Citrobacter koseri and Citrobacter amalonaticus isolates carry highly divergent β-lactamase genes despite having high levels of biochemical similarity and 16S rRNA sequence homology

Sarah Underwood and Matthew B. Avison*

Bristol Centre for Antimicrobial Research and Evaluation, Department of Pathology & Microbiology, University of Bristol, School of Medical Sciences, University Walk, Bristol BS8 1TD, UK

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Objectives: Isolates previously identified as Citrobacter diversus are now known as Citrobacter koseri. We measured sequence variation at the β-lactamase structural gene among a group of clinical isolates originally identified as C. diversus by API 20E profiling.

Methods: β-Lactamase and 16S rRNA genes were amplified by PCR and sequenced by standard methods. β-Lactamase induction was attempted in liquid-grown cultures using cefoxitin. Nitrocefin hydrolysis assays were performed using a spectrophotometer.

Results: Analysis of 16S rRNA gene sequences showed that Citrobacter spp. isolates with an inducible β-lactamase gene, cdiA, closely related to 'C. koseri' NF85 and ULA27 are actually Citrobacter amalonaticus. C. koseri isolates, whose identities were confirmed by 16S rRNA sequencing, produce a class A β-lactamase, Cko, constitutively at low levels. The cko and cdiA β-lactamase genes share <45% identity.

Conclusions: We have confirmed that cko is a β-lactamase gene carried by C. koseri, and that isolates previously identified as 'C. koseri', but carrying the cdiA β-lactamase gene are C. amalonaticus. Thus, β-lactamase-gene-specific PCR may provide a valuable tool to differentiate these biochemically homogeneous Citrobacter species.

Keywords: Citrobacter, β-lactamases, phylogenetics, induction

Introduction

The genus Citrobacter is currently divided into 11 species, and some can be opportunistic pathogens of the immunocompromised or debilitated host, often following prophylactic β-lactam therapy. In the 1970s, two biochemical profiles were ascribed to the genus Citrobacter. Isolates fitting one profile were named Citrobacter freundii, but there was considerable confusion concerning the name of isolates having the other profile, with three possibilities being put forward: Citrobacter diversus, Citrobacter koseri and Levinea malonaticus. In general, however, the name C. diversus held sway.

Both C. freundii and C. diversus were shown to express single, chromosomally encoded, inducible β-lactamases, but the two have quite different substrate profiles and come from different molecular classes. C. freundii produces a class C β-lactamase, AmpC, which hydrolyses cephalosporins, including later generation compounds, and some penicillins. In contrast, C. diversus was shown to produce a class A β-lactamase, CdiA, hydrolysing aminopenicillins and carboxypenicillins. β-Lactamase genes from two C. diversus isolates, NF85 and ULA27, have been cloned and sequenced. The two cdiA genes are 98% identical.

In 1982, another species was introduced into the genus Citrobacter, named Citrobacter amalonaticus, which was only differentiated from C. diversus by the fact that the former is not able to utilize malonate as sole carbon and energy source. Isolates ULA27 and NF85 were identified as C. diversus using biochemical profiles set down in 1977, before the introduction of C. amalonaticus. At that time, it was acknowledged that C. diversus was variably positive for malonate utilization, with 12% of isolates being negative.

In 1993, C. diversus was rejected as a species name in favour of C. koseri because of an error in the naming of C. diversus, and the previously (and correctly) proposed name, C. koseri, for isolates with the C. diversus/C. koseri/L. malonaticus biochemical profile took over. This helped clarify the taxonomy of Citrobacter spp. at a time when eight new genomospecies were being proposed. At this time, all nucleotide database entries originally designated containing
sequences from *C. diversus* were automatically altered to state that the sequence came from *C. koseri*.

The *C. diversus* NF85 and ULA27 *cdiA* nucleotide sequence database entries (EMBL accession numbers CAA54738 and CAA44485) currently state that these isolates are *C. koseri*, and so CdiA β-lactamase is now thought of as being a *C. koseri* enzyme. However, in the first report characterizing *C. diversus* ULA27 and NF85 β-lactamases, differences from a β-lactamate, said to be expressed by a strain identified as *C. koseri*, were reported. Given that all these isolates are now known as *C. koseri* enzyme as around 5.0.7 Given that all these isolates are now known as *C. diversus*, it is possible that they are in fact *C. diversus*, a species that has a distinct 16S rRNA gene sequence that does not match perfectly with the 16S rRNA gene sequences from other *Citrobacter* spp. type strains. These isolates are not actually *C. koseri* isolates. Since they were identified as *C. diversus* before the separation of *C. amalonaticus* from *C. diversus*, it is possible that they are in fact *C. amalonaticus*, and that the automatic renaming of these isolates as *C. koseri* by the NCBI database was in error.

