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MexCD-OprJ Multidrug Efflux System of *Pseudomonas aeruginosa*: Involvement in Chlorhexidine Resistance and Induction by Membrane-Damaging Agents Dependent upon the AlgU Stress Response Sigma Factor▽

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The biocide chlorhexidine (CHX) as well as additional membrane-active agents were shown to induce expression of the *mexCD-oprJ* multidrug efflux operon, dependent upon the AlgU stress response sigma factor. Hyperexpression of this efflux system in *nfxB* mutants was also substantially AlgU dependent. CHX resistance correlated with efflux gene expression in various mutants, consistent with MexCD-OprJ being a determinant of CHX resistance.

Pseudomonas aeruginosa is an opportunistic human pathogen characterized by an innate resistance to multiple antimicrobials (13), resistance increasingly attributable to the operation of broadly specific, tripartite multidrug efflux systems of the resistance-nodulation-division (RND) family (35, 36). One of these, MexCD-OprJ, was originally identified as a determinant of fluoroquinolone resistance (17) but is known to accommodate a variety of clinically relevant antimicrobials (35, 36) as well as biocides (5), dyes, detergents, and organic solvents (27, 45, 46). MexCD-OprJ is typically quiescent in wild-type cells (20, 46), with expression following mutation of the *nfxB* gene (16, 22, 23, 50) that is divergently transcribed from the *mexCD-oprJ* operon and encodes a repressor of *mexCD-oprJ* expression (37). Little is known about the signal(s) to which this regulator responds in naturally promoting efflux gene expression, although *mexCD-oprJ* is inducible by the biocides benzalkonium chloride and chlorhexidine (CHX) (33). These biocides are known to interact with and disrupt bacterial membranes (8), with the possibility that *mexCD-oprJ* expression is a response to membrane damage/envelope stress. Envelope stress responses (ESRs) are well documented in bacteria (40, 41), with the extracytoplasmic sigma factor RpoE being a key regulator of ESRs in *Escherichia coli* and other gram-negative bacteria (1, 40, 41). The RpoE homologue in *P. aeruginosa* is AlgU, first identified as a regulator of alginate production in mucoid isolates recovered from the lungs of cystic fibrosis patients (15, 28) and shown to be functionally interchangeable with RpoE (51). This study was undertaken to assess the contribution of MexCD-OprJ to biocide resistance in *P. aeruginosa* and its possible regulation as part of an ESR.

Bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were cultivated at 37°C in Luria broth (LB) (34) supplemented with antibiotics to maintain plasmids as needed (for pEX18Tc and derivatives, tetracycline was used [10 μg/ml for *E. coli* and 50 to 100 μg/ml for *P. aeruginosa*]; for pMMB206 and derivatives, chloramphenicol was used [10 μg/ml for *E. coli* and 150 μg/ml for *P. aeruginosa*]; for pK18MobSacB and derivatives, kanamycin was used [50 μg/ml for *E. coli* and 750 to 1,500 μg/ml for *P. aeruginosa* as indicated]; for miniCTX-lacZ and derivatives, tetracycline was used [10 μg/ml for *E. coli* and 25 μg/ml for *P. aeruginosa*]; and for pUC19 and derivatives, ampicillin was used [100 μg/ml for *E. coli*]). AlgU-encoding plasmid pSF02 was constructed by amplifying the *algU* gene from the chromosome (isolated as described previously [3]) of *P. aeruginosa* K767 via PCR using Vent DNA polymerase (NEB) and cloning it into pMMB206 (primers and parameters available upon request). To construct the *algU* strains of *P. aeruginosa*, an in-frame deletion of the gene was first engineered in the gene replacement vector pK18MobSacB following amplification and cloning ca. 1-kb portions upstream and downstream of the *algU* sequences being deleted (primers and parameters available upon request). The resultant vector, pSF01, was mobilized into *P. aeruginosa* (46), and transconjugants were selected on LB agar containing kanamycin (1,500 μg/ml) and imipenem (0.5 μg/ml). Those harboring a chromosomal deletion of *algU* were subsequently recovered on sucrose plates (46) and screened for the loss of *algU* using colony PCR (39). The Δ*mexB*-Δ*mexXY* mutant strain K1542 was constructed by introducing the *mexXY* deletion of plasmid pCSV05 into Δ*mexB* strain K1523 as described previously (7). A Δ*mexCD-oprJ* derivative of *P. aeruginosa* K1542 was constructed using plasmid pRSP05 as described previously (46), with initial selection of the vector in strain K1542 made on kanamycin (1,000 μg/ml) and imipenem (0.5 μg/ml). Putative Δ*mexCD-oprJ* mutants were recovered from sucrose plates and screened for the loss of *mexCD-oprJ* using colony PCR (39). A chromosomal *mexC-lacZ* transcriptional fusion was generated using a previously described procedure (19). A 700-bp region containing the *mexCD-oprJ* promoter region ($P_{mexCD\text{-}oprJ}$) was PCR amplified from the chromosome of *P. aeruginosa* K767 and cloned upstream of the promoterless

