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Promoter and Riboswitch Control of the Mg^{2+} Transporter MgtA from *Salmonella enterica*[▽]†

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The MgtA protein from *Salmonella enterica* serovar Typhimurium mediates Mg^{2+} uptake from the periplasm into the cytoplasm. Here we report that the PhoP/PhoQ two-component regulatory system, which responds to periplasmic Mg^{2+}, governs *mgtA* transcription initiation at all investigated Mg^{2+} concentrations and that the Mg^{2+}-sensing 5′ leader region of the *mgtA* gene controls transcription elongation into the *mgtA* coding region when *Salmonella* is grown in media with <50 μM Mg^{2+}. Overexpression of the Mg^{2+} transporter CorA, which is believed to increase cytoplasmic Mg^{2+} levels, decreased *mgtA* transcription in a manner dependent on a functional *mgtA* 5′ leader.

Expression of the Mg^{2+} transporter gene *mgtA* from *Salmonella enterica* serovar Typhimurium is regulated at both the transcription initiation and elongation steps. Transcription initiation is dependent on the two-component regulatory system PhoP/PhoQ (9), which is activated in response to low Mg^{2+} (8), acid pH (16), and antimicrobial peptides (1) sensed in the periplasm by the PhoQ protein. Transcription elongation into the *mgtA* coding region is controlled by the 5′ leader region of the *mgtA* transcript, which functions as a Mg^{2+}-sensing device or riboswitch (5) and renders the transcript susceptible to degradation by RNase E (20). The *mgtA* leader region can adopt alternative stem-loop structures that favor or hinder transcription elongation into the *mgtA* coding region at low and high Mg^{2+}, respectively (5). In addition, overexpression of the regulatory gene *rob* promotes *mgtA* transcription from a site located 44 nucleotides downstream of the PhoP-dependent transcription start site (2), thereby generating an *mgtA* transcript with a shorter leader region that could lack some of the Mg^{2+}-sensing elements.

To examine the contributions that the PhoP-dependent *mgtA* promoter and the *mgtA* riboswitch make to the Mg^{2+}-regulated expression of the *mgtA* gene, we constructed a set of four isogenic strains with alterations in the promoter and/or riboswitch regions of the chromosomal copy of the *mgtA* gene, as well as a *lac* transcriptional fusion at position 977 in the *mgtA* open reading frame (position 1 corresponds to the PhoP-dependent transcription start site in the wild-type strain) (Fig. 1A). All four strains are derived from wild-type strain 14028s and have a *cat* cassette (conferring resistance to chloramphenicol) upstream of the promoter, which does not alter *mgtA* expression and was used as a selectable marker when moving mutations into different genetic backgrounds. One strain—YS773—retains the wild-type PhoP-dependent promoter and *mgtA* 5′ leader region. The second strain—YS783—contains nucleotide substitutions in the DNA sequence corresponding to positions 151 to 160 of the *mgtA* 5′ leader region, where the sequence AGAUGUUUC replaced the original GUAAGAC AGU, which was anticipated to interfere with the formation of stem-loop B, a structure normally formed in cells experiencing high Mg^{2+} (5). The third strain—YS802—lacks the PhoP-dependent wild-type promoter and harbors a derivative of the *lac* promoter—designated $p_{lac1\text{-}6}$—(12) that responds neither to PhoP nor to Mg^{2+} (5). (This strain retains the normal Mg^{2+} response of the *mgtA* riboswitch despite lacking the first 31 nucleotides of the *mgtA* leader region [5].) The fourth strain—YS812—combines the PhoP-independent $p_{lac1\text{-}6}$ promoter present in strain YS802 with the mutant *mgtA* 5′ leader of strain YS783. The construction of strains YS773 and YS802 has been reported elsewhere (5). Strains YS783 and YS812 were made by the one-step gene disruption method (6), using chromosomal DNA from strains YS773 and YS802, respectively, as templates to create PCR-generated DNA fragments for substitution using primers 4416 (5′-TGATTTCCCTAC GCCGCTCAGGCGGGCGATGTCTTTGATAG TGTAGGCTGGAGCTGCTTC) and 4479 (5′-CCTTGCCC GATGAGCAATGTTTAAATAAAAACAGGGACGTTA TTGTGTCGAAAACATCTACACCGGTAAGACAGCAG AGG). The resulting DNA was integrated into the chromosome of strain EG9521 (8), harboring a *lac* transcriptional fusion in the *mgtA* coding region.

