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Exploring the *in situ* evolution of nitrofurantoin resistance in clinically derived uropathogenic *Escherichia coli* isolates

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Background: Nitrofurantoin has been re-introduced as a first-choice antibiotic to treat uncomplicated acute urinary tract infections in England and Wales. Highly effective against common uropathogens such as *Escherichia coli*, its use is accompanied by a low incidence (<10%) of antimicrobial resistance. Resistance to nitrofurantoin is predominantly via the acquisition of loss-of-function, step-wise mutations in the nitroreductase genes *nfsA* and *nfsB*.

Objective: To explore the *in situ* evolution of Nit^R in *E. coli* isolates from 17 patients participating in AnTIC, a 12-month open label randomized controlled trial assessing the efficacy of antibiotic prophylaxis in reducing urinary tract infections (UTIs) incidence in clean intermittent self-catheterizing patients.

Methods: The investigation of Nit^R evolution in *E. coli* used general microbiology techniques and genetics to model known Nit^R mutations in Nit^S *E. coli* strains.

Results: Growth rate analysis identified a 2%–10% slower doubling time for nitrofurantoin resistant strains: Nit^S: 20.8 ± 0.7 min compared to Nit^R: 23 ± 0.8 min. Statistically, these data indicated no fitness advantage of evolved strains compared to the sensitive predecessor (*P*-value = 0.13). Genetic manipulation of *E. coli* to mimic Nit^R evolution, supported no fitness advantage (*P*-value = 0.22). In contrast, data argued that a first-step mutant gained a selective advantage, at sub-MIC (4–8 mg/L) nitrofurantoin concentrations.

Conclusion: Correlation of these findings to nitrofurantoin pharmacokinetic data suggests that the low incidence of *E. coli* Nit^R, within the community, is driven by urine-based nitrofurantoin concentrations that selectively inhibit the growth of *E. coli* strains carrying the key first-step loss-of-function mutation.

Introduction

Nitrofurantoin is a broad-spectrum antibiotic that has been used since the mid-1950s to manage uncomplicated urinary tract infections (UTIs).[1] With the introduction of trimethoprim and modern β-lactam antibiotics its popularity waned in the 1970s.[1,2] However, due to increased antimicrobial resistance the 2015 England and Wales NICE recommendations replaced trimethoprim with nitrofurantoin as the front-line antibiotic treatment for uncomplicated lower UTIs. [3] Reassuringly, increases in nitrofurantoin prescriptions have not only been associated with decreased trimethoprim resistance, but also with no apparent changes in nitrofurantoin resistance, consistently, below 10%. [4]

Nitrofurantoin is effective against a variety of common uropathogenic bacterial species, including *E. coli*, *Enterococcus* spp., *Enterobacter* spp. and *Klebsiella* spp.[5,6] It has been established that many of these uropathogens carry genes encoding oxygen insensitive nitroreductases, which convert nitrofurantoin into electrophilic intermediates that attack bacterial ribosomal proteins, thereby inhibiting protein synthesis and facilitating microbial death.[5] When nitrofurantoin is present at high concentrations, these intermediates can also interfere in nucleic acid synthesis and aerobic metabolism targeting the citric acid cycle.[5] Bacterial nitroreductases are not essential proteins, allowing for the acquisition of chromosomally derived loss-of-function mutations generating nitrofurantoin resistance (Nit^R).[7] In *E. coli*, the

inactivation, via deletion or point mutation, of the genes *nfsA* and *nfsB*, is the common route to Nit^R.[7] Other pathways leading to Nit^R include the horizonal acquisition of the efflux system *oqxAB* or chromosomal mutations in *ribE*.[8]

Inactivation of *nfsA* and *nfsB* follows a two-step evolutionary pathway: *nfsA* before *nfsB*.[9] Supporting this evolutionary pathway, genetic surveillance studies of Nit^R uncovered *nfsA*$^-$ *nfsB*$^+$ isolates, but seldom the reciprocal *nfsA*$^+$ *nfsB*$^-$ combination.[8,10] Sandegren *et al.* provided insight into potential factors contributing to the low incidence of Nit^R among *E. coli* isolates suggesting the growth rate of Nit^R isolates to be 3%–6% slower compared to Nit^S isolates.[11] These authors concluded there was a fitness cost to uro-associated *E. coli* of inactivating *nfsA*/*nfsB*.[11] This loss of fitness may impact the establishment of resistant isolates in the urinary tract, explaining the low incidence of nitrofurantoin resistance among urinary *E. coli* isolates.[11]

