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A rule governing the FtsH-mediated proteolysis of the MgtC virulence protein from *Salmonella enterica* serovar Typhimurium[§]

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A tightly controlled turnover of membrane proteins is required for lipid bilayer stability, cell metabolism, and cell viability. Among the energy-dependent AAA$^+$ proteases in *Salmonella*, FtsH is the only membrane-bound protease that contributes to the quality control of membrane proteins. FtsH preferentially degrades the C-terminus or N-terminus of misfolded, misassembled, or damaged proteins to maintain physiological functions. We found that FtsH hydrolyzes the *Salmonella* MgtC virulence protein when we substitute the MgtC 226th Trp, which is well conserved in other intracellular pathogens and normally protects MgtC from the FtsH-mediated proteolysis. Here we investigate a rule determining the FtsH-mediated proteolysis of the MgtC protein at Trp226 residue. Substitution of MgtC tryptophan 226th residue to alanine, glycine, or tyrosine leads to MgtC proteolysis in a manner dependent on the FtsH protease whereas substitution to phenylalanine, methionine, isoleucine, leucine, or valine resists MgtC degradation by FtsH. These data indicate that a large and hydrophobic side chain at 226th residue is required for protection from the FtsH-mediated MgtC proteolysis.

Keywords: *mgtC*, FtsH protease, post-translational regulation

Introduction

A *Salmonella* MgtC virulence protein encoded by the *mgtC* gene in the *mgtCBR* operon that is belonged to SPI-3 (Blanc-Potard and Groisman, 1997; Blanc-Potard and Lafay, 2003). The presence of MgtC protein is conserved in many other intracellular bacterial pathogens including *Brucella suis* (Lavigne *et al.*, 2005), *Burkholderia cenocepacia* (Maloney and Valvano, 2006), *Mycobacterium tuberculosis* (Buchmeier *et al.*, 2000), and *Yersinia pestis* (Alix and Blanc-Potard, 2007; Grabenstein *et al.*, 2006). The two-component regulatory system PhoP/PhoQ controls transcription initiation of the *mgtCBR* operon (Soncini *et al.*, 1996) in response to a limitation of magnesium ions (Garcia Vescovi *et al.*, 1996), low pH (Prost *et al.*, 2007), and antimicrobial peptides (Bader *et al.*, 2005) that are similar to those predicted in the phagosomal environment. The *mgtC* leader RNA promotes transcription elongation of the *mgtCBR* operon in response to ATP and charged tRNA^{Pro} levels (Lee and Groisman, 2012a, 2012b; Lee *et al.*, 2014). Because the *Salmonella mgtC* deletion mutant shows a severe defect in both mouse virulence and macrophage infection model (Blanc-Potard and Groisman, 1997; Lee *et al.*, 2013) and increases cytoplasmic ATP levels (Lee *et al.*, 2013; Pontes *et al.*, 2015), MgtC seems to have a versatile role in both bacterial virulence and bioenergetics.

Salmonella has five AAA$^+$ proteases, ClpXP, ClpAP, HslUV, Lon, and FtsH. Among those, FtsH is an ATP-dependent zinc metalloprotease (Langklotz *et al.*, 2012) and has two transmembrane domains (Tomoyasu *et al.*, 1993). Due to FtsH's location anchoring in the bacterial inner membrane, FtsH is expected to be the only protease responsible for degrading membrane proteins and is essential for *Salmonella* (Langklotz *et al.*, 2012; Kim *et al.*, 2013). Although specific catalytic sites have not been described yet, FtsH recognizes either ends of peptides, N- or C- terminus with short protruding residues (Chiba *et al.*, 2002) and preferentially targets misfolded, damaged, misassembled proteins with integral or cytosolic peptides (Ito and Akiyama, 2005), suggesting that FtsH tightly controls a membrane protein turnover in an ATP-dependent manner.

