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Overexpression of AmpC and Efflux Pumps in *Pseudomonas aeruginosa* Isolates from Bloodstream Infections: Prevalence and Impact on Resistance in a Spanish Multicenter Study▽

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The prevalence and impact of the overexpression of AmpC and efflux pumps were evaluated with a collection of 190 *Pseudomonas aeruginosa* isolates recovered from bloodstream infections in a 2008 multicenter study (10 hospitals) in Spain. The MICs of a panel of 13 antipseudomonal agents were determined by microdilution, and the expressions of *ampC*, *mexB*, *mexY*, *mexD*, and *mexF* were determined by real-time reverse transcription (RT)-PCR. Up to 39% of the isolates overexpressed at least one of the mechanisms. *ampC* overexpression (24.2%) was the most prevalent mechanism, followed by *mexY* (13.2%), *mexB* (12.6%), *mexF* (4.2%), and *mexD* (2.2%). The overexpression of *mexB* plus *mexY*, documented for 5.3% of the isolates, was the only combination showing a significantly ($P = 0.02$) higher prevalence than expected from the frequencies of the individual mechanisms (1.6%). Additionally, all imipenem-resistant isolates studied (25 representative isolates) showed inactivating mutations in *oprD*. Most of the isolates nonsusceptible to piperacillin-tazobactam (96%) and ceftazidime (84%) overexpressed *ampC*, while *mexB* (25%) and *mexY* (29%) overexpressions gained relevance among cefepime-nonsusceptible isolates. Nevertheless, the prevalence of *mexY* overexpression was highest among tobramycin-nonsusceptible isolates (37%), and that of *mexB* was highest among meropenem-nonsusceptible isolates (33%). Regarding ciprofloxacin-resistant isolates, besides the expected increased prevalence of efflux pump overexpression, a highly significant link to *ampC* overexpression was documented for the first time: up to 52% of ciprofloxacin-nonsusceptible isolates overexpressed *ampC*, sharply contrasting with the 24% documented for the complete collection ($P < 0.001$). In summary, mutation-driven resistance was frequent in *P. aeruginosa* isolates from bloodstream infections, whereas metallo-β-lactamases, detected in 2 isolates (1%) producing VIM-2, although with increasing prevalences, were still uncommon.

The increasing prevalence of nosocomial infections produced by multidrug-resistant (MDR) *Pseudomonas aeruginosa* strains severely compromises the selection of appropriate treatments and is therefore associated with significant morbidity and mortality (17, 21, 25). The growing threat of antimicrobial resistance in *P. aeruginosa* results from the extraordinary capacity of this microorganism for developing resistance to almost any available antibiotic by the selection of mutations in chromosomal genes and from the increasing prevalence of transferrable resistance determinants, particularly those encoding class B carbapenemases (or metallo-β-lactamases [MBLs]) or extended-spectrum β-lactamases (ESBLs), frequently cotransferred with genes encoding aminoglycoside-modifying enzymes (18, 19). Among the mutation-mediated resistance mechanisms, particularly noteworthy are those leading to the repression or inactivation of the carbapenem porin OprD, the hyperproduction of the chromosomal cephalosporinase AmpC, or the upregulation of one of the several efflux pumps encoded in the *P. aeruginosa* genome (11, 13, 22, 32). Furthermore, the accumulation of many of these chromosomal mutations can lead to the emergence of MDR (or even panantibiotic-resistant) strains, which eventually may be responsible for notable outbreaks in the hospital setting (7). Nevertheless, except for some seminal French studies (13), there is still scarce information on the large-scale (suprahospital) epidemiology of *P. aeruginosa* resistance mechanisms, and there is much less information on the impact of these mechanisms on resistance prevalence. Therefore, a national-scale study was conducted in Spain to determine the prevalence and impact of AmpC and efflux pump overexpression and OprD mutations in *P. aeruginosa* bloodstream isolates on resistance.

