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A mechanism of carbapenem resistance due to a new insertion element (ISPa*133*) in *Pseudomonas aeruginosa*

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Summary. This study explored the evolutionary mechanism by which the clinical isolate PA110514 yields the imipenem-resistant derivative PA116136. Both isolates were examined by PFGE and SDS-PAGE, which led to the identification of a new insertion sequence, ISPa*133*. This element was shown to have distinct chromosomal locations in each of the original isolates that appeared to explain the differences in imipenem susceptibilty. In strain PA110514, ISPa133 is located 56 nucleotides upstream of the translational start codon, which has no effect on expression of the porin OprD. However, in strain PA116136 ISPa*133* it is located in front of nucleotide 696 and, by interrupting the coding region, causes a loss of OprD expression, thus conferring imipenem resistance. In vitro experiments mimicking the natural conditions of selective pressure yielded imipenem-resistant strains in which ISPa133 similarly interrupted *oprD*. A mechanism is proposed whereby ISPa*133* acts as a mobile switch, with its position in *oprD* depending on the degree of selective pressure exerted by imipenem. [**Int Microbiol** 2011; 14(1):51-58]

Keywords: *Pseudomonas aeruginosa* · protein OprD · carbapenems · imipenem · insertion elements · antimicrobial resistance

Introduction

Infections by *Pseudomonas aeruginosa* are a serious clinical problem, particularly in immune compromised hosts in hospital settings [9,29,33]. Moreover, the treatment of these infections is often difficult because of the limited number of effective antimicrobial agents, due to the intrinsic resistance of *P. aeruginosa* strains and their different modes of growth [10]. These properties reflect the synergy between the bacterium's low outer-membrane permeability [2,6,38], its chromosomally encoded AmpC β-lactamase [14], and its broadly specific drug efflux pump [11,17,22,23]. Furthermore, *P. aeruginosa* readily acquires resistance to most antimicrobials through mutations in its chromosomal genes and through extrachromosomal elements carrying resistance determinants [14,25]. Although there are several antimicrobials (carbapenems, cefepime, ceftazidime, tobramycin and amikacin) that continue to be effective against *P. aeruginosa,* in the last few years the bacterium's increasing resistance to many others has been reported [4,13,18,28,30].

Carbapenems are a class of β-lactam antibiotics with good antimicrobial activity against *P. aeruginosa* but the emergence and spread of acquired carbapenem resistance in this species have challenged the success of therapeutic and control efforts. Since carbapenems, especially imipenen, are

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widely used in the clinical setting [26], investigation of the molecular mechanisms leading to resistance is crucial. Imipenem resistance can involve low permeability, the activity of an inducible β-lactamase [15], and multidrug efflux systems, but the most widely accepted mechanism involves the loss of the porin OprD from the outer membrane [37], which can occur at the transcriptional or translational level or through the emergence of mutations in the *oprD* gene [20]. For example, in one report, the down-regulation of *oprD* transcription in clinical isolates of *P. aeruginosa* was shown to have occurred by a mechanism involving inactivation of the gene due to the presence of insertion sequence elements (IS) [36]. Additional mechanisms of carbapenem resistance include repressed OprD expression, as is the case in *nfxC*-type mutants, which simultaneously overexpress the MexEF-oprN efflux pump.

The present study resulted from the isolation of two strains of *P. aeruginosa* from the same patient in the course of a chronic respiratory infection. The first strain was obtained soon after the patient was admitted to the University Hospital of Bellvitge (L'Hospitalet, Barcelona, Spain). The infection was successfully treated with imipenem. The second strain was obtained 6 days later, from the patient's rectum. Pulsed-field gel electrophoresis (PFGE) profiles of the two isolates were identical, as were the results of biochemical tests aimed at their identification. However, determination of antibiotics susceptibility demonstrated that the isolates were identical for all antibiotics tested except imipenem: the first isolate was imipenem susceptible whereas the second was imipenem resistant. Thus, aim of this study was to search for underlying changes in the isolates' DNA that could account for the difference in imipenem susceptibility, starting from the hypothesis that resistance was mediated by a loss of OprD expression on the outer membrane.

