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La réponse de Brucella abortus au stress alkylant en culture et en infection

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Brucella abortus response to alkylating stress in culture and in infection

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La réponse *de Brucella abortus* au stress alkylant en culture et en infection

ROBA, Agnès

Résumé

Le patrimoine génétique de tout organisme est constamment sous la menace d'être endommagé. Les agents alkylants sont un exemple de cause possible de dommages à l'ADN. Pour se protéger contre les effets néfastes des dommages alkylants à l'ADN, les organismes vivants possèdent des systèmes de réparation dédiés aux dommages alkylants. Cela est vrai aussi pour la bactérie intracellulaire *Brucella abortus*, responsable d'une zoonose mondialement répandue, la brucellose. Lorsqu'elle infecte une cellule, on pense que *B. abortus* est sujette à différents stress, parmi lesquels l'exposition à des composés génotoxiques. De ce fait, les systèmes de réparation de l'ADN de *B. abortus* pourraient contribuer de manière importante à sa survie en milieu intracellulaire. Toutefois, il n'a jamais été démontré que *B. abortus* est réellement exposée à des composés alkylants génotoxiques en cours d'infection. Dans le présent travail, nous avons investigué l'alkylation de l'ADN de *B. abortus* au moyen d'un système rapporteur sensible aux dommages alkylants, qui permet une détection au niveau d'une seule bactérie. Nous avons d'abord démontré que ce système rapporteur était activé en milieu riche, après exposition à des agents alkylants. Nous avons ensuite cherché à caractériser la sensibilité et la spécificité du rapporteur en culture. Ensuite, nous avons également montré que le système rapporteur s'activait lors d'infection de deux types cellulaires, les cellules HeLa et les macrophages RAW 264.7. En conclusion, ces résultats suggèrent que *B. abortus* est bel et bien sujette à des dommages alkylants en cours d'infection.

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Abstract

DNA damage is a constant threat for every living organism. Alkylating agents are an example of genotoxic molecules susceptible to provoke DNA damage. To counteract the harmful effects of DNA alkylation damage, living organisms possess dedicated DNA repair systems. This holds true for *Brucella abortus,* an intracellular bacterium that is responsible for the worldwide zoonotic disease brucellosis. Throughout survival inside its host cells, *Brucella* is thought to endure many stresses, which possibly include exposure to genotoxic molecules. In that regard, DNA repair systems could play an important role in the survival of *B. abortus* inside its host. However, the occurrence of alkylating genotoxic assault during infection has never been established yet in *B. abortus*. In this work, we investigated DNA alkylation in *B. abortus*, using a new alkylation-responsive fluorescent reporter system which allows detection at the single-bacterium level. First, we showed that the reporter system was activated in rich medium culture, following treatment with alkylating agents. We then characterized the specificity and the sensitivity of the reporter system in culture. Second, we showed that during infection of HeLa cells and RAW 264.7 macrophages, the reporter system was turned on. These results therefore suggest that *B. abortus* actually faces alkylating stress during infection.

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"In this world, there are things you can only do alone, and things you can only do with somebody else. It's important to combine the two in just the right amount."

Haruki Murakami

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ABBREVIATIONS

Ada-C	C-terminal domain of Ada
Ada-N	N-terminal domain of Ada
AP site	Apurinic/apyrimidinic/abasic site
BCV	*Brucella*-containing vacuole
BER	Base excision repair
Cys38	Cysteine 38
Cys321	Cysteine 321
EMS	Ethyl methanesulfonate
ER	Endoplasmic reticulum
GFP	Green fluorescent protein
IFN-γ	interferon-γ
iNOS	Inducible Nitric Oxide Synthase
LPS	Lipopolysaccharide
MMR	Mismatch repair pathway
MMS	Methyl methanesulfonate
MNNG	Methylnitronitrosoguanidine
MNU	N-methylnitrosurea
MPT	Methyl phosphotriester
N1meA	N1-methyladenine
N3meA	N3-methyladenine
N3meC	N3-methylcytosine
N7meG	N7-methylguanine
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
O2meC	O2-methylcytosine
O2meT	O2methylthymine
O4meT	O4-methylthymine
O6meG	O6-methylguanine
PFA	Paraformaldehyde
PI	Post-infection
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SAM	S-adenosylmethionine
WT	Wild-type

INTRODUCTION

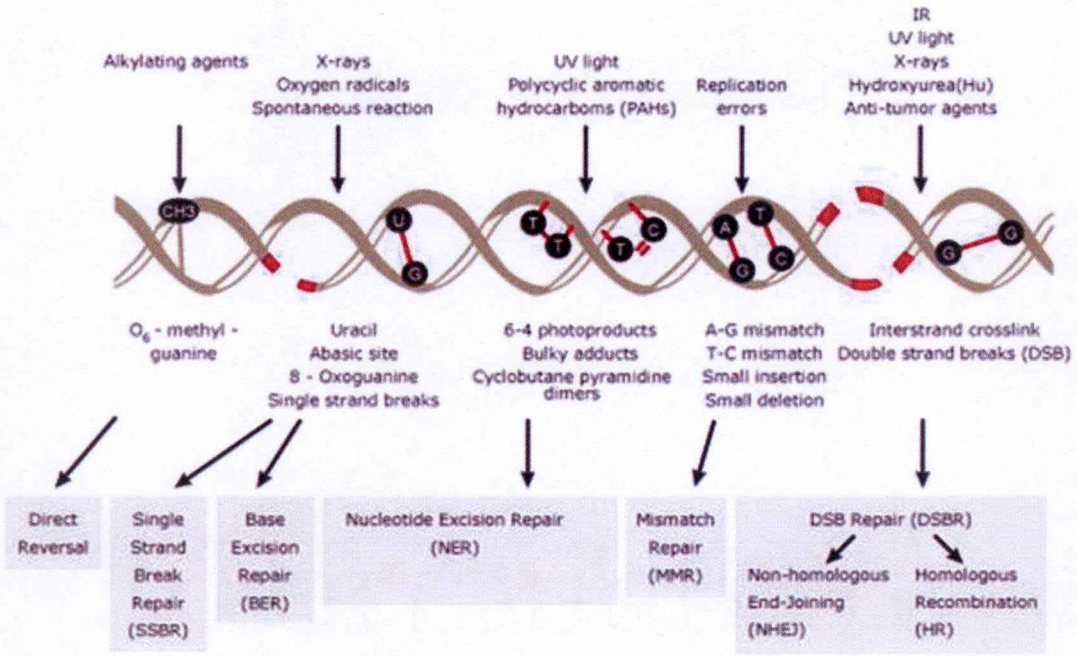


Figure 1 – DNA damage and repair mechanisms. Common DNA damaging agents (top); examples of DNA lesions induced by these agents (middle); and most relevant DNA repair mechanism responsible for the removal of the lesions (bottom). Abbreviations: (6–4)PP and CPD, 6–4 photoproduct and cyclobutane pyrimidine dimer, respectively (both induced by UV light); BER and NER, base- and nucleotide-excision repair, respectively; HR, homologous recombination; EJ, end joining (modified after Hoeijmakers, 2001).

INTRODUCTION

1. Protecting the genome

1.1 A question of survival

DNA is the repository of genetic information. Every living organism has to maintain the integrity of its genome to survive and successfully replicate. However, like every biological macromolecules, DNA is not inert and can be damaged by a variety of factors. If not repaired, damage caused to DNA can lead to cell death or can provoke mutation, and therefore loss of genetic information. Still, DNA is constantly exposed to assaults from external sources (chemicals, radiation) or endogenous sources (reactive by-products of the metabolism) (Drabløs et al. 2004).

The DNA double helix can be subject to a great variety of lesions. Those lesions can range from single base modifications, such as the addition of a methyl group to a DNA base, to single or double-strand breaks, in which one or both strands of the double helix are severed. Other DNA lesions include DNA crosslinks, which occurs when two bases are covalently joined, aberrant base pairing, apyrimidinic or apurinic (AP) sites and bulky adduct formation (Figure 1) (van der Veen and Tang 2015).

In order to counteract the deleterious effects of DNA damage, every living organism has evolved multiple DNA repair mechanisms. Given their fundamental importance, these mechanisms are generally conserved from bacteria to human. The repair of double-strand breaks is mediated by non-homologous end joining (NHEJ) or by homologous recombination. DNA crosslinks and bulky adducts are commonly reversed by the nucleotide excision repair (NER) pathway. Aberrant base pairings or "mismatches" are repaired by the mismatch repair pathway (MMR). Finally, single-base modifications are either removed by direct repair, or by the base excision repair (BER) pathway, which also repairs single-strand breaks. Unlike all the other repair mechanisms, direct repair occurs without breakage of the DNA backbone (Figure 1) (Ahmad, Nay, and Connor 2013). Importantly, even though these mechanisms are often presented as operating independently, some of these pathways can have overlapping functions. The consequent redundancy in function further helps to ensure survival (van der Veen and Tang 2015).

1.2 DNA repair in pathogenic bacteria: a virulence factor?

During colonization and persistence inside a host, pathogenic microorganisms are exposed to a hostile environment resulting from the host immune defenses. A key element of the innate immune response is the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which damage pathogen molecules, especially DNA (van der Veen and Tang 2015). In that regard, maintenance of genome integrity is of crucial importance for pathogens. Especially, microorganisms that live in close association with their host for long periods are under the constant threat of DNA damaging agents (Gorna, Bowater, and Dziadek 2010).

In the context of infection, pathogenic bacteria are classically studied for their virulence properties. Therefore, much attention has been paid to their adhesion and invasion molecules, toxins and secretion systems, which are considered as "classical virulence factors." However, the success of a bacterial pathogen does not rely solely on its ability to promote virulence. To successfully survive and replicate inside a host, pathogenic bacteria need to be well adapted to specific microenvironments at distinct stages of infection. The adaptation of bacteria to such microenvironments can be enabled by classical virulence factors, but also by "housekeeping"

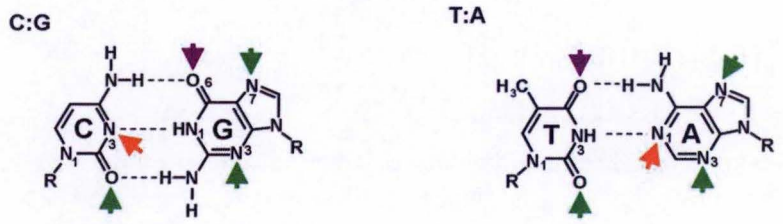


Figure 2 – Sites of methylation on the DNA bases. Purple arrows indicate sites repaired by Ada. Green arrows indicate sites repaired by AlkA, and orange arrows indicate sites repaired by AlkB (adapted from Ahmad, Nay, & Connor, 2013 and Sedgwick & Lindahl, 2002).

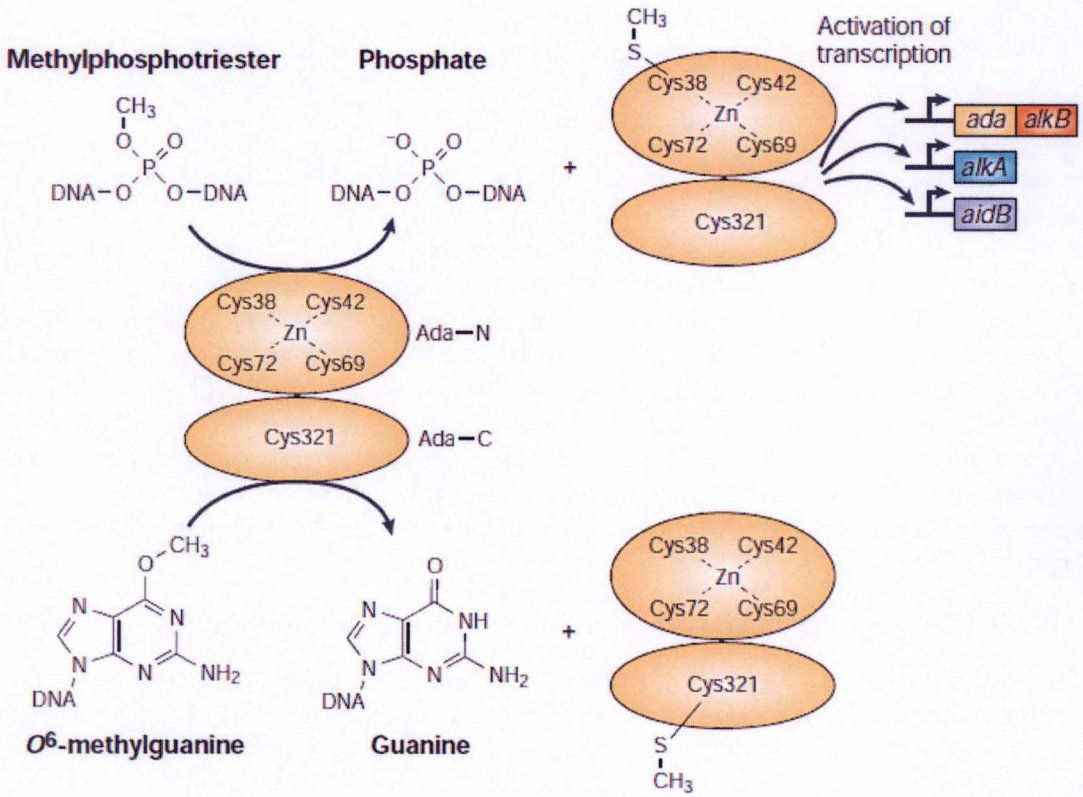


Figure 3 - Demethylation of stable O-methylated-DNA lesions by the *Escherichia coli* Ada DNA-methyltransferase. Ada has two active domains (Ada-N and Ada-C), which are separated by a hinge region. The carboxy-terminal domain (Ada-C) directly demethylates O6-methylguanine (O6-meG) in DNA to regenerate guanine (lower part). It transfers the methyl adduct from O6-meG to its active-site cysteine 321 residue. The resulting self-methylated Ada-C domain is irreversibly inactivated. The amino-terminal domain (Ada-N) contains a tightly bound zinc ion that is coordinated by four Cys residues in a zinc-thiolate centre. The metal-ligand activates the Cys38 residue. Ada-N demethylates methylphosphotriesters in DNA by transferring the methyl group to the metalloactivated Cys38 residue (upper part). Ada that is self-methylated at Cys38 becomes a gene activator. It binds to the promoters of the genes (*ada-alkB*, *alkA* and *aidB*) that are induced in the adaptive response to methylating agents, and causes their transcription. Methylphosphotriesters in DNA are therefore the inducing signal of this response (Sedgwick 2004).

functions, that usually receive less interest. For example, DNA repair systems are housekeeping activities that are highly conserved in bacteria, but some pathogenic bacteria have specialized DNA repair systems that allow their adaptation to genotoxic stresses during infection (van der Veen and Tang 2015). The analysis of how genomic integrity of pathogenic bacteria is maintained during infection is thus an interesting area of research.

The importance of such DNA repair systems can be highlighted using mutants lacking specific DNA repair genes. For example, in the enteropathogenic bacteria *Salmonella typhimurium*, a double mutant for two components of the BER pathway, Nfo and XthA, showed an increased sensitivity to nitric oxide, an RNS, and was also attenuated in mice (Richardson et al. 2009). Interestingly, the BER pathway mostly repairs single-base modifications, which are notably caused by the oxidative damage resulting from ROS and RNS (van der Veen and Tang 2015). These single-base modifications are thereby more likely to occur in the context of infection. Aside from oxidative stress, single-base modifications can also arise from alkylating stress.

2. How to fight alkylating stress?

2.1 Alkylating agents

Alkylating agents comprise a wide family of DNA damaging agents. These chemicals can transfer alkyl groups (e.g. methyl or ethyl groups) onto DNA, thereby altering its structure and function. Alkylating agents are present in the environment, notably as components of tobacco smoke and fuel combustion products (Ballschmiter 2003; Hecht 1999). Endogenously, alkylating agents are proposed to arise as by-products of the metabolism or from cellular methyl donors such as the cofactor S-adenosylmethionine (SAM) (Rydberg 1982; Taverna and Sedgwick 1996).

Alkylating agents can generate a wide range of DNA lesions. They can react with the oxygen (O) and nitrogen (N) atoms of the DNA bases as well as the O atoms of the phosphodiesters. The resulting lesions can be cytotoxic, mutagenic or neutral to the cell, depending on the position of the DNA adducts. Major products of DNA alkylation include N7-methylguanine (N7meG), N3-methyladenine (N3meA) and O6-methylguanine (O6meG) (Figure 2). While the most frequent lesion N7meG is relatively innocuous for the cell, the N3meA lesion blocks DNA replication and is therefore cytotoxic (Fu, Calvo, and Samson 2012). By contrast, the O6meG site tends to mispair with thymine during DNA replication and is therefore mutagenic. Other sites of DNA alkylation comprise N1-methyladenine (N1meA), N3-methylcytosine (N3meC), O4-methylthymine (O4meT) and methyl phosphotriesters (MPT) where the methyl group is added on the O atoms of the phosphodiesters. (Drabløs et al. 2004; Fu, Calvo, and Samson 2012; Sedgwick and Lindahl 2002). The pattern of DNA lesions depends on the nature of the alkylating agent, its reaction mechanism, the type of alkyl group added and the DNA substrate (single-stranded or double-stranded). For example, the two sites N1A and N3C are protected by base-pairing in double-stranded DNA (Figure 2) (Fu, Calvo, and Samson 2012; Sedgwick and Lindahl 2002).

2.2 Repair of DNA alkylation damage – the adaptive response in *E. coli*

To counteract the harmful effect of alkylating agents, living organisms have evolved dedicated DNA repair systems. Owing to the diversity of DNA alkylation lesions, these DNA repair systems are numerous and complex. DNA alkylation response has been extensively studied in the model bacterium *Escherichia coli*. When *E. coli* cells are exposed to low doses of alkylating agents, they mount an inducible response that increases their resistance to those same alkylating agents (Lindahl and Sedgwick 1988). This so-called adaptive response involves four genes whose expression is increased following DNA alkylation damage: *ada*, *alkB*, *alkA* and *aidB*

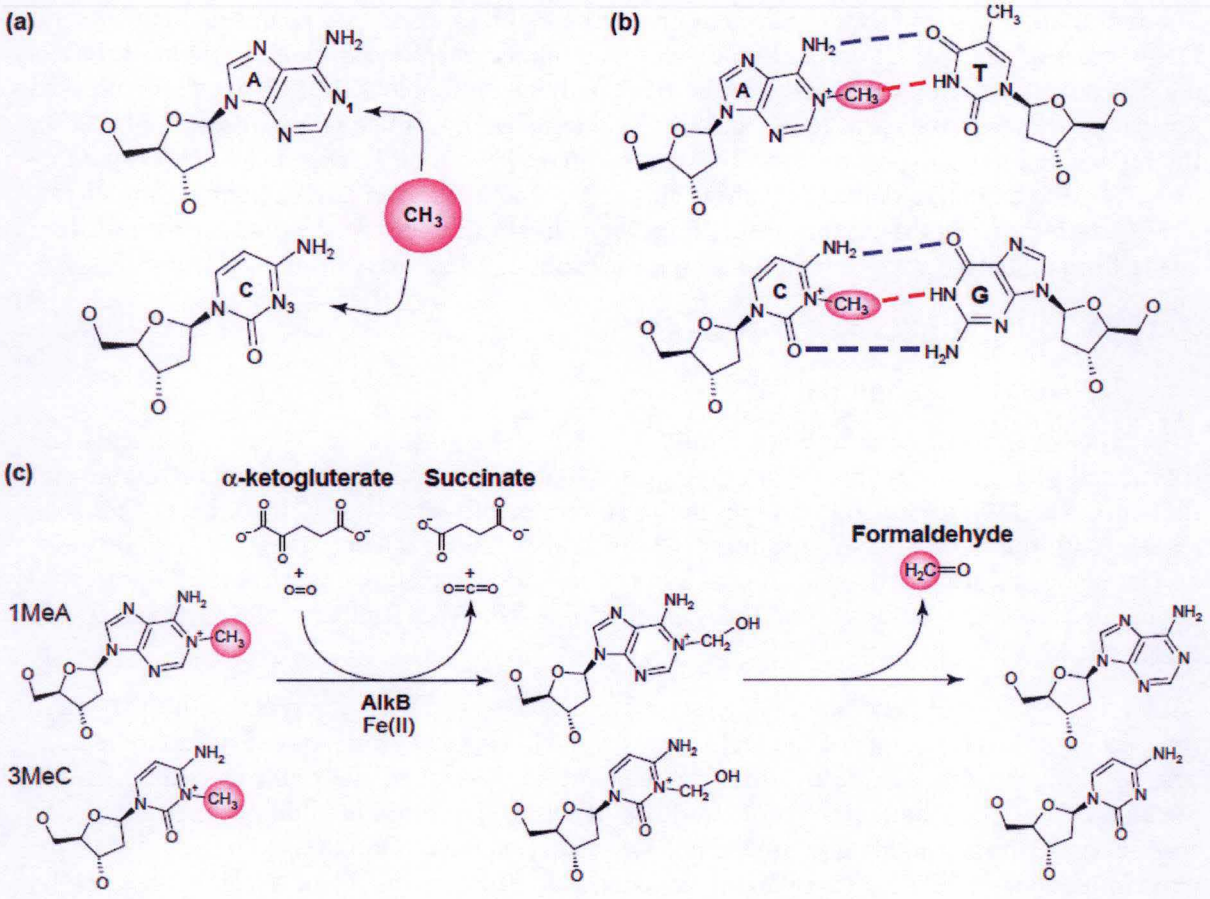


Figure 4 - N1-methyladenine (1MeA) and N3-methylcytosine (3MeC) lesions repaired by AlkB. The N1 and N3 positions of adenine and cytosine are equivalent because: (a) in single stranded (ss) DNA they are both susceptible to attack by a methylating agent, and (b) in double stranded (ds) DNA they are both shielded from attack. 1MeA and 3MeC lesions can form in regions of ss DNA and, upon re-annealing of the double helix, these lesions can be found in ds DNA. Methyl lesions (pink) are buried within the double helix of DNA but should disrupt hydrogen bonding with the complementary strand. Broken blue lines represent hydrogen bonds and broken red lines represent disrupted hydrogen bonds. (c) 1MeA and 3MeC lesions in DNA are repaired by AlkB-catalyzed oxidative demethylation. The cytotoxic methyl group is shown in pink for each lesion. The reaction requires α-ketogluterate, O_2 and Fe(II) and generates succinate and CO_2. Oxidized methyl groups on 1MeA and 3MeC are removed in the form of formaldehyde, generating the normal DNA bases adenine (top) and cytosine (bottom) by a direct reversal mechanism (Begley and Samson 2003).

