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Characterization of RarA, a Novel AraC Family Multidrug Resistance Regulator in *Klebsiella pneumoniae*

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Transcriptional regulators, such as SoxS, RamA, MarA, and Rob, which upregulate the AcrAB efflux pump, have been shown to be associated with multidrug resistance in clinically relevant Gram-negative bacteria. In addition to the multidrug resistance phenotype, these regulators have also been shown to play a role in the cellular metabolism and possibly the virulence potential of microbial cells. As such, the increased expression of these proteins is likely to cause pleiotropic phenotypes. *Klebsiella pneumoniae* is a major nosocomial pathogen which can express the SoxS, MarA, Rob, and RamA proteins, and the accompanying paper shows that the increased transcription of *ramA* is associated with tigecycline resistance (M. Veleba and T. Schneiders, Antimicrob. Agents Chemother. 56:4466–4467, 2012). Bioinformatic analyses of the available *Klebsiella* genome sequences show that an additional AraC-type regulator is encoded chromosomally. In this work, we characterize this novel AraC-type regulator, hereby called RarA (Regulator of antibiotic resistance A), which is encoded in *K. pneumoniae*, *Enterobacter* sp. 638, *Serratia proteamaculans* 568, and *Enterobacter cloacae*. We show that the overexpression of *rarA* results in a multidrug resistance phenotype which requires a functional AcrAB efflux pump but is independent of the other AraC regulators. Quantitative real-time PCR experiments show that *rarA* (MGH 78578 KPN_02968) and its neighboring efflux pump operon *oqxAB* (KPN_02969_02970) are consistently upregulated in clinical isolates collected from various geographical locations (Chile, Turkey, and Germany). Our results suggest that *rarA* overexpression upregulates the *oqxAB* efflux pump. Additionally, it appears that *oqxR*, encoding a GntR-type regulator adjacent to the *oqxAB* operon, is able to downregulate the expression of the *oqxAB* efflux pump, where OqxR complementation resulted in reductions to olaquindox MICs.

Multidrug resistance in Gram-negative bacteria is a significant issue in the treatment of infectious diseases. Research associated with reduced antimicrobial susceptibility is largely focused on specific mechanisms that confer high levels of antibiotic resistance and are generally acquired extrinsically (18). In contrast, genes that encode transcriptional regulators such as MarA, SoxS, Rob, and RamA confer a low-level multidrug-resistant (MDR) phenotype by increasing levels of efflux pump expression (1).

The study of these intrinsic genetic mechanisms is key to the understanding of the development and persistence of antimicrobial resistance, as these intrinsic systems are generally encoded by transcription factors that control a plethora of genes. In *Escherichia coli* and *Salmonella* spp., two major systems, namely, the *marAB* and *soxRS* systems, have been extensively studied (3, 25). In numerous studies involving multidrug-resistant clinical strains, it has been shown that mutations that alter the levels of the MarA or SoxS proteins also lead to increased levels of the efflux system AcrAB (20, 30). Generally, mutations that result in derepression or activation generate the constitutive expression of the MarA/SoxS proteins; however, transient induction of these intrinsic proteins can also occur through interaction with ligands such as salicylate (5) or menadione (13).

Chromosomally intrinsic systems in *E. coli* and *Klebsiella pneumoniae* such as MarRAB and SoxRS have been shown to control efflux pumps, particularly the AcrAB pump, which confers low-level resistance to a variety of antibiotics and disinfectants (22). Additionally, several global transcriptional profiling studies have shown that other less-well-characterized efflux pumps and other genes involved in cellular metabolism are also regulated by these intrinsic systems (3, 24). Accordingly, studies show that the virulence profiles and infectivity potential of bacteria can be altered on the basis of the expression levels of these intrinsic regulators (4). Addressing the role of accessory genes or operons associated with or involved in antimicrobial resistance is an integral part of understanding the microbial response to antibiotic challenge, as antibiotics have been shown to function as signaling molecules that trigger specific transcriptional signals (7). Hence, it is evident from these studies that the intrinsic resistome of bacteria consists of genes that may be involved in a wide variety of cellular functions but which also provide protection against antibiotic challenge.

Klebsiella pneumoniae is a commonly encountered major nosocomial pathogen that is increasingly multidrug resistant (12). As in *E. coli* and *Salmonella* spp., transcription factors that mediate multidrug resistance have been identified in *K. pneumoniae* (16, 27, 28). To date, most studies that have identified the role of transcriptional regulators in the multidrug resistance phenotype have shown that, in clinical *K. pneumoniae* isolates, increased expression of a *marA*-like gene, *ramA*, has been associated with upregulation of the *acrAB* efflux pump and multidrug resistance (27, 28). In order to determine whether other intrinsic systems exist that

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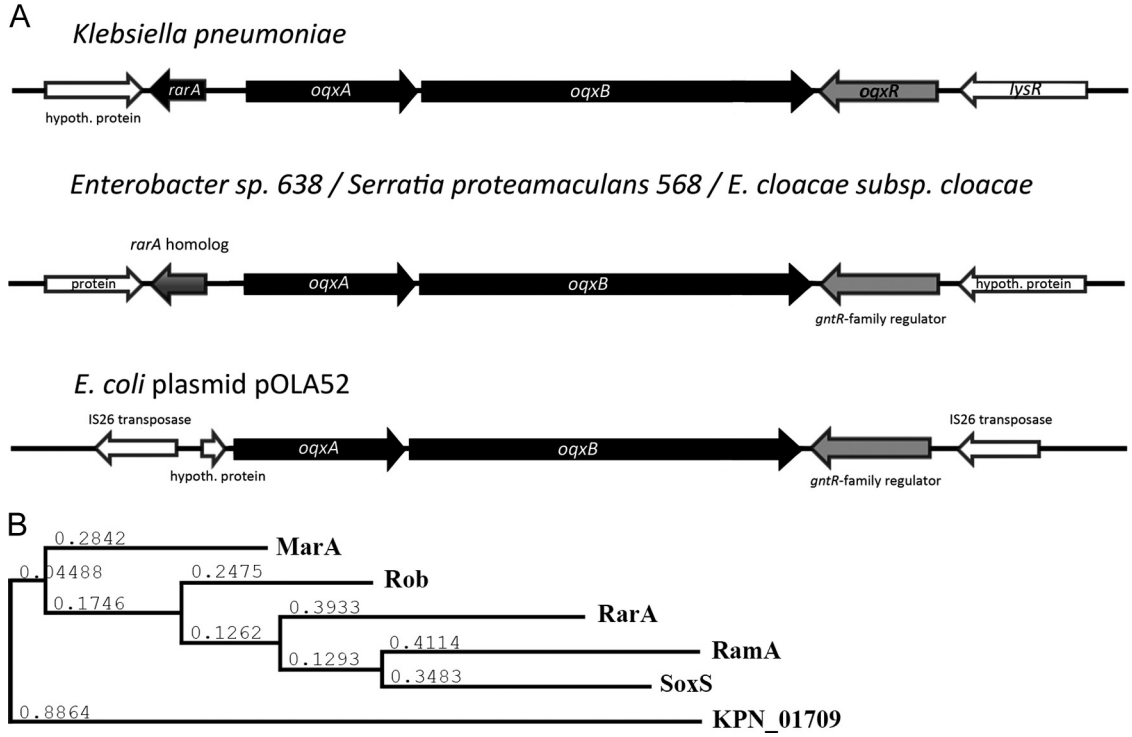

