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An *In Vitro* Deletion in *ribE* Encoding Lumazine Synthase Contributes to Nitrofurantoin Resistance in *Escherichia coli*

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Nitrofurantoin has been used for decades for the treatment of urinary tract infections (UTIs), but clinically significant resistance in *Escherichia coli* is uncommon. Nitrofurantoin concentrations in the gastrointestinal tract tend to be low, which might facilitate selection of nitrofurantoin-resistant (NIT-R) strains in the gut flora. We subjected two nitrofurantoin-susceptible intestinal *E. coli* strains (ST540-p and ST2747-p) to increasing nitrofurantoin concentrations under aerobic and anaerobic conditions. Whole-genome sequencing was performed for both susceptible isolates and selected mutants that exhibited the highest nitrofurantoin resistance levels aerobically (ST540-a and ST2747-a) and anaerobically (ST540-an and ST2747-an). ST540-a/ST540-an and ST2747-a (aerobic MICs of >64 µg/ml) harbored mutations in the known nitrofurantoin resistance determinants *nfsA* and/or *nfsB*, which encode oxygen-insensitive nitroreductases. ST2747-an showed reduced nitrofurantoin susceptibility (aerobic MIC of 32 µg/ml) and exhibited remarkable growth deficits but did not harbor *nfsA/nfsB* mutations. We identified a 12-nucleotide deletion in *ribE*, encoding lumazine synthase, an essential enzyme involved in the biosynthesis of flavin mononucleotide (FMN), which is an important cofactor for NfsA and NfsB. Complementing ST2747-an with a functional wild-type lumazine synthase restored nitrofurantoin susceptibility. Six NIT-R *E. coli* isolates (NRCI-1 to NRCI-6) from stools of UTI patients treated with nitrofurantoin, cefuroxime, or a fluoroquinolone harbored mutations in *nfsA* and/or *nfsB* but not *ribE*. Sequencing of the *ribE* gene in six intestinal and three urinary *E. coli* strains showing reduced nitrofurantoin susceptibility (MICs of 16 to 48 µg/ml) also did not identify any relevant mutations. NRCI-1, NRCI-2, and NRCI-5 exhibited up to 4-fold higher anaerobic MICs, compared to the mutants generated *in vitro*, presumably because of additional mutations in oxygen-sensitive nitroreductases.

Nitrofurantoin belongs to the group of synthetic nitrofurans and is an antibiotic that has been available for clinical use for more than 60 years (1). Upon oral administration, it is rapidly absorbed in the small intestine and is excreted by the kidneys into urine, where it reaches high therapeutic concentrations (200 µg/ml) (2). For this reason, nitrofurantoin is prescribed solely for the treatment of uncomplicated lower urinary tract infections (UTIs) (1). It is active against a wide spectrum of Gram-positive and Gram-negative bacteria, including most urinary tract pathogens (3). The antibacterial effect of nitrofurantoin is activated by reduction of the drug into toxic intermediate compounds (4). These intermediates can interact with protein and DNA, thereby inhibiting multiple bacterial enzymes involved in DNA, RNA, and protein synthesis and carbohydrate metabolism (1). The reduction of nitrofurantoin is mediated by nitroreductase systems of the bacterial host. In *Escherichia coli*, there are two types of nitroreductases, based on their sensitivities to oxygen, namely, type I (oxygen-insensitive) nitroreductases and type II (oxygen-sensitive) nitroreductases (5). Nitrofurantoin resistance in *E. coli* was shown previously to result primarily from stepwise mutations in *nfsA* and *nfsB* genes, encoding oxygen-insensitive nitroreductases (5, 6). While the majority of these changes constituted insertions of insertion sequence (IS) elements into the genes, deletions and missense mutations have also been described (5). Mutations in genes encoding oxygen-sensitive nitroreductases have not been described as yet.

Although nitrofurantoin has been used for decades, clinically significant resistance in *E. coli* remains uncommon (4). This might

be ascribed to an associated fitness cost, especially in the presence of nitrofurantoin at high therapeutic concentrations in the urine (7). As mentioned earlier, most of the nitrofurantoin is absorbed in the small intestine; however, 6 to 13% of the drug reaches the colon (1, 8). Therefore, due to low concentrations of nitrofurantoin in the gastrointestinal tract, selection of nitrofurantoin-resistant (NIT-R) strains in this niche is highly probable. Additionally, low oxygen levels in the gastrointestinal tract might favor mutations in genes encoding oxygen-sensitive nitroreductases. NIT-R strains were found previously in the fecal flora with very low prevalence rates (0.6 to 2%) (9–11); however, these strains were not investigated molecularly. In this study, two nitrofurantoin-susceptible (NIT-S) intestinal *E. coli* strains were used as parent strains for *in vitro* generation of NIT-R mutants under aerobic and anaerobic growth conditions. Known and novel nitrofurantoin resistance determinants were identified by whole-genome se-

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quencing and *in vitro* confirmational experiments, and the growth rates of these strains were assessed. Finally, we compared the resistance determinants and growth rates of the mutants generated *in vitro* with those of six clinical NIT-R *E. coli* strains recovered from the fecal flora of four UTI patients who were treated with nitrofurantoin, cefuroxime, ciprofloxacin, or norfloxacin.

