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New System Based on Site-Directed Mutagenesis for Highly Accurate Comparison of Resistance Levels Conferred by SHV β-Lactamases

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We developed a system based on site-directed mutagenesis that allows a precise comparison of SHV enzymes under isogenic conditions. In addition, the influences of two different, naturally occurring promoters were examined for each SHV derivative. The system comprised two separately cloned DNA fragments, each the size of 3.6 kb. Both fragments encoded an SHV gene originating from clinical isolates but with different promoters. The structural genes were made identical by site-directed mutagenesis. Other mutations were then introduced into both fragments by means of site-directed mutagenesis, resulting in the SHV derivatives SHV-1, SHV-2, SHV-2a, SHV-3, and SHV-5. The amino acid exchange of glutamic acid at position 235 for lysine in SHV-5 resulted in the highest resistance levels. SHV-3, differing from SHV-2 by the exchange of arginine at position 201 for leucine and previously described as indistinguishable from SHV-2, was shown to cause slightly higher resistance to ceftazidime and lower resistance to ceftriaxone, cefotaxime, and cefepime than SHV-2. The point mutation in SHV-2a, with the leucine-to-glutamine replacement at the unusual position 31, previously considered almost insignificant, proved to increase resistance to ceftazidime but reduced the MICs of all other cephalosporins tested when compared with those for SHV-2. For all clones harboring SHV derivatives, resistance was increased by a stronger promoter, in some cases masking the effect of the point mutation itself and demonstrating the importance of regulatory mechanisms of resistance.

Bacterial resistance to β-lactam antibiotics in the family *Enterobacteriaceae* is often caused by the presence of β-lactamases belonging to class A (1), such as the plasmid-mediated TEM and SHV-type enzymes (13, 25). Amino acid substitutions caused by point mutations in the genes of these enzymes can broaden the substrate specificity, causing problems in the therapy of infections treated with β-lactams (for a review, see reference 28).

In β-lactam-resistant members of the *Enterobacteriaceae*, TEM-1 and its derivatives are most common (19), whereas SHV enzymes, for reasons yet unknown, are found predominantly in *Klebsiella* species (14). Derivatives of SHV-1 are able to hydrolyze third-generation cephalosporins such as cefotaxime because of a glycine-to-serine substitution at position 234 (glycine [234] → serine) (11). The numbering of the amino acids in β-lactamases is, according to Sutcliffe, for TEM-1, counting 2 amino acids less for SHV enzymes (32) and including the NH_2-terminal sequence consisting of 21 amino acids which is no longer present in the mature protein. In addition to the glycine (234) → serine substitution, further point mutations of the original SHV-1 β-lactamase resulting in increased resistance of the host strain for third-generation cephalosporins have been identified and the enzymes have been termed SHV-2 to SHV-7 (2–4, 7, 16, 22). Most of these mutations have been found to be associated with the active site of the enzyme (31). As an exception, an SHV variant (SHV-2a) which carries a lysine-to-glutamine substitution at position 31 of the protein, a region not usually affected, has been described (27). The increased level of resistance due to this enzyme has been ascribed to a strong promoter situated upstream from the β-lactamase (*bla*) gene rather than to the amino acid substitution (26).

In order to examine the phenotypical role of amino acid substitutions in known SHV β-lactamases and to evaluate the effect of the promoter regions on the level of resistance, we developed a system based on site-directed mutagenesis which allows a direct comparison of SHV derivatives under isogenic conditions.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Klebsiella pneumoniae* KPZU-3 harboring plasmid pKPZU3 was a clinical isolate from the University Hospital of Zürich, Switzerland (see below). *Escherichia coli* C600 containing plasmid pBP60, which originated from *Klebsiella ozaenae* (*K. pneumoniae* subsp. *ozaenae*) 2180 (16), was kindly given by B. Wiedemann. *E. coli* HK225 (*E. coli* K-12, rifampin- and streptomycin-resistant [15]) was used as a recipient in conjugation experiments. *E. coli* DH5α (8) was used as a recipient in transformation experiments with cloned β-lactamase genes using the vector plasmid pTZ18R (33). *E. coli* TG-1 (6, 33) was used as a host for transformation with phage M13mp18 and M13mp19 single-stranded and double-stranded DNA (Boehringer GmbH, Mannheim, Germany).

