Characterization of MgtC, a Virulence Factor of Salmonella enterica Serovar Typhi

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Abstract

The MgtC is a virulence factor in Salmonella Typhimurium that is required for growth at low-Mg\(^{2+}\) concentrations and intramacrophage survival. This gene is codified in a conserved region of the Salmonella pathogenicity island 3 (SPI-3), and is also present in the chromosome of other Salmonella serovars. In this study we characterized the MgtC factor in S. Typhi, a human specific pathogen, by using mgtC and SPI-3 mutant strains. We found that MgtC is the most important factor codified in the SPI-3 of S. Typhi for growth in low-Mg\(^{2+}\) media and survival within human cells. In addition, by using reporter genes we determined that the low-Mg\(^{2+}\) concentration, acidic media and PhoP regulator induce mgtC expression in S. Typhi. We suggest that MgtC is the most important virulence factor codified in the SPI-3 of S. Typhi.

Introduction

The Salmonella enterica genome has at least five DNA regions associated with pathogenicity, referred to as the Salmonella pathogenicity islands (SPI). One such island, SPI-3, is located in the selC locus of S. Typhimurium and contains ten ORFs [1], among which some have been experimentally associated with virulence functions of this bacterium. This is the case for the mgtCBA operon, for which there is evidence of involvement in intramacrophage survival and virulence in mice [2,3]. This operon is codified in all Salmonella serovars in a very conserved SPI-3 region [4]. The mgtC sequence seems to encode a virulence factor that has been repeatedly acquired by horizontal gene transfer throughout bacterial evolution, since it has also been associated with virulence in Mycobacterium tuberculosis and Brucella suis [5–7]. MgtC is a protein of unknown function of about 25 kDa in size. In S. enterica serovar Typhimurium (S. Typhimurium), the experimental evidence suggests that MgtC participates in adaptation to low-Mg\(^{2+}\) environments, supporting bacterial invasion and proliferation in macrophages [2]. Although the mgtCBA operon is co-transcribed with mgtB, which encodes a Mg\(^{2+}\) transporter, MgtC is not required for MgtB function [8]. Indeed, a recently described polypeptide encoded by the mgtCBA operon, named MgtR, promotes MgtC degradation by a bacterial protease, acting as a negative feedback that limits the amount of MgtC under certain conditions [9]. In addition, it has been shown that the two-component system PhoP-PhoQ induces the expression of mgtC, in response to low Mg\(^{2+}\) levels and acidic pH [8,10].

Another SPI-3 gene involved in bacterial pathogenicity is misL, which encodes an autotransporter protein involved in the adhesion of S. Typhimurium to the extracellular matrix in mice and chicks, thereby acting as an intestinal colonization factor [11,12]. It has also been shown that marT, another sequence present in SPI-3, encodes a transcriptional regulator that induces the expression of misL [13]. There is no additional information on other SPI-3 ORFs, all of them remaining until now as sequences encoding conserved hypothetical proteins with unknown function [14].

S. Typhimurium is a wild host range serovar and has been extensively studied in a murine model of systemic infection to indirectly elucidate some microbiological and immunological traits of typhoid fever in humans [15], a life-threatening and systemic infection caused by the S. enterica serovar Typhi (S. Typhi). The latter, a human-restricted pathogen, is a facultative intracellular bacterium responsible for significant morbidity and mortality worldwide, and there are an estimated 21.5 million cases per year, most of which occur in developing countries [16].

The aim of this work was to characterize the role of the MgtC factor in the virulence of S. Typhi by comparing the growth and survival of mgtC and SPI-3 mutant strains in different stressful conditions, and determining the signals and transcriptional regulators that command MgtC expression. We demonstrated that MgtC is the most important factor in S. Typhi SPI-3 for bacterial growth in a low-Mg\(^{2+}\) environment and for bacterial survival inside human cells. In addition, the PhoP regulator participates in inducing the expression of mgtC in S. Typhi.

Materials and Methods

Bacterial strains and growth conditions

All Salmonella Typhi strains used in this study are derived from STH2370, a Chilean clinical isolate described previously [15]. Unless otherwise stated bacteria were grown at 37°C in Luria Bertani (LB) broth or in M9 minimal medium supplemented with either 10 μM or 10 mM MgCl\(_2\), 0.2% glucose, tryptophan and cysteine (50 μg/mL each). When necessary, the pH was adjusted...
to pH 7.0 (NaHPO₄/NaH₂PO₄ 25 mM) or 5.0 (citric acid/sodium citrate 0.1 M) and the following antibiotics were added: chloramphenicol (Cam; 20 μg/mL), kanamycin (Kan; 50 μg/mL), ampicillin (Amp; 100 μg/mL) and gentamicin (Gem; 50 μg/mL).