MgtC has five transmembrane domains and its cytoplasmic C-terminus that is partially structured (Rang *et al.*, 2007). For MgtC to be degraded, the MgtR regulatory membrane peptide binds to MgtC, leading to the FtsH-mediated proteolysis (Alix and Blanc-Potard, 2008). The previous study demonstrated that MgtC 226th tryptophan residue is conserved in other intracellular pathogens and critical for protecting MgtC from degradation by FtsH (Choi *et al.*, 2015). In this paper, we determined a requirement that influences MgtC proteolytic regulation. We replaced the MgtC 226th Trp amino acid to other amino acids such as Phe, Met, Ile, Leu, Val, Ala, Gly, and Tyr. Substitution of Trp226 to Phe, Met, Ile, Leu, or Val protects MgtC from proteolysis whereas *Salmonella* strains with Trp226 to Ala, Gly, or Tyr substitution promote FtsH-mediated MgtC proteolysis and behave just like the *mgtC* deletion mutant. Therefore, these data suggest that the side chain of the 226th residue should be large and hydrophobic to protect MgtC from degradation by FtsH.

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Materials and Methods

Bacterial strains, oligodeoxynucleotides, and growth conditions

Bacterial strains used in this study are listed in Supplementary data Table S1. All *Salmonella enterica* serovar Typhimurium strains are derived from the wild-type strain 14028s (Fields *et al.*, 1986) and were constructed by phage P22-mediated transductions as described (Davis, 1980). All DNA oligonucleotides are listed in Supplementary data Table S2. Bacteria were grown at 37°C in Luria-Bertani broth (LB), N-minimal medium (pH 7.7) (Snavely *et al.*, 1991) supplemented with 0.1% casamino acids, 38 mM glycerol and the indicated concentrations of $MgCl_2$. Ampicillin was used at 50 μg/ml, kanamycin at 50 μg/ml, or tetracycline at 10 μg/ml. Isopropyl-β-D thiogalactopyranoside (IPTG) was used at 0.0625 mM.

Quantitative real time-polymerase chain reaction (RT-PCR)

Total RNA was isolated using RNeasy Kit (Qiagen) according to the manufacturer's instructions. The purified RNA was quantified using a Nanodrop machine (NanoDrop Technologies). cDNA was synthesized using PrimeScript™ RT reagent Kit (TaKaRa). The mRNA levels of the *mgtC* and *rrsH* genes were measured by quantification of cDNA using SYBR Green PCR Master Mix (TOYOBO) and appropriate primers (*mgtC* coding: 7530/7531) and monitored using a 7500 Fast Real-Time PCR system (Applied Biosystems). Data were normalized to the levels of 16S ribosomal RNA amplified with primers 6970 and 6971.

Western blot analysis

Bacteria were grown in 15 ml N-minimal medium containing 10 mM or 0.01 mM Mg^{2+} to $OD_{600} = 0.5$. Crude extracts were prepared in TBS (Tris-buffered saline) buffer by sonication and analyzed as described (Lee and Groisman, 2010). The data are representative of two independent experiments, which gave similar results.

Construction of chromosomal mutant strains with the *mgt*C with the Trp codon at position 226 replaced by various amino acids

For *mgtC* 226th Trp codon substitution to various amino acids, we used the fusaric acid based counter selection method as described previously (Lee and Groisman, 2010). We prepared DNA fragments containing various amino acid substitutions by two rounds of PCR reactions. For the first PCR, we used two sets of primer pairs 8118/KHU774 and KHU747/1259 (for $mgtC^{W226M}$), 8118/KHU773 and KHU745/1259 (for $mgtC^{W226I}$), 8118/KHU772 and KHU743/1259 (for $mgtC^{W226L}$), 7554/KHU771 and KHU741/1259 (for $mgtC^{W226V}$), 7554/KHU770 and KHU769/1259 (for $mgtC^{W226G}$), 8118/KHU704 and KHU703/1259 (for $mgtC^{W226M}$), and 14028s genomic DNA as a template. For the second PCR, we mixed the two PCR products from the first PCR as templates and amplified DNA fragments using primers 8118/1259 (for $mgtC^{W226M}$, $mgtC^{W226I}$, $mgtC^{W226L}$, and $mgtC^{W226Y}$) and 7554/1259 (for $mgtC^{W226V}$ and $mgtC^{W226G}$). The resulting DNA fragments were purified and introduced into the EG18804 (for $mgtC^{W226M}$, $mgtC^{W226I}$, $mgtC^{W226L}$, and $mgtC^{W226Y}$) and EG18750 (for $mgtC^{W226V}$ and $mgtC^{W226G}$) electrocompetent cells and selected against Tet^R using a fusaric

