Differential contribution of AcrAB and OqxAB efflux pumps to multidrug resistance and virulence in Klebsiella pneumoniae

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Objectives: In Klebsiella pneumoniae, overexpression of the AcrAB efflux pump and the more recently described OqxAB efflux pump has been linked to an antibiotic cross-resistance phenotype, but the mechanisms of regulation are largely unknown. Moreover, while AcrAB has been shown to participate in K. pneumoniae virulence, the contribution of OqxAB has not yet been assessed.

Methods: In the present study we investigated a K. pneumoniae clinical isolate (KPBj1+E+), displaying cross-resistance to quinolones, chloramphenicol and cefoxitin, and its phenotypic revertant (KPBj1 Rev, susceptible to antibiotics) by using whole-genome sequencing, RT–PCR, complementation and a Caenorhabditis elegans virulence model.

Results: We detected a point mutation in the oqxR repressor gene of KPBj1+E+, which overexpressed genes rarA, encoding a transcriptional regulator, and oqxAB, but not acrB. Complementation with wild-type oqxAB restored antibiotic susceptibility and normalized rarA and oqxAB expression levels. Whole-genome sequencing showed that KPBj1 Rev had lost the entire rarA-oqxABR locus, situated close to an integration hot spot of phage P4. This large deletion seemed responsible for the significantly lower virulence potential of strain KPBj1 Rev compared with KPBj1+E+. Moreover, we found that KPBj1+E+ ΔacrB was significantly less virulent than its parental strain.

Conclusions: This work demonstrates the role of the overexpression of efflux pump OqxAB, due to a mutation in gene oqxAB, in the antibiotic resistance phenotype of a clinical isolate, and suggests that the presence of AcrAB, associated with overexpression of OqxAB, is required for high virulence potential.

Keywords: K. pneumoniae, efflux systems, antimicrobial resistance

Introduction

Klebsiella pneumoniae is an important pathogen responsible for a wide range of nosocomial infections.1 It can also cause invasive community-acquired infections, including liver abscess, pneumonia, severe bacteraemia and meningitis.2,3 Among the various mechanisms of resistance displayed by K. pneumoniae, efflux system overexpression has recently been shown to induce low-level cross-resistance to β-lactams (mainly cefoxitin) and to antibiotics belonging to other families, such as quinolones and chloramphenicol.4 The efflux system that has been the most extensively studied in K. pneumoniae and other members of the Enterobacteriaceae family, with regard to antibiotic resistance, is the pump AcrAB.5–11

However, the chromosome of K. pneumoniae harbours various other genes and operons encoding putative efflux systems, among which the rarA-oqxABR locus has recently been characterized.12 The oqxAB operon was originally described on plasmid pOLA52, carried by an Escherichia coli strain isolated from swine manure.13 It has been shown to encode an efflux pump conferring resistance to olaquindox, a growth enhancer widely used in pig farming, as well as to chloramphenicol and quinolones.14 Since then, the presence of oqxAB has been reported in K. pneumoniae isolates from various regions of the world.12,15–18 Moreover, Veleba et al.19 have recently described the two genes flanking the oqxAB operon in the chromosome of K. pneumoniae reference strain MGH 78578: rarA, encoding an AraC-type transcriptional activator, and oqxR, encoding a GntR-type
transcriptional repressor. Their results suggest that rarA up-regulates, whereas oqXR down-regulates, the expression of the oqxA/B efflux pump operon.12

We have previously shown that three K. pneumoniae clinical isolates, presenting an antibiotic resistance pattern indicative of the overexpression of an efflux system and transcription levels of gene acrB either equal to or only slightly increased compared with those of their spontaneous phenotypic revertants, were significantly more virulent than the latter in a Caenorhabditis elegans model.15 Our aim in the present study was to investigate the possible role of the rarA-oqxA/B operon in the antibiotic resistance phenotype and in the increased virulence potential of one of these three isolates and to get insight into the regulatory mechanisms of this novel efflux pump.

