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Diversity of β-lactam resistance mechanisms in cystic fibrosis isolates of *Pseudomonas aeruginosa*: a French multicentre study

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Objectives: To investigate the resistance mechanisms of β-lactam-resistant *Pseudomonas aeruginosa* isolated from cystic fibrosis (CF) patients in France.

Methods: Two-hundred-and-four *P. aeruginosa* CF isolates were collected in 10 French university hospitals in 2007. Their susceptibility to 14 antibiotics and their resistance mechanisms to β-lactams were investigated. Their β-lactamase contents were characterized by isoelectric focusing, PCR and enzymatic assays. Expression levels of efflux pumps and the intrinsic β-lactamase AmpC were quantified by reverse transcription real-time quantitative PCR. Genotyping was performed using multiple-locus variable number of tandem repeats analysis (MLVA). The *oprD* genes were sequenced and compared with those of reference *P. aeruginosa* strains. To assess deficient OprD production, western blotting experiments were carried out on outer membrane preparations.

Results: MLVA typing discriminated 131 genotypes and 47 clusters. One-hundred-and-twenty-four isolates (60.8%) displayed a susceptible phenotype to β-lactams according to EUCAST breakpoints. In the 80 remaining isolates, resistance to β-lactams resulted from derepression of intrinsic cephalosporinase AmpC (61.3%) and/or acquisition of secondary β-lactamases (13.8%). Efflux pumps were up-regulated in 88.8% of isolates and porin OprD was lost in 53.8% of isolates due to frameshifting or nonsense mutations in the *oprD* gene.

Conclusions: β-Lactam resistance rates are quite high in CF strains of *P. aeruginosa* isolated in France and not really different from those reported for nosocomial strains. Development of β-lactam resistance is correlated with patient age. It results from intrinsic mechanisms sequentially accumulated by bacteria isolated from patients who have undergone repeated courses of chemotherapy.

Keywords: OprD, efflux systems, AmpC, antibiotic resistance surveillance, molecular typing

Introduction

Pseudomonas aeruginosa contributes to the decline of respiratory function in cystic fibrosis (CF) patients. Shortly after infection of the CF airways, the microorganism is able to develop various strategies to evade the host's immune defences and to resist antibiotics of different classes, including β-lactams.[1] These latter agents are essential to control infectious exacerbations and are administered intravenously (e.g. ceftazidime, cefepime, piperacillin/tazobactam and meropenem) or by aerosol (e.g. aztreonam). The persistence of *P. aeruginosa* in the CF lung is believed to result from both transient (adaptive) and stable (mutational) mechanisms.[2] For example, the slow growth or dormancy of bacteria living in biofilms under low oxygen tension has been proposed to strongly impair the *in vivo* efficacy of β-lactams, as these drugs mostly kill actively dividing cells.[3] The spatial induction of the intrinsic β-lactamase AmpC by β-lactams (e.g. ceftazidime and imipenem) would provide protection to those bacteria located in microaerophilic zones of the biofilm.[4] On the other hand, the accumulation of mutations in persistent clones is known to gradually increase the resistance of *P. aeruginosa* to antibiotics during the chronic stage of infection. It is now well established that this evolution is amplified in a majority of CF patients by alteration of the bacterial system of DNA repair and the emergence of hypermutator populations.[5] Some of these mutations have been reported to

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promote β-lactam resistance through the stable overexpression of AmpC,[6,7] the constitutive up-regulation of Mex efflux systems (mainly MexXY/OprM)[8,9] or the loss of the carbapenem-specific porin OprD.[10] According to previous studies, the horizontal acquisition of genes coding for secondary β-lactamases seems to be a rather infrequent event in CF strains.[11–14] Whether alterations in penicillin-binding proteins[15] or lipopolysaccharides[16] contribute to the decreased susceptibility to β-lactams remains unclear.

Only a few studies have systematically investigated the resistance mechanisms of *P. aeruginosa* to β-lactams in the CF context.[13,17] This prospective multicentre study was set up to improve our knowledge on how the pathogen adapts to antibiotic treatments in this pathology.

Materials and methods

Bacterial strains and culture conditions

Between October 2006 and April 2007, consecutive, non-repetitive, respiratory isolates of *P. aeruginosa* were collected from CF patients in 10 French university hospitals (CHU or CHRU). Eighteen, 19, 20 and 27 isolates were collected by one, one, seven and one participating centres, respectively, leading to a total of 204 isolates. The participating centres were CHU Rangueil (Toulouse), CHU Hôtel Dieu (Nantes), CHU Robert Debré (Paris), CHU Cochin (Paris), CHRU Arnaud-de-Villeneuve (Montpellier), CHRU Calmette (Lille), CHRU Gabriel-Montpied (Clermont-Ferrand), CHU Côte-de-Nacre (Caen), CHU Trousseau (Paris) and CHRU Jean Minjoz (Besançon). The isolates were sent to two coordinating centres for further investigations together with patient data (Bégin Military Hospital, Saint-Mandé and CHRU Jean Minjoz, Besançon). The bacterial isolates were kept frozen at −70°C until analysed. The well-characterized mutants PT629,[18] MutGR1,[9] EryR[19] and PAO7H,[20] which all derive from wild-type reference strain PAO1, were used as positive controls in reverse transcription real-time quantitative PCR (RT–qPCR) experiments to identify clinical isolates overexpressing the MexAB-OprM, MexXY/OprM, MexCD-OprJ and MexEF-OprN pumps, respectively. Bacterial cultures were performed at 37°C on Mueller–Hinton medium (MH agar, bioMérieux, Marcy l'Étoile, France).

