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Identification and Molecular Characterization of the Mg^{2+} Stimulon of *Escherichia coli*

Shu Minagawa,[1] Hiroshi Ogasawara,[1] Akinori Kato,[1] Kaneyoshi Yamamoto,[1] Yoko Eguchi,[1] Taku Oshima,[2] Hirotada Mori,[2] Akira Ishihama,[3] and Ryutaro Utsumi[1]*

Department of Bioscience and Biotechnology, Graduate School of Agriculture, Kinki University, 3327-204 Nakamachi, Nara 631-8505,[1] Research and Education Center for Genetic Information, Nara Institute of Science and Technology, Ikoma 630-0101,[2] and Division of Molecular Biology, Nippon Institute for Biological Science, Ome, Tokyo 198-0024,[3] Japan

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Transcription profile microarray analysis in *Escherichia coli* was performed to identify the member genes of the Mg^{2+} stimulon that respond to the availability of external Mg^{2+} in a PhoP/PhoQ two-component system-dependent manner. The mRNA levels of W3110 in the presence of 30 mM $MgCl_2$, WP3022 (*phoP* defective), and WQ3007 (*phoQ* defective) were compared with those of W3110 in the absence of $MgCl_2$. The expression ratios of a total of 232 genes were <0.75 in all three strains (the supplemental data are shown at http://www.nara-.kindai.ac.jp/nogei/seiken/array.html), suggesting that the PhoP/PhoQ system is involved directly or indirectly in the transcription of these genes. Of those, 26 contained the PhoP box-like sequences with the direct repeats of (T/G)GTTTA within 500 bp upstream of the initiation codon. Furthermore, S1 nuclease assays of 26 promoters were performed to verify six new Mg^{2+} stimulon genes, *hemL*, *nagA*, *rstAB*, *slyB*, *vboR*, and *yrbL*, in addition to the *phoPQ*, *mgrB*, and *mgtA* genes reported previously. In gel shift and DNase I footprinting assays, all of these genes were found to be regulated directly by PhoP. Thus, we concluded that the *phoPQ*, *mgrB*, *mgtA*, *hemL*, *nagA*, *rstAB*, *slyB*, *vboR*, and *yrbL* genes make up the Mg^{2+} stimulon in *E. coli*.

The two-component system is the most prevalent signal transduction mechanism that mediates bacterial responses to environmental stimuli. The two-component system typically consists of a sensor protein and a regulatory protein. Each sensor monitors a particular environmental signal and responds by modifying the phosphorylated state of its response regulator. The affinity of the response regulator for promoters is controlled by phosphorylation, ultimately leading to transcription activation of a distinct set of the stress response genes. Eubacterial species such as *Escherichia coli* and *Bacillus subtilis* harbor >30 two-component systems, each of which responds to a different signal in the environment (2, 9). However, the specific ligand that is recognized by each sensor protein remains unidentified.

The PhoP/PhoQ two-component system was first recognized in *Salmonella enterica* serovar Typhimurium as a regulatory system that monitors the availability of extracellular Mg^{2+} (3, 5). The PhoQ protein functions as an Mg^{2+} sensor (3) and, in the presence of micromolar concentrations of Mg^{2+}, phosphorylates the PhoP regulator. Phosphorylated PhoP activates the transcription of some 30 different genes (13, 14). The PhoP/PhoQ system is present in many nonpathogenic, gram-negative bacteria, suggesting that it plays a fundamental physiological role in the response to Mg^{2+} starvation (4, 5). However, the mechanism by which the PhoP transcription factor regulates the Mg^{2+} response genes remains poorly understood. Previously, we identified the tandem direct repeats of the sequence (T/G)GTTTA, which we designated the PhoP box, in promoters of the Mg^{2+}-responsive *phoPQ*, *mgtA*, and *mgrB* genes of *E. coli* K-12 (7). A search of the entire *E. coli* genome sequence for the (T/G)GTTTA-5bp-(T/G)GTTTA or TAGTTA-5bp-(T/G)GTTTA motif detected four additional genes, *vboR*, *ydcD*, an *fimD* homolog, and *yrbL* (7). In order to identify the member genes of the Mg^{2+} stimulon in *E. coli*, we carried out in this study a genomewide transcription profile analysis in the presence or absence of $MgCl_2$ by using a DNA microarray. The dependency on the PhoP/PhoQ two-component system was also examined by using *E. coli* mutants lacking *phoP* or *phoQ*. The sequence-activity relationship was analyzed for all of the Mg^{2+} response promoters herein identified.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains used in this work were *E. coli* K-12 derivatives (Table 1). WP3022 (W3110 *phoP2146*::Tn*10*dCam^r) and

TABLE 1. Bacterial strains used in this study

Strain	Description[a]	Source or reference
MC4100	F$^-$ Δ(*argF-lac*)*U169 araD139 rpsL150*	2a
MP4022	MC4100 *phoP2146*::Tn*10*dCam^r	7
MQ4007	MC4100 *phoQ608*::Tn*10* dCam^r	7
W3110	Wild type	Laboratory stock
WP3022	W3110 *phoP2146*::Tn*10*dCam^r	W3110 × P1(MP4022)→Cam^r
WQ3007	W3110 *phoQ608*::Tn*10*dCam^r	W3110 × P1(MQ4007)→Cam^r

[a] Cam^r, chloramphenicol resistance.

