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Peptide-assisted degradation of the *Salmonella* MgtC virulence factor

Eric Alix[1,2] and Anne-Béatrice Blanc-Potard[1,2*]

[1] Inserm, ESPRI 26, Avenue J.F. Kennedy, 30908 Nîmes cedex 02, France

[2] Université Montpellier 1, UFR de Médecine, Avenue J.F. Kennedy, 30908 Nîmes cedex 02, France

Running title: Peptide-assisted degradation of MgtC in *Salmonella*

*Corresponding author. Institut National de la Santé et de la Recherche Médicale, Espri 26, UFR de Médecine, CS 83021, Avenue J.F. Kennedy, 30908 NIMES Cedex 02, France. Tel : 33 4 66 02 81 47 ; Fax : 33 4 66 02 81 48 ; E-mail: ablancpotard@univ-montp1.fr

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Abstract

MgtC is a virulence factor common to several intracellular pathogens that is required for intramacrophage survival and growth in magnesium-depleted medium. In *Salmonella enterica*, MgtC is coexpressed with the MgtB magnesium transporter and transcription of the *mgtCB* operon is induced by magnesium deprivation. Despite the high level of *mgtCB* transcriptional induction in magnesium-depleted medium, the MgtC protein is hardly detected in a wild-type *Salmonella* strain. Here, we show that down-regulation of MgtC expression is dependent on an hydrophobic peptide, MgtR, which is encoded by the *mgtCB* operon. Our results suggest that MgtR promotes MgtC degradation by the FtsH protease, providing a negative regulatory feedback. Bacterial two-hybrid assays demonstrate that MgtR interacts with the inner-membrane MgtC protein. We identified mutant derivatives of MgtR and MgtC that prevent both regulation and interaction between the two partners. In macrophages, over-expression of the MgtR peptide led to a decrease of the *Salmonella* replication rate. This study highlights the role of peptides in bacterial regulatory mechanisms and provides a natural antagonist of the MgtC virulence factor.

Keywords: FtsH / MgtC / regulatory peptide / *Salmonella enterica* serovar Typhimurium

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Introduction

Bacteria have evolved sophisticated regulatory pathways to adapt to environmental changes. The control of protein stability allows the cell to adjust to changing conditions by changing the rate of turnover of proteins. Regulated proteolysis has been well characterized for few proteins, including proteins involved in stress response mechanisms (Gottesman, 2003). Whereas the complex ubiquitin ligase machinery provides a target for regulating proteolysis in eukaryotes, no ubiquitin tagging exists in bacteria. A small number of ATP-dependent proteases (AAA+ proteases) are responsible for degradation of specific proteins, frequently by directly interacting with the substrate protein (Baker and Sauer, 2006). An additional layer of potential specificity and regulation is provided by adaptor proteins, that enhance or alter the substrate-recognition properties of AAA+ proteases (Dougan *et al*, 2002; Baker and Sauer, 2006). The ClpXP and Lon AAA+ proteases have been implicated in bacterial virulence (Yamamoto *et al*, 2001; Frees *et al*, 2003; Takaya *et al*, 2003; Kwon *et al*, 2004) and in the degradation of transcriptional regulators that control virulence gene expression (Takaya *et al*, 2005).

MgtC is a virulence factor common to several intracellular pathogens that plays a key role in intramacrophage survival (Alix and Blanc-Potard, 2007). MgtC was first described in *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) where it is required for intramacrophage multiplication and long-term systemic infection in mice (Blanc-Potard and Groisman, 1997; Lawley *et al*, 2006). MgtC is also a critical factor for the intramacrophage growth of *Mycobacterium tuberculosis*, *Brucella suis*, *Yersinia pestis* and *Burkholderia cenocepacia* (Buchmeier *et al*, 2000; Lavigne *et al*, 2005; Grabenstein *et al*, 2006; Maloney and Valvano, 2006). Phylogenetic analysis suggested that *mgtC* has been acquired by horizontal gene transfer repeatedly throughout bacterial evolution (Blanc-Potard and Lafay,

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2003). In *S*. Typhimurium, the *mgtC* gene is carried by the SPI-3 pathogenicity island (Blanc-Potard and Groisman, 1997). The biochemical function of MgtC remains unknown and the role of MgtC might differ in host and non-host environments (Rang *et al*, 2007). Heterologous expression of MgtC protein in *Xenopus laevis* oocytes has significant effect on cellular ion homeostasis *via* a modulation of eukaryotic Na^+,K^+-ATPase activity (Gunzel *et al*, 2006). However, the physiological relevance of these results during bacterial infection is currently unknown.

MgtC is also involved in adaptation to low Mg^{2+} environments (Alix and Blanc-Potard, 2007). The regulation of *mgtC* in response to Mg^{2+} deprivation has been well studied at the transcriptional level in *S.* Typhimurium. The *S*. Typhimurium *mgtC* gene is cotranscribed with *mgtB*, encoding a Mg^{2+} transporter (Snavely *et al*, 1991). Both MgtC and MgtB are inner-membrane proteins. The *mgtCB* operon is regulated by the PhoP-PhoQ two-component system and is highly induced by low Mg^{2+} concentration (García-Véscovi *et al*, 1996). In addition, the *mgtCB* operon is also regulated at the transcriptional level by a PhoPQ-independent mechanism that depends on the secondary structure of the 5'UTR (Groisman *et al*, 2006). The MgtC protein could be detected at short times after Mg^{2+} starvation (Moncrief and Maguire, 1998; Adkins *et al*, 2006). Surprisingly, despite the high transcriptional induction, MgtC is undetectable during prolonged Mg^{2+} starvation in a *mgtC*$^+$ *mgtB*$^+$ strain, although large amounts of MgtB are observed (Moncrief and Maguire, 1998). However, MgtC was detected in a *mgtC*$^+$ strain that lacks the *mgtB* sequence, suggesting that MgtC is not produced or is not stable in the presence of the MgtB protein (Moncrief and Maguire, 1998). In the present study, we further investigated the regulation of MgtC expression. We demonstrate that the amount of MgtC protein is down-regulated by a 30 amino-acid peptide that is encoded downstream of the *mgtB* gene. We show that this peptide, termed MgtR, interacts with MgtC *in vivo* and promotes the degradation of MgtC by the FtsH AAA+

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protease. Identification of MgtR highlights the regulatory role of peptides and very small proteins that are often missed in genomic and proteomic analyses.

Results

MgtC expression is negatively regulated by sequences 3' of mgtB

Previous work has shown that even though the *mgtCB* operon is highly induced in low Mg^{2+} environment, the MgtB, but not the MgtC, protein is detected in a wild-type *Salmonella* strain upon prolonged Mg^{2+} starvation (Moncrief and Maguire, 1998). In addition, it has been suggested that *mgtB* sequence has a negative effect on MgtC expression (Moncrief and Maguire, 1998). To explain the role of *mgtB* on MgtC protein level, we have transformed an *E. coli* strain, which does not contain the *mgtCB* operon, with plasmids containing only the *mgtC* gene (pEG9094) or the entire *mgtCB* operon (pEG9091) (Figure 1A). Consistently with previous data, MgtC was clearly expressed from pEG9094, but to a much lesser extent from pEG9091 (Figure 1B). A frameshift mutation at the beginning of the *mgtB* sequence did not produce higher MgtC levels, indicating that the lack of MgtC expression from pEG9091 is not due to the production of MgtB protein per se, but rather to other elements in the *mgtB* sequence (Figure 1B). This result agrees with the previous report that a functional MgtB protein was not required to repress MgtC expression (Moncrief and Maguire, 1998). We carried out successive deletions on pEG9091 plasmid to characterize the DNA region responsible for down-regulation of MgtC expression (Figure 1A). Deletions within the *mgtB* gene did not increase MgtC levels (Figure 1B). However, a deletion of the region downstream of the *mgtB* coding region (pEG9091 del4) gave a pattern similar to the one observed with

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pEG9094, indicating that the 3' region of *mgtB* is involved in the regulation of MgtC expression.

A 150 bp region downstream of *mgtB* was subcloned into the pBBR1MCS vector plasmid, which is compatible with pEG9094, to produce the pMgtR plasmid. The pMgtR plasmid was introduced in *E. coli* strain carrying pEG9094. Western Blot analysis clearly showed a decrease of MgtC expression in the presence of pMgtR but not in the presence of the pBBR1MCS vector (Figure 1C). This result indicates that the region downstream of *mgtB* acts *in trans* to regulate MgtC expression. Taken together, these results indicate that a small DNA region, present at the 3' end of the *mgtCB* operon, negatively regulates the amount of MgtC protein.

MgtR: a peptide that down-regulates MgtC expression

The 150 bp regulatory region described above includes a 90 bp region (position 3961428-3961518 of the *S*. Typhimurium LT2 genome) that could encode a small RNA and/or an open reading frame (ORF) of 30 amino-acids, preceded by a shine-dalgarno consensus sequence (Figure 2A). This sequence, which is not annotated in the *S*. Typhimurium genome, was named *mgtR* (GenBank accession number EU154350). To distinguish between the RNA and protein hypotheses, we examined the phenotype of strains with several site-directed mutants in the pMgtR plasmid. First, we generated two mutations (Mut 1 and Mut 2) that disrupt the predicted RNA secondary structure without changing the predicted amino-acid sequence (Figure 2A). As shown in Figure 2B, regulation of MgtC expression was maintained in the presence of both mutations. Secondly, we performed mutagenesis of the initial ATG of the putative ORF (Mut 3) or introduced a frameshift mutation (Mut 4), without changing the stability of the predicted RNA secondary structure (Figure 2A). The amount of MgtC was highly increased when pMgtR plasmid carried these mutations (Figure 2B), indicating that

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these mutations prevented the down-regulation of MgtC expression. Taken together, these results indicate that a peptide, and not a small RNA, is involved in MgtC regulation.

To further explore the role of *mgtR* in *S*. Typhimurium, we have carried out a precise chromosomal deletion of *mgtR*. As expected, MgtC is expressed in the Δ*mgtR* mutant strain and expression is turned down by introduction of the pMgtR plasmid (Figure 3A). The *mgtR* gene was cloned into pQE30 to produce an IPTG-inducible N-terminal His-tagged peptide. The resulting plasmid, pQEMgtR, produces a functional peptide since it complemented the *S*. Typhimurium Δ*mgtR* mutant strain upon IPTG induction by down-regulating MgtC expression (Figure 3B). Upon IPTG induction, the decrease in the level of MgtC was correlated with the detection of the 5 kDa His_6-MgtR at the bottom of the gel. The detection of His_6-MgtR in this Western Blot experiment is due to the fact that MgtC antibodies recognize the His epitope. The 5 kDa band is also detected upon IPTG induction in a strain that encodes His_6-MgtR but not MgtC, indicating that it is not related with degradation fragments of MgtC (data not shown). Taken together, these results demonstrate that a 30 amino-acid peptide encoded downstream of *mgtB* negatively regulates the levels of MgtC protein.

