ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 1994, p. 104–114
0066-4804/94/$04.00+0

Vol. 38, No. 1

Sequence Analysis of PER-1 Extended-Spectrum β-Lactamase from *Pseudomonas aeruginosa* and Comparison with Class A β-Lactamases

PATRICE NORDMANN[1,2]* AND THIERRY NAAS[2]

Service de Microbiologie, Hôpital Raymond Poincaré, Faculté de Médecine Paris-Ouest, Université Paris 5, 92380 Garches, France,[1] and Abteilung Mikrobiologie, Biozentrum der Universität Basel, 4056 Basel, Switzerland[2]

Received 28 June 1993/Returned for modification 14 September 1993/Accepted 28 October 1993

We have determined the nucleotide sequence (EMBL accession number, Z 21957) of the cloned chromosomal PER-1 extended-spectrum β-lactamase gene from a *Pseudomonas aeruginosa* RNL-1 clinical isolate. bla_{PER-1} corresponds to a 924-bp open reading frame which encodes a polypeptide of 308 amino acids. This open reading frame is preceded by a −10 and a −35 region consistent with a putative *P. aeruginosa* promoter. Primer extension analysis of the PER-1 mRNA start revealed that this promoter was active in *P. aeruginosa* but not in *Escherichia coli*, in which PER-1 expression was driven by vector promoter sequences. N-terminal sequencing identified the PER-1 26-amino-acid leader peptide and enabled us to calculate the molecular mass (30.8 kDa) of the PER-1 mature form. Analysis of the percent GC content of bla_{PER-1} and of its 5′ upstream sequences, as well as the codon usage for bla_{PER-1}, indicated that bla_{PER-1} may have been inserted into *P. aeruginosa* genomic DNA from a nonpseudomonad bacterium. The PER-1 gene showed very low homology with other β-lactamase genes at the DNA level. By using computer methods, assessment of the extent of identity between PER-1 and 10 β-lactamase amino acid sequences indicated that PER-1 is a class A β-lactamase. PER-1 shares around 27% amino acid identity with the sequenced extended-spectrum β-lactamases of the TEM-SHV series and MEN-1 from *Enterobacteriaceae* species. The use of parsimony methods showed that PER-1 is not more closely related to gram-negative than to gram-positive bacterial class A β-lactamases. Surprisingly, among class A β-lactamases, PER-1 was most closely related to the recently reported CFXA from *Bacteroides vulgatus*, with which it shared 40% amino acid identity. This work indicates that non-*Enterobacteriaceae* species such as *P. aeruginosa* may possess class A extended-spectrum β-lactamase genes possibly resulting from intergeneric DNA transfer.

Among the known β-lactamases in *Pseudomonas aeruginosa*, induced and derepressed Ambler class C chromosomal cephalosporinases may lead to failure of therapeutic regimens which include extended-spectrum β-lactam antibiotics such as cefotaxime, ceftazidime, ceftriaxone, and aztreonam. In *Enterobacteriaceae* species, plasmid-mediated extended-spectrum β-lactamases (ESbla) have been extensively reported; they hydrolyze extended-spectrum β-lactams, and their enzymatic activity is totally or partially inhibited by β-lactam inhibitors such as clavulanic acid and sulbactam (45). These ESbla are Ambler class A β-lactamases differing from TEM-1, TEM-2, and SHV-1 restricted-spectrum β-lactamases by a few amino acids near their active sites, thus explaining the extension of their hydrolytic activity (24, 45, 52, 53). ESbla, primarily found in *Klebsiella pneumoniae*, were later reported in most of the *Enterobacteriaceae* species responsible for nosocomial outbreaks of multiple-resistant strains (45).

We have recently described the presence of an ESbla, PER-1 β-lactamase, in a *P. aeruginosa* clinical isolate (42). Cloning of this ESbla gene into an *Escherichia coli* plasmid vector isolated it from the chromosomal cephalosporinase gene of *P. aeruginosa* and therefore led us to analyze the strong hydrolytic activity of this ESbla towards ceftriaxone, cefotaxime, ceftazidime, and, to a lesser extent, aztreonam (42). PER-1 activity was inhibited by clavulanic acid, sulbactam, and, uncommonly for an ESbla, by moxalactam and imipenem. Surprisingly, a 1.1-kb internal probe of the cloned fragment from *P. aeruginosa* RNL-1 which encoded PER-1 failed to hybridize with plasmids that encode β-lactamases of the TEM, SHV, OXA, and CARB-PSE types and with the *ampC* gene from *P. aeruginosa*. These negative hybridization results suggested that the PER-1 sequence did not derive from any of the described β-lactamases in *P. aeruginosa*. We, thus, sequenced the DNA of the chromosomal PER-1 gene.

This report shows that, although a member of Ambler class A β-lactamases, the PER-1 protein sequence differs not only from all sequenced ESbla but also from the class A β-lactamases found in gram-negative and gram-positive bacteria. Moreover, DNA sequence analysis of the PER-1 gene and of its upstream and downstream DNA sequences suggested that PER-1 gene insertion within *P. aeruginosa* genomic DNA may have resulted from an intergeneric gene transfer.

MATERIALS AND METHODS

Bacterial strains and plasmids. *P. aeruginosa* RNL-1, which encodes PER-1 β-lactamase, was isolated from the urinary tract of a hospital patient in France (42). The recombinant plasmid pPZ1 has previously been described

* Corresponding author. Present address: Service de Microbiologie, Hôpital Raymond Poincaré, 104 Bd Raymond Poincaré, 92380, Garches, FRANCE. Phone: 33 1 47 10 79 00 ext. 5461. Fax: 33 1 47 10 78 23.

(42) (see Fig. 1). It contains a 2.1-kb *Sau*3A fragment from the chromosome of *P. aeruginosa* RNL-1 cloned into the *Bam*HI site of pACYC 184 (8). Plasmid pPZ1 in the *E. coli* JM109 reference strain expresses a β-lactamase with hydrolytic properties towards extended-spectrum cephalosporins similar to those described for the *Enterobacteriaceae* ESbla (42). As previously described (42), the pRAZ1 recombinant plasmid was constructed by cloning of a *Sna*BI fragment from pPZ1 into *Sma*I-digested multicopy plasmid pK19 (see Fig. 1). This construct was used to determine the sequence of the PER-1 leader peptide. An additional construct, pRAZ2, was made by using standard molecular techniques (34) (see Results).

Media and chemicals. Luria-Bertani (LB) broth and agar and trypticase soy broth and agar were from Diagnostics Pasteur (Marnes-la-Coquette, France); reverse transcriptase was from Promega (Madison, Wis.). Restriction enzymes and T4 DNA ligase were from New England Biolabs (Beverly, Mass.), [^{32}P]dATP and [^{35}S]dATP were from Amersham (Buckinghamshire, England), and the random primer DNA labeling kit was from Bio-Rad (Richmond, Calif.). Routine chemicals were from Merck (Darmstadt, Germany). Ampicillin (100 μg/ml) and chloramphenicol (25 μg/ml) were from Sigma.

Plasmid DNA preparation. Recombinant plasmid DNA was obtained from LB broth (500-ml) cultures grown with ampicillin overnight at 37°C. The DNA plasmids were purified by alkaline lysis according to the Qiagen protocol (Diagen, Hilden, Germany).

