The sheathed flagellum of Brucella melitensis is involved in persistence in a murine model of infection

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Summary
Persistence infection is the keystone of the ruminant and human diseases called brucellosis and Malta fever, respectively, and is linked to the intracellular tropism of Brucella spp. While described as non-motile, Brucella spp. have all the genes except the chemotactic system, necessary to assemble a functional flagellum. We undertook to determine whether these genes are expressed and are playing a role in some step of the disease process. We demonstrated that in the early log phase of a growth curve in 2YT nutrient broth, Brucella melitensis expresses genes corresponding to the basal (MS ring) and the distal (hook and filament) parts of the flagellar apparatus. Under these conditions, a polar and sheathed flagellar structure is visible by transmission electron microscopy (TEM). We evaluated the effect of mutations in flagellar genes of B. melitensis encoding various parts of the structure, MS ring, P ring, motor protein, secretion apparatus, hook and filament. None of these mutants gave a discernible phenotype as compared with the wild-type strain in cellular models of infection. In contrast, all these mutants were unable to establish a chronic infection in mice infected via the intraperitoneal route, raising the question of the biological role(s) of this flagellar appendage.

Introduction
Brucella organisms are Gram-negative cocco-bacilli that cause brucellosis: a zoonotic disease that has a significant impact on both animal and human health worldwide (Alton et al., 1975). These bacteria are exquisitely well adapted to survival and replication inside eukaryotic cells which is one of the basis for the well-known but still poorly explained chronicity of Brucella infection (Ficht, 2003). The genus Brucella is a member of the alpha subclass of Proteobacteria and belongs to the redefined family Rhizobiaceae that contains bacteria such as Sinorhizobium, Mesorhizobium, Agrobacterium and Ochrobactrum species (Yanagi and Yamasato, 1993). In contrast to most Rhizobiaceae, Brucella have long been described as non-motile (Alton et al., 1975). Nevertheless, open reading frames (ORFs) encoding homologues of several flagellar-related proteins were described in Brucella abortus (Hal- ling, 1998). Even though the genome sequences of Brucella melitensis and Brucella suis revealed the presence of three clusters of flagellar genes (DelVecchio et al., 2002; Paulsen et al., 2002), these genes were considered to be cryptic remnants (Moreno and Moriyon, 2002) because some were truncated and others encoding crucial components of the putative flagellar apparatus (e.g. P ring, L ring) were described as missing (DelVecchio et al., 2002).

Surprisingly, a flagellar gene was identified in a signature-tagged mutagenesis (STM) screen of B. melitensis 16M performed in our laboratory using a mouse infection model (Lestrate et al., 2000). One of the attenuated mutants has a transposon insertion in a gene encoding a protein similar to FlIF, the monomer of the MS ring, a very basal part of the flagellar apparatus (Lestrate et al., 2003). The attenuation of this mutant leads us to hypothesize that the flagellar genes are expressed under some specific conditions encountered during the infectious cycle.

Up to now, only a few major virulence factors of Brucella have been identified, i.e. the O-chain of the lipopolysaccharide (LPS) (Godfroid et al., 1998), the Omp25 (Jubier-Maurin et al., 2001), the type IV secretion system (T4SS) (Boschirolì et al., 2002) and the BvrS–BvrR two-component system that seems to be involved in the homeostasis of the outer membrane (Guzman-Verri et al.,...
2002). These factors are mostly localized at the bacterial surface and are important in the interaction of Brucella with its target eukaryotic cell and, for the LPS and the Omp25, with the host immune system (Forestier et al., 2000; Jubier-Maurin et al., 2001). Other steps of the Brucella infectious process are also only partially understood, such as: adhesion, invasion, escape from infected cells, modulation of the immune response probably contributing to the persistence inside the host. These types of interactions with the host have often been linked to the expression of functional flagella in both pathogenic and symbiotic bacteria (Josenhans and Suerbaum, 2002). Moreover, the flagellar system, known to be phylogenetically related to the type III secretion apparatus (T3SS) (Blocker et al., 2003), has been shown to be involved in virulence factor secretion (Young et al., 1999; Ghelardi et al., 2002; Song et al., 2004).

We report here that B. melitensis in the early log phase of a growth curve in 2YT broth expressed genes corresponding to the basal (MS ring) and the distal (hook and filament) components of the flagellar apparatus. Under these conditions, a complete polar flagellar structure surrounded by a LPS bearing sheath is visible by transmission electron microscopy (TEM). We evaluated the effect of mutation of several flagellar genes of B. melitensis encoding various parts of the flagellar structure (MS ring, P ring, motor protein, secretion apparatus, hook, filament). None of these mutants gave a discernible phenotype when compared with the wild-type (WT) strain in cellular models of infection. In contrast, all these mutants were unable to persist in a murine intraperitoneal model of infection. Finally, we put forward hypothesis about the biological function(s) of Brucella flagellar apparatus. This apparatus richly deserves further attention as, in the very least, it appears involved in the persistence of the infection.

