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Biochemical Characterization of PER-2 and Genetic Environment of $bla_{\mathrm{PER-2}}$ ▽

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PER-2 was the first detected and the second most prevalent extended-spectrum β-lactamase in clinical pathogens isolated in Argentina and was also reported only in other South American countries. *Citrobacter freundii* 33587 was isolated in 1999 in Buenos Aires and was resistant to all tested β-lactams except cephamycins and carbapenems. The strain produced both plasmid-borne TEM-1 and PER-2 (pI 5.4), which could be transferred by conjugation. By PCR screening, thermal asymmetric interlaced PCR, and DNA sequencing, we detected an IS*Pa12*/IS*1387a* insertion sequence upstream of $bla_{\mathrm{PER-2}}$, previously reported as also being associated with $bla_{\mathrm{PER-1}}$. The presence of similar structures upstream of $bla_{\mathrm{PER-1}}$ and $bla_{\mathrm{PER-2}}$ suggests a common origin and mobilization. Compared to $bla_{\mathrm{PER-1}}$ genes, an additional putative promoter for $bla_{\mathrm{PER-2}}$ was found. PER-2 kinetic analysis showed its high hydrolysis efficiencies toward both CTX and CAZ (k_{cat}/K_m, 0.76 and 0.43 $\mu M^{-1} \cdot s^{-1}$, respectively).

Up to 10% of *Escherichia coli* and 35 to 45% of *Klebsiella pneumoniae* isolates in Argentina are resistant to oxyimino-cephalosporins (Sistema Informático de la Resistencia [SIR], SADEBAC, AAM, http://www.aam.org.ar). Even if CTX-M-2 (and related enzymes) is the most frequently detected extended-spectrum β-lactamase (ESBL) in Argentina (26–28), accounting for the ESBL-producing profiles in 77% of *E. coli*, 60% of *K. pneumoniae*, and more than 90% of *Proteus mirabilis* isolates, a significant number of isolates are PER- and SHV-derived ESBL producers (26). For unknown reasons, TEM-derived ESBLs have not been reported, as was the case for most other countries (4).

Among the members of the PER family, PER-1 (first identified in 1991) (16) has been responsible for the ESBL profiles in clinically important enterobacteria and nonfermenter gram-negative bacilli isolated in different locations around the world: France (*Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Providencia stuartii*) (12, 16, 22, 23), Italy (*P. aeruginosa*, *Alcaligenes faecalis*, and *P. mirabilis*) (17, 18, 20), Turkey (*Salmonella enterica* serovar Typhimurium, *A. baumanii*, and *P. aeruginosa*) (34–37), Belgium (*P. aeruginosa*) (7), and Korea (*A. baumannii*) (10, 39).

Recently, the genetic environment of the $bla_{\mathrm{PER-1}}$ gene has been elucidated for different species. In some strains, it is part of composite transposons bracketed by different arrangements of insertion sequences (IS), depending on whether it is chromosome or plasmid borne (13, 22). Although there is clear redundancy in the nomenclature for the same IS elements bracketing $bla_{\mathrm{PER-1}}$, which should be clarified in the future, we will retain both throughout the paper, as their descriptions were almost simultaneous.

Biochemical analysis and the crystal structure of PER-1 have also been reported (3, 16, 20, 33).

On the other hand, although PER-2 was first identified in 1996 from a *Salmonella* serovar Typhimurium isolate (1), there is evidence for its presence as early as 1989, from *P. mirabilis* isolated in Argentina, although a different name (ARG-1) was proposed for the enzyme at that time (30a). PER-2, sharing 86% amino acid sequence with PER-1, accounts for 10% and 5% of the oxyimino-cephalosporin-resistant *K. pneumoniae* and *E. coli* isolates (26). Since its first report, it has been found in Argentina in other species, such as *K. pneumoniae*, *Enterobacter cloacae*, *Enterobacter aerogenes*, and *Vibrio cholerae* (14, 19, 21, 26), and in a few locations around the world (6, 29).

PER-2 was circulating as early as 1991 in community-acquired enteropathogenic *E. coli* isolates coproducing TEM-116 even before being responsible for nosocomial outbreaks in Montevideo (38).

The aim of this study was to determine the biochemical properties of PER-2, including a kinetic characterization, and to analyze the regulation and genetic structures associated with the plasmid-borne $bla_{\mathrm{PER-2}}$.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Citrobacter freundii* 33587 was isolated in 1999 in Buenos Aires, Argentina. The strain was identified using standard biochemical criteria and commercial systems (API 20E; BioMérieux, France). *E. coli* CAG12177 (Tet^r Nal^r; *E. coli* Genetic Stock Center) was used as a recipient cell for mating experiments; *E. coli* Top10 F′ (Invitrogen) and *E. coli* BL21(DE3) (kindly provided by Bernard Joris, Liège, Belgium) were hosts for transformation experiments.

