Molecular and Biochemical Characterization of VEB-1, a Novel Class A Extended-Spectrum β-Lactamase Encoded by an *Escherichia coli* Integron Gene

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A clinical isolate, *Escherichia coli* MG-1, isolated from a 4-month-old Vietnamese orphan child, produced a β-lactamase conferring resistance to extended-spectrum cephalosporins and aztreonam. In a disk diffusion test, a typical synergistic effect between ceftazidime or aztreonam and clavulanic acid was observed along with an unusual synergy between cefoxitin and cefuroxime. The gene for VEB-1 (Vietnamese extended-spectrum β-lactamase) was cloned and expressed in *E. coli* JM109. The recombinant plasmid pRLT1 produced a β-lactamase with a pI of 5.35 and conferred high-level resistance to extended-spectrum (or oxyimino) cephalosporins and to aztreonam. V_{max} values for extended-spectrum cephalosporins were uncommonly high, while the affinity of the enzyme for ceftazidime and aztreonam was relatively low. bla_{VEB-1} showed significant homology at the DNA level with only bla_{PER-1} and bla_{PER-2}. Analysis of the deduced protein sequence showed that VEB-1 is a class A penicillinase having very low levels of homology with any other known β-lactamases. The highest percentage of amino acid identity was 38% with PER-1 or PER-2, two uncommon class A extended-spectrum enzymes. Exploration of the genetic environment of bla_{VEB-1} revealed the presence of gene cassette features, i.e., (i) a 59-base element associated with bla_{VEB-1}; (ii) a second 59-base element just upstream of bla_{VEB-1}, likely belonging to the *aacA1-orfG* gene cassette; (iii) two core sites (GTTRRRY) on both sides of bla_{VEB-1}; and (iv) a second antibiotic resistance gene 3′ of bla_{VEB-1}, *aadB*. bla_{VEB-1} may therefore be the first class A extended-spectrum β-lactamase that is part of a gene cassette, which itself is likely to be located on a class 1 integron, as sulfamide resistance may indicate. Furthermore, bla_{VEB-1} is encoded on a large (>100-kb) transferable plasmid found in a *Klebsiella pneumoniae* MG-2 isolated at the same time from the same patient, indicating a horizontal gene transfer.

Although naturally susceptible to extended-spectrum cephalosporins, *Escherichia coli* strains may become resistant to these β-lactams by several mechanisms, mainly alterations in outer membrane proteins, overproduction of cephalosporinase (chromosomal or plasmid mediated), or production of an extended-spectrum β-lactamase (ESBL). ESBLs are enzymes capable of hydrolyzing oxyimino cephalosporins and monobactams.

Analysis of the known β-lactamase sequences has resulted in their being divided according to their amino acid sequences into four classes, designated A to D (1). Most of the ESBLs found so far in *Enterobacteriaceae* are Ambler class A β-lactamases. They are plasmid encoded, and the enzymes most commonly observed in *E. coli* are TEM derivatives and to a lesser extent SHV derivatives (28). The extension of their hydrolysis properties results from single amino acid changes within their catalytic sites. Epidemiological studies have revealed that these ESBLs are now disseminated worldwide (28). In addition to these ESBLs, non-SHV non-TEM derivative enzymes have been detected in *E. coli*: FEC-1 (27), CTX-M1 (MEN-1) (5, 6), CTX-M2 (6), PER-2 (7), and TOHO-1 (19). The structurally related CTX-M1, TOHO-1, and CTX-M2 have

been isolated among rare enterobacterial isolates in Europe, while the spread of PER-2 among *Enterobacteriaceae* family members has been limited so far to South America (7). FEC-1, which was found in an *E. coli* strain in Japan, has so far only been biochemically characterized (27).

Along with a plasmid location, some β-lactamase genes may be encoded in gene cassettes that are present in the variable region of the integrons (15, 38, 45). The gene cassettes are discrete mobile units, and each comprises a gene, normally an antibiotic resistance gene and a recombination site that is recognized by an integrase (12, 38). The cassette-associated recombination sites known as 59-base elements (59-be) are located downstream of the genes and are of variable length (12, 46). The most highly conserved features of 59-be are a 7-bp core site with the consensus site GTTRRRY located at the right-hand end of the element (furthest from the 3′ end of the cassette-encoded gene) and an inverse core site with the consensus site RYYYAAC at the left-hand end (12, 46). The integrons most commonly isolated from antibiotic-resistant clinical isolates from members of the family *Enterobacteriaceae* and *Pseudomonads* belong to class 1. These class 1 integrons possess two conserved regions located on either side of the integrated gene cassettes. The 5′ conserved segment includes a gene, *int1*, encoding the integrase; *attI*, the cassette integration site; and the promoter *Pant*, which is responsible for expression of cassette genes. The 3′ conserved segment includes, along with two other open reading frames (ORFs), the sulfamide

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype	Source or reference[a]
Strains		
E. coli JM109	endA1 hsdR17 gyrA96 Δ(lac proA) recA1 relA supE44 thi F′ (lacI^q lacZΔM15 proAB$^+$ traD36)	52
In vitro-obtained ciprofloxacin-resistant E. coli JM109	Ciprofloxacin resistant	37
E. coli NCTC 50192	154-, 66-, 48-, and 7-kb reference plasmids	37
E. coli MG-1	Extended spectrum cephalosporin resistant	This study
Plasmids		
pBK-CMV phagemid	Neomycin^r, kanamycin^r	Stratagene (La Jolla, Calif.)
pBR322	Recombinant plasmid containing the 560-bp SspI-PstI internal fragment of bla$_{TEM-1}$	48
pHUC37	Recombinant plasmid containing the 435-bp PstI-NotI internal fragment of bla$_{SHV-3}$	32
pPZ1	Recombinant plasmid containing the 1.1-kb SnaBI internal fragment of bla$_{PER-1}$	34
pPL1	Recombinant plasmid containing the 450-bp SnaBI internal fragment of oxa18	37
pRLT1	Recombinant plasmid containing a 1.3-kb Sau3A fragment with bla$_{VEB1}$	This report
pNLT1	Natural plasmid from E. coli MG-1 containing bla$_{VEB1}$. Resistance phenotype: ESBL, Kan, Tm, AN, Net, Tet, Cm, Sul	
pNLT2	Natural plasmid from E. coli MG-1 containing bla$_{TEM-1}$. Resistance phenotype: TEM, Cm	
pNLT3	Natural plasmid from K. pneumoniae MG-2 containing bla$_{VEB1}$. Resistance phenotype: ESBL, Kan, Tm, AN, Net, Tet, Cm, Sul	
pRLT50	Recombinant plasmid containing 1.4-kb genomic Sau3A fragment containing bla$_{TEM-1}$	This report

[a] Abbreviations: Kan, kanamycin; Tm, tobramycin; Net, netilmicin; AN, amikacin; Cm, chloramphenicol; Tet, tetracycline; Sul, sulfamide.

resistance determinant (sulI) (38). The existence of integrons and integron-associated genes explains how plasmids may accumulate a diversity of resistance genes. While some class D ESBLs are found on integrons (14, 37, 38), no class A ESBL has yet been found on the variable region of integrons (15).

The β-lactamase described in this report is a novel plasmid-encoded ESBL from an E. coli clinical isolate recovered from a 4-month-old Vietnamese child. E. coli MG-1 displayed resistance to both extended-spectrum cephalosporins and to aztreonam and showed typical synergy during a double disk assay when ceftazidime or aztreonam was placed next to a clavulanic acid disk on an agar plate. Its gene was cloned and sequenced, and the deduced protein sequence was compared with those of other class A β-lactamases. The enzymatic properties of the enzyme included high-level hydrolytic activity against extended-spectrum cephalosporins. We also characterized its plasmid determinant and its transferability to other Enterobacteriaceae spp. In addition, we provided evidence of gene transfer from E. coli to Klebsiella pneumoniae. Finally, its gene is located on a gene cassette which may itself be present on an integron.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this work are listed in Table 1.