PCR amplifications and construction of mutant strains

PCR amplifications were performed in a standard volume of 25 μL. Reaction mixes contained 1× PCR buffer, 1.5 mM MgCl₂, each deoxynucleoside triphosphate (200 μM), primers (1 μM), 100 ng of template DNA, and 2 U of Taq (Fermentas) DNA polymerase. Standard conditions for amplification were an initial step of 95°C for 5 min, 30 cycles of incubation at 96°C for 40 s, 60°C for 40 s, and 72°C for 2 min, followed by a final extension step at 72°C for 10 min. Template S. Typhi chromosomal DNA was prepared by the phenol chloroform extraction method [17].

The ΔmgtC::FRT (ΔmgtC) mutant strain was constructed using the lambda Red recombinase system [18]. Briefly, the CamR cassette (chloramphenicol resistance, codified in the pKD3 plasmid) was amplified using the primers MGW1 (5’-ATGGAG-GAAGCAGTATTGATACTTTTCATTATAAAATTGTACAAGCTGGAAC-3’) and MGW2 (5’-TGACCCACGCAGGACCTGAGGTGCTTAGATT-3’). The ΔmgtC::CmR cassette (cycA cassette resistance, codified in the pKD4 plasmid) using the primers SPW1 (5’-AACGACGGCTACGTTGTGATAGTGAGCCCTAATTCTTGTTA-3’) and SPW2 (5’-GCTAAATAGCAGCTACATTCTTCCAGAAAAATGGACATAGTTATCCTTCTTA-3’). Once the ΔmgtC::CmR mutant strain was obtained, the CamR determinant was removed and substituted by the “FRT scar” [18], and the resulting colonies were tested by PCR to confirm the mgtC deletion.

The ΔmgtC::FRT (ΔmgtC) was constructed with the same procedure, amplifying the KanR cassette (kanamycin resistance, codified in the pKD4 plasmid) using the primers SPW1 (5’-AACGACGGCTACGTTGTGATAGTGAGCCCTAATTCTTGTTA-3’) and SPW2 (5’-GCTAAATAGCAGCTACATTCTTCCAGAAAAATGGACATAGTTATCCTTCTTA-3’). Once the ΔmgtC::CmR mutant strain was obtained, the KanR determinant was substituted by the “FRT scar” as described previously [18].

With the mutant strain EG14598 (S. Typhimurium 14028s ΔphoP::cat) [19], a P22 HT105/1 int201 phage lysate was made [20] and used for generalized transduction over S. Typhi strains.

Phenotypic analysis of the S. Typhi mutant strains

Growth in a Mg²⁺-limiting environment was evaluated as described previously [21] with some modifications. Briefly, an overnight culture grown in M9 minimal media with 10 mM MgCl₂ was washed three times with Mg²⁺-free medium, diluted 1/200 in culture media containing either 10 μM or 10 mM MgCl₂, and incubated with shaking at 37°C for different lengths of time. Growth was measured with a spectrophotometer at an optical density of 600 nm (OD₆₀₀).

To evaluate the effect of pH, overnight cultures were grown in LB broth at pH 7, then washed three times with LB at the desired pH (5 or 7), diluted 1/200 in the same medium and incubated with shaking at 37°C for different periods of time. Growth was measured with a spectrophotometer at OD₆₀₀.

The infection assays using monocytic (U937) and epithelial (HEp-2) human cells were carried out as described previously [15,22], with the following modifications. Cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% (vol/vol) fetal bovine serum, seeded into 24-well tissue culture plates at a concentration of 10⁴ cells per well, and then incubated at 37°C in 5% CO₂ until confluent growth was achieved. Later the cells were centrifuged and washed three times with PBS. Approximately 2×10⁶ to 5×10⁶ CFU of exponential-phase (OD₆₀₀ 0.15 to 0.20) anaerobically grown bacteria was pelleted, washed twice with PBS, and resuspended in 1 mL of PBS. Aliquots (100 μL) of bacteria were added to cells at a multiplicity of infection of 50:1(U937) and 100:1 (HEp-2). After 1 h of infection, cells were centrifuged and washed three times with PBS, and the medium was replaced with Dulbecco’s modified Eagle’s medium supplemented with 10% (vol/vol) fetal bovine serum containing gentamicin (200 μg/mL). After additional incubation for 1 and 24 h (times 2 and 24 h respectively), U937 and HEp-2 cells were washed three times with PBS and lysed with 0.5% deoxycholate, and the titers of intracellular bacteria were determined by serial dilution of cell lysates on agar plates. The percentage of survival was calculated at 2 h considering the initial inoculate as 100%, and at 24 considering the CFU counted at 2 h as 100%.

