

Requirement of MgtC for *Brucella suis* Intramacrophage Growth: a Potential Mechanism Shared by *Salmonella enterica* and *Mycobacterium tuberculosis* for Adaptation to a Low-Mg²⁺ Environment

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A *Brucella suis* *mgtC* mutant is defective for growth within macrophages and in low-Mg²⁺ medium. These phenotypes are strikingly similar to those observed with *mgtC* mutants from *Salmonella enterica* and *Mycobacterium tuberculosis*, two other pathogens that proliferate within phagosomes. MgtC appears as a remarkable virulence factor that would have been acquired by distantly related intracellular pathogens to contribute to the adaptation to a low-Mg²⁺ environment in the phagosome.

Intracellular bacterial pathogens such as *Salmonella enterica*, *Mycobacterium tuberculosis*, and *Brucella* species have evolved virulence strategies to modulate host cell biology and survive within the harsh environment of the phagosome (1, 8, 17). *S. enterica* serovar Typhimurium and *M. tuberculosis*, two unrelated intracellular pathogens producing different diseases, both require the MgtC protein for survival inside macrophages and growth in a low-Mg²⁺ medium (2, 5). The macrophage survival defect of an *S. enterica* serovar Typhimurium *mgtC* mutant can be partially suppressed by adding Mg²⁺ to the cell culture medium, suggesting that the growth defect is related to a rate-limiting Mg²⁺ concentration inside the phagosome and that MgtC somehow has a role in Mg²⁺ uptake (2). Other studies based on *mgtC* transcriptional induction within macrophages have suggested that the *Salmonella*-containing phagosome is a Mg²⁺-deprived environment (6, 18), and we have proposed that *M. tuberculosis* also faces a low-Mg²⁺ environment inside phagosomes (5). MgtC is a predicted inner membrane protein of unknown function that contains an N-terminal transmembrane domain and a C-terminal soluble domain (19). In *S. enterica* serovar Typhimurium, *mgtC* is cotranscribed with *mgtB*, encoding a Mg²⁺ transporter (19). The *mgtC* gene is not essential for membrane insertion or transport function of MgtB (20), and MgtC does not appear itself as a cation transporter (14).

Genes encoding MgtC-like proteins are found in a limited number of eubacterial genomes, including those of *Brucella* spp., and analysis of the phylogeny of MgtC-like proteins and *mgtC* chromosomal regions suggests that *mgtC* has been acquired by horizontal gene transfer repeatedly throughout bacterial evolution (3). Phylogenetic analysis shows that the *S. enterica* serovar Typhimurium and *M. tuberculosis* MgtC are grouped with a subclass of MgtC-like proteins, which includes

that of *Brucella melitensis*. In *B. melitensis*, MgtC is encoded on an 8-kb region of the chromosome II that is absent in the genome of the closely related bacterium *Mesorhizobium loti* (3). This region is adjacent to a tRNA gene, arguing in favor of the hypothesis of a region acquired by horizontal gene transfer (7). The origin of *mgtC* remains unknown, and genes linked to *mgtC* are not conserved with the exception of *mgtB* that is found both in *S. enterica* serovar Typhimurium and *Brucella* spp. (3). We have proposed that MgtC-like proteins phylogenetically close to the *S. enterica* serovar Typhimurium and *M. tuberculosis* MgtC proteins might exhibit a similar function and play a role in the virulence of pathogens such as *Brucella* spp., which also replicate within a phagosome (4). In the present study, we investigate the role of the *Brucella suis* MgtC protein for growth in macrophages and low-Mg²⁺ medium.

Construction of a *mgtC* mutant in *B. suis*. The chromosome II region of *B. suis* harboring *mgtC* is schematized in Fig. 1. The MgtC protein of *B. suis*, which is 100% identical to that of *B. melitensis*, exhibits 40% overall sequence identity with the MgtC proteins of *S. enterica* serovar Typhimurium and *M. tuberculosis*, with the highest level of homology (65%) found in the N-terminal part of the protein. Some differences in genetic organization are evident, for example in *B. suis* *mgtC* is found downstream of *mgtB* (Fig. 1) whereas a *mgtCB* operon is found in serovar Typhimurium (no clear MgtB homologue is found in *M. tuberculosis*). The intergenic region (770 bp) contains two putative open reading frames, and it is not clear whether the *mgtB* and *mgtC* genes are grouped in the same operon in *Brucella* spp. A transposon insertion mutation in *mgtB* has been recovered in a genetic screening of *B. melitensis* mutants deficient for growth in macrophages (12). If *mgtB* and *mgtC* are organized as an operon, the attenuation might be due to a polar effect on *mgtC*. To investigate the role of *mgtC* in *B. suis*, we have constructed a mutant harboring a deletion and a kanamycin resistance cassette in the *mgtC* gene (Fig. 1).

