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NOTES

Influence of Mutations in the *mexR* Repressor Gene on Expression of the MexA-MexB-OprM Multidrug Efflux System of *Pseudomonas aeruginosa*

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Several *nalB*-type multidrug-resistant mutants of *Pseudomonas aeruginosa* overexpressed MexAB-OprM and carried mutations in the local regulatory gene, *mexR*. Others, dubbed *nalC* types, carried mutations elsewhere and overexpressed MexAB-OprM less extensively than the *nalB* strains. Available evidence showed that MexR acted solely as repressor. Disruption of the *mexR* gene at various places suggested that the 5′ end of *mexR* may be a part of the *mexAB-oprM* promoter.

The intrinsic antibiotic resistance of *Pseudomonas aeruginosa* is attributable both to the limited permeability of the organism's outer membrane (12) and to the activity of broadly specific antibiotic efflux systems such as MexAB-OprM (3, 14, 15). MexAB-OprM is a member of a family of multidrug efflux systems of which there are several examples in *P. aeruginosa* including MexCD-OprJ (13), MexEF-OprN (6), and MexXY-OprM (1, 10). Mutants hyperexpressing MexAB-OprM and exhibiting an elevated multidrug-resistant (MDR) phenotype have been described (9, 22). Isolated in the laboratory (9, 22) and from patients after antibiotic therapy (4, 24), these so-called *nalB* mutants often carry mutations in a gene, *mexR*, which occurs immediately upstream of the efflux genes (4, 17, 24) and encodes a repressor of *mexAB-oprM* expression (17). Still, a *mexR* null mutant constructed in vitro, though MDR and expressing elevated levels of MexAB-OprM, was more antibiotic susceptible and exhibited reduced expression of *mexAB-oprM* compared with previously described *nalB* strains (17). One hypothesis stated that MexR functions as both repressor and activator, and those mutations in *mexR* which yield a *nalB* phenotype render MexR in an activator form. To assess, then, the role(s) of MexR in regulating *mexAB-oprM* expression, several *nalB* and *mexR* mutants were isolated, and their influence on MexAB-OprM was examined. We report here that MexR functions solely as a repressor in controlling *mexAB-oprM* expression and that *nalB* strains are simply derepressed for *mexAB-oprM* expression.

Methods. Strains and plasmids used in this study are described in Table 1. Mutants hyperexpressing MexAB-OprM were selected on Luria broth (L-broth; Miller's Luria broth base [Difco] and 2 g of NaCl per liter of H_2O) plates containing 0.2 μg of ciprofloxacin and 12 μg of cefoperazone per ml. MexAB-OprM hyperexpression was confirmed by Western immunoblotting with antibodies to MexB and OprM. Antibiotics were included in growth media as required at the following concentrations: tetracycline, 10 μg/ml (*Escherichia coli*) or 100 μg/ml (*P. aeruginosa*); chloramphenicol, 50 μg/ml (*E. coli*) or 200 μg/ml (*P. aeruginosa*); and mercuric chloride, 15 μg/ml.

The *mexR* gene was amplified from *P. aeruginosa* strains by PCR by using chromosomal DNA as template and primers MEXRF1 (5′-GCGAGAATTCCGTTCGTTGCATAGCGTTGTC-3′) and MEXRB1 (5′-GCGAGAATTCCGAAGGCATTCGCCAGTAAGC-3′). The sequences of *mexR* and the *mexR-mexA* intergenic region were determined by sequencing of the PCR products directly and after cloning them into pRK415. The open reading frame (ORF) downstream of *mexR* (ORF2, Fig. 1) was also amplified by using primers K3 (5′-TACGGGATCCCGCGCAACCGCTTGAGATA-3′) and K4 (5′-GCATGCGCATGCCCTGTGGATGCGCGAACTGAG-3′) and then sequenced. ORF2 from *P. aeruginosa* K767 was cloned into pMMB206 (yielding pRSP67) after digestion of the PCR product with *Bam*HI and *Hin*dIII (site present downstream of ORF2) and sequenced. PCR reaction mixtures (100 μl), including 2.5 U of *Taq* DNA polymerase (Life Technologies), 1× PCR buffer (Life Technologies), 0.3 μM concentrations of each primer, 0.2 mM concentrations of deoxynucleoside triphosphates, 2 mM $MgCl_2$, 10% (vol/vol) dimethyl sulfoxide, and 10 ng of template DNA, were heated for 1 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 56°C, and 1 min at 72°C, before finishing with 10 min at 72°C.

