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Expression of the Multidrug Resistance Operon *mexA-mexB-oprM* in *Pseudomonas aeruginosa*: *mexR* Encodes a Regulator of Operon Expression

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The region upstream of the multiple antibiotic resistance efflux operon *mexA-mexB-oprM* in *Pseudomonas aeruginosa* was sequenced, and a gene, *mexR*, was identified. The predicted MexR product contains 147 amino acids with a molecular mass of 16,964 Da, which is consistent with the observed size of the overexpressed *mexR* gene product. MexR was homologous to MarR, the repressor of MarA-dependent multidrug resistance in *Escherichia coli*, and other repressors of the MarR family. A *mexR* knockout mutant showed a twofold increase in expression of both plasmid-borne and chromosomal *mexA*-reporter gene fusions compared with the MexR$^+$ parent strain, indicating that the *mexR* gene product negatively regulates expression of the *mexA-mexB-oprM* operon. Furthermore, the cloned *mexR* gene product reduced expression of a plasmid-borne *mexA-lacZ* fusion in *E. coli*, indicating that MexR represses *mexA-mexB-oprM* expression directly. Consistent with the increased expression of the efflux operon in the *mexR* mutant, the mutant showed an increase (relative to its MexR$^+$ parent) in resistance to several antimicrobial agents. Expression of a *mexR-lacZ* fusion increased threefold in a *mexR* knockout mutant, indicating that *mexR* is negatively autoregulated. OCR1, a *nalB* multidrug-resistant mutant which overproduces OprM, exhibited a greater than sevenfold increase in expression of a chromosomal *mexA-phoA* fusion compared with its parent. Introduction of a *mexR* knockout mutation in strain OCR1 eliminated this increase in efflux gene expression and, as expected, increased the susceptibility of the strain to a variety of antibiotics. The nucleotide sequences of the *mexR* genes of OCR1 and its parental strain revealed a single base substitution in the former which would cause a predicted substitution of Trp for Arg at position 69 of its *mexR* product. These data suggest that MexR possesses both repressor and activator function in vivo, the activator form being favored in *nalB* multidrug-resistant strains.

The phenomenon of bacterial multidrug resistance is well reported in the literature (see reference 38 and references cited therein). In many instances, such resistance has been attributed to efflux systems exhibiting broad substrate specificity (for reviews, see references 18, 33, and 38). In *Pseudomonas aeruginosa*, multidrug resistance is an intrinsic feature of the organism, although instances of acquired elevated multidrug resistance are increasingly common (3, 17, 29, 46, 47, 51). The contribution of efflux to intrinsic and acquired multidrug resistance in *P. aeruginosa* has been demonstrated (19–21), and an operon, previously called *mexA-mexB-oprK*, was described which contributes to this process (21, 42). Although previously identified as the product of the third gene of this operon, the OprK protein overproduced in the multiply resistant strain K385 (42) is now known to be identical to OprJ (40), an outer membrane protein overproduced in *nfxB* multidrug-resistant mutants (30) and the product of the third gene of the newly described *mexC-mexD-oprJ* operon (40). Recent studies have confirmed that OprM, the outer membrane protein overproduced in *nalB* multidrug-resistant mutants (29, 30), is the product of the third gene of the *mexA-mexB-oprK* operon (12), which has been appropriately renamed *mexA-mexB-oprM.*

Localized to the cytoplasmic (MexA, MexB) (41) and outer (OprM) (11) membranes, the products of the *mexA-mexB-oprM* operon function as a non-ATPase efflux pump with broad substrate specificity (21). A member of the so-called RND (for resistance, nodulation, and cell division) family of exporters (49), MexB apparently functions in the proton motive force-driven efflux of antibiotics across the cytoplasmic membrane (21, 32). MexA, a cytoplasmic-membrane-associated lipoprotein (6) and a member of the membrane fusion protein (MFP) family (49), is proposed to link MexB to the outer membrane porin-like OprM, thereby facilitating one-step efflux of drugs out of the cell (26). The MexA-MexB-OprM proteins are highly homologous to the products of the *acrAB* (previously called *acrAE*) (24, 26, 58a) and *acrEF* (previously called *envCD*) (16, 26) loci of *Escherichia coli* and the *mtrCDE* locus of *Neisseria gonorrhoeae* (13), as well as the recently described *mexC-mexD-oprJ* products (40). An OprM homolog, OpcM, has also been identified in *Burkholderia cepacia*; it is apparently part of a multidrug resistance system likely to be similar to MexA-MexB-OprM (7). The *mtr* locus is predicted to facilitate gonococcal resistance to the antimicrobial effects of certain fatty acids and bile salts bathing mucosal surfaces (53), a function also attributed to the stress- and stationary phase-inducible *acrAB* locus (25). Interestingly, AcrAB also plays a major role in the multiple antibiotic resistance phenotype of *E. coli* Mar mutants (34). Despite suggestions of a role for MexA-MexB-OprM in siderophore export (41), this now appears unlikely, and the pump may play a more general role in the export of secondary metabolites (38).

