Role of $\text{oxyR}^{KP}$, a novel LysR-family transcriptional regulator, in antimicrobial resistance and virulence in *Klebsiella pneumoniae*

Vijaya Bharathi Srinivasan, Amitabha Mondal, Manjunath Venkataramaiah, Neeraj Kumar Chauhan and Govindan Rajamohan

*Klebsiella pneumoniae* is a Gram-negative bacillus that causes serious infections in immunocompromised human hosts and exhibits significant multidrug resistance. In this study, we identified a novel lysR-family regulator (designated $\text{oxyR}^{KP}$) in the genome of *K. pneumoniae* NTUH-K2044 whose functions have remained enigmatic so far. Functional characterization of the putative lysR regulator $\text{oxyR}^{KP}$ with respect to cellular physiology and antimicrobial susceptibility was performed by generating an isogenic mutant, $\Delta\text{oxyR}^{KP}$ in a hypervirulent clinical isolate of *K. pneumoniae*. The *K. pneumoniae* $\text{oxyR}^{KP}$ mutant was sensitive to hyperosmotic and bile conditions. Disruption of $\text{oxyR}^{KP}$ increased the susceptibility of *K. pneumoniae* to oxidative (0.78947 mM hydrogen peroxide) and nitrosative (30 mM acidified nitrite) stress by ~1.4-fold and ~10-fold, respectively. Loss of the *Klebsiella* regulator led to a decrease in the minimum inhibitory concentrations for chloramphenicol (10-fold), erythromycin (6-fold), nalidixic acid (~50-fold) and trimethoprim (10-fold), which could be restored following complementation. The relative change in expression of resistance–nodulation–cell division super family (RND) efflux gene *acrB* was decreased by approximately fivefold in the $\text{oxyR}^{KP}$ mutant as evidenced by qRT-PCR. In a *Caenorhabditis elegans* model, the $\text{oxyR}^{KP}$ mutant exhibited significantly ($P<0.01$) lower virulence. Overall, results detailed in this report reflect the pleiotropic role of the $\text{oxyR}^{KP}$ signalling system and diversity of the resistance determinants in hypervirulent K1 serotype *K. pneumoniae* NTUH-K2044.

INTRODUCTION

Signal transduction pathways enable bacterial cells to continuously monitor their surrounding environment and elicit an appropriate adaptive cellular response (Bourret *et al.*, 1991; Jung *et al.*, 2012). Apart from the two-component signal transduction regulatory systems, bacterial genomes encode other regulatory proteins belonging to different families such as ArsR, AsnC, Crp, DeoR, GntR, IclR, LacI, LuxR, XylS, MarR, MerR, NtrC, TetR, YedF and YhdG. Among these, the LysR-type transcriptional regulator (LTTR) is the most abundant and best-characterized type of regulatory cascade in the prokaryotic kingdom (Knapp & Hu, 2010). Proteins of this family have a unique structure with an N-terminal DNA-binding helix–turn–helix motif and a C-terminal co-inducer binding domain (Maddocks & Oyston, 2008). Superoxide and hydrogen peroxide ($\text{H}_2\text{O}_2$) are the two partially reduced reactive oxygen species (ROS) generated during aerobic metabolism that mediate oxidative stress in bacterial pathogens as they get reduced to hydroxyl radical (•OH), the most reactive oxygen species, which can damage numerous cellular components (Zheng *et al.*, 1998). Therefore, in order to survive such conditions, all bacteria are equipped with effective antioxidant defence systems that convert ROS to harmless products, as well as regulatory systems that sense ROS and regulate the genes for these defence systems in response to ROS, for example the *oxyR* system that belongs to the LTTR family (Storz & Imlay, 1999). OxyR is a 34 kDa protein known to induce the expression of a set of defensive genes, including *dps* (a DNA- and iron-binding protein), *gorA* (GSH reductase), *grxA* (glutaredoxin), *katG* (peroxidase), *ahpCF* (alkylhydroperoxide-NADPH oxido-reductase) and *fur* (an iron-binding repressor of iron transport), including those involved in virulence, metabolism, quorum sensing and motility (Pomposiello & Demple, 2001).

An important environmental assailant that pathogenic bacteria are exposed to often is the pressure of antibiotics, and previous studies have shown that antibiotics do induce

**Abbreviations:** CCCP, carbonylcyanide 3-chlorophenylhydrazone; CPS, capsular polysaccharide; EtBr, ethidium bromide; LTTR, LysR-type transcriptional regulator; ROS, reactive oxygen species; RT-PCR, reverse transcription PCR; SNP, sodium nitroprusside.
oxidative stress in bacterial cells (Kohanski et al., 2007, 2008). Homologues of oxyRKP are present in several bacterial genomes, e.g. Escherichia coli, Salmonella spp., Enterobacter spp., Campylobacter spp., Acinetobacter spp. and Pseudomonas spp., including the extremely drug-resistant Klebsiella pneumoniae. Previous studies have reported the role of oxyR in regulating antibiotic resistance (Deretic et al., 1997; Hassett et al., 2000; Rosner & Storz, 1994); however, its role in regulating drug resistance in K. pneumoniae has never been explored (Imlay, 2008; Vinckx et al., 2010).

K. pneumoniae is a non-motile, Gram-negative bacillus and can give rise to severe diseases such as septicaemia, pneumonia, urinary tract infections and soft tissue diseases (Rapp & Urban, 2012). Thus, Klebsiella infections may serve as a paradigm for hospital-acquired infections (Highsmith & Jarvis, 1985). Klebsiella is well known to most clinicians as a cause of community-acquired bacterial pneumonia occurring particularly in chronic alcoholics and showing characteristic radiographic abnormalities (Brown & Seidler, 1973). Carbapenems and cephalosporins are the most common drugs of treatment for K. pneumoniae illness; however, the prevalence of multidrug-resistant strains that are resistant to two or more classes of antibiotics has led to failure of antibiotic therapy (Hirsch & Tam, 2010).

