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The *Salmonella selC* locus contains a pathogenicity island mediating intramacrophage survival

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Pathogenicity islands are chromosomal clusters of horizontally acquired virulence genes that are often found at tRNA loci. The *selC* tRNA locus of *Escherichia coli* has served as the site of integration of two distinct pathogenicity islands which are responsible for converting benign strains into uro- and enteropathogens. Because virulence genes are targeted to the *selC* locus of *E.coli*, we investigated the homologous region of the *Salmonella typhimurium* chromosome for the presence of horizontally acquired sequences. At this site, we identified a 17 kb DNA segment that is both unique to *Salmonella* and necessary for virulence. This segment harbors a gene, *mgtC*, that is required for intramacrophage survival and growth in low Mg^{2+} media. The *mgtC* locus is regulated by the PhoP/PhoQ two-component system, a major regulator of virulence functions present in both pathogenic and non-pathogenic bacterial species. Cumulatively, our experiments indicate that the ability to replicate in low Mg^{2+} environments is necessary for *Salmonella* virulence, and suggest that a similar mechanism is responsible for the dissemination and acquisition of pathogenicity islands in enteric bacteria.

Keywords: intramacrophage survival/magnesium/pathogenicity island/PhoP/*Salmonella*

Introduction

Pathogenicity islands are chromosomal clusters of virulence genes present in pathogenic organisms but absent from related non-pathogenic bacteria (Groisman and Ochman, 1996; Hacker *et al.*, 1997). Pathogenicity islands constitute a major driving force in the evolution of bacterial pathogens because their acquisition often determines the virulence properties of a microorganism. For example, enteropathogenic strains of *Escherichia coli* harbor a 35 kb pathogenicity island, termed LEE, that encodes the ability to form attaching and effacing lesions in intestinal epithelial cells (McDaniel *et al.*, 1995), and introduction of LEE into a benign strain of *E.coli* renders it capable of producing these lesions (McDaniel and Kaper, 1997).

In contrast, *Salmonella typhimurium* has a complex life cycle in infected animals and requires a large number of virulence genes. While many of these virulence determinants are also present in non-pathogenic species, several others are encoded in regions of the chromosome that are specific to *Salmonella* (Groisman and Ochman, 1994). For example, the vast majority of the genes conferring the ability to invade epithelial cells reside within SPI-1, a 40 kb pathogenicity island at 63 minutes in the *S.typhimurium* chromosome (Mills *et al.*, 1995; Galán, 1996). A second pathogenicity island, SPI-2, harbors genes that are indispensable for survival within macrophages and to cause systemic disease (Ochman *et al.*, 1996; Shea *et al.*, 1996). In addition to SPI-1 and SPI-2, other *Salmonella*-specific regions have been implicated in virulence: For example, the *pagC* and *msgA* genes at 25 minutes are required for intramacrophage survival (Gunn *et al.*, 1995; Hohmann *et al.*, 1995) and the *spv* genes in the large virulence plasmid are necessary for replication in extraintestinal tissues (Gulig *et al.*, 1993). Despite their foreign origin, many of these virulence determinants are regulated by PhoP/PhoQ (Miller *et al.*, 1989; Bajaj *et al.*, 1996; Heithoff *et al.*, 1997), a two-component system that is present in both pathogens and non-pathogens (Groisman *et al.*, 1989). The PhoP/PhoQ regulatory system governs the adaptation to low Mg^{2+} environments (García Véscovi *et al.*, 1996; Soncini *et al.*, 1996) and, in *S.typhimurium*, it is essential for intramacrophage survival and virulence in mice (Fields *et al.*, 1986, 1989; Miller *et al.*, 1989).

Pathogenicity islands are often located next to tRNA genes (Groisman and Ochman, 1996; Hacker *et al.*, 1997). For example, the PAI-2, PAI-4 and PAI-5 pathogenicity islands are integrated at the $tRNA^{leuX}$, $tRNA^{pheV}$ and $tRNA^{pheR}$ genes of *E.coli*, respectively (Blum *et al.*, 1994; Swenson *et al.*, 1996), and the SPI-2 island of *S.typhimurium* is adjacent to the $tRNA^{Val}$ locus (Hensel *et al.*, 1997). In *E.coli*, the *selC* tRNA locus has been targeted by two different pathogenicity islands, LEE and PAI-1, which determine whether *E.coli* is rendered an entero- or a uropathogen (Blum *et al.*, 1994; McDaniel *et al.*, 1995). Moreover, *selC* has been shown also to be the attachment site of the retronphage ΦR73 in *E.coli* (Inouye *et al.*, 1991).

Because two pathogenicity islands reside at the *selC* locus of *E.coli*, we investigated the *selC* locus of *S.typhimurium* for the presence of horizontally acquired sequences. In this study, we identify a new pathogenicity island that is necessary for intramacrophage survival and establish that the ability to grow in Mg^{2+}-limiting environments is essential for *Salmonella* virulence.

Results

A Salmonella-specific DNA segment is present at the selC locus of Salmonella typhimurium

The *selC* tRNA gene of *E.coli* is the site of insertion of two pathogenicity islands (Blum *et al.*, 1994; McDaniel *et al.*, 1995) and the retronphage ΦR73 (Inouye *et al.*, 1991; Sun *et al.*, 1991). In *E.coli* K-12, *orf307*, an open

