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Tn*5393*d, a Complex Tn*5393* Derivative Carrying the PER-1 Extended-Spectrum β-Lactamase Gene and Other Resistance Determinants

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In *Alcaligenes faecalis* FL-424/98, a clinical isolate that produces the PER-1 extended-spectrum β-lactamase, the bla_{PER-1} gene was found to be carried on a 44-kb nonconjugative plasmid, named pFL424, that was transferred to *Escherichia coli* by electroporation. Investigation of the genetic context of the bla_{PER-1} gene in pFL424 by means of a combined cloning and PCR mapping approach revealed that the gene is associated with a transposonlike element of the Tn*3* family. This 14-kb element is a Tn*5393* derivative of original structure, named Tn*5393*d, which contains the transposition module and the *strAB* genes typical of other members of the Tn*5393* lineage plus additional resistance determinants, including the bla_{PER-1} gene and a new allelic variant of the *aphA6* aminoglycoside phosphotransferase gene, named *aphA6b*, whose product is active against kanamycin, streptomycin, and amikacin. Tn*5393*d apparently originated from the consecutive insertion of two composite transposons into a Tn*5393* backbone carrying the *aphA6b* and the bla_{PER-1} genes, respectively. The putative composite transposon carrying bla_{PER-1}, named Tn*4176*, is made of two original and nonidentical insertion sequences of the IS*4* family, named IS*1387*a and IS*1387*b, of which one is interrupted by the insertion of an original insertion sequence of the IS*30* family, named IS*1066*. In pFL424, Tn*5393*d is inserted into a Tn*501*-like mercury resistance transposon. Transposition of Tn*5393*d or modules thereof containing the bla_{PER-1} gene from pFL424 to small multicopy plasmids or to a bacterial artificial chromosome was not detected in an *E. coli* host harboring both replicons.

Class A serine β-lactamases with an extended substrate specificity, including specificity for oxyiminocephalosporins and monobactams, also referred to as extended-spectrum β-lactamases (ESBLs), are among the most important resistance determinants emerging in gram-negative bacterial pathogens (4). Acquisition of similar enzymes by members of the family *Enterobacteriaceae*, *Pseudomonas aeruginosa*, and other nonfastidious gram-negative nonfermenters greatly reduces the repertoire of active β-lactams, often leaving a narrow choice of therapeutic options (6, 41).

Several types of class A ESBLs are encountered in the clinical setting, including the TEM- and SHV-type derivatives and the so-called "unconventional" ESBLs, such as the CTX-M-, PER-, VEB-, and GES/IBC-type enzymes (4, 41). The TEM-, SHV-, and CTX-M-type enzymes are mostly or exclusively found in the *Enterobacteriaceae*, while the PER-, VEB-, and GES/IBC-type enzymes are apparently more promiscuous and can be encountered both in the *Enterobacteriaceae* and in gram-negative nonfermenters (2, 4, 41).

PER-1 is an enzyme of notable clinical importance due its powerful ESBL activity (3, 26) and dissemination in various geographic areas of Europe and Asia (see references 27 and 41 and references therein, as well as reference 42). It has been detected in several microbial species including *P. aeruginosa* (see reference 41 and references therein), *Acinetobacter* spp. (40, 42), *Alcaligenes faecalis* (29), and *Enterobacteriaceae* (*Salmonella enterica* serovar Typhimurium [39], *Proteus mirabilis* [28], *Providencia rettgeri* [1], and *Providencia stuartii* and *Escherichia coli* [M. Perilli, F. De Santis, B. Caporale, F. Luzzaro, S. Stefani, A. Toniolo, G. M. Rossolini, and G. Amicosante, Abstr. 44th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 1330, 2004]). PER-1-producing isolates of *P. aeruginosa* and *Acinetobacter* spp. were also shown to be associated with a poor clinical outcome (38).

In PER-1-producing *P. aeruginosa*, the ESBL gene was found to be either inserted in the chromosome (27, 41) or carried on large conjugative plasmids (11). A plasmid location of the bla_{PER-1} gene was also reported in *Salmonella* (7), while a chromosomal location was reported in *Acinetobacter baumannii* (42). The relatively broad host range exhibited by the bla_{PER-1} gene and its heterogeneous genetic location underscore a notable potential for the mobility of this resistance determinant. However, the nature of the genetic element(s) carrying the bla_{PER-1} gene has not been investigated in detail.

In this report we describe the structure of a plasmid-borne transposonlike element of the Tn*3* family carrying the bla_{PER-1} gene from an *A. faecalis* clinical isolate. The element is a Tn*5393* derivative of original structure, named Tn*5393*d, which also contains additional determinants for resistance to aminoglycosides.