Materials and methods

Bacterial strains and plasmids

All bacterial strains and plasmids used and/or constructed in this study are listed in Table 1.

Antibiotic susceptibility testing

Susceptibility to nalidixic acid, ciprofloxacin, chloramphenicol, tigecycline and cefoxitin was determined in triplicate by the broth dilution method according to the guidelines of EUCAST.20 A difference between MICs was considered to be significant starting from two dilutions (i.e. a 4-fold MIC variation).

Sequence studies

Genes rarA and oqXR, as well as the intergenic region situated between rarA and oqxa (inter-rar), containing the promoters of both genes, were amplified and sequenced using the primers listed in Table S1 (available as Supplementary data at JAC Online) and following the PCR and sequencing procedures described previously.21

Real-time RT–PCR for analysis of gene expression

Real-time RT–PCR for analysis of the expression of genes rarA and oqXR was carried out and interpreted as described previously.21 Briefly, after RNA extraction and DNaseI pre-treatment, total RNA concentrations were determined using a NanoDrop Spectrophotometer and adjusted to 100 mg/L. Amplifications were performed in duplicate on a LightCycler using the one-step LightCycler® RNA Master SYBR Green I kit (Roche Applied Science, Meylan, France). Data were analysed by the 2–ΔΔCT method; the pnpB gene was chosen as a reference and strain ATCC 13883 as a calibrator. All the primers used are listed in Table S1.

Cloning and transformation of the oqXR gene

The 677 bp wild-type oqXR gene of strain ATCC 13883 [amplified using primers oqXR-F and oqXR-R (Table S1)] was cloned into pSC-A-amp/kan by using the StrataClone PCR Cloning Kit (Agilent Technologies, Massy, France). After verification of the construct by sequencing, the recombinant plasmid was electroporated into competent strain KB1 E + and the transformants were selected on LB agar plates containing 50 mg/L kanamycin. The empty plasmid pSC-A-amp/kan-E1 was used as a negative control.

Gene replacement experiments

Inactivation of the chromosomal acrB gene and replacement of a native oqXR allele by a mutated one were both achieved following a strategy adapted from Datsenko and Wanner.22 Briefly, recipient strains were transformed with plasmid pKOBEG199, containing the ß Red region under the control of the arabinose-inducible promoter, the araC gene and a tetracycline resistance cassette.23 Concurrently, appropriate linear DNA fragments were synthesized. For acrB inactivation, an FRT-flanked kanamycin resistance cassette (kan) was amplified from the template plasmid pKD4 using primers acrBForFRTKm and acrBRevFRTKm (Table S1) containing 76–79 nt extensions homologous to the N-terminal and C-terminal flanking regions of the acrB gene, respectively. The PCR product was digested with DpnI (New England BioLabs, Evry, France) in order to remove the remaining template plasmid, and purified using the