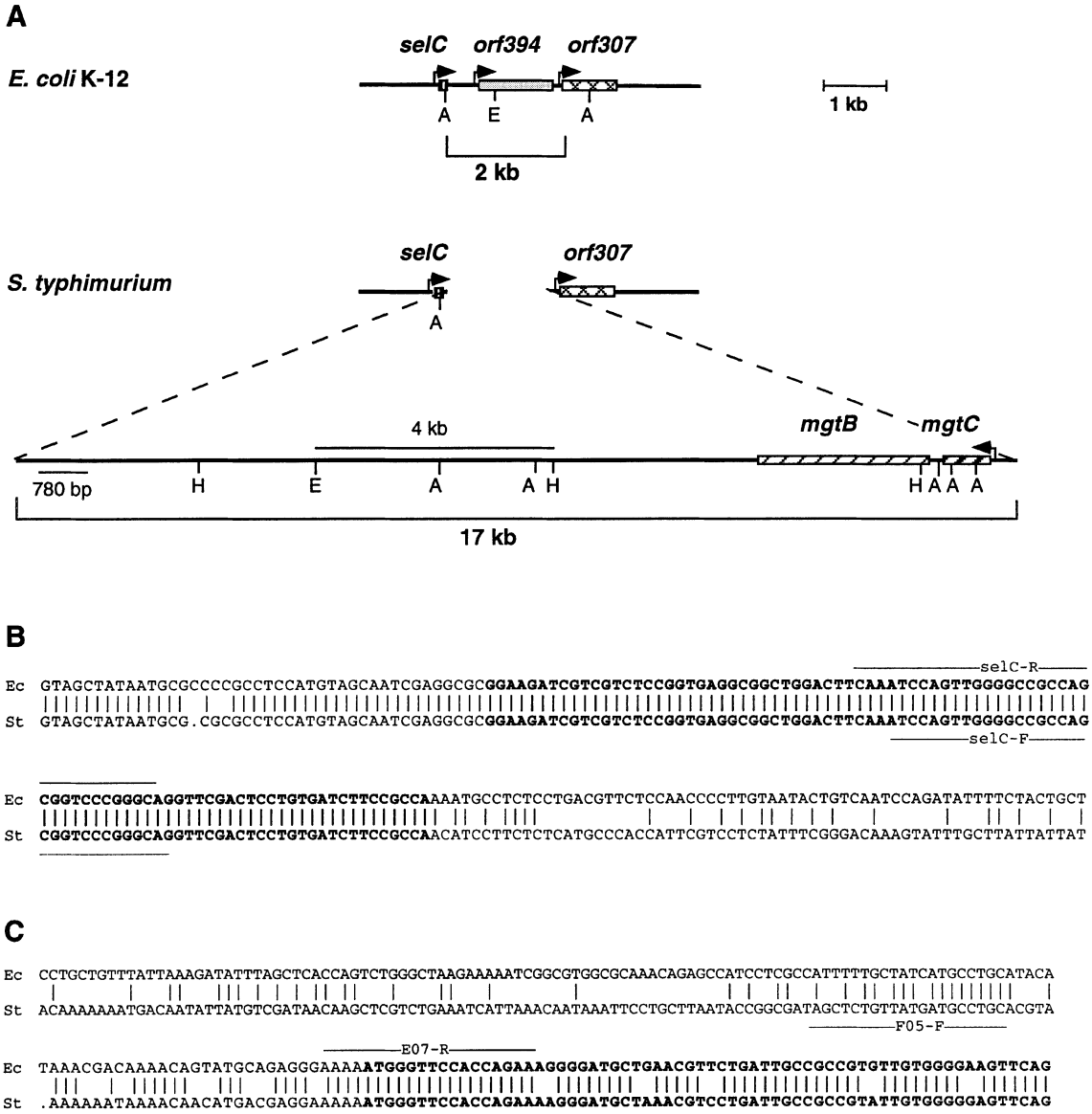

Fig. 1. Physical and genetic maps of the *S.typhimurium* and *E.coli* K-12 *selC–orf307* chromosomal regions. (**A**) The *E.coli* K-12 map is based on reported nucleotide sequences (DDBJ/EMBL/GenBank accession number L10328). The *S.typhimurium* map is based on restriction analysis of plasmid pEG9106, which carries both *selC* and *mgtCB* genes, and on sequence data on the *mgtCB* locus (DDBJ/EMBL/GenBank accession number J05728). A, *Ava*I; E, *Eco*RI; H, *Hin*dIII. The distance between *selC* and the beginning of *orf307* is 17 kb in *S.typhimurium* whereas it is only 2 kb in *E.coli* K-12. The positions of the 4 kb *Eco*RI–*Hin*dIII and 780 bp fragments used as probes in Southern hybridization experiments are shown. The orientation of the various open reading frames is indicated by arrows. (**B**) Nucleotide sequence upstream and downstream of the *selC* gene. Alignment of the corresponding DNA sequences of the *S.typhimurium* (DDBJ/EMBL/GenBank accession number Y13864) and *E.coli* K-12 chromosomes (DDBJ/EMBL/GenBank accession number L10328). The *selC* gene, which is 100% identical between *S.typhimurium* and *E.coli* K-12, is indicated by bold letters. The discontinuity between *Salmonella* and *E.coli* K-12 sequences starts 11 bp downstream of the *selC* gene. (**C**) Nucleotide sequence near the *orf307* gene. Alignment of the corresponding DNA sequences of the *S.typhimurium* (DDBJ/EMBL/GenBank accession number J05728) and *E.coli* K-12 chromosomes (DDBJ/EMBL/GenBank accession number L10328). The 5′ end of the *orf307* gene is indicated in bold letters. The discontinuity between *Salmonella* and *E.coli* K-12 sequences appears 30–50 bp upstream from the beginning of *orf307*. Alignments were performed with the GCG program GAP. The position of the primers (selC-F, selC-R, F05-F and E07-R) used in our studies is indicated by horizontal lines.

reading frame of unknown function, is located 2 kb downstream of the *selC* gene (Figure 1A). However, the *selC* and *orf307* genes are >70 kb apart in uropathogenic strains of *E.coli* harboring the PAI-1 pathogenicity island (Blum *et al.*, 1994), >35 kb apart in enteropathogenic *E.coli* carrying the LEE island (McDaniel *et al.*, 1995) and >12.7 kb apart in ΦR73 lysogens (Inouye *et al.*, 1991; Sun *et al.*, 1991). Thus, we hypothesized that *S.typhimurium* may contain horizontally acquired sequences at the *selC* locus and that these sequences might contribute to the virulence properties of this intracellular pathogen.

To determine the distance between the *selC* and *orf307* genes in *S.typhimurium*, we carried out PCR reactions using primers corresponding to *selC* and *orf307* (Figure 1B and C). The *selC* primer was based on a 32 bp segment of the *E.coli* K-12 *selC* tRNA gene because the sequence of the *Salmonella selC* gene was not available and tRNA genes are highly conserved. We searched the *Salmonella* genome for *orf307* homologs and identified a 94 bp

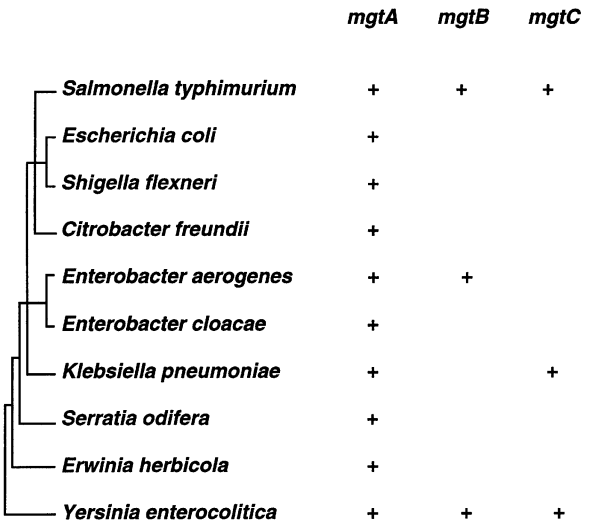

Fig. 2. Distribution of *mgtA*, *mgtB* and *mgtC* sequences among bacterial species. Southern hybridization experiments were carried out using PCR-generated, gene-specific probes for the *S.typhimurium mgtA*, *mgtB* and *mgtC* genes, and total DNA from the bacterial species indicated in the figure. + indicates the presence of a positive hybridization signal in the designated species. Relationships among species follow those presented in Ahmad *et al.* (1990) and Ochman and Groisman (1994).

segment upstream of the *mgtCB* operon that was 91% identical to the 5′ end of *orf307* in *E.coli* K-12. The *orf307* primer corresponded to a 21 bp portion of this segment that is 100% conserved between the *Salmonella* and *E.coli* K-12 genomes. [Both *mgtCB* and *selC* map to the 82 minute region of the *S.typhimurium* chromosome (Sanderson *et al.*, 1995).] When *E.coli* K-12 DNA was used as template, the expected 2 kb PCR product was obtained; however, no fragment could be amplified with *Salmonella* DNA as template (data not shown). These results suggested that the distance between *selC* and *orf307* may be too large in *Salmonella* to be amplified under the PCR conditions used.

We examined the possibility that the DNA segment located between *selC* and *orf307* harbored sequences specific to *Salmonella* by investigating 10 bacterial species for the presence of the *mgtCB* operon. Using gene-specific probes, *mgtC*- and *mgtB*-hybridizing sequences were not detected in *E.coli* K-12 and seven other bacterial species (Figure 2). However, the hybridization patterns obtained with these probes were not identical: *mgtC*-hybridizing sequences were also found in *Klebsiella pneumoniae* and *mgtB*-hybridizing sequences were present in *Enterobacter aerogenes*; yet, *Yersinia enterocolitica* DNA gave positive signals with both probes (Figure 2). In contrast, the *mgtA* gene, which maps to a different chromosomal location than *mgtCB* and is known to be present in *E.coli* K-12 (Tao *et al.*, 1995), was detected in all enteric species tested (Figure 2). (The *mgtA* and *mgtB* genes of *S.typhimurium* encode similar Mg^{2+} transporters and are 57% identical at the DNA level.) The narrow and sporadic distribution of the *mgtC* and *mgtB* genes suggests that the DNA segment harboring the *mgtCB* operon was acquired by horizontal gene transfer. This DNA segment was acquired prior to the diversification of all extant serovars of *Salmonella* because *mgtCB*-hybridizing sequences were detected in all eight subspecies encompassing the genus *Salmonella* (Boyd *et al.*, 1996; data not shown).

Analysis of the junctions of the Salmonella-specific segment harboring mgtCB

To determine the genetic structure of the *selC–orf307* region in *S.typhimurium*, we isolated plasmids containing both *selC* and *mgtCB* genes. First, we screened a library by colony hybridization using an *mgtB*-specific probe and recovered six positive clones. Two of these clones also gave positive signals with a *selC*-specific probe, indicating that the distance between the *mgtB* and *selC* genes of *Salmonella* must be <31 kb, because this is the maximun insert size obtained by the mini-Mu cloning system used to generate the plasmid library (Groisman and Casadaban, 1986).

We constructed restriction maps of $mgtCB^+$ $selC^+$ and $mgtCB^+$ $selC^-$ plasmids and compared them with the published sequence of the *mgtCB* locus. We established that *selC* and *orf307* are separated by 17 kb in *S.typhimurium* whereas these genes are only 2 kb apart in the *E.coli* K-12 chromosome (Figure 1A). To determine whether the *Salmonella*-specific sequences extended to the *selC* end of the 17 kb region, we investigated the DNA from 10 different bacterial species for the presence of sequences hybridizing to a 4 kb *Eco*RI–*Hin*dIII restriction fragment located in the middle of the 17 kb region and to a 780 bp fragment located 260 bp downstream of the *selC* gene (Figure 1A). Apart from hybridizing to the 17 kb region of *S.typhimurium*, the 4 kb probe detected a second weak band in *S.typhimurium* that was similar in intensity to a single band detected in *Citrobacter freundii* (data not shown). On the other hand, sequences hybridizing to the *selC*-proximal probe were not detected in bacteria other than *S.typhimurium* (data not shown). Cumulatively, these results support the notion that the 17 kb region is specific to *Salmonella*.

