Mécanismes de persistance de Bartonella dans son hôte réservoir

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Mechanisms of *Bartonella* persistence in its reservoir host

Jury

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Abstract

Each *Bartonella* species appears to be highly adapted to one or a limited number of reservoir hosts, in which it establishes a long-lasting intraerythrocytic bacteremia as the hallmark of infection. Although the course of *Bartonella* infection has been precisely described, the molecular mechanisms of host specific erythrocyte infection and the stages of precedent the arrival in the bloodstream are poorly understood. In this thesis we purposed to identify the mechanisms of erythrocyte infection by *Bartonella* and characterize the possible locations of *Bartonella* during the days before the intraerythrocytic stage.

By the establishment of an *in vitro* model of adhesion and invasion of erythrocytes by *Bartonella* spp., we demonstrated that host specificity was determined by the interaction between bacteria and erythrocytes. By screening signature-tagged mutagenesis (STM) library of *B. birtlesii in vivo* and *in vitro* and ectopic expression, we revealed that type IV Trw locus was required for host-restricted adhesion to erythrocytes in a wide range of mammals. After that, we further characterized that only TrwJ1 and TrwJ2 were expressed and present on the surface of the bacteria and had the ability to bind to mouse erythrocytes, and the receptor of them was erythrocyte band3 by different technology (phage display, electron microscopy, far western blot and adherence and invasion inhibition assay). By the model of experimental infection of laboratory normal Balb/C mice and splenectomized mice with *B. birtlesii*, we showed that during the first 7 days, no bacteria were recovered from lymph nodes, bone marrow and brain, but in the spleen, transient in the liver, And bacteremia was the same in both infection models during the first 7 days, thereafter, bacteremia was 10 fold higher in splenectomized mice than in normal mice and lasted 2 weeks longer. This suggested that the spleen was able to retain *Bartonella*.

In conclusion, the host specific adhesion between *Bartonella* and erythrocyte was mediated by Trw and erythrocytic band 3, and spleen had a role in retention *Bartonella*.

INTRODUCTION GENERALE

INTRODUCTION GENERALE

Les bactéries du genre *Bartonella* spp. sont des alpha-protéobactéries réparties dans le monde entier [1]. Ces bactéries, Gram-, sont principalement transmises par des vecteurs arthropodes ou par contact direct avec un animal infecté [2].

A ce jour, au moins 24 espèces de *Bartonella* spp. ont été décrites [3]. Chaque espèce semble être très adapté à un ou quelques hôtes réservoirs, dans lequel la bactérie établit une bactériémie intra-érythrocytaire pouvant durée plusieurs mois [2, 4]. Deux espèces de *Bartonella* ont un réservoir humain: *B. bacilliformis* et *B. quintana*, toutes les autres ont pour réservoir des animaux sauvages ou domestiques. *B. bacilliformis* est considéré comme le seul représentant d'une lignée ancestrale à partir de laquelle les autres espèces de *Bartonella* auraient évolué [5, 6]. Les infections à *B. bacilliformis* ne se produisent que dans les régions andines de l'Amérique du Sud [7, 8], et parmi toutes les espèces, c'est celle qui induit les maladies les plus graves (Des taux de mortalité jusqu'à 80% ont été décrites chez des patients ne recevant pas de traitement antibiotique [9]). Les infections à *B. quintana* sont réparties dans le monde entier, elles ont d'abord été reportées au cours de la Première Guerre mondiale sous la forme de la fièvre des tranchées (ou fièvre quintane). L'intérêt médical pour *B. quintana* s'est estompé après la Seconde Guerre mondiale et a été ravivé à la fin du XXe siècle. En effet, *B. quintana* est réapparu comme une bactérie d'importance en santé publique avec une reconnaissance de son rôle dans des bactériémies persistantes et des endocardites chez les sans-abris (fièvre des tranchées urbaines).

De nombreux animaux domestiques et sauvages, y compris les ruminants, les chats, les chiens et les rongeurs peuvent servir d'hôtes réservoirs pour les 22 autres espèces de *Bartonella* [2]. Au moins 13 de ces espèces sont des agents de zoonoses et l'infection chez l'homme est à l'origine de manifestations cliniques aiguës variées alors, que chez l'animal, l'infection est le plus souvent

asymptomatique.

Parmi ces espèces zoonotiques, *B. henselae* est celle qui a l'impact le plus important en santé publique. L'infection provoque une grande variété de manifestations cliniques, y compris des adénopathies (maladie des griffes du chat), malaises, fièvres et splénomégalie qui peut persister pendant plusieurs mois. Moins fréquemment (25% des cas) [10], des manifestations plus graves peuvent survenir, y compris des angiomatoses (chez les patients immunodéprimés), des troubles oculaires, des encéphalites, méningites, glomérulonéphrites et des endocardites [11].

En ce qui concerne le cycle de vie des Bartonelles, il est admis qu'après pénétration dans son hôte vertébré réservoir, par le biais d'une morsure ou d'une piqûre par un arthropode, *Bartonella* spp. colonise une niche primaire encore inconnue à ce jour. Puis, par vagues successives, elle colonise le sang, infecte les érythrocytes dans lesquels la bactérie se multiplie pour atteindre environ 8 bactéries par cellule et survit ainsi jusqu'à la mort naturelle du globule rouge [12]. Cette caractéristique semble vraie pour tous les représentants du genre, sauf pour *B. bacilliformis* qui se multiplie jusqu'à la lyse de l'érythrocyte infecté. La colonisation intra-érythrocytaire est une étape clé qui assure à la fois la multiplication et le maintien de la bactérie, à l'abri des défenses immunitaires, et sa transmission à un vecteur arthropode au moment de son repas sanguin [13]. Les essais d'infection d'espèces de mammifères non réservoirs par des espèces hétérologues de *Bartonella* aboutissent à une bactériémie très fugace, suivi d'une élimination de la bactérie du sang circulant. L'étude des mécanismes liés à la persistence de *Bartonella* spp. dans son hôte réservoir et plus précisément à la persistence intra-érythrocytaire a fait l'objet de cette thèse.

En guise d'introduction générale de ce travail de thèse, nous avons réalisé une revue blibliographique récapitulant les connaissances actuelles concernant les strategies utilisées par *Bartonella* spp. pour infecter son hôte reservoir.

Le premier chapitre de cette thèse est consacré à l'identification des gènes essentiels à l'induction de la bactériémie en utilisant un modèle murin d'infection

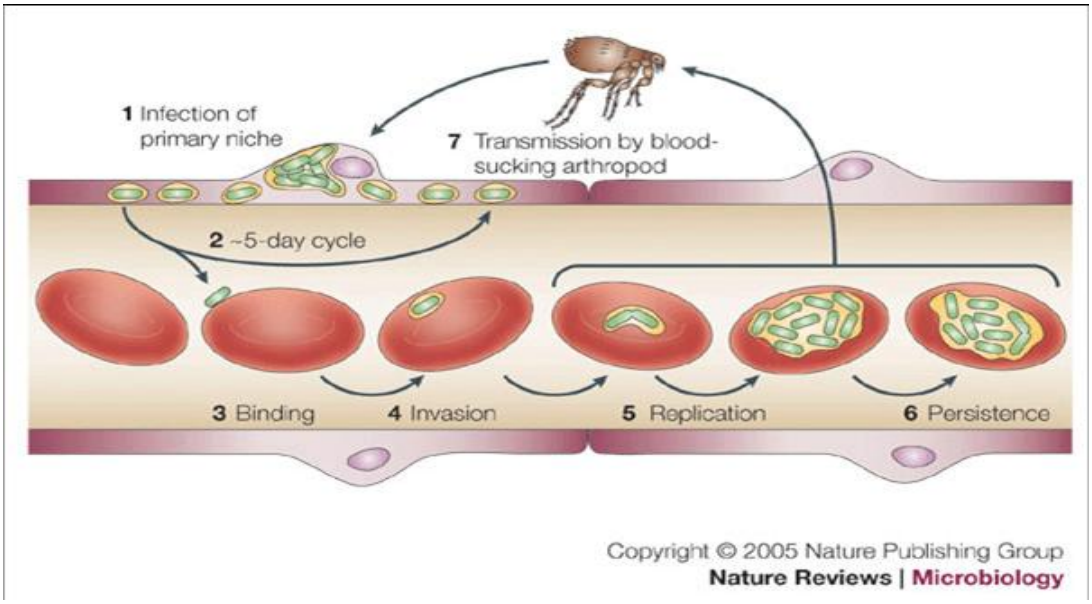
par *B. birtlesii,* grâce à l'utilisation d'une banque de mutants de cette bactérie. Nous avons ensuite mis au point un système d'infection *in vitro* d'érythrocytes par *Bartonella* spp. ce qui nous a permis d'identifier parmi les mutants qui ont perdu la capacités à induire une bactériémie chez la souris, ceux qui sont altérés dans leur capacité à infecter les globules rouges. Cette étude nous a permis de mettre en évidence le rôle clé de la reconnaissance du globule rouge dans la spécificité d'hôtes de *Bartonella* spp. et d'identifier le système de sécrétion de type IV Trw de *Bartonella* spp, comme l'élément clé de la reconnaissance hôte-spécifique du globule rouge.

Le deuxième chapitre de cette thèse est consacré à l'identification des composants de ce système de sécrétion qui sont responsable de l'adhesion de *Bartonella* spp. aux érythrocytes et leur récepteur érythrocytaire.

Dans cette thèse, nous avons participé à l'élucidation des mécanismes de reconnaissance des globules rouges par *Bartonella* spp. Toutefois, les étapes plus précoces de l'infection, avant l'arrivée de *Bartonella* spp dans le sang, sont beaucoup moins connues. L'opinion courante est que les cellules endothéliales vasculaires seraient une des cibles des bactéries avant qu'elles n'infectent les globules rouges [12, 14-18]. D'autres cellules, comme les précurseurs des globules rouges, ont également été proposées comme niche précoce de *Bartonella* spp [19]. Dans le troisième chapitre de cette thèse, nous avons exploré les localisations de *Bartonella* spp. pendant les premiers jours suivant l'infection en utilisant le modèle d'infection de souris par *B. birtlesii*.

Review

Strategies of exploitation of mammalian reservoirs by *Bartonella* species



Review

Strategies of exploitation of mammalian reservoirs by *Bartonella* species

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Strategies of exploitation of mammalian reservoirs by *Bartonella* species

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Abstract

Numerous mammal species, including domestic and wild animals such as ruminants, dogs, cats and rodents, as well as humans, serve as reservoir hosts for various *Bartonella* species. Some of those species reservoired by non-human mammals have zoonotic potential. Our understanding of interactions between bartonellae and reservoir hosts has been greatly improved by the development of animal models of infection and the use of molecular tools allowing large scale mutagenesis of *Bartonella* species. By reviewing and combining the results of these, and other approaches, we can obtain a comprehensive insight into the molecular interactions that underlie exploitation of reservoir hosts by *Bartonella* species, particularly the well-studied interactions with vascular endothelial cells and erythrocytes.

Keywords: Bartonellae, human, animal, pathogenesis, reservoir hosts.

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1. INTRODUCTION

Bartonella species are small, curved, pleomorphic, fastidious, hemotropic Gram-negative bacteria that have specifically adapted to infect mammals. Although 26 *Bartonella* species or sub-species have been formally validated to date, many more, as yet partially characterized, await formal proposal. Bartonellae have been encountered in mammals all over the world, however, the prevalence of infections and their public health and veterinary importance vary according to species and geographical region.

Bartonella species are transmitted between mammalian hosts by blood-feeding arthropods. Numerous, diverse arthropods have been implicated in the transmission of bartonellae, but each *Bartonella* species appears to exploit only one, or few, arthropod species. The role of arthropods in the natural cycle of bartonellae may extend beyond more vectors; there is some evidence to support their role as additional reservoirs for the bacteria. Each *Bartonella* species appears to be highly adapted to one or few mammalian reservoir hosts [1, 2], in which *Bartonella* establishes a long-lasting intra-erythrocytic bacteraemia as the hallmark of infection [3]. This bacteraemia does not appear to cause immediate detriment to the host. In general, bartonellae provoke acute clinical manifestations only when accidentally introduced into the wrong host or when encountering immunocompromised individuals among reservoir populations.

The *Bartonella* genus lies among the alpha proteobacteria in proximity to the genus *Brucella*. Both genera are classified in the family *Rhizobiales* that also embraces a large number of taxa of plant-associated and environmental bacteria. Twenty four *Bartonella* species have been validly described to date, with one species, *B. vinsonii*, subdivided into three subspecies (Table 1). Inference of the phylogenetic relationships within the genus reveals a profound

divergence of *B. bacilliformis*, which lies alone on an “ancient” ancestral lineage (lineage 1), apart from the other *Bartonella* species (Figure 1). The remaining “modern” species form two further lineages, with lineage 2 containing the four ruminant-associated species (*B. bovis, B. capreoli, B. chomelii and B. schoenbuchensis*), and lineage 3 containing the remaining 19 species (Figure 1). Within lineage 3, *B. clarridgeiae* is the outlier, and phylogenetic studies that have also included as yet only partially characterised bartonellae have suggested that this species is a representative of a fourth lineage within the genus [4, 5]. The complete genome sequences of six *Bartonella* species (*B. bacilliformis, B. clarridgeiae, B. grahamii, B. henselae, B. quintana* and *B. tribocorum*) have been published to date, although efforts to sequence the genomes of all remaining species and numerous partially-characterised bartonellae are also underway. The sizes of the published genomes range from 1.45Mb to 2.62Mb [4, 6].

B. bacilliformis and *B. quintana* are the two *Bartonella* species that appear to exploit humans as reservoir hosts (Table 1). Infections with *B. bacilliformis* only occur in the Andean region of South America [7, 8], and this specific geographical distribution correlates with the range of the *Lutzomyia* species that are thought to transmit infections [8]. *B. bacilliformis* appears to be potentially the most pathogenic *Bartonella* species, provoking a remarkably bi-phasic disease referred to as bartonellosis. Acute bartonellosis manifests as Oroya fever, and is characterized by infection, then haemolysis, of nearly 100% of erythrocytes. Fatality rates of up to 80% have been described in patients not receiving antibiotic treatment [9]. *B. bacilliformis* infections can also manifest as verruga peruana, characterized by vascular tumors that result from the massive proliferation of endothelial cells, and which can persist for more than one year [7, 8]. Verruga peruana was probably recognized during pre-Columbian times, but its etiology was not resolved until 1905, when *B. bacilliformis* was first isolated by Barton in 1909. *B. quintana* infections were

first recognized during World War I in the form of trench fever (or five-day fever), but the bacterium was not isolated until 1961. Medical interest in *B. quintana* waned after World War II as infections became rare, but, at the end of the twentieth century, *B. quintana* re-emerged as a bacterium of public health importance with recognition of its role in persistent bacteraemia in the homeless, drug and alcohol addicts ("urban" trench fever) and refugees. Infection is usually characterized by a chronic intra-erythrocytic bacteraemia with few, mild, self-limiting symptoms [10], although more severe manifestations such as endocarditis and bacillary angiomatosis have also been reported [10]. *B. quintana* is transmitted by the human body louse (*Pediculus humanus humanus*), an ectoparasite that is specific to humans but which only emerges when clothes remain unchanged and unwashed [11]. Thus, as with *B. bacilliformis*, vector behavior is a key determinant in the epidemiology of *B. quintana* infections.

Numerous domestic and wild animals, including ruminants, felids, canids and rodents serve as reservoir hosts for various *Bartonella* species (Table 1) [2]. To date, evidence of zoonotic potential has been reported for 10 of these species (Table 1). In these reports, no evidence of an intra-erythrocytic presence of zoonotic bartonellae was recorded in humans [12]. Of the zoonosis-associated *Bartonella* species, *B. henselae* is the most frequently associated with human disease. *B. henselae* exploits felids, including domestic cats as reservoir hosts, between which infection is transmitted by cat fleas (*Ctenocephalides felis*) [13]. As is typical for reservoir hosts, cats usually do not develop any apparent symptoms of infection, which can persist for months or years [14]. *B. henselae* transmission from cats to humans is thought to occur primarily by the inoculation of infected flea faeces via cat scratches or bites [13], although there is also some evidence that infection can also be acquired from ticks [15, 16]. *B. henselae* can provoke a wide variety of clinical manifestations in humans, including, most commonly, lymphadenopathy (cat

scratch disease), malaise, fever and splenomegaly that can persist for several months. Less frequently (25% of cases) [17], more serious manifestations can occur, including angiomatosis (in immunocompromised patients), ocular disorder, encephalitis, meningitis, glomerulonephritis and endocarditis [12].

The other nine zoonosis-associated *Bartonella* species exploit a range of reservoir hosts; for example, *B. vinsonii* subspecies *berkhoffii* parasitizes canids (domestic dogs, coyotes and gray foxes) and *B. alsatica* parasitizes rabbits. Others, such as *B. grahamii* are associated with woodland rodents (Table 1). In addition to their potential as human pathogens, numerous *Bartonella* species have been implicated in veterinary infectious diseases, including, most frequently, *B. henselae* and *B. vinsonii* subspecies *berkhoffii*. Many of the manifestations observed in cats and dogs are akin to those observed in humans.

2. DYNAMICS OF INFECTION IN MAMMALIAN RESERVOIR HOSTS

The life cycle of bartonellae in their reservoir hosts has been deduced through observations of naturally infected mammals and through experimental infections. In a seminal study [3], the evolution of infection of *B.tribocorum* in laboratory rats was monitored using a green fluorescent protein (GFP)-tagged bacterial strain in conjunction with microscopy and flow cytometry. This study revealed that, following intravenous injection (models incorporating a natural route of infection still remain generally elusive), bacteria were cleared from circulating blood within few hours but bacteraemia reappeared approximately five days later. In the blood, bacteria were observed adhering to and invading mature erythrocytes within two days. An erythrocyte was usually invaded by a single bacterium, which, once inside, replicated in a membrane-bound compartment over a period of several days until, on average, eight daughter cells were created. Replication then ceased, and infected erythrocytes were

shown to persist in circulation for several weeks. This process did not provoke symptoms in the infected rat [3]. Experiments using other animal models, such as the *B. birtlesii*-mouse model [18-20], the *B. henselae*-cat model [21] have yielded results that are akin to those observed in the *B. tribocurum*-rat model, suggesting a common infection mode for all *Bartonella* species in their respective animal reservoirs [22]. Furthermore, that the kinetics of bacteraemia observed in these models were similar to those observed captured naturally-infected animals (unpublished observations), supports the status of the models, including their artificial routes of inoculation, as faithful and therefore meaningful reproductions.

Our understanding of bartonellae/host interactions has been greatly improved by the development of molecular tools allowing large scale mutagenesis of *Bartonella* species such as *B. tribocorum* and *B. birtlesii*. The use of these tools in conjunction with judicious screening in relevant animal models (rats and mouse respectively) has resulted in the identification of numerous genetic sequences the integrity of which is necessary for the establishment of bacteraemia by bartonellae [4, 19]. Those genetic sequences can be classified in six groups: (1) genes previously implicated in *Bartonella* infection of its mammalian hosts, (2) genes implicated in cell envelope integrity, (3) genes involved in metabolism, (4) phage-related genes, (5) genes encoding proteins of unknown function and (6) intergenic regions. These data, when combined with the results of *in vitro* studies, allow us to draw the picture of the molecular basis of the strategy used by *Bartonella* species to exploit reservoir hosts.

3. STEP 1: INFECTION PRIOR TO BACTERAEMIA.

As mentioned above, inoculation of a susceptible host appears to be primarily mediated by the introduction of infected vector faeces into cuts or scratches on the skin. However, the fate of infecting bacteria immediately following

inoculation, prior to their appearance in the bloodstream, remains uncertain. However, it is clear that bartonellae can colonize highly vascularized tissues like liver and spleen during the first days of infection as well as the vascular bed of the skin (for *B. bacilliformis*) [23]. Current opinion is that the vascular endotheliature serves as a primary niche for bartonellae prior to them entering the bloodstream. Indeed, bartonellae have been shown to have the unique ability to induce vasoproliferation, manifesting as verruga peruana (*B. bacilliformis*) or bacillary angiomatosis/peliosis hepatis (*B. henselae* and *B. quintana*). In many *in vitro* studies, bartonellae have been shown to be able to invade endothelial cell lines and/or interfere with their physiology [24-28] (see below). However, although the endothelial vasculature undeniably plays a role in the early stages of infection, there is some experimental evidence that other putative cell types, such as erythrocytic precursors, may also serve as a niche for infecting bartonellae [29]. This hypothesis, however, conflicts with data obtained from experiments with the GFP-*B.tribocorum*/rat model that clearly indicated that encounter with, and invasion of, erythrocytes occurs in the bloodstream [3]. In support of this observation, we have been unable to find any evidence for the presence of bartonellae in erythrocyte precursors isolated from the bone marrow of *B. birtlesii*-infected mice, despite rigorous efforts to do so (unpublished observations).

It should also be borne in mind that evidence for vascular endothelial cell involvement *in vivo* is drawn primarily from pathological observations of *B. bacilliformis*-induced verruga peruana, and bacillary angiomatosis, a rare manifestation of *B. henselae* and *B. quintana* infections of humans. Endothelial cell colonization has not yet been reported in asymptomatically infected reservoir hosts. Furthermore, pathological study of the vasculoproliferative lesions that characterize verruga peruana and bacillary angiomatosis suggest bartonellae are concentrated in proximity to the external surface of the endothelial cells rather within them. Thus, although we devote much of this

review to the molecular basis of bartonellae-vascular endothelial cell interactions, we do not discount the possibility that an as yet unidentified alternative primary niche may exist.

Exploitation of vascular endothelial cells by bartonellae involves binding to the cell surface, possible internalization, then persist within (or adjacent to) cells. To date, two major bacterial factors that play crucial roles in interacting with endothelial cells have been identified, namely the BadA/Vomp/Brp proteins and the VirB/D4 type four secretion system (T4SS) and its effectors. Other bacterial surface proteins, including the heme-binding protein A (pap31) or Omp43, have also been shown to interact with these cells. All of these factors have also been shown to be essential for the establishment of bacteraemia, as revealed by signature-tagged mutagenesis (STM) screening in both *B. birtlesii*-mouse and *B. tribocorum*-rat infection models, demonstrating their essential function in mammalian host infection and underlying the notion that bacteraemia is not the initial stage of the infection process.

3.1. The BadA/Vomp/Brp adhesions

These proteins have different names in different *Bartonella* species, reflecting their concurrent discovery by independent groups of researchers. These proteins are referred to as *Bartonella* adhesin A (BadA) in *B. henselae* [30], the variably-expressed outer membrane proteins (Vomps) in *B. quintana* [31], and *Bartonella* repetitive protein A (BrpA) in *B. vinsonii* [32]. They are outer membrane proteins belonging to the trimeric autotransporter adhesin (TAA) family [33], that also includes the yersinia adhesin A (YadA) in *Yersinia enterocolitica* [34], the *Haemophilus influenzae* adhesin (Hia), the haemophilus surface fibrils (Hsf) in *H.* influenzae [35], and the ubiquitous surface protein A (UspA) in *Moraxella catarrhalis* [36], all of which are considered to be virulence factors. All TAA family members share similar modular architectures, consisting of a head, a neck, a stalk, and C-membrane

anchor domains [34]. The C-membrane anchor domains define the TAA family and form trimers [37, 38]. The size of these proteins varies from one species to another due to the number of TAA neck/stalk repeats, which can differ by up to four-fold. In *B. henselae*, the monomeric form of Bad A is 328 kDa in size [30]. In *B. quintana*, Vomps are encoded by a family of four genes, three of which are very similar to *badA* (the exception being *vompD*). Although BrpA remains little studied, the functions of BadA and Vomp have been extensively explored using *in vitro* assays and experimental infections of laboratory animals. This work has demonstrated the pleiotropy of these proteins, implicating them in (1) mediation of binding of *Bartonella* spp to extracellular matrix proteins (collagens and fibronectin) and to endothelial cells, via α-5β-1-integrins (2) circumvention of phagocytosis (3) mediation of angiogenesis via activation of hypoxia-inducible factor 1 (the key transcription factor in angiogenesis) in infected endothelial cells and via provocation of secretion of proangiogenic cytokines (e.g. vascular endothelial growth factor) [30, 39]. BadA/Vomps are also involved in bacterial auto-aggregation [31, 40]. Interestingly, there is marked variation in the expression of BadA between different *B. henselae* strains [41] and it is known that high number of *in vitro* passages of isolates results in the loss of BadA expression. Different genetic processes, such as single base deletions or insertions, or recombination events, can affect BadA expression resulting in the coexistence of phase variants expressing or not expressing BadA. This characteristic, when coupled with the observation that BadA-expressing strains grow slower than non-expressing strains, suggests that expression of BadA (which is an enormous molecule) is a highly energy-consuming process, and one which bartonellae will only continue whilst required to do so.

3.2. The VirB/D4 type IV secretion system (T4SS) and its effector proteins

T4SSs consist of a multiprotein channel spanning inner and outer

Gram-negative bacteria membranes and a surface filament extending from the bacterial envelope. The system mediates the transfer of protein or DNA substrates from a bacterial donor cell into various cell types (e.g. transfer of DNA into other bacteria by conjugation or transfer of bacterial effector molecules into eukaryotic cells). In pathogenic bacteria, this protein complex can be compared to a microscopic syringe that is used to inject effector proteins into target cells in order to subvert their physiology. T4SSs serve as key virulence factors for many important human pathogens including *Helicobacter pylori*, *Legionella pneumophila, Bordetella pertussis*, and *Brucella melitensis* [42]. Among T4SSs, VirB/D4 is a macromolecular complex of at least 10 components termed VirB2 to VirB11 and an associated substrate recognition receptor known as the T4 coupling protein (T4CP), named VirD4 (Figure 2).

The VirB/D4 T4SS was first identified in *Bartonella* species in 2000 as a result of characterization of the locus that encodes a 17kDa immunodominant protein in *B. henselae* [43]. Genetic comparison of this locus revealed that the 17kDa protein it encoded was a VirB5 homolog and further exploration of the locus revealed the presence of homologes of other members of the T4SS upstream and downstream of VirB5 [43, 44]. The putative promoter region of the operon was also identified and its expression was shown to be induced when *B. henselae* was cultivated with human microvascular endothelial cells [45]. The operon was subsequently identified in other *Bartonella* species, and its necessity for host interaction was demonstrated using the *B. tribocorum*-rat and, subsequently, the *B. birtlesii*-mouse infection models [4, 19, 46]. Experimentation using these models went on reveal that although the VirB/D4 system was essential for exploitation of the primary niche, it was dispensable for the subsequent erythrocytic infection [47]. Subsequent studies have also characterized seven genes encoding for effector proteins, the *Bartonella* effector proteins (Beps), named BepA to BepG that are translocated by the

T4SS into endothelial cells [48] and are responsible for subverting their physiology. Indeed, *in vitro* experiments have indicated that VirB/D4 and its effector proteins mediate a range of profound changes to parasitized endothelial cells [49, 50], including (i) massive rearrangements of the actin cytoskeleton, resulting in the formation and internalization of large bacterial aggregates by the invasome structure; (ii)NF-κB-dependent pro-inflammatory activation, leading to cell adhesion molecule expression and chemokine secretion; and (iii) inhibition of apoptotic cell death, resulting in enhanced endothelial cell survival. Internalisation of bartonellae via a unique "invasome" structure, initially characterized by the formation of a bacterial aggregate on the cell surface, which is subsequently engulfed and internalized by an actin-dependant mechanisms [26]. This process appears to be specific to bartonellae, and is dependent on three VirB/D4 effectors, BepC, BepG, and BepF [51, 52]. BepA has been shown to inhibit EC apoptosis through upregulation of cAMP levels in cytosol [48] and it also promotes capillary sprout formation in an endothelial spheroid infection model, whereas BepG inhibits such sprouting [53]. The functions of BepB, BepD and BepE are still to be elucidated. VirB/D4 appears to be part of the regulon of the BatR/S two component regulatory system, a global regulator that may be a key mediator of the physiological transition of bartonellae as they associated with endothelial cells [54].

A homolog of the VirB/D4 system, Vbh, has also been identified and all *Bartonella* species, except *B. bacilliformis,* possess at least one of these two T4SSs [4]. Comparative genomics have indicated that these systems were acquired by a common ancestor of lineage 2 and 3 *Bartonella* species following its divergence from lineage 1 that carries *B. bacilliformis* (Figure 1) [5]. Given the key roles attributed to these T4SSs, it is clear that its acquisition has resulted in fundamentally different bases of host exploitation by *B. bacilliformis* and the other *Bartonella* species. It has been proposed that

species possessing the VirB/D4 or Vbh systems have attenuated virulence compared to *B. bacilliformis*, although this view remains controversial. Indeed, given that VirB/D4 and Vbh are so important for endothelial cell interaction in all *Bartonella* species, it is intriguing that the species in which it is absent, *B. bacilliformis*, is the species for which *in vivo* endothelial cell subversion is most apparent.

