Fluoroquinolone-specific resistance trajectories in E. coli and their dependence on the SOS-response

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Fluoroquinolone-specific resistance trajectories in *E. coli* and their dependence on the SOS-response

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Abstract

Background Fluoroquinolones are indispensable antibiotics used in treating bacterial infections in both human and veterinary medicine. However, resistance to these drugs presents a growing challenge. The SOS response, a DNA repair pathway activated by DNA damage, is known to influence resistance development, yet its role in fluoroquinolone resistance is not fully understood. This study aims to unfold the mechanisms of fluoroquinolone resistance by investigating the impact of the SOS response on bacterial adaptation.

Results We exposed *Escherichia coli* to four fluoroquinolones—ciprofloxacin, enrofloxacin, levofloxacin, and moxifloxacin. Using a *recA* knockout mutant, deficient in the SOS response, as a control, we assessed how the presence or absence of this pathway affects resistance development. Our findings demonstrated that the rate of resistance evolution varied between the different fluoroquinolones. Ciprofloxacin, enrofloxacin, and moxifloxacin exposures led to the most evident reliance on the SOS response for resistance, whereas levofloxacin exposed cultures showed less dependency. Whole genome analysis indicated distinct genetic changes associated with each fluoroquinolone, highlighting potential different pathways and mechanisms involved in resistance.

Conclusions This study shows that the SOS response plays a crucial role in resistance development to certain fluoroquinolones, with varying dependencies per drug. The characteristic impact of fluoroquinolones on resistance mechanisms emphasizes the need to consider the unique properties of each antibiotic in resistance studies and treatment strategies. These findings are essential for improving antibiotic stewardship and developing more effective, tailored interventions to combat resistance.

Keywords Fluoroquinolones, Antibiotic resistance, De novo resistance, SOS response, Experimental evolution

Background

Fluoroquinolones are broad-spectrum antibiotics commonly used to treat infections in both human and veterinary medicine [1, 2]. Classified under the World Health Organization's (WHO) AWaRe framework as high-priority critically important antimicrobials, these drugs are crucial due to their efficacy but pose a heightened risk for resistance development [3, 4]. Despite their importance and our broad knowledge of their mechanisms of action and corresponding bacterial resistance mechanisms, our understanding of how bacteria evolve to become resistant or adapt to fluoroquinolones remains limited.

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The mode of action of fluoroquinolones revolves around targeting essential bacterial enzymes critical for DNA replication and repair, i.e. DNA gyrase (GyrA and GyrB) and DNA topoisomerase IV (ParC and ParE) [5]. Mutations in gyrA are essential for development of high level fluoroquinolone resistance in *E. coli* [6]. The bacterial resistance mechanisms include target site alterations, efflux pumps, and changes in membrane permeability [7]. Since fluoroquinolones cause DNA damage and consequently have been shown to activate the bacterial SOS response, inhibiting the SOS response has been proposed as a potential approach to combat resistance evolution [8, 9].

The core of the SOS response is the activation of the RecA protein, induced by its interaction with single-stranded DNA (ssDNA) [10, 11]. Once activated, RecA coordinates the self-cleavage of the SOS transcriptional repressor LexA [12], thereby initiating the transcriptional cascade of the SOS response. However, knocking out RecA does not entirely abolish antibiotic-induced mutations in *Escherichia coli* upon beta-lactam exposure, indicating the existence of a LexA/RecA-independent pathway capable of triggering the SOS response that has yet to be fully understood [13].

Utilizing an experimental evolution approach, we sought to study the dynamics governing the adaptation of *E. coli* to four fluoroquinolone antibiotics – ciprofloxacin, enrofloxacin, levofloxacin, and moxifloxacin – using a SOS-deficient mutant as biological control. The selection of these fluoroquinolones was based on their different chemical structures (see Figure S1) and widespread use in both veterinary, enrofloxacin, and human healthcare, the other three, settings, allowing for a broad exploration of the adaptive landscape. Yet, how and if they differ in inducing cellular changes that lead to de novo resistance is unknown. Through this study, we aimed to clarify the genetic and phenotypic changes occurring in both the wild type and SOS-knockout strain, thus highlighting the interplay between the induction of the SOS response and the evolution of resistance. Our research specifically focused on the role of the SOS response in adaptation to each fluoroquinolone and the distinct adaptation patterns associated with each antibiotic.

Results

Dependency on *recA* for resistance evolution differs between fluoroquinolones

We could observe a clear pattern in both strains by comparing the onset of clinically relevant resistance between the tested antibiotics. Both MG1655 and Δ*recA* acquired resistance the fastest when exposed to moxifloxacin. The slowest resistance development was observed during ciprofloxacin exposure. An overview of the number of transfers it took to reach the EUCAST breakpoints can be found in Table 1.

Relative to the wild-type strain, the knockout mutant (Δ*recA*) displayed a higher inconsistency in adaptation rates, defined as numbers of two-fold increases over time, to the tested fluoroquinolones between the replicates. In general, the Δ*recA* mutant showed an unpredictable response to antibiotic exposure, often leading to the loss of cultures after incubation in antibiotic concentrations they already adapted to before (see Table S4). The starting antibiotic concentrations for the evolution experiment with the knockout mutant were lower, reflective of the decreased minimum inhibitory concentration (MIC) of the naive mutant observed during initial MIC testing. The starting concentrations, which equalled ¼ of the starting MICs can be found in Table S2.

