A Novel *Pseudomonas aeruginosa* Strain with an *oprD* Mutation in Relation to a Nosocomial Respiratory Infection Outbreak in an Intensive Care Unit

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Seven imipenem-resistant *Pseudomonas aeruginosa* isolates were recovered from the sputum samples of pneumonia patients in southwestern China. They had identical antibiotic resistance patterns and indistinguishable pulsed-field gel electrophoresis profiles. Nucleotide sequence analysis revealed a 4-bp (AGTC) insertion in the *oprD* gene, resulting in a frameshift in the cognate open reading frame. These isolates became imipenem susceptible when the chromosomal *oprD* lesion was complemented, indicating that the 4-bp insertion in the *oprD* gene resulted in imipenem resistance.

Carbapenems are considered to be excellent antimicrobial agents and are frequently used for the treatment of hard-to-manage *Pseudomonas aeruginosa* infections. However, carbapenem resistance in *P. aeruginosa* has been reported to increase steadily over the years across the United States (1). In China, 10.6% of *P. aeruginosa* species are resistant to imipenem, most of which have been multidrug-resistant isolates based on Mohnarín 2004 to 2005 (2). From April to September 2009, the Emergency Intensive Care Unit (EICU) in a provincial hospital in southwestern China had an outbreak of imipenem-resistant *P. aeruginosa* (IRPA) in seven patients. In this study, we reviewed patient medical records, as well as the phenotypes and genotypes of the seven isolates recovered from the patients, and we investigated the main mechanism of resistance to imipenem.

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Hospital setting and patients. The following data were recorded from the medical charts of patients with *P. aeruginosa* infection: age, gender, number of patient-days in the hospital, diagnosis, susceptibility pattern, and clinical outcome. Only one sample with *P. aeruginosa* per patient was enrolled in this study. This study was approved by the research ethics committee of the Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital.

Bacterial strains and antimicrobial susceptibility testing. Nonduplicated IRPA clinical isolates recovered in 2009 were included in this study. Those isolates were identified and antimicrobial susceptibility testing performed using the BD Phoenix 100 automatic microbial analysis system (Becton Dickinson Microbiology Systems, Sparks, MD, USA). The MIC of imipenem was determined by using the Etest technique (AB Biodisk, Solna, Sweden). The results were expressed as susceptible (S) or resistant (R), according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (3). *P. aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 were used as reference strains in the antimicrobial susceptibility testing. *P. aeruginosa* strain PAO1 was used as an *OprD*-positive reference strain to detect and analyze the *oprD* gene and *OprD* outer membrane porin protein.

Molecular typing by pulsed-field gel electrophoresis. A total of 19 out of 392 IPRA strains with the same antimicrobial phenotypes were suspected to be clonally transmitted. Pulsed-field gel electrophoresis (PFGE) was employed to differentiate the genotypes, as previously described (4).

PCR amplification and sequencing of *oprD*. *P. aeruginosa* genome DNA extraction and PCR amplification of the *oprD* gene was performed as previously described (5). Distilled water and PAO1 genomic DNA derived from a reference strain of *P. aeruginosa* were used as negative and positive controls, respectively. Sequencing of the amplicons was done on both strands with an ABI Prism 377A sequencer analyzer (Applied Biosystems). The nucleotide sequences were determined bidirectionally and compared with those of the reference strain *P. aeruginosa* PAO1.

***oprD* complementation tests and outer membrane protein analysis.** The pAU48 plasmid, which carries the wild-type *oprD* gene and its promoter region of *P. aeruginosa* PAO1 in vector pAU47, was employed for the complementation tests. The construction of pAU48 was described in detail in a previous report (6). The standard protocol for competent cell preparation and transformation (7) was used for the introduction of pAU47 and pAU48 into strains of *P. aeruginosa*, as indicated in that study. The transformants were selected on the LB plates containing tetracycline (100 µg/ml), and the presence of plasmids in the resulting recombinant strains was confirmed by plasmid preparation and restriction digestion. Outer membrane proteins were isolated according to the procedures of Carlone et al. (8).

