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The MexR Repressor of the *mexAB-oprM* Multidrug Efflux Operon in *Pseudomonas aeruginosa*: Characterization of Mutations Compromising Activity

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Mutations in *mexR* yield a multidrug resistance phenotype in *nalB* mutants of *Pseudomonas aeruginosa* as a result of derepression of the *mexAB-oprM* multidrug efflux operon. MexR produced by several *nalB* strains carried single amino acid changes that compromised MexR stability or its ability to dimerize. Changes at residues L95 and R21, however, produced a stable MexR protein capable of dimerization and, thus, likely compromised DNA binding.

Bacterial multidrug efflux pumps play an important role in the antimicrobial resistance of gram-negative pathogens (15, 17), particularly *Pseudomonas aeruginosa*, where five multidrug efflux systems (MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY-OprM, and MexJK-OprM) have been described to date (16, 18, 21). Of particular significance is the MexAB-OprM efflux system, which is expressed constitutively, thereby contributing to the well-known intrinsic resistance of this organism to multiple antimicrobials (11, 12, 14, 23), and is also hyperexpressed in *nalB* multidrug-resistant mutants as a result of mutations in the *mexR* gene (8, 19, 20, 24, 26). The *mexR* gene is transcribed divergently from the *mexAB-oprM* genes and encodes a repressor of the MarR family of regulators (19). MarR is responsible for regulating expression of the *marRAB* (*mar*) operon associated with a multiple antibiotic resistance (mar) phenotype in *Escherichia coli* (2). MexR has been shown to bind, possibly as a dimer, to two sites in the *mexR-mexA* intergenic region, overlapping promoters for *mexR* and *mexAB-oprM* (6), consistent with its observed repression of these promoters (19, 24). To elucidate the effect of *mexR* mutations on MexR repressor activity and thereby identify functionally important residues in the protein, the influence of *mexR* mutations in *nalB* strains on MexR production and dimerization was assessed.

Bacteria (Table 1) were cultivated at 37°C in Luria-Bertani (LB) broth (Difco) supplemented as needed with tetracycline (10 μg/ml for *E. coli*; 100 μg/ml for *P. aeruginosa*), ampicillin (50 μg/ml), chloramphenicol (30 μg/ml), and mercuric chloride (15 μg/ml). Mutated *mexR* genes were PCR amplified from several *P. aeruginosa nalB* strains using primers MEXRF1 (5′-GCGAGAATTCCGTTCGTTGCATAG-CGTTGTC-3′; *Eco*RI site underlined) and MEXRB1 (5′-GCGAGAATTCCGA AGGCATTCG-CCAGTAAGC-3′; *Eco*RI site underlined). PCR mixtures (100 μl) contained 1 μg of chromosomal DNA (prepared as described elsewhere [4]), 2.5 U of Vent DNA polymerase (New England Biolabs, Mississauga, Ontario, Canada), a 0.2 mM concentration of each deoxyribonucleotide triphosphate, 2.5 mM $MgSO_4$, a 0.3 μM concentration of each primer, 1× ThermoPol buffer (New England Biolabs), and 10% (vol/vol) dimethyl sulfoxide. Mixtures were heated for 2 min at 94°C followed by 30 cycles of 94°C for 30 s, 56°C for 1 min, and 72°C for 1 min, before finishing with 72°C for 10 min. PCR fragments were electrophoresed on and recovered from 1.2% (wt/vol) agarose gels using the Prep-a-Gene DNA purification kit (Bio-Rad Laboratories) as outlined by the manufacturer. Following digestion with *Eco*RI and cloning into pRK415 (Table 1), *mexR* sequences were validated by nucleotide sequencing using primer PMSLA25 (5′-GGATTCGTC TGTTGCAGG-3′). The cloned *mexR* genes were then mobilized into *P. aeruginosa* via triparental mating (25) with transconjugants selected on tetracycline (100 μg/ml) and imipenem (0.5 μg/ml; for counterselection). Wild-type and mutant *mexR* genes derived from *nalB* strains were also PCR amplified (as above) and cloned into the bacterial two-hybrid vectors pDP804 and pMS604 (Table 1). For cloning into pDP804, primers *Xho*ILA7 (5′-GCAG-CTCGAGATGAACT ACCCCGTGAATCC-3′; *Xho*I site underlined) and *Bgl*II-ILA11 (5′-GCGGAGATCTAATATCCTCAAGCGGTTGC-GCG-3′; *Bgl*II site underlined) were used, while for cloning into pMS604, primers *Bst*EIILA8 (5′-GAGCGGTGACCATG AACTACCCC-GTGAATCC-3′; *Bst*EII site underlined) and *Xho*ILA12 (5′-GAGGCTCGAGAATATCCTCA-AGCGTT GCGCG-3′; *Xho*I site underlined) were used. Reaction mixtures were formulated and heated as above, with the exception that wild-type *mexR* was amplified from plasmid pRSP55 (20 ng). PCR products were purified as above, restricted with *Xho*I and *Bgl*II or *Bst*EII and *Xho*I, and cloned into *Xho*I-*Bgl*II-digested pDP804 or *Bst*EII-*Xho*I-digested pMS604, as appropriate. Following cloning, the *mexR* sequences were validated by nucleotide sequencing using primer PMSLA25. Plasmid DNAs were prepared using the Qiagen miniprep kit as recommended by the manufacturer.