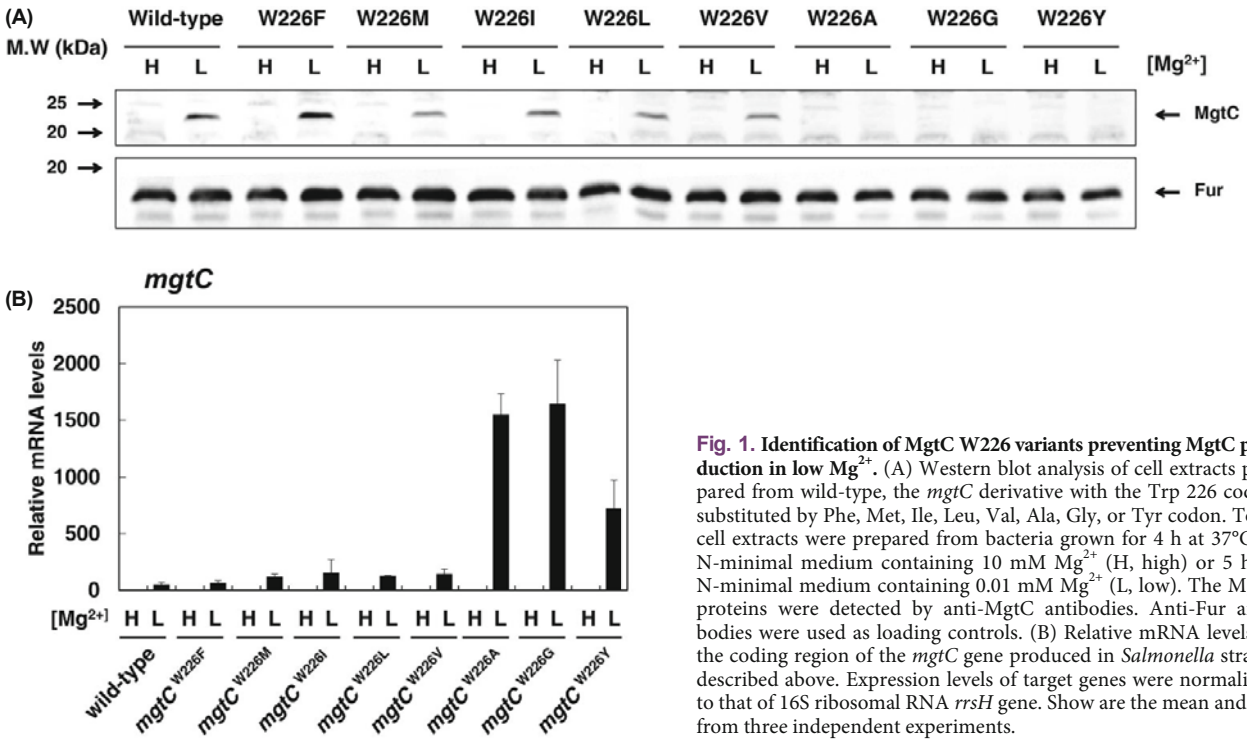

Fig. 1. Identification of MgtC W226 variants preventing MgtC production in low Mg^{2+}. (A) Western blot analysis of cell extracts prepared from wild-type, the *mgtC* derivative with the Trp 226 codon substituted by Phe, Met, Ile, Leu, Val, Ala, Gly, or Tyr codon. Total cell extracts were prepared from bacteria grown for 4 h at 37°C in N-minimal medium containing 10 mM Mg^{2+} (H, high) or 5 h in N-minimal medium containing 0.01 mM Mg^{2+} (L, low). The MgtC proteins were detected by anti-MgtC antibodies. Anti-Fur antibodies were used as loading controls. (B) Relative mRNA levels of the coding region of the *mgtC* gene produced in *Salmonella* strains described above. Expression levels of target genes were normalized to that of 16S ribosomal RNA *rrsH* gene. Show are the mean and SD from three independent experiments.

