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Genetic Markers of Widespread Extensively Drug-Resistant *Pseudomonas aeruginosa* High-Risk Clones

Gabriel Cabot,[a] Alain A. Ocampo-Sosa,[b] M. Angeles Domínguez,[c] Juan F. Gago,[a] Carlos Juan,[a] Fe Tubau,[c] Cristina Rodríguez,[b] Bartolomé Moyà,[a] Carmen Peña,[c] Luis Martínez-Martínez,[b] and Antonio Oliver[a] on behalf of the Spanish Network for Research in Infectious Diseases (REIPI)

Servicio de Microbiología y Unidad de Investigación, Hospital Universitario Son Espases, Palma de Mallorca, Spain[a]; Servicio de Microbiología, Hospital Universitario Marqués de Valdecilla-IFIMAV, Santander, Spain[b]; and Servicios de Microbiología y Enfermedades Infecciosas, Hospital Universitario de Bellvitge, Barcelona, Spain[c]

Recent reports have revealed the existence of widespread extensively drug-resistant (XDR) *P. aeruginosa* high-risk clones in health care settings, but there is still scarce information on their specific chromosomal (mutational) and acquired resistance mechanisms. Up to 20 (10.5%) of 190 bloodstream isolates collected from 10 Spanish hospitals met the XDR criteria. A representative number (15 per group) of isolates classified as multidrug-resistant (MDR) (22.6%), resistant to 1 to 2 classes (moderately resistant [modR]) (23.7%), or susceptible to all antibiotics (multiS) (43.2%) were investigated in parallel. Multilocus sequence typing (MLST) analysis revealed that all XDR isolates belonged to sequence type 175 (ST175) ($n = 19$) or ST111 ($n = 1$), both recognized as international high-risk clones. Clonal diversity was higher among the 15 MDR isolates (4 ST175, 2 ST111, and 8 additional STs) and especially high among the 15 modR (13 different STs) and multiS (14 STs) isolates. The XDR/MDR pattern in ST111 isolates correlated with the production of VIM-2, but none of the ST175 isolates produced acquired β-lactamases. In contrast, the analysis of resistance markers in 12 representative isolates (from 7 hospitals) of ST175 revealed that the XDR pattern was driven by the combination of AmpC hyperproduction, OprD inactivation (Q142X), 3 mutations conferring high-level fluoroquinolone resistance (GyrA T83I and D87N and ParC S87W), a G195E mutation in MexZ (involved in MexXY-OprM overexpression), and the production of a class 1 integron harboring the *aadB* gene (gentamicin and tobramycin resistance). Of particular interest, in nearly all the ST175 isolates, AmpC hyperproduction was driven by a novel AmpR-activating mutation (G154R), as demonstrated by complementation studies using an *ampR* mutant of PAO1. This work is the first to describe the specific resistance markers of widespread *P. aeruginosa* XDR high-risk clones producing invasive infections.

The increasing prevalence of nosocomial infections produced by multidrug-resistant (MDR) or extensively drug-resistant (XDR) *Pseudomonas aeruginosa* strains severely compromises the selection of appropriate treatments and is therefore associated with significant morbidity and mortality (29, 36, 44). This growing threat results from the extraordinary capacity of this pathogen for developing resistance to nearly all available antibiotics by the selection of mutations in chromosomal genes and from the increasing prevalence of transferable resistance determinants, particularly those encoding class B carbapenemases (metallo-β-lactamases [MBLs]) or extended-spectrum β-lactamases (ESBLs), frequently cotransferred with genes encoding aminoglycoside-modifying enzymes (31, 32, 45).

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 (20, 38, 47, 48, 49). Furthermore, the accumulation of several of these chromosomal mutations can lead to the emergence of XDR/MDR strains which may eventually be responsible for notable outbreaks in the hospital setting (12, 54). Likewise, multiple reports over the last decade have warned of the epidemic dissemination of XDR/MDR strains producing acquired resistance mechanisms (particularly integrons bearing MBL and aminoglycoside-modifying-enzyme genes) in multiple hospitals (42, 43, 46, 57, 59, 62).

Even more worrisome are recent reports which have provided evidence of the existence of MDR/XDR clones disseminated in several hospitals worldwide that have been denominated high-risk clones (60). Among them, ST235, ST111, and ST175 are likely those more widespread (9, 13, 14, 15, 30, 34, 51, 58). Nevertheless, there is still scarce information on the chromosomal (mutational) and acquired resistance mechanisms specific to these high-risk clones. Much less information is available on whether these resistance markers are conserved across the different lineages of a given high-risk clone detected in different hospitals (indicating interhospital dissemination of MDR/XDR high-risk clones) or whether we are, in contrast, facing an independent parallel evolution of XDR/MDR profiles in frequent (originally susceptible) high-risk clones in different settings. In order to gain insights into these major epidemiological and clinical questions, we performed a detailed genetic analysis of the epidemiology and resistance markers of XDR/MDR *P. aeruginosa* clones causing bloodstream infections in 10 different Spanish hospitals.

MATERIALS AND METHODS

Bacterial strains, susceptibility testing, and definition of resistance profiles. A total of 190 *P. aeruginosa* isolates recovered from bloodstream infections in a 2008-2009 multicenter study (10 hospitals from different

Received 9 July 2012 Returned for modification 18 August 2012
Accepted 30 September 2012
Published ahead of print 8 October 2012
Address correspondence to Antonio Oliver, antonio.oliver@ssib.es.

doi:10.1128/AAC.01388-12

TABLE 1 Primers used in this work for amplification or cloning of resistance determinants