In 2009, 1,178 *P. aeruginosa* isolates were recovered in the hospital, and 392 (33.3%) were imipenem resistant. The molecular typing of the 19 isolates revealed 9 subgroups (A to I) in PFGE,

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TABLE 1 Clinical characteristics of seven EICU patients infected with IRPA^a

Case	Isolate	Gender/age (yr)	Length of hospitalization (days)	Day of isolation	Diagnosis	Antibiotic(s) administered	Outcome
1	85	Male/78	>365	40	Obstructive pulmonary emphysema	Ceftazidime, levofloxacin, ertapenem	Improvement
2	373	Male/73	7	17	Pneumonia, II respiratory failure, pulmonary encephalopathy	Amikacin, ciprofloxacin, imipenem, piperacillin-tazobactam, azithromycin, roxithromycin	Improvement
3	483	Male/40	107	39	Primary brain stem injury	Teicoplanin, amikacin, meropenem, voriconazole	Improvement
4	599	Male/83	24	11	Obstructive pulmonary emphysema II respiratory failure	cefoperazone-sulbactam, teicoplanin, amikacin	Death
5	2703	Male/82	17	7	Lung aspiration pneumonia	Meropenem, teicoplanin	Cured
6	2860	Male/80	38	30	Obstructive pulmonary emphysema	Meropenem	Improvement
7	2876	Male/70	24	15	Extensive abdominal abscess	Meropenem, amikacin	Cured

^a These 7 patients all experienced ventilator, deep-vein catheterization, and a urine catheter in the EICU. All patients had mixed infections after *P. aeruginosa* was isolated the first time. The most common pathogens isolated from mixed infection were methicillin-resistant *Staphylococcus aureus*, *A. baumannii*, *E. coli*, *Klebsiella pneumoniae*, and *Candida albicans*.

with the most frequent (subgroup F) being represented by seven isolates (*P. aeruginosa* clinical isolates 85, 373, 483, 599, 2703, 2860, and 2876). All were recovered from sputum specimens from patients with hospital-acquired pneumonia in the EICU from April to September 2009, which indicated clonal transmission in the EICU.

The clinical characteristics of seven patients from whom *P. aeruginosa* was isolated are shown in Table 1. PCR was first conducted to verify the existence of *oprD* in the clinical isolates recovered from the seven EICU patients. Sequence analysis of the *oprD* genes revealed that nucleotide sequences of the 5' and 3' ends of *oprD* were highly conserved among all strains of *P. aeruginosa* available in the NCBI database. Accordingly, two oligonucleotide primers with conserved sequences covering these two regions of *oprD* were designed and applied for PCR. All strains tested positive (data not shown), indicating the presence of *oprD* in the genome.

To further characterize the genetic lineage of *oprD* in the seven isolates, the nucleotide sequences of *oprD* from the isolates were determined. Sequence alignment indicated that all these *oprD* genes possess a completely identical sequence. A BLAST search against the NCBI database showed that the coding sequence of this *oprD* gene is identical to that in *P. aeruginosa* strain MTB-1 (GenBank accession no. CP006853) (9) except for a 4-bp AGTC insertion after nucleotide position 752 in the *oprD* gene found this study. The amino acid sequence of MTB-1 OprD of 441 residues exhibited 93% sequence identity to that of the reference strain PAO1 of 443 residues. In comparison, the 4-bp insertion in the mutated *oprD* gene (whose product is designated OprD-M) resulted in a reading frameshift and premature termination in the translated peptide of 264 residues in length, which was truncated after the putative loop 4 of the OprD protein. These data suggest that the truncated OprD-M forms a nonfunctional outer membrane porin in the seven EICU isolates of this study.

