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Nucleotide Sequence and Phylogeny of SHV-2 β-Lactamase

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We determined the nucleotide sequence of the $bla_{SHV-2(pBP60-1)}$ gene from *Klebsiella ozaenae* which confers resistance to broad-spectrum cephalosporins. The structural gene encodes a polypeptide product of 286 amino acids, and the estimated molecular weight of the mature protein is 28,900. Amino acid sequence comparison of the SHV-$2_{pBP60-1}$ enzyme with all known class A β-lactamases and homology studies showed that the residues were highly conserved. Furthermore, SHV-$2_{pBP60-1}$ was clearly related to SHV-1, LEN-1, and OHIO-1. The SHV-$2_{pBP60-1}$ enzyme differed from SHV-1 isolated from *Klebsiella pneumoniae* by seven amino acid substitutions. One of these substitutions, the Gly→Ser substitution at position 234, is probably a key region for the novel activity of cefotaxime hydrolysis. A phylogenetic tree was constructed by using all class A β-lactamases of known sequences by a progressive alignment method. The data suggested that the β-lactamases of gram-positive *Streptomyces*, *Staphylococcus*, and *Bacillus* species appeared early in evolution, followed by the PSE and CARB enzymes of *Pseudomonas* species and, more recently, by the SHV-type and TEM-type enzymes found in enteric bacteria. Larger evolutionary distances separated clusters of the gram-positive β-lactamases than separated clusters of the gram-negative enzymes. Results of this phylogenetic study suggested that extended-spectrum enzymes are recent derivatives that are selected by the use of new cephalosporins.

Transferable resistance to broad-spectrum cephalosporins was first reported in the Federal Republic of Germany in a few bacterial strains (27). A plasmid-mediated β-lactamase capable of hydrolyzing cefotaxime was isolated and characterized from one of these isolates (*Klebsiella ozaenae* 2180). The enzyme was named SHV-2 on the basis of its biochemical and genetic similarity with the well-known SHV-1 β-lactamase (25). Since its discovery in the Federal Republic of Germany, SHV-2 has been reported in many other countries, including Argentina, Chile, the People's Republic of China, Greece, France, Switzerland, and Tunisia; and a growing number of plasmid-mediated β-lactamases capable of hydrolyzing broad-spectrum cephalosporins has been characterized (37). These enzymes are mostly derivatives of TEM (TEM-3 to TEM-9 [37]) or SHV (SHV-3 to SHV-5 [8, 19, 22]).

Barthélémy et al. (5) have published the amino acid sequence of the SHV-2 β-lactamase isolated from *Escherichia coli* 2302. This enzyme differed from SHV-1 isolated from *E. coli* p453 by only one amino acid substitution, Gly→Ser, at position 213 of the mature protein. They proposed that this change was the result of a single base substitution in the DNA structure. The sequences of other extended-spectrum SHV-type β-lactamases have been published recently (37) and show that these enzymes also differ from SHV-1 by a few amino acid substitutions.

In order to understand the structural basis of this novel extended-spectrum activity better, we determined the nucleotide sequence of the $bla_{SHV-2(pBP60-1)}$ gene isolated from *K. ozaenae* 2180. Also, to provide insight into the origin of SHV-$2_{pBP60-1}$ and other β-lactamases, we determined the phylogeny of the SHV-$2_{pBP60-1}$ enzyme in relation to those of other class A enzymes.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and phages. *E. coli* HB101 [F⁻ *ara-14 galK2 hsdS20 (hsdR hsdM) lacY1 leu mtl-1 proA2 recA13 rpsL20 supE44 thi xyl-5*] (7) was the recipient for plasmid pMON39, and *E. coli* JM101 [*supE thi* Δ(*lac-proAB*) F′ (*traD36 proAB lacI*^{q}ZΔM15)] (33) was used to prepare M13 phages and pTZ18R single-stranded DNA. Growth conditions have been described elsewhere (31). Plasmid DNA was from pMON39, a 7.5-kilobase (kb) plasmid originating from pBP60-1 (25), by deleting five *Ava*I fragments of 0.66, 0.68, 1.15, 1.2, and 1.8 kb (32). Plasmid pBP60-1 was a recombinant resulting from the cloning of the *Eco*RI fragments containing the bla_{SHV-2} gene of plasmid pBP60 isolated from *K. ozaenae* 2180 into the *Eco*RI site of pACYC184 (25). This plasmid was kindly provided by B. Wiedemann (Institut für Medizinishe Mikrobiologie und Immunologie der Universität Bonn, Bonn, Federal Republic of Germany). The production of single-stranded DNA for DNA sequencing was done according to the suggestions of the manufacturer for M13mp18 phage (Pharmacia, Dorval, Quebec, Canada) and with the helper phage M13K07 for plasmid pTZ18R (Pharmacia) as described by Vieira and Messing (45).