FIG 1 (A) Comparison of genomic organizations of *rarA* (KPN_02968) locus in *Klebsiella pneumoniae* strain MGH 78578 to those of other *Enterobacteriaceae* harboring the locus. (B) Predicted phylogenetic tree of MarA-type protein homologues in *Klebsiella pneumoniae* determined using PhyML 3.0 (14). The amino acid sequences for MarA (KPN_01624), Rob (KPN_04851), RarA (KPN_02968), RamA (KPN_00556), SoxS (KPN_04462), and KPN_01709 were used in Muscle alignment software to generate the phylogenetic tree. All amino acid sequences were obtained from the genome sequence of MGH 78578 (NC_009648). Of note, different branch lengths denote relative amino acid identities.

can confer multidrug resistance in *K. pneumoniae*, we performed bioinformatic analyses to mine the three sequenced *K. pneumoniae* genomes for AraC-type regulators. As a result of these analyses, we have identified and characterized a novel AraC-type regulator, KPN_02968 (designation based on MGH 78578 genome sequence), that confers a multidrug-resistant phenotype, which we now call RarA (Regulator of antibiotic resistance A). Bioinformatic analyses show that the *rarA* gene is encoded in *K. pneumoniae*, *Enterobacter* sp. 638, *Serratia proteamaculans* 568, and *E. cloacae* (Fig. 1A).

MATERIALS AND METHODS

Bacterial strains. A complete list of all bacterial strains and plasmids used and constructed is shown in Table 1. The propagation of all strains was either performed in LB broth at 37°C with shaking or LB agar at 37°C. The targeted genetic mutants were generated from *K. pneumoniae* Ecl8 by the use of a modified protocol as described by Merlin et al. (21). Briefly, flanking regions of *rarA* were amplified using PCR primers (Co-Ci and No-Ni 02968) (Table 2), and the products were then reamplified using the outer flank primers, Co and No, which generated a PCR fragment that contained both flanking regions but not *rarA*. This fragment was restriction digested by the use of PstI and SalI (NEB, England) and cloned into the pTOF25 replacement vector (21). The kanamycin resistance cassette was excised from the recombinant pTOF3 plasmid by the use of NotI excision and cloned between the flanking regions in the recombinant pTOF25. This recombinant plasmid was electroporated into *K. pneumoniae* Ecl8, and a kanamycin-resistant variant was selected after growth on selection plates at 30°C. The insertion was verified by PCR amplification using the primers 02968delchkF and 02968delchkR. Similarly, for the *acrAB* gene knockout, we used Co-acrB/Ci-acrB and No-acrA/Ni-acrA to create the flanks. In this case, the overlap PCR product was restricted using XhoI and PstI (NEB, England) prior to cloning and gene replacement as described above.

Bioinformatic analyses. Amino acid identity was analyzed using NCBI's Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov) and the MarA amino acid sequence (KPN_01624) from the NCBI entry *Klebsiella pneumoniae* subsp. *pneumoniae* MGH 78578 (accession number NC_009648.1). To assess the genetic relatedness of the AraC regulators such as Rob, RamA, RarA, and SoxS, a phylogenetic tree was generated using the Phylogeny Analysis tool at LIRMM Methodes et Algorithmes pour la Bio-informatique (http://www.phylogeny.fr/version2_cgi/simple_phylogeny.cgi) (14) by inputting sequences of Rob (KPN_04851), RamA (KPN_00556), RarA (KPN_02968), SoxS (KPN_04462), and putative helix-turn-helix (HTH) AraC-type regulator KPN_01709.

Antimicrobial susceptibility testing. Susceptibility testing was undertaken as described by the British Society for Antimicrobial Chemotherapy (BSAC) guidelines using the agar dilution method (2). Overnight cultures were briefly diluted 10^4 in PBS medium, and 5 μl of each dilution was spotted on the LB agar plates supplemented with antibiotics. Plates were incubated overnight at 37°C prior to scoring for growth. All MIC tests were performed in triplicate.

Cloning and transformations. All primers used are shown in Table 2. A recombinant plasmid containing the gene of interest, *rarA* (KPN_02968), was constructed. Due to conflicting antibiotic resistance determinants in the chromosomal gene knockouts, two plasmid recombinants (pACrarA-1 and pACrarA-2) harboring the *rarA* gene were constructed. The fragment for cloning into pACYC177 was amplified using 02968FB and 02968RS and restricted using the BamHI and ScaI enzymes (New England BioLabs, United Kingdom). Similarly, the fragment for cloning into pACYC184 was amplified using 02968HR and 02968BF and

