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A New SHV-Derived Extended-Spectrum β-Lactamase (SHV-24) That Hydrolyzes Ceftazidime through a Single-Amino-Acid Substitution (D179G) in the Ω-Loop

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A new SHV-derived extended-spectrum β-lactamase (SHV-24) conferring high-level resistance to ceftazidime but not cefotaxime and cefazolin was identified in Japan. This enzyme was encoded by a transferable 150-kb plasmid from an *Escherichia coli* clinical isolate. The pI and K_m for CAZ of this enzyme were 7.5 and 30 μM, respectively. SHV-24 was found to have a D179G substitution in the Ω-loop of the enzyme.

TEM- and SHV-derived extended-spectrum β-lactamases (ESBLs) that give resistance to the oxyimino-β-lactams such as ceftazidime (CAZ) and/or cefotaxime (CTX) have become a general concern (5, 9), and at least 12 SHV-derived ESBLs and more than 70 TEM-derived ESBLs have been enrolled in the EMBL-GenBank and SWISS-PROT databases. Several CTX-resistant *Escherichia coli* isolates producing CTX-M-related β-lactamases such as Toho-1 have been reported so far in Japan (8, 15); however, the presence of TEM- or SHV-derived ESBLs has not been demonstrated. Thus, we made a preliminary survey of ESBL-producing gram-negative rods in Japan (16). Prior to this survey, we isolated a unique *E. coli* strain that shows a high-level resistance to CAZ (MIC, >128 μg/ml). In this study, we report the character of a new SHV-derived ESBL that has a single-amino-acid substitution in the Ω-loop of SHV-type β-lactamases (7, 10).

CAZ-resistant *E. coli* strains were isolated from urine samples of an inpatient in Chiba Prefecture in June 1996. *E. coli* strain HKY453 showed high-level resistance to CAZ (MIC, >128 μg/ml); however, this strain was susceptible to CTX (MIC, 4 μg/ml) and cefazolin (MIC, 4 μg/ml). Strain HKY453 showed intermediate resistance to chloramphenicol (MIC, 16 μg/ml), while the same strain was susceptible to minocycline, streptomycin, gentamicin, nalidixic acid, fluoroquinolones, trimethoprim, and rifampin.

Conjugal transfer of CAZ resistance from *E. coli* strain HKY453 to *E. coli* CSH2 (F^- *metB*, resistant to both nalidixic acid and rifampin) was done by a method described elsewhere (14). *E. coli* CSH2 was provided by T. Sawai, Chiba University School of Medicine, Chiba, Japan. The *E. coli* strain HKY453 and an *E. coli* XL1-Blue clone that harbors recombinant plasmid pSHV001 carrying a 3.1-kb *Bam*HI insert were subjected to a double-disk diffusion test using three disks containing CAZ, CTX, or amoxicillin plus clavulanate. Strain HKY453, an *E. coli* CSH2 transconjugant that received a large plasmid, pCAZR001 (approximately 150 kb in size), carrying a CAZ resistance gene (bla_{SHV-24}), and the clone *E. coli* XL1-Blue (pSHV001) were also subjected to antibiotic susceptibility testing by the agar dilution method according to the protocol recommended by NCCLS (11), and the results are shown in Table 1, as are those obtained with *E. coli* CSH2 and *E. coli* XL1-Blue. *E. coli* CSH2(pCAZR001) showed intermediate resistance to chloramphenicol (MIC, 16 μg/ml). Both the transconjugant and the clone were susceptible to CTX and cefazolin, and resistance levels to CAZ in these strains were lower than that of the parental strain HKY453 (Table 1). Since CAZ resistance was blocked by the presence of 4 μg of clavulanate per ml in both the parental strain and the clone, it was suggested that HKY453 produces, rather than class C or class B β-lactamases, a class A β-lactamase belonging to Bush's class 2be.

