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Molecular Characterization of the PhoP-PhoQ Two-Component System in *Escherichia coli* K-12: Identification of Extracellular Mg^{2+}-Responsive Promoters

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We identified Mg^{2+}-responsive promoters of the *phoPQ*, *mgtA*, and *mgrB* genes of *Escherichia coli* K-12 by S1 nuclease analysis. Expression of these genes was induced by magnesium limitation and depended on PhoP and PhoQ. The transcription start sites were also determined, which allowed us to find a (T/G)GTTTA direct repeat in their corresponding promoter regions.

PhoP-PhoQ is a two-component regulatory system that controls several virulence properties in the gram-negative bacterium *Salmonella typhimurium* (6, 8, 11). Recently, extracellular Mg^{2+} has been identified as a stimulus that affects the PhoP-PhoQ system. The PhoQ protein is a Mg^{2+} sensor that changes conformation in the presence of periplasmic Mg^{2+} (6). PhoP is a regulatory protein that is necessary to transcribe some 25 different loci, many of which are essential for growth at low Mg^{2+} concentration (21).

The PhoP-PhoQ system is also present in *Escherichia coli* (7, 12), *Shigella flexneri*, *Yersinia enterocolitica*, and *Yersinia pestis* (8), where a basic physiological role in response to Mg^{2+} starvation has been proposed (6). The PhoP and PhoQ proteins of *E. coli* and *S. typhimurium* are 93 and 86% identical, respectively, indicating a high degree of structural and functional similarity (7, 12). Divalent cations seems to bind to an acidic cluster (148EDDDDAE154) of the *E. coli* PhoQ sensor domain and stabilize a conformation inactive in signaling (23). PhoP-PhoQ in *E. coli* is a promising system for studying ligand-induced signal transduction because it is one of the few two-component systems whose ligands have been identified. However, the physiological role of the PhoP-PhoQ system in *E. coli* is not understood. Here, we identified extracellular Mg^{2+}-responsive genes and promoters in *E. coli* and investigated the regulation of their expression by the PhoP-PhoQ system.

Isolation and identification of Mg^{2+}-responsive genes. To isolate Mg^{2+}-responsive genes, *E. coli* MC4100 (Table 1) was infected with λp*lac*Mu55 and λpMu507 as described by Bremar et al. (3), and the resulting blue colonies were selected as *lac* gene transcriptional fusion strains on a Luria-Bertani (LB) medium plate (18) containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 40 μg/ml) and kanamycin (30 μg/ml). Bacteria were always grown at 37°C. Among 2,000 independent *lacZ* gene fusions, Mg^{2+}-responsive clones, which were white on MacConkey plates containing 30 mM $MgSO_4$ and were red in the absence of Mg^{2+}, were selected. A P1 phage lysate prepared from the selected clones was used to infect MC4100 to obtain kanamycin-resistant colonies (MG1301 and MG1601). β-Galactosidase activity of *lacZ* fusion strains (MG1301 and MG1601) was decreased significantly by 30 mM $MgSO_4$ or 30 mM $MgCl_2$ but not by 30 mM Na_2SO_4 (Fig. 1), indicating that expression of the fused genes was repressed by higher concentrations of Mg^{2+}. Fusion junctions were sequenced as follows. λp*lac*Mu55 specialized transducing phage, which carries various amounts of the adjacent host DNA, was prepared by UV irradiation of the fusion strain (19). Aliquots (1.0 μg of DNA) were sequenced directly with a PRISM Dye Terminator Cycle Sequencing Ready Reaction kit with a Mu C-end primer (MU2; 5′-AATAATCCAATGTCCT CCCGG-3′). The PCR (25 cycles of 96°C for 30 s, 57°C for 30 s, and 60°C for 4 min) identified two Mg^{2+}-responsive genes in *E. coli* K-12; one (MG1601) was the *mgtA* gene encoding an ATP-dependent Mg^{2+} transporter at 96.2 min on the chromosome (positions 8267 to 10963 in GenBank entry AE000495), and the other gene (MG1301) was a newly designated gene, *mgrB*, located at 41.2 min (complementary to positions 68 to 211 in GenBank entry AE000277).