TABLE 1 List of strains used in experiments

Strain	Relevant genotype and phenotype[a]	Reference or source
E. coli strains		
AG100	*E. coli* wt strain	11
AG100/pACrarA-1	AG100 + pACrarA-1 (wt *rarA* cloned into pACYC177 (BamHI, ScaI), Kan^r	This work
AG100/pACYC177	AG100 + pACYC177 (Kan^r, Amp^r)	This work
AG100/pACrarA-2	AG100 + pACrarA-2 (wt *rarA* cloned into pACYC184 [BamHI, HindIII]), Cm^r	This work
AG100/pACYC184	AG100 + pACYC184 (Cm^r, Tet^r)	This work
AG100A	*acrAB* efflux pump-deleted strain	28
AG100A/pACrarA-2	AG100A + pACrarA-2 (wt *rarA* cloned into pACYC184 [BamHI, HindIII]), Cm^r	This work
AG100A/pACYC184	AG100A + pACYC184 (Cm^r, Tet^r)	This work
MG1655	*E. coli* K-12 laboratory isolate	In-house strain collection
MG1655/pACrarA-1	MG1655 + pACrarA-1 (wt *rarA* cloned into pACYC177 [BamHI, ScaI]), Kan^r	This work
MG1655/pACYC177	MG1655 + pACYC177 (Kan^r, Amp^r)	This work
MG1655 Δ*marA*	*marA*-deleted strain	S. B. Levy, L. McMurry
MG1655 Δ*marA*/pACrarA-1	MG1655 Δ*marA* + pACrarA-1 (wt *rarA* cloned into pACYC177 [BamHI, ScaI]), Kan^r	This work
MG1655 Δ*marA*/pACYC177	MG1655 Δ*marA* + pACYC177 (Kan^r, Amp^r)	This work
AG100 Δ*soxS* Δ*rob* Δ*marA*	*soxS*-, *rob*-, *marA*-deleted strain	S. B. Levy, L. McMurry
AG100 Δ*soxS* Δ*rob* Δ*marA*/pACrarA-2	AG100 Δ*soxS* Δ*rob* Δ*marA* + pACrarA-2 (wt *rarA* cloned into pACYC184 [BamHI, HindIII]), Cm^r	This work
AG100 Δ*soxS* Δ*rob* Δ*marA*/pACYC184	AG100 Δ*soxS* Δ*rob* Δ*marA* + pACYC184 (Cm^r, Tet^r)	This work
K. pneumoniae strains		
MGH 78578	ATCC 700721	19
Ecl8	*K. pneumoniae* wt strain	8
Ecl8/pACrarA-1	Ecl8 + pACrarA-1 (wt *rarA* cloned into pACYC177 [BamHI, ScaI]), Kan^r	This work
Ecl8/pACYC177	Ecl8 + pACYC177 (Kan^r, Amp^r)	This work
Ecl8Mdr1	Spontaneous MDR mutant of Ecl8	10
Ecl8 Δ*ramA*	*ramA*-deleted strain derived from Ecl8	27
Ecl8 Δ*ramA*/pACrarA-1	Ecl8 Δ*ramA* + pACrarA-1 (wt *rarA* cloned into pACYC177 [BamHI, ScaI]), Kan^r	This work
Ecl8 Δ*ramA*/pACYC177	Ecl8 Δ*ramA* + pACYC177 (Kan^r, Amp^r)	This work
Kp342	*K. pneumoniae* isolated from maize	9
Ecl8 Δ*acrAB*	*acrAB*-deleted strain derived from Ecl8, Kan^r	This work
Ecl8 Δ*acrAB*/pACrarA-2	Ecl8 Δ*acrAB* + pACrarA-2 (wt *rarA* cloned into pACYC184 [BamHI, HindIII]), Cm^r	This work
Ecl8 Δ*acrAB*/pACYC184	Ecl8 Δ*acrAB* + pACYC184 (Cm^r, Tet^r)	This work
Ecl8 Δ*rarA*	*rarA* (KPN_02968)-deleted strain derived from Ecl8, Kan^r	This work
Ecl8 Δ*rarA*/pACrarA-2	Ecl8 Δ*rarA* + pACrarA-2 (wt *rarA* cloned into pACYC184 [BamHI, HindIII]), Cm^r	This work
Ecl8 Δ*rarA*/pACYC184	Ecl8 Δ*rarA* + pACYC184 (Cm^r, Tet^r)	This work
TS152, TS165	*K. pneumoniae* clinical isolates (Turkey)	27
TS180, TS202	*K. pneumoniae* clinical isolates (Chile)	27
GC9, GC12, GC19, GC21	*K. pneumoniae* clinical isolate (Germany)	This work
GC9, GC12, GC19, GC21/pACoqxR	GC isolates + pACoqxR (wt *oqxR* cloned into pACYC177 [BamHI, ScaI]), Kan^r	This work
GC9, GC12, GC19, GC21/pACYC177	GC isolates + pACYC177 (Kan^r, Amp^r)	This work

[a] wt, wild type; Kan, kanamycin; Amp, ampicillin; Cm, chloramphenicol; Tet, tetracycline.

restricted using the BamHI and HindIII enzymes to generate pACrarA-2. Depending on the strain, one of these constructs was then electroporated or heat shock transformed into bacterial strains harboring the different genetic deletions (shown in Table 1). In order to determine the effect of *oqxR* mutations, the open reading frame (ORF) of *oqxR* (amplified using OqxRBamHI and OqxRScaIR) was cloned into pACYC177 by the use of the BamHI/ScaI restriction sites.

RNA extraction. RNA was extracted from relevant strains of *K. pneumoniae* or *E. coli* (Table 1) by the use of the TRIzol extraction method as described previously (27). Briefly, cells were grown to the mid-log phase at 37°C with shaking and then harvested by centrifugation at 3,000 × *g* at room temperature. The cell pellet was then resuspended in TRIzol reagent (Invitrogen, Paisley, United Kingdom) and chloroform and centrifuged to separate the phases. The upper phase was then precipitated using sodium acetate, glycogen, and 100% ethanol. The resulting pellet was washed and resuspended in an appropriate amount of diethyl pyrocarbonate (DEPC)-treated water.

Real-time RT-PCR. Quantitative real-time reverse transcription-PCR (RT-PCR) was performed to assess levels of gene expression for *rarA*, *ramA*, *marA*, *soxS*, and *oqxA*. 16S RNA was used as a control gene to normalize cDNA levels. cDNA was generated using am AffinityScript quantitative PCR (QPCR) cDNA synthesis kit (Agilent, United Kingdom). A Brilliant III Ultra-Fast SYBR green kit (Agilent, United Kingdom) was used for amplification, and experiments were conducted using a Stratagene Mx3005P QPCR system (Agilent Technologies). The strains used as controls when determining *rarA*, *oqxA*, *ramA*, and *acrAB* expression were wild-type and antibiotic-susceptible *K. pneumoniae* strain Ecl8 and its isogenic resistant variant *K. pneumoniae* Ecl8Mdr1 (Table 1). Expression levels were normalized against levels of 16S expression, and these data were calibrated against expression levels of the sensitive Ecl8 strain as the baseline to determine fold changes in expression. All data were analyzed using Agilent MxPro software.