Expression of the *mtr* genes is regulated by the product of a gene, *mtrR*, located upstream of *mtrCDE* (14, 35). Mutations within the coding or promoter regions of *mtrR*, a putative repressor gene, as well as *mtrR* deletion mutations cause an increase in drug resistance, concomitant with an increase in expression of the efflux genes (13, 14). Genes encoding MtrR

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Source or reference
Strains		
P. aeruginosa		
K199	PAO1 wild type	R. E. W. Hancock, University of British Columbia
K335	Spontaneous streptomycin-resistant derivative of K199	Laboratory strain
K831	K335 carrying a chromosomal *mexA-phoA* fusion	This study
K854	K335 *mexR*::ΩHg (MexR$^-$)	This study
K863	K854 carrying a chromosomal *mexA-phoA* fusion	This study
K870	Spontaneous streptomycin-resistant derivative of K767	This study
K904	K870 *mexR*::ΩHg (MexR$^-$)	This study
K767	PAO1 wild type	N. Gotoh, Kyoto Pharmaceutical University
OCR1	*nalB*-type multidrug-resistant derivative of K767	22
K784	Spontaneous streptomycin-resistant derivative of OCR1	This study
K832	K784 carrying a chromosomal *mexA-phoA* fusion	This study
K855	K784 *mexR*::ΩHg (MexR$^-$)	This study
K864	K855 carrying a chromosomal *mexA-phoA* fusion	This study
K372	*met-9011 amiE200 rpsL pvd-9 pchR*	15
K758	K372 *mexR*::ΩHg (MexR$^-$)	This study
E. coli		
5K	*thr lacZ rpsL thi ser hsdR hsdM*	44
S17-1	*thi pro hsdR recA* Tra$^+$	54
Plasmids		
pMP190	Broad-host-range, low-copy-number *lacZ* fusion vector; Cam^r Sm^r	55
pAK1900	*E. coli-P. aeruginosa* shuttle cloning vector; Ap^r Cb^r	R. Sharp, Department of Microbiology and Immunology, Queen's University
pSUP202	pBR325 derivative carrying the Mob (mobilization) site of plasmid RP4; Ap^r Tc^r Cam^r	54
pQF60	*E. coli-P. aeruginosa* shuttle *phoA* fusion vector; Ap^r Cb^r	9
pHP45ΩHg	Derivative of pHP45:Ω in which Sm^r-Spc^r of the Ω interposon is replaced by the $HgCl_2$ resistance operon of Tn*501*	10
pT7-6	pBR322 derivative carrying an MCS downstream of the strong gene 10 promoter of phage T7; Ap^r	57
pORFA-*pho*	pQF60 derivative carrying the 5′ upstream regions of the divergent *mexA* (referred to previously as ORFA) and *mexR* genes on a 944-bp *Sal*I-*Sac*I fragment cloned upstream of the promoterless *phoA* gene	41
pMXR1	pAK1900 derivative carrying the *mexR* gene on a ca. 3.5-kb *Sal*I fragment	This study
pMXR2	pAK1900 derivative carrying a truncated *mexR* gene on a ca. 2.5-kb *Sal*I-*Sac*I fragment	This study
pMXR3	pAK1900 derivative carrying the *mexR* gene on a ca. 1.1-kb *Fsp*I-*Nru*I fragment	This study
pMXR4	pMP190 derivative carrying the *mexR-mexA* intergenic region on a 944-bp *Sal*I fragment derived from pORFA-*pho*, with the *mexR* promoter oriented towards the promoterless *lacZ* gene	This study
pMXR5	As for pMXR4 except that the *mexA* promoter is oriented towards the promoterless *lacZ* gene	This study
pMXR6	Derivative of pT7-6 carrying the *mexR* gene on a 1.1-kb *Fsp*I-*Nru*I fragment cloned downstream of and in the same orientation as the resident T7 promoter	This study
pMXA1	pSUP202 derivative carrying a *mexA-phoA* fusion liberated from plasmid pORFA-*pho* as a ca. 5-kb *Nde*I-*Sca*I fragment	This study

[a] Ap^r, ampicillin resistant; Cb^r, carbenicillin resistant; Cam^r, chloramphenicol resistant; Tc^r, tetracycline resistant; Sm^r, streptomycin resistant; Spc^r, spectinomycin resistant; MCS, multiple cloning site; *mexR*::ΩHg, *mexR* gene carrying an insertion of the mercury resistance ΩHg interposon.

homologs have been identified upstream of *acrAB* (*acrR*) and *acrEF* (*acrS*, also called *envR*) (35). AcrR, also a repressor, appears to play a modulating role in the regulation of *acrAB* gene expression by global stress signals (23). To determine if a similar gene was to be found upstream of the *mexA-mexB-oprM* operon, this upstream region was sequenced. We report here a gene, *mexR*, which regulates expression of the *mexA-mexB-oprM* operon.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are described in Table 1. In the construction of plasmids pMXR3 and pMXR4 (Fig. 1), the *mexA-mexR* intergenic region was excised from pORFA-*pho* on a ca. 1-kb *Sal*I fragment and cloned in both orientations into the *lacZ* fusion vector pMP190. This fragment encompasses the 950-bp *Sal*I-*Sac*I fragment of pPV2 (Fig. 1), which had previously been cloned into pQF60 to generate pORFA-*pho* (41). Spontaneous streptomycin-resistant derivatives of *P. aeruginosa* were isolated by harvesting 5 ml of an overnight L broth culture and plating the pellet (resuspended in 100 μl) on L agar containing 1,000 μg of streptomycin per ml. Distinct colonies appearing after 24 to 48 h of incubation at 37°C were recovered.