Knocking out *recA* led to a distinct impairment of adaptation to all tested fluoroquinolones (Fig. 1). However, there were notable differences between biological replicates, and there also seemed to be a distinction between the different fluoroquinolones. The strongest dissimilarity between Δ*recA* and the wild-type could be observed upon moxifloxacin exposure ($p = 1.10e\text{-}07$) and enrofloxacin exposure ($p = 2.58e\text{-}07$). While the wild-type could adapt to moxifloxacin concentrations of up to 1024 µg/mL, and enrofloxacin concentrations of 512 µg/mL, Δ*recA* could only grow at a maximum moxifloxacin concentration of 4 µg/mL, and enrofloxacin concentration of 32 µg/mL. Similar patterns were found in the ciprofloxacin cultures ($p = 0.00126$). Two of the three Δ*recA* replicates could not adapt to ciprofloxacin concentrations above 1 µg/mL during the tested time frame. This pattern supports the hypothesis that targeting the SOS response in bacteria could be a valuable approach to prevent the development of antimicrobial resistance to some antibiotics. However, one of the ciprofloxacin-exposed Δ*recA* replicates reached the EUCAST threshold of 0.5 µg/mL after 11 transfers and could survive a final concentration

Table 1 Comparison average transfers until clinical resistance

Fluoroquinolone	MG1655	Δ*recA*	*p*-value MG1655 vs. Δ*recA*
Ciprofloxacin	7.7 ± 0.6	17 ± 5.3	0.0385
Enrofloxacin	5.3 ± 0.6	14.3 ± 2.5	0.00380
Levofloxacin	6.7 ± 1.2	11.3 ± 3.2[a]	0.0771
Moxifloxacin	3.3 ± 0.6	7.7 ± 2.5	0.0438

The table lists the average transfers ± standard deviation until the strains reached the resistance breakpoint according to the EUCAST/CLSI guidelines (see S1 in the supplementary data). Statistical testing was performed using a linear model comparing at which transfer clinical resistance occurred for wildtype and mutants. One replicate could not reach the resistance breakpoint until the experiment ended, indicated by[a]

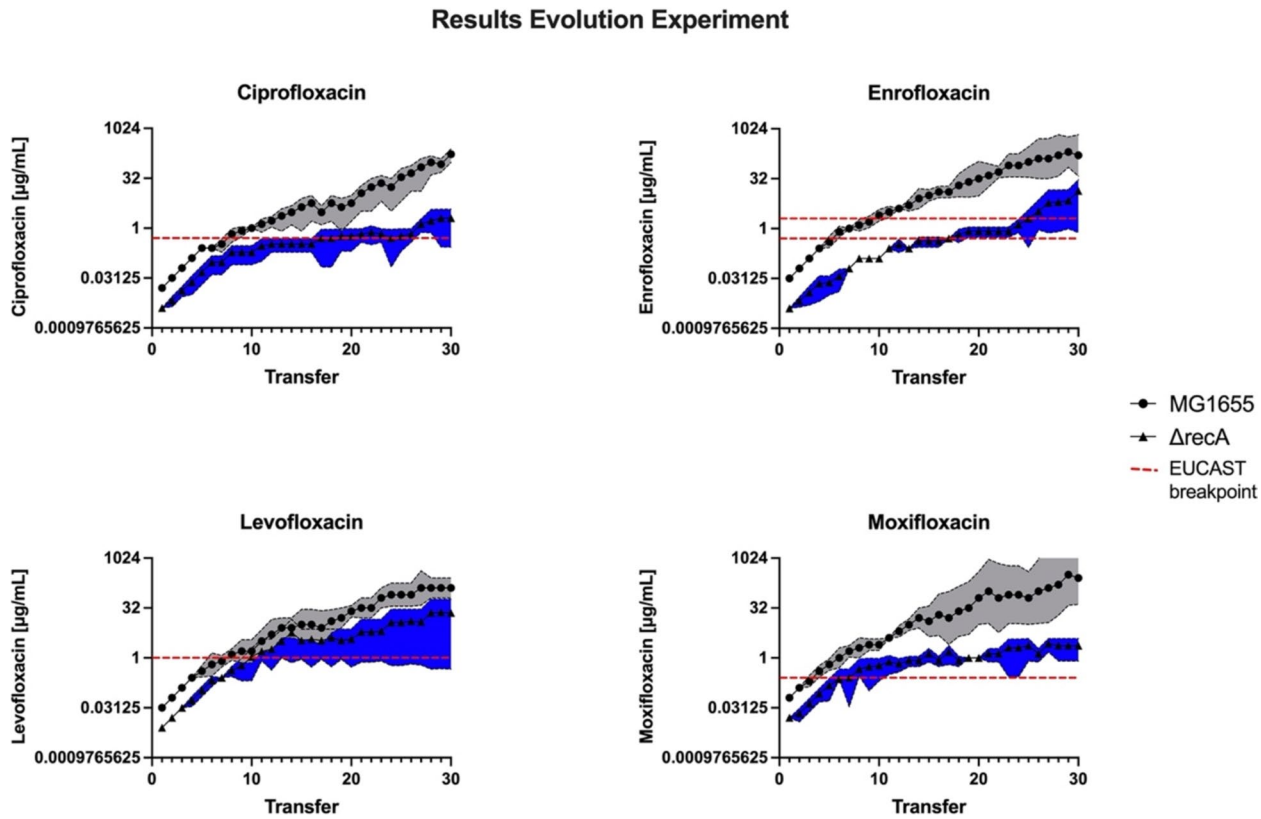

Fig. 1 Results Evolution Experiment. Adaptation patterns of the wild-type and ΔrecA cultures. The shaded areas indicate the standard deviation between the three biological replicates. The EUCAST breakpoints for ciprofloxacin, levofloxacin and moxifloxacin can be found in the supplementary data (Table S1). As enrofloxacin is primarily used for veterinary purposes, the CLSI guidelines values apply for *Horses and **Poultry

