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Evolution of Plasmid-Coded Resistance to Broad-Spectrum Cephalosporins

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A clinical isolate of *Klebsiella ozaenae* with transferable resistance to broad-spectrum cephalosporins produces a β-lactamase determined by plasmid pBP60. The β-lactamase had the same isoelectric point as SHV-1 (7.6). From heteroduplex analysis, an extensive homology between the two *bla* genes could be deduced; therefore, the new β-lactamase was designated SHV-2. Enzymatic studies revealed that SHV-2 was able to hydrolyze broad-spectrum cephalosporins due to an increased affinity of these compounds for the enzyme. The assumption that SHV-2 is a natural mutant of SHV-1 was strongly supported by the isolation of a laboratory mutant of SHV-1 that showed activities similar to those of SHV-2.

The main indications for broad-spectrum cephalosporins are life-threatening infections with gram-negative bacteria like those of the family *Enterobacteriaceae* and *Pseudomonas aeruginosa*. These antibiotics are more or less stable to all β-lactamases produced by these strains. These enzymes mediate resistance to older β-lactam antibiotics. From the work of Sanders and Sanders (16), Seeberg et al. (18), and others, it has been demonstrated that chromosomally mediated β-lactamases from organisms like *Enterobacter cloacae* and *Citrobacter freundii* can be responsible for resistance to broad-spectrum cephalosporins, although these compounds are not hydrolyzed or are only slightly hydrolyzed by these enzymes. A plasmid-mediated mechanism for resistance to broad-spectrum cephalosporins has been found by Knothe et al. (9), who have isolated *Klebsiella* strains (from the University Hospital in Frankfurt, Federal Republic of Germany) that show transferable resistance to cefotaxime, cefuroxime, and other cephalosporins and penicillins as well as gentamicin. In this investigation, we used a strain from the same source, kindly provided by P. Shah, to analyze the plasmid-mediated β-lactamase and the genetic background in more detail. Our main interest was the origin of the β-lactamase gene.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Klebsiella ozaenae* 2180 containing plasmid pBP60 was a clinical isolate from the Innere Medizin der Universitätsklinik Frankfurt, kindly given by P. Shah. Other bacterial strains and plasmids used are listed in Table 1.

Media. Cultures were grown in standard NI broth or standard NI agar (E. Merck AG, Darmstadt, Federal Republic of Germany). For selection of resistant strains, cultures were plated on China blue lactose agar (Oxoid Ltd., London, United Kingdom) containing antibiotics at the following concentrations (micrograms per milliliter): ampicillin, 50; cefotaxime, 1; chloramphenicol, 40; nalidixic acid, 100; streptomycin, 50; sulfonamide, 100; and tetracycline, 10.

Antibiotics. Antibiotics were kindly provided by the following companies: cephaloridine and cephalothin by Eli Lilly, Giessen, Federal Republic of Germany; cefotaxime, cefuroxime, HR 810, PADAC, benzylpenicillin, ampicillin, streptomycin, and tetracycline by Farbwerke Hoechst, Frankfurt, Federal Republic of Germany; cefotiam by Chemie Grünenthal, Stolberg, Federal Republic of Germany; ceftizoxime by Fujisawa Pharmaceutical Co., Japan; ceftriaxone and sulfamethoxazole by Hoffmann-La Roche, Basel, Switzerland; ceftazidime and nitrocefin by Glaxo Group Research, Greenford, United Kingdom; cefotetan by ICI-Pharma, Plankstadt, Federal Republic of Germany; aztreonam by E. R. Squibb & Sons, Princeton, N.J.; imipenem by Merck Sharp & Dome, West Point, Pa.; oxacillin by Bayer AG, Leverkusen, Federal Republic of Germany; cloxacillin by Bristol Laboratories, Syracuse, N.Y.; clavulanic acid by Beecham-Wülfing, Gronau, Federal Republic of Germany; chloramphenicol by Boehringer GmbH, Mannheim, Federal Republic of Germany; and nalidixic acid by Winthrop Laboratories, Neu-Isenburg, Federal Republic of Germany.