acid medium to generate strain EN772, EN1095, EN1093, EN1096, JH118, JH117, and EN1057, respectively (Maloy and Nunn, 1981). The resulting strains were verified by DNA sequencing.

Measuring growth of *mgtC*^{W226} mutant strains

The *mgtC*^{W226} substitution mutant strains were grown in N-minimal medium containing 0.01 mM or 10 mM Mg^{2+} condition. To address whether *Salmonella* strain with the *mgtC* 226th Trp codon replaced by various amino acids could display a similar growth phenotype with the wild-type strain, growth was determined at 37°C for 9 h in a 96-well plate with orbital shaking and absorbance measured at OD_{600} every 2.5 min.

Examining survival inside macrophages

Intramacrophage survival assays were conducted with the macrophage-like cell line J774 A.1 as described (Blanc-Potard and Groisman, 1997).

Results

The *mgtC* chromosomal mutants with the 226th Trp residue substitution show different expression behaviors in a manner dependent on size and polarity of the side chain

The MgtC is a virulence membrane protein and has a crucial role in macrophage survival (Alix and Blanc-Potard, 2007) of intracellular pathogens such as *Brucella suis* (Lavigne *et al*., 2005), *Burkholderia cenocepacia* (Maloney and Valvano, 2006), *Mycobacterium tuberculosis* (Buchmeier *et al*., 2000), *Salmonella enterica* (Blanc-Potard and Groisman, 1997), and *Yersinia pestis* (Grabenstein *et al*., 2006). In *Salmonella* MgtC, four amino acid residues (E84, N92, C99, and W226) were identified to be conserved in other intracellular pathogens (Rang *et al*., 2007). Among them, we further investigated the significance of MgtC 226th tryptophan in detail because we found that the 226th residue has a critical role to protect MgtC degradation by FtsH in the previous study (Choi *et al*., 2015). To determine a requirement for 226th residue to protect MgtC from the FtsH-mediated proteolysis, we created additional *mgtC* chromosomal substitution mutants at 226th position, which are *mgtC*^{W226M}, *mgtC*^{W226I}, *mgtC*^{W226L}, *mgtC*^{W226V}, *mgtC*^{W226G}, and *mgtC*^{W226Y}. Because the PhoP/PhoQ two-component system controls transcription of the *mgtC* gene and thus production of the MgtC protein, we grew *Salmonella* strains in N-minimal media containing 10 mM (PhoP-repressing) or 0.01 mM (PhoP-activating) Mg^{2+} for 5 h to measure amounts of MgtC protein. The tryptophan 226 substitutions to phenylalanine, methionine, isoleucine, leucine, or valine retained MgtC protein levels similar to those observed in wild-type in 0.01 mM Mg^{2+} (Fig. 1A). However, the tryptophan 226 substitution to alanine, glycine, or tyrosine lost the ability to produce MgtC proteins in 0.01 mM Mg^{2+} (Fig. 1A). Considering amino acid structures, these data indicate that large and hydrophobic residues at the 226th position are necessary for protection from MgtC proteolysis whereas small and polar residues promote the proteolysis (Fig. 1A). As previously observed, mRNA levels of strains lacking MgtC protein expression (A, G, or Y substitutions) were elevated relative to those observed in strains expressing MgtC (Fig. 1B) because MgtC depletion results in an increase in ATP levels, which could promote transcription of the *mgtC* gene via the leader RNA (Lee and Groisman, 2012a; Lee *et al*., 2013). As control experiments, MgtC proteins were not detected in the PhoP- repressing condition (Fig. 1A) and protein levels of Fur were unaffected in all conditions tested (Fig. 1A). Cumulatively, we suggest that size and hydrophobicity of the side chain at MgtC 226th residue determine MgtC degradation.

