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Tagging of the BmaC adhesin in Bruce/la abortus

Bonnet, Alexandre

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Tagging of the BmaC adhesin in *Brucella abortus*

Mémoire présenté pour l'obtention
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Alexandre BONNET

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Université de Namur
FACULTE DE MEDECINE
Secrétariat des départements
Rue de Bruxelles 61 - 5000 NAMUR
Téléphone: + 32(0)81.72.41.53
E-mail: katty.lamoline@unamur.be - http://www.unamur.be/

Marquage de l'adhésine BmaC chez *Brucella. abortus*

BONNET Alexandre

Résumé

La bactérie du genre *Brucella* est un pathogène intracellulaire responsable de la Brucellose, une maladie anthropo zoonotique mondiale. Il a été démontré que pour infecter les cellules HeLa, la bactérie devait adhérer à la surface de la cellule hôte. De nos jours, 3 adhésines de *Brucella* ont été identifiées (BmaC, BtaE, BtaF) et il a été démontré qu'elles liaient les composés de la matrice extra cellulaire. Durant ce travaille, nous avons décidé de nous concentrer sur BmaC, un auto-transporteur monomérique qui se lie à la fibronectine.
Il a été récemment montré que l'infectiosité de *Brucella* varie le long de son cycle cellulaire où ce serait principalement la nouvelle génération de bactéries filles (appelées « *Newborns* ») qui infecte les cellules hôtes. Notre hypothèse est que ce serait les *nemborns*, comparées aux autres types du cycle bactérien, qui exprimeraient préférentiellement les adhésines à leurs surfaces. Pour tester cette hypothése, nous avons décidé de marquer BmaC avec un épitope myc au moyen d'un remplacement allélique. Nous avons essayé de détecter la présence de la protéine à la surface de la bactérie par le biais d'une immuno fluorescence contre l'épitope myc. Malheureusement les expériences menées ne nous ont pas apportées de données exploitables.

Mémoire de master en sciences biomédicales

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Promoteur: X. De Bolle

Université de Namur
FACULTE DE MEDECINE
Secrétariat des départements
Rue de Bruxelles 61 - 5000 NAMUR
Téléphone: + 32(0)81.72.41.53
E-mail: katty.lamoline@unamur.be - http://www.unamur.be/

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Abstract

Bacteria from the Brucella genus are intracellular pathogens responsible for brucellosis a worldwide anthropozoonotic disease. It was previously shown that, to infect HeLa cells, Brucella must adhere to the surface of the host cell. So far, three adhesins of Brucella have been identified (BmaC, BtaE, BtaF) and shown to bind to extracellular matrix components. During this work, we have decided to concentrate on BmaC, a monomeric autotransporter that binds to fibronectin.

It has recently been shown that the infectiosity of Brucella varies along its bacterial cell cycle where the newly generated daughter bacteria (the "newborns") mostly infect host cells. Our hypothesis is that the newborns could preferentially express adhesins as compared to others bacterial cell types. To test this, we tagged BmaC with a myc epitope by allelic replacement in Brucella abortus and we tried to detect the presence of the protein on the bacterial surface by performing immunofluorescence against the myc-tag. Until now, the myc-BmaC fusion is undetectable, probably because of its very low production level.

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Tagging of the BmaC adhesin in *Brucella abortus*

Bonnet A[1], Sternon J-F[1], De Bolle X[1]
[1] Unit in Biology of Microorganisms (URBM), University of Namur

Abstract

Bacteria from the* Brucella *genus are intracellular pathogens responsible for brucellosis a worldwide anthropozoonotic disease. It was previously shown that, to infect HeLa cells,* Brucella *must adhere to the surface of the host cell. So far, three adhesins of* Brucella *have been identified (BmaC, BtaE, BtaF) and shown to bind to extracellular matrix components. During this work, we have decided to concentrate on BmaC, a monomeric autotransporter that binds to fibronectin.
It has recently been shown that the infectiosity of* Brucella *varies along its bacterial cell cycle where the newly generated daughter bacteria (the "*newborns*") mostly infect host cells. Our hypothesis is that the* newborns *could preferentially express adhesins as compared to others bacterial cell types. To test this, we tagged BmaC with a myc epitope by allelic replacement in* Brucella abortus *and we tried to detect the presence of the protein on the bacterial surface by performing immunofluorescence against the myc-tag. Until now, the myc-BmaC fusion is undetectable, probably because of its very low production level.

