JOURNAL OF BACTERIOLOGY, Jan. 2007, p. 131–141 Vol. 189, No. 1
0021-9193/07/$08.00+0 doi:10.1128/JB.00712-06

FtcR Is a New Master Regulator of the Flagellar System of *Brucella melitensis* 16M with Homologs in *Rhizobiaceae*[▽]

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Received 18 May 2006/Accepted 5 October 2006

The flagellar regulon of *Brucella melitensis* 16M contains 31 genes clustered in three loci on the small chromosome. These genes encode a polar sheathed flagellum that is transiently expressed during vegetative growth and required for persistent infection in a mouse model. By following the expression of three flagellar genes (*fliF*, *flgE*, and *fliC*, corresponding to the MS ring, hook, and filament monomer, respectively), we identified a new regulator gene, *ftcR* (*f*lagellar *t*wo-*c*omponent *r*egulator). Inactivation of *ftcR* led to a decrease in flagellar gene expression and to impaired *Brucella* virulence. FtcR has a two-component response regulator domain as well a DNA binding domain and is encoded in the first flagellar locus of *B. melitensis*. Both the *ftcR* sequence and its genomic context are conserved in other related α-proteobacteria. During vegetative growth in rich medium, *ftcR* expression showed a peak during the early exponential phase that paralleled *fliF* gene expression. VjbR, a quorum-sensing regulator of the LuxR family, was previously found to control *fliF* and *flgE* gene expression. Here, we provide some new elements suggesting that the effect of VjbR on these flagellar genes is mediated by FtcR. We found that *ftcR* expression is partially under the control of VjbR and that the expression in *trans* of *ftcR* in a *vjbR* mutant restored the production of the hook protein (FlgE). Finally, FtcR binds directly to the upstream region of the *fliF* gene. Therefore, our data support the role of FtcR as a flagellar master regulator in *B. melitensis* and perhaps in other related α-proteobacteria.

Flagella are highly complex bacterial organelles that are usually well conserved among diverse bacterial species (17, 39, 46). In addition to motility, the bacterial flagellum is involved in a variety of interactions between the bacterium and its environment (e.g., adhesion, biofilm formation, secretion, and modulation of the host reponse) (7, 16, 20, 22, 30, 36, 42, 50, 53, 57). Flagellar expression involves about 50 flagellar genes, distributed into three or four classes as observed in *Escherichia coli* (1) and *Salmonella enterica* serovar Typhimurium (31) or in *Caulobacter crescentus* (38), *Pseudomonas aeruginosa* (10), and *Vibrio cholerae* (41). Complex regulation processes control this hierarchical system, allowing for the expression of the genes of one downstream class if the genes from the upstream class have been expressed.

At the top of the flagellar regulatory cascade, one or more distinct master genes encode the transcriptional regulators responsible for turning on and off flagellar synthesis, in response to environmental factors and cell cycle-related signals. In γ-proteobacteria, this so-called class I is represented by a heterotetrameric complex, $FlhD_2C_2$ (lateral flagellar systems), or a sigma 54-associated transcriptional activator of the NtrC family (polar flagellar systems) (4, 52). The situation is somewhat different in α-proteobacteria. In *C. crescentus*, a two-component response regulator called CtrA controls a part of the asymmetric cell cycle as well as the initiation of the flagellar cascade (49), whereas in *Sinorhizobium meliloti*, two LuxR type regulators, VisR and VisN, are at the top of the flagellar hierarchy (51). Direct binding of VisR or VisN to a promoter of a class II gene (e.g., *fliF*) has never been demonstrated, and so, the involvement of other unidentified effectors acting in concert with VisN/VisR at the top of the hierarchy cannot be excluded (51).

Recent studies have shown that *Brucella melitensis* possesses 31 flagellar genes and builds, under strictly defined conditions, a polar sheathed flagellum required for virulence in a mouse infection model (14). The flagellar hierarchy has not been characterized in *Brucella*. A comparison of the three *Brucella* flagellar clusters with the unique cluster of structural flagellar genes from *S. meliloti* (15) revealed extensive gene synteny, namely, a high conservation in the order of genes. Like the *visNR* operon of *S. meliloti*, two regulator genes are located downstream and in the reverse orientation from a set of flagellar genes in *Brucella*. Nevertheless, only one of these two, called VjbR (11), belongs to the LuxR family. The second regulator is of the TetR family, and its function is presently unknown (11). VjbR was recently described as a quorum-sensing regulator that activates the expression of the *virB* secretion system and the flagellar apparatus of *B. melitensis*, both required for virulence in BALB/c mice. A Δ*vjbR* strain does not express *fliF* (coding for the MS ring monomer) and does not produce the FlgE protein (hook monomer). Moreover, an excess of the quorum-sensing pheromone *N*-dodecanoyl homoserine lactone represses FlgE production, probably by inhibiting VjbR activity (11). VjbR could constitute a second example

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▽ Published ahead of print on 20 October 2006.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
B. melitensis strains		
16M Nal^r	Spontaneous nalidixic acid-resistant strain of *B. melitensis* 16M obtained from A. P. MacMillan, Central Veterinary Laboratory, Weybridge, United Kingdom	Laboratory collection
ftcR mutant	16M Nal^r *ftcR* integrative mutant	This study
vjbR mutant	16M Nal^{r}Δ-*vjbR*	11
E. coli strains		
BL21(DE3)plysS	F$^-$ *ompT gal dcm hsdS*$_j$($r_B^-m_B^-$) (DE3) [pLysS, Cm^r]	Novagen
DH10B	F$^-$ *mcrA* Δ(*mrr hsdRMS mcrBC*) ϕ80*lacZ*Δ*M15* Δ*lacX74* *recA1 endA1 ara*Δ*139* Δ(*ara leu*)*7697 galU galK* λ$^-$ *rpsL* (Str^r) *nupG*	Gibco BRL
S17-1	*thi pro hsdR hsdM*$^+$ *recA* RP4-2 (Tc::Mu-Km::Tn*7*)	48
C. crescentus strain		
NA1000	*syn*-1000, synchronizable variant of strain CB15	13
Plasmids		
pBBCm-*lacZ*	Promoterless *lacZ* vector, Cm^r	14
pBBCm*pfliF-lacZ*	*fliF-lacZ* transcriptional reporter, Cm^r	14
pBBCm*pftcR-lacZ*	*ftcR-lacZ* transcriptional reporter, Cm^r	This study
pBBNrs*pfliF-lacZ*	*fliF-lacZ* transcriptional reporter, Nrs^r	This study
pBB*pfliF-gfp*	*fliF-gfp* transcriptional reporter, Amp^r Cm^r	14
pBBR1MCS-4	Medium-copy broad-host-range cloning vector, Amp^r	27
pBBR-*vjbR*	*vjbR* in pBBR1MCS-4	11
pDONR201	Gateway donor vector	Invitrogen
pDONR*ftcR*	*ftcR* Gateway entry vector	This study
pDONR*pftcR-ftcR*	*pftcR-ftcR* Gateway entry vector	This study
pET15b-GW	T7 expression vector pET15b from Novagen containing *attR* recombination Gateway sequences	B. Vandriessche, unpublished data
pET15b*ftcR*	*ftcR* in pET15b-GW	This study
pFA6a-natMX6	Nourseothricin acetyltransferase donor plasmid	Werner BioAgents
pGEM-T Easy	Cloning vector	Promega
pGEMT*pftcR*	*pftcR* in pGEM-T Easy vector	This study
pMR10CmKm	Low-copy-no. broad-host-range vector, Cm^r, Km^r	43
pMR10-GW	Low-copy-no. broad-host-range vector pMR10 containing *attR* recombination Gateway sequences	R. Hallez, unpublished data
pMR*pftcR-ftcR*	*pftcR-ftcR* in pMR10-GW	This study
pMR*plac-ftcR*	*ftcR* in pMR10-GW	This study
pSKKan	Suicide plasmid, Km^r	14
pSKftcR	*ftcR* disruption suicide plasmid	This study

of the LuxR-type master flagellar regulator as in *S. meliloti*, but its effect is known for only two flagellar components (FliF and FlgE). The direct or indirect nature of this control has not yet been determined (11).