Sequence studies. For sequence analysis, PCR amplification was undertaken for intergenic regions (between *rarA* and *oqxA*—primers 02698intF and 02698intR) to determine whether any promoter-associated or ORF mutations result in increased expression of *rarA*. Additionally, *oqxR* (KPN_02971), a putative regulator of the *oqxAB* efflux pump, was sequenced (primers OqxRF and OqxRR) to identify any mutations

TABLE 2 List of primers used in experiments

Primer	Sequence (5′–3′)[a]
Cloning	
02968FB	CGGGATCCCACTATCGCGGCGATTGTA
02968RS	AAAAGTACTTCATGCGGATCGCTGACG
02968HR	CCCAAGCTTTCATGCGGATCGCTGACG
02968BF	CGGGATCCTTGCACTTTATGTGCGGT
OqxRbamHIF	CGGGATCCTTTACTTCGCAGGCTAAC
OqxRsca1R	AGTACTTCATTTTCTGGTGACGAAAA
Deletion	
Ci-02968	CCGTTCCAAGCGGCCGCAAGAGCGCATCTCGTCA GCGATCCGCATG
Co-02968	AAAAAGTCGACTCAGTCCTGACCCGGCCCATG
Ni-02968	CGCTCTTGCGGCCGCTTGGAACGGTCGCGGCCG GTTAAAAGCATC
No-02968	AAAAACTGCAGGTATGGACTAATTTATCGGTGC
02968delchkF	TCAGCCAGATGGCAACCG
02968delchkR	ACCGCTTCAATGCGACCG
Ci-acrB	CGCTCTTGCGGCCGCTTGGAACGGGGCGCGCCT CTCCTGGTT
Co-acrB	AAAAACTCGAGCCACTTTTGCAAATCCGTAGAG
Ni-acrA	CGCTCTTGCGGCCGCTTGGAACGGGTGTCCAATT TCAAAATGTTC
No-acrA	AAAAACTGCAGGCGTAGCGTCGGG CAGAATTG
AcrABdelchkF	GAGCGAATGTGGCAATGTGC
AcrABdelchkR	CACGGTCATTTCACCGACG
Sequencing	
02968FB	CGGGATCCCACTATCGCGGCGATTGTA
02968RS	AAAAGTACTTCATGCGGATCGCTGACG
02698intF	GACAGATCCTGAATGAT
02698intR	TGAATGTTTCCCCAGGTT
OqxRF	GTCACCAGAAAATGATTAATGCGC
OqxRR	GCCTTTGCCCGTGAAATCAG
Q-RTPCR	
Kp02968F	TGGATCGACAACCATCTTGA
Kp02968R	AAGGACTGCTGGGAGTCAAA
Kp_16SQ_F	GTTACCCGCAGAAGAAGCAC
Kp_16SQ_R	CTACGCATTTCACCGCTACA
02969F	AAGGTGCTGGTGAAGTCGAT
02969R	GGAGACGAGGTTGGTATGGA
Kp_ramAQ_F	AGCCTGGGGCGCTATATT
Kp_ramAQ_R	GTGGTTCTCTTTGCGGTAGG
Kp_marAF	TCGAGGATAACCTGGAATCG
Kp_marAR	ACAAATGGGCTCATTGCTC
oqxRqpcrF	TAACGAAGCCTGCTCTGCTT
oqxRqpcrR	AATGGTTCCGCTAACTCGTG
5′ RACE	
GSP1	ATGTCTGCGCAACAG
GSP2	GCTCGTCGGGCAACGGTGTC
GSP3	CCAGACGGCTATCAAGATGGTTG

[a] Underlined sequences denote restriction enzyme cut sites.

that could result in the upregulation of the *oqxAB* efflux pump (KPN_02969, KPN_02970). Primers 02968FB and 02968RS were used to verify the *rarA* gene in the recombinant plasmids pACrarA-1 and pACrarA-2. Prior to sequencing, the PCR products were purified using a Genejet purification kit (Fermentas, Germany), and the sequencing reactions were performed using a BigDye Terminator 3.1 cycle sequencing kit (Applied Biosystems) prior to the automated runs being performed at the Genomics Core at Queen's University Belfast's Centre for Public Health, City Hospital. The consensus sequence (based on both the forward and reverse strands) were aligned and compared to the wild-type sequences from NCBI using MultAlin software (http://multalin.toulouse.inra.fr/multalin/multalin.html).

Mapping the TSS of *rarA*. The transcription start site (TSS) was mapped according to the manufacturer's instructions as outlined in the 5′ Rapid Amplification of cDNA Ends (RACE) kit (Invitrogen, Paisley, United Kingdom). Briefly, DNase I-digested RNA was converted to cDNA by the use of primer GSP1, prior to SNAP purification and TdT tailing (Invitrogen, Paisley, United Kingdom). The dc-tailed cDNA was amplified using the Abridged anchor primer and GSP2 (Table 2). This was followed by an additional round of amplifications using the Abridged universal anchor primer and GSP3 (Table 2), yielding a product sized at 172 bp. The purified PCR product was TA cloned into pGEMTeasy vector (Promega, Southampton, United Kingdom), and the insertion sequence was verified using bidirectional sequencing. The junction between the C tail and the start site of *rarA* open reading frame was taken to be the transcriptional start site. Predictions of the putative −10 and −35 hexamers were determined using SoftBerry analysis of the intergenic region.

RESULTS

Bioinformatic analyses. In order to mine the *K. pneumoniae* genomes for other uncharacterized AraC-type transcriptional regulators, we used the amino acid sequence for the prototype regulator MarA (KPN_01624) from *K. pneumoniae* MGH 78578. We focused on the top five hits from the BLAST analyses, in descending order of identity, which were Rob (53%), RamA (46%), KPN_02968 (called *rarA* [46%]), SoxS (42%), and another putative AraC-type regulatory protein, KPN_01709 (34%). We decided to focus on *rarA* in further work for two reasons: first, because of its higher level of identity to MarA, and second, because of its predicted size of 121 amino acids being closer to that of the subset of AraC regulators such as RamA, SoxS, and MarA rather than the considerably larger KPN_01709 (326 amino acids). As expected, the phylogenetic tree generated using MUSCLE for multiple alignments of the five AraC regulators (Fig. 1B) shows that KPN_02968 is closely related to the MarA, SoxS, and RamA proteins; however, KPN_01709 appears to be an outlier, as shown by the branch lengths denoting relative sequence similarities.

Antimicrobial susceptibility testing. Given the identity of RarA with the other AraC-type proteins such as MarA and RamA, we hypothesized that RarA might possess similar functional properties in conferring low-level multidrug resistance. In order to address this, we cloned the open reading frame encoding *rarA* with its putative promoter region into pACYC177 or pACYC184 and determined the multidrug resistance phenotype conferred by this regulator in both wild-type *E. coli* and *K. pneumoniae* strains as well as in strains harboring deletions in various loci such as *marA*, *ramA*, and the efflux operon *acrAB* (Table 1). Overexpression of *rarA* in the Δ*marA* strain (*E. coli* K-12 MG1655 Δ*marA*) led to increases in MICs as follows: a 2-fold increase in the tigecycline MIC, 4-fold increases in the ciprofloxacin, norfloxacin, and tetracycline MICs, and an 8-fold increase in the chloramphenicol MIC (Table 3). Similarly, in the AG100 Δ*soxS* Δ*rob* Δ*marA* strain, the increased expression of pACrarA-2 resulted in increases in the olaquindox and ciprofloxacin MICs (2-fold) and the tigecycline and norfloxacin MICs (8-fold) relative to the MICs seen with the vector-only control (Table 3). However, when the pACrarA-2 construct was overexpressed in AG100A, there were no differences in the susceptibility profiles in comparison to the vector-only control, implying that the multidrug-resistant phenotype is dependent on the presence of a functional *acrAB* efflux