To define the borders of the 17 kb region, we determined the nucleotide sequence of the *Salmonella* chromosome upstream and downstream of the *selC* gene and upstream of the *mgtC* gene. The *Salmonella* and *E.coli* K-12 chromosomes exhibit 70% DNA sequence identity in the 420 bp region immediately preceding *selC* and 78% identity in a 870 bp segment upstream of *mgtC* which includes *orf307*. The discontinuity between the *Salmonella* and *E.coli* K-12 chromosomes starts 11 bp downstream of the *selC* gene (Figure 1B) and extends for at least 1 kb. In the *mgtCB* end, the discontinuity starts 30–50 bp upstream of the putative initiation codon for *orf307* (Figure 1C) and extends for at least 4.5 kb. The G+C contents of the 1 kb dowstream of the *selC* gene and the 4.5 kb region upstream of *orf307* are 39.8 and 49.3%, respectively, which are lower than the overall 52–54% G+C content estimated for the *S.typhimurium* chromosome. The incorporation of the 17 kb region into the *S.typhimurium* chromosome might have occurred by a mechanism similar to that resulting in the integration of the LEE pathogenicity island in *E.coli*, because microorganisms that harbor these islands at the *selC* locus are deleted for *orf394* (McDaniel *et al.*, 1995; data not shown), an open reading frame present between *selC* and *orf307* in *E.coli* K-12.

Table I. Mouse virulence properties of *mgt* mutants of *S.typhimurium*

Bacterial strain[a]	Dose	No. of surviving mice/ No. of inoculated mice[b]
Wild type	7.5×10^1	0/5
	7.5×10^2	0/5
mgtA	1.4×10^2	0/5
	1.4×10^3	0/5
mgtB	3.0×10^2	0/5
mgtCB	1.6×10^2	2/4
	1.6×10^3	4/10
	1.6×10^4	1/5
mgtA mgtCB	1.5×10^2	4/4
	1.5×10^3	4/4
	1.5×10^4	8/10
	1.5×10^5	3/5

[a]Strains 14028s (wild-type), EG10174 (*mgtA9226*::MudJ), EG10176 (*mgtCB9232*::MudJ) and EG10178 (*mgtB11*::MudJ) and EG10179 (*mgtA9226*::MudCam *mgtCB9232*::MudJ) were used to inoculate mice.
[b]Bacteria were inoculated by i.p. injection and viability was determined for at least 5 weeks.

The mgtCB operon is necessary for virulence

Salmonella typhimurium causes a lethal infection in mice that resembles typhoid fever of humans. We hypothesized that the 17 kb region harboring the *mgtCB* operon may constitute a pathogenicity island and be required for virulence because: (i) *mgtC* and *mgtB* genes display a sporadic distribution in enteric bacteria (Figure 2); (ii) this region is tightly linked to the *selC* gene, the site of insertion of two pathogenicity islands in *E.coli* (Blum *et al.*, 1994; McDaniel *et al.*, 1995); and (iii) transcription of the *mgtCB* operon is governed by PhoP/PhoQ (García Véscovi *et al.*, 1996; Soncini *et al.*, 1996), the major regulator of virulence functions in *Salmonella* (García Véscovi *et al.*, 1994; Groisman and Heffron, 1995).

To examine the virulence role of the 17 kb region, a strain harboring a mutation in *mgtCB* was used to inoculate BALB/c mice by the intraperitoneal (i.p.) route: 40–50% of the animals survived at doses that were 15–150 times the median lethal dose (LD_{50}) of the wild-type strain (i.e. 10 organisms; Table I). While an LD_{50} could not be clearly determined, the *mgtCB* mutant was not as attenuated as strains harboring mutations in the *phoP* locus (Miller *et al.*, 1989), which is known to control expression of several other virulence determinants in *Salmonella*. A mutant harboring a transposon insertion in the *mgtB* gene was as virulent as the wild-type parent, suggesting that the virulence defect of the *mgtCB* mutant is due to the absence of *mgtC*. Despite the *mgtA* gene being regulated also by the PhoP/PhoQ system, an *mgtA* mutant remained virulent in mice. On the other hand, a strain harboring mutations in both *mgtA* and *mgtCB* genes did not cause a lethal infection when inoculated at doses up to 1500 times the LD_{50} of the wild-type strain (Table I). These results demonstrate that the *mgtCB* operon is required for virulence in *Salmonella*, and define a new pathogenicity island that we have designated SPI-3.

The mgtC gene is required for intramacrophage survival

We reasoned that the *mgtCB* mutant might be defective for intramacrophage survival because the *mgtCB* operon is transcriptionally activated by the PhoP protein (García Véscovi *et al.*, 1996; Soncini *et al.*, 1996) and *phoP* mutants are unable to survive within macrophages (Fields *et al.*, 1986, 1989). We investigated strains harboring MudJ transposon insertions in the *mgtA* and *mgtCB* genes (Figure 3A) for their ability to replicate within the macrophage-like cell line J774. The *mgtCB* mutant behaved like the *phoP* strain: it failed to grow in J774 cells (Figure 4A). In contrast, the *mgtA* mutant is virulent in mice (Table I) and survived in macrophages to the same extent as the wild-type strain (Figure 4A). These results demonstrate that the *mgtCB* locus is necessary for replication within macrophages.

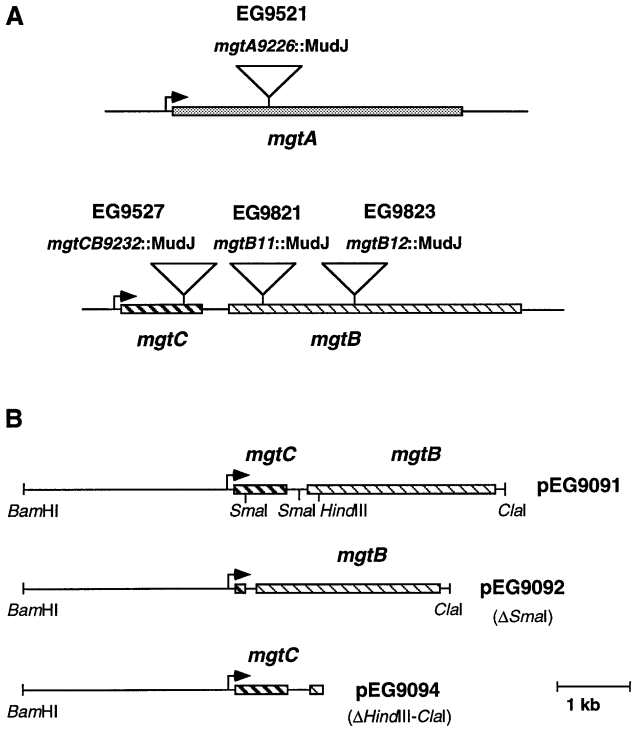

Fig. 3. Structure of chromosomal DNA of *mgt* mutants and of plasmids harboring *mgtCB* genes. (**A**) Position of MudJ insertions in the EG9521, EG9527, EG9821 and EG9823 mutants. Strain EG9527, which carries a MudJ insertion in *mgtC* is phenotypically $mgtC^-$ $mgtB^-$. (**B**) Structure of $mgtC^+$- and/or $mgtB^+$-containing plasmids used in this study. The orientation of the various open reading frames is indicated by arrows.

The *mgtC* and *mgtB* genes form a bicistronic operon (Snavely *et al.*, 1991a; Tao *et al.*, 1995); therefore, a MudJ transposon insertion in the *mgtC* gene is predicted to affect expression of the downstream *mgtB* gene (Figure 3A). To define whether *mgtC*, *mgtB* or both genes are required for intramacrophage survival, we examined the ability of plasmids containing the *mgtC* and/or *mgtB* genes to complement the *mgtCB* mutant (Figure 3B). A plasmid carrying the *mgtC* gene restored the ability to survive in macrophages in the *mgtCB* mutant but one harboring the *mgtB* gene did not (Figure 4B). As expected, a plasmid with the entire *mgtCB* operon rescued the macrophage survival defect of the *mgtCB* strain (Figure 4C).