Besides the TetR-type and the VjbR regulators, there is a third gene coding for a predicted transcriptional regulator within the *Brucella* flagellar loci, with synteny in *S. meliloti*. This gene was called *ftcR* for "*f*lagellar *t*wo-component *r*egulator" (14). The FtcR regulator is in fact predicted to belong to the two-component response regulator family. Because the function of *Brucella melitensis* FtcR and its homologs in other α-proteobacteria is unknown, we set out to elucidate its role in flagellar regulation. In this paper, we demonstrate that FtcR is required in *B. melitensis* 16M for the transcription of the *fliF* gene during vegetative and intracellular growth and for the production of the two structural flagellar components FlgE and FliC during vegetative growth. We also show that an *ftcR* mutant has the same virulence phenotype as previously found with structural flagellar mutants. Furthermore, we show that FtcR directly and specifically binds to the *fliF* upstream region. Finally, we establish that the expression of *ftcR* in *trans* on a plasmid can complement a Δ*vjbR* strain for FlgE production and that *ftcR* is partially activated by VjbR. These data suggest that FtcR could mediate the action of VjbR on flagellar gene expression.

Taken together, these data indicate that FtcR could constitute a master regulator of the *Brucella* flagellar regulon and raises the possibility that FtcR homologs in other α-proteobacteria may play the same role.

MATERIALS AND METHODS

Bacterial strains and culture conditions. All strains and plasmids used in this study are listed in Table 1 and oligonucleotides in Table 2. *B. melitensis* 16M strains were grown with shaking at 37°C in 2YT medium (1% yeast extract, 1.6% peptone, 0.5% NaCl) from an initial optical density at 600 nm (OD_{600}) of 0.05. *C. crescentus* strains were grown at 30°C in PYE medium (40). *E. coli* strains were grown at 37°C in Luria-Bertani (LB) broth (44). Antibiotics were used at the following final concentrations: ampicillin, 100 μg/ml; chloramphenicol, 20 μg/ml; kanamycin, 30 μg/ml; nalidixic acid, 25 μg/ml; and nourseothricin, 50 μg/ml. DNA was transferred into *B. melitensis* or into *C. crescentus* using the *E. coli* S17-1 strain harboring the mobilization plasmid RP4. The *E. coli* strain DH10B

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence (5′ to 3′)	Source
F2C2	ATCTCTAGAGACGACAGAGACATGGTGACT	This study
R2C2	ATCGTCGACCTACGCGAGAATTTCGCGGACATG	This study
p2c2Xamont	ATATCTAGATTCAGCCGCGGCGGGCT	This study
p2c2Baval	ATTGGATCCCCTTCGCGACCGAACCA	This study
1038-attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGATTGTTGTCGTT	This study
1038-attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTGCTCGATATTGATGCA	This study
attB2-pbifR	GGGGACCACTTTGTACAAGAAAGCTGGGTGTTCAGCCGCGGCGGGCTT	This study
attB1-bifR	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGCTTCAGGCTGGGGGACT	This study

was used for the propagation of the plasmids used in this study. BL21(DE3)pLysS was the host strain for the T7 expression system.

Construction of the *ftcR* mutant. The *ftcR* mutant was constructed by integrative disruption. An internal 0.315-kb fragment from nucleotide 16 to nucleotide 340 of the *ftcR* predicted coding sequence (pCDS) was amplified by PCR from genomic DNA of the *B. melitensis* 16M Nal^r strain with the F2C2 and R2C2 primers and cloned into the EcoRV site of pSKKan (14) to generate pSKKan*ftcR*. The pSKKan*ftcR* plasmid was inserted into the *ftcR* pCDS of the *B. melitensis* 16M Nal^r strain by homologous recombination. Transconjugants were selected by selecting for kanamycin resistance. Southern blot analysis was carried out on genomic DNA by using a probe that hybridized to the kanamycin resistance gene to confirm that one plasmid copy had stably integrated into the target gene, as described by Haine et al. (19).

Plasmid construction. Recombinant DNA techniques were carried out using standard protocols (44).

For construction of the reporter *pftcR-lacZ* fusion pBBCm*pftcR-lacZ*, a 0.437-kb fragment containing the *ftcR* promoter (*pftcR*) (including 24 bp of the 3′ end of the upstream hypothetical pCDS, 327 bp of the noncoding region, and 68 bp of the predicted 5′ end of *ftcR*) was PCR amplified from genomic DNA of the *B. melitensis* 16M Nal^r strain with primers p2c2Xamont and p2c2Baval containing XbaI and BamHI sites, respectively. The PCR product was first subcloned into pGEM-T Easy vector (Promega) to generate pGEMT*pftcR* and then inserted into pBBCm-*lacZ* (14) in frame upstream of the promoterless *lacZ* reporter gene, generating pBBCm*pftcR-lacZ*.

The nourseothricin-resistant reporter fusion pBBNr*pfliF-lacZ* was obtained by cloning a 1.028-kb region containing the nourseothricin acetyltransferase gene (recovered from the pFA6a-natMX6 vector [Werner BioAgents] by FspI digestion) into the DraI site of the *cat* gene of pBBCm*pfliF-lacZ* (14).

The FtcR expression vectors pET15b*ftcR*, pMR*ftcR*, and pMR*pftcRftcR* were constructed using Gateway Technology (Invitrogen). Briefly, *ftcR* pCDS was amplified by PCR from *B. melitensis* 16M genomic DNA and tailed with *attB1* and *attB2* sequences using the 1038-attB1 and 1038-attB2 primers. The product was cloned by BP reaction (recombination reaction using BP clonase enzyme mix) into the pDONR201 vector (Invitrogen), generating the pDONR*ftcR* entry vector. In the same way, a 1.132-kb fragment containing the *ftcR* pCDS and its upstream *pftcR* promoter region (*pftcR-ftcR*) was amplified by PCR from *B. melitensis* 16M genomic DNA and tailed with attB1 and attB2 sequences using the attB2-pbifR and attB1-bifR primers and then cloned by BP reaction into the pDONR201 plasmid, generating pDONR*pftcR-ftcR*. All pDONR constructs were verified by sequencing the insert. The *ftcR* pCDS was transferred by LR reaction (recombination reaction using LR clonase enzyme mix) from pDONR*ftcR* into pMR10-GW under the control of the *plac* promoter, generating the pMR*ftcR* plasmid, and into pET15b-GW under the control of the T7 promoter and in frame with an N-terminal histidine tag (His_6) coding sequence, generating the pET15b*ftcR* plasmid. The *pftcR-ftcR* insert was transferred from pDONR*pftcR-ftcR* into pMR10-GW in an orientation opposite of that for the *plac* promoter, generating pMR*pftcR-ftcR*.

