Molecular characterization of a new plasmid-encoded SHV-type β -lactamase (SHV-2 variant) conferring high-level cefotaxime resistance upon *Klebsiella pneumoniae*

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Between 1986 and 1988, multiresistant *Klebsiella pneumoniae* strains exhibiting high-level cefotaxime resistance were isolated from patient specimens particularly of the intensive care units of the Aachen Technical University Hospital. The resistance gene responsible was shown to be encoded on a conjugative 66 kb plasmid designated pZMP1. The MIC values for cefotaxime of the original isolates and the transconjugants were $> 128 \text{ mg l}^{-1}$ and 64 mg l^{-1} , respectively. Isoelectric focusing of protein preparations from the transconjugants showed a β -lactamase with a pI of 7.6. A 3.6 kb *Bam*HI fragment containing the β -lactamase gene was cloned into pLG339 resulting in the recombinant plasmid pZMP1-1. A restriction map of the cloned insert was established and *Pst*I subfragments of the insert were further subcloned into pBGS18. The nucleotide sequence of the complete 3.6 kb fragment was determined. Within 3663 bp an open reading frame of 858 kb was found to show 99% homology to the SHV-2 and -3 nucleotide sequences. The deduced amino acid sequence differed in one and two positions, respectively, from these established SHV enzymes. The 3' noncoding sequence exhibited nearly perfect homology to that of SHV-2, but the 5' upstream sequence showed homology of less than 50% to the corresponding SHV-2 sequence, indicating an altered promoter region of the variant SHV-enzyme. Kinetic analysis of the β -lactamase revealed a 50–100% elevated hydrolytic effectivity on cefotaxime in comparison to other SHV enzymes. Cefoxitin, ceftazidime, aztreonam and imipenem were not hydrolysed by the enzyme. The variant enzyme was inhibited by commonly available β -lactamase inhibitors. Clavulanic acid had the highest affinity for the enzyme and the greatest effectivity in blocking its action. Based on the genetic and kinetic data we propose to classify the enzyme as a new variant β -lactamase of the SHV-type and name it SHV-2a.

Introduction

In general *Klebsiella pneumoniae* is only moderately susceptible to penicillins as a result of a chromosomally encoded β -lactamase (Labia *et al.*, 1979). In addition, strains sometimes contain plasmid-encoded β -lactamases of the SHV type, and less often of the TEM type (Roy *et al.*, 1985).

β -Lactamase SHV-1, first detected by Pitton (1972) and designated as such by Matthew *et al.* (1979),

broadens the resistance of its producer to first-generation cephalosporins. Since the first report (Knothe *et al.*, 1983), the emergence of resistance of *Klebsiella oxytoca* and *K. pneumoniae* isolates to third-generation cephalosporins, namely cefotaxime (CTX) and ceftazidime (CTZ), have been described with increasing frequency [Kliebe *et al.*, 1985 (SHV-2); Jarlier *et al.*, 1988 (SHV-3); Buré *et al.*, 1988 (SHV-4); Gutmann *et al.*, 1989 (SHV-5)]; in Germany these isolates remained rather scarce (Podschun *et al.* 1986). The minimum inhibitory concentrations (MICs) in mg l^{-1} of CTX for the original isolates are as follows: SHV-1, 0.03; SHV-2 and SHV-3, 4; SHV-4 and SHV-5, 8; those of CTZ are: SHV-1, 0.13; SHV-2 and SHV-3, 4; SHV-4 and SHV-5, 64. Mutations of the original SHV-1 enzyme were shown to be the reason for the development of increasing resistance (Barthélémy *et al.* 1988b). Recently the nucleotide sequences of the

Abbreviations: CTX, cefotaxime; CTZ, ceftazidime; IEF, isoelectric focusing; ORF, open reading frame; pHMB, *p*-hydroxy-mercuribenzoate.

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number X53817.

genes of SHV-3 (Nicolas *et al.*, 1989), SHV-2 (Podbielski & Melzer, 1990), an SHV-2 variant (Garbarg-Chenon *et al.*, 1990), SHV-1 (Mercier & Levesque, 1990) and OHIO-1 (Shlaes *et al.*, 1990) were established. Here we characterize another plasmid-encoded β -lactamase and the corresponding gene conferring unusually high-level CTX resistance to its *K. pneumoniae* host (MIC > 128 mg l⁻¹). It first appeared in Aachen University Hospital in 1986, reached its highest prevalence in 1987 and slowly disappeared in 1988, perhaps due to a change of antibiotic regimens in the intensive-care units (Podbielski *et al.*, 1988, 1990).

Methods

Bacterial strains and growth conditions. Nineteen cefotaxime-resistant (CTX^R) *K. pneumoniae* strains were isolated from blood cultures, tracheal aspirates, wound and urine specimens collected predominantly in the intensive care units of our hospital from spring 1987 to summer 1988. Identification to species level was performed by using conventional techniques and API 20 E (api bioMérieux), determination of clusters differing in biochemical markers by using API 50 CHE.

For conjugation experiments a nalidixic-acid-resistant *Escherichia coli* mutant, W3110 (Kliebe *et al.*, 1985), was used, and for transformation experiments an *E. coli* HB 101 derivative, DH5 α (Gibco BRL). Strains were passaged on DST agar (Oxoid) containing 10 mg CTX l⁻¹ and 20 mg tobramycin l⁻¹ in the case of CTX^R *K. pneumoniae* wild-types and 4–20 mg CTX l⁻¹ in the case of transformants or transconjugants. Cultures used for protein preparations for β -lactamase assays were grown in Iso-sensitest broth (Oxoid).

Selection for recombinant clones was performed on H-agar (Boehringer Mannheim) containing X-Gal and ITPG and 4 mg CTX l⁻¹ or 30 mg kanamycin l⁻¹, respectively.