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TABLE 1. Primers used for generation of the probes used for Southern blot experiments for screening the pFL424 library, and/or for PCR mapping of Tn*5393*d[a]

Primer (sequence)	Target	Annealing temp (°C)[b]
BLAPER/f (5′-GGGACARTCSKATGAAT GTCA)	bla_{PER} gene	50
BLAPER/r (5′-GGGYSGCTTAGATAGTG CTGAT)	bla_{PER} gene	50
IR5393/u (5′-TTGGCCARCGATATTCTCC GGTRAGATT)	IRs of Tn*5393*	50 or 60
aphA6/f (5′-GGAAACAGCGTTTTAGA GCC)	*aphA6b* gene	50
aphA6/r (5′-GCTTCACGAGAGACACT GTA)	*aphA6b* gene	50

[a] The locations and orientations of the primers are shown in Fig. 1. The strategy adopted for PCR mapping is described in Materials and Methods.

[b] The annealing temperature used in the PCR experiments. Two values are present for primer IR5393/u: the highest temperature was used when the primer was used alone for long PCR amplification of the entire element; the lowest temperature was used when the primer was used in combination with either the aphA6/r or the BLAPER/f primer.

MATERIALS AND METHODS

Bacterial strains. *A. faecalis* FL-424/98 was a clinical isolate from Varese University Hospital (northern Italy) and was isolated in 1998. The strain was resistant to several antimicrobial agents, including expanded-spectrum cephalosporins and aminoglycosides, and it was found to produce the PER-1 ESBL (29). *E. coli* ATCC 25922 was used for quality control of susceptibility testing. *E. coli* DH5α (33) was used as the host for plasmids. *E. coli* MKD135 (*argH rpoB18 rpoB19 rpsL*; kindly provided by G. Kholodii, Institute for Molecular Genetics, Russian Academy of Sciences, Moscow, Russia) and *P. aeruginosa* 10145/3 (an *rpoB his* derivative of the reference strain ATCC 10145^T) (27) were used as the recipients in the conjugation experiments.

In vitro susceptibility testing. In vitro susceptibility testing was carried out by a broth microdilution method, as recommended by the CLSI (formerly the National Committee for Clinical Laboratory Standards) (23, 24), by using cation-supplemented Mueller-Hinton (MH) broth (Difco Laboratories, Detroit, Mich.) and a bacterial inoculum of 10^5 CFU per well. Antibiotics were from Sigma Chemical Co. (St. Louis, Mo.). Resistance to mercury ions was assessed by growing the bacteria on Luria-Bertani medium (Difco Laboratories) containing $HgCl_2$ (10 μg/ml).

DNA analysis methodology. Extraction of genomic DNA from *A. faecalis* was carried out as described previously (13). Extraction of plasmid DNA from *E. coli* was carried out by the alkaline lysis method (33). Southern blot analysis was carried out directly on dried gels as described previously (37). A probe containing the entire bla_{PER-1} open reading frame (ORF), generated by PCR with primers BLAPER/f and BLAPER/r (Table 1) as described previously (29) and labeled with ^{32}P by the random priming technique with a commercial kit (*Redi*Primer I; Amersham Biosciences, Uppsala, Sweden), was used in Southern blot experiments. The structure of Tn*5393*d was determined by cloning and PCR mapping experiments, as follows. A library of plasmid pFL424 was constructed in the *E. coli* plasmid vector pBC-SK (Stratagene Inc., La Jolla, Calif.) by partial digestion of pFL424 with Sau3AI and cloning of the restriction fragments (fragments of 2 to 8 kb, which were purified by agarose gel electrophoresis) in pBC-SK digested with BamHI. The library, transformed in *E. coli* DH5α, was screened for clones containing the bla_{PER-1} gene by using the same probe used for the Southern blot experiments (see above). Two hybridization-positive clones (pEM-9 and pEM1.2) containing partially overlapping DNA inserts were used for analysis of the sequences of the regions flanking bla_{PER-1} (Fig. 1). A probe generated by PCR with primers BLAPER/r and aphA6/f (Table 1) was then used for a second screening of the pFL424 library, yielding two additional clones (pEM-51 and pEM-43) containing DNA inserts from the region upstream of bla_{PER-1} (Fig. 1). PCR mapping with pFL424 as the template and direct sequencing of PCR products were then used to confirm the authenticity of the assembled sequences and to determine the complete transposon structure. The primers used for PCR mapping of Tn*5393*d are listed in Table 1, and their locations are shown in Fig. 1. Long PCR for amplification of the entire Tn*5393*d sequence with primer IR5393/U was carried out in a 25-μl volume with the Expand 20-kb^{PLUS} PCR system (Roche Biochemicals, Mannheim, Germany) and with the buffer system provided by the manufacturer, 1 enzyme unit, 10 pmol of primer, 10 ng of plasmid template, and the following reaction conditions: initial denaturation at 93°C for 3 min; annealing at 60°C for 30 s, extension at 68°C for 18 min, and denaturation at 92°C for 30 s, repeated for 30 cycles; and then annealing at 60°C for 1 min and extension at 68°C for 30 min. PCR amplifications of smaller fragments of Tn*5393*d were carried out in a 50-μl volume with the Expand PCR System (Roche Biochemicals) and with the buffer system provided by the manufacturer, 3 enzyme units, 40 pmol of primer, 30 ng of plasmid template, and the following reaction conditions: initial denaturation at 96°C for 3 min and then annealing at 50°C for 1 min, extension at 72°C for 1 min/kb, and denaturation at 94°C for 45 s, repeated for 35 cycles. The following primer combinations were used: IR5393/u plus aphA6/r (amplification product, 5,065 bp), aphA6/f plus BLAPER/r (amplification product, 5,162 bp), and BLAPER/f plus IR5393/u (amplification product, 4,978 bp) (Fig. 1). The complete sequences of the inverted repeats (IRs) and the sequences of the regions flanking Tn*5393*d were determined directly on pFL424 as the template. DNA sequences were determined on plasmid templates or PCR products, as described previously (31), by using custom sequencing primers. Both strands were sequenced. Analysis and comparison of the sequence data were carried at the ExPASy server (http://us.expasy.org/) and at the BLAST interface of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). Plasmid pEM-9/rH (Fig. 1), which contained a complete copy of the *aphA6b* gene and which was used for expression experiments with this gene, was constructed by digestion of plasmid pEM-43 (Fig. 1) with HindIII and self-ligation.