Primer	Sequence (5′→3′)[a]	PCR product size (bp)	Target	Reference or source
ampCF	GCGCGCAGGGCGTTCAG	1,467	*ampC*	37
$ampCR_2$	CGGAGGGGCGGGGAAGC			This work
ampDF	GTACGCCTGCTGGACGATG	910	*ampD*	24
ampDR	GAGGGCAGATCCTCGACCAG			
ampRF	GTCGACCCAGTGCCTTCAGG	1,391	*ampR*	24
ampRR	CTCGAGAGCGAGATCGTTGC			
$AmpR_{CL}$F-ERI	TCGAATTCGTCGACCAGTGCCTTCAGG	1,391	*ampR*	This work
$AmpR_{CL}$R-BHI	TCGGATCCCTCGAGAGCGAGATCGTTGC			
dacBF	CGACCATTCGGCGATATGAC	1,721	*dacB*	38
dacBR	CGCGTAATCCGAAGATCCATC			
oprDF	CGCCGACAAGAAGAACTAGC	1,412	*oprD*	17
oprDR	GTCGATTACAGGATCGACAG			
$gyrA_1$	TTATGCCATGAGCGAGCTGGGCAACGACT	364	*gyrA*	41
$gyrA_2$	AACCGTTGACCAGCAGGTTGGGAATCTT			
$gyrB_3$	AGCTCGCAGACCAAGGACAAG	600	*gyrB*	41
$gyrB_4$	GGGCTGGGCGATGTAGATGTA			
$parC_1$	ATGAGCGAACTGGGGCTGGAT	208	*parC*	41
$parC_2$	ATGGCGGCGAAGGACTTGGGA			
$parE_1$	CGGCGTTCGTCTCGGGCGTGGTGAAGGA	592	*parE*	41
$parE_2$	TCGAGGGCGTAGTAGATGTCCTTGCCGA			
INT-R_i	CGCAGTGGCGGTTTTCAT	Variable	Class 1 Integrons	17
qacE-R	CAAGAAAAAGCCAGCCTTTC			
$mexR_{INT}$	GGATGATGCCGTTCACCTG	1,016	*nalB*	18
$mexR_{20}$	CCAGTAAGCGGATAC			
$nalC_1$	TCAACCCTAACGAGAAACGCT	814	*nalC*	33
$nalC_2$	TCCACCTCACCGAACTGC			
$nalD_1$	GCGGCTAAAATCGGTACACT	789	*nalD*	53
$nalD_2$	ACGTCCAGGTGGATCTTGG			
mexZF	ATTGGATGTGCATGGGTG	1,000	*mexZ*	52
mexZR	TGGAGATCGAAGGCAGC			
PA5471F	GATCTACCGTTTCAATCACATGGAT	1,299	*PA5471*	This work
PA5471R	GGCCACCTCCTCGATTACCT			

[a] Sites for restriction endonucleases are underlined.

geographic locations) in Spain were studied. The MICs of ceftazidime, cefepime, piperacillin, piperacillin plus tazobactam (fixed concentration of 4 μg/ml), aztreonam, imipenem, meropenem, ciprofloxacin, gentamicin, tobramycin, amikacin, and colistin were determined by broth microdilution following CLSI guidelines and breakpoints (10) in a previous work (7). The expression of *ampC* and efflux pump-encoding genes (*mexB*, *mexD*, *mexF*, and *mexY*), as well as the presence of acquired β-lactamases, was also determined in the preceding study. Recent consensus recommendations (35) were used to define MDR (nonsusceptible to ≥3 classes of drugs) and XDR (nonsusceptible to all but 1 or 2 classes of drugs) strains, considering the following 7 antimicrobial classes: cephalosporins (ceftazidime or cefepime), penicillin-β-lactamase inhibitor combinations (piperacillin-tazobactam), monobactams (aztreonam), carbapenems (imipenem, or meropenem), fluoroquinolones (ciprofloxacin), aminoglycosides (gentamicin, tobramycin, or amikacin), and polymyxins (colistin). Strains susceptible to all tested antipseudomonal agents were included in the multisusceptible (multiS) category, and those nonsusceptible to at least one agent in 1 or 2 classes were included in the moderately resistant (modR) category. All XDR isolates ($n = 20$) and a representative number of isolates showing the MDR, modR, and multiS profiles (15 isolates per group) were studied in detail in this work.

Molecular typing. Clonal relatedness was evaluated by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST), each performed blindly in independent laboratories. For PFGE, bacterial DNA embedded in agarose plugs prepared as described previously (26) was digested with SpeI. DNA separation was performed in a contour-clamped homogeneous-electric-field DRIII apparatus (Bio-Rad, La Jolla, CA) under the following conditions: 6 V/cm^2 for 26 h with pulse times of 5 to 40 s. DNA macrorestriction patterns were interpreted according to the criteria established by Tenover et al. (55). For MLST analysis, previously described schemes and protocols (11) and available databases and tools (http://pubmlst.org/paeruginosa) (22) were used.

Amplification and sequencing of resistance determinants. All primers used for amplification of resistance genes are listed in Table 1. *ampC*, *ampR*, *ampD*, and *dacB* (PBP4) genes were PCR amplified and sequenced, following previously described protocols (38), in strains presenting *ampC* overexpression. Likewise, genes involved in the regulation of MexAB-OprM (*nalB*, *nalC*, and *nalD*) or MexXY-OprM (*mexZ* and *PA5471*) were amplified and sequenced as previously described in the strains that overexpressed these efflux pumps (18, 33, 52, 53). Additionally, the quinolone resistance-determining regions (QRDR) of *gyrA*, *gyrB*, *parC*, and *parE* were sequenced in ciprofloxacin-nonsusceptible strains (41), while *oprD* (17) was sequenced in those strains nonsusceptible to imipenem (partial *oprD* sequence data were obtained from previous work [7, 40]). Finally, the presence of class 1 integrons and the associated resistance determinants was also evaluated by PCR amplification followed by sequencing using previously described protocols (17). After duplicate PCR amplification, sequencing reactions were performed with the BigDye Terminator kit (PE Applied Biosystems, Foster City, CA), and sequences were analyzed on an ABI Prism 3100 DNA sequencer (PE Applied Biosystems). The resulting sequences were then compared with that of wild-type PAO1 and those available at GenBank (www.ncbi.nih.gov/BLAST).

