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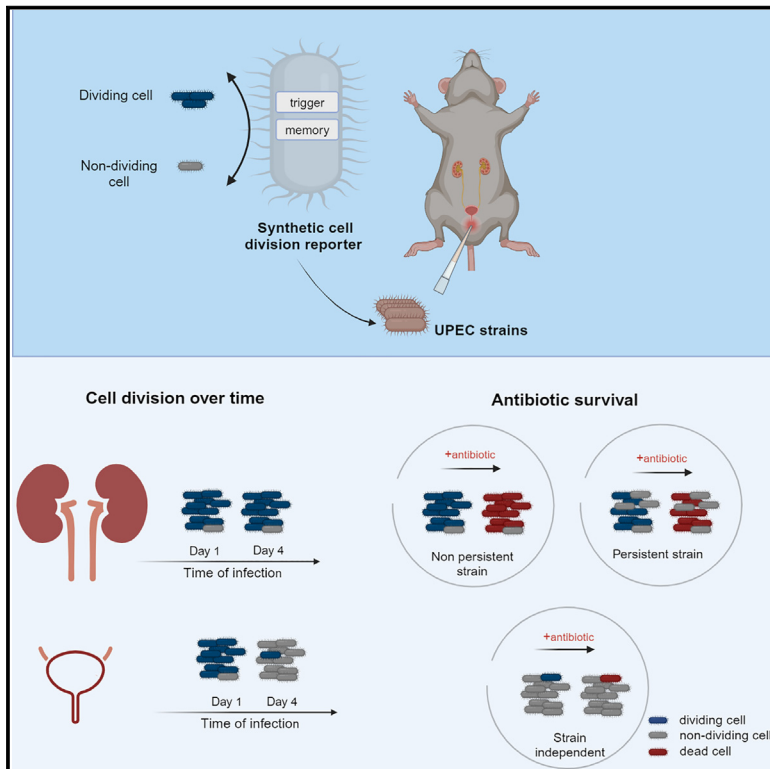


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Cell Host & Microbe

Variability in cell division among anatomical sites shapes *Escherichia coli* antibiotic survival in a urinary tract infection mouse model

Graphical abstract



Authors

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In brief

Amoura et al. assess bacterial division in a UTI mouse model at three sites: the urine, bladder, and kidneys over the course of infection. Using clinical UPEC strains, they dissect the interplay between the infection microenvironment and how it can strongly impact bacterial cell division and survival *in vivo*.

Highlights

- Dividing vs. non-dividing *E. coli* cells are quantified during *in vivo* UTI
- *E. coli* cell's replicative status is distinct between kidneys and bladder
- Non-dividing cells are enriched after antibiotic treatment in kidneys and bladder
- Strain-specific antibiotic persistence *in vitro* correlates with survival in kidneys

Article

Variability in cell division among anatomical sites shapes *Escherichia coli* antibiotic survival in a urinary tract infection mouse model

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SUMMARY

Urinary tract infection (UTI), mainly caused by *Escherichia coli*, are frequent and have a recurrent nature even after antibiotic treatment. Potential bacterial escape mechanisms include growth defects, but probing bacterial division *in vivo* and establishing its relation to the antibiotic response remain challenging. Using a synthetic reporter of cell division, we follow the temporal dynamics of cell division for different *E. coli* clinical strains in a UTI mouse model with and without antibiotics. We show that more bacteria are actively dividing in the kidneys and urine compared with the bladder. Bacteria that survive antibiotic treatment are consistently non-dividing in three sites of infection. Additionally, we demonstrate how both the strain *in vitro* persistence profile and the microenvironment impact infection and treatment dynamics. Understanding the relative contribution of the host environment, growth heterogeneity, non-dividing bacteria, and antibiotic persistence is crucial to improve therapies for recurrent infections.

INTRODUCTION

Bacterial cells within an isogenic population are phenotypically different. This heterogeneity, stochastic or induced, can allow a fraction of the population to transiently survive the sudden appearance of a stressor, such as an antibiotic.^{1,2} One example of a transient survival mechanism is antibiotic persistence, in which a subset of antibiotic-susceptible bacterial cells enters a dormant drug-tolerant state without the expression of antibiotic resistance genes and thus exhibits a slower rate of elimination.³ This state allows a subpopulation to survive and regrow once antibiotics are no longer present, leading to infection relapse. Persistence has historically been derived from the observation of biphasic killing curves.⁴ Antibiotic tolerance allows a bacterial population to temporarily survive longer without growing in the presence of lethal doses of bactericidal antibiotics. It is characterized by a slower killing rate without a resistance mechanism. Antibiotic persistence and tolerance are tightly linked to bacterial cell physiological state and the environment in which the bacteria are present.^{2,5} Both persistence and tolerance levels can also have molecular determinants.^{6–10} Notably, even without geneti-

cally encoded antibiotic resistance, many bacterial infections persist or relapse. As a result, bacterial persistence, and tolerance, in addition to better understood genetically encoded antibiotic resistance mechanisms, likely play a critical role in recalcitrant infections.^{11–13}

Importantly, the environment plays a role in bacterial physiology and, thus, the outcome of infection and treatment. The environment can induce heterogeneous bacterial cell growth and distinct responses to antibiotics, *in vivo*. For instance, upon macrophage internalization of *Salmonella*, phenotypic heterogeneity of the bacteria is induced within the vacuolar environment, leading to the formation of persisters that form a reservoir. In turn, this reservoir contributes to infection relapse after antibiotic therapy.^{14,15} In bladder epithelial cells and mouse macrophages infected with a uropathogenic *Escherichia coli* (UPEC) strain, different antibiotic treatments killed extracellular UPEC to a greater extent than intracellular bacteria though they were highly membrane permeable.^{16,17} Flow cytometry analysis revealed a marked heterogeneity in the intracellular growth of individual bacteria, suggesting that subpopulations of bacteria reached a state of metabolic dormancy.¹⁷ This suggests that

the host microenvironment of the pathogen can profoundly impact bacterial physiology and thus the outcome of an infection after treatment. A better understanding of how bacteria grow and respond to stress, such as antibiotics, *in vivo*, would be beneficial to better target persistent or tolerant populations and limit relapse or recurrent infection.

Urinary tract infections (UTIs) are a major health challenge, characterized by rampant antimicrobial resistance and recurrence.^{18,19} UTIs affect more than 150 million people, worldwide, and are caused by a range of pathogens including *E. coli*, the most common infectious agent. Upon infection, bacteria ascend the urethra and invade the urothelium causing cystitis, or infection of the bladder. Cystitis can progress to pyelonephritis, an infection of the kidneys, if left untreated and in conditions that favor bacterial colonization of the kidneys.¹⁸ In rare cases, pyelonephritis can lead to bacteremia and urosepsis, which can be fatal.²⁰ Recurrent UTI, with the same founder strain, occurs for many reasons, including host response factors and the persistence of bacteria in the urinary tract.^{16,21} Bacterial physiology in the context of cystitis is better understood than bacterial growth and antibiotic response in the kidneys.²² To support the development of effective therapies, quantitative assessment of spatial and temporal bacterial cell division and heterogeneity *in vivo*, in different anatomical sites, is needed.

In mouse models and in mammalian cell cultures, techniques to assess bacterial growth in organs often use fluorescent-based reporters.^{15,23–25} While these techniques bring an enormous advantage in localizing bacteria within complex tissues, they require special equipment and do not always accurately reflect the state of the bacteria *in vivo*—as fluorescence can persist after bacteria are killed. Bacterial growth rate can also be measured based on plasmid segregation and on the coordination between chromosome replication and cell division, such as differential genome quantification (*ori:ter*).^{22,26} In addition, recent work using d-amino-acid-based probes has been used to assess bacterial division in the gut.²⁷ Importantly, these approaches are largely unable to report a “memory” of an earlier event, primarily due to their continuative nature and require specific treatment of the samples and complex data analysis. To surmount these challenges, we adopted a synthetic biology approach using a genetic toggle switch based on the lambda phage *cl/Cro* system, with a trigger and a memory element.²⁸ Previously, in a model in which mice are implanted with an *E. coli*-infected pin in the femur, leading to chronic infection,² use of this genetic circuit unexpectedly showed that antibiotic treatment does not preferentially eliminate actively dividing bacteria and that non-dividing bacteria were not enriched, as was predicted.²⁹ Thus, the *in vivo* response of bacteria to antibiotics cannot necessarily be predicted by *in vitro* assays. Given that UTI recur with a great frequency and bacteria are subjected to diverse stresses in different compartments of the urinary tract, we used the genetic toggle switch approach to follow the dynamics of infection at multiple anatomical sites in a mouse UTI model treated with antibiotics.

Using this model, we quantified the infection dynamics, replicative state, and impact of three classes of antibiotics (beta-lactams, fluoroquinolones, and fosfomycin) in three different environments, the kidneys, bladder, and urine up to 22 days post-infection. Using several relevant UPEC strains, we found

that the fraction of dividing bacteria in the kidneys and urine was greater than that observed in the bladder. Notably, bacteria that survived antibiotic treatment were consistently non-dividing in all three sites of infection, demonstrating that the microenvironment broadly impacts infection dynamics. We showed that while response to antibiotics in the kidneys correlated with the strain survival *in vitro*, bacterial persistence in the bladder was observed among all of the different strains.

