mexEF-oprN Multidrug Efflux Operon of *Pseudomonas aeruginosa*: Regulation by the MexT Activator in Response to Nitrosative Stress and Chloramphenicol[▽]

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A null mutation in the *mexS* gene of *Pseudomonas aeruginosa* yielded an increased level of expression of a 3-gene operon containing a gene, *xenB*, whose product is highly homologous to a xenobiotic reductase in *Pseudomonas fluorescens* shown previously to remove nitro groups from trinitrotoluene and nitroglycerin (D. S. Blehert, B. G. Fox, and G. H. Chambliss, J. Bacteriol. 181:6254, 1999). This expression, which paralleled an increase in *mexEF-oprN* expression in the same mutant, was, like *mexEF-oprN*, dependent on the MexT LysR family positive regulator previously implicated in *mexEF-oprN* expression. As nitration is a well-known result of nitrosative stress, a role for *xenB* (and the coregulated *mexEF-oprN*) in a nitrosative stress response was hypothesized and tested. Using *s*-nitrosoglutathione (GSNO) as a source of nitrosative stress, the expression of *xenB* and *mexEF-oprN* was shown to be GSNO inducible, although in the case of *xenB*, this was seen only for a mutant lacking MexEF-OprN. In both instances, this GSNO-inducible expression was dependent upon MexT. Chloramphenicol, a nitroaromatic antimicrobial that is a substrate for MexEF-OprN, was shown to induce *mexEF-oprN* but not *xenB*, again dependent upon the MexT regulator, possibly because it resembles a nitrosated nitrosative stress product accommodated by MexEF-OprN.

Pseudomonas aeruginosa is an opportunistic human pathogen characterized by an innate resistance to multiple antimicrobials (15), a resistance that is increasingly attributable to the operation of broadly specific, tripartite multidrug efflux systems of the resistance-nodulation-division (RND) family (39, 40). Several RND family multidrug efflux systems have been described for *P. aeruginosa*, although the major systems contributing to intrinsic and/or acquired multidrug resistance include MexAB-OprM, MexXY-OprM, MexEF-OprN, and MexCD-OprJ (39, 40). Unlike MexAB-OprM and MexXY-OprM, which contribute to intrinsic resistance (1, 7, 26, 32), the MexEF-OprN and MexCD-OprJ systems are typically quiescent in wild-type cells (under usual laboratory growth conditions) (19, 28, 50), with expression and, therefore, a contribution to antimicrobial resistance following a mutational upregulation of the efflux genes (13, 20, 30, 41, 48). In the case of MexEF-OprN, this involves the reversion of mutations present in the *mexT* gene of a number of so-called wild-type strains (29, 30) or a mutation of the *mexS* gene (also known as *qrh* [25]), encoding an oxidoreductase of unknown function (48). *mexT*, which occurs upstream of *mexEF-oprN* and downstream of *mexS*, encodes a LysR family positive regulator that promotes *mexEF-oprN* (25, 28, 36) and *mexS* (25) expression. *mexEF-oprN* expression and modest multidrug resistance have also been reported for a mutant disrupted in

the *mvaT* gene (53), encoding a global regulator of virulence gene expression (8).

Originally identified as a determinant of fluoroquinolone resistance (12, 13), MexEF-OprN accommodates a variety of antimicrobials, including trimethoprim and chloramphenicol (28), with chloramphenicol readily selecting multidrug-resistant *mexEF-oprN*-hyperexpressing *mexS* mutants *in vitro* (48). Enhanced resistance to imipenem, which is also seen for *nfxC* (28) and *mexS* (48) strains, results not from MexEF-OprN expression but from the concomitant decrease in levels of the outer membrane protein OprD in these mutants (12, 31, 48). OprD is an imipenem channel and a primary route of entry of this antibiotic in *P. aeruginosa* (52), whose absence is often seen in imipenem-resistant strains of *P. aeruginosa* (3, 27). Like other tripartite RND family pumps, the MexEF-OprN efflux system consists of an inner membrane (IM) drug-proton antiporter (the RND component) (MexF), an outer membrane (OM) channel-forming component (OprN), and a periplasmic membrane fusion protein (MFP) (MexE) (38, 54).

