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Evaluating the level of nitroreductase activity in clinical *Klebsiella pneumoniae* isolates to support strategies for nitro drug and prodrug development

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ABSTRACT

To understand the potential utility of novel nitroreductase (NR)-activated prodrugs, NR enzyme activity was assessed in clinical *Klebsiella pneumoniae* isolates using a NR-activated fluorescent probe. NR activity was constant throughout the bacterial growth cycle, but individual *K. pneumoniae* isolates exhibited a wide range of NR activity levels. The genes of major NR enzymes (*nfsA* and *nfnB*) showed a number of sequence variants. Aside from a C-terminal extension of NfnB, which may be responsible for lower NR activity in specific isolates, the genetic differences did not explain the variation in activity. Analysis of important clinical strains (ST11, ST258, ST14 and ST101) showed significant variation in NR activity between isolates within the same sequence type despite conservation of *nfsA*/*nfnB* sequences. Addition of methyl viologen (MV), a known activator of *soxRS*, caused a significant increase in NR activity for all strains, with proportionally larger increases in activity seen for strains with low uninduced NR levels. Real-time PCR on selected strains following exposure to MV showed upregulation of *soxS* (15–32-fold) and *nfsA* (5–22-fold) in all strains tested. Expression of *nfnB* was upregulated 2–5-fold in 4/6 strains tested. High levels of NR activity in the absence of MV activation correlated with nitrofurantoin susceptibility. These data provide evidence that NR gene mutations and regulatory pathways influence NR activity in *K. pneumoniae* isolates and this is likely to impact treatment efficacy with novel nitro-containing drugs or prodrugs.

1. Introduction

Klebsiella pneumoniae is a major public-health issue, particularly with the increasing global prevalence of carbapenemase-producing strains [1]. Many clinical isolates produce a specific carbapenemase, the so-called *K. pneumoniae* carbapenemase (KPC), which has achieved global spread via isolates characterised as multilocus sequence type 258 (ST258) [2,3]. Few options for antimicrobial therapy exist for carbapenem-resistant *K. pneumoniae* infections [4,5].

Given the lack of treatment options, approaches enabling a broader range of chemical entities to be brought to market are worth investigating. Prodrugs, with inactive precursors activated only after uptake into bacterial cells, potentially enable the use of compounds that would otherwise have an unacceptable therapeutic index as well as reducing the impact and selective pressure on commensal bacteria [6,7]. Nitro-group-modified prodrugs have been developed previously for cancer using a directed-enzyme prodrug therapy (DEPT) with bacterial nitroreductase (NR) family enzymes [8]. Nitro-group-containing prodrug antibiotics such as nitrofurantoin, a NR-activated nitrofuran derivative, are commonly used for urinary tract infections. NR enzymes are found in all bacteria, but few studies have described enzyme activity, protein structure and regulation, or the physiological role of these enzymes. NRs enable bacteria to grow in environments with nitro-compound contamination, particularly toxic nitroaromatic compounds, which are a human creation [9]. It is unclear whether this selective advantage is enough to maintain the enzymes in isolates from uncontaminated environments or whether NRs play another role. Levels of NR activity are linked to resistance to nitro-group containing prodrugs, mediated by mutations in one or more of the NR enzymes [10].

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Klebsiella pneumoniae genomes (e.g. MGH 78578) contain two to four members of the NR family. Two of these NRs (NfsA and NfnB) are homologues of the major *Escherichia coli* enzymes NfsA and NfsB [9,11]. In *E. coli, nfsA* is co-ordinately regulated with other oxidative stress responses via the action of the *soxRS* and *marRA* regulatory systems, and is upregulated by the action of methyl viologen (MV) [11]. This suggests that the enzyme is involved in defence against free radical oxygen species [9].

No systematic studies describe the relative levels of NR activity expressed under normal conditions in specific pathogens and there is no evidence to understand the role of NRs in mediating susceptibility/resistance to other nitro-group-containing antibiotics (e.g. furazolidone), which are inactivated by elevated enzyme activity.

A fluorescent probe, based on a nitro-group-modified resorufin backbone, was generated [12] and was used to assess the levels of NR activity exhibited by different clinical *K. pneumoniae* strains as an easily measurable surrogate for a NR-activated prodrug. The primary sequences of NfsA and NfnB did not correlate with variance in NR activity. Rather, the study suggests that there is regulation of these enzymes via MV and SoxRS, overlaying different basal levels of NR activity in different strains. These are likely to impact on the use of nitro-group-containing drugs and prodrugs for *K. pneumoniae*.

2. Materials and methods

2.1. Nitroreductase activity assay and nitrofurantoin resistance in *Klebsiella pneumoniae*

The *K. pneumoniae* isolates have been described previously [13,14]; additional strains with defined sequence types (STs) were supplied by the Antimicrobial Resistance and Healthcare Associated Infection (AMR-HAI) Reference Laboratory of Public Health England–Colindale (London, UK). Strains were inoculated and grown for ≥16 h with aeration at 200 rpm at 37 °C in tryptic soy broth (TSB). The optical density at 600 nm (OD_{600}) was measured, cultures were diluted to an OD_{600} of 0.1 in fresh media and then 100 µL aliquots were dispensed into a microtitre plate. A caged fluorescent probe (hereafter Probe 1), designed to be activated by bacterial NR enzymes, was synthesised essentially as described previously [12] (Supplementary material). The probe was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 5 mM, was diluted to 25 µM in TSB and then 100 µL was added to the diluted bacterial suspension. Fluorescence (excitation 544 nm, emission 590 nm) was measured every 5 min for up to 20 h in a FLUOstar plate reader (BMG Labtech). Then, 4 µg/mL methyl viologen dichloride hydrate 98% (Sigma, St Louis, MO, USA) (the highest concentration of MV that did not affect *K. pneumoniae* growth; data not shown) was added as required.