Results

Brucella melitensis and B. suis were devoid of chemotactic genes but possessed all the genes to build a flagellum and to make it rotate

The availability of the B. melitensis 16M and B. suis 1330 genome sequences (DelVecchio et al., 2002; Paulsen et al., 2002) allowed us to draw up an inventory of the flagellar genes. A comparison of the three Brucella flagellar clusters with the unique cluster of structural flagellar genes from both Sinorhizobium mellotli and Mesorhizobium loti (Kaneko et al. 2000a,b; Barnett et al., 2001; Capela et al., 2001; Finan et al., 2001; Galibert et al., 2001) revealed extensive gene synteny and gene names have been assigned by similarity to S. mellotli homologues (Sourjik et al., 1998; Galibert et al., 2001). We identified 31 ORFs encoding flagellar and motor proteins distributed in three clusters on the small chromosome. Two coding sequences (CDS), namely fliF and flhA, are interrupted by a UAG (amber) stop codon in the B. melitensis genome (at position 243 for fliF, encoding the monomer of the MS ring and at position 298 for flhA, encoding a component of the export apparatus). These premature stop codons, reported in the published B. melitensis genome, were validated by sequencing (Table 1). No genes for chemotactic receptors or transducers were detected in the B. melitensis genome (DelVecchio et al., 2002), but, in contradiction with previous claims, all the structural genes needed to assemble and to move a flagellum were present.

The expression of Brucella flagellar genes, proteins or apparatus was growth phase dependent

The attenuated phenotype of a transposition mutant (called 9C6) disrupted in the gene encoding the MS ring (fliF) (Lestrate et al., 2003) prompted us to determine whether this gene is actually expressed under laboratory conditions. When B. melitensis 16M harbouring a pflIF-lacZ translational fusion on a plasmid is grown in 2YT broth, the putative fliF promoter is transiently induced during the early exponential phase as measured by a β-galactosidase assay (Fig. 1A). Usually, the assembly of a flagellar structure from the cytoplasmic membrane outward is a sequential process facilitated by a hierarchy of expression by which distal components are synthesized only after proper assembly of proximal substructures (for review, see Aldridge and Hughes, 2002; Macnab, 2003; Soutourina and Bertin, 2003). Having demonstrated the expression of the basal part of a putative flagellar apparatus (i.e. the MS ring), we undertook to determine whether the terminal parts (i.e. the hook or the flagellin) were also produced under similar conditions. To this end, a Western blot (WB) analysis was performed on whole-cell extracts from B. melitensis pellets harvested along a growth curve of another culture. Specific antisera (see Experimental procedures) to FlgE and to FlIC allowed us to visualize the expression of both the hook (FlgE) and the flagellin (FlIC) monomers on the onset of the exponential phase (after 4 h, 8 h and 12 h of culture) (Fig. 1B). These two proteins were not detected at later time points which is in agreement with the pattern of expression of the fliF promoter.

The detection of both the hook monomer and the flagellin protein made it seem likely that a complete flagellar apparatus was indeed assembled. By TEM of negatively stained B. melitensis cells harvested from a early log phase culture, a seemingly polar flagellar structure was observed (Fig. 2A and B). Immunogold labelling of Brucella smooth lipopolysaccharide (S-LPS) was performed on similar samples to confirm the bacterial genus and...
B. melitensis flagellum is a virulence factor in mice

689

species; the gold-labelling was detected not only on the cell surface but also on the flagellar structure, showing that the flagellum of Brucella is sheathed by an outer membrane (Fig. 2C–E) as also described for Vibrio cholerae, Helicobacter sp. and Bdellovibrio sp. (Thomashow and Rittenberg, 1985; Fuerst and Perry, 1988; Hernandez and Monge-Najera, 1994).

The fliF promoter was induced inside eucaryotic cells

We use a B. melitensis 16M strain bearing the pBBpflif-gfp plasmid to infect eucaryotic cells in order to determine whether the pfliF promoter was expressed intracellularly.

While no fluorescence was detectable for bacteria grown on bacteriological solid medium, green fluorescent protein (GFP) fluorescence was detected at 24 and 48 h after HeLa cells infection (Fig. 3A and B). At 24 h after infection in HeLa cells, most of the intracellular Brucella labelled with anti-LPS antibodies display pfliF expression (Fig. 3C). The pfliF promoter was also induced in bovine macrophage cell line (data not shown).

An independent confirmation came from the screen of a library of B. suis containing transcriptional fusions of chromosomal DNA to gfp. This library was screened to identify B. suis promoters strongly induced inside J774 murine macrophages (Kohler et al., 1999). Among new

Table 1. Predicted CDS (pCDS) identified in the three flagellar loci of B. melitensis 16M.

| Locus I | pCDS number | Intergenic region length downstream the pCDS | Name | E-value of E. coli homologue | E-value of S. meliloti homologue | Predicted function |
|---------|-------------|---------------------------------------------|------|-----------------------------|-------------------------------|------------------|
| BMEII0150 | 203 | fliC | 1e-9 | 2e-48 | Flagellin |
| BMEII0151-0152 | 3 | fliF (1 and 2) | 2e-26/7e-11 | 3e-62/3e-43 | MS ring monomer |
| BMEII0153 | -3 | HP | | 9e-14 | NA |
| BMEII0154 | -3 | motB | | 2e-16 | Motor |
| BMEII0155 | -3 | motC | | 8e-39 | Motor |
| BMEII0156 | 8 | motD | | 5e-6 | Motor |
| BMEII0157 | 328 | HP | | 1e-64 | NA |
| BMEII0158 | 411 | ftcr | | 1e-76 | Two-component response regulator |
| BMEII0159 | 124 | flgE | 1e-38 | 7e-88 | Hook monomer |
| BMEII0160 | 5 | flgK | 3e-12 | 5e-84 | Hook-associated protein |
| BMEII0161 | 131 | flgL | 7e-3 | 1e-50 | Hook-associated protein |
| BMEII0162 | 1 | flaF | | 3e-27 | Flagellin biosynthesis regulator |
| BMEII0163 | -4 | flgT | | 9e-39 | Flagellin biosynthesis repressor |
| BMEII0164 | 11 | flgD | 1e-8 | 2e-31 | Hook-capping protein |
| BMEII0165 | 160 | flkQ | 1e-5 | 6e-25 | Export apparatus |
| BMEII0166-167 | 15 | flhA (1 and 2) | 3e-19/1e-93 | 3e-32/0.0 | Export apparatus |
| BMEII0168 | NR | fltR | 5e-18 | 1e-50 | Export apparatus |

