Magnesium transport in Salmonella typhimurium: regulation of mgtA and mgtCB during invasion of epithelial and macrophage cells

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Salmonella typhimurium contains two inducible Mg\(^{2+}\) transport systems, MgtA and MgtB, the latter encoded by a two-gene operon, mgtCB. Mg\(^{2+}\) deprivation of S. typhimurium increases transcription of both mgtA and mgtCB over a thousandfold and a similar increase occurs upon S. typhimurium invasion of epithelial cells. These increases are mediated by the phoPQ two-component signal transduction system, an essential system for S. typhimurium virulence. It was therefore hypothesized that expression of MgtA and MgtCB is increased upon invasion of eukaryotic cells because of a lack of intravacuolar Mg\(^{2+}\). However, when S. typhimurium was grown at pH 5.2, the capacity of the constitutive CorA transporter in mediating Mg\(^{2+}\) was greater than that at pH 7.4. Furthermore, induction of mgtA and mgtCB transcription was greater in the presence of a wild-type corA allele than in its absence. This implies that intravacuolar S. typhimurium could obtain sufficient Mg\(^{2+}\) via the CorA system. The effect of acid pH on mgtA and mgtCB transcription was also measured. Compared to induction at pH 7.4, exposure to pH 5.2 almost completely abolished induction of mgtA at low Mg\(^{2+}\) concentrations but diminished induction of mgtCB only twofold. Adaptation of cells to acid pH by overnight growth resulted in normal levels of induction of mgtA and mgtCB at low Mg\(^{2+}\) concentrations. These results imply an additional level of regulation for mgtA that is not present for mgtCB. Conversely, repression of mgtA and mgtCB expression by increased extracellular Mg\(^{2+}\) was relatively insensitive to acid. Transcription of both loci was strongly induced upon invasion of the Hep-2 or CMT-93 epithelial-like or J774 macrophage-like cell lines. However, the presence or absence of functional alleles of either or both mgtA or mgtCB had no effect on invasion efficiency or short-term survival of S. typhimurium within the eukaryotic cells. It was concluded that the strong Mg\(^{2+}\)-dependent induction of mgtA and mgtCB upon invasion of eukaryotic cells is not required because S. typhimurium lacks sufficient Mg\(^{2+}\) during eukaryotic cell invasion and initial intravacuolar growth.

Keywords: magnesium transport and regulation, bacterial pathogenesis, phoPQ two-component system, Salmonella typhimurium, pH

INTRODUCTION

Salmonella typhimurium has three distinct Mg\(^{2+}\) transport systems, the constitutive CorA transporter and two P-type ATPase transport systems for Mg\(^{2+}\), MgtA and MgtB (Hmiel et al., 1986, 1989; Snavely et al., 1989; Smith et al., 1993). Both CorA and MgtA are encoded by single genes while mgtB forms part of a two-gene

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operator, mgtCB. MgtC is a 22.5 kDa membrane protein of unknown function and its absence has no effect on the transport properties of MgtB (Snayl, 1991a; Tao et al., 1995). Neither MgtA nor MgtB are normally expressed significantly in cells grown in normal laboratory media, but their transcription is increased several thousandfold by growth of the bacteria in medium containing limiting Mg²⁺ (Snayl, 1991b; Tao et al., 1995). Transcription of mgtA and mgtCB, as well as that of several other genes, is under control of the two-component regulatory system PhoPQ, an important virulence factor (Grosman et al., 1989; Gunn & Miller, 1996; Miller et al., 1989; Soncini et al., 1996). We have previously shown that transcription of mgtCB is markedly increased when S. typhimurium invades the MDCK epithelial cell line (Garcia-del Portillo et al., 1992). This increase in transcription is apparently due to limiting Mg²⁺ in the epithelial cell vacuole within which the bacterium initially resides. This result suggests the hypothesis that mgtA and/or mgtCB transcription is induced upon invasion to scavenge Mg²⁺ for replication of S. typhimurium within the eukaryotic cell.

