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Molecular Basis of the Magnesium Deprivation Response in *Salmonella typhimurium*: Identification of PhoP-Regulated Genes

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The PhoP-PhoQ two-component system is essential for virulence in *Salmonella typhimurium*. This system controls expression of some 40 different proteins, yet most PhoP-regulated genes remain unknown. To identify PhoP-regulated genes, we isolated a library of 50,000 independent *lac* gene transcriptional fusion strains and investigated whether production of β-galactosidase was regulated by PhoP. We recovered 47 *lac* gene fusions that were activated and 7 that were repressed when PhoP was expressed. Analysis of 40 such fusions defined some 30 loci, including *mgtA* and *mgtCB*, which encode two of the three Mg^{2+} uptake systems of *S. typhimurium*; *ugd*, encoding UDP-glucose dehydrogenase; *phoP*, indicative that the *phoPQ* operon is autoregulated; and an open reading frame encoding a protein with sequence similarity to VanX, a dipeptidase required for resistance to vancomycin. Transcription of PhoP-activated genes was regulated by the levels of Mg^{2+} in a PhoP-dependent manner. Strains with mutations in *phoP* or *phoQ* were defective for growth in low-Mg^{2+} media. The *mgtA* and *mgtCB* mutants reached lower optical densities than the wild-type strain in low-Mg^{2+} liquid media but displayed normal growth on low-Mg^{2+} solid media. Six PhoP-activated genes were identified as essential to form colonies on low-Mg^{2+} solid media. Cumulatively, our experiments establish that the PhoP-PhoQ system governs the adaptation to magnesium-limiting environments.

PhoP-PhoQ is a two-component regulatory system that controls expression of several virulence properties in the gram-negative bacterium *Salmonella typhimurium* (13, 31, 32). The PhoQ protein is a sensor kinase that responds to changes in environmental magnesium concentration by modifying the ability of the PhoP protein to transcribe several genes (18). Virulence properties governed by the PhoP-PhoQ system include the ability to survive inside macrophages (14), to resist killing by host defense antimicrobial peptides (13, 22, 23), to withstand an acid pH (15), to invade epithelial cells (5), to form spacious vacuoles (1), and to alter antigen presentation (48).

It has been estimated that PhoP controls expression of some 40 different proteins (32). However, only four PhoP-regulated loci have been characterized at the molecular level: *phoN*, which encodes a nonspecific acid phosphatase that localizes to the periplasmic space (24, 28); *pagC*, which encodes an outer membrane protein necessary for intramacrophage survival (35); *pagD*, encoding an envelope protein with no apparent role in virulence (25); and *prgHIJK*, a PhoP-repressed operon encoding components of a secretion apparatus required for invasion of epithelial cells (5, 34).

While the PhoP-PhoQ system is essential for *Salmonella* pathogenicity, there are two lines of evidence suggesting that this system is also required in environments encountered outside animal hosts. First, *phoP* homologs have been found in *Escherichia coli* and other bacterial species which are normally nonpathogenic (20, 22). Second, the majority of PhoP-regulated loci identified to date, including some which are *Salmonella* specific, are not required to cause a lethal infection in mice (17, 21).

In this paper, we report the identification and characterization of a novel set of PhoP-regulated loci. We demonstrate that magnesium is the signal that controls transcription of the PhoP regulon, and we establish that different sets of PhoP-activated genes are required for growth in solid and liquid magnesium-limiting environments.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were grown at 37°C in Luria broth (LB) (30) or in N-minimal medium containing 0.1% Casamino Acids, 38 mM glycerol, and 10 μM or 10 mM $MgCl_2$ (41). N-minimal medium–agarose plates were prepared as indicated previously (18). Ampicillin and kanamycin were each used at 50 μg/ml. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and isopropyl thiogalactopyranoside (IPTG) were used at final concentrations of 70 μg/ml and 0.1 mM, respectively.

The ability of cells to grow in low-Mg^{2+} liquid media was evaluated as follows. Overnight cultures grown in N-minimal medium supplemented with 10 mM $MgCl_2$ were washed three times with Mg^{2+}-free medium and diluted to an optical density at 600 nm (OD_{600}) of 0.010 in their respective culture media. The OD_{600} was determine at different times postinoculation. To determine the number of CFU, cells diluted in cold phosphate-buffered saline (PBS) were spread on LB agar plates. To test for bacterial viability, we used the fluorescence-based LIVE/DEAD *Bac*Light viability assay (Molecular Probes, Inc.) as instructed by the manufacturer.

Genetic and molecular biology techniques and enzymatic assays. Phage P22-mediated transductions were performed as described previously (10). 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 (37). Genomic libraries were constructed by ligating *Sal*I-digested chromosomal DNA to *Sal*I-cut plasmid pUC19. Clones harboring the junction fragments were selected as kanamycin (MudJ)- and ampicillin (pUC19)-resistant colonies. (We cloned the junction fragments for 40 mutants but were unable to isolate the junction fragments corresponding to 14 other mutants.) To determine the chromosomal DNA sequence adjacent to each MudJ insertion, we used a primer complementary

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TABLE 1. Bacterial strains and plasmids used

