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

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Received 12 March 2004/Returned for modification 23 May 2004/Accepted 15 July 2004

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*<sub>CTX-M-30</sub> 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*<sub>CTX-M</sub> show high nucleotide similarity to the chromosomal β-lactamase genes of *Klebsiella* spp. (2, 10). This study identified a new *bla*<sub>CTX-M</sub> gene, *bla*<sub>CTX-M-30</sub>, 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 Cf12, Cf27, Cf28, Cf29, and Cf30. Strain identification was initially achieved using Vitek (Vitek AMS; BioMérieux Vitek Systems Inc., Hazelwood, Mo.) and API 20E strips (Bi-oMérieux 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 (GCAGCACCATTTGATGGG) and CTX-MIR (GCTGGGTGAAGTAAAGTGGC) (accession number X92950) and 46°C to obtain the full-length amplified product by use of primers CTX-M3FLF (CGTCTCTCAGTTAAGAAG G) and CTX-M3FLR (GTGTCCCCATTTGGTTGCGG) (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 tCf29.

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*<sub>CTX-M-30</sub> had 11 separate nucleotide changes positioned randomly throughout the gene compared to *bla*<sub>CTX-M-3</sub>. Two nucleotide changes resulted in two amino acid substitutions, Thr16Ala and Asn117Asp. In addition, *bla*<sub>CTX-M-30</sub> had seven separate nucleotide changes positioned randomly throughout the gene compared to *bla*<sub>CTX-M-29</sub>. 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*<sub>CTX-M-30</sub> was found not to be self-transmissible; therefore, Southern analysis was performed to determine the location of *bla*<sub>CTX-M-30</sub>.

Plasmid DNA was extracted by alkaline lysis from strains Cf12, Cf27, Cf28, Cf29, and Cf30 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 ultraviolet light with a Kodak EDAS 290 system. Southern analysis was performed as described by the manufacturer (Boehringer Mannheim, Indianapolis, Ind.). The *bla*<sub>CTX-M-30</sub>-specific probe was synthesized using PCR by incorporating digoxigenin-11-dUTP into the product by use of primers CTX-M1F and CTX-M1R.

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*<sub>CTX-M-30</sub> was encoded on a 3-kb plasmid and was not chromosomally encoded (data not shown). The gene *bla*<sub>CTX-M-30</sub> was also detected on the pXL-Topo plasmid transformed into tCf29.

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...
Cefotaxime MICs were 32 μg/ml for C. freundii, 14 μg/ml for E. coli Top 10. Cefotaxime and clavulanic acid together were used for testing resistance. Aztreonam MICs were 0.12 μg/ml. Ceftriaxone MICs were 0.25 μg/ml. Cefepime MICs were 1 μg/ml. Cefoxitin MICs were 0.12 μg/ml. Cefdinir MICs were 0.25 μg/ml for C. freundii and 0.5 μg/ml for E. coli Top 10. Cefpime and clavulanic acid MICs were ≤0.03 μg/ml. Cefpime and clavulanic acid MICs were ≤0.03 μg/ml. Cephaloridine MICs were 0.03 μg/ml. Cefpime and clavulanic acid MICs were ≤0.03 μg/ml. Cephalosporin MICs were 0.25 μg/ml. Cephalosporin MICs were 0.5 μg/ml. Cefoxitin MICs were ≤4 μg/ml. Imipenem MICs were 0.12 μg/ml. Amoxicillin and clavulanic acid MICs were 16 μg/ml. Piperacillin MICs were >256 μg/ml. Piperacillin and tazobactam MICs were 1 μg/ml. Cefotaxime MICs were 32 μg/ml for C. freundii, 14 μg/ml for E. coli Top 10. Cefazidime MICs were 0.5 μg/ml for C. freundii, 0.25 μg/ml for E. coli Top 10. Ceftriaxone MICs were 0.25 μg/ml. Cefepime MICs were 1 μg/ml. Cefoxitin MICs were 0.25 μg/ml. Cephaloridine was not inducible (data not shown). No bands were detectable when sonicates of the transformant, tCf29, were subjected to analytical IEF. The pl(s) of the β-lactamases produced by C. freundii were 5.4, 8.0, and 8.9. The pI (pl) of the β-lactamase produced by tCf29 was 8.0.

Relative hydrolysis rates of CTX-M-30

<table>
<thead>
<tr>
<th>Substrate</th>
<th>C2F/29 pl of β-lactamases; 5.4, 8.0, and 8.9</th>
<th>tC2F/29, pl of β-lactamase, 8.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cephaloridine</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Penicillin</td>
<td>58</td>
<td>59</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>14</td>
<td>19</td>
</tr>
<tr>
<td>Cefazidime</td>
<td>NC*</td>
<td>NC</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>NC</td>
<td>NC</td>
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<tr>
<td>Cefepime</td>
<td>3.4</td>
<td>5.3</td>
</tr>
<tr>
<td>Imipenem</td>
<td>NC</td>
<td>NC</td>
</tr>
</tbody>
</table>

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

**REFERENCES**

donor-specific phage propagation, and pilus production require the same
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