**MASTER IN BIOCHEMISTRY AND MOLECULAR AND CELLULAR BIOLOGY**

# Characterization of the major outer membrane proteins Omp25 and Omp25 in the pathogen Brucella abortus

Izquierdo Lafuente, Beatriz

*Award date:*
2018

*Awarding institution:*
University of Namur

UNIVERSITÉ DE NAMUR

Faculté des Sciences

# Characterization of the major outer membrane proteins Omp25 and Omp2b in the pathogen *Brucella abortus*

**Mémoire présenté pour l'obtention**

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

Beatriz IZQUIERDO LAFUENTE

Janvier 2018

**Université de Namur**
**FACULTE DES SCIENCES**
Secrétariat du Département de Biologie
Rue de Bruxelles 61 - 5000 NAMUR
Téléphone: + 32(0)81.72.44.18 - Téléfax: + 32(0)81.72.44.20
E-mail: joelle.jonet@unamur.be - http://www.unamur.be

# Characterization of the major outer membrane proteins Omp25 and Omp2b in the pathogen *Brucella abortus*

IZQUIERDO LAFUENTE Beatriz

Abstract

*Brucella* are pathogens, which mainly infect animals and accidentally humans. *Brucella abortus* is a facultative intracellular Gram-negative bacterium. Their cell envelope is thus composed of two membranes, the inner membrane and the outer membrane (OM). The OM contains proteins called Omps and its outer leaflet is composed of lipopolysacharide (LPS). Omp25 and Omp2b are the major Omps of *B. abortus*. Omp25 is a major non-essential protein, well conserved among the Rhizobiales. Surprisingly, the function of Omp25 is not well characterized. We have created a deletion mutant for *omp25* in *B. abortus* 544 (Δ*omp25*). This mutant was not impaired in growth in rich medium. However, under stress conditions with Triton X-100, bacteria were more susceptible than the wild type, suggesting a role of Omp25 in the membrane stability or an alteration of OM composition in the Δ*omp25* mutant. The Δ*omp25* bacteria are smaller in size than the wild type (WT). Interestingly, WT bacteria exhibit different Omp25 localization patterns in the OM according to the growth phase. 10% of the total bacterial population were unlabelled in the stationary phase. These data could be explained by a differential regulation of the *omp25* gene, which is a direct target of CtrA, a major regulator of the cell cycle, according to ChIP-seq (chromatin immunoprecipitation-sequencing) data. In contrast to Omp25, Omp2b is an essential porin, poorly conserved in Rhizobiales, displaying a patchy distribution in the *B. abortus* outer membrane. Since previous data from our laboratory have shown that patches of Omp2b partially colocalize with patches of R-LPS, we attempted to determine the existence of a potential interaction between R-LPS and the porin. Nevertheless, it was not possible to confirm any interaction between these two molecules using co-immunoprecipitation under the tested conditions.

Mémoire de master 120 en biochimie et biologie moléculaire et cellulaire
Janvier 2018
**Promoteur: X. De Bolle**

**Université de Namur**
**FACULTE DES SCIENCES**
Secrétariat du Département de Biologie
Rue de Bruxelles 61 - 5000 NAMUR
Téléphone: + 32(0)81.72.44.18 - Téléfax: + 32(0)81.72.44.20
E-mail: joelle.jonet@unamur.be - http://www.unamur.be

# Charactérisation des protéines majeures Omp25 et Omp2b chez *Brucella abortus*

IZQUIERDO LAFUENTE Beatriz

Résumé

Les *Brucella* sont des bactéries pathogènes qui infectent principalement les animaux, et accidentellement l'humain. *Brucella abortus* est une bactérie intracellulaire facultative, à Gram-négatif. Leur enveloppe cellulaire est donc composée de deux membranes, les membranes interne et externe. La membrane externe (OM) contient des protéines appelées Omps et son feuillet externe est composé de LPS (lipopolysacharide). Omp25 et Omp2b sont les principales Omps de *B. abortus*. Omp25 est une protéine majeure non essentielle, et bien conservée chez les Rhizobiales. Etonnamment, la fonction de Omp25 n'est pas bien caractérisée. Nous avons créé un mutant de délétion pour *omp25* dans *B. abortus* 544 (Δ*omp25*). Ce mutant n'était pas altéré pour sa croissance en milieu riche. Cependant, dans certaines conditions de stress avec Triton X-100, les bactéries étaient plus sensibles que la souche sauvage (WT), suggérant un rôle d'Omp25 dans la stabilité de l'OM ou une altération de la composition de l'OM dans le mutant Δ*omp25*. Les bactéries Δ*omp25* sont plus petites que la WT. Curieusement, les bactéries WT présentent une disposition de l'Omp25 en OM qui variable en fonction de la phase de croissance. 10% de la population bactérienne n'étaient pas marquées en phase stationnaire. Ces données pourraient être expliquées par une régulation différentielle du gène *omp25*, qui est une cible directe de CtrA, un régulateur majeur du cycle cellulaire, d'après les données de ChIP-seq (immunoprécipitation chromatinienne suivie de séquençage à haut débit). Au contraire de l'Omp25, Omp2b est une porine essentielle, peu conservée chez les Rhizobiales qui présente une répartition en grappes (patchs), non-homogène dans la membrane externe de *B. abortus*. Etant donné que des données précédentes du laboratoire ont montré que les patchs d'Omp2b colocalisent partiellement avec des patchs de R-LPS, nous avons tenté de tester l'existence d'une interaction potentielle entre R-LPS et Omp2b. Néanmoins, il n'a pas été possible de confirmer cette possible interaction entre ces deux composants de l'OM par co-immunoprécipitation, dans les conditions testées.

Mémoire de master 120 en biochimie et biologie moléculaire et cellulaire

Janvier 2018

**Promoteur: X. De Bolle**

**ACKNOWLEDGEMENTS**

First of all, I would like to thank all the members of the URBM team and Gemo for make me feel part of their team during these 10 months.

I would like to thank Xavier de Bolle for giving me the opportunity to undertake the master thesis in his group. For his dedication and help. I really appreciate the time you spent to give me ideas for new experiments and discuss together different hypothesis every time I had a non-expecting result.

I would like to express my gratitude to Victoria Vassen, my mentor, Vicky for friends. Thanks to you I have learned how to work in a laboratory like a German scientist. You taught me how to be precise, how to be independent in my work and how to think as a scientist. Thank you for all the time you spent in teaching me everything. You were a good teacher.

I should also mention the Xa team, Mathilde, Katy, Georges, J-F, Phoung, Aurore and the new incorporations Pierre, Angy and Agnes. Thank you for all the advice and help. Thank you Kevin for all your help in the BL3 and for the musical ambience you created every time you made me sing a Spanish song or just a song. Thank you Francesco for being always smiley with me.

Thank to all the memos. Girls, you were there, every time I needed you. Thank you Audrey, Sarah and Pauline, I really enjoyed all our meetings in the office. Thanks Elias, for your Spanish conversations and the Friday nights in the lab. Thanks Thomas, for being so nice to me and helping me with all my questions and doubts. But especially, thank you Carolina, for believe in me and listening to my crazy ideas in science. You are a really good friend. I wish you all the best in the future.

I would like to thank my family, for being that supportive and for the continuous encouraging. Thank you for being there even if you are 1645km away. Thank you Luis for just simply being around, you were my home here in Belgium.

Finally, I would also like to thank to the members of my jury (Th. Arnould, S. Gillet, M. Jadot and E. Muraille) to take the time for reading this manuscript.

*You never fail until you stop trying*
Albert Einstein

## TABLE OF CONTENTS

## ABBREVIATIONS

| | |
|---|---|
| APS | Ammonium Persulfate |
| BCV | *Brucella*-containing vacuole |
| CFU | Colony-forming unit |
| ChIP-seq | Chromatin immunoprecipitation-sequencing |
| Cm | Chloramphenicol |
| Co-IP | Co-immunoprecipitation |
| DAP | Diaminopimelic |
| DMEM | Dulbecco's Modified Eagle's |
| ERES | ER exist sites |
| FCS | Fetal Calf Serum |
| FRET | Förster Resonance Energy Transfer |
| GFP | Green fluorescdnt protein |
| IF | Immunofluorescence |
| IM | Inner membrane |
| IP | Immunoprecipitation |
| IPTG | Isopropyl β-D-1-thiogalactopyranoside |
| IS | Insertion sequences |
| Kan | Kanamycin |
| LAMP-1 | Lysosomal Associated-Membrane Protein-1 |
| LB | Luria-Bertani |
| LPS | Lipopolysaccharide |
| Nal | Nalidixic acid |
| OD | Optical density |
| OM | Outer membrane |
| oN | Overnight |
| PAL | Peptidoglycan associated proteins |
| PBS | Phosphate-buffered saline |
| PCR | Polymerase chain reaction |
| PFA | Paraformaldehyde |
| PG | Peptidoglycan |
| R-LPS | Rough LPS |
| RE | Endoplasmic Reticulum |
| RT | Room Temperature |
| RTqPCR | Reverse transcription PCR |
| S-LPS | Smooth LPS |
| SDS | Sodium dodecyl sulfate |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| T4SS | Type IV secretion system |
| TEMED | Tetramethylethylenediamine |
| Tm | Melting Temperature |
| TRSE | Texas red-X succinimidyl ester |
| X-Gal | 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside |
| WT | Wild type |

# INTRODUCTION

## 1. Cell envelope

### 1.2 Bacteria classification

Bacteria are mainly classified in two large groups: Gram-negative and Gram-positive bacteria. This classification comes from 1884 when Christian Gram's developed a staining technique, which allowed the classification of the bacteria into these two groups (*Gram, 1884*). Gram-positive bacteria are those bacteria, which retain the Christian stain whereas the Gram-negative bacteria do not. The Christian's stain is composed of a primary stain, the crystal violet and a secondary stain, the carbol fuchsin or saffranin. This differential stain is based on the composition and structure of the cell envelope (*Beveridge, 1999; Silhavy, Kahne and Walker, 2010*).

Both groups of bacteria possess a polymer structure called the peptidoglycan (PG), which differs in width between Gram-positive and Gram-negative bacteria. In fact, Gram-positive bacteria present many layers of peptidoglycan, which are 30-100 nm of thickness. Gram-negative bacteria contain only few layers and less thick than those of Gram-positive bacteria (*Silhavy, Kahne and Walker, 2010*). This is crucial for the differential staining as the peptidoglycan from Gram-positive bacteria is coloured by the Christian's stain (Crystal violet) whereas the Gram-negative bacteria lose the stain when washed and only retains the secondary stain, which colours bacteria in red (*Beveridge, 1999*).

However, the most defining feature of Gram-negative bacteria is the presence of two different membranes, the inner and the outer membrane. This outer membrane is absent in the Gram-positive bacteria and it acts as a selective permeability barrier. This outer membrane is composed by a lipopolysaccharide called the LPS, which makes Gram-negative bacteria more resistant than Gram-positive bacteria (*Beveridge, 1999; Silhavy, Kahne and Walker, 2010*) (**Figure 1**). Gram-negative and Gram-positive bacteria form distinct phylogenetic groups, except for *Mycobacteria*, that are phylogenetically Gram-positive but have selected the presence of an outer membrane (*Nikaido and Brennan, 1995*).

### 1.2 Gram-negative bacteria

#### 1.2.1 Cell envelope components

In Gram-negative bacteria, the inner and the outer membranes delimit an aqueous compartment called the periplasm. This aqueous compartment is more viscous than the cytoplasm as it presents a lower rate of protein diffusion (*Mullineaux et al., 2006*). It is composed of a dense pack of proteins. Many proteins found in the periplasm are called the periplasmic binding proteins and they participate in processes such as the transport of sugar and amino acids, detoxification, chemotaxis and the envelope biogenesis (*Silhavy, Kahne and Walker, 2010*).

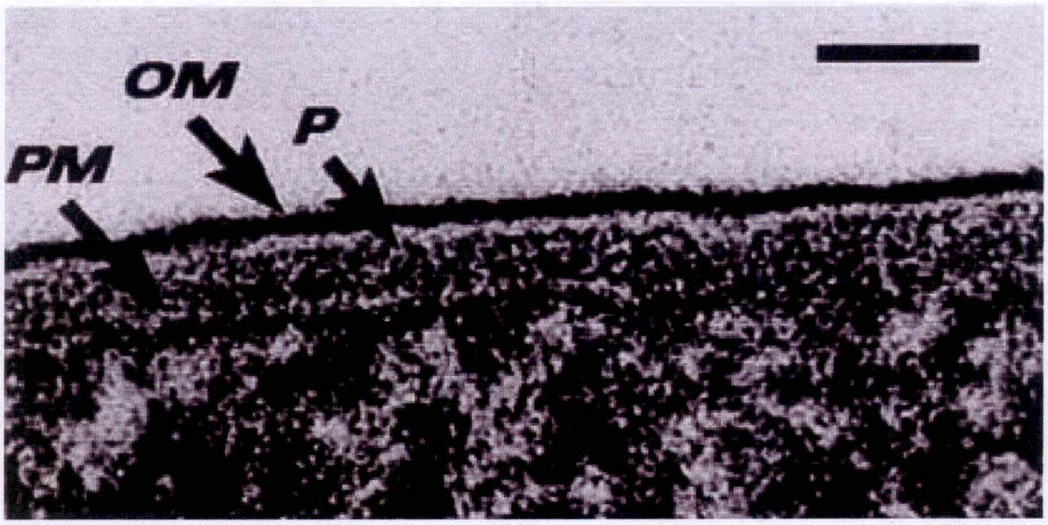



**Figure 1. Cell envelope of *E. coli* K-12.** Image from transmission electron microscopy shows the cell envelope of a Gram-negative bacterium. Outer membrane (OM), periplasm (PM) and the peptidoglycan (P) are indicated with black arrows. Scale bar 100 nm (*Beveridge, 1999).*

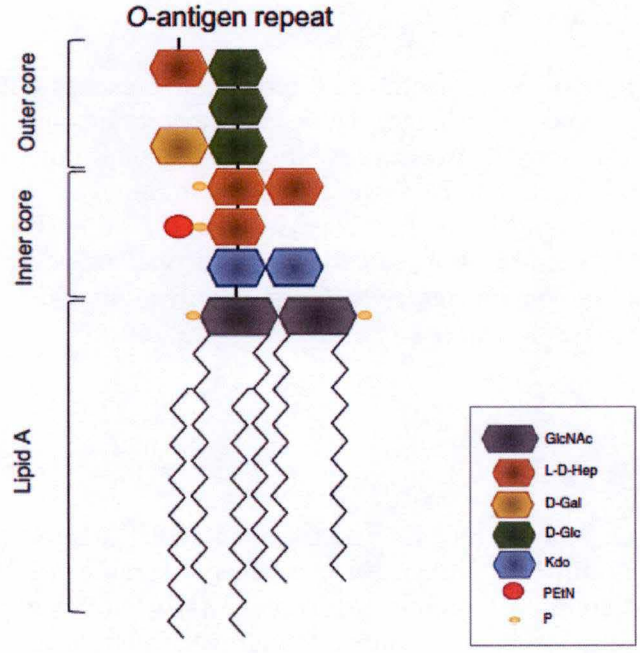



**Figure 2. LPS structure.** LPS consist of a lipid A structure followed by a core of oligosaccharides which is then extended in a long chain of polysaccharides called the O-antigen or O-chain. GlcNAc, N-acetylglucosamine; Kdo, 3-deoxy-D-manno-oct-2-ulosonic acid; Hep, L-glycero-D-manno-heptose; Glc, D-glucose; Gal, galactose: P, phosphate; PEtN, Pentaerythritol tetranitrate. (*Sperandeo, Martorana and Polissi, 2016).*

Besides the periplasmic proteins, the periplasm also contains the peptidoglycan. The peptidoglycan is a huge polymer of repeating units of the monosaccharides N-acetyl glucosamine and N-actyl muramic acid, which are linked by tetrapeptide side chains. This peptidoglycan stabilizes the cell envelope against the high internal osmotic pressure, gives rigidity and determines the cell shape (*Silhavy, Kahne and Walker, 2010*).

The outer membrane (OM) and the inner membrane (IM) are different in composition. The inner membrane consists of a phospholipid bilayer whereas the outer membrane is an asymmetric bilayer formed by a phospholipidic inner leaflet and an outer leaflet of lipopolysaccharides (LPS). LPS is an amphipatic molecule only present in the OM of the Gram-negative bacteria. LPS is comprised of a hydrophobic membrane anchor, lipid A, which is followed by a core of oligosaccharides. It is extended with a chain of polysaccharides, the O-antigen, which can present different lengths (*Bos, Robert and Tommassen, 2007; Silhavy, Kahne and Walker, 2010; Simpson et al., 2015; Konovalova, Kahne et al., 2017*) (**Figure 2**). LPS was considered to be essential for cell survival. Many bacteria such as *Escherichia coli, Salmonella typhimurium* and *Pseudomonas aeruginosa* cannot survive without LPS. However, other bacteria like *Neisseria meningitidis, Acinetobacter baumannii* and *Moraxella catarrhalis* do (*Simpson et al., 2015; Bos and Tommassen, 2005; Moffat, 2010; Peng et al., 2005; Steeghs et al., 2002; Steeghs et al., 1998*). Since the core and the O-chain are not essential for growth, bacteria can synthesize a short version of LPS, the rough LPS (R-LPS) containing the lipid A and the core but lacking the O-chain and opposite to the full version termed as smooth LPS (S-LPS) (*Delcour, 2009; Silhavy, Kahne and Walker, 2010; Zhang, Meredith and Kahne, 2013; Simpson et al., 2015; Sperandeo, Martorana and Polissi, 2016*).

The phospholipid inner leaflet of the OM presents the same lipid composition than the inner membrane, which is composed of phospholipids, mainly phosphatidyl-ethanolamine (70-80%), phosphatidylglycerol and cardiolipin, all equally distributed among the inner and outer leaflet of the IM (*Koebnik, Locher and Van Gelder, 2000*).

Furthermore, the OM contains proteins, the outer membrane proteins (referred as Omps) and the lipoproteins. A fraction of these Omps are integral proteins, which have different roles such as passive diffusion (porins), the specific uptake of nutrients and the incorporation of other Omps into the outer membrane. Unlike the proteins found in the inner membrane, which are consisting out of α-helix, these integral proteins consist of antiparallel amphipatic β-strands disposed in the form of a barrel with a hydrophilic interior but a hydrophobic exterior, in contact with the hydrophobic fatty acids of LPS and phospholipids (*Koebnik, Locher and Van Gelder, 2000; Luirink et al., 2005; Bos, Robert and Tommassen, 2007; Delcour, 2009; Simpson et al., 2015;*). On the contrary, lipoproteins are anchored to the outer membrane by the lipid part covalently linked to the N' terminus (generated by signal cleavage at the IM). They mainly participate in OM and PG biogenesis and envelope stress adaptation. Strikingly, in most of the lipoproteins, the function remains unknown (*Koebnik, Locher and Van Gelder, 2000; Konovalova, Kahne and Silhavy, 2017*).

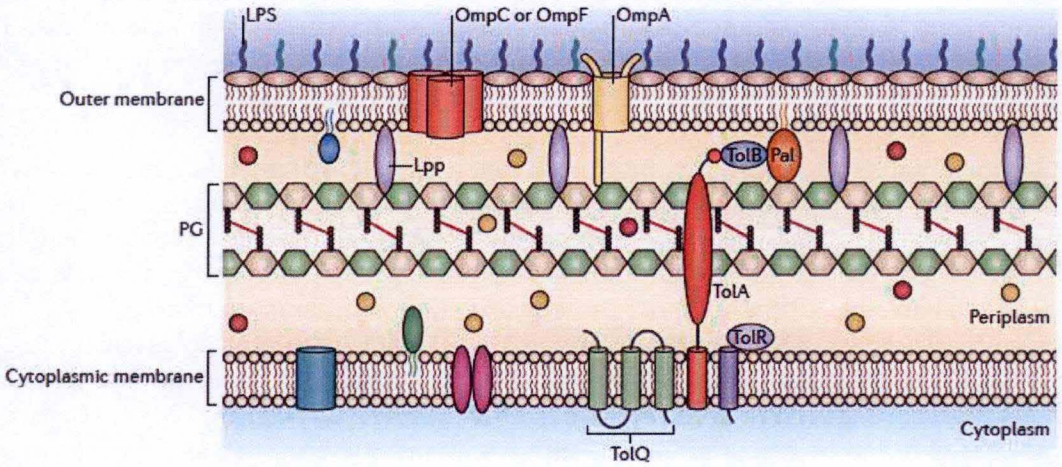



**Figure 3. Gram-negative cell envelope.** The cell envelope of Gram-negative bacteria consists of a cytoplasmic (inner) and an outer membrane. The inner membrane is a phospholipid bilayer whereas the outer membrane comprises an inner leaflet of phospholipids and an outer leaflet of LPS. Between these two membranes, the periplasm is found and immersed in it, the peptidoglycan. The peptidoglycan is a huge polymer consisting of repeating sugar units linked by tetrapeptide side chains. Moreover, there are envelope proteins called periplasmic proteins because they are present in the periplasm (red and oranges spheres); lipoproteins present in the outer membrane (blue spheres) and integral outer membrane proteins (OMPs). The stability of the membrane is due to interaction between the lipoprotein (blue oval) and the OmpA (yellow barrel) proteins with the peptidoglycan. The Tol-Pal (peptidoglycan-associated lipoprotein) complex, which is composed of TolA, TolB, TolQ, TolR and Pal, also binds to the peptidoglycan (orange oval). In the inner membrane there are proteins related to the incorporation of the outer membrane components into the outer membrane and specific autotransporters.
(*Schwechheimer and Kuehn, 2015*).

The OM is stabilized by the presence of some of these lipoproteins anchored to PG. The most abundant protein (about 1 million copies per cell) presents in the OM is the Braun's lipoprotein (Lpp) (*Braun, 1975*). This lipoprotein is homogenously distributed over the entire cell wall and a third of them are covalently bound to the PG by the ε-amino group of the carboxyterminal lysine residue (*Silhavy, Kahne and Walker, 2010*). The Tol-Pal complex (peptidoglycan associated lipoprotein), which consists of TolA, TolB, TolQ, TolR and Pal proteins, participates in the invagination of the outer membrane during cell division and also interacts with the PG (*Beveridge, 1999; Schwechheimer and Kuehn, 2015*).

In addition, OmpA, a major outer membrane protein in *E. coli* thought to contribute to membrane integrity, it contains a peptidoglycan-binding site for the diaminopimelic acid (DAP, part of the peptidoglycan) in its C-terminal domain, outside of the β-barrel structure (*Ishida, Garcia-Herrero and Vogel; 2014*). OmpA presents pore-forming properties but its main function is the membrane stability. Indeed, OmpA exhibits two conformations, a closed conformation, which expose a periplasmic domain and a less abundant form which conforms an open β-barrel with a similar diffusion than the major general porin, OmpF (*Beveridge, 1999; Delcour, 2009; Silhavy, Kahne and Walker, 2010*; *Schwechheimer and Kuehn, 2015*) (**Figure 3**).

### 1.2.2 Membrane permeability

The outer membrane acts as a protective barrier against the harmful substances present in the environment and the host. Gram-negative bacteria are resistant to host defences such as lysozyme, B-lysin and bile salts, which are toxic for the Gram-positive bacteria. Furthermore, this protective barrier limits the permeability of the envelope for several antibiotics, which are effective against other bacteria such as macrolides, novobiocin, rifamycins, lincomycin, clindamycin, and fusidic acid (*Niakido and Vaara, 1985).*

The Omps together with the LPS give the peculiar permeability properties to the Gram-negative bacteria. LPS molecules form a tightly packed layer due to the acyl chains present in the lipid A, which are highly saturated, and the lateral interactions mediated by the divalent cations $Mg^{2+}$ and $Ca^{2+}$, which neutralize the negative charges of the phosphates groups present in the lipid A (*Niakido and Vaara, 1985; Silhavy, Kahne and Walker, 2010; Sperandeo, Martorana and Polissi, 2016*).

Therefore, the OM becomes a strong barrier against the hydrophobic molecules as shown by the resistance to hydrophobic antibiotics (e.g., macrolides, rifampicins) and detergents (e.g., Sodium dodecyl sulfate (SDS), bile salts, Triton X-100) (*Leive, 1974; Nikaido and Nakae, 1979; Niakido and Vaara, 1985*).

However, small hydrophilic molecules can easily pass across the OM thanks to Omps. Some of the most abundant Omps display a β-barrel structure that allows the formation of a pore. This pore can be either open or closed. When the pore is open, as in the general porins, an inner folded extracellular loop (L3) restricts the constriction site limiting the pore size and the permeation properties of the porin. These porins act as non-specific channels that give access to small hydrophilic molecules smaller than 600 Daltons (*Delcour, 2009*). Slightly hydrophobic substances are hardly able to go through the pores of the porin thanks to the nature of the pore (*Niakido and Vaara, 1985*).

Moreover, the IM is missing these porins and these hydrophilic substances that easily diffuse through the porins, cannot pass into the cytoplasm unless they are recognized by specific auto-transporters. Phospholipid layers are permeable to hydrophobic molecules. Thus, LPS retains these molecules, which can pass through the inner membrane, in the outer membrane. If necessary, among these Omps, there are some that act as specific channels for the transport of certain solutes. This opposite permeability characteristic of these two membranes allows bacteria to adapt to harsh environments (*Niakido and Vaara, 1985; Bos, Robert and Tommassen, 2007; Simpson et al., 2015*).

### 1.2.3 LPS interactions with Omps

In order that the OM remains a selective barrier, LPS molecules and Omps have to tightly interact to maintain the structure of a dense pack (*Niakido and Vaara, 1985*).

The first indication about this interaction was observed when the levels of Omps decreased in mutants with deficiency in LPS in *E. coli* and *S. typhimurium.* This decrease was most pronounced in the general porins OmpF and OmpC but less significant in the OmpA structural protein (*Koplow and Goldfine, 1974, Ames et al., 1974; Niakido and Vaara, 1985*). LPS-Omps interaction has also been later confirmed in *E. coli* and other different species such as *Yersinia pestis (Vakorina et al., 2003; Arunmanee et al., 2016; Patel et al., 2016*).