Gene transfer experiments. Conjugation assays were carried out in MH agar for 8 h at 37°C. Donor-to-recipient ratios of 1:10 or 2:5 were used with the *E. coli* and the *P. aeruginosa* recipients, respectively. Transconjugants were selected on MH agar containing rifampin (250 μg/ml) plus ceftazidime (50 μg/ml). With either recipient, the detection sensitivity of the conjugation assays was $\geq 10^{-8}$ transconjugants/recipient. *E. coli* HB101(pRP1.2) (15) and HB101(pRP1.2/pAX22) (pAX22 is a mobilizable plasmid carrying the metallo-β-lactamase gene bla_{VIM-1}) (30) were used as positive control plasmid donors in conjugation assays with *E. coli* MKD135 (transfer frequency, approximately 2×10^{-2} transconjugants/recipient, with selection for tetracycline resistance) and *P. aeruginosa* 10145/3 (transfer frequency, approximately 3×10^{-5} transconjugants/recipient, with selection for tobramycin resistance), respectively. Electroporation of plasmid DNA from FL-424/98 into *E. coli* DH5α was carried out with approximately 250 ng of whole genomic DNA of FL-424/98 by using a Gene Pulser apparatus (Bio-Rad, Richmond, Calif.) under the experimental conditions recommended by the manufacturer. Transformants were selected on MH agar containing ceftazidime at 50 μg/ml.

Transposition assays. Transposition of Tn*5393*d or modules thereof containing the ESBL determinant was assayed by following a strategy similar to that successfully used to demonstrate the transposition of Tn*5393* and Tn*5393*c (9, 18). Briefly, high-copy-number plasmid pBC-SK, low-copy-number plasmid pACYC184 (8), or the single-copy bacterial artificial chromosome pBeloBAC11 (17) was transformed into *E. coli* DH5α(pFL424). The resulting strains were grown aerobically at 25°C either in Super Broth (33) (for strains containing pFL424 plus pBC-SK or pFL424 plus pACYC184) or in MH broth (for strain containing pFL424 plus pBeloBAC11) containing chloramphenicol (85 μg/ml) and ceftazidime (50 μg/ml); and plasmid DNA was extracted after approximately 15, 30, 45, and 60 generations. Plasmid preparations were analyzed by agarose gel electrophoresis to screen for the presence of plasmid bands of modified size. Plasmid preparations were also diluted to a concentration of approximately 10 ng/μl and transformed into *E. coli* DH5α by electroporation. Ceftazidime-resistant transformants (selected on MH agar containing ceftazidime 50 μg/ml) were replica plated on MH agar containing chloramphenicol (85 μg/ml), and transformants resistant to both antimicrobial agents were analyzed for their plasmid contents.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper have been submitted to the EMBL/GenBank database and assigned accession number AJ627643.

RESULTS AND DISCUSSION

Characterization of plasmid pFL424 from *A. faecalis* FL-424/98 carrying the bla_{PER-1} gene. *A. faecalis* FL-424/98 is a clinical isolate that was previously shown to produce the PER-1 ESBL (29). Mating experiments, carried out with FL-424/98 as the donor and either *E. coli* MKD135 or *P. aeruginosa* 10145/3 as the recipient, failed to demonstrate the transferability of the ESBL determinant by conjugation. However, a

