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Community-Onset Disease Caused by *Citrobacter freundii* Producing a Novel CTX-M β-Lactamase, CTX-M-30, in Canada

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Strains of *Citrobacter freundii* intermediate to cefotaxime but sensitive to ceftazidime were isolated from four different patients in Canada. Sequencing of PCR products by use of CTX-M–specific primers revealed a new combination of four amino acid substitutions. This new gene was designated *bla*$_{\text{CTX-M-30}}$ and was encoded on a 3-kb plasmid. The pI of CTX-M-30 was 8.0.

Organisms producing CTX-M β-lactamases have been identified throughout the world in Asia, South America, Europe, Canada, and the United States (4, 5, 11, 13, 18). CTX-Ms are class A β-lactamases in which the majority of enzymes are more active against cefotaxime than against ceftazidime (3, 7, 17). The genes encoding *bla*$_{\text{CTX-Ms}}$ show high nucleotide similarity to the chromosomal β-lactamase genes of *Kluyvera* spp. (2, 10). This study identified a new *bla*$_{\text{CTX-M}}$ gene, *bla*$_{\text{CTX-M-30}}$, within clinical strains of *Citrobacter freundii* isolated from patients from communities in Canada.

Five strains of *C. freundii* intermediate to cefotaxime (CTX) were isolated from the urine samples of four different patients over a 2-month period during 2002. The strains were designated *Cf* 12, *Cf* 27, *Cf* 28, *Cf* 29, and *Cf* 30. Strain identification was initially achieved using Vitek (Vitek AMS; BioMérieux Vitek Systems Inc., Hazelwood, Mo.) and API 20E strips (BioMérieux Inc.) and confirmed by 16S rRNA sequencing using a MicroSeq 500 16S rDNA bacterial sequencing kit (Applied Biosystems; Foster City, CA). The resulting sequences were analyzed with MicroSeq analysis software (Applied Biosystems). The 16S rDNA analysis confirmed that the strains were *C. freundii*.

DNA templates for PCR were prepared as previously described using annealing temperatures of 55°C for primers CTX-M1F (GCAGCACCAGTAAAGTGATGG) and CTX-M1R (GCTGGGTGAAGTAAGTGACC) (accession number X92506) and 46°C to obtain the full-length amplified product by use of primers CTX-M3FLF (CGTCTCTTCCAGAATAAGG) and CTX-M-3FLR (GTTTCCCCATTCCGTTTCCGC) (accession number AF550415) (15). Sequencing using an ABI Prism 3100-Avant genetic analyzer was carried out by automated-cycle sequencing.

The full-length PCR product was cloned into pXL-Topo and transformed into *Escherichia coli* Top10 (Invitrogen) as recommended by the manufacturer. The resulting transformant was designated t*Cf* 29.

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Sequencing data revealed that all strains had identical nucleotide sequences. Computer-generated amino acid analysis using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/BLAST.cgi) identified a unique combination of four amino acid substitutions (Thr16Ala, Asn117Asp, Gly242Asp, and Asn289Asp) which had not been previously described. Therefore, this new CTX-M β-lactamase was designated CTX-M-30. The gene *bla*$_{\text{CTX-M-30}}$ had 11 separate nucleotide changes positioned randomly throughout the gene compared to *bla*$_{\text{CTX-M-3}}$. Two nucleotide changes resulted in two amino acid substitutions, Thr16Ala and Asn117Asp. In addition, *bla*$_{\text{CTX-M-30}}$ had seven separate nucleotide changes positioned randomly throughout the gene compared to *bla*$_{\text{CTX-M-29}}$. Two of these nucleotide changes resulted in two additional amino acid substitutions, Gly242Asp and Asn289Asp. Conjugation and transformation experiments were performed as previously described (4, 9). The gene *bla*$_{\text{CTX-M-30}}$ was found not to be self-transmissible; therefore, Southern analysis was performed to determine the location of *bla*$_{\text{CTX-M-30}}$.

Plasmid DNA was extracted by alkaline lysis from strains *Cf* 12, *Cf* 27, *Cf* 28, *Cf* 29, and *Cf* 30 as previously described (15), and one-half of each plasmid sample was treated with plasmid-safe DNase (Epicentre Technologies) to remove contaminating chromosomal DNA. The plasmids were separated by electrophoresis in a 0.6% agarose gel. Plasmid profile gels were stained with ethidium bromide (10 mg/ml) and visualized by ultra-violet light with a Kodak EDAS 290 system. Southern analysis was performed as described by the manufacturer (Boehringer Mannheim, Indianapolis, Ind.). The *bla*$_{\text{CTX-M-30}}$–specific probe was synthesized using PCR by incorporating digoxigenin-11-dUTP into the product by use of primers CTX-M-1F and CTX-M-1 R.

Plasmid profiles revealed that all the clinical strains had the same three plasmids (3, 6, and 16 kb) (data not shown). Southern analysis indicated that *bla*$_{\text{CTX-M-30}}$ was encoded on a 3-kb plasmid and was not chromosomally encoded (data not shown). The gene *bla*$_{\text{CTX-M-30}}$ was also detected on the pXL-Topo plasmid transformed into t*Cf* 29.

