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Mutations that increase expression of the EmrAB-TolC efflux pump confer increased resistance to nitroxoline in *Escherichia coli*

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Objectives: To determine the mechanism of resistance to the antibiotic nitroxoline in *Escherichia coli*.

Methods: Spontaneous nitroxoline-resistant mutants were selected at different concentrations of nitroxoline. WGS and strain reconstruction were used to define the genetic basis for the resistance. The mechanistic basis of resistance was determined by quantitative PCR (qPCR) and by overexpression of target genes. Fitness costs of the resistance mutations and cross-resistance to other antibiotics were also determined.

Results: Mutations in the transcriptional repressor *emrR* conferred low-level resistance to nitroxoline [nitroxoline MIC (MIC_{NOX})=16 mg/L] by increasing the expression of the *emrA* and *emrB* genes of the EmrAB-TolC efflux pump. These resistant mutants showed no fitness reduction and displayed cross-resistance to nalidixic acid. Second-step mutants with higher-level resistance (MIC_{NOX}=32–64 mg/L) had mutations in the *emrR* gene, together with either a 50 kb amplification, a mutation in the gene *marA*, or an IS upstream of the *lon* gene. The latter mutations resulted in higher-level nitroxoline resistance due to increased expression of the *tolC* gene, which was confirmed by overexpressing *tolC* from an inducible plasmid in a low-level resistance mutant. Furthermore, the *emrR* mutations conferred a small increase in resistance to nitrofurantoin only when combined with an *nfsAB* double-knockout mutation. However, nitrofurantoin-resistant *nfsAB* mutants showed no cross-resistance to nitroxoline.

Conclusions: Mutations in different genes causing increased expression of the EmrAB-TolC pump lead to an increased resistance to nitroxoline. The structurally similar antibiotics nitroxoline and nitrofurantoin appear to have different modes of action and resistance mechanisms.

Introduction

Nitroxoline (5-nitro-8-hydroxyquinoline) is an antibiotic that has been clinically used in certain European countries for the treatment of acute and recurrent urinary tract infections (UTIs) in adults and children.[1,2] Nitroxoline is assumed to primarily work by chelating and sequestering biologically important divalent ions such as Mn^{2+}, Mg^{2+}, Fe^{2+} and Zn^{2+}, which subsequently results in bacterial death.[3–5] This chelating property has been found to inhibit the function of bacterial RNA polymerase, inhibit biofilm formation of multiple pathogens and reduce bacterial adhesion to bladder epithelial cells and catheters.[6–10]

In vitro experiments performed with clinical isolates, as well as clinical studies, have identified nitroxoline as an attractive alternative therapeutic agent for the treatment of uncomplicated UTIs. This is largely based on its broad spectrum of activity, good safety profile and the low frequency of resistance development in *Escherichia coli*.[1,9,11–13] Several studies have reported that nitroxoline has a broad spectrum of activity against different susceptible and resistant Gram-negative and Gram-positive uropathogens, as well as diploid fungi and yeast isolates,[9,14,15] but lacks any appreciable activity against *Pseudomonas aeruginosa*.[1,13] Furthermore, nitroxoline was described as a safe drug based on an individual-patient meta-analysis that looked at >11 000 patients,[12] with mild adverse effects that included gastrointestinal disorders, minor allergic reactions and a small impact on the microbiome.[11,16] In this meta-analysis, nitroxoline was also found to have a comparable eradication rate to co-trimoxazole and norfloxacin (>90%), two commonly used drugs for the treatment of UTIs that are beginning to have decreased clinical use owing to an increased frequency of

antimicrobial resistance development and the negative collateral effects attributed to fluoroquinolones.[12] Most recently, nitroxoline was reported to be equally active to nitrofurantoin in an *in vitro* study of 3012 urinary clinical isolates.[13]

A recent study performed in geriatric patients to evaluate treatment of lower UTIs with nitroxoline reported the development of nitroxoline-resistant isolates after only a few days of treatment;[17] however, the mechanism of resistance was not investigated. This study challenges the observations of other studies that have reported the development of antimicrobial resistance to nitroxoline to be rare,[9,18] and suggests that certain patient groups might not benefit from its use.[2]

In this study, we investigated the genetic and mechanistic basis for nitroxoline resistance in *E. coli*, one of the most common microorganisms causing UTIs. We isolated nitroxoline-resistant mutants *in vitro* at low (4× the WT MIC) and high (8× to 16× the WT MIC) levels of drug and determined the fitness costs of resistance and cross-resistance to other antimicrobial agents. Our genomic analysis of these mutants identified increased efflux activity, mediated by the efflux pump EmrAB-TolC, as being solely responsible for the decreased susceptibility to nitroxoline.