Antimicrobial agents and MIC determinations. The antimicrobial agents used in this study were obtained in the form of standard laboratory powders and were used immediately after their solubilization. The agents and their sources were as follows: amoxicillin, clavulanic acid, and ticarcillin (Smith-Kline-French-Beecham, Nanterre, France); aztreonam and cefepime (Bristol-Myers Squibb, Paris La Défense, France); ceftazidime, cefuroxime, and cephaloridine (Glaxo, Paris, France); cefamandole, cefalotin, and moxalactam (Eli Lilly, Saint-Cloud, France); piperacillin and tazobactam (Lederle, Oullins, France); sulbactam and cefoperazone (Pfizer, Orsay, France); cefotaxime and cefpirome (Hoechst-Roussel, Paris, France); cefoxitin and imipenem (Merck Sharp and Dohme-Chibret, respectively, Paris, France); ceftriaxone (Hoffmann-La-Roche, Neuilly-sur-Seine, France); benzylpenicillin (Specia, Paris, France); and ciprofloxacin (Bayer, Paris, France). Antibiotic disks were used for routine antibiograms (Sanofi-Diagnostics Pasteur, Marnes-la-Coquette, France).

MICs were determined by an agar dilution technique on Mueller-Hinton agar (Diagnostics Pasteur) with an inoculum of 10^4 CFU per spot. All plates were incubated at 37°C for 18 h. MICs of β-lactams were determined alone or in combination with a fixed concentration of clavulanic acid (2 μg/ml).

Hybridization and PCR analyses. Dot blots and Southern hybridizations were performed as described by the manufacturer by using the ECL nonradioactive kit (Amersham, Les Ulis, France). The probes (Table 1) consisted of the 1.1-kb SnaBI fragment from recombinant plasmid pPZ1 for bla$_{PER-1}$, the 450-bp PstI-NotI fragment from recombinant plasmid pHUC37 for bla$_{SHV-3}$, the 560-bp

SspI-PstI fragment from plasmid pBR322 for bla$_{TEM-1}$, or the 450-bp PstI-NotI fragment from recombinant plasmid pPL1 for oxa18. Standard PCR experiments were performed as described previously (40).

Plasmid content and mating-out assays. Plasmid DNAs of E. coli MG-1 and K. pneumoniae MG-2 were prepared with the Qiagen plasmid DNA maxi kit (Qiagen, Courtaboeuf, France). Plasmid DNAs were analyzed by electrophoresis on a 0.8% agarose gel (Gibco-BRL-Life Technologies, Eragny, France) containing 0.15 μg of ethidium bromide (Pharmacia-Biotech, Orsay, France)/ml. Standard sizes of plasmid DNAs were extracted from E. coli NCTC 50192.

The extracted plasmid DNAs from either E. coli MG-1 or K. pneumoniae MG-2 were subjected to electroporation into E. coli JM109 according to the manufacturer's instructions (Bio-Rad, Ivry-sur-Seine, France). Recombinant bacteria were plated onto Trypticase soy agar plates containing 100 μg of amoxicillin/ml. The plasmids were again extracted by using the Qiagen maxi columns kit (Qiagen), and the sizes were estimated by restriction endonuclease digestions (Pharmacia Biotech).

Direct transfer of resistance into ciprofloxacin-resistant E. coli JM109 obtained in vitro was attempted by liquid and solid mating-out assays at 30 and 37°C. Transconjugant selection was performed on Trypticase soy agar plates containing ciprofloxacin (3 μg/ml) and amoxicillin (100 μg/ml).

Cloning experiments and analysis of recombinant plasmids. Genomic DNA of E. coli MG-1 was extracted as described previously (34). Fragments from Sau3AI partially digested genomic DNA were ligated into the BamHI site of pBK-CMV phagemid (Stratagene, La Jolla, Calif.) as previously described (34). The restriction enzymes as well as the ligase were from Pharmacia Biotech.

Recombinant plasmid DNA was prepared by using Qiagen columns (Qiagen), and plasmid maps were determined after double restriction analysis (40). Fragment sizes were estimated by comparison to the molecular weight standard 1-kb DNA ladder (Gibco-BRL-Life Technologies).

β-Lactamase preparation. Cultures of E. coli expressing bla$_{VEB-1}$ were grown overnight at 37°C in 100 ml of Trypticase soy broth with amoxicillin (100 μg/ml). Bacterial suspensions were disrupted by sonication (four times for 20 s at 20 KHz) and centrifuged (30 min, 20,000 × g, 4°C). The supernatant contained the crude enzyme extract. The enzyme was further purified by ion exchange chromatography with AGMP-1 resin (Bio-Rad) (34). The resin, in the form of ion chloride, was first treated with 0.1 M ammonia in water and then washed extensively with water. After absorption of the extracts, elution was performed with a 0.1 M NaCl solution. The active fractions were pooled, dialyzed extensively, and lyophilized. The relative molecular mass of partially purified β-lactamase obtained from E. coli JM109 harboring recombinant plasmid pRLT1 was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (24).

Isoelectric focusing. Crude β-lactamase extracts were subjected to analytical isoelectric focusing on an ampholine polyacrylamide gel (pH 3.5 to 9.5) (Pharmacia Biotech) for 36 h at 10 W of constant power on a flatbed apparatus (FBE-3000; Pharmacia). The β-lactamase was visualized with an overlay of agar-iodine starch gel containing benzylpenicillin (0.01% [wt/vol]) in 0.1 M phosphate buffer (pH 7.0) (4). The pI of VEB-1 was determined by comparison to those of known β-lactamases.

Kinetic measurements. Kinetic measurements were performed with a purified β-lactamase preparation extracted from E. coli harboring recombinant plasmid pRLT1. The kinetic constants of preparations were determined by the online computerized microacidimetric method at pH 7.0 and 37°C as previously de-

scribed (23). As assessed by isoelectric focusing and sequencing, the enzyme preparation contained only a single β-lactamase activity. The K_m was expressed in micromolar concentrations, and V_{max} was expressed relative to that of benzylpenicillin ($V_{max} = 100$). In the case of substrates with low or undetectable V_{max} values, enzyme substrate affinity was measured as K_i (inhibition constant) rather than K_m with cefotaxime as the substrate.

Inhibition of β-lactamase activity. Various concentrations of clavulanic acid, sulbactam, tazobactam, imipenem, cefoxitin, and moxalactam were preincubated with the enzyme for 10 min at 37°C before testing the rate of cefotaxime hydrolysis and calculating the inhibition constant (K_i) (23).

DNA sequencing and protein analysis. The 1.2-kb cloned DNA fragment from pRLT1 and the 1.4-kb cloned DNA fragment from pRLT50 were sequenced on both strands by using an Applied Biosystems sequencer (ABI 311). The nucleotide sequence and the deduced protein sequence were analyzed with software available over the Internet at the National Center of Biotechnology Information website (30a) and at Pedro's BioMolecular Research Tools website (35a). Multiple sequence alignment of deduced peptide sequences was carried out over the Internet at the University of Cambridge website using the program ClustalW. The following 19 class A β-lactamases were compared to VEB-1: SHV-2 (18), TEM-3 (44), PSE-4 (8), SME-1 (30), NMC-A (29), KOXY (2), CTX-M-1 (6), TOHO-1 (19), CITDI (36), YENT (41), BLIP (31), CAKCC (25), ROB-1 (22), PC-1 (11), PER-1 (34), PER-2 (7), CFXA (35), CEPA (39), and CBLA (43). A dendrogram was derived from the multiple sequence alignment by a parsimony method using the phylogeny package PAUP (Phylogenetic Analysis Using Parsimony) version 3.0 (49).

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the GenBank nucleotide database under the accession no. AF010416.

RESULTS

Origin of the *E. coli* MG-1 isolate. *E. coli* MG-1 was isolated in 1996, at the Hôpital Antoine Béclère, Clamart (a suburb of Paris), France, from the pus of a 4-month-old Vietnamese boy hospitalized for severe respiratory problems. He was previously hospitalized in an intensive care unit in Vietnam. Antimicrobial regimens before admission were not documented, and the patient did not receive any antibiotic treatment prior to the isolation of the strain at the Hôpital Antoine Béclère. A routine antibiogram revealed high levels of resistance of *E. coli* MG-1 to amino, carboxy, and ureido-penicillins and to restricted and extended-spectrum cephalosporins (Table 2). *E. coli* MG-1 was also resistant to kanamycin, chloramphenicol, tetracycline, gentamicin, tobramycin, netilmicin, amikacin, trimethoprim, and trimethoprim-sulfamethoxazole. In the course of a systematic multiresistant-bacteria rectal screening, the same *E. coli* MG-1 was isolated along with a *K. pneumoniae* MG-2 strain presenting a similar resistance profile.