The tryptophan to alanine, glycine, or tyrosine substitution at the 226th residue promotes MgtC degradation in a manner dependent on the presence of the FtsH protease

FtsH is a membrane-bound protease that has ATP-dependent catalytic activity (Langklotz *et al*., 2012) and hydrolyzes MgtC via the MgtR regulatory peptide (Alix and Blanc-Potard,

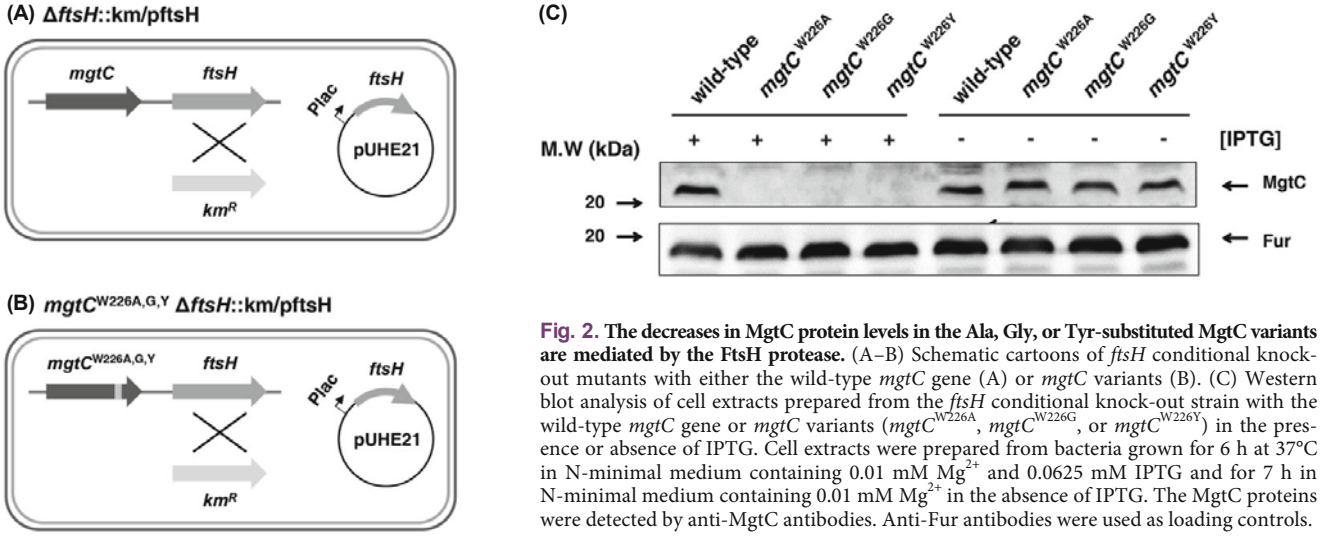

Fig. 2. The decreases in MgtC protein levels in the Ala, Gly, or Tyr-substituted MgtC variants are mediated by the FtsH protease. (A–B) Schematic cartoons of *ftsH* conditional knock-out mutants with either the wild-type *mgtC* gene (A) or *mgtC* variants (B). (C) Western blot analysis of cell extracts prepared from the *ftsH* conditional knock-out strain with the wild-type *mgtC* gene or *mgtC* variants (*mgtC*^{W226A}, *mgtC*^{W226G}, or *mgtC*^{W226Y}) in the presence or absence of IPTG. Cell extracts were prepared from bacteria grown for 6 h at 37°C in N-minimal medium containing 0.01 mM Mg^{2+} and 0.0625 mM IPTG and for 7 h in N-minimal medium containing 0.01 mM Mg^{2+} in the absence of IPTG. The MgtC proteins were detected by anti-MgtC antibodies. Anti-Fur antibodies were used as loading controls.