Plasmid DNA from *C. freundii* 33587 (pCf587) was extracted by the Hansen-Olsen methodology (9). Plasmid vectors pCR2.1-TOPO (TOPO TA Cloning, Invitrogen) and kanamycin-resistant pET28a(+) (Novagen, Germany) were used for routine cloning experiments and as production vectors.

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TABLE 1. Oligonucleotide primers used in this study

Primer name	Nucleotide sequence (5′→3′)	Target DNA	Purpose or use	Source
PER-2A	ATGAATGTCATCACAAAATGTGT	*bla*$_{\text{PER-2}}$	Screening of *bla*$_{\text{PER-2}}$ and	This study
PER-2B	TCAATCCGGACTCACTGCAG	*bla*$_{\text{PER-2}}$	DNA sequencing	This study
PER2-FS[a]	AATAACGAGCTCAGTCGTATGAAT	*bla*$_{\text{PER-2}}$	Cloning in pET28a(+) for	This study
PER2-RX[b]	CAGATCATGACTCGAGTTTATACC	*bla*$_{\text{PER-2}}$	PER-2 production	This study
ISPa12A	ACAATCGCTGATATACATCG	*tnpA* of IS*Pa12*/IS*1387a*	PCR mapping and sequencing	22
ISPa12B	GATCTCGCTTTACATTTACC	*tnpA* of IS*Pa12*/IS*1387a*	PCR mapping and sequencing	22
ISPa13A	TAACCATATGCACTCAACGG	*tnpA* of IS*Pa13*/IS*1387b*	PCR screening	22
ISPa13B	GGTATCCACCACATATGGGC	*tnpA* of IS*Pa13*/IS*1387b*	PCR screening	22
ISPa14A	AATCAAATGTCCAACCTGCC	*tnpA* of IS*Pa14*/IS*1012*$_R$	PCR screening	22
ISPa14B	GCCTAATTCGATGCCTTAT	*tnpA* of IS*Pa14*/IS*1012*$_R$	PCR screening	22
ISPrst1A	ATTTCTGGAACTTTAACGAC	*tnpA* of IS*Prst1*	PCR screening	22
ISPrst1B	GACAGTCATTTTTTCAAGGC	*tnpA* of IS*Prst1*	PCR screening	22
PerP1	ATCGCCCTGATGATCTTT	*bla*$_{\text{PER-2}}$	5′-RACE cDNA synthesis	This study
PerP2	GGCGACCAGGTATTTTGTAA	*bla*$_{\text{PER-2}}$	Target cDNA amplification	This study
PER2-UP1	CATCATTGGCGACCAGGT	*bla*$_{\text{PER-2}}$	TAIL-PCR and sequencing	This study
PER2-UP2	CCCACACTGCTACACCTACA	*bla*$_{\text{PER-2}}$	TAIL-PCR and sequencing Nested amplification	This study
PER2-UP3	ATCAGCAGAGCAGAAGCGG	*bla*$_{\text{PER-2}}$	TAIL-PCR and sequencing Nested amplification	This study
PER2-DN1	CCACAGGACCACAGCGGT	*bla*$_{\text{PER-2}}$	TAIL-PCR and sequencing	This study
PER2-DN2	AACTGCGGCGACTAATGATGC	*bla*$_{\text{PER-2}}$	TAIL-PCR and sequencing	This study
PER2-DN3	ACTCTCTGCAGTGAGTCCGG	*bla*$_{\text{PER-2}}$	TAIL-PCR and sequencing	This study
AD1	NGTCGASWGANAWGAA	Arbitrary random target	TAIL-PCR	11
AD3	AGWGNAGWANCAWAGG	Arbitrary random target	TAIL-PCR	11
AD6	WGTGNAGWANCANAGA	Arbitrary random target	TAIL-PCR	11
OPA-2	TGCCGAGCTG	Arbitrary random target	TAIL-PCR	11
AAP	GGCCACGCGTCGACTAGTACGGGII GGGIIGGGIIG	*bla*$_{\text{PER-2}}$	Target cDNA amplification	This study
AUAP	GGCCACGCGTCGACTAGTAC	*bla*$_{\text{PER-2}}$	Nested amplification	This study

[a] Includes a SacI restriction site (underlined).
[b] Includes an XhoI restriction site (underlined).

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Antimicrobial susceptibility. MICs were determined by the agar dilution method, following CLSI guidelines (15), using a Steers' multipoint inoculator. Detection of ESBLs was performed by a double-disk synergy test, using ampicillin/clavulanate disks (10 plus 10 μg) placed between 30-μg cefotaxime and ceftazidime disks. All the disks were from Britania, Argentina.