Hybridizations and cloning of the ESBL gene. Preliminary hybridization experiments indicated that *E. coli* MG-1 harbored a TEM-derived resistance gene as indicated by a positive hybridization signal detected by a bla_{TEM-} probe (data not shown). bla_{SHV-3}, $oxa18$, and bla_{PER-1} probes failed to give positive hybridization signals. PCR amplification using TEM-1 intragenic primers and direct sequencing of the PCR product showed 100% identity with bla_{TEM-1}. Since TEM-1 is not an ESBL, its presence might not explain the uncommon resistance phenotype. DNA from *E. coli* MG-1 was partially digested with restriction endonuclease *Sau*3AI and ligated to *Bam*HI-digested plasmid pBK-CMV. The ligation product was transformed into *E. coli* JM109 by electroporation. Several recombinant colonies expressing one of the following two phenotypes were obtained: (i) a high level of resistance to amoxicillin, cephalothin, and ticarcillin, which was inhibited by clavulanic acid; or (ii) an extended-spectrum phenotype. The recombinant plasmids expressing each β-lactamase resistance phenotype were extracted and analyzed. The insert sizes ranged from 1.2 to 15 kb. Restriction maps were generated for both plasmids pRLT1 and pRLT50 containing, respectively, a 1.2-kb insert expressing the ESBL and a 1.3-kb insert expressing a penicillinase (pRLT50) (Fig. 1).

TABLE 2. MICs of β-lactams for *E. coli* MG-1, *E. coli* JM109 harboring recombinant plasmids pRLT1 and pRLT50, and reference strain *E. coli* JM109

Antibiotic(s)	MIC (μg/ml) for:			
	E. coli MG-1[a]	*E. coli* JM109 (pRLT1)[b]	*E. coli* JM109 (pRLT50)[c]	*E. coli* JM109
Amoxicillin	>512	>512	>512	2
Amoxicillin-Cla[d]	128	8–16	256	1
Ticarcillin	>512	>512	>512	2
Ticarcillin-Cla	256	4	256	2
Piperacillin	256	16	512	1
Piperacillin-Cla	4	2	64	1
Cephalothin	128	128	256	4
Cephalothin-Cla	4	4	4	4
Cefamandole	32	32	32–64	0.5
Cefuroxime	64	128	8	<2
Cefuroxime-Cla	4	4	4	<2
Cefoxitin	4	8	4	4
Cefoxitin-Cla	4	4	2	2
Ceftazidime	256	256	1	0.25
Ceftazidime-Cla	0.25	0.25	1	0.25
Cefotaxime	2	2	0.12	0.06
Cefotaxime-Cla	0.06	0.06	0.06	0.06
Cefepime	1	1	0.12	0.06
Cefepime-Cla	0.03	0.06	0.03	0.06
Ceftriaxone	2	2	0.12	0.06
Moxalactam	0.25	0.12	0.12	0.12
Moxalactam-Cla	0.25	0.12	0.12	0.25
Aztreonam	32	32	0.25	0.12
Aztreonam-Cla	0.12	0.12	0.25	0.06
Imipenem	0.25	0.25	0.12	0.12

[a] *E. coli* MG-1 produces VEB-1 along with a TEM-1 penicillinase.
[b] *E. coli* JM109 harboring recombinant plasmid pRLT1 produced the VEB-1 β-lactamase.
[c] *E. coli* JM109 harboring recombinant plasmid pRLT50 produced the TEM-1 β-lactamase.
[d] Cla, clavulanic acid at fixed concentration of 2 μg/ml.

Biochemical properties of VEB-1. MICs of β-lactams for *E. coli* JM109 harboring recombinant plasmid pRLT50 showed mainly resistance to penicillins, while pRLT1 gave high MICs of aztreonam, ceftazidime, moxalactam, and cefuroxime. All β-lactam antibiotic MICs were lower in the presence of clavulanic acid (2 μg/ml).

Kinetic parameters of purified VEB-1 β-lactamase, obtained from an *E. coli* JM109 culture harboring recombinant plasmid pRLT1, showed strong hydrolytic activity against most antibiotics tested (Table 3). The activity against expanded-spectrum cephalosporins in general was very high except for ceftazidime and aztreonam (Table 3), while the hydrolytic activities against penicillins were much lower. The kinetic parameters of VEB-1 are characterized by low K_m values for all the β-lactams tested (Table 3) except for ceftazidime and aztreonam. Steady-state inhibitory kinetic parameters (K_i) of VEB-1 β-lactamase with cefotaxime as substrate were as follows: cefoxitin, 15 nM; moxalactam, 18 nM; imipenem, 25 nM; clavulanic acid, 10 nM; sulbactam, 20 nM; tazobactam, 20 nM.

Analytical isoelectric focusing revealed that *E. coli* MG-1 had two β-lactamase activities with pIs of 5.4 and 5.35. *E. coli* JM109 harboring the recombinant plasmid pRLT1 had one β-lactamase activity with a pI of 5.35 (data not shown), while the recombinant plasmid pRLT50 had a β-lactamase activity with a pI of 5.4. The relative molecular mass of the cloned mature β-lactamase expressed from *E. coli* JM109 harboring pRLT1 was estimated by SDS-PAGE to be 30 kDa (data not shown).
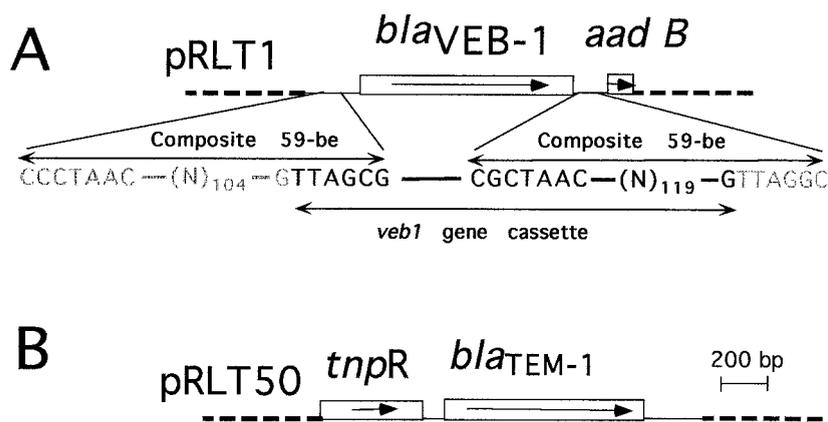
FIG. 1. Schematic map of the recombinant plasmids pRLT1, encoding VEB-1, (A) and pRLT-50, encoding TEM-1 (B). The thin line represents the cloned inserts from *E. coli* MG-1 while the dotted lines indicate the vector pBK-CMV. The open boxes represent the genes, and the arrows indicate their translational orientation. The designations of the gene names are provided in the text. The core sites (GTTRRRY) along with the inverse core sites (RYYYAAC) are indicated along with the composite 59-BEs. The boundaries of *veb1* gene cassette are indicated in bold, while the surrounding sequence is in gray.

Structural properties of bla_{VEB-1} and of its deduced protein sequence. The cloned 1.3-kb genomic DNA of pRLT1 was sequenced on both strands. Analysis of coding regions revealed a sufficiently large ORF of 897 bp encoding a 299-amino-acid preprotein approximately 33 kDa in size. The DNA sequence of this gene, along with flanking sequences, is shown in Fig. 2. A BLAST search against the GenBank database using the DNA sequence of this gene revealed significant identity scores (54% over 260 bp) with bla_{PER-1} and bla_{PER-2} (7, 34). No other scores for known β-lactamase genes were found. The overall GC content of this gene, 45%, is typical of *Enterobacteriaceae*. The translation stop codon (TAA), found at positions 1071 to 1073, corresponded to the most common codon in *E. coli* and enterobacterial genes. No putative promoter sequence was detected by sequence analysis upstream of the β-lactamase gene.

Within the deduced protein sequence, a serine-valine-methionine-lysine tetrad (S-X-X-K) was found at positions 70 to 73 (Fig. 2); it included the conserved serine and lysine amino acid residues characteristic of penicillin-binding proteins (20) or β-lactamases possessing a serine active site (21). Several other structural elements characteristic of class A β-lactamases were found, e.g., serine-aspartate-asparagine (S-D-N) at positions 130 to 132 and lysine-threonine-arginine (K-T-G) at positions 234 to 237 (Fig. 2). The deduced peptide sequence showed less than 20% amino acid identity with most known class A enzymes, with the highest percentage of identity being 38% with PER-1 and PER-2 (see Fig. 4), two ESBLs found primarily in *P. aeruginosa* (13, 34, 51) and in several *Enterobacteriaceae*, respectively (7). The enzyme is therefore a novel class A β-lactamase and was named VEB-1 (for Vietnamese extended-spectrum β-lactamase). A dendrogram analysis of VEB-1 with 17 class A β-lactamases showed that VEB-1 clearly clustered with PER-1, PER-2, CBLA, and CEPA, and to a lesser extent with CFXA.