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TABLE 1. Bacterial strains used in this study

Strain or plasmid	Description[a]	Source or reference
P. aeruginosa		
K767	PAO1 prototroph	29
K1521	K767 Δ*mexCD-oprJ*	46
K1536	K767 *nfxB*	18
K1523	K767 Δ*mexB*	18
K1542	K767 Δ*mexB* Δ*mexXY*	This study
K2443	K767 Δ*algU*	This study
K2895	K1536 Δ*algU*	This study
K2896	K1542 Δ*mexCD-oprJ*	This study
K2897	K1542 Δ*algU*	This study
K2898	K2896 Δ*algU*	This study
K2899	K1542 carrying a $P_{mexCD\text{-}oprJ}$ -*lacZ*[b] transcriptional fusion at the *attB* site	This study
K2900	K1542 carrying a promoterless *lacZ* gene at the *attB* site	This study
K2901	K2897 carrying a $P_{mexCD\text{-}oprJ}$ -*lacZ* transcriptional fusion at the *attB* site	This study
K2902	K2897 carrying a promoterless *lacZ* gene at the *attB* site	This study
K2888	PAO1 prototroph	26
K2890	K2888 *mexD*::mini-Tn*5-luxCDABE*	26
K2889	CHX-passaged K2888	This study
K2891	CHX-passaged K2890	This study
E. coli		
DH5α	ϕ80d*lac*ZΔM15 Δ(*lacZYA-argF*) *endA1 recA1*	2
S17-1	*thi pro hsdR recA* Tra^+	43
Plasmids		
pK18MobSacB	Broad-host-range gene replacement vector; *sacB* Km^r	43
miniCTX-lacZ	Integration vector with promoterless *lacZ*; *oriT*$^+$ Tc^r	4
pFLP2	Source of Flp recombinase; Ap^r/Cb^r	19
pMMB206	*P. aeruginosa* and *E. coli* shuttle cloning vector; Cm^r	32
pEX18Tc	Gene-replacement vector; *sacB* Tc^r	19
pCSV05	pEX18Tc::Δ*mexXY*	7
pRSP05	pK18MobSacB::Δ*mexCD-oprJ*	46
pSF01	pK18MobSacB::Δ*algU*	This study
pSF02	pMMB206::*algU*	This study
pAJC03	miniCTX-$P_{mexCD\text{-}oprJ}$-*lacZ*[b]	This study

[a] Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Cb^r, carbenicillin resistance; Km^r, kanamycin resistance; Tc^r, tetracycline resistance.
[b] The *mexCD-oprJ* promoter region upstream of a promoterless *lacZ* gene.

