Emergence of Carbapenem Resistance in *Pseudomonas aeruginosa* Isolates from a Patient with Cystic Fibrosis in the Absence of Carbapenem Therapy

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The emergence of carbapenem-resistant *Pseudomonas aeruginosa* in the lung of a patient with cystic fibrosis was evaluated. A single strain of *P. aeruginosa* persisted during a 3-year study despite antipseudomonal treatment. A stepwise decrease in carbapenem susceptibility leading to resistance was observed in the absence of carbapenem treatment. These data suggest that chronic exposure to unrelated drug classes may be an important determinant for the emergence of carbapenem resistance in *P. aeruginosa*.

Chronic colonization and persistent infections with *Pseudomonas aeruginosa* have been associated with the progression of lung damage and increased morbidity and mortality among patients with cystic fibrosis [1]. Despite prolonged antibiotic exposure to multiple drug classes, *P. aeruginosa* is rarely eradicated from the lungs of patients with cystic fibrosis, in part because of biofilm production and antimicrobial resistance mechanisms.

P. aeruginosa undergoes several genotypic and phenotypic changes in response to the dynamic environment of the lungs in patients with cystic fibrosis, including mucoid conversion and antibiotic resistance [1–3]. Smith et al. [4] observed numerous genetic changes in a *P. aeruginosa* isolate from a patient with cystic fibrosis over an 8-year period, including mutations in genes responsible for antimicrobial resistance. Through genetic alterations, *P. aeruginosa* uses a variety of resistance mechanisms, including target site mutations, altered permeability, and inactivating enzymes, in response to constant therapeutic selective pressures. In addition, environmental triggers, such as oxidative stress, have been shown to select for hypermutable *P. aeruginosa,* with increased rates of spontaneous mutations [5, 6], which may aid in the development of antibiotic resistance [7].

Resistance to meropenem in *P. aeruginosa* is associated with the overproduction of the MexAB-OprM efflux pump in addition to the loss of the carbapenem-specific porin OprD [8]. Although susceptibility to meropenem is reduced only when the mechanisms occur independently of each other, resistance to imipenem is associated with the absence of OprD. Loss of OprD has been shown to occur at the transcriptional or translational level through mutations or insertional inactivation of the structural gene [9, 10]. An alternative pathway of *oprD* regulation involves the overexpression of the *mexEF-oprN* efflux pump. In many cases, mutants that overexpress *mexEF-oprN* have a concomitant loss of OprD because of regulatory factors that influence the expression of both gene products [11, 12].

Because mutations frequently occur in *P. aeruginosa* isolates from patients with cystic fibrosis in response to various selective pressures unique to the lung environment of such patients, resistance to a particular drug class, such as the carbapenems, may emerge even when the patient has never been treated with that drug class. The goal of this study was to examine the *P. aeruginosa* population of a patient with cystic fibrosis over time with respect to carbapenem susceptibility in the absence of carbapenem therapy.

Materials and methods. The patient provided sputum samples every 3 months for 3 years (quarters 1–4). *P. aeruginosa* was isolated from sputum samples, and antimicrobial susceptibility testing was performed by agar dilution according to the methodology of the Clinical and Laboratory Standards Institute [13]. Pulmonary function tests were performed using a Jaeger Masterscope spirometer (Masterscope software, version 4.51).

The genetic relatedness of the strains was determined using PFGE in accordance with the Centers for Disease Control and Prevention PulseNet protocol [14]. Restriction patterns were analyzed using BioNumerics software, version 4.6 (Applied Maths), with unweighted pair group arithmetic averages and Dice coefficients.

RNA was isolated from logarithmic cultures of *P. aeruginosa,* and expression of 2 efflux pumps *(mexAB-oprM* and *mexEF-oprN)* and the porin *(oprD)* were measured by real-time RT-

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PCR, as described elsewhere [15]. Primers were designed for the detection of *mexA, mexE,* and *oprD* transcripts. Expression of the control gene *rpsL* was used to normalize the data. Primer efficiency was tested by real-time PCR, and primers were considered to be efficient if a ≤ 2 threshold cycle difference was observed for the clinical isolates, compared with control strain PAO1. Outer membrane protein analysis, DNA template preparation, PCR amplification, and sequencing were conducted as described elsewhere [15].

