Molecular Genetics of Resistance to Both Ceftazidime and β-Lactam–
β-Lactamase Inhibitor Combinations in *Klebsiella pneumoniae* and In Vivo
Response to β-Lactam Therapy

Louis B. Rice, Lenore L. Carias, Robert A. Bonomo, and David M. Shlaes

The number of plasmid-mediated extended-spectrum cephalosporinases has increased dramatically since the clinical introduction of ceftazidime [1]. The molecular basis of the extended spectrum involves point mutations within plasmid-mediated β-lactamase genes resulting in either single or multiple amino acid changes in the encoded enzymes. Variants of the TEM-1 plasmid-mediated β-lactamase have been most commonly identified in outbreaks of extended-spectrum β-lactamase-producing bacteria [2–5]. SHV-1-derived extended-spectrum β-lactamases are less commonly identified [1]. In general, plasmid-mediated extended-spectrum enzymes have remained susceptible to inhibition by the clinically available β-lactamase–β-lactamase inhibitor combinations ampicillin-sulbactam, and tazobactam. Exceptions to this rule include rare examples of plasmid-mediated AmpC-type enzymes, since the AmpC enzymes confer resistance to ceftazidime and remain resistant to inhibitors [6, 7].

In vivo studies have documented the efficacy of the β-lactam–β-lactamase inhibitor combinations against a TEM-26-producing *Klebsiella pneumoniae* strain [8]. Mutational analysis of the TEM-1 enzyme suggests that resistance to either extended-spectrum cephalosporins or the β-lactam–β-lactamase inhibitor combinations are possible [9]. However, a combination of mutations within a single gene resulting in resistance to both classes of compounds has not been achievable in the laboratory or discovered in nature [1, 10, 11].

Resistance to β-lactam–β-lactamase inhibitor combinations in Enterobacteriaceae is most frequently achieved via the overproduction of a normally susceptible enzyme [12–14]. It has also been documented that some β-lactamase inhibitors, notably sulbactam, are less effective against SHV-1-producing *Escherichia coli* strains than they are against those that produce TEM-1 [15]. In vitro resistance to both extended-spectrum cephalosporins and β-lactam–β-lactamase inhibitor combinations should therefore be achievable by either the overproduction of an extended-spectrum β-lactamase or the production of an extended-spectrum TEM-type β-lactamase in combination with an SHV-1 enzyme. Jarlier et al. [16] reported that the production of two enzymes (either TEM-1 and SHV-2 or TEM-1 and TEM-3) within *K. pneumoniae* or *E. coli* strains resulted in elevated levels of resistance to the combination of amoxicillin with either sulbactam or clavulanic acid. More recently, a clinical *E. coli* isolate was found to produce high levels of TEM-1 and an extended-spectrum SHV-type enzyme (SHV-7), a combination that conferred in vitro resistance to both extended-spectrum cephalosporins and the β-lactam–β-lactamase inhibitor combinations [17]. The clinical importance of these elevated levels of inhibitor resistance has not been determined.

At our institution, we have recently experienced a dramatic rise in the percentage of *K. pneumoniae* isolates expressing resistance to ceftazidime. The rate of ceftazidime resistance, which was 5% in 1992, rose to 15% in 1993 and to ~30% in the first 2 months of 1994. The majority of the ceftazidime-resistant strains retained in vitro susceptibility to the β-lactam–β-lactamase inhibitor combinations ampicillin-sulbactam and...
piperacillin-tazobactam. A subgroup of strains expressed resistance to both classes of compounds. Here we report molecular analysis of 2 ceftazidime-resistant *K. pneumoniae* isolates with differing susceptibilities to the β-lactam–β-lactamase inhibitor combinations and examine the in vivo efficacy of ceftazidime, ampicillin-sulbactam, and piperacillin-tazobactam in the treatment of rat intraabdominal abscesses caused by these strains.

Materials and Methods

Identification of resistant isolates. Data on resistance and susceptibility of clinical isolates was obtained from the Microbiology Laboratory at the VA Medical Center in Cleveland. Ceftazidime-resistant *K. pneumoniae* isolates were first identified using a gram-negative identification card (Vitek GNI; BioMérieux Vitek, Hazelwood, MO). This system measures susceptibility to ceftazidime concentrations of 4, 8, and 64 μg/mL. MICs ≤8 μg/mL are considered to indicate susceptible isolates and those ≥32 μg/mL are considered to indicate resistant isolates. Subsequent susceptibilities of selected strains and transconjugants were determined by an agar dilution technique (inoculum = 10^5 cfu/spot) for a variety of antibiotics [18]. High-inoculum MICs were determined by a broth macrodilution method using Mueller-Hinton broth [18]. β-lactam–β-lactamase inhibitor combinations were used in concentrations consistent with the clinical preparations (ampicillin-sulbactam = 2:1; piperacillin-tazobactam = 8:1) Screening susceptibility tests for some non-β-lactam antimicrobials was done by disk diffusion [19].

