Plasmidic qnrA3 Enhances Escherichia coli Fitness in Absence of Antibiotic Exposure

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Abstract

The widespread presence of plasmid-mediated quinolone resistance determinants, particularly qnr genes, has become a current issue. By protecting DNA-gyrase from quinolones, Qnr proteins confer a low level quinolone resistance that is not sufficient to explain their emergence. Since Qnr proteins were hypothesized to act as DNA-binding protein regulators, qnr genes could have emerged by providing a selective advantage other than antibiotic resistance. We investigated host fitness of Escherichia coli isogenic strains after acquisition of the qnrA3 gene, inserted either alone onto a small plasmid (pBR322), or harbored on a large conjugative native plasmid, pHe96(qnrA3) found in a clinical isolate. The isogenic strains were derived from the susceptible E. coli CFT073, a virulent B2 group strain known to infect bladder and kidneys in a mouse model of pyelonephritis. In vitro experiments included growth analysis by automatic spectrophotometry and flow cytometry, and competitions with CFU enumeration. In vivo experiments included infection with each strain and pairwise competitions in absence of antimicrobial exposure. As controls for our experiments we used mutations known to reduce fitness (rpsL K42N mutation) or to enhance fitness (tetA deletion in pBR322). E. coli CFT073 transformed with pBRAM(pBR322-qnrA3) had significantly higher maximal OD than E. coli CFT073 transformed with pBR322 or pBR322ΔtetA, and in vivo competitions were more often won by the qnrA3 carrying strain (24 victories vs. 9 loss among 42 competitions, p = 0.0001). In contrast, when pHe96(qnrA3) was introduced by conjugation in E. coli CFT073, it exerted a fitness cost shown by an impaired growth observed in vitro and in vivo and a majority of lost competitions (33/35, p<0.0001). In conclusion, qnrA3 acquisition enhanced bacterial fitness, which may explain qnr emergence and suggests a regulation role of qnr. However, fitness was reduced when qnrA3 was inserted onto multidrug-resistant plasmids and this can slow down its dissemination without antibiotic exposure.

Introduction

Fluoroquinolones are antibacterial drugs that bind to type II topoisomerases (DNA gyrase and topoisomerase IV) and inhibit DNA re-ligation after enzyme cut [1,2]. These drugs are very useful, especially for treatment of urinary tract infections due to Enterobacteriaceae [1]. Fluoroquinolone resistance rate has increased much for the last years and is mostly due to their large use [3,4]. 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resistance can be driven by Darwinian selection for improved fitness, and not only by the antibiotic use [26]. We hypothesized that Qnr proteins have an effect on bacterial growth and fitness, which may have contributed to the emergence of qnr genes in commensal bacteria.

The aim of our study was to evaluate the impact of the qnr gene acquisition on bacterial fitness. Therefore we compared the fitness of isogenic strains of *Escherichia coli* with and without the *qnrA3* gene, whether alone onto a small plasmid or carried onto a large conjugative multi-drug-resistant native plasmid. Growth and competitive performances were studied in *vitro* and in *vivo* using a mouse model of pyelonephritis.

**Results**

**Description of the isogenic systems expressing qnr or not**

Two systems of isogenic strains were derived from *E. coli* CFT073, a virulent strain belonging to the phylogenetic group B2 and whose genome has been sequenced [32]. This strain was originally used to set the murine model of pyelonephritis used in this study [33]. We selected a streptomycin resistant mutant (SmR) of *E. coli* CFT073 in order to have a resistance marker for the recipient strain after acquisition of the plasmid pHe96, which is a multidrug resistant plasmid not mediating streptomycin resistance. This mutant was selected using 160 μg/ml streptomycin at a proportion of ca.10⁻⁹ and harbored a *rlpK* K42N mutation which is consistent with its high level of resistance (MIC=512 μg/ml) and the stability of this resistance. Although pHe96 contains an *ant3*-I gene known to confer streptomycin resistance [34], this gene is truncated and we confirmed that pHe96 does not confer streptomycin resistance by transferring pHe96 into *E. coli* J53. The MIC of streptomycin was 4 mg/l for this transconjugant and was stable.

The first isogenic system included five strains: *E. coli* CFT073, *E. coli* CFT073 (pBR322) and *E. coli* CFT073 transformed with three other plasmids derived from pBR322 and described in *Figure S1*: pBRAM1 where the tetracycline resistance gene (*tetA*) was deleted, pBRAM1 where the *qnrA3* gene was cloned including the 24-bp DNA motif upstream from *qnrA3*, and pBRAM2 where *qnrA3* was cloned including the 233-bp DNA motif upstream. In both pBRAM1 and pBRAM2, *qnrA3* was inserted into pBR322 by inactivating the *tetA* gene. Minimal inhibitory concentrations (MIC) of quinolones performed on the five strains showed that *qnrA3* expressed quinolone resistance equally (Table 1) with an increase of 4-, 8-, 10- and 16-fold for nalidixic acid, ofloxacin, ciprofloxacin and norfloxacin, respectively.

