



## Short Communication

# Identification of a novel mutation at the primary dimer interface of GyrA conferring fluoroquinolone resistance in *Clostridium difficile*



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## ABSTRACT

The aim of this study was to determine whether alternative resistance mechanisms, other than mutation in the quinolone resistance-determining region (QRDR) of DNA gyrase, could confer fluoroquinolone resistance in *Clostridium difficile*. An in vitro-generated *C. difficile* mutant exhibiting increased fluoroquinolone resistance was isolated through antibiotic selection on ciprofloxacin. The QRDR of this mutant was investigated by chain-termination sequencing and was found to be devoid of mutation. To determine the nature of the non-QRDR resistance mechanism in this strain, the genomes of the mutant and wild-type strains were sequenced. The *gyrBA* region from a collection of clinical isolates exhibiting variable fluoroquinolone resistance levels was also sequenced and was compared with that present in 918 publicly available *C. difficile* genomic data sets. Whole-genome sequence analysis of the fluoroquinolone-resistant mutant revealed a single non-synonymous substitution (Ala384Asp) at the predicted primary dimer interface of GyrA, far beyond the classically defined QRDR. This novel mutation caused increased resistance to ciprofloxacin, ofloxacin, levofloxacin and moxifloxacin while conferring hypersusceptibility to novobiocin. Several novel extra-QRDR polymorphisms in *C. difficile* DNA gyrase were identified among clinical isolates, whilst observed fluoroquinolone resistance in strains devoid of *gyrBA* mutations confirmed the existence of DNA gyrase-independent resistance mechanisms in this species. In conclusion, we report the first non-QRDR mutation to confer fluoroquinolone resistance in *C. difficile*. Although the Ala384Asp substitution was not detected in clinical isolates, this study revealed a diversity of alternative extra-QRDR polymorphisms in DNA gyrase whose association with fluoroquinolone resistance warrants further investigation.

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## 1. Introduction

*Clostridium difficile* is a major intestinal pathogen with a disease spectrum ranging from mild diarrhoea to life-threatening pseudomembranous colitis [1]. An established risk factor for *C. difficile* infection (CDI) is prior antibiotic exposure, which causes reduced microbial diversity in the gut and the consequent loss of colonisation resistance [2]. Antibiotic resistance is a common characteristic of endemic *C. difficile* strains. Outbreaks in the 1990s are attributed to the emergence and international

spread of virulent clindamycin-resistant strains, whilst globally disseminated strains of the O27/BI/NAP1 lineage exhibit resistance to the fluoroquinolones [1,3].

Fluoroquinolone resistance is caused by mutations in DNA gyrase or topoisomerase IV that abrogate drug-target binding [4]. In *C. difficile*, resistance is linked to a homoplasic Thr82Ile substitution in the quinolone resistance-determining region (QRDR) of the GyrA DNA gyrase subunit, a hallmark of globally disseminated O27/BI/NAP1 strains [5]. Several additional mutations that map to the QRDR of *gyrA* and *gyrB* have also been reported in *C. difficile*, whereas the type IV topoisomerase is absent in *C. difficile* as well as several other pathogenic species [6]. Molecular characterisation of fluoroquinolone resistance in *C. difficile* also suggests the existence of undetermined mechanisms among resistant strains lacking QRDR mutations [7,8]. Whilst the roles of alternative resistance mechanisms such as extra-QRDR

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mutations have been speculated upon, direct evidence for their involvement in fluoroquinolone resistance is lacking. We employed a forward genetic approach to reveal a novel mutation causing fluoroquinolone resistance in *C. difficile* and investigated its presence among clinical strains.

## 2. Materials and methods

### 2.1. Bacterial strains used in this study

Strains used in this study were isolated at St James's Hospital (Dublin, Ireland) between 2010 and 2011 from cases of CDI as part of routine diagnostic testing. Toxin production was confirmed in all strains with the Premier™ Toxin A and B Enzyme Immunoassay (Meridian Bioscience Inc., Cincinnati, OH). Strains were subjected to PCR-based ribotyping performed at the Anaerobe Reference Laboratory (Cardiff, UK) as previously described [9].

