

# Contribution of the PhoP-PhoQ and PmrA-PmrB Two-Component Regulatory Systems to $Mg^{2+}$ -Induced Gene Regulation in *Pseudomonas aeruginosa*

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When grown in divalent cation-limited medium, *Pseudomonas aeruginosa* becomes resistant to cationic antimicrobial peptides and polymyxin B. This resistance is regulated by the PhoP-PhoQ and PmrA-PmrB two-component regulatory systems. To further characterize  $Mg^{2+}$  regulation in *P. aeruginosa*, microarray transcriptional profiling was conducted to compare wild-type *P. aeruginosa* grown under  $Mg^{2+}$ -limited and  $Mg^{2+}$ -replete conditions to isogenic *phoP* and *pmrA* mutants grown under  $Mg^{2+}$ -limited conditions. Under  $Mg^{2+}$ -limited conditions (0.02 mM  $Mg^{2+}$ ), approximately 3% of the *P. aeruginosa* genes were differentially expressed compared to the expression in bacteria grown under  $Mg^{2+}$ -replete conditions (2 mM  $Mg^{2+}$ ). Only a modest subset of the  $Mg^{2+}$ -regulated genes were regulated through either PhoP or PmrA. To determine which genes were directly regulated, a bioinformatic search for conserved binding motifs was combined with confirmatory reverse transcriptase PCR and gel shift promoter binding assays, and the results indicated that very few genes were directly regulated by these response regulators. It was found that in addition to the previously known *oprH-phoP-phoQ* operon and the *pmrHFIJKLM-ugd* operon, the PA0921 and PA1343 genes, encoding small basic proteins, were regulated by  $Mg^{2+}$  in a PhoP-dependent manner. The number of known PmrA-regulated genes was expanded to include the PA1559-PA1560, PA4782-PA4781, and *feoAB* operons, in addition to the previously known PA4773-PA4775-*pmrAB* and *pmrHFIJKLM-ugd* operons.

*Pseudomonas aeruginosa* is an important opportunistic pathogen that is capable of infecting a large number of hosts, including nematodes, insects, plants, animals, and especially humans. It is the third-leading cause of nosocomial infections and is also the leading cause of morbidity and mortality in cystic fibrosis (CF) patients (33). *P. aeruginosa* is also noted for its metabolic diversity, which allows it to colonize a large number of environmental habitats. The versatility of this organism is believed to be related to the large number of regulatory proteins found in its genome (469 of 5,570 open reading frames) (43).

The two-component response regulators constitute one of the larger families of regulatory proteins in *P. aeruginosa* (43). These systems typically contain a sensor protein that responds to some chemical or physical stimulus, which leads to phosphorylation of the sensor protein at a conserved histidine residue, thus altering the conformation of the sensor and promoting phosphotransfer to a cognate response regulator protein (9). The phosphorylated response regulator then recognizes and binds to a specific DNA sequence, leading to modulation of transcription from that promoter. Although this is often the mechanism, regulation may also occur through phosphatase activity of the sensor kinase with the response regulator (37) or

through integration into the signaling cascade of multiple signals from other proteins (32). In *P. aeruginosa*, there are 64 response regulators and 63 histidine kinases, as well as 16 atypical kinases (36). The functions of the majority of these regulatory proteins have not been established yet.

In *P. aeruginosa*, two separate two-component regulatory systems, PmrA-PmrB (26) and PhoP-PhoQ (21), are known to respond to the presence of limiting concentrations of  $Mg^{2+}$  and to separately regulate certain operons. The PhoPQ system autoregulates the *oprH-phoP-phoQ* operon (21) under  $Mg^{2+}$ -limiting growth conditions and is also involved in resistance to cationic antimicrobial peptides and polymyxin B and in virulence, as *phoQ* mutants exhibit increased resistance to cationic antimicrobial peptides and polymyxin B and have reduced virulence (20). Similarly, the PmrAB system regulates resistance to cationic antimicrobial peptides and polymyxin B in response to limiting concentrations of  $Mg^{2+}$ . This regulatory redundancy occurs because both the PhoPQ and PmrAB systems separately contribute to regulation of the *pmrHFIJKLM-ugd* operon (PA3552 to PA3559) in response to limiting concentrations of  $Mg^{2+}$  (26). The PmrAB system also autoregulates the PA4773-PA4775-*pmrAB*-PA4778 operon under  $Mg^{2+}$ -limiting conditions, and there is evidence that the first three genes in this operon, PA4773 to PA4775, also contribute to cationic antimicrobial peptide and polymyxin B resistance (26). Previous work has established that within the promoters controlled by PhoPQ- and PmrAB-containing operons there are related but distinct putative binding motifs that have been implicated in autoregulation (21, 26).

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TABLE 1. *P. aeruginosa* strains and plasmids used in this study

Strain or plasmid	Genotype or characteristics	Reference
H103	Wild-type <i>P. aeruginosa</i> PAO1	21
H851	<i>phoP::xylE-aacCI</i> ; Gm <sup>r</sup>	21
H974	PA4773:: <i>luxCDABE</i> derivative of H103; Tc <sup>r</sup>	26
H988	<i>pmrA::xylE-aacCI</i> ; Gm <sup>r</sup>	26
H993	<i>pmrH::xylE-aacCI</i> ; Gm <sup>r</sup>	This study
H1034	<i>feoA::luxCDABE</i> derivative of H103	19a
pphoP-His <sub>6</sub>	His <sub>6</sub> - <i>phoP</i> cloned into pET28a	This study
ppmrA-His <sub>6</sub>	His <sub>6</sub> - <i>pmrA</i> cloned into pET28a	This study

Because of the importance of Mg<sup>2+</sup> regulation via the PhoP-PhoQ and PmrA-PmrB genes to virulence and cationic antimicrobial peptide resistance in *P. aeruginosa*, a transcriptional analysis of *P. aeruginosa* PAO1 under Mg<sup>2+</sup>-deficient conditions was performed. Mutants with mutations in PhoP and PmrA were also examined to determine the contributions of each of these regulatory systems to regulation by limiting concentrations of Mg<sup>2+</sup>. In addition to this transcriptional profiling, in silico searches were carried out to identify promoters that contain the previously identified PhoP and/or PmrA regulatory motifs. This analysis was conducted independent of the transcriptional profiling to allow a comparison of these different approaches for identifying and characterizing targets of the regulatory proteins. Using both techniques, several novel PhoP- and PmrA-regulated genes were identified and characterized phenotypically.

#### MATERIALS AND METHODS

**Bacterial strains, primers, and growth conditions.** The bacterial strains and plasmids used in this study are described in Table 1. The sequences of DNA primers used in this study are available by request from us. All primers were synthesized by AlphaDNA (Montreal, QC, Canada). Cultures were routinely grown in Luria-Bertani broth or BM2-glucose minimal medium containing a low MgSO<sub>4</sub> concentration (20 μM) or a high MgSO<sub>4</sub> concentration (2 mM) (11). Antibiotics for selection were used at the following concentrations: tetracycline, 100 μg/ml; and gentamicin, 50 μg/ml. Routine genetic manipulations were carried out by using standard molecular biology procedures (23).

**RNA extraction, cDNA synthesis, and hybridization to DNA microarrays.** For each condition microarray experiments were performed with five independent cultures. *P. aeruginosa* PAO1 was grown for 18 h in BM2-glucose medium supplemented with either 2 mM MgSO<sub>4</sub> (high concentration) or 20 μM MgSO<sub>4</sub> (low concentration) in acid-washed glassware. Cultures were diluted 1/100 into fresh media, and cells were harvested at the mid-log phase (optical density at 600 nm [OD<sub>600</sub>] for the high Mg<sup>2+</sup> concentration, 0.5 to 0.6; OD<sub>600</sub> for the low Mg<sup>2+</sup> concentration, 0.3 to 0.4). RNA was isolated using a QIAGEN RNeasy midi RNA isolation kit according to the manufacturer's protocols (QIAGEN Inc., Canada). Contaminating genomic DNA was removed by treatment with a DNA-free kit (Ambion Inc., Austin, TX). RNA was stored at -80°C with 0.2 U/μl of SUPERase-In RNase inhibitor (Ambion Inc., Austin, TX). RNA quality was assessed by agarose gel electrophoresis and spectrophotometrically.

