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A Novel Class C β-Lactamase (FOX-2) in *Escherichia coli* Conferring Resistance to Cephamycins

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An *Escherichia coli* strain resistant to a broad spectrum of β-lactams, including cephamycins, was isolated from a patient suffering from urinary tract infection. A resistance plasmid (pMVP-7) was transferred from the clinical isolate to an *Escherichia coli* recipient. Both strains produce a cefoxitin-hydrolyzing β-lactamase focusing at pI 6.7. The phenotype was similar to that of a *Klebsiella pneumoniae* strain producing cephamycinase FOX-1, so primers were selected from the FOX-1 sequence to amplify the *bla* gene of the transconjugant. The PCR product obtained was sequenced. The percentage of identity of the deduced amino acid sequence with sequences of other AmpC-type β-lactamases was 96.9% with FOX-1, 74.9% with CMY-1, and 67.7% with MOX-1. This new plasmid-mediated enzyme is most closely related to FOX-1 (11 amino acid exchanges). We therefore propose the designation FOX-2.

The majority of *Escherichia coli* strains producing plasmid-encoded extended-spectrum β-lactamases remain susceptible to cephamycins, e.g., cefoxitin (6, 13). An *E. coli* isolate (*E. coli* BW U2206) resistant to a broad spectrum of β-lactam antibiotics, including cephamycins, was isolated recently in a Berlin hospital from a patient suffering from urinary tract infection. We asked the question whether the resistance of this strain is due to the production of a cephamycinase. Our results demonstrate that this enzyme represents a new plasmid-encoded cephamycin-hydrolyzing β-lactamase. The amino acid sequence of the enzyme is different from those of all the cephamycinases so far sequenced (2, 3, 5, 7, 9, 12, 21, 26) and is most closely related (96.9% identity) to that of FOX-1 (9), with 11 amino acid exchanges. We therefore propose the designation FOX-2.

Patient. A 35-year-old male patient who suffered paraplegia from a gunshot wound in Guatemala (26 August 1993) was transported to Berlin, Federal Republic of Germany (28 October 1993). There a multiresistant (Table 1) *E. coli* strain (BW U2206) was isolated from his urine in a titer of $>10^5$ CFU/ml (29 October 1993).

Bacterial strains. The FOX-1-producing reference stain, *E. coli* C600 R^+ (pRYC132), was a gift of F. Baquero, Hospital Ramóny Cajal, Madrid, Spain (9). *E. coli* C600 R^- (MIC of nalidixic acid, 1,024 mg/liter) was the recipient strain for transfer of the resistance factor from *E. coli* BW U2206, and *E. coli* DH5α was the host for transformation of the bla_{FOX-2} gene. *E. coli* transconjugants producing the β-lactamases TEM-16 (17) and SHV-3 (22) were used as pI references for isoelectric focusing.

Antibiotics. Antibiotics were obtained from the respective manufacturers: cefoxitin and imipenem (Merck, Sharp & Dohme, Haar, Federal Republic of Germany); clavulanate, temocillin, and cefepime (SmithKline Beecham Pharma, Munich, Federal Republic of Germany); sulbactam (Pfizer, Karlsruhe, Federal Republic of Germany); cefotetan and meropenem (Zeneca, Plankstadt, Federal Republic of Germany); moxalactam and tobramycin (Eli Lilly, Bad Homburg, Federal Republic of Germany); cefotaxime, cefpirome, gentamicin, and tetracycline (Hoechst, Frankfurt/Main, Federal Republic of Germany); ceftazidime (Cascan, Wiesbaden, Federal Republic of Germany); aztreonam (Bristol-Myers Squibb, Munich, Federal Republic of Germany); tazobactam (Lederle, Wolfratshausen, Federal Republic of Germany); flomoxef (Shionogi, Düsseldorf, Federal Republic of Germany); ciprofloxacin (Bayer, Wuppertal, Federal Republic of Germany); cotrimoxazole (Roche, Grenzach-Wyhlen, Federal Republic of Germany); and chloramphenicol (Boehringer, Mannheim, Federal Republic of Germany). Combinations of cefoxitin with β-lactamase inhibitors were used at proportions of 4:1 (clavulanate), 2:1 (sulbactam), and 8:1 (tazobactam) or according to the National Committee for Clinical Laboratory Standards standard (cefoxitin plus clavulanate or cefoxitin plus sulbactam,