Keywords: Adhesion, *Brucella*, BmaC, Myc-tag, Immunofluorescence

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Introduction

Brucellosis is a worldwide anthropozoonotic disease caused by the bacterial genus *Brucella*[1]. Human brucellosis or Malta fever was discovered by Dr David Bruce in the 19th century[2]. The human incidence of the disease is 500,000[3] new cases per year in the world. The disease is common in the Mediterranean region, Latin America and in the under developed countries where it causes important economic losses[4]. In humans, brucellosis usually causes flu-like symptoms such as muscular pain, undulant fever, sweating, headache, and joint pain[5]. No effective vaccine is currently available for human and current treatments consist in the administration of high doses of antibiotics for several weeks[6] [7]. The duration of the disease is variable, ranging from several weeks to several years, and if not treated properly the disease may evolve into a life-persisting chronic state and in rare cases death[8]. For these reasons, brucellosis can be defined as a debilating disease.
Human is an accidental host of *Brucella* and its infection is highly dependent on promiscuity with infected animals. In fact, people in direct contact with infected animals like farmers, abattoir workers, veterinarians and also laboratory technicians are more susceptible to brucellosis[9]. Another main route of infection is the consumption of contaminated dairy products such as cheese or milk[10].

Bacteria from the *Brucella* genus are class III pathogens and are gram-negative coccobacilli belonging to the alpha-proteobacteria group such as *Rickettsia spp.* or *Bartonnela quitana*[11] [12]. Ten species of *Brucella* have been identified

based on host preferences, among which three have been shown to be pathogenic for human (*B. abortus, B. suis, B. melitensis*)[7]. However, it should be noted that their genome is highly similar, their DNA identity exceeds 95% on average[13].
Brucella is an intracellular pathogen and was shown to replicate within the endoplasmic reticulum of both phagocytic and non-phagocytic cells[14]. However, *Brucella* can survive in the environment for extended periods of time, thus making *Brucella spp.* facultatively extracellular intracellular pathogens [7 15 16].
Recently, it has been shown that the infectiosity of *B. abortus* varies along its cell cycle. In fact, using markers of chromosome replication initiation and growth, research from my host laboratory showed that mostly newly generated daughter bacteria (termed "*newborns*") are able to infect cultured cells (Deghelt M. Mullier C. *et al.*, manuscript is under reviewing) (see figure 1).

Adhesion on the surface of host cell is an important step for numerous pathogenic bacteria[17]. One way to adhere is for example to use macromolecular complexes such as pili, as in *Legionella pneumophila* or *Pseudomonas aeruginosa*[18] [19]. Other bacteria use outer membrane proteins called adhesins, many of which belong to the autotransporter family, in order to adhere onto the surface of host cells. For example, *Yersinia enterocolitica*, the causative agent of yersiniosis, uses several trimeric autotransporters (YadA, YadB, YadC) to adhere to host cells[20]. Also, *Bordetella pertusis* the causative agent of the whooping cough uses pertactin, a monomeric autotransporter, in order to adhere and *in extenso* to invade host cells[21].
Recently, it has been shown that *Brucella* binds to components of the extracellular matrix[22]. So far 3 adhesins (BmaC, BtaE and BtaF) have been identified in *Brucella*. BtaE, a trimeric autotransporter, binds to hyaluronic acid, and BtaF, another trimeric autotransporter, was shown to bind to hyaluronic acid and to confer serum resistance[23] [24]. BmaC, a very large protein (340 kDa) belonging to the monomeric autotransporter group has been reported to bind to fibronectin[25]. Most importantly, it has been shown that the deletion of this protein induces a tremendous decrease of *Brucella* infectiosity in HeLa cell by reducing its adhesion capacity [25]. The link between the expression of adhesins and the preferential infectivity of *newborn Brucella* compared to the other *Brucella* cell types has never been investigated. Therefore, the aim of this study is to test for the preferential expression of BmaC in *newborn Brucella* by detecting the presence of the protein on the bacterial outer membrane using immunofluorescence.

Material and Methods

This "material and methods" section is mostly derived from the master thesis of Jean-François Sternon: " Characterization of the replication and segregation of *Brucella abortus* chromosomes during *in vitro* culture" (2013).

Bacterial strains, growth conditions, and plasmids

Escherichia coli host strain DH10B or S17-1 was cultivated on solid Luria-Bertani (LB) or liquid LB medium at 37°C. *Brucella* abortus 2308 or 544 were cultivated on solid or liquid 2YT medium at 37°C. Antibiotics were used at the following final concentrations: kanamycin 50 μg/ml (for *E. coli*) and 10 μg/ml (for *B. abortus*); nalidixic acid 25 μg/ml. Plasmid used was pNPTS138.

Polymerase Chain Reaction

-Preparative PCR: The mix of PCR was composed of 5X Phusion Buffer (1X), Phusion polymerase (0.02 U/μl, Finnzymes), dNTPS (5 mM each), primers

(20 µM), and template DNA (about 70 ng). After a first denaturation step at 98°C for 30 s, the program was made of 30 amplification cycles composed of a denaturation step (10 s at 98°C) then a primer hybridization step (30 s at a suitable temperature according to primers predicted Tm) and an elongation step at 72°C (30 s/kb, duration depending on the expected amplification product size). A final elongation step was made for 10 min at 72°C. Size-approximation and absence of aspecific amplification products were checked by electrophoresis migration on a 1% agarose gel supplemented with ethidium bromide to visualize DNA using UV light. The fragment size marker used was the GeneRulerTM 1KB DNA Ladder (Fermentas).

