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Intracellular rescuing of a *B. melitensis* 16M *virB* mutant by co-infection with a wild type strain

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ABSTRACT

Brucella is a broad-range, facultative intracellular pathogen that can survive and replicate in an endoplasmic reticulum (ER)-derived replication niche by preventing fusion of its membrane-bound compartment with late endosomes and lysosomes. This vacuolar hijacking was demonstrated to be dependent on the type IV secretion system VirB but no secreted effectors have been identified yet. A *virB* mutant is unable to reach its ER-derived replicative niche and does not multiply intracellularly. In this paper, we showed that, by co-infecting bovine macrophages or HeLa cells with the wild type (WT) strain of *Brucella* melitensis 16M and a deletion mutant of the complete *virB* operon, the replication of Δ*virB* is rescued in almost 20% of the co-infected cells. Furthermore, we demonstrated that co-infections with the WT strains of *Brucella abortus* or *Brucella suis* were equally able to rescue the replication of the *B. melitensis* Δ*virB* mutant. By contrast, no rescue was observed when the WT strain was given 1 h before or after the infection with the Δ*virB* mutant. Finally, vacuoles containing the rescued Δ*virB* mutant were shown to exclude the LAMP-1 marker in a way similar to the WT containing vacuoles.

1. Introduction

Brucella species are Gram negative, facultatively intracellular bacteria that cause chronic infections in a large range of mammals, including humans, and have a high socio-economic impact [19,47]. *Brucella* can survive and replicate within a membrane-bound compartment in professional as well as non-professional phagocytic host cells by escaping the endo-lysosomal degradation pathway [5,17,29,39,40,42].

After its internalization by an LPS-dependent lipid raft-mediated entry, *Brucella* is found in a vacuole (5–10 min post-infection (p.i.)) that interacts with early endosomes, transiently acquiring Rab5 and EEA-1. The vacuole called BCV (*Brucella*-containing vacuole) then matures into an acidic intermediate compartment that accumulates LAMP-1 but avoids extensive interactions with late endosomes and fusion with lysosomes. The intermediate BCV finally interacts with the endoplasmic reticulum (ER) (8–12 h p.i.), generating an ER-derived organelle where the bacteria can replicate (12 h p.i. and later). BCV containing the WT strain excludes LAMP-1 and acquires various ER markers like calnexin, calreticulin and Sec61β, by limited fusion with the ER membrane [6]. Nowadays, bacterial replication is thought to occur through splitting of the BCV into two daughter BCVs via further accretion of ER membranes [27,32,39,45]; for a review see Refs. [5–8].

Little is known about the molecular mechanisms used by *Brucella* to deviate its intracellular trafficking. Nevertheless, some key factors were identified. First, the LPS O-chain of *Brucella* spp. is important for intracellular survival [21] and is involved in the lipid rafts-mediated entry and delayed fusion with lysosomes [5]. By contrast *Brucella* spp. rough strains (which do not possess O-chains) do not seem to enter by interactions with lipid rafts, and their vacuoles fuse rapidly with lysosomes. Other surface components putatively under the control of the two-component system (TCS) BvrS/BvrR could also be involved in these early steps. BvrS/BvrR mutants displayed increased attachment to the cells but a strongly reduced internalization in both professional and non-professional phagocytic cells [48]. Second, in the context of the BCV, periplasmic cyclic β-1, 2-glucans are necessary to avoid fusion of the BCV with lysosomes probably by sequestering cholesterol and disturbing lipid rafts formation [1,4,33].

The type IV secretion system (T4SS) called VirB is the major molecular determinant in the final step of the "organelle robbery". The *virB* genes are not necessary for controlling the first events of *Brucella*/host cells interaction (i.e. attachment, internalization) or for avoiding fusion with the late endosomal-lysosomal compartments [6,15,37]. It rather seems that the *virB* operon is required for regulating intracellular trafficking from the LAMP-1 and Sec61β

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positive intermediate vacuole to the LAMP-1 negative ER-derived replicative organelle, via the interaction of the BCV with ER exit sites [5]. Δ*virB* mutant can deviate the first part of intracellular trafficking but cannot establish a replication niche by interacting with the ER.

T4SS acts generally by secreting proteins and/or DNA-protein complexes [9,14,16,34,38]; for a review see Ref. [2]. Currently, no effector of the *Brucella* T4SS has been identified. If the T4SS secreted effectors are involved in the maturation of the intermediate BCV to the ER-derived replicative niche, we hypothesized that the effectors secreted by a WT strain could rescue the trafficking deficiency of a Δ*virB* mutant during a co-infection in cells. In this work, we demonstrated that when cells were co-infected with the WT strain and the Δ*virB* mutant, the Δ*virB* mutant replicates in almost 20% of the cells. No rescuing was observed if the WT strain was not given at the same time as the Δ*virB* mutant. Furthermore, the trafficking deficiency of the Δ*virB* mutant can be rescued by co-infection with the WT strains of *Brucella melitensis* 16M, *Brucella abortus* 544 and *Brucella suis* 1330. Moreover, the BCV containing a rescued Δ*virB* mutant shares similar markers compared to the BCV containing the WT strain.

