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Emergence of AcrAB-mediated tigecycline resistance in a clinical isolate of *Enterobacter cloacae* during ciprofloxacin treatment

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Abstract

Tigecycline resistance remains rare amongst Enterobacteriaceae in the UK, as elsewhere, but has been associated with up-regulation of the AcrAB efflux system. Using isolates of an *Enterobacter cloacae* strain that developed tigecycline resistance *in vivo* during ciprofloxacin therapy and laboratory-selected mutants, we investigated the role of this pump and the global regulator RamA in this resistance. Laboratory mutants were selected from a susceptible clinical isolate *in vitro* by exposure to increasing concentrations of tigecycline. Expression of the *acrAB* operon and *ramA* gene was monitored by real-time RT-PCR. Overexpression of *ramA* was achieved using the pBAD expression vector, whilst insertional inactivation of *acrB* with a gentamicin resistance cassette was achieved with the bacteriophage λ Red recombination system. Increased tigecycline MICs in the clinical isolate and a laboratory mutant were associated with increases in *acrAB* and *ramA* transcripts. Induction of increased *ramA* expression resulted in increased *acrAB* expression, whilst insertional inactivation of *acrB* restored full susceptibility to tigecycline. Treatment with ciprofloxacin – a substrate of AcrAB in *E. cloacae* – possibly selected for cross-resistance to tigecycline as a result of RamA-mediated AcrAB up-regulation.

1. Introduction

Enterobacter cloacae is the most important nosocomial pathogen in the genus *Enterobacter* and is of increasing concern to clinicians owing to the many isolates that are multi-drug resistant. Tigecycline has good *in vitro* activity against *E. cloacae* and is a welcome addition to the armamentarium (1), however, *E. cloacae* is the least susceptible member of the genus to this compound (2) and cases of emerging resistance have been described (3-5). Where their mechanisms have been investigated, up-regulation of the RND efflux system AcrAB and the global regulator RamA have been implicated (5).

We investigated the role of these components in an *E. cloacae* strain that developed tigecycline resistance during ciprofloxacin treatment and *in vitro*, under tigecycline pressure.

1.1. Case report

In 2006, a patient presented with a ten-day history of progressively increasing back pain, high fever (40°C) and swelling over the spine. He had had two previous operations for stabilisation of a lumbar spinal fracture, 20 months, followed by a revision 16 months, prior to this event. An aspirate revealed two colony variants of *Enterobacter cloacae* which varied in their antibiotic susceptibility patterns. Both appeared sensitive to ciprofloxacin and the initial regimen was changed to ciprofloxacin (400mg 12 hourly iv) on day four after admission. A definitive surgical procedure was performed three days later and one litre of pus and all the metalwork (eight screws and plates) were removed. The same organisms were isolated again but they now showed changes in antibiotic resistance patterns, with one variant now

76 resistant to ciprofloxacin (here called isolate TGC-R) and the other remaining
77 susceptible (isolate TGC-S). This pair of isolates was referred to the Health Protection
78 Agency's Antibiotic Resistance Monitoring and Reference Laboratory, where the
79 ciprofloxacin-resistant isolate was found also to be resistant to tigecycline.
80 Ciprofloxacin was subsequently replaced with meropenem after eight days and the
81 patient slowly recovered after several months of carbapenem therapy. The original
82 source of the infection was not established.

83

84

2. Materials and Methods

2.1. Bacterial isolates, identification, and growth conditions

The organisms were clinical isolates, laboratory-selected mutants, or gene knockout mutants of *E. cloacae* (Table 1). The clinical isolates, designated TGC-S and TGC-R, were identified by API20E (bioMérieux, Marcy l'Etoile, France). PFGE was performed on *Xba*I-digested genomic DNA to determine isolate relatedness. Isolates were propagated in Luria-Bertani (LB) or Iso-Sensitest (Oxoid, Basingstoke, UK) broth or agar at 37°C.

2.2. Antimicrobial susceptibility testing

MICs of tigecycline, tetracycline, minocycline, ciprofloxacin, chloramphenicol, meropenem, and ceftiofur were determined by agar dilution on Iso-Sensitest agar according to BSAC guidelines or by E-test (AB Biodisk, Solna, Sweden) used on Iso-Sensitest agar and MICs were interpreted using BSAC breakpoints (6).