Analysis of the genetic environment of bla_{VEB-1} on pRLT1 revealed key signatures of gene cassettes. The presence of a core site, GTTAGCG, at positions 128 to 134 (Fig. 2) and the presence, 3′ of bla_{VEB-1}, of an inverse core site, CGCTAAC, followed by the remainder of a 59-be strongly suggested that bla_{VEB-1} is encoded on a gene cassette and could thus be part of the variable region of an integron. The *veb1* gene cassette is 1,059 bp long, and its 59-be is 133 bp long (Fig. 1 and 2). Exploration of the genetic environment of the *veb1* gene cassette revealed the presence of a second antibiotic resistance

gene, *aadB*, encoding an aminoglycoside adenyl transferase (17, 42), associated with a consensus 7-bp core site. Furthermore, the sequence upstream to the *veb1* gene cassette is identical to part of a sequence from Tn*2424* submitted to GenBank (AF047479) and not yet published. The alignment is to the end of a cassette (including the entire 59-be) containing two genes, *aacA1* and *orfG*. Using primers specific to *aacA1* and to bla_{VEB-1}, we were able to amplify a 0.9-kb fragment from total DNA of *E. coli* MG-1 (data not shown), indicating that this *aacA1-orfG* cassette is indeed upstream of *veb1* gene cassette.

The cloned 1.4-kb genomic DNA from pRLT50 was entirely sequenced on both strands. Coding region analysis revealed a sufficiently large ORF of 861 bp encoding a 286-amino-acid preprotein. A schematic representation of the ORFs and flanking sequences is shown in Fig. 2. A BLAST search against the GenBank database revealed 100% identity with a gene encoding the TEM-1 β-lactamase. The 1.4-kb insert had perfect homology with a plasmid, pJCD4, found in *Neisseria gonorrhoeae* (unpublished GenBank accession no. U20374) and with plasmid pCFF04 from *K. pneumoniae* (26). Indeed, the analysis of the sequence upstream of bla_{TEM-1} revealed the presence of *tnpR*, the resolvase gene of a Tn*3* or Tn*3*-derived transposon (16). The sequence information did not allow us to discriminate between Tn*3* and Tn*3*-derived transposons. One of these transposons, Tn*1331*, is present on plasmid pCFF04 and encodes *oxa9* in addition to bla_{TEM-1} (50). However, this trans-

TABLE 3. Steady-state kinetic parameters of VEB-1 β-lactamase

Substrate	$V_{max_{rel}}$	K_m (μM)	$V_{max_{rel}}/K_m$[a]
Benzylpenicillin	100	2.8	100
Amoxicillin	110	6.0	50
Ticarcillin	8	1.0	22
Cephalothin	700	6.0	325
Cephaloridine	2,300	12.0	533
Cefamandole	800	5.6	397
Cefoperazone	140	4.5	86
Ceftriaxone	2,900	22.0	366
Cefotaxime	4,300	38.0	314
Cefuroxime	2,000	24.0	230
Ceftazidime	8,000	460.0	47
Aztreonam	400	500.0	2

[a] Values relative to that of benzylpenicillin, which was set at 100.

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GATCAACCGAAATTTGCCCTAACAAATGCTTCAACCTGACAATTCCTGTTGTCATGGTTT    60

GTGCTGTCGCTTTGCTCAGCACAAACCACGCCAAGCCCTTCGGGCCGGAATTGCAGGTTA   120

AGCAAATGTTAGCGGTAATTTAACCAGATAGGAGTACAGACACATATGAAAATCGTAAAAAG  180
                 RBS              M   K   I   V   K   R    6
                                 bla_VEB-1 --->

GATATTATTAGTATTGTTAAGTTTATTTTTTACAATTGTGTATTCAAATGCTCAAACTGA   240
 I   L   L   V   L   L   S   L   F   F   T   I   V   Y   S   N   A   Q   T   D    26

CAACTTAACTTTGAAAATTGAGAATGTTTTAAAGGCAAAAAATGCCAGAATAGGAGTAGC   300
 N   L   T   L   K   I   E   N   V   L   K   A   K   N   A   R   I   G   V   A    46

AATATTCAACAGCAATGAGAAGGATACTTTGAAGATTAATAACGACTTCCATTTCCCGAT   360
 I   F   N   S   N   E   K   D   T   L   K   I   N   N   D   F   H   F   P   M    66

GCAAAGCGTTATGAAATTTCCGATTGCTTTAGCCGTTTTGTCTGAGATAGATAAAGGGAA   420
 Q   S   V   M   K   F   P   I   A   L   A   V   L   S   E   I   D   K   G   N    86

TCTTTCTTTTGAACAAAAAATAGAGATTACCCCTCAAGACCTTTTGCCTAAAACGTGGAG   480
 L   S   F   E   Q   K   I   E   I   T   P   Q   D   L   L   P   K   T   W   S    106

TCCGATTAAAGAGGAATTCCCTAATGGAACAACTTTGACGATTGAACAAATACTAAATTA   540
 P   I   K   E   E   F   P   N   G   T   T   L   T   I   E   Q   I   L   N   Y    126

TACAGTATCAGAGAGCGACAATATTGGTTGTGATATTTTGCTAAAATTAATCGGAGGAAC   600
 T   V   S   E   S   D   N   I   G   C   D   I   L   L   K   L   I   G   G   T    146

TGATTCTGTTCAAAAATTCTTGAATGCTAATCATTTCACTGATATTTCAATCAAAGCAAA   660
 D   S   V   Q   K   F   L   N   A   N   H   F   T   D   I   S   I   K   A   N    166

CGAAGAACAAATGCACAAGGATTGGAATACCCAATATCAAAATTGGGCAACCCCAACAGC   720
 E   E   Q   M   H   K   D   W   N   T   Q   Y   Q   N   W   A   T   P   T   A    186

GATGAACAAACTGTTAATAGATACTTATAATAATAAGAACCAATTACTTTCTAAAAAAAG   780
 M   N   K   L   L   I   D   T   Y   N   N   K   N   Q   L   L   S   K   K   S    206

TTATGATTTTATTTGGAAAATTATGAGAGAAACAACAACAGGAAGTAACCGATTAAAAGG   840
 Y   D   F   I   W   K   I   M   R   E   T   T   T   G   S   N   R   L   K   G    226

ACAATTACCAAAGAATACAATTGTTGCTCATAAAACAGGGACTTCCGGATAAATAATGG   900
 Q   L   P   K   N   T   I   V   A   H   K   T   G   T   S   G   I   N   N   G    246

AATTGCAGCAGCCACTAATGATGTTGGGGTAATTACTTTACCGAATGGACAATTAATTTT   960
 I   A   A   A   T   N   D   V   G   V   I   T   L   P   N   G   Q   L   I   F    266

TATAAGCGTATTTGTTGCAGAGTCCAAAGAAACTTCGGAAATTAATGAAAAGATTATTTC  1020
 I   S   V   F   V   A   E   S   K   E   T   S   E   I   N   E   K   I   I   S    286

AGACATTGCAAAAATAACGTGGAATTACTATTTGAATAAATAAAAAACTACCGCTAACAC  1080
 D   I   A   K   I   T   W   N   Y   Y   L   N   K   *                          299

TGGCTCATAGGCAATGGCGGGTTGAAGTGCAATTTGCAAAGTCGGTAGCCCGCCCGAGCG  1140

TTTTCTCGGTTTGACAGGAAAGGCTCACGCAAACCGCCACTGCCCATAGCCCAAACCGTT  1200

AGGCCGCATGGACACAACGCAGGTCACATTGATACACAAAATTCTAGCTGCGGCAGATGA  1260
         M   D   T   T   Q   V   T   L   I   H   K   I   L   A   A   A   D   E    18
         aadB --->