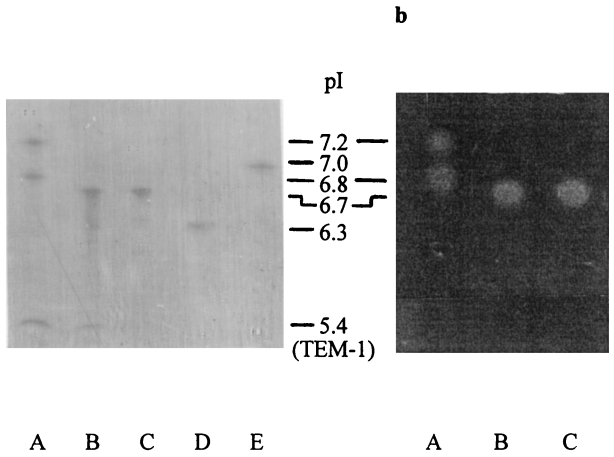

FIG. 1. Isoelectric focusing of β-lactamase FOX-2. (a) The FOX-2-producing transconjugant strain revealed a major band at a pI of about 6.7, lower than those of SHV-3 (7.0) and FOX-1 (6.8) but above that of TEM-16 (6.3). (b) This band was able to hydrolyze cefoxitin, as shown by a bioassay. Lanes: A, *E. coli* pRYC132 producing FOX-1; B, *E. coli* R^+ (BW U2206) producing FOX-2; C, *E. coli* DH5α T^+ producing FOX-2; D, *E. coli* R^+ producing TEM-16; E, *E. coli* R^+ producing SHV-3.

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TABLE 1. Antibiotic susceptibilities of the wild-type *E. coli* BW U2206, the transconjugants *E. coli* C600 R^+ (pMVP-7) and *E. coli* C600 R^+ (pRYC132), the transformant *E. coli* DH5α T^+, and the *E. coli* C600 R^- recipient

Antibiotic	MIC (μg/ml) for:				
	Wild-type *E. coli* BW U2206 (FOX-2)	Transconjugant *E. coli* C600 R^+ (pMVP-7) (FOX-2)[a]	Transformant *E. coli* DH5α T^+ (FOX-2)[b]	Transconjugant *E. coli* C600 R^+ (pRYC132) (FOX-1)	Recipient *E. coli* C600 R^-
Cefoxitin	512	256	256	64	2
+ clavulanate (4:1)	128	128	128	32	2
+ clavulanate (2:1)	64	64	64	32	2
+ sulbactam (1:1)	32	32	32	16	2
+ sulbactam (2:1)	64	64	64	32	2
+ tazobactam (8:1)	128	128	128	32	2
+ tazobactam (4 μg/ml)	128	128	128	32	2
Cefotetan	128	128	64	8	0.13
Moxalactam	2	2	1	0.25	0.06
Flomoxef	4	2	2	0.5	0.06
Cefotaxime	32	16	16	2	0.06
Ceftazidime	64	64	32	4	0.13
Cefpirome	4	2	1	0.25	0.03
Cefepime	0.5	0.25	0.13	0.06	0.016
Aztreonam	4	4	2	0.5	0.06
Temocillin	8	8	8	4	4
Imipenem	0.5	0.5	0.5	0.5	0.5
Meropenem	0.03	0.03	0.03	0.03	0.03
Ciprofloxacin	0.016	0.016	0.016	0.008	0.016
Gentamicin	2	1	0.13	1	0.13
Tobramycin	32	16	0.13	4	0.13
Cotrimoxazole	>512	512	0.5	0.5	0.25
Tetracycline	256	128	1	1	1
Chloramphenicol	512	512	>512	>512	4

[a] FOX-2 plus TEM-1.
[b] Only FOX-2.

2:1; cefoxitin plus tazobactam at a constant concentration of 4 μg/ml).