PCR analyses using several sets of primers for class A β-lactamases, including TEM-derived ESBLs, Toho-1-type β-lactamase, and SHV-derived ESBLs, were done as described elsewhere (16), and only the PCR using SHV-specific primers yielded a band of amplicon. Thus, it was suggested HKY453 produces a plasmid-dependent SHV-derived ESBL. The total DNA preparation from HKY453 was digested with *Bam*HI and ligated with *Bam*HI cleaved pBCSK+. Then *E. coli* XL1-Blue cells were transformed with the resultant recombinant DNA mixture by an electroporation method, and several CAZ-resistant colonies were isolated. Cloning vector pBCSK+ and *E. coli* strain XL1-Blue were purchased from Stratagene (La Jolla, Calif.). The clone *E. coli* XL1-Blue(pSHV001) showed CAZ resistance, and a PCR product was detected when an SHV-specific primer was used. For confirmation of the location of the bla_{SHV-24} gene in pSHV001, the DNA insert was digested from both sides with mung bean nuclease and exonuclease III with a deletion kit (Nippon Gene Co. Ltd., Toyama, Japan), and the bla_{SHV-24} gene was localized in the central region of the insert of pSHV001. Nucleotide sequencing was performed on PCR products from parental strain HKY453 and plasmid DNA carrying the bla_{SHV-24} gene by dye-terminator methods, using six primers for the SHV-derived ESBL gene (16) on both DNA strands. Mutations found in the nucleotide sequence of the bla_{SHV-24} gene were again checked on these deletion mutants. As a result, the *E. coli* HKY453 and the clone were found to produce a new β-lactamase. A single point mutation was found in the coding region of the β-lactamase gene compared with that encoding SHV-6 (3), and this mutation resulted in a single-amino-acid substitution at amino acid residue 179 (D179G) based on the consensus sequence of SHV-derived ESBLs (1). Two nucleotide mutations were also observed in the genes for SHV-24 and SHV-8 (13). The enzymes used for gene manipulation were purchased from Nip-

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TABLE 1. Results of antibiotic susceptibility testing

Strain	MIC (μg/ml)														
	AMP[a]	PIP	CER	CFZ	CFP	CAZ	CAZ+CVA	CTX	CPM	CMZ	CMX	MOX	ATM	SUL+CFP	IPM
E. coli HKY453	>128	>128	16	4	8	>128	4	4	16	1	1	8	4	1	<0.5
E. coli CSH2(pCAZR001)	>128	128	8	2	4	64	1	1	4	1	0.5	2	1	<0.5	<0.5
E. coli CSH2	2	1	1	1	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
E. coli XL1-Blue(pSHV001)	>128	>128	8	4	4	128	1	2	8	1	0.5	2	1	0.5	<0.5
E. coli XL1-Blue	2	2	1	1	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5

[a] Abbreviations: AMP, ampicillin; PIP, piperacillin; CER, cephaloridine; CFZ, cefazolin; CFP, cefoperazone; CAZ, ceftazidime; CAZ+CVA, ceftazidime and clavulanic acid (4 μg/ml); CTX, cefotaxime; CPM, cefpirome; CMZ, cefmetazole, CMX, cefminox; MOX, moxalactam; ATM, aztreonam; SUL+CFP, sulbactam and cefoperazone (1:1); IPM, imipenem.

pon Gene Co. Ltd. (Tokyo, Japan) or TAKARA Co. Ltd. (Kyoto, Japan). The primers for PCR and sequencing analyses were designed by using the GENETYX system, version 5.0.0 (SDC, Tokyo, Japan) and made by TAKARA Co. Ltd. The nucleotide sequences and amino acid sequences of SHV-derived ESBLs were downloaded from the EMBL-GenBank and SWISS-PROT databases, respectively. The nucleotide sequence determined in this study was analyzed by using the GENETYX system, version 10.0. The consensus sequence of SHV-derived ESBLs was referred to a research organization for inclusion in a standard numbering scheme of Ambler et al. (1) and Jacoby (9) (more information can be found elsewhere [http://www
.lahey.org/studies/webt.html]).

An *E. coli* clone carrying bla_{SHV-24} was cultured in 2 liters of Luria-Bertani broth at 37°C overnight, and then bacterial cells were harvested by centrifugation (15,000 × *g* for 20 min). SHV-24 was purified according to a method described previously (15). Purified SHV-24 was subjected to isoelectric focusing by using IPGphor (Amersham Pharmacia Biotech, Uppsala, Sweden). Determination of kinetic parameters was done according to a method described previously (2) using purified SHV-24. The molar extinction coefficients (Δε) used were as follows: for ampicillin (AMP) (235 nm), $\Delta\varepsilon = 1.2\ mM^{-1}\ cm^{-1}$; for aztreonam (315 nm), $\Delta\varepsilon = 0.68\ mM^{-1}\ cm^{-1}$; for cephaloridine (295 nm), $\Delta\varepsilon = 1.0\ mM^{-1}\ cm^{-1}$; for CTX (264 nm), $\Delta\varepsilon = 7.25\ mM^{-1}\ cm^{-1}$; and for CAZ (272 nm), $\Delta\varepsilon = 7.6\ mM^{-1}\ cm^{-1}$. The K_i values of clavulanic acid were determined with AMP and CAZ, respectively.