2. Results

2.1. Construction and characterization of a B. melitensis Δ*virB* mutant

From the literature, it is known that the intracellular behavior of transpositional *virB* mutants could be different whether the mutation is polar or not. For example, a vacuole containing a *virB*10 polar mutant cannot avoid fusion with a lysosome while a vacuole with a *virB*10 non-polar mutant avoids degradation and is recycled to the cell surface [12]. To avoid this drawback, we removed the 12 open reading frames (ORFs) encoding the T4SS of *B. melitensis* 16M by allelic replacement. A two-step selection method was used to obtain a mutant without antibiotic resistance cassette [43] (see Section 4).

In vitro, in a rich medium, there is no detectable growth difference between the Δ*virB* mutant and the *B. melitensis* WT strain (data not shown). The Δ*virB* mutant was attenuated after 48 h (Fig. 1) but not after 1 h of infection (data not shown) in cellular models, showing that this mutant has no internalization defect but could not reach its replication niche. This result confirms the data obtained with the classical transpositional mutants *virB*2, *virB*4 and *virB*9 in a HeLa model [12,15]. In a BALB/c murine model, the Δ*virB* mutant was attenuated at 1 week post-infection as well as at later times (data not shown) as described in the literature for the transpositional *virB* mutants [25,44].

The mutant was complemented by using the medium copy plasmid pBBR-p_{virB}*virB* containing the complete *virB* operon controlled by its own promoter [37]. The complementation was detected by replication of this complemented strain in HeLa cells (Fig. 1A). At 48 h post-infection, the complemented Δ*virB* mutant was able to replicate as well as the WT strain bearing the empty pBBR plasmid (Fig. 1). In addition we showed that no VirB10 proteins were detected by Western blot on extracts from the *in vitro* grown Δ*virB* mutant and that complementation restored VirB10 production (data not shown).

The *B. melitensis* Δ*virB* mutant strain was made constitutively red fluorescent and selectable with an antibiotic marker, by chromosomal insertion of the pSK*oriTsojAdsred* plasmid. This suicide vector integrates at the *sojA* promoter (see Section 4) ensuring a strong DsRed production [49]. The integration of the pSK*oriTsojAdsred* in the chromosomal WT background had no effect on the virulence in cellular models of infection (Fig. 1B).

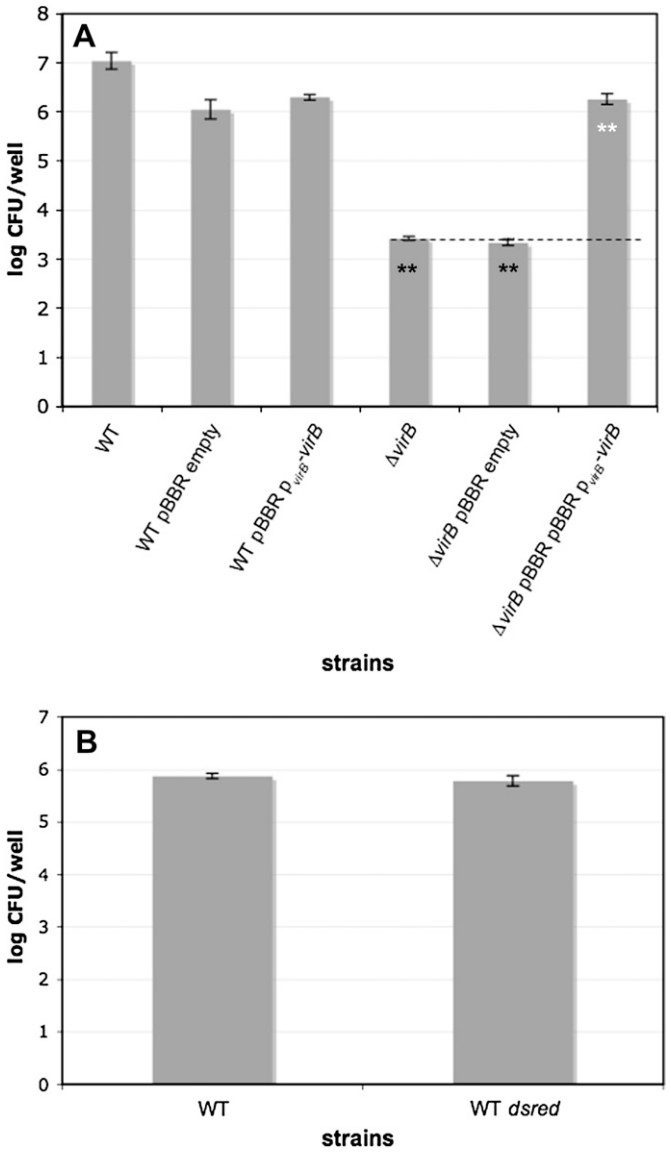

Fig. 1. Intracellular replication of wild type (WT) and Δ*virB* strains at 48 h post-infection in HeLa cells. (A) Complementation of the Δ*virB* mutant by a pBBR-p_{virB}*virB* vector in HeLa cells. The same plasmid was used in the WT *B. melitensis* 16M. Both the WT and the Δ*virB* mutant containing an empty pBBR vector were used as controls. (B) Effect of the pSK*oriTsojAdsred* integrated in the chromosome of the WT strain on the virulence in bovine macrophages. Data shown are the means of three independent assays done in triplicate ± standard deviations. The results were proved to be statistically significant (ANOVA1) after testing the homogeneity of variances (Bartlett test). Post-hoc comparisons have been performed by pairwise Scheffe's test (**$p < 0.01$). The Δ*virB* mutant is compared to the 16M strain and the Δ*virB* pBBR empty mutant is compared to the 16M pBBR empty strain (black asterisks). The Δ*virB* pBBR empty mutant is also compared to the Δ*virB* pBBR-p_{virB}*virB* mutant (white asterisks).

