involved in the transformation of M6P into M1P (figure 8) (Haag *et al.*, 2010). It is important to note that both species exhibit two different phosphomannomutases in their genome, one in the *wbk* region and another ($manB_{core}$) in a region of the chrII (figure 7). Because mutants for *manB* do not show defects in terms of O-chain and core biosynthesis unlike $manB_{core}$ mutants, it has been postulated that *manB* present in the *wbk* region only furnish mannose for the O-chain biosynthesis whereas $manB_{core}$ can provides mannose both for the core and the O-antigen biosynthesis (Gonzalez *et al.*, 2008). While Gmd is a mannosyltransferase transforming GDP-mannose into GDP-4-keto-6deoxymannose, the *per* product is involved in the last step of perosamine formation which is the conversion of 4-keto-6-deoxymannose into GDP-perosamine (figure 8). Eventually, this one received a formyl residue added by the formyltransferase WbkC before being assembled onto bactoprenol to form the final O-chain (figure 7) (Haag *et al.*, 2010). While bactoprenol biosynthesis depends on WbkD and WbkF, 4 glycosyltransferases are involved in the polymerisation of the O-chain : WbkA, WbkE, WboA and WboB (figure 8) (Haag *et al.*, 2010). Once synthesized, the O-antigen is translocated in the periplasm through the inner membrane thanks to an ABC transporter composed of a transmembrane domain (Wzm) and an ATPase (Wzt) and finally ligated to the LPS core (Gonzalez *et al.*, 2008).

7. Adhesion of *Brucella* to host cells

7.1 Adhesins

For many pathogenic bacterial species, adhesion to the host cells is a crucial step for host colonization success. In order to be able to establish a contact with specific host cells molecules and to invade targeted cells, bacteria possess surface-exposed virulence factors (Salcedo *et al.*, 2005). Since newborns selection during infection appears very quickly (as

early as 15 minutes PI), it is postulated that they probably are more adhesive and/or invasive than other bacterial cell types. In order to study newborns surface-exposed components which could be involved early in the infection process, three adhesins recently identified in *B. suis* were deleted in *B. abortus* and infectiosity was assessed in previous experiments (M. Van der Henst & J.-F. Sternon, unpublished data). These three adhesins belong to the autotransporters (AT) superfamily and are named BmaC, a monomeric AT (Posadas *et al.*, 2012), BtaE (Ruiz-Ranwez *et al.*, 2013, a) and BtaF (Ruiz-Ranwez *et al.*, 2013, b), both predicted to be trimeric AT thanks to *in silico* analyses. Unfortunately, single or combined deletion strains for these adhesins genes did not result in a decreased internalization at 2 h post-infection in RAW 267.4 macrophages or HeLa cells (M. Van der Henst & J.-F. Sternon, unpublished data). One likely explanation is that even if *Brucella spp.* have a high percentage of genetic identity (usually >98%), their natural hosts are different, which can lead to the selection of distinct adhesion mechanisms for strain to strain.

7.2 RGD motif

Infection success not only depends on pathogens virulence factors but also on the host cells surface molecules/receptors and their ability to interact with a specific ligand. In order to evade their host cells, several host receptors are frequently used by different intracellular parasites (Campbell *et al.*, 1994). On phagocytic cells, two major receptors can be used for pathogen invasion : the integrins and the mannose-binding receptors. Integrins are receptors present on the surface of mammalian cells able to recognize cytoskeletal elements as well as extracellular ligands (Springer, 1990). Several integrins can interact with their ligands through recognition of peptides containing the RGD (Arginine – Glycine – Aspartate) amino acid sequence (Springer, 1990). Thus, integrins can be used by intracellular pathogens exposing RGD motifs as a front door to invade host cells (Campbell *et al.*, 1994). Several years ago, a binding inhibition assay performed on monocyte-derived macrophages from two population of cows (genetically resistant or susceptible to *B. abortus* infection) revealed that bacterial entry in resistant cells was decreased after pretreatment with RGDS (Arginine – Glycine – Aspartate – Serine) tetrapeptides. Thus, those result support the idea that presence of RGD motifs on the surface of intracellular pathogens could be a potential mechanism of entry in the targeted cells (Campbell *et al.*, 1994). Since it is known that the major outer membrane protein Omp2b (Introduction, section 6) exhibits a RGD motif on the bacteria extracellular surface (Campbell *et al.*, 1994) this one could constitute a good candidate to study the implication of this motif in *B. abortus* adhesion and invasion mechanisms.

8. Tn-seq experiment

In order to find candidates which could be involved in the infection cycle, one year ago a new approach named Tn-seq (van Opijnen et al, 2009) has been performed.
Tn-seq is a method without *a priori* allowing the identification of virtually all essential genome regions of any bacterial species at a few base pairs resolution (Christen *et al.*, 2011) in a given culture condition. This method consists in creating a random transpositional mutants library, then determining the transposon insertion sites (TIS) by high throughput sequencing (Curtis & Brun, 2014) and analyzing the results thanks to bioinformatic tools and biostatistical tests.

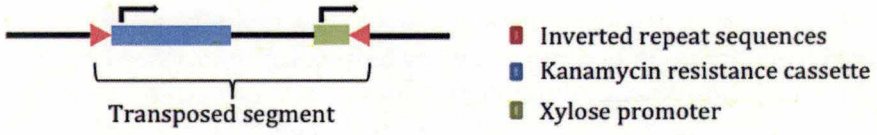

Figure 9 | Modified mini Tn5 transposon. This hyper-active version of a mini Tn*5* transposon is flanked by inverted repeat sequences and composed of a kanamycine resistance cassette as well as a *C. crescentus* xylose promoter.

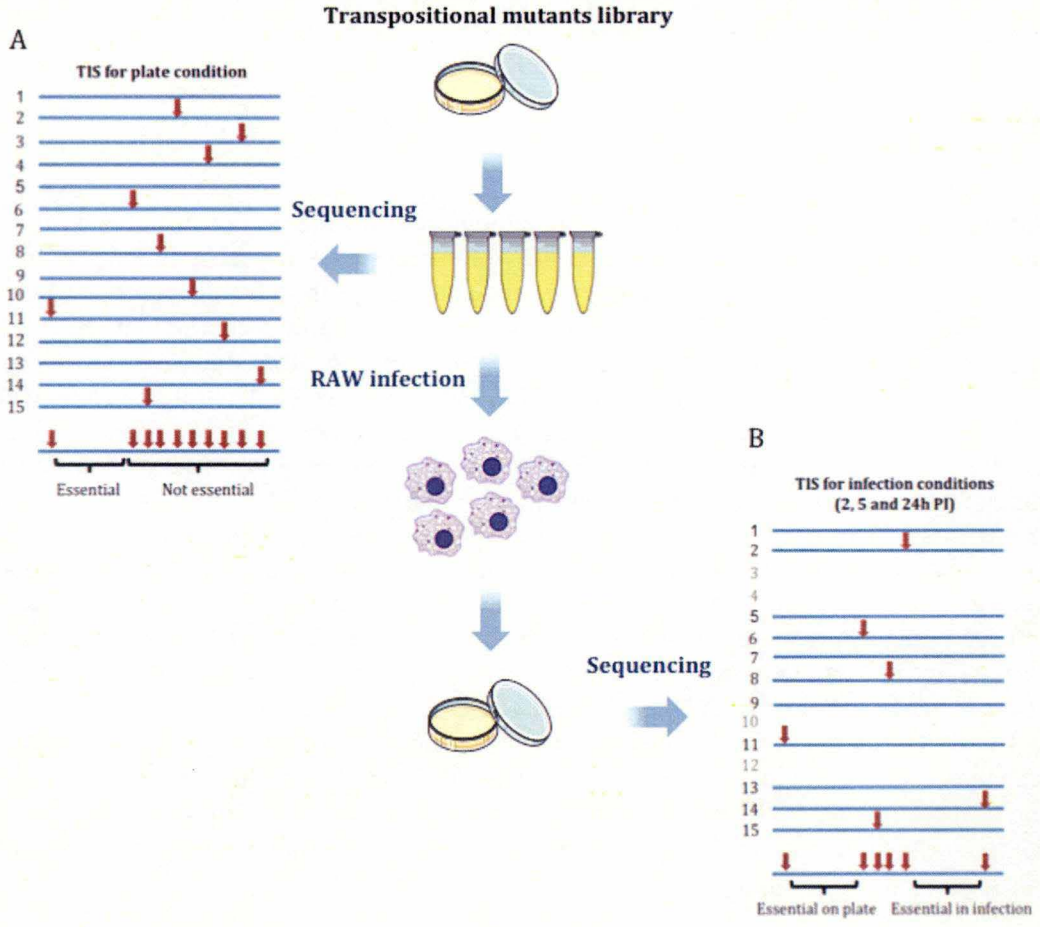

Figure 10 | Tn-seq approach. Firstly, a large transpositional mutant library is generated, about 3.10^6 clones are selected on plates containing kanamycine and colonies are pooled. A part of the mix is used to extract genomic DNA which is then sent for the sequencing of DNA regions flanking the Tn5 transposon in order to identified the transposons insertion sites. The remaining samples are used to infect RAW 264.7 macrophages in order to harvest and plate bacteria at different post-infection times. The genomic DNA of colonies obtained at different post-infection times (2, 5 and 24 hours post-infection) is then sent for sequencing. Finally, the sequencing results are mapped on a reference genome in order to identify the transposon insertion sites (TIS). (**A**) A region is considered as essential to growth on plate if it shows a lack of TIS. (**B**) Regions highlighting a lack of TIS after infection are considered as essential for adhesion to and/or invasion of the host cell (2 hours post-infection), survival (5 hours post-infection) and replication (24 hours post-infection) inside the host cell.