Despite the identification of regulatory genes impacting the expression of *mexEF-oprN*, little is known about the signals to which the regulators respond in promoting efflux gene expression, and in fact, it is far from clear that antimicrobial efflux and resistance are the intended functions of this pump. For example, mutants overexpressing MexEF-OprN were readily recovered from an experimental model of rat pneumonia in the absence of antibiotic selection (22), indicating some advantage of MexEF-OprN expression *in vivo*, independent of antimicrobial export. Transcriptome analysis of *P. aeruginosa* exposed to airway epithelial cells has revealed substantial increases in the levels of expression of *mexEF-oprN* and *mexS* (then called PA2491), apparently in parallel with an increased damage of

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the epithelial cells and, therefore, the release of cell contents (11), although the efflux-recruiting signal(s) released by these cells is unknown. The observation that a mutational loss of the *VsqR* quorum-sensing (QS) and virulence regulator compromises *mexEF-oprN* expression in cells under conditions of oxidative stress suggests that this system may normally be induced in response to this stressor (23). Finally, a synthetic derivative of a natural furanone compound that functions as an antagonist of QS in *P. aeruginosa* was also shown previously to induce *mexEF-oprN* expression (16).

In an effort to elucidate the signals to which *mexEF-oprN* responds and, therefore, to define the natural function of the MexEF-OprN efflux system, attempts were made to identify known genes coregulated with the efflux genes in a *mexS* mutant. These studies identified a xenobiotic reductase (XenB) with a possible link to nitrosative stress, and indeed, nitrosative stress is shown here to induce *mexEF-oprN* (and *xenB*) expression. The implications vis-à-vis the *in vivo* environment impacting efflux gene expression and, therefore, antimicrobial resistance in *P. aeruginosa* are clear.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were cultivated at 37°C in Luria broth (LB), as described previously (35), supplemented with tetracycline (10 µg/ml for *Escherichia coli* and 50 to 100 µg/ml for *P. aeruginosa*) (for plasmids pRK415, pMP220, pEX18Tc, miniCTX1, and their derivatives), chloramphenicol (10 µg/ml for *E. coli* and 200 µg/ml for *P. aeruginosa*) (for plasmid pMMB206 and its derivatives), or kanamycin (50 µg/ml for *E. coli* and 1,500 µg/ml for *P. aeruginosa*) (for plasmid pK18MobSacB and its derivatives) to maintain plasmids as needed. Plasmid pHF003, in which the *mexE* upstream (promoter) region is fused to a promoterless *lacZ* gene, was constructed by amplifying the *mexE* promoter region from the chromosome of *P. aeruginosa* K767 via PCR using Vent DNA polymerase (NEB) and cloning it (as an EcoRI-KpnI fragment) into pMP220. Amplification was achieved by using primers MexEPCo-F (5'-GACTGGAATTCGGCGATCCGGACAGTCTCTG-3' [the EcoRI site is underlined]) and MexEPCo-R (5'-GATCGGTACCCGCCAGGAGAAGTGGGATGA-3' [the KpnI site is underlined]) and heating at 94°C for 2 min, followed by 30 cycles of 1 min at 94°C, 1 min at 68°C (annealing temperature), and 1 min at 72°C, before finishing with a final 10-min elongation step at 72°C. The reaction mixture was formulated as described previously (48), except that dimethyl sulfoxide (DMSO) was included at a 5% (vol/vol) final concentration. The *mexE* promoter-containing fragment was first cloned into pUC19, where it was sequenced to confirm that no errors had been introduced during PCR, before it was cloned into pMP220. Plasmid pHF001, encoding MexT, was constructed by amplifying the *mexT* gene from the chromosome of *P. aeruginosa* K767 via PCR using Vent DNA polymerase (NEB) and cloning it (as a EcoRI-HindIII fragment) into pRK415. Amplification was achieved by using primers MexTEco-F (5'-GACTGGAATTCCTGTATCCGCCATGCCTG-3' [the EcoRI site is underlined]) and MexTHin-R (5'-GACTAAGCTTCGATTTTCCCGTTGCGACG-3' [the HindIII site is underlined]) and reaction conditions and parameters described previously (except that 19 cycles and an annealing temperature of 53°C were employed) for the amplification of the *mexS* gene (48). The *mexT* gene was also cloned into plasmid pMMB206 on an EcoRI-HindIII fragment following its amplification from the chromosome of *P. aeruginosa* K2153 by using primers MexT-F (5'-GACTGAATTCCTGTATCCGCCATGCCTG-3' [the EcoRI site is underlined]) and MexT-R (5'-GACTAAGCTTCGATTTTCCCGTTGCGACG-3' [the HindIII site is underlined]). Reaction mixtures were formulated and processed as described above for *mexE* except that an annealing temperature of 65.5°C was used. The gene was then excised from pMMB206 following EcoRI-HindIII digestion and cloned into plasmid miniCTX1 to generate pHF004, which was mobilized into Δ *mexT* *P. aeruginosa* strains (45). Chromosomal inserts of miniCTX::*mexT* at the phage D113 *attB* site in these strains were selected as described previously (45), with plasmid pFLP then being used to excise miniCTX sequences (45), leaving the *mexT* gene in the chromosome.

