Magnesium transport in *Salmonella typhimurium*: biphasic magnesium and time dependence of the transcription of the *mgtA* and *mgtCB* loci

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*Salmonella typhimurium* has three distinct Mg²⁺ transport systems, the constitutive high-capacity CorA transporter and two P-type ATPases, MgtA and MgtB, whose transcription is repressed by normal concentrations of Mg²⁺ in the growth medium. The latter Mg²⁺-transporting ATPase is part of a two-gene operon, *mgtCB*, with *mgtC* encoding a 23 kDa protein of unknown function. Transcriptional regulation using fusions of the promoter regions of *mgtA* and *mgtCB* to *luxAB* showed a biphasic time and Mg²⁺ concentration dependence. Between 1 and 6 h after transfer to nitrogen minimal medium containing defined concentrations of Mg²⁺, transcription increased about 200-fold for *mgtCB* and up to 400-fold for *mgtA*, each with a half-maximal dependence on Mg²⁺ of 0.5 mM. Continued incubation revealed a second phase of increased transcription, up to 2000-fold for *mgtCB* and up to 10000-fold for *mgtA*. This secondary increase occurred between 6 and 9 h after transfer to defined medium for *mgtCB* but between 12 and 24 h for *mgtA* and had a distinct half-maximal dependence for Mg²⁺ of 0.01 mM. A concomitant increase of at least 1000-fold in uptake of cation was seen between 8 and 24 h incubation with either system, showing that the transcriptional increase was followed by functional incorporation of large amounts of the newly synthesized transporter into the membrane. Regulation of transcription by Mg²⁺ was not dependent on a functional stationary-phase sigma factor encoded by *rpoS*, but it was dependent on the presence of a functional *phoPQ* two-component regulatory system. Whereas *mgtCB* was completely dependent on regulation via *phoPQ*, the secondary late Mg²⁺-dependent phase of *mgtA* transcription was still evident in strains carrying a mutation in either *phoP* or *phoQ*, albeit substantially diminished. Several divalent cations blocked the early phase of the increase in transcription elicited by the decrease in Mg²⁺ concentration, including cations that inhibit Mg²⁺ uptake (Co²⁺, Ni²⁺ and Mn²⁺) and those which do not (Ca²⁺ and Zn²⁺). In contrast, the second later phase of the transcriptional increase was not well blocked by any cation except those which inhibit uptake. Overall, the data suggest that at least two distinct mechanisms for transcriptional regulation of the *mgtA* and *mgtCB* loci exist.

Keywords: magnesium transport, magnesium regulation, *phoPQ* two-component system, *Salmonella typhimurium*

INTRODUCTION

Magnesium is a vital divalent cation in living organisms, functioning as both a cofactor and a regulator of...
numerous proteins and as a stabilizing factor for membranes, ribosomes and other cellular structures (Altura, 1992; Grubbs & Maguire, 1987; Maguire, 1990; Romani et al., 1993). Studies in mammalian and other systems have shown that Mg$^{2+}$ transport is far more active and the intracellular content of Mg$^{2+}$, both free and total, is far greater than previously appreciated (Clausen et al., 1991; Grubbs et al., 1983; Maguire, 1990; Romani et al., 1993). In Salmonella typhimurium, three Mg$^{2+}$ transport systems have been identified and designated as CorA, MgtA and MgtB, each encoded by the respective locus (Hmiel et al., 1989; Snively et al., 1989). Transport via the CorA system is constitutive and of high capacity. This locus encodes a single protein of about 40 kDa lacking homology to any known protein and capable by itself of mediating Mg$^{2+}$ influx (Smith et al., 1993a). In contrast, the mgtA and mgtCB loci encode P-type ATPases (Snively et al., 1991a), and are thus members of a large family of homologous proteins responsible for membrane transport of cations (Pedersen & Carafoli, 1987). Interestingly, the MgtA and MgtB proteins have relatively little similarity to other prokaryotic P-type ATPases but much greater similarity to eukaryotic P-type ATPases, especially the muscle sarcoplasmic reticulum Ca$^{2+}$-ATPases (Snively et al., 1991a; Smith et al., 1993b). The mgtA locus consists of a single gene encoding MgtA whereas the mgtCB locus is a two-gene operon, where mgtC encodes a 23 kDa protein of unknown function and mgtB encodes a Mg$^{2+}$-transporting P-type ATPase. Both loci are tightly repressed under normal laboratory growth conditions. This repression, however, is relieved when Mg$^{2+}$ concentration in the medium is lowered (Snively et al., 1991b) and upon phagocytosis into mammalian epithelial cells (Garcia-del Portillo et al., 1992). Recent evidence has indicated that this repression is mediated at least in part by a two-component regulatory system, phoPQ, with the PhoQ protein being a membrane sensor-kinase for Mg$^{2+}$ (Garcia-Vescovi et al., 1996; Soncini et al., 1996).