Bacterial strains and plasmids. Strains selected for detailed study and the plasmids used in these experiments are listed in table 1. *K. pneumoniae* 21300 and 26139 were clinical isolates obtained from the Microbiology Laboratory at the VA Medical Center in Cleveland. *E. coli* J53-2 (met, pro, Rif') [20] was used as a recipient in mating experiments. *E. coli* DH5α was used as a recipient in transformation experiments [21]. Phagemid pBCSK+ (Stratagene, La Jolla, CA) and plasmids pCRII (Invitrogen, San Diego) and pACYC184 [22] were used as cloning vectors.

Conjugation experiments. Filter matings were done on antibiotic-free Luria-Bertani (LB) agar plates as described [23]. Antibiotic concentrations used for selective plates were ceftazidime, 5 μg/mL; ampicillin, 50 μg/mL; sulbactam, 25 μg/mL; and rifampin, 100 μg/mL.

Plasmid isolation and DNA techniques. Plasmid isolation from transconjugants was done according to the technique described by Takahashi and Nagano [24]. Plasmids were digested with restriction enzymes according to the specifications of the manufacturer (Promega, Madison, WI) and separated on agarose gels. Cloning experiments were done by ligating digested fragments to similarly digested pBCSK+ and transforming *E. coli* DH5α by electroporation with selection on LB agar plates containing either ceftazidime (5 μg/mL) or ampicillin and sulbactam (50 μg/mL + 25 μg/mL). The ceftazidimase gene from pLRM7 was amplified from a crude plasmid preparation using primers designed to amplify the entire TEM-1 gene as described [25] and ligating the polymerase chain reaction product to vector pCRII. The ceftazidimase gene from pLRM8 was cloned directly into pBCSK+ on a 3.8-kb ClaI fragment. blaSHV-1 from pLRM7 was cloned into pBCSK+ on a 3.5-kb BamHI fragment.

Southern transfers were done using a negative pressure transfer apparatus (Pharmacia, Piscataway, NJ) and hybridized using probes labeled with digoxigenin according to the specifications of the manufacturer (Boehringer-Mannheim, Indianapolis). Probing for sequences homologous to *blaSHV-1* was done using a probe derived from the 1.1-kb *TaqI* fragment of plasmid pDS075, which contains the OHIO-1 β-lactamase gene in its entirety [26]. The gene encoding OHIO-1 has been shown to be >95% homologous to the gene encoding SHV-1 [26]. A previously cloned internal amplification product of a TEM-26 gene [25] was used as a probe for TEM-related genes.

DNA sequencing was done on subclones of the above clones using fluorescein-labeled primers and the AutoRead sequencing kit (Pharmacia). Sequence was determined using the ALF DNA sequencer (Pharmacia). Homology comparisons were made using the HIBIO MacDNASIS Pro DNA and protein sequence analysis system (version 2.0; Hitachi Software Engineering America, San Bruno, CA).

Isoelectric (pI) focusing experiments. Analytic isoelectric focusing was done on ceftazidime-resistant *E. coli* transconjugants as described [27].

β-lactamase kinetic studies. *E. coli* strains with the cloned SHV-1 gene (pCWR101) were grown in 500 mL of LB broth to an optical density at 595 nm of 0.5–0.8. Cell pellets were washed three times with 20 mM sodium phosphate buffer, pH 7.4. Pellets were suspended in 10 mL of phosphate buffer and disrupted by agitation with glass beads for 2 h. The suspension was centrifuged at 10,000 g for 15 min, and the resulting supernatant was then centrifuged at 40,000 g for 60 min to remove cell debris.

Hydrolysis of β-lactams was done at 30°C in a temperature-controlled Guiford spectrophotometer. Wavelengths and extinction coefficients used were as described [24]. Substrates were used in various concentrations. Lineweaver-Burk plots were prepared and *Km* and *V* max were calculated from the linear regression. For inhibitor kinetics, the enzyme and inhibitor were preincubated for 10 min at 25°C before the addition of nitrocefin to a final concentration of 100 μM. Standard (1/3) versus (1) (concentration of inhibitor) plots were prepared by using linear regression and the *K* was calculated.