Growth media. L broth (45) and brain heart infusion (BHI) broth (Difco) were employed as the rich media throughout. The iron-deficient succinate minimal medium has been described previously (43). Methionine (1 mM) was added to minimal medium as required. Chloramphenicol (*P. aeruginosa*, 100 [broth] or 200 [agar] μg/ml; *E. coli*, 30 μg/ml), carbenicillin (200 μg/ml), ampicillin (100 μg/ml), streptomycin (500 μg/ml), and $HgCl_2$ (15 μg/ml) were included in growth media

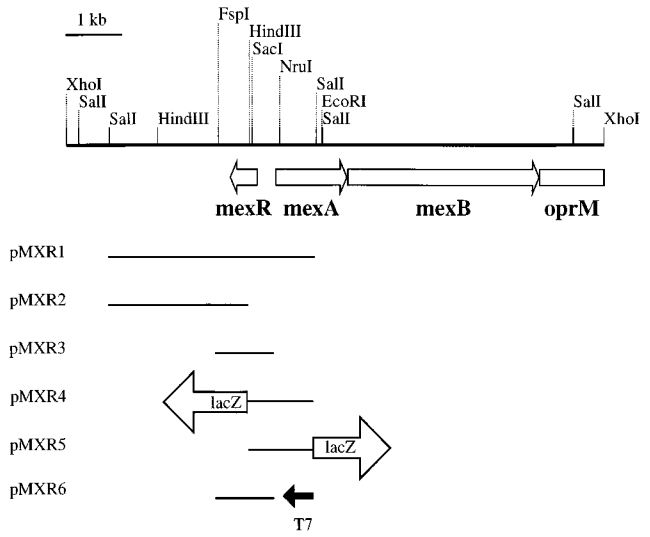

FIG. 1. Physical map of the *mexRAB-oprM* locus in plasmid pPV2 (42). Restriction fragments cloned into plasmid pAK1900 (pMXR1 to pMXR3), pMP190 (pMXR4 and pMXR5), and pT7-6 (pMXR6) are highlighted. The orientations of the promoterless *lacZ* gene in pMXR4 and pMXR5 are indicated by large arrows, and the orientation of the strong T7 promoter in pMXR-6 relative to the indicated restriction fragments is indicated by a black arrow.

as necessary. Solid media were obtained by the addition of agar (BDH; 1.5% [wt/vol]).

DNA methodology. Plasmid DNA was prepared from *E. coli* and *P. aeruginosa* by the alkaline lysis procedure (50). For sequencing purposes, however, such DNA was purified on CsCl gradients (50) or by using the Magic/Wizard Miniprep System (Promega, Madison, Wis.). *P. aeruginosa* genomic DNA for use in PCR was prepared as described previously (4). Transformation of *E. coli* (51) and *P. aeruginosa* (5) with plasmid DNA has been described elsewhere. Restriction endonucleases and T4 DNA ligase were obtained from Gibco-BRL or New England Biolabs and used according to the manufacturer's instructions or as described previously (50). The Klenow fragment of DNA polymerase I was obtained from Pharmacia-LKB and used to fill in 5′ extensions of restricted DNA (50). Restriction fragments were isolated, as required, from agarose gels (0.8% [wt/vol]) by using the Prep-a-Gene glass matrix (Bio-Rad, Mississauga, Ontario) in accordance with a protocol supplied by the manufacturer. Cloning of DNA was carried out first in *E. coli* 5K prior to its introduction into *P. aeruginosa.*

PCR. Amplification of the *mexR* genes of selected *P. aeruginosa* strains was achieved by PCR. For sequencing purposes, the genes were amplified with *Taq* DNA polymerase (Life Technologies Inc., Burlington, Ontario) and primers mexR1 (5′-GGCCGGAACCAGTACACG-3′), which anneals at the 3′ end of *mexA*, and mexR2 (5′-AATATCCTCAAGCGGTTGC-3′), which anneals at the 3′ end of *mexR*. The reaction mixture (100 μl) included 2.5 U of *Taq* DNA polymerase, 0.5 μM each primer, 0.2 mM each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 10% (vol/vol) dimethyl sulfoxide, 100 ng of genomic DNA, and 1× PCR buffer (Life Science Technologies). The mixture was treated for 2 min at 94°C, followed by 35 cycles of 45 s at 94°C, 1 min at 56°C, and 2 min at 72°C, before finishing with 10 min at 72°C. PCR products were examined on 0.8% (wt/vol) agarose gels and purified with the QIAquick-spin PCR purification kit (Qiagen, Inc., Chatsworth, Calif.).

Nucleotide sequencing. Plasmid DNA (double stranded) and PCR products were sequenced by the Centres of Excellence Core Facility for Protein and DNA Chemistry at Queen's University. Overlapping sequences of the cloned, plasmid-borne *mexR* gene were obtained from both strands by using custom-synthesized primers, while the amplification primers were used to sequence PCR products. Nucleotide and deduced amino acid sequences were analyzed with the PC Gene software package (Intelligenetics Inc., Mountain View, Calif.).

In vitro mutagenesis and gene replacement. A 3.5-kb *Sal*I fragment encompassing *mexR* (Fig. 1) was cloned into the unique *Sal*I site in plasmid pSUP202. The resultant recombinant plasmid was linearized with *Sac*I (which cuts within *mexR* and nowhere else in the vector or the 3.5-kb *Sal*I fragment), and the resulting 5′ extensions were filled in with Klenow fragment (see above). An *Sma*I fragment of pHP45ΩHg carrying the mercury resistance interposon ΩHg was ligated to the aforementioned DNA and used to transform *E. coli* 5K. $HgCl_2$- and tetracycline-resistant transformants were recovered and screened to confirm the insertion of ΩHg within *mexR*. Following transformation of *E. coli* S17-1 with *mexR*::ΩHg-containing pSUP202, the vector was mobilized into *P. aeruginosa* via conjugation as described previously (41). To permit counterselection of the donor *E. coli* strain in these experiments, either the recipient *P. aeruginosa* strains were streptomycin resistant or spontaneous streptomycin-resistant derivatives were first isolated as described above. Following conjugation, the mating mixture was plated on L agar containing both streptomycin and $HgCl_2$. Gene replacement *mexR*::ΩHg mutants were streptomycin and $HgCl_2$ resistant but had lost the plasmid-encoded carbenicillin resistance. Disruption of the chromosomal *mexR* gene was confirmed by PCR with the mexR1 and mexR2 primers and *Taq* DNA polymerase as described above.

