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 Mg²⁺ transport systems, MgtA and MgtB, the latter encoded by a two-gene operon, *mgtCB*. Mg²⁺ 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 MgtB is increased upon invasion of eukaryotic cells because of a lack of intravacuolar Mg²⁺. However, when *S. typhimurium* was grown at pH 5.2, the capacity of the constitutive CorA transporter in mediating Mg²⁺ 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 Mg²⁺ 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 Mg²⁺ 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 Mg²⁺ 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 Mg²⁺ 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 Mg²⁺-dependent induction of *mgtA* and *mgtCB* upon invasion of eukaryotic cells is not required because *S. typhimurium* lacks sufficient Mg²⁺ 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 Mg²⁺ transport systems, the constitutive CorA transporter and two

P-type ATPase transport systems for Mg²⁺, 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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operon, *mgtCB*. MgtC is a 22.5 kDa membrane protein of unknown function and its absence has no effect on the transport properties of MgtB (Snaveley *et al.*, 1991a, b; 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 Mg^{2+} (Snaveley *et al.*, 1989, 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 (Groisman *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 Mg^{2+} 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 Mg^{2+} 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 μ M (Altendorf & Epstein, 1994; Dosch *et al.*, 1991; Epstein *et al.*, 1993). The Mg^{2+} transporters of *S. typhimurium* (CorA, MgtA and MgtB) and *E. coli* (CorA and MgtA) do not exhibit these properties, however. The CorA Mg^{2+} transporter has a K_a for uptake of Mg^{2+} of 15–20 μ M whereas MgtA and MgtB have apparent K_a values of 5–30 μ M. CorA also has a high capacity of 0.5–1 nmol min⁻¹ per 10⁸ cells (Hmiel *et al.*, 1986; Snaveley *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 Mg^{2+} and which, at least initially, has an acid pH (Alpulche-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 (Snaveley *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 Mg^{2+} 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 Co^{2+} 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; Snaveley *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 ⁶³Ni²⁺ uptake assays, nitrogen minimal medium (N-minimal medium) was routinely supplemented with 0.1% Casamino acids, 0.4% glucose, 1 μ g FeSO₄ ml⁻¹ and the indicated concentration of Mg^{2+} (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 Mg^{2+} 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 (Snaveley *et al.*, 1991b; Tao *et al.*, 1995, 1998). We have previously determined that the contaminant Mg^{2+} concentration inherent in N-minimal medium as measured by atomic absorption spectrometry is approximately 15 μ M (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 μ M (Snaveley *et al.*, 1991b). There is no significant alteration in *CorA* transcription by the concentration of extracellular Mg^{2+} (Snaveley *et al.*, 1991b; Tao *et al.*, 1998).

⁶³Ni²⁺ 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 Mg^{2+} 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

Table 1. Bacterial strains used

Strain no.	Genotype	Source*
MM731	$\Delta corA501$	This study
MM1265	<i>mgtC9232::MudJ</i> $\Delta corA501$	This study
MM1266	<i>mgtC9232::MudJ</i>	E. Groisman (EG9232)
MM1268	<i>mgtA9226::MudA</i>	E. Groisman (EG9226)
MM1269	14028s wild-type (pathogenic)	ATCC
MM1274	<i>mgtA9226::MudA</i> $\Delta corA501$	This study
MM1314	<i>mgtC9232::MudJ</i> <i>mgtA9226::MudA</i>	This study
MM1364	<i>invA::Tn10phoA</i>	R. Maurer (RM4456)
MM1512	MM1269/pTT-ALux	This study
MM1520	MM1269/pTT-CLux	This study

* 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.

and washed twice in the same volume of N-minimal medium containing no added Mg²⁺. Strains grown in 10 mM Mg²⁺ were washed three times. New subcultures were then started from the washed cells by resuspension in N-minimal medium containing the indicated Mg²⁺ concentration to a final OD₆₀₀ of 0.1. After incubation as indicated for each experiment, cells were collected and washed three times in N-minimal medium without added Mg²⁺ before resuspension in the same medium at a final OD₆₀₀ of 1.0–2.0 for use in the transport assay. Transport was performed as described previously (Snively *et al.*, 1989; Grubbs *et al.*, 1989). Uptake of ⁶³Ni²⁺ was measured for 20 min. Transport was normalized to OD₆₀₀ for each strain. Induction of detectable Mg²⁺ uptake via MgtA or MgtB (when present) requires at least 60 min incubation at 37 °C in the absence of Mg²⁺. This allows measurement of CorA-mediated transport using ⁶³Ni²⁺ 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) CO₂ atmosphere in 25 or 75 cm² plastic tissue culture flasks and passaged every 3 d using Dulbecco's Modified Essential Medium (DMEM) containing penicillin (50 units ml⁻¹), streptomycin (50 µg ml⁻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 confluency. 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 bacterial cell suspension in DMEM without serum or antibiotics to each well. Bacterial cell densities were routinely about 1 × 10⁸ ml⁻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% CO₂ 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⁻¹. Cells were then incubated for 2 h at 32 °C in 5% CO₂. 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 ml 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 (Snively *et al.*, 1991b; Tao *et al.*, 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 Mg²⁺ concentration (Snively *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 Mg²⁺ influx systems for the cell, one hypothesis would be that, in some environments, CorA becomes less functional. For example, within the acid environment of the eukaryotic cell vacuole, CorA might not function adequately. Therefore, we investigated the ability of CorA to transport ⁶³Ni²⁺, used as a surrogate for the unavailable ²⁸Mg²⁺, as a function of extracellular pH (Fig. 1). At pH 5.2, the total amount of uptake via CorA increased substantially after an acute pH shift. In addition the affinity for Mg²⁺ decreased by a factor of about 3 as shown by the shift to the right in the Mg²⁺ 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