lacZ gene in plasmid miniCTX-lacZ. The resultant vector, pAJC03, was mobilized (46) into *P. aeruginosa* strains K1542 and K2897 (K1542 Δ*algU*), and transconjugants carrying chromosomal copies of pAJC03 were selected on tetracycline (25 μg/ml) and imipenem (0.5 μg/ml). The miniCTX plasmid backbone was excised using the pFLP-encoded Flp recombinase as described previously (19), leaving the $P_{mexCD\text{-}oprJ}$ -*lacZ* fusion behind. Control derivatives of K1542 and K2897 harboring a promoterless *lacZ* gene in the chromosome were generated as described above with promoter-free miniCTX-lacZ. β-Galactosidase assays were performed as described previously (31) on cells cultured overnight in LB, diluted 1:49 in fresh LB, and cultured for a further 2 h prior to a 2-h exposure to various membrane-damaging agents (MDAs). *P. aeruginosa* strains with reduced susceptibility to CHX were isolated, following serial passage in LB containing increasing concentrations (1 to 50 μg/ml) of the biocide (1-μg/ml increments up to 20 μg/ml; 2-μg/ml increments from 20 to 50 μg/ml). Bacteria were incubated for 24 h at 37°C at a given CHX concentration before being harvested by centrifugation, washed twice in 5 ml phosphate-buffered saline (34), and used to inoculate LB cultures (1/100 dilution) containing the next-highest CHX concentration. Individual colonies were recovered from the cultures with the highest concentrations of CHX permitting growth by streaking onto L agar, and stable CHX-resistant mutants were recovered following passage (10 times) in biocide-free LB. Susceptibility testing (34) and reverse transcriptase PCR (RT-PCR) using RNA isolated from log-phase cells (44) after a 2.5-h exposure to various MDAs was carried out as described above (primers and parameters available upon request). *rpsL* was used as an internal control in RT-PCR to ensure equal loading of RNA in all lanes.

CHX-induced *mexCD-oprJ* expression is AlgU dependent. Treatment of wild-type *P. aeruginosa* with the cationic biocides CHX and benzalkonium chloride has been shown to induce *mexCD-oprJ* expression (33), a result confirmed here for CHX (Fig. 1, lane 2, cf. lane 1). Additional cationic biocides, including alexidine, poly(hexamethylenebiguanide)hydrochloride (PHMB; Vantocil), and cetrimide were tested and also shown to induce *mexCD-oprJ* expression (Fig. 1, lanes 3 to 5). These agents interact with and disrupt bacterial membranes (8), suggesting that *mexCD-oprJ* induction may be a response to membrane damage and not to the agents themselves. In *E. coli*, membrane disruption with chemical agents (40, 47) or mutation (47) stimulates expression of genes controlled by the RpoE envelope stress sigma factor. AlgU is the *P. aeruginosa* homologue of RpoE, and so the involvement of AlgU in CHX-promoted *mexCD-oprJ* expression was assessed by measuring

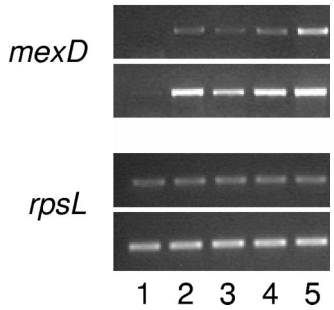

FIG. 1. Biocide induction of *mexCD-oprJ* expression in *P. aeruginosa*. Expression of *mexD* and *rpsL* was assessed in wild-type *P. aeruginosa* PAO1 strain K767 grown in the absence of biocide (lane 1) or after a 2.5-h exposure to a fourth of the MIC of the cationic biocides CHX (2.5 μg/ml) (lane 2), PHMB (1 μg/ml) (lane 2), alexidine (3 μg/m) (lane 2), or cetrimide (100 μg/ml) (lane 2) by semiquantitative RT-PCR. The PCR portion was carried out at 32 (top *mexD* panel) and 34 (bottom *mexD* panel) or 20 (top *rpsL* panel) and 22 (bottom *rpsL* panel) cycles.

the impact of an *algU* knockout. As shown in Fig. 2A, elimination of *algU* obviated CHX-promoted *mexCD-oprJ* expression in the wild-type strain K767 (compare lanes 3 and 4), and this was reversed by the cloned *algU* gene (compare lanes 6 and 7), indicating that AlgU mediates CHX-induced *mexCD-oprJ* expression. *mexCD-oprJ* hyperexpression in an *nfxB* mutant (Fig. 3, lane 3, cf. lane 1) was also compromised in the absence of *algU* (Fig. 3, lane 4, cf. lane 3) and restored with the cloned *algU* gene (Fig. 3, lane 8, cf. lane 7), consistent with AlgU also being involved in mutational *mexCD-oprJ* expression.

