RpoE1, an extracytoplasmic function sigma factor, is a repressor of the flagellar system in Brucella melitensis

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The genome of Brucella melitensis contains genes coding for the sigma factors RpoD, RpoN, RpoH1, RpoH2, RpoE1 and RpoE2. Previously published data show that B. melitensis is flagellated and that an rpoE1 mutant overexpresses the flagellar protein FlgE. In this study, we demonstrate that mutation of rpoE1 causes an overexpression of the flagellar genes flfF, flgE, flfC, flaF and flbT, correlating with the production of a longer filament and thereby demonstrating that RpoE1 acts as a flagellar repressor. Moreover, mutation of rpoE1 increases the promoter activity of the flagellar master regulator ftcR, suggesting that RpoE1 acts upstream of ftcR. Together, these data show that RpoE1 represses the flagellar synthesis and filament length in B. melitensis.

INTRODUCTION

Brucellae are Gram-negative, intracellular pathogenic bacteria that cause brucellosis in a variety of mammals, including humans. For a long time, they were considered as being unflagellated. However, a sheathed flagellum has recently been discovered in Brucella melitensis and flagellar mutants are found to be impaired for infection in vivo (Ferooz & Letesson, 2010; Fretin et al., 2005; Zygmunt et al., 2006).

The bacterial flagellum is a complex organelle used for motility and consists of at least three structural elements, the basal body, the hook and the filament (Macnab, 1999). The basal body, working as a motor, is embedded within the cell envelope and is anchored in the cytoplasmic membrane via the MS-ring structure, while the hook and filament, which function as a universal joint and a propeller, respectively, extend outwards from the cells (Minamino et al., 2008).

Sigma and anti-sigma factors play important roles in the regulation of the flagellar genes (Smith & Hoover, 2009). The genome of B. melitensis has revealed the presence of genes encoding six sigma factors (rpoD, rpoH1, rpoH2, rpoE1, rpoE2 and rpoN) (Delory et al., 2006). The rpoD gene encodes the housekeeping sigma factor, the rpoH1 and rpoH2 genes encode two \( \sigma^{32} \) homologues, rpoE1 and rpoE2 encode two extracytoplasmic function (ECF) sigma factors and the rpoN gene encodes a \( \sigma^{34} \) homologue. The phenotypic characterizations were done on the five non-essential sigma factor mutants \( \Delta rpoH1, \Delta rpoH2, \Delta rpoE1, \Delta rpoE2 \) and \( \Delta rpoN \) (Delory et al., 2006). Interestingly, at early growth phase in rich liquid medium, \( \Delta rpoE1 \) over-produces the flagellar hook protein FlgE, suggesting that the flagellar system synthesis could be downregulated by the ECF sigma factor RpoE1 in B. melitensis (Delory et al., 2006).

In this study, we demonstrate that the ECF sigma factor RpoE1 is a flagellar repressor in B. melitensis and that RpoE1 controls the length of the flagellar filament.

METHODS

Bacterial strains and culture conditions. All strains and plasmids used in this study are listed in Table 1. Brucella strains used in this study were derived from B. melitensis 16M NaI (spontaneous nalidixic acid resistant mutant selected from B. melitensis 16M, received from A. Macmillan, Central Veterinary Laboratory, Weybridge, UK). Bacterial growth was measured by reading OD_{600}. Growth curves of B. melitensis 16M and isogenic mutants were performed on a late-exponential overnight cultures obtained in rich liquid 2YT medium (1 % yeast extract, 1.6 % peptone, 0.5 % NaCl, B. melitensis 16M and mutant strains were grown with agitation at 37 °C in 2YT medium containing appropriate antibiotics from an initial culture, with OD_{600} 0.05. Antibiotics were used at the following final concentrations: 100 \( \mu g \) ampicillin ml \(^{-1} \); 20 \( \mu g \) chloramphenicol ml \(^{-1} \); 50 \( \mu g \) kanamycin ml \(^{-1} \); 25 \( \mu g \) nalidixic acid ml \(^{-1} \).

Construction of the pfgE–lacZ reporter. Recombinant DNA techniques were carried out using standard protocols (Ausubel et al., 1987).
1991). To construct plasmid pJF012, the lacZ coding region with its first 26 nt deleted was cloned into pBBR1MCS as described previously (Fretin et al., 2005). A 589 bp region containing the flgE promoter (including the first 123 bp of the flgE ORF) was amplified from B. melitensis 16M genomic DNA by using the primers in Table 2 (including the first 123 bp of the 16M Nalr genomic DNA by using the primers in Table 2 melitensis were taken at various time points during culture at 37 μ
(Fretin et al., 2011). To construct plasmid pJF012, the
lacZ inserted in-frame upstream of the promoterless was first subcloned into pGEM-T Easy Vector (Promega) and then
into pBBR1MCS to generate the plasmid pJF012.