2.3. Laboratory-selection of mutants

Clinical isolate TGC-S was passaged with serially increasing concentrations of tigecycline in Iso-Sensitest broth, beginning at half the MIC (0.25 mg/L) and the tigecycline concentration doubled every 24 hours until there was no further growth. MICs for mutants were determined daily by E-test. PFGE profiles of the final mutant (TGC-S7) and all intermediates were compared with that of isolate TGC-S in order to confirm parentage.

2.4. DNA manipulations and GenBank accession number

Gel electrophoresis and restriction enzyme digestion were performed as previously described (7). Genomic DNA was isolated using the Wizard Genomic DNA Purification Kit (Promega, Southampton, UK) and was used as the template for PCR. Plasmids were isolated from α -select *Escherichia coli* (Bioline, London, UK) using the PureYield Plasmid Miniprep System (Promega). PCR products were purified using the GeneClean Turbo for PCR (MP Biomedicals, Strasbourg, France). DNA transformations were performed by electroporation (1.8 kV, 25 μ F, 200 Ω) using the Gene Pulser system (Bio-Rad, Hemel Hempstead, UK). Nucleotide sequences were determined with a CEQ 8000 Genetic Analysis System automatic sequencer (Beckman Coulter, High Wycombe, UK). The nucleotide sequence data reported here have been deposited with GenBank with the accession numbers **GU180677** and **GU180678**.

2.5. Real-time RT-PCR

Expression of the *acrAB* operon and the *ramA* gene was monitored by real-time RT-PCR and quantified relative to that of the RNA polymerase β subunit gene, *rpoB*, using oligonucleotide primers specific for *acrB* (forward, 5'-CGA TAA CCT GAT GTA CAT GTC C-3'; reverse, 5'-CCG ACA ACC ATC AGG AAG CT-3'), *ramA* (forward, 5'-CCG TTA CGC ATC GAA GAG AT-3'; reverse, 5'-CCC AGA CTT TCG CCT TTG TA-3'), and *rpoB* (forward, 5'-AAG GCG AAT CCA GCT TGT TCA GC-3'; reverse, 5'-TGA CGT TGC ATG TTC GCA CCC ATC A-3') to amplify products of 185, 92, and 147 bp, respectively.

DNase-treated RNA template was prepared from overnight cultures using the RNeasy Mini Kit (Qiagen, Crawley, UK) and RT-PCR was undertaken using the QuantiFast SYBR Green RT-PCR Kit (Qiagen) on a LightCycler 2.0 Real-Time PCR System (Roche, Burgess Hill, UK) with 10 ng RNA and protocols supplied by the manufacturer. Normalised expression levels of the target gene (relative to *rpoB*) were calculated using the $2^{-\Delta\Delta CT}$ method (8). Reactions were performed in triplicate using template from separate RNA extractions.

2.6. Overexpression of *ramA* in clinical isolate TGC-S

The prokaryotic expression vector pBAD (Invitrogen, Paisley, UK) was modified to replace the unique *NcoI* restriction site with a unique *SmaI* site, yielding plasmid pBAD_S. A 1138 bp DNA fragment containing the kanamycin resistance cassette from pCR2.1 (Invitrogen) was then amplified by PCR using the proofreading *Pfu* DNA polymerase (Promega) (forward primer, 5'-GCT TAC ATG GCG ATA GCT AGA CT-3'; reverse primer, 5'-AGT TCG ATG TAA CCC ACT CGT GC-3'), and cloned into *EcoRV*-digested pBAD_S to give the final vector pBADKM.

The *ramA* gene was amplified by PCR from clinical isolate TGC-R using *Pfu* DNA polymerase and the following primer pair: forward, 5'-ATG ACG ATT TCC GCT CAG GTC-3'; reverse, 5'-TGA AGC TTT CAG TGC GTC CGA CTA TGG TTT TC-3'. The resulting 350-bp product was digested with *HindIII* and directionally cloned into *SmaI/HindIII*-digested pBADKM to produce plasmid pBADKM-R, which was transformed into isolate TGC-S for the *ramA* overexpression studies; vector pBADKM was used as a negative control.