DNA protocols. Standard protocols were used for restriction endonuclease digestions, ligations, transformation, plasmid isolation, and agarose gel electrophoresis, as described previously by Sambrook and Russell (44). Plasmid DNA

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s) ^a	Reference
Strains		
<i>E. coli</i>		
DH5α	φ80 <i>dlacZ</i> Δ <i>M15 endA1 recA1 hsdR17</i> (r _K [−] m _K ⁺) <i>supE44 thi-1 gyrA96 relA1 F[−] Δ(lacZYA-argF)U169</i>	2
S17-1	<i>thi pro hsdR recA Tra⁺</i>	46
<i>P. aeruginosa</i>		
K2153	Clinical isolate	47
K2376	K2153 Δ <i>mexS</i>	This study
K2520	K2153 Δ <i>mexT</i>	This study
K2959	K2520 <i>attB::mexT</i>	This study
K2892	K2153 Δ <i>mexF</i>	This study
K2942	K2153 Δ <i>mexS</i> Δ <i>mexT</i>	This study
K2943	K2153 Δ <i>mexS</i> Δ <i>xenB</i>	This study
K2944	K2153 Δ <i>xenB</i>	This study
K2945	K2153 Δ <i>mexF</i> Δ <i>mexT</i>	This study
K2960	K2945 <i>attB::mexT</i>	This study
K2946	K2153 Δ <i>mexS</i> Δ <i>mexF</i>	This study
K2956	K2153 Δ <i>xenB</i> Δ <i>mexF</i>	This study
Plasmids		
pRK415	<i>P. aeruginosa</i> - <i>E. coli</i> shuttle cloning vector; Tc ^r	24
pHF001	pRK415:: <i>mexT</i>	This study
pK18MobSacB	Broad-host-range gene replacement vector; <i>sacB</i> Km ^r	46
pMLS002	pK18MobSacB::Δ <i>mexT</i>	48
pEX18Tc	Broad-host-range gene replacement vector; <i>sacB</i> Tc ^r	17
pRK010	pEX18Tc::Δ <i>mexS</i>	This study
pCG004	pEX18Tc::Δ <i>mexF</i>	This study
pHF002	pEX18Tc::Δ <i>xenB</i>	This study
pMP220	Transcriptional fusion vector carrying a promoterless <i>lacZ</i> gene; Tc ^r	49
pHF003	pMP220:: <i>mexE-lacZ</i>	This study
pMMB206	<i>P. aeruginosa</i> - <i>E. coli</i> shuttle cloning vector; Cm ^r	34
miniCTX1	<i>P. aeruginosa</i> chromosome integration vector; Tc ^r	18
pHF004	miniCTX:: <i>mexT</i>	This study

^a Tc^r, tetracycline resistance; Km^r, kanamycin resistance; Cm^r, chloramphenicol resistance.

was also prepared from *E. coli* or *P. aeruginosa* cells using a GeneJet plasmid miniprep kit (Fermentas) or a QIAfilter Plasmid Midi kit (Qiagen, Inc., Mississauga, Ontario, Canada) according to protocols provided by the manufacturers. Genomic DNA of *P. aeruginosa* was extracted according to a protocol described previously by Barcak et al. (4). DNA fragments or PCR products used for cloning were purified (directly or from agarose gels as warranted) by using the Wizard SV Gel and PCR Clean-Up system (Promega Corp., Madison, WI). The preparation of competent *P. aeruginosa* (6) and *E. coli* (44) cells was described previously. Plasmid pMP220 and its derivatives were mobilized into *P. aeruginosa* from *E. coli* S17-1 via conjugation as described previously (47), with plasmid-carrying *P. aeruginosa* cells selected on tetracycline (100 µg/ml) and chloramphenicol (5 µg/ml) (to counterselect *E. coli*).

