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An SHV-Derived Extended-Spectrum β-Lactamase in *Pseudomonas aeruginosa*

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A clinical isolate of *Pseudomonas aeruginosa* RP-1 produced the extended-spectrum β-lactamase (ESBL) SHV-2a. Its gene was expressed from a composite promoter made of the −35 region derived from the left inverted repeat of IS*26* and the −10 region from the *bla*$_{\text{SHV-2a}}$ promoter itself. The DNA sequences immediately surrounding *bla*$_{\text{SHV-2a}}$ were homologous to plasmid pMPA2a from *Klebsiella pneumoniae* KpZU-3, while further away and 3′ to the *bla*$_{\text{SHV-2a}}$ gene, a sequence corresponding to the left end of Tn*1721* was detected, thus indicating a likely enterobacterial origin of this ESBL gene.

The so-called extended-spectrum β-lactamases (ESBLs) hydrolyze extended-spectrum cephalosporins such as ceftriaxone, cefotaxime, and ceftazidime and monobactams such as aztreonam, while their activity is inhibited by clavulanic acid. Most of them are penicillinases (Ambler class A β-lactamases) (2), which are members of the 2be group of the Bush functional classification (3, 10), being mainly point mutation derivatives of TEM-1/TEM-2 or SHV-1 (22). They have been extensively described worldwide and are mostly plasmid mediated in members of the family *Enterobacteriaceae* (22).

Pseudomonas aeruginosa possesses inducible, naturally occurring cephalosporinases (3) which confer low-level resistance to aminopenicillins, narrow-spectrum cephalosporins such as cephalothin, and cephamycins such as cefoxitin. These Ambler class C β-lactamases are not inhibited by clavulanic acid (3). The most common mechanism for increased resistance to ceftazidime and other extended-spectrum cephalosporins in *P. aeruginosa* is derepression of the chromosomal class C enzyme, resulting in its overproduction (4, 5, 26).

However, within the last 4 years, three clavulanic acid-inhibitable ESBLs were found in *P. aeruginosa*. Among the 2be Bush group enzymes, two ESBLs are known, PER-1 and TEM-42 (16, 19). PER-1 was originally identified as chromosomally located in a *P. aeruginosa* isolate from the urinary tract of a Turkish patient hospitalized in Paris in 1992 (16). The PER-1 gene was later also identified as plasmid mediated (6). Recently, a Turkish study shows that PER-1 is found in 11% of *P. aeruginosa* hospital isolates and in 43% of *Acinetobacter* sp. strains, underlining its wide spread in this country (27). PER-1 is weakly related to the ESBLs of TEM or SHV derivatives (18). TEM-42 is the second ESBL found in a *P. aeruginosa* isolate in Paris in 1992 and is so far limited to just one isolate (16). It is also plasmid mediated. The only oxacillin-hydrolyzing β-lactamase (Ambler class D) with clavulanic acid-inhibited extended-spectrum properties is OXA-18, identified in a *P. aeruginosa* strain from an Italian patient hospitalized in Paris in 1995 (23). *bla*$_{\text{OXA-18}}$ was chromosomally located.

In this study, we analyzed the β-lactamase content of a *P. aeruginosa* clinical strain for which a slight synergy between the ceftazidime and clavulanic acid discs was found in the double-disc diffusion test on a routine antibiogram.

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P. aeruginosa RP-1 was isolated in 1995 from a bronchoalveolar brush of a 52-year-old patient hospitalized in the intensive care unit at the Raymond Poincaré hospital (Garches, France). This French patient had recently returned from a trip to Tunisia, where he was hospitalized. This laboratory specimen was collected because the patient suffered from pneumonia. The strain was identified by using an API 20NE system (bioMérieux, Marcy l'Etoile, France). According to routine antibiogram results, it was additionally resistant to fluoroquinolones (ciprofloxacin, ofloxacin, and pefloxacin), aminoglycosides (amikacin, isepamicin, netilmicin, and tobramycin), chloramphenicol, and rifampin. The isolated strain showed a slight synergy between ceftazidime and clavulanate discs, which was best evidenced when the discs were put 1 cm from one another, suggesting the presence of an ESBL. Such a synergy test was performed as routine screening for all *P. aeruginosa* isolates in order to detect any ESBL-possessing strains. To search for any other gastrointestinal carriers of *P. aeruginosa* strains with the same unusual β-lactam resistance profile, rectal swab samples were collected from patients in the same hospitalization unit over the same period of time. The negative results ruled out any cross-contamination or any outbreak in this hospitalization unit.

