

## Signal Transduction Cascade between EvgA/EvgS and PhoP/PhoQ Two-Component Systems of *Escherichia coli*

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**Transcriptional analysis of a constitutively active mutant of the EvgA/EvgS two-component system of *Escherichia coli* resulted in enhanced expression of 13 PhoP/PhoQ-regulated genes, *crcA*, *hemL*, *mgtA*, *ompT*, *phoP*, *phoQ*, *proP*, *rstA*, *rstB*, *slyB*, *ybjG*, *yrbL*, and *mgrB*. This regulatory network between the two systems also occurred as a result of overproduction of the EvgA regulator; however, enhanced transcription of the *phoPQ* genes did not further activate expression of the PhoP/PhoQ-regulated genes. These results demonstrated signal transduction from the EvgA/EvgS system to the PhoP/PhoQ system in *E. coli* and also identified the genes that required the two systems for enhanced expression. This is one example of the intricate signal transduction networks that are posited to exist in *E. coli*.**

The two-component signal transduction system is the major system in bacteria for sensing environmental stresses and transducing the information inside the cells for adaptation. This system is basically composed of a histidine kinase sensor residing in the inner membrane and a cognate response regulator in the cytoplasm. In *Escherichia coli*, 29 histidine kinase sensors, 32 response regulators, and one HPt (histidine-containing phosphotransmitter) domain have been found by analyses of the *E. coli* K-12 genome (23). Each sensor responds to individual environmental stresses to cope with the numerous environmental conditions that *E. coli* faces; however, 29 sensors are apparently insufficient, and it is posited that a complex regulatory network between the two-component systems exists.

Transcriptome analysis using the microarray technique is an extremely efficient method for analyzing regulatory networks. Oshima et al. (27) performed a global analysis of two-component regulatory system mutants of *E. coli* K-12 for a single growth condition (Luria-Bertani medium, aerobic, and mid-exponential phase) and proposed the existence of a network of functional interactions, such as cross talk and cascade signal transduction between the systems. In their study, some of the 36 two-component deletion mutants showed little or no change in their mRNA profiles, indicating that these two-component systems were not strongly operating under their culture condition.

One of these low- or nonoperating systems in mid-exponential-phase cells grown in a rich medium is the EvgA/EvgS system. The EvgA/EvgS system in *E. coli* is highly similar to the BvgA/BvgS system, which controls the expression of adhesins, toxins, and other virulence factors in *Bordetella pertussis* (2, 30, 31). Although the environmental signal to which the EvgS

sensor responds is still unidentified, studies using a mutant with a constitutively active EvgS (*evgS1* mutant [12]) or using overexpression of the response regulator EvgA demonstrated that the EvgA/EvgS system confers multidrug resistance to a drug-hypersusceptible strain that lacks constitutive multidrug efflux genes *acrAB* (16) and acid resistance to exponentially growing cells (5, 17, 18, 24–26). In our recent microarray analysis of the mutant with a constitutively active EvgS, we found that as many as 225 open reading frames showed significant increases in transcription and that 144 open reading frames showed significant decreases in transcription (<http://www.nara.kindai.ac.jp/nogei/seiken/array2.html>) (5). Comparison of this result with the systematic analysis of 36 two-component deletion mutants by Oshima et al. (27) revealed a surprisingly strong relationship between the EvgA/EvgS system and another two-component system, PhoP/PhoQ, which senses external Mg<sup>2+</sup> concentration (8). Signal transduction between these two systems is investigated in this study.

### MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The *E. coli* strains and plasmids used in this study are listed in Table 1. The *phoP* strains (KMP1 and KMP2001) and *phoQ* strains (KMQ1 and KMQ2001) were constructed with P1 transduction from donor strains WP3022 and WQ3007 (22) to recipient strains KMY1 (wild type) and KMY2001 (*evgS1* mutant). Bacteria were grown at 37°C in Luria-Bertani (LB) medium (pH 7.5), containing 1% Bacto Tryptone (Difco), 0.5% Bacto Yeast Extract (Difco), and 1% NaCl. MgCl<sub>2</sub> was added to a final concentration of 30 mM when necessary. For induction of the arabinose promoter of pA191, arabinose at a final concentration of 0.2% (wt/vol) was added to the culture at an optical density at 600 nm (OD<sub>600</sub>) of 0.2. Ampicillin, chloramphenicol, kanamycin, and tetracycline were used at 100, 25, 25, and 12.5 μg/ml, respectively.

**Site-directed mutagenesis.** In order to create pOH2001D52A, a low-copy-number plasmid carrying the *evgA* gene with an Asp-to-Ala mutation at position 52, site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Stratagene), primers 5'-GATATCGTCATCATTTGCTGTGCGA TATCCCC-3' and 5'-GGGGATATCGACAGCAATGATGACGATATC-3' (the mutation site is underlined), and pOH2001 carrying the *evgA* and *evgS1* genes.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or description	Reference, construction, or source
<i>E. coli</i> strains		
MC4100	F <sup>-</sup> $\Delta$ ( <i>argF-lac</i> )U169 <i>araD139 rpsL150 ptsF25 fibB5301 rbsR</i>	3
MK12	MC4100 $\Delta$ ( <i>ara-leu</i> )7696	12
KMY1	MK12 $\lambda$ RS45[ $\phi$ ( <i>emrK'</i> - <i>lacZ</i> )]	12
KMY2001	KMY1 <i>evgS1</i>	12
KMP1	KMY1 <i>phoP::cat</i>	KMY1 $\times$ P1(WP3022) $\rightarrow$ Cam <sup>r</sup>
KMP2001	KMY2001 <i>phoP::cat</i>	KMY2001 $\times$ P1(WP3022) $\rightarrow$ Cam <sup>r</sup>
KMQ1	KMY1 <i>phoQ::cat</i>	KMY1 $\times$ P1(WQ3007) $\rightarrow$ Cam <sup>r</sup>
KMQ2001	KMY2001 <i>phoQ::cat</i>	KMY2001 $\times$ P1(WQ3007) $\rightarrow$ Cam <sup>r</sup>
KMY204	KMY1 <i>evgA::cat</i>	12
WP3022	W3110 <i>phoP::cat</i>	22
WQ3007	W3110 <i>phoQ::cat</i>	22
MG1601	MC4100 <i>mgtA::<math>\lambda</math>placMu55</i>	11
Plasmids		
pMV191	pBAD18 derivative carrying an EcoRI-AbaI fragment containing the <i>tetR</i> gene of pBR322	12
pA191	pMV191 derivative carrying a 0.7-kb fragment containing the <i>evgA</i> gene	12
pMW119	Low-copy-number vector	Nippon Gene
pOH2001	pMW119 derivative carrying a 5.2-kb fragment containing the <i>evgA</i> and <i>evgS1</i> genes from KMY2001	Laboratory stock
pOH2001D52A	pOH2001 derivative carrying an <i>evgA</i> D52A gene	This study
pHO119	pMW119 derivative carrying a 3.6-kb fragment containing the <i>phoPQ</i> gene	11