In fact, LPS was revealed to interact in a non-covalently manner with Omps by hydrophobic interactions and van der Waals forces between the acyl groups of the lipid A and the hydrophobic residues from the external wall of the Omp barrel. Basic amino acids form salt bridges with the phosphate groups of LPS. LPS parts display different flexibilities. Lipid A results in the rigid part whereas the O-antigen is more flexible. Bacteria can exhibit heterogeneous systems of LPS with R- and S-LPS versions. In these systems, the adjacent O-antigen chains show weak interactions and more flexibility than those homogeneous systems in which O-antigen chains have the same length. If the O-chain is long, it could shield the opening of the pore thus compromising the function of the porin. Therefore, these heterogeneous systems are quite relevant because they do not compromise the pore opening by shielding it with the O-antigen chains (*Patel et al., 2016*).

## 1.3 Bacterial cell length

Bacteria modulate their size to preserve their viability to survive in the environment. Bacteria need to accommodate all the proteins, structures and machineries to maintain their metabolism. Obligate intracellular pathogens present a reduce size compared to the free-living bacteria presumably because many metabolic activities are supplied by the host. On the contrary, in large bacteria, the problem resides in diffusion. The transport of biomolecules inside bacteria takes place by molecular diffusion. For most molecules, an efficient transport is produced in a short distance of a few microns. If the ratio between surface area and volume is altered, the transport of nutrients becomes then insufficient (*Koch, 1996; Schulz and Jørgensen, 2001; Levin and Angert, 2015; Westfall and Levin, 2017*).

Bacteria can present a wide diversity in size. However, for a given species, individual bacteria maintain a regular range in size (*Deforet, Van Ditmarsch, Xavier, 2015*).

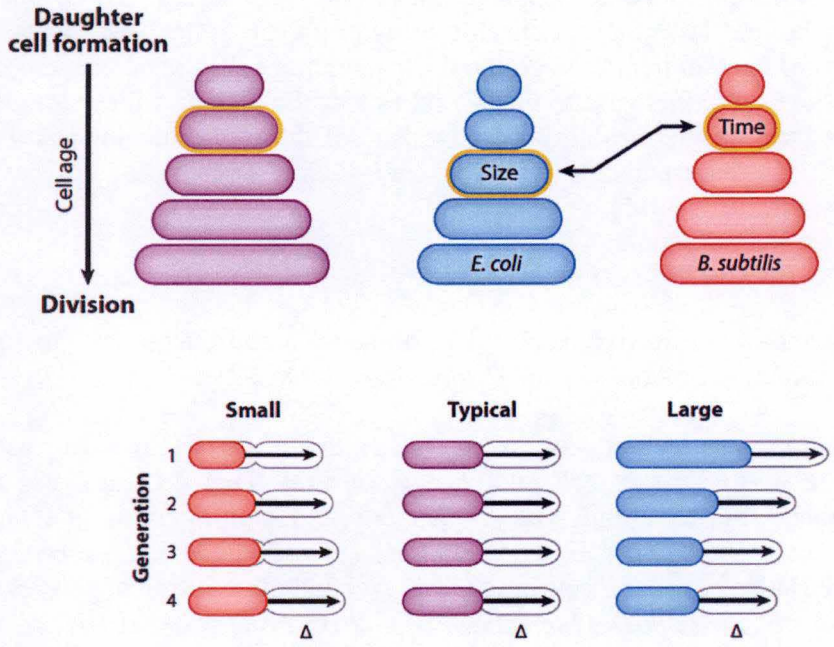



**Figure 4. Cell size regulation.** Cell size can be regulated by time or size. *E. coli* initiates replication when bacteria reach a critical size whereas in *B. subitlis* time control this process. In each generation, bacteria add the same volume regardless their size. After several cell cycles, this size is then normalized (*Westfall and Levin, 2017*).

All bacteria control their size, but the mechanisms they employ differ. In fact, it is well known that in rod-shape bacteria, cell cycle can be controlled by either a regulation on time or a regulation depending on size. Bacteria such as *E. coli* initiate the cell division when they reach a critical size whereas cell cycle in bacteria such as *B. subtilis* (Gram-positive bacteria) depends on time (*Taheri-araghi et al., 2015; Westfall and Levin, 2017*). In both cases, bacteria add a constant amount of volume (Δ) during each cell cycle before they divide. Daughter cells, which are small at the time of division, all add the same amount of volume. This constant addition does not immediately correct the cell size but after several cell cycles, this size is normalized (*Amir, 2014; Westfall and Levin, 2017*) (**Figure 4**).

Moreover, bacterial size is correlated with nutrient availability. Bacteria are significantly larger in rich-nutrient medium conditions in contrast to bacteria cultured under poor-nutrient conditions (*Schaechter, Maaloe, Kjeldgaard, 1958*). In order to maintain the cell size homeostasis, cells growing slowly due to the nutrient limitation adjust the Δ in order to maintain a constant mean size (*Deforet, Van Ditmarsch and Xavier, 2015; Taheri-Araghi et al., 2015*). Growth rate is then related to cell size. During replication, rapidly growing bacteria like *E. coli* initiate one or several replication forks before the previous one is completed. Thus, bacteria after division still present active replication forks. In the case of slowly growing bacteria, the time needed for a complete cell cycle exceeds the time needed for the elongation. Therefore, there are never more than two replicative forks in a cell to ensure that the division is coordinated with the size (*Cooper, 1968; Sharpe et al., 1998; Wear et al., 2007*). This, together with the Δ adjustment, ensures the cell size homeostasis.

## 2. *Brucella abortus*

### 2.1 *B. abortus* is an alphaproteobacterium

*Brucella* is a pathogen that belongs to the α-sub-division of proteobacteria (Gram-negative bacteria). These proteobacteria show great variability in their ecological niches. Their lifestyle differs according to their host relationship. They can be symbiotic or pathogen for plants, insects or animals. Moreover, they can behave as intracellular or extracellular pathogens being either obligate or facultative pathogenic bacteria. Free-living bacteria are also found in soil and water (*Batut, Andersson and O'Callaghan, 2004; Van der Henst et al., 2013*).

Ribosomal 16S RNA similarities positioned *Brucella spp.* in the α-class of proteobacteria (*Moreno and Moriyó, 2006*). *Brucella* are phylogenetically belonging to the order Rhizobiales (*Yanagi, and Yamasato, 1993; Cloeckaert et al., 2002; Cameron, Zupan and Zambryski, 2015*). In this group, diverse bacteria are found, ranging from the plant pathogen *Agrobacterium* and the facultative intracellular pathogens *Brucella* and *Bartonella* to obligate intracellular pathogens of mammals and insects (*Rickettsia, Anaplasma*). Plant symbionts known as Rhizobia (e.g. *Shinorhizobium*) and the opportunistic bacteria *Ochrobactrum* also form part of this group (*Batut, Andersson and O'Callaghan, 2004; Cameron, Zupan and Zambryski, 2015*).

### 2.2. The *Brucella* genus

*Brucella spp.* are intracellular facultative bacteria responsible for the human zoonosis called brucellosis.

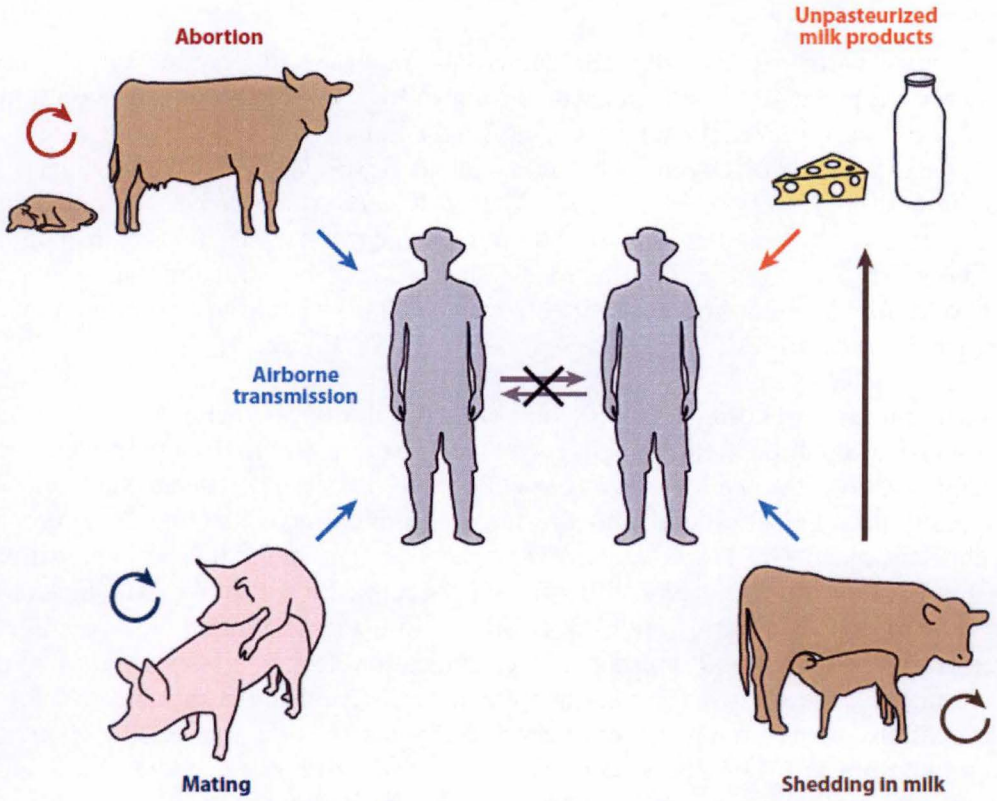



**Figure 5. Transmission of *Brucella* in animals and humans.** The *Brucella* transmission within the natural host occurs via abortion, mating and shedding in milk. Humans can be infected via ingestion of unpasteurized milk or by occupational exposure (farmers and veterinarians). Human-human transmission is a quite rare event (*Atluri et al., 2011*).

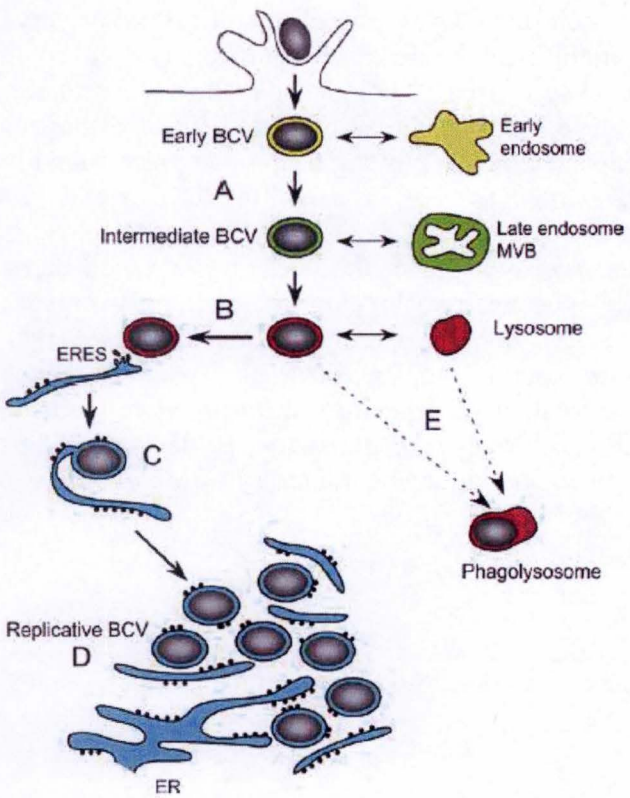



**Figure 6. *Brucella* intracellular trafficking.** After *Brucella* is internalized into the host cell, the *Brucella* containing vacuole (BCV) acquires markers of the early (A) and later late endosomes (shown in yellow and green, respectively) before being fused with the lysosome (shown in red) (E). *Brucella* is able to avoid the fusion with the lysosomes and interact with the ER exit sites (ERES) to fuse with the ER (shown in blue) (B, C and D). Mutants lacking the *virB* operon (encoding the type IV secretion system) are not able to interact with the ERES, leading to the fusion with the lysosome and the killing of *Brucella* (E) (*Starr, et al., 2008*).

The *Brucella* genus comprises six classical species that are mainly classified according to their host infection specificities (*B. melitensis, B. abortus, B. suis, B. neotomae, B. ovis* and *B. canis*). This classification has been enlarged by the inclusion of four more species isolated from marine environments (*Moreno and Moriyó, 2006; Jahans et al., 1997*). Three species are considered as the main pathogenic species: *B. melitensis*, responsible for the ovine and caprine brucellosis, *B. abortus* for the bovine brucellosis and *B. suis* for the swine brucellosis (*Cloeckaert et al., 2002).*

*Brucella* is considered as an intracellular facultative pathogen, because it can multiply in extracellular and intracellular conditions even though they present host preferences. Under suitable environment circumstances of temperature and nutrient availability, *Brucella* can also survive but there is no evidence that they can replicate in an open environment (*Corbel and Brinley-Morgan, 1984; Moreno and Moriyó, 2006*).

### 2.3 Pathogenicity

*Brucella* can infect domesticated, wild and marine animals (*Moreno and Moriyó, 2006*). The infection in animals can lead to abortion in pregnant females and infertility in males (*Van der Henst et al., 2013*). Transmission within the host mainly occurs by close contact, ingestion of milk, via aerosols generated by the abortion in infected females, via semen or by secretions during the mating and parturition (*Atluri et al., 2011*).

Despite the fact that animals are the preferential hosts, *Brucella* can also accidentally infect humans. This infection in humans is more severe and it results in quite diverse symptoms varying from an asymptomatic disease to the Malta fever, characterized by an acute manifestation of undulant fever (*Van der Henst et al., 2013; Cloeckaert et al., 2002; Moreno, 2014 Moreno and Moriyó, 2006;*). If left untreated, this disease can become chronic and it may affect many organs, causing arthritis, orchitis, hepatitis, encephalomyelitis, and endocarditis (*De Figueiredo et al., 2015*). The main source of infection is the occupational exposure either by aerosols when working with infected animals or by direct contact of the placenta or aborted foetus. Moreover, *Brucella* can be transmitted by ingestion of products derived from unpasteurized milk (*Atluri et al., 2011;Van der Henst et al., 2013; De Figueiredo et al., 2015*). Since humans are considered as accidental or dead-end hosts, reports of human-to-human transmission are quite rare (*Atluri et al., 2011*) (**Figure 5**).

### 2.4 *Brucella's* infection and trafficking

*Brucella* can enter into the host by the mucosal membranes of the respiratory, digestive and genital tracts (*von Bargen, Gorvel and Salcedo, 2012*). Once inside the host organism, the bacteria can infect different types of cells from professional and non-professional phagocytes to epithelial cells. The intracellular lifestyle is characterized by two phases, a non-proliferative phase, which occur during the trafficking, and a proliferative phase, which takes place inside the replication niche, an endoplasmic reticulum-derived compartment (*Deghelt et al., 2014*).

After the internalization in the host cell, *Brucella* resides in a membrane-bound compartment named BCV (*Brucella*-containing vacuole) (*Starr, et al., 2008; Myeni et al., 2013*). Intracellular pathogens have the ability to modify the trafficking pathway in order to survive inside the host cells (*Pizarro-Cerdá et al., 1998*).

*Brucella* also alters this pathway to avoid the fusion with the lysosome and reach the Endoplasmic Reticulum (ER), its replication niche.

During the maturation along the endosomal pathway, the BCV is slowly acquiring some markers for the late-endosomal pathways such as Rab7 and LAMP-1 (Lysosomal Associated-Membrane Protein-1) (*Starr, et al., 2008*). In this step, the BCV is also acidified. This acidification is crucial for the replication of the bacteria as the low pH induces the expression of the *virB* operon, encoding the type IV secretion system (T4SS). This is an important virulent factor, which delivers effector molecules to facilitate the trafficking to the ER in the host cell (*Myeni et al., 2013; Boschiroli, 2002*).

Further steps include the progressive exclusion of the LAMP-1 marker from the BCV. *Brucella* is able to control this trafficking and the maturation of the BCV to interact with an organelle derived from the ER, the ER exist sites (ERES) to reach the ER (*Celli et al., 2005*). This process occurs when the BCV finally interacts with the ERES to proliferate massively (**Figure 6**).

### 2.5 *Brucella* type of growth

Bacteria usually display two modes of growth, the zonal or dispersion growth. In the zonal growth, the incorporation of the new cell envelope material comes to specific areas such as the midcell, whereas the dispersed growth spreads this addition in small patches along the membrane of the bacteria. Nevertheless, many rod-shaped bacteria such as Rhizobiales differ from these two growth models. Instead of exhibiting the typical zonal growth, they present a specific type of growth in which the new cell wall synthesis is restricted to a defined area in the mother cell, namely the new pole (*Brown et al., 2012; Cameron, Zupan and Zambryski, 2015*). Once the unipolar growth is finished, the peptidoglycan synthesis is then redirected to the constriction site. Other bacteria also display polar growth (*Howell and Brown, 2016*)

Moreover, unipolar growth seems to be quite conserved among Rhizobiales as it was already shown in species like *Ochrobactrum anthropi* and *Sinorhizobium meliloti* (*Brown et al., 2012)*. In order to analyse this polar growth behaviour, bacteria can be labelled with the amine reactive dye Texas red-X succinimidyl ester (TRSE) to precisely identify the areas of active growth.

In *Brown et al., 2012,* Cells were labelled with TRSE in order to monitore the *Agrobacterium tumefaciens* growth. This compound labels the amine groups present on the surface of the bacterium. Bacteria are first completely labelled with TRSE, washed and then incubated in new fresh medium. Therefore, the new incorporated material will be unlabelled.

When *E. coli* was stained with TRSE, the TRSE compound was homogeneously diluted while the bacteria were growing and it only remained concentrated to the old cell poles because these regions consist of old material.

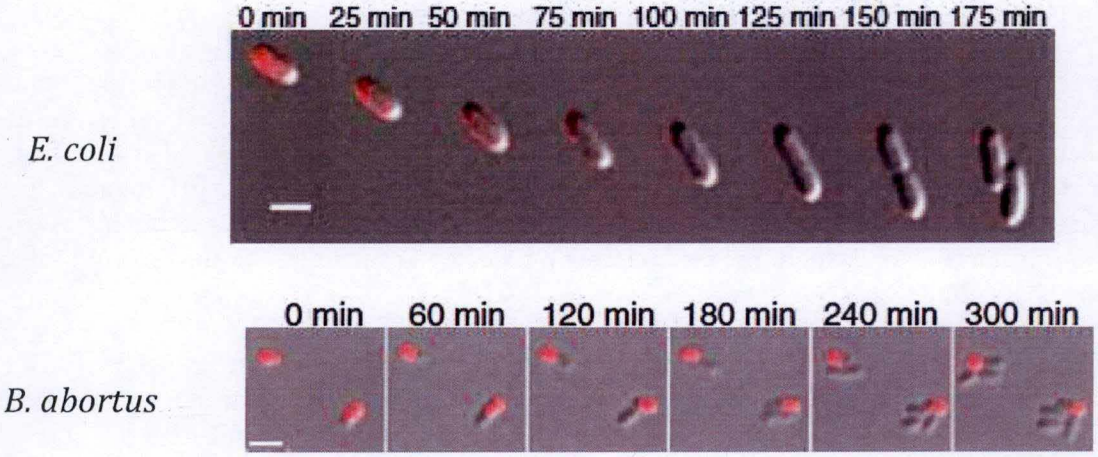



**Figure 7. Growth in α-proteobacteria.** Bacterial growth can be studied by using the dye TRSE that labels the surface of the bacteria completely. Then, bacteria are washed and incubated in new medium to allow new growth. Therefore, areas of new envelope synthesis, which will be unlabelled, can be identified. A) *E. coli* employs a dispersed growth mode in which the new incorporated materials come from different areas along the surface of the bacteria. Thus, the TRSE is diluted while the bacteria are growing. B) Other bacteria, such as Rhizobiales (*B. abortus*), present another mode of growth, the unipolar growth. In this case, the new material rises from one specific pole (*Brown et al., 2012*).

This shows dispersed growth. In contrast in the case of *A. tumefaciens*, the TRSE labelling remained constant in the whole mother cell whereas the new unlabelled material emerged from one specific pole (*Brown et al., 2012*). Studies in *B. abortus* revealed the same pattern of growth when they were also stained with TRSE (*Brown et al., 2012; Van der Henst et al., 2013*) (**Figure 7**).

In addition to this unipolar growth, Rhizobiales divide asymmetrically to produce two different cells in size and morphology as the daughter cell is composed of newly synthesized envelope (*Hallez, Bellefontaine, Letesson and De Bolle, 2004; Van Der Henst et al., 2013*).

This polar growth is strongly coordinated with the cell cycle, which comprises the DNA replication and the chromosome segregation between the daughter and the mother cells (*Cameron, Zupan and Zambryski, 2015*).

### 2.6 *Brucella* cell membrane

#### 2.6.1 LPS in *Brucella*

Although LPS is a quite well conserved structure in all Gram-negative bacteria, differences in its composition exist among species and strains (*Rittig et al., 2003*).

*Brucella* spp. exhibit two LPS phenotypes. They can either express the full-version LPS containing the O-chain (called smooth LPS, S-LPS) or a version with an absent O-chain known as the rough LPS form (R-LPS) (*Rittig et al., 2003*). The most infectious strains of *Brucella*, *B. melitensis, B. suis* and *B. abortus* are characterized as smooth LPS species by classical tests, whereas species such as *B. canis* and *B. ovis,* which are less virulent, are naturally occuring rough species (*Cardoso et al, 2006; Mancilla, 2016*).

Classical LPS is a molecule known to have a profound effect in the human immune system because of its endotoxic properties. However, *Brucella* LPS is poorly endotoxic and inflammatory and it is unable to active the innate system response in a strong way (*Mancilla, 2016*).

In smooth species, rough bacteria can spontaneously emerge and accumulate in culture, under laboratory conditions. The genes responsible for the O-chain synthesis in *Brucella* are found in the same chromosome in two major regions *wbo* and *wbk*. The *wbo* region contains two glycosyltranferase genes (*wboA* and *wboB*) which form part of the genomic island GI-2, an unstable genetic element. On the contrary, the *wbk* genes are mostly involved in the polymerization and the translocation of the O-chain. The *wbk* genes do not form a continuous unit since the region contains several insertion sequences (IS). The genomic islands are maintained under selective pressure but upon changes in the environment, they can be released from the chromosome.

The excision of the island GI-2 can then lead to the loss of O-LPS genes, which are also located in recombination spots. Site-specific recombinase can excise these genes and along with the spontaneous mutations produced in *wbk*, they can trigger the dissociation of S-LPS to R-LPS (*Mancilla et al., 2012, Mancilla, 2016*).

That suggests that the O-chain synthesis is not essential and not metabolically demanding under culture conditions. The presence of the O-chain could be then a selective advantage in infection of the host (*McQuiston et al., 1999; Rittig et al., 2003; Mancilla et al., 2012; Mancilla, 2016*). The dissociation from S-LPS to R-LPS is stimulated by changes in the environmental conditions including nitrogen sources and exposure to D-alanine. D-alanine is an end metabolite that accumulates in the medium along time. Bacteria produce D-amino acids when they reach the stationary phase, These D-amino acid appear to modulate the PG composition. This could be probably a strategy to adapt to the environmental changing conditions (*Lam et al., 2009*). The accumulation of D-alanine decreases the growth of smooth bacteria whereas it increases the proportion of rough bacteria, which are alanine resistant (*Braun et al., 1951; Goodlow et al., 1952*).

LPS also gives *Brucella* some permeability properties, which are not present in other Gram-negative bacteria. *Brucella* is more resistant to cationic peptides (polymyxin B) and chelating detergents (EDTA) than *E. coli* (*Moriyon and Berman, 1982; De Tejada et al., 1995*). In the case of polymyxin B, R-strains are more sensitive to this cationic peptide than the S-strains suggesting that the smooth LPS plays an important role in the resistance of *Brucella* (*De Tejada et al., 1995*).

### 2.6.2 Omps in *Brucella*

The major outer membrane proteins of *Brucella* were first identified in 1980 by detergent extraction of cell envelopes. They are classified according to their apparent molecular mass into three groups: group 1 with proteins from 94 to 88 kDa, group 2 with 33-36 kDa proteins and group 3 with Omps from 31-34 to 25-27 kDa (*Cloeckaert et al., 1996 a; Moriyón and Lopez-Goni, 1998; Cloeckaert et al., 2002; Moreno and Moriyón, 2006).*

The group 2 of Omps consists of porins (Omp2b and Omp2a) whereas the group 3 contains three unrelated proteins, Omp3a (Omp25) Omp3b (Omp22) and Omp31, in which, based on the amino acid composition and similarities, Omp25 is considered the counterpart of OmpA (*E. coli*) (*Moreno and Moriyón, 2006*) except that it does not have a C-terminal PG-binding domain (*Ishida, Garcia-Herrero and Vogel; 2014*). The group 1 of Omps include the Omp85 (also called BamA), which allow the incorporation of the other Omps into the outer membrane. In *E. coli*, this Omp is tightly associated with four lipoproteins and together they form the Bam complex. This complex is responsible for the translocation of the outer membrane proteins from the periplasm into the outer membrane (*Gatsos et al., 2008*).

*Brucella* also contains three minor outer membrane proteins: Omp10, Omp16 and Omp19. Omp16 shows significant similarity to the PALs proteins of Gram-negative bacteria, known for binding the peptidoglycan (peptidoglycan associated lipoproteins) (*Tibor et al., 1996; Tibor and Letesson, 1999*).