Antibiotics. Ampicillin was obtained from SmithKline Beecham Pharmaceuticals, Surrey, England; cefamandole, cephalothin, and tobramycin were from Eli Lilly, Indianapolis, Ind.; cefepime and amikacin were from Bristol-Myers Squibb, New York, N.Y.; cefoperazone and tetracycline were from Pfizer, Groton, Conn.; cefotaxime was purchased from Roussel, Paris, France; cefoxitin, gentamicin, and imipenem were obtained from Merck Sharp & Dohme, Glattbrugg, Switzerland; ceftazidime was from Glaxo, Greenford, England; ceftriaxone was obtained from Hoffmann La Roche, Basel, Switzerland; chloramphenicol was from Calbiochem-Behring, La Jolla, Calif.; netilmicin was from Werthenstein Chemie, Schachen, Switzerland; and rifampin was supplied by Ciba-Geigy, Basel, Switzerland.

Susceptibility tests and biotyping. MICs were determined by the National Committee for Clinical Laboratory Standards microdilution method (20), and inhibition zone diameters were acertained by disc testing according to the guidelines of the National Committee for Clinical Laboratory Standards (21).

E tests (AB Biodisk, Solna, Sweden) were performed on Mueller-Hinton agar plates (Difco, Detroit, Mich.) according to the manufacturer's instructions.

Antibiotic gradient plates were prepared according to Gerhardt et al. (5) with slight modifications. A slant was first poured with 35 ml of antibiotic-free Luria-Bertani agar (29) into disposable inoculum plates (120 by 85 by 13 mm) (Dyna-

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TABLE 1. Oligonucleotides used for site-directed mutagenesis

Enzyme	Nucleotide no.[a]	Oligonucleotide sequence[b]	Corresponding SHV-2a sequence
SHV-1	688	5′-GACCGGAGCT**G**GCGAGCGGG-3′	**A**GC
		Gly	Ser 234
SHV-2	83	5′-AATTAAAC**T**AAGCGAAAGCC-3′	C**A**A
		Leu	Gln 31
SHV-2a	83	5′-AATTAAAC**A**AAGCGAAAGCC-3′	CAA
		Gln	Gln 31
SHV-3	592	5′-TTCGCAAC**T**GCAGCTGCTGC-3′	C**G**G
		Leu	Arg 201
SHV-5	706	5′-CTAGC**A**AGCGGGGTGCGCGC-3′	**G**AG
		Lys	Glu 235

[a] Nucleotide number within the SHV open reading frame which corresponds to the first base of the oligonucleotide.

[b] Oligonucleotides were chemically 5′ phosphorylated. Introduced mutations are indicated in boldface type.

tech, Chantilly, Va.). After solidification, the top layer (containing Luria-Bertani agar with antibiotic) was poured. The inoculum was an overnight bacterial culture diluted to McFarland standard 0.5 turbidity, and inoculation was performed with sterile cotton swabs. Plates were incubated at 35°C for 18 to 20 h and photographed with a Polaroid camera type 667 (professional). Growth was measured in centimeters.

Organisms were identified with API 20 E strips (Biomerieux, Marcy-l'Etoile, France).

DNA preparation. Plasmid DNA was prepared with the Qiagen plasmid kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

Total DNA was extracted following standard protocols (29).

