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

Jonathan Ferooz,[†] Julien Lemaire, Marie Delory, Xavier De Bolle and Jean-Jacques Letesson

Correspondence

Jean-Jacques Letesson
jean-jacques.letesson@fundp.ac.be

Unité de Recherche en Biologie Moléculaire (URBM), Facultés Universitaires Notre-Dame de la Paix Namur (FUNDP), 61 rue de Bruxelles, B-5000 Namur, Belgium

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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 *fliF*, *flgE*, *fliC*, *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 *fltR*, suggesting that RpoE1 acts upstream of *fltR*. 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 σ^{32} homologues, *rpoE1* and *rpoE2* encode two extracytoplasmic function (ECF) sigma factors and the *rpoN* gene encodes a σ^{54} 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, $\Delta rpoE1$ overproduces 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 Nal^r (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 OD₆₀₀. 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 OD₆₀₀ 0.05. Antibiotics were used at the following final concentrations: 100 µg ampicillin ml⁻¹; 20 µg chloramphenicol ml⁻¹; 50 µg kanamycin ml⁻¹; 25 µg nalidixic acid ml⁻¹.

Construction of the *pflgE-lacZ* reporter. Recombinant DNA techniques were carried out using standard protocols (Ausubel *et al.*,

[†]Present address: GlaxoSmithKline Biologicals, 20 Avenue Fleming, B-1300 Wavre, Belgium.

Abbreviations: ECF, extracytoplasmic function; qRT-PCR, quantitative reverse-transcriptase PCR; TEM, transmission electron microscopy.

A supplementary table listing the genes that contain the σ^{E1} binding site in their promoter is available with the online version of this paper.

Table 1. Bacterial strains and plasmids used in this study

Nal^r, Nalidixic acid-resistant; Kan^r, kanamycin-resistant; Cm^r, chloramphenicol-resistant; Amp^r, ampicillin-resistant.

Strain or plasmid	Description	Source
<i>B. melitensis</i>		
16M Nal ^r	Spontaneous nalidixic acid-resistant strain of <i>B. melitensis</i> 16M	Laboratory collection; A. P. MacMillan, Central Veterinary Laboratory, Weybridge, UK
<i>fliC</i> mutant	<i>B. melitensis</i> 16M Δ <i>fliC</i> ::Kan ^r	Ferooz <i>et al.</i> (2011)
<i>rpoE1</i> mutant	<i>B. melitensis</i> 16M Δ <i>rpoE1</i> ::Kan ^r	Delory <i>et al.</i> (2006)
<i>rpoE2</i> mutant	<i>B. melitensis</i> 16M Δ <i>rpoE2</i> ::Kan ^r	Delory <i>et al.</i> (2006)
<i>rpoH1</i> mutant	<i>B. melitensis</i> 16M Δ <i>rpoH1</i> ::Kan ^r	Delory <i>et al.</i> (2006)
<i>rpoH2</i> mutant	<i>B. melitensis</i> 16M Δ <i>rpoH2</i> ::Kan ^r	Delory <i>et al.</i> (2006)
<i>rpoN</i> mutant	<i>B. melitensis</i> 16M Δ <i>rpoN</i> ::Kan ^r	Delory <i>et al.</i> (2006)
<i>E. coli</i>		
DH10B	F ⁻ <i>mcrA</i> Δ (<i>mrr hsdRMS mcrBC</i>) ϕ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74 recA1 endA1 ara</i> Δ 139 Δ (<i>ara leu</i>)7697 <i>galU galK</i> λ^- <i>rpsL</i> (Str ^r) <i>nupG thi pro hsdR hsdM</i> ⁺ <i>recA</i> RP4-2 (Tc::Mu-Km::Tn7)	Gibco-BRL
S17-1		Simon <i>et al.</i> (1983)
Plasmids		
pBBCm- <i>lacZ</i>	Promoterless <i>lacZ</i> vector, Cm ^r	Fretin <i>et al.</i> (2005)
pGEM-T Easy	Cloning vector	Promega
pGEM-T <i>pflgE</i>	<i>flgE</i> promoter in pGEM-T Easy vector	This study
pBBRCmp <i>ftcR-lacZ</i>	pBBRCm- <i>lacZ</i> vector, promoter <i>ftcR-lacZ</i> translational reporter, Cm ^r	Léonard <i>et al.</i> (2007)
pJF011	pBBRCm- <i>lacZ</i> vector, promoter <i>fliC-lacZ</i> translational reporter, Cm ^r	Ferooz <i>et al.</i> (2011)
pJF012	pBBRCm- <i>lacZ</i> vector, promoter <i>flgE-lacZ</i> translational reporter, Cm ^r	This study

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 Nal^r genomic DNA by using the primers in Table 2 containing the *Xba*I and *Bam*HI restriction sites. The PCR product was first subcloned into pGEM-T Easy Vector (Promega) and then inserted in-frame upstream of the promoterless *lacZ* reporter gene into pBBR1MCS to generate the plasmid pJF012.