We determined the β-galactosidase activity produced by the four isogenic strains following 4 h of growth in N-minimal medium, pH 7.4 (19), supplemented with 0.1% Casamino Acids, 38 mM glycerol, and different Mg^{2+} concentrations spanning a 1,000-fold range as described previously (13). There was no β-galactosidase activity in strain YS773 following growth at 5 or 10 mM Mg^{2+}, presumably because the PhoP protein is not activated under such high Mg^{2+} concentrations (8). However, the β-galactosidase levels increased as the Mg^{2+} concentration decreased with a dramatic jump between 50 and 10 μM Mg^{2+} (Fig. 1B; notice log scale of *x* axis), as previously reported (5, 21). In contrast, there was little *mgtA* transcription

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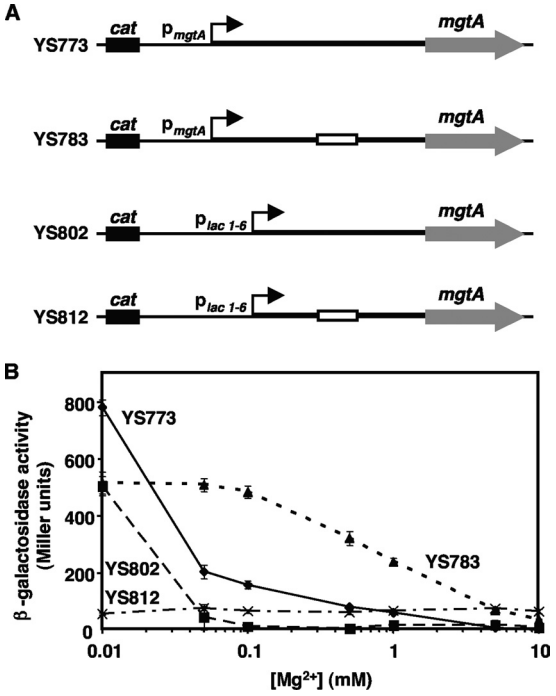

FIG. 1. Distinct roles for the *mgtA* promoter and *mgtA* riboswitch in *mgtA* expression. (A) Schematic representation of four isogenic strains with wild-type (P_{mgtA}) or variant ($P_{lac1\text{-}6}$) promoters and wild-type (black line) or mutant (black line interrupted by white box) *mgtA* leader regions. (B) β-Galactosidase activity produced by the four strains depicted in panel A grown for 4 h in N-minimal medium with the indicated Mg^{2+} concentrations. Data correspond to the average of two experiments conducted in duplicate.

when strain YS802 was grown in media with >50 μM Mg^{2+}, some transcription in 50 μM Mg^{2+}, and maximum levels in 10 μM Mg^{2+} (Fig. 1B). The similar β-galactosidase activity produced by strain YS802 when grown in 10 mM and 100 μM Mg^{2+} reflects the Mg^{2+}-insensitive nature of the $p_{lac1\text{-}6}$ promoter (5). For both YS773 and YS802, the largest difference in *mgtA* expression was observed following growth in 50 versus 10 μM Mg^{2+} (Fig. 1B). Because these strains differ in the promoter transcribing the *mgtA* gene (Fig. 1A), we hypothesized that the shared sequences in the 5′ leader region were likely responsible for the significant *mgtA* derepression displayed in organisms grown in 10 μM Mg^{2+}. Consistent with this notion, strain YS783, with the mutant *mgtA* 5′ leader but wild-type *mgtA* promoter (Fig. 1A), produced similar β-galactosidase activity when grown in media containing 50 and 10 μM Mg^{2+} (Fig. 1B). Finally, strain YS812 made β-galactosidase at the same levels at all seven Mg^{2+} concentrations (Fig. 1B), indicating that it lacks the sequence information to modulate *mgtA* expression in response to changes in the levels of Mg^{2+}.