Cloning and sequencing of the β-lactamase genes. Total DNA or plasmid DNA was digested with restriction enzyme *Bam*HI (Boehringer). T4 DNA ligase, ligation buffer, and calf intestinal phosphatase were obtained from Boehringer, and cloning of the *Bam*HI-fragments into pTZ18R followed by transformation of competent *E. coli* DH5α cells was performed according to Sambrook et al. (29). Clones were initially selected on X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) plates (29) containing 50 μg of ampicillin per ml. Colonies were isolated and purified on Luria-Bertani agar containing ceftazidime (30 μg/ml) or ampicillin (50 μg/ml). The sizes and orientations of cloned DNA in recombinant plasmids were determined by visualizing restriction fragments under UV light after agarose gel electrophoresis (with 0.7% agarose and 0.5 μg of ethidium bromide per liter at 4 V/cm). The gels were photographed with a Polaroid camera type 667 (professional). DNA sequencing was performed with a Sequenase kit, version 2.0 (United States Biochemicals, Cleveland, Ohio), by the dideoxy-chain termination method (30) on phage M13 subclones containing the relevant DNA fragments using deoxyadenosine 5′-[α-^{35}S]thiotriphosphate (Amersham, Little Chalfont, England).

Site-directed mutagenesis and oligonucleotides. Single nucleotide mutations were introduced with a Sculptor in vitro mutagenesis system (Amersham) on β-lactamase genes cloned into phage M13. By sequencing, the introduction of each point mutation was confirmed and the entire gene and promoter region were checked. With the *Bam*HI restriction sites, mutated genes were cloned as 3.6-kb fragments into the vector pTZ18R and transformed into DH5α for expression.

Oligonucleotides for sequencing (15-mers) and site-directed mutagenesis (20-mers) were custom synthesized (Microsynth, Balgach, Switzerland). Oligonucleotides used in site-directed mutagenesis are listed in Table 1.

Nucleotide sequence accession number. The complete sequence of the 3.6-kb *Bam*HI fragment of the ceftazidime-resistant clone MPA-2a is filed in the EMBL Data Library as accession no. X84314.

RESULTS

Properties of KPZU-3. *K. pneumoniae* KPZU-3 harboring pKPZU3 was isolated from the bronchial secretion of a patient hospitalized in the surgical ward of the University Hospital of Zürich in April 1991. KPZU-3 was resistant to penicillins and cephalosporins, including expanded-spectrum cephalosporins, but susceptible to imipenem (0.25 μg/ml). Prior to its isolation, the patient had been treated with ceftazidime and amoxicillin-clavulanic acid. The MIC of ceftazidime was 64 μg/ml, the MICs of ceftriaxone and cefotaxime were >256 μg/ml and 256 μg/ml, respectively, and the MIC of cefepime was 32 μg/ml (Table 2). β-Lactam resistance was transferred to *E. coli* HK225 in broth mating experiments. The rate of transfer was 3.1×10^{-6} per input donor cell. Plasmid DNA from KPZU-3 and transconjugant ECB-1 was extracted and analyzed by gel electrophoresis. A plasmid >40 kb in size was detected in both strains. The plasmid also conferred resistance to aminoglycosides such as amikacin, gentamicin, netilmicin, and tobramycin and to tetracycline and chloramphenicol (data not shown).

Molecular cloning and identification of *bla* genes. Total DNA from KPZU-3 was digested with *Bam*HI and ligated to the *Bam*HI-digested vector plasmid pTZ18R (Amp^r). The mixture was used to transform *E. coli* DH5α, and transformants were preselected for resistance to ampicillin followed by rep-