* Corresponding author. Mailing address: Department of Bioscience and Biotechnology, Graduate School of Agriculture, Kinki University, 3327-204, Nakamachi, Nara 631-8505, Japan. Phone: 81-742-43-7273 (ext. 3309). Fax: 81-742-43-1445. E-mail: utsumi@nara.kindai.ac.jp.

TABLE 2. PCR primers used in this study

Gene	Primer name and sequence	Position	GenBank no.
phoPQ	PH6: 5′-CCG GCT AAC TAT ATT GGT CG-3′ PH3: 5′-CTG CGT CGT CGA CCT GAT GAC CAG-3′	4957–4977 4667–4691	AE000213
mgtA	MGF6: 5′-AAC TGT AGA TTT CCC CAC GC-3′ MGR4: 5′-GTT TTA ATC TCC GTC GAG GG-3′	7826–7846 8096–8116	AE000495
mgrB	BGF2: 5′-TCC ATA CCA GTG CTA TCA GC-3′ BGR2: 5′-CCT GAT CGC ACA TCA TGT TG-3′	395–415 120–140	AE000277
hemL	HEM1: 5′-ATC CAA TGA TCA CTT ATT GG-3′ HEM2: 5′-ACG CCA GTA AAG GCG CGA AC-3′	1595–1615 1303–1323	AE000125
nagA	NAG1: 5′-GCT ACT ACA GCT TTA TGC AC-3′ NAG2: 5′-GAC AGT CAG GGC ATA TTT TG-3′	6048–6068 5694–5714	AE000171
rstA	RST1: 5′-TCG GGA AAA GTG GAA TCA GC-3′ RST2: 5′-AAC CTG CAT ATC ATG TTT TG-3′	3864–3884 4165–4185	AE000256
slyB	SLY1: 5′-AAA GCG GCC CGA TTT CAT AG-3′ SLY2: 5′-ATT GAA ACA ACC AAT ACG CG-3′	7192–7214 7492–7512	AE000259
vboR	BOR1: 5′-GAG AAT TCC GAT GGA TTA CAA ATA-3′ BOR3: 5′-GTG CTA CTG CTG TCT GTT TG-3′	2164–2188 1685–1705	AE000161
yrbL	YRL-F: 5′-CCG AGG CTG AAG CCA ACA GC-3′ YRL-R: 5′-GCC CAG GGG ACT TTG TTC AG-3′	1620–1640 1995–2015	AE000400

WQ3007 (W3110 *phoQ608*::Tn*10*dCam^r) were constructed by P1 transduction from the donors MP4022 and MQ4007 (7) to the recipient W3110, respectively. They were cultured at 37°C in Luria-Bertani (LB) medium in the presence or absence of 30 mM $MgCl_2$.

RNA isolation, cDNA labeling, and hybridization to DNA microarrays. To prepare total RNA for DNA microarray analysis, overnight cultures were diluted 100-fold in 30 ml of LB medium in the presence or absence of 30 mM $MgCl_2$ and grown to an optical density at 600 nm (OD_{600}) of 0.3 to 0.4 (approximately 8×10^7cells/ml) at 37°C. Subsequent purification steps were carried out as described previously (7). The resulting total RNA preparations were treated with RNase-free DNase I (Takara Co.) in accordance with the manufacturer's protocol. The RNA preparations from each sample were used as the template for cDNA preparations, labeled with Cy3-dUTP or Cy5-dUTP (Amersham Pharmacia), and hybridized to *E. coli* DNA microarrays (IntelliGene *E. coli* CHIP Version beta; Takara Shuzo Company, Ohtsu, Japan). The corrected intensities of duplicate spots were averaged as described previously (11, 12).

Preparation of the labeled promoter fragments for S1 nuclease and gel shift assays. The ^{32}P-labeled probes were generated by PCR amplification with the primer pairs shown in Table 2. Primers PH3, MGR4, BGR2, HEM2, NAG2, RST2, SLY2, YRL-R, and BOR3 were labeled with 10 μCi of [γ-^{32}P]ATP (5,000 Ci/mmol) by T4 polynucleotide kinase (Toyobo). *E. coli* W3110 genome DNA (100 ng) was used as the template for Ex *Taq* DNA polymerase (Takara). The PCR product with ^{32}P at its termini was recovered from a polyacrylamide gel and then used for S1 nuclease and gel shift assays.

S1 nuclease assay. RNA was prepared as described previously (7). The labeled promoter fragment was incubated with 100 μg of total RNA in hybridization buffer (80% formamide,0.4 M NaCl, 20 mM HEPES [pH 6.4]) at 75°C for 10 min, followed by further incubation at 37°C overnight and then digested with S1 nuclease. The undigested DNA was precipitated by ethanol, dissolved in formamide dye solution (95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol), and analyzed by electrophoresis on a 6% polyacrylamide gel containing 8 M urea.