Other bartonellae proteins have been shown to interact directly or indirectly with endothelial cells or the extracellular matrix. For instance, the outer membrane lipoprotein Omp43 is one of the bacterial proteins that binds most strongly to human umbilical vein endothelial cells (HUVEC) [55, 56]. The Pap31 protein (heme-binding protein A) of *B. henselae* binds to fibronectin and promotes bacterial adhesion to endothelial cells [57]. As for VirB/D4 and Bad/Vomp, the disruption of these genes in the genomes of both *B. birltesii* and *B. tribocorum* results in the non-appearance of bacteraemia in inoculated animal models [4, 19]. *Bartonella* species also, remarkably, appear to secrete the heat-shock protein GroEL, and this molecule is a potent mitogen of HUVECs [58]. The holistic view of the synergy between all those virulence factors are schematically represent in Figure 3.

4. STEP 2: SEEDING OF BLOOD AND EXTRA CELLULAR SURVIVAL

In various animal models of infection, *Bartonella* bactaeremia appears between two and seven days post infection. It has been proposed that this appearance of bartonellae is orchestrated, with discreet, recurrent waves of seeding occurring during infection rather than continuous stream of bacteria entering the blood [3]. Initially, the bacteria are extracellular, thus their passage must present them with a significant challenge in that they are fully exposed to the host immune system. Recently, we have shown that a *B. birtlesii badA*-knockout (ΔbadA) mutant was sensitive to mouse serum, while the

wildtype *B. birtlesii*, expressing active BadA, was resistant (Figure 4A). As the ΔbadA mutant was not killed by heat-inactivated serum (Figure 4B), we suspect that *B. birtlesii* BadA is involved in resistance to complement. In support of this hypothesis, we have shown that when wildtype *B. birtlesii* is grown in liquid media, the supernatant of this medium has anti-complement activity, but that this activity can be neutralized with anti-BadA antibodies (kindly provided by Professor Volkard Kempf, Goethe-Universität, Frankfurt am Main, Germany). These observations suggest that BadA, or a part of BadA, could be secreted or released by *B. birtlesii* to counter the effects of complement, in a manner akin to that reported for the BadA homolog YadA in *Yersinia enterocolitica* [59]. We have also obtained evidence that bartonellae are capable of binding IgG Fc fragments and hypothesize that by doing so, the bacteria further facilitate their extracellular longevity by subversion of host humoral response. We observed that several *Bartonella* species had the capacity to bind Fc, and most could bind immunoglobins derived from a range of different mammals. Western blotting indicated that the Fc binding capacity was mediated by a protein of approximately 65kDa size, and N-terminal sequencing of this protein demonstrated its identity with the heat shock response protein GroEL. Western blotting of *B. henselae* cellular fractions indicated that GroEL was located in the cytoplasm and in the inner and outer membrane of the cell, as previously demonstrated for *B. bacilliformis* [58]. Expression of recombinant *B. henselae* GroEL conferred an Fc binding capacity on *Eschericha coli* (Figure 5). A further mechanism by which bartonellae may counteract the threat of host immunity is via lipopolysaccharide (LPS) modification. The LPS of *B. henselae* possesses an unusual penta-acylated lipid A with a longchain fatty acid [60]. This feature, also a characteristic of LPS attenuates toll-like receptor (TLR) 4-mediated host response to bartonellae endotoxin [60, 61].

5. STEP 3: ERYTHROCYTE ENCOUNTER AND ADHESION.

The ability of bartonellae to exploit erythrocytes is key to their parasitic strategy and is almost unique amongst bacteria. Once inside erythrocytes, bartonellae occupy a nutrient-rich, immunologically privileged niche that, by virtue of its position in the circulatory system, facilitates not only bartonellae persistence within the reservoir host but also its uptake by hematophagous vectors.

There is no evidence yet that *Bartonella* species are able to sense and specifically move towards circulating erythrocytes, hence we must currently assume that contact between bacterium and erythrocyte results from their chance encounter. Some *Bartonella* species possess flagella, which may facilitate their movement in blood plasma, but their absence from most species suggests they are not essential appendages and indeed, there is evidence that their value in host interaction occurs elsewhere during the course of infection (see below).

Adherence to erythrocytes by most *Bartonella* species appears to be mediated by the Trw T4SS and as yet uncharacterized receptors. Trw is the third T4SS found in bartonellae, and its importance in the establishment of intra-erythrocytic infections by bartonellae in reservoir hosts has been recognized for some time [62-64]. However, evidence for the direct role of Trw in erythrocyte infection has been only recently obtained following the development of an *in vitro* model for erythrocyte adherence and invasion [19]. In this study, we identified *B. birtlesii* genes required for erythrocyte infection by identifying, among STM mutants unable to induce bacteraemia in mice, those which could also not invade erythrocytes *in vitro*. From this screening we identified nine invasion-deficient mutants. In seven of these, genes within the trw operon were disrupted, whereas in the other two, disruptions were located in the invasion-associated *ialA/B* locus (see below) and in *livG*, a putative ABC-transporter encoding gene. The nature of our screening resulted in the

conclusion that the products of all these genes are essential for the adhesion to and/or invasion of erythrocytes rather than replication or persistence within them.

Trw shares homology with the broad-host-range conjugation system of R388 plasmid and has been acquired by horizontal transfer from phylogenetically distant bacterial species [65]. Unlike VirB/D4, the Trw T4SS lacks the coupling protein required for export of effectors, suggesting that it is no longer a secretion system [64]. The *trw* genes of *Bartonella* species are co-linear with the respective genes of plasmid R388 except for the presence of multiple tandem gene duplications of *trwL* (the virB2 homolog) and *trwJ-I-H* (the virB5, virB6 and virB7 homologs). The multiple copies of *trwL* and trw*J* are thought to encode the suface-exposed pilus components of the T4SS, while the products of *trwI* and *trwH* are thought to be involved in pilus elongation and anchorage of the T4SS to the outer membrane (Figure 2). The presence of the multiple copies of these components indicates that they probably participate to the expression of variant pilus forms. It is not know if all *trw* genes are co-expressed and thus if numerous pili variants are concurrently present in the bartonellae population infecting a host or if differential expression of copies of these genes occurs, resulting in different pili variants being present on the bacterial cell surface during different stages of the infection process. It has been hypothesized that the presence of pili variants may facilitate the interaction with different erythrocyte receptors or with the variable forms of a specific receptor found across the breadth of the reservoir host population [63, 66].

Our studies have also yielded evidence that Trw is a key determinant of bartonellae host specificity. As discussed above, although bartonellae can infect non-reservoir hosts, when they do so, they are unable to establish an intra-erythrocytic bacteraemia. However, we were able to confer to *B.*

henselae and *B. quintana* (which are naturally associated with only cats or humans respectively) the ability to interact with rat erythrocytes by transforming them to express the *trw* locus of *B. tribocorum*, a naturally rat-specific species [19].

Even though erythrocyte parasitism is the hallmark of *Bartonella* species, the *trw* genes are not present in all *Bartonella* species, being restricted to all members of lineage 3 with the exception of this lineage's outlier, *B. clarridgeiae* (Figure 1). This distribution suggests that the Trw T4SS was horizontally acquired by a common ancestor of these members of lineage 3 members [4]. Interestingly, the distribution of Trw and flagella among the *Bartonella* species is mutually exclusive (Figure 1), thus it has been proposed that following its acquisition, the function of Trw evolved to replace that performed by flagella. This hypothesis is supported by observations that the flagella of *B. bacilliformis* are involved in adhesion to and entry of erythrocytes [67-69].

6. STEP 4: INVASION OF, AND PERSISTENCE WITHIN, ERYTHROCYTES

Among first virulence factors to be described for bartonellae were those encoded by the *ialA/B* locus in *B. bacilliformis* [70, 71]. The survey of *B. birtlesii* genes involved in erythrocyte adherence/ invasion, described above, also identified *ialA* and *ialB*. Early work demonstrated that the transformation of *E. coli* with *B. bacilliformis ialA/B* conferred the ability to invade erythrocytes [70] and more recently, we have shown that although deletion of *ialB* did not significantly affect adhesion of *B. birtlesii* to erythrocytes, it provoked a 10 fold decrease in bacterial entry into erythrocytes [19]. IalA has been characterized as a (de)nucleoside polyphosphate hydrolase of the MutT motif family and has homologs in other invasive bacteria such as *Yersinia enterocolitica* and *Rickettsia prowazekii* [72]. The precise role of IalA and its homologs is

suspected to be the reduction of stress-induced dinucleotide levels during invasion, thereby enhancing pathogen survival [72]. IalB is a 19.9 kDa protein with about 64% sequence similarity to the *Yersinia enterocolitica* protein Ail, a surface protein that plays a key role in mediating cell entry and serum resistance [59]. Intriguingly, *B.bacilliformis* IalB appears to be localized to the inner membrane [70] so it is unclear how it affects its role in erythrocyte entry. However, more recent work has suggested that in *B. henselae*, the protein is also associated with the outer membrane [71, 73]. To add to this uncertainty, we have been unable to detect IalB on the cell surface of *B. birtlesii* while ial B was detected in *B. birtlesii* cryo-sections supporting ialB is not a cell surface exposed protein (Figure 6). Exploration of the determinants of *ialB* expression in *B. bacilliformis* has been reported and the patterns of expression observed under different conditions (temperature, pH, oxidative stress, hemin limitation) suggest that the gene is upregulated in response to environmental cues signaling passage of the bacterium from vector to host, and also possibly at times when the bacterium is subjected to stress-inducing environmental conditions [74]. Despite the identification of entry-associated virulence factors, we currently have very little idea about how bartonellae enter erythrocytes. Given the unusual structure and physiology of erythrocytes, it is likely that bartonellae employ a mechanism of invasion that is different from those used for microbial entry into other cell types. How akin this mechanism is to, for example, those employed by *Plasmodium* species for erythrocyte invasion, remains to be seen. Microscopy has been used to monitor *B. bacilliformis* entry into erythrocytes, and suggested that the bacteria provoke, then enter, substantial deformations in erythrocyte membranes. Bacteria appeared to drive themselves into deep invaginations then membrane fusion at the necks of these invaginations led to the formation of intracellular vacuoles containing bacteria [67]. This process was considered, at least in part, to be mediated by the action of flagella. As discussed above, only very few *Bartonella* species express flagellae, hence, those without these appendages must have evolved

an alternative entry strategy. There is also evidence that bartonellae produce an extracellular protein, termed deformation factor, which induces extensive invaginations dentations and trenches in erythrocyte membranes [75, 76].

Intra-erythrocytic replication starts within a vacuolar membrane immediately after invasion [3]. After several days, bacterial replication slows down, reaching a plateau maintained for the remaining life span of the infected erythrocytes. Cessation of bacterial replication may result from an active mechanism of growth control not yet identified. Moreover, the intra-erythrocytic environment is inhospitable and the bacteria must cope with oxidative stress. None of molecular mechanisms allowing multiplication, growth control and them persistence of *Bartonella* spp. within erythrocytes have been identified so far. However, genomics have identified potential candidates that may help bacteria to cope with stressors. Proteases IalA and CtpA degrade misfolded proteins that arise from stress. Genes encoding these 2 proteins have been shown to be essential for bacteraemia establishment [4, 19]. Interestingly, the BatR/BatS, a two-component regulatory system serves as a pH sensor, upregulating genes at neutral pH and repressing them at alkaline pH, and is probably used by *Bartonella* species to respond to environmental cues encountered in the context of its host [77]. However, the specific role of this system in *bartonella* adaptation to erythrocytes has still to be demonstrated.

As many pathogenic bacteria, bartonellae utilizes host heme-containing proteins as a source of heme and iron. Bartonellae sources of heme include hemin, hemoglobin and host erythrocytes. Within *Bartonella* genus, it has been shown that acquisition of heme involves paralogous gene family encoding Heme binding proteins (hbp) and a heme uptake locus [78-80]. We have previously shown that disrupting *heme-binding protein* genes (*hbp*) in *B. birtlesii* and *B. tribocorum* lead to the lost of ability of the correponding mutants to induce bacteraemia in their corresponding natural host indicating their

important role in establishment of bacteraemia by *Bartonella* spp [4, 19]. Little is known about molecular acquisition of heme acquisition by *Bartonella* spp. However, it is well known that both *hbp* genes and the hemin associated locus (*hut*) are regulated by Irr, an iron response regulator in response to various stress (temperature shift, oxygen level and hemin concentration) [81, 82]. Further studies of those systems will contribute to understand how bartonellae use the most abundant source of heme in the mammalian host (i.e., hemoglobin) and how it contributes to the success of its persistence within erythrocytes as well as in its different hosts, i.e., arthropod and mammals

The rapid development of transcriptomic technologies should soon provide the means for exploration of the dynamics of bacterial gene expression during host interaction, thus we predict that the transcriptome of intra-erythrocytic bartonellae will soon be available, helping us better understand the molecular means by which bartonellae thrive in their erythrocyte niche.

There is currently no evidence to suggest that infection of erythrocytes by *Bartonella* spp. has a significant effect on their physiology (life span and membrane integrity appear unchanged) [3]. However some subtle changes may occur; for example, during the period of erythrocyte invasion and multiplication, *B. tribocorum*-infected cells were observed to be cleared from circulation more rapidly than uninfected cells. However, once intracellular replication has ceased, this difference in clearance rates disappeared [3]. These observations suggest that there are “recognizable” changes in erythrocyte structure or physiology during the early stages of their parasitism. These changes may result from the effects of deformin, as discussed above, with altered erythrocytes being filtered out by spleen as demonstrated for *Plasmodium* spp. [83].

While most *Bartonella* species appear intent on not significantly altering the

physiology of the circulating erythrocyte population, *B. bacilliformis* has the potential to provoke severe hemolytic anemia [84], although this pathology is not necessarily a consequence of infection. Evidence that hemolysis may be mediated by a contact-dependant hemolysin has been presented [84], but this protein has yet to be fully characterized. A putative hemolysin-encoding gene is present in the *B. bacilliformis* KC583 genome, although the genomes of other *Bartonella* species (*B. henselae*, *B. quintana*, *B. tribocorum* and *B. grahamii*), also contain hemolysin homolog genes.

7. CONCLUSION

The high prevalence of infections in mammals, and the potential threat posed to the health of humans, livestock and companion animals, warrants further exploration of the fundamental biology of *Bartonella* species. Despite a huge effort in the last 20 years to understand mechanisms used by *Bartonella* to exploit their natural reservoir hosts, many areas of uncertainty remain and require further research.

In retrospect, one of the key studies that paved the way for recent advances was that completed a decade ago describing the dynamics of infection, as monitored using fluorescently-labelled bacteria [3]. The identification and quantification of distinct stages of infection, in tandem with the development of reliable tools for the genetic manipulation of bartonellae, has allowed significant advances to be made in our understanding of the molecular basis of bartonellae parasitism. However, despite this progress, and the ever increasing medical importance of bartonellae, it appears that today, fewer and fewer scientists are studying these bacteria. It is unthinkable that research into the molecular basis of bartonellae-host interactions should falter now, with some many important and exciting questions still to be answered. For example, what is the fate of bacteria following inoculation? How do bacteria disseminate around the body and is the endothelial vasculature truly a primary

niche for infection? Furthermore, why, on occasion, do bartonellae provoke angiogenesis? We also know nothing about how bartonellae regulate their intra-erythrocytic replication and persistence, what physiological changes they endure. We know little about the interaction between bartonellae and host immunity and thus little about the extent and importance of immunoregulation. Researchers also need to consider the role of arthropods in the bartonellae natural cycle and should perhaps incorporate “natural” inoculation by arthropod rather than by syringe into relevant animal models. In summary, the challenges for future years, are (1) to understand how, the unique infection strategy of bartonellae contributes to their remarkable epidemiological success in their reservoir hosts, and (2) to better assess if bartonellae have the potential to emerge as new zoonotic pathogens. In such a small field, a constructive means of helping to invigorate stimulating and high level science, is collaboration between those laboratories with expertise in various key technical skills (experimental vector transmission, genetics, animal models) and those with the enthusiasm, but perhaps not all the means, to progress the field. Such initiatives should be encouraged and welcomed by all.

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The authors declare that they have no competing interests.

Author’s contributions: HKD, RB, MVT drafted the manuscript. DLR, CV carried out BadA experiment (described in Figure 4), AR carried out GroEL experiment (described in Figure 5), HD, MVT performed electron microscopy analysis (described in Figure 6). All authors read and approved the final

manuscript.

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Table 1 :

***Bartonella* species**	**proven/suspected reservoir host**	**evidence of human infections?**
B.alsatica	rabbits	yes
B. bacilliformis	humans	yes
B. birtlesii	small rodents	
B.bovis	ruminants	
B. capreoli	ruminants	
B. chomelii	ruminants	
B. clarridgeiae	felids	yes
B. coopersplainensis	rats	
B. doshiae	small rodents	
B. elizabethae	rats	yes
B. grahamii	small rodents	yes
B. henselae	felids	yes
B. japonica	small rodents	
B. koehlerae	felids	yes
B.peromysci	small rodents	
B. queenslandensis	rats	
B. quintana	humans	yes
B. rattaustraliani	rats	
B. schoenbuchensis	ruminants	
B. silvatica	small rodents	
B. talpae	moles	
B. taylorii	small rodents	
B. tribocorum	rats	yes
B. vinsonii subsp. arupensis	small rodents	yes
B. vinsonii subsp. berkhoffii	canids	yes
B. vinsonii subsp. vinsonii	small rodents	yes

Table 1: Validated *Bartonella* species, their reservoir hosts, and their currently perceived medical relevance.

Figures Legend:

Figure 1: Molecular phylogenetic analysis of the 24 extant, validated *Bartonella* taxa inferred from alignment of partial (326bp) *gltA* sequences**.** Evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The tress with the highest log likelihood is shown. Evolutionary analyses were conducted in MEGA5. Imposed on the tree are the disctributions of the three T4SSs (red box= VirB/D4, blue box= Trw, green box: vbh) and flagella (orange box) amongst the taxa. To the right of the tree is an indication of the three evolutionary lineages defined within the genus.

Figure 2: Hypothetical models of the architectures of *Bartonella* Type 4 Secretion System (T4SS), VirB/VirD4 and Trw.

The T4SS VirB/VirD4 system possesses an inner membrane protein VirD4, that is an associate substrate recognition receptor named as T4 Coupling Protein (T4CP). On the external part of the outer membrane, VirB4 and VirB11 energize the secretion process. VirB3, 6, 8, 9 and 10 are considered to build a secretion channel accross the inner membrane and the surface exposed pilus associated components VirB2 and VirB5.

The Bep proteins are secreted through this system.

The T4SS Trw system has the same organization as VirB/D4 system with homologs for all VirB proteins (named Trw D, E, F, G, H, I, J, K, L, M) except that Trw system lacks VirD4 (T4CP) and does not secrete any known substrat. However, Trw system is organized as VirB/D4 with a channel accross the inner membrane and the surface exposed pilus associated components TrwL and TrwJ. Unlike VirB/D4 system, the Trw system express multiple variant copies of TrwL and TrwJ.

EX, extracellular matrix; OM, outermembrane; PP, periplasm; IM, innermembrane; CY, cytoplasm.

Figure 3: Holistic view of bartonellae interactions with endothelial cells and erythrocytes.

Exploitation of vascular endothelial cells by bartonellae involves binding to cell surface via BadA/Vomps proteins as well as VirB/D4 and possible other adhesions as Omp43/pap31. VirB/D4 and its effectors mediate massive rearrangements of the actin cytoskeleton, resulting in the formation of large bacterial aggregates by the invasome structure as well as inhibition of apoptosis leading to enhanced endothelial cells survival.

The mechanisms of the passage of *Bartonella* spp. from endothelial cells to erythrocytes is unknown however, it appears that before infecting erythrocytes, the bacteria is free in the blood. Adherence to erythrocytes appears to be mediated by Trw T4SS (for most *Bartonella* species) and by flagella (for *B. bacilliformis* and bovine-specific species). An erythrocyte is usually infected by one single bacteria; which once inside replicate in a membrane-bound compartment to reach up to eight cells per erythrocytes, and then persist till the natural death of the infected cells. During this process, proteases ialA and CtpA are suspected to degrade misfolded proteins and to protect bacteria against stress.

Figure 4: Role of *B. birtlesii* BadA in complement inactivation

A- The different strains of *B. birtlesii* (wild type: WT, or badA-knockout mutant: ΔbadA) was incubated with calf fetal serum diluted twice (either in Schneider medium, or in supernatant culture medium of *B. birtlesii* WT after 24h of growth) at 35°C in a 5% CO_2 atmosphere for one hour. Different dilutions were then plated on CBA (Columbia blood agar) medium, plates incubated at 35°C in a 5% CO2 atmosphere for 5 days. Colonies forming unit (CFU) were then counted. N= 6 +/- SE

B- The different strains have undergone the same treatments as in A but the serum used has been heated for 56°C for 30 min to inactivated the complement. N=6 +/- SE.

Figure 5: Far-Western blot of (1) E. coli BL21, (2)E. coli BL21 transformed with pET30a, and (3) E. coli BL21 transformed with pET30-AR1 (containing B. henselae groEL insert), probed with rat Fc fragments conjugated to alkaline phosphatase (Jackson Immunoresearch). All strains were grown in LB to log-phase and induction of plasmid expression was achieved using IPTG as per manufacturer's instructions (Novagen).). Construction of pET30a-AR1: The B. henselae groEL ORF was amplified using a PCR incorporating primers BhgroELF (AAG GAG AGG AAG AAA TGG CTG CTA AAG AAG T) and BhgroELR (TCA AGG GCT TAG AAA TCC) then cloned into pCR2.1-TOPO and used to transform E. coli TOP10 cells according to manufacturer's instructions (Invitrogen). The resulting plasmid, pAR1-TOPO, was purified then digested with BamHI and XhoI to yield a 1600 bp fragment that included the groEL ORF. This fragment was cloned into compatible sites of pET30a (Novagen) to generate pET30-AR1, which was used to transform E. coli XL10 cells (Stratagene). pET30-AR1 was recovered from these cells and subcloned into E. coli BL21 cells.

Figure 6: Transmission electron microsopy and immunogold labeling of pellets of B. *birtlesii* using anti-*B. birtlesii* serum (A); negative serum (B); anti-*B. birtlesii* recombinant ialB serum C) or in B*. birtlesii* cryosection stained with anti-B*. birtlesii* recombinant ialB serum (D). Arrows indicated the gold particules corresponding to the detection of the corresponding proteins.