The second isogenic system included three strains: *E. coli* CFT073-SmR, *E. coli* CFT073-SmR(pHe96), and a variant of this transconjugant named *E. coli* CFT073-SmR(pHe96) “R42”, obtained after one passage in the mouse and which showed improved growth in *vitro* and in *vivo* and higher plasmid stability (see below). The evolved variant “R42” had the same phenotype for antibiotic resistance than the original strain CFT073-SmR(pHe96) including for streptomycin resistance. The acquisition of pHe96 conferred, as described previously [16], a 62- and 50-fold increase in the MIC of ciprofloxacin and norfloxacin (Table 1), respectively, because this plasmid harbored the *aac(6’)-Ib-cr* gene in addition to *qnrA3* [16]. In contrast, ofloxacin and nalidixic acid MICs were the same for the transconjugants CFT073-SmR(pHe96) and for *E. coli* CFT073(pBRAM1) and *E. coli* CFT073(pBRAM2) confirming that *aac(6’)-Ib-cr* has no effect on these quinolines, and showing that the expression of *qnrA3* was similar whether it was harbored on the small plasmid derived from pBR322 or on the large clinical plasmid from which *qnrA3* originated [11,16]. The “R42” variant showed similar sensitivity to quinolones as its parental strain.

Clinical *E. coli* isolates carrying a conjugative multidrug resistant plasmid harboring a *qnr* gene were also tested in the *in vitro* experiments along with the strain *E. coli* J53, a K-12 derivative used as a recipient strain for conjugation [35,36]. Description of the strains, their *qnr* allele and the quinolone resistance conferred (increase in quinolone MICs observed for the transconjugants) was done previously [11,13,35,37]. *E. coli* J53 transconjugants harboring *qnr*-positive plasmids were studied in similar *in vitro* experiments as were the two isogenic systems based on *E. coli* CFT073 (Table S1).

*E. coli* CFT073-SmR was used as a negative control for host fitness since it has been shown that streptomycin resistance, and especially the *rlpK* mutation K42N, had a fitness cost [26,38,39]. *E. coli* CFT073(pBRAM1) was used as a positive control with regard to *E. coli* CFT073(pBR322) since the *tetA* gene has been shown to have a cost for growth when carried by pBR322 and constitutively expressed [40,41].

**Table 1. Minimal Inhibitory Concentrations (MIC) of quinolones against the strains of the two isogenic systems derived from *E. coli* CFT073.**

| *E. coli* strains | MIC* (μg/ml) |
|------------------|-------------|
|                  | NAL | NOR | OFX | CIP | AMK | TOB |
| *E. coli* CFT073 | 2   | 0.064 | 0.094 | 0.012 | 1.5 | 0.75 |
| *E. coli* CFT073(pBR322) | 2   | 0.064 | 0.094 | 0.012 | 1.5 | 0.5 |
| *E. coli* CFT073(pBRAM1) | 2   | 0.064 | 0.094 | 0.012 | 1.5 | 0.5 |
| *E. coli* CFT073(pBRAM2) | 2   | 0.064 | 0.094 | 0.012 | 1.5 | 0.5 |
| *E. coli* CFT073-SmR | 6   | 3   | 0.75 | 0.75 | 48 | 32 |
| *E. coli* CFT073-SmR(pHe96) “R42” | 6   | 3   | 0.75 | 0.75 | 48 | 32 |

*Minimal inhibitory concentrations measured by E-test for quinolones and aminoglycosides. NAL, nalidixic acid; NOR, norfloxacin; OFX, ofloxacin; CIP, ciprofloxacin; AMK, amikacin; TOB, tobramycin.

In *vitro* growth capacity of strains harboring *qnrA3*

In *vitro* growth capacity was measured by automated spectrophotometry for the calculation of the maximal growth rate, the doubling time and the maximal optical density, and by flow cytometry for the size of bacterial cells.

Growth parameters of *E. coli* CFT073 and its four isogenic strains harboring the plasmid pBR322 or one of its derivatives (pBRAM1 and pBRAM2) are shown in Table 2. Maximal OD was significantly higher for all strains harboring a *qnrA3* carrying plasmid. This gain was not due to the inactivation of *tetA*, since the increase in maximal OD was significantly higher for *E. coli* CFT073 transconjugants harboring pBRAM1 or pBRAM2 (acquisition of *qnrA3* at the place of *tetA*) than for the transconjugant harboring pBRAMtetA. No significant difference was seen in the doubling time between strains where *qnrA3* was present (*E. coli* CFT073 transformed with pBRAM1 or pBRAM2) and those where *qnrA3* was absent (*E. coli* CFT073 transformed with pBRAMtetA or pBR322).

Acquisition of the plasmid pHe96 by *E. coli* CFT073-SmR was responsible for a significant lengthening of doubling time and
decrease in maximal OD (Table 3), at the opposite of what was shown above for pBRAM plasmids. Similar results were obtained in brain heart infusion and minimal media (data not shown). The “R42” variant of E. coli CFT073-SmR(pHe96) showed significantly better growth parameters than the original transconjugant strain but stayed below the control strain E. coli CFT073-SmR. Comparing results between transconjugant with pHe96 and the variant isolate R42 of this transconjugant strain but stayed below the control strain showed significantly better growth parameters than the original CFT073-SmR. Comparing results between each strains and the corresponding control qnr-negative strain CFT073(pBR1tetA), p<0.05 was considered significant.

