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 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 BamHI 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 PstI 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 SHV-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 effectiveness 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 effectiveness 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 (Barthéléméy et al. 1988b). Recently the nucleotide sequences of the

Abbreviations: CTX, cefotaxime; CTZ, ceftazidime; IEF, isoelectric focusing; ORF, open reading frame; pHMB, p-hydroxymercuribenzoate.
genes of SHV-3 (Nicolaïs 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 \( \beta \)-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\(^{-}\)) \( K. pneumoniae \) strains were isolated from blood cultures, tracheal aspirates, wound and urine specimens collected predomi-
nantly 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), determina-
tion 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\( \alpha \) (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\(^{-}\) \( K. pneumoniae \) wild-types and 4–20 mg CTX l\(^{-1} \) in the case of transconjugants or transconjugants. Cultures used for protein preparations for \( \beta \)-lactamase assays were grown in Iso-sensitest broth (Oxoid).

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

**Disk sensitivity testing, MIC determination and antibiotics.** Conven-
tional 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 \( \beta \)-lactamase inhibitors was screened according to Gutmann et al. (1989). Antimicrobial agents were kindly provided as follows. Benzylpenicil-
lin, ampicillin, cefotaxin, 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 sulfamethoxa-
zole-trimethoprim were from Hoffmann-La Roche; moxalactam and tobramycin were from Lilly; cefotaxin and imipenem were from MSD; cefotetan was from ICN; aztreonam was from Squibb; gentamicin was from E. Merck; cephalexin was from Sigma; cefazolin was from Boehringer Mannheim; clavulanic acid was from Beecham; sulbactam was from Pfizer; tazobactam was from Taibo-Lederle.

\( \beta \)-Lactamase assays. \( \beta \)-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 \( \mu \)m pore size filters and overnight dialysis against 10 mm-\( \text{NaCl} \) 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 \( \beta \)-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 \( \mu \)M-CTX and performing the assays as described below.

Kinetic analysis of \( \beta \)-lactamases were performed at least in duplicate according to the recommendations of Bush & Sykes (1986) using 10, 20, 50, 100 and 200 \( \mu \)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 Seeburg 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 microuicrometric 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 mmol cephaloridine min\(^{-1} \) (ug protein)\(^{-1} \) was placed into the assay; the total assay volume was always 800 \( \mu \)l.

Apparent saturation velocity (\( V \)) and \( K_s \) 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 \( K_s \) values were calculated by the method of Waley (1982) using cephaloridine as a substrate at concentrations mentioned above. \( I_0 \) values were determined by nonlinear regression with 200 \( \mu \)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, 5, 50, 500, 5000 \( \mu \)M-pHMB in the assay, with 10 min preincubation and spectrophotometric measurement of hydrolysis of benzylpenicillin and cephaloridine each at a concentration of 250 \( \mu \)M.

Inhibition by 0.1 and 1 \( \mu \)M-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 Biodyne B nylon membranes by Pall. DNA hybridization (68 °C, 6 × SSC) and non-radioactive detection were performed according to the instructions of the manufacturer of Chemiprobe (Biozyme). Cloning experiments with pLG339 (Stoker et al., 1982) and pBG518 (Spratt et al., 1986) as vectors were carried out as described by Walker (1984), using the modified Kushner method (Hanahan, 1983) for transformation. Restrictionendonuclease sites were determined by single and double digest and in case of \( Escherichia \) coli by time-dependent partial digestion using the 3–6 kb BamHI fragment labelled with \( \gamma \)-\(^{32}\)P-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 Beck-
man TLV 100K rotor for 3 h at 10000 r.p.m. DNA sequencing was performed by the dye-deoxy-chain-termination method (Sanger et al., 1977) using the Pharmacia T\(_7\) sequencing kit and [\( \alpha \]-\(^{35}\)S]-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 pBG58 and two adjacent 2.35 kb \( PstI \) fragments from pBP60 encoding the SHV-2 gene, and pBP60, the natural plasmid from \( Klebsiella oxytoca \) strain 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\textsuperscript{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\textsuperscript{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 BamHI and none for EcoRI, HindIII or PstI. These small plasmids could be mobilized in transconjugation experiments and appeared to be cryptic in their natural host, whereas transformation into E. coli DH5\textalpha{} led to expression of a \( \beta \)-lactamase of pI 4.8. The 66 kb plasmid, which we call pZMPl, 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 BamHI, EcoRI, HindIII and PstI differs completely from that of pBP60, which encodes the SHV-2 \( \beta \)-lactamase (data not shown), but is identical for all of the CTX\textsuperscript{R} Klebsiella isolates tested.