Epidemiological typing

The clinical isolates of this collection were genotyped using a multiple-locus variable number of tandem repeats (VNTRs) analysis (MLVA) assay, based on the analysis of short to long tandemly repeated sequences (also called microsatellites and minisatellites). A total of 15 VNTR markers were studied as described previously.[21] The allelic profile of an isolate with 15 VNTRs was expressed as the number of repeats for each VNTR in the order ms77, ms127, ms142, ms172, ms211, ms212, ms213, ms214, ms215, ms216, ms217, ms222, ms223, ms207, ms209. A new genotype number was given when one difference was observed at any VNTR. The genotype numbers were determined for the present study and were given the prefix 'gerpa'. The MLVA clustering analysis was performed using the categorical coefficient and the unweighted pair group method with arithmetic mean with three reference strains, namely PAO1, PA14[22] and clinical strain C50 from Sweden that belongs to the widely distributed 'clone C'.[23] The MLVA profile of strain 2192[24] was deduced from the genome sequence (Broad Institute, www.broadinstitute.org). For global phylogenetic pattern determination, a cut-off value of 53% was applied to define a cluster, corresponding to seven allelic differences among the 15 markers. In the case of a VNTR that could not be amplified or the size of one VNTR that could not be analysed because of the presence of an insertion sequence (IS) element in the VNTR,[21] the percentage cut-off value for clustering was applied to the 14 remaining VNTRs.

Antibiotic susceptibility testing

The disc diffusion method (Bio-Rad, Marne la Coquette, France) on Mueller–Hinton II (MHA) plates (bioMérieux) was used routinely by the participating laboratories to establish the resistance profiles of isolates. Drug MICs were determined by the reference broth microdilution method,[25] with *P. aeruginosa* ATCC 27853 as a control. The isolates were then categorized as susceptible, intermediate or resistant in accordance with current EUCAST clinical breakpoints for *P. aeruginosa* (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/Breakpoint_table_v_3.1.pdf). These breakpoints are (S breakpoint/R breakpoint, mg/L): ticarcillin, 16/16; ticarcillin/clavulanate, 16/16; piperacillin, 16/16; piperacillin/tazobactam, 16/16; ceftazidime, 8/8; cefepime, 8/8; aztreonam, 1/16; imipenem, 4/8; ciprofloxacin, 0.5/1; gentamicin, 4/4; tobramycin, 4/4; amikacin, 8/16; fosfomycin, 32/32; and colistin, 4/4.

Identification of β-lactamases

All of the isolates resistant to at least one of the antipseudomonal β-lactams tested were screened phenotypically for stable AmpC overproduction by measuring the inhibition zones around the disc of ceftazidime on MHA ± 500 mg/L cloxacillin (1000 mg/L with some highly resistant isolates). An increase of >5 mm in the presence of the AmpC inhibitor was considered as potentially positive.[26] All the resistant isolates were subjected to ultrasonic disruption and then isoelectric focusing (IEF) to determine the isoelectric point (pI) of individual β-lactamases, as published previously.[27] The β-lactamase activities in the lysates were measured spectrophotometrically at 262 nm with nitrocefin as a chromogenic substrate.[27] The expression of gene bla_{AmpC} was assayed by quantitative RT–qPCR with specific primers (Table S1, available as Supplementary data at *JAC* Online) as reported previously.[18] The mRNA levels were normalized to that of housekeeping gene *rpsL* and expressed as a ratio to that of strain PAO1.[28] The RT–qPCR results were interpreted according to the thresholds defined by Cabot *et al.*[28]

In isolates with a negative cloxacillin test, showing additional bands in IEF gels (other than AmpC at pI 8.7–8.9) and/or exhibiting significant β-lactamase activity despite low bla_{AmpC} gene expression (<10-fold versus PAO1), the presence of transferable β-lactamase genes was investigated by real-time PCR in a LightCycler apparatus (Roche Diagnostics) after extraction of total bacterial DNA.[29] The consensus primers used were specific of type PSE, TEM, SHV, VEB, GES, PER, VIM, IMP, OXA-10 group, OXA-1 group, OXA-2 group, CTX-M1 group, CTX-M2 group and CTX-M9 group β-lactamases (Table S1, available as Supplementary data at *JAC* Online). The PCR products were sequenced on both strands. The predicted sequences of AmpC were compared with those of extended-spectrum AmpC (ESAC) PDC-1 (accession number FJ666065) and PDC-2 (accession number FJ666064) for identification of the Ala105 mutation.

