A pmrA Constitutive Mutant Sensitizes Escherichia coli to Deoxycholic Acid

Jamie M. Froelich, Khoa Tran, and Daniel Wall*

Anadys Pharmaceuticals, Inc., San Diego, Calif.

Received 22 August 2005/Accepted 10 November 2005

An Escherichia coli mutant was isolated and shown to be polymyxin B resistant. Mapping and sequence analysis revealed a missense mutation at codon 53 within the pmrA (basR) gene that results in a G-to-V substitution. Fusions of promoters from the pmrC, yibD, and pmrH genes with the lacZ reporter showed that they were constitutively expressed in pmrA53 cells. In pmrA+ strains, these promoters were induced by iron and zinc, while a ΔpmrA mutation blocked induction. The PmrA regulon regulates genes whose products remodel the composition and charge of lipid A and hence the barrier properties of the outer membrane. Along these lines, the pmrA53 mutant was also found to be hypersensitive to the anionic bile detergent deoxycholic acid.

The outer membrane of gram-negative bacteria functions as a barrier to exclude toxic chemicals, such as antibiotics, from entering and killing the cell (18). To meet different environmental challenges, bacteria can physiologically adapt or mutate to remodel the chemical composition of lipopolysaccharides (LPS) and hence the permeability properties of their outer membranes (6, 16). In Salmonella enterica, pmrA constitutive mutants become resistant to the cationic antibiotic polymyxin B with the addition of 4-amino-4-deoxy-L-arabinose (L-Ara4N) and, to a lesser extent, phosphoethanolamine (peEtN) moieties to lipid A (9, 25). The addition of L-Ara4N partly neutralizes the negative charges on lipid A, a component of LPS, and hence reduces the electrostatic interaction with polymyxin B. Similarly, the PmrAB two-component regulon can be induced in wild-type cells by iron, which in turn leads to lipid A modification and polymyxin resistance (28). PmrAB-dependent remodeling of LPS also confers resistance towards cationic peptides and metal ions (4, 16). In Pseudomonas aeruginosa, polymyxin-resistant mutants can be cross resistant to aminoglycosides (12).

Inside the human host, LPS (endotoxin) remodeling plays a key role in bacterial fitness and virulence. L-Ara4N LPS modification not only confers resistance to endogenous antibacterial cationic peptides but also helps bacteria evade the innate immune system by making LPS a poor Toll-like receptor 4 agonist (16). To date, Salmonella has been the primary model organism for understanding the role of LPS remodeling in pathogenesis and antibiotic resistance. Here we identify and characterize a pmrA constitutive mutant of Escherichia coli and show that the mutant confers hypersensitivity to the bile detergent deoxycholic acid.

Strains. During the course of our studies with novel anti-bacterial agents, we isolated a spontaneous resistant mutant named DW137 (Table 1) (D. Wall and J. M. Froelich, unpublished data). This mutation was mapped by Hfr crosses (15) to ∼93 min on the E. coli chromosome. Subsequent bacteriophage P1vir transductions with a set of known Tn10 insertions around 93 min mapped the mutation between zje-2241::Tn10 and cadB2231::Tn10 (15, 17). Because the mutation mapped near the pmrAB (basR5) locus, we tested and found the strain was resistant to polymyxin B and colistin (polymyxin E) (Table 2). A set of PCR primers was then designed, and the entire pmrAB region was sequenced. A single missense mutation (GGG to GTG) was found within codon 53 of pmrA. This mutation results in the substitution of valine for a highly conserved glycine within the receiver domain of the PmrA response regulator. Consistent with this result, Trent and coworkers (24) identified a polymyxin B-resistant pmrA mutant of E. coli that contained a substitution in the identical residue (G53E) and had a second substitution (A42T). The pmrA505 mutation in Salmonella enterica serovar Typhimurium contains a neighboring R81H substitution (19).

To help define the nature of the pmrA53 mutation, we constructed a pmrA deletion mutant using Lambda Red technology (29). Here, the PCR primers used to amplify (20) the cat gene (underlined) pmrA-F (5’TATTACCAG GCTGCCGATGATATTCGTGCAAACCTTGCGAGGAG GTAAGTGACACGTAAAGGGTCCACATTTTACC-3’) and pmrA-R (5’AGCCGTGGCAGCGATATTTGGTCGG CGCAGAAAATGCACTCAGATTCAATTACGCCCGCCC TGCCACTCATCG-3’) were flanked at both ends by short pmrA 5' and 3' sequences. The amplified PCR product was electroporated into MG1655, and chloramphenicol-resistant colonies were isolated. For one transformant, the correct genotype was verified by PCR with primers that resided within and outside the deletion/insertion region and this strain was designated DW169. As shown in Table 2, DW169 (ΔpmrA::cam) was sensitive to polymyxin B, suggesting that the pmrA53 mutant was not from a loss-of-function mutation but instead a gain-of-function mutation.