The mgtR sequence belongs to the mgtCB operon and MgtR acts on MgtC at the post-transcriptional level

The *mgtCB* operon is induced by growth in low Mg^{2+} medium (Figure 4A). To examine *mgtR* transcription, total RNA was extracted from wild-type *S*. Typhimurium strain 14028s grown in NCE medium supplemented with 10 µM or 10 mM Mg^{2+}. RT-PCR experiments showed that, similarly to *mgtC*, the *mgtR* gene is only transcribed in low Mg^{2+} conditions. In contrast, a non-PhoP-regulated gene, *gapA*, is transcribed in both low and high Mg^{2+} (Figure 4B). In addition, RT-PCR fragments were amplified with primers belonging to *mgtB* and *mgtR*

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(Figure 4B). Taken together, these results demonstrate that *mgtR* belongs to the *mgtCB* operon.

The levels of MgtB protein were identical in the presence or absence of MgtR (Supplementary Figure S1), indicating that the effect of MgtR is specific for MgtC and does not affect MgtB. This result further suggests that MgtR does not act on the promoter sequences of the *mgtCB* operon. Accordingly, when the region upstream of *mgtC*, including *mgtC* promoter sequence and *mgtC* 5'UTR, was fused to the *lacZ* gene (Supplementary information), the β-galactosidase activity was independent of the presence of MgtR (data not shown). Moreover, MgtC expression was regulated by MgtR when the *mgtC* coding sequence was under the control of the *lac* promoter (data not shown). Cumulatively, these results indicate that MgtR acts on MgtC at the post-transcriptional level.

MgtR promotes MgtC degradation by FtsH protease

We have studied the stability of MgtC in a *S*. Typhimurium Δ*mgtR* mutant strain that harbors the inducible His_6-MgtR peptide. Bacterial cultures were grown O/N in low Mg^{2+} medium to induce MgtC expression. The transcription of *mgtC* was then stopped by shifting the bacteria to high Mg^{2+} medium. In addition, IPTG was simultaneously added to induce the His_6-MgtR synthesis. In a control experiment, the Δ*mgtR* strain harbored the pQE30 vector and did not express MgtR. The level of MgtC protein was evaluated at time points after the shift (Figure 3C). In the presence of MgtR, MgtC was barely detected 4 h after the shift. On the other hand, MgtC was still detected 4 h after the shift in the absence of MgtR. This result demonstrates that MgtR promotes MgtC degradation, although through a relatively slow process.

FtsH is a AAA+ protease that has been involved in the degradation of membrane proteins (Ito and Akiyama, 2005). To determine whether FtsH plays a role in MgtC degradation, we have studied the level of MgtC protein in the presence or absence of MgtR in

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a *E. coli* thermosensitive *ftsH* mutant or an isogenic wild-type strain. At the permissive temperature of 30°C, the down-regulation of MgtC in the presence of MgtR is similar in the wild-type and *ftsH* mutant (Figure 5). However, at 42°C, the regulation is clearly observed in the wild-type strain but not in the *ftsH* mutant (Figure 5). Hence, a functional FtsH protease is required to mediate the down-regulation of MgtC expression in the presence of MgtR. To address the specificity of the FtsH protease, we have also used a *clpX* mutant of *Salmonella* (Aldridge *et al*, 2003). The regulation of MgtC by MgtR was similar in the wild-type strain and the *clpX1*::Tn*10*d*cam* mutant (Supplementary Figure S2). Taken together, these results indicate that MgtR assists MgtC to be degraded by the FtsH protease.

Identification of MgtR and MgtC mutants that prevent MgtR-assisted MgtC degradation

MgtR is predicted to be an inner-membrane peptide of 3.5 kDa with a short cytoplasmic N-terminus, a highly hydrophobic transmembrane alpha-helix, and a short periplasmic C-terminus. As expected, MgtR is found to be associated with the *S*. Typhimurium membrane fraction (Supplementary Figure S3). We carried out site-directed mutagenesis in the N- and C-terminal parts as well as in the transmembrane helix (Figure 6A). We tested the ability of mutated peptides to regulate MgtC degradation. Mutations were carried out on the pMgtR plasmid (Figure 6B) or on the pQEMgtR plasmid (Figure 6C). Results clearly showed that, among the 11 residues tested, only two residues abolished the degradation of MgtC and are therefore important for MgtR function (Figure 6C). These mutations, L15R and A24R, introduce charged residues in the hydrophobic region. On the other hand, single mutations in the N-terminal or C-terminal extremities had minor or no detectable effect on MgtR function. In the L15R mutant of MgtR, the peptide was poorly expressed and was similarly found in insoluble and soluble fractions, suggesting incorrect MgtR location (Figure 6C and Supplementary Figure S3). The A24R mutant of MgtR appeared mostly membrane associated

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despite the introduction of a charged residue. We have carried out additional mutations, A15P and A24P, that would disrupt the alpha-helix but maintain the hydrophobicity. Both mutations reduced MgtC degradation, with a clearer effect for A24P (Figure 6C).

In the course of previous studies, we have characterized several mutant forms of MgtC substituted in residues that are conserved among MgtC proteins (Rang *et al*, 2007; Rang and Blanc-Potard, unpublished results). We have noticed that mutants E84A, G85A, and N92T, led to a high level of MgtC protein by Western blot analysis in *S*. Typhimurium (data not shown). We hypothesized that these mutations, which are clustered in the cytoplasmic loop between the third and fourth transmembrane domains (TM3 and TM4) of MgtC (Figure 7A), might interfere with MgtR regulation. Plasmids encoding mutant MgtC proteins were introduced in isogenic *E. coli* strains expressing or lacking MgtR. In contrast to the wild-type protein, mutant MgtC proteins were similarly expressed in the presence or absence of MgtR (Figure 7B). Hence, the E84A, G85A, and N92T mutations render MgtC resistant to degradation promoted by MgtR.

Bacterial two-hybrid analysis demonstrates that MgtC and MgtR interact in vivo

Both MgtC and MgtR are predicted to localize in the inner-membrane. To test putative interaction between MgtC and MgtR, we have used the bacterial two-hybrid (BACTH) system that has been validated for detecting specific interactions between inner-membrane proteins (Karimova *et al*, 2005). A derivative of pUT18 was constructed to fuse the T18 fragment of adenylate cyclase to the C-terminal end of MgtC, that is cytoplasmic (Rang *et al*, 2007). A derivative of pKT25Δ*Xba*I was constructed to fuse the T25 fragment of adenylate cyclase to the N-terminal end of MgtR, that is predicted to be cytoplasmic. Plamids encoding the T18 and T25 fusion proteins were introduced in an *E. coli cya* mutant (BTH101) and functional complementation was determined by measuring β-galactosidase activity (Figure 8).

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A high level of β-galactosidase activity was observed when BTH101 was cotransformed with the MgtC-T18 and T25-MgtR plasmids, indicating an interaction between both fusion proteins. Only basal level of β-galactosidase activity was observed with the two vectors or with either fusion protein with the other vector control.

To investigate the correlation between MgtC-MgtR interaction and MgtC down-regulation, we have introduced in the two-hybrid plasmids mutations in MgtR or MgtC that prevent MgtR-assisted MgtC degradation. As shown in Figure 8, the two mutations in the hybrophobic domain of MgtR, L15R and A24R, gave only a basal level of β-galactosidase activity, indicating a lack of MgtR-MgtC interaction, whereas mutations L15P and A24P only lowered the interaction. The substitutions E84A, G85A and N92T in MgtC also lowered the interaction, with a marked effect for the MgtC mutant G85A that exhibited a β-galactosidase activity only 2.5 fold higher than basal level. These effects are not due to a lower expression of the mutated hybrid proteins (data not shown). The effect on the interaction of mutations L15P and A24P in MgtR and E84A and N92T in MgtC appeared much clearer when the β-galactosidase assays were carried out at a more stringent temperature, 37°C, since the level of β-galactosidase was below the threshold for interaction (Supplementary Figure S4). Taken together, these results show that MgtC and MgtR mutants that prevent the degradation of MgtC can be correlated with a decrease of *in vivo* interaction between the two partners.

The fact that MgtC residues located in the cytoplasmic loop between TM3 and TM4 play a role in the interaction with MgtR suggests that TM3 and/or TM4 might interact with MgtR. Both MgtR and TM4 contain cysteine residues. We have shown that the C22 residue of MgtR had no role in regulation (Figure 6B) and a C99A mutation in the TM4 of MgtC had no effect on regulation and interaction (data not shown). MgtR and the TM4 domain of MgtC also harbor an Ala-coil domain, which is an helix-helix interaction motif characterized by small residues (Ala, Gly, Ser) in heptad repeats (Walters and DeGrado, 2006). To test the

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contribution of Ala-coil motifs in MgtC-MgtR interaction, we have substituted small residues of the motif by large hydrophobic residues (Figure 9A). Results clearly showed that the disruption of MgtR Ala-coil prevented the interaction (Figure 9B). The reduced interaction is not linked to inaccurate expression or location of MgtR since, for example, a MgtR peptide carrying mutation S17I is produced and localized like wild-type MgtR (Supplementary Figure S3). Conversely, the change to another small residue (mutation A24G) had no effect (Figure 9B), probably because a correct helix-packing is preserved. A correlation is found between MgtC-MgtR interaction with the BACTH system and regulation of MgtC expression in Western analysis (Figure 9C). On the other hand, single mutations in the Ala-coil domain of MgtC TM4 have no effect on MgtC-MgtR interaction with the exception of G108F (Figure 9 and Figure S4). The loosely interaction in the presence of G108F mutation might be related to a lower expression of the MgtC-T18 hybrid protein (data not shown)

Role of MgtR in intramacrophage survival

MgtC plays a role for *Salmonella* intramacrophage growth (Blanc-Potard and Groisman, 1997). The mutations E84A, G85A and N92T in MgtC produce proteins that can not complement the macrophage growth defect of a *S.* Typhimurium Δ*mgtC* mutant (Rang *et al*, 2007; data not shown). We have compared the intramacrophage survival of a *S*. Typhimurium wild-type strain and a Δ*mgtR* mutant strain. The absence of MgtR had only a slight effect on intracellular growth (Figure 10A), indicating that the stabilization of MgtC has no major effect in this virulence assay. In addition, the Δ*mgtR* mutant strain grew similarly as the wild-type strain in low Mg^{2+} medium (data not shown). On the other hand, we have shown that the over-expression of MgtR in a *S*. Typhimurium wild-type strain significantly reduced the intramacrophage growth (Figure 10B). This intracellular growth defect is not linked to a slow

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down of bacterial growth rate since strains exhibited similar growth curves in LB medium or low Mg^{2+} medium (data not shown).

Discussion

Multiple regulatory pathways control the MgtC protein levels

The *mgtCB* operon is known to be regulated at the transcriptional level by the PhoPQ two-component system in response to external Mg^{2+} concentration (García-Véscovi *et al*, 1996). The PhoP regulatory protein interacts directly with the *mgtCB* promoter (Zwir *et al*, 2005). In addition, the operon is regulated at the transcriptional level by intracellular Mg^{2+} concentration through a PhoPQ-independent mechanism that depends on the secondary structure of its 5'UTR (Groisman *et al*, 2006). In the present study, we describe an additional level of regulation at the post-translational level for the MgtC protein. Expression of the MgtC protein is prevented by the MgtR peptide, which is encoded downstream of the *mgtB* gene. The MgtR peptide directly interacts with MgtC and probably unfolds MgtC to promote its degradation by the membrane-bound AAA+ protease FtsH. This novel regulatory mechanism, which identifies for the first time a virulence factor as FtsH target, is schematised in Figure 11.