The classical concept of a scavenger system for a nutrient is an enzyme or transporter of low capacity but high affinity, while the major metabolic system for that nutrient would exhibit high capacity but relatively low affinity. The K⁺ transport systems of Escherichia coli fit this model with the TRK systems having high capacity and an affinity of 1–2 mM whereas the inducible Kdp transport system has a significantly lower maximal transport rate but an affinity of about 2 μM (Altendorf & Epstein, 1994; Dosch et al., 1991; Epstein et al., 1993). The Mg²⁺ transporters of S. typhimurium (CorA, MgtA and MgtB) and E. coli (CorA and MgtA) do not exhibit these properties, however. The CorA Mg²⁺ transporter has a Kₘ for uptake of Mg²⁺ of 15–20 μM whereas MgtA and MgtB have apparent Kₘ values of 5–30 μM. CorA also has a high capacity of 0.5–1 nmol min⁻¹ per 10⁶ cells (Hmiel et al., 1986; Snayl et al., 1989), although given the extraordinary degree of induction for MgtA and MgtB expression, which may reach 10000-fold for MgtA, expression in some environments would provide significant additional transport capacity. It is also possible that in some environments CorA might not function adequately so that MgtA and/or MgtB could be required. One such environment might be the vacuole which S. typhimurium forms upon invasion of eukaryotic cells (Finlay & Falkow, 1988; Finlay & Cossart, 1997), which is known to be limiting in iron and Mg²⁺ and which, at least initially, has an acid pH (Alpuclhe-Aranda et al., 1992; Garcia-del Portillo et al., 1992, Rathman et al., 1996).

We report here some parameters of regulation of MgtA and MgtCB expression relevant to eukaryotic cell invasion. While, consistent with previous studies (Snayl et al., 1991b; Tao et al., 1998), we find that mgtA and mgtCB exhibit differences in transcriptional regulation, we also conclude that neither P-type ATPase system is induced for the purpose of supplying Mg²⁺ to the bacterium. Thus, the role(s) of MgtA and MgtCB during S. typhimurium pathogenesis remains to be established.

**METHODS**

**Bacterial strains and growth.** The bacterial strains used are listed in Table 1. Strain MM731 is a spontaneous deletion of about 300 bp in corA isolated by screening for Co²⁺ resistance as described previously (Hmiel et al., 1986). The deletion occurs between an internal 5 aa repeat within CorA. The deletion has been confirmed by complete sequencing of a PCR product spanning the entire corA gene. Strains MM1265 and MM1274 were formed by P22 transduction of the MudJ or MudA insertion from MM1266 and MM1268, respectively, into MM731 as recipient. MM1314 was likewise constructed by P22 transduction. Strains MM1512 and MM1520 were constructed by transformation of the appropriate plasmid DNA into MM1269. The pTT-Lux plasmids have been described previously (Tao et al., 1995, 1998).

Bacteria were routinely grown in Luria-Bertani (LB) broth at 37 °C with shaking and with antibiotics supplemented as required as described previously (Hmiel et al., 1989; Snayl et al., 1989). Cultures to be used for invasion assays were grown similarly but without shaking and in the absence of antibiotic. For luciferase, β-galactosidase and ⁶⁸Ni²⁺ uptake assays, nitrogen minimal medium (N-minimal medium) was routinely supplemented with 0.1 % Casamino acids, 0.4 % glucose, 1 μg FeSO₄ ml⁻¹ and the indicated concentration of Mg²⁺ (Hmiel et al., 1989). Luciferase assay buffer was 50 mM sodium phosphate buffer, pH 7.5, containing 0.01 % (v/v) dodecyl aldehyde. A stock solution of 1 % (v/v) dodecyl aldehyde was kept frozen and added immediately before use.

For measurements of transcriptional responses, bacterial cells were grown in N-minimal medium at either pH 7.4 or 5.2. For pH 5.2 experiments, MES replaced Tris as the buffer in N-minimal medium. Control experiments showed that transcriptional response at pH 7.4 was identical in either buffer. Transcriptional responses were measured either after an acute shift from pH 7.4 to 5.2 and further incubation for the indicated time at 37 °C or after 'adaptation' to acid pH by overnight growth at pH 5.2 before resuspension in fresh medium and further incubation for the indicated time. The Mg²⁺ concentration was altered as indicated in each experiment. The buffer concentration was 100 mM which is sufficient to prevent major pH shifts during the assay period.

**Luciferase and β-galactosidase assays.** β-Galactosidase from lacZ expression and luciferase activity from luxAB expression were assayed as described previously (Snayl et al., 1991b; Tao et al., 1995, 1998). We have previously determined that the contaminant Mg²⁺ concentration inherent in N-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 Mg²⁺ concentrations as low as 1–2 μM (Snayl et al., 1991b). There is no significant alteration in CorA transcription by the concentration of extracellular Mg²⁺ (Snayl et al., 1991b; Tao et al., 1998).