MATERIALS AND METHODS

Isolation and characterization of *E. coli* strains from stool and urine samples. Six NIT-R *E. coli* strains (MICs of ≥ 64 $\mu\text{g/ml}$) were isolated from stool samples from four UTI patients who were treated with nitrofurantoin, cefuroxime, ciprofloxacin, or norfloxacin. The recovery of these strains was performed as part of a large clinical trial investigating the emergence of antibiotic-resistant *Enterobacteriaceae* in the fecal flora of UTI patients after antibiotic treatment and the spread of these resistant strains to household members (A. Stewardson, N. Adriaensens, M. Godycki-Cwirko, H. Goossens, C. Lammens, S. Malhotra-Kumar, J. Vervoort, and S. Harbarth, unpublished data). Six intestinal and three urinary *E. coli* isolates with reduced nitrofurantoin susceptibility (MICs of < 64 $\mu\text{g/ml}$) were also included in the study. The *E. coli* strains were identified by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry using the Microflex LT system (Bruker Daltonics, Bremen, Germany) with MALDI Biotyper 3.0 software (database version 5627). Routine susceptibility tests were performed by disk diffusion testing (disks from BD BBL, Franklin Lakes, NJ), according to the CLSI guidelines (12), for the following antibiotics: amoxicillin (plus clavulanate), piperacillin (plus tazobactam), cefotaxime (plus clavulanate), cef-tazidime (plus clavulanate), cefepime, cefoxitin, aztreonam, ertapenem, meropenem, ciprofloxacin, fosfomicin, nitrofurantoin, and trimethoprim-sulfamethoxazole. The nitrofurantoin MICs of all investigated strains were determined by agar dilution, and breakpoints were applied according to CLSI guidelines (susceptible, ≤ 32 $\mu\text{g/ml}$; intermediate, 64 $\mu\text{g/ml}$; resistant, ≥ 128 $\mu\text{g/ml}$), utilizing a multipoint inoculator (Multi-pointelite; MAST Group Ltd., Bootle, United Kingdom) (12). The inoculated nitrofurantoin-containing Mueller-Hinton (MH) plates were incubated for 16 to 20 h at 37°C in an O₂ incubator (Thermo Scientific, Waltham, MA) or Bugbox anaerobic workstation (Ruskinn Technology Ltd., Bridgend, United Kingdom), to determine the MICs under aerobic and anaerobic growth conditions, respectively.

***In vitro* generation of NIT-R mutants.** Two NIT-S *E. coli* strains (MICs of 4 to 16 $\mu\text{g/ml}$), of multilocus sequence types (STs) ST540 and ST2747 (ST540-p and ST2747-p, respectively) (as determined from whole-genome sequencing data), were recovered from stool samples from two UTI patients and were used as parent strains for *in vitro* generation of NIT-R mutants under aerobic and anaerobic growth conditions. A maximum of 3 selection rounds were implemented, to allow isolation of both single-step and multistep mutants. In each selection round, 1×10^8 to 2×10^8 CFU of the parent strains were spread homogeneously on MH agar plates supplemented with increasing nitrofurantoin concentrations (0.5 to 4 times the MIC for the parent strain) and were incubated for 24, 48, and 72 h at 37°C under aerobic (O₂ incubator) and anaerobic (Bugbox) growth conditions. Resistant mutants were counted, and 10 to 20 isolates were subcultured on MH agar supplemented with nitrofurantoin, to confirm the resistance phenotype. Aerobic and anaerobic nitrofurantoin MICs were determined by agar dilution, and strains of interest were utilized for the next selection round.

Whole-genome sequencing to identify known and novel nitrofurantoin resistance determinants. Whole-genome sequencing was performed (Pacific Biosciences, Menlo Park, CA) for ST540-p and ST2747-p and for the mutants generated *in vitro* that exhibited the highest nitrofurantoin resistance levels aerobically (ST540-a, with aerobic and anaerobic MICs of 256 and 32 $\mu\text{g/ml}$, respectively, and ST2747-a, with aerobic and anaerobic MICs of 64 and 16 $\mu\text{g/ml}$, respectively) and anaerobically (ST540-an, with aerobic and anaerobic MICs of 128 and 32 $\mu\text{g/ml}$, respectively, and ST2747-an, with aerobic and anaerobic MICs of 32 and 16 $\mu\text{g/ml}$, respec-

tively). Genomic DNA was isolated (MasterPure complete DNA and RNA purification kit; Epicentre, Madison, WI), and DNA concentrations were measured (NanoDrop; Thermo Scientific). Library preparation and sequencing reactions were performed using a PacBio DNA template prep kit 2.0 (3 kb to 10 kb) and a PacBio DNA sequencing kit 2.0 with C2 chemistry. Sequence runs for six single-molecule real-time (SMRT) cells were performed with a PacBio RS II sequencer with a 1 by 180-min movie time SMRT cell. SMRT Analysis Portal 2.0 was used for filtering of the reads and subreads, with default parameters, and filtered data of ~ 320 Mb for each cell per strain were considered for assembly. The genomes were assembled using the hierarchical genome assembly process (HGAP), which is available with the SMRT Analysis packages and was accessed through SMRT Analysis Portal 2.0 (13).

The genomes from ST540-p, ST540-a, and ST540-an and from ST2747-p, ST2747-a, and ST2747-an were compared and checked for genomic rearrangements using Mauve 2.3.1 (14). Single-nucleotide polymorphisms (SNPs) were identified by Show-SNPs, a script associated with MUMmer 3.23 (15). The presence of IS elements or mutations in *nfsA* and *nfsB* and other genomic changes that might potentially be in as yet unknown resistance determinants were confirmed by PCR sequencing using target-specific primer pairs (primer sequences available on request) (7).

Validation of novel nitrofurantoin resistance determinant. The contribution of lumazine synthase to nitrofurantoin resistance was evaluated by complementing the lumazine synthase mutant generated *in vitro* (ST2747-an) with a functional wild-type lumazine synthase allele. A 559-bp fragment containing the coding sequence of wild-type *ribE*, including 65-bp upstream and 23-bp downstream regions (GenBank accession no. CP007392; bases 1080502 to 1081060) (13), was prepared by PCR amplification, and the PCR fragment was cloned in a pCR2.1 vector by Topo TA cloning (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. The resulting recombinant vector, LMZ-pCR2.1, was electrotransformed in ST2747-an, which was made electrocompetent by successive washing steps with an ice-cold 10% glycerol suspension at log phase. Transformants were selected on LB agar supplemented with kanamycin (50 $\mu\text{g/ml}$) and were screened for the presence of the correct insert by PCR sequencing using M13 primers. The presence of functional and/or mutated lumazine synthase was confirmed by PCR detection using an internal primer set that resulted in PCR fragments with a 12-nucleotide size difference (primer sequences for lumazine synthase primers available on request). Aerobic and anaerobic nitrofurantoin MICs of the complemented strain were determined to detect the restoration of nitrofurantoin susceptibility.

