Molecular Characterization of the PmrA Regulon*

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The two-component system PmrA/PmrB of Salmonella enterica controls expression of several loci including those mediating modifications in the lipopolysaccharide that result in polymyxin resistance. To gain insight in the regulation of polymyxin resistance, we mapped the transcription start sites of the PmrA-regulated genes pmrC, pmrG, pbgPE, and ugd and identified a conserved sequence in the promoter region of the first three genes. His-tagged PmrA protein could gel shift DNA fragments containing the promoters of the pmrC, pmrG, and pbgPE genes but not the ugd promoter. DNase I footprinting analysis of the pmrC, pmrG, and pbgPE promoters indicate that phosphorylated PmrA binds to a 16-base pair imperfect inverted repeat sequence that is conserved upstream from the transcription start sites of the pmrC, pmrG, and pbgPE genes, respectively. Our data suggest that a PmrA dimer activates transcription of the divergent pmrG and pbgPE promoters by binding to a single site in the pmrG-pbgPE intergenic region and that the ugd gene is regulated by the PmrA/PmrB system only indirectly.

To survive in different environments, bacteria modulate expression of their genes often using two-component signal transduction systems (1, 2). These systems typically consist of a sensor histidine kinase and a response regulator. In general, sensor kinases contain conserved carboxyl-terminal histidine residues that are autophosphorylated in the presence of ATP (3). Most sensors are membrane-bound and transfer the phosphate group to an aspartic acid residue in the amino terminus of the response regulator. The phosphorylated response regulator usually mediates control at the transcriptional level by binding to specific DNA sequences. The pmrA and pmrB genes of Salmonella enterica serovar Typhimurium encode products with sequence similarity to DNA binding response regulators and autophosphorylatable histidine kinases, respectively (4). The pmrA locus is required for resistance to polymyxin B and to other antimicrobial compounds in Salmonella (5–7) and controls the production of proteins that mediate the modification of the lipopolysaccharide core and lipid A with ethanolamine and 4-aminoarabinose (8).

Transcription of PmrA-activated genes is induced in response to mild acidic conditions (9) or during growth in a low extracellular magnesium media in a process that requires the PhoP/PhoQ two-component system (10, 11) (Fig. 1). Four PmrA-regulated loci have been identified to date as follows: ugd, encoding UDP-glucose dehydrogenase; the seven-gene operon pbgPE (designated as the pmrF locus by Gunn et al. (12)); pmrG; and the pmrCAB operon, indicating that the pmrA is autoregulated (10, 11). The ugd and pbgPE loci are required for polymyxin resistance A (12, 13), growth in low magnesium positive media (13), and the incorporation of 4-aminoarabinose into lipid A (12, 14). The PmrG protein is homologous to Ais, an aluminum induced protein of Escherichia coli K-12 (12), but its biochemical function as well as that of the putative membrane protein PmrC remain unknown. The pmrG gene is located upstream and transcribed in the opposite orientation of the pbgPE operon. Downstream of the pbgPE operon is the pmrD gene, which may also be PmrA-regulated because when present in a multicopy number plasmid, it confers polymyxin resistance in a PmrA-dependent manner (15).

In this paper, we characterize the PmrA-regulated promoters in Salmonella by determining the transcription start sites of four PmrA-regulated operons and identifying the DNA sequence to which the PmrA protein binds. Our experiments define two classes of PmrA-regulated genes as follows: those that are directly regulated by the PmrA protein (pmrCAB, pmrG, and pbgPE), and those that are regulated indirectly by the PmrA protein (ugd).

MATERIALS AND METHODS

Strains and Plasmids—E. coli JM109 was used as the host for the preparation of plasmid DNA. E. coli BL21(DE3) served as the host for overexpression of the PmrA protein and the cytoplasmic domain of the PmrB (PmrBc) protein. To overproduce the PmrA protein, the chromosomal pmrA gene was PCR-amplified with primer 351 (5′-AAG GAT CCA GGA GAC TAA GCG-3′) and six histidine tag (H6) containing primer 438 (5′-TTC AAG CTT AGT GGT GTG GGT GC TT TTT CCT CAG TGG CAA CC-5′), which is found 605, 80, and 38 nucleotides upstream from the transcription start sites of the pmrC, pmrG, and pbgPE genes, respectively. Our data suggest that a PmrA dimer activates transcription of the divergent pmrG and pbgPE promoters by binding to a single site in the pmrG-pbgPE intergenic region and that the ugd gene is regulated by the PmrA/PmrB system only indirectly.