Disk sensitivity testing, MIC determination and antibiotics. Conventional disk sensitivity testing was done on Mueller–Hinton agar (Oxoid) according to DIN standards using antibiotic disks supplied by Becton–Dickinson. MICs were determined at least in triplicate by a microdilution method according to the NCCLS standard methods (Podbielski *et al.*, 1989) and additionally by the use of the Sensititre system (Radiometer). Restoration of susceptibility by use of β -lactamase inhibitors was screened according to Gutmann *et al.* (1989). Antimicrobial agents were kindly provided as follows. Benzylpenicillin, ampicillin, cefotiam, cefmenoxime and doxycycline were from Grünenthal; cefotaxime (CTX), desacetylcefotaxime and cefpirome were from Hoechst; ceftazidime (CTZ) was from Glaxo; mezlocillin and ciprofloxacin were from Bayer; ceftriaxone and sulfamethoxazole-trimethoprim were from Hoffmann La Roche; moxalactam and tobramycin were from Lilly; cefoxitin and imipenem were from MSD; cefotetan was from ICI; aztreonam was from Squibb; gentamicin was from E. Merck; cephaloridine was from Sigma; ceftazolin was from Boehringer Mannheim; clavulanic acid was from Beecham; sulbactam was from Pfizer; tazobactam was from Taiho-Lederle.

β -Lactamase assays. β -Lactamases were prepared from clinical isolates, transconjugants and transformants after complete disruption of cells by sonication (Vibra Cell VC 300, Sonics & Materials), filtration of cell lysates through 0.22 μ m pore size filters and overnight dialysis against 10 mM-NaCl at 4 °C. Protein content of the crude extracts was determined with BCA reagent (Pierce) according to the instructions of the manufacturer.

Analytical isoelectric focusing (IEF) was performed by the method

of Matthew *et al.* (1975) using TEM-1, TEM-2, PSE-1, PSE-2, OXA-1, SHV-1 and *Enterobacter cloacae* P99 chromosomal β -lactamase as pl standards (Kliebe *et al.*, 1985) and nitrocefin for visualization.

Determination of specific CTX hydrolysis by single bands from IEF was carried out by cutting slabs containing these bands from the gel, placing them as a whole into the cuvette containing phosphate-buffered saline pH 7.2 and 75 μ M-CTX and performing the assays as described below.

Kinetic analysis of β -lactamases were performed at least in duplicate according to the recommendations of Bush & Sykes (1986) using 10, 20, 50, 100 and 200 μ M-substrate dissolved in phosphate-buffered saline of pH 7.2 monitored up to 30 min by a temperature-controlled (37 °C) Beckman DU-7 spectrophotometer. In the case of the cephalosporins appropriate wavelengths for monitoring enzyme activity were according to Seeborg *et al.* (1983); for desacetyl-CTX (264 nm), cefpirome (262 nm) and imipenem (292 nm) according to the manufacturer's information. In the case of penicillins the microacidimetric method of Ross & O'Callaghan (1975) was used. In all cases an aliquot of the crude enzyme extract exhibiting a specific activity of 0.5 nmol cephaloridine min⁻¹ (μ g protein)⁻¹ was placed into the assay; the total assay volume was always 800 μ l.

Apparent saturation velocity (*V*) and *K_m* values were calculated by using the Lineweaver–Burk transformation and linear regression of the data, rejecting sets of measurements with *r* values < 0.98. Apparent *K_i* values were calculated by the method of Waley (1982) using cephaloridine as a substrate at concentrations mentioned above. *I*₅₀ values were determined by nonlinear regression with 200 μ M-cephaloridine as a substrate. All inhibition experiments were performed with 5 min preincubation time for the inhibitor.

Inhibition by *p*-hydroxymercuribenzoate (pHMB, Sigma) was tested with 0.5 mM-pHMB in the assay, with 10 min preincubation and spectrophotometric measurement of hydrolysis of benzylpenicillin and cephaloridine each at a concentration of 250 μ M.

Inhibition by 0.1 and 1 mM-EDTA was monitored as for inhibition by pHMB.

Nucleic acid techniques. Plasmid DNA was extracted according to Birnboim (1983). For determining plasmid patterns, DNA restriction enzyme analysis by 0.7–1.2% agarose gel electrophoresis was used; for Southern blotting onto nylon membranes conventional techniques (Sambrook *et al.*, 1989) were applied. Restriction enzymes were supplied by Boehringer Mannheim, 'DNA' agarose Seakem by Biozyme and Biohyne B nylon membranes by Pall. DNA hybridization (68 °C, 6 $\times$ SSC) and non-radioactive detection were performed according to the instructions of the manufacturer of Chemprobe (Biozyme). Cloning experiments with pLG339 (Stoker *et al.*, 1982) and pBGS18 (Spratt *et al.*, 1986) as vectors were carried out as described by Walker (1984), using the modified Kushner method (Hanahan, 1983) for transformation. Restriction endonuclease sites were determined by single and double digests and in case of *Pst*I by time-dependent partial digestion using the 3.6 kb *Bam*HI fragment labelled with [γ -³²P]ATP (Amersham) at one 5' end (Brooks, 1987). Double-stranded plasmid DNA for sequencing was purified as above, followed by ethidium bromide/caesium chloride density-gradient centrifugation in a Beckman TLV 100K rotor for 3 h at 100000 r.p.m. DNA sequencing was performed by the dideoxy-chain-termination method (Sanger *et al.*, 1977) using the Pharmacia T₇ sequencing kit and [α -³⁵S]dATP (Amersham) for labelling. Sequencing primers were synthesized on a Beckman 200 A DNA synthesizer. DNA sequences were analysed by the use of the PC-GENE program (Genofit/IntelliGenetics).

pBP60-1-2, a recombinant plasmid of pBGS8 and two adjacent 2.35 kb *Pst*I fragments from pBP60 encoding the SHV-2 gene, and pBP60, the natural plasmid from *Klebsiella ozaenae* 2180 containing the SHV-2 gene were kindly provided by C. Kliebe-Frisch, Institute of Medical Microbiology, University of Bonn, FRG.