Reagents and enzymes. Restriction endonucleases, T4 DNA ligase, the Klenow fragment of DNA polymerase I, and sequencing reagents were purchased from GIBCO/BRL (Burlington, Ontario, Canada) or Pharmacia. T7 DNA polymerase was a product of Pharmacia. [^{35}S]dATP was from Amersham Corp. (Oakville, Ontario, Canada). Ampicillin was a product of Sigma Chemical Co. (St. Louis, Mo.).

DNA preparation and nucleotide sequence determination. Plasmid DNA was prepared by the cleared lysate method and was purified by cesium chloride-ethidium bromide gradient ultracentrifugation (18, 38). Plasmid DNA was digested with restriction enzymes by using the conditions recommended by the manufacturers. The restriction fragments used for directional subcloning in the vectors M13mp18 and pTZ18R were isolated from agarose gels by the electroelu-

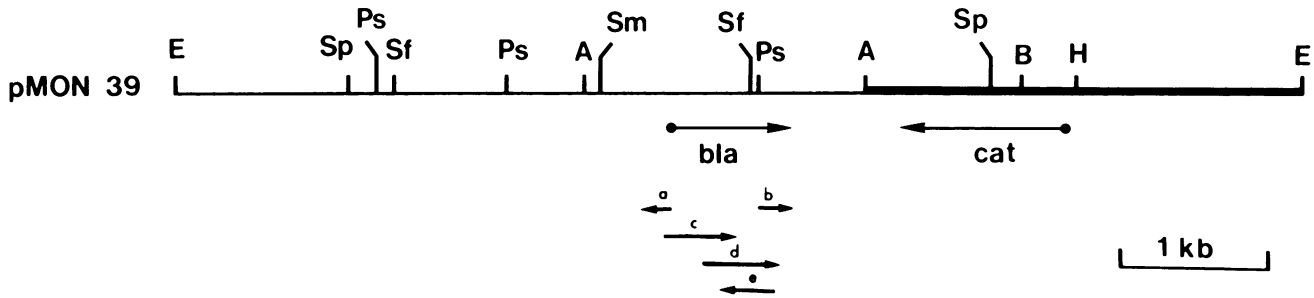

FIG. 1. Structure of pMON39 and sequencing strategy used for the SHV-2 *bla* gene. The thick line represents the vector pACYC184, and the thin line represents the insert. Arrows indicate the direction of nucleotide sequence analyzed by oligonucleotide primers. Arrows a and e indicate the sequence done in M13mp18; and arrows b, c, and d indicate the sequences done in pTZ18R. Abbreviations: A, *Ava*I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; Ps, *Pst*I; Sf, *Sfi*I; Sm, *Sma*I; Sp, *Sph*I; bla, β-lactamase; cat, chloramphenicol acetyltransferase.

tion procedure of Maniatis et al. (29). Subcloning and transformation were carried out as described by Maniatis et al. (29). DNA sequencing was performed by the dideoxy polymerase chain termination method of Sanger et al. (39). DNA fragments were sequenced by using the Klenow fragment of DNA polymerase I or T7 DNA polymerase. We synthesized five 17-mer oligonucleotide primers using phosphite triester chemistry (3) on a Pharmacia Gene Assembler Plus Apparatus. The oligonucleotides were purified on 20% polyacrylamide-urea gels (3) and were used for sequencing.

Computer analysis. Alignment, identity, and cladogram were determined by using the computer progressive alignment method of Feng and Doolittle (15). Briefly, for this method the algorithm of Needleman and Wunsch (34) was used in a three-matrix form (17) and the minimum mutation matrix of Dayhoff (13) was used for scoring to first determine pairwise alignments. The algorithm was used successively on pairs of sequences in search of the best multiple alignment. Based on these data, a distance matrix was calculated and the method of Fitch and Margoliash (16) was used to determine the branching order of the sequences. Finally, by combining the branching order with the distance matrix, a least-squares approach (26) was used to determine the branch lengths. In this study, the best alignment was obtained with a gap penalty of 12, which maximized the numbers of identities and minimized the numbers of gaps introduced into positions corresponding to secondary structure units of *Staphylococcus aureus* PC1 β-lactamase (20). The percentages of β-lactamase identity and branch lengths were calculated on the basis of this alignment.

Nucleotide sequence accession number. The SHV-2 β-lactamase sequence has been submitted to GenBank and assigned accession number M35595.

RESULTS

Physical mapping and subcloning of the bla_{SHV-2} structural gene. To localize the bla_{SHV-2} gene, we mapped pMON39 with the restriction endonucleases *Eco*RI, *Sma*I, *Pst*I, *Ava*I, *Bam*HI, *Sph*I, *Sfi*I, and *Hin*dIII (Fig. 1). A comparison with the physical map of plasmid pMON38 containing the SHV-1 *bla* gene from *E. coli* (32) localized the bla_{SHV-2} gene in a 1.8-kb *Sma*I-*Ava*I fragment on the map. We subcloned a 2.6-kb *Sma*I-*Sph*I fragment into M13mp18 and a 3.1-kb *Sma*I-*Hin*dIII fragment into pTZ18R to have the gene in both orientations for sequencing.

