ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 2006, p. 1633–1641 Vol. 50, No. 5
0066-4804/06/$08.00+0 doi:10.1128/AAC.50.5.1633–1641.2006

Interplay of Efflux System, *ampC*, and *oprD* Expression in Carbapenem Resistance of *Pseudomonas aeruginosa* Clinical Isolates

John Quale,* Simona Bratu, Jyoti Gupta, and David Landman

Division of Infectious Diseases, SUNY-Downstate Medical Center, 450 Clarkson Avenue, Box 77, Brooklyn, New York 11203

Received 17 January 2006/Returned for modification 17 February 2006/Accepted 28 February 2006

Carbapenems are important agents for the therapy of infections due to multidrug-resistant *Pseudomonas aeruginosa*; the development of carbapenem resistance hampers effective therapeutic options. To assess the mechanisms leading to resistance, 33 clinical isolates with differing degrees of carbapenem susceptibility were analyzed for the expression of the chromosomal β-lactamase (*ampC*), the porin that is important for the entry of carbapenems (*oprD*), and the proteins involved in four efflux systems (*mexA*, *mexC*, *mexE*, and *mexX*). Real-time reverse transcriptase PCR was performed using primers and fluorescent probes for each of the target genes. The sequencing of regulatory genes (*ampR*, *mexR*, *nalC*, *nalD*, *mexT*, and *mexZ*) was also performed. Diminished expression of *oprD* was present in all imipenem- and meropenem-resistant isolates but was not required for ertapenem resistance. Increased expression of *ampC* was not observed in several isolates that were overtly resistant to carbapenems. Increased expression of several efflux systems was observed in many of the carbapenem-resistant isolates. Increased efflux activity correlated with high-level ertapenem resistance and reduced susceptibility to meropenem and aztreonam. Most isolates with increased expression of *mexA* had mutations affecting *nalC* and/or *nalD*. Two isolates with mutations leading to a premature stop codon in *mexZ* had markedly elevated *mexX* expressions, although mutations in *mexZ* were not a prerequisite for overexpression. β-Lactam resistance in clinical isolates of *P. aeruginosa* is a result of the interplay between diminished production of *oprD*, increased activity of *ampC*, and several efflux systems.

Both acquired and intrinsic mechanisms of antibiotic resistance make *Pseudomonas aeruginosa* a formidable nosocomial pathogen (19). Carbapenem antibiotics remain important agents for the therapy of serious infections due to multidrug-resistant *P. aeruginosa*; the development of carbapenem resistance severely compromises effective therapeutic options. In the absence of carbapenem-hydrolyzing enzymes, the mechanism leading to carbapenem resistance is usually multifactorial. Increased chromosomal cephalosporinase activity, reduced porin expression, and augmented antibiotic extrusion have all been defined as contributory factors (18, 19). The outer membrane protein OprD allows entry of carbapenems, and its reduced expression is frequently noted in carbapenem-resistant isolates (18, 33).

Several antibiotic efflux systems that belong to the resistance-nodulation-division family and contribute to multidrug resistance have been characterized in *P. aeruginosa*. The MexAB-OprM system is constitutively expressed in virtually all isolates, and substrates for this pump include fluoroquinolones, tetracycline, chloramphenicol, and β-lactams (including carbenicillin, piperacillin, ceftazidime, cefepime, and aztreonam) (17, 27, 40, 46, 50). Imipenem does not appear to be a substrate for MexAB-OprM, but because of its hydrophobic side chain, meropenem can be affected by this system (13, 17, 33). The expression of the MexAB-OprM system is negatively controlled by the repressor MexR, and derepression of the *mexAB-oprM* operon may be caused by mutations in the *mexR* gene (1, 5, 22, 37, 39, 40, 46, 51). However, it is evident that other control mechanisms are involved in *mexAB-oprM* expression (22, 24, 37, 42, 46, 51). Mutational inactivation of a second regulatory gene, *nalC*, increased the expression of an ill-defined protein, PA3719, which then increased MexAB-OprM expression (4). Although selected mutations have been observed in clinical isolates, the same mutations have also been observed in wild-type strains (22). A third regulatory gene of the *mexAB-oprM* operon, *nalD*, has also been identified; mutations in this gene have been observed in clinical isolates overexpressing MexAB-OprM (43).

Fluoroquinolones and the antipseudomonal β-lactams piperacillin, cefepime, and meropenem (but not carbenicillin, aztreonam, ceftazidime, or imipenem) are substrates for the MexCD-OprJ system, although this efflux pump is not typically expressed under normal growth conditions (27, 36). A third efflux system, MexEF-OprN, can export fluoroquinolones, trimethoprim, and chloramphenicol and is positively regulated by *mexT* (12, 14, 26). Although mutations converting MexT from nonfunctional to functional have been recovered in isolates overexpressing *mexEF-oprN*, in some isolates, no changes have been detected (12, 25). The disruption of a second recently described regulator, MexS, can also increase the expression of *mexEF-oprN* (45). MexT can also function as a negative regulator for the expression of other proteins, including OprD (mediating imipenem resistance) and MexAB-OprM (leading to β-lactam hypersusceptibility) (12, 24, 32). Finally, MexXY-OprM expression can be induced with growth in tetracycline or aminoglycosides (2, 28, 29). MexXY-OprM may contribute to fluoroquinolone, aminoglycoside, and selected β-lactam (piperacillin, cefepime, and meropenem but not carbenicillin, ceftazidime, or imipenem) resistance (2, 27, 29, 44, 48). The expression of *mexXY* is negatively regulated by the *mexZ* gene.

* Corresponding author. Mailing address: Division of Infectious Diseases, Box 77, SUNY-Downstate, 450 Clarkson Avenue, Brooklyn, NY 11203. Phone: (718) 270-2148. Fax: (718) 270-2465. E-mail: jquale@downstate.edu.

Mutations involving *mexZ* have been noted in clinical isolates overexpressing MexXY that were obtained from patients with cystic fibrosis (47). However, not all isolates with increased expression of *mexXY* carry mutations in *mexZ*, suggesting that other regulatory mechanisms are involved (44, 47). Antibiotics that inhibit ribosomal function have also been found to induce *mexXY* expression, although additional mechanisms may be involved (10).

Virtually all studies examining the mechanisms of carbapenem resistance in *P. aeruginosa* have involved carefully controlled laboratory-derived strains. Studies involving clinical isolates have not clearly defined the contributions of the AmpC cephalosporinase, porin defects, and efflux systems in carbapenem resistance (9, 15, 33, 51). Indeed, the transcriptional responses of clinical isolates to carbapenem exposure have been described as "unpredictable and chaotic" (15). In this report, we examine the mechanisms of carbapenem (and in particular, ertapenem) resistance in clinical isolates of *P. aeruginosa* from New York City.

MATERIALS AND METHODS

Bacterial isolates. Isolates were gathered from citywide surveillance studies performed from 1997 to 2003 as previously described (16). During these studies, unique patient isolates of *P. aeruginosa* were collected from the microbiology laboratories from hospitals in Brooklyn, NY. Organisms were identified by the individual laboratories by using standard techniques. MICs for carbapenem antibiotics were performed in the central research laboratory using the Etest method. Susceptibility breakpoints were defined according to CLSI (formerly NCCLS) recommendations (30). The following β-lactam antibiotics were tested: imipenem, meropenem, ertapenem, ceftazidime, cefepime, and aztreonam.