TABLE 2. Resistance profiles of strains and mutants

Strain[a]	Plasmid	Enzyme	Promoter	MIC (μg/ml) by E test of[b]:			
				CAZ	CRO	CTX	PM
KPZU-3	pKPZU3	SHV-2a	Strong	64[c]	>256[c]	256[c]	32[d]
ECB-1	pKPZU3	SHV-2a	Strong	32[c]	>256[c]	>256[c]	8
HK225				0.5[c]	0.25[c]	0.5[c]	ND
C600	pBP60	SHV-2	Weak	0.5[c]	8[c]	16[c]	0.019
MPA-1	pMPA1	SHV-1	Strong	2	0.125	0.047	0.38
MPB-1	pMPB1	SHV-1	Weak	1.5	0.094	0.047	0.19
MPA-2	pMPA2	SHV-2	Strong	6	24	>256	4
MPB-2	pMPB2	SHV-2	Weak	4	16	>256	4
MPA-2a	pMPA2a	SHV-2a	Strong	24	64	>256	6[e]
MPB-2a	pMPB2a	SHV-2a	Weak	12	8	16	1.5
MPA-3	pMPA3	SHV-3	Strong	6	8	>256	2
MPB-3	pMPB3	SHV-3	Weak	2	6	>256	1.5
MPA-5	pMPA5	SHV-5	Strong	>256	48	>256	4
MPB-5	pMPB5	SHV-5	Weak	>256	16	>256	1
DH5α				0.094	0.016	0.032	0.016
DH5αptz	pTZ18R	TEM-1	Vector	0.24	0.032	0.047	0.064

[a] All strains are *E. coli*, except KPZU-3, which is *K. pneumoniae*; all MPA and MPB clones are derivatives of DH5α.

[b] Abbreviations: CAZ, ceftazidime; CRO, ceftriaxone; CTX, cefotaxime; PM, cefepime; ND, not determined.

[c] Determined by the microdilution method.

[d] The MIC of cefepime as determined by microdilution is >256 μg/ml for KPZU-3 (see text).

[e] The MIC of cefepime as determined by microdilution is 128 μg/ml for MPA-2a (see text).

lica plating onto Luria-Bertani agar containing 30 μg of ceftazidime per ml. One ceftazidime-resistant clone, MPA-2a, containing plasmid pMPA2a was isolated and analyzed. It consisted of pTZ18R and a 3.6-kb *Bam*HI fragment from the large plasmid in KPZU-3, as confirmed by Southern hybridization (not shown). The entire 3.6-kb fragment, as well as two separate 1.4-kb and 2.2-kb *Bam*HI-*Nhe*I fragments thereof, was subcloned into phage M13, and the complete nucleotide sequences (3,660 bp) of both strands were determined with the universal primer on four starting points and 35 individually synthesized internal primers. The precise sequence and location of the SHV gene were identified, and the sequence was found to be identical to that of SHV-2a, an SHV derivative described previously (27). A nucleotide identity of 99.6% was found over the entire length of the 3.6-kb fragment. The differences found in the regions flanking the structural gene of our fragment comprised six deletions and three insertions of 1 bp and five nucleotide exchanges.

Plasmid pBP60 DNA from *E. coli* C600 was digested with *Bam*HI and ligated into pTZ18R in the same manner as described above. Clone MPB-2 carrying the recombinant plasmid pMPB-2 was isolated, and pMPB-2 was shown to encode SHV-2 on a 3.6-kb insertion fragment by restriction endonuclease mapping (data not shown). Resistance profiles of the strains MPA-2a and MPB-2 were determined by microdilution or by use of the E test and are listed in Table 2.

Site-directed mutagenesis and isolation of mutants. Prior to mutagenesis, the respective fragments were cloned into phage M13. In preliminary experiments SHV-2a, originally encoded on the 3.6-kb *Bam*HI fragment in pMPA-2a, was subjected to site-directed mutagenesis, replacing glutamine at position 31 with leucine. This resulted in SHV-2 being downstream of the SHV-2a promoter. The promoter was designated PA. Together with the equivalent SHV-2 originally located on plasmid pMPB-2 but under the influence of a weaker promoter (PB), the foundation for direct comparative studies was established. The introduction of point mutations into each of these fragments resulted in the β-lactamases SHV-1, SHV-3, and SHV-5 with the amino acid substitutions of serine (234) → glycine, arginine (201) → leucine, and glutamic acid (235) → lysine, respectively. Furthermore, SHV-2 encoded on the fragment with the weaker promoter PB was mutated to SHV-2a by replacing leucine for glutamine at position 31, enabling the examination of this particular point mutation, previously described as having no influence on resistance levels of the host (27). Mutated genes were confirmed by sequencing, beginning about 190 nucleotides upstream of the promoter and terminating approximately 150 nucleotides downstream of the stop codon. Point mutations had been introduced correctly, and no misincorporations of nucleotides in the sequenced areas were detected. Mutated 3.6-kb *Bam*HI-fragments were cloned back into pTZ18R and transformed into DH5α for expression. The resulting clones containing SHV-1, SHV-2, SHV-3, and SHV-5 with the strong promoter were designated MPA-1, MPA-2, MPA-3, and MPA-5, respectively. Mutants harboring the genes for SHV-1, SHV-2a, SHV-3, and SHV-5 downstream of a weaker promoter were designated MPB-1, MPB-2a, MPB-3, and MPB-5, respectively. Studies on the genes SHV-2a with the strong promoter and SHV-2 with a weak promoter were done with the original clones MPA-2a and MPB-2. Restriction endonuclease analysis (data not shown) guaranteed that only clones carrying the engineered 3.6-kb *Bam*HI fragments in the same orientation within the vector were selected for subsequent phenotypic studies. This ensured that expression of the 10 individual promoter-bla_{SHV} constructs occurred under perfectly isogenic conditions.