- Joining PCR: The initial PCR mix was composed of dNTPs (5 mM each), 5X Phusion buffer (1X), Phusion polymerase (0.02 U/µl, Finnzymes), and the two PCR products to be joined. After a first step of denaturation at 98°C during 30 s, the program began by 5 amplification cycles (see "preparative PCR"). The goal of these first rounds of amplification was to join both PCR products thanks to a 15 bp homology sequence present at one end of each PCR products. Then, a pair of primer (20 µM each) was added to the initial mix in order to amplify the entire newly formed template (*i.e.* the two initial PCR products now joined together) and 27 additionnal cycles of amplification are performed. Size-approximation and absence of aspecific amplification products were checked by electrophoresis migration.

- Diagnostic PCR: The PCR mix was composed of dNTPs (5 mM each), primers (20 µM each), GoTaq polymerase (Promega®, Madison, USA), 5X GoTaq buffer (1X) and template DNA (about 70 ng). After a first denaturation step at 94°C for 4 min, program was made of 30 amplifications cycles composed of a denaturation step (30 s at 94°C), a hybridization step (30 s at a suitable temperature regarding primers predicted Tm), and a final elongation step at 72°C (1 min/kb, duration depending on the excepted amplification product size). A final elongation step was performed for 10 min at 72°C. Again, size-approximation and absence of aspecific amplification products were checked by electrophoresis migration.

Purification of product PCR

The PCR products purification on column was made using the MSB SpinPCRapace® (Invitek Berlin Germany). The protocol of the manufactorer was followed.

Ligation into the pNPTS138

The ligation mix was composed of the restricted vector and the insert sequence (volumes used was calculated on respective concentration to have 1/10 ratio), 5X ligase buffer (1X), and T4-ligase (Fermentas).
The final mix was incubated either overnight at 18 °C or 4 hours at room temperature.

Transformation with $CaCl_2$-competent DH10B *E. coli*

50 µl of DH10B *E. coli* competent were put on ice and 5 µl of plasmid (about 100 ng/µl) were added. The mix was incubated during 20 min at 4 °C and then placed at 42 °C for a 2 min thermal shock. The bacteria were then resuspended in 700 µl liquid LB and placed at 37 °C for 45 min. Afterwards, the culture was centrifuged at 5000 rpm during 3 min and the pellet was resuspended in 100 µl of supernatant before being spread onto solid LB containing the appropriate antibiotic for an overnight culture.

Plasmidic DNA minipreparation

The culture were centrifuged at 13,000 rpm during 1 min, we did this twice. The pellet was first resuspended with a P1 solution

(RNAse A 100 µl/ml, TrisHCl 50 mM and EDTA 10 mM) followed by the addition of P2 lysis solution (NaOH 200 mM and SDS 1%) and incubated for 5 min. Then, a P3 solution (KAc 3.0 M) was added to neutralize the P2 lysis solution. The lysat was centrifuged at 13,000 rpm during 10 min. The supernatant was collected and transferred in a new tube and 700 µl of isopropanol were added. The mix was then centrifugated for 10 min at 13,000 rpm.
The supernatant was removed and 400 µl of cold 70 % ethanol (conserved at -20 °C) were added to wash the pellet. After a 5 min centrifugation at 13,000 rpm, the supernatant was carefully removed and the pellet was left to dry at 65 °C for at least 15 min. At last the pellet was resuspended with 20 to 30 µl of ddH20 (milliQ purification system, Millipore).

Enzymatic restriction

DNA was incubated with the appropriate enzyme(s) (10 U/µl) (Roche®) and with 10X appropriate buffer (1X). The mix incubated between 1 h to 1h30,depending on the amount of DNA to be restricted.

Mating

50 µl of conjugative culture *E. coli* S17-1 strain carrying the plasmid of interest were mixed with 1 µl of *B. abortus* 2308 or 544 strains and then centrifuged for 2 min at 7,000 rpm. The supernatant was removed and the pellet was resuspended in 1 ml of fresh 2YT liquid medium. The suspension was centrifuged again for 2 min at 7,000 rpm and the pellet was resuspended into the small part of supernatant before being spread as a single drop onto solid 2YT medium and placed at 37 °C overnight. The next day, bacteria were harvested from the first plate and spread onto a new solid 2YT medium plate supplemented with both kanamycin and nalidixic acid and put at 37 °C for 3 to 5 days. The obtained colonies were streaked on solid 2YT medium supplemented with kanamycin but without nalidixic acid.

Allelic replacement
One *Brucella* colony obtained after mating was incubated in liquid 2YT without antibiotics to permit bacteria to lose the integrated plasmid. 100 µl of this culture was spread onto solid 2YT medium supplemented with sucrose (5 %).
Then, the selection of the clones, which excised the plasmid, was made by spreading candidate colonies on both solid 2YT supplemented with kanamycin and on solid 2YT supplemented with sucrose. Only clones able to grow on 2YT sucrose but not on 2YT kanamycin were selected for diagnostic PCR.