β-Galactosidase assays. β-Galactosidase assays were performed in line with the protocol of Miller (34) as described by Fretin et al. (14).

SDS-PAGE and immunoblotting. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and immunoblotting were performed as previously described (11, 14). Briefly, *B. melitensis* cultures were grown for various times until they reached the appropriate phase of growth (an OD_{600} of 0.1 for the end of latent phase and an OD_{600} of 0.25 for the beginning of exponential phase). Total bacterial cell samples were pelleted and resuspended in SDS sample buffer. The proteins were resolved on a 12% polyacrylamide gel and transferred to Hybond ECL nitrocellulose membranes (Amersham). Immunodetection of proteins was performed using anti-FliC or anti-FlgE polyclonal antibody (14) and an anti-Omp1 monoclonal antibody, A5310B2 (5), as a loading control. The detection of antibodies was performed using donkey anti-rabbit (Amersham) and goat anti-mouse (Dako) horseradish peroxidase-conjugated secondary antibodies as appropriate, and proteins were visualized using the ECL system (Amersham).

Virulence assays. Evaluation of the intracellular survival of the *B. melitensis* 16M wild-type strain and the *ftcR* mutant in cellular models (HeLa cells and bovine macrophages) was performed as previously described by Delrue et al. (12). Briefly, the strains were grown at 37°C in 2YT medium and then inoculated at a multiplicity of infection of 300 to subconfluent monolayers of HeLa cells or bovine macrophages in 96-well tissue culture plates. After a 10-min centrifugation at 1,000 rpm at room temperature in a Jouan centrifuge, the preparations were placed in a 5% CO_2 atmosphere at 37°C for 1 h. The wells were then washed, and 50 μg/ml gentamicin was added to the cells until the end of the infection time (48 h). Cell monolayers were washed and lysed with 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 20 min, and the lysates were serially diluted and plated onto 2YT plates to count viable bacteria.

Virulence assays with the BALB/c mouse model were performed as described by Fretin et al. (14). Briefly, 8-week-old female mice were inoculated intraperitoneally with a PBS suspension containing approximately 10^4 CFU of the appropriate bacterial strain. At different intervals after inoculation (1, 4, 8, and 12 weeks), four mice from each treatment group were sacrificed for spleen collection. Each spleen was homogenized in 2 ml of distilled water, and serial dilutions of it were plated on 2YT agar to determine bacterial survival. Data were expressed as the logarithm (log) of the number of CFU per spleen.

Immunofluorescence assays. Bovine macrophages infected with the *B. melitensis* 16M wild-type strain and the *ftcR* mutant harboring the pBB*pfliF-gfp* plasmid (14) were fixed at various times after infection. Bacteria were detected using primary anti-*Brucella* lipopolysaccharide O-chain monoclonal antibody 12G12 (6) and secondary Alexa 568-conjugated anti-mouse immunoglobulin G antibodies (Molecular Probes) as previously described (14).

Expression and purification of recombinant FtcR. FtcR was overproduced under 1 mM isopropyl-β-D-thiogalactopyranoside induction in *E. coli* BL21(DE3)plysS (Novagen) from the pET15b*ftcR* plasmid as a fusion protein containing an N-terminal six-histidine tag. Protein purification was performed on a 2.5-ml nickel chelation resin column (His-Bind; Novagen) with elution by 6 M urea as recommended by the manufacturer, followed by renaturation of the protein by dialysis using 1× PBS and 0.03 M Tris, pH 7.9. The protein was concentrated to 0.3 mg/ml using a 15-ml Amicon Ultra centrifugal filter unit (Millipore) and finally stored at −20°C in 50% glycerol.

Electrophoretic mobility shift assay (EMSA). Four 40-bp DNA fragments (synthesized and PAGE purified by Sigma-Genosys) corresponding to the upstream region of *fliF* pCDS (probes 1, 2, 3, and 4) (see Fig. 4) were 5′ end radiolabeled using T4 polynucleotide kinase (Biolabs) and [γ-^{32}P]ATP (Amersham). The promoter probes were purified with Sephadex G50 micro columns (ProbeQuant G-50 micro columns; Amersham) and then by QIAEX II gel extraction (QIAGEN) after separation on a 12% polyacrylamide gel. The protein-DNA binding reactions were carried out as follows. Various amounts of recombinant FtcR (from 0 to 200 ng) were first incubated for 10 min at room temperature in the absence of a probe in a 17-μl reaction mixture containing 1 μl dithiothreitol (20 mM), 1 μl bovine serum albumin (10 mg/ml), and 4 μl 5× binding buffer (100 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 0.5 mM EDTA, 50% glycerol). A total of ≈30,000 cpm of probe was then added to the mixture to a final volume of 20 μl with or without a molar excess of an unlabeled competitor probe, and the mixture was incubated for 20 min at room temperature. Samples were then separated on a 6% nondenaturing polyacrylamide gel in 1× TGE buffer (25 mM Tris-acetate, pH 8.3, 190 mM glycine, 1 mM EDTA). To determine the specificity of the binding of recombinant FtcR to the *pfliF* probes, unlabeled probe 1 (data not shown) or 2 or heterologous DNA containing a