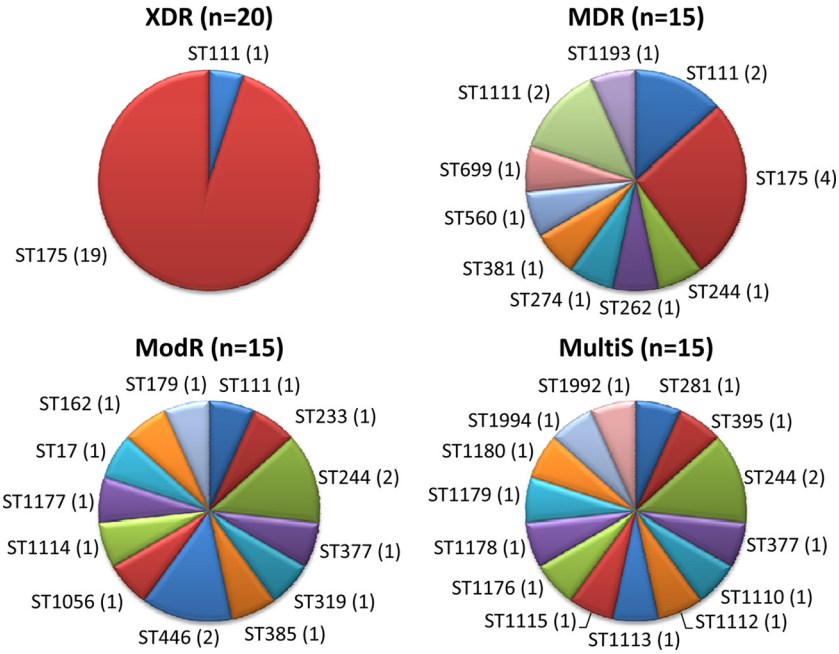

FIG 1 Distribution of STs among the XDR, MDR, modR, and multiS isolates studied. The number of isolates of each ST is shown in parentheses.

Cloning of *ampR* genes and complementation studies. *ampR* genes from wild-type strain PAO1 and from ST175 XDR clinical isolate PAmb12 (G154R AmpR mutant) were PCR amplified using primers listed in Table 1. PCR products were digested with EcoRI and BamHI and ligated to pUCP24 to obtain $pUCPAR_{WT}$ and $pUCPAR_{G154R}$ plasmids that were transformed into *E. coli* XL1-Blue made competent by $CaCl_2$. Transformants were selected in gentamicin (5 μg/ml)–MacConkey agar plates. The cloned genes obtained from three independent experiments were fully sequenced to ascertain the absence of mutations introduced during PCR amplification. Plasmids $pUCPAR_{WT}$ and $pUCPAR_{G154R}$ were then electroporated into a previously constructed (38) *ampR* knockout mutant of PAO1 (PAΔR). Transformants were selected on gentamicin (50 μg/ml)–Luria-Bertani (LB) agar plates. The effect of the G154R AmpR mutation in β-lactam resistance and *ampC* expression and induction was evaluated in transformants and control strains through (i) determination of ceftazidime and imipenem MICs in Mueller-Hinton (MH) agar using Etest strips, (ii) quantification of basal and induced (50 μg/ml cefoxitin) *ampC* expression through real-time RT-PCR following previously described protocols (24), and (iii) phenotypic determination of AmpC inducibility through the assessment of the presence of antagonism between imipenem and ceftazidime disks (separated by 5 to 30 mm) on MH agar plates, as described previously (24).

RESULTS

Clonal diversity according to susceptibility profiles. A total of 20 of the 190 isolates (10.5%) met the XDR criteria, whereas 22.6% were categorized as MDR (excluding XDR), 23.7% as modR (resistant to 1 or 2 antimicrobial classes), and 43.2% as multiS (susceptible to all tested antipseudomonal agents). Clonal relatedness was evaluated by MLST in all XDR isolates and in a representative number (15 per group) of MDR, modR, and multiS isolates. As shown in Fig. 1, all XDR isolates belonged to MLST sequence type 175 (ST175) ($n = 19$) or ST111 ($n = 1$), both previously identified as internationally spread high-risk clones. Notably, XDR ST175 isolates were detected in 7 of the 10 hospitals, with a wide geographical distribution covering all 4 regions participating in the study. On the other hand, clonal diversity was much higher among the 15 MDR isolates evaluated (4 ST175, 2 ST111, and 8 additional STs) and especially among the 15 modR (13 different STs) and 15 multiS (14 STs) isolates (Fig. 1). Thus, a total of 33 different STs were detected among the 65 isolates evaluated, 16 (49%) of which were contributed to the MLST database as new clones (http://pubmlst.org/paeruginosa). Besides high-risk-clones ST175 (19 XDR and 4 MDR isolates) and ST111 (1 XDR, 2 MDR, and 1 modR), only the clone ST244, not particularly associated with resistance, was detected in more than two isolates (1 MDR, 2 modR, and 2 multiS). Three additional STs were detected in two isolates, whereas the 27 remaining STs were detected in single isolates. Only 3 pairs of STs were identified as single-locus variants (SLVs) through BURST analysis, whereas all 27 other STs (including ST175 and ST111 high-risk clones) were classified as singletons. A similar diversity was observed through PFGE analysis and, notably, 21 of the 23 ST175 isolates were independently identified as the same clone by this approach (not shown).

Genetic markers of widespread XDR/MDR high-risk clones. The susceptibility profiles and resistance mechanisms of the XDR, MDR, and modR isolates studied are shown in Table 2. The presence of acquired β-lactamases was only detected in the 4 isolates belonging to the ST111 high-risk clone. Namely, the 3 XDR/MDR ST111 isolates produced a VIM-2 MBL and/or an OXA-46 oxacillinase located in different class 1 integrons, whereas the modR isolate produced the narrow-spectrum penicillinase PSE-1, also encoded in a class 1 integron. Interestingly, the 3 XDR/MDR ST111 isolates (recovered from 2 distantly located hospitals) additionally showed the same specific inactivating mutation in OprD (W339X), as well as two QRDR mutations (GyrA T83I and ParC S87L) conferring high-level fluoroquinolone resistance.