TABLE 3 Susceptibility profiles of *E. coli* strains transformed with pACrarA and vector-only control[a]

Strain	MIC (μg/ml)					
	CHL	OQX	TET	TIG	NOR	CIP
AG100	4	16	4	< 0.500	0.125	0.031
AG100/pACrarA-1	**16**	**32**	**8**	**2**	**1**	**0.062**
AG100/pACYC177	4	16	4	< 0.500	0.125	0.031
AG100/pACrarA-2	*	**32**	*	**2**	**0.250**	**0.062**
AG100/pACYC184	*	16	*	< 0.5	0.125	0.031
AG100A	*	8	*	0.250	0.008	0.004
AG100A/pACrarA-2	*	8	*	0.250	0.008	0.004
AG100A/pACYC184	*	8	*	0.250	0.008	0.004
AG100 Δ*soxS* Δ*rob* Δ*marA*	*	16	*	0.500	0.032	0.031
AG100 Δ*soxS* Δ*rob* Δ*marA*/pACrarA-2	*	**32**	*	**4**	**0.250**	**0.062**
AG100 Δ*soxS* Δ*rob* Δ*marA*/pACYC184	*	16	*	0.500	0.031	0.031
MG1655	4	16	4	4	0.500	0.062
MG1655/pACrarA-1	**16**	**32**	**8**	**8**	**1**	**0.125**
MG1655/pACYC177	4	16	4	4	0.500	0.062
MG1655 Δ*marA*	4	8	4	4	0.250	0.031
MG1655 Δ*marA*/pACrarA-1	**32**	**16**	**16**	**8**	**1**	**0.125**
MG1655 Δ*marA*/pACYC177	4	8	4	4	0.250	0.031

[a] A pACYC177 or pACYC184 backbone was used to clone the *rarA* open reading frame. Depending on the resistance cassettes already *in situ*, the appropriate recombinant constructs would be used. Asterisks denote that MIC testing for that particular antibiotic was not done due to presence of a conflicting antibiotic resistance cassette on the plasmid and/or construct. Entries in bold denote increased MICs over those seen with wild-type/parental strains. CHL, chloramphenicol, OQX, olaquindox, TET, tetracycline, TIG, tigecycline, NOR, norfloxacin, CIP, ciprofloxacin.

pump. Regardless of the absence of *marA*, *soxS*, or *rob*, either singly or in combination, overexpression of *rarA* resulted in a low-level multidrug resistance phenotype in *E. coli* (Table 3).

Correspondingly, in *K. pneumoniae* Ecl8 Δ*ramA*, the absence of *ramA* did not affect the multidrug-resistant phenotype when *rarA* was overexpressed (Table 4). The increases in MIC ranged from 2-fold for tetracycline and norfloxacin to 4-fold for olaquindox, ciprofloxacin, and tigecycline and 8-fold for chloramphenicol. The deletion of the *rarA* open reading frame (Ecl8 Δ*rarA*) resulted in a 2- to 8-fold reduction in MIC (Table 4). As expected, the complementation of the *rarA* regulator in *trans* resulted in MIC levels higher than that observed for the parental strains. Similar to the experiments in *E. coli*, overexpression of *rarA* in *K. pneumoniae* Ecl8 Δ*acrAB* did not result in an MDR phenotype (Table 4).

TABLE 4 Susceptibility profiles of *K. pneumoniae* strains transformed with pACrarA and vector-only control[a]

Strain	MIC (μg/ml)					
	CHL	OQX	TET	TIG	NOR	CIP
Ecl8	4	16	4	2	0.250	0.031
Ecl8/pACrarA-1	**8**	**32**	**8**	**4**	**0.500**	**0.062**
Ecl8/pACYC177	4	16	4	2	0.250	0.031
Ecl8 Δ*ramA*	0.5	8	1	1	0.250	0.016
Ecl8 Δ*ramA*/pACrarA-1	**4**	**32**	**2**	**4**	**0.500**	**0.062**
Ecl8 Δ*ramA*/pACYC177	0.5	8	1	1	0.250	0.016
Ecl8 Δ*rarA*	*	8	*	0.500	0.031	0.016
Ecl8 Δ*rarA*/pACrarA-2	*	**64**	*	**4**	**0.250**	**0.062**
Ecl8 Δ*rarA*/pACYC184	*	8	*	0.500	0.031	0.016
Ecl8 Δ*acrAB*	*	2	*	0.25	0.031	0.016
Ecl8 Δ*acrAB*/pACrarA-2	*	**64**	*	0.25	0.031	0.016
Ecl8 Δ*acrAB*/pACYC184	*	2	*	0.25	0.031	0.016

[a] A pACYC177 or pACYC184 backbone was used to clone the *rarA* open reading frame. Depending on the resistance cassettes already *in situ*, the appropriate recombinant constructs would be used. Asterisks denote that MIC testing for that particular antibiotic was not done due to presence of a conflicting antibiotic resistance cassette on the plasmid and/or construct. Entries in bold denote increased MICs over those seen with wild-type/parental strains. CHL, chloramphenicol; OQX, olaquindox; TET, tetracycline; TIG, tigecycline; NOR, norfloxacin; CIP, ciprofloxacin.

Gene expression levels of *rarA* in clinical multidrug-resistant isolates of *K. pneumoniae*. In order to establish a role for *rarA* in clinical resistance, we determined by quantitative real-time RT-PCR whether *rarA* was upregulated in clinical isolates of *K. pneumoniae* obtained from various geographical locations. In our survey, we included 17 multidrug-resistant strains collected from Chile, Turkey, and Germany, where our results show that of the 17 strains tested, 7 overexpressed *rarA*-specific transcripts compared to the sensitive *K. pneumoniae* strain Ecl8 (see Fig. 3), including two Turkish isolates, TS152 (6.62-fold) and TS165 (3.77-fold), as well as one from Chile (TS202; 7.37-fold). Most of the isolates from Germany also showed overexpression of the *rarA* regulator (GC9, 9.6-fold; GC12, 7.91-fold; GC19, 8.24-fold; GC21, 8.09-fold). For those strains that overexpressed *rarA*, we were also able to demonstrate that the levels of either *marA* or *ramA* were also elevated (4- to 5-fold) among all isolates showing expression (data not shown). Furthermore, analyses of *rarA* and *oqxA* levels in the constitutive *rarA* expresser, *K. pneumoniae* strain Ecl8/pACrarA-1 (Table 1), demonstrated the increased transcription of both genes (see Fig. 3).

