ANTIBIOTIC RESISTANCE

Heterogeneity at the β -lactamase structural gene *ampC* amongst *Citrobacter* spp. assessed by polymerase chain reaction analysis: potential for typing at a molecular level

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Summary. Considerable biochemical diversity and polynucleotide sequence variation have been reported amongst strains of *Citrobacter* spp. However, sequence heterogeneity has not been investigated at gene loci of clinical relevance. In this study, sequence heterogeneity in the β -lactamase structural gene, *ampC*, amongst 91 clinical isolates of *Citrobacter* spp. that showed resistance to various third-generation cephalosporins was investigated. Variation was examined by high-stringency polymerase chain reactions (PCR) with primers homologous to the known *ampC* sequences of *C. freundii* strains OS60 and I113, and *C. diversus* NF85. If an isolate contained an *ampC* gene homologous to one of these three characterised *ampC* genes, a single PCR band of a predictable size was generated with the appropriate primer set; 50 (60%) of isolates gave a PCR product of the expected size with the OS60 primer set and nine (10%) gave a product with the I113 primer set. All these 59 isolates were identified as *C. freundii* by API-20E strips. Six isolates (7%) gave a product with the *C. diversus* NF85 primer set but only four of these were identified as *C. diversus* in API-20E tests; the other two isolates were identified as *C. freundii*. Of the 91 isolates, 28 (31%), were identified as either *C. freundii* or *C. diversus*, but gave no PCR product with any primer set tested. Five of these showed no homology to any of the reference strain *ampC* PCR products in hybridisation tests. Nevertheless, all showed β -lactamase activity. Overall, this method allowed the identification of novel *ampC* gene loci, which may serve as a basis for the identification of *Citrobacter* spp. rapidly at a molecular level.

Introduction

The genus *Citrobacter* comprises facultatively anaerobic, gram-negative rods and is divided into three species: *C. freundii* (biotypes a and b), *C. diversus* and *C. amalonaticus*. They are found commonly in water, soil, food and in the intestinal tracts of animals and man.¹ Previously regarded as harmless, they can be opportunist pathogens in the hospital environment and are associated with various infections in man, most often in the urinary tract, respiratory tract, deep tissue sites, superficial wounds and peritonitis.^{1–3} *C. diversus* is associated with sporadic and epidemic neonatal meningitis.^{4,5} A recent study in the UK, in which 5116 non-fastidious bacteria isolated from blood or urine cultures at 20 geographically diverse hospitals were examined, found that 54 (1%) were *Citrobacter* spp.⁶

Increasing numbers of immunocompromised patients in hospitals, combined with the widespread use of β -lactam antibiotics, may have led to an increase in the incidence of disease caused by *Citrobacter* spp.,⁷ and may have selected further drug resistance in the genus, particularly to penicillins and cephalosporins.^{8,9} In 1980, Lipsky *et al.* reported that c. 50% of *Citrobacter* strains were sensitive to ampicillin² whereas most clinical isolates are now resistant to ampicillin and to some first-generation cephalosporins. Some clinical isolates of *Citrobacter* spp. are resistant to ampicillin at 1000 mg/L and show high-level resistance also to third-generation cephalosporins, such as ceftazidime. Resistance can be mediated by several mechanisms, but one important source is production of a chromosomal β -lactamase encoded by a structural gene designated *ampC*.¹⁰ Expression of these genes is commonly inducible but mutation may give constitutive hyperproduction.^{11,12}

Our experience has shown that several strains of

Fig. 1. Positions of nucleotides homologous to primers used within the reference *ampC* genes.

Citrobacter with high-level β -lactam resistance are classified as "atypical" or incorrectly classified by widely used identification kits such as API-20E. Perhaps underlying this observation, considerable biochemical divergence occurs within this genus and much variation in polynucleotide sequence and genome size has been reported, particularly amongst biochemically "atypical" strains.¹³

Recently, restriction fragment length polymorphisms (RFLPs)¹⁴ and rapid amplified polymorphic DNA (RAPD) analysis by polymerase chain reaction (PCR)¹⁵ have provided alternative methods of typing Enterobacteriaceae. In this study, known sequence data for the β -lactamase structural genes, *ampC*, of *C. freundii* strains OS60¹⁶ and I113 (a biochemically "atypical" isolate) and *C. diversus* NF85,^{17,18} were used to investigate 91 clinical isolates of *Citrobacter* spp., and the homology of their *ampC* genes to those of the reference strains was assessed by PCR analysis. Isolates that appeared to lack a typical *ampC* gene were investigated further.