Growth rate measurements. Growth rates of susceptible, resistant, and complemented mutant strains were measured in the absence and presence of nitrofurantoin. Each strain was analyzed in duplicate by adding 20 μl of a 0.5 McFarland standard suspension to 180 μl of LB broth, with final concentrations of nitrofurantoin ranging from 0 to 512 $\mu\text{g/ml}$, in a flat-bottomed 96-well plate (Greiner Bio-One, Monroe, NC). Automatic measurements of the optical density at 600 nm (OD₆₀₀) were performed every 4 min for at least 21 h at 37°C, with a Multiskan GO microplate spectrophotometer (Thermo Scientific). The fitness of the strains, in terms of individual growth rate deficits, was evaluated by comparing the times of onset of growth, the slopes of the log phase, and the maximum OD₆₀₀ values reached at fixed time points.

RESULTS

NIT-R mutants generated *in vitro*. We generated mutants *in vitro* by plating two NIT-S intestinal *E. coli* strains (ST540-p and ST2747-p) on MH agar supplemented with nitrofurantoin at up to 32 $\mu\text{g/ml}$ and incubating the strains under aerobic and anaerobic growth conditions. ST540-p had aerobic and anaerobic MICs of 16 and 4 $\mu\text{g/ml}$, respectively, while ST2747-p showed heteroresistance to nitrofurantoin, with aerobic and anaerobic MIC ranges of 4 to 16 and 2 to 8 $\mu\text{g/ml}$, respectively (Table 1). The mutants from the first selection round that were derived from ST540-p and

TABLE 1 Aerobic and anaerobic nitrofurantoin MICs and substitutions, insertions, and deletions in *NfsA*, *NfsB*, and lumazine synthase of NIT-S intestinal *E. coli* strains (ST540-p and ST2747-p), mutants that showed the highest nitrofurantoin resistance levels aerobically (ST540-a and ST2747-a) and anaerobically (ST540-an and ST2747-an), and a complemented mutant strain (ST2747-an/cm)

Strain	Nitrofurantoin MIC ($\mu\text{g/ml}$)		Substitutions, insertions, and deletions ^a		
	Aerobic	Anaerobic	<i>NfsA</i>	<i>NfsB</i>	Lumazine synthase
ST540-p	16	4	WT	WT	WT
ST540-a	256	32	Ins of IS1	Ins of IS1	WT
ST540-an	64	16	Stop at position 100	WT	WT
ST2747-p	4–16	2–8	WT	WT	WT
ST2747-a	128	32	Stop at position 20	Stop at position 57	WT
ST2747-an	32	16	WT	WT	Del of positions 131–134
ST2747-an/cm	16	8	WT	WT	WT plus del of positions 131–134

^a WT, wild type; ins, insertion; del, deletion.

ST2747-p had maximum aerobic and anaerobic MICs of 64 and 16 $\mu\text{g/ml}$ (ST540-p mutants) and 16 and 4 $\mu\text{g/ml}$ (ST2747-p mutants), respectively (see Fig. S1 in the supplemental material). A set of five mutants with different MICs (aerobic MICs of 16 to 64 $\mu\text{g/ml}$ and anaerobic MICs of 4 to 16 $\mu\text{g/ml}$) were used as starting strains for the second selection round, on MH agar supplemented with nitrofurantoin at up to 128 $\mu\text{g/ml}$. These mutants, derived from first-round mutants of ST540-p and ST2747-p, had maximum aerobic and anaerobic MICs of 256 and 32 $\mu\text{g/ml}$ (ST540-p mutants) and 32 and 16 $\mu\text{g/ml}$ (ST2747-p mutants), respectively. Finally, a set of seven mutants from the second selection round with different MICs (aerobic MICs of 16 to 128 $\mu\text{g/ml}$ and anaerobic MICs of 16 to 32 $\mu\text{g/ml}$) were used as starting strains for the third selection round, on MH agar supplemented with nitrofurantoin at up to 512 $\mu\text{g/ml}$. These mutants, derived from second-round mutants of ST540-p and ST2747-p, had maximum aerobic and anaerobic MICs of 256 and 32 $\mu\text{g/ml}$ (ST540-p mutants) and 128 and 32 $\mu\text{g/ml}$ (ST2747-p mutants), respectively.

Both ST540-p and ST2747-p, together with their mutants that showed the highest nitrofurantoin MICs aerobically (ST540-a and ST2747-a) and anaerobically (ST540-an and ST2747-an), were subjected to whole-genome sequencing (13). Full genomes were compared to identify mutations in the known resistance determinants *nfsA* and *nfsB*, as well as to check for other genomic changes indicative of as-yet-undescribed nitrofurantoin resistance determinants. The aerobically isolated mutant ST540-a had aerobic and anaerobic MICs of 256 and 32 $\mu\text{g/ml}$, respectively, and both