The signalling pathways which enable Klebsiella to evolve diverse drug resistance mechanisms and cause severe infection remain an important field of research. Genome sequence analysis of highly capsulated and hypervirulent K. pneumoniae strain NTUH-K2044 from a Taiwanese liver abscess patient (accession number NC_012731) reveals the presence of >466 putative signalling proteins (http://mistdb.com) which include an uncharacterized oxyR homologue (Wu et al., 2009). Hennequin & Forestier (2009) have recently demonstrated the role of oxyR in biofilm formation, fimbrial synthesis, intestinal colonization, resistance to several gastrointestinal stresses and resistance to H2O2 exposure in K. pneumoniae LM21 that belongs to the K35 serotype. However, the function of oxyR from this hypervirulent K1 serotype has never been demonstrated. Therefore, in the present study, we characterized the functions of the putative oxyR (denoted oxyRKP) from the multidrug-resistant K. pneumoniae strain NTUH-K2044 and, to our knowledge, provide the first elucidation of the role of this LysR-type regulator in bacterial physiology in general and antibiotic resistance in particular.

**METHODS**

**Bacterial strains, plasmids and media.** K. pneumoniae NTUH-K2044 (from the blood of a previously healthy individual who was diagnosed with a community-acquired primary liver abscess and metastatic meningitis) was kindly provided by Dr Jin-Town Wang of the National Taiwan University Hospital, Taipei, Taiwan (Fang et al., 2004). Escherichia coli SM10p pir, which carries the F plasmid and encodes π protein essential for replication of pUT-Km, was used for cloning experiments (Chuang et al., 2006). Bacterial cultures were grown in Luria–Bertani (LB) broth or on LB agar (Difco, Becton Dickinson) at 37 °C with constant shaking (220 r.p.m.) and supplemented with kanamycin (100 μg ml⁻¹) where required. Mobilization of plasmids into K. pneumoniae cells was performed as described previously (Srinivasan et al., 2012a).

**DNA cloning.** DNA fragments used for cloning were extracted from agarose gels using a QIA quick gel extraction kit (Qiagen). PCR products were purified using a QIA quick PCR purification kit (Qiagen) and, when cloned, sequenced to confirm the correct sequences (Applied Biosystems). Primers used in the present study were custom-synthesized (Eurofins MWG operons).

**Construction of oxyRKP deletion mutant in K. pneumoniae strain NTUH-K2044.** The MisT2 database (www.mistdb.com) shows the presence of 5262 proteins in the 5,726,772 bp (GC content: 57.4 %) genome sequence of the K1 serotype (accession no: AP006725.1) (Wu et al., 2009). The putative oxyR homologue, KP1_0011 (denoted oxyRKP) is located from nucleotides 113 365 bp to 113 282 bp (oxyRKP, 918 bp, 305 aa) in the genome of K. pneumoniae NTUH-K2044 (Wu et al., 2009). To construct ΔoxyRKP, a 365 bp internal fragment was amplified by PCR using primers ΔoxyRKP-F and ΔoxyRKP-R with genomic DNA of strain NTUH-K2044 as template (Table 1). The PCR product was ligated into an EcoRI-digested (blunted by klenow reaction) plasmid, pUT-Km, that contains the kanamycin resistance gene and transformed into Escherichia coli SM10p pir. The resulting recombinant plasmid harbouring the internal fragment of oxyRKP was designated pUT-oxyRKP. The plasmid pUT-oxyRKP was mobilized into recipient K. pneumoniae NTUH-K2044 from donor Escherichia coli.

Briefly, K. pneumoniae was inoculated into 10 ml LB and was incubated for 2–3 h to an optical density (OD600) of 0.2. For matings, recipient and donor cultures were mixed in a ratio of 1:2, respectively, pelleted and spotted onto the centre of an LB agar plate. After 3 h of growth at 37 °C the cells were plated on Klebsiella selective agar (HiMedia HiCrome Klebsiella Selective Agar Base cat. no. M1573; Klebsiella Selective Supplement cat. no. FD225) containing 100 μg kanamycin ml⁻¹ and 5 μg chlorhexidine ml⁻¹ to select for

**Table 1. Primers used in this study**

<table>
<thead>
<tr>
<th>Primer name</th>
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<tr>
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<tr>
<td>ΔoxyRKP-R</td>
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</tr>
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<td>NT-5</td>
<td>TATCTGGCCCTCTGGACCGTGCAAGGCGCA</td>
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<td>CT-6</td>
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colonies. It is expected that colonies appearing on the selective plate would be transconjugants resulting from one DNA exchange event in which the whole suicidal plasmid was incorporated into the genome. Disruption of the oxyRKP gene was confirmed in the selected transconjugant by PCR and DNA sequencing using gene-specific and genome flanking primers, and the deleted mutant was denoted ΔoxyRKP.

The intact oxyRKP gene was amplified along with its promoter using primers NT-5 and CT-6 and cloned into a pCRITOPO-CAT plasmid (Table 1). The selected recombinant plasmid harbouring the intact oxyRKP gene was transformed into the oxyRKP isogenic mutant strain by electroporation. Complemented strains were selected on LB agar plates supplemented with 100 μg kanamycin ml⁻¹ and 100 μg chloramphenicol ml⁻¹ and the transcomplemented strain was designated ΔoxyRKPΔoxyRKP. Mutant and complemented strains generated in this study were characterized and their phenotypes compared with control strain K. pneumoniae NTUH-K2044.