β-Galactosidase assay

The mgtC promoter activity was evaluated by a transcriptional fusion to the Lac reporter, as described previously [23,24] using the pCE36 plasmid. β-Galactosidase activity was measured by a modification of the Miller’s method [25]. Fifty microliters of the bacterial culture were grown in 590 μL of Z buffer (60 mM NaHPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, 50 mM β-mercaptoethanol, pH 7.0). Bacteria were permeabilized with 10 μL chloroform and 10 μL 0.1% SDS, incubated at 30°C for 10 min, and 200 μL of o-nitrophenyl-β-D-galactopyranoside (4 mg/mL) was added. Reactions were stopped by addition of 500 μL 1 M Na₂CO₃. β-Galactosidase activity was calculated in Miller units, using the formula 10²×(OD₄₂₀−1.75×OD₅₅₀)/(mL×min×OD₆₀₀).

RNA isolation and RT-PCR

Total RNA was extracted and purified using Trizol and was treated with RNase-free DNase I (amplification grade; Gibco-BRL). RT-PCR was performed with 500 ng of DNase-treated RNA using the Superscript reverse transcriptase (Invitrogen). Amplification was performed for 30 cycles (94°C for 40 s, 55°C for 40 s, and 72°C for 1.5 min, followed by a 10 min extension at 72°C). The primers used were RTMGC1 (5’-TGGCGTGTTATGCGGCCTTA-3’) and RTMGC2 (5’-AGGCCCTGTCCCCAGAGCAGCAG-3’). In addition, the universal primers 8F and 5’-CACGGCGTAAACGG-GAGCCAG-3’. A translational fusion of three copies of FLAG epitope (3×FLAG) with the MgtC sequence was constructed using the method described by Uzzau et al. [26]. The 3×FLAG epitope was cloned on the pSUB11 plasmid and amplified using the primers FMG1 (5’-CGATAATATCACCGAATTCTTTATAGCCCTGTTGTTATGCGGCTTA-3’) and FMG2 (5’-ACT-TGACCCACGCAGGACCTGAGGTGCTTAGATT-3’). Once inserted immediately preceding the translation stop signal, the mgtC-3×FLAG fusion was confirmed by PCR and the functionality of the protein was verified by the growth of the strain in low-Mg²⁺ medium.

MgtC epitope tagging and immunoblot assay

A translational fusion of three copies of FLAG epitope (3×FLAG) with the MgtC sequence was constructed using the method described by Uzzau et al. [26]. The 3×FLAG epitope was cloned on the pSUB11 plasmid and amplified using the primers FMG1 (5’-CGATAATATCACCGAATTCTTTATAGCCCTGTTGTTATGCGGCTTA-3’) and FMG2 (5’-ACT-TGACCCACGCAGGACCTGAGGTGCTTAGATT-3’). Once inserted immediately preceding the translation stop signal, the mgtC-3×FLAG fusion was confirmed by PCR and the functionality of the protein was verified by the growth of the strain in low-Mg²⁺ medium.

The MgtC epitope tagging was detected by an immunoblot assay using the anti-FLAG M2 monoclonal antibody (Sigma), as previously described [24]. After the samples were resuspended in
1 mL of 100 mM Tris–HCl (pH 8) and sonicated, the total protein was quantified by the Bradford method. The SDS-PAGE was made using 10 ng of total protein per sample.

Statistical analysis

Statistical analysis was performed using the one way ANOVA and Student’s t-test for independent samples. Values of P<0.05 were considered significant. These tests were performed using Microsoft Excel® software.

Results

1. MgtC is required for S. Typhi growth in a low-Mg\(^{2+}\) medium

To elucidate the role of mgtC in S. Typhi, we investigated growth in low-Mg\(^{2+}\) minimal media as evaluated previously in S. Typhimurium [2,6]. The Mg\(^{2+}\) concentrations used were 10 μM or 10 mM, representing the intracellular and extracellular environment respectively [2,27]. At the 10 μM concentration, the ΔmgtC mutant strain grew significantly less than the wild type strain (p<0.05), reestablishing its phenotype when complemented with mgtC cloned in pBBR-5 plasmid. In contrast, at 10 mM Mg\(^{2+}\) there was no difference among the tested strains (Fig. 1A and 1B). These results are in accordance with reports of the role of MgtC in the virulence of S. Typhi [2,8] and other bacteria [5,6].

