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Molecular Characterization of the Gene Encoding SHV-3 β-Lactamase Responsible for Transferable Cefotaxime Resistance in Clinical Isolates of *Klebsiella pneumoniae*

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In *Klebsiella pneumoniae* 86-4, cefotaxime resistance was due to a transferable broad-spectrum β-lactamase, SHV-3. The plasmid-borne gene encoding SHV-3 has been cloned, and the primary structure of the enzyme was deduced from its nucleotide sequence. SHV-3 differs from SHV-1 in two positions. The extended substrate profile of SHV-3 probably results from the substitution of Ser-213 for Gly, as in SHV-2, whereas replacement of Arg-180 by Leu resulted in a decrease in the pI from 7.6 to 7.0. The bla_{shv-3} gene is highly homologous (92% DNA sequence identity) with the chromosomal gene coding for LEN-1 β-lactamase of *K. pneumoniae*, suggesting that the origin of the SHV-encoding genes now present on many plasmids may be chromosomal.

The intensive use of antibiotics is followed sooner or later by the emergence of resistant clinical isolates (8). An important phenomenon recently described in members of the family *Enterobacteriaceae* is the existence of transferable β-lactamase-mediated resistance to the broad-spectrum cephalosporins and monobactams (15, 16, 24, 26). Until 1983, these drugs were known to be active against all strains of *Enterobacteriaceae* except those which overproduced the chromosomally encoded cephalosporinase AmpC (23) or those in which low-level β-lactamase production was associated with altered cell wall permeability (18). From 1983 onwards, significant resistance to the newer β-lactams was increasingly encountered in *Klebsiella pneumoniae* and *Escherichia coli* (22), two species which are not normally associated with chromosomally specified resistance. This resistance was subsequently found to be plasmid mediated and to involve β-lactamases derived from two types of class A enzymes, TEM and SHV, as described previously (15, 27).

The present study reports the genetic characterization of a broad-spectrum β-lactamase that was believed to belong to the SHV family, which includes SHV-1 and SHV-2 (13, 15). The enzyme described here, previously known as the SHV-2 type, was renamed SHV-3 because, although it shows extended substrate and inhibitor profiles indistinguishable from those of SHV-2, it displays a distinct isoelectric point (pI), 7.0 instead of 7.6 (13). The clinical isolates producing the SHV-3 β-lactamase were all *K. pneumoniae*, were involved in an outbreak of nosocomial infections in intensive care units, and seemed to be confined to our hospital (unpublished data), where SHV-2 β-lactamase was also recently described (13).

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* J53-2(pUD18) is a transconjugant producing SHV-3 β-lactamase as a result of a mating between *K. pneumoniae* clinical isolate 86-4 (13) and *E. coli* J53-2 (*metF63 proB22* Rif^r).

Plasmid pACYC184 (6), which confers resistance to chloramphenicol and tetracycline, was used as a vector for cloning. Recombinant plasmids were constructed by standard procedures (25) and introduced by transformation into *E. coli* MΔ15 [Δ(*lac-pro*) *ara rpsL recA thi* Φ80d(*lacZ* ΔM15)].

Media and antibiotic susceptibility testing. For routine purposes, strains were grown at 37°C in L broth (25) or on L agar plates containing ampicillin (Sigma Chemical Co., St. Louis, Mo.) at a concentration of 250 μg/ml, cefotaxime (Roussel, Paris) at 2 μg/ml, and chloramphenicol (Sigma) at 25 μg/ml. MICs of β-lactam antibiotics were determined at 37°C by a serial twofold dilution method in Mueller-Hinton agar with an inoculum of 10^5 CFU. Antimicrobial agents were kindly provided as follows: aztreonam, E. R. Squibb & Sons, Princeton, N.J.; cefoxitin, Merck, Sharp, Dohme & Chibret; ceftazidime, Glaxo Pharmaceuticals, Ltd., Greenford, United Kingdom; clavulanic acid, Beecham Laboratories, Bristol, Tenn.; moxalactam, Eli Lilly & Co., Indianapolis, Ind.; and piperacillin, Lederle Laboratories, Pearl River, N.Y. Clavulanic acid was used at a concentration of 2 μg/ml.

