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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**

Mirabella, A.; Terwagne, M.; Zygmunt, M. S.; Cloeckaert, A.; De Bolle, X.; Letesson, J. J.

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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

A. Mirabella,<sup>a</sup> M. Terwagne,<sup>a</sup> M. S. Zygmunt,<sup>b,c</sup> A. Cloeckaert,<sup>b,c</sup> X. De Bolle,<sup>a</sup> J. J. Letesson<sup>a</sup>

Unité de Recherche en Biologie Moléculaire (URBM), NARILIS, University of Namur (FUNDP), Namur, Belgium<sup>a</sup>; INRA, UMR1282 Infectiologie et Santé Publique, Nouzilly, France<sup>b</sup>; Université François Rabelais de Tours, UMR1282 Infectiologie et Santé Publique, Tours, France<sup>c</sup>

*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 *fltR*. 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). These multilayered 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  $\beta$ -(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 sulfatation 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  $\alpha$ -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 appendices, 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

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Address correspondence to J. J. Letesson, jean-jacques.letesson@fundp.ac.be.

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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/BvrR TCS, which is orthologous to ExoS/ChvI, 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 (29). Fifth, a transcriptional regulator with 61% identity to *S. meliloti* MucR was identified during a screen of *Brucella melitensis* transposon mutants (40). The *mucR* transposon mutant had decreased virulence in both cellular and mouse models of infection but was otherwise uncharacterized. More recently, the protective efficiency 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 detergents). 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-gene 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/BvrR 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<sup>-1</sup>, 16 g liter peptone<sup>-1</sup>, 5 g liter NaCl<sup>-1</sup>) containing the appropriate antibiotics from a stationary-phase overnight culture (2YT; 10 ml) back diluted to an optical density at 600 nm (OD<sub>600</sub>) of 0.05.

For RNA extraction, 10 ml of bacteria was harvested from a 200-ml 2YT culture grown to mid-log phase (OD<sub>600</sub> = 0.5). The 10-ml cultures were used to follow GFP(ASV) (green fluorescent protein) production from *B. melitensis* pBBRPmucRgfp(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 MgSO<sub>4</sub> and 2.5 mM CaCl<sub>2</sub> 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, gen-

tamicin, and nalidixic acid were used at 20, 50, and 25 (8 for *S. meliloti*) µg ml<sup>-1</sup>, respectively. Growth curves were monitored using a Bioscreen system (Thermo Fisher, Erembodegem-Aalst, Belgium), which continuously monitors OD<sub>600</sub> 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  $\Delta$ *mucR* deletion mutant was constructed by allelic replacement using a two-step strategy. Briefly, 500-bp upstream and downstream regions 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  $\Delta$ *mucR* fragment was cloned into pGEM-T Easy (Promega) to generate the intermediate vector pGEMT $\Delta$ *mucR*. The  $\Delta$ *mucR* fragment was excised by NotI restriction, subcloned into the final vector pJQ200-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  $\Delta$ *mucR*KI, we used the same recombination strategy using a plasmid carrying the *mucR* gene with its upstream and downstream regions.

**Construction of the complementation plasmid pBBR*mucR*.** The *mucR* gene was amplified by PCR from *B. melitensis* genomic DNA using the primers (Eurogentec) *mucR* XhoI F and *mucR* ClaI R, which contain XhoI and ClaI restriction sites, respectively. The PCR product was cloned into pGEM-T Easy (Promega) to generate the intermediate vector pGEMT*mucR*. After sequencing, the fragment *mucR* was excised by a ClaI and XhoI double restriction digest and subcloned into a previously XhoI-ClaI-restricted plasmid, pBBRMCS1, to obtain pBBR*mucR*. The vector was then transferred to the  $\Delta$ *mucR* strain to obtain the complemented  $\Delta$ *mucR* pBBR*mucR* strain.

**Construction of the reporter plasmid pBBRP*mucRgfp*(ASV).** The region containing the putative *mucR* promoter was amplified by PCR from *B. melitensis* genomic DNA using the primers XhoIp*mucRR* and BamHIp*mucRF*, which contain XhoI 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 pBBRP-*mucRgfp*(ASV).

**Cellular infection.** Evaluation of the intracellular survival of *B. melitensis* wild-type (WT) and  $\Delta$ *mucR* 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% CO<sub>2</sub> 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<sup>-1</sup> gentamicin<sup>-1</sup> at 37°C under 5% CO<sub>2</sub> 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 × 10<sup>4</sup> 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 log<sub>10</sub> CFU per spleen. Data were statistically