In order to investigate essential genes of *B. abortus* 2308 for growth on plates and at different post-infection time points in macrophages, a random transpositional mutants library was created using a hyper-active version of the Tn*5* transposon (Christen *et al.*, 2011). This transposon is flanked of inverted repeat sequences and composed of a kanamycin resistance cassette and an outward pointing "xylose" promoter (figure 9). This "xylose" promoter was found to be active in *B. abortus*, in the absence of xylose in the culture medium (J.-F. Sternon). Once the library was formed (approximately 3.10^6 mutants, meaning one TIS every base pairs, statistically), clones were selected on plates containing kanamycine in order to only select clones carrying the resistance cassette inserted in their genome. The obtained mutants library was then pooled and split in two different conditions. On the one hand, the majority of the mutants pool was inactivated, genomic DNA was extracted and the regions flanking the transposon were determined by Illumina high throughput sequencing (figure 10). Mapping of the sequencing reads allowed to identify regions of the *B. abortus* genome which could tolerate transposons insertion and still be able to grow on plates, and thus identify which were non-essential for this growth condition. At this stage, genes essential for growth on plates or toxic for the bacteria when mutated are characterized by a lack of transposons insertion sites (figure 10A). On the other hand, in order to identify genes essential for infection of RAW 264.7 macrophages, the remaining of the mutants pool was used to infect those host cells (figure 10). Briefly, RAW 264.7 macrophages were first infected, and bacteria were then extracted and plated. In principle, each colony able to grow at this step originated from a mutant which was not impaired for infection. Genomic DNA from these colonies was then isolated and sent for high throughput Illumina sequencing of regions flanking the transposon. In order to identify genes required at given steps of the infection process, the infected cells were split in 3 samples, differing by the duration of the infection time prior to bacterial extraction (2 h, 5 h and 24 h post-infection respectively). Firstly, a 2 hours post infection (PI) sample was performed to identify mutants unable to adhere to and/or invade macrophages or to survive to early trafficking. Secondly a 5 hours PI sample was performed to identify mutants unable to survive to trafficking inside host cells prior to reach their replication niche. Finally, a 24 h PI time sample was performed to identify mutants unable to access their replication niche or to replicate inside host cells (figure 10B). After sequencing, the obtained reads were mapped onto a reference genome in order to generate a transposons tolerance map (TTM).

Objectives

Objectives

The objective of this master thesis was first to analyze the reads for the control (or "plate") and the 2 h PI conditions using homemade bioinformatic tools and the genome mapping softwares SeqMonk® and Artemis® and then to compare the TTM from these 2 conditions in order to identify regions showing a lack or a decrease of TIS frequency in the 2 h PI condition but not in the control condition. Indeed, genomic regions showing such characteristics are likely to have a function in adhesion and/or invasion and/or early survival in RAW 264.7 macrophages.

The second objective of this master thesis was to challenge the Tn-seq results by mutating 4 candidates generated by the analysis using a disruption vector to mimic the transposon disruption effect and to assess the infectiosity of these mutants by CFU (colony-forming units) countings at 2 h PI in RAW 264.7 macrophages.

Finally, the last objective of this master thesis was to chose interesting candidates amoung the candidate genes indentified by the analysis and to investigate them by mutation using a disruption vector and to study their residual infectiosity through RAW 264.7 macrophages infections and CFU countings.

Results

Table 1

Tn-seq characteristics

Condition	*Number of sequencing reads mapped on genome*	*Number of TIS*	*Theoretical distance between each TIS*
Control	154,630,306	544,094	6 bp
2 h PI	183,451,638	415,778	8 bp

Remark : One transposon insertion site (TIS) corresponds to a site where at least one transposon read has been found.

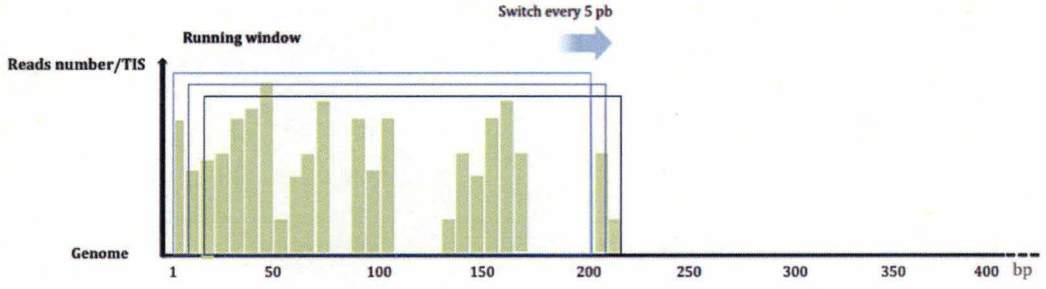

Figure 11 | Running window. In order to facilitate the Tn-seq results analysis, a 200 bp running window was shifted every 5 bp generating 655,662 different R200 values. The number of reads per TIS is shown in green.

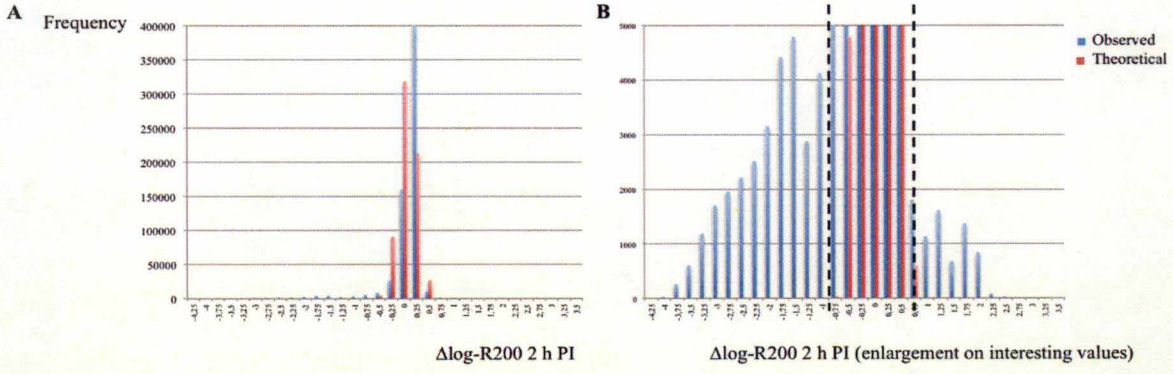

Figure 12 | Frequency distribution. (**A**) The actual distribution fits with the theoretical distribution which allows to define a negative (-0,75) threshold and a positive (+0,75) threshold. (**B**) Enlargement of the lower frequencies (up to 5000) shows that the observed frequencies for the extreme values are much higher compared to the theoretical frequencies. Thus, under- and over-representation of many windows is not due to chance.

Results

1. Statistical analysis

The Tn-seq experiment on plate (control condition) and after 2 hours of infection (« 2 h PI » condition) were performed as described in the Introduction (section 8). Thanks to the genome mapping software SeqMonk, the Tn-seq characteristics (table 1) as well as the TTM for each condition were obtained. To facilitate the analyses, *B. abortus* genome was split using a 200 base pairs (bp) running window shifted every 5 bp (figure 11) thus generating 655,662 different windows. Since almost all TIS were mapped with more than one sequencing read, we defined a parameter called R200, that is equal to the number of reads mapped for a given window of 200 pb. In the control condition (on plates), the average reads frequency for R200 was 9,481, thus 3.98 on a $\log_{10}$ scale ($\log_{10}$R200). A TTM with all $\log_{10}$R200 values was created to be visualized with the Artemis program. The $\log_{10}$R200 values were adapted by adding 1 to the R200 value because many regions of the genome, being essential, do not tolerate a single transposon and thus have a R200 equal to 0.

In order to compare the 2 h PI time to the control condition, a computing named « delta analysis » has been performed. For each window, $\log_{10}$R200 values from the control were substracted to $\log_{10}$R200 from the 2 h PI time point, generating a « Δlog-R200 » value for each window. The frequency distribution of the Δlog-R200 values was mostly centred on 0 (meaning that most sites are neither over-represented nor under-represented after infection) (figure 12A), but about 36,000 windows displayed a lower Δlog-R200 value (figure 12B). The apparently random variation around a Δlog-R200 of 0 was estimated using a theoretical normal distribution using the average of the Δlog-R200 values (-0.061). A standard deviation (0.18) was manually selected to allow the fitting of this theoretical curve with the observed frequency distribution of Δlog-R200 values (figure 12). Stringent cutoffs were chosen (-0.75 and +0.75) to identify the regions having an abnormal Δlog-R200 value at 2 h PI (figure 12B). According to the average and the (abritrary) standard deviation of the theoretical distribution, we calculated that Δlog-R200 values contain 0.11% of false positive windows, *i.e.* that are outside the cutoff values by chance. This percentage of false positive is for each window separately considered thus if several windows are taken together, the probability to have false positive windows is very low. With this analysis, we found 36,006 windows below the -0.75 Δlog-R200 cutoff, and 5811 windows above the 0.75 Δlog-R200 cutoff. The 36,006 windows correspond to 77 candidate genes (annex 2) that have a lower $\log_{10}$R200 value at 2 h PI compared to the $\log_{10}$R200 value obtained for the control. This suggests that those 77 genes are necessary and non-redundant for the bacteria to be internalized in macrophages and/or to survive for up to 2 h in the macrophages. The 5811 windows above the cutoff of 0.75 correspond to mutants that are proportionally more abundant at 2h PI compared to the control condition, suggesting that they have a strong advantage to be internalized or to survive inside host cells. These 5811 windows map to 8 genes (annex 3) that will be described later (Results, section 8).

2. Tn-seq results validation

The goal of the Tn-seq experiment was to identify genes which could be essential for the adhesion to, the survival and the proliferation inside host cells. The statistical analysis of the Tn-seq results brought out 77 genes at 2 h PI (annex 2) which could be involved in the

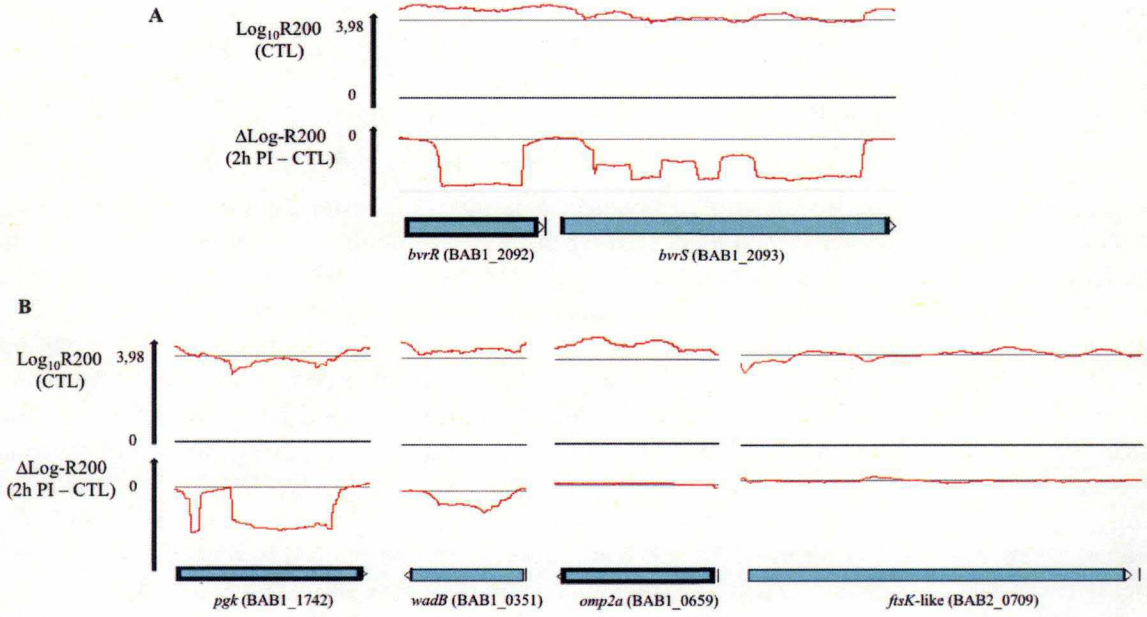

Figure 13 | Log_{10}R200 (control condition) and Δlog-R200 (2 h PI-CTL) values. Log_{10}R200 and Δlog-R200 values show that there is a decrease of the log_{10}R200 values at 2 hours post-infection compared to the control condition for *bvrR/S* (**A**), *wadB* and *pgk* (positive controls – **B**). On the contrary, the log_{10}R200 values of *omp2a* and *ftsK*-like gene (negative controls - **B**) stay stable at 2 hours post-infection compared to the control condition. (Images from Artemis) **Abbreviations : CTL**, control ; **PI**, post-infection.