To correlate with the observed levels of NR activity, nitrofurantoin susceptibility was assessed both in aerobic and anaerobic conditions by the disk diffusion assay (50 µg disks; Oxoid Ltd., Basingstoke, UK) on trypticase soy agar plates. Zones of clearance were measured from at least two replicate experiments.

Sequences of the *nfsA* and *nfnB* genes were determined using whole-genome sequencing (WGS) as previously described [15] and were verified by Sanger sequencing using the primers listed in Supplementary Table S1.

2.2. Quantitative PCR (qPCR)

Expression of *nfsA, nfnB* and *soxS* was assessed by qPCR, with and without MV, for selected *K. pneumoniae* strains that showed differing levels of NR probe activation. The primers are listed in Supplementary Table S1. Triplicate overnight cultures grown in TSB were back-diluted to 0.1 OD_{600} and were harvested using RNAprotect® Bacteria Reagent (QIAGEN) at mid-log phase ($OD_{600} = 0.5$). For extraction in the presence of MV (4 µg/mL), cultures were back-diluted to an OD_{600} of 0.25 and were incubated for 30 min at 37 °C with shaking before RNA was extracted using an RNeasy® Mini Kit (QIAGEN), including on-column DNase treatment according to the manufacturer's instructions. In addition, 5 µg of RNA was treated with a DNA-free™ Kit (Ambion) and then 0.2 µg of RNA was reverse transcribed using a SuperScript™ III First-Strand Synthesis System (Invitrogen, UK). qPCR was carried out at least three times on each sample using a StepOnePlus™ Real-Time PCR system (Life Technologies) and Fast SYBR® Green Master Mix (Life Technologies). Data were analysed using Expression Suite Software v.1.0.3 (Life Technologies) using *gapA*, *rpoB* and *infB* as endogenous controls and taking primer efficiency into account.

2.3. Homology modelling

Homology modelling of NRs used SWISS-MODEL webserver [16–20] with FASTA format amino acid sequences. The crystal structure of *E. coli* NfsA (PDB; 1YLR) was used as the template; sequence identity between target (*K. pneumoniae* strain MGH 78578) and the template were NfsA 83% and NfnB 87%. The homodimeric NRs contained a flavin mononucleotide (FMN) cofactor in each monomer. PyMOL was used to generate mutant structures, with an appropriate rotamer of mutated amino acid that does not lead to any steric clash with the neighbouring residues.

2.4. Molecular docking

Molecular docking was performed to generate several distinct binding orientations and to determine binding affinity, with the lowest binding free energy considered as the most favourable binding mode. AutoDock SMINA [21], using the AutoDock Vina scoring function as default, was used for blind docking of Probe 1 to the NfsA and NfnB subunits. SMINA was performed with default settings, which samples nine ligand conformations using the Vina docking routine of stochastic sampling. The binding site was located close to the FMN co-factor by the SMINA molecular docking and GOLD molecular docking [22,23]. Flexible molecular docking was applied to the docking of Probe 1 to the SMINA-located best binding site of the NR subunits. Based on the fitness function scores and ligand binding positions, the best-docked poses for the Probe 1 were selected.

2.5. Molecular dynamics (MD) simulations

Following molecular docking, 25-ns MD simulations were performed for each complex and the final equilibrated distance between FMN and the probe in native and mutant complexes was monitored. All MD simulations were carried out using the AMBER 12.0 package. Each system was solvated by using an octahedral box of TIP3P water molecules. During each simulation, all bonds in which the hydrogen atom was present were considered fixed, and all other bonds were constrained to their equilibrium values by applying the SHAKE algorithm [24]. The force-field parameters for Probe 1 were generated using the ANTECHAMBER module of the AMBER program.

A cut-off radius of non-covalent interactions was set to 12 Å for the protein and complex. Each minimisation and equilibration phase was performed in two stages: first, ions and all water molecules were minimised for 500 cycles of steepest descent followed by 500 cycles of conjugate gradient minimisation; and second, the whole system was minimised for a total of 2500 cycles without restraint, wherein 1000 cycles of steepest descent were followed by 1500 cycles of conjugate gradient minimisation. In the

second stage, the systems were equilibrated for 500 ps while the temperature was raised from 0 K to 300 K, and equilibration was then performed without a restraint for 100 ps while the temperature was kept at 300 K. Sampling of reasonable configurations was conducted by running 25-ns simulations with a 2 fs time step at 300 K and 1 atm pressure. A constant temperature was maintained by applying the Langevin algorithm, whilst the pressure was controlled by the isotropic position scaling protocol used in AMBER [25].

