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Short communication

Partial characterisation of the *acrAB* locus in two *Citrobacter freundii* clinical isolates

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Abstract

We studied the mechanisms of resistance to fluoroquinolones in two *Citrobacter freundii* strains (1.44 and 1.38) isolated from the same patient and belonging to the same clone by pulsed-field gel electrophoresis. This study allowed partial characterisation of the *acrA* and *acrB* genes of this microorganism. As previously reported, the two strains showed the same substitutions in the GyrA and ParC proteins (Thr-83 → Ile and Asp-87 → Tyr in GyrA and Ser-83 → Ile in ParC). However, differences were observed in the amount of ciprofloxacin accumulated, with strain 1.38 showing less accumulation. Expression of genes in both strains was analysed using DNA microarrays for *Escherichia coli*. Ten genes were overexpressed in strain 1.38 compared with strain 1.44, including genes *acrA* and *acrB*. Nucleotide similarity between the partially sequenced *acrA* and *acrB* genes of *C. freundii* and *E. coli* was 80.7% and 85%, respectively. The *acrA* and *acrB* genes of *C. freundii* are similar to those described in *E. coli* and their overexpression may play an important role in modulating the final minimum inhibitory concentration of fluoroquinolones in collaboration with mutations in the *gyrA* and *parC* genes.

Keywords: *Citrobacter freundii*; *acrA*; *acrB*

1. Introduction

Citrobacter freundii are Gram-negative bacilli causing a range of infections such as urinary tract infections, neonatal sepsis, brain abscess, meningitis, bloodstream infections, intra-abdominal sepsis and pneumonia [1]. Invasive *Citrobacter* infections are associated with a high mortality rate, with 33–48% of patients presenting with *Citrobacter* bacteraemia [2]. This high mortality rate may be due to ineffective empirical antibiotic therapy. With the use of broad-spectrum antibiotics, *C. freundii* has become increasingly resistant to antimicrobial agents [3].

Fluoroquinolones are a group of antimicrobial agents with good activity against Gram-negative bacteria, including *C. freundii*. Fluoroquinolones act by inhibiting the activity of type II topoisomerases (DNA gyrase and topoisomerase IV) [4]. Development of fluoroquinolone resistance in Gram-negative bacteria is due to two main factors: (i) mutations in the topoisomerases [4,5]; and (ii) decreased intracellular accumulation of the antimicrobial agent by decreased cell wall permeability or increased efflux pump expression [4]. Moreover, the presence of a plasmid carrying the *qnr* gene can contribute to the acquisition of quinolone resistance, mainly in *Klebsiella* spp. and *Escherichia coli* [4]. A single mutation in the amino acid codon Ser-83 of the *gyrA* gene is associated with decreased susceptibility or low-level resistance to fluoroquinolones [4], whereas double mutations in the amino acid codons Ser-83 and Asp-87 of the *gyrA* gene are associated with high levels of resistance [4]. On the other hand, accumulation of amino acid changes in GyrA with the simultaneous presence of alterations in ParC contribute to an increase in quinolone resistance [4]. In *C. freundii*, mutations in *gyrA* and/or *parC* appear to be the main mechanism of resistance to quinolones [5,6].

Gram-negative bacteria contain multidrug transporters to fluoroquinolones belonging to five different families: the multidrug and toxic compound extrusion (MATE)

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family; the major facilitator superfamily (MFS); the resistant–nodulation–division (RND) family; the small multidrug resistance (SMR) family; and the ATP-binding cassette (ABC) family [7]. The AcrAB pump is a member of the RND family, which has been investigated in depth in some Enterobacteriaceae [7]. First described in *E. coli*, this transporter consists of three elements: AcrB, an integral inner membrane protein; AcrA, a periplasmic lipoprotein; and an outer membrane channel, thought to be TolC [7]. The role of AcrAB in resistance to quinolones has been shown in other Enterobacteriaceae such as *Salmonella* [4,7,8], *Klebsiella* spp. [4,7,9] and *Enterobacter* spp. [4,7,10].

In this study, we characterised the *acrA* and *acrB* genes in *C. freundii* and investigated expression of these genes in two isogenic *C. freundii* strains isolated from the same patient and showing the same mutations in the *gyrA* and *parC* genes but with different levels of resistance to several antimicrobial agents.