Construction of gene deletions in *P. aeruginosa*. To introduce in-frame gene deletions into strains of *P. aeruginosa*, deletion constructs were first prepared in plasmid pEX18Tc by cloning PCR-amplified 1-kb DNA fragments corresponding to the regions upstream and downstream, respectively, of the gene sequences to be deleted. Typically, these constructs were amplified from the chromosome of *P. aeruginosa* strain K767 and cloned individually into pEX18Tc, where they were sequenced to verify the absence of PCR-introduced mutations before being cloned together in the same vector to generate the deletion construct. PCR mixtures were formulated as described above for the amplification of the *mexE* promoter region, except that 10% (vol/vol) DMSO was used for amplifying the

FIG. 1. Influence of *mexS* and *mexT* mutations on *mexEF-oprN* and *xenB* expression. Expression levels of *mexEF-oprN*, *xenB*, and/or *rpsL* in *P. aeruginosa* strains K2153 (wild type) (lane 1), K2376 (*MexS*[−]) (lane 2), K2492 (*MexS*[−] *MexT*[−]) (lane 3), K2492 carrying plasmid pRK415 (*MexS*[−] *MexT*[−]) (lane 4), and K2492 carrying *mexT* plasmid pHF001 (*MexS*[−] *MexT*⁺) (lane 5) (A to C) as well as strains K2153 (lane 1), K2376 (lane 2), and K2946 (*MexS*[−] *MexF*[−]) (lane 3) (D and E) were assessed by using RT-PCR. The *rpsL* reaction served as an internal control that ensured that equal amounts of RNA were employed in all of the RT-PCRs shown. The PCR portion of the reactions was carried out for 31 cycles (top) or 34 cycles (bottom) in all instances except in C, where 21 cycles (top) and 23 cycles (bottom) were used, and E, where 24 cycles (top) and 26 cycles (bottom) were used. Data are representative of at least 2 independent experiments.

ΔmexF fragments. The amplification of the *ΔmexS* fragments was achieved by heating at 94°C for 3 min followed by 34 cycles of 94°C for 0.5 min, 60°C for 0.5 min, and 72°C for 3 min, finishing with 72°C for 5 min. The amplification of the *ΔmexF* fragments was achieved by heating at 95°C for 3 min followed by 34 cycles of 94°C for 0.75 min, 62°C for 0.75 min, and 72°C for 3.5 min, finishing with 72°C for 10 min. The deletion-carrying pEX18Tc derivatives were mobilized into *P. aeruginosa* from *E. coli* S17-1 (47), and transconjugants carrying the plasmids in the chromosome were selected on L agar plates containing tetracycline (50 μg/ml) and chloramphenicol (5 μg/ml) (to counterselect *E. coli*). Tetracycline-resistant colonies were streaked onto L agar plates containing 10% (wt/vol) sucrose, and sucrose-resistant colonies were screened for the presence of the respective deletions using colony PCR (42). The construction of *ΔmexT* derivatives of *P. aeruginosa* was carried out by using pMLS002 as described previously (48).

Antimicrobial susceptibility testing. The antimicrobial susceptibilities of *P. aeruginosa* strains were assessed by using a 2-fold serial microtiter broth dilution method described previously (21), with an inoculum of 5×10^5 cells per ml. MICs were recorded as the lowest concentration of antibiotic inhibiting visible growth after an 18-h incubation at 37°C. In some experiments *s*-nitroglutathione (GSNO) (5 mM) was included in the growth medium used to determine MICs in order to assess its impact on antimicrobial susceptibility.

β-Galactosidase assay. *P. aeruginosa* strains harboring *lacZ* fusion vector pMP220 or its derivatives were cultured overnight in L broth containing tetracycline (100 μg/ml), diluted 1:49 in the same medium, and grown to log phase before being assayed for β-galactosidase activity as described previously (33). In some experiments pMP220-carrying strains were exposed to antibiotics at one-eighth the MIC (chloramphenicol, 16 μg/ml; ciprofloxacin, 0.0625 μg/ml; gentamicin, 0.5 μg/ml) for 90 min.