Animal experiments. Rat intraabdominal abscess experiments were done as described [28–30] using a 10^5 cfu inoculum of either organism implanted into the rat peritoneum along with sterilized rat cecal contents and killed *Bacteroides fragilis* ATCC 25285 in a 1:2:1 ratio. Therapy was administered via continuous intravenous infusion into the internal jugular vein and continued for 3 days. Doses administered were ampicillin, 1000 mg/kg/day, with sulbactam, 500 mg/kg/day; piperacillin, 1500 mg/kg/day, with tazobactam, 187.5 mg/kg/day; imipenem, 300 mg/kg/day; ceftazidime, 400 mg/kg/day; and cefoxitin, 500 mg/kg/day. Untreated control animals were included with each experiment. Serum was sampled for determination of antimicrobial concentrations on day 2 of therapy. Serum concentrations of ampicillin, piperacillin, imipenem, ceftazidime, and cefoxitin were determined by bioassay using *Bacillus subtilis* ATCC 6633 spores or using *E. coli* ATCC 25922 [31]. Sulbactam concentrations were determined by bioassay with *Pasteurella haemolytica* ATCC 43823.
Table 1. Bacterial strains and plasmids.

<table>
<thead>
<tr>
<th>Bacterial strain or plasmid</th>
<th>Resistance profile</th>
<th>Origin (reference)</th>
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</thead>
<tbody>
<tr>
<td><em>K. pneumoniae</em> 21300</td>
<td>Caz&lt;sup&gt;a&lt;/sup&gt;, A/S&lt;sup&gt;a&lt;/sup&gt;, P/T&lt;sup&gt;a&lt;/sup&gt;, Gm&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Clinical isolate (this study)</td>
</tr>
<tr>
<td><em>K. pneumoniae</em> 26139</td>
<td>Caz&lt;sup&gt;a&lt;/sup&gt;, Gm&lt;sup&gt;a&lt;/sup&gt;, Su&lt;sup&gt;a&lt;/sup&gt;, Tp&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Clinical isolate (this study)</td>
</tr>
<tr>
<td><em>Escherichia coli</em> J53-2</td>
<td>met, pro, Rif&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Recipient in mating experiments [20]</td>
</tr>
<tr>
<td><em>E. coli</em> DH5α</td>
<td>F-800 lacZ (lacZYA-argF&lt;sup&gt;F&lt;/sup&gt;) U169 endA1 recA1</td>
<td>Transformation recipient strain [21]</td>
</tr>
<tr>
<td>pLRM7</td>
<td>Caz&lt;sup&gt;a&lt;/sup&gt;, A/S&lt;sup&gt;a&lt;/sup&gt;, P/T&lt;sup&gt;a&lt;/sup&gt;, Gm&lt;sup&gt;a&lt;/sup&gt;</td>
<td><em>K. pneumoniae</em> 21300 (this study)</td>
</tr>
<tr>
<td>pLRM8</td>
<td>Caz&lt;sup&gt;a&lt;/sup&gt;, Gm&lt;sup&gt;a&lt;/sup&gt;, Su&lt;sup&gt;a&lt;/sup&gt;, Tp&lt;sup&gt;a&lt;/sup&gt;</td>
<td><em>K. pneumoniae</em> 26139 (this study)</td>
</tr>
<tr>
<td>pACYC184</td>
<td>Cm&lt;sup&gt;a&lt;/sup&gt;, Tc&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Cloning vector [22]</td>
</tr>
<tr>
<td>pBCSK+</td>
<td>Cm&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Cloning plasmid (Stratagene)</td>
</tr>
<tr>
<td>pCRII</td>
<td>Ap&lt;sup&gt;a&lt;/sup&gt;, Km&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.5-kb BamHI fragment of pLRM7 (SHV gene) cloned into pBCSK+ (this study)</td>
</tr>
<tr>
<td>pCWRI101</td>
<td>A/S&lt;sup&gt;a&lt;/sup&gt;, Cm&lt;sup&gt;a&lt;/sup&gt;, P/T&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1-kb PCR product from pLRM7 (TEM gene) cloned into pCRII (this study)</td>
</tr>
<tr>
<td>pCWRI112</td>
<td>Caz&lt;sup&gt;a&lt;/sup&gt;, Km&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1-kb insert from pCWRI12 cloned into EcoRI site of pACYC184 (this study)</td>
</tr>
<tr>
<td>pCWRI122</td>
<td>Caz&lt;sup&gt;a&lt;/sup&gt;, Tc&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.4-kb ClaI fragment of pLRM8 (TEM-6 gene) cloned into pBCSK+ (this study)</td>
</tr>
<tr>
<td>pCWRI155</td>
<td>Caz&lt;sup&gt;a&lt;/sup&gt;, Tc&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.8-kb PatI subfragment of pCWRI101 cloned into pBCSK+ (this study)</td>
</tr>
<tr>
<td>pCWRI158</td>
<td>Cm&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.9-kb PatI subfragment of pCWRI101 cloned into pBCSK+ (this study)</td>
</tr>
<tr>
<td>pCWRI159</td>
<td>Cm&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.3-kb ClaI fragment of pLRM8 (which hybridizes to OHIO-1 probe) cloned into pBCSK+ (this study)</td>
</tr>
<tr>
<td>pCWRI178</td>
<td>Cm&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.9-kb ClaI/Rem fragment of pCWRI178 cloned into pBCSK+ (this study)</td>
</tr>
<tr>
<td>pCWRI191</td>
<td>Cm&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13-kb plasmid from clinical isolate encoding OHIO-1 β-lactamase gene [26]</td>
</tr>
</tbody>
</table>