Nucleotide sequence of the bla_{SHV-2} gene. The nucleotide sequencing strategy for the bla_{SHV-2} gene is illustrated in Fig. 1. First, by using the nucleotide sequence of the SHV-1 *bla* gene (32), we synthesized four oligonucleotide primers in both orientations and determined the sequences of 326-, 625-, 403-, and 248-base-pair segments. Then, we synthesized a final oligonucleotide primer and sequenced a 405-base-pair segment in one orientation.

Analysis of the complete nucleotide sequence of the coding regions revealed only one open reading frame that was long enough to encode the expected protein of 286 amino acids (Fig. 2). By comparison with the amino acid sequence of the protein published by Barthélémy et al. (5), the mature protein started at position 250 and terminated with stop codon TAA at positions 1045 to 1047. A putative ATG initiation codon found at positions 187 to 189 suggested the presence of a β-lactamase secretion signal of 21 amino acids. This signal peptide was composed of a positively charged amino-terminal region followed by a hydrophobic core typical of this kind of signal (36). The ATG initiation codon was preceded by the putative ribosome-binding site AGGATG at positions 171 to 176 and the possible −10 region TATTCT (positions 124 to 129) and the −35 region TTGTGA (positions 100 to 105) of a promoter. These sites were also found in the sequences of LEN-1 and SHV-1 β-lactamases (2, 32) and in OHIO-1 β-lactamase from *Enterobacter cloacae* (40). We found a Ser-Thr-Phe-Lys tetrad (STFK) characteristic of the β-lactamase-active site at amino acid positions 66 to 69. Comparison with the published amino acid sequence of the SHV-2 enzyme from *E. coli* (5) revealed a single inversion of Thr-Ala for Ala-Thr at positions 136 to 137.

Homology with other class A β-lactamases. The deduced amino acid sequence of the bla_{SHV-2} structural gene revealed that this enzyme is a typical class A β-lactamase (1). Indeed, comparison of the amino acid sequence of $SHV\text{-}2_{pBP60-1}$ with the prototype class A enzyme TEM-1 showed 65% identity. In order to understand the structural basis of its novel extended-spectrum activity better, we compared its amino acid sequence with those of all known class A β-lactamases. Alignment of class A β-lactamases was performed by using the Align program of Feng and Doolittle (15) with a gap penalty of 12 (Fig. 3). This penalty gave the best alignment when the highest overall identities and potential α-helix and β-sheet motifs were taken into consideration (horizontal boxes delimited the secondary structures of PC1 in Fig. 3). This alignment was slightly different from previously published ones for class A β-lactamases (6, 23, 24), because of