MICs. MICs were determined by an agar dilution technique with Mueller-Hinton agar (Difco, Augsburg, Federal Republic of Germany). The inoculum of 10^4 CFU per spot was deposited on the agar by a multipoint inoculator (Denley, Billinghurst, United Kingdom). MICs were read after 16 h of incubation at 35°C. *E. coli* ATCC 25922 was used as a quality control reference strain.

Transfer of resistance determinants. The wild-type and recipient strains (10^9 CFU/ml for each strain) were suspended in Mueller-Hinton broth (Difco) and incubated for 18 h at 35°C. Transconjugants were selected on MacConkey agar (Unipath, Wesel, Federal Republic of Germany) supplemented with nalidixic acid (64 μg/ml) and cefoxitin (64 μg/ml) to inhibit the growth of the donor strain or the recipient strain, respectively.

Cloning of the bla_{FOX-2} gene into *E. coli* DH5α. The bla_{FOX-2} gene was amplified from the R plasmid (pMVP-7) with specific primers carrying recognition sites for *Eco*RI or *Xba*I. The resulting PCR product of 1,313 bp was purified, digested with *Eco*RI and *Xba*I, ligated into vector pBC, and transformed into *E. coli* DH5α (24). The transformation was verified by sequencing of the bla_{FOX-2} gene.

Isoelectric focusing of β-lactamases. Crude homogenates of β-lactamases were prepared as described previously (1). For isoelectric focusing, the procedure of Matthew et al. (19) was modified (1).

Assignment of β-lactamase activity within the lanes. After isoelectric focusing, the gel was covered with an agar overlay

TABLE 2. Percentage of identity of amino acid sequences of FOX-2 and other class C β-lactamases

β-Lactamase (reference)	% Identity with:								
	FOX-2	FOX-1	CMY-1	MOX-1	CMY-2	LAT-1	BIL-1	ACT-1	*P. aeruginosa* AmpC
FOX-2	100	96.9	74.9	67.7	42.2	41.9	41.4	42.4	53.7
FOX-1 (9)		100	73.6	66.7	42.5	42.2	41.7	42.6	53.4
CMY-1 (3)			100	89.5	41.7	41.4	40.9	41.5	54.4
MOX-1 (12)				100	38.1	37.8	37.3	38.6	49.5
CMY-2 (2)					100	98.9	98.4	75.3	41.3
LAT-1 (26)						100	97.4	74.5	41.0
BIL-1 (7)							100	73.9	41.0
ACT-1 (5)								100	44.7
P. aeruginosa AmpC (16)									100