That the macrophage survival defect of the *mgtCB*::MudJ mutant is due to the lack of MgtC protein is substantiated further by the phenotype of *mgtB* mutants, which replicated within macrophages but to a lesser extent than the wild-type strain (Figure 4A). The mild defect

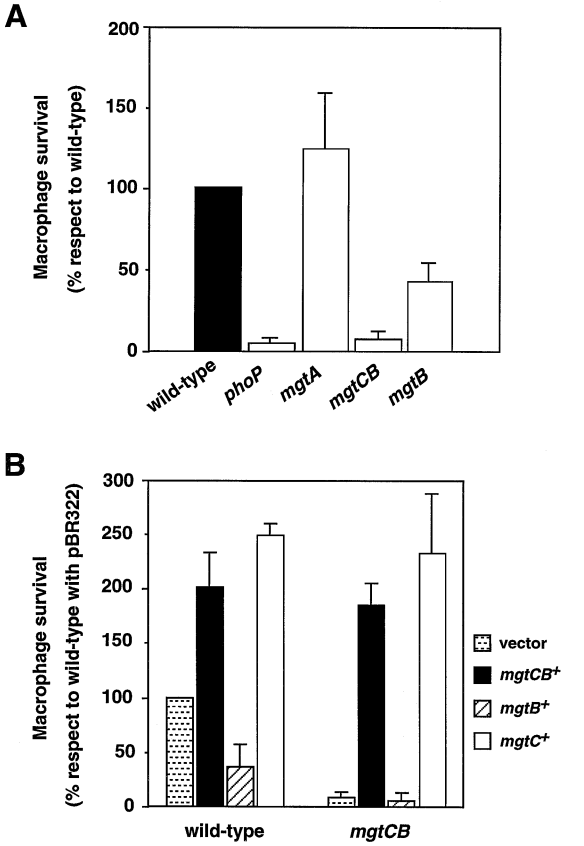

Fig. 4. The *mgtC* gene of *S.typhimurium* is required for intramacrophage survival. (**A**) Intramacrophage survival properties of 14028s (wild-type), MS7953s (*phoP7953*::Tn*10*), EG9521 (*mgtA9226*::MudJ), EG9527 (*mgtCB9232*::MudJ) and EG9821 (*mgtB11*::MudJ) in J774 cells. Macrophage survival was determined as described in Materials and methods 18 h after infection. For each mutant, the percentage of survival relative to the wild-type strain is presented. (**B**) Intramacrophage survival of 14028s (wild-type) and EG9527 (*mgtCB9232*::MudJ) harboring plasmids with $mgtC^+$ and/or $mgtB^+$ genes. The percentage of macrophage survival relative to the wild-type strain harboring pBR322 is presented. Plasmids used for complementation tests, $mgtCB^+$ (pEG9091), $mgtB^+$ (pEG9092) and $mgtC^+$ (pEG9094), are derivatives of pBR322 (Figure 3B). Values represent the mean of three independent experiments ± SD.

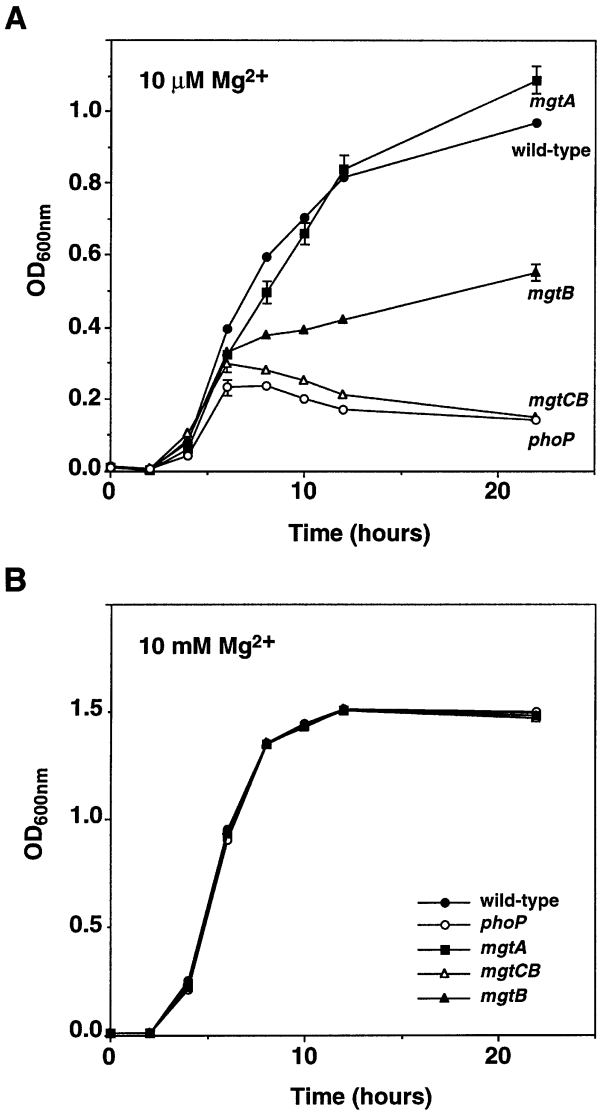

Fig. 5. *mgtCB* mutants of *Salmonella* are defective for growth in low Mg^{2+} NCE liquid medium. Optical density (OD_{600}) achieved by 14028s (wild-type), MS7953s (*phoP7953*::Tn*10*), EG9521 (*mgtA9226*::MudJ), EG9527 (*mgtCB9232*::MudJ) and EG9821(*mgtB11*::MudJ) at different times following inoculation in NCE medium containing 10 μM or 10 mM $MgCl_2$. The data correspond to a representative experiment done in duplicate. Error bars are indicated only if larger than the symbols.

displayed by the *mgtB* mutant is probably due to an indirect effect on *mgtC* expression because the *mgtC*-containing plasmid could restore wild-type macrophage survival to the *mgtB* mutant (data not shown). Taken together, these results establish that the *mgtC* gene is essential for intramacrophage survival.

Identification of a defined medium that reproduces the macrophage survival phenotype of the mgt mutants

We have established previously that both *mgtA* and *mgtCB* are required for optimal growth in liquid N-minimal medium supplemented with 10 μM Mg^{2+} (Soncini *et al.*, 1996); yet, *mgtA* and *mgtCB* mutants differ in their ability to survive within macrophages (Figure 4A). We identified a defined medium that reproduces the macrophage survival phenotype of these mutants: when grown in NCE medium supplemented with 10 μM Mg^{2+}, the *mgtCB* and *phoP* mutants reached an optical density (OD) that was lower than the ODs achieved by the wild-type and *mgtA* strains (Figure 5A). The *mgtB* mutant, which is slightly defective for macrophage survival, reached an OD that was in between the ODs of the wild-type and *mgtCB* strains (Figure 5A). The growth defect of the *phoP*, *mgtCB* and *mgtB* mutants is due to a low Mg^{2+} concentration in the medium because these mutants grew like the wild-type strain in the presence of 10 mM Mg^{2+} (Figure 5B). Equivalent levels of Ca^{2+} and Mn^{2+} could not rescue the mutants, even though these divalent cations can modulate expression of PhoP-activated genes (García Véscovi *et al.*, 1996).

While there are several differences between NCE and N-minimal media, a lower pH in NCE medium (i.e. pH 7.0 versus pH 7.5 in N-minimal medium) may account for the ability of the *mgtA* mutant to grow like the wild-type strain in NCE medium. When the pH of N-minimal

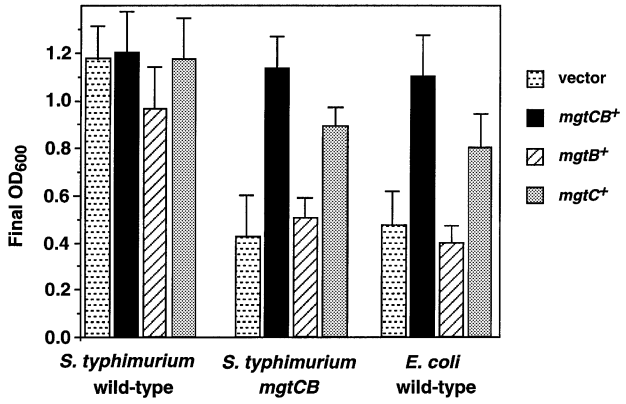

Fig. 6. The *mgtCB* genes allow *E.coli* K-12 to grow in low Mg^{2+} NCE medium. OD_{600} of *S.typhimurium* 14028s (wild-type) and EG9527 (*mgtCB9232*::MudJ), and *E.coli* MG1655 (wild-type) harboring pEG9091 (*mgtCB*$^+$), pEG9092 (*mgtB*$^+$), pEG9094 (*mgtC*$^+$) or pBR322 following 20 h in NCE liquid medium containing 10 μM $MgCl_2$. Values represent the mean of at least three independent experiments ± SD.

