AAC Journals.ASM.org

Fluoroquinolone Resistance Does Not Impose a Cost on the Fitness of *Clostridium difficile In Vitro*

François Wasels,[a] Sarah A. Kuehne,[b] Stephen T. Cartman,[b] Patrizia Spigaglia,[a] Fabrizio Barbanti,[a] Nigel P. Minton,[b] Paola Mastrantonio[a]

Department of Infectious, Parasitic and Immune-Mediated Diseases, Istituto Superiore di Sanità, Rome, Italy[a]; Clostridia Research Group, Centre for Biomolecular Sciences, School of Life Sciences, Nottingham Digestive Diseases Centre, NIHR Biomedical Research Unit, The University of Nottingham, University Park, Nottingham, United Kingdom[b]

Point mutations conferring resistance to fluoroquinolones were introduced in the *gyr* genes of the reference strain *Clostridium difficile* 630. Only mutants with the substitution Thr-82→Ile in GyrA, which characterizes the hypervirulent epidemic clone III/027/NAP1, were resistant to all fluoroquinolones tested. The absence of a fitness cost *in vitro* for the most frequent mutations detected in resistant clinical isolates suggests that resistance will be maintained even in the absence of antibiotic pressure.

Modifications within the quinolone resistance-determining regions (QRDRs) of the DNA gyrase subunits GyrA and GyrB are the only identified mechanism of resistance to fluoroquinolones in *Clostridium difficile* (1, 2). The substitution Thr-82→Ile in GyrA characterizes the epidemic clone III/027/NAP1, and it is prevalent in isolates resistant or showing a reduced susceptibility to moxifloxacin (3, 4). The substitution Thr-82→Val has also been detected, although rarely (3, 5). In GyrB substitutions Asp-426→Asn and Asp-426→Val are some of the most common changes found in resistant isolates (2, 6).

The main parameters that are likely to affect the emergence, maintenance, and diffusion of mutations conferring antibiotic resistance are the mutation rate itself and the subsequent burden imposed on the fitness of the bacterium. To this day, nothing is known concerning this burden in *C. difficile*, despite the strong association between the use of fluoroquinolones and *C. difficile* infection (CDI).

In this study, fluoroquinolone-resistant mutants were generated by introduction of point mutations into either the *gyrA* or *gyrB* QRDR domains of the fluoroquinolone-susceptible reference strain *C. difficile* 630, using a two-step allele exchange recently developed (7). These mutants were characterized to determine the impact of the introduced mutations in terms of resistance and fitness cost *in vitro*.

All strains used in this study are listed in Table 1. *C. difficile* was grown at 35°C under anaerobic conditions on brain heart infusion (BHI [Oxoid]) supplemented with 0.5% yeast extract and 0.1% L-cysteine (BHIS). When appropriate, BHIS plates were supplemented with D-cycloserine (250 mg/liter), cefoxitin (8 mg/liter), and thiamphenicol (15 mg/liter). *C. difficile* minimal medium (CDMM) supplemented with 5-fluorocytosine (5-FC) was used to select for loss of pMTL-SC7315 (7). *Escherichia coli* was grown aerobically at 37°C in LB. Chloramphenicol was used at final concentrations of 12.5 and 25 mg/liter in broth and agar, respectively.

Genomic DNA was extracted from *C. difficile* using the NucleoBond AXG columns and NucleoBond buffer set III (Macherey-Nagel). Plasmid DNA isolation from *E. coli* was carried out using the NucleoSpin plasmid kit (Macherey-Nagel). DNA was purified from agarose gels using the NucleoSpin extract II kit (Macherey-Nagel). All kits were used according to the manufacturer's recommendations.