MgtR, a regulatory peptide that interacts with MgtC and promotes its degradation by the FtsH protease

MgtC is an inner-membrane protein that harbors five transmembrane segments in the first half of the protein (Rang *et al*, 2007). MgtR is an inner-membrane peptide with a single highly hydrophobic transmembrane domain. Using the bacterial two-hybrid BACHT system, which

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appears as the technique of choice to study interaction between bacterial proteins within biological membranes (Karimova *et al*, 2005; Schneider *et al*, 2007), we could demonstrate interaction between MgtC and MgtR. The strength of this interaction correlates with the ability of MgtR to mediate MgtC degradation. The hydrophobicity and the alpha-helical structure of MgtR are important for its function. The interaction between membrane-imbedded helices remains hard to predict solely on the basis of amino-acid sequences but some motifs can drive helix-helix interactions (Schneider *et al*, 2007). MgtR harbors an Ala-coil motif that has been implicated in the association of membrane-imbedded helices (Walters and DeGrado, 2006) This Ala-coil motif appears essential for MgtC-MgtR interaction and MgtR-mediated MgtC degradation. We identified mutations in MgtR, as S17I or A24L, that do not prevent the expression or membrane location of the peptide, and completely abolish its regulatory function and interaction with MgtC. We have also identified mutations in MgtC that prevented its down-regulation by MgtR and lowered the interaction of MgtC with MgtR in the BACHT system. These mutations are located in a cytoplasmic loop between the third and fourth transmembrane domains (TM3 and TM4) of MgtC. The context of a transmembrane helix is essential for its correct topology and loops can contribute to the stability of helix interactions (Schneider *et al*, 2007). Hence, loop mutations could disrupt the helical packing in TM3 and/or TM4 and thereby prevent interaction with MgtR. Interestingly, the fourth helical segment of MgtC harbors an Ala-coil motif. However, mutagenesis of single residues did not indicate a contribution of this motif in the interaction with MgtR. The interacting domain of MgtC might be complex and involve more than a single TM domain.

A kinetic experiment demonstrated that MgtR promotes MgtC degradation, although through a relatively slow process. MgtR acts specifically on MgtC since no effect was detected on MgtB. The fact that both MgtC and MgtR are encoded by the same operon is rather surprising and raises the question of timing for MgtC expression and the regulatory

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peptide expression. Previous studies have indicated that MgtC can be detected after limited time of Mg^{2+} starvation (2 – 4 h) (Moncrief and Maguire, 1998; Adkins *et al*, 2006; data not shown) but MgtC is not expressed anymore after 16 h Mg^{2+} starvation (Moncrief and Maguire, 1998; Figure 3A). Our results suggest that upon induction of the *mgtCBR* operon, MgtR does not affect MgtC production, MgtC being similarly expressed in the presence or absence of MgtR after short time induction (data not shown), but that the production of MgtR promotes MgtC degradation after few hours.

We have shown that the degradation of MgtC is dependent on the presence of a functional FtsH protease. FtsH is the only membrane-bound AAA+ protease in *E. coli* and this protease plays an important role in the quality control of membrane proteins (Ito and Akiyama, 2005). We propose that the interaction between MgtC and MgtR might unfold MgtC and make it a substrate for FtsH-mediated degradation. FtsH recognizes unstructured peptide sequences, which are typically located at the N- or C-terminal end of a target protein, and degrades target protein processively. Internal loops also contribute to the degradation of substrates by FtsH and other AAA+ proteases (Datta *et al*, 2005; Hoskins and Wickner, 2006; Okuno *et al*, 2006). To date, the precise mechanism of degradation of MgtC by FtsH remains unknown.

To our knowledge, MgtR is the first example of an α-helical hydrophobic peptide that modulates degradation of a bacterial membrane protein by an AAA+ protease. It is possible that the participation of peptides in the regulation of protein stability might be a more common process that has not been characterized yet due to the lack of appropriate screening / detection methods. To date, the only other reported case of a peptide that modulates AAA+ protease activity is the SpoVM peptide from *Bacillus subtilis*, which, in contrast to MgtR, is an amphipathic helix (Cutting *et al*, 1997; Prajapati *et al*, 2000). SpoVM has been involved in sporulation and has an inhibitory action on the FtsH protease. In addition, several adaptor

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proteins modulate proteolysis by enhancing or inhibiting interaction between specific substrates and AAA+ proteases (Dougan *et al*, 2002; Baker and Sauer, 2006). Most adaptor proteins interact directly with the substrate in complex with the AAA+ partner protein and they usually modulate recognition of the substrate. Adaptors are generally small proteins encoded by genes that are rarely cotranscribed with the genes encoding their target(s). MgtR differs from previously described adaptors in its size and coexpression with its target.

Physiological role of MgtR during infection

MgtR is conserved in other *Salmonella enterica* serovars (*S.* Typhi and *S.* Choleraesuis), but is not found in other bacterial species that encode MgtC. Hence, it seems that *Salmonella* has developed a specific regulatory system that acts as a negative feedback to limit the amount of MgtC protein. We propose that this feedback regulation is linked to the high transcriptional induction of the *Salmonella mgtC* gene under certain conditions. In other bacteria, the *mgtC* gene might be expressed at lower levels, preventing the need for a feedback regulatory mechanism. MgtC has likely been acquired by horizontal gene transfer and is known to be differentially regulated in various species (Alix and Blanc-Potard, 2007). MgtC is required for intramacrophage growth (Alix and Blanc-Potard, 2007) and the *Salmonella mgtCB* operon is highly induced in macrophages (Smith *et al*, 1998; Eriksson *et al*, 2003). However, a proteomic analysis of *S*. Typhimurium isolated from macrophages identified MgtB but not MgtC (Shi *et al*, 2006). This suggests that the intracellular level of MgtC protein is low and/or that there is a detectable expression at infection times that were not experimentally tested. To better understand the physiological role of MgtR during *Salmonella* infection, we have investigated the growth phenotype of a Δ*mgtR* mutant in macrophages. The Δ*mgtR* mutant is only slightly attenuated for growth within macrophages, indicating that MgtR does not play a major role in this virulence assay. On the other hand, overexpression of MgtR in a wild-type

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strain of *S.* Typhimurium reduces significantly the ability of the strain to grow within macrophages. This might be linked to a high degradation level of the MgtC virulence factor. Hence, this result suggests that MgtR could act as an antagonist of the MgtC virulence factor under certain conditions.

Conclusion

The completion of bacterial genomes has significantly improved the description of regulatory networks. However, small ORFs are not annotated on genome maps. In addition, functional small ORFs are rarely detected by genetic screening due to the low frequency of mutational or insertion events in these regions. To date, studies on small ORFs have mainly focused on the identification of small regulatory RNAs. In the present study, we have identified a regulatory peptide involved in the degradation of a virulence factor. In *Salmonella*, a recent peptidomics analysis, which did not include peptides encoded by non-annotated ORFs, has suggested a targeted protein degradation under phagosome-mimicking culture conditions (Manes *et al*, 2007). Further peptidomics research is required to characterize additional functional bacterial peptides, which can be encoded by small ORFs or can be generated by the proteolytic cleavage of proteins.

Material and methods

Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table I. All *S.* Typhimurium strains are derived from wild-type 14028s with the exception of TH6767 that is derived from LT2. *E. coli* thermosensitive *ftsH1* mutant (Begg et al, 1992) is derived from W3110 and has been provided by T. Ogura. *E. coli* DH5α (Hanahan, 1983) was used as host in cloning

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experiments. Bacteria were grown in Luria Broth (LB) supplemented with suitable antibiotics to maintain plasmid DNA (ampicillin (Amp) at 100 µg ml^{-1}, kanamycin (Kan) at 25 µg ml^{-1}, chloramphenicol (Cm) at 10 µg ml^{-1}). Growth in low Mg^{2+} liquid medium was carried out in NCE-minimal medium (Maloy, 1990) supplemented with 0.1% casamino acids, 38 mM glycerol and 10 µM $MgCl_2$. Growth in high Mg^{2+} liquid medium was carried out in the same medium supplemented with 10 mM $MgCl_2$.

Plasmids construction

Plasmids purified with Qiaprep spin kit (Qiagen) were introduced into *S.* Typhimurium strains by electroporation using a Bio-Rad apparatus and into *E. coli* strains by chemical transformation using standard procedures. Plasmids used in this study are listed in Table I and primers are listed in supplementary Table S1.

Deletions of sequences downstream of *mgtC* were carried out on the pEG9091 plasmid that harbors the *mgtCB* operon (Figure 1A). PCR amplification was performed with primers flanking the region to delete. Amplified fragments were phosphorylated and self-ligated.

To clone *mgtR*, a 1200 bp *Hind*III-*Pst*I restriction fragment from pEG9091del3, which carries a 270 bp-region downstream of *mgtB*, was first subcloned into pBBR1MCS, which is a Cm^R vector compatible with pBR322 derivatives. Deletions were subsequently made at the 3' (with primers sup3'-F and sup3'-R) and 5' (with primers sup5'-F and MgtB-del-F) extremities of the insert to keep a 150 bp region downstream of *mgtB* that includes the last 6 codons of *mgtB*. The resulting plasmid was called pMgtR.

To produce a His-tagged MgtR peptide, we cloned the *mgtR* gene between the *Bam*HI and *Hind*III sites of the pQE30 vector (Qiagen). The cloned fragment resulted from the annealing of long oligonucleotides (MgtR-BamHind-F and MgtR-HindBam-R) corresponding to the entire *mgtR* sequence with cloning sites at the ends. The resulting plasmid, pQEMgtR,

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encodes an N-terminal His-tagged MgtR. Overexpression of MgtR was not well tolerated by bacteria and pQEMgtR was maintained in strains that harbor the *lac*I^{q} gene on pREP4 plasmid. Expression of His$_6$-MgtR was induced by adding 0.1 mM IPTG.

Site-directed mutagenesis was conducted on plasmids using the Quickchange® II kit (Stratagene) according to manufacturer's instructions. Sets of primers were designed for each engineered mutation including sufficiently long flanking regions. Sequencing was conducted to confirm the presence of the desired mutation and the absence of additional mutations.

Western Blot analysis

Bacteria were grown overnight in low Mg^{2+} medium (to induce *mgtC* transcription) supplemented with suitable antibiotics to maintain plasmid DNA. To prepare whole-cell extracts, cultures were normalized for the number of cells, centrifuged, resuspended in 100 µl Laemmli buffer and lysed by 10 min boiling. Samples were run on 12 % SDS-PAGE and transferred to PVDF membrane (Millipore) for immunoblotting. Anti-MgtC antibodies were raised in rabbits against the His-tagged C-terminal domain of the MgtC protein (Rang *et al*, 2007). Hence, these antibodies react both with MgtC protein and His-tagged proteins. Mouse anti-DnaK antibodies (Tebubio) used at 1:5000 dilution were used as control. Rabbit anti-MgtB antibodies, kindly provided by M. Maguire, were used at 1:1000 dilution. Secondary horseradish peroxidase-conjugated anti-rabbit or anti-mouse antiserum (Sigma) were used at 1:3000 dilution. The blots were developed with the Immobilon™ Western kit (Millipore).