Strain YS802 synthesized less β-galactosidase than the double mutant YS812 when grown in the presence of 100 μM to 10 mM Mg^{2+} (Fig. 1B), which may reflect the dampening effect exerted by the riboswitch at high Mg^{2+}. This effect is highlighted also by the consistently higher levels of *mgtA* transcription displayed by strain YS783 than strain YS773, both of which share the PhoP-activated *mgtA* promoter but differ in that the latter harbors a functional riboswitch but the former does not, when bacteria were grown in the presence of 50 μM to 1 mM Mg^{2+} (Fig. 1B). Yet, at 10 μM Mg^{2+}, the strains with the wild-type *mgtA* leader region produced higher β-galactosidase activity than their respective isogenic strains with a mutant *mgtA* leader (Fig. 1B). This indicates that the *mgtA* leader region both promotes transcription of the *mgtA* coding region in low Mg^{2+} and decreases its transcription in high Mg^{2+}. Cumulatively, our data demonstrated that the PhoP-dependent *mgtA* promoter controls *mgtA* expression over the whole range of Mg^{2+} concentrations tested (i.e., 10 μM to 10 mM Mg^{2+}) whereas the *mgtA* riboswitch exerts its regulatory effect primarily when bacteria experience <50 μM Mg^{2+}.

Because transcription elongation into the *mgtA* coding region responds to cytoplasmic Mg^{2+} via the *mgtA* riboswitch, we reasoned that synthesis of the MgtA protein might also take place under other conditions promoting a drop in the cytoplasmic Mg^{2+} concentration. In other words, the MgtA protein might be observed earlier if *Salmonella* is grown in media with a Mg^{2+} concentration of <10 μM but at later times if the Mg^{2+} concentration is >10 μM, as one would anticipate that Mg^{2+} would be exhausted at earlier and later times, respectively. To test this hypothesis (and because anti-MgtA antibodies were not available), we engineered a *Salmonella* strain expressing a C-terminal FLAG-tagged MgtA protein from its normal promoter and chromosomal location and harboring the wild-type 5′ leader region. The FLAG epitope was introduced as described previously (22) by amplifying the *cat* gene from plasmid pKD3 by the PCR with the following primers: MgtA-FLAG, 2048 (5′-GTTGGTGAAAGGGTTTTACAGCAGAC GTTATGGCTGGCAGGACTACAAGGACGACGATGAC AAGTAACATATGAATATCCTCCTTAG-3′) and 2049 (5′-TCGGGGATTAAGCACGCTGGCGAATCCCCGACGAA AGTGTGTGTAGGCTGGAGCTGCTTC-3′). Addition of the FLAG tag does not appear to disrupt normal MgtA protein function because the *corA mgtB mgtA*$^{+}$*-FLAG* strain MJC116 grew as well as the isogenic *corA mgtB mgtA*$^{+}$ strain EG10983 in LB medium (data not shown), whereas the *corA mgtB mgtA* triple mutant did not grow in LB unless supplemented with high concentrations of Mg^{2+}, as described previously (11).

The MgtA-FLAG-expressing strain EG13250 was grown in 10 mM Mg^{2+} and then harvested at different times after organisms were switched to media with different Mg^{2+} concentrations. Western blot analysis was carried out with crude cell extracts prepared from the different cell cultures and developed with anti-FLAG antibodies (Fig. 2). We determined that the lower the Mg^{2+} concentration, the earlier the MgtA-FLAG protein was produced. For instance, MgtA-FLAG was detected after 2.5 h in organisms switched to 2 μM Mg^{2+}, after 4 h when *Salmonella* was grown in 20 μM Mg^{2+}, and only after 5 h in organisms experiencing 40 μM Mg^{2+} (Fig. 2). As expected, the levels of the Mg^{2+} transporter CorA, which was used as control because CorA expression is PhoP/PhoQ independent and nonresponsive to changes in the concentration of Mg^{2+} in the growth media (3), were similar at the investigated times and Mg^{2+} concentrations (Fig. 2). Thus, even though the PhoP-activated *mgtA* and *phoP* promoters are bound by the PhoP protein at the same time and transcription of the *mgtA* leader sequence happens concurrently with that of the *phoP* gene (17), production of the MgtA protein takes place >4 h later than that of the PhoP protein (Fig. 2) (17).