Allele exchange cassettes were generated by PCR using the primers listed in the Table S1 in the supplemental material and cloned into the PmeI site of pMTL-SC7315. Ligation products were used to transform *E. coli* α-select competent cells. Plasmid DNA was then extracted, Sanger sequenced, introduced in *E. coli* CA434, and transferred to *C. difficile* by conjugation, as described previously (7). Transconjugants were streaked on BHIS plates with 4 mg/liter of moxifloxacin to directly select allele exchange events between the vector and the chromosome. The colonies obtained were grown again on the same medium and incubated for at least 48 h to allow subsequent plasmid loss. Colonies were then patch plated onto BHIS with thiamphenicol and CDMM supplemented with 5-FC (7). Desired mutations in thiamphenicol-susceptible and 5-FC-resistant colonies were confirmed by Sanger sequencing of the *gyrA* and *gyrB* genes. Three separate clones of each mutant were isolated, each from independent conjugations.

MICs were determined using the Etest (AB Biodisk) as recommended by the Clinical and Laboratory Standards Institute (8). The breakpoint used for moxifloxacin, levofloxacin, ciprofloxacin and gatifloxacin was 8 mg/liter, in accordance with the guidelines established by the CLSI (8).

Bacterial fitness was estimated by competition assays performed as already described (9). The number of viable cells was determined at the end of every cycle by plating aliquots on BHI agar plates containing 4 mg/liter moxifloxacin or without antibiotic. The competition index (CI) was calculated as the CFU ratio of the resistant and susceptible strains at time t_1 divided by the

Received 8 October 2014 **Returned for modification** 4 November 2014
Accepted 15 December 2014

Accepted manuscript posted online 22 December 2014

Citation Wasels F, Kuehne SA, Cartman ST, Spigaglia P, Barbanti F, Minton NP, Mastrantonio P. 2015. Fluoroquinolone resistance does not impose a cost on the fitness of *Clostridium difficile in vitro*. Antimicrob Agents Chemother 59:1794–1796. doi:10.1128/AAC.04503-14.

Address correspondence to Patrizia Spigaglia, patrizia.spigaglia@iss.it.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/AAC.04503-14.

doi:10.1128/AAC.04503-14

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Source or reference(s)
Strains		
E. coli		
α-select competent cells	F$^-$ *deoR endA1 recA1 relA1 gyrA96 hsdR17*(r_k^- m_k^+) *supE44 thi-1 phoA* Δ(*lacZYA-argF*)*U169* ϕ80*lacZ*ΔM15 λ$^-$	Bioline, United Kingdom
CA434	HB101 [F$^-$ *mcrB mrr hsdS20*(r_B^- m_B^-) *recA13 leuB6 ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20*(Sm^r) *glnV44* λ$^-$] with plasmid R702	16, 17
C. difficile		
630 (wild type)	PCR ribotype 012	18
ISST82Ia, ISST82Ib, ISST82Ic	630 *gyrA*(Thr-82→Ile)	This study
ISST82Va, ISST82Vb, ISST82Vc	630 *gyrA*(Thr-82→Val)	This study
ISSD426Na, ISSD426Nb, ISSD426Nc	630 *gyrB*(Asp-426→Asn)	This study
ISSD426Va, ISSD426Vb, ISSD426Vc	630 *gyrB*(Asp-426→Val)	This study
Plasmid		
pMTL-SC7315	*E. coli-C. difficile* shuttle vector (pCB102 *catP* ColE1 *traJ codA*)	7

same ratio at time t_0. The selection coefficient s was calculated as $s = \ln(CI)/[t \times \ln(2)]$. Fitness of the susceptible strain was set to 1, and the relative fitness of the mutants was determined as $1 + s$. Each independent mutant was tested versus the wild-type strain three times, for a total of nine independent assays for each single mutation analyzed. Statistical analyses were performed using the GraphPad software and an unpaired Student's t test.