Materials and methods

Selection of nitroxoline-resistant mutants

The mutants examined in this study were selected using DA4201, an *E. coli* MG1655 WT, as the parental strain. The MIC of nitroxoline for the WT DA4201 was 4 mg/L. To isolate first-step mutants, 0.1 mL of bacteria from each of 20 independent overnight cultures of the WT strain grown in lysogeny broth (LB) were plated on LB agar plates containing nitroxoline concentrations of 8, 16 and 32 mg/L and incubated at 37°C. The appearance of spontaneous nitroxoline-resistant mutant colonies was monitored at 24 and 48 h. The resistant colonies were first re-streaked on nitroxoline agar plates containing the same drug concentration as those from which they were selected, incubated at 37°C and subsequently grown in liquid culture with selection for subsequent freezing at −80°C. Second-step mutants with higher levels of resistance were selected in a similar manner by plating the parental mutant strain DA62285 on five independent agar plates with a concentration of 24 and 32 mg/L of nitroxoline.

Strain constructions

Target mutations identified in first-step resistant mutants were reconstructed in DA4201 using the scar-less Dup-In recombineering approach.[19] Briefly, this method consists of the generation of a tandem duplication using *acatsacB* in the proximity of the mutated gene. An *acatsacB* cassette was amplified with Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific Inc.) with 35 nt overhangs with homologies to the target region for duplication. The *acatsacB* cassette was inserted in the mutant strains via recombination and the antibiotic resistance cassette was selected by plating cells on LB agar plates supplemented with 12.5 mg/L chloramphenicol to generate a duplication. The duplication was transduced back into the WT background with a P1 phage and plated on no salt LB agar plates containing 50 mg/L sucrose for segregation. Duplications are unstable genetic modifications that resolve by homologous recombination and thereby leave the mutation of interest in the recipient strain without any scar sequence. PCR and sequencing were performed to confirm that the mutations were correctly reconstructed.

To determine the effect of the *emrR* mutations (selected in this study) on conferring nitrofurantoin resistance, two different *emrR* mutations were combined in the double-knockout Δ*nfsAB* mutant (DA65117). This was performed by transferring the desired *emrR* mutations from the above-constructed strains using a P1 transduction protocol and selecting for transductants on LB agar plates containing 15 mg/L chloramphenicol. Segregants containing the desired mutation were then selected for by streaking the transductants on LB agar plates containing sucrose. The desired mutations were confirmed by performing local sequencing of the *emrR* gene.

Antibiotic susceptibility tests

MICs were determined using two different assays: a broth microdilution method (for nitroxoline and colistin) and Etests (for chloramphenicol, tetracycline, kanamycin, streptomycin, nalidixic acid, erythromycin, rifampicin and nitrofurantoin). For the broth microdilution assay to determine susceptibility to nitroxoline, a nitroxoline stock solution of 12 mg/mL was prepared in 100% DMSO and diluted in Mueller–Hinton (MH) medium (Difco) to obtain a solution of 256 mg/L. This was then used to prepare serial dilutions of the antibiotic in a 96-well plate, each well containing 50 μL of the antibiotic solution. Fresh colonies were used to generate a 0.5 MacFarland suspension in 0.9% NaCl and diluted 1:100 in MH medium (~1×10^6 cfu/mL). Fifty microlitres of the bacterial suspension was inoculated in the wells containing the nitroxoline serial dilutions and the plate was incubated at 37°C for 16–20 h. The results were determined by visual inspection and the MIC was read as the lowest concentration at which no growth was observed. The broth microdilution test for colistin was performed in a similar manner using CAMHB (Difco) and a solution with a starting concentration of 2 mg/L colistin. Following EUCAST and CLSI recommendations for the determination of the MIC of colistin, a resistant (*mcr-1*-positive) strain and a susceptible quality control (QC) ATCC 25922 *E. coli* strain were included.[20,21] For Etests, fresh colonies were used to obtain a 0.5 MacFarland suspension in 0.9% NaCl. The suspension was spread on MH agar plates using a cotton swab. The Etest strips were placed on the plates and incubated at 37°C; the MIC was recorded after 16–20 h incubation.

Determination of growth rates

Growth rates of the mutant strains were determined in LB medium using a Bioscreen C reader (Oy Growth Curves Ab Ltd). Three independent cultures for each mutant and six independent cultures for the WT reference strain were grown overnight and diluted to 5×10^6 cfu/mL the next day. Three hundred microlitres of this suspension was inoculated in duplicate into the wells of the honeycomb plates. The plates were incubated in the Bioscreen C analyser at 37°C with shaking for 24 h. The OD at 600 nm wavelength (OD_{600}) value was measured every 4 min. Exponential growth was calculated from OD measurements in the range of 0.02–0.055. All exponential growth rates were normalized to that calculated for the WT strain.