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 OD₆₀₀. 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

Table 2. Oligonucleotide sequences used in this study

Gene	Sequence (5'→3')	
	Forward primer	Reverse primer
qRT-PCR		
<i>fliF</i> (BMEII0151)	ATGAGCTGTTTCGACAATATGGG	CTCCTTCGAGCGCGC
<i>flgE</i> (BMEII0159)	TTCGCTGAAGAACGCTGC	GAAACGAGATCGCCCCGT
<i>fliC</i> (BMEII0150)	GTCGATCGTCGCTAATATCAACT	TCGGTCGTCGGGCC
<i>flbT</i> (BMEII0163)	AACCTCTGAACGATGCGACAT	AAAGCTGGCGCAGCG
<i>flaF</i> (BMEII0162)	AGTTGCGCTACGAAGATGTCA	CAATGGTGCGGTCGAAA
<i>lacZ</i> reporter*		
<i>flgE</i> promoter	ATATCTAGAGGGTTCGATCCTATCGATT	ATTGGATCCCGTTTCCGCGCGCTTGTA

*The underlined sequences in the forward and reverse primers are the *Xba*I and *Bam*HI restriction sites, respectively.

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 $\Delta rpoE1$ 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 μ l 10 % SDS and 20 μ l 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 min at 4 °C. Then, an equal volume of 70 % ethanol was added to the aqueous phase and mixed by vortexing. The sample was transferred to the RNA spin cartridge (Invitrogen) and centrifuged at 12 000 g 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 μ l 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 μ l 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 $\Delta fliC$ 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 $\Delta rpoE1$ mutant ($\Delta rpoE1$) cultures were harvested after 4, 8, 24 and 38 h of growth in 2YT growth medium. FliC and FlgE migrate at estimated molecular masses of 29 and 41 kDa, respectively. (c, d) Expression of the *pflgE* (c) and *pflfC* (d) translational fusions in *B. melitensis* 16M wild-type (white bars) and $\Delta rpoE1$ (grey bars) strains. Cells were grown at 37 °C in 2YT rich medium and harvested after the indicated times for the *pflfC-lacZ* and *pflgE-lacZ* fusions. The β -galactosidase activity is expressed as the mean $\pm$ 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 $\Delta rpoH2$ mutant did not produce FliC, while the $\Delta rpoE1$ mutant overproduced it (Fig. 1a) (Delory *et al.*, 2006). Because the $\Delta rpoH2$ 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 $\Delta rpoE1$ 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 $\Delta rpoE1$ (Fig. 1b). In parallel, we also tested the activity of the *flgE* and *fliC* promoters in $\Delta rpoE1$ compared with the wild-type strain along the growth curve by quantification of β -galactosidase (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 $\Delta rpoE1$, 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 $\Delta rpoE1$ 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 $\Delta rpoE1$ 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 $\Delta rpoE1$ 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 downregulates 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 $\Delta rpoE1$ mutant using TEM. Samples of the $rpoE1$ mutant were stained with

Fig. 2. (a) The amount of *fliF*, *flgE*, *fliC*, *flaF* and *flbT* transcripts was measured in $\Delta rpoE1$ and compared with transcripts measured in the wild-type strain (WT). Cells were grown at 37 °C until the early phase of growth (OD₆₀₀ 0.2). The fold change in gene expression in the *rpoE* 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 $\Delta rpoE1$ 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 $\pm$ SD from three independent experiments.

Fig. 3. Negative-staining TEM images of the sheathed polar flagellum from *B. melitensis* wild-type strain (a) and $\Delta rpoE1$ (b) stained with uranyl acetate 2%. (c) Image of a flagellum from the *rpoE* mutant. These images are representative of the population observed. Bars, 1 μ m.

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 $\Delta rpoE1$ mutant (Fig. 3b) and we observed more disconnected free flagella in the medium (Fig. 3c).

DISCUSSION

It has been recently 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*, *fliC*, *flaF* and *flbT* flagellar genes and that RpoE1-mediated repression probably occurs by inhibiting expression of the flagellar master regulator *fltR*.

In *B. melitensis*, the promoter activity of the master regulator *fltR* is higher in $\Delta rpoE1$ compared with the wild-type, suggesting that RpoE1 acts upstream of *fltR*. 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 *fltR* in *B. melitensis*.

The homologue of *B. melitensis* RpoE1 in *Bradyrhizobium japonicum* genome is σ^{EcfG} (Gourion *et al.*, 2009). By analysing the whole genome of *B. melitensis* with the σ^{EcfG} 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; Martínez-Salazar

et al., 2009; Sauviac *et al.*, 2007). Among the potential genes regulated by RpoE1 detected in our *in silico* analysis, BMEI0372 and BMEI0720 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 *fltR* or other flagellar genes.

Finally, TEM experiments indicated that the flagellum is longer in $\Delta rpoE1$ 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 expression 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 FlhA 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 $\Delta rpoE1$. Despite the presence of a sheath surrounding the flagellum, FlaF and 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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