ORIGINAL ARTICLE

Genetic analysis of a multiresistant strain of *Pseudomonas aeruginosa* producing PER-1 β-lactamase

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ABSTRACT

A multiresistant strain of *Pseudomonas aeruginosa*, PA2345, belonging to serotype O:1, was isolated at the Teaching Hospital of Besançon, France. Resistance to β-lactams, including third-generation cephalosporins, depended upon a chromosomally-located composite transposon carrying the bla_{PER-1} gene encoding extended-spectrum β-lactamase PER-1. PA2345 was unrelated genotypically to two previous PER-1-producing isolates of *P. aeruginosa*. Sequence analysis of the transposon in PA2345 revealed the presence of two insertion sequences (IS*Pa23* and IS*Pa24*) with very different predicted transposases (TnpA1, TnpA2), which were both bordered by closely related 16-bp inverted repeats. High resistance of PA2345 to aminoglycosides was caused, in part, by a chromosomal class-I integron containing gene cassettes *aadB*, encoding an ANT(2″) enzyme, and *aadA11*, encoding a new ANT(3″) enzyme with 281 amino-acids that conferred elevated resistance to streptomycin and spectinomycin. Stable overproduction of efflux system MexXY contributed to resistance to amikacin, while mutations in the quinolone resistance-determining regions of *gyrA* and *parC* accounted for the high resistance of PA2345 to fluoroquinolones. The study indicates that multidrug resistance in *P. aeruginosa* might arise from sequential acquisition of a variety of mechanisms provided by both horizontal gene transfers and mutations in chromosomal genes.

Keywords Composite transposon, extended-spectrum β-lactamase, multiresistance, PER-1, *Pseudomonas aeruginosa*, resistance

INTRODUCTION

Acquisition of multidrug resistance by *Pseudomonas aeruginosa* is becoming a matter of concern in many hospitals worldwide [1,2]. The mechanisms leading to the emergence of strains resistant to almost all the anti-pseudomonal antibiotics available for parenteral administration are often multiple and complex. Thus, resistance may evolve through the transfer of foreign genes from other Gram-negative species, or through the development of intrinsic resistance mechanisms (e.g., up-regulation of efflux systems and AmpC β-lactamase, target alterations, loss of porins) subsequent to spontaneous mutations [3].

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In this context, integrons and transposons have been detected repeatedly in multidrug resistant *P. aeruginosa* isolates [4,5]. Integrons are genetic elements encoding the components of a site-specific recombination system that recognises and captures mobile gene cassettes, mostly resistance determinants [6]. Such elements may be located within transposons, which in turn contribute actively to the dissemination of resistance determinants to aminoglycosides and β-lactams among Gram-negative species [7]. Moreover, it has been demonstrated that integrons and transposons are associated with the spread of resistance to third-generation cephalosporins when they encode extended-spectrum β-lactamases (ESBLs) such as VEB-1 [8], GES-1 [9] or TEM-21 [4].

Pagani *et al.* [10] reported that bla_{PER-1}, the gene encoding ESBL PER-1, was situated close to the gene of a putative transposase in epidemic strains of *P. aeruginosa*. PER-1, which is responsible for

high-level resistance to ceftazidime, has been found in *Acinetobacter* spp. and *P. aeruginosa* isolates from Turkey [11,12], Belgium [13], Italy [10,14] and France [15]. However, the genetic structures bearing *bla*_{PER-1} in all these bacteria have not been elucidated completely. The present study describes the mechanisms responsible for multidrug resistance in a strain of *P. aeruginosa* isolated at the Teaching Hospital of Besançon, France.