Purification of PhoP. His-tagged PhoP for gel shift and DNA footprinting assays was purified as described previously (16).

Gel shift assay. The ^{32}P-labeled probes for the S1 nuclease assay were also used in the gel shift assay. The probe was incubated at 37°C for 10 min with the purified His-tagged PhoP (0 to 20 pmol) and bovine serum albumin (BSA; 100 pmol) in binding buffer (5 mM $CaCl_2$, 3 mM $MgCl_2$). After addition of the DNA dye solution (40% glycerol, 0.025% bromophenol blue, 0.025% xylene cyanol), the mixtures were directly subjected to 6% polyacrylamide gel electrophoresis (pH 6.0).

DNase I footprinting assay. The ^{32}P-labeled probe was incubated at 37°C for 10 min with purified, His-tagged PhoP in 25 μl of 50 mM Tris-HCl (pH 7.8)–50 mM NaCl–3 mM magnesium acetate–5 mM $CaCl_2$–0.1 mM EDTA–0.1 mM dithiothreitol–25 μg of BSA per ml. After incubation for 10 min, DNA digestion was initiated by the addition of 5 ng of DNase I (Takara). After digestion for 30 s at 25°C, the reaction was terminated by the addition of 45 μl of DNase I stop solution (20 mM EDTA, 200 mM NaCl, 1% sodium dodecyl sulfate, 250 μg of yeast tRNA per ml). Digested products were precipitated by ethanol, dissolved in formamide dye solution, and analyzed by electrophoresis on a 6% polyacrylamide gel containing 8 M urea.

Computer search for the PhoP box. The DNA sequences 500 bp upstream of the initiation codon of 232 genes, which were estimated by DNA microarray analysis, were obtained from GenoBase (http://ecoli.aist-nara.ac.jp). The PhoP box (TGTTTANNNNN TGTTTA) was searched for within their sequences with GENETYX-MAC (Software Development Co., Ltd.) set up with a 2-bp mismatch.

RESULTS AND DISCUSSION

DNA microarray-based identification of Mg^{2+}-responsive genes controlled by the PhoP/PhoQ two-component system. To search for the member genes of the Mg^{2+}stimulon of *E. coli*, wild-type strain W3110 was grown in LB medium in the presence or absence of 30 mM $MgCl_2$ and genomewide transcription profiles were analyzed with DNA microarrays. On addition of external Mg^{2+}, the mRNA levels for a number of genes were markedly reduced. To identify whether these genes are under the control of the PhoP/PhoQ two-component system, the microarray assay was also performed for both WP3022 (*phoP* disruptant) and WQ3007 (*phoQ* disruptant) in the absence of $MgCl_2$ and the transcription profiles were compared with that of W3110. A total of 232 genes, whose expression ratios were <0.75, were found to be repressed by the addition

of external $MgCl_2$ and in a PhoP/PhoQ-dependent manner (for details, see the supplemental data at http://www.nara.kindai.ac.jp/nogei/seiken/array.html). After sequence analysis of 500-bp-long promoter regions upstream from the ATG initiation codon of all of these genes, a total of 26 genes, including the previously identified *phoPQ*, *mgtA*, and *mgrB* genes (7), were found to possess a PhoP box with the consensus sequence (T/G)GTTTA-N_5-(T/G)GTTTA (Table 3).

To validate these putative Mg^{2+}-responsive genes regulated by PhoP/PhoQ, an S1 nuclease assay was performed for all 26 genes. In addition to the previously identified *phoPQ*, *mgtA*, and *mgrB* genes (7), six other genes (*hemL*, *nagA*, *rstAB*, *slyB*, *vboR*, and *yrbL*) were found to be repressed in the presence of high concentrations of extracellular Mg^{2+} (Fig. 1). mRNAs for these genes were detected in W3110 but not in WP3022 or WQ3007. In each case, Mg^{2+}-dependent repression required the presence of both PhoP and PhoQ (Fig. 1). In the promoter regions of all of these Mg^{2+}-responsive genes, the consensus PhoP box was identified (Fig. 2). The corresponding genes of *S. enterica* serovar Typhimurium (*phoPQ*, *mgtA*, STM1839, *hemL*, *nagA*, *rstAB*, *slyB*, and STM35040) also carry the PhoP box sequence in the respective promoter regions (Fig. 2).