While genome surveillance studies support Nit^R being linked to point mutations and deletions of the *nfsA*/*nfsB* genes,[8,10] they are unable to explain the factors that drive resistance phenotype selection. Understanding these factors is key to informing prescribing guidelines that underpin UTI treatments and future urology antibiotic stewardship programmes. AnTIC was an open label randomized controlled trial that assessed the efficacy of antibiotic prophylaxis in reducing the incidence of symptomatic UTIs in clean intermittent self-catheterized patients over a period of 12 months.[12] Forty-five isolates from 17 patients experiencing persistent uro-associated *E. coli* colonization were analysed by WGS [13] and five isolates from two patients (PAT1646 and PAT2015) supported Nit^R acquisition during the trial period. Clinical data associated with these cases confirmed exposure to nitrofurantoin. One case provided an opportunity to investigate the *in situ* evolutionary dynamics driving nitrofurantoin resistance and address aspects of the Nit^R phenotype that underpin the low incidence of Nit^R observed among community isolates of *E. coli*.

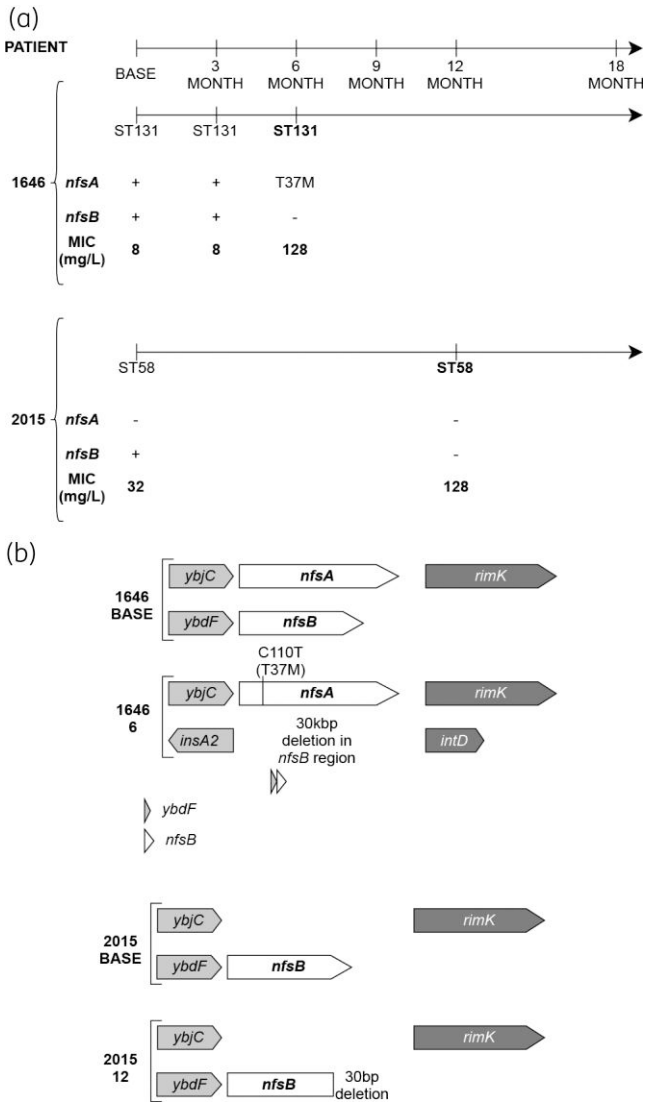

Figure 1. Acquisition of the Nit^R phenotype in PAT1646 and PAT2015. (a) Timeline for each patient indicating the temporal isolation of each strain, the sequence type identified via MLST genotyping and the MIC for each isolate. (b) *nfsA* and *nfsB* genotype for the BASE and Nit^R isolates from PAT1646 and PAT2015.

Materials and methods

Bacterial strains

Strains used or constructed in this study are shown in the Supplementary materials (Table S1, available as Supplementary data at *JAC* Online). Antibiotics for selection were used at concentrations described previously.[14]

pBKK plasmid construction

Five pBKK plasmids carrying different DNA constructs of the *nfsA* or *nfsB* region (*nfsA* 1646B, Δ*nfsA*, Δ*nfsA-rimK*, Δ*rimK* and Δ*nfsB*) were generated using Gibson assembly, with pBKK as the vector backbone. The pBKK vector was constructed using Gibson assembly, with pBlueScript II SK (Ampicillin resistant) as the vector backbone and the kanamycin resistance gene from pKD4 as the insert. All plasmids were sequenced and primers are defined in Table S2.