Bacterial strain or plasmid	Description[a]	Reference
S. typhimurium		
14028s	Wild type	14
MS7953s	*phoP7953*::Tn*10*	13
MS5996s	*phoQ5996*::Tn*10*	13
TT10288	*hisD9953*::MudJ *hisA9944*::MudI	26
EG9521	*mgtA9226*::MudJ	18
EG9523	*mgtA9226*::MudJ *phoP7953*::Tn*10*	18
EG9227	*pbgB1*::MudJ	This work
EG9085	*pbgB1*::MudJ *phoP7953*::Tn*10*	This work
EG9524	*ugd9228*::MudJ	18
EG9526	*ugd9228*::MudJ *phoP7953*::Tn*10*	18
EG9229	*pbgD1*::MudJ	This work
EG9110	*pbgD1*::MudJ *phoP7953*::Tn*10*	This work
EG9230	*pbgE1*::MudJ	This work
EG9112	*pbgE1*::MudJ *phoP7953*::Tn*10*	This work
EG9231	*pbgF1*::MudJ	This work
EG9115	*pbgF1*::MudJ *phoP7953*::Tn*10*	This work
EG9527	*mgtC9232*::MudJ	18
EG9529	*mgtC9232*::MudJ *phoP7953*::Tn*10*	18
EG9238	*pbgM1*::MudJ	This work
EG9136	*pbgM1*::MudJ *phoP7953*::Tn*10*	This work
EG9240	*pbgO1*::MudJ	This work
EG9165	*pbgO1*::MudJ *phoP7953*::Tn*10*	This work
EG9241	*pbgP1*::MudJ	This work
EG9168	*pbgP1*::MudJ *phoP7953*::Tn*10*	This work
EG9248	*pbgW1*::MudJ	This work
EG9212	*pbgW1*::MudJ *phoP7953*::Tn*10*	This work
EG9249	*pbgX1*::MudJ	This work
EG9215	*pbgX1*::MudJ *phoP7953*::Tn*10*	This work
EG9250	*pbgY1*::MudJ	This work
EG9218	*pbgY1*::MudJ *phoP7953*::Tn*10*	This work
EG9277	*pcgD1*::MudJ	This work
EG9278	*pcgD1*::MudJ *phoP7953*::Tn*10*	This work
EG9279	*pcgE1*::MudJ	This work
EG9280	*pcgE1*::MudJ *phoP7953*::Tn*10*	This work
EG9530	*pcgF9281*::MudJ	18
EG9558	*pcgF9281*::MudJ *phoP7953*::Tn*10*	18
EG9532	*pcgG9283*::MudJ	18
EG9534	*pcgG9283*::MudJ *phoP7953*::Tn*10*	18
EG9312	*pcgH1*::MudJ	This work
EG9313	*pcgH1*::MudJ *phoP7953*::Tn*10*	This work
EG9327	*pcgJ1*::MudJ	This work
EG9328	*pcgJ1*::MudJ *phoP7953*::Tn*10*	This work
EG9331	*pcgL1*::MudJ	This work
EG9332	*pcgL1*::MudJ *phoP7953*::Tn*10*	This work
EG9335	*pcgN1*::MudJ	This work
EG9336	*pcgN1* MudJ *phoP7953*::Tn*10*	This work
EG9339	*pcgP1*::MudJ	This work
EG9340	*pcgP1*::MudJ *phoP7953*::Tn*10*	This work
EG9341	*pcgQ1*::MudJ	This work
EG9342	*pcgQ1*::MudJ *phoP7953*::Tn*10*	This work
EG9632	*psgA1*::MudJ	This work
EG9633	*psgA1*::MudJ *phoP7953*::Tn*10*	This work
EG9635	*psgB1*::MudJ	This work
EG9636	*psgB1*::MudJ *phoP7953*::Tn*10*	This work
EG9638	*psgC1*::MudJ	This work
EG9639	psgC1::MudJ *phoP7953*::Tn*10*	This work
EG9641	psgD1::MudJ	This work
EG9642	psgD1::MudJ *phoP7953*::Tn*10*	This work
EG9185	psgE1::MudJ	This work
EG9186	psgE1::MudJ *phoP7953*::Tn*10*	This work
EG9188	psgF1::MudJ	This work
EG9189	psgF1::MudJ *phoP7953*::Tn*10*	This work
EG9223	psgG1::MudJ	This work
EG9224	psgG1::MudJ *phoP7953*::Tn*10*	This work
E. coli JM109	F′ *traD36 lacI*^{q} Δ(*lacZ*)*M15 proA*$^+$*B*$^+$/ *e14*$^-$ (*McrA*$^-$) Δ(*lac-proAB*) *thi gyrA96* (Nal^r) *endA1 hsdR17*	49
Plasmids		
pUHE21-2lacI^q	rep$_{pMB1}$ Ap^r *lacI*^{q}	43
pEG9014	rep$_{pMB1}$ Ap^r *lacI*^{q} *phoP*	43
pUC19	rep$_{ColE1}$ *lacZa* Ap^r *lacI*^{q}	49

[a] Gene designations are as summarized by Sanderson et al. (38).

to the left end of MudJ and the plasmid clones harboring the junctions as templates.

β-Galactosidase activity was determined as described by Miller (30) on cells grown in LB broth or N-minimal medium and harvested in the stationary phase.

Electron microscopy. A 10-μl drop of bacterial suspension in PBS was applied to a carbon-coated grid and incubated for 2 min. Excess fluid was removed, and the specimen was rinsed with distilled water. Negative staining was performed as described previously (44) with 0.5% (wt/vol) aqueous uranyl acetate for 1 min.