<sup>a</sup> Cam<sup>r</sup>, chloramphenicol resistance.

**RNA preparation and S1 nuclease mapping.** Total RNA was extracted with hot phenol from a mid-log-phase culture (OD<sub>600</sub> of about 0.8) at 60°C, as described by Aiba (1). Total RNA of EvgA-overexpressing strains was prepared from cells grown for an additional 30 min at 37°C after reaching an OD<sub>600</sub> of about 0.8 for ample induction of EvgA from the arabinose promoter. S1 mapping was performed by the method of Kato et al. (12). Briefly, a <sup>32</sup>P-end-labeled probe was prepared by PCR using the primers described by Minagawa et al. (22) and the primers listed in Table 2, *E. coli* MC4100 genomic DNA as the template, and ExTaq DNA polymerase (Takara). A mixture of <sup>32</sup>P-end-labeled probe and 100  $\mu$ g of total RNA was incubated for 10 min at 75°C, then gradually cooled to 37°C, and incubated overnight for hybridization. This was followed by S1 nuclease (Takara) digestion for 10 min at 37°C. Undigested RNA-probe DNA was extracted with phenol, precipitated with ethanol, and subjected to electrophoresis on a 6% (wt/vol) polyacrylamide sequencing gel. The radioactivity of the transcripts was measured by BAS1000 Mac (Fuji film) and analyzed by MacBAS2.2 (Fuji film).

**PhoP detection.** Cells were grown to an OD<sub>600</sub> of about 0.8 at 37°C in LB medium with constant shaking, collected by centrifugation, and resuspended in a solution of 25% sucrose and 40 mM Tris-HCl (pH 8.0). After treatment with 1 mM EDTA and 0.5 mg of lysozyme per ml at 0°C for 10 min, cells were lysed by adding 0.5% Brij 58. The Brij 58 lysate was supplemented with 0.01 M MgCl<sub>2</sub> and 0.2 M KCl, digested at 37°C for 10 min with 20  $\mu$ g of RNase A per ml in the presence of 1 mM phenylmethylsulfonyl fluoride, and sonicated. The supernatant after centrifugation for 30 min at 20,000  $\times$  g was used as the cell lysate. The protein concentration of cell lysates was determined by the Bradford method

(protein assay kit; Bio-Rad). Equal amounts of protein (16  $\mu$ g/lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were then transferred to a polyvinylidene difluoride membrane (Immobilon-P transfer membranes; Millipore), probed with anti-PhoP serum, and visualized by alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G, 5-bromo-4-chloro-3-indolylphosphate, and nitroblue tetrazolium (immunoblot kit; Bio-Rad).

**$\beta$ -Galactosidase assay.** Duplicate samples of cells grown to an OD<sub>600</sub> of about 0.8 at 37°C in LB medium were subjected to  $\beta$ -galactosidase assay, and the results were expressed in Miller units (21). The data shown are means and standard deviations for three individual cultures.

## RESULTS

**DNA microarray-based analysis of the interaction between EvgA/EvgS and other two-component systems.** To search for two-component systems interacting with the EvgA/EvgS system, we first selected up-regulated genes of other two-component systems by picking genes that were down-regulated by deletion of each individual two-component system ([http://ecoli.aist-nara.ac.jp/xp\\_analysis/2\\_components](http://ecoli.aist-nara.ac.jp/xp_analysis/2_components)) (27). We then matched them with the up-regulated genes by the activation of

TABLE 2. PCR primers

Gene	Primer	Primer sequence	GenBank accession no.	Nucleotide positions
<i>crcA</i>	crcAF	5'-TGG TGT TAG CTG CGG CTG TG-3'	AE000167	4147-4166
	crcAR	5'-GGC TGT TGC CAG GTT TGT GC-3'	AE000167	4631-4612
<i>ybjG</i>	ybjGF	5'-GGC CCC AGC AGC CAT TGT AA-3'	AE000186	5782-5763
	ybjGR	5'-CCA GCA TAA CTT CCC GAC GC-3'	AE000186	5271-5290
<i>proP</i>	proPF	5'-GTG GTT TGC CTC TTC GAC CG-3'	AE000483	4827-4846
	proPR	5'-CCC AGT GAT GCT GCG GTA AT-3'	AE000483	5398-5379
<i>ompT</i>	ompTF	5'-GCG GCC CAC GAC TTA GAA GT-3'	AE000161	8964-8945
	ompTR	5'-GCA ATA GGG GTT GTC AGG AC-3'	AE000161	8479-8498