Mutations introduced in the genome of *C. difficile* 630 resulted in the amino acid changes Thr-82→Ile or Thr-82→Val in GyrA and Asp-426→Asn or Asp-426→Val in GyrB. Genome sequencing of the mutants ISST82Ia and ISST82Va and the progenitor strain 630 (GATC, Constance, Germany, for ISST82Ia and progenitor 630; DEEPSEQ, the University of Nottingham, Nottingham, United Kingdom, for ISST82Va) validated the efficiency of the mutation strategy. Single nucleotide polymorphism (SNP) analysis was performed using the deposited genome sequence of *C. difficile* 630 (GenBank accession no. NC_009089) as the reference. Overall genome coverage levels were 324.2-, 283.0-, and 795.0-fold for strains 630, ISST82Ia, and ISST82Va, respectively. All strains contained the same six SNPs compared to the reference sequence. As expected, the C→T mutation introduced at position 6310 was detected in ISST82Ia, while the AC→GT substitutions at positions 6309 to 6310 were detected in ISST82Va, resulting in the Thr-82→Ile and Thr-82→Val substitutions in the encoded GyrA sequence, respectively (data not shown). In addition, a G→T mutation was detected at position 3949724 in ISST82Va only, leading to an Arg-113→Ser substitution at a nonconserved position of a magnesium-transporting ATPase. We anticipated that this substitution would affect neither antimicrobial resistance nor fitness of the bacterium.

MIC values for moxifloxacin, ciprofloxacin, levofloxacin, and gatifloxacin were identical between replicate clones containing the same mutation. All mutants were highly resistant to ciprofloxacin, levofloxacin, and gatifloxacin, except for mutants with the substitution Asp-426→Val in GyrB, which showed reduced susceptibility to the last antibiotic (MIC, 6 mg/liter) (Table 2). While mutants with Thr-82→Ile substitution in GyrA were highly resistant to moxifloxacin, the other mutants showed a MIC of 4 mg/liter, in accordance with the observation that substitution Thr-82→Ile is generally associated with high-level resistance (MIC, ≥32 mg/liter) (6). Notably, mutant ISST82V, which had undergone two mutations in the triplet ACT coding for Thr-82, showed decreased MIC values to moxifloxacin and gatifloxacin compared to mutant ISST82I (Table 2).

Even if Asp-426→Val has been associated with higher MICs to fluoroquinolones than Asp-426→Asn, which is associated with intermediate and high levels of resistance (4, 6), both substitutions conferred to strain 630 mutants high levels of resistance only to levofloxacin and ciprofloxacin (Table 2).

Substitution Thr-82→Ile in GyrA had no detectable cost on the fitness of *C. difficile* 630, while substitution Thr-82→Val reduced the fitness of the bacterium (Table 3). The fitness cost imposed by mutations occurring in corresponding positions of GyrA in other

TABLE 2 MICs of *C. difficile* 630 and the mutants obtained in this study

Strain(s)	MIC (mg/liter) of[a]: MXF	GAT	LVX	CIP
630 (wild type)	0.5	0.75	3	6
ISST82Ia, ISST82Ib, ISST82Ic	32	≥32	≥32	≥32
ISST82Va, ISST82Vb, ISST82Vc	4	8	≥32	≥32
ISSD426Na, ISSD426Nb, ISSD426Nc	4	8	≥32	≥32
ISSD426Va, ISSD426Vb, ISSD426Vc	4	6	≥32	≥32

[a] MXF, moxifloxacin; GAT, gatifloxacin; LVX, levofloxacin; CIP, ciprofloxacin.

