New insights into the infection strategy of lineage 3 and lineage 4 *Bartonella* species

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Statement of my Thesis

This work was carried out in the group of Prof. Christoph Dehio in the focal area of Infection Biology at the Biozentrum of the University of Basel, Switzerland.

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My thesis is written in a cumulative format. It consists of an introduction covering the major aspects related to my work. It is followed by two unpublished manuscripts comprising the following parts: title page, abstract, introduction, results, material and methods and discussion. Finally, I close this thesis report by a global conclusion summarizing of all major findings of this study.

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General Introduction

During evolution, the arms race between pathogens and host has been the driving process for bacteria to develop specific infection strategies and acquired variety of weapons to fight or avoid host immune responses and to establish successful infections. Regarding the *Bartonella* genus, the comprised pathogens evolved a "stealth strategy" characterized by an intraerythrocytic colonization and persistence.

1.1. Prevalence and epidemiology of *Bartonella*

Bartonella species have been discovered in a wide range of mammals all over the world and can be the causative agents of multiple human diseases. The prevalence of *Bartonella* species in wild animals is very high all over the world. Studies revealed that most of deers tested in France and California and more than 60% of rodent in central Europe were PCR positive for *Bartonella* [1, 2]. *B. henselae*, the agent of cat-scratch disease (CSD), is widely distributed among human pets such as cats and dogs. A recent study in Germany revealed that over 95% of cats were PCR positive for *Bartonella* without being bacteremic [3]. Another study reported that in Europe 50 out of 94 (53%) stray cat blood samples gave a positive culture for *Bartonella* species (spp.) [4]. The presence of *Bartonella* among bees, bats, cattle, gorilla, horses and many other indicate that *Bartonella* spp. are highly incident in mammals [5-7] . Important to note, coinfection with several *Bartonella* spp. has been reported in many instances [8].

Bartonella species can be also causative agents of numerous clinical manifestations and diseases in humans. These include Carrion's disease, CSD, chronic lymphadenopathy, trench fever, chronic bacteremia, culture-negative endocarditis, bacilliary angiomatosis, bacillary peliosis, vasculitis and uveitis [9-14]. Recently, *Bartonella* spp. have been also associated with more diverse manifestations such as weight loss, hallucinations, muscle fatigue, partial paralysis or neurological manifestations such as pediatric acute-onset neuropsychiatric syndrome (PANS) [11, 13, 15]. The severity of the clinical manifestation is usually correlated with the immune status of the patient [9]. The diversity of *Bartonella* species and associated diseases, the large spectrum of reservoir hosts and arthropod vectors that can transmit these bacteria among mammals and humans turn out to be major causes for public health concern and call for more researches on the infection mechanism of the underappreciated *Bartonella* genus.

1.2. The *Bartonella* genus

The Gram-negative α-proteobacterial genus *Bartonella* comprises more than 45 fastidious facultative intracellular bacteria that cause acute and chronic infections in a broad spectrum of mammals such as canids, rodents, ruminants, felids, and humans [16]. Based on a genetic analysis, a robust phylogenic tree of the genus *Bartonella* has been established classifying *Bartonella* species into 4 distinct lineages. The ancestral lineage 1 composed of the deadly *B. bacilliformis* and the recently discovered *B. ancashensis* (both pathogens associated with Carrion's disease); Lineage 2 comprises ruminant-specific *Bartonella* species; Lineage 3 consists of closely related *Bartonella* species; and the most recent and species-rich lineage 4 [17-19]. The broad spectrum of hosts for lineage 3 and lineage 4 indicates a higher capacity for adaptation compared to lineage 1 and lineage 2, which are limited to human and ruminants, respectively (Fig. 1).

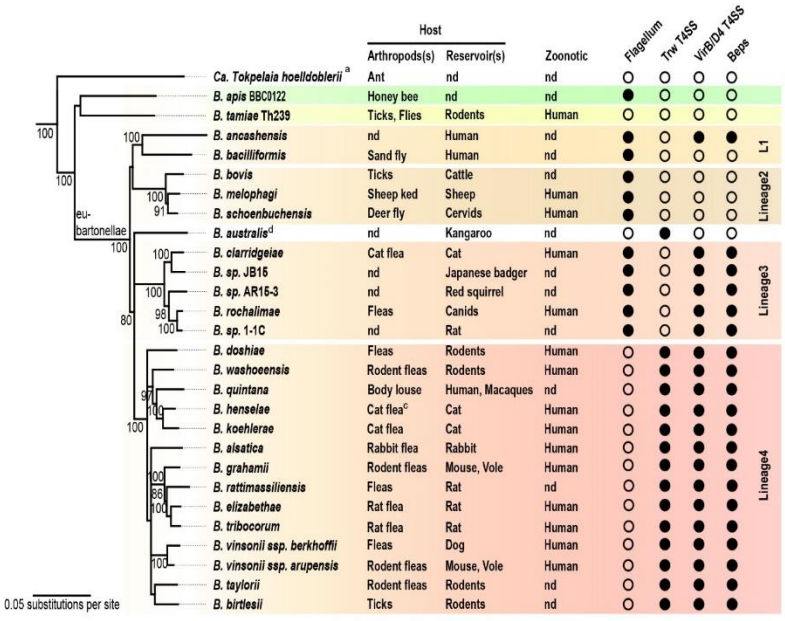

Figure 1. Phylogenetic structure and distribution of the virulence factors in the genus *Bartonella*

Phylogeny of the genus *Bartonella* with (a) *candidatus tokpelaia hoelldoblerii* as outgroup taxon. The phylogenic tree was based on analysis of concatenated alignment of 5 core protein sequences. Three *Bartonella* clades have been identified: *B. apis* (green), *B. tamiae* (yellow) and eubartonellae. Eubartonellea are further classified into 4 lineages (shades of orange/pink). The presence or absence of the virulence factors are reported by full or empty circles, respectively. Values above branches show bootstrap support values (≥70%). Taken from Segers et *al.* 2017. *ISME journal*

Host specificity during infection is one hallmark of the genus *Bartonella.* Each *Bartonella* species can establish a long-lasting intraerythrocytic bacteremia in a group of closely related mammalian reservoir hosts [20, 21]. Infection course has been described in natural and experimental animal models, such as the *B. birtlesii*-mouse, *B. tribocorum*-rat and *B. henselae*-cat models [22]. Even if the exact process of *Bartonella* infection is still enigmatic, many studies of the infection course demonstrated similar outlines suggesting a common infection cycle shared by all *Bartonella* species in their specific and respective reservoir host(s) [23-26]. Following dermal inoculation, the fastidious pathogen *Bartonella*

colonizes two sequential undetermined niches, the “dermal” and “blood-seeding niche”. After several days of replication and a suggested reprogramming in the “blood-seeding niche”, *Bartonella* are released in the bloodstream where they invade erythrocytes until being ingested during an arthropod blood meal and subsequently being transmitted to another host (Fig. 2) [27-30].

During the following paragraphs, I will describe in detail the infection cycle of *Bartonella* species, from the transmission by arthropods to the colonization of erythrocytes of the infected host, and describe the different virulence factors involved in these consecutive stages of infection.

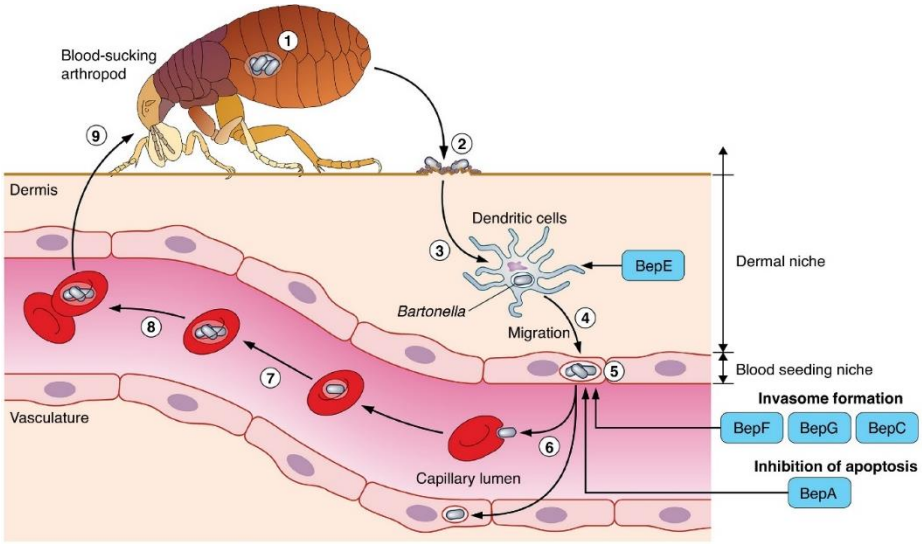

Figure 2. Schematic description of the *Bartonella* infection cycle

(1) *Bartonella* replicate in the mid-gut of the hematophagous arthropod. (2) During a blood meal, *Bartonella* are shed with the faeces onto the mammalian host skin. When the arthropod aspirate blood, it triggers irritation followed by scratching and subsequently by superficial inoculation of the bacteria in the dermis of the host. (3) *Bartonella* colonize DCs defined as the “dermal niche” which will disseminate bacteria from the inoculation site to the fenestrated lymphatic circulation providing an access to the “blood seeding niche” (4). Process that strictly depends on BepE. (5) It is believed that *Bartonella* colonize ECs as the replicative “blood seeding niche” and require the action of BepA, BepC, BepF and BepG. (6) From the “blood seeding niche”, *Bartonella* are periodically seeded into the blood stream where they colonize erythrocytes and reinfect a new blood seeding niche for future reinfection waves. (7) *Bartonella* replicate a limited number of times and (8) stay in a slow physiological state. (9) *Bartonella* are ingested during an arthropod blood meal leading to a new cycle of infection in another host. Taken from Siamer et *al.* 2015. *Curr. Opin. Microbiol.*

1.3. Infection cycle

1.3.1. Transmission

Bartonella spp. are mainly transmitted between mammals via direct contacts such as scratches and bites or by numerous hematophagous arthropods vectors including lice, keds, flies, sand flies, fleas and potentially mites, ticks and spiders [31-34]. Current information suggest a highly adapted vector-species association between *Bartonella* and the vector, explaining the specificity observed between *Bartonella* species and their vectors (Fig. 1) [35]. When ingested by the vector, *Bartonella* replicate within the mid-gut lumen of the arthropods and are shed either with the faeces onto the mammalian skin or by the oral [35-38].

So far two types of transmission have been demonstrated among the genus *Bartonella* dependent on the feeding behavior of the vector. *B. bacilliformis* from lineage 1 and the ruminants-specific lineage 2 species share a common transmission by diptera, sandflies, and keds bites [39, 40]. The aggressive feeding behavior of biting arthropods provokes skin damage of the host and bleeding of capillaries providing a direct access to the blood for *Bartonella* by oral shedding [40]. Oral shedding of *Bartonella* via cat biting has also been reported [41].

In contrast to this aggressive mode of feeding, arthropods vectors spreading lineage 3 and lineage 4 *Bartonella* spp. such as fleas, lice or ticks use a less damaging blood feeding behavior preventing *Bartonella* to have direct access to the bloodstream [42-47]. In this configuration of transmission, *Bartonella* excreted in arthropod's faeces relies onto the mammalian skin. When the arthropod aspirates blood, it triggers irritations followed by scratching and subsequently by superficial inoculation of the bacteria into the derma of the host [28, 29, 34, 48]. Unlike *B. bacilliformis* and lineage 2 species that have a direct access to the blood, lineage 3 and lineage 4 species have to travel through the derma to reach the blood [27]. This step of the infection is named "dermal stage".

1.3.2. Dermal stage

Studies on lineage 4 species suggest that the dermal stage of *Bartonella* infection is characterized by the colonization of two consecutive undetermined replicative niches named "dermal niche" and "blood-seeding niche" (Fig. 2) [27, 49].

Up to date, this "dermal niche" remains unclear. However, it has been shown *in vitro* that lineage 4 *Bartonella* species such as *B. tribocorum* or *B. henselae* have a tropism for dendritic cells (DCs) [27, 30].

Therefore, DCs were proposed to be the “dermal niche” and might play a role for systemic dissemination from the dermal site of inoculation to the “blood-seeding niche” [50]. Indeed, it is believed that infected DCs carry *Bartonella* species into the permeable lymphatic circulation and subsequently to the bloodstream where *Bartonella* can colonize the “blood-seeding niche” [27, 28, 50]. The lymphatic circulation allows the bacteria to bypass the endothelial barriers, constituted by membrane matrix and endothelial cells (ECs), facilitating the access to the bloodstream [50]. The colonization of a “dermal niche” is dependent on the feeding behavior of the arthropod. Since the feeding behavior of lineage 2 and *B. bacilliformis*-vectors provide a direct access to the bloodstream and subsequently to the “blood-seeding niche”, these *Bartonella* species might not have to colonize a “dermal niche” [51]. In contrast, because lineage 3 and lineage 4 species are superficially inoculated into the derma by scratching, it is believed that the colonization of the “dermal niche” is a crucial step to travel through the dermis and reach the “blood-seeding niche”.

Following the infection of the “dermal niche” *Bartonella* colonize a “blood-seeding niche” which is also not fully determined *in vivo* [27, 28, 52]. *In vitro* experiments have shown the tropism of *Bartonella* spp. for ECs and proposed ECs to be the replicative “blood-seeding niche” [27, 29, 50, 51, 53]. In contrast to the dermal niche, the colonization of the “blood-seeding niche” is shared by all *Bartonella* lineages. The existence and the essentiality during *Bartonella* infection of this replicative niche have been supported by intravenous rat infection using *B. tribocorum* where bacteremia appeared only 5 days post inoculation [51]. It strongly suggests that a replicative niche might be present allowing a physiological reprogramming of *Bartonella* to afterward successfully colonize erythrocytes [51].

In both niches, *Bartonella* need to invade mammalian nucleated cells. The initial steps in the infection of nucleated cells are the adherence of the bacteria to the extracellular matrix and to the host plasma membrane [54, 55]. Such interactions involve surface proteins mediating contact between bacterial and host cell surfaces such as the trimeric autotransporter adhesin BadA (*Bartonella* adhesin A) of *B. henselae* and variably expressed outer membrane proteins (Vomps) of *B. quintana* [56, 57]. It has also been demonstrated for *B. henselae* that $HbpA_{Bhe}$ (hemin-binding protein) interacts with the membrane of ECs facilitating the entrance of the bacteria [58]. This observation argues in favor of ECs as the “blood seeding-niche” *in vivo.* In addition to promote ECs invasion, hemin binding proteins appear to be crucial for *Bartonella* to survive. Indeed, since *Bartonella* is a heme auxotroph α-proteobacteria, hemin binding and hemin uptake proteins allow the bacteria to process hemin in their environments such as the bloodstream of the host and the flea gut [59-61]. Furthermore, hemin binding proteins protect the bacteria from toxic concentrations of heme [62].

Following this adherence step, *Bartonella* subvert cellular signaling pathway allowing the persistent colonization of the targeted cell. To do so, *Bartonella* translocate a cocktail of effectors (*Bartonella*

effector proteins – Beps) via a type 4 secretion system (T4SS): the VirB/D4 T4SS [17, 61, 63]. The function of the VirB/D4 T4SS and the role of Beps during *Bartonella* infection will be discussed in more detail in paragraph 1.4.1.1 (p. 8) and 1.4.1.2 (p. 10).

1.3.3. Blood stage

The process of erythrocyte parasitism by *Bartonella* spp. has been mainly studied in rats infected with *B. tribocorum* [51]. In this model, after residing within the "blood-seeding niche", the bacteremia onset occurs 5-6 days post-infection by a synchronous wave of bacterial release in the bloodstream (Fig. 3) [51]. In general, erythrocytes colonization by *Bartonella* is characterized by four successive steps: adhesion, deformation, entry, and replication [22, 52, 64].

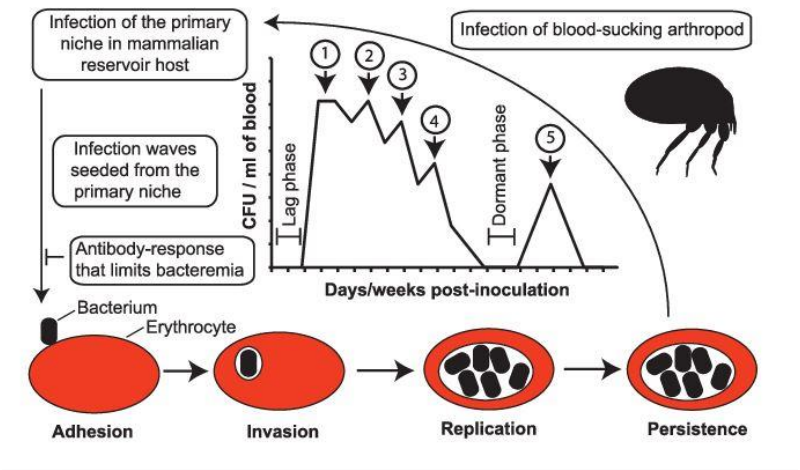

Figure 3. Schematic description of the cyclic waves of *Bartonella* spp. intraerythrocytic bacteremia in a mammalian reservoir host

After several abacteremic days post-inoculation (lag phase), bacteremia is initiated with a high number of bacteria in the bloodstream (arrow 1) with bacteria invading erythrocytes and a new blood seeding niche. It is believed that blood seeding niches releases synchronously bacteria in the bloodstream at regular intervals (arrows 2-4) until a specific antibody overcome the infection. In some cases, bacteremia may peak (arrow 5) after several abacteremic days (dormant phase). The intraerythrocytic lifestyle of Bartonella allow its transmission to other mammalian host mediated by blood sucking arthropods. Taken from Pulliainen et *al.* 2011. *FEMS*

During erythrocytes adhesion, studies on *B. bacilliformis* suggested *Bartonella* spp. to target proteins localized at the surface of the red blood cell such as spectrin, Band3, and glycophorins A and B [65, 66]. Interaction between the pathogen and these membrane proteins and attachment to erythrocytes are mediated either by the Trw type 4 secretion system (Trw T4SS) or by the flagellum [22, 32, 55, 64]. These two virulence factors will be discussed in more details in paragraphs 1.4.2.2 (p. 12) and 1.4.2.3 (p. 13), respectively.

Upon attachment, *B. bacilliformis* and *B. henselae* cause a severe deformation of the red blood cell membrane, which might facilitate the uptake of the bacteria [67, 68]. A small hydrophobic molecule

named deformin, which is able to bind serum proteins such as albumin, triggers erythrocyte-membrane deformation [69].

The exact process of *Bartonella* entry into erythrocytes remains unclear and, unlike nucleated cells infected during the dermal stage, there is no evidence for active membrane transport in erythrocytes [55]. However, since *Bartonella* are able to bind Band3, it has been speculated that the bacteria might disturb the overall organization of the red blood cell membrane and its integrity, therefore, facilitating the entrance within the cell [32, 66]. A similar invading process has been described for *Plasmodium falciparum*, causative agent of malaria [29]. Furthermore, one study revealed the presence in *B. bacilliformis* of an invasion-associated locus A and B (*ialAB*) encoding for two membrane proteins named IalA and IalB. This locus has been shown to confer an erythrocyte-invasive phenotype when expressed in *E. coli*, indirectly implicating its role in *B. bacilliformis* erythrocyte invasion [70, 71]. IalA is a (di)nucleoside polyphosphate hydrolase which helps bacterial survival during erythrocyte colonization by reducing levels of stress-induced dinucleosides. During invasion, IalB is exported to the pathogen surface and functions as a crucial invasion factor [70, 71].

Following adhesion and deformation of the erythrocyte membrane, bacteria are internalized into a membrane-bound compartment where they replicate until the critical limit of eight bacteria per erythrocyte is reached, which is constant during the remaining life span of the invaded erythrocytes [51]. It has been shown that *B. tribocorum* colonize mature erythrocytes of different ages with similar efficiency [51]. After the synchronous erythrocyte invasion on 5-6 days post-infection, the number of infected erythrocytes decreases over time due to erythrocyte turnover [51]. However, the initial wave of erythrocyte infection is followed by reinfection waves occurring in intervals of 3-6 days suggesting that *Bartonella*, when released in the bloodstream, might re-infect a new “blood-seeding niche” as a bacterial reservoir for a future release (Fig. 3) [51]. This cyclic bacterial release in the bloodstream is in accordance with cyclic fever relapses in patients affected by the trench fever (five days fever) caused by *B. quintana* [72]. The intraerythrocytic localization has also been demonstrated for other *Bartonella* species such as *B. grahamii* in mouse, *B. henselae* in cat or *B. bacilliformis* in human [24, 67, 73, 74].

This hemotropism represents a clear advantage for *Bartonella* to survive in the host by being hidden from the immune system and subsequently by being imbibed during an arthropod blood meal, and thus transmitted to another host [29].

1.4. Virulence factors

1.4.1. The VirB/D4 T4SS and its *Bartonella* effector proteins

1.4.1.1. The VirB/D4 T4SS

The VirB/D4 T4SS is a multi-protein translocation channel that spans the entire cell envelope. The Type 4 secretion machinery in *Bartonella* spp. is composed of 11 proteins present in multiple copies (VirB2-11) and expressed from a conserved operon (Fig. 4). An additional Type 4 coupling protein (T4CP), named VirD4 protein, is also expressed but unlike the *virB* operon, *virD4* is expressed under its own promoter [75]. It has been reported that the *virB* operon is under the control of the BatR/BatS two-component system [76]. BatR expression is controlled by the sigma factor RpoH1, whom levels are regulated by the stringent response components DksA and SpoT [76]. By using a sigma factor, *Bartonella* are able to regulate the expression of their virulence factors in response to environmental changes such as nutrient availability or pH variation [77-79]. Furthermore, it has been also demonstrated that BatR/BatS has an optimum activity at the physiological pH of the blood (pH 7.4) [75].

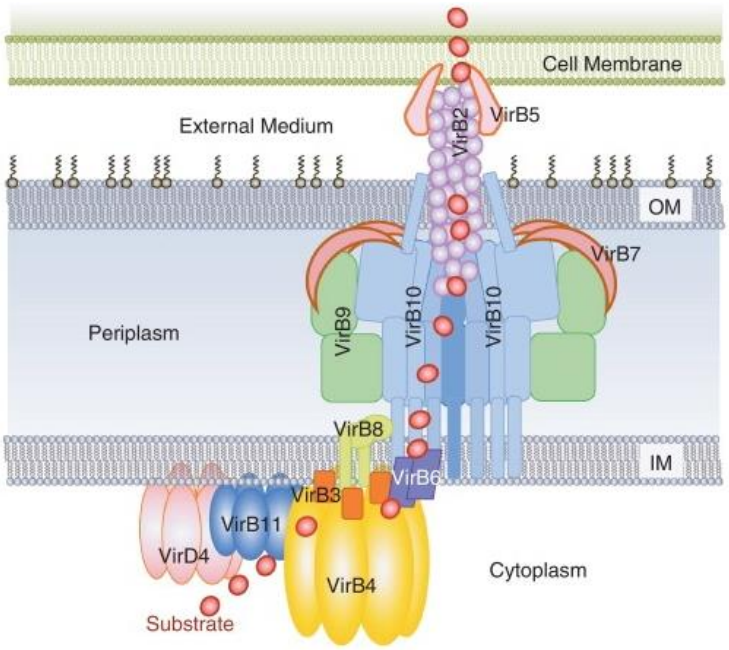

Figure 4. Schematic description of the overall organization of the VirB/D4 T4SS

VirD4 (pink), VirB4 (yellow) and VirB11 (blue) are ATPases and compose the cytoplasmic inner membrane complex with VirB3 (orange), VirB6 (purple) and VirB8 (light green). The core complex consists of VirB7 (brown), VirB9 (green) and VirB10 (light blue). The pilus is composed of VirB2 (light purple) and VirB5 (light pink). The path taken by substrates through the VirB/D4 T4S machinery is indicated by red dots. The VirB/D4 T4SS spans the entire cell envelope and allows the translocation of substrates into the target cell. Taken from Waksman et *al.* 2014. *Curr. Opin. Microbiol*

The general organization of the VirB/D4 T4SS is divided into 3 major complexes: The core complex (CC), the cytoplasmic inner membrane (CIM) and the extracellular pilus (Fig. 4) [80-83]. The CC is a large complex spontaneously assembled and consisting of VirB7, VirB9 and VirB10 forming a channel

spanning both membranes (inner membrane -IM- and outer membrane –OM-). The CC creates a channel through the periplasm of the bacteria and connects the CIM to the extracellular pilus [84]. The CIM is composed of two ATPases, VirB4 and VirB11, that provide the energy for the assembly of the different parts of the CC and for Bep translocation. The VirB3, VirB6 and VirB8 proteins anchor VirB4 into the IM and connect the CIM and the CC. The coupling protein VirD4, another ATPase, recruits, and transfers cognate substrates to VirB11, which delivers the substrate to the machinery. The three ATPases VirD4, VirB4, and VirB11 shared a conserved triphosphate-binding site called walker A motif [85]. Any mutation within this sequence arrests substrate transfer suggesting that these subunits energize the transfer reaction in an ATP-dependent manner [86-88]. Unlike VirB4 and VirB11, VirD4 exclusively provides energy for substrate translocation [85]. The extracellular pilus is composed of VirB2 and VirB5. Numerous copies of VirB2 that form the filament and VirB5 proteins are localized at the extremity of the pilus and might function as adhesin that mediates binding to specific host receptors such as integrin or fibronectin [89].

The similarity of the VirB/D4 T4SS with the bacterial conjugation system suggests that the *virB/D4* locus was acquired via horizontal gene transfer from a plasmid. In the current model, the *virB/D4 T4SS* locus was independently acquired in all lineage 3 and lineage 4 species and in the recently discovered *B. ancashensis* (lineage 1) with a single primordial effector [17, 90, 91]. Consistently, there is no trace of this machinery in the lineage 2 species and in *B. bacilliformis* (lineage 1) suggesting that they never acquired the machinery [92]. Remarkably, these latter species share a similar mode of transmission by biting diptera [63].

The VirB/D4 T4S machinery has been shown to be crucial for lineage 4 *Bartonella* species to establish a successful colonization of the host. Rat infections with *Bartonella* strains deficient for *virB4* and *virD4* show an abacteremic phenotype suggesting the essentiality of the machinery to establish bacteremia [93]. These observations are consistent with the fact that the VirB4 protein is a crucial component of the T4S machinery and the coupling protein VirD4 is essential for the delivery of the substrate to the machinery [93, 94]. Wild type phenotype was recovered when the mutants were *trans*-complemented confirming that the abacteremic phenotype is associated with deletion of *virB4* or *virD4.* Interestingly, the plasmid used for complementation was lost during the blood stage of the infection without any impact on the infection. This strongly suggests that VirB/D4 T4SS is only needed during trafficking from the “dermal niche” to the “blood-seeding niche”, before the erythrocyte colonization [93]. This assumption is also supported by the correlation between the presence of the VirB/D4 T4SS and the blood-feeding behavior [63]. Indeed, *Bartonella* spp. lacking VirB/D4 T4SS (*B. bacilliformis* and lineage 2 species) have direct access to the blood and do not have to pass through the dermis (Fig. 1) [17].

During *Bartonella* infection, the main role of the VirB/D4 T4SS is to translocate Beps and the success of *Bartonella* infection relies on the subversion of host cellular process by these effectors [93, 95].

1.4.1.2. *Bartonella* effector proteins (Beps)

The diversity of Beps observed in lineage 3 and lineage 4 is the result of two parallel radiative evolutions after an independent acquisition in the lineage 3 and 4 of the VirB/D4 T4SS with a single primordial effector suggested to be Bep1 or BepA in lineage 3 and lineage 4, respectively [17, 61]. Concerning *B. ancashensis* (lineage 1), an independent acquisition of the VirB/D4 T4SS locus with a single Bep also occurred [18]. A succession of effector gene duplications and functional diversifications evolved into complex effector repertoires (Fig. 5) [17].