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Table 2. In vitro growth parameters for the strains of the first isogenic system based on E. coli CFT073 carrying pBR322 derivatives harboring qnrA3 or not.

| E. coli strains       | Maximal growth rate in log(OD)/h. | Doubling time in min | Maximal OD |
|-----------------------|-----------------------------------|----------------------|------------|
|                       | mean (SD)                          | mean (SD)            | mean (SD)  |
| CFT073[pBR1tetA]      | 0.96 (+/− 0.02)                    | 18.9 (+/− 0.3)       | 1.07 (+/− 0.02) |
| CFT073[pBR322]        | 0.94 (+/− 0.02)                    | 19.2 (+/− 0.3)       | 1.05 (+/− 0.06) |
| CFT073[pBRAM1]        | 0.94 (+/− 0.01)                    | 19.2 (+/− 0.3)       | 1.11 (+/− 0.02) |
| CFT073[pBRAM2]        | 0.94 (+/− 0.02)                    | 19.2 (+/− 0.5)       | 1.10 (+/− 0.02) |

Enhanced fitness after acquisition of qnrA3 onto pBR322 derived plasmids

In vitro competitive assays were run six times by cultivating E. coli CFT073[pBRAM1] (qnrA3+) or E. coli CFT073[pBRAM2] (qnrA3+) with E. coli CFT073[pBR322] (tetA+) or E. coli CFT073 in a 1:1 ratio. Population increase was measured by CFU counting. Relative Fitness was calculated as the ratio of the increase of each population (see material and methods for details) [42–45]. Comparing with E. coli CFT073[pBR322], estimated Relative Fitness (RF) was close to 1 (1.03+/−0.19), meaning that none of the two competing strains had a selective advantage upon the other in vitro. Comparing with E. coli CFT073, RF was estimated at 1.12+/−0.2, showing that the entire plasmid had no significant biological cost. The mean plasmid loss was less than 3% after 30 generations for all the strains harboring pBR322-derivatives.

We performed in vivo experiments with the strain E. coli CFT073[pBRAM2] rather than with E. coli CFT073[pBRAM1] in order to be closer to clinical conditions (233-hp vs. 24-hp upstream sequence). In competitive infections (Figure 2A), E. coli CFT073[pBRAM2] (qnrA3+) had a significant advantage upon E. coli CFT073[pBR322] (tetA+) with 22 organs/25 where it had takeover versus only 2/25 where it had lost competition (p<0.0001). This selective advantage was confirmed when competitions were run against E. coli CFT073[pBRAtetA] (qnr−) (Figure 2B). Indeed among 37 competitions, 24 were won by the qnrA3-positive strain, 4 were judged as tie because the ratio of the two populations was 1+/−0.2 and 9 were lost (p=0.01). At day 10, a median competitive index was calculated for qnr-positive strains as described previously [29]. It was 1.27+/−0.16 in bladders and 13.9+/−7.2 in kidneys.

Table 3. In vitro growth parameters for the strains of the second isogenic system based on acquisition of the multidrug resistant qnrA3-positive plasmid pHe96.

| E. coli strains       | Maximal growth rate in log(OD)/h. | Doubling time in min | Maximal OD |
|-----------------------|-----------------------------------|----------------------|------------|
|                       | mean (SD)                          | mean (SD)            | mean (SD)  |
| CFT073-SmR            | 0.70 (+/− 0.01)                    | 25.9 (+/− 0.5)       | 1.08 (+/− 0.01) |
| CFT073-SmR(pHe96)     | 0.67 (+/− 0.02)                    | 27.1 (+/− 0.9)       | 0.94 (+/− 0.03) |
| CFT073-SmR(pHe96) R42 | 0.70 (+/− 0.01)                    | 26.1 (+/− 0.8)       | 0.97 (+/− 0.02) |

Enhanced fitness due to qnr

The comparison was performed with a Wilcoxon test between each strains and the corresponding control qnr-negative strain CFT073-SmR, p<0.05 was considered significant.

The difference between the “R42” variant and E. coli CFT073-SmR (pHe96) was significant (p = 0.04).

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Measurements were made separately, not in a competitive assay.

Plasmid loss measured for the "R42" variant was similar with an index of 0.68 when relative fitness index (RF) of E. coli CFT073[pBR322] was significantly less than 1 (0.67)

Stability of pBR322-derived plasmids in in vitro and in vivo experiments

In vitro, no plasmid loss was observed after daily culture of E. coli CFT073[pBR322], E. coli CFT073[pBRAM2] and E. coli CFT073[pBRAM1]. In vivo, the mean loss of the plasmids pBR322 and pBRAM2 in single strain infections (data not shown) was 47% and 67%, respectively, which was not statistically different. In the competitive experiments, the global plasmid loss was higher when we used E. coli CFT073[pBR322] as a control (37% at day 5 and 80% at day 10) than when we used E. coli CFT073[pBRAM1] (3% at day 5 and 11% at day 10). This suggests that the high plasmid loss observed in the competition with tetA-bearing cells corresponded mainly to the loss of pBR322.

In the competitive assays where E. coli CFT073[pBRAM1] was used for control, among 45 organs studied, 4 (9%) organs showed only ampicillin-susceptible colonies corresponding to a plasmid loss of 100%. When E. coli CFT073[pBR232] was used as control, it was 10/35 (29%) organs showing only ampicillin-susceptible colonies. Although we excluded these organs in the final counting, it showed that pBR322-derived plasmids were more stable in vivo when they were carrying a qnrA3 gene that when they did not (p = 0.038), even considering the biological cost of tetA.