Since the phoPQ system is an important virulence factor in Salmonella and other species, the ability of Mg$^{2+}$ to control a two-component regulatory system that in turn regulates Mg$^{2+}$ transport is of interest. Here we report that the derepression of the promoters for both the mgtA and mgtCB loci has a biphasic time and Mg$^{2+}$ concentration dependence, that such derepression results in extremely large increases in both gene transcription and translation of functional transport protein even in the absence of cell growth, and that this response appears to involve a second regulatory pathway, in addition to the phoPQ system.

**METHODS**

**Plasmids and strains.** These are shown in Table 1.

**Buffers.** Luria–Bertani (LB) broth was used for routine cultures with antibiotics supplemented as required (Hmiel et al., 1989; Snively et al., 1989). For luciferase assays as well as $^{65}$Ni$^{2+}$ transport assays, nitrogen minimal medium supplemented with 1 mM leucine, 0.1% Casamino acids, 0.4% glucose, 1 µg FeSO$_4$ ml$^{-1}$ and the indicated concentration of Mg$^{2+}$ was used as culture broth (Hmiel et al., 1989). Luciferase assay buffer is 50 mM sodium phosphate buffer, pH 7.5, containing 0.01% (v/v) dodecyl aldehyde.

**Luciferase assay.** Previous work (Maguire et al., 1992; Smith et al., 1993b; Snively et al., 1991b, Tao et al., 1995) has shown that a decrease in the Mg$^{2+}$ concentration in minimal medium greatly increases transcriptional activity at both the mgtA and mgtCB loci. Previous studies were performed using mgtA::lacZ and mgtA::lacZ fusions, measuring β-galactosidase activity at a single 6 h time point. The use of the luciferase reporter constructs allows easier continuous monitoring of much smaller volumes of individual cultures over any incubation period. Further, since at 37 °C the luciferase protein is unstable, a time course of luciferase activity can give some measure of the temporality of transcriptional response, unlike β-galactosidase measurements which give only a sum of activity over time without necessarily indicating when the activity occurred. The constructs were tested over a range of supplemental Mg$^{2+}$ concentrations from 0.3 µM to 100 mM Mg$^{2+}$. We have previously determined that the contaminant Mg$^{2+}$ concentration inherent in nitrogen minimal medium as measured by atomic absorption spectrometry is approximately 15 µM (Hmiel et al., 1986, 1989). However, this amount appears to be bound completely since we can detect effects of added Mg$^{2+}$ concentrations as low as 1–2 µM (Snively et al., 1991b).

The luciferase assay has been previously described (Tao et al., 1995). Briefly, an aliquot of cells (5–50 µl) is mixed with 500 µl luciferase assay buffer at room temperature in a 0.7 ml clear microfuge tube. The capped tube is mixed for 10 s, placed in a carrier scintillation vial, and luciferase activity determined immediately by light counting in a Beckman LS7000 liquid scintillation counter for 0.5 min using a full channel setting. To correct for coincidence detection in the scintillation counter, net c.p.m. activity is calculated as the square root of the c.p.m. detected in the counter. All data are normalized for cell number as represented by simultaneous measurement of turbidity as OD$_{600}$. Luciferase activity was linear over cell densities in the scintillation counter (as OD$_{600}$) from 0.001 to at least 1.0 as long as the total c.p.m. was less than 1 x 10$^{10}$. Activity greater than this amount were measured by dilution so that the total c.p.m. per cell aliquot used was less than 1 x 10$^{10}$.

It is important to note that the luciferase enzyme is temperature sensitive, being most stable at 28–30 °C. Transcriptional data in this report were obtained with cells grown both at 30 °C and at 37 °C. Control experiments indicated that qualitatively similar results were obtained with cells grown at either temperature although the maximal response obtained at 37 °C was somewhat less and slightly more variable, presumably because of ongoing denaturation and proteolysis of the luciferase. The growth temperature also has some effect on the temporal dependence of the transcriptional response, with lower temperatures resulting in a lengthening in the lag period before increases in transcription were observed. Finally, estimation of the fold increase in transcription (and transport itself) is inherently variable because of the extremely low, possibly zero, levels of activity seen in the presence of high extracellular Mg$^{2+}$ concentrations, thus making the denominator in the calculation a small, variable number. However, both the baseline and maximal responses varied slightly with inoculum density and from experiment to experiment. Overall, the most consistent presentation of the data is by fold increase. Representative absolute values for responses are given in the figure legends; $K_{95}$ values are
Table 1. S. typhimurium strains and plasmids used in this work