Locus II

| pCDS number | Intergenic region length downstream the pCDS | Name | E-value of E. coli homologue | E-value of S. meliloti homologue | Predicted function |
|-------------|---------------------------------------------|------|-----------------------------|-------------------------------|------------------|
| BMEII080 | -4 | fliP | 3e-53 | 1e-83 | Export apparatus |
| BMEII081 | 12 | fliI | | 1e-15 | Basal-body-associated protein |
| BMEII082 | -4 | fliH | 7e-14 | 1e-47 | L ring monomer |
| BMEII083 | -4 | motE | | 4e-32 | Motor |
| BMEII084 | 295 | flgi | 2e-75 | 1e-117 | P ring monomer |
| BMEII085 | 62 | flgA | <0.01 | 1e-25 | Basal-body P ring biosynthesis protein |
| BMEII086 | 65 | flgG | 1e-54 | 3e-98 | Basal-body rod protein |
| BMEII087 | -2 | fljE | <0.01 | 2e-18 | Hook basal-body complex protein |
| BMEII088 | 2 | flgG | 8e-23 | 8e-44 | Basal-body rod protein |
| BMEII089 | NR | flgB | <0.01 | 1e-24 | Basal-body rod protein |

Locus III

| pCDS number | Intergenic region length downstream the pCDS | Name | E-value of E. coli homologue | E-value of S. meliloti homologue | Predicted function |
|-------------|---------------------------------------------|------|-----------------------------|-------------------------------|------------------|
| BMEII105 | -30 | fliI | 5e-64 | 1e-111 | Flagellum ATP synthase |
| BMEII106 | 205 | HP | – | – | NA |
| BMEII107 | -1 | flgF | 2e-09 | 1e-38 | Basal-body rod protein |
| BMEII108 | 46 | HP | | 6e-69 | NA |
| BMEII109 | -1 | motA | 7e-58 | 1e-109 | Motor |
| BMEII110 | -20 | fljM | | 1e-12 | Motor switch |
| BMEII111 | 23 | HP | – | – | NA |
| BMEII112 | 16 | fljN | 1e-15 | 1e-23 | Motor switch |
| BMEII113 | 290 | fljG | 1e-08 | 1e-23 | Motor switch |
| BMEII114 | NR | fljB | 3e-32 | 3e-76 | Export apparatus |

a. Intergenic regions smaller than 40 nucleotides are expected to be part of an operon (Salgado et al., 2000)
b. In agreement with the name given for flagellar genes of E. coli, Bacillus subtilis or S. meliloti.
c. Indicate the CDS with a premature stop codon. The numbers 1 and 2 refer, respectively, to the N- and C-terminal parts of complete homologous genes.
d. Conserved in alphaproteobacteria. BMEI1111 and BMEII0169 could be badly predicted pCDS.

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clones fluorescing exclusively intracellularly, a clone containing a DNA fragment corresponding to the 526 bp sequence located upstream from the fliF ORF, and the 129 bp 5′ end of this ORF was identified (S. Köhler, unpublished results).

No attenuation could be demonstrated for Brucella flagellar mutants in cellular models of infection

In order to evaluate the contribution of the flagellar genes to Brucella virulence, we constructed mutants in genes encoding flagellar proteins involved in different structural components. As targets for mutation, we chose genes coding for homologues of basal body components similar to T3SS (MS ring monomer: FliF and flagellar export apparatus: FlhA), P ring monomer (FlgI), the motor protein (MotB), the hook monomer (FlgE) and the flagellin monomer (FliC), and these mutants were compared with the WT strain for their ability to invade and replicate inside cells as described previously (Delrue et al., 2001).

Fig. 1. A. Brucella bearing a plasmid with the translational fusion pflif::lacZ was grown in 2YT nutrient broth. The activity of the fliF promoter was followed using β-galactosidase assay. Black circles: optical density at 600 nm; empty squares: β-galactosidase activity expressed as mean ± SD of three replicates. This figure is representative of several independent cultures; in all cases the optimal expression was reached around OD600 of 0.2.

B. FliC and FlgE production in Brucella harvested at 4, 8, 12, 24 and 36 h of growth in 2YT nutrient broth evaluated by SDS-12% PAGE of cell lysates, followed by Western blotting with FlgE-specific (B1) or with FliC-specific (B2) antisera respectively. Omp1 (89 kDa) detected by an anti-Omp1 monoclonal antibody was used as loading control (B1) and is indicated by a star. Recombinant proteins (rFlgE and rFliC) were run as positive controls. The calculated molecular mass (kDa) of FlgE and rFlgE is: 41 and 46. The calculated molecular mass (kDa) of FliC and rFliC is: 29 and 32.

Fig. 2. Transmission electron microscopy of Brucella melitensis cells harvested in early log phase.

A–D. Negative staining with uranyl acetate showing a polar flagella; bars = 1 μm.