Materials and methods

Bacterial strains. The two studied clinical isolates of *P. aeruginosa* were isolated from a patient at the Servei de Microbiologia of the Hospital Universitari de Bellvitge. PA110514 was isolated before imipenem treatment was started, and strain PA116136 after the patient had received the full course of the antibiotic. *P. aeruginosa* PA9 was used as the positive control for amplification of the *oprD* gene. Strains PA132325 and PA138635, susceptible and resistant to imipenem, respectively, were used as controls in preparing extracts of outer membrane proteins (OMPs). *P. aeruginosa* strains PAFL2, PAFL4, PAFL8, PAFL12, and PAFL16 are the resistant mutants obtained in this study. All of the bacterial strains were cultured in trypticase soy broth (TSB) or on trypticase soy agar (TSA). Bacteriological media were purchased from Scharlab (Barcelona, Spain).

Pulsed-field gel electrophoresis. To compare the different strains, PFGE was carried out as follows: DNA was extracted and purified as described elsewhere [28,31]. *Spe*I, a low-frequency restriction enzyme, was used according to the manufacturer's specifications (New England Biolabs, Beverly, MA, USA). The *Spe*I DNA restriction fragments were separated in a CHEF-DR III unit (Bio Rad, Hercules, CA, USA) for 20 h at 14ºC and 6 Volts/cm, with pulse times ranging from 0.5 to 25 s. Strain relatedness was assigned in accordance with published criteria [32]. *Pseudomonas aeruginosa* PAO1 was used as the control in sequence analysis (accession number Z14065.1)

Minimum inhibitory concentration (MIC) determinations. Antimicrobial susceptibility was tested using the microdilution method, with Mueller-Hinton broth (Scharlab, Barcelona, Spain), according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI). The following antimicrobials were tested: piperacillin, piperacillin/tazobactam, ticarcillin, aztreonam, gentamicin, tobramicin, amikacin, trimethoprim/sulfametoxazol, ciprofloxacin, ofloxacin, ceftazidime, cefepime, imipenem, and meropenem. The antibiotics were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Outer membrane preparations and SDS-PAGE. Whole bacterial proteins and OMPs were obtained as described elsewhere [24,27]. SDS-PAGE was performed, using a modification of the method of Laemmli, in a Bio-Rad apparatus (Mini-Protean II). Gels were stained with 0.25% Coomassie brilliant blue, destained, and then dried on a gel dryer (Biorad 543) [8,12].

Gene *oprD* amplification. To obtain *P. aeruginosa* genomic DNA, 5 ml of Luria broth inoculated with a single colony was incubated overnight at 37°C. Bacteria were harvested by centrifugation and suspended in 180 µl of ATL buffer from the DNeasy Tissue kit (Qiagen, Germany), which was used for DNA purification following the manufacturer's instructions. A modified version of the PCR assay previously described [36] was used to amplify *oprD*. Reaction mixtures had a final volume of 50 µl and contained 2 µl (1/10th volume) of genomic DNA, 0.5 µM of the OprDSEQF1 forward primer (5′-CTACGCAGATGCGACATGC-3′), 0.5 µM of the OprDSEQR1 reverse primer (5′-CCTTTATAGGCGCGTTGCC-3′) (Invitrogen, USA), 1 U of Taq DNA polymerase (Fermentas, Vilnius, Lithuania), 0.2 mM of each deoxynucleoside triphosphate (Fermentas, Lithuania), 1× PCR buffer, and 2 mM $MgCl_2$. Amplification was achieved in a Techne (Staffordshire, UK) thermocycler model TC-312 during 25 cycles, each consisting of 30 s at 95°C, 30 s at 56°C, and 2 min at 72°C. Final extension was performed at 72°C for 10 min. PCR products were separated by agarose-gel (1.5% w/v) electrophoresis in Tris-borate buffer (TBE) at 9 Volts/cm for 1.5 h and visualized using ethidium bromide.

DNA sequencing and analysis of sequence data. Sequence data for *oprD* were obtained by using the primers OprDSEQF1 and OprDSEQR1. PCR products were purified with the MinElute PCR purification kit (Qiagen, Germany) prior to sequencing. The ABI PRISM BigDye Terminator (version 3.1) Cycle Sequencing Ready Reaction kit and ABI PRISM(R) 3700 DNA analyzer were used (Applied Biosystems, USA). Database searches were conducted using the basic local alignment search tool (BLAST) [1] and multiple alignments carried out with CLUSTAL W [34].