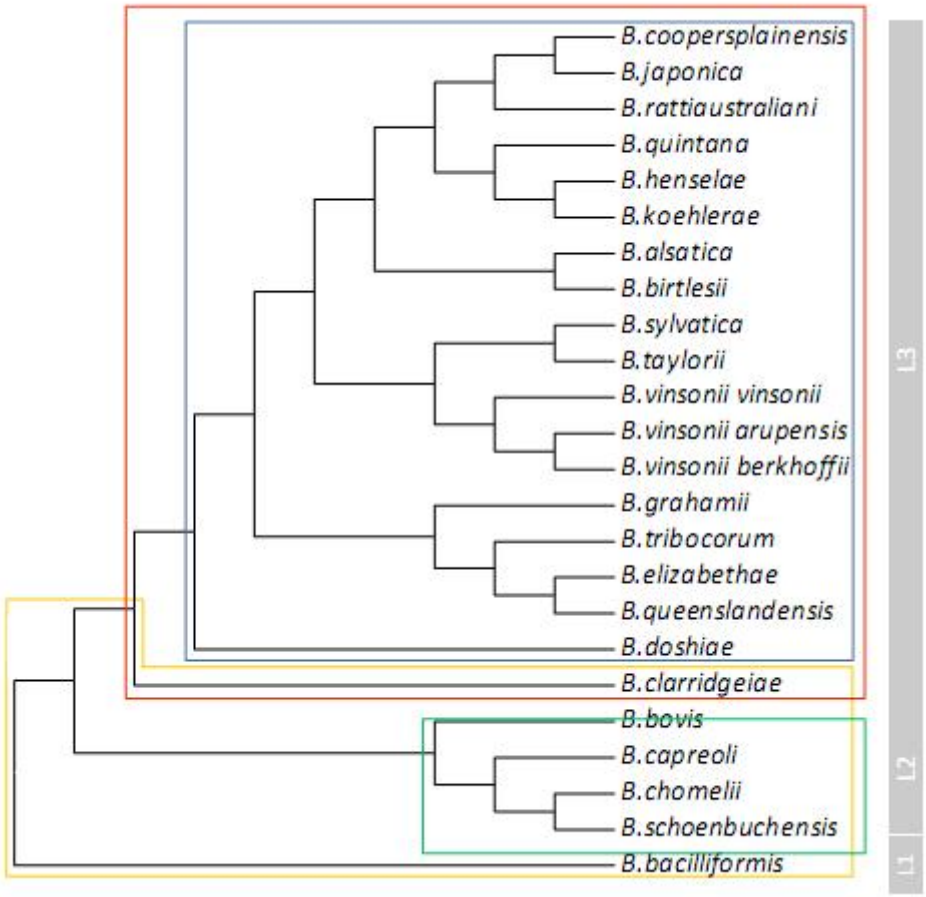


Figure 1 -Deng et al. 2011

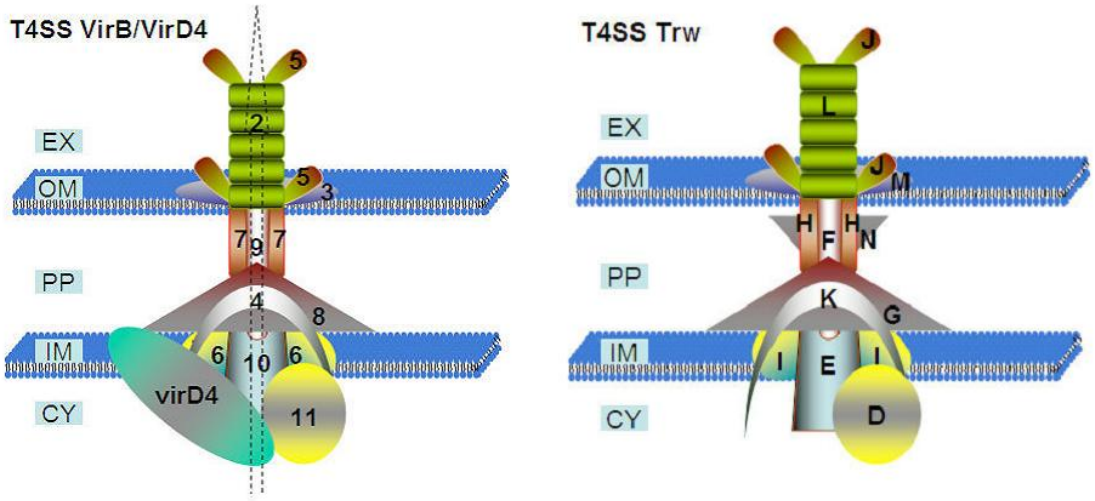


Figure 2 -Deng et al. 2011

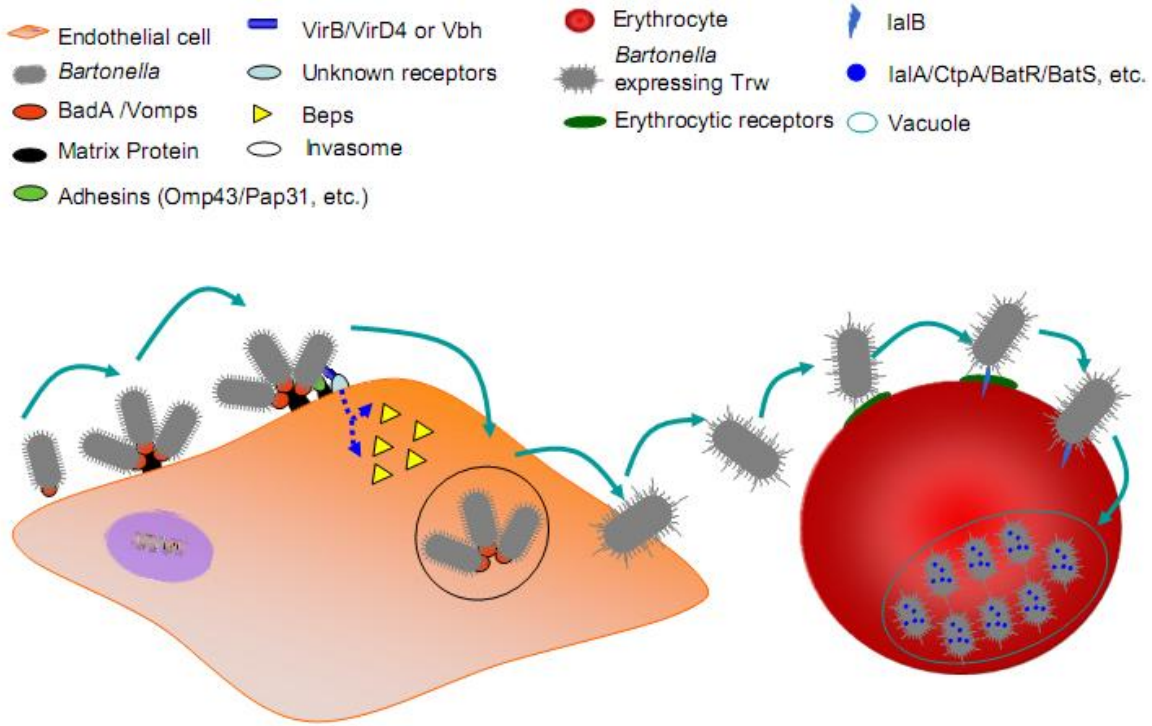


Figure 3 -Deng et al. 2011

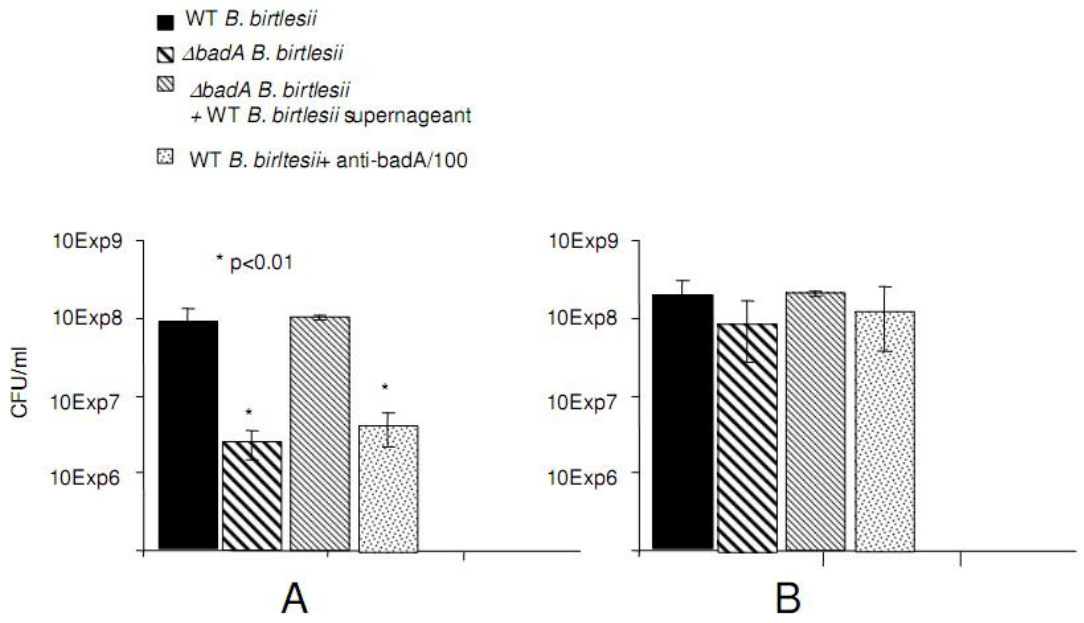


Figure 4 -Deng et al. 2011

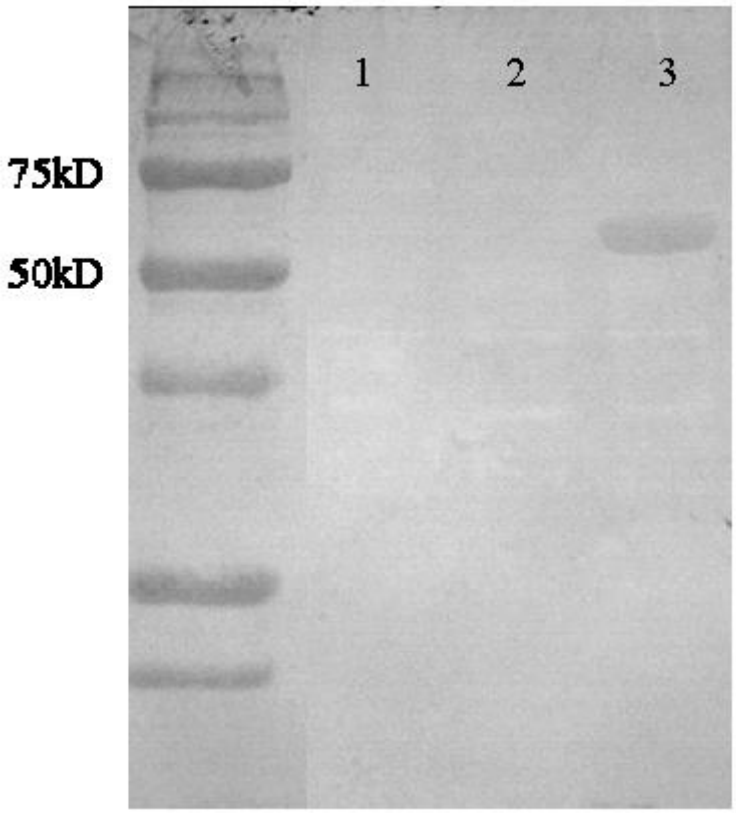


Figure 5 -Deng et al. 2011

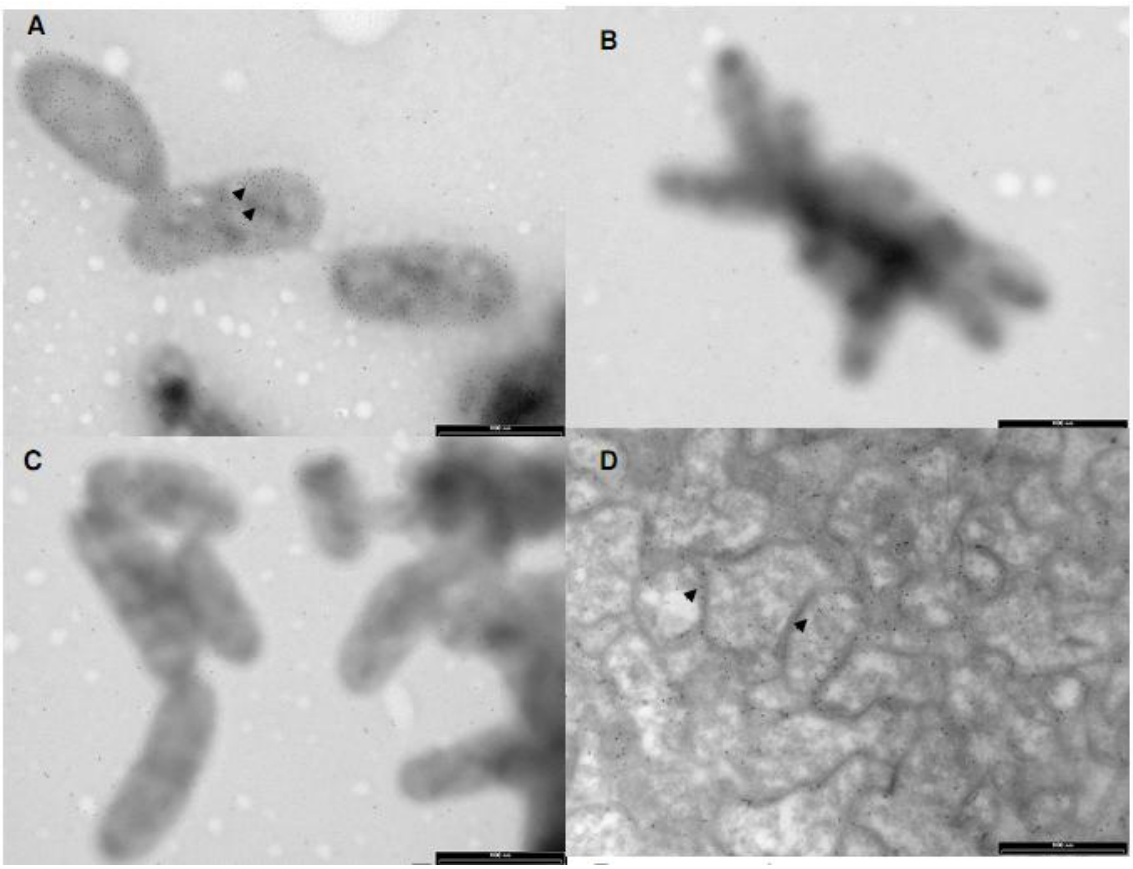
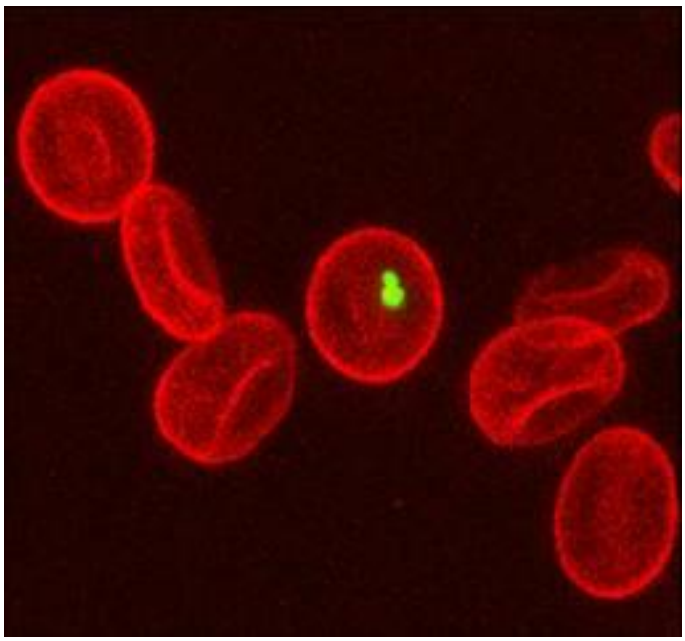


Figure 6 -Deng et al. 2011

CHAPTER I

The Trw Type IV Secretion System of *Bartonella* Mediates Host-Specific Adhesion to Erythrocytes



Commentaires Article-1 (Chapitre 1)

Chaque espèce de *Bartonella* spp. n'est capable d'infecter qu'un ou quelques espèces de mammifères, dans lesquels la bactérie va induire une bactériémie intra-érythrocytaire persistante. Chez les hôtes accidentels (=non réservoirs), cette phase bactériémique est inexistante ou de très courte durée. Ainsi, nous avons fait l'hypothèse que si la bactérie ne colonise pas le sang d'hôtes non réservoirs, c'est parce qu'elle est incapable d'infecter ou de reconnaître les globules rouges de l'espèce en question.

Les travaux présentés dans l'article ci après ont pour but de confirmer cette hypothèse. Pour ce faire, nous avons :

- mis au point un modèle d'infection *in vitro* des globules rouges de différentes espèces de mammifères par différentes espèces de *Bartonella* spp. Ainsi nous avons confirmé que les espèces de bartonelles ne sont capables d'infecter *in vitro* que les globules rouges provenant de leurs hôtes réservoirs.
- Nous avons ensuite entrepris d'identifier les gènes bactériens impliqués dans la reconnaissance des globules rouges. Ceci a été réalisé en 2 étapes : (1) en criblant une banque de mutants de *B. birtlesii,* construits par la technique de Signature-Tagged Mutagenesis, afin les mutants qui ont perdu la capacité à induire une bactériémie persistante chez la souris, hôte naturel de *B. birltesii* ; (2) en criblant *in vitro* les mutants, dits « abactériémiques », en utilisant le système mis au point dans la première partie de nos travaux. Ainsi nous avons mis en évidence que le système de sécrétion de Type IV Trw était essentiel à l'adhésion de *B. birtlesii* aux globules rouges murins.
- Enfin, nous avons prouvé que la reconnaissance hôte-spécifique du globule rouge par *Bartonella* spp. était portée par le système de sécrétion de type IV Trw, en exprimant les gènes codant le système de sécrétion de

type IV Trw d'une espèce de *Bartonella* infectant le rat (*B. tribocorum*), chez *B. henselae* (spécifique du chat) *et B. quintana* (spécifique de l'homme). Cette expression ectopique a pour effet de changer le tropisme de *B. henselae* et *B. quintana* qui deviennent infectieuses, *in vitro*, pour les globules rouges de rat.

Par ailleurs, les analyses phylogénétiques menées dans le cadre de cette étude montrent que les gènes codant ce système de sécrétion ont été acquis par *Bartonella*, au cours de l'évolution grâce à des mécanismes de transfert latéral de gènes, puis se sont diversifiés au sein du genre bactérien pour faciliter l'infection de différentes espèces de mammifères.

L'émergence de maladies causées par ces bactéries, chez l'Homme et/ou l'animal, pourrait donc voir le jour, après transfert des gènes codant ces complexes macromoléculaires de bactéries pathogènes vers des bactéries à l'origine non infectantes pour l'Homme et qui, du fait de cette acquisition de gènes, deviendraient pathogène pour l'Homme.

ARTICLE- 1

The Trw Type IV Secretion System of *Bartonella* Mediates Host-Specific Adhesion to Erythrocytes

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Abstract

Bacterial pathogens typically infect only a limited range of hosts; however, the genetic mechanisms governing host-specificity are poorly understood. The α-proteobacterial genus *Bartonella* comprises 21 species that cause host-specific intraerythrocytic bacteremia as hallmark of infection in their respective mammalian reservoirs, including the human-specific pathogens *Bartonella quintana* and *Bartonella bacilliformis* that cause trench fever and Oroya fever, respectively. Here, we have identified bacterial factors that mediate host-specific erythrocyte colonization in the mammalian reservoirs. Using mouse-specific *Bartonella birtlesii*, human-specific *Bartonella quintana*, cat-specific *Bartonella henselae* and rat-specific *Bartonella tribocorum*, we established *in vitro* adhesion and invasion assays with isolated erythrocytes that fully reproduce the host-specificity of erythrocyte infection as observed *in vivo*. By signature-tagged mutagenesis of *B. birtlesii* and mutant selection in a mouse infection model we identified mutants impaired in establishing intraerythrocytic bacteremia. Among 45 abacteremic mutants, five failed to adhere to and invade mouse erythrocytes *in vitro*. The corresponding genes encode components of the type IV secretion system (T4SS) Trw, demonstrating that this virulence factor laterally acquired by the *Bartonella* lineage is directly involved in adherence to erythrocytes. Strikingly, ectopic expression of Trw of rat-specific *B. tribocorum* in cat-specific *B. henselae* or human-specific *B. quintana* expanded their host range for erythrocyte infection to rat, demonstrating that Trw mediates host-specific erythrocyte infection. A molecular evolutionary analysis of the *trw* locus further indicated that the variable, surface-located TrwL and TrwJ might represent the T4SS components that determine host-specificity of erythrocyte parasitism. In conclusion, we show that the laterally acquired Trw T4SS diversified in the *Bartonella* lineage to facilitate host-restricted adhesion to erythrocytes in a wide range of mammals.

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Introduction

The successful infection of a mammalian host by a bacterial pathogen typically involves a series of intimate host-pathogen interactions. On the molecular level this is reflected by specific receptor-ligand interactions between bacterial virulence factors and their targeted host factors [1]. Adaptation of a bacterial virulence factor to a host factor that displays variability within the host population can restrict the host range that is susceptible to infection. The resulting host-specificity is an inherent feature of most bacterial pathogens of humans, including *Helicobacter pylori*, *Listeria monocytogenes*, *Neisseria gonorrhoae*, *Salmonella typhi*, *Streptococcus pyogenes* and *Staphylococcus aureus*. However, remarkably little is known about the molecular determinants of host specificity in bacterial infections, with the only exception of *L. monocytogenes* for which the conjugated action of two distinct host-specific invasion proteins was shown to be critical for fetoplacental listeriosis [2,3,4].

Bartonellae represent an interesting but largely unexplored model for host specificity. These facultative intracellular bacteria use arthropod transmission and hemotropism as mammalian parasitism strategies [5]. As the result of an adaptive radiation each of the 21 species infects only one or a few closely related mammalian reservoir host(s), which is highlighted by their capacity to cause a long-lasting intraerythrocytic bacteremia [6]. Non-reservoir hosts may get incidentally infected without developing an intraerythrocytic infection [7]. Two *Bartonella* species are human-specific: *Bartonella bacilliformis* causes the biphasic Carrion's disease, with acute Oroya fever followed by the chronic verruga peruana, and *Bartonella quintana* causing trench fever. The life-threatening Oroya fever and the much milder course of trench fever represent

Author Summary

Pathogens are—as the result of adaptive evolution in their principal host(s)—typically limited in the range of hosts that they can infect successfully. However, infrequently such host-restricted pathogens may undergo a spontaneous host switch, which can lead to the evolution of pathogens with altered host specificity. Most human pathogens evolved this way, and animal-specific pathogens have thus to be considered as an important reservoir for the emergence of novel human pathogens. Despite host-specificity representing a common feature of pathogens, the underlying molecular mechanisms are largely unknown. In this study we have used bacterial pathogens of the genus *Bartonella* to identify bacterial factors involved in the determination of host specificity. The bartonellae represent an excellent model to study host-specificity as each species is adapted to cause an intracellular infection of erythrocytes exclusively in its respective reservoir host(s). Using a genetic approach in combination with erythrocyte infection models *in vitro* and *in vivo* we demonstrate that a surface-located bacterial nanomachine—a so-called type IV secretion system—determines host specificity of erythrocyte infection. Our work sheds light on the molecular basis of host specificity and establishes an experimental model for studying the evolutionary processes facilitating spontaneous host shifts.

the characteristic intraerythrocytic stages of these pathogens. The other 19 species cause intraerythrocytic infections in various non-primate mammalian reservoirs. At least seven of them are recognized as zoonotic pathogens which incidentally infect humans. Commonly, *B. henselae* is associated with cat scratch disease [7].

The life cycle of *Bartonella* in the reservoir host has been analyzed in detail in rats experimentally infected with *B. tribocorum* [8]. Following intravenous inoculation, bacteria initially infect a primary niche outside of circulating blood, which is considered to comprise the vascular endothelium and possibly other cell types. Approximately on day five of infection, large numbers of bacteria are released into the bloodstream where they bind to and invade mature erythrocytes. Bacteria then replicate in a membrane-bound compartment until reaching a critical number. For the remaining life span of the erythrocytes the intracellular bacteria remain in a non-dividing state [8]. Monitoring of bacteremia in other animal models, such as the *B. birtlesii*-mouse [9] and *B. henselae*-cat models [10], or in captive naturally infected animals has yielded results that match those observed in the *B. tribocorum*-rat model, suggesting a common mode of infection of the different species in their respective animal reservoirs [11]. The only exception is *B. bacilliformis*, which causes lysis of the infected human erythrocytes, eventually resulting in a severe hemolytic anemia.

The *B. tribocorum*-rat model was further explored to identify bacterial pathogenicity factors that are required for colonization of the mammalian reservoir host. A signature-tagged mutagenesis (STM) screen identified 98 essential bacterial loci [12], including genes encoding components of two distinct type IV secretion systems (T4SS), VirB/VirD4 and Trw, the invasion-associated locus B (IalB) protein, the trimeric autotransporter adhesin BadA, as well as further members of the autotransporter family [6]. Whether any of the identified genes is critical for host-specificity is unknown, although it is conceivable to assume that host-specificity loci are essential for infection and may thus be represented among the hits of the performed STM screen.

Experimental infections of different mammalian hosts by a given *Bartonella* strain have reproduced the species-specificity of erythrocyte invasion as observed in natural infections [11,13,14,15]. However, despite their availability, *in vitro* erythrocyte infection assays [16,17] have not been investigated for the study of host specificity. Here, we demonstrate for the first time that host specificity is reflected by the exclusive capacity of *Bartonella* species to adhere to erythrocytes isolated from their natural host(s). Second, by performing STM in *Bartonella birtlesii* followed by screening in mice *in vivo* and in isolated erythrocytes *in vitro* we identified the T4SS Trw as the molecular determinant of host-specific erythrocyte infection.

Results

An *in vitro* erythrocyte colonization assay to study host-restricted infection

Based on described *in vitro* models of human and feline erythrocyte infection by *B. bacilliformis* and *B. henselae*, respectively [16,17], we established for *B. birtlesii* an *in vitro* infection model for erythrocytes isolated from the murine reservoir host. Balb/C mice were used as the source of erythrocytes as they are known to develop a long lasting intraerythrocytic infection upon experimental infection with *B. birtlesii* [9]. The intraerythrocytic presence of bacteria was evaluated over a period of three days using the gentamicin protection assay (Fig. 1A). Bacterial entry into erythrocytes was dependent on the number of bacteria per erythrocyte (multiplicity of infection, MOI; tested MOI range: 0.01 to 10) and time of infection (days post infection, DPI; tested time range: 1 to 3 DPI). The highest intraerythrocytic bacterial content over time was obtained for MOI = 0.1 and 1, with approximately 2×10^5 colony forming units (CFU) per 10^{10} erythrocytes (≈0.002% infected erythrocytes) at 3 DPI. Given that mouse blood contains approximately 10^{10} erythrocytes/ml, this value corresponds well to the bacteremia reported for experimentally infected Balb/C mice (≈1×10^5 CFU/ml; 0.001% infected erythrocytes) [9]. For MOI = 10, erythrocytes were infected at 1 DPI, but lysed entirely by 3 DPI. At MOI = 0.01, only low numbers of intraerythrocytic bacteria were detected over time. Based on these data, MOI = 1 was used for all subsequent erythrocyte infection assays. To evaluate whether the increase of intraerythrocytic bacteria over time was mainly due to continued bacterial invasion, or to intraerythrocytic bacterial multiplication, or to a combination of both, erythrocytes were infected with *B. birtlesii* for one day in the absence of gentamicin, followed by incubation in the continuous presence of gentamicin to kill extracellular bacteria. Fig. 1B shows that the number of intracellular bacteria increased over time in the presence of gentamicin, albeit to a lesser extent than in the untreated control. Bacteria thus appear to enter erythrocytes beyond 1 DPI and, moreover, to replicate in an intra-erythrocytic location.

Invasion of erythrocytes by *Bartonella* is preceded by bacterial adhesion to the erythrocyte surface [18]. To quantify erythrocytes infected by adherent extracellular and/or intracellular bacteria, we used GFP-expressing bacteria in combination with flow cytometry (Fig. 1C, D.). Similar as described for intraerythrocytic bacteria in the gentamicin protection assay, erythrocyte colonization revealed by flow cytometry was dependent on time (Fig. 1.C) and MOI (Fig. 1.D). However, the rate of erythrocyte colonization evaluated by flow cytometry (55% for MOI = 1 at 3 DPI) was approximately 20′000-fold higher than erythrocytes invasion determined by the gentamicin protection assay (compare Fig. 1.A), indicating that the vast majority of bacteria detected by flow cytometry were associated extracellularly with erythro-

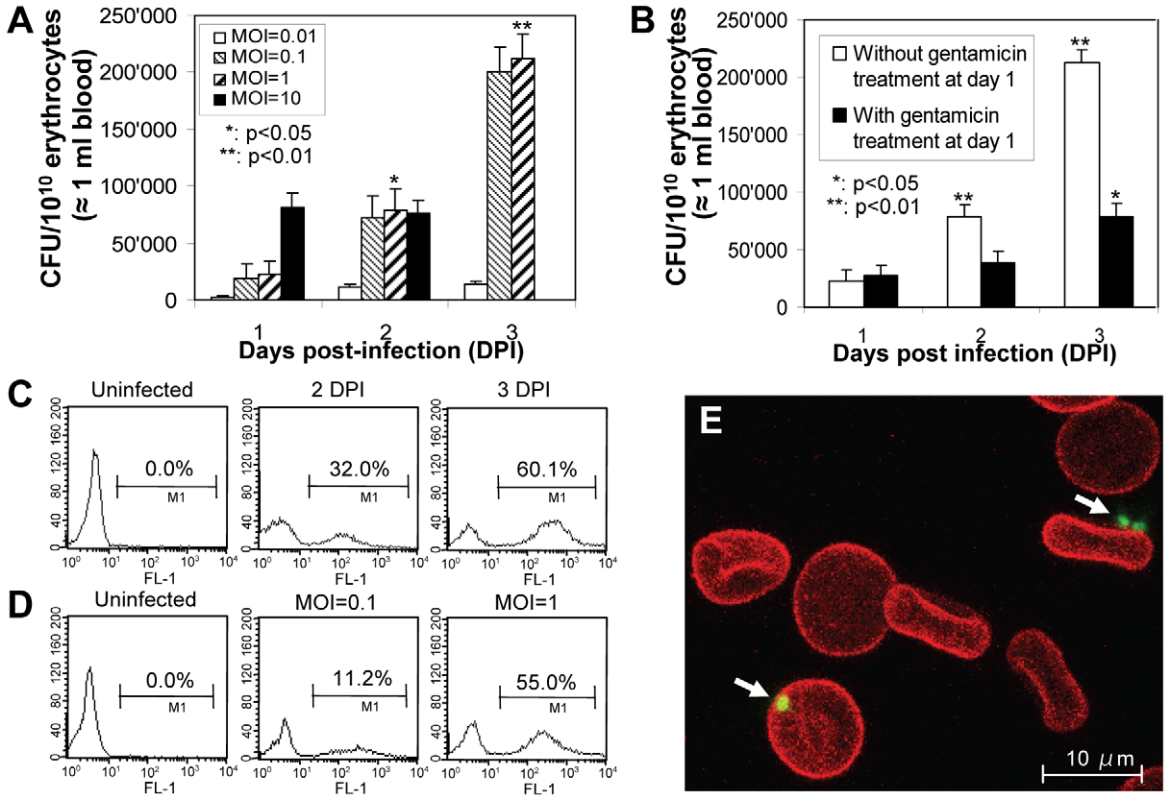

Figure 1. *B. birtlesii* invades murine erythrocytes *in vitro*. (**A**, **B**) Time- and bacterial number-dependency of *B. birtlesii* invasion of murine erythrocytes determined by the gentamicin protection assay. (**A**) Freshly isolated murine erythrocytes were infected with *B. birtlesii* at the indicated multiplicity of infection (MOI) and the numbers of intra-erythrocytic bacteria (colony forming units, CFU) was determined by the gentamicin protection assay at 1, 2 and 3 days post infection (DPI); n = 6, mean +/-SD; *, **: significant difference of data compared to 1 DPI. (**B**) Freshly isolated murine erythrocytes were infected with *B. birtlesii* at MOI = 1. At 1 DPI, gentamicin was added to half of the samples, and growth was continued in the continous presence of gentamicin through 3 DPI to kill extracellular bacteria. The other half of the infected erythrcyotes was not treated with gentamicin. For both untreated and gentamicin treated samples, numbers of intra-erythrocytic bacteria were determined by the gentamicin protection assay at 1, 2 and 3 DPI (n = 6; mean +/−SD, *, **: significant difference in gentamicin treated samples compared to 1 DPI). (**C**) Time- and (**D**) bacterial number-dependency of *B. birtlesii* associated to murine erythrocytes determined by flow cytometry. Freshly isolated murine erythrocytes were infected with *B. birtlesii-gfp* (MOI = 1, detection at 1 and 3 DPI in C and MOI = 0.1 or 1, detection at 3 DPI in D). The percentage of erythrocytes associated with bacteria were quantified by flow cytometric analysis at 2 and 3 DPI. Representative data for the fluorescence (FL-1) of 10′000 erythrocytes are shown as histogram plots. (**E**) Confocal microscopic analysis of murine erythrocytes infected for 2 days with GFP-expressing *B. birtlesii* (MOI = 1). Arrows point to bacteria found in close association with erythrocytes.
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cytes. Confocal microscopy confirmed the predominant extracellular localization of erythrocyte-associated bacteria (Fig. 1.E).

Next we investigated whether *Bartonella* species differ in their capacity to interact *in vitro* with erythrocytes of different mammalian origin, and whether this capacity may reflect the host-restriction displayed during natural infection. First, mouse erythrocytes were infected with either *B. birtlesii*, *B. vinsonii arupensis* (both mouse-specific), *B. alsatica* (rabbit-specific), *B. vinsonii berkhoffii* (dog-specific), *B. henselae* (cat-specific), *B. quintana* (human-specific), or *B. tribocorum* (rat-specific). Erythrocyte invasion was quantified by the gentamicin protection assay (Fig. 2). *B. vinsonii arupensis* displayed invasion rates similar to *B. birtlesii*, while none of the other strains tested resulted in significant erythrocyte invasion. Using a corresponding set of strains expressing GFP, consistent results were obtained for the flow cytometric determination of bacterial adhesion to mouse erythrocytes (Fig. 3 and Fig. S1). These findings indicate that specificity for the mouse reservoir *in vivo* correlates with efficient adhesion to and invasion of mouse erythrocytes *in vitro*.

Next, we tested whether - similarly as observed for mouse erythrocytes and *B. birtlesii* – the capacity of *B. henselae*, *B. quintana* and *B. tribocorum* to adhere to erythrocytes *in vitro* is also restricted to erythrocytes from their natural reservoir host, i.e. cat, human and rat, respectively. GFP-expressing bacteria were used for erythrocyte infection, and adhesion was quantified by flow cytometric analysis. Fig. 3 and Table 1 illustrate that all tested *Bartonella* species were able to efficiently adhere to erythrocytes isolated from their respective reservoir hosts, while they essentially did not adhere to erythrocytes from non-reservoir hosts. The only exception is *B. quintana*, which further to erythrocytes from the human reservoir also colonized cat erythrocytes. Together, these data indicate that the established *in vitro* model of erythrocyte colonization reflects well the host restriction as observed during natural infection.

