Detection of clonally related *Escherichia coli* isolates producing different CMY β-lactamases from a cystic fibrosis patient

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**Objectives:** This study reports details on *Escherichia coli* isolates recovered from a cystic fibrosis (CF) patient in order to understand how this pathogen adapts to and resists broad-spectrum antipseudomonal therapy in this context.

**Methods:** Five *E. coli* isolates were obtained from various clinical samples (airways, urine or dialysis catheter) over a 7 month period covering a double-lung transplantation. All isolates were analysed in terms of clonality [enterobacterial repetitive intergenic consensus (ERIC)-PCR and multilocus sequence typing], virulence profiles (phylogroup and search for 15 virulence genes), growth patterns (morphotype, biofilm-forming ability and growth rate), hypermutability and antimicrobial susceptibility, with molecular characterization of β-lactamases and porins.

**Results:** The five isolates shared similar ERIC-PCR profiles and sequence types (ST1193) and exhibited the same virulence profile. The respiratory isolates were strong mutators, exhibited mucoid or small-colony morphotypes, exhibited strong biofilm-forming ability and grew slowly compared with the others. All isolates were highly resistant to ceftazidime. The respiratory isolates showed reduced susceptibility to cefepime and high resistance to aztreonam. The patient had received a 31 day course of ceftazidime/aztreonam until transplantation. All isolates harboured the same wild-type chromosomal AmpC. A CMY-2 enzyme was detected in the non-respiratory isolates. The respiratory isolates harboured L293S and V211A/L293S new CMY-2 variants, which were designated CMY-94 and CMY-95, respectively. OmpF porin loss was observed in the non-respiratory isolates.

**Conclusions:** Our study shows that, similarly to *Pseudomonas aeruginosa*, *E. coli* can undergo phenotypic and genomic changes in the CF context. For the first time, we identified an in vivo expanded-spectrum evolution of the CMY-2 β-lactamase, during bacterial persistence in the CF lung.

**Keywords:** adaptive process, mucoidy, hypermutators, expanded-spectrum cephalosporinases

**Introduction**

Cystic fibrosis (CF) causes multisystem complications and, generally, patients succumb to respiratory failure at an early age. The CF patient lungs become colonized throughout childhood with a variety of microorganisms.¹ Selection pressure, either intrinsic to the lung or induced by antimicrobial treatments, results in the selection of pathogens more suited to the inflamed lung tissue. *Pseudomonas aeruginosa*, with its remarkable genetic and metabolic flexibility, is the major cause of morbidity and mortality among CF patients.

*Escherichia coli* is rarely isolated from sputum cultures of CF patients and the pathogenicity of this species in CF airways has not been documented.²⁻⁴ However, in vitro experimental studies have shown that in *E. coli*, phenotypic diversifications can emerge under conditions of nutrient depletion or stress.⁵⁻⁷ In particular, it has been shown that in *E. coli* K-12, a mucoid phenotype might be an adaptive response to osmotic stress.³ Furthermore, hypermutable strains of *E. coli* have been involved in various diseases, such as urinary tract infections and, while at lower frequency than for *P. aeruginosa*, in CF lung infections (~1% versus 20%).⁵⁻⁶ Such hypermutable phenotypes are particularly worrying, since they may generate antibiotic-resistant mutants, while multidrug resistance in *E. coli* is an ever-increasing serious problem worldwide. In *E. coli*, decreased susceptibility to cephalosporins is mostly due to expanded-spectrum β-lactamase production, but can also be induced by overproduction of the chromosomal AmpC β-lactamase, with...
Evolution of the CMY enzyme in CF E. coli isolates

Materials and methods

**Bacterial isolates**

The five E. coli isolates (Jt1, Jt2, Jt3, Jt4 and Jt5) were collected over a 7 month period covering a double-lung transplantation. The times and sites of isolation are given in Table 1.

**Susceptibility testing**

Antimicrobial susceptibility was determined by the disc diffusion method, according to the 2011 EUCAST guidelines. The MICs were determined by the Etest method (bioMérieux, Marcy l’Etoile, France).

**Phylogenetic typing and virulence genotyping**

Phylogenetic groups were determined as previously described. Fifteen virulence genes (fimH, papGII, papGIII, sfa/foc, iucC, hly, hra, cnf1, neuB, ibeA, sat, vat, usp, csgA, and pgaA) were searched for, as described previously.

**Molecular typing**

The relatedness between the five isolates was assessed by enterobacterial repetitive intergenic consensus (ERIC)-PCR, as previously described. Multilocus sequence typing (MLST) was performed by using the primers and protocol specified at the E. coli MLST web site (http://mlst.ucc.ie/mlst/dbs/Ecoli).

**Determination of bacterial growth rates**

The growth rates of bacteria cultured at 37°C in Mueller–Hinton (MH) broth were determined from absorbance readings at 620 nm in a microplate reader.

**Biofilm formation assay**

As previously described, cultures of E. coli were inoculated in microtitre plates and incubated for 48 h at 37°C. Biofilm formation, assessed by crystal violet staining, was quantified at OD560.

**Determination of mutation frequencies**

This was performed as described by Galan et al., using MH agar plates with and without rifampicin (100 mg/L). Mutation frequency values, estimated as the mean ratio of rifampicin-resistant colonies to the total number of cfu, classified the strains as strong mutants (>5 × 10⁻⁷), weak mutants (≤5 × 10⁻⁷ to >5 × 10⁻⁸) or normomutables (≤5 × 10⁻⁸).

**Molecular characterization of β-lactamases and porins**

Boiled lysates were used as template DNA. Acquired β-lactamase genes (blaTEM, blaCTX-M, blaSHV, and blaCMY) were sought by PCR using specific primers. The chromosomal ampC promoter and gene and both porin genes, ompC and ompF, were PCR amplified and sequenced, as described previously. Sequences of the ampC promoter and gene were compared with those of E. coli K-12 (GenBank accession no. J01611).