The 33 isolates chosen for study were selected based on their carbapenem susceptibilities. Isolates with differing degrees of carbapenem susceptibility were selected, including some more resistant to imipenem than to meropenem, some more resistant to meropenem than to imipenem, some resistant only to ertapenem, and some susceptible to all three carbapenems. Isolates were analyzed according to their expression of *oprD*, *ampC*, and the efflux systems. For all experiments, *P. aeruginosa* ATCC 27853 served as the control microorganism. Strain relatedness was determined by ribotyping using the RiboPrinter microbial characterization system (Qualicon, Wilmington, DE) as previously described (16).

β-Lactamase analysis. Isoelectric focusing of β-lactamases was performed according to previously established techniques (38). Crude cell extracts were analyzed on 3% polyacrylamide gels containing ampholytes (pH range, 3 to 10). The β-lactamases were detected by overlaying the gel with nitrocefin (0.5 mg/ml). PCR investigations regarding β-lactamases (TEM, SHV, OXA, VIM, and IMP) were performed by using primers and conditions as previously described (8, 31, 34).

Crude cell extracts from selected isolates were also analyzed for β-lactamase activity. Extracts were prepared following 3 h of incubation in LB broth with or without cefoxitin (50 μg/ml) as previously described (11); results were expressed as nanomoles of nitrocefin hydrolyzed per minute per milligram of protein. Protein concentrations were determined by using the Bradford reagent. Extracts from selected isolates were also assessed spectrophotometrically (at an optical density at 299 nm) for evidence of carbapenem hydrolysis. Carbapenem hydrolysis was analyzed using imipenem (100 μM) in 50 mM HEPES buffer (pH 7.5) supplemented with Zn^{2+} (50 μM).

Outer membrane protein analysis. Bacterial outer membrane proteins were examined using previously reported methods (38). Following sonication, membranes were collected by ultracentrifugation at 100,000 × *g* for 35 min. Outer membrane proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and gels were stained with Coomassie blue.

Real-time RT-PCR studies. The expression of *rpoD*, *ampC*, *oprD*, *mexA*, *mexC*, *mexE*, and *mexX* was determined by using real-time reverse transcriptase PCR (RT-PCR). An overnight culture was diluted 1:100 in Mueller-Hinton broth and grown to the late log phase of growth (6). RNA was isolated using the RNeasy kit (QIAGEN, Inc.) and treated with DNase according to the manufacturer's instructions. Total RNA concentration was determined fluorometrically by using RiboGreen (Molecular Probes, Eugene, OR). Real-time RT-PCRs were performed and analyzed by using the MX3000P real-time PCR system (Stratagene, La Jolla, CA). The primers and hydrolysis probes used for real-time RT-PCR studies are given in Table 1. Real-time RT-PCR experiments were performed using the Brilliant quantitative RT-PCR master mix (Stratagene). Samples were run in triplicate and contained 50 ng of RNA per reaction. Virtually all individual results were within 0.5 cycle threshold units of the average triplicate value. Controls run without reverse transcriptase confirmed the absence of contaminating DNA in any of the samples. For the probes, the reporter dye was 6-carboxyfluorescein (DFAM) and the quencher dye was 6-carboxytetramethylrhodamine (DTAM); carboxy-X-rhodamine was included as a reference dye. Primer and probe concentrations were adjusted to provide amplification efficiencies of approximately 90 to 110% for all experiments. The expression of mRNA for the genes of interest was normalized to that of the housekeeping gene *rpoD*. This gene has been demonstrated to be consistently expressed in *P. aeruginosa* (41), and preliminary experiments demonstrated stable expression in our isolates (data not shown). Normalized expression of each gene of interest was calibrated against corresponding mRNA expression by *P. aeruginosa* ATCC 27853; results are given as the relative expression of the mRNA compared to that of *P. aeruginosa* 27853.

To insure that nonspecific amplification did not occur, products were resolved on a 1% agarose gel and stained using ethidium bromide. No additional PCR products were noted for any of the reactions.

Regulatory gene studies. To search for possible mutations affecting important regulatory genes, the following genes were sequenced: *ampR*, *mexR*, *nalC*, *nalD*, *mexT*, and *mexZ*. The primers used to amplify and sequence the regulatory genes and the intergenic regions are listed in Table 1. For *mexR* and *mexT* amplifications, the PCR conditions were 95°C for 15 min followed by 35 cycles of 95°C for 30 s, 56°C for 90 s, and 72°C for 60 s; this was followed by a final extension at 72°C for 10 min. PCR conditions for the amplification of *nalC* included 94°C for 10 min followed by 30 cycles of 95°C for 60 s, 56°C for 60 s, and 72°C for 60 s; this was followed by a final extension at 72°C for 10 min. Conditions for the amplification of *ampR*, *nalD*, and *mexZ* were as previously described (3, 43, 44). For selected isolates, amplification of the *oprD* gene was performed by using previously established primers and conditions (35).

Amplified products underwent bidirectional sequencing using the automated fluorescent dye terminator sequencing system (Applied Biosystems, Foster City, CA). Sequences were compared to those of the PAO1 strain of *P. aeruginosa* by using the BLAST program from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST/). Sequences of *mexT* were compared to the genomic sequences of PAO1 characterized by Kohler et al. (12).

Statistical methods. Fisher's exact test and Student's *t* test were used to compare groups. A two-tailed *P* value of ≤0.05 was considered significant. For statistical analysis, the following values were considered to represent overexpression compared to the control strain: for *ampC*, ≥10-fold; for *mexA*, ≥2-fold, and for *mexX*, ≥10-fold. These values are within the ranges noted in other studies (9, 22, 33, 49).

This study was approved by the Institution Review Board at SUNY-Downstate.

RESULTS AND DISCUSSION

The 33 clinical isolates originated from eight different hospitals in Brooklyn, NY. Susceptibility results, ribotyping data, and genetic expression data (relative to control strain *P. aeruginosa* ATCC 27853) are given in Table 2. Based on the RT-PCR results, isolates were placed into three groups.

Group 1 isolates were those with diminished *oprD* and increased *ampC* expression. Diminished OprD production was defined primarily according to the real-time RT-PCR studies. For the isolates that failed to have amplification of *oprD*, the presence or absence of an ~46-kDa protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of outer membrane proteins was used to classify isolates. Group 2 isolates were those with diminished *oprD* and unchanged *ampC* expression, and group 3 isolates were those with unchanged *oprD* and unchanged *ampC* expression.