MATERIALS AND METHODS

Bacterial strains and plasmids

P. aeruginosa PA2345, belonging to serotype O:1, was isolated in October 2001 from the sputum of a male aged 52 years who was hospitalised in the medical intensive care unit of the Teaching Hospital of Besançon, France. Two other PER-1 producing strains of *P. aeruginosa* were used for genotyping comparison, namely RNL-1, isolated in France in 1991 [16], and PABG, isolated in Belgium in 1998 [13]. The wild-type strain PAO1, which has been sequenced completely [17], and its MexXY-overproducing mutant MutGr1 [18], served as controls for quantification of *mexX* expression by quantitative real-time RT-PCR. DNA cloning experiments were performed in *Escherichia coli* DH5α with plasmid vectors pUC18 [19] (coding for ampicillin resistance, Ap^r) and pK18 [20] (a pUC18 derivative coding for kanamycin resistance, Km^r). In Southern blot experiments, plasmid pSTI1 (81 MDa) [21] was used as a positive control for determining the plasmid location of *bla*_{PER-1}.

Determination of drug susceptibilities

Production of ESBL by *P. aeruginosa* PA2345 was detected initially with the double-disk synergy test (typical enlargement of the inhibition zone between disks of ceftazidime and ticarcillin-clavulanic acid), performed according to the guidelines of the French Society for Microbiology [22]. MICs were determined by the microdilution method in Mueller-Hinton broth (MHB) with adjusted concentrations of Ca^{2+} and Mg^{2+} (BBL, Cockeysville, MD, USA) and an inoculum of 2.5×10^5 CFU/mL [23]. Tobramycin was provided by Eli Lilly (Indianapolis, IN, USA), and amikacin by Bristol-Meyers Squibb (Paris, France); all other antibiotics were obtained from Sigma (Saint-Quentin Fallavier, France).

Analytical isoelectric focusing

After culture in Tryptone Soya Broth (Oxoid, Basingstoke, UK), supplemented with yeast extract 5 g/L and glucose 10 g/L, bacteria were disrupted by sonication. Unbroken cells and cell debris were removed by centrifugation. Isoelectric focusing was performed on polyacrylamide gels (acrylamide 7% w/v, bis-acrylamide 0.2% w/v) containing ampholines with a pH range of 3.5–10 (Pharmacia Diagnostics, Saint Quentin Yvelines, France). Migration was performed at 500 V overnight in a LKB 2117 Multiphor apparatus (Pharmacia Diagnostics, Uppsala, Sweden). The β-lactamase bands were

visualised in the gels by the iodine starch procedure [24]. β-Lactamases of known pI (TEM-1, pI 5.4; TEM-2, pI 5.6; TEM-3, pI 6.3; TEM-24, pI 6.5; SHV-1, pI 7.6; SHV-4, pI 7.8; CTX-M-1, pI 8.4) were used for gel calibration.

Analysis of genomic DNA by pulsed-field gel electrophoresis (PFGE)

Genomic DNA was digested with *Xba*I and analysed by PFGE for 20 h at 180 V with a pulse ramping from 15 to 5 s using a CHEF-DR2 apparatus (Bio-Rad, Marnes la Coquette, France) as described previously [25]. PFGE patterns were interpreted according to the criteria of Tenover *et al.* [26].

DNA isolation, amplification and sequencing

DNA from strain PA2345 was extracted as described by Sambrook *et al.* [27]. Plasmids were prepared using a Plasmid Midi kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. Purified DNA samples were digested with *Bam*HI, *Sac*I or *Hin*dIII (Roche, Meylan, France), and the resulting fragments were separated by electrophoresis in agarose 0.8% w/v gels [27].

The *gyrA*, *gyrB*, *parE* and *parC* quinolone resistance-determining regions (QRDRs) of *P. aeruginosa*, as well as the coding sequence of the *mexZ* repressor gene and the *mexZ-mexXY* intergenic region, were amplified by PCR with the primers listed in Table 1. Cloned fragments and PCR products were sequenced on both strands by Genome Express (Roche) on a capillary autosequencer (Applied Biosystems, Foster City, CA, USA). GenBank searches were performed using the BLASTN Align algorithm (http://www.ncbi.nlm.nih.gov). Deduced protein sequences were edited with BLASTP (http://www.ncbi.nlm.nih.gov) and aligned with top hits by using Clustal W v.1.8 [28].