In vitro generation of imipenem-resistant mutants of *Pseudomonas aeruginosa*. Approximately 10^5 *P. aeruginosa* PA110514 cells were used to inoculate 20 ml of MHB medium with imipenem concentrations ranging from 0.5 to 16 µg/ml. After 24 h of incubation at 37°C, resistant derivatives (MICs = 6–16 µg/ml) were selected by spreading

Table 1. Susceptibilities to antimicrobial agents tested against *Pseudomonas aeruginosa* PA110514 and PA116136

	Strain PA110514		Strain PA116136	
Antibiotics	MIC*	Susceptibility	MIC	Susceptibility
piperacillin	<16	S	<16	S
piperacillin/tazobactam	<16	S	<16	S
ticarcillin	<16	S	<16	S
aztreonam	2	S	2	S
gentamicin	<4	S	<4	S
tobramicin	<4	S	<4	S
amikacin	<8	S	<8	S
trimethoprim/sulfametoxazol	<2/38	S	>2/38	R
ciprofloxacin	<0.12	S	<0.12	S
ofloxacin	<0.5	S	<0.5	S
ceftazidime	<1	S	<1	S
cefepime	<1	S	<1	S
imipenem	<1	S	>8	R
meropenem	<4	S	<4	S

*MICs are expressed in μg/ml.

0.01 ml of the overnight culture onto MHA plates (Scharlab, Barcelona, Spain) containing the appropriate concentration of imipenem. Repeated exposure to the antibiotic was continued with the most resistant derivatives until an increase in the minimum inhibitory concentration (MIC) of imipenem to 16 μg/ml was achieved.

Nucleotide sequences accession numbers. The nucleotide sequences reported herein have been submitted to GenBank databases. The accession numbers for the ISPa*133* found in strains PA110514 and PA116136 are FJ387165.2 and FJ387166.2, respectively.

Results

Minimum inhibitory concentration determinations. Antimicrobial susceptibilities were determined in MIC tests, with the results shown in Table 1. *P. aeruginosa* strain PA110514 was found to be susceptible to all tested antibiotics while *P. aeruginosa* strain PA116136 was resistant to trimethoprim/sulfamethoxazole and imipenem.

Outer-membrane protein profile. Electrophoretic comparisons of OMPs from both strains demonstrated, as expected, that the susceptible strain, *P. aeruginosa* PA110514, but not the resistant strain, PA116136, showed a visible band corresponding to the porin OprD in the outer membrane (Fig. 1). To investigate the mechanism responsible for the loss of OprD expression, the *oprD* genes of both strains were amplified and sequenced and their sequences compared.

Gene *oprD* amplification. The amplicons obtained from the *oprD* gene region of strains PA110514 and PA116136 were larger than that of strain PA9 used as positive control (Fig. 2). The predicted length of *oprD* is 1586 bp, while the amplicons from PA110514 and PA116136 were 3000 and 2000 bp, respectively. Sequence analysis of both amplicons revealed the presence of a long insert, approximately 1300 bp, with a 59.6% G + C content. The insert, designated ISPa*133*, showed high homologies with the mobile elements of the IS*3* family of insertion sequences. The similarities were not only in terms of the length of the ISPa*133*, which for members of the IS*3* family is typically between 1200 and 1550 bp, but due to the presence of two characteristic open reading frames (ORF), *orfA* and *orfB*, which, unusually, did not overlap (Fig. 3). The insertion element was flanked by 25-bp terminal inverted repeats (IRs), with TG at the 5′ end and CA at the 3′ end, as in other members of the IS*3* family. Another general feature of IS elements is that, on insertion, most generate short directly repeated sequences (DR) of the target DNA flanking the IS, but these were not detected in ISPa*133*.