Materials and methods

Bacterial strains

The following reference strains were used: *C. freundii* OS60,¹⁶ *C. freundii* I113 and *C. diversus* NF85.^{17,18} All other isolates used were from hospitals in the UK and Europe; replicates were excluded. Isolates were identified at the source hospital with API-20E strips (API System, Monalieu, Vercieu, France) and were re-identified by the same method upon receipt by the authors.

Bacterial storage, culture and PCR template preparation

Isolates were stored on Dorset's egg slopes at room temperature. Malonate phenylalanine medium was used as a test of the ability of an organism to utilise malonate as a sole carbon source. Positive results were indicated by the de-amination of phenylalanine, resulting in a colour change from green to blue. For

PCR template preparation, cultures were streaked on to nutrient agar containing ampicillin 25 mg/L and incubated aerobically overnight at 37°C. Single colonies were picked-off and suspended in 15 μ l of sterile water. The suspension was boiled for 5 min to lyse the cells and the lysate was clarified by microcentrifugation. The supernate was used directly as the DNA template source.

ampC-specific DNA primers

All primers were synthesised in the Department of Biochemistry at the University of Bristol. Three primer sets were chosen—OS60 (primers 1 and 2, amplifying a 0.87-kb PCR product), I113 (primers 1 and 3, amplifying a 0.87-kb PCR product) and NF85 (primers 4 and 5, amplifying a 0.62-kb PCR product). Fig. 1 indicates the regions within each reference *ampC* gene homologous to each primer. Thus primer 1, sequence 5'-ATTCCGGGTATGGCCGTG-3', was complementary to the 5' region of both the *C. freundii* OS60 and the *C. freundii* I113 *ampC* genes; primer 2, sequence 5'-GATGGAATCAGCTTTCAG-3', was complementary to the 3' region of the *C. freundii* OS60 *ampC* gene; primer 3, sequence 5'-AACTATGTCGGCTTTCAC-3', was complementary to the 3' region of the *C. freundii* I113 *ampC* gene; primer 4, sequence 5'-ACAGGTCCAACAAAAGCTGG-3', and primer 5, sequence 5'-GTTTATCGCCAACCACCCA-3', were complementary to the 5' and 3' regions, respectively, of the *C. diversus* NF85 *ampC* genes. Each primer pair amplified only a region from the homologous gene type, and thus was *ampC*-type specific.

Conditions of amplification

Amplification reactions were performed in a reaction mixture containing 10 mM Tris-HCl (pH 9.0), 1.5 mM MgCl₂, 50 mM KCl; gelatin 0.1% w/v, Triton X-100 0.1% w/v, 200 μ M each of dATP, dGTP, dCTP and dTTP (LKB-Pharmacia, Milwaukee, WI USA), 0.1 μ M of each primer in the desired set and 0.1 unit of Super-Taq DNA polymerase (Stratagene, Scientific, Luton); 10 μ l of cell extract supernate were added and

Fig. 2. Examples of bands obtained from PCR amplification with *ampC*-specific primers. Tracks 1, molecular size markers (λ -*Bgl*II digest); 2-4, *C. freundii* OS60, ABGN5 and I113, respectively, tested with the OS60 primer set; 5-7, *C. freundii* I113, C525 and OS60, respectively, tested with the I113 primer set; 8-10, *C. diversus* NF85, C13 and *C. freundii* I113, respectively, tested with the NF85 primer set.

the volume was made up to 50 μ l. The mixture was overlaid with two drops of filtered liquid paraffin. PCR amplifications were performed in a Hybaid thermal cycler (Hybaid, Teddington, Middlesex) with temperature conditions as follows: 94°C for 3 min to denature the template DNA; followed by 30 high-stringency cycles, each of 94°C for 1 min, 68°C for 1 min and 72°C for 1 min; finally the mixture was held at 72°C for 5 min.

Analysis of PCR products

From each PCR reaction mixture, 10 μ l were re-

moved, avoiding the overlaid oil, and mixed with 4 μ l of bromophenol blue 0.25% w/v and sucrose 40% w/v in double-distilled water. Samples were analysed by electrophoresis in agarose 0.8% w/v gels with ethidium bromide 10 μ g/L. TBE buffer (0.089 M Tris base, 0.089 M orthoboric acid, 0.02 M EDTA, pH 8.0) was used both in the gel and as the running buffer. DNA amplification products were visualised by viewing over a UV-transilluminator (LKB-Pharmacia). Results were recorded on Polaroid type 665 instant positive/negative film.