Tests for hypermucoviscosity. The WT (control strain: NTUH-K2044), ΔoxyRKP and ΔoxyRKPΔoxyRKP were streaked onto LB agar plates and incubated at 37 °C overnight. A standard bacteriologic loop was used to stretch a mucoviscous string from the colony. Hypermucoviscosity was defined by the formation of viscous strings >5 mm in length when a loop was used to stretch the colony on the agar plate (Pinsky et al., 2009). The strains to be tested were cultured for 12 h in LB broth at 37 °C and subjected to centrifugation at 3200 g for 3 min to check reduction in mucoidy appearance. For exopolysaccharide analysis (Shemesh et al., 2010), cells were grown to late-exponential phase in shaking culture and stained with crystal violet followed by treatment with 20% CuSO₄ solution (Anthony’s work station). Capsular polysaccharide (CPS) was extracted from 12-hour-old bacterial suspensions adjusted to ~10⁶ cells ml⁻¹ with Zwittergent 3-14 detergent. The amount of uronic acid was then measured according to the method described previously (Srinivasan et al., 2012b). Each experiment was performed in triplicate.

Bacterial growth curves. The growth kinetics of WT, ΔoxyRKP and ΔoxyRKPΔoxyRKP at mid-exponential phase (10⁵ cells ml⁻¹) were monitored in 96-well, clear, flat-bottom polystyrene microplates (Costar; Corning) with LB at different pH (3.0, 5.0, 6.0, 7.0, 7.5, 8.0, 10.0 and 12.0). Optical densities were measured for 10 h at 37 °C with shaking using a Synergy H1 Hybrid microplate reader (BioTek Instruments) at 600 nm and automatically recorded for each well after every 15 min. The experiment was performed with freshly autoclaved medium in triplicate at least three independent times.

Growth inactivation assay. The growth inactivation assay to assess the impact on drug efflux capacity was performed as described previously with slight modifications (Srinivasan et al., 2009). The WT and ΔoxyRKP cultures at mid-exponential phase (10⁵ cells ml⁻¹) were inoculated into LB broth containing antibiotic (0.005 μg chloramphenicol ml⁻¹, 0.005 μg erythromycin ml⁻¹ or 0.005 μg tetracycline ml⁻¹) and disinfectants (0.01 μg benzalkonium chloride ml⁻¹, 0.01 μg chlorhexidine ml⁻¹ or 0.001 μg triclosan ml⁻¹) in independent experiments, either alone or with the efflux pump inhibitor carbonylcyanide 3-chlorophenylhydrazone (CCCP, 10 μg ml⁻¹; Sigma) (Srinivasan et al., 2009; Zhang et al., 2010). The growth profile of WT and ΔoxyRKP thereafter at 37 °C was analysed by measuring the absorbance at OD₆₀₀ periodically in a Synergy H1 Hybrid microplate reader. These experiments were performed more than three times.

Gastrointestinal stress challenge assays. Different stress assays were performed as described previously (Srinivasan et al., 2012b). Briefly WT, ΔoxyRKP and ΔoxyRKPΔoxyRKP strains were grown to mid-exponential phase, and cultures were spread onto LB and LB Kan (100 μg kanamycin ml⁻¹) agar plates containing different concentrations of bile (0.2, 0.5, 0.75, 1.0 and 2.0%) and NaCl (0.075, 0.15, 0.25, 0.5, 0.75, 1 and 2 M). The results are expressed as the ratio of the number of c.f.u obtained from LB cultures containing different concentrations of substrates to the number of c.f.u. obtained from control cultures (LB agar alone). These experiments were performed at least three times. The WT, ΔoxyRKPΔoxyRKP and ΔoxyRKPΔoxyRKP strains were exposed to different temperatures of heat shock (37, 42 and 60 °C) for 1 h and survival was checked on LB and LB Kan plates.

Stress response to different structurally related compounds. The assays were performed as described previously (Srinivasan et al., 2012b). Briefly WT, ΔoxyRKPΔoxyRKP and ΔoxyRKPΔoxyRKP strains were grown to mid-exponential phase, and cultures were spread onto LB and LB Kan agar plates containing different concentrations of SDS (1,024, 2,048, 4,096, 8,192 or 16,384 mg ml⁻¹), deoxycholate (32, 64, 128, 256, 512, 1,024 or 2,048 μg ml⁻¹), ethidium bromide (EtBr) (2, 8, 64, 128, 256 or 512 μg ml⁻¹), rhodamine (2, 8, 64, 128, 256 or 512 μg ml⁻¹) or aclarivine (2, 8, 64, 128, 256 or 512 μg ml⁻¹). The results are expressed as the ratio of the number of c.f.u. obtained from LB cultures containing different concentrations of substrates to the number of c.f.u. obtained from control cultures (LB agar alone). These experiments were performed at least three times.

Oxidative stress tolerance assays. In the oxidative stress susceptibility test, small Whatman 3MM paper discs (6 mm) were impregnated with different amounts of H₂O₂ (78.94 μM, 263.15 μM, 789.47 μM) and later air-dried (Coudeyras et al., 2008). The WT, ΔoxyRKP and ΔoxyRKPΔoxyRKP strains were grown to mid-exponential phase (10⁵ cells ml⁻¹) and were uniformly spread over an LB agar plate. Next, filter paper discs impregnated with specific concentrations of H₂O₂ were placed onto the agar surface at the centre of the plate. The culture was then incubated at 37 °C for 12–24 h. The diameter of a zone of inhibition was measured (in millimetres), which is a qualitative measure of the inhibitory activity of a compound. The data represent the diameter of the zone of inhibition. The sensitivity of WT, ΔoxyRKP and ΔoxyRKPΔoxyRKP cells to oxidative stress was tested by exposing stationary-phase bacteria diluted in LB medium (10⁵ cells ml⁻¹) at 37 °C to 0.07894 mM, 0.7894 mM, 1.5788 mM, 2.3682 mM and 3.1576 mM H₂O₂ for 1 h. Viable cells were counted by plating them onto agar plates before and after exposure to H₂O₂, and results are expressed as survival percentages.