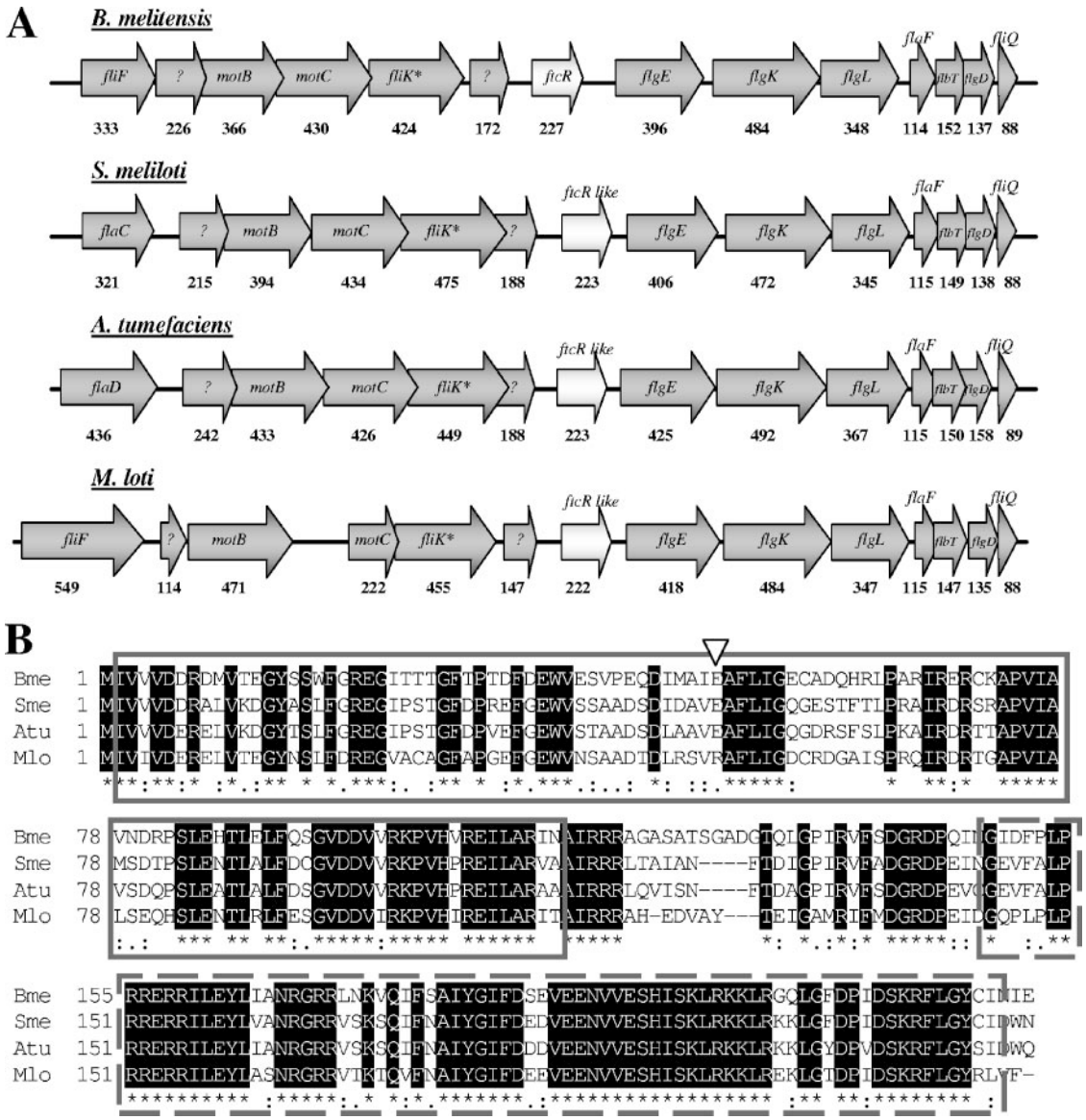

FIG. 1. Analysis of the FtcR sequence. (A) Synteny between *B. melitensis* 16M, *A. tumefaciens*, *M. loti*, and *S. meliloti* 1021 in the genomic region containing the *ftcR* CDS. The *ftcR* CDS is indicated by a white arrow, as is the homologous CDS encoding a predicted response regulator from a two-component system (*ftcR like*). The names of the CDSs are written in the arrows. The length in amino acid residues of each CDS is indicated under the arrows. The CDS regions conserved without predicted function (hypothetical conserved) are labeled with a question mark. This genomic region is conserved in *Brucella suis* and *Brucella abortus*. *fliK**, *fliK* was previously called *motD*. (B) CLUSTALW multiple alignment of the deduced amino acid sequences of the pCDSs located upstream of the *flgE* gene in *B. melitensis* (Bme; AAL53399.1), *S. meliloti* (Sme; AAG48153.1), *A. tumefaciens* (Atu; NP_353599.1), and *M. loti* (Mlo; NP_104158.1) (GenBank accession numbers are in parentheses). Residues marked with an asterisk and shaded black correspond to identical residues. Residues marked with points belong to a conserved group of amino acids defined as weak (one point) or strong (two points) by CLUSTALW 1.83. The frames indicate the response regulator domain (continuous line) and the transcriptional regulatory domain (discontinuous line) as recognized in Pfam. The white inverted triangle indicates the classical position of the conserved aspartate in the phosphorylation site replaced by another residue in *B. melitensis*, *S. meliloti*, *A. tumefaciens* (E), and *M. loti* (R).

mammalian Oct binding site (CV1048, 5′-TGTCGAATGCAAATCACTAGA A-3′) was included in the competition assay.

Statistical analysis. Data were analyzed by one-way analysis of variance after testing of the homogeneity of variance. Post hoc comparisons were performed by a pair-wise Scheffé test.

RESULTS

The *ftcR* gene encodes a predicted two-component response regulator conserved in *Rhizobiaceae*. BMEII0158 on the *B. melitensis* 16M small chromosome corresponds to a pCDS of unknown function. This sequence is located downstream from a predicted operon encoding the MS ring monomer (FliF), three motor proteins (MotB, MotC, and MotD), and two hypothetical proteins and upstream of the *flgE* pCDS encoding the hook monomer (Fig. 1A) (14). The deduced 227-residue polypeptide sequence (25.7 kDa) of FtcR is well conserved in other members of the α-subgroup of proteobacteria (Fig. 1B). In *Sinorhizobium meliloti*, *Agrobacterium tumefaciens*, and *Me-*

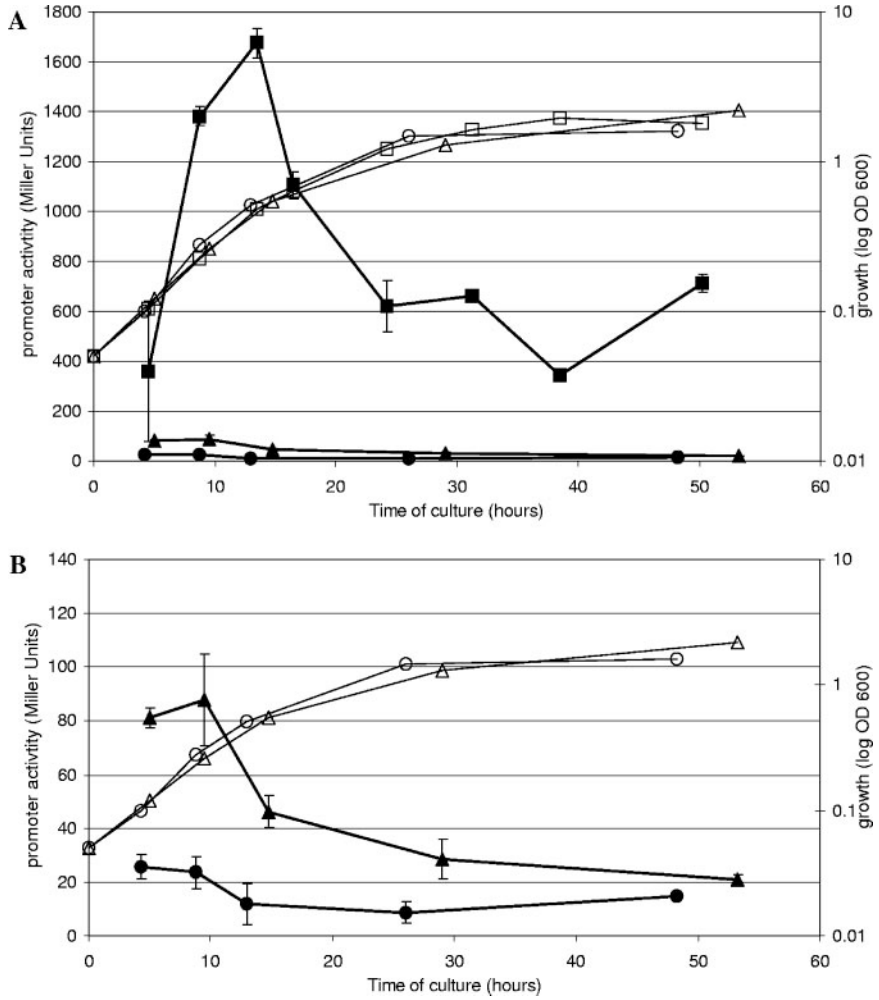

FIG. 2. Activity of the *fliF* promoter region in different *Brucella* strains. (A) Activity of the *fliF* promoter region in the wild-type strain (black squares), the *ftcR* mutant strain (black circles), and the *vjbR* mutant strain (black triangles). A *pfliF-lacZ* fusion plasmid was transmitted by conjugal transfer into the three strains, each was cultivated in 2YT nutrient broth, and samples were recovered at intervals during bacterial growth to test the *pfliF* activity using β-galactosidase assays. The β-galactosidase activity is expressed as the mean ± standard deviation from three replicates. White squares, circles, and triangles correspond to the optical density at 600 nm for the wild-type strain, the *ftcR* mutant, and the *vjbR* mutant, respectively. (B) Same results as those in panel A shown with a larger scale for the abscissa axis to facilitate the distinction between results for the *ftcR* and *vjbR* mutants. Only the activity of the *fliF* promoter in the *ftcR* mutant strain and the *vjbR* mutant strain are visible.