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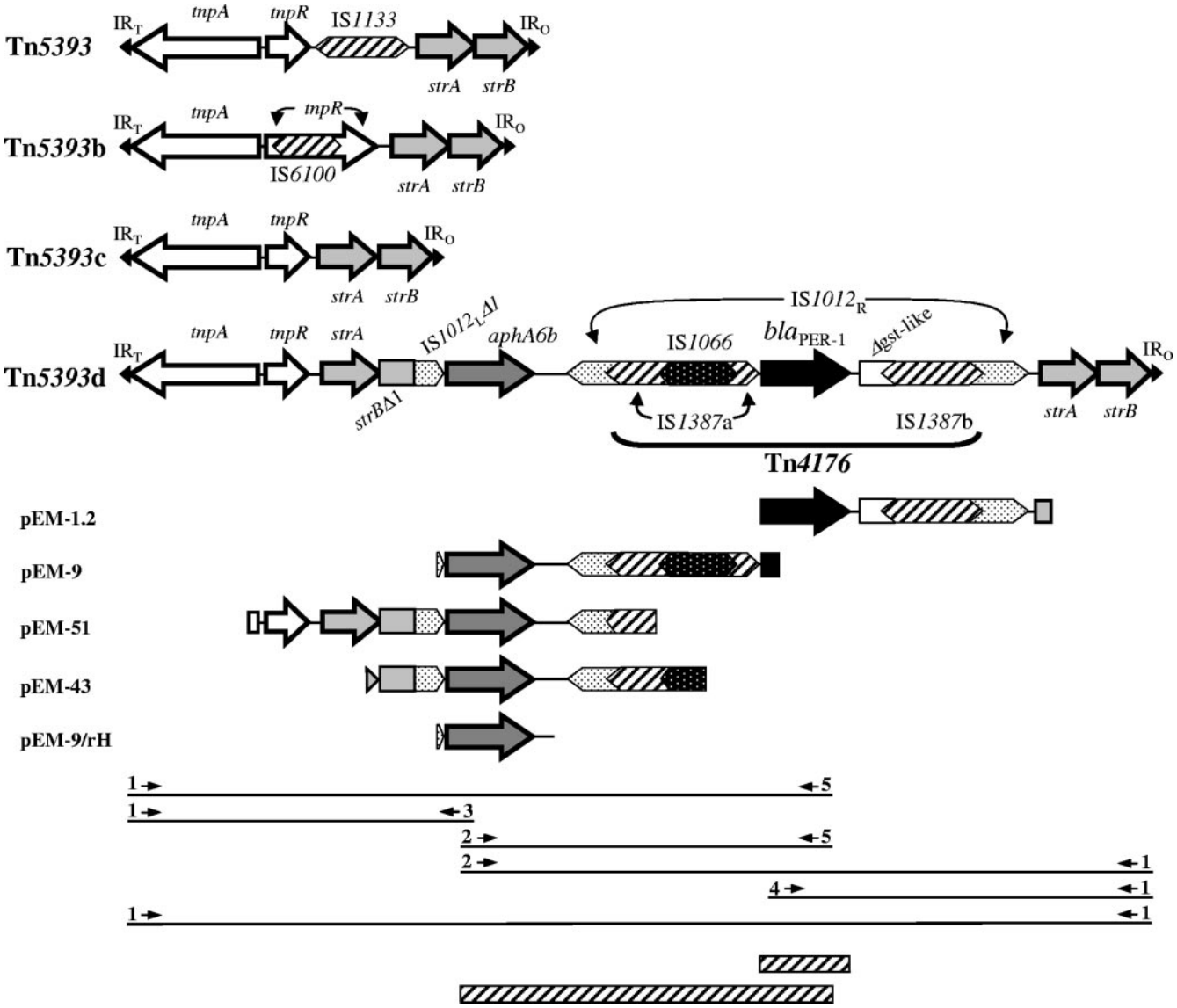

FIG. 1. Schematic representation of the structure of Tn*5393*d, the Tn*5393* derivative from plasmid pFL424 which carries the bla_{PER-1} gene. The structures of other known Tn*5393* derivatives (9, 18, 35) are shown above for comparison. The compositions of the inserts of plasmids pEM-1.2, pEM-9, pEM-51, and pEM-43, selected from the pFL424 library and used to determine the structure of the genetic element, and that of plasmid pEM-9/rH, constructed to perform expression experiments with the *aphA6b* gene, are also shown below the Tn*5393*d map. The positions of the primers used for PCR mapping experiments are indicated by numbered arrows (1, IR5393/u; 2, aphA6/f; 3, aphA6/r; 4, BLAPER/f; 5, BLAPER/r), and the corresponding PCR products are indicated by thin horizontal bars. The primer sequences are reported in Table 1. The positions of the probes used to screen the pFL424 library are shown by hatched bars.

bla_{PER-1}-containing plasmid could be transferred to *E. coli* DH5α by electroporation with total genomic DNA of FL-424/98. The plasmid was named pFL424, and its size was estimated to be approximately 44 kb according to restriction analysis. Southern blot analysis confirmed the presence of the bla_{PER-1} gene in pFL424 (data not shown).

Analytical isoelectric focusing of a crude extract of DH5α(pFL424) revealed a β-lactamase band of pI 5.4 (data not shown). This finding was consistent with the expression of PER-1 (26).

Compared to DH5α, DH5α(pFL424) was resistant to several β-lactams, including extended-spectrum cephalosporins and aztreonam (Table 2), in agreement with the production of the PER-1 ESBL. It also exhibited reduced susceptibility to some aminoglycosides (streptomycin and kanamycin) (Table 2), suggesting that pFL424 also carried one or more determinants for resistance to these drugs.