2.2. A Δ*virB* mutant is rescued by the WT strain in a cellular model

In *Brucella*, we know that the classical transpositional *virB* mutants are still able to modulate the first part of their intracellular trafficking but do not replicate either in HeLa cells or in bovine macrophage cell lines [15]. The T4SS of *Brucella* is crucial to gain access to the replicative niche while its precise role is still unknown. Nevertheless we could hypothesize that this system secretes putative effectors that act on the BCV and modulates its trafficking properties as demonstrated for the *Legionella pneumophila* containing vacuole [11,13,16,31,34,36]. If this holds true, the effectors secreted by the T4SS of a WT strain could compensate for

the impairment of a Δ*virB* mutant. So, to address this question, we co-infected HeLa cells and bovine macrophages with an equal mixture of the Δ*virB dsred* mutant and the WT strain. As controls, we also used the couples Δ*vjbR*/WT, *mTn5::virB2*/WT, *mTn5::bvrR*/WT (Fig. 2A). Data in Fig. 2A illustrate that the Δ*virB* mutant as well as the transpositional *virB2* mutant are able to replicate when used in co-infection with the WT strain. VjbR is a transcriptional activator of the *virB* operon, and a Δ*vjbR* mutant, being blocked at the same maturation state as the Δ*virB* mutant, does not replicate in the cells [15]. We also observed a rescuing of the Δ*vjbR* mutant used in co-infection with the WT strain.

On the contrary, a mutant in the gene encoding the two-component regulator *bvrR* [30,48] is unable to replicate even during co-infection with the WT strain. The transpositional *bvrR* mutant has a defect in internalization. This step of trafficking occurs in a T4SS-independent manner [3,12,30] and cannot be complemented by co-infecting cells with the WT strain.

Furthermore, we demonstrate that rescuing of the Δ*virB* mutant by co-infecting cells with the WT strain is also able to occur in HeLa cells (Fig. 2B), murine macrophages J774 and trophoblasts (data not shown). Nevertheless, the Δ*virB* mutant is not rescued when the co-infection is done with a killed WT strain (data not shown).

Rescuing appears to be species-independent because the *B. melitensis* 16M Δ*virB* mutant is rescued by co-infecting cells with *B. abortus* 544 or *B. suis* 1330 WT strain. The same holds true when we rescue a Δ*vjbR* mutant of *B. abortus* 544 with a *B. melitensis* WT strain (Table 1).

From the above results, it appears that the lack of intracellular multiplication of the Δ*virB* or Δ*vjbR* mutant is not an intrinsic failure to replicate but a defect in the maturation of the BCV to become an adequate replication niche. The observed rescuing is dependent on the T4SS and could be mediated by the secretion of "effectors" by the WT strain. The effectors should be similar in the different species as the three tested WT species rescued the replication of the Δ*virB* mutant. This means that the mutant BCV matures into a vacuole permissive for replication that should be similar to WT BCV.

2.3. The rescued ΔvirB containing vacuole is similar to WT BCV

We first confirmed that the WT strain and the Δ*virB* mutant are both able to infect around 20% of the cells by counting bacteria in ≈500 cells (data not shown). Furthermore, by counting bacteria in ≈1000 cells, we observed that when the Δ*virB* mutant was internalized alone, we never found more than 10 bacteria in the cell at 48 h post-infection. Therefore the Δ*virB* mutant was unable to replicate in infected cells (data not shown).

In the literature, it is already described that up to three bacteria could be internalized in the same cell [41]. At 34 h post-infection,