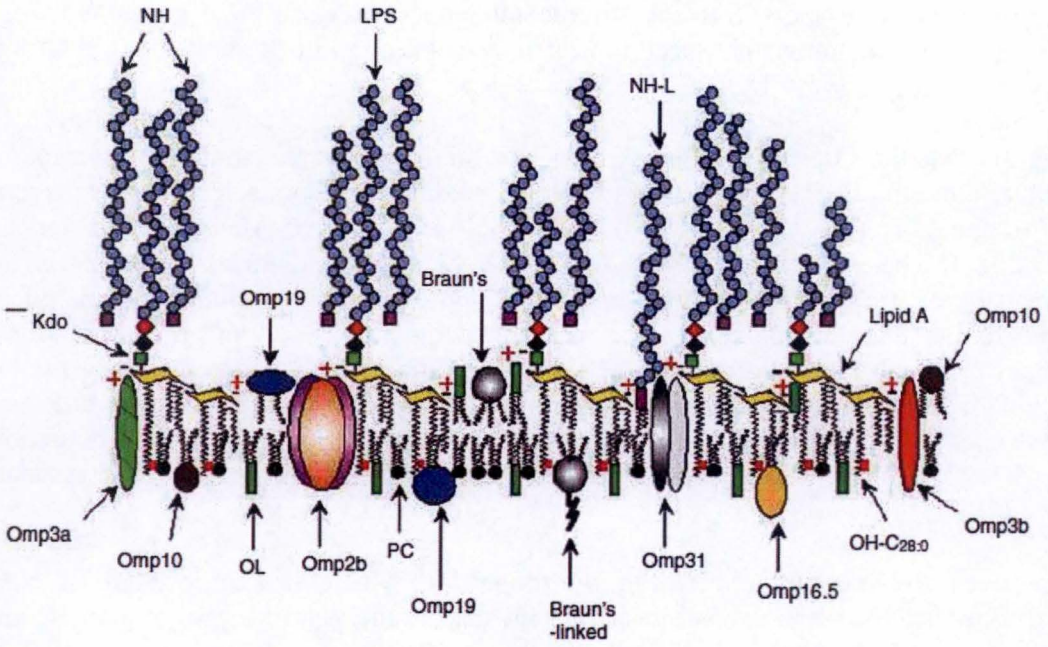



**Figure 8. Representative model of the outer membrane of *Brucella*.** Major outer membrane proteins are indicated and named by their apparent molecular mass. Lipoproteins showing the acyl groups anchored to the membrane. Braun's lipoprotein may be linked to peptidoglycan or free in the outer membrane. The lipid A (represented by yellow trapezoids) is linked to fatty acids (black zigzag structures). The red squares at the bottom of the structure mark the hydroxyl groups of the LPS. Dark circles represent phospholipids (*Moreno and Moriyón, 2006*).

Omp10 and Omp19 have no homologs yet described, and their function is not well studied. These three Omps contain in their sequences conserved signal typical of bacterial lipoprotein suggesting the presence of a lipidic anchor, which was later confirmed experimentally (*Tibor, Decelle and Letesson, 1999; Moreno and Moriyón, 2006*) (**Figure 8**).

### 2.6.3 Group 2 Omps

The group 2 comprises the genes *omp2a* and *omp2b,* which encode proteins associated to porin activity (*Verstreate et al., 1982; Douglas et al., 1984; Marquis and Ficht, 1993*). Hybridization and DNA sequences analysis revealed the presence of two duplicated genes, named as *omp2a* and *omp2b* (*Ficht et al., 1988; Ficht et al., 1989; Ficht et al., 1990; Marquis and Ficht, 1993*). Both genes share 85% of identity at the amino acid level. Moreover, they are found in close proximity, separated by 850 bp and oriented in opposite directions (*Ficht et al., 1989; Marquis and Ficht, 1993;Cloeckaert et al., 1996 a; Moriyón and Lopez-Goni, 1998Cloeckaert et al., 2002*). This gene arrangement was studied by Southern blot with genomic DNA cut by specific restriction enzymes. Specific probes were used to detect *omp2b* and *omp2a* genes. The *omp2a* and *omp2b* location appeared to be highly conserved in all *Brucella* species even though the predicted size of the two porins was highly diverse (*Ficht et al., 1990; Ficht et al., 1996*). However, *B. suis* presented two gene copies more related to *omp2a* than to *omp2b.* These observations lead to the hypothesis that this *omp2* polymorphism was due to genetic conversion. Indeed, *omp2a* was considered as the progenitor copy, which would serve as a template for the *omp2b* conversion. These corrections may have occurred over evolution in *Brucella,* resulting in the different size variants of the *omp2* genes (*Ficht et al., 1990; Ficht et al., 1996; Paquet et al., 2001*).

According to their nucleotide sequences, *omp2b* would encode a 36-kDa protein and *omp2a* would encode a smaller version due to a deletion of 108 bp in the coding sequence. The expression of *omp2a* and *omp2b* was analysed in *B. abortus*. Only the 36 kDa protein corresponding to the Omp2b porin was detected and thus it was assumed that the *omp2a* gene was not expressed in *B. abortus* (*Paquet et al., 2001; Ficht et al., 1989*). This was further confirmed by RNA sequence analysis in which *omp2b* seemed to be expressed whereas *omp2a* does not (*Marty Roop, unpublished data*). Omp2a protein was only observed when the promoter controlling the *omp2* region was inversed in *E. coli (Ficht et al., 1989).*

Omp2b is able to form oligomers resistant to SDS denaturation, a characteristic of porins as such from *E. coli* and *S. typhimurium*. When the *omp2a* gene was expressed in *E. coli*, an increasing hydrophobicity was observed, as it also occurs with other porins (*Marquis and Ficht, 1993; Cloeckaert et al., 2002*). Both porins in *B. abortus* are similar in amino acid composition to *E. coli* porins and they exhibit similar internal pore diameters (*Douglas et al., 1984; Cloeckaert, 1996; Mobasheri et al., 1997; Moriyón et Lopez-Goni, 1998; Paquet et al., 2001*).

The predicted topology of Omp2b consists of a trimer in which each monomer contains 16-stranded β-barrels with long external loops (*Paquet et al., 1999; Moriyón et Lopez-Goni, 1998; Cloeckaert et al., 2002*). The main difference between the two Omps is the presence of two insertion/deletions in the beginning of the external loops L3 and L5 of Omp2a.

Omp2b exhibits a well-defined homotrimeric structure whereas Omp2a forms monomer pores, which are smaller than those in Omp2b (*Mobasheri et al., 1997; Paquet et al., 2001*). The pore activity resides in the L3 external loop in most porins, restricting the sugar permeability (*Jap et Walian, 1996; Paquet et al., 2001*). Omp2a L3 external loop is more negatively charged, suggesting that it could have an impact in the pore activity. Omp2b of *B. abortus* displays similar sugar permeability than Omp2a. In fact, the external loop L5 is also shorter in the Omp2b of *B. abortus* and it is responsible for the smaller size of the pore, which appears to be crucial for the sugar permeability. This was confirmed by Omp2b of *B. suis,* which presents a larger L5 external pore and a consequent lower sugar permeability. Therefore, this small deletion could enlarge the opening of the pore, contributing to the determination of the pore diameter (*Paquet et al., 2001; Cloeckaert et al., 2002*).

Furthermore, Omp2b is essential in *B. melitensis* (*Laloux et al., 2010*). Regarding recent Tn-seq data generated in the host laboratory (*PhD project J-F Sternon*), it is suggested that *omp2b* also seems to be essential in *B. abortus* 2308 wild type strain.

### 2.6.4 Group 3 Omps

The group 3 of Omps in *Brucella* comprises three major Omps (Omp25 (Omp3a), Omp31a and b) and other 5 homologous (minor) proteins, the Omp25-like proteins (Omp25b, c, d) and Omp22 (Omp3b) (*Cloeckaert et al., 1996 a ; Moriyón and Lopez-Goni, 1998; Cloeckaert et al., 2002; Salhi et al., 2003Moreno and Moriyón, 2006; Caro-Hernández et al., 2007*).

Little is known about the minor five Omps, even though they have been detected experimentally in at least one *Brucella* species. Omp31b has been identified in *B. abortus* and *B. suis* and the Omp25-like proteins, Omp25b and c in *B. suis* and *B. mellitensis,* Omp25c was also identified in *B. abortus* (*DelVecchio et al., 2002; Guzmán-Verri et al., 2002; Salhi et al., 2003*). The Omp25d evidence is less clear as it was only weakly detected in a mutant lacking *omp25* gene in *B. suis* (*Salhi et al., 2003*).

Omp31 protein is produced in all *Brucella* species except *B. abortus. B. abortus* carries a deletion of 10 kb which includes the Omp31a gene and probably other genes still not characterized (*Vizcaino et al., 1997; Moreno and Moriyón, 2006*). Moreover, the absence in *B. abortus* suggests that this protein is not essential, at least in this species (*Moreno and Moriyón, 2006*). Omp31 is able to form oligomers resistant to SDS denaturation as well as porins can do. Therefore, Omp31 could act as a porin in those *Brucella* species in which there have been reported a reduced expression of the genes from group 2 (porins) (*Cloeckaert et al., 1996 a; Vizcaíno et al., 1996*).

The amino acids sequences of Omp25 and Omp31 share 34% of identity. The predicted structure of Omp31 shows an eight-stranded β-barrel with external loops larger than those present in Omp25 (*Cloeckaert et al., 1996 a; Moriyón et Lopez-Goni, 1998; Cloeckaert et al., 2002; Vizcaino, 2007*).

Omp25 is a non-essential protein with a predicted structure composed by eight transmembrane β-strands connected by external loops exposed to the surface (*Cloeckaert et al., 2002*). It is highly conserved among all *Brucella* species (*Cloeckaert et al., 1996 a and b*).

OmpA from *E. coli* was previously proposed to be the counterpart of Omp25 based on the amino acid sequence (*Verstreate et al., 1982*). Omp25 shows some similarities in the secondary structure of *E. coli* OmpA, but they are different in size (*Baldermann, et al., 1998; Moriyón et Lopez-Goni, 1998*). However, sequencing the *omp25* gene in *B. abortus* revealed no significant homology to others OmpA of Gram-negative bacteria (*de Wergifosse et al., 1995*). Although OmpA share a low similarity to Omp25, Omp25 could have a similar role in the OM of *Brucella* (*Moriyón et Lopez-Goni, 1998*). OmpA is a major Omp considered to have a significant role in the membrane integrity as mutants lacking OmpA show membrane instability (*Wang, 2000*).

Furthermore, Omp25 seems to be involved in the virulence of *B. melitensis* as bacteria lacking the *omp25* gene are attenuated during infection in mice. BALB-C mice were infected intravenously with *Brucella* lacking *omp25* gene and results showed a significant decrease in the CFU/spleen counts (*Edmonds et al., 2002*). Omp25 was also proposed to inhibit the TNF-α release from infected human macrophages (*Jubier-Maurin et al., 2001; Salhi et al., 2003*), but the mechanisms behind this phenotype are unclear and could result from indirect effects, such as envelope alteration in the *omp25* mutant compared to the wild type strain, having an effect on host cell signalling pathways.

## 3. Objectives

In the present study, we will investigate the function of the most abundant Omps of *B. abortus*, Omp25 and Omp2b. We chose a genetic approach to investigate their functions. Therefore, we will generate a deletion mutant of *omp25* and a depletion mutant of *omp2b* by allelic replacement, since *omp2b* is essential.

Omp25 is a major non-essential Omp but its function is not well defined. It has been shown that CtrA, a cell regulator crucial for the cell division in *B. abortus*, could control the expression of the *omp25* gene. When CtrA is depleted, the abundance and location of Omp25 is altered (*Francis et al., 2017*). We will create a deletion mutant (Δ*omp25*) of *B. abortus* 544 in order to analyse the possible effects of the absence of Omp25 on the membrane morphology when bacteria undergo different type of stresses. Moreover, as Omp25 is a major component in the outer membrane, the type of growth and the cell size will be studied as well as the composition of the outer membrane when Omp25 is missing. As mentioned above, mutants in *B. melitenis* lacking *omp25* are attenuated during mice infection (*Edmonds et al., 2002)*. Here, the virulence in *B. abortus* will be also studied by infection in non-activated RAW 264.7 macrophages, as a first step of its characterization in infection models.

Since Omp2b is an essential protein in *B. melitensis* (*Laloux et al., 2010*) and a previous study in the host laboratory showed that Omp2b is also essential in *B. abortus* (*PhD project J-F Sternon*), we will create a depletion strain of *B. abortus* 544, Δ*omp2b* $p_{lac}$-*omp2b*, in order to study its effect on the growth and pathogenicity of the bacterium. Interestingly, R-LPS was shown to be partially colocalized with the essential Omp2b porin (*V. Vassen, on going PhD thesis*). This potential interaction between Omp2b and R-LPS will be examined in a biochemical approach by the analysis of a possible copurification of R-LPS and S-LPS with Omp2b.

In addition, some bioinformatics analyses will be performed to characterize Omp2b and Omp25 at the structural level. Furthermore, possible PG-associated proteins will be studied in *silico* to predict PG binding site(s) in these Omps of *B. abortus*.

## MATERIALS AND METHODS

### Bacterial strains and growth conditions

*Brucella abortus* 544 wild type strain was used for the described experiments. *Brucella* was grown in 2YT rich medium (1 % yeast extract, 1.6 % peptone, 0.5 % NaCl) at 37°C. *Brucella* was spontaneously modified to be resistant to nalidixic acid ($Nal^R$).
*Escherichia coli* DH10b was used for plasmid constructions and *E. coli* S17-1 for conjugation with *B. abortus* 544. *E. coli* S17-1 strain was used for conjugation as it has conjugal transfer functions. Both *E. coli* strains were grown in LB (Luria-Bertani) medium at 37°C. If necessary, antibiotics were added to the medium with the following concentrations: kanamycin (10 µg/ml), chloramphenicol (20 µg/ml) or nalidixic acid (25 µg/ml). To select *B. abortus* clones having excised the plasmid by homologous recombination in the context of gene deletion, the medium was supplemented with 5% of sucrose.

### Plasmids

pNPTS138 (also called pNPTS hereunder), which has a kanamycin resistant marker ($Kan^R$) and sucrose-sensivity marker (*sacB*), was used for the mutant constructions. pNPTS contains an origin of conjugative transfer (oriT) and it cannot replicate in *Brucella*. pBBR1 plasmid with a chloramphenicol resistance marker ($Cm^R$) was used for the complementation of the Δ*omp25* strain. pBBRI ($Cm^R$) with a lac-promoter inducible with IPTG (Isopropyl β-D-1-thiogalactopyranoside) was used as the plasmid containing the rescue copy for the depletion Δ*omp2b* $p_{lac}$-*omp2b* strain.

Plasmid constructions were named as: pNPTS_Δ*omp25* and pNPTS_Δ*omp2b* for the construction of the deletion of *omp25* and *omp2b* in *Brucella* respectively pBBR1_*omp25* for the complementation in Δ*omp25* strain and pBBRI_*omp2b* for the creation of the rescue copy for *omp2b*.

### PCR and cloning

In order to construct the plasmid for the creation of deletion mutants, the fragments up- and downstream of the gene of interest were amplified by polymerase chain reaction (PCR). PCR mix contained Q5 high-fidelity DNA polymerase (100U, 1000bp/30s, Biolabs), Q5 buffer (5x, Biolabs), dNTPs (1.25 mM each), genomic DNA (60 ng/µl), distilled $H_2O$ and forward and reverse primers (20 µM each) to have a final volume of 50 µl. Forward primer from the downstream region contains a flanking sequence corresponding to the complementary terminal fragment of the upstream region.

Amplified fragments from upstream and downstream regions were then joined by joining-PCR. PCR products corresponding to the upstream and downstream regions were hybridized thanks to the flanking sequence from the downstream region (complementary to the upstream region ending).

Then, the resulting fragment was amplified by PCR. PCR mix was unchanged except for primers forward and reverse that were used to amplify the whole deletion fragment (upstream and downstream region joined) and added after 5 cycles of alignment of the upstream and downstream regions.

PCR programs with Q5 polymerase were composed of a first DNA denaturation step of 98°C for 30 seconds followed by 30 amplification cycles. Each cycle was composed of a DNA denaturation step (98°C for 10 seconds), a primer hybridization step (temperature set according to primers Tm (melting temperature), for 30 seconds), and an elongation step at 72°C (duration depending on the length of the PCR product to be amplified). Eventually, after the 30 amplification cycles, a final elongation step was performed at 72°C during 5 minutes. In the particular case of joining PCR, a classical PCR program (without primers added) of 5 cycles was performed to join the PCR products of the upstream and downstream region. Then, primers were added into the mix to amplify the entire product (composed of the joined initial products) for 30 cycles. PCR products were purified with the NucleoSpin or Gel and PCR Clean-up kit (Nucleospin).

The pNPTS138 ($Kan^R$, $sacB^S$) plasmid was used for the cloning of the PCR product containing the upstream and downstream regions. The plasmid and the PCR product were digested the corresponding restriction enzymes (BamHI and XhoI, Thermo Fisher fast digest pack) and ligated. 1 µg of DNA was restricted with the appropriate enzymes (1 U) in fast digest buffer and incubated for 15 min at 37°C. The plasmid and the insert were then ligated with T4 ligase in the T4 ligase buffer (Invitrogen) and incubated either at 18°C overnight (oN) or at room temperature (RT) for 1 h.

To construct the rescue copy for the depletion mutant for *omp2b*, the *omp2b* gene was amplified by PCR and cloned into the pBBRI plasmid using BamHI and XhoI as restriction enzymes. In the case of the complementation strain for the Δ*omp25* strain, *omp25* gene was cloned into pBBR1 using KpnI and BamHI for the restriction sites. Primers used for the construction of the mutants are detailed in **Table S1**.

**Transformation by heat-shock**

Transformation was performed with the competent cells (transformed by $CaCl_2$ method) of *E. coli* DH10b and *E. coli* S17-1 strains (stored at -80°C). 300 ng of the purified plasmid or the ligation approach were mixed with the cells and incubated around 20 min on ice. Then, cells were incubated 1 min at 42°C for the heat shock and 2 min on ice. To recover, LB was added and cells were agitated for 45 min at 37°C. Cells were later plated with the corresponding antibiotics. IPTG (24 µg/ml) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (20 µg/ml) were added if blue-white screening was performed.

Colonies were screened in order to check the presence of the plasmid of interest by using screening PCR. PCR mix included Taq polymerase enzyme ($10^{-4}$U, 1000bp/60s, Promega), GoTaq buffer (5x, Promega), dNTPs (1.25mM each), picked colony, $H_2O$, forward and reverse primers (20 µM each) to have a final volume of 20 µl.

The PCR programme was composed of a first DNA denaturation step (94°C, during 5 minutes), followed by 30 cycles containing an initial denaturation step (94°C, during 30 seconds), followed by a hybridization step (Tm according to primers during 30 seconds), and eventually an elongation step (72°C for a time dependent on the length of the PCR product). The programme was then ended by a final elongation step of 72°C for 5 minutes.

**Construction of mutants by allelic replacement**

In this work, two mutants will be constructed: the deletion strain *B. abortus* 544 Δ*omp25* and the depletion strain *Brucella abortus* Δ*omp2b* $p_{lac}$-*omp2b* strain. Moreover, *Brucella abortus* 544 Δ*omp25* strain will be complemented with a plasmid containing the *omp25* gene (Δ*omp25*-pBBR1_ *omp25,* named Δ*omp25*$^{+}$).

*Conjugation*

Conjugation is an adequate genetic engineering tool to transfer genetic material from one bacterium to another. It has some advantages over the other techniques such as transformation as it has a minimum effect in the disruption of the cell envelope.

Fifty µl of an oN culture of *E. coli* S17-1, containing either pNPTS_Δ*omp25* or pNPTS_Δ*omp2b* plasmids, were mixed with 1 ml of stationary phase of *B. abortus* 544 and then centrifuged at 7000 rpm for 2.5 min. Two washing steps with phosphate-buffered saline (PBS, Biowhittaker®, LONZA) were performed by centrifugations at 7000 rpm for 2.5 min and the pellet was resuspended in 2YT medium. A concentrated mix of both bacteria was plated on a 2YT plate and then incubated oN at 37°C for integrative plasmid. Mix of bacteria was collected and resuspended in PBS. The bacterial suspension was again plated but with the corresponding antibiotic (Kan) and nalidixic acid to kill *E. coli* and incubated for 2-3 days at 37°C. First homologous recombination leading to the integration of the plasmid in *Brucella* was verified by screening PCR with primers binding in the genome and the backbone of the plasmid. Colonies were streaked in 2YT agar plates containing Kan without Nal. Then, selected clones with the pNPTS integrated in the genome were incubated oN without antibiotics.

Diluted oN culture (x5) was plated on plates with 5% sucrose to promote the excision of the plasmid backbone in the genome. *Brucella* clones sensitive to kanamycin but resistant to sucrose were selected as possible mutants. The second recombination to excise the plasmid from the genome was also verified by diagnostic PCR.

For the construction of the depletion Δ*omp2b* $p_{lac}$-*omp2b* mutant, additional steps before the selection with sucrose were performed. *E. coli* S17-1 with the replicative rescue copy (pBBRI_*omp2b*) was mixed with *B. abortus* 544 containing the integrated plasmid pNPTS_Δ*omp2b*. Mix of the bacteria was washed with 2YT medium as explained above and resuspended bacteria were incubated at 37°C during 4 h. Cells were again plated but in 2YT with the corresponding antibiotic (Cm) and nalidixic acid to kill *E. coli* and incubated for 2-3 days. The following steps were performed as described before. For the sucrose selection, medium was supplemented with IPTG to allow a high expression of the gene from the rescue copy.

IPTG is an analogue of the allolactose, which indirectly starts the transcription of the lac operon from the lac promoter by inhibiting the repression operated by the Lac repressor (encoded by lacI, also present on the pBBRI plasmid backbone). IPTG is thus used to induce the expression of genes under the control of the lac promoter.

To construct the complementation strain of Δ*omp25, E. coli* S17-1 containing pBBR1_Δ*omp25* was mixed with *B. abortus 544* Δ*omp25*. Instead of incubating the mix on plate at 37°C oN, bacteria were incubated only 4h at RT, because the complementation plasmid is replicative.

**Growth at the single cell level (TRSE labelling)**

1 ml of oN cultures of *B. abortus* 544 wild type and Δ*omp25* were washed twice with PBS by centrifugation at 7,000 rpm, 2.5 min. The resulting pellets were resuspended in 1 ml of PBS containing TRSE (Texas red goat anti-mouse IgG (H+L), Invitrogen) (1 µg/ml) and incubated for 15 min at RT in the dark. Cells were then washed twice with PBS by centrifugation at 7000 rpm, 2.5 min and incubated in fresh 2YT medium for 2 h. Small portion of the suspensions (2 µl) were kept for microscopy on PBS agarose pads (1% agarose) as time= 0h. After 2h growth, samples were washed two times and 2 µl were used on PBS agarose pads (1% agarose) for the observation with the fluorescence microscope. Images were visualized by microscope Nikon eclipse 80 and analysed by Nis-element, Imaging software (Nikon).

**Growth at the population level (Bioscreen) in different mediums**

200µl of a diluted oN culture of *B. abortus* 544 and *B. abortus* 544 Δ*omp25* with an optical density (OD) of 0.1 were loaded on a 96-well plate. Bacteria were incubated in different media: 2YT, 0.5% 2YT Triton X-100 (VWR chemical), 1% 2YT Triton X-100, 2YT Sodium dodecyl sulfate 0.1% (SDS, VWR chemical) and 2YT polymyxin B (PMB, Sigma) at concentrations from 1µg/ml to 20µg/ml. Bioscreen measured optical density during 72h every 30 min at 37 °C. Bacteria were analysed by technical and biological triplicates.

**Sensitivity tests in plates**

Plates containing different stress agents were prepared as following: 2YT 1% Triton X-100, 2YT 0.1% Sodium dodecyl sulfate (SDS), 2YT 1 mg/ml deoxycholate (DOC) and 2YT 1µg/ml PMB. 2YT plates without any additives were used as control.

Serial dilutions of exponential phase (OD=0.3) culture of *B. abortus* 544, Δ*omp25* and Δ*omp25*$^{+}$ were plated. Experiments were performed with continuing cultures allowed to reach the stationary phase after 5h at 37°C.

**Immunofluorescence (IF)**

50 µl of an exponential phase (OD=0.3) culture of *B. abortus* 544 and Δ*omp25* strains were washed twice with PBS by centrifugation at 7,000 rpm, 2.5 min. The resulting pellets were then resuspended in 50 µl of the corresponding first antibody (A68/04B10/F05, undiluted supernatant from hybridoma culture containing anti-Omp25 antibody) and incubated with agitation for 40 min at RT. Samples were twice washed with PBS by centrifugation at 4000 rpm during 2.5 min at 4°C. The pellets were resuspended carefully in 50 µl of the secondary antibody anti-mouse Alexa fluor ™ 488 goat anti-mouse IgG (H+L) diluted 1:500 in PBS and incubated for 40 min at RT with agitation and protected from light. Samples were washed twice with PBS by centrifugation at 4000 rpm, 2.5 min at 4°C. Final pellets were resuspended in PBS and 2 µl of the solution were used for visualization by fluorescence microscopy on PBS agarose pads (1% agarose). Bacterial cultures were incubated further for 5h to reach stationary phase (OD= 0.9-1.2) and the experiment continued as explained above.

Images were visualized by use of the fluorescence microscope Nikon eclipse 80 and analysed by Nis-element, Imaging software (Nikon) and ImageJ MicrobeJ software.

**Co-labelling of R-LPS and S-LPS by IF**

50 µl of an exponential phase (OD=0.3) culture of *B. abortus* 544 and Δ*omp25* strain were washed twice with PBS by centrifugation at 7000 rpm, 2.5 min. The resulting pellets were resuspended in 50 µl of the corresponding first antibody against R-LPS (A68/3F3/D5, undiluted supernatant from hybridoma culture) and incubated with agitation for 40 min at RT. Then, samples were twice washed by centrifugation at 4000 rpm during 2.5 min at 4°C. The pellets were resuspended in 50 µl of the first antibody against S-LPS (B66/4F9, undiluted supernatant from hybridoma culture) and incubated for 40 min by agitation at RT. Bacterial cultures were washed again two times with PBS.

The pellets were resuspended carefully in 50 µl of the secondary antibodies anti- mouse recognizing specific isotypes from the two first antibodies used (S-LPS: Alexa fluore ™ 594 goat anti-mouse IgG2a (y2a), R-LPS: Alexa fluor ™ 488 goat anti-mouse IgG2b (y2b)) diluted 1:500 in PBS and incubated for 40 min at RT with agitation and protected from light. Samples were washed twice with PBS by centrifugation at 4000 rpm, 2.5 min at 4°C. Final pellets were resuspended in PBS and 2 µl of the solution were used for visualization on fluorescence microscopy on PBS agarose pads (1% agarose). Bacterial cultures were incubated for 5h to reach stationary phase (OD= 0.9-1.2) and the experiment was repeated as explained above.