Fitness cost due to acquisition of conjugative multidrug resistance plasmids harboring qnr

Reduced growth capacity of E. coli after pHe96 acquisition suggested a fitness cost. This was confirmed by the results of the in vitro competitive assays (eight repeated experiments growing together E. coli CFT073-SmR and E. coli CFT073-SmR[pHe96]) where relative fitness index (RF) of E. coli CFT073-SmR[pHe96] was significantly less than 1 (0.67+/−0.17). The relative fitness of the “R42” variant was similar with an index of 0.68+/−0.14. Plasmid loss measured for E. coli CFT073-SmR[pHe96] was 65% after 30 generations. In contrast, plasmid loss was only 3% for its variant isolate R42 suggesting a compensatory mutation in the chromosome or the plasmid. To investigate whether the reduced growth observed with pHe96 exists for other qnr genes and other qnr-positive MDR plasmids, we studied the in vitro growth capacity of E. coli J53 and of its transconjugants carrying five different qnr-positive and MDR plasmids previously described [13,33,37]: pHm13 (qnrA1), pHm477 (qnrA3), pHS805 (qnrS1), and pU1696 (qnrB4). No difference was seen between growth parameters of the strain E. coli J53 and those of the five qnr-positive transconjugants (Table S1).

When mice were inoculated by the strain E. coli CFT073-SmR[pHe96], the bacterial density in bladders and kidneys was significantly lower than with the control strain, either at day 2, 5 or 10 (Figure 3). In addition, the number of mice where the strain failed to induce a persistent UTI was higher with the transconjugant than with the parental strain: 3 versus 0 for bladders and 7 versus 2 for kidneys (p = 0.003). The results were similar for the “R42” variant carrying the plasmid pHe96 but less than with the original transconjugant. This suggests that a compensatory mutation might have occurred in this variant when it was cultivated into the mouse for the first passage. In competitive experiments, E. coli CFT073-SmR[pHe96] lost the competition in 35 out of 35 organs (p<0.05) with regard to the control strain E. coli CFT073-SmR[pHe96] confirming the fitness cost of pHe96 acquisition (Figure 4A). A similar rate of lost experiments was found for the “R42” variant (Figure 4B). The mean loss of pHe96 was less than 20% for the strains E. coli CFT073-SmR[pHe96] and its variant “R42”, which was expected since these plasmids were coming from clinical isolates. The median value of the competitive index for the transconjugants carrying pHe96 was 0.0012+/−0.001. No competition has been run opposing the two pHe96 transconjugants because it was not possible to distinguish them.

We also tested, in competitive experiments against the E. coli CFT073 strain and using the same UTI mouse model, two qnr-positive
uropathogenic clinical isolates: *E. coli* Hm13 (*qnrA1*) and *E. coli* PS105 (*qnrS1*) (Figure S2). In these non-isogenic competitions, the susceptible strain *E. coli* CFT073 won 53 of 57 competitions (kidneys and bladders) upon *E. coli* Hm13 (*qnrA1*) and 40 of 65 competitions upon *E. coli* PS105 (*qnrS1*) (p<0.001). This confirmed that the fitness cost attributed to the acquisition of pHe96 was not specific to this plasmid but was similarly observed with other *qnr*-positive MDR plasmids.

**Discussion**

The interplay between resistance and fitness is a challenging issue when increase of antibiotic resistance is observed worldwide [26]. Selection pressure due to the increase in antibiotic prescription explains the increase in resistance in most of the settings [3] and quinolone resistance was so far associated to a fitness cost when it was due to mutations either in the topoisomerase genes or in the efflux operons [27–29,45–49]. The emergence of plasmid-mediated resistance determinants questioned about links between acquisition of resistance and selective advantage. Although plasmid acquisition brings a fitness cost, the stability of native plasmids varied according to the host and the presence of drug addiction systems may compensate this plasmid cost [50,51]. For some antibiotic resistance determinants described recently, such as CTX-M beta-lactamases [52,53], we
Figure 3. Single strain urinary tract infections with the isogenic system of *E. coli* CFT073-SmR harboring or not the multidrug resistance plasmid pHe96 (*qnrA3*).

Part A: Bacterial density (log_{10}CFU/g of tissue) in bladders collected two, five and ten days after inoculation by *E. coli* CFT073-SmR (*qnr*—, purple plot), *E. coli* CFT073-SmR(pHe96) (*qnrA3*+, light green plot) and *E. coli* CFT073-SmR(pHe96) R42 variant (*qnrA3*+, dark green plot). At day 2, bacterial density was 6.79+/−0.35, 4.95+/−0.8 (p<0.0001), and 4.9+/−0.65 (p<0.0001), respectively; at day 5, it was 4.61+/−0.25, 3.24+/−0.3 (p<0.0001) and 3.79+/−0.5 (p=0.03), respectively; and at day 10, 5.47+/−0.8, 2.91+/−0.6 (p=0.0004) and 2.85+/−0.5 (p=0.001), respectively. At least 10 mice were studied per group.

Part B: Bacterial density (log_{10}CFU/g of tissue) in kidneys collected two, five and ten days after inoculation by *E. coli* CFT073-SmR (*qnr*—, purple plot), *E. coli* CFT073-SmR(pHe96) (*qnrA3*+, light green plot) and *E. coli* CFT073-SmR(pHe96) R42 variant (*qnrA3*+, dark green plot). Results were respectively 4.29+/−0.53, 3.14+/−0.64 (p=0.005) and 3.53+/−0.46 (p=0.053) at day 2; 4.29+/−0.61, 2.94+/−0.6 (p=0.02), and 4.23+/−0.43 (p=0.06) at day 5; and 4.81+/−0.6, 3.26+/0.63 (p=0.012 and 3.3+/−1.59 (p=0.04) at day 10. At least 10 mice were studied per group.