Recently, Oshima et al. (11) published the transcription profile for an *E. coli* mutant defective in the PhoP/PhoQ two-component system as determined by DNA microarray analysis. The expression of at least 28 genes, including *rstAB*, *slyB*, *vboR*, and *yrbL*, was decreased in the *phoP*/*phoQ* mutant, but it remained to be determined whether these genes respond to the availability of Mg^{2+} in the external environment. In an S1 nuclease assay, the mRNA levels of three genes, *sfcA*, *gppA*, and *srlD*, decreased in the presence of Mg^{2+}, but this reduction was not observed even with the *phoP* and *phoQ* mutants (data not shown). These genes are not under direct control of the PhoP/PhoQ system. In fact, the PhoP box sequence is not present in these promoters. These results, together, indicate that the presence of a PhoP box is important for PhoP/PhoQ-dependent transcription of the Mg^{2+} stimulon genes.

Gel shift assay of PhoP-binding activity for the Mg^{2+}-responsive gene promoters. Recently, we found that the purified PhoP protein specifically binds in vitro to the PhoP box located within the *mgtA* promoter region (16). To check whether the PhoP protein can also bind to the PhoP box associated with the newly identified Mg^{2+}-responsive genes, we carried out gel shift assays with DNA fragments from the promoters from these nine genes (*phoPQ*, *mgtA*, *mgrB*, *hemL*, *nagA*, *rstA*, *slyB*, *vboR*, and *yrbL*). All nine of the DNA probes used were retarded to form a single band of the probe DNA-PhoP protein complex (Fig. 3). The minimum concentration of PhoP protein required to convert all of the input probes to PhoP complexes was, however, different among the nine promoters, suggesting differences in their affinity to PhoP. We quantified the amount of PhoP needed to bind half of the probe amount used in gel shift assay (Table 4). PhoP binds the promoters of two genes (*mgtA* and *vboR*) with the highest affinity. Among the remain-

TABLE 3. DNA microarray analysis results[a]

Gene	Δ*phoP* (−)/wild (−)			Δ*phoQ* (−)/wild (−)			Wild (+)/wild (−)		
	Ratio	Ratio	Avg	Ratio	Ratio	Avg	Ratio	Ratio	Avg
ais	0.51	ND[d]	0.51	ND	ND	ND	ND	ND	ND
argD	0.55	ND	0.55	ND	ND	ND	ND	ND	ND
carA	0.67	0.61	0.64	0.42	0.40	0.41	0.21	ND	0.21
crcA	0.47	0.77	0.62	0.46	ND	0.46	ND	ND	ND
dfp	0.64	0.53	0.58	0.56	0.55	0.56	0.27	0.26	0.26
dinG	0.69	0.81	0.75	0.56	0.90	0.73	0.46	ND	0.46
gcvT	0.77	0.68	0.73	0.60	0.55	0.58	0.33	0.37	0.35
gppA	0.68	0.64	0.66	0.61	0.66	0.63	0.49	0.44	0.46
hemL	0.32	0.41	0.37	0.35	0.35	0.35	0.41	0.44	0.43
mgrB	0.21[b]	0.17[b]	0.19[b]	—[c]	—	—	—	—	—
nagA	0.55	0.53	0.54	0.59	0.70	0.64	0.52	0.68	0.60
phoP	0.22	0.21	0.21	0.67	0.50	0.59	0.34	0.45	0.40
rstA	0.54	0.46	0.50	0.47	0.29	0.38	0.13	ND	0.13
sfcA	0.38	0.35	0.36	0.41	0.27	0.34	0.26	0.21	0.24
slyB	0.29[b]	0.27[b]	0.28[b]	—	—	—	—	—	—
srlD	0.71	0.50	0.60	0.77	0.66	0.71	0.43	0.31	0.37
sucB	0.31	0.25	0.28	0.75	0.56	0.66	0.10	ND	0.10
vboR	0.21	0.12	0.17	ND	ND	ND	ND	ND	ND
xylA	0.69	ND	0.69	ND	ND	ND	ND	ND	ND
411#13 b2362	0.63	ND	0.63	ND	ND	ND	ND	ND	ND
ybjX	0.12	0.10	0.11	0.13	0.09	0.11	0.10	0.18	0.14
yfcS	0.46	0.94	0.70	ND	ND	ND	ND	ND	ND
yfdC	0.57	ND	0.57	ND	ND	ND	ND	ND	ND
ygeW	0.23	ND	0.23	ND	ND	ND	ND	ND	ND
yijF	0.43	ND	0.43	ND	ND	ND	ND	ND	ND
yrbL	0.12	0.10	0.11	0.15	0.14	0.15	0.55	0.60	0.58

[a] *mgtA* was not spotted on the DNA microarray used in this experiment. The symbols + and − mean presence and absence of $MgCl_2$, respectively. Δ*phoP,* WP3022; Δ*phoQ*, WQ3007; wild, W3110. The underlined genes were identified as parts of the Mg^{2+} stimulon. The values shown are individual intensities of duplicate spots and the average of the two preceding values.

[b] The data were obtained with *E. coli* DNA microarrays fabricated by Mori et al., in which N-minimal medium (3) was used.

[c] —, no experiments were done.

[d] ND, fluorescent signal too weak for detection.