**Flow cytometry reverse**

250 µl of an exponential phase (OD=0.3) culture of *B. abortus* 544, Δ*omp25* and Δ*omp25*$^{+}$ were fixed with 2% paraformaldehyde (PFA) during 20 minutes at RT. Initial bacterial cultures were incubated for 5h to reach stationary phase (OD= 0.9-1.2) and bacteria were also fixed with PFA. Then, the co-labelling explained above was performed. Secondaries antibodies used were Alexa fluor ™ 647 goat anti-mouse IgG2a (y2a) and Alexa fluor ™ 488 goat anti-mouse IgG2b (y2b) for S-LPS and R-LPS respectively. Afterwards, 300 µl of the flow cytometry buffer were added to the samples and a flow cytometry analysis was carried out with BD FACS reverse machine.

Bacteria only incubated with the secondaries antibodies were used as negative control for each strain. Samples were visualized on fluoresencence microscope to confirm the labelling of the antibodies to the corresponding molecules.

**Infection of RAW264.7 macrophages**

Non-activated RAW264.7 macrophages, cultivated in DMEM (Dulbecco's Modified Eagle's Medium, Thermo Fisher) supplemented with 5% of FCS (Fetal Calf Serum) medium, were seed in a concentration of $1x10^5$ cells/ml in a 24-well plate. Cells were incubated at 37°C, 5% $CO_2$ oN. oN bacterial cultures of *B. abortus* 544 and Δ*omp25* were diluted to reach a multiplicity of infection (MOI) of 50 in DMEM medium. DMEM medium from the cells was discarded and 500 µl medium containing bacteria were added. Plates were centrifuged at 1200 rpm during 10 min at 4°C. After this step, the plates were incubated at 37°C, 5% $CO_2$. After 1h of incubation, cells were washed twice with pre-warmed DMEM. DMEM with gentamycin (50 µg/ml) was added to kill the extracellular bacteria. After 2h post-infection, wells were washed two times with PBS. DMEM with gentamycin (10 µg/ml) was added to the cells incubated until later timepoint PI and plates were incubated for 24h post infection and 48h post infection at 37°C 5% $CO_2$.

At every desired time post infection, cells were washed two times with PBS. 500 µl of Triton X-100 0.1% diluted in PBS was added to lyse the eukaryotic cells and the plates were incubated at 37°C during 10 min. Afterwards, every well was flashed 15 times and the solution was recovered to prepare several dilutions to plate the CFU. 20 µl of every dilutions of interest from the wild type and the mutant strain were plated on 2YT agar plates and incubtaed for 2-3 days at 37°C.

**OD/CFU experiment**

oN cultures of *B. abortus* 544, Δ*omp25* and Δ*omp25*$^+$ were diluted to an OD of 0.1 in 50 ml of 2YT liquid media. Cultures were incubated at 37°C and ODs were measured from 0-9h, 24-33h and 48-57 h every three hours. Dilutions of CFUs for each timepoint were plated and incubated for 2-3 days at 37°C. Technical replicates n=9, biological replicate n=1.

**Immunoprecipitation of Omp2b**

*Extraction of Omps from cell envelope*

Extraction of Omps from *B. abortus* 544 was carried out to prepare the co-immunoprecipitation of Omp2b with R-LPS.

High volume of exponential phase culture of *B. abortus* 544 (100 ml) with an OD of 0.3 was concentrated by performing several centrifugation steps at 7000 rpm, 2.5 min. For subsequent peptidoglycan digestion, cells were mixed with DNAse (1 mg/ml, Roche) and lysozyme (5 mg/ml, Roche) in TES buffer (Tris-HCl 10 mM pH 7.5, EDTA (Ethylenediaminetetraacetic acid) 1 mM, NaCl 100 mM). For the physical breakage of the cells, a Zirconia/Silica beads mix was used (0.1 and 0.5 mm beads 1:1). Bacteria were lysed with cell disruptor (Digital Disruptor Genie, Scientific industries) at maximum amplitude (2800) for 60 min at RT. Two washing steps were performed by centrifugation at 7,000 rpm, 2.5 min and the sample was filtered with a 0.2 µm filter. Samples before and after filration were kept for western blot analysis to test the presence of Omp2b and R-LPS during the extraction.

*Co-localization R-LPS with Omp2b*

The filtered sample from the cell lysis was incubated with the same volume of specific monoclonal antibody against Omp2b (A68/4D11/G11, undiluted supernatants of hybridoma cell culture) and with TES buffer used as negative control during 2h in agitation on a rotator wheel. 20 µl of magnetic beads covalently linked to protein A Sepharose, which can bind to the IgG, were washed four times with PBS-Tween 20 (PBS-T) and then twice with TES buffer. Beads and the samples were mixed and incubated on the rotator wheel for 2 hours. The samples were then washed six times with TES buffer-Tween (1%) and beads were recovered by using magnets. Antibodies were eluted from the beads when SDS loading buffer (20% β-mercaptoethanol, 8% SDS, 0.12M Tris-HCL pH6.8, 40% glyrecol and 0.05 Bromophenol blue) (1x) was added to the samples. The suspension was then incubated 10 min at 70°C. Supernatants containing Omp2b with the possible binding partners were kept and boiled at 95°C during 10 min for SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis).

**Western blot for *Brucella* lysate**

A liquid culture of *Brucella* was concentrated to reach an OD of 10 by centrifugation at 13000 rpm during 5 min. Cells were killed by incubation for 1 h at 80°C. SDS-loading dye was added (1:4) and samples were boiled at 95 °C for 10 min. 20 µl of each sample was loaded on a SDS-PAGE two-parts gels, the running gel and the stacking gel (**Table S2**). The gel was run at 40 mA, 300 V. Transfer of the proteins from the gel to the nitrocellulose membrane was performed with a semi-dried principle (Transblot turbo Bio-Rad). Transfer was run at 14 V, 112 mA during 30 min. Then, blocking of the membrane was carried out with 5% of milk in PBS-T (0.5%) oN at 4°C or 1h at RT. S-LPS, R-LPS, Omp25 and Omp2b proteins were detected by specific monoclonal antibodies (LPS: A68/24D87/G9, Omp25: A68/04B10/F05, Omp2b: A68/15B06/C8 or A68/4D11/G11, undiluted supernatants of hybridoma cell culture) diluted 1:1000 in the 1% milk PBS-T.

Incubation with the primary antibody was done for 1h at RT. Omp10 was used as a loading control if necessary and detected by a specific monoclonal antibody (A68/7G11/C10, 1:100, undiluted supernatant of hybridoma cell culture). Subsequently, the membrane was washed with PBS-T three times for 10 min each and then incubated during 1 h with the secondary antibody anti-mouse IgG (1:5000) conjugated with HRP (Horseradish peroxidase) diluted in 1% milk PBS-T. The membrane was washed again three times with PBS-T. Proteins were visualized by adding a mix of the peroxide solution and luminol/enhancer solution (1:1) to the membrane (Clarity $^{TM}$ ECL Western Substrate, Bio-Rad). Images were revealed with Amersham imager 6000.

**Bioinformatic analysis**

*Omp2b and Omp25 alignments*

Sequences for Omp2b and Omp25 proteins were retrieved from GenBank database and aligned to homologs from the order Rhizobiales, found in a Blastp search on NCBI website. Putative homologs of Omp25 (OmpA) and Omp2b (OmpF) from *E. coli* were added to the multiple alignment (Clustal Omega).

Possible Omp25-like proteins supposed to be present in the *Brucella* genome were identified by delta blastp tool (NCBI).

*Peptidoglycan-associated proteins*

Lipoproteins from *B. abortus* were analysed by multiple alignment (Clustal Omega) with the Pal, MotB and OmpA proteins known to bind to the peptidoglycan in *E. coli* (motif: NX2LSX2RAX2VX3L) (*Koebnik, 1995*). Braun's protein, the only protein recognized as covalently binding protein to the peptidoglycan (*Braun, 1975*), was also aligned with the *Brucella* lipoproteins to find a possible consensus sequence. Omp2b and Omp25 were added then to these analyses to search for a binding site to the peptidoglycan.

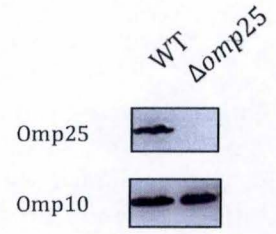



**Figure 9. Omp25 presence in the Δ*omp25* mutant and WT strain.** Omp25 was detected by a specific monoclonal antibody. Omp10 was used as a loading control.

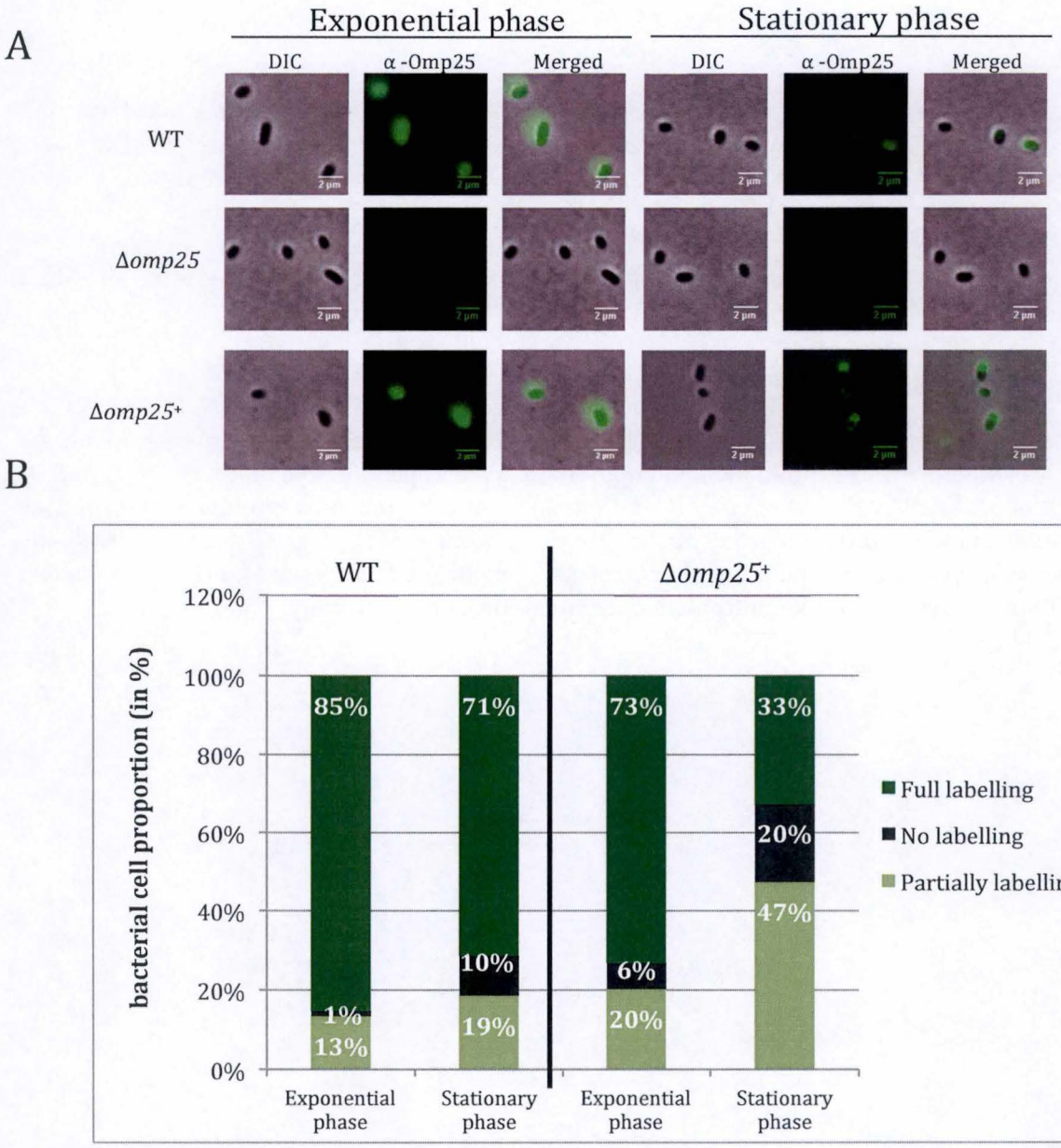



**Figure 10. Omp25 fluorescence in WT, Δ*omp25* and Δ*omp25*$^{+}$.** A) Immunofluorescence was performed in the exponential (OD=0.3) and early stationary phase (OD=0.9-1.2) from the same bacteria culture of the wild type, the mutant and the complementation strains. Presence of Omp25 on the bacterial surface was detected by specific monoclonal antibody and visualized by a fluorescent secondary antibody anti-mouse Alexa488. Scale bar 2µm. B) Wild type and complementation strains microscope images were analysed with MicrobeJ software and defined as fully labelled, partially labelled or not labelled. Total number of bacteria in exponential phase n=881, stationary phase n=1743, in the wild type. Total number of bacteria in exponential phase n=726, stationary phase n=666 in the complementation strain. Total bacteria analysed come from the three experiments performed. Statistical analysis by chi$^2$ test for the homogeinity of bacterial cell proportions in the different strains and conditions were performed (p value $10^{-15}$).

## RESULTS

### Characterization of *B. abortus* Δ*omp25*

#### *B. abortus* Δ*omp25* confirmation

The function of Omp25 is unclear despite its high abundance in the *B. abortus* OM. It was recently shown that the *omp25* gene (BAB1_0722) could be a direct target of the CtrA transcription factor, a cell cycle regulator crucial for cell division in *B. abortus* (*Francis et al., 2017*). It was noticed that when CtrA is depleted, the abundance of Omp25 was decreased and its localization on the bacterial cells is affected (*Francis et al., 2017*). As indicated in the Objectives section, a genetic approach was followed to try to propose new functions for Omp25.

We have created a Δ*omp25* mutant by allelic replacement in the *B. abortus* 544 $Nal^R$ (wild type, WT) strain. The resulting mutant strain does not contain a marker (such as an antibiotic resistance cassette) inserted at the deletion site. We have also created a complementation strain Δ*omp25*-pBBR1_ *omp25* (Δ*omp25*$^+$) with a plasmid carrying the *omp25* coding sequence under the control of a *E. coli lac* promoter ($Cm^R$) in the Δ*omp25* strain.

Monoclonal antibodies have been raised against several outer membrane proteins, including Omp25 (*Cloeckhaert et al., 1996*). Since the expression of the different *omp25*-like genes may differ from one strain to another, it was important to check the specificity of the A68/04B10/F05 monoclonal antibody against Omp25. It was predicted that in the Δ*omp25* strain, antibody binding should be absent if this monoclonal is specific for this Omp25 protein. The A68/04B10/F05 monoclonal antibody was used to detect Omp25 in a crude bacterial cell lysate. Omp10 was used as loading control. Western blot images showed that the band corresponding to the expected size of Omp25 protein (25-kDa) was only present in the wild type (**Figure 9**).

#### Detection of Omp25 on the surface of *B. abortus* 544 and Δ*omp25*

Omp25 detection on the surface of the bacteria was analysed by immunofluorescence. WT and Δ*omp25* bacteria grown until stationary phase (OD=0.9-1.2) were labelled with the same specific antibody directed against Omp25 in order to detect the presence of the protein on the surface of the bacteria. Results revealed that the wild type bacteria were labelled, whereas the bacteria of the mutant strain Δ*omp25* were unlabelled. Interestingly, some wild type bacteria were partially labelled under these experimental conditions. Thus, wild type bacterial cultures were analysed in the exponential (OD=0.3) and early stationary growth phases (OD= 0.9-1.2) to determine if the fluorescence coverage was different between both growth phases. This experiment was additionally performed in the mutant to further confirm the absence of Omp25 and in the complementation strain to verify that changes in the Omp25 labelling were due to the absence of the protein. (**Figure 10A**).

The fluorescence coverage of the wild type and Δ*omp25*$^+$ bacteria was analysed by MicrobeJ, a plug-in of the ImageJ software (*Ducret et al., 2016*). Bacteria were classified in three categories: unlabelled, partially labelled and labelled. Then, the fluorescence coverage was analysed.

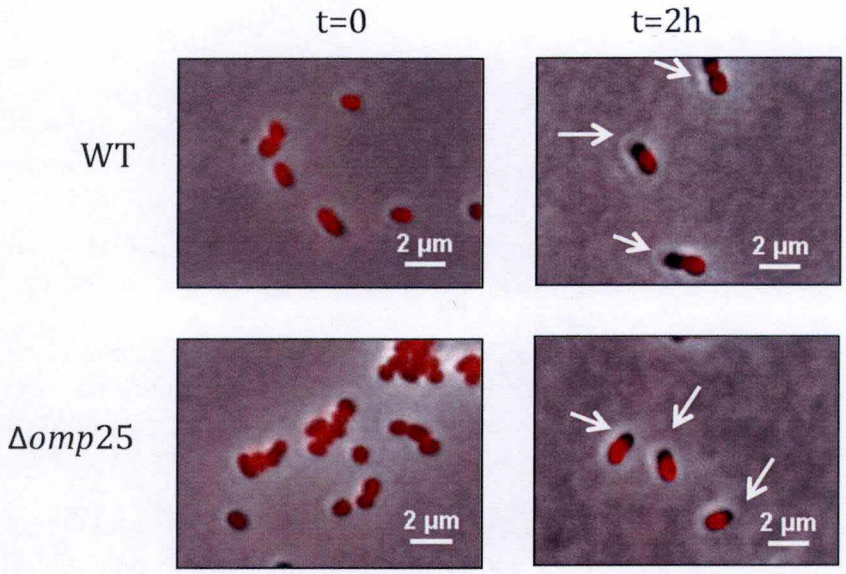



**Figure 11. Growth behaviour of *Δomp25* and wild type strain visualized by TRSE labelling.** Cells were labelled with TRSE, washed and then incubated for 2 h in fresh medium. Arrows indicate the new synthesized material. Scale bar 2 µm.

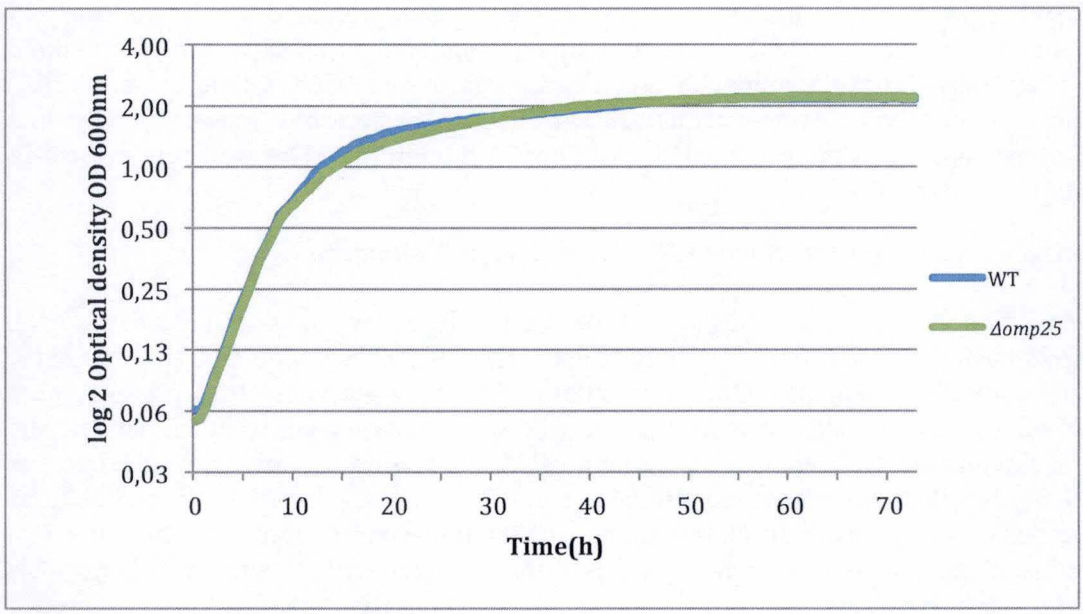



**Figure 12. Growth behaviour of *Δomp25* and WT strains**. Bacterial growth in rich medium was analysed by measuring optical density during 72h every 30 min at 37 °C. n=3.

A threshold of 0.9 of fluorescence coverage was established for the bacteria defined as fully labelled, the bacteria with a fluorescence coverage from 0 to 0.9 being defined as partially labelled bacteria and those with a value of 0 coverage, as unlabelled bacteria.

Results suggested that the proportion of partially labelled wild type bacteria in stationary phase rises up from 13% to 19% when compared to the exponential phase. The difference was more drastic in the case of unlabelled bacteria in which the proportion rises from 1% to 10%. In the case of the complemented bacteria (*Δomp25*$^{+}$), the percentage of unlabelled bacteria increased from 10% in the exponential phase to 20% in the stationary phase. This increase was also observed in the partially labelled bacteria. In the exponential phase, partially bacteria constituted a 19% of the total population whereas the percentage is 47% in the stationary phase (**Figure 10B**). Statistics analyses were performed by a $\chi^2$ test of independence between the proportions of cell types and the strains/culture conditions used (p-value $<10^{-15}$). It is therefore likely that our complementation does not produce enough Omp25 to cover the whole bacterial surface in the stationary phase of culture.

**Study of the growth mode**

*B. abortus* belongs to the order Rhizobiales, which present a specific type of growth, the unipolar growth (*Van der Henst et al., 2013; Brown et al., 2012)*. In order to study if the mutant either exhibits or not the same pattern of growth, TRSE labelling was carried out. This dye binds to the amine groups of the surface of the bacteria. Bacteria were stained with TRSE, washed and then incubated in fresh medium in the absence of the dye. This allows the study of the bacterial growth by the incorporation of new material, which will be unlabelled. In Rhizobiales, the unlabelled material emerges from one pole, named as the new pole. Fluorescent microscopy images showed that the characteristic unipolar growth of *Brucella* was not altered by the deletion of the *omp25* gene (**Figure 11**).

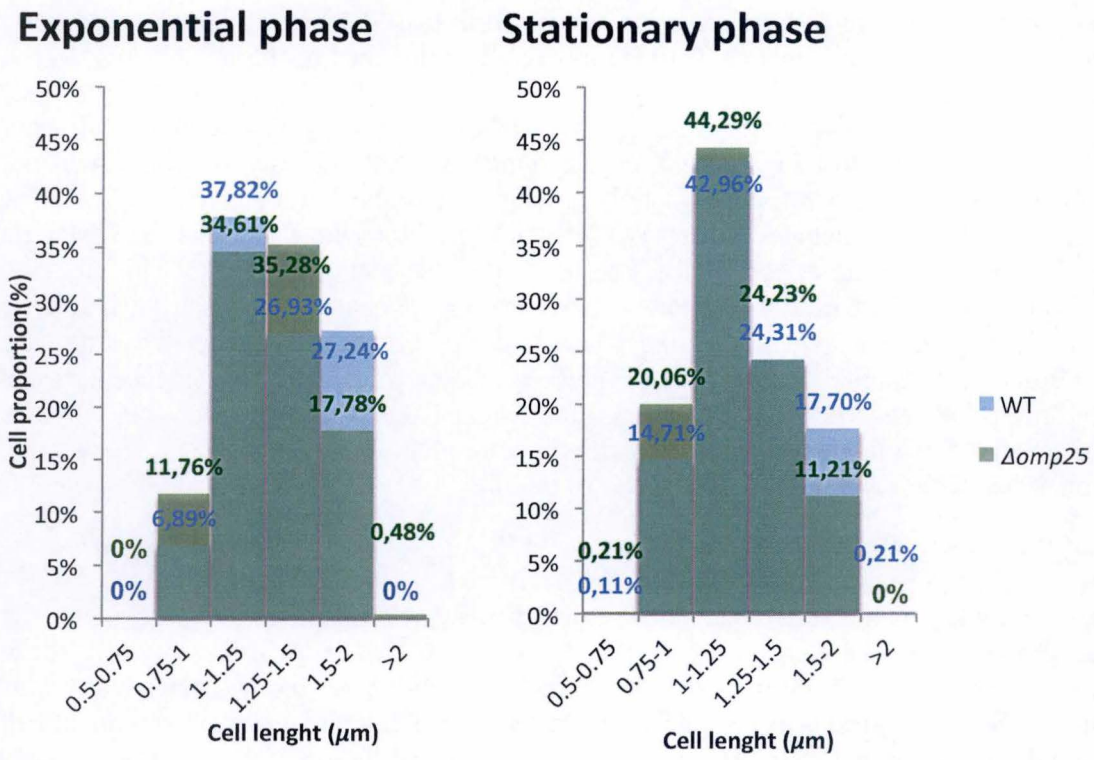
Furthermore, the growth behaviour was studied in rich medium measuring the optical density of the bacterial culture. Growth curves did not show any difference comparing WT and the mutant strains. The doubling time of both strains was also similar ($t_d$ WT= 2.92 h, $t_d$ *Δomp25*=2.86 h) in the exponential phase of culture (**Figure 12**).

**Comparison of bacterial cell length**

Initial microscopic observations suggested that the absence of Omp25 in the *Δomp25* strain could lead to a reduction of size in the bacterial length. Analysed images were recorded in the immunofluorescence experiments performed in the exponential and the stationary phases from the same culture. Images were analysed by the imaging software ImageJ with the plug-in MicrobeJ to measure the length of the wild type, the *Δomp25* and the *Δomp25*$^{+}$ bacteria. Bacterial length was classified in six ranges: 0.5-0.75μm, 0.75-1μm, 1-1.25μm, 1.25-1.5μm, 1.5-2μm and >2μm (**Figure 13**).

Results suggested that there was a higher proportion of the cell population with a smaller cell length in the mutant than in the wild type. This could be observed in both growth phases.