RESULTS

Bacteria replicate in the kidneys and urine, while rapidly becoming non-replicative in the bladder

To follow the division status during UTI, we first used the PAS133 murine strain (B2 phylogroup, sequence type [ST] 998, serotype O2:H6) expressing the genetic circuit (Figure 1A). This circuit is a bistable genetic toggle switch based on the *cl/Cro* element from lambda phage. If a bacterium is dividing upon anhydrotetracycline (ATC) exposure, the *cl* concentration will decrease due to dilution over 3 to 4 cell divisions, permitting expression of LacZ (LacZ+). The LacZ+ phenotype is retained even after ATC is removed, leading to “memory” of bacterial division at the time of ATC exposure. Bacteria that are not dividing when exposed to ATC will remain LacZ-. Of note, in the absence of ATC, there is no expression of LacZ. To add clinical relevance to our data, we constructed the genetic circuit composed of the trigger and memory elements (TM) in two archetypal UPEC strains, CFT073 (B2 phylogroup, ST73, O6:H1) and UTI89 (B2 phylogroup, ST95, O18:H7), pyelonephritis and cystitis isolates, respectively.^{30,31} During active growth, there is a switch from a LacZ- phenotype (non-dividing) to a LacZ+ phenotype (dividing) upon exposure to ATC (Figure 1A). To verify the infectivity of the PAS133 strain and the genetically altered UPEC strains, cohorts of mice were inoculated with a bacterial suspension of 10⁸ colony-forming units (CFUs) of each of the three strains. There was no difference in bacterial counts between mice infected with wild-type CFT073 and CFT073+TM, demonstrating that the circuit does not affect *in vivo* fitness (Figure 1B). We then wondered whether ATC had an effect on bacterial counts *in vivo*. Upon ATC exposure, we observed that bacterial counts were not significantly different in mice treated vs. not treated with ATC at day 7 post-infection (p.i.), allowing us to conclude that at the low doses used *in vivo*, ATC does not have an antibiotic effect (Figure 1C).

To assess the division status of *E. coli* in an ascending pyelonephritis UTI mouse model, 8-week-old female CBA/J mice were intravesically infected as above with 10⁸ CFU of each strain and treated with ATC 0.08 mg intraperitoneally twice on the day prior to sacrifice. We measured total bacterial counts in the kidneys, bladder, and urine and the fraction of dividing/non-dividing bacteria at each site by plating the organs of X-gal plates and enumerating the number of blue and white colonies (Figure 1D). Most of the bacteria in the kidneys and urine were dividing over the course of the infection, up to 22 days, indicating active division of all three strains. By contrast, in PAS133-infected mice, the dividing fraction in the bladders decreased significantly after 24 h p.i., and most bacterial cells were in a non-dividing state (LacZ+ at 24 h p.i. 66% vs. 23% at 48 h p.i., $p = 0.003$) (Figure 1D;

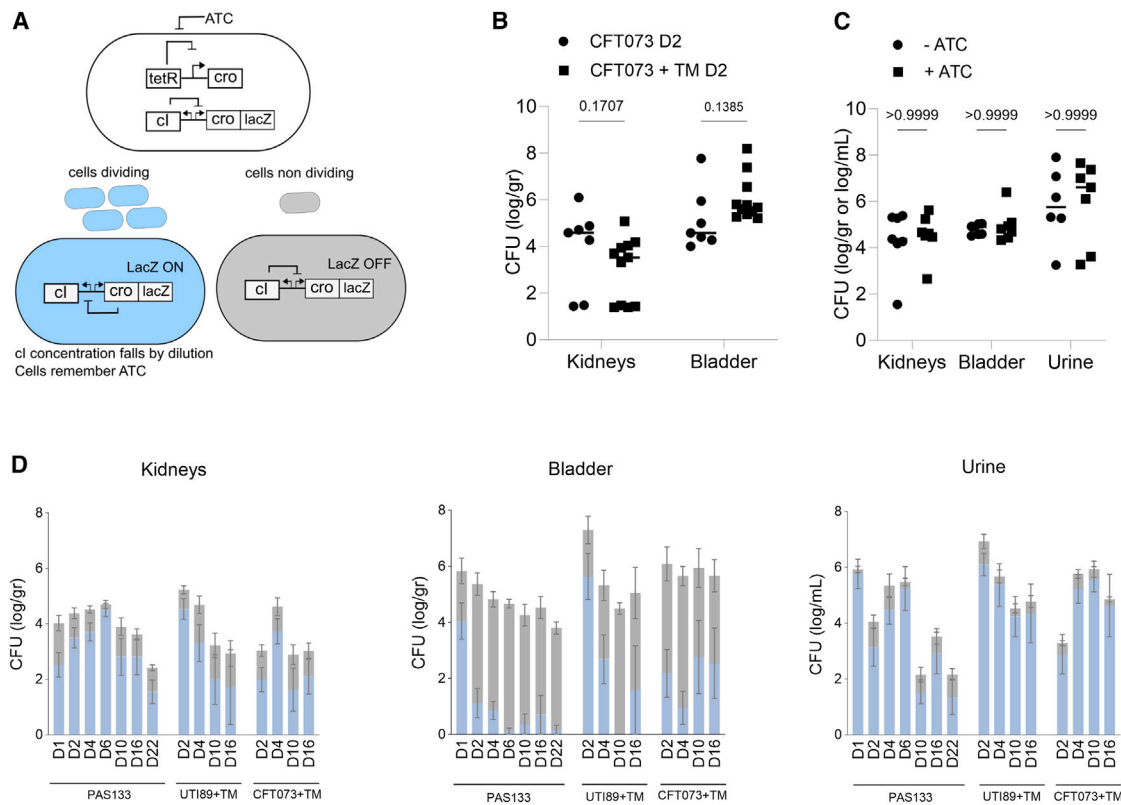


Figure 1. Bacteria actively replicate in the kidneys and urine over the course of a UTI, while rapidly becoming non-dividing in the bladder
(A) Cartoon of the genetic toggle switch and trigger (adapted from Certain et al.²⁵).
(B) Bacterial counts in the kidneys and bladder on day 2 p.i. of mice infected with wild-type (WT) CFT073 or CFT073+TM. Data are pooled from three experiments, $n = 3\text{--}5$ mice per experiment.
(C) Bacterial counts in the kidneys, bladder, and urine on day 7 p.i. of mice infected with PAS133 and treated or not with anhydrotetracycline (ATC) 0.08 mg twice on the day prior to sacrifice. Data are from two experiments, $n = 7$ mice. Each dot represents one mouse; lines are means. Significance was determined using the nonparametric Mann-Whitney test with the Bonferroni method to adjust for multiple comparisons. All p values are shown.
(D) Bacterial counts and dividing fraction in the kidneys, bladder, and urine during the course of a UTI in mice infected with PAS133, UTI89+TM, or CFT073+TM. Bacterial counts are plotted on the ordinate (means and bars are SEM) and within each histogram, the LacZ+ fraction is plotted in blue. Data are pooled from three to eight experiments for each strain, $n = 3\text{--}5$ mice per group per experiment.

Table S1). Similar dynamics of cell division were observed for all three strains PAS133, UTI89+TM, and CFT073+TM.

Antibiotics kill dividing bacteria and enrich non-dividing bacteria in the urinary tract

We next aimed to characterize the responses of the different strains to antibiotics *in vivo* and correlate this response with the cell division status of the cells. We first determined the minimal inhibitory concentrations (MICs) of ciprofloxacin (quinolone), fosfomycin (phosphonic antibiotic), and cefotaxime (beta-lactam) for all three strains *in vitro*. These antibiotics are currently used to treat UTI and act on different cell targets. Cefotaxime is recommended as first-line treatment for acute pyelonephritis requiring hospitalization,²⁰ fosfomycin is a first-line antibiotic recommended for uncomplicated cystitis,^{32,33} and ciprofloxacin is an antibiotic widely used in the treatment of UTI.³⁴ PAS133, UTI89+TM, and CFT073+TM were susceptible to each of these antibiotics (Table 1).

Having established that all three strains were susceptible to these antibiotics, we asked whether the strains would be sus-

ceptible to antibiotic killing *in vivo*. We infected mice with each strain. At 48 h p.i., a control group was sacrificed just before treatment (early control). A cohort of the remaining mice were treated for 24–48 h, depending on the antibiotic,^{35–38} and sacrificed 24 h after antibiotic treatment. A third group of mice, which were infected and not treated with antibiotics, were sacrificed at the end of the treatment (late control) at the same time as the treated groups to control for decreases in bacterial counts due to spontaneous clearance and host immunity (Figure 2A). We measured total bacterial counts and the fraction of dividing/non-dividing bacteria in the kidneys, bladder, and urine.

In mice treated with cefotaxime, we observed a significant decrease in bacterial counts in the kidneys and urine but not in the bladders compared with late controls (Figure 2B–2D; Table S2). Of the surviving bacteria, the majority were non-dividing (Figures 2E; Table S2). In mice treated with fosfomycin, we also observed a significant reduction of bacterial counts in the kidneys and urine but not in the bladder. Similar to cefotaxime-treated mice, a majority of surviving bacteria were non-dividing (Figures 2B–2E; Table S2).

Table 1. Minimal inhibitory concentrations of antibiotics for the PAS133, CFT073+TM, UTI89+TM, NILS 69+TM, and NILS 77+TM *E. coli* strains

| Bacterial strains | Cefotaxime (mg/L) | Fosfomycin (mg/L) | Ciprofloxacin (mg/L) |
|-------------------|-------------------|-------------------|----------------------|
| PAS133 | 0.0625 | 2 | 0.008 |
| CFT073+TM | 0.0625 | 2 | 0.008 |
| UTI89+TM | 0.0625 | 2 | 0.008 |
| NILS 69+TM | 0.25 | 4 | 0.031 |
| NILS 77+TM | 0.0625 | 2 | 0.016 |

The results are expressed as medians. Three replicates were performed for each experiment.