NOTE. Ap, ampicillin; A/S, ampicillin/sulbactam; Caz, ceftazidime; Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; P/T, piperacillin/tazobactam; Su, sulfamethoxazole; Tc, tetracycline; Tp, trimethoprim. PCR, polymerase chain reaction.

**Statistical analysis.** Results of animal studies were compared using Student’s *t* test for comparing independent variables.

**Results**

**Susceptibility results.** The in vitro broth dilution susceptibility results for the study strains are shown in table 2. Strain 21300 expressed resistance to ceftazidime as well as to ampicillin-sulbactam and piperacillin-tazobactam. Resistance to ampicillin-sulbactam was increased almost 10-fold when the inoculum was increased from 2 × 10⁵ to 2 × 10⁶ cfu/mL and the resistance to piperacillin-tazobactam was increased 4-fold under similar conditions. Strain 26139 expressed resistance to ceftazidime but was susceptible to both ampicillin-sulbactam and piperacillin-tazobactam.

**Analysis of resistance-encoding plasmids.** Ceftazidime resistance was transferable to *E. coli* recipient strain J53-2 at low frequency (<10⁻⁷/recipient cfu) from both 21300 and 26139. One ceftazidime-resistant transconjugant from each donor strain containing a single large plasmid (>100 kb) was selected for further analysis. The plasmid originating in 21300 was designated pLRM7 and that originating in 26139 was designated pLRM8.

Susceptibility testing of the transconjugants was consistent with results obtained with the donor strains (table 2). J53-2(pLRM7) expressed resistance to both ceftazidime and ampicillin-sulbactam. The MIC for piperacillin-tazobactam fell into the susceptible range but was higher than that for J53-2(pLRM8). J53-2(pLRM8) expressed resistance to ceftazidime but remained highly susceptible to the inhibitor combinations. In addition, both plasmids conferred resistance to gentamicin. In contrast to pLRM7, pLRM8 also conferred resistance to trimethoprim and sulfisoxazole (table 2).

Restriction digestion of pLRM7 and pLRM8 suggested that they were related, with several similarly sized BamHI and ClaI restriction bands (figure 1). Hybridization of plasmid digests with the TEM-type probe indicated that both plasmids possessed TEM-type genes on ClaI, EcoRI, and HindIII fragments of identical size (data not shown). Hybridization with the OHIO-1 probe indicated that both plasmids also possessed se-
Table 2. In vitro susceptibility of *K. pneumoniae* and *Escherichia coli* strains to selected antimicrobial agents and disk susceptibilities to non-β-lactam antibiotics.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Ampicillin (μg/mL)</th>
<th>Ampicillin/ sulbactam (2:1)</th>
<th>Piperacillin (μg/mL)</th>
<th>Piperacillin/ tazobactam (8:1)</th>
<th>Imipenem (μg/mL)</th>
<th>Cefoxitin (μg/mL)</th>
<th>Ceftazidime (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>K. pneumoniae</em> 21300</td>
<td>&gt;4096</td>
<td>128*</td>
<td>1024</td>
<td>64†</td>
<td>≤0.5</td>
<td>4</td>
<td>256</td>
</tr>
<tr>
<td><em>E. coli</em> J53-2 (pLRM7)</td>
<td>4096</td>
<td>64</td>
<td>512</td>
<td>16</td>
<td>≤0.5</td>
<td>8</td>
<td>256</td>
</tr>
<tr>
<td><em>K. pneumoniae</em> 26139</td>
<td>&gt;4096</td>
<td>≤16</td>
<td>1024</td>
<td>16</td>
<td>≤0.5</td>
<td>4</td>
<td>512</td>
</tr>
<tr>
<td><em>E. coli</em> J53-2 (pLRM8)</td>
<td>4096</td>
<td>≤16</td>
<td>256</td>
<td>≤16</td>
<td>≤0.5</td>
<td>16</td>
<td>256</td>
</tr>
<tr>
<td><em>E. coli</em> DH5α-TEM-6 (pCWR122)</td>
<td>ND</td>
<td>16</td>
<td>ND</td>
<td>16</td>
<td>&lt;0.5</td>
<td>ND</td>
<td>&gt;256</td>
</tr>
<tr>
<td><em>E. coli</em> DH5α-SHV-1 (pCWR101)</td>
<td>ND</td>
<td>&gt;128</td>
<td>ND</td>
<td>&gt;128</td>
<td>≤0.5</td>
<td>ND</td>
<td>≤0.5</td>
</tr>
</tbody>
</table>