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OP50</td>
<td>avirulent control strain</td>
<td>34</td>
</tr>
<tr>
<td>ATCC 13883</td>
<td>wild-type reference strain</td>
<td>44</td>
</tr>
<tr>
<td>KP1</td>
<td>MDR clinical isolate</td>
<td>4</td>
</tr>
<tr>
<td>KP1 Rev</td>
<td>antibiotic-resistant revertant of KP1 E+</td>
<td>19</td>
</tr>
<tr>
<td>KP1 M3 Lev</td>
<td>ramR-deleted mutant derived from KP1 Rev</td>
<td>37</td>
</tr>
<tr>
<td>KP1 E+ ΔacrB</td>
<td>acrB-deleted strain derived from KP1 E+, KAN'</td>
<td>this work</td>
</tr>
<tr>
<td>KP1 E+ ΔacrB TqoxR-ATCC</td>
<td>complemented with plasmid pSC-A-amp/kan-oqXR-ATCC, AMP', KAN'</td>
<td>this work</td>
</tr>
<tr>
<td>KP1 E+ TqoxR-ATCC</td>
<td>pSC-A-amp/kan-oqXR-ATCC, AMP', KAN'</td>
<td>this work</td>
</tr>
<tr>
<td>LM21</td>
<td>wild-type clinical strain</td>
<td>35</td>
</tr>
<tr>
<td>LM21 RqoxR-KP1 E+</td>
<td>LM21 with the oqXR allele of strain KP1 E+</td>
<td>this work</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
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<tr>
<td>pSC-A-amp/kan-oqXR-ATCC</td>
<td>pSC-A-amp/kan plasmid vector containing the cloned wild-type oqXR gene, AMP', KAN'</td>
<td>this work</td>
</tr>
<tr>
<td>pSC-A-amp/kan-E1</td>
<td>empty control plasmid vector, AMP', KAN'</td>
<td>21</td>
</tr>
<tr>
<td>pKOBEG199</td>
<td>pBR322 derivative containing the ß Red region and araC gene of pKOBEG, TET'</td>
<td>23</td>
</tr>
</tbody>
</table>

TET, tetracycline; KAN, kanamycin; AMP, ampicillin.

Table 1. List of bacterial strains used in this study
Role of efflux pumps in *Klebsiella pneumoniae* resistance and virulence

QiAmp PCR Purification Kit (Qiagen, Courtaboeuf, France). For oqxR allelic replacement, a 175 bp fragment was amplified from the DNA of strain KBPj1 E+ using primers oqxR-175-F and oqxR-175-R (Table S1). Finally, the PCR products were electroeluted into competent cells carrying pKOBEG199 in which the expression of α Red recombinase had been induced by arabinose, and transformants were selected on LB agar plates containing either 50 mg/L kanamycin, for *acrB* inactivation, or 16 mg/L chloramphenicol, for *oqxR* allelic replacement. The correct insertion of the kan cassette, leading to αcrb gene knockout, was verified by PCR amplification using the primer pairs acrBupF/kt and acrBdoR/k2 on the one hand (expected to be positive) and acrBupF/acrBupR and acrBdoR/acrBdoF on the other hand (expected to be negative) (Table S1). Similarly, allelic replacement of gene *oqxR* was checked by PCR and sequencing. Moreover, we obtained pKOBEG199-free transformants following successive subcultures on LB plates without tetracycline antibiotic.

**Whole-genome sequencing**

Total DNA of strains KBPj1 E+ and KBPj1 Rev was extracted using the QIAamp DNA Blood Midi Kit (Qiagen). Genomic sequencing was performed by GATC Biotech (Konstanz, Germany) on an Illumina HiSeq-2000 sequencer, using Nextera technology for library construction and a ≥100 nt paired-end strategy. All reads were pre-processed to remove low-quality or artefactual nucleotides. First, all nucleotides occurring at the 5′ and 3′ ends and supported by a Phred quality score <30 were trimmed off using Sickle (github.com/najoshi/sickle). Second, contaminant oligonucleotides (i.e. library adapters) were detected and trimmed off using AlienTrimmer.24 Third, reads shorter than 60 nt after the above cleaning steps were discarded, as well as those containing >20% nucleotides with a Phred score <30. Finally, the program fastDuplicate (ftp.posteur.fr/pub/gensoft/projects/fqtools) was used to discard every duplicate read pair. Resulting reads were assembled using clc_assembler from the CLC Genomics Workbench analysis package (www.clcbio.com/products/clc-genomics-workbench/). All contigs of size ≥500 nt were reordered and reoriented, using as reference the genomic sequence of strain MGH 78578, with Mauve Contig Mover.25 The reordered contigs were subsequently imported into the Bacterial Isolate Genome Sequence Database (BIGSdb) platform14 and analysed for the presence of known efflux systems and regulators. Alleles corresponding to the seven MLST genes were also extracted from both genomic sequences in order to determine the ST of the strains. Furthermore, the two genomes were annotated and compared using the RAST server.28 Finally, the reads corresponding to strain KBPj1 Rev were mapped against the contig containing the *rarA-oqxABR* locus in the genome assembly of strain KBPj1 E+ using BWA29 and SAMtools30 and the read alignments on the reference sequence were visualized using Artemis.31 Comparison of the genomic region surrounding the *rarA-oqxABR* locus in strains KBPj1 E+, KBPj1 Rev and KCTC 22422 was achieved using Easyfig version 2.1.33

