Acquisition of multidrug resistance transposon Tn6061 and IS6100-mediated large chromosomal inversions in *Pseudomonas aeruginosa* clinical isolates

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Pseudomonas aeruginosa is a major human opportunistic pathogen, especially for patients in intensive care units or with cystic fibrosis. Multidrug resistance is a common feature of this species. In a previous study we detected the *ant(4')-IIB* gene in six multiresistant clinical isolates of *P. aeruginosa*, and determination of the environment of the gene led to characterization of Tn6061. This 26 586 bp element, a member of the Tn3 family of transposons, carried 10 genes conferring resistance to six drug classes. The *ant(4')-IIB* sequence was flanked by directly repeated copies of ISCR6 in all but one of the strains studied, consistent with ISCR6-mediated gene acquisition. Tn6061 was chromosomally located in six strains and plasmid-borne in the remaining isolate, suggesting horizontal acquisition. Duplication-insertion of IS6100, that ended Tn6061, was responsible for large chromosomal inversions. Acquisition of Tn6061 and chromosomal inversions are further examples of intricate mechanisms that contribute to the genome plasticity of *P. aeruginosa*.

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INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous species able to survive and adapt in diverse environments such as aquatic habitats, soils, animals and human flora, where it can contribute to pathological outcomes. It is responsible for acute infections such as septicaemia, meningitis and infection of skin and soft tissue, and chronic infections of the urinary tract or lungs are especially problematic, most notably nosocomial ventilator-assisted and chronic pneumonia in cystic fibrosis (CF) patients. *P. aeruginosa* is intrinsically resistant to several drugs and possesses an extraordinary propensity to develop new resistances. Intensive care units and the CF lung are particular niches for selection of multidrug resistant (MDR) organisms, and

P. aeruginosa isolates resistant to all available antibiotics used in therapy are not uncommon (Bonomo & Szabo, 2006). Resistance occurs by one or, more often, a combination of the following mechanisms: decrease in the intracellular drug concentration due to impaired permeability or active efflux, e.g. imipenem resistance by OprD2 porin mutation and multidrug resistance by overexpression of MexAB-OprM or MexXY efflux pumps; drug inactivation, notably by β -lactamases and aminoglycoside-modifying enzymes; and modification or protection of the target, such as rRNA methylation and protection of the gyrase, conferring resistance to aminoglycosides and fluoroquinolones, respectively (Bonomo & Szabo, 2006).

The large size of the genome of *P. aeruginosa*, from 5 to 7 Mb, composed of a core of more than 5000 genes and of a variable accessory part, provides mechanisms for intrinsic resistance and adaptability to various environments (Mathee *et al.*, 2008). Members of this species can further evolve by mutation in endogenous genes or by acquisition of foreign DNA. Hypermutator *P. aeruginosa* with a defective mismatch repair system has been isolated in CF lungs, where the increased mutation frequency may have aided rapid adaptation to this particular niche (Oliver *et al.*, 2000). *P. aeruginosa* has developed other genetic tools, such as insertion sequence transposition and large chromosomal inversion (Kresse *et al.*, 2003, 2006), which, by reorganizing the genome, constitute adaptive mechan-

Abbreviations: CF, cystic fibrosis; CS, conserved sequence; IR, inverted repeat; MDR, multidrug resistant; SGI1, *Salmonella* genomic island 1; TAIL-PCR, thermal asymmetrical interlaced-PCR.

The GenBank/EMBL/DDBJ accession number for the sequence of Tn6061 from *P. aeruginosa* BM4530 is GQ388247, that for the ISCR sequence from BM4531 is GU475047, those for the insertion sites of Tn6061 in BM4492, BM4530 and BM4534 are GU475054, GU475055 and GU475053, respectively, and those for the boundaries of IS6100 copies in BM4530 are GU475050 and GU475052, and in BM4492 are GU475048, GU475049 and GU475051.