Results

Disk susceptibility testing, biochemical properties and plasmid pattern

Our attention was drawn to the CTX^R *K. pneumoniae* isolates by the uniform resistance pattern they showed in agar diffusion tests. The values of the disk susceptibility testing were in agreement with the MIC values shown in Table 1. The common antibiotic resistance pattern of these strains was paralleled by the results of biotyping. They all exhibited a homogeneous API 20 E code (5215773) and, with the exception of one isolate for tagatose, a uniform carbohydrate fermentation pattern when tested by the API 50 CHE biochemical test system.

All the CTX^R *K. pneumoniae* isolates possessed a 2.6 kb and a 66 kb plasmid, some additionally contained a plasmid of 4.4 or 4.8 kb, and occasionally another one larger than 50 kb. The small plasmids of 2.6 and 4.4 kb were at least partially homologous, since they hybridized to each other (data not shown). Both had one restriction site for *Bam*HI and none for *Eco*RI, *Hind*III or *Pst*I.

These small plasmids could be mobilized in transconjugation experiments and appeared to be cryptic in their natural host, whereas transformation into *E. coli* DH5 α led to expression of a β -lactamase of pI 4.8. The 66 kb plasmid, which we call pZMP1, is conjugative, and after conjugation or transformation it conferred resistance to cefotaxime, but not to doxycycline and tobramycin, as monitored by MIC determination (see below). Its restriction endonuclease pattern for *Bam*HI, *Eco*RI, *Hind*III and *Pst*I differs completely from that of pBP60, which encodes the SHV-2 β -lactamase (data not shown), but is identical for all of the CTX^R *Klebsiella* isolates tested.

β -Lactamase patterns

All the CTX^R *K. pneumoniae* isolates showed a β -lactamase of pI 7.6 in IEF; 13 isolates showed an additional one of pI 5.9.

Only the enzyme of pI 7.6, and not that of pI 5.9, hydrolysed CTX when using the corresponding gel slab

Table 1. MICs for selected CTX^R *K. pneumoniae* isolates, and for *E. coli* transformants, transconjugants and recipients of conjugation

Antibiotic	MIC (mg l ⁻¹) of antibiotics for*:				
	Kpr 14	Kj 14	Iso 1	DH5 α	W3110NaI ^R
Ampicillin	>128	>128	>128	2	1
Mezlocillin	>128	>128	>128	0.5	1
Piperacillin	>64	>64	>64	0.25	1
Cefazolin	>128	>128	>128	2	2
Cefotiam	>128	>64	32	1	0.5
Cefoxitin	32	4	2	1	1
Cefotetan	2	2	1	0.5	0.5
Moxalactam	4	2	1	0.25	0.25
Cefotaxime (CTX)	>128	32	64	0.25	0.25
Desacetylcefotaxime	>128	64	128	0.5	0.5
Ceftazidime (CTZ)	64	16	8	0.25	0.25
Ceftriaxone	>128	16	16	0.25	0.25
Cefmenoxime	>128	16	16	0.25	1
Cefpirome	32	4	8	0.125	0.125
Aztreonam	16	0.5	2	0.25	0.25
Imipenem	1	0.5	0.25	0.125	0.25
Gentamicin	2	0.5	1	0.25	0.5
Tobramycin	>128	4	1	0.25	0.25
Ciprofloxacin	0.25	0.25	0.06	0.06	0.125
Doxycyclin	>128	2	0.5	0.5	1
Trimethoprim/ sulfamethoxazole	3.2/ 60.8	0.8/ 15.2	0.025/ 0.48	0.025/ 0.48	0.025/ 0.48

* Kpr 14, CTX^R *K. pneumoniae* isolate; Kj 14, transconjugant harbouring original plasmid pZMP1 from Kpr 14; Iso 1, transformant harbouring recombinant plasmid pZMP1-1-1; DH5 α , *E. coli* recipient of transformation; W3110NaI^R, *E. coli* recipient of conjugation.

Fig. 1. Analytical IEF of β -lactamases produced by CTX^R *K. pneumoniae* isolates from clinical specimens and recipients of transformants. Lane 1, pI reference β -lactamases (from bottom to top): TEM-1 (pI 5.4), TEM-2 (pI 5.6), OXA-1 (pI 7.4), SHV-1 (pI 7.6), *Enterobacter cloacae* P99 chromosomal β -lactamase (pI 8.8); lanes 2 to 5, CTX^R *K. pneumoniae* isolates (Kpr 12, Kpr 14, Kpr 18, Kpr 9). Lane 6, *E. coli* DH5 α transformant harbouring natural plasmid pZMP1 (which encodes the new SHV-type β -lactamase) from Kpr 12 = tf 12 (the other transformants displayed identical bands, not shown). Lane 7, *E. coli* DH5 α , recipient of transformation; lane 8, *E. coli* DH5 α harbouring recombinant plasmid pZMP1-1-1 (containing a 2.2 kb cluster of adjacent *Pst*I fragments of pZMP1-1)

which was excised from the IEF gel and tested in a photometric β -lactamase assay.

All transconjugants and transformants, irrespective of whether they had been transformed with the original plasmid pZMP1 or a recombinant derivative, possessed only the β -lactamase of pI 7.6 (Fig. 1).

The expression of the pI 5.9 β -lactamase could be transferred only by conjugation. The transconjugants remained free of plasmids; thus this enzyme is presumed to be chromosomally encoded. It hydrolysed penicillin and ampicillin fairly well, cephaloridine only slowly ($K_m > 1000 \mu\text{M}$) and CTX and CTZ not at all when crude lysates of the specific transconjugants were monitored. It was not investigated further.