**Feeding behaviour assays**

For occupancy assays, each bacterial strain was cultured overnight in LB medium, spotted as a circular lawn on NGM plates and dried at room temperature for 20 min. Approximately 20 L4 nematodes were carefully placed in the centre of the bacterial lawn. The number of worms inside or outside each lawn was counted after 16 h. The number of bacteria within the C. elegans digestive tract was obtained as previously described.34 Briefly, worms were picked at 72 h and the surface bacteria were removed by washing the worms twice in M9 medium containing 25 mg/L gentamicin. The nematodes were then mechanically disrupted in M9 medium containing 1% Triton X-100. Serial dilutions of the sample were then plated on LB agar and the colonies were counted after 24 h. For both types of experiment, at least three replicates were performed for each condition.

**Statistical analysis**

To compare the entire survival curves in nematode killing assays, Cox regression was used. To perform pairwise comparison between two different strains, we used a log rank test. The analysis was carried out using SPSS 6.1.1 (SPSS Inc., Chicago, IL, USA).

**Results**

**Contribution of AcrAB to the efflux phenotype of strain KBPj1 E+**

In order to determine whether the AcrAB efflux pump was responsible for the antibiotic resistance phenotype of KBPj1 E+, we constructed an *acrB* deletion mutant of this clinical isolate. The MICs of antibiotics belonging to different families and known to be affected by efflux system overexpression were measured for the *acrB*-deleted and parental strains (Table 2). The results show that the *acrB* knockout conserved its efflux phenotype, since the MIC values of all the antibiotics tested were not significantly different from those measured for strain KBPj1 E+. Consequently, the MDR phenotype of this strain is unrelated to AcrAB and is probably due to overexpression of another efflux pump.

**Genetic determinants of the antibiotic resistance phenotype of strain KBPj1 E+**

Subsequently, we wanted to test the implication of the recently characterized *rarA-oqxABR* locus in the MDR phenotype of KBPj1 E+. For this purpose, we measured in this strain the expression levels of genes *acrB*, *oqxB* and *rarA*. Compared with the reference strain ATCC 13883T, KBPj1 E+ considerably overexpressed *oqxB* (89.7-fold) and *rarA* (1537-fold) (Table 2). Moreover, in contrast to what had been found previously, KBPj1 E+ did not seem to overexpress, even slightly, gene *acrB* (Table 2). Subsequently, amplification and sequencing of genes *oqxR* and *rarA*, as well as of the *rarA-oqxA* intergenic region, were carried out in strains KBPj1 Rev, KBPj1 E+ and ATCC 13883T. In strain KBPj1 Rev, the PCRs using the primers *rarA-infF* and *rarA-intR*, on the one hand, and *oqxRF* and *oqXR*, on the other hand, were negative, suggesting deletion of these loci. The sequences of the *rarA* gene of strains KBPj1 E+ and ATCC 13883T displayed only some silent polymorphisms. As regards the *oqxR* gene, we detected a T305G mutation specific to strain KBPj1 E+, which was responsible for the replacement of Val-102, located in the C-terminal effector-binding and oligomerization domain of the OqxR protein, by Gly.
Complementation with the wild-type oqxR gene

We hypothesized that the Val-102→Gly mutation identified in OqxR was responsible for the loss of its repressor function and for the subsequent overexpression of the OqxAB efflux pump, resulting in an MDR phenotype. Transformation of KPBj1 E+ with pSC-A-amp kan-oqxAB-ATCC considerably lowered the transcription level of gene oqxAB and the MICs of nalidixic acid, ciprofloxacin, chloramphenicol and cefoxitin, but not that of tigecycline (Table 2). Furthermore, introduction of the wild-type oqxR gene resulted in a decrease in rarA expression level, suggesting that OqxR is also a repressor of rarA. In contrast, complementation with control plasmid pSC-A-amp kan-E1 had no significant effect on either gene expression levels or antibiotic susceptibilities (Table 2).