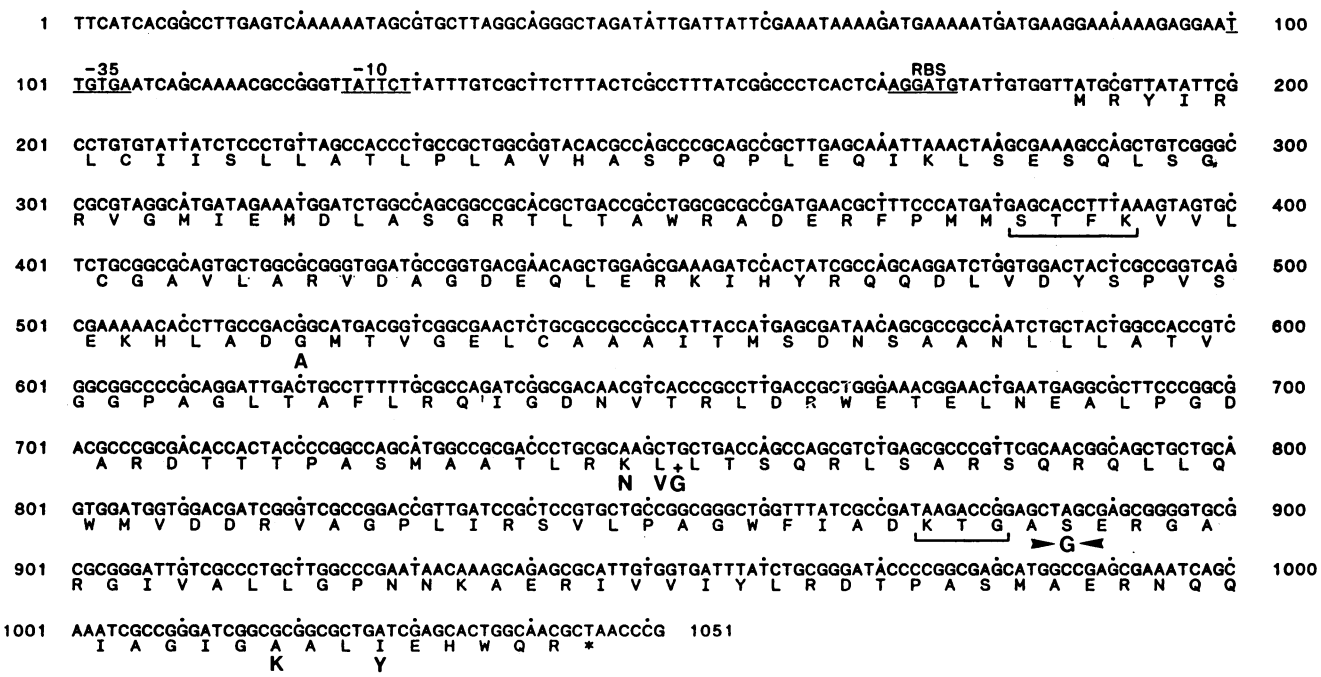

FIG. 2. Nucleotide sequence of the SHV-2 β-lactamase gene. The proposed ribosome-binding site (RBS) is underlined. The deduced amino acid sequence is designated by one-letter code. The active site tetrad STFK and the KTG triad are underlined, as are the −35 and −10 regions of the β-lactamase promoter. Differences with the polypeptide SHV-1 sequence from *K. pneumoniae* (32) are indicated below each amino acid. Arrowheads indicate significant differences.

the sequences that were added and the new progressive alignment method that was used. Indeed, the alignment was obtained directly from the computer, and no refinement was permitted by hand. This alignment showed 27 highly conserved amino acids in all class A enzymes (labeled by asterisks in Fig. 3). The seven conserved regions described by Joris et al. (23) for the penicillin-interactive proteins are indicated by roman numerals in Fig. 3. Boxes I, II, IV, and VII all included highly conserved residues.

The identities that we measured based on the alignment shown in Fig. 3 had the highest homology between the SHV-type enzyme group comprising LEN-1, OHIO-1, SHV-1, and SHV-$2_{pBP60-1}$ (Table 1). Indeed, SHV-$2_{pBP60-1}$ had 97.9% identity with SHV-1, 92.5% identity with OHIO-1, and 89.6% identity with LEN-1. The TEM enzymes were also strongly related to the SHV group, since we found 63 to 65% identity between the TEM and SHV enzymes. The group of PSE and CARB enzymes was the next most strongly related group, with about 40 to 44% identity with the TEM and SHV groups, followed by the enzyme group of gram-positive bacteria, which had less than 40% identity with the TEM and SHV groups.

Phylogeny of class A β-lactamases. A phylogenetic tree was constructed for amino acid sequences of 18 class A β-lactamases. The cladogram shown in Fig. 4 has important characteristics. First, this is an unrooted phylogenetic tree, which means that the common root can be placed along any of the branches. Second, by this method a present-day sequence is used as a temporary ancestor for the purpose of determining the correct phylogenetic tree topology. The topology in Fig. 4 shows the order in which the branches connect. Third, the numbers on the branches represent relative evolutionary distances calculated by a least-squares approach by using the values from the distance matrix (26).

We observed two major groups of proteins, the β-lactamases of gram-positive bacteria, including BCE5H, BCE5B, BLIP, BCEZ, CACAOI, PC1, SABLA, and ALBUS, that presumably appeared early in evolution, followed by the β-lactamases of gram-negative bacteria, such as CARB-4, CARB-3, PSE-4, LEN-1, OHIO-1, SHV-1, SHV-$2_{pBP60-1}$, TEM-1, and TEM-3. The gram-negative bacteria were further subdivided into two main groups: the PSE and CARB enzymes of *Pseudomonas* species followed by the SHV- and TEM-type enzymes commonly found in members of the family *Enterobacteriaceae*. Furthermore, as shown in Fig. 4, branch lengths indicate larger evolutionary distances for β-lactamases from gram-positive bacteria than those for enzymes from gram-negative bacteria.

In the gram-positive group of bacteria, we noted at the bottom of the phylogenetic tree the presence of the β-lactamases SABLA and ALBUS from *Streptomyces* species followed by the well-known β-lactamase of *Staphylococcus aureus*. Surprisingly, following the PC1 enzyme, we noted the branching off of the plasmid-mediated ROB-1 β-lactamase from *Haemophilus influenzae*, a gram-negative bacterium, followed by the β-lactamase of *Streptomyces cacaoi*. Finally, at the top of the gram-positive group branch of the phylogenetic tree, we found the β-lactamases of *Bacillus* species, indicating a more recent derivation of these enzymes.

In the gram-negative bacteria group, the PSE and CARB enzymes of *Pseudomonas aeruginosa* presumably branched off early, starting with CARB-4. Following the appearance of the PSE and CARB enzymes, we noted the recent divergence of the SHV group. LEN-1, the chromosomal β-lactamase from *Klebsiella* species, appeared first, followed by OHIO-1, a recently characterized SHV-type enzyme that is restricted geographically within the state of Ohio (41). SHV-1 and SHV-$2_{pBP60-1}$ enzymes were separated by a very short evolutionary distance, indicating that SHV-$2_{pBP60-1}$ is

