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Veleba, M., & Schneiders, T. (2012). Tigecycline Resistance Can Occur Independently of the *ramA* Gene in *Klebsiella pneumoniae*. *Antimicrobial Agents and Chemotherapy*, *56*(8), 4466-4467. https://doi.org/10.1128/AAC.06224-11

Published in:
Antimicrobial Agents and Chemotherapy

Document Version:
Publisher's PDF, also known as Version of record

AAC Journals.ASM.org

Tigecycline Resistance Can Occur Independently of the *ramA* Gene in *Klebsiella pneumoniae*

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Tigecycline resistance in *Klebsiella pneumoniae* results from *ramA* upregulation that causes the overexpression of the efflux pump, AcrAB-TolC. Tigecycline mutants, derived from Ecl8Δ*ramA*, can exhibit a multidrug resistance phenotype due to increased transcription of the *marA*, *rarA*, *acrAB*, and *oqxAB* genes. These findings support the idea that tigecycline or multidrug resistance in *K. pneumoniae*, first, is not solely dependent on the *ramA* gene, and second, can arise via alternative regulatory pathways in *K. pneumoniae*.

Tigecycline resistance in *Klebsiella pneumoniae* is on the increase, with reported cases in Greece (9), India (3), and Saudi Arabia (1), and its effectiveness as a therapeutic agent is uncertain, with patients treated with tigecycline showing persistent bacteremia caused by *Escherichia coli*, *Acinetobacter baumanii*, and *K. pneumoniae* (2).

The AcrAB-TolC pump complex is a clinically relevant efflux system activated by several AraC-type transcriptional regulators such as MarA, SoxS, and RamA (7). Genetic studies have shown that resistance to tigecycline is mediated by the increased expression of the *ramA* gene, which subsequently results in the upregulation of the efflux pump *acrAB* in *K. pneumoniae* (10, 11) and *Enterobacter cloacae* (6). It has been shown that the increased expression of the *ramA* gene is linked to mutations within the cognate repressor, *ramR*, that is divergently transcribed from the *romA-ramA* genes (10). However, in a recent study (10), it was shown that high-level tigecycline resistance is exhibited in clinical strains of *K. pneumoniae* that do not overexpress *ramA* or *acrAB*; hence, we hypothesized that alternative pathways to tigecycline resistance must exist in *K. pneumoniae*. Accordingly, we searched the *Klebsiella* genome for AraC-type transcriptional regulators that had a size similar to those of the other multidrug resistance regulators such as *marA*, *soxS*, and *ramA*. The bioinformatic analyses of the genome of *Klebsiella* MGH78578 (NC_09648) located an additional regulator, which we have termed *rarA* (Fig. 1). In an accompanying paper, we report the characterization of this novel multidrug-resistant (MDR) regulator (13), which, when overexpressed, exhibits a MDR phenotype independently of *marA-soxS-rob* and *ramA*. In *K. pneumoniae* and *Enterobacter* spp., the chromosomally encoded *rarA* regulator lies downstream of the efflux pump, *oqxAB* (Fig. 1), which has been previously linked to decreased susceptibility to olaquindox, ciprofloxacin, and chloramphenicol (5). Interestingly, in *E. coli*, the gene locus encoding the efflux pump has been shown to be located on the pOLA52 plasmid in strains isolated from pig manure (12), where the locus is flanked by two IS*26* insertion sequences, highly suggestive of its genetic mobility.

Our research issue was whether alternative pathways to tigecycline resistance exist in *K. pneumoniae*. Accordingly, we used a genetically modified *Klebsiella pneumoniae* Ecl8Δ*ramA* strain, with a markerless deletion of the *ramA* gene, in a tigecycline selection experiment. The markerless deletion of the *ramA* gene was constructed as described previously (8). Briefly, Ecl8Δ*ramA* from an overnight culture was grown overnight at 37°C on agar plates containing tigecycline concentrations of 0.5 μg/ml, 1 μg/ml, 2 μg/ml, 4 μg/ml, and 8 μg/ml. The overnight culture was also diluted in phosphate-buffered saline (PBS) and grown on LB plates without antibiotic to establish baseline growth levels. After overnight incubation, we picked three mutants (TGC1-3) from the plate with 4 μg/ml for further analyses. Accordingly, we found that the mutational frequency of Ecl8Δ*ramA* with respect to tigecycline was 0.466×10^{-6}. MIC testing (performed in triplicate) of tigecycline, ciprofloxacin, norfloxacin, tetracycline, and olaquindox was undertaken as described in the British Society for Antimicrobial Chemotherapy (BSAC) guidelines (4). Additionally, quantitative real-time PCR analyses were carried out to assess expression levels of genes *rarA*, *oqxB*, *acrA*, *marA*, and *soxS* in the 3 mutant strains by the use of cDNA (generated by AffinityScript [Agilent]) and a Brilliant III kit (Agilent) for amplification. Experiments were conducted using a Stratagene Mx3005P system (Agi-