Constructing and testing PmrA-dependent reporters. To test if the pmrA53 mutant was a constitutively active transcriptional activator, we cloned the putative promoter regions from three E. coli genes, pmrC, pmrH, and yibD. These genes are Salmonella enterica serovar Typhimurium homologs that contain PmrA-dependent promoters (14, 23, 27). Each of these putative E. coli regulatory elements contains a PmrA binding box which is characterized by the consensus sequence CTTAAT(T/G)
TABLE 1. Bacteria and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristic(s)</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td><strong>E. coli</strong> strains</td>
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<tr>
<td>MG1655</td>
<td>Wild type</td>
<td>ATCC</td>
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<tr>
<td>MC4100</td>
<td>Δ(arg-lac)U169</td>
<td>ATCC</td>
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<tr>
<td>DW137</td>
<td>MG1655 pmrA53</td>
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<tr>
<td>DW169</td>
<td>MG1655 ΔpmrA::cam</td>
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<tr>
<td>DW194</td>
<td>MC4100 ΔpmrA::cam</td>
<td>This study</td>
</tr>
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<td>MC4100 cadB2231::Tn10 pmrA53</td>
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</tr>
<tr>
<td>DW200</td>
<td>MC4100 cadB2231::Tn10 pmrA+</td>
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<td>pJM22</td>
<td>pRL124, pmrC::lacZ</td>
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* ATCC, American Type Culture Collection.

(G/A)T(T/A)(C/A)(C/T)TAAT (1, 14). The actual sequences are CTTAAGGTGGCTTAAAT (pmrC), CTTAAGGTGAAGTAAAT (pmrH), and CTTAATGTTTCTTAAAT (yibD). Sequences that are shared with the consensus are underlined.

To construct reporter plasmids, these elements were PCR amplified from MG1655 chromosomal DNA and cloned into pRL124 (Table 1) (13). These elements were amplified with primer pairs pmrC-Sall, 5’-ACCGCGTACGTCACCTCTGATGGTCATACCCGC-3’; pmrC-EcoRI, 5’-CCGGAAATTCGCAAGTTTCAACG-3’; pmrH-Sall, 5’-ACCGCGTACGTCACCTCTGATGGTCATACCCGC-3’; pmrH-EcoRI, 5’-CCGGAAATTCGCAAGTTTCAACG-3’; and pmrH-yibD, 5’-CCGGAAATTCGCAAGTTTCAACG-3’. Stop codons (bold) were engineered into the primers to ensure that protein fusions were not created with the reporter, and induced restriction sites are underlined. PCR products were then restricted with Sall-EcoRI and cloned into the corresponding sites of pRL124. Each clone was checked by sequence analysis and was then transformed into three isogenic strains: MC4100, DW194 (ΔpmrA::cam), and DW199 (yibD-2231::Tn10 pmrA53) (Table 1). DW194 and DW199 were constructed by P1 transduction. For DW199, the pmrA53 allele was cotransduced into MC4100 by first selecting for Tet’ from cadB2231::Tn10 and then scoring polymycin B resistance (~50% linked).

Ferric iron has been shown to induce the PmrAB regulon in *Salmonella enterica* serovar Typhimurium (28) and has recently been reported in *E. coli* (8, 27). As illustrated in Fig. 1, the pmrC, pmrH, and yibD reporters of *E. coli* were induced 10- to 100-fold by iron in a pmrA- strain. This induction was PmrA dependent since expression was abolished by the ΔpmrA::cam mutation (Fig. 1). In contrast, all three promoters were constitutively expressed at high levels in an isogenic strain containing the pmrA53 mutation and, moreover, were not further induced by iron (Fig. 1). These results suggest that the putative PmrA cognate histidine kinase, PmrB, cannot further activate PmrA53 upon ligand binding and that PmrA53 is locked into a constitutively active conformation. Quantitatively, the expressions from the pmrC and yibD constructs were 3- to 10-fold higher, respectively, in the pmrA53 mutant than in the strain with induced pmrA+. In contrast, we repeatedly observed that the pmrH promoter could be induced to higher levels in a strain carrying pmrA+ than in a strain carrying pmrA53 (Fig. 1C). These results suggest that there could be physiological differences between a ligand-activated PmrAB regulon and a regulon genetically activated by PmrA53 (see below). It is also possible that the G53V substitution could alter the promoter recognition or transcriptional activation properties of PmrA.

Other reported inducers of the PmrAB regulon include ZnSO₄ and NH₄NO₃ (8, 10, 21, 30). Here we show that these chemicals do indeed induce pmrC, pmrH, and yibD expression in a PmrA-dependent manner (Fig. 1D and data not shown). FeSO₄, which in solution primarily consists of oxidized ferric iron (28), was found to be a more potent inducer than ZnSO₄. Although the induction levels observed for NH₄NO₃ were greater than those for FeSO₄, this finding was of little physiological significance because ammonium metavanadate was used at a high concentration that resulted in significant growth inhibition and, moreover, metavanadate likely activates the PmrAB regulon by a nonspecific mechanism (21, 30). Additionally, we found that low Mg²⁺ concentrations (27), CaCl₂, mouse serum, and sublethal concentrations of polymyxin B did not induce the PmrAB regulon (data not shown).