Allelic replacement of the oqxR gene

In order to definitely demonstrate that the point mutation detected in the oqxR gene of strain KPBj1 E+ was the cause of the efflux phenotype of this strain, we performed an allelic replacement experiment: the oqxR allele originally present in the antibiotic susceptible strain LM21 was replaced by the mutated allele of strain KPBj1 E+. This allelic change resulted in a sharp increase in the MICs of the antibiotic tested (nalidixic acid, ciprofloxacin, chloramphenicol and cefoxitin) except for tigecycline, as well as in the transcription levels of genes rarA and oqxAB (Table 2).

Genome studies

The genome sequences of strains KPBj1 E+ and KPBj1 Rev were assembled into 277 and 934 contigs, respectively. Gene-by-gene analysis revealed that both strains belonged to the same sequence type, ST461, which is quite unusual. Genes acrB, marA, marR, ramA, ramR, soxR, soxS, rob, sdiA, fis and envR, coding for efflux system regulators, were detected in the two genomes with exactly the same nucleotide sequence, confirming our unpublished Sanger sequencing data. Genes oqxAB and rarA were present only in strain KPBj1 E+, whereas gene oqxA was truncated (148/480 bp) in KPBj1 Rev. Mapping of the reads corresponding to strain KPBj1 Rev against the sequence of KPBj1 E+, taken as a reference, showed the deletion of a region of nearly 17.4 kb from the KPBj1 Rev genome. The deleted region comprised genes oqxA, oqxB and rarA, as well as a cluster of genes lying downstream of rarA and corresponding to a putative NAD(P)H-flavin oxidoreductase, a putative LysR-family transcriptional regulator, a putative plasmid-related protein and a number of putative phage P4-related proteins (Figure 1).

The rarA-oqxABR locus in complete public K. pneumoniae genomes

Among the nine complete K. pneumoniae genomes available at the NCBI genome sequence repository (www.ncbi.nlm.nih.gov/ genome/) as of 17 December 2013, eight contained the oqxA, oqxB, oqxr and rarA genes. The rarA-oqxABR locus was absent from the genome of strain HS11286, an MDR clinical isolate from China. In only one of the eight remaining genomes, that of strain KCTC 2242, an isolate used for industrial 2,3-butanediol production, a group of genes annotated as putative phage P4-related proteins was detected. However, this gene cluster was homologous but not identical to that present in the genome of strain KPBj1 E+. A comparison of the genomic region surrounding the rarA-oqxABR locus in strain KCTC 2242 and in the two strains sequenced in the present study is depicted in Figure 1. In the genome of strain JM45, another cluster of phagic origin, having no homology with those found in strains KCTC 2242 and KPBj1 E+, was inserted after gene smrB. In the six other complete public K. pneumoniae genomes, either a completely different gene cluster was present (n=2) or smrB was adjacent to the gene encoding a putative plasmid-related protein (n=4). In all the genomes considered, a BLAST search identified a sequence corresponding to the tmRNA gene (ssrA; locus tag KP1_6100 in the genome of strain NTUH K2044) immediately downstream of gene smrB.

Virulence studies

In order to determine the respective roles of AcrAB and OqxAB in virulence, several K. pneumoniae strains, differentially expressing both efflux pumps, were tested in the C. elegans model. Consistent with previous findings, strain KPBj1 E+ was significantly more virulent than both strain KPBj1 Rev (P<0.001) and strain ATCC 13883 (P<0.001), which showed the same virulence profile.

