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ANTIBIOTIC TOLERANCE IN QUIESCENT UROPATHOGENIC ESCHERICHIA COLI

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ANTIBIOTIC TOLERANCE IN QUIESCENT UROPATHOGENIC ESCHERICHIA COLI

BY

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ABSTRACT

Uropathogenic *Escherichia coli* (UPEC) are the leading cause of urinary tract infections (UTIs). In about a quarter of these cases the patient experiences reoccurring infection after treatment with antibiotics. The prevalence of reoccurrence may be attributed to the ability of some UPEC populations to enter a growth state where they are tolerant to antibiotic treatment. In reduced metabolic, dormant, and quiescent states bacteria are less susceptible to antibiotics that target critical growth pathways, allowing for prolonged survival. In Manuscript I, we investigate antibiotic tolerance in the quiescent UPEC strain CFT073 in vitro. We demonstrate that during quiescence UPEC are tolerant to first line therapies used in the treatment of UTIs, as well as various other broad spectrum treatment options. We also show that introducing a molecule that promotes proliferation, such as succinate or a mixture of amino acids L-Met and L-Lys, in conjunction with antibiotic exposure enhances susceptibility in quiescent UPEC populations. Our findings demonstrate that quiescent UPEC cells are tolerant to various antibiotics in vitro and that susceptibility can be restored in these populations through a combination treatment of an antibiotic and a proliferant cue.

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PREFACE

This thesis has been prepared in the Manuscript Format according to the guidelines set forth by the Graduate School of the University of Rhode Island. Manuscript I, "Antibitoic tolerance in quiescent uropathogenic *Escherichia coli*" is formatted for and will be submitted to *Journal of Bacteriology* for publication.

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ABSTRACT

Uropathogenic *Escherichia coli* (UPEC) are the leading cause of urinary tract infections (UTIs) worldwide. UPEC can also invade bladder epithelial cells (BECs) and ascend into the kidneys causing pyelonephritis. Approximately a quarter of patients will experience a reoccurring UTIs (rUTIs) after antibiotic treatment [1]. This high percentage of reoccurrence may be attributed to the ability of some UPEC populations to exist in an antibiotic tolerant state and persist inside bladder cells. Reduced metabolic, dormant, and quiescent states of bacteria can enhance tolerance to antibiotics as cells are less reliant on critical growth pathways. Here, we demonstrate that the metabolite-dependent quiescent state exhibited by the UPEC ST-73 isolate CFT073, which is elicited by cultivation on glucose at low cell density, enhances tolerance to a wide range of antibiotics. We show that the extent of this tolerance is not limited to antibiotics commonly prescribed to treat UTIs, but extends to various drug families including beta-lactams, protein synthesis inhibitors, and DNA replication inhibitors. Molecular cues that reverse quiescence and promote proliferation, including the metabolite succinate and the amino acids L-methionine and L-lysine, restore antibiotic susceptibility. Our findings demonstrate that quiescent UPEC cells are tolerant to various antibiotics in vitro and that susceptibility can be restored in these populations through a combination treatment of an antibiotic and a proliferant cue.

IMPORTANCE

Urinary tract infections (UTIs) are usually caused by a bacterial infection that colonizes the epithelial cells that line the bladder. If left untreated this infection can later ascend to the kidneys and cause systemic infection. Uropathogenic *Escherichia coli* (UPEC) are the leading cause of urinary tract infections, and upon colonization of bladder cells UPEC can establish quiescent intracellular reservoirs where they can evade antibiotic treatment and may contribute to reoccurring UTIs. Here, we demonstrate using an in vitro approach that quiescent UPEC are tolerant to first line therapies as well as various other antimicrobials. We also show that this antibiotic tolerance is reduced when cells are treated with a combination of a proliferant cue and an antibiotic.

INTRODUCTION

Bacterial antibiotic resistance is of growing concern within the medical community and, with few new antibiotics in development, there is a growing threat to public health. According to the 2019 Antibiotic Resistance Threats Report from the Centers for Disease Control[1], at least 2.8 antimicrobial-resistant infections occur each year, and over 35,000 people die from these infections[1]. UTIs are the most common outpatient bacterial infection, with approximately 404 million cases of UTIs occurring worldwide in 2019[2]. These infections are responsible for hundreds of millions of dollars in health care expenses each year in the United States alone[2].

Uropathogenic *Escherichia coli* (UPEC) cells are responsible for 65%-90% of urinary tract infections (UTIs)[3,4]. During active UTIs, UPEC are capable of invading bladder epithelial cells (BECs) and forming intracellular bacterial communities (IBCs)[5]. It is estimated that between 40-50% of women will experience a UTI at some point in their lifetime[6,7]. In addition to a high initial infection rate, the prevalence of recurring UTIs (rUTIs) after antibiotic treatment is significant, and current evidence suggests that an initial infection increases the risk potential for subsequent infections by the same pathogen[8]. Data indicates that approximately a quarter of patients treated for a UTI experience a recurring infection within six months of antibiotic treatment[8]. The recurrent nature of infections suggests that UPEC can either become tolerant to the prescribed antibiotic or can elude the cytotoxic effect of the drug without developing antibiotic-specific resistance at the genetic level. One theory on how UPEC may elude antibiotic treatment suggests that while infecting BECs, they can enter a state of quiescence, a non-proliferating, dormant state,

which aids in their ability to evade the host immune response and potentially the cytotoxic effect of antimicrobials[5]. This quiescent state is reversible with cues such as peptidoglycan peptides, amino acids, and metabolites[9]. These quiescent intracellular reservoirs (QIRs) are thought to play an important role in the development of rUTIs[5,10]. Unfortunately, there are currently no treatments available that prevent rUTIs, and this leaves patients with few options to alleviate the cycle of infections[11].

Other bacteria have been found to enter a state of quiescence when challenged with growth-limiting stress conditions, most notably *Mycobacterium tuberculosis*[12,13]. Under growth limiting-stress induced by the host immune response, much of the population will slow or arrest growth completely and can remain viable in non-replicating state for years[13]. Similarly, UPEC QIRs in vivo may also exist in a non-proliferative state for months[5]. In about 5-10% of cases where *M. tuberculosis* has entered quiescence, they exit and cause an active disease state[12]. Treatment of an active *M. tuberculosis* infection in vivo requires lengthy administration of antibiotics to prevent relapse[13]. Reduced efficacy of antibiotics demonstrated by the longevity of treatment regimens is not unique to *M. tuberculosis* and is often attributed to slowly replicating or quiescent populations in other disease states[13]. Metabolic adaptation is highly prevalent in clinical pathogens and can arise in response to antibiotic treatment; in turn this confers resistance[14]. Antibiotics target cellular processes necessary for cell growth, however, because quiescent UPEC populations are non-proliferating, this would implicate that they would be less susceptible to treatment. Understanding what processes are necessary for regulation or maintenance of quiescence

will help develop new approaches for the eradication of these non-replicating bacterial populations.

One of the current strategies for eliminating quiescent populations in other species is to re-sensitize them to antibiotics[13]. In *M. tuberculosis* antibiotic efficacy is reduced as a result of altered central carbon metabolism, directing cellular resources away from the tricarboxylic acid (TCA) cycle[15]. Restoration of antibiotic susceptibility in *M. tuberculosis* was demonstrated by increasing TCA cycle flux[15]. Similarly, UPEC cells are able to enter into a state of antibiotic tolerance by altering their TCA cycle flux, and this can also be reversed through supplementation of specific nutrients utilized in central carbon metabolism[16,17]. Quiescence can be induced in vitro by plating the UPEC lineage CFT073, an ST-73 sequence type, at a cell density below 10^6 colony forming units (CFU) ml^{-1} on defined minimal medium containing glucose[9,18]. However, when CFT073 cells are plated at a density of $>10^6$ CFU ml^{-1} they proliferate robustly, suggesting that proliferation in nutrient limiting conditions is quorum-dependent[9,18]. Of the UPEC clinical isolates tested to date, more than a quarter enter quiescence in this quorum-dependent manner, including 80% of ST-73 strains tested[18]. This quiescent population of CFT073 exhibits reduced metabolic activity and an increased tolerance to the beta-lactam antibiotic ampicillin, when compared to an actively growing culture[17,18]. Tolerance to commonly prescribed treatments is not well characterized, and further investigation into UPEC antibiotic tolerance in the quiescent state will guide the development of new therapies for rUTIs.