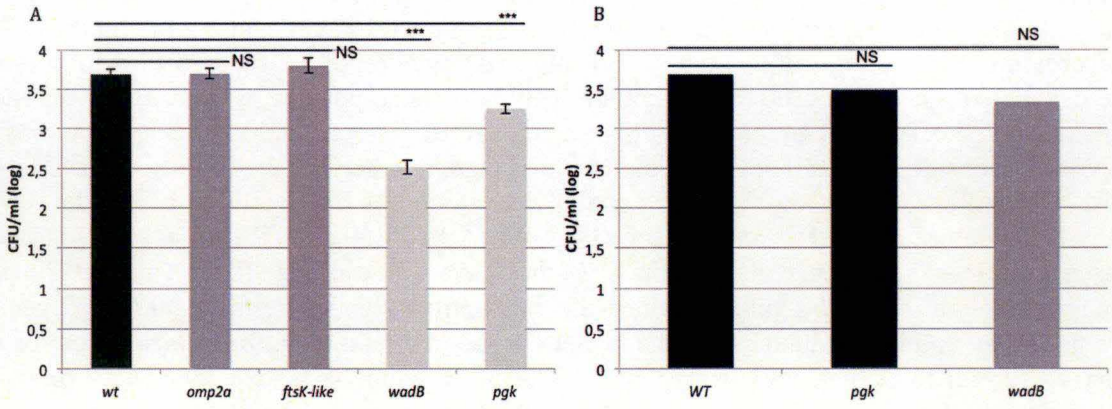

Figure 14 | CFUs countings after (A) RAW 264.7 macrophages infection and (B) sensitivity test to decomplemented bovine serum. (**A**) Infection of RAW 264.7 macrophages have been performed with the wild-type strain *B. abortus* 2308 as well as with *omp2a* and *ftsK*-like gene disruption strains (negative controls) and *wadB* and *pgk* disruption mutants (positive controls). After 2 hours of infection, bacteria were extracted, plated and colonies were counted 4 days later. Here, bacteria able to form a colony are expressed in CFU/ml (log scale). (**B**) *wadB* disruption strain sensitivity to bovine serum has been tested in conditions close to infection conditions. The wild-type strain *B. abortus* 2308 as well as the *pgk* disruption mutant have been used as negative controls for this experiment. Bacteria able to form a colony after the test are expressed in CFU/ml (log scale).

bacterium internalization and/or short-term survival inside the macrophages (Results, section 1).

In the first place, in order to have a preliminary opinion on the reliability of the Tn-seq results, we decided to look after the two genes coding for the two-component regulatory system BvrR/BvrS among our candidate genes list (Introduction, section 6). Indeed, since it has been shown that this system is involved in *B. abortus* cell invasion (Sola-Landa *et al.*, 1998), we expected that *bvrR* and *bvrS* would display a lower $\log_{10}$R200 values at 2 h PI compared to the control condition. As expected, both genes exhibit the profile we were looking for (figure 13A) and thus consistute good preliminary positive controls. Then, in order to validate the Tn-seq results, *i.e.* to check that genes identified at 2 h PI indeed generate attenuation when mutated, we decided to mutate four different genes (2 positive and 2 negative controls) using a disruption vector to mimic the transposon disruption effect, and to assess the infectiosity of these mutants by CFU countings at 2 h PI in RAW 264.7 macrophages.

First, we looked for two positive controls, meaning genes known for inducing an attenuation in infection when mutated. Among our candidates list, we chose *wadB* (BAB1_0351), which codes for a glycosyltransferase involved in the synthesis of a branch in the core of the LPS. In fact a *wadB* deletion mutant was described to be attenuated at 48 h PI in bone marrow derived dendritic cells (2 h PI time has not been tested in this paper) (Gil-Ramirez *et al.*, 2014). The second candidate chosen is *pgk* (BAB1_1742, not in the candidate genes list because selected before the completion of the statistical analysis) which encodes a phosphoglycerate kinase involved in glycolysis as well as in gluconeogenesis. In fact, it was reported that a *pgk* deletion mutant are attenuated from 2 h PI in bone marrow derived macrophages (Trant *et al.*, 2010). These two candidates are relevant as positive controls for the Tn-seq experiment because they show a decrease of their $\log_{10}$R200 values at 2 h PI compared to the plate (control) condition (figure 13B).

Then, we looked for negative controls, meaning genes which do not show a difference of the $\log_{10}$R200 value in the 2 h PI condition compared to the plate condition in our data sets. For this purpose, *omp2a* (BAB1_0659) and a *ftsK*-like gene (BAB2_0709) have been chosen (figure 13B). The *omp2a* is proposed to be silent (not transcribed) in *B. abortus* but codes for a porin in other *Brucella* species (Cloeckaert *et al.*, 1996) (Introduction, section 6) whereas the *ftsK*-like gene encodes a putative ATPase with a C-terminal domain homologous to FtsK, a protein putatively involved in chromosomes segregation during cell division.

In order to create a disruption mutant for each of these 4 candidates, a plasmid containing a central part of the gene of interest as well as a kanamycin resistant cassette was inserted in the genome of *B. abortus* 2308 (Experimental procedures, section 8). Once the mutants were generated and selected on kanamycine plates (Experimental procedures, section 8), they were used to infect RAW 264.7 macrophages in order to evaluate their ability to be internalized and survive for 2 h in this cell line. Living bacteria were enumerated by counting CFU. In parallel, macrophages infection as well as CFU countings were also performed with the wild type (WT) *B. abortus* 2308 strain. As expected, the CFU countings for the disruption mutants for *omp2a* and *ftsK*-like did not show any difference compared to the results obtained for the WT strain (figure 14A). Conversely, the disruption mutants for *wadB* and *pgk* did show a decrease of the number of CFU compare to the WT (figure 14A). It is important to underline that this decrease is stronger for the *wadB* disruption strain compared to the *pgk* disruption mutant (figure 14A). However, we noticed that *pgk* disruption mutant colonies were smaller than those of the 3 others disruption strains and of the WT even if there is not apparent growth defect for *pgk* disruption mutant in liquid culture. However, the Tn-seq in the control condition indicates that the transpositional mutants in *pgk* are slightly less frequent that

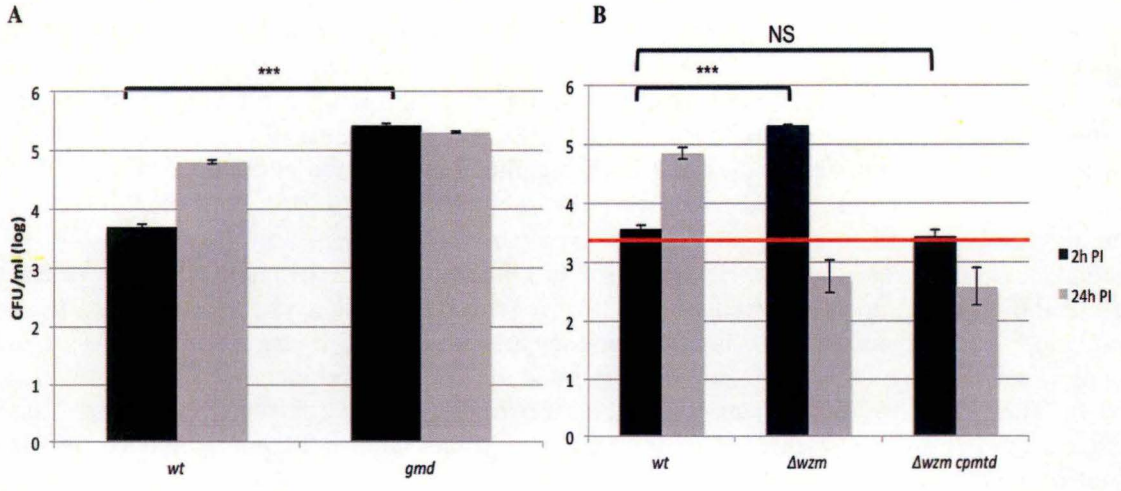

Figure 15 | RAW 264.7 macrophages infection and CFUs counting. (**A**) Infection of RAW 264.7 macrophages have been performed with the wild-type strain *B. abortus* 2308 as well as with *gmd* disruption strain. (**B**) RAW 264.7 macrophages infections were performed with the wild-type strain *B. abortus* 544 as well as with a Δ*wzm* mutant and a Δ*wzm* complemented strain. For (**A**) and (**B**) after 2 hours and 24 hours post-infection bacteria were harvested and plated. Here, bacteria able to form a colony are expressed in CFU/ml (log scale). The detection threshold of CFUs countings is represented in red (3,9). **Abbreviation : cpmtd**, complemented.

mutants in other regions of the genome (figure 13B), suggesting that pgk mutant have some problems to grow optimally on rich medium plates.

An additional experiment has been performed with the *wadB* disruption mutant. Indeed, its sensitivity to decomplemented bovine serum (present in macrophages culture medium) was tested since it has been shown that deletion mutants for *wadB* were more sensitive to bovine serum (Gil-Ramirez *et al.*, 2014), and thus the attenuation of the *wadB* mutant could be due to its increased sensibility to the serum rather than to a decreased internalization or an increased killing by the macrophages. To test this, the *wadB* disruption strain was incubated during one hour in macrophages culture medium before performing CFU countings. In parallel, the same experiment was performed on *pgk* disruption strain and on WT strain, used as negative controls. With this experiment, we observed that decomplemented serum has a weak effect on *wadB* disruption mutant (figure 14B) as shown for the deletion strain (Gil-Ramirez *et al.*, 2014) which does not account for the phenotype observed in the previous experiment.