Most antibiotics act on actively dividing bacteria,³⁹ which could explain the heterogeneity in the response and enrichment of non-dividing cells. However, fluoroquinolones, such as ciprofloxacin retain some activity on bacteria in stationary phase^{39,40} (Figure S1). Complicating treatment, part of the UPEC pathogenic cycle is intracellular—which may protect bacteria from antibiotic killing.^{30,41–43} However, ciprofloxacin can penetrate eukaryotic cells.¹⁶ To test whether the variable response to antibiotics observed with cefotaxime and fosfomycin was due to cell division or intracellular status of the bacteria, we used the same infection protocol and treated mice with ciprofloxacin. Despite its ability to penetrate eukaryotic cells, ciprofloxacin treatment did not reduce bacterial counts in the bladder when compared with control mice at the end of the treatment, similar to previously reported results (Figures 2B–2E; Table S2).^{16,17,36}

An explanation for the lack of bacterial clearance after ciprofloxacin treatment could be that we did not reach inhibitory concentrations *in vivo*. We performed a pharmacokinetic analysis of ciprofloxacin *in vivo* in infected mice. 48 h p.i. with the PAS133 strain, we administered a single dose of ciprofloxacin at 2.5 milligram per kilogram (mg/kg) and measured the concentration of the antibiotic in blood, bladder, and kidneys over time. The area under the curve (AUC) of ciprofloxacin concentrations was not different between the kidneys and bladder although the bacterial response to the treatment was different (Figure S3A). However, in the plasma, the AUC/MIC ratio was lower than 125, which is the pharmacokinetic/pharmacodynamic (PK/PD) target predicting fluoroquinolone efficacy.³⁶ Thus, we increased the concentration of ciprofloxacin to 10 mg/kg, which was shown to have an AUC of 10.48 to reach the PK/PD objective of AUC/MIC ratio superior to 125 in Guillard et al.⁴⁴ This dosing regimen provides the same AUC as treatment with 250 mg × 2 per day of ciprofloxacin in humans.⁴⁵ With this higher concentration of ciprofloxacin, we reduced the bacterial count in the bladder by 0.53 log when compared with the late control group ($p = 0.0642$). However, this difference did not appear to be clinically relevant as we never sterilized the bladder, and there was no significant difference in bacterial counts between bladders treated with 2.5 mg/kg ciprofloxacin or 10 mg/kg ciprofloxacin ($p > 0.9999$). By contrast, we did significantly decrease the survival fraction in both the kidneys and the urine with 2/7 and 6/7 sterile kidneys and urine, respectively (Figures 2B–2E; Table S2).

The significant decrease in bacterial counts in the kidneys and urine vs. no response in the bladder after treatment with cipro-

floxacin 10 mg/kg was confirmed when we infected the mice with the two uropathogenic strains CFT073+TM and UTI89+TM (Figure S2; Table S2). The enrichment of the non-dividing fraction among the surviving bacteria was consistent with that observed with the PAS133 strain (Figure S2).

We then wondered whether an earlier treatment, e.g., 24 h after the onset of infection, could clear the infection better, as bacteria in the bladder are still actively dividing at this time. Although bacterial counts in the bladder were reduced by 10-fold when compared with the early control, 24 h p.i. treatment with ciprofloxacin did not significantly reduce the number of bacteria in the bladder compared with the late controls and the bacterial counts were comparable to the treatment at 48 h p.i. (Figure 2F).

Bladder reservoirs are not fully eradicated despite bladder permeabilization and multiple cycles of ciprofloxacin

Since bacteria can reside in endosomes,⁴³ we wondered whether the failure of ciprofloxacin in the bladder could be explained by a decreased activity of this antibiotic at acidic pH. We performed MIC analysis in various pH conditions and found that the MIC of ciprofloxacin increased from 0.008 mg/L at pH 7 to 2 mg/L at pH 5 for the *E. coli* PAS133 strain. Therefore, we treated mice with 10 mg/kg delafloxacin, a quinolone with better activity at acidic pH⁴⁶ and as cell permeable as others quinolones,⁴⁷ which generated pharmacokinetics parameters (AUC and peak concentration) close to those observed in humans at the recommended dose of 300 mg twice daily.^{48,49} Despite bladder concentrations greater than 1,000 times the MIC (Figure S3B), treatment with delafloxacin also failed to reduce bacterial counts in the bladder (Figures 2B–2D).

The bladder mucosa is known to have a very strong permeability barrier. Therefore, antibiotics that are effective against intracellular bacteria in cell culture assays, such as quinolones, may not be effective *in vivo*. To test this hypothesis, we performed an *ex vivo* experiment where we collected UTI89+TM infected bladders from mice treated with ATC for circuit activation. On one hand, infected bladders were placed with 100 × MIC ciprofloxacin or 100 × MIC delafloxacin for 4 h before homogenization and plating. On the other hand, infected bladders were homogenized with Triton X-100 to permeabilize the bladder mucosa and then treated with 100 × MIC ciprofloxacin or 100 × MIC delafloxacin for 4 h before plating. Permeabilization allowed better efficacy of both ciprofloxacin and delafloxacin, confirming the role of the bladder mucosa in antibiotic non-response in the bladder.¹⁶ However, a subset of non-dividing bacteria survived treatment with both quinolones despite permeabilization, confirming the presence of tolerant bacteria in the bladder (Figure 2G). Interestingly, treatment with delafloxacin appeared to be more efficient (although not statistically significant) than ciprofloxacin in both experiments, which could be explained by a better efficacy on stationary phase bacteria (Figure S1).

As women with recurrent UTI often receive several cycles of antibiotic treatment, we wondered whether repeated antibiotic therapy would eradicate *E. coli* in the bladder. To test this, groups of mice received 1, 2, or 3 cycles of 48-h treatment with ciprofloxacin every 5 days. Multiple treatments did not

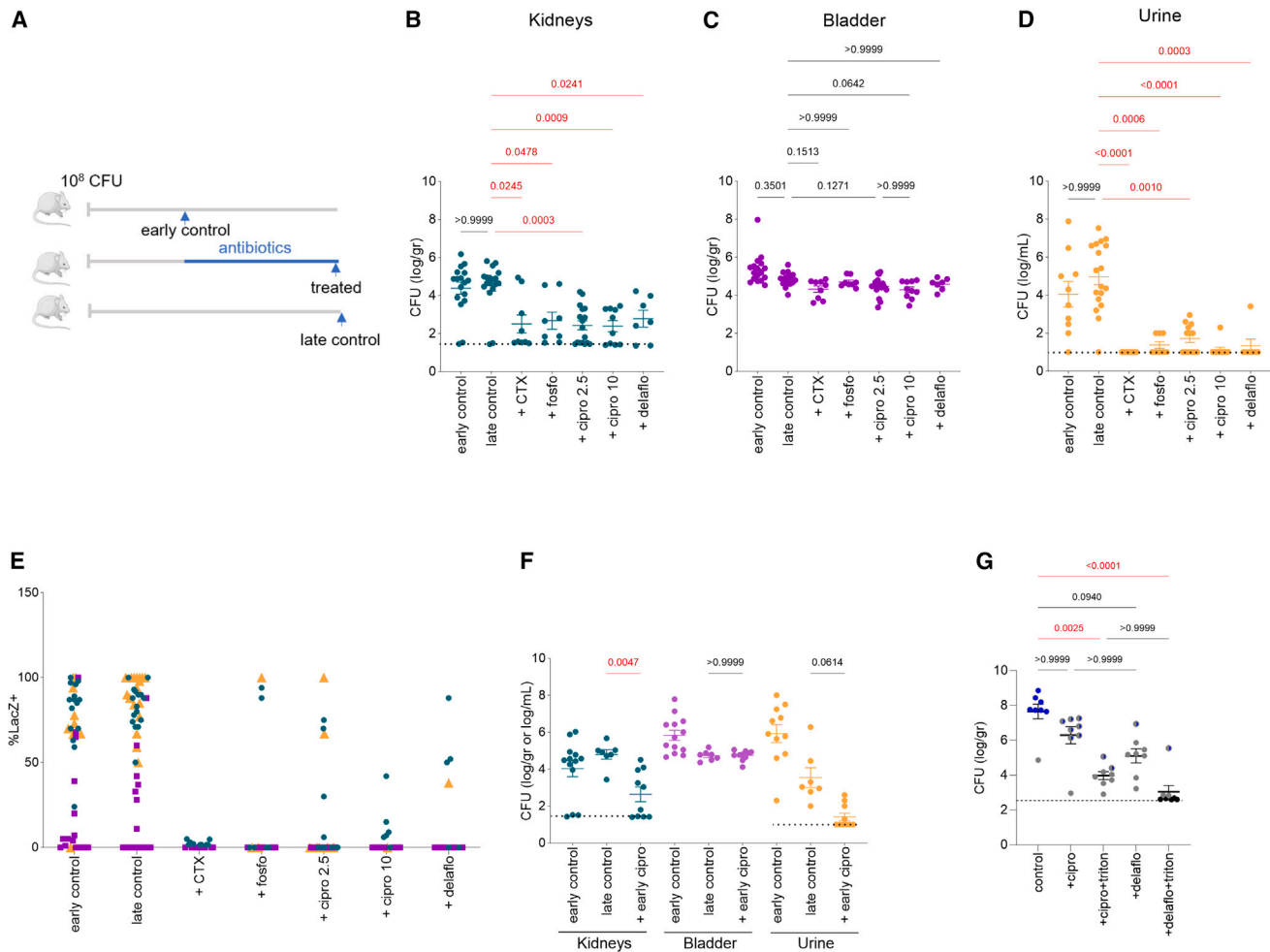


Figure 2. Antibiotics kill actively dividing bacteria in the kidneys and urine but fail to clear the bacteria in the bladder

(A) Experimental scheme used for infections shown in (B)–(D). 8-week-old female CBA/J mice were infected with 10^8 CFU of the PAS133 strain. One group of mice was sacrificed before the start of treatment (early controls), and a second group of mice was sacrificed at the end of antibiotic treatment (late controls). Mice were treated with cefotaxime, fosfomycin, ciprofloxacin, or delafloxacin. Mouse-gray icon by Servier <https://smart.servier.com/> is licensed under CC-BY 3.0 Unported <https://creativecommons.org/licenses/by/3.0/>.

