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Nitrofurantoin resistance mechanism and fitness cost in *Escherichia coli*

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***Objectives*: The biological fitness cost of antibiotic resistance is a key parameter in determining the rate of appearance and spread of antibiotic-resistant bacteria. We identified mutations conferring nitrofurantoin resistance and examined their effect on the fitness of clinical *Escherichia coli* isolates.**

***Methods*: By plating bacterial cells on agar plates containing nitrofurantoin, spontaneous nitrofurantoin-resistant *E. coli* mutants were isolated. The fitness of susceptible and resistant strains was measured as growth rate in the presence and absence of nitrofurantoin in rich culture medium. Time–kill kinetics of the resistant mutants was compared with the susceptible strains. Resistance mutations were identified by DNA sequencing.**

***Results*: Spontaneous resistant mutants of initially susceptible clinical *E. coli* appeared with a rate of 10^{-7}/cell/generation, and these mutants showed a reduction in the growth rate compared with the susceptible parent strain. Similarly, comparison of a set of susceptible and resistant clinical isolates of *E. coli* showed that the average growth rate of the resistant mutants was ~6% lower than the susceptible strains. Furthermore, the bacterial growth rate in the presence of nitrofurantoin at therapeutic levels was greatly reduced even for nitrofurantoin-resistant mutants. The resistance-conferring mutations were identified in the *nsfA* and *nfsB* genes that encode oxygen-insensitive nitroreductases.**

***Conclusions*: Nitrofurantoin resistance confers a reduction in fitness in *E. coli* in the absence of antibiotic. In the presence of therapeutic levels of nitrofurantoin, even resistant mutants are so disturbed in growth that they are probably unable to become enriched and establish an infection.**

Keywords: urinary tract infections, growth inhibition, clinical isolates

Introduction

Nitrofurans are a group of compounds characterized by the presence of one or more nitro-groups on a nitroaromatic or nitroheterocyclic backbone. Examples of compounds belonging to this group include furazolidone, nitrofurazone and nitrofurantoin: drugs that all display antimicrobial activity and are used clinically to treat different types of infections. Nitrofurantoin is taken orally, rapidly absorbed and excreted in the urine to generate high therapeutic concentrations.[1] Nitrofurantoin is prescribed for use against uncomplicated lower urinary tract infections (UTIs) in, on average, 7% of the cases in Western Europe and Canada, but prescription varies greatly between countries as exemplified by Spain (1%) and the Netherlands (27%).[2]

Although the specific mode of action of nitrofurans is still unknown, studies of *Escherichia coli* extracts have shown that strains resistant and susceptible to nitrofurans differ in their ability to reduce the compounds,[3,4] suggesting that nitrofurans need to be activated by this reducing activity to show their antibiotic effect. Furthermore, it has been shown that there are two types of nitroreductase activities in *E. coli*: one type insensitive to oxygen (type I) and the other inhibited by oxygen (type II). Chemical analyses indicate that in type I nitroreduction, the nitro-moiety of the parent compound is reduced via a sequence of intermediates, including a nitroso and hydroxylamine state, to a biologically inactive end product where one of the intermediates is thought to be responsible for toxicity.[5] The specific identity of the bio-reactive intermediate is not known, but it binds

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and disrupts bacterial DNA and protein and is believed to be hydroxylamine.[5–7]

Isolation *in vitro* of mutants resistant to nitrofurans has shown that increasing resistance is caused by step-wise mutations, where increased resistance is accompanied by a decrease in the activity of their reductive capacity.[6,8–12] Sequential increase in resistance was genetically shown to result from sequential inactivation of the different nitro-reducing activities present in *E. coli*. The mutations were genetically mapped and named *nfsA* and *nfsB*.[9,10] The direct link between these genes, and the sequential loss of nitro-reducing activity, was established by mutant isolation and sequencing of *nsfA* and *nsfB*.[12] Nitrofuran resistance has been mapped only to type I nitroreductase genes,[8,10,12] and *in vitro*-isolated single-step mutations have been found only in the *nfsA* gene.[10,12] Resistant strains (clinical isolates and *in vitro*-isolated) have been found to have increased susceptibility under anaerobic conditions, which is thought to be because the oxygen-sensitive reduction system is active under anaerobic conditions.[8,10,12]

E. coli is the causative agent in ~90% of all lower UTIs, and since for the most commonly used antibiotics, aminopenicillins, fluoroquinolones, co-trimoxazole and trimethoprim there are increasing problems with resistance,[2,13] further investigations into the mechanisms and effects of resistance to other less commonly used antibiotics are required. In Europe, clinical resistance to nitrofurantoin is observed in, on average, 1.2% of the cases, where the frequencies of resistance are the highest in Portugal (5.8%) and Spain (4.2%) and lowest in Finland (0.5%) and Sweden (0%).[13] Bacterial strains are considered to be resistant to nitrofurans if they display an MIC value greater than 32 mg/L, and susceptible strains have MICs below 16 mg/L.