**Oligonucleotide sequences used in this study**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5′→3′)</th>
</tr>
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<tbody>
<tr>
<td>qRT-PCR</td>
<td></td>
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<tr>
<td>fliE (BMEII0151)</td>
<td>ATGAGC/CGTTGCAACAATATGGG</td>
</tr>
<tr>
<td>flgE (BMEII0159)</td>
<td>TTTCC/CGTAA/ACGGCACTG</td>
</tr>
<tr>
<td>fliC (BMEII0150)</td>
<td>GTTCGATCGC/CTAG/GAAATCAACT</td>
</tr>
<tr>
<td>flbT (BMEII0163)</td>
<td>AACCT/CCTGAAAG/CGCATG</td>
</tr>
<tr>
<td>flaF (BMEII0162)</td>
<td>AGT/GCGCTACAG/AAATG</td>
</tr>
<tr>
<td>lacZ reporter*</td>
<td></td>
</tr>
<tr>
<td>flgE promoter</td>
<td>ATATC/CTAGGGGT/CTGACCT/CTGATT</td>
</tr>
</tbody>
</table>

*The underlined sequences in the forward and reverse primers are the XbaI and BamHI restriction sites, respectively.

**Table 2. Oligonucleotide sequences used in this study**

**Table 1. Bacterial strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Source</th>
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</thead>
<tbody>
<tr>
<td><em>B. melitensis</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16M Nal'</td>
<td>Spontaneous nalidixic acid-resistant strain of <em>B. melitensis</em> 16M</td>
<td>Laboratory collection; A. P. MacMillan, Central Veterinary Laboratory, Weybridge, UK</td>
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<td>fliC mutant</td>
<td><em>B. melitensis</em> 16M ΔfliC::Kan'</td>
<td>Ferooz et al. (2011)</td>
</tr>
<tr>
<td>rpoE1 mutant</td>
<td><em>B. melitensis</em> 16M ΔrpoE1::Kan'</td>
<td>Delory et al. (2006)</td>
</tr>
<tr>
<td>rpoE2 mutant</td>
<td><em>B. melitensis</em> 16M ΔrpoE2::Kan'</td>
<td>Delory et al. (2006)</td>
</tr>
<tr>
<td>rpoH1 mutant</td>
<td><em>B. melitensis</em> 16M ΔrpoH1::Kan'</td>
<td>Delory et al. (2006)</td>
</tr>
<tr>
<td>rpoH2 mutant</td>
<td><em>B. melitensis</em> 16M ΔrpoH2::Kan'</td>
<td>Delory et al. (2006)</td>
</tr>
<tr>
<td>rpoN mutant</td>
<td><em>B. melitensis</em> 16M ΔrpoN::Kan'</td>
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<td>DH10B</td>
<td>F− mcrA Δ(mrr hsdRMS mcrBC) φ80lacZΔM15 ΔlacX74 reca1 endA1 araΔ139 Δ(ara leu)7697 galU galK Δ2c rpsLΔ3 lacI1597 rpsLΔ3 lacI1597 recA RP4-2 (Tc::Mu-Km::Tn7)</td>
<td>Gibco-BRL</td>
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<td>S17-1</td>
<td>thi pro hsdR hsdM+ recA RP4-2</td>
<td>Simon et al. (1983)</td>
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<td><strong>Plasmids</strong></td>
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<td>Promoterless lacZ vector, Cm'</td>
<td>Fretin et al. (2005)</td>
</tr>
<tr>
<td>pGEM-T Easy</td>
<td>Cloning vector</td>
<td>Promega</td>
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<td>flgE promoter in pGEM-T Easy vector</td>
<td>This study</td>
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<td>pBBR lacZ vector, promoter ftrC-lacZ transpositional reporter, Cm'</td>
<td>Léonard et al. (2007)</td>
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<td>pfJ011</td>
<td>pBBCm-lacZ vector, promoter fliC-lacZ transpositional reporter, Cm'</td>
<td>Ferooz et al. (2011)</td>
</tr>
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<td>pfJ012</td>
<td>pBBCm-lacZ vector, promoter flgE-lacZ transpositional reporter, Cm'</td>
<td>This study</td>
</tr>
</tbody>
</table>