Genetic context of the bla_{PER-1} gene carried on pFL424, the Tn*5393*d transposonlike element. The genetic context of the bla_{PER-1} gene carried on plasmid pFL424 was investigated by a cloning and PCR mapping approach as described in Materials and Methods. The sequence data assembled revealed that bla_{PER-1} was embedded in a transposonlike element of the Tn*3* family derived from Tn*5393*c (18), which contains a large in-

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TABLE 2. Antimicrobial susceptibilities of *A. faecalis* FL-424/98, *E. coli* DH5α(pFL424), and *E. coli* DH5α containing various pFL424 subclones[a]

Antimicrobial agents	MIC (μg/ml)[b]				
	FL-424/98 (*strAB*, *aphA6b*, bla_{PER-1})	DH5α(pFL424) (*strAB*, *aphA6b*, bla_{PER-1})	DH5α(pEM-43) (*aphA6b*)	DH5α(pEM-9/rh) (*aphA6b*)	DH5α[c] (none)
Ampicillin	>128	>128	—[d]	—	2
Piperacillin	8	64	—	—	0.5
Cephalothin	64	>128	—	—	8
Cefotaxime	128	>128	—	—	≤0.12
Ceftazidime	>128	>128	—	—	≤0.12
Cefepime	>128	64	—	—	≤0.12
Aztreonam	>128	>128	—	—	≤0.12
Chloramphenicol	16	0.5	—	—	0.5
Tetracycline	4	0.25	—	—	0.25
Streptomycin	>128	32	2	2	1
Spectinomycin	>128	16	16	16	16
Kanamycin	>128	4	32	16	1
Gentamicin	16	0.12	0.25	0.12	0.12
Sisomicin	32	0.25	0.25	0.25	0.25
Tobramycin	>128	1	0.5	0.5	0.5
Netilmicin	16	0.25	0.25	0.25	0.25
Amikacin	>128	0.5	2	1	0.5

[a] For details on the subclones, see Fig. 1. The antimicrobial susceptibility of DH5α is also shown for comparison.
[b] The presence of the resistance gene(s) investigated in this work is reported in parentheses.
[c] The MICs of aminoglycosides for *E. coli* DH5α(pBC-SK) were identical to those for DH5α.
[d] —, not determined.

sertion of genetic material of original structure (Fig. 1). This new Tn*5393* derivative is 14,184 bp and was named Tn*5393*d. Tn*5393*d has 81-bp IRs and a transposition module identical to those of Tn*5393*c (18). Compared to Tn*3* (12), the *tnpA* and *tnpR* genes of Tn*5393*d exhibit 47.2% and 47.7% sequence identities, respectively, while the IRs are longer (81 versus 38 nucleotides) and are 42% divergent in the homologous region.

A partial copy of the *strAB* genes (*strABΔ1*) is present downstream of *tnpR*, while a complete copy of the *strAB* genes is present upstream of IR-5393o, resulting in a partial duplication of the *strAB* resistance module typical of the Tn*5393* elements (Fig. 1). In *strABΔ1*, most of the *strB* gene is deleted, and the sequence merges directly into a partially deleted copy of IS*1012* (named IS1012_L*Δ1*), an insertion sequence of the IS*1* family that has previously been described, although partially, in plasmid pKLH205 from an *Acinetobacter* environmental isolate (EMBL/GenBank accession no. AJ459234) (16). A complete copy of IS*1012* (named IS1012_R), in the same orientation as IS1012_L*Δ1*, is present upstream of the complete set of *strAB* genes (Fig. 1). IS1012_R reveals, for the first time, the complete structure of IS*1012* (Table 3). The region between the two copies of IS*1012* contains an allelic variant of the *aphA6* gene that was previously detected in a plasmid from an *A. baumannii* clinical isolate and that encodes an APH(3′) aminoglycoside phosphotransferase (20). This *aphA6* allelic variant, named *aphA6b*, encodes a protein which is 93% identical to AphA6 and which retains the three highly conserved motifs typical of APH(3′) enzymes (Fig. 2). The putative promoter sequences and ribosome-binding site previously identified upstream of *aphA6* (20) were found to be fully conserved in the sequence upstream of *aphA6b*. However, a better putative promoter could be at positions 4819 to 4824 (−35 hexamer) and 4842 to 4847 (−10 hexamer). Whether a similar promoter is also conserved upstream of *aphA6* remains unknown, since the sequence upstream *aphA6* is not available up to that point (20).

IS1012_R is interrupted by the presence of a putative composite transposon, named Tn*4176*. Tn*4176* is made of two original insertion sequences of the IS*4* family, named IS*1387*a and IS*1387*b, which bound to a region containing the bla_{PER-1} gene and an ORF which encodes a protein that exhibits the highest similarity (25% amino acid identity) to a putative glutathione-*S*-transferase of *Ralstonia solanacearum* (32). This ORF starts 73 bp downstream the bla_{PER-1} gene and is truncated at the carboxy terminus by the presence of IS*1387*b (Fig. 1). IS*1387*a and IS*1387*b are not identical to each other (65% nucleotide sequence identity) but exhibit the same size, the same orientation, and very similar terminal IRs (Table 3). Tn*4176* is inserted into the putative transposase gene of IS1012_R, and an 8-bp duplication (GGCGTAAA) flanks the site of insertion, suggesting insertion by transposition. No direct repeats flanking the individual IS*1387*a and IS*1387*b insertion sequences were detectable, a finding which would support the composite transposon nature of Tn*4176*. IS*1387*a is interrupted by an original insertion sequence of the IS*30* family, named IS*1066* (Fig. 1). IS*1066* is inserted into the putative transposase gene of IS*1387*a, and a 4-bp duplication (GTTG) flanks the site of insertion, suggesting insertion by transposition. Interestingly, in PER-1-producing *P. aeruginosa* isolates from northern Italy (27), the bla_{PER-1} gene was found to be associated with an element identical to Tn*5393*d but lacking IS*1066* (E. Mantengoli and G. M. Rossolini, unpublished results). It should also be noted that in Tn*5393*d the sequence upstream of bla_{PER-1} is identical to that found upstream of bla_{PER-1} in the RNL-1 PER-1-producing index strain (25) for the first 27 bp, but thenceforth, the two sequences abruptly diverge from each other. The point at which the two sequences diverge falls inside the IR_R of IS*1387*a. Altogether, these find-