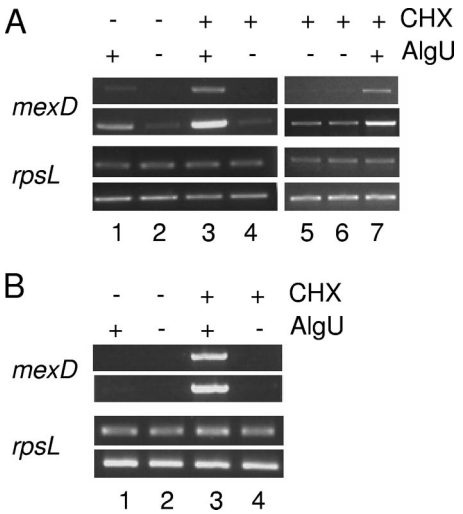

FIG. 2. Impact of CHX and AlgU on *mexCD-oprJ* expression in *P. aeruginosa*. Expression of *mexD* and *rpsL* was assessed in *P. aeruginosa* strains K767 (wild type) (lanes 1 and 3), K2443 (Δ*algU*) (lanes 2, 4, and 5), and K2443 carrying pMMB206 (lane 6) or pSF02 (pMMB206::*algU*) (lane 7) (A) or in strains K1542 (Δ*mexB* Δ*mexXY*) (lanes 1 and 3) and K2897 (Δ*mexB* Δ*mexXY* Δ*algU*) (lanes 2 and 4) grown in the absence of CHX (−CHX) or after a 2.5-h exposure to a quarter of the MIC of this biocide (2.5 μg/ml for AlgU$^+$ strains and 0.33 μg/ml for AlgU$^-$ strains) (+CHX) by semiquantitative RT-PCR (B). The PCR portion was carried out at 33 (top *mexD* panel) and 35 (bottom *mexD* panel) or 23 (top *rpsL* panel) and 25 (bottom *rpsL* panel) cycles.

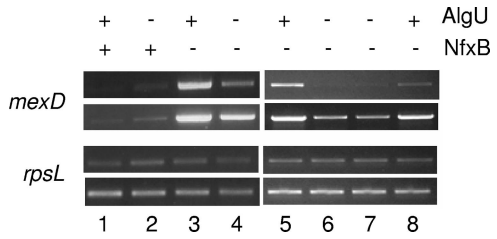

FIG. 3. Impact of *algU* on *mexCD-oprJ* expression in *nfxB* strain K1536. The expression of *mexD* and *rpsL* was assessed in *P. aeruginosa* strains K767 (lanes 1), K2443 (Δ*algU*) (lane 2), K1536 (*nfxB*) (lanes 3 and 5), K2895 (*nfxB* Δ*algU*) (lanes 4 and 6), and K2895 carrying pMMB206 (lane 7) or pSF02 (pMMB206::*algU*) (lane 8) by semiquantitative RT-PCR. Cycle numbers are as per the legend to Fig. 2.

Additional membrane-active agents induce *mexCD-oprJ*. If membrane damage is a signal for *mexCD-oprJ* induction, additional MDAs should promote expression of this efflux operon. To assess this, *P. aeruginosa* was treated with sub-MIC levels of several agents known to target and disrupt the cytoplasmic and/or outer membranes of this organism, including solvents (ethanol [10, 21, 42], hexane, and xylene [38, 42, 48]), a detergent (sodium dodecyl sulfate [SDS]) (14, 42, 49), EDTA