Analytical isoelectric focusing. Crude extracts were obtained from overnight cultures in LB supplemented with 100 μg/ml ampicillin and resolved by isoelectric focusing as described previously (30).

PCR screening of the *bla*$_{\text{PER-2}}$ gene and insertion sequences. The PER-2-encoding gene was amplified using PER-2A and PER-2B primers, and *bla*$_{\text{PER-1}}$-associated IS elements were screened using specific primers (Table 1). All PCRs were performed using pCf587 (50 ng) as a template, and PCR amplicons were resolved in 1% agarose gels, using commercial standards (1-kb DNA Ladder; MBI-Fermentas, Lithuania).

Conjugative transfer of pCf587. Conjugative mobilization of pCf587 was attempted by agar medium mating to recipient *E. coli* CAG12177 cells. Briefly, equivalent volumes of log-phase cultures of *C. freundii* 33587 and *E. coli* recipient cells were deposited on nitrocellulose membranes lying on Mueller-Hinton agar plates. After 4 h of incubation at 37°C, the mating mixture was spread on LB agar plates supplemented with 5 μg/ml ceftriaxone, 10 μg/ml tetracycline, and 30 μg/ml nalidixic acid.

Determination of *bla*$_{\text{PER-2}}$ flanking sequences. Two strategies were used for studying the *bla*$_{\text{PER-2}}$ flanking regions. A PCR mapping approach was attempted by combining specific primers for *bla*$_{\text{PER-2}}$ and the IS, which were detected by PCR screening, in order to assess the gene arrangement. When the *bla*$_{\text{PER-2}}$ surroundings could not be detected by PCR mapping, a thermal asymmetric interlaced (TAIL)-PCR strategy was followed as described previously (11). TAIL-PCR consisted of three consecutive amplifications using nested primers complementary to *bla*$_{\text{PER-2}}$, named PER2-DN1, PER2-DN2, and PER2-DN3, and each of the arbitrary degenerate primers (in separate reactions) AD1, AD3, AD6, and OPA-2, which randomly hybridize to adjacent sequences (Table 1). The resulting fragments were resolved in 1% agarose gels, and those above 1 kb were cloned in a pCR2.1-TOPO vector and sequenced, starting with universal primers.

Recombinant DNA methodology. Basic recombinant DNA procedures were carried out as described by Sambrook et al. (31). For cloning *bla*$_{\text{PER-2}}$, a PCR was performed with pCf587, using 3 U *Pfu* polymerase (Promega) and 1 μM PER2-FS and PER2-RX primers (Table 1), and the purified amplicon was ligated in a pCR2.1-TOPO vector. The identity of the *bla*$_{\text{PER-2}}$ gene, as well as the absence of aberrant nucleotides, was checked by double-strand sequencing of the insert using the same primers. The resulting recombinant plasmid (pT2P-C5) was digested with SacI and XhoI, and the released insert was cloned in a pET28a(+) vector. The ligation mixture was used to first transform competent *E. coli* Top10 F′ cells, and after selection of recombinant clones, a second transformation was performed in *E. coli* BL21(DE3) cells in LB agar plates supplemented with 30 μg/ml kanamycin. Positive recombinant clones were screened by PCR with *bla*$_{\text{PER-2}}$-specific primers, and the *E. coli* BLEP281-1 clone (harboring the pEPC281 plasmid) was used for the PER-2 production experiments.

Determination of transcription initiation sites. *C. freundii* 33587 total RNA was extracted using an RNeasy Midi kit (QIAGEN) according to the manufacturer's recommendations. 5′ Rapid amplification of cDNA ends reactions were performed with 3.5 μg total RNA and a 5′-RACE system kit (Invitrogen), following the manufacturer's guidelines. cDNA synthesis was primed with PerP1-specific primer; dC-tailing at the cDNA 3′ end was performed according to the manufacturer's instructions. Amplification of target cDNA was performed with tailed cDNA as templates using PerP2 and AAP primers. A nested amplification was carried out using PER2-UP2 or PER2-UP3 and AUAP primers.

DNA sequencing and sequence analyses. DNA sequences were determined in both strands by the automated dideoxy chain termination method of Sanger et al. (32) in an automated sequencer (ABI 3100; Applied Bio-System, Spain). Nucleotide and amino acid sequence analyses were performed with NCBI (http://www.ncbi.nlm.nih.gov/) and European Bioinformatics Institute (http://www.ebi.ac.uk/) analysis tools.