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ings suggest the existence of some diversity among bla_{PER-1}-containing elements.

A summary of the features of the new insertion sequences associated with Tn*5393*d is reported in Table 3.

Functional analysis of the *aphA6b* aminoglycoside phosphotransferase gene product. Compared to *E. coli* DH5α, DH5α(pEM-43) and DH5α(pEM-9/rH), which contained a cloned copy of the *aphA6b* gene on a multicopy plasmid vector (Fig. 1), exhibited reduced susceptibilities to kanamycin and amikacin but not to gentamicin, tobramycin, netilmicin, sisomicin, or spectinomycin. Susceptibility to streptomycin was only slightly affected (Table 2). Thus, the spectrum of activity of the enzyme was, overall, consistent with that reported for AphA6 from *A. baumannii* BM2580 (19).

The amikacin and kanamycin MICs for *E. coli* DH5α carrying the cloned *aphA6b* gene were notably lower that those reported for *E. coli* HB101 carrying the cloned *aphA6* gene (19). This could be the case for one or more of several reasons, including (i) an inefficient activity of the *aphA6b* promoter in *E. coli* which could be different from that present upstream of the *aphA6* gene (see above), (ii) poor stability of the AphA6b enzyme when it is expressed in this *E. coli* host, or (iii) low specific phosphotransferase activity of the AphA6b enzyme toward those aminoglycoside substrates. However, given the strong similarity between AphA6b and AphA6 and the possible divergence of the sequences upstream of the two genes, differences at the level of gene expression would seem to be the more plausible reason that accounts for this observation. The fact that DH5α(pFL424) exhibited kanamycin and amikacin MICs lower than those of DH5α(pEM-43) and DH5α(pEM-9/rH) (Table 2) likely reflects the lower gene dosage present in the former strain due to a lower copy number of pFL424. The fact that a similar effect was not observed for the streptomycin MIC [which was actually higher for DH5α(pFL424)] is likely due to the additional presence of the *strAB* streptomycin resistance module in the original plasmid.

The insertion site of Tn*5393*d. Sequence analysis of the regions flanking Tn*5393*d, spanning 776 bp upstream of IRt and 413 bp downstream of IRo, revealed that the transposon was inserted into a Tn*501* backbone (Fig. 3). Tn*501* is a mercury resistance transposon of the Tn*3* family originally found in a *P. aeruginosa* plasmid (5, 34). The insertion site of Tn*5393*d is located in orf2 of Tn*501*, which encodes a conserved protein of unknown function (EMBL/GenBank accession no. Z00027), and is flanked by a 5-bp direct repeat (Fig. 3), suggesting that the presence of Tn*5393*d in this position was the result of a transposition event. Sequence analysis confirmed the presence of an orf2, part of orf1, and part of the *res* site identical to those of Tn*501* (EMBL/GenBank accession no. Z00027) (Fig. 3). The structure of the Tn*501*-like element present in pFL424 was not further investigated in this work. However, the fact that, unlike DH5α, *E. coli* DH5α(pFL424) was able to grow on mercury-containing medium (10 μg/ml) suggested that a mercury resistance module was also present on the plasmid.

The insertion of Tn*5393*d in orf2 of a Tn*501*-like backbone, in proximity of the *res* site and associated with a 5-bp target duplication, could relate to the serine recombinase-targeting mechanism previously described for Mu-related transposons such as Tn*402*/Tn*5090* (14) and Tn*5053* (21).