Nineteen ribotypes were identified. Eight isolates belonged to ribotype A; five of the eight were nonsusceptible to all three carbapenems. Three ribotypes consisted of three isolates each,

TABLE 1. DNA sequences used in the real-time RT-PCR experiments and regulatory gene characterization studies

Study and gene	Primer or probe	Primer or probe	Reference
Real-time RT-PCR experiments			
rpoD	For	5′-GGGCTGTCTCGAATACGTTGA-3′	This study
	Rev	5′-ACCTGCCGGAGGATATTTCC-3′	
	Probe	[DFAM]TGCGGATGATGTCTTCCACCTGTTCC[DTAM]	
ampC	For	5′-CGCCGTACAACCGGTGAT-3′	This study
	Rev	5′-CGGCCGTCCTCTTTCGA-3′	
	Probe	[DFAM]TCAGCCTGAAAGGAGAACCGCATTACTTC[DTAM]	
oprD[a]	For	5′-CTACGGCTACGGCGAGGAT-3′	This study
	Rev	5′-GACCGGACTGGACCACGTACT-3′	
	Probe	[DFAM]CACCACGAAACCAACCTCGAAGCC[DTAM]	
mexA	For	5′-AACCCGAACAACGAGCTG-3′	This study
	Rev	5′-ATGGCCTTCTGCTTGACG-3′	
	Probe	[DFAM]CATGTTCGTTCACGCGCAGTTG[DTAM]	
mexC	For	5′-GGAAGAGCGACAGGAGGC-3′	This study
	Rev	5′-CTGCACCGTCAGGCCCTC-3′	
	Probe	[DFAM]CCGAAATGGTGTTGCCGGTG[DTAM]	
mexE	For	5′-TACTGGTCCTGAGCGCCT-3′	This study
	Rev	5′-TCAGCGGTTGTTCGATGA-3′	
	Probe	[DFAM]CGGAAACCACCCAAGGCATG[DTAM]	
mexX	For	5′-GGCTTGGTGGAAGACGTG-3′	This study
	Rev	5′-GGCTGATGATCCAGTCGC-3′	
	Probe	[DFAM]CCGACACCCTGCAGGGCC[DTAM]	
Regulatory gene characterization studies			
ampRC2	For	5′-CTCGAGAGCGAGATCGTTGCGGCACG-3′	3
amprRC1	Rev	5′-GTCGACCAGTGCCTTCAGGCGATCC-3′	
mexR	For	5′-TGTTCTTAAATATCCTCAAGCGG-3′	This study
	Rev	5′-GTTGCATAGCGTTGTCCTCA-3′	
nalC	For	5′-TCAACCCTAACGAGAAACGCT-3′	This study
	Rev	5′-TCCACCTCACCGAACTGC-3′	
nalD	For	5′-GCGGCTAAAATCGGTACACT-3′	42
	Rev	5′-ACGTCCAGGTGGATCTTGG-3′	
mexT	For	5′-AAAACCACCCGTCGTTATTG-3′	This study
	Rev2	5′-CAGTTCGTCGGTGTAGCTGA-3′	
	For2	5′-TCAGCTACACCGACGAACTG-3′	
	Rev	5′-GGGATGACTGTTCCAT-3′	
mexZ	For	5′-ATTGGATGTGCATGGGTG-3′	43
	Rev	5′-TGGAGATCGAAGGCAGC-3′	

[a] For two isolates with nonamplifiable *oprD*, the nucleotide sequence corresponding to the forward primer was (changes underlined) GGGCCTCTACGGCGAG GAC; that corresponding to the reverse primer was GGCCGGCCTGGACGACGTACT; and that corresponding to the probe was CACCACGAGACCAACCTG GAAGCC.

1 ribotype had two isolates, and the remaining 14 ribotypes had one isolate each.

β-Lactamase studies. Most isolates demonstrated a β-lactamase with a pI of >8, which is consistent with the class C chromosomal cephalosporinase. Seven of the eight isolates belonging to ribotype A possessed additional β-lactamases with pI values of 7.2 and 7.8. These β-lactamases did not amplify with primers specific for the TEM-, SHV-, IMP-, VIM-, and OXA-type enzymes. None of these isolates demonstrated spectrophotometric evidence of carbapenem-hydrolyzing activity (data not shown); the identification of these β-lactamases remains unknown. One additional isolate possessed a β-lactamase with a pI of 5.4; this enzyme was subsequently identified as TEM-1.

TABLE 2. Ribotyping, susceptibility, SDS-PAGE of outer membrane proteins, and mRNA expression studies for the clinical isolates of *P. aeruginosa*

Group and isolate	Ribotype	Susceptibility value[a] (μg/ml) for:						46-kDa protein present[b]	mRNA expression[c] for:					
		IPM	MEM	Ertapenem	CAZ	FEP	ATM		*oprD*	*ampC*	*mexA*	*mexC*	*mexE*	*mexX*
Group 1														
AM602	J	4	8	>32	>32	>256	>256	No	0.71	29	3.5	1.8	0.19	0.06
FI506	H	8	2	16	4	1	2	No	0.16	66	0.72	0	0.01	0.95
BK546	A	>32	16	>32	>32	32	8	ND[d]	0.04	480	0.52	0.07	0.20	6.7
HW311	A	4	8	>32	>32	>256	>256	No	0.70	970	2.8	0.17	0.01	19
DB322	A	32	16	>32	>32	>256	64	No	0.17	2,400	3.5	0.46	0.22	20
AM339	E	32	32	>32	>32	>256	>256	No	0.28	290	5.0	2.5	0.66	23
BK553	A	32	4	>32	16	>256	2	No	0.09	610	0.41	0.08	0	24
BK501	A	32	16	>32	>32	64	32	No	0.09	930	1.4	4.0	0.76	29
AM609	I	8	0.5	>32	2	16	8	No	NA[e]	2,600	1.9	0.17	0.05	29
BK545	A	>32	16	>32	>32	32	4	No	0.13	270	1.1	0.87	0.24	32
DB513	N	2	2	>32	8	64	32	Yes	0.46	1,500	3.1	2.0	0.60	43
CK575	F	32	16	>32	>32	16	4	No	0.56	170	0.88	2.9	0.41	53
DB537	A	>32	16	>32	>32	16	2	No	0.14	380	0.87	0.20	0	88
Group 2														
CK505	C	2	0.5	8	4	2	4	No	0.19	0.70	1.5	9.0	1.1	13
UL118	C	8	16	>32	2	8	4	No	0.02	1.3	0.96	0.67	0	9.6
CK558	D	16	2	>32	4	16	4	No	0.38	2.0	1.0	0.84	1.4	18
UL517	E	>32	32	>32	16	64	16	No	0.35	2.4	0.90	0.95	1.8	3.8
BK555	G	32	4	>32	2	8	4	No	NA	2.4	1.6	0.15	0	31
CK550	D	8	2	>32	4	4	16	Yes	0.27	6.2	1.4	0	2.8	73
Group 3a														
MD519	O	2	≤0.12	1	2	8	2	Yes	NA	2.1	1.0	1.0	0.23	35
MD504	P	2	0.25	2	2	2	4	Yes	NA	1.8	1.8	1.8	0.65	5.7
CK516	R	2	≤0.12	2	2	1	2	Yes	NA	3.5	2.6	1.4	0.66	1.1
MD521	Q	2	0.25	2	2	2	4	Yes	NA	3.8	1	1.6	0.67	1.1
CK503	S	1	≤0.12	2	2	2	4	Yes	1.02	4.0	1.8	9.4	1.7	4.0
DB504	A	2	0.25	2	4	2	2	Yes	0.943	7.5	1.4	4.4	0.71	4.4
Group 3b														
MD119	M	2	4	>32	4	8	8	Yes	1.2	2.7	2.4	2.2	0	5.7
CK188	B	2	4	>32	8	16	16	Yes	4.1	0.31	3.9	3.0	0.43	5.8
CK140	B	2	4	>32	8	16	32	Yes	11	5.5	2.2	2.1	0.21	6.9
MD104	B	2	2	>32	8	16	16	Yes	10	0.41	2.2	16	0.55	16
UL140	L	2	4	>32	4	16	8	Yes	2.4	5.1	13	9.3	1.6	330
AM128	K	2	4	16	2	4	8	Yes	NA	3.6	0.71	0.29	0.41	2.8
MD510	C	2	1	16	1	2	2	ND	0.81	2.6	0.97	2.7	0.40	5.0
DB311	D	2	2	>32	2	4	4	Yes	1.8	1.4	1.7	0.77	1.2	6.6