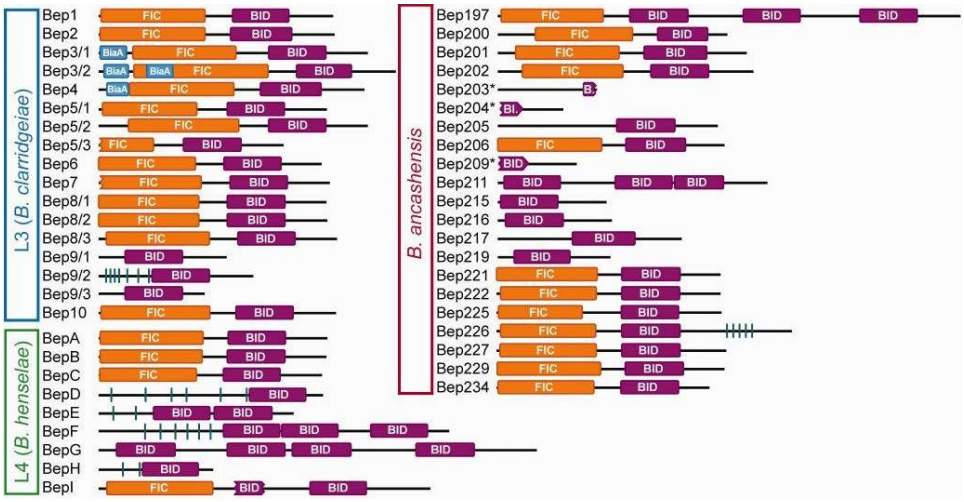

Figure 5. Representation of the three Bep repertoires among *Bartonella* lineages

The diversification of Beps from *B. ancashensis* (lineage 1), lineage 3 and lineage 4 species results from gene duplication and functional diversification of a primordial effector horizontally acquired with the *virB* locus. It is believed that the acquisition of the *virB* locus and the ancestral *bep* occurred independently in *B. ancashensis* (lineage 1), lineage 3 and lineage 4. All Beps among *Bartonella* spp. share a bipartite Type 4 secretion signal composed of a C-terminal Bep intracellular delivery (BID) domain (purple) and a positively charged C-tail. The presence of the FIC domain (orange) and the tyrosine phosphorylation (pY) motifs (vertical lines in cyan) is Bep-dependent. Taken from Harms et *al.* 2017. *Genome Biol. Evol.*

Despite these radiative evolutions and diversifications, all Beps among *Bartonella* spp. share a bipartite T4S signal composed of a C-terminal Bep intracellular delivery (BID) domain and a positively charged C-tail (Fig. 4) [28, 96]. Because translocation into host cells via the T4SS evolved from DNA conjugation machineries, it is believed that the primordial *Bartonella* effector acquired this bipartite signal from relaxases of alphaproteobacterial conjugation systems to facilitate translocation into the targeted cell [95, 97]. Furthermore, beyond being only a secretion signal several studies highlight the ability of the

BID domain to interact with host cellular function [27, 98]. A striking example is the BID domain of BepA from *B. henselae* that interacts with host adenylyl cyclase and subsequently disturbs cyclic AMP production, a second messenger involved in a multitude of cellular functions such as apoptosis [99, 100].

In addition to this T4S signal, most of the Beps harbor a N-terminal filamentous induced cAMP (Fic) domain mediating post-transcriptional modification such as AMPylation, phosphorylation or phosphocholination (Fig. 5) [101-103]. Interestingly, Beps lacking the Fic domain harbor tandem-repeated pY-motifs mimicking eukaryotic host proteins. These Beps are phosphorylated by host cellular kinases allowing the interaction of the effectors with SH2 domain proteins, which are key elements in host signaling [95, 104]. Furthermore, Beps-containing pY-motifs are present in lineages 1,3 and 4suggesting pY-motifs evolved in all three lineages and this motif plays a fundamental role during infection (Fig. 5) [96].

Up to date, the study of Beps has been mainly performed on Beps from *B. henselae* and *in vitro* studies have demonstrated a variety of cellular phenotypes associated with these *Bartonella* effectors [105]. It is believed that BepE plays a crucial role in infected-DCs migrating from the infection site to the "blood-seeding niche" by counteracting the effect of BepC, which may otherwise trigger cell fragmentation [27]. BepA prevents apoptosis and protects infected DCs from being cleared by the immune system of the host [106]. By hijacking the filamentous actin (F-actin) organization of DCs, BepC, BepF, and BepG allow the engulfment of a bacterial cluster within the *Bartonella*-containing vacuole tightly surrounded by actin fibers named "invasome" [27, 29, 100]. Furthermore, *in vitro*, by interacting with the signal transducer and activator of transcription 3 (STAT3), BepD inhibits, on one hand, the secretion of the pro-inflammatory cytokine TNFα by DCs and macrophages and, on the other hand, stimulates the production of interleukin 10 (IL-10), an anti-inflammatory cytokine. (Unpublished data from Y-Y Lu, C. Schmutz, and I. Sorg). Of note, all these experiments have been performed *in vitro*.

Taken together, all these findings strongly suggest the significance of the VirB/D4 T4SS and the translocation of *Bartonella* effector proteins during the infection cycle from the inoculation site to the "blood-seeding niche". However, the exact process of the *in vivo* infection remains unclear and needs further investigation to clarify the exact role of Beps during *Bartonella in vivo* infection.

1.4.2. Trw Type 4 secretion system

The Trw T4SS, restricted to lineage 4 and *B. australis*, is a multiprotein complex that spans the bacterial inner and outer membrane [90]. Unlike VirB/D4, the Trw T4SS does not harbor a coupling protein necessary for translocation of substrates and is thus believed to have lost this function during evolution [54]. The Trw T4SS of *Bartonella* spp. is highly homologous to the enterobacterial Trw conjugation system encoded by the enterobacterial antibiotic-resistance plasmid pR388 [107]. Genetic studies revealed a rather recent lateral acquisition of the *trw* locus in *Bartonella* spp. which was followed by functional diversification [97]. Consistent with the inability to translocate proteins, host specificity allocated to the Trw T4SS is not reliant on effector translocation but on the exposure of specific proteins on bacterial surface.

Indeed, the highly conserved *trw* T4SS loci are collinear except for the presence of numerous tandem repeat gene duplications in *B. tribocorum*. *TrwJ, trwI, trwH,* and *trwL* are present in variable copy numbers in the *trw* locus (Fig. 6) [22]. *TrwL* and *trwJ*, homologs of *virB2* and *virB5* respectively, encode for different TrwL and TrwJ proteins, the main components of the pilus, which mediates host attachment to erythrocytes and subsequently determine the host specificity (Fig. 6) [22, 32, 52, 108]. Indeed, TrwJ paralogs bind specifically glycoprotein Band3 at the surface of red blood cells (RBCs). Furthermore, variability of this gene among *Bartonella* species has a key role in adaptation to new mammalian hosts and thus in the adaptive radiation of *Bartonella* species [22, 52, 109]. Based on the essential role of the Trw system for erythrocyte invasion it is conceivable that these variant pili may facilitate the interaction with different erythrocyte receptors, either within the reservoir host population (e.g. different blood group antigens), or among different reservoir hosts [54]. At present, the erythrocytic receptor of TrwL remains unknown [32].

Remarkably, in the modern lineage 4, the acquisition of the Trw T4SS correlates with the loss of the flagella suggesting that Trw T4SS might functionally replace flagella during infection. The significance of the Trw T4SS to establish erythrocyte infection has been demonstrated for several *Bartonella* species such as *B. henselae, B. tribocorum, B. quintana* or *B. birtlesii* [22, 32, 61, 108, 110]. For each *Bartonella* spp., colonization and intra-erythrocytic persistence are limited to a specific reservoir host, where its specificity is defined by the capacity of *Bartonella* species to adhere exclusively to erythrocytes of their reservoir host in a Trw T4SS-dependent manner. A Trw-T4SS deficient *Bartonella* species or a host-unspecific Trw-T4SS lead to a defective intra-erythrocytic infection establishment [22].

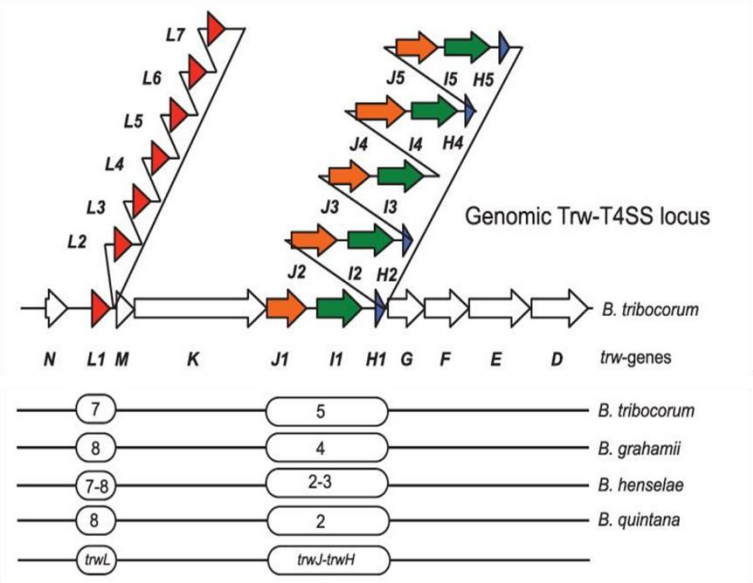

Figure 6. Genetic organization of the *trw* locus in *B. tribocorum*

Host specificity is determined by the Trw-T4SS, which mediates erythrocytes adhesion. This scheme represents the genetic organization of the chromosomal *trw* locus encoding the Trw-T4SS in *B. tribocorum* . One major characteristic of the *trw* locus in *Bartonella* spp. is the partial expansion by gene duplications and differentiation of *trwL, trwj, trwI* and *trwH*, which are determining in host specificity. Numbers under the scheme of *trw* locus in *B. tribocorum* indicate the copy number of duplicated genes in other *Bartonellae*. Taken from Pulliainen et *al*. 2011. *FEMS*.

1.4.3. Flagella

The flagellum is a rotating semi-rigid helical filament that is anchored within the bacterial membrane and driven by the influx of protons or Na^+ ions direction confers motility to the bacteria in a liquid environment. Flagella are regularly associated with virulence mechanism in many bacterial pathogens such as biofilm formation, Pathogen-associated molecular pattern (PAMP), adherence or invasion (*Escherichia coli, Pseudomonas aeruginosa*, *Helicobacter pylori* or else *Salmonella enterica serovar*) [111-114]. Differences in flagella number, position, and structures among bacterial species underlie the bacterial adaptation to a wide range of encountered environment.

Among *Bartonella* genus, the flagellum is expressed in the ancestral *B. bacilliformis* from lineage 1, in lineage 2 and in lineage 3 species. The role of flagella during *Bartonella* infection has been extensively described in *B. bacilliformis* [64, 65, 67, 115]. *In vitro*, when erythrocytes are infected with *B. bacilliformis*, a reversible binding of the bacteria to red blood cells is observed. This interaction initiates irreversible membrane trenches and invagination of erythrocyte induced by the small hydrophobic molecule deformin [67, 115]. For *B. bacilliformis*, combination of the deformin activity with bacterial motility leads to a forced endocytosis of the bacteria within a Bartonella-vacuole compartments in the erythrocyte cytoplasm [67, 68]. *In vitro*, a non-motile *B. Bacilliformis* bound poorly, if at all, to erythrocytes. Erythrocytes invasion was reduced by approximatively 98% with an antiflagellin serum confirming the key role of flagella in erythrocyte invasion for *B. bacilliformis* and very likely for other flagellated *Bartonella* spp. [64, 108]. However, is still unclear if the flagella directly bind to RBCs or the

flagella-dependent mobility of the bacteria enhances the interaction between the bacteria and the RBCs.

1.4.4. Vbh Type 4 secretion system

As the VirB/D4 T4SS system, the Vbh T4SS is closely related to the conjugative T4S-machineries of other Rhizobiales such as the AvhB T4SS on pAT of *Agrobacterium tumefaciens* [61, 90, 92, 95].

In *Bartonella* spp., the Vbh T4SS and its coupling protein TraG are either encoded on a plasmid, like pVbh of *B. schoenbuchensis,* pBGR3 of *B. grahamii,* or chromosomally in a genomic island related to these plasmids [92]. As the sole T4S-machinery in lineage 2 and the fact that its substrate is a toxin (VbhT) similar to Beps, it is assumed that this machinery plays a role in pathogenesis. Interestingly, the plasmid pVbh of *B. schoenbuchensis* encoding for the machinery and the T4CP acts as a classical conjugation system translocating the toxin VbhT into the targeted cell associated with the relaxase suggesting VbhT as an interbacterial rather than an interkingdom effector [92]. Up to date, studies demonstrated that VbhT inactivates gyrase and topoisomerase IV by AMPylation [116]. These two targets belong to the class of type II isomerase and play a crucial role in DNA topology [116]. Since the biological role of the resulting post-transcriptional modifications (PTMs) remains unclear, it needs further investigations [116].

Vbh T4SS, the only secretion machinery presents in lineage 2 and some lineage 4-species, is considered as a pathogenic factor crucial for *Bartonella* infection for these lineages (Figure 1.1) [29]. However, *vbh* locus vestiges with deleterious mutations are observed in some lineage 4 species and *traG* or/and *traA,* a crucial component in the transfer and replication (Dtr) machinery, are also missing [92]. Vbh T4SS appears to be functional only in species that do not encode a functional VirB/D4 T4SS [92]. The chromosomally *vbh* locus represents vestiges of a deteriorating conjugation system and suggests that the Vbh T4SS tends to disappear [18].

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Aims of the thesis

The work of my thesis, started in September 2014, can be separated in two parts.

The first part intended to establish an *in vivo* infection model for *Bartonella* lineage 3 species by using *Bartonella 1-1C*, previously described as a rat-specific pathogen. After characterization of the course of infection in rats with intradermal and intravenous delivery of the bacteria, I aimed to study the contribution of the virB/D4 T4SS, the lineage 3-specific *Bartonella* effector proteins, and the flagellum during the infection process. To do so, I infected rats via the two routes of infection with different *B1-1C* mutants. Additionally, I investigated the ability of *B1-1C* to colonize host erythrocytes by performing gentamicin protection assay and FACS analysis on rat infected blood.

The second part aimed to clarify the contribution of *Bartonella* effector proteins during *in vivo* infection of lineage 4 by using the well-characterized *Bartonella tribocorum* infection model. As a first step, I infected intradermally and intravenously rats with a strain deficient for all Beps. Following the bacteremic phenotype observed with this Bep-free mutant, I reoriented my research on a putative additional effector named YopJ. To gain a deeper understanding of the role of this effector, I performed *in-silico* analysis on its prevalence among the *Bartonella* genus and I infected rats with *B. tribocorum* strains lacking YopJ.

Research Article I

Bartonella 1-1C: Establishment of an *in vivo* Infection Model for a Lineage 3 Species

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Abstract

The Gram-negative α-proteobacterial genus *Bartonella* comprises arthropod-borne pathogens that trigger a long-lasting intraerythrocytic bacteremia. *Bartonella* infection is characterized by two successive stages: the dermal stage characterized by the subversion of dendritic cells and the invasion of the lymphatic circulation and, the blood-stage characterized by the colonization of erythrocytes. *Bartonella* species are classified into four different lineages based on a phylogenetic analysis and on the nature of the virulence factors involved in the infection process. Studies on lineage 4 *Bartonella* species revealed the involvement of two Type 4 secretion systems (T4SS) during the infection process: the VirB/D4 T4SS might assist *Bartonella* during the dermal stage and the Trw T4SS is suggested to contribute to host-specific infection of erythrocytes. Lineage 3 and lineage 4 species harbor a VirB/D4 T4SS but each lineage translocates a different set of effectors and lineage 3 species harbor flagella instead of a Trw T4SS, which is confined to lineage 4 species.

In this study, investigations on the rat-specific lineage 3 strain *Bartonella 1-1C* (*B1-1C*) allowed the establishment of a lineage 3 *in vivo* infection model in rats by developing appropriate growth conditions for intravenous (*i.v.*) and intradermal (*i.d.*) infections. Compared to the well-studies lineage 4, rat infected with *B1-1C* develop bacteremia with a delayed-onset and a shorter duration. FACS analysis combined with a gentamicin protection assay on infected blood revealed the inability of *B1-1C* to colonize erythrocytes explaining the short bacteremia. This finding is unexpected since one hallmark of *Bartonella* pathogenicity is a persistent intraerythrocytic bacteremia. Finally, in this study we perform genetic engineering on *B1-1C* to generate *B1-1C* Δ*flaA* and *B1-1C* Δ*virD4* in order to characterize the role of the flagella and the VirB/D4 T4SS during the lineage 3 *B1-1C* strain infection process. As results, *in vivo* infections with *B1-1C* mutants revealed an attenuation of the bacterial fitness preventing any conclusion on their contribution during the infection.

Introduction

The facultative intracellular α-proteobacterial genus *Bartonella* comprises Gram-negative arthropod-borne pathogens characterized by a long-lasting intraerythrocytic parasitism in their specific mammal reservoir host(s) [1-7]. From the dermal inoculation to the bloodstream, *Bartonella* species are known to invade two sequential niches, the dermal niche and the blood-seeding niche considered to comprise dendritic and endothelial cells, respectively [8, 9]. When *Bartonella* is released into the bloodstream, it colonizes erythrocytes and replicates within a vacuolar membrane-bound compartment until reaching the critical number of height bacteria per cell. Then, *Bartonella* persists intracellularly in a non-proliferating state for the remaining lifetime of the erythrocyte, an immune-privileged environment [3, 9]. According to the nature of the virulence factors involved during the infection process and based on a phylogenetic analysis *Bartonella* spp. are divided into 4 different lineages [10, 11]. The ancestral lineage 1 composed of the deadly *B. bacilliformis* and the recently discovered *B. ancashensis* (pathogens associated with the Carrion's disease); Lineage 2 comprises ruminant-specific *Bartonella* species; Lineage 3 consists of closely species; and the most recent and species-rich lineage 4 [12].

Based on the well-characterized infection cycle of the lineage 4 strain *Bartonella tribocorum*, two Type 4 secretion systems (T4SS) are engaged during the infection process: the VirB/D4 T4SS involved during the dermal stage and the Trw T4SS involved during the blood stage. Translocation of *Bartonella* effector proteins (Beps) via the VirB/D4 T4SS has been identified as a critical virulence mechanism for a successful infection [13, 14]. Horizontal acquisition of the same *virB/D4 T4SS* locus with a single ancestral effector has occurred independently in lineage 3, lineage 4 and in *B. ancashensis* (lineage 1), and was followed by chromosomic integration [12, 15-17]. The parallel evolution of these sets of effectors, driven by the pathoadaptation to the host, led to the emergence of three distinct arsenals [10, 12, 18].

The success of the infection relies also on the capacity of bacteria to colonize erythrocytes. Adhesion of the bacteria to erythrocytes is mediated by the Trw T4SS in *Bartonella* lineage 4 or by the flagella for species of the other lineages. During infection it has been speculated that the interaction between the bacteria and membrane protein Band3 disturbs the integrity of the red blood cell membrane promoting the entrance of *Bartonella* [1, 19-23]. Remarkably, the genomic analysis highlighted that the Trw T4SS is confined to lineage 4 spp. and it is assumed that it functionally replaced the flagella as a host-specific virulence factor for erythrocytes invasion [1, 5, 19, 22]. Acquisition of the Trw T4SS may have led to an increased capacity for host adaptation explaining the extensive adaptive radiation observed for lineage 4 species compare to other lineages [5, 12, 15].

Concerning host specificity, we know that in lineage 4 it is also mediated by the Trw T4SS [1, 24, 25]. Although the Trw T4SS is lacking in lineage 3 species, host-specificity in these lineage 3 species has been reported [12, 26]. Up to date, we know that *B. rochalimae* (*Bro*) is Canidae-like specific such as dog or foxes [27] while, *B. clarridgeiae* (*Bcl*) is cat-specific [28] and *B. AR15-3* (*BAR15*) is red squirrel-specific [29]. So far the exact process of host specificity and erythrocyte colonization remains unknown for lineage 3 species but since they bear flagella instead Trw T4SS, it is conceivable to believe that the flagellum plays a major role in erythrocyte adhesion and/or colonization as a mechanical force or as an adhesion mechanism [30]. Studies on *B. bacilliformis* (lineage 1) reported that obstruction of flagella by antibodies decreased significantly adhesion to erythrocytes and flagella deficient *B. bacilliformis* strain does not bind to erythrocyte [21]. Remarkably, flagella from *B. bacilliformis* alone do not attach to erythrocytes indicating that the interaction between flagellated *Bartonella* and erythrocytes remains unclear [31].

Since lineage 3 species harbor a different arsenal of Beps from lineage 4 spp. [10, 12, 18] and possesses a flagellum instead of a Trw T4SS [5, 12], we were interested in studying the infection cycle of this lineage. *Bartonella 1-1C* (*B1-1C*) appeared to be the best lineage 3-candidate to work *in vivo* for several reasons: firstly, phylogenetic analysis on housekeeping genes classifies *B1-1C* as a lineage 3 species like *B. clarridgeiae* (*Bcl*)*, B. AR15-3* (*BAR15*) and *B. rochalimae* (*Bro*) [32]. Secondly, *B1-1C* harbors a set of effectors named Bep1 to Bep10 which are different from lineage 4 species. Thirdly, *Bartonella 1-1C* was isolated from *Rattus norvegicus* in Taiwan suggesting *B1-1C* to be the only lineage 3 species known so far having rat as a natural reservoir host, which was convenient for our *in vivo* laboratory studies since we worked with Wistar rats, a strain of *Rattus norvegicus domestica* [33].

In this study, we establish the appropriate growth conditions for successful intravenous (*i.v.*) and intradermal (*i.d.*) infection with the lineage 3 *B1-1C* strain. We then compared the infection cycle with the one of the well-characterized lineage 4 *Bartonella tribocorum* and gentamicin protection assay and FACS analysis of infected blood were performed to evaluate erythrocyte colonization. Finally, we genetically manipulate *B1-1C* to generate *B1-1C* Δ*flaA* and *B1-1C* Δ*virD4* to have a better understanding of the role of the flagella and the VirB/D4 T4SS during the lineage 3 *B1-1C* species infection process.

Results

As there was no experimental infection model for lineage 3 to date, the interest in first place was to characterize the infection kinetic.

Determination of optimal laboratory growth conditions for reliable intradermal (*i.d.*) and intravenous (*i.v.*) rat infections

Our first goal was to establish more appropriate laboratory growth conditions for reliable *i.d.* and *i.v.* rat infections. In the literature, *Bartonella* spp. are mainly grown on enriched-media such as HIA (Heart infusion agar), CBA (Columbia blood agar) or TSA (Tryptic Soy agar) media supplemented with blood at 35°C in a moist atmosphere with 5% CO_2. Adding blood to the media provides a crucial source of hemin since *Bartonella* spp. are unable to synthetize this growth factor [34, 35]. In previous rat infections with lineage 3 species performed in our group, P. Engel cultivated bacteria for 3-5 days on TSA plates supplemented with 5% of defibrinated sheep blood [12]. Taking into account that there are medium- and temperature-dependent growth differences *in vitro* between diverse isolates of the *Bartonella* genus, we compared two different growth media (HIA and TSA) and different cultivation times to optimize the laboratory growth conditions [36]. We decided to test HIA rather than CBA because HIA is the growth medium generally used for the well-described *B. tribocorum in vivo* infections. To note, *Bartonella* spp. can be grown between 35-37°C but with a significantly higher growth rate at 35°C explaining our choice to grow bacteria at this temperature in a humidified environment [36].

Groups of 5 rats were intradermally infected with *B1-1C* WT strain grown either on HIA for 3 days (Fig. 1A), 7 days (Fig. 1B) or 10 days (Fig. 1C) or on TSA for 3 days (Fig. 1D). A correlation between the cultivation time on HIA plates and the rate of infection was observed, showing that the longer was the cultivation on plates the higher was the infection rate (Fig. 1A, B, C). Thus, we assumed that over time the nutrient availability on plate decreased, thereby inducing a stringent response in the bacteria leading to the expression of the VirB/D4 T4SS [6]. This assumption was supported by previous data showing that the expression of the VirB/D4 T4SS in *Bhe* (Lineage 4 species) is under control of the stringent sigma factor RpoH1 and the BatR/BatS two-component system [37].

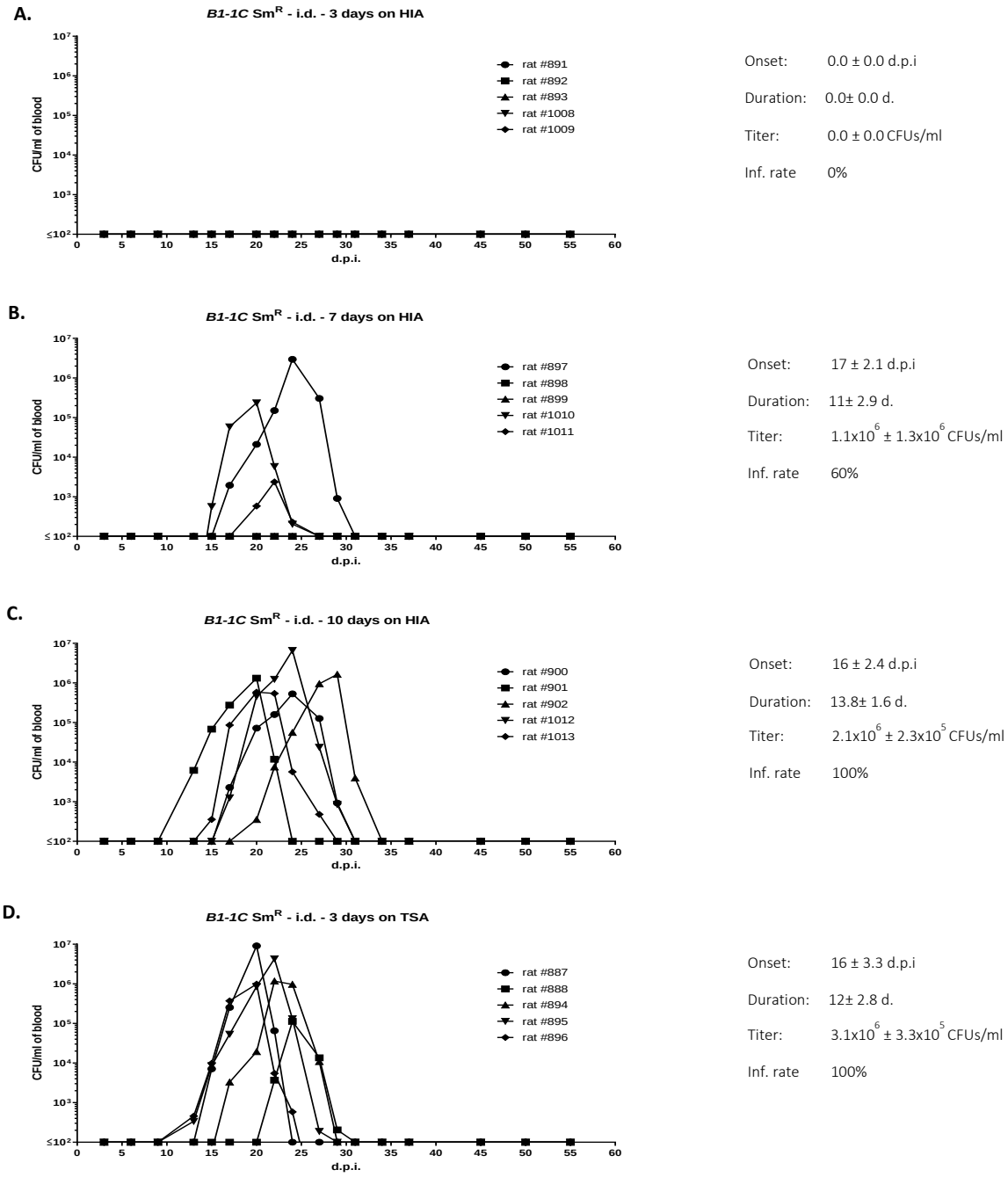


Figure 1: Determination of the appropriate growth conditions for reliable *i.d.* and *i.v.* rat infections

Groups of 10 weeks old Wistar rats were intradermally infected (inoculum $1x10^7$ CFUs, n = 5) in the ear with *B1-1C* WT. Bacteria were grown for 3 days at 35°C and 5% CO_2 on either HIA or TSA plates supplemented with defibrinated sheep blood. Plotted graphs represent time course of single animals (indicated by the different animal numbers) from which bacteria were recovered at a given time point post-infection. Each value corresponds to an average (CFU/ml of ml of blood) acquired from serial dilutions. Graphs (A), (B) and (C) represent bacteremia triggered by *B1-1C* WT grown on HIA plate for 3, 7 or10 days, respectively. Graph (D) represent bacteremia triggered by *B1-1C* WT grown on TSA for 3 days. Table next to the graphs indicates the mean of the onset, the duration, the maxmimum titer and the infection rate of the bacteremia.

media in order to reduce the number of animals for experimentation (Reduce, Replace, Refine – Animal welfare).