RESULTS

Identification of PhoP-regulated genes. The environmental signal controlling the PhoP-PhoQ system was unknown when we started our experiments. Therefore, we searched for PhoP-regulated genes by using a strategy based solely on the differential transcriptional activities of individual genes in the presence and absence of PhoP. First, we constructed a strain (MS7953s/pEG9014) harboring a Tn*10* transposon insertion in the chromosomal copy of *phoP* and a plasmid with the wild-type *phoP* gene under control of the *lac* promoter. Then, we mutagenized strain MS7953s/pEG9014 with MudJ, a derivative of bacteriophage Mu that harbors a gene conferring resistance to kanamycin and a promoterless *lac* operon for the generation of transcriptional fusions (7). MudJ was delivered to MS7953s/pEG9014 by phage P22-mediated transduction (26), and kanamycin-resistant transductants were isolated on LB agar plates containing kanamycin and ampicillin. Purified colonies were patched onto two LB agar plates containing kanamycin and X-Gal. One of these plates also contained IPTG to promote *phoP* transcription from the *lac* promoter. Clones displaying differences in β-galactosidase activity between the two plates were candidates for harboring MudJ insertions in PhoP-regulated genes.

We tested 50,000 mutants and found 54 that exhibited PhoP-regulated expression of β-galactosidase. Forty-seven mutants displayed higher levels of β-galactosidase when PhoP was induced and thus harbored insertions in PhoP-activated genes. Seven mutants expressed lower levels of β-galactosidase upon PhoP induction, indicating that they carried MudJ insertions in PhoP-repressed loci. Phage P22 lysates were prepared in each of the 54 mutants, and MudJ was transduced into wild-type and *phoP*::Tn*10* strains. We investigated the Lac phenotypes of the resulting isogenic strains by streaking them side by side on LB X-Gal plates; in all mutants but one, Lac expression was PhoP regulated. This experiment confirmed that the MudJ insertions were indeed in PhoP-regulated loci: PhoP had a regulatory effect not only when expressed from the *lac* promoter in plasmid pEG9014 but also when expressed from its own promoter in the chromosome. The mutant that exhibited PhoP-regulated expression only when *phoP* was expressed from the plasmid harbored a MudJ insertion in the promoter region of the *phoPQ* operon, indicating that this locus is transcriptionally autoregulated (43). (We have followed the nomenclature pro-

TABLE 2. PhoP-regulated genes identified in this study

Gene[a]	Map location[a]	Comment[b]	Reference(s)
phoPQ	27.4	Two-component system	43
mgtA	96.7	Mg^{2+} transporter	45
pbgB		*mar* locus	8
ugd	44.9	UDP-glucose dehydrogenase	3, 4
mgtCB	82.1	Mg^{2+} transporter	42
pcgF		*orf1-nhoA*	47
pcgL		Similarity to *vanX* gene product	36

[a] As summarized by Sanderson et al. (38).
[b] Refers to properties of gene or encoded gene product.

posed by Miller et al. [31], who referred to PhoP-activated and -repressed genes as *pag* and *prg*, respectively. We have used the prefixes *pbg* and *pcg* for PhoP-activated gene fusions and *psg* for PhoP-repressed fusions to prevent designation of different genes with the same name.)

Molecular characterization of PhoP-regulated loci. To establish the identity of the PhoP-regulated loci, we cloned the DNA fragments harboring the junction between the left end of MudJ and the *Salmonella* chromosome for 40 mutants. Then, we determined the nucleotide sequence of the region adjacent to the MudJ and used that sequence to search the sequence databases for related genes or proteins. Eleven mutants harbored MudJ insertions in the *mgtA* gene, and one harbored MudJ insertions in the *mgtCB* locus (Table 2). The *mgtA* and *mgtB* genes encode two of the three Mg^{2+} uptake systems of *S. typhimurium* (42, 45). The *mgtC* gene, which precedes *mgtB* in the *mgtCB* operon, encodes a protein of unknown function that is not required for Mg^{2+} uptake (45). The discovery that two Mg^{2+} transporters are transcriptionally controlled by PhoP led to the identification of Mg^{2+} as the signal sensed by the PhoQ protein (18).

One mutant harbored a MudJ insertion in an open reading frame encoding a protein homologous to the UDP-glucose dehydrogenase of *Streptococcus pneumoniae* (11, 12). This gene, which we have designated *ugd*, is located 143 bp upstream of *cld* in the *rfb* gene cluster at the 44.9-min region of the *S. typhimurium* chromosome. We recovered a MudJ in a region that exhibits 85% sequence identity with the *E. coli mar* locus (8). This locus comprises 27 putative genes and controls resistance to several antibiotics (19). However, the MudJ inserted between two open reading frames (ORF180 and ORF66 in reference 8) whose role in antibiotic resistance has not been investigated. We localized a MudJ insertion to an open reading frame encoding a protein with sequence similarity to VanX (36). The *vanX* gene, which is part of the enterococcal transposon Tn*1546*, encodes a dipeptidase that is necessary for resistance to vancomycin. Finally, two mutants harbored MudJ insertions at different positions of an open reading frame located 284 bp upstream of the *nhoA* gene of *S. typhimurium*. Whereas *nhoA* encodes an *N*-hydroxyarylamine *O*-acetyltransferase (47), the function of the PhoP-regulated open reading frame remains unknown.