Two supplementary figures, showing alignment of transposases of ISCR elements displaying the highest identity with ISCR6 and PFGE of total *Hind*III- or *Xba*I-digested DNA of some of the *P. aeruginosa* strains examined in this study, are available with the online version of this paper.

isms. Horizontal gene transfer, a common feature in *P. aeruginosa*, confers new functions by the acquisition of plasmids, transposons or pathogenicity islands. To survive in the hospital environment, notably by achieving multidrug resistance, *P. aeruginosa* has combined and accumulated these various mechanisms.

Seven epidemiologically unrelated clinical strains of *P. aeruginosa* isolated between 1992 and 1998 in Bulgaria have been described previously for their resistance to aminoglycosides (Sabtcheva *et al.*, 2003). A new O-aminoglycoside adenyltransferase gene, *ant(4')-IIb*, has been characterized and shown to confer resistance to amikacin and tobramycin but not to gentamicin. The gene was present in six strains, adjacent to a sequence homologous to an ISCR element. The seventh strain lacked *ant(4')-IIb* but was genetically related to another isolate. The gene was chromosomally located in five strains and plasmid-borne in one. Preliminary study of the genetic context has shown that *ant(4')-IIb* was part of a region homologous to the MDR portion of the *Salmonella* genomic island 1 (SGI1) (Boyd *et al.*, 2001). Taken together, these data suggest mobility of an *ant(4')-IIb* carrying element and dissemination among non-clonal clinical isolates. Study of the genomic environment of *ant(4')-IIb* led to the characterization of MDR transposon Tn6061, the description of ISCR6, and indicated that insertion sequence IS6100, present at one end of the transposon, mediates large chromosomal inversions in the *P. aeruginosa* isolates.

An initial report of this work was presented at the 49th Interscience Conference on Antimicrobial Agents and Chemotherapy (Coyne *et al.*, 2009).

METHODS

Strains and growth conditions. Seven *P. aeruginosa* isolates, BM4492, and BM4529–BM4534, were isolated from 1992 to 1998 at the National Oncology Center in Sofia, Bulgaria (Sabtcheva *et al.*, 2003). All strains, except BM4530 and BM4531, could be distinguished by PFGE after *SpeI* digestion; however, the common bands indicated that the seven strains were related (Sabtcheva *et al.*, 2003). Cells were grown at 37 °C in Luria–Bertani (LB) broth and on LB agar (Difco Laboratories).

Susceptibility testing. Antibiotic susceptibility was determined by disk diffusion on Mueller–Hinton agar (Bio-Rad) and MICs were determined by E-test (AB Biodisk). The MICs of NaCl and chromate were determined by inoculating strains grown to mid-exponential phase into a 96-microwell plate containing LB broth with twofold increasing concentrations of NaCl or chromate.

DNA manipulation. *P. aeruginosa* genomic DNA was extracted as described elsewhere (Sambrook & Russell, 2001). Amplification of DNA was performed in a GeneAmp PCR system 9700 (Perkin-Elmer Cetus) with *Taq* (MPbio) or Phusion (Finnzymes) DNA polymerases, as recommended by the manufacturers. Amplification of large DNA fragments was achieved using the Expand Long Template PCR system (Roche) according to the manufacturer's recommendations. PCR elongation times and temperatures were adjusted according to the expected size of the PCR products and the nucleotide sequences of the primers, respectively. Thermal asymmetrical interlaced-PCR (TAIL-

PCR), a technique of chromosomal walking giving access to unknown sequences that flank a known sequence, was performed to determine the insertion sites of Tn6061 and of IS6100 copies. Nested PCRs using successively four specific primers were carried out in combination with each of four arbitrary degenerate primers, as described elsewhere (Liu & Whittier, 1995). The PCR products were sequenced on a CEQ 2000 DNA Analysis System automatic sequencer (Beckman Instruments). Flanking regions obtained by TAIL-PCR were reamplified, and the sequence of the products was verified. Homology searches were carried out using the BLAST suite of programs and ORFs were detected with ORF Finder via the National Center for Biotechnology Information (NCBI) website (<http://www.ncbi.nlm.nih.gov/>). Comparison of Tn6061 with SGI1 and AbaR1 was represented using the Artemis Comparison Tool (Carver *et al.*, 2005). Alignment of sequences was performed and represented using CLUSTAL W and BoxShade, respectively. Southern blot hybridization using an IS6100 internal probe was performed as described elsewhere (Sambrook & Russell, 2001).