\( \beta \)-Lactamase patterns

All the CTX\textsuperscript{R} K. pneumoniae isolates showed a \( \beta \)-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\textsuperscript{R} K. pneumoniae isolates, and for E. coli transformants, transconjugants and recipients of conjugation

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC (mg l\textsuperscript{-1}) of antibiotics for*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kpr 14</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>&gt;128</td>
</tr>
<tr>
<td>Mezlocillin</td>
<td>&gt;128</td>
</tr>
<tr>
<td>Piperacillin</td>
<td>&gt;64</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>&gt;128</td>
</tr>
<tr>
<td>Cefotiam</td>
<td>&gt;128</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>32</td>
</tr>
<tr>
<td>Cefotetan</td>
<td>2</td>
</tr>
<tr>
<td>Moxalactam</td>
<td>4</td>
</tr>
<tr>
<td>Cefotaxime (CTX)</td>
<td>&gt;128</td>
</tr>
<tr>
<td>Desacetylcefotaxime</td>
<td>&gt;128</td>
</tr>
<tr>
<td>Cefazidime (CTZ)</td>
<td>64</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>&gt;128</td>
</tr>
<tr>
<td>Cefmenoxime</td>
<td>&gt;128</td>
</tr>
<tr>
<td>Cefpirome</td>
<td>32</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>16</td>
</tr>
<tr>
<td>Imipenem</td>
<td>1</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>2</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>&gt;128</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.25</td>
</tr>
<tr>
<td>Doxycyclin</td>
<td>&gt;128</td>
</tr>
<tr>
<td>Trimethoprim/</td>
<td>3.2/</td>
</tr>
<tr>
<td>sulfamethoxazole</td>
<td>60/8</td>
</tr>
</tbody>
</table>

* Kpr 14, CTX\textsuperscript{R} K. pneumoniae isolate; Kj 14, transconjugant harbouring original plasmid pZMPl from Kpr 14; Iso 1, transformant harbouring recombinant plasmid pZMPl-1-1; DH5\textalpha{}, E. coli recipient of transformation; W3110Nal\textsuperscript{R}, E. coli recipient of conjugation.
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 (Kₘ >1000 μM) 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 BamHI, and the resulting fragments were cloned into the BamHI site of pLG339. The recombinant plasmids were used to transform E. coli DH5α 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 BamHI fragment was established for EcoRV, MraI, PstI, SalI and StuI (Fig. 2). BglII, EcoRI, HindIII, HpaI, SacI, ScaI, SphI, XbaI and XhoI did not cut within the 3.6 kb fragment.

Further subcloning of the partially or completely PstI-digested 3.6 kb BamHI fragment into pBGS18 led to CTX® clones when the three adjacent PstI fragments of
0.84, 0.81 kb hybridized to the cloned SHV-2 gene under stringent conditions (data not shown).

Nucleotide sequencing of the total 3.6 kb BamHI 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 potential open reading frames (ORFs) according to a computerized Fickett analysis, of which the largest, of 160 nucleotides immediately upstream from the start codon, shows homology to the 35 and 5 boxes of the bluLEN-l promoter, Shine–Dalgarno sequence, and stop codon and putative transcription termination sequences are underlined.

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 et al., 1989); SHV-2a, this paper.

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 region in comparison to the corresponding largest 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>
<thead>
<tr>
<th>Nucleotide position†</th>
<th>β-Lactamase*</th>
<th>92</th>
<th>153</th>
<th>402</th>
<th>602</th>
<th>705</th>
<th>759</th>
<th>762</th>
<th>786</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHV-2</td>
<td>T (L)</td>
<td>C</td>
<td>G</td>
<td>G (R)</td>
<td>C</td>
<td>G</td>
<td>C</td>
<td>T</td>
<td>C</td>
</tr>
<tr>
<td>SHV-2/variant</td>
<td>A (Q)</td>
<td>G</td>
<td>G</td>
<td>G (R)</td>
<td>T</td>
<td>G</td>
<td>C</td>
<td>T</td>
<td>G</td>
</tr>
<tr>
<td>SHV-3</td>
<td>T (L)</td>
<td>C</td>
<td>A</td>
<td>T (L)</td>
<td>C</td>
<td>A</td>
<td>G</td>
<td>G</td>
<td>C</td>
</tr>
<tr>
<td>SHV-2a</td>
<td>A (Q)</td>
<td>G</td>
<td>G</td>
<td>G (R)</td>
<td>C</td>
<td>G</td>
<td>C</td>
<td>T</td>
<td>G</td>
</tr>
</tbody>
</table>

