The importance of the magnesium transporter MgtB for virulence of *Yersinia pseudotuberculosis* and *Yersinia pestis*

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Mg$^{2+}$ has been shown to be an important signal controlling gene regulation via the PhoPQ two-component regulatory system for a range of Gram-negative bacteria, including *Yersinia pestis* and *Yersinia pseudotuberculosis*. The magnesium ion transporter MgtB is part of the complex PhoPQ regulon, being upregulated in response to low Mg$^{2+}$. Despite the presence of other Mg$^{2+}$ transport systems in *Yersinia*, inactivation of mgtB had a significant effect on the ability of the bacteria to scavenge this crucial ion. Whereas inactivation of PhoPQ is reported to adversely affect intracellular survival, we show that *Y. pestis* and *Y. pseudotuberculosis* ΔmgtB mutants survived equally as well as the respective parent strain within macrophages, although they were more sensitive to killing in the *Galleria* model of infection. Surprisingly, despite MgtB being only one member of the Mg$^{2+}$ stimulon and PhoPQ controlling the expression levels of a range of genes including mgtB, the *Yersinia* ΔmgtB mutants were more highly attenuated than the equivalent *Yersinia* ΔphoP mutants in mouse models of infection. MgtB may be a suitable target for development of novel antimicrobials, and investigation of its role may help elucidate the contribution of this component of the PhoPQ regulon to pathogenesis.

INTRODUCTION

There are three human pathogenic species in the genus *Yersinia*. Two of these, *Yersinia pseudotuberculosis* and *Yersinia enterocolitica*, are gastrointestinal pathogens, while the third, *Yersinia pestis*, is the aetiological agent of plague. Plague can manifest in three forms: bubonic, pneumonic and septicaemic (Perry & Fetherston, 1997). Bubonic and septicaemic plagues arise following the bite of an infected flea, whereas primary pneumonic plague arises from inhalation of infectious aerosols. Secondary pneumonia can arise from bubonic and septicaemic plague and all pneumonic cases pose a serious risk to close contacts, and thus plague patients must be quarantined to contain outbreaks (Dennis et al., 1999). Antibiotic therapy and prophylaxis are effective, but treatment must be initiated early in infection to be effective. Without antibiotics the mortality rate for bubonic plague is approximately 50%, and pneumonic and septicaemic plagues are almost always fatal. Due to the serious nature of the disease, the potential for human-to-human spread and the high mortality rate, plague is a notifiable disease. For similar reasons, *Y. pestis* has also been regarded as an agent of concern in biodefence (Inglesby et al., 2000).

*Y. pestis* appears to have evolved from *Y. pseudotuberculosis* relatively recently (Achtman et al., 1999). It has adapted from the enteropathogenic lifestyle to become a fleavectored systemic pathogen by a complex process of gene acquisition and gene loss. However, the two species still retain high levels of genetic similarity and *Y. pseudotuberculosis* is often used as a model to study some pathogenic aspects of *Y. pestis*. However, as the pathogens produce such different diseases, observations from *Y. pseudotuberculosis* must be validated experimentally in *Y. pestis*, rather than extrapolated.

Much emphasis has been placed on the multiple systems possessed by pathogens including *Y. pestis* for the acquisition of iron in vivo. The plague bacillus has multiple systems for acquiring iron, and inactivation of these systems is attenuating (reviewed by Fetherston et al., 2012). However, other ions are also important for bacteria, such as manganese. Because of functional redundancy between MntH and the Yfe system, mainly regarded as an iron transport system, a single deletion to inactivate MntH was not attenuating, and both MntH and Yfe systems were
required to be inactivated before attenuation was observed in the mouse (Perry et al., 2012).

