**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 (H₂O₂) 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...
oxidative stress in bacterial cells (Kohanski et al., 2007, 2008). Homologues of oxyR<sup>KP</sup> 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 infections (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 & Forester (2009) have recently demonstrated the role of oxyR in biofilm formation, fimbrial synthesis, intestinal colonization, resistance to several gastrointestinal stresses and resistance to H<sub>2</sub>O<sub>2</sub> 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 oxyR<sup>KP</sup>) 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 SM10<sup>pir</sup> pir, which carries the F plasmid and encodes <i>π</i> 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<sup>−1</sup>) 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 agrose 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 oxyR<sup>KP</sup> deletion mutant in K. pneumoniae strain NTUH-K2044.** The MisT2 database (www.mistdb.com) shows the presence of 5262 proteins in the 5472672 bp (GC content: 57.4%) genome sequence of the K1 serotype (accession no. AP006725.1) (Wu et al., 2009). The putative oxyR homologue, KP1_0115 (denoted oxyR<sup>KP</sup>) is located from nucleotides 1132365 bp to 113282 bp (oxyR<sup>KP</sup>: 918 bp, 305 aa) in the genome of K. pneumoniae NTUH-K2044 (Wu et al., 2009). To construct ΔoxyR<sup>KP</sup>, a 365 bp internal fragment was amplified by PCR using primers ΔoxyR<sup>KP</sup>-F and ΔoxyR<sup>KP</sup>-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 SM10<i>pir</i>. The resulting recombinant plasmid harbouring the internal fragment of oxyR<sup>KP</sup> was designated pUT-oxyR<sup>KP</sup>. The plasmid pUT-oxyR<sup>KP</sup> 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 (OD<sub>600</sub>) 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<sup>−1</sup> and 5 µg chlorhexidine ml<sup>−1</sup> to select for

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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 oxyR<sup>KP</sup> 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 ΔoxyR<sup>KP</sup>.

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

**Tests for hypermucoviscosity.** The WT (control strain: NTUH-K2044), ΔoxyR<sup>KP</sup> and ΔoxyR<sup>KP</sup>ΔoxyR<sup>KP</sup> 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 % CuSO<sub>4</sub> solution (Anthony’s late-exponential phase in shaking culture and stained with crystal violet) and later air-dried (Coudeyras et al., 2008). The WT, ΔoxyR<sup>KP</sup> and ΔoxyR<sup>KP</sup>ΔoxyR<sup>KP</sup> strains were grown to mid-exponential phase (10<sup>7</sup> cells ml<sup>-1</sup>) and uniformly spread over an LB agar plate. Next, filter paper discs impregnated with specific concentra-tions of H<sub>2</sub>O<sub>2</sub> (78.94 M) and later air-dried (Coudeyras et al., 2008). These experiments were performed at least three times.

**Oxidative stress tolerance assay.** In the oxidative stress susceptibility test, small Whatman 3MM paper discs (6 mm) were impregnated with different amounts of H<sub>2</sub>O<sub>2</sub> (78.94 μM, 263.15 μM, 789.47 μM) and later air-dried (Coudeyras et al., 2008). The WT, ΔoxyR<sup>KP</sup> and ΔoxyR<sup>KP</sup>ΔoxyR<sup>KP</sup> cells to 0.07894 mM, 1.5788 mM, 3.1576 mM H<sub>2</sub>O<sub>2</sub> and acidified nitrite were used to generate nitrosative stress in our experiments. These experiments were performed at least three times.

**Nitrosative stress tolerance assay.** Sodium nitroprusside (SNP) and acidified nitrite were used to generate nitrosative stress in our recent study (Steavin 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, ΔoxyR<sup>KP</sup> and ΔoxyR<sup>KP</sup>ΔoxyR<sup>KP</sup> strains were grown aerobiocally in LB medium up to OD<sub>560</sub>=0.01 (10<sup>8</sup> cells ml<sup>-1</sup>). The cells were then treated with different concentrations (5, 10, 15, 20 or 30 mM) of SNP and growth was monitored at OD<sub>600</sub> at intervals of every 15 min in a Synergy H1 Hybrid microplate reader. To check the response of cultures against acidified nitrite, growth profiles of different strains, were determined at pH 6.0 in buffered LB medium supplemented with 5, 10, 15, 20 or 30 mM sodium nitrite and compared with the WT by observing OD<sub>600</sub> periodically as described above. Each experiment was repeated three times.