MATERIALS AND METHODS

Strains, molecular typing, and susceptibility testing. A total of 190 *P. aeruginosa* isolates recovered from bloodstream infections in a 2008 multicenter study (10 hospitals from different geographic locations) in Spain were studied. Clonal relatedness was evaluated through repetitive extragenic palindromic PCR (REP-PCR) according to previously described protocols (4). The MICs of ceftazidime (Sigma-Aldrich, Madrid, Spain), cefepime (Aventis Pharma, Madrid, Spain), piperacillin (Sigma-Aldrich), piperacillin plus tazobactam (fixed concentration of 4 μg/ml) (Sigma-Aldrich), aztreonam (Sigma-Aldrich), imipenem (Merck, Sharp & Dohme, Madrid, Spain), meropenem (Astra-Zeneca, Madrid, Spain), ciprofloxacin (Sigma-Aldrich), levofloxacin (Roussel Uclaf, Paris, France), gentamicin (Sigma-Aldrich), tobramycin (Sigma-Aldrich), amikacin (Sigma-Aldrich), and

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TABLE 1. Activities of antipseudomonal agents against *P. aeruginosa* bloodstream isolates from Spanish hospitals

Antibiotic[a]	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	MIC range (μg/ml)	% S (% S according to according to EUCAST breakpoints)[b]	% I (% I according to according to EUCAST breakpoints)[b]	% R (% R according to according to EUCAST breakpoints)[b]
CAZ	4	32	0.25–≥128	76.3	6.8 (0.0)	16.9 (23.7)
FEP	8	32	0.25–≥128	61.6	20.0 (0.0)	18.4 (38.4)
ATM	8	32	0.25–≥128	67.4 (1.6)	21.6 (87.4)	11.0
PIP	8	128	1–≥128	80.5 (65.8)		19.5 (34.2)
PIP-Tz	8	128	1–≥128	86.3 (72.1)		13.7 (27.9)
IMP	2	32	0.12–≥64	67.9	9.5	22.6
MER	1	16	0.06–≥64	77.4 (70.0)	7.4 (14.8)	15.2
CIP	0.25	32	0.03–≥32	71.6 (65.8)	1.6 (5.8)	26.8 (28.4)
LEV	1	32	0.03–≥32	68.4 (62.6)	2.6 (5.8)	29.0 (31.6)
GEN	2	64	0.06–≥64	78.9	2.1 (0.0)	19.0 (21.1)
TOB	0.5	64	0.06–≥64	81.6	1.1 (0.0)	17.3 (18.4)
AMK	4	8	0.12–≥128	98.4 (93.7)	0.5 (4.7)	1.1 (1.6)
COL	0.5	1	0.03–8	96.8 (98.9)	2.1 (0.0)	1.1

[a] CAZ, ceftazidime; FEP, cefepime; ATM, aztreonam; PIP, piperacillin; PIP-Tz, piperacillin-tazobactam; IMP, imipenem; MER, meropenem; CIP, ciprofloxacin; LEV, levofloxacin; GEN, gentamicin; TOB, tobramycin; AMK, amikacin; COL, colistin.

[b] Percentage of isolates susceptible (S), intermediate (I), or resistant (R) according to CLSI breakpoints (5). When different, the corresponding percentages of S, I, and R isolates according to EUCAST breakpoints (www.eucast.org) are also indicated in parentheses for comparative purposes.

colistin (Sigma-Aldrich) were determined by broth microdilution according to CLSI guidelines and breakpoints (5).