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Characteristics <sup>a</sup>	Source or reference
<i>B. melitensis</i>		
16M	WT; Nal <sup>r</sup> ; parental strain	A. Macmillan, Central Veterinary Laboratory, Weybridge, UK
16M pBBR-gfp(ASV)	Nal <sup>r</sup> Cm <sup>r</sup> ; WT carrying pBBR-gfp(ASV)	This study
16M pBBRpmucRgfp(ASV)	Nal <sup>r</sup> Cm <sup>r</sup> ; WT carrying pBBRpmucRgfp(ASV)	This study
$\Delta$ mucR	Nal <sup>r</sup> ; deletion strain for <i>mucR</i> gene	This study
$\Delta$ mucR KI	Nal <sup>r</sup> ; chromosomal insertion of the <i>mucR</i> gene in the $\Delta$ mucR strain	This study
$\Delta$ mucR pBBRMCS1	Nal <sup>r</sup> Cm <sup>r</sup> ; $\Delta$ mucR strain carrying the plasmid pBBRMCS1	This study
$\Delta$ mucR pBBRmucR	Nal <sup>r</sup> Cm <sup>r</sup> ; $\Delta$ mucR strain carrying the complementation plasmid pBBRmucR	This study
$\Delta$ mucR pBBRpmucRgfp(ASV)	Nal <sup>r</sup> Cm <sup>r</sup> ; $\Delta$ mucR strain carrying pBBRpmucRgfp(ASV)	This study
$\Delta$ mucRKI	Nal <sup>r</sup> Cm <sup>r</sup> ; $\Delta$ mucR KI strain carrying pBBRpmucRgfp(ASV)	This study
$\Delta$ vjbR	Nal <sup>r</sup> $\Delta$ vjbR::Kan <sup>r</sup> (referred to as CD100 in reference 29)	27
<i>S. meliloti</i>		
Rm1021	Nal <sup>r</sup> Sm <sup>r</sup> ; WT	42
Rm1021::exoY	Rm1021 <i>exoY</i> ::Tn5 (formerly Rm7210); Nal <sup>r</sup> Sm <sup>r</sup> Neo <sup>r</sup>	43
Rm2011	Nal <sup>r</sup> Sm <sup>r</sup> ; wild type	44
Rm101	Rm2011 <i>Spc</i> <sup>r</sup> cassette inserted into the <i>P</i> macl site of <i>mucR</i>	45
<i>E. coli</i>		
DH10B	F <sup>-</sup> <i>mcrA</i> $\Delta$ ( <i>mrr-hsdRMS-mcrBC</i> ) $\Phi$ 80 <i>lacZ</i> $\Delta$ M15 $\Delta$ <i>lacX74</i> <i>recA1</i> <i>endA1</i> <i>araD139</i> $\Delta$ ( <i>ara leu</i> )7697 <i>galU</i> <i>galK</i> <i>rpsL</i> <i>nupG</i> $\lambda^-$ (Sm <sup>r</sup> )	Gibco BRL
S17-1	<i>recA</i> <i>thi</i> <i>pro</i> <i>hsdR</i> <sup>-</sup> M <sup>+</sup> RP4:2-Tc:Mu:Km Tn7 $\lambda$ <i>pir</i> ; allows plasmid mobilization	46
Plasmids		
pBBR1MCS1	Broad-host-range cloning vector; high copy number; Cm <sup>r</sup>	47
pGEM-Teasy	Amp <sup>r</sup>	Promega
pJQ200-uc1	<i>sacB</i> Gtm <sup>r</sup>	48
pBBR-gfp(ASV)	Cm <sup>r</sup>	M. Terwagne
pJQ $\Delta$ mucR	<i>sacB</i> Gtm <sup>r</sup> strain containing the $\Delta$ mucR fragment; used to construct the deletion strain	This study
pJQmucR	<i>sacB</i> Gtm <sup>r</sup> strain containing the <i>mucR</i> gene flanked by its upstream and downstream regions	This study
pBBRmucR	Cm <sup>r</sup> ; complementation plasmid	This study
pBBRpmucRgfp(ASV)	Cm <sup>r</sup> ; reporter plasmid bearing the transcriptional fusion <i>P</i> mucRgfp(ASV)	This study

<sup>a</sup> Nal<sup>r</sup>, nalidixic acid resistance; Sm<sup>r</sup>, streptomycin resistance; Neo<sup>r</sup>, neomycin resistance; Cm<sup>r</sup>, chloramphenicol resistance; *Spc*<sup>r</sup>, spectinomycin resistance; Amp<sup>r</sup>, ampicillin resistance; Gtm<sup>r</sup>, gentamicin resistance.

analyzed via a Mann-Whitney statistical test using the program Prism. The animal-handling and study procedures were in accordance with the current European legislation (directive 86/609/EEC) and in agreement with the corresponding Belgian law, *Arrêté royal relatif à la protection des animaux d'expérience du 6 avril 2010 publié le 14 mai 2010*. The complete protocol was reviewed and approved by the Animal Welfare Committee of the Facultés Universitaires Notre-Dame de la Paix (FUNDP), Belgium (permit number 05-558).