2008). To address whether the inability to detect MgtC in the strains with alanine, glycine, or tyrosine substitutions at MgtC 226th residue is due to degradation mediated by the FtsH protease, we used a ftsH conditional knockout strain as described previously (Fig. 2A and B)(Choi *et al.*, 2015). We grew cells in low Mg^{2+} media containing 0.0625 mM IPTG to induce FtsH expression from the IPTG-inducible plasmid. MgtC proteins from strains with MgtC Trp226 to Ala, Gly, or Tyr substitution were not detected similarly to those presented in Fig. 1A (Fig. 2C). However, when IPTG was removed from the medium to deplete FtsH, MgtC proteins from the strains with MgtC Trp226 to Ala, Gly, or Tyr substitution were accumulated similar to those in wild-type (Fig. 2C). This demonstrated that the disappearance of MgtC proteins in the W226A, G, or Y-substituted *mgtC* gene in low Mg^{2+} is due to the action of the FtsH protease. As a control, the protein levels of Fur were not affected in the presence or absence of IPTG (Fig. 2C).

The *mgtC* chromosomal mutants with the Trp226 to Ala, Gly, or Tyr substitution show defects in growth in low Mg^{2+} and survival inside macrophages

The *Salmonella mgtC* deletion mutant displays two distinct phenotypes: a growth defect when cells are grown in low Mg^{2+} media and a defect in replicating inside macrophages (Blanc-Potard and Groisman, 1997). We tested whether the *mgtC* variants with alanine, glycine, or tyrosine substitution showed similar defects observed in the *mgtC* deletion mutant due to the FtsH-mediated proteolysis. When we measured the optical density in N-minimal media containing 0.01 mM Mg^{2+}, the *mgtC* substitution mutants with the 226th residue to Ala, Gly, or Tyr grew similarly to the wild-type up to about OD 0.3 but then showed a decrease in OD, that is a distinctive growth pattern observed in the *mgtC* deletion mutant. The growth behaviors observed in the Trp226 to Ala, Gly, or Tyr-substituted *mgtC* mutants are due to lacking MgtC protein expression because i) other *mgtC* substitution mutants expressing MgtC (Phe, Met, Ile, Leu, or Val) showed similar growth profiles to wild-type (Fig. 3A) and ii) there are no significant differences in growth curves when cells were grown in N-minimal media containing 10 mM Mg^{2+}, a PhoP-repressing condition (Fig. 3B).

Likewise, when we measured *Salmonella*'s replication efficiency within macrophage-like J774 A.1 cells, the Ala, Gly, or Tyr substitution at the *mgtC* 226th residue decreased survival inside J774 A.1 macrophages compared to that of wild-type (Fig. 3C). The defects in macrophage survival were similar to the strain deleted the *mgtC* gene (Fig. 3C). By contrast, the Leu or Ile substitution at the *mgtC* 226th residue did not affect macrophage survival, consistent with the fact that *Salmonella* strains with the Leu or Ile substitution at the *mgtC* 226th residue produce MgtC proteins in low Mg^{2+} just like wild-type *Salmonella* (Fig. 1A).

Discussion

We have established a requirement for determining degradation of the MgtC virulence protein. Specifically, we have identified that the size and hydrophobicity of the 226th residue at the C-terminal end of MgtC are critical to determine whether MgtC is hydrolyzed by FtsH or not (Figs. 1 and 2). The side chains to promote the FtsH-mediated MgtC proteolysis decrease *Salmonella*'s growth in low Mg^{2+} and survival inside macrophages due to lacking MgtC expression (Fig. 3). Based on our findings, we would like to propose a simple model to explain our data (Fig. 4). Amino acid substitutions with large and hydrophobic side chains at the MgtC 226th residue protect MgtC from FtsH-mediated proteolysis. This might be due to that large and hydrophobic side chains, such as Trp, Phe, Met, Ile, Leu, and Val, block the access of the FtsH active site and thus inhibit the FtsH-mediated proteolysis (Fig. 4A). However, amino acids with small side chains including Ala and Gly might be fitted in the active