TABLE 3. Features of the original insertion sequences detected in Tn*5393*d

Insertion sequence	Family	Size (bp)	Inverted repeat[a]	Putative transposase
IS*1012*	IS*1*	788	GGTGGTGTTTCAAAAAGTATGCTGA-738 bp-TCAGCATACTTTTTGAAACACCACC	233 aa[b]; closest relative: IS*1* transposase of *Salmonella enterica* serovar Typhimurium (GenBank accession no. AAR05766; 28.5% amino acid identity)
IS*1387*a	IS*4*	1,387	TCATACGTATGTCTTTAG-1,350 bp-CTAAAGA(T)CATACGTATGA	421 aa; closest relatives: putative transposase of IS*1387*b (62.4% amino acid identity) and putative transposase from marine psychrotrophic bacterium Mst37 (GenBank accession no. CAC84124; 33.3% amino acid identity)
IS*1387*b	IS*4*	1,387	TCATACGTATGACGTTAA-1,351 bp-TTAAAGATCATACGTATGA	423 aa; closest relatives: putative transposase of IS*1387*a (62.4% amino acid identity) and putative transposase of marine psychrotrophic bacterium Mst37 (GenBank accession no. CAC84124; 34.6% amino acid identity)
IS*1066*	IS*30*	1,066	CCTGAATTCAACACATAAGTGCAACGCT-1,010 bp-AGCGTTGCGCTTATTCTATGAATTCAGG	320 aa; closest relative: putative transposase of an IS*30* family insertion sequence form *Aeromonas punctata* (GenBank accession no. AAS66625; 45.1% amino acid identity)

[a] The nucleotide sequences of the inverted repeats; residues in parentheses are insertions which do not match in the other IR; underlined residues indicate mismatches between the two IRs.
[b] aa, amino acid.

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AphA6      1 MELPNIIQQFIGNSVLEPNKIGQSPSDVYSFNRNNETFFLKRSSTLYTETTYSVSREAKM
AphA6b     1 MELPNIIQQFIGNSVLEPNKIGQSPSDVYSFNRNNETFFLKRSSTLYTETTYSVSREAKM
             ************************************************************

AphA6     61 LSWLSEKLKVPELIMTFQDEQFEFMITKAINAKPISALFLTDQELLAIYKEALNLLNSIA
AphA6b    61 LSWLSDKLKVPELIMTFQDEQFEFMITKAINAKSISALFLTEQELLAIYKETLNQLNAVA
             ***** *************************** ******* ********* ** **  *

AphA6    121 IIDCPFISNIDHRLKESKFFIDNQLLDDIDQDDFDTELWGDHKTYLSLWNELTETRVEER
AphA6b   121 IIDCPFISSIDHRLKESKFFIDNQLLDEIDQDDFEAELWGDHKTYISLWNELNETRVEER
             ******** ****************** ******  ********* ****** *******

AphA6    181 LVFSHGDITDSNIFIDKFNEIYFLDLGRAGLADEFVDISFVERCLREDASEETAKIFLKH
AphA6b   181 LVFSHGDITDSNIFIDKSGEIYFLDLGRAGLADEFVDISFVERCLREDVSEETAKIFLKH
             *****************  ***************************** ***********
             [ Motif 1  ]          [        Motif 2         ]