B. birtlesii genes required for intra-erythrocytic bacteremia in mice

As a basis for identifying genetic factors involved in host-restricted erythrocyte colonization, we identified a comprehensive set of *B. birtlesii* genes required for establishing intraerythrocytic bacteremia in mice. To this end, an STM library of *B. birtlesii* was constructed as previously described for *B. tribocorum* [6,12,19].

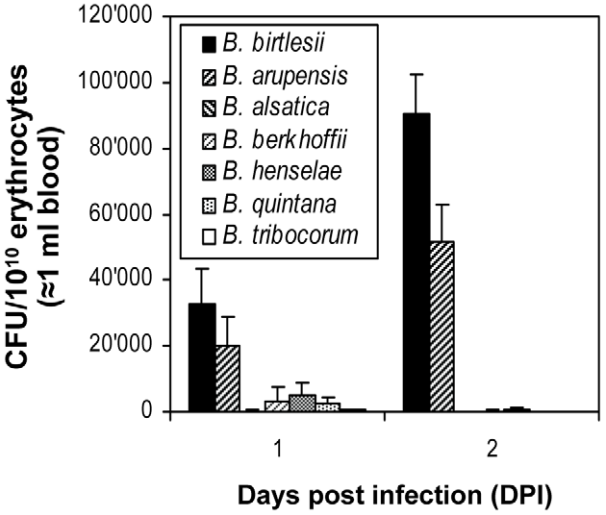

Figure 2. Efficiency of *in vitro* invasion of murine erythrocytes by different *Bartonella* species. Freshly isolated murine erythrocytes were infected with the indicated *Bartonella* species with a MOI = 1. The numbers of intra-erythrocytic bacteria (colony forming units, CFU) was determined by the gentamicin protection assay at 1 and 2 days post infection (DPI); mean +/-SD of triplicate samples.
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From each conjugation assay, we selected 96 single kanamycin-resistant colonies and assembled an STM mutant library of 3456 mutants. We then identified mutants that have lost the capacity to cause intraerythrocytic bacteremia by screening the library in the mouse infection model [9]. Of 1456 mutants tested in the input pools, 98 were not detected in the output pools from mice at days 7 and 14 post infection and were thus classified as abacteremic mutant candidates. All 98 abacteremic mutant candidates were retested by reassembling them into 49 pools of 9 mutants, each pool containing two abacteremic mutant candidates and an invariable set of seven mutants displaying wild-type behavior (bacteremic mutants). The rescreen confirmed an abacteremic phenotype for 48 of the initial 98 abacteremic mutant candidates, corresponding to 3.3% of the total number of mutants screened. Growth of all of the 48 confirmed abacteremic mutants on solid media was similar to the parental wild-type strain (data not shown).

We determined the transposon insertion sites for all 48 abacteremic mutants by direct sequencing out of the transposon into the flanking chromosomal region and mapping of the derived sequences onto the draft genome sequence of *B. birtlesii* (S. Cescau, H.M. Yang, J. Wang, M. Vayssier-Taussat, A. Danchin, and F. Biville, unpublished data). Three mutants harboring two separate transposon insertions were not considered further in the analysis. *Table S1* lists the loci inactivated by single transposon insertion in the remaining 45 abacteremic mutants. Five mutants carried the transposon insertion in an intergenic region: one (83D04) was near a gene encoding a tRNA; three of them (04A01, 86C05, 69B07) were upstream of genes encoding proteins of unknown function and one (69C09) was in proximity to a putative transcriptional regulator gene. In these mutants, the transposon may have thus disrupted a promoter or another regulatory sequence. 40 transposon insertions were mapped to the coding region of 38 different protein-encoding genes. In 8 mutants the insertions were found in genes encoding a conserved protein of unknown function, among them three putative surface proteins. Sixteen mutants carried insertions in genes previously implicated in bacterial

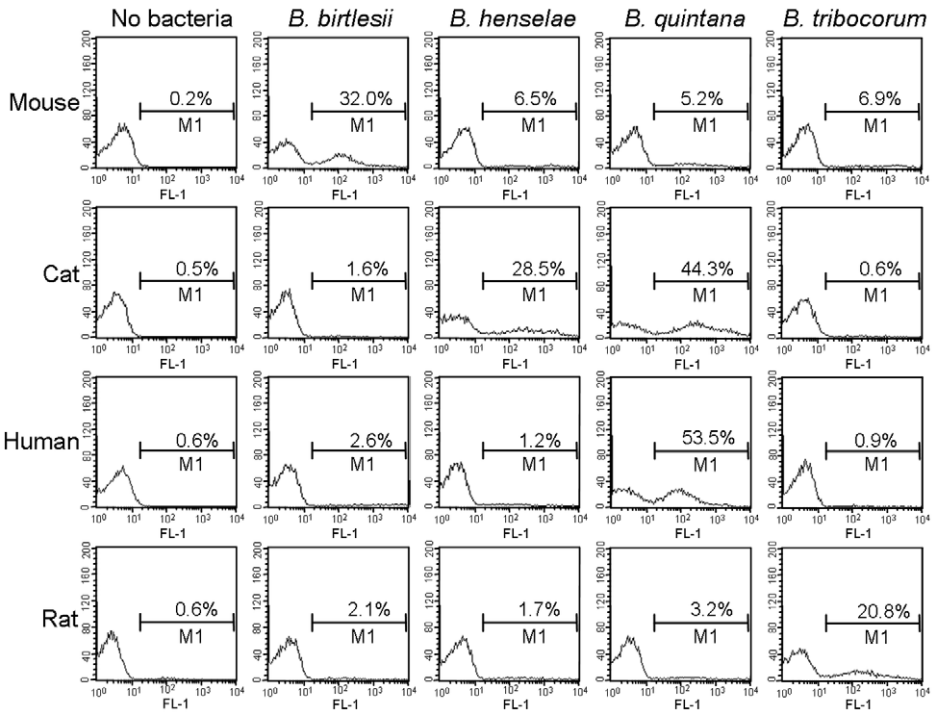

Figure 3. Efficiency of interaction between erythrocyte and *Bartonella* sp. according to host origin and *Bartonella* species. Freshly isolated erythrocytes from mouse, cat, human or rat were infected with *gfp*-expressing bacteria of the indicated *Bartonella* species (MOI = 1). The percentages of infected erythrocytes were determined by flow cytometry at two DPI. Representative histogram plots for GFP-fluorescence (FL-1) of 10′000 erythrocytes are shown.
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Table 1. Efficiency of erythrocyte colonization according to host origin and *Bartonella* species.

	B. birtlesii		*B. henselae*		*B. quintana*		*B. tribocorum*	
	in vivo	*in vitro* (%)*	*in vivo*	*in vitro* (%)*	*in vivo*	*in vitro* (%)*	*in vivo*	*in vitro* (%)*
Mouse	+[a]	26.3 +/− 2.2	-[b]	1.3 +/− 0.5	n.r.	0.8 +/− 0.3	n.r.	1.6 +/− 0.4
Cat	n.r.	1.6 +/− 0.8	+[c]	28.5 +/− 4.1	n.r.	42.2 +/− 3.1	n.r.	0.5 +/− 0.3
Human	n.r.	2.7 +/− 1.0	n.r.	1.3 +/− 0.3	+[d]	58.4 +/− 1.2	n.r.	0.9 +/− 0.2
Rat	n.r.	2.0 +/− 1.3	n.r.	1.8 +/− 0.7	n.r.	3.5 +/− 1.1	+[e]	20.7 +/− 2.8

[a][9], [b][15], [c][41,42], [d][8,43,44], [e][8].

*Freshly isolated erythrocytes from mouse, cat, human or rat were infected with *gfp*-expressing bacteria of the indicated *Bartonella* species (MOI = 1). The percentages of colonized erythrocytes were determined by flow cytometry at day two post infection. Data for 10′000 erythrocytes per time-point were analyzed (n = 6 for tests with homologous species and n = 3 for tests with heterologous species, mean +/− SD). For previously described infections of the respective mammalian hosts with the indicated *Bartonella* species the presence (+) or absence (-) of intraerythrocytic bacteremia is indicated (n.r. = not reported).

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pathogenicity, either in *Bartonella* (*virB/D4*, *trw*, *ialA/B*, *badA*, *omp43*, *iba*) or other pathogenic bacteria (i.e. loci encoding heat shock proteins) [5]. Moreover, mutant genes encoding proteins involved in transport and metabolism, as well as phage-related function were also identified.

B. birtlesii genes required for erythrocytic infection *in vitro*

The 45 confirmed abacteremic mutants with single transposon insertion were individually tested for their capacity to invade murine erythrocytes using the gentamicin protection assay (*Table S1*). Nine mutants were found to be impaired in murine erythrocyte invasion (Fig. 4). Complementary erythrocyte adhesion assays based on flow cytometric analysis of antibody-stained bacteria demonstrated that seven of these nine invasion-deficient mutants are also deficient in erythrocytes adhesion. All seven mutants harbor a mutation in the operon encoding the T4SS Trw (two in *trwD*, *trwE*, *trwF*, *trwJ2*, *trwL1*, *trwL2)*, which was previously shown to be important for establishing an intraerythrocytic bacteremia in *B. tribocorum* [20]. Compared to wild-type, both *trwD* mutants (04B03 and 41C12) showed a five-fold decrease in invasion/adhesion efficiency. All other *trw* mutants failed to invade erythrocytes and were severely impaired in their capacity to adhere to erythrocytes (Fig. 4). These data demonstrate that the Trw system is required for erythrocyte invasion by mediating specific adhesion to erythrocytes. In contrast, mutants harboring an insertion in the invasion-associated locus *ialA/B* showed normal erythrocyte adhesion but impaired invasion (10-fold reduced, $p<0.01$), confirming the previously suggested role of this locus in erythrocyte invasion [21,22]. Equally, an insertion mutant (25A02) inactivating *livG* (encoding an amino acid ABC-transporter) showed normal adhesion but a specific defect in invasion (4-fold, $p<0.05$) compared to wild-type (Fig. 4). None of the other abacteremic mutants appeared to be involved in erythrocytes invasion indicating that they probably are required for an earlier step of infection, i.e. for colonization of the primary niche.

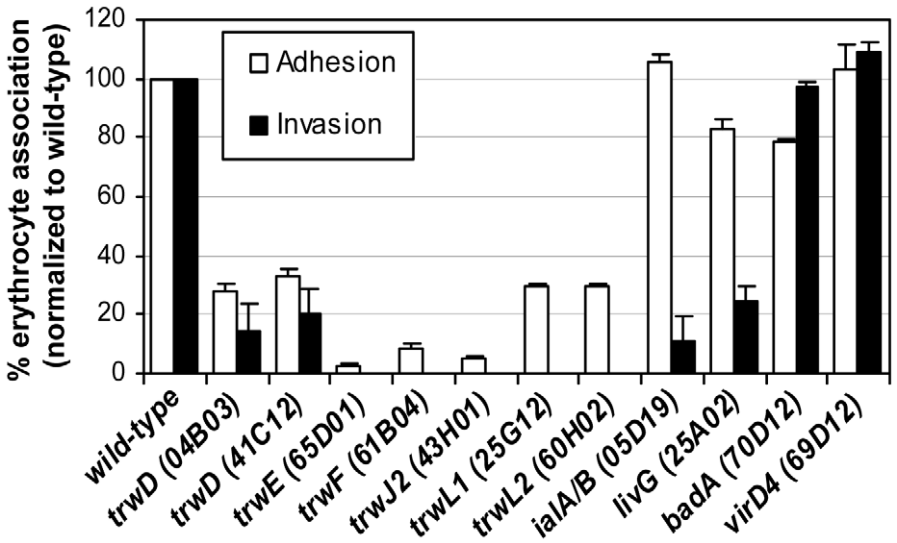

Figure 4. Role of Trw in erythrocyte infection. Efficiency of *in vitro* invasion of murine erythrocyte by abacteremic mutants of *B. birtlesii*. The *in vitro* erythrocyte adhesion or invasion phenotype of abacteremic mutants identified in the STM screen was evaluated at 2 DPI by flow cytometry after immunocytochemical staining of bacteria (see Figure S2) or at 1 DPI by the gentamicin protection assay, respectively. The efficiency of erythrocyte adhesion or invasion of each tested mutant is expressed as percentage of erythrocyte adhesion or invasion of the isogenic wild-type strain (mean +/- SD of triplicate samples). All mutants listed in *Table S1* that do not appear in this figure display wild-type phenotype in regard of *in vitro* erythrocyte invasion.

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Role of Trw T4SS in host-specific infection of erythrocytes

Next we tested whether the Trw system shown here to be essential for erythrocyte infection *in vitro* and *in vivo* may be directly involved in determining host-specificity. To this end we introduced pAB2, a plasmid encoding the *trw* locus of rat-specific *B. tribocorum* [20], into cat-specific *B. henselae*, human-specific *B. quintana* and *B. tribocorum* (control). We then compared the capacity of these recombinant strains to infect rat erythrocytes with their parental strains. Among the parental strains, only *B. tribocorum* mediated invasion of rat erythrocytes (Fig. 5), which is consistent with the erythrocyte adhesion data presented in Fig. 2. The pAB2-mediated ectopic over-expression of *trw* in *B. tribocorum* resulted only in a slight increase of invasion, indicating that the endogenous level of trw expression is sufficient to mediate efficient bacterial entry. Strikingly, ectopic expression of the *B. tribocorum trw* locus in *B. henselae* and *B. quintana* rendered these pathogens capable of infecting rat erythrocytes. These data clearly demonstrate a direct role of the Trw system in determining host-specificity of erythrocyte infection.

To further assess which components of the Trw T4SS may mediate host specificity we analyzed the molecular evolution of different *trw* genes of *B. birtlesii* and related species. Candidate genes for mediating host specificity are surface exposed components, i.e. the T4SS pilus components TrwL and TrwJ. As shown by Nystedt *et al.* [23] for other *Bartonella* species, *trwL* and *trwJ* genes have been amplified and diversified several times during evolution. The *trw* locus of *B. birtlesii* also displays amplification of *trwL* (five copies) and co-amplification of *trwJ* together with *trwH and trwI* (two copies) (Fig. S3, panel A). Phylogenetic analyses and calculation of the non-synonymous (*d*N) and synonymous (*d*S) substitution frequencies of different *trw* genes further showed that *trwJ* and *trwL* homologs have diversified to much higher degree than other components of the Trw T4SS, within and among different species [Fig. S3, panel B-G, and [23]].

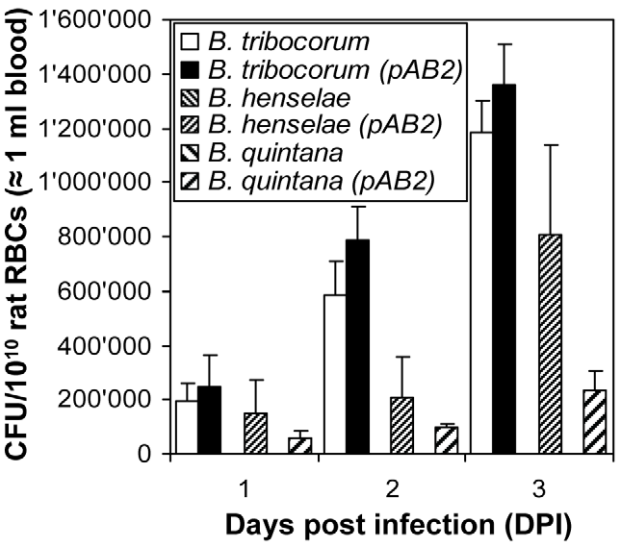

Figure 5. Role of Trw T4SS in mediating host-specific erythrocyte invasion. Freshly isolated rat erythrocytes were infected with *B. tribocorum, B. tribocorum* (pAB2), *B. henselae, B. henselae* (pAB2), *B. quintana*, or *B. quintana* (pAB2) at a MOI = 1. Intra-erythrocytic bacteria were enumerated at 1, 2 and 3 days post infection (DPI) by the gentamicin protection assay (n = 3; mean +/− SD).
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Discussion

Host-specificity is a prominent feature of pathogenic bacteria that reflects the host range susceptible to infection. Subtle changes in the molecular mechanisms that govern host-specificity may result in spontaneous host shifts, which represent a major risk for the emergence of novel human pathogens from animal reservoirs. Striking examples for this evolutionary scenario are the bartonellae, which cause host-restricted intra-erythrocytic infections in their mammalian reservoirs. In conjunction with repeated host shifts, the large number of *Bartonella* species evolved by adaptive radiation [6], including the human-specific pathogen *B. quintana* that evolved from cat-specific *B. henselae* [24]. Here we explored the bacterial genetic basis for host-restricted infection of erythrocytes. The establishment of an *in vitro* model of erythrocyte adherence and invasion allowed us to demonstrate for the first time a direct correlation of host-restricted erythrocyte infection *in vivo* and *in vitro*, demonstrating that host-specificity is determined by the capacity of bacteria to adhere to erythrocytes. In order to identify the bacterial factors critical for host-restricted erythrocyte infection we have used a two-step experimental protocol. First, we performed an STM screen for *B. birtlesii* in mice which allowed us to identify 45 abacteremic mutants defective in establishing intra-erythrocytic infection. Among the corresponding set of 38 protein-encoding genes, 13 loci were also indentified in a similar STM screen performed in the *B. tribocorum*-rat model [6]. This indicates extensive similarities in the repertoire of pathogenesis factors in these closely related organisms as well as robustness of the performed genetic screens. Second, rescreening of the entire set of 45 abacteremic *B. birtlesii* mutants in the *in vitro* mouse erythrocyte infection model resulted in the identification of nine mutants impaired in erythrocte invasion. The other mutants (36 of 45 = 80%) displaying a wild-type phenotype in this assay are therefore not directly involved in erythrocyte infection, but rather may contribute to the establishment of infection in the primary niche. Prominent examples are the *virB/virD4* genes encoding the VirB/VirD4 T4SS, which is known to be required for primary niche infection in the *B. tribocorum*/rat model (Schulein, 2002). Moreover, a recent study inferred the VirB/VirD4 T4SS as major bacterial factor facilitating bacterial adaptation to novel hosts [6]. The nine mutants impaired in *in vitro* erythrocyte invasion differ in their capacity to adhere to erythrocytes. Transposon insertions in the invasion locus (*ialA/B*) previously implicated in erythrocyte invasion [21,22] and *livG* encoding an amino acid ABC-transporter displayed wild-type like adherence to erythrocytes. IalA/B and LivG should thus represent invasion factors. The remaining seven invasion-deficient transposon mutants were all severely impaired in erythrocyte adhesion. Strikingly, all these mutants carry insertions in components encoding the T4SS Trw, which thus represents an erythrocyte adherence system that is critical for erythrocyte invasion. Trw is known to be required for establishing intra-erythrocytic infection in the *B. tribocorum*-rat model [5,20,25], however, evidence for a direct role of the Trw system in erythrocyte adhesion as provided here was lacking so far. Based on the presumable surface location of components of Trw [20] this T4SS may directly interact with the erythrocyte surface and thus may restrict the host range of erythrocyte infection. To test the hypothesis that Trw determines host range we have expressed Trw of rat-specific *B. tribocorum* in cat-specific *B. henselae* and human-specific *B. quintana*. Strikingly, this genetic manipulation resulted in an extension of the host range for *in vitro* erythrocyte infection towards rats, demonstrating that Trw indeed represents a major determinant of host-specificity of erythrocyte infection. Thus, this finding establishes a new experimental model

to study the molecular mechanisms governing host restriction - further to the molecular paradigm of host-specificity exemplified by the interaction of two surface proteins of *L. monocytogenes*, InlA and InlB, with their respective host receptors [2,3,4].

The Trw locus was laterally acquired during evolution of the bartonellae. It is present in the largest sub-branch of the genus tree, comprising 13 species that are adapted to diverse mammalian reservoir hosts, while it is absent from human-specific *B. bacilliformis*, cat-specific *Bartonella clarridgeiae* and the species of the ruminent-specific sub-branch, which all diverted early during evolution of the bartonellae [6]. Interestingly, the acquisition of Trw by the modern lineage correlates with the loss of flagella, which are know to represent a major pathogencity factor for the invasion of erythroctes by *B. bacilliformis* and probably other flagellated bartonellae [25]. The Trw system of *Bartonella* represents an interesting example of a pathogenesis-related T4SS that evolved rather recently by functional diversification of a laterally acquired bacterial conjugation system. The *trw* locus displays characteristic features of a pathogenicity island and shares extensive similarity with the *trw* locus of IncW broad-host range plasmid R388 encoding a genuine conjugation system. The *trw* loci of *Bartonella* and R388 are colinear, except for multiple tandem gene duplications of *trwL* and *trwJ-trwH* in *Bartonella*. Complementation of R388 derivatives carrying mutations in different *trw* genes with their *Bartonella* homologues allowed to demonstrate functional interchangability for some T4SS components [20,26], underscoring the structural and functional conservation of individual subunits of these functionally diversified T4SSs. However, a major difference between these homologous systems is the lack of the coupling protein TrwB in *Bartonella*, which in R388 is required for export of T4SS substrates. The lack of TrwB in *Bartonella* thus indicates that its Trw system may not be capable of translocating substrates. However, the multiple copies of *trwL* and *trwJ* in the *Bartonella trw* locus encode variant forms of surface-exposed pilus components, which probably are all co-expressed [20], indicating that the primary function of the *Bartonella* Trw system may be the formation of variant pilus forms [25]. Based on the essential role of the Trw system for adhesion to erythrocyte and its role in determining host range it is conceivable to assume that these variant pili may facilitate the specific interaction with polymorphic erythrocyte receptors, either within the reservoir host population (e.g. different blood group antigens), or among different reservoir hosts. Phylogenetic analyses and calculation of the non-synonymous (*d*N) and synonymous (*d*S) substitution frequencies of different *trw* genes indeed demonstrated that *trwJ* and *trwL* homologs have diversified to much higher degree than other components of the Trw T4SS, within and among different species [23]. Together with the notion that the number of tandem repeats of *trwL* and *trwJIH* are variable among different *Bartonella* species these findings indicate that *trwL* and *trwJ* genes have been amplified and diversified several times during evolution. Horizontal transfer of such genes from a different bartonellae – similarly as we have demonstrated here for the entire *trw* operon of rat-specific *B. tribocorum* resulting in an extension of the host range of cat-specific *B. henselae* or human-specific *B. quintana* to rat – or alternatively pre-adaption of superfluous copies of *trwL* and *trwJ* may represent realistic molecular evolutionary scenarios for host shifts and thereby the evolution of pathogens with an altered host-specificity as it has happened repeatedly during the evolution of the bartonellae. Future studies should identify the nature of the erythrocyte receptors targeted by the Trw system and their specific interaction that facilitate host-specific erythrocyte infection.

Materials and Methods

Ethics statement

Animals were handled in strict accordance with good animal practice as defined by the relevant European (European standards of welfare for animals in research), national (Information and guidelines for animal experiments and alternative methods, Federal Veterinary Office of Switzerland) and/or local animal welfare bodies. Animal work performed at the Biozentrum of the University of Basel was approved by the Veterinary Office of the Canton Basel City on June 2003 (licence no. 1741), and animal work performed at the Ecole Nationale Vétérinaire d'Alfort (ENVA/AFSSA) was approved by the institute's ethics committee on September 2005.

Bacterial strains and growth conditions

B. alsatica (IBS 382^T, CIP 105477^T) [27], *B. birtlesii* (IBS 135^T, CIP 106691^T) [28], *B. henselae* (Houston-1, ATCC 49882^T), *B. quintana* (Fuller^T, ATCC VR-358^T), *B. tribocorum* (IBS 506^T, CIP 105476^T) [29], *B. vinsonii subsp. berkhoffii* (ATCC 51672^T), *B. vinsonii subsp arupensis* (ATCC 700727) [30] were grown for 5 days on Columbia agar containing 5% defibrinated sheep blood (CBA) in a humidified atmosphere with 5% $C0_2$ at 35°C.

Construction of bacterial strains

B. tribocorum-gfp containing a chromosomally-integrated *gfp*-expression cassette [8] was used as GFP-expressing *B. tribocorum* strain. GFP-expressing bacteria of other *Bartonella* species were obtained by electroporation with plasmid pJMBGFP as previously described [31,32]. This plasmid was extracted and purified from *B. quintana* using a Midi Prep Kit (Qiagen). The electroporation procedures was described previously [31]. Transformed bacteria were selected by plating on CBA-Km. A signature-tagged mutant library of *B. birtlesii* IBS135^T was constructed as described for *B. tribocorum* [6,12]. Cosmid pAB2 encoding the entire *trw* locus of *B. tribocorum* [20,33,34] was introduced into *B. henselae* and *B. quintana* by three parental mating [34,35].

In vitro infection of erythrocytes

Erythrocytes from peripheral blood of mice (Balb/C), cats, rats (Wistar) and humans were isolated and purified by Ficoll gradient centrifugation. After washing in PBS, they were maintained in F12 modified medium [supplemented with 10% fetal calf serum, 2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM Hepes, 257 mM histidine, 0.1 mg/ml hematin/histidine, non-essential amino acid (Gibco, FRANCE)] at 2×10^8/ml. For *in vitro* infection experiments, *Bartonella* species were grown on CBA or CBA-km (*Bartonella-gfp* and STM mutants) plates. After 5 days of culture (10 days for GFP-expressing *Bartonella*), bacteria were harvested, washed, suspended in PBS, and added to erythrocytes at a multiplicity of infection (MOI, calculation based on 1 $OD_{600\ nm} = 3\times10^9$ bacteria/ml) varying from 0.01 to 10 and incubated at 35°C in 5% $C0_2$ for various periods of time (from 1 to 3 days).

Detection of erythrocyte-associated bacteria

Colonization of erythrocytes by *Bartonella* was assessed and quantified by the gentamicin protection assay, flow cytometry and confocal microscopy. For the quantification of intracellular bacteria by gentamicin protection, 100 µl were withdrawn from the invasion mixtures after 1, 2 or 3 days of *in vitro* infection. Mouse erythrocytes were separated from non-associated bacteria by washing 3 times with PBS and centrifuged at 500 g for 5 min. Erythrocytes were then incubated for 2 h at 35°C with gentamicin sulfate (250 µg/ml) to kill residual extracellular bacteria. Erythro-

cytes were then washed three times in PBS to remove the antibiotic and intracellular bacteria were released from erythrocytes by hypotonic lyses of erythrocytes in 10 µl of sterile water by freezing at −20°C for 15 min. After thawing, serial dilutions of bacteria in PBS were inoculated onto CBA plates and incubated at 35°C for 5 days before being counted. For data presentation, all measurements were expressed as the number of CFU/10^{10} erythrocytes (corresponding to ≈1 ml of blood).

For flow cytometric detection of erythrocyte-associated with GFP-expressing bacteria, measurements were performed at day 1, 2, 3 after *in vitro* infection of erythrocytes with ten days old bacterial cultures. 100 µl of the infection mixtures was washed 3 times in PBS and fixed for 10 min with 0.8% paraformaldehyde and 0.025% glutaraldehyde. After fixing, erythrocytes were analyzed by flow cytometry (FACScan, Becton Dickinson Bioscience, France). For flow cytometric detection of erythrocytes associated with bacteria that do not express GFP (abacteremic mutants), measurements were performed at day 2 after *in vitro* infection. 100 µl of the infection mixtures was washed 3 times in PBS and fixed for 10 min with 0.8% paraformaldehyde and 0.025% glutaraldehyde. After fixing, erythroctes/mutants association was revealed with mouse anti-*B. birtlesii* serum and anti-mouse FITC antibodies (Santa Cruz Biotechnology, Santa Cruz, CA,USA) and analyzed by flow cytomtry. Data were analyzed using the CellQuestPro software, version 4.0.2. Data for 10′000 gated erythrocytes were collected and analyzed.

For confocal microscopy, 100 µl of the infection mixtures was washed three times in PBS and the erythrocytes cell surface was stained using goat anti-mouse GPA antibodies (Santa Cruz Biotechnology, California, USA) and labelled with anti- goat-PE-antibodies (ImmunoQuest Antibody, North Yorkshire, UK). Samples were viewed with a Nikon Eclipse C1 Plus confocal laser scanning microscope (Nikon, Amstelveen, Netherlands) with detection in channel 1 (GFP fluorescence) and channel 2 (PE fluorescence) at original magnification x100.

STM library

The transposon vectors pHS006-Tag-001 to pHS006-Tag-036 each contained an *oriT* for conjugative transfer, the *Himar1* transposon, a kanamycin resistant marker, a hyperactive transposase and one of 36 distinct signature-tags [6]. These 36 signature-tagged mariner transposon vectors were separately transferred from *E. coli* β2155 to *B. birtlesii* by two-parental mating as previously described [35]. From each mating, 96 single kanamycin-resistant *B. birtlesii* transconjugants were transferred to a 96-well plate with cryo-medium and stored at −80°C.