of 4 µg/mL. Yet, this final concentration was considerably lower than the final concentrations of the wild-type replicates, i.e. 128 µg/mL and 256 µg/mL of ciprofloxacin. For levofloxacin, the difference between the adaptation rates between the wildtype and the mutant was less statistically significant ($p = 0.077$). A detailed account of the concentrations at each transfer cycle is available in the supplementary data (Table S3 and S4).

Different fluoroquinolones result in distinct mutational patterns

To study the influence of the four tested fluoroquinolones on the mutational trajectories of the wild-type and the mutant, the genome of every culture was sequenced before the evolution experiment as well as after 14 and 30 transfer cycles. MG1655 and Δ*recA* both acquired mutations that have commonly been described in fluoroquinolone-resistant strains, i.e. in the genes *gyrA*, *gyrB*, *parC* and *parE* [7, 14, 15], with the highest frequency of mutations in *gyrA*. Both strains also developed mutations that have been described to accelerate antibiotic resistance (*acrR*, and *marR*) [7, 15, 16]. Mutations in these genes were the most prevalent and abundant overall, along with mutations in *soxR* and *dinG*. In addition, both strains gained mutations in genes specific for either the wild-type or the knockout mutant. Genes that were only mutated in one replicate were ignored in this analysis. After this filtering, the specific mutations for the strains occurred in *rpoC*, *mprA*, *eptB*, and *rrlF* for MG1655, and *dinG* and *rbsR* for Δ*recA*, respectively. Mutations in *acrR*, *gyrA*, *gyrB*, and *marR* occurred in both strains before transfer 14. Mutations in *parC* and *parE* were acquired by single replicates before transfer 14 but were more abundant at the end of the experiment (Fig. 2). This early appearance of *gyrA* mutations and the later onset of *parC* mutations is coherent with earlier observations [17, 18]. Single nucleotide polymorphisms (SNPs) were the most common type of mutations observed, with 375 SNP's of which 41 were synonymous.

Distinct patterns could be found at the end of the experiment when examining the individual antibiotic-associated mutations (see network in Fig. 2, and Fig. 3). Whereas no *acrR* mutations could be observed in any of the ciprofloxacin-exposed strains, it was the only