2. MgtC is required for growth of S. Typhi within epithelial and monocytic human cells

To verify the role of MgtC in the intracellular survival of S. Typhi, we tested the ΔmgtC strain in infection assays using both HEp-2 epithelial and U937 monocytic cell lines. Two post-infection times were evaluated, 2 h and 24 h, representing the early and late survival abilities respectively. As expected, MgtC is required for infection of monocytic cells, with significant differences (p<0.05) among wild type and ΔmgtC strains (Fig. 2A). Remarkably, inside HEp-2 epithelial cells there was a significant impairment (p<0.05) in the invasive phenotype of the ΔmgtC mutant strain (Fig. 2B), suggesting that MgtC participates during the infection of this kind of human cell.

3. MgtC reestablishes the wild type phenotype of a SPI-3 mutant strain both in low Mg\(^{2+}\) media and inside monocytic cells

Previously it has been shown that MgtC can restore the wild type intramacrophage survival phenotype of a S. Typhimurium mgtCB mutant strain [2]. In S. Typhi we wanted to determine whether MgtC is required for intracellular survival and growth at low-Mg\(^{2+}\) concentrations in the context of a SPI-3 deletion. Therefore, we constructed the ΔSPI-3 mutant strain, the complemented ΔSPI-3/pBmgtC (mgtC\(^+\)) and ΔSPI-3/pBBR5 (mgtC\(^-\)) strains, and repeated the assays of growth in low Mg\(^{2+}\) media (Fig. 3A) and survival inside human monocytic cells (Fig. 3B). The results suggest that MgtC can restore the phenotypes observed in a SPI-3 mutant strain by itself and can be considered the most important product codified on the S. Typhi SPI-3 island for bacterial response to those experimental conditions.

4. The PhoP regulator controls mgtC expression in a low Mg\(^{2+}\) and acidic environment

In S. Typhimurium, the PhoP-PhoQ two-component system regulates the expression of many genes when bacteria are exposed to the intracellular environment, including SPI-2 and SPI-3 associated effectors [10,19]. The signals sensed by this regulatory system are the extracellular pH and Mg\(^{2+}\) concentrations. PhoQ is the sensor component that phosphorylates the PhoP regulator, which then modifies gene expression. By using a STH2370 ΔphoP::cat mutant strain (ΔphoP\(^-\)), we examined whether PhoP regulates MgtC in S. Typhi. The results obtained by β-galactosidase and RT-PCR assays show that mgtC transcription is induced in a phoP-dependent manner by either low Mg\(^{2+}\) (10 μM, data not shown) or pH 5 (Fig. 4A and 4B). Moreover, immunoblot assay shows an increase in the MgtC levels under the same conditions (Fig. 4C and 4D), a finding that differs from the

Figure 1. MgtC is necessary for growth at a low-Mg\(^{2+}\) concentration. Strains WT (STH2370 wild type), ΔmgtC (mgtC\(^-\)), ΔmgtC/pBmgtC (mgtC\(^+\)) and ΔmgtC/pBBR-5 (mgtC\(^+\)) were grown in M9 minimal medium supplemented with 10 mM (A) or 10 μM (B) MgCl\(_2\). The OD\(_{600}\) was measured at the indicated times. Values represent the mean of three independent experiments ±SD (*p<0.05). doi:10.1371/journal.pone.0005551.g001
reported situation in *S. Typhimurium* where MgtC translation is not detected in many hours [8,9].

**Discussion**

In previous reports the *Salmonella* SPI-3 island has been associated with intramacrophage invasion by supporting survival when Mg$^{2+}$ is scarce, a condition that seems a common strategy of the host to avoid the growth of intracellular bacteria [1,2,8]. Mg$^{2+}$ is a divalent ion essential for living organisms that works as a regulator and co-factor in many proteins, stabilizing membranes, ribosomes and other cellular structures [28]. *Salmonella* contains several transport systems, both inducible and constitutive, that have functional complementarities with the aim of adjusting the Mg$^{2+}$ concentration in different environmental conditions [29,30]. In addition, these systems are controlled by transcriptional and post-transcriptional regulatory networks to maintain strict control of the Mg$^{2+}$ balance [31,32], stabilizing its concentrations as required for biological processes in *Salmonella*. In this context, MgtC seems to be the most important SPI-3 factor that supports the survival and growth of *Salmonella* in low-Mg$^{2+}$ concentrations, as observed in previous reports for *S. Typhimurium* and in this work with *S. Typhi*. This factor is codified in a SPI-3 conserved region [4] and probably exerts the same, although yet unknown, function in all *Salmonella* serovars.

