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Cloning of SHV-2, OHIO-1, and OXA-6 β-Lactamases and Cloning and Sequencing of SHV-1 β-Lactamase

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Molecular cloning of DNA fragments permitted the isolation of structural genes coding for SHV-1, SHV-2, OHIO-1, and OXA-6 β-lactamases. DNA probes were constructed for SHV-1, and under conditions of high stringency, hybridization was observed only between SHV-1 and SHV-2. Oligonucleotide typing with a 15-mer SHV-1 probe was capable of discriminating between SHV-1 and SHV-2 but not OHIO-1. The nucleotide sequence of the SHV-1 β-lactamase gene from plasmid R974 has been determined. The structural gene encodes a polypeptide product which differs by 9 residues from the p453 (SHV-1) PIT-2 enzyme determined by peptide sequencing. The significance of each mutation was assessed by alignment of amino acid sequences and comparisons with the *Staphylococcus aureus* PC1 penicillinase crystal structure. Structural similarities between SHV-1 and class A β-lactamases are extensive, with amino acid identities of 88.9% between SHV-1 and LEN-1, 91.8% between SHV-1 and OHIO-1, and 63.7% between SHV-1 and TEM-1.

Plasmid-mediated β-lactamases (EC 3.5.2.6) from *Klebsiella* species share interesting features at the biochemical and molecular levels. Early reports indicated that a high proportion of these enzymes were of the SHV type and unique in their responses to inhibition by the sulfhydryl group reagent *p*-chloromercuribenzoate (4, 26, 28). Purification and further biochemical studies indicated that SHV-type β-lactamases have a wide spectrum of hydrolytic activity towards penicillins and cephalosporins (4). SHV-1 has been associated with an uncharacterized transposon of 14.25 kilobases (kb) (29).

Recent evidence indicates that extended-spectrum β-lactamases capable of hydrolyzing new cephalosporins have been derived not only from TEM-type but also from SHV-type enzymes (13, 22, 31). Titration curves of SHV-1-SHV-4 and SHV-2-SHV-4 pairs suggest the replacement of an acidic amino acid in the former β-lactamases by a neutral one in the latter enzyme of each pair (36). Complete amino acid sequencing of the p453 plasmid-mediated PIT-2 (SHV-1) and an *Escherichia coli* SHV-2 capable of hydrolyzing cefotaxime identified a single amino acid substitution of a serine for glycine at position 234 (3). All of the extended-spectrum SHV-type β-lactamases retained the active-site serine at position 66.

This work reports the nucleotide sequence of the prototype SHV-1 *bla* gene isolated from the *Klebsiella* sp. plasmid R974. We also present data on intragenic DNA probes and the genetic relatedness of SHV-1 to other SHV-type enzymes, LEN-1, OHIO-1, and other class A β-lactamases.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial plasmids used are described in Table 1. *E. coli* HB101 (F⁻ *hsdS20 recA13 ara-14 proA2 leu lacY1 galK2 rpsL20 xyl-55 mtl-1 supE44*) was used for cloning, and *E. coli* JM101 [*supE thi* Δ(*lac-proAB*) (F′ *traD36 proAB lacI*^{q} *Z* Δ*M15*)] was utilized as the recipient for M13mp18 and M13mp19 recombinant bacteriophages for nucleotide sequencing (7, 38). Growth conditions and preparation of DNA for hybridization and cloning were as described previously (25).

DNA cloning and related techniques. Plasmid DNA was cleaved with restriction endonucleases under conditions recommended by the manufacturers. Selected transformants were analyzed for antibiotic susceptibility by using the Kirby-Bauer disk diffusion method and β-lactamases identified by isoelectric focusing (12). Probe DNA fragments were obtained from plasmid pMON31 digested with restriction endonucleases and prepared by electroelution (6). Synthesis, purification, and labeling of oligonucleotides have been described previously (12). Nucleic acid hybridization was usually performed with purified DNA dotted or transferred to nitrocellulose membranes (18, 25). Hybridization and washings were done under stringent conditions with 50% formamide at 42°C and at 60°C for oligonucleotides (18).