**Kirby–Bauer assay.** Antibiotic susceptibility of WT, ΔoxyR<sup>KP</sup> and ΔoxyR<sup>KP</sup>ΔoxyR<sup>KP</sup> strains was examined using the following commercial discs: AMP (10 μg ampicillin ml<sup>-1</sup>); CHL (30 μg chloramphenicol ml<sup>-1</sup>); CAZ (30 μg ceftazidime ml<sup>-1</sup>); CAR (10 μg carbenicillin ml<sup>-1</sup>); CIP (5 μg ciprofloxacin ml<sup>-1</sup>); CST (10 μg colistin ml<sup>-1</sup>); CPM (30 μg cepime ml<sup>-1</sup>); CTB (30 μg ceftriaxone ml<sup>-1</sup>); ENX (10 μg enrofloxacin ml<sup>-1</sup>); KAN (30 μg kanamycin ml<sup>-1</sup>); LXY (5 μg

http://mic.sgmjournals.org
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 oxyRKP 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 oxyRKP, an oxyRKP 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 oxyRKP, required for the synthesis of a functional regulatory protein. PCR followed by DNA sequencing was conducted to confirm the disruption of the gene in *K. pneumoniae*. RT-PCR analysis confirmed that the mutations abolished the transcription of oxyRKP (data not shown).

**Disruption of oxyRKP decreases CPS production**

The oxyRKP 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 ΔoxyRKP 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 ΔoxyRKP cells formed a compact pellet (Fig. 1b). To determine the role

![Fig. 1](link-to-image)

**Fig. 1.** Phenotypic characterization of oxyRKP mutant. (a) The oxyRKP mutant (ΔoxyRKP) had different colony morphology with smaller (0.85 ± 0.1 mm) and fewer regular colonies than the WT (3.0 ± 0.5 mm). (b) The precipitation test was carried out on strains grown for 12 h in LB broth at 37 °C, and each pellet was evaluated after centrifugation at 3200 g for 3 min. The WT and ΔoxyRKP showed profound differences in the compactness of the pellet. (c) The hypermucomucosity string test was performed by measuring the formation of a viscous string stretched between the bacterial colony and the inoculation loop. The mean lengths of string in WT and ΔoxyRKP were 55 ± 1 mm and 75 ± 1 mm, respectively. (d) Cells were grown to late-exponential phase in shaking culture and stained with crystal violet followed by treatment with 20% copper sulphate solution. The WT and ΔoxyRKP were visualized in an Olympus microscope work station.
of \textit{oxyR}^{KP} 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 \Delta\textit{oxyR}^{KP} were 55 \pm 5 mm and 7.5 \pm 1 mm, respectively (Fig. 1c). Visualization of cultures using 20% CuSO₄ as per Anthony’s capsule staining methodology revealed that a thinner capsule was observed surrounding mutant cells, reflecting a decreased exopolysaccharide production in \Delta\textit{oxyR}^{KP} cells (Fig. 1d); complementation restored the defect. Quantification of uronic acid content (WT: 19.27 \pm 1.29; \Delta\textit{oxyR}^{KP}: 6.54 \pm 2.21; and \Delta\textit{oxyR}^{KP}::\textit{oxyR}^{KP}: 18.42 \pm 1.06 \mu g per10⁹ c.f.u.) reconfirmed the same observation. These data suggest that \textit{oxyR}^{KP} contributes significantly towards capsule production in \textit{K. pneumoniae} NTUH-K2044.