GCGAAATCTGCCGCTCTGGATC  1282
 R   N   L   P   L   W   I      25
```

FIG. 2. Nucleotide sequence of the 1,282-bp fragment of pRLT1 containing the VEB-1 β-lactamase coding region. The deduced amino acid sequence is designated in single-letter code below the nucleotide sequence. Conserved motifs for class A enzymes are underlined. The start and stop codons of the various genes are shown in boldface and underlined. RBS, putative ribosomal binding site of bla_{VEB-1}. The core sites (GTTRRRY) are double underlined, while the inverse core sites (RYYYAAC) are underscored with a dashed line. The composite 59-be's are italicized.

poson was ruled out because the plasmid pNLT2 expresses only one β-lactamase, corresponding to TEM-1, as seen by isoelectric focusing (data not shown).

Plasmid analysis. Plasmid DNA preparation from *E. coli* MG-1 revealed the presence of two distinct plasmids, pNLT1 and pNLT2. pNLT2 encoded bla_{TEM-1} and was not further characterized, whereas pNLT1 was >100 kb and coded for bla_{VEB-1}, as shown by hybridization. Both plasmids were transferred by electroporation into *E. coli* JM109, resulting in the following phenotypes: ESBL, gentamicin, kanamycin, tobramycin, netilmicin, amikacin, chloramphenicol, tetracycline, and sulfamide resistance (for pNLT1) and penicillinase and chloramphenicol resistance (for pNLT2). These results indicated that both genes were plasmid borne and confirmed the hybridization results in the sense that the plasmid pNLT1 encoded bla_{VEB1}. Furthermore, both plasmids were transferred by conjugation at a very high frequency (10^{-3} to 10^{-4}) into an *E. coli* JM109 recipient strain (data not shown).

Rectal screening for multiresistant bacteria revealed the presence of the same *E. coli* MG-1 and a *K. pneumoniae* MG-2 presenting a similar resistance profile. PCR analysis using intragenic primers of bla_{VEB-1} indicated the presence of this gene in the *K. pneumoniae* strain. Furthermore, a single plasmid, pNLT3, similar in size to pNLT1 was present in the *K. pneumoniae* MG-2 strain. pNLT3, once conjugated into *E. coli* JM109 had the same associated markers and an identical restriction digestion pattern as pNLT1 (data not shown). These results indicate that the same plasmid is present in both enterobacterial strains.

DISCUSSION

This work was initiated with the observation that an *E. coli* clinical isolate showed an extended-spectrum resistance phenotype with a marked synergistic effect between clavulanic acid and ceftazidime. The enzyme responsible for the observed phenotype, VEB-1, has important functional similarities with ESBLs found in *Enterobacteriaceae*. The mature form of VEB-

```
              1        10        20      -><-              30        40        50        60
              |         |         |                        |         |         |         |
TEM-3   ---MSIQHFRVALIPFFAAFCLPVFAHP---------------ETLVKVKDAEDKLGARVGYIELDLNSGKILESFRP
PER-1   -MNVIIKAV----VTASTLLMVSFSSFETSAQSP--------LLKEQIESIVIGKKATVGVAVWGPDDLEPLL-INP
PER-2   -MNVITKCV----FTASALLMLGLSSFVVSAQSP--------LLKEQIETIVTGKKATVGVAVWGPDDLEPLL-LNP
CEPA    -----MQKR----LIHLSIIFFLLCPALVVAQNS--------PLETQLKKAIEGKKAEIGIAVI-IDGQDTIT-VNN
CBLA    ------MKAY------FIAILTLFTCIATVVRAQQMS-------ELENRIDSLLNGKKATVGIAVW-TDKGDMLR-YND
VEB-1   --MKIVKR------ILLVLLSLFFTIVYSNAQTDN--------LTLKIENVLKAKNARIGVAIFNSNEKDTLK-INN
CFXA    MEKNRKKQIVVLSIALVCIFILVFSLFHKSATKDSANPPLTNVLTDSISQIVSACPGEIGVAVI-VNNRDTVK-VNN
                                                                        .Box-I

              70        80        90        100       110       120       130
              |         |         |         |         |         |         |
TEM-3   EERFPMMSTFKVLLCGAVLSRVDAGQEQLGRRIHYSQNDLVK--YSPVTEKHLTD--GMTVRELCSAAITMSDNTAA
PER-1   FEKFPMQSVFKLHLAMLVLHQVDQGKLDLNQTVIVNRAKVLQNTWAPIMKAYQGDEFSVPVQQLLQYSVSHSDNVAC
PER-2   FEKFPMQSVFKLHLAMLVLHQVDQGKLDLNQSVTVNRAAVLQNTWSPMMKDHQGDEFTVAVQQLLQYSVSHSDNVAC
CEPA    DIHYPMMSVFKFHQALALADYMHHQKQPLETRLLIKKSDLKPDTYSPLRETYPQGGIEMSIADLLKYTLQQSDNNAC
CBLA    HVHFPLLSVFKFHVALAVLDKMDKQSISLDSIVSIKASQMPPNTYSPLRKKFPDQDFTITLRELMQYSISQSDNNAC
VEB-1   DFHFPMQSVMKFPIALAVLSEIDKGNLSFEQKIEITPQDLLPKTWSPIKEEFPNG--TTLTIEQILNYTVSESDNIGC
CFXA    KSVYPMMSVFKVHQALALCNDFDNKGISLDTLVNINRDKLDPKTWSPMLKDYSGPVISLTVRDLLRYTLTQSDNNAS
              Box-II                                 Box-III Box-IV

              140       150       160       170       180       190       200
              |         |         |         |         |         |         |
TEM-3   NLLLT-TIGGPKELTAFLHNMGDHVTRLDRWEPELNEAIPNDERDTTMPAAMATTLR-KLLTGELLTLAS-RQQLID
PER-1   DLLFE-LVGGPAALHDYIQSMGIKETAVVANEAQMHADDQVQYQNWTSMKGAAEILK-KFEQKTQLSETS-QALLWK
PER-2   DLLFE-LVGGPQALHAYIQSLGVKEAAVVANEAQMHADDQVQYQNWTSMKAAAQVLQ-KFEQKKQLSETS-QALLWK
CEPA    DILFN-YQGGPDAVNKYLHSLGIRECAVIHTENDMHKNLEFCYQNWTTPLAAAKLLE-IFRNENLF-DKEYKNFIYQ
CBLA    DILIE-YAGGIKHINDYIHRLSIDSFNLSETEDGMHSSFEAVYRNWSTPSAMVRLLR-TADEKELFSNKELKDFLWQ
VEB-1   DILLK-LIGGTDSVQKFLNANHFTDISIKANEEQMHKDWNTQYQNWATPTAMNKLLIDTYNNKNQLLSKKSYDFIWK
CFXA    NLMFKDMVNVAQTDSFIATLIPRSSFQIAYTEEEMSADHNKAYSNYTSPLGAAMLMN-RLFTEGLIDDEK-QSFIKN
                                          Box-V

              210       220       230       240       250       260       270
              |         |         |         |         |         |         |
TEM-3   WMEADKVAGPLLRS-ALPAG-WFIADKSGAS-------ERGSRGIIAALGPDGKPSRIVVIYTTGSQATMDERNRQI
PER-1   WMVETTTGPERLKG-LLPAG-TVVAHKTGTSGIKA-GKTAATNDLGIILLPDGRPLLVAVFVKD-SAESSRTNEAII
PER-2   WMVETTTGPQRLKG-LLPAG-TIVAHKTGTSGVRA-GKTAATNDAGVIMLPDGRPLLVAVFVKD-SAESERTNEAII
CEPA    TMVECQTGQDRLIAPLLDKK-VTMGHKTGTGDRNAKGQQIGCNDIGFILLPDGHAYSIAVFVKD-SEADNRENSEII
CBLA    TMIDTETGANKLKG-MLPAK-TVVGHKTGSSDRNADGMKTADNDAGLVILPDGRKYYIAAFVMD-SYETDEDNANII
VEB-1   IMRETTTGSNRLKG-QLPKN-TIVAHKTGTSGINN-GIAAATNDVGVITLPNGQLIFISVFVAE-SKETSEINEKII
CFXA    TLKECKTGVDRIAAPLLDKEGVVIAHKTGSGYVNENGVLAAHNDVAYICLPNNKGNKSQA-----SQYVAHISAVVY
         Box-VI                         Box-VII