**Oxidation-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  $5 \times 10^5$  CFU ml<sup>-1</sup> in PBS.

In 96-well plates, 50  $\mu$ l of the bacterial suspension was supplemented with 50  $\mu$ l of H<sub>2</sub>O<sub>2</sub> (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  $\mu$ l of PBS (without H<sub>2</sub>O<sub>2</sub>) 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  $\mu$ l 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  $\mu$ g ml<sup>-1</sup> (bioMérieux). *Brucella* was adjusted to an OD<sub>750</sub> 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  $\Delta$ mucR mutant, and the complemented  $\Delta$ mucR 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-

ware (Applied Biosystems) to generate PCR products ranging from 80 to 100 bp and are listed in Table S2 in the supplemental material. Reactions performed without reverse transcriptase were used as negative controls to test for DNA contamination. Real-time PCRs were performed with SYBR green mix (Applied Biosystems) in 96-well optical reaction plates (Applied Biosystems). Ratios were calculated using the  $\Delta\Delta CT$  method for each primer in an Applied Biosystems Step One Plus real-time PCR instrument. The results for each target mRNA were normalized to 16S rRNA transcript levels and averaged.

**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<sub>600</sub> 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 (Amersham) for Western blotting (54). 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).

**Western blotting.** Nitrocellulose membranes were blocked overnight in PBS containing 5% nonfat dry milk. After being washed three times in PBS-0.05% Tween 20 for 10 min, the membranes were incubated for 1 h with the primary antibodies diluted in PBS (0.05% Tween 20, 1% dry milk), washed three times in PBS-0.05% Tween 20 for 10 min, incubated for 1 h with the secondary antibody diluted in PBS (0.05% Tween 20, 1% dry milk), and finally washed three times for 10 min in PBS-0.05% Tween 20. The blots were developed using an enhanced chemiluminescence (ECL) system (100 mM Tris-HCl, pH 8.5, 0.009% H<sub>2</sub>O<sub>2</sub>, 0.2 mM coumaric acid, and 1.25 mM luminol).

**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-FlaC (diluted 1:3,000) or anti-FlaE (1:5,000) rabbit polyclonal antibodies (26). PrlR, which was used as a loading control, was detected using anti-PrlR 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 i80 fluorescence microscope through a 100 $\times$  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 a FACScalibur using CellQuest software (Becton Dickinson) as previously described (59). Bacteria were gated according to size and scatter to eliminate debris from analysis. Then, 50,000 individual events were excited with a 488-nm argon ion laser, and emission light was detected through a 530-nm bandpass filter.

**Scanning electron microscopy.** Bacterial aggregates of *B. melitensis* overexpressing *mucR* (pBBR*mucR*) 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<sup>-1</sup>; 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<sub>10</sub> CFU per well, on the survival percentage values, or on the  $\Delta CT$  values, respectively. When needed, a Scheffé's comparison test was performed, and statistical significance at a *P* value of <0.05 was accepted.

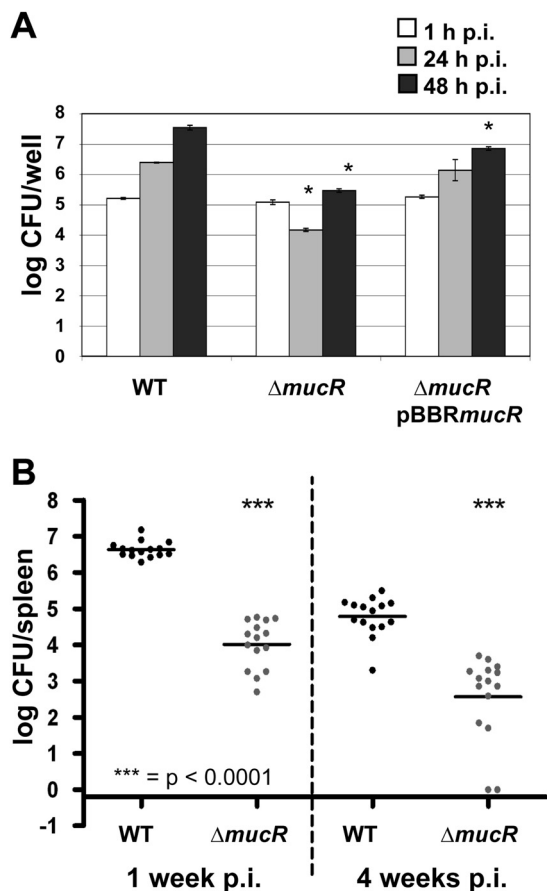
## RESULTS

**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 BMEI1364 (referred as  $\Delta$ *mucR*) 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  $\Delta$ *mucR* 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  $\Delta$ *mucR* strain was almost restored to WT levels in the complemented  $\Delta$ *mucR* 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  $\Delta$ *mucR* 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  $\Delta$ *mucR* 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  $\Delta$ *mucR* 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. in BALB/c mice infected with the  $\Delta$ *mucR* strain. Decreased persistence of the  $\Delta$ *mucR* strain was also consistently observed at 4 weeks p.i. (Fig. 1B). Moreover, this virulence attenuation was associated with reduced splenomegaly (data not shown).

**The *mucR* mutant enters prematurely into stationary phase in bacterial cultures.** The attenuation reported above could be caused by an *in vitro* growth defect that is exacerbated in intracellular environments; we therefore examined the growth rates of the  $\Delta$ *mucR* and WT strains in rich 2YT broth.