### 2.2. In vitro antibiotic selection conditions

Between  $10^8$  and  $10^9$  cells of cultured clinical isolate SJH\_18 [ciprofloxacin minimum inhibitory concentration (MIC) = 8 mg/L] were plated onto pre-reduced brain–heart infusion agar supplemented with 0.5% yeast extract, 0.1% cysteine, 0.1% taurocholate and 32 mg/L ciprofloxacin (Sigma–Aldrich, Dublin, Ireland). Plates were incubated at 37 °C under anaerobic conditions (10% H<sub>2</sub>, 10% CO<sub>2</sub> and 80% N<sub>2</sub>) until resistant colonies emerged (48–72 h).

### 2.3. Antibiotic sensitivity testing

Sensitivity to antibiotics was investigated using Etest strips (bioMérieux, Basingstoke, UK). Strains were assayed for sensitivity to ciprofloxacin, moxifloxacin, levofloxacin and ofloxacin as previously described, adopting the previous definition of 'highly resistant' (MIC  $\geq$  32 mg/L) for ciprofloxacin and moxifloxacin in the absence of clinical breakpoints in *C. difficile* [8]. The relative levels of novobiocin sensitivity between strains were compared using antibiotic disks (5 µg/disc; Oxoid Ltd., Basingstoke, UK).

### 2.4. PCR amplification and sequencing of the QRDR and gyrBA open reading frames (ORFs)

DNA was extracted from *C. difficile* using a High-Pure PCR Template Preparation Kit (Roche Diagnostics, West Sussex, UK). The QRDRs of *gyrB* (nucleotides 1059–1448) and *gyrA* (nucleotides 71–460) were amplified by PCR as described previously [6]. To investigate the entire *gyrBA* region, beyond the defined QRDR, primers flanking the entire ORFs of *gyrB* and *gyrA* were designed. Resultant amplicons were purified using QIAquick PCR Clean Up and Gel Extraction Kits (QIAGEN, West Sussex, UK) and were sequenced externally (Source Biosciences, Dublin, Ireland). Primers used for amplification and sequencing reactions are listed in Supplementary Table S1.

### 2.5. Whole-genome sequencing

Sequencing of *C. difficile* genomic DNA was performed on an Illumina MiSeq platform at the TrinSeq sequencing facility (Trinity College Dublin, Ireland). This generated ca. 500,000,150-bp paired-end reads per strain. Short-read data obtained for *C. difficile* SJH\_18 and SJH\_18R have been deposited in the European Nucleotide Archive (ENA) under study accession no. [PRJEB5003](#).

### 2.6. Analysis of whole-genome short-read sequence data

Sequencing reads from SJH\_18 were assembled de novo using the NSilico Simplicity™ pipeline (Simplicity™ v.1.2) [10]. This generated an assembly for SJH\_18 against which reads from SJH\_18R were mapped with Burrows–Wheeler short-read aligner [11]. The SAMtools analysis suite was used to detect single nucleotide variants (SNVs) present in SJH\_18R [12]. Publicly available short-read data from 918 *C. difficile* strains was retrieved from the ENA to investigate the prevalence of non-synonymous SNVs in DNA gyrase among the broader *C. difficile* population. Data from 918 strains (Supplementary Table S2) was mapped to *C. difficile* 630 ([AM180355.1](#)) and variants in the *gyrBA* ORFs were called for each sequenced isolate. Non-synonymous SNVs in *gyrB* and *gyrA* were annotated with SNPdat [13].