Nitrosative stress tolerance assays. Sodium nitroprusside (SNP) and acidified nitrite were used to generate nitrosative stress in our current study (Stevanin et al., 2000). Growth of cultures against SNP was determined as described previously (Srinivasan et al., 2012b) with the modification that absorbance was measured in a Synergy H1 Hybrid microplate reader. Briefly, WT, ΔoxyRKP and ΔoxyRKPΔoxyRKP cells were exposed to oxidative stress by exposing stationary-phase bacteria diluted in LB medium (10⁵ cells ml⁻¹) at 37 °C to 0.07894 mM, 0.7894 mM, 1.5788 mM, 2.3682 mM and 3.1576 mM H₂O₂ for 1 h. Viable cells were counted by plating them onto agar plates before and after exposure to H₂O₂, and results are expressed as survival percentages.

Kirby–Bauer assay. Antibiotic susceptibility of WT, ΔoxyRKP and ΔoxyRKPΔoxyRKP strain was examined using the following commercial discs: AMP (10 μg ampicillin ml⁻¹); CHL (30 μg chloramphenicol ml⁻¹); C2H (30 μg ceftazidime ml⁻¹); CAR (10 μg carbenicillin ml⁻¹); CIP (5 μg ciprofloxacin ml⁻¹); CST (10 μg colistin ml⁻¹); CPM (30 μg cepime ml⁻¹); CTR (30 μg ceftriaxone ml⁻¹); ENX (10 μg enrofloxacin ml⁻¹); KAN (30 μg kanamycin ml⁻¹); LXX (5 μg nitrofurantoin ml⁻¹); GENT (30 μg gentamicin ml⁻¹). The Kirby–Bauer assay was performed as described by the National Committee for Clinical Laboratory Standards (2001). The diameter of the zone of inhibition was measured (in millimetres), which is a qualitative measure of the inhibitory activity of the antibiotic. The data represent the diameter of the zone of inhibition.
levofoxacin ml⁻¹); NAL (30 μg nalidixic acid ml⁻¹); OFX (10 μg ofloxacin ml⁻¹); PMB (300 μg polymyxin B ml⁻¹); RIF (5 μg rifampicin ml⁻¹); STR (10 μg streptomycin ml⁻¹); TET (30 μg tetracycline ml⁻¹); TGC (15 μg tigecycline ml⁻¹); TOB (10 μg tobramycin ml⁻¹); TMP (5 μg trimethoprim ml⁻¹) (Hi Media), as described previously according to the interpretation criteria recommended by CLSI (CLSI, 2010).

**RESULTS**

**Statistical analysis.** All data are presented as mean ± SEM. Plotting and calculation of the standard deviation were performed in Microsoft Excel. Statistical analysis was performed on crude data by using a paired Student t-test as well as ANOVA in Microsoft Excel 2007. P values of <0.05 were considered significant.

**Construction of oxyR<sup>KP</sup> deletion mutant**

The nucleotide sequence spanning nucleotides 112 365 bp to 113 282 bp, encoding the 918 bp DNA fragment obtained from *K. pneumoniae* NTUH-K2044, shared >95% identity with the OxyR regulatory protein in other Gram-negative pathogens. To determine the role of oxyR<sup>KP</sup>, an oxyR<sup>KP</sup> mutant was created by conjugation in the WT *K. pneumoniae* NTUH-K2044. This strain was selected due to its high virulence in a murine model of pneumonia (Lin et al., 2009). We used insertion-duplication mutagenesis to interrupt oxyR<sup>KP</sup>, required for the synthesis of a functional regulatory protein. PCR followed by DNA sequencing was done to confirm the disruption of the gene in *K. pneumoniae*. RT-PCR analysis confirmed that the mutations abolished the transcription of oxyR<sup>KP</sup> (data not shown).

**Disruption of oxyR<sup>KP</sup> decreases CPS production**

The oxyR<sup>KP</sup> mutant had a different colony morphology when compared to the WT. *K. pneumoniae* NTUH-K2044 produced larger (3.0 ± 0.5 mm) and heavily mucoid colonies while ΔoxyR<sup>KP</sup> colonies were smaller (0.85 ± 0.1 mm) and non-mucoid, indicating a direct decrease in CPS production (Fig. 1a). To deduce the effect on CPS production, the precipitation test was carried out on cultures grown for 12 h in LB broth at 37 °C. The WT did not form a dense pellet after centrifugation at 3200 g for 3 min while the ΔoxyR<sup>KP</sup> cells formed a compact pellet (Fig. 1b). To determine the role
of oxyR<sup>KP</sup> in CPS production, the hypermucoviscosity string test was performed; this test assesses the formation of a viscous string (Pinsky et al., 2009). The lengths of the strings for WT and ΔoxyR<sup>KP</sup> were 55 ± 5 mm and 7.5 ± 1 mm, respectively (Fig. 1c). Visualization of cultures using 20% CuSO<sub>4</sub> as per Anthony’s capsule staining methodology revealed that a thinner capsule was observed surrounding mutant cells, reflecting a decreased exopolysaccharide production in ΔoxyR<sup>KP</sup> cells (Fig. 1d); complementation restored the defect. Quantification of uronic acid content (WT: 19.27 ± 1.29; ΔoxyR<sup>KP</sup>: 6.54 ± 2.21; and ΔoxyR<sup>KP</sup>ΔoxyR<sup>KP</sup>: 18.42 ± 1.06 µg per 10<sup>9</sup> c.f.u.) reconfirmed the same observation. These data suggest that oxyR<sup>KP</sup> contributes significantly towards capsule production in <i>K. pneumoniae</i> NTUH-K2044.