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype/plasmid</th>
<th>Reference/source</th>
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<tbody>
<tr>
<td>MM281</td>
<td>DEL485(LeuB) mgtB10::MudJ corA45::MudJ mgtA21::MudJ zjb1628::Tn10(Cam')</td>
<td>Hmiel et al. (1989)</td>
</tr>
<tr>
<td>MM387</td>
<td>DEL485(LeuB) corA185::MudJ zjb1628::Tn10(Cam')</td>
<td>Smith et al. (1993a)</td>
</tr>
<tr>
<td>MM101</td>
<td>MM387/pTTS5E</td>
<td>This study</td>
</tr>
<tr>
<td>MM102</td>
<td>MM387/pTT6SE</td>
<td>This study</td>
</tr>
<tr>
<td>MM103</td>
<td>MM387/pTT-CALux</td>
<td>This study</td>
</tr>
<tr>
<td>MM104</td>
<td>MM387/pTT-ALux</td>
<td>This study</td>
</tr>
<tr>
<td>MM106</td>
<td>MM387/pTT-CLux</td>
<td>This study</td>
</tr>
<tr>
<td>MM1270</td>
<td>pheS5170::MudJ (14028s)</td>
<td>E. Groisman</td>
</tr>
<tr>
<td>MM1270</td>
<td>pheS5172::MudJ (14028s)</td>
<td>E. Groisman</td>
</tr>
<tr>
<td>MM1270</td>
<td>pheQ5170::MudJ (14028s)</td>
<td>ATCC</td>
</tr>
<tr>
<td>MM1512</td>
<td>MM1269/pTT-ALux</td>
<td>This study</td>
</tr>
<tr>
<td>MM1511</td>
<td>pheS5170::MudJ/pTT-ALux</td>
<td>This study</td>
</tr>
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<td>pheQ5172::MudJ/pTT-ALux</td>
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<td>pheQ5172::MudJ/pTT-CLux</td>
<td>This study</td>
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Fig. 1 shows control data for the promoter constructs pTTS5E and pTT6SE and for the promoter plasmid containing the corA promoter sequence, pTT-CALux. These plasmids were derived from pTrc99A (Pharmacia) and carry the trc promoter under lacO control. There is no significant alteration in corA transcription by the concentration of extracellular Mg2+. Likewise, transcription from the trc promoter of the parental construct was not affected by Mg2+ whether expressed relative to the negative control plasmid or to its activity at 100 mM extracellular Mg2+.

Control experiments for cation toxicity. Cell exposure to high concentrations of many divalent cations is toxic; therefore, in experiments to determine cation specificity of transcriptional response, we used three parameters to gauge toxicity. First, cells carrying the various plasmids were tested by a disk inhibition assay in which a filter paper disk was placed in the centre of a lawn of cells. Cells that are not inhibited by the test ion grow up to the disk while those that are sensitive form a ring some distance from the disk with no visible growth within the ring (Hmiel et al., 1986, 1989). Second, in liquid cultures, we have used the cell density as a guide. In nitrogen minimal medium containing no added Mg2+, cell density (OD657) usually increases by a factor of about 3–5 with a starting inoculum of OD657 0.1 over the first several hours of incubation and remains constant for the remainder of the 24 h incubation period. (Toxicity results were similar at initial densities of OD657 0.02–0.2.) Cations were considered as non-toxic if, at the tested concentration, they did not significantly affect final cell density in liquid culture or cause formation of a centre ring devoid of growth on plates. Based on these considerations (data not shown), Ca2+, Ni2+, Mn2+ and Zn2+ were toxic at approximately the same or slightly higher concentrations than those at which they diminished transcription of the luxAB fusions. Ba2+ and Sr2+ showed no significant toxicity. The effect of Ca2+ depends on the system being tested. In cells dependent on the MgtB Mg2+ transport system for Mg2+ uptake (e.g. mgtA corA double mutants), Ca2+ is highly toxic (Hmiel et al., 1986, 1989). In contrast, in cells dependent on the MgtA system (mgtCB corA double mutants), even very
high concentrations of extracellular Ca\(^{2+}\) have no effect on growth.

As a third test of possible cation toxicity, we measured the ability of the various cations to alter transcription of the treR promoter carried on pTT-DLux. The treR gene is adjacent to and transcribed in the opposite direction from mgtA (Tao et al., 1995), with their promoters carried on the same segment of DNA between the two genes. This is probably the most informative assay because it measures the same parameter (transcription) and utilizes the same segment of DNA as the Mg\(^{2+}\)-sensitive promoter carried on pTT-ALux (see above). Using transcription of treR as a guide, we concluded (data not shown) that regardless of any effect of divalent cations to inhibit growth, no cation is severely toxic based on inhibition of treR transcription. Only Ni\(^{2+}\) represses transcription from pTT-DLux more than 50% after 24 h incubation.