*P. aeruginosa* PAO1 microarray slides were provided by The Institute for Genomic Research (TIGR) Pathogenic Functional Genomics Resource Center (<http://pfgrc.tigr.org/>). Ten micrograms of total RNA was treated using a Microbe Express kit with the *Pseudomonas* module (Ambion) to remove rRNA. Two micrograms of cDNA was reverse transcribed using random hexamers (Invitrogen Canada Inc., Burlington, ON, Canada), deoxynucleoside triphosphates (Invitrogen), dUTP (Ambion), and Superscript II reverse transcriptase (Invitrogen) with a PTC-225 Peltier thermal cycler (MJ Research). cDNA labeling, purification, and analysis of the labeling reaction mixture were performed by using the TIGR "microbial RNA aminoallyl labeling" protocol ([pfgrc.tigr.org/protocols/M007.pdf](http://pfgrc.tigr.org/protocols/M007.pdf)). Treated cDNA was labeled with cyanine-5 (GE Healthcare Canada), and control cDNA was labeled with cyanine-3 (GE Healthcare Can-

ada). Labeled cDNA was hybridized by using the TIGR "hybridization of labeled DNA probes" protocol ([pfgrc.tigr.org/protocols/M008.pdf](http://pfgrc.tigr.org/protocols/M008.pdf)). Briefly, 200 pmol of each cyanine-labeled cDNA was combined and hybridized to the array slides for 18 h in a solution containing 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate, and 0.6 μg/μl salmon sperm DNA at 42°C in chambers in which the humidity was maintained at 100%. Following hybridization, the slides were washed, dried, and scanned using a ScanArray Express scanner and software (Packard BioScience BioChip Technologies).

**Analysis of DNA microarrays.** Images from microarray slides were quantified using the ImaGene 6.0 Standard Edition software (BioDiscovery, Inc., El Segundo, CA). Assessment of slide quality, normalization, detection of differential gene expression, and statistical analysis were carried out with ArrayPipe (version 1.7), which is web-based, semiautomated software specifically designed for processing of microarray data (<http://koch.pathogenomics.ca/cgi-bin/pub/arraypipe.pl>) (16), using genome annotation from the *Pseudomonas* genome database (51). The following processing steps were performed: (i) flagging of markers and control spots; (ii) subgrid-wise background correction, using the median of the lower 10% foreground intensity as the foreground intensity as an estimate for the background noise; (iii) data shifting, to rescue most of the negative spots; (iv) printTip LOESS normalization; (v) merging of replicate spots; (vi) two-sided one-sample Student's *t* test for the log<sub>2</sub> ratios within each experiment; and (vii) averaging of biological replicates to obtain overall fold changes for each treatment group. Only genes that exhibited a change compared to the control of twofold or more and a *P* value of ≤0.05 were considered in this study.

**Identification of PhoP and PmrA binding sites.** Sequence logos for the proposed PhoP binding sites upstream of the *oprH-phoPQ* and *pmrH-ugd* operons, as well as the proposed PmrA binding sites upstream of the PA4773-*pmrB* and *pmrH-ugd* operons, were first constructed using Weblogo ([weblogo.berkeley.edu/logo.cgi](http://weblogo.berkeley.edu/logo.cgi)) (5) (Fig. 1). Frequency matrices were then constructed using the Emboss program "Prophecy" ([emboss.sourceforge.net/apps/prophecy.html](http://emboss.sourceforge.net/apps/prophecy.html)). These matrices were used to parse the *P. aeruginosa* genome for additional matching binding sites, using a conservative cutoff score of 0.82 to 0.85. Any sites that were in a promoter between convergent genes were excluded from further analysis based on the likelihood that they did not represent real promoters. Regulation of the putative target genes was then examined by semiquantitative PCR (qPCR) for strains H103 (wild type), H851 (*DphoP*), and H988 (*ΔpmrA*) in response to a limiting concentration of Mg<sup>2+</sup>. Genes identified as genes that were regulated by PhoP and/or PmrA were then used to refine the binding motif and carry out a second iteration of frequency matrix construction and genomic searches for matching motifs.

**His<sub>6</sub>-PhoP and His<sub>6</sub>-PmrA purification.** The constructs used to overexpress and purify His<sub>6</sub>-PmrA and His<sub>6</sub>-PhoP were created by PCR amplifying the *phoP* or *pmrA* genes and cloning them separately into pET28a (Invitrogen, Carlsbad, CA) as NdeI-BamHI fragments. The plasmids containing the His<sub>6</sub>-*phoP* or His<sub>6</sub>-*pmrA* genes were transformed into *Escherichia coli* BL21. Cells were grown to an OD<sub>600</sub> of 0.5 before they were induced with 1 mM isopropyl-β-D-thiogalactopyranoside for 3 h. Cells were harvested and resuspended in sonication buffer containing 500 mM NaCl, 5 mM MgCl<sub>2</sub>, 50 mM sodium phosphate buffer (pH 7.8), and 10 mM imidazole. Cells were lysed by sonication on ice (three 1-min treatments). Cell debris and unbroken cells were removed by centrifugation at 7,500 × *g*. The supernatant was filtered through a 0.8-μm filter (Nalgene, Rochester, NY). The filtered supernatant was mixed with Ni<sup>2+</sup>-nitrilotriacetic acid resin (QIAGEN, Mississauga, ON, Canada) and gently shaken at 23°C for 1 h. The resin was washed sequentially with 5-ml portions of sonication buffer containing 30, 50, 100, and 200 mM imidazole. His<sub>6</sub>-*phoP* was eluted with sonication buffer containing 300 mM imidazole and 15% glycerol. Protein was collected on ice, aliquoted, and frozen in a dry ice-ethanol bath. Frozen aliquots were stored at -80°C.

**qPCR assays.** Using RNeasy mini columns (QIAGEN, Mississauga, ON, Canada), total RNA was isolated from mid-log-phase *P. aeruginosa* cultures grown in BM2-glucose minimal medium with 20 μM Mg<sup>2+</sup> or 2 mM Mg<sup>2+</sup>. RNA samples were treated with DNase I (Invitrogen, Carlsbad, CA) to remove contaminating genomic DNA. Four micrograms of total RNA was added to a reaction mixture containing each deoxynucleoside triphosphate at a concentration of 0.5 μM, 500 U/ml SuperaseIN (Ambion, Austin, TX), and 10 μM dithiothreitol in 1× reaction buffer and reverse transcribed for 1 h at 37°C and for 2 h at 42°C with 10,000 U/ml Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). The RNA was subsequently destroyed by addition of 170 mM NaOH and incubation at 65°C for 10 min. The reaction mixture was then neutralized by addition of HCl, and the cDNA was used as a template for PCR. The number of cycles used to amplify each gene of interest was chosen to ensure that the PCR was not

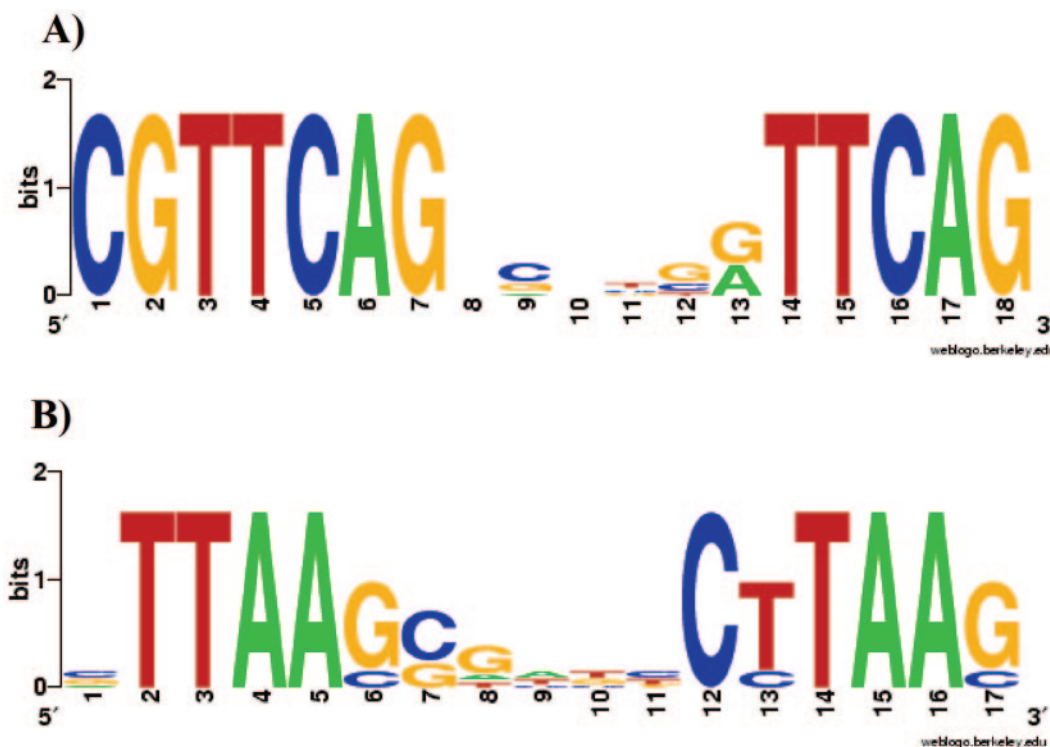


FIG. 1. Weblogos generated from the conserved sequences identified in the promoters of the PhoP- and PmrA-regulated genes. (A) PhoP weblogo. (B) PmrA weblogo.

saturated. All reactions were normalized to the *rpsL* gene encoding the 30S ribosomal protein S12.