Nucleotide sequence determination and computer analysis. Restriction fragments from pMON31 were subcloned into M13mp18 and M13mp19 vectors, and the dideoxynucleotide T7 polymerase chain termination method of Sanger et al. (33) was used for nucleotide sequencing. Analysis of the DNA sequence data was performed with software packages from International Biotechnologies Inc. (Toronto, Ontario, Canada), and from the University of Wisconsin Genetics Computer Group (11, 32).

RESULTS

Isolation of *bla* structural genes. Plasmids coding for SHV-1, SHV-2, OHIO-1, and OXA-6 β-lactamases (Table 1) were digested with *Ava*I, *Bam*HI, or *Hin*dIII and ligated with pACYC184 treated with the same endonuclease, and the mixtures were transformed into *E. coli* HB101. Recombinants were selected for resistance to ampicillin and chloramphenicol. DNA minipreparations were screened with endonucleases to identify the smaller recombinants coding for each prototype β-lactamase confirmed by isoelectric focusing. The SHV-1 (R974), OHIO-1 (pDS075), and OXA-6

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TABLE 1. Plasmids used

Plasmid	β-Lactamase	pI	Relevant phenotype[a]	Reference
R974	SHV-1	7.6	Ap^r Cm^r Sm^r Su^r Tc^r Lac	26
pBP60-1	SHV-2	7.6	Ap^r Cm^r Tc^r	20
pDS075	OHIO-1	7.0	Ap^r Gm^r Km^r	21, 34
pBR322	TEM-1	5.4	Ap^r Tc^r	35
pMON60	TEM-1	5.4	Ap^r Cm^r	25
pMK20::RP4	TEM-2	5.6	Ap^r Km^r	24
pMG204b	TLE-1	5.55	Ap^r Cm^r Km^r Sm^r Su^r	27
pMON300	OXA-1	7.4	Ap^r Cm^r	25
pMON20	OXA-2	7.7	Ap^r Cm^r Sp^r Su^r	6
pMON922	OXA-3	7.1	Ap^r Cm^r	25
pMON100	OXA-4	7.5	Ap^r Cm^r	25
pMON53	OXA-5	7.62	Ap^r Cm^r Sm^r Su^r	25
pUZ8::pMG39	OXA-6	7.68	Ap^r Gm^r Km^r Sm^r Su^r Tm^r	24
pMG202	OXA-7	7.65	Ap^r Cm^r Gm^r Km^r Su^r Tm^r Hg^r	27
pMON810	PSE-1	5.7	Ap^r Cm^r	25
pMON230	PSE-2	6.1	Ap^r Cm^r	25
pMON225	PSE-3	6.9	Ap^r Cm^r	25
pMON709	PSE-4	5.3	Ap^r Cm^r	5a
pMON41	CARB-3	5.75	Ap^r Cm^r Sm^r	25
pMON1025	CARB-4	4.3	Ap^r Cm^r Gm^r Km^r Su^r Tm^r	25
pMON80	LCR-1	6.5	Ap^r Cm^r	25
pMON510	AER-1	5.9	Ap^r Cm^r	25
pMON401	ROB-1	8.1	Ap^r Cm^r	25
pNU81	*ampC*	9.8	Ap^r Km^r	16
pACYC184			Cm^r Tc^r	8
pMK20			Km^r	19
pCR1			Km^r	19
R388			Su^r Tp^r	37
pUZ8			Km^r Tc^r Hg^r	23

[a] Abbreviations: Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Gm^r, gentamicin resistance; Hg^r, mercuric chloride resistance; Km^r, kanamycin resistance; Lac, ability to ferment lactose; Sm^r, streptomycin resistance; Sp^r, spectinomycin resistance; Su^r, sulfonamide resistance; Tc^r, tetracycline resistance; Tm^r, tobramycin resistance; Tp^r, trimethoprim resistance.