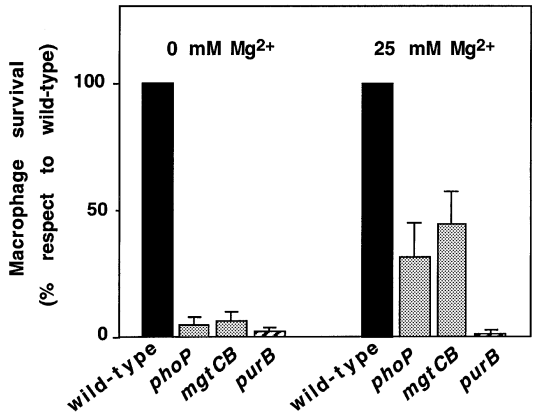

Fig. 7. Effect of Mg^{2+} addition on intramacrophage survival of *phoP* and *mgtCB* mutants. Survival properties of 14028s (wild-type), MS7953s (*phoP7953*::Tn*10*), EG9527 (*mgtCB9232*::MudJ) and EG9652 (*purB877*::Tn*10*) in J774 cells in the absence or presence of Mg^{2+} (25 mM) added to the DMEM, after internalization of the bacteria by the macrophages. The *purB* mutant is a purine auxotroph that is defective for intramacrophage survival and could not be rescued by the addition of Mg^{2+}. Values represent the mean of at least three independent experiments ± SD.

medium was lowered to 7.0, the *mgtA* mutant grew like wild-type *Salmonella* (data not shown). In contrast, the *mgtCB* mutant was defective in N-minimal medium at both pH 7.0 and 7.5. These results demonstrate that the *mgtCB* locus is essential for optimal growth in Mg^{2+}-limiting environments.

The Salmonella mgtCB locus confers growth in low Mg^{2+} upon wild-type E.coli

We reason that wild-type *E.coli* would be impaired for growth in low Mg^{2+} because this bacterial species is missing the *mgtCB* operon (Figure 2) and *mgtCB* is required for growth in low Mg^{2+} (Figure 5A). Wild-type *E.coli* K-12 reached an OD that was lower than that of wild-type *S.typhimurium* and similar to the OD of the *Salmonella mgtCB* mutant when grown in NCE liquid medium supplemented with 10 μM Mg^{2+} (three different *E.coli* K-12 strains exhibited the same behavior; data not shown). The limited growth of *E.coli* was due to the low Mg^{2+} concentration in NCE medium because *E.coli* and *Salmonella* reached the same OD in 10 mM Mg^{2+}.

The absence of *mgtCB* appears to be responsible for the limited growth of *E.coli* in low Mg^{2+} medium because a plasmid harboring the *Salmonella mgtCB*$^+$ operon allowed *E.coli* to reach the same OD as that of wild-type *Salmonella* or the *mgtCB* mutant transformed with the *mgtCB*$^+$ plasmid (Figure 6). An *mgtC*$^+$-containing plasmid conferred partial growth in low Mg^{2+}, but a plasmid carrying the *mgtB*$^+$ gene had no effect. These results further support the notion that the *mgtCB* locus is essential for optimal growth in low Mg^{2+}. Apart from *mgtC*, several *Salmonella*-specific genes are required for intramacrophage survival (Gunn *et al.*, 1995; Ochman *et al.*, 1996), and, as predicted, the *mgtCB*$^+$ plasmid could not confer macrophage survival properties upon *E.coli* (data not shown).

Mg^{2+} rescues growth of mgtCB mutants in macrophages

If the macrophage survival defect of *mgtCB* mutants is due to their inability to grow in low Mg^{2+} environments, one would anticipate that addition of Mg^{2+} to host cells might restore intramacrophage survival. Indeed, growth of both *mgtCB* and *phoP* mutants was improved when Mg^{2+} was added to the tissue culture medium after bacteria had been internalized by the macrophage (Figure 7). Mg^{2+} exerted its action after phagocytosis because it could rescue the mutants even when added 4 h after infection of J774 cells. Moreover, the effect was specific to the *phoP* and *mgtCB* mutants because growth of a *purB* strain (a purine auxotroph that cannot replicate in macrophages) was not affected by the addition of Mg^{2+}. These results indicate that the *Salmonella*-containing vacuole is an Mg^{2+}-limiting environment, which had been suggested previously by the transcriptional induction of PhoP-activated genes both in host cells (Garcia-del Portillo *et al.*, 1992; Heithoff *et al.*, 1997) and in low Mg^{2+} media *in vitro* (García Véscovi *et al.*, 1996; Soncini *et al.*, 1996).

Discussion

Pathogenicity islands are chromosomal clusters of horizontally acquired virulence genes that are often found at tRNA loci (Groisman and Ochman, 1996; Hacker *et al.*, 1997). These clusters typically encode complete functional units and their incorporation into a benign strain can render it pathogenic. We have now identified a pathogenicity island in *S.typhimurium* that mediates intramacrophage survival, virulence in mice and growth in low Mg^{2+}.

The evolution of Salmonella virulence

Two pathogenicity islands have been identified in *Salmonella*: SPI-1, at 63 minutes in the *S.typhimurium* chromosome, governs the ability to invade epithelial cells (Mills *et al.*, 1995; Galán, 1996); and SPI-2, at 31 minutes, is necessary for intramacrophage survival (Ochman *et al.*, 1996; Shea *et al.*, 1996). We have now established that the 17 kb region harboring the *mgtCB* operon at 82 minutes constitutes a third pathogenicity island, designated SPI-3, because: (i) the *mgtC* gene is necessary for full virulence in mice and intramacrophage survival (Table I; Figure 4); (ii) the *mgtCB* sequences exhibit a restricted

Table II. Horizontally acquired sequences at the *selC* locus

Organism	Element	Size (kb)	Distance from *selC* (bp)
E.coli	ΦR73	12.7	9
E.coli	PAI-1	70	16
E.coli	LEE	35	16
S.typhimurium	SPI-3	17	11

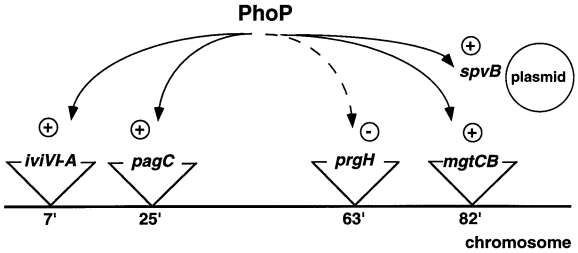

Fig. 8. The PhoP/PhoQ system regulates expression of horizontally acquired virulence genes. The *prgH* locus resides within the SPI-1 invasion island and the *mgtCB* operon is part of the SPI-3 island described in this study. + and – indicate PhoP-activated and -repressed loci, respectively.

distribution in eubacteria (Figure 2); and (iii) SPI-3 is located immediately downstream of *selC* (Figure 1), a tRNA gene that is the site of insertion of two pathogenicity islands in *E.coli* (Blum *et al.*, 1994; McDaniel *et al.*, 1995).

The identification of SPI-3 provides the first example for the incorporation of horizontally acquired sequences at the *selC* gene in two different bacterial species. Whereas SPI-3 inserted 11 bp downstream of *selC* in *S.typhimurium* (Figure 1B), the PAI-1 and LEE pathogenicity islands are present 16 bp 3′ to the *selC* gene in *E.coli* (Blum *et al.*, 1994; McDaniel *et al.*, 1995) (Table II). In contrast to PAI-1, which is flanked by short direct repeats (Blum *et al.*, 1994), no repeats are found at the insertion sites of SPI-3 (Figure 1) and LEE (McDaniel *et al.*, 1995). The utilization of *selC* as integration target in two different species suggests that a similar mechanism is responsible for the incorporation of horizontally acquired sequences at this tRNA locus. This mechanism may be phage mediated because *selC* is the attachment site for the retronphage ΦR73 of *E.coli* (Inouye *et al.*, 1991; Sun *et al.*, 1991) and an ΦR73-related integrase is encoded within the pathogenicity island PAI-1 of *E.coli* (Hacker *et al.*, 1997).

The nutritional environment of the Salmonella-containing phagosome

To replicate within phagocytic cells, *Salmonella* must adapt to the microbicidal and nutrient-poor environment of the phagosome. This requires the manufacture of nutrients not available from host tissues and the co-ordinate regulation of genes that protect the bacterium from the oxygen-dependent and -independent killing mechanisms of the phagocytic cell. The *Salmonella*-containing phagosome is limiting for purines, pyrimidines, histidine and methionine because auxotrophs for these compounds are not able to replicate in macrophages (Fields *et al.*, 1986). This environment appears also to be low in glutamine because strains defective in both the biosynthesis and transport of glutamine are unable to grow within macrophages (Klose and Mekalanos, 1997). Our experiments now indicate that the Mg^{2+} concentration in the phagosome is also limiting since *phoP* and *mgtCB* mutants cannot survive in macrophages (Figure 4A), are impaired for growth in low Mg^{2+} liquid media (Figure 5A), and in both cases growth can be ameliorated by the addition of Mg^{2+} (Figures 5B and 7). That the *Salmonella*-containing phagosome might be low in Mg^{2+} was suggested previously by the transcriptional induction of PhoP-activated genes in host cells (Garcia-del Portillo *et al.*, 1992; Heithoff *et al.*, 1997) and in low Mg^{2+} media *in vitro* (García Véscovi *et al.*, 1996; Soncini *et al.*, 1996), but a direct biochemical determination of the Mg^{2+} concentration in *Salmonella*-containing phagosomes has yet to be reported.