[a] MEM, meropenem; CAZ, ceftazidime; FEP, cefepime; ATM, aztreonam; IPM, imipenem.
[b] Determined by SDS-PAGE of outer membrane proteins.
[c] Relative to the expression of *P. aeruginosa* ATCC 27853, which is assigned a value of 1.
[d] ND, not determined.
[e] NA, not amplifiable.

Porin OprD studies. Seven isolates, including four that were susceptible to the three carbapenems, did not have detectable *oprD* mRNA expression. Amplification of the entire *oprD* gene from template DNA, using primers distinct from the internal primers used for the RT-PCR studies, was unsuccessful in five isolates, suggesting the presence of multiple mutations in this gene. Two isolates without detectable *oprD* mRNA expression had the entire *oprD* gene amplified and sequenced. Mutations were evident in the region targeted by the RT-PCR primers (Table 1). Therefore, for these seven isolates with undetectable mRNA *oprD* expression, it is likely that mutations in the *oprD* gene prevented the measurement of expression. Isolates also had OprD production assessed by SDS-PAGE of outer membrane proteins; 13 of the 14 imipenem-nonsusceptible isolates lacked an ~46-kDa protein, which is compatible with OprD (the one isolate with this protein had an MIC of imipenem of 8 μg/ml). Of the seven isolates that had nonamplifiable *oprD* genes during the RT-PCR studies, five were susceptible to carbapenems and had the 46-kDa protein by SDS-PAGE. The other two isolates were nonsusceptible to imipenem and lacked this protein.

Relationship of carbapenem resistance with OprD, AmpC, and efflux pump activity. Nineteen isolates were considered to have reduced *oprD* expression, including 17 isolates with relative *oprD* expressions of ≤70% of those of *P. aeruginosa* ATCC 27853 and two isolates with nonamplifiable *oprD* and the absence of the 46-kDa protein as shown by SDS-PAGE (Table 2). All 17 isolates that were nonsusceptible to imipenem and/or meropenem had reduced expressions of *oprD* relative to 2 of 16 isolates that were susceptible to both antibiotics ($P <$

0.001). While ertapenem-resistant isolates were also more likely to have reduced *oprD* expressions ($P = 0.003$), eight resistant isolates had normal expressions of this porin. Similarly, of the 25 ertapenem-resistant isolates that had outer membrane proteins analyzed by SDS-PAGE, 11 had the 46-kDa protein evident (data not shown).

Of the 19 isolates with reduced *oprD* expression, 13 isolates (group 1) (Table 2) also had increased *ampC* expression. All 13 were resistant to ertapenem, and 10 and 9 were nonsusceptible to imipenem and meropenem, respectively. Most group 1 isolates had increased *mexX* and some had increased *mexA* expression, although their contributions to carbapenem resistance are difficult to assess in this group. Nine and 12 isolates were resistant to ceftazidime and cefepime, respectively, but only 6 were resistant to aztreonam. The six aztreonam-resistant isolates were more likely to have increased expression of *mexA* than were the seven susceptible isolates ($P = 0.005$).

Six isolates (group 2) (Table 2) had diminished *oprD* activity without increased expression of *ampC*. Five of the six were nonsusceptible to imipenem and/or meropenem, and all were resistant to ertapenem in this group. Five of six group 2 isolates had increased expression of *mexX*, but there was no consistent increased activity of the other efflux systems. Most remained susceptible to cephalosporins and aztreonam.

Fourteen isolates had *oprD* and *ampC* expressions similar to those of the control. Six isolates were susceptible to all β-lactams tested (group 3a) (Table 2); efflux activity also tended to be similar to that of the controls in this group. The eight remaining isolates with *oprD* and *ampC* expressions similar to that of the control (group 3b) (Table 2) were resistant to ertapenem, had uncharacteristically elevated MICs of meropenem (compared to imipenem), and tended to have higher MICs of aztreonam. Five of the eight had increased *mexA* expression. These isolates also tended to have increased expression of *mexC* (at least twofold times that of the control) and/or *mexX*. Compared to group 3a, group 3b isolates were more likely to have increased expression of two or more efflux systems (five of eight versus none of six; $P = 0.03$). For these isolates with normal *oprD* and *ampC* expressions, a global increase of efflux activity was associated with reduced susceptibility to ertapenem, meropenem, and aztreonam. Two isolates (AM128 and DB311) did not have increased efflux pump expressions; the mechanisms of resistance in these two isolates remain undefined.

Regulatory gene studies. Ten isolates had various combinations of mutations in the regulatory gene *ampR* leading to amino acid substitutions (Table 3); none of the changes correlated with altered *ampC* expression. Similarly, three isolates had single base substitutions of either 87 or 143 nucleotides upstream from the *ampC* start codon that were not correlated with *ampC* expression. Nine isolates failed to have an induction of *ampC* following incubation with cefoxitin; seven of these isolates belonged to a single ribotype (Table 3). Mutations in AmpR did not correlate with the ability of *ampC* to be induced following exposure to cefoxitin.

There were no mutations in the regulatory gene *mexR* that correlated with increased *mexA* expression. Eleven isolates had a Val_{126}→Glu change in MexR; this amino acid substitution has been found in clinical isolates of *P. aeruginosa* and has been considered insignificant (22, 51). There were no mutations detected in the purported −10 and −35 promoter regions for either *mexR* or *mexA* (5). Four isolates had mutations in the distal binding site for *mexR* (5); however, *mexA* expression in these isolates was similar to that in isolates lacking the mutation. Two isolates had 5-bp and 4-bp deletions leading to a frameshift following Ala_{145} and Gln_{162}, respectively, in NalC; both of these isolates had increased expressions of *mexA*. Six isolates had mutations affecting NalD that correlated with increased *mexA* expressions; one isolate with an Asp_{147}→Asn change did not have altered *mexA* expression. Overall, of the 11 isolates with increased expressions of *mexA*, eight had mutations in *nalD* and/or frameshift mutations in *nalC* that could explain the increased expressions. The remaining three isolates had *mexA* activity just above the limit defining increased expression (a 2.2- to 2.6-fold increase over that of the control).