Rapid qualitative detection of β -lactamase activity

In a well of a sterile microtitration plate, 30 μ l of an overnight broth culture were diluted with 50 μ l of 10 mM sodium phosphate buffer, pH 7.0. To this, 20 μ l, of toluene in ethanol 2% were added. This treatment destroyed the permeability barrier formed by the outer membrane. After 5 min, 400 μ l of nitrocefin (50 mg/L; Glaxo) were added. A colour change from yellow to red indicated the presence of a β -lactamase.

In-situ transfer of DNA from bacterial colonies on to nylon filters

Bacterial colonies were transferred on to nutrient agar with toothpicks. After overnight incubation at 37°C, the surface of the agar was dried for 1 h by removing the lid and incubating the inverted dish at 37°C. A sheet of GeneScreen Plus hybridisation membrane (Dupont) was cut to the same shape as the petri dish and gently pressed over the surface of the agar with a glass spreader to ensure good contact. The membrane was then removed and placed, bacterial cells uppermost, on a stack of Whatmann 3MM blotting paper which had been wetted with SDS 5%, to lyse the cells. After 5 min, the membrane was removed and placed, colony-side upwards, on to a stack of Whatmann 3MM papers wetted with denaturing solution (0.5 M NaOH, 1.5 M NaCl). After 5 min, the membrane was removed, blotted to remove excess denaturing solution, and floated, colony side upwards, on neutralising solution containing 3 M NaCl, 0.5 M Tris-HCl, pH 7.0, for 5 min. The membrane was then submerged and bacterial debris gently brushed off. After 5 min, the membrane was removed, blotted to remove excess moisture, and then submerged in 6 $\times$ SSC (0.9 M NaCl, 1 M sodium citrate; pH 7.0) for 20 min before baking at 80°C for 2 h.

Hybridisation

ampC PCR products from each reference strain were gel-purified by the method of Ausebel *et al.*,¹⁹ then labelled with [α^{32} P] dCTP by means of a commercial kit (Multiprime Kit; Amersham International Amersham, Buckinghamshire) used according to the manufacturers' instructions. High-stringency hybridisation procedures were as described by Ausebel *et al.*²⁰ All apparatus used in these procedures was supplied by Hybaid. Radioactivity was detected by overnight exposure of the filter to Hyperfilm[®]-MP (Amersham) at -70°C.

Results

Use of a high-stringency annealing temperature (68°C) eliminated non-specific PCR products, allowing clear results to be obtained with each of the

91 isolates. Either the PCR reaction yielded a specific band of the appropriate size or no product was obtained. Typical PCR products obtained from the primer sets are shown in fig. 2.

Fifty isolates (55%) gave bands of the appropriate size with the OS60 primer set. All these organisms had been identified as *C. freundii* with API-20E kits (table I). Nine isolates (10%) gave appropriate bands with the I113 primer set; seven of these had been identified as *C. freundii* by API-20E kits (table II) and the other two, initially identified as *Escherichia coli*, were found, on retyping with the API-50CHB kit, to be "atypical" *C. freundii*. One isolate, from Italy, gave a larger-than-expected band of 0.9 kb with the I113 primer set and was designated as having an I113+ *ampC* gene. Six isolates (7%) gave products with the NF85 primer set (table III). Four of these were identified as *C. diversus*

Table I. Isolates that gave a 0.87-kb PCR product with the OS60 primer set

Number of isolates	API profile no.	Identity	Confidence (%)
13	3604772	<i>C. freundii</i>	99.9
3	1604572	<i>C. freundii</i>	99.9
2	3604572	<i>C. freundii</i>	99.9
2	1604572	<i>C. freundii</i>	99.9
2	3404772	<i>C. freundii</i>	99.9
3	5404572	<i>C. freundii</i>	99.8
3	3404773	<i>C. freundii</i>	99.7
4	3604773	<i>C. freundii</i>	99.6
1	1404772	<i>C. freundii</i>	99.5
5	1604772	<i>C. freundii</i>	97.9
3	1604773	<i>C. freundii</i>	97.9
2	1404512	<i>C. freundii</i>	95.9
3	1404562	<i>C. freundii</i>	93.1
2	1404563	<i>C. freundii</i>	93.1
2	1004572	<i>C. freundii</i>	40.9