Mouse infections

Eight weeks old female Balb/C mice from Charles River Laboratories were housed in an animal facility (2 animals/cage) and allowed to acclimate to the facility and the diet for at least 5 days prior infection. Food and water were provided *ad libitum*. 36 differently signature-tagged mutants were grown separately from the transposon library for each input pool. They were pooled in PBS immediately before infection, and used to infect two mice with a total inoculum of 5×10^7 colony forming units (10 µl of $OD_{595}=1$) in the ear dermis of Balb/C mice. The remainder of the input pools was heated at 100°C for 10 min and used as template for PCR detection. Fifty µl of blood were taken from the tail vein of the infected mice when bacteremia is peaking (days 7 and 14 post-infection) [9]. Bacteria released from erythrocytes by a freeze/thaw cycle were plated on CBA-km. After 10 days, bacterial colonies (output pool) were counted, harvested in PBS, suspended to $OD_{595}=1$ and heated at 100°C for 10 min to be used as template for PCR detection. The rescreen was done following the same protocol using pools of nine mutants (two abacteremic mutants and seven mutants displaying a wild-type phenotype).

PCR detection of abacteremic mutants

For signature-tag identification, the generic primer Srev01 corresponding to a sequence in the transposon and a set of tag-specific primer were used for amplification of a fragment of approximately 600 bp [6]. The conditions for the PCR were as follows: a first denaturation step at 95°C for 5 min, followed by 30 cycles of PCR with denaturation at 95°C for 1 min, annealing for 30 s at 52°C, and extension at 72°C for 1 min. The program was completed by an extension step at 72°C for 5 min. The amplified fragments were displayed on a 1% agarose gel. Mutants that were detected in the input pools and absent from the out put pools (days 7 and 14) in both mice were considered as abacteremic mutants.

Identification and analysis of transposon insertion sites

Genomic DNA from abacteremic mutants, regrown from the library, was prepared with the ROCHE Genomic DNA Isolation Kit. Genomic DNA was sent to QIAGEN for sequencing with primers Tn_{start} and Tn_{end} [6]. The sequences obtained by the genomic sequencing were compared by BlastN to the nr data base of NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The exact transposon insertion sites were found by comparing the genomic sequences to contigs of the ongoing *B. birtlesii* genome sequencing project by BlastN.

Screening of abacteremic mutants for their capacity to infect murine erythrocytes

Mutants displaying an abacteremic phenotype were tested for their capacity to invade murine erythrocytes using the gentamicin protection assay. Each mutant was tested at MOI = 1 in at least two independent experiments performed in triplicate samples. For mutants displaying an impaired erythrocyte invasion phenotype, invasion assays were performed at least three times in triplicate samples and adhesion assays were tested at day 2 post infection by flow cytometric detection once in triplicate samples.

Statistical analysis

Numerical data are reported as the mean of at least 3 replicate samples +/- standard errors of the means. Statistical significance of the data was measured by use of Student's t test. A p-value <0.05 was considered significant.

Phylogenetic and evolutionary analysis

The sequence of the *B. birtlesii trw* locus was deposited under the EMBL-EBI accession no. FN555106. Sequence alignments were calculated with ClustalW as implemented in MEGA4. Phylogenetic trees were inferred by maximum likelihood methods with Paup 4.0 [36] and 100 bootstrap replicates were calculated. To select an appropriate substitution model the Akaike information criterion of Modeltest 3.7 was used [37]. The models obtained were general time reversible (GTR) + I for *trwFED* and *trwN*, transversion model (TVM) + I for *trwI*, and TVM + I + G for *trwJ* and *trwL*. Nonsynonymous (dN) and synonymous (dS) substitution frequencies were calculated using the method of Yang and Nielson [38] as implemented in the PAML package [39,40].

Supporting Information

Figure S1 Efficiency of *in vitro* adhesion of murine erythrocytes to *Bartonella* sp. Freshly isolated murine erythrocytes were infected

with *Bartonella* sp.-GFP (MOI = 1, detection at two DPI). The percentage of erythrocytes associated with bacteria were quantified by flow cytometric analysis. Representative data for the fluorescence (FL-1) of 10'000 erythrocytes are shown as histogram plots.
Found at: doi:10.1371/journal.ppat.1000946.s001 (0.08 MB TIF)

Figure S2 Efficiency of in *vitro* adhesion of murine erythrocytes to abacteremic mutants. Freshly isolated murine erythrocytes were infected with *B. birtlesii* abacteremic mutants (MOI = 1, detection 2 DPI). Association between erythrocytes and bacteria was revealed with mouse anti-*B. birtlesii* polyclonal serum and labelled with anti-mouse FITC antibody. The percentage of erythrocyte associated with bacteria was quantified by flow cytometric analysis. Representative data for the fluorescence (FL-1) of 10'000 erythrocytes are shown as histogram plots.
Found at: doi:10.1371/journal.ppat.1000946.s002 (0.20 MB TIF)

Figure S3 Genetic organization of the *Bartonella trw* locus, and phylogenies and synonymous (*d*S) vs. nonsynonymous (*d*N) substitution frequencies of the encoded trw genes. (A) Gene order structure of the trw locus of *B. birtlesii* and comparison to other *Bartonella* species. The copy number of amplified genes or segments in other *Bartonella* species is indicated within brackets. Maximum Likelihood phylogenies of (B) the concatenated nucleotide alignments of *trwF, trwE,* and *trwD*, the nucleotide alignments of (C) *trwJ* copies, (D) *trwI*, (E) *trwL* copies, and (F) *trwN* of *B. birtlesii (Bb)*, *B. grahamii (Bg)*, *B. henselae (Bh)*, *B. quintana (Bq)*, and *B. tribocorum (Bt)*. For *trwJ* (C) and *trwL* (E), the range of pairwise *d*N/*d*S ratios of different phylogenetic subclusters (shaded areas) are indicated at the upper right of each cluster. For *trwL1*, the range of pairwise *d*N/*d*S ratios is indicated as well, although they do not cluster. (G) The pairwise *d*N/*d*S ratios of orthologous trw genes and the two adjacent genes *ubiH* and *sdhA* of *B. birtlesii* and *B. grahamii*, *B. henselae*, *B. quintana*, or *B. tribocorum* are plotted according to their gene order. For the tandem repeated genes *trwL*, *trwJ*, *trwI*, and *trwH* only *trwL5*, *trwJ1*, *trwI1*, and *trwH1* are shown, since ortholog assignment is difficult for the others due to copy number variation and the occurrence of recombination among different species [23].
Found at: doi:10.1371/journal.ppat.1000946.s003 (0.46 MB JPG)

Table S1 Genotypic characterization of abacteremic mutants of *B. birtlesii* obtained by signature-tagged mutagenesis (STM). The columns BARBAKC, BH, BQ and BT list the extensions of systematic names of orthologous genes from the published genomes of *B. bacilliformis* (accession no. CP000524), *B. henselae* (accession no. BX897699), *B. quintana* (accession no. 897700) and *B. tribocorum* (accession no. AM260525), respectively. * The *in vitro* erythrocate invasion phenotype of each mutant was determined by the gentamicin protection assay after 1 day of infection (triplicate samples) and categorized as normal (>70% of wild-type), reduced (<70% of wild-type but >1% of wild-type) or none (<1% of wild-type). Mutants with reduced or none in vitro invasion were tested again (n = 3) and the resulting mean and SD of all three experiments are represented in Figure 4.
Found at: doi:10.1371/journal.ppat.1000946.s004 (0.15 MB DOC)

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Author Contributions

Conceived and designed the experiments: MVT CD. Performed the experiments: MVT DLR HKD GM MM LA MQ HS. Analyzed the data: MVT EL PE HS CD. Contributed reagents/materials/analysis tools: MVT FB SC AD HJB HY JW PE HS CD. Wrote the paper: MVT CD.

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Table S1

Supporting Table S1: Genotypic characterization of abacteremic mutants of *B. birtlesii* obtained by signature-tagged mutagenesis (STM). The columns BARBAKC, BH, BQ and BT list the extensions of systematic names of orthologous genes from the published genomes of *B. bacilliformis* (accession no. CP000524), *B. henselae* (accession no. BX897699), *B. quintana* (accession no. 897700) and *B. tribocorum* (accession no. AM260525), respectively. * The *in vitro* erythrocate invasion phenotype of each mutant was determined by the gentamicin protection assay after 1 day of infection (triplicate samples) and categorized as normal (>70% of wild-type), reduced (<70% of wild-type but >1% of wild-type) or none (<1% of wild-type). Mutants with reduced or none *in vitro* invasion were tested again (n=3) and the resulting mean and SD of all three experiments are represented in Figure 4.

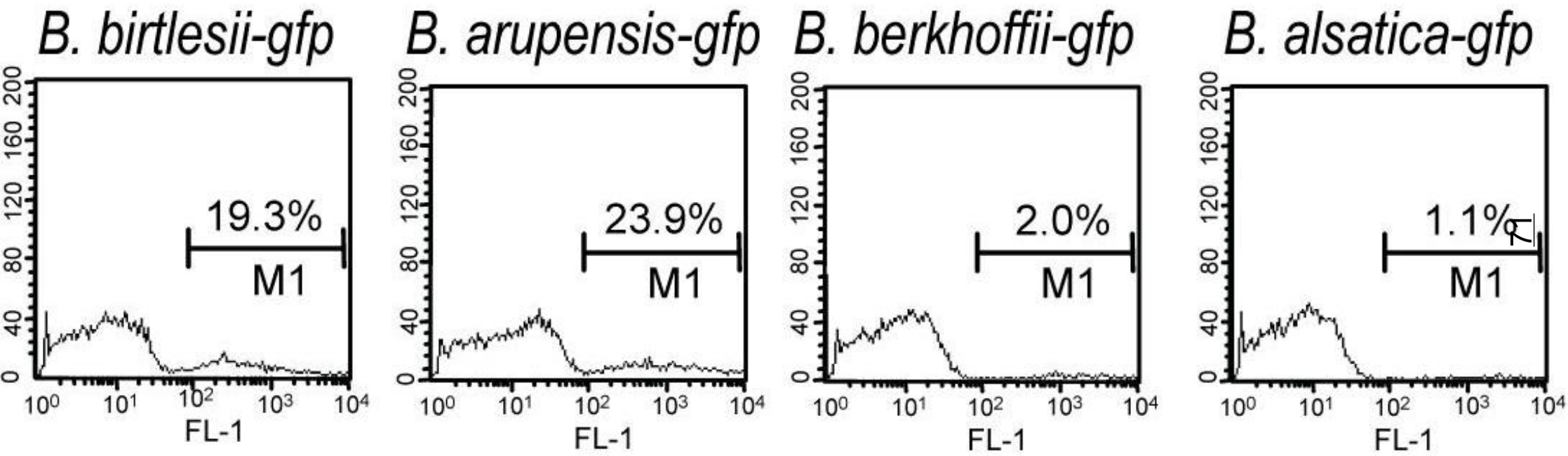
Gene Name	Mutant	Putative function	BARBAKC	BH	BQ	BT	*In vitro* infection*
Adhesion/Invasion							
badA	05A04	adhesin	583_0314	01490 01510	01390 01400 01410	0168	normal
badA	70D12	adhesin	583_0314	01490 01510	01390 01400 01410	0168	normal
ialA/ialB	05D10	invasion associated gene B	583_0326	01650	01550	0181	reduced
ibaA	44H12	putative inducible autotransporter	583_1132	13160	10410	1655	normal
omp43	43G09	outer membrane protein, adhesin	583_0447	12500	09890	1902	normal
trwD	04B03	T4SS component, VirB11 homolog	-	15760	12680	2533	reduced
trwD	41C12	T4SS component, VirB11 homolog	-	15760	12680	2533	reduced
trwE	65D01	T4SS component, VirB10 homolog	-	15750	12670	2532	none
trwF	61B04	T4SS component, VirB9 homolog	-	15740	12660	2531	none
trwJ2	43H01	T4SS component, VirB5 homolog	-	15670 15700	12590 12620	2519 2522 2524 2526 2528	none
trwL1	25G12	T4SS component, VirB2 homolog	-	15570 15580 15590 12600 12610	12490 12500 12510 12520 12530	2511 2512 2513 2514 2515	none

				12620	12540	2516	
				12630	12550	2516a	
				12640	12560		
trwL2	60H02	T4SS component, VirB2 homolog	-	15570	12490	2511	none
				15580	12500	2512	
				15590	12510	2513	
				12600	12520	2514	
				12610	12530	2515	
				12620	12540	2516	
				12630	12550	2516a	
				12640	12560		
virB4	61H02	T4SS component, VirB4 homolog	-	13280	10550	1691	normal
virD4	69D12	T4SS component, VirD4 homolog	-	13380	10640	1701	normal
virD4	79C12	T4SS component, VirD4 homolog	-	13380	10640	1701	normal
	61C01	autotransporter protein	-	13030	10290	1796	normal
Iron uptake							
hutA	5B10	outer membrane heme receptor	583_0460	04970	04160	0774	normal
Transport function							
ilvE	45B03	amino acid transporter	583_0747	10010	07730	1376	normal
livF	04A08	amino acid transporter	-	08250	06330	1144	normal
livG	25A02	amino acid transporter	-	08260	06320	1145	reduced
livH	45C07	ABC transporter	-	08280	06300	1147	normal
phaA	05G09	K+/H+ transmembrane protein	583_0030	16460	13360	2670	normal
Cell stress response							
ibpA (*hsp20*)	86B07	chaperon	583_0614	07300	05230	1333	normal
hslO (*hsp33*)	41A03	chaperon	583_1292	01080	00990	0118	normal
Metabolism / cell integrity							
carD	41C07	transcriptional regulator factor	583_0123	15240	12150	2444	normal
glnE	70D02	glutamate ammonialigase adenyl transferase	-	4800	04000	0707	normal
ftsK	15G10	cell division transmembrane protein	583_0291	03840	02850	0572	normal
cobS	44G10	cobalamin biosynthesis	583_0080	15880	12800	2554	normal
lpcC	69H08	lipopolysaccharide core biosynthesis mannosyltransferase	583_0983	11690	09300	0746	normal
mfd	41B10	transcription repair coupling factor	583_0798	08750	05840	1197	normal
Unknown function							
	43H05	unknown function	-	03150	-	0332 0505	normal

						1229	
						1275	
						1394	
						1812	
						2614	
BA0981	65D04	putative exported protein	-	02590	02450	0286	normal
BA1484	15A08	putativemembrane protein	583_1009	11960	09380	0713	normal
BA1559	35D02	helicase/methyltransferase	-	15450	-	0164	normal
						0455	
						0541	
						1006	
						1021	
						1035	
						1053	
						1080	
						1105	
						2491	
BA1819	41C02	unknown function	-	09350	-	0466	normal
						1089	
						1090	
						2281	
						2282	
BA1566	61D04	unknown function	-	-	-	1926	normal
	05H01	conserved/putative (Tm helices) membrane protein	583_0492	05300	04480	0812	normal
	44G12	putative efflux transport protein	-	12560	-	1909	normal
Phage origin							
BA1301	86C10	putative anti-repressor protein	583_1070	06900	-	0325	normal
				02990		0355	
				03430		0372	
				03240		0373	
				03670		0431	
				03690		0470	
						0486	
						0494	
						0556	
						0557	
						0954	
						0976	
						2290	

BA1052	60B07	Putative anti-repressor protein	-	02890 03020 03250 03440 0345 03460 03470	-	2301 0475	normal
Intergenic region							
	83D04	ig	583_1301/ 583_1302	00970/ 00960	00900/ 0089	tRNA- BT000 1/BT0 082	normal
	69C09	ig, close to putative transcriptional regulator	583_1248	13590 14160 14370 14380 14970	02090	2389 2390 2397 2399 2400	normal
	04A01	ig	583_1132	13140 or 13160	10380	1660 or 1661	normal
	69B07	ig	583_0094/ 583_0093	15500/ 15510	12420/ 12430	2497/ 2504	normal
	86C05	ig	583_1019/ 583_1020	12050/ 12060	09460/ 09470	1641/ 1642	normal

Figure S1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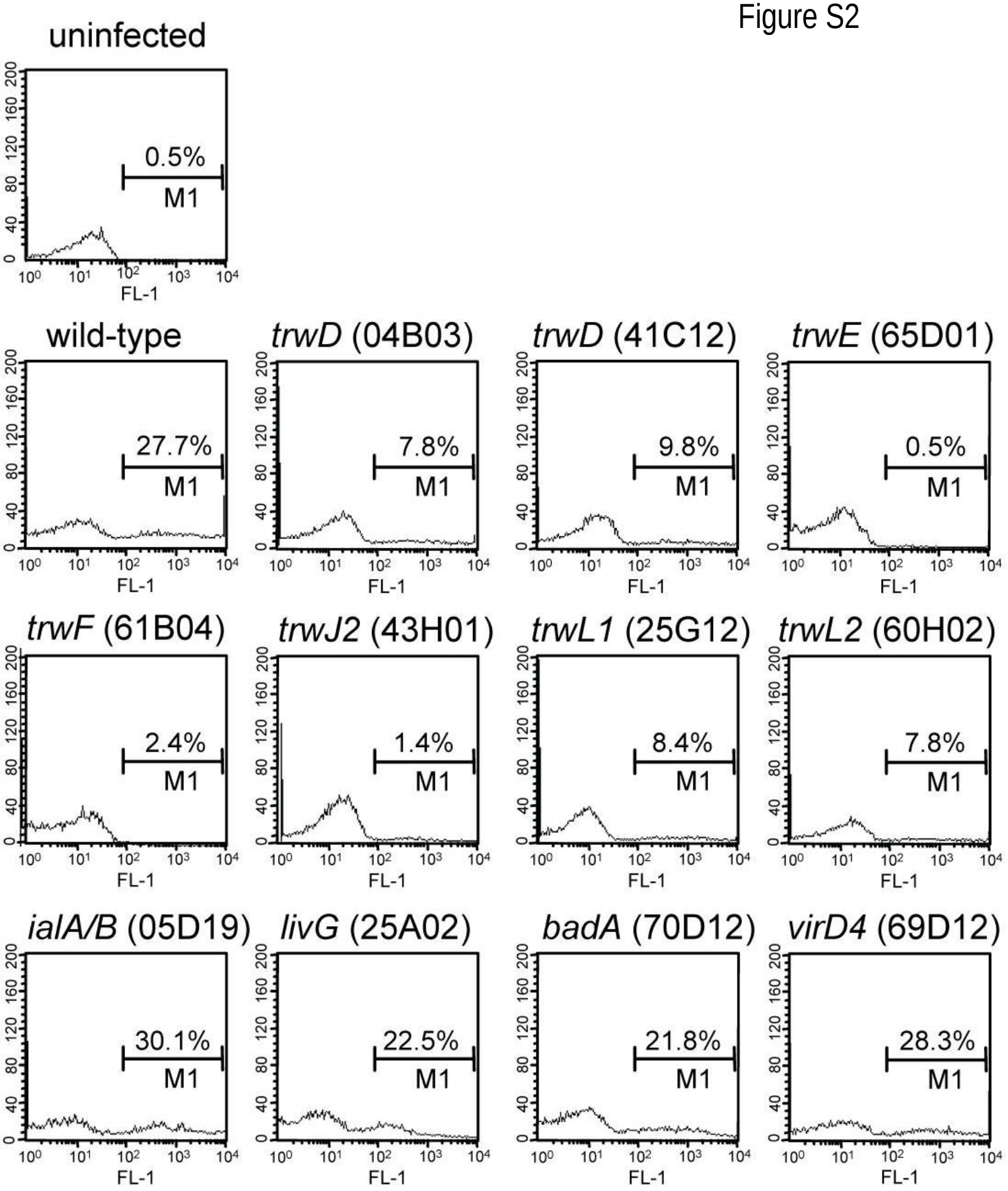
FL-1
B. arupensis-gfp
23.9%
M1
FL-1
B. berkhoffii-gfp
2.0%
M1
FL-1
B. alsatica-gfp
1.1%
M1
FL-1

Figure S2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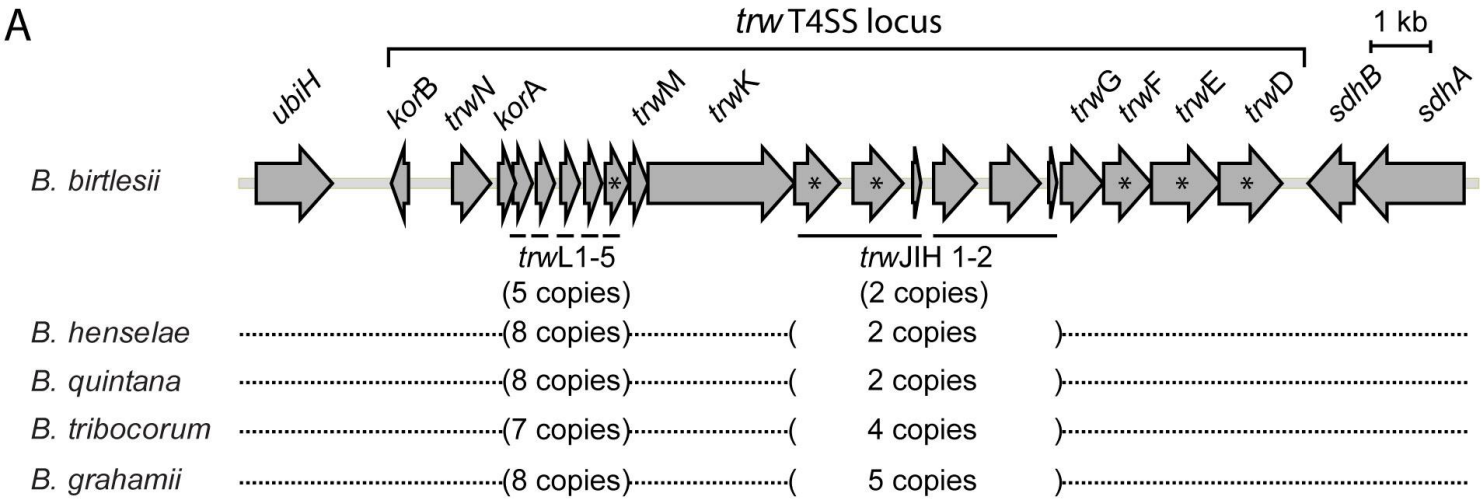
M1
wild-type
27.7%
M1
trwD (04B03)
7.8%
M1
trwD (41C12)
9.8%
M1
trwE (65D01)
0.5%
M1
trwF (61B04)
2.4%
M1
trwJ2 (43H01)
1.4%
M1
trwL1 (25G12)
8.4%
M1
trwL2 (60H02)
7.8%
M1
ialA/B (05D19)
30.1%
M1
livG (25A02)
22.5%
M1
badA (70D12)
21.8%
M1
virD4 (69D12)
28.3%
M1
FL-1

Figure S3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trw T4SS locus
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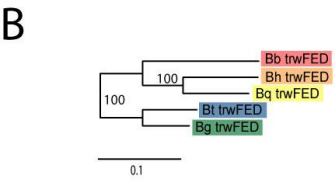
ubiH
korB
trwN
korA
trwM
trwK
trwG
trwF
trwE
trwD
sdhB
sdhA
B. birtlesii
trwL1-5
(5 copies)
trwJIH 1-2
(2 copies)
B. henselae (8 copies) (2 copies)
B. quintana (8 copies) (2 copies)
B. tribocorum (7 copies) (4 copies)
B. grahamii (8 copies) (5 copies)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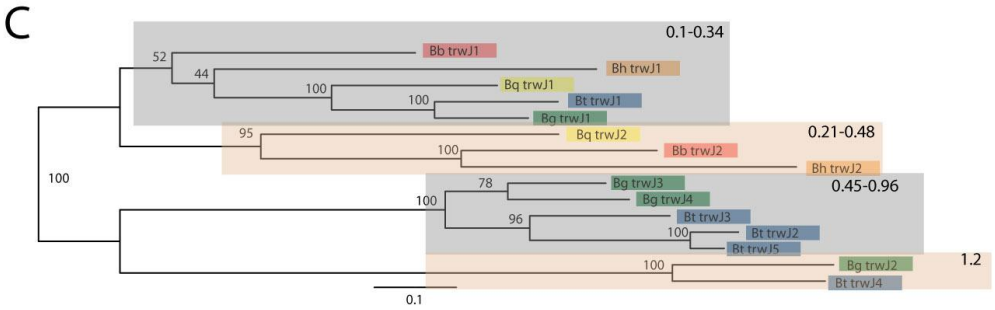
Bh trwFED
Bq trwFED
Bt trwFED
Bg trwFED


C
0.1-0.34
0.21-0.48
0.45-0.96
1.2
Bb trwJ1
Bh trwJ1
Bq trwJ1
Bt trwJ1
Bg trwJ1
Bq trwJ2
Bb trwJ2
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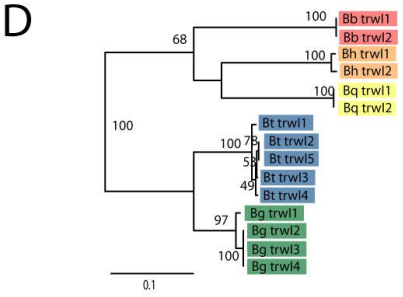
Bt trwJ4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Bg trwI2
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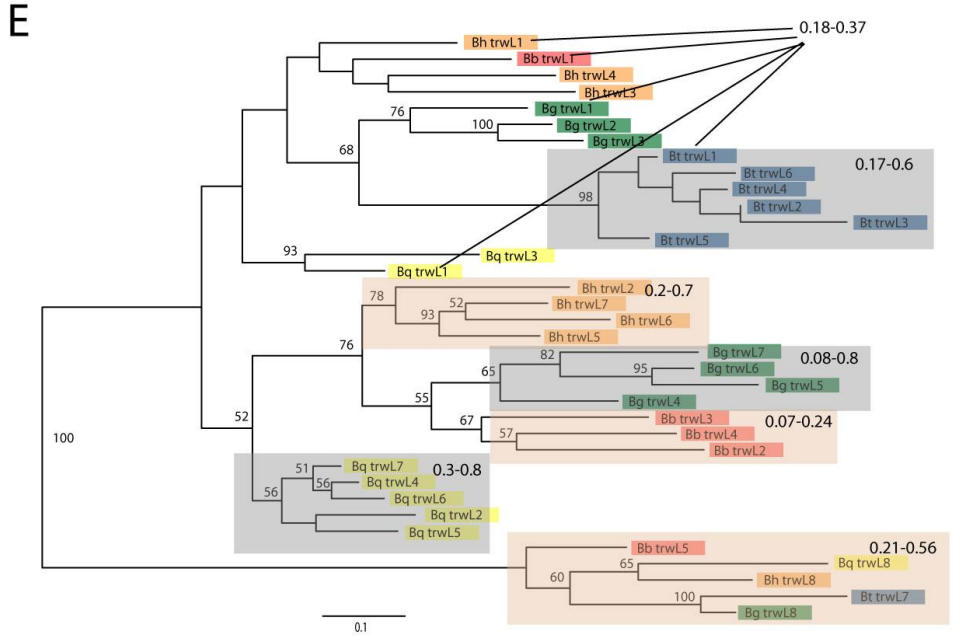
Bg trwI4


E
0.18-0.37
0.17-0.6
0.2-0.7
0.08-0.8
0.07-0.24
0.3-0.8
0.21-0.56
Bh trwL1
Bb trwL1
Bh trwL4
Bh trwL3
Bg trwL1
Bg trwL2
Bg trwL3
Bt trwL1
Bt trwL6
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Bt trwL3
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Bb trwL2
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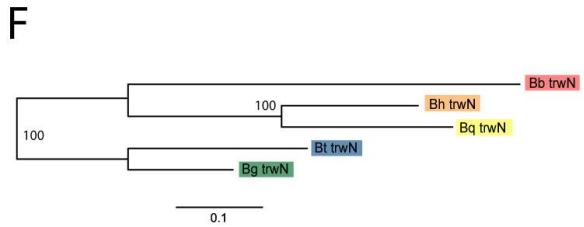
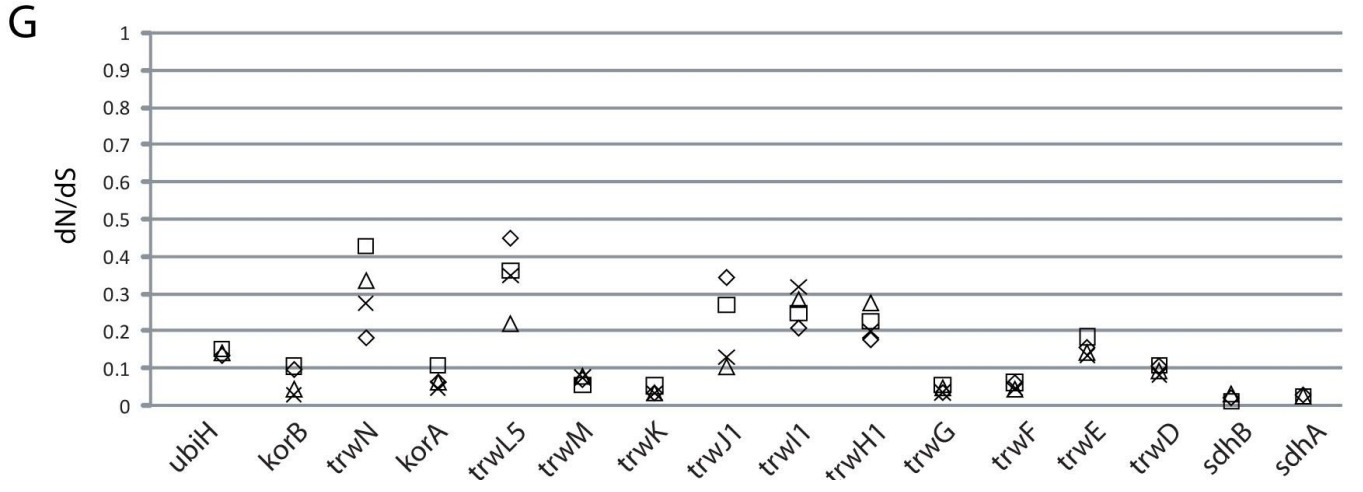

F
Bb trwN
Bh trwN
Bq trwN
Bt trwN
Bg trwN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dN/dS
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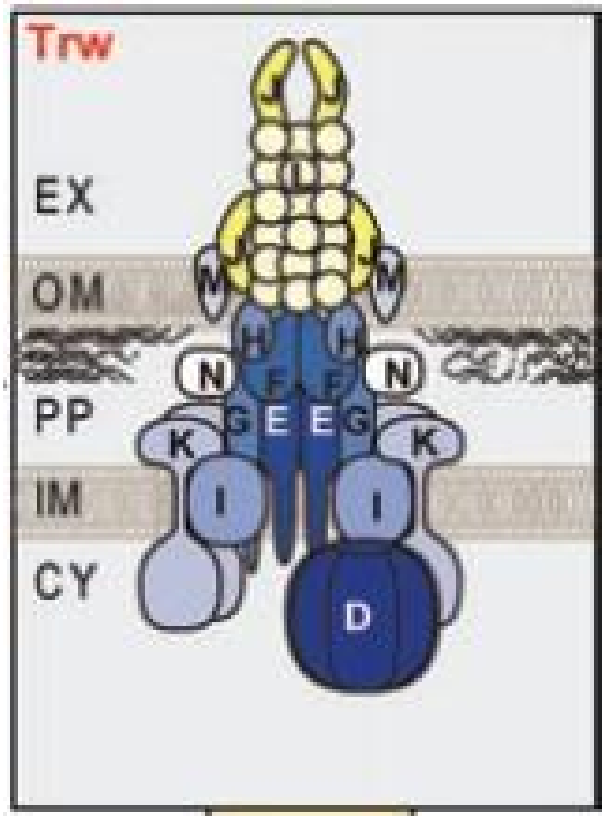
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
ubiH
korB
trwN
korA
trwL5
trwM
trwK
trwJ1
trwI1
trwH1
trwG
trwF
trwE
trwD
sdhB
sdhA
B. henselae
B. quintana
B. tribocorum
B. grahamii

CHAPTER II

Identification of *Bartonella* Trw Host- specific receptor on erythrocyte



Commentaires Article-2 (Chapitre 2)

Dans la première partie du travail de thèse, nous avons identifié le système de sécrétion de Type IV Trw de *Bartonella* spp. comme étant le complexe permettant l'adhésion de la bactérie au globule rouge.