Mutation frequency measurement. Mutation frequency was determined as described by Rodríguez-Rojas & Blázquez (2009) using fosfomycin instead of rifampicin or streptomycin, since the strains were highly resistant to these drugs ($>600 \mu\text{g ml}^{-1}$ and $>500 \mu\text{g ml}^{-1}$, respectively). Briefly, one colony was resuspended in 20 ml LB and grown at 37 °C overnight. Aliquots from successive dilutions were plated onto LB plates with or without fosfomycin ($300 \mu\text{g ml}^{-1}$). Colony counting was performed after 48 h of incubation at 37 °C, and the mutation frequency calculated.

UV radiation resistance. Aliquots (10 μl) of serial dilutions of an overnight culture were plated on an LB agar plate and irradiated with a UV lamp (model TL-900, Camag) ($\lambda=254 \text{ nm}$). Cells were counted to determine the ratio of irradiated versus non-irradiated c.f.u. Four replicates were performed for each strain.

Heat-stress resistance. Overnight cultures were diluted 1:100, incubated at 37 °C until the OD₆₀₀ reached 0.5, and shifted to a shaking water bath at 37, 42, 50 or 53 °C for 30 min. Viable cell counts were determined on LB agar plates after appropriate dilutions.

Biofilm formation. Biofilm formation was quantified as described by Rodríguez-Rojas & Blázquez (2009). Overnight cultures were diluted 1:100 in LB broth, poured into a 96-microwell polystyrene plate (Greiner Bio-One) and incubated for 4 h at 37 °C. After crystal violet staining, the A₅₉₀ was measured using a multiwell spectrophotometer (Labsystems Multiskan RC). Eight replicates were carried out for each strain.

Tn6061 designation. The designation of Tn6061 was assigned by the UCL Eastman Dental Institute website (<http://www.ucl.ac.uk/eastman/tn/>).

RESULTS AND DISCUSSION

Characterization of Tn6061

The genomic environment of the *ant(4')-IIb* gene for an aminoglycoside adenyltransferase in strain BM4492 was determined by sequencing the flanking regions obtained by cloning a 12 kb *HindIII* fragment and by TAIL-PCR. Transposon Tn6061 of 26 586 bp carried resistance to β -lactams (*bla*_{VEB-1}, *bla*_{OXA-10}), aminoglycosides [*ant(2')-Ia*, *ant(3'')-Ia*, *ant(4')-IIb*], tetracycline [*tet(G)*], rifampicin (*arr-2*), chloramphenicol (*cmlA5*, *floR*) and sulfonamides

Fig. 1. Schematic representation of Tn6061 in BM4492, BM4529, BM4530 (GenBank accession no. GQ388247), BM4532 and BM4533. Genetic rearrangements observed in BM4531 and BM4534 are represented (GenBank accession nos GU475047 and GU475053, respectively). Open arrows indicate coding sequences and direction of transcription. Blue, resistance genes; red, transposition module; yellow, integrase genes; green, insertion sequences; orange, ISCR elements; grey, other ORFs. Horizontal black lines delimit class 1 integrons; black bent arrows and open circles, *oriS* and *terS* sequences of ISCR elements, respectively; vertical red bar, initial IR of Tn6061 (*IRiTn*); vertical black bars, initial (*IRiInt*) and terminal (*IRtInt*) IRs of the integron; the scale bar is in kilobase pairs.

(*sulI*) (Fig. 1, Table 1). The presence of the 10 genes accounted for the high-level resistance to various unrelated antibiotic classes (Table 2). Other mechanisms, such as mutations in type II topoisomerases, diminished permeability, overexpression of efflux systems, or additional acquired genes, are likely to be associated to achieve multi-drug resistance. Tn6061 was found by PCR mapping in the six other related *P. aeruginosa* clinical isolates and entirely resequenced in BM4530. A previous study had assigned *ant(4')-IIb*, and thus Tn6061, to a chromosomal location, except in BM4534, in which it is carried by a plasmid larger than 320 kb (Sabtcheva *et al.*, 2003). The fact that Tn6061 is plasmid-borne in BM4534, in contrast to the chromosomal location in the six other strains, is consistent with mobility and dissemination of the element among *P. aeruginosa* isolates.