Gene amplification screen

IPTG-inducible plasmids containing target genes were isolated from specific strains from the ASKA collection[22] using the Plasmid DNA Mini Kit I (VWR: D6943-02). Four specific target genes were investigated: *parE*, *parC*, *tolC* and *mdaB*. The plasmids were transformed in the reconstructed mutant DA62285 by electroporation. MICs of nitroxoline for the transformed strains were determined by broth microdilution assay as described above and were done in replicates. IPTG (100 μM) was used for induction of the plasmids.

Determination of gene expression

Overnight cultures of the mutant strains were used to inoculate 1 mL of LB, which was grown to $OD_{600} = 0.2$. From this culture, 500 μL was added to 1 mL of RNAprotect Bacteria Reagent (QIAGEN) and incubated for 5 min at room temperature. The suspension was centrifuged at 7200 rpm for 10 min and the supernatant was discarded. The resulting pellet was used for total RNA extraction using the RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. Degradation of any remaining DNA in

the sample was performed with the Turbo DNA-*free* Kit (Ambion). After DNAse treatment, 500 ng of RNA was used for reverse transcription with the High-capacity Reverse Transcription Kit (Applied Biosystems) following the manufacturer's protocol. A real time quantitative PCR (qRT-PCR) was performed to determine the expression levels of *emrA*, *emrB* and *tolC* using the Eco Real-Time PCR system (Illumina). All the primers were designed with Primer3Plus and are listed in Table S1 (available as Supplementary data at *JAC* Online). The total 10 μL reaction mix contained 5 μL 1× PerfeCTa SYBR Green SuperMix (QuantaBio), 0.5 μL (10 mM) of forward and reverse primers and 4 μL of 10× and 100× cDNA of each sample. The PCR programme was as follows: 10 min of polymerase activation at 95°C, followed by amplification for 40 cycles of 10 s at 95°C and 30 s at 60°C. The expression levels were calculated by normalizing to the geometrical mean of the levels of housekeeping genes *cysG* and *idnT* using the following formula: $2^{[Cq(geometrical\ mean\ reference\ genes)-Cq(target\ gene)]}$, where Cq stands for the quantitation cycle. The expression levels are given as the fold-change relative to the WT strain. Two biological replicates were used in each case. Only 2-fold or more expression level change was considered to be biologically significant.

DNA extraction and WGS

Genomic DNA was extracted using an Epicentre MasterPure DNA Purification Kit (Epicentre) and following the manufacturer's instructions. An Illumina Nextera XT kit was used to make libraries (2×300 bp) to be sequenced with MiSeq. Samples were dual-indexed and pooled together. The paired-end sequence reads were trimmed for quality and mapped to the WT parental strain (NZ_CP032667) using CLC Genomics Workbench 8. Structural variant changes were determined with CLC Genomics Workbench and with breseq.[23]

Determining the stability of nitroxoline resistance mutations

The stability of nitroxoline resistance mutations was investigated in two first-step low-level nitroxoline-resistant mutants (DA62277 and DA62285) and in two second-step high-level nitroxoline-resistant mutants (DA63527 and DA63538). These mutants were grown for 50 generations by transferring 1 μL of an overnight culture into 1 mL of fresh antibiotic-free medium for 5 days (1000-fold dilution every day for 5 days). Three biological replicates were used for each strain. At the end of the experiment, 50 individual colonies were patched from each replicate population for each strain onto LB agar plates with either no nitroxoline, with nitroxoline at a concentration of 16 mg/L or at a concentration of 32 mg/L. In each case the WT strain (DA4201) and the starting strain were patched as controls.

Results

Selection of first-step nitroxoline-resistant mutants

To select for nitroxoline-resistant mutants, 20 independent overnight cultures of the WT strain were plated at concentrations of 2×, 4× and 8× the nitroxoline MIC of the parental strain (4 mg/L). The resistance mutation rate obtained at a nitroxoline concentration of 8 mg/L (median method) was 5.4×10^{-8}/cell/generation, and at a nitroxoline concentration of 16 mg/L (P_0 method) was 5.9×10^{-10}/cell/generation. Twelve mutants were chosen for further study (11 isolated at 2× and 1 isolated at 4× WT MIC) and relative exponential growth rates were measured (Table 1). No significant differences in relative growth values were observed for any of the mutants. The MICs of nitroxoline for all these mutants were determined by the broth microdilution assay and were 16 mg/L for all mutants (Table 1). Given that nitroxoline is a 5-hydroxy-8-quinoline, a cross-resistance check was performed against structurally similar antibiotics that included ciprofloxacin, a fluoroquinolone, and nitrofurantoin. None of the mutants showed cross-resistance to these antibiotics (Table 1).