Efflux and OprD deficiency

The overexpression of genes *mexB*, *mexY*, *mexC* and *mexE* was assayed by RT–qPCR with specific primers (Table S1, available as Supplementary data at *JAC* Online) as reported previously.[18] Transcripts of gene *oprD* were quantified by RT–qPCR in isolates exhibiting imipenem resistance (MIC >4 mg/L) and full-length OprD coding sequences. Because of the high sequence polymorphism of *oprD*, specific primers were designed in two consensus regions (ch1-oprD and ch2-oprD, Table S1, available as Supplementary data at *JAC* Online) deduced from the *oprD* sequences of the selected isolates. The mRNA levels were normalized to that of housekeeping gene *rpsL* and expressed as a ratio to PAO1 (value of 1

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by definition). The thresholds for interpretation were those defined by Hocquet *et al.*[30]

Immunodetection of OprD

Outer membranes were isolated, subjected to SDS–PAGE and analysed by western blotting with an OprD-specific polyclonal antiserum (diluted 1:10000) as reported previously.[31] OprD bands were visualized with alkaline phosphatase conjugated to an antirabbit secondary antibody by using a colorimetric AP substrate reagent kit (Promega, Charbonnières, France).

Multiple sequence alignments and phylogenetic analyses

The *oprD* gene sequences from clinical and reference *P. aeruginosa* strains (PAO1, PA14, PA5, PA7, PP2 and LESB58) were compared with MUSCLE software.[32] The phylogenetic analysis was performed with SEAVIEW[33] and the distances were estimated with the two-parameter substitution model of Kimura.[34] The tree was reconstructed using BIONJ, an improved version of the neighbour-joining method,[35] on alignment positions without a gap. One thousand bootstrap replicates were computed. The root of the tree was considered as the barycentre of the tree.

Results

Molecular epidemiology

A total of 204 isolates of *P. aeruginosa* were collected in 10 French hospitals from 156 CF patients (78 females, 78 males; 1–45 years of age, median age 21) (Table S2, available as Supplementary data at *JAC* Online). The MLVA identified 131 different genotypes with 47 clusters (defined as identical size alleles for at least eight VNTR). Genotypes are listed in Table S2, available as Supplementary data at *JAC* Online and are represented in the phylogenetic tree (Figure S1, available as Supplementary data at *JAC* Online). The largest cluster contained 14 isolates from five centres (centres 3, 5, 6, 8 and 10) and included genotypes 33–36. Cluster C (corresponding to MLVA profile C50) included 12 isolates (5.9%) from eight different centres (centres 1–6, 9 and 10). Cluster PA14 contained eight isolates from four centres (centres 5 and 7–9). Isolates 1-7 and 3-16 had a genotype similar to that of strain 2192, which was recovered from a CF patient.[24] The last 168 CF isolates belonged to 112 different genotypes. No isolate appeared to be genotypically related to the epidemic clone Liverpool LESB58.[36]

Antimicrobial susceptibility

The drug susceptibility rates of the isolates are indicated in Table 1. Overall, 124/204 (60.8%) isolates displayed a phenotype susceptible to β-lactams according to EUCAST breakpoints (Table S2, available as Supplementary data at *JAC* Online and Table 1). Susceptibility rates to piperacillin/tazobactam, ceftazidime and imipenem were 77.1%, 81% and 80%, respectively, higher than those reported previously (ceftazidime susceptibility rate: 60.4%).[37] Fifteen of the 204 isolates (7.35%) exhibited concomitant resistance to ceftazidime, imipenem, ciprofloxacin and aminoglycosides (tobramycin, gentamicin and/or amikacin). Colistin remained active against all these 15 isolates. In contrast to colistin and fosfomycin, resistance to ciprofloxacin, aminoglycosides, piperacillin/tazobactam, ceftazidime and cefepime tended to increase with patient age (Table 1). Overall, no correlation could be established between the resistance profiles and the different MLVA genotypes.

Table 1. Susceptibility rates of the 204 *P. aeruginosa* isolates

	MICs (mg/L)			Susceptibilities (%)[a]			
					Patient age (years)		
Antibiotics	MIC_{50}^{b}	MIC_{90}^{b}	range	total, $n=204$	0–6, $n=15$	7–18, $n=71$	>18, $n=118$
Ticarcillin	16	256	0.125–2048	66.8	80.0	71.8	62.7
Ticarcillin/clavulanate	16	256	0.25–2048	67.3	80.0	73.2	62.7
Piperacillin	4	256	0.25–1024	74.1	93.3	77.4	69.5
Piperacillin/tazobactam	4	128	0.125–256	77.1	93.3	77.4	73.7
Ceftazidime	2	32	0.064–128	81.0	100	84.5	75.4
Cefepime	4	32	0.064–256	81.5	100	81.7	77.9
Aztreonam	2	64	0.032–512	42.9	40.0	38.0	44.9
Imipenem	1	16	0.032–64	80.0	86.6	83.1	77.1
Ciprofloxacin	0.5	4	0.016–32	65.9	93.3	69.0	58.4
Gentamicin	4	32	0.064–512	60.5	73.3	63.3	56.7
Tobramycin	2	16	0.125–512	82.0	100	85.9	77.1
Amikacin	8	32	0.125–512	58.0	66.6	60.5	55.0
Fosfomycin	64	512	0.25–1024	35.6	40.0	29.5	39.0
Colistin	1	4	0.064–4	100	100	100	100