3. Results

3.1. Differing levels of nitroreductase activity in clinical Klebsiella pneumoniae isolates

A NR-activated fluorescent probe (Probe 1) was synthesised to enable real-time measurement of NR activity in *K. pneumoniae* cells during standard growth. Molecular simulation modelled the docking of Probe 1 with the two predominant NR enzymes in *K. pneumoniae* (NfsA and NfnB) from strain MGH 78578. The data suggested that the probe would be recognised by both enzymes with a similar binding energy and predicted distance between the FMN cofactor and the enzyme cleavage site (Fig. 1; Supplementary material). The NR activity of a variety of *K. pneumoniae* clinical isolates with differing antibiotic resistance mechanisms and different sequence types was assessed. All strains were able to activate the probe, with activation detected above the threshold (based on 3 standard deviations above background cell fluorescence) at between 1.5 h and 4.7 h post-addition, with a median time of 2.3 h to activation (data not shown). The endpoint fluorescence (20 h) was measured for each isolate and large differences between activation levels were observed between different strains (Fig. 2). To assist in interpretation, levels of activation were grouped into high (top 25%), medium (mid 50%) and low (bottom 25%) activity based on mean normalised endpoint fluorescence. No significant differences in cell growth were observed for the majority of the strains tested in the presence or absence of the probe, with the OD_{600} at 20 h showing <10% variance (data not shown). The outlier was Murray strain M433, a strain originally isolated in 1940 [14], that had an endpoint OD_{600} typically 50% of the mean of the other isolates in the presence and absence of Probe 1. Low cell densities have been observed to artificially reduce the signal in resorufin/resazurin assays [26].

To understand whether the level of NR activity is linked to a particular growth phase, bacteria were isolated at different time intervals (representing lag, mid-log and late-log phase) from a selection of high, mid and low NR activators. Cell pellets were normalised for cell numbers and the amount of NR activity was assessed by measuring probe activation after cell lysis with Triton X-100 (Supplementary Fig. S1). No significant difference was seen between the different time points for all strains tested, suggesting that the observed NR activity is not affected by the growth phase.

3.2. Differences in nfsA and nfnB gene sequence only partially explain different levels of nitroreductase activity

The sequences of *nfsA* and *nfnB* were analysed and were compared with the reference strain MGH 78578 (Table 1). NfsA has an asparagine residue in place of the lysine residue found in the majority of other strains at position 222. The lysine is predicted to form part of the active site of the equivalent *E. coli* enzyme [27]. Similarly, most other strains had a conservative replacement of an alanine for valine at position 196 in NfnB. The majority of strains with low probe activation had a significant sequence alteration in one of the two NR genes. Strain NCTC 13443 (ST14) had an elongated 24-amino acid C-terminal section in NfnB, strain M433 (ST93) had a frameshift mutation resulting in a severe truncation in NfsA, and the ST258 strains (NCTC 13438, 51851 and 46704) also had a severe truncation to NfsA. These three ST258 strains also had a P190R substitution in NfnB. Strains 2609 and 2619 (ST11) had identical NfsA and NfnB sequences to the ST258 strains but had significantly higher probe activation levels ($P < 0.001$), suggesting that sequence variation is not the only factor affecting probe activation levels. Two isolates, the intermediate probe activator strain 16 (ST101) and the low probe activator strain 18 (ST15), entirely lacked one of the NR genes (*nfnB*). Strain 16 had a unique mutation in NfsA (R203C) that, in terms of overall activity, might partly compensate for the loss of NfnB in this strain. However, other ST101 isolates contained an *nfnB* gene and showed similar levels of probe activation to strain 16.

Molecular docking using homology-modelled NRs was used to study the impact on the interaction with the probe of three of the variants (P190R from NfnB; and D162V and R203C from NfsA) identified in the clinical isolates (Supplementary Tables S2–S7). The results suggested, both in the P190R NfnB variant and the R203C NfsA variant, that the probe was positioned more closely to the FMN cofactor than in the wild-type enzyme, and this may increase activation of the fluorescent probe.

Resistance to nitrofurantoin is linked to mutations in *nfsA* and *nfsB* in *E. coli* and in the current study susceptibility to nitrofurantoin was determined by the disk diffusion method both under aerobic and anaerobic conditions (Table 1). In general, probe activation levels gave an indication as to the level of susceptibility to nitrofurantoin based on measured zones of clearance. All strains showing high levels of probe activation were susceptible to nitrofurantoin, with zones of clearance between 8 mm and 28 mm under aerobic conditions and between 14 mm and 28 mm under anaerobic conditions. Strains with low levels of probe activation were resistant to nitrofurantoin, with all low-activating strains showing no zone of clearance, with the exception of M433 that grows poorly under test conditions, and strain 18 (ST15) that showed a small zone of clearance under anaerobic conditions. The intermediate group showed sporadic strains, e.g. NCTC 13439 (ST54), that were resistant to nitrofurantoin and others that were susceptible, e.g. M6 (ST23), NCTC 9633 (ST3) and KPW8 (ST317), despite having similar ranges of probe activation. All ST11 and ST258 strains with a predicted non-functional NfsA were resistant to nitrofurantoin (no zone of clearance), as was strain 16 (lacking NfnB). The role of other nitrofurantoin resistance determinants, such as the presence/absence of other efflux pumps and regulators, e.g. *oqxAB, acrAB* and *marAR*, was not examined as part of this study given the diversity of strain backgrounds and the difficulty in interpreting the data. The gene encoding regulator *ramAR* is located very close to the *nfnB* gene in *K. pneumoniae* and is missing in all strains that lack *nfnB*. Whether this has a stronger role in regulating NR activity was not investigated.

