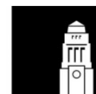
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The effect of fluoroquinolone resistance mutation Thr-82→Ile on *Clostridium difficile* fitness

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Abstract

Objectives

Fluoroquinolone (FQ) resistance is common among epidemic *Clostridium difficile* PCR ribotype (RT) 027, and may have contributed to outbreaks of *C. difficile* infection (CDI). We investigated the impact of FQ mutations on the bacterial fitness (BF) of *C. difficile* RT027 isolates.

Methods

BF of seven RT027 mutants with reduced susceptibility to moxifloxacin (4-32 mg/L) was compared with their susceptible (<2 mg/L) progenitor strains in competitive batch culture (CBC), cell cytotoxicity and maximal growth rate assays. Comparative fitness dynamics of one *gyrA* Thr-82→Ile harbouring isolate, CD3079M versus parent strain (CD3079) were also investigated in a continuous co-culture (CC) chemostat model. Mutant and parent strain populations were assessed every 24hrs over eight days using selective and non-selective agars. Sequencing was performed using NEBNext® Ultra™ chemistry and Illumina®HiSeq3000 technologies.

Results

BF was significantly increased in all Thr-82→Ile exhibiting isolates (w=1.08-1.22) in CBC assays (p=0.002). Gly-429→Val and Gln-434→Lys (*gyrB*), also showed no burden to fitness (w=1.24 and 1.18, respectively), but, Asp-71→Tyr conferred reduced fitness (w=0.80). CC results for strains CD3079 and CD3079M (Thr-82→Ile) supported CBC findings; mutant to parent ratios differed significantly by 96hrs ($\bar{x}$=1.80; p=0.025).

Conclusions

37 The absence of a fitness cost associated with the most prevalent FQ resistance

38 mutations may have contributed to the success of RT027. Furthermore, a demonstrable

39 *in vitro* advantage over FQ susceptible parent strains in CC may contribute to the

40 maintenance of RT027, even in the absence of FQ selection pressure.

41 **Introduction**

42 Fluoroquinolones have been associated with *Clostridium difficile* infection (CDI) as a
43 predisposing risk factor, [1, 2] and have been strongly implicated in major international
44 outbreaks of PCR ribotype 027. [2, 3] A recent retrospective analysis reported correlations
45 between a reduction in fluoroquinolone prescribing and CDI caused by fluoroquinolone
46 resistant strains. [4] However, the potential for *C. difficile* strains to retain the resistant
47 phenotype in the absence of antimicrobial pressure remains unclear. The fitness
48 associated with these resistant determinants may be a factor in the maintenance of
49 fluoroquinolone resistance in this important nosocomial pathogen.

50 The impact of resistance determinants may not always be constrained to an antibiotic
51 susceptibility phenotype. Several reports have linked resistance-conferring amino acid
52 substitutions to a demonstrable burden on bacterial fitness. [5-8] Whilst typically related
53 to a reduction in growth rates, the effects on the functionality of crucial pathways, such
54 as sporulation and toxin production in *C. difficile* can also be appropriate measures of
55 fitness. Investigations of fluoroquinolone resistance mutations have demonstrated
56 diverse responses across a host of bacterial species, [9-11] with some more detrimental to
57 fitness than others. Both transferable elements and chromosomal mutations have been
58 correlated with fitness disadvantages in *C. difficile*, with *ermB* [8] and *rpoB* [12] respectively.
59 However, the relationship is not always straightforward; rifamycin resistance-
60 conferring mutations in different locations on the *rpoB* gene have demonstrated
61 minimal effect on *C. difficile* fitness. [13] Similarly, Wasels *et al.* observed no impairment
62 with the common fluoroquinolone resistance, Thr-82→Ile mutation in isogenic mutants
63 created via allelic exchange, whilst the rarer valine substitution at the same codon
64 imposed a significant fitness burden. [14]

65 We investigated the impact of *gyrA* and *gyrB* mutations on *C. difficile* PCR ribotype 027
66 fitness, through growth rate analysis and, for the first time, using a competitive co-
67 culture assay.

68

69 Material and methods

70 Test Isolates

71 Seven moxifloxacin susceptible (1-2 mg/L) PCR ribotype 027 isolates (parent); [15] (Table
72 1) and seven corresponding moxifloxacin-resistant mutants were generated through
73 mutability testing. Briefly, one millilitre of 6 h brain heart infusion (BHI) broth culture
74 of each moxifloxacin-susceptible, parent strain was inoculated across ten moxifloxacin-
75 incorporated (4x MIC) Braziers agar plates. After subsequent anaerobic incubation at
76 37°C for 48 h, colonies were removed and MICs confirmed using an agar incorporation
77 method. [16]

78 Agar-incorporation Minimum Inhibitory Concentration Testing

79 A moxifloxacin dilution series was created (0.125-128 mg/L) in sterile water and added
80 to molten Wilkins-Chalgren anaerobe agar. Overnight Schaedler's broth cultures were
81 diluted (1:10) in sterile saline and a multi-point inoculator was used to transfer ~$1x10^4$
82 cfu to the moxifloxacin-incorporated agar dilution series, prior to incubation. MICs were
83 defined as the lowest concentration at which growth was markedly inhibited.