Accordingly, the aims of this study were: first to definitively identify ULA27 and NF85 is CKO+ve isolates ULA27 and NF85 is considerably different from *C. diversus* NF85 *cdiA* β-lactamase gene;7 primers *rRNA +ve* (5’-TCAGATTG- AACGCTGGCCGCA-3’) and *rRNA –ve* (5’-CCTATTCCGGG- GCCTGTCGCCAC-3’) for amplification of a 500 bp hypervariable region from the 16S rRNA gene. All PCR products were cleaned and sequenced as described previously. DNA sequence analysis, alignment and phylogenetic mapping was performed using the suite of programs, Lasergene (DNA-Star, Madison, WI, USA). For the phylogenetic analysis, an alignment was produced using the CLUSTAL W algorithm, applying a BLOSUM matrix with a gap-opening penalty of 10 and a gap-extension penalty of 0.1. The resultant alignment was analysed using a maximal likelihood method with the standard parameters in Lasergene.

**Materials and methods**

**Bacterial strains and materials**

Test isolates (see Table 1) were collected from faecal samples of inpatients at Southmead Hospital, Bristol, during 1980–1984. The identity of each isolate was determined as *C. diversus* using API 20E profiling, and to determine the sequence of the β-lactamase gene carried by each, and its level of expression.

**Polymerase chain reaction protocols**

16S rRNA and β-lactamase gene-specific PCRs were used, with genomic DNA from boiled colonies and the method described previously,10 with primers, ‘CKO +ve’ (5’-TTATCCATAGACTGCGAGTG-3’) and ‘CKO –ve’ (5’-CTTTATCAAGCTGCGAGTG-3’) for amplification of the entire *C. koseri* CK4eko β-lactamase gene (903 bp) (EMBL accession number AF477396); primers *CDIA +ve* (5’-ACAGGTCCAACA- AACGCTGG-3’) and *CDIA –ve* (5’-GTTTTATCGCCAACAC- CCA-3’) for amplification of a 650 bp internal fragment of the *C. diversus* NF85 *cdiA* β-lactamase gene;7 primers *rRNA +ve* (5’-GATTGGG- AAGCTGGCAGC-3’) and *rRNA –ve* (5’-CTGTATTCCGGG- GCCCTGTCGCCAC-3’) for amplification of a 500 bp hypervariable region from the 16S rRNA gene. All PCR products were cleaned and sequenced as described previously. DNA sequence analysis, alignment and phylogenetic mapping was performed using the suite of programs, Lasergene (DNA-Star, Madison, WI, USA). For the phylogenetic analysis, an alignment was produced using the CLUSTAL W algorithm, applying a BLOSUM matrix with a gap-opening penalty of 10 and a gap-extension penalty of 0.1. The resultant alignment was analysed using a maximal likelihood method with the standard parameters in Lasergene.

**Results**

Given that the sequence of *cdiA* carried by ‘*C. koseri’ isolates ULA27 and NF85 is considerably different from *cko*, carried by the biochemically confirmed *C. koseri* isolate CK4,9 we investigated the possibility that ULA27 and NF85 were erroneously identified as *C. koseri* by using 16S rRNA sequencing to identify them definitively. The sequence of a 500 bp hypervariable region of the gene was determined following PCR from both isolates. The sequences were then aligned with the equivalent regions of 16S rRNA genes from database entries representing *Citrobacter* spp. type strains. These data, presented as a phylogenetic tree in Figure 1(a), clearly show that the two isolates are not *C. koseri* at all, but actually represent *C. amalonaticus*.

Next, we set out to determine which of nine test clinical isolates, collected in the early 1980s, and originally identified as *C. diversus* by API 20E (Table 1) actually represent *C. koseri* and which represent *C. amalonaticus*. To do this, we used the 16S rRNA hypervariable sequencing method described above. Comparisons of the sequences obtained with the equivalent 500 bp regions of all *Citrobacter* spp. type strain 16S rRNA gene database entries (Figure 1a), revealed that four of the isolates, C13, C25, C28 and C32, are *C. amalonaticus* and four, C38, C45, C48 and C50, are *C. koseri*. The other isolate, C37, has a distinct 16S rRNA gene sequence that does not match perfectly with any of the *Citrobacter* spp. type strain sequences.