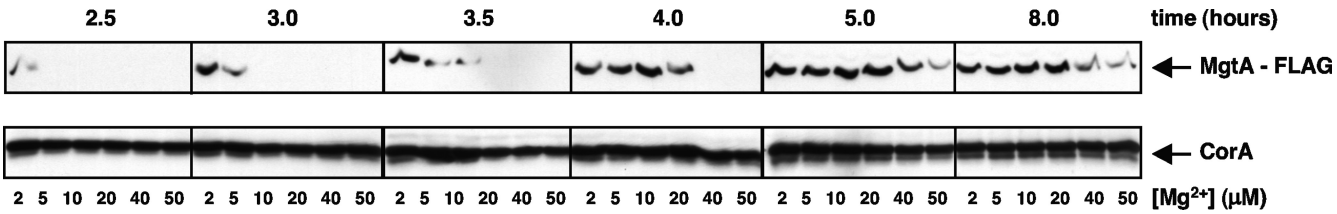

FIG. 2. Synthesis of the MgtA protein is determined by the length of time and the Mg^{2+} concentration in which *Salmonella* is grown. Western blot analysis of crude extracts prepared from strain EG13250 coding for an MgtA-FLAG protein following growth in N-minimal medium with the indicated Mg^{2+} concentrations and incubation times. Blots were probed with both anti-FLAG (top) and anti-CorA (bottom) antibodies.

If MgtA synthesis is triggered when the cytoplasmic Mg^{2+} drops below a certain threshold, as one would expect from MgtA being regulated by a Mg^{2+}-responding riboswitch, artificially increasing cytoplasmic Mg^{2+} levels may hinder synthesis of the MgtA protein. To explore this possibility, we examined the chromosomally encoded MgtA-FLAG protein levels by Western blot analysis in bacteria carrying plasmid pUC-*corA*, which expresses the Mg^{2+} transporter gene *corA* from the vector p*lac* promoter, or the plasmid vector pUC19 (23). MgtA-FLAG could be detected in the latter but not in the former strain (Fig. 3A). (As expected, there was no reactivity in extracts prepared from the wild-type strain lacking the FLAG tag [Fig. 3A].) Likewise, when streaked onto 1% agar plates containing N-minimal media, pH 7.4, supplemented with 0.1% Casamino Acids, 38 mM glycerol, 10 μM $MgCl_2$, and the chromogenic LacZ substrate X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 60 μg/ml; Gold Biotechnology, Inc.), strain EG9170, harboring a chromosomal *mgtA-lac* transcriptional fusion, formed blue colonies when containing pUC19 but white colonies when carrying pUC-*corA* (Fig. 3B).

The silencing effect of the *corA*-expressing plasmid is exerted on the *mgtA* riboswitch because the pUC-*corA* plasmid had no effect on the expression of strain EG9220 harboring a *lac* fusion at the sixth nucleotide of the *mgtA* 5′ leader region and thus lacking the *mgtA* riboswitch (Fig. 3B), but could silence *mgtA* expression in strain EG9170 with a *lac* fusion at position 2190. Moreover, *mgtA* expression in strain EG17425, with a defective *mgtA* riboswitch due to replacement of the 100-bp sequence corresponding to positions 148 to 247 in the wild-type *mgtA* 5′ leader (5) by the 84-bp "scar" sequence (6), was refractory to repression by the pUC-*corA* plasmid (Fig. 3B).

In sum, we have established that production of the Mg^{2+} transporter MgtA is ultimately governed by the Mg^{2+} levels in the cytoplasm, which are sensed by the *mgtA* riboswitch. Expression of the *mgtA* gene is also controlled at the transcription initiation step via the two-component system PhoP/PhoQ responding to periplasmic Mg^{2+}. Because the PhoP/PhoQ system is a major regulator of virulence functions (9) and the *mgtA* gene is not required for pathogenicity, the *mgtA* riboswitch may enable *Salmonella* to produce the MgtA protein only when the cytoplasmic Mg^{2+} concentration falls below a certain level and thus differentially from other gene products belonging to the PhoP regulon. Indeed, there is no *mgtA* expression when *Salmonella* experiences acid pH (4) even though the PhoP/PhoQ system is activated by acid pH (16). The *mgtA* expression behavior is in contrast to that of the Fe^{2+} transporter gene *feoB*, which is turned on via the PhoP-activated RstA protein when *Salmonella* faces acid pH but not in response to low Mg^{2+} (4). As *rstA* transcription is also induced in low Mg^{2+} (14, 20, 24), these findings suggest that acid pH is necessary for activation of the RstA protein.