In all the strains where *rarA* overexpression was noted, we sought to determine the molecular basis of upregulation by initially focusing on identifying changes within the promoter and associated regions of the *rarA* regulator by (i) determining the transcriptional start site of *rarA*, (ii) mapping the changes within the intergenic region between *rarA* and the *oqxAB* operon relative to the transcription-relevant sequences (Fig. 2), and (iii) determining the sequence of the *rarA* regulator itself.

DNA sequence analyses of the intergenic region and ORF of the *rarA* regulator. (i) Intergenic region. Sequence analyses

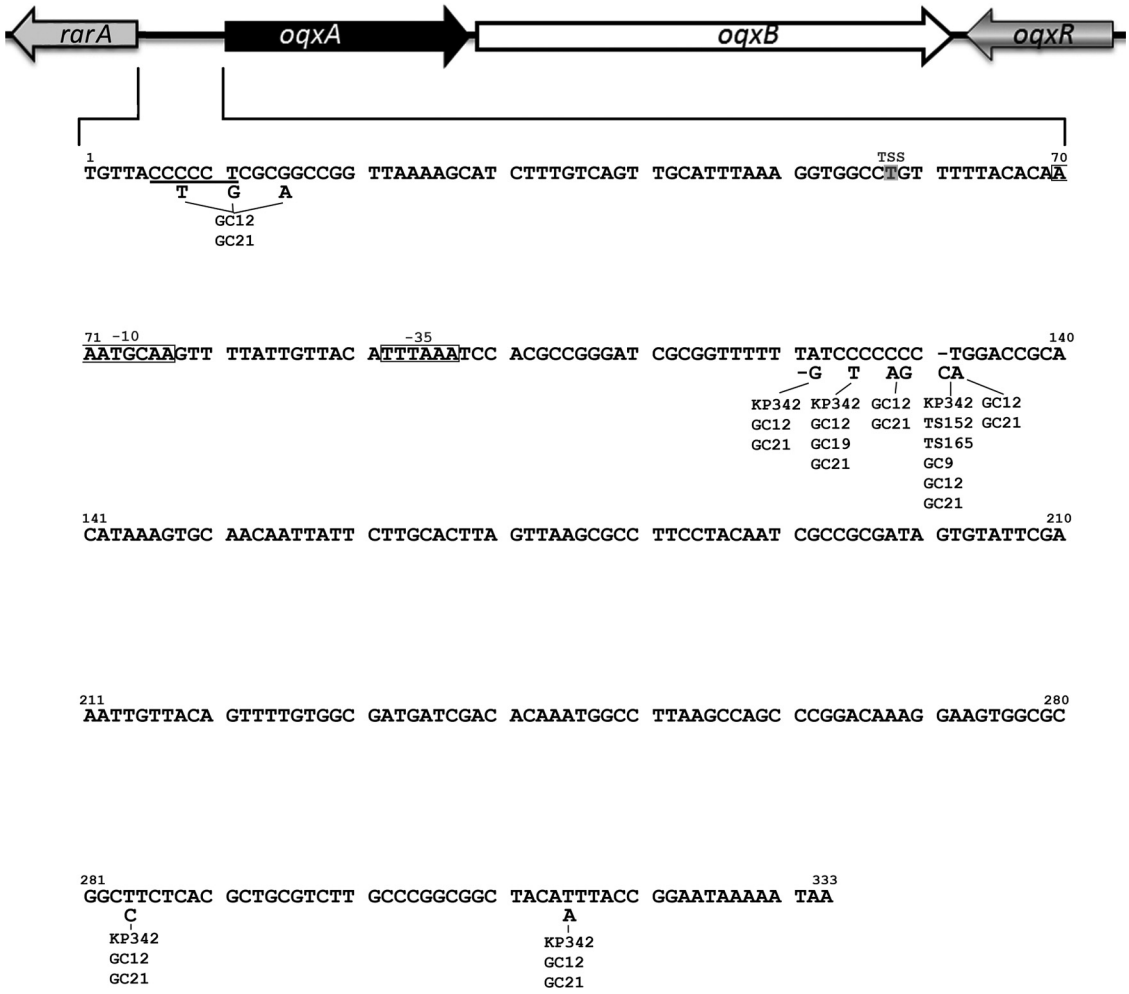

FIG 2 Nucleotide changes within the intergenic region between KPN_02968 (*rarA*) and KPN_02969 (*oqxA*). Changes observed within the intergenic region between *rarA* and *oqxA* in the different clinical strains tested are shown. The numbering scheme is based on the first nucleotide before the ATG of *rarA* as position 1. The transcription start site of *rarA*, determined by 5′ RACE analysis, is labeled TSS. The Shine-Dalgarno sequence is underlined; putative −10 and −35 promoter regions determined through Softberry analysis are shown boxed and labeled accordingly.

within the intergenic region for detection of polymorphisms highlighted changes within many of the *rarA*-overexpressing strains (Fig. 2). To clarify the possible significance of these intergenic changes, we performed 5′ RACE experiments using strain *K. pneumoniae* Ecl8Mdr1 to determine the transcription start site (TSS), which maps 58 bp upstream of the open reading frame of *rarA* (Fig. 2). Notably, none of the *rarA*-overexpressing clinical strains showed changes within regions, e.g., the *rarA* TSS, −10 or −35 hexamer, relevant to gene transcription (Fig. 2). In contrast, all of the *rarA*-overexpressing strains (TS152, TS165, Kp342, GC9, GC12, GC19, and GC21), with the exception of Ecl8MDR1 and TS202, harbored changes approximately 121 to 131 bp upstream of the *rarA* open reading frame (Fig. 2), where the most common change was a C insertion at 131 bp (Fig. 2). However, two of the *rarA* overexpressers (GC12 and GC21) showed identical changes (C8→T and T11→G) within the putative Shine-Dalgarno sequence (Fig. 2). Interestingly, no changes were found in multidrug-resistant strains Ecl8Mdr1 and TS202, but those strains still overexpressed *rarA*. Our findings suggest that the molecular basis for *rarA* upregulation may not be linked to the changes identified within the intergenic region.