To construct *mexR*::ΩHg insertion mutations, a 3.4-kb *mexR*-containing *Eco*RI-*Sma*I fragment of pPV2 was first cloned into pEX18Tc, yielding pRSP64. *mexR* was disrupted at its *Sst*I site by digesting pRSP64 with *Sst*I, followed by treatment with T4 DNA polymerase (New England Biolabs) and ligation with the *Sma*I-restricted ΩHg fragment of pHP45ΩHg, yielding pRSP65. Similarly, *mexR* and ORF2 were disrupted at their *Mlu*I and *Tth*111I sites, respectively, following digestion of pRSP64 with either *Mlu*I or *Tth*111I, treatment with the Klenow fragment (New England Biolabs), and ligation with the ΩHg *Sma*I fragment, creating pRSP70 and pRSP72. An internal deletion of *mexR* was constructed by digesting pRSP64 with *Sst*I, treatment with T4 DNA polymerase, and digestion with *Mlu*I. The pEX18Tc-containing DNA was purified free of the *Sst*I-*Mlu*I fragment, treated with Klenow fragment and ligated to yield pRSP75. These pEX18Tc-derived plasmids were mobilized from *E. coli* S17-1 into *P. aeruginosa*

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Source or reference
E. coli		
5K	*thr lacZ rpsL thi ser hsdR hsdM*	16
S17-1	*thi pro hsdR recA* Tra^+	19
P. aeruginosa		
K767	PAO1 prototroph	8
OCR1	*nalB*-type MDR mutant of K767	8
K1454 to K1461	MexAB-OprM hyperexpressing MDR mutants of K767	This study
H103	PAO1 prototroph	R. E. W. Hancock, University of British Columbia, Canada
K1462 to K1468	MexAB-OprM hyperexpressing MDR mutants of H103	This study
K1482	K767 carrying a chromosomal ΩHg insertion in the *Sst*I site of *mexR*	This study
K1483	K1454 (*nalC*) carrying a chromosomal ΩHg insertion in the *Sst*I site of *mexR*	This study
K1485	K767 carrying a chromosomal ΩHg insertion in the *Mlu*I site of *mexR*	This study
K1486	K1454 (*nalC*) carrying a chromosomal ΩHg insertion in the *Mlu*I site of *mexR*	This study
K1488	K767 carrying a chromosomal ΩHg insertion in the *Tth*111I site of ORF2	This study
K1489	K1454 (*nalC*) carrying a chromosomal ΩHg insertion in the *Tth*111I site of ORF2	This study
K1491	K767 Δ*mexR*; in-frame deletion	This study
K1494	K767 carrying a chromosomal *mexA-phoA* fusion	This study
K1495	K1454 (*nalC*) carrying a chromosomal a *mexA-phoA* fusion	This study
K1496	K1455 (*nalB*) carrying a chromosomal a *mexA-phoA* fusion	This study
K1497	K1482 (*mexR*::ΩHg [*Sst*I]) carrying a chromosomal a *mexA-phoA* fusion	This study
K1498	K1483 (*nalC* + *mexR*::ΩHg [*Sst*I]) carrying a chromosomal a *mexA-phoA* fusion	This study
K1500	K1485 (*mexR*::ΩHg [*Mlu*I]) carrying a chromosomal a *mexA-phoA* fusion	This study
K1501	K1486 (*nalC* + *mexR*::ΩHg [*Mlu*I]) carrying a chromosomal a *mexA-phoA* fusion	This study
Plasmids		
pRK415	Broad-host-range cloning vector; p*lac* MCS, Tc^r	5
pMMB206	Broad-host-range cloning vector; *lacI* p*lac* MCS, Cm^r	11
pEX18Tc	Gene replacement vector; Mob^+ *sacB* Tc^r	H. Schweizer, Colorado State University
pMP190	Broad-host-range, low-copy-number *lacZ* fusion vector; Cm^r Sm^r	20
pHP45ΩHg	pHP45Ω derivative carrying the $HgCl_2$ resistance operon of Tn*501*	2
pMXR5	pMP190 derivative carrying the *mexR-mexA* intergenic region, with the *mexA* promoter oriented toward the promoterless *lacZ* gene	17
pPV2	pAK1900 derivative carrying *mexAB*, *mexR* and ORF2 on a 7.7-kb *Xho*I fragment, Ap^r Cb^r	17
pMXA1	pSUP202 derivative carrying a *mexA-phoA* fusion, Tc^r	17
pRSP55	pRK415::$mexR_{K767}$ (i.e., *mexR* from K767)	This study
pRSP56	pRK415::$mexR_{K1454}$	This study
pRSP58	pRK415::$mexR_{K1455}$	This study
pRSP60	pRK415::$mexR_{K1456}$	This study
pRSP64	pEX18Tc derivative carrying *mexR* on a 3.4-kb *Eco*RI-*Sma*I fragment	This study
pRSP65	pRSP64::ΩHg; ΩHg insertion in the unique *Sst*I site of *mexR*	This study
pRSP67	pMMB206::ORF2; ORF2 in the same orientation as p*lac*	This study
pRSP70	pRSP64::ΩHg; ΩHg insertion in the unique *Mlu*I site of *mexR*	This study
pRSP72	pRSP64::ΩHg; ΩHg insertion in the unique *Tth*111I site of ORF2	This study
pRSP75	pEX18Tc::Δ*mexR*; carries a 261-bp *Sst*I-*Mlu*I deletion	This study
pRSP83	pRK415 derivative carrying the *mexR*::ΩHg (*Sst*I) gene of pRSP65 on a 5.5-kb *Pst*I fragment	This study