Sequencing

The sequencing of the plasmid was made by Beckman Coulter Genomics®.

Microscopy

The microscope used in this study was a Nikon Eclipse E1000 (objective 100X, plan Apo) connected to a Hamamatsu ORCA-ER camera. DF type immersion oil (Nikon oil) with a refraction indice of 1.5150 +/- 0,0002 was used to visualize bacteria.

Preparation for the immunofluorescence

First, the glass slide (Thermo Scientific®) was washed with both water and ethanol (70 %). Once dried, the slide was spotted with 20 µl of poly-L Lysine (0.01 mg/ml) and incubated at room temperature for 20 min. The slide was then washed 3 times with PBS (Phosphate Buffered Saline, Lonza® Verviers Belgium) and 20 µl of bacterial culture were put on the poly-L-lysine spot and incubated for 20 min. The slide was washed 3 times with PBS and 20 µl of primary staining mix was added on the slide. This mix was composed of anti-myc mouse antibody (Roche®,

concentrations ranging from 1 to 10 µg/ml), and BSA (Bovine Serum Albumin (5%) diluted in PBS. After 45 min of incubation, the slide was washed 10 times with PBS and then 20 µl of secondary staining mix was added. This mix was composed of anti-mouse goat antibody conjugated to TxRed (Molecular Probes®), and BSA (5%) diluted in PBS. Finally, after 10 wash with PBS, coverslips were mounted onto the slide using Slowfade®. The slide was then conserved in the dark at 4 °C up to one week prior to observation.

Inactivation of Brucella

The culture was first washed twice in PBS. Then, the suspension was centrifuged and the pellet was resuspended in 1 ml PFA (paraformaldehyde, 2 %) for 15 min at 37 °C.

Results

To localize BmaC on the surface of *Brucella*, we tagged the protein with a myc epitope (EQKLISEEDL peptidic sequence) and performed Immunofluorescence staining against the myc-tag.

Contruction of pNPTS *bmaC*-myc 75

We decided to insert the myc tag 75 aa downstream of the start codon (*i.e.* 3 aa downstream of the predicted atypical autotransporter signal peptide cleavage site) not to disturb the cleavage site. To build such a strain, we constructed an allelic replacement vector containing a fragment of the *bmaC* gene carrying the myc epitope (see figure 2). To do this, we used a joining PCR strategy consisting in 3 PCRs. A first PCR was designed to amplifiy 750 bp upstream of the myc-tag insertion site while carrying the myc-tag sequence it the reverse primer (see figure 2 B). Then, a second PCR was designed to amplify 750 bp downstream of the myc-tag insertion site while carrying the myc-tag sequence in the forward primer (see figure 2 C). Finally, both PCR products were used to perform a third PCR (*i.e.* the joining PCR, see figure 2 D). The resulting amplicon, consisting in a fragment of the *bmaC* gene carrying a myc epitope after residue 75, was then cloned in an *Eco*RV-restricted pNPTS138 vector (see figure 2 F) that was transformed in $CaCl_2$ competent DH10B *E. coli*. It should be noted that the two 750 bp sequences flanking the myc-tag will be used to allow the site specific integration of the plasmid in *B. abortus* genome thanks to homologous recombination.
The plasmid (pNPTS138 *bmaC*-myc 75) was extracted, checked by diagnostic restriction, and sequenced. Once validated, the plasmid was transformed into the conjugative S17-1 *E. coli*, which was then used to mate with *Brucella*, thus allowing allelic replacement to occur (see figure 3).

Observation of *B. abortus bmaC*-myc 75

Once the strain *B. abortus bmaC*-myc 75 was obtained, we performed immunofluorescence in order to detect a signal corresponding to the presence of BmaC on the surface of the bacterium (see "material and methods"). Unfortunately, our observations did not allow us to detect any exploitable signal.

To perform immunofluorescence, we confronted our sample with a positive control. This last consisted in binding the outer membrane protein Omp1, a protein on the surface of *Brucella,* with an anti-Omp1 antibody and to use the same secondary antibody for the immunofluorescence (see figure 4 A). When we compared our sample with the positive control using the same LUTs parameters[a], we observed no fluorescence with our sample (see figure 4 B) unlike our

