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Brucella melitensis MucR, an Orthologue of Sinorhizobium meliloti
MucR, Is Involved in Resistance to Oxidative, Detergent, and Saline Stresses and Cell Envelope Modifications

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Brucella spp. and Sinorhizobium meliloti are alphaproteobacteria that share not only an intracellular lifestyle in their respective hosts, but also a crucial requirement for cell envelope components and their timely regulation for a successful infectious cycle. Here, we report the characterization of Brucella melitensis mucR, which encodes a zinc finger transcriptional regulator that has previously been shown to be involved in cellular and mouse infections at early time points. MucR modulates the surface properties of the bacteria and their resistance to environmental stresses (i.e., oxidative stress, cationic peptide, and detergents). We show that B. melitensis mucR is a functional orthologue of S. meliloti mucR, because it was able to restore the production of succinoglycan in an S. meliloti mucR mutant, as detected by calcofluor staining. Similar to S. meliloti MucR, B. melitensis MucR also represses its own transcription and flagellar gene expression via the flagellar master regulator ftcR. More surprisingly, we demonstrate that MucR regulates a lipid A core modification in B. melitensis. These changes could account for the attenuated virulence of a mucR mutant. These data reinforce the idea that there is a common conserved circuitry between plant symbionts and animal pathogens that regulates the relationship they have with their hosts.

The bacterial envelope is a bacterium’s first point of contact with its challenging environment, and in the case of symbionts or pathogens, it is the main target for antibacterial host defenses (1). Thesemultilayered structures (cytoplasmic membrane and cell wall and possibly the outer membrane, polysaccharide capsule, and proteinaceous S layer) have both protective and adaptive functions that require tightly regulated gene expression to control their biosynthesis and to adjust their properties in response to changing environments (2–4). In the alphaproteobacteria, Sinorhizobium meliloti is the paradigmatic model for studying the crucial role surface components play and how their production is finely tuned to respond appropriately to the environmental signals (e.g., carbon and nitrogen sources, phosphate starvation, and plant signals) and various stresses (osmolality, ionic strength, and oxidation) related to either their free-living state or their partnership with leguminous hosts. For example, the establishment of rhizobium-legume symbiosis requires the timely and spatially regulated bacterial synthesis (or modification) of four classes of envelope-associated polysaccharides: outer membrane lipopolysaccharides (LPS), periplasmic cyclic β-(1,2)-glucans and external capsular polysaccharides (K), and the exopolysaccharides (EPS) (succinoglycan [EPS I] and galactoglucan [EPS II]) (5–7). The regulated production of EPS is particularly well described and involves an intricate regulatory network (for a review, see reference 2). Briefly, the inner-membrane sensor histidine kinase ExoS and the cytoplasmic transcriptional regulator ChvI constitute a two-component system (TCS) that controls succinoglycan production (8). A third partner, the periplasmic protein ExoR (believed to sense calcium and ammonium), is involved in this regulatory cascade (9, 10). Both ExoR and ExoS are involved in regulating EPS I production, and S. meliloti mutants with mutations of the genes involved in LPS sulfation and flagellum biosynthesis (11–13), exoR and exoS, overproduce EPS I and are symbiotically deficient (14–16). Finally, the zinc finger protein MucR appears to couple the two EPS biosynthetic pathways by positively regulating succinoglycan biosynthesis genes and repressing the synthesis of galactoglucan (17–20). It remains to be determined how mucR becomes active, but it has also been shown to repress flagellar-gene expression (21). In addition, most of these signaling pathways are influenced by the quorum-sensing (QS) hierarchy of S. meliloti (22). S. meliloti belongs to the order Rhizobiales in the α-2 subdivision of the class Proteobacteria and, although a plant symbiont, is very closely related to the animal pathogens belonging to the genus Brucella (23). Brucella spp. are considered to be facultative intracellular parasites that cause brucellosis, a chronic globally widespread zoonotic disease that affects a broad range of mammals, including livestock and humans (24).

Most Brucella virulence determinants have been associated with the bacterial surface as either permanent or transient structural components (e.g., the envelope and its appendages, the virB type IV secretion system [25], the flagellum [26], or their respective regulators [27–29]). Sinorhizobium and Brucella not only lead to similar intracellular chronic infections within a host-derived membrane-bound compartment of their respective hosts (30), but also share similar requirements for establishing a relationship with their dedicated hosts (31). In Brucella spp., cell envelope-associated polysaccharides and their regulated production also...
play a crucial role during the interaction with the host. First, the LPS O chain is required to resist complement-mediated lysis (32), avoid intracellular killing, mediate early steps in vacuolar trafficking (33), and inhibit host cell apoptosis (34). Second, cyclic glucans allow the bacteria to prevent phagosome-lysosome fusion and reach their final replicative compartment (35). Third, the BvRS/BvR TCS, which is orthologous to EtxS/ChrI, is also critical to the infectious cycle and is clearly involved in the homeostasis of the outer membrane (OM) (36, 37). Fourth, among the targets of this TCS, which were identified by transcriptomic analysis (38), is the QS regulator VjbR, which was previously demonstrated to be a major regulator of outer-membrane organization (OM proteins, flagellum, and type IV secretion system) (39). Notably, in medium containing yeast extract, VjbR mutants have an aggregative phenotype that has been suggested to be linked to EPS production in both cellular and mouse models of infection but was otherwise uncharacterized. More recently, the protective efficacy of this mutant was evaluated as a live attenuated vaccine (41).