The first ORF (*orfA*) encodes a transposase (Fig. 3). In the search for conserved domains [16], we determined that

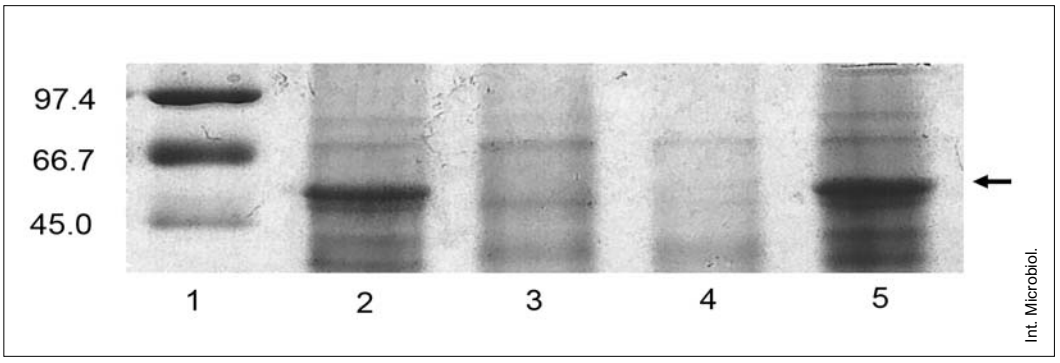

Fig. 1. Outer membrane protein profiles. Black arrows indicate the position of OprD. Lane 1: SDS-PAGE standards, (Low Range, Bio-RAD, USA); lane 2: strain PA110514; lane 3: strain PA116136; lane 4: strain PA138635 (imipenem-resistant control); lane 5: strain PA132325 (imipenem-susceptible control).

the protein includes a helix-turn-helix motif (HTH), a family of DNA-binding domains unique in bacteria, and that it showed high identity to the transposase 8 family, some of which are members of the IS3 family. The second ORF, *orfB*, encodes the central catalytic domain of an integrase from the rve super-family [http://pfam.sanger.ac.uk/family PF00665]. Integrase mediates the integration of a DNA copy of the viral genome into the host chromosome. The enzyme is composed of three domains. The amino-terminal zinc-binding domain (pfam02022) is the central catalytic domain. The carboxyl terminal domain is a non-specific DNA binding domain (pfam00552). The catalytic domain acts as an endonuclease when two nucleotides are removed from the 3′ ends of the blunt-ended viral DNA resulting from reverse transcription. This domain also catalyzes the DNA-strand-transfer reaction of the 3′ ends of the viral DNA to the 5′ ends of the integration site.

While the large insert located in PA116136 was 99% identical to that found in PA110514, its location in *oprD* was different. In PA110514, ISPa133 was located 56 nucleotides upstream of the translational start codon, but in PA116136 it occurred immediately before nucleotide position 697, effectively replacing this nucleotide and causing the removal of the first 232 amino acids of the porin OprD (Fig. 4).

In vitro generation of imipenem-resistant mutants of *Pseudomonas aeruginosa*. Repeated exposure of *P. aeruginosa* PA110514 to imipenem allowed the selection of several defective OprD mutants. When *oprD* was amplified using DNA from cultures resistant to imipen-