In conclusion for this part of the Results, it seems that the attenuation of genes suggested by the Tn-seq experiment generated data, is indeed observed by the analysis of individual mutants, even if they are not mutated with a Tn*5* but by the insertion of a plasmid.

3. Rough mutants

In addition to the the 77 candidates showing a decrease of their $\log_{10}$R200 values at 2 h PI compared to the control, the statistical analysis of the Tn-seq results also brought out 8 genes (annex 3) showing an increase of their $\log_{10}$R200 values at 2 h PI compared to the plate condition (Results, section 1). These regions are interesting because they suggest that these transpositional mutants have a strong advantage in term of internalization and/or survival inside RAW 264.7 macrophages. These 8 genes are involved in the LPS O-chain synthesis and 5 of them belong to the *wbk* region (Introduction, section 6). Since these genes are involved in the LPS O-chain biosynthesis, transpositional mutants for these genes are probably rough mutants (Introduction, section 6). It is known that rough mutants entry is increased compared to their corresponding smooth strains (Jimenez de Bagues *et al.*, 2005), thereby, *gmd* gene belonging to *wbk* locus was chosen in order to built a disruption mutant and to assess its infectiosity in RAW 264.7 macrophages by CFU countings at two PI times (2 h and 24 h PI) in order to see if this rough mutant would enter better than its corresponding WT smooth strain. As for the control candidates, the disruption mutant was created thanks to the integration of a plasmid containing a central part of the gene in the *B. abortus* 2308 genome (Experimental procedures, section 8). Furthermore, RAW 264.7 macrophages infections were also performed with the WT strain in parallel. As expected, CFU countings at 2 h PI for the *gmd* disruption mutant showed an increase of the number of CFU compared to the WT (figure 15A). At 24 h PI, the number of CFU for the disruption strain remains stable compared to 2 h PI whereas the number of CFU for the WT has increased (figure 15A). These results show that this mutant enter better in host cells and thus corroborate with the Tn-seq results and with the litterature concerning rough mutant strains entry. Interestingly, the CFU number at 24 h PI indicate that the mutant is not killed inside the macrophages, or that its potential killing is compensated by its growth.

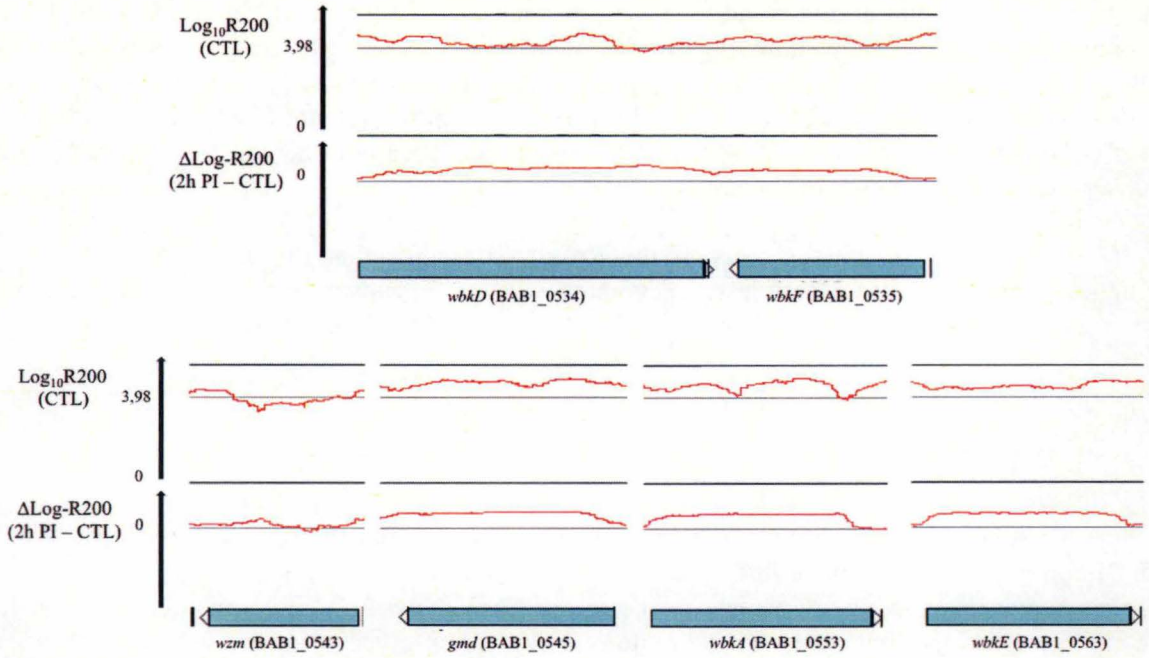

Figure 16 | Log_{10}R200 (control condition) and Δlog-R200 (2 h PI-CTL) values of 4 genes of the *wbk* locus. Log_{10}R200 and Δlog-R200 values show that there is an increase of the log_{10}R200 values at 2 hours post-infection compared to the control condition for *wbkD*, *wbkF*, *gmd*, *wbkA* and *wbkE*. The *wzm* log_{10}R200 has also increased at 2 h PI but weakly compared to the 3 other genes of the same locus. **Abbreviations : CTL**, control ; **PI**, post-infection.

Tn-seq results show that *wzm*, that also belongs to the *wbk* region and is involved in the O-chain transport (Introduction, section 6), does not show the same log_{10}R200 increase at 2 h PI compared to the 5 other genes of the same locus (figure 16) and thus is not on our candidate genes list (annex 2). But interestingly, unlike the 5 other genes of the *wbk* region, *wzm* transpositional mutants seem to be less frequent on plates (control condition). This would make sense because these mutants, unable to transport O-chain from the cytoplasm to the perisplasm, would accumulate O-chain in the cytoplasm, which could be toxic, generating a low growth phenotype on plates. Thus, if *wzm* transpositional mutant do not grow well on plates but enter better in the host cells, the phenotype observed at the plate condition (weak log_{10}R200 values) for this gene can be compensated at 2 h PI if these mutants enter better in host cells and then this compensation lead to a higher Δlog-R200 values at 2 h PI. This would explain why the ΔlogR200 profile is different for *wzm* than the 5 other genes of the *wbk* locus. Therefore, to see if the mutation of *wzm* leads also to a better enter in host cells, the infectiosity of a deletion mutant of *B. abortus* 544 *wzm* (previously generated by V. Vassen) was assessed in RAW 264.7 macrophages in order to count CFU at 2 h and 24 h PI. In parallel, infections were also performed with *B. abortus* 544 WT and Δ*wzm* complemented strains (generated by V. Vassen). Compared to the WT and the complemented strains, CFU counting increased at 2 h PI for Δ*wzm* (figure 15B). At 24 h PI, CFU number increased for the WT strain whereas it strongly decreased for Δ*wzm* (figure 15B). However, it is important to notice that no statistical test could be performed on the results obtained at 24 h PI for Δ*wzm* since its CFU number is below the experiment detection threshold (figure 15B). Furthermore, because the CFU number at 2 h PI for the Δ*wzm* complemented strain is close to the detection threshold, it is impossible to estimate if its decrease at 24 h PI is weak or strong compared to the CFU number obtained at 2 h PI.
The results obtained here suggest that Δ*wzm* enter better than the WT and the complemented strain and thus act identically as the *gmd* disruption strain in term of entry. Furthermore, we can see that the Δ*wzm* complemented strain only partially acts as the WT strain. Indeed, while the entry of both strains is almost similar, on the the contrary its seems that the complemented strain does not replicate inside the host cells as the WT does. Interestingly, the ability to survive intracellularly is very different between the *wzm* and *gmd* mutants. While the CFU of the *gmd* mutant remain stable between 2 h and 24 h PI, the CFU of the *wzm* mutant dramatically decrease. Both mutants being rough, one possible explanation would be that the *wzm* mutant accumulates O-chain in the cytoplasm, which intoxicates the bacterial cell and impairs its intracellular survival. Curiously, the *wzm* complemented strain is unable to allow intracellular replication like the wild type strain. This could be due to the potentially low expression of *wzm* in the pSRK vector within the host cells, thus generating an incomplete complementation of one of the *wzm* mutant phenotypes.

4. RGD motif investigation

In the previous section, we report that two different rough mutants have an increased internalization/survival in host cells, at 2 h PI. It is known that the presence of RGD motifs on the surface of the bacterium could be involved in the entry of rough bacteria in host cells (Campbell *et al.*, 1994) (Introduction, section 7.2). Thus, in order to evaluate if RGD motifs are involved or not in *Brucella* adhesion and entrance mechanisms, the RGD loop of Omp2b was modified into a QGQ loop (Glutamine – Glycine – Glutamine) in *B. abortus* 2308 WT strain (*B. abortus* 2308 Omp2b-QGQ) as well as in *B. abortus* 2308 *gmd* disruption mutant (*B. abortus* 2308 *gmd* disrupt-Omp2b-QGQ) and in *B. abortus* 544 Δ*wzm* strain (*B. abortus*

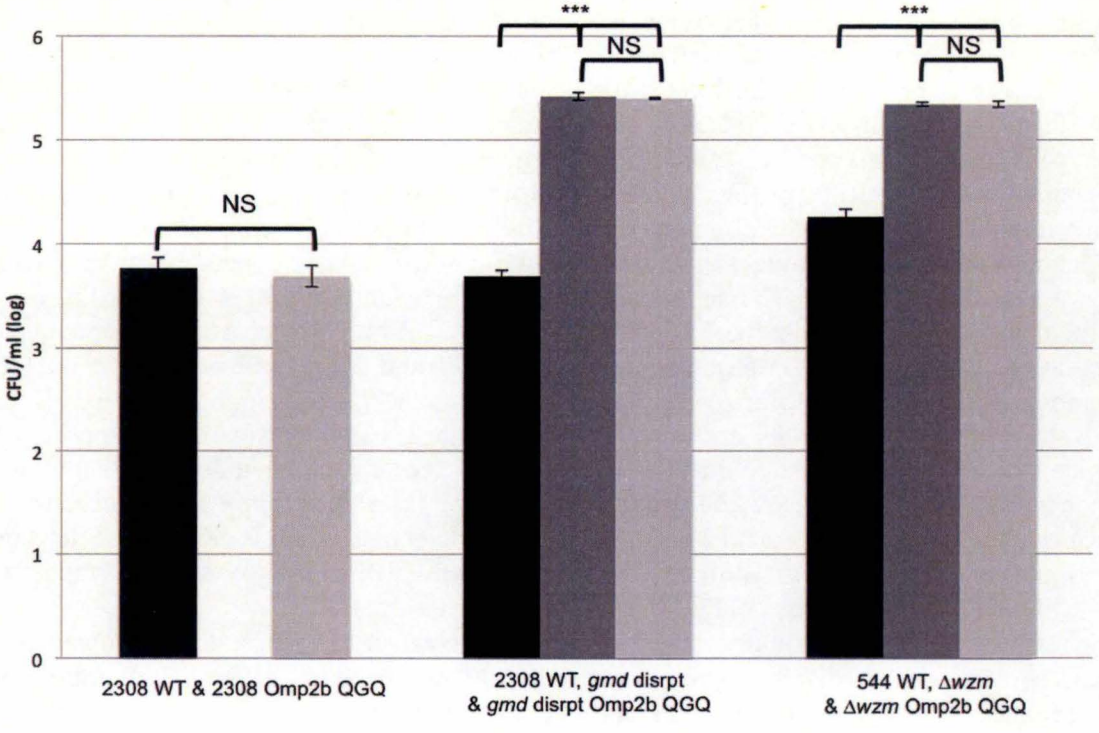

Figure 17 | RAW 264.7 macrophages infection and CFUs counting. Three RAW 264.7 macrophages infections have been performed for 2 hours with different strains. For the first infection, *B. abortus* 2308 WT and *B. abortus* 2308 Omp2b-QGQ were used. Second infection was performed with *B. abortus* 2308 WT strain, *gmd* disruption mutant and *B. abortus* 2308 *gmd* disrupt-Omp2b-QGQ. The last infection has been performed with *B. abortus* 544 WT strain, Δ*wzm* and *B. abortus* 544 Δ*wzm*-Omp2b-QGQ. For each infection, bacteria were harvested at 2 hours post-infection and plated. Here, bacteria able to form a colony are expressed in CFU/ml (log scale).

