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Characterization of β-Lactamase Gene *bla*$_{PER-2}$, Which Encodes an Extended-Spectrum Class A β-Lactamase

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Plasmidic extended-spectrum β-lactamases of Ambler class A are mostly inactive against ceftibuten. *Salmonella typhimurium* JMC isolated in Argentina harbors a *bla* gene located on a plasmid (pMVP-5) which confers transferable resistance to oxyiminocephalosporins, aztreonam, and ceftibuten. The β-lactamase PER-2 (formerly ceftibutenase-1; CTI-1) is highly susceptible to inhibition by clavulanate and is located at a pI of 5.4 after isoelectric focusing. The *bla*$_{PER-2}$ gene was cloned and sequenced. The nucleotide sequence of a 2.2-kb insert in vector pBluescript includes an open reading frame of 927 bp. Comparison of the deduced amino acid sequence of PER-2 with those of other β-lactamases indicates that PER-2 is not closely related to TEM or SHV enzymes (25 to 26% homology). PER-2 is most closely related to PER-1 (86.4% homology), an Ambler class A enzyme first detected in *Pseudomonas aeruginosa*. An enzyme with an amino acid sequence identical to that of PER-1, meanwhile, was found in various members of the family *Enterobacteriaceae* isolated from patients in Turkey. Our data indicate that PER-2 and PER-1 represent a new group of Ambler class A extended-spectrum β-lactamases. PER-2 so far has been detected only in pathogens (*S. typhimurium*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*) isolated from patients in South America, while the incidence of PER-1-producing strains so far has been restricted to Turkey, where it occurs both in members of the family *Enterobacteriaceae* and in *P. aeruginosa*.

Plasmidic extended-spectrum (ES) β-lactamases of Ambler class A (1) include in their spectra of activity oxyiminocephalosporins and aztreonam. Ceftibuten, a new oral cephalosporin (12), is stable against most plasmidic Ambler class A ES β-lactamases (5). Recently, an ES β-lactamase active against ceftibuten was identified in *Salmonella typhimurium* (3). This enzyme (PER-2) is encoded by a plasmidic gene readily transferable among members of the family *Enterobacteriaceae*. We cloned and sequenced the *bla*$_{PER-2}$ gene and analyzed the relationship of its amino acid sequence with those of other plasmidic or chromosomal β-lactamases of Ambler class A. The results indicate characteristic elements of Ambler class A (16) but only a low degree of homology with plasmidic ES β-lactamases known so far (e.g., TEM, SHV, or CTX-M). A close relationship (86.4% homology in its amino acid sequence) was, however, detected with PER-1, a β-lactamase first detected in *Pseudomonas aeruginosa* (8, 18, 19).

MATERIALS AND METHODS

Bacterial strains. *S. typhimurium* JMC (O antigens: 4 and 5; H antigens: phase 1, i; phase 2, 1 and 2) was isolated in April 1990 by José Maria Casellas from the feces of a patient suffering from gastroenteritis. The strain was resistant to ceftibuten; however, it was susceptible to cefoxitin. *Escherichia coli* C600 R$^-$ resistant to nalidixic acid (MIC, 1,024 mg/liter) was the recipient strain for the transfer of resistance determinants. *E. coli* DHα was the host strain for the cloning experiments.

Vector. The vector pBluescript KS (Stratagene, Heidelberg, Germany) carrying an ampicillin resistance gene was used for the cloning of the *bla*$_{PER-2}$ gene.

Antibiotics. Ceftazidime (Cascan GmbH & Co. KG, Wiesbaden, Germany), cefotaxime, cefpirome, and tetracycline (Hoechst AG, Frankfurt am Main, Germany), clavulanate (SmithKline Beecham Pharmaceuticals, London, United Kingdom), cefepime and aztreonam (Bristol-Myers Squibb, Munich, Germany), ceftibuten (Schering-Plough Corporation, Kenilworth, N.J.), cefoxitin, and imipenem (Merck Sharp & Dohme, Munich, Germany), sulfamethoxazole and trimethoprim (Hoffmann-La Roche Inc., Basel, Switzerland), tobramycin (Eli Lilly GmbH, Bad Homburg, Germany), gentamicin (Merck, Darmstadt, Germany), and chloramphenicol (Boehringer, Mannheim, Germany) were used in the study. Combinations of β-lactams with clavulanate were used at a proportion of 4 + 1.

