JOURNAL OF BACTERIOLOGY, Nov. 2006, p. 7707–7710 Vol. 188, No. 21
0021-9193/06/$08.00+0 doi:10.1128/JB.00644-06

An RpoH-Like Heat Shock Sigma Factor Is Involved in Stress Response and Virulence in *Brucella melitensis* 16M▽†

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Received 5 May 2006/Accepted 16 August 2006

***B. melitensis* 16M genome analysis revealed the presence of six putative sigma factor-encoding genes: *rpoD*, *rpoH1*, *rpoH2*, *rpoE1*, *rpoE2*, and *rpoN*. We mutated all these genes except *rpoD*. Phenotypic analysis of the mutants reveals that a strain carrying an *rpoH2* null mutation (Δ*rpoH2*) is impaired for growth at 21 and 42°C and shows increased sensitivity to hydrogen peroxide. Compared to the wild-type strain, the Δ*rpoH2* mutant is attenuated in all virulence models tested. Three other null mutants (Δ*rpoH1*, Δ*rpoE1*, and Δ*rpoE2* mutants) are also defective for survival in mice at 4 weeks postinfection. We also demonstrated that *rpoH2* deletion strongly reduces the expression of two major virulence factors in *B. melitensis*, the type IV secretion system and the flagellum.**

During their infectious cycle, pathogenic bacteria are exposed to a wide variety of environments. Associations between alternative σ factors and RNA polymerase provide one efficient mechanism for appropriately modifying the transcriptional profile of the bacterium in response to changing environments (10). Alternative σ factors contribute to bacterial resistance to environmental stress conditions, such as high temperature, oxidative stress, carbon starvation, and low pH, and therefore contribute to virulence of pathogenic bacteria. Alternative σ factors may also be involved in the regulation of more "specific" virulence genes (4, 11). Among the σ factors involved in bacterial virulence, there are stress response σ factors (σ^B and σ^S), flagellar σ factors (σ^{28}), extracytoplasmic function σ factors (RpoE, AlgU), and σ^{54}. Up to now, very few examples of heat shock sigma factor (σ^{32}) being involved in bacterial virulence have been reported (13).

Brucellae are gram-negative, intracellular pathogenic bacteria that cause brucellosis in a variety of mammals, including humans. The availability of the complete *Brucella melitensis* 16M genomic sequence allowed us to identify six putative σ factors in this organism (Fig. 1A), a housekeeping σ factor (σ^{70}, encoded by *rpoD*), two σ^{32} homologues (σ^{H1} and σ^{H2}), two extracytoplasmic function (ECF) σ factors (σ^{E1} and σ^{E2}), and a σ^{54} homologue (σ^N). Unlike enteric bacteria and pseudomonads, *B. melitensis*, as well as the other α-proteobacteria (14), does not possess an *rpoS*-like gene coding for the general stress σ factor, σ^S.

The genomic organization of the σ coding sequences (CDS) in *B. melitensis* 16M is depicted in Fig. 1B. Some interesting observations may be made. The *rpoH1* CDS is found close to CDS potentially involved in adaptation to heat shock (a zinc metalloprotease homologue and a heat shock protein 15 homologue). The *rpoH2* CDS is located close to the *rpoE1* CDS, which is next to genes coding for proteins belonging to a two-component regulatory system, TcaR (response regulator) and TcaS (sensory transduction histidine kinase). This locus is well conserved in α-proteobacteria. TcaR contains an unusual N-terminal DNA binding domain compared to other response regulators, displaying homology to the RpoE domain (COG1595), which is usually found in σ^{ECF} factors. The predicted peptidic sequence for BMEII0072 presents similarity to the Sigma_r2 domain from Pfam (E value = 1.3e−14), and it is also similar to a predicted σ^{ECF}, CarQ from *Myxococcus xanthus* (6). These data suggest that BMEII0072 may encode a phylogenetically distant σ^{ECF} homologue. The *rpoN* CDS is close to genes coding for a σ^{54} modulation protein and a nitrogen-regulatory protein, which suggests that there may be a functional relationship between RpoN and these two gene products. None of the σ CDS is predicted to be part of a transcriptional unit with other CDS.