β-Galactosidase assays. Bacteria harboring plasmid pMP190 (55) or its derivatives were cultured overnight at 37°C in BHI broth supplemented with chloramphenicol and subsequently diluted 100-fold into fresh BHI broth without chloramphenicol. Following growth to late log phase (A_{600}, 0.8 to 1.0), cultures were assayed for β-galactosidase activity as described previously (31).

Construction of chromosomal *mexA-phoA* fusions. The *mexA-phoA* fusion of plasmid pORFA-*pho* was excised as a ca. 5-kb *Nde*I-*Sca*I fragment, blunt ended (at the *Nde*I site) with Klenow fragment, and cloned into the gene replacement vector pSUP202 at the blunt-ended *Eco*RI site. The resultant vector, pMXA1, was then transformed into *E. coli* S17-1 and mobilized into *P. aeruginosa* via conjugation as described previously (41). Strains with a chromosomal insertion of pMXA1 were selected on tetracycline-containing L agar and tested for expression of the now chromosome-borne *mexA-phoA* fusion on L agar plates containing the chromogenic alkaline phosphatase substrate XP (250 μg/ml; Sigma Chemical Co., St. Louis, Mo.).

Alkaline phosphatase assays. *P. aeruginosa* strains carrying chromosomal or plasmid-borne (pORFA-*pho*) *mexA-phoA* fusions were assayed for alkaline phosphatase activity as described previously (39) with modifications. Strains were cultured overnight in L broth with (in the case of pORFA-*phoA*-containing strains) or without (in the case of chromosomal fusions) carbenicillin, and an aliquot (300 μl) was harvested by centrifugation in a microcentrifuge and resuspended in 600 μl of 0.1 M Tris-HCl (pH 8.0). Following permeabilization with sodium dodecyl sulfate and $CHCl_3$ (39), cells (500 μl) were transferred to fresh tubes containing an equal volume of *p*-nitrophenyl phosphate (2 mg/ml in 0.1 M Tris-HCl [pH 8.0]) and incubated at room temperature for 15 to 20 min, at which time the reaction was stopped by the addition of NaOH (0.1 N final concentration). Following centrifugation to remove cell debris, alkaline phosphatase-mediated release of *p*-nitrophenyl was measured at 405 nm and reported relative to the quantity of cells used in the assay (determined by measuring the A_{600} of the initial cell cultures).

Antimicrobial agent susceptibility testing. The susceptibility of *P. aeruginosa* to antimicrobial agents was determined by the broth dilution method as described previously (42). The MIC was defined as the lowest concentration inhibiting visible growth, as assessed following 18 h of incubation at 37°C.

Nucleotide sequence accession number. The *mexR* sequence has been deposited with Genbank under the accession number U23763.

RESULTS

Nucleotide sequence of the *mexR* gene. The *mexAB* genes and most of *oprM* were previously cloned on an 8.5-kb *Xho*I fragment on plasmid pPV2 (41) (Fig. 1). Sequencing of the region upstream of *mexA* revealed an open reading frame of 441 bp, called *mexR*, in the opposite orientation (Fig. 2) capable of encoding a polypeptide 147 amino acids in length with a predicted molecular mass of 16,964 Da. Consistent with this, overexpression of *mexR* with the phage T7-based expression system of Tabor and Richardson (57) yielded a product with a molecular mass of ca. 16 kDa (data not shown). Putative −10 and −35 promoter regions were identified upstream of *mexR*, although a consensus ribosome binding site (Shine-Dalgarno sequence) was not seen. There are at least three possible ATG start codons at the predicted 5′ end of *mexR*, although the largest open reading frame is likely to be correct, in light of the similarity between the largest deduced MexR product and other bacterial proteins (see below). A ca. 1-kb *Sal*I-*Sac*I fragment (Fig. 1), encompassing the region upstream of *mexR*, directed substantial *lacZ* expression when fused to a promoterless *lacZ* gene in plasmid pMP190 (Table 2; compare vectors pMXR4 and pMP190), consistent with there being an active promoter upstream of this gene. This confirmed that the indicated direction of transcription of *mexR* is opposite that of the *mexA-mexB-oprM* operon.