Cell envelope proteins were prepared as described previously (22), except that 15 ml of cell culture was harvested in stationary phase. Soluble proteins were recovered by saving the supernatant following centrifugation of cell envelopes. Protocols for sodium dodecyl sulfate-polyacrylamide gel electro-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[c]	Reference or source
E. coli strains		
DH5α	*endA hsdR17 supE44 thi-1 recA1 gyrA relA1* Δ(*lacZYA-argF*)*U169 deoR* [ϕ80d*lac*Δ(*lacZ*)M15]	4
SU202	*lexA71*::Tn*5 sulA211 sulA*::*lacZ* Δ(*lacIPOZYA*)*169* F′ *lacI*^{q} *lacZ*ΔM15::Tn*9*	5
P. aeruginosa strains		
K767	PAO1 prototroph; $MexR_{WT}$[a]	14
K1491	K767 Δ*mexR*	24
OCR1	K767 *nalB*; $MexR_{R70W}$	14
PAO503	*met-9011*; $MexR_{WT}$	9
K1647	PAO503 *nalB* [Pa10c][b]; $MexR_{A110T}$	B. Wretlind, Stockholm, Sweden
K1648	PAO503 *nalB* [T3]; $MexR_{G58E}$	9
K1649	PAO503 *nalB* [T4]; $MexR_{L95F}$	9
K1651	PAO503 *nalB* [T6]; $MexR_{T69I}$	9
K1652	PAO503 *nalB* [T7]; $MexR_{R21W}$	9
K1657	PAO503 *nalB* [K3]; $MexR_{R91H}$	9
K1658	PAO503 *nalB* [K4]; $MexR_{L13M}$	9
H103	PAO1 prototroph; $MexR_{WT}$	R. E. W. Hancock, Vancouver, Canada
K1462	H103 *nalB*; $MexR_{L57R}$	24
K1463	H103 *nalB*; $MexR_{T130P}$	24
Plasmids		
pRK415	Broad-host-range cloning vector; Tc^r	10
pRSP55	pRK415::$mexR_{767}$ (wild-type MexR)	This study
pLK501	pRK415::$mexR_{1649}$ (L95F)	This study
pLK503	pRK415::$mexR_{1652}$ (R21W)	This study
pLK505	pRK415::$mexR_{1462}$ (L57R)	This study
pLK507	pRK415::$mexR_{1658}$ (L13M)	This study
pMS604	$LexA_{1–87}$WT-Fos zipper fusion; *ori* (ColE1) Tc^r	5
pDP804	$LexA_{1–87}$408-Jun zipper fusion; *ori* (P15A) Ap^r	5
pRK2013	Broad-host-range helper vector; Tra$^+$ Km^r	7
pLK451	pDP804::$mexR_{767}$ (wild-type MexR)	This study
pLK452	pMS604::$mexR_{767}$ (wild-type MexR)	This study
pLK453	pMS604::$mexR_{1647}$ (A110T)	This study
pLK454	pMS604::$mexR_{1648}$ (G58E)	This study
pLK455	pMS604::$mexR_{1649}$ (L95F)	This study
pLK457	pMS604::$mexR_{1652}$ (R21W)	This study
pLK458	pMS604::$mexR_{1657}$ (R91H)	This study
pLK459	pMS604::$mexR_{1658}$ (L13M)	This study
pLK460	pMS604::$mexR_{1462}$ (L57R)	This study
pLK461	pMS604::$mexR_{1463}$ (T130P)	This study