Received 22 November 2011 **Returned for modification** 2 January 2012
Accepted 6 March 2012
Published ahead of print 29 May 2012
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doi:10.1128/AAC.06224-11
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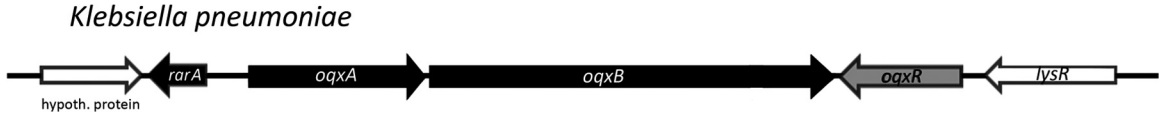

FIG 1 Organization of *rarA* locus in *K. pneumoniae*. This genomic organization is conserved in *Enterobacter* sp. 638, *Serratia proteamaculans* 568, and *Enterobacter cloacae*.

TABLE 1 MIC and QPCR measurements of tigecycline mutants (TGC1, TGC3, and TGC5)

Strain	MIC[a]					Fold expression increase vs Ecl8Δ*ramA*			
	NOR	CIP	TET	OQX	TIG	*rarA*	*oqxA*	*marA*	*acrB*
Ecl8	0.250	0.031	4	16	2				
Ecl8Δ*ramA*	0.063	0.016	2	8	1				
TGC1	8	0.25	16	32	16	4.59	6.06	6.06	55.72
TGC3	8	0.25	8	32	8	17.15	13.93	12.13	103.97
TGC5	8	0.25	8	16	8	2.14	3.03	5.28	27.86

[a] NOR, norfloxacin; CIP, ciprofloxacin; TET, tetracycline; OQX, olaquindox; TIG, tigecyline.

lent) and analyzed using MxPro software (Agilent). MIC susceptibility testing demonstrated that all 3 clones exhibited reduced susceptibility to the antibiotics tested in comparison to the parental strain, Ecl8Δ*ramA* (see Table 1). We also found that the transcriptional levels of *rarA* (4.59-fold, 17.15-fold, and 2.14-fold, respectively) and *marA* (6.06-fold, 12.13-fold, and 5.28-fold) as well as those of the efflux operons *oqxAB* (6.06-fold, 13.93-fold, and 3.03-fold) and *acrAB* (55.72-fold, 103.97-fold, and 27.86-fold) were higher than the expression levels seen with the parental strain (Ecl8Δ*ramA*) (see Table 1). Of note, *soxS* levels were found to be unaltered in comparison to those seen with the parental strain (Ecl8Δ*ramA*).

Our work shows that tigecycline exposure to Ecl8ΔramA can generate mutants that exhibit low-level multidrug resistance. However, in order to pinpoint the individual contributions of *rarA*, *marA*, and *oqxAB*, individual gene deletions are essential. We surmise that both *rarA* and *marA* provide alternative pathways for the emergence of multidrug resistance in *K. pneumoniae* in the absence of the *ramA* gene. Additionally, we demonstrate that both the *acrAB* and the newly described *oqxAB* efflux pump can contribute to the MDR phenotype. From our findings, it is evident that a functioning *ramA* gene is not always needed to confer tigecycline resistance in *K. pneumoniae.*

ACKNOWLEDGMENTS

This work was funded by MRC grant G0601199 and studentship support for M.V. by the Department for Employment and Learning (Northern Ireland).

We thank S. McAteer and D. Gally for the construction of Ecl8Δ*ramA*.

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