(Figure 3). The induction of those genes increase the cell's resistance to alkylating agents because Ada, AlkB and AlkA are enzymes that catalyze the repair of alkylated DNA. The function of AidB is still unclear in *E. coli*, but it is thought to contribute to resistance against alkylating agents by destroying the alkylating agents rather than by DNA repair (Mielecki and Grzesiuk 2014). In the present section, the adaptive response and the different mechanisms of DNA alkylation repair in *E. coli* will be briefly introduced.

2.2.1 The Ada protein and the adaptive response

The Ada protein is a bifunctional protein that on the one hand repairs methylated bases and on the other hand regulates the whole adaptive response. Ada is indeed named after "adaptive". This protein comprise two domains that function independently and that are separated by a hinge region susceptible to proteolytic cleavage (Mielecki and Grzesiuk 2014). The 19kDa C-terminal domain of Ada (Ada-C) repairs the highly mutagenic lesions O6meG and O4meT. This domain directly demethylates the lesion and transfers the aberrant methyl group onto its cysteine 321 (Cys321) residue (Figure 3). Importantly, this transfer is irreversible and stoichiometric, meaning that the DNA-methyltransferase can only act once. The 20kDa N-terminal domain (Ada-N) transfers the methyl group from phosphotriesters onto its cysteine 38 (Cys38) residue, also in an irreversible manner. The irreversible methylation of Cys38 converts Ada protein into a strong transcriptional activator of the inducible genes of the Ada response, *ada, alkB*, *alkA* and *aidB* (Figure 3). Therefore, Ada-N acts as a chemosensor that coordinates the adaptive response depending on the alkylation status of *E. coli* DNA (Mielecki and Grzesiuk 2014). In the absence of DNA methylation, *E. coli* contains on average only one Ada molecule per cell (Uphoff et al. 2016). Upon alkylation of the DNA and consequently activation of the adaptive response, Ada is 1000-fold amplified (Mielecki, Wrzesinski, and Grzesiuk 2015). As a result, cellular resistance to alkylating agents is greatly enhanced. The Ada response is eventually repressed by high concentration of unmethylated Ada protein (>200 molecules per cell), which accumulates when all MPT are repaired (Saget and Walker 1994).

In *E. coli*, the mutagenic O6meG lesion is also repaired by another enzyme, Ogt (**O6**-methyl**g**uanine-DNA methyl**t**ransferase) which is expressed constitutively (Sedgwick 2004). Interestingly, Ada-N is the only enzyme known to repair MPT. Because the MPT lesion is innocuous and is not repaired constitutively by the cell, it serve as a good signal for the induction of the adaptive response (Sedgwick and Lindahl 2002). Consistent with this, eukaryotes, which do not display any adaptive response to alkylating agents (with the exception of one known organism that will be discussed later in the text), do not possess any methyltransferase that repairs the MPT. As this lesion is neither mutagenic nor cytotoxic, it is possible that MPT repair is not required in organisms that do not have an inducible response (Sedgwick and Lindahl 2002).

2.2.2 AlkB – an oxidative demethylase

The two genes *ada* and *alkB* are organized into an operon in *E. coli* (Kondo et al. 1986). AlkB catalyzes the reversion of the two cytotoxic lesions N1meA and N3meC to adenine and cytosine respectively, through an oxidative demethylation mechanism. To perform the reaction, AlkB uses non-heme iron Fe(II) as cofactor and α-ketoglutarate and O_2 as cosubstrates (Figure 4) (Sedgwick and Lindahl 2002). The enzyme first catalyzes the hydroxylation of the aberrant methyl group to form an unstable hydroxymethyl intermediate, which then spontaneously decomposes to release the native base as well as formaldehyde (Figure 4) (Aravind and Koonin 2001). In *E. coli*, AlkB is the only enzyme to perform oxidative demethylation of N1meA and N3meC. No other constitutively expressed enzyme backs up this activity (Sedgwick and Lindahl 2002).

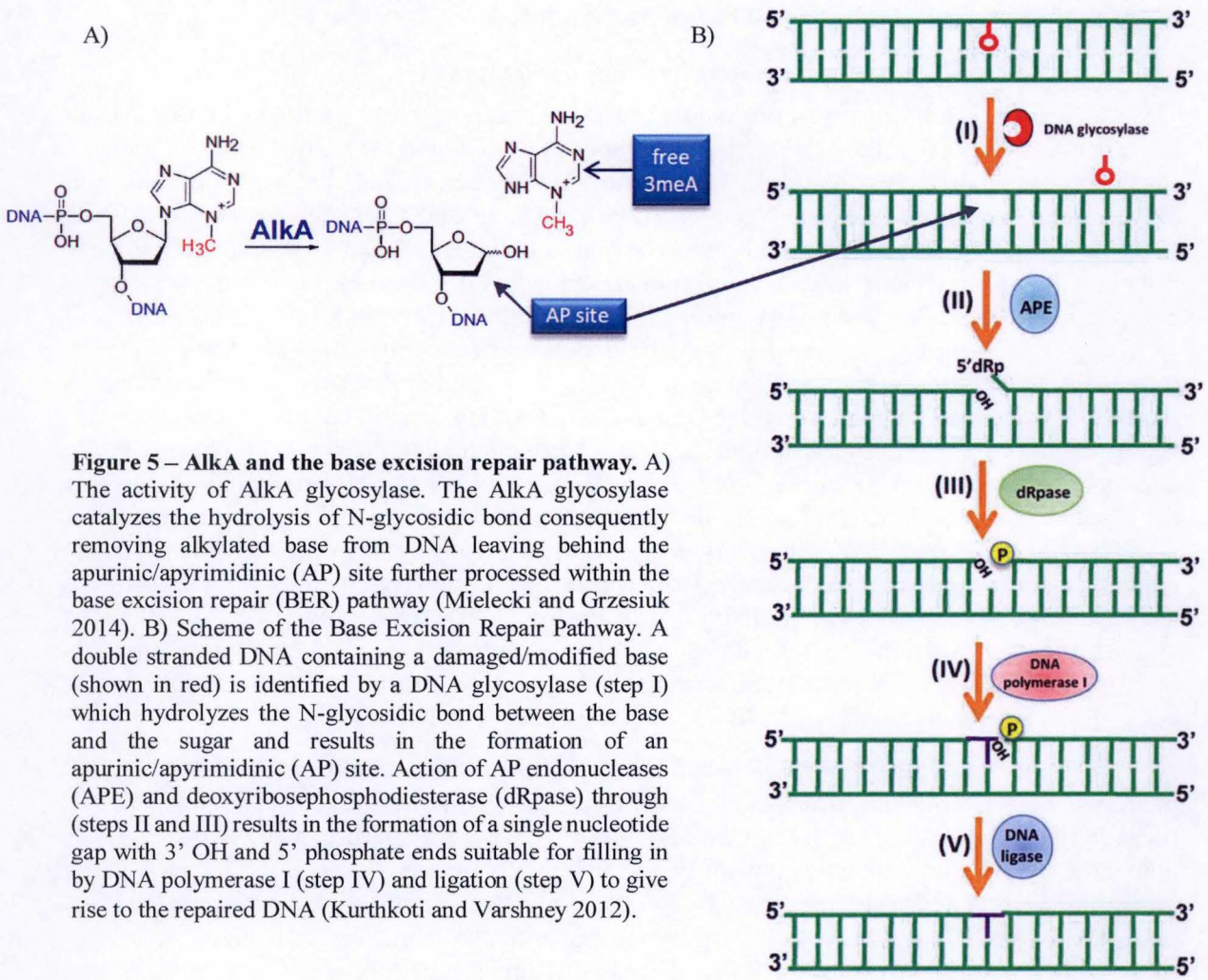


Figure 5 – AlkA and the base excision repair pathway. A) The activity of AlkA glycosylase. The AlkA glycosylase catalyzes the hydrolysis of N-glycosidic bond consequently removing alkylated base from DNA leaving behind the apurinic/apyrimidinic (AP) site further processed within the base excision repair (BER) pathway (Mielecki and Grzesiuk 2014). B) Scheme of the Base Excision Repair Pathway. A double stranded DNA containing a damaged/modified base (shown in red) is identified by a DNA glycosylase (step I) which hydrolyzes the N-glycosidic bond between the base and the sugar and results in the formation of an apurinic/apyrimidinic (AP) site. Action of AP endonucleases (APE) and deoxyribosephosphodiesterase (dRpase) through (steps II and III) results in the formation of a single nucleotide gap with 3' OH and 5' phosphate ends suitable for filling in by DNA polymerase I (step IV) and ligation (step V) to give rise to the repaired DNA (Kurthkoti and Varshney 2012).

2.2.3 AlkA – a DNA glycosylase initiating the BER pathway

In *E. coli*, the most cytotoxic lesion N3meA is repaired by the DNA glycosylase AlkA. In contrast to enzymes such as Ada and AlkB that directly reverse the methylation, AlkA is only initiating a broader process which is the BER pathway. AlkA first cleaves the glycosidic bond linking the methylated base and the sugar-phosphate backbone, thereby leaving an AP site which is then repaired by the other enzymes of the BER pathway (Figure 5). *E. coli* also possesses a constitutively expressed enzyme encoded by the *tag* gene which performs the same activity. The Tag protein is however much more specific for the repair of N3meA than AlkA, which also recognizes minor lesions such as 3meG, O2meC and O2meT (Sedgwick 2004).

2.3 The adaptive response in other (micro)organisms

Independently, the inducible genes of the adaptive response to alkylation are broadly conserved across both prokaryotes and lower and higher eukaryotes. But this is not necessarily the case for the adaptive response and its Ada-mediated regulation. Indeed, the vast majority of the eukaryotes, including mammals, do not display any adaptive response. Nevertheless, such a response has been observed in the lower eukaryote and human pathogen *Aspergillus fumigatus* (O'Hanlon et al. 2012). In prokaryotes, the adaptive response is not a generality. It has been experimentally observed notably in *Pseudomonas aeruginosa Bacillus subtilis* and *Xanthomonas maltophilia*, whereas it doesn't seem to occur in *Haemophilus influenzae*, *Salmonella typhimurium*, *Staphylococcus aureus* and *Caulobacter crescentus,* to name but a few (Colombi and Gomes 1997; Mielecki, Wrzesinski, and Grzesiuk 2015; Sedgwick and Lindahl 2002). In this section, we will briefly discuss the response against DNA alkylation in human and in the human pathogen *Mycobacterium tuberculosis*.

2.3.1 Human homologues of DNA alkylation repair genes

As previously mentioned, mammals do not show any adaptive response to alkylating agents. However, they do possess different homologues of the inducible genes of the adaptive response in *E. coli*. The fact that these genes have been conserved from bacteria to humans further highlights their crucial importance for survival. Repair of O6meG is a function that is extremely well conserved across prokaryotes and eukaryotes, including humans. The human homologue of *ada-C/ogt* is termed MGMT (for O6-**m**ethyl**g**uanine-DNA **m**ethyl**t**ransferase) (Ahmad, Nay, and Connor 2013; Drabløs et al. 2004). However, the repair of MPT, which is performed by Ada-N in *E. coli*, has not been observed in eukaryotes (Ahmad, Nay, and Connor 2013). *alkB* homologues are also found in almost all living organisms. Notably, *alkB* is conserved from bacteria to humans who even possess 9 homologues of *alkB,* ALKBH1-8 and FTO (Duncan et al. 2002; Gerken et al. 2009; Kurowski et al. 2003). Although simple eukaryotes like *Saccharomyces cerevisiae* have an AlkA homologue, mammals do not appear to have one. AlkA function is indeed performed by an unrelated DNA glycosylase in mammals (Drabløs et al. 2004).

2.3.2 DNA alkylation response in the intracellular pathogen Mycobacterium tuberculosis

The causative agent of tuberculosis, *Mycobacterium tuberculosis*, is an intracellular pathogen which survives and replicates inside human macrophages. *M. tuberculosis* is primarily found inside phagosomes, and is able to prevent fusion of phagosomes with lysosomes (Gorna, Bowater, and Dziadek 2010). Within its intracellular niche, *M. tuberculosis* can persist for decades (Gorna, Bowater, and Dziadek 2010). In the hostile environment of activated macrophages, *M. tuberculosis* is probably exposed to a variety of DNA damaging agents (Gorna, Bowater, and Dziadek 2010). Accordingly, DNA repair systems of *M. tuberculosis* are expected to be of great importance for long-term survival of the bacterium. Inside phagosomes,

M. tuberculosis is probably exposed to alkylating agents, resulting from nitrosation reactions between nitrous anhydride and amines or amides. However, the existence of such a stress in this context has never been shown until now (Durbach et al. 2003).

In order to determine the relevance of DNA alkylation repair in *M. tuberculosis*, homologs of the gene involved in DNA repair systems described in section 2 have been investigated. All the genes involved in the adaptive response in *E. coli* have homologs in *M. tuberculosis* (Yang et al. 2011). However, the genomic organization of those genes is different. For example, the two functional domains of Ada are found on two separated proteins, AdaA and AdaB, which remove the methyl of MPT and O6meG respectively (Miggiano et al. 2013). Furthermore, the AdaA domain is fused to AlkA in *M. tuberculosis*, and this fusion constitutes an operon with the *adaB* gene (Yang et al. 2011).

An inducible response to alkylating agents has been observed in *M. tuberculosis*, as the expression of *adaA-alkA* and *adaB* was increased after exposure to an alkylating agent, methylnitronitrosoguanidine (MNNG). However, the expression of *aidB* and *alkB* was not significantly upregulated after treatment of the bacteria with this same agent (Yang et al. 2011).

Mutants lacking the operon comprising *adaA-alkA* and *adaB* showed increased sensitivity to alkylating agents *in vitro* but displayed no growth impairment *in vivo* (Durbach et al. 2003). Consistent with this, *M. tuberculosis* also possesses two genes coding for putative N3meA glycosylase, *tagA* and *mpg*, which could substitute for AlkA activity (van der Veen and Tang 2015).

3. *Brucella abortus*

3.1 An overview of the brucellosis

Bacteria of the genus *Brucella* are responsible for a worldwide zoonosis termed brucellosis, which affects a wide range of mammals including humans (Pappas et al. 2006). The brucellae are Gram-negative pathogens that belong to the class of the α-proteobacteria. This class also includes bacteria of the genera *Bartonella, Rickettsia, Agrobacterium, Sinorhizobium,* and *Caulobacter*. In animals, *Brucella* spp. mainly affect the reproductive system (Moreno and Moriyon, 2006). *Brucella abortus, Brucella melitensis* and *Brucella suis* respectively cause infertility and abortion in cattle, goats and sheep, and swine (Moreno and Moriyon, 2006). Those three strains can also accidentally infect humans and cause a debilitating disease named "undulant fever" or Malta fever (Moreno and Moriyon, 2006). The main clinical manifestations of this disease are undulant fever and weakness. In the absence of treatment, human brucellosis becomes chronic and can lead to musculoskeletal, cardiac and neurological complications (Dean et al. 2012). However, human brucellosis is usually not transmitted from person to person and can be treated with antibiotics. Humans are typically infected by ingestion of dairy products or by direct contact with infected animals (Pappas et al. 2006).

Brucella spp. are often referred to as facultative intracellular parasites, but it would be more appropriate to term them "facultatively extracellular intracellular parasites" (Moreno and Moriyon 2002). Indeed, even if they are relatively easy to cultivate on rich media, these bacteria are not found free-living in the environment and are almost exclusively found intracellularly inside their host (Moreno and Moriyon, 2006). *Brucella* is able to survive and replicate inside a variety of host cells. These bacteria notably successfully replicate inside professional phagocytes such as macrophages or dendritic cells (Archambaud et al. 2010). On the one hand, these phagocytes provide *Brucella* with a replicative niche protected from components of the immune system such as antibodies and complement, and on the other hand, *Brucella* uses these cells as vehicles for their systemic dissemination (Roop et al. 2009). *Brucella* also invades and

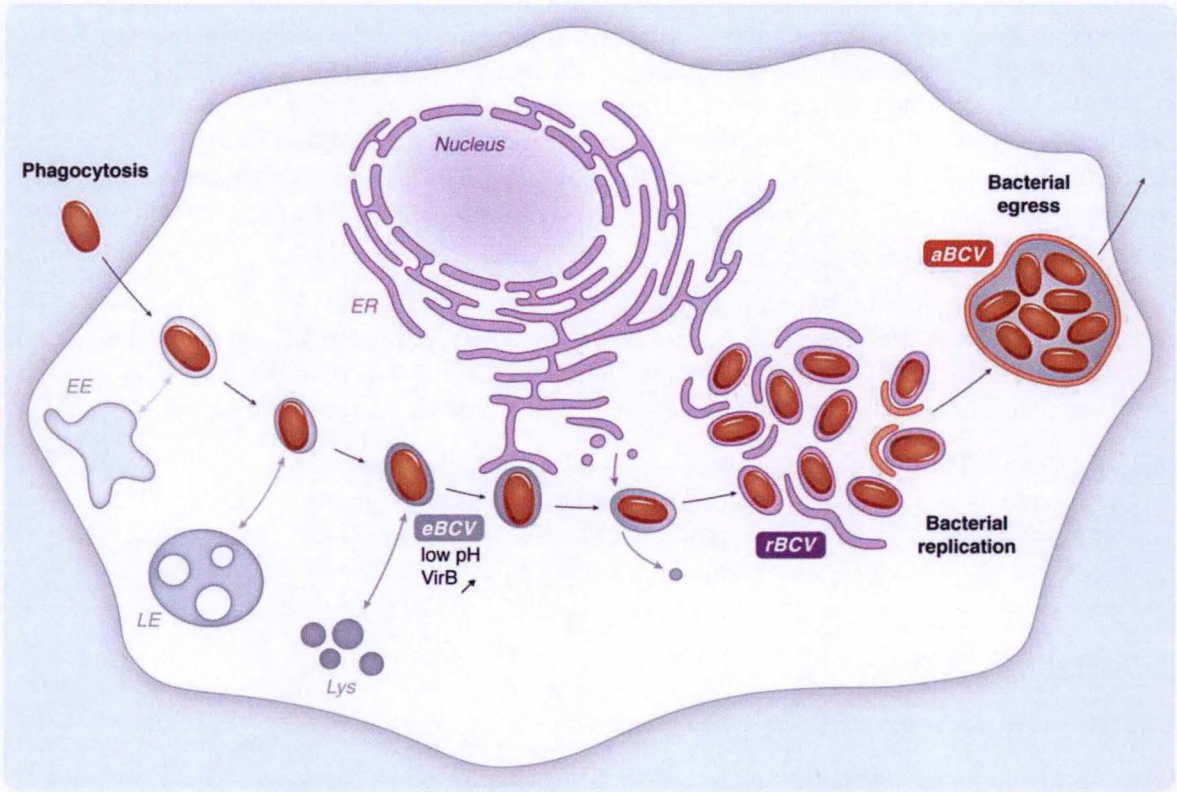


Figure 6 - Model of *Brucella* intracellular trafficking in mammalian cells. Upon entry, *Brucella* resides within the BCV, which undergoes interactions with early endosomes (EE), late endosomes (LE) and partially fuses with lysosomes (Lys) to become the eBCV. The eBCV provides cues for induction of the VirB T4SS, which delivers effector proteins that control eBCV interactions with ER exit sites (ERES). The small GTPases Sar1 and Rab2 (via its interaction with the *Brucella* effector RicA) are required for rBCV biogenesis and subsequent bacterial replication in rBCVs. Following replication in the ER, rBCVs are converted into autophagic aBCVs via a process involving the autophagy initiation proteins Beclin1, ULK1 and ATG14L. aBCVs promote completion of the *Brucella* intracellular cycle by facilitating bacterial egress (modified after Celli, 2015).

replicates inside placental trophoblasts in pregnant mammals. Extensive proliferation of the bacteria in the placenta of a pregnant mammal can ultimately cause its disruption, leading to abortion or the birth of a weak and infected offspring (Roop et al. 2009). This pathogen is usually studied in cellular models of infection such as macrophages, trophoblasts but also HeLa cells. Mouse models of infection have also been developed (Moreno and Moriyon 2006).