**DNA binding assays.** DNA binding assays were performed by using previously described methods (15). PCR-amplified promoter fragments of the entire intergenic region of the genes of interest were purified by excision from agarose gels using the Qiaquick column purification system (QIAGEN, Mississauga, ON, Canada). These fragments were labeled with digoxigenin (DIG) using a DIG gel shift kit (second generation) obtained from Roche Applied Science. The purified protein (His<sub>6</sub>-PhoP or His<sub>6</sub>-PmrA) was mixed with 40 µg of DIG-labeled probe in buffer consisting of 20 mM HEPES, 1 mM EDTA, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1 mM dithiothreitol, 0.2% Tween 20, 30 mM KCl, and 0.5 mM MgCl<sub>2</sub>. For samples containing unlabeled probe, 100 ng of the probe was allowed to bind for 15 min at 20°C before addition of DIG-labeled probe and incubation for another 20 min. All other samples were incubated for 20 min at 20°C before electrophoresis. Following electrophoresis, the samples were blotted onto nylon membranes and exposed to film overnight at 23°C.

**Growth curves.** Overnight cultures of *P. aeruginosa* PAO1 strains H103 (wild type) and H1034 (*feoA::luxCDABE*) were grown in HEPES-buffered minimal medium containing 20 mM glucose, 2 mM MgSO<sub>4</sub>, and 10 µM FeSO<sub>4</sub>. These cultures were washed in sterile saline (0.85% NaCl) and diluted to obtain an OD<sub>600</sub> of 0.2 in saline. Five-microliter portions of these cultures were added to 200 µl of minimal medium containing a high concentration of MgSO<sub>4</sub> (2 mM) or a low concentration of MgSO<sub>4</sub> (20 µM) and 10 µM FeSO<sub>4</sub> or FeCl<sub>3</sub>. Medium containing FeSO<sub>4</sub> was also supplemented with 1 mM sodium ascorbate to maintain the iron in the ferrous form and with 300 µM desferrioxamine mesylate to chelate any contaminating ferric ions. Similarly, medium containing ferric iron was supplemented with 300 µM 2,2'-dipyridyl to chelate ferrous ions. The growth of the cultures at 37°C was monitored with a TECAN Spectrofluor Plus by determining the A<sub>620</sub> every 20 min for 36 h. Growth experiments were carried out twice, and eight replicates were used in each experiment.

## RESULTS

**Microarray analysis of regulation by Mg<sup>2+</sup> limitation, PhoP, and PmrA.** To determine the subsets of Mg<sup>2+</sup>-regulated genes that were regulated by PhoP and/or PmrA, microarray

analyses were performed. For these studies we utilized quite stringent criteria, and the results presented below reflect only those genes that exhibited significantly altered expression (*P* values of <0.05 as determined by Student's *t* test) in five separate experiments. Growth of *P. aeruginosa* in BM2-glucose medium supplemented with either 20 µM Mg<sup>2+</sup> (low concentration) or 2 mM Mg<sup>2+</sup> (high concentration) led to a twofold or greater change in the expression of 158 genes (Table 2), which represented approximately 3% of the coding capacity of the genome.

As expected, Mg<sup>2+</sup> limitation induced the operons that encode PhoPQ and PmrAB, the two previously characterized (20, 26, 29) regulatory systems involved in resistance to cationic antimicrobial peptides and polymyxin B. The previously described *pmrHFJKLM-ugd* operon, which is responsible for the addition of aminoarabinose to lipid A (29), was similarly up-regulated (4- to 13-fold) with Mg<sup>2+</sup> limitation, consistent with our previous observations (26).

Several genes involved in divalent cation uptake were up-regulated under Mg<sup>2+</sup> limitation conditions; these genes included PA0913 (*mgtE*) and the PA4826-PA4822 operon containing *mgtA*. The homologs of both *mgtE* and *mgtA* are involved in Mg<sup>2+</sup> uptake in enteric organisms (24). In addition, the *feoAB* (PA4359-PA4357) operon, which has been proposed to be involved in ferrous iron uptake in other bacteria, was upregulated under Mg<sup>2+</sup> limitation conditions (24), suggesting that divalent cation signaling has a potential role in iron uptake.

Interestingly, Mg<sup>2+</sup>-deficient growth conditions led to 2.5- to 4-fold upregulation of the *algU-mucAB* genes that encode the

TABLE 2. Selected *P. aeruginosa* genes that are regulated by growth in the presence of a limiting concentration of Mg<sup>2+</sup> in wild-type (H103), *phoP::xylE* (H851), and *pmrA::xylE* (H988) strains sorted by category<sup>a</sup>

PA no.	Gene designation	Comparison of growth					
		H103 with low Mg <sup>2+</sup> concn vs H103 with high Mg <sup>2+</sup> concn		H851 with low Mg <sup>2+</sup> concn vs H103 with high Mg <sup>2+</sup> concn		H988 with low Mg <sup>2+</sup> concn vs H103 with high Mg <sup>2+</sup> concn	
		Fold change	<i>P</i> value	Fold change	<i>P</i> value	Fold change	<i>P</i> value
<b>Positively Mg<sup>2+</sup> regulated</b>							
PA0762 <sup>b</sup>	<i>algU</i>	4.1	0.04	1.9	<0.01	2.6	<0.01
PA0763 <sup>b</sup>	<i>mucA</i>	3.4	<0.01	2.3	0.08	1.8	0.05
PA0764 <sup>b</sup>	<i>mucB</i>	2.5	<0.01	1.9	0.01	4.2	0.02
PA1297		3.3	<0.01	3.1	<0.01	3.0	<0.01
PA3552	<i>pmrH</i>	7.2	<0.01	5.6	<0.01	6.9	<0.01
PA3553	<i>pmrF</i>	12.5	<0.01	5.3	0.05	7.6	0.05
PA3554	<i>pmrI</i>	11.9	<0.01	10.4	<0.01	11.6	<0.01
PA3555	<i>pmrJ</i>	3.8	<0.01	3.0	<0.01	3.7	<0.01
PA3556	<i>pmrK, arnT</i>	10.6	<0.01	9.6	<0.01	7.8	<0.01
PA3557	<i>pmrL</i>	3.7	<0.01	1.6	0.63	-1.3	0.37
PA3558	<i>pmrM</i>	3.6	0.01	2.0	<0.01	1.8	0.01
PA3559	<i>ugd</i>	8.0	<0.01	5.3	<0.01	3.9	<0.01
PA3920		5.4	<0.01	8.6	0.01	6.2	<0.01
<b>Negatively Mg<sup>2+</sup> regulated</b>							
PA2260	<i>kguE</i>	-3.3	0.03	-1.9	<0.01	-2.9	<0.01
PA2261	<i>kguK</i>	-5.4	<0.01	-6.7	<0.01	-7.8	<0.01
PA2262	<i>kguT</i>	-3.4	0.01	-2.6	<0.01	-3.3	<0.01
PA2265	<i>gad</i>	-2.8	<0.01	-2.2	0.04	-2.1	0.01
PA2266		-2.7	<0.01	-2.3	<0.01	-2.6	<0.01
PA2321		-5.2	0.01	-3.2	<0.01	-4.2	<0.01
PA2322	<i>gntT</i>	-16.9	<0.01	-16.4	<0.01	-9.5	<0.01
PA2323		-11.1	<0.01	-9.2	<0.01	-10.7	<0.01
PA4236	<i>kata</i>	-2.5	0.05	-2.7	<0.01	-3.1	<0.01
PA4463		-6.6	<0.01	-4.7	0.01	-4.6	<0.01
PA4761	<i>dnaK</i>	-2.9	0.02	-2.8	0.03	-1.8	<0.01
PA4835		-2.2	0.01	-2.4	<0.01	-2.4	<0.01
PA4836		-2.1	0.02	-4.6	<0.01	-3.0	<0.01
<b>Positively Mg<sup>2+</sup> and positively PhoP regulated</b>							
PA0265	<i>gabD</i>	5.2	<0.01	1.4	0.06	4.0	0.01
PA0266	<i>gabT</i>	5.1	<0.01	1.2	0.09	4.0	0.05
PA0921		2.2	<0.01	-1.1	0.57	2.6	<0.01
PA1178	<i>oprH</i>	7.6	<0.01	-1.8	0.01	18.4	0.03
PA1179 <sup>b</sup>	<i>phoP</i>	19.8	<0.01	-1.2	0.29	39.8	<0.01
PA1180 <sup>b</sup>	<i>phoQ</i>	5.9	<0.01	-1.2	0.09	8.0	<0.01
PA3522	<i>mexQ</i>	16.4	<0.01	3.1	<0.01	7.9	<0.01
PA3649		2.5	<0.01	-1.8	0.80	2.3	<0.01
PA3885 <sup>b</sup>		2.7	0.05	-1.5	0.05	1.4	<0.01
PA4010		7.0	<0.01	-1.1	0.08	4.7	<0.01
PA4011		2.0	0.01	-1.1	0.47	2.3	<0.01
PA4453		2.8	<0.01	-1.3	0.04	4.9	<0.01
PA4454		2.2	<0.01	-1.3	0.07	3.1	<0.01
PA4455		2.6	<0.01	-1.2	0.08	1.8	<0.01
<b>Negatively Mg<sup>2+</sup> and negatively PhoP regulated</b>							
PA0836 <sup>b</sup>	<i>ack</i>	-4.0	<0.01	-1.5	0.04	-5.7	<0.01
PA1196 <sup>b</sup>		-3.1	<0.01	-1.3	0.07	-4.2	<0.01
PA3309		-3.2	<0.01	1.6	0.14	-8.6	<0.01
PA4918		-2.5	0.02	-1.4	0.85	-2.8	<0.01
<b>Not Mg<sup>2+</sup> regulated and positively PhoP regulated</b>							
PA4366	<i>sodB</i>	-1.3	0.26	-5.2	<0.01	-1.1	0.49
<b>Positively Mg<sup>2+</sup> regulated and positively PmrA regulated</b>							
PA0201		2.6	0.03	2.8	<0.01	-2.4	0.08
PA0202		2.2	<0.01	1.1	0.12	-1.2	0.26
PA0282	<i>cysT</i>	3.6	0.01	2.7	<0.01	-1.2	0.02
PA0546	<i>metK</i>	2.7	<0.01	1.8	0.06	-1.0	0.60
PA0913	<i>mgfE</i>	2.8	0.01	2.7	<0.01	-1.2	0.61
PA1559		4.0	0.01	13.6	<0.01	2.4	<0.01

