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Extended-Spectrum β-Lactamases in Clinical Isolates of *Enterobacter gergoviae* and *Escherichia coli* in China

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Resistance to ceftazidime, detected in isolates of *Escherichia coli* 5518 and *Enterobacter gergoviae* 3773 from our hospital, was transferred, together with resistance to aminoglycosides, trimethoprim, sulfonamide, and other β-lactam antibiotics, by conjugation to *E. coli* JP559. Both *E. coli* transconjugants were resistant to ampicillin, all cephalosporins, and aztreonam but remained susceptible to cefoxitin and imipenem. The enzymes of the two transconjugant strains readily hydrolyzed cephalosporins in a spectrophotometric assay. Hybridization results suggested that the extended-spectrum β-lactamase produced by *E. coli* 5518 was a non-TEM, non-SHV enzyme, the origin of which is currently unknown. The β-lactamase produced by *E. gergoviae* 3773 was of the SHV type and was further proved to be SHV-2 by DNA sequencing. Thus, extended-spectrum β-lactamases are occurring in China as well as in other parts of the world.

The extended-spectrum cephalosporins and other new β-lactams, such as monobactams, were once thought to be poorly hydrolyzable by most β-lactamases. Thus, they have been used extensively clinically. In some species, such as *Pseudomonas aeruginosa* and *Enterobacter cloacae*, emergence of resistance during therapy with one of these new β-lactams by overproduction of chromosomally mediated β-lactamase was documented (17, 25, 26). The first extended-spectrum β-lactamase, designated SHV-2, was reported in Germany in 1983 (3, 14). Since then, more and more extended-spectrum β-lactamases have been detected across the world (11, 15, 22). In this study, we describe two extended-spectrum β-lactamases in multiresistant isolates of *Enterobacter gergoviae* 3773 and *Escherichia coli* 5518 from Peking Union Medical College Hospital.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains and plasmids used in this study are listed in Table 1. *E. gergoviae* 3773 and *E. coli* 5518 (harboring resistant plasmids pC3773 and pC5518, respectively) were isolated from abdominal abscesses in two postsurgery patients at Peking Union Medical College Hospital in 1991. *E. coli* HB101 was used for cloning. *E. coli* JM101 was utilized as the recipient for M13mp19 recombinant bacteriophage for nucleotide sequencing. Strains were identified by the API 20E system (BioMérieux, Marcy l'Etoile, France).

Antibiotics. Antibiotics used were rifampin, cephaloridine, and cefazolin (Tuobin Pharmaceutical Works); nalidixic acid (Boshan Pharmaceutical Works); ampicillin (Shanghai no. 4 Pharmaceutical Works); cefuroxime and ceftazidime (Glaxo Pharmaceuticals, Ltd.); cephradine (E. R. Squibb & Sons Inc.); cefoperazone (Pfizer Inc.); ceftriaxone (F. Hoffmann-La Roche Inc.); cefotaxime (Changzheng Pharmaceutical Works); cefoxitin (Merck Sharp & Dohme); and clavulanate (Beecham Laboratories).

Susceptibility testing. MICs of β-lactam antibiotics were determined by the agar dilution method in Mueller-Hinton agar with an inoculum of 10^5 to 10^6 CFU, alone or in combination with clavulanate (2 μg/ml). Plates were incubated at 37°C for 18 h, and the MIC was recorded as the lowest concentration that totally inhibited growth, disregarding a single colony or a faint haze caused by the inoculum (19). Agar disk diffusion susceptibility tests were performed according to the guidelines of the National Committee for Clinical Laboratory Standards (20).