Determination of MICs. MICs were determined by the microdilution test, with Isosensitest broth (Oxoid) and an inoculum of 10^5 CFU/ml. After incubation at 37°C for 18 to 20 h, the MICs were read.

TABLE 1. Bacterial strains and plasmids used in this study

Strains and plasmids	Relevant markers[a]	Reference
Escherichia coli strains		
W3110	Nal^r, *lac*$^+$	4
JC2926	Str^r, *recA*, *thi*, *thr*, *arg*, *his*, *leu*, *lac*	1
Plasmids		
pBP60	Cx^r, Cm^r, Sm^r, *tra*$^+$	This paper
p453	Ap^r, Su^r, Sm^r, Tc^r Cm^r, *tra*$^+$	10, 15
pBP1	Sm^r, Su^r	22
pBP65	Ap^r, Sm^r, Su^r	This paper
pACYC184	Cm^r, Tc^r	2
pBP60-1	Cx^r, Cm^r, Tc^r	This paper
pUC8	Ap^r	24
pBP60-1-1	Ap^r, Cx^r	This paper
pBP65-1	Cx^r, Sm^r, Su^r	This paper

[a] Abbreviations of resistance phenotype symbols: Ap, ampicillin; Cx, cefotaxime; Cm, chloramphenicol; Nal, nalidixic acid; Str or Sm, streptomycin; Su, sulfonamide; Tc, tetracycline.

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TABLE 2. MICs of various drugs for strains producing SHV-1, SHV-2, or SHV-1_{mut}

Drug	MIC (μg/ml) for:				
	E. coli W3110	SHV-1[a]	SHV-2[a]	SHV-2[b]	SHV-1_{mut}[a]
Cephaloridine	4	16	64	64	64
Cephalothin	8	32	128	128	256
Cefotiam	0.125	0.12	4	4	4
Cefmenoxime	0.03	0.03	2	4	2
Cefuroxime	2	8	8	8	32
Cefotaxime	0.03	0.03	4	4	4
Ceftazidime	0.125	0.125	4	2	2
Ceftriaxone	0.03	0.03	4	4	4
Cefotetan	0.06	0.06	0.25	0.125	0.125
HR 810	0.015	0.03	1	1	1
Benzylpenicillin	8	512	>2,048	>2,048	1,024
Ampicillin	2	1,024	>2,048	>2,048	2,048
Imipenem	0.5	0.5	0.5	1.0	0.5
Aztreonam	0.06	0.03	1	1	1

[a] Host strain, *E. coli* W3110.
[b] Host strain, *K. ozaenae* 2180.

Matings, plasmid preparation, endonuclease digestion, cloning and transformation procedures, and electron microscopy. Matings, plasmid preparation, endonuclease digestion, cloning and transformation procedures, and electron microscopy have been described before by Meyer et al. (13).

Isolation of cefotaxime-resistant mutants with hydroxylamine. Cells were grown to an optical density of 1.0 at 546 nm at 37°C and incubated with 0.1 mM hydroxylamine for 4 h. Subsequently the cells were spread on cefotaxime-containing agar plates (0.5 and 1.0 μg/ml).

Preparation of crude β-lactamase extracts. Cultures were grown in the absence of antibiotics and disrupted by sonication (16).

Analytical isoelectrical focusing. The isoelectric points (pI's) of the enzymes were determined by the method described by Vesterberg (23) and Seeberg et al. (18). TEM-1 (pI, 5.4), SHV-1 (pI, 7.6), and three chromosomal β-lactamases of *Enterobacter cloacae* with pI's of 7.8, 8.0, and 9.1 were chosen as standards.