Plasmid DNA extractions from *P. aeruginosa* RP-1 failed, despite repeated attempts using four different extraction methods (23). Conjugation assays performed as previously described (23), by using as the recipient strain *P. aeruginosa* PU21 or in vitro-obtained ciprofloxacin-resistant *Escherichia coli* JM109, also failed. Genomic DNA from *P. aeruginosa* RP-1 was then prepared as described previously (23). Preliminary dot blot hybridizations were performed with probes consisting of several class A or D β-lactamase genes, i.e., *bla*$_{\text{PER-1}}$,

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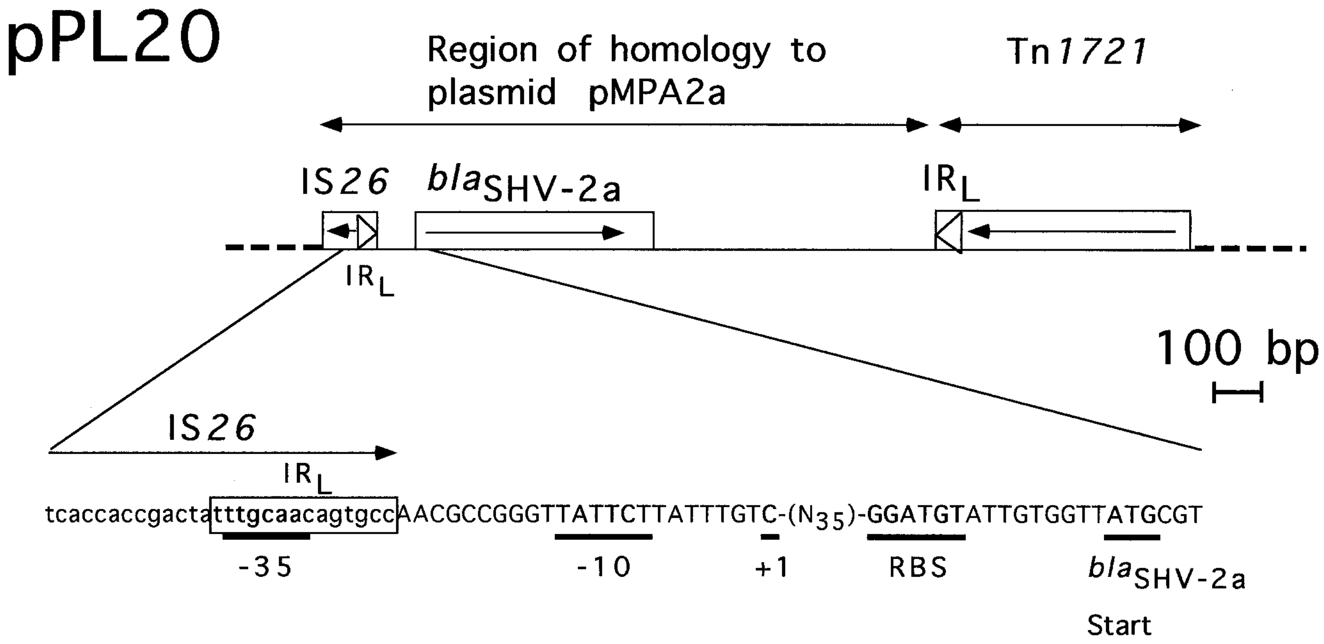

FIG. 1. Schematic map of recombinant plasmid pPL20 which possesses *bla*$_{\text{SHV-2a}}$. The thin, solid line represents the cloned insert from *P. aeruginosa* RP-1, while the dotted lines indicate the pBK-CMV cloning vector. The open boxes represent genes, and the arrow indicates their translational orientation. pMPA2a (20) and Tn*1721* (1) homology regions are indicated. Details of the nucleotide sequence of the *bla*$_{\text{SHV-2a}}$ promoter region are shown below. The boxed sequence corresponds to the left inverted repeat (IR$_{\text{L}}$) of IS*26*, the −35, −10, and +1 promoter sequences of *bla*$_{\text{SHV2-a}}$ are those described by Podbielski et al. (24). RBS, ribosome-binding site.