**Detection of FlgE and FliC proteins by Western blot analysis.**

SDS-PAGE and immunoblotting were performed as described previously (Léonard et al., 2007). Briefly, *B. melitensis* culture samples were taken at various time points during culture at 37 °C in 2YT broth until they reached the appropriate growth phase. Samples of bacterial culture were inactivated for 1 h at 80 °C and standardized according to OD 600. Total bacterial 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). The immunodetection of proteins was performed using anti-FliC or anti-FlgE rabbit polyclonal antibodies at dilutions of 1:3000 and 1:5000, respectively (Fretin et al., 2005). The detection of primary antibodies was performed using donkey anti-rabbit (Amersham) horseradish-peroxidase-conjugated secondary antibodies as appropriate, and visualized using the ECL system (Amersham). The measured molecular masses of FlgE and FliC are 41 and 29 kDa, respectively. Films were scanned using a Canon CanoScan 3200F and images were analysed using the GIMP 2.6.1.
software. The FlgE and FliC polyclonal antisera cross-reacted with a protein which was used as internal control for equal protein loading.

**RNA preparation.** Total RNA was extracted from *B. melitensis* 16M and the isogenic ΔrpoE1 mutant (all cultured in triplicate) as follows: 45 ml culture was centrifuged at 3500 r.p.m. (Thermo Electron rotor AB50.10A; 1500 g) for 15 min. Bacterial pellets were resuspended in 100 µl 10 % SDS and 20 µl proteinase K (20 mg ml⁻¹) and incubated at 37 °C with shaking for 1 h. Five millilitres of TRIzol reagent (Invitrogen) was added and suspensions were vigorously shaken. After 10 min incubation at 65 °C, 1 ml chloroform was added to the suspensions and the mixtures were shaken and incubated at room temperature for 5–10 min. Samples were centrifuged at 14 000 r.p.m. for 15 s at room temperature. The RNA spin cartridge was washed with buffers I and II supplied with the kit. RNA was eluted from the RNA spin cartridge with 100 µl RNase-free water and stored at −80 °C. The integrity of the RNA and the absence of DNA were checked by gel electrophoresis. RNA quantity was measured using a NanoDrop spectrophotometer (ND-1000, Thermo Fisher Scientific).

**Quantitative real-time RT-PCR.** RNA samples were treated for DNA digestion with DNase I (Fermentas) and reverse-transcription performed with transcriptor first strand complementary DNA (cDNA) synthesis kit (Roche). cDNA samples were used as template in real-time PCRs. Primers were designed using PrimerExpress 2.0 (Applied Biosystems; sequences are listed in Table 2). PCR products ranged from 80 to 100 bp. Real-time PCRs were performed using SYBR Green mix (Applied Biosystems) in 96-well optical reaction plates (Applied Biosystems). Relative quantification using a standard curve method was performed for each set of primers in an Applied Biosystems 7900HT real-time PCR instrument (absolute quantification method). Results for each target mRNA were normalized to BMEI0861 mRNA and averaged as described previously (Uzureau *et al.*, 2010).

**Visualization of the flagella by transmission electron microscopy (TEM).** Bacteria were grown in rich medium at 37 °C to OD₆₀₀ 0.25 as described previously (Ferooz & Letesson, 2010). The bacteria were centrifuged at 1000 r.p.m. for 20 min in a Jouan centrifuge, washed in PBS and fixed for 20 min in 50 µl 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 % Alcian blue for 5 min, rinsed five times in water and then placed on a drop of bacterial suspension for 10 min. Adherent cells were negatively stained with a 2% aqueous solution of uranyl acetate for 10 s. Samples were examined with a transmission electron microscope (Technai 10, Philips).

**RESULTS**

**Production of flagellin in *B. melitensis* sigma factor mutants**

We firstly analysed the impact of the mutation of sigma factors on the production of flagellin FliC (filament monomers) in *B. melitensis*. To identify whether the sigma factors RpoN, RpoH1, RpoH2, RpoE1 and RpoE2 are involved in FliC synthesis, we performed a Western blot analysis with the anti-FliC antibody on the five sigma factor mutants. A sample of each mutant and the