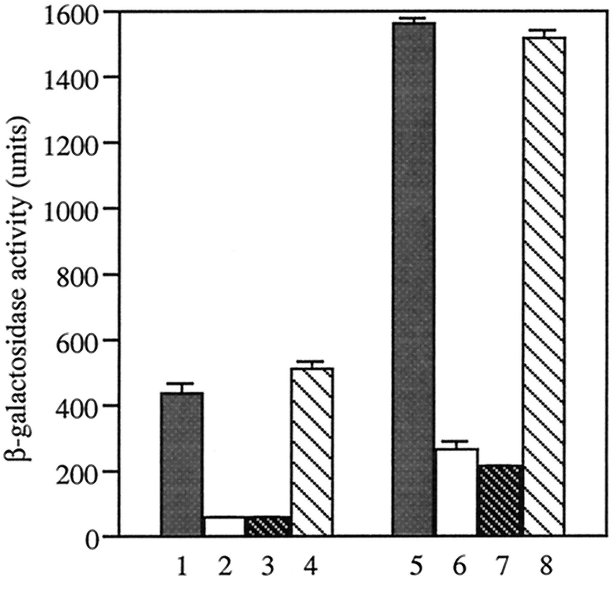

FIG. 1. Gene expression dependent on Mg^{2+}. *E. coli* MG1601 (lanes 1 to 4) and MG1301 (lanes 5 to 8) were grown in LB medium and LB medium containing 30 mM $MgSO_4$ (lanes 2 and 6), 30 mM $MgCl_2$ (lanes 3 and 7), or 30 mM Na_2SO_4 (lanes 4 and 8). An early-log-phase (OD_{600}, 0.4) culture was used to determine β-galactosidase activity (18). Data are the means of triplicate values with standard deviations.

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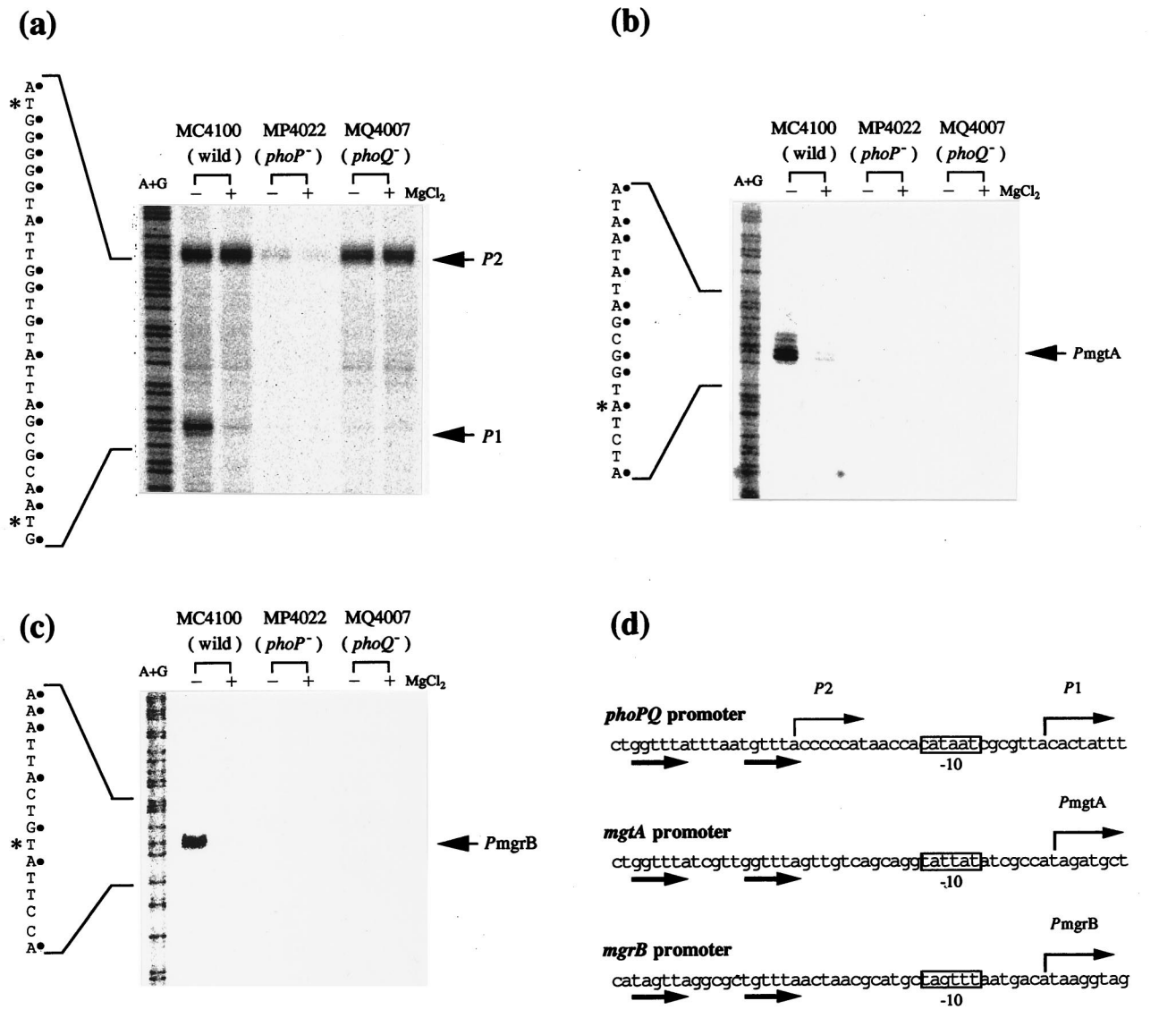