* Data from the following sources: SHV-2, Podbielski & Melzer (1990); SHV-2 variant, Garbag-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.
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

<table>
<thead>
<tr>
<th>Strain†</th>
<th>Ampicillin (8 mg l(^{-1}))</th>
<th>Mezlocillin (8 mg l(^{-1}))</th>
<th>CTX (1 mg l(^{-1}))</th>
<th>CTZ (1 mg l(^{-1}))</th>
<th>Cefpirome (1 mg l(^{-1}))</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Cla</td>
<td>Sul</td>
<td>Taz</td>
<td>Cla</td>
<td>Sul</td>
</tr>
<tr>
<td>Kpr 14</td>
<td>8</td>
<td>128</td>
<td>&gt;128</td>
<td>1</td>
<td>&gt;128</td>
</tr>
<tr>
<td>Kj 14</td>
<td>8</td>
<td>16</td>
<td>8</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Iso 1</td>
<td>2</td>
<td>16</td>
<td>8</td>
<td>4</td>
<td>8</td>
</tr>
</tbody>
</table>

* Cla, clavulanic acid; Sul, sulbactam; Taz, tazobactam.
† See Table 1.

Table 4. \(V_{\text{m}}\), \(K_{\text{m}}\), and \(V/K_{\text{m}}\) values of SHV enzymes for selected antibiotics

<table>
<thead>
<tr>
<th>β-Lactamase</th>
<th>Penicillin</th>
<th>Ampicillin</th>
<th>Mezlocillin</th>
<th>Cephaloridine</th>
<th>Cefotiam</th>
<th>CTX</th>
<th>desacetylCTX</th>
<th>Cefpirome</th>
</tr>
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<tbody>
<tr>
<td>SHV-2a*</td>
<td>33</td>
<td>17</td>
<td>1.9</td>
<td>30</td>
<td>1.7</td>
<td>100</td>
<td>73</td>
<td>100</td>
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<td></td>
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<tr>
<td>SHV-1†</td>
<td>42</td>
<td></td>
<td></td>
<td>58</td>
<td>1.2</td>
<td>7</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td>58</td>
<td></td>
<td>71</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>SHV-2*</td>
<td>32</td>
<td>20</td>
<td>1.6</td>
<td>58</td>
<td>1.2</td>
<td>7</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td></td>
<td></td>
<td>58</td>
<td></td>
<td>71</td>
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<td>16</td>
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<td></td>
<td></td>
<td></td>
<td>3</td>
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<tr>
<td>SHV-5†</td>
<td>11</td>
<td></td>
<td></td>
<td>77</td>
<td>1.1</td>
<td>9</td>
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<td>100</td>
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<td></td>
<td>77</td>
<td></td>
<td>45</td>
<td></td>
<td>10</td>
</tr>
</tbody>
</table>

* Data for SHV-2a were obtained from *E. coli* DH5a transformant harbouring recombinant plasmid pZMP1-1-1 by using a crude protein extract with a specific activity of 0.5 nmol cephaloridine min\(^{-1}\) (µg 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 Garbagh-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* 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 cephemycins, 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* 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 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.

<table>
<thead>
<tr>
<th>β-Lactamase</th>
<th>Cla (μM)</th>
<th>I$_{50}$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cla</td>
<td>Sul</td>
</tr>
<tr>
<td>SHV-2a*</td>
<td>0.08</td>
<td>0.47</td>
</tr>
<tr>
<td>SHV-1†</td>
<td>0.19</td>
<td>1.70</td>
</tr>
<tr>
<td>SHV-2*</td>
<td>0.16</td>
<td>0.36</td>
</tr>
<tr>
<td>SHV-5†</td>
<td>0.10</td>
<td>0.18</td>
</tr>
</tbody>
</table>

* Data for SHV-2a were obtained from E. coli DH5α 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 μM 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).
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 cephemycins 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; Burè 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 acylurideo penicillins as well as to methoxyimino-cephalosporins but not to cephemycins, monobactams or carbapenems. The preference for methoxyimino-cephalosporins over cephemycins 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 pl 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 values and the apparent *K*ᵦ for sulbactam or tazobactam are generally lower than those for SHV-1 but higher than those for SHV-2 and SHV-5, respectively. The hydrolytic effectiveness 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 ceftoxin 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 μM), 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*ᵦ values and of their effectiveness upon the enzyme action expressed by the *I*₅₀ values parallels 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*ᵦ 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).
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.

Fig. 4: Dendogram of the nucleotide sequence relatedness of the structural genes of SHV-type $\beta$-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 (Staph. aureus) $\beta$-lactamase, for which the crystal structure is known (Herzberg & Moul, 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).

**References**


