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Mutations Affecting DNA-Binding Activity of the MexR Repressor of *mexR-mexA-mexB-oprM* Operon Expression

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We have isolated 25 MexR mutants that retained their dimerizing ability but were unable to bind *mexOP* DNA. Surprisingly, 20 mutations were located in the hydrophobic core region at α4, W1, α2, α3, and β2, and only 3 were in positively charged residues. These results verified that DNA binding is mediated by distinct regions of MexR and showed the importance of the hydrophobic core region of the DNA-binding domain.

Wild-type cells of *Pseudomonas aeruginosa* express a low level of MexAB-OprM transporter, which provides decreased susceptibility to multiple species of antipseudomonal antibiotics (5, 7, 9). Mutations in the *mexR* (*nalB*) gene confer high resistance to the same antibiotics (10, 11, 13, 16, 18). It was reported that the MexR repressor, a member of the newly recognized *marR* family (1, 8), coregulates expression of the divergently transcribed *mexA-mexB-oprM* and *mexR* by binding to their shared operator-promoter region (*mexOP*), located between *mexA* and *mexR* (3, 13, 15). MexR, consisting of 147 amino acid residues, forms a homodimer for DNA binding. The three-dimensional structure of MexR has been solved by X-ray crystallography (6), and the structure predicted distinct regions for dimerization (N- and C-terminal helices) and DNA binding (winged helix-turn-helix motif). Since the structure of MexR cocrystallized with MexOP-DNA remains unresolved, the specific details of its interaction with DNA remain unclear. To identify residues in the MexR structure that are important for DNA binding, we isolated mutants that are unable to repress expression of the *mexAB-oprM* operon and show a dominant-negative phenotype relative to wild-type MexR. Mutation sites were mapped on the structure of MexR, suggesting how the key residues might impair DNA binding.

The strain used for DNA manipulation was *Escherichia coli* DH5α (Takara). The wild-type *P. aeruginosa* strain used was PAO4290 (17). Mutant strains used were TNP076 lacking *mexA-mexB-oprM* (17) and TNP030#10 carrying a mutation in *mexR* (12). Plasmids used were pET19 (Novagen) and the shuttle vector pMMB67HE (4). Cells were grown in L broth throughout this study except that MICs of antibiotics were determined in Mueller-Hinton agar at 37°C as reported previously (12). The gene encoding MexR was amplified by PCR using the primer pair 5′-GATGCCATGGGCAACTACCCCG TGAATCCCGAC-3′ and 5′-GCGCAACCGCTTGAGGATA TTTGGCACCATCACCATCACCATTAAGGATCCCG-3′, and the product was subcloned into pET19 (Novagen). The DNA fragment containing the *mexR* gene with codons for a six-histidine tag at the carboxyl-terminal end was transferred onto the *P. aeruginosa* shuttle vector, pMMB67HE, yielding pMEXR-His. Next, we generated random mutations on the plasmid-borne *mexR* using the mutator strain XL1-Red according to the manufacturer's instructions (Stratagene). Mutants were selected for increased antibiotic resistance due to the production of inactive *mexR* and consequent production of a derepressed level of the MexAB-OprM efflux pump. *P. aeruginosa* PAO4290 cells having chromosomal native *mexR*$^+$ were transformed with the mutant *mexR* library, and cells with the dominant-negative phenotype relative to native *mexR*$^+$ were selected for resistance against 6.25 μg of aztreonam/ml and 150 μg of sulbenicillin (for a plasmid marker)/ml on Luria-Bertani agar containing 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG).