65Ni\(^{2+}\) transport. MM281 was transformed by electroporation with plasmids carrying inserts to be tested. The resulting strains were grown overnight in LB broth supplemented with appropriate antibiotics. Subcultures (20 ml) were started by 1:50 inoculation of nitrogen minimal medium containing 1 mM Mg\(^{2+}\). After 8 h growth, the cells from the subculture were collected by centrifugation at 1000 g for 5 min and washed twice in the same volume of nitrogen minimal medium containing no added Mg\(^{2+}\). New subcultures were then started from the washed cells by resuspension in nitrogen minimal medium containing the indicated Mg\(^{2+}\) concentration to a final OD\(_{600}\) of 0.1. After incubation for the indicated time, cells were collected and washed three times in nitrogen minimal medium without added Mg\(^{2+}\) before suspension in the same medium at a final OD\(_{600}\) of 1.0 for use in the transport assay (Snävely et al., 1989; Grubbs et al. 1989).

**RESULTS**

**Regulation of mgtCB**

Regulation of the mgtCB promoter (pTT-CLux) over the first several hours of incubation at 37 °C is shown in Fig. 2. An increase in transcription can be seen as early as 1 h after resuspending cells in low Mg\(^{2+}\) concentrations. A marked increase occurs beginning after about 2 h incubation, reaching about 200-fold for mgtCB by 6 h. The apparent half-maximal (extracellular) Mg\(^{2+}\) concentration \(K_{0.5}\) at which this increase in transcription occurs is 0.5–1 nM. Continued incubation for a total of 24 h provided an additional increase in transcription of mgtCB (Fig. 3). Beginning at approximately 7 h incubation, a second phase of transcriptional activity ensues, reaching a maximum of 1500–2000-fold in most experiments. The \(K_{0.5}\) for Mg\(^{2+}\) of this second phase is about 10 μM, significantly lower than that of the initial phase. The time course of the increase in activity is clearly at least biphasic, as shown in the inset to Fig. 3.

**Regulation of mgtA**

Identical experiments with the mgtA promoter construct (pTT-ALux) in cells grown at 37 °C gave results qualitatively comparable to those with the mgtCB promoter.
3–4 h incubation is delayed with \textit{mgtA} until 5–6 h incubation. While the overall activity of \textit{mgtA} during this initial phase is significantly higher than that of \textit{mgtCB}, the $K_{95}$ for Mg$^{2+}$ is similar, about 0.5–1 mM. As with the initial response, the second phase of the transcriptional response was delayed for \textit{mgtA} compared to \textit{mgtCB}, occurring after at least 10 h incubation, and was greater for \textit{mgtA} (Fig. 5) than for \textit{mgtCB} (Fig. 3). Increases of up to 10000-fold have been routinely measured. As with \textit{mgtCB}, the $K_{95}$ of Mg$^{2+}$ for this second phase is significantly lower than for the first phase. The variability in the assay and their lengthy time course make determination of the second $K_{95}$ for \textit{mgtA} more difficult, but the $K_{95}$ values for both \textit{mgtA} and \textit{mgtCB} appear similar at approximately 10 µM Mg$^{2+}$.

Previous data on \textit{mgtA} using the \textit{mgtA::lacZ} fusion showed only the initial phase of the Mg$^{2+}$-dependent increase in transcription (Snively et al., 1991b). However, the second phase was probably not seen since β-galactosidase activity was measured only at a single 6 h time point, well before the second phase of the increase in transcription occurs with \textit{mgtA}.

### Regulation of Mg$^{2+}$ transport

The question of whether these large increases in transcription result in a similarly large translation into functional protein was addressed using $^{65}$Ni$^{2+}$ uptake as surrogate for the unavailable $^{28}$Mg$^{2+}$ (Snively et al., 1989, 1991b). We have previously shown that activation of the (single-copy) chromosomally encoded \textit{mgtCB} locus results in significant increases in $^{40}$Mg$^{2+}$ and $^{65}$Ni$^{2+}$ uptake when the Mg$^{2+}$ concentration of the medium is decreased (Snively et al., 1991b). Since the luciferase reporter plasmids assayed above are pBR322-based and therefore have a relatively high copy number, $^{65}$Ni$^{2+}$ uptake was assayed under conditions as similar as possible to the promoter plasmids using pDS107 (Smith et al., 1993b), a pBR322-based plasmid expressing the intact \textit{mgtCB} operon, carried in MM281. Since the chromosomal Mg$^{2+}$ transport genes have been inactivated in MM281 (Hmiel et al., 1989, Snively et al., 1989), the strain is dependent on MgtB protein encoded by pDS107 for Mg$^{2+}$ uptake and Mg$^{2+}$-independent growth. When extracellular Mg$^{2+}$ is lowered, Mg$^{2+}$ uptake measured as $^{65}$Ni$^{2+}$ accumulation markedly increases, with a time course similar to that of the transcriptional increase (Fig. 6). Similar increases in transport via the \textit{mgtA} system are also seen under these incubation conditions (data not shown). The degree of increase is a function of the extracellular Mg$^{2+}$ concentration (Maguire et al., 1992; data not shown). Estimation of the exact fold increase is difficult however because of the low initial level of uptake.