In vitro characterization of σ mutants indicates that σ^{H2} is involved in adaptation to heat, cold, and oxidative stress. Each nonessential *rpo* gene (coding for a σ factor) was replaced by a kanamycin resistance gene, using a previously reported strategy (1) with the oligonucleotides listed in Table S1 in the supplemental material. Classical polar effects probably do not occur in these mutants, since none of these genes forms part of a predicted operon (Fig. 1). All the mutants displayed a smooth phenotype, as detected with the crystal violet colony staining method, suggesting that lipopolysaccharide O chain is present. Indeed, rough variants are frequent, and attenuation may be due to this character instead of the absence of a σ factor. The σ mutants (Δ*rpoH1*, Δ*rpoH2*, Δ*rpoE1*, Δ*rpoE2*, and Δ*rpoN* mutants) were characterized with regard to their sensitivity to oxidative and heat stresses.

The σ mutants were tested for survival upon oxidative stress (H_2O_2) using a disk sensitivity assay (3). Briefly, 100 μl of *B. melitensis* cultures adjusted to an optical density at 600 nm (OD_{600}) of 0.4 were plated on 2× yeast extract-tryptone (2YT) agar, and sterile paper disks (5-mm diameter) saturated with 10 μl of H_2O_2 at a concentration of 5 M were layered on top

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† Supplemental material for this article may be found at http://jb.asm.org/.

▽ Published ahead of print on 25 August 2006.

A.

CDS numbers	Genes [a]	Proteins	E-value for *E. coli* homologues [b]	Mutants
BMEI0280	*rpoH1*	σ^{H1}	275/311 3e-33	Δ*rpoH1*
BMEI0378	*rpoH2*	σ^{H2}	265/303 1e-49	Δ*rpoH2*
BMEI0371	*rpoE1*	σ^{E1}	127/190 6e-10	Δ*rpoE1*
BMEII0072	*rpoE2*	σ^{E2}	136/378 3.8	Δ*rpoE2*
BMEI1789	*rpoN*	σ^{N}	485/500 5e-77	Δ*rpoN*

B.

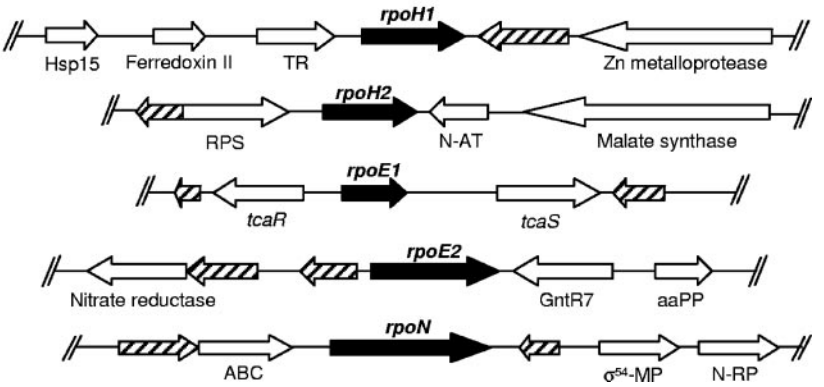

FIG. 1. Predicted σ-factor CDS identified in the *B. melitensis* 16M genome. A. CDS numbers and gene, protein, and mutant names used in this study. a, in agreement with the names given to σ-coding genes in *E. coli*; b, numbers before the E values correspond to the fraction of the predicted protein that is aligned with the *E. coli* homologue, and the second number is the predicted protein length. B. Genomic organization around the *B. melitensis* σ CDS. Each predicted CDS is indicated by an arrow. The CDS for putative σ factors are black, and the hypothetical proteins are hatched. Abbreviations: Hsp15, heat shock protein 15; TR, transcriptional regulator; RPS, ribosomal large-subunit pseudouridine synthase; N-AT, phosphinothricin *N*-acetyltransferase; aaPP, leucine-, isoleucine-, valine-, threonine-, and alanine-binding protein precursor; GntR7, transcriptional regulator GntR family (8); σ^{54}-MP, putative σ^{54} modulation protein; N-RP, nitrogen-regulatory protein; ABC, ATP-binding protein of the ATP-binding cassette transporter.

prior to incubation at 37°C. The Δ*rpoH2* mutant showed significantly increased sensitivity to H_2O_2. The assay was performed on three separate plates, and the diameter of growth inhibition for the Δ*rpoH2* mutant was 5.52 cm (±0.20 cm [standard deviation]) compared with 4.18 cm (±0.04 cm) for the wild-type strain, which is a highly significant difference (Student's *t* test, $P < 0.01$). Sensitivity of the Δ*rpoH2* mutant to H_2O_2 is reduced when this strain carries a complementation plasmid, pMR-*rpoH2* (data not shown). pMR-*rpoH2* is a low-copy-number plasmid (RK2-derived pMR10, made compatible for Gateway cloning to give pRH001; R. Hallez, unpublished data) carrying the *rpoH2* coding sequence. Oligonucleotides used for the construction of complementation plasmids are available in Tables S2 and S3 in the supplemental material. The other σ mutants did not display a reproducible and significantly altered sensitivity to H_2O_2.