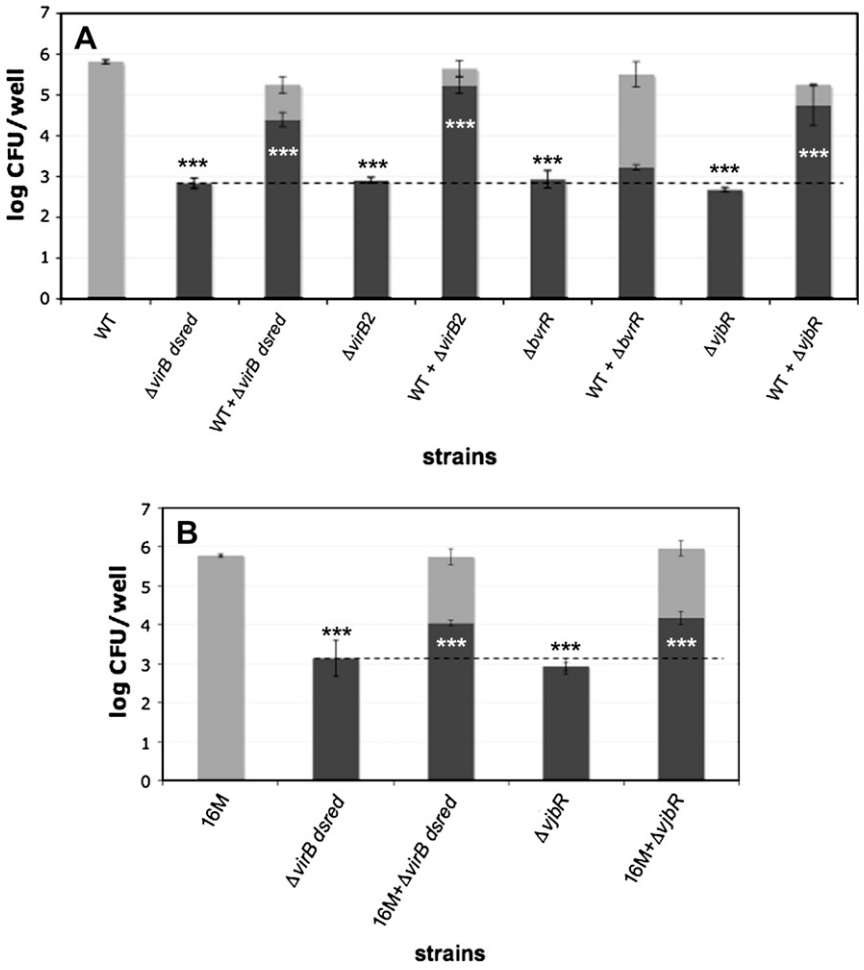

Fig. 2. Analysis of intracellular rescuing of the selected mutants by the wild type strain *B. melitensis* 16M 48 h post-infection, in (A) SV40 bovine macrophages or (B) HeLa cells. The colony-forming units (CFU) were determined on 2YT medium (grey) and on 2YT medium with kanamycin (black). Cells were infected as described in Section 4. Data shown are the means of three independent assays done in triplicate ± standard deviations. The results were proved to be statistically significant (ANOVA1) after testing the homogeneity of variances (Bartlett test). Post-hoc comparisons were performed by pairwise Scheffe's test (***$p < 0.001$ and *$p < 0.05$). The different mutants were first compared to the WT strain (black asterisks). The different mutants were also compared to their respective co-infection data on 2YT medium with kanamycin (white asterisks).

Table 1
Analysis of intracellular rescuing of the selected mutants by the different wild type strains: *B. melitensis* 16M, *B. abortus* 544 and *B. suis* 1330 48 h post-infection in bovine macrophages SV40

Mutant tested	WT strain tested			
	No WT strain	*B. melitensis* 16M	*B. abortus* 544	*B. suis* 1330
CFU WT strain		6.33 ± 0.16	6.07 ± 0.34	5.54 ± 0.07
CFU mutant				
Δ*virB* in *B. melitensis* 16M	2.97 ± 0.06	$5.07 \pm 0.06^{***}$	$5.09 \pm 0.11^{***}$	$4.62 \pm 0.06^{***}$
Δ*vjbR* in *B. melitensis* 16M	3.77 ± 0.01	$5.60 \pm 0.13^{***}$	$5.42 \pm 0.01^{**}$	ND
Δ*vjbR* in *B. abortus* 544	3.19 ± 0.37	$5.63 \pm 0.06^{***}$	$5.14 \pm 0.06^{***}$	ND

ND: not done.
Cells were infected as described in Section 4. Data shown are the means of three independent assays done in triplicate ± standard deviations and are expressed as the logarithm of the CFU. The results were proved to be statistically significant (ANOVA1) after testing the homogeneity of variances (Bartlett test). Post-hoc comparisons were performed by pairwise Scheffe's test ($^{***}p < 0.001$ and $^{**}p < 0.01$). The CFU of the Δ*virB* mutant or the Δ*vjbR* mutants used alone were compared to those obtained by the selection on 2YT medium with kanamycin after the co-infections with the different WT strains.

bacteria in about 500 cells containing both the WT strain and Δ*virB* mutant were counted. Almost 20% of these cells show a rescuing of the Δ*virB* mutant. The presence of WT bacteria is a necessary condition for the rescuing of the Δ*virB* mutant (Fig. 3A) but is not *per se* sufficient. Indeed, in about 80% of the cells, WT and Δ*virB* strains were present in the same cell but while we observed a replication of the WT strain, the Δ*virB* mutant was not rescued (Fig. 3B).

During intracellular trafficking, the WT BCV acquires the LAMP-1 marker and excludes it when the BCV interacts with the ER [15]. This is not the case with the Δ*virB* mutant BCV, which cannot exclude the LAMP-1 marker at late infection times [5]. Therefore LAMP-1 constitutes a good marker to check if the BCV is maturing in a permissive replication vacuole.

By analyzing about 500 cells containing rescued Δ*virB* mutants, we demonstrated that the LAMP-1 marker is lost by the rescued Δ*virB* mutant BCV as described for the WT BCV. On the contrary, the Δ*virB* BCV keeps the LAMP-1 marker. So, it appears that the late steps of intracellular trafficking of a rescued Δ*virB* mutant are similar to the characteristics of the WT BCV in bovine macrophages (Fig. 4).

*2.4. Only simultaneous co-infection allows Δ*virB *rescue by a WT strain*

To determine whether synchronous uptake of the Δ*virB* mutant and of the WT strain was necessary for rescuing, we infected macrophages for 2 h with the Δ*virB* mutant. Afterwards we removed the non-internalized bacteria by washing and by 1 h incubation in a culture medium supplemented with gentamicin, before infecting the macrophages with the WT strain. We also did this in the reverse way, the WT strain first, followed by the mutant. No rescuing was observed if the cells were not incubated with the mutant and WT strains at the same time (Fig. 5).