MexR shows similarity to MarR and other regulatory proteins. The deduced MexR sequence was compared with sequences present within the Genbank databases by using the BLAST algorithm (1). Interestingly, no homology was detected

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                <mexA
  1  CATAGCGTTGTCCTCATGAGCGAAAGCGGCCGATGCAGCGTGAAGGCGCTGCACCGGCTC   60
     MET
                    -10                     -35
 61  GAAGAAGGCAGGCAAAATACTTACATTCATAGGTGTTTGTAAACGTCCGAAAGCCTCGCG   120

121  TGAAAACACCTGAAACGTTTTAGCTCGATGGCCGGTTATCCACCCGGCGCCATGGCCCAT   180
              -35                     -10
181  ATTCAGAACCTGAAACAAGGTTGATAAGGTCAACTAAAATAAGCAAATAGTTGACTGGAT   240
                                      mexR>
241  CAACCACATTTACATTAGGTTTACTCGGCCAAACCAATGAACTACCCCGTGAATCCCGAC   300
                                     METAsnTyrProValAsnProAsp
                                                        SacI
301  CTGATGCCCGCGCTGATGGCGGTCTTCCAGCATGTGCGGACGCGCATCCAGAGCGAGCTC   360
     LeuMETProAlaLeuMETAlaValPheGlnHisValArgThrArgIleGlnSerGluLeu
                                                 HindIII
361  GATTGCCAGCGACTCGACCTGACCCCGCCCGACGTCCATGTATTGAAGCTTATCGACGAA   420
     AspCysGlnArgLeuAspLeuThrProProAspValHisValLeuLysLeuIleAspGlu

421  CAACGCGGGCTGAACCTGCAGGACCTGGGACGCCAGATGTGCCGCGACAAGGCACTGATC   480
     GlnArgGlyLeuAsnLeuGlnAspLeuGlyArgGlnMETCysArgAspLysAlaLeuIle
        T
481  ACCCGGAAGATCCGCGAGCTGGAGGGAAGAAACCTGGTCCGCCGCGAGCGCAACCCCAGC   540
     ThrArgLysIleArgGluLeuGluGlyArgAsnLeuValArgArgGluArgAsnProSer
        Trp
541  GACCAGCGCAGCTTCCAGCTCTTCCTCACCGACGAGGGGCTGGCCATCCACCAGCATGCG   600
     AspGlnArgSerPheGlnLeuPheLeuThrAspGluGlyLeuAlaIleHisGlnHisAla

601  GAGGCCATCATGTCACGCGTGCATGACGAGTTGTTTGCCCCGCTCACCCCGGTGGAACAG   660
     GluAlaIleMETSerArgValHisAspGluLeuPheAlaProLeuThrProValGluGln

661  GCCACCCTGGTGCATCTCCTCGACCAGTGCCTGGCCGCGCAACCGCTTGAGGATATTTAA   720
     AlaThrLeuValHisLeuLeuAspGlnCysLeuAlaAlaGlnProLeuGluAspIle---

721  GAACATTCTTTTCGAAG
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FIG. 2. Nucleotide sequence and translation of the *mexR* gene of *P. aeruginosa* PAO. The start sites of *mexR* and the previously described *mexA* (42, 43) are indicated along with their putative −10 and −35 promoter regions. Selected restriction sites are shown. The single base pair change in the *mexR* gene of the multidrug-resistant strain OCR1 and the corresponding change in the translated product are also highlighted.

between MexR and AcrR, AcrS (EnvR), or MtrR, although substantial similarity to MarR, the repressor of MarA-dependent multidrug resistance in *E. coli* (2), was detected. Alignment of the two sequences (MexR, 147 amino acids; MarR, 143 amino acids) revealed that there were 26.6% identical residues and 10.5% conserved changes (Fig. 3). The regions of similarity are concentrated somewhat within the C-terminal portion of the proteins, with 38.3% identity observed over the final 60 amino acid residues of MarR and 50% identity observed over the final 24 residues of this protein (Fig. 3). Several additional proteins, including MprA (identified initially as a repressor of the synthesis of Microcins B17 and C7 in *E. coli* [8] and now called EmrR, a repressor of the EmrAB multidrug resistance pump [22]), HpcR (the putative repressor of the *E. coli* C homoprotocatechuate degradation operon [48]), the *hpr* gene product (a repressor of protease production and sporulation in *Bacillus subtilis* [36]), PecS (47a), and, finally, a predicted 17-kDa polypeptide whose gene resides within the *papprs* gene clusters involved in pilin biosynthesis (27) showed varying degrees of homology to MexR. These proteins, of similar size, each contained a conserved region of 14 amino

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MEXR   MNYPVNP-DLMPALMAVFQHVRTRIQSELDC--QRLDLTPPDVHVLKLID   47
       |         .  |  .   |  .    |      ||.|     ||  |
MARR   MKSTSDLFNEIIPLGRLIHMVNQKKDRLLNEYLSPLDITAAQFKVLCSIR   50

MEXR   EQRGLNLQDLGRQMCRDKALITRKIRELEGRNLVRRERNPSDQRSFQLFL   97
         .  .| . .  |  .|| .  |  .  | |  || | |   . |
MARR   CAACITPVELKKVLSVDLGALTRMLDRLVCKGWVERLPNPNDKRGVLVKL   100

MEXR   TDEGLAIHQHAEAIMSR-VHDELFAPLTPVEQATLVHLLDQCLAAQPLED   146
       | | ||      ..  .| ||  ||  | |||  ||   |
MARR   TTGGAAICEQCHQLVGQDLHQELTKNLTADEVATLEYLLKKVL          143

MEXR   I   147
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FIG. 3. Homology of MexR and MarR proteins. Amino acid sequences of MexR and MarR were aligned with the PALIGN program of the PC Gene software package (Intelligenetics, Inc.). Identical residues (|) and conserved changes (.) are indicated.

acids within the C-terminal half of the protein highlighted by five invariant and four conserved residues (Fig. 4). This region did not correspond to the extreme C termini of the proteins, where homology between MarR and MexR was greatest and where no homology to or among the other proteins was observed. The existence of this conserved region suggests that these proteins may form a new family of regulatory (perhaps repressor) proteins.