DNA sequencing and protein analysis. The nucleotide sequence was determined by the dideoxy polymerase chain termination method (49) with the Sequenase Version II Sequencing Kit (United States Biochemical Corp., Cleveland, Ohio). Custom 18-mer oligonucleotide primers were synthesized at the Biocenter of Basel University (Switzerland) with phosphoramidite chemistry on an Applied Biosystems 380B DNA synthesizer. Part of the 2.1-kb cloned fragment from pPZ1 was sequenced on both complementary strands. The nucleotide sequence and the deduced protein sequence were analyzed with GCG software (Biotechnology Center, University of Wisconsin—Madison, Madison) on a VAX computer from Digital Corp. (14). The hydrophobicity profile of the deduced protein was predicted by using the GCG program Pepplot, which uses the Kyte and Doolittle method (28). Known DNA and protein sequences were taken for comparison of β-lactamase from European Molecular Biology Laboratory and Swiss-Prot data bases. Multiple alignment of deduced peptide sequences was carried out with the GCG program Pileup using a simplification of the progressive alignment method of Feng and Doolittle (15). This progressive alignment approach utilizes the three-matrix form of the Needleman and Wunsch algorithm (40), which uses the minimum mutation matrix of Dayhoff in its scoring. Among the so-far-sequenced class A β-lactamases, 10 were compared with PER-1: SHV-2 and TEM-3 from *E. coli* (20, 53), since they are commonly found ESbla from *Enterobacteriaceae* species; MEN-1 from *E. coli* (3), the first sequenced non-TEM non-SHV ESbla from *Enterobacteriaceae* species; BLA I from *Yersinia enterocolitica* (50) and ROB-1 from *Haemophilus influenzae* (27), whose gram-negative class A β-lactamase protein sequences differ significantly from those of TEM or SHV β-lactamases; PSE-4 from *P. aeruginosa* (5), a restricted-spectrum β-lactamase commonly found in *P. aeruginosa*; BLIP from *Bacillus licheniformis* (41), CAKCC from *Streptomyces cacaoi* (31) and PC1 from *Staphylococcus aureus* (6) as representing gram-positive class A β-lactamases isolated from phylogenetically unrelated organisms; and CFXA from *Bacteroides vulgatus* (44), since it is the class A β-lactamase with which PER-1 shows the highest amino acid identity. A dendrogram was derived from these multiple β-lactamase alignments by a parsimony method using the phylogeny package PAUP (Phylogenetic Analysis Using Parsimony) version 3.0 (57).

Determination of the transcription start of bla_{PER-1} in *P. aeruginosa* and *E. coli*. mRNAs were extracted according to the modified method from Chomczynski and Saachi (9). *E. coli* JM109 harboring pPZ1, pRAZ1, or pRAZ2 (recombinant plasmids) and *P. aeruginosa* RNL-1 were grown overnight in 5 ml of ampicillin-containing LB broth. These cultures were diluted 1:100 and further grown for 6 h in LB containing ampicillin. Portions (5 ml) of each culture were centrifuged for 10 min at 5,000 × *g*, and the pellets were resuspended in 200 ml of solution A made up of 1 volume of 4 M guanidium thiocyanate, 25 mM sodium citrate (pH 7), 0.5% sarcosyl, 0.1 M 2-mercaptoethanol, 1 volume of saturated phenol in Tris-EDTA buffer, and 0.1 volume of 2 M sodium acetate (pH 4). Then, 40 ml of CH_3Cl was added and the suspension was vigorously vortexed for 15 s, placed on ice for 5 min, and spun down for 15 min at 12,000 × *g*. The aqueous phase was transferred to a new tube, and 2 volumes of ethanol was added; the tubes were then held for 1 h at −70°C and centrifuged for 15 min at 12,000 × *g*. The pellets were suspended in 200 ml of 0.2 NaCl. Two volumes of ethanol was added, and the tubes were held at −20°C for 30 min and then spun down for 15 min at 15,000 × *g*. The pellets were washed in 70% ethanol, spun down for 10 min, Speed Vac dried, and kept at −70°C. Primer extension was then carried out according to the method Geliebter et al. (16). The probe consisted of a 23-mer oligonucleotide (5′-GGATTGCGCT GAGGTTTCGAATG-3′) hybridizing at position 370 to 393 (see Fig. 2).

N-terminal protein sequencing. In order to determine the size of the PER-1 β-lactamase leader peptide, *E. coli* JM109 harboring the pRAZ1 recombinant plasmid was grown overnight at 37°C in 5 ml of ampicillin-containing LB broth. The suspension was then centrifuged at 5,000 × *g* for 5 min at 4°C, resuspended in 500 ml of distilled water, and disrupted by sonication (4 × 20 s at 20 Hz). The suspension was centrifuged (30 min, 20,000 × *g*, 4°C), and the supernatant containing the crude enzyme extracts was isolated. Crude extracts and marker proteins were subjected to electrophoresis in a 12.5% gel (20 mA, 5 h, room temperature) (30). Proteins were then electrotransferred onto a Problot membrane (Applied Biosystems, Foster City, Calif.) by using the Mini Protean II transfer cell (8 by 7.3 cm) (Bio-Rad) in Tris-glycine blotting buffer (25 mM Tris-HCl, 192 mM glycine, MeOH and H_2O in the ratio 10:90 [vol/vol]) at 40 V (300 mA) for 2 h. The membrane was then stained with a solution made of 0.1% Coomassie blue, 1% acetic acid, and 40% methanol for 5 min and destained in MeOH and H_2O (10:80 [vol/vol]) and acetic acid and H_2O (10:80 [vol/vol]). The protein band corresponding to the only known band with β-lactamase activity (42) was then excised with a razor blade and resuspended overnight in distilled water. The PER-1 amino-terminal sequence was determined with an automated Edman sequencer on a model 477A gas phase sequencer (Applied Biosystems) (19, 35).

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the EMBL nucleotide data bases under the accession number Z 21957.

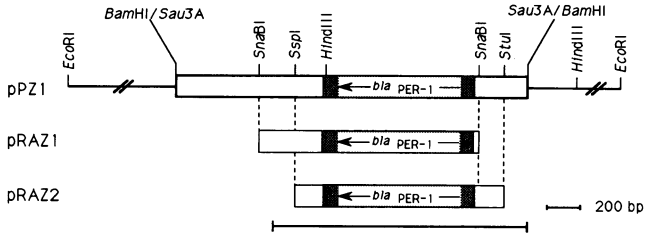

FIG. 1. Restriction endonuclease map of recombinant plasmids which include bla_{PER-1}. pPZ1 is a recombinant plasmid including a 2.1-kb *Sau*3A fragment from *P. aeruginosa* RNL-1 cloned into the *Bam*HI site of pACYC184. From this plasmid, subcloning of a *Sna*BI fragment into a *Sma*I-digested pK19 plasmid was performed, giving pRAZ1. In addition, for transcription analysis, pRAZ2 was constructed, resulting from cloning of *Stu*I-*Ssp*I from pPZ1 into *Ava*I-*Hin*dIII-digested pACYC184. The horizontal bar at the bottom indicates the DNA segment sequenced and shown in Fig. 2.

RESULTS

Subcloning of the PER-1 gene. In order to determine the transcriptional start for PER-1 mRNA in *E. coli* from sources additional to the pPZ1 recombinant plasmid, the PER-1 gene was subcloned, giving rise to pRAZ2 (Fig. 1). This plasmid resulted from cloning of the 1.3-kb *Stu*I-*Ssp*I from pPZ1 into a 2.9-kb *Ava*I-*Hin*dIII-digested blunt-ended pACYC184 vector. This modified cloning vector was made in order to remove the entire coding region of the *tet* gene from pACYC184 but to leave the promoter intact. *E. coli* JM109 harboring pRAZ2 or pPZ1 led to PER-1 expression in *E. coli* JM109, as indicated by antibiotic disc susceptibility testing (data not shown). However, insertion of the 1.3-kb *Stu*I-*Ssp*I fragment into the pACYC184 derivative vector could be obtained in only one direction (*Stu*I side in front of the *Hin*dIII site from pACYC184), giving rise to a recombinant plasmid encoding the β-lactam resistance phenotype.

Sequence analysis of *P. aeruginosa* bla_{PER-1}. The determined nucleotide sequence, which included the PER-1 structural gene and flanking sequences, was 1,519 bp and is shown in Fig. 2. This sequence was included within the 2.1-kb *Sau*3A fragment from *P. aeruginosa* RNL-1 cloned into the *Bam*HI site of pACYC184 (pPZ1). Analysis of the 1,519-bp sequence for coding regions revealed a sufficiently long 924-bp open reading frame which encoded a 308-amino-acid protein, which corresponded in size to a 33.5-kDa protein. Within this protein, a serine-threonine-valine-lysine tetrad (S-V-F-K) (underlined in Fig. 2) was found; it included the conserved serine and lysine amino acid residues characteristic of β-lactamases possessing a serine active site (25). In this open reading frame, two possible ATG initiation codons were found at positions 309 to 311 and 354 to 356 (Fig. 2). No typical *E. coli* consensus promoter sequences were found upstream of these initiation codons. However, a