## 3. Results

### 3.1. Forward genetic identification of an extra-QRDR mutation conferring fluoroquinolone resistance in *C. difficile*

Following in vitro antibiotic selection on ciprofloxacin, the stable phenotypic mutant SJH\_18R (ciprofloxacin MIC  $\geq$  32 mg/L) was derived from strain SJH\_18 (ciprofloxacin MIC = 8 mg/L). In addition to ciprofloxacin, the SJH\_18R mutant exhibited increased resistance to other fluoroquinolones including ofloxacin, levofloxacin and moxifloxacin while displaying concomitant hypersusceptibility to the aminocoumarin novobiocin (Table 1). As chain-termination sequencing revealed the QRDRs from the mutant and wild-type to be identical, the presence of a QRDR-independent mutation in SJH\_18R was suspected. Whole-genome sequencing of SJH\_18 and SJH\_18R identified a single SNV difference between strains causing an Ala384Asp substitution in GyrA in SJH\_18R (Table 1). This extra-QRDR mutation was thus identified as the cause of the pleiotropic antibiotic resistance phenotype observed in SJH\_18R.

### 3.2. *C. difficile* clinical isolates exhibit QRDR-independent ciprofloxacin resistance

To investigate QRDR-independent fluoroquinolone resistance in clinical isolates, we sequenced and compared the QRDRs of *gyrB* and *gyrA* from 31 additional isolates exhibiting variable fluoroquinolone resistance phenotypes (Table 2). Comparator strains SJH\_18 and SJH\_26 exhibited relatively lower ciprofloxacin MICs and were devoid of QRDR mutations, in line with their susceptibility profiles

**Table 1**  
Antibiotic resistance profile of SJH\_18 and SJH\_18R.

Strain	Ribotype	Antibiotic MIC (mg/L)					Non-synonymous DNA gyrase mutations
		CIP	OFX	LEV	MXF	NOV <sup>a</sup>	
SJH_18	Type 110	8	8	4	0.5	7.3 ± 1	– <sup>b</sup>
SJH_18R	Type 110	$\geq$ 32	$\geq$ 32	8	1	13.7 ± 1	GyrA, A384D

MIC, minimum inhibitory concentration; CIP, ciprofloxacin; OFX, ofloxacin; LEV, levofloxacin; MXF, moxifloxacin; NOV, novobiocin.

<sup>a</sup> Expressed as zone diameter around antibiotic disc (mm).

<sup>b</sup> – Indicates no observed change relative to *Clostridium difficile* 630 ([AM180355.1](#)).

**Table 2**Comparison of fluoroquinolone minimum inhibitory concentrations (MICs) and non-synonymous mutations in DNA gyrase among *Clostridium difficile* clinical isolates.

Strain	Ribotype	Antibiotic MIC (mg/L)		QRDR		Extra-QRDR <sup>a</sup>	
		CIP	MXF	GyrA	GyrB	GyrA	GyrB
SJH_18	Type 110	8	0.5	– <sup>b</sup>	–	–	–
SJH_26	Type 010	8	1	–	–	–	–
SJH_5	Type 020	≥32	1	–	–	–	I139R
SJH_10	Type 020	≥32	1	–	–	–	I139R
SJH_27	Type 020	≥32	1	–	–	–	I139R
SJH_33	Type 020	≥32	1	–	–	–	I139R
SJH_43	Type 020	≥32	1	–	–	–	I139R
SJH_50	Type 020	≥32	1	–	–	–	I139R
SJH_24	Type 014	≥32	1	–	–	–	I139R
SJH_48	Type 020	≥32	1	–	–	–	V130I
SJH_14	Type 011	≥32	1	–	–	–	V130I
SJH_20	Type 015	≥32	1	–	–	L345I	V130I
SJH_17	Type 081	≥32	1	–	–	–	–
SJH_23	Type 254	≥32	1	–	–	–	–
SJH_41	Type 001	≥32	1	–	–	–	–
SJH_31	Type 003	≥32	1	–	–	–	–
SJH_69	Type 002	≥32	1	–	–	–	–
SJH_28	Type 017	≥32	1	–	S366A	na <sup>c</sup>	na
SJH_16	Type 078	≥32	8	A118T	S366V	na	na
				–	S416A	na	na
SJH_34	Type 087	≥32	16	–	D426N	na	na
SJH_1	Type 027	≥32	≥32	T82I	–	na	na
SJH_3	Type 027	≥32	≥32	T82I	S464T	na	na
SJH_4	Type 027	≥32	≥32	T82I	S464T	na	na
SJH_32	Type 027	≥32	≥32	T82I	S464T	na	na
SJH_51	Type 027	≥32	≥32	T82I	S464T	na	na
SJH_61	Type 027	≥32	≥32	T82I	S464T	na	na
SJH_64	Type 027	≥32	≥32	T82I	S464T	na	na
SJH_25	Type 078	≥32	≥32	T82I	–	na	na
SJH_45	Type 078	≥32	≥32	T82I	–	na	na
SJH_47	Type 078	≥32	≥32	T82I	–	na	na
SJH_57	Type 078	≥32	≥32	T82I	–	na	na
SJH_58	Type 078	≥32	≥32	T82I	–	na	na