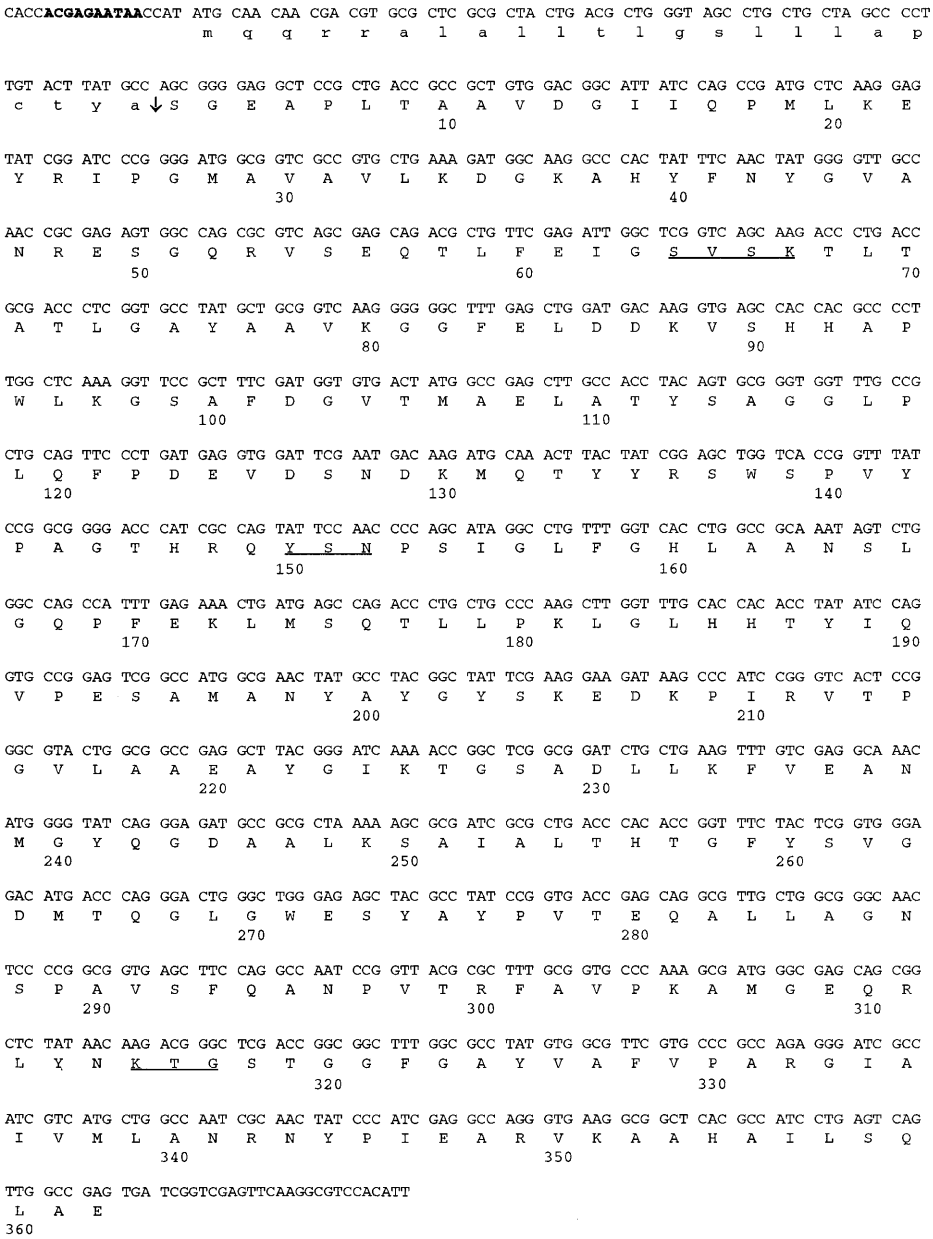

FIG. 2. Nucleotide sequence of the *bla*$_{FOX-2}$ gene (pMVP-7). The deduced amino acid sequence of FOX-2 is shown in the line below the nucleotide triplets. Amino acids of the signal peptide are written in lowercase letters, and the putative cleavage site of the signal peptidase is marked by an arrow. The β-lactamase active site S-V-S-K, the conserved triad K-T-G, and the class C typical motif Y-S-N are underlined. The putative ribosome binding site upstream of the start codon is indicated by bold letters.

containing cefoxitin (16 μg/ml). After incubation at 35°C for 2 h a second overlay with a susceptible *E. coli* strain (MIC of cefoxitin, 2 μg/ml) was applied. Following overnight incubation, growth of the indicator strain at the spot where cefoxitin had been hydrolyzed allowed specific localization of the cephamycinase band (1).

Plasmid DNA preparation. Cells grown overnight in 150 ml of tryptic soy broth (Difco) were used to prepare R plasmids or recombinant plasmids. DNA was obtained by alkaline lysis (4). Plasmid DNA in the lysate was purified with an anion exchange column (Qiagen, Hilden, Federal Republic of Germany) according to the recommendations of the manufacturer.

Sequencing of the *bla*$_{FOX-2}$ gene. Two primers were selected from the FOX-1 sequence (9) for amplification of the *bla*$_{FOX-2}$ gene of the *E. coli* transconjugant strain: FOX-1H (CACCAC GAGAATAACCAT; nucleotides 683 to 700) and FOX-1F (ATGTGGACGCCTTGAACT; nucleotides 1874 to 1857). The PCR product was sequenced with consecutive primers by the dideoxy chain termination procedure of Sanger et al. (25) with an automatic sequencer (373A; Applied Biosystems, Weiterstadt, Federal Republic of Germany).