In vivo in a murine model, QIRs have been reported to occur in the underlying immature cells under the BECs that constitute the superficial layer of the urothelium[19]. It is thought that as these infected immature cells differentiate and migrate towards the surface of the bladder, the internalized UPEC can then begin proliferating again upon rearrangement of the actin cytoskeleton causing reoccurring infection[19]. This quiescent state has previously been demonstrated in vitro by our lab and in more than a quarter of UPEC clinical isolates tested to date, where the entire population is able to shift to a non-proliferating phenotype on nutrient-limiting agar in a quorum-dependent manner[9]. Mini transposon screens have previously identified that the genes *zapE*, *ihfB*, and *sdhA* are critical for quiescence in the UPEC strain CFT073[17,18]. ZapE is a late-stage cell division protein that has been reported to be important for cell division under stress and acts to bundle FtsZ polymers in vitro[20]. IhfB is one part of the heterodimer that makes up the DNA-binding IHF complex, which has been reported to increase persister cell formation and direct substrates into the glyoxylate shunt, away from the TCA cycle[21]. Recently our lab has been able to identify that quiescence appears to be mediated by metabolic flux, specifically through the TCA cycle[17]. It has also been shown that the metabolite succinate is able to reverse this quiescent state and that ZapE interacts with the succinate dehydrogenase (SDH) complex in vitro linking cell division and metabolism[17]. Evidence suggests that UPEC quiescence is widespread among the population and their ability to metabolically adapt is important for establishing chronic infection.

Current antibiotic treatments that are recommended as first line therapies for uncomplicated UTIs include fosfomycin, nitrofurantoin, and the widely used first line

antimicrobial trimethoprim-sulfamethoxazole (Fig. 1)[22,23]. The fluoroquinolones ciprofloxacin and levofloxacin are considered to be alternative treatments for uncomplicated UTIs and should not be used as first line therapies for non-extended spectrum beta lactamase (ESBL) producing organisms as resistance to these drugs is increasing in UPEC populations[22,23]. This development is quickly depleting the number of drugs we have at our disposal to treat these infections, and without the development of new therapies this poses a significant threat. Therefore, to prevent further development of antibiotic resistance it is imperative that we carefully monitor antibiotic treatment and test antibiotic susceptibility.

It has been previously shown that in *E. coli* antibiotic lethality is not growth rate dependent but instead reliant on metabolic state at the time of treatment[24]. This has been attributed to sequential downstream metabolic events after the drug takes effect, enhancing the lethality of the drug. Lethality may be diminished by reducing the toxic byproducts such as reactive oxygen species that are generated during cellular respiration[24]. As quiescent cells exhibit a marked decrease in metabolic activity based on previous findings, it would naturally raise questions about their ability to evade antibiotic treatment[17]. Regulation of metabolism is a strategy cells use to cope with stress conditions. Some toxin-antitoxin systems allow for arrest of macromolecule synthesis to protect the cell in starvation conditions. HipA is a serine/threonine kinase and part of the HipAB toxin-antitoxin system in *E. coli*[25]. Free HipA toxin is responsible for arrest of protein synthesis, and the entrance into dormancy[25]. The previously isolated *hipA7* allele is a gain-of-function mutant that enhances HipA kinase activity, thus promoting a metabolically dormant state[26].

Mutants with *hipA7* and similar alleles have also been linked to antibiotic tolerance in rUTIs caused by UPEC[25].

In the current study, we investigated the effects of different antibiotic treatment options on quiescent populations of the UPEC strain CFT073. We demonstrate that actively proliferating populations of UPEC are susceptible to a variety of antimicrobial treatments, however, during the quiescent state UPEC cells are tolerant to a wide variety of antibiotics in vitro. We also demonstrate that this antibiotic tolerance is mediated by metabolic adaptation to circumvent the cytotoxic effect of antimicrobial drugs. We show that it is possible to reduce antibiotic tolerance in quiescent UPEC by increasing TCA cycle flux through supplementation of the metabolite succinate or by the addition of other proliferants that prevent quiescence.

RESULTS

Quiescent *E. coli* exhibits an increased level of tolerance to antibiotics. To identify conditions for comparing antibiotics sensitivities across various *E. coli* strains and growth states, we first evaluated the minimum inhibitory concentration (MIC) of antibiotics representing multiple classes and first line UTIs therapies for wild type UPEC (CFT073) and K-12 (MG1655) strains of *E. coli* (Fig. 2). We cultured *E. coli* strains in rich medium (LB broth), to support robust proliferation, in the presence of increasing concentrations of antibiotics and determined the MIC of each to be: ampicillin 25 μg ml^{-1}; cephalexin 10 μg ml^{-1}; fosfomycin 12.5 μg ml^{-1}; azithromycin 5 μg ml^{-1}; tetracycline 3.13 μg ml^{-1}; nalidixic

acid 15μg ml^{-1}; ciprofloxacin 62.5 ng ml^{-1}; nitrofurantoin 12.5 μg ml^{-1}; trimethoprim 6.25 μg ml^{-1}. Observed MICs were similar for CFT073 and MG1655 strains in rich medium for all antibiotics tested, except for cephalexin, which had a lower MIC against CFT073 (10 μg ml^{-1}) than against MG1655 (20 μg ml^{-1}) (Fig. 2).

We previously reported that UPEC cells (CFT073) in a quiescent state are highly tolerant to ampicillin in vitro, whereas non-quiescent cells are ampicillin sensitive[17]. To determine if the quiescent state exhibited by the UPEC cells contributes to an increased level of tolerance across a range of antibiotics, we performed antibiotic tolerance assays in quiescent and actively growing cells. First, we induced the quiescent state by plating cells (CFT073) at low density ($<10^6$ CFU ml^{-1}) on mixed cellulose ester disks (2.5 cm) and then placed the disks on minimal agar containing glucose (0.2%), or on rich medium to prevent quiescence and promote growth. After incubating the plates at 37 °C for 3 h, disks were transferred to glucose minimal or LB agar plates containing an antibiotic, as required to maintain the growth state. Plates were incubated for 20 h to expose cells to the antibiotic, and then cells were harvested and enumerated via spread plating on LB agar.

We first investigated two top-line therapeutics that are commonly prescribed to treat UTIs, trimethoprim and fosfomycin (Fig. 1)[22]. Trimethoprim halts folate synthesis via inhibition of the enzyme dihydrofolate reductase, responsible for the NADPH-dependent catalysis of dihydrofolate to tetrahydrofolate[27]. We recovered cells and observed that a large percentage (16.2%) of the quiescent UPEC population survived exposure to

trimethoprim (6.25 μg ml^{-1}) for 20 h; however, only 0.25% of the actively proliferating population remained viable (Fig. 3A). Fosfomycin, which prevents cell wall synthesis via inhibition of MurA, a transferase responsible for the synthesis of UDP *N*-acetylmuramic acid (UDP-MurNAc)[28], produced a similar result as observed for trimethoprim. We observed that over half (56.38%) of quiescent UPEC survive exposure to fosfomycin for 20 h, compared to the 0.26% surviving in the actively proliferating population (Fig. 3B).

To determine if tolerance in the quiescent state was exclusive to ampicillin, we tested another beta-lactam antibiotic cephalexin. Cephalexin disrupts cell wall synthesis and maintenance by preventing transpeptidation[29]. After repeating the assay with 10 μg ml^{-1} cephalexin, we observed a 1.46% survival in population and <0.01% survival in the population on rich medium (Fig 4A). We then looked at the effects of the beta-lactam antibiotic ampicillin, which was previously reported to have reduced cytotoxicity against quiescent UPEC cells[17], and cephalexin. We quantified the percent survival of actively proliferating UPEC after incubation with 12.5 μg ml^{-1} of ampicillin but detected no survivors (<0.01%) (Fig. 4B). Conversely, under conditions that promote quiescence, we observed 24.4% survival.

We also tested various antibiotics that compromise other vital cell processes, including tetracycline to inhibit protein synthesis. Tetracycline binds the A site of the 30S ribosomal subunit, thereby blocking new aminoacyl-tRNAs from entering[30]. This assay yielded a similar result where the quiescent population saw a 1.06% percent survival,

compared to the actively proliferating population with <0.01% percent survival (Fig. 5A). We then inhibited protein synthesis using the macrolide antibiotic azithromycin at 5 μg ml^{-1}. Azithromycin binds to the peptide exit tunnel on the 50S ribosomal subunit and sterically blocks the emerging peptide, thus halting protein synthesis[31]. Azithromycin is of relevance for treating urothelial infections as it readily penetrates cell membranes and demonstrates excellent intracellular accumulation[32,33]. Again, the quiescent population of UPEC had a higher percent survival at 5.10% compared to the active population with a 0.86% (Fig. 5B). Based on these results inhibition of protein synthesis appears to be an ineffective strategy for targeting these quiescent populations in vitro.