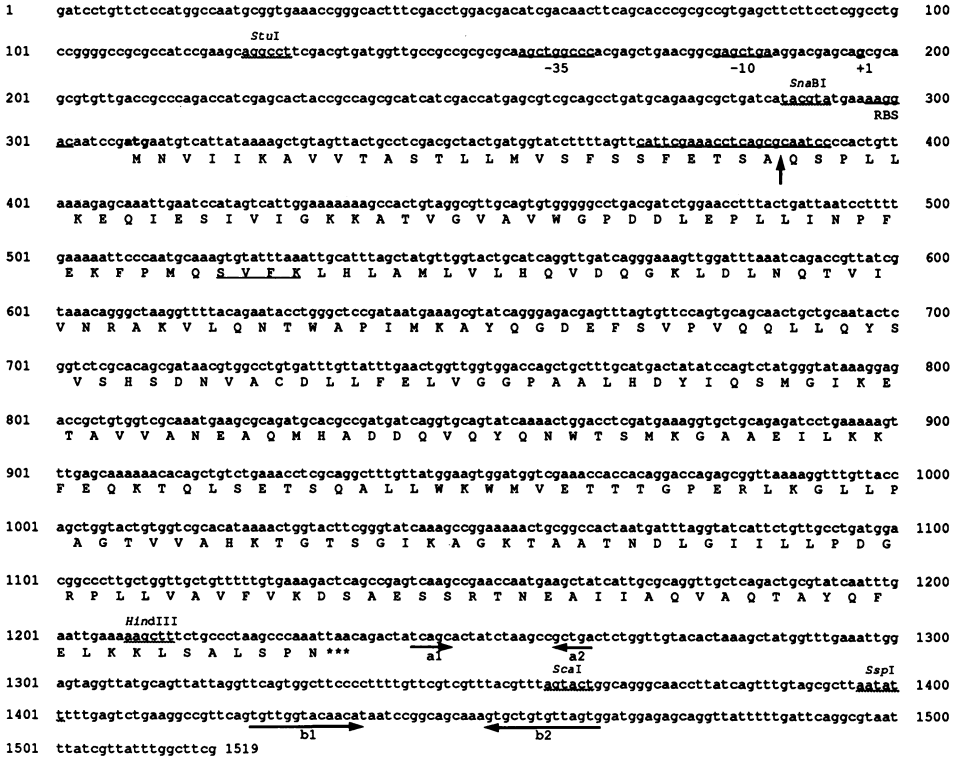

FIG. 2. Nucleotide sequence of the 1,519-bp fragment of pPZ1 containing the PER-1 coding region. The nucleotide sequence is numbered throughout. The deduced PER-1 amino acid sequence is designated in single-letter code below the nucleotide sequence. The PER-1 transcription start in *P. aeruginosa* RNL-1 is indicated by +1. The derived promoter sequence is represented by −10 and −35 regions. RBS indicates a potential ribosome binding site. The dotted underlined sequences indicate restriction endonuclease recognition sites. Reversed arrows indicate inverted repeat sequences (a1-a2 and b1-b2) acting as possible Rho-independent terminators. The PER-1 signal peptide extends from amino acid residues 1 to 26, and the proposed cleavage site is indicated by a vertical arrow. The conserved residues in serine β-lactamases (S-V-F-K) are underlined. The bla_{PER-1} stop codon is indicated with three asterisks. The underlined nucleotide sequence (370 to 392) corresponds to the primer-annealing region for mRNA primer extension analysis.

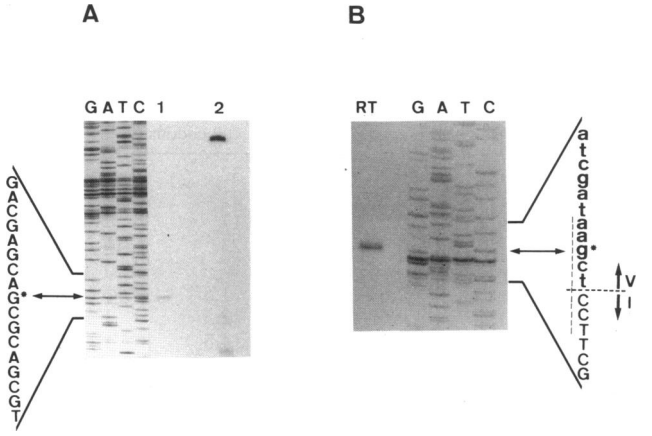

FIG. 3. Mapping of the 5′ end of bla_{PER-1} by primer extension analysis according to a modified method from Geliebter et al. (16). The primer extension products were analyzed on a sequencing gel. (A) Lane 1 corresponds to the mRNA extracted and extended from *P. aeruginosa* RNL-1, and lane 2 corresponds to that from *E. coli* harboring pRAZ2, which contained the cloned bla_{PER-1} gene. The nucleotide sequence on the left corresponds to that of the complementary strand, which has been deduced from the sequencing reaction obtained by using the same primer as this for mRNA extension and by using pPZ1 as template. The star on this nucleotide sequence indicates (corresponding to nucleotide 196, Fig. 2) the mRNA 5′ end of PER-1 as determined in *P. aeruginosa* RNL-1. (B) Lane RT (RNA template) corresponds to the same reaction as that described for lane 2 in panel A. The sequencing reaction was performed with the same primer for mRNA extension and with pRAZ2 as template. The nucleotide sequence on the right corresponds to the complementary strand. The star indicates the mRNA start of PER-1, V stands for vector sequence, and I stands for insert sequence. At the horizontal dotted line, the nucleotide sequence of the insert at position 125 (Fig. 2) starts. On the left of the V and I signs (vertical dotted line), the filling region which has occurred during the cloning experiment to obtain the pRAZ2 recombinant plasmid is shown. The sequencing gel shows a clear compression zone which did not interfere with the mRNA start determination.

purine-rich sequence of six nucleotides (AAGGAC) upstream from the first ATG codon may correspond to a putative ribosome binding site, designated RBS in Fig. 2 (51). The open reading frame was preceded by a region consistent with a −10 region (GAGCTGA) and a −35 region (AGCTGGCCC) of a putative *P. aeruginosa* promoter (Fig. 2). This promoter may correspond to the consensus sequence YTGCTTR, RRNTGGGCAT, of the *rpoN* promoter type from *P. aeruginosa* (47). As shown in Fig. 3A, primer extension analysis indicated that the proposed −10 and −35 promoter regions were the active promoter sequences in *P. aeruginosa* RNL-1. However, the mRNA start for PER-1 β-lactamase, as found in *E. coli* JM109 harboring the pPZ1 recombinant plasmid, did not correspond to the base pair designated +1 in Fig. 2. In this case, the transcriptional start could not be precisely mapped because it was too far away from the extension primer oligonucleotide fixation site, located on the pACYC184 sequence (Fig. 3A) (data not shown). In order to find the location of this promoter, pRAZ2 mRNA was analyzed and its transcription start was located upstream of the base pair designated +1 in Fig. 2 (Fig. 3A). The PER-1 mRNA start in pRAZ2 was exactly located 70 bp upstream from the +1 position in Fig. 2 within the *tet* gene promoter sequence (Fig. 3B). Similarly, with pRAZ1 which resulted from cloning of a *Sna*BI fragment from pPZ1 into *Sma*I-digested plasmid, the PER-1 mRNA start was located within the *lacZ* promoter sequence (data not shown). Thus, the promoter of the proposed −10 and −35 regions was active in *P. aeruginosa* but not in *E. coli* with all the recombinant plasmids used. Analysis of the downstream regions of bla_{PER-1} revealed two palindromic sequences (designated a1-a2 and b1-b2 in Fig. 2) (48). These palindromic sequences may form weak mRNA hairpin loops corresponding to possible Rho-independent transcription terminator signals. The ΔG values for the a1-a2 and b1-b2 putative terminators were −13 kcal/mol (ca. −54 kJ/mol) and −21 kcal/mol (ca. −88 kJ/mol), respectively.