Resistance to antibiotics is most often accompanied by a biological cost, observed as a decrease in fitness, i.e. a reduced growth rate or virulence.[14,15] The biological cost plays a vital role in determining the extent and rate by which a resistant mutant can appear and spread within a population. Furthermore, in the absence of selective antibiotic pressure, a resistant mutant can be outcompeted by its susceptible relatives because of its lower fitness. This is particularly relevant for the bladder and urinary system that follows a sequence of filling and emptying, where a uropathogen has to grow fast enough to maintain itself and not be flushed out and disappear. This can be mathematically modelled, and the growth rate required for continued maintenance in the bladder is referred to as the Gordon–Riley criterion.[16] In this report, we investigate the mechanism and rate of formation of mutations to nitrofurantoin resistance in *E. coli* and characterize the fitness and properties of *in vitro*-isolated resistant strains and clinical isolates. The results suggest that the reduction in the bacterial growth rate caused by nitrofurantoin at therapeutic levels will prohibit the establishment of an infection even for nitrofurantoin-resistant mutants.

Materials and methods

Clinical isolates resistant to nitrofurantoin

In a study published in 2000,[13] 2478 isolates of *E. coli* from lower UTIs in women were examined. These isolates were obtained between 1999 and 2000 in 16 European countries and Canada, and were tested for resistance to 12 antibiotics commonly used to treat UTIs, one of which was nitrofurantoin. Twenty-one nitrofurantoin-resistant isolates and 24 susceptible isolates obtained from the previous study were examined here.

In vitro isolation of nitrofurantoin-resistant strains

Nitrofurantoin-resistant mutants of susceptible clinical strain DA10708 were isolated by plating 2×10^8 cfu from 20 independent cultures on 20 separate LB-agar plates containing 12 mg/L nitrofurantoin (2 × MIC), and colonies representing different sizes were isolated after 24, 48 and 72 h of incubation at 37°C. Mutants were verified on LB agar plates containing the same amount of nitrofurantoin, and subsequent overnight cultures were frozen in 10% dimethyl sulfoxide. Double-step mutants were isolated by plating 2×10^8 cfu from 20 independent cultures of strains DA13815 and DA13824 on LB agar plates containing nitrofurantoin 48 mg/L (3–4 × MIC). Mutants were isolated after 24 h at 37°C, verified on 48 mg/L plates, restreaked and frozen. The isolation of triple-step mutants was attempted by plating 5×10^8 cfu from 20 independent cultures of two-step mutant strains DA13957 (MIC 128 mg/L) and DA13992 (MIC 192 mg/L) on LB agar plates containing 200 and 500 mg/L antibiotic. No mutants were detected on either concentration after 96 h of incubation.

Mutation frequency determination

The mutation frequency of bacteria to nitrofurantoin resistance was calculated by the median method of Lea and Coulson[17] as the median of the number of colonies on the 20 antibiotic plates after 24 h divided by the number of cfu plated on each plate as determined by a viable count of five cultures chosen at random.

MIC measurement

MICs of mutants were measured using Etest from AB Biodisk, as described by the manufacturer. Aerobic MICs were determined at 37°C in normal atmosphere, while anaerobic MICs were determined at 37°C with plates incubated in anaerobic jars using the Becton Dickinson BBL GasPak Plus system.

Mutant prevention concentration (MPC) measurement

MPC was established by plating 3×10^{10} cfu of strain DA10708 from five independent cultures on LB agar plates containing 48, 64 and 96 mg/L nitrofurantoin. After 96 h, visible colonies were examined by restreaking on the concentration of antibiotic they had been isolated from to examine their putative resistance.

Growth rate measurements

Growth rates of strains were measured at 37°C in LB, with 0.2% glucose with or without antibiotic present, using a BioscreenC reader (Labsystems), taking measurements at OD_{600} every 4 min. The calculation was based on OD_{600} values between 0.02 and 0.1, where growth was observed to be exponential. The relative growth rate was calculated as the derived growth rate divided by the growth rate of DA10708 from the same experiment.

Time–kill kinetics

The killing kinetics of nitrofurantoin for DA10708, two first-step *in vitro*-isolated mutants (DA13815 and DA13824), two second-step *in vitro* mutants (DA13957 and DA13992) and two clinical resistant

isolates (DA10626 and DA10627) were investigated. A tube containing LB medium (1 mL) was inoculated with 3×10^6 cells at an overnight culture and supplemented with 0, 32, 64, 128, 256 and 512 mg/L nitrofurantoin and incubated at 37°C. Viable counts were performed prior to the addition of antibiotic and subsequently determined on the samples 2, 4 and 8 h after antibiotic addition.

DNA sequencing

Genes of interest were PCR amplified using primer sequences selected in the area 100–150 bp upstream and downstream of the coding sequence. When a gene could not be amplified with these primers, primer sequences from ~600 bp upstream and downstream of the gene were used. PCR products were then purified from solution using the Illustra GFX DNA and Gel Band Purification Kit (GE Healthcare), lyophilized with one of the amplification primers and sent to MWG-Biotech in Martinsried, Germany, for sequencing.