Another antibiotic class inhibits transcription and repair of DNA via inhibition of DNA gyrase. Quinolones such as nalidixic acid, and the fluoroquinolone ciprofloxacin, inhibit the re-ligation of DNA by GyrA leading to double stranded breaks and eventual fragmentation of the entire chromosome[34]. Here we investigate the effects of quinolone antibiotics on quiescent UPEC populations. After exposure to the fluoroquinolone ciprofloxacin, we observed that 3.30% of the quiescent population survives treatment, however less than 0.01% survives on the rich medium, the condition that promotes proliferation (Fig. 6A). When these two populations of UPEC were treated with nalidixic acid we observed the quiescent population had a 47.44% percent survival, whereas the actively proliferating population had a survival rate of 31.20% (Fig. 6B).

Another antimicrobial that is commonly used to treat tuberculosis infections is the drug rifampin. Rifampin inhibits RNA synthesis by sterically blocking the emerging mRNA transcript from RNA polymerase. This drug demonstrates specificity for the bacterial RNA polymerase and has no effect on the mammalian RNA polymerase[35,36]. We investigated the effect a 12.5 μg ml^{-1} treatment of rifampin has on quiescent UPEC populations. We observed that 103.6 % of quiescent cells and 28.44% of proliferating cells survive treatment (Fig. 7).

Proliferant molecules reduce antibiotic tolerance in quiescent UPEC populations. We then tested if antibiotic susceptibility could be restored in quiescent UPEC populations by the administration of antibiotics in combination with a known proliferant molecule or cue. Our lab previously identified molecules that promote proliferation in quiescent UPEC, including the metabolite succinate and the amino acids L-lysine and L-methionine[9,17,18]. Trimethoprim alone and in combination with proliferant were tested for survival. A combination treatment of trimethoprim and succinate showed a 0.14% population survival which is a significant decrease from the 17.22% survival population after antibiotic treatment alone (Fig. 8). When trimethoprim was administered in combination with an amino acid mixture of L-Met and L-Lys we observed a 0.03% survival, which is 2-log lower than the 3.76% surviving from the group treated with trimethoprim alone (Fig. 8). The addition of a proliferant to antibiotic treatment increased susceptibility to trimethoprim in quiescent UPEC populations.

Non-quiescent mutants have reduced tolerance to antibiotics. Previously, the global gene regulator the IHF complex, a heterodimer of IhfA and IhfB, and the cell division protein ZapE have been reported to be regulators of quiescence and antibiotic tolerance in UPEC[17]. Here we tested CFT073 strains that have deletions of either the *zapE* or *ihfB* genes for antibiotic tolerance of trimethoprim. The percent survival of CFT073 Δ*zapE* cells that survived treatment on glucose minimal agar are 0.02%, similar to what we observed on LB agar at 0.04% survival (Fig. 9A). The *ihfB* deletion strain showed 0.85% survival on glucose minimal agar, while 0.06% of the population survived on LB agar (Fig. 9B). In both cases we see less than one percent survival of these non-quiescent strains whereas survival of the wild-type strain is 16.22% (Fig. 3A). Finally, we also evaluated if an *sdhA* mutant strain (CFT073 *sdhA*::Tn5), which is also defective for entering a quiescent state, has reduced tolerance[17,18]. We observed that cells on glucose minimal agar and cells on LB had similar survivability, 1.31% and 0.74%, respectively (Fig 9C). These results show that genes essential for quiescence are also important for tolerance to trimethoprim.

Tolerance to nitrofurantoin is reduced in nutrient limiting conditions. Finally, nitrofurantoin is associated with very low antibiotic resistance rates (Fig. 1). We investigated the effects of nitrofurantoin on quiescent UPEC cells and observed no survivors after exposure. Interestingly, when we observed the effect of nitrofurantoin on actively proliferating UPEC on LB medium we observed 86.82% survival (Fig S1A). Upon this finding we performed the same assay with a non-quiescent mutant of CFT073, Δ*zapE*, which showed a survival rate of 0.03% on glucose minimal agar and 52.54% on LB (Fig. S1B) and a K-12 lab strain, MG1655, with a 0.05% and 92.83% survival on glucose

minimal and LB, respectively (Fig. S1C). Our findings suggest that the increased efficacy we see with nitrofurantoin is not specific to quiescence but is attributed to the low nutrient environment.

Constructing a fluorescent CFT073 reporter strain to visualize intracellular UPEC cells. To visualize UPEC cells that have invaded BECs we constructed a reporter strain of CFT073 that uses a constitutive tetracycline promoter[37] to express *gfp*$_{uv3}$ from the chromosome. GFP$_{uv3}$ contains the mutations F64L, S65T, and Q80R to enhance maturation rate[38], and the cycle 3 mutations F99S, M153T, V163A which enhance fluorescence by 45-fold compared to the standard GFP[39]. GFP$_{uv3}$ was amplified out of the pBAD24- *GFP*$_{uv3}$ plasmid using primer extension to add the tetracycline promoter, then ligated back into a pBAD-24 backbone for maintenance. We then used λ red recombination to remove *rpnC* from the CFT073 genome and insert a chloramphenicol resistance gene and the *parE* toxin under the control of the rhamnose promoter and plated on chloramphenicol for selection, the resulting strain was DB021 (CFT073 Δ*rpnC*::*parE-cat*). We used λ red recombination again to insert our gene construct (*pTet-gfp*$_{uv3}$) into the *rpnC* locus and plated on minimal agar containing rhamnose for selection (Fig. 10A). The resulting strain DB030 (CFT073 Δ*rpnC*::P$_{tet}$-*gfp*$_{uv3}$) was then visualized using flouresence microscopy (Fig. 10B). We confirmed that the quiescent phenotype was preserved after genetic manipulations by plating on glucose minimal agar at 10^4 CFU ml^{-1}, and that the strain responded to the proliferant L-methionine and L-lysine (Fig. 10C).

Using the DB030 strain that expresses gfp_{uv3} from the chromosome and the human bladder epithelial cell carcinoma line HTB-4, we performed an invasion assay as described in Methods. Briefly, UPEC cells were incubated with BECs and allowed to infect for 1 hour at a (multiplicity of infection) MOI of 15:1. Cultures were then treated with 100 μg ml^{-1} of gentamicin to kill any remaining extracellular or adherent bacteria. Visualization of UPEC cells inside of BECs was done according to microscopy described in Methods and monitored up to 72 hours post-infection. Our results indicate that the DB030 strain can colonize and persist inside bladder cells (Fig. 10D). Phalloidin-594 was used to stain and visualize BEC actin cytoskeletal structure, while DAPI was used to stain genetic material. We observed that after 72 hours posit infection that most remaining bacterial cells were present in the cytoplasm of BECs and occasionally found in proximity to the nucleus.

DISCUSSION

Here we describe a strategy UPEC cells may use to tolerate antibiotics. We demonstrate that UPEC cells cultured on rich medium are susceptible to current recommended treatments for UTIs, as well as other various antimicrobials. We show that UPEC cells cultured at low cell density on glucose minimal agar, which promotes quiescence, are tolerant to these first line therapies, such as fosfomycin and trimethoprim in vitro. We have seen that these quiescent UPEC populations are also tolerant to other drug classes such as the beta-lactams ampicillin and cephalexin, the protein synthesis inhibitors tetracycline and azithromycin, the DNA replication inhibitors ciprofloxacin and nalidixic acid, and the antimycobacterial drug rifampin. We have seen that these populations of quiescent UPEC are tolerant to a myriad of antibiotic treatments in vitro.

We know that quiescence is reversible, therefore if an infection is not cleared it would be possible for QIRs to begin proliferating and cause reoccurrence of the disease state. This antibiotic tolerance during quiescence may provide an explanation for the high percentage of patients who experience reoccurring infection after antibiotic treatment.

UPEC quiescence has also been reported to be modulated by succinate levels[17]. Quiescent cultures of CFT073 have been shown to have decreased metabolic activity compared to non-quiescent cultures[17]. Reduced metabolic activity has been correlated with increased antibiotic tolerance, conversely increasing activity of central carbon metabolism has been shown to sensitize tolerant cells to antibiotics[24,40]. The CFT073 mutant *sdhA*::Tn5 has previously been identified as non-quiescent through a mini transposon screen[18]. SdhA is directly involved in the TCA cycle and deletion of the gene would disrupt the succinate dehydrogenase complex, responsible for the conversion of succinate to fumarate. In turn, this would lead to an accumulation of succinate inside the cell, preventing quiescence[17]. We wanted to investigate if this mutant, incapable of entering quiescence, would exhibit the same level of antibiotic tolerance seen in the wild type. CFT073 *sdhA*::Tn5 showed reduced tolerance to the commonly prescribed antibiotic trimethoprim, indicating that *sdhA* is important for UPEC antibiotic tolerance (Fig. 9C).