Production and purification of PER-2. Overnight cultures of recombinant *E. coli* BLEP281-1 (containing the bla_{PER-2} gene) were diluted (1/50) in 400 ml LB containing 30 μg/ml kanamycin and grown at 37°C to an optical density at 600 nm of 0.8. In order to induce β-lactamase expression, 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was added, and cultures were grown at 37°C for 3 h. Crude extracts were obtained by freeze-thawing using a dry-ice–ethanol mixture. After centrifugation, clear supernatants containing PER-2 were dialyzed against 20 mM Tris-HCl buffer, pH 7.6, and loaded onto a Q-Sepharose column (XK 16/10; Pharmacia), connected to an ÁKTA-purifier (GE Healthcare) equilibrated with the same buffer. The column was extensively washed to remove unbound proteins, and β-lactamases were eluted with a linear gradient of NaCl (0 to 1 M) in the same buffer. β-Lactamase active fractions (detected by an iodometric system using 500 μg/ml ampicillin as a substrate) were pooled and loaded onto a Sephadex G-100 column (2.0 by 20 cm; Pharmacia-LKB, Sweden) equilibrated with 20 mM phosphate buffer, pH 7.5. Elution was performed with the same buffer, and active fractions were collected. After purification, samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 12% polyacrylamide gels, in order to assess the degree of purification.

Determination of β-lactamase activity. β-Lactamase activity was determined spectrophotometrically by measuring the hydrolysis of 100 μM nitrocefin as a substrate. One unit of β-lactamase activity was defined as the amount of enzyme, determined by the Bio-Rad Protein Assay kit (Bio-Rad), that hydrolyzes 1 μmol of substrate per min (in 20 mM phosphate buffer, pH 7.0) at 30°C.

Mass spectrometry. The molecular mass of PER-2 was determined by high-performance liquid chromatography–mass spectrometry, using a C_4 Vydac column (30 by 1.0 mm) in an LCQ Duo ESI-Ion Trap (Thermo Fisher Scientific, Inc. [formerly Finnigan], Waltham, MA).

Determination of kinetic parameters. Hydrolysis of β-lactam antibiotics by purified PER-2 was monitored by following the absorbance variation, using a Shimadzu UV-2101 spectrophotometer equipped with thermostatically controlled cells. Reactions were performed in a total volume of 500 μl at 30°C. For good substrates, the steady-state kinetic parameters (K_m and k_{cat}) were determined under initial-rate conditions as described previously (2). In cases of low K_m values, or for poor substrates and inactivators, apparent K_m values were determined as competitive inhibitor constants (K_i) by monitoring the residual activity of the enzyme in the presence of the drug and 100 μM nitrocefin as a reporter substrate, while the k_{cat} for poor substrates was determined by analyzing the complete hydrolysis time courses (8). Tested drugs, along with the wavelengths and extinction coefficients used, were the same as those described previously (24).

Nucleotide sequence accession number. Sequence data were deposited in the GenBank/EMBL nucleotide databases under the accession number AM409516.

RESULTS AND DISCUSSION

Antimicrobial susceptibility and preliminary biochemical analysis. *C. freundii* 33587 was resistant to all tested β-lactams, except cephamycins and carbapenems. The isolate was also resistant to kanamycin, chloramphenicol, and trimethoprim-sulfamethoxazole (Table 2). By double-disk tests, clavulanate was able to substantially increase cefotaxime and ceftazidime inhibition zones, suggesting the presence of at least one ESBL.

Analytical isoelectric focusing showed a single band of pI 5.4, which hydrolyzed both ampicillin and ceftriaxone when they were used as revealing substrates (data not shown). This result, supported by the MICs, suggested the presence of at least one enzyme with extended-spectrum activity, compatible with PER β-lactamases or extended-spectrum TEM variants.

The absence of additional bands compatible with AmpC enzymes (basic pI), even when extracts were obtained under induction conditions, is consistent with the unusual behavior of the isolate toward cefoxitin, which could be due to malfunctions in the *ampC* regulatory system.

PCR screening, sequencing of *bla* genes and IS elements, and genetic location. An ~900-bp amplicon was obtained by PCR screening with bla_{PER-2}-specific primers from DNA extracts that yielded a large-molecular-size plasmid (pCf587), which was estimated as >54 kb, compared to plasmids of known size (25). DNA sequencing yielded a 927-bp fragment with 100% nucleotide identity with the bla_{PER-2} gene. By PCR, we were also able to detect a bla_{TEM} gene from the same preparation (data not shown).