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2 The abbreviations used are: PCR, polymerase chain reaction; H6, six-histidine tag; bp, base pair(s); RT, room temperature; nt, nucleotide(s).
7.7, containing either 10 μl or 10 mM MgCl₂ or N-minimal medium, pH 5.8, containing 10 mM MgCl₂. Total RNA was extracted from mid-exponential phase cultures (A₅₅₀, 0.4–0.6) with Trizol (Life Technologies, Inc.) according to the manufacturer's specifications. Primers used for cDNA synthesis were 935 (5'-AGA CGG CAG CAT AAA AGG AA-3'), located 56 bp downstream of the pmrG start codon; 955 (5'-CAT TAA CCT CTC AGG CAG AC-3'), situated 14 bp downstream of the start codon of the first gene in the pbgPE operon; 1069 (5'-ATA ACG GTG AGG GTA AAT GC-3'), located 5 bp downstream of the pmrG start codon; and 1007 (5'-GCA ATG ACA AAA CCA TTA GA-3'), located 36 bp downstream of the ugd initiation codon. The cDNA synthesis was performed with 30 μg of total RNA, 2 pmol of primer labeled with T4 polynucleotide kinase, and [γ-³²P]ATP and 50 units of SuperScriptTM II RnaseH reverse transcriptase (Life Technologies, Inc.). The extension products were analyzed by electrophoresis on a 6% polyacrylamide, 7.5 M urea gel and compared with sequence ladders initiated with primers 935, 955, 1069, or 1007.

Purification of the PmrA and PmrB Proteins—Histidine-tagged PmrA and PmrB proteins were expressed in E. coli BL21(DE3) containing either plasmid pT7-PmrA-H6 (EG10067) or pT7-PmrBc-H6 (EG11751). Cells were grown in 500 ml of LB broth at 30 °C to an A₅₅₀ of 0.6. Then, isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 1 mM, and incubation was continued for 4 h. Cells were harvested and suspended in 10 ml of buffer A (50 mM NaH₂PO₄, pH 8.0; 300 mM NaCl, and 10 mM imidazole) and disrupted by sonication. Cell debris was pelleted by centrifugation at 3,000 g for 15 min.

Iterative gel filtration with a 2.5 × 50 cm column of 50% nickel-nitrilotriacetic acid was added to the supernatant and incubated for 1 h at 4 °C on a rocking platform. The PmrA-H6 and PmrBc-H6 proteins were washed 4 times with 10 ml of buffer A containing 50 mM imidazole. The proteins were eluted with 4 ml of buffer containing 250 mM imidazole.

Gel Mobility Shift Assays—DNA fragments used for gel mobility shift assays were amplified by the PCR using S. enterica serovar Typhi murium chromosomal DNA as a template. Prior to the PCR, primers 935, 955, and 1007, which anneal to the coding strand of pmrC, pbgPE, and ugd, respectively, were labeled with T4 polynucleotide kinase and [γ-³²P]ATP. The pmrC promoter region was amplified using primer 454 (5'-TGG AAT TCG ATC ACC GGC CTC-3') and labeled primer 935. The pbgPE/promrG promoter region was generated by PCR with primer 767 (5'-TGG CCG GAC GGG AGA AAG GC-3') and labeled primer 955. The ugd promoter region was amplified with primer 977 (5'-CGG CAT CCG GCC TTT TTT TCT C-3') and labeled primer 1007. Approximately 25 amol of labeled DNA and 0, 10, 20, 30, 50, 100, or 250 pmol of PmrA-H6 protein in a 100-µl volume were incubated at room temperature for 15 min. The binding buffer used for protein-DNA incubations was 20 mM Tris, pH 7.4, 5 mM MgCl₂, 50 mM KCl, 50 μM Mg²⁺ bovine serum albumin, 2.5 μg/ml salmon sperm DNA, and 10% glycerol. Samples (20 μl) were run on a 2% non-denaturing Tris glycerol polyacrylamide gel at 2 °C. After electrophoresis the gel was dried and autoradiographed.