TABLE 3. Interaction between EvgAS and other two-component systems

TCS <sup>a</sup>	Ratio <sup>b</sup>	TCS <sup>a</sup>	Ratio <sup>b</sup>
<i>phoPQ</i> .....	14/27	<i>nrBC</i> .....	1/8
<i>rssB</i> .....	3/23	<i>arcA</i> .....	0/38
<i>envZ ompR</i> .....	2/54	<i>arcB</i> .....	3/112
<i>basSR</i> .....	0/12	<i>baeSR</i> .....	0/8
<i>narXL</i> .....	0/4	<i>cpxRA</i> .....	1/16
<i>narQ</i> .....	2/25	<i>creBC</i> .....	1/1
<i>yojN</i> .....	0/17	<i>kdpDE</i> .....	0/10
<i>hydHG</i> .....	0/13	<i>phoBR</i> .....	0/8
<i>yfhA</i> .....	1/17	<i>rstAB</i> .....	2/22
<i>rcsB</i> .....	0/11	<i>torSR</i> .....	1/24
<i>uhpAB</i> .....	0/3	<i>yedWV</i> .....	0/7
<i>yfhK</i> .....	0/8	<i>ygiXY</i> .....	0/6
<i>narP</i> .....	0/9	<i>barA</i> .....	1/24
<i>uvrY</i> .....	1/37	<i>cusRS</i> .....	1/7
<i>ypdAB</i> .....	1/18	<i>fimZ</i> .....	1/22
<i>citAB</i> .....	1/23	<i>cheABY</i> .....	1/28
<i>yehUT</i> .....	0/7	<i>atoSC</i> .....	0/32
<i>dcuSR</i> .....	2/29		

<sup>a</sup> TCS, two-component system.

<sup>b</sup> Ratio, number of up-regulated genes in the constitutively active *evgS1* mutant among the genes up-regulated by the corresponding system to the number of up-regulated genes by the corresponding system ([http://ecoli.aist-nara.ac.jp/xp\\_analysis/2\\_components](http://ecoli.aist-nara.ac.jp/xp_analysis/2_components)) (27).

the EvgS sensor (<http://www.nara.kindai.ac.jp/nogei/seiken/array2.html>) (5). As shown in Table 3, expression of 14 of 27 PhoP/PhoQ up-regulated genes was enhanced in the *evgS1* mutant, clearly indicating interaction between the EvgA/EvgS and PhoP/PhoQ systems. The expression ratio of the 14 PhoP/PhoQ-regulated genes, which were up-regulated by the constitutively active *evgS1* mutation, is shown in Table 4. Of these 14 genes, 9, namely, *hemL*, *mgtA*, *phoP*, *phoQ*, *rstA*, *rstB*, *slyB*, *yrbL*, and *mgrB*, were members of the PhoP/PhoQ-dependent Mg<sup>2+</sup> stimulon reported by Minagawa et al. (22). These genes are directly regulated by PhoP via phosphorylation from the PhoQ sensor.

**Enhanced expression of PhoP regulons by EvgA/EvgS.** To further analyze the promoter regions of EvgA/EvgS-enhanced PhoP regulons, S1 mapping was performed for the 14 genes listed in Table 4. Enhanced expression of 13 genes in the *evgS1* mutant (note that the *phoP* and *phoQ* genes and the *rstA* and *rstB* genes form operons) was confirmed (Fig. 1, lanes 2), validating our microarray data. Expression of these transcripts was dependent on PhoP/PhoQ, since deletion of *phoP* or *phoQ* resulted in a prominent decrease in transcription, even in the *evgS1* mutants (Fig. 1, lanes 3 to 6). This decrease in transcription was complemented by pHO119, a plasmid carrying the *phoPQ* operon with its native promoter (data not shown). Thus, the enhancement of these promoters by EvgA/EvgS is clearly dependent on PhoP/PhoQ, indicating signal transduction from the EvgA/EvgS system to the PhoP/PhoQ system. As for the remaining *copA* gene, transcripts were searched for in a range of 1,000 bp upstream of the translational start codon, but no transcript was detected in our S1 mapping for unknown reasons.

**Promoters of the PhoP regulon activated by EvgA/EvgS.** All nine genes reported by Minagawa et al. (22) share a consensus PhoP binding sequence [consensus sequence of PhoP box, (T/G)GTTTAnnnnn(T/G)GTTTA (n is any nucleotide) (Fig. 2)] at their -35 region with a maximum mismatch of two nucleotides. Promoter regions of the four remaining genes, *crcA*, *ybjG*, *proP*, and *ompT*, are shown in Fig. 2 in comparison

with several PhoP regulons. PhoP boxes with a mismatch of two and three nucleotides were found in the -35 regions of *ybjG* and *crcA*, respectively, suggesting that these two genes may be directly regulated by PhoP. On the other hand, PhoP boxes were not found in the promoter regions of *proP* and *ompT*, suggesting an indirect regulation of these genes by PhoP.

**Enhanced expression of the PhoP regulon by overproduced EvgA.** EvgA was overexpressed from an arabinose promoter in the wild-type strain, and the expression of EvgA/EvgS-enhanced PhoP regulons was determined. As shown in Fig. 3, lanes 1, overexpression of EvgA in a wild-type EvgS background also caused an increase in transcription from 10 of the 11 promoters under investigation. Furthermore, pOH2001D52A, which harbors *evg4D52A* (mutation in the phosphorylation site of *evg4*) and *evgS1* genes and their native promoter was transformed into

TABLE 4. Up-regulation of PhoPQ regulated genes by constitutively active *evgS1* mutation

PhoPQ-regulated gene	Expression ratio <sup>a</sup>
<i>copA</i> .....	10.0, 9.3
<i>crcA</i> .....	58.9, H <sup>b</sup>
<i>hemL</i> .....	3.5, 3.2
<i>mgtA</i> .....	14.7, 18.6
<i>ompT</i> .....	5.4, 7.0
<i>phoP</i> .....	7.2, 7.2
<i>phoQ</i> .....	4.5, 6.3
<i>proP</i> .....	13.3, 17.8
<i>rstA</i> .....	3.6, 5.4
<i>rstB</i> .....	5.0, 3.5
<i>slyB</i> .....	3.0, 3.6
<i>ybjG</i> .....	5.9, 5.3
<i>yrbL</i> .....	2.9, 2.5
<i>mgrB</i> .....	5.6, 2.5

<sup>a</sup> Ratio of normalized signal intensities of Cy5-dUTP (strain KMY2001) against Cy3-dUTP (strain KMY1) from DNA microarray analysis. Two ratios are shown for each gene (<http://www.nara.kindai.ac.jp/nogei/seiken/array2.html>) (5).