The kinetic parameters of SHV-24 are shown in Table 2. These data suggest that SHV-24 can hydrolyze AMP like SHV-1. It is also suggested that the affinity of CAZ to SHV-24 was similar to that of AMP. This enzyme, however, hydrolyzes CAZ with a very low catalytic rate. Cephaloridine and aztreonam were also hydrolyzed detectably, but hydrolysis of CTX by SHV-24 was too poor to calculate the kinetic parameters. Four micrograms of clavulanic acid per milliliter blocked SHV-24, an effect that was observed for the other ESBLs. According to the kinetic parameters of SHV-24, this enzyme is able to hydrolyze CAZ with a low catalytic rate, but the parental strain, *E. coli* HKY453, demonstrated a relatively high level of resistance to CAZ, as shown in Table 1. A similar observation was reported for SHV-6, and this finding may implicate other factors, such as alterations in the bacterial cell membrane, in the high-level CAZ resistance in HKY453. This speculation was supported by the fact that the MIC of CAZ for the *E. coli* XL1-Blue clone producing SHV-24 was lower than that for parental strain HKY453 despite multicopy expression of the *bla* gene in the clone. Hence, both production of a large amount of SHV-24 and decreased permeation of CAZ through the bacterial outer membrane might be implicated in the high level of CAZ resistance in *E. coli* strain HKY453.

TABLE 2. Kinetic parameters of SHV-24

β-Lactam antibiotic[a]	V_{max}[b] (μM/min)	K_m (μM)	V_{max}/K_m	Relative V_{max}/K_m	K_i[c] (nM)
AMP	2.00	32	0.0625	100	57
CER	2.37	210	0.0113	18.1	ND[d]
ATM	0.735	500	0.00147	2.35	ND
CAZ	0.043	30	0.000143	0.23	37

[a] AMP, ampicillin; CER, cephaloridine; ATM, aztreonam; CAZ, ceftazidime.
[b] Calculated with 5.46 μg of purified SHV-24.
[c] K_i was calculated using clavulanic acid.
[d] ND, not done.

A single-amino-acid substitution (D179G) was found in the newly identified SHV-24. Since Asp179 makes a salt bridge to Arg164 across the neck of the Ω-loop (10, 13), an amino acid substitution from Asp to Gly that has no anionic R side chain will cancel the salt bridge and cause the distortion of the Ω-loop at the active site of this enzyme, and this may well result in a change in the enzyme substrate specificity. The same amino acid substitution (D179G) has been documented in an OHIO-type β-lactamase that also hydrolyzes CAZ (4). Similar single-amino-acid substitutions at residue 179 are observed in SHV-6 (D179A) (3) and SHV-8 (D179N) (13). SHV-24 had an isoelectric point (pI = 7.5) similar to that of SHV-6 (pI = 7.6). Hence, from the viewpoint of enzyme kinetics and molecular features, it seems reasonable to separate the group of SHV-6, SHV-8, and SHV-24 from the group of SHV-2, SHV-3, SHV-4, and SHV-5, which have a common amino acid substitution (G238S) between two β-strands, B3 and B4 (7, 10).

SHV-24 has not been found so far in Europe or the United States, where many SHV-derived ESBLs have been identified. Much clinical use of CAZ in Japan might lead to the emergence of this kind of new enzyme that enables bacteria to survive in a high-level concentration of CAZ such as was found in the urine sample of an inpatient. Strain HKY453 was isolated from a urine sample and demonstrates high-level CAZ resistance (MIC, >128 μg/ml), but it was apparently susceptible to cefazolin (MIC, 4 μg/ml), as shown in Table 1. Emergence of this kind of enzyme may reflect the trend of antibiotic use in Japan, where the broad-spectrum cephems tend to be preferentially used rather than the first-generation cephems and penicillins.

Various *Klebsiella pneumoniae* and *E. coli* strains producing plasmid-mediated TEM- or SHV-derived ESBLs have been found worldwide. In Japan, however, no TEM- or SHV-derived ESBL producer had been recognized until our recent survey on ESBL producers (16), although several outbreaks of *E. coli* that produce Toho-1-type class A enzyme had been reported (8, 15). It has been speculated that this phenomenon

may have something to do with the widespread use of cephamycins and carbapenems as first-line drugs in Japan. Recently, however, restricted use of these agents was recommended for several clinical settings to prevent further dissemination of IMP-1-type metallo-β-lactamase-producing gram-negative bacteria such as *Serratia marcescens* (12) and *Pseudomonas aeruginosa* (14), and this may contrarily induce proliferation of CAZ- or CTX-resistant bacteria that produce TEM- or SHV-derived ESBLs, as well as CTX-M-type enzymes, including Toho-1 and MEN-1, in Japan hereafter.

Nucleotide sequence accession number. The nucleotide sequence of the coding region of the β-lactamase gene has been deposited in the EMBL-GenBank nucleotide sequence data banks through the DNA Data Bank of Japan (accession number AB023477).

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