Molecular cloning of the β -lactamase gene, restriction enzyme mapping and nucleotide sequencing

pZMP1 was digested with *Bam*HI, and the resulting fragments were cloned into the *Bam*HI site of pLG339. The recombinant plasmids were used to transform *E. coli* DH5 α with selection by CTX and tetracycline. Only one fragment of 3.6 kb encoded the β -lactamase.

A restriction map of the cloned 3.6 kb *Bam*HI fragment was established for *Eco*RV, *Mra*I, *Pst*I, *Sal*I and *Stu*I (Fig. 2). *Bgl*II, *Eco*RI, *Hind*III, *Hpa*I, *Sac*I, *Sca*I, *Sma*I, *Sph*I, *Xba*I and *Xho*I did not cut within the 3.6 kb fragment.

Further subcloning of the partially or completely *Pst*I-digested 3.6 kb *Bam*HI fragment into pBGS18 led to CTX^R clones when the three adjacent *Pst*I fragments of

Fig. 2. Restriction endonuclease map of the 3.6 kb *Bam*HI-fragment encoding the new SHV-type β -lactamase and sequencing strategy exerted on it. Restriction sites are as follows: B, *Bam*HI; E, *Eco*RV; M, *Mra*I; P, *Pst*I; Sa, *Sal*I; St, *Stu*I. Numbers mark nucleotide positions starting from the 5' end of the fragment. Arrows indicate the direction and length of individual sequencing assays. Horizontal bars above the map represent the extent and corresponding position of the nucleotide sequences of the LEN-1, SHV-2, SHV-3 genes and SHV β -lactamase structural gene.