(B–D) Bacterial counts in the kidneys (B), bladder (C), and urine (D) of untreated vs. treated mice. Each dot represents one mouse; lines are means and bars SEM. Black dotted lines represent the limit of detection (1 colony per 100 μ L). Significance was determined using the Kruskal-Wallis test with adjustment for multiple comparisons. All p values are shown; statistically significant p values (<0.05) are in red. Data are pooled from eight experiments, $n = 3$ –8 mice per group per experiment.

(E) Percentage of LacZ+ bacteria in the kidneys, bladder, and urine of control and treated mice. In each non-sterile organ or urine, the number of dividing and non-dividing bacteria was counted and the percentage of LacZ+ bacteria was calculated.

(F) Bacterial counts in the kidneys, bladder, and urine of untreated vs. treated mice. Treatment with ciprofloxacin was started 24 h after infection. Each dot represents one mouse; lines are means and bars SEM. Black dotted lines represent the limit of detection (1 colony per 100 μ L). Significance was determined using the Kruskal-Wallis test with adjustment for multiple comparisons. All p values are shown; statistically significant p values (<0.05) are in red. Data are from three experiments, $n = 3$ –8 mice per group per experiment.

(G) Antibiotic susceptibility in permeabilized bladders. 8-week-old female CBA/J mice were infected with 10^8 of the UT189+TM strain and sacrificed at day 2 p.i. $n = 8$ bladders were homogenized in saline + 0.025% Triton X-100 to permeabilize the bladder urothelium. Initial total CFU including the fraction of dividing cells were determined (control). These homogenates were then treated with ciprofloxacin or delafloxacin for 4 h, and CFUs were determined (+cipro+triton and +delaflo+triton). Separately, 16 ($n = 8$ and $n = 8$) bladders were treated with ciprofloxacin and delafloxacin respectively for 4 h before homogenization (+cipro and +delaflo). Each dot represents one bladder; lines are means and bars SEM. Black dotted lines represent the limit of detection (1 colony per 100 μ L). Significance was determined using the Kruskal-Wallis test with adjustment for multiple comparisons. All p values are shown; statistically significant p values (<0.05) are in red.

Data are from one experiment with 8 mice per group. Blue dots represent LacZ+ fraction $> 75\%$, gray dots LacZ+ fraction $< 25\%$, and mixed dots LacZ+ fraction between 25% and 75%.

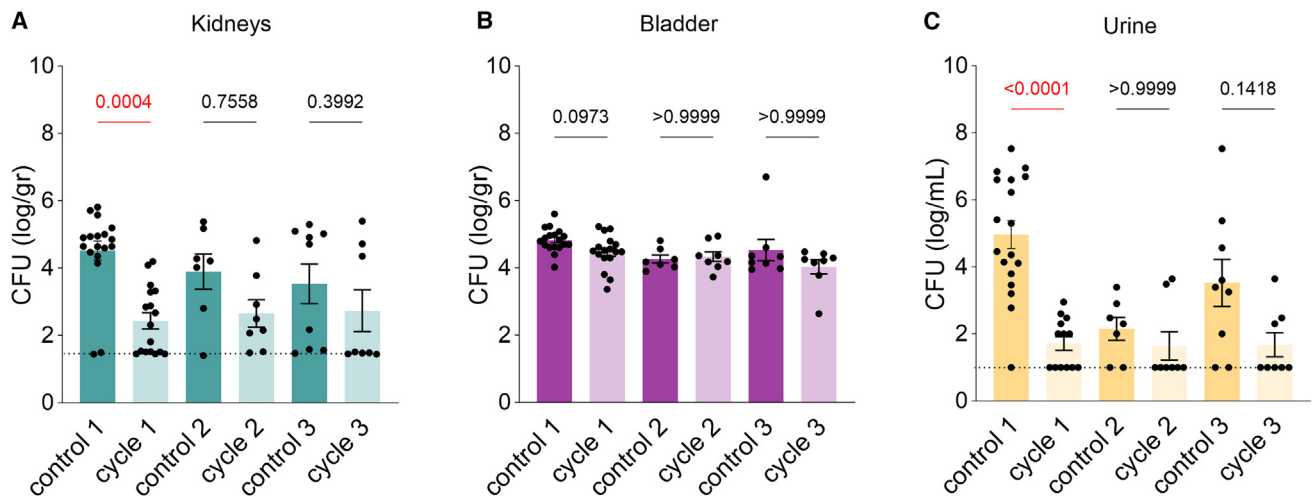


Figure 3. Multiple cycles of ciprofloxacin treatment fail to clear the bacteria in the bladder

(A–C) Bacterial counts in the kidneys (A), bladder (B), and urine (C) of untreated (late control) vs. multiple cycles of ciprofloxacin-treated mice. 8-week-old female CBA/J mice were infected with 10^8 CFU of the PAS133 strain. Treated mice were given ciprofloxacin 2.5 mg/kg subcutaneously twice daily for 48 h starting 48 h after the onset of infection. Treatment was repeated every 5 days such that mice received 1, 2, or 3 cycles of 48-h treatment with ciprofloxacin. Each dot represents a mouse. Lines and bars represent means \pm SEM. Black dotted lines represent the limit of detection (1 colony for 100 μ L). Significance was determined using the Kruskal-Wallis test with adjustment for multiple comparisons. All *p* values are shown; statistically significant *p* values (<0.05) are in red. Data are pooled from seven experiments, *n* = 3–8 mice per group per experiment.

significantly reduce bacterial counts in the bladders compared with the control group at the end of each treatment (Figure 3).

Phenotypic and environmental factors influence bacterial survival in the different sites of infection

The reporter of cell division circuit links the replicative status with antibiotic survival. In contrast to previous reports,²⁹ we observed an enrichment for non-dividing bacteria after antibiotic treatment in all treated mice. This observation was consistent in the kidneys, bladder, and urine, for all antibiotics tested even if they belonged to different classes and for all three bacterial strains (Figures 2 and S2). We next asked if bacterial survival resulted from the emergence of resistance. We performed antibiotic susceptibility testing on the surviving bacteria in the presence of the different antibiotics used in this study and on dividing and non-dividing colonies from all three strains. We did not observe genetic resistance to the antibiotics used for treatment in any condition. Antibiotic tolerance and persistence enable bacterial cells to survive lethal antibiotic treatments without an increase in MIC.⁵ They have been overshadowed by the more prominent narrative of antibiotic resistance but represent interesting ways in which bacteria can escape treatments. We also found similar kill dynamics in comparison with the original strains (data not shown).

We naturally asked whether cells could have acquired mutations that led to higher tolerance or persistence profile. To this effect, we performed whole-genome sequencing on surviving colonies from bladder and kidneys of infected mice that underwent multiple ciprofloxacin treatments (3 to 4 cycles) or one course of cefotaxime treatment and untreated mice. We sequenced the genomes of 8 to 15 colonies per organ and after mapping, variant calling, and thorough filtering, we only found 26 mutations. Most of the mutations were low-frequency polymorphisms, and there was no evidence of convergence toward resistance or tolerance/

persistence genes or convergence within organs of a specific mouse or across different mice Table S4).

In vitro replicative status and persistence levels to ciprofloxacin correlate with non-response to antibiotics in the kidneys

Tolerance and persistence can be associated with quiescence and slow or non-growth. As we did not find mutations conferring resistance and persistence/tolerance in the surviving clones, we tested if pre-existing tolerance and persistence observed in clinical isolates correlated with survival *in vivo* in our UTI model. This correlation between persistent strains and treatment failure has been observed in *Pseudomonas* isolates in a mouse model of lung infection.⁵⁰ We chose a UPEC strain, the NILS 69 isolated from patient urine⁵¹ (B2 phylogroup, ST7340, and Oneg:H4), susceptible to ciprofloxacin (Table 1), with a different growth profile as shown in the OD_{600nm} and growth curve differences observed in comparison with the PAS133, CFT073+TM, and UTI89+TM strains (Figure 4A) and with a higher survival *in vitro* as shown by the time-kill curve in Figure 4A. After inserting the circuit into this clinical strain, we infected and treated mice with ciprofloxacin. The proportion of dividing bacteria in the organs of control infected mice were lower compared with the PAS133, CFT073+TM, and UTI89+TM strains (Figure 4B) In addition, treatment with similar doses of ciprofloxacin did not reduce bacterial counts not only in the bladder but also in the kidneys, unlike what was observed with the PAS133, CFT073+TM, and UTI89+TM strains (Figure 4C). Consistent with the other strains, the survivors were essentially non-dividing bacteria (Figure 4D).