Sequence analysis. Related β-lactamases were identified by comparison with EMBL and Swissprot databases done with the Fasta program. Multiple alignment was calculated with Clustal V (10, 11).

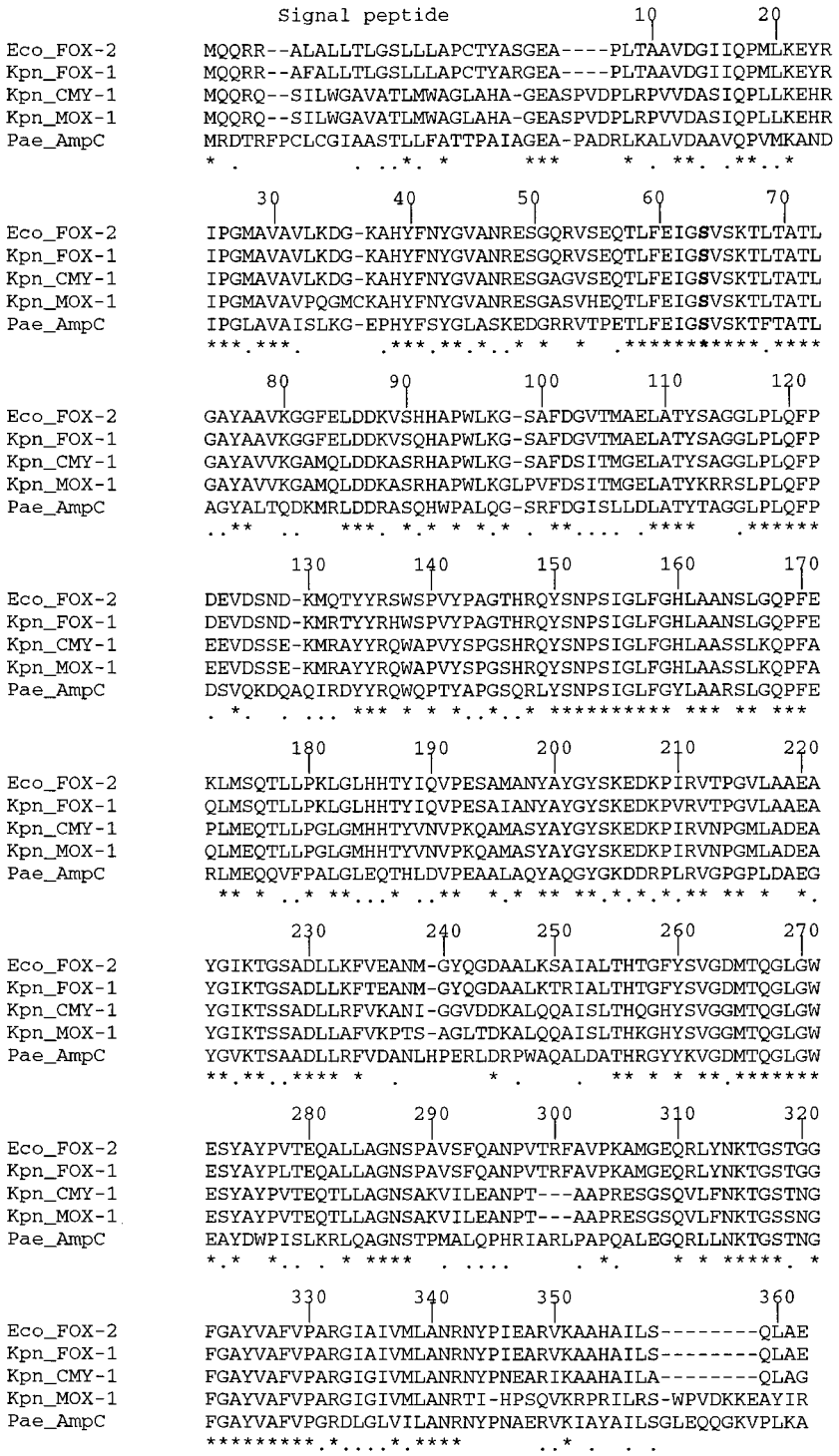

FIG. 3. Multiple sequence alignment of the amino acid sequences of FOX-2, FOX-1, CMY-1, MOX-1, and AmpC of *P. aeruginosa*. Identical amino acids are marked with an asterisk, and conservative exchanges are indicated by a dot. Active-site serine (position 64) is marked by bold letters.