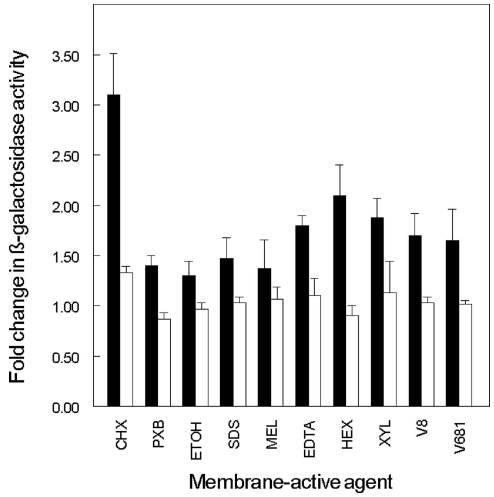

FIG. 4. Impact of membrane-active agents on $P_{mexCD\text{-}oprJ}$-*lacZ* expression in *P. aeruginosa*. *P. aeruginosa* K2899 (K1542::$P_{mexCD\text{-}oprJ}$-*lacZ*) (black bars) and AlgU$^-$ derivative K2901 (K2897::$P_{mexCD\text{-}oprJ}$-*lacZ*) (white bars) were grown to log phase in the presence or absence of CHX, polymyxin B (PXB), ethanol (ETOH), SDS, melittin (MEL), EDTA, *n*-hexane (HEX), *p*-xylene (XYL), and the cationic antimicrobial peptides V8 and V681 and assayed for β-galactosidase activity. Values for activity measured were subsequently adjusted for background levels seen for promoter-free, *lacZ*-containing strains K2900 (K1542::promoterless *lacZ*) and K2902 (K2897::promoterless *lacZ*). The results shown are the fold change in activity observed in the presence versus the absence of the indicated agent and are the mean ± the standard deviation of at least two independent experiments performed in triplicate. MDAs were used under the following conditions: CHX, 1/8 MIC (1.25 and 0.16 μg/ml for K2899 and K2901 derivatives, respectively); polymyxin B, 1/4 MIC (0.125 μg/ml); ethanol, 1/8 MIC (0.5% [vol/vol]); SDS, 1/16 MIC (3.1 μg/ml); melittin, 1/4 MIC (15 μg/ml); EDTA, 1/16 MIC (11.6 μg/ml); *n*-hexane, 1/4 MIC (1.25% [vol/vol]); *p*-xylene, 1/8 MIC (0.0156% [vol/vol]); antimicrobial peptide V8, 1/8 MIC (8 μg/ml); and antimicrobial peptide V681, 1/8 MIC (16 μg/ml)]. Various MDA concentrations were tested, and results are shown for concentrations that did not adversely impact growth.

TABLE 2. Contribution of AlgU and MexCD-OprJ to CHX resistance in *P. aeruginosa*[a]

Strain	Relevant properties[b]	CHX MIC (μg/ml)
K1542	CDJ$^+$ AlgU$^+$	10
K2896	CDJ$^-$ AlgU$^+$	2.5
K2897	CDJ$^+$ AlgU$^-$	1.25
K2898	CDJ$^-$ AlgU$^-$	0.625
K767	CDJ$^+$ AlgU$^+$	10
K1521	CDJ$^-$ AlgU$^+$	5
K2443	CDJ$^+$ AlgU$^-$	2.5
K2443 + pMMB206	CDJ$^+$ AlgU$^-$	2.5
K2443 + pSF02[c]	CDJ$^+$ AlgU$^+$	10
K1536	NfxB$^-$ AlgU$^+$	20
K2895	NfxB$^-$ AlgU$^-$	2.5
K2895 + pMMB206	NfxB$^-$ AlgU$^-$	2.5
K2895 + pSF02[c]	NfxB$^-$ AlgU$^+$	20
K2889	CHX passaged; CDJ$^+$	>50[d]
K2891	CHX passaged; CDJ$^-$	10

[a] The MICs shown are for the most resistant isolates recovered.
[b] CDJ$^+$, *mexCD-oprJ* present and inducible by CHX but not otherwise expressed in untreated cells; CDJ$^-$, Δ*mexCD-oprJ*; AlgU$^+$, *algU* present; AlgU$^-$, Δ*algU*; NfxB$^-$, *nfxB* repressor gene inactivated; CHX passaged, strains recovered following serial passage in LB containing increasing concentrations of CHX.
[c] pSF02, plasmid pMMB206 carrying *algU*.
[d] Limit of CHX solubility.