              280       290
              |         |
TEM-3   AEIGASLIKHW-----------
PER-1   AQVAQTAYQFELKKLSALSPN
PER-2   AQVAQAAYQFELKKLSAVSPD
CEPA    AEISRIVYEYVTQQID-----
CBLA    ARISRMVYDAMR---------
VEB-1   SDIAKITWNYYLNK-------
CFXA    SLLMQTSVKS-----------
```
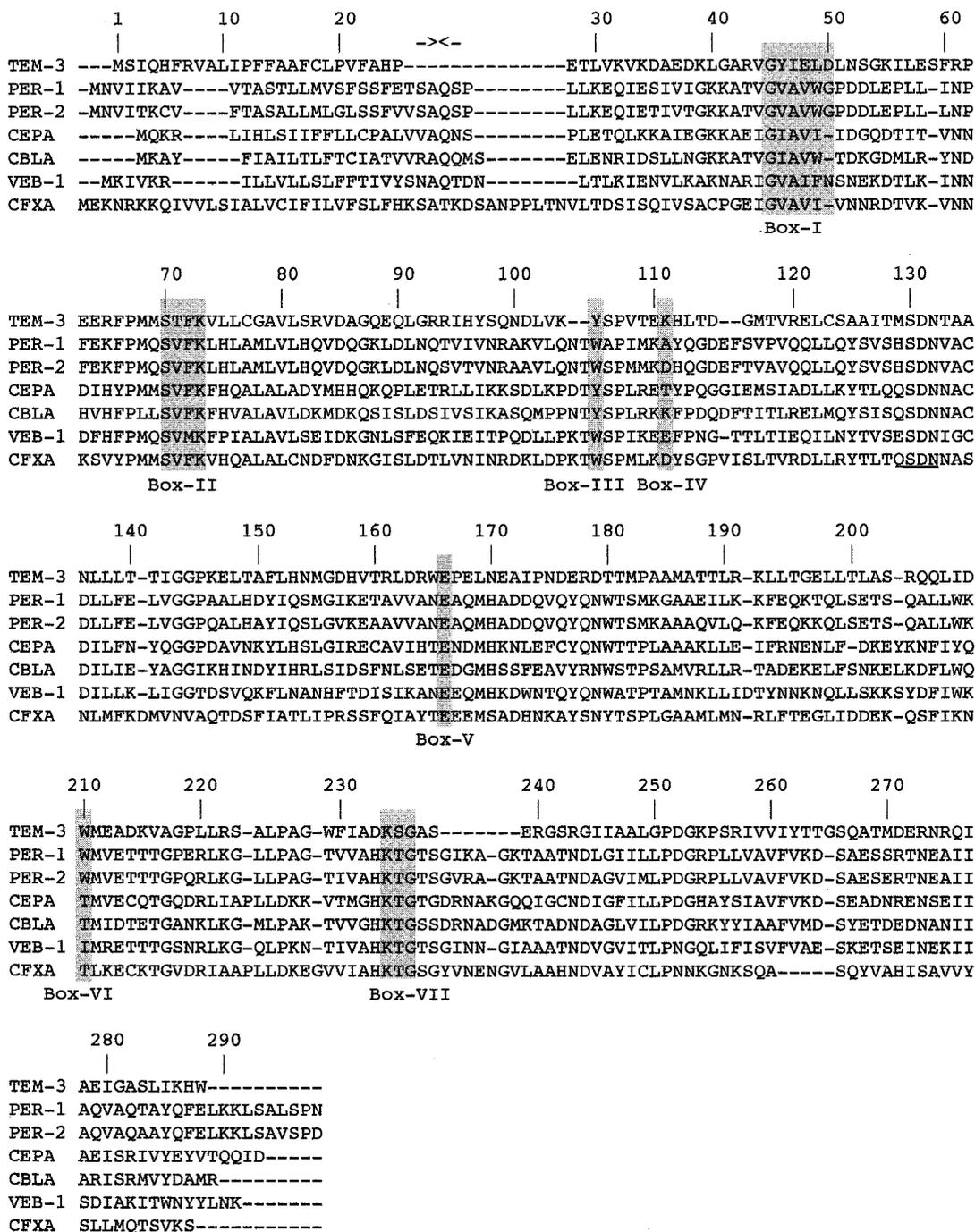
FIG. 3. Alignment of the amino acid sequence of VEB-1 with those of the closely related class A β-lactamases PER-1 (34), PER-2 (7), CBLA (43), CEPA (39), and CFXA (35) and to that of TEM-3. Dashes indicate gaps within the alignment. Standard numbering for class A enzymes according to Ambler (1) is indicated. The Roman numerals below the shaded boxes indicate conserved regions within the class A β-lactamases according to Joris et al. (21). The two facing arrows near the top of the figure indicate the cleavage site of the PER-1 leader peptide (34).

1 had a molecular mass of about 30 kDa as determined by SDS-PAGE and it belongs to Ambler class A β-lactamases (1) and to the Bush 2be class of enzymes (10).

Several interesting features emerged from the analysis of the sequence of bla_{VEB-1} and of the surrounding sequence. No obvious E. coli promoter sequence was identified 5′ to the coding sequence but instead, bla_{VEB1} displayed gene cassette features (15, 38). Based on sequence analysis and comparison to con-

sensus sequences, several gene cassette signatures were identified. bla_{VEB1} is flanked at its 5′ end by the core site GTTA GCG (Fig. 1 and 2) and at its 3′ end by an imperfect inverted repeat of 127 bp called 59-be, possessing a perfect inverse core site immediately after the 3′ end of bla_{VEB1} CGCTAAC (38, 46), which corresponds to the start of the 59-be. The 59-be's constitute a loosely related family of imperfect inverted repeats which differ from each other by their sequences and lengths
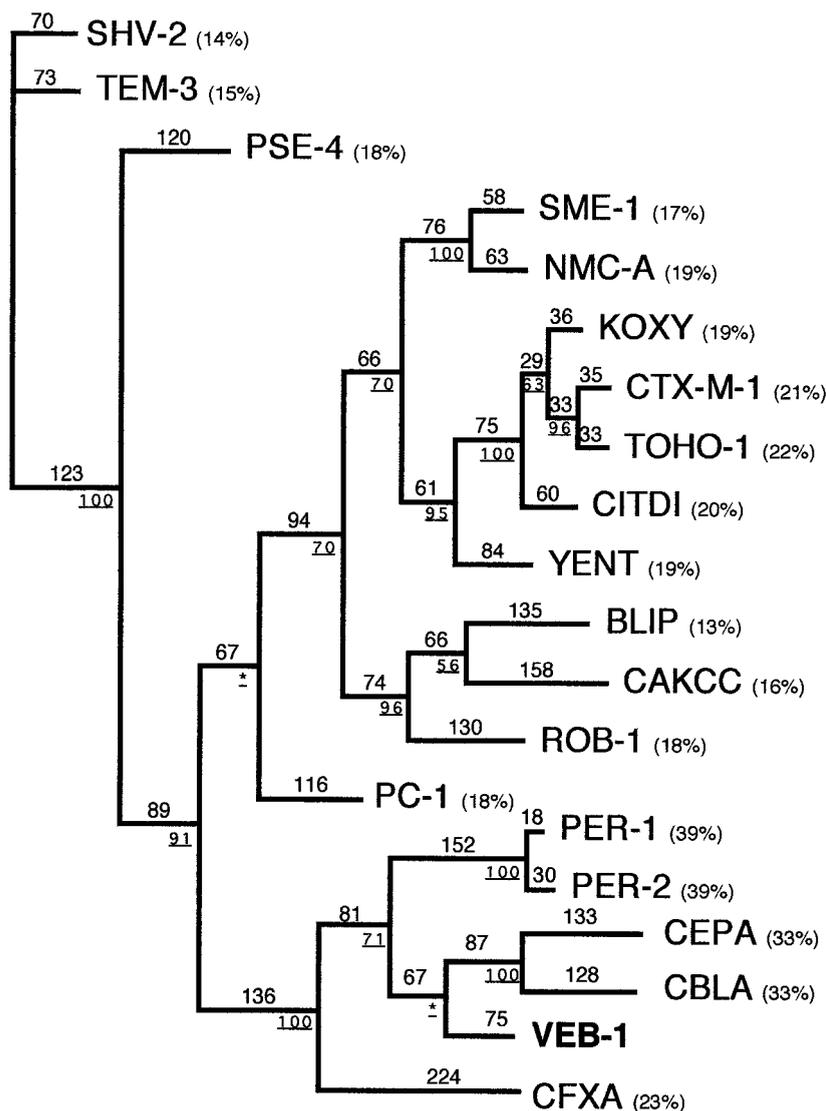
FIG. 4. Dendrogram obtained for 20 class A β-lactamases according to the parsimony method (49). Branch lengths are drawn to scale and are proportional to the number of amino acid changes. The percentages at branching points (underlined) refer to the number of times a particular node was found in 100 bootstrap replications (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 Materials and Method section. Percentages in parentheses are amino acid identities to VEB-1.

(38). For the *veb1* gene cassette, a longer than usual imperfect inverted repeat was found which has only 30% identity with the consensus 59-be sequence (46). Next to *veb1*, another gene cassette, *aadB* (17, 42), which confers resistance to gentamicin, kanamycin, and tobramycin, was found in same orientation as bla_{VEB1}. This cassette starts with a perfect core site GTTA GGC and has 100% identity over the first 120 bp with other sequenced *aadB* gene cassettes. In fact, these cassettes are widespread and are mostly found on the variable region of integrons (38). Upstream of bla_{VEB-1}, another 59-be belonging to the *aacA1-orfG* gene cassette was identified. The presence of gene cassette features, the lack of any obvious *E. coli* promoter in front of bla_{VEB1}, the fact that bla_{VEB-1} is surrounded by two other gene cassettes, and the fact that pNLT1 confers resistance to sulfamides makes it likely that bla_{VEB1} is the first class A ESBL from *E. coli* located on the variable region of a class 1 integron. So far, only some extended-spectrum oxacillinase genes from *Pseudomonas aeruginosa* (14, 37) and one

class B enzyme, IMP-1 (3), were found to be located on the integron. In order to have a conclusive answer, further analysis will be necessary to characterize the integrase and the nature of the integron.

In the *E. coli* MG-1 clinical strain, two natural plasmids were found. One of them, pNLT2, encoded the TEM-1 enzyme, which is part of a transposon, a derivative of Tn*3* (26). This plasmid was not further analyzed. The second plasmid, pNLT1, encoded bla_{VEB-1}. Both plasmids were transferable by conjugation into *E. coli* JM109. This is worrisome, since integrons and their associated gene cassettes have a tendency to spread rapidly, especially when they are located on conjugative plasmids. The finding of bla_{VEB-1} in a *K. pneumoniae* strain is a good illustration of the spread of this resistance gene to other bacteria.

The protein alignment with 19 class A β-lactamases showed that VEB-1 shares the highest sequence identity (38%) with PER-1 and PER-2 (Fig. 3 and 4) from *Salmonella typhimurium*,

Klebsiella sp., *E. coli*, and *Proteus mirabilis* strains from South America (7), and from *S. typhimurium*, *Acinetobacter* sp. (51), and *P. aeruginosa*, respectively (33). In addition, VEB-1 shares significant sequence identity with CBLA and CEPA, found in *Bacteroides uniformis* (43) and in *Bacteroides fragilis*, respectively (39). Interestingly, all these enzymes are ESBLs themselves and are plasmid mediated. VEB-1 is not a simple point mutant derivative from any known β-lactamase as are most of the described ESBLs from *E. coli* but rather belongs to a novel family or subgroup of class A ESBLs consisting of PER-1, PER-2, CEPA, and CBLA. The leader peptide cleavage site of PER-1 was found to be located between alanine and glutamine residues at ABL positions 22 and 23 (33). Interestingly, even though the leader peptides are very different, the two residues are conserved in the VEB-1 family members (Fig. 3), indicating that these amino acids may be important in the leader peptide cleavage site as well. As for PER-1, VEB-1 possesses highly conserved amino acid residues of the active-site serine enzymes that interact with β-lactam compounds (20, 21) (Fig. 3) and the SDN motif, which is known to be a structural block of the active site. It is interesting to note these homology regions are accountable for the observed homologies between VEB-1 and all the other class A enzymes.