Genetic analysis of the first-step mutants' identified mutations in the repressor emrR

WGS analysis of the 12 above-mentioned resistant mutant strains identified different mutations in the gene *emrR* (previously referred to as *mprA*) in all the isolates (Table 1). The *emrR* gene encodes a transcriptional repressor of the multidrug efflux pump EmrAB.[24–26] Other mutations that were identified across different strains included mutations in the gene *ycfK*, which was present in all of the 12 isolates, in the genes *fimE* and *fimA* in 2 isolates, and an IS*5*

Table 1. Characteristics of the first-step mutants

	MIC (mg/L)				
Strain	nitroxoline	nitrofurantoin	ciprofloxacin	Relative growth rate	Mutation in *emrR*
DA4201	4	4–8	0.016	1	—
DA60440	16	6	0.016	1.02	V128fs
DA60441	16	4	0.016	1.11	E119*
DA60443	16	6	0.016	1.00	S177*
DA60447	16	6	0.016	0.95	L64R
DA60451	16	6	0.023	1.11	ΔL115_H137
DA60452	8	6	0.023	1.02	F58fs
DA60453	16	6	0.023	1.02	L113P
DA60454	16	6	0.023	1.02	L61W
DA60455	16	6	0.023	1.01	G121fs
DA60456	16	6	0.023	1.01	ΔL115_H137
DA60458	16	4	0.023	1.01	S78insS
DA60460	16–32	6	0.023	0.99	A60*

—, no changes; fs, frame shift; *, stop codon; Δ, deletion; ins, insertion.

insertion between the genes *yjiC* and *yjiD* in 1 isolate. From this set of genes, mutations in the *emrR* gene were hypothesized to be responsible for the resistant phenotype against nitroxoline as it regulates the expression of an efflux pump, which is a known mechanism of antibiotic resistance. Previously, a point mutation in *emrR* was shown to lead to the development of antibiotic resistance in *Chromobacterium violaceum*.[27] To confirm this for our resistant mutants, five different mutations in *emrR* were reconstructed in the WT *E. coli* background using a scar-less recombineering approach.[19] The MICs of nitroxoline for these reconstructed mutants were all 16 mg/L (Table 2), confirming our hypothesis that mutations in *emrR* were solely responsible for the observed resistance phenotype and that the other mutations were non-selected random changes.

Cross-resistance to other antibiotics for the reconstructed mutants with an increased expression of the EmrAB efflux pump

The efflux pump EmrAB-TolC has been shown to confer resistance to a range of structurally unrelated molecules.[28,29] Given that EmrAB is a pump not commonly associated with antibiotic resistance, we investigated the cross-resistance of three reconstructed first-step mutants against a set of different antibiotics belonging to different families of antibiotics, including chloramphenicol, tetracycline, kanamycin, streptomycin, nalidixic acid, erythromycin, rifampicin, nitrofurantoin and colistin (Table 3). A small increase in MIC was observed for nalidixic acid only.

Table 2. Susceptibility to nitroxoline of the first-step mutants and the reconstructed mutants

		MIC (mg/L)	
Strain	Mutation in *emrR*	first-step mutant	reconstructed mutant
DA4201	—	4–8	NA
DA62277	V128fs	16	16
DA62271	L64R	16	16
DA62269	ΔL115_H137	16	16
DA62275	L113P	16	16
DA62285	A60*	16–32	16–32

—, no changes; NA, not applicable; fs, frame shift; Δ, deletion; *, stop codon.

Selection of high-level nitroxoline-resistant mutants

In order to investigate the evolution of higher levels of resistance, the reconstructed strain DA62285 with a 352 bp deletion in *emrR* was used for selection of high-level nitroxoline-resistant mutants. An overnight culture of DA62285 consisting of $\sim 10^{10}$ cells was plated at nitroxoline concentrations of 6× (24 mg/L) and 8× (32 mg/L) the WT MIC. Mutation rates of 1.6×10^{-9}/cell/generation and 9.6×10^{-11}/cell/generation to resistance were obtained on 24 and 32 mg/L nitroxoline plates, respectively. MICs of nitroxoline for 13 isolates were measured and they were all in the range of 32–64 mg/L (Figure 1). Relative growth rate was also measured for these isolates and, as is seen in Figure 1, the relative exponential growth rate was reduced to 0.88–0.96 in the different mutants. Measurements of cross-resistances against the structurally similar antibiotics ciprofloxacin, a fluoroquinolone, and nitrofurantoin showed a one-step increase in the MIC of nitrofurantoin for the second-step mutants, with only strain DA65538 appearing to be highly susceptible to nitrofurantoin (Figure 1). Only DA63533 had a slight increase in resistance to ciprofloxacin, but no cross-resistance was observed for the other strains.