Conjugation. Conjugation experiments were carried out by the membrane filter mating technique (5). The recipient was *E. coli* K-12 JP559 (Rif^r Nal^r). A 0.5-ml volume of the donor culture and 1.0 ml of the recipient culture were mixed in a microcentrifuge tube, and the tube was centrifuged at 12,352 × *g* for 3 min. The cell pellet was resuspended in 50 μl of brain heart infusion (BHI) broth, transferred onto a 2.5-cm-diameter, 0.45-μm-pore-size filter on a prewarmed BHI agar plate, and incubated at 37°C for 4 to 6 h. The filter was then immersed in 2 ml of BHI broth, the cells were resuspended by scraping with a wire loop, and 0.2 ml of the resuspension was spread on transconjugant-selecting plates containing ceftazidime (12 μg/ml), rifampin (380 μg/ml), and nalidixic acid (380 μg/ml).

β-Lactamase assay. β-Lactamases were obtained from transconjugants as follows (23, 28, 31). Overnight cultures in BHI broth were diluted 100-fold with fresh broth containing ampicillin (20 μg/ml) or ceftazidime (12 μg/ml) and incubated with shaking for 20 h at 37°C. Cells were harvested by centrifugation at 4°C, washed with 0.1 M phosphate buffer (pH 7.0), and resuspended in 1/50 of the original volume. After sonication at 0°C, crude extracts were obtained by ultracentrifugation at 45,000 × *g* at 4°C.

The β-lactamase assay was performed spectrophotometrically by measuring the change in absorbance at the appropriate wavelength for each substrate. The wavelengths were 255 nm for cephaloridine; 265 nm for cefazolin and cefuroxime; 275 nm for cefoperazone; 257 nm for ceftriaxone, cefotaxime, and ceftazidime; and 260 nm for cefoxitin. β-Lactamase activity was determined in 3 ml of a 100 μM substrate in 10 mM phosphate buffer, pH 7.0. Substrate blanks were recorded for each reaction.

One unit of activity was defined as the amount of enzyme that hydrolyzed 1 μM cephaloridine per min at 37°C and pH

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TABLE 1. Bacterial strains and plasmids used in this study

Strain	Plasmid	Relevant markers[a]	Source
E. coli 5518	pC5518	Caz^r Kan^r Str^r Gen^r Tmp^r	Clinical isolate
E. gergoviae 3773	pC3773	Caz^r Kan^r Str^r Tmp^r	Clinical isolate
E. coli JP559		Rif^r Nal^r	J. Pittart
E. coli C5518	pC5518	Caz^r Kan^r Str^r Gen^r Tmp^r Rif^r Nal^r	This work
E. coli C3773	pC3773	Caz^r Kan^r Str^r Gen^r Tmp^r Rif^r Nal^r	This work
E. coli HB101	pMON38	Amp^r (SHV-1) Cm	G. A. Jacoby
E. coli C600	pBR322	Amp^r (TEM-1) Tet^r	G. A. Jacoby
E. coli HB101	pCHEN1	Tet^r Caz^r	This work
E. coli HB101			C. F. Huang
E. coli JM101			C. F. Huang
E. coli ATCC 25922			American Type Culture Collection

[a] Abbreviations: Caz^r, resistance to ceftazidime; Tmp^r, resistance to trimethoprim-sulfamethoxazole; Kan^r, resistance to kanamycin; Str^r, resistance to streptomycin; Gen^r, resistance to gentamicin; Rif^r, resistance to rifampin; Nal^r, resistance to nalidixic acid; Amp^r, resistance to ampicillin; Cm, resistance to chloramphenicol; Tet^r, resistance to tetracycline.

7.0. The rates of hydrolysis calculated were relative to that of cephaloridine (100%).

DNA agarose gel electrophoresis. Plasmid DNA was extracted from clinical isolates and transconjugants by the rapid procedure of Takahashi and Nagano (29), and the crude extract was used directly for electrophoresis, which was performed in a 0.7% agarose gel for 2 h at 10 V/cm. The size of the plasmids was roughly determined by using pMON21 (7.5 kb).