The *mgtC* gene was amplified by PCR from *B. suis* 1330 (ATCC 23444T) genomic DNA with MgtCF (5'-TAATCCAGAAAGGCCACCGG-3') and MgtCR (5'-CGGCGATACGC

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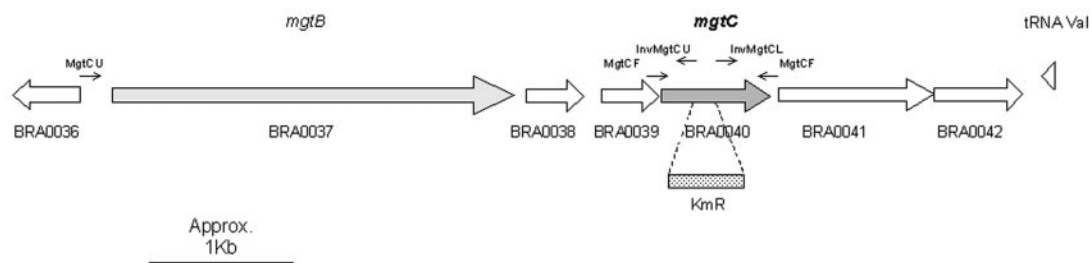


FIG. 1. Schematic representation of the *B. suis* 1330 chromosome II region around *mgtC* (16). Orientation of the different genes is indicated by large arrows. The orientation and the location of the different primers used in this study are indicated by small arrows. The position of the resistance cassette (KmR) inserted during the construction of the *mgtC*::Kan mutant by allelic replacement is indicated.

AGTGGCAGG-3') primers. A similar fragment was amplified when genomic DNA from *B. melitensis*, *B. ovis*, *B. canis*, and *B. abortus* was used as template, showing that the gene is conserved throughout the *Brucella* genus (not shown). The PCR product from *B. suis* 1330 was cloned into the pGEMT vector (Promega, Madison, WI), which confers resistance to ampicillin and is unable to replicate in *Brucella* spp. A fragment of 152 bp within the *mgtC* gene was deleted by an inverse PCR using InvMgtCU (5'-TGCAGAGGCTCAGGTGCGCG-3') and InvMgtCL (5'-CGCCGAAAGCAGCCAACA-3') primers. The 152-bp fragment was replaced by a 1.2-kb *HincII* fragment carrying the kanamycin resistance gene from plasmid pUC4K (Pharmacia, Peapack, NJ), giving pGEMT-*mgtC*::kan. *B. suis* was transformed with this suicide plasmid by electroporation (10). Double recombination events between the chromosomal *mgtC* gene and the inactivated *mgtC* gene on the suicide plasmid led to a *mgtC*::Kan^r ampicillin-sensitive strain. The chromosomal insertion within *mgtC* was verified by Southern blot analysis (data not shown). To complement the *mgtC*::Kan mutant, the *mgtC* gene was cloned into pBBR1MCS (11), which confers resistance to chloramphenicol and replicates in *Brucella*. Because the location of the *mgtC* promoter is unknown, we cloned a region upstream of *mgtC* that includes the *mgtBC* intergenic region and the *mgtB* gene and its putative promoter region (Fig. 1). The *mgtBC* region was amplified by PCR from *B. suis* genomic DNA with MgtCU (5'-GGT CAGGCGGCTCAGGAAAGCC-3') and MgtCR primers and was first cloned into pGEMT easy (Promega). An *EcoRI* digestion fragment harboring the *mgtBC* region was then subcloned into pBBR1MCS, giving pBBR1-*mgtBC*.

A *B. suis* *mgtC* mutant is attenuated for growth within J774 macrophages. The role of *B. suis* *mgtC* gene in intracellular survival was evaluated in murine J774 macrophage-like cells as described previously (15). Briefly, 24-well plates were seeded with 5×10^5 cells in RPMI 1640 medium supplemented with 10% fetal calf serum and infected 24 h later with *B. suis* at a multiplicity of infection of 20. After 30 min, cells were washed three times with phosphate-buffered saline and reincubated in cell medium supplemented with $30 \mu\text{g ml}^{-1}$ gentamicin. The number of intracellular bacteria was measured at 2, 5, 24 and 48 h postinfection. As shown in Fig. 2, no significant difference was found at 2 h or 5 h postinfection between the wild type and the *mgtC*::Kan mutant, indicating that *mgtC* is not involved in the early stages of intramacrophage growth. However, the *mgtC*::Kan strain showed significantly lower levels of intracellular multiplication at 24 h and 48 h postinfection (Fig. 2). The multiplication defect of the *mgtC*::kan mutant can be com-

pletely suppressed by complementation with the pBBR1-*mgtBC* plasmid, since the complemented strain multiplies at a rate similar to that of the wild-type strain (Fig. 2). These results indicate that MgtC is required for the intramacrophage growth of *B. suis*.