In this work, the decreased ability to survive within human monocytic cells observed with a *S. Typhi* ΔSPI-3 strain could be overcome with an mgtC-containing plasmid, which restored the wild type phenotype at 2 and 24 hours post-infection. This means that MgtC is a virulence factor playing a major role that is not

![Figure 2. MgtC has an important role in the growth of *S. Typhi* within human cells.](image)

Figure 2. MgtC has an important role in the growth of *S. Typhi* within human cells. Infection assays in U937 (A) and HEp-2 (B) human cells using WT (STH2370 wild type), ΔmgtC (ΔmgtC$^-$), ΔmgtC/pBmgtC (mgtC$^+$) and ΔmgtC/pBBR-5 (mgtC$^-$) strains. Culture cells were infected at a MOI of 50:1 (U937) and 100:1 (HEp-2), respectively. Colonies were counted at time 2 h and 24 h and expressed as a percentage of intracellular survival. Values represent the mean of at least three independent experiments ±SD (*p<0.05).

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![Figure 3. MgtC can restore the WT phenotype in a SPI-3 mutant strain.](image)

Figure 3. MgtC can restore the WT phenotype in a SPI-3 mutant strain. Strains WT (STH2370 wild type), ΔSPI-3 (ΔSPI-3$^-$), ΔSPI-3/pBmgtC (mgtC$^+$) and ΔSPI-3/pBBR-5 (SPI-3$^-$). (A) For growth in 10 μM MgCl$_2$ strains were incubated in M9 minimal medium and the OD$_{600}$ was measured at the indicated times. (B) U937 cells were infected at a MOI of 50:1. Colonies were counted at time 2 h and 24 h and expressed as a percentage of intracellular survival. Values represent the mean of at least three independent experiments ±SD (*p<0.05).

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supplied by any other bacterial factor codified either inside SPI-3 or in the entire chromosome of Salmonella.

A characteristic phenotype associated with S. Typhi MgtC was demonstrated by the early survival phenotype inside HEp-2 epithelial cells, in which the S. Typhi mgtC mutant strain showed a significant lower survival (p < 0.05) than the wild type strain (Fig. 2B). This difference has not been reported previously in any Salmonella serovar and suggests that S. Typhi requires the MgtC function from the initial infective phase, when it colonizes the intestinal epithelium. Whether this requirement responds to particular conditions during bacterial entry or when bacteria are inside human epithelial cells are questions that remain to be elucidated.

However, these findings are indicating that both epithelial and monocytic human cells represent a variety of conditions that require the MgtC function for the bacterial survival. This hypothetical “multi-requirement” of MgtC is in accordance to several reports suggesting a connection of this virulence factor with the structural stability of Mg$^{2+}$ channels in the bacterial cell membrane [7], linked to survival in Mn$^{2+}$ depleted environments, or modifying the membrane potential of the host cell and affecting the host-pathogen interaction [33,34]. Since single amino acid substitutions can affect one role of MgtC without affecting others [35], it seems possible to assume a diversity of functions in which this internal membrane protein participates during infection. In addition, MgtC is important in other bacterial species that are able to invade, live and proliferate within host cells, as for Mycobacterium tuberculosis, Brucella melitensis and Yersinia pestis [5,7].

The expression assays showed that mgtC transcription and translation are induced at low Mg$^{2+}$ concentrations and acidic pH, and that PhoP is the global regulator that participates in this process. These signals stimulate the expression of many genes associated with pathogenicity by inducing the PhoP-PhoQ system [10], and MgtC of S. Typhi is one of these. In addition, mgtC and mgtB are co-transcribed in S. Typhi (data not shown), suggesting the almost identical functionality of these sequences between S. Typhimurium and S. Typhi, and probably in all Salmonella serovars that contain the mgtCB operon in their chromosomes. Previously it has been shown in S. Typhimurium that MgtR induces the degradation of MgtC but not of MgtB, resulting in a downregulation of MgtC when the operon is expressed [9]. Our results suggest that in S. Typhi this regulation could be different, since MgtC is detected after acidic or low magnesium stimuli.

In conclusion, in this work we determined that MgtC in S. Typhi represents a mechanism of pathogenicity codified inside the SPI-3 that has a relevant role in the intracellular survival of bacteria, induced by the PhoP global regulator in response to a low-Mg$^{2+}$ concentration and acidic pH. These findings suggest that MgtC is a key factor in most, if not all, pathogenic Salmonella serovars.

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Author Contributions

Conceived and designed the experiments: PR GCM. Performed the experiments: PR MCR GCM. Analyzed the data: PR GCM. Wrote the paper: PR GCM.
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