The growth of σ mutants was compared to that of the wild-type strain on 2YT agar plates at temperatures of 21, 37, and 42°C. Serial dilutions of bacterial cultures adjusted to an OD_{600} of 0.4 were spotted on 2YT plates and incubated at these temperatures. Growth at 21 and 42°C was completely abolished for the Δ*rpoH2* mutant (Fig. 2A). Complementation of the Δ*rpoH2* mutant with pMR-*rpoH2* restored a growth comparable to the wild-type strain at 21 and 42°C (Fig. 2A). We also compared the growth curve of the wild-type strain, the Δ*rpoH2* mutant, and the complemented strain at 37 and 42°C in liquid 2YT medium, starting with an OD_{600} of 0.1. At 37°C, the Δ*rpoH2* mutant reached an optical density slightly higher than the wild-type strain in late exponential and stationary phases. Following a temperature shift to 42°C, the Δ*rpoH2* mutant was unable to grow, while the wild-type strain grew, as shown in Fig. 2B. Complementation of the Δ*rpoH2* mutant with pMR-*rpoH2* partially restored the growth at 42°C in liquid medium (Fig. 2B). The reduced growth of the complemented strain observed at 37 and 42°C (Fig. 2B) compared to that of the wild-type strain is probably due to the lack of appropriate regulation of *rpoH2*, which is fused to an *Escherichia coli lac* promoter in the pMR10 vector. The growth curves of the Δ*rpoH1*, Δ*rpoE1*, Δ*rpoE2*, and Δ*rpoN* mutants were also monitored, but no differences was observed compared to the wild-type strain (data not shown). Measuring CFU at different culture phases indicated that *rpoH2*, *rpoE1*, *rpoE2*, and *rpoN* are not required for survival in stationary phase (data not shown).

σ^{H2} is required for survival of *B. melitensis* in several models of infection. The pathogenicity of *Brucella* spp. is critically dependent on its ability to infect and to multiply within both professional and nonprofessional phagocytes (2). We tested the ability of σ mutants to invade and survive within J774 macrophages and epithelial (HeLa) cells, using a previously described protocol (9), with 10^5 cells/well. A Δ*vjbR* mutant was used as a positive control for attenuation (1). The CFU were counted after 48 h of infection. The results showed that the Δ*rpoH2* mutant was strongly attenuated in both J774 and HeLa