Magnesium has been shown to be one of the most abundant divalent cations within the cells of a diversity of living organisms and as such is involved in many fundamental cellular processes; with insufficient cellular Mg\(^{2+}\) ribosomal subunits dissociate and membranes become leaky (reviewed by Smith & Maguire, 1998). In bacteria, magnesium has been shown to be involved in regulation and stabilization of membranes and a cofactor of several enzymes (reviewed by Tao et al., 1995). As magnesium is an essential nutrient, bacteria possess multiple transporters for Mg\(^{2+}\). The primary Mg\(^{2+}\) transporter in *E. coli* and *Salmonella* is CorA, and orthologues of CorA are widespread (reviewed by Moncrief & Maguire, 1999). In contrast, the other Mg\(^{2+}\) transporters identified in *E. coli* and *Salmonella enterica*, MgtA and MgtB, have a more limited distribution, although the presence of *mgtB* appears to be enriched in pathogens over non-pathogens (Stubben et al., 2009). Mg\(^{2+}\) has been shown to be an important signal controlling gene regulation via the PhoPQ two-component regulatory system for a range of Gram-negative bacteria, including *Y. pestis* and *Y. pseudotuberculosis* (Grabenstein et al., 2004; Oyston et al., 2000). The PhoPQ regulon has been evaluated in *Y. pestis* and shown to involve over 400 differentially regulated genes (Grabenstein et al., 2006; Li et al., 2008; Perez et al., 2009; Zhou et al., 2005) including *mgtB*. However, the specific role of this transporter in these species and the contribution of MgtB to the phenotypes observed following inactivation of the PhoPQ regulon has not been studied. We have previously shown that mutational inactivation of *mgtB* in *Y. pseudotuberculosis* IP32953 caused a high degree of attenuation in mice, with the mutant having a competitive index of 0.01 relative to wild-type *in vivo* (Stubben et al., 2009), indicating an important role for MgtB within the *Yersinia* PhoPQ regulon. We report here the generation of an isogenic *ΔmgtB* mutant of *Y. pestis* and the characterization of the *ΔmgtB* mutants of *Y. pestis* and *Y. pseudotuberculosis*, *in vitro* and *in vivo*, to provide new insight into the importance of magnesium transport for *Yersinia*.

**METHODS**

**Bacterial strains, plasmids and primers.** Plasmids and bacterial strains are listed in Table 1. *Y. pseudotuberculosis* strains were maintained in Luria–Bertani (LB) broth or on LB agar, incubated at 28 °C. LB broth or agar plates supplemented with either kanamycin or chloramphenicol (both at 50 mg ml\(^{-1}\)) were used to culture the *Y. pseudotuberculosis* IP32953 *ΔmgtB* mutant or the complemented strain, respectively. *Y. pestis* GB was cultured at 28 °C in blood agar base (BAB) broth or on BAB agar, supplemented as required with 0.025% haemin, 10 mM MgCl\(_2\) and 50 μg kanamycin ml\(^{-1}\). For selection of bacteria carrying pAJD434, media were supplemented with 100 μg trimethoprim ml\(^{-1}\). For growth at 37 °C, media were supplemented with 2.5 mM CaCl\(_2\) to avoid loss of pYV/pCD1.

**Construction of the *Y. pestis* Δ*mgtB* mutant.** Construction of the *Y. pestis* Δ*mgtB* mutant was carried out as previously described using the λRed recombinase method that had been used to create the *Y. pseudotuberculosis* Δ*mgtB* mutant (Stubben et al., 2009). Briefly, primers mgtB1H and mgtB2H (Table 1) were designed to amplify the kanamycin resistance gene and incorporate 50 bp gene-specific flanks for *mgtB*. Excess template was removed by *DpnI* digestion before purification with Microcon YM100 centrifugal filters (Millipore). PCR products were electroporated into *Y. pestis* GB carrying the λRed recombinase helper plasmid, pAJD434, to facilitate homologous recombination with the chromosomal gene copy. Following overnight incubation in broth supplemented with 0.8% arabinose, transformants were selected on agar supplemented with kanamycin (50 μg ml\(^{-1}\)) and trimethoprim (100 μg ml\(^{-1}\)) for 48 h. Transformants were screened for mutation by PCR with gene-specific primers, *YPmgtB* for and *YPmgtB* rev. To cure the pAJD434 plasmid, broth cultures supplemented with 2.5 mM CaCl\(_2\) were incubated at 37 °C: the pAJD434 plasmid is temperature sensitive and lost at this temperature, while the CaCl\(_2\) avoids loss of pYV by abrogating the *in vitro* growth restriction phenotype. Loss of pAJD434 was confirmed by PCR using primers against the *gum* gene (Table 1). Presence of the pCD1 plasmid was determined by PCR using primers against the plasmid-encoded *yseC* gene (Table 1).