<sup>b</sup> H, signal not detected in strain KMY1 but signal was significantly detected in strain KMY2001.

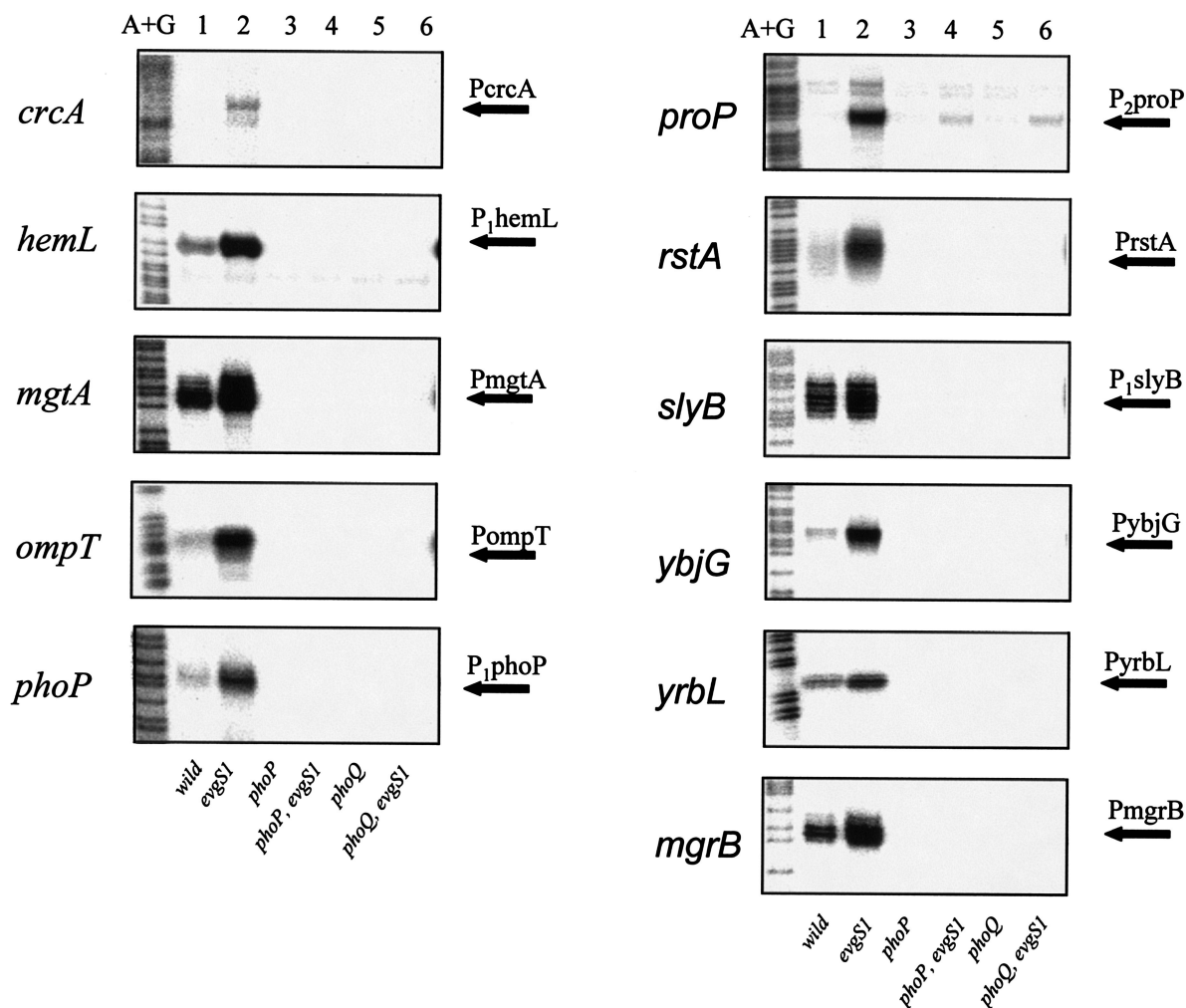


FIG. 1. S1 mapping of EvgA/EvgS-regulated PhoP regulons of *E. coli* strains. Lanes: A+G, Maxam-Gilbert sequencing ladder; 1, KMY1; 2, KMY2001 (*evgS1*); 3, KMP1 (*phoP*); 4, KMP2001 (*phoP evgS1*); 5, KMQ1 (*phoQ*); 6, KMQ2001 (*phoQ evgS1*). Transcripts with increased expression in the *evgS1* mutants are indicated by arrows labeled with the gene promoter.

an *evgA* mutant and the wild-type strain (Fig. 3, lanes 3 and 4). Although introduction of the *evgS1* gene did not enhance transcription of the PhoP-regulated genes in the absence of a wild-type *evgA* (Fig. 3, lanes 3), one copy of the wild-type *evgA* in the chromosome of the host strain resulted in enhanced transcription of the PhoP regulons (Fig. 3, lanes 4). These results clearly indicate that phosphotransfer from phospho-EvgS to EvgA is essential for the increased transcription of the EvgA/EvgS-enhanced PhoP regulons. They also show that cross talk from EvgS to PhoP is not the major pathway.

**Effect of enhanced transcriptional level of *phoP* on the expression of the PhoP regulon.** Transforming pHO119 to an *evgA* mutant and the wild-type strain caused an increase in *phoP* transcription to a level similar to that found in the *evgS1* mutant (Fig. 4a, lanes 1 and 3). Immunoblotting with anti-PhoP serum confirmed that the increase in PhoP protein in the pHO119-transformed strains was also similar to the level found in the *evgS1* mutant (Fig. 4b). However, the pHO119 transformants did not show increased expression of EvgA/EvgS-enhanced PhoP regulons, except for *phoPQ*, in both the

*evgA* mutant and the wild-type strain (Fig. 4a, lanes 1 and 3). Thus, a simple increase in the level of PhoP was not involved in the signal transduction from EvgA/EvgS to PhoP/PhoQ.