3.2 *Brucella*'s trafficking

The mechanism underlying the entry of *B. abortus* into macrophages and dendritic cells is still largely unknown. Nevertheless, interactions with the O-chain of smooth lipopolysaccharide (LPS) and lipid rafts have been suggested to be required for successful replication and survival inside the cells. Rough mutants which lack the O-chain do not enter via the lipid rafts and are rapidly degraded by phagosome-lysosome fusion (Naroeni and Porte 2002; Porte et al. 2003).

Following successful entry into its host cell, *B. abortus* is enclosed in a membrane-bound compartment named *Brucella*-containing vacuole (BCV). In macrophages and epithelial cells, the BCV transiently and sequentially interacts with the endocytic pathway, resulting in the acquisition of markers of the early and late endosomes (Celli et al. 2003; Starr et al. 2008). The BCV also undergoes acidification to pH 4-4.5, but lysosomal hydrolases have never been detected inside the BCV, suggesting that interactions between BCV and lysosomes are restricted, but not completely inhibited (Celli 2015). BCV acidification is determinant for further survival of *Brucella* inside the cell because it provides a signal for the induction of a major virulence factor, the VirB type IV secretion system (Starr et al. 2008). This apparatus allows *Brucella* to reach its replicative niche. Indeed, following BCV acidification and subsequent *Brucella virB* expression, the BCV segregates from the endosomal pathway and undergoes sustained interaction with the endoplasmic reticulum (ER). *B. abortus* eventually replicates inside those ER-derived vacuoles (Figure 6), in most cell types tested so far (Celli et al. 2003; Starr et al. 2008).

One peculiarity of *B. abortus* intracellular trafficking is that it can be divided into two distinct phases: one non-proliferative and one replicative. During the first phase of the infection, *Brucella* traffics along the endocytic pathway, interacting with endosomes (Celli et al. 2003; Starr et al. 2008). This phase is characterized by growth arrest of the bacteria and their blockage in the G1 step of their cell cycle (Deghelt et al. 2014). The second step of infection is characterized by proliferation of *Brucella* in ER-derived vacuoles. This biphasic trafficking has been observed in professional phagocytes such as RAW 264.7 macrophages, as well as in HeLa cells. Because the trafficking of *B. abortus* has been well characterized in HeLa cells and seems to recapitulate what was observed in RAW 264.7 macrophages, although with a slightly different kinetics, HeLa cells can be considered as a good cellular model of infection as these cells are easier to manipulate for microscopy experiments (Celli 2015).

Two hypotheses currently exist to explain the egress and the cell-to-cell spreading of *Brucella*. The first hypothesis states that the host cell would simply rupture when bacteria become too numerous. Consequently released bacteria would then re-infect surrounding cells (Moreno and Moriyon 2006). The second hypothesis suggests that *Brucella* would subvert some components of the autophagy machinery to complete its intracellular cycle and infect neighboring cells (Figure 6) (Starr et al. 2012). It should be noted that these two hypotheses are not mutually exclusive.

3.3 Repair of alkylation damage in *B. abortus*

Although *Brucella* controls the trafficking of the BCV inside macrophages and epithelial cells, the bacterium probably encounters important stresses along infection, such as nutrient

Table 1 - *B. abortus* homologues of the DNA alkylation repair genes of *E. coli*. % identity was calculated between the *E. coli* reference and the *B. abortus* homologue, at the protein level

Gene	ORF	Identity	Putative function
ada1	BAB1_0398	51%	Repair of MPT/O6meG?
ada2	BAB2_0347	33%	Repair of MPT/O6meG?
ogt	BAB1_0185	37%	Repair of O6meG
alkA	BAB1_1661	29%	N3meA DNA glycosylase
alkB	BAB2_0704	63%	Oxidative demethylation of N1meA and N3meC
aidB	BAB2_0642	45%	Unknown (destruction of alkylating agents?)

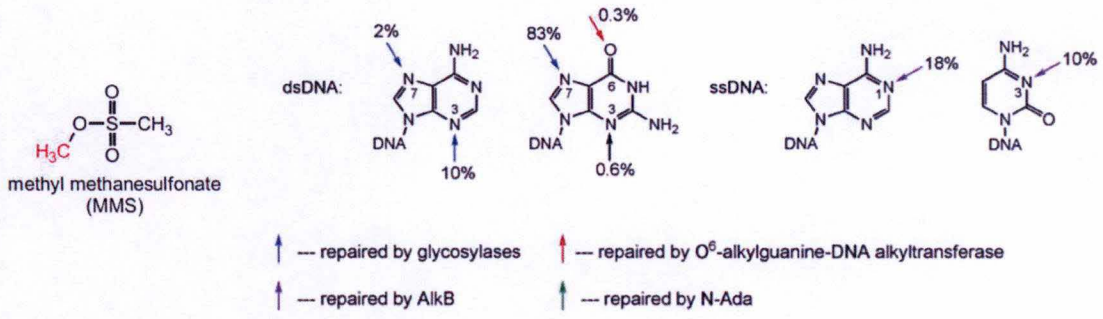
starvation, acidic pH, reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Roop et al. 2009). ROS and RNS are unstable and toxic compounds that can react with diverse macromolecules including proteins and DNA. Accordingly, DNA repair systems could play an important role in the intracellular persistence of *B. abortus* as they would protect its genome from surrounding genotoxic molecules. Furthermore, *B. abortus* can survive inside its host for decades, like *M. tuberculosis*. We therefore could imagine that these bacteria require robust DNA repair mechanisms to persist in long-term close association with their host.

According to genomic data, all the genes involved in the previously described adaptive response in *E. coli* have homologs in *B. abortus* (Table 1) (Katy Poncin, unpublished data). Interestingly, the *ada* gene is even duplicated in *B. abortus*. The genomic organization of those genes is also different from that of *E. coli* as *ada* and *alkB* are not organized into an operon in *B. abortus*. Currently, little is known about the regulation of the expression of those alkylation response genes in *B. abortus*.

In the α-proteobacterium *Caulobacter crescentus*, neither *ada* nor *alkB* is induced by MMS methanesulfonate (MMS, Box 1), which suggests that an adaptive response is absent in this bacterium (Colombi and Gomes 1997). *C. crescentus* possess two homologs of the *ada* gene, which slightly differ to that of *E. coli* (Martins-Pinheiro, Marques, and Menck 2007). In *E. coli*, the regulatory domain Ada-N comprises two protein motifs that are thought to be involved in DNA binding and transcription activation, respectively an ada-zinc binding site and an HTH-AraC motif (Myers et al. 1992; Rhee et al. 1998). In the two *ada* genes of *C. crescentus,* each Ada-N regulatory domain lack either one of the protein motifs or the other (Martins-Pinheiro, Marques, and Menck 2007). Consistent with this, preliminary data suggest that *B. abortus* does not display an adaptive response either. Indeed, contrarily to what was observed in *E. coli* (Schendel et al. 1978), *B. abortus* does not show increased resistance to alkylating agents after a first sub-lethal exposure (Katy Poncin, unpublished data). Taken together, those data would suggest that the function of Ada in *C. crescentus* and *B. abortus* is primarily to repair the O6meG lesion. It is likely that the repair of MPT would have been selected in some bacteria as a sensor mechanism to mount an adaptive response, and would not have been selected in other organisms because MPT are innocuous.

Box 1 | Methyl Methanesulfonate (MMS)

MMS is a mono-functional methylating agent, reacting in a S_N2-type nucleophilic substitution mechanism. Major lesions caused by MMS involve N7meG, N3meA, N7meA, N3meG and O6meG in double-stranded DNA; and N1meA and N3meC in single-stranded DNA (Mielecki, Wrzesinski, and Grzesiuk 2015; Mishina, Duguid, and He 2008).



Aside from the genomic information, functional information on the DNA alkylation repair systems of *B. abortus* remains sparse. So far, different mutants for enzymes involved in DNA alkylation repair have been constructed and characterized *in vitro* and in different models of infection in order to decipher the importance of those repair mechanisms in the pathogen *B. abortus* (Katy Poncin, unpublished data). Interestingly, two of these mutants showed an attenuation during infection of HeLa cells and RAW 264.7 macrophages: *ΔalkA* and *ΔalkB*. **Of particular interest is *ΔalkB***, because this mutant grows like the wild-type (WT) in culture, but is strongly impacted by the addition of MMS to its culture medium, suggesting that this strain is sensitive to exogenous alkylating agents. The *B. abortus ΔalkA* mutant has already a growth defect in culture in rich medium without MMS, which could indicate that it has a role in protecting the bacterium against endogenous alkylating stress. Interestingly, bacterial loads of the *ΔalkB* mutant were slightly decreased when compared to the WT *B. abortus* strain in the spleen of mice after 60 hours post-infection (PI). Furthermore, previous random large-scale genetic screens identified two *Brucella* mutants theoretically defective for DNA alkylation repair as attenuated in different models of infection (Delrue et al. 2004). Indeed, a *B. abortus* mutant with a mini-transposon in the *aidB* gene was found to be attenuated in macrophages (Lestrate et al. 2003) and a *B. melitensis* mutant lacking the *alkA* gene was identified as attenuated in mice (Kohler et al. 2002).

OBJECTIVES

The bacterium *B. abortus* is an intracellular pathogen which lives in long-term close relationship with its host. Throughout survival inside its host cells, *B. abortus* is thought to endure many stresses, which possibly include exposure to genotoxic molecules. In this work, we will focus on one type of DNA damage which is alkylating damage. Indeed, previous data indirectly suggest that *B. abortus* could be subject to alkylating stress during infection.

With the aim of monitoring DNA alkylation in the pathogen *B. abortus*, we propose to design a new alkylation-responsive fluorescent reporter, based on the adaptive response of *E. coli*. In a first part, we will try to characterize the activation, the specificity and the sensitivity of the reporter system in rich culture medium. In order to determine whether *B. abortus* encounters alkylating stress during infection of mammalian cells, we also plan to use our reporter system in the HeLa cell and the RAW 264.7 macrophage infection model.

RESULTS

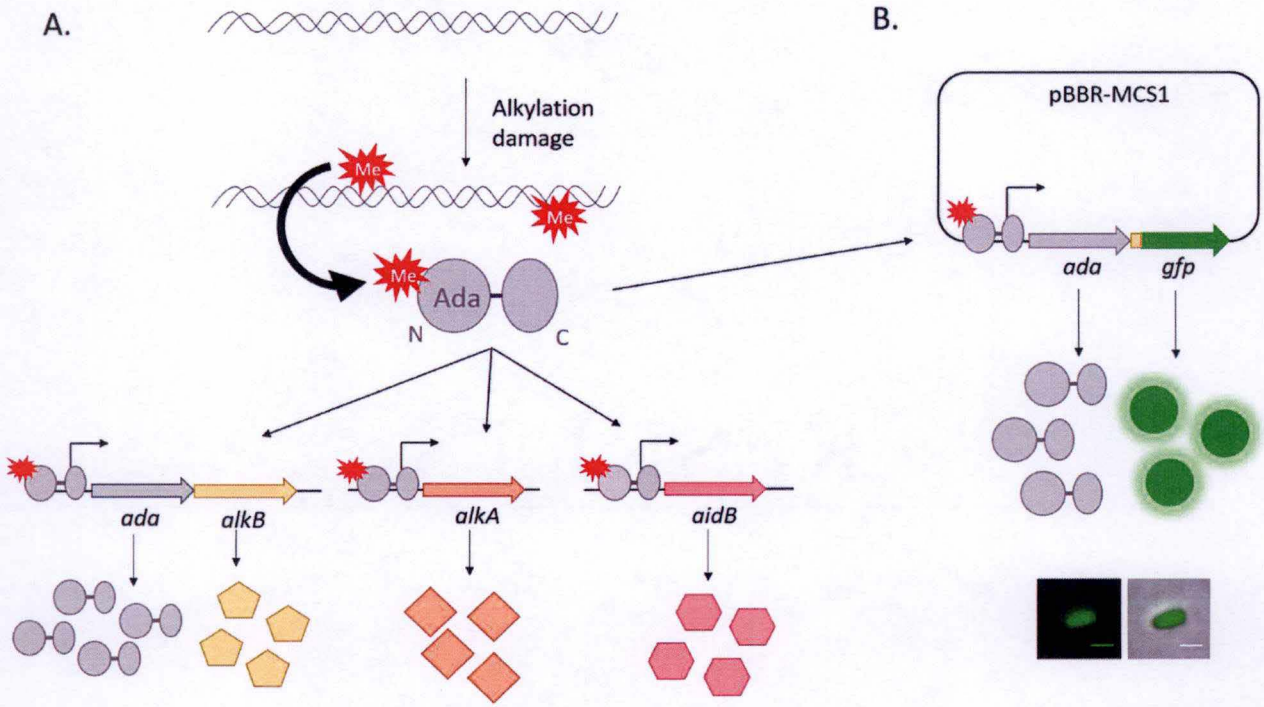


Figure 7 – A. Schematic representation of the adaptive system in *E. coli*. Ada protein coordinates the adaptive response to alkylation. Following alkylation of *E. coli* DNA, Ada protein gets methylated and becomes a strong transcriptional activator. Methylated Ada-N induces the expression of *alkB, alkA* and *aidB* genes as well as its own expression. Ada, AlkB, AlkA and AidB increase the resistance of *E. coli* against alkylating stress. **B. Schematic representation of the pBBR-MCS1 P_{ada}-*ada-gfp* reporter system**. For this reporter system, we relied on the fact that Ada should become a strong transcriptional activator once its N-terminal domain gets methylated. In *E. coli*, *ada* and *alkB* genes are organized in an operon and for the reporter system, the *alkB* coding sequence has been replaced by the *gfp* coding sequence (with the codon bias of *B. abortus*). The very first amino acids of *alkB* coding sequence were conserved upstream of the *gfp* gene. When Ada gets methylated, it should bind to its promoter region on the plasmid and strongly induce the expression of *ada* and *gfp*. Newly synthesized Ada proteins could in turn amplify the reporter activation in the context of DNA alkylation. Induced expression of *gfp* can be visualized by fluorescence microscopy. Scale bars, 1 µm.

RESULTS

1. The overall strategy: the reporter system

In order to detect DNA alkylation in the pathogenic bacterium *B. abortus*, a new reporter system was designed, based on the adaptive response of *E. coli* (Figure 7). For this reporter system, *ada* and *gfp* coding sequences have been ligated to form a single transcriptional unit, under the control of the native *ada* promoter. The *ada* coding sequence and its native promoter originate from *E. coli* genome. For the *gfp* coding sequence, the codon usage bias has been adapted to *B. abortus*. The reporter system, which is called P_{ada}-*ada-gfp*, was inserted in a medium copy pBBR-MCS1 plasmid. In principle, under non-stressing conditions, *ada* and *gfp* genes have a low transcriptional activity (Uphoff et al. 2016). Following alkylation of bacterial DNA and formation of MPT, Ada should get methylated and in turn amplify the transcription of the two genes by binding onto its promoter region (P_{ada}) on the plasmid. Importantly, this fluorescent reporter enables us to work at the single-bacterium level, which is an important aspect because at the first stage of a *B. abortus* cellular infection, there are very few bacteria (usually one or two) per infected cell. The same fluorescent reporter was also constructed on a pMR10 low-copy plasmid. Because the signal provided by this construction was lower, we decided to focus on the pBBR-MCS1 to bear our reporter system.

2. Detection of DNA alkylation following MMS treatment in culture

Alkylating agents are toxic compounds that have both cytotoxic and mutagenic properties. To monitor the level of DNA damage generated by alkylating agents in bacterial cells, we assessed the activity of the reporter system in rich medium cultures of *B. abortus,* using a fluorescence microscopy approach.

Prior to its use in *Brucella*, the activity of the reporter system was investigated in cultures of *E. coli* and *Salmonella enterica* serovar Typhimurium (*S. typhimurium*). A strong induction of GFP fluorescence was observed following treatment with MMS in the three bacterial species (Figure 8). The experiment was also carried out on bacteria expressing *cfp* instead of *gfp* (Figure S1). The brightness of the cells seemed however lower when compared to those expressing *gfp*. We thus decided to focus on the *gfp* reporter system for the rest of the experiments. *E. coli* cells were also treated with ethyl methanesulfonate (EMS) and we could also observe an activation of the reporter system (Figure S1). Anticipating a future use of this system in a model that requires fixation, bacterial cultures were also treated with paraformaldehyde (PFA) 2% for 20 minutes after MMS challenge to see if the signal was conserved. The fluorescence of the *gfp* reporter decreased marginally after fixation of *S. typhimurium* with PFA (data not shown).

The activation of the reporter system was assessed at different concentrations and different timings of exposure to MMS in *B. abortus* (Figure 9). The sensitivity of the reporter system to the exogenous alkylating agent MMS appeared to be limited to concentrations equal or superior to 0.5 mM, at least after 90 minutes of stress. Time also appeared to be a limiting factor for the activation of the reporter system. Indeed, even at high concentrations of MMS such as 1 or 2 mM, an activation of the reporter system was barely observable after 30 minutes of exposure, whereas after 90 minutes of MMS treatment, *gfp* expression increased drastically.

Because the activation of the reporter system implies mRNA and protein synthesis, one can imagine that its activation could be dependent on the bacterial growth phase.