3.3. Both the nfsA and nfnB genes are regulated by the SoxRS regulon in Klebsiella pneumoniae

With the genetic sequence of the *nfsA* and *nfnB* alleles not fully explaining the differences in levels of probe activation, the study explored the transcriptional regulation of the two genes. In *E. coli, nfsA* expression is induced by addition of MV via the *soxRS* regulon as part of the oxidative stress response [28], and *E. coli nfsB* has a predicted SoxS binding site in its promoter. All isolates tested showed increased levels of probe activation when exposed to MV (4 μg/mL), irrespective of the basal levels of probe activation (Fig. 3). This was true in strains that lacked NfnB (strains 16 and 18) and those expected to have a non-functional NfsA (ST258 and ST11 strains), suggesting that both *nfsA* and *nfnB* are regulated by SoxRS. Across all sequence types, strains with low probe activa-

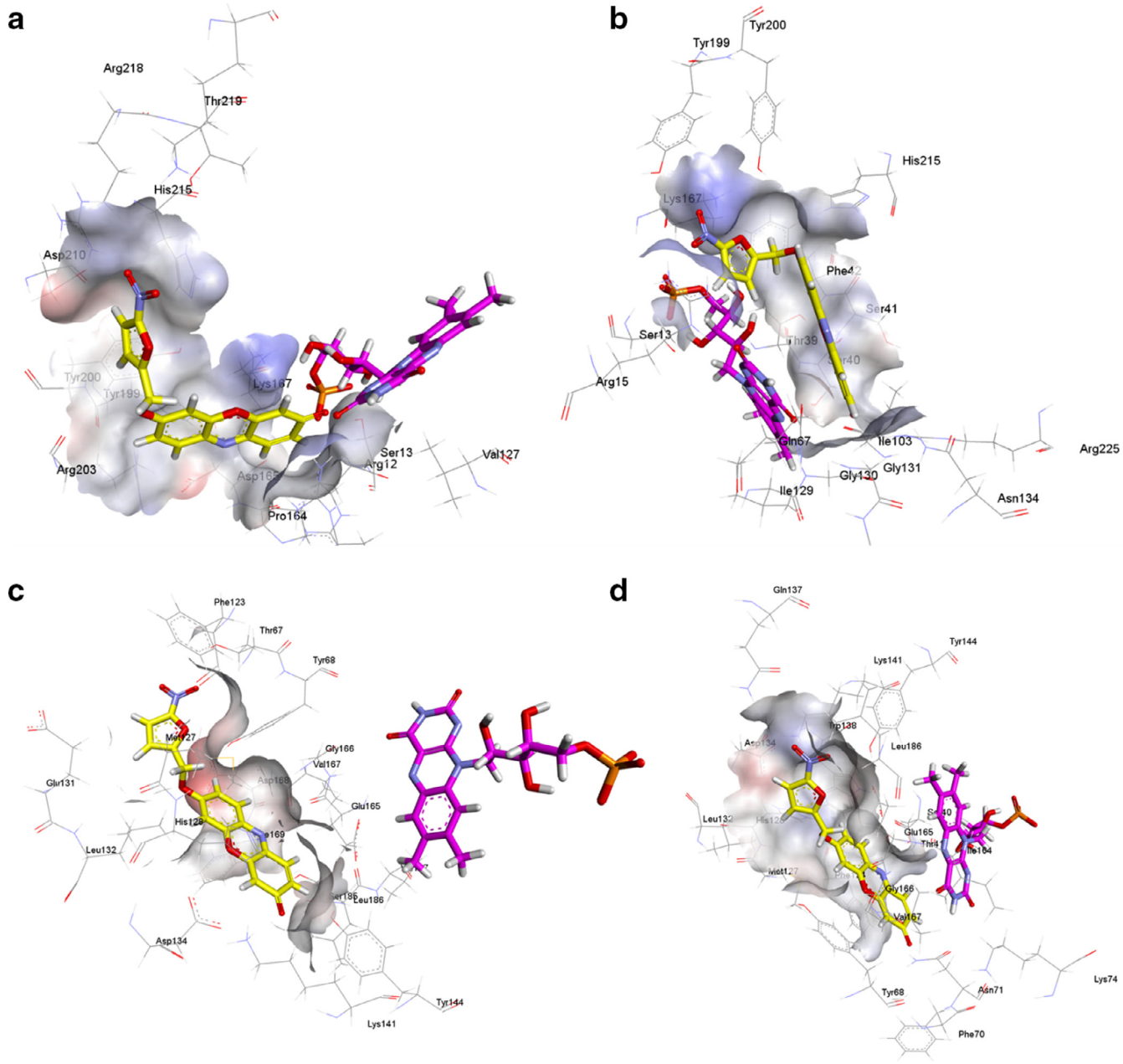

Fig. 1. Molecular models showing interactions of Probe 1 (yellow) with the *Klebsiella pneumoniae* MGH 78578 NfsA subunit A (a) and subunit B (b), and MGH 78578 NfnB subunit A (c) and subunit B (d) showing close proximity with the flavin mononucleotide cofactor (purple).

tion in the absence of MV showed a greater increase in NR activity with an mean of 2.90-fold increase in activity upon addition of MV (range 1.86–4.55-fold) compared with 1.67-fold (range 1.01–2.96-fold) and 1.12-fold (range 1.02–1.25-fold) for intermediate and high probe activators, respectively (Supplementary Tables S8–S10).