[a] The MexR protein (wild-type or mutant) produced by each strain, including the nature of any mutation, is indicated.
[b] Strain designation of Jalal et al. (9) is shown in square brackets.
[c] The *nalB* strain from which the *mexR* gene was cloned is indicated by a subscript, and the mutant version of MexR that is produced is shown in parentheses.

phoresis and Western immunoblotting have been described previously (22). For gels immunoblotted and developed with an anti-MexR antiserum, 2-mercaptoethanol (2.5% [vol/vol] final concentration) was included in the gel loading buffer (prepared as a 4× stock solution) and the gels were thicker (3 mm) than usual to permit more sample to be loaded. Antibody to MexR was prepared in New Zealand White rabbits (by L. Mutharia, University of Guelph, Guelph, Ontario, Canada) using purified histidine-tagged MexR (6) as antigen, and it was adsorbed against whole-cell lysates of *P. aeruginosa* K1491 prior to use. The protocol for antimicrobial susceptibility has been described elsewhere (22).

Two-hybrid systems are routinely used to assess protein-protein interactions, and a recently developed bacterial version of this system has proven useful in this regard (5). The system is based on LexA, a protein that binds only as a dimer to the *sulA* operator and in so doing downregulates transcription. Using vectors (pMS604 and pDP804) engineered to encode only the DNA-binding domain of LexA (hereafter called LexA′) and a reporter strain with a *lacZ* gene controlled by a *sulA* operator (*E. coli* SU202), one simply clones genes and sequences predicted to encode interacting proteins and domains as fusions to *lexA′* and screens for repression of *lacZ* (i.e., reduction in β-galactosidase activity). Such repression requires dimerization of the LexA DNA-binding domain, which will occur only if the protein sequences fused to LexA′ are able to interact. Since the system was originally designed to assess interactions between heterologous proteins, the *lexA′* sequence in one plasmid (pMS604) was altered such that its LexA′ bound to an altered operator sequence and the reporter strain carried a hybrid *sulA* operator, such that only LexA′ heterodimers would bind and repress *lacZ*. The two-hybrid vectors also contain sequences encoding Jun and Fos zipper motifs (known to interact) fused to *lexA′*, such that *E. coli* SU202 carrying these vectors demonstrates substantial repression of *lacZ* (Table 2). The unaltered vectors thus provide a positive control for the system, although the Jun and Fos zipper-encoding sequences will be disrupted upon cloning test

TABLE 2. β-galactosidase activity of *E. coli* SU202 isolates expressing wild-type and mutant MexR proteins in the LexA-based two-hybrid system[a]