On the other hand, transcriptional activity of *mgtA*, a well-characterized member of the PhoP regulon (11, 36), was assayed in the presence of 30 mM MgCl<sub>2</sub> (an inactivating condition for the PhoQ sensor [11]). Although transcription in the wild-type strain was repressed to 30% of that in the absence of MgCl<sub>2</sub> (Fig. 5a, lanes 1 and 2) (ratio calculated from the radioactive intensity of the corresponding bands), transcription in the *evgS1* mutant grown in the presence of 30 mM MgCl<sub>2</sub> was still nearly twice that of the wild-type strain grown in the absence of MgCl<sub>2</sub> (Fig. 5a, lanes 1 and 4).

Furthermore, these results were confirmed by reporter assays (Fig. 5b). Plasmids pOH2001, carrying the *evgA-evgS1* operon and its native promoter, and pHO119, carrying the *phoPQ* operon and its native promoter, were transformed into strain MG1601. The expression of the *mgtA* promoter was assayed by  $\beta$ -galactosidase activity. Introduction of *evgS1* enhanced the *mgtA* expression over that of the control (Fig. 5b,



Consensus sequence of PhoP box: **(T/G) GTTTAnnnnn (T/G) GTTTA**

FIG. 2. Promoter analysis of *crcA*, *ybjG*, *proP*, and *ompT* compared with other PhoP regulons. Transcription start sites (thin arrows labeled with the gene promoter) and direct repeats (PhoP box) (thick arrows) are indicated. The  $-10$  region of each promoter and the  $-35$  region of the *ompT* promoter are underlined. The  $-10$  and  $-35$  hexamers, start sites, and the direct repeats are indicated in capital letters.

bars 3 and 4), but increase in *phoP* expression did not enhance *mgtA* expression (Fig. 5b, bars 5 and 6).

## DISCUSSION

Through a combination of microarray analysis and S1 mapping, we found evidence of signal transduction between EvgA/EvgS and PhoP/PhoQ systems. This signal transduction was observed to occur as a result of both EvgS activation and EvgA overproduction (Fig. 1 and 3). We also demonstrated that phospho-EvgA was essential to this signal transduction (Fig. 3). Although the level of *phoPQ* transcription increased as a result of activation of EvgS, transcription of the *phoPQ* genes from a heterologous promoter in a wild-type EvgS background did not promote expression of the PhoP regulons, except in the case of *phoP* (Fig. 4a).

One hypothesis presented to explain the mechanism underlying signal transduction between EvgA/EvgS and PhoP/PhoQ was that the phospho-EvgA directly regulated the PhoP/PhoQ-regulated genes in cooperation with PhoP. Masuda and Church (18) reported that EvgA binds to an 18-bp consensus sequence (5'-TTCPyTACA-3' and its inverted repeat, 5'-TGTAPuGAA-3', separated by two random bases [Py is a pyrimidine, and Pu is a purine]) for the direct regulation of the EvgA regulons. This 18-bp consensus sequence was not found in any of the PhoP/PhoQ-regulated genes enhanced by the EvgA/EvgS system. We also performed an in vitro transcription assay of the *mgtA* gene using DNA templates containing the promoter region of *mgtA*, RNA polymerase, acetyl phosphate, PhoP, and EvgA and found no additive effect of EvgA (data not shown). Thus, we were not able to find any evidence of a

direct interaction between EvgA and the PhoP/PhoQ-regulated genes.

Another possibility was the indirect interaction of EvgA in cooperation with PhoP, where another transcriptional factor regulated by the EvgA/EvgS system directly interacted with the promoters of PhoP/PhoQ-regulated genes. If this were the case, a consensus sequence for the binding site of the unknown transcriptional factor should have been present in the 11 promoter regions of the PhoP/PhoQ-regulated genes. A search for such a consensus sequence was performed using Genespring software for positions  $+1$  to  $-500$  of each transcriptional start site of the 11 promoters but failed to find any such sequence. Therefore, direct and indirect interaction of EvgA with the 11 promoters in cooperation with PhoP also could not explain the signal transduction under investigation.

Taking all the aforementioned results together, the only possibility left was the increase in the level of phospho-PhoP by the EvgA/EvgS system. PhoQ of the PhoP/PhoQ system responds to extracellular  $Mg^{2+}$  levels (6, 7, 33). At a high concentration of  $Mg^{2+}$ , its phosphatase activity is enhanced, resulting in a lower level of cellular phospho-PhoP and thus repression of PhoP regulon transcription (4). In the presence of 30 mM  $MgCl_2$ , *mgtA* transcription in the wild-type strain was actually repressed to 30% of that in the absence of  $MgCl_2$  (Fig. 5). However, *mgtA* transcription in the *evgS1* mutant grown in the presence of 30 mM  $MgCl_2$  was still nearly twice that of the wild-type strain grown without  $MgCl_2$  (Fig. 5). In other words, compared to the wild-type strain at a low  $Mg^{2+}$  concentration, a higher level of phospho-PhoP was present in the *evgS1* mutant even at a high  $Mg^{2+}$  concentration, where the phospho-