TABLE 2. Antimicrobial susceptibilities

Antibiotic	MIC (μg/ml)		
	C. freundii 33587	*E. coli* 33587-TC9	*E. coli* CAG12177
Ampicillin	>512	>512	0.25
Ampicillin/clavulanate	4	4	0.125
Piperacillin	256	256	0.063
Cephalothin	512	>512	0.5
Cefoxitin	1	0.125	≤0.063
Cefotaxime	64	64	≤0.063
Cefotaxime/clavulanate	0.063	0.063	≤0.063
Ceftazidime	128	>128	≤0.063
Ceftazidime/clavulanate	1	2	≤0.063
Cefepime	16	8	≤0.063
Aztreonam	256	256	≤0.016
Imipenem	0.063	0.125	≤0.063
Gentamicin	2	2	≤0.25
Kanamycin	>128	>128	≤0.25
Tetracycline	0.5	32	32
Nalidixic acid	2	>128	>128
Chloramphenicol	>32	>32	2
Trimethoprim/sulfamethoxazole	>8/152	>8/152	≤1/19

After attempting the detection of specific IS elements usually associated with bla_{PER-1}, we obtained compatible amplicons only when IS*Pa12*/IS*1387a tnpA* (transposase) gene-specific primers were used, suggesting that the other described bla_{PER-1}-associated IS elements are absent in PER-2-producing strains.

We succeeded in transferring pCf587 by conjugation to *E. coli* CAG12177 host cells. The antimicrobial susceptibility of a selected transconjugant clone (*E. coli* 33587-TC9) is shown in Table 2. It is noteworthy that β-lactam resistance was cotransferred along with kanamycin, chloramphenicol, and TMS resistance, suggesting that these markers are harbored by the same plasmid.

Plasmid extraction on a transconjugant clone showed the presence of a plasmid having the same electrophoretic mobility as pCf587 from the donor *C. freundii* isolate. By PCR on a plasmid obtained from the *E. coli* 33587-TC9 clone, we were able to detect the presence of bla_{PER-2}, as well as associated elements, such as the IS*Pa12*/IS*1387a tnpA* gene.

These results confirm the plasmid location of both bla_{PER-2} and associated structures, as well as the ability to be transferred by conjugation, along with other resistance determinants.

***bla*$_{PER-2}$ is associated with IS*Pa12*/IS*1387a*.** By a combination of PCR-mapping and TAIL-PCR strategies, we were able to analyze the genetic environment of the bla_{PER-2} gene.

Figure 1 shows the architecture of the PER-2-encoding gene and neighboring sequences, covering 3,294 bp. The structure is homologous to those associated with plasmid-borne bla_{PER-1} in *Salmonella* serovar Typhimurium and *A. baumannii* isolates (5, 22, 23).

Upstream of bla_{PER-2}, we found a 1.4-kb sequence including

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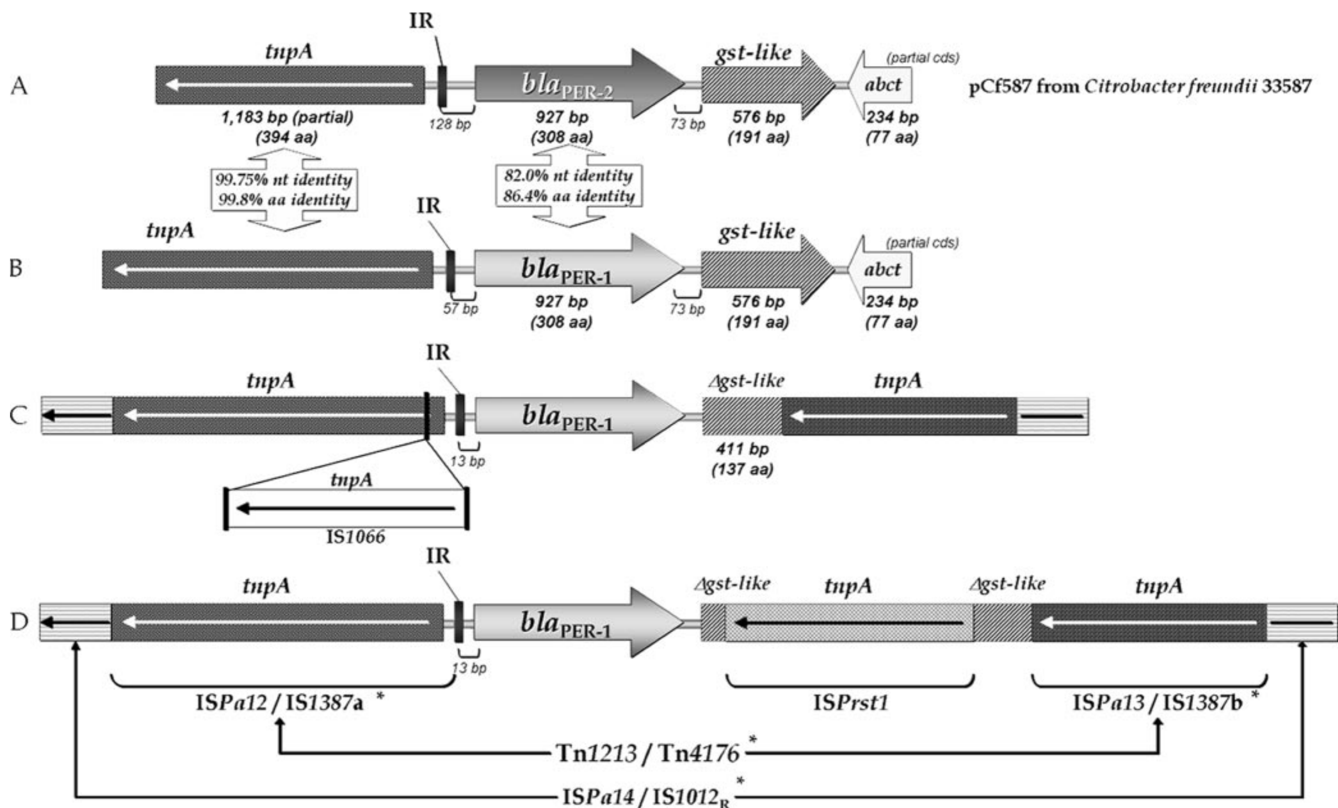