Southern hybridisation experiments

A digoxigenin-labelled probe for the detection of *bla*_{PER-1} was obtained by adding DIG-11-dUTP to the PCR mix with the Per-1/Per-2 primers (Table 1) as recommended by the manufacturer (Roche). DNA was extracted from bacteria by the method of Kieser [29] and subjected to electrophoresis in a horizontal agarose 0.8% w/v gel, calibrated with reference plasmids from *E. coli* V517 [30]. The DNA was then capillary blotted [27] to a Hybond-N+ nylon membrane (Amersham, Orsay, France). Southern blot hybridisation was performed under highly stringent conditions, with a DIG DNA detection kit (Roche) used for colourimetric detection of *bla*_{PER-1} on the membrane.

Cloning of the integron

A *Sac*I genomic library of strain PA2345 was constructed in *E. coli* DH5α with the plasmid vector pK18 (Kan^r). The resulting transformants were screened for aminoglycoside resistance on Mueller-Hinton agar (MHA; Bio-Rad, Ivry-sur-Seine, France) containing gentamicin 4 mg/L. Two gentamicin-resistant clones were selected for further analysis, both of which were found to contain the same recombinant plasmid, named pAD1, containing a 5.8-kb *Sac*I fragment. The *aadA11* gene was subcloned into vector pUC18, previously cleaved

Table 1. Primers used for DNA amplification

Primers	5′ → 3′ nucleotide sequences	Annealing T°C	Reference
Detection of mutations in the quinolone resistance-determining regions			
GyrA1	TTATGCCATGAGCGAGCTGGGCAACGACT	57°C	[56]
GyrA2	AACCGTTGACCAGCAGGTTGGGAATCTT	57°C	[56]
GyrB1	GCGCGTGAGATGACCCGCCGT	65°C	[57]
GyrB2	CTGGCGGTAGAAGAAGGTCAG	65°C	[57]
ParC1	ATGAGCGAACTGGGGCTGGAT	57°C	[32]
ParC2	ATGGCGGCGAAGGACTTGGGA	57°C	[32]
ParE1	CGGCGTTCGTCTCGGGCGTGGTGAAGGA	55°C	[58]
ParE2	TCGAGGGCGTAGTAGATGTCCTTGCCGA	55°C	[58]
Detection of mutations in mexZ and the mexZ-mexXY intergenic region			
SeqZ1	GCAGCCCAGCAGGAATAG	61°C	This study
SeqZ2	GCCTGTCGGTGCTCTACATC	61°C	This study
Detection of the integron			
L1	GGCATCCAAGCAGCAAG	52°C	[43]
R1	AAGCAGACTTGACCTGA	52°C	[43]
Subcloning of aadA11			
SubE1	GGG**GGATCC**GGCTTACCTTGGCCG (*Bam*HI)	52°C	This study
SubE2	GGG**AAGCTTT**GTGCTTAGTGCATC (*Hin*dIII)	52°C	This study
Amplification of bla$_{PER-1}$			
Per-1	ATGAATGTCATTATAAAAGC	52°C	[11]
Per-2	AATTTGGGCTTAGGGCAGAA	52°C	[11]
Perm-2	GCGATAACGTGGCCTGTG (712–729 in *bla*$_{PER-1}$)	52°C	This study
39 BP	CCACTGAACCTAATAACTGC (1311–1330 in *bla*$_{PER-1}$)	52°C	This study
Detection of tnpA1			
Per-324	AAAACGGTCTGACGACCATC	60°C	This study
Per-1304	TGTTTAAGGGGCAACCTGAC	60°C	This study
Detection of tnpA2			
Per-190	GTAGCGGCTGACAGAGTCCT	60°C	This study
Per-1085	TCAGTAGCGTCGAGGCAGTA	60°C	This study
Degenerate primers			
CEKG2A	GGCCACGCGTCGACTAGTACNNNNNNNNNNAGAG	Variable	[31]
CEKG2B	GGCCACGCGTCGACTAGTACNNNNNNNNNNACGCC	Variable	[31]
CEKG2C	GGCCACGCGTCGACTAGTACNNNNNNNNNNGATAT	Variable	[31]
CEKG4	GGCCACGCGTCGACTAGTAC	65°C	[31]

with *Bam*HI and *Hin*dIII, by ligating a 914-bp PCR product generated with primers SubE1 (which created a *Bam*HI restriction site) and SubE2 (which created a *Hin*dIII site) designed from the sequence of the pAD1 insert. The DH5α clones containing the new construct, designated pADD, were selected on MHA containing streptomycin 32 mg/L.