Le système de sécrétion de Type IV Trw de *Bartonella* spp. est un complexe macro-moléculaire similaire au système de conjugaison IncW du plasmide R388 de *E. coli*. Les gènes trw de *Bartonella* spp. sont colinéaires avec leurs homologues respectifs du plasmide R388 sauf pour TrwL et TrwJ qui sont en multi-copies (nombre de multi-copies dépendant de l'espèce de *Bartonella* considérée). Ces gènes codent des variants des composants des pili. Etant donné le rôle clé du système Trw dans la reconnaissance hôte-spécifique des globules rouges, il est concevable d'imaginer que ces variants permettraient l'interaction avec le ou les récepteurs érythrocytaires. Toutefois, la nature des composants de Trw associés à l'interaction avec les érythrocytes et celle des récepteurs érythrocytaires sont encore inconnues. Dans cette étude, en combinant différentes technologies et en utilisant le modèle B. birtlesii / érythrocytes murins, nous avons identifié que, parmi les composants trw, seuls TrwJ1 et TrwJ2 sont exprimés à la surface bactérienne et pouvait se lier à la membrane érythrocytaire via la glycoprotéine transmembranaire majeure : la Band-3.

Cette glycoprotéine a déjà été impliqué dans l'adhésion de *B. bacilliformis* et de *Plasmodium* spp. aux globules rouges suggérant un rôle essentiel dans la reconnaissance des globules rouges par divers agents pathogènes à tropisme érythrocytaire.

ARTICLE-2

Identification of *Bartonella* Trw Host- specific receptor on erythrocyte

In Revision to PLoS ONE

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Identification of *Bartonella* Trw Host-specific receptor on erythrocyte

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Abstract

Each *Bartonella* species appears to be highly adapted to one or a limited number of reservoir hosts, in which it establishes long-lasting intraerythrocytic bacteremia as the hallmark of infection. Recently, we identified Trw as the bacterial system involved in recognition of erythrocytes according to their animal origin. The T4SS Trw is characterized by a multiprotein complex that spans the inner and outer bacterial membranes, and possesses a hypothetical pilus structure. *TrwJ, I, H* and *trwL* are present in variable copy numbers in different species and the multiple copies of *trwL* and *trwJ* in the *Bartonella trw* locus are considered to encode variant forms of surface-exposed pilus components. We therefore aimed to identify which of the candidate Trw pilus components were located on the bacterial surface and involved in adhesion to erythrocytes, together with their erythrocytic receptor. Using different technologies (electron microscopy, phage display, invasion inhibition assay, far western blot), we found that only TrwJ1 and TrwJ2 were expressed and localized at the cell surface of *B. birtlesii* and had the ability to bind to mouse erythrocytes, and that their receptor was band3, one of the major outer-membrane glycoproteins of erythrocyte, (anion exchanger). According to these results, we propose that the interaction between TrwJ1, TrwJ2 and band 3 leads to the critical host-specific adherence of *Bartonella* to its host cells, erythrocytes.

Introduction

Bartonella species (*Bartonella* spp.) are small, curved, pleomorphic, fastidious, hemotropic, Gram-negative bacteria, mainly transmitted by arthropod vectors or via direct contact [1]. Until now, 24 species or subspecies, 13 of which being involved in human disease, have been formally validated [2]. Each of them appears to be highly adapted to a limited number of mammalian reservoir hosts, which results in relatively strict host specificity [1, 3].

Bartonella infection can cause many human and animal diseases. For example, *B. bacilliformis* causes Carrión's disease, *B. quintana* causes trench fever and *B. henselae* causes a variety of clinical manifestations in humans: the main disease in immunocompetent individuals is cat scratch disease (CSD), whereas in immunocompromised patients it causes bacillary angiomatosis (BA) and bacillary peliosis (BP).

Bartonella spp., along with *Plasmodium spp.*, *Babesia spp.* and *Anaplasma marginale,* is one of the few infectious agents to infect erythrocytes [4]. The remarkableness, in contrast to other infectious agents infecting erythrocytes, is that all *Bartonella* spp. described to date, with the exception of the deadly *B. bacilliformis*, are maintained within the erythrocytes without having a significant effect on their physiology [5].

The dynamics of erythrocyte infection have been monitored in rats infected with fluorescently labelled *B. tribocorum*. After a primary phase, corresponding to the infection of a still unknown primary niche, potentially vascular endothelial cells [5-10] or erythrocytic precursors [11]), *Bartonella* spp. reached the blood stream where they adhered to and invaded mature erythrocytes within 2 days. After infection, intracellular replication started immediately in a membrane-bound compartment, continuing over a period of several days until

a steady number of intracellular bacteria was reached, the infected erythrocytes persisting in circulation for several weeks [5].

Bartonellae play an active role during erythrocyte invasion requiring both respiration and proton motive force [12], whereas treatment of erythrocytes with proton-motive force inhibitors has no effect on *Bartonella* adhesion. This suggests that erythrocytes play a passive role in invasion [13-15] and that *Bartonella* spp. are the main active participants in erythrocyte invasion.

The successful infection of a mammalian reservoir host erythrocyte by a *Bartonella sp.* typically involves a series of intimate host-pathogen interactions. On the molecular level this is reflected by attachment between *Bartonella* ligands and the erythrocyte receptors. The flagella of *B. bacilliformis* was identified to mediate initial erythrocyte adhesion [12]. This was supported by the reduction of the erythrocyte-binding ability of *B. bacilliformis* with anti-flagellin antibodies [16], and the poor adherence of non-motile variants and flagellin-minus mutant [17, 18]. Erythrocyte receptors for attachment to flagella have been partially characterized for *B. bacilliformis*. Buckles and McGinnis hill [19] demonstrated that *B. bacilliformis* was able to bind to several erythrocyte proteins: α and β subunits of spectrin, band 3 protein, glycophorin A, and glycophorin B. In addition, Iwaki-Egawa and Ihler [20] demonstrated that spectrin, actin and the other potential erythrocyte membrane proteins from different sources (human, cat, sheep) were able to bind to *B. bacilliformis* and *B. henselae*.

However, within the *Bartonella* genus, 13 *Bartonella spp.* are represented as a major phylogenic sub-branch of flagella-free *Bartonella*. All these flagella-free *Bartonella* possess a Trw Type 4 Secretion System (T4SS). T4SSs are supra-molecular transporters ancestrally related to bacterial conjugation [21]. In *Bartonella* spp., 2 T4SS, the VirB/D4 and Trw have been described and

identified as pathogenicity factors required for bacterial colonization [22, 23]. Interestingly, the distribution of Trw and flagella among *Bartonella* spp. is mutually exclusive suggesting that, after its acquisition by horizontal transfer, the function of Trw evolved to replace that performed by flagella. In a recent study, using an *in vitro* model of erythrocyte adherence and invasion we demonstrated the direct role of Trw in erythrocyte recognition [23].

The *trw* genes of *Bartonella* species are collinear except for the presence of multiple tandem gene duplications of *trwL* and *trwJIH*. The multiple copies of *trwL* and *trwJ* are considered to encode variant forms of surface-exposed pilus components which are postulated to have a role in host-interaction with various surface structures of erythrocytes in different species. In contrast, the other duplicated genes, *trwI* and *trwH* are considered to encode the components required for pilus elongation and for pilus anchorage to the outer membrane, respectively [24].

Although the Trw locus has been identified as one of the *Bartonella* spp. factors involved in erythrocytic host-specific recognition, which of the Trw components are associated with the attachment, and the identity of the erythrocytic receptors are still unknown. In this study, combining different technologies and using the *B. birtlesii* / mouse erythrocytes model, we first identified that among the Trw components, only TrwJ1 and TrwJ2 were expressed at the bacterial surface and could bind to the erythrocyte membrane. Using Far Western blot we identified the major erythrocyte transmembrane glycoprotein Band3 as the receptor of the type IV TrwJ component.

Material and Methods

Bacterial strains and growth conditions

Bartonella birtlesii (*B. birtlesii*) (IBS 325^T, CIP 106691^T) were grown for 5 days on Columbia agar containing 5% defibrinated sheep blood (CBA) in a humidified atmosphere with 5% CO_2 at 35°C.

E.coli TOP10 (Invitrogen, USA), BL21 Star (Invitrogen, USA) and BL21 pLysS (Novagen, Germany) were grown overnight in Luria-Bertani (LB) broth or on LB agar plates supplemented when needed with carbenicillin (50µg/mL) at 37°C.

Animals and Ethics statement

Animals were handled in strict accordance with good animal practice as defined by the relevant European animal welfare body. Animal work was approved by our institute's ethics committee. The protocol was approved by the Committee on the Ethics of Animal Experiments of the National Veterinary School of Alfort (Permit Number: 08/03/11-3).

Six-week old OF1 or Balb/C female mice were housed in an animal facility (5 mice per cage) for blood sampling or immunization with recombinant proteins. White New Zealand male rabbits (16 weeks old) were used to produce polyclonal antibodies against murine band 3 extracted from erythrocytic membrane.

Isolation of erythrocytes

Erythrocytes from the peripheral blood of mice were isolated and purified by Ficoll gradient centrifugation as previously described [25]. After washing in PBS, erythrocytes were maintained in F12 modified medium (F12 medium supplemented with 10% foetal calf serum, 2mM glutamine, 1mM sodium pyruvate, 0.1mM Hepes, 257mM Histidine (His), 0.1mg/ml Hematin/His, and non-essential amino acid) (Gibco, France) before being used for further

analysis (erythrocyte invasion assay, phage binding assay).

Trw proteins expression and purification

Genomic DNA was isolated from *B.birtlesii* using the Roche high pure PCR template preparation kit (Roche, Switzerland). Based on the entire *trwJ1, trwJ2, trwL1, trwL2 ,trwL3, trwL4* and *trwL5* sequences (F. Biville, unpublished data), DNA inserts corresponding to *trw* genes were amplified by PCR from genomic *B. birtlesii* as template and the corresponding specific primers (shown in table 1). PCR consisted of an initial denaturation step at 98°C for 2 min followed by 30 cycles of denaturation at 98°C for 20s, annealing at 55°C for 50s and extension at 72°C for 50s, and a final extension step at 72°C for 10min. All PCR reactions were performed in a MyCycler™ thermocycler (Biorad, USA) with the Phusion high-fidelity DNA polymerase (New England Biolabs, USA).

PCR products were ligated to the PET-102 expression vector (Invitrogen, USA). This vector allows expression of recombinant protein containing a thioredoxin epitope followed by an enterokinase recognition site at the N-terminal end and a 6-His tag at the C-terminal end. After propagation of the recombinant plasmids in *E.coli* TOP10, they were then transformed into BL21 Star and BL21 pLysS by electroporation. Expression was obtained for *trwJ1* and *trwJ2* in *BL21 Star* incubated with 0.5mM IPTG (isopropyl β-D-thiogalactoside) for 4 hours at 22°C, and for *trwL2*, *trwL3*, *trwL4*, and *trwL5* in *BL21 pLysS* incubated with 0.5mM IPTG for 4h at 37°C. As TrwJ1 was recovered in inclusion bodies, an 8M urea treatment was performed for this protein before purification.

The recombinant fusion proteins were purified by affinity chromatography using the nickel-nitrilotriacetic acid (Ni-NTA) resin following the manufacturer's protocol (Qiagen, Germany) under native conditions or denaturing conditions according to their properties. For mice immunization, the thioredoxin parts of the recombinant proteins were cut off by enterokinase (Invitrogen, USA). The digestion reactions were performed in the Ni-NTA-protein mixture overnight at

37°C, under shaking, in 1ml containing 10X enterokinase buffer and 25U of enterokinase and were followed by 3 washes in PBS. In each case the recombinant proteins were eluted from the resin in 400µl elution buffer (300mM NaCl, 50mM NaH_2PO_4, 250mM imidazole, pH 8.0).

The purified recombinant proteins were analyzed by a 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), followed by gel staining with Coomassie brilliant blue R-250 (Sigma, USA). The SigmaMarker™ low range (Sigma; USA) was used as reference for the molecular weights.

Production of murine polyclonal antibodies against recombinant Trw proteins

Balb/C mice were injected twice subcutaneously with 10µg of each recombinant protein mixed in oil Montanide® adjuvant ISA-70 (Seppic, France) at 2-weeks-interval with the same antigen dose. Sera were collected 15 days after the last immunization and stored at -20 °C. The titres of polyclonal antibodies were determined by dot-blot analysis using the corresponding recombinant proteins.

Western blot (WB) analyses

B. birtlesii (1.10^8 UFC from 5 days growth on CBA plates) and 0.1 µg of rTrwJ1, rTrwJ2, rTrwL2, rTrwL3, rTrwL4, rTrwL5 recombinant proteins were reduced with 100mM DTT, resolved by a Tris-Glycine 15% SDS-PAGE gel and blotted onto PVDF membranes (GE Healthcare, UK) at 15V for 12min by Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell Instruction (Biorad, USA) in Towbin transfer buffer (25mM Tris, 192mM glycine, 20% methanol, pH 8.3). The PVDF membranes were blocked in 1X blocking buffer (50mM Tris, 150mM NaCl pH 7.4 and 0.05% Tween-20, 5% non-fat dried milk) for 1h at 37°C and then incubated with 1/1000 dilution of mouse anti-Trw proteins polyclonal antibodies for 1h at 37°C in blocking buffer. Anti-Trw labelling assays were revealed with an anti-mouse IgG (H+L) alkaline phosphatase (AP)-goat antibody (1:10,000;

Jackson ImmunoResearch Laboratories, USA) for 1h at 37°C, and a 10ml solution of NBT (Nitro blue tetrazolium chloride)/BICP (5-Bromo-4-chloro-3-indolyl-phosphate p-toluidine salt) (Sigma, Germany).

Electron microscopy and immunolocalization of Trw components

Pellets of bacteria were fixed for 30 min with 2% paraformaldehyde solution in PBS, then centrifuged and washed in PBS. The bacteria were collected onto 400 mesh formvar-coated nickel grids. Grids were quenched with NH4Cl 50mM in PBS, blocked with PBS containing 1% BSA, and 0.1% BSA-c™ (BioValley, France). Antibodies (anti-TrwJ1, anti-TrwJ2, anti-TrwL2-L5, naïve mouse serum) were added at a 1/100 dilution in PBS containing 1% BSA, 0.1% BSA-c™ and incubated over night at +4°C. The grids were then washed twice for 3 min in PBS -1% BSA, 0.1% BSA-c™ and goat anti-mouse IgG (1/50 dilution) coupled to 10 nm colloidal gold particles (British Biocell International – TEBU, France) added for 1 hour. The grids were again washed twice with PBS -BSA, twice with PBS, and fixed for 5 min with 2.5% glutaraldehyde in PBS. Finally, the grids were washed three times with distilled water and air dried.

The grids were then examined with a Zeiss EM902 electron microscope operated at 80kV (Carl Zeiss – France), and images were acquired with a charge-coupled device camera (Megaview III) and analysed with ITEM Software (Eloïse, France) MIMA2 Platform, INRA-CRJ (http//MIMA2@jouy.inra.fr)

Expression of TrwJ1 and TrwJ2 on T7 phage

The T7 select 10-3b Cloning kit (Novagen, Germany) containing the T7 select 10-3b *EcoRI*/*HindIII* vectors and T7 packaging extracts was used to display TrwJ1 and TrwJ2 on T7 phage. Briefly, *trwJ1* and *trwJ2* genes were amplified by PCR using the specific primers described in table 1. To allow insertion in T7 phage sequences, the forward primer contained an *EcoRI* restriction enzyme site while the reverse primer contained a *HindIII* restriction enzyme site.

After PCR amplification, the PCR products were digested by *EcoRI* (TaKaRa, Japan) and *HindIII* (TaKaRa, Japan) and purified by PCR clean-up Gel extraction Kit (MACHEREY-NAGEL, Germany), before being packaged, titered and amplified following the procedures outlined in T7Select system.

Phage binding assay with mouse erythrocytes

Mouse erythrocytes were resuspended in PBS at 1×10^8 cells/ml, and incubated with 1×10^9 PFU TrwJ1-T7 or TrwJ2-T7 phages with shaking for 4 hours at 35°C. The bound phages were separated as previously described with slight modification [26]. Briefly, 300µl of the cell-phage mixtures were gently transferred to the top of a non-miscible dibutyl phthalate/cyclohexane (9:1 [v:v]) organic lower phase (600µl) and centrifuged at 10,000g for 10 min. The supernatants were drawn-off. Bound phages were eluted from cells for 10 minutes at room temperature by adding 500µl of 1% SDS. The titres of the bound phages were then determined by following the procedures outlined in T7Select system.

Effect of anti-Trw antibodies on *in vitro* infection of mouse erythrocytes by *B. birtlesii*

The effect of the different mouse anti-Trw antibodies on the invasion capacity of erythrocytes by *B. birtlesii* was measured *in vitro* as described [23]. Briefly, after culturing *B. birtlesii* for 5 days on CBA plates, the bacteria were harvested, washed in PBS and suspended in F12 modified medium. Anti-Trw antibodies (1/100 dilution) or serum from a non-immunized mouse (1/100 dilution) were then incubated with bacteria at 35°C for 4h, while the control was incubated with F12 modified medium. In each case, bacteria were then added to mouse erythrocytes at a multiplicity of infection (MOI, calculation based on 1 OD_{600} nm=3×10^9 bacteria /ml) of 1 and incubated at 35°C. After 48h of invasion, the erythrocytes were separated from the non-associated bacteria by washing 3 times with PBS and centrifuged at 1500rpm for 10 min. The erythrocytes were

then incubated with 50µl gentamicin sulfate (125µg/ml) for 2h at 35°C to kill any residual extracellular bacteria, washed three times in PBS to remove the antibiotic and then any intracellular bacteria were released by hypotonic lyses of the erythrocytes in 20µl of sterile water by freezing at -20°C for 15 min. After thawing, serial dilutions of bacteria in PBS were inoculated onto CBA plates and incubated for 5 days before being counted. The impact of anti-Trw antibodies on invasion capacity was then evaluated by comparing the numbers of intra-erythrocytic bacteria with or without antibodies.

Identification of TrwJ2 erythrocytic receptor by Far-Western blot

Lysates of erythrocytic membranes were prepared from frozen blood (5×10^9 erythrocytes) samples, thawed, resuspended in stabilization solution (ID-CellStab, Diamed) and washed in 0.9% NaCl (B. Braun Medical). Membranes were prepared at 0-4°C by hypotonic lysis with 5P8 buffer (5mM Na_2HPO_4, pH8.0 and 350µM EDTA, pH 8.0), stripped by incubation with 10mM NaOH and finally solubilized with an equal volume of 4X LDS Sample buffer (Invitrogen). Erythrocytic membrane lysates were reduced with 100mM DTT, resolved by Tris-Glycine 8% SDS-PAGE and transferred onto PVDF membranes at 20V for 25min by Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell. The PVDF membranes were blocked in 1X blocking buffer and incubated for 2 hours at RT with 200µg of rTrwJ2 in 10ml blocking buffer, immunodetected with 1/1000 dilution of anti-TrwJ2 polyclonal antibodies and 1/10,000 dilution of AP-goat anti-mouse IgG (H+L) (1/10,000), then stained with a solution of NBT/BICP as above. The PVDF membranes were similarly reprobed with a 1/100 dilution of anti-band3 (C-17) monoclonal antibodies (Santa Cruz Biotechnology, USA) and a 1/30,000 dilution of AP-goat anti-donkey IgG (Jackson ImmunoResearch Laboratories, USA), and stained with a solution of NBT/BICP as above.

Inhibition of *Bartonella*-erythrocytes interaction using anti-band 3

polyclonal antibodies

As commercially available anti-band3 antibodies did not recognize all the surface part of band3, polyclonal antibodies raised against the entire sequence of murine erythrocytic band3 were produced as follows: lysates of erythrocytic membrane were resolved by Tris-Glycine 8% SDS-PAGE gels, the band corresponding to the size of band3 (90kDa) was cut from the gels, grinded and resuspended in PBS.

Two rabbits were injected subcutaneously with 200µg of murine erythrocytic band 3 mixed in Montanide® oil adjuvant ISA-70. Second and third injections were given at 2-week-interval with the same antigen dose. Sera were collected 15 days after the final immunization and stored at -20 °C. The titres of polyclonal antibodies were determined by dot-blot analysis using the purified erythrocytic band3 protein.

The effect of anti-band3 polyclonal antibodies on the interaction between TrwJ1-T7 or TrwJ2-T7 phages and mouse erythrocyte was assessed by incubating 1×10^8 mouse erythrocytes with anti-band3 antibodies (1/100 dilution) or serum from a non-immunized rabbit (1/100 dilution) at 35°C for 4h, while the control was incubated with PBS. Then 1×10^9 PFU of TrwJ1-T7 or TrwJ2-T7 phages were added and phage binding assays with mouse erythrocytes were performed as described above.

In parallel, the effect of anti-band3 polyclonal antibodies on the invasion capacity of erythrocytes by *B. birtlesii* was measured by incubating anti-band3 antibodies (1/150 dilution) or serum from a non-immunized rabbit (1/150 dilution) in *B. birtlesii*-erythrocyte mixture at 35°C for 4h, while the control was incubated with F12 modified medium. The intracellular bacteria were quantified as described above.

Results

1- Identification of Trw components that are expressed at the *B. birtlesii* cell surface:

Candidate genes for mediating Trw interaction with erythrocytic receptors encoded surface-exposed components. Among the Trw components, the T4SS pilus components TrwL (L1 to L5) and TrwJ (J1 and J2) were shown to be putative surface proteins [27]. We checked whether they were indeed expressed at the *B. birtlesii* surface by first producing polyclonal antibodies which reacted specifically with each of the corresponding recombinant proteins.

Recombinant soluble proteins rTrwJ2, rTrwL2, rTrwL3, rTrwL4 and rTrwL5 were expressed and recovered from the supernatant of lysated *E. coli*, while recombinant rTrwJ1 was recovered as an insoluble form in the inclusion body of *E. coli*. Despite many assays using different *E. coli* strains and different culture conditions, we failed to express rTrwL1.

After purification, a single band corresponding to each purified recombinant protein was identified on SDS-PAGE on a Coomassie stained acrylamide gel. The observed molecular mass corresponded to the predicted size of the recombinant protein with the addition of 13 kDa corresponding to the thioredoxin motif and 3 kDa corresponding to the V5 and 6×His-tag motifs, i.e. 43.5 kDa for rTrwJ1, 42 kDa for rTrwJ2, 23.5 kDa for rTrwL2, 23.5 kDa for rTrwL3, 23.5kDa for rTrwL4 and 23.5 kDa for rTrwL5 (Figure 1A).

The thioredoxin-free recombinant proteins were used to produce polyclonal antibodies from immunized Balb/C mice. The obtained polyclonal antibodies reacted with the corresponding recombinant proteins as shown in Figure 1B.

Recognition of native proteins by antibodies was then evaluated by western blot on proteins extracted from *B. birtlesii* culture on agar plates and separated on SDS-PAGE. As shown in Figure 1C, only TrwJ1 and TrwJ2 were detected, while Trw L2, L3, L4, L5 were not (Figure 1C). The molecular mass observed

for TrwJ1 corresponded approximately to the one calculated from the sequence (27.5 kDa). On the contrary, the molecular mass observed for TrwJ2 was higher than was expected from the sequence (26 kDa) with signal peptides around 32kDa. This difference would suggest the presence of aggregates or post-translational modifications.

The different putative Trw surface components were localized by immunostaining *B. birtlesii* whole bacteria with the different anti-Trw polyclonal antibodies and electron microscopy observations. TrwJ1 and TrwJ2 were detected at the *B. birtlesii* cell surface whereas none of the TrwL proteins was detected at the cell surface (Figure 2).

2 – TrwJ1 and TrwJ2 interact with erythrocytes

Two complementary analyses were performed to see whether TrwJ1 and TrwJ2 were able to bind to mouse erythrocytes: the first analysis consisted of measuring the capacity of TrwJ1-T7 as well as TrwJ2-T7 phages to bind to mouse erythrocytes; the second consisted of evaluating the capacity of anti-TrwJ1 and anti-TrwJ2 polyclonal antibodies to inhibit erythrocyte invasion by *B. birtlesii.* As shown in Figures 3A and 3B, T7 phage displaying *B. birtlesii* TrwJ1 and TrwJ2 were able to bind to mouse erythrocytes. The amounts of TrwJ1-T7 and TrwJ2-T7 phages which were able to bind to 1×10^8 mouse erythrocytes were 1.7×10^7 PFU and 2×10^7 PFU respectively, while wild-T7 phages were unable to bind to mouse erythrocytes. Addition of anti-TrwJ1 and anti-TrwJ2 polyclonal antibodies to the *B. birtlesii*-erythrocytes invasion mixture, significantly reduced the invasion of mouse erythrocytes by *B. birtlesii* by 60% and 55.7% respectively, while serum from a non-immunized mouse did not reduce invasion.

3- Identification of erythrocytic receptor of TrwJ2

As TrwJ1 was expressed as an insoluble form, identification of the receptor was only conducted with TrwJ2 by Far-Western blotting. When recombinant

rTrwJ2 is incubated with mouse erythrocytic membrane proteins, antibodies against TrwJ2 react with a single band which corresponds to a mouse erythrocytic membrane protein with an estimated size of 90 kDa (Figure 4A). This band corresponds to the size of the erythrocytic band3 as validated by immunoblot with goat anti-band3 monoclonal antibody (Figure 4B).

To further demonstrate that band 3 was or was not a receptor of *B. birtlesii* TrwJ, we then evaluated the capacity of rabbit anti-band3 polyclonal antibodies to inhibit erythrocyte binding with *B. birtlesii* TrwJ1-T7 or TrwJ2-T7 phages and erythrocyte invasion by *B. birtlesii*. As shown in Figures 5A and 5B, adding anti-band3 polyclonal antibodies to the TrwJ1-T7 or TrwJ2-T7 phages-erythrocytes binding mixture significantly reduced phage binding capacity by 62% and 64% respectively, and significantly reduced *B. birtlesii* invasion capacity by 62%, while serum from non-immunized rabbit had only a very slight influence on both adherence and invasion.