To investigate the hypothesis that *cdiA* is a *C. amalonaticus*-specific β-lactamase gene, and that *cko* is a *C. koseri*-specific β-lactamase gene, we used PCR with primers specific for an internal portion of *cdiA*, or for the entire *cko* gene, together with stringent annealing conditions. In fact, five isolates gave appropriately sized amplicons (650 bp) with the *cdiA* primers, and four gave amplicons of the correct size (903 bp) with the *cko* primers (not shown). All four
C. amalonaticus isolates were positive for cdiA and the sequences were >99% identical across the region sequenced, and all four C. koseri isolates were positive forcko, with the sequences being >97% identical. The isolate that could not be identified by 16S rRNA sequencing, C37 (Figure 1a), gave an amplicon with the cdiA primers, but the sequence was only 85% identical to those of the other cdiA amplicons. These sequence homologies are represented in the phylogenetic tree drawn in Figure 1(b), which also includes the database Citrobacter spp. class A β-lactamase sequences. This analysis confirms that the β-lactamase genes carried by the test isolates fall into two major groups, with one group clustering around the C. koseri CK4cko, and one clustering around the C. amalonaticus NF85cdiA. There is only ~40% identity between these two clusters. The variant cdiA sequence from isolate C37 does not cluster more closely with any other known Citrobacter spp. β-lactamase sequence than with cdiA (Figure 1b).

Previously, it has been reported that CdiA-producing isolates like ULA27 and NF85 produce the enzyme inducibly, or, if the induction system is mutated, then at high levels4,7 and that Cko-producing isolates like CK4, do so constitutively at low levels.9 To test whether the level/inducibility of β-lactamase production is generally consistent with the β-lactamase gene carried (and so the actual Citrobacter species), β-lactamase induction was attempted in all nine test clinical Citrobacter spp. isolates, and β-lactamase production was quantified as the ability of cell extracts to hydrolyse nitrocefin (Table 1). All the isolates that have a cdiA-like β-lactamase gene (including isolate C37) express an inducible enzyme, except isolate C13, which expresses the enzyme constitutively at high levels (Table 1). Of the four isolates that have ackoβ-lactamase gene, three produce a β-lactamase enzyme constitutively at low levels, and one, C45, produces an enzyme constitutively at high levels (Table 1). This high-level nitrocefin hydrolysing activity in isolate C45, was found by PCR to be due to the presence of an acquired TEM-1 β-lactamase, whereas no evidence of an acquired β-lactamase was found for isolate C13 (data not shown).

### Discussion

The genus Citrobacter is biochemically and genotypically diverse. To aid differentiation, 11 species have so far been identified,1 with the
**Citrobacter amalonaticus and Citrobacter koseri \(\beta\)-lactamas**es

Table 1. Identification and \(\beta\)-lactamase activity data for clinical Citrobacter spp. isolates used in this study

<table>
<thead>
<tr>
<th>Isolate</th>
<th>API 20E profile</th>
<th>ID (current)</th>
<th>Uninduced activity</th>
<th>Induced activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>C13</td>
<td>3344513</td>
<td>C. koseri/amalonaticus (99.9%)</td>
<td>0.92(^a)</td>
<td>0.90</td>
</tr>
<tr>
<td>C25</td>
<td>1144513</td>
<td>C. koseri/amalonaticus (59.2%)</td>
<td>0.16</td>
<td>0.25</td>
</tr>
<tr>
<td>C28</td>
<td>3344513</td>
<td>C. koseri/amalonaticus (99.9%)</td>
<td>0.18</td>
<td>0.50</td>
</tr>
<tr>
<td>C32</td>
<td>1344533</td>
<td>C. koseri/farmerii (93.2%)</td>
<td>0.15</td>
<td>0.29</td>
</tr>
<tr>
<td>C37</td>
<td>3644513</td>
<td>Citrobacter youngae (97.9%)</td>
<td>0.18</td>
<td>0.26</td>
</tr>
<tr>
<td>C38</td>
<td>3344551</td>
<td>C. koseri/amalonaticus (79.3%)</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>C45</td>
<td>3144713</td>
<td>C. koseri/amalonaticus (99.9%)</td>
<td>1.09</td>
<td>0.86</td>
</tr>
<tr>
<td>C48</td>
<td>3344513</td>
<td>C. koseri/amalonaticus (99.9%)</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>C50</td>
<td>3344713</td>
<td>C. koseri/amalonaticus (99.9%)</td>
<td>0.01</td>
<td>0.01</td>
</tr>
</tbody>
</table>

\(^a\)The identification given by bioMérieux, Inc., on 9 September 2003 (with the percentage confidence value) using the same profile. The original identification for all isolates was C. diversus.