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know that they have been transferred from environmental bacteria harboring the resistance genes as chromosomal-borne, to human commensal bacteria, mainly E. coli, through mobile elements such as plasmids [54]. For quinolone resistance, such transfer may have occurred since similar qnr genes are chromosome-borne in environmental bacteria [18]. However, the persistence of qnr genes on plasmids is not fully explained by the quinolone selective pressure since there are numerous other effective mechanisms to obtain quinolone resistance such as stepwise chromosomal mutations in the target genes [55] and in the several efflux systems present in E. coli [56]. We hypothesized that qnr genes confer a selective advantage outside the quinolone exposure.

To study the impact on fitness of qnr acquisition, we compared in vitro growth curves, in vitro pairwise competition, and in vivo single culture and pairwise competition, which are usual methods found in literature [26,28,29,42–45,57]. Pairwise competitions are usually more sensitive than single cultures to reveal a fitness change, and in vivo assays are more relevant than in vitro assays since the complex growth environment is closer to reality [26]. This justifies the conclusions we drew on qnr impact on fitness, which were based on the results of in vivo competitions assays. We chose the mouse model of pyelonephritis for in vivo experiments because it was well validated and recently used for studying the interplay in fluoroquinolone resistance mutations and bacterial fitness [29]. Marcusson and colleagues showed that although the

Figure 4. Reduced fitness observed after pHe96 acquisition in competitive infections in absence of antimicrobial exposure. Each symbol represents the ratio (number of CFU for the qnr-positive strain/number of CFU for the qnr-negative isogenic strain) in organs (blue diamond = kidneys, red circle = bladder), collected five and ten days after inoculation of a 1:1 mix of the two strains. Part A: competition experiments opposing E. coli CFT073-Sm(qnr) and E. coli CFT073-Sm(pHe96) (qnrA3+). Twenty mice were inoculated, 19 bladders and 16 pairs of kidneys were efficiently infected. Competition was lost 33 times by E. coli CFT073-Sm(pHe96) (qnrA3+), and won only 2 times (p<0.0001). Part B: competition experiments opposing E. coli CFT073-Sm(qnr) and E. coli CFT073-Sm(pHe96) variant “R42” (qnrA3+). The R42 variant was selected from kidneys that were infected by E. coli CFT073-Sm(pHe96). Twenty mice were inoculated, 18 bladders and 16 pairs of kidneys were efficiently infected. Competition was lost 33 times by the qnrA3-positive strain with only one won (p<0.0001).

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first quinolone resistance mutations (mostly gyrA) had fitness cost, latest compensatory parC mutations could provide increase of both resistance and fitness, suggesting that a higher level of resistance could be selected in absence of antimicrobial exposure [29].

Our findings, obtained using an isogenic system in E. coli where the gyr gene was cloned onto a simple replicative plasmid such as pBR322, showed that gyr acquisition enhanced the bacterial fitness in vitro and in vivo. Indeed when the only difference between two E. coli strains was the presence of gyr with its flanking region, the strain that acquired the gyr gene took over the susceptible strain in absence of quinolone exposure. Several studies [13,14] suggested that the low level quinolone resistance is relevant when the host is exposed to quinolones in clinical situations. This could give a partial explanation for widespread but not for emergence. Several environment bacterial species, such as Shewanella algae or Vibrio splendidus, have been shown to harbor chromosomal qnr-like genes and are supposed to be the reservoir of qnrA3 and qnrS1, respectively [15–18]. The enhancement of fitness we observed after the acquisition by E. coli CFT073 of qnrA3 without antimicrobial exposure could explain how gyrA had first emerged. This could have been the following scenario: the transfer from the chromosome of S. algae to a mobile element replicating in E. coli, or another member of the Enterobacteriaceae family, due to gyr giving a selective advantage. gyrD is the last gyr gene that was discovered in 2009 and it is carried onto a small non conjugative plasmid [58]. Although we did not test the fitness conferred by this small replicative gyr-positive plasmid, similar to pBR322, we hypothesized that the first step of the gyr emergence could have been through mobilization onto small plasmids, which may have resulted in an enhanced fitness as we showed in our in vivo experiments. In addition, there might be low concentrations of quinolones in the environment at some places which could have contributed to the resilience of these bacteria harboring gyr genes [59].

To investigate if their impact on fitness could also explain the widespread of gyr genes, we measured the fitness after acquisition of qnrA3 when included in a multidrug resistant plasmid as those usually observed in clinical gyr-positive isolates [10,11,36,60]. When the gene was acquired with the whole multi drug resistant plasmid pHe96, the host fitness was lessened. This could be explained by the presence of another fitness impairing gene on the plasmid. However, this fitness cost was not specific of pHe96 since competitive experiments between E. coli CFT073 and two other E. coli clinical strains with gyr containing MDR plasmids showed the same fitness cost. Our experiments with an evolved clone of the E. coli CFT073(pHe96) transconjugant suggest that the fitness cost can be reduced by the host. Further explorations are needed to investigate the mechanism of this compensation. The difference in the impact on fitness when gyrA is included into a large plasmid or into a small non conjugative plasmid could also be a consequence of a different level of gyr expression. However, MICs of quinolones were the same in the two plasmids.