C–E. Immunogold labelling of Brucella S-LPS illustrating the sheathed nature of B. melitensis 16M flagellum. (C and D) Bar = 0.5 μm. (E) Magnification of the sheathed filament. The core filament is clearly seen surrounded by the sheath. Bar = 50 nm.
B. melitensis flagellum is a virulence factor in mice

Regardless of the cellular models tested and the conditions used for the cellular infection (see Experimental procedures), no clear difference between the WT and the mutants was evident when internalization or intracellular replication was examined (data not shown). In contrast to a newly constructed MS ring (fliF) mutant, the transpositional fliF (9C6) mutant was consistently attenuated (data not shown). This discrepancy is probably linked to an additional unidentified mutation in the B. melitensis strain bearing the transpositional 9C6 mutation as the WT B. melitensis strains used in this study and in the paper describing the 9C6 mutant (Lestrate et al., 2003) are not of the same origin.

The lack of attenuation of the flagellar mutants in cellular models of infection could either mean that the flagellar genes, while expressed intracellularly, are not needed to infect and replicate inside laboratory cell lines or that the role of these genes are not apparent using these kinds of assays.

Being unable to assign a role to the flagellar structure using <<in vitro>> models of infection and knowing that these models are only partial mimics of the natural infectious process, we used mice as a classical animal model for Brucella infection.

All Brucella flagellar mutants were attenuated in mice at 4 weeks after infection

Groups of BALB/c mice were infected via the intraperitoneal (IP) route either by the WT B. melitensis 16M strain or by flagellar mutants corresponding to the MS ring (fliF), the P ring (flgI) and the filament (fliC), encompassing all three structural levels of the flagellum. The mice were sacrificed 1, 4, 8 and 12 weeks after infection to determine bacterial counts in the spleen. One week after infection, none of the mutants was significantly attenuated as compared with the virulent parental Brucella strain (Fig. 4A). On the contrary, as the infection progressed, the mean spleen counts from mice infected with mutants fliF, flgI and fliC were significantly lower than the number of bacteria in the spleen of mice infected with the WT strain. Most of the mice cleared the flagellar mutants 12 weeks after infection. As a confirmation, new groups of mice were infected with fliF, motB, flgE and fliF mutants and mice were sacrificed 4 weeks after infection. At this time, all the mutants were attenuated to the same extend (Fig. 4B). Altogether, these data indicated that B. melitensis requires the expression of a flagellar structure to cause a persistent disease in the murine model.

Discussion

Brucella is described as non-motile (Alton et al., 1975).

Fig. 3. Expression of GFP from the fliF promoter in HeLa cell. HeLa cells were infected at a ratio of 300 bacteria per cell with B. melitensis 16M harbouring pBBpflIF-gfp. GFP production was monitored by fluorescence microscopy after 24 h (A) and (C) or 48 h (B) of infection. In (C), the GFP expression appears in yellow because Brucella were immunostained in red with a monoclonal antibody raised against LPS O-chain and detected using Alexa 568-conjugated anti-mouse IgG.

Fig. 4. Virulence of B. melitensis 16M Nalr and flagellar mutant strains in BALB/c mice. Mice were infected by intraperitoneal injection. Values are means (log number of cfu per spleen) ± standard deviations (error bars) (n = 4).

A. Mice infected with the 16M strain or the fliF, flgI and fliC mutants and sacrificed at 1, 4, 8 and 12 weeks after infection. The ratios above the columns indicate the proportion of mice that cleared the infection at the indicated time.

B. Mice infected with the 16M strain or the fliF, motB, flgE and fliF mutants and sacrificed at 4 weeks after infection.

*Significant (P < 0.05), **Highly significant (P < 0.01).
The lack of demonstrated motility, the presence of truncated flagellar genes and the absence of chemotactic genes support the view that the flagellar genes identified in the genome of various Brucella species are cryptic remnants (DelVecchio et al., 2002; Abdallah et al., 2003). Along with the lack of resident plasmids in Brucella, the lack of functional flagella was taken as additional evidence of the inability of Brucella to survive or replicate for prolonged periods of time in the environment, leading to its description as a <<facultatively extracellular, intracellular pathogen>> (Moreno and Moriyon, 2002). Here we established unambiguously that B. melitensis was able to build up a complete flagellar apparatus which appeared to be polar and sheathed, at least in vitro. The synthesis and assembly of this structure was tightly regulated both in vitro and in vivo and appears essential for the long-term survival of Brucella in mice.

By itself, the presence of flagellar genes, even a complete set of them, in a pathogenic bacterium previously considered as non-motile is not unique. Other non-motile pathogens (e.g. Shigella flexneri and Aeromonas salmonicida) were shown to have flagellar genes and to assemble a flagellum, although rarely, under strictly defined conditions (Tominaga et al., 1994; Umelo and Trust, 1997).

Two points are very surprising with Brucella. First, as opposed to the above-mentioned non-motile pathogens, Brucella needed its flagellum for a normal infectious cycle: actually, the inability to assemble a complete flagellum, even the very distal component, led to a severe attenuation of the mutated Brucella in a mouse model of infection.

Second, to our knowledge, it is the first time that a functional flagellar apparatus is described without the existence of either chemotactic receptors or signal transducers. These two peculiarities make the biological function(s) of this structure in Brucella very intriguing.

Brucella melitensis has all the genes needed for and is effectively able to construct a complete flagellar structure

Taking into account the known departure of rhizobial flagellum from the enterobacterial flagellar paradigm (Scharf et al., 2001; Scharf and Schmitt, 2002), the genomic analysis uncovered 26 structural genes and five genes involved in the motor function in the B. melitensis genome (Table 1). Homologues of several ORFs strictly typical of Rhizobiaceae flagellar systems were present: (i) homologues of the genes encoding three novel motility proteins, MotC, MotD and MotE, that are essential for the control of flagellar rotary speed in S. meliloti (Platzter et al., 1997; Eggenhofer et al., 2004), (ii) an homologue of fliT encoding a post-transcriptional regulator of flagellin synthesis in Caulobacter crescentus (Anderson and Gober, 2000) and (iii) several ORFs of unknown function conserved in S. meliloti (Sourjik et al., 1998) (see Table 1).