**Complementation of Δ*mgtB* in *Y. pseudotuberculosis* and *Y. pestis.*** A plasmid was designed to facilitate complementation of the deletion. The *mgtB* ORF was amplified using a proofreading enzyme (pfx, New England Biolabs), from genomic DNA of *Y. pseudotuberculosis* IP32953 using primers mgtB5COMP and mgtB3COMP (Table 1), which amplified the gene from the start ATG to terminal stop codon, with a C-terminal His\(_{(x6)}\) tag prior to the terminal TGA, amplified the gene from the start ATG to terminal stop codon, with a C-terminal His\(_{(x6)}\) tag prior to the terminal TGA, amplified the gene from the start ATG to terminal stop codon, with a C-terminal His\(_{(x6)}\) tag prior to the terminal TGA, amplified the gene from the start ATG to terminal stop codon, with a C-terminal His\(_{(x6)}\) tag prior to the terminal TGA. The PCR product was then digested with *SacI* and 3′ *XbaI* sites, purified PCR products and pBAD33 were digested with *SacI* and *XbaI* and ligated. Ligations were transformed by electroporation into competent *Y. pseudotuberculosis* IP32953Δ*mgtB* or *Y. pestis* GB*ΔmgtB* cells. Transformants were selected on LB agar or BAB haemin agar supplemented with chloramphenicol. Constructs were induced with arabinose and lysates separated by SDS-PAGE and probed with anti-His antibodies to check for expression of the His-tagged protein.

**Effect of Mg\(^{2+}\) supplementation on growth.** To assay the effect of inactivation of the MgtB Mg\(^{2+}\) transporter in *Y. pseudotuberculosis*, the wild-type, mutant and complemented mutant were grown in a defined medium (Zahorachk & Brubaker, 1982) with Mg\(^{2+}\) at 0 mM, 50 μM, 100 μM and 1 mM. For *Y. pestis*, BAB broth was supplemented with 10 mM Mg\(^{2+}\). Growth was followed by measuring the optical density of cultures at 600 nm (OD\(_{600}\)).

**Macrophage infection assay.** Macrophage infection assays were performed using the I77A4.1 mouse macrophage cell line using published methods (Oyston et al., 2000; Papp-Wallace & Maguire, 2008). For *Y. pseudotuberculosis* infections an m.o.i. of 100:1 was used. For *Y. pestis*, cultures with no visible aggregates were diluted and vortexed in media and for infections an m.o.i. of 10:1 was used. Assays were performed in triplicate. Invasion was assayed at 3 h post-infection for *Y. pseudotuberculosis* and at 1 h post-infection for *Y. pestis*, and survival of both species was evaluated at 24 h. Outputs were analysed by unpaired *t*-test with Welch’s correction (two-tailed) using Prism 6.02.

**Galleria infection assay.** *Galleria melonella* (greater wax moth) infection assays were performed as described by Champion et al. (2009), with minor modifications. Briefly, *Y. pseudotuberculosis* was grown overnight at 37 °C in LB broth supplemented with CaCl\(_2\). Aliquots containing 10\(^7\) c.f.u. bacteria in a volume of 10 μl were injected into the first right pro-leg. Groups of 10 larvae were used per experiment, and the experiment was performed three times. Viable counts of inocula were determined retrospectively. *G. melonella* larvae were scored at 24, 48 and 72 h for survival and melanin production.
Significance was analysed by unpaired t-test with Welch’s correction (two-tailed) using Prism 6.02.