TABLE 2. Recombinant plasmids carrying various β-lactamase genes

Plasmid	Relevant characteristics[a]	β-Lactamase	pI
pMON31	Ap^r Cm^r; 3.2-kb *Bam*HI fragment from R974	SHV-1	7.6
pMON34	Ap^r Cm^r; deletion of a 1.5-kb *Ava*I fragment from pMON31	SHV-1	7.6
pMON36	Cm^r; deletion of a 0.64-kb *Pvu*II fragment from pMON31	SHV-1	7.6
pMON38	Ap^r Cm^r; deletion of a 1.6-kb *Cla*I fragment from pMON34	SHV-1	7.6
pMON39	Ap^r Cm^r; *Ava*I deletions of 7 kb from the 13-kb plasmid pBP60-1	SHV-2	7.6
pMON1100	Ap^r Cm^r; 5-kb *Bam*HI fragment from pDS075	OHIO-1	7.0
pMON1300	Ap^r Cm^r; 4-kb *Bam*HI fragment from pUZ8::pMG39	OXA-6	7.68

[a] All pACYC184 derivatives. Abbreviations: Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance.

(pUZ8::pMG39) β-lactamase structural genes were isolated by subcloning into pACYC184 as 3.2-, 5-, and 4-kb *Bam*HI fragments, respectively (Table 2). Deletion of seven *Ava*I fragments in pBP60-1 carrying SHV-2 reduced its size from 13 kb to 7.5 kb, to give pMON39 (Table 2).

Because of our interest in *bla* probes and structure-activity relationships, we localized the SHV-1 structural gene by constructing a series of deletion plasmids. By producing the *Pvu*II and *Ava*I plasmids pMON34 and pMON36, the *bla* gene was localized in the *Bam*HI-*Cla*I region of pMON31. Further deletion of a 1.6-kb *Cla*I fragment localized the *bla* gene in the *Cla*I-*Sma*I region of pMON38 (Fig. 1). For uniformity in nomenclature, we refer to the SHV-1 structural gene in pMON38 as bla_{SHV-1}.

Nucleotide sequence of SHV-1 β-lactamase. Physical mapping of pMON31 suggested that SHV-1 was encompassed by three fragments: 640-, 360-, and 150-base-pair (bp) *Pvu*II fragments. These fragments and a 600-bp *Pvu*II-*Ava*I fragment were subcloned into the phage vectors M13mp18 and M13mp19, and the nucleotide sequence was determined (Fig. 2). The dideoxy termination polymerase chain reaction was used to sequence each fragment at least twice from a minimum of two independent clones. Nucleotide sequencing was completed with 17-mer oligonucleotide primers. The sequence was determined for both strands and accounted for more than 95% of the whole sequence.

The entire nucleotide sequence obtained was 1,372 bp long and is shown in Fig. 3. Analysis for coding regions showed only one open reading frame encoding a putative peptide of 286 residues. We found an STFK tetrad and a KTG triad (underlined in Fig. 3) characteristic of β-lactamases (17). This open reading frame had one ATG initiation codon (positions 125 to 127) and a TAA termination codon (positions 986 to 988), and the upstream sequences included the typical −35 and −10 regions and the putative ribosome-binding site. Use of this initiation codon and comparisons with the polypeptide sequences of SHV-1 (2) and SHV-2 (3) identified a secretion signal of 20 amino acids. The mature protein has a calculated molecular mass of 28,800 daltons, and the active-site serine is at position 66.

Sequence homologies with other known β-lactamases. The nucleotide sequence of the SHV-1 structural gene had less than 50% nucleic acid identity with previously published β-lactamases, except LEN-1. However, comparisons of amino acid sequences between the deduced SHV-1 reported here and the amino acid sequences of SHV-1 and SHV-2 indicated nine distinct residues (Fig. 3). Differences from the previously reported SHV-1 include a Gly-112 for Ala, a Thr-136 for Ala, an Ala-137 for Thr, a Lys-188 for Asn, a Leu-189 for Val, an insertion of Gly-190, an Ala-269 for Lys, an Ile-272 for Tyr, and a Ser-234 (in SHV-2) for Gly-235 (2, 3). A dot plot graphic with the Staden amino acid comparison table showed important similarities between SHV-1 and LEN-1 (1), TEM-1 (35), and *Staphylococcus aureus* PC1 (9, 36) β-lactamases, with calculated identities of 88.9, 63.7, and 27%, respectively. In contrast, no regions of the PSE-2 enzyme (15) and the OXA-1 (30) and OXA-2 (10) class D enzymes were homologous with SHV-1 (data not shown).