TABLE 1. Effect of *mexR* mutation on MICs of antibiotics for *P. aeruginosa*

Description of strain(s)	*mexR* allele on pMEXR-His$_6$[a]	MIC of antibiotic (μg/ml)[b,c]				No. of isolates
		Aztreonam		Ofloxacin		
		−IPTG	+IPTG	−IPTG	+IPTG	
Wild-type, PAO4290	None	6.25	6.25	0.78	0.78	
	Wild-type	3.13	0.2	0.78	0.1	
Dominant-negative mutants	L45P	6.25	12.5	1.56	3.13	1
	I46N	6.25	25	1.56	3.13	1
	L57P	6.25	25	1.56	3.13	1
	L57R	6.25	25	1.56	3.13	1
	T69I	6.25	12.5	1.56	3.13	1
	I72N	6.25	25	1.56	3.13	9
	L75P	6.25	25	1.56	3.13	6
	L75R	6.25	25	1.56	3.13	1
	R83C	6.25	6.25	1.56	3.13	1
	R91C	6.25	12.5	1.56	3.13	2
	R91H	6.25	12.5	1.56	3.13	1
Δ*mexAB-oprM* mutant, TNP076	None	0.2	0.2	0.1	0.1	
	L57P	0.2	0.39	0.1	0.1	
	R91H	0.2	0.2	0.1	0.1	
mexR-deficient mutant, TNP030#10	None	25	25	6.25	6.25	
	L57P	50	50	6.25	6.25	
	R91H	50	50	6.25	6.25	

[a] Number refers to amino acid residue of the mutation site.
[b] MIC was determined by the agar dilution method with Mueller-Hinton agar (Becton-Dickinson).
[c] MexR-His$_6$ was induced in the presence (+) or absence (−) of 1 mM IPTG.

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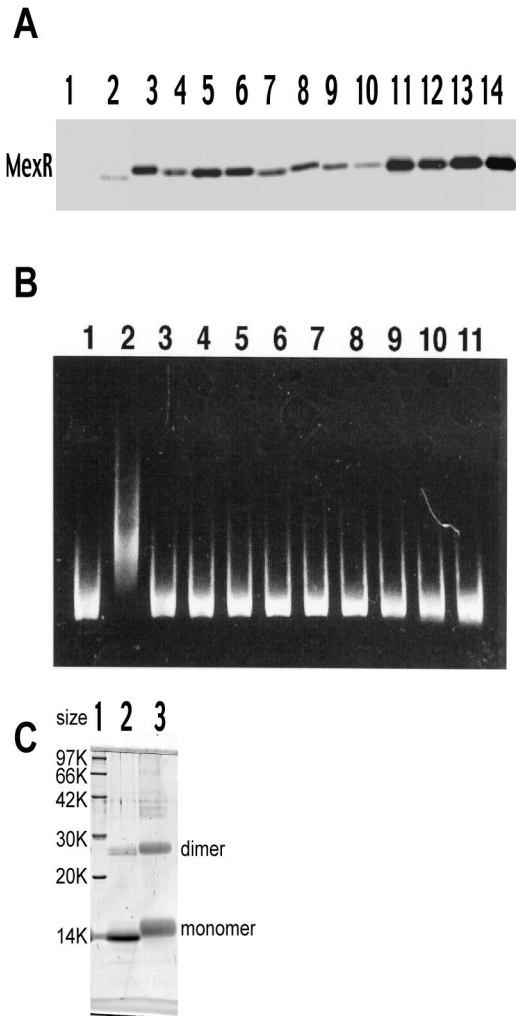