Two-hybrid plasmid combination		β-galactosidase activity[b] (Miller units)
pMS604 derivative	pDP804 derivative	
None	pDP804	1,171 ± 152
pMS604	None	1,370 ± 99
pMS604	pDP804	96 ± 24
pLK452 ($mexR_{WT}$)[c]	pLK451 ($mexR_{767}$; wild-type MexR)	77 ± 12
pLK453 ($mexR_{1647}$; A110T)	pLK451 ($mexR_{767}$; wild-type MexR)	1,520 ± 197
pLK454 ($mexR_{1648}$; G58E)	pLK451 ($mexR_{767}$; wild-type MexR)	614 ± 82
pLK455 ($mexR_{1649}$; L95F)	pLK451 ($mexR_{767}$; wild-type MexR)	89 ± 19
pLK457 ($mexR_{1652}$; R21W)	pLK451 ($mexR_{767}$; wild-type MexR)	87 ± 21
pLK458 ($mexR_{1657}$; R91H)	pLK451 ($mexR_{767}$; wild-type MexR)	570 ± 25
pLK459 ($mexR_{1658}$; L13M)	pLK451 ($mexR_{767}$; wild-type MexR)	1,760 ± 212
pLK460 ($mexR_{1462}$; L57R)	pLK451 ($mexR_{767}$; wild-type MexR)	684 ± 53
pLK461 ($mexR_{1463}$; T130P)	pLK451 ($mexR_{767}$; wild-type MexR)	615 ± 32
pLK455 ($mexR_{1649}$; L95F)	pDP804	1,117 ± 65
pLK457 ($mexR_{1652}$; R21W)	pDP804	1,265 ± 96

[a] *E. coli* SU202 harboring the indicated two-hybrid vectors was assayed for β-galactosidase activity as described in the text.
[b] β-Galactosidase activities are the means of at least three independent determinations, with the standard deviations indicated.
[c] The strain from which the *mexR* gene was cloned is identified by a subscript. Any changes in MexR encoded by these genes are also highlighted.

sequences into the two-hybrid vectors (and, thus, *lacZ* repression will be dependent upon interaction of protein sequences encoded by the cloned DNA).

In utilizing this system to assess MexR dimerization, then, the *mexR* gene was cloned into pDP804 and pMS604, and *E. coli* SU202 isolates carrying these vectors was cultured overnight in antibiotic-supplemented LB broth and diluted 1:99 in fresh LB medium supplemented with appropriate antibiotics and isopropyl-β-D-thiogalactopyranoside (1 mM). Once cultures had reached an optical density at 600 nm of 0.4 to 0.8, β-galactosidase assays were performed as described by Miller (14a). As seen in Table 2, *E. coli* SU202 carrying one or the other of the two-hybrid vectors demonstrated substantial β-galactosidase activity, consistent with the absence of LexA′ dimerization. When wild-type *mexR* genes were cloned into both two-hybrid vectors, *E. coli* SU202 harboring the resultant plasmids (pLK451 and pLK452) showed a substantial decrease in β-galactosidase activity below that seen for the vectors-only positive control, indicating that LexA′ and, thus, MexR, was dimerizing. Dimerization has been reported for MarR, whose crystal structure is known (3), and the recent MexR crystal structure confirms the dimeric nature of MexR (13).

Mutations in *mexR* compromise MexR repressor activity, leading to MexAB-OprM hyperproduction in *nalB* mutants. Loss of activity might result, however, from protein instability, loss of dimerization, or defects in DNA binding. Initially, then, several *nalB* strains were screened for production of MexR. Intriguingly, although *nalB* strains show increased *mexR* gene expression owing to the characteristic *mexR*-negative autoregulation (19), many *nalB* strains produced little or no detectable MexR protein (Fig. 1). While this might be expected for mutants carrying frameshift mutations in *mexR* (see reference 24), those MexR proteins shown in Fig. 1 all carry single amino acid changes only. Some clustering of these mutations is evident (including residues 59 to 83), although the significance of this is not clear. Several *nalB* strains did, however, produce substantial levels of MexR, far above what was seen in wild-type strains (Fig. 1, lanes 1, 4, and 17) and comparable to levels seen in a *nalC* mutant (Fig. 1, lane 2) which hyperexpresses *mexAB-oprM* and *mexR* as a result of mutation(s) in an as-yet-unknown gene(s) (24). Thus, loss of repressor activity in these MexR proteins must be due to changes other than protein instability.