Characterisation of the transposon carrying *bla*$_{PER-1}$

Sequencing of the entire transposon in strain PA2345 was achieved by a genome walking strategy with a two-step semi-degenerate PCR, essentially as described by Jacobs *et al.* [31]. Partially overlapping DNA sequences of *c*. 200–400 bp, upstream of and downstream of the *bla*$_{PER-1}$ gene, were amplified with a set of specific primers anchored in the sequences surrounding *bla*$_{PER-1}$, and with semi-degenerate primers with a defined tail (Table 1). In the first round of PCR, amplifications were performed in a final volume of 20 μL with *c*. 100 ng template DNA, 0.4 U Red*Taq* DNA polymerase (Sigma), 0.2 mM dNTPs, 2.5 mM MgCl$_2$, 0.5 μM of a specific primer designed to anneal to the 5′- or 3′-end of *bla*$_{PER-1}$, and 0.34 μM each of the semi-degenerate primers CEKG2A, CEKG2B and CEKG2C (Table 1). Amplification involved 5 min at 94°C, six cycles of 30 s at 94°C, 40 s at 40°C (1°C decreasing at each cycle) and 3 min at 72°C, 25 cycles of 30 s at 94°C, 40 s at 60°C and 3 min at 72°C, and 7 min at 72°C. Each first-round amplicon (1 μL) was used to prime a second-round PCR under the same conditions with 0.5 μM of primer CEKG4 (Table 1), complementary to the tail sequence shared by the three random primers, and 0.5 μM of a nested primer overlapping the end of the anchored primer. PCR conditions comprised 5 min at 94°C, 30 cycles of 30 s at 94°C, 40 s at 60°C

and 2 min at 72°C, and 7 min at 72°C. The amplicons were separated by electrophoresis in an agarose 1% w/v gel, and then extracted (QIAquick Gel Extraction Kit; Qiagen) and sequenced. New specific primers were then designed (data not shown) for subsequent PCR amplifications in order to walk the chromosome.

Quantitative real-time PCR

Quantitative data for *mexX* transcripts were obtained in triplicate and normalised to *rpsL* gene expression as an internal control, as described previously [32]. The expression levels of *mexX* in strain PA2345 were then divided by those found in wild-type strain PAO1 in order to yield relative results.

RESULTS AND DISCUSSION

A new ESBL-producing strain of *P. aeruginosa*

Strain PA2345 was detected initially because of its high resistance to many anti-pseudomonal antibiotics, especially ceftazidime (Table 2). Production of an ESBL by this isolate was first suspected from a standard antibiogram, and was then confirmed with a *bla*$_{PER-1}$-specific PCR, in accordance with the finding of several PER-1-producing *P. aeruginosa* isolates in Europe [10,13–15] and Turkey [11,33] during the past decade. A single

Table 2. Susceptibilities to antimicrobial agents

Strains	MICs (mg/L)													
	TIC	AZT	IMP	CAZ	CAZ + CLA	FEP	KAN	AMK	GEN	TOB	NET	STR	SPT	CIP
Pseudomonas aeruginosa														
PAO1	16	4	1	2	nd	2	128	4	2	1	2	16	512	0.12
PA2345	512	512	0.5	512	8	64	1024	64	> 1024	512	32	> 1024	> 1024	8
RNL-1	512	512	0.5	128	4	256	1024	64	1024	256	> 1024	> 1024	512	4
PABG	256	256	8	256	4	256	512	32	256	128	> 1024	> 1024	> 1024	32
Escherichia coli														
DH5α(pUC18)	nd	nd	nd	nd	nd	2	4	1	0.5	0.25	0.06	2	8	0.06
DH5α(pK18)	nd	nd	nd	nd	nd	2	512	1	0.5	0.25	0.06	2	8	0.06
DH5α(pAD1)	nd	nd	nd	nd	nd	1	512	1	1024	8	0.25	> 1024	1024	0.06
DH5α(pADD)	nd	nd	nd	nd	nd	1	4	1	0.5	1	0.12	1024	1024	0.06