![Fig. 1. Western blots of SDS-PAGE protein gels probed with anti-FliC and anti-FlgE polyclonal antisera. (a) Early exponential-phase cultures were harvested from 2YT growth medium. The Δflc mutant was used as a negative control. Flagellin migrates at an estimated molecular masses of 29 kDa. (b) The *B. melitensis* wild-type strain (WT) and ΔrpoE1 mutant (ΔrpoE1) cultures were harvested after 4, 8, 24 and 38 h of growth in 2YT growth medium. FlgE and FlgE migrate at estimated molecular masses of 29 and 41 kDa, respectively. (c, d) Expression of the flgE (c) and fliC (d) translational fusions in *B. melitensis* 16M wild-type (white bars) and ΔrpoE1 (grey bars) strains. Cells were grown at 37 °C in 2YT rich medium and harvested after the indicated times for the pflc–lacZ and pflgE–lacZ fusions. The β-galactosidase activity is expressed as the mean ± SD from three independent experiments.**
B. melitensis wild-type strain were harvested at the early growth phase in rich medium (Fig. 1a). As demonstrated previously, flagellar expression only occurs at the early growth phase in B. melitensis (Fretin et al., 2005). Our results showed that the ΔrpoH2 mutant did not produce FliC, while the ΔrpoE1 mutant overproduced it (Fig. 1a) (Delory et al., 2006). Because the ΔrpoH2 mutant presents a pleiotropic phenotype (Delory et al., 2006) rather than a specific action on the flagellar genes, we did not further characterize the flagellar phenotype of this mutant, and focused our research on the rpoE1 mutant.

Mutation of rpoE1 induces overproduction of the flagellar system

Interestingly, Western blot analysis showed that FliC is overproduced in the ΔrpoE1 mutant, even during stationary phase (Fig. 1b). To confirm that the rpoE1 mutation bypasses the flagellar repression, Western blot analysis was performed with the anti-FlgE (hook monomers) and anti-FliC antibodies on the samples harvested throughout growth (Fig. 1b). Compared with the wild-type strain, FlgE and FliC are produced in higher amounts and for a longer time in ΔrpoE1 (Fig. 1b). In parallel, we also tested the activity of the flgE and fliC promoters in ΔrpoE1 compared with the wild-type strain along the growth curve by quantification of β-galactosidase activity (Fig. 1c and d, respectively) produced by strains bearing a lacZ fusion to a promoter of interest. Though the β-galactosidase activity slightly decreased at stationary phase in ΔrpoE1, the β-galactosidase activity is still between 7 and 12 times higher than in the wild-type strain, demonstrating that flagellar expression clearly persists later in the rpoE1 mutant compared with the wild-type strain. This also indicated that the rpoE1 mutation affected the promoter activity of flgE and fliC.

We also tested the effect of the rpoE1 mutation on other flagellar genes using quantitative RT-PCR (qRT-PCR). We quantified the transcript of fliF, flgE, fliC, flbT and flaF genes in ΔrpoE1 compared with the wild-type strain (Fig. 2). FliF is the monomer of the MS-ring that is the first part assembled in the flagellum structure, while FlbT and FlaF are two flagellin regulators (Ferooz et al., 2011). Expression of all the flagellar genes tested was found to be higher in ΔrpoE1 compared with the wild-type strain (Fig. 2a).

RpoE1 represses the expression of the flagellar master regulator ftcR

The results obtained from qRT-PCR analysis showed that all flagellar genes tested were downregulated in the presence of RpoE1 and, since FtcR stimulates the expression of all other flagellar genes, it is likely that the observed repression of other flagellar genes occurs as a result of the repression of ftcR (Léonard et al., 2007). To further evaluate the effect of RpoE1 on ftcR, we used the ftcR–lacZ fusion (Léonard et al., 2007) and quantified β-galactosidase activity in ΔrpoE1 compared with the wild-type strain. The results of this experiment revealed that the expression of ftcR was reduced in the presence of RpoE1, suggesting that RpoE1 down-regulates the expression of flagellar genes by repressing ftcR expression at the transcriptional level (Fig. 2b).

RpoE1 controls the length of the filament

We next examined the phenotype of the ΔrpoE1 mutant using TEM. Samples of the rpoE1 mutant were stained with

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**Fig. 2.** (a) The amount of fliF, flgE, fliC, flaF and flbT transcripts was measured in ΔrpoE1 and compared with transcripts measured in the wild-type strain (WT). Cells were grown at 37 °C until the early phase of growth (OD_{600} 0.2). The fold change in gene expression in the rpoE1 mutant is compared with the gene expression in the B. melitensis wild-type strain. The level of expression of the gene of interest in the wild-type was set up at 1. The figure represents average results from three experiments. (b) Expression of the ftcR translational fusion in B. melitensis 16M wild-type and ΔrpoE1 strains. Cells were grown at 37 °C in 2YT rich medium and harvested after 8 h of growth. The β-galactosidase activity is expressed as the mean ± SD from three independent experiments.
2% uranyl acetate and examined at different time points. No obvious differences in cell shape or number of flagella were noted. However, compared with the wild-type strain (Fig. 3a), the filament often seems to be longer in the ΔrpoE1 mutant (Fig. 3b) and we observed more disconnected free flagella in the medium (Fig. 3c).