MexR as a repressor of the *mexA-mexB-oprM* operon. Given the homology of the deduced MexR product to a number of repressor proteins and its proximity to the *mexA-mexB-oprM* operon, it was deemed likely that MexR negatively regulated expression of this operon. To test this directly, a mutant defective in *mexR* (K758) was constructed by gene replacement mutagenesis, and expression of the operon (measured as a function of expression of a *mexA-lacZ* fusion on plasmid pMXR5 [Fig. 1]) was assessed in the mutant and its MexR$^+$ parent. Insertional inactivation of *mexR* yielded a reproducible ca. twofold increase in *mexA-lacZ* expression compared with the parent strain (Table 2). Similar results were obtained with the *mexA-phoA* fusion vector pORFA-*pho* (data not shown). Consistent with the increased expression of the efflux operon, the *mexR* mutant showed a modest but reproducible increase in resistance to a variety of antibiotics (Table 3).

To determine whether *mexR* repressed the operon directly or whether its effect on operon expression was mediated by a third gene (perhaps an activator), the *mexA-lacZ* fusion vector pMXR5 was moved into *E. coli* and the influence of the cloned *mexR* gene on expression of the fusion was examined. Initially, the *mexR* gene was cloned on a 3.5-kbp *Sal*I fragment (plasmid pMXR1 [Fig. 1]) and introduced into plasmid pMXR5-containing *E. coli*, where it repressed expression of the fusion 1.6-fold (Table 4; compare pAK1900 and pMXR1). To rule out the possible involvement of additional genes present on pMXR1 in the observed repression, a 2.5-kb *Sal*I-*Sac*I fragment encompassing the same portion of *P. aeruginosa* DNA

TABLE 2. Expression of *mexR*- and *mexA-lacZ* fusions in *P. aeruginosa*[a]

Strain	Vector	β-Galactosidase activity (Miller units)[b]
K372	pMP190	52 ± 27
	pMXR4	2,160 ± 60
	pMXR5	5,470 ± 261
K758 (MexR$^-$)	pMXR5	9,520 ± 470

[a] *P. aeruginosa* strains carrying plasmid pMP190 or pMP190 derivative pMXR4 (*mexR-lacZ*) or pMXR5 (*mexA-lacZ*) (see Fig. 1) were grown to log phase in BHI broth and assayed for β-galactosidase activity.

[b] Data are reported as the means of three determinations (for duplicate cultures) ± the standard deviations.

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MEXR     88  SDQRSFQLFLTDEG  101   (147)
MARR     91  NDKRGVLVKLTTGG  104   (143)
MPRA    108  NDRRCLHLQLTEKG  121   (176)
17KDA   107  EDRRAKKISLTSEG  120   (164)
PECS    108  GDRRSVNIQLTDEG  121   (166)
HPCR     92  NDQRKLYISLTKEG  105   (148)
HPR      97  NDKRNTYVQLTEEG  110   (203)
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FIG. 4. Conserved region in MexR and a number of bacterial regulatory proteins. Multiple alignment of the indicated sequences was carried out with the CLUSTAL program of the PC Gene software package. Invariant (*) and similar (.) residues are indicated. The numbers at the beginning and end of each sequence represent the positions of the first and last amino acids, respectively, in the primary sequence of the intact protein. The total number of residues in each protein is shown in parentheses. Descriptions of these proteins are contained within the text.

TABLE 3. Influence of a *mexR* mutation on susceptibility of *P. aeruginosa* to antimicrobial agents

Strain	MexR phenotype	MIC[a] (μg/ml) of:				
		TET	CAM	NAL	CIP	CEF
K372	+	1.25	12.5	62.5	ND[b]	5
K758	−	5	25	250	ND	10
K335	+	3.13	ND	ND	0.6	12.5
K854	−	6.25	ND	ND	1.2	25
K784	+	12.5	100	125	0.3	25
K855	−	3.13	50	62.5	0.15	12.5

[a] Minimum inhibitory concentrations of the indicated antibiotics are reported for the indicated pairs of isogenic $MexR^+/MexR^-$ strains. TET, tetracycline; CAM, chloramphenicol; NAL, naladixic acid; CIP, ciprofloxacin; CEF, cefotaxime.

[b] ND, not determined.

present on pMXR1 but lacking an intact *mexR* gene (pMXR2 [Fig. 1]) was also introduced into the pMXR5-containing *E. coli* strain. In this instance, no repression of the fusion was observed (Table 4; compare pAK1900 and pMXR2), suggesting that the *mexR* gene on pMXR1 was responsible for the observed repression of the *mexA-lacZ* fusion in *E. coli*. Consistent with this, cloning of *mexR* alone on a ca. 1.1-kb *Fsp*I-*Nru*I fragment (pMXR3) (Fig. 1) resulted in a 2.3-fold decrease in *mexA-lacZ* expression (Table 4).