Here, we report a detailed characterization of the transcriptional regulator MucR and show that it modulates bacterial surface properties and resistance to environmental stresses (i.e., oxidative stress, cationic peptide, and detergent). Using heterospecific complementation, we show that B. melitensis mucR is a functional orthologue of S. meliloti mucR based on its ability to restore succinoglycan production in the S. meliloti Rm101 mucR mutant. In addition, similar to S. meliloti, B. melitensis MucR inhibits flagellar expression via the flagellar master regulator and negatively regulates its own transcription. More surprisingly, we demonstrate that MucR regulates a lipid A core modification in B. melitensis. In addition to the BvRS/BvR TCS (37), this is the second transcriptional regulator of Brucella spp. shown to modulate the lipid A core component of LPS. Considering the strong conservation of the mucR gene in alphaproteobacteria and the link generally established between the gene and altered host-bacterial interaction, it would be worthwhile to examine LPS alterations associated with mucR mutations in other alphaproteobacteria.

**MATERIALS AND METHODS**

**Strains, plasmids, and culture conditions.** All strains and plasmids used in this study are listed in Table 1.

Classically, Brucella strains were grown with shaking at 37°C in 2YT medium (10 g liter yeast extract, 16 g liter peptone, 5 g liter NaCl/l) containing the appropriate antibiotics from a stationary-phase overnight culture (2YT; 10 ml) back diluted to an optical density at 600 nm (OD600) of 0.05.

For RNA extraction, 10 ml of bacteria was harvested from a 200-ml 2YT culture grown to mid-log phase (OD600 = 0.5). The 10-ml cultures were used to follow GFP(ASV) (green fluorescent protein) production from B. melitensis pBBRmucRgfp(ASV) and from B. melitensis harboring the vector pBBR-gfp(ASV). GFP(ASV) is an unstable variant of GFPmut3 and is a useful reporter gene for monitoring transient gene expression because of the reduced half-life of the reporter gene (49). Escherichia coli strains were routinely grown in Luria-Bertani (LB) medium with appropriate antibiotics at 37°C. S. meliloti strains were cultivated in LB broth with 2.5 mM MgSO4 and 2.5 mM CaCl2 at 30°C. Matings were performed by mixing E. coli S17-1 donor cells with Brucella or S. meliloti recipient strains on 2YT or LB medium, respectively, for 3 to 4 h. The mixed population was plated on medium containing the appropriate antibiotics to select for B. melitensis and S. meliloti conjugants. Chloramphenicol, gentamicin, and nalidixic acid were used at 20, 50, and 25 (8 for S. meliloti) μg ml⁻¹, respectively. Growth curves were monitored using a Bioscreen system (Thermo Fisher, Erembodegem-Aalst, Belgium), which continuously monitors OD600 readings in a multiwell format.

**Molecular techniques.** DNA manipulations were performed using standard molecular techniques (50). Restriction enzymes were purchased from Roche, and primers were purchased from Eurogentec. Primer sequences are listed in Table S1 in the supplemental material.

**Mutant construction.** The B. melitensis 16M ΔmucR deletion mutant was constructed by allelic replacement using a two-step strategy. Briefly, 500-bp upstream and downstream fragments flanking the mucR gene were amplified by PCR from B. melitensis genomic DNA using the primers PmucRf and PmucRr and TmucRf and TmucRr, respectively. For each construction, a second PCR was used to join the two PCR products using the primer pairs PmucRf and TmucRr. Finally, the ΔmucR fragment was cloned into pGEM-T Easy (Promega) to generate the intermediate vector pGEMTΔmucR. The ΔmucR fragment was excised by NotI restriction, subcloned into the final vector pIQ200-uc1, and used to construct a Brucella mutant following a previously described strategy (51). Gene replacement was confirmed by PCR using the following primers: mucR upstream and mucR downstream. To construct the strain ΔmucR1, we used the same recombinations strategy using a plasmid carrying the mucR gene with its upstream and downstream regions.

**Construction of the complementation plasmid pBBRmucR.** The mucR gene was amplified by PCR from B. melitensis genomic DNA using the primers (Eurogentec) mucRF and mucRP Clal R, which contain Xhol and Clal restriction sites, respectively. The PCR product was cloned into pGEM-T Easy (Promega) to generate the intermediate vector pGEMTmucR. After sequencing, the fragment mucR was excised by a Clal and Xhol double restriction digest and subcloned into a previously Xhol-Clal-restricted plasmid, pBBRMC51, to obtain pBBRmucR. The vector was then transferred to the ΔmucR strain to obtain the complemented ΔmucR pBBRmucR strain.

**Construction of the reporter plasmid pBBRpBmpucRgfp(ASV).** The region containing the putative mucR promoter was amplified by PCR from B. melitensis genomic DNA using the primers XholpumpucR and BamHIpumpucR, which contain Xhol and BamHI restriction sites, respectively. The PCR product was first cloned into the vector pGEM-T Easy. The fragment was then inserted in frame upstream of the promoterless gfp(ASV) reporter gene in pBBR1MCS to generate the plasmid pBBRpBmpucRgfp(ASV).