84 Sequencing

85 Sequencing of all seven moxifloxacin susceptible parent and seven resistant mutant
86 isolates was performed by the University of Leeds, Next Generation Sequencing facility
87 using NEBNext® Ultra™ chemistry (New England Biolabs) and Illumina HiSeq3000

88 technology. Genomes were assembled using CLC Genomics Workbench (Qiagen) [17] and
89 annotated by the Rapid Annotation using Subsystem Technology (RAST) service. [18]
90 Alignment and single nucleotide polymorphism detection of the *gyrA* and *gyrB* genes
91 was accomplished using Clustal Omega. [19]

92

93 **Sanger sequencing**

94 Sanger sequencing of the QRDRs of both *gyrA* and *gyrB* genes was used to confirm
95 amino acid substitutions. Briefly, DNA was extracted using a QIAxtractor and the
96 QIAamp Fast DNA Kit. Primer sequences were obtained from previously published
97 literature, [20, 21] and synthesised by Metabion international AG (Germany). The QRDR
98 sections of the gyrase genes were amplified using the primer pairs: gyrAF (5´-
99 AATGAGTGTTATAGCTGGACG-3´), gyrAR (5´-TCTTTTAACGACTCATCAAAGTT-3´) and
100 gyrBF (5´-AGTTGATGAACTGGGGTCTT-3´), gyrBR (5´-TCAAAATCTTCTCCAATACCA-3´)
101 generating 390bp region of both *gyrA* and *gyrB,* respectively. PCR amplification was
102 performed with an initial denaturation stage of 94°C for 15 minutes, followed by 30
103 cycles of denaturation for 30 seconds at 94°C, annealing for 30 seconds at 58°C (*gyrA*)
104 and 54°C (*gyrB*) and a 30 second extension phase at 72°C. A final extension stage of 5
105 minutes at 72°C was implemented. PCR clean-up was performed using the QIAquick
106 PCR Purification Kit (Qiagen). Sequencing was performed by the Leeds Teaching
107 Hospitals Trust, Molecular Microbiology Department, using BigDye™ Terminator Kit
108 v.3.1 (Thermofisher) and an ABI 3130xl genetic analyser. Sequence data was analysed
109 in comparison to the *gyrA* and *gyrB* sequences of the CD630 reference genome, [22] using
110 CLC genomics workbench.

111

112 **Maximal Growth Rate Determination**

113 Maximal growth rates were determined from bacterial growth curves, as previously
114 reported. [8] Briefly, optical densities (OD_{595}) of 18 hour anaerobic (37°C) shaking
115 cultures, standardised by spectrophotometry and dilution with BHI broth to OD_{600} 0.5
116 (±0.05), were measured hourly in triplicate for 11 hours (Tecan infinite F200 pro plate
117 reader). Maximal growth rates were calculated using the following formula:

118 $$\ln (N_t/N_0) = \alpha (t-t_0),$$

119 where, *ln* is natural logarithm, *Nt* is the absorbance at log phase end point, *N0* is
120 absorbance at the beginning of the log phase, α is the growth rate constant and t is time.
121 [23, 24] Relative growth rate of the mutant strain to its progeny was determined as ***parent***
122 ***α / mutant α.***

123

124 **Assessment of Toxin Production by Cytotoxigenic Culture**

125 *C. difficile* isolates were cultured in BHI broth for 48 hours. Toxin titres were
126 determined by serial ten-fold dilution of culture supernatant (12,000 g) and inoculation
127 of Vero cell cytotoxicity assay, with *Clostridium sordellii* antitoxin neutralisation.
128 Positive titres were recorded when cell rounding was >50%.

129

130 **Competitive Batch Culture**

131 Independent overnight BHI cultures of parent and mutant strains were standardised as
132 for Maximal Growth Rate Determination, and combined in equal concentrations, prior to
133 inoculation of 1 mL in to fresh BHI broth. Population balance was measured at zero and

134 24 hour time points, via serial dilution and enumeration of total viable counts (TVC) and
135 mutant populations on Braziers agar supplemented with 2% defibrinated horse blood
136 (Braziers) and Braziers with moxifloxacin (0.5x MIC). All strains were tested in both
137 biological and technical triplicate. *In vitro* fitness was calculated as

138 $$s = \ln(CI) / [t \times \ln(2)],$$

139 where, s is the selection coefficient, CI is the competition index and t is the number of
140 generations. [8]

141 Number of generations was calculated as

142 $$(\log_{10} N_t - \log_{10} N_0)/\log_{10}2,$$

143 where, N_t is the total population at 24 hours and N_0 is the total population at time point
144 zero. [25]

145 Competition index was calculated as

146 $$(R_{(t1)} / S_{(t1)})/(R_{(t0)} / S_{(t0)}),$$

147 where $R_{(t0)}$ is resistant (mutant) population (cfu) at time point zero, $R_{(t1)}$ is the resistant
148 population at 24 hours and S is the susceptible (progenitor) population (cfu). Fitness of
149 the parent strain was set at 1 and relative fitness of mutant in competition per
150 generation (w) was defined as $\boldsymbol{w = 1 + s}$. Based on a lower limit of bacterial detection of
151 50 cfu/mL, the limits of resolution for the assay were between w=0.23-2.23.