Nucleic acid techniques. For large-scale preparations, plasmid DNA was extracted from a 1-liter culture and purified by ultracentrifugation in cesium chloride-ethidium bromide discontinuous gradients (24). Plasmid DNA was digested with restriction endonuclease according to the recommendations of the manufacturers. The 420-bp *Bgl*I-*Hin*cII fragment and the 360-bp *Pvu*II-*Pvu*II fragment (internal to the structural genes for TEM-1 of pBR322 and for SHV-1 of pMON38, respectively) and the 450-bp *Pvu*II-*Cla*I fragment (containing the partial structural gene for SHV-1 of pMON38) were used as probes (10, 18). DNA was labeled with [α-^{32}P]ATP by nick translation, and hybridization was carried out under stringent conditions after DNA was transferred to nitrocellulose sheets by the method of Southern (10). Cloning and subcloning were carried out as described by Sambrook et al. (24). DNA sequencing was performed by the dideoxy polymerase chain termination method of Sanger et al. (8, 27).

RESULTS

Antibiotic susceptibilities. The resistance phenotypes of the clinical isolates of *E. gergoviae* 3773 and *E. coli* 5518 were determined by the disk diffusion assay (results not shown). These two isolates were highly resistant to ampicillin, piperacillin, carbenicillin, cephalothin, cephaloridine, cefazolin, and cefuroxime. *E. gergoviae* 3773 was moderately susceptible to

TABLE 2. MICs of β-lactams for *E. coli* and its derivatives producing different plasmid-mediated β-lactamases

β-Lactam[a]	MIC (μg/ml) for strain:					
	JP559(pC3773)	JP559(pC5518)	HB101(pMON38)	JP559	HB101	ATCC 25922
Ampicillin	>2,048	>2,048	2,048	4	4	4
Ampicillin plus clavulanate	16	32	4	2	2	2
Cefazolin	256	512	8	2	2	1
Cefazolin plus clavulanate	1	1	0.5	0.5	0.5	0.5
Cephradine	256	256	16	8	8	8
Cephradine plus clavulanate	16	8	8	8	8	8
Cefuroxime	128	256	4	4	1	4
Cefuroxime plus clavulanate	8	4	2	2	1	2
Ceftazidime	16	128	1	0.125	0.06	0.06
Ceftazidime plus clavulanate	1	1	0.125	<0.06	<0.06	<0.06
Ceftriaxone	32	>64	<0.06	<0.06	<0.06	<0.06
Ceftriaxone plus clavulanate	<0.06	<0.06	<0.06	<0.06	<0.06	<0.06
Cefotaxime	32	>64	<0.06	<0.06	<0.06	<0.06
Cefotaxime plus clavulanate	<0.06	<0.06	<0.06	<0.06	<0.06	<0.06
Cefoperazone	64	64	4	0.125	0.125	0.25
Cefoperazone plus clavulanate	0.5	0.25	<0.06	<0.06	<0.06	<0.06
Cefoxitin	1	2	2	2	2	2
Aztreonam	8	32	—[b]	—	—	—
Imipenem	<0.5	<0.5	—	—	—	—

[a] The concentration of clavulanate is a constant 2 μg/ml.

[b] —, not done.

TABLE 3. Hydrolysis of β-lactams by crude extract β-lactamases

Source of enzymes	Enzyme activity (U/ml)	Rate of hydrolysis relative to that of CLR (%)[a]							
		CLR	CZL	CRX	CFP	CTR	CTX	CAZ	FOX
E. coli pc5518	6.1	100	93	72	87	77	75	66	5
E. coli pc3773	1.4	100	89	69	82	75	67	61	3
E. coli HB101(pMON38)	6.9	100	70	12	65	8	4	6	6

[a] Abbreviations: CLR, cephaloridine; CZL, cefazolin; CRX, cefuroxime; CFP, cefoperazone; CTR, ceftriaxone; CTX, cefotaxime; CAZ, ceftazidime; FOX, cefoxitin.

ceftazidime and ceftriaxone (inhibition zone diameters of 17 and 14 mm, respectively) and resistant to chloramphenicol, and *E. coli* 5518 was resistant to ceftazidime and ceftriaxone. Both of them were also resistant to streptomycin, kanamycin, gentamicin, and trimethoprim-sulfamethoxazole.