GGATCCAGGC GCAGTATGCG GACAAGGCCA GCAGCGAAGC GGTGGAACAG CTGTTTGGCC CTCTTGGCCG CGCCCTGAAG CGCAACGGAT TTACCCGCTT TATTGTGGCC GGAGGGGAAA 120
 CGTCGAGCAT TGTGGCGCAG ACCCTGGGGG TTGAGGCGTT CCATATTGGG CGACCATCT CCCTTGGCGT GCCCTGGGTG CGTGACACCC GCCAGCCGCT CTCCCTGGCG CTGAAGTCAG 240
 GTAACCTCGG CGATATCCAG TTCTTTGGCC GTGCCAGCA GGAATTTGCT CATGACTGAG CAACAACATGC GAGAGGAAAT GGTGCAGATT GGCCTGCTGT TGTTTAGCCG CGGCTATGCC 360
 ACCGGCTCCG TTGGCAATCT GTGCTGCTGT CTGCCGAGC GCAACTGCTT GGGCAGCCCG ACCGCGCCCT CCCTCGGCCA ACTGCAGGCT CAGCGTTGTG CGGTGGTGAC CTTGCAAGGG 480
 GAATGATCT CGGCGACAA ACCGTCGAAA GAGGTCACTT TTCACCGGGC GGTCTATTGT CACAACCCGG CCTGCAAGGC GATCGGTCCA CTGTCACAGC CACTATCTGA CCGCGCTCTC 600
 CTGCTGCGAG GGGCTTCGAT CCGCACAAC GGGCAGCTGT TGCAGAGTTA GCGATGAGGC AGCCTTTTGT CTTATTCAAA GGCCTTACAT TTCAAAAACCT CTGCTTACCA GGGCGATTTC 720
 GCCCAGGGGA TCACCATAAT AAAATGCTGA GGGCTGGCCT TTGCTAGTGC CACGCATCAC CTCAATACCT TTGATGGTGG CGTAAGCCGT CTTCTAGGAT TTAAATCCCA CGGTGGCGCC 840
 GATTATCCGT TTCAGTTTGC CATGATCGCA TTCAATCAGC TTGTTCCGGT ACTTAATCTG TCGGTGTTCA ACCTCAGACG GGCACCGGCC TTCCGCTTTG AGCAGAGCAA GCGCGCGACC 960
 ATAGCGGGC GCTTTATCCG TGTGGATGA ATCGCGGGAT CTGCCACTTC TTCACGTTGT TGAGGATTTT ACCCAGAAAC CGGTATGCGC CTTTGTCTGT ACACGGGGAG GAGAGATAAA 1080
 AATCGACAGT GCGGCCCCGG CTGTGCGAGC CCGGCTACAG ATACGCCAG CGGCCATTGA CTTTACAGTA GGTTCATCC ATGTGCCACG GGCAGAGATC GGAAGGGTTA CGCCAGTACC 1200
 AGCGCAGCCG TTTTTCATT TCAGCGCAT AACGCTGAAC CCAGCGGTAA ATCGTGGAGT GATCGACATT CACTCCGCGT TCAGCCAGCA TCTCCTGCAG CTCACGGTAA CTGATGCCGT 1320
 ATTTGCGTA CCAGCGTACG GCCACAGAA TGATGTCAGC CTGAAAATGC CGGCCCTTGA ATGGGTTTAT GTGCAGCTCC ATCAGCAAAA GGGGATGATA AGTTTATCAC CACCGACTAT 1440
 TTGCAACAGT GCCAACCGCG GGTATTCTT ATTTGTCGCT TCTTTACTCG CCTTTATCGG CCTTCACTCA AGGATGTATT GTGGTTATGC GTTATATTCG CCGTGTGATT ATCTCCCTGT 1560
 TAGCCACCTT GCCCTGGCG GTACACGCCA GCCCGCAGCC GCTTGAGCAA ATTAACAAA GCGAAAGCCA GCTGTGGCG CGCTAGGCA TGATAGAAAT GAATCTGGCC AGCGGCCGCA 1680
 CGTGACCCG CTGGCGCGCC GATGAACGCT TTCCCATGAT GAGCACCTTT AAAGTAGTGC TCTGCGCGC AGTGTGCGCG CGGGTGATG CCGGTGACGA ACAGCTGGAG CGAAAGATCC 1800
 ACTATCGCCA GCAGGATCTG GTGACTACT CGCCGGTCAG CGAAAACAC CTTGCCGAGC GCATGACGGT CGGCGAAGTC TCGCCCGCCG CCATTACCAT GAGCGATAAC AGCGCCGCCA 1920
 ATCTGCTGCT GGCCACCGCT GCGGCCCCCG CAGGATTGAC TGCCCTTTTG CGCCAGATCG GCGCAACGT CACCCGCTT GACCGCTGGG AAACGGAAT GAATGAGCGC CTTCCCGCGC 2040
 ACGCCCGCGA CACCACTACC CCGGCCGACA TGCCCGCAAG GTCTGACCA CCGCATGCTC GAGCGCCGT TCAGCAACGGC AGCTGTGCA GTGGATGGTG GACGATCGGG 2160
 TCGCCCGACC GTTGATCCG TCGCTGCTGC CCGCGGGCTG GTTTATCGCC GATAAGACCG GAGCTAGCGC GCGGGGTGCG CCGCGGATTG TCGCCCTGCT TGGCCGAAT AACAAAGCAC 2280
 AGCGCATTTG GGTGATTAT CTGCGGATA CTGCGGCGAG CATGGCCGAG CGAAATCAGC AAATCGCCGG GATCGCGCG CGCTGTGCG AGCACTAAGC GCGGGTGGC 2400
 GCGCGCTGTA TCGGCGCCG AGCAGCTCGC AGCGGTGCGG GCGCATATGA CTGCGGCGCG CATCGGAAAG ATGCGCGTGC GTAATGATGG TGGTGAACCG GGTCAAAGGT AACGCCATAA 2520
 ACCTGCGCAC CTGATTGTAT TTGCAACTGT CCGACAGCAG GATGCTTCTC GCGCTGACCT GGCTGACGGT CTCCTTGACG GTAACTTTGT TCTCATCAGG GGTGAATATC CCGCGACTGT 2640
 CCGACCCCTT GCGCGAGATA AAGCGCGTAT CGATAGCCAG GTGGCGTAAC GTACGCGCGC CCGATTGCGC CACGAGGAG CGGTTCTCCC GGCACAGAGT GGCGCCGGTG TGGATCACGC 2760
 CGCACTGGCT GGCATCGATC AGCAGCTGGG TTATCTCAA ATCATTGGTG ACCACCTGGA GATCGTTCCG GTCGAGGATC GCCCGGCCA GCGCCAGGCT GGTAGTCCCG GCATCCAGAT 2880
 AGATGCAACT GTTTTATAGC ATATGACTCG CCGCCAGCGC GCGATGCGC TGTTTCTCT CACTCTGCGC CGTGCTTTTC ACCAGATGAC TGGGTTCCCG GGCAGCCGG CTGACGCGCG 3000
 GTACGCCGCC CGAGACGCTG ACAGCAGCCG CCGTCTCTC CAGTTTACTG ACCTGCGGAC GAGTGGTCT ATGGGACAG CCGAGGATCT CCGTCAGCTC GTTAATGCTT ACCGCCACG 3120
 CTGCTCCACC AGGGCTAAAA TAGCTGATG TCGTTCTATT GGAATCAGCG CTCTCCCTT ACCATTTTTT CACACAGCG GTCACCAACC AGGCTACGCG CCGTGGGACA CCGGGTGTG 3240
 TTATCGCGCT TTGCGCTGT TTTTACGCT ATTTTAGGGC AAGAATCGCC GCCTGTGCG CCTCTTTCCG CCGTGAATT TTTTATATC ATGTGGGTGA TCGGTGATA CTCTCACATA 3360
 TAATTCATG ATAACGCTTT ATCTATCAT TAATTAACAT TAATTAACAT GAGAACAGC ATGCTGACG ACTAATGCTT GGAATGCTT GGAATGCTT CAATGGAGAT 3480
 GGGCGCCGCC CCGCCTGCC TGGAGCGGG CCGTAACACC TGGGCGCTG ACATCAATCC CGCAACTGT CCGCGACTGC TGGCGCGGG CGCCAAAGCG GGGGCCCGAG CCGCGTGGC 3600
 TTCGCGCGG AACTGGATG AGTTGTGCTG CTGGTGTCA ATGCCGCCA GGTGCGGGG TCC

Fig. 3. Complete nucleotide sequence of the 3663 bp DNA fragment containing *bla*_{SHV-2a}. Sequences (partially) homologous to the -35 and -10 boxes of the *bla*_{LEN-1} promoter, Shine-Dalgarno sequence, start and stop codon and putative transcription termination sequences are underlined.

0.84, 0.81 and 0.53 kb were cloned as a continuous insert (pZMP1-1-1; MIC values shown in Table 1) and to CTX^S clones when the single fragments were cloned as independent inserts. Only the *Pst*I fragments of 0.84 and 0.81 kb hybridized to the cloned SHV-2 gene under stringent conditions (data not shown).