Based on these data, growing *B1-1C* WT on TSA plates supplemented with defibrinated sheep blood for 3 days at 35°C with 5% CO_2 turned out to be the more appropriate growth conditions for our i*n vivo* experiments. Therefore, we didn't test CBA media in order to save animals.

Generation and analysis of the streptomycin-resistant *B1-1C* strain (*B1-1C* Sm^R)

During the procedure of gene replacement, we used a suicide mutagenesis vector harboring a kanamycin cassette for the positive selection and a streptomycin sensitive cassette for the negative selection (described in the materials and methods section). Thus, we generated the streptomycin-resistant strain *B1-1C* Sm^R to allow the selection of bacteria having lost the mutagenesis plasmid during the second homologous recombination on HIA plates supplemented with streptomycin.

Streptomycin resistance is mediated either by mutations in the *rpsL* gene encoding the ribosomal S12 protein or, less frequently, in the *16s rRNA* gene [38]. We generated a spontaneous streptomycin-resistant *B1-1C* mutant by spreading bacteria on TSA plates containing streptomycin at a concentration of 100 µg/ml. Grown bacteria were sequenced and we selected a mutant carrying the K43R mutation in the S12 protein encoded by the *rpsl* gene. The K43R mutation was chosen because of its high frequency in nature and its reliability in both genetic selection and complementation [39, 40].

To assess a potential effect of the streptomycin resistance on bacterial fitness of *B1-1C* WT, we infected intradermally groups of rats with either *B1-1C* WT or *B1-1C* Sm^R and monitored bacteremia (Fig. 2A, B). Compared to *B1-1C* WT, animals infected with *B1-1C* Sm^R developed bacteremia with no difference for neither the onset, the duration nor the titer (Fig. 2D). A two-way ANOVA test comparing bacteremia curves of *B1-1C* WT and *B1-1C* Sm^R confirmed that there was no significant difference (*p*-value = 0.7731). Therefore, *B1-1C* Sm^R was used for further genetic manipulations and animal infections in this study.

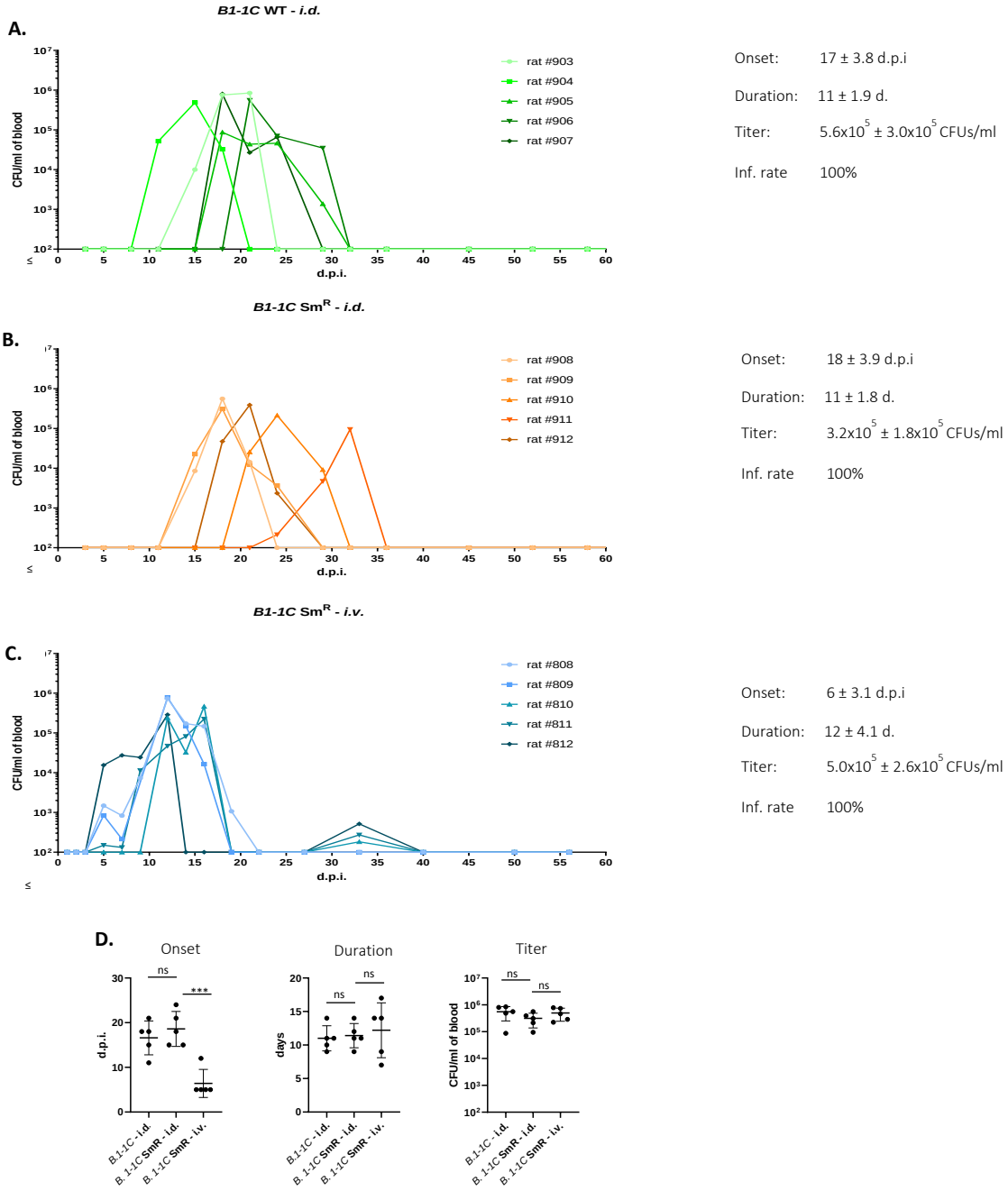


Figure 2: *B1-1C* Sm^R triggers bacteremia not significantly different from *B1-1C* WT

Bacteria were grown for 3 days at 35°C and 5% CO_2 on TSA plates supplemented with 5% of defibrinated sheep blood. Plotted graphs represent time courses of single animals (indicated by the different animal numbers) from which bacteria were recovered at a given time point post-infection. Each value corresponds to an average (CFU/ml of blood) acquired from serial dilutions. (A, B) Groups of 10 weeks old Wistar rats were intradermally infected (inoculum 10^7 CFUs, n = 5) with either (A) *B1-1C* WT or (B) *B1-1C* Sm^R. Animals infected with *B1-1C* Sm^R showed no significant difference with animals infected with *B1-1C* WT. (C) Groups of 10 weeks old Wistar rats were intravenously infected (inoculum $2x10^8$ CFUs, n = 5) with *B1-1C* Sm^R. Values indicated next to the graphs correspond to the means and standard deviations of the onset, the duration, the maximum titer and indicate the infection rate of bacteremia induced by the same *Bartonella* strain in different animals (n = 5). (D) t-test were performed to compare bacteremia onset duration and maximum titer between *B1-1C* WT (*i.d.*) vs. *B1-1C* Sm^R (*i.d.*) and *B1-1C* Sm^R (*i.d.*) vs. *B1-1C* Sm^R (*i.v.*).

Compared to *i.v.* infection, bacteremia induced by *B1-1C* Sm^R via *i.d.* route shows a delayed onset

The *Bartonella* infection cycle is characterized by two consecutive stages, the dermal stage, and the blood stage. Intradermal injections mimic the natural way of infection, while intravenous injections bypass the dermal stage. We used these two routes of infection to clarify at which stage of the infection the virulence factors are required, such as the VirB/D4 T4SS, the Beps or the flagellum. Therefore, we analyzed the bacteremia course caused by *B1-1C* Sm^R via the two different infection routes (*i.d.* / *i.v.*)

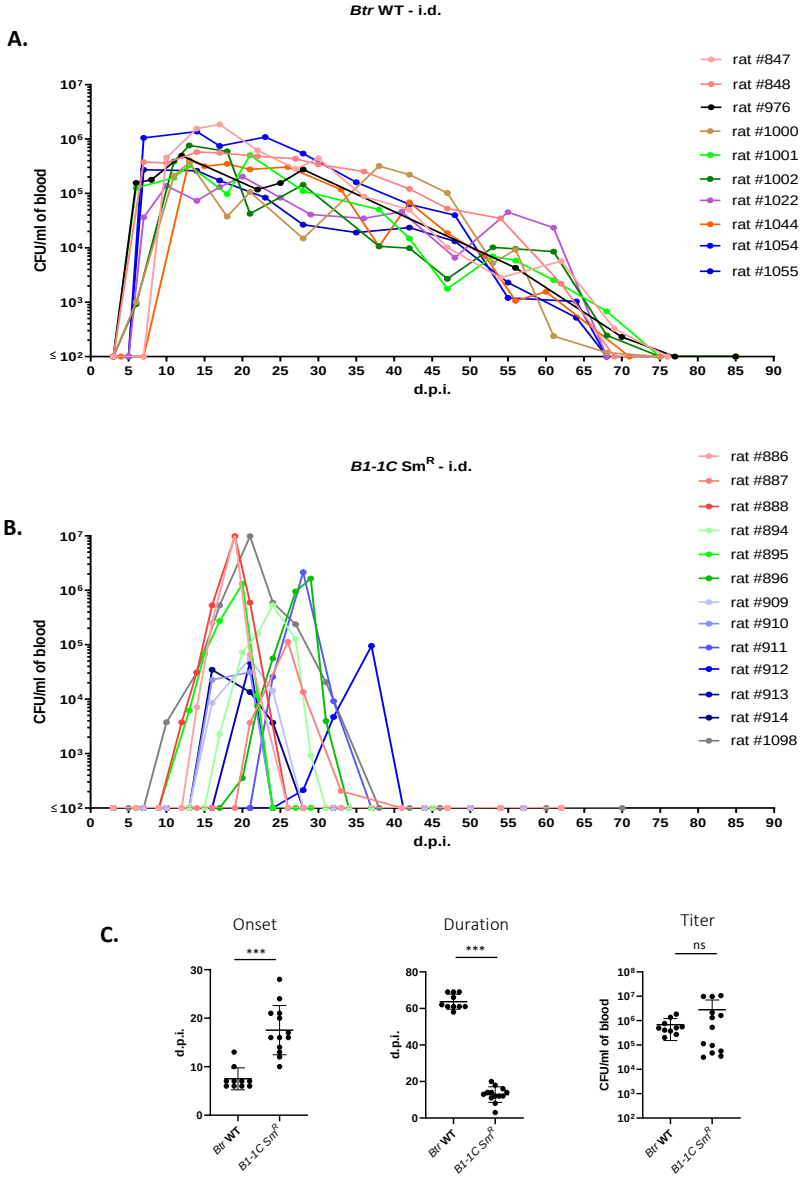
A group of rats was intravenously infected with *B1-1C* Sm^R and bacteremia was monitored (Fig. 2C) and compared to the group of rats intradermally infected with the same strain (Fig. 2B). The onset was significantly different between both groups (*p*-value = 0.0006) (Fig. 2D) and the delayed-onset observed for the *i.d.* group was most likely due to the time needed for *Bartonella* to reach the bloodstream from the dermal inoculation site (Fig. 2B, C). In accordance with published data for lineage 4, after *i.v.* infection bacteria were detected in the blood 5 days post-infection following colonization of a blood-seeding niche [8, 9]. No significant difference was observed regarding duration and titer of the bacteremia between the two strains. (Fig. 2D)

To notice, we assumed that the second peak observed in the *i.v.* group at days 33 post-infection for 3 rats out of 5 was related to a contamination during plating.

Compared to *Btr* WT (lineage 4), *B1-1C* Sm^R triggers a delayed and shorter bacteremia

Since we had now established *B1-1C* as an appropriate *in vivo* infection model for lineage 3, we compared its bacteremia to the most studied lineage 4-strain *B. tribocorum* allowing us to analyze any difference in the infection cycle of *B1-1C*.

Rats were intradermally infected with either *Btr* WT (Fig. 3A) or *B1-1C* Sm^R (Fig.3 B). By comparing bacteremia of these two strains, we first observed a high heterogeneity of bacteremia titer for *B1-1C* Sm^R oscillating between 10^4 to 10^7 CFUs/ml of blood, whereas the *Btr* WT bacteremia titer oscillated between 10^5 and 10^6 CFUs/ml of blood. Despite this heterogeneity, the bacteremia titer did not significantly differ (*p*-value = 0.1329) whereas the bacteremia onset and duration were significantly different (*p*-value < 0.0001 for both features) (Fig 3C). These impairments of the bacteremia triggered by *B1-1C* (delayed onset and shorter duration) suggested that lineage 3 *B1-1C* strain might behave differently *in vivo* than the lineage 4 *Btr* species.



Onset:	8 ± 2.3 d.p.i
Duration:	64 ± 4.1 d.
Titer:	$8.2x10^5 \pm 7.0x10^5$ CFUs/ml
Inf. rate:	100%

Onset:	17 ± 5.1 d.p.i
Duration:	14 ± 3.5 d.
Titer:	$4.5x10^6 \pm 5.3x10^6$ CFUs/ml
Inf. rate:	100%

Figure 3: Compared to *Btr* WT, *B1-1C* Sm^R triggers a delayed and shorter bacteremia

Groups of 10 weeks old Wistar rats were intradermally infected in the ear with either (A) *Btr* WT (inoculum 1x10^7 CFUs, n = 10) or (B) *B1-1C* Sm^R (inoculum 1x10^7 CFUs, n = 13). During 3 days, *Btr* WT was grown on HIA plates and *B1-1C-Sm^R* on TSA plates supplemented with defibrinated sheep blood. Plotted graphs represent time courses of single animals from which bacteria were recovered at a given time point post-infection. Each value corresponds to an average (CFU/ml of blood) acquired from serial dilutions. Each color shade represents a cohort of animal infected the same day with bacteria from the same bacterial solution and the tables next to the graph correspond to the mean of the onset, duration, maximum titer and indicate the infection rate between all bacteremia. (C) t-test were performed to compare bacteremia onset (C), duration (D) and maximum titer (E) between *Btr* WT (i.d.) vs. *B1-1C* Sm^R (i.d.).

B1-1C Sm^R does not invade rat erythrocytes

Bartonella tribocorum, like other *Bartonella* species, use an infection strategy characterized by a long-lasting intraerythrocytic colonization providing protection against the host's immune system and enabling transmission by blood sucking arthropods [1, 5, 6]. In lineage 4 species, the host specific colonization of red blood cells is mediated by the Trw T4SS [1].

Since *B1-1C* expresses a flagellum instead of a Trw T4SS, we aimed to explore the ability of *B1-1C* to invade erythrocytes. We used flow cytometry to analyze infected-blood samples and to subsequently detect bacteria association with erythrocytes. Thus, we generated a *B1-1C-gfp* Sm^R strain by inserting a transposon containing a *gfp* expression cassette under the control of a constitutive promoter into the *B1-1C* Sm^R chromosome. GFP (Green Fluorescence Protein) expression was validated by flow cytometry (Fig. 4). Then, we intravenously infected one rat with of *B1-1C-gfp* Sm^R to monitor bacteremia (Fig. 5). Compared to *B1-1C* Sm^R (Fig. 2B) there was no significant difference for the onset (*p*-value = 0.8711), the duration (*p*-value = 0.5041) and the maximum titer (*p*-value = 0.2981).

Since our *B1-1C-gfp* Sm^R strain exhibited a bacterial fitness comparable to *B1-1C* Sm^R, we performed intravenous rat infections with either *B1-1C-gfp* Sm^R or *Btr- gfp*. At day 11 post-infection, blood from infected animals was collected from the tail vein, diluted in PBS and analyzed by flow cytometry. Recorded GFP-positive events were gated into two populations according to the size (FSC-A): "Single bacteria" and "Blood cell-associated bacteria" (Fig. 6A, B). Then, we compared the distribution of *B1-1C-gfp* Sm^R and *Btr-gfp* between these two populations (Fig. 7A, B). In addition to the 1:80 dilution, we also performed a 1:500 dilution of the blood discriminating doublets from our analysis. In that analysis, we name doublets when a bacterium and a red blood cell pass through the laser at the exact same time and are counted as associated events while it is not the case. By increasing the dilution rate, we reduced the probability of this event.

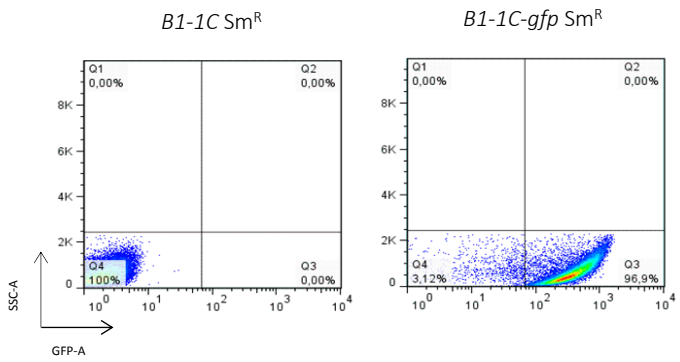


Figure 4: FACS validation of the constitutive expression of GFP in *B1-1C-gfp* Sm^R

B1-1C- gfp Sm^R was harvested after 5 days of cultivation on TSA plates and diluted in PBS (1:1000 dilution). The diluted sample of *B1-1C- gfp* Sm^R was analyzed by flow cytometry. SSC-A versus GFP-A dot blot analysis allowed the validation of GFP expression for *B1-1C- gfp* Sm^R characterized by a shift on the GFP x-axis. SSC-A (Side-scattered light) corresponds to the cell granularity.

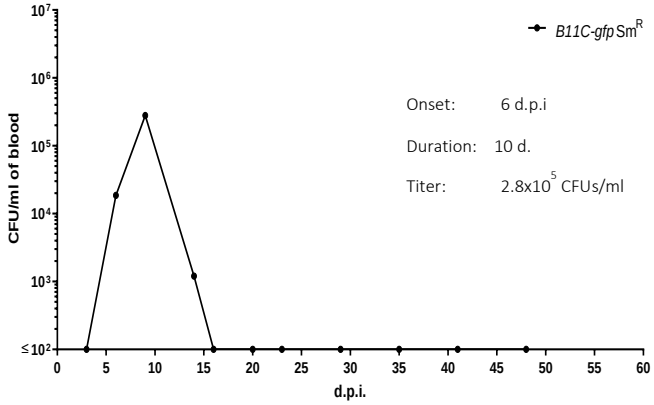


Figure 5: Bacteremia of the *B1-1C-gfp* Sm^R strain

One 10 weeks old Wistar rats was intravenously infected (inoculum 2x10^8 CFUs, n = 1) in the tail vein with *B1-1C-gfp* Sm^R. *B1-1C-gfp* was cultivated for 3 days at 35°C and 5% CO_2 on TSA plates supplemented with defibrinated sheep blood. Plotted graphs represent time course of single animals from which bacteria were recovered at a given time point post-infection. Each value corresponds to an average (CFU/ml of blood) acquired from serial dilutions. One rat out of 3 showed a bacteremia comparable to *B1-1C* Sm^R and was therefore used for further experiments. Values indicated next to the graphs correspond to the onset, the duration and the maximum titer of bacteremia. A statistical t-test was performed to compare bacteremia onset, duration and maximum titer between *B1-1C-gfp* Sm^R and *B1-1C* Sm^R.

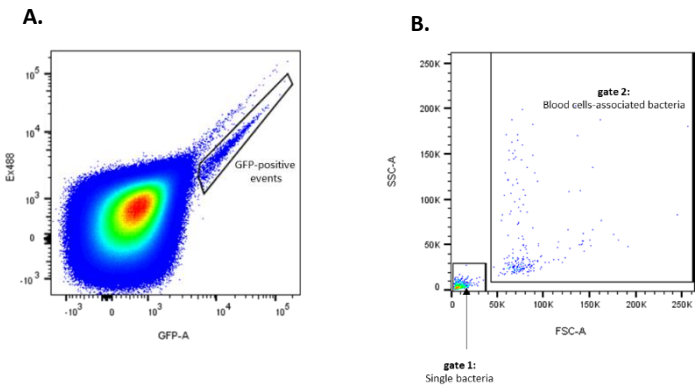


Figure 6: Analytic process of FACS data

(A) Blood samples were analyzed by flow cytometry. Ex 488 versus GFP-A dot blot analysis allowed the isolation of population of GFP-positive events. (B) SSC-A versus FSC-A analysis of the previously gated GFP-positive events allowed distinction of single bacteria contained in gate 1 and blood-associated bacteria contained in gate 2. FACS pictures are from 1:500 dilution data. To notice, FSC-A is proportional to cell-surface size.

In accordance with the intraerythrocytic lifestyle of *Btr* WT [9], 84.5% (1:80 dilution) and 86% (1:500 dilution) of *Btr-gfp* were gated as blood cell-associated bacteria and 11% (1:80 dilution) and 12% (1:500 dilution) were gated as single bacteria. A t-test confirmed that there was no significant difference between the two dilution for each group of bacteria. In contrast, 63.5% of *B1-1C-gfp* Sm^R positive events were gated as blood cell-associated bacteria for the 1:80 dilution and dropped to 22% for the 1:500 dilution. Consistently, the percentage of *B1-1C-gfp* Sm^R positive events gated as single bacteria increased from 31% for the 1:80 dilution to 76.5% for the 1:500 dilution. Statistical analysis confirmed that there was a significant difference between both dilutions for single bacteria (p-value = 0.0078) and blood cell-associated bacteria (p-value = 0.0053). Taken together, these observations suggested that *B1-1C* Sm^R might not be able to colonize erythrocytes.

To confirm the hypothesis that *B1-1C* Sm^R has a non-intraerythrocytic life style, we performed a gentamicin protection assay. Gentamicin is an aminoglycoside antibiotic that cannot penetrate intact eukaryotic cell membranes and therefore kills only extracellular bacteria while intracellular bacteria are protected [41]. Here, we intravenously infected rats with either *B1-1C* Sm^R or *Btr* WT and collected blood at different time points after infection. Then blood samples were treated with gentamicin and erythrocytes were lysed as mentioned in the material and method section. For each infected animal, CFUs were counted and presented as graph column (Fig. 8A, B, C, D). In contrast to *Btr* WT (Fig 8A, B), when we performed a gentamicin treatment on blood samples from rats infected with *B1-1C* Sm^R (Fig. 8C, D) we did not observe any CFU after plating confirming the incapability of *B1-1C* Sm^R to invade erythrocytes at any time during the infection.

Taken together, FACS data and Gentamicin protection assay results strongly suggest a non-intraerythrocytic life style for *B1-1C* Sm^R.

Genetic engineering on *B1-1C* Sm^R leads to unspecific attenuation

Bartonella 1-1C translocate via the VirB/D4 T4SS a different set of effectors compared to lineage 4 species and harbors a flagellum instead of the Trw T4SS. Therefore, to analyze the role of these virulence factors during *B1-1C* infection we generated two *B1-1C* Sm^R mutants: *B1-1C* Δ*virD4* Sm^R harboring an *in frame* deletions of *virD4* and *B1-1C* Δ*flaA* Sm^R harboring *in frame* deletions of *flaA*. For each mutant, we infected rats intradermally or intravenously and monitored bacteremia (Fig. 9A, B).

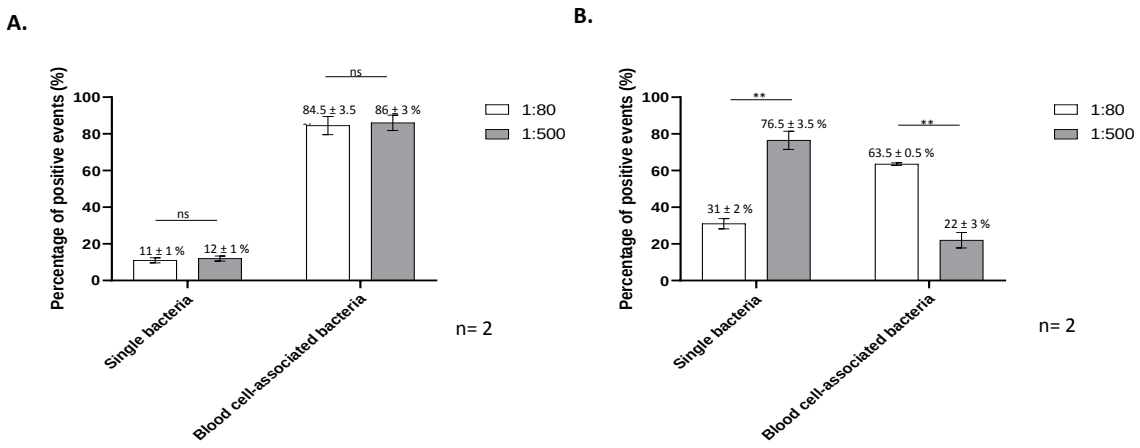


Figure 7: FACS analysis of blood infected with *Btr-gfp* or *B1-1C-gfp* Sm^R

Groups of 10 weeks old Wistar rats were intravenously infected in the tail vein (inoculum 2x10^8 CFUs, n = 2) with either *Btr-gfp* or *B1-1C-gfp* Sm^R. At day 11 post-infection, blood from infected animals was collected from the tail vein, diluted in PBS and analyzed by flow cytometry. GFP-positive events were gated into two different populations: "Single bacteria" and "Blood cells-associated bacteria". We performed two dilutions 1:80 (white columns) and 1:500 (grey columns). For each graph, values the represent the mean of the percentage of GFP-positive events from the two animals infected by either (A) *Btr-gfp* or (B) *B1-1C-gfp* Sm^R. Our data suggested a non-intraerythrocytic life style for *B1-1C* Sm^R compared to *Btr^R* WT. A t-test was performed to compare the two dilution. Statistical was performed by using a T-test.

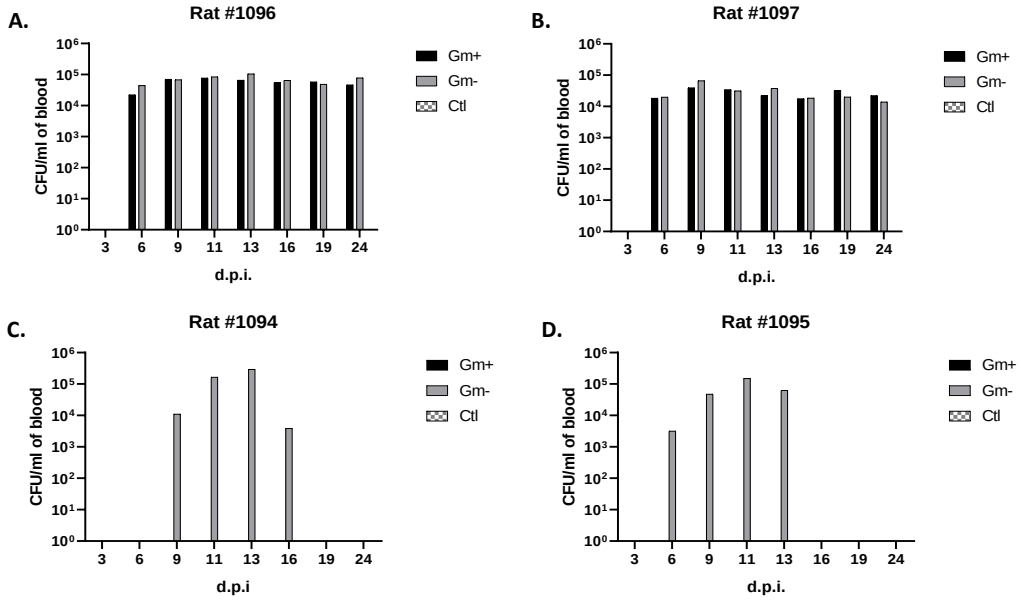


Figure 8: Gentamicin protection assay

Ten weeks old Wistar rats were intravenously infected (inoculum 2x10^8 CFUs, n = 2) in the tail vein with either *Btr* WT (A, B) or *B1-1C* Sm^R(C, D). During 3 days, *Btr* WT was cultivated on HIA plates and *B1-1C* Sm^R on TSA plates supplemented with defibrinated sheep blood. Blood from infected rats was collected at different time points and treated with gentamicin as mentioned in the material and methods section. For each infected animal, treated and untreated blood were plated on HIA plates and CFUs were counted and presented as graph column. Grey columns show untreated blood, black columns show gentamicin-treated blood and patterned columns show the control. Our data suggested that *B1-1C* Sm^R was non-intraerythrocytic. Efficiency of gentamicin was proven by the absence of CFU from the control.