Expression of PhoP-regulated genes in LB media. To determine the magnitude of the PhoP regulatory effect, we examined the transcriptional activity of 30 different *lac* gene fusions to PhoP-regulated loci in isogenic wild-type and *phoP* strains. We chose these 30 fusions because they represent different loci as determined by sequence analysis and/or map location in the chromosome. The β-galactosidase activity expressed by PhoP-regulated genes ranged from 7 to 524 Miller units for PhoP-activated genes (Fig. 1A) and from 5 to 81 Miller units for PhoP-repressed loci (Fig. 1B). The ratio of β-galactosidase activities between isogenic wild-type and *phoP* strains ranged from 4 to 100 (median value of 19) for PhoP-activated genes (Fig. 1A) but was only 1.5 to 7 for PhoP-repressed loci (Fig. 1B). These experiments establish that the PhoP protein can regulate transcription over a wide range.

Mg^{2+} governs expression of the PhoP regulon. We investigated whether the PhoP-regulated genes identified in our screening displayed the same regulatory behavior as *mgtA* and *mgtCB*, which are known to be transcriptionally activated under Mg^{2+}-limiting conditions (18, 41). Except for *pcgJ*, all PhoP-activated genes were transcriptionally regulated by the concentration of Mg^{2+} in the medium (Fig. 2). The ratio of β-galactosidase activity for cells grown in defined medium with 10 μM Mg^{2+} versus 10 mM Mg^{2+} ranged from 4 to 287 (mean value of 81). The activation that results from Mg^{2+} limitation is PhoP dependent because it was not observed in isogenic

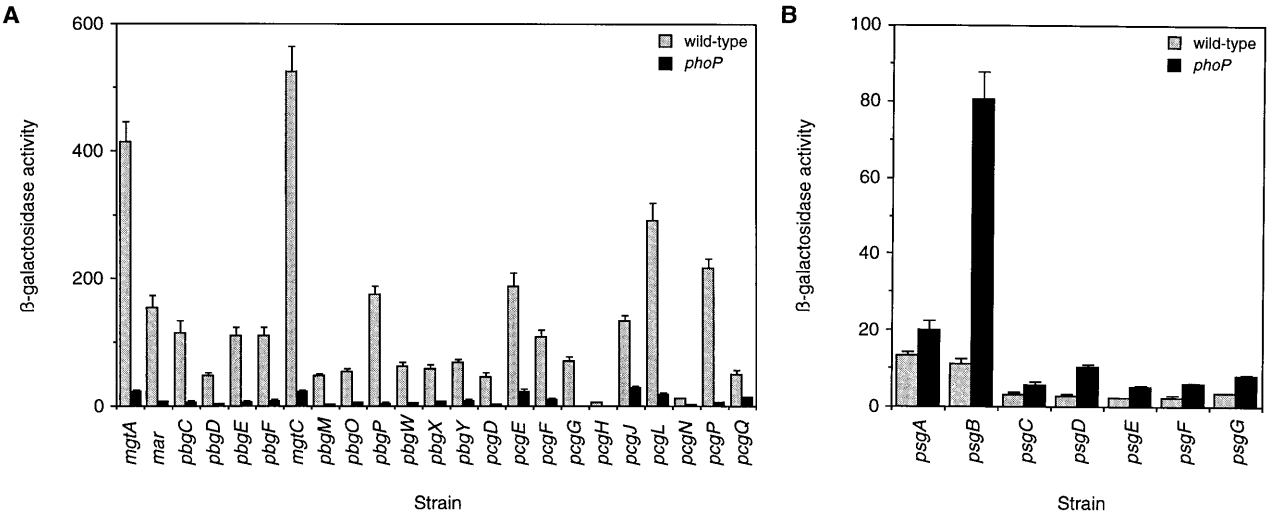

FIG. 1. Transcriptional activities of PhoP-regulated genes. β-Galactosidase activities (Miller units) expressed by strains grown in LB broth were determined in mutants harboring a *lac* transcriptional fusion to PhoP-activated genes (A) or to PhoP-repressed genes (B). Transcriptional activity was investigated in two different backgrounds, wild type and *phoP*. The data correspond to mean values of at least three independent experiments done in duplicate.