We then questioned whether the replicative status is solely responsible for ciprofloxacin survival *in vivo*. For this we chose to focus on the RpoB S531L mutant, selected by plating the UTI89+TM strain on agar plates containing 100 mg/L of rifampicin. This non-lethal mutation in the *rpoB* gene encoding the β

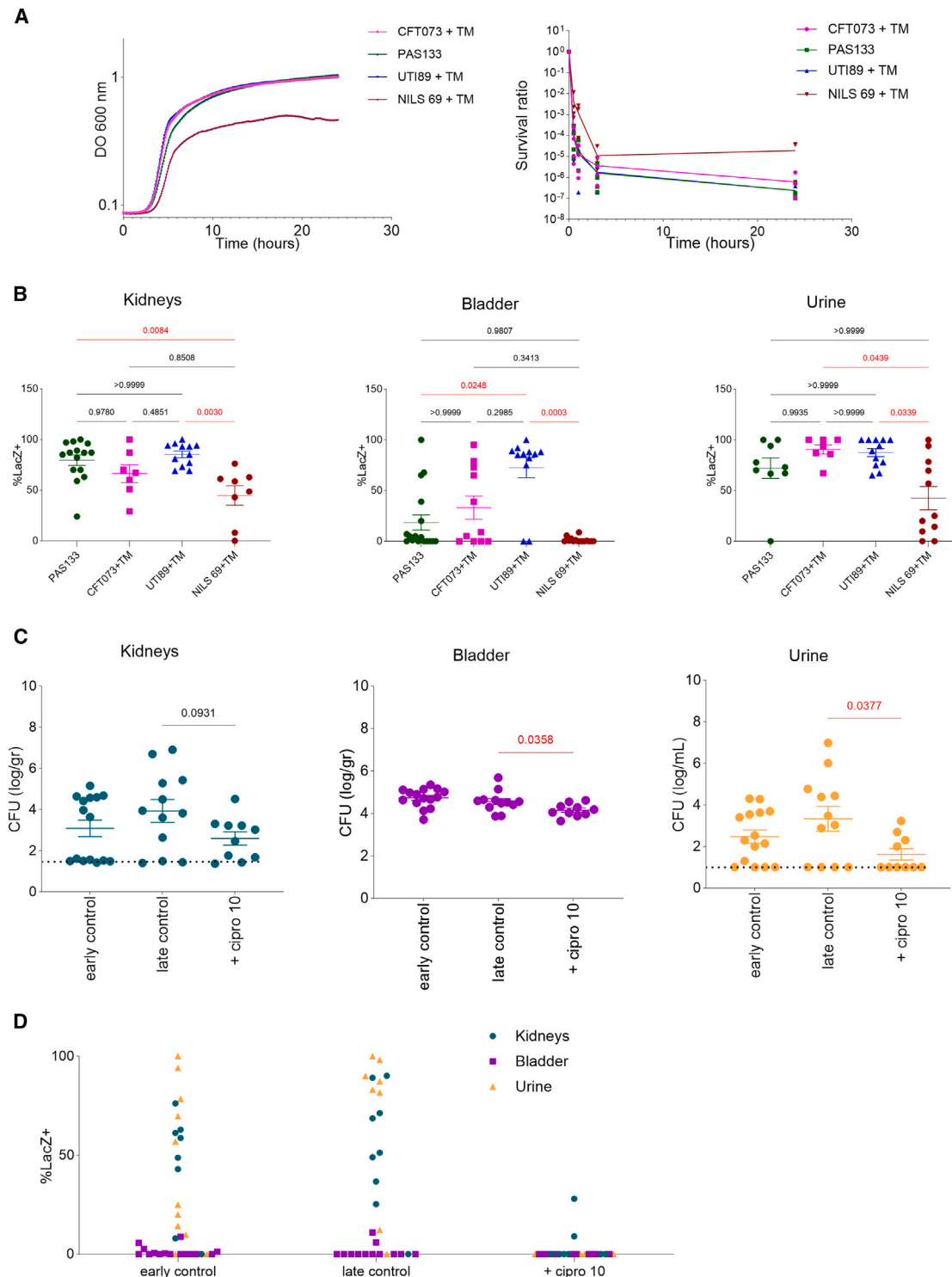


Figure 4. *In vitro* slow division and persistence to ciprofloxacin correlate with non-response to antibiotics in the kidneys

(A) Growth and time-kill curves performed on the CFT073+TM, PAS133, UTI89+TM, and NILS 69+TM strains. Four to six experiments were performed for each condition.

(B) LacZ⁺ fraction in the kidneys, bladder, and urine at day 2 p.i of mice infected with 10⁸ CFU of the PAS133, CFT073+TM, UTI89+TM, or NILS 69+TM strains. Each dot represents one mouse. Lines and bars represent means ± SEM. Significance was determined using the Kruskal-Wallis test with adjustment for multiple comparisons; statistically significant *p* values (<0.05) are in red.

(C) Bacterial counts in the kidneys, bladder, and urine of untreated vs. treated mice. 8-week-old CBA/J mice were infected with 10⁸ CFU of the NILS 69+TM strain. One group of mice was sacrificed before the start of treatment (early controls) and a second group of mice was sacrificed at the end of antibiotic treatment (late

(legend continued on next page)

subunit of bacterial RNA polymerase affected the growth profile of the strain (maximum growth rate 1.161 h^{-1} vs. 1.532 h^{-1} of the UTI 89+TM, $p = 0.0286$) (Figure 5A). The strain was rapidly killed by ciprofloxacin *in vitro* (Figure 5B). Interestingly, after mouse infection, we observed a lower fraction of dividing bacteria, confirming a slower replication rate *in vivo* (Figure 5C). Additionally, ciprofloxacin treatment effectively killed the UTI89+TM RpoB S531L strain in the kidneys (Figure 5D), confirming the absence of effect on persistence that we also observed *in vitro*.

We then wanted to investigate the implication of other genetic mechanisms in *in vivo* survival. We chose a ciprofloxacin-persistent strain isolated from patient with a UTI (NILS 77: B2 phylogroup, ST7344, O158:H34).⁵¹ The NILS 77+TM strain showed increased persistence to ciprofloxacin *in vitro*, and its growth was comparable to UTI89 in Mueller-Hinton (MH) media (Figures 5E and 5F). However, *in vivo*, the TM circuit allowed us to observe a lower fraction of dividing cells in the kidneys (Figure 5G), confirming the necessity of an *in vivo* UTI model, in which complex environments can be studied. Interestingly, higher survival to ciprofloxacin was observed in the kidneys as observed *in vitro* (Figure 5H).

This result shows that both the strain's replicative status and persistence level are important in survival in the kidneys. Finally, in the bladder, despite a statistically significant decrease of survival for the RpoB S531L mutant and NILS 77+TM, the reduction of bacterial loads was modest and comparable (0.4 log for UTI89+TM and NILS 69+TM, 0.5 log for PAS133, 0.7 log for CFT073+TM and NILS 77+TM and 1.2 log for UTI89+TM RpoB S531L) between all the strains (Figures 2, 4, 5, and S2).

Consistent with our previous findings, the surviving bacteria were mostly non-dividing in all sites of infection. Overall, our results emphasize the relevance of cell division and antibiotic persistence *in vivo* in different sites of infection in the context of UTI.

DISCUSSION

Here, we employed synthetic biology techniques using a strain with a division status reporter to track heterogeneity in bacterial division over time. Unlike fluorescent reporters, the circuit we incorporated records the state of division at the moment of exposure to ATC, allowing events to be recorded before the mice are sacrificed and longitudinal monitoring to be performed, particularly in urine.^{28,29} This is critical for analyzing the replicative status of bacteria before, during, and after treatment. We followed the dynamics of bacterial counts and division in the kidneys, urine, and bladder for up to 22 days after infection with and without antibiotic treatment. Heterogeneity in division and antibiotic response varied significantly among the anatomical sites, which influenced bacterial survival. The factors underlying non-response to UTI *in vivo* are crucial to understand, considering the high frequency and recurrent nature of these infections.¹⁸

The study reveals that in the bladder, bacteria primarily undergo active division within the first 24 h but subsequently enter a dormant state. During the initial 24-h period, the rapid replication of bacteria is associated with the formation of intracellular bacterial communities (IBCs).⁴¹ Simultaneously or subsequently, quiescent intracellular bacterial reservoirs are formed in the deeper layers of the urothelium.⁴³ Fluorescence and electron microscopy investigations have identified these dormant reservoirs within late endosomes.⁴³ These intracellular reservoirs are somehow protected from both antibiotics and the immune system. A recent study *in vitro*¹⁷ showed that ciprofloxacin killed 99.9% of extracellular bacteria at concentrations around the MIC, while for intracellular bacteria, concentrations more than 100 times the MIC were required to achieve bactericidal activity. Intracellularly, time-kill curves showed biphasic kinetics suggesting the presence of a drug-tolerant subpopulation. The presence of subpopulations in a state of metabolic dormancy was confirmed by TIMERbac fluorescence. Thus, bacteria within bladder cells are not only mechanically protected from antibiotics, but the intracellular environment also modifies bacterial metabolism, leading to the formation of persisters *in vitro*. These intracellular bacteria reside in the late endosome, which is characterized by an acidic pH (pH 5).⁴³ Ciprofloxacin may have reduced activity in such acidic environments.⁵² However, the *in vitro* use of fleroxacin¹⁷ or the *in vivo* use of delafloxacin in our study, both of which are quinolones designed to be active at acidic pH,⁴⁶ did not result in reduced bacterial counts. This suggests that the non-response to antibiotics is likely multifactorial and cannot be attributed solely to the effect of pH. To target actively dividing bacteria before they enter a dormant state, we tried early treatment with ciprofloxacin. However, this early treatment did not effectively reduce the number of bacteria in the bladder. This finding is consistent with the concept that intracellular reservoir formation occurs early, within the first 24 h of infection, and concomitantly with IBCs, as demonstrated in a bladder organoid model.⁵³ As the bladder mucosal barrier is very strong, we investigated the delafloxacin activity on permeabilized bladders. While we could not fully eradicate bacterial cells, delafloxacin seemed to be a better candidate than ciprofloxacin to kill quiescent non-growing bacteria.

The presence of IBCs and filamentous bacteria in urine samples has been observed in woman with cystitis and a child with recurrent UTI.^{54,55} Intracellular UPEC and other species have also been detected in bladder tissue biopsies from patients with recurrent cystitis,^{56,57} highlighting the accuracy of the mouse model to study UTI.