To further test if metabolic adaptation via TCA cycle regulation is important for antibiotic tolerance, we increased TCA cycle flux in quiescent wild type CFT073 through supplementation of succinate, and in combination treated with trimethoprim. We saw that

quiescent cells treated with succinate and trimethoprim had a lower survivor population by 2-log compared to cells treated with trimethoprim alone (Fig. 8). In addition, the supplementation of another previously identified proliferant, L-methionine with L-lysine, also reduced antibiotic tolerance to trimethoprim in quiescent UPEC populations by 2-log (Fig 8). Therefore our results suggest that UPEC quiescence contributes to increased antibiotic tolerance in vitro and that susceptibility is restored in the presence of a proliferant molecule.

Previously it has been reported that UPEC quiescence is, in part, regulated through the IHF complex[17]. IHF is also known to regulate the *fim* operon which is crucial for production of type 1 pili, necessary for the colonization and persistence within the bladder[41]. IHF is also a repressor of *sucAB*, both of which are part of the α-ketoglutarate dehydrogenase complex, absence of *ihfB* would lead to higher expression of *sucAB*, and the prevention of quiescence[17]. We demonstrate that deletion of the gene Δ*ihfB*, thereby disrupting the complex and preventing quiescence, leads to a one log reduction in antibiotic tolerance to trimethoprim when compared to quiescent populations in vitro (Fig 9B). This result suggests that IHF and quiescence are important for UPEC antibiotic tolerance in vitro.

The cell division protein ZapE is reported to be important for cell division under stress conditions such as high temperatures and low oxygen environments[20]. ZapE has also been reported to be essential for UPEC to enter quiescence, however the mechanism of this

is not understood. Here we demonstrate that the CFT073 Δ*zapE* mutant exhibits greater than a 2-log decrease in survivor population compared to quiescent wild type populations after trimethoprim treatment suggesting that *zapE* plays a role in UPEC antibiotic tolerance in vitro.

Nitrofurantoin is a bactericidal, synthetic antimicrobial that was approved in the 1950s to treat UTIs, however, it lost prevalence as modern treatments such as trimethoprim-sulfamethoxazole and other beta-lactams became widely available[42]. Resistance to nitrofurantoin is rare and current data puts the UPEC population resistance at 1.6% making it a viable option for treatment[43]. The mechanism of action is poorly understood given that this compound has been around for over 70 years. Nitrofurantoin is metabolized by the nitroreductases NfsA and NfsB inside the cell, forming nitroaromatic anion radicals, which, in the presence of oxygen, can generate superoxide anions[44]. Superoxide anions are converted to hydrogen peroxide by superoxide dismutases. Through Fenton chemistry hydrogen peroxide reacts with ferrous iron to generate hydroxyl radicals which cause extensive DNA and RNA damage within the cell[45]. These reactive oxygen species (ROS) then compromise vital cellular processes including protein synthesis, DNA replication, and cause DNA damage[42]. The large difference between survivor populations that we observe when cells are treated with nitrofurantoin in nutrient limiting environments but not in nutrient rich environments may be attributed to induced auxotrophy for branched chain amino acids (BCAA). BCAA biosynthesis is shut down inside the cell as a response to ROS as they inactivate the iron-sulfur clusters of ilvD and LeuC/LeuD[45]. As nitrofurantoin is metabolized inside the cell it causes oxidative damage to the cell, in turn

this could be shutting down biosynthesis of vital amino acids and leading to increased cell death when amino acids are not available for uptake.

The UPEC pyelonephritis isolate CFT073, is from phylogenetic group B2, sequence type 73 (ST73), which has been used extensively to study UPEC pathogenesis[18]. The CFT073 genome encodes five distinct cryptic prophage genomes that include a multitude of virulence-associated genes, known as pathogenicity islands (PAIs)[46]. These PAIs contain a range of genes important for overcoming host immune defenses and establishing infection within the bladder[47]. GFP_{uv3} fluorescence was observed, and we were also able to visualize the bacteria though DIC images and DAPI staining. The gene expression of CFT073 is greatly altered once they invade BECs, and many carbohydrate metabolism genes, such as *sucCD* and *icdA*, are downregulated. This suggests that they are decreasing metabolic flux through the TCA cycle[48]. Downregulation of these genes would in turn, decrease succinyl-CoA levels and may promote the quiescent state. This alteration in gene expression may help explain the reduction in fluorescence we see after invasion. Future directions include visualizing these UPEC after antibiotic treatment to observe any phenotypical changes in the population within BECs.

METHODS

Cell lines, bacterial strains, plasmids, and growth conditions

E. coli strains and plasmids used in this study are included in table 1. *E. coli* strains were grown in LB (Lennox)[49], Luria-Bertani broth with NaCl 5 g L^{-1} and/or on LB agar

(Lennox), plus ampicillin (100 μg ml^{-1}), chloramphenicol (35 μg ml^{-1}), and kanamycin (50 μg ml^{-1}) when necessary. UPEC strains were grown in either LB medium or M9 minimal medium and on agar containing 0.2% glucose as previously described[18]. Culture densities were measured and standardized using OD_{600}. All genetic manipulations were confirmed through Sanger Sequencing[50]. Human bladder epithelial cell carcinoma line HTB-4 (ATCC T24) was cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with heat-inactivated fetal bovine serum to a final concentration of 10% and 1% penicillin and streptomycin at 37°C with 5% CO_2.

The conditionally replicative plasmids, pKD3 and pKD267 and all variations of the two were maintained in the *pir*$^+$ host BW25141[51]. Site-directed mutagenesis of pKD3 and pKD267 plasmids were carried out using the QuikChange II XL mutagenesis system (Agilent) using the primers PstIsitepkd3For and PstIsitepkd3Rev to add a PstI restriction site on pKD3, and EraNdeIpkd267F and EraNdeIpkd267R to remove the NdeI site on pkd267 yielding the plasmids pKD3/PstI and pKD267/-NdeI, respectively. *parE* under the control of the rhamnose promoter was amplified from pKD267 using primers rhaParEcloningFor and rhaParEcloningRev, the resulting PCR product was double digested with PstI (HiFi) and NdeI, purified via gel purification, and ligated into a PstI/NdeI digested pKD3/PstI plasmid. The resulting pKD367 plasmid containing a new *cat-parE* cassette was transformed into chemically competent BW25141 cells and confirmed through sequencing.

To achieve constitutive expression of eGFP from the chromosome of CFT073, *eGFP* was amplified out of pBAD24-gfp_{uv3} and put under the control of a tetracycline promoter lacking the tetracycline repressor gene[37]. This was accomplished using the Ptet*eGFP*For and Ptet*eGFP*Rev primers, the resulting PCR product was then digested with the restriction enzymes EcoRI(HiFi) and HindIII (HiFi), purified via gel purification, and ligated into an EcoRI/HindIII digested pBAD24 (lab stock). Ligation reactions were transformed into XL10 gold ultracompetent cells (Agilent) and verified by sequencing, resulting in the plasmid pBAD24-$P_{tet}gfp_{uv3}$ (no RBS). A strong ribosome binding site was inserted after the tetracycline promoter on the plasmid using the Q5 site-directed mutagenesis kit (NEB) and the Q5RBSinsptetfor and Q5RBSinsptetrev primers. The resulting plasmid, pBAD24-$P_{tet}gfp_{uv3}$ was transformed into XL10 gold ultracompetent cells (Aligent) and verified by sequencing.

Gene deletions were performed using λ red recombination as previously described[51]. Briefly, strains of interest containing pKD46 were grown to an OD_{600} of approximately 0.3 in the presence of ampicillin (100 μg ml^{-1}) and L-arabinose (0.36%). Cells were then electroporated with a PCR amplified insert and recombinants were selected for via spread plating LB agar supplemented with kanamycin (50 μg ml^{-1}) or chloramphenicol (35 μg ml^{-1}). To construct CFT073 Δ*rpnC*::P_{tet}-gfp_{uv3} recombinants were selected for by spread plating on M9 minimal agar containing 1% L-rhamnose and confirmed by sequencing.

Preparation of chemically competent BW25141 using the rubidium chloride method

An overnight culture of BW25141 was grown shaking in 3 ml of LB broth at 37° C. 100 ml of LB broth was inoculated with 1 ml of the overnight culture and grown to an OD_{600} of 0.5. Cells were then chilled on ice for 10 minutes and centrifuged at 3000 x g for 10 minutes at 4° C. Pellets were then washed twice with 5 ml of cold TFB1 (1M rubidium chloride, 1M calcium chloride, 15% glycerol, 50mM $MnCl_2$, and 30 mM potassium acetate) and centrifuged for 10 minutes as previously. Pellets were resuspended in 1 ml of cold TFB2 at pH 7 (10 mM sodium MOPS (pH7), 1M calcium chloride, 1M rubidium chloride, 15% glycerol), aliquoted, and snap frozen in liquid nitrogen.