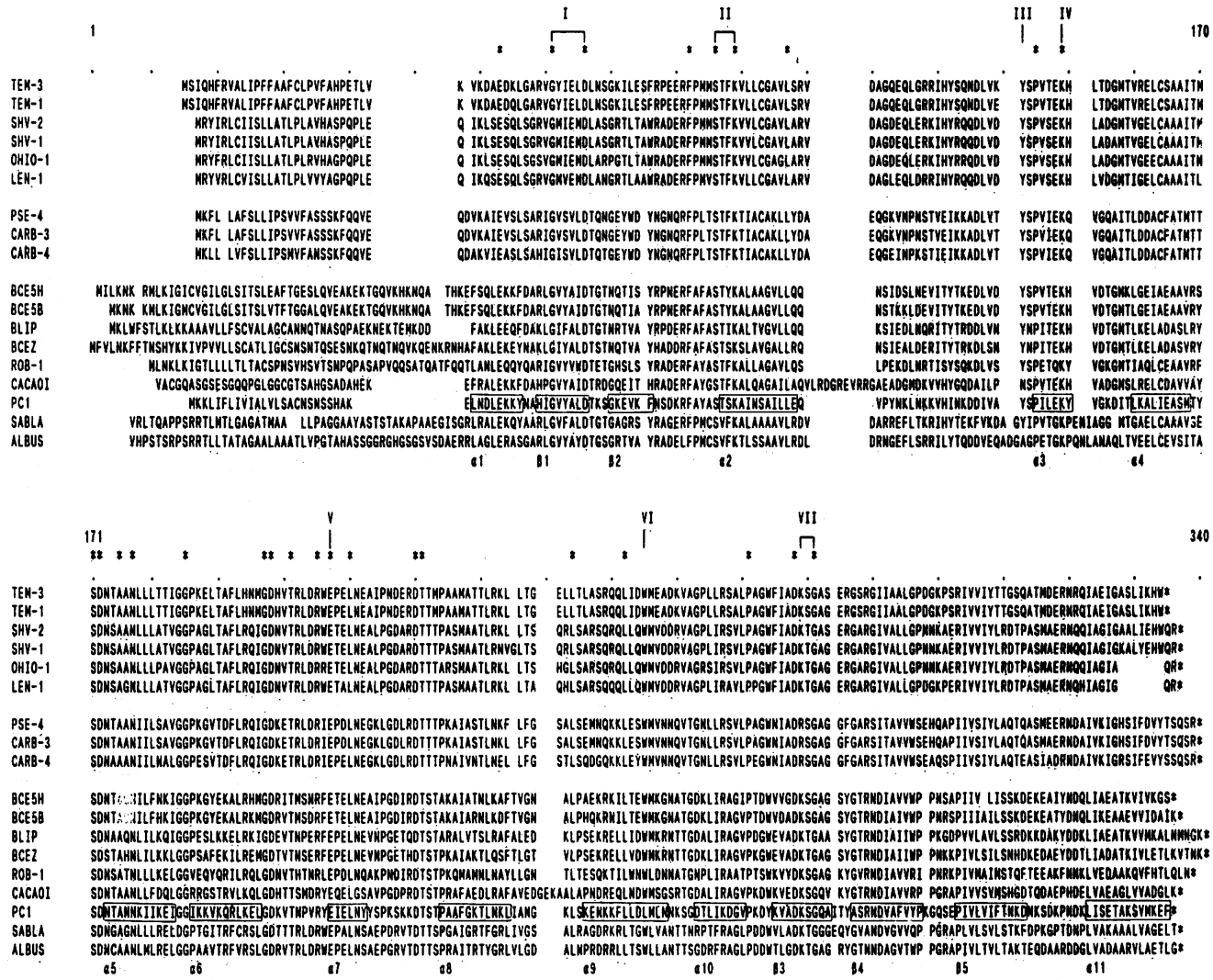

FIG. 3. Alignment of the SHV-2 amino acid sequence with the sequences of other class A β-lactamases. The alignment was done by using the protein sequence alignment program of Feng and Doolittle (15) with a gap penalty of 12. Abbreviations: ALBUS, β-lactamase (*bla*) of *Streptomyces albus* G (14); SABLA, *bla* of *Streptomyces aureofaciens* (J. P. Reynes, D. Drocourt, and G. Tiraby, EMBL data library, accession number X13597); PC1, *bla* of *Staphylococcus aureus* PC1 (12); CACAOI, chromosomal *bla* of *Streptomyces cacaoi* KCC-SO352 (28); ROB-1, *bla* of *Haemophilus influenzae* type b strain F990 (24); BCEZ, *bla* III of *Bacillus cereus* 569/H (21); BLIP, *bla* of *Bacillus licheniformis* 749/c (35); BCE5B, *bla* I of *Bacillus cereus* 5/B (46); BCE5H, *bla* I of *Bacillus cereus* 569/H (42); CARB-4, *bla* of *Pseudomonas aeruginosa* P83 372 (N. Bejaoui and R. C. Levesque, unpublished data); CARB-3, *bla* of *Pseudomonas aeruginosa* Cilote (J. Lachapelle and R. C. Levesque, submitted for publication); PSE-4, *bla* of *Pseudomonas aeruginosa* Dalgleish (6); LEN-1, *bla* of *Klebsiella pneumoniae* LEN-1 (2); OHIO-1, *bla* of *Enterobacter cloacae* 075 (40); SHV-1, *bla* of *Klebsiella pneumoniae* R974 (32); SHV-2, *bla* of *Klebsiella ozaenae* 2180; TEM-1, *bla* of *Salmonella paratyphi* B (44); TEM-3, *bla* of *Klebsiella pneumoniae* CF104 (43). Asterisks indicate conserved residues in all sequences. Roman numbers designate boxes I (positions 71 to 76), II (positions 96 to 99), III (position 143), IV (position 149), V (position 207), VI (position 255), and VII (positions 279 to 281) described by Joris et al. (23).