**Cellular infection.** Evaluation of the intracellular survival of B. melitensis wild-type (WT) and ΔmucR strains in RAW 264.7 murine macrophages was performed as previously described (52). Briefly, bacterial strains were grown overnight in 2YT medium and then inoculated at a multiplicity of infection (MOI) of 300 into cell monolayers in 24-well plates. After a 10-min centrifugation at 1,000 rpm at room temperature, the plates were placed in a 5% CO2 atmosphere at 37°C for 1 h. Afterward, the cells were washed with phosphate-buffered saline (PBS) and incubated in medium containing 50 μg ml gentamicin at 37°C under 5% CO2 until the end of the infection (48 h). The cells were then washed and lysed in sterile MilliQ water for 10 min, and serial dilutions of lysates were plated on 2YT solid medium to enumerate CFU. The data are expressed as CFU per well on a logarithmic scale.

**Mouse infections.** Virulence assays using BALB/c mice were performed as described previously (26). Briefly, 8-week-old mice were inoculated intraperitoneally with 500 μl of a suspension containing 4 × 10⁴ CFU of the appropriate bacterial strain. At 1 and 4 weeks postinoculation, mice from each group were sacrificed, and spleens were collected. The spleens were homogenized in 2 ml of PBS–0.1% Triton X-100, and serial dilutions of the homogenates were plated on 2YT solid medium to determine the bacterial load. The lysis of spleen homogenates with 0.1% Triton X-100 does not affect the survival of mucR mutants, as similar results were obtained with a lysis protocol using distilled water (data not shown). The data are expressed as the log10 CFU per spleen. Data were statistically
After exposure for 1 h in a 37°C shaking incubator, the cells were rapidly diluted with PBS and plated onto 2YT medium. After 5 days at 37°C, CFU were enumerated, and the survival of each bacterial strain was determined with the correction tool of PowerPoint software. Polymyxin B sensitivity was determined using an Etest containing a preformed gradient covering a continuous MIC range from 0.064 to 1,024 μg/ml (bioMérieux). *Brucella* was adjusted to an OD 750 of 0.109 (1 McFarland standard) in 2YT medium. The suspension was spread onto Mueller-Hinton II (cation-adjusted) broth (BD Difco) plates using a cotton swab, and the Etest strips were then applied. The plates were incubated for 72 h at 37°C. For each strain, the MIC was determined as the concentration at which the ellipse intersects the concentration scale printed on the Etest strip. Three independent tests were performed.

**Oxidative-resistance assay.** Oxidation resistance assays were performed according to previously described protocols with some modifications (53). Cells were grown overnight in 10 ml of 2YT medium with shaking and adjusted to a concentration of 5 × 10^8 CFU ml^-1 in PBS.

In 96-well plates, 50 μl of the bacterial suspension was supplemented with 50 μl of H_2O_2 (freshly diluted in PBS) at final concentrations of 1 mM, 2.5 mM, and 5 mM. A negative-control experiment was performed by adding 50 μl of PBS (without H_2O_2) to the same bacterial suspension. After exposure for 1 h in a 37°C shaking incubator, the cells were rapidly diluted with PBS and plated onto 2YT medium. After 5 days at 37°C, CFU were enumerated, and the survival of each bacterial strain was determined as a percentage of the negative control.

**Detergent sensitivity, polymyxin B sensitivity, and Congo red staining.** Bacteria from an overnight culture in 2YT medium were spotted on tryptic soy broth (TSB) agar medium (Difco) containing 2% SDS, 0.1% Triton X-100, or 0.01% Congo red in triplicate (20 μl per spot) and incubated at 37°C for 4 days. Images were captured using a Canon A430 camera, and the contrast and brightness of the complete image were optimized with the correction tool of PowerPoint software. Polymyxin B sensitivity was determined using an Etest containing a preformed gradient covering a continuous MIC range from 0.064 to 1,024 μg/ml (bioMérieux). *Brucella* was adjusted to an OD 750 of 0.109 (1 McFarland standard) in 2YT medium. The suspension was spread onto Mueller-Hinton II (cation-adjusted) broth (BD Difco) plates using a cotton swab, and the Etest strips were then applied. The plates were incubated for 72 h at 37°C. For each strain, the MIC was determined as the concentration at which the ellipse intersects the concentration scale printed on the Etest strip. Three independent tests were performed.

**Quantitative real-time reverse transcription-PCR (qRT-PCR).** Total RNA samples were prepared as previously described (39) for *B. melitensis* 16M, the ΔmucR mutant, and the complemented ΔmucR* pBBRmucR* mutant. DNA was removed from the samples using DNase (Kit Fermentas), and samples were reverse transcribed with SuperScript II Reverse Transcriptase (Invitrogen) using random oligonucleotide hexamers, as recommended in the manufacturer’s protocol. RNA and cDNA quantities were measured using a NanoDrop spectrophotometer (ND-1000; Thermo Fisher Scientific). The resulting cDNA samples were used as the template in real-time PCRs. Primers were designed using PrimerExpress 2.0 soft-
Total extraction and SDS-proteinase K extraction of LPS. Total extracts were prepared from *B. melitensis* strains cultivated in 2YT medium. Bacteria were concentrated to obtain an OD$_{600}$ equivalent of 10 and inactivated at 80°C for 1 h. Total extracts were used for SDS-proteinase K extraction of LPS as previously described (54). Samples were loaded onto a 16% or 15% polyacrylamide gel for SDS-PAGE analysis. The gels were then silver stained or transferred onto a nitrocellulose membrane (Amer sham) for Western blotting (34). In the silver-stained gel, some contaminating (or LPS-linked) proteins were also observed, and the most common contaminants had masses of 25,000 to 27,000 Da (35).