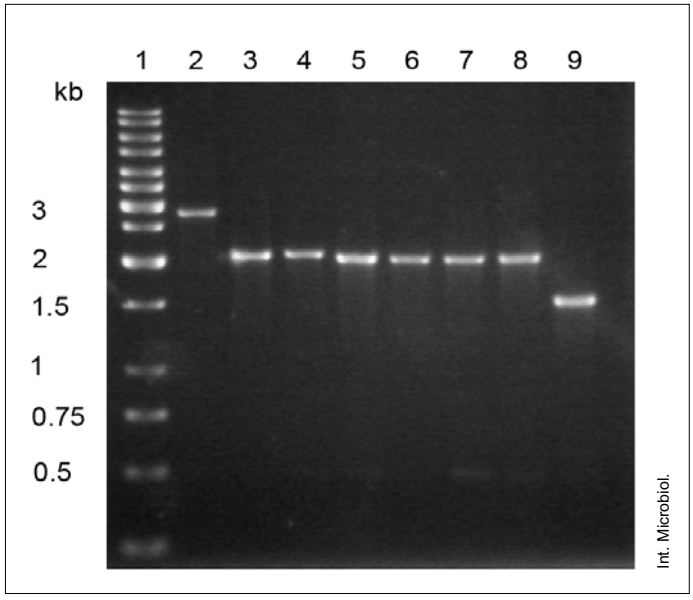

Fig. 2. PCR amplification products obtained with primers OprDSEQF1 and OprDSEQR1. Fragments were separated by electrophoresis through a 1.5% agarose gel. Lane 1: GeneRuler 1 kb DNA Ladder (Fermentas, Vilnius, Lithuania). Lane 2: strain PA110514. Lane 3: strain PA116136. Lane 4: strain PAFL2. Lane 5: strain PAFL4. Lane 6: strain PAFL8. Lane 7: strain PAFL12. Lane 8: strain PAFL16. Lane 9: strain PA9, used as positive control.