A




B

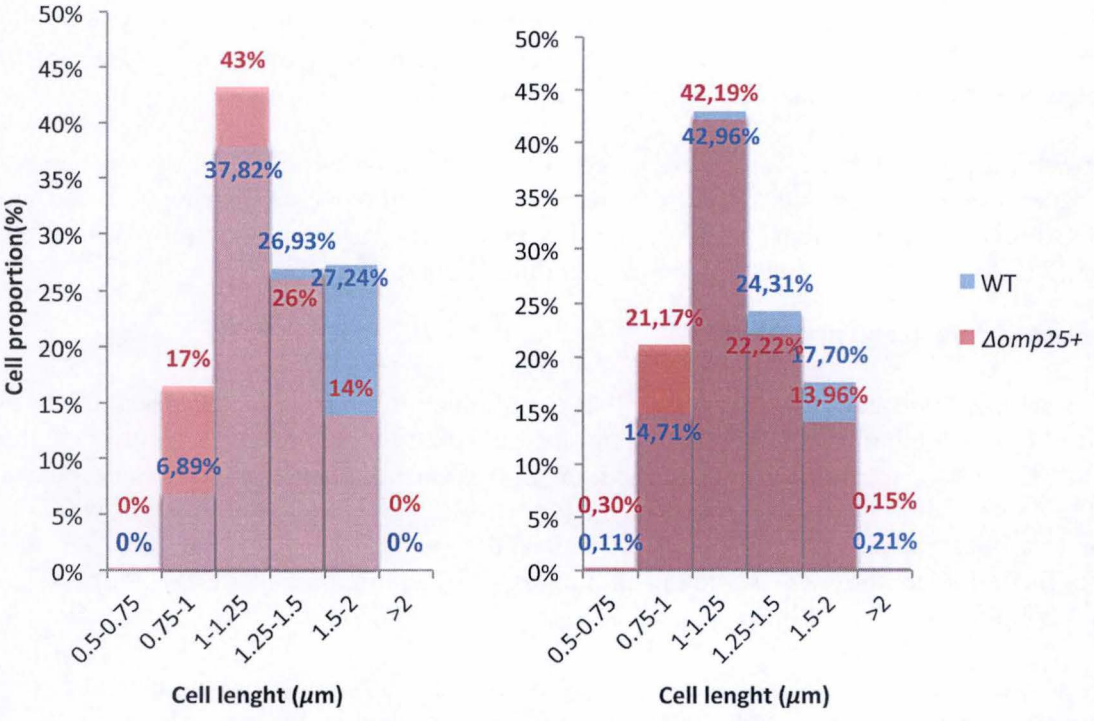



**Figure 13. Cell length analysis in the wild type, *Δomp25* and *Δomp25* $^{+}$.** Bacteria cell length in exponential and stationary phase was measured in the wild type, mutant and complementation strains with the software MicrobeJ. Blue bars correspond to WT strain, green bars to *Δomp25* and pink bars *Δomp25+*. Overlapping colours refer to the proportion of bacteria with the same length in both strains.5Total number of population comes from three different experiments. Total number of wild type bacteria n=973 in exponential phase and n=938 in stationary phase. Total number of bacteria in the mutant strain n=1046 in exponential phase, n= 937 in stationary phase. Total number of bacteria in the complementation strain n=726 in exponential phase, n=666 in stationary phase. Results were analysed by $X^2$ test of independence (p-value< $10^{-15}$).

The proportion of bacteria with a length of 0-1.5μm was higher in the mutant than in the WT, especially in the exponential phase, whereas the WT culture showed a higher proportion of larger bacterial cells (**Figure 13A**). These results were also observed in the complementation strain when cell length was compared to wild type cell length (**Figure 13B**), suggesting that this cell length phenotype was independent of Omp25 or not complemented if the complementation plasmid did not allow a sufficient expression of *omp25*.

**OD/CFU experiment**

Since the Δ*omp25* mutant is forming smaller cells, it is likely that the OD/CFU ratio could be different between the wild type strain and the Δ*omp25* mutant. WT and Δ*omp25* cultures with a starting OD=0.1 were incubated during 57h in rich medium. Every three hours the OD of each strain was measured and cultures at the same time point were also plated to count CFU. Plates were incubated during 4 days at 37°C.

Growth curves did not display any difference between the WT and Δ*omp25* strains in the exponential phase, in the stationary phase, Δ*omp25* OD was decreased (**Figure 14A**). When the CFU for each OD measured was analysed, main differences were observed in the stationary phase (**Figure 14B**). All OD values reaching the stationary phase (OD >0.9) were collected and the number of bacteria per OD was calculated for each strain. Whiskers box were used to represent the dispersion of the data. However the dispersion of the data was really high in the WT and Δ*omp25* strains suggesting there was no difference in the numbers of bacteria/ml between the strains (**Figure 14C**).

**Sensitivity test in *B. abortus* 544 wild type and Δ*omp25***

Omp25 is one of the most abundant outer membrane proteins in *B. abortus* (*Moreno and Moriyón, 2006*) suggesting that Omp25 could also have a role in the membrane integrity. Thus, we studied the sensitivity of the Δ*omp25* strain to the cationic peptide detergent polymyxin B (PMB) and several detergents including sodium dodecyl sulfate (SDS), Triton X-100 and deoxycholate (DOC).

*Brucella* are known to be resistant to polymyxin B (*Martínez de Tejada et al., 1995*). Therefore, first tests were performed with PMB stress in order to determine if Δ*omp25* was more susceptible to this cationic peptide. Bacteria were incubated during 10 min with PMB at several concentrations (29 μg/ml, 117 μg/ml and 468 μg/ml). Then, serial dilutions were plated to count bacterial colonies. The colony forming units (CFU) results in the two highest concentrations of PMB tested (117 and 468 μg/ml) showed a decrease of a 2 log compared to the WT. The mutant displayed the same phenotype when compared to the wild type (**Figure 15**).

Next, other stress agents were used to study the sensitivity of both strains in a long-time exposure to the stress. SDS 1%, 0.5% and 0.1%, Triton X-100 1% and 0.5% and PMB, at different concentrations from 1 μg/ml to 100 μg/ml, were used. Growth profiles were analysed by bioscreen in which bacteria underwent these stress conditions in liquid medium for 72 hours. Both strains did not grow in any of the stress agents at any tested concentration (data not shown) except in the Triton X-100 stress. Under both Triton X-100 stress conditions (1% and 0.5%), already the wild type strain presented a growth defect showed by the fact that it reaches the stationary phase at OD=1.4 whereas in rich medium it can reach an OD of 2.

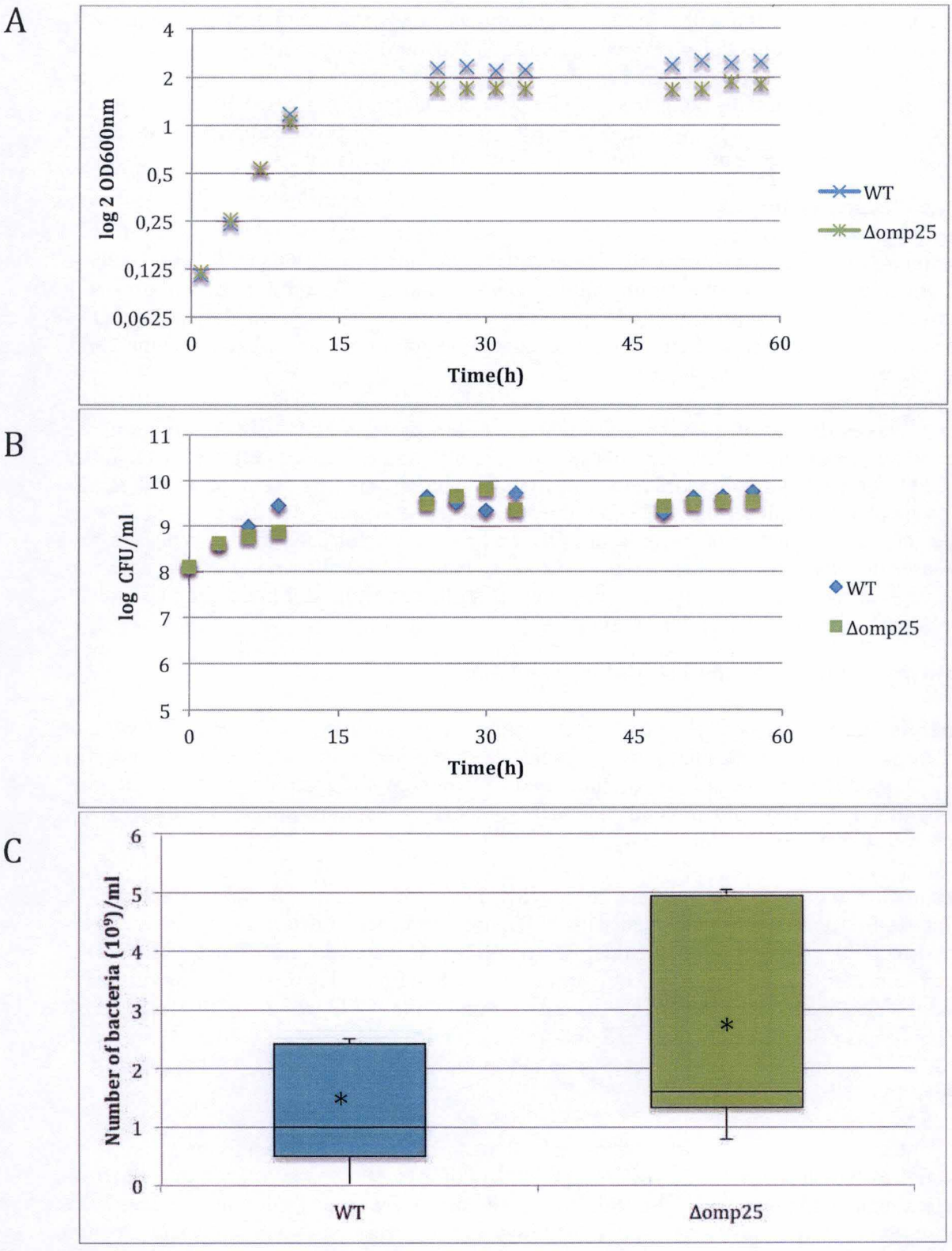



**Figure 14. Relation between OD and CFU in the WT and Δ*omp25*.** Bacteria with an initial OD of 0.1 were incubated in rich medium at 37°C. Every three hours OD was measured and the respective CFU were counted. A) Graph representing the OD at the desired time points. B) CFU were counted at the desired points. C) Whiskers box plot to show the number of bacteria/Od from the CFU when the OD <0.9 (stationary phase). Asterisk (*) represents the mean of the number of bacteria/OD of each strain.

Growth curves also revealed that the wild type displayed a slower grwoth compared to the growth curve in 2YT ($t_d$ WT= 3.91 h instead of 2.92h). When Δ*omp25* was incubated under the same Triton X-100 stress conditions, the growth defect was even higher. Bacteria grew better in the exponential phase ($t_d$ Δ*omp25*=2.36h) than the WT. At stationary phase, bacteria could grow but the mutant did not grow as well as the wild type as shown by a decrease in OD (OD=1). Growth curves showed that the difference in the stationary phase was higher with an increasing Triton X-100 concentration (**Figure 16**).

Different dilutions of the Δ*omp25* and wild type strains were also plated on 2YT agar plates containing specific concentrations of the detergents (100 μg/ml polymyxin B, 2% and 0.1% SDS, 0.1mg/ml DOC, 1% Triton X-100). Bacteria were incubated on plates for 4 days at 37°C. Images of the agar plates showed no difference in the sensitivity to DOC of the Δ*omp25* when compared to the wild type (**Figure 17A**).

However, in the case of the Triton X-100 stress, wild type dilutions were comparable to those in 2YT. In the case of Δ*omp25,* a strong growth defect was observed especially in the three last dilutions ($10^{-3}$- $10^{-5}$) in the mutant being also unable to form WT-like colonies at the $10^{-2}$ dilution. Complementation strain displayed the same profile than the mutant, except for the exponential phase condition, in which a slight complementation was observed (**Figure 17B**). SDS and PMB stress were not possible to analyse because no colonies could grow for the wild type at the tested concentrations (data not shown).

**Infection of RAW 264.7 macrophages in the Δ*omp25* and wild type**

Infection of RAW264.7 macrophages was carried out to examine the intracellular survival of the mutant at different times post infection (PI). Non-activated RAW 264.7 macrophages were infected with the wild type and the Δ*omp25* strains. Bacteria were extracted at 2 h, 5 h, 24 h and 48 h PI and CFUs were counted. No significant difference could be observed at neither of the time points studied under this experimental condition (**Figure 18**).

**R- and S-LPS content in the wild type and Δ*omp25* strains**

*Brucella* species can present either a S-LPS or a R-LPS phenotype (*Cardoso et al., 2006; Caro-Hernandez et al.2007*). Previous data in the host laboratory showed that *B. abortus* possess a heterogeneous OM (PhD project V. Vassen). Indeed, S-LPS is homogenously distributed whereas R-LPS molecules are mostly present in patches.

If Omp25 is missing in the outer membrane, the composition of the membrane could have changed. In order to examine in further detail this hypothesis, co-labelling of the R-LPS and S-LPS was performed on the wild type and the Δ*omp25* strains in exponential phase (OD=0.3) and early stationary phase (OD= 0.9-1.2) following one bacterial culture. Specific antibodies against R-LPS and S-LPS were used and were detected by different secondary antibodies recognizing their specific isotypes of the primary antibodies.

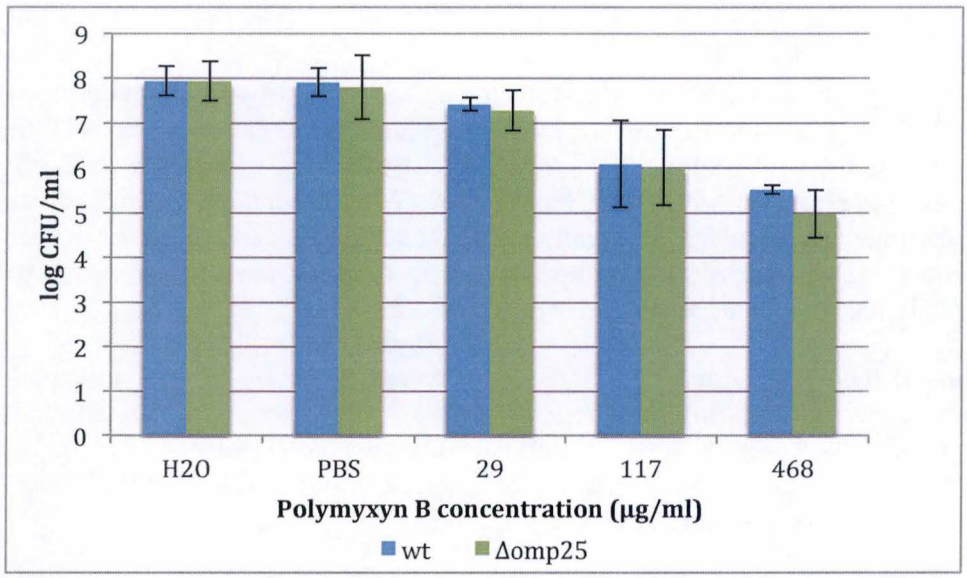



**Figure 15. Sensitivity test Polymyxyn B.** Bacteria in exponential phase were incubated during 10 min at 37°C with different concentrations of Polymyxyn B. The CFU were enumerated after this stress condition. n=3.

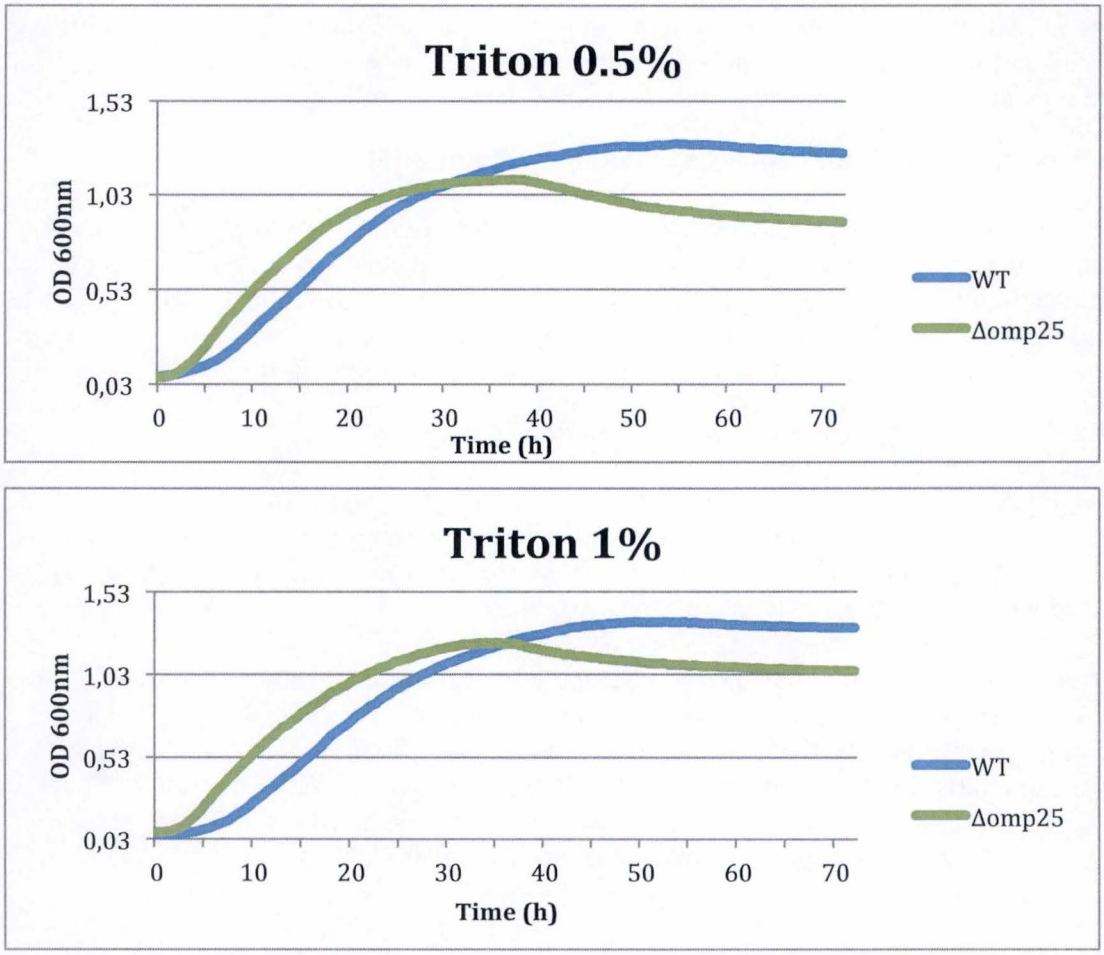



**Figure 16. Growth behaviour of *Δomp25* and WT strains in stress conditions.** Bacterial growth in Triton X-100 1% and 0.5% diluted in 2YT medium was analysed by measuring optical density during 72h every 30 min at 37 °C. n=3.

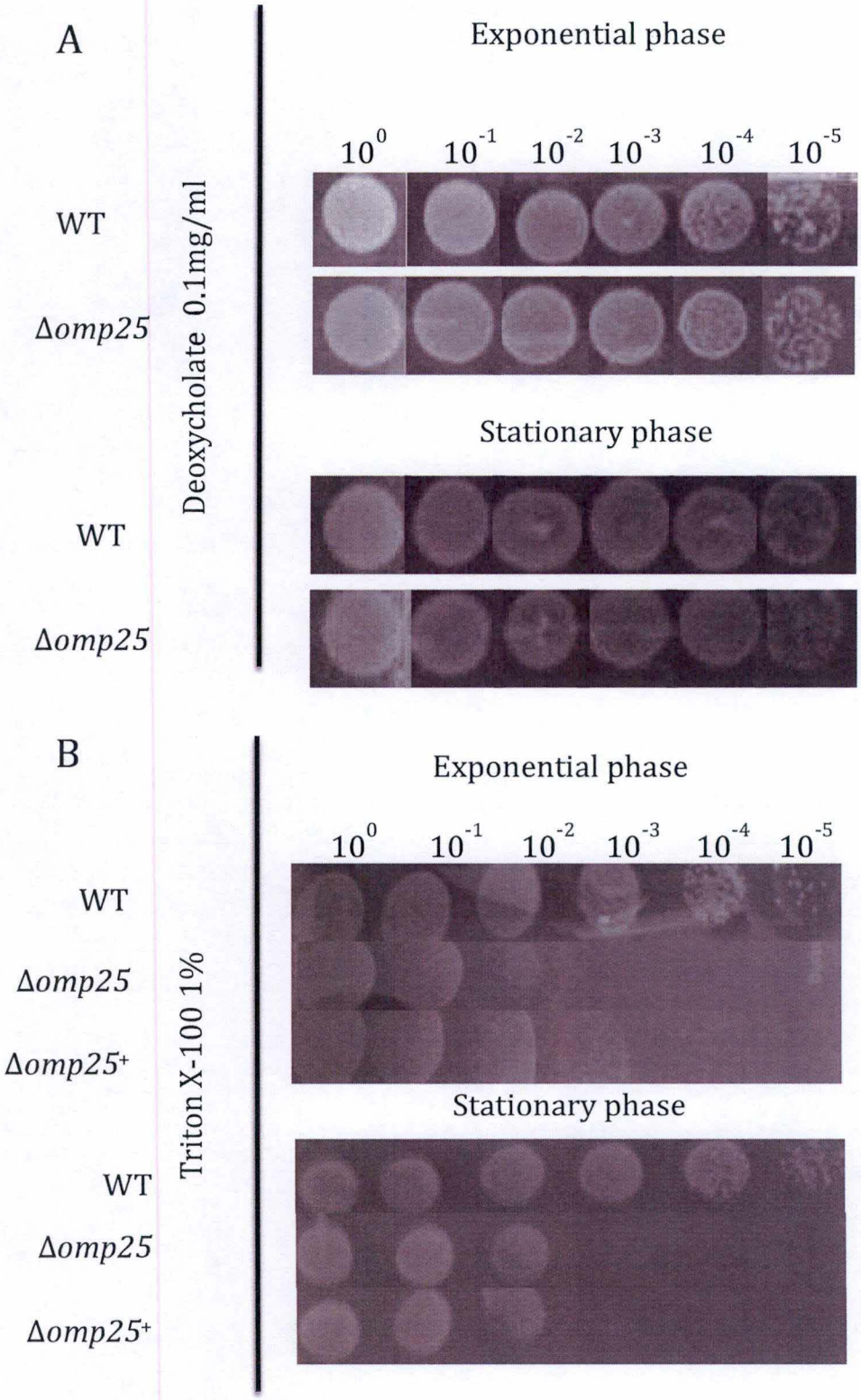



**Figure 17. Sensitivity tests of the wild type and *Δomp25*.** Bacteria in exponential and stationary phases were plated on deoxycholate 0.1 mg/ml (A) or Triton X-100 1% (B) 2YT agar plates at different dilution and incubated at 37°C for 4 days. The *Δomp25*$^+$ corresponds to the complementation strain.

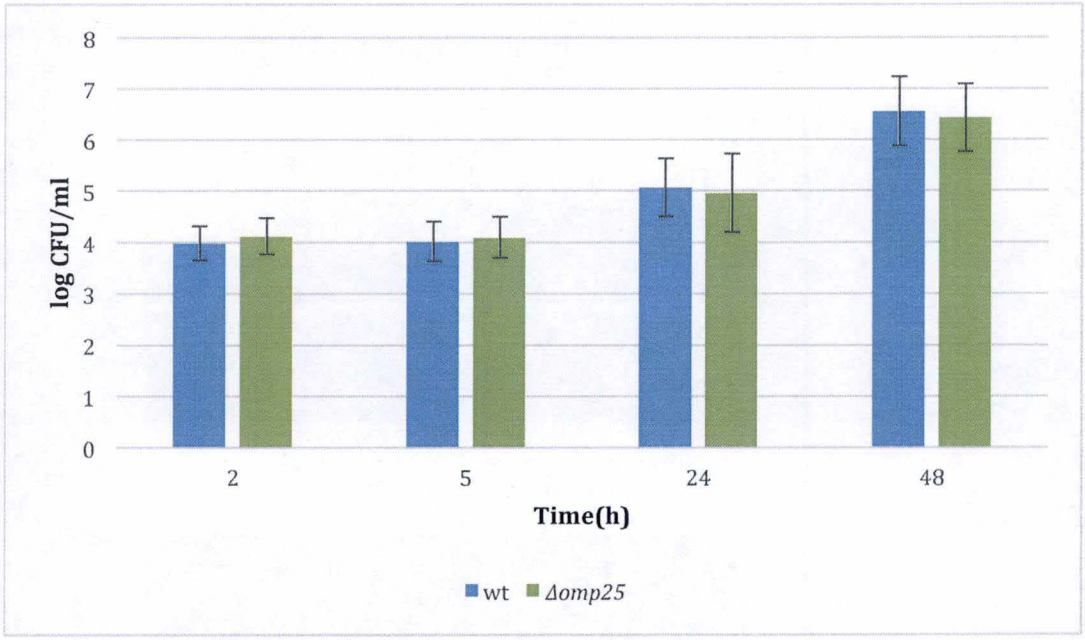



**Figure 18. Infection of RAW 264.7 macrophages with Δ*omp25* and WT strains.** Isolated bacteria were plated for the time points 2h, 5h, 24h and 48h post-infection. The experiment was performed in technical and biological triplicates.

| | | WT | Δomp25 |
|---|---|---|---|
| Exponential phase | R-LPS | 2005.02 | 2709.10 |
| | S-LPS | 2416.16 | 2587.19 |
| Stationary phase | R-LPS | 1801.06 | 2174.50 |
| | S-LPS | 1024.88 | 878.40 |
| | Ratio R-LPS | 1.11 | 1.25 |
| | Ratio S-LPS | 2.36 | 2.95 |

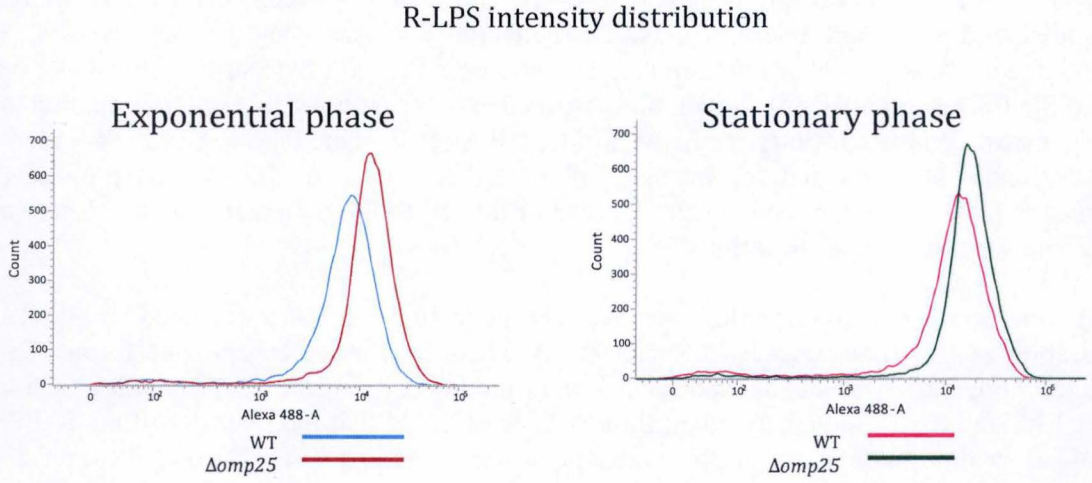
**Figure 19. R-LPS and S-LPS labelling in the wild type and Δ*omp25*.** Bacteria in exponential and stationary phase were labelled with specific antibodies against R-LPS or S-LPS. MicrobeJ software was used to analyse the Mean Fluorescence Intensity (FMI) of the R-LPS and S-LPS. Intensity values for the R-LPS and S-LPS are collected in this Table. Ratio of LPS between the exponential phase and the stationary phase was also calculated (Ratio R-LPs and S-LPS). Total number of bacteria comes from three independent experiments. WT n= 675 and Δ*omp25*, n= 589 in exponential phase, in stationary phase WT n=698, and Δ*omp25*, n= 729.