The above results suggest that WT *B. melitensis* are able to rescue intracellular replication of the Δ*virB* mutant only if they establish residence at the same time within the macrophage.

3. Discussion

Brucella is a broad host range, facultative intracellular pathogen that can survive and replicate in the hostile environment of host cells by preventing fusion of their membrane-bound compartment with lysosomes. Alteration of vacuolar trafficking by intracellular pathogen relies on either surface determinants [20] or specialized secretion apparatus [2,18,46].

Numerous bacteria use a T4SS to translocate macromolecular substrates to bacteria, plant or animal cells. The T4SS functions include (i) conjugal transfer of DNA by cell-to-cell contact, (ii) translocation of effector molecules to eukaryotic target cells, and (iii) DNA uptake from or release to the extra cellular medium (for reviews, see Refs. [10,18]).

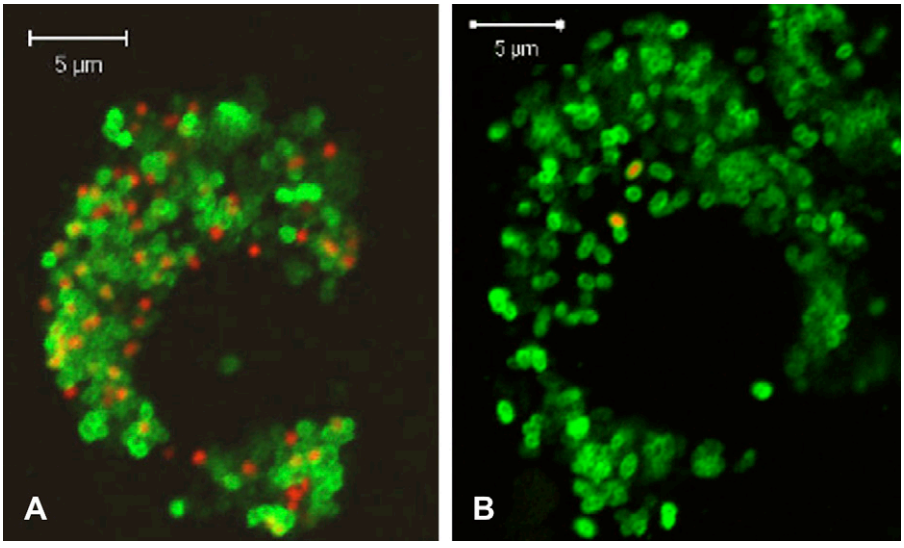

Fig. 3. Co-infection of bovine macrophages SV40 with the wild type strain *B. melitensis* 16M and Δ*virB dsred* (*ΔvirB* strain in which the DsRed coding sequence was inserted under the control of *sojA* promoter, see text for details). The LPS of both bacteria is labeled green and the Δ*virB dsred* mutant appears in red. Rescuing of the replication of the Δ*virB dsred* in the presence of the WT strain is observed in (A) but not in (B).