Although the moderate effect of the cloned *mexR* gene on *mexA-lacZ* expression could be explained by poor expression of *mexR* in *E. coli*, it is similar to the modest ca. twofold effect of a *mexR* mutation on fusion expression in *P. aeruginosa*. However, to rule out the possibility that the minimal effect results from titration of MexR in the $MexR^+$ parent strain due to the presence of multiple copies of the *mexA* promoter on fusion vector pMXR5 (leading to high expression of *mexA-lacZ*) or to induction of the fusion by chloramphenicol (which is a substrate of the MexA-MexB-OprM pump and which is included in growth media to maintain pMXR5), it was decided that a *mexA*-reporter gene fusion would be introduced into the chromosome of *P. aeruginosa* and that the influence of a *mexR* knockout mutation would be reexamined. The *mexA-lacZ* fusion was not readily recovered from pMXR5, and thus the *mexA-phoA* fusion from pORFA-*pho* was chosen. Furthermore, to rule out any strain-dependent effects which might have been influencing *mexA*-reporter gene activity, the *mexA-phoA* fusion was introduced into the chromosomes of strain K335 (to yield K831) and its *mexR* derivative, K854 (to yield K863). Despite the single-copy nature of the fusion in these strains, elimination of *mexR* elevated *mexA-phoA* expression

TABLE 4. Influence of the cloned *mexR* gene on expression of a plasmid-borne *mexA-lacZ* fusion in *E. coli*[a]

Vector	β-Galactosidase activity (Miller units)[b]
pAK1900	2,730 ± 60
pMXR1	1,650 ± 32
pMXR2	2,660 ± 58
pMXR3	1,200 ± 95

[a] *E. coli* 5K harboring the *mexA-lacZ* fusion vector pMXR5 (Fig. 1) was transformed with plasmid pAK1900 or pAK1900 derivative pMXR1 ($MexR^+$), pMXR2 ($MexR^-$), or pMXR3 ($MexR^+$), grown to late log phase in BHI broth, and assayed for β-galactosidase activity.

[b] The data are reported as the means of three determinations (for duplicate cultures) ± the standard deviations.

TABLE 5. Expression of a chromosomal *mexA-phoA* fusion in *P. aeruginosa*[a]

Strain	Description[b]	Alkaline phosphatase activity (A_{405}/A_{600})[c]
K831	PAO1	0.21 ± 0.01
K863	PAO1 *mexR*::ΩHg	0.40 ± 0.02
K865	PAO1[d]	0.31 ± 0.04
K832	OCR1	2.14 ± 0.03
K864	OCR1 *mexR*::ΩHg	0.16 ± 0.01

[a] The indicated *P. aeruginosa* strains carrying chromosomal *mexA-phoA* fusions were cultured overnight in L broth and assayed for alkaline phosphatase activity as described in Materials and Methods.

[b] Although a number of manipulations were necessary to introduce the *mexR*::ΩHg mutation and *mexA-phoA* fusion into the strains indicated on the left (see Materials and Methods), these strains are ultimately derived from PAO1 and its multidrug-resistant derivative OCR1. For clarity in interpreting the data, this derivation is highlighted. A more complete description of the strains is available in Table 1.

[c] 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.

[d] This PAO1 strain, the parent from which OCR1 was derived, displays a lower intrinsic resistance to chloramphenicol than the previous PAO1 strain. Because of this, OCR1 was compared to its parental PAO1 strain and not the existing laboratory strain of PAO1.

only twofold (Table 5), with an attendant modest increase in resistance to a variety of antibiotics (Table 3).

Nature of *mexR* in the *nalB* strain OCR1. *nalB* multidrug-resistant strains of *P. aeruginosa* are characterized by an overproduction of the outer membrane protein OprM and, thus, are likely to overexpress the *mexA-mexB-oprM* operon. To confirm this and to assess the nature of the overexpression, a *mexA-phoA* fusion was introduced into the chromosome of the *nalB* strain OCR1 (to yield K832) and its PAO1 parent strain, K767 (to yield K865). Expression of the fusion was increased 7.4-fold in the multidrug-resistant strain, a result reproduced with pORFA-*pho* (data not shown). These results indicate that the lesion in OCR1 is neither *cis* dominant (e.g., a *mexA-mexB-oprM* promoter up mutation) nor a *mexR* null mutation. Insertional inactivation of *mexR* in OCR1 resulted in a 13-fold decrease in expression of a chromosomal *mexA-phoA* fusion (Table 5; compare K864 with K832) with a corresponding increase in drug susceptibility (Table 3). While this confirmed a role for *mexR* in the overexpression of *mexA-mexB-oprM* in OCR1, it suggested that MexR was functioning as an activator in this strain.

To assess the nature of the mutation in *mexR* resulting in overexpression of *mexA-mexB-oprM*, the *mexR* genes of OCR1 and its parent strain, K767 (PAO1), were amplified by PCR and sequenced. Both sequences were identical to the *mexR* sequence shown in Fig. 2 with the exception of a single C-to-T base substitution at position 484 in the OCR1 gene, which converts an arginine at position 69 in MexR to a tryptophan (Fig. 2).

MexR negatively regulates its own expression. To determine if *mexR* is subject to autoregulation, the *mexR-lacZ* fusion vector pMXR4 (Fig. 1) was introduced into PAO1 wild-type strain K767 and a *mexR* knockout derivative (strain K870) and β-galactosidase activity was assessed. Elimination of *mexR* in K870 caused a greater than threefold increase in β-galactosidase activity, indicating that MexR represses its own expression (Table 6). The multidrug-resistant strain OCR1 also showed a ca. threefold increase in expression of the *mexR-lacZ* fusion compared with K767, although elimination of *mexR* in

TABLE 6. Influence of a MexR knockout mutation on *mexR-lacZ* expression in *P. aeruginosa* PAO1 and OCR1[a]

Strain	β-Galactosidase activity (Miller units)
K767 (PAO1)	654 ± 30
K870 (K767 MexR$^-$)	2,170 ± 636
OCR1	1,880 ± 163
K855 (OCR1 MexR$^-$)	909 ± 249

[a] *P. aeruginosa* strains carrying the *mexA-lacZ* fusion vector pMXR5 were grown to log phase in L broth containing chloramphenicol and assayed for β-galactosidase activity. Values reported represent the means of three determinations (for duplicate cultures) ± the standard deviations.

this strain (yielding K855) actually reduced *mexR* expression to levels observed for K767 (Table 6).

