ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 1988, p. 1243–1246
0066-4804/88/081243-04$02.00/0

Vol. 32, No. 8

Novel Plasmid-Mediated β-Lactamase from *Escherichia coli* That Inactivates Oxyimino-Cephalosporins

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Received 11 January 1988/Accepted 29 April 1988

A highly cephem-resistant *Escherichia coli* strain, FP1546, isolated from the fecal flora of laboratory dogs previously administered β-lactam antibiotics was found to produce a β-lactamase, FEC-1, of 48-kilodalton size and pI 8.2. FEC-1 hydrolyzed cefuroxime, cefotaxime, cefmenoxime, and ceftriaxone, as well as the enzymatically less-stable antibiotics cephaloridine, cefotiam, and cefpiramide. Of the oxyimino-cephalosporins, ceftizoxime was fairly stable to FEC-1. FEC-1 differed notably from chromosomal *E. coli* cephalosporinase, especially in its broad-spectrum substrate profile and its high inhibition by clavulanic acid, sulbactam, and imipenem. A conjugation study revealed that FEC-1 was encoded by a 74-megadalton plasmid, pFCX1. This may be the first instance of a plasmid-mediated oxyimino-cephalosporinase from *E. coli*.

Over 30 plasmid-mediated β-lactamases (mostly penicillinases) have been found in gram-negative bacteria and classified according to characteristics such as substrate specificity, isoelectric point, and molecular weight. Common are the TEM, OXA, SHV, and PSE types (10, 17–19, 24), with the TEM type of penicillinase being the most frequently isolated plasmid β-lactamase from such gram-negative bacteria as members of the family *Enterobacteriaceae*, *Pseudomonas aeruginosa*, *Haemophilus influenzae*, and *Neisseria gonorrhoeae*. On the other hand, there have been only two reports of plasmid-mediated cephalosporinases, from *Proteus mirabilis* (2) and *Achromobacter* spp. (14).

We evaluated the possible resistance mechanisms of oxyimino-cephalosporin-resistant *Escherichia coli* isolated from the fecal flora of laboratory dogs, and we identified a β-lactamase which hydrolyzes oxyimino-cephalosporins such as cefuroxime, cefotaxime, cefmenoxime, and ceftriaxone in addition to cephaloridine. The physiological properties of this enzyme were quite distinct from those of chromosomal cephalosporinases from *E. coli* (20, 23). We confirmed that this enzyme was plasmid mediated and had properties similar to those of oxyimino-cephalosporinase type I (7).

MATERIALS AND METHODS

Bacterial strains. *E. coli* FP1546 was isolated from the fecal flora of a laboratory dog being used for pharmacokinetic studies of β-lactam antibiotics. The nalidixic acid-resistant derivative of *E. coli* CSH2 (*metB* F^-) was kindly provided by T. Yokota of Juntendo University.

Antibiotics. Commercially available cephaloridine, cephalothin, cefamandole, cefotiam, cefmetazole, cefsulodin, cefuroxime, cefotaxime, cefmenoxime, ceftriaxone, ceftazidime, cefoperazone, cefpiramide, cefoxitin, moxalactam, and imipenem were used. Cefazolin and ceftizoxime were from Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan. Clavulanic acid, sulbactam, and nitrocefin were synthesized in our laboratories.

Susceptibility testing. Antibacterial activity of test antibiotics was determined by the agar dilution method. Hundredfold dilutions of overnight cultures in Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) were inoculated with a multipoint replicating apparatus onto Mueller-Hinton agar plates containing serial twofold dilutions of antibiotic. MICs were read after incubation at 37°C for 18 h.

Preparation of β-lactamase. Exponentially growing cells of a test strain in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) were harvested by centrifugation, washed once, and suspended in 0.067 M potassium phosphate buffer (pH 7.0). The cell suspension was sonicated, yielding a lysate, and then partially purified by gel filtration (Sephadex G100; Pharmacia AB, Uppsala, Sweden), using a previously described method (25).

Assay of β-lactamase activity. β-Lactamase activity was determined spectrophotometrically (Hitachi 220A) at 37°C in 0.067 M phosphate buffer (pH 7.0).

β-Lactamase inhibitor susceptibility. Serial dilutions of a β-lactamase inhibitor were incubated with enzyme solution for 10 min at 37°C. Residual β-lactamase activity was determined spectrophotometrically, using the chromogenic substrate nitrocefin at 482 nm. The concentration inhibiting 50% of activity was calculated.

Isoelectric focusing. Isoelectric focusing of the cell lysate, using Ampholine (LKB Stockholm, Bromma, Sweden)-polyacrylamide gel, was done. β-Lactamase bands were detected by zymogram, using nitrocefin (22).