Both strains were abacteremic in *i.d.* and *i.v.* infections suggesting either a key role of these two bacterial components during infection or a potential polar effect of the deletion. Of note, the lack of *in vitro* infection model for *B. 1-1C* hampered us to assess any growth defect due to the deletion.

To investigate further the role of these virulence factors *in vivo*, complementation experiments were performed by expressing *virD4* or *flaA in trans* on a plasmid under the control of the natural promoter in *B1-1C* Δ*virD4* Sm^R or *B1-1C* Δ*flaA* Sm^R, respectively. Rats were infected intradermally or intravenously and bacteremia was monitored (Fig. 9C, D). Bacteremia was not restored independently of the infection route indicating that complementation of the mutant was not successful and preventing any conclusion about the role of the two virulence factors during the infection of *B1-1C* Sm^R. The lack of an *in vitro* infection protocol for *B1-1C* hampered the analysis of the complementation.

Finally, to track down any impact of genetic engineering on bacterial pathogenicity, we infected intravenously rats with three revertant clones of either *B1-1C* Δ*virD4* Sm^R and *B1-1C* Δ*flaA* Sm^R and bacteremia was monitored (Fig 9E, F). Interestingly, bacteremia was not restored indicating that genetic manipulation and/or successive *in vitro* passages affects negatively *B1-1C* Sm^R fitness.

Of note, we named revertant strain when the mutant restores its WT genomic situation following the excision of the suicide plasmid by the second homologous recombination (described in the materials and methods section).

B1-1C Sm^R does not trigger bacteremia in BALB/c mice indicating host specificity in trw-lacking species

Host specificity has been shown *in vitro* to be mediated by the Trw T4SS in lineage 4 species [1]. Since lineage 3 comprises Trw-lacking species we were interested to investigate the host specificity in *B1-1C.* Therefore, we intradermally infected BALB/c mice with *B1-1C* Sm^R (Fig. 10). None of the infected mice developed bacteremia indicating that host specificity is maintained in lineage 3 species even if they are lacking the Trw T4SS [6]. To notice, it confirmed a previous experiment demonstrating host specificity in lineage 3 where *B. 1-1C* was the only species triggering homogenous bacteremia in rats [12].

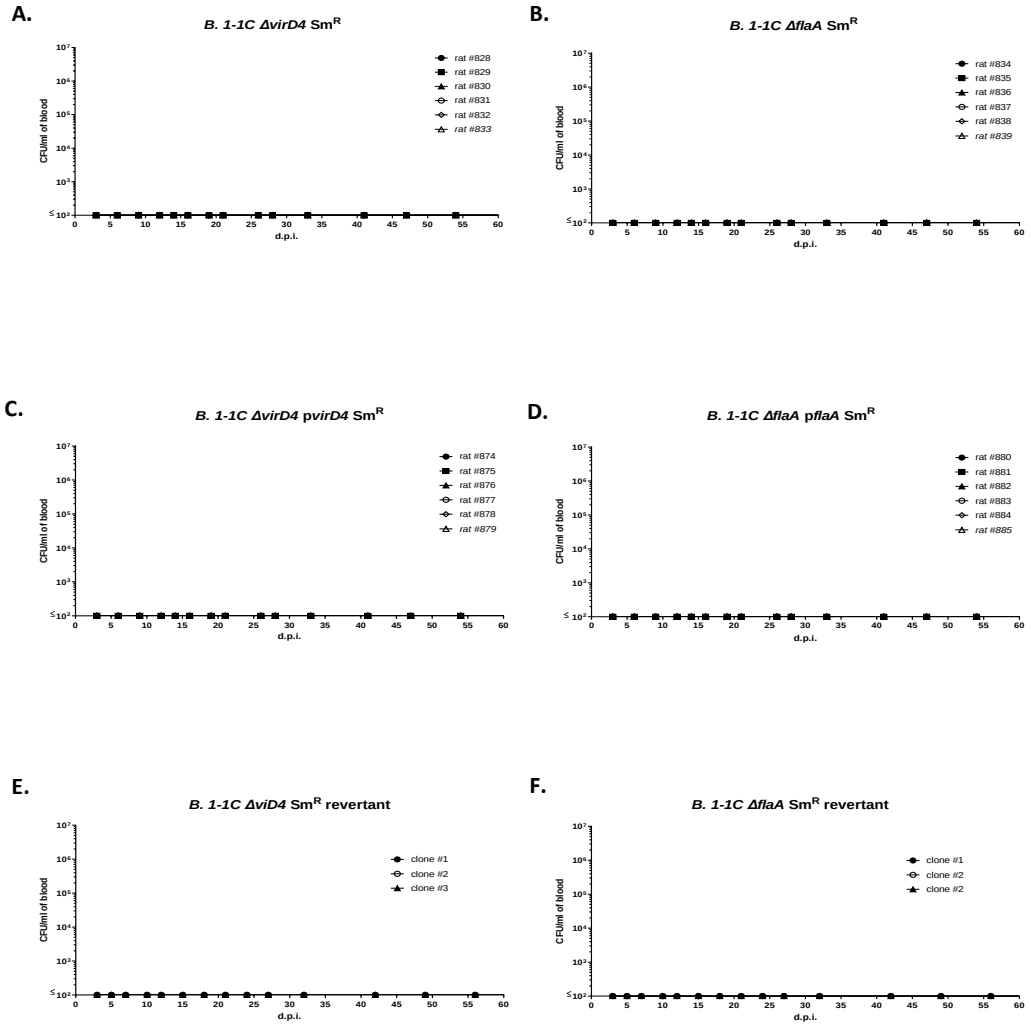


Figure 9: Determination of the role of the VirB/D4 T4SS and the flagellum during *B.1-1C-Sm^R* infection

Bacteria were grown for 3 days at 35°C and 5% CO_2 on TSA plates supplemented with defibrinated sheep blood. Plotted graphs represent bacteria which were recovered from single animals at a given time point post-infection. Each value corresponds to an average (CFU/ml of blood) acquired from serial dilutions. (A, B) Groups of 10 weeks old Wistar rats were intradermally (inoculum 10^7 CFUs, n = 3, filled symbols) or intravenously (inoculum $2x10^8$ CFUs, n = 3, unfilled symbols) infected with either (A) *B1-1C ΔvirD4* Sm^R or (B) *B1-1C ΔflaA* Sm^R. No infected animals showed any bacteremia suggesting a fitness loss for *B1-1C* Sm^R associated with the deletion of either *virD4* or *flaA*. (C, D) Groups of 10 weeks old Wistar rats were intradermally (inoculum 10^7 CFUs, n = 3, filled symbols) or intravenously (inoculum $2x10^8$ CFUs, n = 3, unfilled symbols) infected with either (C) *B1-1C* Sm^R *ΔvirD4*-p*virD4* or (D) *B1-1C* Sm^R *ΔflaA*-p*flaA*. Both complemented strains were abacteremic preventing any conclusion about the role of the VirB/D4 T4SS and the flagellum during *B1-1C* Sm^R infection cycle. (E, F) Groups of 10 weeks old Wistar rats were intravenously (inoculum $2x10^8$ CFUs, n = 1) infected with one of the three clones of either (E) *B1-1C ΔvirD4* Sm^R revertant or (F) *B1-1C ΔflaA* Sm^R revertant. Each rat was infected with one clone. No infected animals showed any bacteremia suggesting a fitness loss for *B1-1C* Sm^R associated with genetic engineering and/or *in vitro* passages.

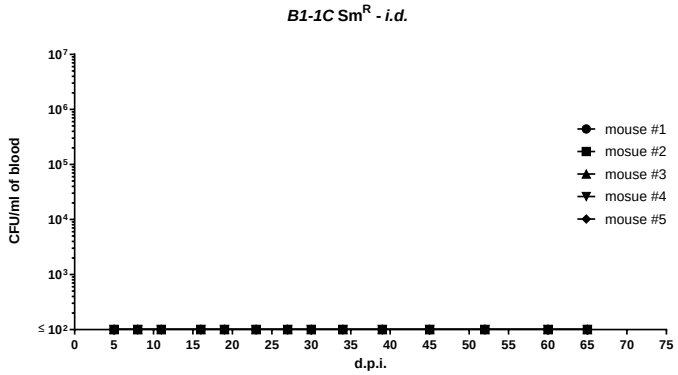


Figure 10: Assessment of *B1-1C* Sm^R's ability to infect BALB/c mice

Bacteria were grown for 3 days at 35°C and 5% CO_2 on TSA plates supplemented with defibrinated sheep blood. Plotted graphs represent bacteria which were recovered from single animals at a given time point post-infection. Group of BALB/C mice were intradermally infected (inoculum 10^7 CFUs, n = 5) with *B1-1C* Sm^R. No infected animals showed any bacteremia suggesting *B1-1C* was incompetent to infect BALB/c mice.

Materials and Methods

Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 1. *Bartonella 1-1C* strains were grown either on Tryptic soy agar plates containing 5% defibrinated sheep blood (TSA plates) or on heart Infusion Agar containing 5% defibrinated sheep blood (HIA plates) at 35°C in a moist atmosphere under 5% CO2 for, if no contraindications, 5-6 days. *Bartonella tribocorum* strains were grown on HIA plates at at 35°C in a moist atmosphere under 5% CO2 for 2-3 days. When indicated, media were supplemented with 30 µg/ml kanamycin, 100 µg/ml streptomycin, 10 µg/ml gentamicin, and/or 500 µM isopropyl-β-Dthiogalactoside (IPTG, http://www.applichem.de). E. coli strains were cultivated in LuriaBertani liquid medium (LB) or on Luria-Bertani agar on plates (LA) at 37°C overnight. When indicated, media were supplemented with 50 µg/ml kanamycin, 200 µg/ml ampicillin, 20 µg/ml gentamicin, 500 µM IPTG, and/or 1 mM diaminopimelic acid (DAP).

Animal experimentation

Animal handling was performed in accordance with the Swiss Animal Protection law and local animal welfare bodies. The animal experiments in this work were approved by the Veterinary Office of the Canton Basel-Stadt (License number 1741). All animals were kept at SPF (specific pathogen-free) conditions. Animals were obtained from Janvier labs at the age of 8-10 weeks. After two weeks of adaptation, rats were infected with bacteria harvested and diluted in phosphate-buffered saline (PBS) at OD595nm = 1. On anesthetized rats (1 – 3% isoflurane/O_2 mixture), we performed either intradermal injection in the dermis of the ear (10 µl of the bacterial solution; inoculum 10^7 CFUs) or intradermal injection in the tail vein (200 µl of the bacterial solution; inoculum $2x10^8$ CFUs).

Blood samples were drawn and diluted (9:1) in PBS supplemented with 3.8 % of sodium-citrate, to prevent coagulation, and kept at -80°C for at least 1 hour. After thawing, blood samples were serial diluted in PBS and plated on appropriate agar plates (HIA plates). Then for counting, bacteria were incubated at 35°C and 5% CO2 for either 5-6 days for *Btr* or 10 days for *B1-1C*.

Medium composition

Media	Composition	Quantity (g) for 1L
Tryptic Soy Agar (TSA) BD™	Pancreatic Digest of Casein	15.0
	Papaic Digest of Soybean Meal	5.0
	Sodium Chloride	5.0
	Agar	15.0
Heart Infusion Agar (HIA) BD™	Beef Heart infusion from 500g	10.0
	Tryptose	10.0
	Sodium Chloride	5.0
	Agar	15.0
Luria-Bertani Broth (LB) BD™	Tryptone	10.0
	Yeast extract	5.0
	NaCl	10.0
Luria-Bertani Broth (LB) BD™	Tryptone	10.0
	Yeast extract	5.0
	NaCl	10.0
	Agar	15.0

The powder mixed with 600 ml of ddH_2O was autoclaved and supplemented with 4.2% (v/v) sterile defibrinated sheep's blood

TSA and HIA plates were supplemented with 5% of defibrinated sheep blood (Thermos Fisher™)

Generation of spontaneous streptomycin-resistant strains

We generated the spontaneous streptomycin-resistant *B1-1C* mutant by spreading 100µl (OD_{595nm} = 1) bacteria on TSA plates containing streptomycin at a concentration of 100 µg/ml. The *rspL* gene was amplified by colony-PCR primers prCB020 (Frw) and prCB021 (Rev) then sequenced with prCB020, prCB021 and prCB022. Nucleotide sequence alignment allowed to isolate the streptomycin-resistant *B1-1C* strain (CB0126). Generation of the spontaneous streptomycin-resistant *Btr* strain RSE149 (CB0165) has been previously described [9].

Plasmid Construction

All primers used for the elaboration of this report are listed in table. The suicide mutagenesis vector pTR1000 used for gene replacement in this study was described previously [36]. The vector pBZ485 was used as template for complementation.

pCB001 used for generating the *Bartonella 1-1C-gfp* strain (CB0140) was constructed as follow. The 951-bp fragment *gfp-mut3* was amplified from pCD353#5 by using the oligonucleotide primers prCB013 and prCB014. The amplified product was digested with AvrII and PacI and cloned into the Tn mutagenesis vector pPE0012, digested with AvrII and PacI, to yield the *gfp*-transposon vector pCB001

pTRΔ*flaA* and pTRΔ*virD4* were used to generate *B1-1C* Δ*flaA* Sm^R (CB0147) and *B1-1C* Δ*virD4* Sm^R (CB0151), respectively. Plasmid construction and gene replacement were already described [40].

The complementation vector pJK024 used for generating *B1-1C* Δ*flaA* p*flA* Sm^R (CB0139) was generated as follow. The *flaA* gene was amplified from CB0131 (*B1-1C* Sm^R) with the oligonucleotide primers prJK221 and prJK222.The 1800 bp-amplified product was digested with KpnI and BamH1 and cloned into the complementation plasmid pBZ485a, digested by KpnI and BamH1, to yield pJK024.

The complementation vector pJK025 used for generating *B1-1C* Δ*virD4* p*virD4* Sm^R (CB0138) was generated as follow. The *virD4* gene was amplified from *B1-1C* Sm^R (CB0131) with the oligonucleotide primers prJK223 and prJK224.The 2600 bp-amplified product was digested with KpnI and BamH1 and cloned into the complementation plasmid pBZ485a, digested by KpnI and BamH1, to yield pJK025.

pJK011 used for generating *B11C* Δ*bep1 in-frame* mutant was generated as follows. The HR1, corresponding to the 1030 bp downstream *bep1* locus*,* was amplified from *B11C* Sm^R (CB0131) with the oligonucleotide primers prJK113 and prJK115. The HR2, corresponding to the 1046 bp upstream *bep1* locus, was amplified from *B11C* Sm^R (CB0131) with the oligonucleotide primers prJK114 and prJK116. The amplified products were merged by SOEing PCR with the oligonucleotide primers prJK113 and prJK116. The 2076 bp-product was digested with BamH1 and XbaI and clones into the suicide vector pTR1000, digested with XbaI and BamH1, to yield pJK011. Insertion of the SOEing fused-product within pTR1000 was confirmed by PCR with the oligonucleotide primers prCB116 and prCB117 spanning the insertion site.

pJK012 used for generating *B11C* Δ*bep2 in-frame* mutant was generated as follows. The HR1, corresponding to the 1053 bp downstream *bep2* locus*,* was amplified from *B11C* Sm^R (CB0131) with the oligonucleotide primers prJK120 and prJK123. The HR2, corresponding to the 1136 bp upstream *bep2* locus, was amplified from *B11C* Sm^R (CB0131) with the oligonucleotide primers prJK121 and prJK123. The amplified products were merged by SOEing PCR with the oligonucleotide primers prJK120 and prJK123. The 2189 bp-product was digested with BamH1 and XbaI and clones into the suicide vector pTR1000, digested with XbaI and BamH1, to yield pJK012. Insertion of the SOEing fused-product within pTR1000 was confirmed by PCR with the oligonucleotide primers prCB116 and prCB117 spanning the insertion site.

pJK020 used for generating *B11C* Δ*bep10 in-frame* mutant was generated as follows. The HR1, corresponding to the 1072 bp downstream *bep10* locus, was amplified from *B11C* Sm^R (CB0131) with the oligonucleotide primers prJK192 and prJK193. The HR2, corresponding to the 1140 bp upstream *bep10* locus, was amplified from *B11C* Sm^R (CB0131) with the oligonucleotide primers prJK194 and prJK195. The amplified products were merged by SOEing PCR with the oligonucleotide primers prJK192 and prJK195. The 2212 bp-product was digested with BamH1 and XbaI and clones into the suicide vector pTR1000, digested with XbaI and BamH1, to yield pJK020. Insertion of the SOEing fused-product within pTR1000 was confirmed by PCR with the oligonucleotide primers prCB116 and prCB117 spanning the insertion site.

Procedure for gene replacement and confirmation of the mutants

Following the mobilization of the suicide mutagenesis vector into the appropriate *Bartonella* strain, kanamycin was used as a positively selection step to isolate cointegrates. Growing bacteria that integrated the suicide mutagenesis vector by homologous recombination were selected by colony PCR spanning the two homology regions with the appropriate oligonucleotide primers. Then, cointegrates were negatively selected on a media supplemented with streptomycin. The surviving bacteria have lost the mutagenesis vector by a second homologous recombination leading to two situations; either the clone harbor the *in-frame* deletion or the restoration of the wild type conformation. Colony PCR spanning the region of interest was performed to isolate clones carrying the deletion by comparing the size of the generated PCR products.

Construction of GFP-expressing *Bartonella 1-1C*.

The plasmid pCB001 containing a transposable kanamycin-*gfp* cassette, was introduced into *B1-1C* (CB0131) as a suicide vector via two-parental mating with JKE217 as the donor strain. Transconjugants displayed a light green colony phenotype. Twenty clones were harvested. FACS analysis of these clones confirmed GFP expression and 3 clones were intravenously injected in 3 rats. The clone triggering a similar bacteremia to *B1-1C SmR* (CB0131) was selected and used in all subsequent experiments reported in this study. Transposon insertion site was localized by semi-random PCR.

Gentamicin protection assay

Blood 100 µl of blood samples were withdrawn from infected rats with a syringe filled with 50 µl of PBS supplemented with 3.8% of sodium citrate to prevent blood coagulation. To isolate erythrocytes from non-associated bacteria, the mixture was centrifuged at 500 g for 5 min. The pellet was resuspended in 100 µl of PBS and equally split into two tubes which were then centrifuged at 2100 g for 4 min. One pellet was resuspended in 50 µl of PBS supplemented with gentamicin sulfate (150 µg/ml) while the other pellet was resuspensed in 50 µl of PBS. After 2 hours of incubation at 35°C, samples were washed three times in PBS to remove the antibiotic. In a final volume of 20 µl of PBS, samples were freezed at -80°C for at least 20 min to release bacteria by hypotonic lyse of erythrocytes. After thawing, bacteria were serial diluted in PBS and plated onto HIA plates. Finally, bacteria were incubated at 35°C and 5% CO2 for either 5-6 days for *Btr* or 10 days for *B1-1C* before being counted. Results were annotated as CFU/ml of blood, where 1 ml of blood corresponds approximatively at 10^9 erythrocytes.

Fluorescence Activated Cell Sorting (FACS) analysis

Flow cytometry was used to assess the amount of GFP-expressing bacteria associated to erythrocytes. At day 11 post-infection, blood samples were withdrawn from rat with a syringe filled with 50 µl of PBS supplemented with 3.8% of sodium citrate to prevent blood coagulation. On each blood sample was performed a 1:80 (12.5 µl) and a 1:500 (2 µl) dilution in 1ml of PBS. Then samples were analysed by flow cytometry and data for 10^6 events per sample were collected and analyzed.

Data analysis

Statistical analysis of the obtained data was performed using GraphPad Prism SoftwaRE. Depending on the experiment I used either the T-test or the two-way ANOVA to compare bacteremia curves. This information is indicated in the figure legends. P-

value$_{t\text{-}test}$ indicates the p-value calculated by the t-test and P-value$_{ANOVA}$ indicates the p-value calculated by the two-way ANOVA.

Plasmid	Genotype or relevant characteristics	Reference
pCD351	Contains the *gfp-mut3*	[9]
pPE0012	Tn mutagenesis vector	[59]
pTR1000	Basic suicide mutagenesis vector for *Bartonella*	[7]
pBZ485a	Plasmid used for complementation of B1-1C mutants	Harms, A.
pJK024.2.0.	Encoding *flaA*$_{B1\text{-}1C}$	This study
pJK025.2.0.	Encoding *virD4*$_{B11C}$	This study
pJK008	*B sp. 1-1C* Mtase	[40]
pTRΔ*flaA*	Suicide mutagenesis vector for *flaA* deletion in CB0126	[40]
pTRΔ*virD4*	Suicide mutagenesis vector for *virD4* deletion in CB0126	[40]

Primers	Sequence	Reference
prCB0113	ATGCTTAATTAAGGTTCTGGCAAATATTCTGA	This study
prCB0114	ATGTCCTAGGGCCTGCAGTTATTTGTATAG	This study
prJK113	GAGCGGGGATCCTTTCTCATTATTCATATTGCGG	This study
prJK114	TAGCTTGATACAATAACTATTTTTCTTCTTTCATTATTCTCTCCTTATAGTGTAAT	This study
prJK115	GAAAAATAGTTATTGTATCAAGCTAAAAATATG	This study
prJK116	GCCCTCGGATCCATGTATCTTAGAAACAGCAATCAGC	This study
prJK120	GAGCGGGGATCCTAAACTTAAATACAAACCGCTCATC	This study
prJK121	AATGGAAGAACACATTTTAACAAGCAAAATACACCTTTCCGTTTACAATAA	This study
prJK122	GCTTGTTAAAATGTGTTCTTCCA	This study
prJK123	GCCCTCGGATCCTTCGTTTGGAGAATGACATC	This study
prJK192	GAGCGGGGATCCAAGAACTAGGCTTATTAAGCACAAC	This study
prJK193	TATCGACATGATTCCTCCTCTAC	This study
prJK194	TCGTAGAGGAGGAATCATGTCGATAGCCAGTTAATGACGTAAACCTT	This study
prJK195	GCCCTCGGATCCTTAAAAACATTCAACAGCATGTC	This study
prJK221	GAGCGGGGATCCGCTGTTCCATTGCCTTTTAT	This study
prJK222	CTCCCGCGGTACCCTAATTACGAAACAAGGCTAAAATATT	This study
prJK223	GAGCGGGGATCCGAACGGATTATAAATTCTGCTAAA	This study
prJK224	CTCCCGCGGTACCTCAGTCTTTCTTTCCTTTGG	This study

Strains	Genotype or relevant characteristics	Reference
Bartonella 1-1C		
PEE0266	Wild Type	[32]
CB0131	Spontaneous Sm^R strain, Stocked after one passage in rat	This study
CB0140	Chromosomal integration of gfp, derivative of CB0131 Tn::5 transposon integration	This study
CB0151	ΔvirD4, derivative of CB0131	[40]
CB0138	Δ*virD4* p*virD4*, derivative of CB0131	This study
CB0350	Δ*virD4* revertant, derivative of CB0131	This study
CB0147	Δ*flaA*, derivative of CB0131	[40]
CB0139	Δ*flaA* p*flaA*, derivative of CB0131	This study
CB0344	Δ*flaA* revertant, derivative of CB0131	This study
CB0153	Δ*bep1*, derivative of CB0131	This study
CB0155	Δ*bep2*, derivative of CB0131	This study
CB0157	Δ*bep10*, derivative of CB0131	This study
CB0158	Δ*bep10* revertant, derivative of CB0131	This study
Bartonella tribocorum		
RSE149	Spontaneous Sm^R strain, used as *Btr* WT in this study	[9]
Escherichia coli		
JKE217	MFDpri ΔT IV lacIq, harbor pJK008	[40]

Discussion

During the establishment of the infection model, our experiments revealed the requirement of a specific medium for reliable intradermal infection with *B1-1C*. Indeed, 3 days of growth on TSA medium were sufficient to trigger an infection rate of 100 % while 10 days were necessary for bacteria grown on HIA plates to display a similar rate. It is known that during the early stage of infection, lineage 4 and lineage 3 *Bartonella* species require the expression of the VirB/D4 T4SS to subvert host immune cell response and subsequently travel through the dermis and create an immune-silent environment in the lymphatic nodes [42]. Studies on *B. henselae* (Lineage 4) have shown that expression of the VirB/D4 T4SS and its effector proteins are under the control of a dual activation of the BatR/BatS two-component system (TCS) and the sigma factor RpoH1. On one hand, BatR/BatS is a pH sensor mechanism displaying an optimal activity at the physiological pH (7.4). On the other hand, RpoH1 activation is modulated by the stringent response component SpoT and DksA involved in stress responses such as starvation. Therefore, we can assume that TSA medium induces a stringent response explaining why 3 days of growth is sufficient for reliable intradermal infection. Consistently, growing bacteria 10 days on HIA plates might decrease the nutrient availability over time impoverishing the environment and subsequently inducing a stringent response. It would explain the infection rate of 100 % observed after 10 days of growth on HIA plates. However, since both media are considered as rich media for bacterial growth and both stabilize at a pH of 7.4 it is difficult to explain why TSA is more adapted. This kind of sensitivity, but less pronounced, for the growth media has also been reported in mice infections with *Bartonella birtlesii.* Indeed, mice develop bacteremia when intradermally infected with 10^2 CFUs of bacteria grown on TSA medium whereas 10^5 CFUs is the minimum inoculum to induce bacteremia when bacteria are grown on CBA medium (data not shown).

Since we had now established appropriate conditions for *in vivo* infections with *B1-1C*, we compared its bacteremia to the lineage 4 strain *B. tribocorum* which provides most of our knowledge concerning the infection cycle of *Bartonella* spp. [6]. Comparison with *Btr* WT revealed a 9 days-delayed onset and a shorter bacteremia for *B1-1C* Sm^R (14 days instead of 64 days for *Btr* WT).

Firstly, the 9 days delay observed for *B1-1C* can be explained by the different growth rate between *B1-1C* and *Btr*. Indeed, independently of the growth media *in vitro*, it takes at least 5-6 days to observe *B1-1C* Sm^R colonies whereas 2-3 days are sufficient for *Btr* WT. It is known that during *Bartonella* infection, a large number of bacteria are simultaneously seeded from the lymphatic circulation into the bloodstream [42]. Even if the mechanism remains unknown, it is believed that *Bartonella* has to replicate until reaching a critical concentration inducing the release. Thus, we can assume that *B1-1C* requires more time to reach this critical concentration in comparison with *Btr* explaining the delay of

the bacteremia onset. As another hypothesis, *B1-1C* is not well adapted to rat resulting in an inefficient subversion of the immune response. Thus, *B1-1C* might require more time to travel through the dermis and reach the bloodstream (15-16 days) compared to other robust *Bartonella* models such as *B. tribocorum*, *B. taylorii* or *B. birtlesii* where the bacteremia appears 5-6 days post-inoculation [6, 8, 43]. Remarkably, we also reported for both strains a delayed onset for *i.d.* infection compared with *i.v.* infection. This delay could correspond to the time needed for *Bartonella* to travel from the inoculation site to the blood-seeding niche and conceivable interactions of *Bartonella* with the immune cells slowing down the progression through the dermis.