Genetic analysis of the high-level nitroxoline-resistant mutants

Six high-level nitroxoline-resistant mutants were selected for WGS and the identified genetic changes are shown in Table 4. Four of these mutants had an amplification (5-fold increase in copy number estimated from sequence coverage) of a 50 kb region containing a total of 52 genes (Table S2). In the other two strains, mutations in the *marR* gene (DA63533) and an IS upstream of the *lon* gene (DA63538) were observed. Mutations in these genes have previously been reported to induce the function of the global regulator MarA,[30,31] which controls the expression of over 80 genes involved in MDR.[32,33] MarR negatively autoregulates the *marRAB* operon,[34] whereas Lon has a protease function where one of its substrates includes MarA.[35] MarA in turn activates expression of the gene *tolC*, which encodes the outer membrane protein that

Table 3. Cross-resistance to other antibiotics for the reconstructed *emrR* mutants

		MIC (mg/L)								
Strain	Mutation in *emrR*	CHL	TET	KAN	STR	NAL	ERY	RIF	NIT	CST[a]
DA4201	—	12	1.5	1.5	4	7.3	48	13.3	7	0.25
DA62271	L64R	10	1.2	1.3	3	13.3	42.7	17.3	7	0.5
DA62275	L113P	12.7	1.2	1.3	3.3	14.7	48	17.3	8	0.25–0.5
DA62285	A60*	10.7	1.3	1.2	3	24	58.7	16.7	7	0.5

MIC values were determined with Etests and are presented as the mean value of three independent cultures.
CHL, chloramphenicol; TET, tetracycline; KAN, kanamycin; STR, streptomycin; NAL, nalidixic acid; ERY, erythromycin; RIF, rifampicin; NIT, nitrofurantoin; CST, colistin; —, no changes; *, stop codon.
[a]Broth microdilution test. Complying with standard procedure, we determined the MIC value of a colistin-resistant strain (*mcr-1* positive) to be >1 mg/L and of a colistin-susceptible QC strain ATCC 25922 to be 0.75 mg/L.

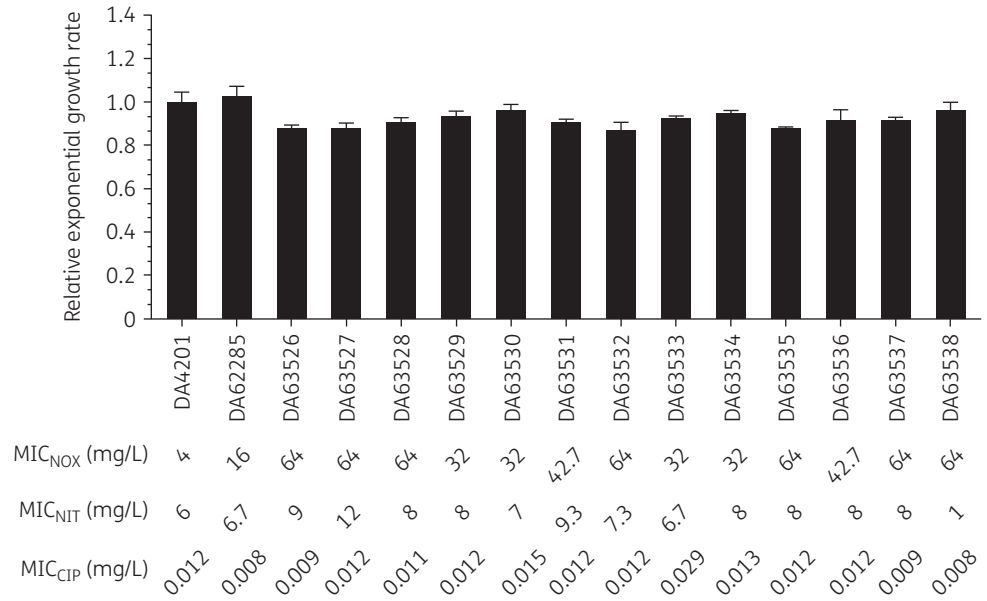

Figure 1. Relative exponential growth rate normalized to the WT strain DA4201 and susceptibility of second-step resistant mutants to nitroxoline (NOX), nitrofurantoin (NIT) and ciprofloxacin (CIP). Error bars represent standard deviation.