152

153 **Competitive culture in a Continuous Culture Chemostat Model**

154 A one litre sealed, glass chemostat model was set up with a continuous supplementation
155 of BHI culture medium (42 mL/hr). Temperature and pH were constantly monitored

156 and maintained at 37°C and pH 6.8 (±0.2). The culture vessel was continually stirred
157 and sparged with nitrogen to maintain anaerobicity. A syringe vacuum sample port was
158 used to minimise contamination risk of the sealed environment (Figure 1).

159 CD3079 and CD3079M (Thr-82→Ile substitution) were inoculated in equivalent (0.5 mL)
160 concentrations after culture standardisation. Population dynamics were again
161 determined using the previously described viable count method at zero hour and every
162 24 hours for a further 192 hours. Further mutant development was assessed through
163 enumeration of populations on 32 and 64 mg/L moxifloxacin-containing Braziers agar.
164 Population proportions were measured and fitness calculated as before. Three
165 independent model replicates were used to generate the data.

166 Statistical analyses were carried out using IBM SPSS Statistics v.21.0.0.1. The Shapiro-
167 Wilk test statistic was used to assess normality of data distribution, whilst a one-tailed,
168 paired t-test compared mutant to parent population ratios. The Wilcoxon Signed-Rank
169 test was performed to compare Thr-82→Ile containing mutant to parent ratios.

170 **Results**

171 **Maximal Growth Rate Determination**

172 Maximal growth rates of parent and moxifloxacin-resistant progeny were similar (Table
173 2), ranging between 0.0050-0.0065 OD_{600} min^{-1} ($\bar{x}$ = 0.0060) and 0.0053-0.0070 OD_{600}
174 min^{-1} ($\bar{x}$ = 0.0061), respectively. Individual parent to mutant growth rate comparisons
175 demonstrated no significant differences, ranging 0.0003-0.0007 OD_{600} min^{-1} ($\bar{x}$ =
176 0.0005).

177

178 **Assessment of Toxin Production by Cytotoxigenic Culture**

179 All parent and mutant strains produced *C. difficile* toxin, to a titre of 10^{-4} (Table 2). No

180 differences were observed between parent and mutant isolates.

181

182 **Competitive Batch Culture**

183 Fitness (w) responses to fluoroquinolone resistance ranged between w=0.80 and 1.24

184 ($\bar{x}$ = 1.13), relative to parent fitness (1) (Figure 2). Fluoroquinolone resistant, mutant

185 progeny (n=6) exhibited a fitness advantage when competitively cultured with parent

186 strains in batch culture experiments. All mutants containing a Thr-82→Ile substitution

187 displayed significantly elevated fitness level, with 24 h mutant to parent ratio scores

188 significantly higher than the zero time point scores; Wilcoxon Signed-Rank Test, z -78,

189 p=0.002. Isolate CD9946, containing a Gly-429→Val substitution, demonstrated the

190 largest fitness benefit (w=1.24). Notably, a single fluoroquinolone resistant isolate

191 exhibiting the Asp-71→Tyr mutation (CD3809) demonstrated a burden to fitness, with

192 relative fitness defined as w= 0.80, but this was not significant. All strain comparisons of

193 final and initial parent to mutant population ratios identified a statistically significant

194 variation between the ratios of fluoroquinolone susceptible parents and resistant

195 mutants at zero and 24 hour time points; (Paired t-test; t(20) = 4.307, p<0.001).

196

197 **Competitive culture in a Continuous Culture Chemostat Model**

198 In the continuous co-culture model, fluoroquinolone resistant mutant to parent ratios

199 increased gradually throughout the experiment, averaging at $\bar{x}$ = 1.43, with a peak at

200 1.80 at 96 h (Figure 3). Mutant to parent population ratios were significantly different

201 between the zero ($\bar{x}$ = 1.1574, SD=0.3769) and 96 hour time points ($\bar{x}$ = 2.7407,

202 SD=1.9154); t(8)=-2.294, p=0.025. Proportional disparity was briefly reduced between

203 120 and 144 h, before rising again towards peak levels. Populations of highly

204 moxifloxacin-resistant bacteria peaked after 24 h, with counts on 32 mg/L moxifloxacin

205 and 64 mg/L moxifloxacin-containing agars at 7.34×10^4 and 4.44×10^1 $\log_{10}$ cfu/mL,

206 respectively. By 72 h, >32 mg/L moxifloxacin mutant counts decreased to stable

207 populations for the remainder of the model duration, ranging between 7.89×10^3 to 1.17

208 $\times 10^4$, $\bar{x} = 9.54 \times 10^3$ $\log_{10}$ cfu/mL. High level moxifloxacin-resistant (>64 mg/L) mutants

209 were undetectable between 48 and 96 h, with sporadic low level detection towards the

210 end of the experiment. All colonies isolated on 64 mg/L moxifloxacin agars had

211 moxifloxacin MICs of 128 mg/L. Colonies present on 32 mg/L moxifloxacin agars from

212 early (days 1-2) and late (days 6-8) stages of each model had moxifloxacin MICs of 64

213 mg/L.