Dose Escalation Assay

E. coli K12 strain MG1655 and UPEC strain CFT073 were exposed to various antibiotic concentration gradients to determine a minimal effective dose. This was done in 96 well plates with 200 µl of LB medium in each well. The target antibiotic was then titrated via serial dilution with the starting well containing a high concentration. OD_{600} measurements of overnight cultures of both CFT073 and MG1655 grown in LB medium at 37° C were standardized to the lowest culture. The wells were then inoculated at an OD_{600} of 0.02 with either CFT073 or MG1655. Plates were incubated at 37°C for 20 hours then read at 595nm on a microplate reader.

Antibiotic Tolerance Assay

UPEC strains were cultured in 2 ml of M9 minimal medium + 0.2% glucose and LB medium in parallel, shaking at 37°C for 24 hours. The OD_{600} was standardized to the lower of the two cultures and serial diluted to a culture density of $1x10^5$ CFU ml^{-1}. Two 13 mm nitrocellulose membranes with a pore size of 0.22 μm were placed onto M9 minimal + 0.2% D-glucose agar plates or LB agar for each of their respective liquid cultures and pre-warmed to 37°C. 10 μl of the dilution was spotted onto the membranes and all filters were incubated at 37°C for 3 hours to induce the quiescent state for CFT073 on M9 minimal + 0.2% D-glucose agar. After incubation the filters were aseptically transferred to experimental LB agar and M9 minimal + 0.2% D-glucose agar plates for antibiotic exposure. Two filters were assigned to each culture, one was exposed to antibiotic treatment on a new respective agar plate, while the other was transferred to a fresh agar plate of the respective medium. The filters were incubated on the experimental plates at 37°C for 20 hours. Filters were then sterilely transferred to conical tubes containing 1 ml of LB medium and rigorously vortexed to remove cells from the filters. Filters were aseptically removed from the conical tubes using forceps, serial diluted, and spread plated to quantify CFU ml^{-1}. Percent survival was then calculated by dividing the number of viable cells on plates containing antibiotic by the number of viable cells on plates without antibiotic.

Invasion Assays

UPEC strains were grown in static LB broth at 37°C for 24 hours. HTB-4 bladder epithelial cells were seeded in a 24-well plate at an approximate density of 5×10^4 cells per well and grown at 37°C with 5% CO_2 to ~95% confluency. Bladder epithelial cells were

infected with CFT073 at a multiplicity of infection (MOI) of 15:1. The plates were centrifuged at 600 x g for 5 minutes to localize bacteria to host cells. Plates were incubated for 1 hour, washed three times with PBS, then treated with 100 μg ml^{-1} gentamicin for 1 hour, killing extracellular bacteria. Post gentamicin wash, fresh medium containing 10 μg ml^{-1} gentamicin was added, and cells were incubated until ready for microscopy.

Quiescence Assay

UPEC strains were grown on LB agar plates incubated at 37°C overnight. A loopful of cells was used to inoculate 2 ml of M9 minimal media supplemented with 0.2% glucose and incubated at 37°C overnight shaking. Cultures were diluted out to a cell density of 10^6 CFU ml^{-1}, 10 μl of this dilution was suspended in 3 ml of top agar containing 0.75% agar and 0.2% glucose. This was poured on M9 minimal glucose agar plates to create an agar overlay and allowed to dry for 1 hour at room temperature. Proliferants were spotted on the overlay when necessary; they include a mixture of 1mM L-methionine and 1 mM L-lysine, or 0.5% succinate, or 5 μl of mutanolysin digested peptidoglycan.

Microscopy

Cells were visualized via fluorescence microscopy on a Zeiss 700 LSM confocal fluorescence microscope with an excitation and emission wavelength of 488/508 nm for eGFP, 405/461 nm for DAPI, and 581/609 nm for Phalloidin-594. Differential interference contrast (DIC) images were acquired using a Nomarski Prism. Images were captured on an AxioCam digital camera with ZEN 2012 software. Coverslips were treated overnight with 1M HCl in 6 well plates, washed repeatedly with sterile dH_2O to remove remaining HCl,

100% ethanol was added to each well, and the cover was left open to allow evaporation. Coverslips were then treated with 0.1% poly L-lysine for 15 minutes, washed 3 times with 1x PBS, allowed to air dry, and finally treated with UV radiation for 15 minutes. BECs were grown on treated glass coverslips prior to infection. Each well containing one coverslip was seeded at a cell density of approximately 300,000 cells and incubated in DMEM supplemented with 10% FBS at 37°C with 5% CO_2 for 24 hours. The BECs were then infected with UPEC cells at an MOI of 15:1 following the same procedure indicated in the invasion assay. Following treatment with 100 μg ml^{-1} gentamicin, wells were washed with 1x PBS and fresh media containing 10 μg ml^{-1} gentamicin was added to the wells. At the desired timepoints the media was aspirated off the coverslip, washed 3 times with 1x PBS, and then fixed with 4% paraformaldehyde for 30 minutes. The coverslip was then washed 3 times with 1x PBS and treated with phalloidin-594 for 45 minutes. After, the coverslip was washed 3 times with 1x PBS, treated with DAPI at 1 μg ml^{-1} for 15 minutes, and placed face down on a glass slide for visualization.

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Table 1. E. coli strains

Strain or Plasmid	Relevant Genotype	Source, reference, or construction
Strain		
CFT073 Str^r	Spontaneous streptomycin-resistant mutant of CFT073	([18])
DB021	CFT073 Δ*rpnC*::*parE-cat*	This study
DB030	CFT073 Δ*rpnC*::P_{tet}-gfp_{uv3}	This study
MG1655	LAM- -*rph*1	([52])
JM028	CFT073 Str^r Δ*ihfB*::*parE-kan*	([17])
JM023	CFT073 Str^r Δ*zapE*::*parE-kan*	([17])
PC358	CFT073 Str^r *sdhA Tn5:kan*	([18])
BW25141	F-, *Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), Δ(phoB-phoR)580, λ-, galU95, ΔuidA3::pir+, recA1, endA9(del-ins)::FRT, rph-1, Δ(rhaD-rhaB)568, hsdR514*	([51])
Plasmid		
pKD3	*amp, cat*	([51])
pKD3/PstI	*amp, cat*	This study
pKD46	*amp*	([51])
pDK267	*kan*	B. Wanner[a]
pKD267/-NdeI	*kan*	This study
pKD367	*amp, cat*	([17])
pBAD24	*amp*	([53])
pBAD24-gfp_{uv3}	*amp*	
pBAD24-$P_{tet}gfp_{uv3}$ (no RBS)	*amp*	This study
pBAD24-$P_{tet}gfp_{uv3}$	*amp*	This study

[a]J. Teramoto, K. A. Datsenko, and B. L. Wanner, unpublished.

Table 2. Primer list

Oligonucleotide	Sequence
Ptete*GFP*For	5'ATCAAGAATTCTCCCTATCAGTGATAGAGATTGACATCCCTATCAGTGATAGAGATACTGAGCACATCAGCAGGACGCACTGAATGAGTAAAGGAGAAGAACT 3'
Ptete*GFP*Rev	5' ATCACAAGCTTTTATTTGTAGAGCTCATCCA 3'
Q5RBSinsptetFor	5' GGAGAAAGGTACCCATGAGTAAAGGAGAAGAACTTTTCAC 3'
Q5RBSinsptetRev	5'- TCTTTAATGAATTCGTCAGTGCGTCCTGCTGAT -3'
PstIsitepkd3For	5' CTTCCCGGTATCAACAGGGACTGCAGGATTTATTTATTCTGCGAAG 3'
PstIsitepkd3Rev	5' CTTCGCAGAATAAATAAATCCTGCAGTCCCTGTTGATACCGGGAAG 3'
EraNdeIpkd267F	5' CAGCAGGATCACTGTATGACGGCCTAC 3'
EraNdeIpkd267R	5' GTAGGCCGTCATACAGTGATCCTGCTG 3'
rhaParEcloningFor	5' CGGACTGCAGGGAACTAGTCGAGGCCATGG 3'
rhaParEcloningRev	5' GATTCATATGCAGCCCTTGAGCCTGTCGGC 3'
367DelRpnCfor	5' ACCATGACTGGATACTGCCTTCACGCAAGACCACACGGAAGGCAGGGAAGGTGTAGGCTGGAGCTGCTTC 3'
367DelRpnCrev	5' GGTCAGTTAACAATAACAATTATCTGGCTAAAACTGATAATGCAGATAACCAGCCCTTGAGCCTGTCGGC 3'
PteteGFPKIRev	5'GGTCAGTTAACAATAACAATTATCTGGCTAAAACTGATAATGCAGATAACTTATTTGTAGAGCTCATCCA 3'
PteteGFPKIrpnCFor	5' ACTGCCTTCACGCAAGACCACACGGAAGGCAGGGAAGTCCCTATCAGTGATAGAGATTGACATCCCTATC 3'