Hybridization with SHV-1 probes. To study more closely the DNA homology between SHV-1 and the other β-lactamase structural genes, we initially used as a probe the 640-bp *Pvu*II fragment from pMON31 (Fig. 1). This probe contains only 251 bp internal to the SHV-1 structural gene but

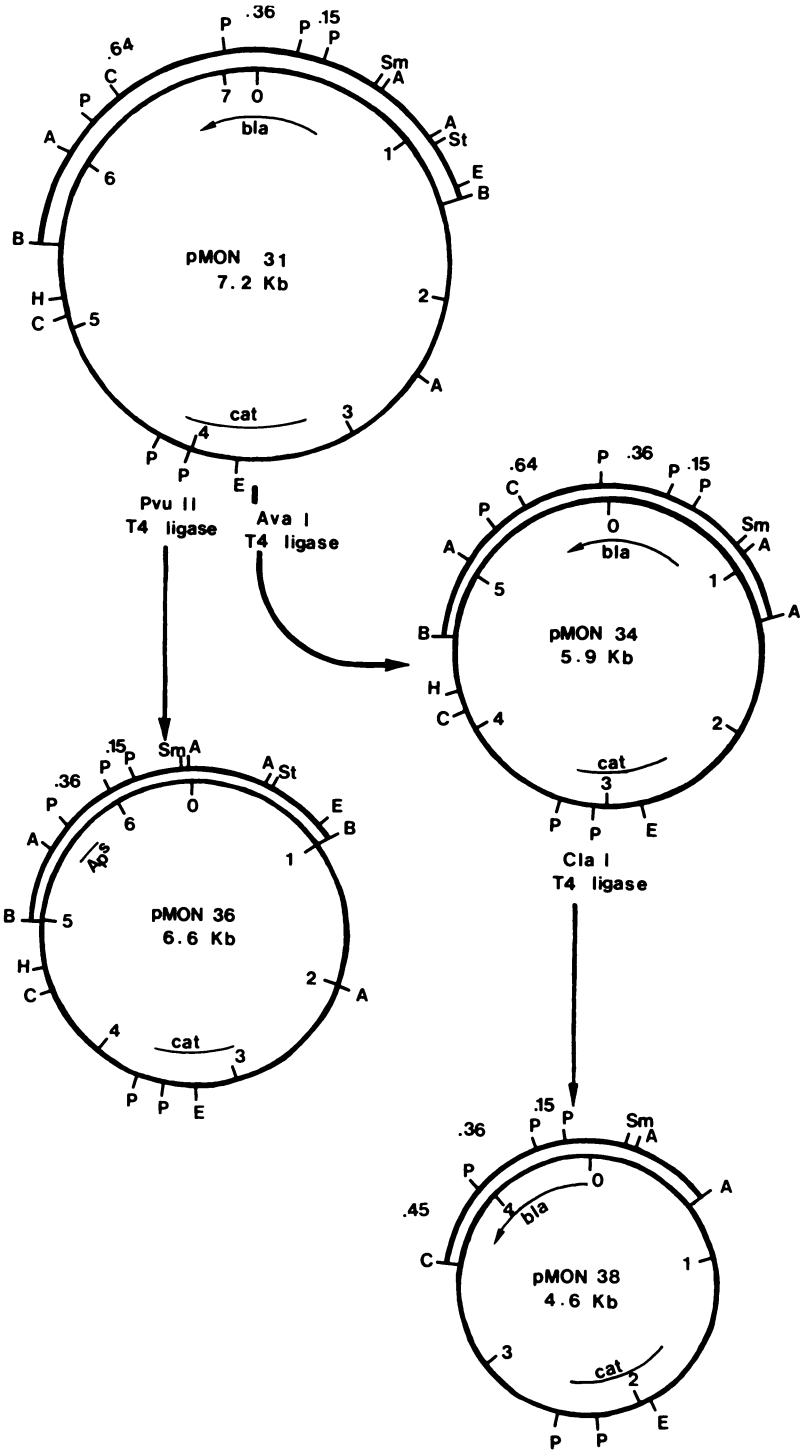

FIG. 1. Molecular cloning of the *blaS1* SHV-1 β-lactamase and construction of the recombinant plasmids pMON31, pMON34, pMON36, and pMON38 with plasmids R974 and pACYC184 as the cloning vehicles. Coordinates are indicated in kilobases, open boxes represent cloned DNA fragments, and thin lines represent the pACYC184 vector moiety. Abbreviations: Ap^s, ampicillin susceptible; bla, β-lactamase; cat, chloramphenicol acetyltransferase; A, *Ava*I; B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; P, *Pvu*II; Sm, *Sma*I; St, *Sst*I. The direction of transcription is indicated by arrows.

hybridized solely with SHV-1 at high-stringency conditions (data not shown). We also found that when hybridization was done with SHV-2, the same *Pvu*II-*Pvu*II fragment cross hybridized, suggesting similar flanking sequences (Fig. 4). Additional testing with the 352-bp *Pvu*II intragenic probe fragment gave cross hybridization with SHV-1 and SHV-2 only (data not shown). Finally, we tested a 15-mer synthetic oligonucleotide probe that was expected to be capable of

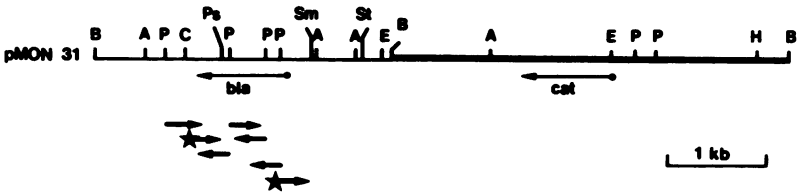