**Deoxycylacid sensitivity.** Since PmrAB-dependent remodeling of LPS confers resistance to cationic agents, reducing the negative charge on lipid A, we tested whether the pmrA53 mutant compromised the innate resistance of cells to anionic agents. Of particular note, we found that strain DW137 was indeed hypersensitive to the anionic detergent deoxycholic acid (Table 2). Previously, cryptic *E. coli* polymyxin B-resistant mutants have been reported to be hypersensitive to deoxycholate (5). Importantly, PmrA mutant in *Salmonella* was not hypersensitive to deoxycholate (7, 26). Here, we show that deoxycholic acid hypersensitivity was specific to the pmrA53 allele, since the ΔpmrA::cam allele did not confer sensitivity (Table 2). Figure 2 shows that DW137 was, in fact, rapidly killed by deoxycholic acid while exhibiting polymyxin B resistance. Conversely, MG1655 was susceptible to polymyxin B killing and was resistant to deoxycholic acid (Fig. 2A). To verify that the pmrA53 mutation was indeed the cause of the deoxycholic acid sensitivity, we tested and found that the DW199 (pmrA53) transductant was deoxycholic acid sensitive, while the isogenic transductant DW200 (Table 1, pmrA+) was polymyxin B sensitive and deoxycholic acid resistant (data not shown). We conclude that pmrA53 is the cause for the changes in susceptibilities since both phenotypes cotransduce.

Since the PmrAB regulon was activated by iron, we tested whether the deoxycholic acid sensitivity could be phenocopied...
under a variety of conditions by wild-type cells grown in iron. Under no condition could we phenocopy deoxycholic acid sensitivity (data not shown). The lack of iron-induced sensitivity to deoxycholic acid suggests there could be physiological differences between the activation of the PmrAB regulon by pmrA and the growth of wild-type cells in iron (Fig. 1). In support of this view, Hagiwara and coworkers (8) showed, by microarray analysis, that a significant fraction of the E. coli iron (FeSO₄)-regulated genes are, in fact, independent of PmrAB. This includes 32 genes that were induced by FeSO₄ in the absence of iron (FeSO₄).

FIG. 1. PmrA-dependent gene expression. pmrC::lacZ (A), yibD::lacZ (B), and pmrH::lacZ (C) reporter plasmids were transformed into isogenic MC4100 (carrying pmrA⁺; circles), DW194 (carrying ΔpmrA::cat; squares), and DW199 (carrying pmrA53; triangles) strains. The indicated amounts of iron sulfate were added to log phase cultures grown in LB for 2 h at 37°C before cells were harvested and assayed for β-galactosidase activity (in Miller units [15]). (D) Isogenic MC4100 (striped bars) and DW194 (filled bars) strains containing the pmrC::lacZ reporter plasmid were grown during the log phase in LB with the indicated additives at 200 μM (zinc and iron sulfate) and 25 mM (ammonium metavanadate).

FIG. 2. Polymyxin B and deoxycholic acid kill curves. E. coli strains MG1655 (A) and DW137 (B) were grown to mid-log phase and then back diluted in LB or with the addition of polymyxin B (PmB, 1 μg/ml) or deoxycholic acid (DA, 10 mg/ml).
PmrAB (8). These results demonstrate that there are physiological differences between iron exposure and a constitutively active PmrA mutant. Additionally, it has been shown that zinc, which activates the PmrA regulon (Fig. 1) (10), induces an efflux pump (MdtABCD) that was implicated in deoxycholate resistance (3). Thus, we hypothesize that although iron exposure may render the outer membrane more susceptible to deoxycholate, it may also induce the mdtABCD operon or other gene products that nullify the former effect.

**Conclusion.** Here, we report the isolation of the pmrA53 mutant that confers moderate polymyxin resistance while simultaneously sensitizing cells to the anionic bile detergent deoxycholic acid. The constitutive expression of the PmrAB regulon and the resulting remodeling of lipid A likely explains the susceptibility changes. Although the addition of 1-Ara4N and pEtN to lipid A is known to confer polymyxin resistance, their possible role in sensitizing cells to deoxycholic acid remains to be tested. It is plausible, for example, that the addition of 1-Ara4N, which reduces the net negative charge on LPS, compromises the outer membrane to select anionic agents. These results highlight the environmental challenge that E. coli faces in its normal habitat in the mammalian intestinal tract. Here, both antimicrobial cationic peptides and high concentrations (~2%) of bile detergents reside (7, 22). In this respect, it is interesting to note that the Salmonella enterica serovar Typhimurium PhoQ sensor was recently shown to recognize antimicrobial peptides (2), which in turn leads to PmrA activation and LPS remodeling (6). In E. coli, the PhoQ-P system is not coupled to PmrA (27). Future studies are needed to understand how antibacterial peptides and other intestinal track cues are recognized and integrated by enteric bacteria to build an effective outer membrane barrier.

This work was supported in part by a grant from the National Institute of Allergy and Infectious Disease (AI51104).

**REFERENCES**