Quantification of gene expression by RT-PCR. The levels of expression of *ampC*, *mexB*, *mexD*, *mexY*, and *mexF* were determined by real-time reverse transcription (RT)-PCR according to previously described protocols (15, 26). Briefly, strains were grown in 10 ml of LB broth at 37°C and 180 rpm to the late log phase (optical density at 600 nm [OD_{600}] of 1) and collected by centrifugation. Total RNA was isolated by using the RNeasy minikit (Qiagen), dissolved in water, and treated with 2 U of Turbo DNase (Ambion) for 30 min at 37°C to remove contaminating DNA. The reaction was stopped by the addition of 5 μl of DNase inactivation reagent to the mixture. A 50-ng sample of purified RNA was then used for one-step reverse transcription and real-time PCR amplification using the QuantiTect SYBR green RT-PCR kit (Qiagen) with a SmartCycler II instrument (Cepheid). Previously described primers (15, 26) were used for the amplification of *ampC*, *mexB*, *mexD*, *mexY*, *mexF*, and *rpsL* (used as a reference to normalize the relative amount of mRNA). Strains were considered positive for *ampC*, *mexD*, *mexF*, or *mexY* overexpression when the corresponding mRNA level was at least 10-fold higher than that of PAO1, negative if lower than 5-fold, and borderline if between 5- and 10-fold. Strains were considered positive for *mexB* overexpression when the corresponding mRNA level was at least 3-fold higher than that of PAO1, negative if lower than 2-fold, and borderline if between 2- and 3-fold. All PCRs were performed in duplicate. Strains showing mRNA values of >5-fold for *ampC*, *mexD*, *mexF*, or *mexY* or >2-fold for *mexB* in a first experiment were subjected to two additional independent RNA extractions and duplicate PCRs. Mean values (± standard deviations) of mRNA levels obtained in three independent duplicate experiments were considered. Previously obtained PAO1 mutants overexpressing these mechanisms were used as controls (22, 23, 29).

Detection of genes encoding acquired β-lactamases. Preliminary phenotypic tests included the Etest MBL and double-disk synergy test (DDST) using ceftazidime and amoxicillin-clavulanate (distance from 10 to 30 mm) for the detection of ESBLs. The potential presence of genes encoding acquired β-lactamases was additionally explored by PCR and sequencing for all isolates resistant to imipenem and ceftazidime (MBLs) or ceftazidime (ESBLs). Previously described primers and conditions were used to amplify the genes encoding VIM-, IMP-, PER-, CTX-M-, SHV-, TEM-, and OXA-type β-lactamases (6, 11, 27). After PCR amplification, sequencing reactions were performed with the BigDye Terminator kit (PE Applied Biosystems, Foster City, CA), and sequences were analyzed with an ABI Prism 3100 DNA sequencer (PE Applied Biosystems). The resulting sequences were then compared with those available at the GenBank database (www.ncbi.nih.gov/BLAST).

PCR amplification and sequencing of the *oprD* gene. The presence of inactivating mutations in *oprD* was investigated with 25 representative isolates (representing 22 different clones) resistant to imipenem (MIC > 8 μg/liter) through the PCR amplification of the entire gene with specific primers followed by sequencing, as described previously (31). The nucleotide sequences were transcribed into the amino acid sequence using Vector NTI Advanced 9.0.0 (InforMax; Invitrogen). Nucleotide and amino acid sequences were compared with those of reference strain PAO1.

OMP analysis. Cultures of *P. aeruginosa* were grown overnight at 37°C in 5 ml of LB medium and then diluted 100-fold into fresh medium. Bacterial cells were incubated for approximately 5 h with shaking at 37°C to yield late-logarithmic-phase cells. Outer membrane protein (OMP) profiles were examined for the 25 above-mentioned strains using a previously reported method (24). OMPs were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie blue. OprD profiles from clinical isolates were compared with those of reference strain PAO1 and a PAO1-derivative *oprD*-deficient mutant.

Statistical analysis. Categorical and quantitative variables were compared by using a χ^2 test and a Mann-Whitney U test, respectively. A P value of <0.05 was considered statistically significant.