Mutant generation

nfsA/*nfsB* mutants were generated using a two-step recombination strategy involving the lambda-red and CRISPR systems.[15,16] The mutagenesis protocol has been previously described.[17] During CRISPR mutagenesis, *cat* was replaced with a DNA construct carrying a modified version of the *nfsA*/*nfsB* region (Δ*nfsA*, Δ*nfsA-rimK*, Δ*rimK*, *nfsA* T37M, Δ*nfsB*, Δ*nfsB30*). Where necessary the target mutant was amplified from the pBKK plasmids or directly from chromosomal DNA (*nfsA* T37M and Δ*nfsB30*). Double mutants were generated by first replacing the *nfsA* region with the appropriate DNA construct followed by the *nfsB* region. Confirmation of mutants was conducted through colony PCR and sequencing.

MIC and growth assay and analysis

Using standard microbiology protocols MIC and growth curve assays were performed using sterile Greiner Bio-One clear 96-well flat-bottom plates. Technical details are reported in the Supplementary material. For MIC assays, no-growth was defined as an average optical density (OD_{600}) reading over three independent repeats as <0.05. If an OD_{600} >0.05 was identified, the MIC was taken as the next upper concentration. The

maximum doubling time of each isolate was derived from the maximum slope calculated using a moving 'frame' of eight timepoints using basic R coding.[18] Statistical analysis of the doubling times was performed using either *t*-tests or ANOVA. For *t*-tests, the Bonferroni correction was considered to determine the threshold *P*-value for significance.

Viability and competition assays

Overnight bacterial cultures were diluted to achieve a starting density of 50–100 cfu/mL in 6 mL of MHB media and incubated for 16 hours at 37°C. Cultures were plated onto CPSE plates, incubated overnight at 37°C, photographed and colonies counted with the image processing package ImageJ. Competition assays were performed using the same protocol with adjusted volumes to incorporate the addition of two strains. Visual differentiation between strains was achieved by deleting *uidA*, the β-glucuronidase that leads to *E. coli* being red on CPSE plates. The competitive index was defined as $\log_{10}$(strain A cfu/mL: strain B cfu/mL).

Ethical approval

Permission to use clinical isolates and data from the AnTIC clinical study was derived from prior ethical approvals (ethics 19/NS/0024, IRAS Project ID 243903, Ref. 2586/2016).[13]

Results

In situ development of nitrofurantoin resistance amongst clinical E. coli isolates

Forty-five (45) uro-associated isolates from 17 AnTIC patients with persistent *E. coli* colonization were previously analysed using WGS.[13] Five isolates from two patients (PAT1646 and PAT2015) provided examples that supported *in situ E. coli* acquisition of nitrofurantoin resistance following exposure to the antibiotic. MLST and cgMLST genotyping confirmed stable *E. coli* colonization in both patients (Figure 1a). PAT1646 was colonized by ST131, whereas PAT2015 was colonized by ST58 (Figure S1). The Nit^S (MIC 8 mg/L) baseline isolate of PAT1646, 1646 BASE, possessed wild-type *nfsA* and *nfsB* genes (Figure 1b). The 6-month isolate, 1646 6, was Nit^R (MIC 128 mg/L: Figure 1a) and had a T37M point mutation in *nfsA* and a complete deletion of *nfsB*, spanning 30 kb upstream of the start codon. The baseline isolate of PAT2015, 2015 BASE, was Nit^S (MIC 32 mg/L) and carried a complete deletion of *nfsA*, but an intact, wild-type *nfsB* (Figure 1b). The 12-month isolate, 2015 12, was Nit^R (MIC 128 mg/L) having gained a partial deletion in *nfsB* (Figure 1b).