All of the clinical isolates possessed a Leu_{26}→Val substitution in the MexT regulatory protein, which likely did not affect MexT function (25). The second half of the *mexT* gene and the intergenic region could not be amplified in seven isolates (despite attempts with different nonoverlapping primers), suggesting major mutations affecting this region. Of the seven isolates with altered MexT, all belonged to ribotype A. Compared to those of the other strains, the seven isolates with alterations in the terminal *mexT* gene had reduced expressions of *mexE* (0.20 ± 0.27 versus 0.71 ± 0.68; $P = 0.006$), which is consistent with the positive regulatory effect of a functional MexT. The expressions of *mexA* were similar in the seven isolates with aberrant MexT; however, *oprD* expression was actually decreased in the seven isolates (0.27 ± 0.30 versus 2.0 ± 3.29; $P = 0.04$). Finally, 10 isolates had a single base substitution in the region of the *nod* box-like intergenic region, where MexT is likely to bind to initiate *mexEF-mexEF-oprN* transcription (12). However, the expression of *mexE* was not significantly different in these 10 isolates relative to the isolates that did not have this nucleotide substitution.

Fifteen isolates had no detectable mutations in *mexZ* or in the *mexZ-mexX* intergenic region (wild type). Two isolates had nucleotide substitutions leading to premature stop codons, and both had elevated levels of expression of *mexX*. One isolate had a nucleotide insertion causing an out-of-frame MexZ, also resulting in an increased expression of *mexX*. Although isolates with these major mutations in *mexZ* had increased expressions of *mexX*, their presence was certainly not a prerequisite for increased *mexX* transcription.

Concluding remarks. Carbapenem antibiotics are important for treating nosocomial infections due to *P. aeruginosa*, and the development of resistance severely hampers therapy. Understanding the mechanisms leading to carbapenem resistance in clinical isolates will be important in the development of new antimicrobial strategies. Our method of incorporating specific primers and probes for real-time RT-PCR allowed quantification of the expression of multiple genes. This is the first study to analyze the contribution of six potential mechanisms of carbapenem resistance in a large number of clinical isolates.

In the absence of an efficient carbapenemase, carbapenem resistance in *P. aeruginosa* has been felt to be an interplay between AmpC hyperproduction, loss of OprD, and increased expression of *mexAB-oprM* (13, 20). The most consistent finding in carbapenem-resistant isolates has been the loss of OprD (33). For imipenem, increased AmpC activity and the loss of

TABLE 3. Correlation between mutations in purported regulatory genes and target gene expression

Group and isolate	Ribotype	AmpR amino acid substitution	Inducible AmpC	Expression of *ampC* mRNA[a]	Mutation(s) in:			Expression of *mexA* mutation[a]	MexT amino acid substitution	Expression of *mexE* mutation[a]	MexZ amino acid substitution	Expression of *mexX* mutation[a]
					MexR	NalC	NalD					
Group 1												
AM602	J	Gly_{283}→Glu; Met_{288}→Arg	No	29	Val_{126}→Glu	Gly_{71}→Glu; Ala_{145}→Val; Ser_{209}→Arg	His_{154}→Pro; deletion Pro_{155} through Thr_{158}	3.5	Leu_{26}→Val	0.19	None	0.06
FI506	H	None	Yes	66	None	Gly_{71}→Glu	None	0.72	Leu_{26}→Val	0.01	Leu_{138}→Arg; Leu_{196}→Ile	0.95
BK546	A	None	No	480	None	Gly_{71}→Glu; Ser_{209}→Arg	None	0.52	Leu_{26}→Val; partial[b]	0.20	Insert His-Gly between Gly_{50}→His_{51}	6.7
HW311	A	None	No	970	None	Gly_{71}→Glu; Ser_{209}→Arg	Pro_{196}→Gln	2.8	Leu_{26}→Val; partial	0.01	None	19
DB322	A	None	No	2,400	None	Gly_{71}→Glu; Ser_{209}→Arg	Insert Ile between Ile_{15}-Leu_{16}	3.5	Leu_{26}→Val; partial	0.22	Insert His-Gly between Gly_{50}→His_{51}	20
AM339	E	Glu_{114}→Ala; Gly_{283}→Glu; Met_{288}→Arg	No	290	Val_{126}→Glu	Gly_{71}→Glu; Ala_{145}→Val; Ser_{209}→Arg	Cys_{149}→Arg	5.0	Leu_{26}→Val	0.66	Arg_{13}→Gln	23
BK553	A	None	No	610	None	Gly_{71}→Glu; Ser_{209}→Arg	None	0.41	Leu_{26}→Val; partial	0	None	24
BK501	A	None	No	930	None	Gly_{71}→Glu; Ser_{209}→Arg	None	1.4	Leu_{26}→Val; partial	0.76	Insert His-Gly between Gly_{50}→His_{51}	29
AM609	I	None	Yes	2,600	Val_{126}→Glu	Gly_{71}→Glu	None	1.9	Leu_{26}→Val	0.05	Leu_{128}→Met	29
BK545	A	None	No	270	None	Gly_{71}→Glu; Ser_{209}→Arg	None	1.1	Leu_{26}→Val; partial	0.24	Insert His-Gly between Gly_{50}→His_{51}	32
DB513	N	None	Yes	1,500	Val_{126}→Glu	Gly_{71}→Glu; frameshift at Gln_{162}	None	3.1	Leu_{26}→Val	0.60	None	43
CK575	F	None	Yes	170	None	Gly_{71}→Glu; Ser_{209}→Arg	None	0.88	Leu_{26}→Val	0.41	Tyr_{49}→Stop	53
DB537	A	None	No	380	None	Gly_{71}→Glu; Ser_{209}→Arg	None	0.87	Leu_{26}→Val; partial	0	Insert His-Gly between Gly_{50}→His_{51}	88
Group 2												
CK505	C	Arg_{244}→Trp; Gly_{273}→Glu	Yes	0.70	None	Gly_{71}→Glu; Ser_{209}→Arg	None	1.5	Leu_{26}→Val	1.1	None	13
UL118	C	Arg_{244}→Trp; Gly_{273}→Glu	Yes	1.3	None	Gly_{71}→Glu; Ser_{209}→Arg	None	0.96	Leu_{26}→Val	0	None	9.6
CK558	D	None	Yes	2.0	Val_{126}→Glu; Val_{132}→Arg	Gly_{71}→Glu; Ser_{209}→Arg	None	1.0	Leu_{26}→Val	1.4	Frameshift at Ala_{47}	18
UL517	E	Glu_{114}→Ala; Gly_{283}→Glu; Met_{288}→Arg	Yes	2.4	Val_{126}→Glu	Gly_{71}→Glu; Ala_{145}→Val; Ser_{209}→Arg	None	0.90	Leu_{26}→Val	1.8	Arg_{13}→Gln	3.8
BK555	G	None	Yes	2.4	Val_{126}→Glu	Gly_{71}→Glu	None	1.6	Leu_{26}→Val	0	None	31
CK550	D	None	Yes	6.2	Val_{126}→Glu; Val_{132}→Arg	Gly_{71}→Glu; Ser_{209}→Arg	None	1.4	Leu_{26}→Val	2.8	None	73
Group 3a												
MD519	O	None	Yes	2.1	None	Gly_{71}→Glu; Ser_{209}→Arg	Asp_{147}→Asn	1.0	Leu_{26}→Val	0.23	Frameshift at Arg_{158}	35
MD504	P	None	Yes	1.8	None	Gly_{71}→Glu; Ser_{209}→Arg	None	1.8	Leu_{26}→Val	0.65	None	5.7
CK516	R	Glu_{114}→Ala; Ile_{251}→Val; Gly_{283}→Glu; Met_{288}→Arg	Yes	3.5	None	Gly_{71}→Glu; Ser_{209}→Arg	None	2.6	Leu_{26}→Val	0.66	None	1.1
MD521	Q	Glu_{114}→Ala; Ile_{251}→Val; Gly_{283}→Glu; Met_{288}→Arg	Yes	3.8	None	Gly_{71}→Glu; Ser_{209}→Arg	None	1	Leu_{26}→Val	0.67	Ala_{35}→Val	1.1