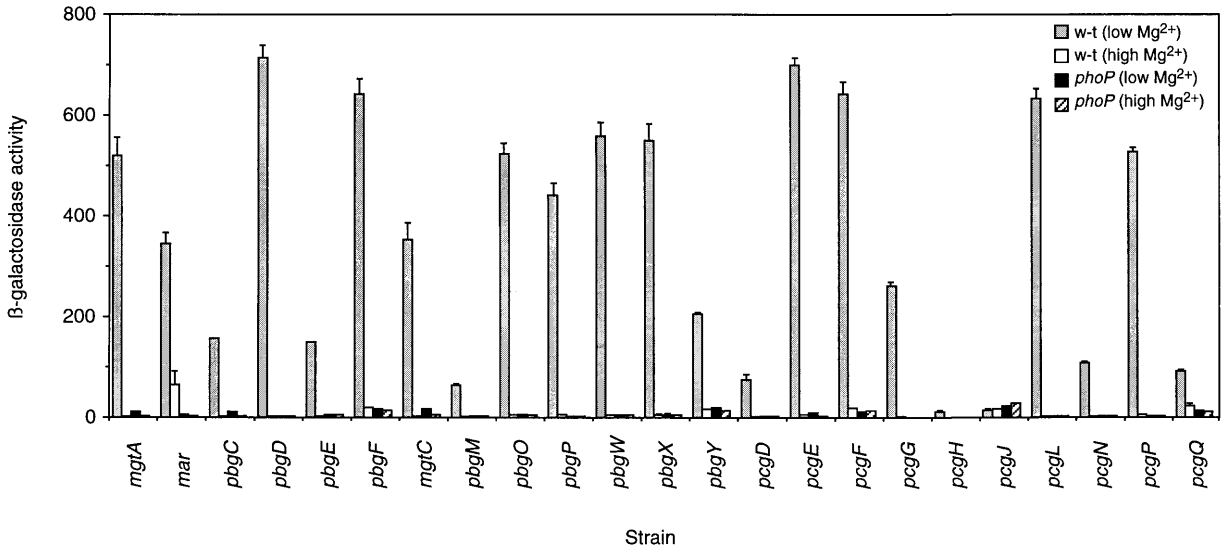

FIG. 2. Mg^{2+} controls expression of PhoP-activated genes. β-Galactosidase activity expressed by cells grown in N-minimal medium with the addition of 10 μM or 10 mM $MgCl_2$ was determined in strains harboring a *lac* transcriptional fusion to PhoP-activated genes. The transcriptional activity was investigated in two different backgrounds, wild type (w-t) and *phoP*. Assays were performed as described in the legend to Fig. 1.

phoP derivatives. Moreover, the β-galactosidase activity displayed by isogenic *phoP* mutants (grown in either 10 μM or 10 mM Mg^{2+}) is similar to that exhibited by *phoP*$^+$ cells grown in 10 mM Mg^{2+}. These results demonstrate that Mg^{2+} limitation is the signal that induces transcription of PhoP-activated genes.

The *psgB* and *psgD* genes were chosen to investigate the regulatory effects of Mg^{2+} on PhoP-repressed loci because they displayed the highest ratio of expression between *phoP* and wild-type cells grown in LB broth (Fig. 1B). By definition, PhoP-repressed genes should be maximally expressed under conditions that result in minimal expression of PhoP-activated genes: either in the absence of PhoP or in the presence of millimolar concentrations of Mg^{2+}. However, we did not find significant derepression of *psgB* or *psgD* when these mutants were grown in millimolar Mg^{2+} concentrations (data not shown).

Different PhoP-activated genes are required for normal growth in solid and liquid low-Mg^{2+} media. We have previously demonstrated that neither *phoP* nor *phoQ* mutants can form colonies on N-minimal medium–agarose plates containing <40 μM Mg^{2+} (18). To identify the PhoP-regulated gene(s) responsible for this phenotype, we streaked individual mutants onto N-minimal medium–agarose plates containing different concentrations of Mg^{2+}. Surprisingly, strains with mutations in either *mgtA* or *mgtCB* exhibited wild-type growth on low-Mg^{2+} media despite these genes encoding high-affinity magnesium transporters, and even a mutant with transposon insertions in both *mgtA* and *mgtCB* grew like the wild-type strain in low-Mg^{2+} media (data not shown). On the other hand, strains harboring mutations in either of six other PhoP-activated loci (*ugd*, *pbgE*, *pbgM*, *pbgN*, *pcgD*, and *pcgP*) could not form colonies on media containing <40 μM Mg^{2+}. However, these mutants grew better than the *phoP* strain when tested at higher Mg^{2+} concentrations (Fig. 3).

We noticed that *phoP* mutants reached a lower optical density than the wild-type strain when grown in micromolar concentrations of Mg^{2+} liquid media. We investigated the behavior of the *phoP* mutant and established that its growth rate was indistinguishable from that of the wild-type strain during logarithmic growth (Fig. 4A). However, at 10 h postinoculation, the OD_{600} of the wild-type strain continued to increase but that of the *phoP* mutant reached a plateau and then decreased from a maximal value of 0.25 to 0.18. This defect was due to the low concentration of Mg^{2+} in the media because wild-type and *phoP* strains had identical growth phenotypes in 10 mM Mg^{2+} media (Fig. 4B).

The growth defect displayed by the *phoP* mutant was not entirely surprising since we had previously shown that it could not form colonies in low-Mg^{2+} solid media. However, all six PhoP-regulated genes that were essential to form colonies in low-Mg^{2+} solid media were dispensable for growth in low-Mg^{2+} liquid media (Fig. 4A and data not shown). On the other hand, strains with mutations in the *mgtA* or *mgtCB* locus ex-

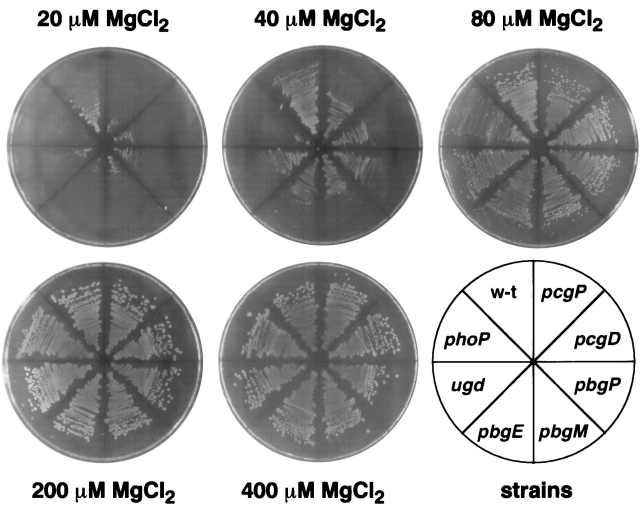

FIG. 3. Growth properties of mutants harboring insertions in PhoP-activated genes on low-Mg^{2+} solid media. Bacterial strains (wild type [w-t; 14028s], *phoP* [MS7953s], *ugd* [EG9524], *pbgE* [EG9230], *pbgM* [EG9238], *pbgP* [EG9241], *pcgD* [EG9277], and *pcgP* [EG9339]) were streaked onto N-minimal medium–agarose plates, with the addition of $MgCl_2$ to a final concentration of 20, 40, 80, 200, or 400 μM, and incubated at 37°C for 36 h before photography.