Susceptibility testing. MICs were determined by an agar dilution technique on Mueller-Hinton agar (Difco, Augsburg, Germany). An inoculum of 10^4 CFU per spot was delivered with a multipoint inoculator (Denley, Billinghurst, United Kingdom) to a series of agar plates which contained antibiotics in twofold dilutions. Incubation was for 16 h at 35°C. The MIC was determined as the lowest concentration of antibiotic at which no visible growth or the growth of three or fewer colonies was observed. *E. coli* ATCC 25922 was used as the reference strain for determination of MICs.

Transfer of resistance determinants. Cells of the donor strain and the recipient strain (10^9 CFU/ml per strain) were mixed in Mueller-Hinton broth (Difco), and the mixture was incubated for 18 h at 35°C. Transconjugants were selected on MacConkey agar (Unipath GmbH, Wesel, Germany) supplemented with nalidixic acid (64 mg/liter) to inhibit the growth of the donor strain and ceftibuten (4 mg/liter) to inhibit the growth of the recipient strain.

Isoelectric focusing of β-lactamases. Crude homogenates of β-lactamases were prepared as described previously (4). For isoelectric focusing the procedure described by Matthew et al. (17) was modified (4).

Assignment of the β-lactamase activity within the lane. After isoelectric focusing the polyacrylamide gel was covered with a 0.75% tryptic soy agar (Difco) overlay containing ceftibuten (4 mg/liter), and the mixture was incubated for 2 h at 35°C. A second layer with *E. coli* susceptible to ceftibuten (MIC, 0.25 mg/liter; 1.2×10^7 CFU of *E. coli* per ml) was then applied. Following overnight incubation at 35°C, visible growth on the spot of the gel where ceftibuten had been hydrolyzed allows for the specific localization of the ceftibutenase band.

Plasmid DNA preparation. Cells were grown overnight in 150 ml of tryptic soy broth (Difco) supplemented with ceftazidime (1 mg/liter). DNA preparation was performed by alkaline lysis as described by Birnboim and Doly (7). The plasmid DNA in the lysate was purified with an anion exchange column (tip 100; Qiagen, Hilden, Germany) according to the recommendations of the manufacturer.

Cloning and sequencing of the *bla*$_{PER-2}$ gene. Cloning experiments were performed by following the standard procedures of Sambrook et al. (23). All enzymes used were purchased from Boehringer. The resistance plasmid (pMVP-5) carrying the *bla*$_{PER-2}$ gene was isolated from the *E. coli* C600 transconjugant strain and was digested with *Sal*I. Ligation to the vector pBluescript and then transformation of $CaCl_2$-treated *E. coli* DH5α and selection on Mueller-Hinton agar supplemented with ceftibuten (8 mg/liter) resulted in ceftibuten-resistant *E. coli* transformants harboring a recombinant plasmid with a 9.4-kb insert (pMVP-5-1). A *Hin*dIII subclone with a 2.2-kb fragment in the vector pBluescript (pMVP-5-2) was used for sequencing. Sequencing was performed by the dideoxy

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TABLE 1. Antibiotic susceptibilities of the wild-type and transconjugant strains producing β-lactamase PER-2 and the recipient strain

Antibiotic	MIC (μg/ml)		
	Wild-type *S. typhimurium* JMC	Transconjugant *E. coli* C600 R^+ (*S. typhimurium* JMC)	Recipient *E. coli* C600 R^-
Ceftibuten	256	128	0.25
Ceftibuten plus clavulanate	1	0.5	0.25
Ceftazidime	4,096	256	0.13
Ceftazidime plus clavulanate	4	1	0.13
Cefotaxime	256	32	0.06
Cefotaxime plus clavulanate	1	0.25	0.03
Cefpirome	128	8	0.03
Cefpirome plus clavulanate	1	0.5	0.03
Cefepime	64	8	0.016
Cefepime plus clavulanate	1	0.25	0.016
Aztreonam	1,024	256	0.06
Aztreonam plus clavulanate	2	0.25	0.06
Cefoxitin	2	2	4
Imipenem	0.5	0.25	0.25
Tobramycin	16	2	0.13
Gentamicin	16	2	0.13
Chloramphenicol	512	512	4
Tetracycline	128	128	1
Sulfamethoxazole	>512	512	32
Trimethoprim	0.25	0.13	0.06