FIG. 2. Structure of pMON31 and sequencing strategy used. The vector and insert fragments are indicated by the thick and thin lines, respectively. Arrows indicate the direction of nucleotide sequencing. Stars indicate the oligonucleotide primers synthesized. Abbreviations: A, *Ava*I; B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; P, *Pvu*II; Ps, *Pst*I; Sm, *Sma*I; St, *Sst*I; bla, β-lactamase; cat, chloramphenicol acetyltransferase.

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                                    -35                 -10
1    AATAAAAGATGAAAAATGATGAAGGAAAAAAGAGGAATTGTGAATCAGCAAAACGCCGGGTTATTCTTATTGTCGCTTCTTTACTGGCCTTTATGGCC  100
RBS
101  CTCACTCAAGGATGTATTGTGGTTATGCGTTATATTCGCCTGTGTATTATCTCCCTGTTAGCCACCCTGCCGCTGGCGGTACACGCCAGCCCGCAGCCGC  200
                  M  R  Y  I  R  L  C  I  I  S  L  L  A  T  L  P  L  A  V  H  A  S  P  Q  P  L

201  TTGAGCAAATTAAACTAAGCGAAAGCCAGCTGTCGGGCCGCGTAGGCATGATAGAAATGGATCTGGCCAGCGGCCGCACGCTGACCGCCTGGCGCGCCGA  300
       E  Q  I  K  L  S  E  S  Q  L  S  G  R  V  G  M  I  E  M  D  L  A  S  G  R  T  L  T  A  W  R  A  D

301  TGAACGCTTTCCCATGATGAGCACCTTTAAAGTAGTGCTCTGCGGCGCAGTGCTGGCGCGGGTGGATGCCGGTGACGAACAGCTGGAGCGAAAGATCCAC  400
       E  R  F  P  M  M  S  T  F  K  V  V  L  C  G  A  V  L  A  R  V  D  A  G  D  E  Q  L  E  R  K  I  H

401  TATCGCCAGCAGGATCTGGTGGACTACTCGCCGGTCAGCGAAAAACACCTTGCCGACGCAATGACGGTGGGCGAACTCTGCGCCGCCGCCATTACCATGA  500
       Y  R  Q  Q  D  L  V  D  Y  S  P  V  S  E  K  H  L  A  D  A  M  T  V  G  E  L  C  A  A  A  I  T  M  S
                                                          G