CIP, ciprofloxacin; MXF, moxifloxacin; QRDR, quinolone resistance-determining region.

<sup>a</sup> Polymorphisms occurring outside the QRDR of GyrA (nt position 25–153) or GyrB (nt position 353–483).<sup>b</sup> – Indicates no observed change relative to *Clostridium difficile* 630 (AM180355.1).<sup>c</sup> na, not assayed (the wider *gyrA* and *gyrB* ORFs of strains in which QRDR mutations were detected were not further investigated).

(Table 2). In contrast, 15 strains that also lacked mutations in the QRDRs of either *gyrA* or *gyrB* none the less exhibited ciprofloxacin MICs of ≥32 mg/L (Table 2). Thus, in 50% of highly resistant strains (15/30), the observed ciprofloxacin MIC of ≥32 mg/L was not attributable to QRDR mutations. This suggested the presence of QRDR-independent resistance mechanisms. In addition, ribotyping combined with sequence analysis of *gyrBA* QRDR regions revealed high-level co-resistance to both ciprofloxacin and moxifloxacin (MIC ≥ 32 mg/L) in 027 and 078 strains harbouring the classical Thr82Ile substitution in GyrA (Table 2). Other non-synonymous changes in the QRDR were also observed in strains belonging to ribotypes 087 (GyrB, Asp426Asn), 078 (GyrA, Ala118Thr; GyrB, Ser366Val, Ser416Ala) and 017 (GyrB, Ser366Val). In addition to the Thr82Ile mutation, six ribotype 027 strains also harboured a Ser464Thr substitution in GyrB (Table 2).

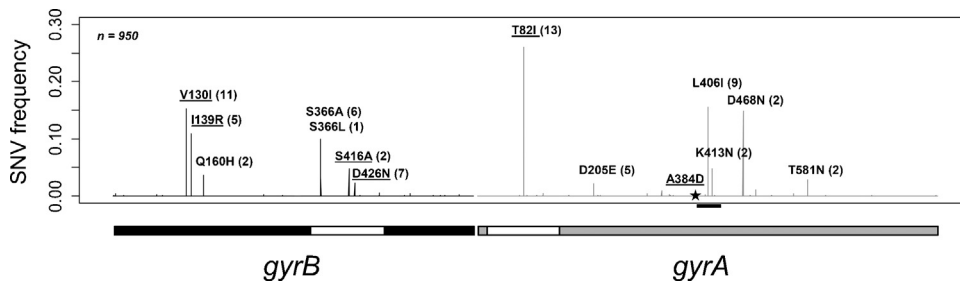
### 3.3. Characterisation of non-synonymous extra-QRDR polymorphisms in ciprofloxacin-resistant *C. difficile* isolates