**Nucleotide sequence accession numbers**

The nucleotide sequences of the CMY-94 and CMY-95 β-lactamases and of porins OmpC and OmpF (full-length and truncated form) were deposited in GenBank under accession numbers JX514368, JX514369, KC192041, KC192042 and KC243388, respectively.

Results and discussion

Few reports have described the recovery of E. coli isolates in CF patients. In general, species of Enterobacteriaceae are believed to be transient colonizers and are usually not addressed by antibiotic treatments. Molecular typing, by both ERIC-PCR and MLST, of the five E. coli isolates investigated in this study showed that the CF patient was persistently colonized/infected by a single E. coli clone belonging to ST1193, a single-locus variant of ST14 complex. The emergence of ST1193 fluoroquinolone-resistant E. coli isolates has been recently documented in Australia. Our five isolates exhibited the same virulence profile (phylogenetic group B2, fimH, iucC, sat, vat, usp, csgA and pgaA), similar to that described for the Australian ST1193 isolates. This high level of homogeneity between ST1193 strains from different geographical areas indicates a probable recent divergence from a common ancestor.

Here, we show that our ST1193 clone generated subclonal variants. Thus, the three E. coli isolates recovered from the airways (Jt1, Jt2 and Jt5) showed intrapulmonary pathoadapted phenotypes with impaired growth, formation of mucoid or small colonies when grown on agar plates and production of biofilm in vitro (Figures S1, S2 and S3, available as Supplementary data at JAC Online). In comparison, the Jt3 and Jt4 isolates (found in the urine and a catheter, respectively) did not exhibit such CF-niche specialization. It is very likely that the variations observed were consequences of a trade-off between self-preservation and nutritional capability. Changes through the ompF/ompC system provide control over this nutrition/protection balance. Hence, the Jt3 and Jt4 isolates lacked the porin OmpF as a consequence of the deletion of 11 nucleotides at position 367, resulting in an early stop codon. We can hypothesize that this loss of permeability protected the bacteria from bactericidal effects, whereas it would be deleterious in the nutrient-poor CF lung. Finally, we showed that this bacterial adaptability was promoted by a hypermutator genetic background, particularly for the Jt1, Jt2 and Jt5 isolates (mutation frequency of 2.03 × 10⁻⁷, 1.48 × 10⁻⁶ and 0.98 × 10⁻⁶, respectively), while the Jt3 and Jt4 isolates were considered as weak mutants (mutation frequency of 1.34 × 10⁻⁷ and 2.02 × 10⁻⁷, respectively).

All isolates were multiresistant, but exhibited variations in their β-lactam resistance phenotypes (Table 1). Taking into account their hypermutable phenotypes, we looked for mutational β-lactam resistance development. The expanded-spectrum hydrolysis profiles of the isolates were not explained by the overproduction or extension of the hydrolytic activity of the chromosomally encoded β-lactamase. The five isolates harboured the same ampC promoter (polymorphisms at positions

or without an extension of the spectrum of activity of the enzyme. Acquired AmpC β-lactamases, such as CMY enzymes, can also contribute to resistance to ceftazidime.
273, 228, +17 and +81) and gene (amino acid substitutions Q175K, P193S, S282I, A300P and D351A). All isolates coproduced a TEM-1 penicillinase and a CMY-type cephalosporinase; but, strikingly, the Jt3 and Jt4 isolates harboured a CMY-2 enzyme, whereas new CMY-2 variants, designated CMY-94 (L293S) and CMY-95 (L293S and V211A), were carried by the isolates from the airways. Furthermore, in the Jt3 and Jt4 isolates, the loss of the porin OmpF probably induced a slight increase in the ertapenem MICs. The family of CMY-type $\beta$-lactamases is the largest group of mobile AmpC. The CMY-2 enzyme is the most widespread worldwide and may also be the precursor of the other CMY variants. Thus, expanded-spectrum CMY-2 variants have already been reported. In our study, the CMY-94 and CMY-95 $\beta$-lactamases, carrying the L293S substitution in the R2 loop, exhibited increased activity against cefepime. Of note, other changes at this position (L293P mutation or amino acid deletions) have been described in the chromosomal AmpC of Enterobacter aerogenes and E. coli, and in the CMY-33 or CMY-44 $\beta$-lactamases. Furthermore, in our study, both CMY-95 enzymes carried an additional V211A substitution in the V loop, which certainly provided an increase in aztreonam resistance and a simultaneous decrease in cefepime resistance. In fact, other authors have shown that the CMY-30 and CMY-42 $\beta$-lactamases, carrying the V211G and V211S substitutions, respectively, exhibited enhanced activity against ceftazidime and aztreonam, through the replacement of the bulky side chain of valine.

We suppose that the CMY variants identified in our hypermutable E. coli evolved from CMY-2 by stepwise mutations. The patient was infected with P. aeruginosa and, before transplantation, received a 31 day course of aztreonam plus ceftazidime at high doses. This treatment, ineffective on the Jt1 isolate, probably induced the V211A substitution.

In conclusion, this report provides evidence of the tremendous ability of E. coli to diversify and adapt in the CF lung environment. The clinical significance of such pathogens in CF is still subject to debate. However, the persistence of such bacteria in the CF lung makes them a reservoir of new resistance mechanisms. The in vivo selection of CMY-2 mutants in this study offers a unique insight into one of the main drawbacks of aggressive antimicrobial therapeutic strategies, which remain the cornerstone of CF therapy.

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### Transparency declarations

None to declare.
Supplementary data
Figures S1, S2 and S3 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References