Table II. Isolates that give a 0.87-kb PCR product with the I113 primer set

Number of isolates	API profile no.	Identity	Confidence (%)
1	1604532	<i>C. freundii</i>	99.9
1	3604533	<i>C. freundii</i>	99.9
1	1404513	<i>C. freundii</i> *	99.6
2	1404512	<i>C. freundii</i>	95.9
1	1444553	<i>C. freundii</i>	90.0
3	1444503	<i>C. freundii</i>	59.2

* Gave an 0.9-kb PCR product, designated I113+.

Table III. Isolates that gave a 0.62-kb PCR product with the NF85 primer set

Number of isolates	API profile no.	Identity	Confidence (%)
1	3644513	<i>C. diversus</i>	99.9
3	3344513	<i>C. diversus</i>	99.8
1	1504513*	<i>C. freundii</i>	99.0
1	1404452*	<i>C. freundii</i>	95.9

* Gave 0.65-kb PCR products, designated NF85+.

with API-20E kits but two were identified as *C. freundii*. Both these latter organisms gave a band of c. 0.65 kb with the NF85 primer set, which is slightly larger than the expected size of 0.62 kb. This profile was designated NF85+. Twenty-six isolates (29%) failed to yield bands with any of the three primer sets (table IV); 17 of these were identified as *C. freundii* and nine as *C. diversus*. Nevertheless, all these 26 isolates were found to hydrolyse nitrocefin and so to express β -lactamase(s) and 21 had *ampC* genes that hybridised with one of the PCR products (table IV). The remaining five isolates, all of which were identified as *C. diversus* with API-20E strips and were able to utilise malonate as a sole carbon source (see *Discussion*), showed no homology to the *ampC* PCR products from *C. freundii* strains OS60 or I113 or *C. diversus* NF85.

Discussion

The first chromosomally encoded β -lactamase structural gene, *ampC*, to be sequenced from within the Enterobacteriaceae was that of *E. coli* K12²¹ followed by that of *Enterobacter cloacae* P99.²² Since then, many *ampC* genes have been characterised for several species, including *C. freundii* OS60¹⁶ (EMBL Accession Number X03866) and, more recently, *C. diversus* NF85^{17,18} (EMBL Accession Number X77657). The AmpC β -lactamase from *C. freundii* OS60 is a class C cephalosporinase, whereas the *C. diversus* NF85 AmpC enzyme is a class A penicillinase. Recently, the *ampC* gene from *C. freundii* I113 (EMBL

Accession Number X76636) has been sequenced in our laboratory and shown to encode a class C cephalosporinase, but to have only 82% nucleotide sequence identity to the *C. freundii* OS60 *ampC* sequence. The β -lactamase gene from *C. diversus* NF85 has only 28% nucleotide sequence identity to the *ampC* genes of *C. freundii* OS60 and I113. Nevertheless, the characterised *ampC* genes from these reference strains may not be representative of their species. Therefore, the relative abundance of the OS60, I113 and NF85-like *ampC* gene types was assessed amongst *Citrobacter* isolates.

With sequence-specific primer sets under high-stringency conditions, *ampC*-specific fragments were amplified by PCR. For many isolates these reactions rapidly identified the gene type present. If no PCR product was obtained with any primer set, further *ampC* sequence variation was assumed and hybridisation reactions were performed to detect whether the gene was present at all.

Over half of the isolates (54 of 91) were identified as typical *C. freundii* or as *C. diversus* and possessed β -lactamase structural genes with a high degree of homology to those of *C. freundii* OS60 or *C. diversus* NF85, respectively. Two isolates identified as *C. freundii* yielded an atypically large (c. 0.65 kb) PCR product, designated NF85+, with the NF85 primer set, but no product with either *C. freundii* primer set. This result suggests that these *ampC* genes have an unidentified insertion but, more importantly, that these strains of *C. freundii* possess an *ampC* homologous to the class A enzyme of *C. diversus* NF85.