Primers used for amplification and sequencing were as follows: *E. coli nfsA*: Fwd 5′-TTTTCTCGGTGTTTTGCTCA-3′, Fwd1 5′-ATTTTCTCGGCCAGAAGTGC-3′, Fwd2 5′-TCTTGCCCCACA GCTGATG-3′, Rev 5′-GCTGTATAGCGGCTTCACG-3′, Rev1 5′-A GAATTTCAACCAGGTGACC-3′, Rev2 5′-CTTACACGAATAGA GCGTTCC-3′; *E. coli nfsB*: Fwd 5′-CCCGCTAAATCTTCAACCT G-3′, Fwd1 5′-CAACAGCAGCCTATGATGAC-3′, Fwd2 5′-TGCA AATCAGGAGAATCTGAG-3′, Rev 5′-AAAAGAGTGCGTCCAG GCTA-3′, Rev1 5′-CTTCGCGATCTGATCAACG-3′, Rev2 5′-TGG TCTGGCTAAACGCGATC-3′.

Results

Isolation of mutants

We isolated a set of nitrofurantoin-resistant mutants *in vitro* by plating independent overnight cultures of the clinical *E. coli* isolate DA10708 on LB agar plates supplemented with nitrofurantoin 12 mg/L. A subset of these isolates is listed in Table 1. The number of visible colonies on the plates showed a typical Luria–Delbruck distribution of mutation occurrence. Using the median number of colonies per plate, the mutation frequency to nitrofurantoin resistance was calculated to be 1.8×10^{-7}/cell and generation. Mutants were also selected at higher antibiotic concentrations, and it was found that mutants could be isolated from plates with nitrofurantoin up to 48 mg/L, but not above that concentration level, even when plating as many as 10^{11} cells. The MPC value was therefore established as 64 mg/L. We then used two of the *in vitro*-isolated mutant strains, DA13815 and DA13824, as starting strains for the isolation of second-step mutants with higher levels of resistance. The third-step mutant selection was attempted using two second-step mutants (DA13957 and DA13992) as starting strains. However, no third-step mutants could be isolated on either nitrofurantoin 200 or 500 mg/L.

Characterization of mutants

Along with the set of *in vitro*-isolated nitrofurantoin-resistant mutants, we acquired a set of clinical isolates of *E. coli* strains isolated from patients with uncomplicated lower UTIs, representing 21 nitrofurantoin-resistant and 24 -susceptible strains. The aerobic MIC for nitrofurantoin was tested both for the *in vitro*-selected mutants (Table 1) and for the clinical isolates (Table 2). The MIC for the susceptible strain DA10708 that was used for selecting *in vitro* mutants was 6 mg/L, and the median MIC for the susceptible clinical strains was 14 mg/L. The MIC for resistant clinical strains ranged from 32 to 192 mg/L, with a median value of 96 mg/L. First-step *in vitro*-isolated resistant mutants showed a median MIC of 8 mg/L and ranged from 8 to 16 mg/L, and were therefore not classified as clinically resistant. The median MIC for both sets of double mutants, however, were 96 mg/L (Table 1), the same as for the clinically resistant isolates tested here. Thus, in two selection steps, even the susceptible bacteria with the lowest MIC can gain an MIC similar to the highest level clinically resistant isolates.

The MIC in an anaerobic environment was also measured for all clinical isolates and a subset of the *in vitro* mutants representing the whole range of aerobic MICs (Tables 1 and 2). MICs were consistently lower anaerobically. DA10708 showed an MIC of 3 mg/L, and the non-resistant clinical isolates of *E. coli* were distributed between 3 and 6 mg/L. Interestingly, the *in vitro*-isolated mutants not only displayed a much lower MIC anaerobically, but were also in fact similar, 1.5–6 mg/L, to the parental DA10708. This was not the case for the clinical isolates that showed anaerobic MICs between 8 and 256 mg/L with a median value of 24 mg/L, which is lower than the aerobic values but clearly higher than that for the susceptible isolates.

We also investigated the bactericidal effect of nitrofurantoin on the susceptible strain DA10708, the two first-step *in vitro*-isolated mutants DA13815 and DA13824, the two second-step *in vitro*-isolated strains DA13957 and DA13992 and the two clinical isolates DA10626 and DA10627. We incubated 3×10^6 cfu of each strain in 1 mL LB supplemented with 0, 32, 64, 128, 256 and 512 mg/L nitrofurantoin and determined the viability of the culture as a function of time (Figure 1). For DA10708 and for the first-step *in vitro*-isolated mutants, a bactericidal effect was observed already at 32 mg/L, whereas for the second-step *in vitro* mutants, growth was completely inhibited at 128 mg/L and a bactericidal effect was observed at 256 mg/L and above. The clinical isolates of *E. coli* were able to grow in concentrations up to 128 mg/L and showed a moderate killing above that.

Identification of resistance mutations in in vitro-isolated mutants

Prior investigations of nitrofurantoin resistance in *E. coli* have identified the genes *nfsA* and *nfsB* as important resistance determinants.[10,12] We sequenced the single-step mutants in the coding sequences and 150 bp upstream and downstream of the coding sequences of *nfsA* and *nfsB* to identify potential resistance mutations.