Tn6061 is a member of the Tn3 family of transposons. It contains at its left side transposase (*tnpA*) and resolvase (*tnpR*) genes. TnpR is identical to the resolvase of Tn1403 (Stokes *et al.*, 2007), and TnpA differed by the single Leu₉₇₉Phe substitution from the transposase of Tn1403. The two transposons shared the same organization, i.e. a 38 bp initial inverted repeat (IR) (IR_{Tn}) followed by a transposition module and a class 1 integron inserted into the *res* site. Tn6061 was thus closely related to Tn1403 but in all strains lacked the 38 bp terminal IR (IR_{Tn}). The transposon was present at different chromosomal loca-

tions, based on the hybridization of an *ant(4')-IIb* probe to a PFGE gel after *SpeI* digestion (Sabtcheva *et al.*, 2003). It could have been acquired and transferred either with its two flanking IRs and the IR_{Tn} copy lost in a second step, or by a one-ended transposition mechanism. Initial and terminal IRs are required for efficient transposition of the element, but one-ended transposition has been described with a 100-fold lower frequency for two Tn3-like transposons, Tn21 and Tn1721 (Avila *et al.*, 1984; Motsch & Schmitt, 1984). Deletion of IR_{Tn} could therefore have dramatically decreased, but not abolished, the transposition frequency of Tn6061.

All resistance genes within Tn6061 were part of a complex class 1 integron terminated by an IS6100 element, *floR*, *tet(G)* and *ant(4')-IIb* being bracketed by two integron structures (Fig. 1). This organization was similar to that of an In4-related MDR complex integron of SGI1 (Boyd *et al.*, 2001) and to part of the AbaR1 resistance island of *Acinetobacter baumannii* AYE (Fournier *et al.*, 2006), in which a *floR*–*tet(G)* region is flanked by two class 1 integrons (Fig. 2). The Tn6061 MDR region differed from that of SGI1 and AbaR1 in several aspects. (i) Content of the integrons: the first one contained six antibiotic-resistance genes preceded by an IS1999 copy that brought a strong promoter (Fig. 1) (Aubert *et al.*, 2003). This integron, already described in *P. aeruginosa* (Girlich *et al.*, 2002), is also present in AbaR1 but at another location

Table 1. Composition of transposon Tn6061

ORF	Gene	G + C (mol%)	Function
1	<i>tnpA</i>	65	Transposase (Tn1403)
2	<i>tnpR</i>	65	Resolvase (Tn1403)
3	<i>intI</i>	61	Integrase of class 1 integron
4	<i>tnpA</i>	48	Transposase (IS1999)
5	<i>bla_{VEB-1}</i>	31	Extended spectrum β -lactamase
6	<i>ant(2')-Ia</i>	58	Aminoglycoside 2'-O-nucleotidyltransferase
7	<i>arr-2</i>	46	Rifampicin ADP-ribosylating transferase
8	<i>cmlA5</i>	55	Chloramphenicol efflux pump
9	<i>bla_{OXA-10}</i>	42	Class D β -lactamase
10	<i>ant(3'')-Ia</i>	52	Aminoglycoside 3''-O-nucleotidyltransferase
11	<i>qacEA1</i>	50	Quaternary ammonium compound resistance (truncated)
12	<i>sulIIA</i>	63	Dihydropteroate synthetase (truncated)
13	<i>floR</i>	59	Florfenicol/chloramphenicol efflux pump
14	<i>tetR</i>	59	Tetracycline repressor
15	<i>tet(G)</i>	57	Tetracycline transporter
16	<i>orf1</i>	62	LysR-type transcriptional regulator
17	ISCR6 Δ	69	ISCR element (truncated)
18	<i>orfA</i>	60	Multicopper oxidase domain-containing protein
19	<i>ant(4')-IIb</i>	61	Aminoglycoside 4'-O-nucleotidyltransferase
20	ISCR6	69	ISCR element
21	<i>groEL/intI</i>	63	GroEL-like/integrase
22	<i>sulI</i>	62	Dihydropteroate synthetase
23	<i>orf5</i>	65	Putative acetyltransferase
24	<i>orf6Δ</i>	64	Hypothetical protein (truncated)
25	<i>tnpA</i>	61	Transposase (IS6100)