MICs and substrate profile. MICs of β-lactams, alone and combined with clavulanate (2 μg/ml), against *E. coli* transconjugants harboring pC3773 or pC5518 compared with those against *E. coli* harboring pMON38 (SHV-1) are shown in Table 2. MICs of the extended-spectrum cephalosporins for *E. coli* harboring pC5518 were slightly higher than those for *E. coli* harboring pC3773. *E. coli* transconjugants harboring pC5518 or pC3773 also showed decreased susceptibility to aztreonam (MIC, 16 to 32 μg/ml) but remained susceptible to cefoxitin (MIC, 1 to 2 μg/ml) and imipenem (MIC < 0.5 μg/ml). Activities of ampicillin, cefazolin, cephradine, cefuroxime, ceftazidime, ceftriaxone, cefotaxime, and cefoperazone were enhanced by the addition of 2 μg of clavulanate per ml. The substrate profile of β-lactamase obtained from *E. coli* transconjugants harboring pC3773 or pC5518 was compared with that of SHV-1 and showed an extended-spectrum profile (Table 3). The rates of hydrolysis of agents tested were very high (>61%), with the exception of that of cefoxitin (3 to 5%).

Characterization of the specifying plasmid. Plasmid DNA from the clinical isolates of *E. gergoviae* 3773 and *E. coli* 5518 and their *E. coli* transconjugants was analyzed by agarose gel electrophoresis (Fig. 1). A plasmid of 60 kb was isolated from *E. gergoviae* 3773 and its transconjugant. *E. coli* 5518 harbored two plasmids, and only the smaller one (pC5518) of <10 kb, responsible for the extended-spectrum activity, was transferable.

In the *E. coli* transconjugant harboring pC3773, β-lactam resistance was cotransferred with resistance to aminoglycosides (kanamycin and streptomycin), trimethoprim-sulfamethoxazole, and chloramphenicol. In the *E. coli* transconjugant harboring pC5518, β-lactam resistance was associated with resistance to aminoglycosides (kanamycin, streptomycin, and gentamicin) and trimethoprim-sulfamethoxazole.

Under stringent conditions, an intragenic SHV-1-derived probe hybridized with the largest (19 kb) fragment of the *Bam*HI digest of pC3773; however, no fragment of the *Eco*RI digest of pC5518 hybridized with either the intragenic SHV-1-derived probe or the intragenic TEM-1-derived probe (Fig. 2).

Cloning and nucleotide sequencing of SHV β-lactamase gene from pC3773. For further studies with the SHV-type extended-spectrum β-lactamase of pC3773, we cloned *Eco*RI fragments of pC3773 into the *Eco*RI site of pBR322. The cloned DNA was used to transform *E. coli* HB101 with selection for ceftazidime. From one of the transformants, we isolated a plasmid, designated pCHEN1, that contained the 4.4-kb *Eco*RI fragment of the vector and a 6.5-kb *Eco*RI fragment from pC3773 including the extended-spectrum β-lactamase gene.

There are SHV-type enzymes that include SHV-1 through SHV-6, but there are four molecular sequences described for the SHV-2 enzyme. They differ from one another by point mutations at amino acid positions 201, 234, and 235, which were located in the 0.36-kb *Pvu*II-*Pvu*II fragment and the 0.45-kb *Pvu*II-*Cla*I fragment of pMON38 (18). By using these two fragments of pMON38 as probes, hybridization was per-

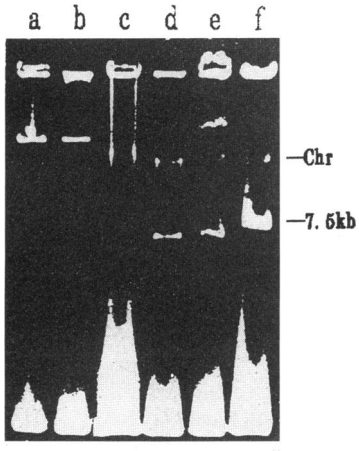

FIG. 1. Agarose gel electrophoresis of crude lysates of donors, recipient, and transconjugants. Lanes: a and b, *E. coli* transconjugant and *E. gergoviae* 3773, respectively; c, recipient, *E. coli* JP559; d and e, *E. coli* transconjugant and *E. coli* C5518, respectively; f, marker, pMON21. Chr, chromosomal DNA.