214 Total *C. difficile* counts peaked after 24 hours (1.23×10^8 $\log_{10}$ cfu/mL), with the

215 population levelling out by 48 hours until the completion of the model ($\bar{x} = 7.93 \times 10^7$

216 $\log_{10}$ cfu/mL); (Data not shown). Spore counts demonstrated an exponential increase

217 between 24 and 48 h (4.07×10^3 – 6.32×10^6 $\log_{10}$ cfu/mL), corresponding with the

218 initial reduction and subsequent stabilisation of total *C. difficile* counts.

219 Moxifloxacin resistant mutant strain fitness remained relatively consistent throughout

220 the experiment (w= 1.11–1.45, $\bar{x}$ = 1.25).

221 **Discussion**

222 The effect of fluoroquinolone resistance determinants on an organism's fitness has been
223 widely investigated in a host of bacterial species. [5, 10, 11, 26, 27] While fitness burdens are
224 regularly reported, commonly associated with a decreased growth rate, [5-7] beneficial
225 effects of gyrase and topoisomerase mutations have also been observed. [9-11] However,
226 there is little information regarding the fitness effect of fluoroquinolone mutations in *C.*
227 *difficile*, particularly ribotype 027. Fluoroquinolone resistance was a notable
228 characteristic of PCR ribotype 027 strains involved in large outbreaks of severe disease
229 around the world and may have been a factor in the predominance of this ribotype. [1, 4, 28]

230 Crucially, *in vitro* batch culture experiments with *C. difficile* may not yield clinically
231 relevant results, whereas chemostat models have been used successfully to predict
232 clinical CDI risk and treatment response. [29-33] The competitive batch culture findings of
233 this investigation demonstrated evidence of both benefit and burden to bacterial fitness,
234 with amino acid substitution dependent responses observed. Where the majority of
235 mutations exhibited a significant advantageous effect ($p<0.001$), an Asp-71→Tyr
236 mutation in isolate CD3809 demonstrated a fitness burden (Figure 2).

237 Wasels *et al.* reported that Thr-82→Ile mutations did not confer a burden on *C. difficile* in
238 PCR ribotype 012. [14] However, one less common mutation, Thr-82→Val, demonstrated a
239 statistically significant impairment in co-culture investigations. Lee *et al.* reported
240 epidemiological shift in Korean hospitals aligned with this fitness dynamic, representing
241 a shift from Thr-82→Val to Thr-82→Ile. [34] Nonetheless, there are other factors that
242 potentially had a major impact on strain prevalence, including the fluoroquinolone MICs
243 and additional antibiotic class resistances of the emerging strains.

244 The present study expands these findings and demonstrates that a significant advantage
245 is conferred upon isolates with Thr-82→Ile mutations in parent-mutant co-culture.
246 Continuous culture modelling of *C. difficile* population dynamics consistently
247 demonstrated that CD3079M, (Thr-82→Ile *gyrA*), gradually increased in ratio
248 divergence from its progenitor (CD3079), strain population. The mean average fitness
249 of the mutant was established as w = 1.25, supporting the results of the batch culture
250 investigation of this strain (w = 1.15), indicating a fitness advantage conferred by this
251 amino acid substitution.

252 High level moxifloxacin-resistant mutants (>64 mg/L) were sporadically isolated
253 throughout the experiment (Figure 3) reflecting observations of high level resistance
254 reversion in previous *C. difficile* models by Saxton *et al.* [33] It is possible that these
255 mutants may become burdened with additional mutations and unable compete
256 efficiently at high levels. Intriguingly, Saxton *et al.* also demonstrated low level toxin
257 production prior to detectable germination, during investigations with moxifloxacin and
258 ribotype 027. The presence of a minority, resistant sub-population, able to germinate in
259 the presence of high moxifloxacin concentrations may explain this phenomenon. This
260 concept may be evident in the continuous culture model, where early detection of highly
261 resistant colonies was observed. [33]

262 The Thr-82→Ile *gyrA*, common fluoroquinolone resistance substitution has been
263 identified in ribotype 027 isolates associated with major outbreaks, [2, 3] suggesting that
264 resistance acquisition without fundamental hindrance to fitness may contribute to
265 clonal expansion. A recent retrospective analysis of local and national data provided
266 further insight into the influence of fluoroquinolones on CDI rates. [4] Dingle *et al*.
267 identified greater reductions in CDI caused by fluoroquinolone resistant versus