Secondly, in addition to the delayed onset, another striking difference with *Btr* WT is the short bacteremia duration induced by *B1-1C*. Gentamicin protection assays and FACS analysis revealed that *B1-1C* is not intraerythrocytic which could justify this surprising bacteremia duration. Considering that erythrocytes colonization is a pivotal event for *Bartonella* pathogenicity by providing an immune-privileged environment [42, 44], we can assume that *B1-1C* is no more protected against immune system and therefore is rapidly cleared from the blood explaining this short bacteremia duration. Interestingly, it has been reported that in an accidental host *Bartonella* can invade replicative niches such as DCs and ECs but is unfit for establishing intraerythrocytic parasitism [45]. Interestingly, the *B1-1C* strain used in our experiments was isolated in 1 out of 58 rats (*R. norvegicus*) tested by Lin et *al.* [32]. The small sample size of animals tested and the very low prevalence of *B1-1C* prevents any conclusion on *R. norvegicus* as the natural reservoir host of *B1-1C* [32]. Therefore, we cannot exclude that *B1-1C* was isolated from an accidental host explaining the extraerythrocytic localization reported in our rat infection.

In lineage 4 species, host-specific adhesion to erythrocyte is mediated by the Trw T4SS [1, 24, 25]. Since in *B. bacilliformis* colonization of erythrocyte is flagella-dependent and lineage 3 species bear flagella instead of the Trw T4SS, we were interested in studying the role of the flagella during *B1-1C* infection. Therefore, rats were infected with either a flagellin-deficient strain *B1-1C* Δ*flaA* or a *flaA trans*-complemented strain *B1-1C* Δ*flaA* p*flaA* or *B1-1C* Δ*flaA* revertant. To notice, FlaA protein (flagellin) is the main component of the filament and disruption of this gene prevents flagella assembly [35]. All strains have shown to be abacteremic preventing any conclusion concerning the role of the flagella during *B1-1C* infection. Furthermore, the abacteremic phenotype of the revertant strain indicates that genetic manipulation might have an impact on the fitness of the bacteria. Other *B1-1C* mutants were generated such as *B1-1C* Δ*virD4, B1-1C* Δ*virD4* p*virD4, B1-1C* Δ*virD4* revertant in order to study the role of the VirB/D4 T4SS during infection and *B1-1C* Δ*bep1, B1-1C* Δ*bep2, B1-1C* Δ*bep10* and *B1-1C* Δ*bep10* revertant strain to clarify the role of lineage 3-Beps during infection. Remarkably, similarly to *flaA*

mutants, all were abactermic (Unpublished data) supporting the hypothesis that there is an attenuation of bacterial fitness associated with genetic manipulations or/and successive *in-vitro* passages.

Attenuation of the ability to infect laboratory animals after successive *in vitro* passages or gene manipulation has been reported in the literature for different species such as *Borrelia burgdorferi* or *Mycobacterium tuberculosis*. *B. burgdorferi* lost its ability to colonize laboratory animals after 10 *in vitro* passages and the virulent *M. tuberculosis* strain H37Rv was no longer virulent for common laboratory animals after numerous *in vitro* passages [46-48]. Another striking example of infectivity attenuation associated with *in vitro* passages is the use of this technic to attenuate pathogens to *in fine* produce vaccines or safe laboratory model pathogens [49-51].

Concerning *Bartonella* spp., the loss of the virulence factor BadA, a trimeric autotransporter adhesin, has been reported for *B. henselae* after numerous *in vitro* passages disturbing the capacity of the bacteria to successfully infect its host [52]. Of note, *Bartonella* spp. are fastidious bacteria with a very weak *in vitro* growth rate. *Bartonella 1-1C* is no exception to the rule and any genetic engineering requires several *in vitro* passages added to long growth time on plate. Taken together, we can assume that recurrent genetic manipulations on *B1-1C* and/or successive *in vitro* passages attenuate the infectivity of the original strain explaining the abacteremic phenotype reported for all *B1-1C* mutant strains.

Since we assume that genetic engineering and/or *in vitro* passages attenuate bacterial infectivity it was surprising to observe no significant difference between bacteremia curves induced by *B1-1* WT and *B1-1C* SmR. Even if antibiotic resistance acquisition is frequently associated with a fitness cost [53-55], it has been reported that the fitness cost of resistance acquisition such as streptomycin can be compensated by a secondary mutation [56-58]. Therefore, it could explain the similarity between bacteremia triggered by *B1-1C* WT and the streptomycin resistant strain. Furthermore, acquisition of a streptomycin resistant strain requires only one supplementary *in vitro* passage, which might be insufficient to induce infectivity attenuation. Furthermore, the chromosomic insertion of a transposon harboring a *gfp* cassette within Bartonella *1-1C* has a weak impact on the bacterial fitness. Transposition is a genetic technique where a transposable element is inserted within the genome and requires only a few *in vitro* passages.

Taken together, all the findings concerning *B1-1C* compared to what is known about *Bartonella* infection, drove us to reconsider the rat as the natural specific host of *B1-1C*. Indeed, this would explain why (i) *B1-1C* is unable to colonize rat's erythrocytes in our experiments, which has a pivotal role in *Bartonella* pathogenicity; (ii) that genetic engineering and/or successive *in vitro* passages have a strong

impact on the infectivity; (iii) the high sensitivity for the growth media for reliable infection; and (iv) the long-time needed to *B1-1C* to travel through the dermis from the inoculation site to the bloodstream likely due to the lack of appropriate and specific tools to subvert host immune cells. Nevertheless, if we assume that the rat is not the natural host of *B1-1C*, the real natural host might be evolutionary closed to *Rattus norvegicus* explaining why *B1-1C* is able to trigger a short infection of the host similarly to *BAR15* that induces bacteremia in rat while it is a red squirrel specific pathogen [12].

In summary, in this study we have developed an *in vivo* infection model in rat for lineage 3 *Bartonella* species. By using *i.d.* and *i.v.* infections with *B1-1C* we reveal a delayed and shorter bacteremia kinetic compared to the lineage 4. The major finding of this study is the inability of *B1-1C* to colonize erythrocytes which rise to question the specific relation between rat and this *Bartonella* species.

To provide more significance to our *in vivo* results such as the non-intraerythrocytic life style and to confirm our genetic engineering it seems crucial to establish an *in vitro* infection model. Finally, we have to keep in mind that lineage 3 species could use a different infection strategy compared to other lineages and it needs further investigation on bacteremia kinetic of other lineage 3 species such as *B. clarridgeiae* or *B. AR15-3*. Therefore, if the extraerythrocytic life style of *B 1-1C* is confirmed, the murine model described in this study should provide an experimental basis for further investigations on lineage 3 and hasten our understanding of *Bartonella* infection strategies.

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Research Article II

Bartonella Infection: Identification of a YopJ-like Protein, a Putative T4SS Effector Involved in Bacteremia Establishment

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Abstract

Many bacterial pathogens translocate through their membrane numerous effectors to subvert host cellular signaling pathways. These effectors often act in concert to efficiently infect the host. The α-proteobacterial genus *Bartonella* uses a VirB/D4 Type 4 secretion system (T4SS) to translocate a set of *Bartonella* effector proteins (Beps) into targeted host cells. The significance of the VirB/D4 T4SS for *Bartonella* pathogenicity has been shown previously but, up to date, the role of the effectors was extensively studied *in vitro* but not *in vivo*.

Therefore, in this study we investigated the contribution of the *Bartonella* VirB/D4 T4SS and its translocated proteins during *in vivo* infection process. To do so, we used the rat-specific *Bartonella tribocorum* (*Btr*) infection model. Our investigations highlighted a bacteremic phenotype of the Bep-deficient *Btr* Δ*bepA-I* mutant characterized by a bacteremia with a shorter duration and a delayed onset compared to *Btr* WT. This fitness lost was increased at low inoculum (10^5 CFUs/ml) where the *Btr* Δ*bepA-I* was unable to trigger bacteremia whereas the WT strain was still infectious. Even though this phenotype was inoculum-dependent, the ability of the Bep-deficient *Btr* to cause bacteremia whereas a VirB/D4 T4SS-deficient strain was abacteremic raised questions on the central role of the Beps. The phylogenetic analysis of *Bartonella* species suggested the translocation of an additional effector named YopJ via the VirB/D4 T4SS. Previously described as a Type 3 secretion effector in *Yersinia pestis*, our results suggest YopJ to have evolved as a Type 4 secretion effector in *Bartonella* species. We also showed that a single *yopJ* was independently acquired by the last common ancestors of lineage 3 and lineage 4, two lineages bearing the VirB/D4 T4SS, and evolved in several *Bartonella* species via parallel rounds of gene duplication and differentiation events. Finally, the decreased bacterial fitness of *Btr* Δ*yopJ* and *Btr* Δ*bepA-I* Δ*yopJ* strains in rat infection model confirmed the implication of YopJ in *Bartonella* pathogenesis.

Introduction

During evolution, pathogenic bacteria have developed a wide variety of highly specialized virulence factors. Secretion of proteins via macromolecular nanomachine is one strategy shared by many bacteria in order to subvert immune signaling pathways of the host [1]. Depending on their structures, secretion mechanism and specificity, translocation apparatus can be categorized into different classes from type 1 to type 9 secretion systems [2]. Among Gram-negative and Gram-positive bacteria, the type IV secretion systems are extensively spread. The number of bacteria using these systems during infection process continues to expand and includes some clinically-important human pathogens such as *Helicobacter pylori*, *Legionella pneumophila*, *Bordetella pertussis*, *Brucella* and *Bartonella* species [3-5]. One striking example of host signaling pathway subversion is the translocation of CagA by *Helicobacter pylori* T4SS. This effector disturbs both innate and adaptive immune responses of the host allowing *H. pylori* to infect the mucous membrane of the human stomach [6].

The facultative intracellular α-proteobacterial genus *Bartonella* comprises arthropod-borne Gram-negative bacteria that typically cause a long-lasting intraerythrocytic infection in their specific mammalian host. Based on phylogenetic analysis and properties of the virulence factors, *Bartonella* genus is divides into four different lineages named lineage 1 to 4. *Bartonella* infection cycle is characterized by the infection of two distinctive niches: the dermal niche and the blood seeding niche. Following the dermal inoculation via a blood sucking arthropod, *Bartonella* invades the dermal niche characterized by the colonization of dendritic cells and the lymphatic circulation [7, 8]. Then, *Bartonella* is released into the bloodstream where it infects endothelial cells and erythrocytes. Within red blood cells bacteria undergo few rounds of replication before staying in a non-dividing state for the remaining life time of the red blood cells [8, 9]. Thus, *Bartonella* can be ingested during the arthropod blood-meal and subsequently being transmitted to another host.

In the *Bartonella* genus, translocation of *Bartonella* effector proteins (Beps) via the VirB/D4 T4SS has been identified as a critical virulence mechanism for a successful infection [1, 10]. Horizontal acquisition in lineage 3, lineage 4 and in *B. ancashensis* of the same *virB/D4 T4SS* locus with a single ancestral effector has occurred during three independent events, which were followed by chromosomal integration [11-14]. The parallel evolution of these sets of effectors, driven by the pathoadaptation to the host, led to the emergence of three distinct arsenals resulting in a high ability to infect a broad spectrum of mammalian hosts [11, 15, 16]. Despite the radiative evolution and diversification, a bipartite signal consisting of a C-terminal Bep-intracellular delivery (BID) domain and a positively charged C-tail is shared by all the Beps [17, 18]. It is believed that a primordial effector acquired this bipartite signal to promote translocation by the VirB/D4 T4SS into the targeted cell.

Moreover, it has been also revealed that the BID domain can interfere with host signaling pathways [7, 19]. In addition to the bipartite signal, around 70% of all of the Beps in the *Bartonella* genus display a N-terminal filamentous induced cAMP (Fic) domain shown to promote post-transcriptional modification such as AMPylation, phosphorylation or phosphocholination [20-22]. The other Beps, lacking the Fic domain, bear instead additional BID domains or/and tandem-repeated tyrosine (pY)-motifs [17].

Previously, the role of *Bartonella* effector translocation *in vivo* was analyzed via intravenous infection of rats with either *Btr* Δ*virD4* or *Btr* Δ*virB4.* The VirB4 ATPase, located in the cytoplasmic side, is a crucial component of the VirB/D4 T4SS that provides energy for pilus biogenesis and effector translocation. Therefore, *Btr* Δ*virB4* does not express a functional translocation machinery [23, 24]. Unlike VirB4, the coupling protein VirD4 is involved exclusively in effector translocation by recognizing the bipartite T4S signal harbored by effectors and by providing energy for the process [24]. Therefore, *Btr* Δ*virD4* expresses a machinery that lacks the ability to recruit and translocate effectors. Rats infected with *Btr* Δ*virD4* or *Btr* Δ*virB4* did not develop bacteremia indicating the dependency of *Bartonella* on the VirB/D4 T4SS and on the effector translocation process to establish bacteremia [25].

In this study, we showed a bacteremic phenotype of a *Bartonella tribocorum* strain deficient for all Beps questioning the significance of the Beps during the infection process. Furthermore, genetic analysis performed in this study revealed the presence of another effector widely distributed among *Bartonella* species harboring the VirB/D4 T4SS: a YopJ-like effector. The YopJ effector has been previously characterized as a Type 3 secretion effector (T3SE) in *Yersinia pestis*. Interestingly, our researches suggest that YopJ evolved as a T4S effector in *Bartonella* species.

Results

Protein translocation by the virB/D4 T4SS is a crucial process for *Bartonella* to establish a successful infection bacteremia. Previous work showed that *Bartonella* loses its infectivity when we prevent either the assembly of the virB/D4 T4SS (*Btr* Δ*virB4*) or the translocation by the coupling protein (*Btr* Δ*virD4*) [25]. To analyze the role of the virB/D4 T4SS and the Beps during infection at the molecular level, I generated a Bep free mutant, *Btr* Δ*bepA-I*, and compared it to *Btr* WT. Furthermore, rats were infected via two different infection routes in order to have a more precise overview at which stage of the infection the VirB/D4 T4SS and the Beps could play a role. The intradermal infection route (*i.d.*) mimics the natural way of infection whereas the intravenous route (*i.v.*) bypasses the first dermal stage of the infection.

Btr Δ*bepA-I* triggers impaired bacteremia after *i.d.* injections

The goal in first step was to compare bacteremia kinetics induced by *Btr* WT (Fig. 1A) and *Btr* Δ*bepA-I* (Fig. 1B) via intradermal route.

Compared to *Btr* WT, *Btr* Δ*bepA-I* showed bacteremia impairments indicating a fitness decrease. Indeed, in the group of rats infected with *Btr* Δ*bepA-I*, bacteremia onset and duration were significantly different from *Btr* WT (*p*-value = 0.0015 and 0.0001, respectively) (Fig. 1C). Taken together, these results indicate that Beps facilitate bacteremia establishment and maintain a long-lasting infection. Moreover, the infection rate of *Btr* Δ*bepA-I* was 60% whereas the infection rate of *Btr* WT was 100% confirming a fitness loss for the Bep-free mutant. However, the fact that the maximum titer reached by bacteremia was not significantly different from *Btr* WT (*p*-value = 0.4879) indicates that Beps are not involved in bacterial replication during the infection process (Fig. 1C). Remarkably, rat #849 (Fig. 1A) and rat #1023 (Fig. 1B) showed defective bacteremia curves. We assume that *Bartonella* faced a strong immune response making the infection more challenging in these infected rats.

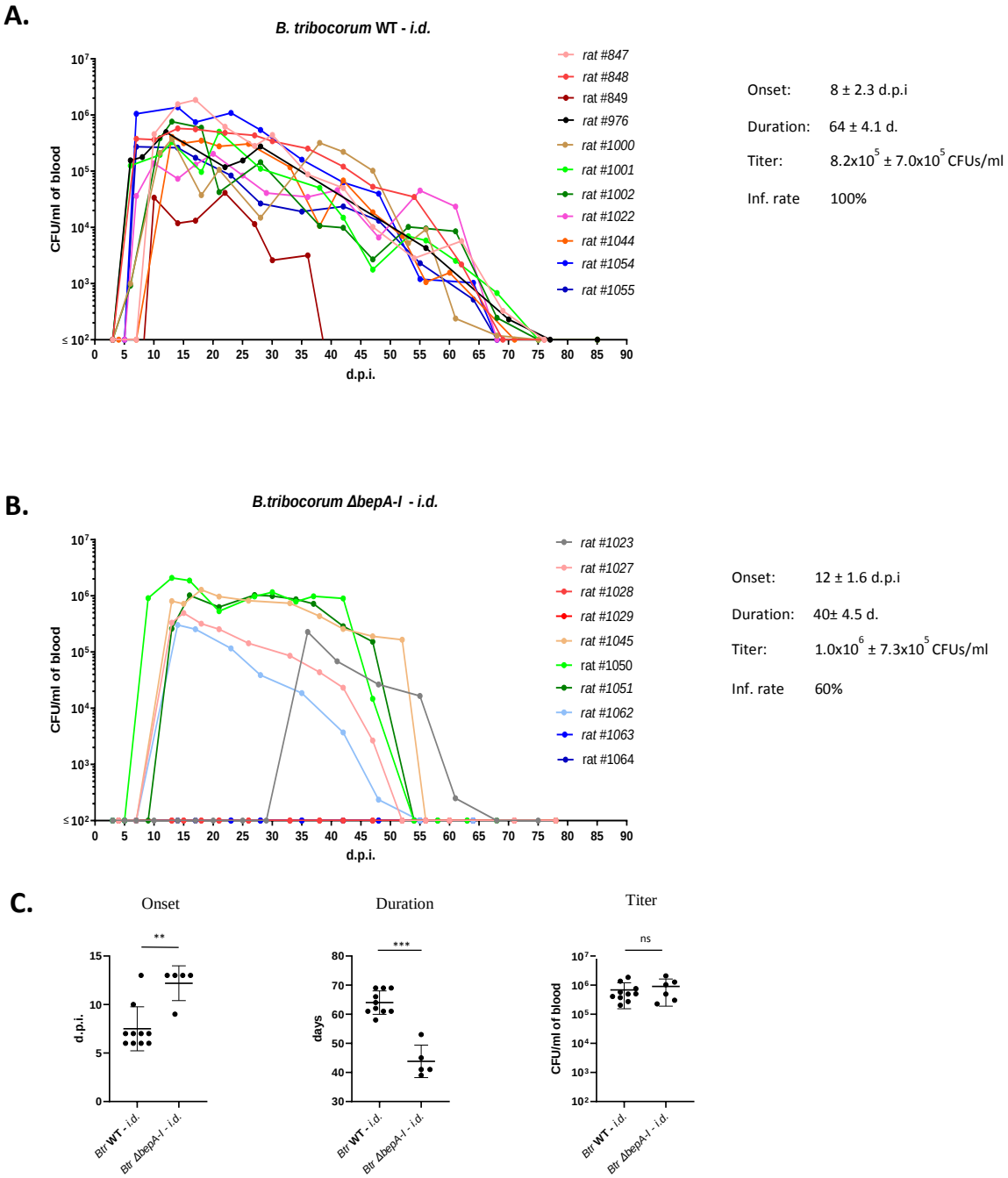


Figure 1. Bacteremia analysis in rats intradermally infected with *Btr* WT and *Btr ΔbepA-I*

Bacteria were grown for 3 days at 35°C and 5% CO_2 on Heart Infusion Agar (HIA) plates supplemented with defibrinated sheep blood. Plotted graphs represent time course of single animals (indicated by the different animal numbers) from which bacteria were recovered at a given time point post-infection. Each value corresponds to an average (CFU/ml of ml of blood) acquired from serial dilutions. Each color shade represents a cohort of animal infected the same day with bacteria from the same bacterial solution. Tables correspond to the mean ± SD of the onset, duration and maximum titer between all bacteremia of the associated graph. The infection rate is also indicated (A) Groups of 10 weeks old Wistar rats (10^7 CFUs, n = 11) were intradermally infected with *Btr* WT. (B) Groups of 10 weeks old Wistar rats were intradermally infected (10^7 CFUs, n = 10) with *Btr ΔbepA-I.* (C) A statistical t-test analysis between the two strains was performed on bacteremia onset, duration and maximum titer.

Btr Δ*bepA-I* triggers impaired bacteremia after *i.v.* injections

Since we observed a fitness loss in *i.d.* injections, we were interested to determine if there is also a fitness decrease after intravenous infection. Therefore, we infected rats with either *Btr* WT (Fig. 2A) or *Btr* Δ*bepA-I* (Fig. 2B) strain via intravenous route.

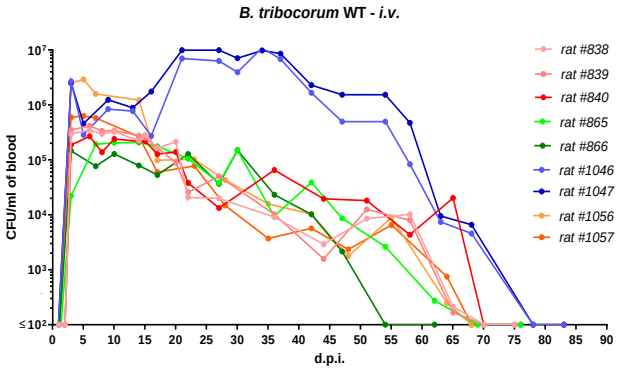
Similarly to *i.d.* infection, rats intravenously infected with *Btr* WT developed impaired bacteremia with a delayed onset and a shorter duration statistically different from *Btr* WT (*p*-value = 0.0021 and 0.0002, respectively) (Fig. 2C). Furthermore, we observed that *Btr* Δ*bepA-I* displayed an infection rate of 83% instead of 100% for *Btr* WT. No significant difference was observed for the maximum titer reached by the bacteremia (Fig. 2C). Therefore, consistently to *i.d.* infection, these observations indicate that (i) Beps are not crucial but facilitate bacteremia establishment and maintain a sustainable bacteremia and, (ii) Beps are not involved in bacterial replication during the infection.

Remarkably, rats #1046 and #1047 (Fig. 2A, blue lines) showed an unusual high titer (reached 10^7 CFUs/ml) and a longer duration (75 days). Interestingly, these two rats belonged to the same cohort of animal. Therefore, we suggested that these rats must have had a weaker immune system to face the bacteria and subsequently to clear the infection. To notice, we termed cohort rats belonging to the same animal order.

Furthermore, when we compared the two routes of infection for either *Btr* WT or *Btr* Δ*bepA-I*, we detected a delayed onset after *i.d.* injections, which might correspond to the time needed for the bacteria to travel from the dermis to the bloodstream.

Taken together, even if the bep-free mutant is still bacteremic, these results clearly showed a loss of infection fitness of *Btr* Δ*bepA-I* compared to *Btr* WT. However, compared to the natural condition, the high inoculum used for infection is not relevant and could have an effect on *Bartonella* infection *in vivo*. To assess this hypothesis, we decided to test lower inocula.

A.

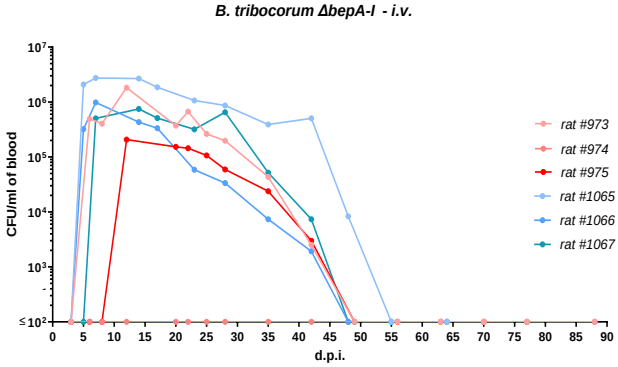


Onset: 3 ± 0.0 d.p.i

Duration: 64 ± 5.8 d.

Titer: $2.7x10^5 \pm 1.1x10^5$ CFUs/ml

B.



Onset: 7 ± 2.9 d.p.i

Duration: 43 ± 4.7 d.

Titer: $1.3x10^6 \pm 9.9x10^5$ CFUs/ml

C.

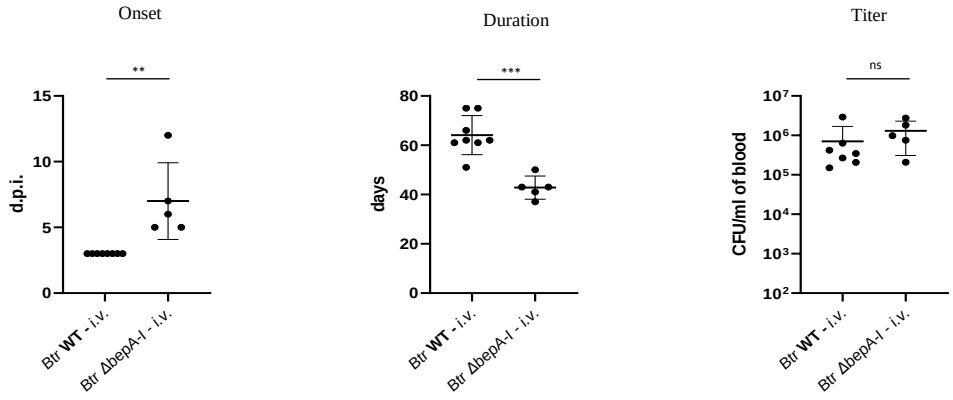


Figure 2. Bacteremia analysis in rats intravenously infected with *Btr* WT and *Btr ΔbepA-I*

Bacteria were grown for 3 days at 35°C and 5% CO_2 on Heart Infusion Agar (HIA) plates supplemented with defibrinated sheep blood. Plotted graphs represent time course of single animals (indicated by the different animal numbers) from which bacteria were recovered at a given time point post-infection. Each value corresponds to an average (CFU/ml of ml of blood) acquired from serial dilutions. Each color shade represents a cohort of animal infected the same day with bacteria from the same bacterial solution. Tables correspond to the mean ± SD of the onset, duration and maximum titer between all bacteremia of the associated graph. The infection rate is also indicated (A) Groups of 10 weeks old Wistar rats (10^7 CFUs, n = 9) were intravenously infected with *Btr* WT. (B) Groups of 10 weeks old Wistar rats were intravenously infected (10^7 CFUs, n = 6) with *Btr ΔbepA-I.* (C) A statistical t-test analysis between the two strains was performed on bacteremia onset, duration and maximum titer.

Btr Δ*bepA-I* bacterial fitness is dose-dependent

In the wild, the amount of bacteria needed to establish bacteremia is hardly assessable but probably is significantly lower than the dose used in laboratory (10^7 CFUs for *i.d.*). The high dose used could explain the infectivity of *Btr* Δ*bepA-I* and to assess this hypothesis, we decided to infect rats with lower inocula, 10^5 and 10^2 bacteria, and to compare with bacteremia kinetics observed with the inocula 10^7 bacteria.

Groups of rats were infected with different doses of *Btr* WT and bacteremia was monitored and compared (Fig. 3A). We observed a correlation between the inoculum and bacterial fitness: with lower inoculum, bacteremia appearance was delayed and bacteremia duration was shorter. We also noticed that with an inoculum of 10^2 bacteria the infection rate dropped to 33% instead of 100% for the two others. This suggests that in natural conditions the amount of *Bartonella* for a successful infection probably has to be above a certain threshold comprised between 10^2 and 10^5 CFUs. Interestingly, in all the conditions tested, bacteremia reached a maximum titer of around 10^6 CFUs/ml implying that the bacteremia titer was not dose-dependent and that following an unknown mechanism, *Bartonella* were released synchronously in the bloodstream after several rounds of replication in an undefined replicative niche.

Groups of rats were infected with different doses of *Btr* Δ*bepA-I* and bacteremia was monitored and compared (Fig. 3B). We also observed a correlation between the inoculum and bacterial fitness but with a more pronounced phenotype compared to *Btr* WT. Indeed, infection rates dropped to 0% for 10^5 and 10^2 CFUs instead of 100% for 10^7 CFUs. Compared to *Btr* WT, results clearly showed for a decrease in *Btr* Δ*bepA-I* fitness represented by a shift to the right of the infective dose 50 (ID_{50}) (Fig. 3C). ID_{50} corresponds to the estimated number of pathogens required to produce an infection in 50% of the animals exposed to the pathogen by a given route. Thus, in our experiment, a higher number of *Btr* Δ*bepA-I* has to be injected compared to *Btr* WT to establish successful bacteremia, showing that the bacteremic phenotype of *Btr* Δ*bepA-I* is dose-dependent.