β-Lactamase assays. β-Lactamase activity for cephalosporins was determined by the spectrophotometric method of O'Callaghan (14) with a temperature-controlled spectrophotometer (model DG-G; Beckman Instruments, Inc., Fullerton, Calif.) at 37°C; the absorption maxima of the cephalosporins was as described by Seeberg et al. (18). For measuring the activity with penicillins, the microiodometric method (20) was used. One unit of enzymatic activity was defined as the amount of enzyme that hydrolyzes 1 μmol of substrate in 1 min at 37°C in 0.05 M sodium-potassium buffer (pH 7.0). Protein concentration for calculating specific activity was measured by the method of Lowry et al. (11).

Inhibition of β-lactamase. By using *p*-chloromercuribenzoate, cloxacillin, and clavulanic acid as inhibitors, the inhibition of the β-lactamase activity was tested after preincubation of enzyme and inhibitor for 10 min at 37°C.

I_{50} determination. The concentrations at which substrate hydrolysis was inhibited by 50% (I_{50}) were determined by using PADAC (17) as substrate and cephalosporins as inhibitors. PADAC, a chromogenic cephalosporin, had its absorption maximum at 578 nm (in 0.05 M Tris hydrochloride buffer [pH 7.3] at 37°C). At this wavelength, the decomposition of the inhibitor did not affect the rate of hydrolysis of PADAC. PADAC hydrolysis rates were measured for different inhibitor concentrations. The I_{50} was calculated from the inhibition curve (percentage of inhibition as a function of the inhibitor concentration).

RESULTS

A cefotaxime-resistant clinical isolate, *K. ozaenae* 2180, was tested for its susceptibility to cephalosporins of the expanded spectrum, penicillins, and monobactams (Table 2). *K. ozaenae* 2180 was at least relatively resistant to all compounds tested, with the exception of cefotetan. In mating experiments with *K. ozaenae* 2180 and *Escherichia coli* W3110, it was demonstrated that the resistance was plasmid mediated. Resistance to cefotaxime was transferred at a frequency of 2.6×10^{-5} related to cell number of the donor strain. The transconjugant strain showed the same MICs as did the donor strain (Table 2). We determined the pI's of β-lactamases from crude enzyme extracts of *K. ozaenae* and *Escherichia coli* transconjugant. Figure 1 shows the position of the enzymes in the pH gradient. It can be seen that the β-lactamases produced by *K. ozaenae* and the transconjugant have the same pI as β-lactamase SHV-1, i.e., 7.6. From these findings, we assumed a similarity between SHV-1 and the *K. ozaenae* β-lactamase.

Characterization of pBP60. From a transconjugant, we isolated a plasmid of 46 kilobases (kb). It coded for cefotaxime, chloramphenicol, and streptomycin resistance (Table 1). The plasmid was further analyzed by restriction endonuclease digestion with *Pst*I, and 14 fragments sizes from 11.5 to 0.5 kb were found.

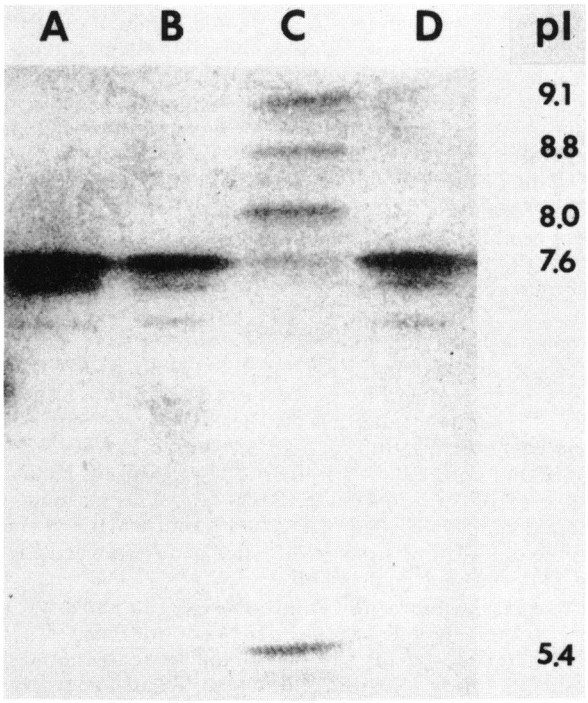

FIG. 1. Isoelectric focusing of β-lactamases produced by the following: lane A, *K. ozaenae* 2180 (SHV-2; pI, 7.6); lane B, *E. coli* pBP60 (SHV-2; pI, 7.6); lane C, markers TEM-1 (pI, 5.4), SHV-1, (pI, 7.6), and *E. cloacae* β-lactamases (pI's, 8.0, 8.8, and 9.1); lane D, *E. coli* p453 (SHV-1; pI, 7.6).