To assess the possible impact of the *mexR* mutation in each of these mutants on protein dimerization, the corresponding genes were amplified by PCR and cloned into the two-hybrid vectors described above. As seen in Table 2, the bulk of the mutations yielding a stable MexR protein interfered with MexR-MexR interaction with, e.g., *E. coli* SU202 isolates ex-

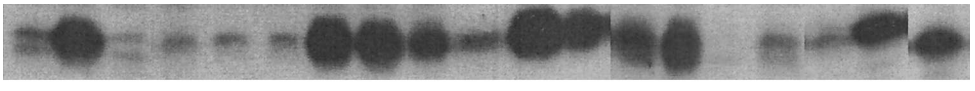

FIG. 1. Expression of MexR in *nalB* mutants of *P. aeruginosa*. Soluble extracts of MexAB-OprM hyperexpressing multidrug-resistant strains of *P. aeruginosa* carrying mutations in *mexR* (and their parental strains) were electrophoresed on sodium dodecyl sulfate–polyacrylamide (15% [wt/vol]) gels and immunoblotted using antibodies to MexR. Lane 1, *P. aeruginosa* PAO1 strain K767 ($MexR_{WT}$); lane 2, strain K1454 (*nalC*); lane 3, OCR1 ($MexR_{R70W}$); lane 4, *P. aeruginosa* PAO503 ($MexR_{WT}$); lane 5, K1655 ($MexR_{R83H}$); lane 6, K1656 ($MexR_{R83H}$); lane 7, K1657 ($MexR_{R91H}$); lane 8, K1658 ($MexR_{L13M}$); lane 9, K1647 ($MexR_{A110T}$); lane 10, K1646 ($MexR_{R59C}$); lane 11, K1648 ($MexR_{G58E}$); lane 12, K1649 ($MexR_{L95F}$); lane 13, K1651 ($MexR_{T69I}$); lane 14, K1652 ($MexR_{R21W}$); lane 15, K1653 ($MexR_{L80P}$); lane 16, K1654 ($MexR_{R70W}$); lane 17, *P. aeruginosa* PAO1 strain H103 ($MexR_{WT}$); lane 18, K1462 ($MexR_{L57R}$); lane 19, K1463 ($MexR_{T130P}$). The impact of the *nalB* mutation on MexR in each strain is indicated in parentheses, with the exception of K1455, which is a *nalC* strain that hyperproduces MexAB-OprM as a result of an unknown mutation. The gel as presented is a composite assembled from lanes of the same gel.

pressing A110T (pLK453) or L13M (pLK459) MexR proteins demonstrating substantial levels of β-galactosidase activity (i.e., comparable to that seen for the single-vector negative controls). Changes at position 58 (G58E, pLK454), 91 (R91H, pLK458), 57 (L57R, pLK460), and 130 (T130P, pLK461) produced β-galactosidase activities that were half that of the negative controls though substantially above that seen for wild-type MexR (Table 2). Presumably, these MexR proteins interact only weakly or associate abnormally, such that LexA′ dimers are not positioned in such a way as to bind the *sulA* operator. Thus, while the aforementioned mutations compromise normal dimer activity by MexR, it is not at all clear that these changes identify residues directly involved in dimerization. The A110T change, for example, is far from sites of interaction between MexR monomers in the crystal structure (13), where any impact on dimerization or dimer structure would have to occur via conformational changes that interfere with dimerization (or formation of normal dimers).