FIG. 2. Transcriptional regulation of *phoPQ*, *mgtA*, and *mgrB*. When grown to mid-log phase (OD_{600}, 0.5 to 0.8) in LB medium in the presence (+) or absence (−) of 30 mM $MgCl_2$, cells of the indicated *E. coli* strains were collected, and then RNA preparation and S1 nuclease assay were done as described in the text. Probes A (*Sal*I-*Hinc*II [380-bp] DNA fragment of pHO19P), B (*Alu*I-*Dde*I [400-bp] fragment of pMGA19P), and C (*Eco*RV-*Bst*UI [600-bp] fragment of pMGB19P) were used to determine start sites of *phoPQ* (a), *mgtA* (b), and *mgrB* (c), respectively. Electrophoresis was done with a 6% (a) or 4% (b and c) acrylamide sequencing gel. Lanes A+G represent Maxam-Gilbert sequence reactions. P1, P2, *P*mgtA, and *P*mgrB point to the corresponding protected transcripts. Transcription start sites are marked with asterisks. (d) DNA sequence (coding strand) around the promoter of *phoPQ* (complementary to positions 4786 to 4837 of GenBank entry AE000213), *mgtA* (positions 7961 to 8012 of AE000495), and *mgrB* (complementary to positions 229 to 280 of AE000277). Thin and bold arrows indicate starts and directions of transcription and direct repeats, respectively. The putative −10 region of each promoter is boxed.

Isolation of *phoPQ*-defective strains by using Tn*10*dCam. To determine that expression of Mg^{2+}-responsive genes was controlled by both PhoP and PhoQ, *E. coli* MG1301 was infected with λNK1324 grown on *E. coli* BD25 as described by Kleckner et al. (13). Chloramphenicol-resistant (Cam^r) colonies (about 30,000) were selected on MacConkey plates containing chloramphenicol (20 μg/ml), from which white colonies were isolated. A P1 phage lysate prepared from one of them was used to infect MG1301, and Cam^r colonies were isolated. We determined the Tn*10*dCam insertion site by sequencing the genomic region adjacent to Tn*10*dCam amplified by thermal asymmetric interlaced PCR (15, 16). Consequently, three *phoP* mutants (MG1320, MG1322, and MG1323) and four *phoQ* mutants (MG1303, MG1306, MG1307, and MG1321) were identified. The mutant strains defective in *phoP* (MG1622) and *phoQ* (MG1607) were constructed by P1 transduction from MG1322 and MG1307 to MG1601, respectively (Table 1). When β-galactosidase activity was assayed in *phoP*- and *phoQ*-defective strains, neither *mgtA* nor *mgrB* was expressed, irrespective of Mg^{2+} concentration (data not shown).

Identification of the promoter region of Mg^{2+}-responsive genes. We determined the transcriptional start sites of *mgtA* and *mgrB* as well as *phoPQ* by S1 nuclease assay as follows. *E. coli* cells were grown in LB medium in the absence of $MgCl_2$ overnight, then diluted 100-fold into 20 ml of LB medium in the presence or absence of 30 mM $MgCl_2$, and grown to mid-log phase (optical density at 600 nm [OD_{600}], 0.5 to 0.8) to prepare total RNA (1). The S1 nuclease assay was conducted

TABLE 1. Bacterial strains, phages, and plasmids used in this study[a]