501  GCGATAACAGCGCCGCCAATCTGCTACTGGCCACCGTCGGCGGCCCCGCAGGATTGACTGCCTTTTTGCGCCAGATCGGCGACAACGTCACCCGCCTTGA  600
       D  N  S  A  A  N  L  L  L  A  T  V  G  G  P  A  G  L  T  A  F  L  R  Q  I  G  D  N  V  T  R  L  D
                                    T  A

601  CCGCTGGGAAACGGAACTGAATGAGGCGCTTCCCGGCGACGCCCGCGACACCACTACCCCGGCCAGCATGGCCGCGACCCTGCGCAACGTTGGCCTGACC  700
       R  W  E  T  E  L  N  E  A  L  P  G  D  A  R  D  T  T  T  P  A  S  M  A  A  T  L  R  N  V  G  L  T
                                                                              ►K  L  +◄

701  AGCCAGCGTCTGAGCGCCCGTTCGCAACGGCAGCTGCTGCAGTGGATGGTGGACGATCGGGTCGCCGGACCGTTGATCCGCTCCGTGCTGCCGGCGGGCT  800
       S  Q  R  L  S  A  R  S  Q  R  Q  L  L  Q  W  M  V  D  D  R  V  A  G  P  L  I  R  S  V  L  P  A  G  W
                  OLiGO
801  GGTTTATCGCCGATAAGACCGGAGCTGGCGAGCGGGGTGCGCGCGGGATTGTCGCCCTGCTTGGCCCGAATAACAAAGCAGAGCGCATTGTGGTGATTTA  900
       F  I  A  D  K  T  G  A  G  E  R  G  A  R  G  I  V  A  L  L  G  P  N  N  K  A  E  R  I  V  V  I  Y
                                    ►S◄

901  TCTGCGGGATACCCCGGCGAGCATGGCCGAGCGAAATCAGCAAATGCCGGGATCGGCAAGGCGCTGTACGAGCACTGGCAACGCTAACCCGCGTGGCCG  1000
       L  R  D  T  P  A  S  M  A  E  R  N  Q  Q  I  A  G  I  G  K  A  L  Y  E  H  W  Q  R  *
                                                          A     I

1001 CGCGTTATCCGGCCCGCAGCACCTCGCAGCGTGCCGGGCGATATGACTGGCGGCGGCATCGGAAAGATGCCGGTCGGTAATGATGGTGGTGAACCGGGTC  1100

1101 AAAGGTAACGCCATAAACGTGGCCACCTGATTGTATTTCGAACTGTCGCACGACGGATAGCTCTCGCGCTGACCTGGCTGACGTCTCCTTGACGGTAACC  1200

1201 TTGTTCTCATCAGGGTGAATATCCCGCGACTGTCCCAGCCGCTGGCGGAGATAAAGGCCGTATCGATAGCCAGGTGGCGTAACGTACGCGCCGCCGATTC  1300

1301 GCCCACGCAGGAGCGGTTCTCCGCCACAGAGTGCCGCCGGTGTGGATCACGCCGCACTGGCTGGCATCGATC  1372
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FIG. 3. Nucleotide sequence of the SHV-1 β-lactamase gene. The deduced amino acid sequence is designated by the one-letter code. The active-site tetrad STFK and the box VII KTG triad are underlined. The −10 and −35 regions, the putative ribosome-binding site (RBS), and the 15-mer oligonucleotide probe are overlined. Differences with the published polypeptide SHV-2 sequence are indicated below each amino acid. Arrowheads indicate significant differences from SHV-2. +, An inserted amino acid.