The available set of genes is sufficient to build a complete flagellar apparatus. This was demonstrated not only by the co-ordinate expression of both the fliF gene and of the FliE and FliC proteins when Brucella was grown to early log phase in 2YT broth, but also, and much more convincingly, by the TEM observation of a flagellar structure. In vitro, the expression of the flagellum is transient (see Fig. 1). Actually, the flagellar proteins detected by WB in the early course of the growth curve could be assumed either to progressively disappear diluted by the increasing number of non-flagellated newly formed cells in the growing population or to be degraded or ejected from the cells. The hook protein and the flagellin were no more detected in samples harvested at 24 h (OD$_{600}$: 1.2) while there gave strong bands from 12 h old cells (OD$_{600}$: 0.49). During this period of time the OD increased less than threefold meaning a maximum threefold dilution of pre-existing proteins which should still be detected at least faintly. Concomitantly with the disparition of the FlgE band we observed also an increase of a lower molecular weight (MW) band which could be a degradation product (see Fig. 1). The total disappearance of these proteins probably suggests that a more active process is taking part in this process as it has been observed in other bacteria. The flagellar components of S. meliloti also disappear rapidly in vitro (B. Scharf, pers. comm.). This is also reminiscent of the C. crescentus story where the MS ring, composed of FliF multimers, is subjected to proteolysis leading to ejection of the flagellum (Jenal and Shapiro, 1996). Actually, both Brucella and Sinorhizobium share with Caulobacter numerous aspects of the cell cycle including an asymmetrical division and proteins partners of the phosphorelay controlling the cycle and expression of cell appendages (Hallez et al., 2004). In addition, the sheathed flagellum of Bdellovibrio spp. is dropped before prey invasion (Thomashow and Cotter, 1992). Several points make the flagellum of Brucella quite peculiar among Rhizobiaceae or even alphaproteobacteria. As opposed to the majority of bacterial species which have flagellar filaments made from a single protein (flagellin) (Armitage, 2004), the alphaproteobacteria (e.g. Agrobacterium tumefaciens, C. crescentus, S. meliloti, Rhizobium lupini, Rhodobacter sphaeroides) have various flagellins as subunits to construct a complex rigid flagellum (Scharf et al., 2001; Armitage, 2004). It is noteworthy that Brucella sp. had only one gene (flIC) encoding the filament. Although the Rhizobiaceae have mostly peritrichous flagella (Armitage, 2004), Brucella, much more like C. crescentus, appears to build a polar (monotrichous) flagellum, at least in the condition tested. Finally, as demonstrated by the immunogold labelling with anti-LPS O-chain monoclonal antibody (mAb), Brucella has a

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sheathed flagellum similar to that described in *V. cholerae* (Fuerst and Perry, 1988), *Bdellovibrio* sp. and *Helicobacter* sp. (Thomashow and Rittenberg, 1985; Hernandez and Monge-Najera, 1994). This is the first description of synthesis of a sheathed flagellum in alphaproteobacteria.

Last, but not least, a full-length flagellar structure is assembled, even if the *fliF* and *flhA* CDS are interrupted by a UAG (amber) stop codon in the *B. melitensis* genome (see Table 1), which are essential for the flagellar biosynthesis and assembly. These interrupted CDS must therefore produce functional products. We hypothesize that either truncated portions of *fliF* and *flhA* are expressed and functional, or there is some degree of suppression of the amber codons allowing translation of full-length FliF and FlhA products. Note that the corresponding codons in the *B. suis* homologous genes correspond to CAG (encoding a Gin residue) for the *fliF* gene and GAG (encoding a Glu residue) for the *flhA* gene.

The complete flagellar structure of *B. melitensis* is strictly required for the long-term survival of *Brucella* in mice

The only insight to the biological function(s) of the *Brucella*’s flagellar apparatus has been gained by comparing the residual virulence of the WT strain and flagellar mutants after an IP inoculation in mice.

From these experiments, there are three major observations: first, no attenuation [in terms of colony-forming units (cfu) per spleen] was recorded after 1 week of infection; second, the mean spleen count of the mutants is nevertheless reduced after 4 weeks and later times; and third, all the mutants behaved very similarly.

Injected by IP in mice, *B. melitensis* reaches a maximal splenic count at 5–6 days after the infection, afterwards bacterial numbers stabilize over the next 4–5 weeks and then decrease slowly. The observation that, no matter what mutant was tested, there was no reduction in the splenic count observed after 1 week of infection is indicative that, at least via the IP route, *B. melitensis* did not need its flagellar apparatus either for the initial capture event, for its dissemination to the target organs or even for its intracellular replication process in these infected organs where *Brucella* are found intracellularly within macrophages (Meador et al., 1986).

This lack of detectable effect during the *in vivo* replicative phase of the infection is consistent with the failure to identify any defect of the flagellar mutants using *in vitro* cellular models of infection. These data are also in agreement with the fact that, despite the large number of strains tested, no flagellar genes have been identified in random large screens for attenuated *Brucella* mutants either *in vitro* (Foulongne et al., 2000; Delrue et al., 2001; Kohler et al., 2002; Kim et al., 2003) or in the acute phase of infection in mice (Lestrate et al., 2000). The lack of detectable attenuation in cellular models of infection or after 1 week of infection in mice as compared with the WT strain and the fact that all the mutants tested demonstrated the same behaviour make very unlikely a pleiotropic effect of the mutations on the membrane integrity which could explain the reduced virulence at 4 weeks in the mouse model.