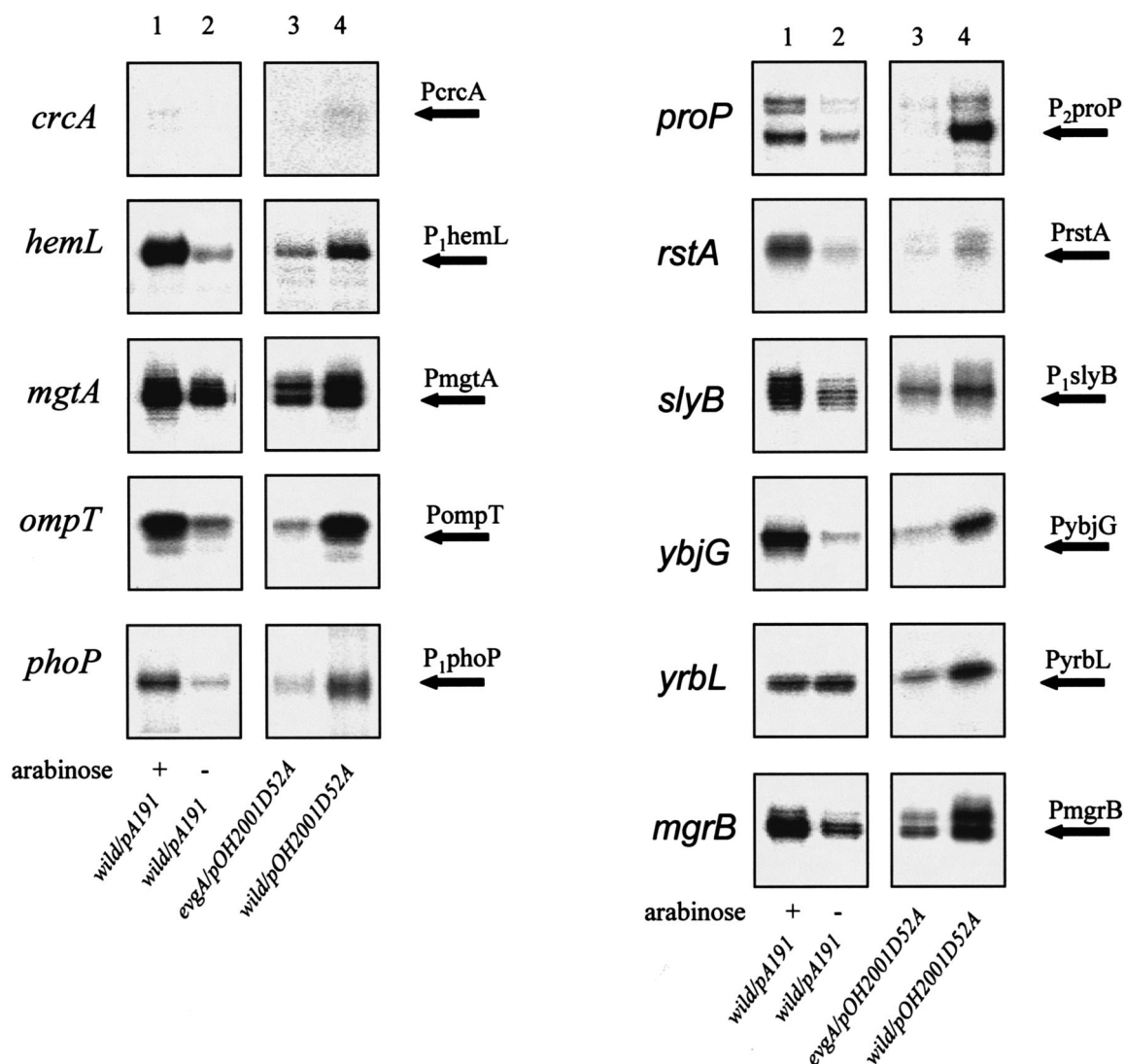


FIG. 3. Effect of overproduction of EvgA and significance of the phosphorylation site of EvgA. S1 mapping of EvgA/EvgS-regulated PhoP regulons of *E. coli* strains was performed. Lanes 1, KMY1 (wild type) carrying plasmid pA191 in the presence of arabinose; 2, KMY1/pA191 grown without arabinose; 3, KMY204 (*evgA*) carrying pOH2001D52A (*evgA*D52A *evgS*); 4, KMY1/pOH2001D52A (*evgA*D52A *evgS*). Transcripts with increased expression in the *evgS* mutants are indicated by arrows labeled with the gene promoter.

tase activity of PhoQ should have been enhanced. The increase in the phospho-PhoP level may be due to inhibition of the phosphatase activity of PhoQ or to activation of the kinase activity of PhoQ by a mechanism requiring further investigation.

In *Salmonella enterica* serovar Typhimurium, a similar cascade signal transduction between the PhoP/PhoQ and PmrA/PmrB systems has been reported (9, 29). In signal transduction from PhoP/PhoQ to PmrA/PmrB, an increase in the phospho-PmrA levels by phospho-PhoP was suggested from the following two results: (i) transcription of the *pmrAB* genes from a heterologous promoter cannot restore expression of PmrA-regulated genes to strains lacking *phoP* or *phoQ*, and (ii) the *pmrCAB* operon is transcriptionally autoregulated (29). We also obtained similar results with signal transduction from EvgA/EvgS to PhoP/PhoQ in *E. coli*: transcription of the *phoPQ* genes from plasmid pHO119 could not restore expres-

sion of PhoP-regulated genes (Fig. 4a), and the *phoPQ* operon was transcriptionally autoregulated (Fig. 1). In *Salmonella*, a small protein, PmrD, was found to mediate activation of the PmrA/PmrB system by the PhoP/PhoQ system (14), and the PmrA/PmrB system negatively controls the expression of PmrD (13). However, it is not yet known how PmrD activates the PmrA/PmrB system. A similar signal transduction model between the EvgA/EvgS and PhoP/PhoQ systems may exist in *E. coli* and involve other factors. In addition to the PhoQ sensor sensing Mg<sup>2+</sup> and Ca<sup>2+</sup>, PhoQ in *E. coli* was recently reported to be inactivated by acetate in a sensor domain-independent mechanism (15). Thus, there is a possibility that PhoQ in *E. coli* may sense stimulants other than Mg<sup>2+</sup> and Ca<sup>2+</sup>, which may involve the EvgA/EvgS system.