No sequence changes were found in the *nfsB* genes for the single-step mutants. Instead, all single-step *in vitro* isolates were found to have a mutation in *nfsA*. For some strains, no PCR product could be obtained with any of the primer combinations, suggesting the presence of larger rearrangements in the *nfsA* gene in these strains. For mutants where a PCR product could be obtained, sequence changes were located within the coding sequence of the gene and consisted of deletions, substitutions and insertions, and almost all changes resulted in either a nonsense or frame-shift mutation (Table 1). Although no measurement of nitroreductase activity was performed on the

Table 1. *In vitro*-isolated nitrofurantoin-resistant mutants

Strain	MIC (mg/L)		Growth rate	*nfsA* (*240 aa*)	*nfsB* (*217 aa*)
	aerobic	anaerobic			
First-step mutants					
DA13815	16	2	1.01	Del C356 (frame-shift A119)	*wt*
DA13816	8	1.5	0.99	large deletion	*wt*
DA13819	8	2	0.98	R15C	*wt*
DA13822	12	3	1.01	W77Stop	*wt*
DA13824	12	2	1.02	Del 399–483 (frame-shift N134)	*wt*
DA13826	8	2	0.98	W212Stop	*wt*
DA13916	12	2	1.01	large deletion	*wt*
DA13917	8	2	1.04	Y45C	*wt*
DA13918	12	1.5	0.96	Del C199 (frame-shift Q67)	*wt*
DA13919	8	2	1.00	large deletion	*wt*
DA13921	8		1.02	Ins G380 (frame-shift V127)	*wt*
DA13922	12		1.00	large deletion	*wt*
DA13923	16		0.95	large deletion	*wt*
DA13924	8		0.97	Del 399–483 (frame-shift N134)	*wt*
DA13926	12		1.07	R203L	*wt*
DA13927	8		1.03	large deletion	*wt*
DA13928	8		1.02	large deletion	*wt*
DA13929	8		1.04	Ins C440 (frame-shift Q147)	*wt*
DA13930	8		1.04	Del 364–376 (frame-shift L122)	*wt*
DA13932	8		1.07	Del 148–162 (Del 50–54)	*wt*
DA13934	12		1.05	Del A338 (frame-shift M113)	*wt*
DA13935	16		1.03	Del T128 (frame-shift L43)	*wt*
Second-step mutants					
DA13936	128		1.01	Del C356 (frame-shift A119)	G166D
DA13938	64		0.89	Del C356 (frame-shift A119)	R107H
DA13941	96		0.97	Del C356 (frame-shift A119)	Q44L
DA13942	96		1.00	Del C356 (frame-shift A119)	Del A223 (frame-shift K74)
DA13944	128		0.96	Del C356 (frame-shift A119)	Del T635 (frame-shift N211)
DA13946	96		0.98	Del C356 (frame-shift A119)	G192A
DA13947	64	4	0.98	Del C356 (frame-shift A119)	Y183Stop
DA13950	96		1.00	Del C356 (frame-shift A119)	large deletion
DA13952	96		1.02	Del C356 (frame-shift A119)	V93G
DA13954	48	3	1.00	Del C356 (frame-shift A119)	Del 229–273 (Del 79–93)
DA13955	96	3	1.00	Del C356 (frame-shift A119)	large deletion
DA13957	192	3	1.00	Del C356 (frame-shift A119)	Del C130 (frame-shift S43)
DA13961	96	3	1.01	Del C356 (frame-shift A119)	large deletion
DA13990	96	3	0.99	Del 399–483 (frame-shift N134)	Del 259–339 (Del 86–112)
DA13991	96	4	0.90	Del 399–483 (frame-shift N134)	W94Stop
DA13992	128	6	0.97	Del 399–483 (frame-shift N134)	Q101Stop
DA13993	96	4	0.95	Del 399–483 (frame-shift N134)	Del 303–470 (Del 102–157)
DA13994	96	4	0.96	Del 399–483 (frame-shift N134)	Del 545–546 (frame-shift G182)
DA13995	48		0.96	Del 399–483 (frame-shift N134)	W138C
DA13998	96		0.94	Del 399–483 (frame-shift N134)	large deletion
DA14001	96		0.95	Del 399–483 (frame-shift N134)	large deletion
DA14002	96		0.97	Del 399–483 (frame-shift N134)	large deletion
DA14003	96		1.00	Del 399–483 (frame-shift N134)	Del G466 (frame-shift A156)
DA14004	64		0.97	Del 399–483 (frame-shift N134)	W94Stop
DA14005	128		0.99	Del 399–483 (frame-shift N134)	large deletion
DA14006	96		1.03	Del 399–483 (frame-shift N134)	large deletion
DA14007	64		0.99	Del 399–483 (frame-shift N134)	E165A
DA14008	128		0.97	Del 399–483 (frame-shift N134)	G192D

wt, wild-type.