3.4. Understanding variation in nitroreductase activity in epidemic strains with identical NfsA and NfnB

To understand whether there is variation in NR activity within sequence types, a wider panel of important clinical strains (ST14, ST101, ST11 and ST258) were tested (Fig. 4). For ST14, there was clear partitioning of low versus high levels of probe activation based on the presence/absence of the C-terminal extension to NfnB; six strains that showed low levels of activation (0.1–0.25) contained a frameshift within the stop codon at the end of *nfnB* and one strain contained a deletion within *nfnB* (KPTR8), whilst five isolates with higher levels of activation (0.53–0.95) contained no such mutation (Fig. 4a, solid bars) (Supplementary Table S11). Strains with high and intermediate probe activation all showed zones of clearance with nitrofurantoin, providing a clear relationship between NR activity, NfnB activity and nitrofurantoin susceptibility in this strain background. All ST101 strains had an identical NfsA sequence (R203C variant) and, with the exception of strain 16, contained a predicted functional NfnB (Supplementary Table S12). All strains showed low levels of probe activation with a maximal endpoint fluorescence of 0.4 (strain 16) (Fig. 4a, solid bars) and were resistant to nitrofurantoin. All ST11 and ST258 isolates tested had an identical profile of *nfsA* (frameshift) and *nfnB* (P190R, A196V) sequences (Supplementary Tables S13 and S14), with the exception of strain CFI_131_KPC2 that lacked *nfnB*. However, they showed a range of relative probe activation. In ST11 isolates this

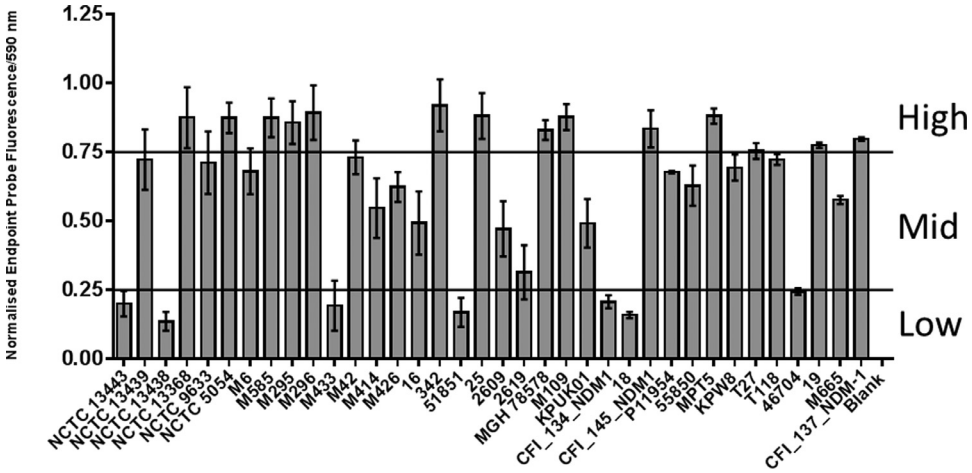

Fig. 2. Nitroreductase (NR) activity showing distinct variation within a panel of *Klebsiella pneumoniae* clinical isolates. Cultures were diluted down to an OD_{600} of 0.1 (early log-phase) and bacterial NR activity was observed by measuring fluorescence (590 nm) of Probe 1 every 5 min for 20 h. Results are shown as the 20-h endpoint values and are the mean ± standard deviation of three independent experiments. The top quartile and lower quartile are indicated, with mean values defining the isolates as low, mid and high probe activators as indicated. OD_{600}, optical density at 600 nm.

Table 1
Sequence variation in NfsA and NfnB compared with *Klebsiella pneumoniae* MGH 78578 (standard) for all *K. pneumoniae* isolates tested.

Isolate	MLST	Sequence variation		Zone of clearance with NIT (mm)[a]		NR activity
		NfsA	NfnB	Aerobic	Anaerobic	
MGH 78578	ST38	None	None	10	20	High
M585	ST3	N222K	A196V	28	28	
CFI_145_NDM1	ST15	N222K	A196V	16.5	22	
MPT5	ST20	N222K	A196V	16	18	
M109	ST23	N222K	A196V	14	23	
T27	ST27	A20V, Q68R, N222K	A196V	17	20	
25	ST34	N222K	A196V	16	20	
M295	ST35	Q195K, N222K	A196V	14	24	
M296	ST36	A55T, N222K	A196V	14	28	
19	ST48	A55T, N222K	None	8	14	
NCTC 5054	ST82	N222K	A24G, A196V	22	28	
342	ST146	R59Q, T117I, E144A, R180H, E191D, E194D, N222K	G25S, A196V	18	24	
NCTC 13368	ST489	E29A, Q94E, Q147K, E194D, N222K	Q181K, A196V	12	14	
CFI_137_NDM1	ND	N222K	A196V	19	20	
NCTC 9633	ST3	N222K	A196V	20	28	Intermediate
2609	ST11	Insertion (G) after nt 70 (frameshift/premature termination)	P190R, A196V	0	0	
2619	ST11	Insertion (G) after nt 70 (frameshift/premature termination)	P190R, A196V	0	0	
KPUK01	ST15	N222K	Absent	0	18	
M6	ST23	N222K	A196V	18	18	
NCTC 13439	ST54	D162V, N222K	A196V	0	14	
M414	ST82	N222K	A24G, A196V	26	34	
16	ST101	R203C, N222K	Absent	0	0	
55850	ST147	A112V, N222K	M1R, A196V	0	15	
KPW8	ST317	N222K	None	15	18	
T118	ST461	A190G, N222K	A196V	19	23	
P11954	ST628	Q195L, N222K	A196V	15.5	19	
M42	ND	L132V, N222K	A24G, A196V	34	38	
M426	ND	N222K	A196V	0	16	
M665	ND	N222K	A24G, A196V	20	24	
NCTC 13443	ST14	N222K	A196V, STOP218L (elongated C-terminal section)	0	0	Low
CFI_134_NDM1	ST15	N222K	A196V	0	0	
18	ST15	N222K	Absent	0	9	
M433[b]	ST93	V78A, Δg379 (frameshift and premature termination)	A196V	26	36	
NCTC 13438	ST258	Insertion (G) after nt 70 (frameshift/premature termination)	P190R, A196V	0	0	
46704	ST258	Insertion (G) after nt 70 (frameshift/premature termination)	P190R, A196V	0	0	
51851	ST258	Insertion (G) after nt 70 (frameshift/premature termination)	P190R, A196V	0	0	