**⁶⁸Ni²⁺ transport.** Test strains were grown in LB broth supplemented with appropriate antibiotics overnight. Twenty millilitre subcultures were started by 1:50 inoculation of N-minimal medium containing 1 or 10 mM Mg²⁺ in various experiments. No significant differences were seen under either growth condition. After 8 h of growth, the cells from the subculture were collected by centrifugation at 1000 g for 5 min.
and washed twice in the same volume of N-minimal medium containing no added Mg\textsuperscript{2+}. Strains grown in 10 mM Mg\textsuperscript{2+} were washed three times. New subcultures were then started from the washed cells by resuspension in N-minimal medium containing the indicated Mg\textsuperscript{2+} concentration to a final OD\textsubscript{600} of 0.1. After incubation as indicated for each experiment, cells were collected and washed three times in N-minimal medium without added Mg\textsuperscript{2+} before resuspension in the same medium at a final OD\textsubscript{600} of 1.0–2.0 for use in the transport assay. Transport was performed as described previously (Snavely et al., 1989; Grubbs et al., 1989). Uptake of \textsuperscript{65}Ni\textsuperscript{2+} was measured for 20 min. Transport was normalized to OD\textsubscript{600} for each strain. Induction of detectable Mg\textsuperscript{2+} uptake via MgtA or MgtB (when present) requires at least 60 min incubation at 37 °C in the absence of Mg\textsuperscript{2+}. This allows measurement of CorA-mediated transport using \textsuperscript{65}Ni\textsuperscript{2+} in the presence of functional alleles of mgtA and mgtB.

**Tissue culture.** Eukaryotic cell lines were grown at 37 °C in a 10% (v/v) CO\textsubscript{2} atmosphere in 25 or 75 cm\textsuperscript{2} plastic tissue culture flasks and passed every 3 d using Dulbecco’s Modified Essential Medium (DMEM) containing penicillin (50 units ml\textsuperscript{-1}), streptomycin (50 μg ml\textsuperscript{-1}), 10% (v/v) heat-inactivated foetal bovine serum, 50 μM 2-mercaptoethanol, 10 mM HEPES, pH 7.4, 0.25 mM (v/v) non-essential amino acids (Gibco-BRL) and 1 mM sodium pyruvate.

For measurement of *S. typhimurium* invasion, cells were passaged in the same medium into 24-well plates at a moderately high density and grown for 24 or 48 h just to confluence. The cell culture medium was changed 24 h in advance with penicillin and streptomycin omitted. Cells were prepared by aspirating medium, washing the plates once in DMEM without serum or antibiotics and adding 0.1 ml of bacterial cell suspension in DMEM without serum or antibiotics to each well. Bacterial cell densities were routinely about 1 × 10\textsuperscript{8} ml\textsuperscript{-1}. The plates were centrifuged at 800 r.p.m. for 10 min at room temperature and incubated for 50 additional min at 32 °C in a 5% CO\textsubscript{2} atmosphere. The medium was removed, 0.5 ml PBS (Gibco-BRL) was added to wash the cells and then aspirated. The wash was repeated three times before addition of 1 ml DMEM containing 0.1 mg gentamicin ml\textsuperscript{-1}. Cells were then incubated for 2 h at 32 °C in 5% CO\textsubscript{2}. The medium was removed, the cells washed three times in PBS as above, 1 ml DMEM was added and the plate was returned to the incubator for the indicated time. To lyse the eukaryotic cells at any time point and obtain bacteria for plating, plates were washed three times in PBS, 0.2 ml 1% (v/v) Triton X-100 in PBS was added and after 5 min at 32 °C 0.8 ml PBS was added with vigorous pipetting to lyse the cells. The entire mixture was then diluted with 9 ml PBS. To determine the number of bacteria present, serial dilutions of this final solution were plated in triplicate on LB containing appropriate antibiotics and viable colonies were counted after 24–36 h incubation at 37 °C. To measure luciferase activity from bacteria after invasion of eukaryotic cells, 0.5 ml of the final 1:0 lysate was added to 1 ml luciferase assay buffer in a 4 ml scintillation vial. After capping and vortexing the vial for 5 s, light emission was determined as described previously (Snavely et al., 1991a, b; Tao et al., 1995, 1998). Activity was normalized to bacterial cell number.

**RESULTS**

**Effect of pH on CorA-mediated transport**

Although MgtA and MgtCB expression and transport capacity are greatly increased upon exposure to limiting extracellular Mg\textsuperscript{2+} concentration (Snavely et al., 1991a, b; Tao et al., 1995, 1998), their increased transport capacity is still similar in magnitude to the capacity of the constitutively expressed CorA. Thus, if MgtA and/or MgtCB were to become the dominant Mg\textsuperscript{2+} influx systems for the cell, one hypothesis would be that, in some environments, CorA becomes less functional. For example, within the acidic environment of the eukaryotic cell vacuole, CorA might not function adequately. Therefore, we investigated the ability of CorA to transport \textsuperscript{65}Ni\textsuperscript{2+}, used as a surrogate for the unavailable \textsuperscript{65}Mg\textsuperscript{2+}, as a function of extracellular pH (Fig. 1). At pH 5.2, the total amount of uptake via CorA increased significantly after an acute pH shift. In addition the affinity for Mg\textsuperscript{2+} decreased by a factor of about 3 as shown by the shift to the right in the Mg\textsuperscript{2+} inhibition curve (Fig. 1). Cells adapted to an acid environment by overnight growth at pH 5.2 showed a decrease in uptake capacity compared to cells acutely