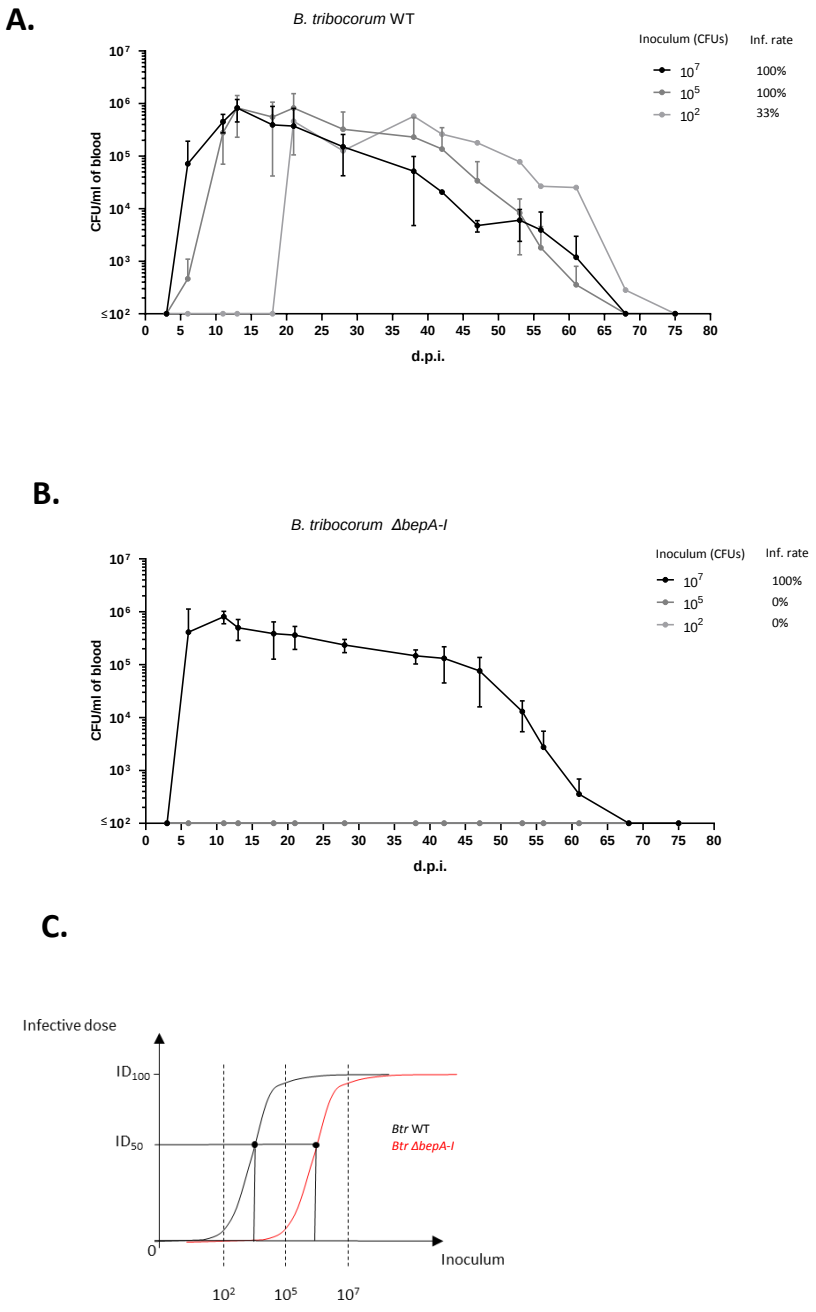


Figure 3: Bacterial fitness of *Btr* WT and *Btr ΔbepA-I* decreases with low inoculum

Bacteria were grown for 3 days at 35°C and 5% CO_2 on Heart Infusion Agar (HIA) plates supplemented with defibrinated sheep blood. Plotted graphs represent an average CFU/ml of blood in animal groups from which bacteria were recovered at a given time point post-infection. (A) Groups of 10 weeks old Wistar rats (n = 3 for each inoculum) were intradermally infected in the ear with 10^7, 10^5 or 10^2 CFUs of *Btr* WT. To notice, only one rat was bacteremic for the inoculum 10^2 CFUs. (B) Groups of 10 weeks old Wistar rats (n = 3 for each inoculum) were intradermally infected in the ear with 10^7, 10^5 or 10^2 CFUs of *Btr bepA-I.* (C) Graph representing the infective dose curves for *Btr* WT (black line) and *Btr ΔbepA-I* (red line). ID_{50} correspond to the number of bacteria required to induce bacteremia in 50% of infected rats. As result, we observed a shift to the right of *Btr ΔbepA-I* curve meaning a higher dose of bacteria is needed to trigger bacteremia in 50% of infected rats.

A functional VirB/D4 T4SS is crucial for bacteremia establishment

The abacteremic phenotype of *Btr* Δ*virD4* and *Btr* Δ*virB4* previously described in the literature suggested a key role of Beps translocation by the VirB/D4 T4SS to establish successful bacteremia [25]. However, the *Btr* Δ*bepA-I* bacteremic phenotype we obtained at high inoculum contrast with the essential trait of Beps during infection. To further characterize the role of the VirB/D4 T4SS and Beps during infection, we generated two mutants: *Btr* Δ*bepA-I* Δ*virD4* and *Btr* Δ*bepA-I* Δ*virD4* Δ*virB.* By infecting rats with these mutants, we aimed to answer multiple questions: (i) Is there a toxic accumulation of Beps within *Btr* Δ*virD4* explaining its abacteremic phenotype? (ii) Has the T4SS a pathogenic effect on its own? (iii) Does the T4SS translocate proteins different from Beps?

To elucidate the significance of the VirB/D4 T4SS during *Btr* infection, we first tried to reproduce the results published by Schulein et *al.* by infecting intradermally and intravenously rats with *Btr* Δ*virD4* (Fig. 4A) [25]. The strain appeared abacteremic with both infection routes (*i.d.* and *i.v.*), as previously described, implying that translocation mediated by the coupling protein VirD4 is crucial for bacteremia establishment. To exclude any polar effect of the deletion, we also infected intradermally rats with the complemented strain *Btr* Δ*virD4* p*virD4* (Fig. 4B). By expressing *in-trans virD4*, the WT phenotype was restored concerning bacteremia duration and titer but with a short delay in bacteremia onset (14 d.p.i.) compared to *Btr* WT (8 d.p.i.) (Fig. 1A). Considering that *Btr* Δ*bepA-I* was still bacteremic, could the abacteremic phenotype of *Btr* Δ*virD4* be due to a toxic accumulation of Beps?

To determine if a toxic accumulation of Beps could explain the abacteremic phenotype of *Btr* Δ*virD4*, we generated a strain deficient for VirD4 and the Beps by removing *virD4* in a Bep-free *Btr* strain: *Btr* Δ*bepA-I* Δ*virD4.* Therefore, we infected rats with this strain via both *i.d*. and *i.v*. routes. Then bacteremia kinetic was monitored by sampling over time the blood of infected rats (Fig. 4C). We observed that *Btr* Δ*bepA-I* Δ*virD4* was abacteremic excluding the assumption of a toxic effect of an accumulation in *Btr* Δ*virD4*. Furthermore, since *Btr* Δ*bepA-I* Δ*virD4* still express the T4S machinery, we can assume that the VirB/D4 T4SS did not trigger any pathogenic effect on its own.

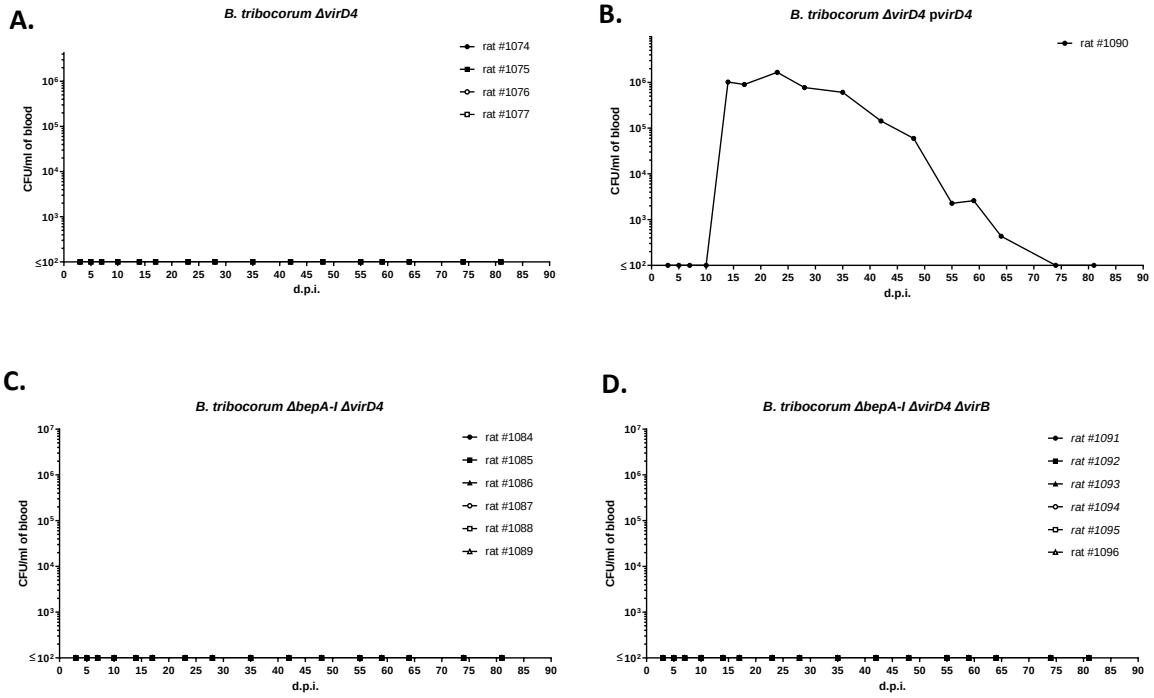


Figure 4: Bacteremia analysis in rats intradermally infected with *Btr ΔvirD4*, *Btr ΔvirD4* p*virD4*, *Btr ΔbepA-I ΔvirD4* and *Btr ΔbepA-I ΔvirD4 ΔvirB*.

Bacteria were grown for 3 days at 35°C and 5% CO_2 on Heart Infusion Agar (HIA) plates supplemented with defibrinated sheep blood. Plotted graphs represent time course of single animals (indicated by the different animal numbers) from which bacteria were recovered at a given time point post-infection. Each value corresponds to an average (CFU/ml of blood) acquired from serial dilutions. (A) Groups of 10 weeks old Wistar rats were intradermally (inoculum $1x10^7$ CFUs, n = 2, filled symbols) or intravenously (inoculum $2x10^8$ CFUs, n = 2, unfilled symbols) infected with *Btr* Δ*virD4.* (B) One 10 weeks old Wistar rats was intradermally (inoculum 10^7 CFUs, n = 2) infected *Btr* Δ*virD4* p*virD4*. (C) Groups of 10 weeks old Wistar rats were intradermally (10^7 CFUs, n = 3, filled symbols) or intravenously (inoculum $2x10^8$ CFUs, n = 3, unfilled symbols) infected with *Btr ΔbepA-I* Δ*virD4.* (D) Groups of 10 weeks old Wistar rats were intradermally (inoculum 10^7 CFUs, n = 3, filled symbols) or intravenously (inoculum $2x10^8$ CFUs, n = 3, unfilled symbols) infected with *Btr ΔbepA-I* Δ*virD4 virB.*

To clarify the role of the VirB/D4 T4SS and its Beps during *Btr* infection, we decided to generate *Btr* Δ*bepA-I* Δ*virD4* Δ*virB.* To generate this strain, we did a one-step deletion of 20 kb including the *virB* locus, the *bep* locus, and the coupling protein *virD4*. In *Btr* genome, these loci are successive to each other, making this one-step deletion feasible. Then we studied bacteremia kinetic by infecting rats via *i.d.* and *i.v.* routes (Fig. 4D). In both conditions, we did not observe bacteremia confirming that the T4S machinery, with its associated coupling protein VirD4, is crucial for *Btr* to establish infection. In combination with the bacteremic phenotype of *Btr* Δ*bepA-I*, these results indicate the translocation of unknown substrate(s) by *Btr* to establish a successful infection.

YopJ: a novel putative VirB/D4 Type 4 effector protein

To subvert eukaryotic signaling pathway, *Y. pestis* translocates six type 3 secretion effectors (T3SEs), including YopJ, that are part of the Yop virulon encoded on a 70-kb plasmid named pCD [26]. The T3SE YopJ is an acetyltransferase targeting the Mitogen-activated protein kinase (MAPK) and the nuclear factor κB (NFκB) signaling pathways involved in the innate immune response [27]. The 288 amino acid YopJ protein is divided into three regions: The N-terminal domain carrying the T3S signal, a central region harboring a catalytic triad and the C-terminal domain regulating effector activity. The catalytic triad of YopJ is formed by 3 amino acids: His^{109}, Glu^{128} and Cys^{172} [28]. In a previous study, the presence of YopJ in *Bartonella quitana* species has been revealed [29]. Therefore, we wanted to analyze the prevalence of YopJ among *Bartonella* spp. and more specifically within *Btr*.

Lineage-specific acquisition of *yopJ*

In order to evaluate the occurrence of YopJ among *Bartonella* spp., we decided to blast the amino acid sequence of the well-described YopJ of *Yersinia pestis* with *Bartonella* genus (Protein BLAST) [30]. We noticed that YopJ was well-represented among *Bartonella spp*. but only in lineage 3 and lineage 4, two lineages expressing the VirB/D4 Type 4 machinery (Fig. 5). In contrast, *B. ancashensis* (lineage 1) also carries a VirB/D4 T4SS, but no YopJ protein was detected. Two hypotheses could be made of it: *B. ancashensis* may have lost YopJ during evolution or may simply never acquired the protein. Furthermore, we found that the number of *yopJ* varied between one to three homologous genes depending on the lineage 3 and lineage 4 *Bartonella* species. Concerning our strain of interest, *B. tribocorum* had three copies of *yopJ*. Interestingly, some lineage 4 *Bartonella* spp. did not possess *yopJ* such as *B. henselae* and *B. koehlerae* (lineage 4). It could be that they lost the protein during evolution.

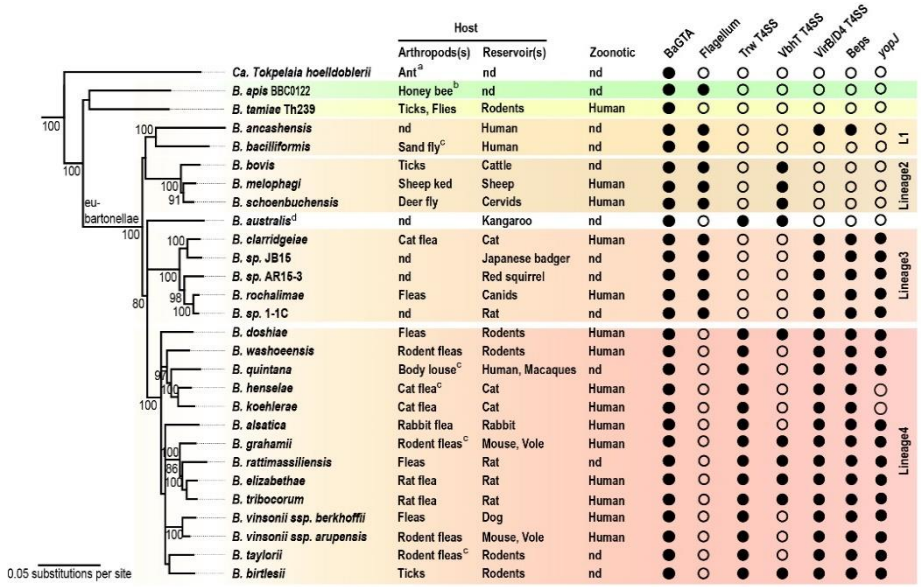


Figure 5. YopJ proteins are widely represented in *Bartonella* species harboring the VirB/D4 T4SS

Phylogeny of the genus *Bartonella* with (a) *candidatus tokpelaia hoelldoblerii* as outgroup taxon. The phylogenetic tree was based on analysis of concatenated alignment of 5 core protein sequences. Three *Bartonella* clades have been identified: *B. apis* (green), *B. tamiae* (yellow) and eubartonellae. Eubartonellea are further classified into 4 lineages (shades of orange/pink). The presence or absence of the virulence factors are reported by full or empty circles, respectively. Values above branches show bootstrap support values (≥70%). Taken from Segers et *al*. 2017. *ISME journal*

Since YopJ was widely distributed among lineage 3 and lineage 4 *Bartonella* spp., we were interested in studying the genomic localization of *yopJ*. The mapping of *yopJ* loci in *Btr* (lineage 4) and *B1-1C* (lineage 3) genomes indicated that they were not situated in a similar genomic region in both lineages (Fig. 6A). Furthermore, *in silico* analysis on *Bartonella* sequenced-species by using Geneious v.9.1.5 allowed the establishment of a preliminary *yopJ* synteny for lineage 4 and lineage 3 (Fig. 6B and C). Interestingly, we noticed that the *yopJ* synteny was conserved and specific for the two lineages. In lineage 4 species *yopJ* homologous gene(s) were localized in one locus and always nearby *groL*, *fumC*, a gene encoding for a lysozyme and other uncharacterized genes sharing more than 80% of identity (dashed lines). However, in *B. quintana*, *B. vinsonii* and *B. taylorii* we did not detect *groL* and *fumC.* For *B. taylorii* this could be explained by the fact that this species is not fully sequenced so far. In *B. quintana* and *B. vinsonii*, these two genes were identified in another locus resulting from presumable genomic rearrangements. In lineage 3 species, three *yopJ* genes were found in two different loci where *yopJ1* and *yopJ2* were conserved on the same locus and *yopJ3* was isolated on a different one.

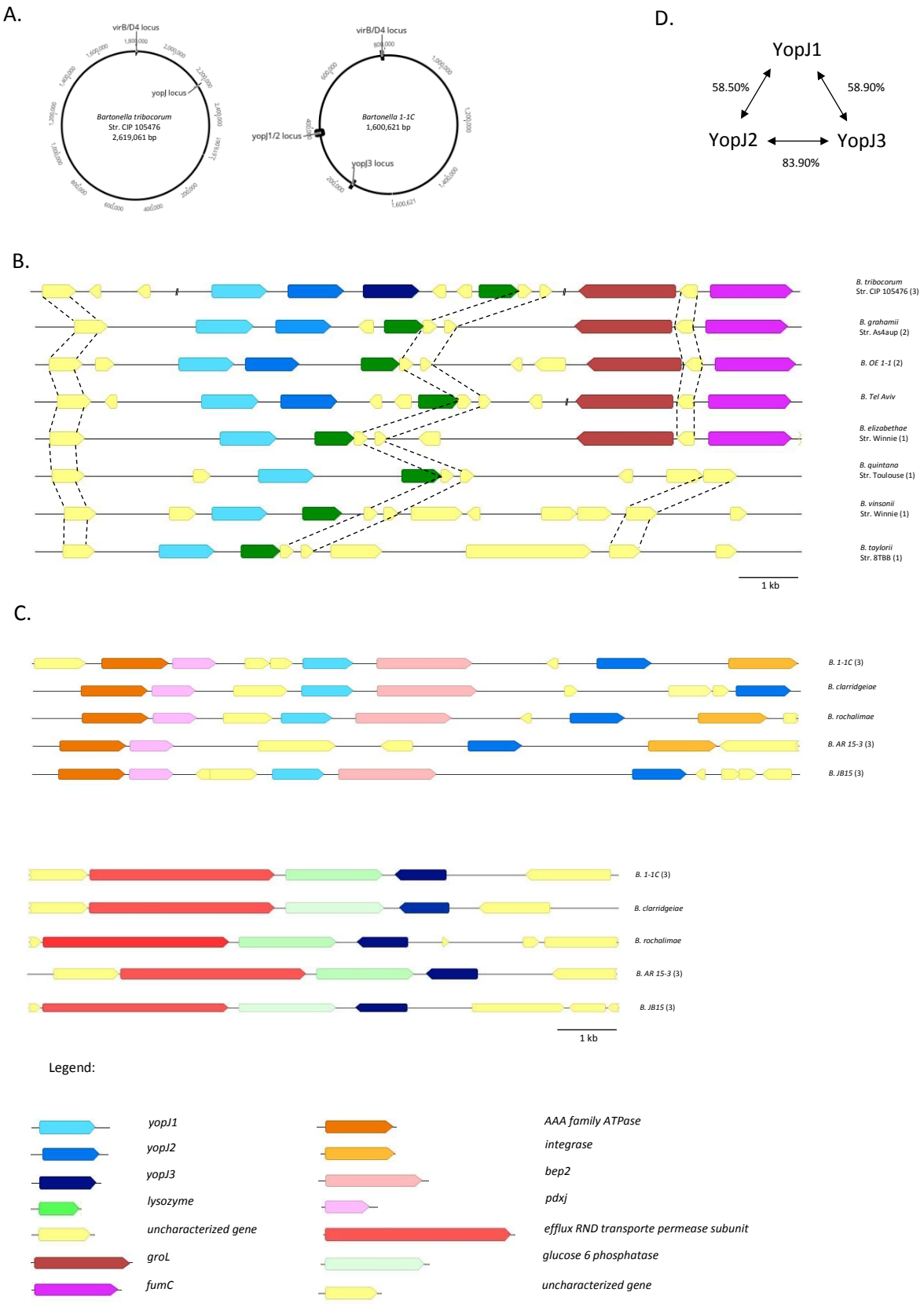


Figure 6. ***yopJ* was acquired independently in lineage 3 and lineage 4 *Bartonella* species and evolved via parallel duplication and diversification events**

(A) Genome maps of *Btr.* and *B1-1C*. (B) Synteny of *yopJ* genes in lineage 4 sequences species. (C) Synteny of *yopJ* genes in lineage 3 sequences species. Numbers in brackets correspond to the number of *yopJ* harbored by the species (D) Percentage of identity between YopJs from *Btr*.

The fact that there was a conserved lineage-specific synteny indicated that *yopJ* was independently acquired by the last common ancestors of lineage 3 and lineage 4. This assumption was supported by the fact that lineage 1 and lineage 2 were lacking *yopJ* and by the different localization of *yopJ* loci between lineages (Fig 5 and 6A).

Remarkably, in lineage 3 a *bep* identified as *bep2* was flanked by *yopJ1* and *yopJ2*. Since *bep2* is assumed to be a duplication of *bep1* in *Bartonella* spp., it is unlikely that *yopJ* and *bep* were acquired together (Fig. 6C). However, we cannot exclude that *bep2* was displaced downstream to *yopJ1* by genomic rearrangement and might act subsequently as a functional unit. This assumption could be supported by the fact that *in B. AR 15-3 yopJ1* and *bep2* were missing.

Furthermore, *in silico* analysis using BLAST implemented in Geneious v.9.1.5 with the amino acid query sequences of YopJ from *Bartonella* sequenced-species permitted to set up a phylogenetic tree of YopJ from lineage3 and lineage 4 *Bartonella* species (Fig.7). The main finding was the clear separation between lineage 3 and lineage 4 YopJ proteins supporting the assumption that *yopJ* was acquired independently in the last common ancestors of each lineage and was followed by duplication and diversifications. Furthermore, the fact that all lineage 3 species displayed the same number of *yopJ* genes with conserved synteny indicates that these events occurred in the common ancestor prior speciation. On the contrary, lineage 4 species possessed one to three copies suggesting that the duplication events occurred after speciation.

Indeed, since for *B. washoeensis* YopJ2 was not clustered with other YopJ2 we assumed an independent duplication of *yopJ1*. Furthermore, based on the phylogenetic tree established by A. Segers *et al.* [31] we know that *B. tribocorum*, *B. grahamii* and *B. rattumassiliensis* are closely related (Fig. 5). In our phylogenetic tree YopJ2 of these species were grouped. Therefore, we can suppose that the duplication of *yopJ1* occurred in the common ancestor of these three *Bartonella* species. However, we have to be careful with this assumption since *B. elizabethae*, which is also suggested to be closely related to these three species, carried only one *yopJ*. It is possible that (i) this species lost *yopJ2* or, (ii) within the three others closely related *Bartonella* species *yopJ1* was duplicated independently.

Finally, *B. tribocorum* is so far the only known species bearing three *yopJ* homologous genes. Amino acid sequences alignment of the three YopJs of *Btr* with Geneious v.9.1.5. shown 83.90% identity between YopJ2 and YopJ3 and 58.50% and 58.90% with YopJ1, respectively (Fig. 6D). Furthermore, in the phylogenetic tree YopJ2 is closely related to YopJ3. Therefore, we believe that *yopJ2* appeared after a first duplication event of *yopJ1*, and a more recent second duplication event of *yopJ2* gave rise to the appearance of *yopJ3*.

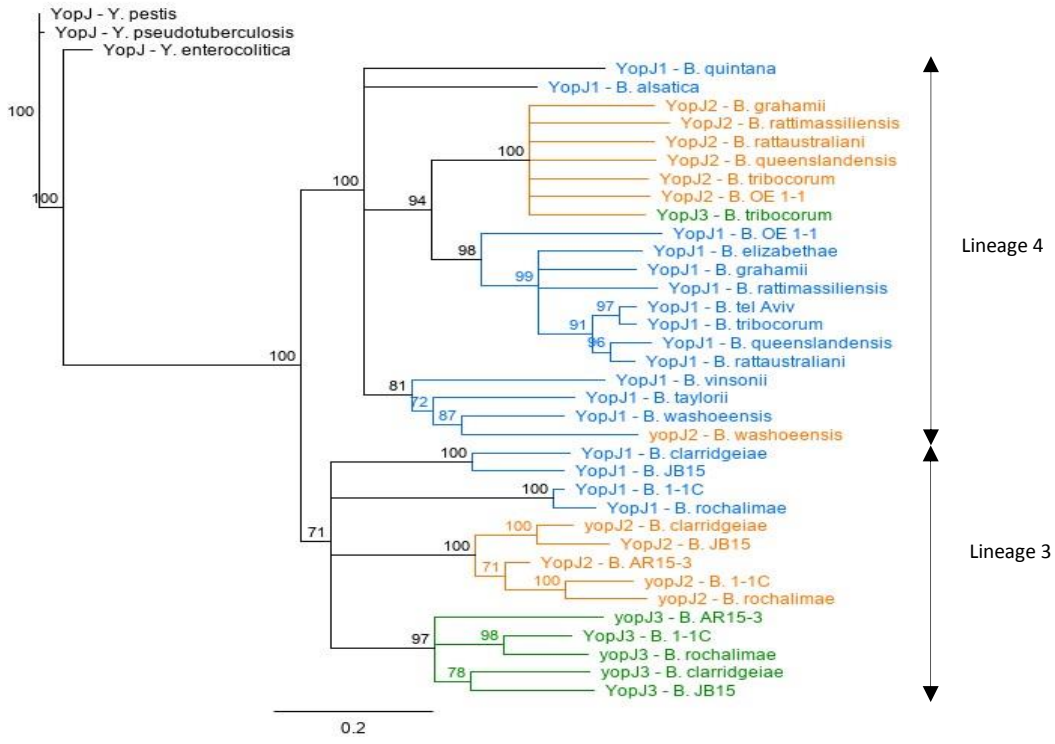


Figure 7: Phylogenetic distribution of YopJ proteins among *Bartonella* species

Phylogeny of YopJ proteins among the genus *Bartonella* with YopJ of *Y. pestis* as outgroup taxon (black). YopJ1 distribution is showed in blue. YopJ2 and YopJ3 distribution is showed in orange and green, respectively. Values above branches show bootstrap support values (≥70%).