TIC, ticarcillin; AZT, aztreonam; IMP, imipenem; CAZ, ceftazidime; CAZ + CLA, ceftazidime + clavulanic acid 2 mg/L; FEP, cefepime; KAN, kanamycin; AMK, amikacin; GEN, gentamicin; TOB, tobramycin; NET, netilmicin; STR, streptomycin; SPT, spectinomycin; CIP, ciprofloxacin; nd, not determined.

925-bp amplicon was obtained, with a sequence that was 100% identical with those generated from two other bla_{PER-1}-carrying *P. aeruginosa* isolates from France in 1991 (strain RNL-1, serotype O:2) [16] and Belgium in 1998 (strain PABG, serotype O:12) [13]. Genotyping analysis of the three strains by PFGE showed very distinct profiles, which indicates that this ESBL gene may spread among isolates of different geographical origins. In contrast to other PER-1-producing *P. aeruginosa* strains involved in hospital outbreaks in Italy [10], Turkey [11] and Belgium [13], strain PA2345 did not disseminate and remained confined to a single patient in this institution.

The isoelectric point for this enzyme (pI 5.2) was close to that reported by Nordmann *et al.* [16] (pI 5.4). In addition, strain PA2345 produced basal levels of chromosomally-encoded AmpC β-lactamase (pI 8.2).

Characterisation of the genetic element carrying bla_{PER-1} in PA2345

Unlike the PER-1-producing strains of *Salmonella* Typhimurium described by Vahaboglu *et al.* [21] and Casin *et al.* [34], PA2345 did not appear to harbour a plasmid after DNA extraction, and failed to transfer ceftazidime resistance by conjugation to other bacterial hosts (results not shown). Consistent with these findings, Southern blot experiments with a specific bla_{PER-1} probe revealed that the gene was located on the chromosome of this strain (Fig. 1), as also reported previously for *P. aeruginosa* RNL-1 [16], PABG [13] and a variety of epidemic strains isolated in Italy [10].

In order to learn more about the genetic environment of the bla_{PER-1} gene, the sequence of the flanking region in PA2345 was established
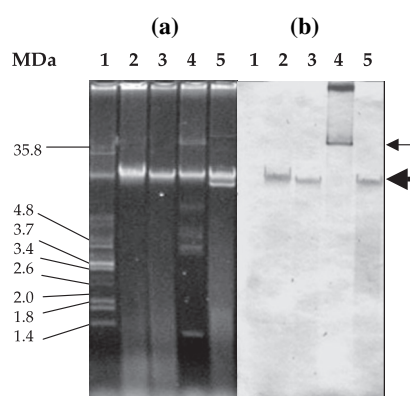

Fig. 1. Detection of the bla_{PER-1} gene by Southern hybridisation. (a) Agarose gel electrophoresis of DNA. (b) Colourimetric detection following Southern blotting of the bla_{PER-1} gene on plasmid pSTI1 (small arrow) and on the *Pseudomonas aeruginosa* chromosome (large arrow). Lanes: 1, *Escherichia coli* V517 used for plasmid size calibration; 2, $bla_{PER-1}{}^{+}$ *P. aeruginosa* strain PA2345; 3, $bla_{PER-1}{}^{+}$ *P. aeruginosa* strain PABG; 4, *Salmonella* Typhimurium (pSTI1) used as a positive control for the plasmid location of bla_{PER-1}; 5, $bla_{PER-1}{}^{+}$ *P. aeruginosa* strain RNL-1.