Table 2. Clinical *E. coli* isolates

Strain	MIC (mg/L)		Growth rate	Inactivating mutations	
	aerobic	anaerobic		*nfsA* (*240 aa*)	*nfsB* (*217 aa*)
Nit-resistant					
DA10623	48	8	0.95	Del 231–239 (aa 77–79)	nd
DA10624	96	12	0.86	nd	nd
DA10625	128	32	1.02	Y181Stop	—
DA10626	128	256	0.57	nd	nd
DA10627	192	96	0.99	start ATG to ATT	—
DA10628	192	16	0.98	Del 231–239 (aa 77–79)	nd
DA10630	96	16	0.95	R203C	Q101Stop
DA10631	128	24	1.02	Del 281–284 (frame-shift P94)	Del 110–121 (aa 37–40)
DA10634	64	48	1.00	nd	—
DA10635	96	48	1.02	Q195Stop	Del T501 (frame-shift F167)
DA10636	96	32	1.04	Del 376–463 (frame-shift G126)	Del 333–363 (frame-shift E112)
DA10637	96	24	0.99	Q182Stop	K9Stop
DA10638	128	24	0.95	Q147Stop	Ins IS5 (1199 nt) after nt 43
DA10639	192	24	0.99	G131D	Del A632 (frame-shift N211)
DA10640	64	24	0.96	R15C	—
DA10641	48	24	0.98	W77Stop	—
DA10642	48	48	0.92	Ins IS1 (767 nt) after nt 420	—
DA10643	128	16	1.04	nd	nd
DA10644	64	12	1.02	Del A381 (Y128Stop)	start ATG to ATA
DA10645	48	32	0.99	Ins Tn10 (1339 nt) after nt 286	—
DA10646	32	32	1.04	S33R	—
Nit-sensitive					
DA10629	8	4	1.01	—	—
DA10687	16	6	1.03	—	—
DA10688	16	4	0.92	N/A	N/A
DA10689	16	6	1.04	N/A	N/A
DA10690	16	4	0.96	N/A	N/A
DA10691	16	6	1.05	N/A	N/A
DA10692	16	6	1.00	N/A	N/A
DA10693	16	4	1.05	N/A	N/A
DA10694	12	6	1.07	—	—
DA10695	8	3	0.94	—	—
DA10696	12	4	1.02	—	—
DA10697	16	6	1.04	N/A	N/A
DA10698	12	4	1.08	—	—
DA10699	16	4	1.00	N/A	N/A
DA10700	24	6	1.02	N/A	N/A
DA10701	8	3	1.08	—	—
DA10702	6	6	1.08	—	—
DA10703	16	4	1.07	N/A	N/A
DA10704	8	3	1.11	—	—
DA10705	12	4	1.06	—	—
DA10706	16	6	1.03	N/A	N/A
DA10707	8	6	1.05	—	—
DA10708	6	3	1.00	—	—
DA10709	12	4	0.92	—	—

nd, not determined (failed to amplify).
N/A, not analysed.
—, no deleterious change found.