### Deletion of oxyR<sup>KP</sup> results in stunted growth in <i>K. pneumoniae</i>

To decipher the involvement of oxyR<sup>KP</sup> in <i>K. pneumoniae</i> growth under conditions of different pH, the growth kinetics of ΔoxyR<sup>KP</sup> were compared with those of the WT strain. The growth characteristics of the WT and ΔoxyR<sup>KP</sup> strains were determined over a period of ~8 h in LB medium with different pH (3.0, 5.0, 6.0, 7.0, 7.5, 8.0, 10.0 and 12.0) and subsequent data analysis revealed unique patterns. At pH 5.0, the ΔoxyR<sup>KP</sup> mutant exhibited ~1.4-fold reduced growth (± 0.223) compared to the WT after 4 h (P = 0.07206). At pH 6.0, the oxyR<sup>KP</sup> mutant exhibited >1.5-fold reduced growth (± 0.351) compared to the WT after 4 h (P = 0.0458). The apparent growth of ΔoxyR<sup>KP</sup> at pH 7.0 was ~1.09-fold lower (± 0.23) compared to the WT after 4 h (P = 0.0035), whereas it was ~1.15-fold lower (± 0.16) compared to the WT after 4 h (P = 0.0023) at pH 7.5. At pH 8.0, ΔoxyR<sup>KP</sup> exhibited ~1.12-fold stunted growth compared to the WT after 4 h (P = 0.0142) (Fig. 2). The other conditions tested (pH 3.0, 10.0 and 12.0) were toxic to both the cultures (data not shown). These results demonstrate that oxyR<sup>KP</sup> influences growth of <i>K. pneumoniae</i> significantly at physiological pH.

### Loss of oxyR<sup>KP</sup> renders <i>K. pneumoniae</i> cells sensitive to different stress conditions

To determine the role of WT, ΔoxyR<sup>KP</sup> and ΔoxyR<sup>KP</sup>ΔoxyR<sup>KP</sup> under conditions relevant to intestinal colonization, cells underwent specific gastrointestinal related challenges. In the bile resistance assay, WT, ΔoxyR<sup>KP</sup> and ΔoxyR<sup>KP</sup>ΔoxyR<sup>KP</sup> strains were exposed to different concentrations of bile (physiological concentration is 0.2 to 2%) (Gunn, 2000). When mid-exponential-phase cultures were exposed to different concentrations of bile it was observed that the total c.f.u. count of the WT (measure of surviving capacity) was higher compared to the ΔoxyR<sup>KP</sup> mutant. The ability of the WT to grow in the presence of 0.5, 0.75, 1 and 2% bile was 1.25 (± 0.025)-, 1.3 (± 0.047)-, 1.6 (± 0.014)- and 2.6 (± 0.45)-fold higher, respectively, than ΔoxyR<sup>KP</sup> (P = 0.0177), while the transcomplemented ΔoxyR<sup>KP</sup>ΔoxyR<sup>KP</sup> strain restored the ability to tolerate bile stress (P = 0.0217) (Fig. 3a). The ability of the WT to grow in the presence of NaCl (physiological concentration being 150 mM; Coudeyras et al., 2008) at 0.25, 0.5 and 0.75 M was 1.4 (~ 0.022)-, 1.7 (~ 0.022)- and 5.3 (~ 0.24)-fold higher, respectively, than ΔoxyR<sup>KP</sup>, regardless of the inoculum size (P = 0.0141), while the transcomplemented ΔoxyR<sup>KP</sup>ΔoxyR<sup>KP</sup> strain restored the phenotype (P = 0.0229) (Fig. 3b).

To deduce the role of oxyR<sup>KP</sup> in temperature tolerance, we performed a heat-shock assay. The temperature-dependent assay showed that the oxyR<sup>KP</sup> mutant displayed 10% reduced survival compared to the WT at 42°C (Fig. 3c), thereby demonstrating that oxyR<sup>KP</sup> may have an indirect role in temperature stress (P = 0.009).

The WT and ΔoxyR<sup>KP</sup> cultures in this study were tested for their ability to withstand high concentrations of different substrates that are structurally related to antibiotics. Upon exposing the cells to different concentrations of SDS, it was observed that the total c.f.u. count of the WT was higher than that of ΔoxyR<sup>KP</sup> (P = 0.0149) (Fig. 3d). The ability of ΔoxyR<sup>KP</sup> to withstand different concentrations of deoxycholate was reduced at higher concentrations [P = 0.00248] (Fig. 3e). The ability of ΔoxyR<sup>KP</sup> to withstand different concentrations of EtBr (P = 0.008764) (Fig. 3f), rhodamine (P = 0.00516) (Fig. 3g) and acriflavine (P = 0.02698) (Fig. 3h) was also decreased when compared to the WT, respectively. Overall, these results imply that oxyR<sup>KP</sup> has a contributory role towards varied stress tolerance in <i>K. pneumoniae</i>.

### Role of oxyR<sup>KP</sup> in modulating oxidative and nitrosative stress responses

To deduce the role of oxyR<sup>KP</sup> in oxidative stress, we performed H<sub>2</sub>O<sub>2</sub> challenge assays. Oxidative disc assay showed that the oxyR<sup>KP</sup> mutant exhibited 1.4-fold greater sensitivity to 789.47 µM H<sub>2</sub>O<sub>2</sub> (inhibition zone = 48 ± 1.8 mm) than the WT strain (inhibition zone = 33 ± 0.0 mm) (Fig. 4a) (P = 0.015), clearly demonstrating the role of <i>K. pneumoniae</i> oxyR<sup>KP</sup> in oxidative stress. The sensitivity of stationary-phase cultures to oxidative stress was tested by exposing them to a range of H<sub>2</sub>O<sub>2</sub> concentrations (0.07894, 0.7894, 1.5788, 2.3682 and 3.1576 mM) for 1 h. Only 27 and 0% of the ΔoxyR<sup>KP</sup> cells survived upon treating with 0.07894 mM or 0.7894 mM H<sub>2</sub>O<sub>2</sub> as compared to the 87 and 77% survival observed in WT cells, respectively (P = 0.005) (Fig. 4b).

To test whether the presence of <i>K. pneumoniae</i> oxyR<sup>KP</sup> provides any protection against NO donor and nitrosative stress, we compared the growth profiles of WT and ΔoxyR<sup>KP</sup> strains in the presence of different concentrations of the NO donor SNP and acidified nitrite. In the presence of 5, 10, 15 and 30 mM SNP, growth kinetics of ΔoxyR<sup>KP</sup> cells were ~1.24-fold (P = 0.004), ~1.27-fold (P = 0.0034), ~1.40-fold (P = 0.0027), ~1.39-fold (P = 0.002) and ~1.67-fold (P = 0.001) lower than WT cells, respectively, after 4 h (Fig. 4c;
In the presence of 10, 20 and 30 mM acidified nitrite, the growth kinetics of ΔoxyR<sup>KP</sup> cells were ~5.721-fold (~3.24-fold) and ~6.5-fold lower than WT cells after 4 h (Fig. 4d). These results indicated the protective effect mediated by oxyR<sup>KP</sup> against NO toxicity.