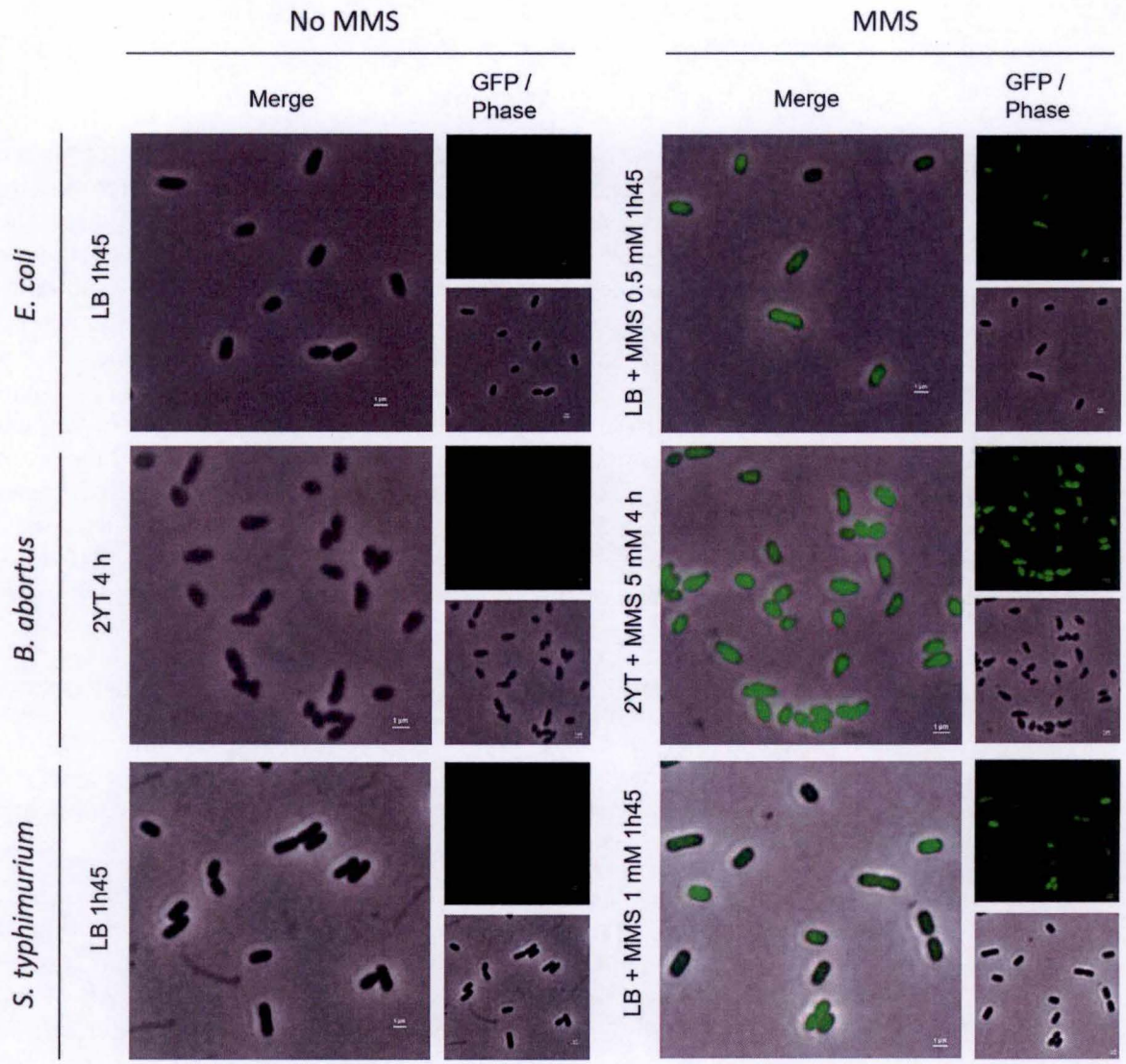


Figure 8 – Characterization of the pBBR-MCS1 - P_{ada}-*ada-gfp* reporter system in *Brucella abortus*, *Salmonella typhimurium* and *Escherichia coli* cultures upon MMS treatment. Exponential cultures of *E. coli, B. abortus* and *S. typhimurium* carrying the plasmid borne P_{ada}-*ada-gfp* reporter system were treated with 0.5, 5 and 1 mM of MMS, respectively, for 1h45, 4 h and 1h45. Scale bars, 1 μm.

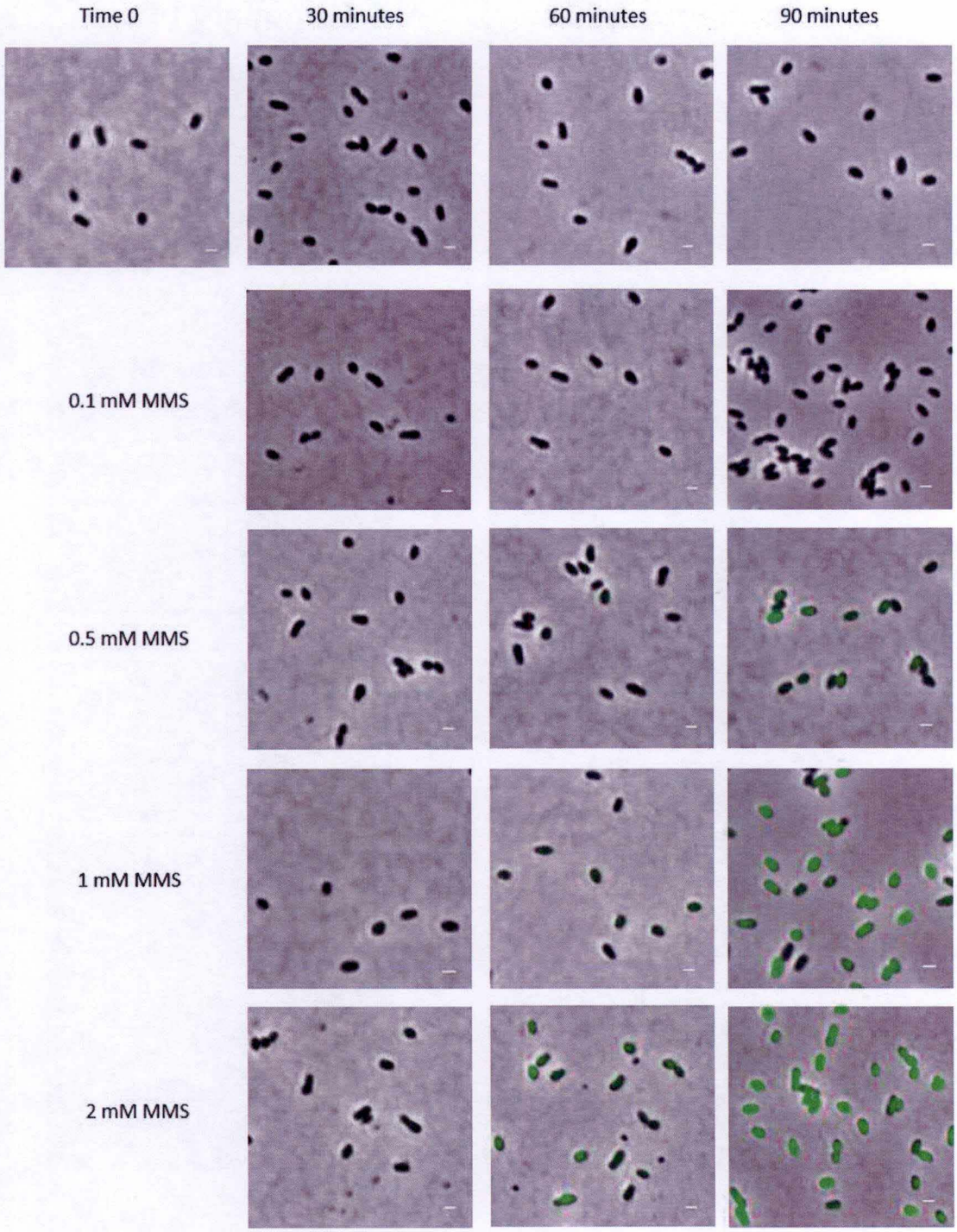


Figure 9 – Kinetics of activation of the pBBR-MCS1 - P_{ada}-*ada-gfp* reporter system in *B. abortus* cultures, upon exposure to increasing doses of MMS. Exponential phase cultures of *B. abortus* carrying the plasmid borne P_{ada}-*ada-gfp* reporter system were treated with 0.1, 0.5, 1 and 2 mM of MMS, for a duration of 30, 60 and 90 minutes. Scale bars, 1 μm.

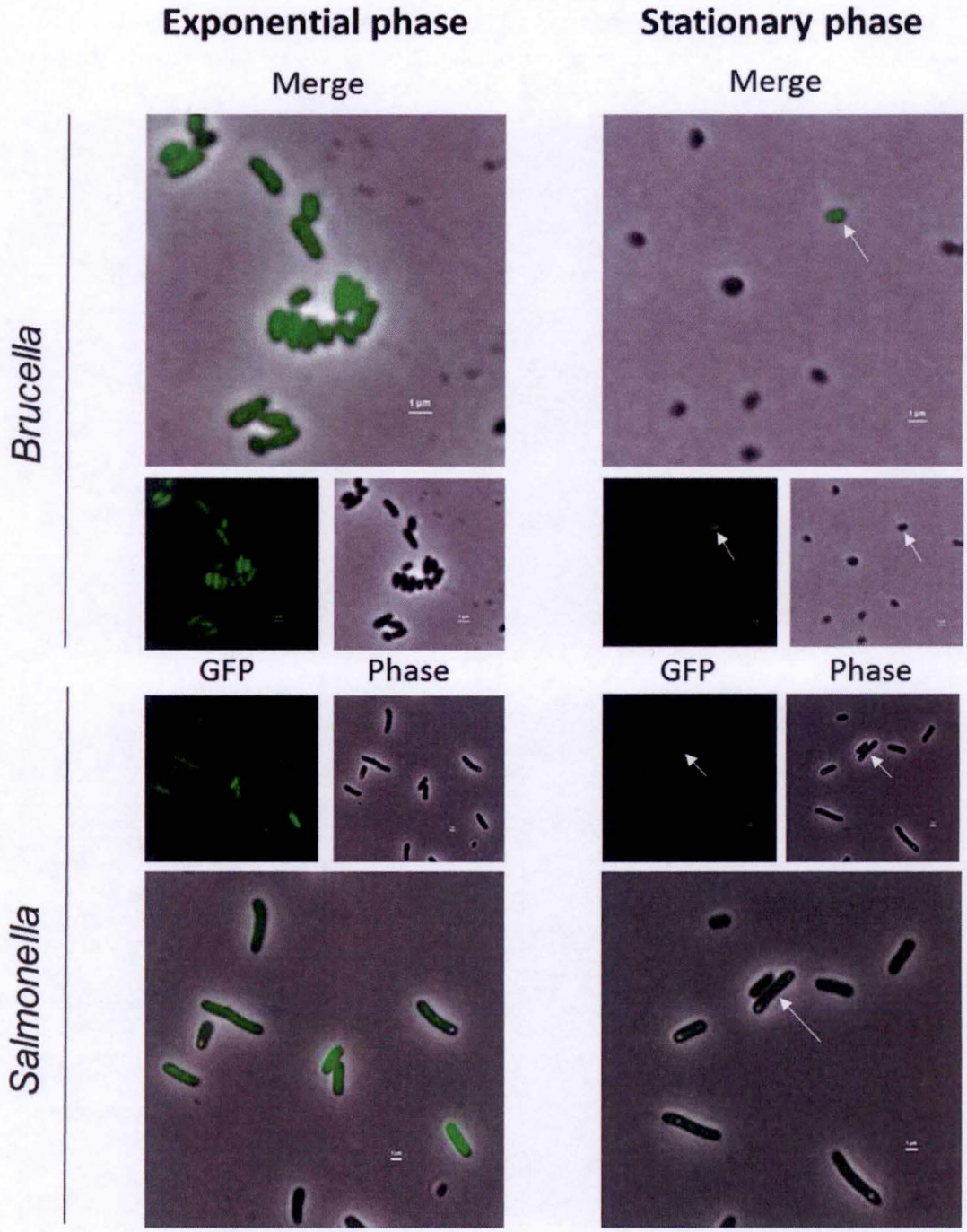


Figure 10 – Characterization of *Brucella abortus* and *Salmonella typhimurium* pBBR-MCS1 - P_{ada}-*ada-gfp* reporter strains in exponential and stationary phase. *B. abortus* cells were grown in 2YT liquid medium. Either exponential or stationary phase cultures were then stressed with MMS at a final concentration of 5 mM during 1h45. *S. typhimurium* cells were grown in LB liquid medium. Either exponential or stationary phase cultures were stressed with MMS at a final concentration of 0.5 mM for about 2h40. Arrows indicate bacteria that show a slight activation of the reporter system. Scale bars, 1 µm.

Indeed, during stationary phase, protein synthesis is globally downregulated by the alarmone ppGpp, which is synthesized in response to nutrient limitation (Dalebroux and Swanson 2012). Besides, in order to face starvation, this molecule is able to redirect RNA polymerase to certain promoters, and thereby prevent transcription of many other genes (Dalebroux and Swanson 2012). We could therefore expect that the activation of the reporter system would be limited during stationary phase. Thus, *Brucella* and *Salmonella* cultures in stationary and exponential phase were treated with MMS. As expected, bacteria in stationary phase showed a decreased expression of *gfp* in the presence of MMS compared to exponentially growing bacteria (Figure 10), but the GFP fluorescence induction was still detectable compared to the control made in the absence of MMS.

Even though the adaptive response is known to be specific for the repair of DNA alkylation damage (Lindahl and Sedgwick 1988), we wanted to confirm that our reporter system was specific of DNA alkylation, and would not be induced by other genotoxic stresses. Therefore, we treated the reporter strain with acid pH and hydrogen peroxide (H_2O_2) (Figure 11) which are known to cause damage to DNA (van der Veen and Tang 2015). Predictably, only MMS triggered the induction of *ada* and *gfp* expression. In this experiment, bacteria exposed to MMS for 90 minutes were either incubated in 2YT rich medium or PBS. Not surprisingly, when exposed to equal concentrations of MMS, the fluorescent reporter activation was greater when bacteria were incubated in 2YT rich medium than in PBS (Figure 11). This is indeed consistent with the observation that GFP fluorescence induction is lower in stationary phase bacteria. More surprisingly, with the combined presence of acid PBS and MMS, we could not detect any *gfp* signal (Figure 11). A possible interpretation of this last result is developed in the Discussion.

To further confirm that the activation of the reporter system was specifically activated by alkylating stress, we checked that *gfp* expression was strictly dependent on the transactivation capacity of methylated Ada. Indeed, it is possible that the signal we observed via our reporter system could be somehow contaminated by a background signal due to basal expression of *gfp*. As previously explained, methylation of a single residue, the Cys38 of Ada-N, is responsible for the conversion of Ada into a strong transcriptional activator. We therefore mutated this Cys38 residue by PCR site-directed mutagenesis to prevent Ada from becoming a transcriptional activator. Thereby the actual signal corresponding to the level of alkylating stress was supposed to be suppressed. This negative control reporter was obtained by replacing the Cys38 of the *ada* gene of the reporter by an alanine or a serine residue. The resulting mutated reporter was again inserted in a medium copy pBBR-MCS1 plasmid and introduced into *B. abortus*. By fluorescence microscopy, we validated that both point mutations in the *ada* gene were sufficient to inhibit the transactivation activity of Ada (Figure 12). Indeed, following MMS challenge, the strains carrying the mutated reporter systems showed no detectable *gfp* expression, whereas the WT P_{ada}-*ada-gfp* reporter was normally activated (Figure 12). Only the pictures of P_{ada}-*adaC38A-gfp* are shown on this figure, but similar observations were obtained for P_{ada}-*adaC38S-gfp*. The negative control reporter thus provides us with a tool to properly discriminate between the potential basal *gfp* expression and the actual signal reflecting the level of alkylating stress.

As mentioned in the introduction, *B. abortus* possesses two homologs of the *ada* gene of *E. coli*, namely *ada1* and *ada2* (Katy Poncin, unpublished data). The two isoforms of Ada encoded by those genes could potentially constitutively repair DNA alkylation, and thereby would decrease the levels of DNA alkylation monitored by the fluorescent reporter system. Moreover, although unlikely, we cannot exclude that Ada1 or Ada2 could bind to P_{ada} from *E. coli* and perturb the reporter system.

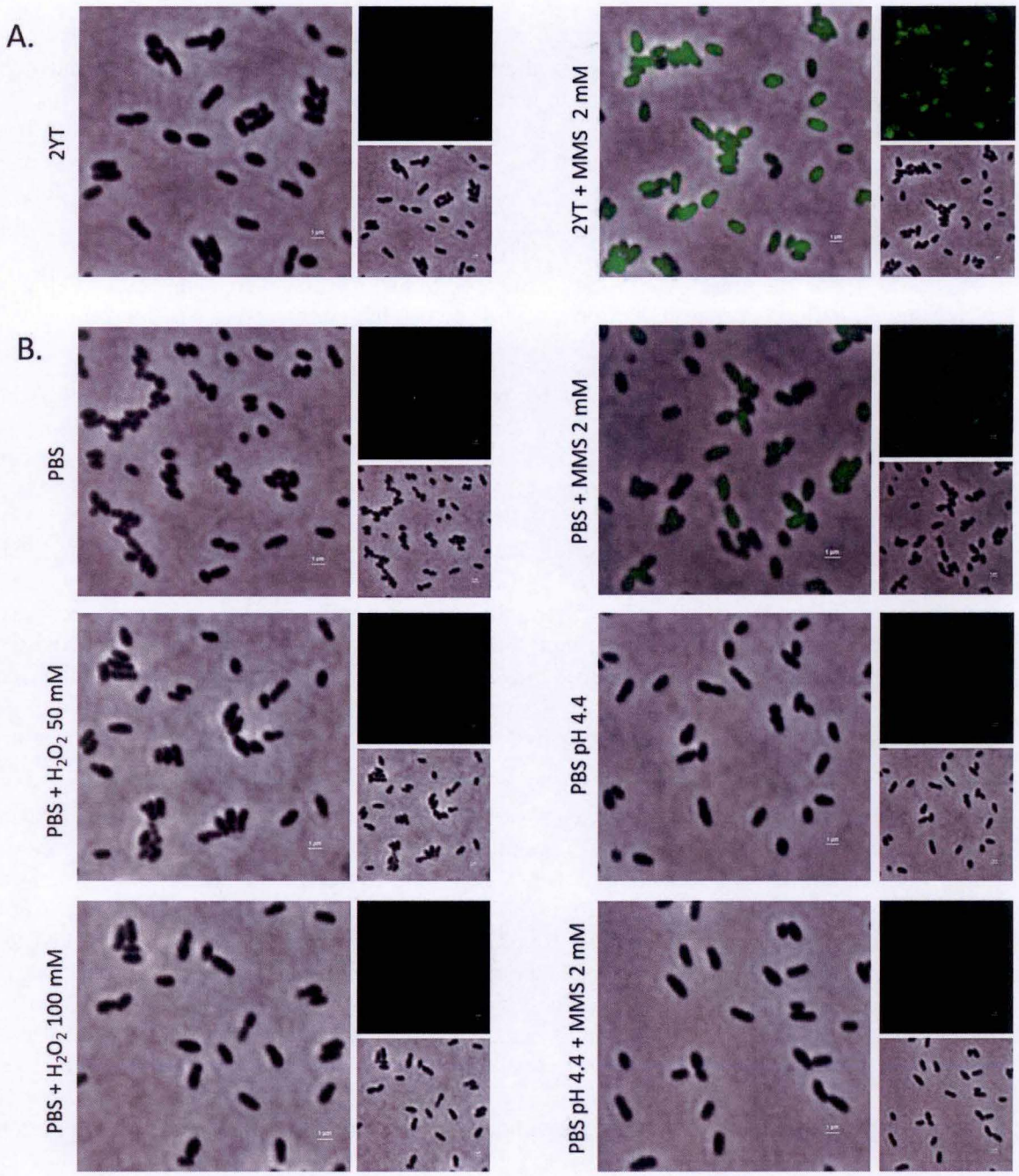


Figure 11 – Validation of the specificity of the pBBR-MCS1 - P_{ada}-*ada-gfp* reporter system to alkylating agents in *B abortus* cultures. Bacteria carrying the P_{ada}-*ada-gfp* reporter strain were grown in 2YT rich medium until they reached exponential phase. Exponential phase cultures of *B. abortus* were washed twice with PBS and resuspended either in 2YT rich medium (A.) or in PBS (B.), in the presence of different genotoxic molecules. Detailed composition of each medium is indicated next to the corresponding pictures. Pictures were taken after 90 minutes of incubation at 37°C with shaking in the different indicated conditions. Scale bars, 1 µm.