LB broth (10 ml) containing 50 mg/L kanamycin was seeded with 100 µl of an overnight culture of TGC-S harbouring either pBADKM or pBADKM-R and incubated with shaking (225 rpm) at 37°C to mid-log phase ($OD_{600} = 0.5$). Expression of *ramA* was then induced for 4 hours by the addition of L-arabinose to final concentrations of 0.2, 0.02, 2×10^{-3} , and 2×10^{-4} % w/v. RNA extractions were then performed and expression of *acrAB* was monitored by real-time RT-PCR.

2.7. Insertional inactivation of *acrB*

The *acrB* gene was inactivated by the insertion of a gentamicin resistance cassette using methods described by Pérez et al. (9) with some modifications. The helper plasmid pKOBEG (10) was introduced into TGC-R and transformants selected on LB agar plates containing 100 mg/L chloramphenicol during overnight incubation at 30°C. A single such transformant was made electrocompetent as follows: 20 ml of modified LB broth (without NaCl) containing 100 mg/L chloramphenicol was seeded with 200 µl of an overnight culture of TGC-R harbouring pKOBEG and incubated at 30°C with shaking (200 rpm). When the OD_{600} reached 0.2 the phage λ Red genes encoded by pKOBEG were induced by the addition of L-arabinose to a final concentration of 0.2 % w/v. When the OD_{600} reached 1.0 the culture was centrifuged at 4000 g at 4°C for 10 minutes, the supernatant removed and the pellet re-suspended in 40 ml of cold sterile water and re-centrifuged as before. The final pellet was suspended in 90 µl of 15 % sterile glycerol.

An internal 1558-bp fragment of the *acrB* gene was amplified by the Expand High Fidelity Plus PCR system (Roche) from TGC-R genomic DNA using primers 5'-CAT CCA GCA CTT TCT GCG TA-3' (forward) and 5'-CGA TAA CCT GAT GTA CAT

GTC C-3' (reverse) and cloned into pCR2.1 to generate plasmid pCRBK-1. The gentamicin resistance cassette from cloning vector pBBR1MCS-5 was amplified by PCR using *Pfu* DNA polymerase and primers 5'-TGG CGG CGT TGT GAC AAT TTA C-3' (forward), and 5'-TGG ACG CAC ACC GTG GAA ACG G-3' (reverse) (11). The resulting 854-bp fragment was cloned into *Nru*I-digested pCRBK-1 (within the *acrB* fragment) to generate pCRBK-2. The linear DNA fragment employed to interrupt *acrB* was then amplified from pCRBK-2 using the internal *acrB* fragment primers detailed above and the Expand High Fidelity Plus PCR system. The resulting PCR product was introduced into TGC-R::pKOBEG, and transformants were selected on LB agar plates containing 10 mg/L gentamicin. Chromosomal integration of the gentamicin resistance cassette was confirmed by PCR and sequencing across the insertion site.

3. Results and Discussion

MICs of ciprofloxacin and tigecycline were eight-fold higher for the clinical isolate TGC-R (4 mg/L for both compounds) than for TGC-S (both 0.5 mg/L; Table 1).

Although this clinical pair did not have identical PFGE profiles, analysis of the intermediate laboratory-selected mutants derived from TGC-S showed an analogous transition from the tigecycline-susceptible to the tigecycline-resistant profile; moreover, the PFGE profile of the final mutant TGC-S7 (ciprofloxacin MIC 4 mg/L; tigecycline MIC 32 mg/L) was indistinguishable from that of clinical isolate TGC-R (Figure 1). MICs of chloramphenicol, minocycline, and tetracycline also were raised for TGC-R and TGC-S7 relative to TGC-S, whereas those of β -lactams were unaffected, although that of ceftiofame was high already (8 mg/L), possibly reflecting AmpC-activity, as based on interpretive reading of wider antibiogram data (data not shown).

Expression of *acrAB* and *ramA* was analysed by real-time RT-PCR. Relative to the susceptible isolate, TGC-S expression of *acrAB* and *ramA* was increased four- and 276-fold, respectively, in isolate TGC-R and nine- and 407-fold, respectively, in the laboratory mutant TGC-S7.