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**Table 1. Bacterial strains used**

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Genotype</th>
<th>Source*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MM731</td>
<td>ΔcorA501</td>
<td>This study</td>
</tr>
<tr>
<td>MM1265</td>
<td>mgtC9232::MudJΔcorA501</td>
<td>This study</td>
</tr>
<tr>
<td>MM1266</td>
<td>mgtC9232::MudJ</td>
<td>E. Groisman (EG9232)</td>
</tr>
<tr>
<td>MM1268</td>
<td>mgtA9226::MudA</td>
<td>E. Groisman (EG9226)</td>
</tr>
<tr>
<td>MM1269</td>
<td>14028s wild-type (pathogenic)</td>
<td>ATCC</td>
</tr>
<tr>
<td>MM1274</td>
<td>mgtA9226::MudAΔcorA501</td>
<td>This study</td>
</tr>
<tr>
<td>MM1314</td>
<td>mgtC9232::MudJmgtA9226::MudA</td>
<td>This study</td>
</tr>
<tr>
<td>MM1364</td>
<td>invA::Tn10phoA</td>
<td>R. Maurer (RM4456)</td>
</tr>
<tr>
<td>MM1512</td>
<td>MM1269/pTT-ALux</td>
<td>This study</td>
</tr>
<tr>
<td>MM1520</td>
<td>MM1269/pTT-CLux</td>
<td>This study</td>
</tr>
</tbody>
</table>

* ATCC, American Type Culture Collection, Rockville, MD, USA; E. Groisman, Washington University School of Medicine, St Louis, MO, USA; R. Maurer, Case Western Reserve University, Cleveland, OH, USA.
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Effect of CorA on induction of MgtA and MgtCB

Even though the CorA transporter is functional at acid pH, the induction of MgtA and/or MgtCB expression during invasion and exposure to acid might still be required to supply sufficient Mg^{2+}. If so, the degree of their induction might be affected by the presence or absence of CorA itself. However, induction of transcription of mgtA or mgtCB at low Mg^{2+} concentration measured using lacZ fusions was significantly greater in the presence of a wild-type corA allele than in its absence (Table 2). Similar data were obtained with the corresponding luxAB fusions (data not shown).

Effect of acid pH on MgtA and MgtCB expression

Upon invasion, S. typhimurium is exposed to acid pH in the vacuole within epithelial and macrophage cells, although in the epithelial cell this low pH may be modulated upwards with time (Alpulche-Aranda et al., 1992; Garcia-del Portillo et al., 1992; Rathman et al., 1996). This acid exposure also alters gene expression extensively (Bearson et al., 1997; Foster & Spector, 1995; Riesenberg-Wilmes et al., 1996). We therefore tested the effect of acute and chronic acid exposure on the ability of decreased Mg^{2+} to induce mgtA and mgtCB expression using lacZ fusions to monitor transcription (Snively et al., 1991a, b). Similar data (not shown) were also obtained with luxAB fusions carried on plasmids (Tao et al., 1995). Such transcriptional data probably reflect actual protein production since we have previously shown that the large increase in mgtCB transcription at low Mg^{2+} concentrations results in a correspondingly large increase in functional MgtB transport protein.

When corA^{+} cells grown at pH 7.4 were acutely shifted to both acid (pH 5.2) and no added extracellular Mg^{2+},

may therefore be concluded that CorA is functional at acid pH.

Table 2. Effect of CorA on transcription of MgtA and MgtCB

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Mg^{2+} (mM)</th>
<th>β-Galactosidase activity (Miller units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MM1268 or MM1266</td>
<td>corA^{+}</td>
<td>10</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>MM1274</td>
<td>mgtA::lacZ corA^{-}</td>
<td>0</td>
<td>460 ± 25</td>
</tr>
<tr>
<td>MM1268</td>
<td>mgtA::lacZ corA^{+}</td>
<td>0</td>
<td>1235 ± 80</td>
</tr>
<tr>
<td>MM1265</td>
<td>mgtB::lacZ corA^{-}</td>
<td>0</td>
<td>170 ± 110</td>
</tr>
<tr>
<td>MM1266</td>
<td>mgtB::lacZ corA^{+}</td>
<td>0</td>
<td>1450 ± 210</td>
</tr>
</tbody>
</table>

exposed to pH 5.2 and were more similar in CorA uptake to cells grown at pH 7.4 (Fig. 1). Chronic acid exposure did not alter the slightly decreased cation concentration dependence compared to acute acid exposure. Because the apparent affinity for cation is slightly decreased, the change in capacity upon shift to pH 5.2 is actually even greater than is apparent. Calculation of the change in apparent V_{max} ranged from a five- to tenfold increase in various experiments (data not shown). It
Mg\textsuperscript{2+} and acid regulation in S. typhimurium