While the flagellar genes were not involved in the *Brucella* intracellular trafficking towards its replicative niche, the *fliF* gene was induced intracellularly. The intracellular expression of flagellar genes has to serve some crucial and currently unknown function that is worthy of the high energy costs demanded in the synthesis and assembly of this macromolecular structure. It probably means that the flagellar synthesis cascade is initiated intracellularly and could be essential at some later point of the infectious process as demonstrated for *Legionella pneumophila* (Byrne and Swanson, 1998). This intracellular pathogen and *Brucella* sp. share some virulence traits. For both of them a T4SS is necessary for their access to the replicative vacuole and their replicative vacuole is also endoplasmic reticulum (ER) derived (Scott et al., 2003). *Legionella*’s flagellum appears to be involved in the escape from the infected cells. The models we used for *Brucella* probably do not allow us to demonstrate an effect either *in vitro* or *in vivo* (Ficht, 2000; Jacobs et al., 2000). We propose that the flagellum of *Brucella* is used to somehow subvert the specific immune response which could be more efficient when the flagellum is lacking. Considering the known immunomodulatory role of flagellin via TLR5 signalling the putative *in vivo* role of this *Brucella* patho-
gen-associated molecular pattern (PAMP) will certainly
deserve further attention so as the role of the sheath
which was suggested preventing the release of flagellin
monomers into the surrounding environment (Ramos
et al., 2004).

Despite the fact that the exact reason(s) of this late
attenuation are not understood, the observation that the
flagellar mutants are not cleared during the early course
of the infection in mice but are nevertheless cleared at
later time points makes them potential valuable vaccine
candidates, as also suggested by preliminary results
(J. Godfroid, pers. comm.).
The observation that the \textit{fliC} mutant (lacking only the
filament of the flagellum) has the same phenotype as
other deeper flagellar mutants (i.e. \textit{flgE}, \textit{flgL}, \textit{fliF} mutants)
is a strong indication that even \textit{in vivo} a complete flagellar
structure is built and needed for a full function.
The above-reported data are important cues indicating
that the \textit{B. melitensis} flagellar genes do not encode a
\textit{bona fide} T3SS as it has been suggested previously
(Abdallah \textit{et al}., 2003; Karlin \textit{et al}., 2003). However, as
examplified by the case of \textit{Yersinia} \textit{(Young \textit{et al}., 1999)} or
\textit{Campylobacter} \textit{(Song \textit{et al}., 2004)}, an involvement of the
\textit{Brucella} flagellar apparatus in the secretion of virulence
factor(s) cannot be ruled out.

\textbf{What signifies the absence of a chemotactic system?}

Finally, one of the most striking observations with regard
to the \textit{Brucella} flagellar genes is the absence of genes
encoding both membrane chemoreceptors and proteins
of the signal transduction pathway to the flagellar motor.
Actually, an homologous set of proteins (chemoreceptors,
\textit{CheW}, \textit{CheY}, \textit{CheA}, \textit{CheB} and \textit{CheR}) governs chemotaxis
in all motile species of \textit{Bacteria} or even \textit{Archea}
whose genome is complete or available (Szurmant and
Ordal, 2004). This pathway leads either to the switching
of the sense of rotation, from right-handed to left-handed,
and vice versa, according to the enterobacterial paradigm,
or to the modulation of the rotary speed as described in
\textit{S. meliloti}. Moreover, the genome of several alphaproteo-
bacteria contain multiple homologues of chemotactic
genes distributed in several regions (Hauwaerts \textit{et al}.,
2002) and the signalling pathway is more elaborate and
more complex than that of the classical pathway found in
\textit{Escherichia coli}. The absence of such a system in
\textit{Brucella}, as an actual exception among bacteria with a func-
tional flagellar apparatus, raises the intriguing question
whether all or part of its biological function(s) involves
movement and, hence, a rotary process.

Accessory roles described for other flagellated bacteria
(e.g. adhesion, toxin secretion) \textit{(Young \textit{et al}., 1999)} are
always in addition to the motility \textit{<per se>} function
(Josenhans and Suerbaum, 2002). So, except for rotation
and movement there is no need for a complete complex
structure. Actually the fact that a motor mutant (\textit{motB}) has
the same phenotype in mice as structural mutants is indic-
ative that rotation could be involved.

The chemotactic signalling pathway is usually involved
in controlling the directionality of cell movements in
response to environmental stimuli. At least for some intra-
cellular pathogens, could it be that movement is more
important than direction? If \textit{Brucella} is indeed rotating its
flagellum and if the resulting movement is important for
some part of the infectious process, this would recall
the observation that \textit{Salmonella typhimurium} and \textit{Vibrio
anguillarum} mutants of the chemotactic pathway, which
are still able to build and rotate their flagella, are more
invasive than the WT strain (Jones \textit{et al}., 1992; Larsen
and Boesen, 2001). This leads to the hypothesis that
\textit{Brucella}, when becoming more and more restricted to its
intrahost niche, did not need the chemotactic pathway any
more and discarded it. Whatever the exact function(s) of
the \textit{Brucella} flagellum may be, it certainly deserves further
investigations to uncover the step(s) of the infection in
which it is involved, the signal(s) which turn on its expres-
sion and the regulatory network leading to the finely tuned
synthesis of such a complex structure which seems to
play a crucial role in establishment of a chronic infection.