indeed a very recent derivative of SHV-1. The alignment in Fig. 3 shows that these two β-lactamases differ by only seven amino acid substitutions. Differences between SHV-$2_{pBP60-1}$ and the SHV-1 enzyme isolated from *E. coli* include Ala-112 for Gly, Asn-188 for Lys, Val-189 for Leu, a deletion of Gly-190, Gly-235 (in SHV-1) for Gly-234, Lys-278 (in SHV-1) for Ala, and Tyr-281 (in SHV-1) for Ile. The more recent class A β-lactamases found in the cladogram are the TEM enzymes commonly found in enteric bacteria that are highly related to SHV-type β-lactamases. A very short evolutionary distance also separated TEM-1 from TEM-3 because of three amino acid substitutions (Fig. 3).

DISCUSSION

We sequenced the complete bla_{SHV-2} structural gene from *K. ozaenae* and 190 base pairs of its flanking nucleotides. Amino acid sequence comparison showed that SHV-$2_{pBP60-1}$ is a typical class A β-lactamase by the classification scheme of Ambler (1) and is highly related to the SHV-type enzymes LEN-1, OHIO-1, and SHV-1. A very high level of homology was observed between the nucleotide sequences upstream of the coding region of SHV-type enzymes, emphasizing the fact that these β-lactamases had the same origin or ancestor. Alignment of the SHV-$2_{pBP60-1}$ amino acid sequence with all

TABLE 1. Identities between class A β-lactamases

β-Lactamase	% Identity[a]																
	TEM-3	TEM-1	SHV-2	SHV-1	OHIO-1	LEN-1	PSE-4	CARB-3	CARB-4	BCE5H	BCE5B	BLIP	BCEZ	ROB-1	CACAOI	PC1	SABLA
TEM-1	98.95																
SHV-2	65.14	65.14															
SHV-1	63.03	63.73	97.90														
OHIO-1	60.29	61.01	92.47	91.76													
LEN-1	63.18	63.90	89.61	88.89	84.95												
PSE-4	40.07	40.43	43.66	44.01	43.32	43.68											
CARB-3	40.43	40.78	44.37	44.37	44.04	44.40	99.31										
CARB-4	37.23	37.59	42.25	42.61	41.88	41.88	86.11	86.81									
BCE5H	34.29	34.29	34.05	33.57	34.93	35.29	33.33	33.33	32.26								
BCE5B	34.88	34.88	34.52	34.04	35.04	36.13	33.45	33.45	31.67	85.86							
BLIP	33.21	33.57	33.21	32.74	34.07	34.80	28.01	28.01	30.50	52.00	52.65						
BCEZ	31.67	32.03	31.67	31.56	32.48	34.67	30.99	30.99	31.34	52.29	53.92	64.82					
ROB-1	36.30	37.01	37.01	36.52	35.04	38.69	33.57	33.92	33.92	43.81	43.52	39.13	40.66				
CACAOI	33.57	33.22	34.28	33.80	33.70	34.42	28.62	28.62	26.86	47.20	46.88	43.90	42.71	39.93			
PC1	31.41	31.05	28.16	26.98	25.93	27.78	34.66	35.02	34.30	38.13	37.99	41.01	39.78	37.28	37.99		
SABLA	34.29	35.00	35.71	35.23	34.07	37.73	28.93	29.29	28.93	35.91	36.67	33.00	34.65	38.67	38.33	29.96	
ALBUS	35.34	35.69	36.04	35.56	34.42	36.96	29.33	30.04	30.04	33.22	33.99	37.00	36.60	35.64	37.24	29.39	55.81

[a] Percent identities as the number of identities per 100 aligned residues. They were calculated by using the progressive alignment method of Feng and Doolittle (15). β-Lactamase abbreviations are given in the legend to Fig. 3.