RESULTS

REP-PCR revealed a remarkable clonal diversity, with up to 116 different patterns identified among the 190 isolates. The activities of the tested antipseudomonal agents against the collection of *P. aeruginosa* bloodstream isolates from Spanish hospitals are shown in Table 1. Among β-lactams, activity was lowest for cefepime (62% susceptible) and highest for piperacillin-tazobactam (86% susceptible) according to CLSI breakpoints. It should be noted, however, that the application of EUCAST breakpoints (resistant at >16 μg/ml) would drop the susceptibility of the latter antibiotic to 72% (Table 1). Among carbapenems, the activity was higher for meropenem (77% susceptible) than for imipenem (68% susceptible). As for non-β-lactams, nearly 30% of the isolates were resistant to fluoroquinolones (ciprofloxacin and levofloxacin), and 20% were resistant to the aminoglycosides gentamicin and tobramycin. Twelve isolates (6.3%) were resistant to all first-line antibiotics (all β-lactams and fluoroquinolones). The only antibiotics conserving activity in most of the isolates were amikacin (98% susceptible) and colistin (97% susceptible).

Two of the isolates were found to produce the MBL VIM-2, whereas ESBLs were not detected in any of the isolates. The prevalence of AmpC and efflux pump overexpression is shown in Table 2. Up to 39% of the isolates overexpressed at least one of the mechanisms. *ampC* overexpression (24.2%) was the

TABLE 2. Prevalences of clinical isolates showing AmpC or efflux pump overexpression

Resistance gene(s)	No. (%) of isolates with:		
	Overexpression[a]	Borderline expression	No overexpression
Any gene	74 (39.0)	21 (11.1)	95 (49.9)
ampC	46 (24.2)	6 (3.2)	138 (72.6)
mexY	25 (13.2)	10 (5.3)	155 (81.5)
mexB	24 (12.6)	8 (4.2)	158 (83.2)
mexF	8 (4.2)	10 (5.3)	172 (90.5)
mexD	4 (2.1)	3 (1.6)	183 (96.3)
Most frequently found combinations			
mexB + *mexY*	10 (5.3)	3 (1.6)	177 (93.2)
ampC + *mexY*	10 (5.3)	2 (1.1)	178 (93.7)
ampC + *mexB*	8 (4.2)	2 (1.1)	180 (94.7)
ampC + *mexB* + *mexY*	3 (1.6)	2 (1.1)	185 (97.4)

[a] Mean values (and ranges) of overexpression (fold increase compared to PAO1) were 419.7 (14.5 to 3,586.1) for *ampC*, 20.9 (10.2 to 37.4) for *mexY*, 5.6 (3.1 to 13.3) for *mexB*, 20.5 (14.2 to 33.2) for *mexF*, and 20.7 (13.3 to 38.4) for *mexD*.

most prevalent mechanism, followed by *mexY* (13.2%) and *mexB* (12.6%). The prevalences of *mexF* (4.2%) and *mexD* (2.2%) overexpression were considerably lower. Additionally, a certain fraction of the isolates, ranging from 1.6% for *mexD* to 5.3% for *mexY* and *mexF*, were considered to present borderline expression. The means and ranges of values for the overexpression of *ampC*, *mexB*, *mexD*, *mexF*, and *mexY* for the studied collection are shown in Table 2.

The prevalences of the different combinations of resistance mechanisms are also shown in Table 2. The only combination showing a significantly ($P = 0.02$ by χ^2 test) higher prevalence than expected from the frequencies of the individual mechanisms was *mexB* plus *mexY*, documented for 5.3% of the isolates (1.6% expected).

REP-PCR analysis of isolates showing *ampC* or efflux pump overexpression also revealed a high level of clonal diversity. For instance, a total of 29 different patterns were detected among the 46 isolates showing *ampC* overexpression. Nevertheless, a certain degree of intra- and interhospital clonal dissemination was evidenced: two of the clones showing *ampC* overexpression were each detected in 6 isolates, in one case from a single institution and in the other case from two different hospitals in the Barcelona area.