CK503	S	None	Yes	4.0	None	Gly_{71}→Glu; Ser_{209}→Arg	None	1.8	Leu_{26}→Val	1.7	None	4.0
DB504	A	None	Yes	7.5	None	Gly_{71}→Glu; Ser_{209}→Arg	None	1.4	Leu_{26}→Val	0.71	None	4.4
Group 3b												
MD119	M	None	Yes	2.7	None	None	None	2.4	Leu_{26}→Val	0	None	5.7
CK188	B	None	Yes	0.31	None	Gly_{71}→Glu; Ser_{209}→Arg; Pro_{210}→Leu	Thr_{188}→Ala	3.9	Leu_{26}→Val	0.43	None	5.8
CK140	B	None	Yes	5.5	None	Gly_{71}→Glu; Ser_{209}→Arg; Pro_{210}→Leu	Thr_{188}→Ala	2.2	Leu_{26}→Val	0.21	None	6.9
MD104	B	None	Yes	0.41	None	Gly_{71}→Glu; Ser_{209}→Arg	None	2.2	Leu_{26}→Val	0.55	None	16
UL140	L	Glu_{114}→Ala; Gly_{283}→Glu; Met_{288}→Arg	Yes	5.1	Val_{126}→Glu	Gly_{71}→Glu; frameshift at Ala145	None	13	Leu_{26}→Val	1.6	Tyr_{49}→Stop	330
AM128	K	Gly_{283}→Glu; Met_{288}→Arg	Yes	3.6	Val_{126}→Glu	Gly_{71}→Glu; Ser_{209}→Arg	None	0.71	Leu_{26}→Val	0.41	Leu_{138}→Arg	2.8
MD510	C	Arg_{244}→Trp; Gly_{273}→Glu	Yes	2.6	None	Gly_{71}→Glu	None	0.97	Leu_{26}→Val	0.40	None	5.0
DB311	D	None	Yes	1.4	Val_{126}→Glu; Val_{132}→Arg	Gly_{71}→Glu; Ser_{209}→Arg	None	1.7	Leu_{26}→Val	1.2	None	6.6
ATCC 27853		None	Yes	1	None	Gly_{71}→Glu; Ser_{209}→Arg	None	1	Leu_{26}→Val	1	None	1

[a] Relative to the expression of *P. aeruginosa* ATCC 27853, which is assigned a value of 1.
[b] Only the initial 462 bp could be amplified and sequenced.

OprD can lead to MICs of 16 μg/ml (18). However, increased expression of AmpC had little effect on meropenem, and MICs in isolates that were deficient in OprD reached only 2 to 4 μg/ml (18). Meropenem is a known substrate of the MexAB-OprM system, but increased expression of this system has had various effects on meropenem susceptibility (9, 13, 33). Several conclusions can be made regarding β-lactam resistance in our clinical isolates. For imipenem and meropenem, decreased *oprD* expression was found in all resistant isolates. Most imipenem-/meropenem-resistant strains also had increased *ampC* and efflux pump expressions. For ertapenem, efflux pump activity appeared to be an important contributor to resistance. Most isolates that were resistant to ertapenem alone had increased activity of multiple efflux systems. Although the role of efflux systems as contributors to high-level carbapenem resistance has been questioned, our data clearly support their role in the development of ertapenem resistance. The isolates that were resistant to ertapenem alone also had reduced susceptibilities to meropenem (in relation to imipenem). This suggests a role of efflux systems for the reduced susceptibility to meropenem in this group. The contribution of efflux systems to ertapenem resistance has similarly been suggested in *P. aeruginosa* isolates that are susceptible to other β-lactams (21). Finally, increased MexAB-OprM activity was also associated with aztreonam resistance in our isolates. Meropenem and aztreonam are known substrates for MexAB-OprM (27, 50).

In some isolates, it was not possible to fully explain the basis for β-lactam resistance. It is possible that other mechanisms of resistance may be present in these isolates. A reduction in the outer membrane protein OprF in *P. aeruginosa* has been associated with increased MICs of ceftazidime and aztreonam; whether this protein is important for the entry of ertapenem remains to be determined (7).

In carefully controlled in vitro experiments involving laboratory-derived isogenic mutants, the role of regulatory genes and the contribution of efflux pumps to antibiotic resistance have been assessed. However, these carefully designed experiments may not mimic conditions in the clinical setting. Mutations affecting *mexR*, selected by in vitro exposure to antibiotics (generally a fluoroquinolone and a β-lactam) (1, 37, 39, 40, 46), have not been consistently observed in clinical isolates overexpressing *mexAB-oprM* (9, 22, 33, 51). Our isolates overexpressing *mexAB-oprM* also did not appear to possess clinically significant mutations in *mexR* that explained the increased activity. However, most of our isolates with substantially increased expression of *mexAB-oprM* did have significant mutations affecting two recently described regulatory genes, *nalC* and *nalD* (4, 43).

The roles of other regulatory proteins in clinical isolates remain to be defined. In our isolates, it is evident that regulators other than *ampR* (e.g., *ampD*) are involved with *ampC* expression (11, 23). MexT, a positive regulator of the MexEF-OprN system, has been implicated in the down-regulation of OprD (12). However, isolates in this study that possessed an incomplete *mexT* gene had depressed expression of *oprD*, suggesting that other factors are involved. While disabling mutations in *mexZ* correlated with increased expression of *mexX*, many of our isolates that were hyperexpressing this efflux system did not have mutations in *mexZ*. It is noteworthy that

many of our isolates had increased expression of several efflux systems.

ACKNOWLEDGMENT

This study was supported by a grant from Merck & Co., Inc.