FIG. 1. Schematic representation of the bla_{PER-2} gene and neighboring sequences compared to those for bla_{PER-1} genes. (A) *C. freundii* 33587 (plasmid borne). (B) *Salmonella* serovar Typhimurium and *A. baumannii* (plasmid borne). (C) *P. aeruginosa*, *A. baumannii* (chromosome encoded), and *A. faecalis* (plasmid borne). (D) *P. stuartii* (chromosome encoded). The patterns represent different genetic backgrounds. *, alternative names given in different references; nt, nucleotide.

part of the IS*Pa12 tnpA* gene, also known as IS*1387a tnpA1*. This genetic element has also been found associated with all of the bla_{PER-1}-harboring structures analyzed so far in either chromosomal or plasmid locations (13, 22). The *C. freundii* IS*Pa12*/IS*1387a* transposase shares 99.8% amino acid sequence with that of the bla_{PER-1}-harboring microorganisms, suggesting a relatively recent association of this IS with both bla_{PER} genes. Homologous left inverted repeats (IR) were found upstream of all *tnpA* genes, with the consensus sequence AAGATCATACGTATGA**G** in the bla_{PER-2}-associated structure compared to AAGATCATACGTATGA**A** (the single mutation is in boldface) in bla_{PER-1}-producing *P. aeruginosa* and *A. faecalis* isolates (12, 13); in another bla_{PER-1}-producing *P. aeruginosa* isolate, the homologous IR consensus sequence was proposed to include only 11 bp (CATACGTATGA) (22), but it is nevertheless within the hypothetical 17-bp consensus sequence of the other structures. The left IR, representing the boundary of IS*Pa12*/IS*1387a*, is located at different positions upstream of bla_{PER} genes (Fig. 1), the farthest in our case (128 bp). As others have suggested, this could be evidence that different sites directed the IS*Pa12*/IS*1387a* insertion (22) and could also be related to differences in bla_{PER} expression (see below).

Seventy-three nucleotides downstream of bla_{PER-2} lies a 576-bp open reading frame, *gst*-like, encoding a hypothetical protein having 51% amino acid identity to a putative glutathione *S*-transferase from the water microorganisms *Alteromonas macleodii* and *Marinobacter aquaeloei*. This *gst*-like gene is also present in the plasmid-borne bla_{PER-1} from *Salmonella* serovar Typhimurium and *A. baumannii* (22).

A second open reading frame, *abct*, is located downstream of the *gst*-like gene in both *C. freundii* 33587 and some isolates producing plasmid-borne PER-1 (Fig. 1). The deduced amino acid sequence possesses 87% identity with a putative ABC transporter from *Shewanella oneidensis*. Whether the *abct* gene is also present in the bla_{PER-1}-harboring structures is not known, due to lack of sequence information far beyond the database entries.

Two transcripts may be involved in bla_{PER-2} expression. An initial +1 transcription start site was located 227 bp upstream from the bla_{PER-2} start codon (Fig. 2), embedded within IS*Pa12*/IS*1387a*. We deduced a −35 consensus sequence (TT CAAA) separated by 17 bp from a −10 box (TAATTT), constituting the bla_{PER-2} P_{Cf1} promoter (Fig. 2). These elements are homologous to that described for bla_{PER-1} in different *P. aeruginosa* isolates (12, 22), differing only in the +1 start site location (112 bp upstream from the bla_{PER-1} ATG) and the −10 consensus sequence (TAATCT).