RT-PCR. Total bacterial RNA was isolated from log-phase *P. aeruginosa* L broth cultures by using the Ribopure Bacteria kit (Ambion) and Turbo DNase (Ambion) according to protocols provided by the manufacturer. In some in-

stances, log-phase cultures were exposed to chloramphenicol (32 μg/ml), florfenicol (64 μg/ml), GSNO (5 mM), glutathione (GSH) (5 mM), or diethylamine triamine NONOate (DETA) {also known as (Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazene-1-ium-1,2-diolate} (5 mM) for various time periods prior to RNA extraction. Reverse transcription (RT)-PCR was performed with ca. 500 ng RNA using the Qiagen One Step RT-PCR kit according to a protocol provided by the manufacturer. Primer pairs *mexE*-F (5′-GCGGGTGTCTGGGC TACATC-3′) and *mexE*-R (5′-CGGCGTCTGAAGTAGGCGTAG-3′), *xenB*-F (5′-GGCCGAGTACTACGCCAGC-3′) and *xenB*-R (5′-GCTGTCTGGAG GAATGGTC-3′), PA2811-F (5′-CCATCGTCTACCGGAGGTG-3′) and PA2811-R (5′-CACGAAGGTCGGGATGATC-3′), PA3229-F (5′-GAAACTT GCCTCTGTCTGTGTC-3′) and PA3229-R (5′-CCATTCGAGGAAGCGGAT-3′), and PA4881-F (5′-GAAAGCTTCCTGATCCTCG-3′) and PA4881-R (5′-CAGGTCGAAACGGCTCATG-3′) were used to assess the expression of the *mexE* (as a measure of *mexEF-oprN*), *xenB*, PA3229, and PA4881 genes, respectively. The reaction mixtures were incubated for 30 min at 50°C, followed by 15 min at 95°C and various cycles (see figures for details) of 0.5 min at 95°C; 0.5 min at 60°C (*mexE*), 63°C (*xenB* and PA2811), or 62.5°C (PA3229 and PA4881); and 1 min (*mexE*, *xenB*, and PA2811) or 0.5 min (PA3229 and PA4881) at 72°C, before finishing with a final 10-min elongation step at 72°C. Primers and reaction conditions for assessing *rpsL* expression were described previously (10).

RESULTS AND DISCUSSION

Coregulation of *mexEF-oprN* and a xenobiotic reductase gene, *xenB*, in a *ΔmexS* mutant. The transposon disruption of the *mexS* (also known as PA2492) gene that lies adjacent to *mexEF-oprN* regulatory gene *mexT* was previously shown to enhance *mexEF-oprN* expression and multidrug resistance in *P. aeruginosa* (48). This result was confirmed here by using an in-frame knockout of *mexS* (Fig. 1A, lane 2, and Table 2) and was further shown to be dependent upon *MexT* (Fig. 1A, lanes 3 and 5, and Table 2). The *mexS* gene encodes a “conserved hypothetical” product of unknown function annotated only as an oxidoreductase. Thus, it was impossible to predict the impact of its loss on the cell and the nature of the resultant recruitment of *MexEF-OprN*. In an attempt to gain some insights into the circumstances of *mexEF-oprN* expression in *ΔmexS* mutant strain K2376, a DNA array study of parent strain K2153 and strain K2376 was carried out. The expression of several genes was shown to be enhanced in the *ΔmexS* strain, including the 3-gene operon PA4354-PA4355-*xenB* (results were confirmed by using RT-PCR) (Fig. 1B and D, lane 2). The *xenB* expression level remained elevated in a *ΔmexS* *ΔmexE* double mutant (Fig. 1D, lane 3), indicating that its expression was directly linked to the loss of *mexS* and was not a secondary effect of *MexEF-OprN* pump overproduction (the expression of this efflux system has been shown, for example, to

TABLE 2. Impact of the *ΔmexS* mutation on antibiotic susceptibility of *P. aeruginosa*

Strain	Relevant genotype	MIC (μg/ml) of ^a :			
		CAM	NAL	NOR	TMP
K2153	Wild type	128	256	2	256
K2376	<i>ΔmexS</i>	1,024–2,048	1,024	4	1,024
K2942	<i>ΔmexS</i> <i>ΔmexT</i>	64	128	1	128
K2942	<i>ΔmexS</i> <i>ΔmexT</i>	64	128	1	256
(pRK415) ^b					
K2942	<i>ΔmexS</i>	>2,048	512–1,024	2	1,024–2,048
(pHF001) ^{b,c}					

^a CAM, chloramphenicol; NAL, nalidixic acid; NOR, norfloxacin; TMP, trimethoprim.

^b Strain K2942 carrying the indicated plasmid.

^c pHF001 equals pRK415::*mexT*.