[a] MCS, multiple cloning site; Tc^r, tetracycline resistant; Cm^r, chloramphenicol resistant; Sm^r, streptomycin resistant; Ap^r, ampicillin resistant; Cb^r, carbenicillin resistant; ΩHg, mercury resistant Ω interposon.

(14), and transconjugants carrying the plasmids in the chromosome were selected on L-agar containing tetracycline. *mexR*::ΩHg mutants were then selected on L-agar containing 10% (wt/vol) sucrose and $HgCl_2$, while strains harboring the *mexR* deletion were selected on 10% (wt/vol) sucrose and screened for the *mexR* deletion by PCR.

Restriction digests, ligations, and transformations were carried out as described previously (18). Plasmid DNA was isolated with the aid of a plasmid Maxi Kit (Qiagen). DNA fragments used in cloning were purified from agarose gels with Prep-A-Gene (Bio-Rad). pRK415-, pMMB206-, and pMP190-derived vectors were introduced into *P. aeruginosa* from *E. coli* by triparental mating (22, 23). Cell envelopes were isolated as described previously (21) and resolved on sodium dodecyl sulfate-polyacrylamide gels (10% [wt/vol]). Gels were Coomassie blue stained or else electroblotted and developed with anti-MexB (21) or anti-OprM (23) antibodies. The antibiotic susceptibility of *P. aeruginosa* strains was assessed by using the broth dilution assay (7). β-Galactosidase assays were carried out as described elsewhere (17). The *mexA-phoA* fusion plasmid, pMXA1, was introduced into the chromosome of *P. aeruginosa* strains via conjugation from *E. coli* S17-1 as described previously (17). Fusion-containing strains were grown to an A_{600} of 1 in L-broth, concentrated twofold in 0.1 M Tris-HCl (pH 8.0) and assayed for alkaline phosphatase activity as described earlier (17).

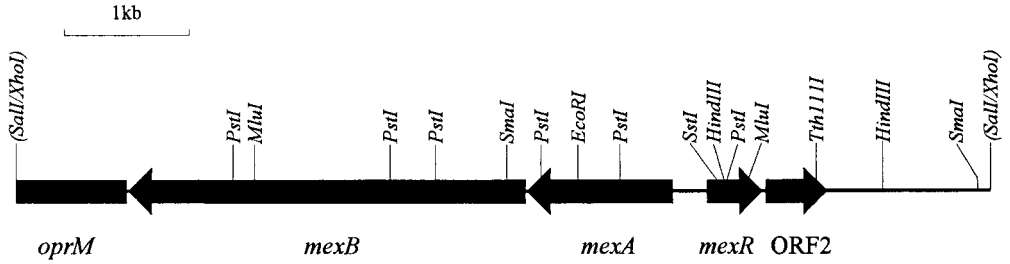

FIG. 1. Restriction map of the *mexRAB-oprM* locus in plasmid pPV2. The *Sal*I-*Xho*I junction that is not digested by either enzyme is within parenthesis.