Construction of a ΔmgtR Salmonella strain

A chromosomal deletion in the *mgtR* gene was constructed using λRed mediated site-specific recombination (Datsenko and Wanner, 2000). The kanamycin resistance gene of plasmid pKD13 (Datsenko and Wanner, 2000) was amplified by PCR using primers delta-MgtR-F and

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delta-MgtR-R. The resulting PCR product was used to replace 76 bp of *mgtR* in strain MA6897 (Uzzau *et al*, 2001) by homologous recombination catalysed by the λRed recombinase encoded by plasmid pKD46. The kanamycin resistance cassette inserted at the chromosomal *mgtR* locus was transferred by P22 transduction into a wild-type 14028s strain. The temperature-sensitive plasmid pCP20 encoding FLP recombinase (Datsenko and Wanner, 2000) was transformed in the resulting strain to allow loss of the kanamycin resistance cassette leaving behind a single FRT site. The chromosomal deletion was verified by colony PCR using primers MgtB-del-F and MgtRseqR followed by sequencing.

RNA extraction and RT-PCR

Overnight culture of wild-type 14028s strain was diluted 1:50 into 3 ml of NCE medium containing 10 mM Mg^{2+} and grown at 37°C until OD_{600}=0.7. Bacteria were harvested by centrifugation, washed three times with NCE medium containing 10 µM Mg^{2+} and cultivated 30 min at 37°C in 3 ml of the same medium before RNA extraction. Total RNA was isolated using the SV Total RNA Isolation System (Promega) and treated with RNase-free DNase I following the protocol provided by the manufacturer. RT-PCR were performed with the AccessQuick™ RT-PCR System (Promega) following manufacturer's instructions. Primers used are RT-GapA-F and RT-GapA-R for *gapA*, MgtC-RT-F2 and MgtC-RT-R2 for amplification 1, MgtR-RT-F and MgtR-RT-R for amplification 2, MgtB-del-F and MgtR-RT-R for amplification 3, MgtBR-RT-F and MgtR-RT-R for amplification 4. Reverse transcription was done by 45 min incubation at 45°C, and PCR as followed: 2 min initial denaturation at 95°C, 28 repetitions of 30s at 95°C, 30s at 50°C and 30s at 72°C and a final amplification of 5 min at 72°C. To exclude possible DNA contamination, the same reactions were performed without reverse transcriptase.

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Bacterial two-hybrid analysis

We used the Bacterial Adenylate Cyclase Two-Hybrid (BACTH) system (Karimova *et al*, 1998). The *mgtC* gene was PCR amplified using pEG9091 as template and primers MgtC-pUT18-F and MgtC-pUT18-R. The PCR fragment was cloned at the *Sma*I site of the pUT18 vector, to produce a fusion protein MgtC-T18. Expression of MgtC-T18 hybrid protein and mutant derivatives was checked by Western analysis. The pKT25 vector (Karimova *et al*, 1998) was modified by deleting the *Xba*I site to produce pKT25Δ*Xba*I that allows in frame cloning at the *Bam*HI site. The *mgtR* gene was amplified by colony PCR from 14028s with *Pfu* polymerase (Promega) using primers MgtR-pKT25-F and MgtR-pKT25-R. The blunt-ended PCR fragment was digested by *Bam*HI and cloned into the pKT25Δ*Xba*I vector, previously digested by *Eco*RI, treated with Kleenow and digested by *Bam*HI, to produce a fusion protein T25-MgtR.

Recombinant plasmids carrying *mgtC* and *mgtR* genes were cotransformed into BTH101 bacteria. Transformants were plated on LB Ap Kn X-gal medium at 30°C for 30 h. To quantify the interaction between hybrid proteins, bacteria were grown overnight either at 30°C (as recommended in the BACHT protocol) or at 37°C (where interaction is usually weaker comparatively to 30°C) in LB Ap Kn liquid medium supplemented with 0.5 mM IPTG. Before the β-galactosidase assay, cultures were diluted 1:3 in LB medium. Beta-galactosidase assays were carried out as described previously (Miller, 1972) and activities are expressed in arbitrary Miller units. Values are average from at least six independent cultures. A level of β-galactosidase activity at least fivefold higher than that measured for vectors indicates an interaction.

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Macrophage infection experiments

The rate of intramacrophage replication after 18 h infection was performed in J774 mouse macrophages as described previously (Rang et al., 2007).

Accession number

The *mgtR* sequence has been submitted to GenBank database under accession number EU154350.

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Figure legends

Figure 1 The levels of the MgtC protein are regulated by the 3' region of the *mgtCB* operon. (**A**) Schematic representation of sequences carried by plasmids pEG9091 (*mgtC*$^+$*mgtB*$^+$), pEG9094 (*mgtC*$^+$) and pEG9091 derivatives. (**B**) Western Blot analysis of *E. coli* strains transformed with pEG9091, pEG9094 or pEG9091 derivatives. Total extracts were prepared from bacteria grown 16 h in low Mg^{2+} medium and were blotted with anti-MgtC antibodies or anti-DnaK antibodies. The band detected above MgtC appears as non-specific since it is also found with *E. coli* strains or *S.* Typhimurium strain NM14. (**C**) Western Blot analysis of *E. coli* strains transformed with pEG9094 and pBBR1MCS vector or pMgtR. The pMgtR plasmid is a pBBR1MCS derivative that carries a sequence of 150 bp downstream of *mgtB* (sequence in bold in panel A).

Figure 2 The 3' region of the *mgtCB* operon encodes a peptide that is involved in MgtC down-regulation. (**A**) The 150 bp regulatory region at the 3' end of the *mgtCB* operon includes a 90 bp sequence that could encode a small RNA or a peptide. One of the secondary structures of a putative RNA predicted by Mfold server (http://bioweb.pasteur.fr/seqanal/interfaces/mfold-simple.html) is shown (dG=-15,5 kcal mole^{-1}). Site-directed mutagenesis was performed on pMgtR plasmid at positions 1 (TCGCvCAGA) and 2 (GGTGvAGTC) to destabilize RNA structure without affecting protein sequence. The mutations 3 (TvC) and 4 (ΔC) affected synthesis of a putative ORF without affecting predicted RNA structure. (**B**) Western Blot analysis of *E. coli* strains transformed with pEG9094 and mutated pMgtR plasmids. The pBBR1MCS vector and pMgtR plasmid were used as controls. Total extracts were prepared from bacteria grown 16 h in low Mg^{2+} medium and were blotted with anti-MgtC antibodies or anti-DnaK antibodies.

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Figure 3 Down-regulation of MgtC by the induction of a His_6-MgtR peptide in a *Salmonella* Δ*mgtR* mutant. Western Blot experiments were carried out on *Salmonella* extracts using anti-MgtC antibodies. (**A**) Characterization of a *Salmonella* Δ*mgtR* mutant and complementation with the pMgtR plasmid. Western Blot on *S. typhimurium* wild-type and Δ*mgtR* mutant strains. The pMgtR plasmid or the pBBR1MCS vector were introduced into the Δ*mgtR* mutant strain. Extracts were blotted with anti-DnaK antibodies as control. (**B**) Induction of His_6-MgtR expression by addition of 0.1 mM IPTG decreased expression of MgtC in a *Salmonella* Δ*mgtR* mutant strain. The lower band is the His_6-MgtR peptide (approximately 5 kDa) which cross reacts with anti-MgtC antibodies. (**C**) Kinetic of MgtC degradation by His_6-MgtR. After overnight growth without IPTG in conditions of *mgtC* transcription (10 µM Mg^{2+}), bacteria were shifted to a medium containing 10 mM Mg^{2+} to shut-down *mgtC* transcription and IPTG to induce His_6-MgtR expression. In each case, samples were prepared for Western Blot at several times after the shift (0, 2 h, and 4 h). Identical volumes corresponding to a constant number of cells were loaded independently of the time point. (**D**) Quantification of the levels of MgtC in panel C.

Figure 4 The *mgtR* sequence belongs to *mgtCB* operon. RT-PCR experiments were performed on total RNA of wild-type *S.* Typhimurium extracted after 30 min growth with 10 µM Mg^{2+} (L) or with 10 mM Mg^{2+} (H). (**A**) Schematic representation of the fragments amplified by RT-PCR. (**B**) Analysis of DNA fragments amplified by RT-PCR on agarose gel. RT-PCR of *gapA* is used as control. No band was amplified in the absence of reverse transcriptase (not shown).

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Figure 5 The FtsH protease is involved in MgtC negative regulation mediated by MgtR. The thermosensitive *E. coli ftsH* mutant and the isogenic *ftsH*$^+$ strain were transformed by plasmids pEG9094 (*mgtC*$^+$) or pEG9091 del3 (*mgtC*$^+$ *mgtR*$^+$) (Figure 1A). Strains were cultured in a low Mg^{2+} medium for 36 h at 30°C or for 7 h at 30°C before a 18 h shift at 42°C. Total extracts were blotted with anti-MgtC antibodies or anti-DnaK antibodies.

Figure 6 Amino-acid residues of MgtR important for MgtC degradation. (**A**) Position of the mutations on MgtR sequence. The predicted transmembrane domain is shaded. Changes that lead to loss of function are highlighted with circles. (**B**) Western Blot analysis of crude extracts prepared from strains with mutant MgtR encoded by pMgtR derivatives. Extracts were prepared from *E. coli* strains carrying the pEG9094 plasmid (*mgtC*$^+$) and the mutated pMgtR plasmids. The pBBR1MCS vector and pMgtR plasmid were used as control. Total extracts were blotted with anti-MgtC antibodies or anti-DnaK antibodies. (**C**) Western Blot analysis of crude extracts prepared from strains with mutant MgtR encoded by pQEMgtR derivatives. Extracts were prepared from *S. typhimurium* Δ*mgtR* strain carrying the mutated pQEMgtR plasmids. The pQE30 vector and pQEMgtR plasmid were used as control. Total extracts were blotted with anti-MgtC antibodies that recognize both MgtC and His_6-MgtR.

Figure 7 Identification of MgtC mutants that are insensitive to down-regulation by MgtR. (**A**) Schematic representation of MgtC protein topology. Mutations that promoted MgtC expression on Western Blot are clustered in the second cytoplasmic loop. (**B**) Western Blot analysis of MgtC mutants. MgtC mutations were carried out on the pNM12 plasmid. Extracts were prepared from *E. coli* strains carrying pMgtR plasmid or the pBBR1MCS vector and the pNM12 plasmid (*mgtC*$^+$) or the mutated pNM12 derivatives.

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Figure 8 Analysis of *in vivo* interaction between MgtC and MgtR using the BACHT system. The *E. coli* BTH101 strain was cotransformed with plasmids encoding MgtC-T18 and T25-MgtR fusions. Assays were carried out at 30°C. Four mutations, L15R, L15P, A24R and A24P, were introduced in the T25-MgtR fusion. Three mutations, E84A, G85A and N92T were introduced in the MgtC-T18 fusion. The basal level of β-galactosidase activity measured with vector is approximately 60 Miller units.

Figure 9 Role of Ala-coil motifs in MgtC-MgtR interaction. **(A)** Ala-coil motifs and mutagenesis on MgtR and MgtC TM4 sequences. **(B)** Effect of Ala-coil mutations on the interaction between MgtC and MgtR *in vivo* using the BACHT system. Assays were carried out at 30°C. **(C)** Effect of Ala-coil mutations in MgtR on MgtC expression. Extracts were prepared from *S. typhimurium* Δ*mgtR* strain carrying the mutated pQEMgtR plasmids and blotted with anti-MgtC antibodies. The pQE30 vector and pQEMgtR plasmid were used as control.