**Immunodetection.** Immunodetection was performed with primary mouse monoclonal antibodies (MAbs) (undiluted hybridoma culture supernatant) against the O antigen of *Brucella* (A76/12G12/F12 (55) and against the LPS core (A68/24G12/A8 and A68/24D8/G9) (56) for LPS detection. Flagellar protein was detected using anti-FliC (diluted 1:3,000) or anti-FlgE (1:5,000) rabbit polyclonal antibodies (26). PrtR, which was used as a loading control, was detected using anti-PrtR polyclonal rabbit antibodies (1:1,000) (57). Secondary antibodies consisted of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Amersham catalog no. NA931; 1:10,000 dilution) and HRP-conjugated donkey anti-rabbit IgG, HRP-linked whole antibody (Amersham catalog no. NA934; 1:5,000 dilution).

**Fluorescence microscopy.** Bacteria were spotted onto a microscope slide layered with a 1% agarose pad containing PBS (58). These slides were placed on a microscope stage at room temperature. The samples were observed on a Nikon 80i fluorescence microscope through a 100× differential interference contrast (DIC) (Nomarski) or phase-contrast objective with a Hamamatsu Orca-ER LCD camera. Image acquisition and processing were performed with NIS element software (Nikon).

**Fluorescence-activated cell sorter (FACS) analysis.** Bacterial suspensions were washed in PBS and fixed in 2% paraformaldehyde (PFA), pH 7.4, at room temperature for 20 min. After an additional wash in PBS, the bacteria were used for flow cytometric analysis of GFP*ASV* production with the stresses linked to stationary phase, which would be relevant. The hypothesis is that the mucR mutant enters prematurely into stationary phase (34). The mucR mutant had a growth rate similar to that of the WT until 22 h (log phase) (Fig. 2). At this time point, the *ΔmucR* mutant transitioned into stationary phase earlier, more abruptly, and at a lower bacterial density than the WT (Fig. 2). One possible hypothesis is that the *ΔmucR* mutant is poorly adapted to cope with the stresses linked to stationary phase, which would be rele-

**RESULTS**

**MucR is required for a successful *B. melitensis* infection in both RAW264.7 macrophages and BALB/c mice.** To characterize the role of mucR in virulence, we constructed a deletion mutant of BME11364 (referred to as *ΔmucR*) in *B. melitensis* 16M by allelic replacement and characterized it phenotypically. We first analyzed the ability of the newly constructed mutant to multiply within cultured macrophages. As shown in Fig. 1A, the intracellular bacterial load of the ΔmucR strain was significantly reduced at 24 h and 48 h postinfection (p.i.) in murine RAW 264.7 macrophages compared to the WT control. The intracellular replication of the ΔmucR strain was almost restored to WT levels in the complemented ΔmucR strain. These results are consistent with the reduced intracellular growth of a transposon mutant described previously using the mouse macrophage-like cell line J774.A1 (40). The difference between the ΔmucR and WT strains cannot be due to the mutant’s reduced capacity to invade the cells, because the number of intracellular bacteria at 1 h p.i. was the same for each strain. On the other hand, all strains displayed the same growth rate between 24 h p.i. and 48 h p.i. (Fig. 1A). The ΔmucR strain also showed reduced virulence in HeLa cells (data not shown). Taken together, these results confirm the importance of MucR in *B. melitensis* 16M intracellular infection, especially during the first hours of infection.

To verify that, as previously described for the transposon mutant (40), the mucR gene is involved in the successful infection process of *B. melitensis in vivo*, we compared the behaviors of the ΔmucR and WT strains in a mouse model of infection at 1 and 4 weeks p.i. (Fig. 1B). We observed a large reduction (more than a 2-log-unit difference) in the splenic bacterial load at just 1 week p.i. (Fig. 1B). Moreover, this virulence attenuation was associated with reduced splenomegaly (data not shown).

**Fluorescence microscopy.** Bacterial aggregates of *B. melitensis* overexpressing mucR (pBBRMucR) and WT *B. melitensis* were observed by scanning electron microscopy. The two strains were grown for 72 h in 2YT medium. Glass coverslips were treated with poly-L-lysine (0.05 mg ml$^{-1}$; Sigma) for 1 h at 4°C. For each strain, 1-ml cell suspensions were centrifuged onto the pretreated coverslips at 1,000 rpm for 10 min at room temperature. The medium was removed, and adherent cells were fixed for 20 min with 2% PFA in PBS. After the incubation, samples were washed with PBS and dehydrated twice 10 min in 25, 50, 75, 85, and 100% ethanol at room temperature. Dehydrated samples were then prepared by critical-point drying (Balzer; CPD 030) and covered with a thin layer of gold (25 nm). Examinations were performed using a scanning electron microscope (Jeol 7500F) at the University of Namur, Namur, Belgium.

**Statistical analysis.** For the mouse experiments, we used a Mann-Whitney test included in the program GraphPad Prism to statistically analyze our results. *P* values of <0.05 were considered to represent a significant difference.