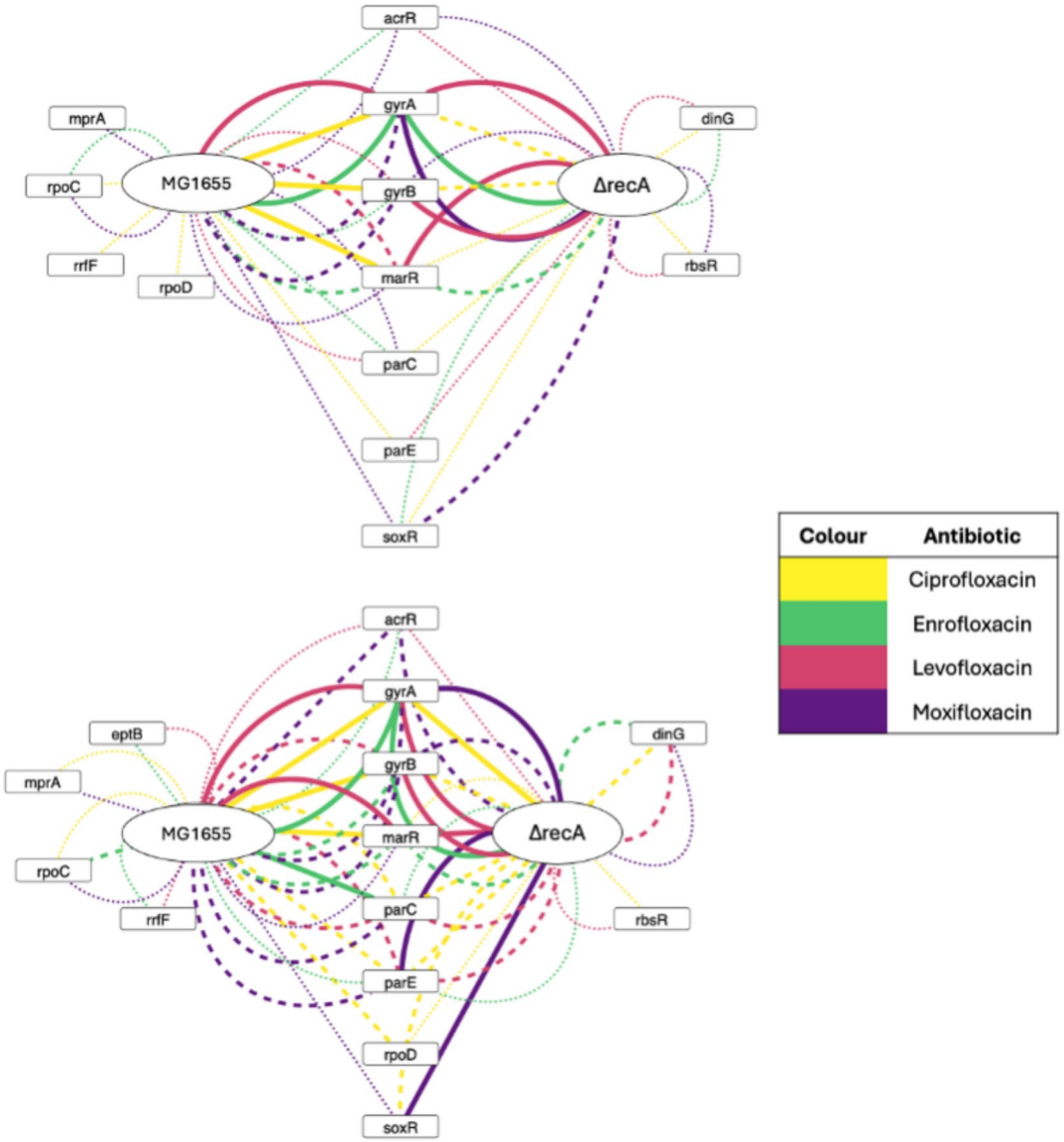

Fig. 2 Network Graph of Antibiotic-Mutation Correlation. Transfer 14 (upper) and Transfer 30 (lower). The weight of the edges corresponds to the number of replicates with a mutation in each gene. A thin dotted line reflects one replicate, a thick dotted line two, and a thick continuous line equals three replicates. Only lines starting and ending in a box are to be considered. Those going below a box and continuing can be ignored for that box

fluoroquinolone that could be associated with *rpoD* mutations. Next to moxifloxacin, it was also the only fluoroquinolone linked to *soxR* mutations. Overall, *soxR* mutations were more abundant in the knockout strains than in the wild-type. Mutations in *gyrB* were the least common in enrofloxacin-exposed strains, with no occurrence in any of the knockout replicates. In contrast, all levofloxacin-exposed Δ*recA* replicates had at least a single mutation in *gyrB*. Exposure to levofloxacin led to mutations in *marR* in all cultures, whereas exposure to moxifloxacin resulted in a mutation in this gene for only one of the wild-type replicates. Adaptation to moxifloxacin was possible for all Δ*recA* replicates without mutations in *parC*. Noteworthily, one of the moxifloxacin-exposed wild-type replicates could adapt to a concentration of 512 µg/mL without any *gyrA*, *parC,* or *parE* mutation.

In the wild-type exposed to the four fluoroquinolones, the evolved strains showed a higher prevalence of D87 than S83 mutations in GyrA (see Table S5). 79% of the strains had a D87 mutation while only 29% of the evolved strains had a mutation in S83. This pattern was