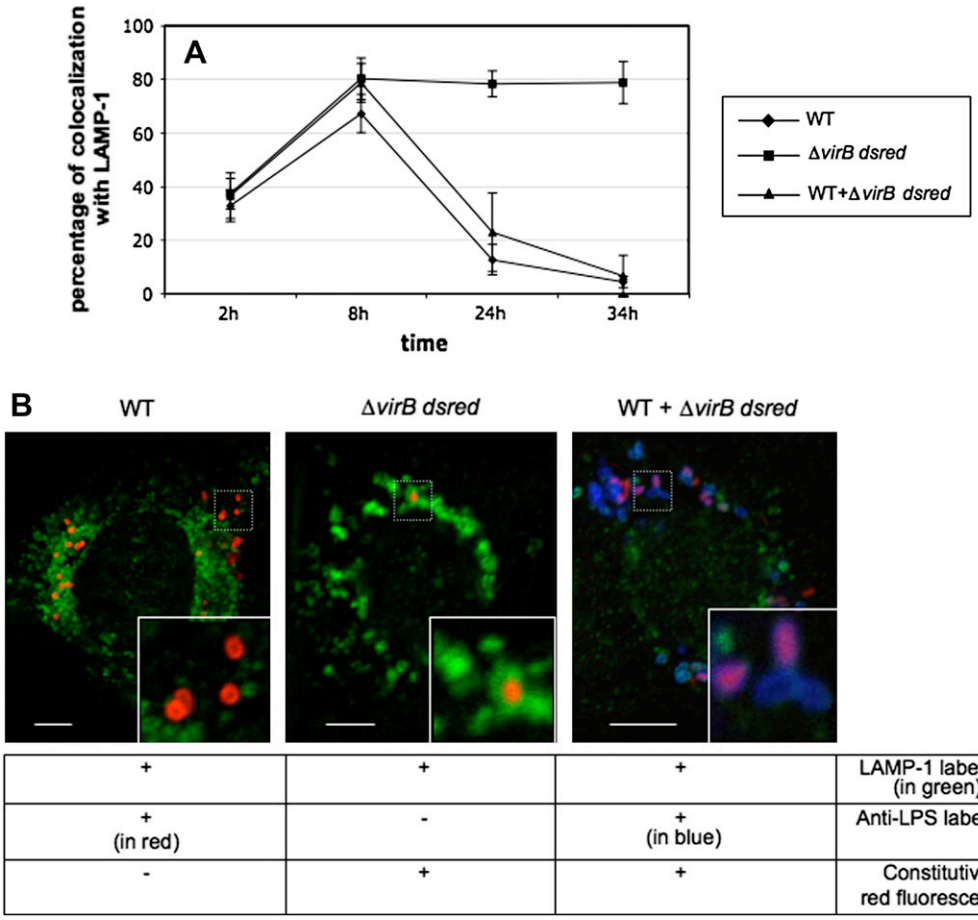

Fig. 4. The vacuole of the rescued Δ*virB dsred* mutant excludes the LAMP-1 marker. (A) Percentage of infected cells presenting LAMP-1 positive vacuoles at several times post-infection with the WT strain *B. melitensis* 16M, the Δ*virB dsred* and co-infection with both strains in SV40 bovine macrophages. The results were proved to be statistically significant (ANOVA1) after testing the homogeneity of variances (Bartlett test). Post-hoc comparisons were performed by pairwise Scheffe's test ($p < 0.01$). (B) Pictures showing the 24 h time point post-infection. The LAMP-1 labeling appears in green in all pictures. In the left panel, the WT was labeled with an anti-lipopolysaccharide (anti-LPS) and appears in red. In the middle panel, the Δ*virB dsred* mutant appears in red. In the right panel, the Δ*virB dsred* mutant appears in red and the LPS of both the WT strain and the Δ*virB dsred* mutant is labeled in blue. Small dotted squares represent the zoom inlets at the right bottom of each picture. Bars correspond to 5 μm.

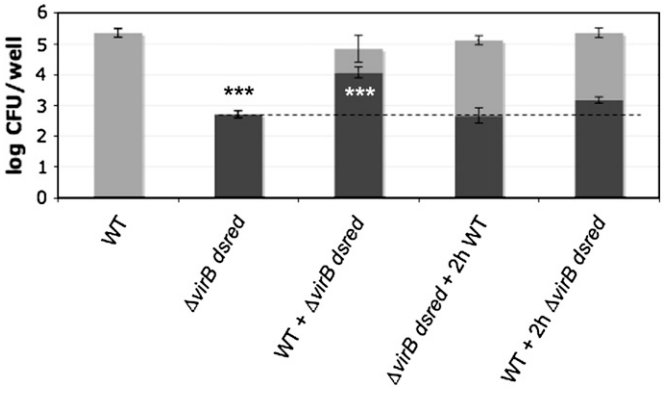

Fig. 5. Analysis of the rescuing of the Δ*virB* mutant in SV40 bovine macrophages co-infected at different times with the WT. The colony-forming units (CFU) were determined on 2YT medium (grey) and on 2YT medium with kanamycin (black). Data shown are the means of three independent assays done in triplicate ± standard deviations. The results were proved to be statistically significant (ANOVA1) after testing the homogeneity of variances (Bartlett test). Post-hoc comparisons were performed by pairwise Scheffe's test (***$p < 0.001$). The Δ*virB dsred* mutant was first compared to the WT strain (black asterisks). The Δ*virB dsred* mutant was also compared to the co-infections data on the 2YT medium with kanamycin (white asterisks).

The implication of the VirB apparatus in the maturation process of the *Brucella*-containing vacuole in host cells is demonstrated [12,15]. Even though the putative molecules exported by the *Brucella* T4SS remain unknown, it is proposed that VirB T4SS exports effectors. The eukaryotic targets of the molecules exported by the T4SS also remain unknown. The putative secreted proteins could act either on the bacteria-containing vacuoles themselves or on the global vacuolar trafficking [11,51].

In our rescuing experiments, we observed that the Δ*virB* mutant is rescued by the WT strain only when the cells are co-infected with both strains (Fig. 1A). Furthermore, we demonstrated that Δ*virB* mutants have no intrinsic replication problems because they can replicate in 20% of the cells co-infected with both the WT strain and the Δ*virB* mutants. The transpositional *virB2* mutant, Δ*vjbR* and Δ*virB* mutants are blocked at the same stage during the intracellular trafficking [15]. In our results (Fig. 2A), we also observed rescuing of all these mutant strains during a co-infection with the WT strain. No rescue was observed during a co-infection with a transpositional *bvrR* mutant and the WT strain. The transpositional *bvrR* mutant is described to present a strong internalization defect in the host cell and a higher sensitivity to bactericidal substances present in the cellular vacuoles [22,23,48]. These data suggest a model in which the VirB apparatus secretes some effectors that act on the intermediate BCV to remodel it in an organelle permissive for bacterial replication. Afterwards, nutrients

and additional membrane necessary for bacterial replication are provided by the host cell, especially by the ER [6]. The fact that rescuing is observed in only around 20% of the co-infected cells is probably linked to the rapidity of *Brucella* entry and of BCVs maturation. This maturation is a stepwise process involving step by step regulation/modulation of the BCV and we propose that rescuing is only achieved when both the WT and the mutant strains establish their infection in a quite narrow time window. This means that both vacuoles should be in the very same maturation stage in order to allow the effector(s) secreted by the WT T4SS to act on the mutant BCV. Accordingly, Coers et al. [11] demonstrated that the *L. pneumophila* WT strain is able to rescue the type IV mutant *dotA* only if they establish residence in the same compartment within the first 5 min of uptake. They proposed a model, similar to our model, in which factors transported by the *L. pneumophila* Dot/Icm transporter act to remodel the phagosome into a specialized organelle permissive for bacterial replication.