class A β-lactamases showed many interesting features. We observed 27 residues that have been highly conserved in evolution. The residues present in boxes II, III, IV, V, and VII described by Joris et al. (23) have already been implicated in the structure and function of the active-site-serine penicillin-recognizing enzymes. The new conserved residues identified in this report must also have an important role in the structure and function of these enzymes. The few regions with gaps that were inserted in the alignment were mostly located between the secondary structures found on the PC1 crystal structure. A long gap corresponding to seven residues was inserted in the N-terminal amino acid sequences of LEN-1 and OHIO-1 compared with the sequences of SHV-1 and SHV-$2_{pBP60-1}$. This gap was directly located in the region corresponding to α-helix 11 of the PC1 crystal structure. The shorter amino acid sequences of OHIO-1 and LEN-1 must be due to an error in the sequencing of the 3′ end of these genes. Boissinot and Levesque (6) have observed three specific substitutions for the PSE-4 enzyme sequence compared with the sequences of class A β-lactamases. First, the triad Lys-Thr/Ser-Gly of box VII described by Joris et al. (23) was replaced by Arg-Ser-Gly, and second, the conserved amino acid Pro at positions 215 and 297 was replaced by amino acids Leu and Ser, respectively, in the PSE-4 enzymes (Fig. 3). These substitutions were also found in the CARB-3 and CARB-4 enzymes, suggesting the strong specificity of these residues to the PSE and CARB enzymes of *Pseudomonas* species.

The alignment presented in this report showed that the SHV-$2_{pBP60-1}$ β-lactamase differs by seven amino acid substitutions from the SHV-1 enzyme isolated from *Klebsiella pneumoniae* (32). These mutations allowed the new SHV-$2_{pBP60-1}$ β-lactamase to hydrolyze broad-spectrum cephalosporins such as cefotaxime. The SHV-2 enzyme described here could not have arisen from SHV-1 isolated from *K. pneumoniae* simply by mutation. The amino acid sequence of a SHV-1 β-lactamase isolated from *E. coli* has also been published (4) and showed three amino acid substitutions from SHV-$2_{pBP60-1}$: Ser-234 for Gly and inversion of Ala-Thr for Thr-Ala at positions 136 and 137. Amino acid comparison of SHV-$2_{pBP60-1}$ isolated from *K. ozaenae* with SHV-2 isolated from *E. coli* also showed this inversion. These inversions might represent an error in the amino sequencing of these two β-lactamases. Thus, bla_{SHV-1} genes are heterogeneous and $bla_{SHV-2(pBP60-1)}$ seems to be more related to the SHV-1 isolated from *E. coli*. The recently published crystal structure of the class A β-lactamase PC1 from *S. aureus* has shown that many amino acid residues are implicated in the interaction of β-lactam antibiotics with β-lactamase (20). One of these residues, alanine at position 238, has been suggested to interact directly with the side chain of the β-lactam antibiotic. Since position 238 of the *S. aureus* enzyme corresponds to position 234 of SHV enzymes, Barthélémy et al. (5) have suggested that Ser-234 found on SHV-2 β-lactamase could generate a new hydrogen bond in the enzyme-substrate complex and increase the catalytic activity toward cefotaxime. The six other amino acid substitutions found on the SHV-$2_{pBP60-1}$ enzyme compared with those found on SHV-1 isolated from *K. pneumoniae* must be silent mutations, since they did not appear in any important structural regions of the enzyme. The nucleotide sequencing and analysis done in this study will permit testing of these observations by site-specific mutagenesis.

Two other extended-spectrum SHV-type enzymes (SHV-3 and SHV-4) have recently been characterized at the structural level (37). They possessed a Gly→Ser substitution at position 234 and an Arg→Leu substitution at position 201 when they were compared with the SHV-1 sequence. In addition, SHV-4 has a Glu→Lys substitution at position 235 and hydrolytic activity for ceftazidime. The substitution at position 235 has recently been suggested (37) to facilitate electrostatic interactions with negative charges on the ceftazidime molecule and extends the substrate profile of SHV-4 for this antibiotic.

The origin of the bla_{SHV-2} gene was studied by constructing a phylogenetic tree for all known class A β-lactamases. Interestingly, the structural subgroupings that appeared by doing this phylogenetic analysis are directly related to the biochemical properties of these enzymes. Indeed, the SHV and TEM enzyme groups are members of the broad-spectrum enzymes of class 2b and 2b′ described by Bush (9–11), the PSE and CARB groups are carbenicillinase enzymes of