544 Δ*wzm*-Omp2b-QGQ). Glutamine was chosen to replace the arginine (positively charged) and the aspartate (negatively charged) because unlike these two amino acids, glutamine is polar but does not exhibit a net charge. We chose Omp2b because Omp2b and Omp25 are the two major outer membrane proteins (Moreno & Moriyon, 2006) (Introduction, section 6) and since there is no RGD motif in Omp25 (data not shown), only Omp2b displays a RGD motif. An allelic exchange plasmid with a pNPTS138 backbone was constructed by insertion of the *omp2b* gene with two mutations (Experimental procedures, section 8), themselves inserted by PCR-based site-directed mutagenesis (Experimental procedures, section 2.1). The complete coding sequence of *omp2b* was inserted in the allelic exchange plasmid because the *omp2b* was shown to be essential (Tn-seq data not shown) (Laloux *et al.*, 2010). The genotype of the strains carrying a *omp2b*-QGQ allele were checked by PCR (Experimental procedures, section 2.2). The ability of each mutant to enter in host cells was assessed through RAW 264.7 macrophages infections and CFU countings for bacteria harvested at 2 h PI. In parallel, the same experiments were performed with *B. abortus* 2308 and 544 WT bacteria as well as with *B. abortus* 2308 *gmd* disruption strain and *B. abortus* 544 Δ*wzm* mutant. The comparison of CFU number of *B. abortus* 2308 WT strain with *B. abortus* 2308 Omp2b-QGQ showed no difference (figure 17). Then it was observed that CFU number for *B. abortus* 2308 *gmd* disrupt-Omp2b-QGQ and *B. abortus* 2308 *gmd* disruption both showed an increase compared to *B. abortus* 2308 WT strain (figure 17). The same results was also observed for *B. abortus* 544 Δ*wzm*-Omp2b-QGQ and *B. abortus* 544 Δ*wzm* compared to *B. abortus* 544 WT strain (figure 17). Thus these results seem to underline that this RGD motif does not play a role in *B. abortus* adhesion to host cells.

Discussion and perspectives

Discussion and perspectives

1. Tn-seq results validation

In order to discover new essential genes involved in the infection process of *B. abortus* at given time points, a Tn-seq experiment has been performed. After statistical analysis of the control condition and the 2 h PI time results, we observed that 77 candidate genes (annex 2) showed a decrease of their log_{10}R200 values at 2 h PI compared to the plate condition. These genes are probably involved in the adhesion to and/or invasion of the host cells as well as in the survival of the bacteria at the early stages of infection.

For preliminary Tn-seq validation, we looked after *bvrR* and *bvrS* among our candidate genes because we knew both genes are involved in *B. abortus* cell invasion (Sola-Landa *et al.*, 1998). As expected, both genes show a lower log_{10}R200 values at 2 h PI compared to the control condition and thus can constitute two prelimnary good positive controls. Then to further validate the Tn-seq results, *wadB* and *pgk* have been chosen has positive controls because it is known that a deletion mutant for *wadB* is attenuated at 48 h PI in dentritic cells and mice (Gil-Ramirez *et al.*, 2014) and that deletion of *pgk* lead to an attenuation in bone-marrow derived macrophages (as early as 2 h PI) as well as in mice (Trant *et al.*, 2010). Here, CFU countings for disruption mutants, created using an integrative disruption vector for each control, show a decrease compared to the *B. abortus* 2308 WT strain (figure 14A) and thus show that the Tn-seq experiment results are reliable. However, two interesting points deserve further discussion.

On the one hand, the decrease of the CFU number is weaker for *pgk* than for *wadB* (figure 14A). However, it is important to underline that colonies formed by *pgk* disruption mutant are smaller than the 3 others controls (*wadB*, *omp2a* and *ftsK*-like) and than the WT strain. A likely explanation for this would be that the *pgk* disruption mutant grows slower than the others strains. The mechanisms hidden behind the slow growth of *pgk* disruption strain on plate is not clear. A first hypothesis would be that because *pgk* is an enzyme of the central metabolism of *Brucella* (glycolysis and gluconeogenesis), a mutation of this gene could slow down this metabolism and consequently the bacterial growth. Another possible explanation could be the accumulation of a metabolic intermediate which could be toxic for the bacterium. Indeed, it has been shown in a proteomic study that in order to adapt to low-oxygen conditions (similar to infection conditions), *B. suis* upregulates its glycolysis (Al Dahouk *et al.*, 2009). Thus, if *B. abortus* reacts in the same way, we could hypothesize that a metabolic intermediate accumulation could be possible during glycolysis and could be toxic for the bacteria and thus lead to a low number of CFU and to smaller colonies for *pgk* disruption strain.

On the other hand, it has been shown that the deletion mutant for *wadB* is more sensitive to decomplemented bovine serum than the WT strain (Gil-Ramirez *et al.*, 2014). Thus, it was important to test sensitivity of *wadB* disruption strain to the medium used for macrophages infections. The results obtained for the sensitivity test for *wadB* disruption mutant compared to the WT strain show a slight decrease of CFU numbers after 1 h incubation (figure 14B), as seen for the *wadB* deletion strain (Gil-Ramirez *et al.*, 2014). However, this effect alone does not completely explain the CFU decrease observed at 2 h PI for the *wadB* disruption mutant. Thus it is likely that *wadB* disruption mutant is more sensitive to bactericidal arsenal used by the macrophages, like cationic peptides. Indeed, it is suggested in the litterature that WadB, which is a glycosyltransferase involved in the synthesis of a lateral chain in the core of LPS, could be involved in cationic peptides resistance in *Brucella* (Gil-Ramirez *et al.*, 2014). This

is interesting because it suggests that bacteria have already been agressed by the macrophages during the first 2 h of infection, but also that all other mutants generated by the analysis of Tn-seq data at 2 h PI compared to the control could be either essential for entry or survival in macrophages. Therefore, it could be interesting to identify other genes which could be involved in bacteria early survival inside the macrophages. An interesting way to do this could be to perform a new Tn-seq experiment but in a medium enriched in cationic peptides and to compare the candidate genes obtained with those obtained here at 2 h PI.

2. Rough mutants

In order to see if transpositional mutants of the *wbk* region showing an increase of their $\log_{10}$R200 values are rough mutants and thus better enter in host cells (Jimenez de Bagues *et al.*, 2005), a *B. abortus* 2308 *gmd* disruption mutant as well as a *B. abortus* 544 Δ*wzm* (generated by V. Vassen) were tested in infection. As expected, CFU counting for each mutant, after plating bacteria harvested at 2 h PI, showed an increase compared to their corresponding WT strains (figure 15). In the litterature it has been reported that rough mutants would be more phagocyted by macrophages than the smooth strains (Haag *et al.*, 2010). Thus, according to our results, one could speculate that *B. abortus* O-chain would be an essential element to inhibit professional macrophages phagocytosis. To test this hypothesis and to discover mechanisms hidden behind this speculative inhibition, different experiments could be performed. Firstly, infection of other cell types including HeLa cells and trophoblasts with *gmd* disruption strain as well as with the Δ*wzm* mutant could be interesting since unlike macrophages, they are not professional phagocytic cells. Thus, in the absence of O-chain, the results should show a lower entry of the rough mutants since those cell types are in theory not able to ingest exogenous particles by phagocytosis. Another possible experiment would be the use of a molecule able to specifically inhibit phagocytosis and see if rough mutants entry is prevented. Eventually, a last expirement which could be interesting would be to cover beads with *Brucella* purified O-chain, incubate them with RAW 264.7 macrophages and see if these beads are uptaken by phagocytosis, using the uptake of untreated beads as a control.

For each rough mutant (*gmd* disruption mutant and Δ*wzm*) CFU were also counted for 24 h PI time, but no difference was observed for the *gmd* disruption strain compared to the CFU counting at 2 h PI (figure 15). For the WT strain, an increase of the CFU number was observed at 24 h PI compared to 2 h PI (figure 15). On the contrary, Δ*wzm* show a decrease of the CFU number at 24 h PI compared to the 2 h PI results whereas the *B. abortus* 544 WT strain show an increase of the CFUs number at 24 h PI compared to 2 h PI (figure 15).
On the one hand, the increase of the CFUs number for each WT strain show, as expected, that both WT strains are proliferating in the host cells. On the other hand, these results show that rough mutants are able to enter better in the host cells, which was expected, but that their survival inside the macrophages is dependent of the type of mutation generated in the *wbk* locus. Indeed, *gmd* is involved in the synthesis of the monomers N-formylperosamine which are the precursor of the polysaccharide O-chain whereas *wzm* is involved in the transport of the O-chain (Introduction, section 6). Then, while a *gmd* mutant will lead to O-chain synthesis inhibition, in a *wzm* mutant, the O-chain is still produced and only its transport is inhibited. Thus, a *wzm* mutant can lead to an accumulation of O-chain in the cytoplasm of the bacterium which could be toxic. This possible toxicity would explained why in our experiments, at 24 h PI, there is a lower CFU number for Δ*wzm* compared to *gmd* disruption strain. This likely

toxicity could also explained why in the Tn-seq experiment the log_{10}R200 values for *wzm* in the plate condition is already low (figure 16).