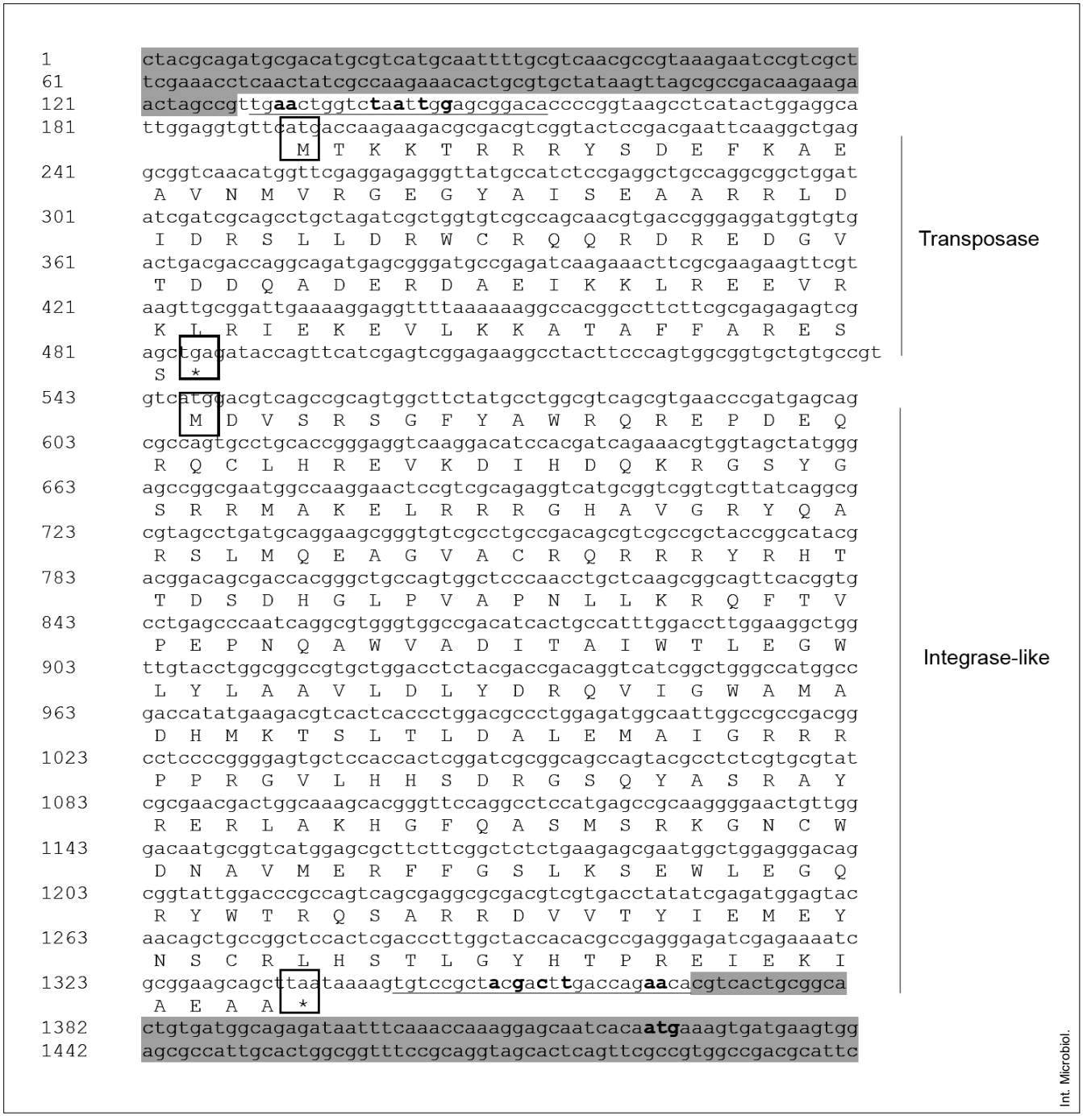

Fig. 3. Nucleotide sequence of de novel IS*3*-like element, ISPa*133*, present in strain PA110514 and its proposed derivative PA116136. The underlined sequences represent the 25-bp inverted repeats (IRs). Bold letters within the IRs indicate mismatches between left and right IRs. Translational start and stop codons of both *orf*s encoded by the mobile element are boxed; the amino acid sequence of the two open reading frames are shown below the sequence. Shaded regions represent the sequence of the *oprD* gene flanking the IS element.

em concentrations 2, 4, 8, 12, and 16 times higher than the MIC value for PA110514, an amplicon of 2000 bp was obtained, i.e., identical in size to the amplicon of the resistant clinical isolate PA116136. Sequencing of the amplicon revealed the presence of ISPa*133*. The position of the insertion element in the mutants was not the same as in PA110514 and PA116136. In the mutant strain PAFL2, ISPa*133* was located immediately before nucleotide position 667, replac-

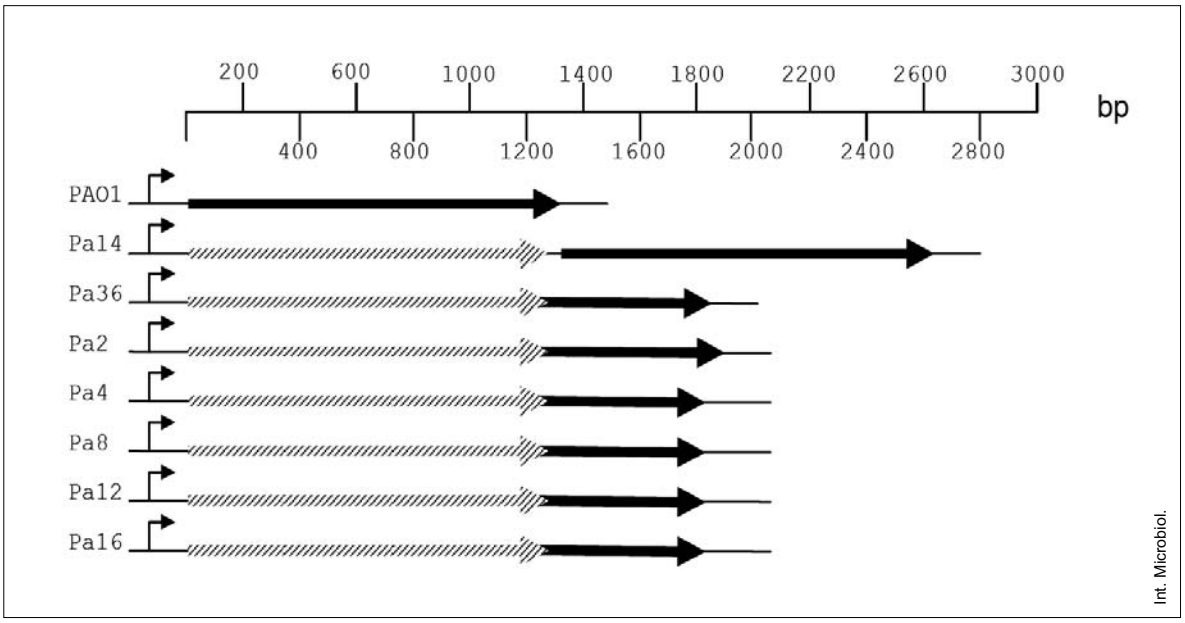

Fig. 4. Schematic diagram of the locations of the ISPa*133* element with respect to the *opr*D gene. The solid arrow represents the *oprD* structural gene while the striped arrow represents ISPa*133*.

ing the first 222 amino acids of the porin, but in PAFL4 and in the rest of resistant strains, it was located just before nucleotide 703, causing the removal of the first 234 amino acids of OprD (Fig. 4).