(ii) *rarA* regulator. Only 4 (GC19, Ecl8, Ecl8Mdr1, and Kp342) of the 10 strains sequenced (9 *rarA* overexpressers and the sensitive Ecl8 strain) harbored changes within the *rarA* regulator. Identical mutations in *rarA* leading to a Glu96→Arg substitution were found in both nonexpresser *K. pneumoniae* Ecl8 and *rarA* overexpresser *K. pneumoniae* Ecl8Mdr1. This change is located within the α-helix in the predicted HTH binding site (http://bioinf.cs.ucl.ac.uk/psipred/); however, its presence in both strains implies that it is not a crucial residue. Several substitutions not present in other strains were identified in *K. pneumoniae* Kp342: Ala31→Ser, Lys57→Gln, Ile63→Val, Val111→Ala, Ala112→Glu, and Thr114→Ala. This strain also harbors a mutation at position Arg117→STOP which leads to a premature stop codon (TGA). Of all the other clinical isolates that overexpress *rarA*, only GC9 showed a unique change, Gln99→Lys, within the sequence between helices 6 and 7, proximal to the C-terminal end of the protein.

The *oqxAB* (KPN_02969 and KPN_02970) efflux operon lies downstream from the *rarA* regulator where the *oqxAB* pump has been associated with reduced susceptibility to olaquindox, ciprofloxacin, and chloramphenicol (15). Interestingly, all clinical

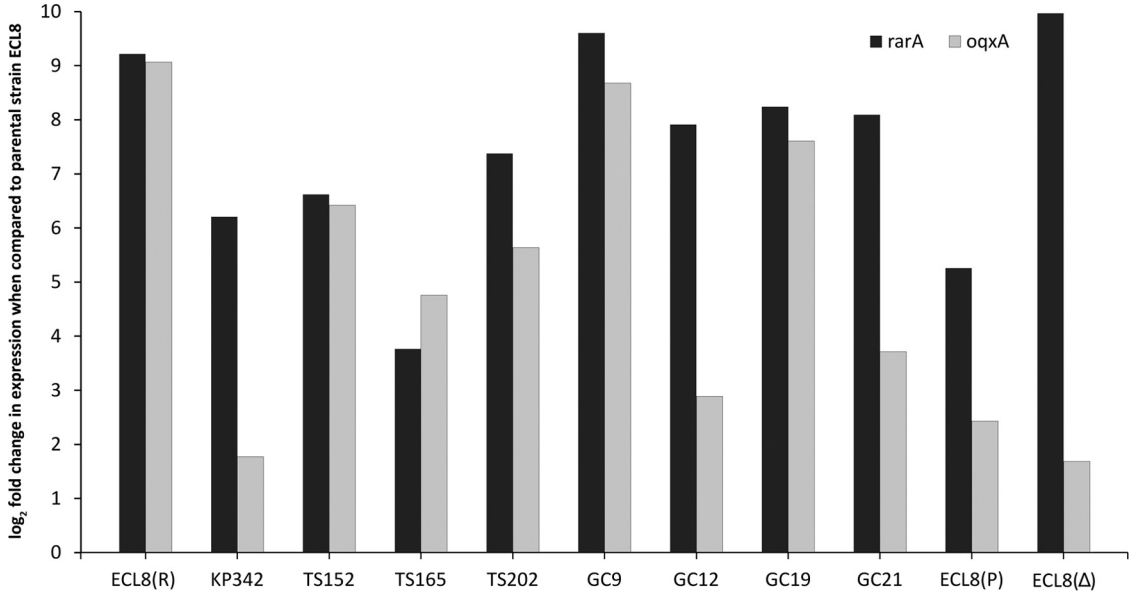

FIG 3 Fold change in expression levels of *rarA* and *oqxA* among clinical isolates compared to *K. pneumoniae* Ecl8. All QPCR experiments were performed as outlined in Materials and Methods. For strains Ecl8Mdr1 [represented by EcL8(R)], Kp342, TS152, TS165, TS202, GC9, GC12, GC19, and GC21, fold change values were generated after normalizing to 16S levels for each strain and then using *oqxA* and *rarA* levels derived from the sensitive *K. pneumoniae* Ecl8 strain. To assess whether *rarA* could activate the expression of the efflux pump *oqxAB*, transcription of *oqxA* was assessed in Ecl8/pACrarA-1 [EcL8(P)] and Ecl8 Δ*rarA*/pACrarA-2 [EcL8(Δ)]. In this case, the fold change was normalized as mentioned before but calibrated against either Ecl8/pACYC177 or Ecl8Δ*rarA*/pACYC184.

strains that showed upregulation of *rarA* also demonstrated increased expression of KPN_02969 (*oqxA*) (Fig. 3). In order to dissect the molecular basis for this upregulation, we determined the following: (i) whether there was an association between *rarA* and *oqxAB* upregulation and (ii) whether the GntR-type regulator (OqxR_KPN_02971) encoded downstream of the *oqxAB* operon would function as a repressor.

Our results show that plasmid-mediated overexpression of *rarA* resulted in increased levels of *oqxA* in *K. pneumoniae* Ecl8 and Ecl8Δ*rarA* (Fig. 3). Additionally, a reproducible (1.5-fold, mean of 4 experiments) increase in *acrAB* levels was also observed in the same strains. Therefore, we surmise that *rarA* may function as a positive regulator of *oqxAB* and *acrAB* levels.

Putative regulator OqxR. (i) Sequencing results. We first sequenced all the *rarA*- and *oqxAB*-overexpressing strains and found that not all strains (e.g., TS202) that overexpressed *oqxA* (KPN_02969) harbored changes within the *oqxR* gene (Table 5). However, there were several recurring changes, namely, Gln11→Leu (GC12 and GC21), Asp95→Glu (GC12 and GC21), Val113→Ile (GC12, GC21, and and KP342) and Val130→Ala (TS152 and TS165). Based on alignments with other GntR family regulators, Gln11→Leu is located within the DNA-binding domain of the winged helix-turn-helix (WHTH) of OqxR. The other mutations were located within the predicted C-terminal effector binding and oligomerization domain (26).

(ii) *oqxR* overexpression effects on 02969 (*oqxA*) levels. In order to confirm that the mutations noted within OqxR would be directly associated with increased expression of the *oqxAB* efflux

TABLE 5 Sequence analysis of OqxR and *rarA*/*oqxA* expression levels of clinical *K. pneumoniae* isolates[a]

Strain	OqxR change(s)	Expression level of *rarA* calibrated against Ecl8	Con/Comp $\log_2$ expression level(s)[b]	
			rarA	*oqxA*
152	**Val130→Ala**	6.62	—	6.42/—
165	Arg25→His, **Val130→Ala**	3.77	—	4.76/—
202	Ala133→Thr	7.37	—	5.64/—
GC9	Phe6→Ser	9.60	17.97/17.53	8.68/−1.4
GC12	**Gln11→Leu**, **Asp95→Glu, V113→Ile**	7.91	21.77/23.6	2.89/0.64
GC19	Frameshift Δ, aa 73-77	8.24	21.06/20.84	7.6/0.74
GC21	**Gln11→Leu**, **Asp95→Glu**, **Val 113→Ile**	8.09	23.34/21.75	3.72/0.01
Kp342	Asn38→Thr, **Asp95→Glu, Val113→Ile**, His159→Leu	6.21	21.76/22.03	1.77/−0.735
Ecl8Mdr1	Frameshift Δ, aa 88-94	10.46	17.88/17.44	9.06/0.28

[a] Strains whose names are underlined were complemented with pACoqxR. Boldface font denotes amino acid changes present in several strains. Expression levels of *rarA* calibrated against sensitive *K. pneumoniae* strain Ecl8 are shown.