[a]Percentages of susceptibility are in accordance with EUCAST breakpoints: ticarcillin/clavulanate, 16/16; piperacillin/tazobactam, 16/16; ceftazidime, 8/8; cefepime, 8/8; aztreonam, 1/16; imipenem, 4/8; ciprofloxacin, 0.5/1; gentamicin, 4/4; tobramycin, 4/4; amikacin, 8/16; fosfomycin, 32/32; and colistin, 4/4.
[b]MIC_{50} and MIC_{90}: concentrations of antibiotics that inhibit 50% and 90% of isolates, respectively.

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Mechanisms of resistance to β-lactams

Eighty isolates, belonging to 56 different MLVA profiles, showed a non-susceptible phenotype to at least one of the β-lactams tested. The resistance mechanisms found in these isolates are listed in Table 2. Interestingly, a high proportion of isolates (71/80; 88.8%) was found to overproduce one or more active efflux systems, including MexAB-OprM (36/80; 45%) and MexXY/OprM (65/80; 81.25%). MexEF-OprN (7/80; 8.75%) and MexCD-OprJ (2/80; 2.5%) overproducers were less prevalent in the collection. Of the 71 efflux overproducers, 29 and 5 overexpressed two or three efflux systems simultaneously, respectively.

Stable derepression of β-lactamase AmpC was identified in 49/80 isolates (61.3%). While 40/49 of these isolates were categorized as resistant to ceftazidime according to EUCAST breakpoints (MIC >8 mg/L), 9/49 appeared susceptible to this antibiotic (MIC 4–8 mg/L), suggesting deficient MexAB-OprM efflux systems counterbalancing the effect of AmpC production (TicHS phenotype).[8] Indeed, while ceftazidime is not a good substrate of MexAB-OprM,[38] deficient production of this pump in general results in a 2-fold decrease in the ceftazidime MIC.

Transferable β-lactamases occurred in only 11 isolates (13.8%) (isolated from seven patients of 13, 19, 20, 23, 24, 31 and 35 years of age), namely 1 VIM-2 carbapenemase and 10 narrow-spectrum penicillinases (1 TEM-2, 1 PSE-1, 4 OXA-47 and 4 OXA-2). Of note, 3/4 OXA-47- and 4/4 OXA-2-producing isolates were clonally related, respectively. All of the 11 isolates displayed high-level resistance to aminoglycosides (gentamicin MICs ≥16 mg/L).

Multiple combinations of enzymatic and non-enzymatic resistance mechanisms were observed in the CF isolates that increased with patient age (Figure 1). Two or more mechanisms were evidenced in 67/80 isolates, with 19 isolates harbouring at least four mechanisms. As expected, accumulation of these mechanisms was associated with higher β-lactam MICs in isolates (Table 3).

Resistance to carbapenems

Forty-two isolates were non-susceptible to imipenem (Table 4), of which 26 were also resistant to ceftazidime. As indicated above, one isolate was found to produce the metallo-β-lactamase VIM-2, while 27 overexpressed the intrinsic enzyme AmpC. Sequencing of the bla_{AmpC} genes in these latter isolates indicated that 25 of them contained the T105A change proposed to extend the hydrolytic activity of AmpC to carbapenems.[39] Ten out of the 42 isolates were resistant or of intermediate susceptibility to imipenem only, as compared with the other β-lactams tested. Sequence analysis of the entire *oprD* gene from all of the

Table 2. Mechanisms involved in β-lactam resistance among the CF isolates

Identified mechanisms of resistance								
efflux systems[a]					β-lactamases			
MexAB-OprM	MexXY/OprM	MexCD-OprJ	MexEF-OprN	OprD[b]	AmpC[a]	transferable	unknown	Number of isolates (n=80)
+	−	−	−	+	−	−	−	1
+	−	−	−	+	+	−/PSE-1	−	1/1
+	−	−	+	−	+	−	−	1
+	+	−	−	+	−	−	−	5
+	+	−	−	+	+	−	−	6
+	+	−	−	−	−	−/VIM-2	−	3/1
+	+	−	−	−	+	−	−	10
+	+	−	−	+	−	OXA-2/OXA-47	−	1/1
+	+	+	−	−	+	−	−	1
+	+	−	+	+	+	−	−	1
+	+	−	+	−	+	−	−	3
−	+	−	−	+	−	−/OXA-47	−	2/2
−	+	−	−	−	−	OXA-2	−	1
−	+	−	−	−	+	−/OXA-2/TEM-2	−	10/1/1
−	+	−	−	+	+	−/OXA-2	−	6/1
−	+	−	+	+	+	−	−	1
−	+	−	−	−	−	−	−	8
−	−	+	−	−	+	−	−	1
−	−	−	+	+	−	−	−	1
−	−	−	−	+	−	OXA-47	−	1
−	−	−	−	+	+	−	−	4
−	−	−	−	−	−	−	−	2
−	−	−	−	+	−	−	+	2

[a]+, stable up-regulation of efflux or β-lactamase AmpC; −, basal uninduced levels.
[b]+, presence of wild-type porin; −, inactivation or down-regulation of *oprD*.