Table IV. Isolates that give no PCR product with any primer set; homology to any of the PCR products is indicated in the final column

Strain no.	Strain source	API profile no.	Identity	Confidence (%)	Hybridisation*
D526	Bristol, UK	3404553	<i>C. freundii</i>	99.7	OI
D330	Bristol, UK	5104572	<i>C. freundii</i>	99.6	OI
C29	Bristol, UK	1404573	<i>C. freundii</i>	99.6	OI
C21	Bristol, UK	1404513	<i>C. freundii</i>	99.6	OI
C15	Bristol, UK	1404513	<i>C. freundii</i>	99.6	OI
C11	Bristol, UK	1404573	<i>C. freundii</i>	99.6	OI
D564	Bristol, UK	1404572	<i>C. freundii</i>	99.2	OI
ABGN5	ISGAR Study, Europe	1407552	<i>C. freundii</i>	96.8	OI
1715	Bristol, UK	3404512	<i>C. freundii</i>	94.8	OI
GUGN20	ISGAR Study, Europe	1504553	<i>C. freundii</i>	93.7	OI
C43	Bristol, UK	1504553	<i>C. freundii</i>	93.7	OI
C42	Bristol, UK	1104553	<i>C. freundii</i>	88.1	OI
STH48902	London, UK	3004532	<i>C. freundii</i>	86.1	OI
C49	Bristol, UK	3004532	<i>C. freundii</i>	86.1	OI
C19	Bristol, UK	1704552	<i>C. freundii</i>	60.9	OI
C33	Bristol, UK	1104542	<i>C. freundii</i>	48.6	OI
C14	Bristol, UK	1004572	<i>C. freundii</i>	40.8	OI
C50	Bristol, UK	3344713	<i>C. diversus</i>	99.6	U
GUGN29	ISGAR Study, Europe	1344513	<i>C. diversus</i>	99.4	U
C38	Bristol, UK	3344551	<i>C. diversus</i>	99.2	U
GUGN27	ISGAR Study, Europe	3144713	<i>C. diversus</i>	99.0	OI
C45	Bristol, UK	3144713	<i>C. diversus</i>	99.0	U
C32	Bristol, UK	1344533	<i>C. diversus</i>	97.0	N
BBGN13	ISGAR Study, Europe	1144513	<i>C. diversus</i>	88.3	OI
C25	Bristol, UK	1144513	<i>C. diversus</i>	88.3	N
BUGN15	ISGAR Study, Europe	1144513	<i>C. diversus</i>	88.3	U

* Homology to PCR product of: O, strain OS60; I, strain I113; N, strain NF85; U, no hybridisation found.

Of the nine isolates that gave products with the I113 specific primer set, seven originated from the same hospital in Bristol, where they were isolated over an 8-year-period. The other two isolates that gave PCR products with the I113 *ampC*-type originated from one hospital in Italy. One of these isolates gave an atypically large I113 PCR product, designated I113+, and came from the same hospital as the isolate with the NF85+ *ampC* type.

Of the 13 *C. diversus* isolates tested, four yielded a PCR product of the expected size with the NF85 primer set, but the other nine gave no PCR products with any primer set.

When the PCR products generated from the *ampC* loci of the three reference strains were used as hybridisation probes, it was possible to identify those isolates that possessed an *ampC* gene with homology to one of these reference strains (table IV). DNA from all but five of the isolates that were identified as *Citrobacter* spp. with a high degree of certainty showed homology to either the NF85 probe or to both the I113 and OS60 probes. The *C. freundii* I113 and OS60 *ampC* genes were sufficiently homologous for their probes to be cross-reactive. Five isolates that gave no product were all identified as *C. diversus*. A possible explanation is that *C. diversus* is distinguished from *C. amalonaticus* by the ability to utilise malonate as a

carbon source and this property is not tested with the API-20E kit. However, all these strains could utilise malonate and so were clearly *C. diversus* but had *ampC* genes that were not homologous to the gene of strain NF85.

In summary, most of the *C. freundii* isolates possessed an *ampC* locus homologous to OS60 or I113; but variation from the reference strain NF85 was greater amongst *C. diversus* isolates with five (33%) of 13 lacking typical *ampC* genes. Moreover, this method identified considerable sequence variation at the *C. diversus ampC* locus and indicated that the published *ampC* sequence of *C. diversus* NF85 may not be representative of all *C. diversus* isolates.

PCR analysis, with the primer sets described has provided an excellent tool for preliminary investigations into variation at the *ampC* locus. The method may locate potentially novel *ampC* genes for further identification and may form the basis of a rapid, reliable and cheap procedure to enable typing of these organisms at a molecular level.

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