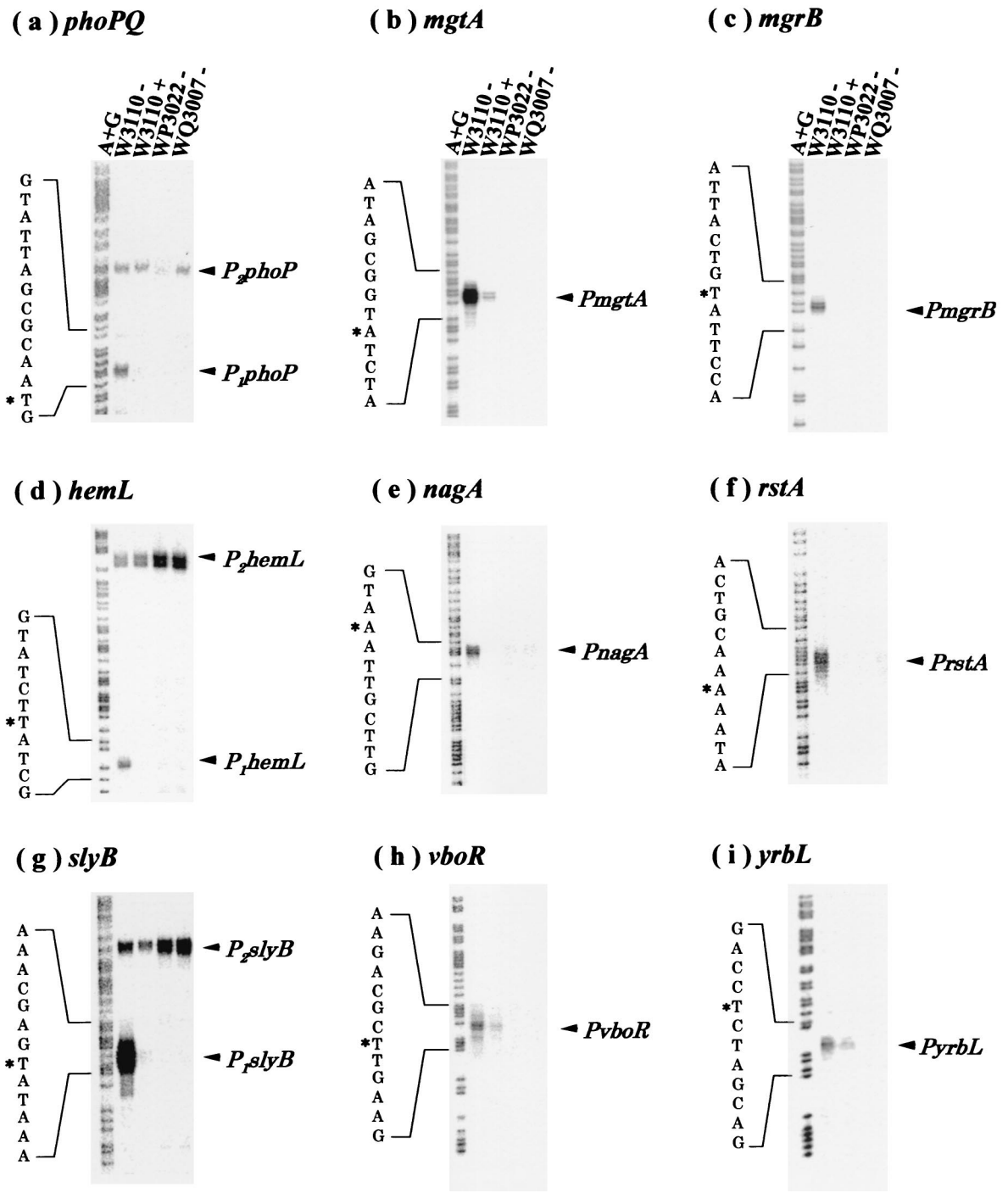

FIG. 1. S1 nuclease assays. *E. coli* W3110 (wild type), WP3022 (*phoP* defective), and WQ3007 (*phoQ* defective) were grown in LB medium to early exponential phase (OD_{600}, 0.3 to 0.4) in the presence (+) or absence (−) of 30 mM $MgCl_2$. S1 nuclease mapping was carried out to determine the transcription start sites of *phoPQ* (a), *mgtA* (b), *mgrB* (c), *hemL* (d), *nagA* (e), *rstAB* (f), *slyB* (g), *yrbL* (h), and *vboR* (i). Electrophoresis was performed with a 6% acrylamide sequencing gel. Lanes A+G represent Maxam-Gilbert sequencing reactions. Arrowheads show the transcripts protected from nuclease digestion. Transcription start sites are marked with asterisks.

ing six promoters, probes of *hemL* and *nagA* showed the lowest affinity.