A comparison of the *Pst*I restriction pattern of pBP60 with that of p453 (determining SHV-1) demonstrated that eight fragments, with sizes of 2.1, 1.6, 1.15, 1.0, 0.8, 0.76, 0.61, and 0.52 kb, were identical. These findings were further evidence for the assumption that the genes for the SHV-1 β-lactamase and the β-lactamase coded by pBP60 might be related.

Cloning of the *bla* gene from pBP60. For further studies with the β-lactamase gene (*bla*) of pBP60, we cloned *Eco*RI fragments of pBP60 into the *Eco*RI site of pACYC184 (2). The cloned DNA was used to transform *Escherichia coli* JC2926 with selection for cefotaxime and tetracycline. From one of the transformants, we isolated a plasmid, designated pBP60-1, that contained the 4.0-kb *Eco*RI fragment of the vector and a 9.0-kb *Eco*RI fragment from pBP60, including its *bla* gene.

pBP60-1 was further subcloned with *Pst*I, with pUC8 (24) as vector. The resulting plasmid, pBP60-1-1, harbored a 2.7-kb *Pst*I fragment for the vector and two *Pst*I fragments with sizes of 1.6 and 0.8 kb, which mediated cefotaxime resistance.

Cloning of the SHV-1 gene. The SHV-1 gene from p453 (10, 15) was cloned into plasmid pBP1 (22) with *Eco*RI. Plasmid DNA of a representative clone was digested with *Eco*RI, yielding the 6.0-kb fragment of pBP1 and a 7.6-kb fragment of p453 coding for SHV-1 β-lactamase. This clone was designated pBP65. A comparison of the *Pst*I restriction patterns of pBP60-1 and pBP65 (Fig. 2) showed that seven *Pst*I fragments (6.4 kb) were identical.

Heteroduplex between pBP65 and pBP60-1-1. To determine the homology between the *bla* genes of pBP65 (SHV-1) and pBP60-1-1 (new β-lactamase), the plasmids were subjected to heteroduplex analysis. The electron micrograph (Fig. 3) revealed that both plasmids hybridize over a length of 2.4 kb, completely corresponding to the cloned fragment of pBP60-1 in pUC8. Thus, the heteroduplex indicated that the genes for the β-lactamases mediated by pBP60-1-1 and pBP65 are homologous. Because of this close homology and the identical pI's of the two enzymes, we designated the β-lactamase mediated by pBP60 as SHV-2.

Isolation of mutants of an SHV-1-producing strain to cefotaxime resistance. To verify our assumption that the SHV-2 gene is a mutation of the SHV-1 gene, we tried to mutate the gene coding for SHV-1 towards resistance to broad-spectrum cephalosporins. Cells (10^9) of an SHV-1-producing strain (JC2926 containing pBP65) were plated on cefotaxime-containing agar, but no resistant colonies were detected. However, by treating JC2926(pBP65) with hydroylamine, cefotaxime-resistant clones were obtained with a frequency of 10^{-8} related to total cell number. The resistance of one clone was transferred by transformation to *Escherichia coli* W3110 and correlated with the production of a β-lactamase with a pI of 7.6, designated SHV-1_{mut}. The plasmid with mutated gene was called pBP65-1. From the MICs (Table 2) and the identical pI's observed, similarities between SHV-2 and SHV-1 could be deduced.