(12, 30), and several cationic antimicrobials (polymyxin B, melittin, and antimicrobial peptides V8 and V681 [9, 11, 24, 52]). A strain lacking the MexAB-OprM and MexXY-OprM efflux systems, K1542, was used in these studies to avoid possible problems with the export of MDAs by these efflux systems, compromising their membrane-damaging activities (solvents and SDS are, for example, known efflux substrates [27, 45]). Initially, CHX inducibility of *mexCD-oprJ* was assessed in this strain and, as in strain K767, it was seen to be AlgU dependent (Fig. 2B). Using a chromosomal $P_{mexCD\text{-}oprJ}$-*lacZ* reporter to assess the impact of MDAs on *mexCD-oprJ* expression subsequently revealed that efflux gene expression was induced by all MDAs tested, though CHX was the most effective inducer (Fig. 4). Although the effects seen were modest (ca. 1.5- to 3-fold), the increased *mexCD-oprJ* expression revealed by the reporter fusion correlated with increased resistance of MDA-treated K1542 (but not its Δ*mexCD-oprJ* derivative K2896) to MexCD-OprJ substrate antimicrobials (a 2- to 4-fold increase in norfloxacin MICs and a 4- to 16-fold increase in erythromycin MICs [data not shown]). As with CHX-treated K767, the loss of *algU* compromised MDA induction of *mexCD-oprJ* in K1542 (Fig. 4) as well as MDA-promoted antibiotic resistance (data not shown). These data are consistent with AlgU and MexCD-OprJ playing a role in the ESR of *P. aeruginosa*, possibly orchestrating membrane changes necessary for adaptation to MDAs.

Salmonella enterica RpoE, like AlgU, is also linked to membrane damage and resistance to MDAs, as the sigma factor is inducible by cationic antimicrobial peptides and is required for antimicrobial peptide resistance (6). Similarly, carbon source starvation, which apparently causes membrane stress that induces *rpoE* in this organism, also promotes resistance to polymyxin B that is RpoE dependent (25).

MexCD-OprJ and CHX resistance. Despite the earlier report of CHX induction of *mexCD-oprJ*, a possible contribution of this efflux system to CHX resistance was not examined (33). Compared to their MexCD-OprJ$^+$ parents K1542 and K767, mutants lacking *mexCD-oprJ* (K2896 and K1521) were more susceptible to this biocide, while an *nfxB* mutant hyperexpressing this efflux system, K1536, was more resistant than its parent, K767 (Table 2), consistent with MexCD-OprJ contributing to CHX resistance. Consistent with AlgU's role in the hyperexpression of *mexCD-oprJ* in an *nfxB* mutant, the loss of *algU* in this mutant markedly enhanced CHX susceptibility (Table 2; compare K1536 and K2895). Similarly, Δ*algU* strains K2897 and K2443 were also more susceptible to CHX than their parents K1542 and K767, consistent with AlgU contributing to CHX-promoted *mexCD-oprJ* expression. In all instances, the increased CHX susceptibility of Δ*algU* strains was reversed by the cloned *algU* gene (Table 2). Interestingly, Δ*algU* derivatives K2897 and K2443 were more susceptible to CHX than the Δ*mexCD-oprJ* derivatives (K2896 and K1521), and a double mutant lacking both loci (K2898) was more susceptible still (Table 2). These data are consistent with an additional AlgU-regulated gene(s) contributing to CHX resistance. Still, MexCD-OprJ appears to be the most important AlgU-regulated determinant of CHX resistance and, indeed, highly CHX-resistant mutants selected following serial passage of *P. aeruginosa* in CHX-containing media were recoverable only from a MexCD-OprJ$^+$ strain (Table 2). The presence or absence of *mexCD-oprJ* or *algU* did not impact susceptibility to any of the other MDAs examined in this study (data not shown), consistent with the more modest influence of these agents on *mexCD-oprJ* expression or their being, possibly, poor substrates for this efflux system.

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