Continued on following page

TABLE 2—Continued

PA no.	Gene designation	Comparison of growth					
		H103 with low Mg <sup>2+</sup> concn vs H103 with high Mg <sup>2+</sup> concn		H851 with low Mg <sup>2+</sup> concn vs H103 with high Mg <sup>2+</sup> concn		H988 with low Mg <sup>2+</sup> concn vs H103 with high Mg <sup>2+</sup> concn	
		Fold change	P value	Fold change	P value	Fold change	P value
PA1560		3.2	<0.01	8.0	<0.01	2.9	<0.01
PA2359 <sup>b</sup>		3.4	0.01	1.7	<0.01	1.0	0.51
PA3446		2.3	0.02	2.8	0.01	-1.6	0.06
PA4357		4.6	0.03	1.4	0.03	-1.3	0.06
PA4358	<i>feoB</i>	3.3	<0.01	5.4	<0.01	-1.7	0.03
PA4359	<i>feoA</i>	4.0	<0.01	2.2	0.10	-2.3	0.01
PA4773		4.1	<0.01	3.2	0.04	1.0	0.45
PA4774		6.2	0.02	19.0	<0.01	1.0	0.70
PA4775		1.0	0.22	1.1	0.08	1.0	0.25
PA4776 <sup>b</sup>	<i>pmrA</i>	3.3	<0.01	4.5	<0.01	-1.3	0.05
PA4777 <sup>b</sup>	<i>pmrB</i>	6.7	<0.02	2.8	<0.01	-1.1	0.49
PA4778 <sup>b</sup>		4.4	<0.01	2.7	<0.01	1.1	0.02
PA4781 <sup>b</sup>		2.2	<0.01	2.0	<0.01	1.1	0.67
PA4782		12.7	<0.01	6.2	<0.01	-1.1	0.02
PA4822		7.7	<0.01	6.9	<0.01	-1.9	<0.01
PA4823		5.8	<0.01	9.0	<0.01	-1.0	0.69
PA4824		8.1	<0.01	13.3	<0.01	-1.1	0.02
PA4825	<i>mgtA</i>	10.0	<0.01	30.5	<0.01	-2.4	<0.01
PA4826		5.2	<0.01	3.6	0.02	-1.4	0.03
Negatively Mg <sup>2+</sup> regulated and positively PmrA regulated							
PA0527 <sup>b</sup>	<i>dnr</i>	-3.1	0.01	-1.7	0.01	-9.8	<0.01
Positively Mg <sup>2+</sup> regulated and negatively PmrA regulated							
PA2064	<i>pcoB</i>	3.2	0.01	2.0	0.04	4.4	<0.01
PA2065	<i>pcoA</i>	3.2	0.02	2.0	0.01	18.6	<0.01
PA3515		6.1	<0.01	7.3	0.02	150.0	<0.01
PA3516		3.6	0.01	1.3	0.16	5.9	0.02
PA3517		13.7	<0.01	11.2	<0.01	22.1	<0.01
PA3518		8.0	<0.01	7.2	0.01	55.5	<0.01
Not Mg <sup>2+</sup> regulated and negatively PmrA regulated							
PA2274		1.2	0.18	1.5	0.21	5.4	<0.01
PA4205	<i>mexG</i>	1.4	0.05	1.2	0.16	6.5	<0.01
PA4206	<i>mexH</i>	1.3	0.06	1.3	0.05	5.0	<0.01
PA4207	<i>mexI</i>	1.2	0.17	1.2	0.19	2.3	<0.01

<sup>a</sup> Only selected Mg<sup>2+</sup> genes that were not affected by *phoP::xylE* or *pmrA::xylE* mutations are included. All results were standardized to expression of the wild-type strain in the fully repressed state (i.e., in the presence of 2 mM Mg<sup>2+</sup>).  
<sup>b</sup> Known or putative regulatory gene.

alternative sigma factor AlgU (AlgT) and its cognate anti-σ factor MucA (39), which combine to regulate alginate production, a common adaptation found in CF isolates of *P. aeruginosa* (25). This seems especially relevant since *P. aeruginosa* isolates from the lungs of CF patients, who suffer from chronic infection by mucoid *Pseudomonas*, have lipid A modifications that are equivalent to those of cells grown under Mg<sup>2+</sup> limitation conditions (7).

Two groups of genes with similarity to genes encoding gluconate and 2-ketogluconate uptake systems were strongly downregulated when organisms were grown under Mg<sup>2+</sup>-limiting conditions. PA2260 to PA2263 (*kguEKT D*) are required for utilization of 2-ketogluconate in *P. aeruginosa* (44). Additionally, PA2321 (*gntK*, a homologue of the gene encoding gluconokinase), PA2322 (*gntT*, a homologue of the gene encoding high-affinity gluconate permease), and PA2323 (encoding a predicted glyceraldehyde phosphate dehydrogenase) were also downregulated 5- to 16-fold. While this system has

not been well characterized in *P. aeruginosa*, mutants of the high-affinity gluconate uptake system in *E. coli* exhibit dramatically longer doubling times when they are grown aerobically on gluconate (8).

As expected based on previous studies, a number of known PhoP-PhoQ- and PmrA-PmrB-regulated genes were observed to be upregulated in response to a limiting concentration of Mg<sup>2+</sup>, including the *oprH-phoPQ*, *pmrHF IJKLM-ugd*, and PA4773-PA4775-*pmrAB*-PA4778 operons. To determine which Mg<sup>2+</sup>-regulated genes were independently regulated by each system, microarray analyses were performed with both *phoP* and *pmrA* mutants under Mg<sup>2+</sup>-limiting conditions. These analyses indicated that each system was responsible for the regulation of a subset of the total genes induced under Mg<sup>2+</sup>-limiting conditions in *P. aeruginosa*, indicating that other, as-yet-unidentified regulatory systems may also be involved in the response of *P. aeruginosa* to divalent cation limitation. Consistent with this concept, in addition to the previously described two-component regulatory

TABLE 3. PhoP-like promoters identified by consensus sequence searching, patterns of regulation detected by qPCR, and results of gel shift assays

Sequence or operon	Sequence <sup>a</sup>	Genome coordinates	Mg <sup>2+</sup> regulated <sup>b</sup>	PhoP regulated <sup>b</sup>	Gel shift
Consensus sequence	CGTTCAGNNNNR <sup>T</sup> TCAG				
<i>oprH-phoP-phoQ</i>	CGTTCAGCCCGGGTTCAG	1276906-1276923	Yes	Yes	Yes <sup>d</sup>
	CGTTCAGGGCGGGTTCAG	1276928-1276945			
<i>pmrH-ugd</i>	CGTTCAGTCTTCATTCAG	3979746-3979763	Yes	Yes	Yes
	CGTTCAGGAGCATTCAG	3979789-3979806			
PA1343	CGTTCAGAAATTGTTTCAG	1457051-1457068	Yes	Yes	Yes
	CGTTCAGGCCGATTCAG	1457073-1457109			
PA0921	CGTTCAGCGATGGTTCAG	1006780-1006797	Yes	Yes	Yes
PA4457-PA4461	CGTTCAGCTFGGATTCAG	4989024-4989041	No	NT <sup>c</sup>	NT
PA1851	CGTCCAGGCTGTTCAG	2010955-2010972	No	NT	NT
PA3925	CGTTCAGACCCATCCAG	4400087-4400104	No	NT	NT
PA0918	CA <sup>T</sup> TCAGGCTGGC <sup>T</sup> TCAG	1002608-1002591	No	NT	NT
PA2775-PA2774	C <sup>T</sup> TCAGGATGATTCAG	3133288-3133271	No	NT	NT
<i>spuABCD</i>	CGTTGAGGCCGT <sup>T</sup> TCAG	334565-334582	No	NT	NT

<sup>a</sup> Nucleotides that differ from the nucleotides in the consensus sequence are indicated by boldface type. Abbreviations: N = A, C, G, or T; R = A or G.