On the other hand, all but 1 of the 23 (19 XDR and 4 MDR) high-risk-clone ST175 isolates had a similar resistance profile, which included cephalosporins, penicillin-β-lactamase inhibitor

TABLE 2 Susceptibility profiles and resistance mechanisms of the studied isolates

Profile	PFGE	ST	ID[a]	MIC (μg/ml) and clinical category (CLSI breakpoint)[b]																				Resistance mechanism(s)[c]				
				ATM		CAZ		FEP		PTZ		IMP		MER		GEN		TOB		AMK		CIP		COL		*ampC*[d]	*oprD*	*gyrA*	*parC*	Integrons
XDR	3	111	238	64	R	128	R	128	R	32	S	64	R	64	R	64	R	64	R	128	R	32	R	2	S	N	W339X[e]	T83I	S87L	(1) *aacA7-bla*$_{VIM-2}$-*aac(6′)-II* (2) *aac(6′)-Ib-bla*$_{OXA46}$
	1	175	12	16	I	16	I	16	I	32	S	16	R	16	R	64	R	16	R	4	S	32	R	0.5	S	P	Q142X[f]	T83I, D87N	S87W	
	1	175	27	32	R	64	R	128	R	64	S	8	I	64	R	64	R	64	R	2	S	32	R	2	S	P	Q142X	T83I, D87N	S87W	
	2	175	43	16	I	64	R	32	R	128	R	32	R	16	R	64	R	64	R	8	S	32	R	1	S	P	Q142X	T83I, D87N	S87W	
	1	175	93	32	R	64	R	32	R	128	R	16	R	16	R	64	R	64	R	4	S	32	R	0.25	S	P	Q142X	T83I, D87N	S87W	
	1	175	123	32	R	32	R	32	R	128	R	16	R	32	R	64	R	32	R	4	S	32	R	0.12	S	P	Q142X	T83I, D87N	S87W	
	1	175	147	16	I	32	R	32	R	64	S	32	R	16	R	64	R	64	R	4	S	32	R	4	I	P	Q142X	T83I, D87N	S87W	
	1	175	179	16	I	32	R	32	R	128	R	32	R	16	R	64	R	32	R	4	S	32	R	1	S	P	Q142X	T83I, D87N	S87W	
	1	175	207	16	I	32	R	16	I	64	S	16	R	16	R	64	R	8	I	0.5	S	32	R	0.12	S	P	Q142X	T83I, D87N	S87W	
MDR	3	111	19	4	S	64	R	16	I	32	S	64	R	64	R	64	R	16	R	8	S	16	R	0.5	S	N	W339X	T83I	S87L	*aacA7-bla*$_{VIM-2}$-*aac(6′)-II*
	3	111	81	8	S	4	S	8	S	8	S	64	R	16	R	64	R	64	R	16	S	32	R	1	S	N	W339X	T83I	S87L	*aac(6′)-Ib-bla*$_{OXA46}$
	1	175	67	8	S	8	S	8	S	16	S	8	I	4	S	64	R	16	R	8	S	32	R	0.25	S	P	Q142X	T83I, D87N	S87W	
	2	175	75	16	I	8	S	16	I	16	S	2	S	1	S	64	R	32	R	4	S	32	R	0.06	S	N	WT	T83I, D87N	S87W	
	1	175	245	8	S	8	S	4	S	64	S	32	R	8	I	32	R	2	S	0.25	S	32	R	0.125	S	P	Q142X	T83I, D87N	S87W	
	1	175	258	2	S	8	S	16	I	8	S	32	R	2	S	64	R	64	R	8	S	32	R	0.5	S	P	Q142X	T83I, D87N	S87W	
	13	244	205	16	I	8	S	16	I	16	S	2	S	8	I	4	S	1	S	4	S	32	R	0.5	S	N		T83I	S87L	
	14	262	60	32	R	8	S	32	R	32	S	64	R	64	R	4	S	1	S	16	S	1	S	1	S	N	nt$_{673}$Ins(T)			
	15	274	148	64	R	128	R	128	R	256	R	2	S	1	S	16	R	4	S	16	S	0.25	S	1	S	P				
	20	381	187	16	I	128	R	64	R	32	S	1	S	0.25	S	2	S	0.5	S	2	S	32	R	0.03	S	P		T83I, D87N	S87L	
	25	560	89	16	I	4	S	8	S	16	S	8	I	0.25	S	2	S	1	S	16	S	0.5	S	1	S	N	nt$_{1113}$Ins(AT)			
	26	699	37	4	S	2	S	32	R	4	S	8	I	0.5	S	4	S	1	S	8	S	4	R	2	S	N	WT	T83I	WT	
	29	1111	59	16	I	64	R	32	R	128	R	32	R	8	I	4	S	0.5	S	4	S	0.12	S	0.5	S	P	MP[g]			
	29	1111	62	16	I	16	I	16	I	64	S	16	R	32	R	2	S	0.25	S	2	S	8	R	1	S	N	nt$_{457}$Ins(A)	T83I	WT	
	39	1193	20	128	R	128	R	64	R	256	R	2	S	2	S	1	S	1	S	8	S	0.06	S	0.5	S	P				
ModR	4	17	191	4	S	2	S	16	I	8	S	0.5	S	0.5	S	4	S	1	S	8	S	32	R	0.25	S	N		T83I	S87L	
	5	111	131	0,25	S	1	S	4	S	1	S	0.12	S	0.06	S	8	I	16	R	32	I	8	R	0.12	S	N		T83I	S87W	*aacA4-bla*$_{PSE-1}$-*aadA2*
	6	162	17	8	S	4	S	4	S	8	S	32	R	8	I	1	S	0.25	S	2	S	0.12	S	0.25	S	N	nt$_{482}$Δ17			
	7	179	54	4	S	2	S	16	I	8	S	0.5	S	0.25	S	1	S	1	S	4	S	0.12	S	2	S	N				
	8	233	97	16	I	2	S	4	S	16	S	16	R	8	I	2	S	0.5	S	4	S	0.06	S	0.25	S	N	nt$_{1163}$Δ2			
	10	244	16	2	S	2	S	4	S	8	S	2	S	4	S	0.25	S	0.12	S	2	S	32	R	0.25	S	N		T83I	S87L	
	12	244	125	16	I	4	S	8	S	16	S	1	S	2	S	1	S	0.5	S	2	S	0.5	S	1	S	N				
	17	319	48	4	S	4	S	2	S	4	S	4	S	1	S	0.5	S	0.25	S	4	S	0.25	S	8	I	N				
	18	377	25	8	S	4	S	4	S	16	S	32	R	8	I	1	S	1	S	2	S	0.5	S	4	I	N	nt$_{433}$Δ14			
	N/D	385	181	4	S	4	S	16	I	4	S	2	S	0.5	S	2	S	0.5	S	4	S	0.12	S	0.25	S	N				
	22	446	63	8	S	16	I	8	S	64	S	16	R	4	S	2	S	0.5	S	4	S	0.06	S	1	S	P	MP			
	23	446	199	16	I	32	R	16	I	64	S	4	S	0.5	S	2	S	0.5	S	2	S	0.06	S	0.5	S	P				
	27	1056	186	8	S	2	S	16	I	4	S	4	S	0.12	S	4	S	1	S	8	S	2	I	0,5	S	N		T83I	WT	
	31	1114	120	4	S	4	S	2	S	4	S	64	R	2	S	1	S	0.5	S	2	S	0.12	S	0.125	S	P	MP			
	34	1177	132	32	R	32	R	16	I	32	S	0,5	S	0.5	S	2	S	0.5	S	4	S	0.25	S	0.125	S	P				