**DISCUSSION**

It has recently been demonstrated that mutation of the sigma factor rpoE1 in *B. melitensis* increased the production of FlgE at the early exponential growth phase (Delory et al., 2006). In this work, we demonstrate that mutation of rpoE1 also increases the expression of the fliF, flgE, flaC, flaF and flbT flagellar genes and that RpoE1-mediated repression probably occurs by inhibiting expression of the flagellar master regulator ftcR.

In *B. melitensis*, the promoter activity of the master regulator ftcR is higher in ΔrpoE1 compared with the wild-type, suggesting that RpoE1 acts upstream of ftcR. In *Pseudomonas aeruginosa*, the flagellar master regulator FleQ controls the flagellum biosynthesis (Arora et al., 1997). In this bacterium, the alternative sigma factor AlgT upregulates the expression of the fleQ transcriptional repressor ArmZ leading to the repression of flagellar expression (Garrett et al., 1999; Tart et al., 2005, 2006). Similar to *P. aeruginosa*, an intermediate RpoE1-dependent regulator could inhibit the expression of ftcR in *B. melitensis*.

The homologue of *B. melitensis* RpoE1 in *Bradyrhizobium japonicum* genome is σECG (Gourion et al., 2009). By analysing the whole genome of *B. melitensis* with the σECG consensus sequence from *Bradyrhizobium japonicum*, we have detected 46 genes potentially regulated by RpoE1 based on the DNA pattern search program RSAT (Thomas-Chollier et al., 2008; Turatsinze et al., 2008) (Supplementary Table S1, available with the online version of this paper). Among them, the presence of rpoE1 suggests an autoregulation, as described in *Escherichia coli* and other bacteria (Rhodius et al., 2006). In *Caulobacter crescentus*, *Sinorhizobium meliloti*, *Rhizobium etli* and *Bradyrhizobium japonicum*, the rpoE1 homologues (sigT, rpoE2, rpoE4 and ecfG, respectively) are positively autoregulated (Alvarez-Martinez et al., 2007; Gourion et al., 2009; Martinez-Salazar et al., 2009; Sauviac et al., 2007). Among the potential genes regulated by RpoE1 detected in our in silico analysis, BMEI0372 and BMEII0720 encode a PhyR and an AraC transcriptional regulator, respectively, which could repress the flagellar genes. PhyR is an unusual type of response regulator conserved in alphaproteobacteria and consisting of a receiver domain and an ECF sigma factor-like domain (Francez-Charlot et al., 2009; Gourion et al., 2009). It would be interesting to test whether these candidates are regulated by RpoE1 and repress ftcR or other flagellar genes.

Finally, TEM experiments indicated that the flagellum is longer in ΔrpoE1 than the wild-type strain. The increased length of the filament could be a consequence of the overexpression of flagellar genes. In *Campylobacter jejuni* and *Vibrio cholerae*, mutation of flgM induced an increased flagella length (Correa et al., 2004; Wösten et al., 2010). In *C. jejuni* this increase was correlated with a higher flagellin exposure at 42 °C (Wösten et al., 2010). As soon as the hook–basal body structure is completed, FlgM is secreted through this structure into the environment, releasing FlgM and enabling activation of late genes encoding the flagellin to allow the formation of the filament. No FlgM homologue is present in the genome of *Brucella*; however, the FlaF and FlbT regulators are involved in flagellin synthesis and are also overexpressed in ΔrpoE1. Despite the presence of a sheath surrounding the flagellum, FlbT could also be secreted by the filament and could have a similar effect on flagellar length in *B. melitensis*. The sheath does not impede secretion by the flagellum, since FlgM is secreted by the sheathed flagellum of *V. cholerae* (Correa et al., 2004).

In conclusion, this work demonstrates that the ECF sigma factor RpoE1 is a flagellar repressor in *B. melitensis* and could control the length of the filament. The analysis of the genome of *B. melitensis* with a putative rpoE1 binding motif highlighted several target regulators potentially involved in flagellar repression. Conservation of the genomic organization of the rpoE1 regulon could infer a similar flagellar regulation in other alphaproteobacteria.

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REFERENCES


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