sorhizobium loti, the corresponding pCDSs exhibit 64%, 62%, and 58% identity with FtcR, respectively, at the amino acid level. The *ftcR* genomic context is also conserved in several α-proteobacteria (Fig. 1A). The deduced sequence contains two Pfam domains (PF00486, Trans_reg C; and PF00072, Response reg) which are characteristic of regulators belonging to two-component response regulators (55). This prompted us to rename BMEII0158 "*ftcR*" for "*f*lagellar *t*wo-*c*omponent *r*egulator." However, FtcR is atypical because it lacks the aspartate residue usually conserved as a phosphorylation site in the receiver domain (Fig. 1B). Actually, in *Brucella* spp. and in FtcR homologs in *S. meliloti* and *A. tumefaciens*, the aspartate residue is replaced at position 50 by a glutamate residue, while in the FtcR homolog of *M. loti*, it is replaced by an arginine residue. In addition, an inventory of all genes predicted to encode histidine kinases and response regulators in the *B. melitensis* genome did not reveal the presence of a cognate sensor partner (putative histidine protein kinase) for FtcR (29).

Inactivation of the *ftcR* gene downregulates the activity of the flagellar *fliF* promoter region and the production of both the FlgE and FliC flagellar proteins during vegetative growth. In order to test whether FtcR plays a role in the regulation of flagellar gene expression, we generated an *ftcR* mutant by integrative disruption as described in Materials and Methods. We knew from a previous study that the noncoding region just upstream of *fliF* (*pfliF*), a gene encoding the flagellar MS ring monomer, is transiently active during vegetative growth of the wild-type (WT) *B. melitensis* 16M strain in 2YT medium. In fact, the *pfliF* activity is maximal at the very beginning of the exponential growth phase, and concomitantly, there is transient production of the FlgE and FliC proteins, which correspond to monomers of the flagellar hook and filament, respectively (14). To investigate the contribution of FtcR to flagellar

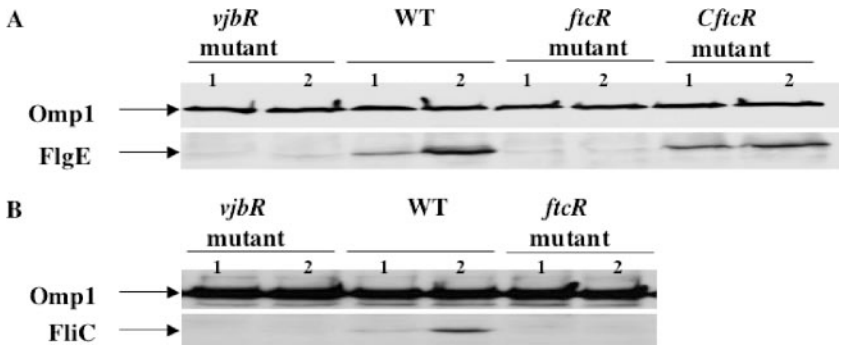

FIG. 3. Western blot of FlgE and FliC expression in the *ftcR* mutant strain, the *vjbR* mutant strain, and the wild-type strain. The strains were cultivated in 2YT nutrient broth, and extracts were prepared from samples harvested at the end of the latent phase of growth (OD_{600} of 0.1) (lanes 1) and at the beginning of the exponential phase of growth (OD_{600} of 0.25) (lanes 2). The extracts were separated by electrophoresis, transferred to nylon membranes, and probed with FlgE-specific (A) or FliC-specific (B) antiserum. A monoclonal anti-Omp1 antibody was used as a loading control. *CftcR*, mutant *ftcR* containing a complementation pMR*pftcR-ftcR* plasmid.

expression during vegetative growth in rich medium, we used a *pfliF-lacZ* translational fusion (plasmid pBBCm*pfliF-lacZ*) to measure *pfliF* activity in the *ftcR* mutant compared to that in the WT strain. A 20- to 160-fold reduction of *pfliF* activity was observed, depending on the growth stage of *pfliF* in the mutant strain (Fig. 2A). The same experiment with a Δ*vjbR* mutant strain showed a 5- to 40-fold reduction of *pfliF* activity as previously described (Fig. 2A) (11). In the same way, FlgE and FliC protein levels were analyzed in WT and *ftcR* mutant strains by Western blot analysis. We used a Δ*vjbR* mutant strain harvested at the same time as a negative control of flagellar protein expression (11). The Omp1 protein detected by a monoclonal antibody was used as a loading control (Fig. 3).

A clear increase in the production of the hook and the flagellin proteins can be seen in the WT extracts between the end of the latent phase of growth and the beginning of the exponential phase. However, almost no FlgE or FliC production was detected in either the *ftcR* mutant or the Δ*vjbR* mutant strain (Fig. 3). The expression in *trans* of the *ftcR* pCDS (using the plasmid pMR*pftcR-ftcR*) in the *ftcR* mutant restores the production of FlgE, indicating that the mutation has no polar effect on downstream genes (Fig. 3A). Taken together, these data show that FtcR is required for the production of at least three flagellar components that are found in distinct locations in the flagellar structure (base, hook, and filament). These results strongly point to FtcR as an important regulatory protein for flagellar gene expression, at least during vegetative growth.

FtcR also regulates the *fliF* promoter region intracellularly. Using a *pfliF-gfp* fusion, it was recently demonstrated that *pfliF* is active during macrophage infection (14). We transferred this *pfliF-gfp* reporter system (pBB*pfliF-gfp*) by conjugation to the *ftcR* mutant and used this strain to infect a monolayer of bovine macrophages. Postinfection observations at different times contrasted with those for the parent strain, with the *ftcR* mutant failing to exhibit *pfliF* activity (data not shown). This result confirms that FtcR is also a transcriptional activator of *pfliF* ex vivo.

The *ftcR* mutant has the same phenotype as structural flagellar mutants in both cellular and murine models of infection. The flagellum of *B. melitensis* is involved in persistence in a murine model of infection, whereas there was no demonstrable need of this appendage during the course of cellular infection (14). Because FtcR positively regulates the production of at least three flagellar components related to distinct locations at the flagellar structural level (see above), it is probable that the absence of functional FtcR leads to the absence of a flagellar structure. Accordingly, an *ftcR* mutant should be attenuated as much as the strains mutated in genes encoding flagellar structural components. To test this hypothesis, cellular and murine infections were carried out with the *ftcR* mutant (see Materials and Methods). In HeLa cells and bovine macrophages, we observed no attenuation of the *ftcR* mutant compared to that in the WT parental strain (data not shown). In BALB/c mice, the *ftcR* mutant was not attenuated after 1 week of infection but was attenuated after 4 weeks of infection (Table 3). These results are in agreement with data obtained with *Brucella* strains mutated in genes encoding various structural flagellar components (14).