<sup>b</sup> Determined by reverse transcription-qPCR.

<sup>c</sup> NT, not tested (no Mg<sup>2+</sup> regulation could be demonstrated, and no regulation was observed in array experiments).

<sup>d</sup> Promoter regions were tested for binding to PhoP and PmrA. Only the *pmrH-ugd* promoter fragment bound to both. The other promoters bound only to PhoP and not to PmrA.

systems PhoP-PhoQ and PmrA-PmrB, several other regulatory proteins were observed to have modulated expression under Mg<sup>2+</sup>-limiting conditions (Table 2). Significantly, the genes encoding regulatory proteins that exhibited Mg<sup>2+</sup>-regulated expression included the alginate regulator genes *algU* and *mucA* described above, *dnr*, which encodes a regulator that responds to nitrogen oxides (35), the RocR-type (4) regulators PA1196 and PA2359, and a MerR-type regulator, PA4778. Another regulated operon contains the PA4782 and PA4781 genes. PA4782 encodes a small protein with an uncharacterized function and is just upstream of PA4781, an uncharacterized response regulator gene. The PA4781-encoded protein is a hybrid protein that contains a typical two-component response regulator receiver domain and a conserved HD-GYP output domain that in some proteins has been proposed to have phosphohydrolase activity and may be involved in cyclic di-GMP signaling (2, 10, 40). These observations indicate that there may be a complex regulatory hierarchy that influences adaptation to growth with a limiting concentration of Mg<sup>2+</sup>.

In order to determine which Mg<sup>2+</sup>-regulated genes are controlled via the PhoP protein, transcriptional profiling of a *phoP::xylE* mutant of *P. aeruginosa* under Mg<sup>2+</sup>-limiting conditions was carried out, and the results were compared to the results for an isogenic wild-type strain grown under Mg<sup>2+</sup>-replete conditions. This analysis revealed that the PhoP protein regulates only a modest number of genes in *P. aeruginosa*. Furthermore, the majority of genes (14/19) that were PhoP regulated appeared to be subject to positive regulation in an Mg<sup>2+</sup>-dependent manner, confirming that PhoP acts primarily as a transcriptional activator. The genes regulated included the previously identified *oprH-phoPQ* operon; three genes of a four-gene operon, the PA4456-PA4453 operon; the *gabD* and *gabT* genes, which are involved in aminobutyrate/putrescine degradation; and PA3885, which encodes a hypothetical serine/threonine phosphatase. Also observed were several hypothetical genes, including PA0921 and the two-gene PA4010-PA4011 operon.

In addition to these positively Mg<sup>2+</sup>- and PhoP-regulated genes, we also found four genes that appeared to be negatively regulated by PhoP. These genes included two regulatory genes, one encoding acetate kinase and one (PA1196) encoding a RocR-type regulator, as well as two hypothetical genes, PA3309 and PA4918. Finally, the expression of the *sodB* gene encoding the Fe<sup>2+</sup>-dependent superoxide dismutase was reduced fivefold when the *phoP* gene was deleted. This was somewhat unusual since this gene was the only PhoP-regulated gene that was not also Mg<sup>2+</sup> regulated.

Transcriptional analysis of a *pmrA::xylE* mutant identified 36 genes with significantly altered expression. As observed for the *phoP::xylE* mutant, the majority (25/36) of these genes were positively regulated by both Mg<sup>2+</sup> limitation and PmrA. These genes included a large number of genes involved in metal transport, such as the *feoAB*-PA4357 operon involved in ferrous iron uptake and the *mgtE* and PA4826-*mgtA*-PA4822 operons involved in Mg<sup>2+</sup> transport. In addition to these transport genes, several regulatory proteins were observed, including the proteins encoded by the previously identified PA4773-PA4775-*pmrAB* operon (26), a RocR-type regulatory protein encoded by PA2659, and the protein encoded by PA4781, a hypothetical two-component response regulator with a CheY-like receiver domain and an HD-GYP motif-containing output domain. PA4778, encoding a MerR-like regulator, was also up-regulated by, and may even be cotranscribed with, the *pmrAB*-containing operon. In addition, we observed that the *dnr* gene, which is involved in sensing nitrogen oxides, was also dysregulated in the *pmrA::xylE* mutant.

One interesting observation was the strong derepression of the PA3515-PA3518 operon observed in the *pmrA::xylE* mutant strain. The function of this operon is unknown, but the genes are similar to genes involved in aliphatic compound catabolism. These genes were also relatively mildly (4- to 16-fold) upregulated under Mg<sup>2+</sup>-limiting conditions. A similar pattern was observed for the *pcoAB* operon, which is involved in resistance to copper (17). Similar observations were made

TABLE 4. PmrA-like promoters identified by consensus sequence searching

Sequence or operon	Sequence <sup>a</sup>	Genome coordinates	Mg <sup>2+</sup> regulated <sup>b</sup>	PmrA regulated <sup>b</sup>	Gel shift
Consensus sequence	NTTAASNNNNCTTAAS				
<i>pmrH-ugd</i>	GTTAAGCGAGCTTAAAC ATTAATGAACCCCTTAA <b>A</b>	3979769-3979785 3979614-3979630	Yes	Yes	Yes <sup>c</sup>
PA4773- <i>pmrAB</i>	GTTAAGCACCTCTTAA <b>G</b>	5361502-5361518	Yes	Yes	Yes
<i>feoAB</i> -PA4357	CTTAAGCGAGCCCTTAA <b>G</b>	4887593-4887577	Yes	Yes	Yes
PA1559-PA1560	ATTAACGGTTCCTTAA <b>G</b>	1697095-1697111	Yes	Yes	Yes
PA4782-PA4781	CTTAAGCGATCCCTTAA <b>G</b>	5370750-5370734	Yes	Yes	ND <sup>e</sup>
PA0327	TTTAAGCTTGGCTT <b>C</b> AG	367370-367386	No	NT <sup>d</sup>	NT
<i>algD</i> -8-44- <i>KEGXLIGFA</i>	CTTAAGGTTTGCTTAA <b>G</b>	3962241-3962257	No	NT	NT
PA0545	GTTAAGGGACAATTA <b>A</b> G	602272-602288	No	NT	NT
PA0053	TTTAAGCATGT <b>C</b> CAAG	69049-69065	No	NT	NT
<i>aprA</i> (PA1249)	TTTAAGTGCAGCTTAA <b>T</b>	1355477-1355493	No	NT	NT
PA2050-PA2051	TTTAAGTGCAGCTTAA <b>T</b>	1355477-1355493	No	NT	NT
PA4500	TTT <b>C</b> AGCATGACTTAA <b>T</b>	5036896-5036912	No	NT	NT
PA2505-PA2506	ATTAAGTGC <b>G</b> AGTTAA <b>G</b>	2824124-2824108	No	NT	NT
PA3868	ATTAAGGCTGCT <b>C</b> AAC	4331298-4331314	No	NT	NT
PA4498-PA4499	TTTAAGCTCGACTTAA <b>A</b>	5036126-5036110	No	NT	NT
PA5106	TTTA <b>C</b> GCACCGCTTAA <b>T</b>	5749388-5749404	No	NT	NT

<sup>a</sup> Nucleotides that differ from the nucleotides in the consensus sequence are indicated by boldface type. Abbreviations: N = A, C, G, or T; S = C or G.  
<sup>b</sup> Determined by reverse transcription-qPCR.  
<sup>c</sup> Promoter regions were tested for binding to PhoP and PmrA. Only the *pmrH-ugd* promoter fragment bound to both. The other promoters bound only to PmrA and not to PhoP.  
<sup>d</sup> NT, not tested (no Mg<sup>2+</sup> regulation could be demonstrated, and no regulation was observed in array experiments).  
<sup>e</sup> ND, not determined.

for the PA2274 and *mexGHI* genes, and loss of *pmrA* led to increased expression of these genes under Mg<sup>2+</sup>-limiting conditions. These results suggest that PmrA has a role in the control of these genes that is more complex than simply causing increased transcription.