Differential MexAB-OprM hyperexpression in *nalB* and *nalC* MDR strains. Many *P. aeruginosa* strains selected on ciprofloxacin and cefoperazone exhibited a MDR profile characteristic of the MexAB-OprM hyperexpressing *nalB* strain OCR1 (17) (Table 2). Western immunoblotting with anti-MexB (Fig. 2A) and anti-OprM (data not shown) demonstrated that the MDR mutants (eight derived from PAO1 strain K767 and seven derived from PAO1 strain H103) hyperexpressed the MexAB-OprM efflux components. MexB production in many of the mutants (Fig. 2A) was comparable to that of OCR1 (Fig. 2A, lane 10). In some, however, MexB production, though elevated, was reduced relative to this *nalB* strain (Fig. 2A). The antibiotic resistance of the MDR strains reflected these differences in MexB levels, with those strains producing less MexB consistently twofold more susceptible to several antibiotics (Table 2).

MexAB-OprM hyperexpression in a variety of *nalB* strains correlates with a mutation in *mexR* (4, 17, 24). The nucleotide sequences of the *mexR* genes from K767 and H103 were identical to the previously published *mexR* sequence (17), and while several of the MDR mutants did carry mutations in *mexR*, others did not (Table 3). These latter mutants, which also lacked mutations in the *mexR-mexA* intergenic region, expressed reduced levels of MexAB-OprM and were less resistant than those MDR strains with *mexR* mutations (Fig. 2A; Table 2). Ziha-Zarifi et al. (24) described a single example of a clinical strain of *P. aeruginosa* hyperexpressing MexAB-OprM also lacking a mutation in *mexR*. To distinguish these from the MexAB-OprM hyperexpressing mutants that carry *mexR* mutations (*nalB*-type), we propose the term *nalC* for these mutants.

The observation that *nalC* strains express reduced levels of MexAB-OprM relative to *nalB* strains contrasts with a previous report (24), although OprM, and not MexB, levels were assessed as a marker of MexAB-OprM expression. Indeed, we also observed no differences in OprM levels between *nalC* and *nalB* strains (data not shown). Since OprM can be expressed and function independently of MexAB (23), a finding consistent with its role as the outer membrane component of additional MDR efflux systems in *P. aeruginosa* (e.g., MexXY [1, 10]), MexB may be a more accurate marker of MexAB-OprM production. Certainly, the antibiotic susceptibility data is consistent with *nalC* strains expressing less MexAB-OprM than the *nalB* strains.

MexR functions solely as a repressor of the *mexAB-oprM* operon. The *mexR* mutations identified in the aforementioned

TABLE 2. Antibiotic susceptibilities of *P. aeruginosa* strains carrying mutations in *mexR*

Strain	Relevant genotype	MIC[a] (μg/ml) of:			
		CAR	CEF	CAM	TET
K767	Wild type	128	2	64	16
OCR1	*nalB*	1,024	8	512	64
K1454[b]	*nalC*	512	4	256	32
K1455[c]	*nalB*	1,024	8	512	64
H103	Wild type	64	2	32	16
K1464[d]	*nalB*	512	8	256	64
K1466[e]	*nalC*	256	4	128	32
K1482	K767 *mexR*::ΩHg (*Sst*I)[f]	256	4	128	32
K1483	K1454 *mexR*::ΩHg (*Sst*I)	256	4	128	32
K1485	K767 *mexR*::ΩHg (*Mlu*I)	1,024	8	512	64
K1486	K1454 *mexR*::ΩHg (*Mlu*I)	1,024	8	512	64
K1488	K767 ORF2::ΩHg (*Tth*111I)	128	2	64	16
K1491	K767 Δ*mexR* (*Sst*I-*Mlu*I)	1,024	8	512	64

[a] CAR, carbenicillin; CEF, cefepime; CAM, chloramphenicol; TET, tetracycline.
[b] K1459, K1460 and K1489 displayed the same MIC values as K1454.
[c] K1456, K1457, K1458, and K1461 displayed the same MIC values as K1455.
[d] K1462, K1463, and K1465 displayed the same MIC values as K1464.
[e] K1467 and K1468 displayed the same MIC values as K1466.
[f] Restriction sites in parenthesis denote either the position of insertion of the Ω interposon (mercury resistant) or the restriction sites that were used to construct defined deletions.