VEB-1 confers high-level resistance to amoxicillin, ticarcillin, piperacillin, cefotaxime, ceftazidime, and aztreonam which is reversed by clavulanic acid. Similar resistance profiles were observed with PER-1 (33, 34) and PER-2 (7). Detailed analysis of the VEB-1 amino acid sequence indicated important residues that may explain the observed ESBL phenotype. As observed for PER-1 (34), VEB-1 possesses only one cysteine, at position ABL 135. Therefore, these enzymes will not be able to form the disulfide bridge from ABL positions 77 to 123, as observed from biochemical and crystallographical observations in TEM (47), SHV, or CARB derivatives, nor the ABL positions 69 through 237 disulfide bridge in NMC-A (29) and SME-1 (30). The Ω loop, which extends from residues ABL 169 to 179, is a structural element encountered only in class A enzymes. This loop, even though present in VEB-1, is totally different from the one found in TEM-1. In this respect, VEB-1, along with PER-1, PER-2, CBLA, and CEPA, has a histidine residue at position ABL 170 instead of an asparagine. This asparagine, together with the glutamate ABL 166 and serine ABL 70, is involved in the positioning of the active-site water molecule. The particular phenotypic properties of VEB-1 and PER-1 may be connected to the presence of this histidine. Site-directed mutagenesis would be necessary to determine the precise function of this histidine. Furthermore, the KTG motif is known to be important in the activity of the enzyme (21). Threonine-serine residues found at positions ABL 237 and 238 are usually found in ESBLs (28) and thus are important in the extension of the substrate profile. However, a recent site-directed mutagenesis study (9) revealed that the S238G mutation has no effect on the activity of PER-1. The histidine at position ABL 233 is observed only in VEB-1 family members and CFXA (Fig. 3) (35). In all other class A enzymes, an aspartate residue is found at this position. In TEM-1, this aspartate 233 forms a salt bridge with arginine 222.

This work gives further insight on the complex genetic variety of β-lactamases and of their potential in spreading. The presence of the same enzyme in two different *Enterobacteriaceae* species from the same patient is a good illustration of how resistance genes can spread in natural conditions by using conjugative plasmids and integrons. Additionally, VEB-1 epidemiology studies among various gram-negative bacteria in Southeast Asian countries should be undertaken. The incidence and spread of other class A ESBLs in distant areas signal the ongoing evolution of novel enzymes beyond the TEM or SHV derivatives.

REFERENCES

1. **Ambler, R. P.** 1980. The structure of β-lactamases. Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci. **289:**321–331.
2. **Arakawa, Y., M. Ohta, N. Kido, M. Mori, H. Ito, T. Komatsu, Y. Fujii, and N. Kato.** 1995. 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. **Arakawa, Y., M. Murakami, K. Suzuki, H. Ito, R. Wacharotayankun, S. Ohsuka, N. Kato, and M. Ohta.** 1995. A novel integron-like element carrying the metallo-β-lactamase gene bla_IMP. Antimicrob. Agents Chemother. **39:**1612–1615.
4. **Barthélémy, M., M. Guionie, and R. Labia.** 1978. β-Lactamases: determination of their isoelectric points. Antimicrob. Agents Chemother. **13:**695–698.
5. **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.
6. **Bauernfeind, A., I. Stemplinger, R. Jungwirth, S. Ernst, and J. M. Casellas.** 1996. Sequences of β-lactamase genes encoding CTX-M-1 (MEN-1) and CTX-M and relationship of their amino acid sequences with those of β-lactamases. Antimicrob. Agents Chemother. **40:**509–513.
7. **Bauernfeind, A., I. Stemplinger, R. Jungwirth, P. Mangold, S. Amann, E. Akalin, O. Ang, C. Bal, and J. M. Casellas.** 1996. Characterization of β-lactamase gene bla_PER-2, which encodes an extended-spectrum class A β-lactamase. Antimicrob. Agents Chemother. **40:**616–620.
8. **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.
9. **Bouthors, A.-T., N. Dagoneau-Blanchard, T. Naas, P. Nordmann, V. Jarlier, and W. Sougakoff.** 1998. Role of residues 104, 164, 166, 238 and 240 in the substrate profile of PER-1 β-lactamase hydrolysing third-generation cephalosporins. Biochem. J. **330:**1443–1449.
10. **Bush, K., G. A. Jacoby, and A. A. Medeiros.** 1995. A functional classification scheme for β-lactamases and its correlation with molecular structure. Antimicrob. Agents Chemother. **39:**1211–1233.
11. **Chan, P. I.** 1986. Nucleotide sequence of the *Staphylococcus aureus* PC1 β-lactamase gene. Nucleic Acids Res. **14:**5940.
12. **Collis, C. M., and R. M. Hall.** 1992. Site-specific deletion and rearrangement of integron insert genes catalyzed by the integron DNA integrase. J. Bacteriol. **174:**1574–1585.
13. **Danel, F., L. M. C. Hall, D. Gur, H. E. Akalin, and D. M. Livermore.** 1995. Transferable production of PER-1 β-lactamase in *Pseudomonas aeruginosa*. J. Antimicrob. Chemother. **35:**281–294.
14. **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.
15. **Hall, R. M., and C. M. Collis.** 1995. Mobile gene cassettes and integrons: capture and spread of genes by site specific recombination. Mol. Microbiol. **15:**593–600.
16. **Heffron, F., B. J. McCarthy, H. Ohtsubo, and E. Ohtsubo.** 1979. DNA sequence analysis of the transposon Tn3: three genes and three sites involved in the transposition of Tn3. Cell **18:**1153–1163.
17. **Hollingshead, S., and D. Vapnek.** 1985. Nucleotide sequence analysis of a gene encoding a streptomycin/spectinomycin adenyltransferase. Plasmid **13:**17–20.
18. **Huletsky, A., F. Couture, and R. C. Levesque.** 1990. Nucleotide sequence and phylogeny of SHV-2 β-lactamase. Antimicrob. Agents Chemother. **34:**1725–1732.
19. **Ishii, Y., A. Ohno, H. Taguchi, S. Imajo, M. Ishiguro, and H. Matsuzawa.** 1995. Cloning and sequence of the gene encoding a cefotaxime-hydrolyzing class A beta-lactamase isolated from *E. coli*. Antimicrob. Agents Chemother. **39:**2269–2275.
20. **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.
21. **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.