Discussion

Bacteria-specific adhesion to host cells can be defined as the selective binding between a specific molecular component on the bacterial surface and a substratum-specific receptor in the host cells. We have previously shown that in *Bartonella* species, the T4SS Trw is involved in erythrocytic recognition [23]. T4SS Trw is characterized by a multiprotein complex that spans the inner and outer bacterial membranes, and possesses a hypothetical pilus structure. *TrwJ* and *trwL* are thought to encode minor and major pilus components, which are considered to be potentially responsible for the interaction with erythrocyte [27]. The aim of the present study was therefore to evaluate the role of TrwJ and TrwL proteins in adhesion to erythrocytes.

B.birtlesii trwJ1, *trwJ2*, *trwL2*, *trwL3*, *trwL4* and *trwL5* were expressed as recombinant proteins and polyclonal antibodies against these proteins were produced, firstly to estimate their expression and secondly to localize them on the cell surface of the bacteria. Immunoblot analysis showed that only TrwJ1 and TrwJ2 could be detected on CBA-cultivated *B. birtlesii*, and not TrwL2, L3, L4, or L5, suggesting that these latter were not expressed in *in vitro* culture, or at an undetectable level. Some studies showed that homologues of TrwJ (VirB5) and TrwL (VirB2) were not detected in wild and complemented *Agrobacterium tumefaciens*, whereas they could be detected in induced complemented cells [28-31]. Similarly the homologue of VirB5 was not detected in *B. henselae* cultivated on cell-free laboratory medium but could be detected in bacteria incubated with HMEC-1 cells [32]. The Trw T4SS has also been identified as being upregulated intracellularly during *B. henselae* interaction with HUVECs or ECs [8]. Finally, *Bartonella* has the ability to infect different hosts (reservoir or incidental mammalian as well as arthropod hosts), and different host cell types, which suggests the existence of different pathogenicity factors on its surface that are presumably controlled by differential gene expression during the course of infection. These findings

suggest that the expression of TrwL, like that of its homologues in other bacteria, might be regulated in response to infection signals, although further work is necessary to unravel the molecular details of this mechanism.

Results obtained by immunoblot analysis were then confirmed by electron microscopic analysis as only Trw J1 and TrwJ2 were detected at the cell surface of *B. birtlesii*. For these reasons, TrwJ1 and TrwJ2 appeared to us as the best potential candidates for *in vitro* interaction between *B. birtlesii* and erythrocytes.

We then investigated whether the surface Trw components TrwJ1 and TrwJ2 were associated with the adherence to erythrocytes, by constructing phage displaying *B. birtlesii* TrwJ1 and TrwJ2. The results showed that *B. birtlesii* TrwJ1-T7 and TrwJ2-T7 phages were able to bind to mouse erythrocytes, while wild-T7 phages showed significantly less binding ability. We then confirmed this result by evaluating the capacity of anti-TrwJ1 and anti-TrwJ2 polyclonal antibodies to inhibit erythrocyte invasion by *B. birtlesii.* We found that incubation with both polyclonal antibodies resulted in inhibition of the invasion of mouse erythrocytes by *B. birtlesii.* These results clearly suggest that TrwJ1 and TrwJ2 are associated with adherence of the bacteria to erythrocytes. As we have previously shown that the Trw T4SS of *Bartonella* mediates host-specific adhesion to erythrocytes, and that *B. birtlesii* is unable to bind and invade cat erythrocytes [23], we performed the same experiment, using cat erythrocytes and showed that *B. birtlesii* TrwJ1-T7 and TrwJ2-T7 phages were not able to bind to cat erythrocytes (data not shown). These results enlarged on those obtained in earlier studies of the relationship between Trw and the host erythrocyte, and now suggest that this host-specific adhesion is mediated by TrwJ1 and TrwJ2.

Although TrwL were not detected on the surface of bacteria, this does not

exclude an interactive role between the bacteria and their host cells. Indeed, after adherence, bacteria use other virulence factors to become more intimately bound to their host cells via specific and stable interactions that can mediate invasion [33]. Mutagenesis of TrwL is reported to lead to inhibition of intraerythrocytic bacteremia in the reservoirs of for *B. tribocorum* and *B. birtlesii* [22, 23], and to loss of the capacity of *B. birtlesii* to infect mouse erythrocyte *in vitro* [23] thus demonstrating that TrwL also has an essential role in erythrocyte invasion, both *in vivo* and *in vitro*. However, in the absence of direct proof, to suggest that TrwL is involved in intimate adhesion rather than in the initial adhesion occurring during infection of the erythrocytes, remains speculative.

By conducting experiments to determine the receptor of TrwJ1 and TrwJ2, we found that TrwJ2 recombinant protein was able to bind a major glycoprotein present in mouse erythrocyte membrane: band3. We also demonstrated that, *in vitro*, polyclonal antibodies raised against mouse Band-3 were able to inhibit the adhesion between TrwJ1-T7 and TrwJ2-T7 phages and mouse erythrocytes and reduce the mouse erythrocyte invasion capacity of *B. birtlesii*. Taken together, all these results clearly suggest an interaction between TrwJ1, TrwJ2 and Band 3 leading to critical adherence of the bacteria to its host cells, the erythrocytes.

Band 3 is a major transmembrane glycoprotein of the erythrocyte membrane and functions in anion transport [34]. It has been suggested to be one of the possible erythrocyte receptors of *B. bacilliformis* [19]. Erythrocytic band 3 has also been suggested to be involved in the malaria parasite invasion of erythrocytes [35-40]. In addition, recent studies have revealed that *P. falciparum* merozoite surface protein 1 (MSP1), an essential parasite protein has a conserved role in the invasion of erythrocytes by *P. falciparum* and *P. chabaudi* [41, 42] and this protein interacts with two nonglycosylated exofacial regions of erythrocyte band 3, designated 5ABC (amino acids 720-761) and 6A (amino acids 807-826) [43]. Two regions of merozoite surface protein 9

(MSP9), which is also known as an acidic basic repeat antigen, interact directly with 5ABC during erythrocyte invasion by *P. falciparum* [44, 45]. Erythrocyte invasion by *P. falciparum* is thought to proceed via two distinct pathways [46, 47]: a sialic acid-dependent pathway mediated by glycophorin A, B and C [48-52], and a sialic acid-independent pathway mediated by band3, as described above. Concerning *Bartonella*, a previous study showed that pre-treatment of feline erythrocytes with neuraminidase and trypsin had no effect on *B. henselae* invasion, indicating that invasion occurs via a sialic acid-independent pathway [53]. As we have identified band 3 as the erythrocyte receptor of *Bartonella*, we attempted to determine whether or not the sialic acid-dependent erythrocyte receptors of *P. falciparum* were also involved in *Bartonella* infection. Preliminary results demonstrated that the anti-mouse N-terminal extracellular domain of glycophorin A polyclonal antibodies reduced invasion of mouse erythrocytes by *B. birtlesii* by approximately 50% (data not shown). This result provides additional informations, which allows us to hypothesize that *Bartonella*-erythrocyte interactions may also be mediated by two distinct pathways, and expands our understanding of the biology and infection course of *Bartonella* spp., which is still far from completely understood. Further studies are needed to elucidate the complete mechanisms involved in erythrocyte invasion by *Bartonella* spp.

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Figure legends:

Figure 1: Expression and detection of the putative surface Trw components.

A - SDS electrophoresis of the purified recombinant Trw proteins.

SDS-PAGE analysis under reduced condition of Trw recombinant proteins expressed in *E. coli* after purification by affinity chromatography and without elimination of the thioredoxin epitope. Lane 1, Low range molecular weight (Sigma); Lane 2, rTrwJ2; Lane 3, rTrwJ1; Lane 4, rTrwL5; Lane 5, rTrwL4; Lane 6, rTrwL3; lane 7, rTrwL2; Lane 8, rTrwL1.

B - Western blot detection of the purified recombinant Trw proteins

Immunoblot analysis of Trw recombinant proteins expressed in *E. coli* after purification by affinity chromatography, without elimination of the thioredoxin epitope, and after separation on SDS-PAGE under reduced conditions, using polyclonal antibodies against rTrwJ2 (lane 2), rTrwJ1 (lane 3), rTrwL5 (lane 4), rTrwL4 (lane 5), rTrwL3 (lane 6), rTrwL2 (lane 7). Lane 1, Prestained molecular weight marker (New England Biolabs).

C - Western blot detection of the *B. birtlesii* Trw proteins

Western blot detection of total *B.birtlesii* proteins separated by SDS-PAGE under reduced conditions, using polyclonal antibodies against rTrwJ1 (lane 2), rTrwJ2 (lane 3), rTrwL2 (lane 4), rTrwL3 (lane 5), rTrwL4 (lane 6) and rTrwL5 (lane 7). Lane 1, Prestained molecular weight marker (New England Biolabs).

Figure 2: Immunogold labelling and transmission electron microscopy of Trw components (in? of the? on the?) *B. birtlesii* surface.

Electron microscopy detection of TrwJ1 and TrwJ2 on the surface of CBA-cultivated *B. birtlesii* using polyclonal antibodies against rTrwJ1 (B) and rTrwJ2 (C) recombinant proteins. (A), naïve mouse serum as negative control.

Figure 3: Identification of TrwJ1 and TrwJ2 interaction with mouse erythrocytes

A – Binding assay between mouse erythrocytes and T7 fusion phages

Assessments of the capacity of TrwJ1-T7 and TrwJ2-T7 phages to bind to mouse erythrocytes were evaluated three times (3 replicates each). Wild-T7 phages were used as control and the values are presented as the mean of three independent experiments.

B – Efficiency of *in vitro* invasion inhibition of mouse erythrocyte by *B. birtlesii* with anti-Trw polyclonal antibodies

Invasion inhibition assays with polyclonal antibodies against rTrwJ1 and rTrwJ2 recombinant proteins and serum from non-immunized mouse were performed three times (3 replicates each). The values are presented as the mean of three independent experiments.

Figure 4: Identification of TrwJ2 erythrocytic receptor

A – Far Western blot analysis of mouse erythrocyte membrane proteins using rTrwJ2 recombinant protein

Mouse erythrocyte membrane proteins were separated on SDS-PAGE under reduced conditions, transferred onto a PVDF membrane, probed with rTrwJ2, and analysed by immunoblot using anti-mouse antibodies (lane 2). Lane 1, prestained molecular weight marker (New England Biolabs). The experiment was conducted twice with qualitatively similar results.

B –Immunoblot analysis of mouse erythrocytic membrane proteins using Band3 monoclonal antibody

Mouse erythrocyte membrane proteins were separated on SDS-PAGE under reduced conditions, transferred onto a PVDF membrane, and analysed by immunoblot using anti-band3 antibodies (lane 2). Lane 1, Prestained molecular weight marker (New England Biolabs).

Figure 5: Role of erythrocytic band3 in adhesion and invasion of mouse

erythrocyte *by B. birtlesii*

A – Analysis of the impact of anti-band3 antibodies on the interaction between mouse erythrocytes and TrwJ1-T7 or TrwJ2-T7 phages.

Phage binding inhibition assays with anti-band3 polyclonal antibodies and serum from non immunized rabbit were performed three times (3 replicates each).The values are presented as the mean of three independent experiments.

B – Analysis of the impact of anti-band3 antibodies on the invasion capacity of mouse erythrocyte by *B. birtlesii*

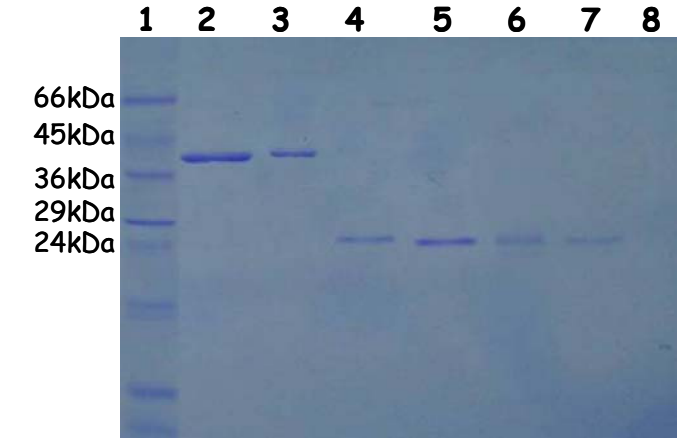
Invasion inhibition assays with anti-band3 polyclonal antibodies and serum from non-immunized rabbit were performed three times (3 replicates each). The values are presented as the mean of three independent experiments.

Table

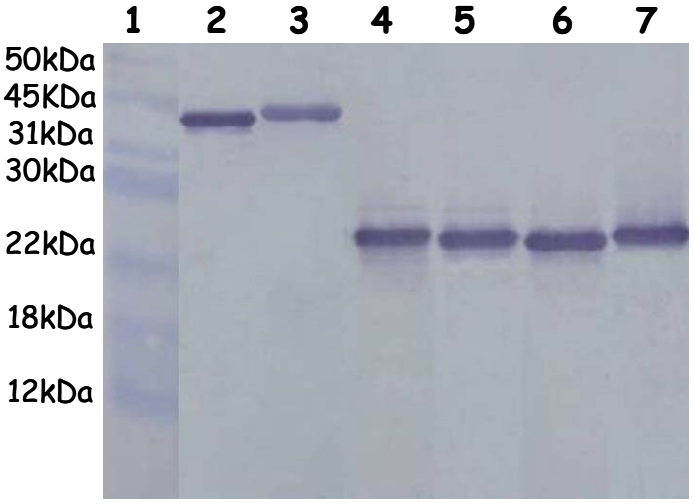
Table 1 Primer sequences and their applications

Primer name	Nucleotide sequence (5'-3')	Applications
J1-F	5′-CACCATGAAAAAGCTGATTAC-3′	Protein expression
J1-R	5′-TCTTATAgTTggCATgCCTC-3′	Protein expression
J2-F	5′-CACCTTTATAGTTGGAGGGAT-3′	Protein expression
J2-R	5′-TCgTATAgATCgTATTATTggC-3′	Protein expression
L1-F	5′-CACCCAAACAACCACAAAAG-3′	Protein expression
L1-R	5′-TTggATgTAAAATAATATATggCTAAT-3′	Protein expression
L2-F	5′-CACCCAACTACAACATGCG-3′	Protein expression
L2-R	5′-ATTAgCTTTgAATAgCATTgCgAC-3′	Protein expression
L3-F	5′-CACCCAAAATACTTTGAAAAAAGC-3′	Protein expression
L3-R	5′-TggCTTAAATAACATCgCgAC-3′	Protein expression
L4-F	5′-CACCGAATTAAAACACGCTAAAAAG-3′	Protein expression
L4-R	5′-TgCATggCCgAACAACATTTTTAC-3′	Protein expression
L5-F	5′-CACCCAAGCACGCGCTTTG-3′	Protein expression
L5-R	5′-TCCGTTTTGAAATAACATAGCGAC-3′	Protein expression
TrxFus Forward	5′-TTCCTCGACGCTAACCTG-3′	Cloning identification
T7 Reverse	5′-TAGTTATTGCTCAGCGGTGG-3′	Cloning identification
TrwJ1-F2	5′-CCggAATTCCATgAAAAAgCTgATTAC-3′	Construction T7 fusion phage
TrwJ1-R	5′-CCCAAGCTTTTATCTTATAGTTGGCAG-3′	Construction T7 fusion phage
TrwJ2-F2	5′-CCggAATTCCATgAAAAAACAggTCA-3′	Construction T7 fusion phage
TrwJ2-R	5′-CCCAAGCTTTTATCGTATAGATCGTA-3′	Construction T7 fusion phage
T7Select up	5′-GGAGCTGTCGTATTCCAGTC-3′	Phage identification
T7Select down	5′-AACCCCTCAAGACCCGTTTA-3′	Phage identification

A-



B-



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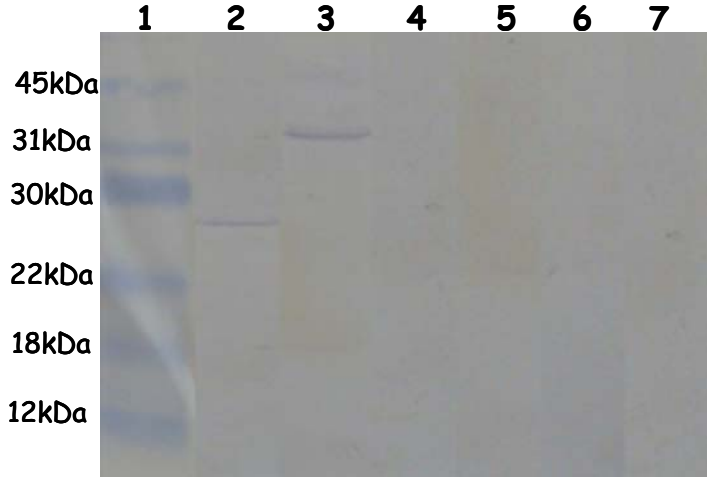


Figure 1 -Deng et al. 2011

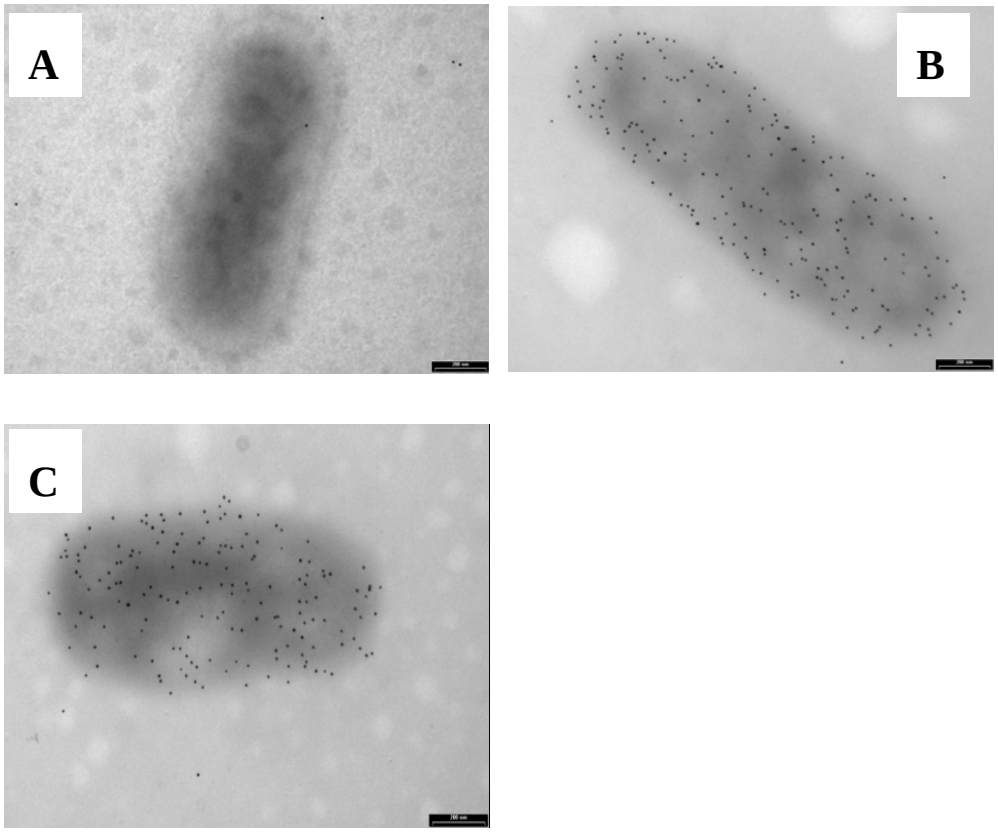
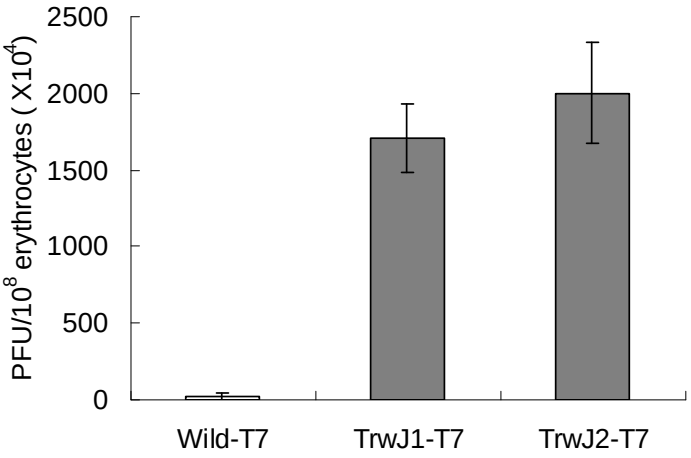


Figure 2 -Deng et al. 2011

A-



B-

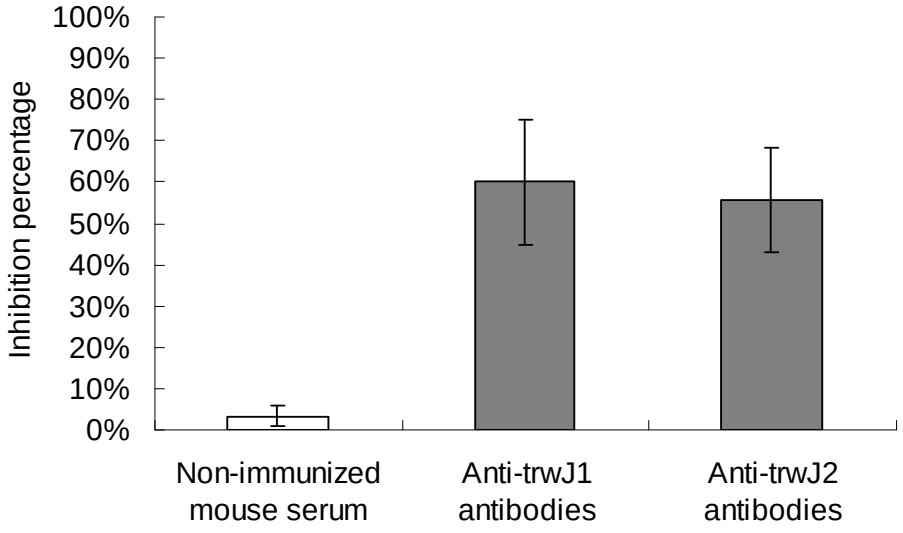


Figure 3 -Deng et al. 2011

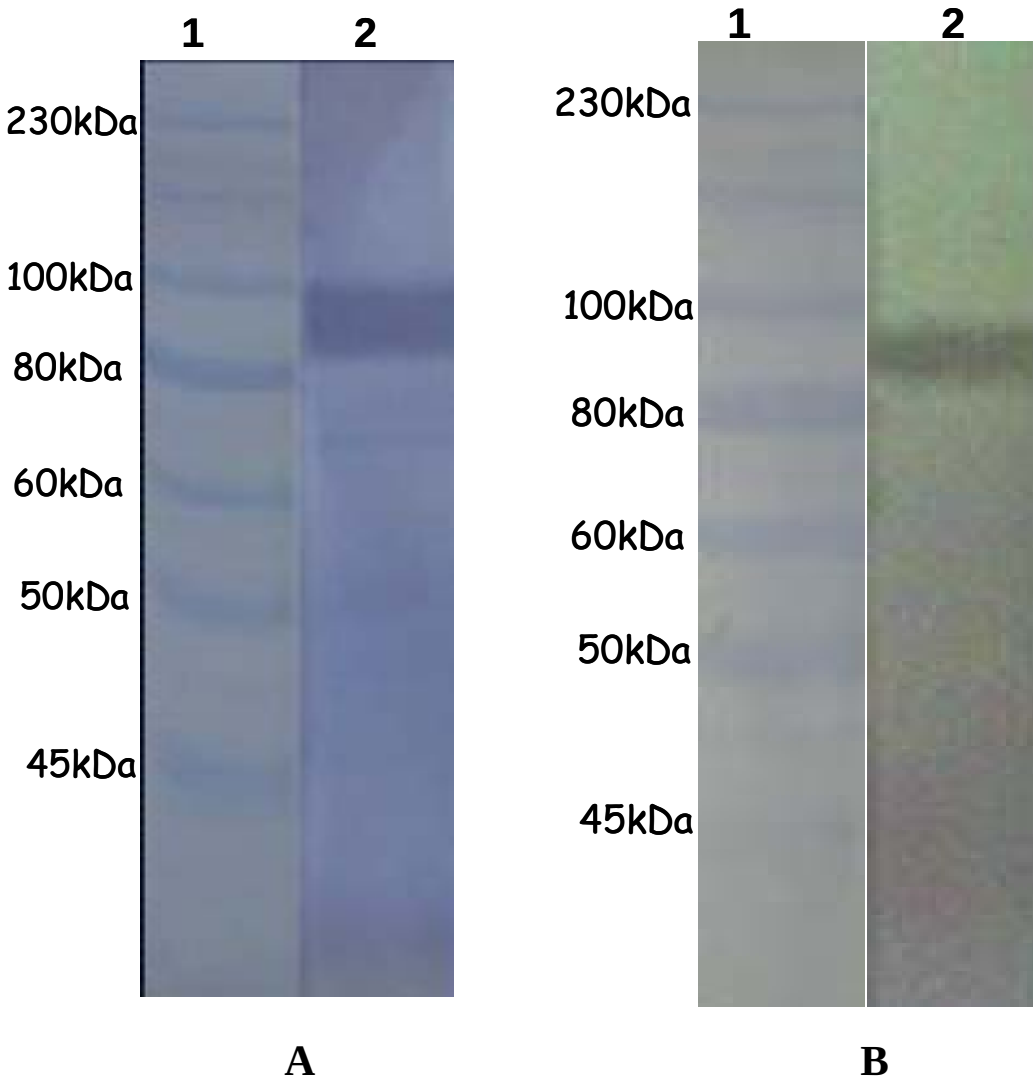
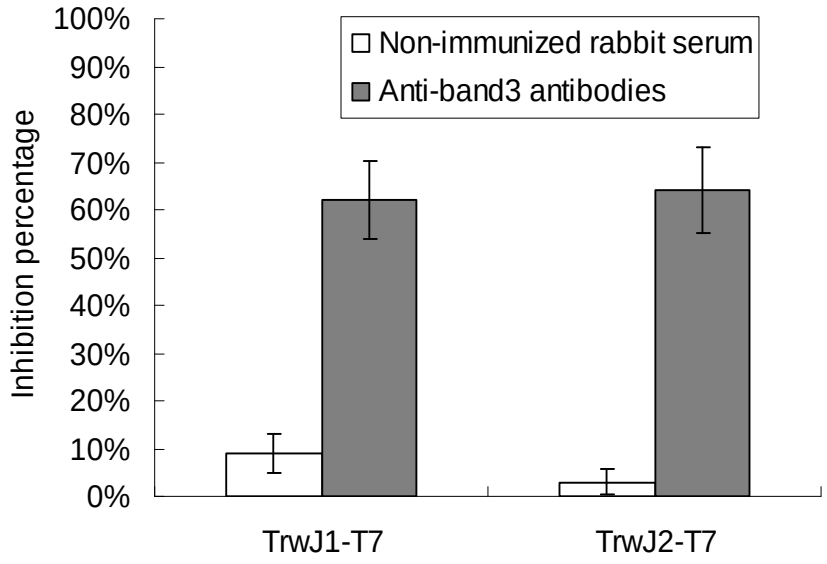


Figure 4 -Deng et al. 2011

A-



B-

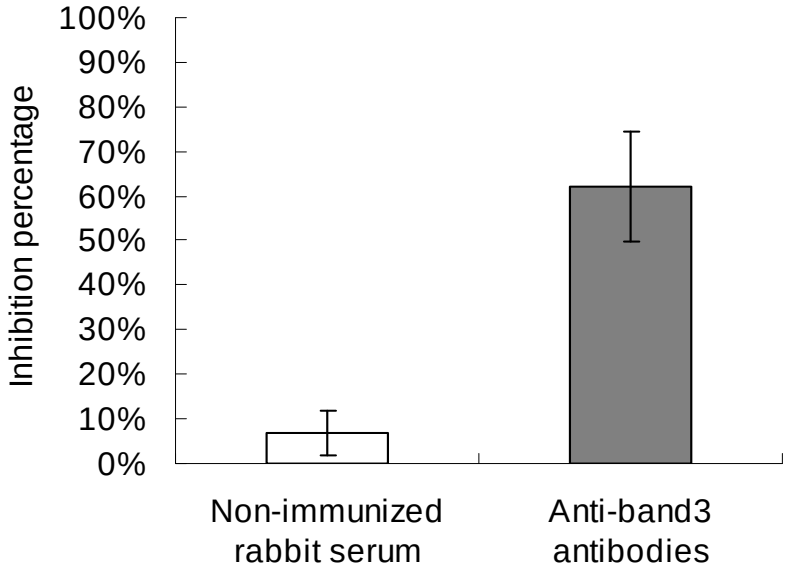
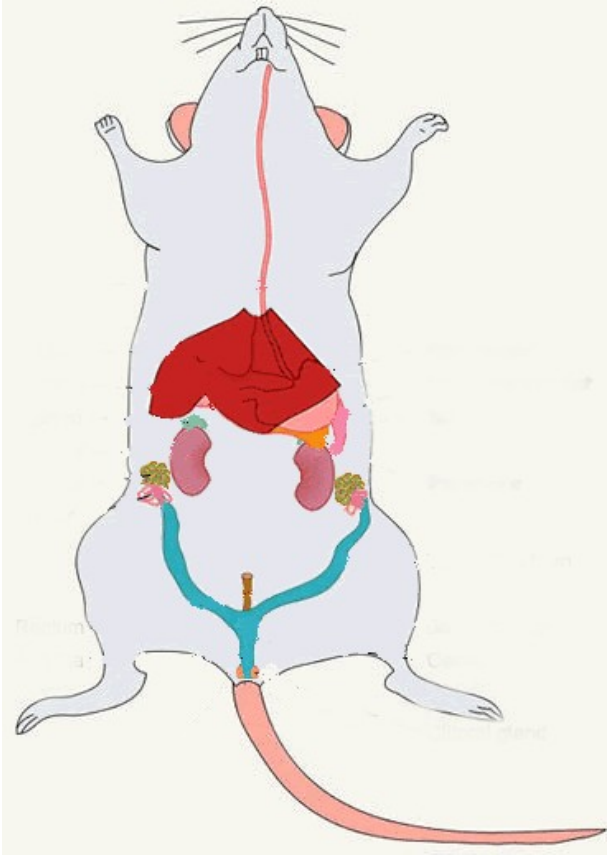


Figure 5 -Deng et al. 2011

CHAPTER III

Role of spleen in *Bartonella* spp. infection



Commentaires Article-3 (Chapitre 3)

Dans les 2 premiers chapitres de cette thèse, nous nous sommes intéressés au stade érythrocytaire de *Bartonella* spp. Toutefois, les stades de l'infection qui précèdent l'arrivée des bartonelles dans le sang sont beaucoup moins bien caractérisés. Il est clair que ces bactéries peuvent coloniser des tissus hautement vascularisés au cours des premiers jours de l'infection comme le lit vasculaire de la peau (pour *B. bacilliformis*). D'ailleurs, l'opinion couramment admise est que l'endothélium vasculaire sert de niche pour les espèces de *Bartonella* avant leur arrivée dans la circulation sanguine. En effet, les bartonelles ont la capacité unique de provoquer une vasoproliferation, se manifestant comme une angiomatose bacillaire ou une péliose hépatique (*B. bacilliformis* et *B. henselae ou B. quintana*). Par ailleurs, dans de nombreuses études *in vitro*, Il a été démontré que *Bartonella* spp envahit des lignées de cellules endothéliales et / ou interfère avec leur physiologie. Cependant, bien que la vascularisation endothéliale joue indéniablement un rôle dans les premiers stades de l'infection dans certains cas particuliers, toujours chez l'homme (pour *B. bacilliformis* ainsi que *B. quintana* ou *B. henselae* chez les patients immunodéprimés), l'implication de ces cellules comme niche primaire chez l'hôte réservoir animal n'a jamais été établi.