Figure 10 Role of MgtR in *S*. Typhimurium intramacrophage growth. The replication of *Salmonella* strains in J774 macrophages was evaluated 18 h after infection. Data represent the mean values plus standard errors from at least three independent experiments. (**A**) Analysis of a Δ*mgtR* mutant strain in comparison with a wild-type strain and a Δ*mgtC* mutant. Values presented are the percentage relative to that of the wild-type S. Typhimurium 14028s. (**B**) Analysis of a wild-type *Salmonella* strain that carries the pMgtR plasmid. Values presented are the percentage relative to that of the wild-type *S*. Typhimurium 14028s with the pBBR1MCS vector.

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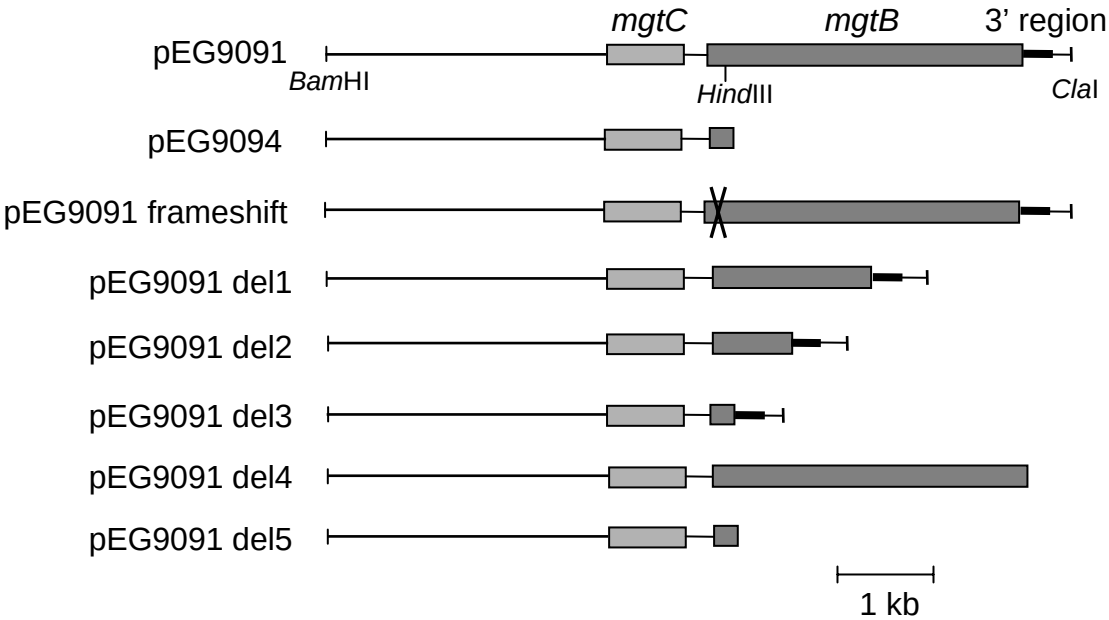
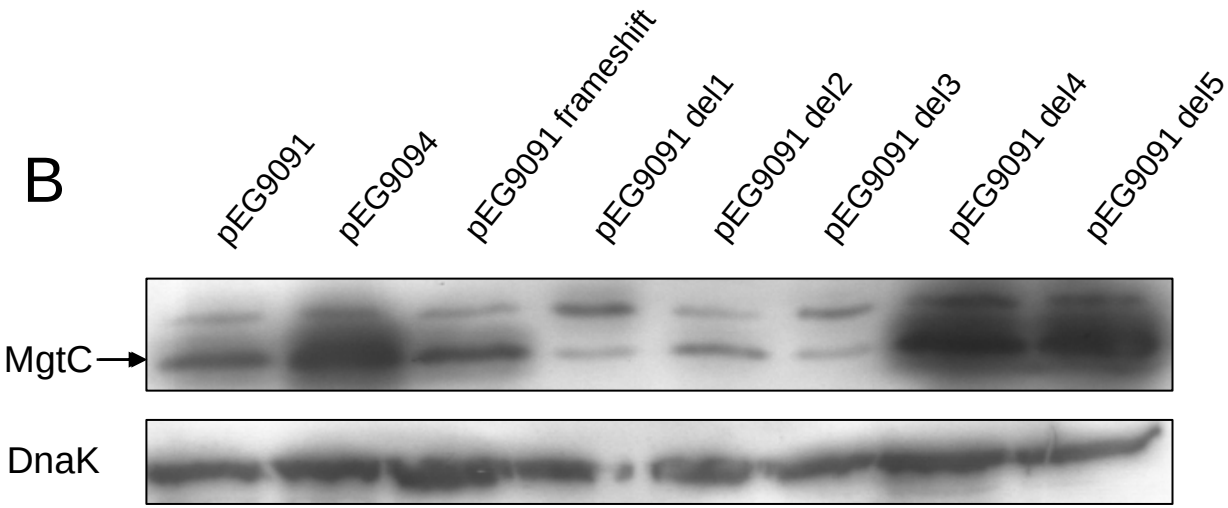
Figure 11 Model for the role of MgtR on MgtC expression: the MgtR peptide binds directly to MgtC at the cytoplasmic membrane. The interaction between MgtC and MgtR would unfold MgtC and makes it a target for degradation by the FtsH protease.

Figure 1

A


mgtC
mgtB
3' region
pEG9091
BamHI
HindIII
ClaI
pEG9094
pEG9091 frameshift
pEG9091 del1
pEG9091 del2
pEG9091 del3
pEG9091 del4
pEG9091 del5
1 kb

B


pEG9091
pEG9094
pEG9091 frameshift
pEG9091 del1
pEG9091 del2
pEG9091 del3
pEG9091 del4
pEG9091 del5
MgtC
DnaK

C

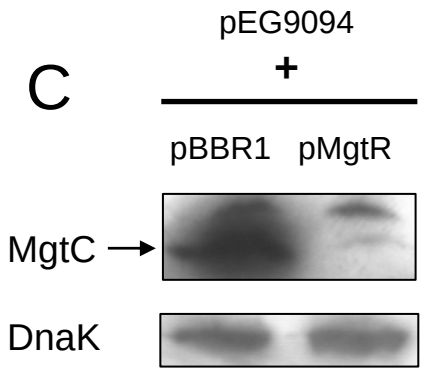
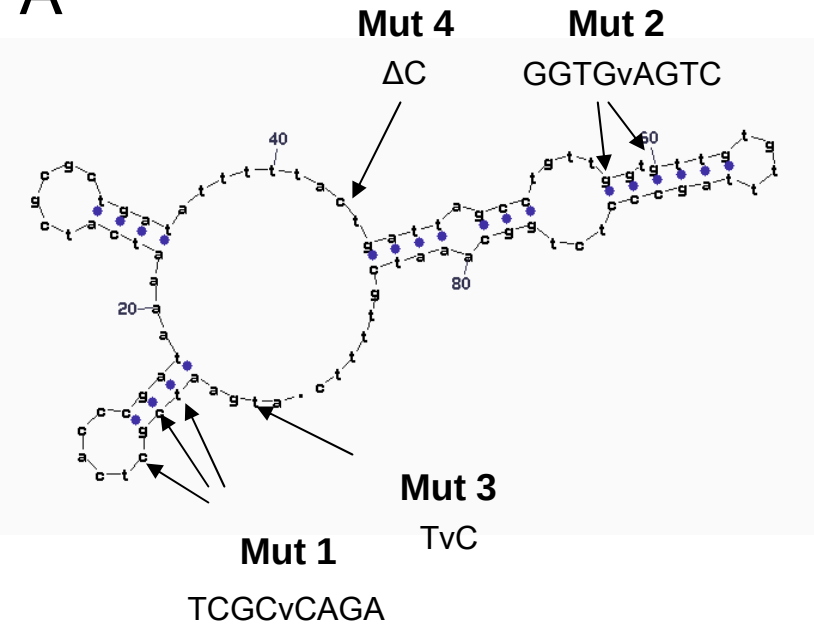

pEG9094
+
pBBR1
pMgtR
MgtC
DnaK

Figure 2

A




```
atgaatcgctcacccgataaaatcatcgcgctgatatttttactgatt
 M  N  R  S  P  D  K  I  I  A  L  I  F  L  L  I
agcctgttggtgttgtgtttagccctctggcaaatcgttttc
 S  L  L  V  L  C  L  A  L  W  Q  I  V  F
```