\textbf{Deletion of \textit{oxyR}^{KP} results in stunted growth in \textit{K. pneumoniae}}

To decipher the involvement of \textit{oxyR}^{KP} in \textit{K. pneumoniae} growth under conditions of different pH, the growth kinetics of \Delta\textit{oxyR}^{KP} were compared with those of the WT strain. The growth characteristics of the WT and \Delta\textit{oxyR}^{KP} strains were determined over a period of \textasciitilde8 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 \textit{oxyR}^{KP} mutant exhibited \textasciitilde1.4-fold reduced growth ([\pm 0.223]) compared to the WT after 4 h ([P=0.07206]). At pH 6.0, the \textit{oxyR}^{KP} mutant exhibited \textasciitilde1.5-fold reduced growth ([\pm 0.351]) compared to the WT after 4 h ([P=0.0458]). The apparent growth of \Delta\textit{oxyR}^{KP} at pH 7.0 was \textasciitilde1.09-fold lower ([\pm 0.23]) compared to the WT after 4 h ([P=0.035]), whereas it was \textasciitilde1.15-fold lower ([\pm 0.16]) compared to the WT after 4 h ([P=0.0223]) at pH 7.5. At pH 8.0, \Delta\textit{oxyR}^{KP} exhibited \textasciitilde1.12-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 \textit{oxyR}^{KP} influences growth of \textit{K. pneumoniae} significantly at physiological pH.

\textbf{Loss of \textit{oxyR}^{KP} renders \textit{K. pneumoniae} cells sensitive to different stress conditions}

To determine the role of WT, \Delta\textit{oxyR}^{KP} and \Delta\textit{oxyR}^{KP}::\textit{oxyR}^{KP} under conditions relevant to intestinal colonization, cells underwent specific gastrointestinal related challenges. In the bile resistance assay, WT, \Delta\textit{oxyR}^{KP} and \Delta\textit{oxyR}^{KP}::\textit{oxyR}^{KP} 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 \Delta\textit{oxyR}^{KP} mutant. The ability of the WT to grow in the presence of 0.5, 0.75, 1 and 2% bile was 1.25 ([\pm 0.025])-1.3 ([\pm 0.047]), 1.6 ([\pm 0.014]) and 2.6 ([\pm 0.45])-fold higher, respectively, than \Delta\textit{oxyR}^{KP} ([P=0.0177]), while the transcomplemented \Delta\textit{oxyR}^{KP}::\textit{oxyR}^{KP} 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; Coudenys et al., 2008) at 0.25, 0.5 and 0.75 M was 1.4 ([\pm 0.022])-1.7 ([\pm 0.022]) and 5.3 ([\pm 0.24])-fold higher, respectively, than \Delta\textit{oxyR}^{KP}, regardless of the inoculum size ([P=0.0141]), while the transcomplemented \Delta\textit{oxyR}^{KP}::\textit{oxyR}^{KP} strain restored the phenotype ([P=0.0229] (Fig. 3b).

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

The WT and \Delta\textit{oxyR}^{KP} 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 \Delta\textit{oxyR}^{KP} ([P=0.0149] (Fig. 3d). The ability of \Delta\textit{oxyR}^{KP} to withstand different concentrations of deoxycholate was reduced at higher concentrations ([P=0.00248] (Fig. 3e). The ability of \Delta\textit{oxyR}^{KP} 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 \textit{oxyR}^{KP} has a contributory role towards varied stress tolerance in \textit{K. pneumoniae}.

\textbf{Role of \textit{oxyR}^{KP} in modulating oxidative and nitrosative stress responses}

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

To test whether the presence of \textit{K. pneumoniae oxyR}^{KP} provides any protection against NO donor and nitrosative stress, we compared the growth profiles of WT and \textit{oxyR}^{KP} 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 \textit{oxyR}^{KP} cells were \textasciitilde1.24-fold ([P=0.004]), \textasciitilde1.27-fold ([P=0.0034]), \textasciitilde1.40-fold ([P=0.00275]), \textasciitilde1.39-fold ([P=0.002]) and \textasciitilde1.67-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 (P=0.00073), ~3.24-fold (P=0.0030) and ~6.5-fold (P=0.0081) lower than WT cells after 4 h (Fig. 4d). These results indicated the protective effect mediated by oxyR<sup>KP</sup> against NO toxicity.