The isolates were screened for hypermutability using the disk diffusion assay developed by Macia et al. [16]. Plates were examined for mutant subpopulations within the inhibition zones for ciprofloxacin, ceftazidime, imipenem, meropenem, and tobramycin (BBL; Becton Dickinson). Strains were considered to be hypermutable if mutant subpopulations (>10 colonies) were present within the zones of ≥ 3 antibiotics [16].

Results. Among the 15 isolates collected over 3 years, 2 major strain groups were present. Eight isolates had identical restriction patterns (group 1A) (table 1), suggesting isolation of the same strain over time. In addition, 4 isolates (PA45, PA108, PA150, and PA107A) had patterns that were highly related (i.e., $\geq 95\%$ similar) to each other and to patterns in group 1A (groups 1B and 1C). The remaining 3 isolates (PA203, PA287, and PA387) possessed patterns that were identical but that differed significantly (<70% similarity) with patterns in isolates from group 1 (group 2).

P. aeruginosa strains isolated during year 1 belonged to group 1 and were susceptible to meropenem and imipenem (MIC, 0.06–2.0 μg/mL) (table 1). Although the first strain, PA150 (group 1B), isolated from year 2, quarter 1, shared a phenotype similar to those of the previous strains, isolation of subsequent group 1A strains from year 2 (quarters 2–3) were 2-fold less susceptible to imipenem and 4–16-fold less susceptible to meropenem than were group 1A strains from year 1. Before the isolation of these strains, the patient was treated with the antipseudomonal drugs ciprofloxacin and tobramycin (table 1). An additional 4-fold reduction in imipenem and meropenem susceptibility was noted for group 1A strains PA323 and PA360 from year 3, quarter 1 and from year 3, quarter 2. According to the Clinical and Laboratory Standards Institute breakpoints, these strains had intermediate resistance to imipenem (MIC, 8 μg/mL) and were at the susceptibility breakpoint for meropenem (MIC, 4 μg/mL). Full resistance to the carbapenems emerged in the group 1A strain PA415, which was isolated from the year 3, quarter 4 sputum sample. In comparison with strain PA77A (year 1), imipenem and meropenem MICs had increased 32- and 256-fold, respectively, in strain PA415. This decrease in susceptibility had occurred in the absence of carbapenem treatment (table 1). Although group 2 strains were exposed to the same treatment regimen, their susceptibility was not altered substantially.

A significant deterioration in pulmonary function was observed at year 2, quarter 3, and repeated testing through year 3, quarter 4 indicated poor pulmonary function (table 1). This decrease in pulmonary function coincided with the isolation of carbapenem-resistant strains. Of note, conversion to the mucoid phenotype occurred in strain PA244 (year 2, quarter 3), and the remaining group 1A strains, PA323 and PA415, continued to exhibit the mucoid phenotype.

The stepwise decrease in susceptibility had occurred over time in epidemiologically related strains belonging to group 1A. Expression of the efflux pump *mexAB-oprM* was evaluated in selected strains of group 1A by real-time RT-PCR to determine its possible role in meropenem resistance. Expression of *mexA* for strains PA77A, PA202, PA244, and PA323 was similar to that of the wild-type laboratory strain PAO1 (table 2). However, strain PA415 expressed *mexA* at 11- and 12-fold higher levels than did strains PAO1 and PA77A, respectively.

The production of the porin OprD was examined in the selected group 1A strains by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The amount of OprD was significantly reduced in strains PA202, PA244, PA323, and PA415, compared with PA77A (data not shown). The low levels of OprD in these strains correlated with a decrease in the amount of *oprD* transcription. Strains PA202 and PA244 had 9- and 11-fold lower levels of *oprD* transcription than did PAO1 and PA77A, respectively (table 2). A decrease in *oprD* expression was associated with the overexpression of the *mexEF-oprN* efflux system in these strains. The strains with intermediate resistance and resistance to imipenem (PA323 and PA415) had a more substantial reduction in *oprD* expression (33- and 50-fold, respectively), compared with PAO1 (table 2). However, these strains were not overexpressing *mexEF-oprN,* suggesting that mechanisms not associated with *mexEF-oprN* overexpression could be responsible for the reduced *oprD* transcription levels.

PCR amplification of *oprD* from strains PA323 and PA415 generated much larger products (~2800 base pairs) than the predicted size of 1648 base pairs from PAO1 (data not shown). Sequence analysis revealed the presence of an insertion sequence (IS) element that was 87 nucleotides upstream of the translational start codon corresponding to the promoter region of the *oprD* gene. The IS element was 1192 base pairs in size and shared 97% identity with ISPa*16* on *P. aeruginosa* plasmid Rms149 (GenBank accession no. AJ877225).