Longitudinal phylogenetic studies reveal that approximately half of recurrent UTI are due to reinfection with different UPEC strains and half are due to relapse with similar UPEC strains, either from the gut or from persistent infection within the urinary tract.^{58–61} Interestingly, Stracy et al.⁶² showed that recurrences gained resistance in 30% of cases after antibiotic

controls). Mice were treated with ciprofloxacin. Bars represent mean \pm SEM. Black dotted lines represent the limit of detection (1 colony per 100 μL). Lines and bars represent means \pm SEM. Black dotted lines represent the limit of detection (1 colony for 100 μL). Significance was determined using the Kruskal-Wallis test with adjustment for multiple comparisons. All p values are shown; statistically significant p values (<0.05) are in red. Data are pooled from four experiments, $n = 3-8$ mice per group per experiment.

(D) Percentage of LacZ⁺ bacteria in the kidneys, bladder, and urine of control and treated mice. In each non-sterile organ or urine, the number of dividing+ and non-dividing bacteria was counted and the percentage of LacZ⁺ bacteria was calculated.

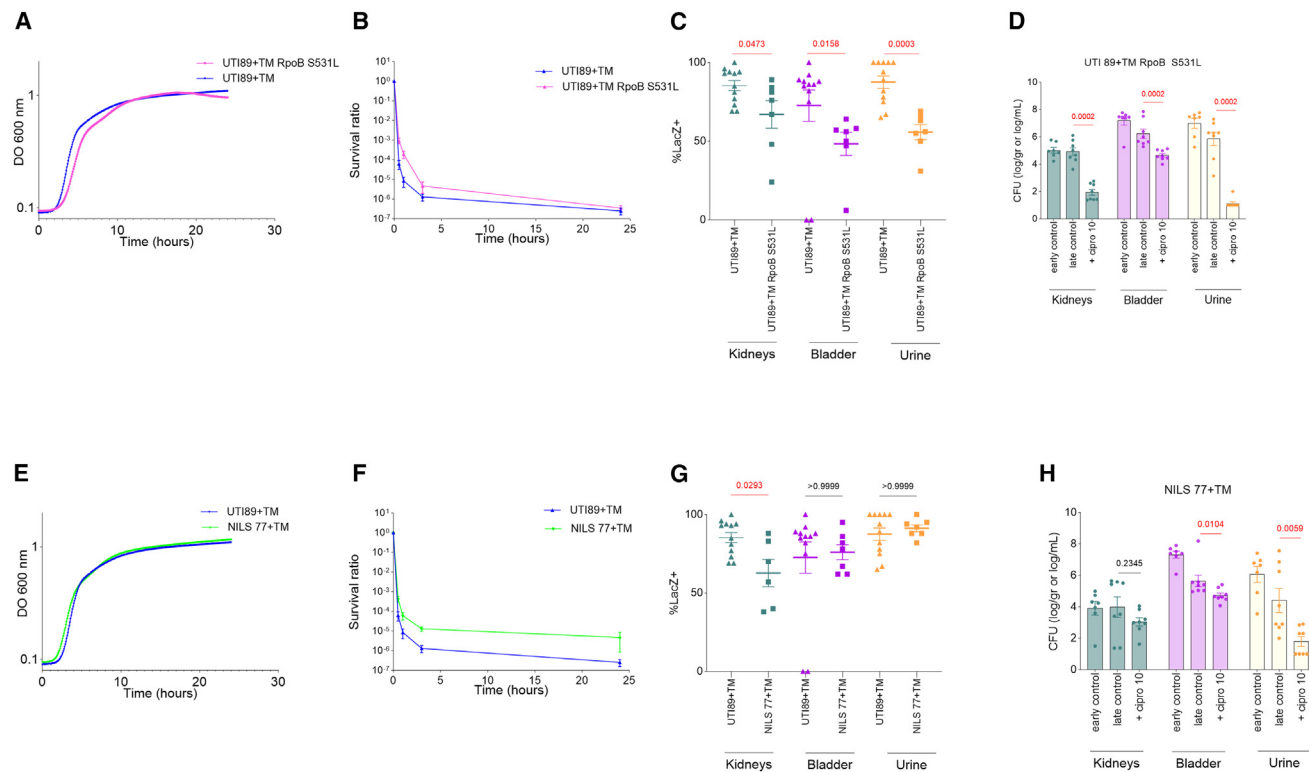


Figure 5. Survival to antibiotics *in vivo* can also be linked to bacterial persistence

(A) Growth curves performed in Mueller-Hinton (MH) media on the UTI89+TM strain and its isogenic mutant, the UTI89+TM RpoB S531L strain. Three experiments were performed for each condition.

(B) Time-kill curves to ciprofloxacin performed on the UTI89+TM and the UTI89+TM RpoB S531L strain in MH media. Four experiments were performed for each condition.

(C) LacZ+ fraction in the kidneys, bladder, and urine at day 2 p.i. of mice infected with 10⁸ CFU of the UTI89+TM or UTI89+TM RpoB S531L. Each dot represents one mouse. Lines and bars represent means \pm SEM. Significance was determined using the Mann-Whitney test. All *p* values are shown; statistically significant *p* values (<0.05) are in red.

(D) Bacterial counts in the kidneys, bladder, and urine of untreated vs. treated mice. 8-week-old CBA/J mice were infected with 10⁸ CFU of the UTI89+TM RpoB S531L strain. One group of mice was sacrificed before the start of treatment (early controls) and a second group of mice was sacrificed at the end of antibiotic treatment (late controls). Mice were treated with ciprofloxacin 10 mg/kg. Bars represent mean \pm SEM. Significance was determined using the Mann-Whitney test. All *p* values are shown; statistically significant *p* values (<0.05) are in red. Data are pooled from two experiments, *n* = 3–5 mice per group per experiment.

(E) Growth curves performed in MH media on the UTI89+TM strain and the NILS 77+TM strain. Three experiments were performed for each condition.

(F) Time-kill curves to ciprofloxacin performed on the UTI89+TM and the NILS 77+TM strain in MH media. Four experiments were performed for each condition.

(G) LacZ+ fraction in the kidneys, bladder, and urine at day 2 p.i. of mice infected with 10⁸ CFU of the UTI89+TM or NILS 77+TM strain. Each dot represents one mouse. Lines and bars represent means \pm SEM. Significance was determined using the Mann-Whitney test. All *p* values are shown; statistically significant *p* values (<0.05) are in red.

(H) Bacterial counts in the kidneys, bladder, and urine of untreated vs. treated mice. 8-week-old CBA/J mice were infected with 10⁸ CFU of the NILS 77+TM strain. One group of mice was sacrificed before the start of treatment (early controls) and a second group of mice was sacrificed at the end of antibiotic treatment (late controls). Mice were treated with ciprofloxacin 10 mg/kg. Bars represent mean \pm SEM. Significance was determined using the Mann-Whitney test. All *p* values are shown; statistically significant *p* values (<0.05) are in red. Data are pooled from two experiments, *n* = 3–5 mice per group per experiment.

treatment and were then due to strain replacement. Cases of reinfections with the same strain remained sensitive to the antibiotic used. Our study confirms that, despite the administration of antibiotics with diverse mechanisms of action and properties, infection persists in the bladder.^{16,36} This persistence is particularly notable because most bacteria in the bladder are in a non-dividing state. Not every case of UTI relapses and known and unknown factors trigger recurrences. For instance, exposure to *Gardenerella vaginalis*, a common member of the vaginal microbial community, induces the emergence of *E. coli* from reservoirs through urothelial exfoliation.⁶³ Importantly, we showed that the non-response observed is not asso-

ciated with the emergence of antibiotic resistance even after multiple cycles of treatment.

Moreover, our observations showed that most bacteria were dividing in the kidneys and urine. We observed an enrichment of non-dividing cells after treatment with antibiotics highlighting the potential role of non-dividing cells in recalcitrant infections. The presence of some bladders with dividing bacteria excludes the fact that ATC did not reach the tissue. The increase in the proportion of non-dividing bacteria raised the suspicion of the emergence of *in vivo* antibiotic tolerance or persistence. Phenotypic tests and sequencing did not show a convergence toward genetic changes leading to resistance, tolerance, or persistence.

Although the number of surviving cells we chose to sequence may be limited, both our phenotypic and genotypic results suggest that bacterial survival in the kidneys and in the bladder is not due to genetic mutations or acquired resistance during the infection but depends largely on the physiological state of the cells in their specific microenvironment. Although we did not examine the interaction between the antibiotic and bacteria at the single-cell level, the significant concentration of ciprofloxacin and delafloxacin detected in the bladder suggests that it likely reached some of the bacteria. Subsequent single-cell analyses and microscopy experiments could be performed to gain a better understanding of the interaction between the antibiotic and bacteria.^{64,65}

The clinical relevance of *in vivo* tolerance, where bacteria exhibit a state of dormancy and are less susceptible to antibiotic treatment, is still a topic of debate. Despite expanding research on the field, most studies are in the context of laboratory strains studied in nutrient rich medium.⁶⁶ However, there is a growing body of evidence showing the importance of considering tolerant or persistent bacteria in the treatment of recurrent infections.^{11,66} For example, in patients with cystic fibrosis, *Pseudomonas aeruginosa* infections are a common occurrence. Over the course the disease and successive antibiotic treatments, patients become infected with more persistent strains.⁶⁷ Similarly, in *Salmonella* sp. infections^{14,68–71} and tuberculosis,²³ tolerant or persistent bacteria are implicated in the development of recurrent infections. Finally, tolerant *E. coli* are common among bloodstream isolates and are associated with an increased risk of reinfection.⁷² In the context of UTI, a screen of library of commensal and UTI *E. coli* isolates from patients identified 23 mutants in the *hipA* gene that conferred a high degree of persistence in these bacteria.⁶ Another study⁷³ found a higher level of tolerance/persistence in clinical isolates from patients with recurrent UTIs compared with those with single episodes of UTI. This suggests that persistent or tolerant bacteria may be more prevalent in recurrent UTI and highlights the need to consider these bacterial populations for effective treatment.