The overall GC content of bla_{PER-1} was 45%, whereas the GC content of *P. aeruginosa* genes (except for pilin genes) ranged from 60.1 to 69.5% (59). Moreover, the pattern of codon usage for bla_{PER-1} was not typical of *P. aeruginosa* (Table 1). Usually, *P. aeruginosa* genes exhibit a strong bias for cytosine or guanine in the wobble position; NNC codons are used 54.5% of the time, NNG codons are used 34.2% of the time, NNT codons are used 6% of the time, and NNA codons are used 5.3% of the time. In bla_{PER-1}, the corresponding values were NNC, 15.3%; NNG, 28.5%; NNT, 29%; and NNA, 15%. In addition, 26 codons have been reported as being used rarely, if at all, by *P. aeruginosa* genes and as representing less than 10% of the codons chosen from a specific synonymous codon group (59). Among them, GGA, GTA, GCA, GTT, GCT, TCA, TCT, AAT, ATA, ATT, ACT, TTG, TTA, TTT, CCA, and CCT were widely used in bla_{PER-1} (Table 1). Moreover, the translation stop codon of bla_{PER-1} (TAA) corresponded to that usually found in *E. coli* and not in *P. aeruginosa* (which has TAG). The codon usage in bla_{PER-1} was more closely related to that of *E. coli* genes than to that of *P. aeruginosa* genes (Table 1). The GC content of the 308-bp upstream sequence from bla_{PER-1} was 63% and that of the 200-bp downstream sequence was 42%, a value similar to that found for bla_{PER-1}.

From the sequencing data, one would expect the first 26 amino acids, which contain numerous hydrophobic residues (MNVIIKAVVTASTLLMVSFSSFETSA) as found by hydrophobicity analysis, to be the leader peptide. As indicated by N-terminal protein sequence analysis of the mature PER-1 β-lactamase isolated from *E. coli* JM109 harboring pRAZ1, this was indeed the case (Fig. 2). Edman analysis (9 cycles) determined the N-terminal sequence of PER-1 mature protein as being XXPLLKEQ, in which X represents unidentified amino acid residues due to protein contamination. The cleavage site was therefore deduced to be just after the arginine residue in the ETSA-QSPLLKEQ amino acid sequence (Fig. 2).

Homology with other β-lactamases. The nucleotide sequence of the PER-1 structural gene has less than 20% DNA identity with other known β-lactamase genes. However, comparison of deduced amino acid sequences using gaps to maximize alignment revealed that PER-1 belonged to Ambler class A β-lactamases. Sequence alignment was performed with 10 class A β-lactamases representative of the different subgroups of these β-lactamases including ESbla. The seven canonical boxes described by Joris et al. (25) were found in PER-1 as well as relatively well-conserved regions (Fig. 4). However, PER-1 showed 7 different amino acid residues among the 25 residues which are known to be highly conserved among class A β-lactamases (11). Pairwise comparison with 10 class A β-lactamases showed that PER-1 has only 30 and 26% amino acid identity with PSE-4 and TEM-3, respectively (Table 2). PER-1 shows very low amino acid

TABLE 1. Codon usage of bla_{PER-1}, *P. aeruginosa* genes (59), and *E. coli* genes (58)

Amino acid	Codon	Codon usage in:						
		bla PER-1			*P. aeruginosa* genes		*E. coli* genes	
		No.[a]	No./1,000 codons[b]	Fraction[c]	No./1,000 codons[b]	Fraction[c]	No./1,000 codons[b]	Fraction[c]
Ala	GCG	6	19.4	0.19	33	0.31	32	0.28
	GCA	4	12.9	0.13	3.3	0.03	21	0.27
	GCT	13	42.2	0.42	7.5	0.07	17	0.18
	GCC	8	25.9	0.26	32.3	0.59	23	0.27
Arg	AGG	1	3.2	0.25	2.2	0.03	1.5	0.05
	AGA	0	0	0	0.5	0.01	2.5	0.12
	CGG	2	6.49	0.50	8.6	0.14	4.5	0.10
	CGA	1	3.25	0.25	2	0.03	3.12	0.10
	CGT	0	0	0	6	0.10	24.4	0.30
	CGC	0	0	0	41.5	0.68	21.5	0.32
Asp	GAT	9	29.2	0.69	8.1	0.14	32.8	0.58
	GAC	4	12.9	0.31	51.9	0.84	22.8	0.41
Asn	AAT	8	25.9	0.73	3.1	0.08	16.4	0.50
	AAC	3	9.7	0.27	35.6	0.92	25.5	0.50
Cys	TGT	1	3.25	1	0.7	0.05	4.7	0.52
	TGC	0	0	0	12.7	0.95	6.3	0.48
Gly	GGG	1	3.2	0.06	5.4	0.06	9.3	0.15
	GGA	7	22.7	0.41	3.1	0.04	6.9	0.11
	GGT	8	25.9	0.47	10.2	0.11	28.2	0.26
	GGC	1	3.2	0.06	70.8	0.79	30	0.48
Gln	CAG	14	45.4	0.64	37	0.86	29	0.64
	CAA	8	25.9	0.36	6	0.14	12.7	0.36
Glu	GAG	7	22.7	0.41	37	0.62	18.9	0.42
	GAA	10	32.4	0.59	23	0.38	43.6	0.58
His	CAT	4	12.9	0.67	5.6	0.22	11.5	0.69
	CAC	2	6.4	0.33	19.6	0.78	10.7	0.31
Ile	ATA	4	12.9	0.25	0.35	0.01	4	0.13
	ATT	6	19.4	0.38	2.3	0.06	26.9	0.50
	ATC	6	19.4	0.38	38.6	0.94	26.6	0.38
Leu	TTG	10	32.4	0.29	8.3	0.08	11	0.10
	TTA	10	32.4	0.29	0.5	0.01	10	0.15
	CTG	12	38.9	0.34	61.4	0.64	52.4	0.43
	CTA	2	6.49	0.06	0.88	0.01	3	0.05
	CTT	1	3.25	0.03	2.1	0.02	10	0.15
	CTC	0	0	0	23.1	0.24	9.5	0.14
Lys	AAG	6	19.4	0.27	34.6	0.89	12	0.37
	AAA	16	51.9	0.73	4.4	0.11	38.2	0.63
Met	ATG	1	29.2	1	21	1	25.6	1
Pro	CCG	1	3.2	0.08	30.2	0.62	23.5	0.38
	CCA	7	22.7	0.54	1.2	0.03	8.3	0.23
	CCT	4	12.9	0.31	8	0.03	6.7	0.29
	CCC	1	3.2	0.08	15.7	0.32	4.2	0.18
Phe	TTT	8	25.9	0.80	0.9	0.03	18.5	0.62
	TTC	2	6.4	0.20	31.1	0.97	17.9	0.38
Ser	AGT	3	9.7	0.14	2.1	0.04	7.3	0.18
	AGC	3	9.7	0.14	23.8	0.40	15.4	0.14
	TCG	6	91.4	0.27	15.7	0.27	7.7	0.23
	TCA	4	12.9	0.18	0.5	0.01	6.8	0.16
	TCT	4	12.9	0.18	0.5	0.01	10.7	0.15
	TCC	2	6.4	0.09	16.4	0.28	9.6	0.14

Continued on following page

TABLE 1—*Continued*

Amino acid	Codon	Codon usage in: *bla* PER-1			*P. aeruginosa* genes		*E. coli* genes	
		No.[a]	No./1,000 codons[b]	Fraction[c]	No./1,000 codons[b]	Fraction[c]	No./1,000 codons[b]	Fraction[c]
Thr	ACG	1	3.2	0.05	5.8	0.13	12.7	0.28
	ACA	2	6.4	0.10	0.7	0.02	6.8	0.11
	ACT	8	25.9	0.40	1.6	0.03	11.6	0.20
	ACC	9	29.2	0.45	38.5	0.83	24	0.40
Trp	TGG	5	16.2	1	11.8	1	13.4	1
Tyr	TAT	4	12.9	0.80	5.4	0.16	16.2	0.64
	TAC	1	3.25	0.20	28.6	0.84	14.1	0.36
Val	GTG	7	22.7	0.41	30	0.62	23.8	0.42
	GTA	6	19.4	0.21	5	0.07	12	0.21
	GTT	10	32.4	0.34	3.1	0.04	20	0.29
	GTC	6	19.4	0.21	33.2	0.47	11	0.22
End	TAG	0	0	0	0.2	0.07	0.2	0.09
	TAA	1	3	1	0.3	0.13	2	0.62
	TGA	0	0	0	2.1	0.80	0.2	0.30

[a] The number of times a codon occurred in bla_{PER-1}.
[b] The number of times a specific codon would occur per 1,000 codons.
[c] The ratio of the number of occurrences of a specific codon to the number of occurrences of all codons in the same synonymous codon group.

identity with class D oxacillin-hydrolyzing β-lactamases (17% for OXA-5) or class C AmpC cephalosporinase from *P. aeruginosa* (20%), which are other β-lactamases found in *P. aeruginosa*. PER-1 differed from the plasmid-mediated ESbla of the TEM-SHV series found in *Enterobacteriaceae* species and from the recently described MEN-1 in *E. coli* (3). Surprisingly, PER-1 had 40% amino acid identity with the recently reported CFXA isolated from *Bacteroides vulgatus* (Table 2) (44). Like CFXA, PER-1 possessed additional amino acid residues at several positions compared with other class A β-lactamases aligned according to class A β-lactamase classification (ABL [1]): downstream from position ABL 103 (Q-N), downstream from position ABL 115 (E-F), and downstream from position ABL 238 (G-I-K-A) (Fig. 4). PER-1 possessed, like CFXA, a deleted amino acid residue at position ABL 251. Moreover, among the 7 amino acids found in PER-1 which differ from the highly conserved 25 amino acids of class A β-lactamases, 4 were identical to those in CFXA at positions ABL 37, 169, 179, and 233. As described by Couture et al. (11), a dendrogram was constructed to relate PER-1 to the other class A β-lactamases (Fig. 5). PER-1 was not more closely related to gram-negative than to gram-positive bacterial class A β-lactamases. PER-1 was mostly related to CFXA, with which it formed a novel class A β-lactamase subgroup.