268 susceptible strains, in the context of a fluoroquinolone restricted environment. Whilst
269 this study highlights the important role of antibiotics in selection and maintenance of
270 certain populations, it does not necessarily explain the whole story. In a three-year pan-
271 European study of *C. difficile* PCR ribotypes and resistance, Freeman *et al.* described the
272 continued prevalence of moxifloxacin-resistant PCR ribotype 027 in Cyprus against a
273 background of decreasing fluoroquinolone use. In contrast, the PCR ribotype 027
274 isolates from Cyprus became progressively less resistant to other antimicrobials tested,
275 inferring no detriment to fitness from the presence of fluoroquinolone resistance
276 mutations in these PCR ribotype 027 isolates. [15] This suggests the involvement of
277 additional factors. Infection control interventions in the Netherlands, without
278 fluoroquinolone restriction, resulted in substantial decreases in ribotype 027
279 prevalence in the aftermath of a series of outbreaks, whilst other types flourished in its
280 absence. [35] The data outlined in the present study suggest that, for Thr-82→Ile mutants
281 in particular, retention of a resistant genotype incurs no detectable fitness cost, and so
282 may be retained in the absence of antibiotic pressure. This may help to explain the
283 continued presence of fluoroquinolone resistant *C. difficile* PCR ribotype 027 in some
284 locations.

285 Although a definitive cause for this fitness advantage is unknown, Marcusson *et al.*
286 suggested that modifications to the genes involved in the supercoiling process may
287 ultimately effect gene expression, [27] eliciting increased gyrase promotor activity and the
288 upregulation of other core processes leading to beneficial adaptations. Whilst it remains
289 unclear as to why sterically promixal mutations can impart such opposing effects, the
290 change in amino acid chemistry may effect the enzyme-nucleic acid binding affinity.

291 The mutations in *gyrB*, conveying low level resistance, also displayed no detectable
292 fitness burden. This correlates with previous findings, [14] where mutations at Asp-426,
293 generating equally moderate MIC reduction (4 mg/L), produced very slight (non-
294 significant) fitness advantages. Since these intermediary steps are an important stage in
295 evolutionary resistance development, it may be that the absence of a fitness cost/slight
296 fitness advantage may shift the population baseline and allow further advantegeous
297 mutations to achieve higher levels of resistance. Whilst *gyrB* mutations may appear of
298 less importance, producing only marginal decreases in fluoroquinolone susceptibility,
299 they may act as a transitional step up the resistance hierarchy.

300 In batch culture, one Asp-71→Tyr containing mutant demonstrated a burden on fitness.
301 Although proximally located to the advantageous mutation, Thr-82→Ile, a contrasting
302 fitness response was observed. Whilst the mechanisms of these polarised responses are
303 not yet understood, there may be a considerable impact on the tertiary structure of the
304 gyrase molecule, affecting replication efficacy. Although the fitness cost of CD3809M
305 alterations may be attributed to the *gyrA* Asp-71→Tyr mutation, as this amino acid
306 substitution was only present in a single instance, further isolates featuring the same
307 modifications are required to substantiate this hypothesis.

308 The ability to outcompete other strains may have been a factor in the successful
309 expansion and maintenance of epidemic ribotype 027 strains. Robinson *et al.*
310 demonstrated a competitive advantage for ribotype 027 versus other non-027 strains,
311 [36] although the precise mechanisms are not clear. The authors postulated the presence
312 of *thyA* genes, encoding for thymidylate synthase enzyme with increased replication
313 rates in RT027, may be a factor. Nonetheless, the capacity to incur fluoroquinolone
314 resistance mutations without detriment, as shown in the present study, may also be a

315 contributory factor. Research in other genera support this notion, with resistance
316 mutations generating the least burden, identified as the most clinically prevalent. [37]
317 While there is evidence that ribotype 027 possesses a competitive advantage over non-
318 027 ribotypes, [36] no link to fluoroquinolone resistance determinants has been made.
319 Robinson *et al.* reported data from an *in vivo* murine model outlining the advantage of
320 027 strains, proffering an explanation of an elevated ability to outcompete others for
321 limited nutrients. [36] Combined with such physiological factors, additional benefits
322 conferred by gyrase mutations, however slight, have the potential to compound any
323 ecological advantages held by this ribotype.

324 Maximal growth rate calculations revealed minimal differences between parent and
325 mutant strains, demonstrating a lack of correlation with co-culture data. This suggests
326 that fitness variability may only become apparent in direct competitive culture. Equally,
327 no effect was observed on strain cytotoxicity. However, the possibly cannot be excluded
328 that a competitive growth advantage could result in increased toxin load.