The prevalences of *ampC* and efflux pump overexpression among the subsets of isolates resistant to different antibiotics are shown in Fig. 1. Most of the isolates nonsusceptible to piperacillin-tazobactam (96%) and ceftazidime (84%) overexpressed *ampC*. The prevalence of *ampC* overexpression was also high for isolates nonsusceptible to other β-lactams, such as cefepime (52%) or meropenem (60%), although efflux pump overexpression gained protagonism among isolates nonsusceptible to these antibiotics, with prevalences of *mexB* and *mexY* overexpression reaching values close to 30%. Regarding ciprofloxacin-resistant isolates, besides the expected increased prevalence of efflux pump overexpression, a highly significant (unexpected) link to *ampC* overexpression was documented: up to 52% of ciprofloxacin-nonsusceptible isolates overexpressed *ampC*, in sharp contrast with the 24% documented for the complete collection ($P < 0.001$ by χ^2 test). Interestingly, the same link to *ampC* overexpression was observed for the aminoglycosides (Fig. 1). As for isolates resistant to all first-line agents (all β-lactams and aminoglycosides), 100% overexpressed *ampC*, 33% overexpressed *mexY*, and 25% overexpressed *mexB*. Finally, it is worth noting that the prevalence of *mexY* overexpression was highest among tobramycin-nonsusceptible isolates (37%), and that of *mexB* overexpression was highest among meropenem-nonsusceptible isolates (33%).

Table 3 shows the activities of the tested antibiotics in the subsets of isolates overexpressing *ampC*, *mexB*, or *mexY*. As could be anticipated, the levels of activity of all β-lactams were low among isolates overexpressing *ampC*, but again, a remarkable unexpected link between *ampC* overexpression and fluoroquinolone and aminoglycoside resistance was noted. For example, up to 60% of isolates overexpressing *ampC* were nonsusceptible to ciprofloxacin (28% for the complete collection), and up to 49% were nonsusceptible to tobramycin (18% for the complete collection). Regarding *mexB*-overexpressing isolates, activity was particularly low for aztreonam, cefepime, the carbapenems, and the fluoroquinolones, and for *mexY*, activity was low for the same antibiotics plus the aminoglycosides gentamicin and tobramycin.

Additionally, the involvement of *oprD* mutations in imipenem resistance was investigated with 25 representative isolates (representing 22 different clones) resistant to this antibiotic. Sequencing of the entire *oprD* gene was carried out, and the expression of OprD was also assessed by an analysis of the OMP profiles in order to correlate the presence of inactivating mutations to the absence or decreasing expression of OprD. Consistent with previously reported observations (11), nearly all isolates presented inactivating mutations in *oprD*. Nine of the isolates (36%) showed point mutations leading to premature stop codons, 8 (32%) showed frameshift mutations originated by the insertion or deletion of 1 bp, 4 (16%) showed larger deletions (10 to 100 bp), and 2 (8%) showed an interruption of the gene by insertion sequences (IS*1471*-like and IS*Pa1328*). One further isolate had a deletion of 5 nucleotides starting at position 1132 and a 1-bp deletion at position 1149, which generated an in-frame deletion resulting in the loss of Gly-378 and Tyr-379 and amino acid substitutions ($K_{380}Y$, $N_{381}A$, $Y_{382}G$, and $G_{383}L$). This isolate also displayed a point mutation in the stop codon leading to a larger (13-amino-acid) predicted protein. Finally, another isolate did not show appar-

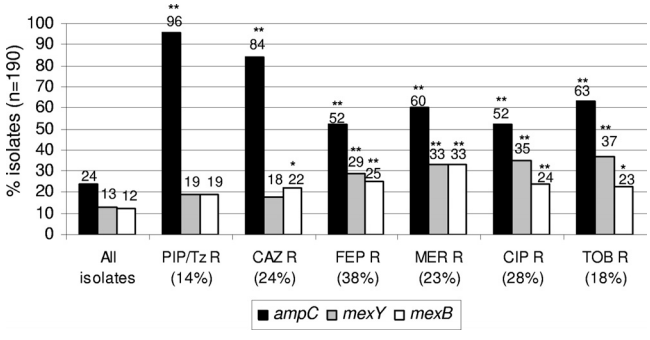

FIG. 1. Prevalences of *ampC*, *mexB*, or *mexY* overexpression in several subsets of isolates nonsusceptible to different antibiotics. * indicates a P value of <0.05 and ** indicates a P value of <0.001 compared to the corresponding subsets of susceptible isolates.