DISCUSSION

Several interesting features emerged from the analysis of the nucleotide sequence and the deduced amino acid sequence of PER-1, an ESbla isolated from a *P. aeruginosa* clinical isolate.

Analyses of percent GC and codon usage of the PER-1 gene suggested that it may not be of pseudomonad origin. The 5′ upstream sequence of PER-1 reveals a GC content of 67%, typical of *P. aeruginosa*, as well as the presence of a promoter which fits the *rpoN* promoter consensus sequence of some *P. aeruginosa* genes. Moreover, analysis of the PER-1 mRNA transcription start indicated that this promoter was active in the original *P. aeruginosa* RNL-1 strain. Comparison of mRNA analysis of PER-1 from pPZ1 in *E. coli* and in *P. aeruginosa* RNL-1 revealed that PER-1 was not transcribed in *E. coli* from its own promoter. Once cloned into the pACYC184 or the pK19 vectors, PER-1 gene expression was driven by vector promoter sequences. In pRAZ2, which resulted from cloning of PER-1 into a derivative pACYC184 vector, PER-1 gene expression was driven by *tet* gene promoter sequences. As previously reported, many cloned genes from *P. aeruginosa* are expressed poorly in *E. coli* (47). As suggested, this may be due to *E. coli* RNA polymerase activity, which has a much higher stringency requirement for promoter consensus sequences than *Pseudomonas* RNA polymerase (47). As reported for a few *P. aeruginosa* genes cloned into *E. coli*, a promoter within the cloning vector may serve as an effective promoter for expression of bla_{PER-1} once cloned and expressed in *E. coli* (47). Comparison with promoters of other β-lactamase genes of the OXA and PSE series found in *P. aeruginosa* cannot be made, as their characterization has been rarely reported (5, 12, 21, 22, 39). However, promoters of bla_{OXA-2} and of bla_{PSE-4} are likely to be typical of *Enterobacteriaceae* (5, 12). The PER-1 gene may have been inserted into *P. aeruginosa* genomic DNA under the control of an active promoter. We are currently performing an extended epidemiological study to detect bla_{PER-1} among other gram-negative bacteria. The mechanism of PER-1 gene insertion into *P. aeruginosa* and the origin of the inserted gene are unknown. This insertion very likely occurred within *P. aeruginosa* RNL-1 genomic DNA, as no plasmid was found to carry or transfer the PER-1 gene and a bla_{PER-1} probe gave a positive signal in hybridization experiments at the position of *P. aeruginosa* RNL-1 chromosomal DNA migration (42). Transposition has been extensively described in *P. aeruginosa* as a source of genetic plasticity. Most of the oxacillin-hydrolyzing β-lactamases and the carbenicillin-hydrolyzing β-lactamase genes isolated from *P. aeruginosa* have been described as being

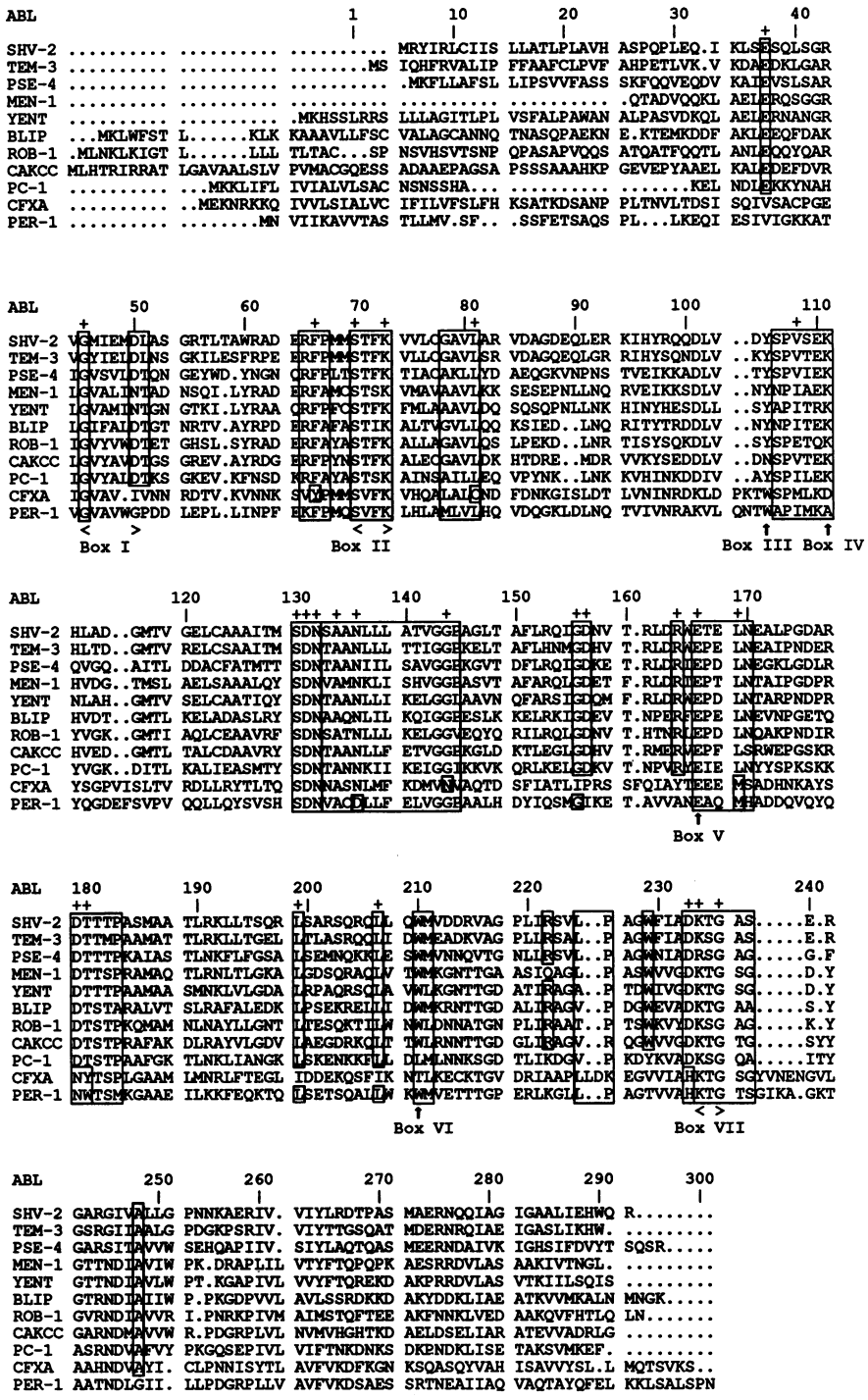

FIG. 4. Alignment of the amino acid sequence of PER-1 (this study) compared with those of 10 class A β-lactamases. Dots indicate gaps inserted within the alignment. The standard numbering scheme of class A β-lactamase is done according to Ambler et al. (1). Roman numbering designates boxes described by Joris et al. (26): box I, positions ABL 45 to 50; box II, ABL 70 to 73; box III, ABL 105; box IV, ABL 111; box V, ABL 166; box VI, ABL 210; box VII, ABL 234 to 236. The β-lactamases included in the alignment are SHV-2 from *E. coli* (20), TEM-3 from *E. coli* (53), MEN-1 from *E. coli* (3), PSE-4 from *P. aeruginosa* (5), BLA I from *Y. enterocolitica* (YENT) (50), BLIP from *Bacillus licheniformis* (41), ROB-1 from *H. influenzae* (26), CAKCC from *Streptomyces cacaoi* (31), PC-1 from *S. aureus* (6), and CFXA from *Bacteroides vulgatus* (44). Plus signs indicate the 25 highly conserved amino acid residues in all the class A β-lactamases, and boxed areas represent relatively well-conserved areas (11).