Molecular weight determination. Enzyme molecular weight was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (13). β-Lactamases and marker proteins were boiled for 2 min in 1% sodium dodecyl sulfate–3% 2-mercaptoethanol before application. A low-molecular-weight calibration kit (Pharmacia AB, Uppsala, Sweden) was used.

Transconjugation method. Previously described broth and filter-mating techniques were used (27). *E. coli* FP1546 and *E. coli* CSH2 Nal^r (recipient) were incubated for 6 h at 37°C before selection of transconjugant.

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TABLE 1. Antibiotic susceptibility of *E. coli* FP1546 and a transconjugant

Antibiotic	MIC (μg/ml) for:		
	Donor (*E. coli* FP1546)	Transconjugant [*E. coli* CSH2(pFCX1)]	Recipient (*E. coli* CSH2)
Cephaloridine	400	400	1.56
Cefazolin	>400	>400	3.13
Cefaclor	>400	>400	1.56
Cefamandole	>400	>400	0.2
Cefotiam	200	400	0.1
Cefsulodin	200	>400	25
Cefoperazone	100	200	<0.025
Cefpiramide	>400	>400	0.1
Cefuroxime	>400	>400	0.78
Ceftizoxime	3.13	1.56	<0.025
Cefotaxime	200	200	<0.025
Cefmenoxime	400	>400	<0.025
Ceftriaxone	400	>400	<0.025
Ceftazidime	25	12.5	0.1
Aztreonam	200	25	<0.025
Cefoxitin	6.25	1.56	1.56
Cefmetazole	3.13	0.78	0.78
Moxalactam	0.78	0.39	0.1
Imipenem	0.39	0.78	0.39
Ampicillin	>400	>400	3.13
Ofloxacin	0.2	0.78	0.78
Streptomycin	1.56	3.13	3.13
Kanamycin	1.56	3.13	3.13
Gentamicin	0.39	0.78	0.78
Amikacin	0.78	1.56	1.56
Minocycline	3.13	1.56	1.56
Tetracycline	1.56	0.78	0.78
Chloramphenicol	12.5	3.13	3.13
$HgCl_2$	1.56	3.13	1.56

Plasmid analysis. Plasmid DNAs were obtained by alkaline treatment and analyzed by 0.8% agarose gel electrophoresis (15). To determine plasmid molecular weight, plasmid DNA was further purified by CsCl-ethidium bromide equilibrium density gradient centrifugation (15) and cleaved by restriction enzymes (*Acc*I, *Hpa*I, *Eco*RI, *Hin*dIII, etc.; Nippon Gene, Toyama, Japan) into fragments of <30 kilobases, which were sized by electrophoresis in 0.3% agarose (agarose H; Wako Junyaku, Osaka, Japan)–0.8% agarose (agarose type I; Sigma Chemical Co., St. Louis, Mo.) in comparison with fragments of a *Hin*dIII digest of lambda DNA.

RESULTS

Antibiotic susceptibility of *E. coli* FP1546. *E. coli* FP1546 was isolated from the fecal flora of dogs, each of which had been previously administered various cephem antibiotics. This strain had typical properties of *E. coli* but was extraordinarily resistant to various cephem antibiotics, including the newer oxyimino-cephalosporins cefotaxime, cefmenoxime, and ceftriaxone (Table 1). Of the oxyimino-cephalosporin MICs, that of ceftizoxime was the lowest against *E. coli* FP1546. MICs of cephamycin-type antibiotics and imipenem were also low.

β-Lactamase production as the mechanism of *E. coli* FP1546 resistance. A chromogenic spot test with nitrocefin showed that *E. coli* FP1546 produced a β-lactamase, suggesting that this was the most probable resistance mechanism. This novel β-lactamase, designated FEC-1 (Fujisawa *E. coli*), was partially purified by gel filtration and characterized to have a pI of 8.2 and a molecular weight of 48,000.

TABLE 2. Kinetic parameters of β-lactamase of *E. coli* FP1546 for β-lactam antibiotics

Substrate	K_m (μM)	V_{max}[a]	V_{max}/K_m[a]
Cephaloridine	152	100	100
Cephalothin	134	198	225
Cefamandole	122	125	156
Cefotiam	38	43	170
Cefoperazone	2.8	2.6	139
Cefpiramide	33	36	167
Cefuroxime	27	32	179
Ceftizoxime	821	12	2.3
Cefotaxime	61	23	59
Cefmenoxime	84	61	110
Ceftriaxone	27	14	80
Ceftazidime	393	0.13	0.05
Ampicillin	30	17	89

[a] Values relative to cephaloridine hydrolysis = 100.