TABLE 3 Competition assays between wild-type *C. difficile* strain 630 and fluoroquinolone-resistant mutants

Mutants tested vs 630	s[a]	Relative fitness/generation[b]	P value[c]
ISST82Ia, ISST82Ib, ISST82Ic	−0.0216	0.9784 ± 0.0757	0.4036
ISST82Va, ISST82Vb, ISST82Vc	−0.0952	0.9018 ± 0.0462	<0.0001
ISSD426Na, ISSD426Nb, ISSD426Nc	0.0877	1.0877 ± 0.0813	0.0052
ISSD426Va, ISSD426Vb, ISSD426Vc	0.0589	1.0590 ± 0.0749	0.0312

[a] Selection coefficient.
[b] Fitness relative to that of the susceptible strain.
[c] Statistical significance of difference in fitness.

pathogens is variable, ranging from disadvantage to benefit (10–15). *In vitro* results obtained in this study suggest that substitution Thr-82→Ile in GyrA can be maintained in the bacterial population even in the absence of antibiotic selective pressure. This is in accordance with the large spread of strains carrying this mutation, in particular the epidemic clone III/027/NAP1. The fact that two nucleotide mutations are required to produce the substitution Thr-82→Val, together with the observation that this substitution reduces both fitness and MICs to fluoroquinolones, may explain its rarity compared to Thr-82→Ile among *C. difficile* clinical isolates (Tables 2 and 3).

Interestingly, both substitutions Asp-426→Asn and Asp-426→Val in GyrB conferred a slight advantage to the mutants *in vitro*, compared to the wild-type strain 630 (Table 3). However, it is interesting to note that, among fluoroquinolone-resistant clinical isolates, mutations in *gyrB* are less frequent than mutations in *gyrA*.

To conclude, allele exchange was successfully used to introduce point mutations in the *gyrA* and *gyrB* genes of *C. difficile* 630. The mutation leading to the substitution Thr-82→Ile in GyrA conferred the highest level of resistance to fluoroquinolones. Moreover, the most frequent mutations detected in fluoroquinolone-resistant clinical isolates of *C. difficile* displayed no fitness cost *in vitro*. These results suggest that resistance to fluoroquinolones could be maintained in *C. difficile* populations in the absence of antibiotic pressure, although *in vivo* studies will be necessary to better characterize the cost of fluoroquinolone resistance in *C. difficile*.

ACKNOWLEDGMENTS

We thank Tonino Sofia for editing the manuscript.

The research leading to these results has received funding from the European Community's Seventh Framework Programme FP7/2007-2013 under grant agreement no. 237942.

The authors report they have no conflicts of interest related to this article.