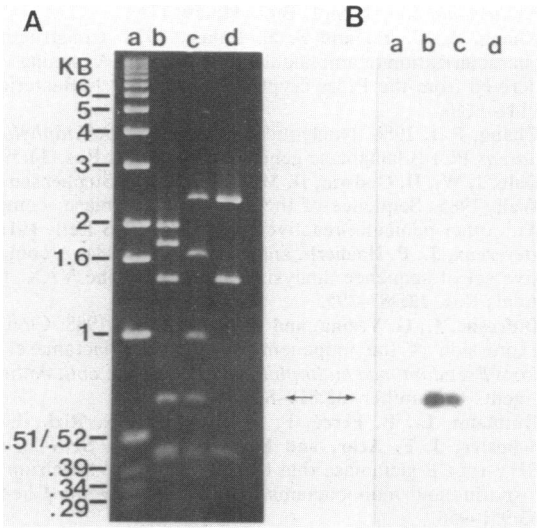

FIG. 4. (A) Agarose gel (0.7%) electrophoresis of *Ava*I-plus-*Pvu*II-digested plasmid DNAs. Lanes: a, ladders; b, pMON31; c, pMON39; d, pACYC184. (B) Autoradiogram obtained after Southern transfer of DNA from the agarose gel in panel A, followed by hybridization with the ^{32}P-labeled 640-bp *Pvu*II SHV-1 probe from pMON31. Homologous fragments in pMON31 (SHV-1) and pMON39 (SHV-2) are indicated by arrows.

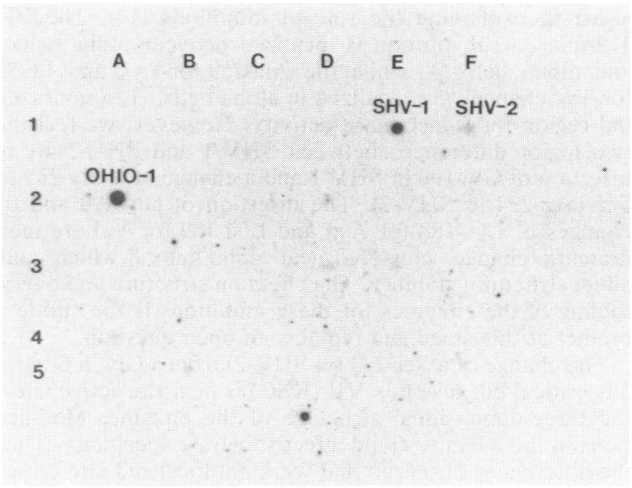

FIG. 5. Oligonucleotide typing with purified recombinant plasmid DNAs carrying cloned β-lactamase genes. The results shown were obtained by using the 15-mer oligonucleotide probe (Fig. 3). Positions are as follows: 1A, pBR322 (TEM-1); 1B, pMON60 (TEM-1); 1C, pMK20::RP4 (TEM-2); 1D, pMG204b (TLE-1); 1E, pMON31 (SHV-1); 1F, pMON39 (SHV-2); 2A, pMON1100 (OHIO-1); 2B, pMON300 (OXA-1); 2C, pMON20 (OXA-2); 2D, pMON922 (OXA-3); 2E, pMON100 (OXA-4); 2F, pMON53 (OXA-5); 3A, pMON1300 (OXA-6); 3B, pMON810 (PSE-1); 3C, pMON234 (PSE-2); 3D, pMON225 (PSE-3); 3E, pMON709 (PSE-4); 3F, pMON41 (CARB-3); 4A, pMON1025 (CARB-4); 4B, pMON401 (ROB-1); 4C, pMON510 (AER-1); 4D, pMON80 (LCR-1); 4E, pNU81 (*ampC*); 4F, pCR1; 5A, pACYC184; 5B, pMK20.

discriminating between SHV-1 and SHV-2 (Fig. 3). Surprisingly, the 15-mer SHV-1 *bla* gene probe hybridized strongly with OHIO-1 but could be differentiated from SHV-2 and other plasmid-mediated β-lactamases (Fig. 5).

DISCUSSION

The identification and differentiation of plasmid-mediated β-lactamases such as SHV-1 (pI, 7.6) and SHV-2 (pI, 7.6) with isoelectric points virtually identical to those of OXA-5 (pI, 7.62), OXA-6 (pI, 7.8), and OXA-7 (pI, 7.65) is a difficult task. In contrast, isoelectric focusing of OHIO-1 (pI, 7.0) β-lactamase would give a band that can be differentiated from these enzymes (34). Because of our interest in SHV-type extended-spectrum β-lactamases and their evolutionary relationships to other plasmid-mediated enzymes, we decided to isolate their *bla* structural genes. The recombinant plasmids constructed provided material for more refined analysis such as nucleic acid homology, nucleotide sequencing, mutagenesis of critical amino acid residues, and computer modeling.