Agarose gel electrophoresis of large plasmids. Plasmid DNA was prepared from *K. pneumoniae* 86-4 or the *E. coli* transconjugant by the rapid method of Wheatcroft and Williams (30), and the crude extract was used directly for electrophoresis, which was performed in agarose gels (0.7%, wt/vol) for 90 min at 50 V. The size of plasmid pUD18 was determined by using pBR322 (4.36 kilobases [kb]), RP4 (54 kb), pIP135 (70 kb), and pIP55 (150 kb) as standards.

Nucleic acid techniques. Restriction endonuclease analysis, agarose gel electrophoresis, Southern transfer, and DNA-DNA hybridization with ^{32}P-labeled probes were performed as previously described (10, 20). Plasmid pBR322 was used as the source of the *tetA*-specific probe, whereas the 6′-*N*-acetyltransferase (type IV) [AAC(6′)-IV]-specific probe was a *Dde*I-*Hae*III fragment of the *aacA4* gene cloned in M13mp18 (29). For large-scale preparations, plasmids were extracted from 1-liter cultures by the method of Ish-Horowicz and Burke (12), concentrated by precipitation with polyethylene glycol (11), and then subjected to ethidium bromide-cesium chloride density gradient centrifugation in a

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TABLE 1. Properties of *E. coli* strains producing SHV-3 β-lactamase[a]

Strain	β-Lactamase	MIC (μg/ml)								Cotransferred resistance markers
		AMP	PIP	CTX	CAZ	CXT	MOX	AZT	CTX+CLA	
J53-2(pUD18)	SHV-3	4,096	256	4	2	1	0.1	1	0.01	A, K, N, T, Tc, Tp
MΔ15	None	8	1	0.03	0.125	4	0.125	0.06	0.06	
MΔ15(pHUC37)	SHV-3	1,024	64	2	1	4	0.25	0.5	0.06	

[a] Abbreviations: A, amikacin; AMP, ampicillin; AZT, aztreonam; CAZ, ceftazidime; CLA, clavulanic acid; CTX, cefotaxime; CXT, cefoxitin; K, kanamycin; MOX, moxalactam; N, netilmicin; PIP, piperacillin; T, tobramycin; Tc, tetracycline; Tp, trimethoprim.

Beckman VTI 65-2 rotor for 16 h at 45,000 rpm. DNA sequences were generated by the modified dideoxy-chain termination method (4), compiled, and analyzed as described previously (10).

RESULTS

Characterization of the resistance plasmid pUD18. It was shown by conjugative transfer between the *K. pneumoniae* clinical isolate 86-4 and *E. coli* J53-2 that resistance to cefotaxime cotransferred with resistance to amikacin, kanamycin, netilmicin, tetracyclines, tobramycin, and trimethoprim (Table 1). Agarose gel electrophoretic analysis of DNA extracted from cefotaxime-resistant *K. pneumoniae* or an *E. coli* transconjugant revealed the presence of a large plasmid, pUD18, of about 180 kilobases (kb) (Fig. 1, lane 5). To construct a restriction map of pUD18 and to locate the gene responsible for cefotaxime resistance, plasmid DNA was purified from crude extracts of *E. coli* J53-2 by ethidium bromide-cesium chloride density gradient centrifugation. From four independently grown cultures, a small plasmid, pHUC86, of about 17 kb (Fig. 1, lane 2) was recovered which was not present in *K. pneumoniae* 86-4 or the *E. coli* transconjugant, as determined by DNA-DNA hybridization (data not shown). This suggested that pUD18 may be unstable. Plasmid pHUC86 could not be established after transformation of a plasmid-free host. Restriction endonuclease analysis of pHUC86 showed four sites for *Bam*HI and no sites for *Hin*dIII, *Xho*I, or *Sal*I.