The lysR regulator oxyR<sup>KP</sup> mediates antimicrobial resistance by altering active efflux

To evaluate the role of oxyR<sup>KP</sup> in drug resistance, antibiotic susceptibilities of WT and ΔoxyR<sup>KP</sup> strains were monitored. The results of disc diffusion assays showed that upon deleting the regulator, the bacterial cells displayed...
significantly altered susceptibility to amoxicillin, chloramphenicol, erythromycin, nalidixic acid, rifampicin and trimethoprim (Fig. 5a). The precise MIC was further evaluated by following the guidelines of the CLSI by E-test. The MIC (represented in µg ml⁻¹) of WT cells for the different antibiotics tested was: amoxicillin, 240; chloramphenicol, 0.1; erythromycin, 60; nalidixic acid, 5; rifampicin, 10; and trimethoprim, 0.1. The MIC for

![Fig. 3. Stress challenge assays. Survival of WT (black bars), ΔoxyR<sup>KP</sup> (grey bars) and ΔoxyR<sup>KP</sup>ΔoxyR<sup>KP</sup> (hatched bars) under different stress conditions. (a) Sensitivity to different concentrations (0.2, 0.5, 0.75, 1.0 and 2.0 %) of bile. In the presence of 0.5, 0.75, 1 and 2 % bile, the tolerance of the WT strain was 1.25-, 1.3-, 1.6- and 2.66-fold higher, respectively, than that of ΔoxyR<sup>KP</sup>. The complemented strain ΔoxyR<sup>KP</sup>ΔoxyR<sup>KP</sup> displayed a phenotype similar to the WT. (b) Sensitivity towards different concentrations (0.075, 0.15, 0.25, 0.5, 0.75, 1.0 and 2.0 M) of NaCl. In the presence of 0.25, 0.5 and 0.75 M NaCl, the tolerance of the WT strain was ~1.396, ~1.724- and 5.317-fold higher, respectively, than that of ΔoxyR<sup>KP</sup>. (c) Survival at 37, 42 and 60 °C after 1 h incubation. (d) Sensitivity towards different concentrations of SDS. In the presence of SDS at 1.024, 2.048, 4.096, 8.192 and 16.834 mg SDS ml⁻¹, the tolerance of the WT strain was ~1.10-, ~1.18-, ~1.12-, ~1.24- and 1.2-fold higher, respectively, than that of ΔoxyR<sup>KP</sup>. (e) Susceptibility towards different concentrations of deoxycholate. In the presence of deoxycholate at 128, 256, 512, 1024 and 2048 µg ml⁻¹, the tolerance of the WT strain was ~1.21-, ~1.43-, ~1.54-, ~3.2- and 3.88-fold higher, respectively, than that of ΔoxyR<sup>KP</sup>. (f) Sensitivity towards different concentrations of EtBr. In the presence of EtBr at 8, 64, 128, 256 and 512 µg ml⁻¹, the tolerance of the WT strain was ~1.21-, ~1.24-, ~1.38-, ~2.05- and 3.33-fold higher, respectively, than that of ΔoxyR<sup>KP</sup>. (g) Sensitivity towards different concentrations of rhodamine. In the presence of rhodamine at 8, 64, 128 and 512 µg ml⁻¹, the tolerance of the WT strain was ~1.18-, ~1.403-, ~1.58- and 2.8-fold higher, respectively, than that of ΔoxyR<sup>KP</sup>. (h) Sensitivity towards different concentrations of acriflavine. In the presence of acriflavine at 8, 64 and 128 µg ml⁻¹, the tolerance of the WT strain was ~1.32-, ~1.52- and ~1.75-fold higher, respectively, than that of ΔoxyR<sup>KP</sup>. The per cent resistance to different stress agents was calculated by comparison to the numbers of viable cells in LB medium alone. Data are the mean ± SEM of measurements made in triplicate performed three times. *Significant difference (P<0.05).

The MIC (represented in µg ml⁻¹) of WT cells for the different antibiotics tested was: amoxicillin, 240; chloramphenicol, 0.1; erythromycin, 60; nalidixic acid, 5; rifampicin, 10; and trimethoprim, 0.1. The MIC for

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\( \Delta \text{oxyR}^{\text{KP}} \) cells (fold decrease in parentheses) for the same antibiotics were: amoxicillin, 60 (4-fold); chloramphenicol, 0.01 (10-fold); erythromycin, 10 (6-fold); nalidixic acid, 0.1 (50-fold); rifampicin, 5 (2-fold); and trimethoprim, 0.01 (10-fold) (Table 2). In summary, deletion of \( \text{oxyR}^{\text{KP}} \) altered the antibiotic susceptibility profile of \( K. \text{pneumoniae} \) belonging to the K1 serotype.