Consistent with the macrophage survival defect, the *mgtCB* mutant was attenuated for virulence in mice (Table I). This suggests that acquisition of the *mgtCB* operon was important in the development of *Salmonella* as an intracellular pathogen because it allowed replication in Mg^{2+}-limiting environments. On the other hand, *E.coli* is an extracellular commensal of mammals that lacks *mgtCB* and exhibits limited growth in low Mg^{2+}. The *Salmonella mgtCB* operon enabled wild-type *E.coli* K-12 to grow in low Mg^{2+} liquid media (Figure 6), but, as predicted, it did not confer intramacrophage survival upon this species since additional *Salmonella*-specific genes are required for this ability (Gunn *et al.*, 1995; Ochman *et al.*, 1996). Because the addition of Mg^{2+} to the macrophage did not fully restore intramacrophage survival to the *mgtCB* mutant, we cannot rule out the possibility of a factor(s) unrelated to the ability to grow in low Mg^{2+} contributing to the macrophage replication defect of this mutant.

Function of the MgtC protein

The macrophage survival defect of the *mgtCB* mutant is due to the lack of *mgtC* (rather than *mgtB*) because this mutant was fully rescued by a plasmid carrying *mgtC*$^+$ (Figure 4B). The MgtC protein is predicted to localize to the inner membrane where it could interact with the Mg^{2+} transport protein MgtB (Snavely *et al.*, 1991a). However, the MgtC protein is not necessary for membrane insertion or transport function of the MgtB protein (Tao *et al.*, 1995), and our results now indicate that MgtC mediates macrophage survival in the absence of the MgtB (Figure 4B) and MgtA proteins (data not shown). Thus, the MgtC protein could be a Mg^{2+} transporter that functions independently of the Mg^{2+} uptake proteins encoded by the *mgtA* and *mgtB* genes.

PhoP/PhoQ: a global regulator of virulence functions

The acquisition of pathogenicity islands offers a rapid way of evolving new functions; however, the incorporated sequences, even when they encode their specific regulators, must coordinate their expression with that of the recipient genome. In addition to the *mgtCB* operon, several virulence genes of foreign origin are regulated by the PhoP/PhoQ regulatory system (Figure 8), which is present in pathogenic and non-pathogenic species (Groisman *et al.*, 1989). These genes include: invasion determinants encoded

Table III. Bacterial strains and plasmids

Bacterial strain or plasmid	Description	Reference or source
S.typhimurium		
14028s	wild-type	ATCC
MM197	*DEL485*(*leuBCD*) *mgtB11*::MudJ	Hmiel *et al*. (1989)
MM198	*DEL485*(*leuBCD*) *mgtB12*::MudJ	Hmiel *et al*. (1989)
MS7953s	*phoP7953*::Tn*10*	Fields *et al*. (1989)
TT282	*purB877*::Tn*10*	J.R.Roth
EG9521	*mgtA9226*::MudJ	García Véscovi *et al*. (1996)
EG9527	*mgtCB9232*::MudJ	García Véscovi *et al*. (1996)
EG9652	*purB877*::Tn*10*	this work
EG9746	14028s/pEG9091	this work
EG9747	14028s/pEG9092	this work
EG9748	14028s/pBR322	this work
EG9755	EG9527/pEG9091	this work
EG9756	EG9527/pEG9092	this work
EG9757	EG9527/pBR322	this work
EG9798	*corA27 mgtA9226*::MudCam *mgtCB9232*::MudJ	this work
EG9821	*mgtB11*::MudJ	this work
EG9823	*mgtB12*::MudJ	this work
EG9902	14028s/pEG9094	this work
EG9905	EG9527/pEG9094	this work
EG9906	EG9821/pEG9091	this work
EG9907	EG9821/pEG9092	this work
EG9908	EG9821/pEG9094	this work
EG9909	EG9821/pBR322	this work
EG10174	*mgtA9226*::MudJ	this work
EG10176	*mgtCB9232*::MudJ	this work
EG10178	*mgtB11*:MudJ	this work
EG10179	*mgtA9226*::MudCm *mgtCB9232*::MudJ	this work
E.coli		
MC1061	F^- *araD139* D(*ara-leu*)*7697* D(*lac*)*X74 thi-1 relA1*?	Casadaban and Cohen (1980)
MG1655	wild-type	laboratory collection
W3110	wild-type	laboratory collection
EG10120	MG1655/pEG9091	this work
EG10121	MG1655/pEG9092	this work
EG10122	MG1655/pEG9094	this work
EG10123	MG1655/pBR322	this work
Plasmids		
pBR322	Ap^R Tc^R rep_{pMB1}	Bolivar *et al*. (1977)
pTT39	Ap^R rep_{pMB1} *mgtCB*$^+$ (pBS derivative)	Tao *et al*. (1995)
pTT39d	Ap^R rep_{pMB1} *mgtB*$^+$ (pBS derivative)	Tao *et al*. (1995)
pEG5005	pBC0::Mud5005	Groisman and Casadaban (1986)
pEG9091	Ap^R rep_{pMB1} *mgtCB*$^+$ (pBR322 derivative)	this work
pEG9092	Ap^R rep_{pMB1} *mgtB*$^+$ (pBR322 derivative)	this work
pEG9094	Ap^R rep_{pMB1} *mgtC*$^+$ (pBR322 derivative)	this work
pEG9106	Mud5005::*selC*$^+$ *mgtCB*$^+$	this work

within the SPI-1 island (Bajaj *et al.*, 1996); the *pagC* gene, required for intramacrophage survival (Hohmann *et al.*, 1995); *iviVI-A*, a gene that is induced specifically in the spleen of *Salmonella*-infected mice (Heithoff *et al.*, 1997); and the plasmid-encoded *spvB* gene (Heithoff *et al.*, 1997), necessary to cause systemic disease.

Horizontally acquired virulence genes may be regulated by the PhoP/PhoQ regulatory system to ensure their proper temporal and spatial expression. The concentration of extracellular Mg^{2+} is the specific regulatory signal that controls the PhoP/PhoQ system (García Véscovi *et al.*, 1996). During the course of infection, *Salmonella* is exposed to millimolar levels of Mg^{2+} in extracellular fluids (Reinhart, 1988) and micromolar concentrations of this divalent cation in host cell vacuoles (Garcia-del Portillo *et al.*, 1992). Thus, the PhoP/PhoQ system contributes to *Salmonella* pathogenicity by both conferring the ability to grow in Mg^{2+}-limiting environments and co-ordinating the expression of different virulence determinants.

Materials and methods

Bacterial strains and growth conditions

Bacterial strains used is this study are listed in Table III. All *S.typhimurium* strains are derived from 14028s, except for MM197, MM198 and TT282 which are derived from LT2. Strains EG9652, EG9821 and EG9823 were constructed by phage P22-mediated transduction (Davis *et al.*, 1980) using lysates grown on TT282, MM197 and MM198, respectively. The position of the MudJ transposon in strains EG9521, EG9527, EG9821 and EG9823 is presented in Figure 3A. Bacteria were grown at 37°C in Luria Broth (LB; Miller, 1972), in NCE-minimal medium (Maloy, 1990) supplemented with 0.1% casamino acids, 38 mM glycerol and either 10 μM or 10 mM $MgCl_2$, or in N-minimal medium (Snavely *et al.*, 1991b) supplemented with 0.1%

casamino acids, 38 mM glycerol and 10 μM $MgCl_2$. Ampicillin and kanamycin were each used at 50 μg/ml.

The ability of the different bacterial strains to grow in low Mg^{2+} liquid media was evaluated as follows: overnight cultures grown in NCE-minimal medium supplemented with 10 mM $MgCl_2$ were washed three times with Mg^{2+}-free medium and diluted 1:200 (Figure 5) or 1:50 (Figure 6) in culture media containing 10 μM or 10 mM $MgCl_2$. The optical density at 600 nm (OD_{600}) was measured at different times (Figure 5) or after 20 h of incubation (Figure 6).