Cloning of the cefotaxime resistance gene. On digestion of pHUC86 with *Bam*HI, four fragments of 8, 3.7, 3.7, and 1.5 kb were generated. To determine whether one of these carried the gene responsible for cefotaxime resistance, bla_{shv-3}, the *Bam*HI fragments were cloned into pACYC184 and used to transform *E. coli* MΔ15 to simultaneous chloramphenicol and cefotaxime resistance. From a suitable transformant, plasmid pHUC37 was isolated and shown by *Bam*HI digestion to contain one of the 3.7-kb fragments. This plasmid conferred an increased MIC of cefotaxime that was abolished by clavulanic acid (Table 1), as has been found with pUD18 in both *K. pneumoniae* and *E. coli* (13). A restriction map of pHUC37 was constructed by performing single and double digests with *Cla*I, *Eco*RV, *Not*I, *Pst*I, and *Sma*I (Fig. 2), and this enabled the bla_{shv-3} gene to be localized more precisely. A series of plasmids was constructed by deleting various restriction fragments from pHUC37, and it was thus established that the smallest fragment capable of conferring cefotaxime resistance was the 1.6-kb *Eco*RV-*Cla*I fragment. Further deletion studies suggested that a *Pst*I site was situated within the coding sequence for SHV-3. This conclusion was reached independently by Bisessar and James (5) from studies on the SHV-1-encoding gene cloned from R1010, although several restriction sites differ in the respective maps.

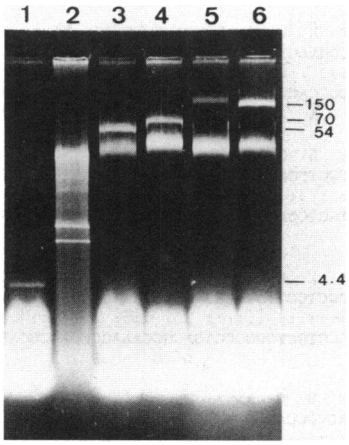

FIG. 1. Agarose gel electrophoresis of DNA preparations extracted from *E. coli* containing the following plasmids. Lanes: 1, pBR322; 2, pHUC86; 3, RP4; 4, pIP135; 5, pUD18; 6, pIP55. With the exception of pHUC86, which was purified on a CaCl gradient, crude preparations were used. The two bands of pHUC86 probably correspond to the open and covalently closed circular forms. The positions of size markers in kilobases are shown at the right.

The antibiotic susceptibility pattern of the *E. coli* transformants indicated that none of the other resistance genes associated with pUD18 was carried by pHUC37 (Table 1). To establish whether any additional coding sequences had been truncated by the *Bam*HI digest used to construct pHUC37, various restriction digests were analyzed by DNA-DNA hybridization using probes specific for the *tetA* and *aacA4* genes. No hybridization was detected, however, although in parallel experiments both probes hybridized to the other 3.7-kb *Bam*HI fragment carried by pHUC86 (data not shown).

Nucleotide sequence of the bla_{shv-3} gene. The precise location of the bla_{shv-3} gene was established by determining the complete nucleotide sequence of a 2.4-kb *Sma*I-*Bam*HI fragment by the M13 cloning-dideoxy sequencing approach. A 1,080-base-pair (bp) segment of this sequence is presented in Fig. 3, where it can be seen that the principal feature is an open reading frame comprising 858 bp, which begins 238 bp downstream of the *Sma*I site. The deduced primary structure of its product agrees almost perfectly with that determined by protein sequencing of the purified SHV-1 β-lactamase (2).

The nucleotide sequence of the chromosomal gene encoding the LEN-1 β-lactamase from *K. pneumoniae*, as determined by Arakawa et al. (1), is aligned with that of bla_{shv-3} in Fig. 3. Extensive sequence identity (>92%) was found in the structural genes and the flanking regions. The majority of differences in the coding sequence is due to transition-type mutations (65%) that occur mainly in the wobble position of the codons. There are, however, 35 nucleotide variations which result in 29 amino acid substitutions. The most striking deviation in the coding sequences occurs near the 3′ end of the genes, where bla_{len-1} has apparently lost 1 base