Because the selection of efflux and porin mutants from the group 1A strains may be attributed to spontaneous mutations associated with hypermutation, group 1A strains were screened for hypermutability by disk diffusion. Mutant subpopulations were not detected for strain PA77A with any of the drugs tested (data not shown). Mutant subpopulations (<20 colonies) were present within the imipenem zone for strain PA202 and within

Table 1. Treatment, strain isolation, and drug susceptibility in a patient with cystic fibrosis.

Year (quarter), date	Drug (no. of days received)	Strain	PFGE group	Susceptibility, MIC, μg/mL				FEV_1
				P-T	CAZ	IPM	MEM	
22 February 2001	Trimethoprim-sulfamethoxazole (20)	…	…	…	…	…	…	…
26 April 2001	Doxycycline (15)	…	…	…	…	…	…	…
4 May 2001	Gentamicin (10)	…	…	…	…	…	…	…
13 May 2001	…	…	…	…	…	…	…	107.4
1 (1)								
5 June 2001	…	PA13	1A	0.5	0.5	1	0.06	…
15 June 2001	…	…	…	…	…	…	…	105
1 (2)								
4 September 2001	Gentamicin (14) plus ciprofloxacin (14)	PA45	1B	1	0.5	1	0.06	83
20 November 2001	Amoxicillin-clavulanate (21)	…	…	…	…	…	…	…
1 (3): 4 December 2001	…	PA77A	1A	0.5	0.5	1	0.06	100.8
		PA77B	1A	1	0.5	1	0.06	…
1 (4)								
5 March 2002	Levofloxacin (14)	PA107A	1C	1	1	2	0.125	93.4
		PA108	1B	1	0.5	0.5	0.125	…
8 April 2002	Clarithromycin (20)	…	…	…	…	…	…	…
24 May 2002	Ciprofloxacin (14)	…	…	…	…	…	…	…
7 June 2002	Gentamicin (14)	…	…	…	…	…	…	…
2 (1)								
18 June 2002	…	PA150	1B	0.5	0.5	0.5	0.06	115.7
9 September 2002	Trimethoprim-sulfamethoxazole (15)	…	…	…	…	…	…	…
23 September 2002	Ciprofloxacin (15)	…	…	…	…	…	…	…
30 September 2002	Tobramycin (28)	…	…	…	…	…	…	…
2 (2): 8 October 2002	…	PA202	1A	0.5	0.5	2	0.25	120.5
		PA203	2	32	8	0.5	1	…
2 (3)								
21 January 2003	Levofloxacin (14) plus azithromycin plus tobramycin (28)	PA244	1A	2	1	2	1	87.7
18 February 2003	…	…	…	…	…	…	…	100
2 (4): 8 April 2003	Clarithromycin (20)	PA287	2	16	4	1	0.5	86.9
3 (1)								
21 July 2003	Levofloxacin (15) plus gentamicin (30)	PA323	1A	8	2	8	4	73.4
19 August 2003	Clarithromycin	…	…	…	…	…	…	…
21 August 2003	Tobramycin (28)	…	…	…	…	…	…	93.8
3 (2)								
14 October 2003	…	PA360	1A	8	2	8	4	80.9
5 December 2003	Levofloxacin (14)	…	…	…	…	…	…	…
4 February 2004	Ciprofloxacin (14)	…	…	…	…	…	…	…
3 (3)								
10 February 2004	…	PA387	2	16	4	4	2	…
20 April 2004	Ciprofloxacin (14)	…	…	…	…	…	…	…
3 (4): 11 May 2004	Tobramycin (28) plus levofloxacin (14)	PA415	1A	32	16	32	16	83.9

NOTE. CAZ, ceftazidime; FEV_1, forced expiratory volume in 1 s (expressed as the percentage of the predicted value using normative data for children from Polgar and Promadhat [17]); IPM, imipenem; MEM, meropenem; P-T, piperacillin-tazobactam.

the imipenem and meropenem zones (<20 colonies) for strain PA323 but not within the zones of the other drugs tested. According to the criteria of Macia et al. [16], these strains did not possess a hypermutable phenotype.