MgtC is required for *B. suis* growth in low-Mg²⁺ medium. To further compare the similarities between the role of MgtC in *B. suis* and that described in serovar Typhimurium and *M. tuberculosis*, we investigated the growth of the *mgtC*::Kan mutant in low-Mg²⁺ liquid medium. Wild-type and *mgtC*::Kan bacteria were grown overnight at 37°C in tryptic soy broth and diluted 1:100 in NCE-minimal medium (13) supplemented with 0.1% Casamino Acids, 1% arabinose, 1% yeast extract, and either 10 μM or 10 mM MgCl₂. Optical density at 600 nm (OD₆₀₀) was measured after 24 h growth. The *mgtC*::Kan mutant is severely impaired for growth in NCE supplemented with 10 μM Mg²⁺ compared to the wild-type strain. The growth defect is due to the low-Mg²⁺ concentration because both strains reached similar OD in the presence of 10 mM Mg²⁺ (Fig. 3A). By using increasing Mg²⁺ concentrations, we found that the *mgtC*::Kan mutant is deficient for growth in Mg²⁺ concentrations below 60 μM (Fig. 3B); that is strikingly similar to what is found with *mgtC* mutants of *S. enterica* serovar

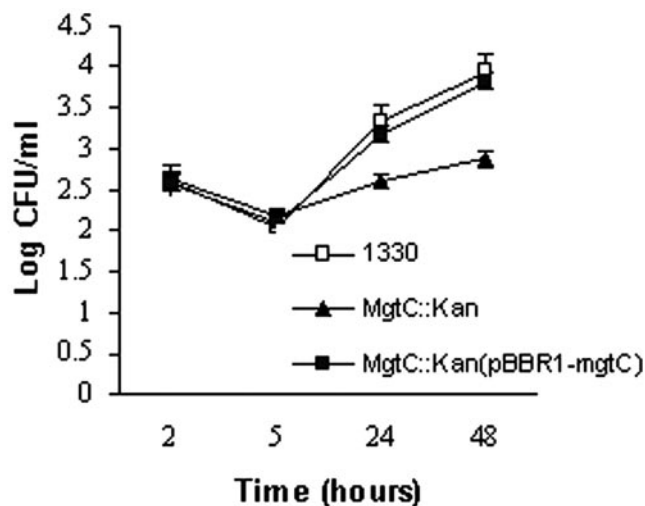


FIG. 2. The *mgtC* gene of *B. suis* is required for intramacrophagic survival. Numbers of CFU were determined at the indicated times. Values are the geometric means \pm standard errors of the results from one representative experiment done in triplicate out of four.