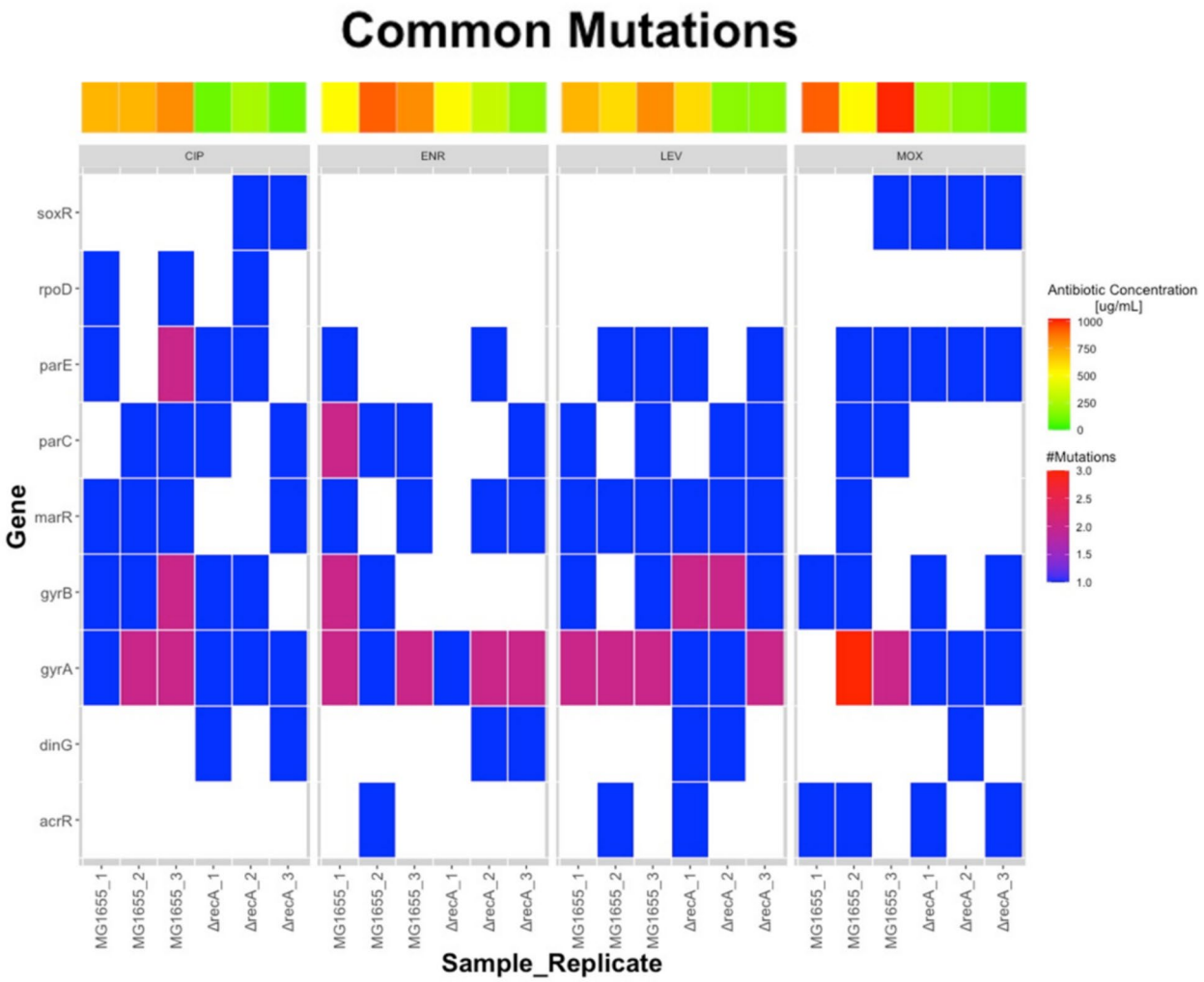

Fig. 3 Frequency of Common Mutations Across Sample Replicates. This heatmap illustrates the frequency of specific genetic mutations across various sample replicates after 30 transfers. Each column represents a sample replicate, while each row corresponds to a gene. The top of the heatmap features a gradient bar displaying antibiotic concentration levels, ranging from low (green) to high (red)

pronounced in the knockout mutant – while 75% of the evolved strains carried a D87 mutation, only a single strain (8%) carried an S83 mutation at the end of the evolution experiment. In addition, 75% of the knockout strains had only a single mutation in GyrA, whereas 83% of the wild-type had two mutations.

Discussion

The observed patterns of mutations associated with the development of resistance varied strongly between the four fluoroquinolones used in this study. Examining the adaptation to fluoroquinolone exposure and its dependency on the SOS response, revealed intriguing dynamics. Notably, both the Δ*recA* knockout and wild-type strains developed clinical levels of resistance most rapidly following moxifloxacin exposure, whereas the slowest adaptation occurred with ciprofloxacin exposure. Furthermore, our analysis revealed that strains exposed to moxifloxacin, enrofloxacin and ciprofloxacin exhibited the strongest reliance on RecA for their adaptation, in stark contrast to those exposed to levofloxacin. These findings suggest that the effectiveness of impairing the SOS response as a strategy to inhibit resistance evolution may depend not only on biological variation but also on the specific fluoroquinolone used. Interestingly, the difference in SOS response induction between different fluoroquinolones is not commonly recognized. Instead, it is often assumed that the cellular response to different antibiotics within the same class is interchangeable [19–25].

Although the differences in the chemical properties of each fluoroquinolone are evident, their impact on cellular uptake and target binding remains unclear. A computational study on quinolone-gyrase binding revealed varying affinities of moxifloxacin, ciprofloxacin, and levofloxacin for gyrase [26]. While the commonly observed S83L mutation in GyrA is known to confer high-level resistance to enrofloxacin, ciprofloxacin and levofloxacin [6, 23], its impact on moxifloxacin resistance appears to be less significant, likely due to moxifloxacin's improved binding to the GyrA protein [26]. Additionally, the study highlighted that mutations at D87 and R121 are more critical binding sites for ciprofloxacin, levofloxacin, and moxifloxacin than the S83 site [26]. Consistent with these findings, the evolved strains showed a higher prevalence of D87 mutations over S83 mutations in the DNA gyrase subunit A (see Table S5), suggesting that the differing binding affinities of these fluoroquinolones lead to distinct impacts on resistance evolution.

The genomic analysis of the resistant cultures revealed distinct mutation profiles when comparing the effects of different fluoroquinolones. We observed fewer mutations in *marR* in moxifloxacin-exposed strains compared to those exposed to other antibiotics, particularly levofloxacin, which induced *marR* mutations in all cultures. The *mar* regulon has been proposed not only to play a role in reduced drug uptake and the regulation of the AcrAB efflux pump but also in the repair of quinolone-induced DNA damage [27–29]. However, it is important to note that changes in *soxS* expression, partially regulated by *soxR*, have been suggested as an alternative to *mar* overexpression in the development of resistance [29]. Our analysis indicates that moxifloxacin exposure results in a higher prevalence of *acrR* and *soxR* mutations compared to the other fluoroquinolones, suggesting a potential shift in AcrAB efflux pump expression in the moxifloxacin-resistant strains via these regulons [30]. Nevertheless, despite this, efflux might not be the primary resistance mechanism in moxifloxacin-resistant cultures, likely due to the chemical complexity and hydrophobic properties of the antibiotic [31].