**Fig. 2.** Effect of oxyR<sup>KP</sup> deletion on general growth characteristics. Effect on bacterial growth was monitored in WT (■), ΔoxyR<sup>KP</sup> (●) and ΔoxyR<sup>KP</sup>VoxyR<sup>KP</sup> (▲) in LB medium at pH 3.0, 5.0, 6.0, 7.0, 7.5, 8.0, 10.0 and 12.0. Patterns representative of growth at pH 5.0 (a), 6.0 (b), 7.0 (c), 7.5 (d) and 8.0 (e) are shown. The data presented are the mean of triplicate measurements performed three times.

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), ΔoxyRKP (grey bars) and ΔoxyRKPΔoxyRKP (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 ΔoxyRKP. The complemented strain ΔoxyRKPΔoxyRKP 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 ΔoxyRKP. (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 ΔoxyRKP. (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 ΔoxyRKP. (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 ~1.24-, ~1.24-, ~1.38-, ~2.05- and 3.33-fold higher, respectively, than that of ΔoxyRKP. (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-, ~1.40-, ~1.58- and 2.8-fold higher, respectively, than that of ΔoxyRKP. (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 ΔoxyRKP. 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).
$\Delta$oxyR<sup>KP</sup> 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 oxyR<sup>KP</sup> altered the antibiotic susceptibility profile of <i>K. pneumoniae</i> belonging to the K1 serotype.

To decipher whether oxyR<sup>KP</sup> confers antibiotic resistance by affecting drug efflux, screening for a potential efflux phenotype was accomplished by determining the growth
profile of WT and ΔoxyR<sub>Kp</sub> strains in the presence of antibiotics and CCCP (10 μg ml<sup>−1</sup>) as described in the methods. In order to evaluate the true impact of oxyR<sub>Kp</sub> 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 ΔoxyR<sub>Kp</sub>. The growth rate of ΔoxyR<sub>Kp</sub> in the presence of the 0.005 μg chloramphenicol ml<sup>−1</sup> was 1.089-fold and 2.48-fold lower than the WT after 4 h and 8 h, respectively (P<0.05). The addition of CCCP drastically reduced the growth of both strains as the action of antimicrobials was restored (Fig. 5b). The growth rate of ΔoxyR<sub>Kp</sub> in the presence of the 0.005 μg erythromycin ml<sup>−1</sup> 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 ΔoxyR<sub>Kp</sub> in the presence of the 0.005 μg tetracycline ml<sup>−1</sup> 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 ΔoxyR<sub>Kp</sub> strains towards different concentrations of popularly used hospital-based disinfectants such as chlorhexidine and benzalkonium chloride (Milstone et al., 2008). The survival of ΔoxyR<sub>Kp</sub> cells was reduced by 18% when exposed to 0.1 μg triclosan ml<sup>−1</sup> (P=0.06) (data not shown). The growth rate of ΔoxyR<sub>Kp</sub> in the presence of 0.01 μg benzalkonium chloride ml<sup>−1</sup> 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 ΔoxyR<sub>Kp</sub> in the presence of 0.01 μg chlorhexidine ml<sup>−1</sup> 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 ΔoxyR<sub>Kp</sub> in the presence of 0.001 μg triclosan ml<sup>−1</sup> 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 ΔoxyR<sub>Kp</sub> 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 oxyR<sub>Kp</sub> in mediating antimicrobial resistance has been demonstrated for the first time in *K. pneumoniae*.

**Role of oxyR<sub>Kp</sub> in virulence in *K. pneumoniae* NTUH-K2044**

The *C. elegans*–*K. pneumoniae* infection model was employed to determine the involvement of oxyR<sub>Kp</sub> 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 ΔoxyR<sub>Kp</sub>ΔoxyR<sub>Kp</sub> strain restored the defect. The *Escherichia coli* strain OP50 was used as negative control. Thus, our findings demonstrate that the oxyR<sub>Kp</sub> mutant kills *C. elegans* more slowly than the WT strain.