using a PCR chromosome walking strategy. The sequence of the 4872-bp genetic element found in this strain contained the 1899-bp fragment described by Pagani *et al.* [10] (Fig. 2a)*.* Database searches revealed that the ORF (1266 bp) located upstream of the bla_{PER-1} gene codes for a putative product of 421 amino-acids that has 37% amino-acid homology with the transposase of an insertion sequence (IS) element from *Aeromonas punctata* (accession number YP067861) [35]. This ORF was arbitrarily named *tnpA1*. The product of the second ORF, designated *tnpA2* (1272 bp), which is transcribed downstream of bla_{PER-1} in the same direction as *tnpA1*, was found to have 37% identity with the transposase of IS*SB1* from the marine psychotrophic bacterium Mst37 (accession
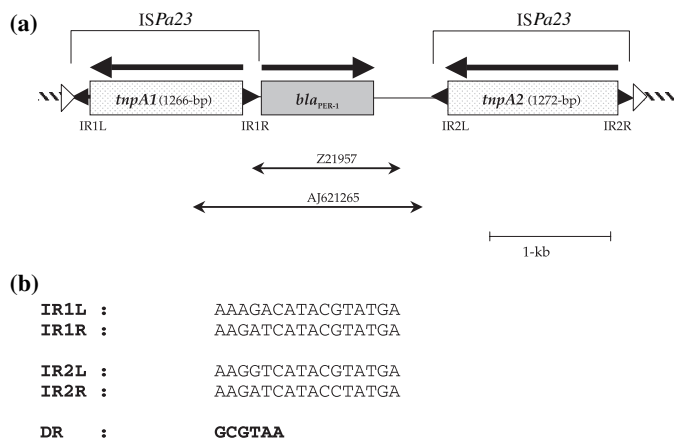
Fig. 2. (a) Schematic representation of composite transposon Tn*2345* carrying *bla*PER-1, based on DNA sequences published by Nordmann *et al.* [55] (RNL-1, EMBL/GenBank database entry Z21957) and Pagani *et al.* [10] (EMBL/GenBank database entry AJ621265). The large solid arrows indicate the direction of transcription. Small solid arrow heads indicate the inverted repeats (IR) flanking ISPa23 and ISPa24. Open arrow heads indicate short 6-bp direct repeat (DR) sequences generated in the target DNA as a consequence of insertion. The whole structure of Tn*2345* is inserted into a copy of IS*1012* (represented by striped dashes). (b) Alignment of the nucleotide sequences of the 16-bp IR and the sequence of the 6-bp DR.

number CAC84124.1). Both *tnpA1* and *tnpA2*, which are bordered by almost identical 16-bp terminal inverted repeats (IR) (Fig. 2b), display the typical structure of IS elements. They have been called ISPa23 and ISPa24 (http://www-is.biotoul.fr/). Moreover, the presence of two 6-bp directly repeated sequences (DRs) flanking IR1L and IR2R (Figs 2a and b), generated in the target DNA as a consequence of transposon insertion, strongly evokes the genetic organisation of composite transposons.

Several composite transposons are known to disseminate resistance to aminoglycosides (Tn*4001*, Tn*5*) [36], chloramphenicol (Tn*9*) [37], tetracycline (Tn*10*) [38] and β-lactams [39]; their IS modules contain transposase genes coding for either strictly identical, functional enzymes (Tn*9*) [40], or closely related enzymes, one of which is functional and the other inactive (Tn*10*, Tn*5*) [41,42]. Interestingly, TnpA1 and TnpA2 demonstrated limited amino-acid sequence homology (45.1% overall identity; 57% overall similarity). To our knowledge, this is the first report of a composite transposon with such dissimilar putative transposases. This new transposon was named Tn*2345*. Amplification of PA2345, RNL-1 and PABG genomic DNA with specific primers targeting *tnpA1* and *tnpA2* (Table 1) generated PCR products of the same length, suggesting very similar, if not identical, structures. Sequencing of the DNA regions upstream and downstream of Tn*2345* revealed that this element was inserted into *insAB* (Fig. 2A), inactivating the gene coding for the putative transposase of IS*1012* (GenBank accession number CAD31055).