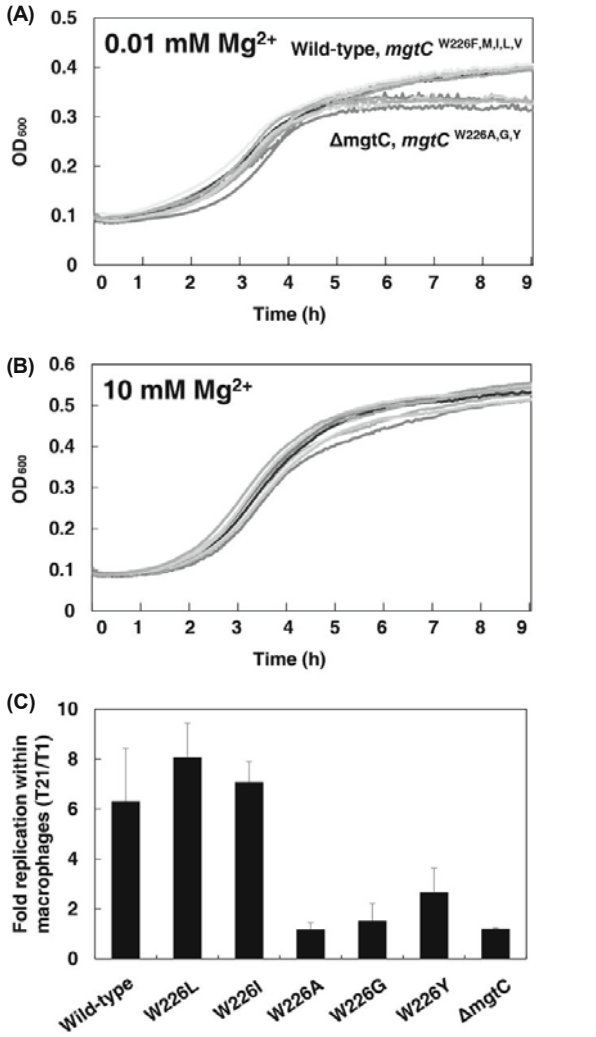

Fig. 3. *mgtC*$^{W226A, G, or Y}$ mutants show a defect in growth in low Mg^{2+} and survival inside macrophages. (A–B) Growth curves of wild-type or *mgtC* derivative *Salmonella* with the Trp 226 codon substituted by Phe, Met, Ile, Leu, Val, Ala, Gly or Tyr codon or the strain deleted the *mgtC* gene. Bacteria were grown at 37°C in N-minimal medium containing 0.01 mM Mg^{2+} (A) or 10 mM Mg^{2+} (B). 96-well plate with orbital shaking was used for bacteria culture and the absorbance at OD_{600} was measured every 2.5 min. (C) Replication within J774 A.1 macrophage-like cells of strains listed above at 21 h after infection.