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42 isolates highlighted a significant polymorphism that could be subdivided into five distinct clusters (not correlated with resistance levels), whose branches were supported by high bootstrap values (Figure 2). The first cluster that grouped most of the *oprD* genes was closely related to the epidemic CF strain LESB58. A second cluster included sequences related to strain PA14 (5-17, 6-20, 9-13, 9-9 and 9-12). The sequences of isolates 2-4, 2-16, 8-9 and 7-18 were similar to that of reference strain PAO1. Those of isolates 10-4, 4-13, 6-5 and 6-11 formed a fourth cluster with the *oprD*-TS allele (strain PP2) and possessed a hypothetical common ancestor with strain PA5. Finally, isolates 1-14, 5-1 and 9-11 exhibited sequences that could be grouped under the same node on the tree. This latter cluster does not include any reference strain. However, it shares a common hypothetical ancestor with the *oprD*-TS/PA5 group.

As already pointed out by other studies on CF *P. aeruginosa*,[17] a high percentage of isolates (76.2% of the 42 imipenem-non-susceptible isolates; 53.8% of the 80 β-lactam-non-susceptible isolates) contained frameshifts or nonsense mutations inactivating the *oprD* gene (Table 4). Of the 10 remaining isolates with intact, full-length coding sequences, six (1-10, 5-11, 7-3, 7-4, 7-7 and 7-20) expressed *oprD* at very low levels. It should be noted that 7-3 and 7-4 were clonally related. These mRNA data correlated with lower amounts of porin OprD in the outer membrane compared with wild-type reference strain PAO1 (Figure 3). Interestingly, the CF isolate 7-20 was found to be a typical *nfxC* mutant overexpressing the MexEF-OprN efflux pump with concomitant down-regulation of OprD.[40] In three of the 10 isolates with intact *oprD* (1-13, 1-17 and 6-20), the porin could not be detected in membrane extracts despite some expression of the gene. Intriguingly, the last isolate, 10-16, appeared to produce significant amounts of intact porin as compared with the control. The reason for its resistance to imipenem (MIC of 8 mg/L) remains unclear so far.

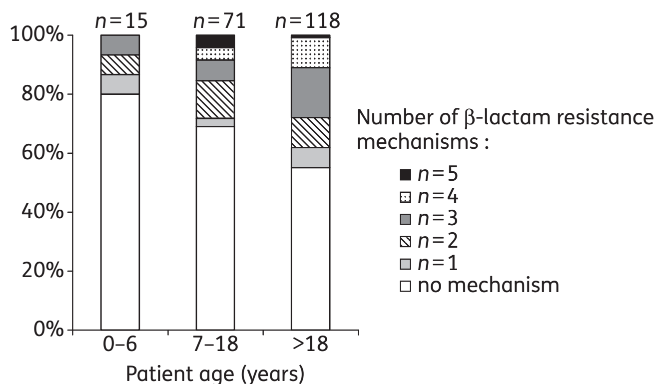

Figure 1. Accumulation of β-lactam resistance mechanisms with patient age. The number of β-lactam resistance mechanisms detected in each isolate is represented for three groups of patients according to their age: 0–6 years (15 isolates); 7–18 years (71 isolates); and >18 years (118 isolates). The different β-lactam resistance mechanisms were AmpC derepression, transferable β-lactamase, MexAB-OprM overproduction, MexCD-OprJ overproduction, MexEF-OprN overproduction, MexXY/OprM overproduction and/or OprD deficiency.

Discussion

The structure of the present collection of CF *P. aeruginosa* isolates is characteristic of the previously described panmictic population with some clonal complexes.[41,42] It reflects the fact that in CF patients the infection is more likely to be community acquired. Clusters C and PA14 correspond to clones previously associated with CF and which might show some ability to colonize the patient airways.[21,43] Cluster C represented 20% of the CF strains isolated in Hanover, Germany.[23] The CF patients were 1–45 years old, with a majority of adolescents and young adults. In longitudinal surveys of CF children, it was shown that

Table 3. MIC_{50}s of β-lactams according to the number of resistance mechanisms identified in the CF isolates