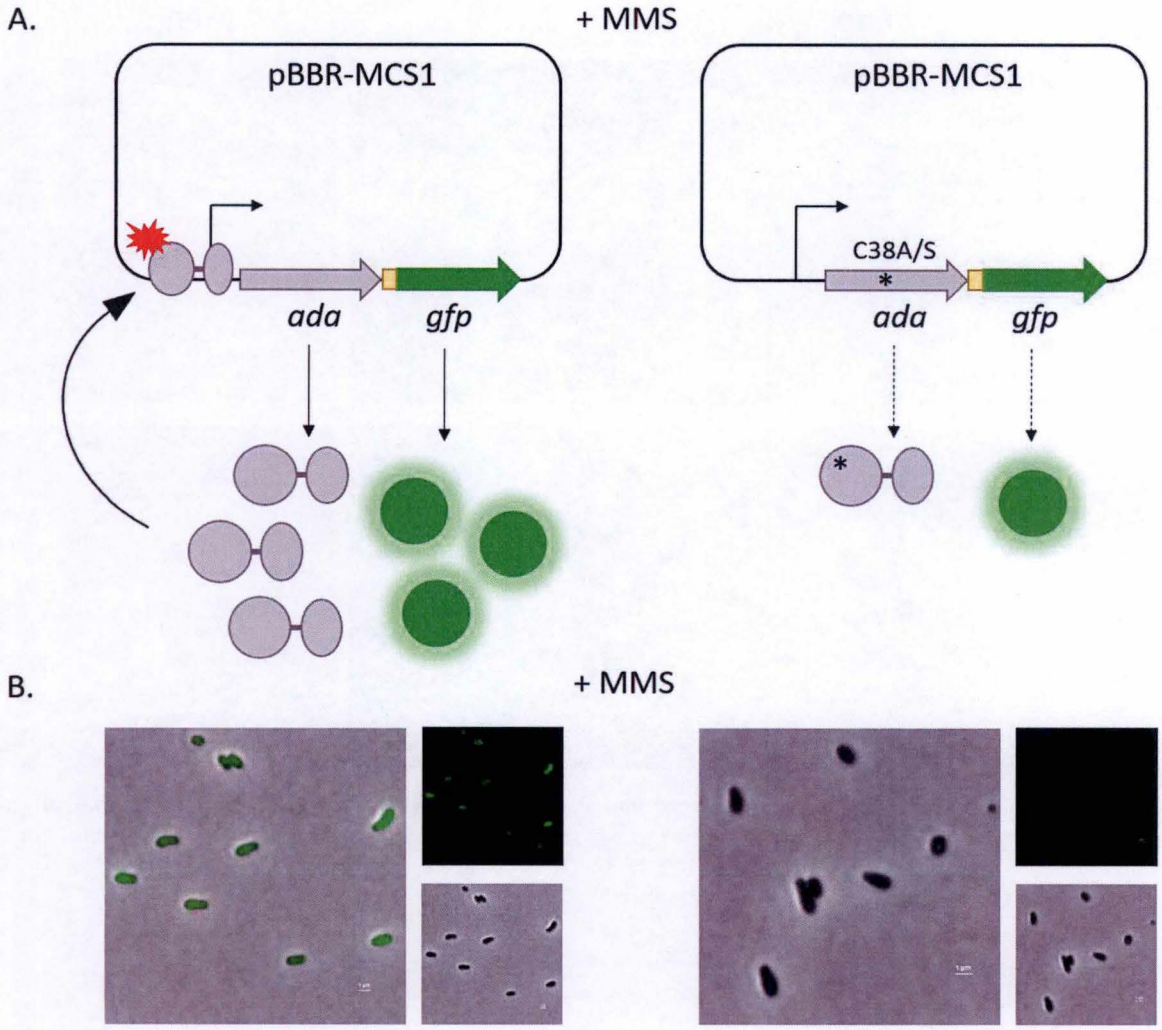


Figure 12 – A. Schematic comparison of the "wild-type" reporter system and the mutated negative control pBBR-MCS1 - P_{ada}-*adaC38A/S-gfp* reporter system. For this reporter system, Ada is supposed to become a strong transcriptional activator once its N-terminal domain gets methylated. Following MMS exposure, Ada should transfer a methyl group from the DNA to itself. The methyl group is transferred onto the Cys-38 residue of Ada-N. Methylated Ada should in turn bind to its promoter region on the plasmid, and strongly induce the expression of *ada* and *gfp*. When the active cysteine Cys-38 is replaced either by an alanine or a serine, conversion of Ada into a strong transcriptional activator is expected to be prevented, even in the presence of MMS. **B. Characterization of the negative control reporter strain following MMS treatment.** *B. abortus* carrying either the wild-type P_{ada}-*ada-gfp* reporter system or the negative control P_{ada}-*adaC38A-gfp*, were grown in 2YT rich culture medium until they reached exponential phase. Bacteria were then treated with 5 mM MMS for 4 hours. Scale bars, 1 µm.

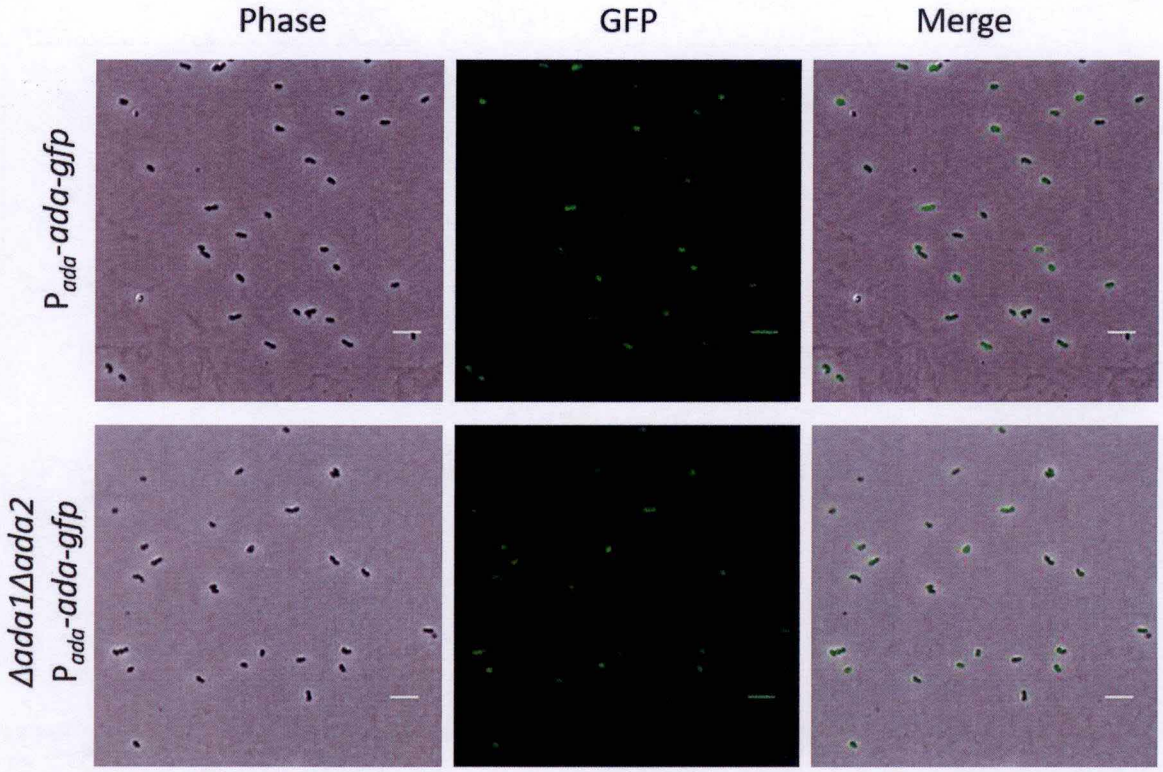


Figure 13 – Characterization of reporter system P_{ada}-*ada-gfp* in the *B. abortus* double deletion mutant *Δada1Δada2* upon MMS treatment. *B. abortus* wild-type 544 reference strain and *B. abortus Δada1Δada2* double deletion mutant carrying the P_{ada}-*ada-gfp* reporter system were grown in 2YT rich culture medium until they reached exponential phase. Bacteria were then treated with 5 mM MMS for 2 hours. Scale bars, 1 µm.

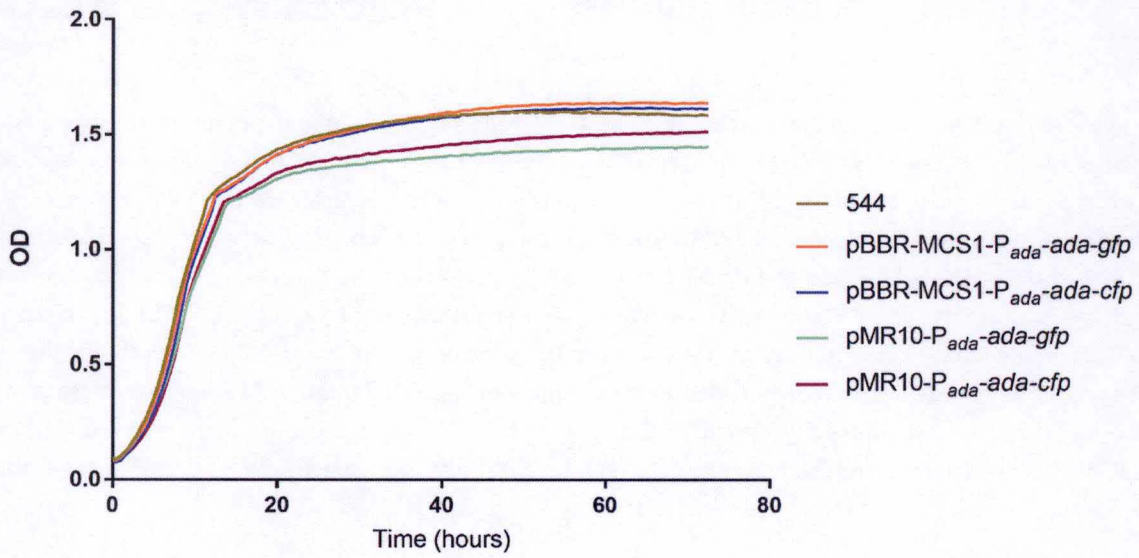


Figure 14 – Growth curves of the different reporter strains in 2YT. Overnight cultures of the different strains were diluted to obtain an optical density at 600nm (OD_{600}) of 0.1. Growth was measured by reading OD_{600} every 30 minutes during 72 h.

To address these problems, we introduced the reporter system in a *B. abortus* double deletion mutant for *ada1* and *ada2* (Katy Poncin, unpublished data) by conjugation. Upon MMS treatment, the *B. abortus* double mutant *Δada1Δada2* carrying the reporter system P_{ada}-*ada-gfp* showed an activation very similar to that observed for the WT reporter strain, which possesses the two *ada* homologs (Figure 13). As a control, we verified that the reporter system had no growth abnormalities in rich medium. To this end, we performed growth curves of the different reporter strains in 2YT rich medium (Figure 14). Growth was measured with a Bioscreen apparatus (C MBR) by reading OD_{600} every 30 minutes for a total of 72 hours. We could conclude from these growth curves that the reporter strains grow normally in 2YT rich medium.

3. Detection of DNA alkylation following HeLa cells infection

As already mentioned in the introduction, previous data suggest that *B. abortus* DNA is subject to alkylating stress during RAW 264.7 macrophages and HeLa cells infections. Indeed, the *ΔalkB* deletion mutant is attenuated at 24 PI in those two cellular models (Katy Poncin, unpublished data). Intriguingly, this mutant grows normally in rich culture medium (2YT), unless we add alkylating agent such as MMS. Those data therefore suggest that the *ΔalkB* mutant is sensitive to exogenous alkylating stress. To explain the attenuation of this mutant during infection, one hypothesis is that it is less able to face DNA alkylation inside the host cell. Still, this data only indirectly suggests that *B. abortus* is subject to alkylating stress during its intracellular trafficking in eukaryotic cells. Hence the reporter system described previously was designed with the aim of monitoring *Brucella* DNA alkylation during infection.

HeLa cells were infected with *B. abortus* carrying the reporter system and were imaged at 6 h and 24 h PI. The infection experiment was also carried out with WT *B. abortus* 544 reference strain as a control for the auto-fluorescence of the bacteria (Figure 15), and the *B. abortus* control strain, carrying the negative control reporter P_{ada}-*adaC38A-gfp*. This latter control represents the basal expression of *gfp* regardless of Ada activation. GFP fluorescence intensity was measured using NIS-element software (Figure 16 A.). Quantitative analysis of GFP fluorescence revealed first of all higher levels of fluorescence for the reporter condition than for the control conditions. Regarding the two control conditions, their mean fluorescence intensities are comparable between them (Figure 16 A.). Thereafter, we will focus on the control reporter P_{ada}-*adaC38A-gfp* for the rest of the experiments, as it is closer to the "test" condition which is the WT P_{ada}-*ada-gfp* reporter. Data resulting from this experiment suggest that *Brucella* actually faces alkylating stress during infection.

To assess whether the reporter was differentially activated along *Brucella* trafficking, we compared the mean fluorescence intensity of the reporter at 6 h and 24 h PI (Figure 16). Two different experiments were performed and provided results somehow divergent. Indeed, the difference between the mean fluorescence of the reporter and WT *B. abortus* was either slightly lower at 6 h PI when compared to 24 h PI (Figure 16 B.), or higher at 6 h PI (Figure 16 D.). However, measures of fluorescence intensity were taken for a higher number of bacteria for the first experiment (Figure 16 A.). Given the high variability of the measure, having a large number of measurements is important. Notably, for the second experiment, another batch of HeLa cells was used, different to that of the first experiment. With the batch of HeLa cells used for the first experiment, very few replication of *Brucella* was observed at 24 h PI, which was unexpected. By contrast, for the second experiment, most measurements were made on bacteria that had largely proliferated inside the HeLa cells. This should be taken into account when looking at the results, as *Brucella*'s trafficking during the first infection could be much slower than during the second one.

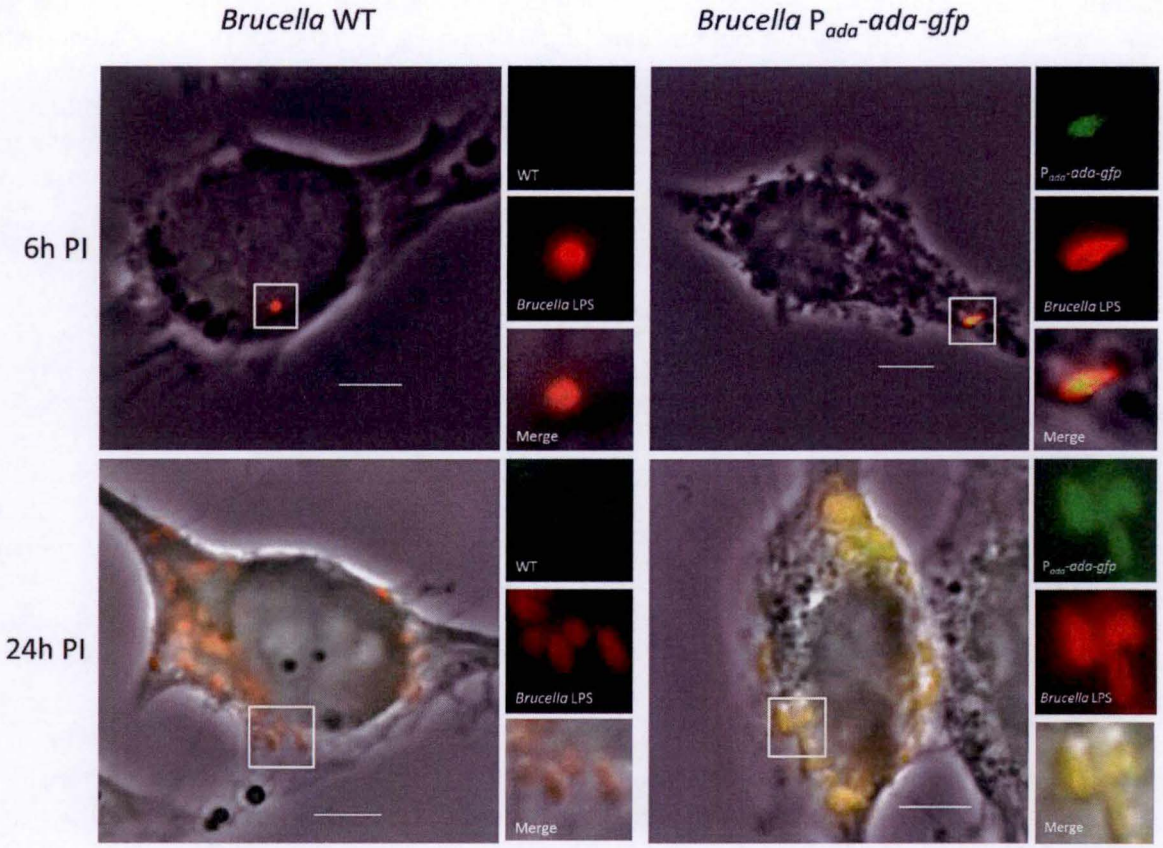


Figure 15 – Characterization of the P_{ada}-*ada-gfp* reporter system in *Brucella abortus* during HeLa cells infection. HeLa cells were infected by the wild-type *Brucella abortus* (*Brucella* WT) or by the reporter strain (*Brucella* pBBR-MCS1 - P_{ada}-*ada-gfp*), and imaged in the same conditions at 6 h and 24 h post-infection (PI). *Brucella abortus* LPS was labelled using the 12G12 monoclonal antibody and a secondary anti-mouse IgG coupled to Texas Red. The fluorescent reporter P_{ada}-*ada-gfp* is presumably activated upon alkylation of *Brucella abortus* DNA. The fluorescence of wild-type bacteria reflects the auto-fluorescence of *Brucella abortus*. Scale bars, 5 µm.

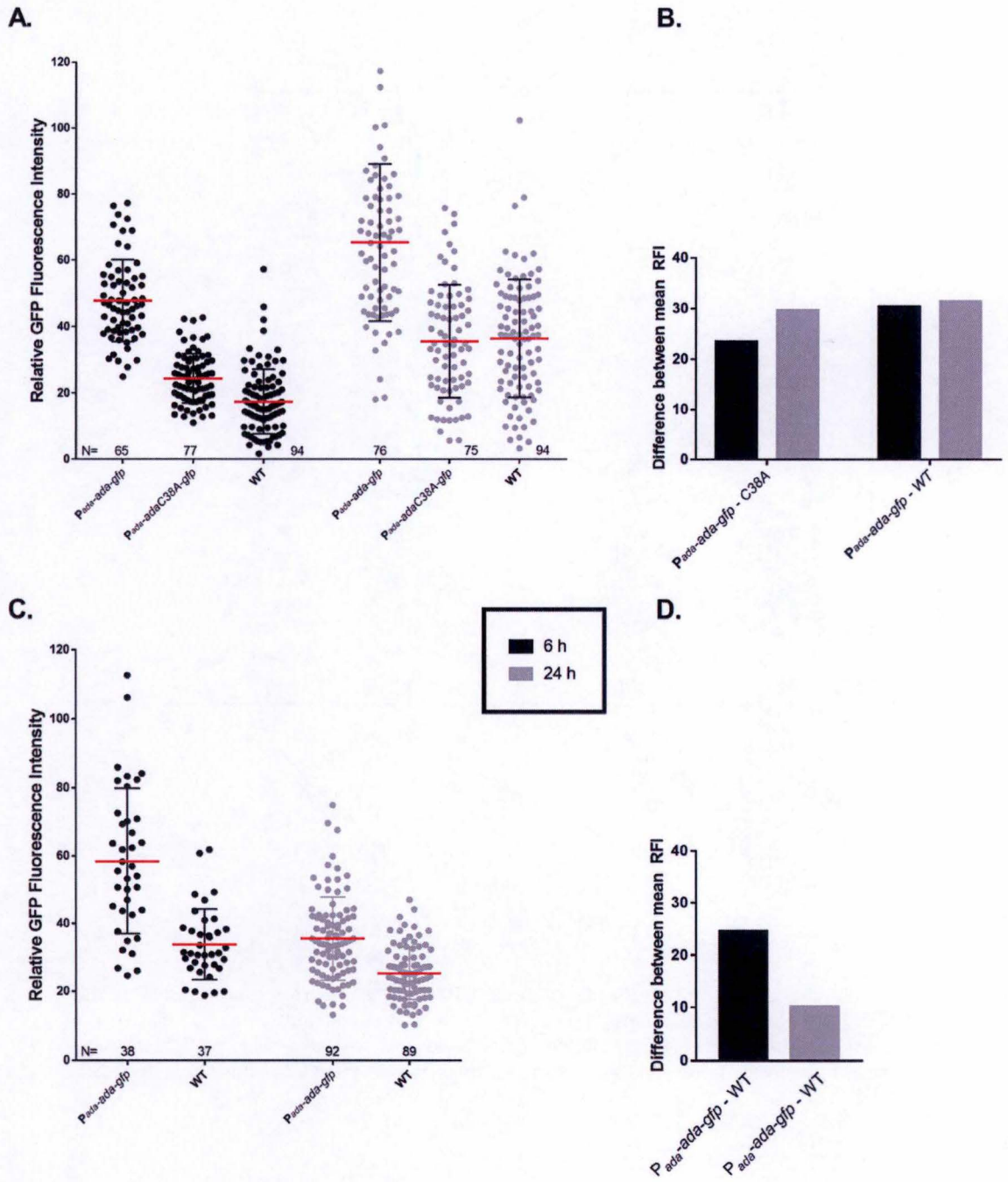


Figure 16 – Quantification of the *B. abortus* P_{ada}-*ada-gfp* reporter strain, P_{ada}-*adaC38A-gfp* negative control strain and wild-type 544 strain during HeLa cells infection. Graphs A. and B. refers to a first experiment. A. Relative GFP fluorescence intensities of each bacterium are plotted at 6 h and 24 h post-infection. Horizontal red bars represent the mean values and error bars represent the standard deviation. **B.** For the same experiment as in A., the difference between mean RFI of the reporter either with the negative control reporter, or with the wild-type, are plotted at 6 h PI and 24 h PI. **Graphs C. and D. refers to a second independent experiment. C.** Relative GFP fluorescence intensities of each bacterium are plotted at 6 h and 24 h post-infection. Horizontal red bars represent the mean values and error bars represent the standard deviation. **D.** Difference between mean RFI of the reporter with the wild-type, are plotted at 6 h PI and 24 h PI.