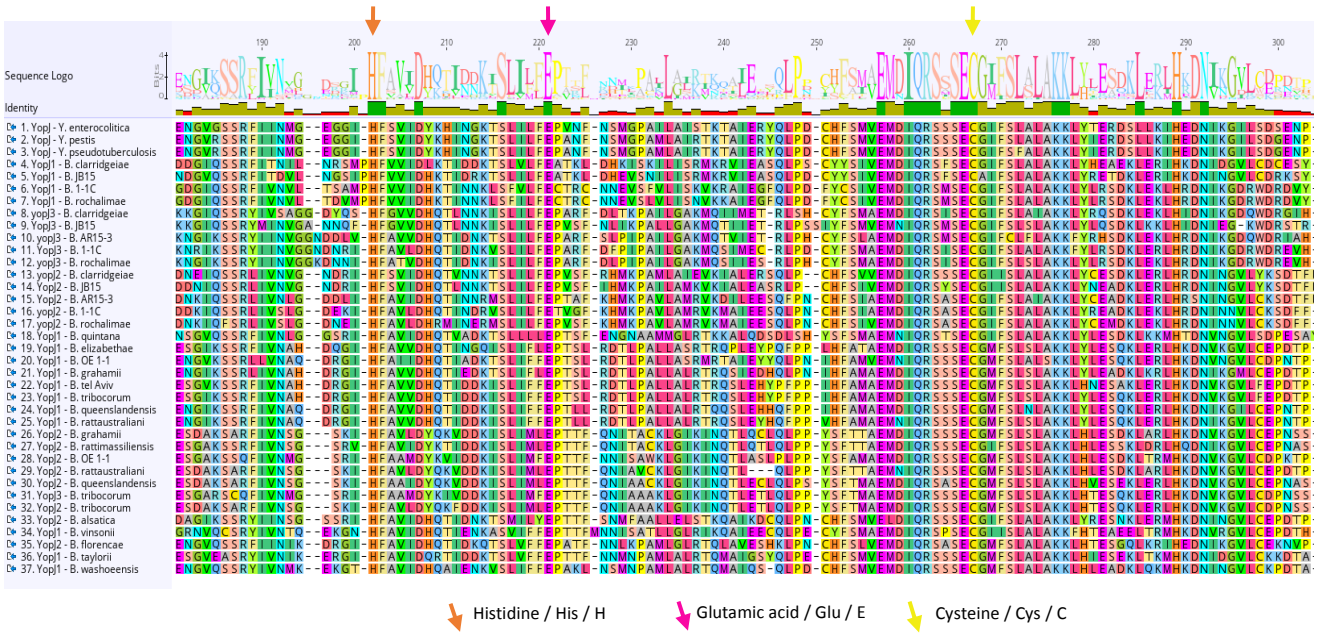


Figure 8. YopJ alignment shows the conservation of the catalytic triad among lineage 3 and lineage 4 *Bartonella* spp.

Comparison of YopJ sequences from *Bartonella* species and three *Yersinia* species (First 3 lines). Colored arrows above the alignment point to amino acids involved in the catalytic triad: His^{109} (orange arrow), Glu^{128} (purple arrow) and Cys^{172+} (yellow arrow). As result, this alignment highlighted that the triad is well conserved among *Yersinia* spp and *Bartonella* spp.

The catalytic triad of YopJ is well conserved in *Bartonella* species

In our *in silico* analysis, we observed that *Bartonella* spp. could have different *yopJ* copies called *YopJ1*, *YopJ2,* and *YopJ3* (Fig. 6B*)*. Although *yopJ* genes within *Bartonella* species undergone duplication and differentiation events we noticed in our amino acid sequences alignment the conservation of the catalytic triad (His/Glu/Cys - colored arrows) (Fig. 8). In *Y. pestis*, this catalytic triad is crucial for the protein activity with an acetyltransferase activity allocated to the cysteine[32].

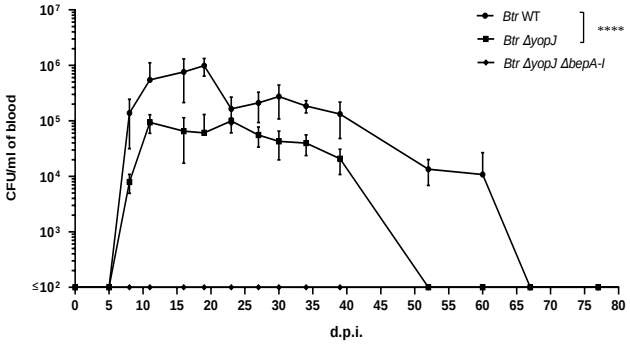
Based on our *in silico* analysis and on the bacteremic phenotype of *Btr.* Δ*bepA-I*, we aimed to examine if YopJ plays a role in *Btr* infection.

Btr* Δ*yopJ* shows a bacterial fitness similar to *Btr* Δ*bepA-I

To elucidate the role of YopJ proteins during Btr *in vivo* infection, we generated a strain where we removed the three *yopJ* genes present in *Btr*. For this purpose, we deleted by a one-step *in frame* deletion the three genes in the *Btr* WT genome. The generated strain, *Btr* Δ*yopJ,* was then intradermally injected in rats (Fig. 9). We observed that the bacteremia kinetic induced by *Btr* Δ*yopJ* was significantly different from *Btr* WT with a shorter duration and an lower titer indicating that YopJ effectors might contribute in *Btr* pathogeneicity.

***Btr* Δ*bepA-I* Δ*yopJ* loses its infectivity**

We showed that *Btr* Δ*bepA-I* was still bacteremic but with a fitness defect compared to *Btr* WT. To investigate further the role of YopJ effectors during *Btr* infection, we performed a one-step in frame deletion of *yopJ* locus in *Btr* Δ*bepA-I*. The strain was then intradermally infected with *Btr* Δ*bepA-I* (Fig. 9). We observed that the strain lost its infectivity, confirming a significant role of YopJs effector during *Btr* infection.



Onset:	8 ± 2.3 d.p.i	*Btr* WT
Duration:	64 ± 4.1 d.	
Titer:	$8.2x10^5 \pm 7.0x10^5$ CFUs/ml	
Inf. rate	100%	
Onset:	8 ± 1.4 d.p.i	*Btr ΔyopJ*
Duration:	41 ± 3.4 d.	
Titer:	$1.1x10^5 \pm 1.3x10^5$ CFUs/ml	
Inf. rate	100%	

Figure 9. Deletion of YopJ triggers bacteremia impairments

Bacteria were grown for 3 days at 35°C and 5% CO_2 on Heart Infusion Agar (HIA) plates supplemented with defibrinated sheep blood. Plotted graphs represent time course of single animals from which bacteria were recovered at a given time point post-infection. Each value corresponds to an average (CFU/ml of blood) acquired from serial dilutions. Groups of 10 weeks old Wistar rats were intradermally infected (inoculum $1x10^7$ CFUs, n = 3) with either *Btr* Δ*yopJ* or *Btr* Δ*yopJ ΔbepA-I* or *Btr WT*.

Materials and Methods

Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 1. *Bartonella tribocorum* strains were grown on HIA plates at at 35°C in a moist atmosphere under 5% CO2 for 2-3 days. When indicated, media were supplemented with 100 µg/ml streptomycin. E. coli strains were cultivated in LuriaBertani liquid medium (LB) or on Luria-Bertani agar on plates (LA) at 37°C overnight. When indicated, media were supplemented with 50 µg/ml kanamycin, 200 µg/ml ampicillin, 20 µg/ml gentamicin, 500 µM IPTG, and/or 1 mM diaminopimelic acid (DAP).

Animal experimentation

Animal handling was performed in accordance with the Swiss Animal Protection law and local animal welfare bodies. The animal experiments in this work were approved by the Veterinary Office of the Canton Basel-Stadt (License number 1741). All animals were kept at SPF (specific pathogen-free) conditions. Animals were obtained from Janvier labs at the age of 8-10 weeks. After two weeks of adaptation, rats were infected with bacteria harvested and diluted in phosphate-buffered saline (PBS) at OD595nm = 1. On anesthetized rats (1 – 3% isoflurane/O_2 mixture), we performed either intradermal injection in the dermis of the ear (10 µl of the bacterial solution; 10^7 CFUs) or intradermal injection in the tail vein (200 µl of the bacterial solution; $2x10^8$ CFUs).

Blood samples were drawn and diluted (9:1) in PBS supplemented with 3.8 % of sodium-citrate, to prevent coagulation, and kept at -80°C for at least 1 hour. After thawing, blood samples were serial diluted in PBS and plated on appropriate agar plates (HIA plates). Then for counting, bacteria were incubated at 35°C and 5% CO2 for either 5-6 days for *Btr*.

Medium composition

Media	Composition	Quantity (g) for 1L
Heart Infusion Agar (HIA) BD™	Beef Heart infusion from 500g	10.0
	Tryptose	10.0
	Sodium Chloride	5.0
	Agar	15.0

Luria-Bertani Broth (LB) BD™	Tryptone	10.0
	Yeast extract	5.0
	NaCl	10.0

Luria-Bertani Broth (LB) BD™	Tryptone	10.0
	Yeast extract	5.0
	NaCl	10.0
	Agar	15.0

The powder mixed with 600 ml of ddH_2O was autoclaved and supplemented with 4.2% (v/v) sterile defibrinated sheep's blood

TSA and HIA plates were supplemented with 5% of defibrinated sheep blood (Thermos Fisher™)

Generation of spontaneous streptomycin-resistant strains

Generation of the spontaneous streptomycin-resistant *Btr* strain RSE149 (CB0165) has been described previously by R. schulein [25]. In this study, the strain has been stocked as CB0165 after one additional *in vitro* passage of RSE149 on HIA agar plate.

Plasmid construction

All primers used for the elaboration of this report are listed in table.
The suicide mutagenesis vector pTR1000 used in this study was described previously [36].

pCB0007 used for generating *Btr* Δ*A-I* Δ*virD4 in-frame* mutant was generated as follows. The homology region 1 (HR1), corresponding to the 1011 bp downstream *virD4,* was amplified from *Btr* Δ*A-I* (CB0265) with the oligonucleotide primers prCB120 and prCB114. The HR2, corresponding to the 978 bp upstream virD4, was amplified from *Btr* Δ*A-I* (CB0265) with the oligonucleotide primers prCB111 and prCB122. The amplified products were merged by SOEing PCR with the oligonucleotide primers prCB120 and prCB122. The 1989 bp-product was digested with XbaI and cloned into the suicide vector pTR1000, digested with XbaI and BamH1, to yield pCB007. Insertion of the SOEing fused-product within pTR1000 was confirmed by PCR with the oligonucleotide primers prCB116 and prCB117 spanning the insertion site.

pCB009 used for generating *Btr* Δ*A-I* Δ*virD4* Δ*virB in-frame* mutant was generated as follows. The HR1, corresponding to the 1054 bp downstream *virB2,* was amplified from *Btr* Sm^R (CB0165) with the oligonucleotide primers prCB121 and prCB110. The HR2, corresponding to the 994 bp upstream *bepI*, was amplified from *Btr* Sm^R (CB0165) with the oligonucleotide primers prCB118 and prCB119. By SOEing PCR the amplified products were merged with the oligonucleotide primers prCB121 and prCB119. The 2048 bp-product was digested with BamH1 and XbaI and clones into the suicide vector pTR1000, digested with BamH1 and XbaI, to yield pCB009. Insertion of the SOEing fused-product within pTR1000 was confirmed by PCR with the oligonucleotide primers prCB116 and prCB117 spanning the insertion site.

pKCB007 used for generating *Btr* Δ*yopJ in-frame* mutants was generated as follows. The HR1, corresponding to the 1024 bp downstream *yopJ* locus*,* was amplified from *Btr* Sm^R (CB0165) with the oligonucleotide primers prJK493 and prJK494. The HR2, corresponding to the 1041 bp upstream *yopJ* locus, was amplified from *Btr* Sm^R (CB0165) with the oligonucleotide primers prJK495 and prJK496. The amplified products were merged by SOEing PCR with the oligonucleotide primers prJK493 and prJK496. The 2089 bp-product was digested with BamH1 and XbaI and clones into the suicide vector pTR1000, digested with XbaI and BamH1, to yield pKC007. Insertion of the SOEing fused-product within pTR1000 was confirmed by PCR with the oligonucleotide primers prCB116 and prCB117 spanning the insertion site.

Procedure for gene replacement and confirmation of the mutants

Following the mobilization of the suicide mutagenesis vector into the appropriate *Bartonella* strain, kanamycin was used as a positively selection step to isolate cointegrates. Growing bacteria that integrated the suicide mutagenesis vector by homologous recombination were selected by colony PCR spanning the two homology regions with the appropriate oligonucleotide primers. Then, cointegrates were negatively selected on a media supplemented with streptomycin. The surviving bacteria have lost the mutagenesis vector by a second homologous recombination leading to two situations; either the clone harbor the *in-frame* deletion or the restoration of the wild type conformation. Colony PCR spanning the region of interest was performed to isolate clones carrying the deletion by comparing the size of the generated PCR products.
For *Btr* Δ*A-I* Δ*virD4 in-frame* mutant, the oligonucleotide primers prCB120 and prCB122 (size expected 1989 bp) were used to select cointegrates after the positive selection and the oligonucleotide primers prMS056 and prPE336 were used to confirm the deletion (size expected 2484 bp). The oligonucleotide primers prMS056, prPE336, and prRS087 were used for sequencing (Microsynth AG).

For *Btr* Δ*A-I* Δ*virD4* Δ*virB in-frame* mutant, the oligonucleotide primers prCB121 and prCB119 (size expected 2048 bp) were used to select cointegrates after the positive selection and the oligonucleotide primers prRS045 and prPE336 were used to confirm the deletion (size expected 2444 bp). The oligonucleotide primers prRS045, prPE336 and prRS042 were used for sequencing (Microsynth AG).

For *Btr* Δ*yopJ in-frame* mutant, the oligonucleotide primers prCB137 and prCB138 (size expected 2137 bp) were used to select cointegrates after the positive selection and the oligonucleotide primers prRS135 and prPE136 (size expected 422 bp) were used to confirm the deletion. The oligonucleotide primers prCB137, prCB138 and prCB135 were used for sequencing (Microsynth AG).

Synteny analysis

The nucleic acid sequences of the three *yopJ* genes of Bartonella tribocorum IBS 506T (annotaded as *yopJ1*, *yopJ2* and *yopJ3*) were compared to all *Bartonella* species of the NCBI database using the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Interesting hits obtained with BLAST were reported in the following tables for the three *yopJ* genes with the corresponding accession numbers. Then, the accession numbers were reported in the UNIPROT database (www.uniprot.org) and to Geneious v.9.1.5. to establish the synteny of the *yopJ* genes in all *Bartonella* species.

Accession numbers

Strains	**YopJ1**	**YopJ2**	**YopJ3**
Lineage 4			
B. tribocorum (str. CIP 105476)	WP_038474176.1	WP_012232336.1	WP_012232337.1
B. tel Aviv	WP_120099831.1	WP_120099832.1	
B. rattaustralian	WP_019224003.1	WP_019224005.1	
B. queenslandensis	WP_083868408.1	WP_039758348.1	
B. grahamii (str. as4aup)	WP_015856898.1	WP_015856899.1	
B. rattismassiliensis (str. 15908)	WP_007346374.1	WP_007346375.1	
B. washoeensis (str. 085-0475)	WP_006926072.1	WP_006926071.1	
B. elizabethae (str. NCTC12898)	WP_005775257.1		
B. taylorii (str. 8TBB)	WP_004857668.1		
B. vinsonii (str. Winnie)	WP_004866265.1		
B. alsatica (IBS 382)	WP_005866866.1		
B. OE 1-1	WP_120121107.1		
B. quintana (str. Toulouse)	WP_011179798.1		
Lineage 3			
B. clarridgeiae (str. 73)	WP_013545275.1	WP_013545271.1	WP_013544601.1
B. 1-1C	WP_100693030.1	WP_100693917.1	WP_100693917.1
B. rochalimae (ATCC BAA-1498)	WP_035006140.1	WP_035006135.1	WP_035006322.1
B. JB15	WP_078719099.1	WP_078719097.1	WP_078718558.1
B. BAR15-3		WP_078679996.1	WP_078679862.1

YopJ proteins phylogenetic analysis

The phylogeny of YopJ proteins among *Barotnella* spp. was built from 23 *Bartonella* spp. and 3 outgroup species (*Y. pestis*, *Y. enterocolitica*, *Y. pseudotuberculosis*) based on the alignment of the amino sequences of the three different YopJ proteins present in Bartonellaceae and in the outgroup species with Geneious v9.1.5. (https://www.geneious.com).

Statistical analysis

Statistical analysis of the obtained data was performed using GraphPad Prism SoftwaRE. Depending on the experiment I used either the T-test or the two-way ANOVA to compare bacteremia curves. This information is indicated in the figure legends. P-value$_{t\text{-}test}$ indicates the p-value calculated by the t-test and P-value$_{ANOVA}$ indicates the p-value calculated by the two-way ANOVA.

Strain	Genotype or relevant characteristics	Reference
Bartonella tribocorum		
RSE149	Spontaneous Sm^R strain, used as WT in this study	[8]
RSE256	Δ*virD4*, derivative of RSE149	[8]
RSE266	Δ*virD4* p*virD4*, derivative of RSE149	[8]
RSE173	Δ*virB4*, derivative of RSE149	[8]
CB0265	Δ*bepA-I*, derivative of RSE149	This study
C83B0320	Δ*bepA-I* Δ*virD4* mutant, derivative of CB0265	This study
CB0322	Δ*bepA-I* Δ*virD4* Δ*virB* mutant, derivative of RSE149	This study
CB0204	Δ*bepA-I*, derivative of RSE149	This study
CB0411	Δ*yopJ*, derivative of RSE149	This study
CB0426	Δ*bepA-I* Δ*yopJ*, derivative of CB0411	This study
Escherichia coli		
JKE217	MFDpri ΔT IV lacIq, DAP auxotroph (1mM)	[33]
CB128	Conjugative strain used for *Bartonella* Strain used for transformation	This study

Plasmid	Genotype or relevant characteristics	Reference
pTR1000	Basic suicide mutagenesis vector for *Bartonella*	[34]
pTRΔ*virD4*	Suicide mutagenesis vector for *virD4* deletion in CB0126	[33]
pCB007	Suicide mutagenesis vector for *virD4* deletion in CB0265	This study
pCB009	Suicide mutagenesis vector for *virD4, virB* locus and *bepA-I* deletion in CB0165	This study
pKCB007	Suicide mutagenesis vector for *yopJ* deletion in CB0165 and CB0265	This study

Primers	**Sequence**	Reference
prCB0013	ATGCTTAATTAAGGTTCTGGCAAATATTCTGA	This study
prCB0014	ATGTCCTAGGGCCTGCAGTTATTTGTATAG	This study
prCB0111	AAGTCTCTATACCCCCCATACACCAA**CCAATAGCGACACCAATC**	This study
prCB0116	GGAGCGGATTTGAACGTT	This study
prCB0117	GTGCTCAACGGGAATCCT	This study
prCB0118	AAGTCTCTATACCCCCCATACACCAA**GTCCTTGCCAAAAGAACAG**	This study
prCB119	ACTGTCTAGATTTCTCTCTAGGGGGATGTT	This study
prCB0120	CGTATCTAGACTTCATTCCACAACGCTCAA	This study
prCB121	CGTATCTAGAGCATGATGATACTGTGTTGGA	This study
prCB122	ACTGTCTAGATTGTTTGCAAGAGCACCTT	This study
prCB126	TTGTTTGCAAGAGCACCTT	This study
prCB135	GCCCTCTAGACTTCTGACAATT	This study
prCB136	GCTTTGAGCCAATAAGAGCTTT	This study
prCB137	CAACACCCAGCCTCCAAT	This study
prCB138	GGTATGACCATAGCCGATTGT	This study
prMS056	GGGTTGAAGGAAAATTTAAGG	This study
prRS045	TTATTAATCTGCTTTCTATCTCA	[25]
prPE336	CAACAAGAGTGCGTGAAGAC	This study
prRS042	TCGCTCAAAAAATTCATTGTT	This study
prRS087	CAAATTTATTGGACTCTTCGTTC	This study
prJK493	GAGCCGGGATCCAACCTTAACACTTGCGATCC	This study
prJK494	TTTCATCATATCAACCTTTTTCTTG	This study
prJK495	GAATAGAGAAAAAAATGTCAATTACATCATT**TTTCATCATATCAACCTTTTTCTTG**	This study
prJK496	GAGCCGGTCGACATTTTCCTCATTTGCTTCTCC	This study

Discussion

Among Gram-negative and Gram-positive bacteria, type 4 secretion systems are extensively spread. The number of newly-discovered bacteria using these systems during infection process continues to expand and includes some clinically-important human pathogens such as *Helicobacter pylori*, *Legionella pneumophila*, *Bordetella pertussis*, *Brucella* and *Bartonella* species [3-5]. In *Bartonella* species, the VirB/D4 T4SS contributes to pathogenicity by translocating Beps. Previous study where rats were intravenously infected with either *Btr* Δ*virD4* or *Btr* Δ*virB4* shows that these strains are abacteremic indicating the dependency of *Bartonella* on the VirB/D4 T4SS and on the effector translocation process to establish infection [25].

So far, *in vitro* studies on ECs have linked several known *Bartonella* phenotypes with Bep translocation [34-36]. BepD modulates host immune response by increasing anti-inflammatory cytokine (IL-10) and by reducing the production of pro-inflammatory cytokines (TNFα) (Unpublished data). BepC, BepF, and BepG trigger cytoskeletal rearrangement resulting in cell invasion in a unique cellular structure called invasome [19, 37]. BepA protects infected cells from apoptosis by increasing cAMP concentration and BepE is required for *Bartonella* dissemination through the dermis [7, 35]. Based on these observations we first thought that Bep were crucial for *Bartonella* to establish bacteremia but the bacteremic phenotype of *Btr* Δ*bepA-I* drove us to reconsider the essentiality of Bep during the infection process.

Beps are not crucial for *Bartonella* dissemination from the inoculation site to the lymphatic circulation and subsequently to the bloodstream. Nevertheless, the delayed bacteremia onset observed in both conditions (*i.d.* and *i.v.*) could be associated with the loss of effectors. Indeed, evasion of the innate immunity is a prerequisite for *Bartonella* to travel from the inoculation site to the bloodstream [38]. By lacking Beps, *Btr* Δ*bepA-I* might be less effective compared to the WT to escape from the immune system and to travel through the dermis making the colonization of the host more challenging. The lost capacity to modulate the immune response might also facilitate the clearance of the pathogen by immune cells, explaining the shorter bacteremia.

After the first bacterial release in the bloodstream, bacteremia reaches the same titer for both strains. Presumably, the first wave of infection corresponds to the release from the lymphatic nodes [39], suggesting that Beps are not crucial for bacterial replication and release in the bloodstream. Furthermore, in our model, bacteria intravenously injected are rapidly cleared from the bloodstream, which remains sterile for at least three days post inoculation. From this observation we believe that bacteria might invade the lymphatic circulation, which represents a crucial step during *Bartonella* infection, before RBC invasion. Indeed, the Trw T4SS, which is essential for erythrocyte colonization, is

under the control of the two-component system BatR/BatS, which displays an optimum activity at the physiological pH 7.4 [40-42]. Therefore, by infecting the lymphatic circulation, we assume that *Bartonella* replicates and is reprogrammed for the invasion of RBCs. Up to date, the exact process leading to the release is still unknown but might be tightly regulated [39, 43]. Indeed, independently of the initial concentration used for infection (inoculum 10^7, 10^5 or 10^2 CFUs), the bacterial load reaches a concentration around 10^6 CFUs/ml when released in the bloodstream.

As described in the introduction, after the first release *Bartonella* invades erythrocytes and endothelial cells, designated as the "blood-seeding" niche. The "blood-seeding" niche is believed to seed additional waves of bacteria in the bloodstream by regular interval (5 days) until the immune response blocks the infection [8, 43]. Another hypothesis to explain the shorter bacteremia is the inability of *Btr* Δ*bepA-I* to invade the replicative "blood-seeding" niche. Thus, following the release from the lymphatic circulation *Bartonella* colonizes only erythrocytes - which is a VirB/D4 TSS independent process [40]- and is progressively cleared with the natural erythrocytes recycling process by the spleen. However, our read-out does not allow us to state the loss of these cyclic-infection waves for *Btr* Δ*bepA-I*. Thus, it would be interesting to test this hypothesis by a biotinylation assay on blood infected with *Btr* Δ*bepA-I gfp*, as described by R. Schulein [8].

Taken together our results demonstrate that Beps are not essential for *Bartonella* to establish bacteremia in the infected host but facilitate the colonization and contribute to maintain a sustainable intraerythrocytic infection. To explain the abacteremic phenotype of *Btr* Δ*virB4* and *Btr* Δ*virD4*, two hypotheses have been submitted (i) Is there a toxic accumulation of Beps within bacteria due to the inability to translocate effectors? (ii) Does the VirB/D4 T4SS translocate another protein(s) different from Bep?

We showed that bacteria expressing a VirB/D4 T4SS without the coupling proteins and the effectors (*Btr* Δ*virD4* Δ*bepA-I*) were abacteremic thereby refuting a potential toxicity associated with an accumulation of Beps. Furthermore, the fact that *Btr* Δ*bepA-I* is bacteremic and *Btr* Δ*bepA-I* Δ*virD4* is abacteremic confirmed the significance of the coupling protein VirD4 for *Bartonella* to establish a successful infection.

Since the coupling protein VirD4 and the VirB/D4 T4SS were shown to be crucial for *Bartonella* to establish bacteremia we assumed that the VirB/D4 T4SS might translocate another protein during infection. Interestingly, a study from 2004 revealed in *B. quintana* the presence of a gene with high similarity to *yopP* (*Yersinia* outer protein P) expressed in *Yersinia enterocolitica* (termed *yopJ* in *Y. pestis* and *Y. pseudotuberculosis*) [29, 44]. *Yersinia* YopJ/P proteins were the first described representatives of an effector family widely distributed among different bacterial genus such as animal pathogen

(*Salmonella* spp.*, Vibrio* spp.*, Aeromonas* spp.*)*, plants pathogens (*Pseudonomas syringae, Xanthomonas* spp.*, Ralstonia* spp.) or plant symbionts (*Rhizobium* spp.) [28, 32, 45]. YopJ and YopJ-related proteins are acetyltransferases and most of these effectors share a catalytic triad consisting of Histidine/Glycine/Cysteine (Fig. 14) [28]. Even though some exceptions have been reported, such as PopP2 from *Ralstonia solanacearum* that exhibits an Asparagine rather a Glycine, all YopJ-related effectors harbor a conserved cysteine [28, 32, 46]. To this conserved cysteine is attributed the acetyltranferase activity of the protein which modifies preferentially serine and threonine of the target by using acetyl-coenzyme A (CoA) as substrate [46]. Although, it has been reported that the YopJ-like effectors PopP2 (*Ralstonia* spp.) and VopA (*Vibrio* spp.) are also able to modify a lysine residue. In our phylogenetic tree of YopJ, we are the first one to show that YopJ is widely distributed among lineage 3 and lineage 4 *Bartonella* species. Furthermore, the protein alignment confirms that all YopJ proteins present in *Bartonella* spp. harbor this characteristic catalytic triad (H/G/C) with the conserved cysteine. Considering that almost all *Bartonella* spp. from lineage 3 and 4 exhibit up to three copies of *yopJ* in their genome and the catalytic triad is well-conserved, we assum that the protein is still functional and might play a role during infection. This assumption is supported by the fitness decrease observed for *Btr* Δ*bepA-I* Δ*yopJ* and *Btr* Δ*yopJ*.

Our *yopJ* synteny analysis in *Bartonella* species suggest that *yopJ* was independently acquired in lineage 3 and lineage 4 species. Indeed, in all lineage 4 species, we observe a conserved synteny where *yopJ* is always nearby a gene coding for a lysozyme. In all lineage 3 species we found three *yopJs* in two different loci where *yopJ1* and *yopJ2* are localized in the same locus whereas *yopJ3* is isolated in a second one. However, similarly to lineage 4 species, we observe in lineage 3 species a common *yopJ* synteny shared by all lineage 3 species for the two loci.

In lineage 4 species, the number of *yopJ* genes varies between one to three copies. Therefore, we suppose that a single copy of *yopJ* was acquired by the common ancestor of lineage 4 species and the duplication and differentiation events occurred after speciation. This assumption is supported by the independent duplication event of *yopJ1* in *B. washoeensis* and in the common ancestor of three species *B. tribocorum*, *B. grahamii* and *B. rattimassiliensis* [31]. In *B. tribococurm*, we believe also that a specific duplication event of *yopJ2* occurred recently leading to the appearance of *yopJ3*. Unlike lineage 4, all lineage 3 species bear three *yopJ* orthologs with a similar synteny suggesting that the duplication and differentiation events occurred before speciation.