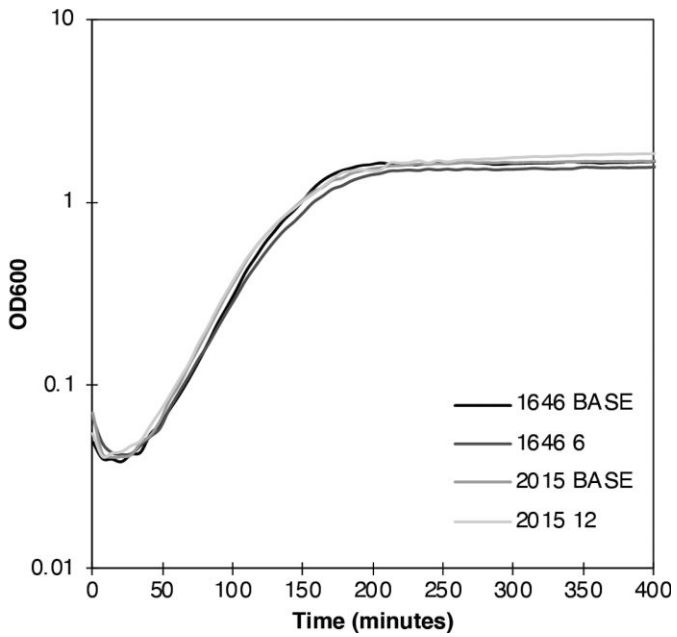

Figure 2. Growth characteristics of the BASE and Nit^R isolates from PAT1646 and PAT2015. The differences between the doubling times are consistent with previous studies of Nit^R but are not statistically significant (ANOVA $P=0.22$). All data was calculated from $n=24$ independent repeats of growth of each strain.

Growth kinetics of the BASE strains, and isolates 1646 6 and 2015 12, resulted in comparable growth curves (Figure 2), which suggested no difference in bacterial fitness. Calculation of the doubling time were comparable for all four strains: 1646 BASE: 20.8 ± 0.7 min; 1646 6 23 ± 0.8 min; 2015 BASE: 21.0 ± 0.7 and 2015 12: 21.4 ± 0.7 (ANOVA *P*-value = 0.13). In the absence of antibiotic, competition between 1646 BASE and 1646 6 was minimal, reflected by a calculated competitive index of 0.22 ± 0.16. These data therefore argued against a fitness advantage playing a significant role in driving the acquisition of Nit^R in UTI patients treated with nitrofurantoin.

Generating nfsA and nfsB mutants from Nit^S isolates

PAT1646 *E. coli* isolates represent a chronological 'snapshot' of the same strain before and after developing nitrofurantoin resistance (Figure 1). To investigate the underlying advantage/disadvantage of acquiring the Nit^R phenotype, mutants in *nfsA* and/or *nfsB*, were generated in 1646 BASE and the Nit^S laboratory *E. coli* strain W3110, a commonly used 'wild-type' model strain (Figure S2).[19] Comparing two isolates of contrasting genetic backgrounds (clinical versus laboratory), provided an opportunity to identify phenotypic effects, if any, of Δ*nfsA*/Δ*nfsB* combinations that were dependent or independent of genetic background. Mutants were generated by targeted mutagenesis rather than *in vitro* selection to minimize the acquisition of spontaneous, compensatory mutations linked to nitrofurantoin exposure, which could distort the phenotypic behaviour of Δ*nfsA*/Δ*nfsB* mutations.

rimK is in an operon with *nfsA* (Figure 1b) and encodes an enzyme involved in the post-translational modification of ribosomal protein S6 via the addition of glutamate residues.[20] There is evidence of clinical Nit^R isolates carrying partial deletions in *rimK*, therefore Δ*rimK* and Δ*nfsA-rimK* mutations were also created (Figure S2).[13] The flexibility of CRISPR–Cas technology allowed 1646 BASE to be engineered to model the impacts of the 1646 6 *nfsA* mutation (*nfsA* T37M) and the 30 kb *nfsB* deletion (Δ*nfsB30*). However, only *nfsA* T37M was modelled in W3110 (Figure S2).

Characterization of ΔnfsA/ΔnfsB mutants

The Nit^R phenotypes of the strains generated were quantitatively assessed via MIC assays (Figure 3). Isolates with a MIC ≥ 64 mg/L were classified as Nit^R [21,22] and strains 1646 BASE, W3110 and 1646 6 were included as Nit^S and Nit^R controls. Single 1646B mutants with Δ*nfsA* or *nfsA* T37M supported MIC values of 32 mg/L