Strain or phage	Description	Reference or source
E. coli strains		
MC4100	F$^-$ Δ(*argF-lac*)*U169 araD139 rpsL150 ptsF25 fibB5301 rbsR deoC relA1*	4
BD25	F$^-$ Δ(*argF-lac*)*U169 supF supE hsdR galK trpR metB lacY tonA*	E. Bremar
MQ4007	MC4100 *phoQ608*::Tn*10*dCam	MC4100 × P1 (MG1307)→Cam^r
MP4022	MC4100 *phoP2146*::Tn*10*dCam	MC4100 × P1 (MG1322)→Cam^r
MG1301	MC4100 *mgrB*::λp*lac*Mu55	This work
MG1307	MG1301 *phoQ608*::Tn*10*dCam[b]	This work
MG1322	MG1301 *phoP2146*::Tn*10*dCam[b]	This work
MG1601	MC4100 *mgtA*::λp*lac*Mu55	This work
MG1607	MG1601 *phoQ608*::Tn*10*dCam	MG1601 × P1 (MG1307)→Cam^r
MG1622	MG1601 *phoP2146*::Tn*10*dCam	MG1601 × P1 (MG1322)→Cam^r
Bacteriophages		
λp*lac*Mu55	Mu *cts*62 *ner*$^+$ *A′* am1093 *′uvrD′* Mu*S′ ′trp′ lacZ*$^+$ *lacY*$^+$ *lacA′* Km^r λ *imm*	3
λpMu507	λ*cIts*857 *S*am7 Mu A$^+$ B$^+$	3
λNK1324	Tn*10*dCam *cIts*857 *P*am80 *nin*5 *b*522 att$^-$	13
Plasmids		
pMW119	Ap^r, *lacZ′*, replication origin derived from pSC101	2
pHO119	pMW119, 3.6-kb *Sal*I-*Eco*RI fragment containing *phoP*$^+$*Q*$^+$ from λ phage clone (Kohara clone 239)[c]	This work
pDA51	PhoP D51A, pHO119	This work
pDN51	PhoP D51N, pHO119	This work
pHR277	PhoQ H277R, pHO119	This work
pUC19	Ap^r, *lacZ′*	23
pHO19P	pUC19, 560-bp *Eco*RI-*Sal*I fragment containing *phoPQ* promoter from pHO119	This work
pMGA19P	pUC19, 820-bp *Eco*RI-*Bam*HI fragment containing *mgtA* promoter from λp*lac*Mu55 phage harboring *mgtA* prepared by UV induction of MG1601	This work
pMGB19P	pUC19, 980-bp *Eco*RI-*Bam*HI fragment containing *mgrB* promoter from λ phage clone (Kohara clone 335)	This work

[a] Ap^r, ampicillin resistance; Km^r, kanamycin resistance; Cam^r, chloramphenicol resistance.
[b] Tn*10*dCam is inserted at position 3003 (MG1307) or 4541 (MG1322) in GenBank entry AE000213.
[c] This fragment corresponds to positions 4319 to 7873 in GenBank entry D90748.

as described previously (1, 6), with the following modification. The RNA (20 to 100 μg) was mixed with the probe DNA in 50 μl of a hybridization buffer (80% formamide, 20 mM HEPES [pH 6.5], 0.4 M NaCl), incubated at 75°C for 10 min, and then incubated at 37°C overnight. Then 220 μl of H_2O and 30 μl of 10× S1 nuclease buffer (0.3 M sodium acetate [pH 4.5], 0.5 M NaCl, 10 mM $ZnSO_4$, 50% glycerol) were added, and the mixture was treated with 50 U of S1 nuclease (Takara) at 37°C for 10 min. The reaction was stopped by adding 300 μl of phenol-chloroform, and the DNAs contained in the aqueous phase were precipitated with ethanol. The precipitate was dissolved in a sequencing loading buffer (80% formamide, 10 mM NaOH, 1 mM EDTA, 0.025% bromophenol blue, 0.025% xylene cyanol) and electrophoresed in a 6 or 4% acrylamide sequencing gel.

In the *E. coli* strain (MC4100) grown in LB medium, two transcripts (P1 and P2) of *phoPQ* were found (Fig. 2a). The transcription of P1 was dependent on extracellular Mg^{2+} concentration and was decreased in *phoP* (MP4022) or *phoQ* (MQ4007) mutants. In MC4100, MP4022, and MQ4007, the P2 transcript was constitutively expressed in the presence or absence of Mg^{2+}. For *mgtA* and *mgrB*, only one transcript (P*mgtA* and P*mgrB*) was found for each; these transcripts were not detected in MP4022 and MQ4007 (Fig. 2b and c). Gene expression in each of these defective mutants was restored at the lower Mg^{2+} concentration upon transformation with pHO119 (data not shown). These results indicated that expression of *mgtA* and *mgrB* as well as *phoPQ* is positively controlled by both PhoP and PhoQ in a Mg^{2+}-dependent manner. In addition, *mgtA* was found to be specifically transcribed during log phase but repressed within 15 min after the addition of 30 mM $MgCl_2$ (Fig. 3).