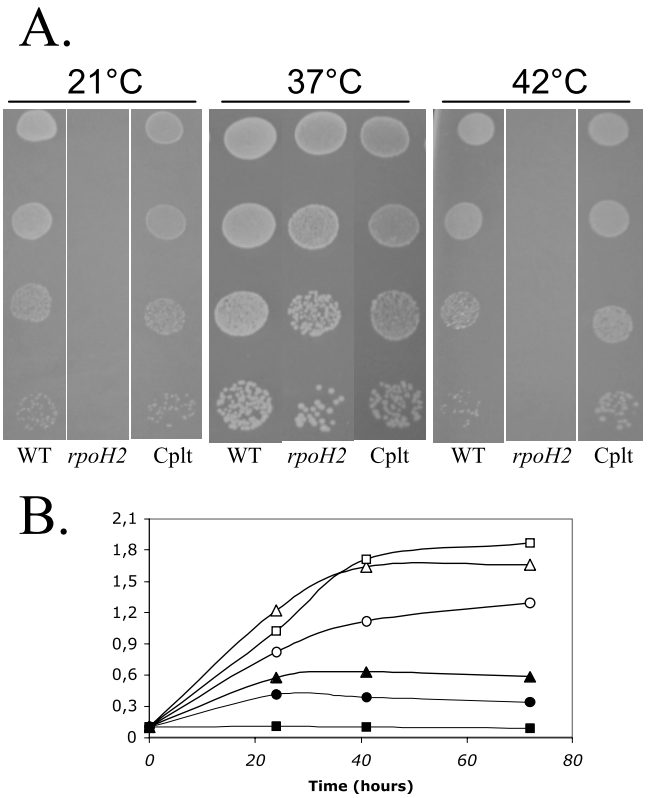

FIG. 2. A. Heat- and cold-sensitive growth phenotype of the *B. melitensis* Δ*rpoH2* mutant. The wild-type strain (WT), the Δ*rpoH2* mutant (*rpoH2*), and the strain complemented with pMR-*rpoH2* (Cplt) were grown overnight in liquid 2YT medium at 37°C. After adjustment to an OD_{600} of 0.4, 10 μl of serial dilutions of these cultures (consecutive 1:10 dilution steps from top to bottom) was spotted onto 2YT agar plates and incubated at the indicated temperature. B. Comparison of the growth of wild-type cells (WT) with the growth of the Δ*rpoH2* mutant and the complemented strain (pMR-*rpoH2*), as measured using OD_{600}. Growth of the wild-type strain (triangles), Δ*rpoH2* mutant (squares), and pMR-*rpoH2*-complemented strain (circles) in liquid medium at 37°C (open symbols) and 42°C (filled symbols) is indicated.

cells. The Δ*rpoE1* mutant was slightly attenuated in both models of infection (Table 1).

The persistence of each mutant was studied in a mouse model of infection, using a previously described protocol (8). Groups of four mice were inoculated intraperitoneally with either a mutant strain or the wild-type strain. The *Brucella* CFU were evaluated 1 and 4 weeks postinfection in the spleens of four animals. After 1 week of infection, the Δ*rpoH2* mutant was recovered from spleens at markedly lower levels (1.9 ± 0.2 log) than the isogenic parental strain (Table 1). By 4 weeks postinfection, the number of Δ*rpoH2* CFU was 3 orders of magnitude lower than that of the parental strain in one mouse, while the three others had cleared the Δ*rpoH2* strain from their spleens. All mutant strains except the Δ*rpoN* strain showed reduced spleen colonization after 4 weeks of infection. The need for four different σ factors suggests that *B. melitensis* faces various environments within this infection model, indicating that this model is rather complex, especially compared to the cellular models of infection tested here. In particular, the requirement for ECF σ factors is in agreement with the major role of the *Brucella* sp. envelope during infection (7). The involvement of four of the five nonessential σ factors predicted from the *B. melitensis* genome indicates that σ factors are key players in the regulation of virulence determinants required for survival in this model of infection. Moreover, our data indicate that σ^{H2} may be required for the acute phase of the infection, while σ^{E1}, σ^{E2}, and σ^{H1} would be required for chronicity of the infection.

σ^{H2} is involved in the regulation of both VirB and FlgE production. Since VirB and flagella are important virulence factors for *Brucella* spp. (5, 12), we investigated the abundance of these structures in the σ mutants compared to that in the wild-type strain, with the hypothesis that σ factors may contribute to the regulation of these structures.

To compare VirB and FlgE production in the mutants and the wild-type strain, we carried out Western blotting analysis of whole-cell extracts by using anti-VirB8 and anti-FlgE polyclonal antisera generated in rabbits (5, 15) (Fig. 3), using a previously described protocol (1). The FlgE protein (flagellar hook monomer homolog) was used for monitoring the production of flagellar proteins. The abundance of VirB8 and FlgE proteins was tested in cells harvested at a phase of the growth curve in 2YT medium where their production is maximal (OD_{600} around 0.3 or 1.5 for the detection of FlgE or VirB8, respectively). The Δ*ftcR* mutant was used as a negative control for FlgE detection, *ftcR* being required for *fliF* expression and FlgE abundance (S. Léonard, unpublished data). We observed that VirB8 is slightly less abundant in the Δ*rpoE1* mutant, almost absent in the Δ*rpoH2* mutant, and overproduced in the Δ*rpoN* mutant compared to the wild-type strain. FlgE is almost absent in the Δ*rpoH2* mutant and overproduced in the Δ*rpoE1* mutant. Comparable effects were observed using a fusion between the *fliF* promoter and *lacZ* in these two mutants (M. Delory, unpublished results), indicating that expression of several flagellar genes is similarly affected by the absence of σ^{H2} or σ^{E1}.

Recently, Delrue et al. identified and characterized VjbR, a LuxR-like transcriptional regulator essential for the expression of the *virB* operon and for the production of FlgE protein (1).