Table 2. Antibiotic susceptibility of *P. aeruginosa* strains

Antimicrobial agent	MIC ($\mu\text{g ml}^{-1}$) for strain:							
	PAO38	BM4492	BM4529	BM4530	BM4531	BM4532	BM4533	BM4534
Chloramphenicol	48	>256	>256	>256	>256	>256	>256	>256
Tetracycline	24	>256	>256	>256	>256	>256	>256	>256
Rifampicin	>32	>32	>32	>32	>32	>32	>32	>32
Piperacillin	4	>256	24	24	64	24	24	>256
Piperacillin/tazobactam	3	128	12	4	32	6	6	>256
Ceftazidime	3	>256	>256	>256	>256	>256	>256	>256
Imipenem	1	16	12	1.5	3	8	8	1.5
Ciprofloxacin	0.125	>32	24	8	12	8	>32	>32
Amikacin	2	>256	>256	>256	1	128	>256	>256
Gentamicin	1	>256	0.5	0.5	0.5	0.5	>256	>256
Netilmicin	2	4	0.25	0.25	0.25	0.25	4	>256
Tobramycin	1	>256	>256	>256	0.25	>256	>256	>256

(Fig. 2). The second integron of Tn6061 was truncated, since no gene cassette was inserted and *qacEΔ1*, which together with *sul1* forms the classical 3'-conserved sequence (3'-CS) of class 1 integrons, was missing (Fig. 1). The 3' portion of the *attI* site was also deleted, thus inactivating the gene capture system (Hansson *et al.*, 1997). (ii) *ant(4')-IIb* was present downstream from the putative multicopper oxidase gene *orfA*; both genes being bracketed by an ISCR6 duplication (see below). (iii) There was a loss of 170 bp that resulted in deletion of the 3' end of *orf6* and the terminal IR (IR_{Int}) of the integron. (iv) There was a deletion of the second IR_{Int} downstream from IS6100. The plasmid-borne Tn6061 of BM4534 exhibited two differences compared with the chromosomal copy in the six other strains: the second IR_{Int} at the right extremity of IS6100 was present and a larger fragment containing the end of *sul1*, *orf5* and *orf6* was absent (Figs 1 and 3). Comparison of the 3'-CSs of integrons showed that this region is less conserved than the 5'-CS (Partridge *et al.*, 2001) (Fig. 3). Conservation of a *floR-tet(G)* region in three distinct genetic elements, Tn6061, SGI1 and AbaR1, from three bacterial species, *P. aeruginosa*, *Salmonella enterica* and *A. baumannii*, respectively, suggests horizontal transfer of an MDR element followed by subsequent evolution in the new hosts (Fig. 2).

Acquisition of ISCR6 and *ant(4')-IIb*

Common regions (ISCRs) are IS91-like transposable elements frequently linked to antibiotic-resistance genes that can mobilize by rolling-circle transposition from an *oriIS* to a *terIS* sequence (Toleman *et al.*, 2006). The MDR region of SGI1 possesses an ISCR3 copy that could have been involved in the construction of the complex class 1 integron (Toleman *et al.*, 2006). In Tn6061, the *ant(4')-IIb* and *orfA* genes were bracketed by a 1660 bp sequence corresponding to a duplication of an ISCR6 element (Fig. 1). The first copy differed by 28 nt from the second one, lacked the first 101 bp, and was thus presumably no longer