chain termination procedure of Sanger et al. (24) by using consecutive primers for both strands with an automatic sequencer (373A; Applied Biosystems, Weiterstadt, Germany).

Sequence analysis. β-Lactamase relatedness was investigated by comparison with the EMBL and Swissprot databases (Fasta). Multiple alignment was calculated by Clustal V analysis (13, 14).

PCR amplification for specific detection of the bla_{PER-2} gene. For specific detection of the bla_{PER-2} gene, primers were chosen from regions with low levels of sequence homology between bla_{PER-2} and related *bla* genes (e.g., PER-1), as follows: PER-2-A, 5′-CGCTTCTGCTCTGCTGAT-3′; PER-2-B, 5′-GGCAG CTTCTTTAACGCC-3′. The PCR mixture contained 25 pmol of each primer, 10 pmol of deoxynucleoside triphosphates, 1 U of *Taq* polymerase, and 5 μl of buffer in a final volume of 50 μl. All reagents were purchased from Perkin-Elmer-Applied Biosystems (Weiterstadt, Germany). Plasmid DNA from a midi-scale alkaline lysis preparation of Sambrook et al. (23) was used as a template (10 μl of 10^{-1}, 10^{-2}, and 10^{-3} dilutions). The PCR program was 3 min of denaturation at 95°C, 25 cycles of 30 s of denaturation at 95°C, 30 s of annealing at 60°C, and 30 s of extension at 72°C, and a final extension period of 3 min at 72°C. The resulting PCR product of 469 bp was detected by agarose gel electrophoresis.

Nucleotide sequence accession number. The nucleotide sequence data reported here will appear in the EMBL database under accession number X93314.

RESULTS

Antibiotic susceptibilities of wild-type and transconjugant strains. The wild-type strain *S. typhimurium* JMC demonstrates resistance to β-lactam antibiotics (MICs between 64 mg/liter for cefepime and 4,096 mg/liter for ceftazidime); however, it remained susceptible to cefoxitin and impenem (Table 1). The acquisition of the bla_{PER-2} gene by *E. coli* C600 increased the MICs of the majority of the β-lactams investigated for the strain between 512 and 4,096 times. Clavulanate reduced the MICs between 16 and 1,024 times. Resistance determinants for aminoglycosides, chloramphenicol, tetracycline, and sulfamethoxazole were cotransferred with the bla_{PER-2} gene.

Isoelectric focusing and identification of the ceftibuten-hydrolyzing band. Isoelectric focusing of crude homogenates indicated a major nitrocefin-hydrolyzing band at a pI of 5.4, similar to that for TEM-1 (Fig. 1a). Ceftibuten was inactivated strongly at this band (Fig. 1b).

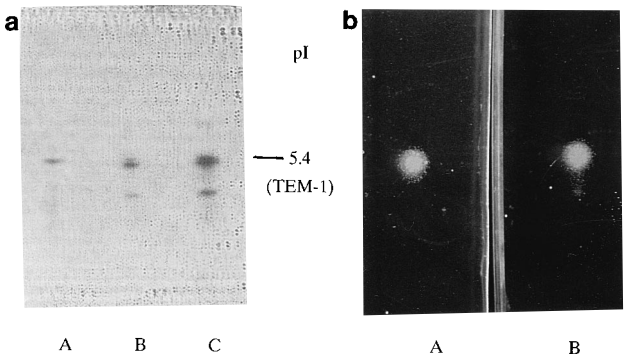

FIG. 1. Isoelectric point of β-lactamase PER-2. (a) Isoelectric focusing of the wild-type and transconjugant strains producing PER-2 revealed two bands, with the most distinct one being at a pI of 5.4, like TEM-1. Lanes: A, *E. coli* TEM-1 (pI 5.4); B, *E. coli* C600 R^+; C, *S. typhimurium* JMC. (b) Only this band at a pI of 5.4 was able to hydrolyze ceftibuten, as shown by a microbiological detection procedure (see Materials and Methods). Lanes: A, *S. typhimurium* JMC; B, *E. coli* C600 R^+.