Table 4. WGS analysis of six nitroxoline-resistant second-step mutants

Strain	Nitroxoline MIC (mg/L)	Relative growth rate	Genomic changes
DA63527	64	0.88	A60*, 50 kb amplification
DA63528	64	0.91	A60*, 50 kb amplification
DA63530	32	0.96	A60*, 50 kb amplification
DA63533	32	0.92	A60*, *marR* N89T
DA63537	64	0.92	A60*, 50 kb amplification
DA63538	64	0.96	A60*, P_{Lon}::IS*186*[a]

*, stop codon.
[a]Identified with breseq. No additional mutations than those listed in the Table were found in these strains.

forms a complex with the *emrAB* efflux pump. Based on this known regulation, we investigated whether overexpression of *tolC* in the low-level resistance *ermR* mutant would allow for further increased resistance to nitroxoline. We transformed the first-step mutant DA62285 with ASKA plasmids containing the gene *tolC*, as well as *parC*, *parE* and *mdaB*, which also were overexpressed genes present in the 50 kb amplification. MIC determination using broth microdilution showed that only overexpression of *tolC* increased the resistance of the mutant DA62285 to 32 mg/L (Table 5). This confirmed our hypothesis that high-level nitroxoline resistance was obtained by increasing the expression of both *emrAB* and *tolC* genes.

We also examined the stability of the resistance in the first-step low-level nitroxoline mutants and the second-step high-level nitroxoline mutants. As is expected, over a period of growth of 50 generations in a drug-free medium, the mutations in the first-step mutants were stable and maintained in the population, while the second-step mutants containing amplifications and IS-element insertions were unstable and therefore lost (Figure S1).

Increased resistance is mediated by an increased efflux pump function

To further demonstrate that nitroxoline resistance, both low level and high level, was mediated by increased expression of *emrAB* and *tolC* genes, a qPCR analysis was performed (Figure 2). We observed significant increased expression of only the *emrAB* genes in the low-level resistance mutant and, as compared with the WT strains, the low-level resistant mutant DA62285 had a 34- and 39-fold increase in expression of *emrB* and *emrA*, respectively (Figure 2). In the high-level resistant mutants, as compared with WT, we observed increased expression of 74- and 67-fold and 79- and 65-fold for *emrA* and *emrB* and 4- and 3-fold *tolC* in DA63533 and DA63538, respectively (Figure 2). These observations confirmed our hypothesis that increased expression of the *emrAB* and *tolC* pump was mediating resistance to nitroxoline.

Nitrofurantoin-resistant mutants show no cross-resistance to nitroxoline

Considering that nitrofurantoin and nitroxoline are structurally similar, we examined whether nitrofurantoin-resistant mutants with inactivated *nfsAB* genes would show any cross-resistance to nitroxoline.[36] Our analysis showed that nitroxoline susceptibility in the different nitrofurantoin-resistant mutants was unchanged (Table 6). Furthermore, we also investigated whether the *emrR* mutations selected in our experiment would increase the nitrofurantoin resistance in the presence of inactivated *nfsAB* genes. As compared with an *nfsAB* double-knockout mutant, the double-knockout mutants with mutations in the *emrR* gene only had a small effect (one-step increase in MIC) on nitrofurantoin resistance (Table S3).

Discussion

Our study demonstrates that resistance to nitroxoline in *E. coli* can be achieved by increasing expression of the MDR pump EmrAB-TolC. The first-step, low-level nitroxoline-resistant mutants had mutations in the DNA-binding domain of *emrR* and these mutations were not associated with any reduction in growth rate. In the second-step high-level resistance mutants, apart from the *emrR* mutations, additional mutations capable of increasing MarA levels were observed, all of which resulted in increased expression of the *tolC* gene and a reduction in fitness. In addition, we tested whether resistance to nitroxoline involved the AcrAB efflux pump and found that AcrAB system knockout mutants, Δ*marR*, Δ*acrR*, Δ*acrA* and Δ*acrB*, showed no change in susceptibility, indicating that nitroxoline is not a substrate for this pump (data not shown).

The EmrAB-TolC pump forms a tripartite efflux system composed of the inner membrane protein EmrB, which belongs to the family of major facilitator superfamily (MFS) transporters, the periplasmically located membrane fusion protein (MFP) EmrA and the outer membrane channel TolC.[37] Its activity is thought to be masked by the broad molecule-binding activity of AcrAB, the most prevalent efflux pump mediating MDR in *E. coli*.[29] EmrAB was shown to form a dimer *in vitro*, and the three-component complex supports the efflux of toxic compounds across the inner and outer membrane, driven by the coupling of the energy stored in cation gradients.[38] EmrR, a transcriptional regulator belonging to the MarR family of repressors, negatively regulates the expression of the *emrAB* operon by directly binding to its promoter region.[25,26] A point mutation (R92H) in the *emrR* gene was recently shown to increase the function of the efflux pump EmrCAB in *C. violaceum* and mediate resistance to nalidixic acid.[27] In *E. coli*, EmrAB-TolC