Keeney et al. have previously demonstrated an association between elevated tigecycline MICs and overexpression of *acrAB* and *ramA* in *E. cloacae* (4). However, no previous experimental evidence has placed *acrAB* definitively in the RamA regulon. In order to investigate this aspect, *ramA* from isolate TGC-R was overexpressed in isolate TGC-S using the pBAD expression vector. Induction of *ramA* expression by increasing the L-arabinose concentration resulted in increased

acrAB expression (Figure 2). When *ramA* was induced with a relatively high (0.2% w/v) concentration of L-arabinose, *acrAB* was expressed at a higher level than in isolate TGC-R, for which the tigecycline MIC was 4 mg/L (relative *acrAB* expression = 7.75 versus 4.43 for TGC-R). Comparison of the nucleotide sequence of *ramA* (partial coding region) in isolates TGC-S and TGC-R revealed a His-95→Arg substitution in TGC-R, although this seems unlikely to be critical as it was not present in TGC-S7.

The *acrB* gene in isolate TGC-R was disrupted by insertional inactivation using the helper plasmid pKOBEG and a linear DNA fragment containing a gentamicin resistance cassette flanked by regions complementary to the target gene. This restored full susceptibility to tigecycline, ciprofloxacin, chloramphenicol, and resulted in a large reduction in the MICs of minocycline (≥ 64 -fold) and tetracycline (16-fold) (Table 1). These data confirm that up-regulation of AcrAB, mediated by RamA activation, was the factor conferring tigecycline resistance in *E. cloacae*.

To our knowledge, this is the first report of emergence of resistance to tigecycline associated with ciprofloxacin therapy, rather than tigecycline therapy. Ciprofloxacin is known to be a substrate of AcrAB in *E. cloacae* (9) and it is possible that exposure to ciprofloxacin selected for AcrAB up-regulation, which resulted in cross-resistance to tigecycline. The potential to select cross-resistance to new antimicrobials such as tigecycline through the use of established front-line therapies is of concern in the clinic.

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Declarations

Funding: This work was supported by an educational grant from Wyeth.

Competing interests: D. M. L. has (i) received research grants from Wyeth, (ii) spoken at meetings organised by Wyeth and (iii) received sponsorship to travel to congresses from Wyeth, as well as from numerous other pharmaceutical and diagnostic companies. He holds shares in GlaxoSmithKline, Schering-Plough, AstraZeneca, Dechra and Pfizer, who have now taken over Wyeth; he acts also as Enduring Attorney for a close relative, managing further holdings in GlaxoSmithKline and EcoAnimal Health. N. W. has received research grants from Wyeth. M. H., M. E., M. D., D. M. L. and N. W. are employees of the HPA and are influenced by its views on antibiotic use and prescribing. G.S. has none to declare.

Ethical approval: Not required.

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Figure Legends

Figure 1

PFGE profiles of clinical isolates TGC-S and TGC-R and all laboratory-selected mutants.

Figure 2

Relative expression (means $\pm$ standard deviations) of *acrAB* in TGC-S harbouring either control plasmid pBADKM (white) or pBADKM-R (black) in the presence of varying concentrations of L-arabinose.

Table 1. Characteristics of *E. cloacae* isolates and derivatives used in this study

Isolate	Origin	MIC (mg/L)							Relative expression of <i>acrAB</i> ($\pm$ SD)	Fold increase in <i>acrAB</i> expression*	Relative expression of <i>ramA</i> ($\pm$ SD)	Fold increase in <i>ramA</i> expression*
		TGC	TET	MIN	CIP	CHL	MEM	CPR				
TGC-S	clinical isolate (pus)	0.5	2	4	0.5	8	0.125	8	1.07 (0.36)	1	0.05 (0.01)	1
TGC-R	clinical isolate (pus)	4	16	>32	4	64	0.125	8	4.43 (1.63)	4	13.78 (2.94)	276
TGC-S7	final lab mutant	32	32	>32	4	>256	0.25	4	9.94 (2.27)	9	20.35 (6.45)	407
TGC-R Δ <i>acrB</i>	<i>acrB</i> knockout mutant	0.25	1	1	0.25	4	0.125	8	not tested	not applicable	not tested	not applicable

* relative to TGC-S

Antibiotic abbreviations: TGC, tigecycline; TET, tetracycline; MIN, minocycline; CIP, ciprofloxacin; CHL, chloramphenicol; MEM, meropenem; CPR, cefpirome.

Figure 1.

Figure 2.