Characterization of the β-lactamases. The substrate profiles of the enzymes SHV-1, SHV-2, and SHV-1_{mut} for several cephalosporins and penicillins were compared with those of other plasmid-mediated broad-spectrum β-lactamases: TEM-1, TEM-2 (6), and HMS-1 (12) (Table 3). The substrate profiles of SHV-1, SHV-2, and SHV-1_{mut} were nearly the same and differed markedly from those of TEM-1, TEM-2, and HMS-1. SHV-2 and SHV-1_{mut} were able to hydrolyze monobactams and broad-spectrum cephalosporins such as cefotaxime. Neither enzymes was active against oxacillin and cefotetan, which correlated in the case of cefotetan with the low MICs of 0.25 μg/ml. The enzyme inhibition with *p*-chloromercuribenzoate, cloxacillin, and clavulanic acid also showed the close relationship between SHV-1, SHV-2, and the mutant enzyme (Table 4). The variation in the inhibition with *p*-chloromercuribenzoate, sensitive in the hydrolysis of cephaloridine and resistant in the hydrolysis of benzylpenicillin, is unique for SHV-1. It was the reason for the name of this enzyme: sulfhydryl variable (12). SHV-2 and SHV-1_{mut} also showed this phenomenon. Since SHV-1_{mut} and SHV-2 were able to hydrolyze cephalosporins that were usually known to be β-lactamase stable (3), we were interested in the kinetic parameters V_{max}, K_m, and I_{50} for cefotaxime, HR 810, cefotetan, and cephaloridine. The data (Table 5) show the close relationship between SHV-2 and SHV-1_{mut} in the ability to hydrolyze cefotaxime and HR 810 and in the corresponding I_{50}. Differences in the three parameters for cefotaxime and HR 810 were detectable in comparisons of SHV-1 with SHV-2 and SHV-1_{mut}.

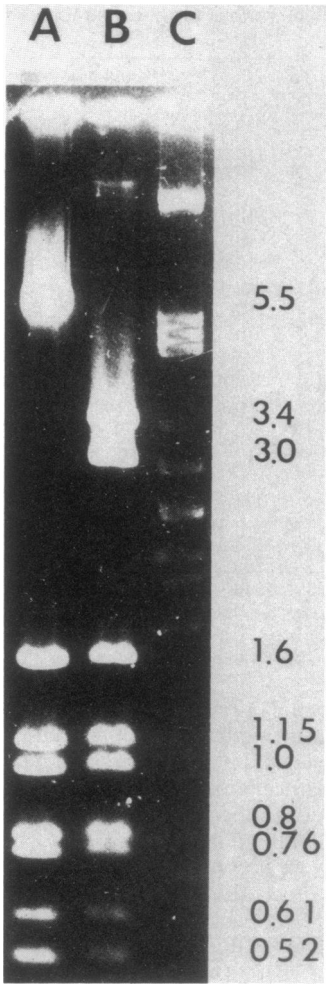

FIG. 2. *Pst*I digestion of plasmid pBP60-1 (lane A), pBP65 (lane B), and phage λ (lane C). Numbers indicate size in kilobases.

DISCUSSION

With the isolation and characterization of SHV-2, encoded by pBP60, from *K. ozaenae*, a transferable resistance mechanism to third-generation cephalosporins is reported. The