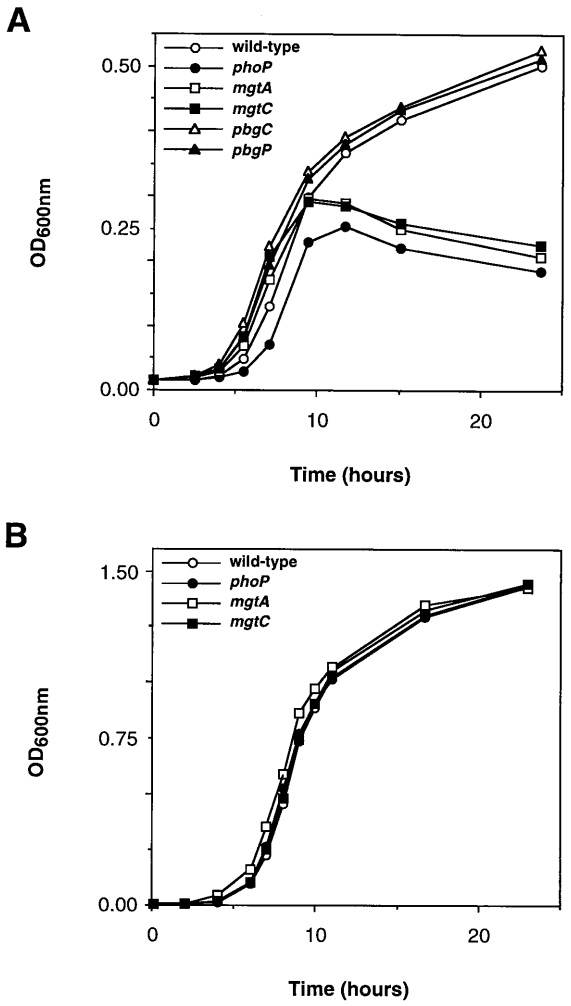

FIG. 4. Growth properties of mutants harboring insertions in PhoP-activated genes in low-Mg^{2+} liquid media. Bacterial strains (wild type [14028s], *phoP* [MS7953s], *mgtA* [EG9521], *ugd* [EG9524], *mgtCB* [EG9527], and *pbgP* [EG9241]) were inoculated in N-minimal medium containing 10 μM (A) or 10 mM (B) $MgCl_2$ to an OD_{600} of 0.010. The data correspond to a representative experiment performed in duplicate.

hibited a defective growth phenotype that was similar to that of the *phoP* mutant (Fig. 4A). (Because the MudJ insertion in *mgtC* is predicted to be polar on *mgtB*, the growth defect of the *mgtC*::MudJ strain could be due to the absence of MgtC and/or MgtB.) Taken together these results indicate that different subsets of PhoP-activated genes are required for growth in solid and liquid low-Mg^{2+} environments.

Mutants defective in *phoP*, *mgtA*, or *mgtCB* adopt an altered cell morphology in low-Mg^{2+} media. We examined the viability of the *phoP*, *mgtA*, and *mgtCB* mutants when these cells reached stationary phase in low (10 μM)-Mg^{2+} media. At 15 h postinoculation, there was a significant decrease in the number of CFU for these mutants which was not reflected in the optical density of the cultures (Table 3). At that time, the mutants lost the ability to form colonies but were still alive, as determined by a fluorescence-based cell viability assay. The inability to give rise to colonies was associated with an altered cell morphology (Fig. 5). Whereas the wild-type strain retained its characteristic rod shape with an average length between poles of 1.6 μm (ranging from 1.35 to 2.25 μm for 42 cells examined), strains with mutations in either *phoP*, *mgtA*, or *mgtCB* were 10 to 20 times larger, with an average length between poles of 4.2 μm (ranging from 2.15 to 7.2 μm for 59 cells examined). A small percentage of the cells in the cultures of *mgtA* and *mgtCB* strains were very elongated, with lengths of up to 120 μm (data not shown). These results show that the inability to express the Mg^{2+} transporters not only impairs growth in low-Mg^{2+} media but also affects the morphology of the cell.

DISCUSSION

The PhoP-PhoQ system was originally identified as necessary for expression of a nonspecific acid phosphatase (29) and was later shown to be essential for virulence in *S. typhimurium* (13, 14, 31). We have now established that PhoP-PhoQ governs expression of a large set of genes that are essential for growth in Mg^{2+}-limiting environments. This conclusion is based on the following findings: (i) the concerted transcriptional regulation of 25 PhoP-activated genes by the Mg^{2+} levels in the growth media; (ii) the identification of the Mg^{2+} transporter genes *mgtA* and *mgtCB* as PhoP regulated; (iii) and the requirement for eight PhoP-activated loci for growth in low-Mg^{2+} media.