Virulence testing of the Y. pestis ΔmgtB mutant in the murine infection model. To determine the effect of inactivation of mgtB on virulence of Y. pestis, Y. pestis ΔmgtB was administered by the subcutaneous route to groups of six female 6-week-old BALB/c mice (Charles River Laboratories). Bacteria were grown overnight with agitation at 28 °C in BAB broth, diluted 1:10 in fresh media and incubated with shaking for 5 h at 28 °C. No visible aggregates were observed in the cultures at this time. Mice were challenged subcutaneously with 100 μl volumes of appropriate dilutions in PBS. Cell suspensions were serially diluted in sterile PBS and plated out dilutions (in triplicate) on BAB agar and BAB agar supplemented with kanamycin to determine the input ratio. After 2 days, spleens were recovered from four mice and passed through 70 μm sieves (Becton Dickinson) to produce a cell suspension in 3 ml PBS. Cell suspensions were serially diluted in sterile PBS and plated onto BAB agar and BAB agar supplemented with kanamycin to determine the output ratio. The competitive index (CI) is defined as the output ratio (mutant/wild-type) divided by the input ratio (mutant/wild-type) (Freter et al., 1981; Taylor et al., 1987).

RESULTS

MgtB is important for growth of Y. pestis and Y. pseudotuberculosis

Isogenic mutants were generated in Y. pestis GB and Y. pseudotuberculosis IP32953 using the zRed recombinase method. The Y. pseudotuberculosis wild-type and mutant strains were grown in defined medium supplemented with Mg²⁺ in the range 0–1 mM (Fig. 1). Without addition of Mg²⁺, the wild-type IP32953 did not grow well. Addition

Table 1. Bacterial strains, plasmids and primers

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description/sequence (primers)</th>
<th>Source or reference/purpose (primers)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y. pseudotuberculosis IP32953</td>
<td>Wild-type: virulent clinical isolate from a human patient</td>
<td>Chain et al. (2004)</td>
</tr>
<tr>
<td>Y. pseudotuberculosis IP32953 ΔmgtB</td>
<td>IP32953, deleted in mgtB, kan', attenuated mutant</td>
<td>Stubben et al. (2009)</td>
</tr>
<tr>
<td>Y. pestis GB ΔmgtB</td>
<td>Wild-type; biovar Orientalis, clinical isolate from a fatal human case of primary pneumonic plague</td>
<td>Parkhill et al. (2001)</td>
</tr>
<tr>
<td>Y. pestis GB ΔmgtB</td>
<td>GB, deleted in mgtB, kan', attenuated mutant</td>
<td>This study</td>
</tr>
</tbody>
</table>

Plasmids

- **pAJD434**: Arabinoose-inducible ‘Red recombinase genes
- **pBAD33**: Arabinoose-induced expression vector, ori_pL; PBAD Cat'
- **pMgt-Comp**: pBAD33 containing the mgtB ORF as a 2.8 kb XbaI/SacI insert

Oligonucleotide primers

- **mgtB5comp**: GATCGAGCTCAGGGAGGGAAGCGTT
  - ATGCGTAAATATGAAAAAAC
- **mgtB3COMP**: GATCTCTAGATCATGATGATGATGATGAGCCA
  - TTCGCCGAAACGGCGGA
- **ysCF**: ACAACTGCTCCTGCTAGA
- **ysCR**: TCACAATAGCGCAAGCTT
- **PBAD5**: TCTCGCTAACCAAACCGGTA
- **mgtB2H1**: TATTTAGGGGATAGCGCTGCGTTTTTGAATTTAAAGAGA
  - GCAGCTtatAG
- **mgtB2H2**: CGGAATGTGCTGACATCAACCGACGGGATTATTGAAC
  - GGCACCCCTCA
- **YPmgtBscr for**: AAGGCCGTAGCAACCCCTCG
- **YPmgtBscr rev**: CGCAGTACTGCTGATTACC
- **gamF**: TGGGAATTCGAGCTACTAGG
- **gamR**: TGGGAATTCGAGCTACTATTCC

Amplification of mgtB, contains engineered SacI site (underlined) and initiation codon (italics)

Amplification of mgtB, contains engineered XbaI site (underlined). The sequence encoding the His6 tag is shown in italics.