The  $\Delta$ *mucR* mutant had a growth rate similar to that of the WT until 22 h (log phase) (Fig. 2). At this time point, the  $\Delta$ *mucR* 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  $\Delta$ *mucR* mutant is poorly adapted to cope with the stresses linked to stationary phase, which would be rele-

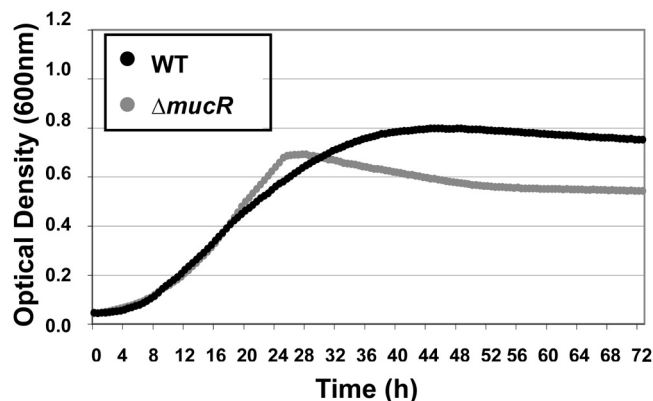


**FIG 1** MucR is crucial for the virulence of *B. melitensis* in both cellular and mouse models of infection. (A) Intracellular replication of WT,  $\Delta mucR$ , and  $\Delta mucR$  pBBRmucR *B. melitensis* strains in Raw 264.7 murine macrophages. The data are the average  $\log_{10}$  CFU per well. The error bars represent the standard deviations of triplicates in one of three representative experiments. Significant differences between the strains are noted by an asterisk ( $P < 0.05$ ). (B) Virulence of WT and  $\Delta mucR$  *B. melitensis* strains in the spleens of BALB/c mice. The graph represents pooled data from two independent experiments and indicates the  $\log_{10}$  CFU per spleen in each mouse. The lines indicate the mean  $\log_{10}$  CFU for each group ( $n = 15$ ). Statistical analysis was performed using the Mann-Whitney test (\*\*\*,  $P < 0.0001$ ).

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  $H_2O_2$ , detergents, and polymyxin B.** As a consequence of their intracellular lifestyle, *Brucella* spp. have to withstand various harsh environmental conditions in their phagosomal compartments within host macrophages, including exposure to reactive oxygen species (ROS) and nutrient deprivation. The successful adaptation of *Brucella* to these stresses has been correlated with stationary-phase physiology (60). Moreover, it has been shown in other bacteria that the limited nutrient availability associated with entry into stationary phase triggers a general stress response that can be cross-protective against heat and oxidative challenges (61, 62).

We therefore evaluated the ability of the  $\Delta mucR$  mutant to resist exposure to exogenous hydrogen peroxide ( $H_2O_2$ ). After a 1-hour exposure to various concentrations of  $H_2O_2$ , the survival rates of the  $\Delta mucR$  strain were significantly reduced compared to



**FIG 2** Growth curves of WT and  $\Delta mucR$  *B. melitensis* strains in 2YT rich medium. Cultures were inoculated from a preculture to an initial  $OD_{600}$  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.

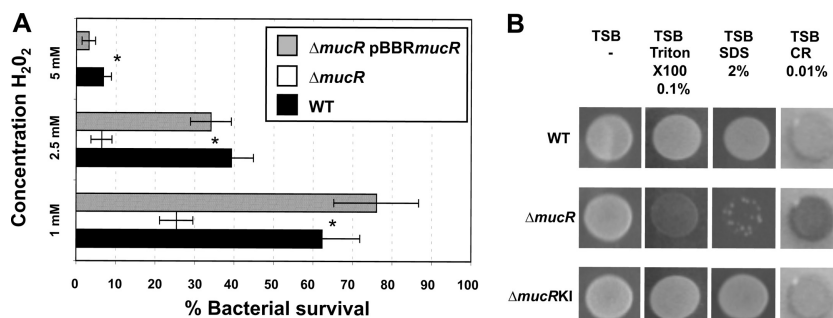
the WT strain under the same conditions (Fig. 3A). The sensitivity of the  $\Delta mucR$  mutant to this exogenous oxidative stress was complemented in *trans* with the plasmid pBBRmucR (Fig. 3A).

We further hypothesized that a *mucR* mutation would generate sensitivity to other stresses. To test this idea, we evaluated bacterial growth on media containing detergents, including 0.1% Triton X-100 and 2% sodium dodecyl sulfate (SDS). While these concentrations did not inhibit the growth of the WT strain, the  $\Delta mucR$  strain had impaired growth/survival in the presence of both detergents (Fig. 3B).

Furthermore, on a medium containing 0.01% Congo red, we observed that the  $\Delta mucR$  strain retains much more of the dye, resulting in a more intense dark-red appearance (Fig. 3B). All these growth or staining phenotypes were restored by supplying *mucR* either on a plasmid or via chromosomal insertion (Fig. 3). Together, these observations suggest that there has been a major cell envelope alteration that affects the susceptibility to these compounds in a *B. melitensis* strain lacking *mucR*.