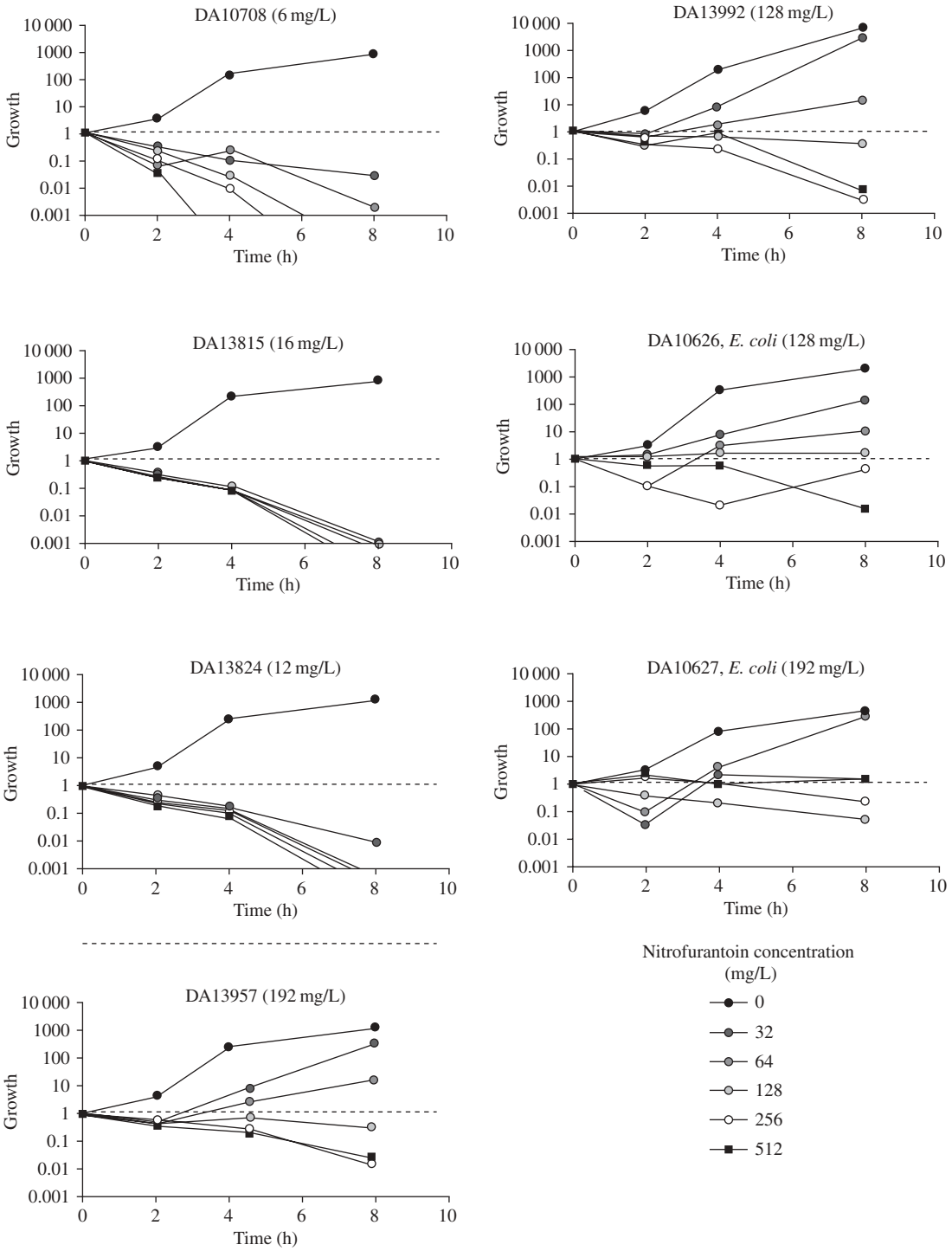

Figure 1. Bactericidal effect of nitrofurantoin. Cell viability over time is plotted for cultures grown at different concentrations of nitrofurantoin. The MIC for each strain is indicated within parentheses. The dotted horizontal line in each graph denotes zero change in viability (bacteriostatic level).

strains, it is likely that these mutations lead to a complete or partial loss of the NfsA nitroreductase activity.

The two nitroreductase genes were also sequenced in the double-step mutants. All double-step strains did contain an additional mutation, deletion or insertion in the *nfsB* gene, apart from the initial *nfsA* mutation (Table 1). As for *nfsA*, several strains did not give a PCR product of the *nfsB* gene with any primer combinations, indicating large rearrangements in the region of the gene. These changes in *nfsB* most likely resulted in a further reduction of the nitroreductase activity in the cell and increased resistance.

Identification of resistance mutations in clinical isolates

The *nfsA* and *nfsB* genes of all resistant *E. coli* clinical isolates and half of the susceptible clinical isolates were PCR amplified

and sequenced (Table 2). As no parental strains were available, only clearly inactivating gene changes or previously characterized resistance mutations can be considered to explain the resistance phenotypes. Sixteen of the 21 resistant isolates contained deletions, insertions or substitutions in *nfsA*, which most likely have an inactivating effect on the enzymatic function or reducing effect on the gene expression. Most of these changes were deletions or mutations, resulting in the premature termination of the protein. Three isolates, DA10630, DA10639 and DA10640, contained substitutions of amino acids of the NfsA active site known to be vital for enzymatic activity.[12,18] Mutations in two of these positions (R15C and R203L) were also identified in *in vitro*-isolated mutants DA13819 and DA13926, respectively. Isolate DA10646 contained a substitution of serine 33 that has not been reported previously to be vital for NfsA function; however, this isolate also had the lowest aerobic MIC of all resistant clinical isolates in this study. In four isolates, the *nfsA* gene could not be amplified with any of the different primer combinations tested, likely indicating larger rearrangements in this region. For *nfsB*, seven strains contained possible inactivating changes, whereas no difference from the susceptible isolates could be found in eight isolates. In five strains, *nfsB* could not be amplified at all. None of the susceptible clinical isolates analysed contained any changes that indicate loss of function for either *nfsA* or *nfsB*.

Five clinical isolates contained putative inactivating changes in both *nfsA* and *nfsB*, and these had aerobic MIC values of 96 mg/L or more. Isolates with changes only in *nfsA* generally had a lower MIC of 48–64 mg/L, with the exception of strains DA10625 and DA10627 which had MICs of 128 and 192 mg/L, respectively, indicating other as yet unknown resistance pathways.

Fitness of mutants

The fitness of all the clinical isolates as well as the *in vitro*-isolated mutants was determined as the growth rate of the tested strain relative to the growth rate of the susceptible strain DA10708 (Tables 1 and 2 and Figure 2). DA10708 had a doubling time of 20.1 min, and the first-step mutant isolates were found to have an average doubling time of 19.8 min (relative growth rate 1.01), not significantly differing from the parental strain DA10708. Both sets of double-step mutants had on average a lower growth rate than DA10708, 1.4% and 3.1%, respectively. Similarly, the clinically isolated resistant *E. coli* strains had a significantly lower growth rate (6% reduction on average) than the clinically isolated susceptible strains. Thus, the average relative growth rate for resistant strains was 0.97 (20.8 min) when compared with 1.03 (19.6 min) (unpaired *t*-test, $P = 0.0095$) for the susceptible. In general, no correlation between MIC value and growth rate in LB for any of the groups of mutants could be established.