[a] ID, strain identification number.

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

[c] Sequencing results are in comparison to those of reference wild-type strain PAO1 (www.pseudomonas.com). All isolates showed wild-type *gyrB* sequence, and a single isolate (ST381) presented a mutation in *parE* (E459K). WT, wild type; nt, nucleotide.

[d] Strains were considered positive for *ampC* 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 (7). P, positive; N, negative.

[e] TGG→T**A**G (premature stop codon) (boldface indicates the mutated nucleotide).

[f] CAA→**T**AA (premature stop codon) (boldface indicates the mutated nucleotide).

[g] MP, multiple polymorphisms of unknown effect compared to PAO1 sequence.

TABLE 3 Sequence analysis of genes involved in MexAB-OprM and MexXY-OprM regulation

ST (resistance profile)	ID[a]	Expression level[b]		Presence of polymorphism(s) in sequence[c]				
				MexAB-OprM regulator			MexXY-OprM regulator	
		mexB	*mexY*	*nalB*	*nalC*	*nalD*	*mexZ*	*PA5471*
175 (XDR/MDR)	Several[d]	V	V	WT	(G71E), A186T	WT	G195E	(L88P), D161G, H182Q, (V243A), V266 M
244 (MDR)	205	P	P	WT	WT	T11N	WT	(L88P), G157D, D161G, H182Q, (V243A)
262 (MDR)	60	P	P	T69P	(G71E), (S209R)	WT	WT	(L88P), D161G, H182Q, (V243A)
560 (MDR)	89	N	P				$nt_{283}\Delta 11$	(C40R), (L88P), (S112N), (D119E), (I237V), (V243A), P244L
699 (MDR)	37	N	P				$nt_{207}\Delta 10$	WT
1111 (MDR)	62	P	N	I68T, (V126E), V132A	(G71E), (S209R)	WT		
179 (modR)	54	N	P				$nt_{630}\Delta 21$	(L88P), D161G, H182Q, (V243A)
244 (modR)	125	P	P	WT	WT	WT	$nt_{292}\Delta 10$	(L88P), G157D, D161G, H182Q, (V243A)
377 (modR)	25	P	N	(V126E)	$nt_{239}\Delta 10$	WT		
1056 (modR)	186	P	P	WT	(G71E), (S209R)	WT	$nt_{278}\Delta 2$	(C40R), (L88P), (S112N), (D119E), (I237V), (V243A)

[a] ID, strain identification number.
[b] Previously defined breakpoints were used (7). Strains were considered positive for *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. V, variable; P, positive; N, negative.
[c] Sequencing results are in comparison (www.pseudomonas.com) to those of reference wild-type strain PAO1; polymorphisms present in reference wild-type strain PA14 are shown in parentheses. WT, wild type; nt, nucleotide.
[d] Regulatory genes were sequenced in all ST175 isolates overexpressing *mexB* ($n = 7$) or *mexY* ($n = 11$), as well as 2 additional isolates not showing overexpression of either of the efflux pumps. The same sequences were documented in all cases, and therefore, they are shown only once.

combinations, monobactams, carbapenems, fluoroquinolones, and aminoglycosides (gentamicin and tobramycin). Borderline susceptibility to cephalosporins, penicillin-β-lactamase inhibitor combinations, and/or monobactams determined that 3 of the ST175 isolates were classified as MDR instead of XDR. The discordant isolate was susceptible to all β-lactams but resistant to ciprofloxacin, gentamicin, and tobramycin. Moreover, in contrast to ST111 isolates, all but this single isolate were documented to overexpress the chromosomal cephalosporinase AmpC instead of producing acquired β-lactamases. Table 2 shows a detailed analysis of the resistance mechanisms detected in 12 representative (from 7 different hospitals) ST175 isolates, including 8 of the XDR isolates and the 4 MDR isolates. As can be observed, in addition to showing AmpC overexpression (see next section for detailed analysis of the genetic mechanisms), all but the single carbapenem-susceptible isolate presented the same specific inactivating mutation in OprD (Q142X), which was not detected in any of the other carbapenem-resistant clones (Table 2). Likewise, high-level ciprofloxacin resistance in ST175 isolates was driven in all cases by a specific combination of 3 QRDR mutations (GyrA T83I and D87N and ParC S87W) not detected in any of the other fluoroquinolone-resistant clones studied (Table 2). Finally, in all ST175 isolates, gentamicin and tobramycin resistance resulted from the production of a class 1 integron harboring *aadB* as a single gene cassette, coding for Ant(2″)-Ia.