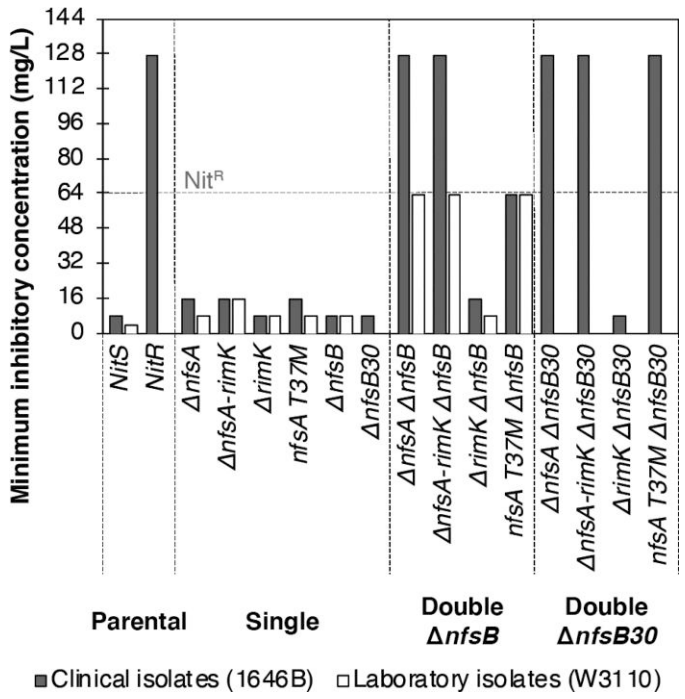

Figure 3. MIC of Δ*nfsA* and Δ*nfsB* mutant combinations in 1646 BASE (filled bars) and the laboratory strain W3110 (open bars). The dashed horizontal line indicates the nitrofurantoin concentration defined as being the point at which strains are defined as clinically resistant to the antibiotic. Data represent a minimum of three independent repeats of the assay for each strain. MIC values represent the nitrofurantoin concentration where the average OD_{600} reached <0.05.

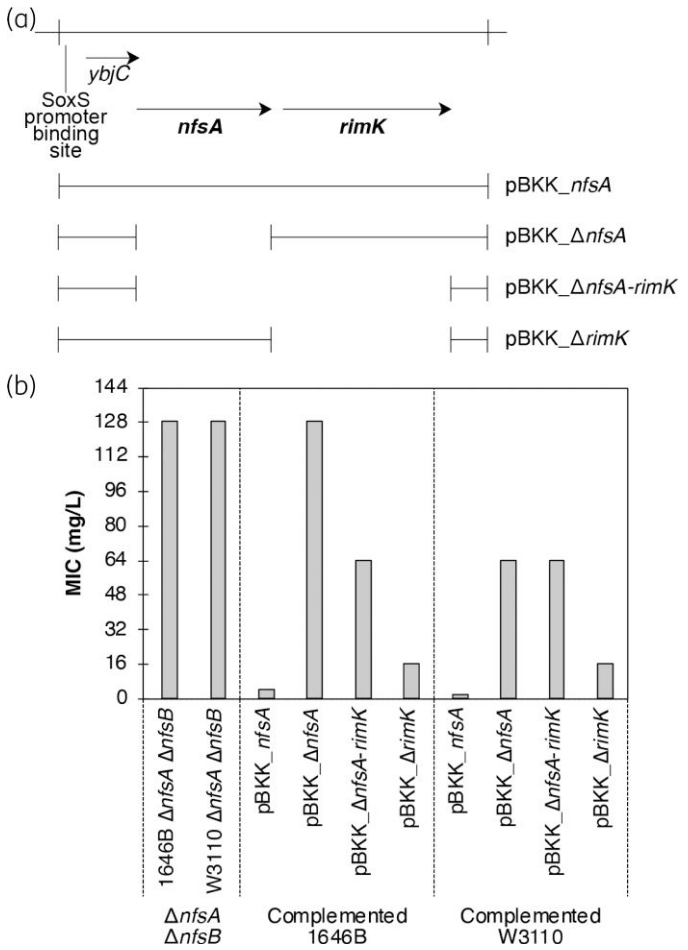

Figure 4. Complementation of Δ*nfsA* with ectopic expression of *nfsA* from pBKK. (a) Schematic diagram of plasmid constructs used in these complementation assays. (b) MIC data for strains used. Data represent a minimum of three independent repeats of the assay for each strain. MIC values represent the nitrofurantoin concentration where the average OD_{600} reached <0.05.

thus still clinically Nit^S (Figure 3). Single mutants Δ*rimK*, Δ*nfsB* and Δ*nfsB30* showed a MIC comparable to their parental background. A similar pattern was observed for the W3110 although the Nit^S MIC of the parent and *nfsA*$^+$ variants was 8 mg/L (Figure 3).

In vitro 1646 6 and its corresponding engineered mutant (*nfsA* T37M Δ*nfsB*) showed MICs of ≥64 mg/L respectively. In contrast, 1646 BASE double mutants in which *nfsA* was inactivated via complete deletion (Δ*nfsA* Δ*nfsB*, Δ*nfsA-rimK* Δ*nfsB*) displayed higher MICs (128 mg/L) (Figure 3). The MICs of the *nfsA*$^+$ Δ*rimK* Δ*nfsB* variants were comparable to their single and parental derivatives (16 or 8 mg/L). Collectively, these data indicated that only single mutants with inactivated *nfsA* had a modest 2-fold MIC increase when compared to their parental isolate while double mutants, including Δ*nfsA*, had substantially higher MICs.