**Identification of conserved PhoP and PmrA binding motifs in the promoters of Mg<sup>2+</sup>-regulated genes.** It seems possible that the analysis of microarray data could have revealed indirectly regulated genes or even missed important directly regulated genes since stringent criteria were used for identification of alterations in gene expression. Therefore, we used an alternative bioinformatic approach to discover genes in the PhoPQ and PmrAB regulons. Putative DNA binding sites for the regulatory proteins PhoP and PmrA were identified in previous studies (21, 26). These sequences were used to generate frequency matrices with the Emboss program Prophecy. These matrices were then used to search the *P. aeruginosa* genome sequence for sequences with high levels of similarity to the PmrA or PhoP matrix. This motif searching was carried out independent of the microarray analysis in order to validate both approaches for identifying Mg<sup>2+</sup>-regulated genes. As new sequences were found and confirmed by qPCR, they were incorporated into the matrix to obtain a larger list of putatively regulated genes. A number of PmrA-like and PhoP-like sequences were identified, suggesting that these genes may be regulated by either of these systems. The promoters identified by this analysis are shown in Table 3 (PhoP) and Table 4 (PmrA). Some operons had single sites, while others had two sites; the *pmrH-ugd* promoter region was unique because it had two PhoP binding sites and two PmrA binding sites.

The transcript levels of the candidate genes in wild-type, *phoP::xylE*, and *pmrA::xylE* strains grown in minimal media were examined under high-Mg<sup>2+</sup> conditions (2 mM Mg<sup>2+</sup>) and low-Mg<sup>2+</sup> conditions (20 μM Mg<sup>2+</sup>). Analysis of the qPCR

results (Fig. 2) confirmed that there was PmrA-dependent Mg<sup>2+</sup> regulation of the *feoAB* (PA4359-PA4358), PA4782, and PA1559-PA1560 operons, as well as the previously identified *pmrH-ugd* (PA3552-PA3559) and PA4773-*pmrAB* operons.

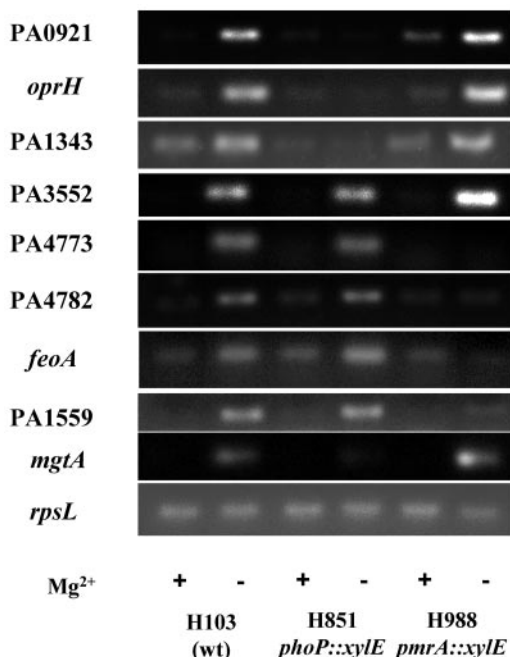


FIG. 2. Selected reverse transcription-qPCR results. Primers were designed to amplify a 100- to 150-bp fragment in the 5' region of each gene indicated. RNA was isolated from strains H103 (wild type [wt]), H851 (*phoP::xylE*), and H988 (*pmrA::xylE*) under Mg<sup>2+</sup>-limiting (-) and Mg<sup>2+</sup>-replete (+) conditions.

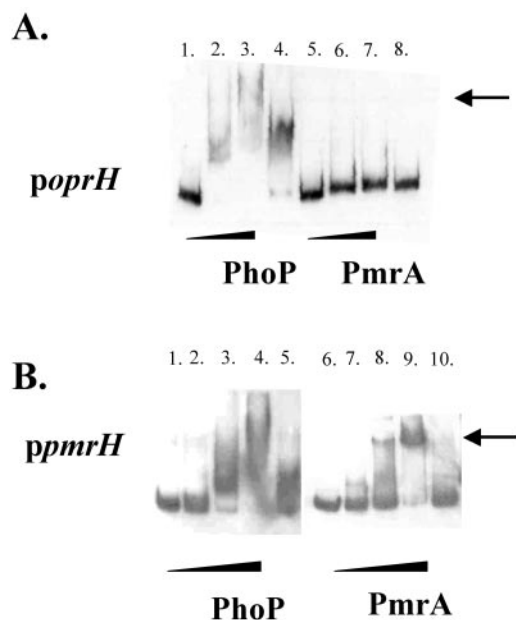


FIG. 3. Demonstration of binding of PhoP and PmrA to selected promoter regions: examples of results of gel shift assays for *oprH* and *pmrH*. (A) The entire intergenic region between *napE* (PA1177) and *oprH* (PA1178) was amplified by PCR and end labeled with DIG. The preparation was incubated with different concentrations of purified His<sub>6</sub>-PhoP or His<sub>6</sub>-PmrA as described in Materials and Methods. All lanes contained 40 pg of labeled probe. Lane 1, no protein; lane 2, 2.5 μg His<sub>6</sub>-PhoP; lane 3, 5 μg His<sub>6</sub>-PhoP; lane 4, 5 μg His<sub>6</sub>-PhoP preincubated with 100 ng unlabeled probe; lane 5, no protein; lane 6, 2.5 μg His<sub>6</sub>-PmrA; lane 7, 5 μg His<sub>6</sub>-PmrA; lane 8, 5 μg His<sub>6</sub>-PmrA preincubated with 100 ng unlabeled probe. (B) The entire intergenic region between *algA* (PA3551) and *pmrH* (PA3552) was amplified by PCR and end-labeled with DIG. The preparation was incubated with different concentrations of purified His<sub>6</sub>-PhoP or His<sub>6</sub>-PmrA as described in Materials and Methods. All lanes contained 40 pg of labeled probe. Lane 1, no protein; lane 2, 1.25 μg His<sub>6</sub>-PhoP; lane 3, 2.5 μg His<sub>6</sub>-PhoP; lane 4, 5 μg His<sub>6</sub>-PhoP; lane 5, 5 μg His<sub>6</sub>-PhoP preincubated with 100 ng unlabeled probe; lane 6, no protein; lane 7, 1.25 μg His<sub>6</sub>-PmrA; lane 8, 2.5 μg His<sub>6</sub>-PmrA; lane 9, 5 μg His<sub>6</sub>-PmrA; lane 10, 5 μg His<sub>6</sub>-PmrA preincubated with 100 ng unlabeled probe.

PhoP-dependent Mg<sup>2+</sup> regulation was observed for PA0921 and PA1343, as well as for the previously identified *oprH-phoPQ* and *pmrH-ugd* operons. We did not observe any Mg<sup>2+</sup> regulation for PA1851, PA3925, PA0918, PA2775, or PA0297 (*spuA*). Similarly, we examined but did not observe Mg<sup>2+</sup> regulation for PA0327, PA0545, PA0053, PA4500, PA2505, PA2506, PA3868, PA4498, PA4499, and PA5106. These results suggest either that these genes are not regulated by Mg<sup>2+</sup> and PhoP/PmrA at all or that their expression is codependent on the presence of a second regulatory signal (e.g., release from repression) that was not present under the conditions used.