TABLE 3. Activities of antipseudomonal agents against *P. aeruginosa* bloodstream isolates with diverse resistance mechanisms

Antibiotic[a]	All isolates (n = 190)			Isolates overexpressing *ampC* (n = 45)[b]			Isolates overexpressing *mexB* (n = 24)			Isolates overexpressing *mexY* (n = 25)		
	% S	MIC_{50}	MIC_{90}	% S	MIC_{50}	MIC_{90}	% S	MIC_{50}	MIC_{90}	% S	MIC_{50}	MIC_{90}
CAZ	76.3	4	32	17.8	32	128	58.7	8	64	68.0	8	32
FEP	61.6	8	32	17.8	32	64	25.0	16	32	16.0	16	32
ATM	67.4	8	32	22.2	16	64	33.3	16	32	40.0	16	16
PIP	80.5	8	128	28.9	128	128	79.2	32	128	68.0	16	128
PIP-Tz	86.3	8	128	44.4	128	256	79.2	16	256	80.0	16	128
IMP	67.9	2	32	37.8	16	32	41.7	8	32	32.0	16	32
MER	77.4	1	16	44.4	8	16	41.7	8	32	44.0	8	16
CIP	71.6	0.25	32	40.0	32	32	45.8	2	32	24.0	32	32
LEV	68.4	1	32	35.6	32	32	33.3	8	32	16.0	32	32
GEN	78.9	2	64	46.7	32	64	66.7	2	64	40.0	32	64
TOB	81.6	0.5	64	51.1	4	64	66.7	1	64	48.0	8	64
AMK	98.4	4	8	100	4	8	100	4	8	100	4	16
COL	96.8	0.5	1	93.3	0.5	1	95.8	0.5	1	100	0.5	1

[a] CAZ, ceftazidime; FEP, cefepime; ATM, aztreonam; PIP, piperacillin; PIP-Tz, piperacillin-tazobactam; IMP, imipenem; MER, meropenem; CIP, ciprofloxacin; LEV, levofloxacin; GEN, gentamicin; TOB, tobramycin; AMK, amikacin; COL, colistin.
[b] One of the isolates showing *ampC* overexpression additionally produced the MBL VIM-2 and was therefore excluded from this analysis.

ent inactivating mutations but presented several polymorphisms compared to PAO1 and lacked OprD expression according to OMP profiles (data not shown).

OprD inactivation is also known to confer reduced susceptibility to meropenem, although clinical resistance to this carbapenem is thought to require additional mechanisms, such as AmpC or MexAB-OprM hyperproduction (10, 11, 30). Consistent with this observation, as shown in Table 4, a high proportion of imipenem-nonsusceptible and meropenem-nonsusceptible isolates overexpressed *ampC* (62.5%) or *mexB* (30%), in contrast to isolates nonsusceptible only to imipenem.

Table 4 also shows the prevalences of *ampC*, *mexB*, and *mexY* overexpression according to the ceftazidime-cefepime resistance phenotype. Consistent with data from previously reported studies (12), a very high prevalence (43%) of *mexY* overexpression was observed for isolates nonsusceptible only to cefepime, whereas *ampC* overexpression was documented for most (86%) of the isolates nonsusceptible to both antibiotics.

Finally, Fig. 2 shows the correlation between the level of *ampC* expression and the level of resistance (MICs) to ceftazidime, cefepime, and piperacillin-tazobactam. While MICs for isolates showing borderline expression (5- to 10-fold higher than that of reference strain PAO1) were not significantly higher than those for negative (<5-fold) isolates, the MICs of the three antibiotics sharply increased at higher expression levels (Fig. 2). As found for *ampC*, the median MICs for isolates with borderline *mexB* overexpression (2- to 3-fold) were nearly identical to those for negative (<2-fold) isolates, but a tendency (not reaching statistical significance) toward higher MICs of cefepime was noted for isolates showing borderline *mexY* expression (not shown).