We also measured the growth rate of DA10708, first-step *in vitro* mutants DA13815 and DA13824, second-step *in vitro* resistant mutants DA13957 and DA13992 and clinically resistant strains DA10627 and DA10635 at increasing concentrations of nitrofurantoin. Growth was measured at nitrofurantoin concentrations of 0, 2, 5, 10, 50 and 100 mg/L (Figure 3). As expected, the resistant isolates were all less inhibited than the wild type at increasing nitrofurantoin concentrations. Furthermore, the first-step *in vitro* mutant isolates had markedly lower growth rate in the presence of antibiotic, compared with the second-step *in vitro* isolates and the clinical isolates, which is not surprising given the difference in MIC values. The growth rate of DA10708 dropped to zero at 10 mg/L, whereas first-step mutants showed zero growth at 50 mg/L, and both the second-step mutants and clinical isolates showed no growth at 100 mg/L.

The minimal concentration of nitrofurantoin present in the bladder during treatment (200 mg/L)[19] is indicated in Figure 3 by a vertical dotted line. At this concentration, all examined strains, including the resistant isolates, showed zero growth, indicating that at the clinically relevant antibiotic concentrations, none of the strains would show any relevant growth. Furthermore, based on previously modelled bladder dynamics, it has been calculated that bacteria must have a doubling time shorter than 36 min to be able to remain in the bladder and not be flushed out. The corresponding growth rate is marked in Figure 3 by a horizontal dotted line. Thus, in order to sustain an infection in the bladder, the bacteria must display growth in the region of Figure 3 above the horizontal line and to the right of the vertical line. This is clearly not the case for any strain,

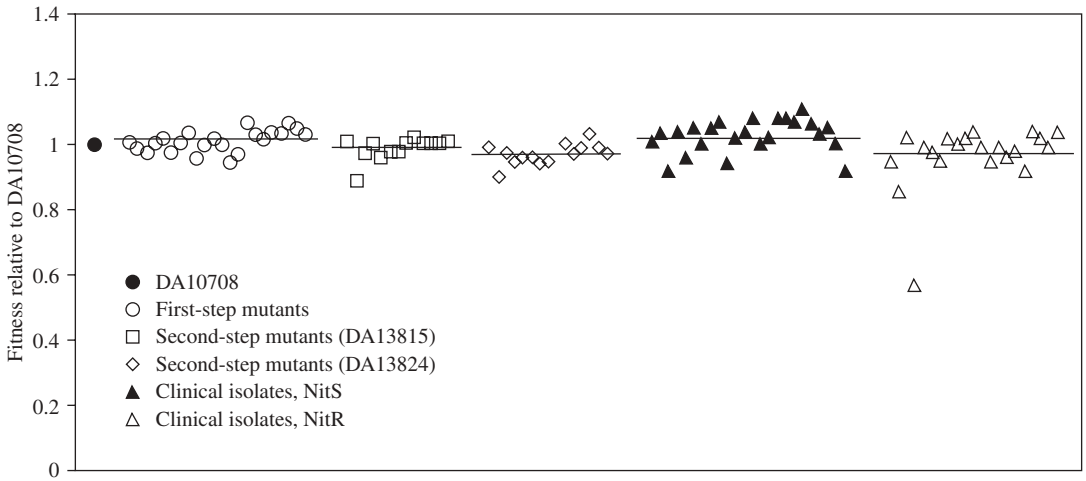

Figure 2. Relative growth rate of isolates when compared with clinically susceptible strain DA10708 that was used as parent for mutation studies and that was set to 1.0. The horizontal line indicates median growth rate for each group.

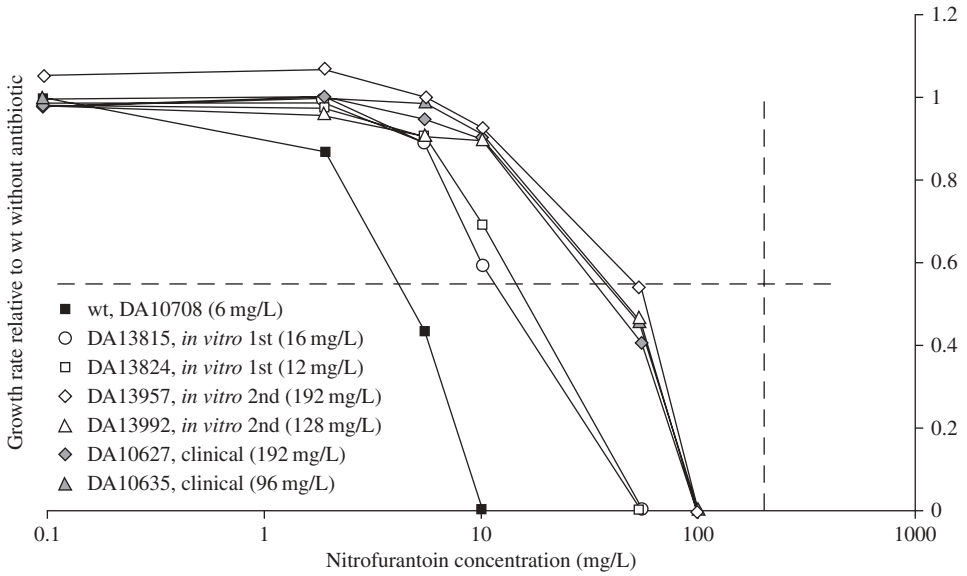

Figure 3. Relative growth rate of isolates as a function of nitrofurantoin concentration. The dotted horizontal line represents the minimum growth rate (36 min doubling time) required for sustained infection according to the Gordon–Riley criterion. The dotted vertical line represents 200 mg/L nitrofurantoin, which is the minimum concentration of nitrofurantoin in the bladder during treatment.[19] The MIC for each strain is indicated within parentheses.

indicating that both susceptible and resistant bacteria would be unable to establish and sustain an infection at therapeutic concentrations of nitrofurantoin.