Zone size (mm)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Amikacin</th>
<th>Gentamicin</th>
<th>Netilmicin</th>
<th>Sulfisoxazole</th>
<th>Tetracycline</th>
<th>Trimethoprim</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> J5-3 (pLRM7)</td>
<td>23</td>
<td>&lt;6</td>
<td>17</td>
<td>25</td>
<td>19</td>
<td>29</td>
</tr>
<tr>
<td><em>E. coli</em> J5-3 (pLRM8)</td>
<td>25</td>
<td>&lt;6</td>
<td>19</td>
<td>&lt;6</td>
<td>19</td>
<td>&lt;6</td>
</tr>
</tbody>
</table>

NOTE. ND, not determined.

* 1024 μg/mL at 2 × 10⁶ cfu inoculum.
† 256 μg/mL at 2 × 10⁶ cfu inoculum.

The sequences homologous to the SHV-1 β-lactamase gene but that these sequences were located on different-sized *BamHI* and *ClaI* fragments (figure 1).

The TEM-related gene of pLRM7 was amplified using polymerase chain reaction and cloned into vector pCRII with selection on LB agar plates containing ceftazidime. This plasmid was designated pCWR112. Since pCWR112 also expresses TEM-1 β-lactamase, the insert from this recombinant plasmid was then separated from pCRII by digestion with *EcoRI* and cloned into the *EcoRI* site of plasmid pACYC184 (with selection on ceftazidime and tetracycline) and designated pCWR122. pCWR122 expressed high levels of resistance to ceftazidime but remained susceptible to the inhibitor combinations (table 2). The SHV-type gene from pLRM7 was cloned into pBCSK+ on a 3.5-kb *BamHI* fragment with selection on ampicillin (50 μg/mL) and sulbactam (25 μg/mL). This plasmid was designated pCWR101. pCWR101 was susceptible to ceftazidime but resistant to the inhibitor combinations (table 2). These results cannot be considered proof that the SHV-type β-lactamase was responsible for ampicillin-sulbactam resistance, since cloning β-lactamase genes into a high-copy number vector is expected to lead to increased levels of resistance to the combinations. In any case, these results were suggestive of a scenario in which ceftazidime resistance was conferred by an extended-spectrum TEM-type enzyme, while resistance to the inhibitor combinations resulted from either production of the SHV-type enzyme or the combination of SHV-type enzyme with the TEM-related extended-spectrum enzyme.

Isoelectric focusing studies. Analytic isoelectric focusing revealed the presence of two β-lactamase enzymes in *E. coli* J53-2(pLRM7). One had a pl of 7.6, consistent with the expected pl of SHV-1. The other had a pl of 5.9, which has previously been described with extended-spectrum TEM variants TEM-4 and TEM-6 [1]. J53-2(pLRM8) elaborated only the pl 5.9 enzyme. These results indicated that pLRM8 was not encoding a functional SHV-1 enzyme despite the fact that it possessed sequences that hybridized with the OHIO-1 probe.