MICs were determined using broth microdilution and E-tests (AB Biodisk, Solna, Sweden) as recommended by the manufacturers. *E. coli* ATCC 25922 was used as the quality control strain. Throughout this study, results were interpreted

TABLE 1. Antimicrobial susceptibilities

Antibiotic (s)	MIC (μg/ml)		
	C. freundii 29[d]	*tCf29*[e]	*E. coli* Top 10
Cefotaxime[a]	32	16	0.12
Cefotaxime and clavulanic acid[a,c]	0.12	0.12	0.12
Cefdinir[b]	64	32	0.25
Ceftazidime[a]	1	0.5	0.25
Ceftazidime and clavulanic acid[a,c]	0.25	0.5	0.25
Ceftriaxone[a]	64	32	0.12
Aztreonam[a]	2	1	0.25
Cefepime[a]	4	1	≤0.06
Cefepime and clavulanic acid[a,c]	≤0.03	≤0.03	≤0.03
Cefpodoxime[a]	64	32	0.5
Cefpodoxime and clavulanic acid[a,c]	2	0.25	0.5
Cefoxitin[a]	≤4	≤4	≤4
Imipenem[a]	0.12	0.12	0.12
Ampicillin[a]	>32	>32	2
Amoxicillin and clavulanic acid[a,c]	16	4	4
Piperacillin[b]	>256	>256	2
Piperacillin and tazobactam[b,c]	1	1	2

[a] MICs determined using microbroth according to NCCLS guidelines.
[b] MICs determined using E-test.
[c] Clavulanate and tazobactam were used at fixed concentrations of 4 μg/ml.
[d] The pI(s) of the β-lactamases produced by *C. freundii* were 5.4, 8.0, and 8.9.
[e] The pI of the β-lactamase produced by tCf29 was 8.0.

using National Committee for Clinical Laboratory Standards (NCCLS) criteria for broth dilution (12). The presence of an ESBL was evaluated using the modified double disk test (MDDT) (14). Cefotaxime MICs were 32 μg/ml for *Cf* 29, 16 μg/ml for t*Cf* 29, and 0.12 μg/ml for *E. coli* Top 10. The ceftazidime MICs were 1 μg/ml for *Cf* 29, 0.5 μg/ml for t*Cf* 29, and 0.25 μg/ml for *E. coli* Top 10 (Table 1). The ceftazidime and cefotaxime MICs for *E. coli* 25922 were within the NCCLS values. All the clinical strains were positive for ESBL production, as determined by the MDDT (14).

Sonicates of both *Cf* 29 and t*Cf* 29 were subjected to analytical isoelectric focusing (IEF) as previously described (1, 16). Analytical IEF revealed the presence of a cefotaxime-hydrolyzing β-lactamase with a pI of 8.0 for both the clinical isolate, *Cf* 29, and the transformant, t*Cf* 29. This band was inhibited by clavulanic acid but not by cloxacillin. In addition, IEF analysis revealed two additional bands in the clinical strain *Cf* 29. One band correlated with a pI of 5.4 and was inhibited by clavulanic acid but not cloxacillin. This band most likely represented TEM-1. The other band correlated with a pI of 8.9 and was inhibited by cloxacillin but not by clavulanic acid. This band most likely represented the chromosomal AmpC of *C. freundii* (data not shown). No bands were detectable when extract from *E. coli* Top 10 was used.

The relative hydrolysis rates were determined spectrophotometrically by using a 100 μM concentration of each antibiotic, with the exception of ceftazidime, for which the concentration used was 50 μM (15). The enzyme preparations from both *Cf* 29 and t*Cf* 29 hydrolyzed cefotaxime (Table 2). Considering the hydrolysis rate of cephaloridin as 100%, the relative hydrolysis rates for the enzymes prepared from the clinical strain *Cf* 29 and the *E. coli* transformant, t*Cf* 29, were comparable, with the highest level of hydrolysis observed for cefotaxime and no hydrolysis detected for ceftazidime. Interestingly, the AmpC β-lactamase of *Cf* 29 was not inducible (data not shown) and cefoxitin MICs were ≤4 μg/ml (Table 1); therefore, hydrolysis due to AmpC of any of the β-lactams tested would be negligible. This is reflected by the relative rates of hydrolysis observed for the transformant, t*Cf*29, in which CTX-M-30 was the only β-lactamase present.

TABLE 2. Relative hydrolysis rates of CTX-M-30

Substrate	Relative hydrolysis rates (%)	
	*Cf*29[a] pIs of β-lactamases; 5.4, 8.0, and 8.9	t*Cf*29[b], pI of β-lactamase, 8.0
Cephaloridine	100	100
Penicillin	58	59
Cefotaxime	14	19
Ceftazidime	NC[c]	NC
Aztreonam	NC	NC
Cefepime	3.4	5.3
Imipenem	NC	NC

[a] Clinical isolate of *C. freundii*.
[b] *E. coli* transformant of strain *C. freundii* 29.
[c] NC, not calculated (rates were too low to obtain reliable values).

The plasmid encoding $bla_{CTX\text{-}M\text{-}30}$ most likely does not encode the genes required to transfer the plasmid, due to the small size of the plasmid. Self-transmissible plasmids encode *tra* genes as well as other genes required for transfer which require at least 15 kb of coding region (6, 8). These data, taken together with all the nucleotide changes (both those silent and those leading to amino acid changes), suggest the emergence of a novel CTX-M in Canada and not simply the transfer of established CTX-M genes from other countries.

The worldwide expansion of CTX-M–producing strains is a major concern. Therefore, it is important for clinical microbiologists to use both ceftazidime and cefotaxime for detecting ESBL-producing organisms. The use of ceftazidime alone may result in false-negative detection of organisms producing CTX-M β-lactamases and the unidentified spread of those ESBL producers.

Nucleotide sequence accession number. The $bla_{\text{-CTX-M-30}}$ gene nucleotide sequence was deposited in the GenBank database with accession number AY292654.

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