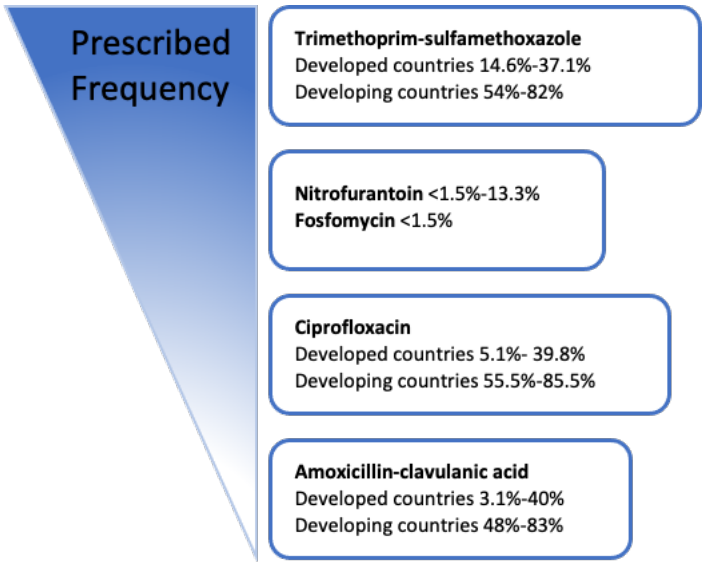
Prescribed Frequency
Trimethoprim-sulfamethoxazole
Developed countries 14.6%-37.1%
Developing countries 54%-82%
Nitrofurantoin <1.5%-13.3%
Fosfomycin <1.5%
Ciprofloxacin
Developed countries 5.1%- 39.8%
Developing countries 55.5%-85.5%
Amoxicillin-clavulanic acid
Developed countries 3.1%-40%
Developing countries 48%-83%

Figure 1. Prescribed antibiotics for UTIs. Illustration showing first line treatments prescribed for UTIs along with the estimated percentages of UPEC populations that exhibit antibiotic resistance in both developed and developing countries. Adapted from Kot, 2019[22].

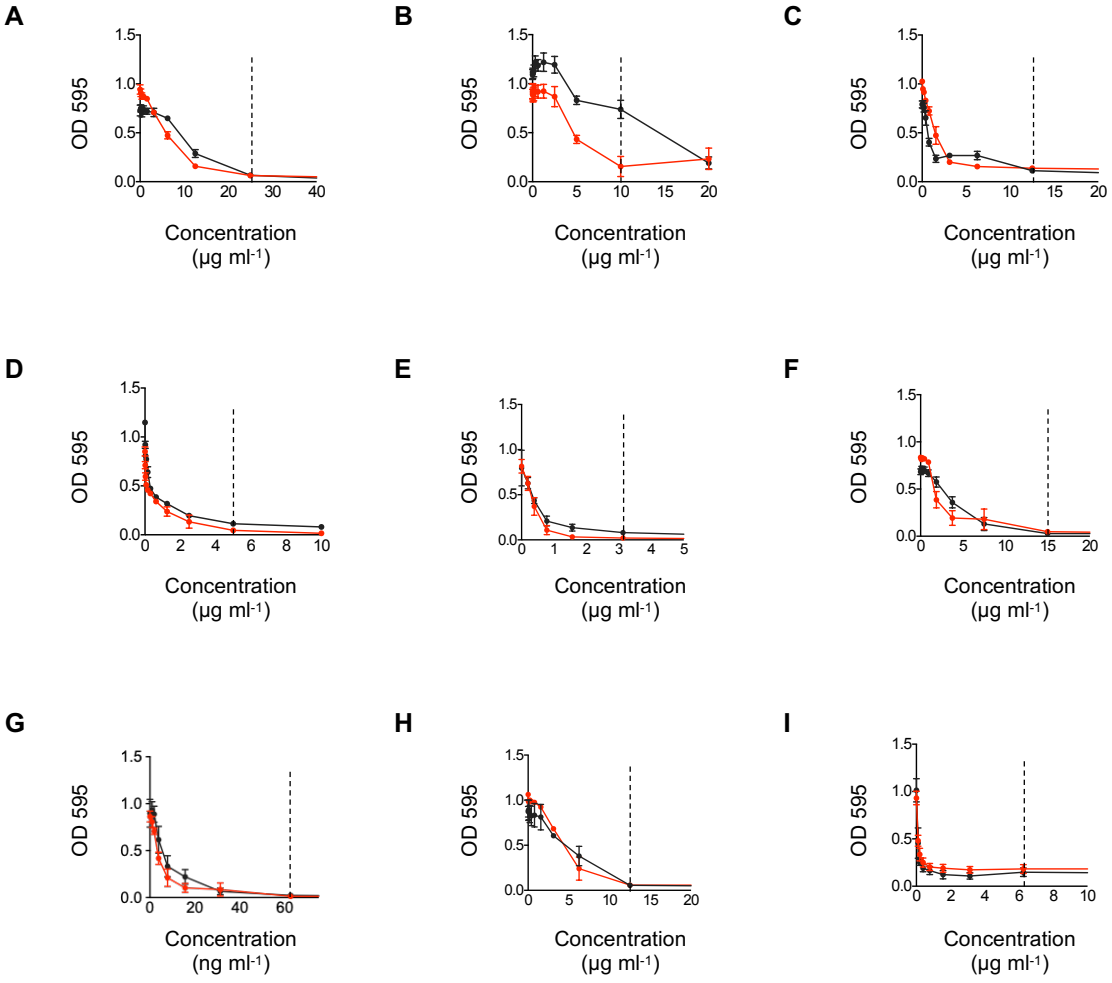
A
B
C
D
E
F
G
H
I
OD 595
Concentration
(µg ml-1)
(ng ml-1)

Figure 2. Minimum inhibitory concentration (MIC) determinations comparing UPEC CFT073 and MG1655 for a variety of antibiotics. Cells were cultured in LB broth for 20 h then read at an OD of 595nm. UPEC is shown in red, MG1655 shown in black. (A) Ampicillin, (B) cephalexin, (C) fosfomycin, (D) azithromycin, (E) tetracycline, (F) nalidixic acid, (G) ciprofloxacin, (H) nitrofurantoin, (I) trimethoprim are shown. Represented are averages from at least 3 biological replicates, error bars represent standard errors of the means (SEM). Dotted line indicates the MIC.

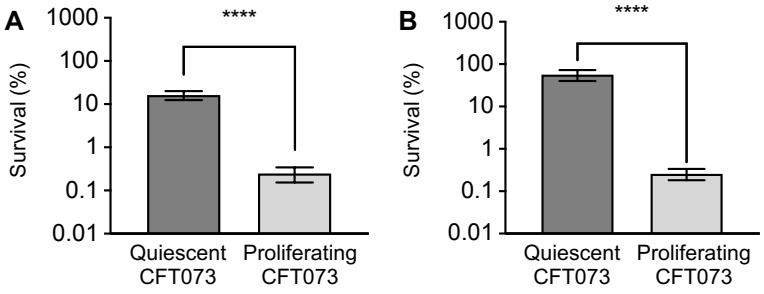
A
1000
100
10
1
0.1
0.01
Survival (%)
****
Quiescent CFT073
Proliferating CFT073
B
1000
100
10
1
0.1
0.01
Survival (%)
****
Quiescent CFT073
Proliferating CFT073

Figure 3. Antibiotic tolerance assays investigating the effects of first line therapies on quiescent compared to proliferating UPEC. (A) Percent survival of quiescent CFT073 on M9 minimal media + 0.2% glucose (16.22 %) and actively proliferating CFT073 on LB (0.25 %) exposed to 6.25 μg ml^{-1} trimethoprim for 20 h. (B) Percent survival of quiescent CFT073 on M9 minimal media + 0.2% glucose (56.38 %) and actively proliferating CFT073 on LB (0.26 %) 6.25 μg ml^{-1} fosfomycin for 20 h. (*p* value, **** ≤ 0.0001) Represented are averages from ten biological replicates, error bars represent standard errors of the means (SEM).