B

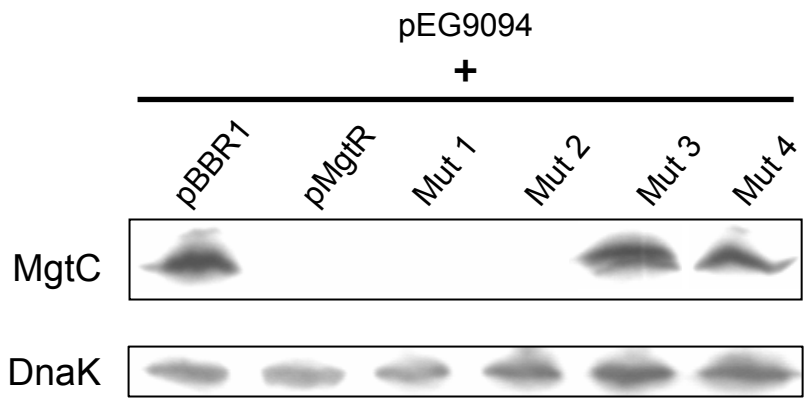


Figure 3

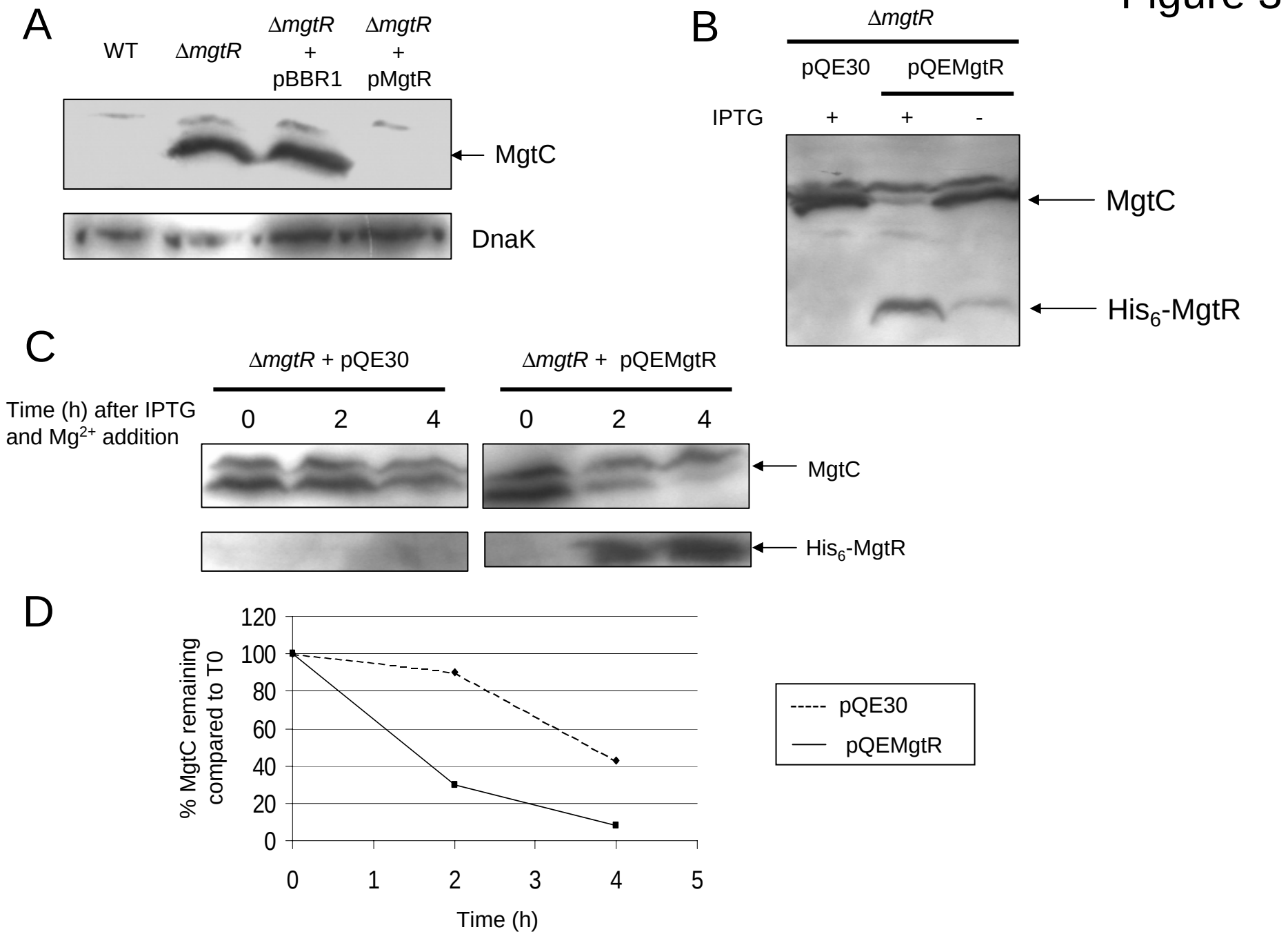

A
WT
ΔmgtR
ΔmgtR + pBBR1
ΔmgtR + pMgtR
MgtC
DnaK
B
ΔmgtR
pQE30
pQEMgtR
IPTG
+
+
-
MgtC
His6-MgtR
C
ΔmgtR + pQE30
ΔmgtR + pQEMgtR
Time (h) after IPTG and Mg2+ addition
0
2
4
0
2
4
MgtC
His6-MgtR
D
% MgtC remaining compared to T0
120
100
80
60
40
20
0
0
1
2
3
4
5
Time (h)
pQE30
pQEMgtR

Figure 4

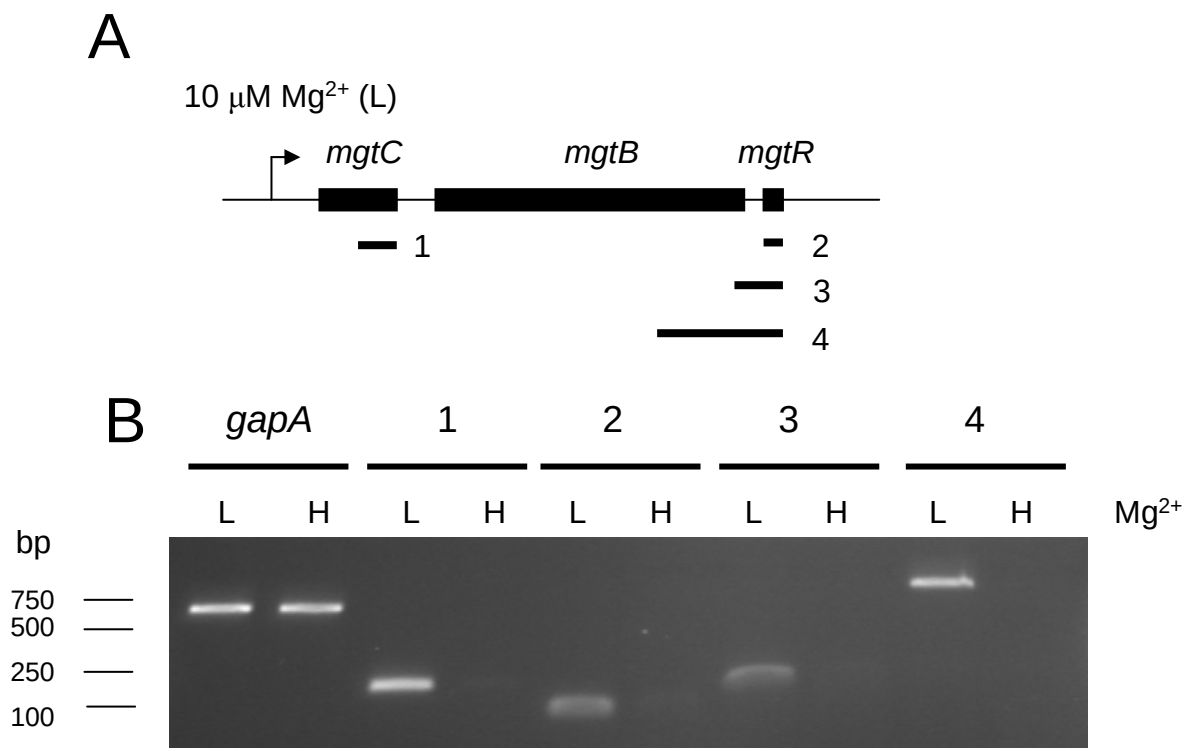

A
10 μM Mg2+ (L)
mgtC
mgtB
mgtR
1
2
3
4
B
gapA
1
2
3
4
L H L H L H L H L H Mg2+
bp
750
500
250
100

Figure 5

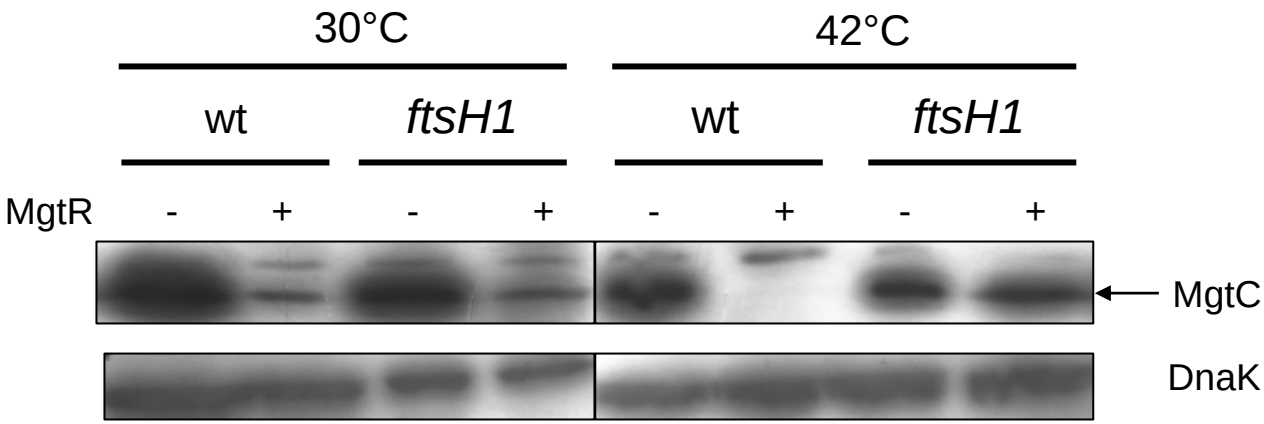
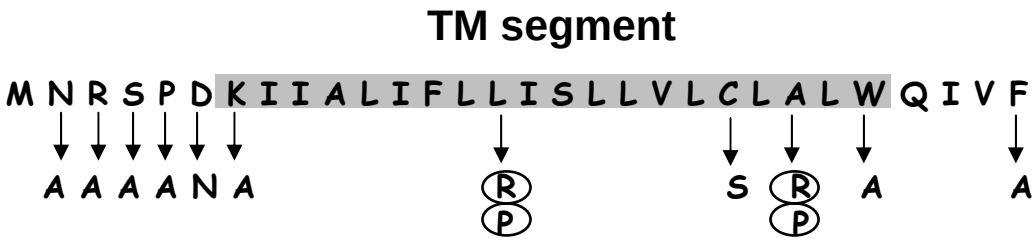
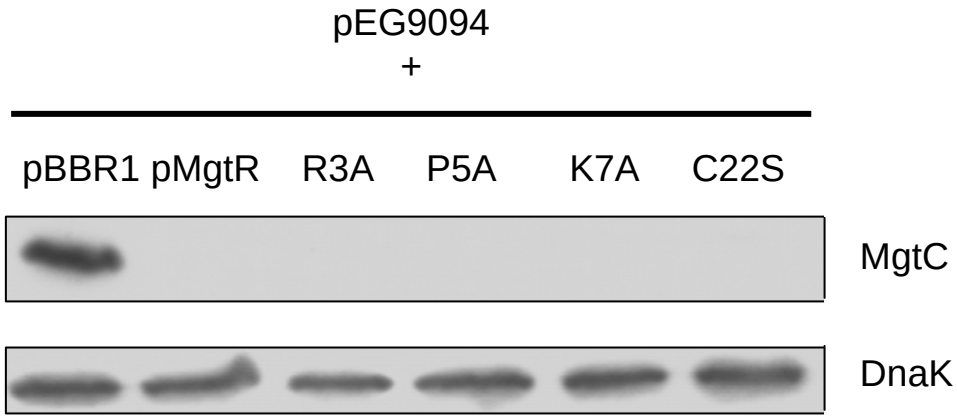