All of the above data strengthen the evidence that FtcR plays a major role in flagellar gene regulation.

FtcR is a direct activator of the *fliF* gene. Like *Brucella*, *Caulobacter crescentus* belongs to the α-subgroup of proteobacteria, but no predicted ortholog of the *ftcR* gene is found in its genome. This fact allowed us to exploit the natural cellular context of *C. crescentus* to study the potential direct transcrip-

TABLE 3. Virulence of *Brucella melitensis* wild-type and flagellar mutant strains in BALB/c mice

Brucella strain	Log CFU/spleen at[b]:			
	1 wk	4 wk	8 wk	12 wk
Wild type	5.61 ± 1.11	5.41 ± 0.61	4.20 ± 0.36	2.55 ± 0.86
fliF mutant[a]	6.22 ± 0.60	3.72 ± 0.39	2.84 ± 0.36	0.43 ± 0.65[d]
ftcR mutant	5.63 ± 0.79	3.01 ± 0.19	1.98 ± 1.10	0.83[c]

[a] Mutant in the gene encoding the basal body and described in reference 14.
[b] For each *Brucella* strain, four mice were intraperitoneally inoculated with about 10^4 CFU. Spleens were collected, homogenized, and plated on 2YT agar after 1, 4, 8, or 12 weeks to determine bacterial survival. Data are means ± standard deviations ($n = 4$).
[c] Three out of four mice cleared the bacteria.
[d] Two out of four mice cleared the bacteria.

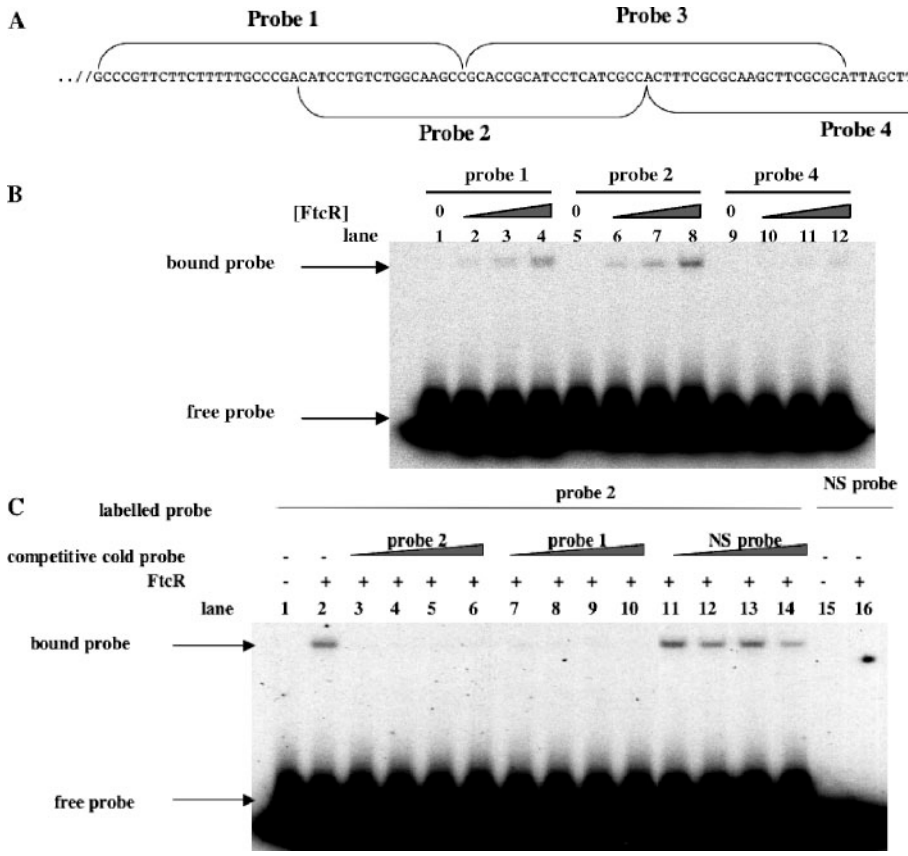

FIG. 4. Analysis of FtcR binding on the *fliF* promoter. (A) Representation of the ~100-nucleotide genomic region containing the *fliF* promoter. The ATG of the *fliF* pCDS is in bold. The four labeled *pfliF* fragments used for the EMSA experiments are indicated (probes 1, 2, 3, and 4). //, predicted transcriptional terminator for the preceding *fliC* gene. (B) Binding of FtcR to *pfliF*. Four different end-labeled probes corresponding to fragments of the *pfliF* genomic region (see panel A) were incubated with increasing quantities of recombinant FtcR for 20 min at room temperature and then subjected to electrophoresis in a nondenaturating polyacrylamide gel. The FtcR quantities used were 0 ng (lanes 1, 5, and 9), 50 ng (lanes 2, 6, and 10), 100 ng (lanes 3, 7, and 11), and 200 ng (lanes 4, 8, and 12) (data for probe 3 not shown). (C) Competitive binding of FtcR to probe 2. End-labeled probe 2 was mixed with unlabeled probe (competitive cold probe) 1 or 2, or a nonspecific (NS) probe and incubated with 200 ng of recombinant FtcR. Samples were then subjected to an electrophoretic mobility shift assay. A competitive cold probe was used in excess of 2.5 ng (lanes 3, 7, and 11), 5 ng (lanes 4, 8, and 12), 15 ng (lanes 5, 9, and 13), and 45 ng (lanes 6, 10, and 14). As controls, labeled probe 2 was tested alone with FtcR (lane 2) or without FtcR (lane 1) and labeled aspecific probe alone was tested with FtcR (lane 16) or without FtcR (lane 15).

tional effect of FtcR on *pfliF*, with both partners being imported from *Brucella*.

The strain NA1000 of *C. crescentus* (Table 1) was first conjugated with a plasmid bearing a *pfliF-lacZ* fusion (pBBNrs*pfliF-lacZ*) and then with a second plasmid which either had no insert as a negative control (pMR10CmKm) or harbored *ftcR* under the control of a constitutive promoter (pMR*plac-ftcR*). β-Galactosidase assays were performed, and the averages from three independent experiments ± standard errors were calculated to determine *pfliF* activity in the two strains. In the absence of *ftcR* in *trans*, 67.2 ± 3.0 Miller units was obtained. In contrast, 620.9 ± 12.8 Miller units was calculated in the presence of *ftcR*. These data correspond to a ninefold induction of *pfliF*, in comparison to what was seen with the negative control, which suggests that FtcR might interact directly with the *fliF* promoter.

This hypothetical interaction was tested using the EMSA technique. In the first instance, increasing amounts of FtcR recombinant protein were mixed with four labeled *pfliF* fragments that were 40 bp in length (designated probes 1, 2, 3, and 4). The probes overlap each other by half of their length and altogether cover a genomic region of 100 nucleotides localized between a predicted transcriptional Rho-independent terminator for the preceding *fliC* gene and the beginning of the *fliF* pCDS (Fig. 4A). Following native gel electrophoresis, a shifted DNA band of increasing intensity appeared when probe 1 or 2 was incubated with increasing amounts of the FtcR protein (Fig. 4B). No clear binding was observed using probe 3 (data not shown) or 4 (Fig. 4B). The specificity of the binding assay was evaluated in a second experiment. A nonspecific DNA probe caused no mobility shift and did not, when unlabeled, compete with labeled probe 1 or 2 for FtcR binding. On the contrary, both cold probes 1 and 2 were able to compete with labeled probe 2 (Fig. 4C) and probe 1 (data not shown) for FtcR binding. This indicates that FtcR binds directly and specifically to the *pfliF* promoter region.