We have now established that transcription of 18 PhoP-activated genes is induced in micromolar Mg^{2+} concentrations and repressed in millimolar concentrations of this ion (Fig. 2A). These genes are in addition to the seven PhoP-activated genes that were previously shown to be transcriptionally regulated by the levels of extracellular Mg^{2+} (18). Several environmental conditions (i.e., oxygen levels, phosphate starvation, and acid pH) had been shown to modulate expression of particular PhoP-regulated genes (2, 5, 6, 20). However, none of these conditions controlled expression of the PhoP regulon in a concerted manner, like Mg^{2+} does. For example, lowering the pH from 7.4 to 6.1 induces transcription of the *psiD* gene (18) but not that of the *mgtA* gene (our unpublished results). Likewise, growth in anaerobic conditions has opposite effects on different PhoP-repressed genes: it stimulates transcription of *prgB* but represses expression of *prgH* (5).

Magnesium limitation induced transcription of PhoP-activated genes to different extents (Fig. 2A), which could reflect differences in the PhoP-binding sites among PhoP-activated genes. For example, the dissimilar expression of five PrfA-regulated genes of *Listeria monocytogenes* has been attributed to the relative affinity of PrfA for its binding sites (39). Alternatively or in addition, expression of certain PhoP-activated genes may require a regulatory factor(s) besides PhoP. This

TABLE 3. Correlation between optical density and CFU for strains grown in low-Mg^{2+} liquid medium

Bacterial strain	Time (h)	OD_{600}	No. of CFU	Cell index[a]
Wild type	7	0.13	1.1×10^8	84.6
	15	0.41	4.2×10^8	102.4
	23	0.50	5.8×10^8	116.0
phoP	7	0.07	6.2×10^7	88.6
	15	0.22	1.7×10^7	7.7
	23	0.18	2.1×10^6	1.0
mgtA	7	0.17	1.4×10^8	82.3
	15	0.25	6.1×10^7	24.4
	23	0.20	2.8×10^7	14.0
mgtCB	7	0.21	1.9×10^8	90.5
	15	0.26	2.5×10^7	9.6
	23	0.23	2.1×10^7	9.1

[a] Determined dividing the CFU by the OD_{600} per 10^7 at each time. The data correspond to a representative experiment performed in duplicate.

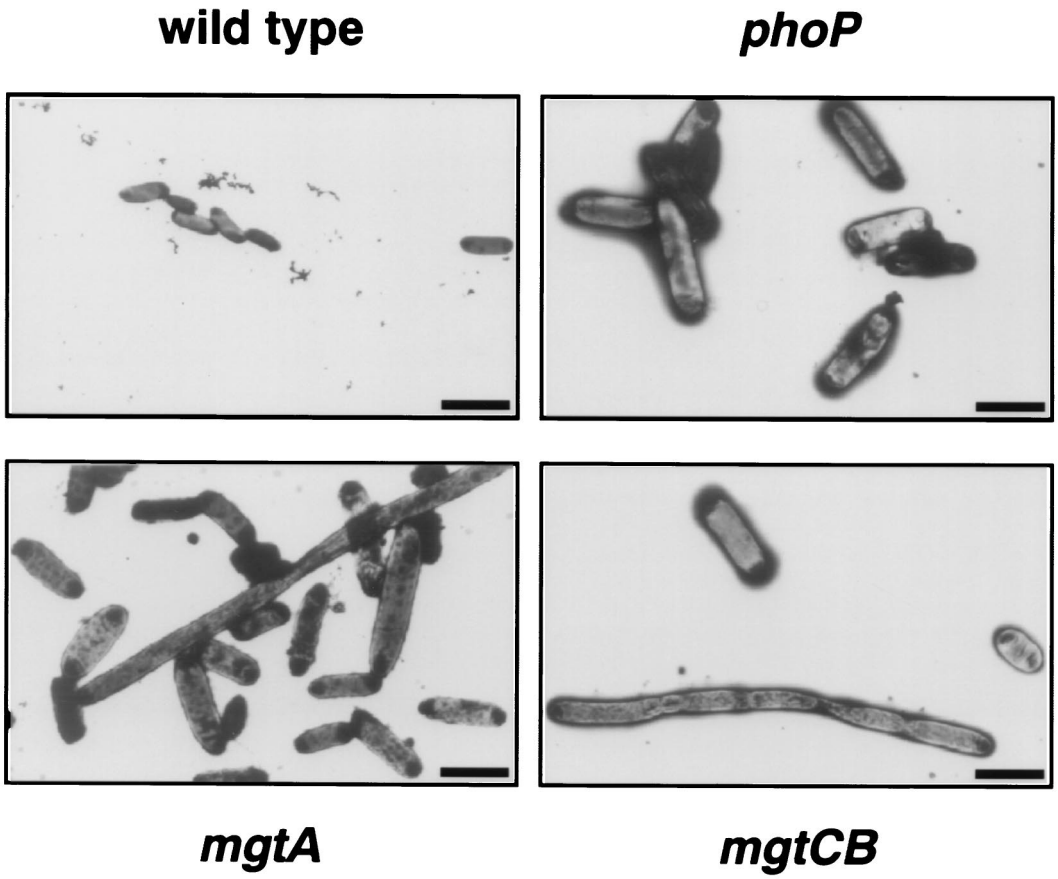

FIG. 5. Morphologies of *Salmonella* strains harboring mutations in the PhoP-controlled Mg^{2+} uptake systems. Electron micrographs show negative-stained strains (wild type [14028s], *phoP* [MS7953s], *mgtA* [EG9521], and *mgtCB* [EG9527]) grown for 36 h in N-minimal liquid medium containing 10 μM $MgCl_2$ at 37°C. Bars, 3 μm. The figure shows differences in size and morphology between the wild-type strain and strains with mutations in the genes encoding the transcriptional regulator PhoP or the Mg^{2+} transporter MgtA or MgtCB.

factor(s) could mediate the response to environmental conditions (other than Mg^{2+}) which are known to modulate transcription of particular PhoP-regulated genes.