The +1 transcription start site of a second putative promoter, named P_{Cf2}, could be located 63 bp upstream from the bla_{PER-2} start codon. Only a putative −10 box could be deduced (TATGAA), whereas a clear −35 consensus sequence

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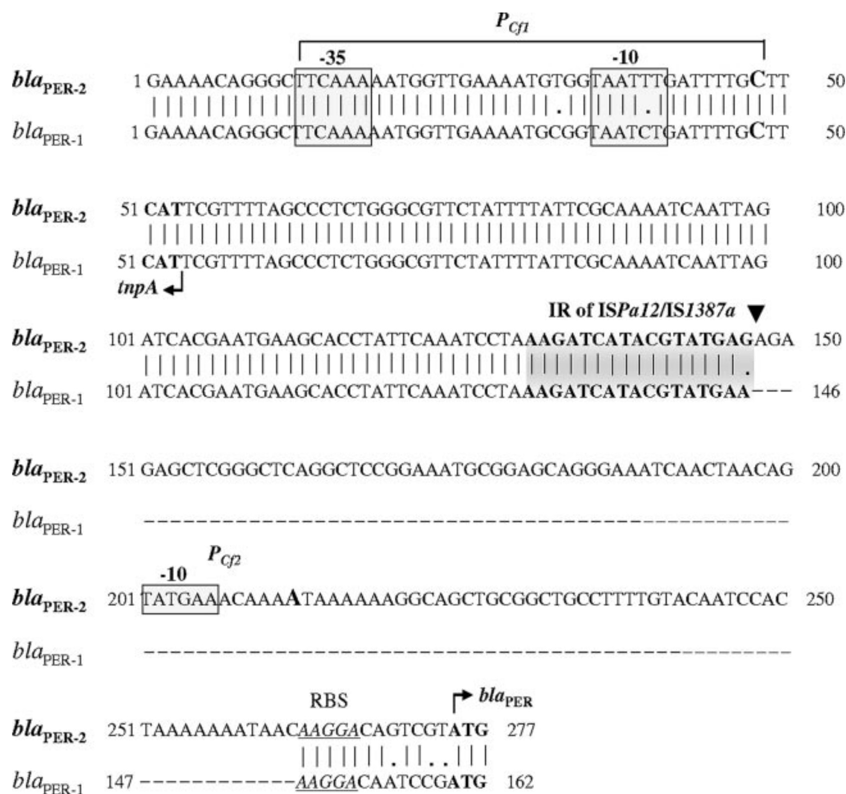

FIG. 2. Comparison of the upstream sequences of bla_{PER-2} from *C. freundii* 33587 and bla_{PER-1} from *P. aeruginosa* RNL-1 (AY779402), comprising the respective promoter regions. The +1 transcription initiation sites are indicated in large boldface letters, respective −10 and −35 boxes are in light-gray boxes, and entire promoters are marked with horizontal brackets. The IR of IS*Pa12*/IS*1387a* is indicated in boldface letters in a shaded box, and the inverted black triangle indicates the IS*Pa12*/IS*1387a* boundary.

could not be inferred (Fig. 2). This promoter would not belong to the IS*Pa12*/IS*1387a* backbone but to an additional 127-bp sequence (between the bla_{PER-2} start codon and the IS IR) that is absent in the *P. aeruginosa* bla_{PER-1} upstream region (Fig. 2).

It was previously suggested that IS*Pa12*/IS*1387a* could drive expression of plasmid-borne bla_{PER-1} genes in *Salmonella* serovar Typhimurium and *P. aeruginosa* strains, provided the respective P_{St} and P_{Pa} promoters are part of the insertion sequence (22). Therefore, although speculative, a putative second promoter (P_{Cf2}) could enhance bla_{PER-2} expression independently of IS*Pa12*/IS*1387a*.

Kinetic analysis of PER-2 revealed high oxyimino-cephalosporinase activity. A total of 9.7 mg (498.5 mU) of highly pure PER-2 β-lactamase was obtained by fast-protein liquid chromatography-based chromatography, with a final yield of 31%. The experimental molecular mass of PER-2 obtained by mass spectrometry was 30,780 Da, in good agreement with the theoretical mass (30,769 Da), and the predicted signal peptide was 26 amino acids long.

The main kinetic parameters of PER-2 are shown in Table 3. According to its extended-spectrum activity, PER-2 showed high catalytic efficiencies (k_{cat}/K_m) toward most of the tested antibiotics, generally characterized by low K_m and high k_{cat} values. Notably, the hydrolytic behavior with both tested oxyimino-cephalosporins was characterized by a sevenfold-higher affinity toward cefotaxime, which is nevertheless contrasted by a turnover constant (k_{cat}) fourfold higher for ceftazidime, resulting in similar catalytic efficiencies and therefore phenotypic high-level resistance to both antibiotics.