Inhibitor kinetic studies. We did inhibitor kinetic analysis on the cloned SHV-type gene (pCWR101). The *Kₘ* for clavulanic acid, sulbactam, and tazobactam were 0.2, 29, and 0.6
TEM-1 SUTCLIFFE)  

<table>
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<th>155</th>
</tr>
</thead>
</table>

TTG GTT GAG TAC TCA ........... CTT GAT CGT TGG GAA
Leu Val Glu Tyr Ser ........... Leu Asp Arg Trp Glu
102

pLRM7

TTG GTT AAG TAC TCA ........... CTT GAT CAT TGG GAA
Leu Val Lys Tyr Ser ........... Leu Asp His Trp Glu
102

pLRM8

................. CTT GAT CAT TGG GAA
Leu Asp His Trp Glu
162

\( \mu M \), respectively. These results were consistent with those anticipated for SHV-1 for clavulanic acid and tazobactam (although slightly higher than normal for sulbactam) and did not suggest the presence of mutations resulting in increased levels of resistance to the \( \beta \)-lactamase inhibitors. We cannot absolutely exclude (without determining the sequence of the SHV-type gene) the presence of a mutation within the gene that would increase resistance to inhibition by sulbactam without altering the isoelectric point, but we consider this possibility very unlikely.

Sequence analysis. Sequence analysis of the TEM-type gene from pLRM7 revealed a G \( \rightarrow \) A change at position 693 (Sutcliffe numbering) of the TEM-1 gene [32], which would be predicted to result in an Arg \( \rightarrow \) His amino acid change at position 162 (figure 2). This change has been described previously in TEM-6 [33]. An additional G \( \rightarrow \) A change was noted at nucleotide 512, resulting in a predicted Glu \( \rightarrow \) Lys change at amino acid position 102, also consistent with TEM-6. We therefore conclude that pLRM7 encodes the extended-spectrum \( \beta \)-lactamase TEM-6. The TEM-type gene from pLRM8 was cloned (pCWR155) and partially sequenced, revealing an identical G \( \rightarrow \) A at position 693. The nucleotide sequence of the region encoding amino acid 102 was not sequenced for this clone. Given the pl results, the identical restriction profile of the two plasmids in the region of the TEM-type gene, and the documented Arg \( \rightarrow \) His change at 162, we are confident that the TEM-type genes in both pLRM7 and pLRM8 are \( \text{bla}_{\text{TEM-6}} \).

We did not pursue the sequence of the SHV-type gene since the pl, inhibitor kinetics, and susceptibility profiles were all consistent with SHV-1. It is worth noting that an SHV-type enzyme (SHV-7) was recently described in \( E. \) coli that has a pl identical to SHV-1 when the pl of the native enzyme (rather than the cloned version) is determined [17]. However, this enzyme conferred resistance to the extended-spectrum cephalosporins. We therefore think it reasonable to conclude that the SHV variant within pLRM7 is SHV-1.

To investigate the apparent inactivation of the SHV-type gene of pLRM8, we cloned the 4.5-kb \( \text{ClaI} \) fragment (which hybridizes to the \( \text{OHIO-I} \) probe) of pLRM8 into pBCSK+. This recombinant plasmid, which did not express ampicillin resistance, was designated pCWR178. Restriction studies indicated that the \( \text{Xmnl} \) site known to be present in the upstream region of the SHV-1 gene was not present in this clone (data not shown). We subcloned a 770-bp \( \text{ClaI/RsaI} \) fragment of pCWR178 into \( \text{ClaI/EcoRV} \)-digested pBCSK+ and designated this plasmid pCWR191. Nucleotide sequence analysis of pCWR191 revealed interruption of the \( \text{bla}_{\text{SHV-1}} \) gene at nucleotide 716, 20 bp upstream of the internal \( \text{PstI} \) site (figure 3) [34]. The sequence upstream of the interruption was identical to one of the ends of insertion sequence ISI5, an IS element widespread within gram-negative bacilli [35, 36]. Hence, it appears that the \( \text{bla}_{\text{SHV-1}} \) gene of pLRM8 is inactivated by insertion of ISI5.

It is intriguing that hybridization to a second band, representing the upstream portion of the SHV-1 gene, is not demonstrable on Southern hybridizations of restriction digests of pLRM8 (figure 1). The probe used encompassed the entire \( \text{OHIO-I} \) gene, so it would be expected to hybridize to the 6- to 700-bp homology represented by the SHV-1 gene upstream of the insertion site [26]. The fact that a second band is not demonstrable suggests
that insertion into the gene has been associated with a deletion of the upstream region. Deletion of the upstream portion of the SHV gene could occur if a copy of IS15 resident within pLRM8 inserted in a replicative fashion into the SHV-1 gene. A secondary recombination event between the two directly repeated IS15 copies could then explain a deletion of the upstream DNA. We initially thought it possible that the presence of IS15 within the SHV gene of pLRM8 may have reflected the insertion of an IS15-based composite transposon conferring resistance to trimethoprim-sulfamethoxazole. However, we have since identified a third plasmid (pLRM17) that confers resistance to ceftazidime but not to the inhibitor combinations or to trimethoprim or sulfamethoxazole. pLRM17 appears identical to pLRM8 by restriction analysis (data not shown). It therefore seems that the order of evolution in these plasmids involves the deletion of the region of the SHV-1 gene, followed in a separate event by the acquisition of resistance determinants for trimethoprim and sulfamethoxazole.