AphA6    241 LKNDRPDKRNYFLKLDELN
AphA6b   241 LKNDMPDKRNYFLKLDELN
             **** **************
                  [ Motif 3 ]
```

FIG. 2. Comparison of the amino acid sequences of the *aphA6* (20) and the *aphA6b* gene products. Identical residues are indicated by asterisks. The conserved motifs typical of APH(3′) enzymes (20) are boxed.

Studies of mobility of Tn*5393*d. Transposition assays were carried out to detect the mobility of Tn*5393*d or modules thereof containing the bla_{PER-1} gene from plasmid pFL424 to plasmid pBC-SK, pACYC184, or pBeloBAC11 by following an experimental strategy that was overall similar to that successfully used to demonstrate the transposition of Tn*5393* and Tn*5393*c (9, 18). By use of this strategy, no transposition events from the donor to the recipient plasmids were detected after up to 60 generations either in *E. coli* DH5α(pFL424/pBC-SK), in *E. coli* DH5α(pFL424/pACYC184), or in *E. coli* DH5α(pFL424/pBeloBAC11).

The apparent lack of mobility of Tn*5393*d could reflect a low transposition efficiency of Tn*5393*d due to its complex structure and/or the genetic context in which it is found in pFL424. However, it could also be due to the fact that the experimental conditions adopted (host, recipient plasmids, and/or culture conditions) were not suitable for the detection of transposition events. However, the presence of a full set of elements involved in the transposition machinery, including a transposition module identical to that of Tn*5393*c, suggests that Tn*5393*d could retain the potential for transposition. The apparent lack of mobility of Tn*4176* could be due to similar reasons, but it could also reflect the insertion of IS*1066* into IS*1387*a, which is one of the insertion sequences that constitute the putative composite transposon.

Hypothetical origin of Tn*5393*d. The structure of Tn*5393*d led us to hypothesize the following steps as an explanation for the genesis of this element. The first step was likely represented by the insertion of a composite resistance transposon (made by two IS*1012* insertion sequences that had captured the *aphA6b* gene) into the region between *tnpR* and *strAB* or between *strAB* and IRo of Tn*5393*c, apparently accompanied

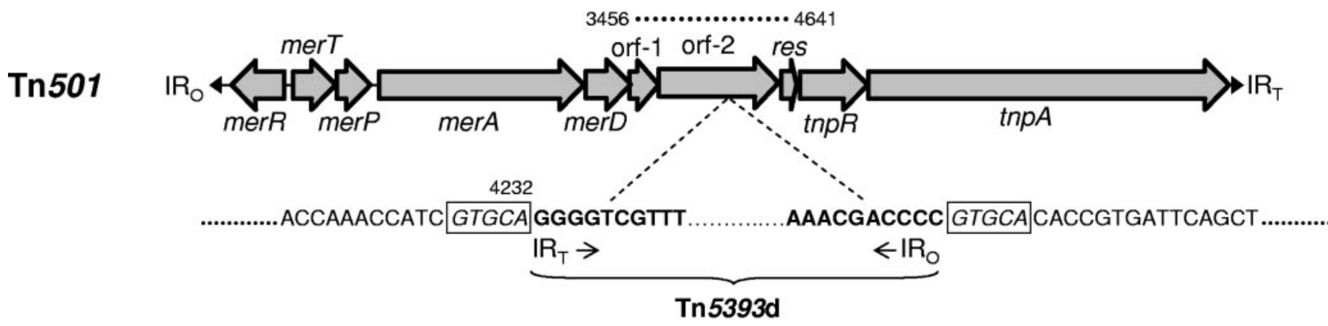

FIG. 3. Insertion site of Tn*5393*d into orf2 of Tn*501*. The partial Tn*5393*d IR sequences are boldfaced. The 5-bp direct repeats flanking the insertion site are boxed. The numbering is based on that for the sequence with EMBL/GenBank accession no. Z00027. A map of Tn*501* (EMBL/GenBank accession no. Z00027) is also shown. The extents of the sequenced regions flanking the insertion site of Tn*5393*d are indicated by a dotted line above the map.

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by a duplication of the *strAB* module, resulting in the presence of two such modules flanking the composite transposon. A deletion involving part of *strB* and part of IS*1012*$_L$ could have then followed, resulting in the fixation of the *aphA6b* aminoglycoside resistance gene while leaving the element with a complete set of the *strAB* module. This hypothesis is consistent with the fact that IS*1012*$_R$ is not flanked by direct repeats. Insertion of Tn*4176*, which carries the bla_{PER-1} gene, into IS*1012*$_L$ could have occurred either before or after the deletion involving *strB* and IS*1012*$_L$, leading to the additional acquisition of the ESBL determinant on the transposon. Insertion of IS*1066* into IS*1387*a likely represents the most recent event. The finding of a Tn*4176* that lacks IS*1066* in a Tn*5393*d-like element from PER-1-producing *P. aeruginosa* clinical isolates from northern Italy (Mantengoli and Rossolini, unpublished) supports this view. The insertion of IS*1066* in this position could affect the mobility of Tn*4176* and/or the expression of the bla_{PER-1} gene. However, the actual role of IS*1066* in this sense remains to be established and will be the subject of future work.

Concluding remarks. Mobile DNA plays a major role in the dissemination of antibiotic resistance determinants. Investigation of the genetic elements involved in the flow of resistance genes from the environmental gene pool to that of pathogenic bacteria and among the latter is important to obtain an understanding of the mechanisms and pathways that lead to the dissemination of clinically relevant resistance genes.

In this work we have described a novel derivative of the Tn*5393* transposon which, in addition to the *strAB* genes, which confer resistance to streptomycin (10), also carries the bla_{PER-1} gene (which encodes a powerful ESBL) and an allelic variant of the *aphA6* aminoglycoside phosphotransferase gene (whose product is active against kanamycin, streptomycin, and amikacin). Tn*5393*d, therefore, can function as a vehicle for at least two clinically important resistance genes; and this association could explain, at least in part, the coresistance to extended-spectrum cephalosporins and amikacin that has often been reported in PER-1-producing clinical isolates (27, 39, 40, 42). To the best of our knowledge, this is the first example of a Tn*5393* derivative that carries clinically relevant resistance genes in addition to the *strAB* module (Fig. 1). Tn*5393*-like transposons have been detected in animal, plant, and human pathogens (22, 36). The present results and the finding of a very similar element in PER-1-producing clinical isolates of *P. aeruginosa* from Italy (Mantengoli and Rossolini, unpublished) underscore the notion that these highly promiscuous elements can play an increasingly important role in the dissemination of relevant resistance determinants in the clinical setting.

As a final remark, the fact that the insertion sequences which constitute Tn*4176* as well as IS*1066* exhibit the strongest similarities with insertion sequences from environmental bacteria living in aquatic environments (Table 3) would suggest, for bla_{PER-1}, a likely origin from some unknown species of bacteria living in similar environments.

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ADDENDUM IN PROOF

After the revised version of this paper had been submitted, another paper reporting on the genetic environment of the bla_{PER-1} gene in some gram-negative bacteria was published (L. Poirel, L. Cabanne, H. Vahaboglu, and P. Nordmann, Antimicrob. Agents Chemother. **49:**1708–1713, 2005). The internal region of Tn*5393*d described in this paper (including the bla_{PER-1} gene and its genetic environment) is closely related, although not identical, to the genetic environments of bla_{PER-1} described by Poirel et al. In particular, the insertion sequences IS*1012*$_R$, IS*1387*a, and IS*1387*b are identical to IS*Pa14*, IS*Pa12*, and IS*Pa13*, respectively, described in that paper, while the composite transposon Tn*4176* corresponds to the composite transposon Tn*1213* described in that paper.

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