Plasmid construction

Plasmids used in this study are listed in Table III. Plasmid DNA was introduced into bacterial strains by electroporation using a Bio-Rad apparatus as recommended by the manufacturer. Recombinant DNA techniques were performed according to standard protocols (Sambrook *et al.*, 1989). Plasmid pTT39 is a pBluescript derivative with a *Bam*HI–*Cla*I insert containing the entire *mgtCB* operon (Tao *et al.*, 1995). pTT39d contains only the *mgtB* gene because of a *Sma*I deletion that removes the 3′ end of the *mgtC* gene (Tao *et al.*, 1995; Figure 3B). Plasmids pEG9091 and pEG9092 were constructed by cloning the *Bam*HI–*Cla*I inserts of pTT39 and pTT39d, respectively, between the *Bam*HI and *Cla*I sites of plasmid pBR322 (Figure 3B). pEG9094 is a derivative of pEG9091 that contains only the entire *mgtC* gene because the *Hin*dIII–*Cla*I segment of the *mgtB* gene has been deleted (Figure 3B). As predicted, pEG9091 and pEG9092 allowed growth of EG9798 (*corA mgtA mgtCB* triple mutant) in LB medium whereas pEG9094-containing EG9798 cells required the addition of 100 mM Mg^{2+} to the LB for growth (Hmiel *et al.*, 1989).

Cloning of the selC–mgtCB chromosomal region

A library from wild-type strain 14028s was constructed by the *in vivo* cloning technique using the mini-Mu replicon element Mud5005 (Groisman and Casadaban, 1986). This method allows the isolation of plasmids with inserts of up to 31 kb. Kanamycin-resistant transductants were screened by colony hybridization for the presence of the *mgtB* gene using a labeled PCR fragment corresponding to the coding region of *mgtB* as probe. Six positive clones were obtained and tested for the presence of the *selC* gene by hybridizing with an oligonucleotide probe corresponding to 32 bp of the *E.coli* K-12 *selC* gene, and two positive clones were recovered. One of these clones harbored a plasmid, pEG9106, that contains an insert of ~20 kb and was used for subsequent molecular analysis.

Molecular biological techniques

PCR reactions were carried out on purified chromosomal DNA with *Taq* polymerase according to the manufacturer's protocol (Gibco BRL). The primer selC-F, 5′-ATCCAGTTGGGGCCGCCAGCGGTCCCGGG-CAG-3′, is complementary to the *E.coli* K-12 *selC* gene. The primer E07-R, 5′-TTTCTGGTGGAACCCATTTTT-3′, corresponds to position 78–98 of the *mgtCB* published sequence [accession number J05728 (Snavely *et al.*, 1991a)]. The sequences of these two primers are 100% identical in the *E.coli* K-12 and *S.typhimurium* chromosomes (Figure 1B and C).

Colony hybridization experiments were performed as described (Buluwela *et al.*, 1989) using either PCR-generated DNA fragments labeled with [α-^{32}P]dCTP and the Ready To Go kit (Pharmacia Biotech) or oligonucleotides labeled with [γ-^{32}P]ATP by the T4 polynucleotide kinase (New England Biolabs) as described (Sambrook *et al.*, 1989). Southern hybridization analysis was carried out using chromosomal DNA digested by *Eco*RI, size fractionated in 1% agarose gels and transferred to nylon membrane by capillarity (Sambrook *et al.*, 1989). Hybridization was performed at 65°C as described (Groisman *et al.*, 1993) with probes corresponding to PCR-generated fragments corresponding to the coding regions of the *mgtA*, *mgtB* and *mgtC* genes, to a 780 bp fragment (Figure 1A) amplified with primers 5′-ACTTACAGGCTCAT-CCTTTCTC-3′ and 5′-AACGTAAGGCTATAGTGCCT-3′ or to a 4 kb *Eco*RI–*Hin*dIII fragment from the 17 kb region (Figure 1A). Prior to autoradiography, washes (two for 15 min at room temperature and one for 5–10 min at 65°C) were performed in 1× SSC, 0.1% SDS.

DNA sequencing was carried out on purified pEG9106 DNA (Qiagen kit) using the dye terminator cycle sequencing kit with AmpliTaq DNA polymerase (Perkin Elmer) and an ABI 373 sequencer. The sequence around the *selC* gene was determined using primers complementary to the *E.coli* K-12 *selC* sequence: primer selC-R (5′-TGCCCGGG-ACCGCTGGCGGCCCCAACTGGATTTG-3′) was used to sequence the region upstream of *selC* and primer selC-F (see above) was used to sequence the region downstream of *selC*. The sequence upstream of the *mgtC* gene was determined using primer F05-F (5′-AGCTCTGTTATG-ATGCCTGC-3′), which corresponds to position 129–148 of the published *mgtCB* sequence (Snavely *et al.*, 1991a). The DNA sequence generated in this work was determined completely on both strands and has been submitted to the EMBL database under accession numbers Y13864 and AJ000509. DNA sequence alignments were conducted with the GCG software packages (University of Wisconsin Biotechnology Center, Madison, WI).

Macrophage survival assay

Macrophage survival assays were conducted with the macrophage-like cell line J774 essentially as described (Buchmeier and Heffron, 1989). Briefly, 5×10^5 macrophages in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 60 μM 6-thioguanine were allowed to adhere for 24 h in duplicate 24-well plates. Bacteria were added at a ratio of 10 bacteria per macrophage, and the plates were centrifuged at 1000 r.p.m. for 10 min at room temperature. The cells were incubated for 20 min at 37°C to permit phagocytosis, and free bacteria were removed by three washes with phosphate-buffered saline (PBS). For the time 0 sample, wells were treated immediately by aspirating the medium and adding 200 μl of 1% Triton X-100 and 800 μl of PBS. The content of each well was then transferred to a new well, appropriate dilutions were made in PBS and the number of bacteria was quantitated by plating for colony-forming units (c.f.u.) on LB agar. For the 18 h samples, DMEM supplemented with 10% FBS, 60 μM 6-thioguanine and 12 μg/ml gentamycin was added, and the cells were incubated at 37°C. When indicated, 25 mM $MgCl_2$ was added to the DMEM supplemented with gentamycin. After 18 h of infection, wells were washed twice with PBS and were treated with Triton X-100 as indicated above. The percentage survival was obtained by dividing the number of bacteria recovered after 18 h by the number of bacteria present at time 0 and multiplying by 100. All experiments were done in duplicate on at least three independent occasions.

Mouse virulence assays

Virulence assays were performed with 7- to 8-week-old female BALB/c mice inoculated i.p. with 100 μl of bacteria diluted in PBS (4–10 mice were used at each dose per mutant strain). The number of organisms injected was quantitated by plating for c.f.u. on LB agar. Viability was recorded for at least 5 weeks. Bacterial strains used for mouse virulence assays were freshly made transductants to ensure that all strains had an identical genetic background and did not accumulate mutations that would interfere with their ability to cause a lethal infection.

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References

Ahmad,S., Weisburg,W.G. and Jensen,R.A. (1990) Evolution of aromatic amino acid biosynthesis and application to the fine-tuned phylogenetic positioning of enteric bacteria. *J. Bacteriol.*, **172**, 1051–1061.

Bajaj,V., Lucas,R.L., Hwang,C. and Lee, C.A. (1996) Co-ordinate regulation of *Salmonella typhimurium* invasion genes by environmental and regulatory factors is mediated by control of *hilA* expression. *Mol. Microbiol.*, **22**, 703–714.

Blum,G., Ott,M., Lischewski,A., Ritter,A., Imrich,H., Tschäpe,H. and Hacker,J. (1994) Excision of large DNA regions termed pathogenicity islands from tRNA-specific loci in the chromosome of an *Escherichia coli* wild-type pathogen. *Infect. Immun.*, **62**, 606–614.

Bolivar,F., Rodriguez,R.L., Greene,P.J., Betlach,M.C., Heyneker,H.L. and Boyer,H.W. (1977) Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. *Gene*, **2**, 95–113.

Boyd,E.F., Wang,F.-S., Whittam,T.S. and Selander,R.K. (1996) Molecular genetic relationships of the salmonellae. *Appl. Environ. Microbiol.*, **62**, 804–808.

Buchmeier,N.A. and Heffron,F. (1989) Intracellular survival of wild-type *Salmonella typhimurium* and macrophage sensitive mutants in diverse populations of macrophages. *Infect. Immun.*, **57**, 1–7.

Buluwela,L., Forster,A., Boehm,T. and Rabbitts,T.H. (1989) A rapid procedure for colony screening using nylon filters. *Nucleic Acids Res.*, **17**, 452.

Casadaban,M.J. and Cohen,S.N. (1980) Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. *J. Mol. Biol.*, **138**, 179–207.

Davis,R.W., Bolstein,D. and Roth,J.R. (1980) *Advanced Bacterial Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Fields,P.I., Swanson,R.V., Haidaris,C.G. and Heffron,F. (1986) Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. *Proc. Natl Acad. Sci. USA*, **83**, 5189–5193.

Fields,P.I., Groisman,E.A. and Heffron,F. (1989) A *Salmonella* locus that controls resistance to microbicidal proteins from phagocytic cells. *Science*, **243**, 1059–1062.