DNA homology among these enzymes was investigated by preparing probes for SHV-1 and examining their hybridization with a collection of recombinant plasmids encoding other β-lactamase types. The 640-bp *Pvu*II SHV-1 probe contains 390 bp extraneous to the structural gene and shares 80% nucleic acid homology with OHIO-1 but under high stringency conditions (where 95% homology is necessary for a positive signal) hybridized only with SHV-1 and SHV-2. This suggested homologous sequences not only within the SHV-1 and SHV-2 *bla* structural genes but also in flanking sequences. A 900-bp *Pst*I fragment from R1010 plasmid has been previously tested as an SHV-1 probe against 17 different β-lactamase types and found to be specific, although the homologous sequence may not be completely within the structural gene (5). Indeed, our sequencing data revealed one *Pst*I site within the structural gene (beginning at position 741) (Fig. 2). At this stage, the 352-bp *Pvu*II fragment is the best SHV-1 probe that we have tested. It shares 88% homology with OHIO-1 but hybridized only with SHV-1 and SHV-2 under stringent conditions. Even though the SHV-2 nucleotide sequence was not yet known when experiments were done, we synthesized oligonucleotide probes whose central nucleotide differences were based on the SHV-1 DNA sequence but corresponded to differences in the SHV-1 and SHV-2 amino acid sequences (2, 3). Surprisingly, the synthetic 15-mer probe enabled us to discriminate between SHV-1 and SHV-2 but not against OHIO-1, indicating conserved sequences near the critical box VII (KTG) at position 234 (2, 17). The 15-mer 5′-GGAGCTGGCGAGCGG-3′ SHV-1 oligo probe that we synthesized did not hybridize to the homologous SHV-2 region (5′-GGAGCTΔGCGAGC GG-3′) because the T-to-A change in SHV-2 was central and destabilized the hybrid. In contrast, the same probe hybridized with OHIO-1 (5′-GGAGCTGGCGAΔCGG-3′) because the G-to-A change was near the 3′ end of the hybrid (Fig. 5).

We sequenced the complete SHV-1 structural gene and compared it with other known β-lactamase sequences. The SHV-1 coding sequence had 90% DNA sequence homology with LEN-1 and 92% homology with OHIO-1. The R974 SHV-1 deduced amino acid sequence differed by 9 amino acids from the peptide sequencing of SHV-1 and SHV-2. Discrete differences in amino acids from the *E. coli* plasmid-mediated p453 PIT-2 (SHV-1), the *E. coli* A2302 SHV-2, and the plasmid R974 SHV-1 β-lactamases reported here are assumed to be silent mutations presumably due to differences in the strains and plasmids used. The Thr-136-for-Ala change was probably due to an inversion during peptide sequencing. Since SHV-1 is a class A β-lactamase, the overall fold of the enzyme can be assumed to be similar to that of the crystallized *S. aureus* PC1 penicillinase and could

assist in explaining the role of mutations (14). The Gly-112-for-Ala substitution is localized between alpha helix 3 and alpha helix 4, while the Ala-269-for-Lys and Ile-72-for-Tyr changes are localized in alpha helix 11, a nonessential region for β-lactamase activity. However, we feel that two major differences between SHV-1 and SHV-2 are an insertion of Gly-190 in SHV-1 and a change of a Gly-235 for Ser (Ser-234 in SHV-2). The insertion of Gly-190 and the changes of Lys-188 for Asn and Leu-189 for Val are more dramatic changes clustered near alpha helix 8 which could affect structural stability. The effect on structure and overall folding of the enzymes for these mutations is too subtle to predict at this stage and remains an open question.

The change of a Ser-234 (in SHV-2) from a Gly in β-barrel 3 is critical because box VII (KSG) is near the active site in the three-dimensional structure of the enzyme. Modifications in this vicinity could affect substrate specificity. Thus, the differences observed that were not localized into critical regions are assumed to be silent mutations. Finally, the structural basis for β-lactamase SHV-1 mutations capable of giving cephalosporin hydrolysis is not understood at the atomic level, and there are no proper models for this. Site-specific mutagenesis combined with computer modeling will be essential to confirm these observations.

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