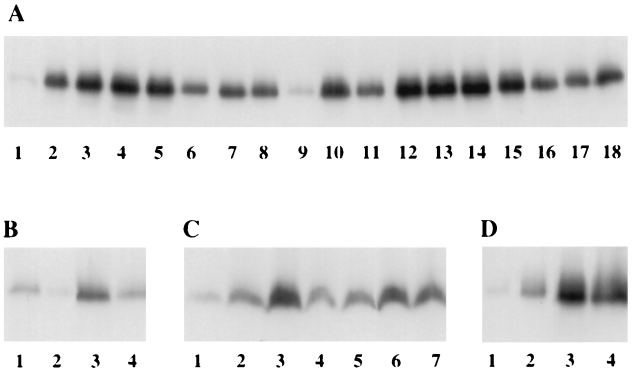

FIG. 2. Expression of MexB in MexAB-OprM hyperexpressing strains of *P. aeruginosa* (A), strains carrying the cloned *mexR* gene (B), *mexR*::ΩHg insertion mutants (C), and *mexR* deletion strains (D). Cell envelopes (10 μg of protein) were subjected to Western immunoblotting with antibodies raised against MexB. (A) Lane 1, H103 (wild type); lane 2, K1462 (*nalB*); lane 3, K1463 (*nalB*); lane 4, K1464 (*nalB*); lane 5, K1465 (*nalB*); lane 6, K1466 (*nalC*); lane 7, K1467 (*nalC*); lane 8, K1468 (*nalC*); lane 9, K767 (wild type); lane 10, OCR1 (*nalB*); lane 11, K1454 (*nalC*); lane 12, K1455 (*nalB*); lane 13, K1456 (*nalB*); lane 14, K1457 (*nalB*); lane 15, K1458 (*nalB*); lane 16, K1459 (*nalC*); lane 17, K1460 (*nalC*); and lane 18, K1461 (*nalB*). (B) Lane 1, K767 carrying pRK415; lane 2, K767 carrying the *mexR* plasmid pRSP55; lane 3, K1482 (*mexR*::ΩHg [*Sst*I]) carrying pRK415; and lane 4, K1482 carrying pRSP55. (C) Lane 1, K767; lane 2, K1454 (*nalC*); lane 3, K1455 (*nalB*); lane 4, K1482 (*mexR*::ΩHg [*Sst*I]); lane 5, K1483 (*nalC* + *mexR*::ΩHg [*Sst*I]); lane 6, K1485 (*mexR*::ΩHg [*Mlu*I]); and lane 7, K1486 (*nalC* + *mexR*::ΩHg [*Mlu*I]). (D) Lane 1, K767 (wild type); lane 2, K904 (*mexR*::ΩHg [*Sst*I]); lane 3, K1455 (*nalB*); and lane 4, K1491 (Δ*mexR*).

TABLE 3. Characteristics of MexAB-OprM hyperexpressing multidrug resistant mutants

Strain	*mexR* mutation[a]	MexR aa[b] change	MexB expression[c]
K767	Wild type		+
OCR1	C_{208} to T	Arg_{70} to Trp	++++
K1454	Wild type		+++
K1455	A_{388} to C	Thr_{130} to Pro	++++
K1456	Deletion from C_{294} to G_{300}	–[d]	++++
K1457	Insertion of T after T_{203}	–	++++
K1458	Deletion from C_{261} to G_{272}	Deletion from Ser_{88} to Arg_{91}	++++
K1459	Wild type		+++
K1460	Wild type		+++
K1461	A_{281} to C	Gln_{94} to Pro	++++
H103	Wild type		+
K1462	T_{170} to G	Leu_{57} to Arg	++++
K1463	A_{388} to C	Thr_{130} to Pro	++++
K1464	Insertion of T after A_{80}	–	++++
K1465	Deletion from T_{71} to A_{425}	–	++++
K1466	Wild type		+++
K1467	Wild type		+++
K1468	Wild type		+++

[a] Changes to the nucleotide (A, G, C, or T) sequence of *mexR* are shown. The numbers in subscript denote the positions of the nucleotides with reference to the A_1TG start codon of *mexR*.

[b] Changes to the amino acid (aa; three-letter code) sequence of MexR are shown. The numbers in subscript denote the positions of the amino acids with reference to the f-Met_1 of MexR.