__Fig. 2. Effect of acute pH 5-2 exposure on induction of mgtA (○, □) and mgtCB (●, ■). Strains MM1266 and MM1268 (both corA\textsuperscript{+}) were grown in N-minimal medium made with MES buffer at pH 7-4 containing 1 mM Mg\textsuperscript{2+} and washed in Mg\textsuperscript{2+}-free medium as described in the legend to Table 2. Aliquots of cells were then acutely resuspended at pH 5-2 (□, ○) or 7-4 (●, ■) in pre-warmed N-minimal medium containing no added Mg\textsuperscript{2+}, incubated for the indicated times and aliquots for β-galactosidase activity taken and frozen for later assay. The results shown are from a single experiment repeated twice with similar results. In addition, similar effects were seen (data not shown) when induction was monitored using plasmid-borne luxAB fusions to mgtA and mgtCB (Tao et al., 1995, 1998).__

**Fig. 3. Effect of prior growth at pH 5-2 on induction of mgtA (○, □) and mgtCB (●, ■). The experiments were conducted identically to those shown in Fig. 2 except that cells were grown overnight at pH 5-2 in the absence of Mg\textsuperscript{2+}. ○, □, acute resuspension at pH 5-2; ■, ■, acute resuspension at pH 7-4. The data presented are from a single experiment repeated twice with similar results.**

mgtCB induction after 2–4 h incubation occurred to about 50% of the level seen without a pH change (Fig. 2). Thus, after an acid shift, a decrease in extracellular Mg\textsuperscript{2+} still elicited a large response in mgtCB transcription. In contrast, an acute shift to acid pH abolished the mgtA transcriptional response at low extracellular Mg\textsuperscript{2+} concentration (Fig. 2). This loss of transcriptional response was evident for at least 8 h after the shift to pH 5-2 (data not shown) and thus did not appear to be simply a lag phenomenon.

When S. typhimurium was grown in 1 or 10 mM Mg\textsuperscript{2+} at acid pH overnight, subsequent acute removal of Mg\textsuperscript{2+} from the medium elicited a large increase in transcription of either system (Fig. 3). Thus, as opposed to the response after acute acid exposure, chronic exposure to acid did not appear to alter the Mg\textsuperscript{2+}-dependent transcriptional response of either locus significantly. Interestingly, the acute reverse pH shift, from pH 5-2 to 7-4, diminished the response of both loci by a factor of about 3, although induction was still over 100-fold (Fig. 3). Thus, S. typhimurium can respond and adapt to chronic acid exposure, consistent with the data of Foster and colleagues (Bearson et al., 1997; Garcia-del Portillo et al., 1992; Foster & Spector, 1995; Riesenberg-Wilmes et al., 1996).

In cells grown overnight at pH 5-2, the absence of a functional corA allele had a marked effect of transcription of these loci. The transcriptional response to removal of extracellular Mg\textsuperscript{2+} was significantly diminished for mgtA and essentially abolished for mgtCB (compare Figs 4 and 3). Acutely shifting from pH 5-2 back to 7-4, concomitant with removal of extracellular Mg\textsuperscript{2+}, further decreased the transcriptional response of mgtA (Fig. 4) as was seen with mgtA in the presence of a functional corA allele (Fig. 3). Overall these data suggest that there is an acid component of the response of these two loci at low extracellular Mg\textsuperscript{2+} concentrations and that a functional corA allele is required for their optimal response to Mg\textsuperscript{2+}.

We also investigated the effect of acid pH on the ability of Mg\textsuperscript{2+} to repress transcription of mgtA and mgtCB. In these experiments, cells were induced by growth in medium at pH 5-2 or 7-4 containing no added Mg\textsuperscript{2+} before acutely adding 10 mM Mg\textsuperscript{2+} to repress tran-
transcription. There was no major difference in the ability of Mg\textsuperscript{2+} to repress transcription of either mgtCB or mgtA after either acute shift to acid or chronic growth in acid (data not shown).