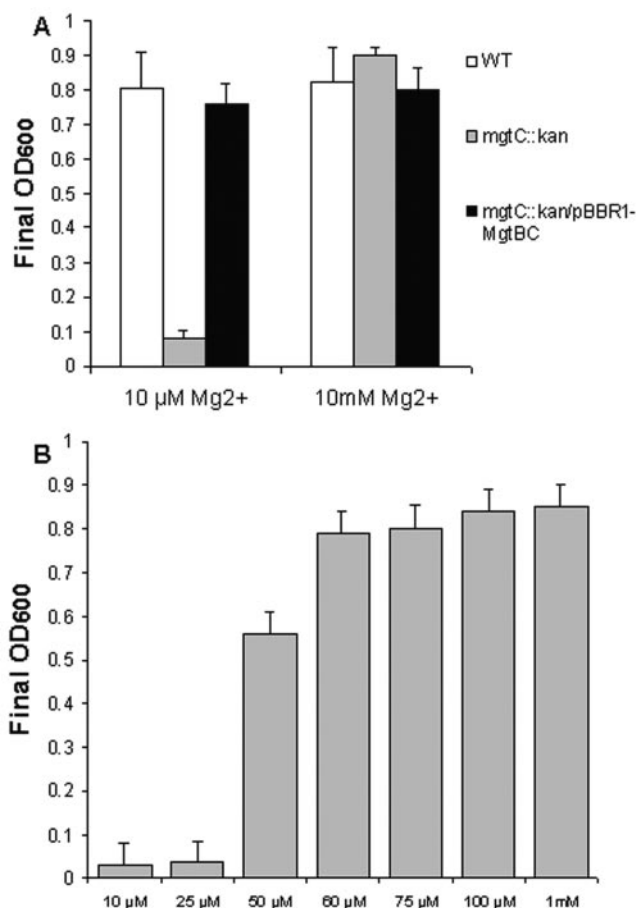


FIG. 3. The *mgtC::Kan* mutant of *B. suis* is unable to grow in low-Mg²⁺ NCE liquid medium. A. OD₆₀₀ reached by wild-type (WT), *mgtC::Kan* mutant, and *mgtC::Kan/pBBR1-mgtBC* strains at 24 h after inoculation with medium containing 10 μM or 10 mM MgCl₂. B. Growth (OD₆₀₀ at 24 h postinoculation) of *mgtC::Kan* mutant in NCE liquid medium supplemented with increasing Mg²⁺ concentrations. Values are the means of three independent experiments plus standard errors.

Typhimurium (data not shown) and *M. tuberculosis* (5). This defect appears specific to Mg²⁺ since neither 1 mM CaCl₂ nor 1 mM MnCl₂ could restore growth (data not shown). On the other hand, growth of the *mgtC::Kan* mutant in low-Mg²⁺ medium was fully restored by complementation with the pBBR1-*mgtBC* plasmid (Fig. 3A). These data show that the *mgtC* gene of *B. suis* is required for growth in low-Mg²⁺ medium. MgtB, however, is not required for growth in low-Mg²⁺ medium both in *B. melitensis* (12) and *S. enterica* serovar Typhimurium (2), suggesting that a magnesium transporter(s) other than MgtB is functional in such conditions.

Mg²⁺ partially rescues the growth defect of the *B. suis mgtC* mutant in macrophages. To test whether the macrophage growth defect of the *mgtC* mutant was linked to its inability to grow in a low-Mg²⁺ environment, we supplemented the tissue culture medium with 25 mM MgCl₂ 2 h after internalization of wild-type or *mgtC::Kan* bacteria. The addition of Mg²⁺ to the tissue culture medium had no significant effect on the wild-type strain, but it significantly improved the intracellular growth of *mgtC::Kan* bacteria, and therefore partially rescued the growth

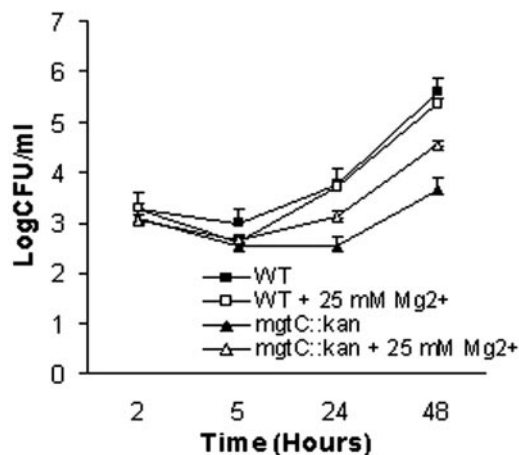


FIG. 4. Effect of the addition of Mg²⁺ to tissue culture medium on the intramacrophage growth of *B. suis mgtC* mutant. MgCl₂ (25 mM) was added to the RPMI 1640 medium 2 h after bacterial internalization. Values are the means of three independent experiments plus standard errors. WT, wild type.

defect of the *mgtC* mutant (Fig. 4) in a manner similar to that described for an *S. enterica* serovar Typhimurium *mgtC* mutant (2). This result suggests that the *Brucella*-containing vacuole is indeed a Mg²⁺-limiting environment.

Conclusion. MgtC appears to be a remarkable factor since, to our knowledge, it provides the only clear example of an acquired intramacrophage growth factor shared by three unrelated intracellular pathogens, *B. suis*, *S. enterica* serovar Typhimurium, and *M. tuberculosis*. It suggests that while each pathogen has evolved specific mechanisms to modulate host cell biology, they also encounter environments with some striking similarities and have developed common virulence strategies. In agreement with MgtC phylogenetic analysis (3), we propose that the MgtC proteins from these pathogens share a similar function, most likely providing the ability to grow in a low-Mg²⁺ environment. The present data strengthen the hypothesis that MgtC-like proteins encoded by other pathogens that replicate within macrophages, such as *Burkholderia* and *Yersinia* species, will be found to be important for intracellular growth (3). The findings also give new information concerning the microenvironment of the *Brucella*-harboring phagosome, which has been shown to be poor in nutrients and characterized by low oxygen tension (9). Our results strongly suggest that the *Brucella*-harboring phagosome also constitutes a Mg²⁺-deprived environment. We propose that the acquisition of *mgtC* is a key evolutionary event in the adaptation of intracellular pathogens to a low-Mg²⁺ niche.

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