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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). 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 β-(1,2)-glucans and external capsular polysaccharides (K), and the exopolysaccharides (EPS) (succinoglycan [EPS I] and galactoglucomannan [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 galactoglucomannan (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 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
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 and reach their final replicative compartment (35). Fourth, among the targets of BvrS/BvrR TCS, which is orthologous to ExoS/ChvI, is also critical and reach their final replicative compartment (35). Fourth, among the targets of BvrS/BvrR TCS, which is orthologous to ExoS/ChvI, is also critical and reach their final replicative compartment (35). Fourth, among the targets of BvrS/BvrR TCS, which is orthologous to ExoS/ChvI, is also critical and reach their final replicative compartment (35). 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TABLE 1 Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Characteristics</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td>B. melitensis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16M</td>
<td>WT; Nal&lt;sup&gt;r&lt;/sup&gt;; parental strain</td>
<td>A. Macmillan, Central Veterinary Laboratory, Weybridge, UK</td>
</tr>
<tr>
<td>16M pBBR-gfp(ASV)</td>
<td>Nal&lt;sup&gt;r&lt;/sup&gt; Cm&lt;sup&gt;r&lt;/sup&gt;; WT carrying pBBR-gfp(ASV)</td>
<td>This study</td>
</tr>
<tr>
<td>16M pBBRPmucRgfp(ASV)</td>
<td>Nal&lt;sup&gt;r&lt;/sup&gt; Cm&lt;sup&gt;r&lt;/sup&gt;; WT carrying pBBRPmucRgfp(ASV)</td>
<td>This study</td>
</tr>
<tr>
<td>ΔmucR</td>
<td>Nal&lt;sup&gt;r&lt;/sup&gt;; deletion strain for mucR gene</td>
<td>This study</td>
</tr>
<tr>
<td>ΔmucR KI</td>
<td>Nal&lt;sup&gt;r&lt;/sup&gt;; chromosomal insertion of the mucR gene in the ΔmucR strain</td>
<td>This study</td>
</tr>
<tr>
<td>ΔmucR pBBRmucR</td>
<td>Nal&lt;sup&gt;r&lt;/sup&gt; Cm&lt;sup&gt;r&lt;/sup&gt;; ΔmucR strain carrying the plasmid pBBRmucR</td>
<td>This study</td>
</tr>
<tr>
<td>ΔmucR pBBRmucRc1</td>
<td>Nal&lt;sup&gt;r&lt;/sup&gt; Cm&lt;sup&gt;r&lt;/sup&gt;; ΔmucR strain carrying the complementation plasmid pBBRmucR</td>
<td>This study</td>
</tr>
<tr>
<td>ΔmucR pBBRmucRc2</td>
<td>Nal&lt;sup&gt;r&lt;/sup&gt; Cm&lt;sup&gt;r&lt;/sup&gt;; ΔmucR strain carrying the complementation plasmid pBBRmucR</td>
<td>This study</td>
</tr>
<tr>
<td>ΔmucRΔvjbRc2</td>
<td>Nal&lt;sup&gt;r&lt;/sup&gt; ΔvjbRc2; referred to as CD100 in reference 29</td>
<td>27</td>
</tr>
<tr>
<td>S. meliloti</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rm1021</td>
<td>Nal&lt;sup&gt;r&lt;/sup&gt; Sm&lt;sup&gt;r&lt;/sup&gt;; WT</td>
<td>42</td>
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<tr>
<td>Rm1021:exoY</td>
<td>Rm1021 exoY::Tn5 (formerly Rm7210); Nal&lt;sup&gt;r&lt;/sup&gt; Sm&lt;sup&gt;r&lt;/sup&gt; Neo&lt;sup&gt;r&lt;/sup&gt;</td>
<td>43</td>
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<tr>
<td>Rm2011</td>
<td>Nal&lt;sup&gt;r&lt;/sup&gt; Sm&lt;sup&gt;r&lt;/sup&gt;; wild type</td>
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</tr>
<tr>
<td>Rm101</td>
<td>Rm2011 Spe&lt;sup&gt;c&lt;/sup&gt; cassette inserted into the Pmacl site of mucR</td>
<td>45</td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
<td></td>
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<tr>
<td>DH10B</td>
<td>F&lt;sup&gt;-&lt;/sup&gt; mucR Δ(mcr-LacR-ermB) Φ80lacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara leu)7697 galU galK rpsL uspG λ&lt;sup&gt;−&lt;/sup&gt; (Sm&lt;sup&gt;r&lt;/sup&gt;)</td>
<td>Gibco BRL</td>
</tr>
<tr>
<td>S17-1</td>
<td>recA thi pro hsdR&lt;sup&gt;−&lt;/sup&gt; M&lt;sup&gt;+&lt;/sup&gt; RP4:2-Tc-Mu Km Tn7 xpir; allows plasmid mobilization</td>
<td>46</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
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<tr>
<td>pBBR1MCS1</td>
<td>Broad-host-range cloning vector; high copy number; Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>47</td>
</tr>
<tr>
<td>pGEM-Teasy</td>
<td>Amp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Promega</td>
</tr>
<tr>
<td>pJQ200-uc1</td>
<td>sacB Gtm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>48</td>
</tr>
<tr>
<td>pBBR-gfp(ASV)</td>
<td>Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>M. Terwagne</td>
</tr>
<tr>
<td>pJQmucR</td>
<td>sacB Gtm&lt;sup&gt;r&lt;/sup&gt; strain containing the ΔmucR fragment; used to construct the deletion strain</td>
<td>This study</td>
</tr>
<tr>
<td>pQmucR</td>
<td>sacB Gtm&lt;sup&gt;r&lt;/sup&gt; strain containing the mucR gene flanked by its upstream and downstream regions</td>
<td>This study</td>
</tr>
<tr>
<td>pBBRmucR</td>
<td>Cm&lt;sup&gt;r&lt;/sup&gt;; complementation plasmid</td>
<td>This study</td>
</tr>
<tr>
<td>pBBRPmucRgfp(ASV)</td>
<td>Cm&lt;sup&gt;r&lt;/sup&gt;; reporter plasmid bearing the transcriptional fusion PmucRgfp(ASV)</td>
<td>This study</td>
</tr>
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</table>