DISCUSSION

The prevalence and impact of the overexpression of the genes encoding the chromosomal cephalosporinase AmpC (*ampC*) and the four major efflux pumps (*mexB*, *mexD*, *mexF*, and *mexY*) on resistance were investigated with a large collection of *P. aeruginosa* bloodstream isolates from a Spanish multicenter study, providing national-scale epidemiologic information on these relevant resistance mechanisms, which was previously available only from preceding studies in France (13). In our study, up to 39% of the isolates overexpressed at least one of the mechanisms, with *ampC* (24.2%), *mexY* (13.2%), or *mexB* (12.6%) being the gene more frequently overexpressed. While the prevalences of *ampC* (20%) and *mexB* (11%) overexpression obtained by the French study were similar, that of *mexY* (36%) was much higher. One factor possibly contributing to this difference is the breakpoints used

TABLE 4. Prevalences of *ampC*, *mexB*, and *mexY* overexpression among isolates showing particular resistance phenotypes

No. (%) of isolates	Resistance phenotype[a]				No. (%) of isolates showing overexpression of:		
	IMP	MER	CAZ	FEP	*ampC*	*mexB*	*mexY*
190 (100)	All	All	All	All	46 (24.2)	24 (12.6)	25 (13.2)
95 (50)	S	S	S	S	4 (4.2)	5 (5.3)	2 (2.1)
21 (11.1)	R	S	All	All	4 (19.0)	1 (4.8)	4 (19.0)
40 (21.1)	R	R	All	All	25 (62.5)	12 (30.0)	13 (32.5)
30 (15.8)	All	All	S	R	1 (3.3)	8 (26.7)	13 (43.3)
43 (22.6)	All	All	R	R	37 (86.0)	10 (23.3)	8 (18.6)
27 (14.2)	R	R	R	R	24 (88.9)	8 (29.6)	8 (29.6)

[a] IMP, imipenem; MER, meropenem; CAZ, ceftazidime; FEP, cefepime. "R" phenotypes include CLSI intermediate and resistant categories. Imipenem-sensitive–meropenem-resistant (n = 3) and ceftazidime-susceptible–cefepime-resistant (n = 2) phenotypes were not included in the table due to the nonsignificant number of isolates. "All" indicates all possible phenotypes (S/I/R).

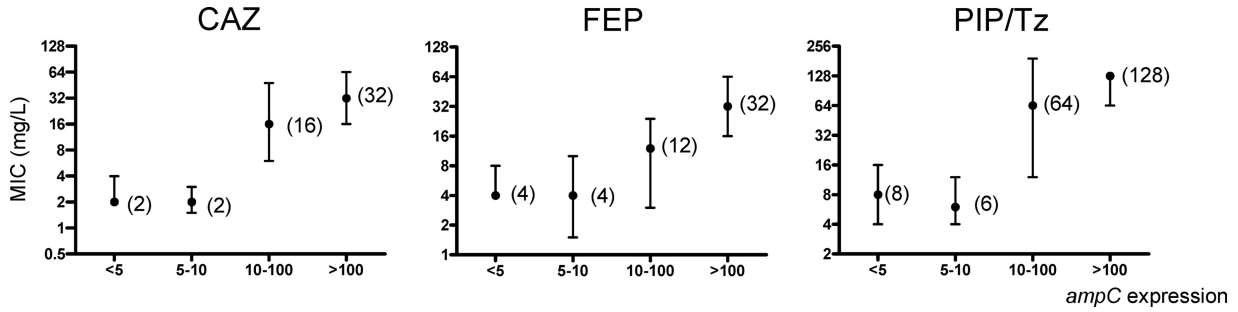

FIG. 2. Effect of the level of *ampC* expression on the MICs of ceftazidime (CAZ), cefepime (FEP), and piperacillin-tazobactam (PIP-Tz). Median MIC values are shown; error bars represent the interquartile ranges.