Interestingly, we also integrated the circuit in two clinical strains with high *in vitro* survival (NILS 69 + TM and NILS 77+TM) and showed high survival *in vivo* (Figures 4 and 5). We were also able to transpose the slow growth observed *in vitro* in the *in vivo* UTI model. There was no bacterial clearance in the bladder for all the strains used. However, higher non-dividing fraction and higher survival were observed in the kidneys of treated mice. This highlights the necessity of considering different environments when assessing bacterial response *in vivo* and hints at different mechanisms that bacteria use to survive in different niches. Further studies involving more clinical isolates should be performed to assess the different roles of *in vivo* survival mechanisms in an *in vivo* UTI model.^{50,68} Finally, depending on the location of the bacteria and their means of survival, a number of different treatment strategies could be considered.^{12,74,75}

Overall, this work contributes to the research efforts to use experimental tools enabling a better assessment of antibiotic division and survival in *in vivo* settings. A multitude of phenotypes can be observed *in vitro* but characterizing bacterial responses in complex *in vivo* environments is required to improve the efficacy of drug treatments. Bacterial responses *in vivo* can be influenced by various factors, including the host immune system, mi-

crobial interactions, and the presence of different tissues and cell types. Therefore, it is essential to bridge the gap between laboratory observations and relevant and complex scenarios to develop more effective antibiotic therapies.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.chom.2024.04.015>.

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AUTHOR CONTRIBUTIONS

I.E.M., A.A., A.L., and B.F. outlined the study and designed the experiments. A.A., C.P., S.D., C.C., A.B. and F.C. performed the experiments. E.B. and M.L. performed antibiotic dosages and pk analyses. M.M. performed whole-genome sequencing. A.B.-N. and M.M. performed the whole-genome sequencing analyses. I.E.M., A.A., A.L., B.F., M.A.I., and E.D. interpreted the data. I.E.M. and A.A. wrote the manuscript with help from all the authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|--|-----------------------------------|-----------------|
| Bacterial strains and plasmids | | |
| Bacterial strains | See Table S3 | N/A |
| pKD46 | See Table S3 | N/A |
| Oligonucleotides | | |
| Primers | See Table S3 | N/A |
| Chemicals, peptides, and recombinant proteins | | |
| Mueller-Hinton (MH) broth | Becton, Dickinson Company | 212322 |
| Agar | Invitrogen | 30391023 |
| Ciprofloxacin | Sigma-Aldrich | 17850-5G-F |
| Cefotaxime | Fisher Scientific | BP2951-1 |
| Fosfomycin | Sigma-Aldrich | P5396-5G |
| Glucose 6 Phosphate | Sigma-Aldrich | G7879-1G |
| Anhydrotetracyclin (ATC) | Sigma-Aldrich | 94664 |
| Delafloxacin | Menarini | 19DEL1 |
| Chloramphenicol | Sigma-Aldrich | C0378-25G |
| Kanamycin | Sigma-Aldrich | K1876-5G |
| X-gal | Fisher Scientific | R0941 |
| Serum | Otec | 600502 |
| Triton X100 | Sigma-Aldrich | 9002-93-1 |
| Experimental models: Organisms/strains | | |
| CBA/J (female; 8-10 weeks old) | Janvier labs Charles-River lab | IMSR_JAX:000656 |
| 1.5 mL tubes with beads | MPBio | 6914801C |
| Cefotaxime | Viatrix | ART-8258-02 |
| Fosfomycin | Delbert | 1752120911E01 |
| Delafloxacin | Menarini | 19DEL1 |
| Ciprofloxacin | Sigma-Aldrich | 17850-5G-F |
| Software and algorithms | | |
| GraphPad Prism V10 | GraphPad Software, Inc. | SCR_002798 |
| BioRender | BioRender Software | SCR_018361 |
| BreSeq ⁷⁶ V0.35.0 | N/A | SCR_010810 |
| Unicycler V0.4.8 | Unicycler software | SCR_024380 |

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Imane El Meouche (imane.el-meouche@inserm.fr).

Materials availability

Strains generated in this study are available from the [lead contact](#) upon request.

Data and code availability

Additional information required to re-analyze the data will be available from the [lead contact](#) upon request. The R script used to filter and analyse the sequencing data has been uploaded to this URL: <https://github.com/A-BN/coliMUTI>.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Bacterial strains and plasmids

All the *in vitro* experiments are performed in Mueller-Hinton (MH) broth or MH broth supplemented with agar (BBL Mueller-Hinton II Broth cation adjusted from Becton, Dickinson Compagny). For the *in vitro* experiments, the antibiotics used were the following: ciprofloxacin (Sigma-Aldrich, Saint-Quentin Fallavier, France), cefotaxime (Sigma-Aldrich, Saint-Quentin Fallavier, France), fosfomycin (Sigma-Aldrich, Saint-Quentin Fallavier, France), anhydrotetracyclin-ATC (Sigma-Aldrich, Saint-Quentin Fallavier, France), delafloxacin (Menarini, Rungis, France).

The PAS133 strain is previously published in Kotula et al.²⁸ and Certain et al.²⁹ This strain, an isolated commensal murine *E. coli* strain, has been engineered with a bistable genetic toggle switch based on the *cl/Cro* element from lambda phage. If a bacterium is dividing upon anhydrotetracycline (ATC) exposure, the *cl* concentration will decrease due to dilution over 3 to 4 cell divisions, permitting expression of LacZ (LacZ+).

The LacZ+ phenotype is retained even after ATC is removed, leading to 'memory' of bacterial division at the time of ATC exposure. Bacteria that are not dividing when exposed to ATC will remain LacZ-. The components were constructed by a combination of PCR, commercial synthesis, and overlap extension PCR, then inserted into *E. coli* TB10 by recombineering. The elements were then transferred into this mouse fecal *E. coli* by P1vir transduction. The circuit comprises both a reporter and a trigger element: the reporter element is the *cl/cro* operon inserted upstream of *lacZ*; the trigger element is the *tetA* promoter upstream of a second copy of *cro*. In the "off" state, the *cl* protein is expressed, inhibiting the expression of Cro and LacZ. Exposure to the trigger anhydrotetracycline (ATC) causes an increase in Cro expression, which in turn represses *cl*, ultimately de-repressing Cro and allowing expression of LacZ, thereby changing the cell from the LacZ- to LacZ+ phenotype. The *cl* protein takes about 3-4 cell divisions to be diluted (8- to 16- fold) which results in a decrease from > 99% *cl* functional activity to about 20%–40% activity,⁷⁷ resulting in Cro expression from the *cl/Cro* toggle switch.

To obtain the *E. coli* CFT073 strain + TM,⁷⁸ the trigger and the memory sequences were PCR amplified (see primers sequence in Table S3) and transferred sequentially by electroporation to the CFT073 containing the pKD46 recombineering helper plasmid expressing the λ -Red recombinase.⁷⁹ The insertion and sequence of the two elements (between *araB* and *araC* promoters for the trigger and upstream *lacZ* for the memory) were confirmed by PCR and Sanger sequencing. The same experiment was done with the UT189, NILS 69 and NILS 77. Strains provenance are listed in Table S3.

The UT189+TM RpoB S531L mutant was obtained by plating an overnight culture of UT189+TM strain on 100mg/L rifampicin MH agar plates and the mutant was whole genome sequenced.

Pyelonephritis mouse model

All animals were housed in regulation cages and given free access to water and food. The pyelonephritis protocol (APAFIS #4950 and 39318) was approved by the French Ministry of Research and the ethics committee for animal experiments. 8-week-old female CBA/J mice (Janvier lab, Charles River, RRID:IMSR_JAX:000656) weighing 20 to 22g were used in the experimental model of pyelonephritis previously described.^{80–82}

Bacterial inocula were obtained by overnight incubation in Luria-Bertani (LB) broth followed by centrifugation at 8000 × *g* for 16 min. Pellets were suspended in 1 to 4 mL of sterile saline solution to a final inoculum of 5.10⁹ CFU/mL. Pyelonephritis and cystitis were induced as previously described.^{80,81} After general anesthesia (with an intraperitoneal administration of 100 mg/kg of body weight of ketamine and 10 mg/kg xylazine), 50 μ L (10⁸ CFU) of the different strains (PAS133, CFT073+TM, UT189+TM, NILS 69+TM, UT189+TM RpoB S531L mutant and NILS 77+TM) were instilled into the bladder through a urethral catheter.

METHOD DETAILS

Growth and antibiotic survival *in vitro*

Growth curves were performed in MH media. Bacteria were grown overnight and diluted 1:10 000 in fresh media. Optical density at 600 nm was measured every 5 minutes during 24 hours at 37°C using an automatic spectrophotometer (Tecan Infinite 107 F200PRO, Männedorf, Switzerland). The Maximum Growth Rate was determined as the maximum value of the derivative of the logOD600 using R software.⁸³

The minimal inhibitory concentrations of ciprofloxacin, delafloxacin and cefotaxime were determined by the broth microdilution method in accordance with EUCAST guidelines.⁸⁴ The minimal inhibitory concentration of fosfomycin was determined using the agar dilution method in accordance with EUCAST guidelines⁸⁴ with 25 μ g/mL glucose-6-phosphate (G6P) in the medium (Sigma-Aldrich, Saint-Quentin Fallavier, France).

Time-kill curves of PAS133, CFT073+TM, UT189+TM, UT189+TM RpoB S531L mutant, NILS 69+TM and NILS 77+TM to ciprofloxacin were performed in MH broth. Bacteria were grown overnight and diluted 1:10 000 in fresh MH. After 4h of regrowth, ciprofloxacin 1 μ g/mL was added to the culture. For the time-kill curve on non-dividing cells, the PAS133 was grown overnight and diluted 1:100 in physiological serum. Viable cells were counted after washing by plating 100 μ L of the appropriate culture dilution on MH agar plates at 0, 0.5, 1, 3 and 24h at 37°C. The lower limit of detection was 1 log₁₀ CFU/mL.