Whatever, this would mean that fitness gain could contribute to the emergence of gyr-bearing plasmids among the bacterial communities living in the environment (soil, water) and their transfer to other bacteria such as those of the human commensal flora [54]. For the widespread observed in the following years, the fluoroquinolone resistance conferred and co selection with other resistance determinant (particularly expanded-spectrum-beta-lactamases, ESBL) would be the predominant selection factor [61,62].

Native functions of Qnr proteins are still unknown. They bind type II topoisomerases even without quinolone binding to their targets [19,20]. Although known Qnr proteins usually do not interfere with E. coli gyrase activity at the concentration they prevent fluoroquinolone inhibition [10,19,21]. Qnr-like proteins inhibit catalytic activity of topoisomerases from their natural host [21,63]. Crystallographic data on the structure of Qnr-like PRPs such as MfpA [22], E6Qnr [23] and AhQnr [24], revealed a right handed β helix structure that mimics a double-strain helix DNA. This could permit interaction with other DNA-linked proteins with a possible regulating function of the activity of these proteins. Mimicking DNA structure could have consequences on cell cycle and replication. Thus, this could explain the impact on fitness.

Limitations
Plasmid instability was significant for some of our plasmid constructions, even the native plasmid pHe96. However our analysis took into account this plasmid loss. Overall, artificial plasmids constructed from pBR322 were less stable than plasmids naturally isolated from clinical strains.

Materials and Methods

Ethics Statement
Animal experiments were performed in accordance with prevailing regulations regarding the care and use of laboratory animals by the European Commission. The experimental protocol was approved by the Departmental Direction of Veterinary Services in Paris, France (agreement N° A 75-18-05, 2005). The procedures are conformed to the Amsterdam protocol on animal protection and welfare and Directive 86/609/EEC on the protection of animals used for experimental and other scientific purposes.

All manipulation of animals were be carried out by qualified personnel. All protocols will be done in accordance with the local institutional review board (Comité d’Ethique en expérimentation animale, Ile de France, Paris Comité 3).

Bacterial strains and plasmids

E. coli CFT073 strain is a clinical isolate susceptible to all antibiotics [32], which was used previously to set the murine model of pyelonephritis [13,33]. The plasmid pBR322 vector (4,361 bp) carries a bla gene encoding ampicillin resistance and a tetA gene encoding tetracycline resistance [64,65]. The plasmid pHe96 was previously isolated in a Vibrio parahaemolyticus cultivated from feces in a patient with cancer. The strain was described before [16] and the plasmid was sequenced for the 10 kb DNA fragment flanking qnrA3 (GenBank accession number EU495237). qnrA3 is a gene homologous to the chromosome-borne gyrA allele found in S. algae strains [18] and was only described as plasmid-borne in the plasmid pHe96 [16].

E. coli J53 strain is a recipient strain widely used for conjugation with plasmid carrying gyrA alleles [6,36]. E. coli J53 transconjugants were obtained after conjugation with the following clinical strains: E. coli pHm477 (gyrA1) [11], E. pneumoniae pH96 (qnrA3) [16], E. coli pPS105 (qnrS1) [13], E. coli pHm13 (gyrA1) [13] and E. coli pU1696 (qnrB4) [37]. These two latter strains, which were uropathogenic clinical isolates harboring multiple drug resistance plasmids containing qnrA1 and qnrS1, respectively, were used for competitive experiments against susceptible E. coli CFT073.

qnrA3 cloning into pBR322
Cloning of the qnrA3 gene and its flanking region was done including either the 24 nucleotides (M24) upstream from qnrA3, resulting in the plasmid pBRAM1, or the 235 nucleotides (M235) upstream, resulting in the plasmid pBRAM2, and 29 nucleotides downstream for both. These upstream regions M24 and M235 are...
known to be conserved in qnrA containing plasmids [16] and are supposed to contain the promoter region [36,66]. Amplification sense primer containing an EcoRV restriction site (underlined) [5'-ATACCTCAGATATCCGCCCTCCCTGATT-3'] for the fragment included in pBRAM1 and 5'-CTGACTGATATCCGCCCTCCCAAATCCACACT-3' for the fragment included in pBRAM2 and a SalI restriction site (underlined) in the reverse primer (GCAAAGCTGCTACAGTGATTGTGCCG) for both DNA amplified fragments. Plasmid DNA of E. coli CFT073 with pBRAM1 or pBRAM2 were selected on MH agar containing streptomycin concentration from 10 to 100 μg/ml. Plasmid DNA of E. coli CFT073-SmR(pHe96), which has been selected from kidneys of an infected mouse. This clone was studied because it caused an infection with a bacterial load in organs higher than with the other clones of E. coli CFT073-SmR(pHe96).

A streptomycin-resistant mutant E. coli CFT073-SmR strain was selected in vitro from E. coli CFT073 by plating 10^6 bacteria onto MH agar containing streptomycin concentration from 10 to 160 μg/ml. The transconjugant E. coli CFT073-SmR[pHe96] was obtained after conjugation between E. coli CFT073-SmR and E. pneumotyphiae He96 [16] after 40 min of mating into MH broth as previously described [13]. After incubation, transconjugants were selected by plating the conjugation mixture on MH agar supplemented with ampicillin (100 μg/ml) plus streptomycin (100 μg/ml). PCR experiments (RAPD and PCR qnrA) were performed as already described to confirm the success of the conjugation [11,67].

E. coli CFT073-SmR[pHe96] “R42” is a clone of E. coli CFT073-SmR[pHe96], which has been selected from kidneys of an infected mouse. This clone was studied because it caused an infection with a bacterial load in organs higher than with the other clones of E. coli CFT073-SmR(pHe96).