[c] Levels of MexB expression are reported qualitatively as determined by visual assessment of Western immunoblots of cell envelopes developed with anti-MexB antibodies.

[d] –, Sequence out of frame; full-length MexR not made.

nalB strains included base substitutions, insertions and deletions, although none of these were described previously (see OCR1, [Table 3] and references 4 and 24), and only one mutation was recovered more than once (i.e., T130P) (Table 3). One mutant, K1465, had 80% of its *mexR* sequence deleted while another, K1464, had a frameshift early in the *mexR* sequence (Table 3). That these expressed levels of MexAB-OprM indistinguishable from that of *mexR* point mutants (Fig. 2A, lanes 4 and 5, compare lanes 2 and 3) strongly suggested that *mexAB-oprM* hyperexpression in *nalB* strains, including those with base substitutions in *mexR*, results from loss of MexR (and its repressor activity). Thus, our previous suggestion that MexR is converted to an activator in *nalB* strains (17) seems unlikely. Consistent with this, the wild-type *mexR* genes of the prototrophic strain K767 (on plasmid pRSP55) and the *nalC* strain K1454 (on plasmid pRSP56; data not shown) reduced expression of a plasmid-borne *mexA-lacZ* fusion (pMXR5) in *E. coli* (from 782 ± 28 Miller units in the absence of the *mexR* plasmid to 108 ± 9 Miller units in the presence of pRSP55), while the mutated *mexR* genes of *nalB* strains K1455 (on plasmid pRSP58) and K1456 (on plasmid pRSP60) had no effect on the *mexA-lacZ* expression (pRSP58, 794 ± 66 Miller units; pRSP60, 609 ± 22 Miller units). Similarly, the wild-type *mexR* gene repressed *mexA-lacZ* expression (data not shown) and MexB production (Fig. 2B, lanes 2 and 4, compare lanes 1 and 3) in *P. aeruginosa*, while the mutated *mexR* genes had no effect on *mexA-lacZ* expression (data not shown). The *mexR* mutations, thus, obviated the repressor activity of MexR without converting it into an activator of *mexAB-oprM* expression.

***mexR cis* effects on *mexAB-oprM* expression.** Disruption of the 5′ end of *mexR* (at the *Sst*I site [27th codon]) (Fig. 1) with a ΩHg cartridge did not enhance *mexAB-oprM* expression and antibiotic resistance to the extent seen in *nalB* strains (17), a result reproduced here (see K1482) (Fig. 2C, lane 4 [see lane 3]; Table 2). One explanation is that the ΩHg insertion exerted a polar effect on a downstream gene(s) required for *mexAB-oprM* hyperexpression and, perhaps, the target of mutation in *nalC* mutants. Although an ORF was identified downstream of *mexR* in strain K767 (ORF2, Fig. 1), no sequence changes were observed in ORF2 of the *nalC* strain K1454. Moreover, the cloned ORF2 failed to restore MexAB-OprM expression in the *mexR*::ΩHg (*Sst*I) derivative of a *nalC* strain (e.g., K1483) to levels seen for the original *nalB* strains (data not shown), and disruption of ORF2 by insertion of the ΩHg cartridge at the *Tth*111I site (Fig. 1) in wild-type strain K767 (yielding K1488; Table 2) or *nalC* strain K1454 (yielding K1489; data not shown) did not alter their drug resistance properties. Finally, disruption of *mexR* with an ΩHg at the *Mlu*I site (codon 114; Fig. 1) in strain K767 (yielding K1485) produced an MDR phenotype reminiscent of *nalB* strains (e.g. OCR1; Table 2) and levels of MexB (as a marker of MexAB-OprM expression) which were higher, like other *nalB* strains, than that seen in the *mexR*::ΩHg (*Sst*I) mutant K1482 (Fig. 2C, compare lanes 4 and 6). Thus, ORF2 is not involved in *mexAB-oprM* expression, and disruption of *mexR* alone is sufficient to produce a *nalB* phenotype.