Galán,J.E. (1996) Molecular genetic bases of *Salmonella* entry into host cells. *Mol. Microbiol.*, **20**, 263–272.

García Véscovi,E., Soncini,F.C. and Groisman,E.A. (1994) The role of the PhoP/PhoQ regulon in *Salmonella* virulence. *Res. Microbiol.*, **145**, 473–480.

García Véscovi,E., Soncini,F.C. and Groisman,E.A. (1996) Mg^{2+} as an extracellular signal: environmental regulation of *Salmonella* virulence. *Cell*, **84**, 165–174.

Garcia-del Portillo,F., Foster,J.W., Maguire,M.E. and Finlay,B.B. (1992) Characterization of the micro-environment of *Salmonella typhimurium*-containing vacuoles within MDCK epithelial cells. *Mol. Microbiol.*, **6**, 3289–3297.

Groisman,E.A. and Casadaban,M.J. (1986) Mini-mu bacteriophage with plasmid replicons for *in vivo* cloning and *lac* gene fusing. *J. Bacteriol.*, **168**, 357–364.

Groisman,E.A. and Heffron,F. (1995) Regulation of *Salmonella* virulence by two-component regulatory systems. In Hoch,J.A. and Silhavi,T.J. (eds), *Two-component Signal Transduction*. ASM Press, Washington, DC, pp. 319–332.

Groisman,E.A. and Ochman,H. (1994) How to become a pathogen. *Trends Microbiol.*, **2**, 289–294.

Groisman,E.A. and Ochman,H. (1996) Pathogenicity islands: bacterial evolution in quantum leaps. *Cell*, **87**, 791–794.

Groisman,E.A., Chiao,E., Lipps,C.J. and Heffron,F. (1989) *Salmonella typhimurium phoP* virulence gene is a transcriptional regulator. *Proc. Natl Acad. Sci. USA*, **86**, 7077–7081.

Groisman,E.A., Sturmoski,M.A., Solomon,F.R., Lin,R. and Ochman,H. (1993) Molecular, functional, and evolutionary analysis of sequences specific to *Salmonella*. *Proc. Natl Acad. Sci. USA*, **90**, 1033–1037.

Gulig,P.A., Danbara,H., Guiney,D.G., Lax,A.J., Norel,F. and Rhen,M. (1993) Molecular analysis of *spv* virulence genes of the *Salmonella* virulence plasmids. *Mol. Microbiol.*, **7**, 825–830.

Gunn,J.S., Alpuche-Aranda,C.M., Loomis,W.P., Belden,W.J. and Miller,S.I. (1995) Characterization of the *Salmonella typhimurium pagC/pagD* chromosomal region. *J. Bacteriol.*, **177**, 5040–5047.

Hacker,J., Blum-Oehler,G., Mühldorfer,I. and Tschäpe,H. (1997) Pathogenicity islands of virulent bacteria: structure, function and impact on microbial evolution. *Mol. Microbiol.*, **23**, 1089–1097.

Heithoff,D.M., Conner,C.P., Hanna,P.C., Julio,S.M., Hentschel,U. and Mahan,M.J. (1997) Bacterial infection as assessed by *in vivo* gene expression. *Proc. Natl Acad. Sci. USA*, **94**, 934–939.

Hensel,M., Shea,J.E., Bäumler,A.J., Gleeson,C., Blattner,F. and Holden,D.W. (1997) Analysis of the boundaries of *Salmonella* pathogenicity island 2 and the corresponding chromosomal region of *Escherichia coli* K-12. *J. Bacteriol.*, **179**, 1105–1111.

Hmiel,S.P., Snavely,M.D., Florer,J.B., Maguire,M.E. and Miller,C.G. (1989) Magnesium transport in *Salmonella typhimurium*: genetic characterization and cloning of three magnesium transport loci. *J. Bacteriol.*, **171**, 4742–4751.

Hohmann,E.L., Oletta,C.A., Loomis,W.P. and Miller,S.I. (1995) Macrophage-inducible expression of a model antigen in *Salmonella typhimurium* enhances immunogenicity. *Proc. Natl Acad. Sci. USA*, **92**, 2904–2908.

Inouye,S., Sunshine,M.G., Six,E.W. and Inouye,M. (1991) Retronphage ΦR73: an *E.coli* phage that contains a retroelement and integrates into a tRNA gene. *Science*, **252**, 969–971.

Klose,K.E. and Mekalanos,J.J. (1997) Simultaneous prevention of glutamine synthesis and high-affinity transport attenuates *Salmonella typhimurium* virulence. *Infect. Immun.*, **65**, 587–596.

Maloy,S.R. (1990) *Experimental Techniques in Bacterial Genetics*. Jones and Bartlett Publishers, Boston, MA.

McDaniel,T.K. and Kaper,J.B. (1997) A cloned pathogenicity island from enteropathogenic *Escherichia coli* confers the attaching and effacing phenotype on *E.coli* K-12. *Mol. Microbiol.*, **23**, 399–407.

McDaniel,T.K., Jarvis,K.G., Donnenberg,M.S. and Kaper,J.B. (1995) A genetic locus of enterocyte effacement conserved among diverse enterobacterial pathogens. *Proc. Natl Acad. Sci. USA*, **92**, 1664–1668.

Miller,J.H. (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Miller,S.I., Kukral,A.M. and Mekalanos,J.J. (1989) A two-component regulatory system (*phoP phoQ*) controls *Salmonella typhimurium* virulence. *Proc. Natl Acad. Sci. USA*, **86**, 5054–5058.

Mills,D.M., Bajaj,V. and Lee,C.A. (1995) A 40 kilobase chromosomal fragment encoding *Salmonella typhimurium* invasion genes is absent from the corresponding region of the *Escherichia coli* K-12 chromosome. *Mol. Microbiol.*, **15**, 749–759.

Ochman,H. and Groisman,E.A. (1994) The origin and evolution of species differences in *Escherichia coli* and *Salmonella typhimurium*. In Schierwater,B., Streit,B., Wagner,G.P. and DeSalle,R. (eds), *Molecular Ecology and Evolution: Approaches and Applications*. Birkhauser Verlag, New York, pp. 479–493.

Ochman,H., Soncini,F.C., Solomon,F. and Groisman,E.A. (1996) Identification of a pathogenicity island required for *Salmonella* survival in host cells. *Proc. Natl Acad. Sci. USA*, **93**, 7800–7804.

Reinhart,R.A. (1988) Magnesium metabolism: a review with special reference to the relationship between intracellular content and serum levels. *Arch. Intern. Med.*, **148**, 2415–2420.

Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanderson,K.E., Hessel,A. and Rudd,K.E. (1995) Genetic map of *Salmonella typhimurium*, edition VIII. *Microbiol. Rev.*, **59**, 241–303.

Shea,J.E., Hensel,M., Gleeson,C. and Holden,D.W. (1996) Identification of a virulence locus encoding a second type III secretion system in *Salmonella typhimurium*. *Proc. Natl Acad. Sci. USA*, **93**, 2593–2597.

Snavely,M.D., Miller,C.G. and Maguire,M.E. (1991a) The *mgtB* Mg^{2+} transport locus of *Salmonella typhimurium* encodes a P-type ATPase. *J. Biol. Chem.*, **266**, 815–823.

Snavely,M.D., Gravina,S.A., Cheung,T.-B.T., Miller,C.G. and Maguire,M.E. (1991b) Magnesium transport in *Salmonella typhimurium*: regulation of *mgtA* and *mgtB* expression. *J. Biol. Chem.*, **266**, 824–829.

Soncini,F.C., García Véscovi,E., Solomon,F. and Groisman,E.A. (1996) Molecular basis of the magnesium deprivation response in *Salmonella typhimurium*: identification of PhoP-regulated genes. *J. Bacteriol.*, **178**, 5092–5099.

Sun,J., Inouye,M. and Inouye,S. (1991) Association of a retroelement with a P4-like cryptic prophage (retronphage ΦR73) integrated into the selenocystyl tRNA gene of *Escherichia coli*. *J. Bacteriol.*, **173**, 4171–4181.

Swenson,D.L., Bukanov,N.O., Berg,D.E. and Welch,R.A. (1996) Two pathogenicity islands in uropathogenic *Escherichia coli* J96: cosmid cloning and sample sequencing. *Infect. Immun.*, **64**, 3736–3743.

Tao,T., Snavely,M.D., Farr,S.G. and Maguire,M.E. (1995) Magnesium transport in *Salmonella typhimurium*: *mgtA* encodes a P-type ATPase and is regulated by Mg^{2+} in a manner similar to that of the *mgtB* P-type ATPase. *J. Bacteriol.*, **177**, 2654–2662.

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