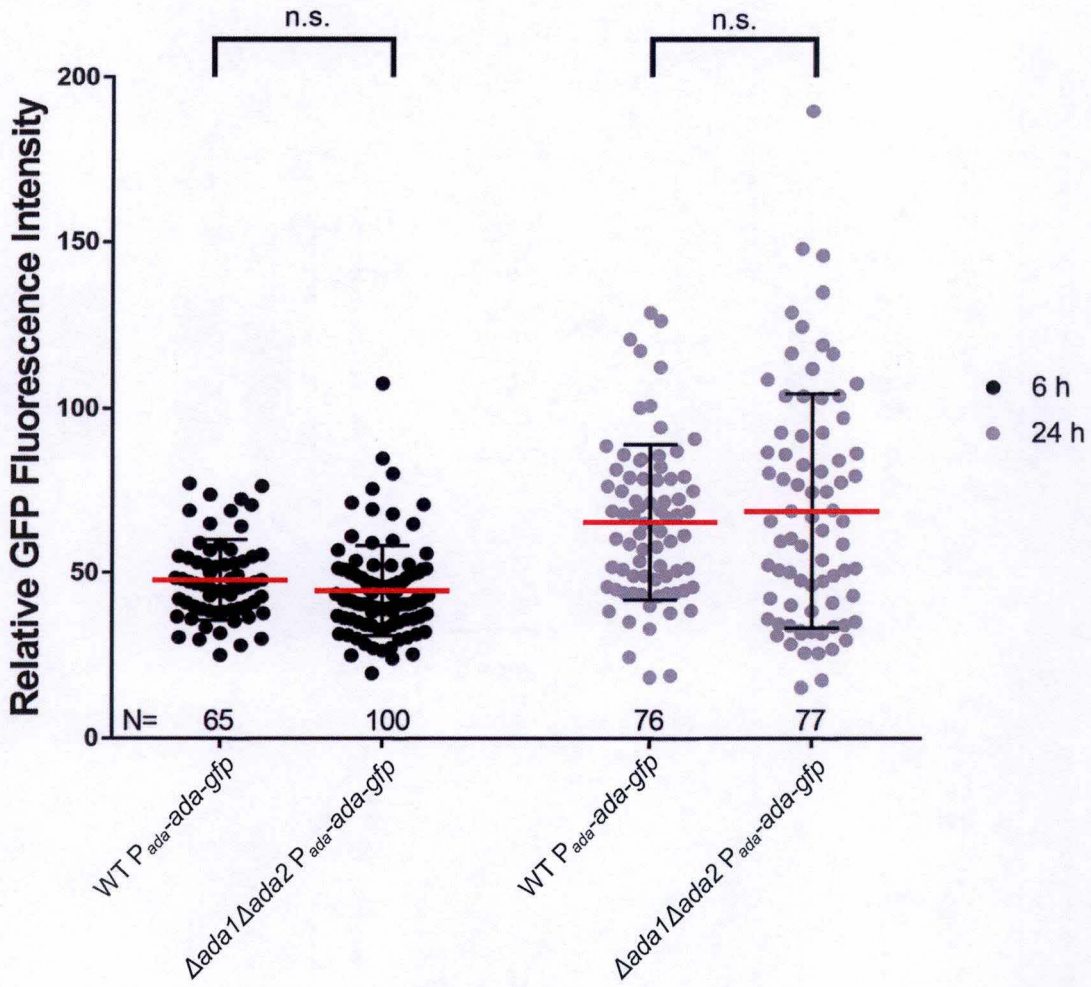


Figure 17 – Quantification of the *B. abortus* wild-type 544 strain and double mutant *Δada1Δada2* bearing the P_{ada}-*ada-gfp* reporter in a HeLa cells infection. Relative GFP fluorescence intensities of each bacterium are plotted at 6 h and 24 h post-infection. Horizontal red bars represent the mean values and error bars represent the standard deviation. Data have been analyzed by Welch's t test. Non-significant differences are denoted as n.s.

Similarly to what has been performed in culture, the activation of the reporter system P_{ada}-*ada-gfp* was assessed in the double mutant *Δada1Δada2* during infection. According to quantitative analysis of GFP fluorescence, the mean fluorescence intensity is not significantly altered in the absence of *B. abortus* isoforms Ada1 and Ada2, either at 6 h or 24 h PI (Figure 17).

Because the *ΔalkB* mutant was attenuated in the same fashion in RAW 264.7 macrophages and HeLa cells, we suspected that the alkylating stress potentially occurring during infection would not necessarily arise from the aggressiveness of the cell type, but rather from a common feature of both models of infection. Therefore, we decided to play on one parameter that is shared by both cellular models: the acidification of the endosomes. The neutralizing agent NH_4Cl was used to prevent endosomes acidification during *B. abortus* infection of HeLa cells (Porte, Liautard, and Kohler 1999). Again, we imaged and measured the GFP fluorescence intensity of the reporter strain and the negative control strain. At 6 h PI, the absence of acidification did not appear to have an impact on the activation of the reporter system (Figure 18). However, GFP fluorescence of the reporter was decreased at 24 h PI when HeLa cells have been treated with NH_4Cl, and was not decreased in untreated cells (Figure 18). The fluorescence of the control reporter was not impacted by the NH_4Cl treatment.

4. Detection of DNA alkylation following RAW 264.7 infection

Although *Brucella* have been shown to infect epithelial cells *in vitro*, evidence of colonization of this cell type *in vivo* remains to be demonstrated (Edgardo Moreno and Moriyon 2006; Roop et al. 2009). By contrast to epithelial cells, it has been well documented that *B. abortus* resides and replicates inside macrophages *in vivo* (Edgardo Moreno and Moriyon 2006). Accordingly, the efficiency of *B. abortus* invasion of cultured cells is lower for epithelial cells when compared to macrophages (Roop et al. 2009). We therefore thought that it would be relevant to use our reporter system in another cellular model of infection which is RAW 264.7 macrophages. Besides, RAW 264.7 can be activated *in vitro* with LPS and interferon-γ (IFN-γ) (Mosser et al. 2008). One of the hallmarks of activated macrophages is the production of nitric oxide (•NO) by the inducible Nitric Oxide Synthase (iNOS) (Mosser et al. 2008).

RAW 264.7 macrophages that were either activated with LPS and IFN-γ or not activated were infected with the *B. abortus* reporter strain and imaged at 4 h and 24 h PI (Figure 19). Since no negative control (either the WT strain or the mutated version of the reporter strain) had been used for this experiment, quantification of fluorescence was not performed.

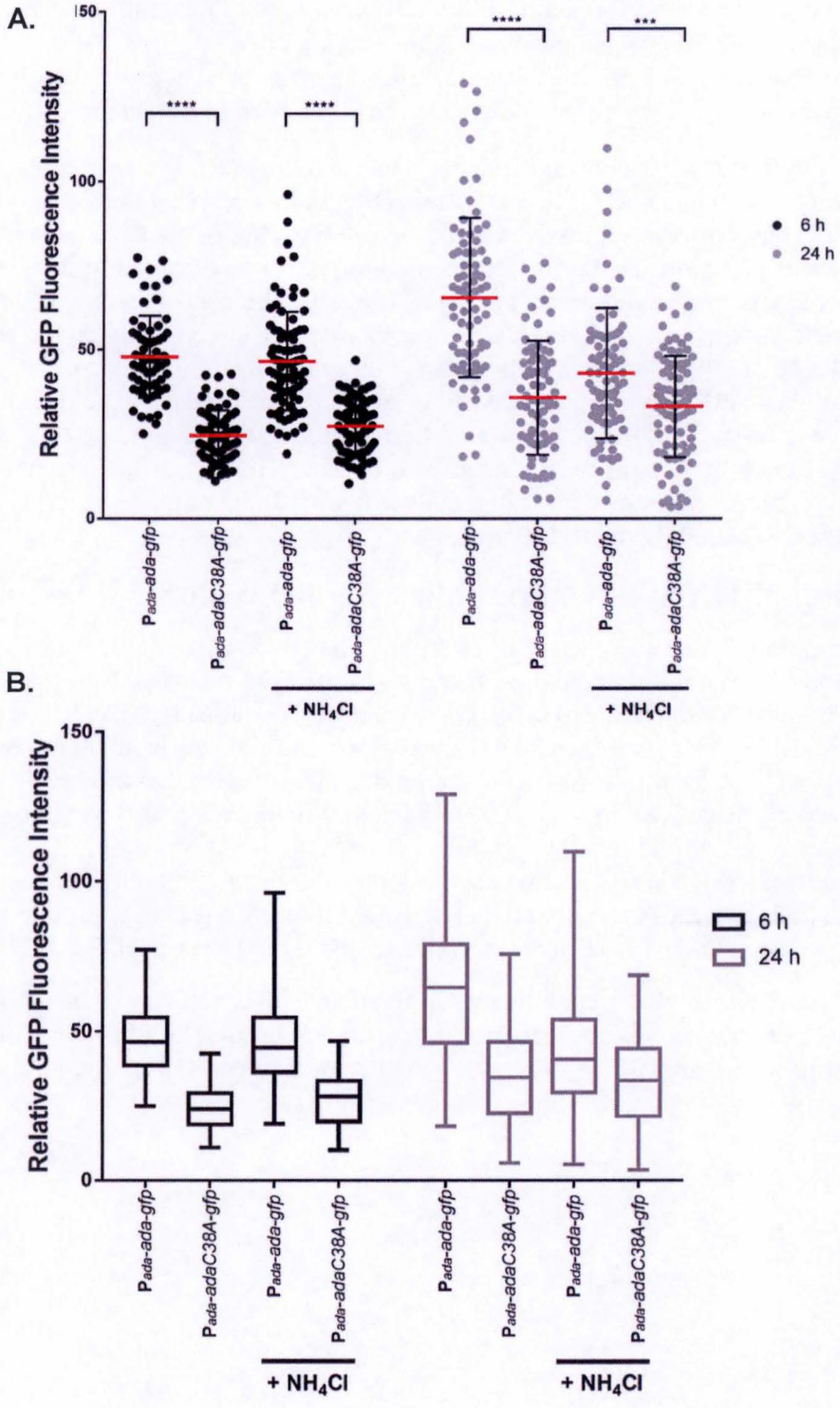


Figure 18 – Effect of NH_4Cl on the fluorescence of the *B. abortus* P_{ada}-*ada-gfp* reporter strain and negative control P_{ada}-*adaC38A-gfp* reporter strain during HeLa cells infection. A. Relative GFP fluorescence intensities of each bacterium were plotted at 6 h and 24 h post-infection. Horizontal red bars represent the mean values and error bars represent the standard deviation. Number of measures (N) are the following from left to right: 65, 77, 87, 94, 76, 77, 76 and 87. Data have been analysed by Welch's t test. **** denotes extremely significant differences ($p<0.0001$), *** denotes highly significant differences ($(0.0001<p<0.001$). **B.** Box plot of at data represented in Figure A. Minimum and maximum values are represented by the whiskers. Boxes represent the 25th, 50th and 75th percentiles.

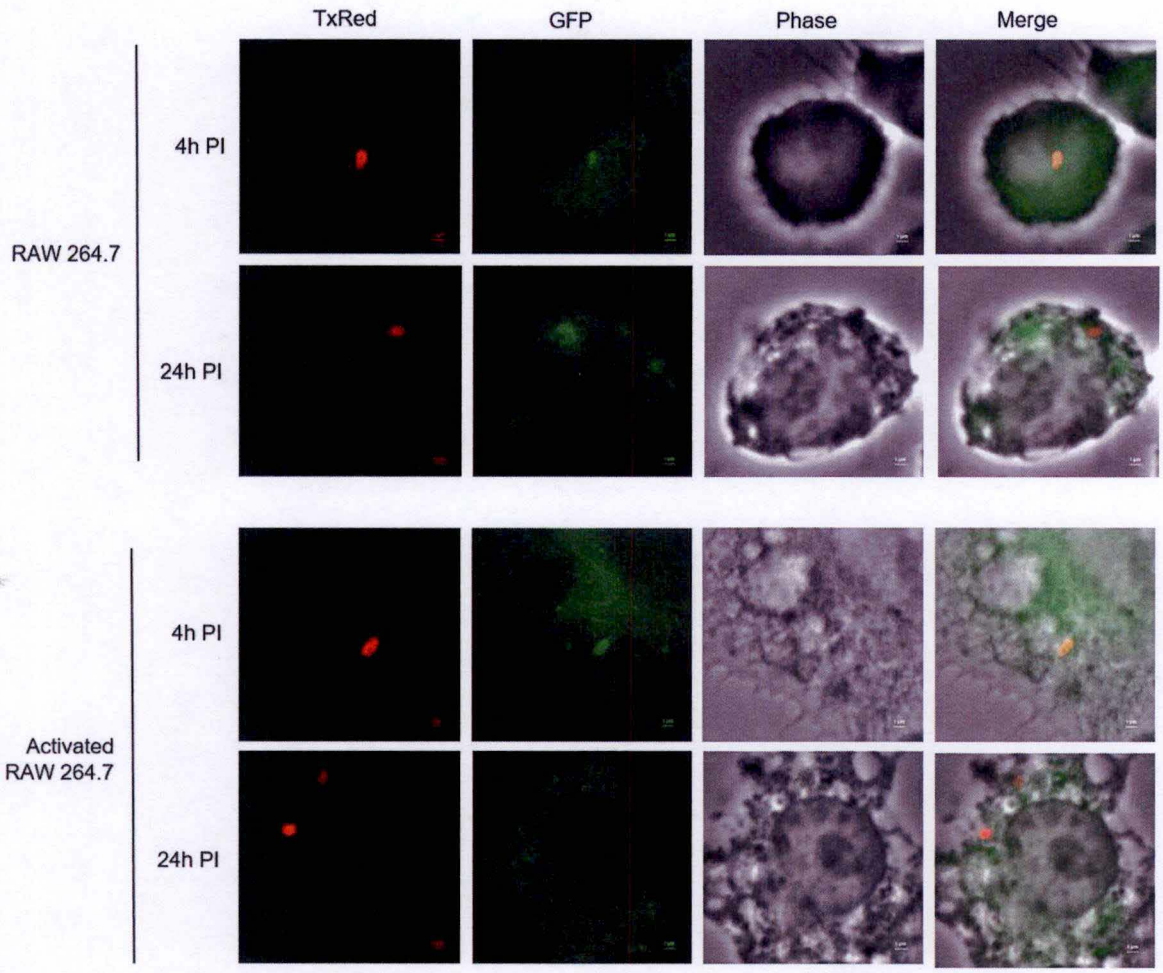


Figure 19 – Infection of RAW 264.7 macrophages and activated RAW 264.7 macrophages with *B. abortus* P_{ada}-*ada-gfp* reporter strain. RAW 264.7 macrophages were infected by the reporter strain (*Brucella* pBBR-MCS1 - P_{ada}-*ada-gfp*), and imaged at 4 h and 24 h PI. RAW 264.7 macrophages were activated with LPS (10 ng ml^{-1}) and IFN-γ (20 ng ml^{-1}). B. abortus LPS was labelled using the 12G12 monoclonal antibody and a secondary anti-mouse IgG coupled to Texas Red. The fluorescent reporter P_{ada}-*ada-gfp* is presumably activated upon alkylation of *B. abortus* DNA. Scale bars, 1 μm.

DISCUSSION

DISCUSSION

Here, we have developed a new fluorescent reporter system based on the adaptive response of *E. coli*. This reporter allows the detection of alkylating stress, at the single-bacterium level, for *B. abortus*. We have seen that the reporter was responsive to the alkylating agent MMS, and was also activated during infection of HeLa cells and RAW 264.7 macrophages.

1. About the reporter system

1.1 Specificity to alkylating agents

This reporter appears to be specifically activated by alkylating agents, as it is activated when *B. abortus* cells are exposed to MMS, and not when exposed to H_2O_2 or acidic pH, which are other conditions known to provoke DNA damage. Peculiarly, when exposed to both MMS and acidic pH, the reporter showed no apparent activation. However, at pH 4.4, MMS stability could be compromised, and it would therefore be less likely to damage DNA. Besides, GFP folding could also be affected by the low pH, which would explain why we could observe no fluorescence.

The specificity of the reporter system was further confirmed by the construction of a negative control reporter, i.e. which is not alkylation-responsive. For this control reporter, the active cysteine of Ada-N, Cys38, was replaced by an alanine or by a serine by PCR site-directed mutagenesis. To our knowledge, the effect of such mutations in the *ada* gene of *E. coli* have not been described. Here, we showed that these two mutations prevented the transcription activation of Ada when bacteria were exposed to MMS, as no GFP fluorescence was detectable anymore. Thereafter, we confirmed that the induction of GFP fluorescence we observed for the WT reporter system was indeed dependent on Ada transactivation.

1.2 An effect of the growth phase on the GFP fluorescence?

Our reporter system, like every technique, has its limitations. Indeed, we showed that the reporter system was less activated in stationary phase cultures, or when bacteria were incubated in PBS. In response to nutrient starvation, bacteria synthesize the alarmone ppGpp, which is known to decrease protein translation globally and to prevent the transcription of numerous genes by redirecting the RNA polymerase to specific promoters (Dalebroux and Swanson 2012). It is therefore not surprising that our fluorescent reporter system is less activated during starvation-like conditions. During its lag phase for the six first hours of the infection, *Brucella* is most likely enduring starvation (Roop et al. 2009). What is more, it has been suggested that *Brucella* is found in a state similar to the stationary phase during this phase of the infection (Roop et al. 2003). Consequently, it is likely that the fluorescent reporter activation is limited because starving *Brucella* restricts mRNA and protein synthesis, even in the presence of alkylating agents.

1.3 An alternative to GFP?

As a future improvement of the reporter system, another fluorescent protein could be used. Indeed, one drawback of the use of GFP is that mammalian cells tend to be autofluorescent when exposed to blue light. The sensitivity of the reporter system in mammalian cells is therefore not optimal, at least in that model of infection. To address this issue, the *gfp* portion of the reporter system should be replaced by another fluorescent protein, for example, the IRFP670 (Shcherbakova and Verkhusha 2016). The construction of this alternative reporter system was started during this master thesis but is still under construction.

1.4 Towards a more quantitative approach

As a future perspective, it would be useful to have a more quantitative approach for the *in vitro* experiments. This could be achieved by using flow cytometry to quantify the fluorescence of the reporter in the different conditions in which the reporter was tested in culture.