Amplification of mutagenesis cassette

Amplification of mutagenesis cassette

Confirmation of presence of pYV

Confirmation of presence of pYV

Confirmation of transformants containing complementation plasmid

Confirmation of mutants

Confirmation of mutants

Confirmation of curing of pAJD434

Confirmation of curing of pAJD434
of 50 μM Mg\(^{2+}\) restored growth of the wild-type, but increasing the concentration to 1 mM resulted in improved growth. In contrast, the *Y. pseudotuberculosis* IP32953ΔmgtB mutant showed reduced growth relative to the wild-type, even in media supplemented with 1 mM Mg\(^{2+}\), and this could be restored by complementation of the mutation.

Similarly, *Y. pestis* GB was able to grow in BAB broth without additional Mg\(^{2+}\). The Mg\(^{2+}\) content of BAB was calculated to be approximately 0.34 mM. Without additional Mg\(^{2+}\), the *Y. pestis* ΔmgtB mutant clumped visibly from the mid-exponential phase of growth, and the aggregates generated towards the end of the exponential phase could not be dispersed by vortexing (data not shown). Supplementation with up to 10 mM Mg\(^{2+}\) resulted in looser aggregates that could be dispersed by vortexing, but typical growth, as seen with the wild-type, was not obtained, even at this level of supplementation. A growth curve was generated by inoculating BAB supplemented with 10 mM Mg\(^{2+}\), and the culture was vortexed prior to each time point to disperse the aggregates. The OD\(_{600}\) was followed and viable counts were enumerated (Fig. 2a). Despite the high level of supplementation, it appears that the mutant was less fit than the wild-type, with a significance of *P*=0.01 at 7 h. Complementation of the *Y. pestis* ΔmgtB mutant partially restored growth (Fig. 2b). However, full complementation was not achieved and the growth defect of the complemented strain versus the wild-type carrying the same plasmid remained significant (*P*<0.01). This was unlikely to be due to the energy required to maintain the plasmid, as there was no significant difference in growth of the wild-type with and without the plasmid. More probably, induced expression with arabinose did not reflect optimal expression of MgtB.

**MgtB is required for macrophage invasion by *Y. pestis* but not *Y. pseudotuberculosis***

The pathogenic *Yersinia* are known to have an intracellular phase during infection, and the inability to survive interaction with macrophages correlates with a reduction in virulence (Klein *et al.*, 2012). Therefore, we aimed to determine the ability of the ΔmgtB mutants to invade and survive within macrophages. No difference was observed in the ability of the *Y. pseudotuberculosis* IP32953 wild-type and mutant to invade the J774.1 macrophage cell line (Fig. 3a). Similarly, survival at 24 h was identical.

The *Y. pestis* mutant showed reduced invasion of J774.1 macrophages compared with the wild-type (Fig. 3b). However, although no visible aggregates were observed, there remains the possibility that small aggregates may have

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**Fig. 1.** Impaired growth of *Y. pseudotuberculosis* following inactivation of mgtB. Defined media lacking Mg\(^{2+}\) or supplemented with MgCl\(_2\) at 0.05, 0.1 or 1 mM were inoculated with the IP32953 wild-type (wt), the mgtB mutant (ΔmgtB) or the complemented mutant strain (ΔmgtB + comp) and growth was monitored for 8 h of incubation. Inactivation of MgtB resulted in a significant growth defect despite Mg\(^{2+}\) supplementation. Data presented are the mean of triplicate experiments ± SEM. *P*<0.05 (one-way ANOVA with Bonferroni’s multiple comparison test).