The direct repeat (T/G)GTTTA was conserved 25 bp upstream of the transcriptional start site of *phoPQ* (P1), *mgtA*, and *mgrB* (Fig. 2d). This direct repeat is also found in the *phoPQ* promoter of *S. typhimurium* (20) and is very similar to one recognized by the regulator PhoB (17). Thus, (T/G)GTTTA is assumed to be a target for PhoP. A search of the entire

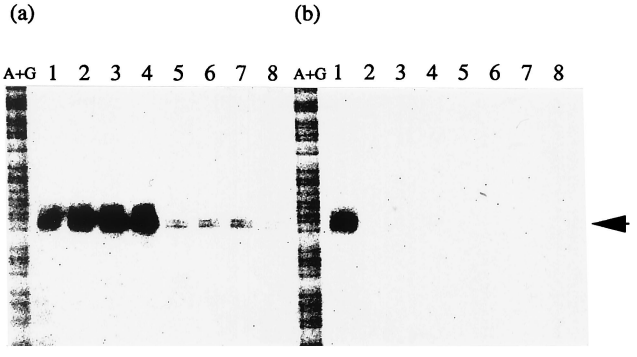

FIG. 3. Growth-phase-dependent transcription of *mgtA*. After growth to exponential phase (OD_{600}, 0.3) in LB medium, cells of *E. coli* MC4100 were further cultivated in the presence (b) or absence (a) of 30 mM $MgCl_2$. Samples were prepared at 0 min (lane 1), 15 min (lane 2), 30 min (lane 3), 1 h (lane 4), 2 h (lane 5), 4 h (lane 6), 6 h (lane 7), and 8 h (lane 8). RNA preparation and S1 nuclease assay were done as described for Fig. 2. Lanes A+G represent Maxam-Gilbert sequence reactions.

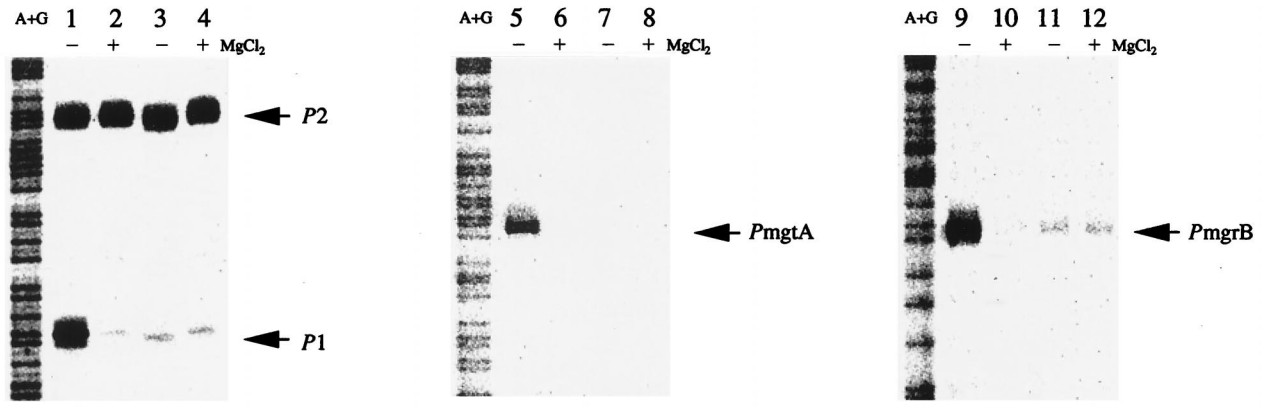

FIG. 4. Effect of PhoQ H277R on transcription of *phoPQ*, *mgtA*, and *mgrB*. *E. coli* MQ4007 in the presence of pHO119 (lanes 1, 2, 5, 6, 9, and 10) or pHR277 (lanes 3, 4, 7, 8, 11, and 12) was grown to mid-log phase in LB medium in the presence (+) or absence (−) of 30 mM $MgCl_2$ to prepare RNA. RNA preparation and S1 nuclease assays were performed as described for Fig. 2.