Phosphorylation of PmrA by PmrBc—The PmrBc-H6 or PmrA-H6 proteins were incubated at room temperature (RT) for 10 or 40 min, respectively, with 50 μCi of [γ-³²P]ATP in phosphorylation buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 2 mM MgCl₂, and 1 mM dithiothreitol). Phosphorylation of PmrA-H6 was accomplished by adding 0.5 pmol of autophosphorylated PmrBc-H6 to 1 pmol of PmrA in phosphorylation buffer. The reaction was stopped after 0.1, 0.5, 1, 2, 5, 10, 20, or 40 min with SDS loading buffer. The samples were run on a 15% SDS-polyacrylamide gel.

DNase I Footprinting—DNase I protection assays were done for both DNA strands using the appropriate labeled primer. PmrA-H6 protein was incubated with phosphorylated or unphosphorylated PmrBc-H6 for 20 min at RT as described above, except that instead of [γ-³²P]ATP, ATP was used. Binding reactions with 0, 4, 8, 16, 32, 64, or 128 pmol of PmrA-H6 protein and 25 amol of labeled DNA were performed as described for the gel mobility shift assay. DNase I (Life Technologies, Inc.) (0.05 units) was added and incubated for 3 min at room temperature. The reaction was stopped by adding 10 μl of 25 mM EDTA. DNA fragments were purified with Wizard DNA cleanup system (Promega, Madison, WI) and resuspended in 30 μl of H₂O. Samples (6 μl) were analyzed by denaturing polyacrylamide (6%) gel electrophoresis by comparison with a DNA sequence ladder generated with the appropriate primer.

Dimerization of PmrA Protein—His-tagged PmrA protein (2 μg) was resuspended in SDS sample buffer with or without 2-mercaptoethanol and electrophoresed on a 15% SDS-polyacrylamide gel.

RESULTS

Mapping the Transcription Start Sites of PmrA-regulated Genes—To characterize the PmrA-regulated promoters, we determined the transcription start sites of the PmrA-activated genes pmrC, pmrG, pbgPE, and ugd. Expression of PmrA-activated genes is promoted during growth in low magnesium media in a process that requires the PhoP/PhoQ two-component system. But transcription of PmrA-activated genes can also be induced by growth in mild acidic conditions in a PhoP/PhoQ-independent manner mechanism (9–11). To distinguish between PhoP/PhoQ-dependent and -independent transcription of PmrA-activated genes, total RNA was isolated from mid-exponential phase cultures of wild-type Salmonella grown in N-minimal media with 10 μM Mg²⁺, pH 7.7, or 10 mM Mg²⁺, pH 5.8, respectively (9). RNA was also isolated from bacteria grown in N-minimal media, 10 mM Mg²⁺, pH 7.7, a condition that does not promote expression of PmrA-activated genes.

A single primer extension product was obtained for the pmrC promoter, corresponding to an A residue located 23 nucleotides upstream of the pmrC start codon, with RNA isolated from bacteria grown under either inducing condition (Fig. 2). Single transcription start sites were also detected for the pmrG and ugd promoters, corresponding to a G residue 104 bp upstream of the pmrG start codon and an A residue 12 bp upstream of the ugd start codon (Fig. 2). In contrast, two major bands located 5 bp apart were observed for the pbgPE promoter (Fig. 2). S1 mapping experiments revealed that the top band, corresponding to an A residue 73 nucleotides upstream of the start codon of the pbgPE1 gene, was the transcription start site (data not shown). The S1 mapping experiments revealed the same transcription start sites (data now shown) identified by primer extension for the pmrC, pmrG, and ugd genes (Fig. 2). On the
other hand, no primer extension products were observed with RNA isolated from bacteria grown at 10 mM Mg^{2+}, pH 7.7 (Fig. 2), consistent with previous results using lac gene fusions (9). That identical transcription start sites were detected when expression of PmrA-activated genes was induced by growth in low magnesium or by mild acidification suggests that the signals act by modulating the activity of the PmrA protein and argue against a model in which different promoters are used in response to different signals.