2. Materials and methods

2.1. Bacterial isolates

Two *C. freundii* clinical isolates (strains 1.44 and 1.38) were consecutively recovered from the stools of a patient during a study of the effect of the use of norfloxacin on intestinal flora of cirrhotic patients treated with this fluoroquinolone in the Clinical Microbiology Laboratory at the Hospital Clínic of Barcelona, Spain. Analysis of the isolates by chromosomal DNA digestion with low-frequency restriction enzyme and pulsed-field gel electrophoresis (PFGE) showed that they belonged to the same clone [11].

2.2. Antimicrobial susceptibility testing

Susceptibility testing was performed by the microdilution method according to the guidelines established by the Clinical and Laboratory Standards Institute [12]. The antimicrobial agents used were ciprofloxacin (Bayer, Barcelona, Spain) and chloramphenicol (Sigma, St Louis, MO). The minimum inhibitory concentrations (MICs) for these isolates were determined either with or without the efflux pump inhibitor phenylalanine arginine β-naphthylamide (PAβN) at 20 μg/mL.

2.3. Microarrays

Total RNA from the *C. freundii* strains was extracted from a mid-exponential phase culture (optical density at 600 nm (OD_{600}) 0.6) using Qiagen RNeasy spin columns (Qiagen, Chatsworth, CA). A total of 20 μg of total RNA was labelled with Cy-3-dUTP (RNA from strain 1.44) or Cy-5-dUTP (RNA from strain 1.38) in a standard reverse transcriptase (RT) reaction, using Superscript II(+) (Gibco BRL, Carlsbad, CA) with 1 μg of random hexamer (Amersham Pharmacia, Piscataway, NJ) primers. After purification through Microcon-30 (Millipore, Billerica, MA), Cy3- and Cy-5-labelled cDNA were combined with SSC (2.5× final; 1× SSC = 0.15 M NaCI, 0.015 M trisodium citrate, pH 7), sodium dodecyl sulphate (0.25%) and 40 μg of *E. coli* rRNA (Boehringer Mannheim, Ingelheim, Germany) in a final volume of 16 μL and hybridised with the DNA microarray for 5 h at 65 °C. The DNA microarray contained 4058 open reading frames (ORFs) representing 95% of *E. coli* ORFs, performed as described in the MGuide (http://cmgm.stanfor.edu/pbrown/mguide/index.html). The glass slide was washed and scanned using an Axon Scanner GENPIX 1.0 (Axon Instruments, Foster City, CA) at 10 μm per pixel resolution. The resulting 16-bit TIFF images were analysed using SCANALYZE software (http://rana.stanford.edu/software/). The reproducibility of the technique was assessed in two separate experiments. A normalised relative Cy5/Cy3 ratio >2 was considered as a significant increase in expression and a normalised relative Cy3/Cy5 ratio >2 was considered as a significant decrease in expression in the two different experiments performed.

2.4. Quantitation of mRNA by RT-polymerase chain reaction (RT-PCR)

Once RNA was pure and free of DNA using RNAwiz (Ambion, Austin, TX), a RT-PCR reaction was performed following the instructions of the SuperScript™ One-Step RT-PCR Kit with Platinum *Taq* (Invitrogen, Barcelona, Spain). The primers used to perform the RT-PCR were designed from the sequences obtained previously as described below. Two sets of primers, facrart and racrart for the *acrA* gene and facrbrt and racrbrt for the *acrB* gene, were used. facrart (5′-CCTCAGGTTAGCGGGATTAT-3′) and racrart (5′-GATTGATGCGTGCAGTTTCTA) amplified a region of 303 bp, whilst facrbrt (5′-TTCGGCTTCTCAATAAATACCC-3′) and racrbrt (5′-GCCATCGCGGAAACAAT-3′) amplified a region of 289 bp. As an internal control for the RT-PCR, the housekeeping gene *gapA* (626 bp) was used. The primers used to amplify this gene were Gap1 (5′-GTATCAACGGTTTTGGCCG-3′) and Gap2 (5′-AGCTTTAGCAGCACCGGTA-3′). The components of the reaction mixture were 2× reaction mix (SuperScript™ One-Step RT-PCR Kit with Platinum *Taq*), 0.5 mM of each primer, 1 U of the RT/platinum *Taq* MIX (SuperScript™ One-Step RT-PCR Kit with Platinum *Taq*), 500 ng of the RNA template and distilled water to a volume of 50 μL. The reaction was performed with two initial steps of 50 °C for 30 min (reverse transcription) and 95 °C for 2 min to activate the *Taq* polymerase, followed by 16 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min.

To quantify a PCR product, it is important to stop the kinetic amplification reaction, usually between cycles 10 and 25, to compare the expression of a gene in differ-