REFERENCES

1. **Adewoye, L., A. Sutherland, R. Srikumar, and K. Poole.** 2002. The MexR repressor of *mexAB-oprM* multidrug efflux operon in *Pseudomonas aeruginosa*: characterization of mutations compromising activity. J. Bacteriol. **184:** 4308–4312.
2. **Aires, J. R., T. Kohler, H. Nikaido, and P. Plesiat.** 1999. Involvement of an active efflux system in the natural resistance of *Pseudomonas aeruginosa* to aminoglycosides. Antimicrob. Agents Chemother. **43:**2624–2628.
3. **Bagge, N., O. Ciofu, M. Hentzer, J. I. A. Campbell, M. Givskov, and N. Hoiby.** 2002. Constitutive high expression of chromosomal β-lactamase in *Pseudomonas aeruginosa* caused by a new insertion sequence (IS*1669*) located in *ampD*. Antimicrob. Agents Chemother. **46:**3406–3411.
4. **Cao, L., R. Srikumar, and K. Poole.** 2004. MexAB-OprM hyperexpression in NalC-type multidrug-resistant *Pseudomonas aeruginosa*: identification and characterization of the *nalC* gene encoding a repressor of PA3720-PA3719. Mol. Microbiol. **53:**1423–1436.
5. **Evans, K., L. Adewoye, and K. Poole.** 2001. MexR repressor of the *mexAB-oprM* multidrug efflux operon of *Pseudomonas aeruginosa*: identification of MexR binding sites in the *mexA-mexR* intergenic region. J. Bacteriol. **183:** 807–812.
6. **Evans, K., and K. Poole.** 1999. The MexA-MexB-OprM multidrug efflux system of *Pseudomonas aeruginosa* is growth-phase regulated. FEMS Microbiol. Lett. **173:**35–39.
7. **Hancock, R. E. W., and W. A. Woodruff.** 1988. Roles of porin and β-lactamase in β-lactam resistance of *Pseudomonas aeruginosa*. Rev. Infect. Dis. **10:**770–775.
8. **Heritier, C. A., L. Dubouix, L. N. Poirel, N. Marty, and P. Nordmann.** 2005. A nosocomial outbreak of *Acinetobacter baumannii* isolates expressing the carbapenem-hydrolyzing oxacillinase OXA-58. Antimicrob. Agents Chemother. **55:**115–118.
9. **Hocquet, D., X. Bertrand, T. Kohler, D. Talon, and P. Plesiat.** 2003. Genetic and phenotypic variations of a resistant *Pseudomonas aeruginosa* epidemic clone. Antimicrob. Agents Chemother. **47:**1887–1894.
10. **Jeannot, K., M. L. Sobel, F. El Garch, K. Poole, and P. Plesiat.** 2005. Induction of the MexXY efflux pump in *Pseudomonas aeruginosa* is dependent on drug-ribosome interaction. J. Bacteriol. **187:**5341–5346.
11. **Juan, C., M. D. Macia, O. Gutierrez, C. Vidal, J. L. Perez, and A. Oliver.** 2005. Molecular mechanisms of β-lactam resistance mediated by AmpC hyperproduction in *Pseudomonas aeruginosa* clinical strains. Antimicrob. Agents Chemother. **49:**4733–4738.
12. **Kohler, T., S. F. Epp, L. K. Curty, and J.-C. Pechere.** 1999. Characterization of MexT, the regulator of the MexE-MexF-OprN multidrug efflux system of *Pseudomonas aeruginosa*. J. Bacteriol. **181:**6300–6305.
13. **Kohler, T., M. Michea-Hamzehpour, S. F. Epp, and J.-C. Pechere.** 1999. Carbapenem activities against *Pseudomonas aeruginosa*: respective contributions of OprD and efflux systems. Antimicrob. Agents Chemother. **43:**424–427.
14. **Kohler, T., M. Michea-Hamzehpour, U. Henze, N. Gotoh, L. K. Curty, and J.-C. Pechere.** 1997. Characterization of MexE-MexF-OprN, a positively regulated multidrug efflux system of *Pseudomonas aeruginosa*. Mol. Microbiol. **23:**345–352.
15. **Kolayli, F., A. Karadenizli, H. Savli, K. Ergen, O. Hatirnaz, E. Balikci, F. Budak, and H. Vahaboglu.** 2004. Effect of carbapenems on the transcriptional expression of the *oprD*, *oprM*, and *oprN* genes in *Pseudomonas aeruginosa*. J. Med. Microbiol. **53:**915–920.
16. **Landman, D., J. M. Quale, D. Mayorga, A. Adedeji, K. Vangala, J. Ravishankar, C. Flores, and S. Brooks.** 2002. Citywide clonal outbreak of multiresistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa* in Brooklyn, N.Y. The preantibiotic era has returned. Arch. Intern. Med. **162:**1515–1520.
17. **Li, X. Z., D. Ma, D. M. Livermore, and H. Nikaido.** 1994. Role of efflux pump(s) in intrinsic resistance of *Pseudomonas aeruginosa*: active efflux as a contributing factor to β-lactam resistance. Antimicrob. Agents Chemother. **38:**1742–1752.
18. **Livermore, D. M.** 1992. Interplay of impermeability and chromosomal beta-lactamase activity in imipenem-resistant *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **36:**2046–2048.
19. **Livermore, D. M.** 2001. Of *Pseudomonas*, porins, pumps, and carbapenems. J. Antimicrob. Chemother. **47:**247–250.
20. **Livermore, D. M.** 2002. Multiple mechanisms of antimicrobial resistance in *Pseudomonas aeruginosa*: our worst nightmare? Clin. Infect. Dis. **34:**634–640.
21. **Livermore, D. M., S. Mushtaq, and M. Warner.** 2005. Selectivity of ertapenem for *Pseudomonas aeruginosa* mutants cross-resistant to other carbapenems. J. Antimicrob. Chemother. **55:**306–311.
22. **Llanes, C., D. Hocquet, C. Vogne, D. Benali-Baitich, C. Neuwirth, and P. Plesiat.** 2004. Clinical strains of *Pseudomonas aeruginosa* overproducing MexAB-OprM and MexXY efflux pumps simultaneously. Antimicrob. Agents Chemother. **48:**1797–1802.
23. **Lodge, J. M., S. D. Minchin, L. J. V. Piddock, and J. W. Busby.** 1990. Cloning, sequencing, and analysis of the structural gene and regulatory region of the *Pseudomonas aeruginosa* chromosomal *ampC* β-lactamase. J. Biochem. **272:**627–631.
24. **Maseda, H., I. Sawada, K. Saito, H. Uchiyama, T. Nakae, and N. Nomura.** 2004. Enhancement of the *mexAB-oprM* efflux pump expression by a quorum-sensing autoinducer and its cancellation by a regulator, MexT, of the *mexEF-oprN* efflux pump operon in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **48:**1320–1328.
25. **Maseda, H., K. Saito, A. Nakajima, and T. Nakae.** 2000. Variation of the *mexT* gene, a regulator of the MexEF-OprN efflux pump expression in wild-type strains of *Pseudomonas aeruginosa*. FEMS Microbiol. Lett. **192:** 107–112.
26. **Maseda, H., H. Yoneyama, and T. Nakae.** 2000. Assignment of the substrate-selective subunits of the MexEF-OprN multidrug efflux pump of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **44:**658–664.
27. **Masuda, N., E. Sakagawa, S. Ohya, N. Gotoh, H. Tsujimoto, and T. Nishino.** 2000. Substrate specificities of MexAB-OprM, MexCD-OprJ, and MexXY-OprM efflux pumps in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **44:**3322–3327.
28. **Masuda, N., E. Sakagawa, S. Ohya, N. Gotoh, H. Tsujimoto, and T. Nishino.** 2000. Contribution of the MexX-MexY-OprM efflux system to intrinsic resistance in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **44:** 2242–2246.
29. **Mine, T., Y. Morita, T. Mizushima, and T. Tsuchiya.** 1999. Expression in *Escherichia coli* of a new multidrug efflux pump, MexXY, from *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **43:**415–417.
30. **National Committee for Clinical Laboratory Standards.** 2002. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7-A5. National Committee for Clinical Laboratory Standards, Wayne, Pa.
31. **Nordmann, P., and L. Poirel.** 2002. Emerging carbapenemases in gram-negative aerobes. Clin. Microbiol. Infect. **8:**321–331.
32. **Ochs, M. M., M. P. McCusker, and M. Bains.** 1999. Negative regulation of the *Pseudomonas aeruginosa* outer membrane porin OprD selective for imipenem and basic amino acids. Antimicrob. Agents Chemother. **43:**1085–1090.
33. **Pai, H., J. W. Kim, J. Kim, J. H. Lee, K. W. Choe, and N. Gotoh.** 2001. Carbapenem resistance mechanisms in *Pseudomonas aeruginosa* clinical isolates. Antimicrob. Agents Chemother. **45:**480–484.
34. **Paterson, D. L., K. M. Hujer, A. M. Hujer, B. Yeiser, M. D. Bonomo, L. B. Rice, R. A. Bonomo, and the International *Klebsiella* Study Group.** 2003. Extended-spectrum β-lactamase in *Klebsiella pneumoniae* bloodstream isolates from seven countries: dominance and widespread prevalence of SHV- and CTX-M-type β-lactamases. Antimicrob. Agents Chemother. **47:**3554–3560.
35. **Pirnay, J. P., D. DeVos, D. Mossialos, A. Vanderkelen, P. Cornelis, and M. Zizi.** 2002. Analysis of the *Pseudomonas aeruginosa oprD* gene from clinical and environmental isolates. Environ. Microbiol. **4:**872–882.
36. **Poole, K., N. Gotoh, H. Tsujimoto, Q. Zhao, A. Wada, T. Yamasaki, S. Neshat, J. Yamagishi, X.-Z. Li, and T. Nishino.** 1996. Overexpression of the *mexC-mexD-oprJ* efflux operon in *nfxB*-type multidrug-resistant strains of *Pseudomonas aeruginosa*. Mol. Microbiol. **23:**345–354.
37. **Poole, K., K. Tetro, Q. Zhao, S. Neshat, D. E. Heinrichs, and N. Bianco.** 1996. Expression of the multidrug resistance operon *mexA-mexB-oprM* in *Pseudomonas aeruginosa*: *mexR* encodes a regulator of operon expression. Antimicrob. Agents Chemother. **40:**2021–2028.
38. **Quale, J., S. Bratu, D. Landman, and R. Heddurshetti.** 2003. Molecular epidemiology and mechanisms of carbapenem resistance in *Acinetobacter baumannii* endemic in New York City. Clin. Infect. Dis. **37:**214–220.
39. **Saito, K., H. Akama, E. Yoshihara, and T. Nakae.** 2003. Mutations affecting DNA-binding activity of the *mexR* repressor of *mexR-mexA-mexB-oprM* operon expression. J. Bacteriol. **185:**6195–6198.
40. **Saito, K., H. Yoneyama, and T. Nakae.** 1999. *nalB*-type mutations causing the overexpression of the MexAB-OprM efflux pump are located in the *mexR* gene of the *Pseudomonas aeruginosa* chromosome. FEMS Microbiol. Lett. **179:**67–72.
41. **Salvi, H., A. Karadenizli, F. Kolayi, S. Gundes, U. Ozbek, and H. Vahaboglu.** 2003. Expression stability of six housekeeping genes: a proposal for resistance gene quantification studies of *Pseudomonas aeruginosa* by real-time quantitative RT-PCR. J. Med. Microbiol. **52:**403–408.
42. **Sawada, I., H. Meseda, T. Nakae, H. Uchiyama, and N. Nomura.** 2004. A quorum-sensing autoinducer enhances the *mexAB-oprM* efflux-pump expression without the MexR-mediated regulation in *Pseudomonas aeruginosa*. Microbiol. Immunol. **48:**435–439.
43. **Sobel, M. L., D. Hocquet, L. Cao, P. Plesiat, and K. Poole.** 2005. Mutations in PA3574 (*nalD*) lead to increased MexAB-OprM expression and multidrug