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Table 2. Antibiotic susceptibility profiles and relative transcription levels of the acrB, oqxAB and rarA genes for the studied K. pneumoniae strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (mg/L)</th>
<th>Gene expression (fold change)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NAL</td>
<td>CIP</td>
</tr>
<tr>
<td>ATCC 13883</td>
<td>4</td>
<td>0.06</td>
</tr>
<tr>
<td>KPBj1 E+</td>
<td>128</td>
<td>0.5</td>
</tr>
<tr>
<td>KPBj1 E+ ΔacrB</td>
<td>128</td>
<td>0.5</td>
</tr>
<tr>
<td>KPBj1 E+ ΔoqxA-ATCC</td>
<td>4</td>
<td>0.03</td>
</tr>
<tr>
<td>KPBj1 E+ ΔrarA</td>
<td>128</td>
<td>0.5</td>
</tr>
<tr>
<td>KPBj1 E+ ΔsoxR</td>
<td>4</td>
<td>0.03</td>
</tr>
<tr>
<td>KPBj1 M3 Lev</td>
<td>32</td>
<td>0.25</td>
</tr>
<tr>
<td>LM21</td>
<td>4</td>
<td>0.03</td>
</tr>
<tr>
<td>LM21 R Δoqxr-KPBj1 E+</td>
<td>128</td>
<td>0.25</td>
</tr>
</tbody>
</table>

NAL, nalidixic acid; CIP, ciprofloxacin; CHL, chloramphenicol; TGC, tigecycline; FOX, cefoxitin; ND, not determined. For gene expression levels, the results of at least two different experiments are indicated as mean ± SD.
Deletion of the acrB gene led to a decrease in the virulence level of strain KPBj1 $E^+$DacrB (LT$_{50}$ 4.0 $\pm$ 0.2 days) compared with strain KPBj1 $E^+$ (LT$_{50}$ 3.0 $\pm$ 0.2 days) ($P = 0.007$) (Figure 2). Furthermore, the same effect was observed for strain KPBj1 M3 Lev (LT$_{50}$ 5.4 $\pm$ 0.2 days), an in vitro selected MDR mutant of KPBj1 Rev overexpressing acrB due to a deletion of the ramR gene, compared with its parental strain (LT$_{50}$ 4.6 $\pm$ 0.2 days) ($P < 0.001$) (Figure 2). To exclude the possibility that the variations in the observed sensitivity of nematodes could be due to an alteration of their feeding behaviour, we performed an occupancy test in which nematodes were placed in the centre of a bacterial lawn and the fractions of worms inside and outside the lawn were quantified after overnight incubation. None of the bacterial strains tested in this study induced strong avoidance behaviour and there was no significant difference in the fraction of nematodes on the bacterial lawn between the different conditions tested (data not shown). We also measured the ingestion and proliferation of bacteria in the digestive tract of nematodes for each condition. Bacterial counts in the digestive tract of the nematodes were similar whatever the strain tested, showing that all the bacteria were actually ingested.

**Discussion**

One of the aims of this work was to explain, at the molecular level, the MDR phenotype of the clinical isolate KPBj1 $E^+$, which had previously been shown to slightly overexpress the acrB gene, compared with strain ATCC 13883$^T$. However, the results obtained in the present study did not confirm this observation and it seems to us that the AcrAB efflux pump is, in fact, not overexpressed in KPBj1 $E^+$. Consistently, the MIC values of nalidixic acid, ciprofloxacin, chloramphenicol, cefoxitin and tigecycline did not change in the acrB-deleted KPBj1 $E^+$ mutant. This suggests that these antibiotics, which are well-known substrates of AcrAB in different enterobacterial species, are probably extruded by another efflux pump in *K. pneumoniae*. Indeed, in KPBj1 $E^+$ we observed high levels of transcription of gene oqxB, as well as of the regulator rarA gene, compared with the wild-type ATCC 13883$^T$ strain.