For the cellular infections, oxidative-resistance assays, and qRT-PCR, after testing for homogeneity of variance (Bartlett test), one-way analysis of variance (ANOVA) was performed on the log$_{10}$ CFU per well, on the survival percentage values, or on the ΔCT values, respectively. When needed, a Scheffé’s comparison test was performed, and statistical significance at a *P* value of <0.05 was accepted.
resist exposure to exogenous hydrogen peroxide (H₂O₂). After a
active against heat and oxidative challenges (61, 62).
limited nutrient availability associated with entry into stationary
ity (60). Moreover, it has been shown in other bacteria that the
these stresses has been correlated with stationary-phase physiol-
phages, including exposure to reactive oxygen species (ROS) and
spp. have to withstand various harsh environmental condi-
polymyxin B.
mutants whose adaptations upon entering stationary
vant to its reduced virulence, as previously suggested for other
Brucella mutants whose adaptations upon entering stationary
phase are affected (60).
The
mucR
mutant is more sensitive to H₂O₂, detergents, and pol
mucR
B. melitensis strains in Raw 264.7 murine macrophages. The data are the average log₁₀ CFU per well. The error bars represent the standard deviations of triplicates in one of three representative experiments.

FIG 1 MucR is crucial for the virulence of B. melitensis in both cellular and mouse models of infection. (A) Intracellular replication of WT, ΔmucR, and ΔmucR pBBRmucR B. melitensis strains in Raw 264.7 murine macrophages. The lines indicate the mean log₁₀ CFU for each group (n = 15). Statistical analysis was performed using the Mann-Whitney test (***, P < 0.0001).

FIG 2 Growth curves of WT and ΔmucR B. melitensis strains in 2YT rich medium. Cultures were inoculated from a preculture to an initial OD₆₀₀ of 0.05 in a Bioscreen plate. Bacteria were grown for 72 h with continuous shaking in 2YT medium. The optical density was measured every 30 min. The graph represents the average OD of technical triplicates for each condition from one of two representative experiments.

The
mucR
mutant displays an altered LPS profile. In the brucellae, LPS is a major virulence factor, and LPS alterations can generally impede the successful infection process (63) and, in particular, resistance to antimicrobial compounds (64). Like the WT strain, the ΔmucR strain was smooth, as determined by crystal violet staining (Fig. 4 and data not shown). Nevertheless, to detect some subtle LPS alterations, we examined the LPS migration patterns for their smooth LPS (S-LPS). Similarly, their reactivities with the anti-O-antigen MAb A76/12G12/F12 in a Western blot were comparable (Fig. 4B).
However, the lipid A core fraction of the ΔmucR mutant migrated faster than the corresponding WT fraction. This observation suggests that there is a modification of the lipid A core. This hypothesis was corroborated by differences in the reactivities of the extracts with anti-core MAb A68/24G12/A8 and A68/24D8/G9 (Fig. 4B) in a Western blot analysis. Normal SDS profiles and epitope detection were restored upon complementation in trans or via chromosomal insertion of the mucR gene (Fig. 4).

The promoter activity of mucR is induced in 2YT medium containing 400 mM NaCl, correlating with morphological alterations of B. melitensis. We have reported previously that Brucella spp. form bacterial aggregates over prolonged culture times when grown in 2YT medium containing 400 mM NaCl (57). Because MucR in S. meliloti regulates EPS I biosynthesis (18, 65) and is somehow involved in environmental-stress response (Fig. 3), we tested whether MucR is required to form these bacterial clumps in B. melitensis 16M. The ΔmucR strain displayed a significant growth defect when cultured in 2YT medium containing 400 mM NaCl (see Fig. S2 in the supplemental material), suggesting that MucR is required for optimal growth under hypersaline conditions.

We used a B. melitensis strain carrying the reporter plasmid pBBRpMucRgfp (ASV) (see Materials and Methods) to monitor mucR promoter (PmucR) activity in B. melitensis at different culture times in unsupplemented 2YT medium or 2YT medium supplemented with 400 mM NaCl. The B. melitensis strain harboring the promoterless vector pBBR-gfp (ASV) was used as a negative control. These strains displayed similar growth curves under both conditions (Fig. 5A). Figure 5C to H represents the fluorescent signal intensities measured by flow cytometry in the reporter strain at different culture times in either 2YT medium or 2YT medium plus 400 mM NaCl. In 2YT medium, PmucR activity is constitutive and gives a mean fluorescent channel intensity (MFI) of approximately 40 to 50, independent of the culture time considered (Fig. 5). Under hypersaline conditions (blue curve), PmucR activity is induced beginning at 8 h p.i. (MFI = 200) and is then strongly enhanced as the culture time increases (MFI = 300, 1,000, and 1,500 for 12, 24, and 48 h p.i., respectively). These data were confirmed by fluorescence microscopy (Fig. 5, insets). The progressive and major morphological changes of the bacteria were most notable when grown at such salt concentrations (Fig. 5, insets). An equatorial bulge can be distinguished after only 8 h of growth in hypersaline 2YT (Fig. 5D). The subsequent increase in the bulge in the ongoing culture results in an increase in cell size, which is also evident in the small MFI shift for the negative control (Fig. 5, compare the green curve to the violet curve). This cell shape alteration seems to be salt specific, because it did not occur under iso-osmolar conditions with sucrose (see Fig. S1 in the supplemental material). At equivalent osmolalities, the NaCl-supplemented medium promotes cell shape alterations and strongly induces PmucR activity compared to cells grown under sucrose-supplemented conditions (see Fig. S1). However, the fluorescent
signal for cells grown for 24 h in sucrose-supplemented 2YT medium seems to be slightly higher than the signal for cells grown in regular 2YT medium (see Fig. S1). Together, these data suggest that the mucR promoter is induced by osmotic stress but even more highly induced by ionic stress in B. melitensis.