bla$_{\text{SHV-3}}$, *bla*$_{\text{TEM-1}}$, and *bla*$_{\text{OXA-18}}$ (23). Only the *bla*$_{\text{SHV-3}}$ probe gave a positive signal with genomic DNA of *P. aeruginosa* RP-1. Partially *Sau3*AI-digested genomic DNA from *P. aeruginosa* RP-1 was then ligated into the *Bam*HI site of a pBK-CMV cloning vector as previously described (23). Ten recombinant plasmids were obtained after selection of the *E. coli* JM109 electroporants on trypticase soy plates containing amoxicillin (100 μg/ml). The recombinant strains had decreased susceptibility to extended-spectrum cephalosporins such as ceftazidime compared to *E. coli* JM109 (data not shown). Analysis of their plasmid content revealed insert sizes ranging from 2.9 to 10 kb. One of them, pPL20 harboring the 2.9-kb insert, was retained for further analysis (Fig. 1).

Susceptibility testing of *E. coli* JM109 harboring pPL20 and of *P. aeruginosa* RP-1 was performed by an agar dilution method as previously described (17, 23). Additionally, MICs were determined for reference strain *P. aeruginosa* ATCC 27853 and its in vitro-obtained, stably derepressed, cephalosporinase-producing mutant. This mutant, obtained after selection on ceftazidime-containing plates, produced an 85-fold increase of cephalosporinase activity determined as described for an *Enterobacter cloacae* isolate (14). This β-lactamase level, as well as the β-lactam MICs (Table 1), corresponded to a *P. aeruginosa* strain with a high basal level of constitutive cephalosporinase production (4). As shown in Table 1, *P. aeruginosa* RP-1 had decreased susceptibility to all of the β-lactams tested except imipenem. The MIC of ceftazidime (32 μg/ml) was reduced to 8 μg/ml in the presence of clavulanic acid. *E. coli* JM109(pPL20) had decreased susceptibility to all of the β-lactams tested except cefoxitin and imipenem; the MICs of the extended-spectrum cephalosporins and aztreonam were markedly reduced in the presence of clavulanic acid, indicating the presence of an ESBL (Table 1). Crude extracts from *P. aeruginosa* RP-1 and from *E. coli* JM109(pPL20) were analyzed by isoelectric focusing (23) and revealed a β-lactamase with a pI of 7.6 in both cases and an additional β-lactamase with a pI of 8.2 (likely corresponding to an AmpC cephalosporinase) found only in *P. aeruginosa* RP-1 extracts (data not shown).

The 2.9-kb cloned fragment from pPL20 was sequenced on both strands with an Applied Biosystems ABI 377 sequencer. Analysis of the sequenced DNA and the deduced protein revealed a sufficiently large open reading frame of 861 bp encoding a 286-amino-acid protein identified as SHV-2a. First described from a *Klebsiella pneumoniae* isolate in Germany (25), SHV-2a is a point mutation derivative of SHV-2 differing only by a leucine-to-glutamine replacement at the unusual position 35 of the Ambler numbering. Compared to the consensus sequence for SHV-1 (21), SHV-2 and SHV-2a possess a G238S change, explaining their extended-spectrum catalytic properties. Although initially considered insignificant (25), the position 35 mutation in SHV-2a compared to SHV-2 increased its resistance to ceftazidime but reduced the MICs of all other cephalosporins (20). The prevalence of SHV-2a in *Enterobacteriaceae* in Western Europe or in the United States is not known, although it was recently reported as being widespread among *K. pneumoniae* strains in Korea (11).

The −35 (5′-TTGCAA-3′) and −10 (5′-TATTCT-3′) promoter sequences of *bla*$_{\text{SHV-2a}}$ present on the 2.9-kb insert of pPL20 (Fig. 1) corresponded exactly to those previously identified for this β-lactamase gene in a *K. pneumoniae* isolate (25). No promoter sequence specific for *P. aeruginosa* genes was found upstream the *bla*$_{\text{SHV-2a}}$ structural gene. The −35 and −10 boxes showed higher homology to *E. coli* promoter sequences described by Hawley and McClure (9) than does the promoter belonging to *bla*$_{\text{SHV-2}}$, thus explaining the *bla*$_{\text{SHV-2a}}$ promoter strength (24). In fact, a detailed analysis of *bla*$_{\text{SHV2a}}$ revealed that the immediate upstream and downstream sequences retained 100% DNA identity with parts of plasmid pMPA2a from *K. pneumoniae* KpZU-3 (20). Analysis of the promoter sequences of *bla*$_{\text{SHV2a}}$ indicated that they resulted