Because envelope defects in *Brucella* have been associated with sensitivity to antimicrobial peptides, such as polymyxin B (28), we used a polymyxin B Etest (bioMérieux) strip to determine the MICs of polymyxin B for all the strains. The WT had a MIC of 64 units, whereas the MIC for  $\Delta mucR$  was 32 units. This result confirms the previous indications that the  $\Delta mucR$  strain has an altered cell envelope.

**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  $\Delta mucR$  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 pattern and the reactivity of the  $\Delta mucR$  strain to anti-LPS MAb compared to the WT strain. Figure 4A shows the SDS-PAGE profiles of SDS-proteinase K-treated extracts of WT *B. melitensis* (lane 1) and the  $\Delta mucR$  mutant (lane 2) strains. As expected, the two strains showed similar 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).



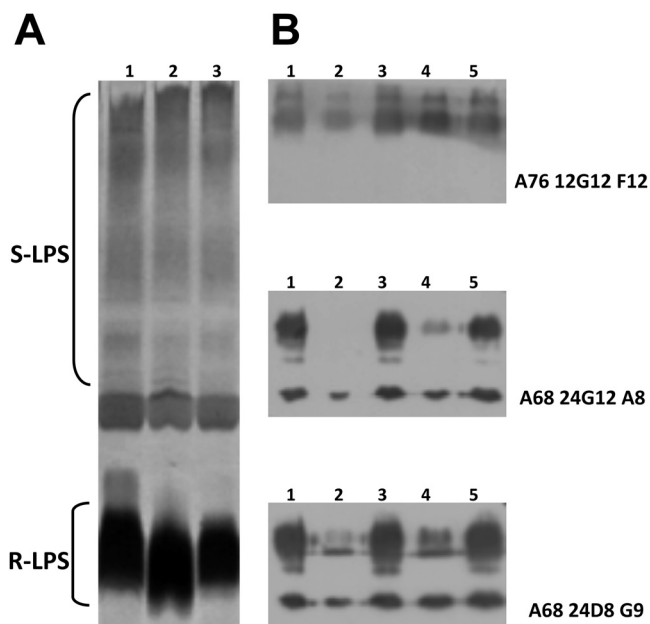
**FIG 3** Sensitivity to oxidative stress and detergents. (A) Survival of WT,  $\Delta$ mucR mutant, and  $\Delta$ mucR pBBRmucR *B. melitensis* at various concentrations of H<sub>2</sub>O<sub>2</sub>. The data are the averages of log<sub>10</sub> CFU per well. The error bars represent the standard deviations of triplicates from one of three representative experiments. Significant differences between the strains are indicated by asterisks ( $P < 0.05$ ). (B) Susceptibility of *B. melitensis* to surfactants and Congo red. Strains from an overnight preculture were spotted onto TSB plates containing the detergent Triton X-100 (0.1%), SDS (2%), or Congo red (CR) (0.01%) in triplicate. The plates were incubated for 4 days at 37°C. One representative spot is shown for each strain. The  $\Delta$ mucR pBBRmucR strain gave the same results as the  $\Delta$ mucRK1 strain (data not shown).

However, the lipid A core fraction of the  $\Delta$ mucR 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 MAbs 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).