Transfer of the plasmid carrying the bla_{FOX-2} gene. Transconjugants resistant to cefoxitin were selected at a frequency of 2.4×10^{-5} per donor cell. Genes for resistance to aminoglycosides, cotrimoxazole, tetracycline, and chloramphenicol were cotransferred (Table 1).

Antibiotic susceptibility. The MICs of selected antibiotics for the wild-type strain, *E. coli* BW U2206; the transconjugant *E. coli* C600 R^+ (pMVP-7); the transformant *E. coli* DH5α T^+, carrying the bla_{FOX-2} gene; the recipient *E. coli* C600 R^-; and the FOX-1-producing transconjugant, *E. coli* C600 R^+ (pRYC132) are shown in Table 1. The MICs of β-lactams for the transconjugant strain producing FOX-2 β-lactamase are 8 to 32 times higher than those for the transconjugant producing FOX-1 β-lactamase, except for temocillin and the

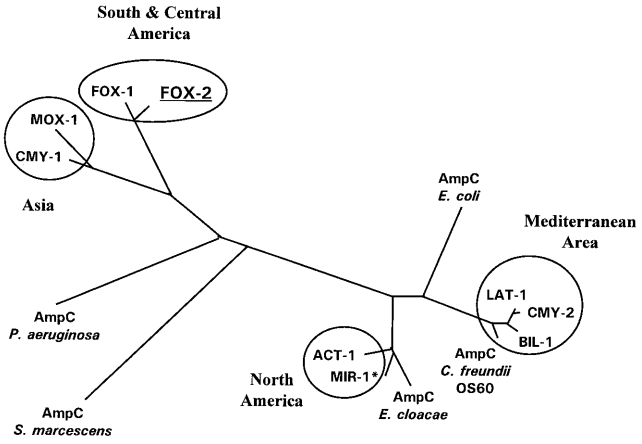

FIG. 4. Dendrogram for 14 mature class C β-lactamases (calculated with Clustal V and the neighbor-joining method of Saitou and Nei [23]). Branch lengths are proportional to the number of amino acid exchanges. The identity between MIR-1 and the AmpC of *Enterobacter cloacae* (marked by an asterisk) cannot be defined exactly, as the published sequence of MIR-1 is restricted to 150 nucleotides.

carbapenems. β-Lactamase inhibitors reduce the MIC of cefoxitin only weakly. Different proportions of cefoxitin and the β-lactamase inhibitors show similar results (Table 1). Comparison between the transconjugant and the transformant harboring the bla_{FOX-2} gene indicates an identical resistance phenotype (i.e., a deviation of MICs of not more than one step of dilution). The resistance phenotype of the FOX-1-producing transconjugant is different from that of the FOX-2-producing transconjugant for non-β-lactam antibiotics as well (e.g., in the organism's susceptibility to cotrimoxazole and tetracycline). This indicates nonidentity of the R plasmids.

Isoelectric point and assignment of cefoxitin-hydrolyzing activity within the lanes. On polyacrylamide gels run with crude homogenates of *E. coli* R^+ (BW U2206), two bands were visualized by nitrocefin: one was at the pI of TEM-1 (5.4), and one was slightly below the pI of the more acid of the two bands of FOX-1 (6.8 and 7.2) at about 6.7 (Fig. 1a). Only the band at pI 6.7 showed activity against cefoxitin as indicated by growth of the cefoxitin-susceptible *E. coli* strain, while at a pI of 5.4 cefoxitin remained unhydrolyzed (Fig. 1b).