TABLE 1. MICs of β-lactams for *P. aeruginosa* RP-1, *E. coli* JM109 harboring recombinant plasmid pPL20, reference strain *E. coli* JM109, reference strain *P. aeruginosa* ATCC 27853, and its stably derepressed cephalosporinase-producing mutant, Mut

Antibiotic(s)	MIC (μg/ml) against:				
	P. aeruginosa RP-1	*E. coli* JM109 (pPL20)	*E. coli* JM109	*P. aeruginosa* ATCC 27853	*P. aeruginosa* Mut
Amoxicillin	>512	512	2	>512	>512
Amoxicillin + CLA[a]	>512	4	2	>512	>512
Ticarcillin	>512	>512	2	2	32
Ticarcillin + CLA	64	4	1	2	32
Piperacillin	256	256	1	1	16
Piperacillin + CLA	32	2	1	1	16
Cephalothin	>512	>512	4	>512	>512
Cephalothin + CLA	>512	4	2	>512	>512
Cefoxitin	>512	8	8	>512	>512
Ceftazidime	32	8	0.25	0.25	8
Ceftazidime + CLA	8	0.25	0.25	0.25	8
Cefotaxime	>512	32	0.06	2	>512
Cefotaxime + CLA	256	0.12	0.06	2	>512
Imipenem	2	0.12	0.06	0.5	0.5
Aztreonam	32	8	0.12	0.5	2
Aztreonam + CLA	16	0.12	0.12	0.5	2

[a] CLA, clavulanic acid at a fixed concentration of 2 μg/ml (MIC for *E. coli* JM109, 16 μg/ml).

from a fusion of the −35 sequence from IS*26* (15) to the −10 sequence of the native *bla*$_{\text{SHV-2a}}$ promoter (Fig. 1). IS*26* was, in fact, previously reported as being the promoter for the expression of an aminoglycoside resistance gene within a multidrug resistance operon (12). A similar hybrid promoter was identified for ESBL gene *bla*$_{\text{TEM-6}}$, for which an IS*1*-like element provided the −35 sequence, thus allowing high-level expression of TEM-6 (8). From a general point of view, it is known that insertion sequences may act as mobile promoters on prokaryotic gene expression (7). Their inverted repeats contain −35 sequences that, upon insertion next to −10 sequences, may boost gene expression (7).

Although *bla*$_{\text{SHV2a}}$ was not plasmid located, our report indicates that the ESBL SHV derivatives may be identified in *P. aeruginosa*. The sequences surrounding *bla*$_{\text{SHV-2a}}$ had strong homology with *K. pneumoniae* plasmid sequences, and the more distantly related downstream sequences from *bla*$_{\text{SHV2a}}$ had homology with Tn*1721*, a transposon often encountered in *Enterobacteriaceae* (1) (Fig. 1). Pulsed-field gel electrophoresis of *Xba*I-restricted genomic DNA of *P. aeruginosa* RP-1 (13), followed by *bla*$_{\text{SHV-2a}}$-specific hybridization, revealed that the fragment containing *bla*$_{\text{SHV-2a}}$ was larger than 300 kb, indicating its likely chromosomal origin (data not shown). We therefore believe that a putative plasmid derived from *K. pneumoniae* (containing *bla*$_{\text{SHV-2a}}$) became integrated into *P. aeruginosa* RP-1 chromosomal DNA either by homologous recombination or by insertion sequence- or transposon-mediated specific cointegration (7).

Our report indicates that the ESBL genes are no longer limited to *Enterobacteriaceae*, from which they may have originated. From a clinical point of view, detection of these ESBLs based on the double-disc synergy test remains difficult in *P. aeruginosa*. This bacterial species may therefore become a hidden reservoir for such ESBLs, as is the case for oxacillinase extended-spectrum derivatives.

Nucleotide sequence accession number. The nucleotide sequence reported in this work will appear in the GenBank nucleotide sequence database under accession no. AF074950.

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