**Fig. 2.** (a) Impaired growth of *Y. pestis* following inactivation of mgtB. BAB broth supplemented with 10 mM MgCl\(_2\) was inoculated with the wild-type GB strain and the mgtB mutant. Growth was monitored for 8 h. Inactivation of MgtB resulted in a growth defect despite Mg\(^{2+}\) supplementation. Data presented are the mean of triplicate experiments ± SEM. Significance was shown for the 7 h time point using an unpaired two-tailed *t*-test using Prism 6.02. (b) Partial restoration of growth of *Y. pestis* by complementation. BAB broth was inoculated with the GB wild-type (wt), the GB wild-type carrying the complementation plasmid (wt + comp), the mgtB mutant (ΔmgtB) or the complemented mutant strain (ΔmgtB + comp). After 7.5 h of incubation, the optical density of the cultures was determined. A highly significant reduction in growth of the mutant relative to the wild type was observed (****P<0.0001). Complementation resulted in partial restoration of growth of the mutant returning towards the wild-type phenotype (**P<0.01). Data presented are the mean of triplicate experiments ± SEM. Significance was determined using one-way ANOVA with Bonferroni’s multiple comparison test.
formed, which may have affected uptake. In this assay, we do not observe significant replication of Y. pestis, but the bacteria survive within the cells and then cell lysis occurs around 48 h post-infection. This was the pattern we observed with the GB wild-type. The \( \Delta \text{mgtB} \) mutant similarly survived to 24 h within the macrophages, although the lower rate of recovery reflected the reduced invasion observed at the early time point. The result was replicated in the RAW macrophage cell line (data not shown), showing the invasion defect to be independent of cell line.

**MgtB is required for virulence**

Previously it had been shown that inactivation of \textit{mgtB} resulted in attenuation of \textit{Y. pseudotuberculosis} IP32953 (Stubben et al., 2009). In an attempt to dissect this attenuation further, \textit{G. mellonella} larvae were injected with the wild-type, the \( \Delta \text{mgtB} \) mutant or the complemented mutant strain. Inactivation of \textit{mgtB} resulted in increased survival of infected larvae \((P=0.0244)\), and this was returned towards wild-type killing by complementation in \textit{trans} (Fig. 4).

The effect of inactivation of the gene on virulence of \textit{Y. pestis} was then determined. Groups of six mice were dosed subcutaneously with dilutions of the \( \Delta \text{mgtB} \) mutant ranging from 0.149 to 1490 c.f.u., as determined by retrospective viable counts of the bacterial suspension. The MLD of GB has been shown to be approximately 1 c.f.u. (Russell et al., 1995), and a control group of six mice were challenged with 216 c.f.u. All mice challenged with the wild-type strain GB died, although the \( \Delta \text{mgtB} \) mutant showed significant attenuation, with only a single mouse dying in the top two challenge groups, and these died late in the experiment (Fig. 5; Table 2). Therefore, the MLD of the mutant was >1490 c.f.u.

To minimize the number of animals used in virulence testing, rather than undertake a repeat of the MLD experiment with a higher dose range, it was decided to obtain an indication of attenuation based on CI. The CI of the \( \Delta \text{mgtB} \) mutant was 0.04. Values below 0.2 are taken to indicate attenuation, and thus this mutant was less fit \textit{in vivo} than the wild-type. The \textit{in vivo} CI of the \textit{Y. pseudotuberculosis} \( \Delta \text{mgtB} \) mutant was previously determined as 0.01 (Stubben et al., 2009).