Sequencing of the oqXR gene revealed a point mutation leading to the amino acid substitution Val-102 $\rightarrow$ Gly in the OqXR protein of strain KPBj1 E+. This substitution is different from those reported by Veleba et al., who described for the first time the rarA and oqXR loci in MDR clinical isolates of *K. pneumoniae*. Complementation of strain KPBj1 E+ with the wild-type oqXR gene dramatically decreased the expression levels of genes oqxB and rarA, and this result suggests that OqXR is a repressor not only of the oqxAB efflux operon, but also of the regulator rarA gene, compared with the wild-type ATCC 13883$^T$ strain.

To exclude the possibility that the variations in the observed sensitivity of nematodes could be due to an alteration of their feeding behaviour, we performed an occupancy test in which nematodes were placed in the centre of a bacterial lawn and the fractions of worms inside and outside the lawn were quantified after overnight incubation. None of the bacterial strains tested in this study induced strong avoidance behaviour and there was no significant difference in the fraction of nematodes on the bacterial lawn between the different conditions tested (data not shown). We also measured the ingestion and proliferation of bacteria in the digestive tract of nematodes for each condition. Bacterial counts in the digestive tract of the nematodes were similar whatever the strain tested, showing that all the bacteria were actually ingested.

**Figure 1.** The rarA-oqxABR locus in some *K. pneumoniae* genomes. Schematic representation of the region encompassing the rarA-oqxABR locus in one *K. pneumoniae* public genome (strain KCTC 2242) compared with the two genomes sequenced in the present study. The large arrows indicate the locations and orientations of the different coding sequences, with annotations displayed below or above the arrows. The black vertical bar represents the limits of contigs in the genomic sequence of strain KPBj1 Rev. The BLAST identity scale is shown in the lower right corner, with the matches ranging from 100% (darkest) to 82% (lightest). 1, abortive infection phage resistance protein; 2, hypothetical protein; 3, phage polarity suppression protein; 4, phage late gene regulator; 5, phage capsid and scaffold protein; 6, hypothetical protein; 7, phage transcriptional regulator; 8, putative phage immunity repressor protein; 9, hypothetical protein; 10, hypothetical phage protein; 11, bacteriophage P4 DNA primase.
and derepressed the expression of genes oqxB and oqxR of the clinical isolate. As expected, substitution of the native allele was performed in strain LM21, a wild-type K. pneumoniae strain previously considered a spontaneous revertant of KPBj1 E+ because of its loss of the cross-resistance phenotype. Our results are consistent with a previous study by Padilla et al., which also demonstrated that the inactivation of gene acrB attenuated the virulence potential of the highly pathogenic K. pneumoniae strain 52145. However, quite unexpectedly, the virulence level of KPBj1 M3 Lev, an acrB-overexpressing in vitro-selected mutant of KPBj1 Rev, was lower than that of its parental strain. From this we conclude that acrB overexpression does not have by itself a positive effect on virulence, although the low virulence of strain KPBj1 M3 Lev might also be due to the fact that this strain carries a

Figure 2. Kinetics of killing of C. elegans infected by the studied K. pneumoniae strains. LT50, corresponding to time (days) required to kill 50% of the nematode population, is represented for each of the studied strains as the mean ± SD of five experiments. E. coli strain OP50 was used as a control. Statistical significance of the differences observed is indicated as follows: *P<0.001; **P=0.007.

was not elevated, which is also different from the findings of Veleba et al., and suggests that the role of RarA as a regulator of acrB is unclear. In order to definitely demonstrate that the point mutation found in the oqXR gene of strain KPBj1 E+ is sufficient to explain its MDR phenotype, an allelic replacement experiment was performed in strain LM21, a wild-type K. pneumoniae clinical isolate. As expected, substitution of the native oqxR allele by the mutated one derepressed the expression of genes oqxB and rarA in strain LM21 RarA-oqxABR.