By using macrophages co-treated with latex-beads and with the *B. suis* WT strain, Naroeni et al. [35] did not observe an effect on the trafficking of the latex-beads towards the lysosomes. They concluded that fusion inhibition was restricted to the pathogen phagosome and that host cell fusion machinery was not altered by the presence of live *Brucella* in the cell [35]. Actually, the data reported here suggest that putative effectors generated by one BCV may act on another BCV. This is not surprising since the rescuing potential of a mutant BCV is suspected to be dependent on the maturation status of this BCV. Latex-beads do not present any maturation like *Brucella* spp., and their trafficking is therefore not affected by the presence of *B. suis* [35]. Accordingly, the heat-killed and *mTn5::bvrR* BCV are probably unable to mature to a stage where rescuing by WT BCV may be effective.

Our data do not rule out the possibility that rescue may act in *cis* on the BCV. Indeed, it is possible that only Δ*virB* sharing the same BCV with a WT strain may be rescued in cellular infections. However, the high proportion of rescue (around 20%) does not fit with the very low efficacy of cellular infections by *Brucella* spp.; in other words vacuole sharing by Δ*virB* and WT bacteria is suspected to be very rare, and therefore rescue in *cis* is unlikely.

The rescuing of the Δ*virB* mutant replication occurs when bovine macrophages, HeLa cells, murine macrophages J774 or trophoblasts are co-infected with both the WT strain and Δ*virB* mutant (data not shown). Furthermore, it seems that at least some of the T4SS secreted effectors are equivalent in the three *Brucella* species tested because the Δ*virB* mutant is rescued when co-infecting cells with *B. melitensis* 16M, *B. abortus* 544 or *B. suis* 1330 (Table 1).

Because the rescuing of the Δ*virB* mutant replication is possible when co-infecting different cellular models with the WT, we tried to rescue the virulence of the Δ*virB* mutant in a BALB/c murine model by co-infecting the mice with the WT strain. We observed that there was no rescuing of the mutant either at 5 days or at 4 weeks post-infection (data not shown). Therefore we propose that the reported failure to isolate *virB* mutants from large signature-tagged mutagenesis (STM) screens at early infection times (5 days post-infection) cannot be linked to "in vivo" rescuing of these mutants [24,28].

In conclusion, we demonstrated that the replication of the Δ*virB* mutant is rescued by co-infecting cells with the *B. melitensis* WT strain. The question whether putative secreted effectors or the T4SS apparatus *per se* are involved in the rescuing is under investigation. Whatever be the situation, the VirB system could only act in *cis* (meaning both the WT and the mutant being in the same vacuole) while the effectors could act in *cis* or in *trans* on the Δ*virB* mutant vacuole. Considering the propensity of *Brucella* spp. to multiply in individual vacuoles [6] in most cell types tested, we favor the hypothesis of an action in *trans*.

4. Experimental procedures

4.1. Bacterial strains and media

B. melitensis 16M resistant to nalidixic acid was provided by Dr. MacMillan, Central Veterinary Laboratory, Weybridge, United Kingdom. *B. melitensis* was grown in a 2YT medium (10% yeast extract, 10 g/l tryptone, 5 g/l NaCl).

Escherichia coli DH10B (Gibco BRL) and S17-1 were grown in Luria–Bertani broth (Invitrogen) and were used for cloning and plasmid mobilization in *B. melitensis* respectively.

When necessary, antibiotics were added to final concentrations of 50 μg/ml for kanamycin or gentamicin, or 25 μg/ml for nalidixic acid.

4.2. Cell culture

SV40 bovine macrophages were cultured in RPMI-1640 (Gibco) supplemented with 10% fetal calf serum in 5% CO_2 at 37 °C. Cells were detached from culture flasks with trypsin, resuspended in fresh culture medium and replated at a density of 1×10^5 in 24-well cell culture plates (Costar). Cells were typically incubated overnight before the addition of bacteria.

HeLa cells were cultured in DMEM (Gibco) supplemented with 10% fetal calf serum in 5% CO_2 at 37 °C. These cells were treated like the bovine macrophages.

4.3. Construction of B. melitensis modified strains

For construction of the Δ*virB* mutant in *B. melitensis* 16M, a pJQ200 uc1-based plasmid was generated using a two-step cloning strategy [43]. The rationale of this selection is that, in the presence of 5% sucrose, the expression of the *sacB* gene carried by the pJQ200 is lethal. This vector is unable to replicate in *Brucella* spp. and contains a gentamicin resistance marker.

In order to delete the *B. melitensis virB* operon, the 500 bp fragments either upstream of *virB1* or downstream of *virB12* were amplified by PCR from the *B. melitensis* 16M genomic DNA with primers *virB1*F: 5′-ggtacccaccggctagctgaaatcca-3′, *virB1*R: 5′-ggaattccgaagatcttcgaggacaaggaatggcaccat-3′ and primers *virB12*F: 5′-gaagatcttcggaattccaagtaacctgcgaggcctat-3′, *virB12*R: 5′-aagcttagatgccctggggcgctt-3′. These two PCR fragments were assembled during a third PCR, in which the short complementarity between the two PCR fragments allowed the generation of a 1000 bp-long fragment that was amplified using *virB1*F and *virB12*R primers.