Figure 6

A


TM segment
M N R S P D K I I A L I F L L I S L L V L C L A L W Q I V F
A A A A N A
R
P
S
R
P
A
A

B


pEG9094
+
pBBR1
pMgtR
R3A
P5A
K7A
C22S
MgtC
DnaK

C

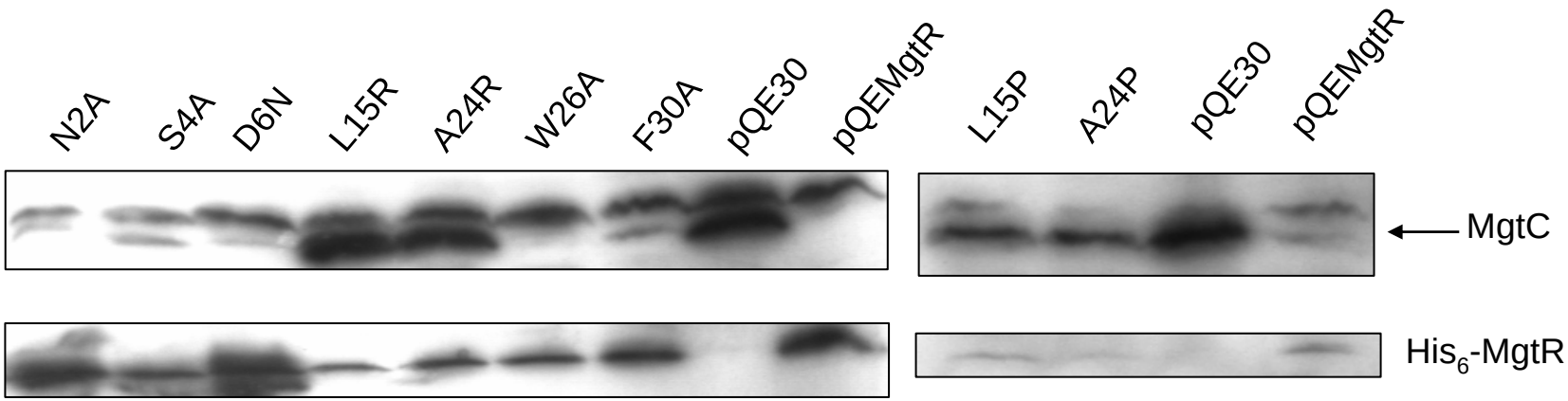
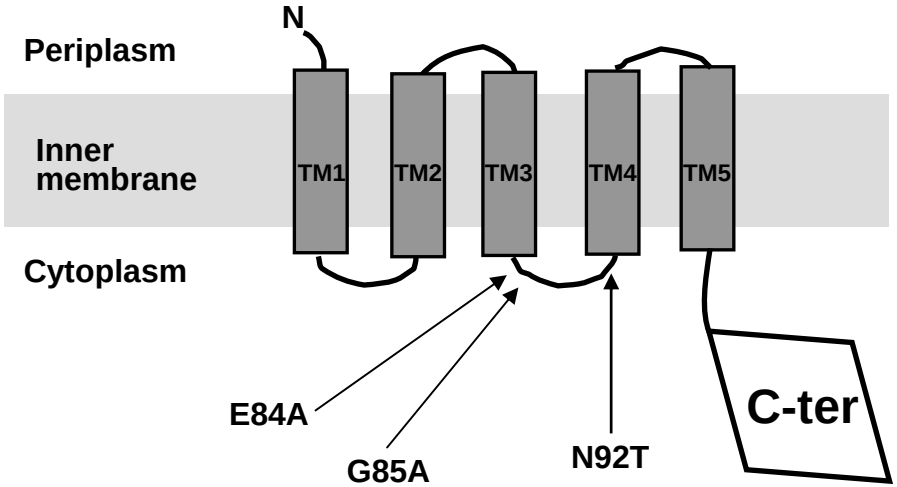

N2A
S4A
D6N
L15R
A24R
W26A
F30A
pQE30
pQEMgtR
L15P
A24P
pQE30
pQEMgtR
MgtC
His6-MgtR

Figure 7

A


N
Periplasm
Inner membrane
Cytoplasm
TM1
TM2
TM3
TM4
TM5
C-ter
E84A
G85A
N92T

B

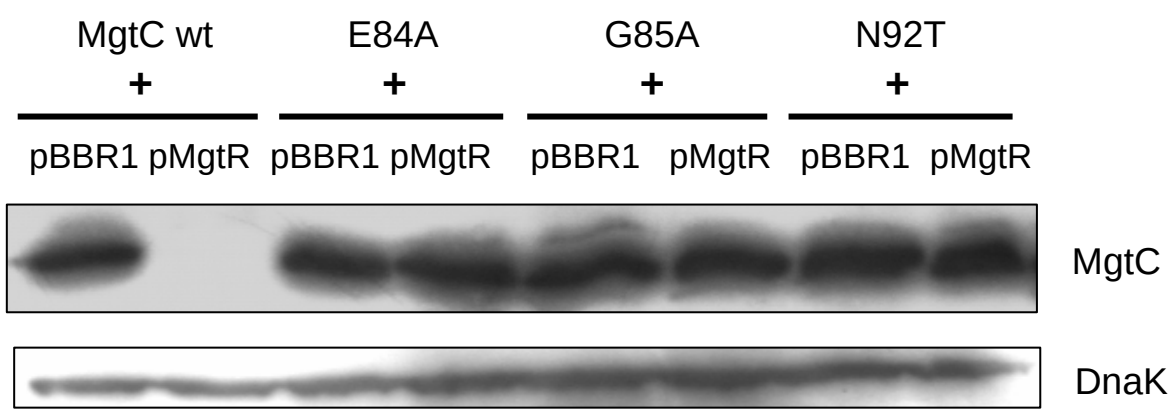

MgtC wt
E84A
G85A
N92T
+
+
+
+
pBBR1 pMgtR
pBBR1 pMgtR
pBBR1 pMgtR
pBBR1 pMgtR
MgtC
DnaK

Figure 8

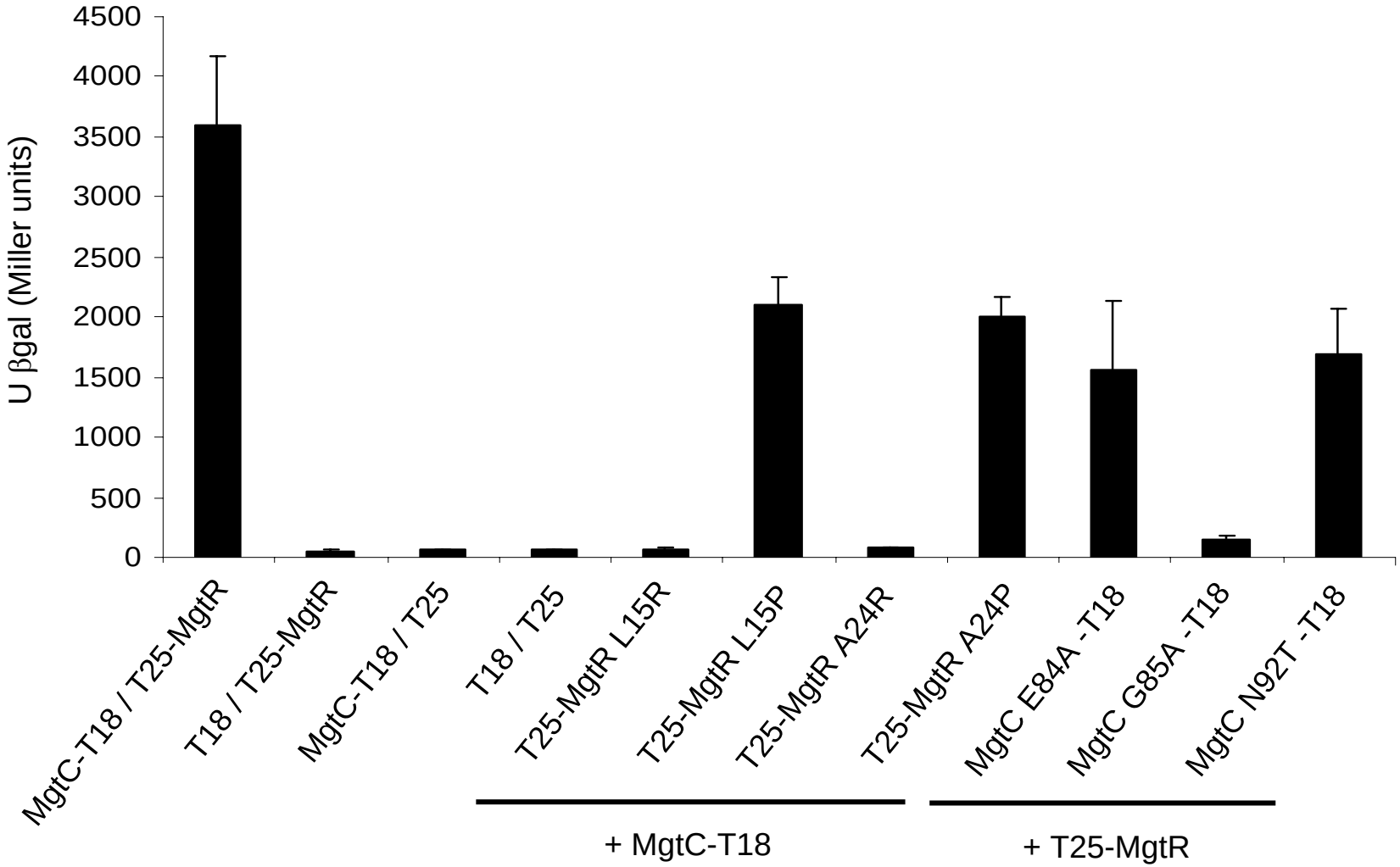
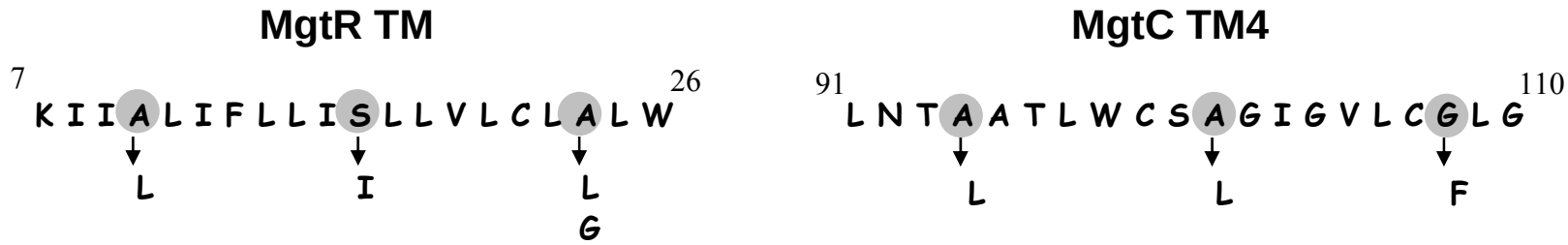
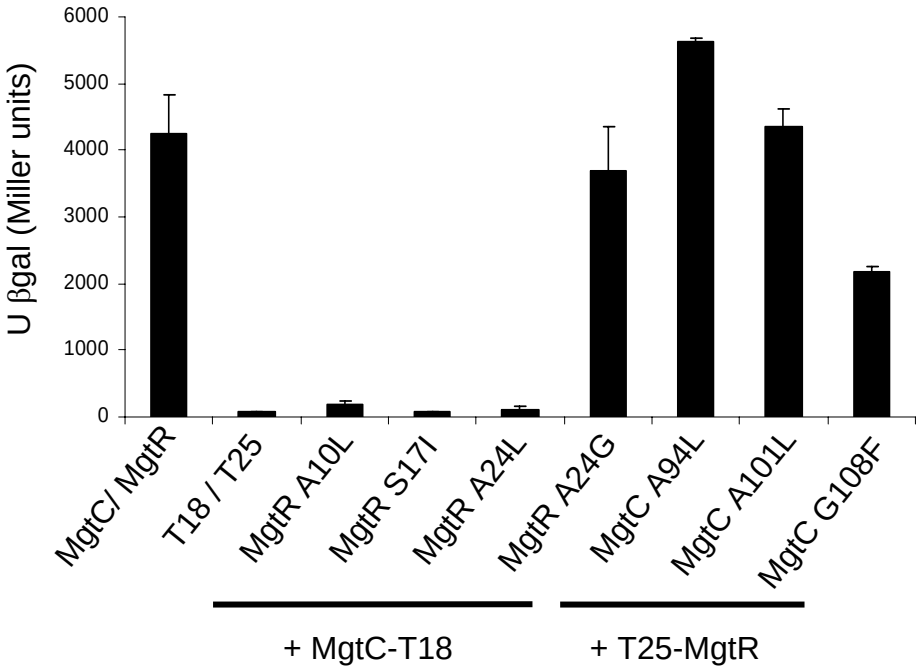