Analysis of the bla_{PER-2} gene. The nucleotide sequence of the 2.2-kb insert of plasmid pMVP-5-2 containing the bla_{PER-2} gene included an open reading frame of 927 bp which was coding for a protein of 308 amino acids (Fig. 2). A possible ribosome-binding site could be identified upstream of the start codon. No obvious promoter consensus sequence could be found by comparison with known promoter sequences. Comparison of the deduced amino acid sequence of the β-lactamase PER-2 demonstrated a low-level relationship with TEM enzymes (as shown for TEM-1) and CTX-M-2 (6), while the degree of homology with β-lactamases produced by *Bacteroides* spp. (CblA of *B. uniformis* [25], CepA of *B. fragilis* [21], and CfxA of *B. vulgatus* [20]) was somewhat higher (29.3 to 38.1%). These enzymes were the most closely related among the β-lactamases whose sequences have been characterized so far. However, the β-lactamase PER-1 detected in *P. aeruginosa* (18) appeared to be closely related to the PER-2 β-lactamase (86.4%; Table 2 and Fig. 3).

Incidence of additional strains carrying the bla_{PER-2} gene. While screening for multiresistant isolates from South America and Turkey for the production of PER-2, various strains resistant to ceftibuten were identified. PCR amplification and sequencing of fragments of their *bla* genes demonstrated the bla_{PER-2} gene in *E. coli*, *Klebsiella pneumoniae*, *Proteus mira-*

TABLE 2. Percent homologies of amino acid sequences of PER-2, PER-1, TEM-1, CTX-M-2, and the chromosomal β-lactamases of *B. uniformis* CblA, *B. fragilis* CepA, and *B. vulgatus* CfxA[a]

	% Homology					
	PER-1	*B. uniformis* CblA	*B. fragilis* CepA	*B. vulgatus* CfxA	TEM-1	CTX-M-2
PER-2	86.4	38.1	34.3	29.3	25.1	24.5
PER-1		37.4	33.3	29.9	24.0	23.4
B. uniformis CblA			42.0	27.6	23.3	21.3
B. fragilis CepA				38.0	23.7	25.3
B. vulgatus CfxA					22.3	20.5
TEM-1						36.7
CTX-M-2						

[a] Percent homologies were calculated by the neighbor joining method of Saitou and Nei (22). The amino sequences of PER-1 (18), TEM-1 and CTX-M-2 (6), *B. uniformis* CblA (25), *B. fragilis* CepA (21), and *B. vulgatus* CfxA (20) were described previously.

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AAAATCCCTAGCAACCCCAAAATGGTTGAAAATGTGGTAATTTAATTTCGCTTCATTCGTTTTAGCCCTCTGGGCGTTCTATTTTATTCGCAAAAT
CAATTAGATCACGAATGAAGCACCTATTCAAATCCTAAAGATCATACGTATGAGAGAGAGCTCGGGCTCAGGCTCCGGAAATGCGGAGCAGGGAAA

                                                                                    RBS
TCAACTAACAGTATGAAACAAAATAAAAAAGGCAGCTGCGGCTGCCTTTTGTACAATCCACTAAAAAAATAACAAGGACAGTCGT  ATG AAT
                                                                                          m   n

GTC ATC ACA AAA TGT GTT TTC ACC GCT TCT GCT CTG CTG ATG CTT GGC TTA AGT TCA TTT GTA GTA TCA GCC
 v   i   t   k   c   v   f   t   a   s   a   l   l   m   l   g   l   s   s   f   v   v   s   a
                                                                        20

CAA TCC CCT TTG TTA AAA GAG CAG ATT GAA ACC ATA GTG ACG GGT AAA AAG GCC ACT GTA GGT GTA GCA GTG
 Q   S   P   L   L   K   E   Q   I   E   T   I   V   T   G   K   K   A   T   V   G   V   A   V
                 30                                              40