We analyzed the DNA sequences 5’ to the transcription start sites of the pmrC, pmrG, pbgPE, and ugd genes by comparing them to known promoter consensus sequences. The promoters of these PmrA-activated genes displayed similarity to E. coli σ^{70} promoters: the −10 regions of these promoters contained three out of the six conserved nucleotides, and the −35 regions contained three or four of the six conserved nucleotides (Fig. 6). The pmrG and pbgPE promoters also contain an 18-bp A + T-rich sequence upstream of the −35 region known as UP element (18).

The PmrA Protein Binds to the Promoter Regions of the pmrC, pmrG, and pbgPE Genes—To examine the ability of the PmrA protein to bind the promoter regions of PmrA-activated genes, we first constructed a derivative of the pmrA gene encoding a protein with six histidine residues at its amino terminus, and we showed that a plasmid encoding this protein was capable of efficient autophosphorylation (Fig. 4). Moreover, it could serve as a phosphate donor for the PmrA-H6 protein. Rapid phosphotransfer from the PmrBc-H6 to the PmrA-H6 protein was observed (Fig. 4). The half-life for phosphorylated PmrA-H6 was 10 min when incubated with the PmrB protein (PmrBc-H6) and investigated its ability to phosphotransfer to the PmrA Protein—We purified the cytoplasmic domain of the PmrB protein (PmrBc-H6) and investigated its ability to autophosphorylate in the presence of ATP. The PmrBc-H6 protein was capable of efficient autophosphorylation (Fig. 4).

Determination of the PmrA-binding Site—To define experimentally the DNA sequence that the PmrA protein recognizes, DNase I footprinting analysis was performed on both the coding and non-coding strands of the pmrC and pbgPE/pmrg genes, and the 261-bp ugd PCR fragment contains 202 bp of the region upstream of the ugd gene. The concentration of PmrA-H6 added to each reaction is indicated at the top of each lane.

The amount of PmrA-H6 protein needed for the gel shift of the pmrC and pbgPE/pmrg DNA fragments was similar: gel shifts were seen with 10 pmol of PmrA-H6 protein, and all of the input pmrC and pbgPE/pmrg DNA was in the complexed state in the presence of 250 pmol of protein. Band retardation was observed despite the presence of a large excess (1,000-fold) of competing salmon sperm DNA, suggesting that PmrA-H6 binds directly and specifically to the pbgPE/pmrg and pmrC promoter regions. In contrast, a 261-bp DNA fragment that includes 202 bp upstream of the ugd start codon could not be gel-shifted by 250 pmol of PmrA-H6 protein (Fig. 3), even when this protein was phosphorylated (data not shown).

Autophosphorylation of the PmrB Protein and Phosphotransfer to the PmrA Protein—We purified the cytoplasmic domain of the PmrB protein (PmrBc-H6) and investigated its ability to autophosphorylate in the presence of ATP. The PmrBc-H6 protein was capable of efficient autophosphorylation (Fig. 4).

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Autophosphorylation of the PmrB Protein and Phosphotransfer to the PmrA Protein—We purified the cytoplasmic domain of the PmrB protein (PmrBc-H6) and investigated its ability to autophosphorylate in the presence of ATP. The PmrBc-H6 protein was capable of efficient autophosphorylation (Fig. 4).

Moreover, it could serve as a phosphate donor for the PmrA-H6 protein. Rapid phosphotransfer from the PmrBc-H6 to the PmrA-H6 protein was observed (Fig. 4). The half-life for phosphorylated PmrBc-H6 was 10 min when incubated with the PmrA-H6 protein in a 1:2 PmrBc-H6/PmrA-H6 stoichiometry. As expected, the PmrA-H6 protein alone showed no evidence of phosphorylation when incubated with ATP (Fig. 4, lane 10).
bp (Fig. 5). In addition, areas of hypersensitivity were observed flanking portions of the protected sites. The location of the hypersensitive site in the phgPE coding strand was dependent on the phosphorylated state of the PmrA-H6 protein. At high protein concentrations, phosphorylated and unphosphorylated PmrA-H6 protected the same region in the different promoters. On the other hand, at low protein concentrations phosphorylated PmrA-H6 protected this region better than unphosphorylated PmrA-H6. The protected sequences of the pmrC, pmrG, and phgPE promoters were highly similar to one another (Fig. 6) and allowed us to define the following PmrA binding sequence: 5′-TTAA(G/T)TTCTA(G/T)GTT-3′, which includes an imperfect inverted repeat (Fig. 6).