Plasmid-based complementation of Nit^R ΔnfsA ΔnfsB mutants

To verify that inactivating *nfsA* was critical in the acquisition of Nit^R, the engineered 1646 BASE and W3110 Δ*nfsA* Δ*nfsB* mutants were transformed with modified pBlueScript II SK (pBKK) plasmids carrying genetic variants of the *nfsA* region (Figure 4a). The pBKK empty vector was used as a negative control. MIC assays were performed on transformed strains (Figure 4b). Complementation was only observed when pBKK plasmids carried functional *nfsA*. Consistent with the role that *nfsA* plays in the Nit^R phenotype, complementation led to a strong 32-fold reduction in MIC compared to the negative control (Figure 4b).

Fitness of nfsA/nfsB mutants

The relative fitness of the mutants was determined by maximum bacterial growth rate in terms of doubling time, with an assumed increase in fitness corresponding to shorter doubling times. In the absence of nitrofurantoin (0 mg/L), there was no significant difference in doubling time between any of the mutants and their corresponding parental isolates (ANOVA 1646 BASE: $P=0.22$; W3110: $P=0.46$) (Tables 1 and 2). Pairwise *t*-test analysis using a Bonferroni corrected significance threshold of $P=0.0009$ agreed with the ANOVA analysis (Tables S3 and S4). These data are consistent with the initial growth analysis of 1646 and 2015 isolates (Figure 2)

ANOVA analysis indicated that at 4 and 8 mg/L nitrofurantoin, there was significance within the bacterial growth data sets (*P*-value range <0.0001–0.02) (Tables 1 and 2) with pairwise

Table 1. Doubling times of mutants and clinical isolates relating to PAT1646 with or without nitrofurantoin

	Nitrofurantoin concentration (mg/L)[a],b							
Strains	0	2	4	8	16	32	64	128
1646 BASE	21.9±0.9	24.7±0.7	29.3±1.1	***43.2±1.8***				
1646 6	25.7±1.1	25.1±0.6	24.5±1.2	25.3±1.1	27.9±0.9	35.2±1	***142±11.3***	
1646B Δ*nfsA*	24.2±0.6	24.3±0.9	23.6±1	31±2	***60.1±1.6***			
1646B Δ*nfsA-rimK*	23.7±0.9	26.2±1	25.4±1.3	32.2±1.9	***64.7±2.4***			
1646B *nfsA* T37M	22.5±0.8	24.2±0.9	24.1±1	30.3±2.1	***55.4±2.2***			
1646B Δ*rimK*	24.1±1.2	25.3±0.4	28.3±1.3	***45.9±1.4***				
1646B Δ*nfsB*	22.2±1.1	25.9±0.8	29.6±1	***45±0.9***				
1646B Δ*rimK* Δ*nfsB*	21.8±1.8	25.3±1.2	30.5±0.8	***43.7±1.6***				
1646B Δ*nfsA* Δ*nfsB*	25.7±0.4	25.6±0.9	25±0.5	24.3±1	26.6±0.3	34.2±1.3	***90.5±5.9***	
1646B Δ*nfsA-rimK* Δ*nfsB*	21.9±1.6	23.4±0.8	22.6±0.7	23.4±1.3	27.7±0.9	33.7±0.8	***88±3.4***	
1646B *nfsA* T37M Δ*nfsB*	24.2±0.8	25±1.1	24.7±0.5	27.7±1	28.7±0.5	37.6±0.9	***131.6±10.8***	

[a]Italics and bold identify significant changes in the doubling time (Table S3).
[b]Data relate to growth, while the MIC is defined as the first concentration having an OD_{600} < 0.05.

Table 2. Doubling times of mutants and the parental strain W3110 with or without nitrofurantoin