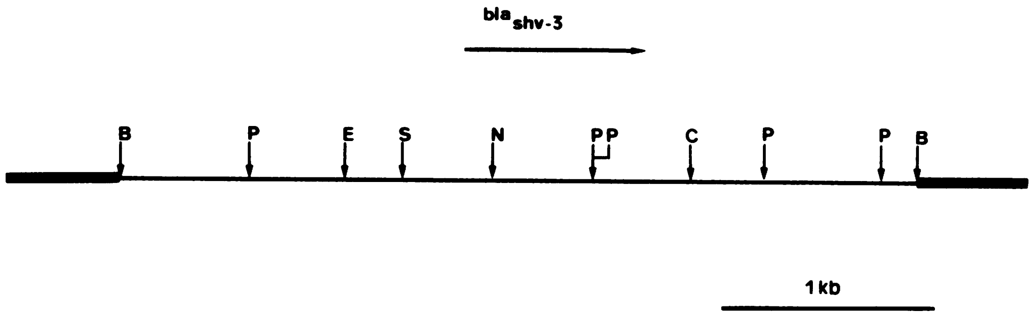

FIG. 2. Restriction map of the bla_{shv-3} region of pHUC37. The thick line indicates the vector pACYC184. The arrow indicates the location and the deduced direction of transcription of the bla_{shv-3} gene. Restriction sites are denoted as follows: B, *Bam*HI; C, *Cla*I; E, *Eco*RV; N, *Not*I; P, *Pst*I; S, *Sma*I.

pair and thus encodes a protein with a slightly shorter COOH terminus.

Features of the SHV-3 protein sequence. Comparison of the deduced SHV-3 sequence with that determined for SHV-1 by protein sequencing (2) revealed two amino acid substitutions, the inversion of two residues, and the presence of an NH_2-terminal signal sequence which is removed upon export of the enzyme to the periplasm. At position 180 of the mature

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TTATAGTTCATCACGGCCTTGAGTCAAAAAATAGCGTGCTTAGGCAGGGCTAGATATTGATTATTCGAAATAAAAGATGAAAAATGATGAAGGAAAAAAGAGGAATTGTGAATCAGCAAA  120
TTATAGTTCATCGCGGCCTTGAGTCAAAAAATAGCGTGCTGAGGCAGGGCTAAATATTGATTATTCGAAATAAAAGATGACAAATGATGAAGGAAAAAAGAGGAATTGTGAATCAGCAAA

M R Y I R L C I I S L L A T L P
ACGCCGGGTTATTCTTATTTGTCGCTTCTTTACTCGCCTTTATCGGCCCTCACTCAAGGATGTATTGTGGTTATGCGTTATATTCGCCTGTGTATTATCTCCCTGTTAGCCACCCTGCCG  240
ACGCCGGGTTATTCTTATTTGTCGCCCCTTTGCTCGCCCTTATCGGCCCTCACTCAAGGAAGTATTGCGGTTATGCGTTATGTTCGCCTGTGTGTTATCTCCCTGTTAGCCACCCTGCCA
V V

L A V H A S P Q P L 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  35
CTGGCGGTACACGCCAGCCCGCAGCCGCTTGAGCAAATTAAACTAAGCGAAAGCCAGCTGTCGGGCCGCGTAGGCATGATAGAAATGGATCTGGCCAGCGGCCGCACGCTGACCGCCTGG  360
CTGGTGGTATACGCCGGTCCACAGCCGCTTGAGCAGATTAAACAAAGCGAAAGCCAGCTGTCGGGCCGCGTGGGGATGGTGGAAATGGATCTGGCCAACGGCCGCACGCTGGCCGCCTGG
V Y G Q V N A

R A D 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 Y R Q Q  75
CGCGCCGATGAACGCTTTCCCATGATGAGCACCTTTAAAGTAGTGCTCTGCGGCGCAGTGCTGGCGCGGGTGGATGCCGGTGACGAACAGCTGGAGCGAAAGATCCACTATCGCCAGCAG  480
CGCGCCGATGAACGCTTTCCCATGGTGAGCACCTTTAAAGTGCTGCTGTGCGGCGCGGTGCTGGCGCGGGTGGATGCCGGGCTCGAACAACTGGATCGGCGGATCCACTACCGCCAGCAG
V L L D R

D L V D Y S P V S E K H L A D G M T V G E L C A A A I T M S D N S A A N L L L A  115
GATCTGGTGGACTACTCGCCGGTCAGCGAAAAACACCTTGCCGACGGCATGACGGTCGGCGAACTCTGCGCCGCCGCCATTACCATGAGCGATAACAGCGCCGCCAATCTGCTACTGGCC  600
GATCTGGTGGACTACTCCCCGGTCAGCGAAAAACACCTTGTCGACGGGATGACGATCGGCGAACTCTGCGCCGCCGCCATCACCCTGAGCGATAACAGCGCTGGCAATCTGCTGCTGGCC
V I L G