active. The second intact copy shared 98 % identity with ISCR14 (Supplementary Fig. S1). Sequence analysis showed that the *terIS*, found upstream only from the second ISCR6 copy, was identical to those of ISCR3 and ISCR14 (Fig. 4a). The *oriIS* of the first copy was identical to those of ISCR3 and ISCR14, whereas the *oriIS* of the second copy was identical to those of ISCR5 and ISCR19A (Fig. 4b). ISCR6 could thus result from recombination between two ISCRs, as has been proposed for ISCR5 (Li *et al.*, 2009). ISCR6 is part of the ISCR3 group (Toleman *et al.*, 2006), of which several members have been found duplicated and flanking antibiotic-resistance genes (Li *et al.*, 2009; Naas *et al.*, 2008; Toleman & Walsh, 2008). These ISCRs are probably responsible for resistance gene mobilization by a rolling-circle mechanism followed by homologous recombination. ISCR6 could have mediated the mobilization and integration of *ant(4')-IIb* and *orfA* into Tn6061. However, only the second ISCR6 copy was probably functional, and its location at the 3' end of the element (Fig. 1) would not allow transposition of the upstream genes. The two ISCR6 could have been initially intact and a deletion could have occurred in the first copy after integration of the *ant(4')-IIb* region. Alternatively, transposition starting from the *oriIS* of the first truncated copy could have been mediated by the transposase provided by the second intact ISCR6.

P. aeruginosa BM4530 and BM4531 are indistinguishable by PFGE after *SpeI* digestion (Sabtcheva *et al.*, 2003). However, BM4531 was susceptible to amikacin, whereas BM4530 was highly resistant (Table 2), and sequence analysis indicated that BM4531 did not possess the *ant(4')-IIb* and *orfA* genes and had a single copy of ISCR3 (Fig. 1) with *oriIS* and *terIS* sequences identical to those of ISCR3 (Fig. 4). BM4531 thus contains a sequence identical to that of the MDR region of SGI-1. These data suggest mobilization of *ant(4')-IIb* and *orfA* by ISCR6 and integration into Tn6061 by homologous recombination with ISCR3. The origin of these genes, which have been found only in *P. aeruginosa*, remains unknown, but their mol% G + C of 60 % is compatible with that of this species

Fig. 2. Schematic representation of Tn6061 in comparison with SGI1 (GenBank accession no. AF261825) and AbaR1 (GenBank accession no. CT025832). Open arrows indicate coding sequences and direction of transcription. Genes are coloured as in Fig. 1. Pairs of homologous ORFs between two strains and graphical representation were generated by BLAST analysis and the Artemis Comparison Tool (Carver *et al.*, 2005), respectively. Shaded areas between the genetic elements indicate homology ($\geq 98\%$ identity).

Fig. 3. Comparison of the backbones of chromosomally located (BM4492, BM4530 and related strains) or plasmid-borne (BM4534) class 1 integrons of Tn6061 with other *Int1*-like integrons. Genes are shown as arrows and IS6100 as an open box. Vertical bars, initial (IR_{iInt}) and terminal (IR_{tInt}) IRs; open squares, *attI* site; 5'-CS and 3'-CS regions are delimited by thin lines.

Fig. 4. (a) Alignment of the 5' sequences of ISCR elements displaying the highest identity with ISCR6. Nucleotides identical to those in ISCR6 are shown on a grey background. Sequences were collected from the following GenBank accession numbers: AF261825 (ISCR3), AM849110 (ISCR5), DQ914960 (ISCR14), DQ517526 (ISCR16) and EU503121 (ISCR19A and B). Asterisks, GTG start codons; inverted arrows, *terIS* sequence composed of a 4 bp IR. (b) Alignment of the 3'-terminal sequences of ISCR elements displaying the highest identity with ISCR6. Asterisks, stop codons; box, *oriIS* sequence.

(Table 1). One can hypothesize that the MDR region of SGI-1 was transferred to *P. aeruginosa*, integrated in a transposon and further evolved, notably by ISCR6-mediated acquisition of *ant(4')-IIb* and *orfA*.