<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; Spe<sup>c</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).

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

In 96-well plates, 50 μl of the bacterial suspension was supplemented with 50 μl 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 μl 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 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<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 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-
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 *Brucella 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 (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 with a 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\(_2\)O\(_2\), 0.2 mM cou maric 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-FlhC (diluted 1:3,000) or anti-FliG (1:5,000) rabbit polyclonal antibodies (26). PrIR, which was used as a loading control, was detected using anti-PrIR 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 (Amer sham 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× objective (Nikon). 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 \( \Delta CT \) values, respectively. When needed, a Scheffe’s comparison test was performed, and statistical significance at a *P* value of <0.05 was accepted.

**RESULTS**

*MucR* is required for a successful *Brucella 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 as Δ*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 Δ*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 Δ*mucR* strain was almost restored to WT levels in the complemented Δ*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 Δ*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 Δ*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 Δ*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 Δ*mucR* strain. Decreased persistence of the Δ*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 Δ*mucR* and WT strains in rich 2YT broth.

The Δ*mucR* mutant had a growth rate similar to that of the WT until 22 h (log phase) (Fig. 2). At this time point, the Δ*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 Δ*mucR* mutant is poorly adapted to cope with the stresses linked to stationary phase, which would be rele-
The **mucR** mutant is more sensitive to H$_2$O$_2$, 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 **ΔmucR** mutant to resist exposure to exogenous hydrogen peroxide (H$_2$O$_2$). After a 1-hour exposure to various concentrations of H$_2$O$_2$, the survival rates of the **ΔmucR** strain were significantly reduced compared to the WT strain under the same conditions (Fig. 3A). The sensitivity of the **ΔmucR** 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 **ΔmucR** 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 **ΔmucR** 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 **ΔmucR** was 32 units. This result confirms the previous indications that the **ΔmucR** strain has an altered cell envelope.

The **mucR** mutant displays an altered LPS profile. In the *Brucella* cell envelope, 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 of WT *B. melitensis* (lane 1) and the **ΔmucR** 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).
Sensitivity to oxidative stress and detergents. (A) Survival of WT, ΔmucR mutant, and ΔmucR pBBRmucR B. melitensis at various concentrations of H$_2$O$_2$. The data are the averages of log$_{10}$ 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 ΔmucR pBBRmucR strain gave the same results as the ΔmucR KI strain (data not shown).

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 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 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 pBBRmucRgfp(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-gfps(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-osmotic 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*.