U βgal (Miller units)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
MgtC-T18 / T25-MgtR
T18 / T25-MgtR
MgtC-T18 / T25
T18 / T25
T25-MgtR L15R
T25-MgtR L15P
T25-MgtR A24R
T25-MgtR A24P
MgtC E84A -T18
MgtC G85A -T18
MgtC N92T -T18
+ MgtC-T18
+ T25-MgtR

Figure 9

A


MgtR TM
7
K I I A L I F L L I S L L V L C L A L W
26
L
I
L
G
MgtC TM4
91
L N T A A T L W C S A G I G V L C G L G
110
L
L
F

B


U βgal (Miller units)
6000
5000
4000
3000
2000
1000
0
MgtC/ MgtR
T18 / T25
MgtR A10L
MgtR S17I
MgtR A24L
MgtR A24G
MgtC A94L
MgtC A101L
MgtC G108F
+ MgtC-T18
+ T25-MgtR

C

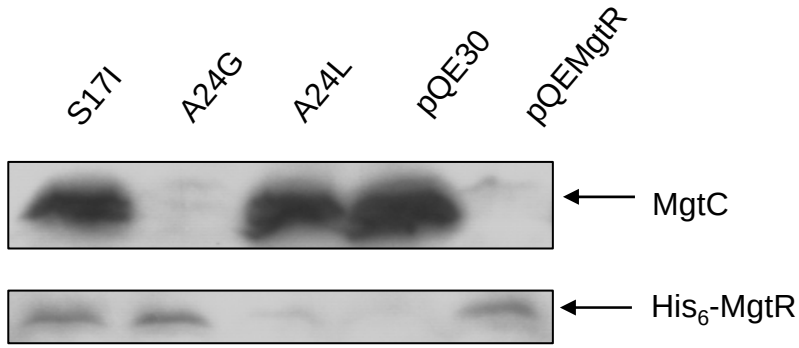
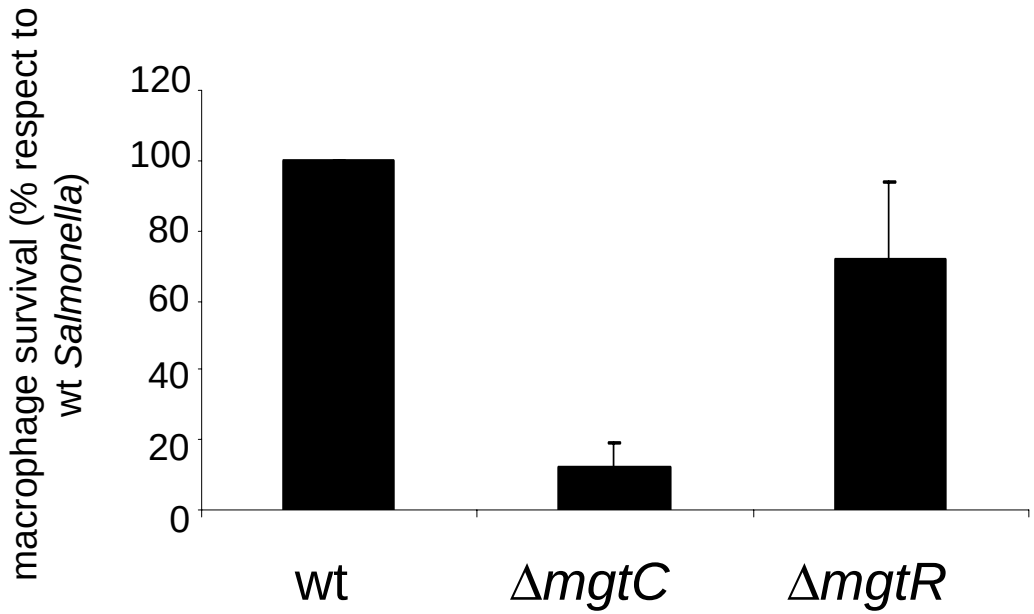

S17I
A24G
A24L
pQE30
pQEMgtR
MgtC
His6-MgtR

Figure 10

A


macrophage survival (% respect to wt *Salmonella*)
120
100
80
60
40
20
0
wt
Δ*mgtC*
Δ*mgtR*

B

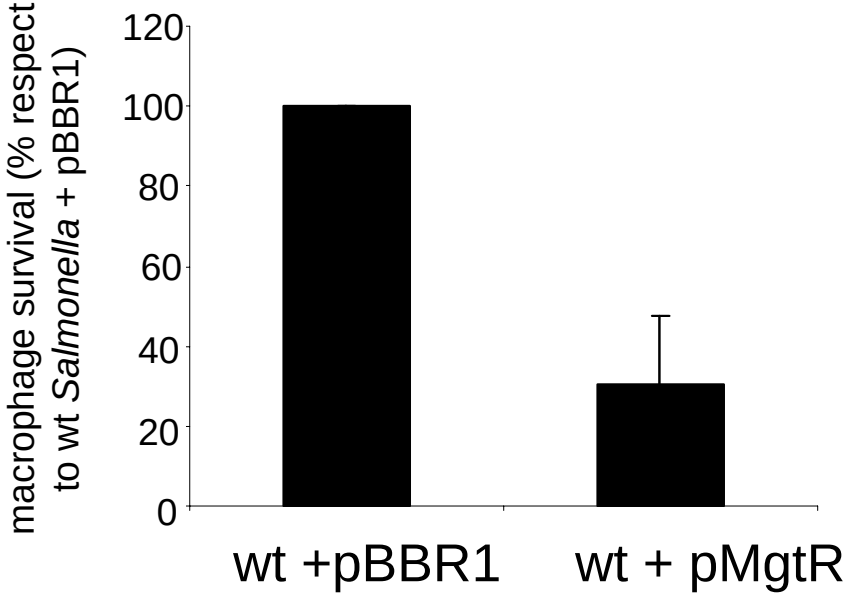

macrophage survival (% respect to wt *Salmonella* + pBBR1)
120
100
80
60
40
20
0
wt +pBBR1
wt + pMgtR

Figure 11

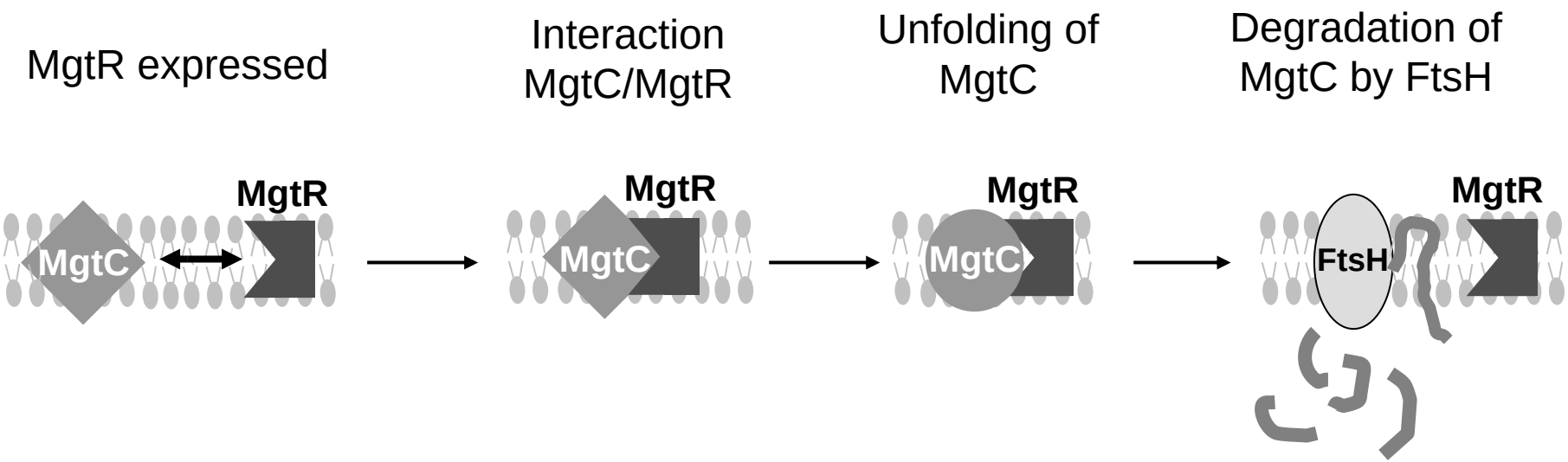

MgtR expressed
Interaction MgtC/MgtR
Unfolding of MgtC
Degradation of MgtC by FtsH
MgtR
MgtC
MgtR
MgtC
MgtR
MgtC
MgtR
FtsH

Table I. Bacterial strains and plasmids used in this study

Strain or plasmid	Description or phenotype	Reference or source
S. Typhimurium		
14028s	Wild type	E.A. Groisman
MA6987	*ilvI3305* ::Tn*10*dTac-*cat*/pKD46	Uzzau *et al* (2001)
TH6767	*clpX1* ::Tn*10*d*cam*	Aldridge *et al* (2003)
NM14	Δ*mgtC*	Rang *et al* (2007)
NM506	Δ*mgtR*::*kan*	This study
NM516	Δ*mgtR*	This study
NM643	*clpX1* ::Tn*10*d*cam* Δ*mgtR*::*kan*	This study
E. coli		
DH5α	F- φ80*lacZ*Δ*M15* Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(rk -, mk+) *phoA supE44 thi-1 gyrA96 relA1* λ-	Invitrogen
AR3307	W3110 *zad220*::Tn*10*	T. Ogura
AR3317	AR3307 *zgj3198*::Tn*10* (kan^R) *ftsH1*(Ts)	T. Ogura
BTH101	F^- *cya-99 araD139 galE15 galK16 rpsL1* (Str^r) *hsdR2 mcrA1 mcrB1*	D. Ladant
Cloning plasmids		
pBR322	Amp^R Tc^R rep_{pMB1}	Bolivar *et al* (1977)
pBBR1MCS	Cm^R	Kovach *et al* (1994)
pQE30	Amp^R rep_{ColE1}	Qiagen
pUT18	Amp^R rep_{ColE1} (pBluescript II KS derivative)	Karimova *et al* (1998)
pKT25Δ*Xba*I	Kan^R rep_{p15A} (pACYC184 derivative)	G. Patey
pREP4	Kan^R rep_{p15A} $lacI^q$	Qiagen
Plasmids		
pEG9094	pBR322 $mgtC^+$	Blanc-Potard and Groisman (1997)
pEG9091	pBR322 $mgtC^+$ $mgtB^+$ $mgtR^+$	Blanc-Potard and Groisman (1997)
pEG9091 del1	pEG9091 $mgtC^+$ $mgtB_{\Delta 439-904}$ $mgtR^+$	This study
pEG9091 del2	pEG9091 $mgtC^+$ $mgtB_{\Delta 235-904}$ $mgtR^+$	This study
pEG9091 del3	pEG9091 $mgtC^+$ $mgtB_{\Delta 60-904}$ $mgtR^+$	This study
pEG9091 del4	pEG9091 $mgtC^+$ $mgtB^+$	This study
pEG9091 del5	pEG9091 $mgtC^+$ $mgtB_{\Delta 60-910}$	This study
pNM12	pBR322 $mgtC^+$	Rang *et al* (2007)
pMgtR	pBBR1MCS $mgtR^+$	This study
pQEMgtR	His_6-MgtR	This study
pUT18-MgtC	MgtC-T18	This study
pKT25Δ*Xba*I-MgtR	T25-MgtR	This study