Insertion sites of Tn6061

The genomic context of Tn6061 was examined in the seven isolates to look for evidence of horizontal transfer. Insertion sites of the transposon were determined by sequencing TAIL-PCR products. (i) In strain BM4492, the *tnpA* end of Tn6061, which is preceded by a 995 bp sequence that did not exhibit similarity to sequences in the databases, was inserted into PSPA7_6052, an ORF of unknown function which is part of a putative 39 kb genomic island in *P. aeruginosa* PA7 (GenBank accession no. CP000744). The IS6100 end of Tn6061 was inserted upstream from the *opdC* gene (PA0162 in PAO1), which encodes a porin of the OprD family involved in histidine uptake (Tamber *et al.*, 2006). Insertion of Tn6061 did not alter transcription of *opdC*, as demonstrated by comparing the level of *opdC* expression in BM4492 and PAO38 by quantitative RT-PCR, using the *rpsL* gene to normalize the data (data not shown). (ii) In BM4530 and related strains BM4529, BM4531, BM4532 and BM4533 (hereafter called BM4530 and related strains), the *tnpA* side of Tn6061 was inserted into PSPA7_6052 at the same position as in BM4492. The IS6100 side was inserted into *oprE* (PA0291 in PAO1) which encodes a porin of the OpdK subfamily. As in the strains studied, *oprE* has been shown to be located close to genes related to arginine and proline metabolism, but a role for OprE in the uptake of these amino acids has not been demonstrated (Tamber *et al.*, 2006). A *P. aeruginosa* mutant with inactivated *oprE* shows chromate susceptibility, suggesting that OprE could participate in metal efflux (Rivera *et al.*, 2008). BM4530 and related strains had increased susceptibility to chromate compared with PAO38 and BM4534, which possess an intact *oprE* (data not shown). (iii) In strain BM4534, Tn6061 was integrated into the plasmid-borne Tn4661, a 12.7 kb transposon which encodes putative metabolic functions and has been previously described in *P. aeruginosa* as part of the genomic island PAGI-4(C) (Klockgether *et al.*, 2004) or carried by a plasmid (GenBank accession no. AB375440). Tn4661 was not present in the six other *P. aeruginosa* isolates studied.

Large chromosomal inversions mediated by duplication-insertion of IS6100

Southern hybridization using an IS6100 internal fragment as a probe showed that IS6100 was present in three copies in BM4530 and related strains and in four copies in BM4492 (Supplementary Fig. S2). One IS6100 copy, ending transposon Tn6061, and its insertion sites have been described above. The insertion site of the remaining IS6100 copies was determined by sequencing TAIL-PCR products. A second copy was inserted in the six strains between the 3' end of *pfpI* (PA0355 in PAO1) and a

truncated ORF, PSPA7_6040 (Fig. 5b). The latter encodes a conserved hypothetical protein and was part of the 39 kb putative genomic island in which Tn6061 was inserted on its *tnpA* side. The PfpI protein, a member of the DJ-1/ThiJ/PfpI superfamily that includes chaperones and peptidases, has been characterized in *P. aeruginosa* as an antimutator factor, providing general stress protection (Rodríguez-Rojas & Blázquez, 2009). A *pfpI*-inactivated variant exhibits a low increase in the mutation frequency, is more sensitive to different stresses such as NaCl, UV radiation and heat, and is deficient in biofilm formation, when compared with its isogenic parental strain (Rodríguez-Rojas & Blázquez, 2009). In clinical isolates BM4492 and BM4530, the small increase in mutation frequency and susceptibility to stress, when compared with PAO38 and BM4534, was not observed. In contrast, BM4492, BM4530 and related strains showed a clear defect in biofilm formation, probably conferred by *pfpI* disruption (data not shown).