**MucR regulates cyclic β-glucan synthase mRNA levels.** The various susceptibilities reported above suggest that there is a major cell envelope alteration in B. melitensis mutants lacking mucR. Susceptibility to detergents (deoxycholic acid, SDS, and Zwittergent) has been described for a cgs mutant of Brucella abortus,
which was unable to produce periplasmic cyclic β-glucans (cβG) (66). To determine whether MucR can transcriptionally regulate cβG synthesis and cgt (encoding the cβG transporter) mRNAs were evaluated by qRT-PCR on RNA purified from the different strains harvested during the exponential growth phase (OD₆₀₀ = 0.5) in 2YT medium. Although a mucR deletion does not affect cgt transcript levels, we found a nearly 2-fold reduction in cgs transcripts in the ΔmucR mutant compared to the WT strain (Table 2). WT levels of cgs mRNA were restored upon complementation with the plasmid pBBRmucR (Table 2). Although additional studies are needed, these results suggest that MucR could be the first regulator of cβG identified in B. melitensis 16M.

Restoration of EPS I production in an S. meliloti mucR mutant through constitutive expression of B. melitensis mucR. The mucR gene is well conserved within Rhizobiales, especially in S. meliloti (61% amino acid identity), where its function has been well studied. In S. meliloti, MucR has been described as a regulator of both flagellar-gene expression and EPS synthesis (21). S. meliloti produces two different types of EPS: succinoglycan, which was first described as a calcofluor-binding acidic exopolysaccharide (EPS I), and galactoglucan (EPS II). Strains producing one versus the other type of EPS can be rapidly discriminated on agar medium: an S. meliloti mucR mutant carrying the wild-type mucR gene of B. melitensis 16M (mucR mutant carrying the wild-type mucR gene of B. melitensis 16M) was spotted in triplicate on LB medium containing 0.02% calcofluor and incubated at 30°C for 2 days before being subjected to UV light.

We therefore examined the putative impact of MucR on flagellar-gene regulation in B. melitensis 16M. Even though they are described as nonmotile, Brucella spp. possess flagellar genes that are expressed only in the early log phase of growth in rich medium (26). Using specific antibodies against FlgE (flagellin), we examined FlgE protein expression at different growth phases in the mucR mutant compared to the WT strain (Fig. 7A). We detected

![Figure 6](https://example.com/figure6.png)

**FIG 6** Heterospecific complementation of an S. meliloti mucR mutant with the mucR gene from B. melitensis on LB plates containing calcofluor. The strains Rm1021 (wild type), Rm1021 exoY:Tn5 (non-EPS I-producing strain), Rm2011 (wild type), Rm101 (mucR mutant), and Rm101 pBBRmucR<sub>rem</sub> (mucR mutant carrying the wild-type mucR gene of B. melitensis 16M) were spotted in triplicate on LB medium containing 0.02% calcofluor and incubated at 30°C for 2 days before being subjected to UV light.

![Figure 7](https://example.com/figure7.png)

**FIG 7** Western blot detection of the flagellar proteins FlgC and FlgE. (A) Detection of flagellin (FlgC) production in B. melitensis WT, ΔflIC, and ΔmucR strains in both early log phase and stationary phase. The strains were cultivated in 2YT broth, and extracts were prepared from samples harvested at the beginning of the exponential phase of growth (OD₆₀₀ = 0.2) (lanes 1) and in the stationary phase (OD₆₀₀ = 1.0) (lanes 2). (B and C) FlgE (B) or FlgC (C) expression in the ΔmucR mutant in stationary phase. Complementation of mucR<sub>rem</sub> in an S. meliloti Rm101 background restored EPS I production (Fig. 6). These data suggest that the mucR gene from B. melitensis 16M encodes a fully functional protein that is at least able to regulate the expression of EPS biosynthesis genes in S. meliloti.

MucR represses flagellar-gene expression by modulating mRNA levels. In addition to regulating EPS production, MucR also regulates flagellar-gene expression in S. meliloti. Indeed, it has been shown that MucR inhibits expression of rem, the flagellar master regulator (67), and consequently, the expression of rem-regulated genes (21). Our laboratory has previously shown that the Brucella flagellar master regulator is orthologous to Rem (68).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Locus tag</th>
<th>ΔmucR WT ratio</th>
<th>ΔmucR pBBRmucR WT ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>flIC</td>
<td>BMEI0150</td>
<td>4.62</td>
<td>0.95</td>
</tr>
<tr>
<td>fígE</td>
<td>BMEI0159</td>
<td>3.78</td>
<td>0.96</td>
</tr>
<tr>
<td>ftC</td>
<td>BMEI0158</td>
<td>4.45</td>
<td>1.35</td>
</tr>
<tr>
<td>flIF</td>
<td>BMEI0151</td>
<td>6.81</td>
<td>1.69</td>
</tr>
<tr>
<td>cgs</td>
<td>BMEI1837</td>
<td>0.55</td>
<td>0.96</td>
</tr>
</tbody>
</table>

*The relative levels of cgs, flIC, fígE, ftC, and flIF mRNAs were determined using quantitative real-time PCR on RNA isolated from bacteria harvested at mid-exponential growth phase in rich medium (2YT). Normalization was performed using 16S rRNA.*

We analyzed the ΔCT values from biological triplicates was used for statistical analysis after testing the homogeneity of variance (Bartlett test). Scheffe’s comparison test was performed, and statistical significance was obtained (at a P value of <0.05) between the ΔCT values of the ΔmucR and WT or complemented strains.