Only the L95F (pLK455) and R21W (pLK457) changes had no apparent impact on MexR dimerization, with *E. coli* SU202 isolates expressing these MexR derivatives demonstrating β-galactosidase activities that were indistinguishable from that of the same strain expressing the wild-type proteins (Table 2). It is likely, therefore, that these mutations ultimately compromised DNA binding, though not necessarily because they define DNA-binding domain(s) of the protein. Indeed, while the L95F change occurs in a region of MexR implicated in DNA binding (13), residue 21 is far from the probable DNA-binding region of the protein. Still, it does occur in a region of MexR where the individual monomers interact with one another. The R21W change may, therefore, alter the positioning of the monomers with respect to one another, altering the proper spacing of the DNA-binding region of each monomer and compromising the ability of MexR dimer to bind to adjacent DNA-binding sites in the *mexAB-oprM* promoter (6).

Nonfunctional mutant MexR proteins that dimerize but are unable to bind DNA should be dominant over wild-type MexR and compromise, to some extent at least, wild-type MexR repressor activity when coexpressed in *P. aeruginosa* (as a result of forming heterodimers). Indeed, negative dominance of *marR* mutations was previously used to define residues implicated in the DNA binding of this repressor protein (1). Thus, the cloned *mexR* genes from *nalB* strains K1649 (pLK501; expresses $MexR_{L95F}$) and K1652 (pLK503; expresses $MexR_{R21W}$) were introduced into wild-type *P. aeruginosa* strain K767, and the impact on *mexAB-oprM* expression was assessed indirectly by measuring the impact on susceptibility to agents known to be substrates of this efflux system. Initial attempts at measuring the impact on expression directly, using Western immunoblotting with a MexB-specific antiserum, were unsuccessful, likely owing to a lack of sensitivity of the method. As seen in Table 3, *P. aeruginosa* K767 isolates carrying pLK501 or pLK503 showed increased resistance to representative antimicrobials relative to that of the vector control or strain K767 expressing wild-type MexR from a plasmid. In contrast, K767 carrying plasmid pLK505 ($MexR_{L57R}$) or pLK507 ($MexR_{L13M}$) did not show any change in drug susceptibility relative to the vector control. These data are consistent with the former but not the latter MexR derivatives negatively impacting the wild-type repressor activity of the chromosomally encoded MexR protein in strain K767, by the formation of heterodimers unable to bind to the *mexAB-oprM* promoter region. These data can be interpreted as suggesting a dimerization defect for the $MexR_{L13M}$ and $MexR_{L57R}$ derivatives. Had these variants retained dimerization ability but interfered with LexA′ binding to *sulA* (see above), thereby compromising LexA′-mediated repression of *lacZ* in the two-hybrid assay, one would have expected them to be also dominant over wild-type MexR and to enhance multidrug resistance. That they were not suggests that these changes compromised MexR dimerization. Recently, a plasmid-encoded $MexR_{T69I}$ variant (from *nalB* strain K1651) has been examined in the two-hybrid system and has also been shown to be competent for dimer formation (data not shown), although its dominance over wild-type MexR has yet to be assessed. Like residue L95, residue T69 occurs in a region of MexR implicated in DNA binding (D. Lim, personal communication).

TABLE 3. Effect of cloned *mexR* mutant genes on the antibiotic susceptibility of wild-type *P. aeruginosa* isolates[a]

Plasmid	MexR[b]	MIC (μg/ml)[c]		
		NOV	CAM	CAR
pRK415	—	256	16	64
pRSP55	Wild type	128	8	32
pLK501	L95F	512	64	256
pLK503	R21W	512	64	256
pLK505	L57R	256	32	64
pLK507	L13M	128	16	64

[a] The antibiotic susceptibilities of *P. aeruginosa* PAO1 strain K767 isolates carrying the indicated *mexR* plasmids were assessed as described in the text.
[b] The alterations in the MexR proteins encoded by the various plasmids are highlighted; —, vector control (no plasmid-encoded MexR).
[c] NOV, novobiocin; CAM, chloramphenicol; CAR, carbenicillin.

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