Adaptation to enrofloxacin was associated with a lower frequency of *gyrB* and *parE* mutations compared to the other fluoroquinolones tested. This observation is particularly interesting given the chemical similarity between enrofloxacin and ciprofloxacin. Despite this, enrofloxacin appears to have a distinct binding affinity and resistance profile compared to ciprofloxacin. The two fluoroquinolones might interact differently with their target enzymes, but further investigation into their unique mechanisms is required. Furthermore, a special case was observed: one wild-type strain exposed to moxifloxacin achieved adaptation to high antibiotic concentrations without acquiring a *gyrA* mutation. These findings suggest that high-level fluoroquinolone resistance can develop without the typically observed *gyrA* mutations, indicating alternative mechanisms or pathways activated that need to be further studied.

The biological variability observed among our replicates could partially be attributed to specific single nucleotide polymorphisms (SNPs). For instance, one of the three moxifloxacin-exposed wild-type replicates acquired an S80I mutation in ParC. This replicate was only able to reach a concentration of 32 µg/mL, compared to the corresponding replicates, which reached 1024 and 512 µg/mL. This S80I amino acid substitution in ParC increases mutation rates in *E. coli* under moxifloxacin exposure by 1300-fold [32], potentially leading to a reduction in population fitness due to the accumulation of deleterious mutations. The fitness effects are intricate, and the effects of single mutations cannot be predicted. For example, the S80I mutation in ParC is fitness-neutral [16]. In combination with the GyrA S83L mutation, it is overrepresented in strains that have evolved fluoroquinolone resistance, suggesting a selective advantage [33]. Once a set of mutations in quinolone resistance determining regions (QRDR) has occurred, the overall fitness costs can be minimal, while still conferring high levels of resistance [34]. The exact set of mutations in the QRDR that gives the optimal combination of fitness and resistance depends on the genetic background of the strains [35].

The abundance of cells with lower fitness in a population can be further diminished by passage through strong bottlenecks (Muller's ratchet) [36–38] or by driving out the most adapted genotype as the population becomes overwhelmed with the mutational backflow [39]. While a higher mutation rate can be beneficial for acquiring resistance through multiple mutations, as is necessary for high-level fluoroquinolone resistance [40, 41], it remains unclear at what point this benefit becomes a disadvantage. Indeed, in natural *E. coli* isolates, weak mutators have been found to have the highest levels of antibiotic resistance [42].

It is noteworthy that Schedletzky and co-workers reported different mutation rates associated with the S80I mutation in ParC, depending on the antibiotic used [32]. Ciprofloxacin exposure resulted in a lower mutation rate increase (40-fold compared to the 1300-fold increase observed during moxifloxacin exposure), which may have had a less detrimental effect on the population's fitness. This finding aligns well with our observed phenotypes. For instance, the wild-type strain that acquired this mutation could only reach a concentration of 32 µg/mL under moxifloxacin exposure, while another wild-type replicate with the same mutation adapted to

a levofloxacin concentration of 256 µg/mL, the highest observed among the replicates. Moreover, moxifloxacin has a greater impact on the growth rate at sub-MIC levels than ciprofloxacin [43], yet it induces the SOS response to a lesser extent [44]. This observation can be explained by the increased SOS response at higher metabolic rates, driven by the elevated ROS levels in faster-metabolizing cells [45].

The mutations identified in this study can be assumed to play vital roles in the development of fluoroquinolone resistance, yet it is important to acknowledge that these mutations may not act independently. The interaction between mutations, known as epistasis, can significantly shape the resistance phenotype [46]. The presence of one mutation may influence the effect of another, leading to non-linear evolutionary trajectories that affect the adaptability and fitness of bacterial populations. Furthermore, the resistance phenotypes observed in this study are likely influenced not only by the specific mutations but also by the broader genetic background of the bacterial strains and the conditions under which the experiments were performed. The impact of a particular mutation can vary significantly depending on the surrounding genomic context, potentially leading to different resistance levels in different strains or under varying environmental stresses.

In addition to directly contributing to resistance, some of the mutations identified in this study may play compensatory roles by modifying the fitness costs associated with fluoroquinolone resistance [47]. Mutations in regulatory genes, for instance, can exert broad effects on gene expression and cellular pathways, potentially rebalancing cellular metabolism that might otherwise be impaired by resistance mutations [47]. These compensatory mutations are crucial for maintaining bacterial fitness in the presence of antibiotics, allowing the organism to sustain growth and survival under selective pressure. Furthermore, they play a crucial role in the emergence of resistance. Without these compensatory changes, resistant cultures would be outcompeted by more fit, susceptible populations in antibiotic-free environments [48].

All strains adapted to some extent to the fluoroquinolones, regardless of whether they possessed a functional *recA* gene. However, it remains unclear whether this adaptation occurred due to RecA-independent activation of the SOS response or through the activation of other pathways. Although ciprofloxacin exposure appears to downregulate the mismatch repair system [49], inactivation of this system alone is not sufficient to acquire ciprofloxacin-resistance-inducing mutations if the SOS response is impaired [50]. It has been proposed that parts of the SOS response can be activated independently of the LexA/RecA-regulon upon beta-lactam exposure, leading to resistance-inducing mutations through the upregulation of *dinB*, an error-prone DNA polymerase that is part of the SOS response [13]. However, our observations indicate that a *dinB* knockout mutant can adapt to amoxicillin and enrofloxacin at nearly the same rate as a wild-type *E. coli* (Teichmann et al., in preparation), suggesting that this DNA polymerase is not essential for adaptation.