its *nfsA* and *nfsB* genes were interrupted by insertion of a mobile IS1 element, at nucleotide positions 579 and 561, respectively (Table 1). The anaerobically isolated mutant ST540-an had aerobic and anaerobic MICs of 64 and 16 $\mu\text{g/ml}$, respectively, and had a nonsense mutation in *nfsA*, leading to a premature stop in *NfsA* at position 100. The *nfsB* gene remained unaffected. The aerobically isolated mutant ST2747-a had aerobic and anaerobic MICs of 128 and 32 $\mu\text{g/ml}$, respectively, and both its *nfsA* and *nfsB* genes harbored a nonsense mutation, leading to premature stops in *NfsA* and *NfsB* at positions 20 and 57, respectively. The anaerobically isolated mutant ST2747-an had aerobic and anaerobic MICs of 32 and 16 $\mu\text{g/ml}$, respectively, but did not exhibit changes in *nfsA* or *nfsB* even though this mutant showed at least 2-fold increases in the MICs for nitrofurantoin, compared to its parent strain. Based on whole-genome sequencing data, we identified a 12-nucleotide deletion in *ribE*, a gene encoding 6,7-dimethyl-8-ribityllumazine synthase (lumazine synthase). Lumazine synthase is an essential enzyme involved in the riboflavin biosynthesis pathway. The 12-nucleotide deletion in *ribE* at nucleotide positions 391 to 402, which was confirmed *in vitro* by PCR and bidirectional sequencing, resulted in a four-amino acid loss (TKAG) in the active site of lumazine synthase, at positions 131 to 134 (Fig. 1) (16). In ST540-a, ST540-an, and ST2747-a, lumazine synthase remained unaffected. An overview of all other proteins with mutations in their nucleotide coding sequences, based on *in silico* comparative genome analysis of parent and mutant strains, is provided in Table S1 in the supplemental material.

```

ST2747-p: 1 MNIIEANVATPDARVAITIA RFNNFINDSLLEG AIDALKRIGQVKDENIT 50
ST2747-an: 1 MNIIEANVATPDARVAITIA RFNNFINDSLLEG AIDALKRIGQVKDENIT 50
          ****

ST2747-p: 51 VVWVPGAYELPLAAGALAKTGKYDAVIALGTVIRGGTAHF EYVAGGASNG 100
ST2747-an: 51 VVWVPGAYELPLAAGALAKTGKYDAVIALGTVIRGGTAHF EYVAGGASNG 100
          *****

ST2747-p: 101 LAHVAQDSEIPVAFVGLTTESIEQAIERAG TKAG NKGAEALTALEMINV 150
ST2747-an: 101 LAHVAQDSEIPVAFVGLTTESIEQAIERAG --- NKGAEALTALEMINV 150
          ****

ST2747-p: 151 LKAIKA. 157
ST2747-an: 151 LKAIKA. 157

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FIG 1 Lumazine synthase sequences for ST2747-p and ST2747-an. *, amino acid residues of the active site. The boxed area shows the deletion of amino acid positions 131 to 134 in ST2747-an.

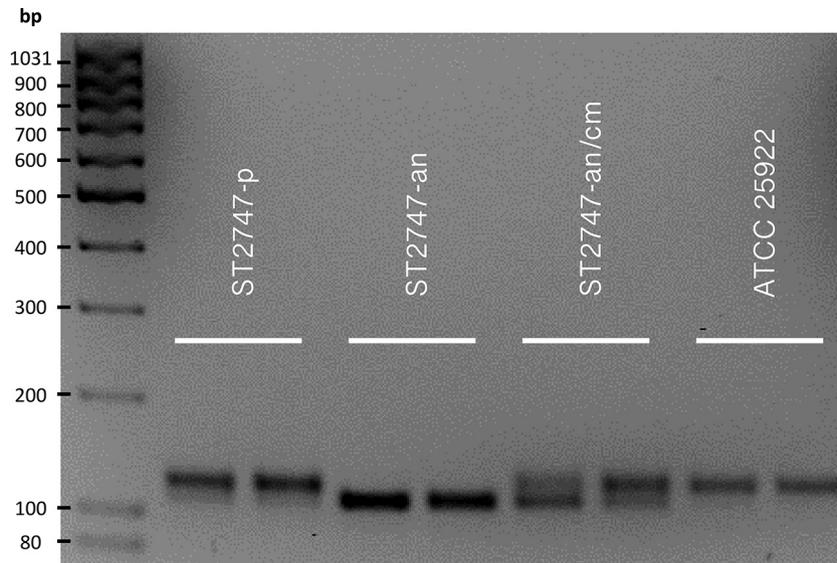


FIG 2 PCR detection of wild-type and/or mutated *ribE* in ST2747-p, ST2747-an, and ST2747-an/cm. ATCC 25922 was included as a positive control for wild-type *ribE*. The PCR fragments were separated, together with a MassRuler low range DNA ladder (Thermo Scientific), on a 2% agarose gel for 1.5 h at 7.5 V/cm with 0.5× Tris-borate-EDTA buffer (40 mM Tris-HCl, 40 mM borate, 1 mM EDTA). The upper band (119 bp) corresponded to the wild-type *ribE* of ST2747-p and the lower band (107 bp) to the mutated *ribE* of ST2747-an.

Identification and validation of novel nitrofurantoin resistance determinant. To confirm the role of the *ribE* deletion in mediating nitrofurantoin resistance in the mutant ST2747-an, we complemented this strain with a recombinant vector encoding a functional wild-type lumazine synthase (LMZ-pCR2.1), which would potentially restore nitrofurantoin susceptibility. Prior to susceptibility testing, we demonstrated the presence of both wild-type and mutated lumazine synthase in the complemented mutant strain (ST2747-an/cm) by PCR detection of *ribE*. Separation of the PCR products of ST2747-an/cm by agarose gel electrophoresis showed the presence of two fragments, with a 12-bp size difference (Fig. 2). MICs for nitrofurantoin were tested by agar dilution and are shown in Table 1. Introducing LMZ-pCR2.1 into ST2747-an resulted in 2-fold decreases of the aerobic and anaerobic nitrofurantoin MICs, from 32 to 16 $\mu\text{g/ml}$ and from 16 to 8 $\mu\text{g/ml}$, respectively. These decreased MIC values were similar to those of the parent ST2747-p strain (Table 1).

Colonies formed by the ST2747-an mutant after 16 to 20 h of incubation on MH agar were smaller than those of ST2747-p, indicating a potential fitness cost associated with the *ribE* deletion. On the other hand, colonies formed by the complemented ST2747-an/cm mutant were similar in size to those of ST2747-p. To quantify differences in growth rates of the strains, we performed automated OD_{600} measurements in the absence and presence of nitrofurantoin (Fig. 3). In the absence of nitrofurantoin, the onset of growth of ST2747-an was clearly delayed and the growth rate was remarkably lower during the log phase, compared to ST2747-p, with an OD_{600} difference of ± 0.2 after 21 h of incubation at 37°C. In contrast, the onset of growth of ST2747-an/cm was identical to that of ST2747-p. The growth rate of ST2747-an/cm remained similar for the first 5 h without significant changes, followed by a slight decrease that ultimately led to an OD_{600} difference of ± 0.1 after 21 h of incubation. In the presence of 16 $\mu\text{g/ml}$ nitrofurantoin, ST2747-an was still able to grow, at a