resistance in laboratory and clinical isolates of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **49:**1782–1786.

44. **Sobel, M. L., G. A. McKay, and K. Poole.** 2003. Contribution of the MexXY multidrug transporter to aminoglycoside resistance in *Pseudomonas aeruginosa* clinical isolates. Antimicrob. Agents Chemother. **47:**3202–3207.
45. **Sobel, M. L., S. Neshat, and K. Poole.** 2005. Mutations in PA2491 (*mexS*) promote MexT-dependent *mexEF-oprN* expression and multidrug resistance in a clinical strain of *Pseudomonas aeruginosa*. J. Bacteriol. **187:**1246–1253.
46. **Srikumar, R., C. J. Paul, and K. Poole.** 2000. Influence of mutations in the *mexR* repressor gene on expression of the MexA-MexB-OprM multidrug efflux system of *Pseudomonas aeruginosa*. J. Bacteriol. **182:**1410–1414.
47. **Vogne, C., J. Ramos Aires, C. Bailly, D. Hocquet, and P. Plesiat.** 2004. Role of multidrug efflux system MexXY in the emergence of moderate resistance to aminoglycosides among *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis. Antimicrob. Agents Chemother. **48:**1676–1680.
48. **Wolter, D. J., E. Smith-Moland, R. V. Goering, N. D. Hanson, and P. D. Lister.** 2004. Multidrug resistance associated with mexXY expression in clinical isolates of *Pseudomonas aeruginosa* from a Texas hospital. Diagn. Microbiol. Infect. Dis. **50:**43–50.
49. **Yoneda, K., H. Chikumi, T. Murata, N. Gotoh, H. Yamamoto, H. Fujiwara, T. Nishino, and E. Shimizu.** 2005. Measurement of *Pseudomonas aeruginosa* multidrug efflux pumps by quantitative real-time polymerase chain reaction. FEMS Microbiol. Lett. **243:**125–131.
50. **Yoneyama, H., A. Ocaktan, M. Tsuda, and T. Nakae.** 1997. The role of *mex*-gene products in antibiotic extrusion in *Pseudomonas aeruginosa*. Biochem. Biophys. Res. Commun. **233:**611–618.
51. **Ziha-Zarifi, I., C. Llanes, T. Kohler, J.-C. Pechere, and P. Plesiat.** 1999. In vivo emergence of multidrug-resistant mutants of *Pseudomonas aeruginosa* overexpressing the active efflux system MexA-MexB-OprM. Antimicrob. Agents Chemother. **43:**287–291.