FtcR mediates the activator effect of VjbR on flagellar genes. VjbR was demonstrated to be the *Brucella* functional counterpart of the *S. meliloti* flagellar master regulator VisR (51), and it is also encoded at the same genetic locus. Notably, the

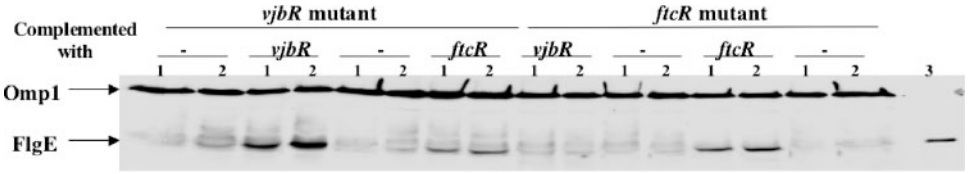

FIG. 5. Expression of FlgE in the *vjbR* and *ftcR* mutants containing expression plasmid *vjbR* pBBR-*vjbR* (*vjbR*), expression plasmid FtcR pMR*pftcR-ftcR* (*ftcR*), or the corresponding empty plasmids pBBR1MCS-4 and pMR10CmKm(−). All strains were cultivated in 2YT broth, and whole-cell extracts were recovered for each strain at the end of the latent phase of growth (OD_{600} of 0.1) (lanes 1) and at the beginning of the exponential phase of growth (OD_{600} of 0.25) (lanes 2). Recombinant FlgE was used as a positive control (lane 3). The extracts were separated by electrophoresis, transferred to nylon membranes, and probed with FlgE-specific antiserum. A monoclonal anti-Omp1 antibody was used as a loading control.

expression levels of both *fliF* and *flgE* were strongly reduced in a *vjbR* mutant (11). A similar result for FliC production was also observed in this study (Fig. 3B). Because this phenotype was globally shared with the *ftcR* mutant described here, we wanted to estimate the relative contribution of FtcR and VjbR with respect to flagellar expression.

As demonstrated, in this study *pfliF* activity was almost abolished in both the *vjbR* and the *ftcR* mutants compared to that in the WT strain but was two- to fourfold higher in the *vjbR* mutant than in the *ftcR* mutant (Fig. 2B). Thus, the absence of FtcR seems to be more deleterious than the absence of VjbR regarding the activation of *pfliF*.

To gain further insight into the respective roles of these two regulators in flagellar expression, we tested the ability of VjbR or FtcR to cross-complement an *ftcR* or a *vjbR* mutant, respectively. To achieve this, an expression plasmid of FtcR (pMR*pftcR-ftcR*) or VjbR (pBBR-*vjbR*) (11) or plasmids without inserts as negative controls (pMR10CmKm and pBBR1MCS-4) were conjugated in the *vjbR* mutant and in the *ftcR* mutant. FlgE protein levels were analyzed in the resulting strains by Western blot analysis. Both the *ftcR* mutant and the *vjbR* mutant produced little or no detectable FlgE with the plasmids used as negative controls, but both were complemented by the expression in *trans* of the corresponding WT gene (positive control). Importantly, while the expression of *vjbR* in *trans* failed to restore FlgE production in the *ftcR* mutant, the expression of *ftcR* in *trans* was able to complement the *vjbR* mutant (Fig. 5). This strongly indicates that the absence of flagellar gene expression in the *vjbR* mutant is actually caused by a reduction in the amount of active FtcR.

In this context, VjbR could be an activator of *ftcR* gene expression. To test this hypothesis, we used a reporter plasmid (pBBCm*pftcR-lacZ*) to compare *pftcR* activity between the *vjbR* mutant and the WT strain of *B. melitensis* 16M along a growth curve. In the WT strain, *pftcR* activity reached a maximum value at the beginning of the exponential phase and then decreased slowly. The same profile was observed in the *vjbR* mutant, but *pftcR* activity was reduced by about half compared to that in the WT strain (Fig. 6). This observation confirms that the expression of *ftcR* is affected (twofold) by the loss of VjbR.

DISCUSSION

The present work focused on *B. melitensis*, describing for the first time a transcriptional regulator called FtcR that directly

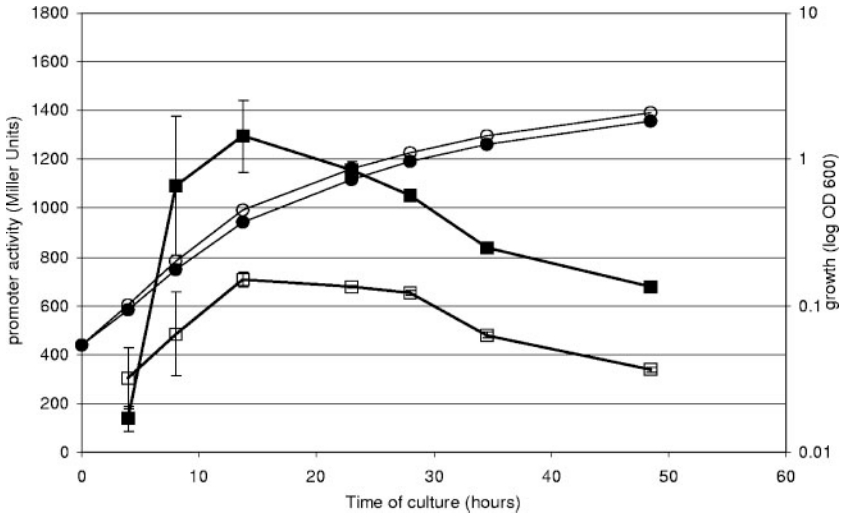

FIG. 6. Activity of the *ftcR* promoter in the *vjbR* mutant (white) and in wild-type *B. melitensis* 16M (black). A *pftcR-lacZ* fusion plasmid was conjugated into the *vjbR* mutant and into the wild-type strain. Both strains were cultivated in 2YT nutrient broth, and samples were harvested along the growth curve to test *pftcR* activity by β-galactosidase assays. Circles, optical density at 600 nm; squares, β-galactosidase activity expressed as the mean ± standard deviation from three replicates.

activates flagellar expression. FtcR is conserved in several symbiotic and pathogenic α-proteobacterial *Rhizobiales*. A *B. melitensis ftcR* mutant is unable to produce the FlgE and FliC flagellar proteins and has almost no *pfliF* activity during vegetative growth or during macrophage infection compared to the parental WT strain. Moreover, observations of this mutant by electron microscopy did not reveal any flagellar structures under growth conditions in which the WT strain was flagellated (14; data not shown). In addition, the *ftcR* mutant is not attenuated in cellular infection models but exhibited a marked virulence defect after 4 weeks or more of infection in BALB/c mice. These virulence phenotypes were reminiscent of the phenotypes observed for strains mutated in genes encoding various flagellar components (14). Taken together, these results suggest the total absence of flagella in the *ftcR* mutant and point to FtcR as a key regulator in switching on the expression of flagella in *Brucella*.