T V G G P A G L T A F L R Q I G D N V T R L D R W E T E L N E A L P G D A R D T  155
ACCGTCGGCGGCCCCGCAGGATTGACTGCCTTTTTGCGCCAGATCGGCGACAACGTCACCCGCCTTGACCGCTGGGAAACGGAACTGAATGAGGCGCTTCCCGGCGACGCCCGCGACACC  720
ACCGTCGGCGGCCCCGCGGGATTAACTGCCTTTCTGCGCCAGATCGGTGACAACGTCACCCGTCTTGACCGCTGGGAAACGGCACTGAATGAGGCGCTTCCCGGCGACGCGCGCGACACC
A

*
T T P A S M A A T L R K L L T S Q R L S A R S Q L Q L L Q W M V D D R V A G P L  195
ACTACCCCGGCCAGCATGGCCGCGACCCTGCGCAAGCTGCTGACCAGCCAGCGTCTGAGCGCCCGTTCGCAACTGCAGCTGCTGCAGTGGATGGTGGACGATCGGGTCGCCGGACCGTTG  840
ACCACCCCGGCCAGCATGGCCGCCACGCTGCGCAAACTACTGACCGCGCAGCATCTGAGCGCCCGTTCGCAACAGCAACTCCTGCAGTGGATGGTGGACGATCGGGTTGCCGGCCCGCTG
A H Q
*
I R S V L P A G W F I A D K T G A S E R G A R G I V A L L G P N N K A E R I V V  235
ATCCGCTCCGTGCTGCCGGCGGGCTGGTTTATCGCCGATAAGACCGGAGCTAGCGAACGGGGTGCGCGCGGGATTGTCGCCCTGCTTGGCCCGAATAACAAAGCAGAGCGGATCGTGGTG  960
ATCCGCGCCGTGCTGCCGCCGGGCTGGTTTATCGCCGACAAAACCGGGGCTGGCGAACGGGGTGCGCGCGGCATTGTCGCCCTGCTCGGCCCGGACGGCAAACCGGAGCGCATTGTGGTG
A P G D G P

I Y L R D T P A S M A E R N Q Q I A G I G A A L I E H W Q R *  265
ATTTATCTGCGGGATACGCCGGCGAGCATGGCCGAGCGAAATCAGCAAATCGCCGGGATCGGCGCGGCGCTGATCGAGCACTGGCAACGCTAACCCGGCGTCGGCCGCGCGCGTTATCG  1080
ATCTATCTGCGGGATACCCCGGCGAGTATGGCCGAGCGTAATCAACATATCGCCGGGATCGGCCA-GCGCTGATCGAGCACTGGCAACGCTAACCCGGCGGTA-CCGTGCGTTAGCGCG
H Q R *
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FIG. 3. Nucleotide sequence of the bla_{shv-3} gene and primary structure of the SHV-3 β-lactamase. The nucleotide sequence of a 1,080-bp segment from the *Sma*I-*Bam*HI fragment starting 100 bp downstream from the *Sma*I site is shown together with the deduced primary structure of the β-lactamase (one-letter code). The bla_{shv-3} sequence is aligned above that of the chromosomal bla_{len-1} gene from *K. pneumoniae*; only those amino acids which differ in LEN-1 are shown. The amino acid substitutions distinguishing SHV-3 from SHV-1 are indicated by *. The amino acid numbering at right refers to the position in the mature protein.

protein, the Arg residue of SHV-1 has been replaced by Leu, whereas position 213 of SHV-3 is occupied by Ser instead of Gly. Two amino acids appear to have been inverted in the sequence of SHV-1 at positions 116 and 117, as the dipeptide Ala-Thr, and not Thr-Ala, is found in both SHV-3 and LEN-1 (Fig. 3). When the protein sequence of SHV-2, which displays the same substrate profile as SHV-3 (13), was determined (3), the single substitution Gly-213 (SHV-1)→Ser (SHV-2) was found. It is, therefore, clear that this amino acid change is responsible for the extended substrate range of the enzyme. In contrast, the substitution of a basic residue, Arg-180 by an uncharged amino acid, Leu, does not appear to affect enzymatic activity, as judged by kinetic parameters (data not shown), but does account for the decrease in the pI from 7.6 (SHV-1 and SHV-2) to 7.0 (SHV-3).