TABLE 2. Percent pairwise identities between 11 class A β-lactamase amino acid sequences[a]

β-Lactamase type	% Identity with β-lactamase of indicated type[b]									
	SHV-2	TEM-3	PSE-4	MEN-1	YENT	BLIP	ROB-1	CAKCC	PC-1	CFXA
SHV-2										
TEM-3	68									
PSE-4	49	45								
MEN-1	40	41	40							
YENT	41	42	36	60						
BLIP	38	39	34	48	44					
ROB-1	39	41	36	43	43	48				
CAKCC	39	41	37	45	43	51	46			
PC-1	32	36	38	36	37	49	42	41		
CFXA	26	24	26	25	26	24	25	21	25	
PER-1	27	26	30	28	26	23	25	24	26	40

[a] The β-lactamase abbreviations and references used are as indicated in the legend to Fig. 4.
[b] Percent identities calculated with entire proteins represent the number of perfect matches divided by the length of the shorter sequence excluding the gaps, according to the progressive alignment method of Feng and Doolittle (15).

part of Tn*21* transposon derivatives (32). These β-lactam resistance genes are usually associated with sulfonamide, aminoglycoside, and mercuric resistance genes which may be present in *P. aeruginosa* RNL1 as suggested by phenotypic analysis of its antibiotic resistance pattern. Therefore, the presence of a transposon carrying bla_{PER-1} cannot be ruled out, and we are currently trying to identify such a potential transposon. However, typical sequences of an integron, which could by itself integrate foreign DNA within a transposon, were not found in the upstream or downstream sequences of bla_{PER-1}, contrary to other β-lactamase genes isolated from *P. aeruginosa* such as OXA and PSE derivatives (43, 55). Analysis of the GC content (45%) of the bla_{PER-1} downstream sequences indicated that these sequences may have been inserted into *P. aeruginosa* RNL-1 along with bla_{PER-1}.

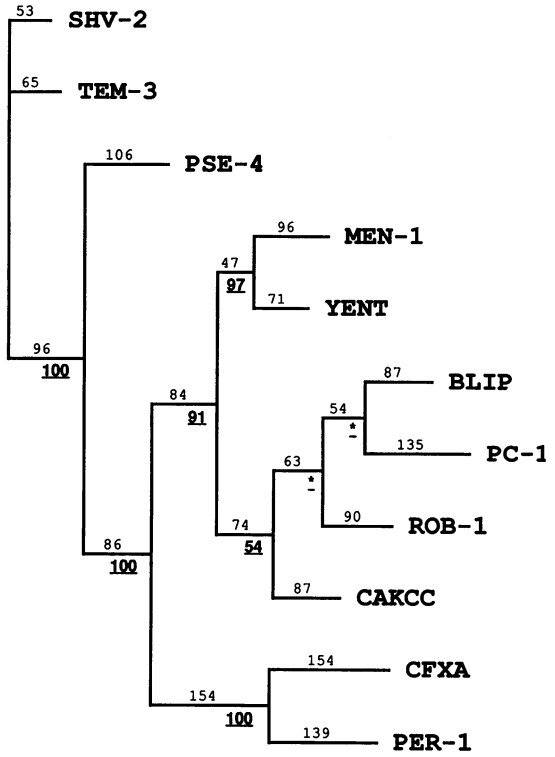

FIG. 5. Dendrogram for 11 class A β-lactamases according to parsimony (57). This graph was performed by using the amino acid sequence of the β-lactamases from position ABL 26 to 302, as the leader peptide of MEN-1 is unknown. Branch lengths are to scale and proportional to the number of amino acid changes. The percentage at branching points refers to the number of times a particular nod was found in 100 bootstrap replications (boldface number; the stars indicate uncertainty of nodes with bootstrap values of less than 50%). The distance along the vertical axis has no significance. Abbreviations for β-lactamases are given in the legend of Fig. 4.

It is interesting to note that for some of the β-lactamase genes such as bla_{PSE-1}, bla_{PSE-2}, bla_{CARB-3}, and bla_{OXA-2} isolated from *P. aeruginosa*, the percent GC was not typical of *P. aeruginosa* but of *Enterobacteriaceae* species (12, 21, 22, 29, 39). It is also noteworthy that some of these genes are also found in *Enterobacteriaceae* species. Among the genes so far sequenced in *P. aeruginosa*, pilin genes possess a GC content of 40 to 45%, which is not typical of *P. aeruginosa* (59). As suggested for the origin of these genes, the presence of bla_{PER-1} in *P. aeruginosa* may also have resulted from an intergeneric transfer from *Enterobacteriaceae*, *Moraxella*, *Neisseria*, or *Bacteroides* species (59). In addition, codon usage of bla_{PER-1} resembled that of *E. coli* class III genes, which correspond to genes inherited from horizontal transfer (such as fimbria or pilin genes) (36).

The second interesting feature of this study is the comparison of PER-1 with other class A β-lactamases. This β-lactamase group is the largest so far described. It comprises β-lactamases found in gram-positive and gram-negative bacteria. PER-1 possesses highly conserved amino acid residues of the active-site serine enzymes that interact with β-lactam compounds, i.e., boxes I to VII (25, 26). Moreover, PER-1 possesses the highly conserved SDN motif, a structural block of the active site of class A β-lactamases (23).

Although isolated from *P. aeruginosa*, PER-1 does not exhibit common features of class A β-lactamases isolated from gram-negative bacteria. By way of an example, class A β-lactamases of the TEM, SHV, and CARB types possess two cysteine residues at positions ABL 77 and 123. Biochemical (46) and crystallographic studies (56) suggest strongly that they are in the form of a disulfide bridge. PER-1 has none of these cysteines but, respectively, an alanine and a leucine residue at these positions. Surprisingly, PER-1 possesses a single cysteine at position ABL 135, close to the SDN motif (starting at position ABL 130). However, other class A β-lactamases isolated from gram-negative bacteria

do not possess the cysteine residues at positions ABL 77 and 123, such as MEN-1 from *E. coli* (3), *Klebsiella oxytoca* E23004 (2), *Y. enterocolitica* BLA I (50), and CFXA from *Bacteroides vulgatus* (44). MEN-1, *K. oxytoca*, and *Y. enterocolitica* β-lactamases all possess a cysteine residue just before box II (position ABL 69). Moreover, with the exception of some ESbla, all class A β-lactamases have an arginine two places before the box V glutamic acid (i.e., R-E, position ABL 164). PER-1 has an alanine in this position. Concerning box VII, all class A β-lactamases have an aspartic acid just before the KS(T)G or RSG [i.e., DKS(T)G or DRSG]. PER-1 has a histidine, which is also observed in CFXA. Surprisingly, this histidine is highly conserved within class C β-lactamases (33). Although PER-1 is a typical ESbla on the basis of its hydrolytic properties, it does not derive by point mutations from ESbla genes of the TEM-SHV series or of the recently described MEN-1. Although seven boxes are known to be highly conserved in class A β-lactamases, boxes III, V, and VII plus the SDN motif are known to play a critical role in the catalytic activity of the enzymes (25, 26). This might be explained by investigation of the X-ray structure of class A β-lactamases from *S. aureus* PC1 (18), *Streptomyces albus* (13), and *Bacillus licheniformis* 749/C (38). All known ESbla have specific mutations close to these strategic positions (10, 24). Inspection of the PER-1 amino acid sequence suggests that the hydrolytic activity towards cefotaxime and ceftazidime may result from the presence of specific residues.