**Intraabdominal abscess studies.** To determine whether the level of resistance expressed to the $\beta$-lactam–$\beta$-lactamase inhibitors was clinically significant, we did rat intraabdominal abscess studies using both *K. pneumoniae* 21300 and 26139. Results are shown in table 3. In general, ceftazidime was ineffective against *K. pneumoniae* 21300, although it did demonstrate a modest effect. Treatment with ampicillin-sulbactam demonstrated no clinical efficacy against 21300 and was associated with a >50% mortality. Piperacillin-tazobactam treatment was not associated with an increased mortality in 21300-infected rats, but the modest effect seen on abscess bacterial counts was statistically indistinguishable from the untreated animals. Imipenem and cefoxitin were highly effective against *K. pneumoniae* 21300 and 26139, with no mortality seen in any animals and untreated controls). Against 26139, the efficacy of ampicillin-sulbactam and piperacillin-tazobactam both approached that of imipenem, with no mortality seen in any group. These results suggest that the increased levels of in vitro and in vivo resistance to the inhibitor combinations are the result of combining the SHV-1 enzyme with the TEM-6 enzyme and that this level of in vitro resistance is likely to have clinical significance.

**Discussion**

The majority of ceftazidime-resistant *K. pneumoniae* isolates reported in this country elaborate one or more of three extended-spectrum enzymes, TEM-10, TEM-12, or TEM-26 [3, 25, 37, 38]. Both TEM-10 and TEM-26 are related to TEM-12 in that they possess the Arg→Ser change at position 162 present in TEM-12 [1]. TEM-10 has an additional Glu→Lys change at position 237, while TEM-26 demonstrates a Glu→Lys change at position 102 [1]. To our knowledge, the results reported here represent the first report of TEM-6–producing isolates in the United States and confirm that this enzyme is capable of contributing to resistance outbreaks as well.

The fortuitous discovery of two different but related plasmids, one of which has an inactivated SHV-type gene, allows us to draw conclusions regarding the mechanism of resistance to both ceftazidime and the $\beta$-lactam–$\beta$-lactamase inhibitor combinations. The TEM-6 gene appears to be solely responsible for resistance to ceftazidime. Inhibitor resistance results from the presence of either SHV-1 or the combination of SHV-1 and TEM-6. Susceptibility profiles of *E. coli* strains possessing the two cloned genes support these conclusions. The essentially normal inhibitor kinetics exhibited by the SHV gene.

**Table 3.** In vivo evaluation of antimicrobial efficacy against clinical *K. pneumoniae* isolates in a rat intraabdominal abscess model.

<table>
<thead>
<tr>
<th>Strain, antibiotic</th>
<th>No. of rats surviving</th>
<th>Mean serum cfulg of abscess (±STD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>21300-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ampicillin/sulbactam</td>
<td>3*</td>
<td>40 (8.9)/14.7 (3.0) 9.98 (0.3)</td>
</tr>
<tr>
<td>Piperacillin/tazobactam</td>
<td>9†</td>
<td>38 (5.2) 8.24 (1.2)</td>
</tr>
<tr>
<td>Imipenem</td>
<td>5</td>
<td>12.6 (2.76) 3.7 (0.4)</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>7</td>
<td>26 (9.6) 4.3 (1.6)</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>10</td>
<td>30 (0.9) 7.3 (2.0)</td>
</tr>
<tr>
<td>No treatment</td>
<td>10§</td>
<td>— 9.5 (0.6)</td>
</tr>
<tr>
<td>26139</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ampicillin/sulbactam</td>
<td>5</td>
<td>38.5 (9)/10 (1.4) 4.2 (0.4)</td>
</tr>
<tr>
<td>Piperacillin/tazobactam</td>
<td>6</td>
<td>52 (17) 3.6 (0.6)</td>
</tr>
<tr>
<td>Imipenem</td>
<td>3</td>
<td>16.5 (0.87) 3.2 (0.1)</td>
</tr>
<tr>
<td>No treatment</td>
<td>5</td>
<td>— 9.45 (2.0)</td>
</tr>
</tbody>
</table>

* Data are results in surviving animals.
† 4/7 (57%) mortality.
§ 1/10 (10%) mortality.
‡ P < .05 for comparison with ceftazidime, ampicillin-sulbactam, piperacillin-tazobactam, and untreated groups.
§ 7/17 (33%) mortality.