Pathogenic *Yersinia* spp. (*Y. pestis, Y. enterocolitica* and *Y. pseudotuberculosis*) differ from non-pathogenic ones by the presence of a 70-kb virulence plasmid names pCD in *Y. pestis* or pYV in *Y. enterolitica* and *Y. pseudotuberculosis* [47, 48]. It encodes the Yop virulon consisting of structural components of the Ycs apparatus, a T3SS, regulatory proteins and effectors including YopJ (to notice,

YopJ is named YopP in *Y. enteroliticolitica*) [49]. Up to date, *yopJ* is the only gene identified in *Bartonella* species from the well-characterized *yop* genes [50]. So far, it is difficult to determine how and when *Bartonella* acquired *yopJ* but we assume it was from a species either encoding the Yop virulon or harbouring a *yopJ*-like gene. Bacteria are known to exchange DNA via horizontal gene transfer (HGT) mechanisms allowing transmission of virulence factor or antibiotic resistances [51]. Since it has been demonstrated that *Yersinia* and many other pathogens are transmitted by fleas like several *Bartonella* spp. [52-54], we cannot exclude that *Bartonella* acquired a copy of *yopJ* in the midgut of an arthropod via a HGT mechanism such as conjugation, transduction, transformation or the gene transfer agent (GTA) [55-57]. Finally, to clarify how *Bartonella* acquired YopJ we need to deepen our phylogenetic analysis (i) by appending more YopJ amino acid sequences from other *Bartonella* species that will be newly sequenced and, (ii) by comparing to *Bartonella* the *yopJ* synteny from other α-proteobacteria with *Bartonella*.

In *Yersinia*, YopJ/P dampens the host immune system by targeting and subsequently inhibiting the activity of the MAPK family and the nuclear factor κB (NF-κB) [28, 58, 59]. Furthermore, it has been demonstrated that the YopJ-related protein AvrA (*Salmonella Typhimurium*) also acts on the MAPK-JNK signaling pathway by interacting with MKK7 and MKK4 and modulates NF-κB production by interacting with TNFα [60-62]. Or still, AopP (YopJ-like protein in *Aeromonas*) also regulates the immune response by disrupting the NF-κB signaling cascade [63].Taking into account the role of these YopJ-like proteins in different species, we presume that YopJ in *Btr* might also interfere with the immune system of the host, justifying the infectious phenotype of *Btr* Δ*bepA-I*. Furthermore, to prevent apoptosis of infected-macrophage by modulating the JNK pathway, AvrA allows rapid dissemination of the bacteria to the spleen [64]. In *Y. pestis*, YopJ promotes dissemination from the inoculation site to the lymph nodes, which takes less than one hour [65, 66]. We can suppose that, in a similar way to $YopJ_{Ypes}$ and AvrA, $YopJ_{Bartonella}$ prevents apoptosis of immune cells during *Bartonella* infection and subsequently allows the dissemination of the bacteria from the inoculation site to the lymphatic nodes.

In *Yersinia*, Yop proteins are Type 3 effectors (T3Es) and translocation signal is related to their 15 residues of the NH2 terminus [67, 68]. Considering YopJ as a T3E and *Bartonella* spp. expressing a T4SS rather than a T3SS [12, 28], different observations lead us to suggest that YopJ evolved as a VirB/D4 T4SS effector. First, *yopJ* is present only in *Bartonella* spp. expressing a virB/D4 T4SS. Secondly, when we disrupt the three copies of *yopJ* in *Btr* Δ*bepA-I* background, *Btr* Δ*bepA-I* Δ*yopJ* loses its infectivity. Finally, when we either disrupt the assembly of the VirB/D4 T4SS (*Btr* Δ*virB4*) or we prevent the expression of the coupling protein VirD4 (*Btr* Δ*virD4*), mutants turn into non-bacteremic confirming the essentiality of VirD4 and the VirB T4SS for YopJ recognition and/or translocation.

Results of preliminary cytokines-measurement experiments performed in Prof. Christoph Dehio's group (unpublished data) support the assumption that YopJ is involved during *Bartonella* infection by modulating the immune system as observed in other species expressing YopJ-like proteins (AvrA *Salmonella*, YopJ *Yersinia*, VopA *Vibrio*…). Indeed, known to be a YopJ lacking species, *B. henselae* modulates the immune response through the action of BepD that on one hand increases anti-inflammatory cytokines production (IL-10) and on the other hand decreases pro-inflammatory cytokines (e.g. TNFα) (data not shown, personal communication) [29]. Interestingly, BepD in *B. taylorii*, which also expresses YopJ, modulates IL-10 production but did not have any effect on TNFα production. Known to decrease the production of NF-kB in *Yersinia* spp., we could assume that YopJ in *B. taylorii* modulates NF-κB by interacting with TNFα. Furthermore, since YopJ is not express in *B. henselae* and that BepD operates at two-level (IL-10 and TNFα production), we suggest that during evolution BepD substitutes the role of YopJ in *B. henselae*.

Finally, it has been shown in *Y. pestis* that YopJ contributes to pathogenicity although without being crucial for its virulence [69]. Similarly to *Y. pestis*, when we infect rat with *Btr* Δ*yopJ* we still observe bacteremia but with a shorter duration indicating that YopJ plays a role but is not essential for bacteremia establishment. Furthermore, the fact that *Btr* Δ*yopJ* and *Btr* Δ*bepA-I* trigger impaired bacteremia in the infected rats whereas *Btr* Δ*yopJ* Δ*bepA-I* lose totally its ability to establish bacteremia point out that YopJ effectors and Beps might be translocated simultaneously and act in synergy to establish an efficient long-lasting infection.

In summary, this study allows to advance our understanding of the infection process of *Bartonella* species. By using *i.d.* and *i.v.* infection models we show that *Bartonella* effectors (Beps) are not essential for bacteremia establishment but play a role for maintaining a sustainable infection in the host. Our *in silico* analysis revealed for the first time that two sister clades (lineage 3 and lineage 4) of the *Bartonella* genus might have acquire independently a single *yopJ* gene encoding for a YopJ-like protein. Depending on the species and lineage we assume that acquisition of *yopJ* was followed by rounds of duplication and differentiation events. Previously described as T3SE, our preliminary results strongly indicate that YopJ evolved as a T4SE in *Bartonella* species and might cooperate with Beps to enable *Bartonella* to successfully infect its host. These preliminary results give a new perspective concerning *Bartonella* infection process but need further studies. First of all, infection with *Btr* Δ*yopJ* complemented strain would be crucial to confirm the involvement of YopJ during infection. Furthermore, it would be relevant to characterize the target of the effector and confirm that the conserved-triad is also carrying the enzymatic activity of the protein in *Bartonella* species. Last but not least, it would be important to confirm that YopJ is translocated via the VirB/D4 T4SS in *Bartonella* species which will provide a new perspective concerning evolution of effectors in bacteria.

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Additional Results

Up to date, Bep targets *in vivo* are not well characterized yet and need further investigation. Since Fic domain-containing Beps are known to catalyze posttranslational modifications such as AMPylation, phosphorylation or phosphocholination we focused our *in vivo* researches on the Fic-containing effecors in *Btr*: BepA, BepC and BepI [1-3]. Therefore, different mutants were generated: *Btr* Δ*bepA*, *Btr* Δ*bepC, Btr* Δ*bepI, Btr* Δ*bepAC, Btr* Δ*bepCI, Btr* Δ*bepACI* and *Btr* Δ*bepDHEF.*

BepA and BepC seem to have a redundant function

Rats infected with either *Btr* Δ*bepA* or *Btr* Δ*bepC* developed bacteremia that was not significantly different from rats infected with *Btr* WT (*p*-value = 0.1845 and 0.8165, respectively) suggesting that there was no impact on the bacterial fitness associated with the loss of either *bepA* or *bepC.* Interestingly, when we intradermally infected rats with *Btr* Δ*bepAC*, we observed strong bacteremia impairments with a delayed onset (16 d.p.i.), a shorter duration (36 days) and a lower maximum titer ($9.9x10^4$ CFUs/ml of blood on average). Statistical analysis indicated that bacteremia curve triggered by *Btr* Δ*bepAC* was significantly different from *Btr* WT. (p-value = 0.0094). Based on these observations, we hypothesized a redundant role between BepA and BepC explaining the similar kinetics of *Btr* Δ*bepA* and *Btr* Δ*bepC* with *Btr* WT and the strong bacteremia impairments observed for *Btr* Δ*bepAC*. Furthermore, BepA and BepC might be involved in bacteremia establishment and duration explaining the delayed onset and the shorter duration.

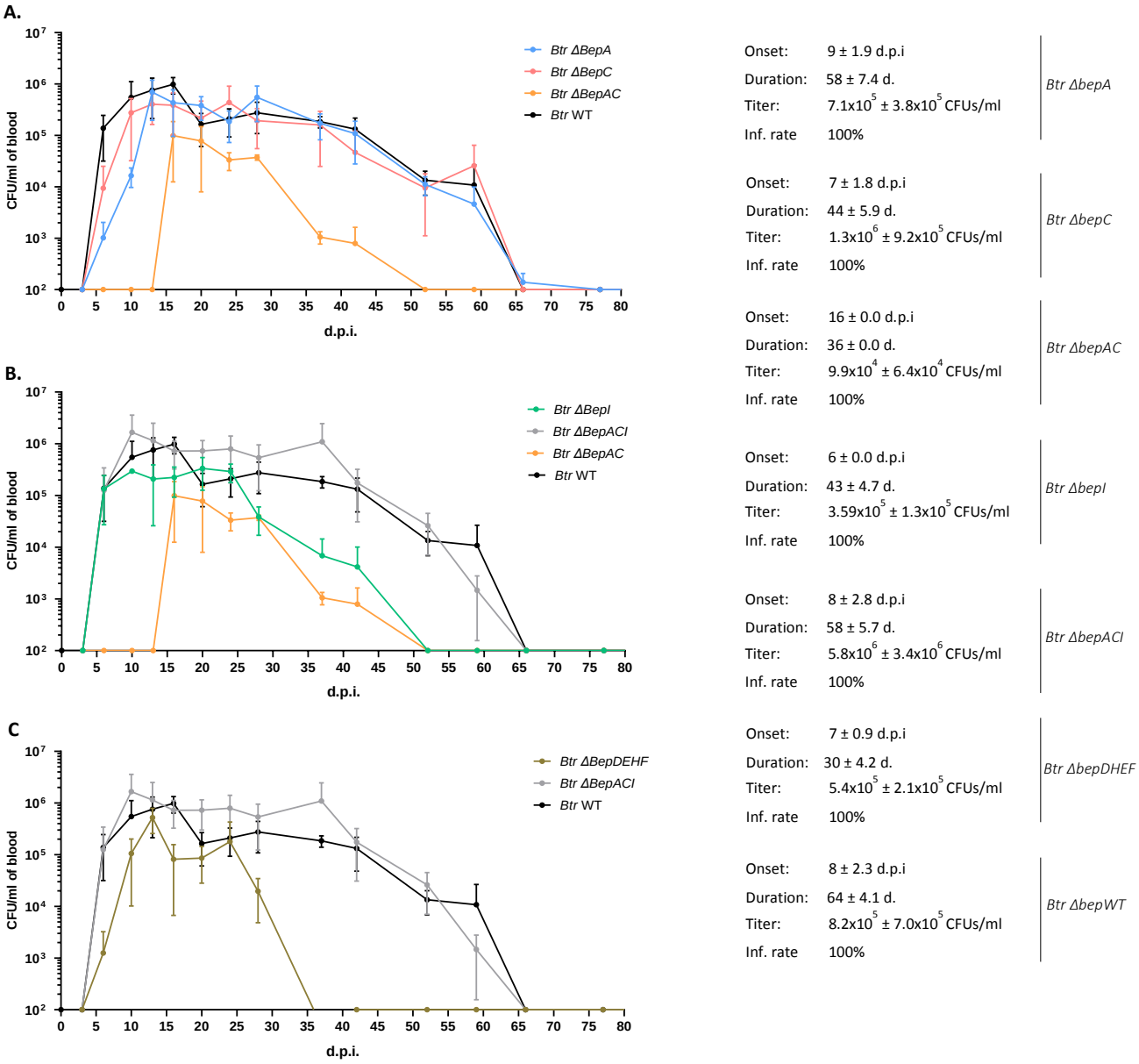

Figure S1. Beps: Redundant activities and collaboration for the establishment of a long-lasting bacteremia

Bacteria were grown for 3 days at 35°C and 5% CO_2 on Heart Infusion Agar (HIA) plates supplemented with defibrinated sheep blood. Plotted graphs represent an average CFU/ml of blood in animals groups (n = 3) from which bacteria were recovered at a given time point post-infection. (A) Groups of 10 weeks old Wistar rats were intradermally infected (inoculum 10^7 CFUs, n = 3) with *Btr* WT*, Btr ΔbepA,* with *Btr ΔbepC or Btr ΔbepAC.* (B) Groups of 10 weeks old Wistar rats were intradermally infected (inoculum 10^7 CFUs, n = 3) with *Btr* WT*, Btr ΔbepA,* with *Btr ΔbepC or Btr ΔbepAC.* Groups of 10 weeks old Wistar rats were intradermally infected (inoculum 10^7 CFUs, n = 3) with *Btr* WT*, Btr ΔbepI,* with *Btr ΔbepAC or Btr ΔbepACI.* (C) Groups of 10 weeks old Wistar rats were intradermally infected (inoculum 10^7 CFUs, n = 3) with *Btr* WT*, Btr ΔbepACI or Btr ΔbepDHEF.* Statistical analysis was performed using two-way ANOVA and results are annotated as p-values.

Beps ACI collaborate to enable a long-lasting bacteremia

To assess the role of BepI during *Btr* infection, we infected intradermally with *Btr* Δ*bepI*. Compared to the bacteremia curve triggered by *Btr* WT, there was a significant decrease of the bacterial fitness associated with the deletion of *bepI* (*p*-value = 0.0127). Since bacteremia onset is similar with WT, BepI is not involved in bacteremia establishment but rather had a role in maintaining a long-lasting bacteremia in the host (43 ± 4.7 d.). Surprisingly, rats infected intradermally with *Btr* Δ*bepACI* and *Btr* Δ*bepCI* developed a bacteremia that was not significantly different from bacteremia induced by *Btr* WT (*p*-value = 0.2522 and 0.4606, respectively). Taken together, we assumed that BepA/BepC and BepI have compensatory effects and might act in synergy to induce a successful and long-lasting bacteremia. This might explain why when we disturbed this balanced collaboration by removing either *bepC/bepA* or *bepI* we affected the fitness of the bacteria and, when we deleted simultaneously these three genes we observed a bacteremia kinetic similar to WT. To confirm this assumption, it would be relevant to infect rat with *Btr* Δ*bepAI*.

Beps DHEF are essential to establish a long-lasting bacteremia

Finally, we generated a strain expressing only beps harboring a Fic domain: *Btr* Δ*bepDEHF.* Rats intradermally infected with *Btr* Δ*bepDEHF* developed bacteremia with significant impairments in the duration compared to *Btr* WT (*p*-value = 0.0005). However, no impact of the deletion on the bacteremia onset was observed. These observations indicate that Beps DEHF have a key role in maintaining a sustainable bacteremia.

Discussion

Our results highlight a putative functional redundancy between BepA and BepC. This assumption is in accordance with a general biological principle, where functional redundancy contributes to an increase of the robustness of relevant mechanisms such as infection process [4]. Furthermore, redundant activities has been previously demonstrated *in vitro* for *B. henselae* where either BepC/F or BepG trigger engulfment and internalization of bacteria cluster within a host cellular structure surrounded by actin named invasome [5-7]. Up to date, we know *in vitro* that BepA prevents DC apoptosis and BepC is involved in cell migration which do not support our assumption of redundancy [8-10]. However, we cannot exclude that there are still other uncharacterized targets for these effectors that could explain this hypothetic redundancy between BepA and BepC. Nevertheless, in accordance to the activities described so far for BepA and BepC, we can assume that without these effectors it is more challenging for the bacteria to travel from the inoculation site to the bloodstream explaining the delayed onset. Our results also indicate that BepI and BepsDHEF might be involved in maintaining a sustainable bacteremia. However, the target of these effectors remains so far undetermined and prevent any conclusion about their contribution during the infection process. Finally, our results show that BepA/C and BepI might have compensatory effects and need to act in synergy to trigger a successful infection.

Based on these preliminary results, we can assume that several Beps might display redundant activities others might act in synergy to increase the chance of *Bartonella* to trigger a sustainable infection. However, the lack of knowledge concerning the targets of Beps prevents any conclusion about their contribution during the *in vivo* infection process and needs further studies to enhance our understanding. Although we have to be cautious with these preliminary results, they provide a good basis for further *in vivo* and *in vitro* investigations.

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General Conclusion and Outlook

The first part of my thesis aimed to establish a lineage 3 *in vivo* infection model by using *B1-1C* to afterward investigate the role of the flagellum and the VirB/D4 T4SS and its lineage 3-specific Beps.

To study at which stage of *Bartonella 1-1C* infection the virulence factors play a role I have established the rat intradermal (*i.d.*) and intravenous (*i.v.*) infection model. The *i.d.* injections reflect the natural route of transmission by arthropods, while *i.v.* injections bypass the dermal stage. Whereas *i.v.* injections are reliable independently of the growth medium, *i.d.* injections require specific growth conditions to trigger reliable infections. Growing bacteria either on TSA for 3 days or on HIA for 10 days at 35 °C with 5% CO_2 in a moist atmosphere is necessary. It is believed that prior invading the host *Bartonella* spp. have to express the VirB/D4 T4SS. Since the expression of this virulence factor is under the control of the stringent response RpoH1 and the BatR/BatS two-component system, TSA medium, as a long-time growth on HIA medium, provide an environment triggering expression of the VirB/D4 T4SS. To confirm this hypothesis, it would be interesting to chromosomally express within *Bartonella* a *gfp* cassette under the control of the natural promoter of the *virB* operon. Therefore, by FACS analysis we could indirectly assess expression over time of the VirB/D4 T4SS on HIA and TSA media. Furthermore, it might be interesting to also test CBA medium.

Since I established appropriate conditions for reliable *i.d.* and *i.v.* infections rats were infected via the two routes. The *i.v.* infected animals develop bacteremia around 6 days post-inoculation while it takes around 18 days for *i.d.* infected animals. The delay observed for *i.d.* injections might be associated with the time needed for the bacteria to travel from the inoculation site to the bloodstream and to subvert immune system of the host. Furthermore, *i.v.* infected animals develop bacteremia 6 days post-inoculation revealing the necessity of *Bartonella* to invade a replicative blood seeding niche.

Compared to the lineage 4 strain *B. tribocorum*, rats intradermally infected with *B1-1C* develop a bacteremia 17 days post-inoculation while it takes 8 days for rats intradermally infected with *Btr*. Because of the lack of knowledge during the early stage of infection and the lack of *in vitro* experiment, it is difficult to explain this delay observed for *B1-1C*. However, if rat is considered as an accidental host we can assume that *B1-1C* is not adapted to rat and might undergo disturbances to reach the bloodstream and/or subvert host immune system.

Furthermore, bacteremia triggered by *B1-1C* persists in the host for around 11 days while bacteremia induced by *Btr* lasts around 64 days. Since *Bartonella* infection is characterized by long-lasting erythrocytic parasitism, this short duration is surprising. This finding suggests that *B1-1C* might use a

different strategy from the stealth strategy used by lineage 4 *Bartonella* species or might not be specific to rat.

Since *B1-1C* is triggering a short bacteremia duration, we analyzed the behavior of the bacteria within the blood by FACS analysis and Gentamicin protection assay. With both analysis I demonstrated that *B1-1C* is unable to colonize rat erythrocytes. Up to date, all *Bartonella* species are able to invade erythrocytes and such inability is associated with the accidental infection of a non-specific host. Furthermore, the disability of *B1-1C* to colonize erythrocytes, which protect bacteria from immune cells, explains the short bacteremia duration. Indeed, by circulating in the blood as a single cell, bacteria is no more protected and is easily cleared from the bloodstream by the immune system. Nevertheless, *B. bacilliformis* is known to have a hemolytic activity [1]. Therefore, it would relevant to establish an *in vitro* model to exclude on one hand that *B1-1C* displays a hemolytic activity and on the other hand to confirm that the bacteria are not able to interact and/or invade rat erythrocyte.

Infections with different *B1-1C* mutants, complemented strains and revertant strains highlight a fitness attenuation associated with genetic engineering preventing the study of the virulence factors such as the flagella, the virB/D4 T4SS and lineage 3-specific Beps. *Bartonella* species are considered as fastidious bacteria and any genetic manipulations require many *in vitro* passages supplemented with long incubation in the appropriate conditions. Therefore, we assume that the loss of infectivity observed for *B1-1C* is associated with genetic engineering and/or successive plating. Establishment of an *in vitro* model would be useful to determine if our genetic engineering has an impact on the bacterial growth and subsequently on the infection process clarifying the abacteremic phenotypes of the mutants.

Bartonella 1-1C strain used in our experiments was isolated by Lin et *al.* in 2008 from 1 rat (*R. norvegicus*) out of 58 tested. The small sample size of animals tested and the very low prevalence of *B1-1C* question the relation between *B1-1C* and rat. Considering rat as an accidental host might explain why:

- *B1-1C* is extremely demanding on the growth media for reliable *i.d.* infection, while such requirement has, up to date, never been mentioned for other *Bartonella* species.
- Genetic engineering has a strong impact on its infectivity.
- *B1-1C* needs around 17 days to travel from the inoculation site to the bloodstream, while other *Bartonella* species take around 1 week to induce bacteremia [2-4]
- *Bartonella 1-1C* is not able to invade erythrocytes, which is one hallmark of *Bartonella* infection even for flagellated species [5].

Although our results suggest that rat is an accidental host for *B1-1C*, it needs further investigations to confirm this hypothesis. Therefore, it seems crucial to characterize the bacteremia kinetic of other lineage 3 species such as the cat-specific *B. clarridgeiae* or the red squirrel-specific *B. AR15-3* strains Then, if these experiments confirm the extraerythrocytic life style of *B1-1C*, our *in vivo* model will be a good experimental basis for further investigations and enhance our knowledge about the infection process of *Bartonella* species.

The second part of my thesis intended to investigate the contribution of the VirB/D4 T4SS and its effector proteins during *Bartonella tribocorum* infection *in vivo*.

The *i.d.* and *i.v.* infected rats with *Btr* Δ*bepA-I* develop an impaired bacteremia characterized by a delayed onset and a shorter duration indicating a fitness loss for the Bep-free mutant. This phenotype indicates that Bep are not crucial for bacteremia establishment but maintain a sustainable bacteremia in the host. The exact *in vivo* contribution of Beps during infection remains unclear and so far, our knowledges are based on *in vitro* experiments. However, Beps might display redundant roles, compensatory effects and synergic activities to trigger a long-lasting bacteremia. Therefore, the next step would to extend *in vivo* infections with other Beps mutants. Thus, by combining *in vivo* and *in vitro* analysis it will provide a more accurate knowledge about Beps functions during the *Bartonella* infection process.

Furthermore, in this study we demonstrate the essentiality of the VirD4/T4SS and its coupling protein VirD4. Since we show that Beps are not essential for establishing bacteremia, we hypothesized the translocation of another effector. Our *in silico* analysis reveal for the first time that two sister clades of the *Bartonella* genus (lineage 3 and lineage 4) might have acquired independently a single *yopJ* gene encoding and, depending on the strain, was followed by duplication and differentiation events. Since our *in silico* analysis is based only on *Bartonella* species that have been so far sequenced, it seems relevant to append to this analysis the future sequenced species in order to have a more accurate overview of the distribution of YopJ proteins among the *Bartonella* genus. Furthermore, it would be relevant to compare *Bartonella yopJ* synteny with other α-proteobacteria to clarify how *yopJ* was acquired by *Bartonella* species.

In this study we suggest the involvement of YopJ during *Bartonella* infection. However, to verify this hypothesis it seems crucial to complement *Btr* Δ*yopJ* and *Btr* Δ*yopJ* Δ*bepA-I* strains by expressing *yopJ* *in trans* as previously described [2, 6]. Therefore, if we restore the WT phenotype for *Btr* Δ*yopJ* p*yopJ* and the *Btr* Δ*bepA-I* phenotype for *Btr* Δ*bepA-I* Δ*yopJ* p*yopJ* it will exclude any pleiotropic effect of the deletion and confirm the contribution of YopJ during *Bartonella* infection.

The abacteremic phenotypes observed for *Btr* Δ*virD4* and *Btr* Δ*virB4* and the finding that YopJ is expressed only in species harboring the VirB/D4 T4SS suggest that YopJ might be translocated by The VirB/D4 T4SS. Since YopJ was previously characterized as a T3S effector in *Y. perstis*, it would be interesting to verify if YopJ evolved as a T4SE in *Bartonella* species by expressing *in-trans* in *Btr* Δ*bepA-I* Δ*yopJ* a chimeric YopJ protein with a tag localized either in the N-terminus or in the C-terminus of the protein. Therefore, *in vitro* infection of nucleated cell combined with an immunofluorescence assay will allow us to determine if the YopJ is translocated via the VirB/D4 T4SS. Furthermore, if the tag is added to the protein extremity harboring the secretion signal it might prevent the translocation and therefore could inform about the secretion signal localization. To notice, in *Y. pestis* the secretion signal is localized in the last 15 residues of the NH2 terminus of Yop effectors [7].

YopJ and YopJ-related proteins are acetyltransferase known to prevent phosphorylation of NFκB [8]. Therefore, it would be interesting to determine *in vitro* if YopJ from *Bartonella* species also prevent NFκB phosphorylation. Furthermore, the catalytic activity of YopJ and YopJ-like proteins is systematically allocated to the cysteine of the catalytic triad and it has been shown that the mutant YopJ (C172A) is catalytically inactive [8-12]. To confirm that the enzymatic activity is also carried by the cysteine in *Bartonella*-YopJ effector, it could be relevant to transfect eukaryotic cells with a plasmid encoding for YopJ protein with a point mutation on the cysteine and assess its enzymatic activity.

To conclude, the discovery of YopJ among *Bartonella* species gives a new perspective on the infection process and further investigations will clarify its contribution. Furthermore, our preliminary results concerning the Beps combine with *in vitro* knowledge provide a good basis for the understanding of their role but need more investigations to *in fine* provide a more accurate overview of the general process of infection of the *Bartonella* genus.

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Abbreviations

Acetyl-Coa	acetyl coenzyme A
B1-1C	*Bartonella 1-1C*
BadA	*Bartonella* adhesin A
BAR15	*Bartonella AR15-3*
Bcl	*Bartonella clarridgeiae*
Beps	*Bartonella* effector proteins
BID	Bep delivery domain
Btr	*Bartonella tribocorum*
Bro	*Bartonella rochalimae*
C/Cys	cysteine
CBA	Columbia blood agar
CC	core complex
Cdc42	cell division control 42
CFU	Colony forming units
CIM	cytoplasmic inner membrane
CSD	cat scratch disease
d.	day(s)
DC	dendritic cell
d.p.i.	day(s) post infection
Dtr	transfer and replication machinery
GTA	gene transfer agent
EC	endothelial cell
FACS	Fluorescence-activated cell sorting
F-actin	filamentous actin
FCS	foetal calf serum
Fic	filamentous induced cAMP
Fig.	figure
FSC	forward scatter
G/Gly	Glycine
GEF	guanine nucleotide exchange factor
GFP	green fluorescence protein
H/His	Histidine
HbpA	hemin binding protein

HGT	horizontal gene transfer
HIA	heart infusion agar
ialAB	invasion-associated locus A and B
i.d.	intradermal infection
IL-10	interleukin 10
IM	inner membrane
Inf. rate	infection rate
i.v.	intravenous infection
ml	milliliter
MAPK	mitogen-activated protein kinase
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
OM	outer membrane
p.	page
PANS	pediatric acute onset neuropsychiatric syndrome
PAMP	pathogen-associated molecular pattern
pdxJ	pyridoxine 5'-phophate synthase
PTMs	post-transcriptional modifications
RBC	red blood cell
SD	standard deviation
Spp.	species
STAT3	signal transducer and activator of transcription 3
SSC	side scatter
T3SE	type 3 secretion effector
T3SS	type 3 secretion system
T4CP	type 4 coupling protein
T4SE	type 4 secretion effector
T4SS	type 4 secretion system
TNFα	Tumor necrosis factor alpha
TSA	tryptic soy agar
Vomps	variably expressed outer membrane proteins
Yop	*Yersinia* outer protein
WT	wild type

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