Class I integron

The high-level resistance of strain PA2345 to all clinically-significant aminoglycosides (Table 2) suggested the production of one or more aminoglycoside-modifying enzyme(s). In order to characterise the genetic determinants of this resistance, a 5.8-kb *Sac*I-*Sac*I genomic fragment from PA2345 was cloned into *E. coli* DH5α. The recombinant plasmid pAD1 conferred resistance to kanamycin, gentamicin and tobramycin, but not to amikacin and netilmicin. Sequencing of the pAD1 insert indicated the presence of a class I integron exhibiting a 5′-conserved segment (5′-CS) carrying the integrase gene *intI1*, and a 3′-CS located downstream of three integrated gene cassettes, namely *aadB*, *orfE* and *aadA11* (Fig. 3a). The 3′-CS itself contained a typical set of three genes, namely *qacE*Δ1, *sul1* and *orf5* [43].

The first gene cassette of the integron, *aadB*, showed a sequence 100% identical to that of the prototype [44] (GenBank accession number L06418), codes for aminoglycoside adenylyltransferase AAD(2″), also designated ANT(2″)-I, and confers high-level resistance to kanamycin, gentamicin and tobramycin. This gene has already been reported to occur in a *P. aeruginosa* strain as a cassette carried by a class I integron,
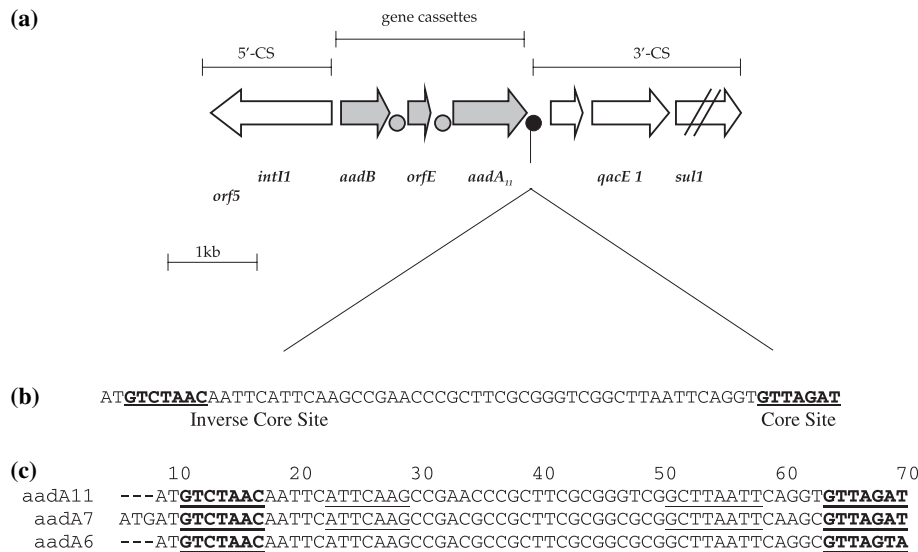
(a)

(b)
ATGTCTAACAATTCATTCAAGCCGAACCCGCTTCGCGGGTCGGCTTAATTCAGGTGTTAGAT
Inverse Core Site   Core Site

(c)

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                 10        20        30        40        50        60        70
aadA11   ---ATGTCTAACAATTCATTCAAGCCGAACCCGCTTCGCGGGTCGGCTTAATTCAGGTGTTAGAT
aadA7    ATGATGTCTAACAATTCATTCAAGCCGACGCCGCTTCGCGGCGCGGCTTAATTCAAGCGTTAGAT
aadA6    ---ATGTCTAACAATTCATTCAAGCCGACGCCGCTTCGCGGCGCGGCTTAATTCAGGCGTTAGTA
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Fig. 3. (a) Schematic representation of the integron carried by PA2345. The 5′-conserved segment (5′-CS) contains the integrase gene *intI1*, while the 3′-CS contains the genes *qacE*Δ1, *sul1* and *orf5* that are usually encountered in class I integrons. The gene cassettes are *aadB*, *orfE* and *aadA11*. The grey circles indicate the *attC* sites, and the black circle indicates the *attC* site associated with *aadA11*. (b) Sequence of the composite 59-base element (*attC*) associated with *aadA11*. The inverse core site and the core site sequences are underlined. (c) Alignment of the 59-base element associated with *aad* genes closely related to *aadA11*. The underlined sequences represent the four conserved regions [49]. GenBank accession numbers are AF224733 for *aadA7* and AF140629 for *aadA6*.

along with an ESBL-determining gene, *bla*VEB-1 [45]. In strain PA2345, no *bla* gene was found in the vicinity of *aadB*.