The PmrA Protein Can Form Dimers—The PmrA-H6 protein was analyzed by SDS-polyacrylamide gel with or without 2-mercaptoethanol in the sample buffer. In the absence of 2-mercaptoethanol the PmrA-H6 protein runs as a 50-kDa species, consistent with the notion that this protein can dimerize (Fig. 7). PmrA harbors a single cysteine at position 27, which may be responsible for disulfide bridge formation between two PmrA molecules. The dimer form of PmrA may bind the inverted repeat in the promoter regions of PmrA-activated genes to promote gene transcription.

DISCUSSION

The two-component system PmrA/PmrB controls transcription of the pmrC, pmrG, phgPE, and ugd genes (9, 12). Some of these genes encode products that mediate the modification of the lipid A by the addition of 4-aminoarabinose, which makes the microorganism 1,000-fold less susceptible to polymyxin B (12, 13). We have defined the promoter region of the PmrA-regulated genes pmrC, pmrG, phgPE, and ugd genes. When compared with known promoter consensus sequences (19), they showed weak identity to the E. coli σ70 promoter consensus sequence, which is typical for activator-dependent promoters (20, 21). PmrA belongs to the OmpR family of response regulators, which are generally transcribed by σ70 RNA polymerase.

![Figure 4](image1.png)

**FIG. 4.** Phosphorylation of the PmrA protein by the cytoplasmic domain of the PmrB protein in vitro. Autophosphorylation of PmrBc-H6 (0.5 pmol) was accomplished by incubation of PmrBc-H6 with [γ-32P]ATP for 10 min at RT. The PmrA protein was incubated for 40 min with [γ-32P]ATP. Time course of phosphotransfer from [32P-PmrBc (0.5 pmol) to PmrA (1 pmol) is indicated at the bottom of the figure. The samples were run on a 15% SDS-polyacrylamide gel, and autophosphorylation and phosphotransfer were visualized by autoradiography.

![Figure 5](image2.png)

**FIG. 5.** DNase I footprinting analysis of the pmrC and phgPE/pmrG promoters. Footprinting analysis of the pmrC and phgPE/pmrG promoter regions was performed on both end-labeled coding and non-coding strands. The amount of phosphorylated [P-PmrA] or unphosphorylated [PmrA] PmrA-H6 protein added to the DNA fragments is indicated at the top of the figures. Solid lines represent the PmrA-binding region. The position of the binding was determined by comparison with sequence ladders, obtained using the same labeled primer as was used for the probe. The hypersensitive DNase I sites are indicated with arrows.
Characterization of the PmrA Regulon

(22). Activator proteins bind at various distances from weak promoters to enhance binding or open complex formation by RNA polymerase (23). In the absence of activator protein, RNA polymerase forms open complexes poorly due to unfavorable interactions with the −35 and −10 elements of the promoter. The pmrG and pbgPE promoters both contain a possible 18-bp UP element located at positions −52 and −66, respectively, relative to the transcription start site, which could be a target for the carboxyl-terminal domain of the RNA polymerase α subunit. Binding of this domain to target promoters can increase promoter activity 2–20-fold (18).

Transcription of PmrA-activated genes is induced in response to mild acidic conditions (9) or by a low extracellular magnesium concentration through interaction with the PhoP/PhoQ two-component system (10, 11). Transcription products for the pmrC, pmrG, pbgPE, and ugd genes were detected only under inducing conditions for the PmrA/PmrB two-component system. Moreover, the same transcription start site was detected whether bacteria were grown in low pH or in low extracellular magnesium. This indicates that the same promoter is used under different activating conditions (as supposed to different promoters being activated in response to different signals).