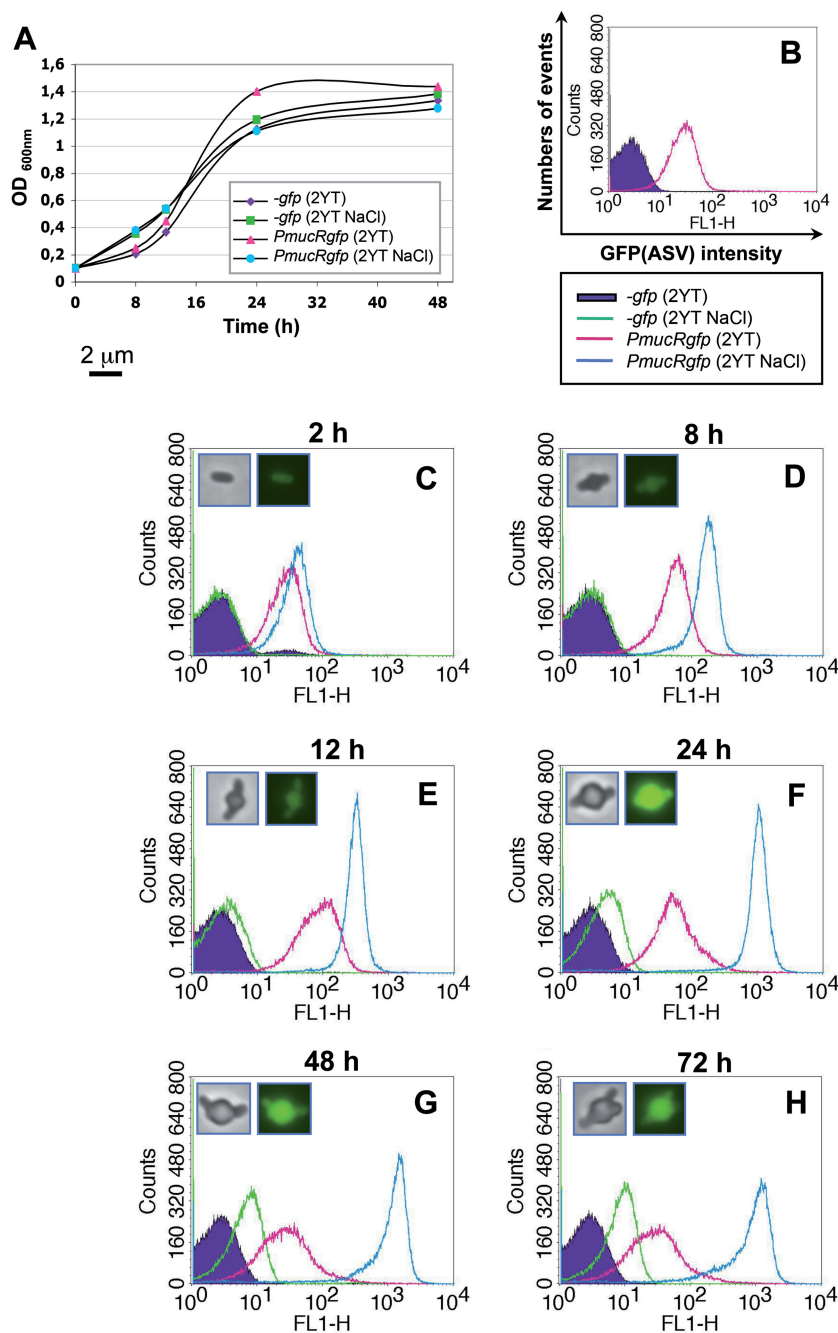
**The promoter activity of *mucR* is induced in 2YT medium containing 400 mM NaCl, correlating with morphological alterations of *B. melitensis*.** We have reported previously that *Brucella*

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  $\Delta$ mucR 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-osmolal 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



**FIG 4** Modification of LPS in the  $\Delta$ mucR strain. (A) Silver staining of SDS-proteinase K-treated extracts following electrophoresis on a 16% acrylamide gel. Lane 1, WT; lane 2,  $\Delta$ mucR; lane 3,  $\Delta$ mucRK1. (B) Western blot with anti-LPS MAbs. Total extracts were separated on a 15% acrylamide gel by electrophoresis, transferred to nitrocellulose membranes, and probed using anti-O-antigen MAb A76/12G12/F12 and anti-core MAbs A68/24G12/A8 and A68/24D8/G9. Lane 1, WT; lane 2,  $\Delta$ mucR; lane 3,  $\Delta$ mucRK1; lane 4,  $\Delta$ mucR pBBRmucR; lane 5,  $\Delta$ mucR pBBRmucR. S-LPS, smooth LPS; R-LPS, rough LPS.



**FIG 5** *PmucR* activity is induced in *B. melitensis* growing in 2YT medium containing 400 mM NaCl. (A) Growth curve of the reporter [*B. melitensis* WT expressing the transcriptional fusion *PmucRgfp*(ASV)] and the control [the WT harboring the plasmid pBBR-*gfp*(ASV)] strains in both 2YT medium (85.5 mM NaCl) and 2YT medium containing 400 mM NaCl. (B) Fluorescence intensity measured by flow cytometry ( $5 \times 10^4$  events acquired) of WT *B. melitensis* expressing the transcriptional fusion *PmucRgfp*(ASV) at the end of preculture in 2YT medium. The WT harboring the plasmid pBBR-*gfp*(ASV) was used as a negative control. (C to H) Fluorescence intensities measured in the reporter strains growing in unsupplemented 2YT medium or 2YT medium supplemented with 314.5 mM NaCl at 2 h (C), 8 h (D), 12 h (E), 24 h (F), 48 h (G), and 72 h (H) postinoculation. The insets are phase-contrast and corresponding fluorescence images of the major morphotype of the reporter strain in 2YT medium containing 400 mM NaCl at the indicated times. Scale bar, 2  $\mu$ m.

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  $\beta$ -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*,

**TABLE 2** MucR regulates the mRNA levels of the flagellar and cyclic  $\beta$ -glucan synthase genes<sup>a</sup>

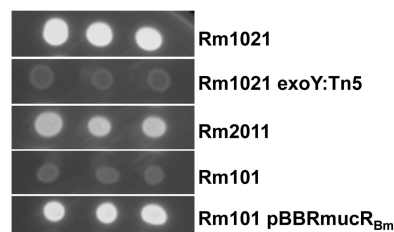
Gene	Locus tag	$\Delta mucR$ /WT ratio	$\Delta mucR$ pBBRmucR/WT ratio
<i>fliC</i>	BMEII0150	4.62	0.95
<i>flgE</i>	BMEII0159	3.78	0.96
<i>flcR</i>	BMEII0158	4.45	1.35
<i>fliF</i>	BMEII0151	6.81	1.69
<i>cgs</i>	BMEI1837	0.55	0.96