FIG. 2. Influence of nitrosative stress on *xenB* expression. Expression levels of *xenB* and *rpsL* in *P. aeruginosa* strains K2153 (wild type) (lanes 1 and 2), K2892 (MexF⁻) (lanes 3 and 4), K2945 (MexF⁻ MexT⁻) (lanes 5 and 6), K2960 (MexF⁻ MexT⁺ [as a result of a chromosomal insertion of *mexT* at the *attB* site]) (lanes 7 and 8), and K2892 (MexF⁻) (lanes 9 and 10) without or with GSNO or DETA (5 mM for 30 min) exposure were assessed by using RT-PCR. The PCR portions of the *xenB* reactions were carried out for 26 cycles (top) or 29 cycles (bottom) (lanes 1 and 2) or for 23 cycles (top) or 26 cycles (bottom) (lanes 3 to 6). The PCR portions of the *rpsL* reactions were carried out for 18 cycles (top) or 20 cycles (bottom) (lanes 1 and 2) or for 17 cycles (top) or 19 cycles (bottom) (lanes 3 to 6). Data are representative of at least 2 independent experiments.

compromise quorum-sensing [QS] signaling and, therefore, the expression of QS-regulated genes [29]). The expression of *xenB* in the Δ *mexS* strain was compromised upon the loss of *mexT* (Fig. 1B, lane 3) and was restored following the introduction of the cloned *mexT* gene (Fig. 1B, lane 5), indicating that its expression, like that of the *mexEF-oprN* operon (Fig. 1A), was dependent upon the MexT activator. The latter result was consistent with the recently reported observation that cloned *mexT* enhances the expression of the *xenB*-containing operon (51). Several additional genes whose expression was enhanced by cloned *mexT*, including the PA2813-PA2812-PA2811 operon, encoding a probable ABC transporter, and PA4881, PA3229, PA4623, PA1970, PA2759, PA2486, and PA1744, encoding small proteins of unknown function (51), were also upregulated in the Δ *mexS* strain, although the involvement of MexT in this was not tested (data not shown).

MexT-dependent nitrosative stress inducibility of *mexEF-oprN* and *xenB*. The *xenB* gene product shows 81.4% identity to the *Pseudomonas fluorescens* XenB protein, an NADPH-dependent flavoprotein oxidoreductase shown to catalyze the removal of a nitro group from nitroglycerin and 2,4,6-trinitrotoluene (5, 37). This link to the metabolism of nitrated molecules suggested that *P. aeruginosa* XenB (and, by association, MexEF-OprN) might play a role in a nitrosative stress response, as nitrated compounds (e.g., nitrated amino acids) are a common product of nitrosative stress (9, 43). The impact of nitrosative stress on *xenB* expression in *P. aeruginosa* K2153 was assessed by exposing cells to the NO-generating compound GSNO. At 5 mM GSNO (ca. one-half the MIC for this compound), no induction of *xenB* was observed after 30 min of exposure (Fig. 2, lane 2, cf. lane 1). Still, GSNO treatment markedly induced *mexEF-oprN* expression (Fig. 3A, lane 2, cf. lane 1), as such induction required as little as a 5-min exposure to 5 mM GSNO (Fig. 3B). *mexEF-oprN* induction was also observed following 30-min (Fig. 3, lane 8, cf. lane 7) and 5-min (data not shown) exposures to a second NO-generating compound, DETA. *xenB* expression was, however, inducible by GSNO and DETA in a MexEF-OprN-deficient Δ *mexF* strain

FIG. 3. Influence of nitrosative stress on *mexEF-oprN* expression. (A) Expression levels of *mexE* and *rpsL* in *P. aeruginosa* strains K2153 (wild type) (lanes 1 and 2), K2520 (MexT⁻) (lanes 3 and 4), K2959 (MexT⁺, as a result of a *mexT* insertion at the chromosomal *attB* site) (lanes 5 and 6), and K2153 (wild type) (lanes 7 and 8) without or with GSNO or DETA (5 mM for 30 min) exposure were assessed by using RT-PCR. The PCR portion of the reactions was carried out for 29 cycles (top) or 32 cycles (bottom) for *mexE* and for 18 cycles (top) or 21 cycles (bottom) for *rpsL*. Data are representative of at least 2 independent experiments. (B) Expression of *mexE* and *rpsL* in *P. aeruginosa* strain K2153 without (lane 1) or with (lanes 2 to 4) GSNO (5 mM) exposure for 5 min (lane 2), 15 min (lane 3), or 30 min (lane 4). The PCR portion of the reactions was carried out for 27 cycles (top) or 30 cycles (bottom) for *mexE* and for 20 cycles (top) or 23 cycles (bottom) for *rpsL*. Data are representative of at least 2 independent experiments.