FIG. 1. Properties of the mutant MexR-His_6 protein. (A) Expression of the MexR protein. PAO4290 cells harboring pMMB67HE with or without *mexR-his*$_6$ were grown in the presence of 1 mM IPTG. Cell lysate was prepared by disintegrating cells in a solution of 10 mM Tris–2% Triton X-100–1 mM $MgCl_2$ (pH 7.9) with ultrasonic oscillation and then centrifuging at 100,000 × *g* for 60 min. About 10 μg of protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12). Preparation of the *mexOP* probe DNA and the in vitro MexR DNA binding assay were done as described elsewhere (12). Lanes: 1, pMMB67HE without *mexR*; 2, pMMB67HE encoding wild-type MexR-His_6; 3, Leu45Pro; 4, Ile46Asn; 5, Leu57Pro; 6, Leu57Arg; 7, Thr69Ile; 8, Ile72Asn; 9, Leu75Pro; 10, Leu75Arg; 11, Arg83Cys; 12, Arg91Cys; 13, Arg91His; 14, purified MexR-His. (B) Gel retardation assays by MexR-His_6. The *mexOP* probe DNA (final concentration, 10 nM) was incubated with homogeneously purified MexR-His_6 derived from *E. coli* DH5α (final concentration, 0.2 μM) in a solution of 4 mM Tris–4 mM HEPES–25 mM KCl–1 mM EDTA–25% glycerol (pH 7.9), and the 7.5-μl mixture was subjected to electrophoresis in 4% polyacrylamide gels (in 0.25× Tris-borate-EDTA). The gel was soaked in 10,000-fold-diluted CYBR Green I (BioWhittaker Molecular Applications), and DNA was visualized with UV light at 254 nm. Lanes: 1, DNA probe without MexR; 2, wild-type MexR-His; 3, Leu57Pro; 4, Leu57Arg; 5, Thr69Ile; 6, Ile72Asn; 7, Leu75Pro; 8, Leu75Arg; 9, Arg83Cys; 10, Arg91Cys; 11, Arg91His. (C) Cross-linking experiment with mutant MexR. A representative mutant MexR (Arg91His) protein, 6 μg/20 μl, was mixed with 10 μg of disuccinimydyl suberate/μl and incubated at 4°C for 30 min. To the mixture was added 10 μl of 500 mM Tris-HCl (pH 8.0) and 30 μl of solubilizer, and the mixture was heated at 100°C for 5 min. A sample containing 2 μg of MexR was applied to a 14% polyacrylamide gel. Lane 1, size markers; lane 2, Arg91His without disuccinimydyl suberate; lane 3, Arg91His with disuccinimydyl suberate. Wild-type MexR and other mutant MexR proteins showed essentially the same gel profile (data not shown). A significant amount of protein bands at the position corresponding to the dimer in the sample without disuccinimydyl suberate also appeared with wild-type MexR (not shown). Retarded mobility of disuccinimydyl suberate-treated MexR is due to bound disuccinimydyl suberates.

The function of the mutant MexR protein was assessed by determining the MICs of antibiotics. To test the influence of His_6 modification, we first determined the antibiotic susceptibility of the cells harboring pMEXR-His_6 (wild-type version of MexR with the His_6 modification) and found that the MIC of aztreonam was 32-fold lower in the presence of IPTG than that without pMEXR, confirming that the His_6 modification did not bother the repressor function. This value was comparable to the MIC of aztreonam in cells lacking MexAB-OprM. In the next experiment, we generated random mutations on the plasmid-borne *mexR* gene using a mutator strain (Table 1). We reasoned that if mutant MexR-His_6 retained its dimer-forming capability but was unable to bind with DNA, its expression in cells with chromosomal *mexR*$^+$ might result in a dominant-negative phenotype. Consequently, wild-type strains harboring such a plasmid were expected to exhibit increased MICs of antibiotics, to a level close to that for *mexR*-negative cells (13).

We selected 25 transformants on aztreonam-impregnated plates, extracted the plasmid DNA, and identified the mutation by sequencing the entire *mexR* gene by the dideoxy chain termination method (10, 14). We found a single amino acid substitution for all 25 plasmid-borne mutant *mexR* genes, which occurred in eight different sites (Table 1). Upon IPTG induction, all MexR-His_6 mutants showed dominant-negative behavior relative to native MexR, increasing resistance to aztreonam and ofloxacin to a level close to that in the *mexR*-deficient strain, except for aztreonam susceptibility of the Arg83Cys mutant (Table 1). These results suggested that the mutant proteins might have an impaired DNA-binding capability but could still form a complex with wild-type MexR protein.