**Effect of mutations in mgtA and mgtCB on invasion**

We have previously shown that mgtCB is induced upon *S. typhimurium* invasion of MDCK epithelial cells (Garcia-del Portillo et al., 1992). Before determining if mutations in either locus had any effect on the ability of *S. typhimurium* to invade epithelial and macrophage cells, we determined whether mgtA is induced after invasion. Two different model epithelial cell lines were used, CMT-93 and Hep-2 cells, as well as a macrophage-like cell line, J774. All three lines have been used extensively as models for bacterial pathogenesis (Harding & Pfeifer, 1994; Hensel et al., 1997; Rhen et al., 1993; Wick et al., 1995). Both mgtA and mgtCB were induced upon invasion of all three cell lines. Representative data during invasion of J774 macrophage-like cells are shown in Fig. 5. While the increase in transcription of mgtCB is clearly greater than that of mgtA, there is at least a 100-fold induction of mgtA within the first hours after invasion. The induction of mgtA and mgtCB after invasion is similar in both time course and magnitude to the first phase of the two phases of induction elicited by a decrease in extracellular Mg\textsuperscript{2+} in *S. typhimurium* in broth culture (Tao et al., 1998).

Fig. 5. Induction of MgtA (○) and MgtCB (■) expression during invasion of J774 cells. Strains MM1512 and MM1520 were grown and invasion of J774 cells was performed as described in Methods. Luciferase activity was measured as described in Methods and normalized for bacterial cell number. Control assays showed no luciferase activity either in J774 cells or J774 cells containing bacterial cells of strain MM1269, which does not carry the luxAB genes. The data are from a single experiment in triplicate. Maximal luciferase activity before normalization for cell number was about 23000 c.p.m. after 5 h compared to a zero time activity of 150 c.p.m. Activity is normalized to the actual number of bacterial cells remaining within the eukaryotic cell at each time point. This ranged from about 6 × 10\textsuperscript{7} ml\textsuperscript{-1} at zero time to as low as 0.7 × 10\textsuperscript{6} ml\textsuperscript{-1} at 5 h in the experiment shown. One additional experiment assayed at slightly different time points gave similar results.

Subsequently, we determined the effect of mutations in either or both mgtA and mgtCB loci on the ability of *S. typhimurium* to invade these eukaryotic cell lines. In a *S. typhimurium* strain carrying mutations in both mgtA and mgtCB, making it dependent on the CorA Mg\textsuperscript{2+} transport system for Mg\textsuperscript{2+} uptake, there is no obvious defect in the ability to invade either CMT-93 (Fig. 6a) or Hep-2 cells (Fig. 6b). Similar data (not shown) were obtained for invasion of J774 cells by the *mgtA mgtCB* double mutant. The time course of invasion also indicated that short-term survival up to 5 h under these assay conditions is also not altered by mutations in mgtA and mgtCB. Invasion of a strain carrying an *invA* mutation is shown as a control. Additional experiments indicated that strains carrying single mutations in either mgtA or mgtCB likewise had no apparent defect in invasion of epithelial or macrophage model cell lines (data not shown). We conclude that, while mgtA and mgtCB transcription is markedly induced during invasion of a eukaryotic cell, such induction is not required for the initial process of invasion.

Fig. 6. Effect of mgtA and mgtCB mutations on invasion. Strains were grown overnight and allowed to infect the cell lines as described in Methods. Bacteria were added to cultures at zero time and the cultures washed at 1 h with the addition of gentamicin. ○, MM1269 (wild-type 14028s strain of *S. typhimurium*); ■, MM1364 (invA::Tn10phoA) used as a control for invasion deficiency; △, MM1314 (mgtA mgtCB). (a) CMT-93 cells and (b) Hep-2 cells. The data for each cell line are from a single experiment in triplicate repeated once. Additional experiments under slightly differing conditions gave comparable results.
DISCUSSION

Mg\(^{2+}\) signalling

Mg\(^{2+}\) regulation of \(mgtA\) and \(mgtCB\) is mediated by the PhoPQ two-component signal transduction system. Mg\(^{2+}\) binding to the PhoQ membrane sensor mediates regulation of multiple genes (Garcia-Vescovi et al., 1996; Soncini & Groisman, 1996; Soncini et al., 1996; Vescovi et al., 1997), including \(mgtA\) and \(mgtCB\) (Soncini et al., 1996, Tao et al., 1998). In normal extracellular Mg\(^{2+}\) concentrations, Mg\(^{2+}\) binds to PhoQ and represses transcription of genes regulated by PhoP. When extracellular Mg\(^{2+}\) concentration falls, such as after entry of \(S.\ typhimurium\) into a eukaryotic cell, transcription is no longer repressed and MgtA and MgtCB and many other proteins are selectively expressed. Since MgtA and MgtB function as Mg\(^{2+}\) influx systems, it is tempting to suggest that their regulation by Mg\(^{2+}\) and PhoPQ serves the purpose of supplying Mg\(^{2+}\) under growth conditions where environmental levels of Mg\(^{2+}\) are low. This hypothesis has several requirements. First, it implies that the constitutive Mg\(^{2+}\) influx system, CorA, cannot function under such conditions; however, since the Mg\(^{2+}\) affinities for CorA, MgtA and MgtB are all similar at approximately 15 \(\mu\)M (Hmiel et al., 1989), MgtA and MgtB are not acting as scavenger systems at low extracellular Mg\(^{2+}\).