Infections and CFU counting at 2 h and 24 h PI for Δ*wzm* mutant were also performed with a Δ*wzm* complemented strain (generated by V. Vassen) in parallel. CFU number for this strain was more or less equivalent to the WT at 2 h PI. On the contrary, its CFU number decreased at 24 h PI and was under the detectability threshold. Furthermore, because the CFU number at 2 h and 24 h PI for the Δ*wzm* complemented strain is close to the detection threshold, it is impossible to estimate if its decrease at 24 h PI is weak or strong compared to the CFU number obtained at 2 h PI. Those results probably underline that the complementation of the Δ*wzm* mutant is partial. Indeed, it seems that only the entry phenotype is complemented but not the survival inside the host cells. This could be due to the potentially low expression of *wzm* in the pSRK vector within the host cells, thus generating an incomplete complementation of one of the *wzm* mutant phenotypes.

3. RGD motif investigation

In order to investigate if RGD motifs are involved in *Brucella* adhesion and entry mechanisms like it is proposed in the litterature (Campbell *et al.*, 1994), the Omp2b RGD loop was modified into a QGQ motif in three different bacteria backgrounds : *B. abortus* 2308 WT bacteria (*B. abortus* 2308 Omp2b-QGQ), *B. abortus* 2308 *gmd* disruption strain (*B. abortus* 2308 *gmd* disrupt-Omp2b-QGQ) and *B. abortus* 544 Δ*wzm* mutant (*B. abortus* 544 Δ*wzm*-Omp2b-QGQ). Infections and CFU countings at 2 h PI time were performed with each strain and in parallel with *B. abortus* 2308 and 544 WT bacteria as well as with *gmd* disruption mutant and Δ*wzm* strain. Unexpectedly, the results show no difference when we compared the CFU number of *B. abortus* 2308 Omp2b-QGQ with the WT strain, *B. abortus* 2308 *gmd* disrupted-Omp2b-QGQ with *B. abortus* 2308 *gmd* disruption strain and *B. abortus* 2308 WT strain and eventually *B. abortus* 544 Δ*wzm*-Omp2b-QGQ with *B. abortus* 544 WT and *B. abortus* Δ*wzm* mutant (figure 17). Indeed, *B. abortus* 2308 Omp2b-QGQ entry is similar to *B. abortus* 2308 WT strain and *B. abortus* 2308 *gmd* disrupt-Omp2b-QGQ as well as *B. abortus* 544 Δ*wzm*-Omp2b-QGQ display the same behavior as their corresponding rough mutant and thus enter better in the host cells than their corresponding WT strains. Therefore, these results suggest that the RGD motif from Omp2b are not involved in *Brucella* adhesion and/or invasion mechanisms. Two hypotheses could explain these results. Firstly, here only the RGD motif of Omp2b was modified because it is the major OMP present on the bacterial surface which displays a RGD loop. However, this does not exclude that there exist other RGD motifs present on other surface elements than Omp2b. A second explanation would be that there maybe exist other motifs able to mimic RGD motifs and thus to be recognized by integrins as well. To further investigate if RGD motifs are important for bacteria adhesion to and/or entry in the host cells, an interesting experiment would be to saturate host cell integrins (which recognize RGD motifs) with a molecule able to bind to these receptors and to observe if the WT bacteria and rough mutants entry is inhibited or not.

4. Tn-seq limitations and perspectives

In order to identify genes involved in the infection cycle of *B. abortus*, a Tn-seq experiment in which samples were harvested at different PI times (2 h, 5 h and 24 h PI) was performed. Here, only the results of the control (plate) condition and the 2 h PI time point results were analyzed and investigated. Since several candidate genes obtained after the statistical analysis as well as the validation corroborate with litterature (for *wadB*, for example), Tn-seq seems to be a reliable method to bring out essential genes in given conditions. However, it is important to notice that in the conditions tested here maybe all the genes involved in the adhesion to and/or invasion of the host cells as well as in the survival of the bacteria at the early stages of infection are not brought out since Tn-seq experiment has two limitations. First, Tn-seq is designed to statistically mutate one locus per strain. Therefore, if an essential function is carried by several regions in parallel, disrupting one of these regions will not appear essential with Tn-seq because there exist other regions able to play a similar role and thus to compensate for the prospective loss of activity of the disrupted region. Secondly, it is possible that essential genes for adhesion and/or invasion are also essential for growth on plate thus are already showing low $\log_{10}$R200 values or an absence of transposons in the control condition. Indeed, one could speculate that *Brucella* may have coupled its infectious cycle with its cell cycle as it probably was a pathogen for several thousands years.

The statistical analysis of the Tn-seq results for the plate condition and the 2 h PI time brought out 77 genes showing a decrease of their Log_{10}R200 values. Among these candidates genes, several were already known but there are still other candidate genes which have to be further investigated and their role in the first step of infection has to be clarified.
Currently, the 5 h and 24 h PI time sequencing results are being analyzed thanks to a similar statistical analysis as the one performed for the 2 first conditions, each time being compared to the control condition. Once candidate genes for the two last conditions will be brought out, it would be interesting to choose positive and negative controls and to create disruption mutants (like it was done for the 2 h PI time) in order to validate the Tn-seq experiment for the later stages of infection. If the Tn-seq experiment is validated for these 2 last times, it would be interesting to study genes which are not yet annotated and to assess their role in *Brucella* trafficking and proliferation within host cells.

For the future, it will be also interesting to perform new Tn-seq experiments but in non professional phagocytic cells such as HeLa cells and trophoblasts. Indeed, since these cells are different from the RAW 264.7 macrophages used here, maybe *B. abortus* displays other strategies to infect these cell types. Thus, these new experiments may bring out new candidate genes involved in *Brucella* infection cycle. Eventually, a Tn-seq experiment performed in mice would be also interesting. In fact, for the Tn-seq experiment here, infections where performed *in vitro*. *In vitro* experiments are important since they allow the study and the discovery of new mechanisms but it is obvious these cellular infection models only imperfectly mimic what happens in living organisms. Therefore, a Tn-seq in mice could highlight different genes which are necessary *in vivo* for *Brucella* infectious cycle. Ultimately a cow infection would be interesting to generate data on the natural host. However, the high cost of these experiments impairs feasability in practice.

To conclude it is interesting to notice that recently, the cell cycle monitoring of *B. abortus* inside host cells showed that newborns are the predominanlty infectious bacterial cell types (Introduction, section 5) (De Bolle *et al.*, 2015). Furthermore, since newborns selection

is very quickly performed, it is postulated that these bacteria are more adhesive and/or invasive than other bacterial cell types and thus own features that other becterial cell types do not exhibit. Then, Tn-seq results can be useful and used to try to characterize newborn cells. Indeed, it would be interesting to see if disruption mutant of a given gene among the 77 candidate genes showing weak $log_{10}R200$ values induce a lower selection of newborns during the first step of infection or if there are still the predominantly infectious cell types. Eventually, since we observed that our two rough mutants (*gmd* disruption strain and Δ*wzm*) enter better in RAW 264.7 macrophages (see Results, section 3) we speculate that rough mutants have an advantage compared to their corresponding smooth strains. Thus, this hypothesis could give hints about why newborn bacteria (even if they are smooth) predominantly enter in host cells. Indeed, one can imagine that newly formed smooth bacteria could exhibit one or several rough spots on their surface. Thus, those rough spots could be an advantage for the bacterium to be internalized in the host cell and explained why newborn cells are predominantly selected. It would be thus interesting to test if smooth newborns display rough patches and an interesting manner to do would be, for example, an immunolabelling of cells owning the two cell cycle monitoring systems (mCherry-ParB and YFP-RepB ; Introduction, section 5) with an anti-rough antibody.

Experimental procedures

Experimental procedures

1. Strains and growth conditions

For the disruption or the motif modification of the different genes, the reference strain *B. abortus* 2308 has been used and grown in liquid or on solid 2YT medium (Luria Bertina or LB 32 g/L Invitrogen®, Yeast Extract 5 g/L and Peptone 6 g/L) at 37°C. For plasmid constructions and for mating in *B. abortus*, *E. coli* DH10B and S17-1 strains have been respectively used. Those strains were cultivated overnight at 37°C in LB medium (Casein Hydrolysate 10 g/L, NaCl 5 g/L, Yeast Extract 5 g/L). When these strains contained the conjugation vector pNPTS 138, kanamycin was added on the medium (50 µg/mL for plasmid replication in *E. coli* and 10 µg/mL for resistance cassette once integrated in *B. abortus* genome).

2. PCR

Two kind of PCR were carried out. On the one hand, preparative PCR were performed for constructing vectors and, on the other hand, diagnostic PCR were used to check if a given DNA fragment was present or not in a specific sample.

2.1 Preparative PCR

The PCR mix was composed of Q5 polymerase (2 U/µL, New England BioLabs® inc.), Q5 buffer (5X), primers (20 µM each), dNTPs (5 mM each), template DNA (about 60 ng) and milliQ water (milliQ purification system, Millipore). The preparative PCR programs consisted in a first DNA denaturation step performed at 98°C for 30 seconds followed by 30 amplification cycles. Each cycle was composed of different steps : DNA denaturation step (98°C for 10 seconds), primer hybridization (for 30 seconds, the temperature being set depending on primer Tm), elongation (72°C, duration according to the length of the PCR product, typically 30 seconds per kilobase). Enventually, a final elongation step was performed (72°C for 5 minutes).
In order to ligate to PCR products toghether (through sequence complementarity), joining PCR was performed. A classical PCR program of 5 cycles was used but without primers. Following this step, primers were added in order to amplify the final product for 25 cycles.

2.2 Diagnostic PCR

The mix contained Taq polymerase (Promega®, Madison, USA), GoTaq buffer (5X), primers (20 µM each), template DNA (about 60 ng) and water. The diagnostic PCR programs consisted in a first DNA denaturation step (94°C for 4 minutes) followed by 30 cycles. Each cycle was composed of DNA denaturation step (94°C for 4 minutes) followed by a primer hybridization step (for 30 seconds, the temperature being set depending on primer Tm) and an elongtaion step (72°C, duration according to the lenght of the PCR sample amplified, typically 1 minute per kilobase). Eventually, a final elongation (72°C for 5 minutes) was performed.

3. PCR product purification and sequencing

The MSB SpinPCRapace (Invitek, Berlin, Germany) kit and the manufacturer's protocol have been used to purify the different PCR amplicons.
Then, the quantity and the size of each PCR product were semi-quantified by correspondence to a DNA ladder of given size and concentration (Gene Ruler 0.1 µg/µL Thermo Scientific), thanks to electrophoresis through agarose gel (1%) containing ethidium bromide. To check the sequence of the different amplicons, Sanger sequencing was performed by Beckman Coulter Genomics.
The QIAquick® Gel Extraction Kit (QUIAGEN) was used to performed DNA gel extraction according to the manufacturer's procedures.