TABLE 1. Attenuation of σ mutants

Mutation	LPS[a]	Attenuation in[b]:		
		HeLa cells	J774 macrophages	BALB/c mice
Δ*rpoH1*	S	−0.4 ± 0.2	0 ± 0.1	1.5 ± 0.1
Δ*rpoH2*	S	1.4 ± 0.3	1.8 ± 0.2	>3
Δ*rpoE1*	S	0.7 ± 0.1	0.9 ± 0.1	2.2 ± 0.2
Δ*rpoE2*	S	−0.6 ± 0.2	0 ± 0.05	1.8 ± 0.2
Δ*rpoN*	S	−0.3 ± 0.2	−0.4 ± 0.3	0.1 ± 0.1
Δ*vjbR*	ND[c]	2.0 ± 0.1	2.6 ± 0.2	ND

[a] Smooth (S) lipopolysaccharide (LPS) was detected by crystal violet colony staining.

[b] Attenuation in cellular models (48 h postinfection) and in BALB/c mice (4 weeks postinfection) is expressed as the difference of the log CFU between the wild-type strain and the mutants ± the standard deviation, which was calculated from values obtained for each mutant in the different models of infection (n = 3 for the cellular models and n = 4 for the mouse model). Cellular infections were performed twice in triplicate, with a multiplicity of infection of 300 bacteria per cell. The Δ*vjbR* mutant was used as a positive control for attenuation in the cellular models (1).

[c] ND, not determined.

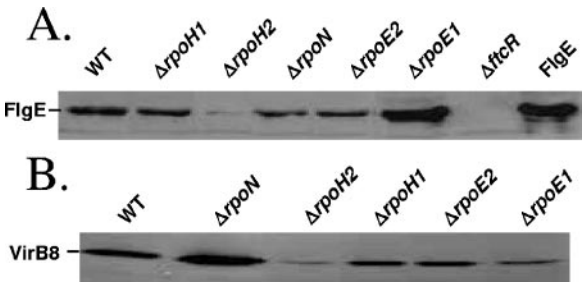

FIG. 3. Western blots of SDS-PAGE protein gels probed with anti-VirB8 and anti-FlgE polyclonal antisera. For blot A, early-log-phase cultures were harvested from 2YT growth medium. The Δ*ftcR* mutant was used as a negative control for FlgE detection. Purified recombinant His_6-FlgE was used as a positive control (lane FlgE). For blot B, cultures were grown in 2YT medium until late log phase. Lanes were loaded with equal cell quantities (determined by OD_{600} before harvesting). VirB8 and FlgE migrate at estimated molecular masses of 31.5 kDa and 41 kDa, respectively.

VjbR being a possible mediator of the regulation of VirB production by a σ factor, we tested the activity of the *vjbR* promoter in the Δ*rpoH2* strain. The activity of *vjbR* promoter was monitored by measuring luciferase activity using a *pvjbR luxAB* fusion carried by the plasmid pJD27-*pvjbR*. The plasmid pJD27-*pvjbR* was conjugatively transferred into the *B. melitensis* 16M Nal^r strain and the mutant Δ*rpoH2* strain. The wild type and the Δ*rpoH2* mutant bearing the pJD27-*pvjbR* plasmid were grown in 2YT medium and harvested during the exponential phase. A luciferase assay was performed as described previously (1). In the Δ*rpoH2* strain, the activity of the *vjbR* promoter was reduced more than 100-fold compared to that in the wild-type strain (1,865 ± 384 [standard deviation] relative light units for the mutant versus 231,411 ± 5,866 for the wild type; data are representative of three independent experiments), suggesting that *vjbR* may be an mediator of the effect of *rpoH2* mutation on the abundance of VirB and FlgE proteins.

In conclusion, using a systematic targeted mutagenesis strategy, we identified a σ factor (σ^{H2}) having multiple roles in *B. melitensis*, since the Δ*rpoH2* mutant is very sensitive to heat, cold, and oxidative stress. The essential role of σ^{H2} in both thermotolerance and cryotolerance is unique among proteins belonging to the RpoH family. It is possible that σ^{H2} is actually involved in generalized cytoplasmic stress response. The molecular mechanisms involving σ^{H2} in the adaptation to low and high temperature, and to oxidative stress, in *B. melitensis* remain to be discovered. The function of the other σ factors should also be studied, since three of them (σ^{H1}, σ^{E1}, and σ^{E2}) are required for virulence in the mouse model of infection.

Marie Delory and Régis Hallez were supported by the Fonds pour la Formation à la Recherche dans l'Industrie et dans l'Agriculture (F.R.I.A.). This work was supported by the Fonds de la Recherche Fondamentale Collective (F.R.F.C., convention no. 2.4521.04).

We thank URPhyM (University of Namur) for DNA sequencing facilities. We thank C. Deschamps for the construction of pJD27-*pvjbR*.

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