lower growth rate, while both ST2747-p and ST2747-an/cm failed to grow.

NIT-R clinical *E. coli* strains. Routine disk diffusion susceptibility testing enabled selection of NIT-R clinical *E. coli* strains (nitrofurantoin zone diameters of ≤ 14 mm) recovered from stool samples from UTI patients. In total, six unique NIT-R *E. coli* strains could be recovered from four UTI patients treated with nitrofurantoin, cefuroxime, ciprofloxacin, or norfloxacin. Aerobic and anaerobic nitrofurantoin MICs were determined by agar dilution and ranged from 64 to 256 $\mu\text{g/ml}$ and from 16 to 128 $\mu\text{g/ml}$, respectively (Table 2). We sequenced the complete coding sequences of the previously identified resistance determinants *nfsA* and *nfsB* and the novel resistance determinant *ribE*. All six isolates were found to show at least one mutation in *nfsA* (Table 2). While we observed missense mutations alone in two isolates (NRCI-1 and NRCI-3), the other four isolates also carried nonsense mutations, leading to a premature stop in NfsA at either position 139 (NRCI-6) or position 147 (NRCI-2, NRCI-4, and NRCI-5). Mutations in *nfsB* were identified in all isolates except one (NRCI-2). For three isolates, these mutations were missense mutations, either exclusively (NRCI-1 and NRCI-6) or in combination with a nonsense mutation (NRCI-3) leading to a premature stop in NfsB at position 138. For the remaining two isolates (NRCI-4 and NRCI-5), the *nfsB* gene was disrupted by insertion of a mobile IS26 element at nucleotide position 249; however, no mutations in *ribE* were observed. We also sequenced the *ribE* gene in six intestinal and three urinary *E. coli* strains that showed reduced nitrofurantoin susceptibility (zone diameters of ≤ 14 mm and MICs of 16 to 48 $\mu\text{g/ml}$). In total, four synonymous substitutions (T108C, C267T, T345G, and C429T) in *ribE* were detected in these strains. In addition to nitrofurantoin resistance, the NRCI-1 to NRCI-6 strains exhibited resistance to ciprofloxacin and trimethoprim-sulfamethoxazole, with zone diameters of ≤ 15 mm and ≤ 10 mm, respectively. Additionally, NRCI-1 and NRCI-2 showed

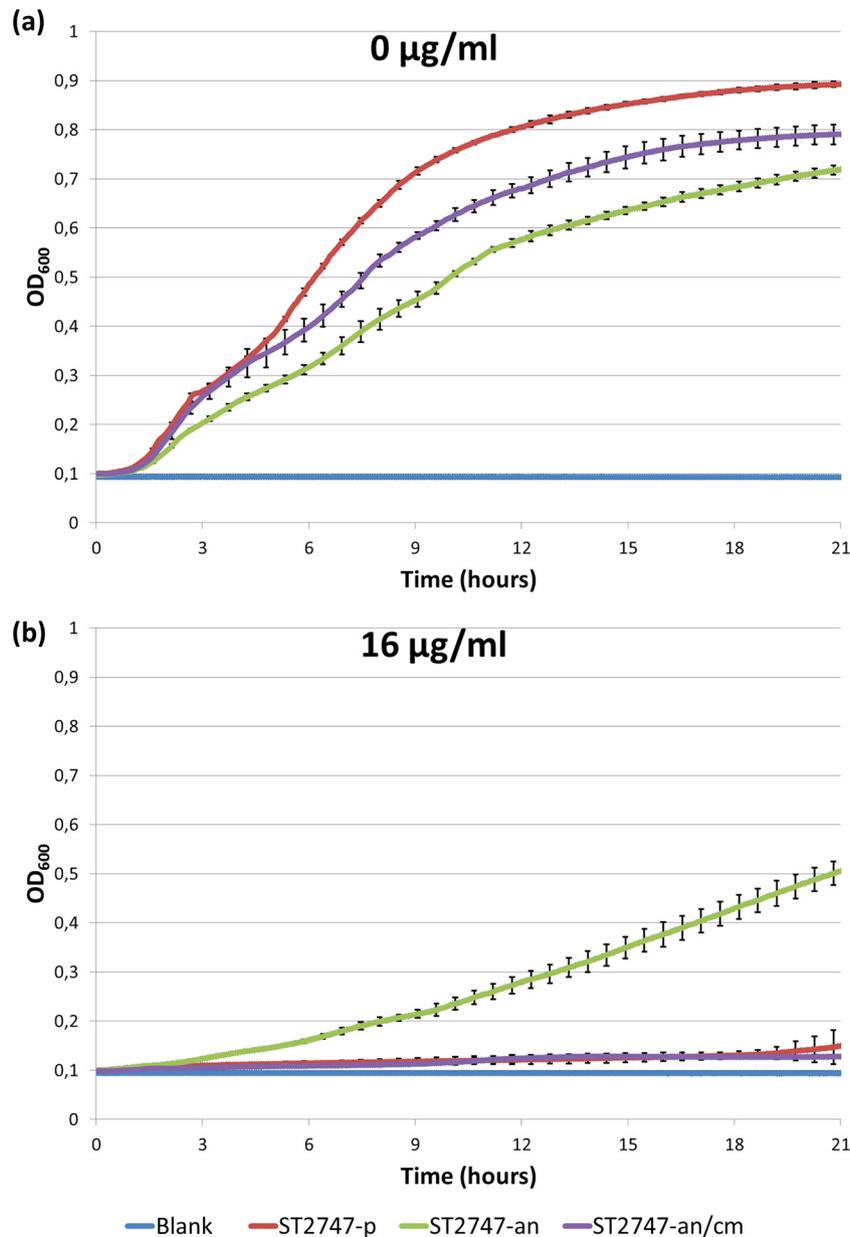


FIG 3 Growth rate measurements (OD₆₀₀ values) for ST2747-p, ST2747-an, and ST2747-an/cm, in the absence (a) and presence (b) of 16 µg/ml nitrofurantoin.

extended-spectrum β -lactamase (ESBL) production by the combination disk method (ceftazidime and cefotaxime zone diameters of ≤ 22 mm and ≤ 27 mm, respectively, with zone diameter increases of ≥ 5 mm when the drugs were combined with clavulanate).

The growth rates of the NIT-R clinical isolates were assessed by automated measurements in the absence and presence of nitrofurantoin and were compared with those of the mutants generated *in vitro* (see Fig. S2 in the supplemental material). Generally, the resistant clinical isolates tended to be more fit than the mutants generated *in vitro* in the absence of nitrofurantoin, exhibiting steeper early log phases and reaching significantly higher OD₆₀₀ values ($P < 0.02$, Student's *t* test) at up to 12 h of incubation (Fig. 4). In the stationary phase, the OD₆₀₀ values exhibited by the clin-

ical isolates were no longer significantly higher. In the presence of nitrofurantoin, the growth curves of the resistant clinical isolates did not differ significantly from those of the mutants generated *in vitro* (data not shown). When the isolates were examined individually, it was remarkable that, even though NRCI-1, NRCI-2, and NRCI-5 had aerobic MICs of 256 µg/ml, only NRCI-5 was able to grow during the growth rate experiments in the presence of 128 µg/ml nitrofurantoin. In the absence of nitrofurantoin, all three isolates showed good growth. The mutant ST540-a generated *in vitro*, with an aerobic MIC of 256 µg/ml, was also able to grow in the presence of 128 µg/ml nitrofurantoin; however, it reached the lowest OD₆₀₀ values in the absence of nitrofurantoin, with an OD₆₀₀ difference from its parent strain ST540-p of almost 0.3 after 21 h of incubation.

TABLE 2 Disk diffusion results, aerobic and anaerobic nitrofurantoin MICs, and substitutions and insertions in NfsA, NfsB, and lumazine synthase of NIT-R clinical *E. coli* strains

Strain	Disk diffusion result ^a																Nitrofurantoin MIC (µg/ml)				Substitution(s) or insertion ^b		Lumazine synthase
	AMX	AMC	PIP	TZP	CTX	CTX-CLA ^c	CAZ	CAZ-CLA ^c	FEP	FOX	ATM	ETP	MEM	CIP	FOF	F/M	SXT	Aerobic	Anaerobic	NfsA	NfsB		