To further investigate the mechanism of ciprofloxacin resistance in the 15 strains devoid of QRDR mutations, extended sequence analysis of the entire *gyrB* and *gyrA* ORFs was performed. This led to the identification of non-synonymous polymorphisms, located far beyond the characterised QRDRs of *gyrB* and *gyrA*, in ten isolates (Table 2). Five strains (SJH\_25, SJH\_45, SJH\_47, SJH\_57 and SJH\_58) were devoid of mutation across the entire *gyrBA* ORF regions confirming the existence of additional, DNA gyrase-independent fluoroquinolone resistance mechanisms in *C.*

*difficile*. Among strains where extra-QRDR mutations were identified, substitutions in GyrB (Ile139Arg and Val130Ile) represented the most frequent non-synonymous changes observed, whilst an extra-QRDR mutation in *gyrA* (Leu345Ile) was observed in a single strain (Table 2).

### 3.4. Extra-QRDR DNA gyrase polymorphisms are present among divergent *C. difficile* lineages

Analysis of the *gyrBA* region in publicly available *C. difficile* genomic data confirmed GyrB substitutions Ile139Arg and Val130Ile to be prevalent among the broader *C. difficile* population (Fig. 1). Publicly available genomic data from 918 strains were analysed, of which 644 harboured at least one non-synonymous polymorphisms in the *gyrBA* region including substitutions occurring outside the QRDR, which were identified in 457 strains. The most prevalent extra-QRDR polymorphisms included Val130Ile, Ile139Arg and Gln160His in GyrB and Leu406Ile, Lys413Asn and Asp468Asn in GyrA, whilst known QRDR mutations including Thr82Ile were also observed (Fig. 1). Although the induced GyrA mutation Ala384Asp was not detected (other than in the in vitro-generated SJH\_18R mutant), other polymorphisms including Leu406Ile and Lys413Asn were present at the predicted primary dimer interface region of GyrA (residues 386–426). Phylogenetic analysis confirmed the presence of extra-QRDR mutations among divergent multilocus sequence typing (MLST) types suggesting homoplasmy (Fig. 1). However, mutations involving more dramatic side chain substitutions (i.e. GyrB, Ile139Arg;



**Fig. 1.** Frequency of non-synonymous variations in the *gyrBA* region among 950 *Clostridium difficile* clinical isolates. Observed non-synonymous single nucleotide variant (SNV) frequencies are plotted against their relative position in the *gyrBA* region. Associated amino acid substitutions are indicated for the most frequently observed SNVs. The SNVs that were also observed in the highly resistant ciprofloxacin isolates from this study are underlined. The number of diverse multilocus sequence typing (MLST) types within which these mutations were observed is indicated in parentheses. The *gyrB* (black) and *gyrA* (grey) open reading frames (ORFs) are represented schematically below the plot, with the locations of the classical quinolone resistance-determining region (QRDR) indicated in white. The location of the novel resistance-conferring mutation in *gyrA* (Ala384Asp) identified in this study is indicated by a black star, and an adjacent black bar underlines the predicted GyrA primary dimer interface region.

GyrA, Asp205Glu, Asp468Asn) were generally restricted to related MLST lineages (Fig. 1; Supplementary Fig. S1; Supplementary Table S2). In addition to Leu406Ile and Lys413Asn, polymorphisms Gly401Arg, Gly401Val and Gln418Lys were observed at lower prevalence within the GyrA primary dimer interface (Supplementary Table S3).