	Nitrofurantoin concentration (mg/L)[a],b							
Strains	0	2	4	8	16	32	64	128
W3110	34.9±0.8	37.8±1	37.7±1.2	***74.7±2.9***				
W3110 Δ*nfsA*	32.7±1.7	35.4±1.2	37.5±1.8	***50.9±2.6***				
W3110 Δ*nfsA-rimK*	33.1±1.3	35.3±1.1	40.4±1.6	***50.4±2.1***				
W3110 *nfsA* T37M	29±2.3	35.8±1.1	38.8±1.3	***51.9±2.4***				
W3110 Δ*rimK*	33.8±1.2	40.3±1.9	44.4±2	***79.5±5.6***				
W3110 Δ*nfsB*	31.5±3.1	34.9±1.7	41.5±1.4	***73.7±3.2***				
W3110 Δ*rimK* Δ*nfsB*	35.8±2.1	37.3±1.4	39.9±2.1	***72±4.3***				
W3110 Δ*nfsA* Δ*nfsB*	31±1.8	33.9±0.8	36±2.1	44.6±1.6	39±2.7	54.4±1.9	***117.2±5.8***	
W3110 Δ*nfsA-rimK* Δ*nfsB*	32.5±1	33.4±0.9	34±2	37.4±1.6	32.8±2.1	50.8±1.4	***97±7.7***	
W3110 *nfsA* T37M Δ*nfsB*	34.4±0.5	37.7±1.3	38.2±2	44.1±0.8	40.3±1.3	***56.7±1.5***		

[a]Italics and bold identify significant changes in the doubling time (Table S4).
[b]Data relate to growth, while the MIC is defined as the first concentration having an OD_{600} < 0.05.

comparisons using *t*-tests identifying specific trends. For example, at 4 mg/L nitrofurantoin an increase in doubling time from 21.9±0.9 to 29.3±1.1 min was observed for the parental strain 1646 BASE with comparable increases for *nfsB* and *rimK* single mutants (Table 1). This increase became statistically significant across all comparisons at 8 mg/L where a doubling time of 43–46 min was observed (Table 1: italics+bold and Table S3). The same trend was observed for W3110, which already had a lower intrinsic resistance to nitrofurantoin (Table 2 and Table S4). At concentrations >8 mg/L, most of these strains were unable to grow indicating that bacterial selection, rather than fitness, was the driving factor.

Similar data for the Δ*nfsA*, double mutants and the control strain 1646 6 was observed at higher concentrations of nitrofurantoin (Table 1). Interestingly, the higher the concentration of antibiotic, the stronger the impact on growth with, for example, 64 mg/L leading to a 5-fold increase in doubling time for 1646 6. The 1646 BASE Δ*nfsA* Δ*nfsB* variant exhibited a 3.5-fold increase in doubling time at this concentration, consistent with the difference defined by the MIC analysis.

Viability of nfsA/nfsB mutants

Growth experiments were initiated with an inoculation of between 0.01–0.05 OD_{600}. This inoculum is equivalent to ~1–5×10^6 cfu/mL, 1 log greater than the diagnostic threshold of microbes detected per millilitre of urine in an acute UTI (1×10^5 cfu/mL).[23] It is well recognized that growth experiments can be influenced by the inoculum effect.[24] Therefore, further growth analysis using a starting inoculum of 50–100 cfu/mL in 0 to 16 mg/L nitrofurantoin was investigated focussing on the viability of the 1646 BASE engineered strains rather than growth kinetics.

All strains tested grew well with or without 2 mg/L nitrofurantoin (Table 3). The 1646 BASE strain did not grow at 4 mg/L while the Δ*nfsB* mutant showed a significant (~6 log-fold) reduction in

Table 3. Viability of test strains in low inoculum growth experiments with sub-MIC concentrations of nitrofurantoin

	Nitrofurantoin concentration (mg/L)				
Strains	0	2	4	8	16
1646 BASE	1.05E+09	1.50E+08	0	0	0
1646 6	6.67E+08	4.03E+08	4.63E+08	2.88E+08	1.56E+08
ΔnfsA	8.37E+08	1.83E+08	3.13E+08	1083	0
nfsA T37M	7.20E+08	5.13E+08	4.36E+08	737	0
ΔnfsB	1.22E+09	3.87E+08	627	0	0
ΔnfsA ΔnfsB	5.47E+08	2.80E+08	2.53E+08	2.62E+08	9.67E+07
nfsA T37M *ΔnfsB*	6.80E+08	2.73E+08	4.50E+08	2.96E+08	1.63E+08

viability (Table 3). Furthermore, single mutants of Δ*nfsA* and *nfsA* T37M behaved in a similar manner, but at double the antibiotic concentration i.e. 8 mg/L. All strains that possessed a Nit^R phenotype grew well at all nitrofurantoin concentrations tested. These data strengthen the argument for selective advantage over fitness for step-wise *nfsA*$^-$ intermediates.

Discussion

High level nitrofurantoin resistance requires the inactivation of two genes, which are located so far apart from each other (287 kb distance) that the likelihood of both being simultaneously inactivated through a single natural genetic event is non-existent.[9] Hence inactivation of these genes is far more likely to occur in a step-wise manner, starting with *nfsA* followed by *nfsB*.