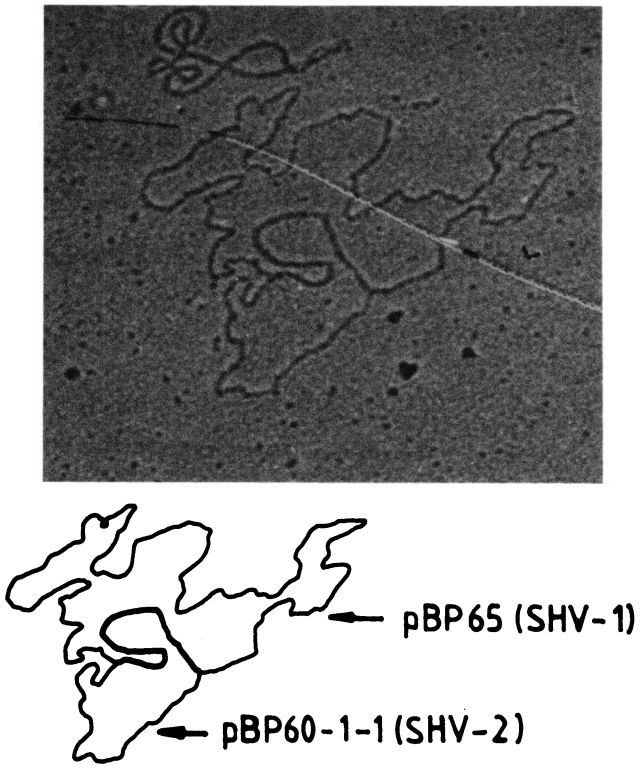

FIG. 3. Electron micrograph and drawing of a heteroduplex between pBP60-1-1 and pBP65, both undigested.

transconjugant gained the ability to produce a β-lactamase with a pI of 7.6, which is identical to that of the SHV-1 enzyme, leading to the assumption that both enzymes might be related. Therefore, we compared the enzymatic character and the genetic basis of the new β-lactamase, SHV-2, with those of SHV-1. The comparison between the genes encoding SHV-1 and SHV-2 was performed by restriction and heteroduplex analysis and showed an extensive sequence homology between both genes, by hybridization in full length. As only minor differences between two genes are not detectable by this method, the close relationship between the SHV-1 and SHV-2 genes led us to the assumption that only a few mutational steps can convert an SHV-1 producing strain into a strain producing a cefotaxime-hydrolyzing enzyme. This view was strongly supported by the isolation of a laboratory mutant from an SHV-1-producing *Escherichia coli* strain that behaved similarly to the SHV-2-producing one. As we have not been able to generate mutants without using mutagenic agents, which implies a mutation rate of less than 10^{-9}, we believe that at least 2 base pairs must change for the conversion of the SHV-1 gene to the SHV-1_{mut} gene. From the enzymatic studies that showed similarities between SHV-1_{mut} and SHV-2, we deduced that the two enzymes are closely related. Therefore, it is tempting to assume that the SHV-1_{mut} gene and the SHV-2 gene are both derived from the SHV-1 gene and that the SHV-2 gene can be regarded as a natural mutation of the SHV-1 gene. Both mutant enzymes were able to hydrolyze third-generation cephalosporins at a considerable rate, and their specific activities increased at least twofold over that of SHV-1 for most antibiotics tested. Other enzymes mediating resistance to these compounds have been isolated in *Escherichia coli* (19), *C. freundii* (21), *Proteus vulgaris* (8), and *Pseudomonas cepacia* (7), but none of these enzymes has any similarities with those described above, and they are all chromosomally mediated. Others (25; B. Wiedemann, R. M. Tolxdorff-Neutzling, J. Dorn, and I. Bömmer, manuscript submitted for publication) have shown that the level of resistance does not depend solely on the rate of hydrolysis but also on the affinity to the β-lactamase in competition with the affinity to the penicillin-binding proteins, the quantity of the enzyme, and the penetration through the outer membrane. For a given β-lactam molecule, the above-mentioned parameters are identical for the three β-lactamase-producing strains, except for the affinity of the substrate to the β-lactamase and the rate of hydrolysis. For cefotaxime, the rate of hydrolysis is considerably higher for SHV-2 and SHV-1_{mut} compared with that for SHV-1. This can be explained by the increase in the affinity by a factor of about 350 for both enzymes, as deduced from the I_{50}. Changes in both of these parameters, the affinity and the rate of hydrolysis, are responsible for the increased MICs. The affinity of cephaloridine and cefotetan for the enzymes is nearly the same with the mutants. HR 810 takes an intermediate position. Both mutant enzymes show a lower rate of hydrolysis than for cefotaxime, which was corresponding to a lower affinity of HR 810 for the enzymes. Thus, it becomes clear that the increase in MICs for the SHV-2- and SHV-1_{mut}-producing strains must be higher for