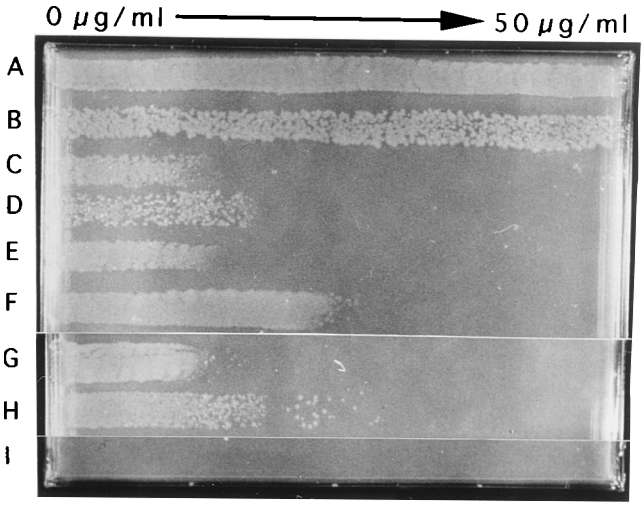

FIG. 1. Growth of extended-spectrum SHV-producing strains and control on agar plates containing a gradient of ceftazidime from 0 to 50 μg/ml. Row A, MPB-5; row B, MPA-5; row C, MPB-3; row D, MPA-3; row E, MPB-2a; row F, MPA-2a; row G, MPB-2; row H, MPA-2; and row I, DH5αptz.

Phenotypical effects of SHV β-lactamases. E tests were performed to obtain a resistance pattern for each individual mutant. The results are summarized in Table 2. Of all the tested extended-spectrum β-lactamases, SHV-5 proved to be the most efficient, followed by SHV-2 and its variant SHV-2a, while mutants with SHV-3 were generally slightly less resistant than those harboring SHV-2, with the exception of cefotaxime, which did not inhibit at a concentration of >256 μg/ml. In the cases of SHV-2 and SHV-2a, two particular details are noteworthy. Mutants harboring SHV-2a with the stronger promoter showed higher resistance levels than mutants carrying SHV-2 with the equivalent promoter. On the other hand, under the control of the weaker promoter, SHV-2 conferred higher resistance levels compared with those of SHV-2a, with the exception of ceftazidime. MICs were even increased for MPA-1 by the promoter PA, especially for ceftazidime whose MIC of 2 μg/ml was equivalent to the value reached by MPB-3. In general, the promoter PA caused higher MICs for all the mutants compared with those caused by the promoter PB, in the case of MPA-2a increasing the MIC of cefotaxime from 16 μg/ml to >256 μg/ml.