The prevalence and genetic markers of efflux pump (MexAB and MexXY) overexpression in XDR/MDR high-risk clones was also investigated, and the results are shown in Table 3. None of the ST111 isolates overexpressed *mexB* or *mexY*. In contrast, 11 of the 23 (48%) XDR/MDR ST175 isolates overexpressed *mexY*. Nevertheless, despite the fact that over 50% of ST175 isolates did not exceed the previously defined *mexY* overexpression threshold of 10-fold (compared to its expression in wild-type PAO1) (7), all of them showed the same *mexZ* sequence, which specifically contained the G195E substitution, previously demonstrated to determine MexXY-OprM overexpression (19). Thus, despite a wide range (from <5- to 37-fold) of *mexY* expression levels being documented for our collection of 23 isolates, the *mexZ* G195E mutation is an additional conserved genetic marker of resistance in XDR/MDR ST175 isolates. In addition to the *mexZ* mutation, all ST175 isolates studied showed several substitutions in gene *PA5471* (also involved in MexXY-OprM regulation) compared to its sequence in PAO1 (Table 3). However, all the substitutions detected were found to be evenly distributed among reference wild-type *P. aeruginosa* genomes (www.pseudomonas.com), suggesting that they are just polymorphisms not involved in MexXY-OprM overexpression. As shown in Table 3, *mexY* overexpression was also frequent in sporadic MDR/resistant clones but, in contrast to ST175, mostly resulted from partial deletions of the *mexZ* coding sequence.

A number of ST175 (7 of 23, 30.4%) isolates were also found to overexpress *mexB* (expression level ≥3-fold compared to that of wild-type PAO1) (7). Nevertheless, as occurred for *mexY*, despite a relatively wide range of *mexB* expression levels (<2- to 6.6-fold), all ST175 isolates studied showed the same sequence for the regulators *nalB* (*mexR*), *nalC*, and *nalD*; *nalB* and *nalD* sequences were identical to those of PAO1, whereas *nalC* showed in all cases two substitutions (G71E and A186T) (Table 3). The G71E substitution is a very frequent polymorphism among wild-type *P. aeruginosa* reference genomes, and it is therefore not thought to be involved in MexAB-OprM overexpression (33). On the other hand, the

TABLE 4 Sequencing of genes involved in AmpC hyperproduction

Profile	ST	ID[a]	*ampC*[b]	Presence of polymorphism(s) in sequence[c]			
				ampC	*dacB*	*ampD*	*ampR*
XDR	175	12	P	WT	WT	G148A, D183Y	G154R
	175	27	P	WT	WT	G148A, D183Y	G154R
	175	43	P	WT	WT	T139 M, G148A, D183Y	WT
	175	93	P	WT	WT	G148A, D183Y	G154R
	175	123	P	WT	WT	G148A, D183Y	G154R
	175	147	P	WT	P59S	G148A, D183Y	G154R
	175	179	P	WT	WT	G148A, D183Y	G154R
	175	207	P	WT	WT	G148A, D183Y	G154R
MDR	175	67	P	WT	WT	G148A, D183Y	G154R
	175	75	N	WT	WT	G148A, D183Y	WT
	175	245	P	WT	WT	G148A, D183Y	G154R
	175	258	P	WT	WT	G148A, D183Y	G154R
	274	148	P	(T105A), (G391A)	A394P	R11L, P41L, G148A	WT
	381	187	P	(T105A), G229S, G248S	WT	G148A, D183Y	(G283E), (M288R)
	1111	59	P	(T105A)	T239S	G156S	WT
	1193	20	P	(T105A), L176R, D233E	Deletion from nt 853	A134V	WT
ModR	446	63	P	(T105A), (V205L),(G391A)	WT	WT	(E114A), (G283E), (M288R)
	446	199	P	(T105A), (V205L), (G391A)	WT	WT	(E114A), (G283E), (M288R)
	1114	120	P	(T105A), (V205L),V356I, (G391A)	Q33H, Q37H, L469 M	WT	A51T, (E114A)
	1177	132	P	(T105A), L176R	Q212X[d]	G148, D183Y	WT

[a] ID, strain identification number.
[b] Strains were considered positive for *ampC* 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 (7). P, positive; N, negative.
[c] Sequencing results are in comparison (www.pseudomonas.com) to those of reference wild-type strain PAO1; polymorphisms present in reference wild-type strain PA14 are shown in parentheses. WT, wild type; nt, nucleotide.
[d] CAG→**T**AG (premature stop codon) (boldface indicates the mutated nucleotide).

A186T substitution is not common among sequenced genomes but has been detected in a few clinical strains (8, 56). However, its role in MexAB-OprM overexpression, if any, still needs to be experimentally demonstrated.

Characterization of a novel AmpR-activating mutation driving AmpC hyperproduction in XDR/MDR *P. aeruginosa* ST175 high-risk clone. In order to determine the genetic markers of AmpC hyperproduction in XDR/MDR ST175 isolates, *ampC* and the genes involved in its regulation (*ampD*, *dacB* [PBP4], and *ampR*) were sequenced in a representative number of isolates from this high-risk clone (8 of the XDR isolates and the 4 MDR isolates, including the single isolate not showing *ampC* overexpression), as well as in all other sporadic MDR/modR clones with documented AmpC hyperproduction (Table 4).

Regarding *ampC*, all ST175 isolates showed a wild-type sequence identical to that of PAO1. Curiously, all other clones showed several AmpC polymorphisms, including the T105A substitution, which has been correlated with more-efficient carbapenem and cefepime hydrolysis (50), although there is still controversy on the real contribution of these polymorphisms, found in wild-type reference strains such as PA14, to the resistance profiles (61).