*MucR regulates the mRNA levels of the flagellar and cyclic β-glucan synthase genes*
MucR is a functional orthologue of S. meliloti MucR. The mucR gene is highly conserved in Rhizobiales (70) and encodes a protein predicted to contain a C$_2$H$_2$-type zinc finger motif. Zinc finger-containing proteins include DNA binding proteins that are able to bind a zinc ion via a conserved structure (e.g., through cysteine and histidine amino acids) (71). In all the bacteria in which MucR orthologues have been characterized, this regulator controls various cell envelope modifications with a common theme of exopolysaccharide synthesis and altered host-bacterial interaction (72–74). In S. meliloti, where MucR (61% identity with B. melitensis MucR) has been the most extensively studied, the regulator couples motility regulation with EPS production (21). The S. meliloti MucR protein appears to be highly specific for its own DNA recognition sequence, because it does not bind to sites recognized by Ros, the orthologous regulator in Agrobacterium tumefaciens (61% identity to B. melitensis MucR) (69, 75). In contrast, as shown on calcofluor plates (Fig. 6), the constitutive expression of the B. melitensis mucR gene in a mucR mutant of S. meliloti (Rm101) was able to restore EPS I (succinoglycan) production. This heterospecific-complementation experiment suggests that the mucR gene of B. melitensis 16M encodes a functional protein able to recognize MucR-specific promoter-targeting sequences, or at least the promoter(s) regulating the expression of EPS biosynthesis genes in S. meliloti.

**B. melitensis MucR also controls flagellar-gene expression and the formation of the aggregative phenotype.** Our results suggest that MucR negatively autoregulates its own transcription, as confirmed by reporter assays (Fig. 8). In addition to pleiotropic complementable phenotypes, which we discuss here and tentatively correlate with the attenuation of the ΔmucR mutant strain. In this study, we report the extensive characterization of an in-frame deletion mutant of B. melitensis mucR. This gene was previously identified as necessary for virulence in a transposon mutagenesis screen of B. melitensis but never characterized (40). Our study has confirmed that a ΔmucR mutant is attenuated in both cellular and murine models of infection. Our study has also shown that the most plausible explanation for its attenuation is a deficiency in intracellular survival, rather than a deficiency in cellular invasion.

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clearly indicate that MucR of *B. melitensis* is also a repressor of flagellar-gene expression and likely acts upstream of FtcR, the flagellar master regulator orthologous to Rem (68) through which MucR of *S. meliloti* acts (21, 67). In *B. melitensis* 16M, flagellar-gene expression is very tightly regulated, given that the QS regulator VjbR (27), the sigma factors rpoE1 (76, 77) and rpoH2 (76), and the cyclic-di-GMP phosphodiesterase BpdA (78) have already been shown to be involved in flagellar regulation. Here, we show that MucR is an additional actor within this complex regulatory network. According to the previous transcriptomic analyses, *mucR* expression is under the control of QS regulators (39). We confirmed these data by qRT-PCR using RNA extracts from Δ*vjbR* and WT strains at the end of log phase in rich medium (data not shown). This indicates that VjbR represses *mucR* expression and thus allows *fcr* expression and subsequent flagellar-gene expression. Together, these results support the hypothesis that MucR plays an intermediate role between VjbR and the regulation of flagellar-gene expression.

It has previously been reported that *B. melitensis* QS mutants form bacterial aggregates in 2YT medium (29, 79). Moreover, we recently described a peculiar growth condition (2YT plus 400 mM NaCl for 72 h) in which WT *B. melitensis* also has a similar aggregative phenotype (57). This behavior could result from the production of EPS and/or a modification of envelope/surface properties. Because MucR is functionally conserved between *S. meliloti* and *B. melitensis* and because MucR in *S. meliloti* regulates EPS I biosynthesis (Fig. 6)(18, 65), we tested whether MucR is involved in the formation of bacterial clumps in *B. melitensis* 16M. Unfortunately, the *ΔmucR* strain displays a growth defect in hypersaline medium (see Fig. S2 in the supplemental material). Therefore, the absence of clumps in the culture could be due to poor growth or to the inability to properly respond to hypersaline stress. These data are consistent with the strong induction of the promoter P*mucR* due to ionic stress, which reinforces the idea that MucR is necessary to optimally respond to the hypersaline stress and could be required to promote aggregation. In this context, we showed that a *mucR*-overexpressing strain, in contrast to the WT strain, developed bacterial aggregates when grown in standard 2YT medium for 72 h (see Fig. S3 in the supplemental material), indicating that MucR could actually be involved in clump formation.

The aggregative phenotype of the *mucR*-overexpressing strain and the cell envelope modifications and flagellar-gene activation of the Δ*mucR* mutant appear to be coordinated phenotypes that are reminiscent of the paradigmatic transition from a “planktonic” to a “sessile” form of life, which often implies the inversely coordinated expression of surface polysaccharide components and the flagellar apparatus (11, 21, 80, 81).