REFERENCES

1. **Dridi L, Tankovic J, Burghoffer Barbut F, Petit JCJ.** 2002. *gyrA* and *gyrB* mutations are implicated in cross-resistance to ciprofloxacin and moxifloxacin in *Clostridium difficile*. Antimicrob Agents Chemother **46:**3418–3421. http://dx.doi.org/10.1128/AAC.46.11.3418-3421.2002.
2. **Spigaglia P, Barbanti F, Mastrantonio P, Brazier JS, Barbut F, Delmée M, Kuijper E, Poxton IR, European Study Group on Clostridium difficile (ESGCD).** 2008. Fluoroquinolone resistance in Clostridium difficile isolates from a prospective study of C. difficile infections in Europe. J Med Microbiol **57:**784–789. http://dx.doi.org/10.1099/jmm.0.47738-0.
3. **Ackermann G, Tang YJ, Kueper R, Heisig P, Rodloff AC, Silva J, Jr, Cohen SH.** 2001. Resistance to moxifloxacin in toxigenic *Clostridium difficile* isolates is associated with mutations in *gyrA*. Antimicrob Agents Chemother **45:**2348–2353. http://dx.doi.org/10.1128/AAC.45.8.2348-2353.2001.
4. **Drudy D, Quinn T, O'Mahony R, Kyne L, O'Gaora P, Fanning S.** 2006. High-level resistance to moxifloxacin and gatifloxacin associated with a novel mutation in *gyrB* in toxin-A-negative, toxin-B-positive *Clostridium difficile*. J Antimicrob Chemother **58:**1264–1267. http://dx.doi.org/10.1093/jac/dkl398.
5. **Huang H, Weintraub A, Fang H, Wu S, Zhang Y, Nord CE.** 2010. Antimicrobial susceptibility and heteroresistance in Chinese *Clostridium difficile* strains. Anaerobe **16:**633–635. http://dx.doi.org/10.1016/j.anaerobe.2010.09.002.
6. **Huang H, Weintraub A, Fang H, Nord CE.** 2009. Antimicrobial resistance in *Clostridium difficile*. Int J Antimicrob Agents **34:**516–522. http://dx.doi.org/10.1016/j.ijantimicag.2009.09.012.
7. **Cartman ST, Kelly ML, Heeg D, Heap JT, Minton NP.** 2012. Precise manipulation of the *Clostridium difficile* chromosome reveals a lack of association between the *tcdC* genotype and toxin production. Appl Environ Microbiol **78:**4683–4690. http://dx.doi.org/10.1128/AEM.00249-12.
8. **Clinical and Laboratory Standards Institute.** 2007. Methods for antimicrobial susceptibility testing of anaerobic bacteria: Approved standard M11-A7. CLSI, Wayne, PA.
9. **Wasels F, Spigaglia P, Barbanti F, Mastrantonio P.** 2013. Clostridium difficile *erm*(B)-containing elements and the burden on the *in vitro* fitness. J Med Microbiol **62:**1461–1467. http://dx.doi.org/10.1099/jmm.0.057117-0.
10. **Komp Lindgren P, Marcusson LL, Sandvang D, Frimodt-Møller N, Hughes D.** 2005. Biological cost of single and multiple norfloxacin resistance mutations in *Escherichia coli* implicated in urinary tract infections. Antimicrob Agents Chemother **49:**2343–2351. http://dx.doi.org/10.1128/AAC.49.6.2343-2351.2005.
11. **Kugelberg E, Lofmark S, Wretlind B, Andersson DI.** 2005. Reduction of the fitness burden of quinolone resistance in *Pseudomonas aeruginosa*. J Antimicrob Chemother **55:**22–30. http://dx.doi.org/10.1093/jac/dkh505.
12. **Marcusson LL, Frimodt-Moller N, Hughes D.** 2009. Interplay in the selection of fluoroquinolone resistance and bacterial fitness. PLoS Pathog **5:**e1000541. http://dx.doi.org/10.1371/journal.ppat.1000541.
13. **Rozen DE, McGee L, Levin BR, Klugman KP.** 2007. Fitness costs of fluoroquinolone resistance in Streptococcus pneumoniae. Antimicrob Agents Chemother **51:**412–416. http://dx.doi.org/10.1128/AAC.01161-06.
14. **Zeitouni S, Kempf I.** 2011. Fitness cost of fluoroquinolone resistance in Campylobacter coli and Campylobacter jejuni. Microb Drug Resist **17:**171–179. http://dx.doi.org/10.1089/mdr.2010.0139.
15. **Kunz AN, Begum AA, Wu H, D'ambrozio JA, Robinson JM, Shafer WM, Bash MC, Jerse AE.** 2012. Impact of fluoroquinolone resistance mutations on gonococcal fitness and in vivo selection for compensatory mutations. J Infect Dis **205:**1821–1829. http://dx.doi.org/10.1093/infdis/jis277.
16. **Hedges RW, Jacob AE.** 1974. Transposition of ampicillin resistance from RP4 to other replicons. Mol Gen Genet **132:**31–40. http://dx.doi.org/10.1007/BF00268228.
17. **Williams DR, Young DI, Young M.** 1990. Conjugative plasmid transfer from *Escherichia coli* to *Clostridium acetobutylicum*. J Gen Microbiol **136:**819–826. http://dx.doi.org/10.1099/00221287-136-5-819.
18. **Wüst J, Sullivan NM, Hardegger U, Wilkins TD.** 1982. Investigation of an outbreak of antibiotic-associated colitis by various typing methods. J Clin Microbiol **16:**1096–1101.