#### 4. Discussion

We report the first confirmed non-QRDR mutation conferring fluoroquinolone resistance in *C. difficile*. The Ala384Asp substitution identified in SJH\_18R is located next to the predicted dimer interface region of GyrA, 231 amino acids outside the classical QRDR. In a previous study investigating fluoroquinolone resistance in *Salmonella enterica*, Blanc-Potard et al. characterised a pleiotropic mutation in *gyrB* causing ciprofloxacin resistance and concomitant novobiocin sensitivity. They subsequently identified an epistatic and compensatory mutation in *gyrA* that restored novobiocin sensitivity to wild-type levels while further augmenting fluoroquinolone resistance [14]. Notably, the epistatic mutation (Thr467Ser) was located at the dimer interface region, thus confirming the ability of mutation at this region in GyrA to influence both ciprofloxacin and novobiocin sensitivity, a finding consistent with the current observations in *C. difficile*. We speculate that the Ala384Asp mutation in *C. difficile* may be functionally analogous to the Thr467Ser mutation in *Salmonella* GyrA that results in a predicted destabilisation of the primary interface favouring a quinolone-resistant DNA gyrase conformation [14].

Having confirmed the ability of an extra-QRDR mutation to modulate resistance in vitro, we investigated whether such mutations were associated with resistance in *C. difficile* clinical isolates. DNA gyrase mutations occurring outside the QRDR are rarely investigated and their prevalence among fluoroquinolone-resistant strains is largely unknown [7,8,15]. Non-synonymous changes present outside the QRDR, including polymorphisms in GyrB (Ile139Arg and Val130Ile) and GyrA (Leu345Ile), were identified among our clinical strains exhibiting elevated ciprofloxacin MICs. In total, one-half (15/30) of the isolates exhibiting increased ciprofloxacin resistance were devoid of QRDR mutations, including ten isolates that harboured non-synonymous, extra-QRDR mutations. Five strains harboured wild-type DNA gyrase alleles, providing the first demonstration that fluoroquinolone resistance can occur in *C. difficile* independent of DNA gyrase mutations. Whilst the precise mechanism of resistance in these strains remains to be determined, these findings add credence to the role of alternative fluoroquinolone resistance mechanisms such as drug efflux or reduced permeability in *C. difficile*, as has been suggested in other studies [7,8].

As in our clinical isolates, the Ala384Asp mutation was not detected among publicly available *C. difficile* genomic data sets, suggesting that it is uncommon. This may be due to its impact on novobiocin susceptibility or to other potentially deleterious effects on strain fitness associated with this DNA gyrase mutation. In contrast, analysis of deposited genomic data revealed a diversity of other extra-QRDR polymorphisms in DNA gyrase including the Val130Ile and Ile139Arg substitutions in GyrB that were also observed in the ciprofloxacin-resistant isolates identified in this study (Table 2). Although found among clonally diverse strains (suggesting homoplasmy), subtle amino acid substitutions such as Val130Ile (GyrB) and Leu406Ile (GyrA) would be unlikely to have a major impact on protein structure. In contrast, polymorphisms Ile139Arg (GyrB), Asp205Glu, Lys413Asn and Asp468Asn (GyrA) embody more dramatic amino acid substitutions. However, these were confined to related MLST groups and thus evidence for homoplasmy and Darwinian selection at these sites is weaker. By comparison, the proven resistance-conferring mutation Thr82Ile in the GyrA QRDR is seen among diverse clonal frames (Fig. 1; Supplementary Fig. S1), consistent with an associated fitness advantage under antibiotic selection [5]. None the less, the current findings highlight the existence of diverse extra-QRDR mutations in *C. difficile* including polymorphisms within the GyrA dimer interface region such as Lys413Asn, Gly401Arg, Gly401Val and Gln418Lys.

Although alteration within the QRDR is clearly a dominant driver of fluoroquinolone resistance in *C. difficile*, the current findings demonstrate the capacity for mutational alteration outside this region to contribute to resistance. Given the confirmed ability of the GyrA Ala384Asp substitution to confer resistance and the numerous polymorphisms identified outside the QRDR of *C. difficile* DNA gyrase (in particular ca. residues 130–160 and 384–468 of GyrB and GyrA, respectively), further investigation of extra-QRDR substitutions and their association with fluoroquinolone resistance is warranted.

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#### Competing interests

None declared.

#### Ethical approval

Not required.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.jgar.2015.09.007](https://doi.org/10.1016/j.jgar.2015.09.007).

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