TGG GGG CCT GAC GAT CTG GAA CCT TTG TTG CTG AAT CCA TTT GAA AAG TTT CCG ATG CAA AGT GTG TTT AAA
 W   G   P   D   D   L   E   P   L   L   L   N   P   F   E   K   F   P   M   Q   S   V   F   K
50                                          60                                          70

CTG CAT TTA GCT ATG TTA GTT CTG CAT CAG GTC GAT CAG GGG AAA CTG GAT TTA AAT CAG TCT GTT ACT GTT
 L   H   L   A   M   L   V   L   H   Q   V   D   Q   G   K   L   D   L   N   Q   S   V   T   V
                         80                                          90

AAT CGT GCT GCA GTA TTA CAA AAT ACC TGG TCG CCA ATG ATG AAA GAT CAT CAG GGC GAT GAA TTT ACT GTT
 N   R   A   A   V   L   Q   N   T   W   S   P   M   M   K   D   H   Q   G   D   E   F   T   V
         100                                             110

GCA GTA CAG CAG TTA CTG CAG TAT TCG GTG TCA CAC AGC GAC AAT GTG GCC TGC GAT TTG TTA TTT GAA CTG
 A   V   Q   Q   L   L   Q   Y   S   V   S   H   S   D   N   V   A   C   D   L   L   F   E   L
     120                                         130                                     140

GTG GGC GGG CCG CAA GCT TTG CAT GCT TAT ATC CAG TCT TTA GGC GTT AAA GAA GCT GCC GTG GTA GCA AAT
 V   G   G   P   Q   A   L   H   A   Y   I   Q   S   L   G   V   K   E   A   A   V   V   A   N
                             150                                         160

GAA GCG CAA ATG CAT GCG GAT GAT CAG GTG CAA TAT CAA AAC TGG ACG TCG ATG AAA GCC GCA GCA CAA GTT
 E   A   Q   M   H   A   D   D   Q   V   Q   Y   Q   N   W   T   S   M   K   A   A   A   Q   V
             170                                         180

CTG CAA AAG TTT GAA CAG AAA AAG CAG TTG TCT GAA ACC TCT CAG GCC TTG TTA TGG AAA TGG ATG GTT GAA
 L   Q   K   F   E   Q   K   K   Q   L   S   E   T   S   Q   A   L   L   W   K   W   M   V   E
190                                         200                                         210

ACC ACC ACA GGA CCA CAG CGG TTA AAA GGC TTG TTA CCT GCT GGT ACT ATA GTG GCG CAT AAA ACC GGT ACT
 T   T   T   G   P   Q   R   L   K   G   L   L   P   A   G   T   I   V   A   H   K   T   G   T
                     220                                         230

TCG GGC GTC AGA GCA GGA AAA ACT GCG GCG ACT AAT GAT GCG GGC GTC ATT ATG TTG CCT GAT GGA CGG CCT
 S   G   V   R   A   G   K   T   A   A   T   N   D   A   G   V   I   M   L   P   D   G   R   P
                 240                                         250

TTA TTG GTG GCG GTA TTT GTC AAG GAT TCG GCT GAA TCA GAA CGA ACC AAT GAA GCT ATT ATT GCG CAG GTT
 L   L   V   A   V   F   V   K   D   S   A   E   S   E   R   T   N   E   A   I   I   A   Q   V
     260                                         270                                     280

GCG CAA GCG GCT TAT CAG TTT GAG CTG AAA AAA CTC TCT GCA GTG AGT CCG GAT TGA  GCAAAAATCAGCACTATC
 A   Q   A   A   Y   Q   F   E   L   K   K   L   S   A   V   S   P   D
                         290                                         300