Discussion

We calculated the mutation frequency to nitrofurantoin resistance for the susceptible clinical strain DA10708 to be $\sim 10^{-7}$/cell and generation. As seen with prior work in *E. coli*, mutations conferring nitrofurantoin resistance were found in the *nfsA* and *nfsB* genes encoding oxygen-insensitive nitroreductases. First-step mutations were found only in *nfsA*, and given that the *nfsA* gene is $\sim$1000 bp long, the per base pair substitution mutation rate in Enterobacteriaceae is around 10^{-10}/cell per generation[20] and that resistance is caused by loss-of-function mutations, the observed mutation rate to resistance was as expected. Most of the two-step mutants listed in Table 1 contained an additional mutation in the *nfsB* gene. This suggests that inactivation of *nfsA* followed by inactivation of *nfsB* is the main mechanism for high-level nitrofurantoin resistance in *E. coli*. The mutations of clinical isolates that cannot be attributed to these nitroreductase genes are at present unknown.

Plasmid-mediated resistance to nitrofurantoin has been described with a moderate increase in MIC (50–70 mg/L), but the mechanism of this resistance remains unknown.[21,22] Whether any of the resistant clinical isolates in this study carry such plasmids is not known; however, our second-step *in vitro*-selected mutants show the same levels of resistance as the resistant clinical isolates, showing that this level of resistance can result from exclusively knocking-out NfsA and NfsB activity.

The lower MIC values observed during the anaerobic growth are most likely explained by the oxygen-sensitive type II nitroreductases, compensating for the lost activity of the type I reductases when oxygen is not present. It is interesting that many resistant clinical isolates still have high MIC values anaerobically. Possibly, they have acquired mutations in oxygen-sensitive reductase genes or other uncharacterized genes, increasing resistance. Resistance without loss of nitroreductase activity has been previously observed,[23,24] and the mechanism could be the same for some of the mutants isolated by Breeze and Obaseiki-Ebor,[8] which did not show a lower nitroreductase activity in spite of their increased resistance. However, this has most likely no clinical relevance as urine is generally quite oxygenated—on average, urine has a dissolved oxygen value of $\sim$4.2 mg/L,[25] and therefore, the most important fitness factor to take into account is the growth rate in the absence and, especially, the presence of nitrofurantoin.

The apparent mutation frequency to nitrofurantoin resistance of 10^{-7} is fairly high, and yet clinically observed resistance and treatment failure are rare. Similar to our previous work on fosfomycin resistance in *E. coli*, this high mutation rate ought to often result in treatment failure,[26] which is, however, not observed. How can this be explained? The growth rate for the second-step *in vitro*-isolated resistant mutants was a few percent lower than that for the parental strain, and the growth rate of the resistant clinical isolates was 6% lower than that of the susceptible isolates. Such a decrease in fitness will certainly impair the ability of the mutants to establish themselves in the bladder. More important, however, is the effect on the growth rate of therapeutic levels (above 200 mg/L[19]) of nitrofurantoin, which will prohibit the selection of resistant mutants according to the Gordon–Riley modelling of bladder dynamics. Thus, even though resistant mutants will appear in the bacterial population in the bladder, they will be unable to become enriched and establish an infection because of their impaired growth at these therapeutic antibiotic concentrations. In those cases where resistant mutants are still selected, we propose that this occurs in patients with poor drug compliance or with suboptimal pharmacokinetics of the drug (e.g. poor absorption and rapid excretion), resulting in lower than expected nitrofurantoin concentrations.

The establishment of breakpoint values is primarily based on the distribution of MICs for members of a supposedly wild-type bacterial population. MICs for nitrofurantoin form a well-defined frequency curve below 32 mg/L[13] with few resistant outliers, indicating that the MIC breakpoint is well defined. Thus, the breakpoint for nitrofurantoin is set to 32 mg/L, meaning that bacterial strains with MICs higher than 32 mg/L are defined as resistant, implying that therapy with the antibiotic should be avoided against such strains. However, our results suggest that at therapeutic concentrations of nitrofurantoin (>200 mg/L), even clinical isolates with the highest levels of resistance (MIC = 192 mg/L) are treatable with a normal dosage regimen. This finding illustrates the difficulties of using a population-based MIC breakpoint as a basis for empirical therapeutic decisions. Furthermore, it is likely that similar reasoning applies for other antibiotics. Thus, factors such as bacterial fitness and growth rates in the absence and presence of antibiotic and the dynamics of the infection (here we used the Gordon–Riley model for bladder dynamics but other types of infections can be modelled following similar principles) greatly affect the therapeutic efficacy of an antibiotic. As resistance is becoming an increasing problem, it ought to be examined whether strains defined as resistant are still treatable with the standard regimen, thus potentially extending the clinical life-time of the antibiotic.

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Transparency declarations

None to declare.

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