Second, MgtA and/or MgtB might be required under some other environmental condition where CorA might not be functional, e.g. in an acid environment such as \(S.\ typhimurium\) encounters after invasion of a eukaryotic cell. At pH 5.2, however, CorA remains a very active, functional Mg\(^{2+}\) influx system (Fig. 1). The capacity of CorA is quite large, at least as large as that of the induced MgtA or MgtB transporters; moreover, induction of the MgtA and MgtB systems is less in the presence of a corA mutation than in the presence of a functional allele. Indeed after adaptation to acid pH, a functional corA allele seems to be required for any substantial response of these two loci to low extracellular Mg\(^{2+}\) concentrations (Fig. 4). If CorA-mediated transport was not sufficient for Mg\(^{2+}\) uptake, the opposite result would be expected. These conclusions are also consistent with the observation (Fig. 6) that initial survival of \(S.\ typhimurium\) during invasion of epithelial-like or macrophage-like cell lines does not depend on functional alleles of either \(mgtA\) or \(mgtCB\). While these arguments are subject to the caveat that the ability of CorA to mediate Mg\(^{2+}\) uptake within eukaryotic cells cannot be directly tested, the overall data indicate that Mg\(^{2+}\) induction of MgtA and/or MgtB expression is not (solely) for the purpose of increasing Mg\(^{2+}\) uptake capacity.

Role of PhoPQ

Discussion of the roles of these proteins in \(S.\ typhimurium\) or during eukaryotic cell invasion is complicated by the interacting regulatory factors for these genes. The PhoPQ regulatory system mediates much of the transcriptional regulation of \(mgtA\) and \(mgtCB\) (Garcia-Vescovi et al., 1996; Soncini et al., 1996). However, \(mgtA\) is not totally PhoPQ-dependent (Tao et al., 1998); Foster and colleagues have shown that under acid culture conditions the phoPQ regulation of \(mgtCB\) may be mediated by H\(^{+}\) rather than Mg\(^{2+}\) (Bearson et al., 1998). Our data are consistent with these observations and indicate that regulation of \(mgtA\) and \(mgtCB\) is sensitive to both Mg\(^{2+}\) and acid during both the induction and repression phases of response.

In the context of PhoPQ regulation of these loci, it is relevant to ask to what extent transcriptional regulation of \(mgtA\) and \(mgtCB\) within the eukaryotic cell is mimicked by direct exposure of bacteria to acid and low Mg\(^{2+}\) concentrations. Comparison of the induction of \(mgtCB\) transcription during invasion with the ability of acid and decreased extracellular Mg\(^{2+}\) suggests an excellent correlation. In contrast, comparison of the induction of \(mgtA\) in these two circumstances (Fig. 5 versus Fig. 2) indicates that its regulation is more complex. After invasion, \(S.\ typhimurium\) is contained within a vacuole that is at least initially acid and low in Mg\(^{2+}\), iron and probably other nutrients (Alpulche-Aranda et al., 1992; Garcia-del Portillo et al., 1992; Foster & Spector, 1995; Rathman et al., 1996). However, at least in the macrophage, this acid environment may become more nearly neutral with time (Alpulche-Aranda et al., 1992). The ability of \(mgtA\) to be induced after eukaryotic cell invasion does not necessarily bring into question the role of acid or Mg\(^{2+}\) in regulation of this or other loci, but presumably reflects some additional and currently unknown regulatory parameter(s) functional within the eukaryotic cell. The lack of perfect correlation between regulation within the eukaryote and ‘free-living’ bacteria serves as a reminder that such regulatory phenomena should be measured in as many contexts as possible. Nevertheless, both acid and Mg\(^{2+}\) appear to have a role in regulation of these loci after invasion of eukaryotic cells.