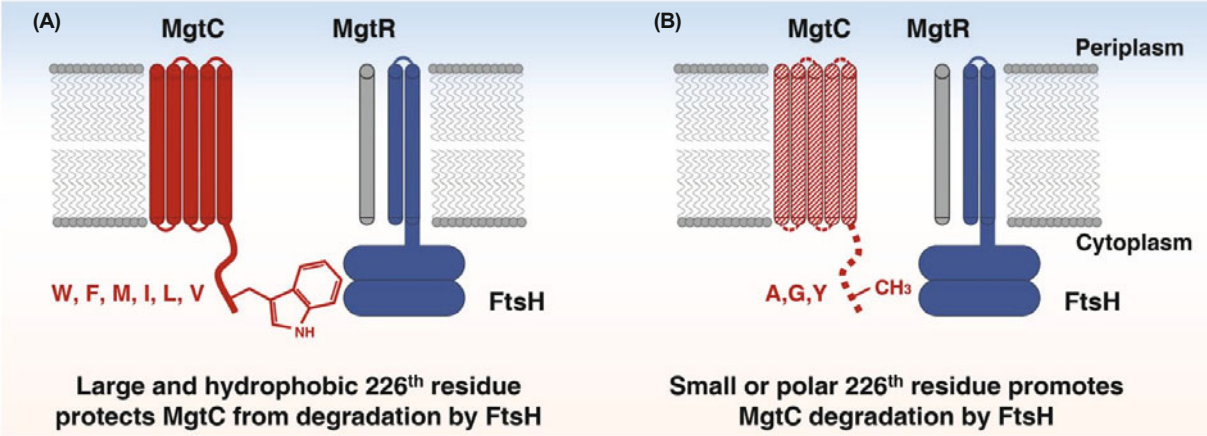

Fig. 4. A model for degradation of MgtC by the FtsH membrane protease. (A) When MgtC has a large and hydrophobic side chain at 226th residue (W, F, M, I, L, or V), it prevents MgtC proteolysis by FtsH. (B) When MgtC has a small or polar side chain at 226th codon (A, G, or Y), it promotes MgtC proteolysis by FtsH. MgtR has an additional role in MgtC degradation because MgtR mediates the FtsH-mediated MgtC proteolysis in both cases (Choi *et al.*, 2015).

site of the FtsH protease and promote MgtC proteolysis (Fig 4B). Likewise, Tyr fails to protect MgtC proteolysis possibly because the polar side chain of Tyr residue induces MgtC binding to the active site of the FtsH protease by an electrostatic interaction (Fig. 4B). To understand how FtsH catalyzes MgtC degradation, a functional or genetic study of the active site in the FtsH protease needs to be carried out in the future. The MgtR regulatory peptide is also involved in MgtC degradation by the FtsH protease (Alix and Blanc-Potard, 2008) (Fig. 4). However, because degradation of the MgtC W226A variant by the FtsH protease takes place independnely of the presence of the MgtR regulatory peptide (Choi *et al.*, 2015), MgtR seems to have an additional role in MgtC degradation besides the amino acid determinant located in the 226th residue of the MgtC protein (Fig. 4).

When *Salmonella* resides within a phagosome inside macrophages, it struggles to survive and endure unfavorable phagosome environments, such as low magnesium concentration, acidic pH, and antimicrobial peptides. To overcome these disadvantageous conditions, *Salmonella* expresses many virulence genes whose expression levels might be critical for survival during infection. Because MgtC is one of such virulence factors that are required for *Salmonella*'s survival inside macrophages (Blanc-Potard and Groisman, 1997), *Salmonella* utilizes several strategies to produce MgtC levels inside host. First, transcription initiation of the *mgtC* gene is controlled by the PhoP/PhoQ two-component regulatory system (Soncini *et al.*, 1996). Because PhoQ senses low Mg^{2+}, acidic pH, or antimicrobial peptides (Garcia Vescovi *et al.*, 1996; Bader *et al.*, 2005; Prost *et al.*, 2007) that might be relevant in the macrophage phagosome, mRNA levels of the *mgtC* gene is highly induced inside macrophages. Second, the *mgtC* harbors a long leader region that is responsible for sensing cytoplasmic ATP levels and charged $tRNA^{Pro}$ levels and controls transcription elongation of the *mgtC* gene (Lee and Groisman, 2012a, 2012b; Lee *et al.*, 2014). The phagosomal environment is expected to be high ATP levels and low charged $tRNA^{Pro}$ levels (Lee *et al.*, 2013, 2014; Nam *et al.*, 2016), the leader region further promotes *mgtC* mRNA levels (Lee and Groisman, 2012a, 2012b). Third, MgtC has the conserved tryptophan residue at the 226th position that protects from the FtsH-mediated proteolysis during infection. Finally, given that MgtC decreases cytosolic ATP levels by targeting F_1F_o ATP synthase (Lee *et al.*, 2013), the activity of the AAA^+ protease FtsH might be lower inside host because FtsH requires ATP for its hydrolysis (Langklotz *et al.*, 2012).

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

EL designed research; EC and JB performed research; EL, EC, and JB wrote the paper. All authors read the paper and contributed to its final form.

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