\textbf{Experimental procedures}

\textit{Bacterial strains and plasmids}

\textit{Brucella melitensis} 16M was obtained from A. MacMillan, Central
Veterinary Laboratory, Weybridge, UK. We selected a spontane-
ous nalidixic acid-resistant (Nal\textsuperscript{R}) mutant of this strain and all
\textit{Brucella} strains used in this study were derived from this Nal\textsuperscript{R}
mutant. \textit{E. coli} DH10B (Gibco BRL), S17-1 (Simon \textit{et al}., 1983)
and BL21(DE3) (Novagen) were used for cloning, plasmid mobi-
лизation in \textit{B. melitensis} and protein overexpression respectively.
\textit{B. melitensis} and \textit{E. coli} strains were grown as described (Tibor
\textit{et al}., 2002). DNA manipulation was performed according to
standard techniques (Ausubel, 1989).

\textbf{Sequence analysis}

Genomic sequence of \textit{B. melitensis} 16M was analysed with the
database of the URBM bioinformatic group: http://
www.serine.urbm.fundp.ac.be/~seqbruce/GENOMES/Brucella,
\textit{melitensis}. Genomic sequences of \textit{S. meliloti} and \textit{M. loti} were
analysed with the Rhizobase: http://www.kazusa.or.jp/rhizobase/.

\textbf{β-Galactosidase assay}

\textit{Construction of plasmid pBBCmpfliF-lacZ}. The \textit{lacZ} coding
region deleted from its 27 first nucleotides was cloned into
pBBR1MCS (Elzer \textit{et al}., 1994). A region containing the \textit{fliF}
promoter (including 137 bp of the 3’ end of the \textit{fliC} ORF, 203 bp of
the \textit{fliC}–\textit{fliF} intergenic region and 65 bp of the predicted 5’ end
of \textit{fliF}) was amplified from \textit{B. melitensis} 16M Nal\textsuperscript{R} genomic DNA

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with the primers FLIFAM (5'-ATATCTAGAGCTCGTCGATGC CGACA-3') and FLIFAV (5'-ATTGTAGCCGCGAGCTTCCCCT TGAGC-3') containing XbaI and BamHI restriction sites respectively. The polymerase chain reaction (PCR) product was first subcloned into pGEM-T Easy Vector (PROMEGA) and then inserted in frame upstream the promoterless lacZ reporter gene into pBBR1MCS.

\[ \beta\text{-Galactosidase enzyme assay.} \]

The plasmid pBBcmpfliF-lacZ was conjunctively transferred into \textit{B. melitensis} 16M Na\(^\oplus\) strain, and samples were harvested at different times during the growth at 37°C in 2YT broth containing chloramphenicol, and \(\beta\)-galactosidase assays were performed on the samples in agreement with the method described by Miller (1972).

**Protein purification, generation of antibodies and immunoblot analysis**

\textit{Brucella melitensis} recombinant FliC and FlgE were overexpressed in \textit{E. coli} as fusion proteins with an N-terminal polypeptide comprising a hexahistidine peptide (Novagen). The \textit{fliC} and \textit{flgE} ORFs were amplified by PCR (the primers contained an \textit{Nde}I site added on the 5' end and a \textit{Bam}HI site on the 3' end of these genes) and cloned first into the EcoRV site of pSK oriT vector. The DNA sequences of the PCR-amplified fragments were determined at this stage. The resulting plasmids were then digested with \textit{Nde}I and \textit{Bam}HI and their insert was subcloned into these sites in pET15b. After overexpression in \textit{E. coli} BL21DE3, protein purification was performed on a 2.5 ml nickel chelation resin column (His-Bind; Novagen) and \textit{B. melitensis} was used as a positive control (Cloeckaert et al., 1993) with 50 \(\mu\)M of purified recombinant proteins to produce specific antibodies to FliC and FlgE. Immunoblot analysis was performed as described (Bellefontaine et al., 2002). Anti-FliC or anti-FlgE polyclonal antibodies were diluted 1:1000 and the anti-OMP1 mAb A5310B2 was used as a positive control (Cloeckaert et al., 1990). These antibodies were revealed using, respectively, donkey anti-rabbit (Amersham) and goat anti-mouse (Amersham) horseradish peroxidase-conjugated secondary antibodies.

**Transmission electron microscopy (TEM) and immunogold labelling**

Bacteria were grown in rich medium at 37°C to an OD at 600 nm of 0.25. Bacteria were centrifuged at 1000 r.p.m. for 20 min (Jouan), washed in PBS and fixed for 20 min in 50 \(\mu\)l of 4% paraformaldehyde pH 7.3, Bacteria were stored at 4°C. A carbon formvar-coated grid was placed on a drop of a solution of 1% paraformaldehyde pH 7.3. Bacteria were stored at 4°C for 10 s. Samples were examined with a transmission electron microscope (Technai 10, Philips).

\[ Immunofluorescence assays \]

For the construction of the pBBpflf-gfp, the sequence located upstream of the \textit{fliF} CDS was amplified by PCR from genomic DNA of \textit{B. melitensis} 16M Na\(^\oplus\) with the primers upfliF (5'-GGTACCCCCTGCTTTCTTTGTTG-3') and lowfliF (5'-TTTGAGGGGAAGAGATGTC-3'). The PCR product was cloned into the EcoRV site of plasmid pSK oriT (Tibor et al., 2002). This vector is mobilizable and is unable to replicate in \textit{Brucella} LPS O-chain mAb 12G12 (Cloeckaert et al., 1993) as described previously (Delrue et al., 2001). Secondary antibodies used were Alexa 568-conjugated anti-mouse IgG (Molecular probes).