After purification, the resulting PCR fragment was first inserted in the pGEM-T Easy vector (Promega) and checked by sequencing. Afterwards, the fragment of interest, removed from the vector by NotI restriction, was ligated in the pJQ200 uc1 linearized by NotI. The resulting pJQ200 uc1-*virB* plasmid was transformed in the *E. coli* S17-1 strain and introduced in *B. melitensis* 16M strain by mating.

The Δ*virB* mutant was selected by a three-step strategy. (i) First, gentamicin resistant candidates were selected on a rich medium supplemented with nalidixic acid and gentamicin. Then, after the growth of the candidates in rich medium without antibiotics, (ii) cells in which resolution of the co-integrate had occurred were selected on a rich medium supplemented with 5% sucrose. (iii) The sucrose resistant and gentamicin sensitive clones were selected and checked by PCR using *virB1*F and *virB12*R primers.

A chloramphenicol resistant derivative of pGEM-T containing an RP4-compatible transfer origin and the upstream region of *virB1* ligated with the downstream region of *virB12* was constructed. The kan^R cassette of pUC4K was inserted between these two regions, and the construct was inserted into *B. melitensis* 16M Nal^R by mating. The nalidixic acid and kanamycin resistant clones that were

chloramphenicol sensitive were selected and checked by PCR as the Δ*virB* mutant without antibiotic resistance.

A previously described rapidly maturing variant of the red fluorescent protein DsRed [49] was cloned into the suicide vector pKS*oriT*-Kan [50] downstream a strong *Brucella* spp. promoter [26]. This construct was conjugated in *B. melitensis* 16M Nal^R, and clones that were kanamycin resistant and fluorescent were further checked by PCR, confirming the insertion of the plasmid at the targeted chromosomal site. This strain was tested in cells and in mouse at different times and revealed no virulence differences compared to the wild type strain.

4.4. Western blotting

Strains were grown in 2YT at 37 °C with shaking overnight. Bacterial cultures were diluted to an initial optical density at 600 nm (OD_{600}) of 0.05 in 50 ml of 2YT and grown at 37 °C. Samples of 10 ml from cultures at an OD_{600} of 1 were inactivated for 2 h at 80 °C. After standardizing for total bacterial extracts using OD_{600} values, the samples were subjected to electrophoresis on 12% SDS-PAGE and transferred to Hybond-C nitrocellulose membranes (Amersham Pharmacia Biotech) by the semi-dry transfer technique. Immunodetection of proteins was performed with polyclonal rabbit anti-VirB10 antisera (a generous gift from C. Baron, McMaster University, Hamilton, Canada) at a dilution of 1/2000. Bound antibodies were detected using chemiluminescence with peroxidase conjugated secondary antibodies and the ECL Western blotting reagents RPN2209 as recommended by the manufacturer (Amersham Pharmacia Biotech).

4.5. Cellular infections

Bacterial strains were grown individually with shaking at 37 °C. Sub confluent monolayers of SV40 bovine macrophages or HeLa cells grown in 24-well cell culture plates were inoculated with a single bacteria suspension or with an equal mixture of two bacterial strains. The cells were inoculated with 500 μl of bacteria at a multiplicity of infection (MOI) of 300 diluted in cell culture medium, centrifuged for 10 min at 1000 r.p.m. at room temperature and placed under a 5% CO_2 atmosphere at 37 °C. After 1 h, the wells were washed three times with phosphate buffered saline (PBS) and were further incubated for 1 h with cell culture medium supplemented with 50 μg/ml of gentamicin. Then, the medium was replaced by the cell culture medium supplemented with 10 μg/ml of gentamicin until the end of the infection time. The number of intracellular viable brucellae was determined at 48 h p.i. To this end, the monolayers were washed three times with PBS and treated with 200 μl of 0.1% Triton X-100 (Roche) in PBS for 10 min. Lysates were serially diluted and plated onto 2YT plates to count viable bacteria (expressed as colony-forming units, CFU). The co-infection with a WT and a kanamycin resistant mutant were plated on 2YT plates and on 2YT plates supplemented with kanamycin.

4.5.1. Immunofluorescence assays

Glass coverslips (13 mm diameter) in 24-well plates were seeded with SV40 bovine macrophages suspended in the culture medium (1×10^5 cells/well). Inoculation and washing were performed as described above. The infected cells were fixed with 3% paraformaldehyde for 20 min at room temperature, at different times after infection for immunofluorescence staining.

4.6. Analytical and quantitative immunofluorescence

The primary antibodies used were the mouse H4A3 LAMP (DSHB product) and the cow anti-LPS (a generous gift from J.P. Gorvel, CIML, Marseille, France). The secondary antibodies were donkey anti-mouse coupled with FITC, goat anti-bovine coupled with Cy-5 or with FITC (Jackson ImmunoResearch).

4.6.1. Intracellular trafficking

At different times p.i., the coverslips were washed to remove non-adherent bacteria and fixed for 20 min in 3% paraformaldehyde, pH 7.4, at room temperature. Immunofluorescence staining and analysis were performed as described previously [41]. To determine the percentage of bacteria that co-localized with the studied intracellular markers, a minimum of 100 infected cells were counted.

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