**DISCUSSION**

Magnesium has been shown to be an essential bacterial nutrient. The ability to acquire Mg\( ^{2+} \) in limited environments, such as in the intracellular niche, is required for virulence of a range of pathogens, and bacteria thus possess in-built redundancy by using multiple Mg\( ^{2+} \) transporters. Mg\( ^{2+} \) transport has been most fully characterized in \textit{E. coli} and \textit{Salmonella}, which possess three systems: CorA, MgtA and MgtB (reviewed by Channongpol & Groisman, 2002). \textit{Y. pestis} and \textit{Y. pseudotuberculosis} possess homologues of these systems. A mutant of \textit{Salmonella} inactivated for all three transporters cannot grow unless media are supplemented with high (10 mM) concentrations of Mg\( ^{2+} \). Although CorA is considered the primary bacterial Mg\( ^{2+} \) transporter, \textit{Salmonella} mutants lacking \textit{mgtA} or \textit{mgtB} are defective for growth in low Mg\( ^{2+} \) media (Soncini et al., 1996) despite CorA being expressed and functional in both high and low Mg\( ^{2+} \) conditions, and having a similar affinity for Mg\( ^{2+} \) as MgtA and MgtB (Snively et al., 1989,
1991). Similarly, we observed that *Y. pseudotuberculosis* and *Y. pestis* required Mg^{2+} supplementation for growth. Previously, the *Y. pseudotuberculosis ΔmgtB* mutant had been shown to have no growth defect in LB medium (Stubben *et al.*, 2009). However, when inoculated into defined media lacking Mg^{2+}, the mutant was unable to grow. The Mg^{2+} composition of LB broth constituents has been estimated (Wee & Wilkinson, 1988) to 200 μM, although lower concentrations have been proposed in the range 30–50 μM (Snively *et al.*, 1989). At 50 μM Mg^{2+} supplementation in defined media, the *Y. pseudotuberculosis ΔmgtB* mutant showed impaired growth relative to the wild-type, suggesting that the higher concentration reported as being present in LB broth is more accurate. The maximum Mg^{2+} concentration evaluated in this study was 1 mM, and even at this elevated concentration the mutant demonstrated a growth defect relative to the wild-type. This may indicate that the organism is more stressed in the defined medium than in LB broth. Complementation of the *Y. pseudotuberculosis ΔmgtB* mutant was able to restore growth to wild-type levels, indicating that the growth defect was specifically due to loss of the MgtB transporter. Similarly, the *Y. pestis* mutant was unable to grow in BAB broth without supplementation with Mg^{2+}. The concentration of Mg^{2+} in this medium is approximately 300 μM (data not shown), and the mutant required supplementation with 10 mM Mg^{2+} before a growth curve could be generated. The growth defect could only be partially reversed by complementation of the *Y. pestis ΔmgtB* mutant, which may be due to suboptimal complementation in this species.

One of the best studied functions of Mg^{2+} in bacteria is as a signal to activate the PhoPQ two-component regulatory system, which has been particularly well characterized in *S. enterica* (García Véscovi *et al.*, 1996). In *Salmonella*, low magnesium culture media induced the expression of a range of proteins, including the MgtA and MgtB ion transporters (Soncini *et al.*, 1996). Inactivation of the PhoPQ two-component regulatory system in *Y. pestis* strain GB resulted in at best a minor increase in MLD (Bozue *et al.*, 2011; Oyston *et al.*, 2000), much less than the attenuation observed in this study with the equivalent mgtB mutant of the same strain. Similarly, a *Y. pseudotuberculosis ΔphoP* mutant was 100-fold attenuated in a mouse model, less than was observed for the *Y. pseudotuberculosis ΔmgtB* mutant (Stubben *et al.*, 2009). Therefore, inactivation of mgtB expression appears more attenuating than inactivation of the regulatory system that induces its upregulation in Mg^{2+}-deprived environments, suggesting a basal level of expression prior to induction. Indeed, induction experiments with the promoter driving expression of mgtCB in *Salmonella* showed a low level of expression in the presence of high Mg^{2+} (Tao *et al.*, 1995).

The macrophage is often suggested as an important Mg^{2+}-limited environment encountered by intracellular pathogens including *Yersinia* and *Salmonella*, and it has been suggested that this is where PhoPQ regulation is key (Garcia-del Portillo *et al.*, 1992; Pollack *et al.*, 1986). Inactivation of PhoP expression in *Y. pestis* resulted in impaired uptake and survival in J774A.1 macrophages (Bozue *et al.*, 2011). Our results indicate that MgtB contributes to the invasion defect observed in the ΔphoP mutant, but not the survival defect. Similarly to our data with the *Y. pseudotuberculosis ΔmgtB* mutant, the *Y. pseudotuberculosis ΔphoP* mutant showed no uptake defect in J774A.1 macrophages (Bozue *et al.*, 2011), although it did show a survival defect. This indicated that the PhoPQ regulon has slightly different contributions to uptake and survival of these two closely related organisms, and similarly it appears that inactivation of MgtB has different