MLST, multilocus sequence typing; NR, nitroreductase; ND, not determined; nt, nucleotide.
[a] Relative nitrofurantoin (NIT) resistance, shown by a zone of clearance with a 50 μg NIT disk, was measured under aerobic and anaerobic conditions.
[b] Strain shows poor growth in culture and this is likely to be the cause of the low NR activity recorded.

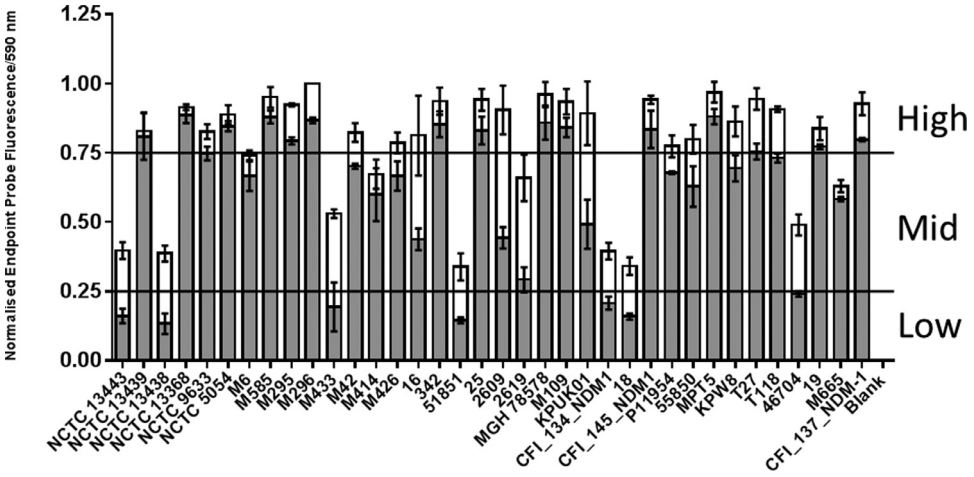

Fig. 3. Nitroreductase (NR) activity was enhanced following exposure to methyl viologen (MV). The NR activity for several *Klebsiella pneumoniae* isolates was observed in the presence (white bars) or absence (grey bars) of MV. Results are shown as the 20-h endpoint values and are the mean ± standard deviation of three independent experiments. Ratios of the NR activity observed ± MV are shown in Supplementary Tables S8–S10.