NRCI-1	R	I	R	I	R	+	R	+	R	I	R	S	S	R	S	R	R	256	64	Q72K	G66D, I75M	WT	
NRCI-2	R	I	R	I	R	+	R	+	I	S	R	S	S	R	S	R	R	256	64	R15L, T117I, stop at position 147, L157F, D187G	WT	WT	
NRCI-3	R	S	R	S	S	-	S	-	S	S	S	S	S	R	S	R	R	64	16	R15C, T117I, D187G	I75M, stop at position 138	WT	
NRCI-4	R	S	R	S	S	-	S	-	S	S	S	S	S	R	S	R	R	64	16	T117I, stop at position 147, L157F, D187G	Ins of IS26	WT	
NRCI-5	R	R	R	R	S	-	R	-	S	S	R	S	S	R	S	R	R	256	128	T117I, stop at position 147, L157F, D187G	Ins of IS26	WT	
NRCI-6	R	I	R	S	S	-	S	-	S	S	S	S	S	R	S	R	R	64	32	A138G, stop at position 139	R121C	WT	

^a R, resistant; I, intermediate; S, susceptible; AMX, amoxicillin; AMC, amoxicillin-clavulanate; PIP, piperacillin; TZP, piperacillin-tazobactam; CTX, cefotaxime; CTX-CLA, cefotaxime-clavulanate; CAZ, ceftazidime; CAZ-CLA, ceftazidime-clavulanate; FEP, cefepime; FOX, ceftoxitin; ATM, aztreonam; ETP, ertapenem; MEM, meropenem; CIP, ciprofloxacin; FOF, fosfomicin; F/M, nitrofurantoin; SXT, trimethoprim-sulfamethoxazole.
^b WT, wild type; ins, insertion.
^c +, ≥5-mm zone diameter increase; -, <5-mm zone diameter increase.

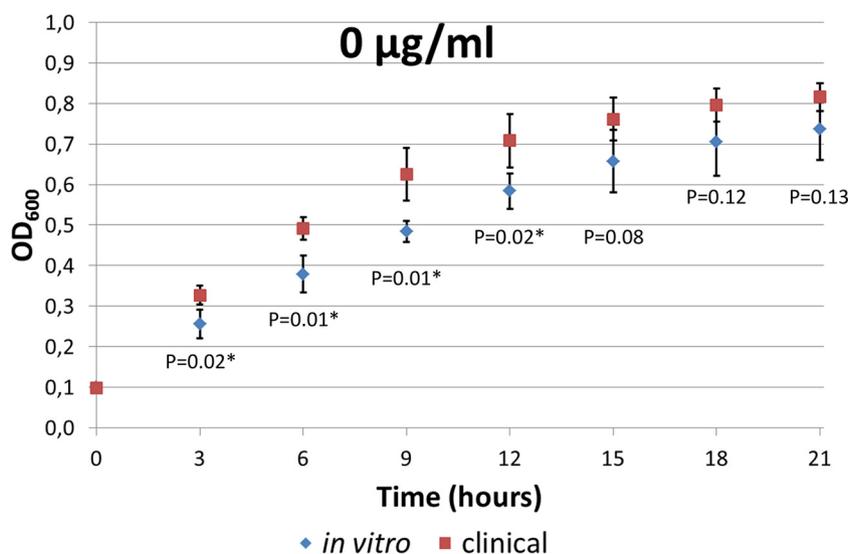


FIG 4 Comparison of growth rates (OD₆₀₀ values) for mutants generated *in vitro* versus NIT-R clinical isolates, in the absence of nitrofurantoin. Error bars, 95% confidence intervals. *, significant difference ($P < 0.02$, Student's *t* test).

DISCUSSION

In this study, we generated NIT-R mutants *in vitro* through stepwise plating of susceptible strains (ST540-p and ST2747-p) on MH plates with increasing nitrofurantoin concentrations, under aerobic and anaerobic growth conditions. This ultimately resulted in the selection of the highly NIT-R strains ST540-a (aerobic and anaerobic MICs of 256 and 32 $\mu\text{g/ml}$, respectively) and ST2747-a (aerobic and anaerobic MICs of 128 and 32 $\mu\text{g/ml}$, respectively). Both resistant strains were generated by successive selection rounds performed exclusively under aerobic growth conditions. Remarkably, only one selection round was necessary for ST540-p to become intermediate resistant (aerobic MIC of 64 $\mu\text{g/ml}$), while ST2747-p remained susceptible (aerobic MIC of 32 $\mu\text{g/ml}$) after two selection rounds (see Fig. S1 in the supplemental material) (12). It was only after the third selection round that ST2747-p achieved a resistant MIC of 128 $\mu\text{g/ml}$ under aerobic growth conditions. There are multiple possible explanations for why the emergence of nitrofurantoin resistance occurred more rapidly in ST540-p. First, ST540-p might have higher intrinsic mutability due to the presence of certain mobile elements that are transposed under the influence of environmental factors such as the presence of antibiotics (17, 18). Indeed, in ST540-a, both *nfsA* and *nfsB* genes were disrupted by insertion of an *IS1* mobile element (Table 1), indicating that, under the influence of nitrofurantoin, *IS1* mobile elements contribute to increased mutability. Additionally, ST540-p might have had preexisting low-level nitrofurantoin resistance mutations that facilitated the acquisition of additional mutations when the cells were placed under selection pressure with nitrofurantoin. Several such strains were described previously (6); they exhibited wild-type resistance to nitrofurantoin but could be mutated to high levels of nitrofurantoin resistance in a single step. Sequence analysis of one of those strains revealed that it had a wild-type *nfsA* gene and a nonsense mutation in *nfsB*, leading to a premature stop in NfsB at position 142 (5). We also isolated mutants that were generated by successive selection rounds performed exclusively under strict anaerobic growth conditions. These isolates, ST540-an and ST2747-an, exhibited aero-

bic and anaerobic MICs of 64 and 16 $\mu\text{g/ml}$ (ST540-an) and 32 and 16 $\mu\text{g/ml}$ (ST2747-an), respectively, which were much lower resistance levels than those generated under aerobic growth conditions. It is possible that an anaerobic environment plays a protective role in the emergence of nitrofurantoin resistance, indicating that growth conditions might be important determinants of bacterial mutability of (18). Moreover, all mutants generated *in vitro* showed anaerobic MIC values that were 2-fold to 8-fold lower than the aerobic MIC values. These results corroborate a previous study (7) and can probably be attributed to the oxygen-sensitive nitroreductase system, which becomes active in the absence of oxygen and hence compensates for the (partial) loss of activity of the oxygen-insensitive nitroreductase system (19, 20). In addition, another study using single- and double-knockout *E. coli* mutants targeting *nfsA* and *nfsB* showed that, at low oxygen levels, the mutants were still able to reduce the nitro compound 7-nitrocoumarin-3-carboxylic acid (7NCCA), indicating that NfsA and NfsB are not required for nitroreduction under anaerobic growth conditions (21).