<sup>a</sup> The relative levels of *cgs*, *fliC*, *flgE*, *flcR*, 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. ANOVA I on  $\Delta CT$  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  $\Delta CT$  values of the  $\Delta mucR$  and WT or complemented strains.

which was unable to produce periplasmic cyclic  $\beta$ -glucans (c $\beta$ G) (66). To determine whether MucR can transcriptionally regulate c $\beta$ G levels, the relative levels of *cgs* (the gene encoding c $\beta$ G synthase) and *cgt* (encoding the c $\beta$ G transporter) mRNAs were evaluated by qRT-PCR on RNA purified from the different strains harvested during the exponential growth phase ( $OD_{600} = 0.5$ ) 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  $\Delta mucR$  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 c $\beta$ G identified in *B. meliloti* 16M.

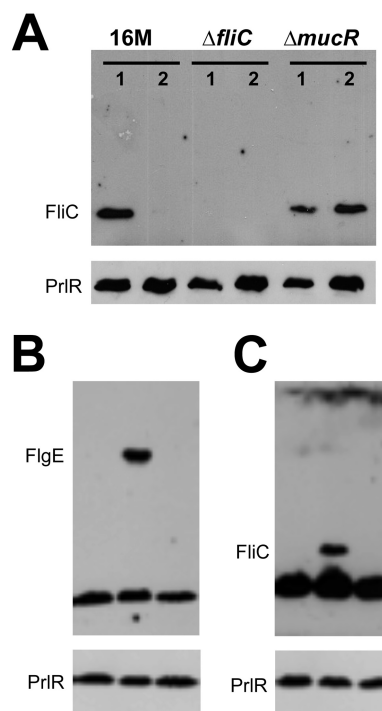
**Restoration of EPS I production in an *S. meliloti* *mucR* mutant through constitutive expression of *B. melitensis* *mucR*.** The *mucR* gene is well conserved within *Rhizobiales*, especially in *S. meliloti* (61% amino acid identity), where its function has been well studied. In *S. meliloti*, MucR 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 containing calcofluor when placed under UV light (65). Under standard laboratory conditions, both wild-type strains, Rm1021 and Rm2011, produce EPS I and appear fluorescent on calcofluor-containing medium, whereas non-EPS I-producing strains do not (Rm1021 *exoY*:Tn5) (Fig. 6). An *S. meliloti* *mucR* mutant (Rm101) forms colonies that are more mucoid and lack the blue-green color characteristic of EPS I-producing strains (18, 65) (Fig. 6). We observed that the constitutive expression of *mucR<sub>Bm</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).

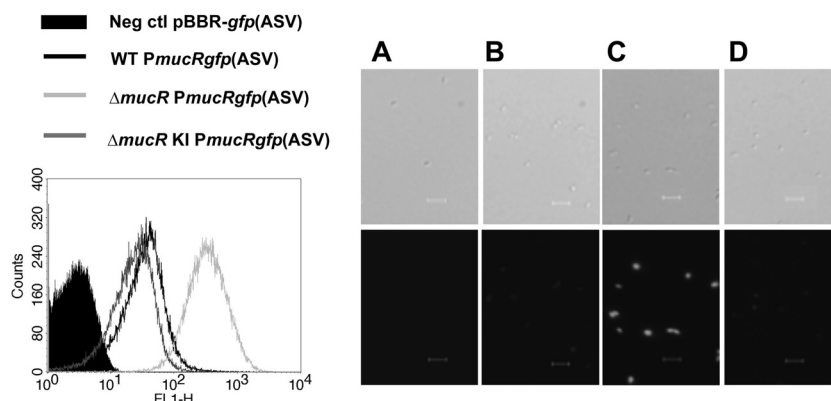


**FIG 6** Heterospecific complementation of an *S. meliloti* *mucR* mutant with the *mucR* gene from *B. melitensis* on LB plates containing calcofluor. The strains Rm1021 (wild type), Rm1021 *exoY*:Tn5 (non-EPS I-producing strain), Rm2011 (wild type), Rm101 (*mucR* mutant), and Rm101 pBBRmucR<sub>Bm</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.

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 FliC (flagellin), we examined FliC protein expression at different growth phases in the *mucR* mutant compared to the WT strain (Fig. 7A). We detected



**FIG 7** Western blot detection of the flagellar proteins FliC and FlgE. (A) Detection of flagellin (FliC) production in *B. melitensis* WT,  $\Delta fliC$ , and  $\Delta mucR$  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 ( $OD_{600} = 0.2$ ) (lanes 1) and in the stationary phase ( $OD_{600} = 1.0$ ) (lanes 2). (B and C) FlgE (B) or FliC (C) expression in the  $\Delta mucR$  mutant in stationary phase. Complementation of *mucR* in *trans* restored the WT phenotype for both FlgE and FliC. The strains were cultivated in 2YT broth, and extracts were prepared from samples harvested in stationary phase ( $OD_{600} = 1.0$ ). Total extracts were separated by electrophoresis, transferred to nitrocellulose membranes, and probed with FlgE-specific or FliC-specific antiserum. A polyclonal anti-PrIR antibody was used to probe PrIR as a loading control.