TAAGCGACTTATTAAAGGTATAAACTGCAGTCATGATCTGAAAATGGAGTAGGTTATGCAGTTATTAGGTTCAGTGG
```

FIG. 2. Nucleotide sequence of 1.3 kb of the 2.2-kb insert of plasmid pMVP-5-2 containing the bla_{PER-2} gene. The deduced amino acid sequence of PER-2 is given on the line below the nucleotide triplets. The amino acids of the signal peptide are indicated by lowercase letters. A possible ribosome-binding site (RBS) can be found upstream of the start codon. No convincing promoter sequence can be identified by simple comparison with the consensus sequences for the −35 and −10 signal in the upstream region.

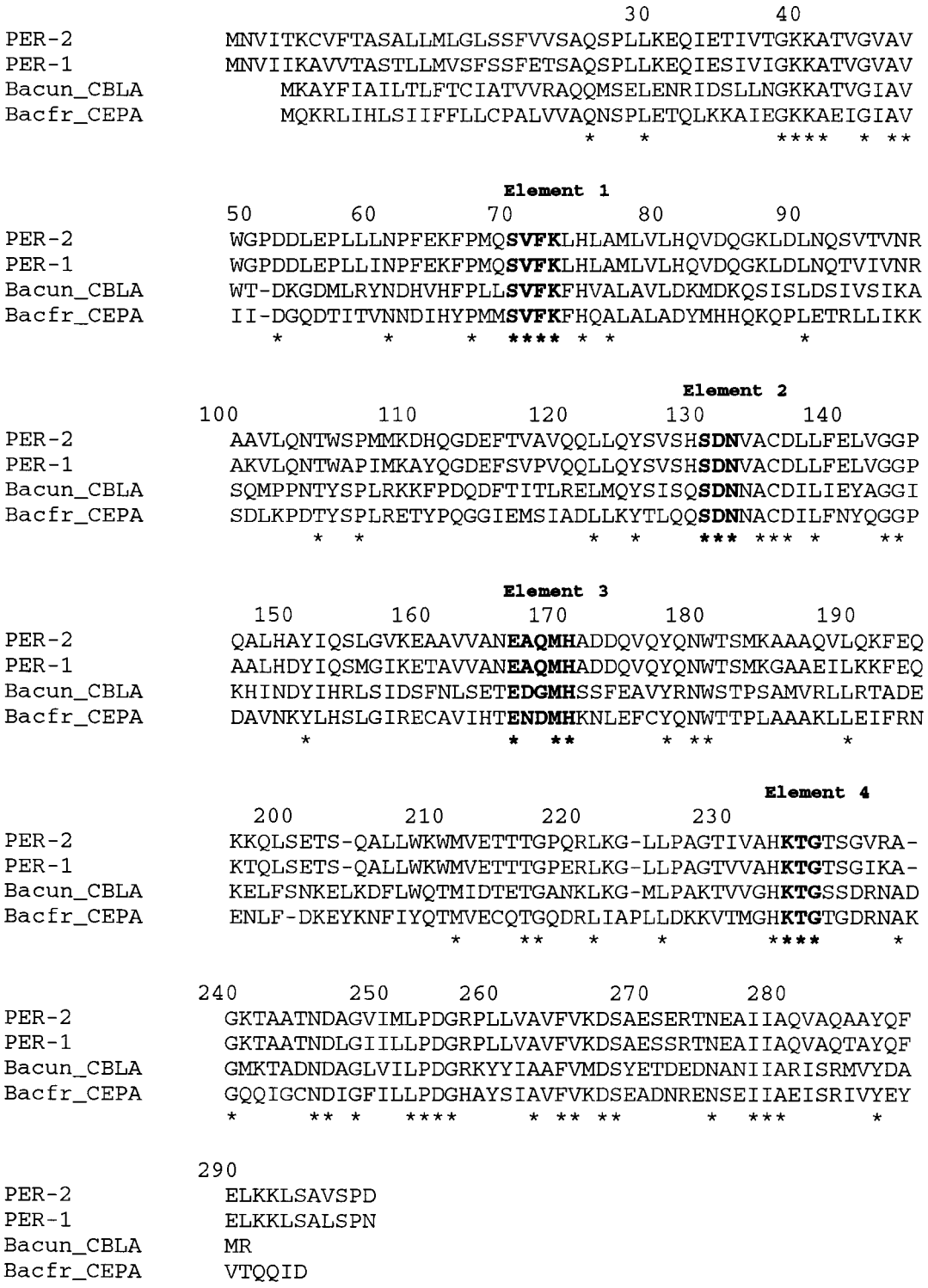

FIG. 3. Multiple sequence alignment of the amino acid sequences of PER-2, PER-1, and related chromosomal β-lactamases of *B. uniformis* (25) and *B. fragilis* (21). Identical amino acids in all four sequences are marked with an asterisk. Numbering is done according to the numbering of Ambler et al. (1). Elements 1 to 4 (indicated by boldface letters) are conserved residues of Ambler class A β-lactamases which surround the active site (16).

bilis, and *S. typhimurium* from Argentina. In contrast, ceftibuten-hydrolyzing *E. coli* and *Salmonella paratyphi* type B strains from Turkey produced the PER-1 type of β-lactamase (5a).

DISCUSSION

The majority of plasmidic ES β-lactamases were shown to be derivatives of TEM-1, TEM-2, or SHV-1, differing from these

ancestors by point mutations in the *bla* genes (10, 15). Furthermore, new ES β-lactamases were detected among the oxacillinases (e.g., OXA-11 [11] and OXA-14 [9]). The number of newly identified plasmidic ES β-lactamases of the TEM or SHV type has decreased in recent years (10). Meanwhile, *bla* genes for ES β-lactamases too unrelated to TEM- or SHV-type genes to represent direct mutational derivatives emerged. Among them the *bla* genes for the cefotaximases CTX-M-1 (MEN-1) and CTX-M-2 were described by Barthélémy et al. (2) and Bauernfeind et al. (6). Their amino acid sequence homologies with the amino acid sequences of TEM and SHV are as low as 36 to 38%. In addition to this new type of *bla* gene reservoir for plasmidic β-lactamases, the PER-2 and PER-1 types of *bla* genes emerged as a second new group within Ambler class A. They are highly unrelated to the CTX-M family (6). The low degree of similarity to the TEM and SHV family has been shown previously for PER-1 from *P. aeruginosa*, in which the $bla_{\text{PER-1}}$ gene was described to be chromosomal (18) as well as plasmidic (8).