The level of the MexR-His_6 proteins in cell extracts was analyzed by the Western blot method using rabbit antibody raised against MexR. Wild-type MexR-His_6 showed a low level of expression (Fig. 1A, lane 2) that is most likely due to its strong repression by plasmid-encoded MexR. In fact, the MIC of aztreonam for the cells expressing plasmid-borne MexR appeared to be 32 times lower than that for the cells without plasmid-borne MexR, suggesting that transcription of *mexAB-oprM* was also strongly repressed by plasmid-borne MexR (Table 1). Cells harboring the plasmid with the *mexR* mutants produced an elevated level of MexR, probably because the transcription was freed from self-repression. Considerable variations in the level of MexR were observed. Mutant MexR-His_6 was purified by His · Bind-Resin (Novagen) chromatography except for Leu45Pro, Ile46Asn, and Leu134Pro (data not shown). The gel retardation assay showed that a 0.2 μM concentration of the wild-type MexR-His_6 formed a complex with the 10 nM *mexOP* DNA as expected (Fig. 1B, lane 2) (12). An attempt to stain MexR with Coomassie blue was unsuccessful, probably due to a limit of assay sensitivity. Specificity

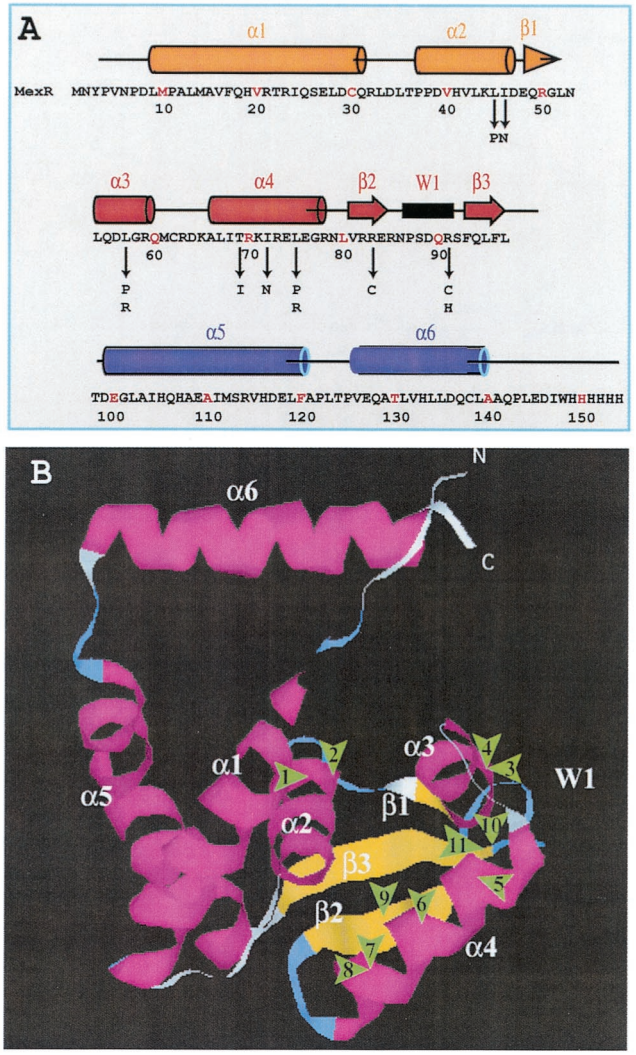

FIG. 2. Mapping of mutations on the MexR structure. (A) Amino acid sequence, secondary structures, and mutation sites. Matching of amino acid sequence and the secondary structure was based on X-ray crystallographic data. Numbers indicate amino acid residues. Arrows mark mutation sites reported in this study. (B) Mutations were localized on the MexR structural model based on X-ray crystallography. Arrowheads indicate the approximate locations of the mutations. Distances are arbitrary. The numbers refer to the mutations as follows: 1, Leu45Pro; 2, Ile46Asn; 3, Leu57Pro; 4, Leu57Arg; 5, Thr69Ile; 6, Ile72Asn; 7, Leu75Pro; 8, Leu75Arg; 9, Arg83Cys; 10, Arg91His; 11, Arg91Cys.