DNase I footprinting of PhoP-binding sites within the Mg^{2+}-responsive gene promoters. Next, we tried to identify the precise site of PhoP binding by using a DNase I footprinting assay. As expected, the purified PhoP protein was found to bind the PhoP box region of *phoPQ*, *mgtA*, *mgrB*, *rstAB*, *slyB*, *vboR*, and *yrbL* (Fig. 4). In the *nagA*, *phoPQ*, *mgtA*, *mgrB*, *rstAB*, *slyB*, and *vboR* promoters, PhoP also bound to the PhoP box in the simultaneous presence of RNA polymerase (data

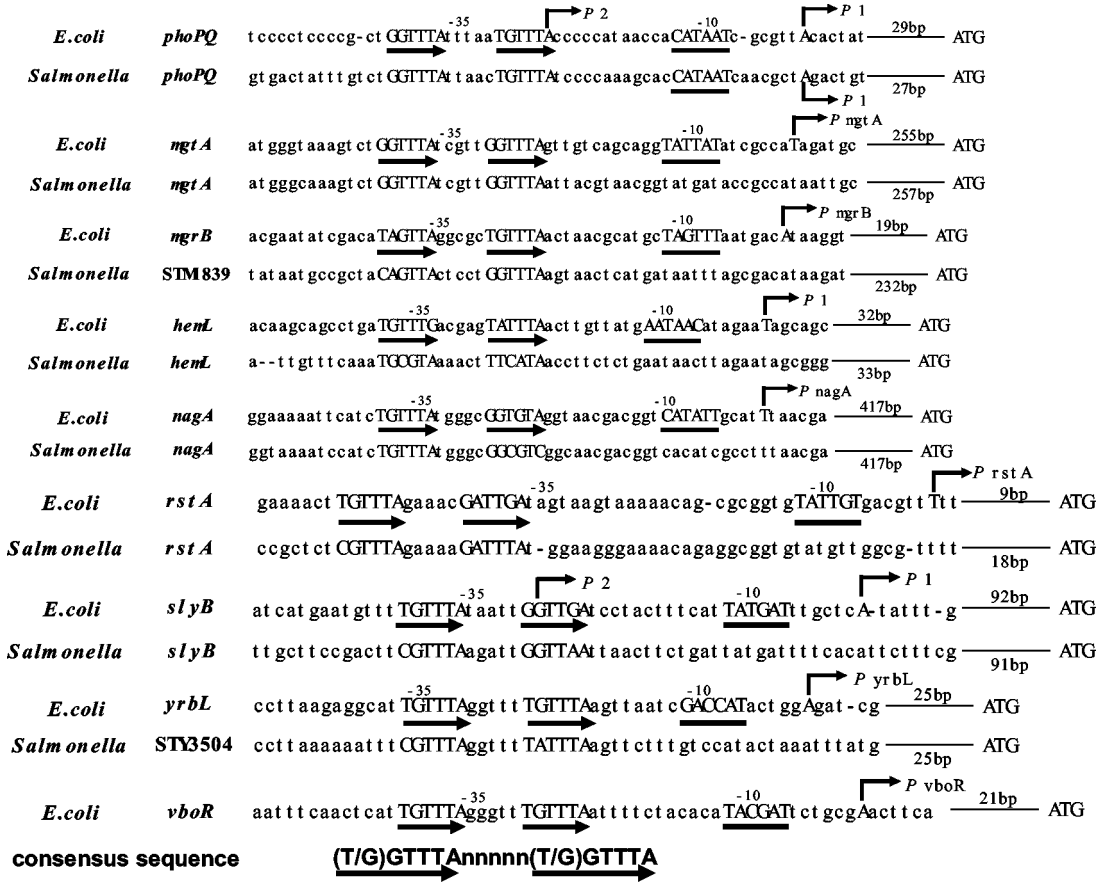

FIG. 2. Mg^{2+}-responsive promoters. The *E. coli* Mg^{2+}-responsive promoters, identified in Fig. 1, are aligned in parallel with the corresponding promoters from *S. enterica* serovar Typhimurium. The following DNA sequences of *E. coli* and *S. enterica* serovar Typhimurium are from the GenBank database (the percentages in parentheses are the homologies of the proteins): *phoPQ*, AE000213 and AE008753 (PhoP, 89%; PhoQ, 79%); *mgtA*, AE000495 and AE008909 (MgtA, 87%); *mgrB*, AE000277 and AE008782 (MgrB, 63%); *hemL*, AE000125 and AE008704 (HemL, 93%); *nagA*, AE000171 and AE008727 (NagA, 92%); *rstAB*, AE000256 and AE008764 (RstA, 80%; RstB, 78%); *slyB*, AE000259 and AE008762 (SlyB, 63%); *yrbL*, AE000400, STY3504, and AE008853 (YrbL, 72%); *vboR*, AE000161. Thin and bold arrows indicate starts and directions of transcription and direct repeats (PhoP box), respectively. The −10 region of each promoter is underlined. In *S. enterica* serovar Typhimurium, no promoters except *phoPQ* have been clarified. The −10 hexamer, start site, and direct repeats are all capitalized.