**Expression analysis of efflux pumps and capsular genes in the oxyR<sub>Kp</sub> mutant**

Quantitative real-time RT-PCR was used to examine expression of the efflux transporter genes in WT and oxyR<sub>Kp</sub> 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<sub>KP</sub> 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<sub>KP</sub> on multidrug-resistant efflux pumps. The downregulation of these pumps could be the molecular mechanism underlying the sensitivity of the _oxyR<sub>KP</sub> mutant to hydrophobic agents tested in this study. As mutation in _oxyR<sub>KP</sub> 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<sub>KP</sub> mutant. The _ΔoxyR<sub>KP</sub>_ 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...
Table 2. Determination of MIC for WT, ΔoxyRKP and ΔoxyRKPΔoxyRKP cells

E-strips were used to determine the precise MIC in μg ml⁻¹ for the following antibiotics: amikacin, ampicillin, cefepime, cefotaxime, ceftazidime, ceftriaxone, chloramphenicol, ciprofloxacin, colistin, erythromycin, kanamycin, nalidixic acid, norfloxacin, ofloxacin, polymyxin, rifampicin, sparflaxin, streptomycin, tetracycline, tobramycin, trimethoprim, and vancomycin following the CLSI guidelines (CLSI, 2010). Representative antibiotics are shown here. The resistance to kanamycin in the ΔoxyRKP strain is due to insertion of a plasmid that carries the kanamycinR gene.

<table>
<thead>
<tr>
<th>Compound</th>
<th>WT MIC (μg ml⁻¹)</th>
<th>ΔoxyRKP MIC (μg ml⁻¹)</th>
<th>Fold change*</th>
<th>ΔoxyRKPΔoxyRKP 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 ΔoxyRKP.

subsequently regulate gene expression based on their surroundings (Hoch, 2000). It is often seen in clinically significant pathogens whereby expression of virulence factors, such as toxins, adhesins, and pili, is regulated in response to the environment (Krachler et al., 2011). The LTTRs comprise the largest family of prokaryotic transcription factors and are recognized as a key mechanism through which bacteria perceive and respond to their environment (Maddocks & Oyston, 2008). Previous studies have reported the role of oxyR in controlling expression of genes required for reactive oxygen defence, virulence, biofilm formation and drug resistance in a broad range of bacteria (Podschun & Ullmann, 1998).

The MisT2 database http://mistdb.com shows the presence of >466 signalling proteins in the 5472672 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. pneumoniae, 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. pneumoniae, 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 ΔoxyR mutant of K. pneumoniae LM21 in the presence of NaCl regardless of the inoculum size and salt concentration. The ability of ΔoxyRKP 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
Antimicrobial resistance is a serious problem creating an alarming menace globally (Kohanski et al., 2007). In this study, we elucidated the role of oxyRKP in antimicrobial resistance. Deletion of oxyRKP rendered cells sensitive to amoxicillin, chloramphenicol, erythromycin, nalidixic acid, rifampicin and trimethoprim. In K. pneumoniae NTUH-K2044, deletion of oxyRKP resulted in loss of drug extrusion capacity. Involvement of efflux pumps in drug resistance has been characterized previously (Ogawa et al., 2005). The oxyRKP deletion reduced the expression levels of efflux pumps such as acrB in the mutant when compared to WT cells, which indicates that oxyRKP 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 oxyRKP in mediating resistance against chlorhexidine and benzalkonium chloride. Besides, we found that the oxyRKP 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 oxyRKP in antimicrobial resistance and virulence.

Bacteria are extremely adaptable. They use the sensory systems such as bacterial oxyRKP 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 oxyRKP 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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**Fig. 6.** Disinfectant challenge assays with WT and ΔoxyRKP.

Growth inactivation assays using (a) benzalkonium chloride (0.01 μg ml⁻¹), (b) chlorhexidine (0.01 μg ml⁻¹), (c) triclosan (0.001 μg ml⁻¹). The efflux pump inhibitor CCCP was used at a concentration of 10 μg ml⁻¹ in the experiment. BENZ, benzalkonium chloride; CHX, chlorhexidine; TRI, triclosan.

oxidative as well as nitrosative stress tolerance of K. pneumoniae NTUH-K2044 has been demonstrated in this study.
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