Downstream of *aadB*, an 'orfE-like' gene was identified that was 90% homologous to various *orfE* genes reported previously [46]. This 'orfE-like' gene flanks an ORF of 846 bp that encodes a novel aminoglycoside resistance gene, named *aadA11*. The *aadA11* sequence showed eight and two nucleotide differences compared with *aadA6* and the first 804-bp of gene fusion *ant(3′′)Ii-aac(6′)-Iid*, respectively [47,48]. Sequence analysis of the *aadA11* flanking regions identified elements typical of gene cassettes. Indeed, immediately adjacent to the gene is an integrase-specific recombination site, *attC*, belonging to the family of '59-base elements' [49], with an inverse core site (GTCTAAC) identical to those flanking *aadA6* and *aadA7*, and a recombinational core site (GTTAGAT) identical to that of *aadA7* (Figs 3b,c).

AadA11 activity was investigated in *E. coli* DH5α transformed with recombinant plasmid pADD, in which the *aadA11* gene was sub-cloned. The recombinant clones showed high resistance to streptomycin and spectinomycin (MICs 1024 mg/L; Table 2), confirming that AadA11 has the same substrate specificity as other AadA enzymes characterised to date, which are referred to as ANT(3′′) [50].

Aminoglycoside efflux and quinolone resistance in PA2345

Products of the *aadB* and *aadA11* genes do not confer resistance to amikacin and cannot account for the reduced susceptibility of PA2345 to this agent [7,44]. As stable overproduction of the MexXY-OprM efflux system may impair the susceptibility of *P. aeruginosa* to virtually all aminoglycosides, including amikacin [51,52], the expression levels of *mexX* were measured by quantitative real-time PCR. PA2345 expressed *mexX* 34-fold more than PAO1, and two-fold more than MutGr1, a MexXY-overproducing mutant derived from reference strain PAO1, which is altered in repressor gene *mexZ* [18]. Sequence analysis of *mexZ* revealed only silent mutations, suggesting that PA2345 is an *agrW* mutant defective in an unknown regulator gene [53]. Non-cystic fibrosis strains of *P. aeruginosa* that overproduce MexXY constitutively and are highly resistant to amikacin (MIC ≥ 64 mg/L) have been reported previously [54]. However, inactivation of *mexXY* in these latter strains left

residual resistance to aminoglycosides. Similarly, disruption of *mexY* by homologous recombination with a central fragment of the gene cloned in suicide vector pUC18 decreased the MIC of amikacin from 64 to 16 mg/L in strain PA2345, and from 4 to 1 mg/L in PAO1 (data not shown). This result indicates that additional unknown mechanisms contribute to the low susceptibility of that strain to amikacin in a synergic interaction with efflux.

Mutations in the QRDRs of *gyrA* (Thr83 → Ile) and *parC* (Ser80 → Leu), together with MexXY efflux, accounted for the high MICs of fluoroquinolones for strain PA2345.

CONCLUSIONS

This study reinforces the concept that multidrug resistance in *P. aeruginosa* may arise from sequential acquisition of a variety of mechanisms provided by multiple genetic exchanges (integrons, transposons) and mutations in chromosomal genes (target alterations, up-regulation of efflux pumps) [3]. The observation that genotypically and geographically diverse *P. aeruginosa* strains have acquired the *bla*$_{PER-1}$ transposon [10] since the initial isolation of RNL-1 in 1991 [16], suggests that contacts with still unknown bacterial reservoirs contribute to the evolution of this pathogen towards multiresistance in Europe and Turkey.

Nucleotide sequences accession numbers

The nucleotide sequences reported in this paper have been submitted to the EMBL and GenBank databases. The accession numbers for the class I integron and *aadA11* gene are AY758206 and AY144590, respectively; the accession number for the putative composite transposon Tn*2345* is AY866517.

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