DISCUSSION

Intrinsic and acquired multiple drug resistance in *P. aeruginosa* is at least partially attributable to the operation of cell envelope-associated efflux pumps. The MexA-MexB-OprM system, which is overproduced in *nalB* multidrug-resistant strains (references 29 and 30 and this study), was initially implicated in siderophore export (41), although more recent data failed to confirm an association with the siderophore system. Indeed, previous reports of iron-regulated expression of *mexA* appear to reflect growth phase-dependent regulation of the *mex* efflux genes (58), and no evidence for iron regulation of *mexR* has been forthcoming (37). The high degree of homology to products of the *acrAB* and *mtrCDE* operons of *E. coli* and *N. gonorrhoeae*, respectively, the former being stress inducible (25) and both seemingly playing roles in resistance to naturally occurring antibacterial fatty acids and bile salts (25, 53), suggests that MexA-MexB-OprM may play a protective role in *P. aeruginosa*. The observation, however, that a variety of antibiotics do not induce expression of *mexA-mexB-oprM* (37) indicates that the natural function of this efflux system is not protection against antibiotics.

Despite the similarity between MexA-MexB-OprM and the products of the *acr* and *mtr* loci, the regulatory gene, *mexR*, identified upstream of *mexA-mexB-oprM* exhibits no obvious similarity to the *mtrR*, *acrR*, or *acrS* regulatory-gene products. Indeed, the greatest similarity to MexR was observed with MarR, a repressor of the *marRAB* operon which controls expression of a variety of genes associated with resistance to multiple antibiotics and oxygen stress in *E. coli* (2). Induction of the *marRAB* operon is inducible by a number of antibiotics and aromatic weak acids (e.g., salicylate), and early indications were that these agents antagonize binding of MarR to *mar* operators, presumably leading to *mar* gene expression (52). More recently, however, studies with purified MarR have shown that while MarR indeed binds to *mar* operator regions, such binding is antagonized only by salicylate (28). Thus, antibiotic induction of the *mar* locus might occur independently of *marR* or via some indirect mechanism.

The antagonism of repressor activity by salicylate or salicylate-like compounds is a feature of several regulatory proteins which are members of a family of proteins with a shared C-terminal signature sequence (Fig. 4) (48, 56). Despite the similarity of MexR to this family of regulators, however, its activity was apparently not antagonized by salicylate, since expression of the MexR-regulated *mexA-mexB-oprM* operon was unaffected by this compound (58). Still, the conservation of aromatic substituents in those drugs known to be substrates for the MexA-MexB-OprM pump is consistent, at least, with a phenolic or salicylate-like compound being the inducer and, perhaps, the natural substrate of the pump.

The observation that a *mexR* knockout mutation (in a non-multidrug-resistant background) consistently yielded an increase in expression of plasmid-borne and chromosomal *mexA*-reporter gene fusions and that the cloned gene decreased expression of such fusions suggests that MexR functions as a repressor. The twofold increase in expression observed in a *mexR* knockout strain, though modest, is similar to the effect of an *mtrR* deletion on *mtr* efflux gene expression in *N. gonorrhoeae*. Indeed, such deletions elicit a less than twofold increase in expression of the efflux genes (as measured by quantitating *mtrC* mRNA levels), and clinical isolates of *N. gonorrhoeae* showing a high-level multidrug resistance phenotype as a result of a single base pair deletion in the *mtrR* promoter region exhibit only a threefold increase in expression (14). Similarly, knockout mutations in *acrR*, which encodes the repressor of *acrAB* expression in *E. coli* (23), also elevate efflux gene expression only two- to threefold (23). One possible explanation for this effect in the case of MexR is that much of MexR in a MexR$^+$ strain may not be functioning fully in a repressor capacity, perhaps as a result of natural derepression under the growth conditions of the assay. The elimination of MexR in a *mexR* null mutant would thus impart only a modest increase in expression of the efflux genes.

Alternatively, and given that a point mutation in *mexR* correlates with high-level expression of *mexA-mexB-oprM* in the multiply resistant strain OCR1, it may be that MexR also functions as an activator in vivo and that the *mexR* mutation in OCR1 favors the activator form of the protein. Still, this does not entirely explain the impact of a *mexR* knockout mutation on expression of a *mexA-phoA* fusion in OCR1. While the observed decrease in *mexA-phoA* expression in an OCR1 *mexR*::ΩHg mutant confirms the need for the altered MexR in the elevated *mexA-mexB-oprM* expression and multidrug resistance of this strain, the fact that *mexA-phoA* expression is lower, by a factor of two, than in the wild type is puzzling. Similarly, elimination of *mexR* in a wild-type strain increases *mexR* gene expression, consistent with negative autoregulation, but actually reverses the elevated *mexR* gene expression seen in OCR1. Thus, increased expression of *mexR* and *mexA-mexB-oprM* in OCR1 cannot be attributed solely, if at all, to loss of MexR repression activity. Consistent with the involvement of a second mutation (gene), we have noted that the cloned *mexR* gene from OCR1 fails to activate expression of a *mexA-lacZ* fusion in *E. coli* or in a *mexR* knockout *P. aeruginosa* strain (59). Given that the *nalB* multidrug resistance phenotype is transducible (47), any additional mutations (genes) must be relatively closely linked to *mexR*. Thus, the region downstream of *mexR* is currently being examined for potential involvement in *mexA-mexB-oprM* expression and multidrug resistance.

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