Some differences arose when the kinetic behavior of PER-2 was compared to the available data for PER-1 (3, 20). The most remarkable difference was the overall behavior toward oxyimino-cephalosporins. According to Bouthors et al. (3), k_{cat}/K_m values for both cephalosporins are 1 order of magnitude lower (0.093 and 0.026 $\mu M^{-1} \cdot s^{-1}$ for cefotaxime and ceftazidime, respectively), due to a 10-fold-higher K_m. On the other hand, kinetic data for the *A. faecalis* PER-1 is more

TABLE 3. Main kinetic parameters of PER-2 β-lactamase

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)	Relative k_{cat}/K_m (%)[b]
Benzylpenicillin	16 ± 4	2 ± 0.08	0.12 ± 0.03	18
Ampicillin	38 ± 4	12 ± 0.7	0.33 ± 0.05	49
Piperacillin[a]	0.2 ± 0.012	0.04 ± 0.002	0.2 ± 0.02	30
Nitrocefin	32 ± 6	48 ± 4	1.5 ± 0.4	224
Cephalothin	9 ± 1.8	6 ± 0.2	0.67 ± 0.16	100
Cefoxitin[a]	0.14 ± 0.005	<0.001		
Cefuroxime	21 ± 2	6 ± 0.12	0.3 ± 0.03	45
Cefotaxime	46 ± 8	34 ± 3	0.76 ± 0.19	113
Ceftazidime	320 ± 50	140 ± 17	0.43 ± 0.12	64
Cefoperazone	5 ± 0.9	0.5 ± 0.02	0.10 ± 0.02	15
Cefepime	16 ± 4	0.39 ± 0.02	0.02 ± 0.006	3
Aztreonam[a]	2 ± 0.04	0.23 ± 0.01	0.12 ± 0.008	18
Imipenem[a]	0.06 ± 0.001	<0.001		

[a] K_m determined as K_i.
[b] Relative to cephalothin.

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similar to that for the *C. freundii* PER-2, with the most important difference being a 10-fold-lower catalytic efficiency for ceftazidime (0.03 $\mu M^{-1} \cdot s^{-1}$), due to a higher K_m value (806 μM) (20).

The most poorly hydrolyzed antibiotics were cefoxitin, cefepime, and imipenem. On the other hand, PER-2 was strongly inhibited by lithium clavulanate and tazobactam, displaying 50% inhibitory concentrations of 0.068 and 0.096 μM, respectively.

Conclusions. Considering the amino acid identity (82%), it is noteworthy that the highly conserved conformational structures of both PER-1 and PER-2 are observed in three-dimensional models (data not shown). It was demonstrated that PER-1 and other related β-lactamases possess a new fold in the Ω loop and an insertion of four residues at strand S3 compared to other class A enzymes, which generate a broader cavity and allow the accommodation of the bulky substituents of oxyimino-cephalosporins and a more efficient hydrolysis of these drugs (33). In addition, a modification at position 242 in PER-1, which represents the counterpart of the Glu240 residue in TEM or SHV β-lactamases, does not seem to result in changes in its kinetic properties, as does occur in TEM/SHV (3). On the other hand, PER-2 displays an amino acid shift at position 242 (Lys242Arg), and we observed some discrepancies in kinetic behavior between PER-2 and PER-1 (see above). If the above hypothesis is correct, then we can assume that other amino acid modifications are probably responsible for the differences in their kinetic properties.

The presence of similar structures upstream of bla_{PER} genes suggests a common history of recruitment and mobilization. However, differences in the lengths of *tnpA*-bla_{PER} intergenic regions are probably evidence of the presence of diverse target sequences used for the insertion of IS*Pa12*/IS*1387a*.

Transcription of the bla_{PER-2} gene seems to be directed at least by a promoter embedded in the IS*Pa12*/IS*1387a* upstream element. A second putative transcription site origin outside the IS backbone could probably also be implicated in the expression of bla_{PER-2}, although the real biological role of this promoter is not known.

The presence of bla_{PER-2} in a conjugative plasmid explains the prevalence of PER-2 in Argentina and neighboring countries (26, 38). It is noteworthy that only PER-1 has been found outside South America. At least two opposing alternatives can account for these results, the first being that primers used for detection of PER-related enzymes are usually based on bla_{PER-1}, explaining the possibilities for being underestimated; the second would be to consider that bla_{PER-1} association with IS*Pa13*/IS*1387b* (absent in our case) or different plasmid backgrounds could be driving its dissemination. The coincidence of the isoelectric points (pI 5.4) could have made it difficult to differentiate it from most TEM-derived ESBLs.

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