**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 × 10⁴ 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 µm.

**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*,
TABLE 2 MucR regulates the mRNA levels of the flagellar and cyclic β-glucan synthase genes

<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>BME11050</td>
<td>4.62</td>
<td>0.95</td>
</tr>
<tr>
<td>flgE</td>
<td>BME11059</td>
<td>3.78</td>
<td>0.96</td>
</tr>
<tr>
<td>fliR</td>
<td>BME11058</td>
<td>4.45</td>
<td>1.35</td>
</tr>
<tr>
<td>flsB</td>
<td>BME11051</td>
<td>6.81</td>
<td>1.69</td>
</tr>
<tr>
<td>cgs</td>
<td>BME11837</td>
<td>0.55</td>
<td>0.96</td>
</tr>
</tbody>
</table>

*The relative levels of cgs, fliC, flgE, fliR, and flsB 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 on 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.

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 which was unable to produce periplasmic cyclic β-glucans (cβG) (66). To determine whether MucR can transcriptionally regulate cβG genes, the relative levels of cgs (the gene encoding cβG synthase) 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 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* 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>16M</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.

**FIG 7** Western blot detection of the flagellar proteins FlgE and FlgC. (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* in trans restored the WT phenotype for both FlgE and FlgC. The strains were cultivated in 2YT broth, and extracts were prepared from samples harvested in stationary phase (OD₆₀₀ = 1.0). Total extracts were separated by electrophoresis, transferred to nitrocellulose membranes, and probed with FlgE-specific or FlgC-specific antisera. A polyclonal anti-PrfR antibody was used to probe PrfR as a loading control.
FIG 8 MucR negatively autoregulates its own transcription. (Left) Fluorescence intensity measured by flow cytometry (5 × 10^4 events acquired) on B. melitensis WT, ΔmucR, and ΔmucRKi 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 pBBRpuncRgfp(ASV). (C) ΔmucR pBBRpuncRgfp(ASV). (D) ΔmucRKi pBBRpuncRgfp(ASV). Scale bars, 3 μm.

**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 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.

In addition to reduced virulence, the ΔmucR strain exhibits pleiotropic complementable phenotypes, which we discuss here and tentatively correlate with the attenuation of the ΔmucR 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

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 fliC 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 strain, including the mRNA levels corresponding to the master regulator FtcR (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, ΔmucR, and ΔmucRKi strains. All the strains harboring the plasmid pBBR-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 ΔmucR mutant was higher (MFI = 350) than that of the WT strain (MFI = 45) or the corresponding complemented ΔmucRKi 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.
clearly indicate that MucR of \textit{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 \textit{S. meliloti} acts (21, 67). In \textit{B. melitensis} 16M, flagellar-gene expression is very tightly regulated, given that the QS regulator VjbR (27), the sigma factors rpoE\textit{I} (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 \textit{\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 \textit{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 \textit{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 \textit{S. meliloti} and \textit{B. melitensis} and because MucR in \textit{S. meliloti} regulates EPS I biosynthesis (Fig. 6) (18, 65), we tested whether MucR is involved in the formation of bacterial clumps in \textit{B. melitensis} 16M. Unfortunately, the \textit{\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 \textit{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 \textit{\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 \textit{S. meliloti} and \textit{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 \textit{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 \textit{in vitro}, the \textit{\Delta}mucR strain enters stationary phase much earlier (and thus at a lower OD\textsubscript{\text{600}}) than the WT strain. As previously demonstrated in a \textit{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 unpublished microarray analysis, the involvement of mucR in stress response mechanisms has been suggested for \textit{B. melitensis} (41). Consistent with this hypothesis, we showed a strong increase in the sensitivity of the \textit{\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.

\textbf{\textit{\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 \textit{\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 \textit{B. melitensis} mutant. A link between reduced virulence and sensitivity to detergents has also been observed in \textit{B. abortus} cyclic β-glucan synthase (cgs) (66) and \textit{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 \textit{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 \textit{B. melitensis} 16M, controlling growth in bacteriological medium, virulence in macrophages and mice, flagellar expression, aggregation, and cell envelope homeostasis. Interestingly, in \textit{B. abortus} 2308, MucR (100% amino acid identity with \textit{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 \textit{Brucella} strains. Moreover, the auto-regulation of mucR also occurs in \textit{B. abortus}, in which MucR as been shown by electrophoretic mobility shift assay (EMSA) to bind its own promoter (100% nucleotide identity with \textit{B. melitensis}) (C. Caswell and R. M. Roop, personal communication). In \textit{B. abortus} 2308, MucR controls the expression of several transcriptional regulators, such as babR and norR (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 Brucella 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 Br. 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 Br. 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 interwoven 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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