**Gel mobility shift assays.** To obtain further evidence that PhoP was directly involved in the PhoP regulation of the PA0921, PA1343, *oprH-phoPQ*, and *pmrH-ugd* operons, gel mobility shift assays were performed using the entire PCR-amplified upstream intergenic region of these operons. When recombinant PhoP was incubated with the promoters upstream of the *oprH*, *pmrH* (Fig. 3), PA0921, and PA1343 genes (Table 3), marked retardation of the mobility of these fragments was observed. The mobility shift was eliminated by preincubating

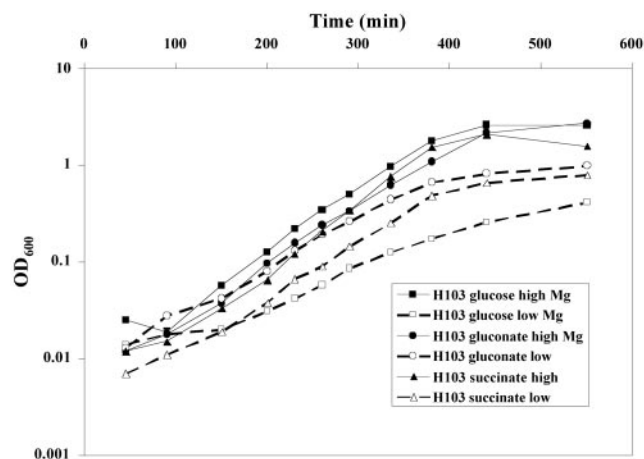


FIG. 4. Effect of Mg<sup>2+</sup> limitation on growth using carbon sources metabolized via the Entner-Doudorhoff pathway. The growth of H103 (wild type) was monitored under Mg<sup>2+</sup>-replete conditions (high Mg or high) and Mg<sup>2+</sup>-limiting conditions (low Mg or low) in BM2 minimal medium with different carbon sources. The carbon sources used included glucose, gluconate, and succinate.

the PhoP protein with excess unlabeled probe. Similarly, mobility shift analysis of the putative PmrA-regulated promoters revealed that the promoters upstream of the *pmrH* (Fig. 3), PA4773, PA1559, and *feoA* (Table 4) genes were all bound by the PmrA protein, and this binding was specifically inhibited by an excess of unlabeled probe. Most promoters regulated by PmrA did not interact with His<sub>6</sub>-PhoP, nor did most PhoP-regulated genes interact with His<sub>6</sub>-PmrA; the only exception was the region upstream of the *pmrH-ugd* operon, which contains both PhoP and PmrA binding motifs (Fig. 3) and is known to be regulated by both regulatory systems (26). These results indicate that the PhoP and PmrA systems of *P. aeruginosa* regulate separate gene sets (with a single overlapping target operon) and that these gene sets independently respond to the same Mg<sup>2+</sup> limitation signal.

**Limiting concentration of Mg<sup>2+</sup> reduces growth on glucose and gluconate.** Due to the strong downregulation of two operons that are probably involved in metabolism of 2-ketogluconate and gluconate, the ability of *P. aeruginosa* to grow on glucose (which in *Pseudomonas* is oxidized to glucose in the periplasm prior to transport), gluconate, and succinate was examined under Mg<sup>2+</sup>-replete and Mg<sup>2+</sup>-deficient conditions. As shown in Fig. 4, in all cases the final density of cells was less when cells were grown in the presence of 20 μM Mg<sup>2+</sup>, but a limiting concentration of Mg<sup>2+</sup> also led to a substantially longer doubling time when cells were grown on either glucose (doubling time with high Mg<sup>2+</sup> concentration, 47 min; doubling time with low Mg<sup>2+</sup> concentration, 70 min) or gluconate (doubling time with high Mg<sup>2+</sup> concentration, 45 min; doubling time with low Mg<sup>2+</sup> concentration, 64 min), but not when they were grown on succinate (doubling time with high Mg<sup>2+</sup> concentration, 44 min; doubling time with low Mg<sup>2+</sup> concentration, 46 min).

**Mutants with mutations in *P. aeruginosa* *feoB* are defective for growth with Fe<sup>2+</sup> as an iron source.** To investigate the potential effect of Mg<sup>2+</sup> regulation of *P. aeruginosa* FeoAB, growth studies were performed in media containing either



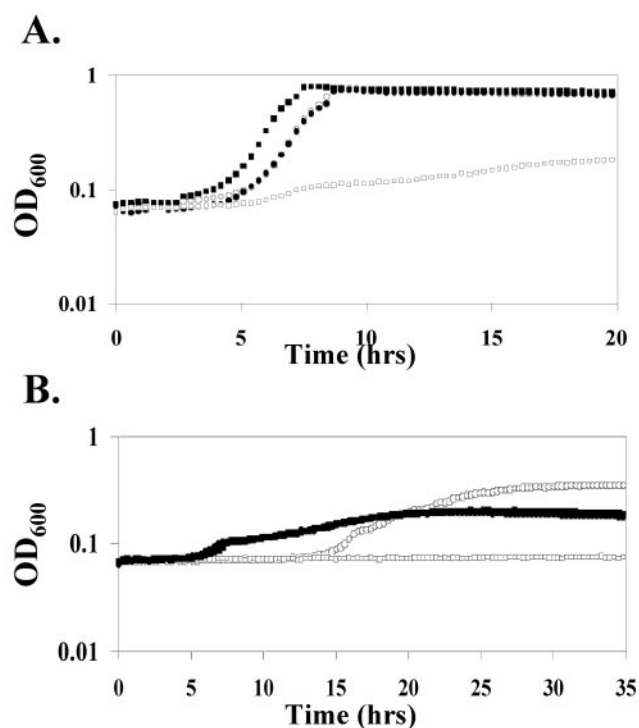


FIG. 5. Involvement of *feoAB* in growth on ferrous iron. The growth of H103 (wild type) and the growth of H1034 (*feoA::luxCDABE*) were measured under Mg<sup>2+</sup>-limiting and Mg<sup>2+</sup>-replete conditions with only Fe<sup>2+</sup> or Fe<sup>3+</sup> available as an iron source. (A) Mg<sup>2+</sup>-replete conditions. (B) Mg<sup>2+</sup>-limiting conditions. Symbols: ●, H103 plus Fe<sup>3+</sup>; ○, H103 plus Fe<sup>2+</sup>; ■, H1034 plus Fe<sup>3+</sup>; □, H1034 plus Fe<sup>2+</sup>.

Fe<sup>2+</sup> or Fe<sup>3+</sup> as the sole source of iron, and a polar knockout mutant with a mutation in the *feoA* gene was compared with the isogenic wild-type strain (Fig. 5). These studies clearly demonstrated that there was no change in the growth of the *feoA* mutant when Fe<sup>3+</sup> was the sole iron source, but there was a severe growth defect when Fe<sup>2+</sup> was provided as the iron source, a result that was consistent with analogous observations made for *Helicobacter pylori* (50). This defect was apparent under both Mg<sup>2+</sup>-limiting and Mg<sup>2+</sup>-replete conditions, but the phenotype was more pronounced when Mg<sup>2+</sup> was limiting, as under these conditions growth of the *feoA* mutant was completely eliminated (Fig. 5).

## DISCUSSION

Here we describe a comprehensive examination of the response of *P. aeruginosa* to growth under Mg<sup>2+</sup>-limiting conditions. Although a large number of genes (158 genes) were found to be regulated by growth in the presence of a limiting concentration of Mg<sup>2+</sup>, much smaller numbers of genes were found to be influenced by mutations in the genes encoding two-component response regulators, *phoP* (19 genes) and *pmrA* (36 genes). This contrasts with the situation in *Salmonella*, in which the PhoP protein regulates, either directly or indirectly, 214 genes located in 189 operons (28), while PmrA regulates at least 47 genes (46). The limited number of proteins that are directly regulated by these regulators in *P. aeruginosa*,

combined with the substantial number of regulatory proteins found to be differentially expressed in the presence of a limiting concentration of Mg<sup>2+</sup>, indicates that there may be other, as-yet-unidentified regulators that contribute to the total Mg<sup>2+</sup> stimulon, and the limited number may also reflect the greater complexity of gene regulation in *P. aeruginosa* (43).

A separate bioinformatic approach was used to search for genes that may be regulated by either the PhoP response regulator or the PmrA response regulator in silico. This study was based on a previous observation that there are conserved binding sites in genes regulated by PhoP or PmrA (21, 26). Using these sequences as a starting point, a bioinformatic search for promoters containing these putative PhoP and/or PmrA binding sites was conducted. Examination of the sequence logos generated (Fig. 2B and 2C) indicated that there was a higher level of plasticity in the PmrA binding site than in the PhoP binding site. It was also clear that the PmrA and PhoP consensus sequences are quite strongly related to one another. Prokaryotic transcription factor binding sites are often direct or inverted repeats, and consistent with this, the PhoP consensus sequence was approximately two GTTCAG half-sites separated by five nucleotides, while the PmrA consensus sequence was approximately two CTTAAG half-sites separated by five nucleotides. The PhoP consensus sequence of *Pseudomonas* is quite different from that of *Salmonella* ([G/T]GTTTA[A/T][G/T]GTTTA[A/T]) (53), while the consensus sequence of PmrA is almost identical ([C/T]YTAA[G/T]-N<sub>5</sub>-[C/T]YTAA[G/T]) (1). This difference in the PhoP binding site may reflect the higher G+C content of *P. aeruginosa* than of *E. coli* and *Salmonella*.