**Construction and characterization of the flagellar mutants**

To construct a \textit{B. melitensis} flgE mutant by allelic replacement, the \textit{flgE} coding sequence (BMEl0159) was amplified by PCR from genomic DNA of \textit{B. melitensis} 16M Na\(^\oplus\) with the primers upflgE (5'-GAAATCCCAAGGGCTACA-3') and lowflgE (5'-TTCCAGGGCGAACGATC-3'). The PCR product was cloned into the EcoRV site of plasmid pSK oriT (Tibor et al., 2002). This vector is mobilizable and is unable to replicate in \textit{Brucella}. A 318 bp Nar fragment was excised from the resulting plasmid and replaced by the 1.3 kb \textit{Bam}HI kanamycin resistance cassette (kan) from pUC4K (Pharmacia), producing plasmid pDHOOK.

The \textit{fliF1}, \textit{flha2}, \textit{flhB}, \textit{flgI}, \textit{motB} and \textit{fliC} mutants were constructed by insertional inactivation. In order to allow the transconjugants maintaining our constructs to be selected in the presence of kanamycin, the \textit{kan} cassette was amplified from the pUC4K plasmids with primers kanamont (5'-GGGATCCCGGTCTTCGTATTTCTG-3') and kanaval (5'-CGCCGTGAACTGGAGGAG-3') and cloned into the SalI site of the bla gene of pSK oriT. This generated the plasmid pSKkan. The internal fragments of the \textit{fliF1}, \textit{flha2}, \textit{flgI}, \textit{motB} and \textit{fliC} coding sequences were amplified by PCR from genomic DNA of \textit{B. melitensis} 16M Na\(^\oplus\) with the following pairs of primers. The primers lowfliF (5'--CCCGATATTGTCGAAACG-3') and upfliF (5'-TCGGCCCATCCTCACGAC-3') amplify from nucleotide (nt) 153 to nt 372 from the \textit{fliF1} ORF (BMEII0159, 729 bp in length). The primers upflhA (5'-CCGCGCTGAACAGGAGAG-3') and lowflhA (5'-GCGGGC CATCCTTGTTGAC-3') amplify from nt 226 to nt 685 from the \textit{flha2} ORF (BMEII0167, 1767 bp in length). Primers upfliC (5'-GGGGCGCCTGAAAGACATC-3') and lowfliC (5'-ACCGGGCG GTGCTGAAATTG-3') allow amplification from nt 113 to nt 635 of the 1290 nt \textit{flgI} ORF. The primers upmotB (5'-GGGCCGCCTGAAAGACATC-3') and lowmotB (5'-CCGCGGCGCCACAGTGGT GTC-3') allow amplification from nt 79 to nt 329 of the 1137 bp \textit{motB} ORF. The primers upfltC (5'-ATATCTAGAGCTCGTCGATGC CGACA-3') and lowfliC (5'-TTATCGGTTCCGCAACATCAGCCGG-3') allow amplification from nt 378 to nt 583 of the 846 nt \textit{fltC} ORF. These amplicons were cloned into the EcoRV site of the pSKkan \textit{fliF1} amplicon was first cloned into pGEM-T easy vector (Promega). These constructs were checked by sequencing of the insert with plasmid templates.

Plasmid mobilization into \textit{B. melitensis}, mutant selection and confirmation of gene inactivation by Southern blot analysis were performed as described by Tibor et al. (2002).
Cell culture and bacterial infection

Survival of Brucella strains was evaluated in an immortalized cell line of bovine peritoneal macrophages (Stabel and Stabel, 1995) and in epithelial human HeLa cells by the procedure described by Delrue et al. (2001).

In addition to these assays, several other infection procedures were applied only to the fliF mutant in comparison to the WT strain. A gentamicin survival assay to quantify Brucella invasion in HeLa cells as described previously (Delrue et al., 2001). An infection of ovine macrophagic cell line at a low multiplicity of infection (moi) (0.1 bacteria per cell) and a follow-up during 7 days as described in studies on Legionella pneumophila flagellum (Dietrich et al., 2001). Infection of fresh bovine blood monocytes in suspension as described for a Brucella infection of human blood monocytes (Rittig et al., 2001).

All the above infections used Brucella cells from an overnight culture. An infection of ovine macrophagic cell line according to the classical protocol (Delrue et al., 2001) was designed to compare Brucella cells harvested in the phase of the growth curve where flagellar genes are known to be expressed and Brucella cells from an overnight culture.

Knowing that flagellar genes are expressed intracellularly, we designed a protocol which used infected cells as inoculum. Briefly, ovine macrophagic cells were infected according to our current protocol (Delrue et al., 2001) and harvested by trypsinization after 48 h. After washing in PBS, infected cells were resuspended at $10^5$ ml$^{-1}$ in complete RPMI medium with gentamicin and layered on a monolayer of fresh non-infected cells of the same origin by centrifugation at 1200 r.p.m. After 1 h incubation at 37°C, wells were washed and incubated in fresh complete RPMI medium.

Virulence assay in the BALB/c mouse model

Eight-week-old female mice were inoculated intraperitoneally with 0.2 ml of a suspension containing around 10$^5$ cfu of each bacterial strain harvested with PBS from a 24-h 2YT liquid culture (exact doses were retrospectively assessed). At appropriate intervals after inoculation, five mice from each treatment group were sacrificed for spleen collection. Bacterial survival was determined following homogenization of the mouse spleens in 2 ml of distilled water. Serial dilutions of the homogenates were plated on 2YT agar to determine bacterial counts. To determine the significance of differences observed in our experiment, pairwise comparisons were performed by Scheffé tests after ANOVA2 providing the residual mean square estimate with the highest available degree of freedom.

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