**FIG 8** MucR negatively autoregulates its own transcription. (Left) Fluorescence intensity measured by flow cytometry ( $5 \times 10^4$  events acquired) on *B. melitensis* WT,  $\Delta$ *mucR*, and  $\Delta$ *mucR*KI strains expressing the transcriptional fusion *PmucRgfp*(ASV) in mid-log-phase culture in 2YT medium. The WT harboring the plasmid pBBR-*gfp*(ASV) was used as a negative control. (Right) DIC (above) and corresponding fluorescence (bottom) images. (A) WT pBBR-*gfp*(ASV). (B) WT pBBRP*mucRgfp*(ASV). (C)  $\Delta$ *mucR* pBBRP*mucRgfp*(ASV). (D)  $\Delta$ *mucR*KI pBBRP*mucRgfp*(ASV). Scale bars, 3  $\mu$ m.

FliC both at the beginning of log-phase growth, as seen in the WT strain, and during stationary phase, when the protein is no longer present in the WT background. Similar results were obtained for FlgE, the hook monomer (data not shown). Complementation of *mucR* in *trans* restored the WT phenotype for both FlgE and FliC (Fig. 7B and C). This result indicates that MucR could also be a repressor of flagellar-gene expression in *B. melitensis* 16M.

To confirm this hypothesis, the relative levels of *fliC* (flagellin), *flgE* (hook monomer), *fliF* (membrane and supramembrane [MS] ring monomer), and *ftcR* mRNAs were determined by qRT-PCR for RNA extracted from the different strains harvested during the exponential growth phase ( $OD_{600} = 0.5$ ) in 2YT medium. We found that a *mucR* deletion resulted in a significant increase in all the mRNA levels tested compared to the WT stain, including the mRNA levels corresponding to the master regulator FtcR (Table 2). For each mRNA, wild-type levels were restored by complementing the  $\Delta$ *mucR* deletion (Table 2). Together, these results clearly indicate that *B. melitensis* MucR, as in *S. meliloti*, is a regulator that acts upstream of the master flagellar regulator FtcR, the orthologue of Rem.

**MucR negatively regulates its own transcription.** In *S. meliloti*, MucR negatively regulates its own transcription (18, 69). To pinpoint a potentially similar self-regulation, *mucR* promoter (*PmucR*) activity was monitored in *B. melitensis* WT,  $\Delta$ *mucR*, and  $\Delta$ *mucR*KI strains. All the strains harboring the plasmid pBBRP-*mucRgfp*(ASV) were grown in 2YT, and the GFP(ASV) fluorescence intensity was measured by flow cytometry at different time points. Figure 8 shows the fluorescence signals measured by flow cytometry (left) and representative epifluorescence micrographs (right) of the different strains in mid-log growth phase. Similar results were obtained for each sampling time point (data not shown). The MFI of the  $\Delta$ *mucR* mutant was higher (MFI = 350) than that of the WT strain (MFI = 45) or the corresponding complemented  $\Delta$ *mucR*KI mutant (MFI = 30) (Fig. 8, left). The *B. melitensis* strain harboring the vector pBBR-*gfp*(ASV) was used as a negative control. *PmucR* is strongly induced when *Brucella* lacks the *mucR* gene, indicating that MucR negatively regulates its own transcription in *B. melitensis* 16M.

## DISCUSSION

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  $\Delta$ *mucR* 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.

In addition to reduced virulence, the  $\Delta$ *mucR* strain exhibits pleiotropic complementable phenotypes, which we discuss here and tentatively correlate with the attenuation of the  $\Delta$ *mucR* strain.

***B. melitensis* 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  $C_2H_2$ -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

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 *rpoEI* (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  $\Delta vjbR$  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 *ftcR* 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  $\Delta mucR$  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 *PmucR* 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  $\Delta mucR$  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  $\Delta mucR$  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* *hfq* 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 unpublished 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  $\Delta mucR$  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.

**$\Delta mucR$  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  $\Delta mucR$  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  $\beta$ -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. Commerci, personal communication).

Further investigations would be necessary to test whether there is a change in cyclic  $\beta$ -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 autoregulation 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. melitensis*) (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*  $\Delta$ *mucR* mutant. In fact, the  $\Delta$ *mucR* 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*  $\Delta$ *mucR* mutant is rough (by crystal violet staining), and this phenotype is complementable (C. Caswell and R. M. Roop, personal communication). Conversely, the *B. melitensis* 16M  $\Delta$ *mucR* 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 could be a more general compensatory mechanism that allows the bacteria to adapt to an altered envelope homeostasis (6). Alternatively, because MucR function is suspected to be intervened into the cell cycle regulatory circuitry of *Caulobacter crescentus* (P. Viollier, personal communication), its differential impact on the envelopes of the two sibling cells is worth investigating in other asymmetrically dividing alphaproteobacteria (95). This opens a new avenue into deciphering why, how (directly or indirectly through other induced membrane changes), and in which part of the bacterial life cycle the lipid A core structure is modulated.

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