Antibiotics	MIC_{50}s (mg/L) for β-lactam-susceptible isolates (*n*=124)	MIC_{50}s (mg/L) for β-lactam-non-susceptible isolates[a] (*n*=80)		
		0 or 1 resistance mechanism[b] (*n*=13)	2 or 3 resistance mechanisms[c] (*n*=48)	4 or 5 resistance mechanisms[c] (*n*=19)
Ticarcillin	4	64	128	512
Ticarcillin/clavulanate	4	32	128	256
Piperacillin	2	16	64	256
Piperacillin/tazobactam	2	16	32	128
Ceftazidime	1	4	8	64
Cefepime	2	8	8	32
Aztreonam	4	16	16	64
Imipenem	0.5	2	4	32

[a]To at least one β-lactam tested.
[b]AmpC derepression, MexAB-OprM overproduction, MexEF-OprN overproduction, MexXY/OprM overproduction or OprD deficiency.
[c]β-Lactam resistance mechanisms among AmpC derepression, transferable β-lactamase, MexAB-OprM overproduction, MexCD-OprJ overproduction, MexEF-OprN overproduction, MexXY/OprM overproduction and OprD deficiency.

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Table 4. Profiles and mechanisms of resistance of the 42 imipenem-non-susceptible CF isolates

Isolates		MICs (mg/L)										β-Lactamases		mRNA expression for[b]:					
Centre	isolate	TIC	PIP	CAZ	FEP	ATM	IPM	CIP	GEN	TOB	AMK	acquired	ESAC[a]	bla_{AmpC}	*oprD*[c]	*mexB*	*mexY*	*mexE*	OprD polymorphism
1	2	1	0.5	1	4	0.125	16	0.125	2	1	8			2.8	0.47	**2.5**	**54.9**	3.1	Δ6 bp (723)
	3	256	512	64	32	128	64	0.5	8	4	32		T105A	**1490**	—	0.6	1.8	5.9	IS Pa (1635)
	5	1024	512	128	32	512	16	0.5	512	32	4		T105A	220	—	**2.8**	**71.8**	3.7	STOP (830)
	10	8	16	2	4	1	16	0.5	8	4	16			1.2	**0.16**	1.4	**27**	1.7	LESB58 variant
	13	1024	256	128	64	128	16	2	8	4	32		T105A	124	0.22	0.3	**16**	7.5	LESB58 variant
	17	128	64	8	16	16	8	2	4	1	8		T105A	90.7	0.64	0.6	**19.8**	2	LESB58 variant
	18	256	512	32	16	64	32	0.25	2	0.5	8		T105A	285	—	**2.7**	**31.6**	5.3	Δ2 bp (482)
2	11	512	256	64	64	64	16	0.125	1	2	1		T105A	42	—	1.0	**60**	39	STOP (642)
3	9	128	128	16	8	16	16	4	4	2	8		T105A	**365.5**	—	**4.0**	**89**	10.6	+4 bp (314)
	10	1	1	1	4	0.25	32	1	2	1	8			7.2	—	0.5	**33**	3.2	STOP (17)
4	18	2	4	8	2	2	16	0.5	2	2	8			0.9	—	**2.5**	**59.4**	5.6	Δ10 bp (319)
5	1	1024	256	64	16	64	32	32	256	512	128	TEM-2	T105A	250	—	1.0	**57**	2.9	STOP (603)
	11	512	512	64	32	64	8	0.5	8	4	16		T105A	338	**0.16**	1.0	**50**	4.6	LESB58 variant
	15	512	256	64	32	64	64	4	128	64	256	VIM-2		1.1	—	1.3	**18.4**	2.8	STOP (711)
	19	2	4	1	4	0.5	16	0.5	4	2	8			0.6	—	1.5	**91.8**	3.6	STOP (711)
6	5	128	256	16	8	8	32	2	4	2	16		T105A	**26**	—	0.9	**48.3**	0.1	Δ2 bp (1145)
	6	16	128	16	8	16	32	1	16	2	32		T105A	**65**	—	**2.8**	**182.7**	23.7	+G (1106)
	14	1024	256	128	64	256	16	4	8	2	16		T105A	**25.5**	—	**2.5**	**50.5**	1.7	ΔC (681)
	15	128	128	16	8	16	32	0.5	2	1	8		T105A	**1144**	—	**2.1**	4.9	**111**	+C (1206)
	20	4	8	8	8	64	32	0.5	8	8	16			4.9	0.25	0.5	1.5	2.4	PA14 variant
7	3	2048	512	64	16	32	32	0.125	512	512	512	OXA-2	T105A	**82**	**0.08**	0.7	**23.8**	5.0	LESB58
	4	512	128	1	1	0.25	8	0.125	128	64	8	OXA-2		1.2	**0.07**	1.9	**53**	12.4	LESB58
	7	16	8	2	4	16	8	0.5	16	4	16			0.3	**0.03**	0.9	**12.3**	0.9	LESB58
	13	256	128	64	64	128	32	4	8	2	16			2.1[d]	—	0.9	**57.1**	13.8	ΔG (1021)
	18	128	128	32	8	32	16	0.5	4	1	8		no	**166**	—	1.3	**17.7**	6.5	ΔG (433)
	20	512	32	32	256	128	16	2	64	32	256		no	**56.8**	**0.07**	**2.0**	**149**	**123**	LESB58
	22	32	64	8	8	2	32	8	16	8	32		T105A	**11.8**	—	0.7	**75**	21.8	+C (1206)
8	10	16	16	4	8	4	64	2	8	4	16			9.2	—	0.5	**30.9**	1.2	ΔA (1166)
	11	128	128	64	128	128	64	2	8	1	32		T105A	**57**	—	**2.8**	**240**	70	ΔA (1166)
	19	128	32	8	8	32	16	2	8	2	16			0.5	—	**6.4**	**66**	6.5	+C (1206)
9	2	32	8	1	4	8	8	32	4	1	8			2.4	—	1.3	**32.3**	10.7	STOP (413)
	9	16	8	4	8	4	16	0.25	8	2	16			7.5	—	0.1	**26.4**	1.6	+AC (657)
	12	512	256	32	16	64	32	0.5	4	1	8		T105A	**407.5**	—	**6.7**	**229**	**364**	Δ11 bp (1001)
	18	1024	128	64	128	128	32	2	32	8	64		T105A	**82**	—	0.7	**22.1**	7.5	STOP (424)
10	2	512	256	128	64	128	64	8	16	2	32		T105A	**158**	—	**6.3**	**152**	49	+C (1206)
	10	1	0.5	0.5	2	0.5	16	1	4	0.5	8		T105A	**33.2**	—	1.5	**84.3**	1.8	Δ11 bp (379)
	11	16	0.5	2	16	0.5	16	2	32	32	128			1.4	—	0.6	1.6	8.0	STOP (195)
	12	512	512	128	32	32	16	4	4	1	16		T105A	**325**	—	**9.2**	**192**	**168**	Δ STOP
	16	32	128	16	32	8	8	0.5	4	1	8		T105A	**13.9**	0.38	**4.4**	**84**	31	LESB58
	17	128	4	32	32	64	16	2	16	4	64		T105A	**12**	—	**2.4**	**37**	11.8	ΔC (736)
	18	512	256	32	32	128	16	4	8	2	16		T105A	**104.5**	—	**3.3**	**90**	12.4	Δ STOP
	19	128	512	64	32	64	64	8	8	4	32		T105A	**39.1**	—	**2.5**	**75**	20.6	ΔG (333)