Time-kill curves on surviving bacteria were performed with ciprofloxacin, cefotaxime and fosfomycin in MH broth. Bacteria were grown overnight then diluted 1:100 in fresh MH containing ciprofloxacin 2.5 μ g/mL, fosfomycin 256 μ g/mL + G6P 25 μ g/mL,

cefotaxime 32 $\mu\text{g}/\text{mL}$. Viable cells were counted by plating 100 μL of the appropriate culture dilutions on MH agar plates at the following time points: 0 h, 1 h or 2 h (depending on the antibiotic) 6h and 24 h. The lower limit of detection was 1 \log_{10} CFU/mL.

In vivo antimicrobial treatment

To determine the bactericidal activity of cefotaxime, fosfomycin, delafloxacin, and ciprofloxacin, at least 8 mice per antibiotic were inoculated with strain PAS133, CFT073+TM strain, UTI89+TM strain, UTI89+TM RpoB S531L mutant, NILS 77+TM or NILS 69+TM strain. For the *in vivo* experiments, the antibiotics used were the following: ciprofloxacin (Sigma-Aldrich), cefotaxime (Viatris), fosfomycin (Delbert), anhydrotetracyclin-ATC (Sigma-Aldrich), delafloxacin (Menarini).

One or two days after inoculation, mice were treated with 2.5 mg/kg or 10 mg/kg of ciprofloxacin injected subcutaneously twice a day for 2 days (four injections). Mice were sacrificed 24 hours after the last dose of ciprofloxacin.

Single-dose pharmacokinetic studies were performed in a previous study after an injection of 10 mg/kg⁴⁴ of ciprofloxacin subcutaneously in infected mice.¹ The dosing regimen of 10 mg/kg provides a peak and an AUC close to human treatment with ciprofloxacin 250 mg twice a day.⁴⁵

Two days after inoculation, mice were treated subcutaneously with 100 mg/kg of cefotaxime 6 hours during 24 hours,³⁵ or 100 mg/kg of fosfomycin every 4 hours during 24 hours,^{37,85} or 10 mg/kg of delafloxacin twice a day for 2 days.^{48,49,35} Mice were sacrificed 24 hours after the last dose of antibiotic.

Mice were injected twice with ATC 0.08 mg intraperitoneally (IP) on the day prior to sacrifice. The second dose preceded euthanasia by at least 16 hours. By waiting 16 hours from the second dose, we ensured that there was not enough residual ATC in the tissues to cause the bacteria to switch to LacZ+ on the plate. Percentage of dividing bacteria before and after centrifugation and washing with sterile saline solution did not differ excluding the hypothesis of residual ATC in the tissue causing the bacteria to switch on the plate.

Urines were collected during spontaneous micturition right before sacrifice. Bladders and kidneys were aseptically removed and homogenized in 1 mL of saline solution. One hundred microliters of the solution or its dilution was spread onto MH agar plates supplemented with kanamycin 25 $\mu\text{g}/\text{mL}$ (Sigma-Aldrich, Saint-Quentin Fallavier, France) and X-gal 60 $\mu\text{g}/\text{mL}$ (ThermoFisher, Asnières-sur-Seine, France) and incubated for 24 to 48 hours. The number of CFU were counted and expressed as the number of CFU/g of tissue. When no growth was observed on agar plates after 48 hours, organs were considered sterile, and the value was considered at the limit of quantification, defined as the \log_{10} CFU per gram of organ corresponding to the growth of 1 CFU per plate and to the weight of organs in the individual mouse.

Ciprofloxacin pharmacokinetics

Single-dose pharmacokinetic studies were performed after an injection of 2.5 mg/kg of ciprofloxacin or 10 mg/kg of delafloxacin subcutaneously in infected mice. 8 weeks-old CBA/J female mice were trans-urethraly inoculated with 10^8 CFU of the PAS133 strain. 48 hours after injection, a single dose of 2.5 mg/kg of ciprofloxacin or 10 mg/kg of delafloxacin was administered subcutaneously. Blood samples of 500 μl were obtained by intracardiac puncturing into vacutainers containing heparin from three to four anesthetized mice at 15, 30, 45, 60, 90, 120, 240 and 360 min after ciprofloxacin or delafloxacin injection. Plasma was separated by centrifugation and stored at -20°C . Bladders and kidneys were also aseptically taken out after sacrifice and homogenized in 1 mL of sterile water. Homogenates aliquots were stored at -20°C . A sensitive method for the quantitative determination of ciprofloxacin was developed and validated by the method of standard addition. Standard additions were used to eliminate matrix effects from a measurement, since it is assumed that the matrix affects is minimized.

Solutions of ciprofloxacin or delafloxacin were prepared at three concentrations in water. For each sample, three aliquots (20 μl) were loaded with 20 μl of ciprofloxacin or delafloxacin solutions at either 30, 60 and 120 ng/ml concentrations. Four hundred microliter of acetonitrile was added for protein precipitation. After centrifugation (15 minutes at 14500 rpm), one microliter of the supernatant was injected onto the LC-MS/MS system. Separation was performed, at 10°C , in binary high pressure gradient mode, at a flow rate of 200 $\mu\text{L}/\text{min}$ using an analytical column (C18 waters BEH 1.7 μm , 50 \times 2.1 mm ID) protected by a guard column (C18 waters BEH 1.7 μm , 10 \times 2.1 mm ID). The mobile phase comprised 0.1% formic acid in water and 0.1% formic acid in acetonitrile for a total runtime of 5 minutes (0% B for 0.5 min, 0 \rightarrow 95% B in 0.5 min, 95% B for 2 min, 95 \rightarrow 0%B in 0.5 min, 0% B for 1.5 min). The column eluent was directed to the electrospray ionization (ESI) source of the MS instrument for MS analysis.

The instrumentation consisted of an Vanquish liquid chromatograph coupled with a Thermo Altis mass spectrometer (Thermo, San Jose, USA). The spray voltage used was 3.5 kV, capillary and vaporizer temperatures were set to 325°C and 350°C respectively. Ciprofloxacin or delafloxacin were detected using positive multiple reaction monitoring (MRM) at unit mass resolution. The MRM transition was 332.1 \rightarrow 288.2 at 18.5 V collision energy with a dwell time of 0.25ms. Data acquisition, integration of MRM chromatograms and standard addition calculation were performed running the Trace finder software version 4.1 platform (Thermo, San Jose, USA). For standard addition, a simple linear least squares analysis is made using the slope and intercept functions.

Bladder permeabilization experiment

Eight-weeks old CBA/J female mice were infected with 10^8 CFU of the UTI89+TM strain and treated twice with ATC 0.08 mg intraperitoneally the day prior to sacrifice. Mice were sacrificed at day 2 post infection. Sixteen Bladders were then put in 1 mL of saline solution with either 1 $\mu\text{g}/\text{mL}$ ciprofloxacin (8 bladders) or 10 $\mu\text{g}/\text{mL}$ delafloxacin (8 bladders) (100 X MIC) and incubated for 4 hours at

37°C with agitation. Bladders were then homogenized and centrifuged at 8000 rpm for 10 minutes to avoid carry-over effect. CFU and LacZ+ fraction were determined by serial dilution plating on LB X-gal agar plates.

Eight bladders were immediately homogenized in saline and 0.025% of Triton X-100 after sacrifice. Initial CFU and LacZ+ fraction were determined by serial dilution plating. Homogenates were then separated in two, and incubated with 1 µg/mL ciprofloxacin for the first half and 10 µg/mL delafloxacin for the second half for 4 hours at 37°C with agitation. Homogenates were centrifuged at 8000 rpm for 10 minutes to avoid carry-over effect. CFU and LacZ+ fraction were determined by serial dilution plating on LB X-gal agar plates.

Whole genome sequencing and bioinformatic analyses

Whole genome sequencing of PAS133 strain was performed using Illumina (Illumina, Cambridge, UK) paired-end sequencing 2x150bp on MiniSeq Platform to generate accurate short reads. In parallel, the genome was sequenced on the Oxford Nanopore Technologies MinION platform using an R9.4 flow cell to generate long reads. A hybrid assembly with Unicycler software V0.4.8 using accurate short reads Illumina and Nanopore long reads provided high-quality assembly creating a 5053320 bp long reference sequence for subsequent genomic analysis.⁸⁶

Colonies isolated from the mice after infection/treatment were sequenced using Illumina paired-end sequencing 2x150bp on NextSeq 500/550 Platform. Briefly, DNA was extracted from 8 to 15 colonies using the genomic DNA NucleoMag tissue kit from Macherey-Nagel and libraries were prepared and indexed using ILMN DNA (M) Tagmentation kit with IDT for Illumina DNA/RNA UD Indexes kit A/B/C/D. The colonies came from the kidneys and bladder from 2 mice treated with one course treatment of cefotaxime, 2 and 3 mice treated with 3 and 4 cycles of ciprofloxacin respectively and 2 controls.

BreSeq⁷⁶ V0.35.0 pipeline was used to identify mutations by aligning the Illumina reads of different strains from the mice organs against the PAS133 reference sequence. In house R script was used to filter and analyse the final data. All relevant scripts have been uploaded to this URL: <https://github.com/A-BN/coliMUTI>.

QUANTIFICATION AND STATISTICAL ANALYSIS

Results were expressed as means +/- SEM for continuous variables. Continuous variables were compared by nonparametric testing (Kruskal–Wallis test with adjustment for multiple comparisons, Mann Whitney test for two groups). A p-value of less than 0.05 was considered significant. Analysis was performed using Graphpad Prism. Refer to figure legends for full, specific statistical details.