Nucleotide sequencing of the total 3.6 kb *Bam*HI fragment was accomplished on both strands. The exact size of the fragment is 3663 bp (Fig. 3). It contains six potential open reading frames (ORFs) according to a computerized Fickett analysis, of which the largest, of 858 bp, spans the major part of the 0.84 kb and a minor part of the 0.81 kb *Pst*I fragments mentioned above (Fig. 2). It starts at nucleotide no. 1527 of the total sequence, ends at nucleotide no. 2384 and shows 99% homology to the known nucleotide sequences of SHV-2 (Podbielski & Melzer, 1990) and SHV-3 (Nicolas *et al.*, 1989) with a difference in 3 and 6 positions, respectively. The nucleotide alterations cluster predominantly within the first 160 positions and the terminal half of the gene. The deduced amino acid sequence of our SHV-type enzyme differs in one and two of 286 positions, respectively, from SHV-2 and SHV-3. The differences in the nucleotide sequence and corresponding amino acid sequence in comparison to SHV-2 and SHV-3 are listed in Table 2. The calculated pI of the new SHV enzyme is exactly the same as that of SHV-2, which corresponds to the results of the IEF gel analysis. The sequence of the 3' noncoding

Table 2. Differences in nucleotide sequences and deduced amino acid sequences of the SHV-2, -2 variant, -3 and -2a β -lactamase genes

β -Lactamase*	Nucleotide position†:								
	92	153	402	602	672	705	759	762	786
SHV-2	T (L)	C	A	G (R)	C	G	C	T	C
SHV-2 variant	A (Q)	G	G	G (R)	T	G	C	T	G
SHV-3	T (L)	C	A	T (L)	C	A	G	C	G
SHV-2a	A (Q)	C	G	G (R)	C	G	C	T	G

* Data from the following sources: SHV-2, Podbielski & Melzer (1990); SHV-2 variant, Garbarg-Chenon *et al.* (1990); SHV-3, Nicolas *et al.* (1989); SHV-2a, this paper.

† Position numbers according to the coding sequence of SHV-3. Letters for nucleotide and amino acids (in brackets) are according to IUPAC Nomenclature.

region in comparison to the corresponding largest known sequence (SHV-2) exhibits only 4 exchanges in 566 nucleotides and no deletions or insertions; thus, the potential transcription termination sequences found for SHV-2 are conserved.

When comparing the 5' upstream sequence of the new β -lactamase gene to the corresponding SHV-2 sequence, 72 of 73 nucleotides immediately upstream from the start codon ATG are identical. This region comprises the Shine-Dalgarno sequence and a putative -10 box

Table 3. Minimum concentrations of β -lactamase inhibitors* (mg l⁻¹) necessary to restore susceptibility to arbitrary concentrations of antibiotics easily achieved as serum levels

Strain†	Ampicillin (8 mg l ⁻¹)			Mezlocillin (8 mg l ⁻¹)			CTX (1 mg l ⁻¹)			CTZ (1 mg l ⁻¹)			Cefpirome (1 mg l ⁻¹)		
	Cla	Sul	Taz	Cla	Sul	Taz	Cla	Sul	Taz	Cla	Sul	Taz	Cla	Sul	Taz
Kpr 14	8	128	>128	1	>128	128	0.5	16	16	0.125	32	16	0.25	16	16
Kj 14	8	16	8	2	4	2	1	8	2	4	4	4	2	4	2
Iso 1	2	16	8	4	8	4	4	8	4	2	8	4	4	8	4

* Cla, clavulanic acid; Sul, sulbactam; Taz, tazobactam.

† See Table 1.

Table 4. V , K_m and V/K_m values of SHV enzymes for selected antibiotics

1, V ($\mu\text{mol l}^{-1} \text{ min}^{-1}$); 2, K_m (μM); 3, above, V/K_m ; below, relative V/K_m (value for ampicillin arbitrarily defined as 100).

β -Lactamase	Penicillin			Ampicillin			Mezlocillin			Cephaloridine			Cefotiam			CTX			desacetylCTX			Cefpirome		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
SHV-2a*	33	17	1.9	47	28	1.7	30	18	1.7	100	30	3.2	58	66	1.2	7	26	0.2	2	45	0.05	20	151	0.14
	112			100			100			188			71					16			3			8
SHV-1†				42			45			201								l.b.a.						
SHV-2*	32	20	1.6	54	27	2.0	37	18	2.0	120	41	2.9	77	71	1.1	9	45	0.19	3	63	0.05	21	130	0.16
	80			100			100			145			55					10			3			8
SHV-5†				11			7			31							7							
				100			116			11							7							

* Data for SHV-2a were obtained from *E. coli* DH5 α transformant harbouring recombinant plasmid pZMP1-1-1 by using a crude protein extract with a specific activity of 0.5 nmol cephaloridine min⁻¹ ($\mu\text{g protein}$)⁻¹. Data for SHV-2 were obtained analogously by the use of an *E. coli* transformant containing pBP60-1-2.

† Data for SHV-1 and SHV-5 are from Gutmann *et al.* (1989). l.b.a., low binding affinity. Values which are not presented were not listed by the authors.

homologous to the promoter of LEN-I (Arakawa *et al.* 1986). Beyond this point the homology drops abruptly below 50%.

Another SHV-2 variant of *Salmonella typhimurium*, recently characterized by Garbarg-Chenon *et al.* (1990), differs in its structural gene from our enzyme in only two nucleotide positions, with no consequence upon the amino acid sequence, whereas the 5' and 3' noncoding regions are identical in 219 of 223 and 23 of 30 positions, respectively. On the basis of these pronounced similarities to the SHV-2 β -lactamases already characterized, our enzyme is considered to be an SHV-2 variant and designated 'SHV-2a'.

Using the five ORFs other than that of the SHV-2a gene as a base for a computer homology search and comparing them to the available sequences of transposases, resolvases and other genes encoded on bacterial transposons as well as to the most recent update of the EMBL databank (release 21), no homology on a minimum level of 60% could be found.

Determination of MICs, reaction to β -lactamase inhibitors and kinetic data

The MIC values for the CTX^R *K. pneumoniae* isolates (Table 1) reveal resistance or intermediate susceptibility to amino- and acylureidopenicillins, and to cephalosporins of the cephalothine, cefuroxime and the methoxyimino groups (with the exception of cefpirome). The isolates are also resistant to tobramycin and tetracyclines. They show susceptibility to the cephamycins, monobactams, carbapenems, quinolones and gentamicin. The MIC values, especially those of CTX and tobramycin, for a given isolate did not vary significantly when tested immediately after isolation and after 30 and 50 passages on selective medium, thus ruling out laboratory-induced enhancement of CTX resistance.