To test the hypothesis that a truncated nonfunctional OprD-M in these isolates was responsible for imipenem resistance, we conducted the following complementation tests in PAO1 and isolates 373 and 599, two of the seven EICU strains. For each strain, plasmids pAU47 and pAU48 (pAU47 carrying the *oprD* gene of PAO1) were introduced into the host, and the MICs of imipenem in these transformants were determined by the broth dilution

method to be 1 µg/ml for PAO1/pAU47, 0.5 µg/ml for PAO1/pAU48, >8 µg/ml for 373/pAU47, 0.5 µg/ml for 373/pAU48, >8 µg/ml for 599/pAU47, and 0.5 µg/ml for 599/pAU48. The apparent >4-fold reduction in the MICs of strains 373 and 599 harboring pAU48 supported a defect in *oprD* as the cause of imipenem resistance in these strains.

The outer membrane porin profiles of these strains were also determined. The outer membrane proteins were separated by SDS-PAGE and stained with Coomassie brilliant blue R-250. Both strains 373 and 599 lacked the corresponding band for OprD in comparison to that of PAO1, and the introduction of pAU48 into these strains exhibited an increased level of OprD (Fig. 1). These results indicated the absence of functional OprD in the clinical isolates of this study.

Imipenem resistance is usually multifactorial. The increased production of carbapenem-hydrolyzing enzymes (mainly metallo-β-lactamases [MBLs]), the production of AmpC chromosome-encoded cephalosporinase, and reduced outer membrane porin OprD expression are known to contribute to imipenem resistance (10). Our previous study showed that the main resistance mechanisms for imipenem in our hospital did not produce carbap-

FIG 1 Outer membrane profiling determined by SDS-PAGE. PAO1, reference strain of *P. aeruginosa*; 373 and 599, representative clinical isolates. PAO1/pAU48, 373/pAU48, 599/pAU48, strains harboring the plasmid-borne *oprD*. M, molecular size marker. The banding position of OprD is indicated by the arrow on the upper right.

enem-hydrolyzing enzymes, AmpC chromosome-encoded cephalosporinase, or active efflux system overexpression (11). The mutational inactivation of the *oprD* gene represents the major cause of OprD loss in ICU *P. aeruginosa* strains (12). In China, it was reported that carbapenem resistance was driven mainly by mutational inactivation of OprD (13).

The *oprD* gene was reported as an interesting genetic marker not only because of its contribution to carbapenem resistance but also its high sequence variability for clonal analysis (14). It was found that although the virtually unlimited number of *oprD* alleles provides high discriminatory power, members of narrow clonal lineages often show identical *oprD* sequences. In this study, several lines of evidence support the conclusion that the carbapenem-resistant phenotype of these clinical isolates was due to the lack of a functional OprD porin. Since the *oprD* coding sequences of these isolates were all identical, it is very likely that they originated from the same clone, which may be very closely related to strain MTB-1, an environmental isolate (9). The results of a BLAST tree analysis revealed additional clan members other than MTB-1. These members include a Spanish carbapenem-resistant isolate, *P. aeruginosa* W64 (GenBank accession no. KF517098), which has a transposon insertion at the 5' end of *oprD*, and several clinical carbapenem-resistant isolates from Lebanon (GenBank accession no. KJ482581, KJ482584, KJ482596, and KJ482597), which have different base substitutions resulting in the introduction of premature termination codons. However, none of these strains had the 4-bp insertion in *oprD* as discovered in this study. This new type of *oprD* mutation as discovered in this study might be the result of selection pressure in clinical settings.

P. aeruginosa OprD is a 443-amino-acid protein that facilitates the uptake of basic amino acids, imipenem, and gluconate across the outer membrane. Huang and colleagues (15) proposed an OprD topology model in which 16 beta-strands were predicted, connected by short loops at the periplasmic side. The eight external loops (L1 to L8) were of varied lengths but tended to be much longer than the periplasmic ones. In this study, the 4-bp insertion mutation at nucleotide (nt) 752 resulted in the OprD protein being truncated after loop 4. It was further confirmed that a novel 4-bp insertion in the *oprD* gene caused the defect in OprD function and resulted in imipenem resistance.

Nucleotide sequence accession number. The nucleotide sequences of *oprD* from the isolates were determined and deposited under GenBank accession no. KM492918.

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