22. **Juteau, J.-M., and R. C. Levesque.** 1990. Sequence analysis and evolutionary perspectives of ROB-1 β-lactamases. Antimicrob. Agents Chemother. **34:**1354–1359.

23. **Labia, R., M. Guionie, M. Barthélémy, and A. Philippon.** 1981. Properties of three carbenicillin hydrolysing β-lactamases (CARB) from *Pseudomonas aeruginosa*: identification of a new enzyme. J. Antimicrob. Chemother. **7:**49–56.

24. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.

25. **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.

26. **Mabilat, C., J. Lourencao-Vital, S. Goussard, and P. Courvalin.** 1992. A new example of physical linkage between Tn*1* and Tn*21*: the antibiotic multiple resistance region of plasmid pCFF04 encoding extended spectrum beta-lactamase TEM-3. Mol. Gen. Genet. **235:**113–121.

27. **Matsumoto, Y., F. Ikeda, T. Kamimura, Y. Yokota, and Y. Mine.** 1989. Novel plasmid-mediated β-lactamase from *Escherichia coli* that inactivates oxy-imino-cephalosporins. Antimicrob. Agents Chemother. **32:**1243–1246.

28. **Medeiros, A. A.** 1997. Evolution and dissemination of β-lactamases accelerated by generations of β-lactam antibiotics. Clin. Infect. Dis. **24**(Suppl.)**:**19–45.

29. **Naas, T., and P. Nordmann.** 1994. Analysis of a carbapenem-hydrolyzing class A β-lactamase from *Enterobacter cloacae* and its LysR type regulatory protein. Proc. Natl. Acad. Sci. USA **91:**7693–7697.

30. **Naas, T., L. Vandel, W. Sougakoff, D. M. Livermore, and P. Nordmann.** 1994. Cloning and sequence analysis of the gene for a carbapenem-hydrolysing class A β-lactamase, Sme-1, from *Serratia marcescens* S6. Antimicrob. Agents Chemother. **38:**1262–1270.

30a.**National Center of Biotechnology Information Website.** 11 December 1998, revision date. [Online.] http://www.ncbi.nlm.nih.gov. [15 January 1999, last date accessed.]

31. **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.

32. **Nicolas, M. H., V. Jarlier, N. Honore, A. Philippon, and S. T. Cole.** 1989. Molecular characterization of the gene encoding SHV-3 β-lactamase responsible for transferable cefotaxime resistance in clinical isolates of *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. **33:**2096–2100.

33. **Nordmann, P., E. Ronco, T. Naas, C. Duport, Y. Michel-Briand, and R. Labia.** 1994. Characterization of a novel extended-spectrum β-lactamase from *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **37:**962–969.

34. **Nordmann, P., and T. Naas.** 1994. Sequence analysis of PER-1 extended-spectrum β-lactamase from *Pseudomonas aeruginosa* and comparison with class A β-lactamases. Antimicrob. Agents Chemother. **38:**104–114.

35. **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.

35a.**Pedrós BioMolecular Research Tools Website.** 16 June 1995, revision date. [Online.] http://www.fmi.ch/biology/research_tools.html. [12 December 1998, last date accessed.]

36. **Perilli, M., N. Franceschini, B. Segatore, G. Amicosante, A. Ovatore, C.**

37. **Duez, B. Joris, and J. M. Frère.** 1991. Cloning and nucleotide sequencing of the gene encoding the β-lactamase from *Citrobacter diversus*. FEMS Microbiol. Lett. **83:**79–84.

37. **Philippon, L. N., T. Naas, A. T. Bouthors, V. Barakett, and P. Nordmann.** 1997. OXA-18, a class D clavulanic-acid inhibited extended-spectrum β-lactamase from *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **41:**2188–2195.

38. **Recchia, G. D., and R. M. Hall.** 1995. Gene cassettes: a new class of mobile elements. Microbiology **141:**3015–3027.

39. **Rogers, M. B., A. C. Parker, and C. J. Smith.** 1993. Cloning and characterization of the endogenous cephalosporinase gene, *cepA*, from *Bacteroides fragilis* reveals a new subgroup of Ambler class A β-lactamases. Antimicrob. Agents Chemother. **37:**2391–2400.

40. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

41. **Seoane, A., and J. M. G. Lobo.** 1991. Nucleotide sequence of new class A β-lactamase from the chromosome of *Yersinia enterocolitica*: implications for the evolution of class A β-lactamases. Mol. Gen. Genet. **228:**215–220.

42. **Shaw, K. J., P. N. Rather, R. S. Hare, and G. H. Miller.** 1993. Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. Microbiol. Rev. **57:**138–163.

43. **Smith, C. J., T. K. Bennett, and A. C. Parker.** 1994. Molecular and genetic analysis of the *Bacteroides uniformis* cephalosporinase gene, *cblA*, encoding the species-specific β-lactamase. Antimicrob. Agents Chemother. **38:**1711–1715.

44. **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.

45. **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.

46. **Stokes, H. W., D. B. O'Gorman, G. D. Recchia, M. Parsekhian, and R. M. Hall.** 1997. Structure and function of 59-base element recombination sites associated with mobile gene cassettes. Mol. Microbiol. **3:**1669–1683.

47. **Strynadka, N. C. J., H. Adachi, S. E. Jensen, K. Johns, A. Sielecki, C. Betzel, K. Sutoh, and N. G. Amyes.** 1992. Molecular structure of the acyl-enzyme intermediate in β-lactam hydrolysis at 1.7 angstroem resolution. Nature (London) **359:**700–705.

48. **Sutcliffe, J. G.** 1978. Nucleotide sequence of the ampicillin resistance gene of *Escherichia coli* plasmid pBR322. Proc. Natl. Acad. Sci. USA **75:**3737–3741.

49. **Swofford, D. L.** 1989. PAUP (version 3.0): phylogenetic analysis using parsimony. Illinois Natural History Survey, Champaign, Ill.

50. **Tolmasky, M. E., and J. H. Crosa.** 1993. Genetic organization of antibiotic resistance genes (*aac*(6′)-*Ib*, *aadA*, and *oxa-9*) in the multiresistance transposon Tn*1331*. Plasmid **29:**31–40.

51. **Vahaboglu, H., R. Ozturk, G. Aygun, F. Coskunkan, A. Yaman, A. Kaygusuz, H. Leblebicioglu, I. Balik, K. Aydin, and M. Otkun.** 1997. Widespread detection of PER-1-type extended-spectrum β-lactamase among nosocomial *Acinetobacter* and *P. aeruginosa* isolates in Turkey: a nationwide multicenter study. Antimicrob. Agents Chemother. **41:**2265–2269.

52. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.