The PhoP regulon candidate genes, *crcA* and *ybjG*, discovered in this study are genes detected only when the PhoP/PhoQ system is enhanced by EvgA/EvgS. These genes were not

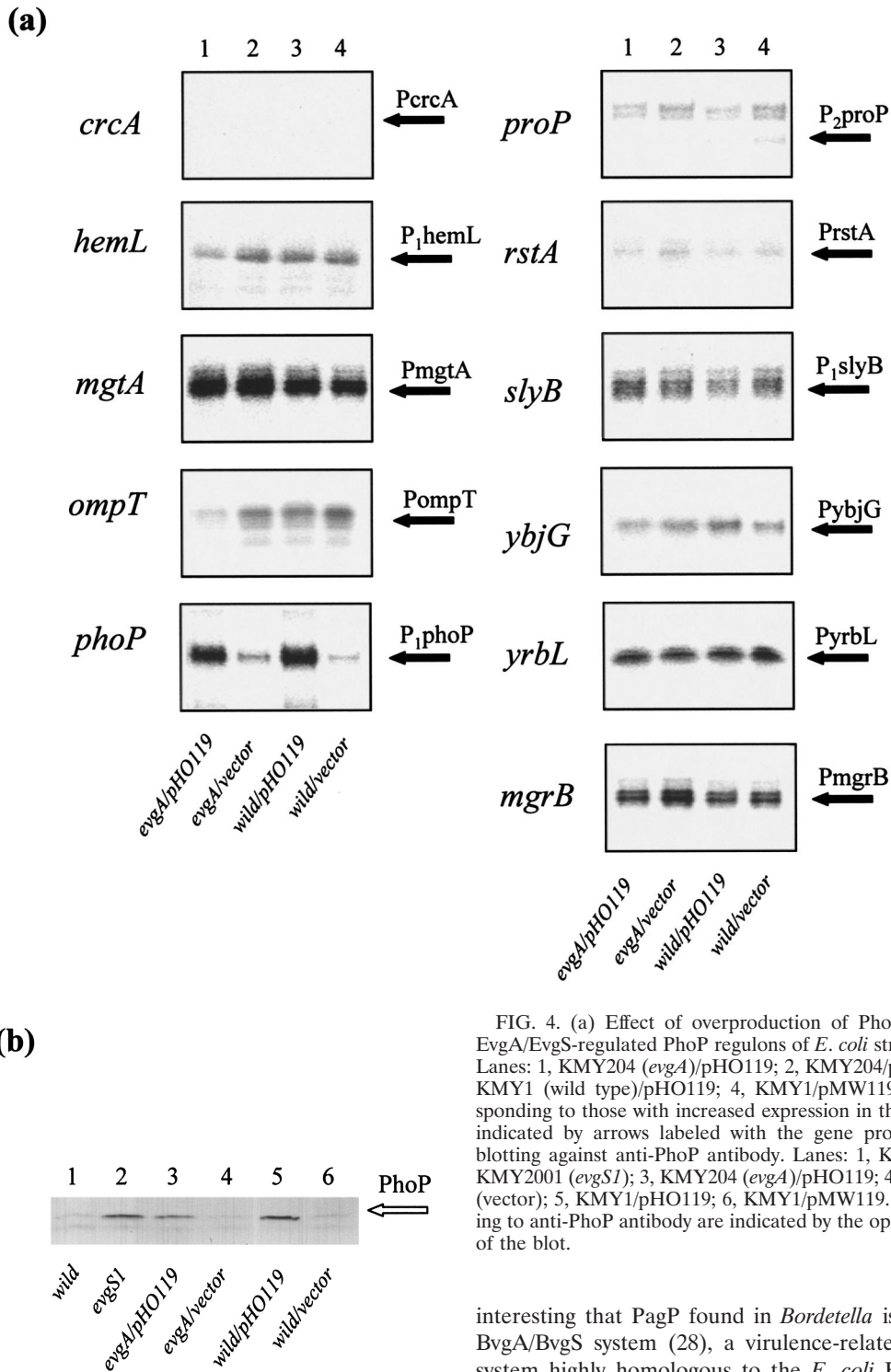


FIG. 4. (a) Effect of overproduction of PhoPQ. S1 mapping of EvgA/EvgS-regulated PhoP regulons of *E. coli* strains was performed. Lanes: 1, KMY204 (*evgA*)/pHO119; 2, KMY204/pMW119 (vector); 3, KMY1 (wild type)/pHO119; 4, KMY1/pMW119. Transcripts corresponding to those with increased expression in the *evgS1* mutants are indicated by arrows labeled with the gene promoter. (b) Western blotting against anti-PhoP antibody. Lanes: 1, KMY1 (wild type); 2, KMY2001 (*evgS1*); 3, KMY204 (*evgA*)/pHO119; 4, KMY204/pMW119 (vector); 5, KMY1/pHO119; 6, KMY1/pMW119. The bands responding to anti-PhoP antibody are indicated by the open arrow to the right of the blot.

found in our earlier search (22) because the expression of these genes is very low without enhancement by the EvgA/EvgS system. The *crcA* gene is a homolog of *pagP* in *Salmonella* and encodes an enzyme located in the outer membrane. PagP is involved in cationic antimicrobial peptide resistance and is regulated by the PhoP/PhoQ system in *Salmonella* (10). It is

interesting that PagP found in *Bordetella* is regulated by the BvgA/BvgS system (28), a virulence-related two-component system highly homologous to the *E. coli* EvgA/EvgS system (31).