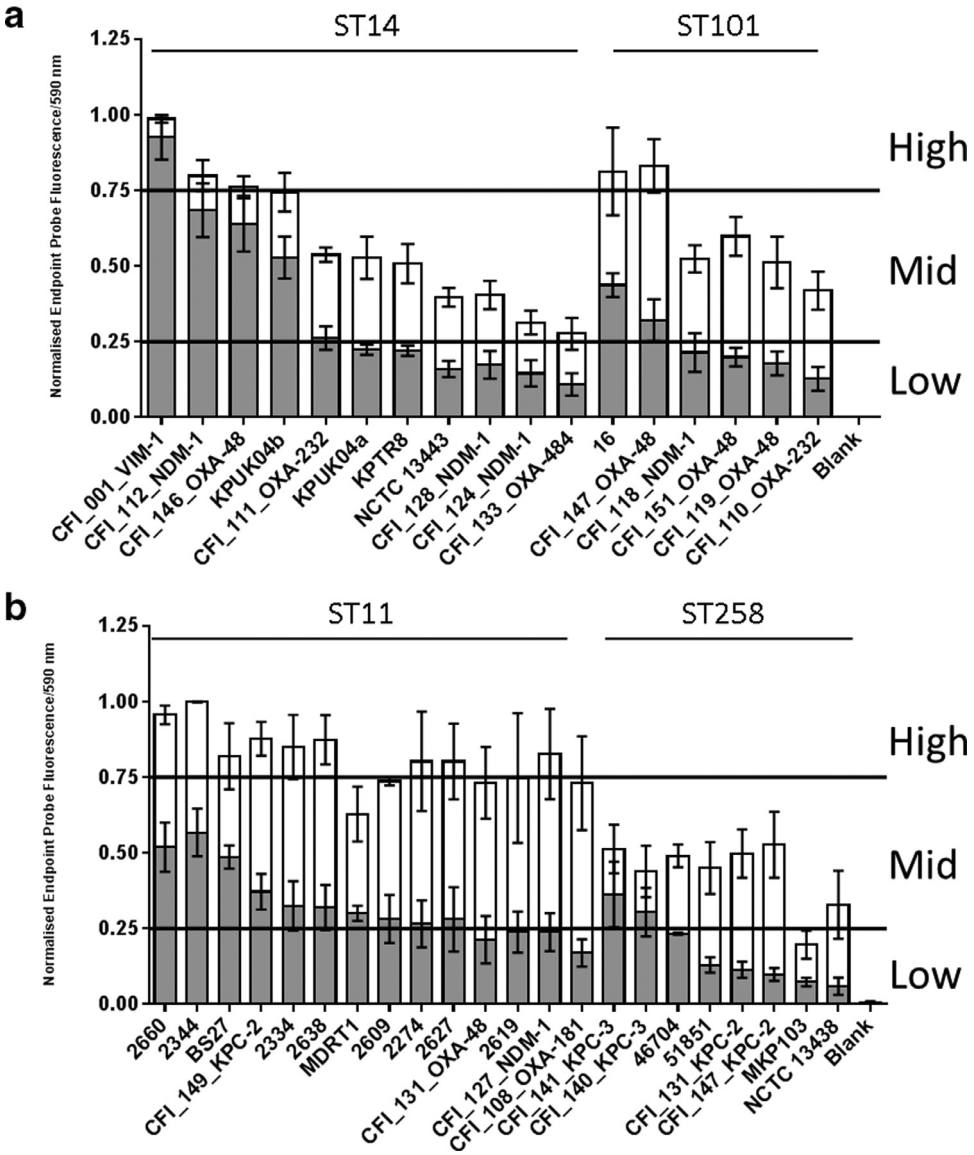

Fig. 4. Nitroreductase (NR) activity showed considerable variation even within the same sequence type (ST). NR activity from a range of *Klebsiella pneumoniae* strains from (a) ST14 and ST101 and (b) ST11 and ST258 was observed in the presence (white bars) and absence (grey bars) of methyl viologen. Results are shown as the 20h endpoint values and are the mean ± standard deviation of three independent experiments.

Table 2
Increase in transcription levels for nitroreductase (NR) genes *nfsA* and *nfnB* and the global regulator *soxS* following exposure to methyl viologen (MV).

Isolate	Fold increase[a]			NR activation level	
	nfsA	*nfnB*	*soxS*	Without MV	With MV
NCTC 13368	7*	3**	32*	High	High
NCTC 13443	9**	2*	29*	Low	Mid
51851	5†	3*	16*	Low	Mid
MGH 78578[b]	23**	No change	17*	High	High
M109	12*	5*	15*	High	High
16	22*	Absent	20*	Mid	High

[a] Statistically significant differences: * $P \leq 0.01$; ** $P \leq 0.05$; and † $P \leq 0.1$.
[b] The *nfnB* gene was present in MGH 78578 but did not show a significant change in expression on exposure to MW.

ranged from 0.17–0.57, with strains 2660, 2344 and BS27 having significantly enhanced probe activation. WGS analysis was unable to identify mutations leading to higher NR activity in these strains compared with other ST11 strains (results not shown). For ST258, all strains were low probe activators (0.05–0.23) except for isolates CFI_141_KPC3 and CFI_140_KPC3 (0.3–0.36). (Fig. 4b, solid bars). Further analysis showed the presence of a truncated *soxR* gene (SoxR Q97STOP), which would result in constitutive upregulation of *soxS* in the absence of MV. Therefore, the higher basal levels of activity are likely to be mediated by SoxS-induced upregulation of *nfnB*. This effect is negated in the presence of MV where all ST258 strains showed similar levels of activation. Whilst the addition of MV had the effect of normalising the activity within each sequence type, the difference in relative levels between ST11 and ST258 isolates was maintained despite the identical NR gene sequences. In addition to this, the transposon library derived from a ST258 strain (MKP103 [29]) was used to investigate the NR activity of a strain that has a transposon inserted in *nfnB* as well as premature termination of *nfsA*. There appeared to be no significant difference in the level of NR activity between MKP103Δ*nfnB* and MKP103 ($P > 0.01$) (results not shown). ST11 and ST258 strains showed no zones of clearance with nitrofurantoin, with the exception of two ST258 strains (CFI_131_KPC2 and CFI-147-KPC2) that showed a zone of clearance under anaerobic conditions only. These strains share the lack of an active NfsA with other ST11 and ST258 strains owing to premature termination, and strain CFI_131_KPC2 also lacked the *nfnB* gene. The strains also carried an identical allele of the OqxAB efflux pump to other ST258 and ST11 strains. The presence/absence of OqxAB was analysed in all strains tested, but again the simple presence of the efflux pump genes did not explain differences in nitrofurantoin susceptibility. We cannot rule out the possibility that the efflux pump is upregulated in some strains but not others, accounting at least part for the difference in nitrofurantoin resistance.

qPCR was used on high and low NR activator strains to confirm upregulation of *soxS, nfsA* and *nfnB* upon exposure to MV. All strains showed highly significant upregulation of *soxS* expression upon the addition of MV, with a range of 15–32-fold upregulation (M109 and NCTC 13368, respectively; $P \leq 0.01$) (Table 2). Levels of *soxS* upregulation did not correspond directly with either the fold increase observed in probe activation with MV nor with the basal level of activity. Hence, NCTC 13368 showed a 32-fold increase in *soxS* expression and a 7-fold increase in *nfsA* expression but little increase in NR activity upon addition of MV. NCTC 13443 and 51851 (ST14 and ST258, respectively) showed a 29- and 16-fold increase in *soxS* expression and 2- and 3-fold increase in expression of *nfnB* (likely to provide the principle NR activity owing to the frameshift mutation in *nfsA*) but showed significant increases in the levels of NR activity (2.48- and 2.32-fold respectively). No upregulation of *marA* upon MV exposure was observed, which was surprising given its role in upregulation of *nfsA* and *nfsB* in *E. coli* [30,31], and might point towards a role for the *ramAR* regulator that is located next to *nfnB* on the chromosome. Nor was an increase in the levels of NR activity observed with the addition of sodium salicylate, a known stimulator of MarA-mediated responses (results not shown).