Table 5. Effect on MIC of nitroxoline by overexpression of *parE*, *parC*, *mdaB* and *tolC*

Strain	IPTG	Nitroxoline MIC (mg/L)
DA4201	—	4
DA62285	—	16
DA63533	—	32
DA63538	—	32
DA62285 + *parE* plasmid	—	16
	100 μM	16
DA62285 + *parC* plasmid	—	16
	100 μM	16
DA62285 + *mdaB* plasmid	—	16
	100 μM	16
DA62285 + *tolC* plasmid	—	16
	100 μM	32

—, no addition.
Overexpression was achieved using the inducible ASKA plasmids and performed in replicates.

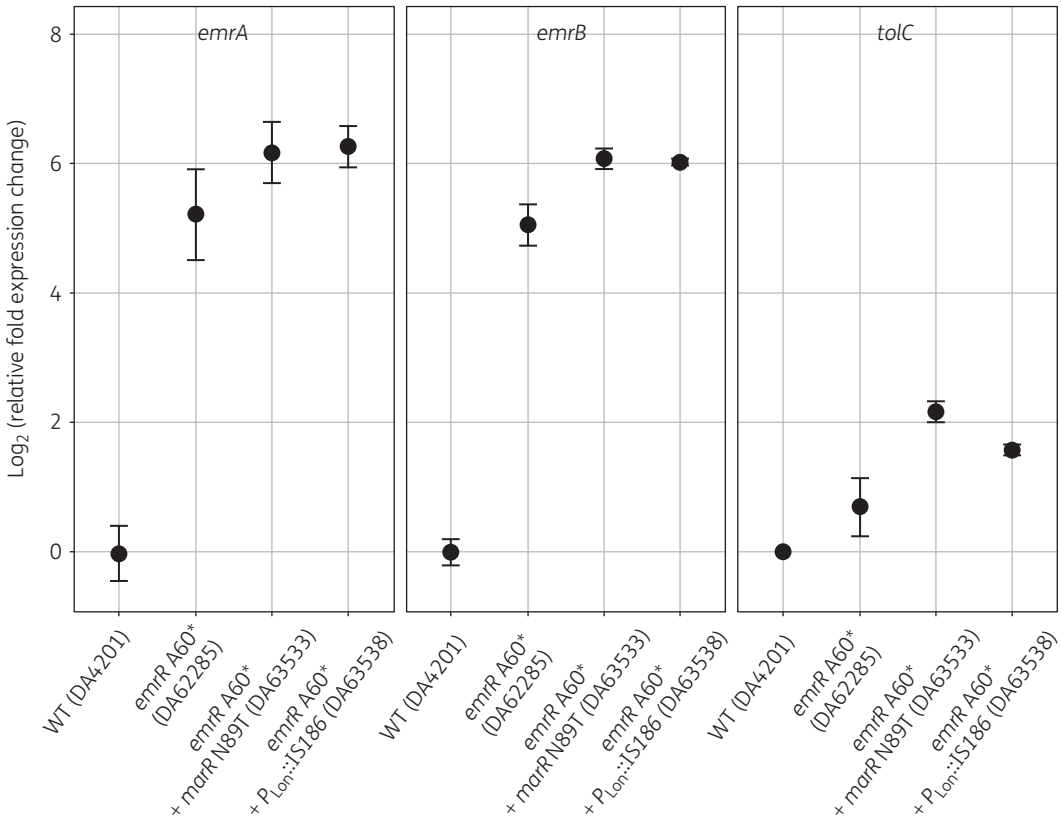

Figure 2. Gene expression-level changes for genes *emrA*, *emrB* and *tolC* in nitroxoline-resistant mutants. All expression levels were $\log_2$-transformed and are normalized to expression levels observed in the WT. Error bars represent standard deviation.

Table 6. Nitroxoline cross-resistance analysis of nitrofurantoin-resistant strains with different inactivating mutations in *nfsAB* genes

Strain	Mutation(s)	Nitrofurantoin MIC (mg/L)[a]	Nitroxoline MIC (mg/L)
DA4201	—	6	4
DA13815	*nfsA* A119fs	16	4
DA13824	*nfsA* N134fs	12	4
DA13957	*nfsA* A119fs, *nfsB* S43fs	192[a]	4
DA13992	*nfsA* N134fs, *nfsB* Q101*	128[a]	4

—, no changes; *, stop codon; fs, frame shift.
[a]As determined by Sandegren *et al.*[36]

has been shown to confer resistance to the antibiotics nalidixic acid and thiolactomycin, and to a set of chemically unrelated antimicrobial substances that act as uncouplers of the proton motive force and hydrophobic compounds.[25,28] Specifically, substrates of the EmrAB-TolC pump such as salicylic acid, carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone, 2,4-dinitrophenol and carbonyl cyanide m-chlorophenylhydrazone, have been demonstrated to directly bind the repressor EmrR and induce the expression of *emrAB*.[39] More work is needed to determine the level of interaction (if any) of the substrate antibiotics with the EmrR repressor.