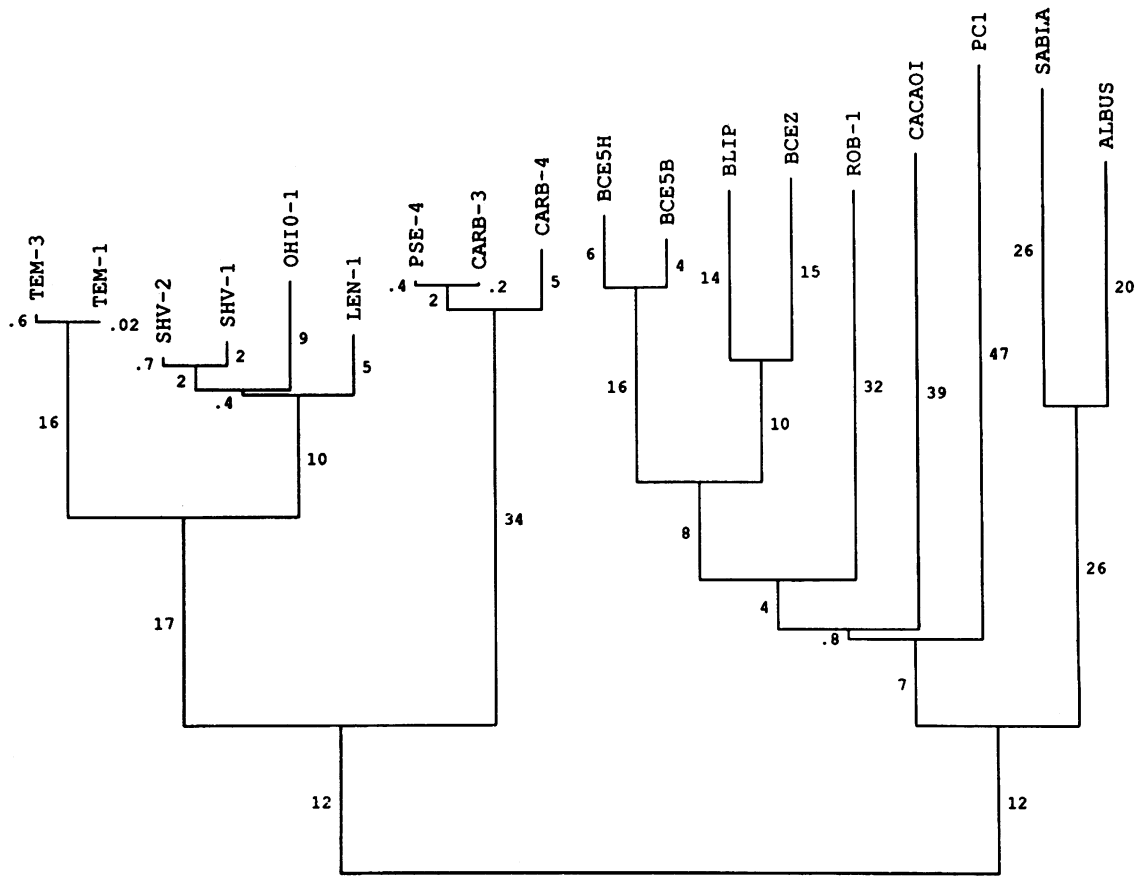

FIG. 4. Cladogram of class A β-lactamases. The cladogram was constructed with the progressive alignment method of Feng and Doolittle (15). Branch lengths were calculated on the basis of the automatic alignment shown in Fig. 3. Branch length values represent relative phylogenetic distances.

class 2c, and the β-lactamases of gram-positive bacteria are penicillinase enzymes of class 2a.

The phylogenetic tree that we obtained showed that β-lactamases of gram-positive bacteria presumably appeared early in evolution. They are mostly of chromosomal origin, except for the plasmid-mediated PC1 enzyme of *S. aureus*. A significant feature of the evolution of these enzymes is the long phylogenetic distances that separate each member of the group when they are compared with those that separate gram-negative enzymes. Exceptions are the β-lactamases from *Bacillus cereus* 569/H (BCE5H) and 5/B (BCE5B) and those from *Bacillus licheniformis* 749/c (BLIP) and *B. cereus* 569/H (BCEZ), which have very high levels of homology. However, no fundamental changes in the substrate spectra of these enzymes were observed, suggesting that the nature of the changes that have occurred on the genotype have not been acted upon by selection (47). In the gram-positive group, the presence of the plasmid-mediated ROB-1 enzyme originating from gram-negative *H. influenzae* could be explained by horizontal gene transfer from a gram-positive bacterium (24).

The class A β-lactamases of gram-negative bacteria that have been sequenced are mostly plasmid mediated (30). A greater heterogeneity in the biochemical properties of these enzymes has been observed. The PSE and CARB carbenicillinases of *Pseudomonas* species presumably branched off early in evolution. They are relatively distant from the SHV and TEM enzymes, but a high level of homology was found within members of this group. In fact, three specific amino acid substitutions, such as the one found at the Arg-Ser-Gly triad, are suggestive of their carbenicillinase activities. Experiments are in progress to verify this hypothesis. The last group of enzymes included the broad-spectrum β-lactamases. The chromosomal β-lactamase LEN-1 could be one of the oldest members of the SHV group, suggesting that it might be the ancestor of these enzymes. Relatively short evolutionary distances were found between each member of the group, and particularly between the SHV-1 and SHV-$2_{pBP60-1}$ enzymes and the TEM-1 and TEM-3 enzymes. SHV-$2_{pBP60-1}$ and TEM-3 possessed extended-spectrum activity against β-lactam antibiotics, suggesting that they could have appeared by discrete mutational steps.

This is the first report that has presented a phylogenetic analysis of class A β-lactamases. The phylogenetic tree that we obtained shows that β-lactamases of gram-positive bacteria presumably appeared earlier in evolution than the enzymes of gram-negative bacteria did and suggests that the broad-spectrum β-lactamases appeared relatively recently by the pressure of new antibiotic use.

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