Expression of the PhoP-activated *pcgJ* and of the PhoP-repressed *psgB* and *psgD* genes provided an interesting exception to the profile of most PhoP-activated loci: transcription of these genes was not regulated by the levels of Mg^{2+} in the media (Fig. 2). PhoP was necessary for *pcgJ* transcription in LB broth (Fig. 1), and no *pcgJ* expression was detected in defined media, even in $phoP^+$ cells (Fig. 2A). These results suggest that, in addition to PhoP, a component of LB (or that is produced in LB) is required for *pcgJ* expression. On the other hand, transcription of the PhoP-repressed *psgB* and *psgD* genes could not be induced by growth at millimolar concentrations of Mg^{2+}. This could be accounted for if this condition produced levels of PhoP that were sufficient to prevent transcription of PhoP-repressed loci but not to induce expression of PhoP-activated genes.

Consistent with Mg^{2+} deprivation being the signal that activates the PhoP-PhoQ system, the *phoP* and *phoQ* mutants are impaired for growth in low-Mg^{2+} media (18). We have now demonstrated that different subsets of PhoP-activated genes are required for growth in liquid and solid media. Strains with mutations in either *mgtA* or *mgtCB* mimicked the behavior of *phoP* mutants in low-Mg^{2+} liquid media: normal logarithmic growth followed by an early onset of stationary phase. All three mutants lost the ability to form colonies once they reached stationary phase (Fig. 4 and Table 3) and adopted an altered cell morphology with single-cell sizes that were 10 to 20 times that of the wild-type strain (Fig. 5). MgtA and MgtB are P-type ATPases that display 50% amino acid sequence identity (75% similarity) to each other (45). Given the ability of MgtA and MgtB to independently take up Mg^{2+}, it is not clear why mutations in just one of these transporters would result in a growth defect. One possibility is that both MgtA and MgtB are necessary to maintain physiological levels of Mg^{2+} in the cytoplasm. Alternatively, the growth defect may be due to the inability of these transporters to take up or export a ligand(s) that is cotransported along with Mg^{2+}.

Growth in low-Mg^{2+} solid media did not require *mgtA* or *mgtCB* but required a different subset of six PhoP-activated genes. That these genes are dispensable for growth in low-Mg^{2+} liquid media suggests that they encode products that are likely to modify the surface (rather than the cytoplasm) of the microbial cell. Indeed, one of these genes, *ugd*, encodes a protein that is homologous to the UDP-glucose dehydrogenase of *Streptococcus pneumoniae*, in which it is necessary for capsule biosynthesis (11, 12). The *ugd* gene maps to one of the gene clusters required for lipopolysaccharide (LPS) biosynthesis in *S. typhimurium*, *E. coli*, and *Shigella flexneri* (3, 4, 33). While *ugd* lies upstream of the *cld* gene, which controls the length of the O-antigen side chain, no differences have been detected between the LPS profiles of wild-type and *phoP S. typhimurium* (16). Thus, the nature of the surface modifica-

tion(s) mediated by *ugd* gene product that allows growth in low-Mg^{2+} solid media is yet to be determined.

The remaining five genes which are essential for growth on low-Mg^{2+} solid media showed no sequence similarity to known genes. While their nucleotide sequences and map positions indicate that they correspond to different loci, it is possible that they encode products that are part of the same complex or metabolic pathway that allows *S. typhimurium* to form colonies on low-Mg^{2+} environments. Magnesium is necessary to stabilize the bacterial outer membrane by interacting with the phosphate groups in the LPS (9). In low-Mg^{2+} environments, charge repulsion between LPS molecules within a cell and between cells are predicted to increase; thus, the PhoP-activated genes *ugd*, *pbgE*, *pbgM*, *pbgN*, *pcgD*, and *pcgP* may encode products that reduce these repulsive forces and allow the formation of bacterial colonies. Examples of changes in the surface charge that modify cell-cell interaction have been found in the yeast *Saccharomyces cerevisiae* (40). The ability of *S. cerevisiae* to flocculate at the end of the fermentation process is due to an increase in both the cell surface hydrophobicity and the Ca^{2+}-mediated lectin-sugar binding sites (40). Another example of modification in cell-cell interaction has been found in neutrophils. In these cells, repulsive forces driven by a negatively charged sialoprotein (CD43) maintain neutrophils at a certain distance from each other in the blood flow. The elimination of this charged protein from the surface of the cell, triggered by inflammatory signals, allows them to interact with each other resulting in adhesion to and migration through endothelial cells (36).

The widespread phylogenetic distribution of the *phoPQ* genes in enteric bacteria (20), including species which are normally nonpathogenic, suggests that the primary role of PhoP-PhoQ system is to mediate the adaptation to low-Mg^{2+} environments rather than virulence per se. Indeed, the PhoP-activated genes *mgtA* and *ugd*, which are required for growth in low Mg^{2+}, are also present in the *E. coli* K-12 genome.

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