Growth curves and antibiotic susceptibility testing

Growth curves of each strain in TS broth were drawn using automatic optical density measurements obtained by reader infinite® M200 (Tecan). After an overnight culture in TS broth at 37°C, bacterial suspensions were diluted in order to reach an Optical Density of 0.5, and then diluted 100 times. Microplates were inoculated with the obtained suspensions. Each combination of strain and medium was run in triplicate. The plates were incubated at 37°C for 24 hours and subject to shaking during 60 s every two minutes. Optical density (OD) at 600 nm was measured every 5 minutes during this 24-hours period. The log10 of each value of OD was calculated. To estimate the doubling time, a slope was calculated between every two consecutive values. After discarding artifacts, a mean of the six highest slopes was calculated, corresponding to the maximal growth rate. Doubling time was calculated as \( \log_{10} 2 \) divided by the maximal growth rate [57,68]. Maximal OD increase was also calculated as the mean of the ten highest values of OD reached during stationary phase. This has been performed five times for each strain.

The Minimal Inhibitory Concentrations (MICs) of nalidixic acid, norfloxacin, olroxacin and ciprofloxacin were determined for each constructed strain and control strain by Etest®, according to the instructions of the manufacturer. MICs of amikacin and tobramycin were also measured in order to evaluate the expression of aac(6\')-Ib-cr. Etest® were performed on MH agar plates and the plates were incubated 18 h at 37°C.

Plasmid stability

Since all the plasmids tested carried an ampicillin resistant determinant, plasmid stability of the transconjugants E. coli CFT073-SmR[pHe96], E. coli CFT073[pBRAM1], E. coli CFT073[pBRAM2], E. coli CFT073[pBRAM2], E. coli CFT073(pBRAM1), E. coli CFT073(pBRAM2) and E. coli CFT073(pBRAM2) was measured in vitro by daily subculture during ten days in antibiotic-free Trypticase Soy (TS) broth with plating onto TS agar containing 100 μg/ml ampicillin and antibiotic free TS agar plates. In vivo, at the end of each experiment, organs were also spread onto ampicillin-containing and antibiotic-free TS agar plates. Plasmid loss was calculated for each organ as the ratio ampicillin resistant CFU/total number of CFU.

Flow cytometry

In order to check that OD variations were not caused by a change of cell size, all the studied strains were also studied in flow cytometry as already described [69]. Flow cytometric measurements were performed on a Guava EasyCyte Plus flow cytometer (Millipore, St Quentin en Yvelines, France) equipped with a 488 nm laser. Overnight cultures were diluted to a cell density of 10^6 CFU/ml. Cells were collected on the forward scatter with logarithmic amplifiers for 5,000 events. The experiment was replicated four times for each strain.

In vitro competitions experiments

Pairwise competition experiments were performed to evaluate the in vitro relative fitness of each qnr-positive strain compared to the qnr-negative control strain. We also performed competition with the wild type strain E.coli CFT073. Each strain of its isogenic system was incubated separately for 18 h at 37°C in TS broth. The cell densities of the suspensions were estimated by flow cytometry [70]. An aliquot containing 10^8 bacteria was taken from each suspension and mixed two by two in a ratio of 1:1 to inoculate 10 ml of TS broth and then incubated for 24 h at 37°C for a competitive growth. The initial and the final densities of each competitive strain were estimated from CFU data by diluting and plating population samples onto TS agar with and without antibiotic. Tetracycline was used to distinguish E. coli CFT073(pBRAM1) from E. coli CFT073(pBRAM2) and E. coli CFT073(pBRAM2) in the first isogenic system. Ampicillin was used to distinguish E. coli CFT073 from E. coli CFT073(pBRAM1) and E. coli CFT073(pBRAM2). In the second isogenic system, Ampicillin was used to distinguish E. coli CFT073-SmR[pHe96] from E. coli CFT073-SmR. With these densities, the Malthusian fitness change of cell size, all the studied strains were also studied in flow cytometry as already described [69]. Flow cytometric measurements were performed on a Guava EasyCyte Plus flow cytometer (Millipore, St Quentin en Yvelines, France) equipped with a 488 nm laser. Overnight cultures were diluted to a cell density of 10^6 CFU/ml. Cells were collected on the forward scatter with logarithmic amplifiers for 5,000 events. The experiment was replicated four times for each strain.

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where $S_{1,dl}$ and $S_{1,do}$ are the CFU densities of the $qnr^+$ strain respectively at the end and at the beginning of the competition experiment, and $S_{2,dl}$ and $S_{2,do}$ their equivalent values for the control strain. A RF score $>1$ meant that the $qnr^+$ strain had a selective advantage upon the control strain whereas a score $<1$ represented a fitness cost for $qnr$ acquisition.

**Mouse model of urinary tract infection**

The ascending unobstructed mouse model of UTI was used as previously described [13,33]. Animal experiments were performed in accordance with prevailing regulations regarding the care and use of laboratory animals by the European Commission. The experimental protocol was approved by the Departmental Direction of Veterinary Services in Paris, France. Eight-week-old immunocompetent female CBA mice (weight, 20 to 23 g) were used. Inocula of different strains were obtained by overnight incubation in BHI broth, washing of the cells by centrifugation at 15,000 $\times$ g for 15 min, and resuspension in TS broth to a final inoculum of $10^{10}$ CFU/ml. Pyelonephritis was induced during general anaesthesia (with an intraperitoneal administration of Ketamin 150 mg/kg and Xylazin 0.5 mg/kg) by injecting 50 $\mu$l (i.e., $5 \times 10^6$ CFU) into the bladder through a urethral catheter.