The transconjugants and transformants containing the original plasmid pZMP1 exhibited a twofold reduced resistance to the methoxyimino-cephalosporins compared to the original CTX^R *K. pneumoniae* strains, but no resistance to tetracyclines or tobramycin.

Each β -lactamase producing strain was susceptible to the action of commonly available β -lactamase inhibitors. The assay according to Gutmann *et al.* (1989) for restoration of susceptibility to arbitrary concentrations of antibiotics demonstrates (Table 3) that the clinical isolate Kpr 14 can tolerate distinctly higher concentrations of sulbactam and tazobactam but not clavulanic acid in comparison to the transconjugant Kj 14 harbouring pZMP1, and transformant Iso 1 harbouring pZMP1-1-1. Furthermore one can deduce that the β -lactamase is most susceptible to clavulanic acid whereas tazobactam

Table 5. Apparent K_i and I_{50} values of SHV enzymes for the β -lactamase inhibitors clavulanic acid (Cla), sulbactam (Sul) and tazobactam (Taz)

β -Lactamase	Apparent K_i (μ M)			I_{50} (μ M)		
	Cla	Sul	Taz	Cla	Sul	Taz
SHV-2a*	0.08	0.47	0.027	0.018	0.68	0.038
SHV-1†	0.19	1.70	0.057	0.057	7.50	0.150
SHV-2*	0.16	0.36	0.040	0.020	0.57	0.049
SHV-5†	0.10	0.18	0.036	0.005	0.40	0.022

* Data for SHV-2a were obtained from *E. coli* DH5 α transformant harbouring recombinant plasmid pZMP1-1-1 by using a crude protein extract with a specific activity of 0.5 nmol cephaloridine min⁻¹ (mg protein)⁻¹ and, for I_{50} determination, 200 μ M-cephaloridine as a substrate. Data for SHV-2 were obtained analogously by the use of an *E. coli* transformant containing pBP60-1-2.

† Data for SHV-1 and SHV-5 are from Gutmann *et al.* (1989).

and sulbactam are generally active at two- to fourfold higher concentrations.

The β -lactamase as expressed by the transconjugant containing the original plasmid was inhibited by pHMB when using benzylpenicillin as a substrate but was not inhibited with cephaloridine as substrate or with either substrate by EDTA at concentrations up to 1 mM. This indicates a lack of involvement of the thiol group normally present in the active site of serine β -lactamases (Dalbadie-McFarland *et al.*, 1986; Arakawa *et al.*, 1989) in the catalytic process, and also a Zn²⁺ independence of the enzyme activity as described for class A enzymes (Ambler, 1980).

The new SHV-type β -lactamase showed higher apparent V values (Table 4) for older cephalosporins than for penicillins or newer cephalosporins. Its substrate affinity as expressed by K_m values was in the same range for ampicillin, cephaloridine and CTX and compared to these substances less for the rest of the cephalosporins tested. The hydrolytic efficiency as expressed by V/K_m ratio was definitely lower for the newer cephalosporins, especially in comparison to that for ampicillin. There was no detectable hydrolytic activity for cefoxitin, CTZ, aztreonam and imipenem even when the reaction was monitored for 30 min at 272 and 260 nm in the case of the CTZ assay. For comparison, values of other SHV enzymes are listed in Table 4.

Analysis of the apparent K_i values revealed that the highest affinity of the enzyme was for clavulanic acid, followed by tazobactam and sulbactam (Table 5). The 50% inhibitory concentration (I_{50}) values were about 50 to 250% higher than the apparent K_i values but they paralleled the order of the apparent K_i values. A comparison with values of other SHV enzymes is presented in Table 5.

Discussion

The new transferable β -lactamase reported here is encoded by a 66 kb plasmid (pZMP1) and confers cefotaxime resistance upon its natural producer *K. pneumoniae* at a level higher than any reported before. Its substrate affinity for methoxyimino-cephalosporins but not cephamycins led to the assumption that it is another member of the emerging SHV β -lactamase family (Pitton, 1972; Kliebe *et al.*, 1985; Jarlier *et al.*, 1988; Buré *et al.*, 1988; Gutmann *et al.*, 1989); the hybridization and nucleotide sequence data presented above confirm this assumption. The small differences in the nucleotide sequence compared to the established SHV-2 sequence (Podbielski & Melzer, 1990) demonstrate that the new enzyme is a variant of this protein. As a consequence we propose to name the enzyme SHV-2a. Like the rapidly increasing family of TEM β -lactamases (Sougakoff *et al.*, 1988a; Mabilat & Courvalin, 1990) the individual members of the SHV β -lactamases differ in the level of resistance for different cephalosporins that they confer upon their producers.

SHV-2a confers high resistance to amino- and acylurido penicillins as well as to methoxyimino-cephalosporins but not to cephamycins, monobactams or carbapenems. The preference for methoxyimino-cephalosporins over cephamycins is explained by an amino acid substitution far from the active site of the enzyme leading to new specific side-chain affinities between enzyme and antibiotic (Labia *et al.*, 1988; Philippon *et al.*, 1989). Within the group of methoxyimino-cephalosporins the MICs decrease from cefotaxime (CTX) to ceftriaxone, cefmenoxime, ceftazidime (CTZ) and cefpirome, a distribution which resembles that of SHV-2 more than that of SHV-5. The MIC for the natural metabolite of CTX, desacetyl CTX, is slightly higher than that for CTX, a finding which appears to be common for *Klebsiella* enzymes (Selwyn & Bakhtiar, 1986).

Differences in membrane permeability between the original *K. pneumoniae* isolates and the *E. coli* transconjugation/transformation recipients probably account for the obvious differences in MIC values for CTX and for the different amounts of the β -lactamase inhibitors sulbactam and tazobactam necessary to restore susceptibility to β -lactams (Yoshimura & Nikaido, 1985). These differences cannot be attributed to the additional chromosomally encoded β -lactamase of pI 5.9 present in some of the *K. pneumoniae* isolates, since this enzyme does not hydrolyse CTX.