4. Extraction of plasmidic DNA

After overnight culture, bacteria were centrifuged during 1 minute at 13,000 rpm. The supernatant was then removed and to increase the amount of material, this first step was repeated. The next step consisted in resuspending the pellet in 300 µL of P1 buffer (RNAase A 100 µg/ml, Tris HCL 50 mM, EDTA 80 mM, pH 8, stored at 4°C). Then, 300 µL of P2 lysis solution (NaOH 100 mM, SDS 1%) were added. After a 5 minutes incubation time at room temperature, 300 µL of P3 buffer (KAc 3M, pH 5.5, stored at 4°C) were added and the mix was centrifuged during 10 minutes at 13,000 rpm. After centrifugation, the supernatant was transfered into a new tube and 700 µL of ice-cold isopropanol were added before the mix was centrifuged again during 10 minutes at 13,000 rpm. The supernatant was then discarded and 400 µL of ethanol 70% (stored at -20°C) were added on the side of the tube where the pellet was located. After a centrifugation time of 5 minutes at 13,000 rpm, the supernatant was delicately removed and in order to remove all traces of ethanol, the tubes were left to dry for 15 minutes in at 55°C incubator. Finally, the pellet was resuspended in 20 µL of desionized water.

5. Enzymatic restriction

Two kinds of enzymatic restrictions were carried out. On the one hand, preparative restrictions were performed for molecular cloning and, on the other hand, diagnostic restrictions were performed to check plasmidic constructions by checking for expected restriction patterns. For each DNA enzymatic restriction, the appropriate restriction enzyme (10U/µL, Roche®) with its corresponding buffer were used. For preparative restrictions, the samples were incubated for 1h to 1h30 in a 37°C water bath whereas the samples were only incubated for 45 minutes in the same bath for diagnostic restriction. Regarding DNA quantity, about 300 ng were used for preparative restriction as opposed to 120 ng for diagnostic restriction.

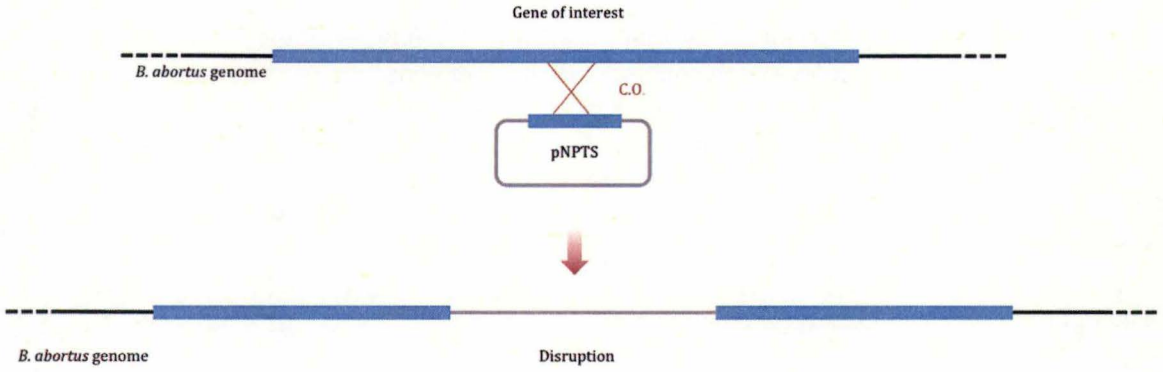

Figure 18 | Generation of disruption mutant using pNPTS138. After a crossing-over event between a central region of a gene present on the pNPTS138 vector (« pNPTS ») and the gene in the genome of the bacterium, the entire plasmid is inserted in the gene of interest. **Abbreviation : C.O.**, crossing-over.

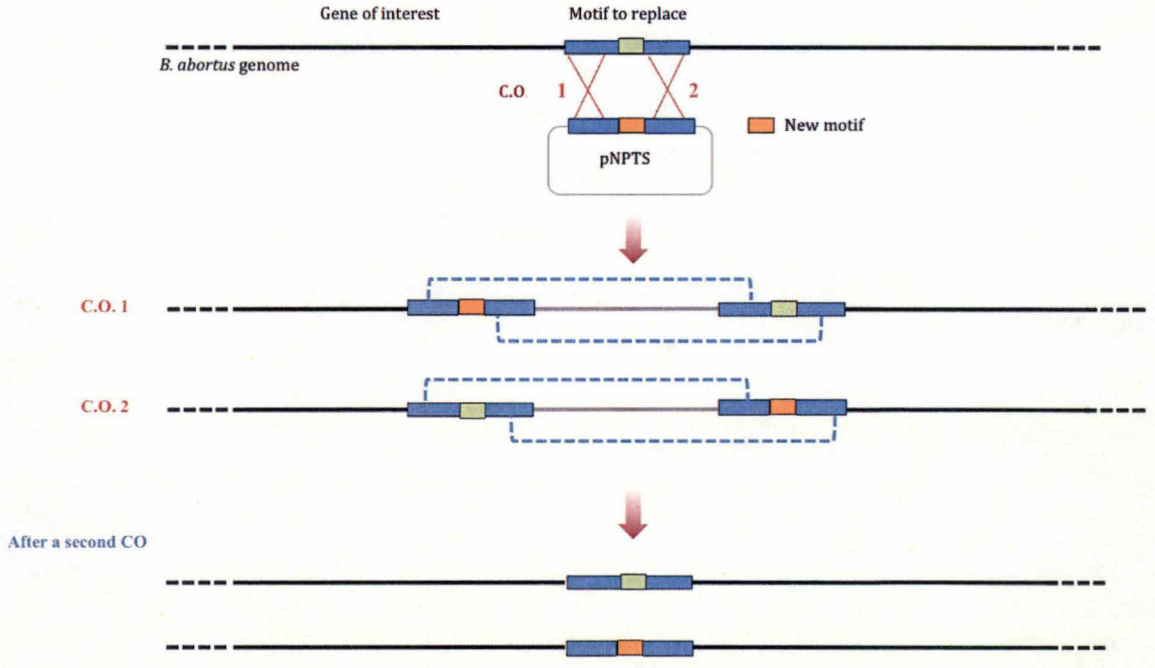

Figure 19 | Motif modification by allelic replacement using pNPTS138. First crossing over can happen upstream (1) or downstream (2) the region to replace, generating two integration possibilities. To complete the allelic replacement, a second crossing over has to take place. Again, two integration possibilites is observed (upstream and downstream the interesting region). If both crossing over occur in the same region, the WT genotype is recreated.

6. Ligation

Insertion of DNA fragments in restricted plasmids were performed in eppendorf tubes using T4-ligase (Fermentas®) and 5X ligase buffer (Invitrogen). The quantity of plasmid and insert used depended on the initial concentration of each partner. The final mix was incubated overnight at 18°C or 1h30 at room temperature.

7. Transformation

Two different competent strains, *E. coli* DH10B and *E. coli* S17-1, stored at -80°C, were used for transformation. Depending on the origin of the DNA, purified plasmid or ligation mix, 120 ng or 300 ng were respectively added to 50 µL of competent cell previously incubated on ice for 3 minutes. Bacteria were then kept on ice for 20 minutes and then heat-shocked in a 42°C water bath for precisely 2 minutes. After heat treatment, 700 µL of LB medium were added and the mix was put at 37°C for 45 minutes under agitation. After incubation, bacteria were centrifuged during 3 minutes at 5,000 rpm. Most of the supernatant was then discarded and the pellet containing bacteria was resuspended in the remaining supernatant (approximately 100 µL). Finally, the bacterial suspension was spreat onto LB agar plates containing the appropriate antibiotic as well as X-gal (0,004%) and IPTG (1 mM), for the blue-white screen if required.

8. Bacterial conjugation

For mating, 50 µL of an overnight culture of the conjugative *E. coli* S17-1 strain containing a plasmid of interest were added to 1 mL of an overnight culture of *B. abortus* 2308. Bacteria were then washed by centrifugation for 2 minutes at 7,000 rpm. Then, the supernatant was discarded and the pellet resuspended in 1 mL of fresh liquid 2YT medium. After repeating this step a second time, almost all the supernatant was removed and the pellet was resuspended in the remaining supernatant (around 100 µL). Then, the bacterial suspension was placed on 2YT agar plate without being spread, to increase the conjugation likelihood. The next day, the half of the obtained lawn was resuspended in 300 µL of fresh 2YT medium and 100 µL of this suspension was spread onto a 2YT agar petri dish containing nalidixic acid (1 µL/mL), necessary to kill *E. coli*, and the appropriate antibiotic depending on the plasmid that had to be mated (10 µg/mL of kanamycin for pNPTS138). The plates were then left in a 37°C incubator during 3-4 days until colonies were present. These colonies were then streaked and incubated at 37°C during 2 days and then kept at room temperature for further utilization (cultures, infections and CFU). The above protocol is suitable for generating disruption strains (figure 18).
For motif modification by allelic replacement (figure 19), following those first steps, one streak was put in liquid 2YT without antibiotic and let overnight at 37°C to favor plasmid loss. The next day, 100 µL of the bacteria culture was plated on 2YT agar plates supplemented with sucrose (5%). This step allows the selection of the bacteria which have lost their plasmid. Indeed, because the plasmid contained a counter-selection marker leading to bacterial death in the presence of sucrose in the medium, bacteria having lost their plasmid are able to survive on medium containing sucrose. The plates were then incubated at 37°C

during 4 days. Several colonies present on those plates where then streaked onto two different 2YT agar plates containing kanamycin (10 µg/mL) and 5% of sucrose, respectively. Finally, only bacteria able to growth on sucrose but not on kanamycine were selected, inactivated at 80°C in PBS during 1 hour, and checked for the motif modification.

9. Infection

RAW 264.7 macrophages have been used to asses the infectivity of different strains of *B. abortus*. The day before infection, *B. abortus* 2308 was cultured at 37°C overnight in 2YT medium containing the appropriate antibiotic if needed. At the same time RAW 264.7 macrophages were diluted at 10^5 cells/mL in DMEM (Dulbecco's modified Eagle's medium) supplemented with decomplemented bovine serum (Gibco®) and placed in 24 wells plate. The next day, the optical density (OD) of the different bacterial cultures was measured and bacteria were diluted in RAW 264.7 macrophages culture medium in order to have a MOI (multiplicity of infection) of 50 (50 bacteria for each cell). Cell culture medium was then removed from RAW 264.7 macrophages and replaced by the appropriate bacterial suspension. The multi-wells plate was then centrifuged at 1,200 rpm for 10 minutes at 4°C in order to synchronize infection, and was then placed in a 37°C incubator (this time point corresponding to the initiation of infection termed time zero). After one hour, all the medium was removed, each well was washed 2 times with DMEM and eventually fresh medium containing gentamycin (50 µg/mL), necessary to kill the remaining extracellular bacteria which did not enter the host cells, was added.