To decipher whether \( \text{oxyR}^{\text{KP}} \) confers antibiotic resistance by affecting drug efflux, screening for a potential efflux phenotype was accomplished by determining the growth...
profile of WT and ΔoxyRKp strains in the presence of antibiotics and CCCP (10 μg ml⁻¹) as described in the methods. In order to evaluate the true impact of oxyRKp deletion on the active efflux capability of cells, the concentrations of the antibiotics used in these assays were below the MIC value (with no deleterious effect on cell growth) for both WT and ΔoxyRKp. The growth rate of ΔoxyRKp in the presence of 0.005 μg chloramphenicol ml⁻¹ was 1.089-fold and 2.48-fold lower than the WT after 4 h and 8 h, respectively (P=0.055). The addition of CCCP drastically reduced the growth of both strains as the action of antimicrobials was restored (Fig. 5b). The growth rate of ΔoxyRKp in the presence of 0.005 μg erythromycin ml⁻¹ was 1.103-fold and 2.12-fold lower than the WT after 4 h and 8 h, respectively (P=0.0033). The addition of CCCP drastically reduced the growth in both strains as the action of antimicrobials was restored (Fig. 5c). The growth kinetics of ΔoxyRKp in the presence of 0.005 μg tetracycline ml⁻¹ was 1.475-fold and 3.247-fold lower than the WT after 4 h and 8 h respectively (P=0.0042). The addition of CCCP drastically reduced the growth in both strains as the action of antimicrobials was restored (Fig. 5d). In independent experiments, growth remained unaltered on the addition of reserpine.

K. pneumoniae is a nosocomial pathogen and has an ability to remain viable on abiotic surfaces for long periods of time (Di Martino et al., 2003); therefore, we tested the susceptibilities of WT and ΔoxyRKp strains towards different concentrations of popularly used hospital-based disinfectants such as chlorhexidine and benzalkonium chloride (Milstone et al., 2008). The survival of ΔoxyRKp cells was reduced by 18% when exposed to 0.1 μg triclosan ml⁻¹ (P=0.06) (data not shown). The growth rate of ΔoxyRKp in the presence of 0.01 μg benzalkonium chloride ml⁻¹ was 1.269-fold and 1.72-fold lower than the WT after 4 h and 8 h, respectively (P=0.00011). The addition of CCCP drastically reduced the growth in both strains as the action of antimicrobials was restored (Fig. 6a). The growth rate of ΔoxyRKp in the presence of 0.01 μg chlorhexidine ml⁻¹ was 1.179-fold and 2.559-fold lower than the WT after 4 h and 8 h, respectively (P=0.07993). The addition of CCCP drastically reduced the growth in both strains as the action of antimicrobials was restored (Fig. 6b). The growth rate of ΔoxyRKp in the presence of 0.001 μg triclosan ml⁻¹ was 0.90625-fold and 1.446-fold lower than the WT after 4 h and 8 h, respectively (P=0.01661). The addition of CCCP drastically reduced the growth in both strains as the action of antimicrobials was restored (Fig. 6c). The ΔoxyRKp strain was found to be sensitive to antibiotics and disinfectants, and much of this is likely due to the decreased capsule production by the oxyR mutant as compared to the mucoidy capsule present in the WT strain. In conclusion, involvement of oxyRKp in mediating antimicrobial resistance has been demonstrated for the first time in K. pneumoniae.

**Role of oxyRKp in virulence in K. pneumoniae NTUH-K2044**

The C. elegans–K. pneumoniae infection model was employed to determine the involvement of oxyRKp in virulence (Fuursted et al., 2012). The WT and mutant strains were examined for their abilities to kill C. elegans. The WT strain displayed 73 and 82% killing at 48 and 72 h, respectively. However, the mutant strain killed only 34 and 57% of the worms after 48 and 72 h, respectively (P=0.0216) (Fig. 7a). The ΔoxyRKpΔoxyVp strain restored the defect. The Escherichia coli strain OP50 was used as negative control. Thus, our findings demonstrate that the oxyRKp mutant kills C. elegans more slowly than the WT strain.

**Expression analysis of efflux pumps and capsular genes in the oxyRKp mutant**

Quantitative real-time RT-PCR was used to examine expression of the efflux transporter genes in WT and oxyRKp mutant strains using specific primers (Table 1) as described previously (Srinivasan et al., 2012a). Compared
to the WT strain, expression of the resistance–nodulation–cell division super family (RND) efflux pumps acrB and eefB was decreased by fivefold (±0.033) in the oxyR<sup>KP</sup> mutant (P<0.0005 Student’s t-test.) (Fig. 7b). No significant difference in expression level was observed for kmrA and eefB efflux genes. These results provide evidence for the additional regulatory role of oxyR<sup>KP</sup> on multidrug-resistant efflux pumps. The downregulation of these pumps could be the molecular mechanism underlying the sensitivity of the oxyR<sup>KP</sup> mutant to hydrophobic agents tested in this study. As mutation in oxyR<sup>KP</sup> results in impairment of capsule synthesis, we monitored the relative expression of capsular synthesis genes (KP1_3706: putative glycosyltransferase wcaI; KP1_3709: GDP-fucose synthase wcaG; KP1_3712: galactoside O-acetyltransferase atf) in the oxyR<sup>KP</sup> mutant. The ΔoxyR<sup>KP</sup> strain showed a decreased expression for wcaI (sixfold), wcaG (fourfold) and atf (twofold) when compared to the WT strain.

**DISCUSSION**

Bacteria are able to sense a variety of environmental stimuli such as temperature, pH, osmolarity and oxygen levels (Krell et al., 2010). They use this information and
depends on the ability of the bacteria to adhere to mucosal
intestinal tract of hosts, and intestinal colonization which includes the
NTUH-K2044 K1 serotype (accession no. AP006725.1), niae
K. pneumoniae
The reservoir for therapeutic options for infected individuals (Bush
infections, and its propensity to acquire novel resistance is known for numerous hospital-acquired
K. pneumoniae
cells were normal with no defect in cell division. of polysaccharide capsule in hypervirulent bacillus was studied for the first time. The
general and antimicrobial susceptibility in particular in this
gene was disrupted and its effect on cellular physiology in
the 5 472 672 bp (encoding 4992 proteins; GC content: 57.4%) genome of K. pneumoniae NTUH-K2044 K1 serotype (accession no. AP006725.1), which includes the oxyR homologue (Wu et al., 2009). The gene was disrupted and its effect on cellular physiology in general and antimicrobial susceptibility in particular in this hypervirulent bacillus was studied for the first time. The sticky appearance of cells is indicative of the over-secretion of polysaccharide capsule in K. pneumonia, which is an important virulence factor that confers resistance to phagocytosis (Podschun & Ullmann, 1998), and in our study, profound differences in capsule synthesis between the K. pneumoniae WT strain and its oxyR mutant were detected. However, it would be worth mentioning here that scanning electron microscopy revealed that oxyR mutant cells were normal with no defect in cell division.