Mean fluorescence intensity (MFI) of the R-LPS and S-LPS was measured in both strains and in both growth phases by the MicrobeJ software (**Figure 19**). The table represents the MFI values for each LPS version and the ratios of R-LPS and S-LPS between each LPS-version in exponential and stationary phases. In both exponential and stationary phases, the R-LPS fluorescence intensity is higher in the mutant strain than in the wild type strain. This is not the case for S-LPS, which does not show a high difference between the wild type and the mutant in both growth phases. Furthermore, the ratio of R-LPS in exponential phase /R-LPS in stationary phase suggests that the intensity of the R-LPS signal in the stationary phase is decreased only in the mutant context. In the case of the S-LPS, the intensity value is lower in the stationary phase in both the strains.

These results were confirmed by flow cytometry (**Figure 20**). Bacteria were again co-labelled with antibodies specific against R-LPS and S-LPS. Then, flow cytometry was performed and the bacteria population was classified according to the intensity of the $2^{nd}$ antibodies used to label R-LPS and S-LPS. Intensity mean fluorescence revealed that the intensity of the R-LPS was higher in the mutant than in the wild type in exponential phase. The R-LPS intensity values are similar in the stationary phase.

However, in the case of S-LPS, the intensity mean fluorescence is higher in the mutant in both growth phases. Moreover, the intensity of the R-LPS increases in both strains when bacteria reach the stationary phase. On the contrary, the S-LPS intensity fluorescence decreases when they reach the stationary phase (**Figure 20 B**).

Graphs representing the distribution of the intensity mean fluorescence corresponding to R-LPS in the total bacteria population suggested that in both growth phases, the intensity of R-LPS in both growth phases was the same when strains were compared. The proportion of bacteria with the same intensity represented by a narrow peak was higher in the *Δomp25* strain (**Figure 20 A**).




| | | WT | *Δomp25* |
|---|---|---|---|
| **Exponential phase** | **R-LPS** | 8576.78 | 14708.83 |
| | **S-LPS** | 57092.50 | 63560.27 |
| **Stationary phase** | **R-LPS** | 17044.05 | 16751.99 |
| | **S-LPS** | 19253.17 | 31379.83 |
| | **Ratio R-LPS** | **0.50** | **0.88** |
| | **Ratio S-LPS** | **2.97** | **2.03** |

**Figure 20. Intensity mean fluorescence measurement by flow cytometry**. Bacteria in exponential and stationary phase were labelled with specific antibodies against R-LPS and S-LPS. Flow cytometry was then performed to analyse the intensity mean fluorescence of both R-LPS and S-LPS in the two strains of interest. n=2.

**Characterization of the Omp2b protein**

In the Δ*omp2b* mutant context, we wanted to create the depletion mutant of *omp2b* in *B. abortus* 544. However, we did not succeed in the construction of this mutant. One possible reason for this could be that the complementation plasmid did not allow a sufficient level of *omp2b* expression to indeed allow the survival of the bacteria after the *omp2b* deletion.

**Co-immunoprecipitation (co-IP) of Omp2b with R-LPS**

Previous results from our group have shown that Omp2b is partially colocalizing with R-LPS (data not shown). Therefore, to test if Omp2b and R-LPS have an interaction, co-immunoprecipitation (co-IP) of Omp2b was performed to identify possible R-LPS copurification with Omp2b in the pull-down samples. Before IP could be performed, we investigated the presence and the enrichment of different Omps in the supernatant and the pellet obtained after centrifugation of the disrupted lysate. Supernatant of hybridoma cultures containing specific monoclonal antibodies against the most abundant Omps of *B. abortus*, Omp25 and Omp2b, were used. LPS was detected in Western Blot analysis by an antibody that can recognize both R-LPS and S-LPS as it binds to the oligosaccharide core with or without the attached O-chain (*Bowden et al., 1995*). A Western Blot was performed to detect the presence of Omp2b in the supernatant as well as in the pellet. Omp25 was used as a control to detect if other Omps different from Omp2b could be also extracted. **Figure 21A** shows that Omp25 and Omp2b could be detected in the supernatants as well as in the pellets after two centrifugation steps. R-LPS molecules are corresponding to lower molecular weight bands representing the lipid A and the core, whereas S-LPS appears as a smear at higher molecular weight corresponding to the different lengths of the attached O-chains. Bands representing Omp2b, Omp25 and LPS from the supernatant were more intense compared to those from the pellet. Therefore, even if some Omps remained in the pellet, there was enough amount of Omp2b in the supernatant to carry out the co-IP.

In order to perform the co-IP of Omp2b with R-LPS, different specific antibodies against Omp2b were tested to pull down Omp2b. A68/4D11/G11 is an antibody, which recognizes the linear form of Omp2b whereas A68/15B06/C8 better recognizes the external loops of the porin (PhD thesis J-Y. Paquet, 2000). As Omp2b could loose its conformation during the lysis procedure, both antibodies were considered for the co-IP.

After bacterial cells were lysed and centrifuged, the supernatant was incubated with the two different specific monoclonal antibodies against Omp2b. An antibody-antigen complex was formed in order to isolate Omp2b and its ligands when binding the provided antibody to protein A sepharose magnetic beads. Afterwards, the immune precipitated samples were analysed by western blot with the different antibodies against Omp2b and also with an antibody against LPS.

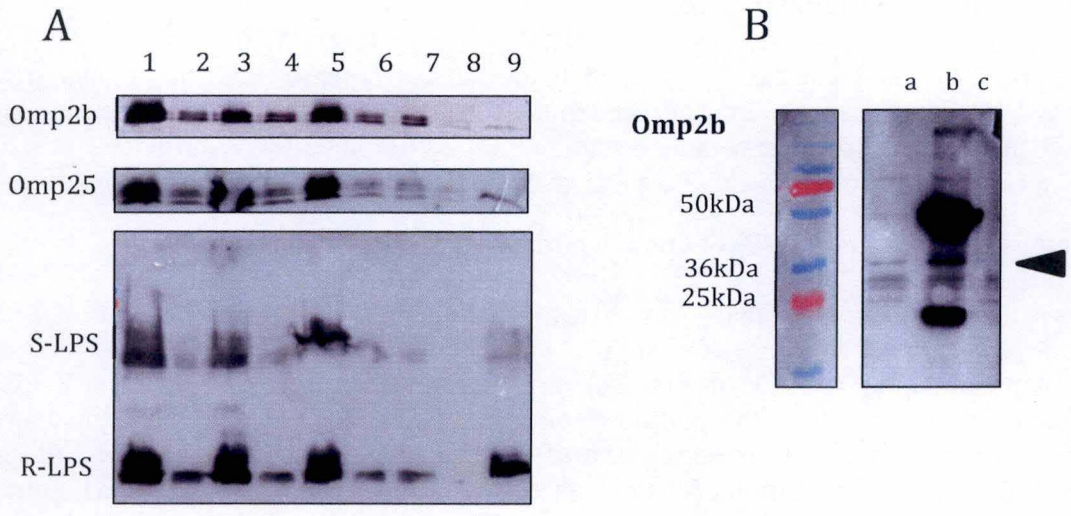



**Figure 21. Co-immunoprecipitation of Omp2b in *B. abortus* 544 wild type strain**. A) Omp extraction by cell lysis. Crude extract refers to not lysed bacteria. Omp2b and Omp25 were detected by specific monoclonal antibodies. R-LPS and S-LPS were detected with an antibody, which can recognize both LPS types. Lane 1: supernatant 1, Lane 2: supernatant 1 (1:100), Lane 3: pellet 1, Lane 4: pellet 1 (1:100), Lane 5: supernatant 2 after 2nd centrifugation of supernatant 1, Lane 6: supernatant 2 (1:100), Lane 7: pellet 2 after 2nd centrifugation of supernatant 1, Lane 8: pellet 2 (1:100), 9: crude cell extract. B) Immunoprecipitation from cell lysates. Lysed samples were incubated with antibodies against Omp2b and mixed with protein A sepharose beads to capture the immune complexes. Two different specific antibodies against Omp2b (A68/15B06/C8 or A68/4D11/G11) were used. Lane a: A68/15B06/C8 antibody Lane b: A68/4D11/G11 antibody. Lane c: TES buffer (negative control).

```
Mesorhizobium_sp_WSM1271_[Mesci_4839]          MEAKLKFARPLAAVFGLLAFAGVANAADVVQEEPPAPAAPMEQPPLNTWSGPYAGVTLGY   60
Rhizobium_miluonense_HAMBI_2971_[GA0061102_108610] MRT---LVTTLMVSAFALGGYTAAYAADAVEQIPQPPVAQEAAPAINNWSGFYIGGEG--   55
Brucella_abortus_544_[BAB1_0722]               MRT---LKSLVIVSAALLPFSATAFAADAIQEQPPVPAPVEVAPQ-YSWAGGYTGLYLGY   56
Phyllobacterium_sp_OV277_[SAMN05443582_10321]  MRT---LKTMILASAAVLTLSAPVLAADIITEQAPAPEAAYQAPQ-ADWSGAYIGAYGGY   56
                                               *.:   :   : .    *     . *** : :    *      *    *:* * *

Mesorhizobium_sp_WSM1271_[Mesci_4839]          GFSGRVKEPGNTVNTDGFIGGGFAGYNYQIDNVVAGVEGDIGYGGLKGDNAGTEVKGGLG  120
Rhizobium_miluonense_HAMBI_2971_[GA0061102_108610] DWNRGHFNRTG--NTYAFGGGAFTGYNWQQGQIVYGVEGDLGYADSETSRNGLTAKNGVN  113
Brucella_abortus_544_[BAB1_0722]               GWNKAKTSTVGSIKPDDWKAGAFAGWNFQQDQIVYGVEGDAGYSWAKKSKDGLEVKQGFE  116
Phyllobacterium_sp_OV277_[SAMN05443582_10321]  GWNKNKTS-AGKLDADGFKGGAYGGYNLQNGQFVYGAEGDIGYSGAKESNFGYRAKQGVE  115
                                               .:.    .  .  .   : .*.: *:* * .:.* *.*** **.  : .. *  .* *.

Mesorhizobium_sp_WSM1271_[Mesci_4839]          GSLRARLGYAVTPDILPYITAGGAAQSVKLTEGGISDKNTMLGWTAGVGADMKVTDNVFA  180
Rhizobium_miluonense_HAMBI_2971_[GA0061102_108610] GSVRGRIGYDFNP-FMLYGTAGLALGQNKLSDSTSSESKTGVGYTVGAGAETFITNNITA  172
Brucella_abortus_544_[BAB1_0722]               GSLRARVGYDLNP-VMPYLTAGIAGSQIKLNNGLDDESKFRVGWTAGAGLEAKLTDNILG  175
Phyllobacterium_sp_OV277_[SAMN05443582_10321]  GSVRARAGIALDP-VLLYGTGGVAIMDSKLSGPLGSESKTHIGWTAGAGAEAKITQNIVG  174
                                               **:*.* *  . * .: * *.* *  . **.    .:.:  :*:*.*.* :  :*:*: .

Mesorhizobium_sp_WSM1271_[Mesci_4839]          RVEYRYTDYGSKEFTTGSG-TRDVDATDHRIQFGVGMKF 218
Rhizobium_miluonense_HAMBI_2971_[GA0061102_108610] RLEYRYTDYGKKTFNLDSG-SFSRGFDEQSVKVGIGVKF 210
Brucella_abortus_544_[BAB1_0722]               RVEYRYTQYGNKNYDLAGT-TVRNKLDTQDIRVGIGYKF 213
Phyllobacterium_sp_OV277_[SAMN05443582_10321]  RVEYRYSDYGKKDYDLGGIDSVSNKITTNEVRVGVGYKF 213
                                               *:****::**.* :   .  :       : ::.*:* **
```

**Figure 22. Conservation of Omp25 protein from *B. abortus* 544 among *Rhizobiales***. Omp25 protein from *B. abortus* 544 was aligned to different similar proteins from specific species from *Mesorhizobium, Rhizobium* and *Phyllobacterium*. Asterisk (*) refers to fully conserved residues, colon (:) indicates conservation between two groups with strongly similar properties and period (.) indicates the two groups with weakly similar properties. The conserved AAD sequence around position 25 corresponds to the end of the signal peptide.

Results showed that Omp2b could be only pulled down from the cell lysate mix when using the antibody A68/4D11/G11 for the IP and could be only detected via western blot with this antibody as a band at 36 kDa. Bands at 25-50 kDa correspond to the denaturated heavy and light chains of the antibody used in the IP. The other antibody (A68/15B06/C8) directed against Omp2b was not able to precipitate a detectable amount of Omp2b. Besides, LPS molecules could not be detected in any of these samples under these experimental conditions, suggesting that some improvements should be done in the experimental procedure (**Figure 21 B**).

In order to make the Omp2b more accessible to the antibody during the procedure, DNAse was added during the cell lysis to reduce the viscosity of the sample due to the DNA extraction. Moreover, the samples were filtered with a 0.2 μm filter in order to keep only the lysed bacteria. In this case, only the A68/4D11/G11 antibody was used for the pull down of the Omp2b as shown in the previous results, because its interaction with Omp2b during the former co-IP. Unfortunately, Omp2b was not pulled down after these new improvements (data not shown).

**Conservation of Omp25 and Omp2b among Rhizobiales**

Omp25 and Omp2b protein sequences were obtained from GenBank database. Both proteins were aligned against the proteome of some representative groups of the order Rhizobiales by a multiple alignment obtained with the Clustal Omega program (**Figures 22** and **23**).

Omp25 (BAB1_0722) was aligned against some phylogenetically related bacteria from the group of Rhizobiales, chosen by their high similarity when E-values from blastp were computed. **Figure 22** shows that Omp25 seems to be conserved among the Rhizobiales as similarity of any protein with Omp25 was around 45-50%. OmpA, considered as the protein homologous to Omp25, in *E. coli* presented a low similarity with Omp25 and a very different length. Therefore, it was not included in the alignment.

In the case of Omp2b (BAB1_0660), alignments showed conserved regions with homologs in all the chosen Rhizobiales. *Ochrobactrum* (55% of similarity with Omp2b) and *Mesorhizobium* (54%) presented more apparent amino acid homology than the other groups as observed in **Figure 23**.

**Omp25-like proteins**

Omp25 is one of the most abundant outer membrane proteins expressed in *Brucella spp.* (*Cloeckaert et al., 2002*). Study from *Salhi et al., 2003* suggested the existence of other Omp25-like proteins in *B. melitensis*.

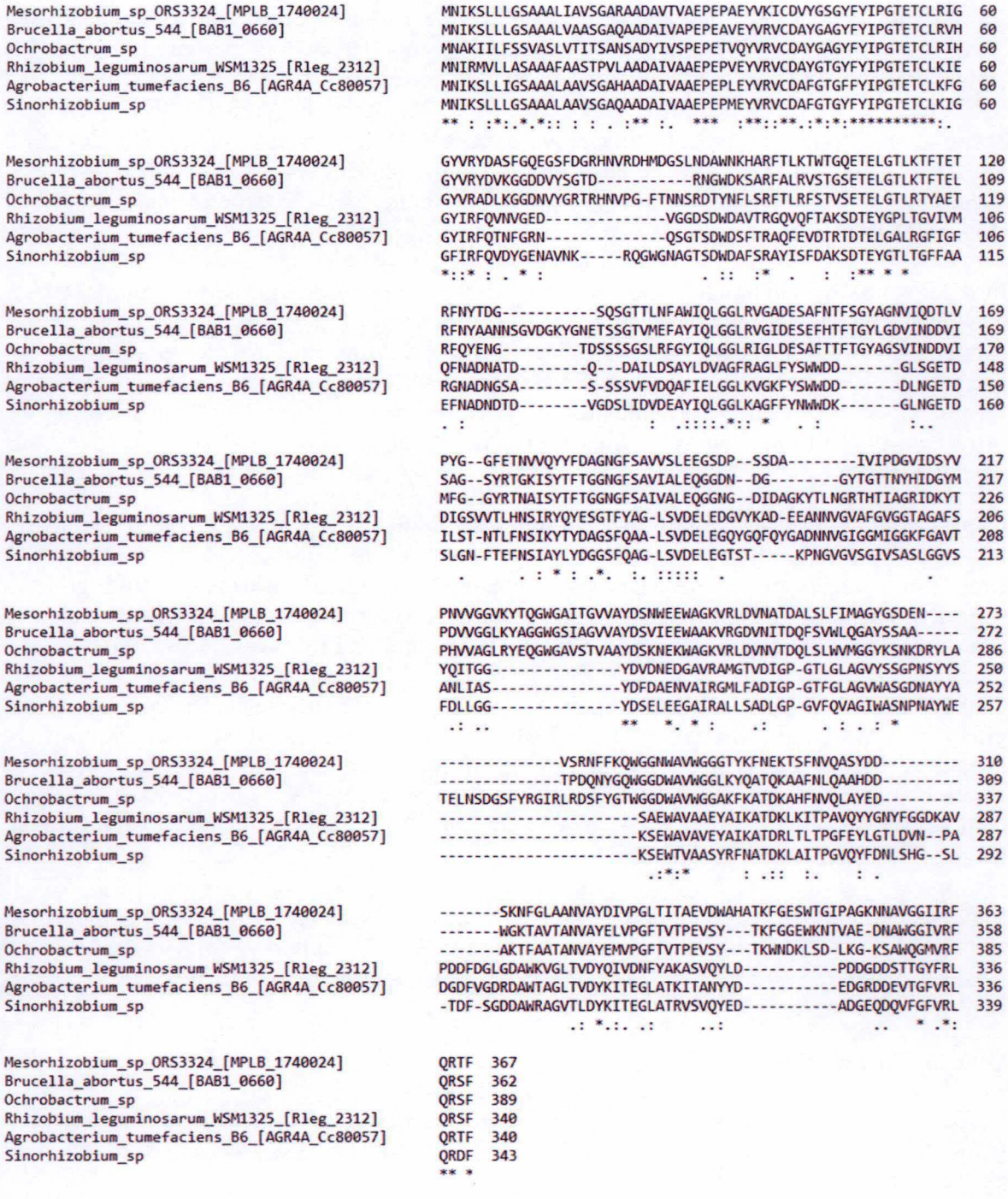



**Figure 23. Conservation of Omp2b protein from *B. abortus* 544 among *Rhizobiales*.** Omp2b protein from *B. abortus* 544 was aligned to different similar proteins from specific species from *Mesorhizobium, Ochrobactrum, Rhizobium, Agrobacterium* and *Sinorhizobium.* Asterisk (*) refers to fully conserved residues, colon (:) indicates conservation between two groups with strongly similar properties and period (.) indicates the two groups with weakly similar properties.

The corresponding Omp25-like proteins present in *B. melitensis* are named as Omp25b (BME1_1007), Omp25c (BME1_1830) and Omp25d (BME1_ 1829). Omp25 protein was aligned with the entire genome of *B. abortus* 544 and two possible Omp25-like proteins were found, BAB1_0115 and BAB1_0116. When the possible Omp25-like proteins were aligned with the Omp25 b-c-d from *B. melitensis*, Omp25c (BAB1_0116) and Omp25d (BAB1_0115) could be identified as the respective homologous in *B. melitensis* (**Figure 24 A**). The Omp25b corresponding homologous could not be found in *B. abortus* genome.

Moreover, Omp25 and the other Omp25-like proteins in *B. abortus* 544 were aligned to determine the similarity among them. Results showed that both Omp25-like proteins share an identity of 39% with Omp25. The similarity between them is only 59% (**Figure 24 B**).

**Search for peptidoglycan-binding site(s) in Omps**

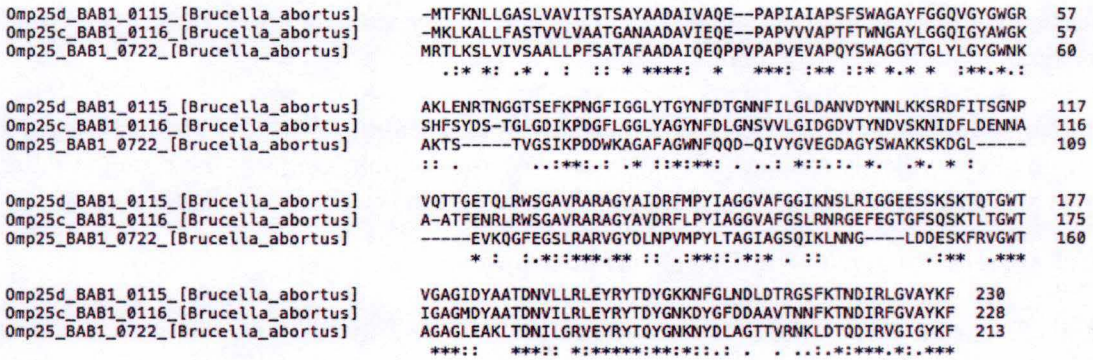
Braun's lipoprotein of *E. coli*, also known as Lpp, is the only known protein from the OM, which is covalently bound to the peptidoglycan. The carbon ε from the C' terminal Lysine can bind to the murein and this Lysine is then covalently bound to the diaminopimelate residue in the peptide chain (*Braun, 1975*).

Omp25 and Omp2b are Omps proposed to bind to the peptidoglycan (*Cloeckaert et al., 1992*). However, when the Omp25 and Omp2b protein sequences were analysed, no Lysine was found in the C' terminal region.

Peptidoglycan associated lipoproteins (PALs) can also interact with the peptidoglycan but they are not covalently linked (*Braun, 1975; Cascales and Lloubès, 2003*). *Koebnik (1995)* predicted a motif present in the α-helix structure from the C' terminal region in OmpA from *E. coli*, which was supposed to interact with the peptidoglycan. When other lipoproteins were aligned, they exhibit the same proposed motif ($NX_2LSX_2RAX_2VX_3L$) including also Omp16 from *B. abortus* (*Koebnik, 1995*).

Therefore, the PAL motif could be alternatively considered to be the possible PG binding site in Omp2b and Omp25. The proteins known so far for binding to peptidoglycan in *E. coli* (OmpA, MotB, Pal) were aligned with their respective homologs in *B. abortus* 544 (*Koebnik, 1995*). The proposed motif was found in MotB and Omp16 from *B. abortus* (**Figure 25**). When the lipoproteins Omp10 and Omp19 from *Brucella* were analysed, the proposed motif could not be found anymore. Finally, Omp25 and Omp2b were added to the alignment and they did not show any related sequence to the proposed PG binding site of the PALs.

A




B

| Omp25-like proteins | *B. abortus* | *B. melitensis* |
|---|---|---|
| Omp25-b | - | BME1_1007 |
| Omp25-c | BAB1_0116 | BME1_1830 |
| Omp25-d | BAB1_0115 | BME1_1829 |

**Figure 24. Omp25-like proteins alignment in *B. abortus* 544.** Sequences of Omp25c and d from *B. melitensis* were identified as Omp25-like proteins in B. *abortus* and then they were aligned to Omp25 to study the similarities among them.

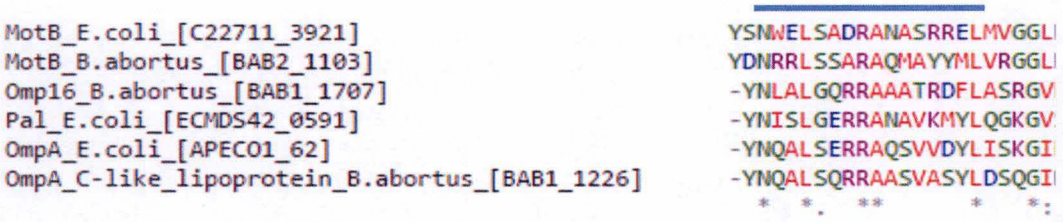



**Figure 25. Peptidoglycan associated proteins in *B. abortus* 544**. Proteins known to bind peptidoglycan in *E. coli* in the motif ($NX_2LSX_2RAX_2VX_3L$) were aligned to their homologous in *B. abortus*.

## DISCUSSION AND PERSPECTIVES

### 1. Characterization of the *B. abortus* Δ*omp25* mutant

Omp25 is a major Omp exposed to the surface of the *B. abortus*. However, its function is not well described. Omp25 is a non-essential Omp. We generated the Δ*omp25* deletion mutant in the *B. abortus* 544 wild type strain. Immunofluorescence microscopy experiment and western blot obviously confirmed the absence of Omp25 in the Δ*omp25* strain (**Figure 9** and **10A**) but also confirm the specificity of the A68/04B10/F05 monoclonal antibody for the Omp25 isoform produced from the deleted coding sequence (BAB1_0722). We could not exclude that the A68/04B10/F05 antibody would recognize several Omp25 isoforms expressed at the same time in the tested conditions. It is still posible that the A68/04B10/F05 antibody is able to recognize other Omp25 isoforms that are not produced in the conditions tested here.