In ST2747-an, mutations in *nfsA* or *nfsB* could not be detected, although a 2-fold MIC increase was observed. Whole-genome sequencing of the strain indicated a 12-nucleotide deletion in *ribE*, a gene encoding lumazine synthase. The *ribE* gene product consists of a 16.2-kDa subunit that builds up lumazine synthase, a hollow icosahedral capsid of 60 subunits with a total mass of about 1 MDa (16). The active site is located close to the inner surface of the capsid, and substrates need to access the active site by penetrating the capsid via narrow channels (22, 23). Lumazine synthase is an essential enzyme involved in the riboflavin biosynthesis pathway, where it catalyzes the following chemical reaction: 1-deoxy-L-glycerotetrol-4-phosphate + 5-amino-6-(D-ribitylamino)uracil \rightleftharpoons 6,7-dimethyl-8-(D-ribityl)lumazine + 2 H₂O + phosphate. Riboflavin, also known as vitamin B₂, is mandatory for the production of flavin mononucleotide (FMN), which is an important cofactor of NfsA and NfsB (5). FMN is tightly associated with NfsA and NfsB, where it serves as a mediator for electron transfer from NAD(P)H to various substrates for nitroreduction (24). En-

zymatic assays with 7NCCA demonstrated that both nitroreductases were greatly dependent on FMN for nitroreduction to proceed (21). The 12-nucleotide deletion in *ribE* resulted in a four-amino acid loss (TKAG) in the active site of lumazine synthase, at positions 131 to 134 (Fig. 1). This region is highly conserved among lumazine synthases of very diverse bacteria, such as *Bacillus subtilis*, *Photobacterium leiognathi*, and *Haemophilus influenzae* (16), suggesting that deletions within this region have major implications for the functionality of the enzyme. This might have ultimately resulted in the inability (or reduced ability) of ST2747-an to synthesize FMN, thereby hindering nitroreduction by both Nfs enzymes, particularly NfsB.

The involvement of lumazine synthase in the development of nitrofurantoin resistance was assessed by complementing the ST2747-an mutant with LMZ-pCR2.1, which encoded a functional wild-type lumazine synthase. This resulted in the restoration of nitrofurantoin susceptibility, which was demonstrated by 2-fold decreases of the aerobic and anaerobic MICs from 32 to 16 $\mu\text{g/ml}$ and from 16 and 8 $\mu\text{g/ml}$, respectively, similar to the MIC values of the parent ST2747-p strain. These results confirm that the reduced nitrofurantoin susceptibility is caused by the TKAG deletion in lumazine synthase. Additionally, the impact of the deletion in lumazine synthase on bacterial growth rate was measured. We found clearly delayed growth onset and a 20% decrease in the bacterial growth of ST2747-an after 21 h of incubation in the absence of nitrofurantoin, compared to the parent strain (Fig. 3). A growth deficit is not entirely unexpected. Gram-negative bacteria are unable to take up riboflavin from the external environment (25), which causes them to be completely dependent on endogenous biosynthesis. As mentioned earlier, lumazine synthase is required for the production of riboflavin. Riboflavin itself does not have any biological activity; however, it is the precursor of FMN and flavin adenine dinucleotide (FAD). Both cofactors are mostly noncovalently bound not only to NfsA and NfsB but also to numerous other flavoproteins, which makes them essential components for cellular metabolism (26). These flavoproteins enable redox reactions involving one- and two-electron transfer processes and catalyze the dehydrogenation of metabolites, as well as oxidation and dehydroxylation reactions (27). Interestingly, the growth rate could be partially restored in the complemented mutant ST2747-an/cm, as the growth onset was not delayed and the rate remained similar to that of ST2747-p for the first 5 h of incubation. At the end of the experiment, ST2747-an/cm showed 10% increased bacterial growth, compared to ST2747-an. As expected, in the presence of 16 $\mu\text{g/ml}$ nitrofurantoin, ST2747-an was still able to grow at a lower growth rate, while both ST2747-p and ST2747-an/cm failed to grow.

Table S1 in the supplemental material gives an overview of proteins carrying mutations, based on *in silico* comparative genome analysis, including two other proteins that are indirectly involved in FMN biosynthesis. In ST540-a and ST540-an, we observed a potential missense mutation in *araB*, which encodes ribulokinase, an enzyme that participates in pentose and glucuronate interconversions. It catalyzes the phosphorylation of L-ribulose to L-ribulose-5-phosphate, which is an essential step in the metabolism of the carbon source L-arabinose (28). In the pentose phosphate pathway, L-ribulose-5-phosphate can be converted into D-ribulose-5-phosphate, a substrate for riboflavin metabolism, ultimately leading to the production of FMN. Additionally, we described a potential missense mutation in *rbsR*, encoding a ribose