The differential effect of ΩHg insertions at the *Sst*I and *Mlu*I on MexAB-OprM production was reflected in the expression of the efflux genes, as assessed by using chromosomal *mexA-phoA* fusions. Disruption of *mexR* at the *Sst*I site (see K1497) yielded a modest ca. twofold increase in expression from the *mexA* promoter, while disruption of this gene at the *Mlu*I site (K1500) produced a fourfold increase in efflux gene expression (Table 4). This suggested a possible *cis* effect of the *Sst*I::ΩHg insertion on *mexAB-oprM* expression, perhaps because sequences beyond the *Sst*I site in *mexR* were needed for full *mexAB-oprM* promoter activity. This was consistent with an earlier observation that a *mexR*::ΩHg (*Sst*I) mutant and a *nalB* strain showed comparably elevated expression of a plasmid-borne *mexA-lacZ* fusion (data not shown), while expression of a chromosomal *mexA-phoA* fusion was reduced in the *Sst*I insertion mutant relative to the *nalB* strain (17). Still, deletion of the *Sst*I-*Mlu*I fragment from *mexR* (in strain K1491) produced a phenotype indistinguishable from that of a *nalB* strain (Fig. 2D, compare lanes 3 and 4) or the *mexR*::ΩHg (*Mlu*I) mutant (Table 3). Although it was possible that a MexR pep-

TABLE 4. Expression of chromosomal *mexA-phoA* fusion in *P. aeruginosa*[a]

Strain	Relevant genotype	Alkaline phosphatase activity (A_{405}/A_{600})[b]
K1494	Wild type	0.54 ± 0.06
K1495	*nalC*	1.15 ± 0.05
K1496	*nalB*	2.26 ± 0.09
K1497	*mexR*::ΩHg (*Sst*I)[c]	0.80 ± 0.05
K1498	*nalC* + *mexR*::ΩHg (*Sst*I)	0.81 ± 0.05
K1500	*mexR*::ΩHg (*Mlu*I)	2.26 ± 0.08
K1501	*nalC* + *mexR*::ΩHg (*Mlu*I)	1.59 ± 0.09

[a] *P. aeruginosa* strains carrying a *mexA-phoA* chromosomal fusion were grown to A_{600} of 1 in L-broth and assayed for alkaline phosphatase activity.

[b] Activity is reported as the amount of *p*-nitrophenyl released from *p*-nitrophenyl phosphate, measured at A_{405}, as a function of the amount of cell material used in the assay, measured at A_{600}. The data are reported as the means of three determinations ± the standard deviations and are representative of three repetitions.

[c] As described in Table 3.

tide resulting from the ΩHg insertion at the *Sst*I site had partial repressor activity, the cloned *mexR* (*Sst*I)::ΩHg fragment from K1482 (pRSP83) did not repress MexAB-OprM expression *in trans* (data not shown). Thus, while the increase in MexAB-OprM and antibiotic resistance seen in strain K1482 (K767 *mexR*::ΩHg [*Sst*I]) likely results from loss of MexR and subsequent derepression of *mexAB-oprM*, failure to see expression at the level typical of *nalB* or *mexR*::ΩHg (*Mlu*I) insertion mutants apparently results from a negative impact of the *Sst*I insertion on *mexA*(*B-oprM*) promoter activity. Perhaps the ΩHg element altered the topology of the DNA at the 5′ end of *mexR* and this region is important for *mexAB-oprM* expression. It is unclear, however, whether this reflects involvement of additional regulator(s) or the need for a specific DNA conformation (for full promoter activity) which requires sequences in the vicinity of *mexR*. The ΩHg insertion at the *Sst*I site of *mexR* in a *nalC* strain (K1483) increased its susceptibility to antibiotics (Table 2) and reduced expression of MexB (Fig. 2C, lane 5) and a *mexA-phoA* fusion (see K1498, Table 4), suggesting that the 5′ region of *mexR* is important for *mexAB-oprM* hyperexpression associated with the *nalC* mutation. We are currently delineating the region required for optimal *mexAB-oprM* expression.

Interestingly, a ΩHg insertion at the *Mlu*I site of *mexR* in the *nalC* strain K1454 (yielding K1486) also caused an increase in antibiotic resistance (Table 2) and MexB expression (Fig. 2C, lane 7), though the latter was less than that seen for a *nalB* strain (K1455; Fig. 2C, lane 3) or a *mexR*::ΩHg (*Mlu*I) derivative of strain K767 (K1485; Fig. 2C, lane 6). This difference in expression was also observed when *mexA-phoA* fusions were employed (Table 4). Thus, while a *nalC* mutation afforded increased MexAB-OprM production and MDR, it also compromised full expression of this efflux system when *mexR* was inactivated. This suggests some interplay between *mexR* and *nalC* in the regulation of *mexAB-oprM*.

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