With regard to antibiotic susceptibility, transformation of KPBj1 E+ with pS-S-A-amp/kan-oqXR-ATCC decreased the MICs of nalidixic acid, ciprofloxacin, chloramphenicol and cefoxitin at least 4-fold, but did not significantly affect the MIC of tigecycline. By using isogenic strains in which the rarA-oqxABR locus is absent and the AcrAB pump is either normally expressed or overexpressed, we showed that the MICs of all of the antibiotics tested were increased in the variant overproducing AcrAB. All these results definitively indicate that nalidixic acid, ciprofloxacin, chloramphenicol and cefoxitin are substrates of both the OqxAB efflux pump and the AcrAB efflux pump, while tigecycline is a substrate of only the AcrAB efflux pump.

Analysis of the genomic sequences of strains KPBj1 E+ and KPBj1 Rev, which is a phenotypic revertant of the former strain that had spontaneously recovered susceptibility to several antibiotics, yielded some interesting observations. First, it confirmed the clonal relatedness of these two strains, which has previously been shown by RAPD (random amplified polymorphic DNA) analysis. Second, the genome of KPBj1 Rev was shown to harbour a deletion of nearly 17.4 kb, encompassing genes oqxA, oqxB and rarA and two-thirds of gene oqxR, as well as a large cluster of genes lying downstream of rarA and comprising a number of putative phage P4-related proteins, when compared with the corresponding region in the genome of strain KPBj1 E+. Among the nine complete K. pneumoniae genomes publicly available at the time period of our study, only one contained a putative phage P4-related gene cluster inserted in the vicinity of the rarA-oqxABR locus, whereas three others harboured a different phage or gene cluster. Gene ssrA, situated in this genomic region just downstream of smpB and encoding a tmRNA, is a known hot spot of integration of mobile genetic elements in Enterobacteriaceae. We suppose that the phage has been excised from the genome of KPBj1 E+, accidentally removing the neighbouring genes and thus leading to strain KPBj1 Rev, previously considered a spontaneous revertant of KPBj1 E+ because of its high MDR phenotype. In our previous study of K. pneumoniae virulence assessed in the C. elegans model, we surmised that the difference in virulence level between strains KPBj1 E+ and KPBj1 Rev was due to a slight variation in the expression of gene acrB. However, since we have shown in the present study that KPBj1 E+ in fact does not overexpress acrB, our current hypothesis is that the high virulence of this strain is related to the overexpression of genes oqxB and/or rarA. With regard to the role of AcrAB in K. pneumoniae virulence, we observed that the acrB deletion mutant was significantly less virulent than its parental strain, KPBj1 E+. Our results are consistent with a previous study by Padilla et al., which also demonstrated that the inactivation of gene acrB attenuated the virulence potential of the highly pathogenic K. pneumoniae strain 52145. However, quite unexpectedly, the virulence level of KPBj1 M3 Lev, an acrB-overexpressing in vitro-selected mutant of KPBj1 Rev, was lower than that of its parental strain. From this we conclude that acrB overexpression does not have by itself a positive effect on virulence, although the low virulence of strain KPBj1 M3 Lev might also be due to the fact that this strain carries a
deletion of the entire ramR gene.\textsuperscript{37} In order to definitely address these questions, further studies are needed and are currently in progress.

To conclude, we found that overexpression of the OqxAB efflux pump, resulting from a point mutation in the repressor oqxAB\textsuperscript{R} gene, was responsible for the MDR phenotype and for the increased virulence potential of one K. pneumoniae clinical isolate. As this pump is widely distributed in K. pneumoniae genomes,\textsuperscript{12,15–18} but not necessarily overexpressed, it would be interesting to determine the extent to which it participates in clinically relevant antimicrobial resistance and in pathogenicity of nosocomial and community-acquired isolates.

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Transparency declarations
None to declare.

Supplementary data
Table S1 is available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References