operon repressor, in ST540-an and ST2747-an. The repressor is a LacI-type transcriptional repressor of the ribose *rbsDACBK* operon. The RbsABC proteins constitute an ATP-binding cassette (ABC)-type high-affinity transporter for the carbon source D-ribose, while RbsD and RbsK are responsible for the conversion of D-ribose into D-ribose-5-phosphate in the pentose phosphate pathway (29). D-Ribose-5-phosphate can be easily converted into D-ribulose-5-phosphate and hence can be utilized for FMN production. A mutation in the inducer recognition site of RbsR might lead to an inability of D-ribose to induce transcription of the *rbsDACBK* operon. However, these mutations were not investigated further and need to be confirmed by *in vitro* analysis.

Routine analysis of strains recovered from stool samples from UTI patients resulted in the detection of six NIT-R clinical *E. coli* strains. These isolates had similar aerobic MICs, compared to the mutants generated *in vitro* (64 to 256 $\mu\text{g/ml}$); for some clinical isolates (NRCI-1, NRCI-2, and NRCI-5), however, the anaerobic MICs were up to 4-fold higher, reaching values of 128 $\mu\text{g/ml}$, compared with 32 $\mu\text{g/ml}$ for the mutants generated *in vitro* (Table 2). In accordance with previous data, these clinical isolates might have acquired mutations in the oxygen-sensitive nitroreductase genes or other uncharacterized genes (7). When the types of mutations observed in *nfsA* and *nfsB* were compared, it was remarkable that all NIT-R clinical isolates had missense mutations in one or both genes, while such mutations were completely absent in the mutants generated *in vitro*. These mutations might have accumulated gradually over a longer time period. One of the mutations in NRCI-3 (R15C) was characterized previously (5) and represents a loss-of-function mutation. Similar to the *in vitro* mutants with aerobic MICs of ≥ 64 $\mu\text{g/ml}$, most NIT-R clinical isolates had nonsense mutations or large insertions of an IS element in *nfsA* and/or *nfsB*, with the exception of NRCI-1, which harbored only missense mutations in both genes. Furthermore, no mutations could be observed in *ribE* in the clinical isolates. Since the deletion in *ribE* was associated with reduced nitrofurantoin susceptibility (MICs of 32 $\mu\text{g/ml}$), we sequenced the *ribE* gene in six intestinal and three urinary *E. coli* isolates, with nitrofurantoin zone diameters of ≤ 14 mm and MICs of 16 to 48 $\mu\text{g/ml}$. Apart from a few synonymous substitutions, the *ribE* gene was largely unaffected in these strains. These results, combined with the growth rate deficit associated with the *ribE* deletion, which is indicative of a fitness cost, might underlie the lack of recovery of such mutants in the community. Interestingly, steeper early log phases and significantly higher OD_{600} values ($P < 0.02$, Student's *t* test) at up to 12 h of incubation in the absence of nitrofurantoin were observed among the resistant clinical isolates, compared to the mutants generated *in vitro* (Fig. 4). These clinical strains might have improved their biological costs of resistance through the acquisition of compensatory mutations (30), although a recent study of NIT-R clinical strains showed a lack of clonal spread of such isolates, reiterating the high fitness costs associated with nitrofurantoin resistance in *E. coli* (31). Presumably, the six NIT-R clinical *E. coli* strains were recovered from the fecal flora of the UTI patients because they showed coresistance to the antibiotics these patients were given, such as ciprofloxacin and cefuroxime.

The individual growth assessment method used to assess fitness costs in this study was not without limitations. Direct *in vitro* competition testing followed by plating for determination of viable counts might have been a more accurate method for fitness estimations (32); however, the minor differences in MICs between

the competing strains precluded the use of a direct competition assay. Finally, while negative *in vitro* competition experiments do not exclude the possibility of fitness costs under *in vivo* conditions, positive *in vitro* results (such as ours) do allow a reasonable assumption that the resistance would engender a fitness deficit in the bacteria under natural conditions, as described previously (33). In conclusion, we identified a novel nitrofurantoin resistance determinant and clearly showed the growth deficits or presumptive fitness costs engendered by this and previously identified nitrofurantoin resistance determinants, thus providing a basis for the lack of widespread resistance to this unusual antibiotic in *E. coli*.

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