Using qPCR and/or microarray analysis, only a subset of the putative PhoP- and PmrA-regulated genes (Table 2) were also observed to be regulated by growth in the presence of a limiting concentration of Mg<sup>2+</sup>. There was an overlap between the promoters that were predicted to contain PhoP and/or PmrA binding sites and the genes that were regulated by a limiting concentration of Mg<sup>2+</sup> in microarray analyses. However, microarray analyses revealed a much larger repertoire of *P. aeruginosa* open reading frames that were Mg<sup>2+</sup> regulated than was predicted by the bioinformatic analyses of PmrA and PhoP binding sites. Conversely, there was substantial overlap between the PhoP and PmrA promoters identified bioinformatically and confirmed by qPCR (Tables 3 and 4) and the PhoP and PmrA promoters shown by microarray analyses to be regulated in the *phoP* and *pmrA* mutants, respectively (Table 2).

In contrast to the situation in *Salmonella enterica* serovar Typhimurium, the microarray analysis of *P. aeruginosa* revealed no overlap between the PhoP and PmrA regulons (Table 2). This was not expected since previous work involving reporter fusions indicated that both systems are involved in regulation of the *pmrH-ugd* (PA3552-PA3559) operon (26), a result consistent with the observation that the *pmrH-ugd* promoter region contained binding sites for both transcription factors and the observation that these binding sites were likely to be functional based on gel shift assays (Fig. 5). However, qPCR and microarray experiments (Fig. 2) indicated that there was not a major decrease in the level of transcript from the *pmrH-ugd* operon in either the *phoP* or the *pmrA* mutant. This indicates that the two activators, PhoP and PmrA, can support activation of this operon to similar extents, so eliminating ei-

ther one could permit altered expression to be observed only by a higher-resolution method, like analysis of transcriptional fusions. Thus, these data illustrate the utility of combining microarray and bioinformatic analyses for determining the contributions of PhoP and PmrA to the total  $Mg^{2+}$  stimulon of *P. aeruginosa*. It is important, however, that the *pmrH-ugd* operon was the only operon for which both PhoP and PmrA binding sites were predicted.

A limiting concentration of  $Mg^{2+}$  leads to increased transcription of the genes encoding several divalent cation transporter proteins, including MgtA and MgtE, and a putative copper-transporting ATPase; PA3920; *pcoAB*, which is involved in resistance to copper; and the *feoAB* operon encoding a ferrous iron transport system. Interestingly, almost all of the  $Mg^{2+}$ -regulated divalent cation transporters required a functional copy of *pmrA* for normal transcription (Table 2). This contrasts with the situation in *S. enterica* serovar Typhimurium, in which the  $Mg^{2+}$  transporter MgtA (22, 47) is regulated by PhoPQ (53). However, no PhoP or PmrA binding motif was identified in the *mgtA* promoter, indicating that the  $Mg^{2+}$  regulation of *mgtA* may be indirectly regulated via PmrA.

MgtE from *Bacillus firmus* OF4 was first described as a multicopy suppressor that was able to complement the deficient growth on  $Mg^{2+}$  of a *Salmonella* strain with mutations in the *corA*, *mgtA*, and *mgtB* genes (41). MgtE is not similar to other types of metal transporters and has limited distribution among the bacterial species that have been examined (49). In *P. aeruginosa*, microarray analysis demonstrated that *mgtE* was induced by a limiting concentration of  $Mg^{2+}$  and that *mgtE* expression was reduced when *pmrA* was deleted, indicating that induction is dependent on this system.

This study also produced some evidence of regulatory processes that are more complicated than those described above. Notably, four genes, PA2274 and the *mexGHI* operon, were shown to be dysregulated in the *pmrA::xylE* mutant but were not regulated by  $Mg^{2+}$  (Table 2). Similarly, the *sodB* gene, encoding an  $Fe^{2+}$ -dependent superoxide dismutase, is dysregulated in the *phoP::xylE* mutant but is not regulated by  $Mg^{2+}$  (Table 2). This suggests that *phoP* and/or *pmrA* mutants have altered physiology, even under  $Mg^{2+}$ -replete conditions. It is also worth noting that the PA2274 and *mexGHI-opmD* genes were identified as the only dysregulated genes in a study examining the role of SoxR in resistance to paraquat in *P. aeruginosa* (30) and that *sodB* has been identified as a gene that is crucially important to *P. aeruginosa* during normal aerobic metabolism and in resistance to paraquat (14).

Since we demonstrated here that PmrA and a limiting concentration of  $Mg^{2+}$  regulated the *feoAB* operon, we investigated the role of *feoAB* in promoting growth in response to limiting iron and limiting  $Mg^{2+}$  conditions. As shown in Fig. 4, strains having a mutation in *feoB* were defective for growth when  $Fe^{2+}$  iron was the only iron source provided. Furthermore, the defect became more prominent when  $Mg^{2+}$  was also limiting. FeoAB is a well-conserved system that is involved in the transport of ferrous iron (24), and *feoB* mutants of *E. coli* (3, 19) and *H. pylori* (50) have reduced virulence and/or colonization activity.

It is not yet clear whether *P. aeruginosa* utilizes  $Fe^{2+}$  or  $Fe^{3+}$  as its primary iron source during infection. There is clear evidence for the presence of  $Fe^{3+}$ -chelating siderophores in the

sputum of CF patients, indicating that iron is limiting in CF lungs (12). Also, mutants with mutations in  $Fe^{3+}$  siderophore, especially ferri-pyoverdine, uptake have somewhat reduced virulence in a number of model systems (27, 45). However, pyoverdine-deficient mutants have been found in CF lungs (6), indicating that other iron uptake systems may be important. Recent results have demonstrated that the thickened and dehydrated mucus in CF lungs has a reduced oxygen tension that approaches complete hypoxia ( $\sim 5$  mm Hg), even though elsewhere in CF lungs the partial  $O_2$  pressure is apparently normal ( $\sim 200$  mm Hg) (52). Consistent with this, *P. aeruginosa* grows anaerobically in CF lungs. The decreased oxygen tension would tend to stabilize iron in the  $Fe^{2+}$  state. Furthermore, the lungs of CF patients have concentrations of ferritins that are 70-fold higher than the normal concentrations (34, 42). Indeed, CF patients have 20-fold-higher proportions of H-type ferritins, which are important for detoxifying  $Fe^{2+}$  ions by oxidizing them to  $Fe^{3+}$  before they are sequestered within the ferritin shell (13). These results collectively indicate that *P. aeruginosa* growing in the CF lung mucus layer may utilize  $Fe^{2+}$  to satisfy its iron requirements. The observation that the lungs of CF patients contain *P. aeruginosa* isolates with lipid A modifications equivalent to those of cells grown under  $Mg^{2+}$ -limiting conditions (7) may thus reflect greater activation of *feoAB* in the CF lungs.

One of the most prominent transcriptional changes in response to  $Mg^{2+}$  limitation that was independent of PhoP and PmrA was the downregulation of two operons that seem likely to be involved in gluconate and 2-ketogluconate metabolism. Both of these carbon sources, as well as glucose, are metabolized via the Entner-Doudorhoff pathway. To enter this pathway, glucose is first taken up across the outer membrane and then oxidized to gluconate in the periplasm via a membrane-bound dehydrogenase (38). The gluconate is then taken up via a gluconate permease (PA2322) (18) or is oxidized further to 2-ketogluconate by a gluconate dehydrogenase (PA2265) (44). The 2-ketogluconate produced is likely taken up via a permease protein, encoded by PA2262, which is in the same operon as the gene encoding gluconate dehydrogenase. Once inside the cell, glucose, gluconate, and 2-ketogluconate are all routed through the intermediate 6-phosphogluconate before they are degraded to pyruvate and glyceraldehyde-3-phosphate (48). As expected from the regulatory patterns observed, growth on glucose or gluconate as a carbon source was substantially slower under  $Mg^{2+}$ -limiting conditions than under  $Mg^{2+}$ -replete conditions. In contrast, there was virtually no difference in the doubling times on succinate, which is independently transported and enters directly into the tricarboxylic acid cycle.

It is not clear why *P. aeruginosa* exhibits this behavior unless growth of *P. aeruginosa* on substrates other than glucose represents a favorable adaptation to in vivo growth conditions. Consistent with this possible explanation and our observations, it was recently demonstrated that gluconate permease (PA2322) and the probable glyceraldehyde-3-phosphate dehydrogenase (PA2323) were downregulated in *P. aeruginosa* growing in CF sputum (31). This phenotype requires further study to elucidate a more detailed mechanism for the regulatory patterns observed; however, this observation and other observations described above are consistent with a role for the

Mg<sup>2+</sup> limitation regulon in adaptation of *Pseudomonas* to the CF lung.

In summary, this work expanded the number of known targets of the PhoP-PhoQ and PmrA-PmrB two-component regulatory systems. Our results demonstrate that these regulatory systems each regulate, either directly or indirectly, only a modest number of genes. The contribution of each system is also relatively small compared to the contribution of the entire Mg<sup>2+</sup> stimulon of *P. aeruginosa*.

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