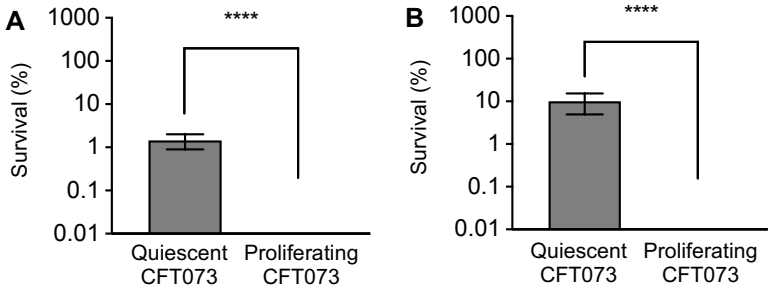
A
1000
100
10
1
0.1
0.01
Survival (%)
****
Quiescent CFT073
Proliferating CFT073
B
1000
100
10
1
0.1
0.01
Survival (%)
****
Quiescent CFT073
Proliferating CFT073

Figure 4. Cephalexin antibiotic tolerance assay and ampicillin antibiotic tolerance assay comparing quiescent and proliferating UPEC. (A) Percent survival of quiescent CFT073 on M9 minimal media + 0.2% glucose (1.46%) and actively proliferating CFT073 on LB (0.00%) exposed to 10 μg ml^{-1} cephalexin for 20 h. (B) Percent survival of quiescent CFT073 on M9 minimal media + 0.2% glucose (24.43%) and actively proliferating CFT073 on LB (0.00%) exposed to 12.5 μg ml^{-1} ampicillin for 20 h. (*p* value, **** ≤ 0.0001) Represented are averages from ten biological replicates, error bars represent standard errors of the means (SEM).

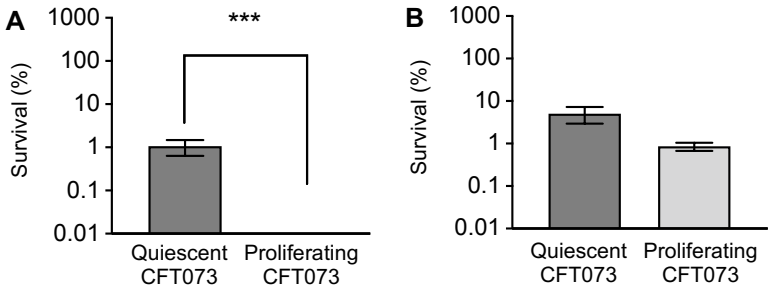
A
1000
100
10
1
0.1
0.01
Survival (%)
***
Quiescent CFT073
Proliferating CFT073
B
1000
100
10
1
0.1
0.01
Survival (%)
Quiescent CFT073
Proliferating CFT073

Figure 5. Tetracycline antibiotic tolerance assay and azithromycin antibiotic tolerance assay comparing quiescent and proliferating UPEC. (A) Percent survival of quiescent CFT073 on M9 minimal media + 0.2% glucose (1.06%) and actively proliferating CFT073 on LB (0.00%) exposed to 3.125 μg ml^{-1} tetracycline for 20 h. (B) Percent survival of quiescent CFT073 on M9 minimal media + 0.2% glucose (5.10 %) and actively proliferating CFT073 on LB (0.86 %) 5 μg ml^{-1} azithromycin for 20 h. (*p* value, *** ≤ 0.001) Represented are averages from ten biological replicates, error bars represent standard errors of the means (SEM).

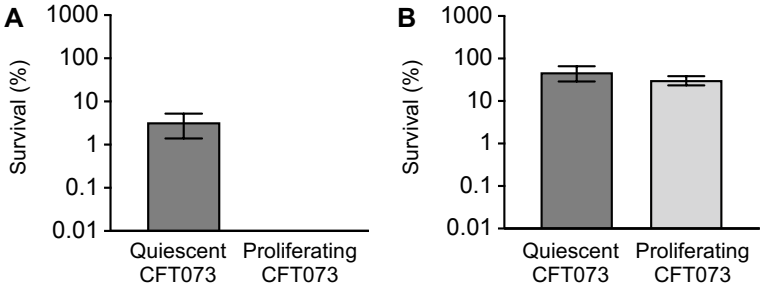
A
1000
100
10
1
0.1
0.01
Survival (%)
Quiescent CFT073
Proliferating CFT073
B
1000
100
10
1
0.1
0.01
Survival (%)
Quiescent CFT073
Proliferating CFT073

Figure 6. Ciprofloxacin antibiotic tolerance assay and nalidixic acid antibiotic tolerance assay comparing quiescent and proliferating UPEC. (A) Percent survival of quiescent CFT073 on M9 minimal media + 0.2% glucose (3.30%) and actively proliferating CFT073 on LB (<0.01%) exposed to 15.6 ng ml^{-1} ciprofloxacin for 20 h. (B) Percent survival of quiescent CFT073 on M9 minimal media + 0.2% glucose (47.44%) and actively proliferating CFT073 on LB (31.19%) 7.5 µg ml^{-1} nalidixic acid for 20 h. Represented are averages from ten biological replicates, error bars represent standard errors of the means (SEM).

A

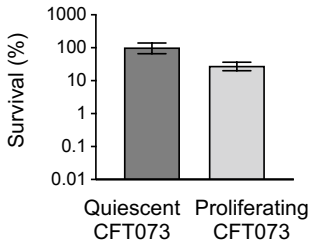
1000
100
10
1
0.1
0.01
Survival (%)
Quiescent CFT073
Proliferating CFT073

Figure 7. Rifampin antibiotic tolerance assay comparing quiescent and proliferating UPEC. Percent survival of quiescent CFT073 on M9 minimal media + 0.2% glucose (103.6%) and actively proliferating CFT073 on LB (28.44%) exposed to 12.5 μg ml^{-1} rifampin for 20 h. Represented are averages from ten biological replicates, error bars represent standard errors of the means (SEM).

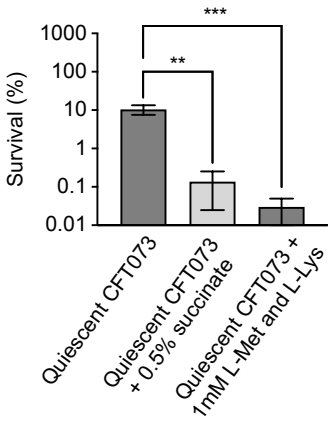
***
**
1000
100
10
1
0.1
0.01
Survival (%)
Quiescent CFT073
Quiescent CFT073 + 0.5% succinate
Quiescent CFT073 + 1mM L-Met and L-Lys

Figure 8. Antibiotic tolerance assay using antibiotic combined with a proliferant molecule. Percent survival of quiescent CFT073 on M9 minimal media + 0.2% glucose treated with 6.25 μg ml^{-1} trimethoprim for 20 h (10.49%), quiescent CFT073 subsequently treated with 0.5% succinate and 6.25 μg ml^{-1} trimethoprim for 20 h (0.14%), and quiescent CFT073 subsequently treated with 1mM L-Met and L-Lys and 6.25 μg ml^{-1} trimethoprim for 20 h (0.03%). (*p* value, ** ≤ 0.01; *** ≤ 0.001) Represented are averages from five biological replicates, error bars represent standard errors of the means (SEM).

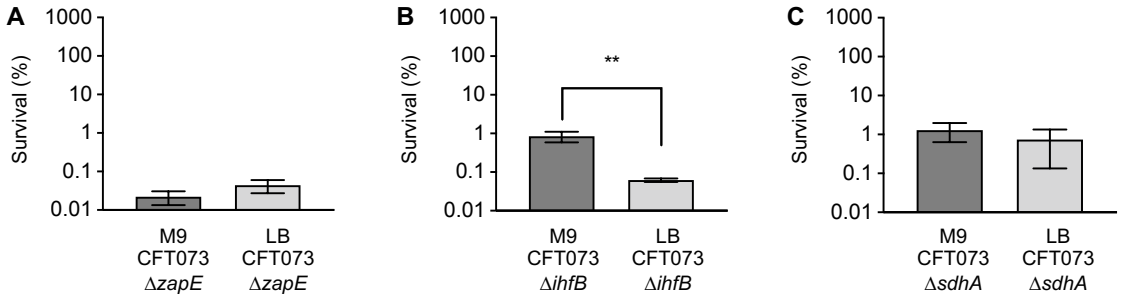
A
B
C
1000
100
10
1
0.1
0.01
Survival (%)
**
M9
CFT073
ΔzapE
LB
CFT073
ΔzapE
M9
CFT073
ΔihfB
LB
CFT073
ΔihfB
M9
CFT073
ΔsdhA
LB
CFT073
ΔsdhA

Figure 9. Trimethoprim antibiotic tolerance assay comparing non-quiescent mutants (Δ*zapE,* Δ*ihfB*, and *sdhA*::Tn5*)* on low nutrient and nutrient rich media. (A) Percent survival of CFT073 Δ*zapE* on M9 minimal media + 0.2% glucose (0.02%) and CFT073 Δ*zapE* on LB (0.04%) exposed to 6.25 μg ml^{-1} trimethoprim for 20 h. (B) Percent survival of CFT073 Δ*ihfB* on M9 minimal media + 0.2% glucose (0.85%) and CFT073 Δ*ihfB* on LB (0.06%) exposed to 6.25 μg ml^{-1} trimethoprim for 20 h. (C) Percent survival of CFT073 *sdhA*::Tn5 on M9 minimal media + 0.2% glucose (1.31%) and CFT073 *sdhA*::Tn5 on LB (0.74%) exposed to 6.25 μg ml^{-1} trimethoprim for 20 h. (*p* value, ** ≤ 0.01) Represented are averages from five biological replicates, error bars represent standard errors of the means (SEM).