Another IS6100 copy separated the 5' end of truncated *oprE* from the 5' end of the *pfpI* gene in the six strains (Fig. 5c). In BM4492, a fourth IS6100 copy was inserted between the 3' end of *oprE* and the 5' end of *opdC* (Fig. 5d). BM4492 exhibited, in common with BM4530 and related strains, a phenotype of chromate susceptibility associated with *oprE* disruption. The bringing together by IS6100 of *P. aeruginosa* chromosomal sequences that are not adjacent in the already sequenced strains is in favour of IS6100-mediated large chromosomal inversions. Transposition of IS6100 is associated with duplication of target DNA at the site of insertion, leading to 8 bp directly repeated sequences at the ends of the element (Chandler & Mahillon, 2002). Analysis of the flanking sequences of every copy indicated that IS6100 had successively inserted into the *pfpI* and *oprE* genes, and sequences upstream from *opdC* (Fig. 5). Due to inversion of the large fragment flanked by two IS6100, the two parts of the target gene are flanked by different copies, themselves bracketed by two different 8 bp sequences (Fig. 5). During IS6100 transposition, duplication and inversion are coupled events (Chandler & Mahillon, 2002). The transposase of IS6100 allows concerted transfer of both ends of the insertion sequence to the target site (Fig. 6a). Resolution of the resulting cointegrate structure leads to duplication of IS6100 in the opposite orientation and concomitant inversion of the chromosomal sequence surrounded by the two copies of IS6100 (Fig. 6c). A model for the locations of Tn6061 and IS6100 in BM4492 and BM4530 and related strains, based on the PCR results and analysis of the 8 bp flanking the IS6100 copies, suggests that Tn6061 first inserted in the 39 kb putative chromosomal island (Fig. 5a). This was followed by insertion of a duplicated copy of IS6100 in the *pfpI* gene, mediating the first chromosomal inversion of approximately 5.6 Mb between *pfpI* and PSPA7_6040 (Fig. 5b). Following a second duplication, another IS6100 copy inserted in the *oprE* gene, leading to the sequence found in BM4530 and related strains, by inversion of approximately 5.7 Mb

Fig. 5. Model for large chromosomal inversions. (a) Insertion of *Tn6061* in the putative 39 kb genomic island. (b) Insertion-duplication of *IS6100* in *pfpI* and chromosomal inversion between the two *IS6100* copies. (c) Second *IS6100*-mediated large chromosomal inversion after duplication and insertion of *IS6100* in *oprE*. This genome portion corresponds to that of BM4530 and related strains. (d) Third chromosomal inversion due to duplication of *IS6100* and insertion upstream from *opdC* in BM4492. Thin line, chromosome; box, *Tn6061*; arrows, coding sequences and direction of transcription; lozenge, *IS6100*, the black portion corresponding to the 3' end. Open circles, triangles and squares represent the 8 bp directly repeated sequences in *pfpI*, *oprE* and upstream of *opdC*, respectively; vertical lines delineate the borders of the large chromosomal inversions.

Fig. 6. Proposed mechanism for the large chromosomal inversion mediated by duplication-insertion of IS6100 (adapted from Badia *et al.*, 1998). (a) In a chromosome carrying the a-b-c-d sequence, the transposase encoded by IS6100 (box containing an arrow) catalyses the cleavage at the 3' ends of the IS. (b) Free 3' OH groups attack a 5' phosphate in the target DNA, leading to an intermediate in which IS6100 is covalently linked to the four chromosomal regions. (c) Replication resolves the cointegrate structure, resulting in the duplication in the opposite direction of IS6100 and in a chromosomal inversion that leads to an a-c-b-d sequence.

between *oprE* and the 5' part of *pfpI* (Fig. 5c). An alternative sequence of events, in which the order of the first two insertion-duplications is inverted, would also lead to the sequence found in BM4530 and related strains. BM4492 underwent a third chromosomal inversion of approximately 5.8 Mb by duplication-insertion of an IS6100 copy upstream from *opdC* (Fig. 5d). An IS6100-mediated chromosomal inversion has been described in SGI1-E, a variant of SGI1 in which *floR* is disrupted by a duplicated IS6100 (Boyd *et al.*, 2002). Large chromosomal inversions are also associated with IS6100 duplication in *P. aeruginosa* clinical strains isolated from CF patients (Kresse *et al.*, 2003). These events, by disrupting genes, have been shown to be involved in phenotypic adaptation of the strains to their particular environment (Kresse *et al.*, 2003).

Bacteria adapt by modification, loss or acquisition of functions. The ability of *P. aeruginosa* to survive in various environments and to switch from a commensal to a pathogenic lifestyle involves an intrinsic adaptability that is further

increased by genome plasticity. The acquisition of Tn6061 and large chromosomal inversions reported in this study are further examples of intricate mechanisms of genome evolution that allow *P. aeruginosa* to adjust to its environment.

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