The superior efficacy of clavulanic acid compared to sulbactam and tazobactam for β -lactamase inhibition documented by the susceptibility restoration assay, especially in the case of the original *K. pneumoniae*

isolates, corresponds to general experience (Jacobs *et al.*, 1986; Kitzis *et al.*, 1988) that clavulanic acid has greater affinity for plasmid-encoded β -lactamases whereas sulbactam and, to a lesser extent, tazobactam bind more avidly to chromosomally encoded enzymes. Analysis of the kinetic data of SHV-2a in comparison to known SHV enzymes discloses an intermediate position of the enzyme concerning the affinity for different penicillins and cephalosporins. These K_m value and the apparent K_i value for sulbactam are generally lower than those for SHV-1 but higher than those for SHV-2 and SHV-5, respectively. The hydrolytic effectivity for CTX is only about 50–100% higher than that of the newer SHV enzymes, so again the much higher MIC of CTX for our *K. pneumoniae* isolates must be due to effects independent of the β -lactamase action. The MICs for cefoxitin, CTZ and aztreonam indicating intermediate susceptibility of our *K. pneumoniae* strains (paralleled by no detectable hydrolysis of these agents by the enzyme) may again be caused by a permeability defect especially since the transconjugants/transformants are fully susceptible to cefoxitin and aztreonam. The slightly elevated resistance to CTZ of the transconjugants/transformants harbouring only the SHV-2a β -lactamase determinant may be ascribed to a trapping phenomenon (Nayler, 1987) exerted on this substance. However, Garbarg-Chenon *et al.* (1990) measured a low basic hydrolytic activity of their SHV-2 variant enzyme for CTZ ($K_m > 150 \mu\text{M}$), so it is possible that our enzyme may also have this activity and we did not detect it due to too low a specific concentration of the enzyme in the crude extract or to (absorptive) interference of other substances contained therein.

The order of affinity for different inhibitors of the enzyme expressed by the apparent K_i values and of their effectivity upon the enzyme action expressed by the I_{50} values parallel those of other authors (Kitzis *et al.*, 1988; Gutmann *et al.*, 1989), showing again that the enzyme has highest affinity for clavulanic acid and is most effectively inhibited by this substance. The two- to fivefold differences between the apparent K_i and I_{50} values of clavulanic acid and tazobactam or sulbactam investigated by the use of protein extracts are in contrast to the more uniform results of the restoration of susceptibility testing, which were achieved by the use of whole bacteria. So the rate of penetration into the periplasmic space may rule out differences in binding affinities of inhibitors to their corresponding enzymes.

The nucleotide sequence of the SHV-2a gene and of the 3' noncoding region classify SHV-2a as a close relative of the plasmid-coded SHV-enzymes. SHV-2a protein differs from SHV-2 protein only in amino acid residue 10, Gln instead of Leu, and from SHV-3 protein additionally in residue 180, Arg instead of Leu (Table 2).

Fig. 4: Dendrogram of the nucleotide sequence relatedness of the structural genes of SHV-type β -lactamases. The horizontal line to LEN-1 represents 10% difference; the rest of the horizontal lines are in scale. Nucleotide sequences were taken from the following sources: LEN-1 (Arakawa *et al.*, 1986), OHIO-1 (Shlaes *et al.*, 1990), SHV-1 (Mercier & Levesque, 1990), SHV-2 (Podbielski & Melzer, 1990), SHV-2 variant (Garbarg-Chenon *et al.*, 1990), SHV-2a (this paper), SHV-3 (Nicolas *et al.*, 1989).

When the SHV-2a protein sequence is aligned with that of the *Staphylococcus aureus* β -lactamase, for which the crystal structure is known (Herzberg & Moulton, 1987; Sougakoff *et al.*, 1988a), the exchange at residue 10 is far from the pouch of the active site; that at residue 180 is nearer to it without directly contacting it. Since neither of the exchanged residues seem to participate in the formation of the active site or its close vicinity the moderately elevated hydrolytic effectivity of SHV-2a for CTX (50% more than SHV-2) may be only the result of experimental variation.

The four- to eightfold higher MIC values of transformants expressing SHV-2a compared to those of strains expressing SHV-2 (Kliebe *et al.*, 1985) may be explained by additional quantitative effects, such as enhanced transcription of the SHV-2a gene. Its 5' upstream sequence differs from all other known SHV nucleotide sequences except the SHV-2 variant of Garbarg-Chenon *et al.* (1990) starting 74 nucleotides upstream from the start codon, a site that is situated between the -10 and -35 box of the LEN-1 promoter which has been described by Arakawa *et al.* (1986) for a chromosomally encoded SHV-type enzyme and which is perfectly conserved in the SHV-2 and SHV-3 sequence. Thus, a mutated promoter region of the SHV-2a gene may partially account for the overall MIC value for CTX of bacteria carrying the complete SHV-2a sequence, a situation which would be comparable to the different TEM operons (Sougakoff *et al.*, 1988b).

Considering the phylogeny of plasmid-coded SHV enzymes in regard to the nucleotide sequences of the structural genes (Fig. 4), all these enzymes have an approximately equal distance from the chromosomally encoded LEN-1 enzyme (Arakawa *et al.*, 1986). All SHV genes have the additional G residue 27 nucleotides in front of their common stop codon TAA, rendering the plasmid-encoded enzymes 7 amino acids longer than their chromosomal counterpart. Within the SHV gene family there are three subgroups: (I) SHV-1, (II) SHV-2 and SHV-3 and (III) SHV-2a and SHV-2. The question of ancestral relationship remains unsolved, since we can give no hints about the way of distribution of the gene between the chromosome and obviously different carrier plasmids.

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