Concerning box V, we observed in PER-1 an alanine residue at position ABL 164, instead of an arginine. Few ESbla have a serine in the corresponding position, as is the case with TEM-5, TEM-7, TEM-8, TEM-9, TEM-12 (TEM-101), CAZ-2, and CAZ-6 (7, 10, 53). Other ESbla have a histidine in this position, as is the case with TEM-6, TEM-16, and CAZ-7 (7, 10). Moreover, many ESbla have a lysine at position ABL 104 (PER-1, a threonine), close to box III. This mutation enhances the effect of serine ABL 164 substitutions on the hydrolytic activity towards extended-spectrum cephalosporins (54). Concerning the vicinity of box VII (starting at position ABL 234), we found a serine residue at position ABL 238 in PER-1, as in TEM-5, TEM-4, TEM-8, SHV-2, SHV-3, SHV-4, and SHV-5, whereas the parent narrow-spectrum β-lactamases have a glycine (7, 10, 37, 53, 54). Among the amino acid residues which are characteristic of TEM-SHV ESbla, only serine at position ABL 238 was present in PER-1.

As suggested by its biochemical properties, PER-1 differs not only from TEM-SHV and MEN-1 but also from the following recently reported ESbla isolated from *P. aeruginosa*. It has been reported that point mutations obtained by in vitro mutagenesis within bla_{PSE-4} and bla_{CARB-4} may lead to extended-spectrum derivatives (4). Similarly, point mutations within bla_{PSE-2} (giving rise to OXA-11, which is, as PSE-2, an oxacillin-hydrolyzing Ambler class D β-lactamase) may confer resistance to extended-spectrum cephalosporins in a *P. aeruginosa* clinical isolate (17). Dendrogram analysis revealed that, on the basis of amino acid pairwise comparison, PER-1 may be part of a special class A β-lactamase subgroup (Fig. 5). Similarities with CFXA from *Bacteroides vulgatus* indicate that these two β-lactamases may be derived from a common ancestor. In this regard, a detailed sequence comparison of PER-1 with CFXA revealed inserted and deleted amino acids at the same positions compared with the other class A β-lactamases.

Seoane and Lobo (50), who analyzed protein sequences of class A β-lactamases, classified them into two groups according to conserved amino acids at determined positions. The first subgroup, called the chromosomal branch, includes *Bacillus* and *Streptomyces* class A β-lactamases, and *K. oxytoca* E23004, *Y. enterocolitica* BLA I, and *S. aureus* PC1 enzymes. The second subgroup, called the transposon branch, includes TEM, SHV, and PSE derivatives as well as *K. pneumoniae* LEN-1. Only the second group of enzymes is believed to be located on transposable elements. Analysis of PER-1 reveals that it cannot be correctly included in any of the subgroups described.

Finally, although ESbla of the TEM and SHV series are commonly reported in *Enterobacteriaceae* species, non-TEM non-SHV ESbla may be isolated from non-*Enterobacteriaceae* gram-negative species of clinical significance such as *P. aeruginosa*. Potential integration of such ESbla genes into *P. aeruginosa* plasmids may lead to their epidemic dissemination, as described for *Enterobacteriaceae* ESbla.

ACKNOWLEDGMENTS

P.N. and T.N. contributed equally to this work. We are very grateful to W. Arber, in whose laboratory part of the work was performed; to R. Labia and P. Roy for precious advice; and to M. Blot for help with the dendrogram analysis.

This study was supported by the Swiss National Science Foundation (no. 31-30040.90), Switzerland, and the Fondation pour la Recherche Médicale, France.

REFERENCES

1. **Ambler, R. P., A. F. W. Coulson, J. M. Frère, J. M. Ghuysen, B. Joris, M. Forsman, R. C. Levesque, G. Tiraby, and S. G. Waley.** 1991. A standard numbering scheme for the class A β-lactamases. Biochem. J. **276**:269–272.
2. **Arakawa, Y., M. Ohta, N. Kido, M. Mori, H. Ito, T. Komatsu, Y. Fujii, and N. Kato.** 1989. Chromosomal β-lactamase of *Klebsiella oxytoca*, a new class A enzyme that hydrolyzes broad-spectrum β-lactam antibiotics. Antimicrob. Agents Chemother. **33**:63–70.
3. **Barthélémy, M., J. Péduzzi, H. Bernard, C. Tancrède, and R. Labia.** 1992. Close amino-acid sequence relationship between the new plasmid-mediated extended-spectrum β-lactamase MEN-1 and chromosomally encoded enzymes of *Klebsiella oxytoca*. Biochim. Biophys. Acta **1122**:15–22.
4. **Bejaoui, N., and R. C. Levesque.** 1992. Structure-function analysis of critical amino acids for extended-spectrum ceftazidimase activity in *Pseudomonas aeruginosa*, abstr. 579. Program Abstr. 32nd Intersci. Conf. Antimicrob. Agents Chemother.
5. **Boissinot, M., and R. C. Levesque.** 1990. Nucleotide sequence of the PSE-4 carbenicillinase gene and correlations with the *Staphylococcus aureus* PC1 β-lactamase crystal structure. J. Biol. Chem. **265**:1225–1230.
6. **Chan, P. I.** 1986. Nucleotide sequence of the *Staphylococcus aureus* PC1 β-lactamase gene. Nucleic Acids Res. **14**:5940.
7. **Chanal, C., M.-C. Poupart, D. Sirot, R. Labia, J. Sirot, and R. Cluzel.** 1992. Nucleotide sequences of CAZ-2, CAZ-6, and CAZ-7 β-lactamase genes. Antimicrob. Agents Chemother. **36**:1817–1820.
8. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. **134**: 1141–1156.
9. **Chomczynski, P., and N. Saachi.** 1987. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162**:156–159.
10. **Collatz, E., R. Labia, and L. Gutmann.** 1990. Molecular evolution of ubiquitous β-lactamases towards extended-spectrum enzymes active against newer β-lactam antibiotics. Mol. Microbiol. **4**:1615–1620.
11. **Couture, F., J. Lachapelle, and R. C. Levesque.** 1992. Phylogeny of LCR-1 and OXA-5 with class A and class D β-lactamases. Mol. Microbiol. **6**:1693–1705.