DISCUSSION

Since 1983, many members of the family *Enterobacteriaceae*, but predominantly *K. pneumoniae* and *E. coli*, have acquired transferable plasmids which carry the genes for extended-spectrum β-lactamases capable of conferring resistance to the newer β-lactam antibiotics. The present study shows that a 180-kb plasmid, pUD18, originally found in clinical isolates of *K. pneumoniae*, carries the bla_{shv-3} gene, a potent agent of cefotaxime resistance. This plasmid, which was unstable in the transconjugant *E. coli*, gave rise to a smaller derivative, pHUC86, bearing the genes for SHV-3, AAC-6′, and tetracycline resistance. The association of these three genes may indicate that they are part of a transposon. In this respect, it may be significant that Nugent and Hedges have demonstrated transposition of the gene encoding SHV-1 (21).

The restriction map obtained for the bla_{shv-3} gene shows several differences from that of a 6-kb *Bam*HI fragment carrying the bla_{shv-1} gene from R1010 (5). To date, no DNA sequence data have been reported for other *bla* genes encoding SHV enzymes, so we have been unable to evaluate the possible extent of genetic variation occurring in nature. Comparison of the SHV-1 and SHV-3 protein sequences clearly indicates that both amino acid exchanges can be accounted for by single point mutations CGG→CTG (Arg-180→Leu) and GGC→AGC (Gly-213→Ser). By aligning the SHV-3 protein sequence (Fig. 3) with that of LEN-1 (1), 29 amino acid differences were found, 4 of which affect the signal peptide. Most of these substututions (21 of 29) are conservative in nature and occur in the 180 NH_2-terminal residues of the enzyme. A more noteworthy difference occurs at the COOH terminus, where LEN-1 lacks the last seven amino acid residues found in the SHV-type enzymes. This could indicate that the COOH-terminal end of the β-lactamase is unimportant for β-lactam hydrolysis, and indirect suppport for this interpretation is provided by the finding that the COOH terminus of TEM-1, which is highly homologous to SHV-1 overall (2), is slightly shorter and more divergent. The extensive nucleotide sequence homology (>92%) between the plasmid-borne bla_{shv-3} and chromosomal bla_{len-1} genes suggests that the origin of the present plasmid-mediated SHV β-lactamases may have been the chromosome of *K. pneumoniae*. Further study of the evolution of the SHV enzymes and their genes is required to understand their line of descent and means of dissemination.

The β-lactamases appear to be capable of extending their substrate profiles. For the inducible chromosomal gene coding for cephalosporinase, this is due to a mutation in one of the regulatory genes (10, 10a, 17, 20), whereas, for the broad-spectrum β-lactamases, this seems to be due to mutations in the structural genes. In the case of the TEM-3 enzyme, cefotaxime resistance is due to a point mutation analogous to that described here which changes Gly-211 of the TEM-2 enzyme to Ser in TEM-3 (27). It is striking that in SHV-2, SHV-3, and TEM-3, this amino acid substitution is located adjacent to the Lys-Thr-Gly motif, which is part of a β-sheet comprising one side of the substrate-binding domain in the *Streptomyces albus* G β-lactamase (7) and in several other β-lactam-reactive enzymes (28). This strongly suggests that the hydroxyl group of Ser-213 plays an important role in cefotaxime binding or hydrolysis. In contrast, position 180, identified by the Arg→Leu substitution in SHV-3, should correspond to an Arg residue within helix 7 of the β-lactamase from *S. albus* G (14). From its crystal structure, it is apparent that this helix and the corresponding helix in *Staphylococcus aureus* PC_1 β-lactamase (helix 9) are close to, but not part of, the β-lactam binding site (7, 9), which is consistent with our conclusions for SHV-3.

Further extended-spectrum derivatives of both the SHV and TEM enzymes can be expected, and the accumulation of silent mutations in the bla_{tem-3} gene strengthens this prediction (27). Perhaps the most intriguing aspect of this resistance phenomenon concerns the elevated mutation rate associated with plasmid-borne bla_{shv} genes compared with the natural chromosomal allele for which no mutations have yet been detected (unpublished data).

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