2. About the timing of activation and extinction of the reporter

Because we followed the activation of our reporter system over time in the cellular infection model, it would be important to characterize its timing of activation and extinction.

By characterizing the kinetics of activation of the reporter at different concentration of MMS (Figure 9), we learned that even with high doses of alkylating agents (here 2 mM of MMS), there was a delay of at least 30 minutes before we could observe an activation of the reporter. This delay was expected because transcription, translation and accumulation of a sufficient amount of GFP to be detected take time. With lower doses (0.1 mM MMS), an activation was not observable, even after 90 minutes of exposure, suggesting that this delay is even longer for milder stresses.

Another important issue to assess is the question of the stability of the GFP, since we followed GFP fluorescence over time. It is indeed possible that a signal measured at a late time PI (e.g. 24 h PI) could somehow be contaminated by residual GFP that was induced earlier in the time-course of infection. Because the stability of the GFP we used is largely unknown in *Brucella*, an important perspective would be to measure it. To this aim, we could use an inhibitor of translation (e.g. puromycin) on a *Brucella* strain bearing a replicative plasmid with a constitutive promoter upstream of a *gfp*. We could therefore follow the decrease in GFP fluorescence over time and have an idea of its half-life in *Brucella*.

The use of unstable variants of GFP is useful to observe rapid changes in expression over time. However, since the signals provided by our reporter system were expected to be low during infection, we decided not to use unstable variants of GFP. Indeed, we feared that with an unstable protein, we could not observe any signal at all with our reporter system. With this in mind, we have to admit that our reporter system is mainly a tool to detect the moment when alkylating stress begins (although a delay is possible between the actual stress and the GFP expression), rather than a tool to detect rapid changes in alkylating stress.

3. Towards a better understanding of *B. abortus* response to alkylating stress

We have seen that in the absence of the two genes *ada1* and *ada2*, the reporter system does not show any increased sensitivity to DNA alkylation in *B. abortus,* both in culture following MMS treatment and during infection. This could suggest that the two Ada1 and Ada2 isoforms of *B. abortus* are not efficient at endogenously repairing MPT. As a reminder, MPT are the inducing signals for the adaptive response, as they are repaired by Ada-N which in turn is converted into a strong transcriptional activator. MPT are thus also the inducing signals for the reporter system to be activated. To explain why the sensitivity of the reporter system is not higher in the double mutant *Δada1Δada2* than in the WT *B. abortus,* we could hypothesize that Ada1 and Ada2 do repair some MPT, but not all of them. Indeed, the two domains of Ada act in a suicidal mechanism, meaning that they can only act once. It is possible that in WT *B. abortus*, Ada1 and Ada2 only repair a fraction of the MPT induced by MMS. Consistent with this, it has been shown that only one Ada molecule could be sufficient to launch an adaptive response in *E. coli* (Uphoff et al. 2016). Thereby, a very small number of MPT left unrepaired could possibly be enough to trigger the adaptive response, and by extension to activate the reporter system. As

mentioned in the introduction, an Ada-dependent adaptive response, such as the one occurring in *E. coli*, has not been observed in *B. abortus* (Katy Poncin, unpublished data). It is therefore possible, as another hypothesis, that the repair of innocuous MPT is not of critical importance in *Brucella*, and that this function would not have been selected in that organism. Accordingly, the vast majority of eukaryotic organisms do not appear to have an enzyme that repairs MPT. It is likely that eukaryotes have lost this activity as they do not have any adaptive response involving Ada (Sedgwick and Lindahl 2002). However, the genes of DNA alkylation repair could be regulated in another way in *B. abortus*. In the α-proteobacterium *C. crescentus,* neither Ada nor AlkB are induced by MMS. Nevertheless, AlkB production is cell cycle regulated in that bacterium. As a future prospect, it would be interesting to gain more insights into the regulation of AlkB and the other DNA alkylation repair genes in *B. abortus*. Interestingly, the *C. crescentus* cell cycle master regulator GcrA could be a potential target to study the regulation of the alkylation repair genes in *B. abortus*. Indeed, in *B. abortus*, detection of GcrA by western-blot was increased following exposure to MMS (Katy Poncin, unpublished data).

To better understand *B. abortus* response to alkylating stress, it would be interesting to monitor the expression of its different DNA alkylation repair genes. This could be achieved through the use of RT-qPCR in different conditions, such as exposure to alkylating agents, or ideally during infection. However, this last perspective could be technically challenging as it would require BCV isolation and purification. Moreover, since no adaptive response was reported until now for *B. abortus*, the expression of alkylation repair genes could be constitutive.

4. About DNA alkylation and *Brucella* trafficking

We expected to observe differential activation of the reporter system along *Brucella* trafficking because this bacterium displays a peculiar intracellular lifestyle. Indeed, the bacterium displays a biphasic trafficking (Deghelt et al. 2014). During the first non-proliferative phase of the infection, *Brucella* traffics along the endocytic pathway, sequentially and transiently interacting with endosomes (Celli et al. 2003; Starr et al. 2008). The second step of infection is characterized by proliferation of *Brucella* in ER-derived vacuoles. This biphasic trafficking has been observed in professional phagocytes such as macrophages, as well as in HeLa cells (Celli et al. 2003; Deghelt et al. 2014).

Differential activation of the reporter along *Brucella* infection in a host cell is difficult to assert in the light of our results. Indeed, two independent experiments gave rise to rather contradictory results regarding the activation of the reporter system at 6 h and 24 h PI. Importantly, in one of the experiments, the number of bacteria whose fluorescence had been measure was quite low, which is not suited given the high variability of the fluorescence measures. In the other experiment, we could only observe little replication of *Brucella* inside the HeLa cells at 24 h PI, which is not consistent with the majority of the infections performed with *Brucella* in HeLa cells (Celli et al. 2003; Pizarro-Cerdá et al. 1998; Starr et al. 2008) (Figure S2). To explain the unexpected low number of proliferating bacteria, one hypothesis would be that their trafficking was somehow delayed, and that they are still in the endosomal phase of the infection. From this point of view, it makes sense that the reporter is activated at 24 h PI. To further confirm this hypothesis, an additional test could be performed to determine *Brucella* subcellular localization, for example by using a specific staining of endosomal markers (such as LAMP1) or ER markers (for example Sec61β). It would be also interesting to have an additional timing for the infection, e.g. 48 h PI. Indeed, at such a late timing PI, *Brucella* would have more likely proliferated. Furthermore, because of the uncertainty regarding the stability of the GFP, it would have been more likely that at 48 h PI, the signal observed would not be due to residual GFP induced earlier in the trafficking. As a control for *Brucella* replication inside HeLa cells, it would be useful to measure CFU counts for the different strains used for the immunofluorescence experiments, at

6 h and 24 h PI. To conclude, if we cannot say that activation of the reporter system vary along infection, we cannot say that it does not vary either. In order to draw a reliable conclusion, these results should be reproduced several times with similar numbers of measures of fluorescence, and with appropriate controls to assess the replication and the actual localization of *Brucella* along the infection.

To gain more insight into the activation of the reporter system along *Brucella* infection, it would be worth using our fluorescent reporter system in a *ΔvirB* mutant. Indeed, this mutant fails to escape from the endocytic pathway and to reach its replicative niche in ER-derived vacuoles (Starr et al. 2008). As a result, this mutant is progressively killed and fails to persist and replicate inside its host cell (Celli et al. 2003; Starr et al. 2008). Therefore, if we observe activation of the reporter system in a *ΔvirB* mutant, it would support the hypothesis that alkylating stress occurs during the first phase of the infection process of *B. abortus*.

As a future control to improve our results, it would also be interesting to assess whether GFP fluorescence, regardless of DNA alkylation, would be influenced by the intracellular localization of *Brucella*. Indeed, measures of GFP fluorescence intensity could be biased due to a difference in their subcellular environment. Furthermore, GFP folding could also be influenced by the subcellular compartment of the bacteria, regardless of DNA damage. Therefore, as another control, it would be interesting to infect HeLa cells with *Brucella* bearing a plasmid with a *gfp* coding sequence inserted downstream of a P_{lac}. This way we could have an idea of the bias existing for GFP fluorescence along *Brucella* trafficking.

5. About the sources of alkylating stress in the host cell

One salient question emerges from the results we obtained so far: what are the sources of alkylating stress inside host cells? In eukaryotic cells as well as prokaryotic cells, alkylating or methylating agents can be generated by nitrosation (addition of a –NO group) of endogenous metabolites such as secondary amines or amides (Taverna and Sedgwick 1996). For instance, N-methylnitrosurea (MNU), a methylating agent, can be generated upon nitrosation of methylurea, itself formed by condensation of carbamoyl phosphate and methylamine (Durbach et al. 2003; Taverna and Sedgwick 1996). Nitrosation occurs in the presence of nitrous anhydride (N_2O_3), a product of the reaction between nitric oxide (•NO) and oxygen (O_2) (Durbach et al. 2003). Inside eukaryotic cells, an important source of nitric oxide is the inducible iNOS (Kröncke, Fehsel, and Kolb-Bachofen 1995).

Because the iNOS enzyme is expressed at higher levels in professional phagocytes such as activated macrophages (Walker, Pfeilschifter, and Kunz 1996), we used our reporter system in activated RAW 264.7 macrophages. Although it seemed that the reporter was indeed activated in RAW 264.7 macrophages, which were either activated or not, we did not perform quantitative analysis on these cells. Indeed, for this experiment we had no negative control such as the WT *B. abortus* or the negative control reporter. Therefore it would not make much sense to quantify only the fluorescence of the reporter. Besides, RAW 264.7 are less amenable to microscopy than HeLa cells, especially given our method of fluorescence quantification (Figure S3). Furthermore, because the *ΔalkB* mutant is attenuated in the same fashion in macrophages and HeLa cells (Katy Poncin, unpublished data), which are no professional phagocytes, it is possible that the iNOS is not the main source of alkylating stress.

Another potential source of nitric oxide is bacterial metabolism. Indeed, it has been shown that in *E. coli,* the bacterial enzyme nitrate reductase could catalyze the formation of nitric oxide due to an aspecific activity of the enzyme (Calmels et al. 1987; Calmels, Ohshima, and Bartsch 1988; Ralt et al. 1988). Besides, this enzyme originally involved in denitrification has been reported to contribute directly to the formation of N-nitroso compounds by catalyzing

nitrosation of amines or amides (Taverna and Sedgwick 1996). Interestingly, nitrosation of metabolites could be favored at low pH (Taverna and Sedgwick 1996). Indeed, while trafficking along the endocytic pathway, BCV undergo acidification (Porte, Liautard, and Köhler 1999). To investigate the hypothesis that nitrosative stress, and consequently alkylating stress, could be promoted by low pH, we treated HeLa cells with NH_4Cl, an agent that prevents endosome acidification, during infection by *Brucella* (Porte, Liautard, and Kohler 1999). NH_4Cl is a lysosomotropic agent, which tends to accumulate inside acidic compartments such as lysosomes and endosomes. Because it is a weak base, it accumulates as a protonated form inside those acidic compartments, thereby increasing the intravesicular pH (Ashfaq et al. 2011; Schwartz and Allen 2006). We therefore infected cells treated with NH_4Cl with *B. abortus* reporter and control strain, and then evaluated the GFP fluorescence at 6 h and 24 h PI. NH_4Cl treatment had no effect on the control reporter fluorescence, and neither had an effect on the reporter fluorescence at 6 h PI. However, interestingly, GFP fluorescence was decreased at 24 h PI when HeLa cells were treated with NH_4Cl.

Different hypotheses could explain this result. It could mean that pH drop can somehow favor the production of alkylating agents, either in the endosomes or inside the bacteria itself. *Brucella* could therefore accumulate DNA alkylating lesions caused by these putative alkylating agents so that the reporter system would be turned on at 24 h PI. When acidification of the endosomes is prevented, the mean fluorescence of the reporter is barely higher than the mean fluorescence of the negative control at 24 h PI, suggesting that DNA alkylation damage is restricted at higher pH. This is consistent with the hypothesis of Taverna and Sedgwick, 1996, which states that nitrosation of metabolites, and by extension formation of N-nitroso compounds, is favored at low pH. In addition, it has been shown that early acidification of the endosomes was required for *Brucella* survival inside macrophages (Pizarro-Cerdá et al. 1998; Starr et al. 2008). Indeed, the number of intracellular viable *B. abortus* and *B. suis* was shown to decrease at 24 h PI following the treatment of host cell with inhibitors of endosome acidification, such as NH4Cl and bafilomycin A1 (Porte, Liautard, and Kohler 1999; Starr et al. 2008). Therefore, the apparent decrease in the reporter fluorescence at 24 PI could be more simply explained by an increased mortality of *Brucella* inside their host cell at 24 h PI.

As a future perspective, it would be worth confirming those results by repeating the experiment, and by performing the same experiment with another inhibitor of endosomes acidification, such as the vacuolar proton ATPase inhibitor bafilomycin A1 (Bowman, Siebers, and Altendorf 1988).

An alternative prospect would be to use the same fluorescent reporter in other bacterial species such as *M. tuberculosis*, *S. typhimurium* or *Legionella pneumophila*. Indeed, those three bacteria display a trafficking inside their host cells which is similar but yet different from that of *B. abortus*. *M. tuberculosis* successfully replicates inside phagosomes and manages to prevent fusion with lysosomes (Warner and Mizrahi 2007). *S. typhimurium* traffics along the endocytic pathway in a controlled way and eventually reaches its replicative niche in vesicles closely associated with the Golgi apparatus. By contrast, *L. pneumophila* replicates in ER-derived vesicles, similarly to *Brucella,* but using a different trafficking to reach the replication niche (Salcedo and Holden 2005). Therefore, comparing the activation of our reporter system in those different bacteria could be a way to better understand the level of alkylating stress in different subcellular compartments.

6. About the double-edged properties of alkylating agents

Alkylating agents can react with a wide variety of atoms of the DNA, giving rise to numerous alkylation lesions. These methyl or ethyl adducts, albeit small, can have various effects on the cell, ranging from no effect at all to mutagenicity or cytotoxicity. To study the effects of alkylating stress on a bacterium such as *B. abortus*, we can therefore assess different outputs. Survival of a bacterial population exposed to alkylating stress can be assessed by CFU counting. The relative importance of each component of the alkylation response can be investigated using mutants for one of the several genes of this response. Nevertheless, DNA repair systems have often redundant, or partially redundant, functions, which makes it difficult to elucidate the role of each gene individually. The cytotoxicity aspect of alkylating stress has already been studied for different mutants in *B. abortus* and revealed that *alkB* was important during infection (Katy Poncin, unpublished data). Indeed, the *ΔalkB* strain, which is theoretically defective for DNA alkylation repair, was attenuated when exposed to alkylating agents and during infection (Katy Poncin, unpublished data).

One aspect that has not been investigated yet in *B. abortus* is the mutagenesis. Since several alkylation lesions of the DNA are mutagenic but not cytotoxic, *Brucella* cells could be subject to an increase in spontaneous mutation frequency, regardless of survival. One interesting perspective for this work would therefore be to assess the mutagenesis that might be induced by exposure to alkylating agents, but also by infection. Evaluating the frequency of spontaneous mutants able to resist to particular drugs such as 5-fluoro-orotate (generated by loss-of-function mutants in *pyrF*, for example), could help us to investigate the mutagenesis induced by alkylating agents. To gain more insights into the induction of mutagenesis along infection, we could perform full genome sequencing of bacterial clones extracted from an infection by the WT, the Δ*alkB* strain or another deletion strain. However, we have to keep in mind that the problem of redundancy is also true in the case.

In the intracellular pathogen *M. tuberculosis*, a "hypermutator" mutant strain has been constructed through deletion of the operon comprising *adaA-alkA* and *adaB* (Durbach et al. 2003). This mutant indeed showed a drastic increase in mutation frequency when compared to the WT after exposure to MNNG, but surprisingly, showed no attenuation of virulence in mice (Durbach et al. 2003). To explain this result, one hypothesis could be that *M. tuberculosis* does not sustain significant alkylation damage *in vivo*. On the other hand, *M. tuberculosis* possesses other DNA glycosylases, TagA and Mpg, which could substitute for AlkA activity during infection. One last hypothesis could be that hypermutability is tolerated during infection, so that proliferation of *M. tuberculosis* inside its host is not impaired (Durbach et al. 2003; Warner, Tonjum, and Mizrahi 2013). More than just being tolerated, it has even been suggested that hypermutability could provide *M. tuberculosis* with a selective advantage inside the host, favoring adaptability in changing environments (Durbach et al. 2003; Warner, Tonjum, and Mizrahi 2013; Yang et al. 2011). These data further confirms the importance of studying mutagenesis and not only survival in the field of DNA alkylation repair. Consistent with what was observed in *M. tuberculosis*, in *B. abortus*, survival of the double mutant *Δada1Δada2* was assessed both in culture following treatment with alkylating agents and during infection (Katy Poncin, unpublished data). Interestingly, no decrease in survival was observed for this strain in the two conditions. Accordingly, the two lesions repaired by Ada-N and Ada-C, MPT and O6meG respectively, are either neutral or mutagenic, but not cytotoxic. Therefore, it is possible that this mutant has a great increase in mutation frequency, but which is not observable regarding survival. Hence, it would be of great interest to monitor the mutagenesis of *B. abortus* during infection. Indeed, it could provide us with new insights into the genome stability and the adaptability of this pathogen inside its host.

MATERIALS AND METHODS

MATERIALS AND METHODS

Bacterial strains and culture

All *E. coli* strains and *S. typhimurium* strains were grown in Luria-Bertani (LB) medium. The DH10B *E. coli* strain was used for plasmid constructions, and the conjugative S17-1 *E. coli* strain was used for mating with *Brucella*. The *B. abortus* 544 reference strain was used and was cultivated in 2YT rich medium (LB 32 g/L,Yeast Extract 5g/L, BD and Peptone 6 g/L) Antibiotics were used at the following concentrations: ampicillin, 100 µg ml^{-1}; chloramphenicol, 20 µg ml^{-1}; kanamycin, 50 µg ml^{-1}; nalidixic acid, 25 µg ml^{-1}.

Plasmids and constructions

DNA manipulations were performed according to standard techniques (Green and Sambrook 2012). Primers sequences are detailed in Table 2.