[b] Con, control data representing either the wild-type or vector-only strains (pACYC177); Comp, complementation data representing increases or decreases of *rarA* or *oqxA* expression levels in the different strains after complementation with pACOqxR. Negative values indicate reduction of *oqxA* levels below the levels noted in the vector-only calibrators. —, strains not complemented due to high levels of innate kanamycin resistance. aa, amino acids.

pump, we performed complementation assays with wild-type *oqxR* on strains (Ecl8Mdr1, GC9, GC12, GC19, GC21, and Kp342) that overexpressed *oqxAB*. QPCR analyses showed that *oqxA* levels were lower in all of the complemented strains than in the vector-only control strains, while *rarA* levels remained unaffected (Table 5). Only two (Kp342 and Ecl8Mdr1) of the six strains were found to show reductions in olaquindox MICs (Table 6). The lack of reduction in olaquinodox MICs noted for the clinical strains (GC9, GC12, GC19, and GC21) may have been due to other mechanisms (Table 6). In all strains (Ecl8Mdr1, GC9, GC12, GC19, GC21, and Kp342) where we expressed the recombinant *oqxR*, we also ascertained that the levels of the AcrA protein (Western blot analyses for the AcrA protein; data not shown) remained identical to those of the vector-only controls. From our results, we surmise that a decrease in *oqxAB* transcription does result in the reduction of olaquindox MICs for some strains.

TABLE 6 Susceptibility profiles of *K. pneumoniae* strains after complementation with pACoqxR[a]

Strain	MICs (mg/liter) for strain transformed with pACoqxR/paCYC177					
	CHL	OQX	TET	TIG	NOR	CIP
GC9	64/64	768/768	16/16	4/4	8/8	8/8
GC12	24/24	768/768	16/16	8/8	8/8	12/12
GC19	256/256	768/768	32/32	4/4	4/4	1/1
GC21	24/24	768/768	16/16	8/8	8/8	12/12
Kp342	**48/64**	**384/512**	32/32	8/8	4/8	**0.375/0.500**
Ecl8Mdr1	32/32	**384/512**	4/4	1/1	8/8	0.500/0.500

[a] MICs show values for strains complemented with pACoqxR/strains complemented with pACYC177. Numbers in bold indicate MIC reductions seen with the complemented strain versus the vector-only control. CHL, chloramphenicol, OQX, olaquindox, TET, tetracycline, TIG, tigecycline, NOR, norfloxacin, CIP, ciprofloxacin.

DISCUSSION

In this work, we have characterized a novel AraC regulator, KPN_02968 (*rarA*), in *K. pneumoniae*. We demonstrate that plasmid-mediated overexpression of *rarA* produces a multidrug-resistant phenotype in either *E. coli* or *K. pneumoniae* independently of the presence of *marA*, *soxS*, or *rob* (29) but requires the presence of a functional efflux pump, *acrAB*, to exhibit the multidrug-resistant phenotype (Table 3 and Table 4). In clinical isolates where we find upregulation of the *rarA* gene, there appears to be a concurrent increase in the levels of transcripts of *oqxAB*, which encodes an efflux pump that has been previously implicated in mediating resistance to ciprofloxacin and olaquindox (15, 17).

In *K. pneumoniae* MGH 78578, *oqxAB* is flanked by genes encoding two regulators, RarA (KPN_02968) and OqxR (KPN_02971). We first tried to ascertain whether plasmid-mediated overexpression of *rarA* in a genetically manipulated *K. pneumoniae* strain would result in the upregulation of the *oqxAB* operon. Our results show that the plasmid-mediated overexpression of *rarA* does result in the increased expression of *oqxAB* (Fig. 3). In contrast, the introduction of wild-type *oqxR* in *trans* repressed the *oqxAB* transcripts (Table 5), which did result in reductions to olaquindox in two strains (Ecl8Mdr1 and Kp342) (Table 6). We surmised that the lack of the reduction in the MICs may be due to AcrA levels which mask the decrease in the MIC or that other alternate mechanisms for olaquindox resistance exist in these strains which may compensate for the reduction of the *oqxAB* levels. Western blot analyses showed that the levels of AcrA protein remained unaltered (data not shown). Taken together, the results suggest that *oqxAB* may be subject to regulation by both RarA (activator) and OqxR (repressor).

The sequencing of the *oqxR* gene in all the strains where *rarA* and *oqxAB* levels were elevated revealed several recurring changes, namely, Gln11→Leu (located in strains GC12 and GC21), Asn95→Glu and Val113→Ile (located in strains GC12, GC21, and Kp342), and Val130→Ala (located in strains TS152 and TS165). On the basis of our complementation studies, we surmise that the changes associated with producing a mutated OqxR protein are Phe 6→Ser, Gln11→Leu, Asp95→Glu, Val113→Ile, and the frameshift deletion spanning positions 73 to 77 or positions 88 to 94. Further experiments are under way to determine whether all other changes reported here are also detrimental to OqxR function.

Seven out of 17 of the clinical multidrug-resistant strains that we tested overexpressed *rarA* as well as either *ramA* or *marA*. While it is clear that decreased susceptibility occurred with *rarA* overexpression in the absence of *marA*, *soxS*, and *rob*, the coexpression of the other AraC regulators in multidrug-resistant clinical strains is not uncommon (6, 23). Despite the small number of isolates reported here that exhibited *rarA* overexpression, we find that *rarA* upregulation is not confined only to clinical isolates from geographically diverse locations but is also noted in the plant endophyte Kp342. Genome analyses of this plant endophyte did indicate that it was multidrug resistant, but the molecular basis of this resistance was not evident from sequence analyses of the commonly encountered mutations (9). Our finding here suggests that the reason for the decreased susceptibility noted in Kp342 may be linked to the upregulation of *rarA*. However, to exactly pinpoint the relative contributions of the transcriptional regulators *rarA*, *ramA*, and *marA* and the efflux pumps *acrAB* and *oqxAB*, multidrug-resistant clinical isolates should be subjected to specific genetic manipulations and complementation experiments. In conclusion, our data show that *K. pneumoniae* encodes another multidrug resistance regulator, RarA, which functions as an activator of the *oqxAB* efflux pump, which is itself negatively regulated by OqxR.

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