The selection of AmpC-hyperproducing mutants during antimicrobial therapy is well known to be driven mainly by *ampD* and/or *dacB* mutations (23, 38, 39). This was evident in sporadic MDR/modR clones, frequently showing inactivating mutations in *dacB* (deletions or stop codons) or mutations in key conserved residues of *ampD* (P41L, G156S, or H157R) (16, 24). The DacB A394P substitution found in one of the isolates has also been previously detected in spontaneous *in vitro* mutants of PAO1 overexpressing *ampC* (39), but the effect of the other *dacB* substitutions, despite affecting conserved residues, still needs to be experimentally explored (Table 4).

The analysis of ST175 high-risk-clone isolates, however, provided quite different and unexpected results (Table 4). All of the isolates (including the one negative for *ampC* overexpression) presented two polymorphisms (G148A and D183Y) in AmpD which are very frequent in wild-type strains and are therefore not involved in *ampC* overexpression, but only two of the 13 isolates studied that overexpressed *ampC* showed mutations in conserved residues of AmpD (T139M) or DacB (P59S). In contrast, all ST175 isolates, except for the one that did not overexpress *ampC* and the AmpD T139M mutant, showed a specific amino acid replacement (G154R) in a highly conserved residue of AmpR. Although other substitutions (A51T, E114A, G283E, or M288R) were detected in some sporadic MDR/modR clones, they were all common polymorphisms found in the genomes of wild-type reference strains, such as PA14 (www.pseudomonas.com). Indeed, specific amino acid substitutions, namely, R86C, G102E, and D135N, characterized in enterobacterial species such as *Citrobacter freundii*, are known to determine a conformational change in the LysR-type AmpR regulator, which alters its interaction with the DNA operator to convert the protein into a transcriptional activator (4, 28). Of them, only the D135N mutation has been detected so far in a single *P. aeruginosa* isolate (1). Thus, in order to explore the effect of the G154R substitution, wild-type and mutant *ampR* genes

TABLE 5 Effect of the G154R AmpR mutation in β-lactam resistance and *ampC* expression

Strain	MIC (μg/ml)[a]		Mean *ampC* expression[b] ± SD	
	CAZ	IMP	Basal	Induced[c]
PAO1	1	1.5	1	52.1 ± 17.9
PAΔR	2	0.25	3.3 ± 1.7	3.2 ± 1.8
PAΔR(pUCPΔR$_{WT}$)	1.5	2	3.0 ± 1.3	42.5 ± 19.2
PAΔR(pUCPΔR$_{G154R}$)	8	2	74.7 ± 25.2	258.8 ± 37.8

[a] CAZ, ceftazidime; IMP, imipenem.
[b] Amount of *ampC* mRNA relative to PAO1 basal levels.
[c] Induction experiments were carried out with 50 μg/ml of cefoxitin.

were cloned in parallel and transformed into the *ampR* knockout mutant of PAO1 (PAΔR). The documented effects of the G154R AmpR mutation in β-lactam resistance and *ampC* expression and induction are shown in Table 5 and Fig. 2 (double-disk AmpC induction test). As expected, the introduction of wild-type AmpR in PAΔR restored *ampC* inducibility and basal imipenem (potent AmpC inducer) susceptibility. On the other hand, the introduction of the G154R mutant not only restored inducibility but also determined a drastic increase in basal *ampC* expression and in the MIC of ceftazidime (weak AmpC inducer). Thus, our results clearly indicate that the G154R mutation converts AmpR into a transcriptional activator.

DISCUSSION

Over the last years, several reports have provided strong evidence for the existence of XDR/MDR *P. aeruginosa* high-risk clones disseminated in several hospitals worldwide. In this work, we report for the first time a detailed analysis of the genetic markers of antibiotic resistance in such lineages in comparison to those in sporadic clones. Among *P. aeruginosa* XDR/MDR high-risk clones, ST235, ST111, and ST175 are those likely to be more widespread (9, 13, 14, 15, 30, 34, 51, 58). Of them, ST235 isolates were not detected in our multicenter study of bloodstream infections, but other recent reports have detected this high-risk clone in Spain, linked either to a large outbreak of GES-5 class A carbapenemase-producing *P. aeruginosa* in a hospital in Madrid (58) or to the class B carbapenemase VIM-13, autochthonous from the Balearic Islands (25). A few of the XDR/MDR isolates from our study belonged to ST111, linked to the MBL VIM-2, but the vast majority (19 of 20) of XDR isolates belonged to ST175, detected in 7 of the 10 hospitals participating in the multicenter study. Likewise, other recent works have revealed that ST175 is also the high-risk clone more widespread in French hospitals (9).

Through a detailed analysis, we determined the genetic markers of antibiotic resistance of the highly disseminated ST175 clone. These markers included an inactivating mutation in OprD (Q142X) that determines carbapenem resistance, a mutation in AmpR (G154R) that drives AmpC hyperproduction (conferring resistance to penicillins, cephalosporins, and monobactams), 3 QRDR mutations leading to high-level fluoroquinolone resistance (GyrA T83I and D87N and ParC S87W), and the production of a class 1 integron harboring the *aadB* gene (gentamicin and tobramycin resistance). All ST175 isolates additionally showed several substitutions in genes involved in the regulation of efflux pumps. Of them, the G195E mutation in MexZ has been clearly demonstrated to be involved in the overexpression of the efflux pump MexXY-OprM (19), which further increases resistance to multiple antipseudomonal agents, including fluoroquinolones, aminoglycosides, and cefepime. Notably, this complex set of resistance markers was conserved in nearly all ST175 isolates studied, which were recovered from 7 different hospitals with a wide geographical distribution covering all 4 regions participating in the study. Nevertheless, we recently reported two large outbreaks of ST175 *P. aeruginosa* producing MBL VIM-2 (59) or VIM-20 (15, 48) in two hospitals in different Spanish cities. Thus, we explored whether the genetic markers were conserved among the ST175 strains from those outbreaks and found that the OprD and AmpR mutations were replaced by the corresponding MBL but that all of them contained the same set of 3 QRDR mutations, suggesting that fluoroquinolone resistance is at the bottom line of the evolution of the XDR/MDR ST175 high-risk clone. Indeed,