Discussion

Carbapenems are a class of β-lactam antibiotics with good antimicrobial activity against *P. aeruginosa*; as such, they are often used as a last resort in infections due to multidrug-resistant strains of the bacterium [21]. However, the emergence and spread of acquired carbapenem resistance have challenged therapeutic and control efforts [5], necessitating a better understanding of the molecular mechanisms underlying resistance. Moreover, it seems likely that several mechanisms are involved in carbapenem resistance [7].

The two clinical isolates of this study, PA110514 and PA116136, were identical in their PFGE profiles and in biochemical tests, suggesting their close relationship. The unique noticeable difference between the isolates was in their susceptibility to imipenem. Our hypothesis, that this difference involved the OMPs, was supported by the results of SDS-PAGE, which revealed the loss of OprD in PA116136 (Fig. 1) and suggested that this strain was a PA110514 derivative. To investigate the mechanism responsible for the loss of the porin, the *oprD* gene of each strain was amplified and sequenced. Examination of these sequences revealed the presence of a new insertion sequence, ISPa*133*, located 56 nucleotides upstream of the *oprD* start codon in PA110514. However, since this isolate is imipenem-susceptible, the presence of ISPa*133* has no obvious effect on the upstream regulatory region of the gene, as *oprD* expression was normal (Fig. 1) as was the expression of the porin OprD. By contrast, in PA116136, the insertion element (99% identity) is located immediately before nucleotide position 696, which causes the removal of the first 232 amino acids of OprD. Consequently, the protein is not expressed and was not detectable in the gels (Fig. 1). Since loss of the protein prevents the entrance of imipenem, strain PA116136 is resistant to the antibiotic.

To date, the presence of ISs in OprD has been reported only once , although these elements have been detected in other genes of *P. aeruginosa* [3,4,28]. In those cases, the ISs were described in resistant isolates and thus assumed to be the cause of resistance, either via gene activation or by inducing the high level expression of a potential resistance gene. Based on the findings in the two clinical isolates analyzed in this study, we propose that the newly identified insertion element ISPa*133* acts as a switch, depending on the degree of selective pressure exerted by imipenem. In the absence of selective pressure, as was the case during isolation of strain PA110514 from an infected, untreated patient, OprD is found

on the OM and the position of ISPa*133* in the genome has no effect on *oprD* expression; however, selective pressure exerted by antibiotic therapy results in the selection of strains in which ISPa*133* moves within the *oprD* gene, such that, in the case of strain PA116136, the first 232 amino acids are removed, thus preventing expression of the gene. A high rate of ISPa*133* jumping would therefore provide a major selective advantage, one that allows the bacterium to survive in the presence of carbapenems.

It has been proposed that ISs without DRs may simply result from the homologous inter- or intra-molecular recombination between two IS elements, each with a different DR sequence, or from the formation of adjacent deletions arising from duplicative intramolecular transposition [35]. For ISPa*133*, this could explain the lack of DRs as well as the absence of a crossover region between *orfA* and *orfB*.

As shown in other species, experiments mimicking natural conditions can provide insight into population phenomena [19]. This was the aim of our experiments in which strain PA110514 was submitted to selective pressure by imipenen in culture flasks in order to obtain spontaneous resistant mutants in vitro with properties similar or identical to those of the strains isolated from a patient hospitalized with a *P. aeruginosa* infection. Indeed, mutants with levels of resistance similar to those of strain PA116136 were easily recovered. Moreover, when the experiments were prolonged, the MICs of the isolates were even higher (16 μg/ml).

Note that, according to the above-proposed mechanism of action, removal of the selective pressure exerted by the antibiotic should restore bacterial susceptibility to carbapenems through the expression of the full-length OprD; whether this is the case remains to be determined. However, this change would not be expected to occur as quickly as the acquisition of resistance, since the selective advantage conferred by imipenem resistance in the presence of the antimicrobial is severe whereas OprD expression is likely to be evolutionarily advantageous only under certain environmental conditions.

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