Finally, there is increasing evidence suggesting the intriguing possibility that signals other than Mg^{2+} may act on the *mgtA* leader region to promote transcription elongation into the *mgtA* coding region. First, mutations in the *mgtA* leader region resulting in heightened *mgtA* expression enhanced the thermo-

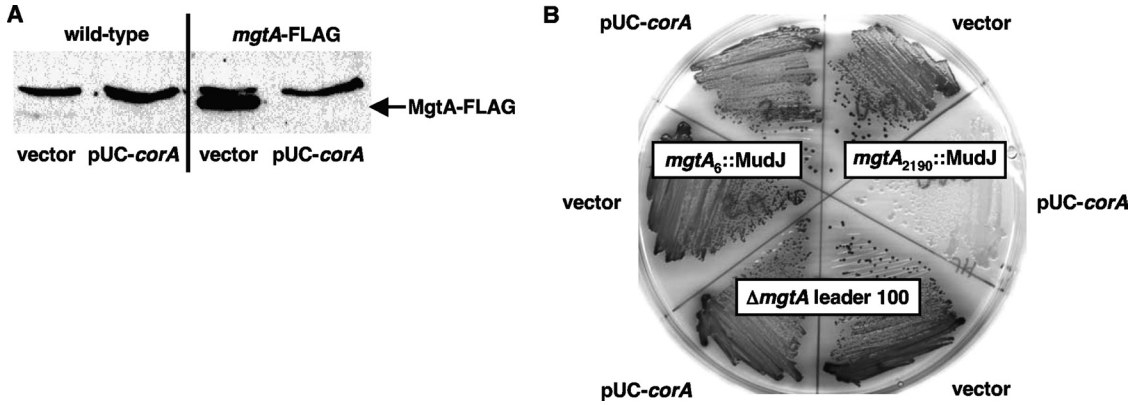

FIG. 3. Overexpression of the Mg^{2+} transporter gene *corA* turns off *mgtA* transcription in an *mgtA* riboswitch-dependent manner. (A) Western blot analysis of crude extracts prepared from strains 14028s (wild type) or EG13250 (*mgtA*$^+$-FLAG) harboring plasmid pUC-*corA* or the plasmid vector pUC19. (B) Lac phenotype of isogenic strains with *lac* transcriptional fusions at positions 6 (EG9220) and 2190 (EG9170) with respect to the PhoP-dependent transcription start site for *mgtA* or at position 977 and with the 100-bp sequence corresponding to positions 148 to 247 in the wild-type *mgtA* 5′ leader replaced by the 84-bp "scar" sequence (6) (EG17425) and harboring plasmid pUC-*corA* or the plasmid vector pUC19.

tolerance of *Salmonella* experiencing high osmolarity (15). Second, *rob* overexpression confers resistance to cyclohexane in an *mgtA*-dependent manner (2). And third, inactivation of the RNA chaperone Hfq promoted *mgtA* expression (7, 18) while decreasing the mRNA levels of other PhoP-activated genes (18). (The *mgtA* gene appears to be regulated in a different manner in *Escherichia coli* because *hfq* inactivation led to increased *mgtA* transcription [10].) Moreover, the dual control of *mgtA* expression at the transcription initiation and elongation steps is unusual for genes regulated by riboswitches, as they are typically transcribed from constitutive promoters. Therefore, we hypothesize that metabolic signals, environmental or cellular cues, and/or regulatory *trans*-acting factors may act on the *mgtA* leader region so that *mgtA* transcription initiated at intermediate Mg^{2+} concentrations in a PhoP-dependent manner (5) (Fig. 1B) can continue into the *mgtA* coding region, resulting in the synthesis of the MgtA protein even if the Mg^{2+} concentration in the cytoplasm is relatively high.

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