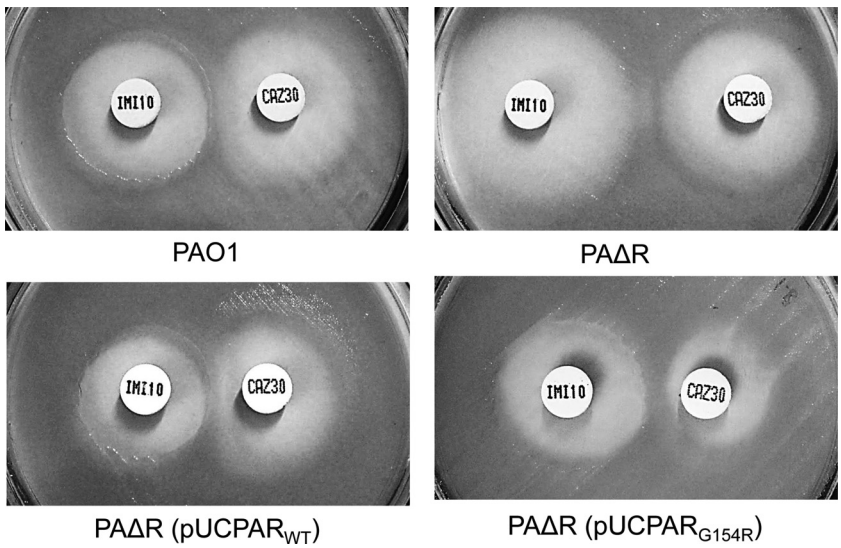

FIG 2 Results for double-disk (imipenem-ceftazidime) AmpC induction test in wild-type PAO1, the *ampR* knockout mutant PAΔR, and PAΔR complemented with wild-type *ampR* (pUCPAR$_{WT}$) or with the G154R mutant (pUCPAR$_{G154R}$).

this adds to growing and worrisome examples of strong linkage of fluoroquinolone resistance to XDR/MDR phenotypes, particularly noteworthy being the extended-spectrum β-lactamases in *Enterobacteriaceae* or methicillin resistance in *Staphylococcus aureus*. Nevertheless, it still needs to be experimentally addressed whether this link is favored by the mutagenic effects of fluoroquinolones (6) and/or genetic capitalism (5). Likewise, whether there are specific features of certain high-risk clones that promote the development of mutation-driven resistance and/or the acquisition of resistance determinants through horizontal gene transfer still needs to be explored.

Among the resistance mechanisms detected in the widespread ST175 clone, a specific mutation (G154R) in the transcriptional regulator AmpR is particularly noteworthy. AmpR is a LysR-type transcriptional regulator required for AmpC induction. Upon exposure to certain β-lactams, such as imipenem or cefoxitin (AmpC inducers), certain cell wall metabolites generated interact with AmpR, determining a conformational change which alters its interaction with the DNA operator to convert the protein from a repressor into a transcriptional activator of *ampC* expression (21). A similar effect (AmpR-mediated activation of *ampC* expression) is obtained through the mutational inactivation of several enzymes involved in peptidoglycan recycling, such as AmpD and/or DacB (PBP4). Indeed, the selection of AmpC-hyperproducing mutants, frequently leading to the failure of antimicrobial therapy with antipseudomonal penicillins or cephalosporins, is known to be driven mainly by mutations that inactivate AmpD and/or DacB, leading to the derepression of the chromosomal cephalosporinase (23, 38, 39). Consistent with this, the sporadic MDR/modR clones that hyperproduced AmpC frequently showed inactivating mutations in *dacB* or *ampD*. There is, however, a third possibility for the activation of *ampC* expression, the acquisition of specific mutations in AmpR that directly provoke the required conformational change. Indeed, specific amino acid substitutions, namely, R86C, G102E, and D135N, characterized in enterobacterial species such as *C. freundii*, are known to produce such an activating effect (4, 28). Here, we demonstrate that the G154R mutation in *P. aeruginosa* also activates AmpR, leading to a drastic increase in basal *ampC* expression and conferring resistance to the weak AmpC-inducer β-lactams, such as ceftazidime. The MIC of ceftazidime for PAO1 increased from 1 to 8 μg/ml when the G154R AmpR mutation was introduced, similar to the effect produced by the inactivation of AmpD (24). The MICs of ceftazidime for ST175 clinical isolates ranged from 8 to 64 μg/ml, perhaps suggesting the presence of additional factors, such as variations in the outer membrane permeability or the expression of penicillin-binding proteins, which modulate the level of resistance (39). Moreover, the implication of such AmpR-activating mutations could extend far beyond the overexpression of *ampC* and β-lactam resistance, since recent works demonstrate that AmpR is a global transcriptional regulator connected to quorum sensing, alginate production, biofilm formation, and the expression of several other virulence factors (2, 3, 27). Thus, ongoing research in our laboratory will explore whether the AmpR-activating mutations, such as G154R, could have an effect on the expression of these relevant pathogenicity traits, perhaps playing a role in the success of the widespread ST175 high-risk clone.

ACKNOWLEDGMENTS

We are grateful to all clinical microbiologists and infectious disease specialists involved in this project from the Hospital Universitario de Bellvitge (Barcelona), Consorci Sanitari Parc Tauli (Barcelona), Hospital Universitario Virgen de la Macarena (Sevilla), Hospital Vall de Hebrón (Barcelona), Hospital Universitari Son Espases (Palma de Mallorca), Hospital de Sant Pau (Barcelona), Hospital Universitario Marqués de Valdecilla (Santander), Hospital Virgen del Rocío (Sevilla), Hospital Reina Sofía (Córdoba), and Hospital Mutua de Tarrasa (Barcelona).

This work was supported by the Ministerio de Ciencia e Innovación of Spain and the Instituto de Salud Carlos III, through the Spanish Network for Research in Infectious Diseases (grants REIPI C03/14 and RD06/0008) and grants PI08/0276, PS09/00033, and PI12/00103.

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