10. Colony Forming Units (CFU)

At a given time post-infection (2 h and 24 h in our case), RAW 264.7 macrophages were washed twice with sterile PBS before being incubated at 37°C for 10 minutes with PBS containing 0.1% of Triton 100X in order to lyse RAW 264.7 macrophages without killing bacteria. After this incubation time, cells were flushed, lysates were collected and serial dilutions were performed. Finally, each dilution was plated on 2YT agar medium containing the appropriate antibiotic (if needed), incubated at 37°C for 3-5 days and CFU were counted.

11. Sensitivity test to decomplemented bovine serum

B. abortus 2308 was cultured in liquid 2YT containing the appropriate antibiotic if needed and placed overnight at 37°C. The next day, the OD of the different bacterial cultures was measured. Then, in order to mimic infection conditions, each bacteria culture was diluted in RAW 264.7 macrophages culture medium to have approximately $7{,}5 \times 10^6$ bacteria/mL. The appropriate bacterial suspensions were incubated during one hour at 37°C. After this incubation time, each dilution was plated on 2YT agar medium with the appropriate antibiotic if needed and the plates were left at 37°C during 4 days. Finally, CFU were counted.

Annexes

Annexes

Annex 1

Genes coding for lipid A biosynthesis in *Brucella melitensis* 16M

Gene name	*Gene product*
lpxA	Acyl-(acyl carrier protein) UDP-N-acetylglucosamine-O acyltransferase
lpxC	UDP-3-O-(3Hydroxymyristoyl) N-acetylglucosamine diacetylase
lpxD	UDP-3-O-(3Hydroxymyristoyl) glucosamine N-acetyltransférase
lpxB	Lipid-A-disaccharide synthetase
lpxK	Tetraacyldisaccharide-I-P4'-Kinase
kdsA	2-dehydro-3-deoxyphosphooctonate aldolase
kdsB	3-deoxy-manno octulosanate cytidylyl transferase
kdtA	3-deoxy-D-manno octulosonic-acid transferase
htrB	Lauroyl/myristoyl acyltransferase

(Adapted from Haag *et al.*, 2010)

Annex 2

Candidates genes (log_{10}R200 values lower at 2 h PI compared to the control) generated by the delta analysis

Gene number	*Gene name*	*Function*
BAB1_0024	*cmk*	Cytidylate kinase
BAB1_0091	*ccmA*	ATP-binding export protein (cytochrome C biogenesis)
BAB1_0092	*ccmB*	Heme ABC transporter permease
BAB1_0093	*ccmC*	Heme export protein
BAB1_0143	*glnD*	Uridylyltransferase
BAB1_0160	*ptsN*	Nitrogen regulatory protein
BAB1_0304	*cenR*	Histidine kinase
BAB1_0351	*wadB*	Glycosyl transferase
BAB1_0386		Copper-translocating P-type ATPase
BAB1_0387		Nitrogen fixation protein
BAB1_0388		Nitrogen fixation protein
BAB1_0391	*ccoO*	Peptidase S41
BAB1_0435		D-2-hydroxyacid dehydrogenase
BAB1_0442	*purD*	Phosphoribosylamine-glycine ligase
BAB1_0491		Invasion protein B
BAB1_0631	*ccmI*	Cytochrome C biogenesis
BAB1_0632	*ccmE*	Cytochrome C-type biogenesis protein
BAB1_0633	*ccmF*	Heme lyase subunit
BAB1_0634	*ccmH*	Ctochrome C biogenesis protein
BAB1_0730	*purN*	Phosphoribosylglycinamide formyltransferase
BAB1_0731	*purM*	Phosphorybosylformylglycinamidine cyclo-ligase
BAB1_0739		ETC complex I subunit region
BAB1_0773		Exopolyphosphatase
BAB1_0855		Glutaredoxin
BAB1_0857	*purL*	Phosphorybosylformylglycinamidine synthase subunit
BAB1_0860	*purQ*	Phosphorybosylformylglycinamidine synthase subunit
BAB1_0862		Phosphorybosylaminoimidazole-succinocarboxamide synthase
BAB1_0868	*purB*	Adenylosuccinate lyase

BAB1_0962		Protein-L-isoaspartate O-methyltransferase
BAB1_1030		Glutathione reductase
BAB1_1038	*mlaE*	Toluene ABC transporter permease
BAB1_1039	*mlaF*	Iron ABC-transporter ATP-binding protein
BAB1_1040	*mlaD (?)*	ABC-transporter substrate-binding protein
BAB1_1041		ABC trnasporter
BAB1_1045		Reductase
BAB1_1115	*tgt*	queuine tRNA-ribosyltransferase
BAB1_1191	*clpA*	ATP-dependent Clp protease ATP-binding protein
BAB1_1437		Aminopeptidase
BAB1_1460	*mntH*	Manganese transporter
BAB1_1485		Membrane protein
BAB1_1553		GTP-binding protein
BAB1_1665	*rpoH2*	DNA-directed RNA polymerase sigma-70 factor
BAB1_1669		Sensor histidine kinase
BAB1_1757		N5-carboxyaminoimidazole ribonucleotide mutase
BAB1_1766		Elongation factor 3
BAB1_1824	*purH*	Bifunctional purine biosynthesis protein
BAB1_1918	*lpdA-2*	Dihydrolipoamide dehydrogenase
BAB1_2006	*cenR*	Transcriptional regulator
BAB1_2025	*dnaJ*	Molecular chaperone
BAB1_2084	*hisH*	Imidazole glycerol phosphate synthase subunit
BAB1_2085	*hisA*	Isomerase
BAB1_2086	*hisF*	Imidazole glycerol phosphate synthase subunit
BAB1_2092	*bvrR*	Transcriptional regulator
BAB1_2093	*bvrS*	Sensor histidine kinase
BAB1_2094	*hprK*	ATP-binding protein
BAB1_2103		Mannose-1-phosphate guanylyltransferase
BAB1_2135	*gshB*	Glutathione synthetase
BAB1_2158	*lnt*	Apolipoprotein N-acyltransferase
BAB1_2159	*hipB*	Cro/Cl family transcriptional regulator
BAB1_2175		Fur family transcriptional regulator
BAB2_0076	*omp10*	Lipoprotein
BAB2_0366	*rpiB (eryL)*	Ribose 5-phosphate isomerase B
BAB2_0367	*tpiA (eryH)*	Triosephosphate isomerase 2
BAB2_0370	*eryC*	Aminotransferase
BAB2_0411		Hypothetical protein
BAB2_0412		Permease
BAB2_0442		Acyl-CoA dehydrogenase
BAB2_0476		Glutamate-cysteine ligase
BAB2_0511		Pyridine nucleotide-disulfide oxidoreductase
BAB2_0656	*ccdA*	Cytochrome C biogenesis protein
BAB2_0668		Phosphatidylcholine synthase
BAB2_0699		Quinone oxidoreductase
BAB2_0701		ABC transporter
BAB2_0702		Peptide ABC transporter permease

BAB2_0703		Peptide ABC transporter ATPase
BAB2_1012	*dapB*	Dihydrodipicolinate reductase
BAB2_1013	*gpm*	Phosphoglyceromutase

Annex 3

Candidates genes (log_{10}R200 values higher at 2 h PI compared to the control) generated by the delta analysis

Gene number	*Gene name*	*Function*
BAB1_0534	*wbkD*	Epimerase/dehydratase
BAB1_0535	*wbkF*	Undecaprenyl glycosyltransferase
BAB1_0545	*gmd*	GDP mannose dehydratase
BAB1_0553	*wbkA*	Mannosyltransferase
BAB1_0563	*wbkE*	Mannosyltransferase
BAB1_0999	*wboA*	Glycosyltransferase
BAB1_1000	*wboB*	Mannosyltransferase
BAB2_0855	*manB*	Phosphomannomutase

Annex 4

Primers table

Gene of interest	*Gene locus tag*	*Primer name*	*Sequence (5' → 3')*
wadB	BAB1_0351	1-0351-disrpt-F	gcatggcaggcgattatc
		1-0351-disrpt-R	aggaaacaaactgcgcaac
pgk	BAB1_1742	pgk-disrpt-F	ccaatggcgatctctatgtg
		pgk-disrpt-R	aagcgcatcgacctttc
omp2a	BAB1_0659	omp2a-disrpt-F	ctgcgcttcaactacacc
		omp2a-disrpt-R	actgaccgtagttctggttc
ftsK-like	BAB2_0709	ftsK-disrpt-F	agtgtgctggaagattttgg
		ftsK-disrpt-R	tcatttcgcggtaaac
gmd	BAB1_0545	gmd-disrpt-F	tgcgaactcagacgcactagg
		gmd-disrpt-R	gcgagcgtgacaaattctcgaac
omp2b	BAB1_0660	omp2b-QGQ-AMF	gaacatcaagagccttctccttg
		omp2b-QGQ-AMR	TTGGCCTTGaaccttggcagcccattcttc
		omp2b-QGQ-AVF	ccaaggttCAAGGCCAAgtcaacatcaccgaccagttc
		omp2b-QGQ-AVR	ggtttctgtatccaatccgtaatg
		omp2bQGQ-FChck	gtccttcagccaaatcagaatgcc
		omp2bQGQ-RChck	agcaagcatctgatgctgcac

Annex 5

Bacterial strains table

E. coli DH10B	pNPTS138 BAB1_0351 disrpt	kanR/sucS
	pNPTS138 pgk disrpt	kanR/sucS
	pNPTS138 omp2a disrpt	kanR/sucS
	pNPTS138 fitsk-like disrpt	kanR/sucS
	pNPTS138 gmd disrpt	kanR/sucS
	pNPTS138 omp2b QGQ loop	kanR/sucS
E. coli S17-1	pNPTS138 BAB1_0351 disrpt	kanR/sucS
	pNPTS138 pgk disrpt	kanR/sucS
	pNPTS138 omp2a disrpt	kanR/sucS
	pNPTS138 fitsk-like disrpt	kanR/sucS
	pNPTS138 gmd disrpt	kanR/sucS
	pNPTS138 omp2b QGQ loop	kanR/sucS
B. abortus 2308	pNPTS138 BAB1_0351 disrpt	kanR/sucS
	pNPTS138 pgk disrpt	kanR/sucS
	pNPTS138 omp2a disrpt	kanR/sucS
	pNPTS138 fitsk-like disrpt	kanR/sucS
	pNPTS138 gmd disrpt	kanR/sucS
	omp2b QGQ loop	

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