329 This study is not without limitations. The continuous culture model does not reflect the
330 complexity of interactions *in vivo*; this may be achieved more successfully by using the
331 clinically reflective gut model by Saxton *et al.* [33] However, even the gut model cannot
332 reproduce host factors, such as immune response and secretions. One study has
333 suggested that the fitness behaviour of an organism maybe different between *in vitro*
334 and *in vivo* experiments, hypothesising that there may be a culture environment specific
335 element to the adaption to a fitness burden. [38] Since resistant mutants detected towards
336 the end of the continuous culture model were not sequenced, it is also possible that
337 compensatory mutations may have occurred, as previously reported. [33, 39]

In summary, fluoroquinolone resistance was a feature of PCR ribotype 027-associated outbreaks of severe disease. This PCR ribotype continues to persist and is highly prevalent in some locations.[3, 40] This study demonstrates a fitness advantage associated with the common Thr-82→Ile *gyrA* mutation, which may have afforded this strain an epidemiological advantage. Without hindrance of an associated fitness impairment, the potential for clonal expansion is increased. Notably, a lack of fitness detriment associated with this phenotype, theoretically allows for the retention of fluoroquinolone resistance in the absence of antimicrobial pressure.

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360 (#P60).

361

362 **Author Contributions**

363 All authors contributed to the project conception and interpretation of data. J.V. led on

364 manuscript construction and writing, whilst J.F. and M.H. contributed to critical revision.

365 All data was generated and analysed by J.V.

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466

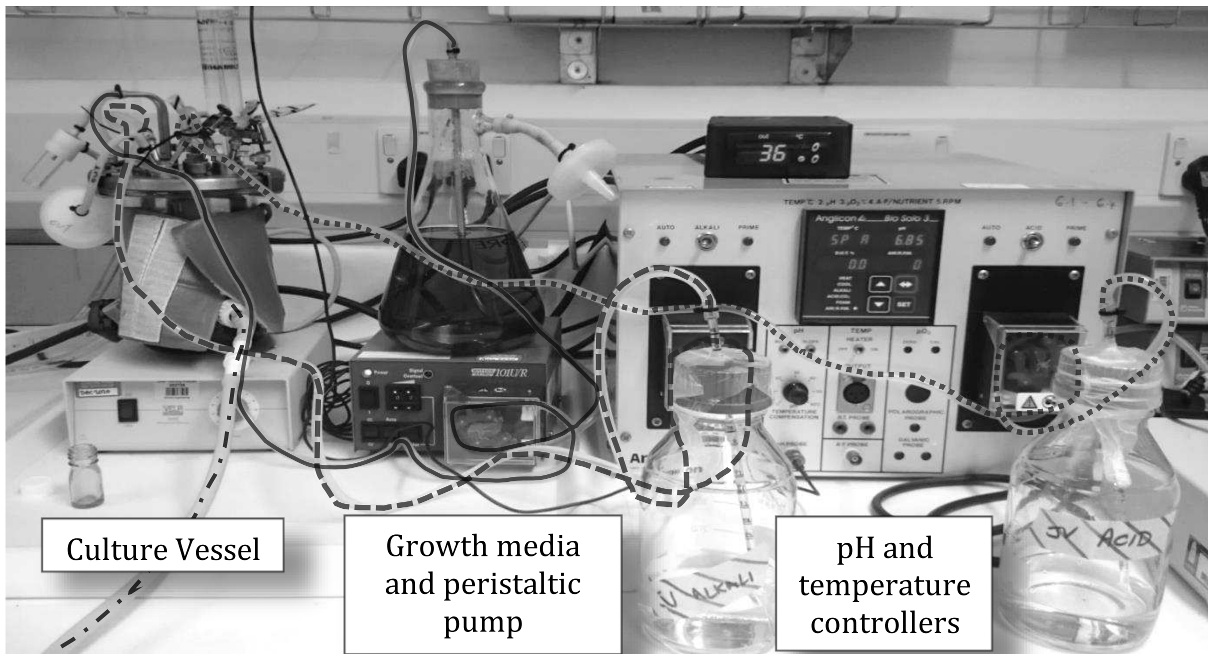
467

Parent Isolate	Parent MXF MIC (mg/L)	Mutant Strain Identifier	Mutant MXF MIC (mg/L)	Amino Acid Substitutions	
				gyrA	*gyrB*
CD3904	1	CD3904M	32	Thr-82→Ile	-
CD3891	2	CD3891M	32	Thr-82→Ile	-
CD9609	1	CD9609M	32	Thr-82→Ile	-
CD9946	1	CD9946M	4	-	Gly-429→Val
CD3809	1	CD3809M	32	Asp-71→Tyr	-
CD3051	1	CD3051M	4	-	Gln-434→Lys
CD3079	1	CD3079M	32	Thr-82→Ile	-