[a] LUTs are lookup tables used to set the thresholds for detection and saturation of the fluorescence signals. Changing these parameters does not modify the data obtained but only changes the way these values appear on the screen. (Nikon NIS-Elements Advanced Research User's guide ver. 3.00, p. 86)

positive control where the fluorescence was observed on the surface of the bacteria (see figure 4 A). This control thus permitted us to confirm that the immunofluorescence was made correctly and that the secondary antibody was functional.
Since a very weak signal would be impossible to see with the first settings, we changed the parameters of the LUTs in order to detect weak signals. With these new settings we observed several foci. However, even though some of them were associated with the bacteria, others were not. Therefore, to know if the foci on the bacteria came from a *bona fide* tagging of BmaC or if they were just part of a background signal, we compared our sample with our negative control, using the same LUTs parameters. The negative control was the anti-myc staining performed on wild type bacteria, with the same anti-myc antibody and the secondary antibody (see figure 4 D).
Even though no counting was performed, we observed no differences between our sample and the negative control condition (see figure 4 C/D). Therefore, we concluded that unfortunately the observed foci on the sample came from the background.
To optimize the quality of the immunofluorescence, we tested another concentration of antibody against myc. The concentration tested was 1 µg/ml instead of 2.5 µg/ml. The obtained results were the same as the first concentration (data not shown).

Also, clear images of the bacteria were difficult to obtain and pictures were blurry. We hypothesized that this could be due to the method used for having bacteria adhering to the glass slide (see "material and methods, preparation for immunofluorescence"). Therefore we tested several dilutions of poly-L Lysine for the purpose of increasing the resolution of the pictures. Unfortunately, the poly-L lysine concentration changes gave no increase on the qualities of the pictures (data not shown).

Discussion

The positive control used permits us to confirm that our immunofluorescence was correctly performed, and that our secondary antibody works properly. Unfortunately it does not allow us to confirm that our primary antibody was efficient. Indeed, a better positive control would be a strain of *Brucella* that is expressing another outer membrane protein tagged with the myc epitope. We cannot rule out that our antibody anti-myc is not functional and does not bind the myc tag.
It would explain why the pictures that we observed of our sample were the same that the negative control.

Strain construction

There are several hypotheses why the localization of the myc-tagged BmaC did not succeed.
One of this is that maybe the adhesin is not present. In fact, the myc tag could prevent the proper folding of the adhesin. The adhesin would then be misfolded and likely destructed by proteases.
A solution to know if the protein is expressed would be to detect the protein by performing a western blot against the myc-tag. However, the high molecular weight of the BmaC monomer (340 kD) renders this western blot very difficult to perform.

Another reason could be that the adhesin is produced but is not present on the outer membrane of the bacteria. It could for example be stuck into the periplasm. Another possibility could be that folding of the protein somehow masks the myc tag that would mean that the antibody does not bind the tag.
To know if the adhesin is functional or not, a solution would be to compare the percentage of cells infected with the strain tagged versus the wild type. If the function

of the adhesin is altered, the percentage of cell infected will be likely lower compared to the wild type strain.

Weak expression of BmaC

There is only a low percentage of bacteria that express the adhesin[24]. Moreover the adhesin is itself weakly expressed[25], thus making it hard to detect. If the tag does not alter the function of the adhesin, one solution would be to introduce several myc tags next to each other. It could permit to increase the visibility of the protein by binding more primary antibody and thus to eventually increase the signal of the immunofluorescence.

Then, if we cannot localize the adhesin, we could also try to change the tag insertion site. It wills maybe permit to give a better access for the antibody. Also, if we cannot have results with the myc tag, another solution could be to change the myc tag with another tag (for example a HA tag or a His tag).

If the observation of the adhesin by immunofluorescence against an inserted tag does not work, one solution would be to produce antibodies directly against the endogenous adhesin. It could help us to observe the adhesin in its natural conformation.

Perspectives

Our hypothesis is that the newly generated daughter bacteria, which mostly infect the host cells, preferentially carry adhesins mediating the initial cobtact between bacteria and host cells. To know if other bacterial cell types can adhere on the surface of the host cell and thus infect, we could overexpress the adhesin. To do this we would switch the promoter of *bmaC* with a stronger promoter, which could lead to an overexpression of the adhesin.

Another thing to do will be to delete all adhesins known in *Brucella* (BmaC, BtaE and BtaF). The strain of *Brucella* with the 3 deletions would then be compared with the wild type strain, to check that it is unable to infect host cells such as HeLa cells. If the triple mutant is still able tyo infect non-professional phagocytes, it could mean that other adhesion molecules are involved in the infection.

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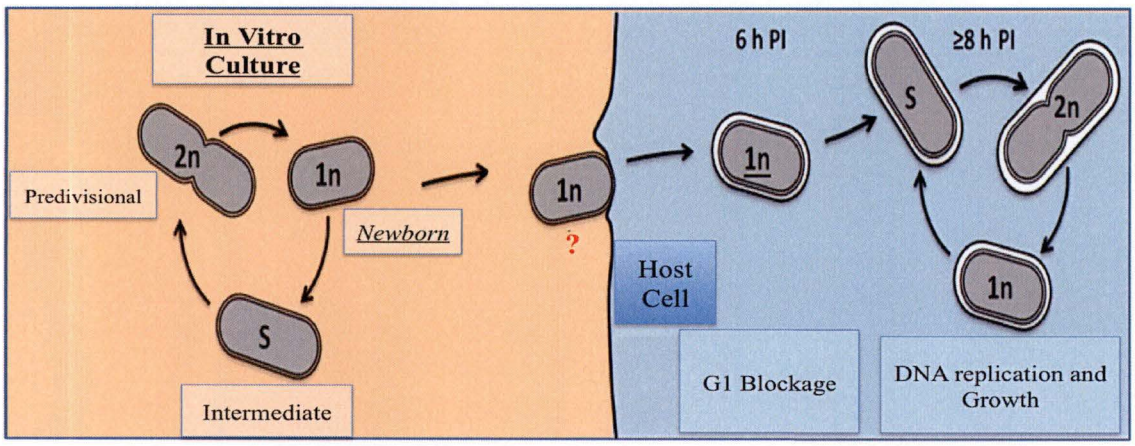
Annexes