Growth on antibiotic gradient plates containing ceftazidime, ceftriaxone, cefotaxime, and cefepime at various concentrations, depending on the level of resistance to be tested, was evaluated. As an example, Fig. 1 shows growth of extended-spectrum β-lactamase-producing mutants on ceftazidime gradient plates. The results are shown in Table 3. In all experiments, strains DH5α and DH5α containing the vector pTZ18R (DH5αptz) were used as controls. Growth in individual lanes was shown not to be influenced by neighboring strains' possible diffusion of β-lactamase into the medium (data not shown). The results were in good agreement with those of the E test (Tables 2 and 3). In all cases, resistance was augmented by the presence of the promoter PA, increasing the length of lanes minimally by 3.6% for the SHV-2-containing strain growing on plates containing ceftriaxone and maximally by 109% for the strain harboring SHV-3 on plates containing cefotaxime. The highest increase in growth caused by promoter PA was observed on cefotaxime plates, except for the strain expressing SHV-1. Growth length was increased by 38% for the SHV-2-harboring mutant, by 82% for the strain carrying SHV-2a, by

TABLE 3. Growth of strains on antibiotic gradient plates

Strain	Enzyme	Promoter	Growth (cm) ± SD of strain with[a]:			
			CAZ	CRO	CTX	PM
DH5α			1.4[b] ± 0.1	1.5[c] ± 0.1	2.6[d] ± 0.3	2.2[c] ± 0.3
DH5αptz		Vector	1.6[b] ± 0.1	1.5[c] ± 0.3	2.6[d] ± 0.1	3.1[c] ± 0.2
MPA-1	SHV-1	Strong	5.0[b] ± 0.2	3.7[c] ± 0.1	3.2[d] ± 0.2	6.0[c] ± 0.4
MPB-1	SHV-1	Weak	4.4[b] ± 0.1	3.3[c] ± 0.1	2.9[d] ± 0.3	4.8[c] ± 0.2
MPA-2	SHV-2	Strong	4.0[e] ± 0.1	5.7[e] ± 0.2	4.7[f] ± 0.3	4.9[g] ± 0.1
MPB-2	SHV-2	Weak	3.0[e] ± 0.2	5.5[e] ± 0.1	3.4[f] ± 0.3	4.2[g] ± 0.1
MPA-2a	SHV-2a	Strong	6.6[e] ± 0.3	7.3[e] ± 0.2	5.1[f] ± 0.2	5.0[g] ± 0.1
MPB-2a	SHV-2a	Weak	3.8[e] ± 0.4	4.5[e] ± 0.5	2.5[f] ± 0.4	3.4[g] ± 0.1
MPA-3	SHV-3	Strong	4.5[e] ± 0.3	5.0[e] ± 0.2	4.4[f] ± 0.3	4.7[g] ± 0.1
MPB-3	SHV-3	Weak	3.2[e] ± 0.4	4.0[e] ± 0.3	2.1[f] ± 0.5	3.0[g] ± 0.1
MPA-5	SHV-5	Strong	7.9[f] ± 0.4	4.8[f] ± 0.2	4.5[f] ± 0.2	6.1[g] ± 0.5
MPB-5	SHV-5	Weak	6.6[f] ± 0.2	3.7[f] ± 0.2	2.6[f] ± 0.2	4.1[g] ± 0.3

[a] Mean value of three independent determinations. Abbreviations are as in note b of Table 1.
[b] The plates contained antibiotic gradients from 0 to 5 μg/ml.
[c] The plates contained antibiotic gradients from 0 to 1 μg/ml.
[d] The plates contained antibiotic gradients from 0 to 0.5 μg/ml.
[e] The plates contained antibiotic gradients from 0 to 50 μg/ml.
[f] The plates contained antibiotic gradients from 0 to 200 μg/ml.
[g] The plates contained antibiotic gradients from 0 to 20 μg/ml.

109% for the SHV-3-producing strain, and by 73% for the strain harboring SHV-5. The strain harboring SHV-3 grew better on plates containing ceftazidime compared with the SHV-2-producing strain, the length of the lane being extended by 12% with the stronger promoter and by 13% with the weaker promoter. On all other plates, the strain expressing SHV-2 grew better than the strain carrying SHV-3.