(Fig. 2, lane 4, cf. lane 10, and lane 3, cf. lane 9). GSNO induction of *mexEF-oprN* and *xenB* was MexT dependent, being abrogated in Δ *mexT* (Fig. 3A, lane 4, cf. lane 3) and Δ *mexF* Δ *mexT* (Fig. 2, lane 6, cf. lane 5) mutants, respectively, and restored in these mutants following the introduction of the cloned *mexT* gene into the chromosomes of these Δ *mexT* strains (Fig. 3A, lane 6, cf. lane 5, and Fig. 2, lane 8, cf. lane 7). Complementation using single-copy chromosomal *mexT* was necessary, since multicopy plasmid-borne *mexT* was shown to markedly enhance the expression of these genes (and increase antimicrobial resistance) in the absence of GSNO exposure (data not shown), in agreement with previously reported results (51). Still, while enhanced *mexT* levels appear to be sufficient for increased *mexEF-oprN* and *xenB* expression levels, GSNO did not induce *mexT* expression (data not shown), and thus, the effect of GSNO treatment cannot be explained by indirect regulation through MexT dosage. Glutathione (GSH), which lacks the reactive NO moiety, failed to induce *mexEF-oprN* or *xenB*, indicating that the recruitment of MexEF-OprN and XenB in response to GSNO was related to the chemical reactivity of the molecule and not its structure. The observation that the nitrosative stress induction of *xenB* was seen only for a Δ *mexF* strain lacking a functional MexEF-OprN pump suggests that the inducer and/or substrate for XenB may well be a substrate for MexEF-OprN and that only in the absence of

FIG. 4. Influence of nitrosative stress on expression of PA2214 (left), PA3229 (middle), and PA4881 (right). (Left) Expression levels of PA2214 and *rpsL* in *P. aeruginosa* strain K2892 (MexF⁻) without (lane 1) or with (lane 2) GSNO (5 mM for 30 min) exposure were assessed by using RT-PCR. The PCR portion of the reactions was carried out for 28 cycles (top) or 31 cycles (bottom) for PA2214 and for 17 cycles (top) or 20 cycles (bottom) for *rpsL*. (Middle and right) Expression levels of PA3229 (middle), PA4881 (right), and *rpsL* (bottom middle and bottom right) in *P. aeruginosa* strain K2153 (wild type) without (lane 1) or with (lane 2) GSNO (5 mM for 30 min) exposure were assessed by using RT-PCR. The PCR portion of the reactions was carried out for 28 cycles (top) or 31 cycles (bottom) for PA3229 and PA4881 and for 20 cycles (top) or 23 cycles (bottom) for *rpsL*. PA2214 was not induced by GSNO in wild-type strain K2153. Data are representative of at least 2 independent experiments.

this efflux system does it accumulate sufficiently to induce the *xenB* operon. These observations were consistent with XenB and MexF-OprN playing a common role in a nitrosative stress response in *P. aeruginosa*. Interestingly, the expressions of the putative ABC transporter operon (PA2813-PA2812-PA2811) as well as the PA4881 and PA3229 genes (as representatives of the MexT-regulated small-protein genes described above) were also induced by GSNO (Fig. 4), suggesting that the previously defined MexT regulon (51) plays a role in the nitrosative stress response of *P. aeruginosa*.

MexT-dependent chloramphenicol inducibility of *mexEF-oprN*. Given the previous observation that chloramphenicol readily selects *mexS* mutants overproducing *mexEF-oprN* *in vitro* (48) and that chloramphenicol is a substrate of the MexEF-OprN pump (28), it was of interest whether MexEF-OprN might naturally respond to this antimicrobial. To assess this, the impact of chloramphenicol exposure on *mexEF-oprN* expression was assessed. As shown in Fig. 5 (lane 2, cf. lane 1) and 6, chloramphenicol (at one-quarter its MIC) induced *mexEF-oprN* expression, and this was dependent on MexT: the

FIG. 5. Influence of chloramphenicol on *mexEF-oprN* expression. Expression levels of *mexE* and *rpsL* in *P. aeruginosa* strains K2153 (lanes 1 and 2) and K2520 (MexT⁻) (lanes 3 and 4) without (odd-numbered lanes) or with (even-numbered lanes) chloramphenicol (32 µg/ml for 90 min) exposure were assessed by using RT-PCR. The PCR portion of the reactions was carried out for 30 cycles (top) or 32 cycles (bottom) for *mexE* and for 21 cycles (top) or 23 cycles (bottom) for *rpsL*. Data are representative of at least 2 independent experiments.