**Figure 3.** Nucleotide sequence of SHV-hybridizing region of pLRM8. Upper sequence is from *bla*SHV-1 [34]. Lower sequence represents homologous region from pLRM8. Sequence identical to gram-negative IS element IS15 is boxed.
suggests that the increased amount of enzyme in the strain with the two functional genes is the primary mechanism of tazobactam resistance. Resistance to ampicillin-sulbactam in these strains may be exacerbated by the intrinsic resistance of SHV-1 to this inhibitor.

The insertion of IS15 into the SHV-type gene of pLRMS is an interesting occurrence that does not result in any obvious selective advantage for the host organism. It is possible that the insertion merely represents a random event. Alternatively, the deleted upstream sequences may have been deleterious to the host organism, providing a selective advantage to the deletion mutant. IS15 has been associated with SHV derivatives in prior reports [39, 40]. Analysis of the sequence upstream of the SHV-2-variant genes from K. pneumoniae and Salmonella typhimurium suggests that the presence of a more active $-35$ promoter region is the result of insertion of IS15 upstream of the structural genes [39, 40]. IS15 has been found in a similar position upstream of a gram-positive ermB erythromycin resistance gene that has found its way into a gram-negative host [41].

Optimal treatment of K. pneumoniae strains expressing extended-spectrum $\beta$-lactamases is controversial. The use of some non-$\beta$-lactam agents is often precluded by the fact that many extended-spectrum $\beta$-lactamases are encoded on plasmids that confer resistance to multiple classes of antibiotics [1]. In limited animal studies done to date, results have suggested that it may be prudent to consider ceftazidime-resistant K. pneumoniae strains to be resistant to all clinically available cephalosporins, including those that appear active in vitro, since increasing the inoculum will frequently unmask resistance to other extended-spectrum cephalosporins [28]. The cefamycins such as cefoxitin may prove to be the only viable therapeutic alternatives of the cephalosporin class. Imipenem remains an option for the treatment of infections caused by these strains, as it maintains its activity against even high inocula of bacteria and has proven efficacious in several animal studies [8, 28]. The emergence of imipenem resistance in Pseudomonas aeruginosa and other gram-negative bacilli is a well-described complication of frequent imipenem use [4, 42].

Mutations in plasmid-mediated $\beta$-lactamases conferring resistance to extended-spectrum cephalosporins have been associated with stable or increased susceptibility to inhibition by the $\beta$-lactamase inhibitors [15]. In addition, mutations associated with elevated levels of resistance to inhibitors have been associated with increased susceptibility to cephalosporins [9, 10]. It would therefore appear that the $\beta$-lactam-$\beta$-lactamase inhibitor combinations are viable alternatives for the treatment of infections caused by extended-spectrum $\beta$-lactamase-producing K. pneumoniae. The results of this study suggest that these combinations will be effective against extended-spectrum $\beta$-lactamase-producing strains that demonstrate in vitro susceptibility to the combinations. A previous study using a TEM-26-producing strain of K. pneumoniae also arrived at this conclusion [8]. However, our results, along with others recently reported [17], provide the cautionary note that plasmid-mediated ceftazidime resistance should not be considered to imply $\beta$-lactam-$\beta$-lactamase inhibitor susceptibility. Moreover, they suggest that strains demonstrating in vitro resistance to the combinations will also demonstrate in vivo resistance, so the susceptibility of both classes of agents should be checked before instituting therapy.

These results highlight the remarkable versatility of bacteria in adapting to the changing demands of the antimicrobial-rich nosocomial environment. In the setting of widespread use of extended-spectrum cephalosporins and increasing use of $\beta$-lactam-$\beta$-lactamase inhibitor combinations, we expect further reports of multiply resistant strains in the future. Plasmids containing combinations of genes for prevalent $\beta$-lactamases should be relatively easy to produce, given the common presence of $\beta$-lactamases on mobile elements and the strong selective pressure exerted by antimicrobial prescribing practices in institutional settings [1, 43, 44]. Prevention and control of outbreaks due to multiple $\beta$-lactamase-producing gram-negative bacilli will require diligent surveillance efforts as well as judicious and parsimonious use of currently available antimicrobial agents.

**Acknowledgment**

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**References**

8. Rice LB, Caras LL, Shlaes DM. In vivo efficacies of $\beta$-lactam-$\beta$-lactamase inhibitor combinations against a TEM-26–producing strain of K. pneumoniae.