12. **Dale, J. W., D. Godwin, D. Mossakowska, P. Stephenson, and S. Wall.** 1985. Sequence of the OXA-2 β-lactamase: comparison with other penicillin-reactive enzymes. FEBS Lett. **191:**39–44.
13. **Dehottay, P., J. Dussart, F. De Meester, B. Joris, J. Van Beeumen, T. Erpicum, J. M. Frère, and J. M. Ghuysen.** 1987. Nucleotide sequence of the encoding *Streptomyces albus* G β-lactamase precursor. Eur. J. Biochem. **166:**345–350.
14. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
15. **Feng, D. F., and R. F. Doolittle.** 1990. Progressive alignment and phylogenetic tree construction of protein sequences. Methods Enzymol. **183:**375–387.
16. **Geliebter, J., R. A. Zeff, R. W. Melvold, and S. G. Nathenson.** 1986. Mitotic recombination in germ cells generated two major histocompatibility complex mutant genes shown to be identical by RNA sequence analysis: K^{bm9} and K^{bm6}. Proc. Natl. Acad. Sci. USA **83:**3371–3375.
17. **Hall, L. M. C., D. M. Livermore, D. Gur, M. Akova, and H. E. Akalin.** 1993. OXA-11, an extended-spectrum variant of OXA-10 (PSE-2) β-lactamase from *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **37:**1637–1644.
18. **Herzberg, O., and J. Moult.** 1987. Bacterial resistance to β-lactam antibiotics: crystal structure of β-lactamase from *Staphylococcus aureus* PC1 at 2.5 A resolution. Science **236:**694–701.
19. **Hewick, R. M., M. W. Hunkapiller, D. Le Hoo, and W. J. Dreyer.** 1981. A gas-liquid solid phase peptide and protein sequenator. J. Biol. Chem. **256:**7990–7997.
20. **Huletsky, A., F. Couture, and R. C. Levesque.** 1990. Nucleotide sequence and phylogeny of SHV-2 β-lactamase. Antimicrob. Agents Chemother. **34:**1725–1732.
21. **Huovinen, P., S. Huovinen, and G. A. Jacoby.** 1988. Sequence of PSE-2 β-lactamase. Antimicrob. Agents Chemother. **32:**134–136.
22. **Huovinen, P., and G. A. Jacoby.** 1991. Sequence of the PSE-1 β-lactamase gene. Antimicrob. Agents Chemother. **35:**2428–2430.
23. **Jacob, F., B. Joris, S. Lepage, J. Dusart, and J. M. Frère.** 1990. Role of conserved aminoacids of the SDN loop (Ser 130, Asp 131, Asn 132) in class A β-lactamase studied by site-directed mutagenesis. Biochem. J. **271:**399–406.
24. **Jacoby, G. A., and A. A. Medeiros.** 1991. More extended-spectrum β-lactamases. Antimicrob. Agents Chemother. **35:**1697–1704.
25. **Joris, B., J. M. Ghuysen, G. Dive, A. Renard, O. Dideberg, P. Charlier, J. M. Frère, J. A. Kelly, J. C. Boyington, P. C. Moews, and J. R. Knox.** 1988. The active-site-serine penicillin-recognizing enzymes as members of the streptomyces R61 DD-peptidase family. Biochem. J. **250:**313–324.
26. **Joris, B., P. Ledent, O. Dideberg, E. Fonzé, J. Lamotte-Brasseur, J. A. Kelly, J. M. Ghuysen, and J. M. Frère.** 1991. Comparison of the sequences of class A β-lactamases and of the secondary structure elements of penicillin-recognizing proteins. Antimicrob. Agents Chemother. **35:**2294–2301.
27. **Juteau, J.-M., and R. C. Levesque.** 1990. Sequence analysis and evolutionary perspectives of ROB-1 β-lactamase. Antimicrob. Agents Chemother. **34:**1354–1359.
28. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157:**105–132.
29. **Lachapelle, J., J. Dufresne, and R. C. Levesque.** 1991. Characterization of bla_{CARB-3} encoding the carbenicillinase-3 β-lactamase of *Pseudomonas aeruginosa*. Gene **102:**7–12.
30. **Laemmli, U. K., and M. Farve.** 1973. Maturation of the head of bacteriophage T4. I. DNA packaging events. J. Mol. Biol. **80:**575–599.
31. **Lenzini, M. V., H. Ishihara, J. Dusart, H. Ogawara, B. Joris, J. Van Beemen, J. M. Frère, and J. M. Ghuysen.** 1988. Nucleotide sequence of the gene encoding the active serine β-lactamase from *Streptomyces cacaoi*. FEMS Microbiol. Lett. **49:**371–376.
32. **Levesque, R. C., and G. A. Jacoby.** 1988. Molecular structure and interrelationships of multiresistance β-lactamase transposons. Plasmid **19:**21–29.
33. **Lodge, J. M., S. D. Minchin, L. J. V. Piddock, and S. J. Busby.** 1990. Cloning, sequencing and analysis of the structural gene and regulatory region of the *Pseudomonas aeruginosa* chromosomal *amp*C β-lactamase. Biochem. J. **272:**627–631.
34. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
35. **Matsudaira, P.** 1987. Sequence from picomole quantities of proteins electroblotted onto polyvinyl difluoride membranes. J. Biol. Chem. **262:**10035–10038.
36. **Medigue, C., T. Rouxel, P. Vigier, A. Henaut, and A. Danchin.** 1991. Evidence for horizontal gene transfer in *Escherichia coli* speciation. J. Mol. Biol. **222:**851–856.
37. **Mercier, J., and R. C. Levesque.** 1990. Cloning of SHV-2, OHIO-1, and OXA-6 β-lactamases and cloning and sequencing of SHV-1 β-lactamase. Antimicrob. Agents Chemother. **34:**1577–1583.
38. **Moews, P. C., J. R. Knox, O. Dideberg, P. Charlier, and J. M. Frère.** 1990. β-Lactamase of *Bacillus licheniformis* 749/C at 2 A resolution. Proteins Struct. Funct. Genet. **7:**156–171.
39. **Mossakowska, D., N. A. Ali, and J. W. Dale.** 1989. Oxacillin-hydrolyzing β-lactamases. A comparative analysis at nucleotide and amino-acids levels. Eur. J. Biochem. **180:**309–318.
40. **Needleman, S. B., and C. D. Wunsch.** 1970. A general method application to the search for similarities in the amino acid sequence of two proteins. J. Mol. Biol. **48:**443–453.
41. **Neugebauer, K. R., R. Sprengel, and H. Schaller.** 1981. Penicillinase from *Bacillus licheniformis*: nucleotide sequence of the gene and implications for the biosynthesis of a secretory protein in a gram-positive bacterium. Nucleic Acids Res. **9:**2577–2588.
42. **Nordmann, P., E. Ronco, T. Naas, C. Duport, Y. Michel-Briand, and R. Labia.** 1993. Characterization of a novel extended-spectrum β-lactamase from *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **37:**962–969.
43. **Ouellette, M., L. Brissonnette, and P. H. Roy.** 1987. Precise insertion of antibiotic resistance determinants into Tn*21*-like transposons: nucleotide sequence of the OXA-1 β-lactamase gene. Proc. Natl. Acad. Sci. USA **84:**7378–7382.
44. **Parker, A. C., and C. J. Smith.** 1993. Genetic and biochemical analysis of a novel Ambler class A β-lactamase responsible for cefoxitin resistance in *Bacteroides* species. Antimicrob. Agents Chemother. **37:**1028–1036.
45. **Philippon, A., R. Labia, and G. Jacoby.** 1989. Extended-spectrum β-lactamases. Antimicrob. Agents Chemother. **33:**1131–1136.
46. **Pollitt, S., and H. Zalkin.** 1983. Role of primary structure and disulfide bond formation in β-lactamase secretion. J. Bacteriol. **153:**27–32.
47. **Ronald, S., M. A. Farinha, B. J. Allan, and A. M. Kropinski.** 1992. Cloning and physical mapping of transcriptional regulatory (sigma) factors from *Pseudomonas aeruginosa*, p. 249–257. *In* E. Galli, S. Silver, and B. Witholt (ed.), *Pseudomonas*: molecular biology and biotechnology. American Society for Microbiology, Washington D.C.
48. **Rosenberg, M., and D. Court.** 1979. Regulatory sequences involved in the promotion and termination of RNA transcription. Annu. Rev. Genet. **13:**319–353.
49. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
50. **Seoane, A., and J. M. G. Lobo.** 1991. Nucleotide sequence of a new class A β-lactamase gene from the chromosome of *Yersinia enterocolitica*: implications for the evolution of class A β-lactamases. Mol. Gen. Genet. **228:**215–220.
51. **Shine, J., and L. Dalgarno.** 1975. Determinant of cistron specificity in bacterial ribosomes. Nature (London) **254:**34–38.
52. **Sougakoff, W., S. Goussard, and P. Courvalin.** 1988. The TEM-3 β-lactamase, which hydrolyses broad-spectrum cephalosporins, is derived from the TEM-2 penicillinase by two amino-acid substitutions. FEMS Microbiol. Lett. **56:**343–348.
53. **Sougakoff, W., A. Petit, S. Goussard, D. Sirot, A. Buré, and P. Courvalin.** 1989. Characterization of the plasmid genes bla_{T-4} and bla_{T-5} which encode the broad-spectrum β-lactamases

TEM-4 and TEM-5 in *Enterobacteriaceae*. Gene **78:**339–348.

54. **Sowek, J. A., S. J. Singer, S. Ohringer, M. F. Malley, T. J. Dougherty, T. Z. Gougoutas, and K. Bush.** 1991. Substitution of lysine at position 104 or 240 of TEM-1 pTZ18R β-lactamase enhances the effect of serine 64 substitution on hydrolysis or affinity for cephalosporin and the monobactam aztreonam. Biochemistry **30:**3179–3188.
55. **Stokes, H. W., and R. M. Hall.** 1989. A novel family of potentially mobile DNA elements encoding site-specific gene-integration functions: integrons. Mol. Microbiol. **3:**1669–1683.
56. **Strynadka, N. C. J., H. Adachi, S. E. Jensen, K. Johns, A. Sielecki, C. Betzel, K. Sutoh, and N. G. Ames.** 1992. Molecular structure of the acyl-enzyme intermediate in β-lactam hydrolysis at 1.7 angstroem resolution. Nature (London) **359:**700–705.
57. **Swofford, D. L.** 1989. PAUP (version 3.0): phylogenetic analysis using parsimony. Illinois Natural History Survey, Champaign, Ill.
58. **Wada, K., S. Aota, R. Tsuchiga, F. Ishibashi, T. Gojobor, and T. Ikamura.** 1990. Codon usage tabulated from the GenBank genetic sequence data. Nucleic Acids Res. **18**(Suppl.):2367–2411.
59. **West, S. E. H., and B. H. Iglewski.** 1988. Codon usage in *Pseudomonas aeruginosa*. Nucleic Acids Res. **16:**9323–9335.