K. pneumoniae is known for numerous hospital-acquired infections, and its propensity to acquire novel resistance determinants results in increased incidences of multidrug-resistant K. pneumonia, which severely limits the therapeutic options for infected individuals (Bush et al., 2011). The reservoir for K. pneumoniae strains is the gastrointestinal tract of hosts, and intestinal colonization depends on the ability of the bacteria to adhere to mucosal surfaces, to form biofilms within the mucus layer, and to resist the varied stresses encountered in the gastrointestinal tract (De Champs et al., 1989; Macfarlane 2008; Selden et al., 1971). Primary studies have shown that colonization in the patient’s gastrointestinal tract is the first stage in nosocomial infections due to K. pneumoniae. The pleomorphic bacillus has to sense and respond to different environmental assailants in order to survive and, consequently, persist in the gastrointestinal tract of the host (Hennequin & Forestier, 2009). The first major barrier encountered following oral consumption is stomach acidity. The bacteria then enter the small intestine, where they encounter stresses associated with volatile fatty acids, variations in pH and osmolarity, and competition with endogenous flora. The behaviour of the WT strain and oxyRKP deficient mutant was further examined under some of these environmental stresses in order to further understand how oxyRKP interacts in the gastrointestinal tract (Hennequin & Forestier, 2009). In the presence of osmotic and bile challenges, the oxyRKP mutant exhibited approximately two- to fivefold lower survival capacities than the WT. Hennequin & Forestier (2009) have shown previously that there was no difference observed in the growth capacities of the DeltaoxyR mutant of K. pneumoniae LM21 in the presence of NaCl regardless of the inoculum size and salt concentration. The ability of DeltaoxyRKP to grow at physiological pH 7.0–7.5 was impaired. It is likely, therefore, that oxyRKP controls functions that are essential for the survival of K. pneumoniae in the upper parts of the gastrointestinal tract, where they encounter adverse environments such as altered pH levels, high osmolarity and high concentrations of bile salts.

ROS such as H2O2, superoxide and hydroxyl radical are toxic to cells because of their ability to damage DNA and proteins (Imlay, 2003). In Gram-negative pathogens, many transcription factors have been found to sense the presence of ROS and induce antioxidant systems (Nordmann et al., 2009). Evidence for the involvement of an oxyRKP system in

<table>
<thead>
<tr>
<th>Compound</th>
<th>WT MIC (µg ml⁻¹)</th>
<th>DeltaoxyRKP MIC (µg ml⁻¹)</th>
<th>Fold change*</th>
<th>DeltaoxyRKP DeltaoxyRKP MIC (µg ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin</td>
<td>240</td>
<td>60</td>
<td>4</td>
<td>&gt;128</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>0.1</td>
<td>0.01</td>
<td></td>
<td>0.1</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>60</td>
<td>10</td>
<td>6</td>
<td>&gt;48</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>5</td>
<td>0.1</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>0.1</td>
<td>0.01</td>
<td></td>
<td>0.1</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>10</td>
<td>5</td>
<td>2</td>
<td>10</td>
</tr>
</tbody>
</table>

*Fold change is the ratio of MICs for WT and DeltaoxyRKP.
Antimicrobial resistance is a serious problem creating an alarming menace globally (Kohanski et al., 2007). In this study, we elucidated the role of oxyR<sup>KP</sup> in antimicrobial resistance. Deletion of oxyR<sup>KP</sup> rendered cells sensitive to amoxicillin, chloramphenicol, erythromycin, nalidixic acid, rifampicin and trimethoprim. In K. pneumoniae NTUH-K2044, deletion of oxyR<sup>KP</sup> resulted in loss of drug extrusion capacity. Involvement of efflux pumps in drug resistance has been characterized previously (Ogawa et al., 2005). The oxyR<sup>KP</sup> deletion reduced the expression levels of efflux pumps such as acrB in the mutant when compared to WT cells, which indicates that oxyR<sup>KP</sup> may have a possible role in regulating its expression; studies to prove this hypothesis are highly warranted. Disinfectants are an essential component of the sterilization practice in clinical settings, helping to prevent the dissemination of pathogenic organisms in the hospital environment (Milstone et al., 2008). Irrespective of the rigorous cleaning procedures in clinical settings, abiotic surfaces have often been described as the source for the continued prevalence/persistence of multidrug-resistant K. pneumoniae (Di Martino et al., 2003). Our study provides direct preliminary evidence for the participation of oxyR<sup>KP</sup> in mediating resistance against chlorhexidine and benzalkonium chloride. Besides, we found that the oxyR<sup>KP</sup> mutant exhibited a reduced ability to kill the nematode C. elegans (probably due to oxygen deficiency in ΔoxyR), demonstrating its key role in virulence. Hennequin & Forestier (2009) have demonstrated the role of oxyR in K. pneumoniae intestinal murine colonization. Overall, studies implicate an important role for oxyR<sup>KP</sup> in antimicrobial resistance and virulence.

Bacteria are extremely adaptable. They use the sensory systems such as bacterial oxyR<sup>KP</sup> to sense various environmental conditions, and utilize the perceived information as the regulatory key to control their bacterial response and physiology to maximize their capacity to survive and propagate in a scenario of adverse environmental surroundings (Hoch, 2000; Krachler et al., 2011; Krell et al., 2010). Understanding the fundamentals of signal transduction cascades is an area of great interest as these sensory components have the potential to serve as targets for novel alternative antibiotic therapeutic strategies.

In conclusion, this study provides primary experimental evidence for the participation of oxyR<sup>KP</sup> in mediating resistance against gastrointestinal stresses and antimicrobial agents in the K. pneumoniae NTUH-K2044 hypervirulent K1 serotype for the very first time.

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