Analysis of the bla_{FOX-2} gene. The nucleotide sequence of the PCR product obtained with consecutive primers and deduced from the FOX-1 sequence (9) revealed an open reading frame coding for a protein of 382 amino acids (Fig. 2). Multiple sequence alignment of the amino acid sequence of FOX-2 was performed with other plasmid-mediated class C β-lactamases as well as with chromosomal AmpC β-lactamases (8, 14–16, 20), among which that of *Pseudomonas aeruginosa* (16) was the most closely related (Fig. 3 and 4 and Table 2). The relationship of the β-lactamase FOX-2 was closest to FOX-1, with 11 amino acid exchanges within the mature protein; four of the exchanges were conservative, and seven were nonconservative (Table 3).

The history of the patient infected with the FOX-2-producing *E. coli* strain indicates that it was acquired in Guatemala and carried with the patient to Berlin, Federal Republic of Germany. There is no evidence that *E. coli* BW U2206 spread into the patient's environment. Comparison of the resistance phenotypes of the transconjugant and the transformant strains as expressed both by MICs and by hydrolysis of cefoxitin on polyacrylamide gels demonstrates that the cephamycin-hydrolyzing activity is due to the expression of the bla_{FOX-2} gene only and remains uninfluenced by the presence of the bla_{TEM-1} gene. The pI of the enzyme (6.7) is different from those of both FOX-1 (6.8 and 7.2) and an AmpC-type β-lactamase described recently (one band at 7.25 [18]).

The analysis of the bla_{FOX-2} gene reveals a close relationship to the gene encoding FOX-1. This may suggest that the bla_{FOX-2} gene is a derivative of the bla_{FOX-1} gene or that they share a recent common ancestor. This hypothesis has some support from the fact that the *Klebsiella pneumoniae* strain harboring the bla_{FOX-1} gene was isolated from a patient in Buenos Aires, Argentina, in 1989 (prior to the isolation of FOX-2), at a location closer to Guatemala than to those of the next most closely related enzymes to FOX-1 and FOX-2, namely CMY-1 (3) and MOX-1 (12) (Fig. 4), which were detected in Asia (CMY-1 in Seoul, Republic of Korea, in 1989 and MOX-1 in Japan in 1991). By their geographical occurrence the plasmid-encoded class C β-lactamases may therefore be subdivided into several clusters: a North American cluster (MIR-1 [20] and ACT-1 [5]), a Central and South American cluster (FOX-1 and FOX-2), an Asian cluster (CMY-1 and MOX-1), and a Mediterranean-Middle East cluster (CMY-2 [2], BIL-1 [7], and LAT-1 [25]).

The amino acid sequence of the β-lactamase FOX-2 is different from that of FOX-1 in 11 positions. Taken together with the differences in resistance phenotype, this appears to justify its designation as a separate β-lactamase. As this β-lactamase is closest in its amino acid sequence to FOX-1, the designation FOX-2 was chosen. Of the 11 amino acid exchanges of FOX-2 in comparison with FOX-1, four are conservative and seven are nonconservative (Table 3). The differences in resistance phenotype between FOX-1 and FOX-2 may be attributed more probably to one or more of the seven amino acids at positions 1, 91, 132, 137, 172, 235, and 251 of the β-lactamase than to the conservative exchanges at positions 196, 210, 250, and 278. Further analysis of this question should contribute to the understanding of the structure-function relationship within class C β-lactamases. It appears that the *ampC* genes display a remarkable variety and a high potential for encoding novel β-lactamases. Therefore, both the spread of *bla* genes through pathogens and patients (causing mostly local epidemics) and their independent emergence at geographically distant locations are major factors in the epidemiology of these β-lactamases.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the EMBL database under accession no. Y10282.

TABLE 3. Amino acid exchanges of β-lactamases FOX-2 and FOX-1 (9)

Amino acid position	Amino acid in:		Type of exchange[a]
	FOX-2	FOX-1	
1	Ser	Arg	N-con
91	His	Gln	N-con
132	Gln	Arg	N-con
137	Ser	His	N-con
172	Lys	Gln	N-con
196	Met	Ile	Con
210	Ile	Val	Con
235	Val	Thr	N-con
250	Ser	Thr	Con
251	Ala	Arg	N-con
278	Val	Leu	Con

[a] N-con, nonconservative exchange; Con, conservative exchange.

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