Altogether, the negative autoregulation of MucR (Fig. 8), its negative impact on both flagellar mRNA and protein levels (Fig. 7 and Table 2), and the ability to form aggregates (Fig. 6; see Fig. S3 in the supplemental material) seem to be conserved between *S. meliloti* and *B. melitensis*. Moreover, in both species, MucR may possibly bind to a similar DNA binding site. This reinforces the idea that these bacterial species have evolved from a common ancestor and share molecular mechanisms for their interactions with their respective hosts (82, 83). Notably, the induction of *PmucR* activity by ionic stress and, to a lesser extent, osmotic stress (see Fig. S1 in the supplemental material) constitute the first factors identified that affect the expression of the autoregulated *mucR* gene in an alphaproteobacterium.

The *mucR* mutant has an altered growth phenotype and an exacerbated sensitivity to oxidative stress. Despite having the same lag phase and a similar growth rate in a rich medium *in vitro*, the Δ*mucR* strain enters stationary phase much earlier (and thus at a lower OD<sub>600</sub>) than the WT strain. As previously demonstrated in a *B. abortus hfg* mutant, stationary-phase physiology plays an important role in the ability of brucellae to establish and maintain long-term intracellular residence in host macrophages (84) and probably also to cope with the stresses of the early, nonreplicative phase (the first 10 h) of intracellular infection (85). A common feature of all these conditions is exposure to oxidative stress either through the accumulation of endogenous oxygen radicals (86–88) or following the oxidative burst inside infected macrophages (89). Based on published microarray analysis, the involvement of *mucR* in stress response mechanisms has been suggested for *B. melitensis* (41). Consistent with this hypothesis, we showed a strong increase in the sensitivity of the Δ*mucR* mutant to exogenous oxidative stress compared to the WT strain. This feature could partially explain the 2-log-unit decrease in survival in macrophages that this and other studies have described (Fig. 1) (40) and that we have also seen in HeLa cells.

Δ*mucR* sensitivity to detergents and polymyxin correlates with cell envelope alterations. In addition to the sensitivity to oxidative stress, an increased susceptibility to detergents (SDS and Triton) and polymyxin B was also observed with the Δ*mucR* strain. These observations and the altered staining of the mutant colonies with Congo red suggest that there are major cell surface alterations in this *B. melitensis* mutant. A link between reduced virulence and sensitivity to detergents has also been observed in *B. abortus* cyclic β-glucan synthase (*cgs*) (66) and *bvrR*-*bvrS* TCS mutants, which are also more sensitive to bactericidal polycationic substances (polymyxin B, melittin, and poly-l-lysine) (28). Mutants in the latter system have a severely altered cell envelope, including lipid A modifications (37, 38, 90).

Consistent with the detergent susceptibility assays, our qRT-PCR data suggest that MucR positively regulates *B. melitensis* cyclic glucan synthase (*cgs*). Unfortunately, we were unable to show modified Cgs protein expression by Western blotting using a previously described antibody (91) because the antibody generated a positive signal only in the *cgs*-overexpressing strain (D. Comerci, personal communication).

Further investigations would be necessary to test whether there is a change in cyclic β-glucan production in the different strains. If so, MucR would be the first regulator of *cgs* identified in brucellae.

In conclusion, we propose that the conserved MucR regulator plays multiple roles in *B. melitensis* 16M, controlling growth in bacteriological medium, virulence in macrophages and mice, flagellar expression, aggregation, and cell envelope homeostasis. Interestingly, in *B. abortus* 2308, MucR (100% amino acid identity with *B. melitensis* MucR) was very recently found (C. Caswell and R. M. Roop, personal communication) to be involved in virulence (in macrophage and spleen colonization in mice) and growth in bacteriological medium, illustrating the similarities between the roles of MucR in these two *Brucella* strains. Moreover, the auto-regulation of *mucR* also occurs in *B. abortus*, in which MucR has been shown by electrophoretic mobility shift assay (EMSA) to bind its own promoter (100% nucleotide identity with *B. meliten- tis*) (C. Caswell and R. M. Roop, personal communication). In *B. abortus* 2308, MucR controls the expression of several transcriptional regulators, such as *babR* and *nolR* (C. Caswell and R. M.
Roop, personal communication), suggesting that it can coordinate the expression of several regulons, which would explain the pleiotropic phenotype of mucR mutants.

Interestingly, we have identified a probable alteration in the core lipid A structure of the *B. melitensis* δmucR mutant. In fact, the δmucR background has a modified Western blot staining pattern for anti-core MAbs compared to a WT background or the complemented mutants. Interestingly, recognition of the free (not linked to the O chain) core lipid A structure is far less altered than the recognition of the O-chain-linked core (Fig. 4B). This staining pattern is reminiscent of a staining pattern described recently for a mutant for a putative core glycosyl transferase (WadC) (92). *wadC* transcript levels were not altered in the *mucR* mutant background (data not shown). Surprisingly, the *B. abortus* δmucR mutant reported here remains smooth, illustrating that there are remarkable differences in MucR function between *Brucella* strains that would be worth investigating. These phenotypic differences could likely be linked to still unidentified differences in the core lipid A proteins of these closely related species (93). For example, differences in the negative charge of the core lipid A (64) could also account for the differences in polymyxin B resistance between *B. abortus* and *B. melitensis* (94). This impact of MucR on the core lipid A has never been reported in the alphaproteobacteria studied previously and is the recipient of a specialization grant from FRIA (Fonds pour la Formation à la Recherche dans l’Industrie et l’Agriculture).

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