Some genes involved in DNA replication and DNA damage tolerance can be activated independently of RecA and LexA under DNA damage. These include genes like *dnaA*, *dnaN*, *nrdAB*, and *iraD*, which are regulated by DnaA in its ATP-bound form, often acting as a repressor [51]. Mechanisms like RIDA (regulatory inactivation of DnaA) might underlie this LexA-independent response, highlighting DnaA's broader regulatory role in managing DNA damage independently of the classic SOS pathway [51]. Furthermore, it has to be noted that some SOS response genes are co-regulated with other stress response systems, such as the stringent response and RpoS-mediated pathways [52, 53].

Alternative adaptive mechanisms, such as adaptive amplification and amplification-mutagenesis, may play a role in fluoroquinolone resistance development [54]. Adaptive amplification involves the temporary increase in gene or chromosomal region copy numbers, which can enhance the expression of genes that confer a survival advantage under selective pressures, such as antibiotic exposure [55]. This mechanism can increase the likelihood of beneficial mutations occurring in these amplified regions. In amplification-mutagenesis, the temporary gene amplification is coupled with mutation, allowing bacteria to experiment with various genetic variations in a short time [56]. If one of these mutations is advantageous, it may become fixed in the genome, while redundant or harmful mutations may be lost as the amplification returns to normal. Together, these mechanisms provide a rapid and flexible way for bacteria to adapt to stressful environments, potentially bypassing reliance on typical mutation pathways like those governed by the SOS response. This alternative adaptability may contribute significantly to fluoroquinolone resistance even in the absence of typical mutations in key SOS-regulated genes.

Conclusion

This study sheds light on the complex dynamics of bacterial adaptation to fluoroquinolones, highlighting the critical role of the SOS response and the unique mutational trajectories linked with different antibiotics within this class. Our findings demonstrate that the pathways leading to resistance are not uniform across all fluoroquinolones; instead, they vary depending on the specific

drug. The differential reliance on the *recA* gene and the distinct mutation profiles observed across fluoroquinolone-exposed cultures emphasizes the necessity of a tailored approach when studying antibiotic resistance mechanisms. This suggests that studies relying predominantly on ciprofloxacin, for example, may not fully capture the broader range of responses provoked by the entire class of fluoroquinolones.

These insights have profound implications for clinical practice and antibiotic stewardship. The variability in the resistance evolution pathways suggests that strategies designed to curb the development of resistance must be tailored to the specific fluoroquinolone in use. Moreover, recognizing distinct mutational profiles associated with different fluoroquinolones can inform more effective screening and monitoring of resistant isolates, potentially leading to improved therapeutic outcomes.

It is important to acknowledge that resistance mechanisms can vary substantially across different bacterial species. The genetic and physiological differences between species may influence how they acquire and express resistance. For example, variations in cell wall structure, efflux pump systems, and DNA repair pathways could result in different evolutionary responses to fluoroquinolones. Thus, while our results provide valuable insights into resistance mechanisms in *E. coli*, further research is necessary to evaluate whether similar patterns of mutation and adaptation occur in other clinically relevant bacteria.

In summary, our research highlights the importance of considering the unique properties of each fluoroquinolone when designing studies and implementing clinical practices. To develop more targeted and effective strategies for combating antibiotic resistance these differences should be explored, particularly in the context of SOS response-independent pathways.

Materials and methods

Strains, growth conditions, and antimicrobial agents

E. coli strain MG1655 was used as the wild-type strain [57]. The single gene knockout mutant JW2669 (ΔrecA636::kan) was chosen from the KEIO collection and ordered from Horizon Discovery Ltd (https://horizondiscovery.com) [58]. The ΔrecA was used because it is deficient in the SOS response. mutant was used The knockout mutant contained a resistance cassette with flanking FLP recognition elements which was removed using the pCP20 method before starting the experiments [59]. The MG1655 strain is widely used as "standard" E. coli but the KEIO parent strain is the BW25113. Both are derived from the W1485 strain background and are therefore closely related. We have performed extensive preliminary physiological control experiments comparing these strains, including growth rate and fitness tests, in minimal and rich medium and performed WGS on both strains. Both the physiological and the genetic differences were negligible. The only major difference was probably the removal of the recA gene, which could have secondary effects. Overall, we are confident that the comparison reported here of experiments using these strains is valid.

Liquid or solid lysogeny broth (LB) (10 g/L NaCl) was used to culture the bacteria. Both strains were grown in 5 mL LB with a starting OD_{600} of 0.1 at 37 °C and 200 rpm overnight. For weekend incubations the starting OD_{600} was reduced to 0.01 and the incubation temperature set to 30 °C to prevent cells from dying prematurely. Fluoroquinolone stocks were purchased from Sigma Aldrich and stored after preparation (10 mM) and filter sterilization either at 4 °C for up to one week or at -20 °C for a maximum of a month.

Minimum inhibitory concentrations (MIC)

MICs were measured twice a week in duplicate for each strain by the broth microdilution method described in [60]. In brief, each strain was inoculated into the wells with a starting OD_{600} of 0.05. Readings were performed in a microtiter plate reader every 10 min with 5 min shaking intervals. The MIC was set to the minimal concentration of the antibiotic which inhibited bacterial growth to an $OD < 0.2$ after 24 h incubation.