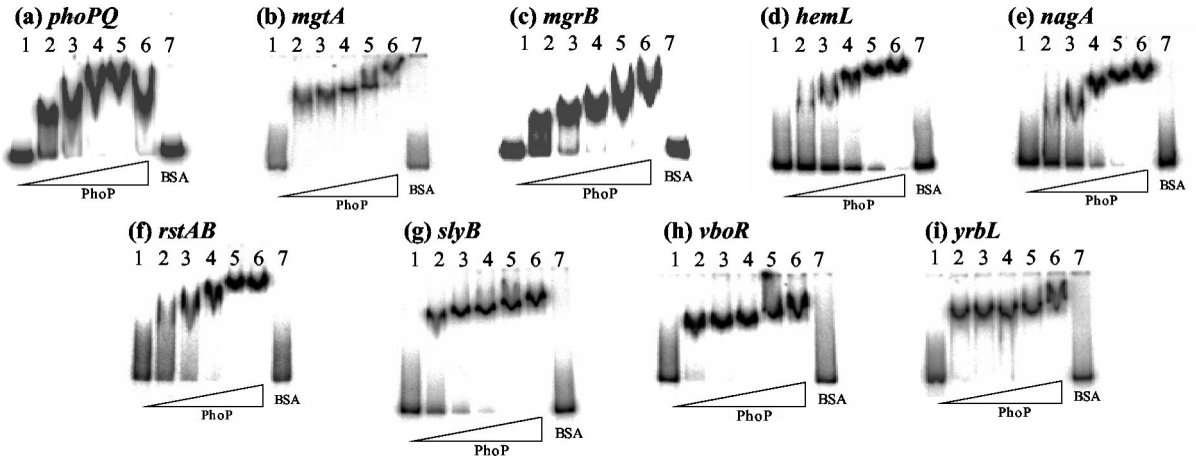

FIG. 3. Gel shift assays. The probes used in Fig. 1 were incubated at 37°C for 10 min with purified PhoP (BSA amounts: lane 1, 0 pmol; lane 2, 1.25 pmol; lane 3, 2.5 pmol; lane 4, 5 pmol; lane 5, 10 pmol; lane 6, 20 pmol; lane 7, 100 pmol). The mixtures were directly subjected to polyacrylamide gel electrophoresis. The sequences of the DNA probes used are described in Fig. 1.

TABLE 4. Differential affinity of PhoP to bind Mg^{2+} stimulon genes[a]

Gene	Amt (pmol) of PhoP
phoPQ	1.54
mgtA	0.88
mgrB	2.25
hemL	9.66
nagA	4.32
rstAB	2.37
slyB	2.30
vboR	0.94
yrbL	1.0

[a] The data were estimated by measuring the density of the probe band (lanes 1 to 6) in Fig. 3.

not shown). However, we failed to detect PhoP binding to the PhoP box of the *hemL* promoter. The lack of stable binding of PhoP alone to the PhoP box of the *hemL* and *nagA* promoters coincides with the results of the gel shift assays and supports the assumption that the affinity of PhoP differs among the nine promoters tested. Taking all of these observations together, we concluded that PhoP is a global transcriptional activator that directly binds to the PhoP box in the promoters of the Mg^{2+} stimulon of *E. coli* (Fig. 5).

The PhoP-PhoQ system responds to changes in the external Ca^{2+} level, as well as changes in the external Mg^{2+} level (3). In fact, we found that the *phoPQ* P1 promoter responds to