E. coli genome sequence (4.64 Mb [4a]) for a (T/G)GTTTA-5 bp-(T/G)GTTTA or TAGTTA-5 bp-(T/G)GTTTA motif detected four genes, a *bor* homolog, *ydcD*, a *fimD* homolog, and *yrbL* (12.5, 33.1, 34.4, and 72 min, respectively, on the chromosome), besides *phoP*, *mgtA*, and *mgrB*. In fact, the promoter of *yrbL* was Mg^{2+} responsive (data not shown). In *S. typhimurium*, this motif was also found in the promoter region of *phoP*, *mgtA*, *mgtBC*, and *pagA*, which are PhoP-activated genes, but many other PhoP-activated genes lack the consensus sequence (11, 21). Recently, the expression of several genes was reported to be under the direct control of the PmrA-PmrB two-component system, in which PhoP participates by activating *pmrAB* (9, 22). These results suggested that some genes are regulated indirectly by PhoP through other regulatory factors.

In *S. typhimurium*, the transcriptional start sites of the *phoPQ* operon were previously identified (6, 20). Two RNA species were detected in an exponentially grown *S. typhimurium* culture. These transcripts define two promoters: P1, which requires both PhoP and PhoQ for activity and is regulated by environmental Mg^{2+}; and P2, which remains active in the absence of PhoP and PhoQ (6, 20). We have obtained similar results for the *E. coli phoPQ* operon.

Identification of amino acid residues involved in the PhoP-PhoQ signaling cascade. To further examine the regulation by *phoPQ*, we introduced a point mutation in the coding region with respect to the putative PhoP phosphorylation site Asp51 and the PhoQ autophosphorylation site His277 on pHO119 as follows. Site-directed mutagenesis was performed by using a QuikChange site-directed mutagenesis kit (Stratagene). pDA51 and pDN51 were derived from pHO119, carrying *phoP* with changes of Asp51 to Ala and Asn, respectively. pHR277 is a derivative of pHO119, carrying *phoQ* with a change of His277 to Arg. Primers for the site-directed mutagenesis were as follows: pDA51, forward (5′GATATTGCGATTGTCGCTCTC GGATTGCC3′) and reverse (5′GGCAATCCGAGAGCGAC AATCGCAATATC3′); pDN51, forward (5′GATATTGCGA TTGTCAATCTCGGATTGCC3′) and reverse (5′GGCAA TCCGAGATTGACAATCGCAATATC3′); and pHR277, forward (5′CCGACCTGACCCGTAGTCTGAAAACGC3′) and reverse (GCGTTTTCAGACTACGGGTCAGGTCGG3′).

As shown in Fig. 4, transcription of *phoPQ* (P1), *mgtA*, and *mgrB* was not observed when PhoQ H277R was expressed in the *phoQ* mutant (MQ4007) (Fig. 4, lanes 3, 4, 7, 8, 11, and 12). When the wild-type PhoQ was expressed, expression of *phoPQ*, *mgtA*, and *mgrB* was observed in the absence of Mg^{2+} but not in the presence of 30 mM Mg^{2+} (lanes 1, 2, 5, 6, 9, and 10). The same result was obtained for the *phoP* mutant strain (MP4022) and pDA51 or pDN51 (data not shown). These results demonstrate that Asp51 of PhoP and His277 of PhoQ are indispensable for activation of PhoP-PhoQ signaling and subsequent expression of *mgtA* and *mgrB*.

Concluding remarks. In this study, we tried to isolate directly a Mg^{2+}-responsive *lacZ* fusion strain of *E. coli*. Among 2,000 independent *lac* gene transcriptional fusions, we identified two PhoP-activated genes, *mgtA* and *mgrB*. *mgtA*, encoding an ATP-dependent Mg^{2+} transporter, is homologous to genes in *S. typhimurium*, but *mgrB* is newly designated in this report. *mgtA* was also found to be specifically transcribed during log phase but repressed through the PhoP-PhoQ system within 15 min after the addition of 30 mM $MgCl_2$. These results suggest that the PhoP-PhoQ-independent P2 promoter of *E. coli* provides a low intracellular concentration of PhoP and PhoQ when magnesium is in excess, and upon shifting to limited magnesium, autophosphorylated PhoQ serves as the phosphate donor for PhoP. Phospho-PhoP autogenously activates the P1 promoter in the *E. coli phoPQ* operon, resulting in activation of *mgtA* gene expression to increase the intracellular Mg^{2+} concentration. Such a signaling cascade seems to be essential for active growth of *E. coli* in a low-Mg^{2+} medium.

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