Fig. 1. Effect of acid pH on CorA cation transport. Strain MM1269 was grown overnight at either pH 7.4 or 5.2 in supplemented N-minimal medium containing 1 mM Mg^{2+} to suppress expression of the MgtA and MgtB transport systems. Cells were washed twice in N-minimal medium containing no added Mg^{2+} at the pH they had been grown in. The final pellet was resuspended in N-minimal medium with no added Mg^{2+} at the indicated assay pH at an OD_{600} of 1.5–2.0 and kept on ice for up to 30 min until uptake was measured using $^{63}Ni^{2+}$ as described in Methods. ●, Cells grown at pH 7.4 and assayed after acute resuspension at pH 5.2; ▲, cells grown at pH 7.4 and assayed at pH 7.4. $^{63}Ni^{2+}$ uptake was measured after a 20 min incubation. Results shown are from a single experiment with duplicate points at each concentration. A second experiment gave similar results.

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

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

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; Riesenber-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+} ,

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

The indicated strains were grown overnight in N-minimal medium containing 1 mM Mg^{2+} at pH 7.4 and then washed twice in N-minimal medium without added Mg^{2+} . The washed cells were resuspended in N-minimal medium containing no added Mg^{2+} at their original pH at an OD_{600} of 0.1 and incubated for 4 h at 37 °C before measurement of β -galactosidase activity resulting from the *lacZ* fusions in *mgtA* or *mgtCB*. The results shown are the mean and range of two experiments. Additional similar experiments at other time points and after growth overnight in 10 mM Mg^{2+} gave similar results.

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

Fig. 2. Effect of acute pH 5.2 exposure on induction of *mgtA* (○, □) and *mgtCB* (●, ■). Strains MM1266 and MM1268 (both *corA*⁺) were grown in N-minimal medium made with MES buffer at pH 7.4 containing 1 mM Mg²⁺ and washed in Mg²⁺-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 Mg²⁺, 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 Mg²⁺. ●, ○, 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 Mg²⁺ still elicited a large response in *mgtCB* transcription. In contrast, an acute shift to acid pH abolished the *mgtA* transcriptional response at low extracellular Mg²⁺ concentration (Fig. 2). This loss of transcriptional

Fig. 4. Effect of prior growth at pH 5.2 on induction of *mgtA* (○, □) and *mgtCB* (●, ■) in a *corA* background. The experiments were conducted identically to those shown in Fig. 3 except that strains MM1274 and MM1265 (both *corA*⁺) were used. ●, ○, acute resuspension at pH 5.2; ■, □, acute resuspension at pH 7.4. The data presented are from a single experiment repeated once with similar results.

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 Mg²⁺ at acid pH overnight, subsequent acute removal of Mg²⁺ 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 Mg²⁺-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; Riesenbergs-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 Mg²⁺ 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 Mg²⁺, 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 Mg²⁺ concentrations and that a functional *corA* allele is required for their optimal response to Mg²⁺.

We also investigated the effect of acid pH on the ability of Mg²⁺ 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 Mg²⁺ before acutely adding 10 mM Mg²⁺ to repress tran-

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 23 000 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 6×10^7 ml⁻¹ at zero time to as low as 0.7×10^6 ml⁻¹ at 5 h in the experiment shown. One additional experiment assayed at slightly different time points gave similar results.

scription. There was no major difference in the ability of Mg²⁺ 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 Mg²⁺ in *S. typhimurium* in broth culture (Tao *et al.*, 1998).

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*::Tn10*phoA*) 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.

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 Mg²⁺ transport system for Mg²⁺ 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.

DISCUSSION

Mg²⁺ signalling

Mg²⁺ regulation of *mgtA* and *mgtCB* is mediated by the PhoPQ two-component signal transduction system. Mg²⁺ 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 Mg²⁺ concentrations, Mg²⁺ binds to PhoQ and represses transcription of genes regulated by PhoP. When extracellular Mg²⁺ 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 Mg²⁺ influx systems, it is tempting to suggest that their regulation by Mg²⁺ and PhoPQ serves the purpose of supplying Mg²⁺ under growth conditions where environmental levels of Mg²⁺ are low. This hypothesis has several requirements. First, it implies that the constitutive Mg²⁺ influx system, CorA, cannot function under such conditions; however, since the Mg²⁺ affinities for CorA, MgtA and MgtB are all similar at approximately 15 µM (Hmiel *et al.*, 1989), MgtA and MgtB are not acting as scavenger systems at low extracellular Mg²⁺. 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 Mg²⁺ 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 Mg²⁺ concentrations (Fig. 4). If CorA-mediated transport was not sufficient for Mg²⁺ 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 Mg²⁺ uptake within eukaryotic cells cannot be directly tested, the overall data indicate that Mg²⁺ induction of MgtA and/or MgtB expression is not (solely) for the purpose of increasing Mg²⁺ 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 Mg²⁺ (Bearson *et al.*, 1998). Our data are consistent with these observations and indicate that regulation of *mgtA* and *mgtCB* is sensitive to both Mg²⁺ 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 Mg²⁺ concentrations. Comparison of the induction of *mgtCB* transcription during invasion with the ability of acid and decreased extracellular Mg²⁺ 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 Mg²⁺, 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 Mg²⁺ 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 Mg²⁺ 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 insertional 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 $^{63}Ni^{2+}$ or $^{57}Co^{2+}$ as alternative substrates for the unavailable $^{28}Mg^{2+}$, 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- (this report) or long-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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