TIC, ticarcillin; PIP, piperacillin; CAZ, ceftazidime; FEP, cefepime; ATM, aztreonam; IPM, imipenem; CIP, ciprofloxacin; GEN, gentamicin; TOB, tobramycin; AMK, amikacin.

[a]Sequencing of the *ampC* gene was performed for isolates showing AmpC overexpression.

[b]Values in bold indicate a significant overexpression (or underexpression for *oprD*) of the corresponding gene according to the defined thresholds.

[c]The relative expression levels of *oprD* were determined in isolates with intact genes.

[d]This isolate was considered as an 'AmpC phenotype' since (i) 1000 mg/L cloxacillin increased the inhibition zones around β-lactams discs, (ii) a high β-lactamase activity was detected with nitrocefin, and (iii) no band other than AmpC was visualized on IEF gels.

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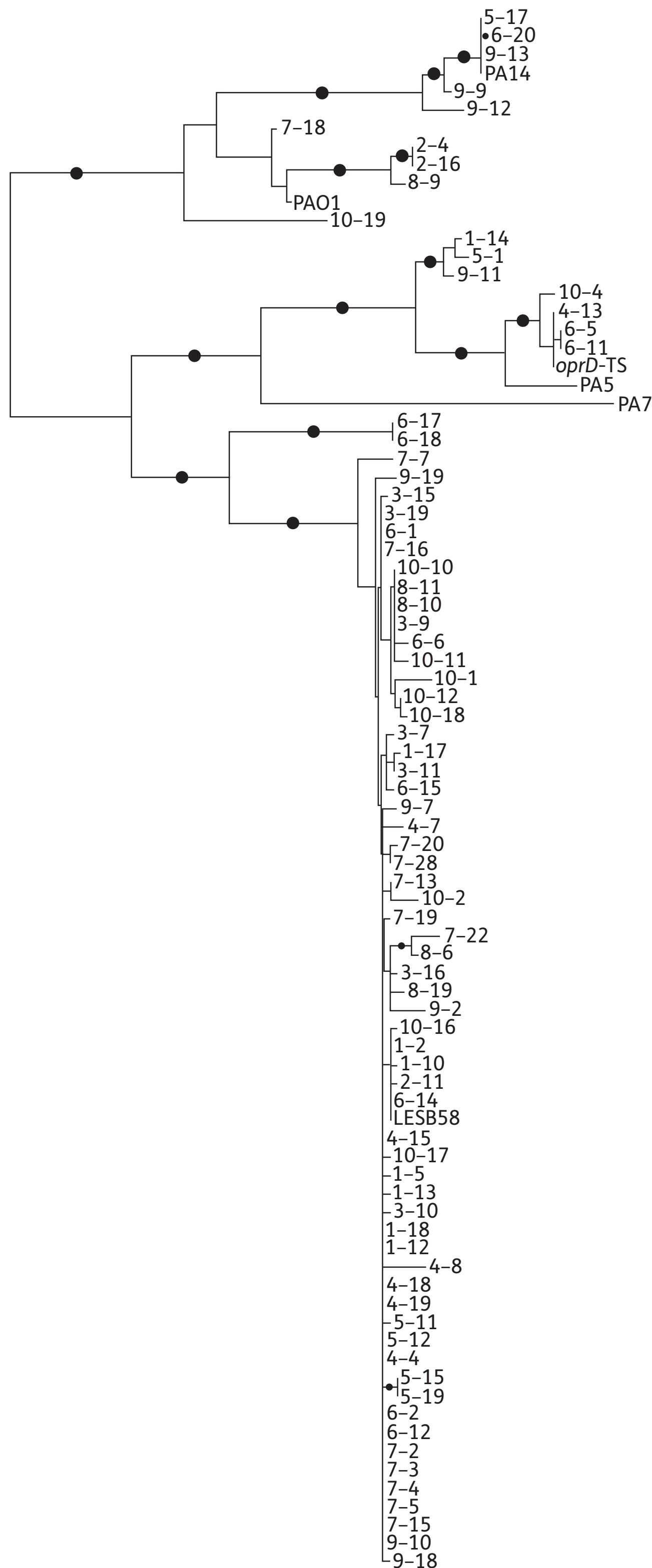