β-Lactamase substrate profile. The kinetic parameters (K_m and relative V_{max}) and hydrolytic efficiency (V_{max}/K_m) of β-lactamase for various β-lactam antibiotics are shown in Table 2. V_{max}/K_m was high for cefuroxime, cefotaxime, cefmenoxime, and ceftriaxone, as well as for the usually less stable cephems cephaloridine, cephalothin, cefamandole, cefotiam, and cefpiramide; that of cefoperazone was also greater than cephaloridine, despite having a lower V_{max} than ceftizoxime. Ceftizoxime was the poorest substrate of the oxyimino-cephalosporins. Ceftazidime was also a poor substrate, although its MIC was higher than that of ceftizoxime. The enzyme did not extensively hydrolyze cephamycins (data not shown). This substrate profile resembles that of chromosomal enzymes from *Proteus vulgaris* (16), *Proteus penneri* (8), *Klebsiella oxytoca* (12), and *Bacteroides* spp. (5, 6, 28) and could thus be classified as an oxyimino-cephalosporinase type I (7).

Enzymatic inhibition. The β-lactamase from *E. coli* FP1546 was greatly inhibited by clavulanic acid, sulbactam, and imipenem, with respective 50% inhibitory concentrations of 0.0093, 0.020, and 0.41 μM. These results also suggest its classification as an oxyimino-cephalosporinase type I enzyme (7).

Transfer of cephem resistance. Because apparent properties of this β-lactamase differ considerably from those of chromosomal cephalosporinases produced by other *E. coli* strains (20, 23), the enzyme is possibly plasmid mediated. Cephem-resistant transconjugants were obtained by both broth and filter-mating methods at frequencies of 10^{-6} and 2.5×10^{-5}, respectively. The MICs of various antibiotics for the donor, recipient, and transconjugant are shown in Table 1. Cephem resistance was fully expressed in the transconjugant, which produced a β-lactamase with the same pI as that of FEC-1.

Identification of plasmid DNA. Plasmid DNA was purified from donor and transconjugant and analyzed by agarose gel electrophoresis. Donor and transconjugant had the same size plasmid, pFCX1. pFCX1 was estimated to be about 78 megadaltons from the sum of the fragments cleaved by restriction enzymes. pFCX1 did not encode a gene for resistance to other antibiotics such as aminoglycosides,

chloramphenicol, sulfamethoxazole, tetracyclines, and trimethoprim (data not shown) or to Hg^{2+} (Table 1).

DISCUSSION

Inactivation by β-lactamases is the most frequent cause of β-lactam antibiotic resistance in clinically isolated gram-negative bacteria (17, 21). In *E. coli*, many plasmid-mediated penicillinases have already been isolated, and elevated levels of chromosomal cephalosporinase also have resulted in resistance (9, 11). However, so-called broad-spectrum oxyimino-cephalosporins such as ceftizoxime and cefotaxime are active against these β-lactam-resistant *E. coli*.

E. coli FP1546 is unusually resistant to various β-lactam antibiotics including the oxyimino-cephalosporins. Characterization of a β-lactamase designated FEC-1 produced by this strain revealed that it is a type I β-lactamase which hydrolyzes oxyimino-cephalosporins (7). Of great interest, FEC-1 is plasmid mediated and can be transferred to another *E. coli* strain by conjugation. Thus, the major mechanism of *E. coli* FP1546 cephem resistance appears to be production of β-lactamase FEC-1, based on the fact that transfer of plasmid pFCX1 produced full expression of β-lactam resistance without resistance to other antibiotics.

Although the origin of this novel plasmid-mediated β-lactamase FEC-1 gene is as yet unknown, quite probably it comes from canine intestinal bacterial flora. Oxyimino-cephalosporinase type I-producing microflora such as *Bacteroides* spp., *K. oxytoca*, and *P. vulgaris* are most likely. Worth consideration is *Bacteroides fragilis*, in which conjugative transfer of β-lactamase in the absence of plasmid has been reported (4), and the possible transfer of a resistance gene to other species cannot be excluded.

Plasmid-mediated β-lactamase FEC-1 could be the result of a "selective pressure" process, arising from the various β-lactam antibiotics which have been given to those dogs used routinely for kinetic experiments; we isolated *E. coli* FP1546 from such animals. This kind of "selection" may possibly be seen eventually in clinical practice, with occurrence of resistant strains such as *E. coli* FP1546 increasing in the future. In fact, plasmid-mediated β-lactamases which hydrolyze broad-spectrum cephalosporins have been reported recently (1, 3, 10, 26), although they are of the penicillinase type.

It is of interest that ceftizoxime has moderate activity againt *E. coli* FP1546 among the tested oxyimino-cephalosporin derivatives, in contrast to the lack of activity seen with cefotaxime, cefmenoxime, and ceftriaxone. The latter three drugs had no activity against this strain, although they have the same structure {7β-[(Z)-2-(2-amino-4-thiazolyl)-2-(methoxyimino)acetamido]} at the 7 position of the cephalosporin nucleus. We need to monitor continuously the trend toward isolation of this kind of resistant strain and its clinical implications.

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