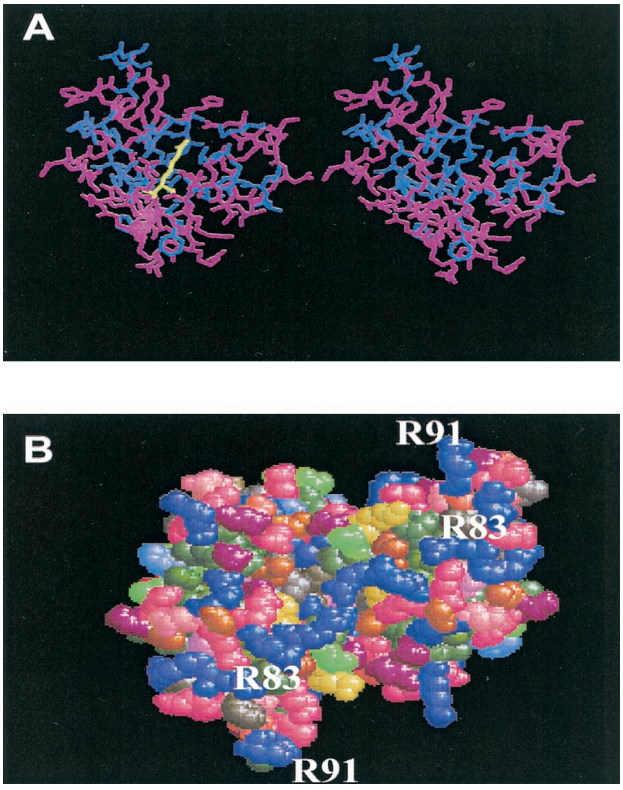

FIG. 3. Computer-aided visualization of mutation. (A) Stereo view molecular model of the Leu75Arg mutation as a representative of mutation in the hydrophobic core region. The mutation was inserted on the crystallographic structure of MexR by Program O for Windows NT, version 7.0. Figures were drawn by using Program Ras Win Molecular Graphics Windows, version 2.6-ucb. Hydrophobic amino acid residues and other residues are shown by blue and red, respectively. Mutation sites are given in yellow. (B) Surface potential of the bottom part of the MexR dimer. Arg residues are emphasized by a purple color in the space-fill model. Distribution of the electrostatic potential was drawn by the Swiss-pdb Viewer, version 3.7. The locations of Arg83 and Arg91 are shown in the region containing the α4, β2, W1, and β3 structures.

of MexR for *mexOP* DNA was confirmed in a previous study (12). On the other hand, all nine of the MexR-His_6 mutants tested failed to bind to the probe DNA (Fig. 1B, lanes 3 through 11), suggesting that the mutations are likely to affect the DNA-binding domain. The possibility that the mutant MexR may have lost its dimerizing capability was ruled out by a cross-linking experiment (Fig. 1C).

The repressor proteins, which regulate the transcription of the multidrug efflux pump, have been assigned for the MarR family (1, 11). Recently the three-dimensional structures of MexR and MarR have been solved by X-ray crystallography (2, 6). We mapped the mutations on the proposed MexR structure. Among 25 mutations, 16 were localized to three specific sites, in the α4-helix (residues 66 to 79) of MexR, suggesting the importance of the α4-helix structure for DNA binding (Fig. 2). In fact, these sites were highly conserved in the DNA-binding region. These sites faced opposite to the charged residues at the α4-helix, which might be important for maintaining the structural integrity of the DNA-binding site. Mutations may disturb the hydrophobic interaction as depicted in Fig. 3A. Changes in the α3-helix (residues 54 to 59) occurred as Leu57Arg and Leu57Pro. Amino acids in the hydrophobic region of the α3-helix, such as Leu54 and Leu57, may interact with the hydrophobic surface of the α4-helix to stabilize the DNA-binding region (Fig. 2B). Arg83 and Arg91 are located at the bottom of the MexR structure and form a line of positive charges that forms the DNA-binding site (Fig. 3B).

In this study, we obtained several classes of MexR proteins with impaired DNA-binding capability by selecting for the dominant-negative phenotype. Mutations were mapped on a

limited region of MexR, which might be important in forming the DNA-binding domain.

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