A single retarded DNA-protein complex was detected for the pmrC and the pbgPE/pmrg promoter fragments indicating that these promoters contain only one PmrA-binding site. This notion is further supported by our DNase I footprinting analysis which showed that the PmrA-binding site is located around nt −40, −38, or −80 of the transcription start sites of pmrC, pbgPE, and pmrg genes, respectively. This location is in agreement with the DNA-binding sites of other activators, which are usually found between nt −30 and −80 with respect to the transcription start site (24). The PmrA protein protected a 16-bp sequence (5′-TTAAKTCTTTAAGGT-3′) from DNase I, partially covering the potential −35 promoter region of the pmrC and pbgPE promoters (Fig. 6). This sequence includes an imperfect 9-bp inverted repeat, similar to the DNA-binding sites of many prokaryotic regulators that consist of 5–10-bp inverted repeat sequences (25). The dyad symmetry in the PmrA-binding sequence suggests that the PmrA protein binds to these sites as a dimer, and consistent with this notion we found that the PmrA protein can dimerize (Fig. 7).

There is single PmrA-binding site in the pbgPE-pmrG intergenic region, and this site is closer to the pbgPE promoter than to the pmrg promoter. A similar phenomenon has been described in the agr locus of Staphylococcus aureus where two divergent promoters, P2 and P3, contain a single binding site for the regulatory protein SarA (26). The SarA location is closer to the P2 promoter, which is the most activated promoter. Further work will be required to examine whether the pbgPE promoter is preferentially activated relative to the pmrg promoter.

Phosphorylation of response regulators is believed to prevent the activity of an inhibitory domain, allowing the amino terminus to dimerize or oligomerize and the carboxyl terminus to bind to the target DNA (27). However, like the PhoP protein of Bacillus subtilis, the PmrA protein dimerizes and binds to DNA regardless of its phosphorylation state (28). Several response regulators also bind to their target promoters efficiently in their unphosphorylated form (29, 30), but others, such as NarL and ComA, bind to their target genes only in phosphorylated form (31, 32). Yet, in all cases, phosphorylation of response regulators affects binding to the target promoters, often by increasing binding affinity such as in the case of the OmpR and PhoB proteins (33, 34). A similar phenomenon is seen for PmrA, where 2–4-fold less protein is needed for full PhoB protein of B. subtilis, the UhpA protein of E. coli, and the BvgA protein of
Bordetella pertussis (1). Phosphorylation of this class of response regulators appears to only change the conformation of these proteins, resulting in transcription activation.

The PmrA protein exhibited specific binding to the pmrC and pbgPE/pmrg promoter fragments. On the other hand, neither phosphorylated nor unphosphorylated PmrA protein could bind to the ugd promoter. This was surprising because genetic analysis of strains harboring ugd::lac fusions demonstrated that ugd transcription requires a functional pmrA gene (9, 12). The ugd gene codes for UDP glucose dehydrogenase which converts UDP-glucose into UDP-glucuronic acid, a precursor in the biosynthesis of the exopolysaccharide colanic acid (35). When grown at low temperature in defined media, the ugd mutant is mucoid presumably because it is unable to synthesize colanic acid. On the other hand, pmrA mutants are mucoid, implying that the ugd gene can be expressed independently of the PmrA protein. This suggests that the PmrA protein regulates transcription of the ugd gene only indirectly and that a different regulator is likely to bind and activate transcription from the ugd promoter. Thus, the ugd gene defines a new class of PhoP/PhoQ-activated genes, a class that can be transcriptionally induced by at least three signals as follows: growth in low magnesium (in a process that requires both the PhoP/PhoQ and PmrA/PmrB two-component systems), growth in mild acid media (in a process that requires PmrA/PmrB but that is independent of PhoP/PhoQ), and a yet undefined condition (in a process that is independent of both PmrA/PmrB and PhoP/PhoQ).

Finally, we analyzed the genome of E. coli K-12 for the presence of PmrA-binding sites and found such sequences in front of the pmrC homologue ydjB, where it covers the inner part of a 14-bp inverted repeat, and in the intergenic region of the homologues of the pbgPE and pmrG genes. The conservation of the binding sites in E. coli and Salmonella is consistent with the high degree of sequence identity that exists between the Salmonella PmrA protein and its homologue in E. coli K-12 BasR (90% at the amino acid level) (36) and suggests that transcription activation of PmrA-regulated genes will be highly similar in these enteric species.

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