TABLE 3. Substrate profiles of SHV, TEM, and HMS enzymes

Drug	Rate of hydrolysis by[a]:					
	SHV-2	SHV-1	SHV-1_{mut}	TEM-1[b]	TEM-2[b]	HMS-1[c]
Ampicillin	145	216	250	106	107	253
Oxacillin	0	0	0	5	5	2
Aztreonam	1.4	0	6	—[d]	—	—
Cephaloridine	32	62	193	76	74	193
Cephalothin	8	15	80	20	20	3
Cefotiam	33	0	70	—	—	—
Cefuroxime	1	2.5	8	2	2	2
Cefotaxime	4	0	23	—	—	—
Cefotetan	0	0	0	—	—	—
HR 810	1.4	0	1.2	—	—	—

[a] The rate for benzylpenicillin, defined as 100, was 0.69 μmol/min per mg for SHV-2, 0.24 μmol/min per mg for SHV-1, and 0.40 μmol/min per mg for SHV-1_{mut}.
[b] Data were obtained from Hedges et al. (6).
[c] Data were obtained from Matthew et al. (12).
[d] —, No data available.

TABLE 4. Enzyme inhibition of SHV, TEM, and HMS enzymes

Inhibitor	Response of[a]:					
	SHV-2	SHV-1	SHV-1_{mut}	TEM-1	TEM-2	HMS-1
Cloxacillin (100 μM)[b]	S	S	S	S	S	S
Clavulanic acid (100 μM)[b]	S	S	S	—	—	—
p-Chloromercuribenzoate (0.5 mM)[c]						
Cephaloridine	S	S	S	R	R	S
Benzylpenicillin	R	R	R	R	R	S

[a] S, Sensitive; R, resistant; —, no data available.
[b] Cephaloridine (0.1 mM) was used as substrate.
[c] Either cephaloridine or benzylpenicillin was used as a substrate.

TABLE 5. Kinetic parameters

Strain containing:	Parameters for:											
	Cephaloridine			Cefotaxime			HR 810			Cefotetan		
	MIC (μg/ml)	V_{max} (U/mg)[a]	K_m (μM)	I_{50} (μM)[b]	MIC (μg/ml)	V_{max} (U/mg)	K_m (μM)	I_{50} (μM)	MIC (μg/ml)	V_{max} (U/mg)	K_m (μM)	I_{50} (μM)	MIC (μg/ml)	V_{max} (U/mg)	K_m (μM)	I_{50} (μM)
SHV-2	64	0.29	20	30	4	0.1	12	18	1	0.012	16	100	0.25	0	ND[c]	200
SHV-1	16	0.32	28	20	0.03	0	ND	7,000	0.03	0	ND	2,700	0.06	0	ND	780
SHV-1_{mut}	64	0.38	15	30	4	0.05	15	14	1	0.012	20	100	0.125	0	ND	150

[a] Specific activity, given in micromoles per minute per milligram.
[b] PADAC was used as substrate.
[c] ND, Not determinable.

cefotaxime than for HR 810, due to the higher affinity of cefotaxime for the enzymes.

Despite the low mutation rate for the conversion of the SHV-1 gene to the SHV-1_{mut} gene, it seems probable that this sort of mutation can be selected in patients, as shown with SHV-2. This implies that similar mutants or even more effective ones can be selected by increasing the use of broad-spectrum cephalosporins. Similar changes from naturally occurring plasmids have not yet been described in the literature. Hall and Knowles (5), however, have produced mutants in the TEM-1 gene, which lead, in parallel with our findings, to an altered enzymatic behavior. As we have demonstrated that bacterial populations have the potential to create transferable resistance to broad-spectrum cephalosporins, these modern compounds should be handled carefully, and the development of resistance to these drugs should be monitored by microbiological means.

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