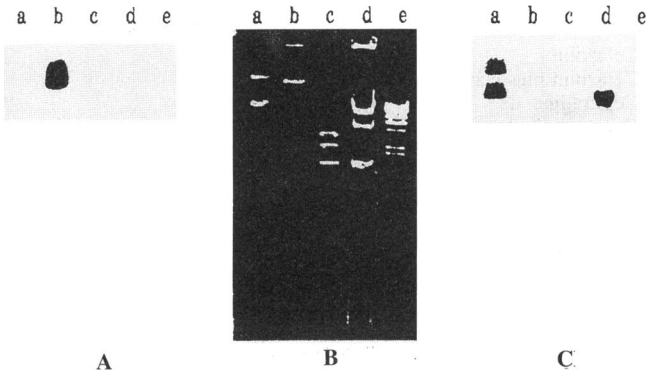

FIG. 2. Southern blot analysis of R-plasmid encoding extended-spectrum β-lactamases. (A) Autoradiogram of gel in panel B probed with TEM-1. (B) Gel stained with ethidium bromide. (C) Autoradiogram of gel in shown in panel B probed with SHV-1. Lanes: a, pMON38 (SHV-1); b, pBR322 (TEM-1); c, pC5518 plus *Eco*RI fragment; d, pC3773 plus *Bam*HI fragment; e, λ DNA plus *Hin*dIII fragment.

TABLE 4. Differences at molecular level between SHV-1 and different SHV-2s

Strain and/or β-lactamase (country)	Codon (amino acid) for amino acid position:					Source or reference
	31	134	183	234	262	
SHV-1	CTA (Leu)	CTA (Leu)	GCC (Ala)	GGC (Gly)	ACC (Thr)	3
SHV-2						
K. pneumoniae 5214773 (United States)			GCG (Ala)	AGC (Ser)		16
K. ozaenae 2180 (Germany)				AGC (Ser)		9
S. typhimurium 122 (France)	CAA (Gln)			AGC (Ser)		4
E. gergoviae 3773 (China)		CTG (Leu)		AGC (Ser)	ACG (Thr)	This work

formed under high-stringency conditions and two homologous fragments, a 0.3-kb *Pvu*II-*Pvu*II fragment and a 0.5-kb *Pvu*II-*Cla*I fragment, were observed on pCHEN1. These two homologous fragments of pCHEN1 were subcloned into the polylinker of the replicative forms of bacteriophage M13 derivative mp19. The recombinant phages were used to transfect *E. coli* JM101, and nucleotide sequences were determined.

The open reading frame for the SHV-type enzyme gene was 858 bp long, encoding a protein of 286 amino acids, of which the first 21 amino acids were a signal peptide and did not appear in the mature enzyme. The partial nucleotide sequence of the SHV-type enzyme gene we report here covered all of the structural gene after codon 86, including the point mutations responsible for the extended-spectrum activity. Comparison of the deduced amino acid sequence of the enzyme from *E. gergoviae* 3773 with that of SHV-1 showed only one substitution of serine for glycine at position 234 (Table 4), suggesting that the extended-spectrum β-lactamase harbored by *E. gergoviae* 3773 was SHV-2. There are another two point mutations at position 134 (CTA to CAG) and position 262 (ACC to ACG) which did not change the amino acids (Table 4).