### Effect of \textit{phoPQ} on transcription

The finding by Groisman and colleagues (Garcia-Vescovi et al., 1996; Soncini et al., 1996) that the \textit{phoPQ} two-component regulatory system, important for virulence in \textit{Salmonella} and other enteric bacteria (Miller et
transport system. Strain MM281 carrying pDS107 (Smith et al., 1993b) was grown overnight in nitrogen minimal medium containing 1 mM Mg2+. The cells were washed twice in the same medium without added Mg2+, resuspended in the same medium at OD600 0.1, transferred into flasks containing medium at 37 °C either without added Mg2+ or containing 1 mM Mg2+, and incubated at 37 °C. At the indicated times, triplicate 1 ml aliquots were withdrawn from each flask, centrifuged for 10 s in a microfuge, resuspended in nitrogen minimal medium containing 100 μM 63Ni2+ and no added Mg2+. Uptake was measured as previously described for 20 min at 37 °C (Snively et al., 1989; Grubbs et al., 1989). The basal level of uptake in cells grown in 1 mM Mg2+ was 100-200 net c.p.m. 63Ni2+ per 10^8 cells over the 20 min incubation period over a scintillation counter background of 100-200 c.p.m. The fold increase at each time point was calculated by dividing the uptake in cells grown without added Mg2+ by uptake in the cells grown with added Mg2+ after normalizing for cell number. The uptake in cells grown in 1 mM Mg2+ varied less than twofold over the time course of the experiment. The variation in uptake at each time point was <5% for cells grown without added Mg2+ and <20% for cells grown in 1 mM Mg2+. The inset shows the same data at the early time points on an expanded scale.

The dose-response curve for Mg2+ and phoP/phoQ transcription from the mgtA and mgtCB promoters measured after 6 h incubation. The parental strain for these studies was S. typhimurium 14028s which carries a wild-type rpoS allele. Transcription was measured as described in Methods and the legend to Fig. 2 after incubation with the indicated Mg2+ concentrations. At each time the data were normalized to cell density (OD600) and to the apparent transcription at 100 mM Mg2+. Absolute values of activity were similar in this experiment to those noted in the legends to Figs 2 and 3. ●, pTT-CLux; ■, pTT-CLux/phoP; ▲, pTT-CLux/phoQ; ●, pTT-ALux; ★, pTT-ALux/phoP; □, pTT-ALux/phoQ.

**Fig. 6.** Time course of induction of Mg2+ uptake by the MgtB transport system. Strain MM281 carrying pDS107 (Smith et al., 1993b) was grown overnight in nitrogen minimal medium containing 1 mM Mg2+. The cells were washed twice in the same medium without added Mg2+, resuspended in the same medium at OD600 0.1, transferred into flasks containing medium at 37 °C either without added Mg2+ or containing 1 mM Mg2+, and incubated at 37 °C. At the indicated times, triplicate 1 ml aliquots were withdrawn from each flask, centrifuged for 10 s in a microfuge, resuspended in nitrogen minimal medium containing 100 μM 63Ni2+ and no added Mg2+. Uptake was measured as previously described for 20 min at 37 °C (Snively et al., 1989; Grubbs et al., 1989). The basal level of uptake in cells grown in 1 mM Mg2+ was 100-200 net c.p.m. 63Ni2+ per 10^8 cells over the 20 min incubation period over a scintillation counter background of 100-200 c.p.m. The fold increase at each time point was calculated by dividing the uptake in cells grown without added Mg2+ by uptake in the cells grown with added Mg2+ after normalizing for cell number. The uptake in cells grown in 1 mM Mg2+ varied less than twofold over the time course of the experiment. The variation in uptake at each time point was <5% for cells grown without added Mg2+ and <20% for cells grown in 1 mM Mg2+. The inset shows the same data at the early time points on an expanded scale.

**Fig. 7.** Effect of Mg2+ and phoP/phoQ mutation on transcription from the mgtA and mgtCB promoters measured after 6 h incubation. The parental strain for these studies was S. typhimurium 14028s which carries a wild-type rpoS allele. Transcription was measured as described in Methods and the legend to Fig. 2 after incubation with the indicated Mg2+ concentrations. At each time the data were normalized to cell density (OD600) and to the apparent transcription at 100 mM Mg2+. Absolute values of activity were similar in this experiment to those noted in the legends to Figs 2 and 3. ●, pTT-CLux; ■, pTT-CLux/phoP; ▲, pTT-CLux/phoQ; ●, pTT-ALux; ★, pTT-ALux/phoP; □, pTT-ALux/phoQ.

transcription, induced by Mg2+ concentrations in the low micromolar range, that remains in the presence of a phoP or phoQ mutation. This dose dependence is also consistent with the concentration dependence of the interaction of Mg2+ with the PhoQ membrane sensor protein, which is in the range of 0.5 mM (Garcia-Vescovi et al., 1996; Vescovi et al., 1997). Together, these data suggest that a second level of transcriptional control exists for mgtA but that a similar second level of control may not exist for mgtCB.