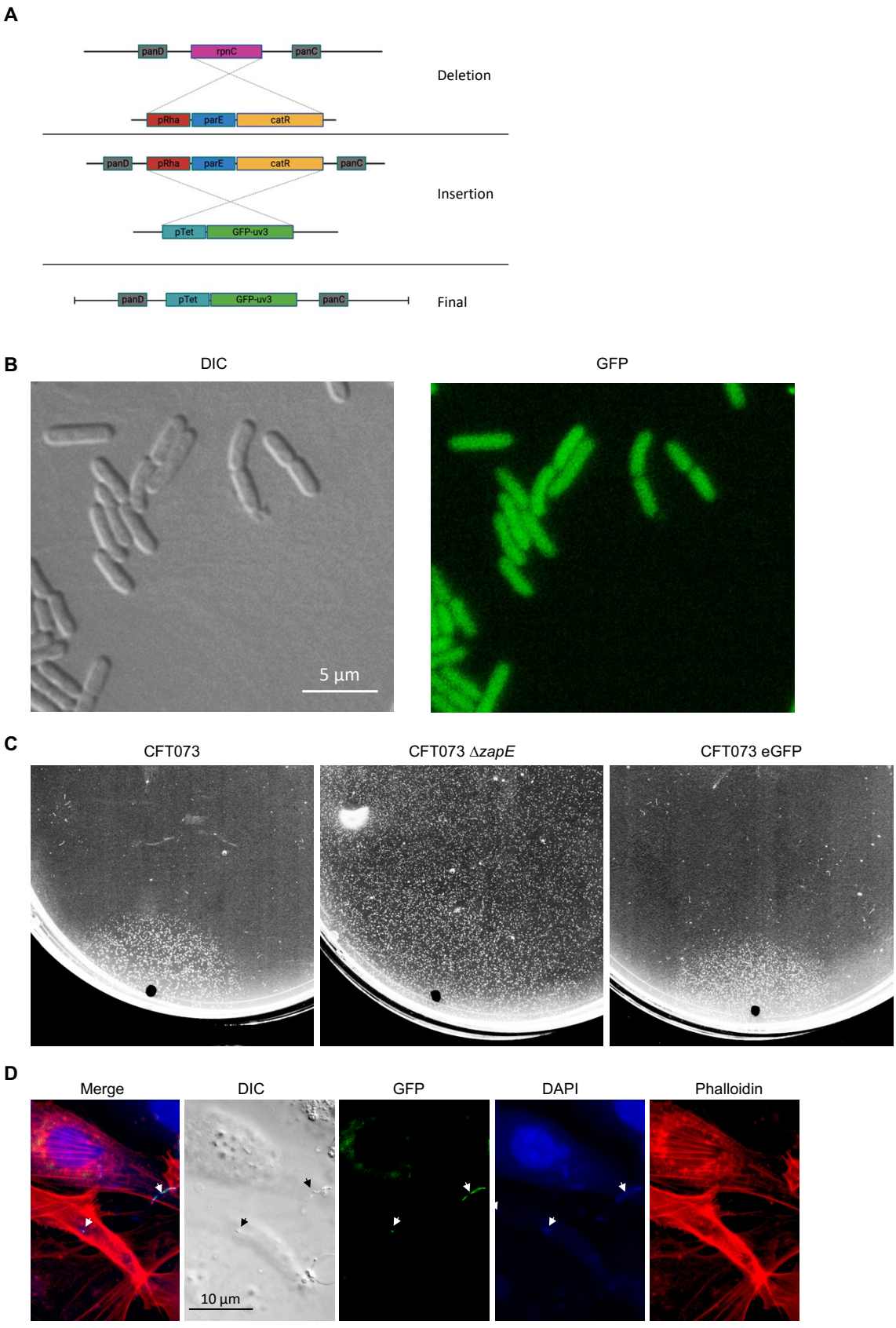
A
panD
rpnC
panC
Deletion
pRha
parE
catR
panD
pRha
parE
catR
panC
Insertion
pTet
GFP-uv3
panD
pTet
GFP-uv3
panC
Final
B
DIC
GFP
5 µm
C
CFT073
CFT073 ΔzapE
CFT073 eGFP
D
Merge
DIC
GFP
DAPI
Phalloidin
10 µm

Figure 10. Strain construction, validation, and invasion microscopy of UPEC reporter strain CFT073 *gfp*$_{uv3}$ colonization of HTB-4 BECs. (A) Illustration showing CFT073 *gfp*$_{uv3}$ reporter strain construction steps and the final gene construct on the chromosome. (B) Differential interference contrast (DIC) and fluorescence microscopy images of reporter strain CFT073 *gfp*$_{uv3}$ (ex. 488nm/em. 507nm). (C) CFT073, CFT073 Δ*zapE*, and CFT073 *gfp*$_{uv3}$ were plated at 10^4 CFU ml^{-1} on 0.2% glucose minimal agar and incubated at 37 °C for 24 h. (D) Fluorescence image overlay of fixed HTB-4 BECs infected with CFT073 *gfp*$_{uv3}$ (ex. 488nm/em. 507nm), actin stained with phalloidin-594 (ex. 555nm/em. 609nm) and DNA stained with DAPI (ex. 405/em. 461nm), 72 h post-infection. DAPI staining shown in Blue, Phalloidin-594 shown in red, and GFP_{uv3} in green. DIC microscopy of fixed HTB-4 BECs infected with CFT073 *gfp*$_{uv3}$, 72 h post infection.

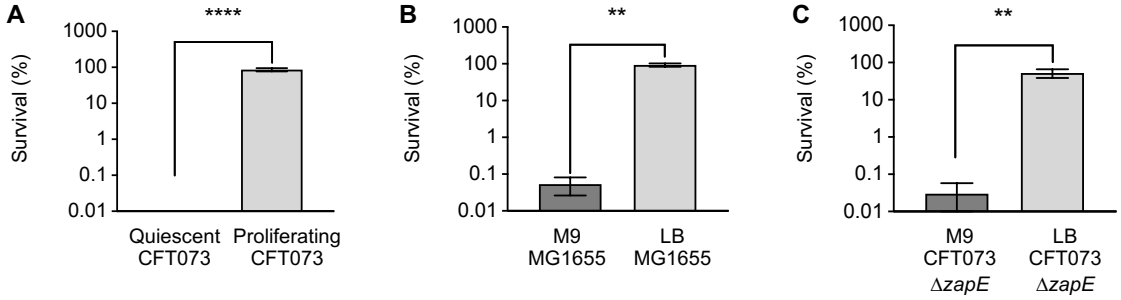
A
****
Survival (%)
1000
100
10
1
0.1
0.01
Quiescent CFT073
Proliferating CFT073
B
**
Survival (%)
1000
100
10
1
0.1
0.01
M9 MG1655
LB MG1655
C
**
Survival (%)
1000
100
10
1
0.1
0.01
M9 CFT073 ΔzapE
LB CFT073 ΔzapE

Figure S1. Nitrofurantoin antibiotic tolerance assay comparing quiescent and proliferating UPEC, MG1655 in low nutrient and nutrient rich media, and a mutant UPEC strain defective for quiescence in low nutrient and nutrient rich conditions. (A) Percent survival of quiescent CFT073 on M9 minimal media + 0.2% glucose (0.00%) and actively proliferating CFT073 on LB (86.82%) exposed to 6.25 μg ml^{-1} nitrofurantoin for 20 h. (B) Percent survival of MG1655 on M9 minimal media + 0.2% glucose (0.05%) and MG1655 on LB (92.83%) exposed 6.25 μg ml^{-1} nitrofurantoin for 20 h.(C) Percent survival of CFT073 Δ*zapE* on M9 minimal media + 0.2% glucose (0.03%) and CFT073 Δ*zapE* on LB (52.54%) 6.25 μg ml^{-1} nitrofurantoin for 20 h. (*p* value, ** ≤ 0.01; **** ≤ 0.0001) Represented are averages from at least five biological replicates, error bars represent standard errors of the means (SEM).