DISCUSSION

According to their molecular structure, plasmid-mediated cephalosporin-hydrolyzing extended-spectrum β-lactamases detected in members of the family *Enterobacteriaceae* may be classified into several groups: the SHV type derived from SHV-1, the TEM type derived from TEM-1 or TEM-2, and the AmpC type derived from the chromosomal AmpC β-lactamases such as CMY-2 (11). The activities of SHV-type and TEM-type extended-spectrum β-lactamases can be inhibited by the classical β-lactamase inactivators clavulanic acid and sulbactam, and these enzymes do not hydrolyze cephamycins and imipenem well. The AmpC type is resistant to the classical β-lactamase inactivators and cephamycins. Recently, two novel plasmid-mediated extended-spectrum β-lactamases were reported to be present in *P. aeruginosa* (6, 21). The first novel β-lactamase is a carbapenem-hydrolyzing metalloenzyme resistant to inhibition by clavulanate and belonging to the class B β-lactamases which confers resistance to imipenem as well as to ceftazidime and moxalactam (21). The second recently reported β-lactamase is a non-TEM, non-SHV enzyme called OXA-11 which is a derivative of a plasmid-mediated oxacillin-hydrolyzing enzyme, OXA-10 (PSE-2), belonging to the class D β-lactamases and whose activity is not inhibited by clavulanate (6). DNA-DNA hybridization shows that the cephalosporin-hydrolyzing extended-spectrum β-lactamase harbored by *E. coli* 5518 is a non-SHV-, non-TEM-type enzyme, and it is unlikely that it is derived from the other β-lactamases mentioned above because of its sensitivity to clavulanate and cefoxitin. This enzyme produced by *E. coli* 5518 has hydrolytic properties that resemble those of the extended-spectrum β-lactamase harbored by *E. gergoviae* 3773 (Tables 3 and 4), and one fragment of pC5518 hybridizes both with the intragenic SHV-1-derived probe and with the intragenic TEM-1-derived probe under low-stringency conditions (results not shown), suggesting that it may be classified within the class A β-lactamases. This needs to be studied further. Recently, a non-SHV, non-TEM plasmid-mediated extended-spectrum β-lactamase, MEN-1, was also reported to be present in *E. coli*, and protein sequence analysis of MEN-1 revealed that it shares 72% identity with the chromosomally mediated β-lactamases of *Klebsiella oxytoca* (2). In addition, pC5518 is less than 10 kb long, too small to be a self-transferable plasmid, and it seems likely that it was mobilized by the larger plasmid in the same strain (30).

A resistant isolate of *Klebsiella pneumoniae* from Hua-Shan Hospital in Shanghai, China, which produced β-lactamase with the isoelectric point and inhibition properties of SHV-2, has been reported (12). SHV-2 has been detected in *Klebsiella ozaenae* and *S. typhimurium* (Table 4), and all the enzymes share the same Gly→Ser change at position 234 in comparison with SHV-1 (4, 9, 11, 16). The nucleotide sequences of SHV-2 variants from different sources have a few unique point mutations at other positions. One of these nucleotide mutations causes an amino acid substitution but does not affect the enzyme activity, and the others do not even affect the amino acids encoded. SHV-2 harbored by *E. gergoviae* 3773 possesses at least two more point mutations at positions 134 and 262 besides the critical mutation at position 234, but these mutations (CTA to CTG for position 134 and ACC to ACG for position 262) do not cause amino acid changes. On the basis of genetic similarity (9), SHV-type enzyme may have evolved from chromosomal β-lactamase LEN-1 of *K. pneumoniae*, and the two enzymes both belong to class A in the scheme of Ambler. Arakawa et al. demonstrated a close evolutionary relationship between TEM β-lactamases and LEN-1 (1). According to the recently published three-dimensional structure of the class A β-lactamase TEM-1 from *E. coli* (7, 13), position 234 is located near the substrate-binding site. For SHV-2, the hydroxyl group of Ser-234 may interact directly with the side chain of the new β-lactams via a hydrogen bond, which therefore increases catalytic activity toward them.

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