Table 2 – Primers used in this work

Name	**5'-3' Sequence**
F-yojL-coli	AAACTAGTTGAGCTACTGAAGTTACCGTTC
R-(serine2)-ada-coli	GACTCGAGCATTACCTCTCCTCATTTT
F1-cfp	GAATTCGATTAATGCTCGAGT
F2-cfp	CTGACCTGGGGCGTGCAGTGCTTC
R1-cfp	GCACTGCACGCCCCAGGTCAGGGTGGTCACC
R2-cfp	TTCTGCAGTCACTTATACAGTTC
F-yojL-coli	AAACTAGTTGAGCTACTGAAGTTACCGTTC
R1-C38A-ada	GTCTGGCGCGGCAAGACGGACGGGCAAAGATGCCTGTGGTACG
F2-C38A-ada	CCACAGGCATCTTTGCCCGTCCGTCTT
R-serine2-ada	CCTTCGACTCGAGGCTCATTACCTCTCCTCATTTTC
F2-alternatif-C38mut	TCTTGCCGCGCCAGACAT
R1-C38S-ada	GTCTGGCGCGGCAAGACGGACGCGAAAAGATGCCTGTGGTAC
F2-C38S-ada	CCACAGGCATCTTTTCGCGTCCGTCTTGCCGCGC

Table 3 – Strains used in this work

Name	Strains	Antibiotic resistance
pBBRMCS1-pada-ada	DH10B	Cm
pBBRMCS1-pada-adaC38A	DH10B	Cm
pBBRMCS1-pada-adaC38A-gfp	DH10B	Cm
pBBRMCS1-pada-adaC38S	DH10B	Cm
pBBRMCS1-pada-adaC38S-gfp	DH10B	Cm
pBBRMCS1-pada-ada-cfp	DH10B	Cm
pBBRMCS1-pada-ada-gfp	DH10B	Cm
pFPV25.1-mCherry	DH10B	Amp
pGEMT-cfp superfolder	DH10B	Amp
pMR10-pada-ada-cfp	DH10B	Kan
pMR10-pada-ada-gfp	DH10B	Kan
pBBRMCS1-pada-adaC38A-gfp	S17	Cm
pBBRMCS1-pada-adaC38S-gfp	S17	Cm
pBBRMCS1-pada-ada-cfp	S17	Cm
pBBRMCS1-pada-ada-gfp	S17	Cm
pMR10-pada-ada-cfp	S17	Kan
pMR10-pada-ada-gfp	S17	Kan
pBBRMCS1-pada-ada-cfp	*S. typhimurium*	Cm
pBBRMCS1-pada-ada-gfp	*S. typhimurium*	Cm
pFPV25.1-gfp	*S. typhimurium*	Amp
pFPV25.1-mCherry	*S. typhimurium*	Amp
pMR10-pada-ada-cfp	*S. typhimurium*	Kan
pMR10-pada-ada-gfp	*S. typhimurium*	Kan
pBBRMCS1-pada-adaC38A-gfp	*B. abortus* 544	Cm
pBBRMCS1-pada-adaC38S-gfp	*B. abortus* 544	Cm
pBBRMCS1-pada-ada-cfp	*B. abortus* 544	Cm
pBBRMCS1-pada-ada-gfp	*B. abortus* 544	Cm
pMR10-pada-ada-cfp	*B. abortus* 544	Kan
pMR10-pada-ada-gfp	*B. abortus* 544	Kan
Δada1Δada2	*B. abortus* 544	
Δada1Δada2 pBBRMCS1-pada-adaC38A-gfp	*B. abortus* 544	Cm
Δada1Δada2 pBBRMCS1-pada-adaC38S-gfp	*B. abortus* 544	Cm
Δada1Δada2 pBBRMCS1-pada-ada-gfp	*B. abortus* 544	Cm

The *ada*-based reporter system was obtained first by PCR amplification of the *ada* coding sequence and its promoter region from *E. coli* genomic DNA (P_{ada}-*ada*), using the F-yojL-coli primer and the R-(serine2)-ada-coli primer.The PCR product was cloned into an *Eco*RV-linearized pBBR-MCS1 plasmid and the resulting plasmid was checked by sequencing. The P_{ada}-*ada* sequence was then excised from the recombinant plasmid using *Xho*I and *Spe*I enzymes.

The Green Fluorescent Protein (GFP) used in this reporter system is a "Superfolder GFP" whose codon usage bias has been adapted to *B. abortus*. This latter was ordered as a "gBlock" (Integrated DNA Technologies, IDT). The *gfp* coding sequence was inserted into an *Eco*RV-linearized pGEM-T and the recombinant plasmid was then checked by sequencing. The fragment coding for the *gfp* was excised using *Pst*I and *Xho*I. The P_{ada}-*ada* fragment and the *gfp* fragment were finally inserted by triple ligation into a pBBR-MCS1 plasmid previously digested with *Pst*I and *Spe*I. This gave rise to pBBR-MCS1- P_{ada}-*ada-gfp*, with the two coding sequences in the opposite orientation to the endogenous P_{lac}. The final construction was transformed into *E. coli* S17-1 and then introduced to *B. abortus* 544 wild-type strain by mating. For *S. typhimurium,* the final plasmid was purified and transferred by electroporation.

The mutated reporter system was obtained by point mutation site-directed mutagenesis. The *ada* gene of *E. coli* was amplified using primers carrying the mutation. First, the upstream region of the mutation was amplified using the primers F-yojL-coli and R1-C38A-ada. Then the downstream region was amplified using the primers F2-C38A-ada and R-(serine2)-ada-coli. A joint PCR between the two PCR products was performed using the primers F-yojL-coli and R-(serine2)-ada-coli. The resulting product was then cloned into an *Eco*RV-linearized pBBR-MCS1 plasmid. Then, the strategy used for the construction of the mutated reporter was the same as for the WT reporter system. The P_{ada}-*ada*C38A/S sequence was excised from the recombinant plasmid using *Xho*I and *Spe*I enzymes and inserted together with the *gfp* fragment into a pBBR-MCS1 plasmid by triple ligation.

For treatment of the bacteria with alkylating agents, the different bacterial strains were grown at 37°C overnight. The liquid culture was diluted ten times the next morning, then after 2h of incubation at 37°C, bacteria were exposed to MMS at final concentrations of 0.1, 0.5, 1, 2 and 5 mM. *E. coli* cells were also exposed to ethyl methanesulphonate (EMS) at a final concentration of 20 nM. Those suspensions were incubated at 37°C with shaking for different incubation times and then dropped on agarose pad for microscope observation.

The different strains used in this study are summarized in Table 3.

Bioscreen

The different *B. abortus* strains were grown in 2YT at 37°C overnight. The next day, bacterial cultures were diluted in 2YT to obtain an optical density of 0.1 at 600 nm (OD_{600}). 200 µL of each properly diluted bacterial cultures were put in wells (Honeycomb 2 plate), and growth was measured by reading OD_{600} every 30 minutes during 72 h (Bioscreen C MBR).

HeLa cells and RAW 264.7 cultures and infections

HeLa cells were cultured at 37°C in a 5% CO_2 atmosphere in DMEM supplemented with 10% Foetal Bovine Serum (FBS, Gibco), 0.1 g l^{-1} non-essential amino acids (Invitrogen) and 0.1 g l^{-1} sodium pyruvate 4.5 g l^{-1}. RAW 264.7 macrophages were cultured at 37°C in a 5% CO_2 atmosphere in DMEM supplemented with 10% FBS (Gibco). Cultures of *Brucella* were prepared in DMEM at a multiplicity of infection (MOI) of 300 for HeLa cells infection and a MOI of 50 for RAW 264.7 infections. Bacteria from an overnight culture were diluted in

DMEM to reach their MOI. They were then centrifuged onto HeLa cells or RAW 264.7 macrophages at 1200 rpm for 10 minutes at 4°C and then incubated 1 hour at 37°C in a 5% CO_2 atmosphere. After one hour of incubation, cell were washed twice with culture medium and then incubated in medium supplemented with 50 µg ml^{-1} gentamicin to kill extracellular bacteria.

For activated RAW 264.7 macrophages, the culture was started in the morning, then after 7 hours, DMEM medium was replaced by medium supplemented with 10 ng ml^{-1} LPS (Sigma Aldrich) and 20 ng ml^{-1} IFN-γ (BD Biosciences).

For the treatment of infected HeLa cells with NH_4Cl, 30 mM of NH_4Cl was added to HeLa cells at 1 h PI, together with gentamicin.

Immunostaining of infected host cells

At specific time points (6 h and 24 h PI), HeLa cells were washed twice with PBS, then fixed in 2% paraformaldehyde (PFA) at 37°C for 20 minutes and permeabilized during 10 minutes with Triton X100 0.1%. Antibodies were prepared in a PBS solution containing bovine serum albumin (BSA) 3% and TritonX100 0.1%. The primary antibody used for the detection of intracellular *Brucella* was an A76-12G12 anti-LPS monoclonal antibody (not diluted), and the secondary antibody was an anti-IgG mouse coupled to Texas Red (Invitrogen) (diluted 500 times). The primary and secondary staining lasted 30 minutes. Coverslips were finally washed three times with PBS, two times with water and then mounted with Mowiol.

Microscopy

For the observation of bacteria, 2 µl of a liquid culture were dropped on agarose pad (solution of 1% agarose in PBS) and sealed with VALAP (1/3 of vaseline, 1/3 of lanoline and 1/3 of paraffin wax). Bacterial samples, as well as infected HeLa cells, were observed with a Nikon 80i (objective phase contrast 100X, plan Apo) connected to a Hamamatsu ORCA-ER camera.

Fluorescence Quantification

Quantification of the relative fluorescence intensity of each bacterium was obtained using NIS-element software (Figure S3). The intensity profile of Texas Red and GFP fluorescence was obtained for each pixel across a line manually drawn on the picture of the infected cell. The mean fluorescence of the peak was defined as the mean fluorescence intensity of 8 pixels in the fluorescence peak corresponding to the bacteria. "Background" fluorescence was calculated with the mean fluorescence intensity of 8 pixels on both sides of the bacteria, inside the host cell. Relative fluorescence intensity of each bacterium, therefore, corresponds to the difference between the mean peak fluorescence and the mean background fluorescence.

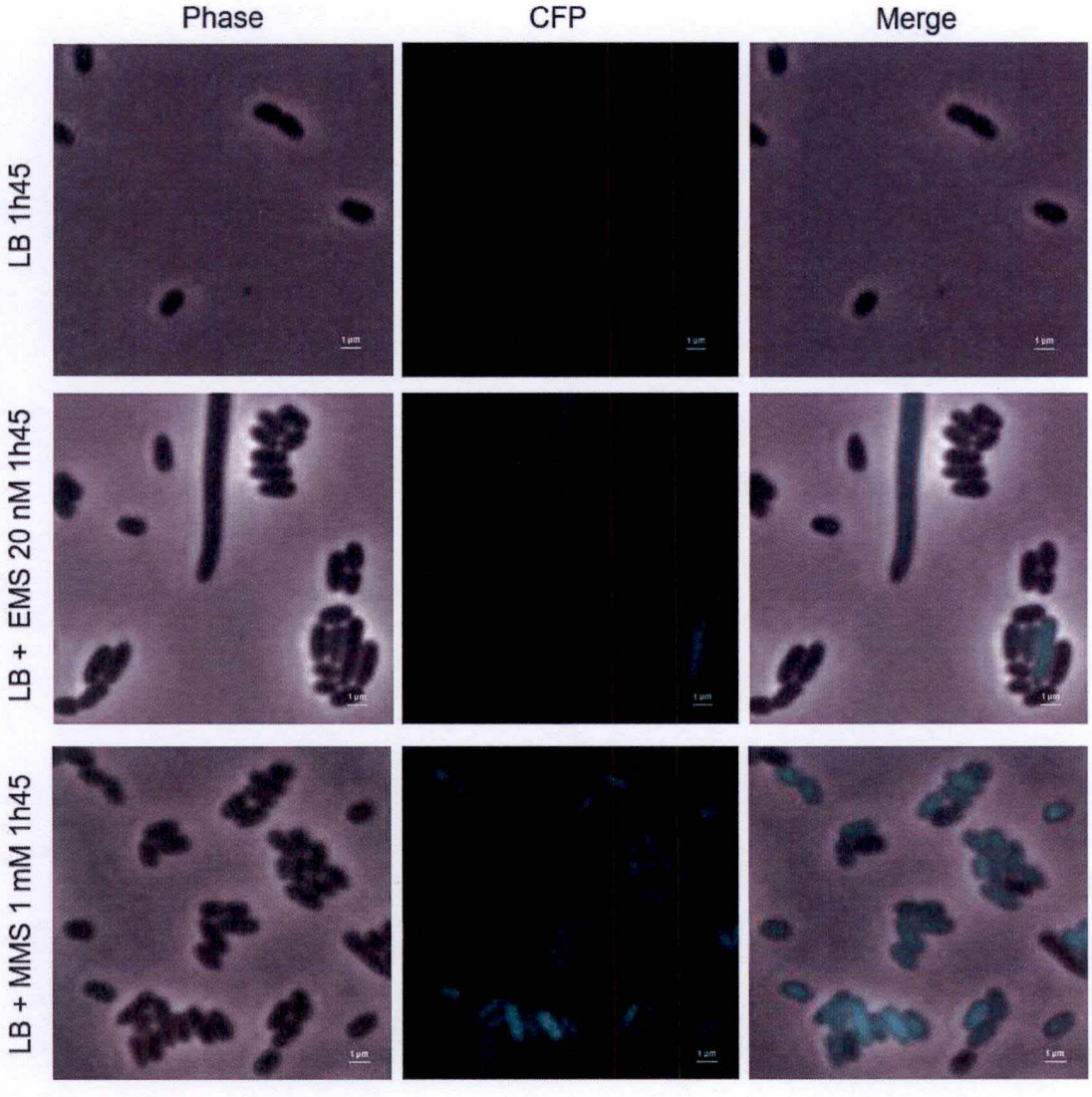


Figure S1 – Characterization of the pBBR-MCS1 P_{ada}-*ada-cfp* reporter in *E. coli* upon MMS and EMS treatment. Exponential phase cultures of *E. coli* carrying the plasmid-borne P_{ada}-*ada-cfp* reporter system were treated with 20 nM of ethyl methanesulfonate (EMS) or 1 mM of methyl methanesulfonate (MMS) for 1h45. Scale bars, 1 μm.

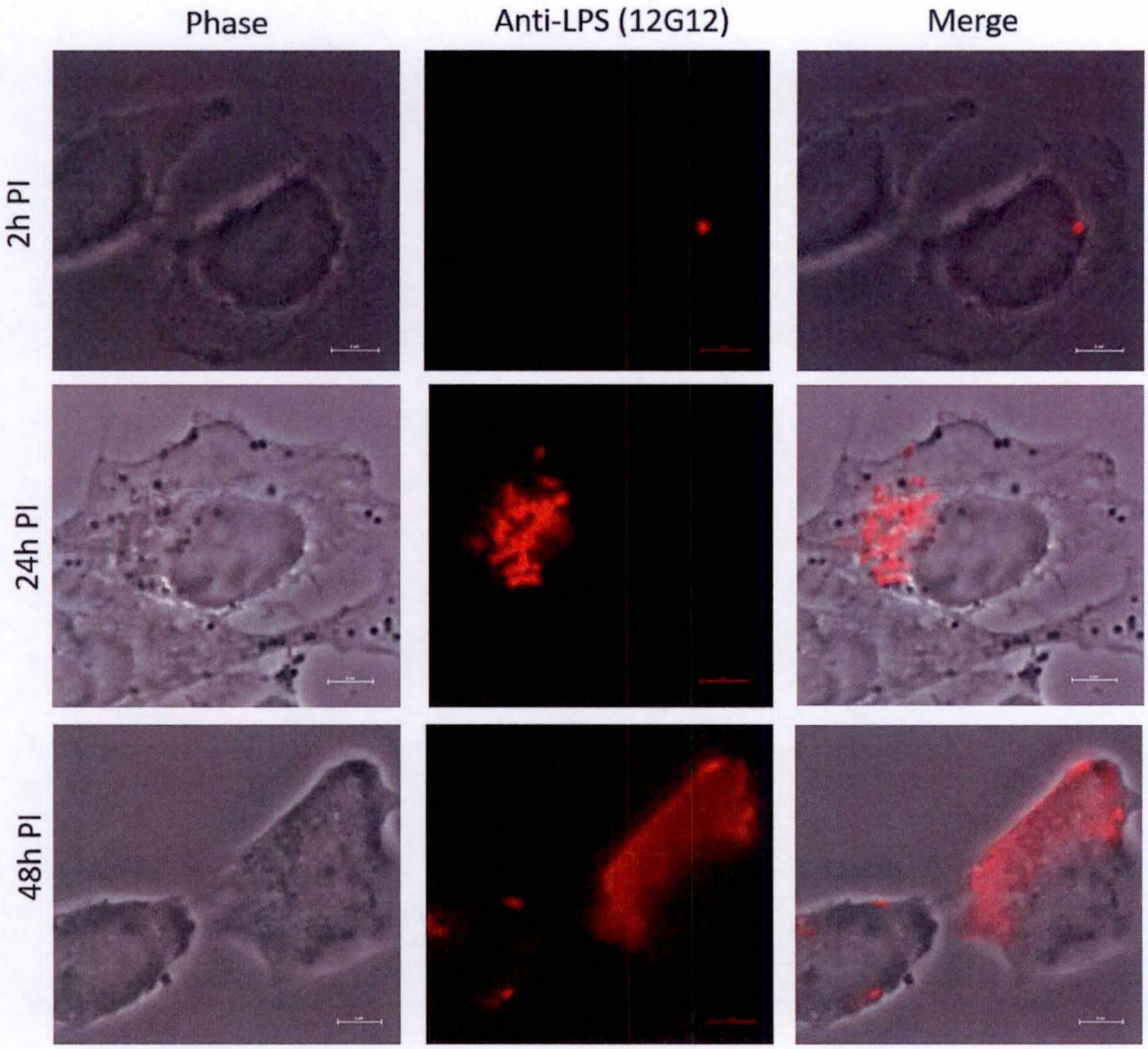


Figure S2 – *B. abortus* replication inside HeLa cells. HeLa cells were infected by *B. abortus* reference strain 544 and imaged at 2 h, 24 h and 48 h post-infection (PI). *Brucella* LPS was labelled using the 12G12 monoclonal antibody and a secondary anti-mouse IgG coupled to Texas Red. Scale bars, 1 μm.

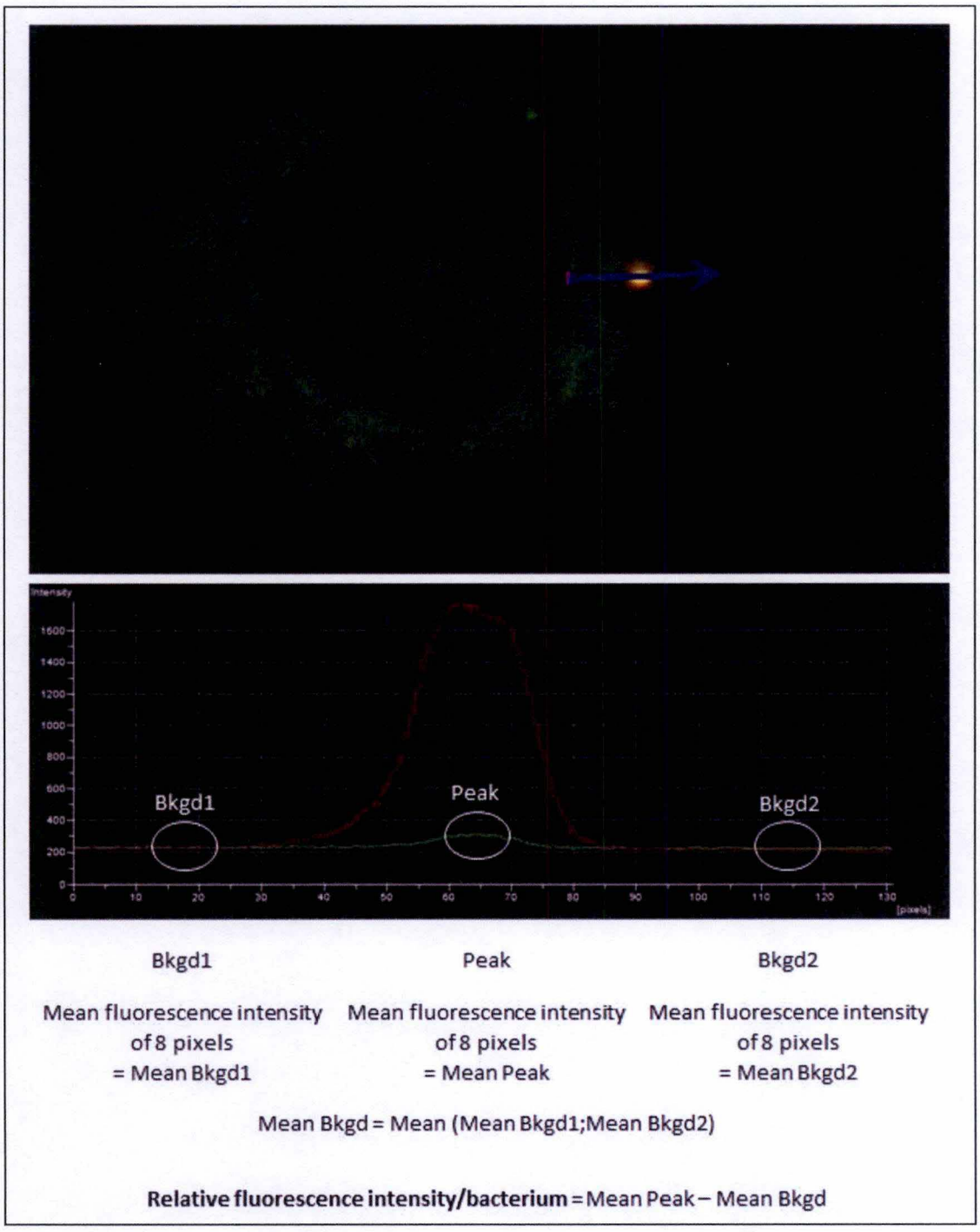


Figure S3 – Explanation of the method of GFP fluorescence quantification for the reporter system during infection of HeLa cells.

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