**Relationship of growth and transcriptional response**

The transcriptional results presented above are somewhat complicated by the fact that at very low Mg2+ concentration in the growth medium, Mg2+ becomes growth limiting. We have examined this by measuring the medium Mg2+ concentration during growth (Fig. 8). In Mg2+ concentrations above about 0.2 mM in the supplemented nitrogen minimal medium described in Methods, Mg2+ is not growth limiting. With cell growth, an initial Mg2+ concentration in the medium of 1 mM falls rapidly to about 150-200 μM within 2 h and remains constant throughout the remainder of the incubation period even though the cells continue to increase in density for at least 6-8 h (data not shown). Cell viability remains constant between 2 and 24 h. In Mg2+ concentrations of 0.1 mM or below, Mg2+ concentration in the medium falls to about 5-10 μM over at least 6 h. With such low Mg2+ concentrations, OD400 doubles within 2-3 h and cell growth ceases even before...
Mg$^{2+}$ has fallen to its lowest level. Again, both Mg$^{2+}$ concentration and cell viability remain constant for at least 24 h (data not shown). The initial cell density had little effect on these time courses, affecting only the final cell density. Interestingly, there was no difference in medium Mg$^{2+}$ level or the time course of its depletion when tested in wild-type versus corA strains. This would imply that the source of Mg$^{2+}$ (CorA versus the Mgt systems) has no direct influence on the cell's Mg$^{2+}$ requirement.

**Regulation by other cations**

Both MgtA and MgtB mediate Mg$^{2+}$ and, non-physiologically, Ni$^{2+}$ influx, but each transporter has a distinct cation inhibition profile. Previous work has also shown that the initial phase of transcription of both mgtA and mgtCB induced by decreasing Mg$^{2+}$ concentration could be blocked by the addition of 1 mM extracellular Ca$^{2+}$ (Snavely et al., 1991b), as measured using mgtB::luxZ fusions. Thus it was of interest to determine how other cations affected transcription of these loci. We therefore tested the Group Iia divalent cations Ba$^{2+}$, Sr$^{2+}$ and Ca$^{2+}$ and the transition metal cations Ni$^{2+}$, Co$^{2+}$, Mn$^{2+}$ and Zn$^{2+}$ for their ability to alter transcription at the mgtA and mgtCB loci. First, no cation could, of itself, elicit an increase in transcription when added in the presence of either low or high Mg$^{2+}$ concentrations (data not shown). Second, a cation's ability to inhibit the increase in transcriptional activity elicited by the absence of extracellular Mg$^{2+}$ depended on the specific cation tested. Of the Group IIA cations, Ca$^{2+}$ but not Sr$^{2+}$ (Fig. 9) blocks the initial increase in mgtA or mgtCB transcription. Ba$^{2+}$ also was unable to block this initial increase (data not shown). About 1 mM Ca$^{2+}$ is required for complete inhibition of transcription at mgtA and mgtCB (data not shown; Snavely et al., 1991b). Since Ca$^{2+}$ is not toxic in strains dependent on Mg$^{2+}$ uptake at concentrations that block the initial increase in transcription, the ability to repress the initial phase of transcription at both the mgtA and mgtCB loci is likely a specific effect of the cation, most probably mediated by binding to PhoQ (see below) which recognizes both Mg$^{2+}$ and Ca$^{2+}$. In contrast to its ability to inhibit the initial phase of increased transcription, Ca$^{2+}$ is largely ineffectual in blocking the second later phase of transcriptional activity completely (Fig. 9).

Among the transition metal cations, Co$^{2+}$ blocks the increase in transcription at either locus (Fig. 9 and data not shown). Significant inhibition can be seen at <10 μM Co$^{2+}$, almost complete inhibition is seen at 100 μM Co$^{2+}$ (data not shown), and both the early and late phases of the transcriptional response are inhibited. Test strain MM387, carrying a corA mutation, is

**Fig. 8.** Mg$^{2+}$ content of growth medium during growth of cells. MM1269 (wild-type) and MM387 (corA) were inoculated at OD$_{600}$ 0.1 into a 25 ml flask with the supplemented nitrogen minimal medium (Hmiel et al., 1989). Wild-type (1 mM Mg$^{2+}$); corA (1 mM Mg$^{2+}$); A, wild-type (100 mM Mg$^{2+}$); B, corA (100 mM Mg$^{2+}$).