FIG. 6. Influence of antimicrobials on *mexEF-oprN* expression. The β-galactosidase activity of *P. aeruginosa* K2153 (wild type [WT]) carrying *mexE-lacZ* fusion vector pHF003 was assessed following exposure to chloramphenicol (CAM) (16 µg/ml), ciprofloxacin (CIP) (0.625 µg/ml), or gentamicin (GEN) (0.5 µg/ml) for 30 min. —, no antimicrobial exposure. The β-galactosidase activity of *P. aeruginosa* K2376 (MexS⁻) (*mexS*) harboring pHF003 is included for comparison purposes. The data have been corrected for the background activity of strains carrying the pMP220 *lacZ* fusion vector without *mexE* and are reported as the means ± standard deviations of data from at least 2 independent experiments. Individual unexposed or wild-type controls are included for each antibiotic-exposed culture of K2153 or K2376.

induction was lost in a Δ*mexT* mutant (Fig. 5, lane 4, cf. lane 3). Another MexEF-OprN antimicrobial substrate, ciprofloxacin, did not induce *mexEF-oprN* expression (Fig. 6). The aminoglycoside gentamicin, which, like chloramphenicol, targets the ribosome, also failed to induce *mexEF-oprN*, indicating that *mexEF-oprN* responds specifically to chloramphenicol and not to ribosome disruption. *xenB* was not induced by chloramphenicol in strain K2153 or its Δ*mexF* mutant strain K2520 (data not shown). As with GSNO as well, the chloramphenicol induction of this efflux operon was seen following as little as 15 min of exposure at one-quarter the MIC (data not shown). The apparently unique induction of *mexEF-oprN* by chloramphenicol (and its ready selection of MexEF-OprN-expressing multidrug-resistant mutants [48]) was suggestive of this antimicrobial being or, more likely, mimicking a natural or intended substrate for this efflux system. Given its nitroaromatic nature and the fact that MexEF-OprN is induced by nitrosative stress, one obvious possibility is that chloramphenicol resembles a nitrosative stress product that may be deleterious to the cell and is thus removed by this efflux system. Consistent with this, an amphenicol derivative resembling chloramphenicol but lacking the nitro moiety, florfenicol, failed to induce *mexEF-oprN* expression (90 min of exposure) (data not shown). In addition, a

mutant lacking *mexF* did not show any increase in florfenicol susceptibility (MICs for the parent strain and mutant were both 256 µg/ml), indicating that unlike chloramphenicol, the “nitro-free” florfenicol is not a substrate for the MexEF-OprN pump.

MexEF-OprN and XenB do not contribute to GSNO resistance. Given their induction by nitrosative stress (i.e., GSNO and DETA), it was of interest whether MexEF-OprN and/or XenB contributed to GSNO “resistance” directly. However, in looking at mutants that overexpress these genes (e.g., Δ *mexS* mutant strain K2376) or lack them individually (Δ *mexEF-oprN* mutant strain K2892 and Δ *xenB* mutant strain K2892) or jointly (strain K2956), no difference in GSNO susceptibility was seen relative to parent strain K2153 (an MIC of 12.5 mM was observed for all strains). This was also true for K2376 derivatives lacking *xenB* (K2943) or *mexEF-oprN* (K2946).

Conclusion. GSNO and DETA, NO-generating agents of nitrosative stress, and chloramphenicol are, with the exception of a furanone analogue, the only known “inducers” of the *mexEF-oprN* efflux operon and the only inducers reported to act via MexT. Still, it is as yet unclear whether they or some product(s) of nitrosative stress or damage caused by reactive nitrogen species like NO is the actual effector. Chloramphenicol being a nitrosated aromatic compound and possible mimic of a nitrosated product of nitrosative stress is certainly consistent with this. From the standpoint of antimicrobial resistance, there is the prospect that nitrosative stress/reactive nitrogen could, *in vivo*, provide a selective pressure for MexEF-OprN-producing multidrug-resistant mutants. Airway epithelial cells are, for example, known to produce NO upon stimulation by bacteria and bacterial products (e.g., lipopolysaccharide [LPS]) (14), and a previously reported *in vitro* transcriptome study of *P. aeruginosa* exposed to normal human epithelial cells revealed that *mexEF-oprN* (and *mexS*) was substantially upregulated (11). In addition, the observation that several genes identified previously as being members of a MexT regulon are induced in response to nitrosative stress suggests that MexT targets are components of a nitrosative stress response in *P. aeruginosa*.

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