Another gene regulated by EvgA/EvgS via PhoP/PhoQ is *proP*, which encodes an integral membrane transporter of proline, glycine betaine, and other osmoprotecting compounds. Two promoters, P1 and P2, have been reported for *proP* (20). EvgA/EvgS up-regulates transcription from the P2 promoter, which has been reported to be completely dependent on RpoS and coactivated by Fis and CRP (cyclic AMP receptor protein)

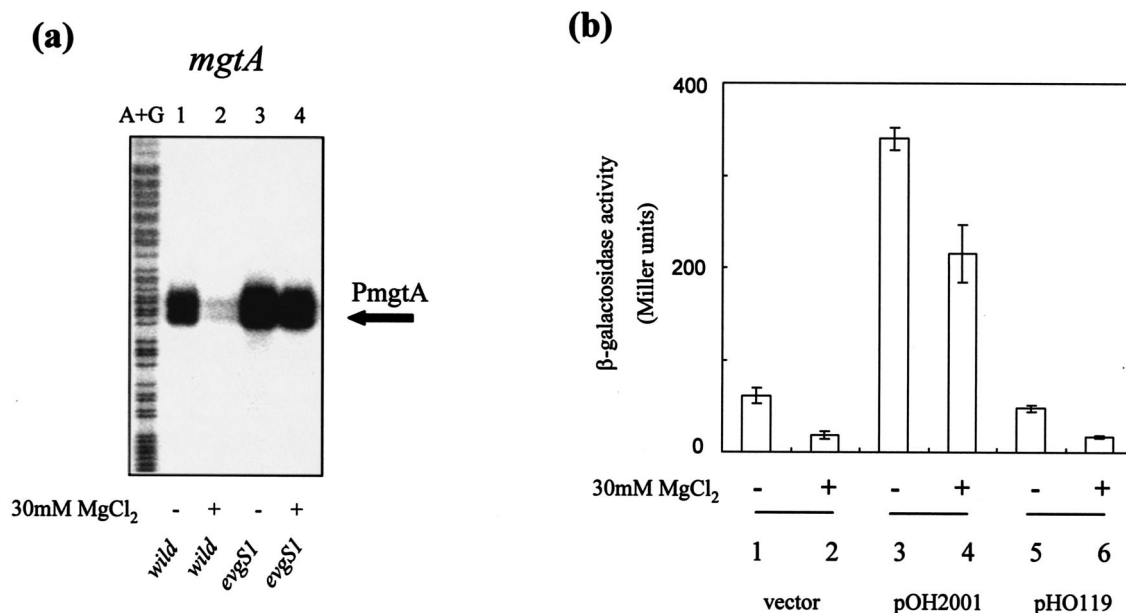


FIG. 5. (a) Effect of high Mg<sup>2+</sup> concentration. S1 mapping of the *mgtA* genes of *E. coli* strains was performed. Lanes: A+G, Maxam-Gilbert sequencing ladder; 1, KMY1 (wild type) without MgCl<sub>2</sub>; 2, KMY1 with 30 mM MgCl<sub>2</sub>; 3, KMY2001 (*evgS1*) without MgCl<sub>2</sub>; 4, KMY2001 with 30 mM MgCl<sub>2</sub>. (b) Effect of high Mg<sup>2+</sup> concentration on the expression of *mgtA* in *evgS1* strains and *phoPQ*-overexpressed strains by reporter assay. *E. coli* MG1601 carrying pMW119 (vector) (bars 1 and 2), pOH2001 (bars 3 and 4), or pHO119 (bars 5 and 6) were grown in the absence (-) or presence (+) of 30 mM MgCl<sub>2</sub>. The data shown are means and standard deviations (error bars) for three individual cultures.

by direct binding to the promoter (19, 34, 35). The absence of the PhoP box in the P2 promoter suggested that the regulation by phospho-PhoP was indirect. Transcript levels of *fis* and *crp* in the *evgS1* mutant compared to the parent strain in our microarray were 0.44 and 0.37, respectively, for *fis* and 0.99 and 0.84, respectively, for *crp* (5), indicating that the changes in transcriptional levels of *fis* and *crp* were not related. It should be pointed out that the wild-type strain that did not overproduce EvgA showed enhanced transcription from the *proP* P<sub>2</sub> promoter (Fig. 3, lane 2), whereas the wild-type strain in Fig. 1 did not (lane 1). This is probably due to the difference in the growth phase of the cells. Although the cultivation of the cells in Fig. 1 was stopped once the cells reached an OD<sub>600</sub> of about 0.8, the cells in lanes 1 and 2 of Fig. 3 were grown for an additional 30 min after the cells reached an OD<sub>600</sub> of about 0.8 for ample overproduction of EvgA. Thus, these cells were collected at an OD<sub>600</sub> of 1.0 to 1.2, possibly in their late log phase, a stage when transcription from the P2 promoter is induced (34). We also performed S1 mapping of *proP* with *rpoS* mutants and found that the EvgA/EvgS-dependent up-regulation of the P2 promoter was completely dependent on RpoS (data not shown).

The difference in growth phase may also explain why transcription of *yrbL* was enhanced without the overproduction of EvgA (Fig. 3, lane 2). Furthermore, in our previous study (5), the EvgA/EvgS-dependent up-regulation of transcription from the P2 promoter of *tolC*, which encodes an outer membrane channel protein, markedly decreased in the absence of *phoP* or *phoQ*, indicating that regulation of *tolC* also involved both EvgA/EvgS and PhoP/PhoQ systems.

Then, why should the EvgA/EvgS system enhance the expression of the PhoP regulons? The PhoP/PhoQ system regu-

lates various genes involved in functions such as modification of the components in the bacterial cell envelope besides those known for the adaptation to Mg<sup>2+</sup>-limiting environments (8). We confirmed that deletion of *phoP* or *phoQ* in *E. coli* decreased the MICs of cholate and deoxycholate (data not shown), as has been reported for *Salmonella* (32). In the natural environment, there may be a situation where *E. coli* requires the activation of the PhoP/PhoQ system at high Mg<sup>2+</sup> concentrations. We believe that this situation may overlap with the unknown signal for the EvgS sensor.

In conclusion, the present study demonstrated a novel interaction between the EvgA/EvgS and PhoP/PhoQ systems in *E. coli* and also identified genes that required the two systems for enhanced expression. This is one example of the intricate signal transduction networks posited to exist in *E. coli*.

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