**Fig. 9.** Effect of various cations on transcription from the mgtCB promoter in cells carrying pTT-Clux. Transcription was measured as described in Methods. Cell aliquots were resuspended in the indicated concentrations of cation, transcription measured after incubation for 6 h (I) or 24 h (II) and the values normalized to transcription at the respective time in the absence of Mg$^{2+}$. Activity of luxAB was measured in triplicate aliquots as described in Methods. The single experiment shown is representative of two such experiments. Triplicate aliquots measured for each individual culture varied ±10% of the absolute value and were similar under all conditions tested. Three independent cell cultures with no added Mg$^{2+}$ were assayed in this experiment and shown as the control with the bars indicating SEM. The percentage transcription relative to the no added Mg$^{2+}$ control is shown by the number above each bar. Absolute values of transcriptional activity were similar to those noted in the legends to Figs 2 and 3.
resistant to Co²⁺ concentrations up to 350 µM in the
growth medium, although growth slows somewhat
above 150 µM Co²⁺. Thus the inhibition of transcription
by Co²⁺ could be a combination of a direct effect on
transcription of these specific genes and an indirect effect
via general cell toxicity, but any toxic effect of Co²⁺ is
probably minimal since Co²⁺ had little effect on treR
transcription (see Methods). By the same argument,
only a portion of the ability of Ni²⁺, Mn²⁺ and Zn²⁺ to
diminish transcription (Fig. 9) can be attributed to
toxicity. Except possibly for Ca²⁺, transcriptional in-
hibition by divalent cations other than Mg²⁺ is rather
unlikely to be physiologically relevant since the con-
centrations required are much greater than the organism
is likely to encounter routinely in its various environ-
ments. Even for Ca²⁺, the levels required for repression
are relatively high.

Since some reports have suggested that sulfate rather
than or in addition to Mg²⁺ may play a role in gene
expression (Gross & Rappuoli, 1989; Kertesz et al.,
1993; Scarlato & Rappuoli, 1991), we tested the effect of
anions on mgtA and mgtCB transcription as a control.
The ability of Mg²⁺ to repress transcription was
independent of chloride or sulfate as the counter-ion;
further, sulfate alone had no effect as seen by the
inability of 10 mM Na₂SO₄ to alter transcription (data
not shown).

**DISCUSSION**

The data presented in this report confirm and extend our
previous work on the ability of Mg²⁺ to regulate transcription of its transport genes. The regulation is
obviously complex. Distinct transcriptional effects can
be seen at different times and different Mg²⁺ concen-
trations. Divalent cations other than Mg²⁺, but not
anions, also alter transcription. Despite some degree of
cell toxicity from other cations, a major proportion of
this transcriptional inhibition is probably a direct
regulatory effect at the mgtA and mgtCB loci. None-
theless, transcriptional inhibition by cations other than
Mg²⁺ is unlikely to be of major physiological significance
since relatively high concentrations of cation are
required.

The mechanism of these effects on transcription is only
partially known. The phoPQ two-component regulatory
system (Garcia-Vescovi et al., 1996; Sontini et al., 1996)
would appear to mediate the initial earlier tran-
scriptional response based on the Mg²⁺ concentration
required for half-maximal induction and the effect of
phoP and phoQ mutations. These data are consistent
with previous data (Miller et al., 1989; Johnston et al.,
1996; Groisman et al., 1989, Gunn & Miller, 1996; Guo
et al., 1997; Sontini et al., 1996; Sontini & Groisman,
1996, Garcia-Vescovi et al., 1996) showing that the
phoPQ system is involved in both induction and
repression of a relatively large set of genes important for
virulence. However, the phoPQ system cannot be the
only regulatory pathway involved. The most direct
evidence for this is the ability of very low extracellular
Mg²⁺ concentrations to induce mgtA transcription in
the presence of a phoP or phoQ mutation. While the
presence of a phoP/Q mutation markedly diminishes
the transcriptional response at mgtA, it is clearly not
abolished and still has a dose dependence on Mg²⁺ (Fig.
7). Additional evidence for a second regulatory pathway
might be deduced from the fact that the second, later
phase of mgtA and mgtCB transcription has a distinct
half-maximal Mg²⁺ concentration dependence, well
below the demonstrated affinity for Mg²⁺ interaction
with phoQ. This interpretation is complicated however
by the results in Fig. 7 showing complete dependence of
mgtCB response on the presence of a functional PhoPQ
system. The cation sensitivity data (Fig. 8) suggest that
the phase of regulation with a half-maximal Mg²⁺
dependence of about 10 µM appears more selective for
Mg²⁺, while that operative at 1 mM Mg²⁺ may be
sensitive to several divalent cations. mgtA and mgtCB
are clearly not the only loci regulated by changes in
extracellular Mg²⁺ concentrations and it would be of
interest to determine if other phoPQ-regulated loci
respond with a biphasic pattern. Other examples of
apparent Mg²⁺ regulation of gene expression have also
been reported (Scarlato & Rappuoli, 1991; Kiyota et al.,
1989; Guzzo et al., 1991; Phinney & Hoober, 1992;
O'Halloran, 1993).