Adapted from Sternon J.F.

Figure 1: Cartoon of *Brucella* life cycle. On the left: *Brucella* cell cycle as described during *in vitro* culture. A particular bacterial cell type (the "*newborns*", displaying a single copy of *Brucella* genome) is mostly able to infect host cells. Our hypothesis is that this phenomenon could be mediated by a preferential adhesion of the newborn bacteria on the host cell surface. It was shown that once internalized, the newborns display a growth arrest and G1 cell cycle block lasting 6 hours post infection prior to intiation of growth. On the right: *Brucella* cell cycle as described during infection, starting at about 8 hours post-infection.

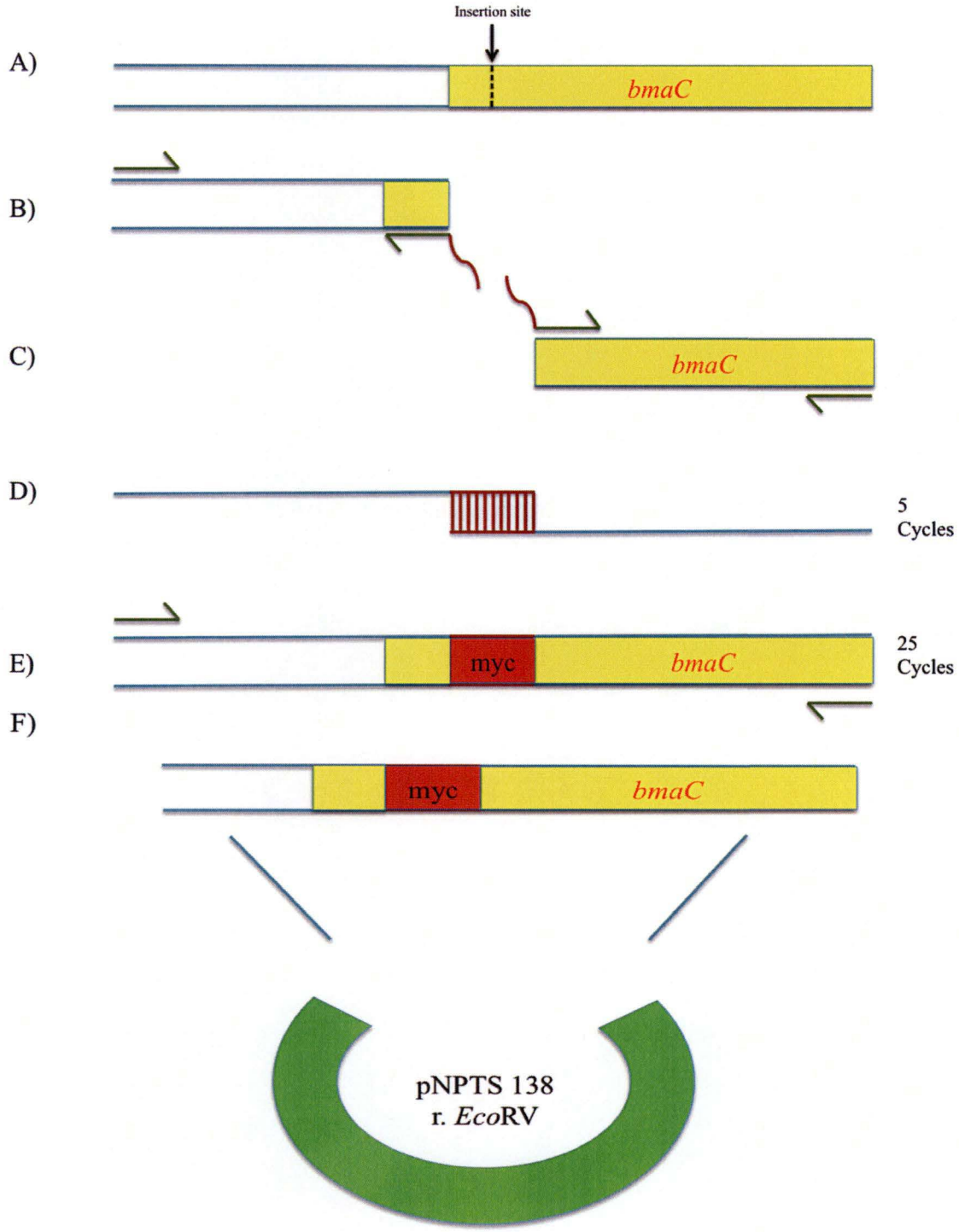


Figure 2: Cartoon displaying the allelic replacement vector construction strategy: A) A fragment of the wild type *bmaC* gene (in yellow), the tag insertion site is shown by the black arrow B) First PCR upstream of the tag insertion site (the reverse primer carries the myc-tag sequence in red). In this figure, all primers are shown with an half arrow at their 3' end. C) Second PCR downstream of the tag insertion site (the forward primer carries the myc-tag sequence in red) D) Five first cycles of the joining PCR where no primers are added. In this step, one strand of each PCR product A and B hybridize with each other, with their 3' end allowing the synthesis of the fusion product. E) The twenty-five last cycles of the joining PCR where only the forward primer of the upstream PCR and the reverse primer of the downstream PCR are added. F) Ligation of the final PCR product in the *Eco*RV restricted pNPTS138 vector.