Role of MgtA, MgtB and MgtC

Blanc-Potard & Groisman (1997) have shown that a functional allele of \(mgtC\) is essential for long-term (18 h) survival of \(S.\ typhimurium\) within the macrophage-like cell line J774 and that the virulence of \(S.\ typhimurium\) carrying an inserional mutation in \(mgtC\) is markedly attenuated. A functional \(mgtB\) allele is not required for virulence or long-term survival within the macrophage, but a mutation in \(mgtB\) elicits a small decrease in survival within the eukaryotic cell. Our data add to these findings. First, transcription of \(mgtCB\) is increased in the macrophage-like J774 cell line (Fig. 5). Likewise, transcription of \(mgtCB\) is greatly increased in two different epithelial-like cell lines, CMT-93 and Hep-2 (data not shown). These data complement our previous demonstration that \(mgtB\) is induced after invasion of the epithelial-like MDCK cell line (Garcia-del Portillo et al., 1992). Second, transcription of the sibling \(mgtA\) is also increased after invasion of eukaryotic cells (Fig. 5), albeit to a lesser extent than for \(mgtCB\). Third,
mutations in mgtA and/or mgtCB have no effect on S. typhimurium invasion of CMT-93 or Hep-2 cells (Fig. 6) or J774 cells (data not shown). Finally, the ability to survive within the eukaryotic cell over short periods of time, up to 5 h, is not markedly affected according to our assays (Fig. 6). Thus, although mgtA, mgtB and mgtC are markedly induced upon invasion of a eukaryotic cell, none are necessary either for invasion or for short-term survival within the eukaryotic host. This implies that the absolute requirement for mgtC and the partial requirement for mgtB for long-term survival within the macrophage is a late effect during the invasion process. Blanc-Potard & Groisman (1997) have suggested that the mechanism of this requirement is that mgtC encodes a Mg\(^{2+}\) transporter functional during invasion, in part because a very high extracellular Mg\(^{2+}\) concentration (25 mM) added to the eukaryotic cell growth medium partially restores survival of an mgtC strain within the macrophage. However, our data indicate that S. typhimurium does not require additional Mg\(^{2+}\) transport capacity during invasion since during invasion the CorA transport system is apparently functional and additional transport capacity is induced in the form of MgtA and MgtB. Preliminary transport data further indicate that, using \(^{65}\)Ni\(^{2+}\) or \(^{57}\)Co\(^{2+}\) as alternative substrates for the unavailable \(^{56}\)Mg\(^{2+}\), no uptake can be detected in cells expressing high levels of MgtC (M. B. C. Moncrief & M. E. Maguire, unpublished data).

These data raise several questions as to the role(s) of mgtA, mgtB and mgtC. With regard to mgtA, the presence of a functional mgtA allele has no significant effect on short-term survival within eukaryotic cells (Blanc-Potard & Groisman, 1997). mgtA appears widely spread within enteric bacteria (Blanc-Potard & Groisman, 1997). This suggests that it performs a normal metabolic function, unassociated with virulence. Nevertheless, it would be premature to assume that this metabolic function is solely involved with Mg\(^{2+}\) uptake. The CorA transporter is functional under a variety of conditions and has high affinity and high capacity. There is little suggestion in these parameters that the cell requires additional uptake capacity for Mg\(^{2+}\). Thus, a functional MgtA transporter could be required under some currently unknown environmental condition and/or MgtA has an additional as yet undiscovered function besides transport of Mg\(^{2+}\).

The same arguments can be made for MgtB function as were made above for MgtA function. However, mgtB is not as widely spread as mgtA, being found only in S. typhimurium, Yersinia enterocolitica and Enterobacter aerogenes among the 10 species of enteric bacteria surveyed by Blanc-Potard & Groisman (1997). Moreover, in S. typhimurium the mgtCB locus appears on an apparent chromosomal insertion near selC, forming part of Salmonella pathogenicity island 3 (SPI-3) (Blanc-Potard & Groisman, 1997; Finlay & Cossart, 1997). Strains deficient in mgtB are partially impaired for long-term intra-macrophage survival and virulence. The cation profile for inhibition of Mg\(^{2+}\) uptake is quite different between the MgtA and MgtB transporters. Ca\(^{2+}\) is a good inhibitor of MgtA transport but without effect on MgtB and there are lesser differences with other cations (Snively et al., 1989). Finally, the data presented herein indicate that MgtB is neither induced nor required during invasion for the purpose of supplying Mg\(^{2+}\) to the bacterium. This strongly implies that MgtB has an additional function besides transporting Mg\(^{2+}\).

MgtC is more problematic. It is required for virulence. The mechanism proposed is that it is an Mg\(^{2+}\) transporter necessary to obtain Mg\(^{2+}\) within the eukaryotic cell (Blanc-Potard & Groisman, 1997). MgtC has no close homologues in the current protein database using BLAST (Altschul et al., 1990) analysis and a small number of more distant homologues give little clue as to function (data not shown). The suggestion that it transports Mg\(^{2+}\) is certainly possible (Blanc-Potard & Groisman, 1997), but the data presented herein indicate that this is not a necessary function of MgtC. While it is possible, even likely, that MgtC is a transporter of some type, either in S. typhimurium or after export to the eukaryotic cell membrane, its substrate is currently unknown. Thus, while the Mg\(^{2+}\) cation and various Mg\(^{2+}\) transport systems are important for virulence, their actual function in either the free-living bacterium or after invasion of a eukaryotic cell remains unsolved.

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