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**Table 2. Survivors following challenge of mice with either the wild-type GB strain or the ΔmgtB mutant**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Dose per mouse (c.f.u.)</th>
<th>Survivors (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Y. pestis</em> GB</td>
<td>216</td>
<td>0</td>
</tr>
<tr>
<td><em>Y. pestis</em></td>
<td>1490</td>
<td>5</td>
</tr>
<tr>
<td>GBΔmgtB</td>
<td>149</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>14.9</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>1.49</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>0.149*</td>
<td>6</td>
</tr>
</tbody>
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*The value of 0.419 c.f.u. was estimated by extrapolation from dilution series and represents a probability of mice receiving 0–1 c.f.u.*

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effects on the interaction of the two species with macrophages. There are similarities between our observations with Yersinia and those in Salmonella, where mutation in mgtB had only a modest effect on long-term survival in the macrophage (Smith & Maguire, 1998).

It has been shown that the outcome of in vitro macrophage infection assays varies depending on the type of cells used (Bozue et al., 2011). We therefore wished to evaluate whether a difference could be observed between the Y. pseudotuberculosis wild-type and the ΔmgtB mutant in an alternative model, as none was observed in the J774A.1 or RAW macrophage cell lines. The Galleria larva model has been proposed as an alternative infection model for screening Y. pseudotuberculosis mutants (Champion et al., 2009). Preliminary experiments with fully virulent plague in Galleria have not found this to be a very reproducible model for this pathogen (K. Hamblin, Dstl, personal communication). Due to the degree of risk associated with such experiments, combined with the poor performance of the model with plaque, this procedure was not only performed with the Y. pseudotuberculosis mutant. Inactivation of PhoP in Y. pestis KIM6+ resulted in attenuation in the Galleria model (Erickson et al., 2011), but screening of a phoP mutant of Y. pseudotuberculosis in this model has not been reported. We have evaluated a naturally arising ΔphoP mutant, Y. pseudotuberculosis YPIII (Sun et al., 2009b), and preliminary results (mean ± SD survival 73 ± 23%) indicate that this strain is less virulent than the IP32953 strain in Galleria (data not shown), and complementation would be required to prove that the effect is due to PhoP. However, we have demonstrated attenuation of the Y. pseudotuberculosis ΔmgtB mutant in Galleria and that this can be partially reversed by complementation. In the wax moth larvae, Y. pseudotuberculosis resides primarily within haemocytes (Champion et al., 2009). Haemocytes perform many of the functions of phagocytic cells in mammals, and like phagocytes can inactivate bacteria by production of superoxide via a respiratory burst. Our results indicate that MgtB is important to intracellular survival in this model of infection, which correlates with the observed attenuation in the mouse model. However, this does not categorically prove that MgtB is mainly important during the intracellular growth phase. While Yersinia are traditionally considered intracellular pathogens, the majority of bacteria during an infection are extracellular (Simonet et al., 1990), and the role of Mg²⁺ transporters during extracellular growth in vivo has yet to be determined. However, mgtB was not identified as being upregulated in Y. pseudotuberculosis or Y. pestis following incubation in human plasma (Chauvaux et al., 2007; Rosso et al., 2008), and indeed for Y. pestis, mgtB appeared to be slightly downregulated (Chauvaux et al., 2007). The normal mean level of Mg²⁺ in the serum of BALB/c mice is reported as 1.38 ± 0.12 mM (Sun et al., 2009a) and would not therefore be expected to be restrictive. Indeed, the PhoPQ regulon responds to low Mg²⁺ and is not considered to be activated extracellularly. However, PhoPQ has been shown to play a role in biofilm formation in the flea, and in subsequent transmission (Rebeil et al., 2013), where the bacteria exist extracellularly. The presence of multiple magnesium transporters indicates that different transporters may be important at different stages of infection, and the interplay of these redundant systems in vivo has not been elucidated.

In summary, MgtB is a key transporter required for survival and virulence of Y. pestis and Y. pseudotuberculosis during infection. The attenuation is not linked to a survival defect in macrophages, although the Y. pseudotuberculosis ΔmgtB mutant was more susceptible to killing in the Galleria model of infection. The increased attenuation following inactivation of MgtB expression compared with inactivation of the PhoQ two-component regulatory system suggests targeting MgtB may be a more effective antimicrobial strategy than targeting the regulon.

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REFERENCES


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