A comparison of SHV-2a and SHV-2 revealed that, with both genes under the control of the weaker promoter, SHV-2a conferred lower resistance to ceftriaxone, cefotaxime, and cefepime, although it was more efficient than SHV-2 in combination with the stronger promoter. Resistance to ceftazidime, however, was always higher for the strain carrying SHV-2a than for the strain expressing SHV-2, regardless of the strength of the promoter.

MICs of the cephalosporins tended to be slightly higher for the strain expressing SHV-1, a broad-spectrum β-lactamase, in the presence of PA compared with PB.

DISCUSSION

Resistance conferred by SHV-2a, an SHV enzyme with a point mutation at an unusual position, has been described as being due solely to the strong promoter situated upstream from the gene (26). This promoter, comprising 5′-TTGCAA-3′ (−35 box) and 5′-TATTCT-3′ (−10 box), shows higher homology to the *E. coli* promoter consensus sequences described by Hawley and McClure (9) than does the promoter belonging to SHV-2, containing 5′-TTGATT-3′ and 5′-AAAAAT-3′ (26).

Since strains harboring SHV-2a were isolated in three locations, Aachen (27), Zürich (see above), and Lausanne, Switzerland (22a), we wanted to develop a perfectly isogenic system for phenotypic comparison of this particular mutation with SHV-2 under the control of either promoter. The second aim was to evaluate the effect of other known SHV mutations on resistance under the same conditions. The system was derived by (i) cloning wild-type SHV-2 (16) and SHV-2a (KPZU-3, see above) with their original promoters, and (ii) the exchange of the mutations in the structural genes by site-directed mutagenesis. The two derivatives containing SHV-2 were then modified by site-directed mutagenesis to contain SHV-1, SHV-3, and SHV-5. Resistance phenotypes were determined with E test strips because they allowed smaller differences to be detected than traditional MIC tests. Furthermore, data from E test strips correlated well with data from MIC tests, with the exception of data for cefepime, for which E test strips yielded much lower MICs (Table 2). Good correlation was observed between E test results and growth on antibiotic gradient plates, the latter test being the best for a direct comparison of strains. The results show that glutamic acid (235) → lysine in SHV-5 gives rise to the highest resistance levels. This amino acid is situated immediately adjacent to the serine at position 234 which is thought to be involved with the binding of oxyimino cephalosporins (17). As opposed to glutamic acid, lysine carries a basic side chain. This major change may well have a catalytic and/or sterical influence on the active site.

SHV-3, although reported to differ from SHV-2 only with respect to the isoelectric point (12, 22), did show minor phenotypical differences. Interestingly, the mutation arginine (201) → leucine in SHV-3 increased resistance to ceftazidime while decreasing resistance to ceftriaxone, cefotaxime, and cefepime. The same pattern, though more distinct, is seen in SHV-2a compared with SHV-2, when the genes are controlled by the weak promoter. This means that both SHV-2a and SHV-3 are better adapted to ceftazidime than is SHV-2. Moreover, the two enzymes have another feature in common. In SHV-2a the mutation leucine (31) → glutamine, located on the protruding NH_2 terminus (10, 18), is far from the active site, as is arginine (201) → leucine in SHV-3. As opposed to SHV-5, SHV-2a and SHV-3 are therefore suggested to adopt a conformation different from that of SHV-2, thus explaining the altered phenotype.

Increased resistance has been ascribed to hyperproduction of β-lactamase (24, 26). That this can be an effect of altered promoter structures is clearly demonstrated in all our derivatives. We conclude that the phenotypical impact of certain point mutations in SHV enzymes is easily characterized in our isogenic system. For the detection of minor differences the antibiotic gradient plate technique is most sensitive and reproducible. The system allows the detection of augmented resistance to specific drugs, a phenomenon that has already been reported in an elegant study on TEM enzymes (23).

Our system provides the potential for testing any known or newly occurring point mutation in SHV genes found in clinical isolates. It could also prove useful for the screening of new drugs for their activity against different extended-spectrum β-lactamase-producing strains.

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