Evolution experiment

Evolution experiments were performed as described previously [61]. At the beginning of the experiment, the MICs of both strains were determined. The starting concentration for each combination of strain and antibiotic equalled ¼ of the MIC for the specific strain and can be found in the supplementary data (Table S4). Cultures were initiated with an OD600 of 0.1, meaning the volume of culture transferred each day was adjusted based on the overnight OD of each specific culture, to start each incubation with the same number of cells. After overnight incubation, the OD_{600} was measured. If the value was above 65% of the OD_{600} of the previous culture, the culture was considered to have adapted to the antibiotic, and the antimicrobial concentration was increased twofold in fresh medium for the next incubation. If the yield was below this threshold, the culture was transferred to a fresh medium with the same antimicrobial concentration as used the previous day. In parallel, each bacterial strain was cultured without antibiotic exposure serving as biological controls. Three biological replicates were performed for each condition. The experiments were stopped after 30 transfers. Differences between the adaptation curves were tested using the Wilcoxon rank sum test in R (version 4.4.2), comparing the differences

between the wildtype and the Δ*recA* strain normalized for the difference in starting MIC between the strains. Differences between wildtype and mutants in terms of average transfers until clinical resistance levels were tested for using a generalized linear model in R, assuming normal distributions.

DNA isolation and whole genome sequencing

The DNA of each culture was isolated at the start, after 14 transfers, and at the end of the evolution experiment (30 transfers). After pelleting, phenol–chloroform-isoamyl alcohol (PCI) (Carl Roth) isolation was used to extract the DNA from the whole population. Genomic DNA libraries were created according to the manufacturer's protocol using NEBNext Ultra II FS DNA Library Prep kit for Illumina (New England BioLabs) in combination with NEBNext multiplex oligos for Illumina (96 Unique Dual Index Primer Pairs; New England BioLabs). The samples were sequenced using a NextSeq 500/550 Mid Output V2.5 kit (Illumina).

Bioinformatic analysis

The bioinformatics analysis pipeline involved several steps to assess the quality of the sequencing data, trim low-quality reads, align the reads to the reference genome, and perform variant calling. First, the quality of the raw reads was evaluated using FastQC v0.11.9 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and MultiQC v1.11 [62]. Next, Trim Galore was used to remove low-quality bases and adapter sequences from the reads (https://github.com/FelixKrueger/TrimGalore). The Trim Galore analysis was performed using v0.6.7 and cutadapt v1.18 [63] with paired-end trimming and settings –phred33 -e 0.1 –quality 28. Afterwards, MultiQC v1.13 [62] was used to create a combined quality report for all samples. The adapter sequence 5' – AGATCGGAAGAG C—3' corresponding to Illumina TruSeq libraries was auto-detected. The maximum allowed error rate was set to the default value of 0.1 during adapter trimming. Additionally, a minimum required adapter overlap of 1 base pair and a minimum sequence length of 20 base pairs for both reads were enforced to ensure stringent trimming criteria. The trimmed reads were then aligned to the reference genome using Snippy v4.6.0, a tool specifically designed for bacterial variant calling from short-read sequencing data (https://github.com/tseemann/snippy). U00096 *Escherichia coli* K-12 sub-strain MG1655, and NZ_CP009273 *Escherichia coli* BW25113, also derived from K-12, were used as reference genomes. The sequences were obtained from the NCBI database. Finally, variant calling was performed using Snippy to identify single nucleotide polymorphisms (SNPs) and small insertions/deletions (indels) in comparison to the reference genome. The final data analysis and visualization were performed in Microsoft Excel, R 4.4.1 and Cytoscape_v3.10.2. Upon comparison with the reference genomes U00096 and NZ_CP009273, we found that both our susceptible *E. coli* strains exhibited notable genetic variations that were considered for further analysis in the framework of the cellular adaptations.

Abbreviations

LB	Lysogeny broth
MIC	Minimum inhibitory concentration
PCI	Phenol-chloroform-isoamyl alcohol
QRDR	Quinolone resistance determining regions
RIDA	Regulatory inactivation of DnaA
SNPs	Single nucleotide polymorphisms
ssDNA	Single-stranded DNA

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12866-025-03771-5.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

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Authors' contributions

LT performed research and wrote the manuscript. Sam Luitwieler carried out experiments and assisted with the analysis. JB-P supervised the bioinformatic analysis and assisted writing. BtK helped design experiments, supervised the project, co-wrote the manuscript and functioned as principal investigator.

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Data availability

Whole genome sequencing data have been deposited in the Sequencing Read Archive under BioProject ID PRJNA1163877 and can be accessed at https://eur04.safelinks.protection.outlook.com/?url=http%3A%2F%2Fwww.ncbi.nlm.nih.gov%2Fbioproject%2F1163877&data=05%7C02%7Cb.h.terkuile%40uva.nl%7Cf38d92a52fcc4ba4599f08dd2929cac6%7Ca0f1cacd618c4403b94576fb3d6874e5%7C0%7C0%7C638711981686822531%7CUnknown%7CTWFpbGZsb3d8eyJFbXB0eU1hcGkiOnRydWUsIlYiOiIwLjAuMDAwMCIsIlAiOiJXaW4zMiIsIkFOIjoiTWFpbCIsIldUIjoyfQ%3D%3D%7C0%7C%7C%7C&sdata=tOYYRHjrRSGYobnBcVe3TpX13Cu1hRUZHcn154Nvdx0%3D&reserved=0. Detailed results from the evolution experiments are provided in the supplementary materials. All materials and strains are available from the corresponding author upon request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests
The authors declare no competing interests.

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