for defining AmpC and efflux pump overexpression, which vary substantially in the literature (8, 11, 13, 26, 34) and still need to be harmonized. In any case, the lower breakpoints used for *mexY* in the French study are comparable to our breakpoints for borderline expression, which would increase the prevalence to 18.5%, still half of that documented by the above-mentioned study. This apparent discrepancy could well reflect differences in antibiotic use in both countries, since the incidence of isolates hyperproducing MexXY-OprM has been shown to be very much influenced by the use of aminoglycosides, fluoroquinolones, and cefepime, as opposed to carbapenems (14). It should be noted, however, that the first large outbreak of a *P. aeruginosa* strain hyperproducing MexXY-OprM was recently reported for a Spanish hospital (28). Indeed, an understanding of the interplay between the specific antibiotic use policies, the local clonal epidemiology, and the dynamics of *P. aeruginosa* resistance is an issue of major relevance that should be further investigated.

Another important issue to consider for an understanding of resistance dynamics is the interconnections between the different resistance mechanisms. A statistically significant link between *mexB* and *mexY* overexpressions was documented for the first time in this study. Our results are in agreement with previously reported data showing that the production of both resistance mechanisms simultaneously is frequently found for *P. aeruginosa* isolates, despite the finding that independent mutations are apparently responsible for the overexpression of each efflux pump (20). Thus, the epidemiological, biological, or mechanistic factors responsible for this link remain to be elucidated. Perhaps, what is more surprising and concerning is the strong statistical link between *ampC* overexpression and fluoroquinolone resistance documented. Indeed, this study adds AmpC hyperproduction in *P. aeruginosa* to the growing and concerning examples of the strong linkage of fluoroquinolone resistance to β-lactam resistance mechanisms, particularly the extended-spectrum β-lactamases in members of the *Enterobacteriaceae* or methicillin resistance in *Staphylococcus aureus*. It still needs to be experimentally addressed whether this link is favored by the mutagenic effects of fluoroquinolones (3) and/or genetic capitalism (a strain resistant to one antibiotic is more likely to acquire resistance to a second antibiotic) (2).

While most isolates nonsusceptible to ceftazidime or piperacillin-tazobactam were found to hyperproduce AmpC, MexAB-OprM, and (particularly) MexX-OprM, overexpression gained protagonism among cefepime-nonsusceptible isolates. In agreement with data from previous works (11, 32, 35), all imipenem-resistant isolates studied were found to be deficient in the carbapenem porin OprD, due mainly to the presence of inactivating mutations in *oprD*. For those isolates additionally resistant to meropenem, a strong link with AmpC and MexAB-OprM overexpression was evidenced, consistent with previously reported findings (10, 11, 30). Moreover, the highest prevalence of *mexB* overexpression (33%) was found among meropenem-nonsusceptible isolates. On the other hand, the highest prevalence of *mexY* overexpression (37%) was found among tobramycin-nonsusceptible isolates. However, it should be noted that the overexpression of MexXY-OprM is known to confer low-level resistance to aminoglycosides (1). Our results indeed indicate that the overexpression of this efflux system is a frequent coadjuvant of aminoglycoside-modifying enzymes in aminoglycoside resistance, as evidenced by its strong linkage to gentamicin and tobramycin resistance, despite most of the isolates being susceptible to amikacin according to CLSI breakpoints.

Finally, 1% of the isolates (4% of those resistant to imipenem) were found to be MBL producers. While this prevalence of MBLs was still much lower than those reported previously for South America (9), the Far East (16), or certain European countries (33), it denotes an approximately 10-fold increase compared to that reported by a multicenter study performed in Spain 5 years earlier (11).

In summary, the overexpression of AmpC and efflux pumps as well as the mutational inactivation of OprD are frequent among *P. aeruginosa* isolates from bloodstream infections in Spanish hospitals, whereas MBL production, although increasing, is still infrequent.

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