After checking that unipolar growth still takes place in the Δ*omp25* mutant (**Figure 11**), we showed that growth kinetics in rich medium is not strongly altered (**Figure 12** and **14A**). However, when cell size was analysed, the Δ*omp25* strain was significantly smaller in size in comparison to the wild type (**Figure 13**). However, the complementation strain displayed the same cell sizes than the mutant. The small size if the bacteria could be caused by a point mutation somewhere else in the genome. It is also possible that the occurrence of such a mutantion is enhanced by the absence of the Omp25 protein, by a genetic suppression effect. The absence of complementation could also be caused by the low expression of the *omp25* gene in the Δ*omp25*$^{+}$ strain, which would not complement all the phenotypes present in the mutant. Indeed, in our complementation plasmid, the *omp25* gene is under the control of the *E. coli* $p_{lac}$ promoter, which could not be strong enough to produce a sufficient amount of Omp25, a very abundant Omp, to complement the phenotype(s) of the Δ*omp25* strain. A western blot against Omp25 in the WT, Δ*omp25* and Δ*omp25*$^{+}$ strains would be interesting to have a first indication about this matter.

Rod-shaped bacteria such as *E. coli* initiate their cell cycle once they reach a critical size (*Taheri-araghi et al., 2015; Westfall and Levin, 2017*). In fact, they add a constant volume (Δ) in each cell cycle before they divide regardless their initial size (*Amir, 2014; Westfall and Levin, 2017*). Moreover, growth rate also controls the bacteria size. Bacteria cultured under rich conditions are significantly larger and grow faster than those cultured under poor-nutrient conditions (*Schaechter, Maaloe, Kjeldgaard, 1958*). In order to coordinate division with growth, bacteria can adjust the Δ to maintain the cell mean size (*Deforet, Van Ditmarsch, Xavier, 2015; Taheri-Araghi et al., 2015*). Our data suggest that growth rate of our mutant strain is not affected compared to the wild type strain (**Figure 12**), a likely hypothesis to explain the smaller size of the Δ*omp25* mutant would be that its divides earlier than the wild type, probably because they reach sooner the critical volume to divide. How *B. abortus* measures this critical volume is of course unknown, but our data suggest that maybe envelope properties could be involved in this phenomenon. Nevertheless, the first step in the future research would be to obtain a satisfactory complementation strain. To test if Δ*omp25* divides earlier than the WT, it could be interesting to analyse the growth in real time. WT and Δ*omp25* bacteria growth could be analysed by time-lapse microscopy to estimate the time between two divisions, using phase contrast microscopy with bacteria cultivated on an agarose pad.

The two strains could even be analysed on the same pad if they are diffently labelled (e.g. mCherry for one, GFP (Green fluorescent protein) for the other).

OmpA from *E. coli* is considered as the counterpart of Omp25 in *Brucella* (*Verstreate et al., 1982*). Although they are different in length and size, Omp25 is similar, regarding the secondary structure, to OmpA (*Baldermann, et al., 1998*). Despite the absence of similarities in the amino acid sequence between both proteins, Omp25 could display the same role than OmpA, which is involved in the membrane stability (*Wang, 2000*). We studied the susceptibility of the mutant when it was exposed to different stress conditions. Indeed, mutants of Δ*ompA* in *Klebsiella pneumoniae* and *Acinetobacter baumannii* are more susceptible to cationic peptides than the wild type (*Lin et al., 2015; Llobet et al., 2008*).

In order to examine this membrane instability, several stress agents were tested. *Brucella* appears to be more resistant to cationic peptides like polymyxin B, than other Gram-negative bacteria due to their hydrophobic surface and LPS core structure (*Martínez de Tejada et al., 1995*; *Jensen and Halling, 2010*). *B. abortus* sensitivity to polymyxin B was therefore studied but we did not detect a difference between the WT and *omp25* mutant strains (**Figure 15**). Under Triton X-100 stress conditions, wild type growth curve seemed to be altered under both concentrations tested (0.5% and 1%) since bacteria doubling time was increased ($t_d$= 3.91h instead of 2.92h in rich medium). Detergents are used for cell lysis as they disrupt the hydrophobic-hydrophilic interactions of the molecules present in the membrane. Therefore, the membrane of the wild type bacteria could be altered, impacting doubling time. Δ*omp25* growth curve was also altered during Triton X-100, the $t_d$ was decreased to 2.35h, bacteria grew better during the expentenial growth phase, but the OD decreased (**Figure 16**). If Omp25 is missing in the cell membrane of bacteria, the stability of the OM could be altered. Thus, under Triton X-100 stressful conditions, bacteria would not be able to face the stress in the same way than the wild type. It should be interesting to study the stress in higher concentrations of Triton X-100 to determine at what level Triton X-100 is altering the membrane and whether this alteration is related to the growth phase. We could also study the permeability of the OM with other different test such as the susceptibility to the hydrophobic antibiotics (macrolides, rifampicins) or larger antibiotics such as Vancomycin.

This susceptibility to Triton X-100 in the mutant was also confirmed in an agar plate assay (**Figure 17B**). Bacteria were plated in 2YT agar containing Triton X-100 1% and the Δ*omp25* mutant displayed a growth defect that was partially complemented as Δ*omp25*+ bacteria were more able to grow in the exponential phase. Partial complementation may be explained by a lower abundance of Omp25 in the complementation strain compared to the wild type strain. In order to better describe the sensitivity of the bacterial envelope of the omp25 mutant, other stress conditions such as temperature, pressure, nutrient availability, pH and other different agents altering the membrane could be also tested, and a new complementation strain should be constructed (e. g. by placing the *omp25* coding sequence under the control of its own promoter). Finally, it should be noted that bacteria are usually extracted with Triton X-100 after cellular infections. The sensitivity of the mutant to Triton X-100 should therefore be taken into account when interpreting these infection data.

Infection of non-activated RAW 264.7 macrophages was performed in order to study the attenuation of the Δ*omp25* mutant, since instability of the OM could lead to a decreased survival in the BCVs, like in the *bvrR* mutant (*Manterola et al., 2005*). Our data did not show any significant difference at any time point post infection (**Figure 18**), suggesting that this deleted *omp25* gene did not have a role in the virulence of the bacteria. This could be caused by the lack of agression by the macrophages. Infection in previously activated macrophages should then be carried out to better investigate Omp25 function in this context.

We wondered if the absence of Omp25 could alter general OM propoperties like the heterogenous (patchy) distribution of R-LPS (PhD project V. Vassen). Immunofluorescence (IF) analysis and flow cytometry were performed to determine the mean fluorescence intensity of the R-LPS and S-LPS in the wild type and Δ*omp25* strains at different growth phases (**Figures 19** and **20**). This will correspond to the presence of these molecules on the surface since they are labelled by IF. Surprisingly, Δ*omp25* displayed more R-LPS fluorescence in the exponential phase (**Figure 19** and **20**). One interpretation could be that the removal of Omp25 would lead to an increase in the incorporation of R-LPS. However, S-LPS fluorescence also seemed to be increased in the flow cytometry experiment. Since it is unlikely that the *omp25* mutant incorporates more R-LPS and S-LPS than the WT, the higher MFI for R-LPS would be explained by the fact that R-LPS molecules are now more accessible to the antibodies. Omp25 secondary structure exhibit long external loops, therefore it is possible that Omp25 could be surrounded by R-LPS molecules which would be covered by these loops and thus no more accessible to the antibodies in the WT context.

Both wild type and Δ*omp25* presented a decrease in the content of S-LPS in the stationary phase as shown by the diminution of the mean intensity fluorescence between exponential and stationary phase (**Figure 19** and **20**). In smooth species, rough bacteria can emerge and accumulate in culture over time (*Mancilla, 2016*). We did not observe this in the culture conditions tested here.

Surprisingly, we also observed that Omp25 presence was variable in the wild type according to the growth phase (**Figure 10**). In exponential phase, all wild type bacteria were labelled with the antibody against Omp25, as expected. However, some bacteria were unlabelled when bacteria reached the stationary phase. Omp25 antibody used was proved to be specific against Omp25, as the Δ*omp25* strain did not show any labelling in any of the growth phases. Results showed that the percentage of unlabelled bacteria in the WT rises from 1% to 10% in the stationary phase. Partially labelled bacteria (bacteria with a fluorescence coverage from 10% to 90% of the whole bacterial surface) also increase from 13 to 19%. The complementation strain did show the same pattern as the wild type but with higher percentage values for unlabelled and partially laballed bacteria, particularly at the stationary phase. This could be due to the expression of the *omp25* gene in the plasmid that could be lower compared to the WT strain, as discussed earlier in this section.

IF results suggest that the expression of *omp25* is varying over time. Indeed, if Omp25 is immobile in the OM, it is likely that it stays at the place of its incorporation during growth of the envelope; thus a partial labelling could suggest that the expression level of *omp25* (and thus the adundance of Omp25 incorporation) is varying with time. CtrA is a cell regulator exclusively found in Alphaproteobacteria (*Brilli et al., 2010*), and it was recently shown that in *B. abortus*, CtrA binds many promoters involved in cell envelope composition including genes involved in LPS biogenesis and export, but also genes involved in OM composition (Omp2b, Omp25) and the Omp incorporation (BamA) (*Francis et al., 2017*). Omp25 expression could be partially controlled by this transcription factor. In *Caulobacter crescentus*, CtrA levels oscillate over cell cycle thus, if this oscillation is also conserved along *B. abortus* cell cycle, the expression of *omp25* could also be modulated according to the CtrA levels. The presence of CtrA is required for the production of normal amounts of Omp25 (*Pini et al., 2015; Francis et al., 2017*).

The transition from the exponential phase to stationary phase is mainly accompanied by morphological and physiological changes in bacteria, which results in a non-division state (*Hirsch and Elliott, 2005*). Therefore, CtrA, as a major regulator of cell division, should be less abundant or less active in stationary phase bacteria and therefore it could alter the production of Omp25. In order to examine this hypothesis, expression analysis such as RT-qPCR should be performed to determine at what level *omp25* expression depends on the growth phase.

The removal of an Omp could trigger a compensatory effect in which proteins of the same group could be expressed to take the place of the missing protein. This compensatory effect was observed in the genus *Bartonella* in the Omps considered as the homologues of the group 3 in *Brucella* (*Minnick et al., 2003*). Moreover, experiments from *Caro-Hernández et al., 2007* showed that Δ*omp31* presented a higher expression of Omp25 in *B. ovis*. *B. abortus* is lacking the outer membrane protein Omp31b, but it displays other Omp25-like proteins. These Omp25-like Omps could be expressed in the Δ*omp25* mutant to take the function of the Omp25 but with a different efficiency, as the sensitivity test suggested an alteration of the outer membrane properties of the Δ*omp25* compared to the wild type. Expression analysis such as proteomics or RNA-sequencing should be then carried out to determine the potential abundance of the other Omp25 proteins or expression of Omp genes in the mutant.

**2. Attempts to characterize Omp2b**

We also wanted to characterize another major outer membrane protein, Omp2b. This protein is associated with porin activity (*Douglas et al., 1984*). Since Omp2b is essential in *B. abortus* (*Laloux et al., 2010* and Tn-seq data), we planned to create a depletion strain in the wild type background. However, it was not possible to obtain it. This could be due to the promoter used in the rescue copy containing the *omp2b* gene. In the pBBRI plasmid, *omp2b* expression is under the control of the *E. coli* $p_{lac}$ promoter. Therefore, the expression of the *omp2b* gene could not be strong enough to complement the deletion of the *omp2b* gene from the genome of *B. abortus* 544. We could use then another regulatory promoter or the endegeneous promoter of *omp2b*.

Previous data from the host laboratory (data not shown) revealed that Omp2b is colocalized with R-LPS. OmpF, considered as the counterpart of Omp2b from *E. coli*, was shown to interact with LPS in a heterogenous system, a R-LPS, S-LPS mixed system. Indeed, the different forms of LPS display different conformations. In *E. coli*, the lipid A consists of the rigid part whereas the O-chain is more flexible. In heterogeneous systems, the adjacent O-chains do not form a highly condensed pack as occurs in homogeneous groups of S-LPS. Thus, in these heterogeneous systems, the flexibility of the O-chains does not compromise the opening of the porin by shielding the pore (*Patel et al., 2016*). Omp2b is also present in a heterogeneous system and strongly colocalized with R-LPS (V. Vassen, PhD). A biochemical approach, the co-immunoprecipitation, was then attempted to detect the potential interaction between Omp2b and R-LPS.

Before the co-IP was performed, a previous test was carried out to show the extraction of Omps from the membrane of the *B. abortus* wild type. Omp2b was successfully extracted as well as LPS (**Figure 21**). After that, Omp2b was pulled down from the sample containing all the cell fragments. Two antibodies were considered for the immunoprecipitation. A68/4D11/G11 is an antibody, which recognizes the linear form of Omp2b whereas A68/15B06/C8 better recognizes the external loops of the porin (PhD thesis J-Y Paquet, 2000). However, Omp2b could only be precipitated when A68/4D11/G11 was used. This might be due to antibodies specificities. The A68/15B06/C8 recognizes the natural confirmation, which could be probably disturbed during the experiment. Antibodies against R-LPS were used to detect the presence of this molecule in the Omp2b extracted sample. Nevertheless, any band corresponding to the R-LPS could be detected, even if DNase was used during the extraction.

The interaction between Omp2b and R-LPS is not a protein-protein interaction but a protein-lipid interaction. Several biochemical and physical methods can be also used for study these types of interaction (*Saliba, Vonkova and Galvin, 2015*). Förster Resonance Energy Transfer (FRET) technique could also be tested. This technique measures the interaction between two biomolecules. The two molecules are coupled to two different fluorescently labelled antibodies. If they are in close proximity, there is an energy transfer from the donor to the acceptor fluorophore. In fact, one of the fluorochrome bound to a first secondary antibody is excited and will emit a photon in the emission spectrum of the fluorochrome of the other secondary antibody, giving a signal if this interaction occurs. We could test monoclonal antibodies against Omp2b and R-LPS with different isotype-specific secondary antibodies, conjugated to FRET-compatible fluorochromes.

**3. Sequence analysis of Omp25 and Omp2b**

Besides, Omp2b and Omp25 were also studied in silico in other Rhizobiales species to determine the conservation of these two proteins. Omp25 seems to be more conserved than Omp2b within the different Rhizobiales (**Figure 22** and **23**).

Rhizobiales porin proteins shared some similarity when aligned with Omp2b (**Figure 23**). The most conserved part was at the beginning of the mature sequences in which there was a highly conserved motif (FYPGTETCL), which could be further analysed in the future. This sequence could play an important role in the function of the porin.

However, it was not found in the OmpF porin from *E. coli*, suggesting that it could be associated to other functions.

Omps are proposed to bind to the peptidoglycan. *Brucella* Omps were only extracted from the outer membrane after lysozyme treatment (*Moriyón et Lopez-Goni, 1998; Cloeckaert et al., 1992*). One attractive hypothesis could be that the FYPGTETCL motif could be part of this binding site. To investigate this, we could create point mutations targeting these conserved residues and then study the association to the peptidoglycan by extracting the Omps in the presence/absence of lysozyme (*Cloeckaert et al., 1992*).

Omp25 seems to be conserved among other similar proteins from Rhizobiales species (**Figure 23**). These proteins were referred as immunogenic proteins in *Rhizobium miluonense* (GA0061102_108610) and *Phyllobacterium* spp (SAMN05443582_10321) suggesting that Omp25 could have an impact in the immune system of the host.

In the *Brucella* genome, there are other genes predicted to encode Omp25-like proteins, which were already identified in *B. melitensis* (Omp25 b-c-d) (*Salhi et al., 2003*). We attempted to identify these Omp25-like proteins in *B. abortus*. Omp25 protein was aligned to the entire genome of *B. abortus* and two Omp25-like proteins sequences were found as potential Omp25-like proteins (BAB1_0116 and BAB1_0115). The two possible Omp25-like proteins were then aligned to the Omp25-like proteins in *B. melitensis* and BAB1_0116 and BAB1_0115 were successfully identified in *B. abortus* 2308 as Omp25c and Omp25d (**Figure 24**). Omp25-b corresponding protein was not found in *B. abortus*. The Δ*omp25* mutant could maybe use these homologous proteins to fulfil the function of Omp25 as suggested before.

Finally, several lipoproteins present in the outer membrane can be associated to the peptidoglycan with a conserved motif present in the α-helix close to the C' terminus. Moreover, OmpA from *E. coli* has been proposed to also bind to peptidoglycan (*Koebnik, 1995; Moriyón et Lopez-Goni, 1998*). Several lipoproteins from *B. abortus* were aligned to the *E. coli* lipoproteins associated to the peptidoglycan (**Figure 25**). However, the conserved motif was only found in Omp16 and MotB. Omp16 has similarities to Pals lipoproteins (*Tibor et al., 1994*). Therefore, it was not surprising that it presents this domain. MotB is a protein related to the flagellar motor (*Garza et al., 1995*). *Brucella* is considered as a non-motile bacterium. However, *Brucella* has the genes to construct a complete functional flagellum, but these genes seemed not to be expressed under normal conditions (*Moreno and Moriyón, 2002; Fretin et al., 2005*). Omp25 and Omp2b are suggested to be also associated to the peptidoglycan but the Pal conserved motif could not be found in their sequence (*Cloeckaert et al., 1992*).

Omp25 is considered as the homologous of OmpA from *E. coli* but they do are different in their amino acid sequences and they are also different in sizes. They share a similar β-barrel structure within the OM (*Baldermann, et al., 1998*). This could suggest that Omp25 may share only a few of the multiple functions of OmpA, and probably not the association to peptidoglycan.

## 4. Conclusions

In conclusion, we have created a deletion mutant lacking *omp25* in *B. abortus* 544. The function of this protein is not well described until now. However, Omp25 is conserved among the order of Rhizobiales and thus, it is expected to have an important function also because it is very abundant in the membrane of the bacteria. The mutant was not impaired for growth under rich medium conditions. Nevertheless, under certain stress conditions, Δ*omp25* bacteria were more susceptible than the wild type suggesting a role of Omp25 in membrane integrity. The removal of the Omp25 protein could also alter the composition of the outer membrane. Mutant bacteria displayed a higher R-LPS intensity, which could be due to higher accessibility to the antibodies, as the external loops present in the Omp25 could cover LPS epitopes in the wild type strain.

Moreover, defining the function of Omp25 was not easy because *omp25* expression in the wild type seemed to be dependent on the growth phase. Some bacteria in the stationary phase were not labelled when they were incubated with specific monoclonal antibodies against Omp25. CtrA, which is a major regulator of the cell cycle, can bind to the promoter of the *omp25* gene. This could result then in a change of the *omp25* expression according to the cell cycle. It should be considered to create point mutations in the CtrA binding box in the promoter of *omp25* in order to examine if the proteins levels are changing over time.

Regarding the second most abundant Omp of *B. abortus*, we studied Omp2b, an essential porin, which is colocalized to R-LPS. LPS molecules can interact in a non-covalent manner with Omps. We tried to confirm if R-LPS and Omp2b were physically interacting by a biochemical approach, the co-immunoprecipitation. Nevertheless, it was not possible to confirm any interaction between these two molecules under the tested conditions. Other techniques such as FRET technique could be used to study this potential protein-lipid interaction.

# References


Ames, G. F., Spudich, E. N., and Nikaido, H. (1974). Protein composition of the outer membrane of Salmonella typhimurium: effect of lipopolysaccharide mutations. J. Bacteriol. 117:406-416

Amir, A. (2014). Cell size regulation in bacteria. *Physical Review Letters*, 112(20), 1–5

Arunmanee, W., Pathania, M., Solovyova, A. S., Le Brun, A. P., Ridley, H., Baslé, A., ... Lakey, J. H. (2016). Gram-negative trimeric porins have specific LPS binding sites that are essential for porin biogenesis. Proceedings of the National Academy of Sciences, 113(34), E5034–E5043

Atluri, V. L., Xavier, M. N., de Jong, M. F., den Hartigh, A. B., & Tsolis, R. M. (2011). Interactions of the Human Pathogenic *Brucella* Species with Their Hosts. *Annual Review of Microbiology*, *65*(1), 523–541

Baldermann, C., Lupas, A., Lubieniecki, J., & Engelhardt, H. (1998). The Regulated Outer Membrane Protein Omp21 from Comamonas acidovorans Is Identified as a Member of a New Family of Eight-Stranded β-Sheet Proteins by Its Sequence and Properties. Journal of Bacteriology, 180(15), 3741–3749

Batut, J., Andersson, S. G. E., & O'Callaghan, D. (2004). The evolution of chronic infection strategies in the α-proteobacteria. Nature Reviews Microbiology, 2(12), 933–945

Beveridge, T. J. (1999). Sructure of fram-negative cell walls and their derived mebrane vesicles. Journal of Bacteriology, 181(16), 4725–4733

Bos, MP,. & Tommassen, J. (2005). Viability of a capsuleand lipopolysaccharide-deficient mutant of Neisseria bacteria. Curr. Opin. Microbiol. 16, 779–785

Bos, MP,. Robert. V., & Tommassen, J. (2007). Biogenesis of the Gram-Negative Bacterial Outer Membrane. Annual Review of Microbiology, 61(1), 191–214

Boschiroli, M. L., Ouahrani-Bettache, S., Foulongne, V., Michaux-Charachon, S., Bourg, G., Allardet-Servent, A., ... Ramuz, M. (2002). Type IV secretion and *Brucella* virulence. Veterinary Microbiology, 90(1), 341–348

Bowden, RA., Cloeckaert, A., Zygmunt, MS., Bernard, S., Dubray, G. (1995).Surface exposure of outer membrane protein and lipopolysaccharide epitopes in Brucella species studied by enzyme-linked immunosorbent assay and flow cytometry. Infect. Immun. 63:3945–3952

Braun, V (1975). Covalent lipoprotein from the outer membrane of Escherichia coli. Biochim Biophys Acta 415:335–377

BRAUN, W., GOODLOW, R. J., KRAFT, M., ALTENBERN, R., & MEAD, D. (1951). The effects of metabolites upon interactions between variants in mixed Brucella abortus populations. Journal of Bacteriology, 62(1), 45–52

Brennan, PJ., Nikaido, H. The envelope of mycobacteria. (1995). Annu Rev Biochem, 64;29-6

Brilli, M., Fondi, M., Fani, R., Mengoni, A., Ferri, L.,Bazzicalupo, M., and Biondi, E.G. (2010) The diversity and evolution of cell cycle regulation in alpha-proteobacteria: a comparative genomic analysis. BMC Syst Biol 4: 52

Brown, P. J. B., de Pedro, M. A., Kysela, D. T., Van der Henst, C., Kim, J., De Bolle, X., ... Brun, Y. V. (2012). Polar growth in the Alphaproteobacterial order Rhizobiales. Proceedings of the National Academy of Sciences, 109(5), 1697–1701

Cameron, T. A., Zupan, J. R., & Zambryski, P. C. (2015). The essential features and modes of bacterial polar growth. Trends in Microbiology, 23(6), 347–353

Cardoso, P. G., Macedo, G. C., Azevedo, V., & Oliveira, S. C. (2006). Brucella spp noncanonical LPS: structure, biosynthesis, and interaction with host immune system. Microbial Cell Factories, 5, 13

Caro-Hernández, P., Fernández-Lago, L., De Miguel, M. J., Martín-Martín, A. I., Cloeckaert, A., Grilló, M. J., & Vizcaíno, N. (2007). Role of the Omp25/Omp31 family in outer membrane properties and virulence of Brucella ovis. Infection and Immunity, 75(8), 4050–4061

Celli, J., Salcedo, S. P., & Gorvel, J.-P. (2005). Brucella coopts the small GTPase Sar1 for intracellular replication. *Proceedings of the National Academy of Sciences*, *102*(5), 1673–1678

Cloeckaert, A., Zygmunt, M. S., de Wergifosse, P., Dubray, G., & Limet, J. N. (1992). Demonstration of peptidoglycan-associated Brucella outer-membrane proteins by use of monoclonal antibodies. *Journal of General Microbiology*, *138*(Pt 7), 1543–1550

Cloeckaert, A., Verger, J. M., Grayon, M., Zygmunt, M. S., & Grépinet, O. (1996). Nucleotide sequence and expression of the gene encoding the major 25-kilodalton outer membrane protein of Brucella ovis: Evidence for antigenic shift, compared with other Brucella species, due to a deletion in the gene. Infection and Immunity, 64(6), 2047–2055.