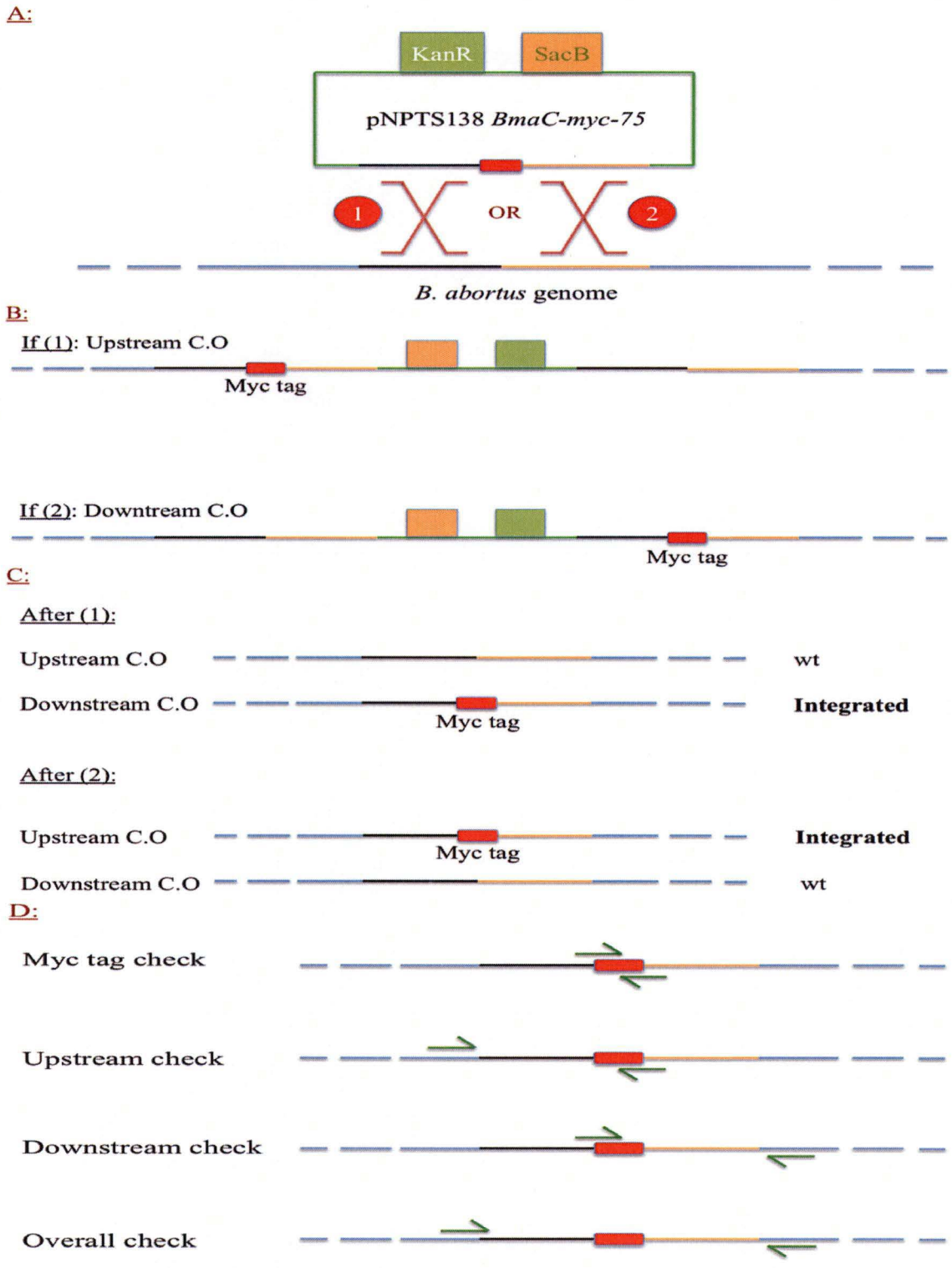


Figure 3: Cartoon of the allelic replacement in *B. abortus* using pNPTS138 *BmaC myc 75*. A) Schematic representation of the two possible theoretical crossing-over events between pNPTS138 *BmaC myc 75* and the genome of *B. abortus*. B) Possible results for plasmid integration, based on the first crossing over (C.O). C) Possible results for the plasmid excision depending on the localization of both first and second crossing over events. D) Positions of the primers hybridization sites used for the different diagnostic PCR allowing to check that the insertion occurred at the correct locus.

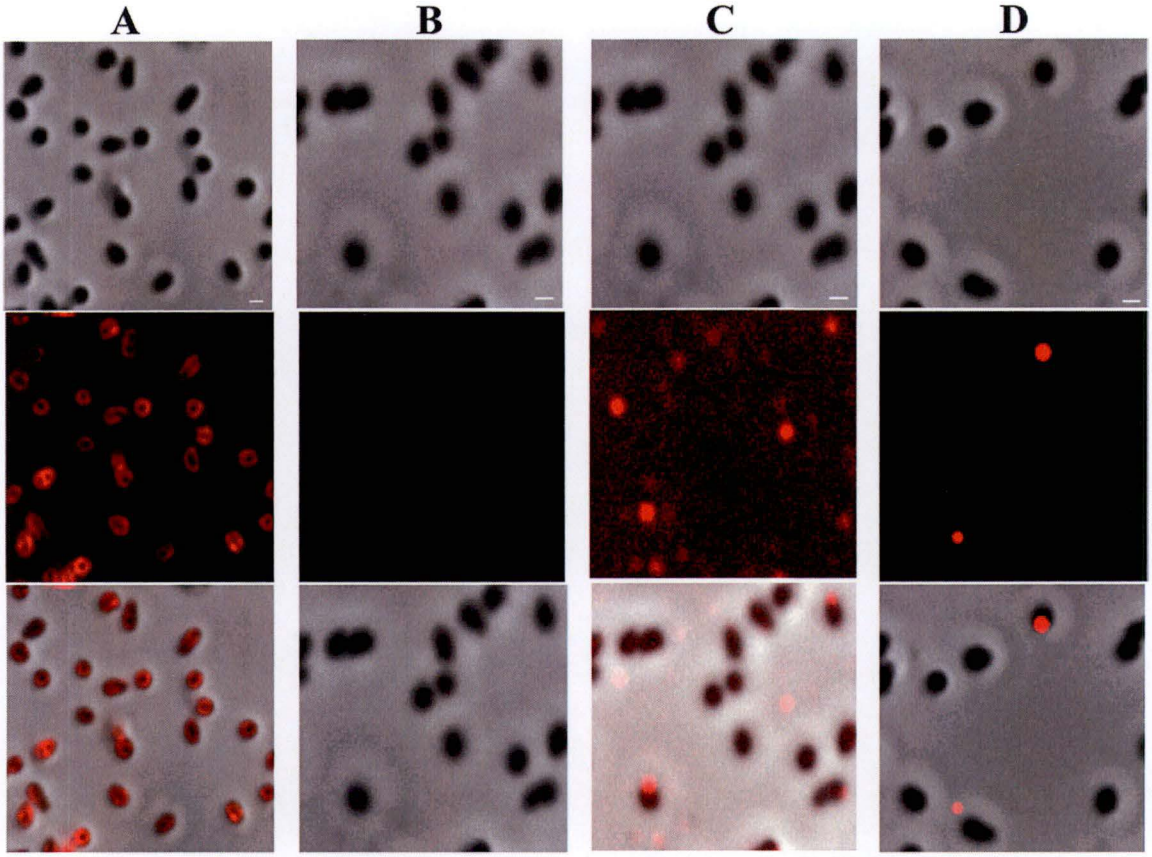


Figure 4 : Immunofluorescence results. For all four conditions, the top picture displays the phase contrast channel, the middle picture displays the Txred fluorescence channel, and the bottom picture shows the merging of the two first pictures. Scale bar is 1 µm. A) The Immunofluorescence performed against Omp1 as a positive control. This staining highlights an overall outer membrane signal. B) Immunofluorescence against myc in *B abortus* 2308 *bmaC*–myc 75. This condition uses the same LUTs settings as for the positive control and no Txred signal is detected. C) Immunofluorescence against myc in *B abortus* 2308 *bmaC*–myc 75 using modified LUTs settings allowing to detect very weak Txred signals. Some foci are associated to bacteria but others are not. D) The negative control, immunofluorescence against myc in wild type *B abortus* 2308 using the same LUTs settings as in condition C. The fluorescence patterns observed are similar to those observed in condition C, indicating that such a signal probably results from the background.