The time courses of the responses of mgtA and mgtCB
suggest that the initial phase of Mg²⁺-dependent tran-
scriptional response occurs during or near the end of
active growth, but that the second phase, seen after
several hours' incubation and at low extracellular Mg²⁺
concentrations, occurs only after cell growth has ceased
(and while extracellular Mg²⁺ concentration remains
constant). The question therefore arises whether the
second phase of response seen with both mgtA and
mgtCB is a stationary-phase response in some part. We
do not believe it is. First, cells grown in moderate levels
of Mg²⁺ to stationary phase do not show induction of
mgtA or mgtCB regardless of how long they are left at
high density. Second, more directly, the stationary-
phase sigma factor encoded by rpoS has no effect of
transcription of mgtA or mgtCB. The experiments
shown in Figs 2–5 were performed in a strain back-
ground using S. typhimurium LT2 as parent. This
nominal wild-type strain carries an rpoS mutation
(Wilmes-Riesenber, 1997). (We have confirmed by
catalase assay and introduction of a wild-type allele of
rpoS that our strain of S. typhimurium LT2 is rpoS.)
In contrast, the data of Fig. 7 were obtained in a strain
background using S. typhimurium 14028s as parent,
which is a virulent strain with a wild-type rpoS allele.
From these data, there is little if any difference in the
transcriptional response of mgtA or mgtCB in wild-type
and rpoS strains. Thus we conclude that neither phase of
the Mg²⁺-associated transcription response at mgtA and
mgtCB is necessarily tied to growth state.

These data also suggest that MgtA and MgtB are not
(solely) scavenger systems for Mg²+. Classically, mul-
tiple transport systems for nutrients consist of a rela-
tively poor-affinity transporter(s) operative during
growth in media rich in that nutrient while a high- 
affinity transporter is expressed in media with a very low 
concentration of that substance. Classic examples are 
the K⁺ transporters of *Escherichia coli*. The TRK system 
has an affinity of 1–2 mM for K⁺ uptake, while in low 
extracellular K⁺ the Kdp P-type ATPase is induced and 
has an affinity for K⁺ of 1–3 μM (Altendorf & Epstein, 
1994; Epstein et al., 1993; Dosch et al., 1991). The MgtA 
and MgtB systems do not fit this model. First, all three 
Mg²⁺ transporters of *S. typhimurium* and *E. coli* have 
affinities for Mg²⁺ of between 5 and 30 μM. Second, as 
noted above, an increase in mgtA and mgtCB tran-
scription is seen at extracellular Mg²⁺ concentrations at 
which the constitutive CorA system is still capable of 
supplying sufficient Mg²⁺. Third, unlike scavenger 
systems, both MgtA and MgtB transport the physi-
ologically relevant cation down its electrochemical grad-
et. Even at very low extracellular Mg²⁺, the 
electrochemical gradient for Mg²⁺ should still be directed 
inward. The purpose of using ATP is therefore unclear. 
Fourth, it is unclear why the cell would possess two 
Mg²⁺ uptake carriers. Although they have similar 
properties and regulation, the nucleotide sequences of 
mgtA and mgtCB, and the amino acid sequences of their 
products, are not exceptionally similar. This suggests 
that the alleles must have been maintained separately 
within *S. typhimurium* for a considerable time in 
evolutionary terms or that one of the ATPases has been 
bound to Salmonella by horizontal transfer. Presumably 
the cell would not maintain two such func-
tionally similar alleles unless one or both had some 
specific, useful property. Thus, while the extraordinarily 
large transcriptional responses of these loci indicate that 
they play a role in providing Mg²⁺ at very low 
extracellular concentrations, this may not be their only 
role. Indeed, it is possible that Mg²⁺ transport is a 
secondary function, with their primary role yet to be 
discovered.

Finally, it must be noted that the function of the mgtC 
gene is currently unknown. The MgtC protein sequence 
yields no clues from current database entries. It is 
entirely possible the Mg²⁺ regulation described herein is 
directed primarily at expression of the MgtC protein 
rather than at MgtA or MgtB. The complex regulation 
of the two loci suggests that these genes are expressed 
under some specific growth or metabolic condition.
Regardless of the mechanism of regulation and the 
growth conditions under which regulation of these loci 
occur, these data demonstrate that extracellular Mg²⁺ 
can have a profound influence on gene expression, 
indicating that Mg²⁺ may play an important role in 
cellular metabolic homeostasis and gene expression.

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REFERENCES


Clausen, T., Van Hardeveld C. & Everts, M. E. (1991). Significance of cation transport in control of energy metabolism and ther-
mogenesis. *Physiol Rev* 71, 733–774.


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