\(^b\)Nitrocefin hydrolytic activity in crude cell extracts is in units of \(\mu\)moles of substrate hydrolysed per min per mg of protein.

\(^c\)Induction was by incubation of cells in 10 mg/L cefoxitin for 2 h prior to cell lysis and \(\beta\)-lactamase assay.

\(^d\)Values presented are averages of data from three experiments using different batches of cells. Variation was no more than 15%. Values have been rounded to three significant figures because of this, although the level of hydrolysis that can be accurately detected by the equipment is 0.001 \(\mu\)mol/min/mg.

old C. diversus being rebranded C. koseri in 1993. Data presented here show that definitively identified (i.e. using 16S rRNA sequencing) C. koseri isolates generally produce a small amount of \(\beta\)-lactamase constitutively. This is in agreement with previous reports, which stated that low-level constitutive production was a property of C. koseri. Knowledge of this constitutive phenotype may affect choice of clinical therapy for infections by confirmed C. koseri isolates. This means that the need to differentiate C. koseri from C. amalonaticus isolates, which produce \(\beta\)-lactamase inducibly, and so have the potential to mutate to high-level expression—thus high-level \(\beta\)-lactam resistance—is not simply an academic one. Indeed, C. amalonaticus isolate UL27 produces CdiA at high levels, presumably because of a mutation in the \(\beta\)-lactamase regulatory system. Overexpression of \(\beta\)-lactamase is also a property of the C. amalonaticus isolate C13, and since we have not been able to confirm the presence of an acquired \(\beta\)-lactamase in this isolate, it is most likely to be CdiA that is being overexpressed. However, in this study, the only C. koseri isolate found to express \(\beta\)-lactamase at high levels, has acquired TEM-1.

The gene encoding the low-level constitutively expressed \(\beta\)-lactamase from C. koseri isolate CK4, cko, was cloned and sequenced by Petrella et al., and the sequence has been deposited on the EMBL database (accession number AF477396). Using this unpublished sequence, we have designed PCR primers, and have amplified a homologue (<3% heterogeneity) from four definitively identified C. koseri isolates. The cko gene is only ~40% identical to cdiA. We await with interest the publication of the full sequence of Cko, and its biochemical characterization.

The data presented here show that at least two nucleotide sequence database entries (cdiA from isolates NF85 and UL27) have been erroneously altered via the NCBI taxonomy database as coming from C. koseri, because it was incorrectly assumed that all C. diversus isolates (the previous identification of NF85 and UL27) have biochemical profiles comparable with C. koseri. The reason for this error is that some isolates were identified as C. diversus prior to the acceptance of C. amalonaticus as a species, at a time when malonate utilization was known to be variable for C. diversus isolates. Thus nothing about the malonate-utilization status of such isolates should be assumed from their prior classification as C. diversus. In fact, we have confirmed here that both NF85 and UL27 are C. amalonaticus. Accordingly, all previous work using NF85 and UL27, of which much has been published, should be reinterpreted in the light of this error in taxonomy.

Given the biochemical variability of the genus Citrobacter, and the difficulty that commercially available systems have in identifying different Citrobacter spp., simple molecular tests may be of considerable value in the diagnostic laboratory. Of course, it is already possible to employ rRNA gene sequencing to identify Citrobacter spp. definitively, as we have done here, but there would be a considerable cost and time advantage if a binary PCR could be developed, which was specific for each species. Data presented here suggest that \(\beta\)-lactamase gene-specific PCR might be suitable for differentiating C. koseri and C. amalonaticus, which are difficult to differentiate biochemically. However, we do not propose that the specific PCR methodology employed here is suitable for such a test, not least because the cdiA PCR does not differentiate between C. amalonaticus cdiA and the homologue carried by isolate C37, which is 15% different.

Indeed, a final, unexpected point to come out of this work is the discovery of this novel partial \(\beta\)-lactamase gene sequence. Fifteen percent divergence between Citrobacter \(\beta\)-lactamase genes is a significant degree of heterogeneity given that Citrobacter sedlakii sedA is 20% different from C. amalonaticus cdiA. In terms of API 20E profile, isolate C37 is currently identified as Citrobacter youngae, but its 16S rRNA gene sequence does not agree with this classification, and does not allow definitive identification of the isolate. All
these data point to the possibility that isolate C37 represents a new *Citrobacter* species, although this remains to be seen.

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**References**