**In vivo growth experiments: single-strain infections**

In order to evaluate their potentials to perform persistent UTI and to reach a high bacterial density in bladder and kidneys, mice were inoculated separately by each of the following strains: *E. coli* CFT073-SmR, *E. coli* CFT073-SmR(pHe96) and *E. coli* CFT073-SmR(pHe96) “R42”. No antimicrobial was administered. Mice were sacrificed at five, ten and five days after inoculation with at least 8 mice per group, and bladders and kidneys were aseptically taken out, weighed and homogenized in 1 ml of saline solution. Bacterial densities were estimated from CFU data by diluting and plating organs samples onto TS agar and were expressed as the number of CFU/g of tissue. The growth rate of each strain was estimated by the bacterial densities in bladder and kidneys and could be compared to the control strain. Plasmid loss on days 5 and 10 was assessed for strains harboring pBR322 or its derivatives, qnrA1 and qnrS1 from its transconjugants, and 2 only were won (p $<0.0001$).

**In vivo fitness measurements: competitive infections**

The in vivo selective value of the $qnr^+$-positive strain compared to the control $qnr$-negative strain was determined by competitive experiments between the two isogenic strains using the same mouse model of UTI. Inocula were prepared the same way that for single-strain infection experiments, except that before mixing the two suspensions, bacterial density was measured by flow cytometry. Proportions of each suspension were adjusted so that inoculum contained the two strains in a 1:1 ratio and that cell density was at least $10^{10}$ bacteria/ml. Inoculum was spread on antibiotic containing plate to count the initial ratio. At least 20 mice were inoculated for each competitive experiment, and were sacrificed at five or ten days after inoculation. After removing and homogenizing bladders and kidneys, they were spread onto TS agar plates without antibiotics and containing the appropriate antibiotic to select one of the two competitive strains among the population. Tetracycline (20 $\mu$g/ml) was used to distinguish between strains harboring pBR322 or pBRAM2, and ciprofloxacin (0.05 $\mu$g/ml) to distinguish strains harboring pBRAM2 or pBRATA. To count the number of pBRATA-bearing cells, we subtracted the number of cells growing onto ciprofloxacin-containing plates from the number of cells growing onto ampicillin-containing plates. Ampicillin (100 $\mu$g/ml) was used to distinguish *E. coli* CFT073-SmR from its transconjugants, *E. coli* CFT073-SmR(pHe96) and *E. coli* CFT073-SmR(pHe96) R42. For competitions between strains carrying pBR322 or its derivatives, bladders and kidneys were also spread onto ampicillin-containing plates, in order to evaluate plasmid loss. A competitive index was calculated for each organ as the ratio of the number of $qnr$-positive cells/number of $qnr$-negative cells, corrected by the initial ratio. Organs where no CFU grew were excluded.

**Statistical analysis**

Results were expressed as mean and 95% confidence interval for continuous variables. Continuous variables were compared by nonparametric (Wilcoxon) tests. Discontinuous variables were compared by Fisher’s exact test. A p value less than 0.05 was considered significant. Analysis was performed using the online free software BioStatgy (http://www.ai707.jussieu.fr/biostatgy).

**Supporting Information**

Figure S1 Scheme of $qnrA3$ cloning from its native plasmid pHe96 into pBR322 and resulting $qnr$-positive (pBRAM1 and pBRAM2) and control plasmids (pBRAtetaA). M24 and M233 are the fragments of 24- and 233-bp upstream from $qnrA3$ and described in pHe96 [16]. Primers (see text for sequences) Fw2, Fw1 and R contain the EcoRV and SalI restrictions sites and were used to amplify the $qnrA3$ DNA fragments.

Figure S2 Reduced fitness observed for clinical isolates of *E. coli* harboring $qnr$-positive mult drug resistance plasmids in competitive infections with *E. coli* CFT073 without antimicrobial exposure. Each symbol represents the ratio (number of CFU for the $qnr$-positive strain/number of CFU for the $qnr$-negative isogenic strain) in organs (blue diamond = kidneys, red circle = bladder), collected five to forty days after inoculation of a 1:1 mix of the two strains. Part A: competition experiments opposing *E. coli* CFT073 ($qnr^+$) and *E. coli* Hm13 ($qnrA1^+$), Thirty mice were inoculated, 27 bladders and 30 pairs of kidneys were efficiently infected. Fifty-three competitions were lost by the $qnr^+$ strain and 2 only were won (p $<0.0001$). Part B: competition experiments opposing *E. coli* CFT073 ($qnr^+$) and *E. coli* PS105 ($qnrS1^+$). Thirty-three mice were inoculated, 33 bladders and 30 pairs of kidneys were efficiently infected. Forty-one competitions were lost by the $qnr^+$ strain, and 17 were won (p $<0.0001$).

**Table S1 In vitro growth parameters of *E. coli* J53 and its transconjugants with $qnr$-positive multidrug resistant plasmids.**

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**Author Contributions**

Conceived and designed the experiments: EC AM BF. Performed the experiments: AM NA FC. Analyzed the data: AM EC BF. Contributed reagents/materials/analysis tools: IP FC. Wrote the paper: AM EC.
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