This study also shows that FtcR binds directly to the region just upstream of the *fliF* pCDS. This region is localized between the transcriptional Rho-independent terminator for the preceding *fliC* pCDS and the beginning of the *fliF* pCDS and so is predicted as a noncoding region. In addition, cloning this sequence upstream of a reporter gene such as *gfp* or *lacZ* provides an expression profile which is modified in response to *ftcR* or *vjbR* mutation in the same way as other flagellar components. All of this evidence suggests that the *fliF* promoter (*pfliF*) is located in this genomic region between the *fliC* and the *fliF* pCDS. In the EMSA, a clear positive and specific signal was observed for probes 1 and 2 (Fig. 4). No signal was observed with probes 3 and 4, suggesting that the FtcR binding site is located in the 20-nucleotide-sized region common to probes 1 and 2. Additional experiments should be carried out to be able to identify and characterize the FtcR binding sequence relative to the transcriptional start of the *fliF* gene.

It has been noted that in all known flagellar hierarchies, the *fliF* gene is considered to be a class II gene, encoding the monomer for the MS ring formation, which is one of the first steps of a classical flagellar assembly. A hierarchical expression of flagellar genes has not yet been demonstrated in *Brucella*, although this is the most probable situation as demonstrated for all other bacterial flagellar systems (except rare spirochetes [3]) investigated to date. The binding of FtcR to *pfliF*, along with several lines of evidence for the control of flagellar gene expression by this regulator, suggests that FtcR could be considered a flagellar class I master regulator (32, 52).

Analysis of the FtcR-predicted amino acid sequence revealed two domains in the protein: a response regulator receiver domain at the N terminus and a C-terminal transcriptional regulatory domain that classifies FtcR as a response regulator belonging to the two-component system (55). The closest homologs of FtcR (more than 30% identity) found in the NCBI nonredundant database are exclusively from *Rhizobiaceae* and are most often encoded by genes located in flagellar loci as in *Brucella*. A unique feature of the FtcR regulator is the absence of a classical phosphorylation site in the response regulator domain. Interestingly, upon multiple alignment (CLUSTALW) of FtcR and its closest homologs with the well-known response regulators CheY and NtrC, the aspartate (D57 in CheY and D54 in NtrC), which has been shown to be the phosphorylated residue of the active site, aligned with the glutamate E50 residue of FtcR (45, 56). The replacement of the classical aspartate residue by a glutamate residue is also observed in *A. tumefaciens* and *S. meliloti*. It has to be noted that the molecular structure of glutamate is very similar to that of aspartate, and like aspartate, glutamate is negatively charged. The E50 glutamate residue in FtcR and its homologs could thus mimic constitutive phosphorylation. Indeed, it has been shown for other transcriptional regulators, such as NtrC (25, 37), CtrA (47), OmpR (28), and RcsB (18), that the experimental replacement of aspartate by a glutamate residue in the phosphorylation active site allows these regulators to bypass, at least partially, the requirement for phosphorylation.

Apart from FtcR and its closest homologs, some other rare bacterial regulators have been discovered which lack the conserved aspartate residue in the phosphorylation site. Examples include FleQ of *Pseudomonas aeruginosa* (9) and *Legionella pneumophila* (21), AdnA of *Pseudomonas fluorescens* (2), FlrA of *Vibrio cholerae* and *Vibrio fischeri* (24, 35), and FlaK and LafK of *Vibrio parahaemolyticus* (23, 54). All of these belong to the σ^{54}-dependent response regulator family, and all are at the top of a flagellar hierarchy without known sensor histidine kinase partners. The absence of a sensor histidine kinase encoded in *Brucella* flagellar loci argues in favor of the hypothesis that FtcR does not need phosphorylation to be active. In fact, genes of two-component systems are often coupled in genomes, as demonstrated in a study by Koretke et al. (26). The hypothesis that FtcR is nonphosphorylated and constitutively active raises the question of how the activity of FtcR is modulated. In *P. aeruginosa*, nonphosphorylated FleQ is regulated at the transcriptional level by the Vfr repressor and its activity is repressed posttranslationally upon binding with the FleN antiactivator (8, 9). The first regulator of FtcR activity in *Brucella*, VjbR, was identified in the present study.

The LuxR-type transcriptional regulator VjbR, which also activates the type IV secretion system, caught our attention principally because it was shown to positively control the activity of the *pfliF* promoter (11), FlgE production (11), and FliC production (this study) in *B. melitensis*. This phenotype, also observed for the *ftcR* mutant, suggests the involvement of FtcR and VjbR in the same flagellar regulatory pathway, making it probable that one of them modulates the expression or the activity of the other to control flagellar expression. In this study, we observed a more attenuated *pfliF* activity in the *ftcR* mutant than in the *vjbR* mutant. In addition, FlgE production was shown to be restored in the *vjbR* mutant when *ftcR* was expressed in *trans* under the control of its own promoter, while the expression in *trans* of *vjbR* did not restore FlgE production in the *ftcR* mutant. These results suggest a more direct regulation of flagellar genes by FtcR. This led us to investigate the influence of VjbR on *pftcR* activity in *Brucella*. While the expression of flagellar genes is almost extinguished in a *vjbR* mutant, the expression of *ftcR* is only twofold reduced. This mild activation of *pftcR* by VjbR could be direct or indirect. In addition, it should also be noted that our data do not exclude a mild activating effect from VjbR directly on *pfliF*, leaving the possibility of a three-gene feed-forward loop motif, including FtcR. This motif has frequently been observed in regulation networks and was defined by Mangan and Alon as a three-gene pattern composed of two input transcription factors, one of which regulates the other and both of which jointly regulate a

target gene (33). That way, a regulation model in which *vjbR* activates *ftcR*, which in turn activates *fliF* and other flagellar genes, could be proposed, without excluding the possibility of a more direct VjbR effect on flagellar genes. More experiments will have to be carried to clarify the situation.

Finally, a comparison of *pfliF* and *pftcR* activities, both under the influence of VjbR, reveals a more abrupt decrease of *pfliF* activity along the growth curve (compare Fig. 2A and 6). This observation, in addition to the partial maintenance of *pftcR* activity in a *vjbR* mutant, suggests the existence of yet-to-be-identified regulators acting beside VjbR. Extra efforts will have to be made to gain further insight into the regulation of the flagellar expression in *Brucella*. Flagellar expression is a complex process that must be precisely tied to the intricacies of the bacterial life cycle. The discovery of the key flagellar regulator, FtcR, is a first step in leading to a better understanding of *Brucella* and, by extension, other α-proteobacteria.

ACKNOWLEDGMENTS

We thank C. Didembourg for technical assistance. We are grateful to the *Brucella* team of the URBM for helpful discussions.

This work was supported by the Commission of the European Communities, contract no. QLK2-CT-1999-00014. V. Haine, S. Léonard, and J. Ferooz hold a specialization grant from the Fonds pour la Formation à la Recherche dans l'Industrie et dans l'Agriculture (FRIA).

ADDENDUM IN PROOF

Rotter et al. recently published the description of the *ftcR* homologue in *Sinorhizobium meliloti* (C. Rotter, S. Mühlbacher, D. Salamon, R. Schmitt, and B. Scharf, J. Bacteriol. **188:**6932–6942, 2006).

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