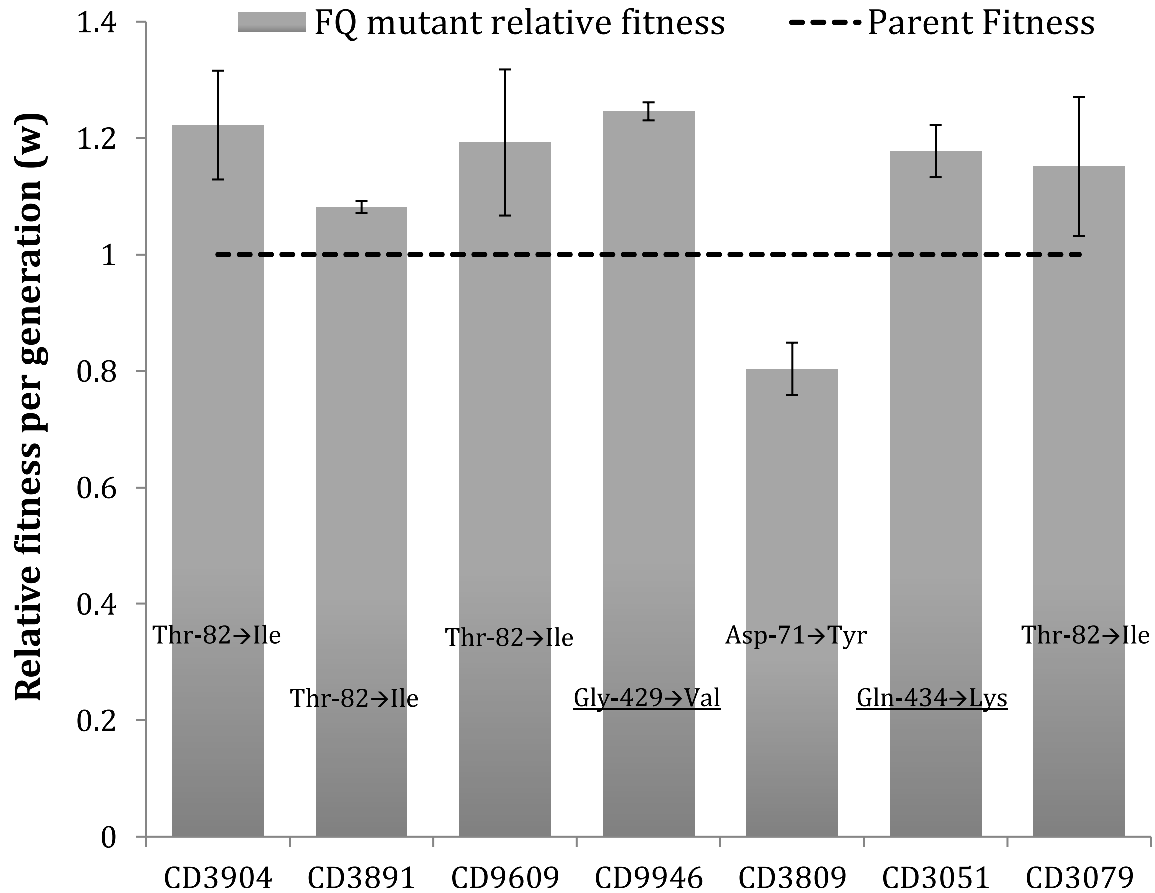
468 ***Table 1: Characteristics of C. difficile RT027 strains tested during fitness***
469 ***investigations.*** *Parent and mutant isolate moxifloxacin (MXF) MICs as determined by*
470 *agar-incorporation assay and mutant strain amino acid substitutions of DNA gyrase*
471 *genes.*

472

473



474 ***Figure 1: Continuous culture chemostat configuration.*** *Dotted line represents acid*
475 *flow, dashed line represents alkaline, solid line represents nutrient media (BHI) and dash*
476 *dot line represents waste output.*