4. Discussion

This study explored the expression levels and regulation of NRs in clinical *K. pneumoniae* isolates in order to understand whether the enzyme is a target to support the development of novel prodrugs to combat antimicrobial resistance. The data suggest that a NR-based prodrug strategy would not be appropriate for this pathogen and revealed insights into both genetic and transcriptional factors that might affect the overall levels of NR activity. The study showed a strong correlation between strains with high levels of NR activity, as measured with the probe, and nitrofurantoin susceptibility. Strains with intermediate and low levels of probe activation did not show a clear relationship between NR activity and nitrofurantoin susceptibility, suggesting that this is multifactorial.

This study revealed wide variance in the ability of clinical strains to cleave a fluorescent probe (Probe 1), an activation that mimics that of nitro-group containing drugs such as nitrofurantoin. There was evidence of a clear reduction in NR activity in the globally important multidrug-resistant ST258 lineage and the closely related ST11. This might have been predicted given the conservation of a frameshift mutation in *nfsA* in all of the ST258 and ST11 strains that were analysed in this study and more widely in these lineages. This study identified strains that lacked NR activity owing to disabling mutations in both major NR genes, as has been described in other pathogens [32,33], and this was replicated using a transposon mutant in a ST258 background, but the study clearly demonstrates that other factors significantly influence the levels of NR activity in cells. Factors affecting maintenance of the NR genes and/or the presence of disabling mutations might include selective pressure from the use of particular antibiotics, either negatively (e.g. nitrofurantoin selecting for loss of NR genes) or positively (e.g. furazolidone selecting for increased NR activity), perhaps counterbalanced by fitness or metabolic advantage of retaining NR activity in some environments. This is the first description of regulation of *nfsA* and *nfnB* by oxidative stress responses likely linked to *soxRS* expression in *K. pneumoniae*, as has been reported previously in *E. coli* [11,32]. In *K. pneumoniae*, the increase in NR gene expression owing to the addition of MV was independent of an increase in *marA* expression, although previously MarA has been shown to regulate *nfsB* gene expression in a salicylate-stimulated response in *E. coli* [31]. Analysis of the ratio of NR activity after MV treatment to the basal level suggests that some strains have constitutively elevated levels of enzyme activity. In two ST258 strains (CFI_141_KPC3 and CFI_140_KPC4), we were able to identify that this was due to a C-terminal deletion in SoxR, likely to permanently derepress *soxS* expression. The ST11 strains 2660 and 2344 did not appear to have unique sequence variations in *nfsA, nfnB* or *soxRS* or in other potential regulatory genes (e.g. *marAR, rob*), and differences in constitutive activation are not explained. This suggests that other unidentified regulatory pathways may also influence NR activity in *K. pneumoniae*. There is growing evidence of a complex regulatory network mediated by small AraC-type regulators, such as SoxS, RamAR and MarA in *Klebsiella*, finetuning expression of a range of targets [34–36]. It is feasible that this network of regulators, perhaps in combination with small RNA species, plays a role in this case.

Resistance to nitrofurantoin owing to loss-of-function mutations in NR genes, typically both *nfsA* and *nfnB*, have been described in *E. coli* [32,33,37]. ST11 and most ST258 isolates were resistant

to nitrofurantoin at the concentration tested and were universally found to be weak activators of the fluorescent probe. Other resistance mechanisms, including efflux via the OqxAB efflux pump, which is widely distributed in *K. pneumoniae*, are known to influence resistance to nitrofurantoin [38]. We were not able to differentiate between nitrofurantoin resistance mediated by reduced NR activity alone, efflux pump expression or other mechanisms; it is likely that all three contribute to the overall level of resistance in the clinic. Conversely, isolates found to have high levels of NR activity could potentially confer some resistance to antimicrobial agents that contain a nitro-aromatic group, as described in *Mycobacterium smegmatis* resistance to benzothiazinones [39]. A number of new compound series with NR-activated prodrugs have proved to be effective for targeting trypanosomes and *Leishmania*, NR-producing eukaryotic parasites [40,41], and have recently been explored for other pathogens [42]. The data presented here suggest that a nitrofurantoin-like prodrug strategy would not be effective for *K. pneumoniae* owing to the intrinsic variance in NR activity and particularly given the low activity in the globally important ST258. Compounds with better efficacy in cells with low NR activity might, however, be useful treatment options for such strains. The fluorescent probe is a useful tool for understanding changes in NR activity and how this contributes to the mechanism of action and/or resistance for new nitro-group containing drugs and prodrugs [43]. This may open up new possibilities for tailoring therapies for bacteria with either very low or very high levels of NR activity, perhaps in combination with probe-based diagnostics.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2019.08.009.

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