The amino acid sequence of the PER-2 β-lactamase that we have described is clearly different from the amino acid sequence of PER-1 (33 amino acid exchanges within the mature β-lactamase; Fig. 3). So far it has been detected in members of the family *Enterobacteriaceae* (*E. coli*, *K. pneumoniae*, *P. mirabilis*, *S. typhimurium*) only and not in *P. aeruginosa* (as PER-1). The spread of PER-2 has been limited to South America (Argentina), where it has persisted over an observation period of 4 years (1990 to 1994; unpublished data). In contrast, the $bla_{\text{PER-1}}$ gene was, until now, identified only in pathogens isolated in Turkey. So, it appears that the incidence of PER-2 or PER-1 at this time is regionally restricted to either South America or Turkey. The identity of the $bla_{\text{PER-1}}$ gene described by Nordmann and Naas (18) and the $bla_{\text{PER-1}}$ gene in members of the family *Enterobacteriaceae* may signal the possibility of an exchange of the gene between members of the family *Enterobacteriaceae* and members of the family *Pseudomonadaceae*. The identity of these two genes indicates that the PER-1 enzyme should hydrolyze ceftibuten as well. This was not noted by Nordmann and Naas (18), since the gene was found in *P. aeruginosa*, which is not susceptible to ceftibuten because of the impermeability of its outer membrane to this compound. However, in homogenates of PER-1-producing *P. aeruginosa* isolates from Istanbul, a band with a pI of 5.4 which hydrolyzed ceftibuten on isoelectric focusing gels was identified (unpublished data). This indicates the similarity of the activities of PER-1 and PER-2 against this substrate.

The $bla_{\text{PER-1}}$ and $bla_{\text{PER-2}}$ genes as well as the $bla_{\text{CTX-M}}$ gene represent additional reservoirs of new ES β-lactamases within Ambler class A with so far unknown variability. Their incidence, spread, and persistence in distant geographical areas signal the ongoing evolution of new ES β-lactamases beyond the TEM and SHV derivatives.

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