Cloeckaert, A., Verger, J.-M., Grayon, M., & Vizcaíno, N. (1996). Molecular and immunological characterization of the major outer membrane proteins of Brucella. FEMS Microbiology Letters, 145(1), 1–8

Cloeckaert, A., Vizcaíno, N., Paquet, J.-Y., Bowden, R., & Elzer, P. (2002). Major outer membrane proteins of Brucella spp.: past, present and future. Veterinary Microbiology, 90(1–4), 229–247

Cooper, S. & Helmstetter, CE.(1968). Chromosome replication and the division cycle of Escherichia coli B/r. J Mol Biol 31:519–540

Corbel, MJ., Brinley-Morgan, WJ. Genus Brucella.(1984) In Bergey's Manual of Systematic Bacteriology Volume 1. Edited by: Krieg NR, Holt JG. The Williams & Wilkins, Baltimore; 1984:377-388

De Figueiredo, P., Ficht, T. A., Rice-Ficht, A., Rossetti, C. A., & Adams, L. G. (2015). Pathogenesis and immunobiology of brucellosis: Review of Brucella-host interactions. American Journal of Pathology, 185(6), 1505–1517

De Tejada, G. M., Pizarro-Cerda, J., Moreno, E., & nnn, I. (1995). The outer membranes of Brucella spp. are resistant to bactericidal cationic peptides. Infection and Immunity, 63(8), 3054–3061

de Wergifosse, P., P. Lintermans, J. N. Limet, and A. Cloeckaert. (1995).Cloning and nucleotide sequence of the gene coding for the major 25-kilodalton outer membrane protein of Brucella abortus. J. Bacteriol. 177:1911–1914

Ducret, A., Quardokus, E. M., & Brun, Y. V. (2016). MicrobeJ, a tool for high throughput bacterial cell detection and quantitative analysis. *Nature Microbiology*, *1*(7), 16077

Deforet, M., Van Ditmarsch, D., & Xavier, J. B. (2015). Cell-Size Homeostasis and the Incremental Rule in a Bacterial Pathogen. Biophysical Journal, 109(3), 521–528

Deghelt, M., Mullier, C., Sternon, J.-F., Francis, N., Laloux, G., Dotreppe, D., … de Bolle, X. (2014). G1-arrested newborn cells are the predominant infectious form of the pathogen Brucella abortus. Nature Communications, 5, 4366

Delcour, A. H. (2009). Outer Membrane Permeability and Antibiotic Resistance. Biochim Biophys Acta., 1794(5), 808–816

DelVecchio, V. G., Kapatral, V., Redkar, R. J., Patra, G., Mujer, C., Los, T., … Overbeek, R. (2002). The genome sequence of the facultative intracellular pathogen Brucella melitensis. Proceedings of the National Academy of Sciences of the United States of America, 99(1), 443–448

Douglas, J. T., Rosenberg, E. Y., Nikaido, H., Verstreate, D. R., & Winter, A. J. (1984). Porins of Brucella species. Infection and Immunity, 44(1), 16–21

Dubray, G., & Bezard, G. (1980). Isolation of three Brucella abortus cell-wall antigens protective in murine experimental brucellosis. Annales de Recherches Vétérinaires, 11(4), 367–373

Edmonds, M. D., Cloeckaert, A. & Elzer, PH. (2002). Brucella species lacking the major outer membrane protein Omp25 are attenuated in mice and protect against Brucella melitensis and Brucella ovis. Vet. Microbiol. 88, 205–221. 10.1016/S0378-1135(02)00110-4

Ficht, T. A., Bearden, S. W., Sowa, B. A., & Adams, L. G. (1989). DNA sequence and expression of the 36-kilodalton outer membrane protein gene of Brucella abortus. Infection and Immunity, 57(11), 3281–3291

Ficht, T. A., H. S. Husseinen, J. Derr, and S. W. Bearden. (1996). Speciesspecific sequences at the omp2 locus of Brucella type strains. Int. J. Syst.Bacteriol. 46:329–331

Ficht, T. A., S. W. Bearden, B. A. Sowa, and H. Marquis. (1990).Genetic variation at the omp2 porin locus of the brucellae:species-specific markers. Mol. Microbiol. 4:1135-1142

Ficht, T. A., S. W. Bearden, B. A. Sowa, and L. G. Adams. (1988). A 36-kilodalton Brucella abortus cell envelope protein is encodedby repeated sequences closely linked in the genomic DNA. Infect. Immun. 56:2036-2046

Francis, N., Poncin, K., Fioravanti, A., Vassen, V., Willemart, K., Ong, T. A. P., … De Bolle, X. (2017). CtrA controls cell division and outer membrane composition of the pathogen Brucella abortus. *Molecular Microbiology*, *103*(5), 780–797

Fretin, D., Fauconnier, A., Köhler, S., Halling, S., Léonard, S., Nijskens, C., Ferooz, J., Lestrate, P., Delrue, R., Danese, I., Vandenhaute, J., Tibor, A., DeBolle, X. and Letesson, J. (2005). The sheathed flagellum of Brucella melitensis is involved in persistence in a murine model of infection. Cellular Microbiology, 7(5), pp.687-698

Garza, A. G., Harris-Haller, L. W., Stoebner, R. A., & Manson, M. D. (1995). Motility protein interactions in the bacterial flagellar motor. Proceedings of the National Academy of Sciences of the United States of America, 92(6), 1970–1974

Gatsos, X., Perry, A. J., Anwari, K., Dolezal, P., Wolynec, P. P., Likić, V. A., … Lithgow, T. (2008).Protein secretion and outer membrane assembly in Alphaproteobacteria. FEMS Microbiology Reviews, 32(6), 995–1009

Gomez-Miguel, M. J., & Moriyon, I. (1986). Demonstration of a peptidoglycan-linked lipoprotein and characterization of its trypsin fragment in the outer membrane of Brucella spp. Infection and Immunity, 53(3), 678–684

GOODLOW, R. J., TUCKER, L., BRAUN, W., & MIKA, L. A. (1952). Effect of the isomeric configuration of the source of nitrogen on changes in population and metabolism in cultures of Brucella. Journal of Bacteriology, 63(5), 681–685

Gram, C. (1884). Ueber die isolirte Firbung der Schizomyceten iu Schnitt-und Trockenpriparaten. Fortschitte der Medicin, Vol. 2, pages 18.5-189

Guzmán-Verri, C., Manterola, L., Sola-Landa, A., Parra, A., Cloeckaert, A., Garin, J., … López-Goñi, I. (2002). The two-component system BvrR/BvrS essential for Brucella abortus virulence regulates the expression of outer membrane proteins with counterparts in members of the Rhizobiaceae. Proceedings of the National Academy of Sciences of the United States of America, 99(19), 12375–12380

Hallez, R., Bellefontaine, A.-F., Letesson, J.-J., & De Bolle, X. (2004). Morphological and functional asymmetry in α-proteobacteria. Trends in Microbiology, 12(8), 361–365

Hirsch, M., & Elliott, T. (2005). Stationary-Phase Regulation of RpoS Translation in Escherichia coli. Journal of Bacteriology, 187(21), 7204–7213

Howell, M., & Brown, P. J. (2016). Building the bacterial cell wall at the pole. *Current Opinion in Microbiology*, *34*, 53–59

Ishida, H., Garcia-Herrero, A., & Vogel, H. J. (2014). The periplasmic domain of Escherichia coli outer membrane protein A can undergo a localized temperature dependent structural transition. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, *1838*(12), 3014–3024

Jahans, K. L., G. Foster, and E. S. Broughton. (1997). The characterization of Brucella strains isolated from marine mmammals. Vet. Microbiol. 57, 373-382

Jap, B. K., and P. J. Walian. (1996). Structure and functional mechanism of porins. Physiol. Rev. 76:1073–1088

Jensen, A. E., & Halling, S. M. (2010). Effect of polymyxin B and environmental conditions on isolation of Brucella species and the vaccine strain RB51. *Comparative Immunology, Microbiology and Infectious Diseases*, *33*(2), 121–131

Jubier-Maurin, V., R.-A. Boigegrain, A. Cloeckaert, A. Gross, M.-T. Alvarez-Martinez, A. Terraza, J. Liautard, S. Kohler, B. Rouot, J. Dornand, and J-P.Liautard. (2001). Major outer membrane protein Omp25 of Brucella suis is involved in inhibition of tumor necrosis factor alpha production during infection of human macrophages. Infect. Immun. 69:4823–4830

Koch, A. L. (1996). WHAT SIZE SHOULD A BACTERIUM BE? A Question of Scale. *Annu. Rev. Microbiol*, *50*, 317–48. https://doi.org/10.1146/annurev.micro.50.1.317

Koebnik, R. (1995). Proposal for a peptidoglycan-associating alpha-helical motif in the C-terminal regions of some bacterial cell-surface proteins. Molecular Microbiology, 16(6), pp.1269-1270

Koebnik, R., Locher, K. P., & Van Gelder, P. (2000). Structure and function of bacterial outer membrane proteins: barrels in a nutshell. Molecular Microbiology, 37(2), 239–253

Konovalova, A., Kahne, D. E., & Silhavy, T. J. (2017). Outer Membrane Biogenesis. Annual Review of Microbiology, 71(1), 539–556. https://doi.org/10.1146/annurev-micro-090816-093754

Koplow, J. & Goldfine, H (1974) Alterations in the outer membrane of the cell envelope of heptose-deficient mutants of Escherichia coli. J Bacteriol 117(2):527–543

Laloux, G., Deghelt, M., de Barsy, M., Letesson, J. J., & de Bolle, X. (2010). Identification of the essential Brucella melitensis porin Omp2b as a suppressor of Bax-induced cell death in yeast in a genome-wide screening. PLoS ONE, 5(10)

Lam, H., Oh, D.-C., Cava, F., Takacs, C. N., Clardy, J., de Pedro, M. A., & Waldor, M. K. (2009). D-amino Acids Govern Stationary Phase Cell Wall Re-Modeling in Bacteria. *Science (New York, N.Y.)*, *325*(5947), 1552–1555

Laub, M.T., Chen, S.L., Shapiro, L., and McAdams, H.H. (2002) Genes directly controlled by CtrA, a master regulator of the Caulobacter cell cycle. Proc Natl Acad Sci USA 99: 4632–4637

Leive, L. (1974). The barrier function of the gram-negative envelope. Ann. N.Y. Acad. Sci. 235:109-127 lipopolysaccharide production. Antimicrob. Agents Chemother. 54, 4971–4977

Levin, P. A., & ;, E. R. (2015). Small but mighty: Cell size and bacteria. *Cold Spring Harbor Perspectives in Biology*, *7*(7), 1–11. https://doi.org/10.1101/cshperspect.a019216

Lin, M., Tsai, P., Chen, J., Lin, Y. and Lan, C. (2015). OmpA Binding Mediates the Effect of Antimicrobial Peptide LL-37 on Acinetobacter baumannii. PLOS ONE, 10(10), p.e 0141107

Llobet, E., March, C., Gimenez, P. and Bengoechea, J. (2008). Klebsiella pneumoniae OmpA Confers Resistance to Antimicrobial Peptides. Antimicrobial Agents and Chemotherapy, 53(1), pp.298-302

Luirink, J., Heijne, G., Houben, E. & Gier, J. (2005). Biogenesis of inner membrane proteins in Escherichi coli. Annual Review of Microbiology, 59(1), pp.329-355

Mancilla, M. (2016). Smooth to Rough Dissociation in Brucella: The Missing Link to Virulence. Frontiers in Cellular and Infection Microbiology, 5(January), 1–7

Mancilla, M., Marín, C. M., Blasco, J. M., Zárraga, A. M., López-Goñi, I., & Moriyón, I. (2012)Spontaneous excision of the O-polysaccharide wbkA glycosyltranferase gene is a cause of dissociation of smooth to rough Brucella colonies. Journal of Bacteriology, 194(8), 1860–1867

Manterola, L., Moriyón, I., Moreno, E., Sola-Landa, A., Weiss, D. S., Koch, M. H. J., ... López-Goñi, I. (2005). The lipopolysaccharide of Brucella abortus BvrS/BvrR mutants contains lipid A modifications and has higher affinity for bactericidal cationic peptides. *Journal of Bacteriology*, *187*(16), 5631–5639

Marquis, H., & Ficht, T. A. (1993). The omp2 gene locus of Brucella abortus encodes two homologous outer membrane proteins with properties characteristic of bacterial porins. Infection and Immunity, 61(9), 3785–3790

Martínez de Tejada, G., Pizarro-Cerdá, J., Moreno, E., & Moriyón, I. (1995). The outer membranes of Brucella spp. are resistant to bactericidal cationic peptides. *Infection and Immunity*, *63*(8), 3054–3061

McQuiston, J. R., Vemulapalli, R., Inzana, T. J., Schurig, G. G., Sriranganathan, N., Fritzinger, D., ... Boyle, S. M. (1999). Genetic characterization of a Tn5-disrupted glycosyltransferase gene homolog in Brucella abortus and its effect on lipopolysaccharide composition and virulence. Infection and Immunity, 67(8), 383Minnick, M. F., Sappington, K. N., Smitherman, L. S., Andersson, S.

G. E., Karlberg, O., & Carroll, J. A. (2003). Five-Member Gene Family of *Bartonella quintana* . *Infection and Immunity*, *71*(2), 814–821

Mobasheri, H., T. A. Ficht, H. Marquis, E. J. Lea, and J. H. Lakey. (1997).Brucella Omp2a and Omp2b porins: single channel measurements and topology prediction. FEMS Microbiol. Lett. 155:23–30

Moffatt JH et al. (2010). Colistin resistance in Acinetobacter baumannii is mediated by complete loss of endotoxin, a potential vaccine candidate. Infect. Immun. 73, 7569–7577

Moreno, E. (2014). Retrospective and prospective perspectives on zoonotic brucellosis. Frontiers in Microbiology, 5(MAY), 1–18

Moreno, E., & Moriyó, I. (2006). The Genus Brucella. Prokaryotes. 5: 315–456

Moriyon I, Lopez-Goni I. (1998) .Structure and properties of the outer membranes of Brucella abortus and Brucella melitensis. Int Microbiol. Mar;1(1):19-26

Moriyon, I., & Berman, D. T. (1982). Effects of nonionic, ionic, and dipolar ionic detergents and EDTA on the Brucella cell envelope. Journal of Bacteriology, 152(2), 822–828

Mullineaux, C. W., Nenninger, A., Ray, N., & Robinson, C. (2006). Diffusion of Green Fluorescent Protein in Three Cell Environments in *Escherichia Coli*. *Journal of Bacteriology*, *188*(10), 3442–3448

Myeni, S., Child, R., Ng, T. W., Kupko, J. J., Wehrly, T. D., Porcella, S. F., ... Celli, J. (2013). Brucella Modulates Secretory Trafficking via Multiple Type IV Secretion Effector Proteins. PLoS Pathogens, 9(8)

Nikaido, H. & Vaara, M.(1985). Molecular Basis of Bacterial Outer Membrane Permeability, 49(1), 1–32

Nikaido, H., and T. Nakae. (1979). The outer membrane of gram-negative bacteria. Adv. Microb. Physiol. 20:163-250

Paquet, J. Y., X. De Bolle, P. Mertens, S. Genevrois, A. Tibor, E. Depireux,J. H. Lakey, and J. J. Letesson (1999). Antigenic properties of Brucella Omp2a and Omp2b porins purified from recombinant Escherichia coli and renatured in vitro. Arch. Physiol. Biochem. 107:B20

Paquet, J., Diaz, M., Genevrois, S., Grayon, M., Verger, J., De Bolle, X., Lakey, J., Letesson, J. and Cloeckaert, A. (2001). Molecular, Antigenic, and Functional Analyses of Omp2b Porin Size Variants of Brucella spp. Journal of Bacteriology, 183(16), pp.4839-4847

Pasqualini, R., & Arap, W. (2004). Hybridoma-free generation of monoclonal antibodies. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(1), 257–259

Patel, D. S., Re, S., Wu, E. L., Qi, Y., Klebba, P. E., Widmalm, G., ... Im, W. (2016). Dynamics and Interactions of OmpF and LPS: Influence on Pore Accessibility and Ion Permeability. Biophysical Journal, 110(4), 930–938

Peng, D., Hong, W., Choudhury, BP., Carlson, RW., Gu, XX. (2005) Moraxella catarrhalis bacterium without Physiol. 20:163-250

Pini, F., De Nisco, N.J., Ferri, L., Penterman, J., Fioravanti, A., Brilli, M., et al. (2015) Cell Cycle Control by the Master Regulator CtrA in Sinorhizobium meliloti. PLoS Genet 11: e1005232

Pizarro-Cerdá, J., Méresse, S., Parton, R. G., Van Der Goot, G., Sola-Landa, A., Lopez-Goñi, I., ... Gorvel, J. P. (1998). Brucella abortus transits through the autophagic pathway and replicates in the endoplasmic reticulum of nonprofessional phagocytes. Infection and Immunity, 66(12), 5711–5724

Rittig, M. G., Kaufmann, A., Robins, A., Shaw, B., Sprenger, H., Gemsa, D., ... Dornand, J. (2003). Smooth and rough lipopolysaccharide phenotypes of Brucella induce different intracellular trafficking and cytokine / chemokine release in human monocytes. Journal of Leukocyte Biology, 74(December), 1045–1055

Roest. H.P., Mulders, I.H.M., Wijffelman, C..4. and Lugtenberg,B.J.J. (1995) Isolation of ropB, a gene encoding a 22-kDa Rhimbium legurninosarum outer membrane protein. Mol.Plant-Microbe Interact. 8, 5766583

Salhi, I., Boigegrain, R.-A., Machold, J., Weise, C., Cloeckaert, A., & Rouot, B. (2003). Characterization of new members of the group 3 outer membrane protein family of Brucella spp. Infection and Immunity, 71(8), 4326–32

Saliba, A.-E., Vonkova, I., & Gavin, A.-C. (2015). The systematic analysis of protein–lipid interactions comes of age. Nature Reviews Molecular Cell Biology, 16(12), 753–761

Schaechter, M., Maaloe, O. & Kjeldgaard, NO (1958). Dependency on medium and temperature of cell size and chemical composition during balanced grown of Salmonella typhimurium. J Gen Microbiol. 19:592–606

Schulz, H. N., & Jørgensen, B. B. (2001). Big bacteria. Annu. Rev. Microbiol., 55, 105–137

Schwechheimer, C., & Kuehn, M. J. (2015). Outer-membrane vesicles from Gram-negative bacteria: biogenesis and functions. Nature Reviews Microbiology, 13(10), 605–619

Sharpe, ME., Hauser, PM., Sharpe, RG., Errington, J. (1998). Bacillus subtilis cell cycle as studied by fluorescence microscopy: constancy of cell length at initiation of DNA replication and evidence for active nucleoid partitioning. J Bacteriol 180:547–555

Silhavy, T., Kahne, D., & Walker, S. (2010). The bacterial cell envelope. Cold Spring Harbor Perspectives in Biology, 2(5), 1–16

Simpson, B.W., May, J.M., Sherman, D.J., Kahne, D., & Ruiz N. (2015) Lipopolysaccharide transport to the cell surface: biosynthesis and extraction from the inner membrane. Phil. Trans. R. Soc. B 370: 20150029

Smith, L. D., & Ficht, T. a. (1990). Pathogenesis of Brucella. Critical Reviews in Microbiology, 17, 209–230

Sperandeo, P., Martorana, A. M., & Polissi, A. (2017). Lipopolysaccharide biogenesis and transport at the outer membrane of Gram-negative bacteria. Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids, 1862(11), 1451–1460

Starr, T., Ng, T. W., Wehrly, T. D., Knodler, L. A., & Celli, J. (2008). Brucella intracellular replication requires trafficking through the late endosomal/lysosomal compartment. Traffic, 9(5), 678–694

Steeghs, L,. De Cock, H,. Evers, E., Zomer, B., Tommassen, J. & Van der Ley, P. (2002) Outer membrane composition of a lipopolysaccharide-deficient Neisseria meningitidis mutant. EMBO J. 20, 6937–6945

Steeghs, L,. De Cock, H,. Evers, E., Zomer, B., Tommassen, J. & Van der Ley, P. (2002) Outer membrane composition of a lipopolysaccharide-deficient Neisseria meningitidis mutant. EMBO J. 20, 6937–6945

Steeghs L, den Hartog R, den Boer, A, Zomer B, Roholl P, van der Ley P. (1998) Meningitis bacterium is viable without endotoxin. Nature 392, 449–450

Taheri-araghi, S., Bradde, S., Sauls, J. T., Hill, N. S., Levin, P. A., Paulsson, J., ... Jun, S. (2015). Cell-Size Control and Homeostasis in Bacteria. Current Biology, 25(3), 385–391

Tibor, A., Decelle, B., & Letesson, J. J. (1999). Outer membrane proteins Omp10, Omp16, and Omp19 of Brucella spp. are lipoproteins. Infection and Immunity, 67(9), 4960–4962

Tibor, A., Saman, E., De Wergifosse, P., Cloeckaert, A., Limet, J. N., & Letesson, J. J. (1996). Molecular characterization, occurrence, and immunogenicity in infected sheep and cattle of two minor outer membrane proteins of Brucella abortus. Infection and Immunity, 64(1), 100–107

Tibor, A., Weynants, V., Denoel, P., Lichtfouse, B., De Bolle, X., Saman, E., Letesson, J. J. (1994). Molecular cloning, nucleotide sequence, and occurrence of a 16.5-kilodalton outer membrane protein of Brucella abortus with similarity to pal lipoproteins. Infection and Immunity, 62(9), 3633–3639

Vakorina, I., Novikova, O. D. , Krasikova, I. N. ,Naberezhnykh G. N. , Solov'ev Yu. T. F. , Ovodov S. (2003) Interaction of porin from Yersinia pseudotuberculosis with different structural forms of endogenous lipopolysaccharide. Biochemistry (Mosc) 68(9):976–983

Van Der Henst, C., De Barsy, M., Zorreguieta, A., Letesson, J. J., & De Bolle, X. (2013). The Brucella pathogens are polarized bacteria. Microbes and Infection, 15(14–15), 998–1004

Verstreate, D. R., Creasy, M. T., Caveney, N. T., Baldwin, C. L., Blab, M. W., & Winter, A. J. (1982). Outer membrane proteins of Brucella abortus: isolation and characterization. Infection and Immunity, 35(3), 979–989

Viljanen, P., & Vaara, M. (1984). Susceptibility of gram-negative bacteria to polymyxin B nonapeptide. *Antimicrobial Agents and Chemotherapy*, *25*(6), 701–705

Vizcaíno, N., Cloeckaert, A., Zygmunt, M. S., & Dubray, G. (1996). Cloning, nucleotide sequence, and expression of the Brucella melitensis omp31 gene coding for an immunogenic major outer membrane protein. Infection and Immunity, 64(9), 3744–3751

Vizcaino, N., Verger, J., Grayon, M., Zygmunt, M. S., & Cloeckaert, A. (1997). Brucella spp .: evidence for a large deletion in Brucella abortus , and other species-specif ic markers DNA polymorphism at the omp-37 locus of. Microbiology, 143(1 997), 2913–29219-2913

von Bargen, K., Gorvel, J. P., & Salcedo, S. P. (2012). Internal affairs: Investigating the Brucella intracellular lifestyle. FEMS Microbiology Reviews, 36(3), 533–562

Wang, Y. (2002). The Function of OmpA in Escherichia coli. Biochemical and Biophysical Research Communications, 292(2), pp.396-401

Weart, R. B., Lee, A. H., Chien, A.-C., Haeusser, D. P., Hill, N. S., & Levin, P. A. (2007). A metabolic sensor governing cell size in bacteria. *Cell*, *130*(2), 335–347

Westfall, C. S., & Levin, P. A. (2017). Bacterial Cell Size□: Multifactorial and Multifaceted. Annual Review of Microbiology, 71, 499–517

Winter, A.J. & Rowe, G.E.(1988). Comparative immune responses to native cell envelope antigens and the hot sodium dodecyl sulfate insoluble fraction (PG) of Brucella abortus in cattle and mice. Vet. Immunol. Immunopathol. 18, 149–163

Yanagi, M., Yamasato, K., (1993). Phylogenetic analysis of the family Rhizobiaceae and related bacteria by sequencing of 16 S rRNA gene using PCR and DNA sequencer. FEMS Microbiol. Lett. 107, 115-120

Zhang, G., Meredith, TC., Kahne, D. (2013) On the essentiality of lipopolysaccharide to Gram-negative bacteria. Curr. Opin. Microbiol. 16, 779–785

## Supplementary data

| Concerned gene | Name | Sequence | |
|---|---|---|---|
| *omp25* (BAB1_0772) | Omp25_up_fw_SpeI | 5'- CTA GAA CTA GTG AAT CAC AGG CGT TTT CA -3' | |
| | Omp25_up_rev | 5'- CGT GTC CAA TTA TGC TAT AAG GCA TTC TCC TTA CAC AA -3' | |
| | Omp25_dw_fw | 5'- TTA TAG CAT AAT TGG ACA CG -3' | |
| | Omp25_dw_rv_SalI | 5'- TGC ACG TCG ACG GAC AAA CAT GAC ACA GA -3' | |
| | Omp25_for_KpnI | 5'- CTA GAG GTA CCA TGC GCA CTC TTA AGT CT -3' (Complementation strain) | |
| | Omp25_rev_BamHI | 5'- TGC ACG GAT CCT TAG AAC TTG TAG CCG ATG -3' (Complementation strain) | |
| *omp2b* (BAB1 0660) | Omp2b_up_fw_SpeI | 5'- CTA GAA CTA GTT AGC CTG GAA AAA CAG AGA TTC AG -3' | |
| | Omp2b_up_rv | 5'- CCT GTC CCC ATC CCC TAA -3' | |
| | Omp2b_dw_fw | 5'- AAT TAG GGG ATG GGG ACA GGT CCA TTA CGG ATT GGA TAC AGA AAC -3' | |
| | Omp2b_dw_rv_SalI | 5'- TGC ACG TCG ACC ATA AAT CGC TTC ATC TGA AAC C -3' | |
| | Omp2b_fw_BamH | 5'- CTA GAG GAT CCT TGT CCT TCA GCC AAA TC -3' | (Rescue copy) |
| | Omp2b_rv_XhoI | 5'- TGC ACC TCG AGT TAG AAC GAA CGC TGG AA -3' | (Rescue copy) |

**Table S1 | Primer table**

### Composition gels

| Stacking gel | Running gel |
|---|---|
| Tris-HCL 0.5M pH 6.6 | Tris-HCL 1.5M pH 8.8 |
| SDS 10% | SDS 10% |
| Ammonium persulfate (APS) 10% | Ammonium persulfate (APS) 10% |
| Acrylamide/ Bis acrylamide 12%, | Acrylamide/ Bis acrylamide 12%, |
| Tetramethylethylenediamine (TEMED) | Tetramethylethylenediamine (TEMED) |
| $H_2O$ | $H_2O$ |

**Table S2 | SDS PAGE gel compsoition**

### Antibodies used

| | | Technique |
|---|---|---|
| LPS | A68/24D87/G9 | WB for OM extraction |
| Omp25 | A68/04B10/F05 | IF, WB for confirmation mutant, WB for OM extraction |
| Omp10 | A68/7G11/C10 | WB loading control |
| Omp2b | A68/15B06/C8 | Co-IP pull down and WB detection |
| Omp2b | A68/4D11/G11 | Co-IP pull down, WB and WB for OM extraction |
| R-LPS | A68/3F3/D5 | IF, Flow citometry |
| S-LPS | B66/4F9 | IF, Flow citometry |

**Table S3 | Antibodies used (undiluted hybridoma culture). Co-IP: co-immunoprecipitation, IF: immunofluorescence, WB: western blot, OM: outer membrane**