D'autres types cellulaires, comme les précurseurs érythrocytaires ont également été proposés comme niche possible de *Bartonella* spp., l'infection intra-érythrocytaire résultant de la présence des bactéries dans les précurseurs. Cette hypothèse n'est toutefois pas confirmée par les données obtenues à partir d'expériences *in vivo* avec le modèle GFP-*B.tribocorum*/rat qui indiquent que la rencontre entre *Bartonella* spp. et les globules rouges se produit dans le sang.

Dans la troisième et dernière partie de la thèse, nous avons entrepris d'explorer les différents sites possibles de colonisation de *B. birtlesii* chez la souris et ceci dans la première semaine suivant l'infection.

Nous avons recherché les bactéries dans les ganglions lymphatiques, les cellules de la moelle osseuse, la rate, le foie, le cerveau et le sang de souris infectées. Nous avons ainsi pu mettre en évidence une accumulation de bactéries dans la rate ainsi qu'une infection passagère du foie. Aucune bactérie n'a été identifiée à partir des autres prélèvements. Afin d'identifier si la rate jouait un rôle de niche pour *Bartonella* spp. ou si au contraire, elle participait à son élimination, nous avons comparé la bactériémie chez des souris normales et splénectomisées. Pendant les 7 premiers jours suivant l'infection, la bactériémie est la même dans les deux lots de souris. Au delà de ces 7 premiers jours suivant l'infection, la bactériémie est 10 fois plus élevée chez les souris splénectomisés par rapport aux souris normales et dure 2 semaines supplémentaires. Ces résultats indiquent que la rate ne joue pas le rôle de niche primaire, mais participe probablement à la rétention des globules rouges infectés.

ARTICLE-3

Role of spleen in *Bartonella* spp. infection

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Role of spleen in Bartonella spp. infection

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Keywords:	spleen, early infection, Bartonella, liver, erythrocytic precursor

Role of the spleen in *Bartonella* spp. infection

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Abstract:

Bartonella spp. are intra-erythrocytic pathogens of mammals. In this study, we investigated the role of spleen, and other tissue and/or organs in *Bartonella* infection. Using an *in vivo* model of mice infection by *Bartonella birtlesii*, we detected accumulation of bacteria in the spleen, transient infection of the liver but failed to detect any bacteria in brain, lymph nodes, lung, or heart. We then compared bacteremia in normal Balb/C mice and in splenectomized mice. Bacteremia in splenectomized mice was 10 fold higher than in normal mice and lasted 2 weeks longer. In conclusion, the spleen seems to retain and filter infected erythrocytes rather than to be a sanctuary for chronic *Bartonella* infection.

The bacterial genus *Bartonella* comprises at least 24 species or subspecies of facultative bacteria that use arthropod transmission and haemotropism as a mammalian parasitism strategy. Each species is highly adapted to infect one or few reservoir hosts in which they induce asymptomatic intra-erythrocytic bacteremia (Vayssier-Taussat*, et al.*, 2009). When they are introduced into an accidental host or into immuno-compromised individuals, infection can result in diverse clinical manifestations. The mode of infection of the reservoir hosts is common to all *Bartonella* species. Following injection, the bacterial inoculum is cleared from circulating blood within a few hours but bacteremia reappears approximately five days later. Bacteria can be observed, adhering to and invading mature erythrocytes in the blood, within two days. An erythrocyte is usually invaded by a single bacterium, which, once inside, replicates in a membrane-bound compartment over a period of several days until eight daughter cells, on average, are created. Replication then ceases, and the infected erythrocytes are shown to persist in circulation for several weeks (Schulein*, et al.*, 2001, Rolain*, et al.*, 2003).

The stages of infection that precede the arrival of bartonellae in the bloodstream have been far less well characterized. The current opinion is that the vascular endothelial cells may serve as a primary niche for *Bartonella* spp. before they enter the bloodstream (Dehio*, et al.*, 1997, Dehio, 1999, Dehio, 2001, Schulein*, et al.*, 2001, Schmid*, et al.*, 2004, Schmid*, et al.*, 2006, Pulliainen & Dehio, 2009). However, other putative cells such as erythrocytic precursors have also been proposed as putative niches *of Bartonella* spp. (Rolain*, et al.*, 2003, Mandle*, et al.*, 2005).

The aim in this study was to explore the different possible locations of *Bartonella* spp. in the first week following infection, using Balb/C mice infected with a rodent-adapted *B. birtlesii* (Boulouis*, et al.*, 2001). For this purpose, an inoculum of $5x10^7$ Colony

Forming Units (CFU) of *B. birtlesii* (IBS 325^T strain) was inoculated into the caudal vein of Balb/C mice. At 0.5, 1, 2, 3 and 7 days post-infection, the mice were euthanized and the lymph nodes, bone marrow, spleen, liver, brain and blood were sampled. Organ samples were mechanically crushed using a Dounce potter in 1mL of F12 medium before being grown for 5 days on Columbia agar containing 5% defibrinated sheep blood (CBA) in a humidified atmosphere with 5% CO_2 at 35°C. Cells from lymph nodes (axillary, inguinal and mesenteral lymph nodes) and bone marrow were isolated by disrupting the lymph nodes, and flushing medium through the bone. The recovered cells and erythrocytes were then lysed by hypotonic shock and the released bacteria were grown on CBA in a humidified atmosphere with 5% CO_2 at 35°C.

As shown in Fig. 1A, no bacteria were recovered from the lymph nodes, bone marrow or brain, indicating that these tissues or organs were not targets for *B. birtlesii* in this animal model of *Bartonella* sp. infection. In contrast, *B. birtlesii* colonized the liver and, to a much larger extent, the spleen during the first 7 days following infection, and before seeding the bloodstream. While liver colonization stopped after this first step, the spleen remained highly infected throughout the duration of bacteremia (data not shown).

In order to investigate whether spleen could provide a possible sanctuary for chronic *Bartonella* infection, as demonstrated for other bacteria (Trulzsch*, et al.*, 2007, Watson & Holden, 2010), or whether the spleen filters infected erythrocytes as shown for *Plasmodium falciparum* (Safeukui*, et al.*, 2008), bacteremia induced by *B. birtlesii* infection was compared in normal Balb/C mice and in splenectomized mice. During the first 7 days, bacteremia was the same in both normal and

splenectomized mice. Thereafter, bacteremia was 10 fold higher in splenectomized mice than in normal mice and lasted 2 weeks longer (Fig. 1B).

Spleen is an important site for host response to bacterial infection (Junt*, et al.*, 2008). Within the spleen, free bacteria may encounter various tissue-resident phagocytes, such as macrophages, dendritic cells and neutrophils, which could eliminate them. In our model, during the first week following infection, (when *B. birtlesii* was not yet inside the erythrocytes), having a spleen or not had no effect on the time of appearance of bacteria in blood or the level of bacteremia. This suggests that the spleen does not have a fundamental role in the first step of infection, based on the model used. Once the bacteria were intra-erythrocytic, the spleen appeared to strangle the infection since bacteremia was 10 fold higher in splenectomized mice. This data suggests that the spleen has a role in clearing *Bartonella*-infected erythrocytes.

The elimination of microorganisms in the spleen is mainly triggered by the clearance of opsonized encapsulated bacteria (such as pneumococci, meningococci, and *Escherichia coli*) and intra-erythrocytic parasites (such as those causing malaria and babesiosis) by the splenic macrophages. However, in the specific case of intra-erythrocytic parasites such as *Plasmodium* sp., the spleen may also retain *Plasmodium*-infected erythrocytes through mechanical sensing of deformed or rigid erythrocytes, which explains the fulminant nature of these infections in persons with anatomic or functional asplenia (Safeukui*, et al.*, 2008). In our study, we have shown that the spleen is able to retain *Bartonella* spp.*,* although the mechanisms of retention have still to be elucidated. First we have to confirm that *Bartonella* is localized within the spleen erythrocytes. If this is so, we will then have to determine the mechanism by which the infected erythrocytes are retained. Indeed, it is widely

accepted that the infection of erythrocytes by *Bartonella* has no effect on their physiology (no changes in life span or in membrane structures,…) (Schulein*, et al.*, 2001). However, a deformation factor, called “deformin”, has been identified in *B. bacilliformis* and *B. henselae,* which causes marked physiological changes (pitting and invagination) of the erythrocyte membranes (Xu*, et al.*, 1995, Iwaki-Egawa & Ihler, 1997). This factor would insert itself into the erythrocyte membrane as a pore-like structure leading to deformation of the erythrocytes. This would explain the differences in clearance between infected and uninfected erythrocytes, the altered erythrocytes being filtered out by spleen as demonstrated, for example, for *Plasmodium* sp. (Buffet*, et al.*, 2011).

It would be interesting to elucidate whether retention of poorly deformable red blood cells also has a crucial impact on *Bartonella* spp. infections.

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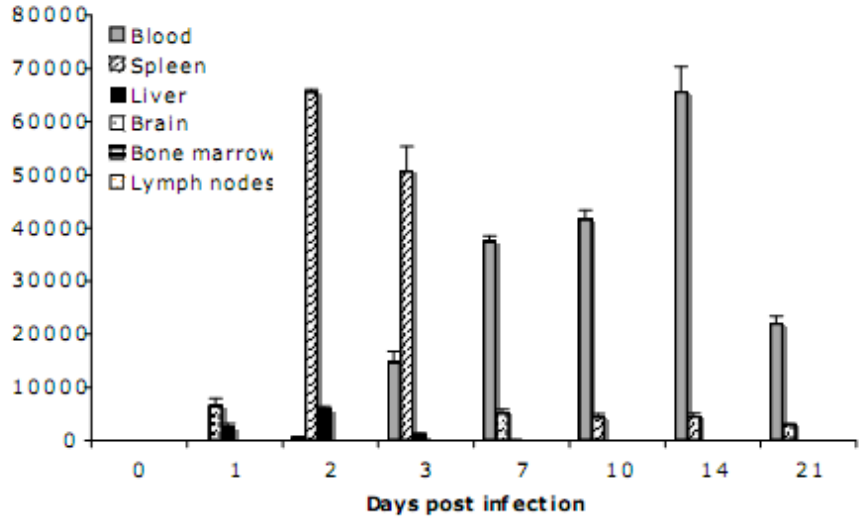
Figure:

Fig. 1: A) Detection of bacteria in different organs, tissues, and in blood during the first 7 days of Balb/C mice infection with *B. birtlesii*. The recovered bacteria are expressed in CFU/ml of blood; CFU/mg of organs, and CFU/10^4 of cells recovered from lymph nodes and bone marrow. N=5 (per day)

B) Bacteremia in normal or splenectomized Balb/C mice infected with *B. birtlesii.* N=10

A-



B-

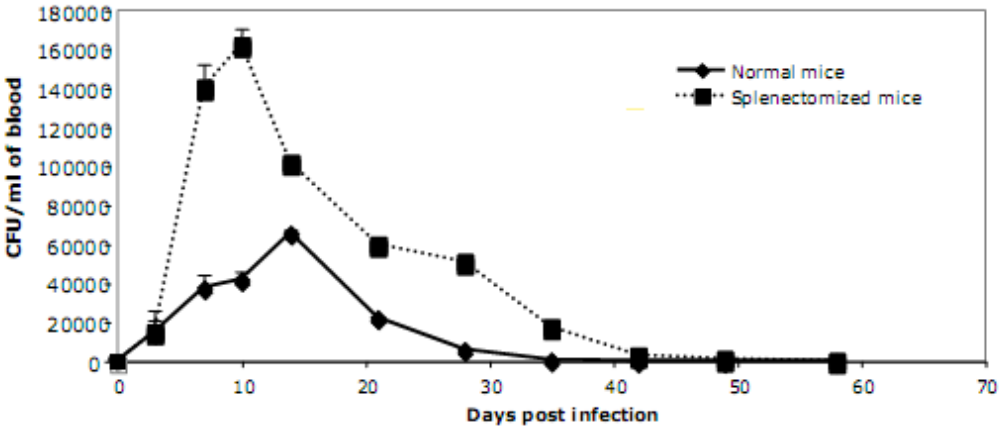


Figure 1 -Deng et al. 2011

Conclusion and perspectives

CONCLUSION ET PERSPECTIVES

Cette thèse présentait comme objectif de mieux identifier les mécanismes liés à l'infection des érythrocytes par *Bartonella* et de mieux caractériser les localisations possibles de la bactérie au cours des premiers jours suivant l'infection et avant l'étape intra-érythrocytaire. Au cours de ce travail, l'étude des interactions bartonellae/globule rouge nous a conduit à mettre en évidence les mécanismes moléculaires liés à la spécificité des espèces de *Bartonella* pour leurs hôtes mammifères.

La spécificité d'hôte est une caractéristique importante des bactéries pathogènes qui restreint l'éventail des hôtes sensibles à l'infection. Des changements subtils dans les mécanismes moléculaires qui régissent cette spécificité d'hôte peuvent entraîner des changements soudain d'hôtes, ce qui représente un risque majeur pour l'émergence de nouveaux agents pathogènes humains à partir des réservoirs animaux.

Des exemples frappant de ce scénario sont les bartonelles, qui causent des infections intra-érythrocytaires dans une large gamme de réservoirs de mammifères, chaque espèce étant adaptée à une espèce de mammifère. L'établissement d'un modèle *in vitro* de l'adhérence et de l'invasion des érythrocytes par *Bartonella spp.* nous a permis de démontrer pour la première fois une corrélation entre la restriction d'hôte et la capacité à infecter les érythrocytes *in vivo* et *in vitro*, montrant que la spécificité d'hôte est déterminée par l'interaction directe des bactéries avec des érythrocytes.

Afin d'identifier les facteurs bactériens responsables de la reconnaissance hôte spécifique des érythrocytes, nous avons utilisé un protocole en deux étapes : Dans un premier temps, nous avons effectué un criblage d'une banque STM de *B. birtlesii* chez les souris qui nous a permis d'identifier des mutants incapables d'établir une infection intra-érythrocytaire. Parmis les 38 protéines essentielles pour l'infection qui ont été identifiées par ce criblage, 13 ont également été

retrouvés comme essentielle pour l'infection intra-érythrocytaires du rat par *B. tribocorum* [6], indiquant des similarités des facteurs moléculaires impliqués dans l'infection par ces deux organismes et la robustesse des criblages effectués. Dans un deuxième temps, un nouveau criblage de l'ensemble des mutants abactéremique de *B. birtlesii* a été réalisé en utilisant le système *in vitro* d'infection des globules rouges. Ceci a abouti à l'identification d'un total de 9 mutants déficients pour l'invasion des globules rouges. Les autres mutants (36/45 = 80%) affichent un phénotype de type sauvage dans cet essai et ne sont donc pas directement impliqués dans l'infection des érythrocytes, mais plutôt contribue à l'établissement de l'infection dans les phases précoces. Les principaux exemples sont les gènes codant pour les virB/virD4 T4SS, qui sont requis pour l'infection de la niche primaire (cellules endothéliales vasculaires) [20].

Parmi les 9 mutants déficients pour l'infection des érythrocytes *in vitro*, 2 (ialA/B et LivG) ont un phénotype sauvage pour l'adherence aux globules rouges, en revanche, ces mutants ont un phénotype d'invasion altéré suggérant qu'ils participent à l'invasion des érythrocytes comme suggéré par d'autres études [21, 22].

Les 7 autres mutants sont tous altérés dans la fonction d'adhésion à l'érythrocyte. Remarquablement, ils sont tous mutés dans le même opéron : le système de sécrétion de type IV Trw (T4SS, Trw). Trw avait été précédemment identifié comme un facteur clé impliqué dans l'établissement de l'infection intra-érythrocytaire dans le modèle *B. tribocorum* / rat [6]. Par ailleurs, l'apparition transitoire de ce mutant dans le sang au début de la bactériémie intraérythrocytaire suggére que le système Trw est dispensable pour la colonisation de la niche primaire, mais est nécessaire pour l'établissement de bactériémie intraérythrocytaire [23]. Toutefois, la preuve directe du rôle de ce système dans le reconnaissance des globules rouges manquait jusqu'ici et est apporté pour la première fois par notre étude. Pour tester si Trw était également impliqué dans la reconnaissance hôte sépcifique des érythrocytes, nous avons

exprimé le Trw d'une espèce spécifique du rat : *B. tribocorum*, dans une espèce spécifique du chat *B. henselae* et de l'homme : *B. quintana*. Cette expression ectopique a provoqué une extension de la gamme d'hôte de *B. henselae* et *B. quintana* pour l'infection des érythrocytes de rats *in vitro*, montrant que l'expression de Trw est suffisante pour déterminer la spécificité d'hôte.

Le système Trw de *Bartonella* donc représente un exemple particulièrement intéressant d'un T4SS liée à la pathogenèse qui a évolué assez récemment par la diversification fonctionnelle d'un système de conjugaison bactérienne latéralement acquis [24]. Dans le genre *Bartonella* spp., il a été acquis pendant l'évolution de ces bactéries, ainsi, il n'est représenté que dans une sous-branche comportant 13 espèces, qui ont divergé assez tôt dans l'évolution de ces bactéries [6]. L'acquisition du système Trw est à corréler avec la perte du flagelle, connu pour être un facteur de virulence important pour l'invasion des érythrocytes chez *B. bacilliformis* et probablement chez les autres espèces flagellées de *Bartonella* [23].

Le système Trw de *Bartonella* spp. possède les caractéristiques d'un îlot de pathogénicité et montre des similitudes avec le système de conjugaison IncW du plasmide R388 (jusqu'à 80% d'identité pour la séquence en acides aminés) initialement isolé à partir Enterobacteriacea. Les gènes *trw* de *Bartonella* spp. sont colinéaires avec les gènes respectifs du plasmide R388, sauf pour la présence de multiples duplications de gènes en tandem : *trwL*, *trwJ-trwH*. La complémentation des gènes de R388 avec leurs homologues de *Bartonella* a permis de démontrer l'interchangeabilité fonctionnelle pour certains composants [23], soulignant le degré élevé de conservation structurale et fonctionnelle des unités individuelles de ce T4SS. Cependant, une différence majeure entre les 2 systèmes est l'absence de TrwB chez les espèces de *Bartonella* spp.. Dans R388, TrwB code la protéine de couplage qui est requis pour l'exportation des substrats véhiculés par le T4SS. L'absence de TrwB chez *Bartonella* indique donc que le système de *Bartonella* Trw a probablement perdu sa capacité de translocation de substrats.

Par ailleurs, les copies multiples de *trwL* et *trwJ* dans le locus *trw* de *Bartonella* spp. code des protéines de pili constituant les parties exposées à la surface bactérienne. Les autres gènes dupliqués, *trwI* et *trwH*, code les composants requis pour l'élongation des pili et leur ancrage à la membrane externe [25]. La présence de multiples copies de ces composants suggèrent la possibilité de la formation de formes variées des pili. Du fait du rôle essentiel du système Trw pour l'adhésion érythrocytaire, il est concevable de penser que ces variants ont pour rôle de faciliter l'interaction avec les différents récepteurs érythrocytaires, soit au sein de la population hôte réservoir (par exemple, différents antigènes de groupe sanguin), ou chez des hôtes réservoirs différents.

Les analyses phylogénétiques et le calcul des fréquences de substitution des différents gènes de l'opéron *trw* montrent que les homologues *trwJ* et *trwL* ont un degré de diversité beaucoup plus élevé que les autres composants du T4SS Trw, au sein et entre les différentes espèces [26]. Par ailleurs, le nombre de répétitions en tandem de *trwL* et *trwJIH* sont variables selon les espèces de *Bartonella* indiquant que les gènes *trwL* et *trwJ* ont été amplifiées et diversifiées à plusieurs reprises durant l'évolution.

Nous avons pu démontré que, dans le cas de culture *in vitro* de *B. birtlesii*, parmi les protéines de surface formant le pilus, seule TrwJ1 et TrwJ2 était exprimées et présentes à la surface de la bactérie, alors que les différentes formes de TrwL n'ont pu être détectées. Il serait intéressant d'évaluer leur expression dans un contexte naturel d'infection *in vivo*, afin d'évaluer leur induction en condition naturelle, soit au moment du passage dans la niche primaire, ou dans la circulation sanguine.

En effectuant des essais pour déterminer le récepteur de TrwJ1 et TrwJ2, nous avons mis en évidence une liaison de la protéine TrwJ2 avec la glycoprotéine majeure transmembranaire, Band3 érythrocytaire qui joue un rôle dans le transport d'anions [27].

Cette protéine avait déjà été identifiée comme un récepteur potentiel de *B. bacilliformis* [28]. Elle a par ailleurs été impliquée dans l'invasion des globules

rouges par l'agent du paludisme [29-34]. Des études récentes ont révélé que la protéine de surface du mérozoïte 1 (MSP1) de *P. falciparum* interagit avec deux régions non glycosylées de la bande 3 des érythrocytes appelé 5ABC (acides aminés 720-761) et 6A (acides aminés 807-826) [35]. D'autres protéines parasitaires, comme MSP9, sont également capables d'interagir avec la même région 5ABC [36, 37].

L'invasion des érythrocytes par *P. falciparum* est assurée par 2 voies distinctes [38, 39]: la voie dépendante de l'acide sialique, nécessitant l'adhésion aux glycophorines A, B et C [40-44], et la voie indépendante de l'acide sialique nécessitant l'interaction avec la Band3 comme décrit ci-dessus. Concernant *Bartonella* spp., des études réalisées dans notre laboratoire ont montré que l'incubation avec des anticorps anti-glycophorine A des globules rouges murins avant l'infection par *B. birtlesii* avait pour effet de décroître de 50% la capacité d'invasion suggérant, que comme pour *P. falciparum*, plusieurs voies d'invasion des globules rouges sont utilisées par *Bartonella* spp. Des études complémentaires doivent être menées pour confirmer ces hypothèses.

Les stades de l'infection qui précèdent l'arrivée des bartonelles dans le sang étant très peu caractérisés, *in vivo*, nous avons utilisé le modèle d'infection expérimentale des souris de laboratoire par *B. birtlesii* pour identifier les différents organes, tissus ou cellules colonisés par *B. birltesii* dans les premiers jours suivant l'infection. Nous avons ainsi pu montré qu'aucune bactérie n'a été récupéré à partir des ganglions lymphatiques, la moelle osseuse et le cerveau, ce qui indique que ces organes, tissus ou cellules ne sont pas des cibles de *B. birtlesii* dans ce modèle animal d'infection. En revanche, *B. birtlesii* colonise le foie et de manière encore plus importante la rate au cours des 7 premiers jours suivant l'infection. Alors que la colonisation du foie s'arrête après cette première étape, la rate reste très infectée pendant toute la durée de la bactériémie.

Nous avons ensuite identifié si la rate pouvait représenter un sanctuaire possible de l'infection à *Bartonella* comme cela a déjà été démontré pour d'autres bactéries [45, 46] ou au contraire, si la rate filtrait les érythrocytes infectés,

comme démontré pour *Plasmodium falciparum* [47]. Nous avons trouvé que durant la première semaine suivant l'infection, alors que *B. birtlesii* n'est pas encore dans les érythrocytes, avoir ou non une rate, ne change pas le moment de l'apparition des bactéries dans le sang ou le niveau de la bactériémie, suggérant que la rate n'a pas un rôle fondamental dans la première étape de l'infection. A partir du moment où les bactéries sont intra-érythrocytaires, la rate apparaît jouer un rôle dans le contrôle de l'infection puisque la bactériémie est 10 fois plus élevée chez les souris ayant subi une splénectomie. Ces données suggèrent que la rate a un rôle dans la rétention des érythrocytes infectés par *Bartonella*.

Dans notre étude, nous avons montré que la rate est en mesure de retenir les *Bartonella*. Cependant, les mécanismes de la rétention doivent encore être élucidés. Dans le futur, nous devrons confirmer la localisation intra-érythrocytaire de *Bartonella* dans la rate. Si, comme supposé, les bactéries sont associées aux érythrocytes, nous aurons à déterminer le mécanisme par lequel les hématies infectées sont reconnues et retenues. En effet, il est largement admis que l'infection des érythrocytes par *Bartonella* n'a aucun effet sur leur physiologie (pas de changement dans la durée de vie, aucun changement dans les structures de la membrane, ...) [12]. Cependant, un facteur de déformation, appelée "deformin", a été identifié dans *B. bacilliformis* provoquant des changements structuraux (piques et invagination) de la membrane érythrocytaire [48, 49]. Ce facteur décrit comme s'insérer dans la membrane bactérienne et créant un pore pourrait déformer les globules rouges, expliquant la différence de clairance des érythrocytes infectés par rapport à ceux non infectés, les érythrocytes altérés étant filtrés par la rate comme cela a été montré pour *P. falciparum* [50].

Le développement de modèles animaux d'infection par *Bartonella* spp. et des outils pour la manipulation génétique de ces bactéries a permis des avancées significatives dans la compréhension des bases moléculaires expliquant les mécanismes d'infection de l'hôte réservoir par *Bartonella*. Cependant, malgré

ces progrès, et l'importance médicale de ces bactéries, de nombreuses questions sont encore sans réponse. Par exemple, quel est le sort des bactéries après inoculation? Comment les bactéries diffusent à partir du point de piqûre via la circulation sanguine, et l'endothélium vasculaire est-il vraiment un site principal de l'infection? En outre, nous ne savons rien sur la façon dont bartonellae se réplique et survie à l'intérieur du globule rouge, et si des changements physiologiques du globule rouge est induit par l'infection. Nous savons peu de choses sur l'interaction entre bartonellae et l'immunité de l'hôte et donc peu de choses sur l'étendue et l'importance de l'immunorégulation. Les recherches futures devront également tenir compte du rôle des arthropodes dans le cycle naturel et *Bartonella* et des infections par inoculation naturelles devront être privilégiées par rapport aux inoculations actuelles par seringue. En résumé, les défis pour les années à venir, sont (1) de comprendre comment, la stratégie unique de l'infection par bartonellae contribue à son succès épidémiologique remarquable dans ses hôtes réservoirs, et (2) d'avoir le maximum d'information concernant la biologie de ces bactéries pour pouvoir évaluer leur potentiel pour devenir de nouveaux pathogènes pour l'homme ou l'animal.

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