To explore the evolutionary mechanism(s) underpinning antibiotic resistance Nit^S and Nit^R uro-associated *E. coli* isolates recovered from UTIs patients treated with nitrofurantoin were exploited. These strains were unique as they reflected *in situ* evolution of *E. coli* over 6 to 12 months in a clinical environment from Nit^S *nfsAB*$^+$ (PAT1646) or *nfsA*$^-$*nfsB*$^+$ (PAT2015) genotypes to resistant genotypes.[12,13] Consistent with the literature, the predominant mutations leading to Nit^R were deletions, although 1646 BASE to 1646 6 evolved Nit^R via the acquisition of a point mutation in *nfsA*: T37M. MIC and growth data provided strong evidence that T37M significantly reduced or inactivated NfsA. However, the behaviour of *nfsA* T37M *ΔnfsB* variants with respect to growth in sub-MIC conditions argues a low level of activity may be retained (Tables 1 and 2). Data also suggested that the method by which *nfs* genes were inactivated (Δ*nfsA*, *nfsA*T37M, Δ*nfsB*, Δ*nfsB*30) did not alter nitrofurantoin resistance with ≥64 mg/L MIC being observed for all combinations. Essentially, gene inactivation *per se* was more important than the mode of inactivation.

Sandegren *et al.* argued that fitness of Nit^R strains plays a key role in driving the low incidence of community AMR to nitrofurantoin.[11] While the bacterial growth data reported in this study were consistent with Sandegren *et al.*, in that a small change in doubling times (2%–10%) was observed, statistical analysis (ANOVA and pairwise *t*-tests) argued that these growth changes were not significant and, alone, could not explain AMR. Moreover, growth analysis relating to 1646 BASE and W3110 strains and their genetic variants in the presence of sub-MIC Nitrofurantoin concentration supports our conclusion against fitness playing a major role in Nit^R acquisition rates (Tables 1 and 2). Bacterial resistance has traditionally been linked to the ability of isolates to grow in antibiotic concentrations that exceed the MIC of sensitive isolates. However, several studies have isolated resistant mutants from growth conditions where antibiotic concentrations were low enough for the proliferation of sensitive isolates.[25–27]

Sub-MIC selective pressure may be a key factor in driving antibiotic resistant phenotypes to successfully establish themselves within a population. However, selective pressures will be determined not only by the pharmacodynamics of an antibiotic, but also by the genetic background of a bacterial species/strain. Findings reported here suggest that the nitrofurantoin selective window in relation to UTIs and uro-associated *E. coli* Nit^R, is wide (>8 mg/L), but this argument is only applicable when comparing Nit^R double mutants against their Nit^S parental isolates. From an evolutionary perspective, this comparison is biased as data suggests the emergence of Nit^R mutants is highly dependent on the ability of intermediate mutants with low level nitrofurantoin resistance to establish themselves within a population of sensitive isolates. In fact, comparing the growth of mutants (Δ*nfsA*, *nfsA* T37M) modelling these intermediate scenarios to their parental isolates (1646 BASE and W3110), argues for a very narrow selective window (4–8 mg/L). Despite these strong selective pressures favouring growth, bacteria may still fail to establish themselves in the urinary environment due to bladder voiding.[28]

Clinically, urinary nitrofurantoin concentrations rarely fall to within this narrow concentration range, which limits the selection of *nfsA* mutants.[29] For example, extrapolating the urinary nitrofurantoin concentration data from Huttner *et al.* suggests the average urine concentration following a standard 50-mg dose may fluctuate between 20 and 40 mg/L during an 8-hour period.[29] Essentially, these conditions inhibit intermediate mutants such as Δ*nfsA* and *nfsA* T37M establishing clinically, which in turn suppresses the emergence of double and hence Nit^R mutants. Therefore, the rarity of Nit^R intermediate mutants undermines the emergence of nitrofurantoin resistance in *E. coli*, explaining the low incidence of resistance.

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A.T., J.H. and P.D.A have no conflicts of interest to declare. M.V. has acted as a consultant for GSK outside the focus of this work, has received speakers fees from Astellas, IPSEN, Mylan and is on an Advisory board for IBSA PHARMA. C. H. has received speakers fees from Astellas, Allergan and Medtronic and is on Advisory boards for Astellas, Teleflex Medical, GSK, Viatris and Convatec. C.H. is also a member of the European Association of urology Female LUTS committee (chair) and the ICS Urodynamic, Mesh complications and Male LUTS committee.

Supplementary data

Figures S1 and S2 and Tables S1 to S4 are available as Supplementary data at *JAC* online.

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