Increased expression of multidrug efflux pumps can result in increased cross-resistance to other similarly structured molecules. Our cross-resistance analysis of the first-step mutants shows that increased expression of the EmrAB-TolC pump causes a 2-fold increase in resistance to nalidixic acid, confirming previous results,[24,26] but several other drugs are unchanged in susceptibility. In contrast, an overexpression analysis of *emrAB*'s ORF demonstrated no increase in resistance to nalidixic acid.[40] In the latter analysis, however, the overexpressed genes also included the repressor *emrR* and it is possible that no activation of the pump was observed owing to a continuous repression of the pump by increased EmrR levels. In the case of the second-step mutants, only a strain with an additional mutation in *marR*, (N89T, DA63533) showed cross-resistance to ciprofloxacin, possibly attributable to changes in the regulation of the MarA-mediated drug resistance.[30] On the other hand, the second-step mutant strain with P_{Lon}::IS*186* (DA63538) was more susceptible to nitrofurantoin, an observation previously made for this type of mutation.[31,41] Importantly, in spite of the structural similarities between nitroxoline and nitrofurantoin there was no indication of cross-resistance between the two drugs. Thus, mutations that up-regulated the EmrAB-TolC pump and conferred nitroxoline resistance had no effect on susceptibility to nitrofurantoin and, conversely, *nfsAB* mutations that caused nitrofurantoin resistance had no effect on susceptibility to nitroxoline. Although we see a small effect on nitrofurantoin resistance when the *emrR* mutations (selected in this study) are combined with *nfsAB*-inactivating mutations, our results generally point at different modes of action and resistance mechanisms for these drugs, which is in accordance with previous studies that suggest that nitroxoline primarily works by chelating biologically important divalent ions and nitrofurantoin by forming a reactive radical.[3–5,35]

Previous reports suggest EmrAB-TolC has no relevant role in mediating resistance to quinolone drugs.[40,42,43] These studies were carried out with different methodologies that could account for the contrasting observations presented in this study. Our findings suggest that the activation of the EmrAB-TolC pump confers increased resistance to nalidixic acid and nitroxoline, a quinolone and a quinoline, respectively. However, this is not the case for other quinolones, as shown by other studies for ciprofloxacin and norfloxacin,[24,40,43] and against ciprofloxacin in our study in Table 1 where low-level resistant mutants with mutations in the repressor *emrR* demonstrated no appreciable change in susceptibility. This suggests resistance mediated by the EmrAB-TolC pump cannot be generalized to the quinolone group, instead only to nalidixic acid. Disruption of the efflux pump function of pumps with a high homology to EmrAB-TolC in other species, EmrCABsm in *Stenotrophomonas maltophilia*,[44] VceAB in *Vibrio cholerae*[45] and EmrCAB in *C. violaceum*,[27] also demonstrated their role in mediating resistance to nalidixic acid.

The present study was performed under laboratory conditions and it remains to be determined whether the observed mechanism of resistance could emerge in clinical settings. As shown by previous studies of fosfomycin and nitrofurantoin resistance in *E. coli*,[36,46] the risk of selection of resistant mutants in the bladder during treatment will depend on the rate of emergence of resistant mutants and their fitness and level of resistance. Combined with the special conditions of the bladder and urinary system, where a sequence of filling and emptying of urine imposes a minimal growth rate required for the uropathogen to be maintained in the bladder (referred to as the Gordon–Riley criterion), this will determine the probability of fixation of a resistant mutant that emerges during treatment.[36,46,47] The concentration of nitroxoline conjugates in urine at steady-state has been reported to lie within a range of 0.8–210.6 mg/L.[2] Considering that the urinary concentration typically reaches levels above the MIC of nitroxoline described for the first-step as well as the second-step mutants, it is reasonable to assume that mutational resistance development to nitroxoline during treatment is unlikely. However, it should be noted that two caveats with this suggestion are that most of the analytical techniques used to measure levels of nitroxoline in urine are outdated and uncertain, and that it remains unclear whether only the unconjugated or both unconjugated and conjugated forms of this drug are active.[2,18]

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Transparency declarations

None to declare.

Supplementary data

Figure S1 and Tables S1–S3 are available as Supplementary data at *JAC* Online.

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