Discussion. A *P. aeruginosa* strain persisted in the lungs of the patient over a 3-year period despite treatment with antipseudomonal agents. Although carbapenems were not used as therapy, a stepwise decrease in susceptibility was observed, leading to a fully resistant phenotype. Low pulmonary function scores corresponded with the isolation of a mucoid-producing strain (PA244) and the emergence of carbapenem resistance. Previous studies have indicated that the presence of mucoidal

Table 2. Efflux and porin expression in 5 strains with identical restriction patterns.

Strain	Year (quarter)	Susceptibility, MIC, μg/mL		Relative fold expression[a]		
		Imipenem	Meropenem	*mexA*	*mexE*	*oprD*
PAO1[b]	…	1	0.5	1.00	1.00	1.00
PA77A	1 (3)	1	0.06	0.89	1.58	0.64
PA202	2 (2)	2	0.25	0.70	1265	0.11
PA244	2 (3)	2	1	0.62	209	0.09
PA323	3 (1)	8	4	1.02	2.12	0.02
PA415	3 (4)	32	16	11.38	1.81	0.03

[a] Transcriptional expression of *mexA, mexE,* and *oprD* as measured by real-time RT-PCR. Values represent the difference (*n*-fold) in expression relative to PAO1.

[b] Reference strain.

P. aeruginosa was the most important risk factor for pulmonary deterioration [18, 19], whereas Lechtzin et al. [20] suggested that antibiotic-resistant *P. aeruginosa* was also associated with a rapid decrease in forced expiratory volume in 1 s.

The selection of carbapenem-resistant *P. aeruginosa* in the absence of carbapenem therapy is perplexing. Oliver et al. [21] reported that 36% of patients with cystic fibrosis were colonized with hypermutable *P. aeruginosa* isolates, whereas no mutator strains were present in patients without cystic fibrosis. Hypermutable strains have a higher rate of spontaneous mutations, leading to the random selection of resistance. This may increase the probability of selecting resistance to a particular drug regardless of its therapeutic use. However, a hypermutability assay [16] indicated that the group 1A strains were not hypermutators, suggesting that the emergence of meropenem resistance was selected (especially in PA415) and was not a result of hypermutability.

The decrease in susceptibility to meropenem was associated with a reduction in the amount of the OprD porin. Lower OprD levels in strains PA202 and PA244 occurred in response to the reduced expression of *oprD* and correlated with *mexEF-oprN* overexpression. *oprD* transcription levels were further reduced in strains PA323 and PA415, without concurrent *mexEF-oprN* overexpression. Disruption of the promoter region of *oprD* through IS element insertion was most likely responsible for decreased *oprD* expression in these strains. Studies have shown a reduction in the transcriptional expression of genes containing IS elements inserted into putative promoter regions [22, 23]. Insertion of the IS element may have been a random event associated with the genetic adaptability of isolates from patients with cystic fibrosis. IS elements have been shown to participate in the evolution of *P. aeruginosa* isolates from patients with cystic fibrosis through insertional mutagenesis, causing genomic rearrangements [24]. In the final strain collected (PA415), clinical resistance to meropenem (MIC, ⩾16 μg/mL) was associated with a loss of OprD in combination with *mexAB-oprM* efflux pump overexpression.

Fluoroquinolones are substrates for both MexEF-OprN and MexAB-OprM and have been shown to select for mutants overproducing these pumps both in vitro [25] and in vivo after fluoroquinolone challenge [26, 27]. *mexEF-oprN* overexpression has been linked to carbapenem resistance through a concomitant loss of OprD [11]. Therefore, the exposure of the patient to ciprofloxacin and levofloxacin before the isolation of the efflux mutants and throughout the 3-year study may have contributed to the selection of carbapenem resistance. In addition to meropenem, trimethoprim and sulfamethoxazole can be exported by the MexAB-OprM efflux pump [28]; however, to our knowledge, a study has not demonstrated the selection of MexAB-OprM–overexpressing mutants with the trimethoprim-sulfamethoxazole combination. Furthermore, trimethoprim-sulfamethoxazole was prescribed to this patient close to year 2, quarter 1, whereas the MexAB-OprM–overexpressing strain PA415 was not isolated until almost 1.5 years later (year 3, quarter 4).

Data in this report support the emergence of carbapenem resistance in *P. aeruginosa* isolates from patients with cystic fibrosis in the absence of carbapenem therapy and independent of hypermutability. Physicians should be aware that the emergence of resistance does not always correlate with treatment and should consider this possibility when choosing therapy for patients with cystic fibrosis. Understanding what factors contributed to the emergence of carbapenem resistance in these *P. aeruginosa* isolates is critical for the continued treatment of this patient population.

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