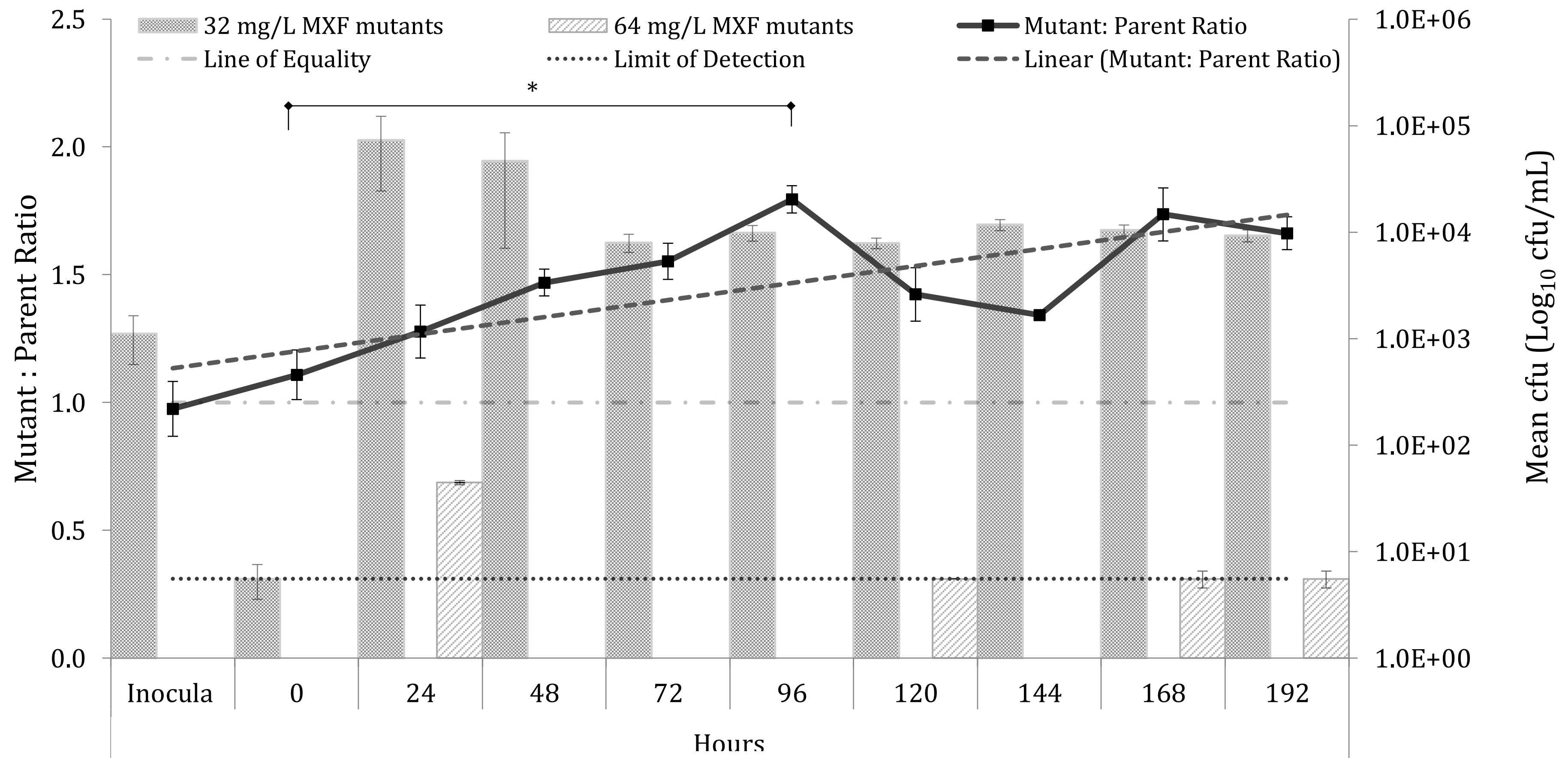
477

478 ***Figure 2: Mean relative fitness (±SE) of fluoroquinolone resistant progeny***
479 ***compared to parent fitness (set to 1) in competitive co-culture batch assays.*** *Values*
480 *are based on three biological replicates. Non underlined text represents gyrA substitutions,*
481 *underlined text represents gyrB substitutions.*

482

Strain Identifier	MXF (4 mg/L) induced Mutation Frequency (proportional)	Maximal Growth Rate (OD_{600} min^{-1})		Competition Assay Parent:Mutant Ratio		Co-culture Relative Fitness (*w*)		Cell Cytotoxicity Assay Status (+ve titre)	
		Parent	FQR Mutant	Zero Hour	24 Hours	Parent	FQR Mutant	Parent	FQR Mutant
CD3904	5.91×10^{-6}	0.0062	0.0059	1.17	0.46	1	1.2224	+ (10^{-4})	+ (10^{-4})
CD3891	2.11×10^{-6}	0.0065	0.0065	0.92	0.68	1	1.0815	+ (10^{-4})	+ (10^{-4})
CD9609	2.73×10^{-6}	0.0063	0.0057	0.94	0.58	1	1.1925	+ (10^{-4})	+ (10^{-4})
CD9946	3.77×10^{-8}	0.0059	0.0053	1.03	0.40	1	1.2459	+ (10^{-4})	+ (10^{-4})
CD3809	3.91×10^{-7}	0.0064	0.0070	0.95	1.20	1	0.8036	+ (10^{-4})	+ (10^{-4})
CD3051	3.02×10^{-8}	0.0059	0.0066	0.97	0.74	1	1.1777	+ (10^{-4})	+ (10^{-4})
CD3079	2.93×10^{-8}	0.0050	0.0057	1.19	0.69	1	1.1512	+ (10^{-4})	+ (10^{-4})

483 ***Table 2: Fitness of seven PCR ribotype 027 strains and fluoroquinolone resistant (FQR) progeny determined by competitive batch***
484 ***culture.***



485

486 ***Figure 3: Progression of mutant (CD3079M) and parent (CD3079) RT027 C. difficile ratios in a continuous culture chemostat***
487 ***model.*** *The linear trend line (purple) demonstrates the increasing divergence between parent and mutant strain populations. Highly*
488 *resistant populations >32 mg/L and >64 mg/L moxifloxacin (MXF) were documented to track further resistance development. The line of*
489 *equality represents the baseline of equivalent mutant and parent strain proportions, data above this signifies a fitness advantage for the*
490 *mutant strain. Data represents mean averages (±SE) of three independent model replicates. * p=0.025, significantly different mutant to*
491 *parent ratios. Line series relate to the left Y axis, bar charts relate to the right.*