Figure 2. Phylogenetic tree of the *oprD* sequences. Sequences of the *P. aeruginosa* reference strains are indicated by the name of the strain (PA14, PA01, PA5, PA7, LESB58). The *oprD*-TS allele belongs to the PP2 strain.[50] One thousand bootstrap replicates were computed; bootstrap values >80% are indicated on branches with filled circles.

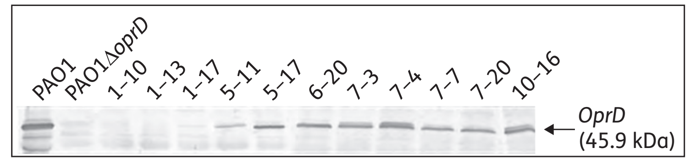

Figure 3. Production of protein OprD in imipenem-resistant CF isolates exhibiting no frameshifting or nonsense mutations in the *oprD* gene. Outer membrane proteins were analysed by western blotting; they were separated by SDS–PAGE, transferred to nitrocellulose membrane and probed with an anti-OprD polyclonal specific antibody. Fourteen micrograms of outer membrane proteins was used per lane for immunodetection of OprD. PAO1 and PAO1Δ*oprD* were used as positive and negative controls, respectively.

primary colonization of young children is often cured but is later followed by stable recolonization with a single strain.[21,44]

To our knowledge, this is the first French multicentre study analysing the antibiotic susceptibility of a large collection of CF *P. aeruginosa*. As already noted previously,[13,37,45] CF strains become gradually more resistant to β-lactams with patient age as a consequence of the increased selective pressure exerted by multiple courses of aerosol and intravenous chemotherapy. Spencker *et al.*[46] suggested that this evolution might be slower for β-lactams than for aminoglycosides and quinolones. The present study confirms that three major mutational mechanisms contribute to the loss of β-lactam susceptibility in the CF context, namely the derepression of β-lactamase AmpC, the overproduction of efflux systems (MexXY/OprM, MexAB-OprM and MexEF-OprN) and the loss of porin OprD.[13] More importantly, we show here that these intrinsic mechanisms often randomly accumulate over time in late isolates, leading to complex resistance phenotypes that cannot be dissected without the help of molecular (e.g. RT–qPCR and DNA sequencing) and enzymatic techniques (e.g. IEF and spectrophotometric assays). In this evolution, the acquisition of secondary β-lactamases plays a modest role despite sporadic reports on extended-spectrum β-lactamases or metallo-β-lactamases in CF *P. aeruginosa*,[12,14,47] as is the case here with metallo-β-lactamase VIM-2. In spite of intensive investigations, the β-lactam resistance of a minority of isolates (2/80) could not be elucidated, suggesting other mechanisms specific of CF strains.

Carbapenem resistance mostly relies on mutations resulting in deficient production of the specific porin OprD. As already described for CF strains, no correlation could be established between *oprD* disruption or underexpression and the isolate genotype.[13,48] In this collection, seven isolates overexpressing the MexEF-OprN efflux pump were identified, reinforcing the notion that these mutants are more represented among clinical strains than anticipated previously.[49] The repression of porin OprD that is concomitant with MexEF-OprN activation accounts for the higher resistance of these bacteria to carbapenems. Further investigations will be needed to better understand the emergence of these *nfxC* mutants in the clinical context.

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for DNA sequencing of *oprD*. We are grateful to Chantal Gros, Pascale McGill and Rodolphe Suspène for technical assistance.

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Transparency declarations

None to declare.

Supplementary data

Table S1, Table S2 and Figure S1 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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