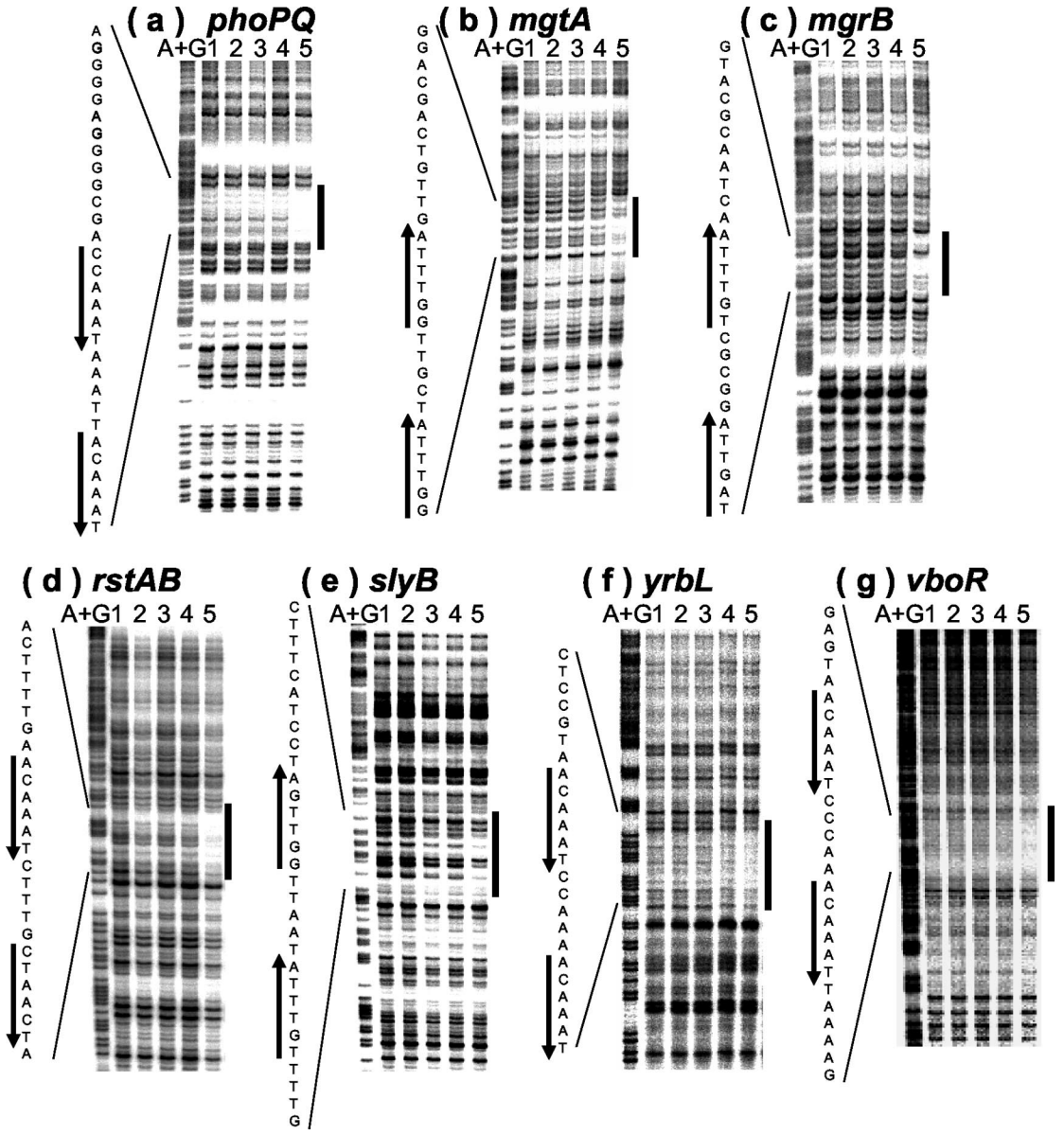

FIG. 4. DNase I footprinting assays. Coding (b, *mgtA*; c, *mgrB*; e, *slyB*) and noncoding (a, *phoPQ*; d, *rstAB*; f, *yrbL*; g, *vboR*) strands containing the PhoP box region were labeled with ^{32}P at the 5′ end, incubated with various amounts of purified PhoP (lanes 1, 2, 3, 4, and 5 contain 0, 10, 20, 40, and 80 pmol, respectively), and subjected to DNase I footprinting assays. The DNA probes described in Fig. 1 were used, except that MGF6, BGF2, and SLY1 were labeled with [γ-^{32}P]ATP. Lanes A+G represent the Maxam-Gilbert sequence reaction. DNA sequences from the bottom (5′) to the top (3′) are shown. Black boxes and bold arrows indicate PhoP-binding regions and PhoP boxes, respectively.

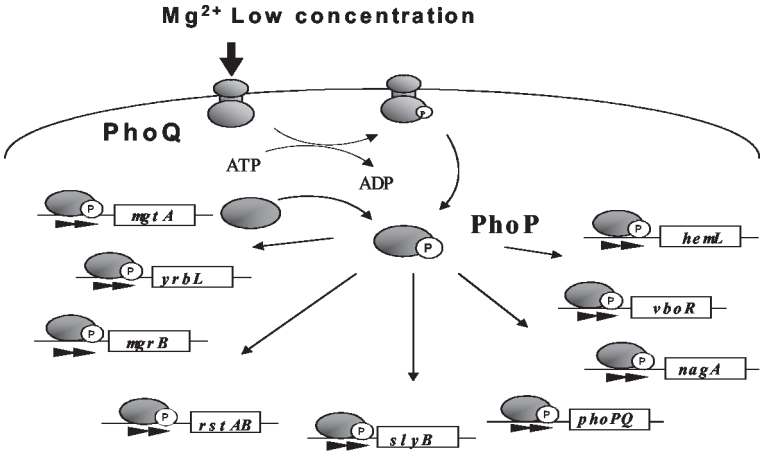

FIG. 5. Proposed Mg^{2+} stimulon of *E. coli*.

changes in the extracellular Ca^{2+} level in a PhoP/PhoQ-dependent manner (data not shown). The response of *E. coli* to changes in extracellular Fe^{3+}, Cu^{2+}, Ag^{+}, and Ni^{2+} levels was found to be under the control of the two-component systems PmrB/PmrA (1, 15), CusS/CusR (10), SilS/SilR (6), and NrsS/NrsR (8), respectively. These metal-responsive promoters also contain inverted or direct repeat sequences encompassing 5 bp. The binding of the respective regulatory proteins to these sequences is currently being examined. The signal transduction systems triggered by extracellular metal ions should be good models with which to obtain insights into the global network of bacterial gene expression.

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