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ramR Mutations in Clinical Isolates of *Klebsiella pneumoniae* with Reduced Susceptibility to Tigecycline▽

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Five *Klebsiella pneumoniae* isolates with reduced susceptibility to tigecycline (MIC, 2 μg/ml) were analyzed. A gene homologous to *ramR* of *Salmonella enterica* was identified in *Klebsiella pneumoniae*. Sequencing of *ramR* in the nonsusceptible *Klebsiella* strains revealed deletions, insertions, and point mutations. Transformation of mutants with wild-type *ramR* genes, but not with mutant *ramR* genes, restored susceptibility to tigecycline and repressed overexpression of *ramA* and *acrB*. Thus, this study reveals a molecular mechanism for tigecycline resistance in *Klebsiella pneumoniae*.

Klebsiella pneumoniae is an important pathogen of nosocomial infections, including urinary tract infections, pneumonia, wound infections, and sepsis (22). *Klebsiella pneumoniae* rapidly acquires resistance to most commonly used beta-lactam antibiotics by different mechanisms, including expression of extended-spectrum beta-lactamases (ESBLs), plasmid-mediated AmpC beta-lactamases, and recently also carbapenemases (29). Isolates are also frequently nonsusceptible to fluoroquinolones and aminoglycosides, leaving only few if any treatment options, and even infections with untreatable, panresistant strains have been reported (6). Infections with such multidrug-resistant (MDR) pathogens represent an important field of application for treatment with the recently introduced antibiotic tigecycline (8, 14, 20, 30), the first member of the novel class of glycylcyclines (19). It has an extraordinarily broad spectrum of antibacterial activity, covering most Gram-positive, Gram-negative, and anaerobic pathogens, including vancomycin-resistant enterococci (VRE), methicillin-resistant *Staphylococcus aureus* (MRSA), and ESBL- and carbapenemase-producing strains (8). Unfortunately, the emergence of resistance to tigecycline in *Klebsiella pneumoniae* isolates has already been reported (25, 28).

Overexpression of RamA, which is a positive regulator of the AcrAB efflux system, has been observed in tigecycline-resistant *Klebsiella pneumoniae* strains (2, 25, 28) and also in tigecycline-resistant *Enterobacter cloacae* isolates (11). Furthermore, AcrAB and related efflux pumps which confer resistance to multiple antibiotics, including tetracyclines, fluoroquinolones, chloramphenicol, and others (21, 23), have been implicated in resistance to tigecycline in several other species (4, 5, 9–12, 16, 26, 27, 32). The overexpression of *ramA* seemed to be causative for overexpression of AcrAB in *Klebsiella pneumoniae* and *Enterobacter cloacae*, but the molecular basis of *ramA* upregulation could not be defined in these species. We were recently able to show that upregulation of *ramA* and consecutively AcrAB in a tigecycline-resistant *Salmonella enterica* isolate was due to an inactivating mutation in *ramR*, a repressor of *ramA* (1, 13, 17, 24) in *Salmonella* (9). How *ramA* is regulated in bacteria other than *Salmonella* is currently unknown.

We collected five independent *Klebsiella pneumoniae* isolates from our diagnostic service, and they exhibited suspiciously small disk diffusion zone diameters (<19 mm), and further analyzed these strains. For tigecycline, MICs were determined by broth microdilution with a commercially available tigecycline panel (Merlin Diagnostika GmbH, Bornheim-Hersel, Germany) using freshly prepared (<12 h old) Mueller-Hinton II broth (BBL, BD Bioscience, Sparks, MD). For ciprofloxacin and chloramphenicol, MICs were determined by Etest (AB Biodisk, Solna, Sweden). MICs were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints (for tigecycline, ≤1.0 μg/ml is susceptible, 2.0 μg/ml is intermediate, and >2.0 μg/ml is resistant; for ciprofloxacin, ≤0.5 μg/ml is susceptible, 1.0 μg/ml is intermediate, and >1.0 μg/ml is resistant; for chloramphenicol, ≤8.0 μg/ml is susceptible and >8.0 μg/ml is resistant). All five isolates exhibited MICs of 2 μg/ml, which was interpreted as intermediate. Testing of 12 randomly collected *Klebsiella pneumoniae* patient isolates with disk diffusion zone diameters of >19 mm uniformly revealed MICs of 0.25 μg/ml. Resistance to tigecycline in *Klebsiella pneumoniae* has previously been linked to overexpression of *ramA* (28). Because we recently found in a tigecycline-resistant *Salmonella* isolate that *ramA* overexpression was due to a mutation in *ramR*, a known negative regulator of *ramA* (9), we asked whether a similar mechanism is instrumental in *Klebsiella*. A BLAST search identified a predicted *Klebsiella pneumoniae* protein (accession number YP_001334235) with 63% identity to *Salmonella* RamR (NP_459572.1). Strikingly, the gene for this protein is located directly upstream of the *Klebsiella pneumoniae ramA* gene (YP_001334236.1) in a head-to-head arrangement (Fig. 1), a genomic organization reminiscent of the respective situation in *Salmonella*. The intergenic region between *ramR* and *ramA* additionally harbors a predicted gene, *romA*, with homology to beta-lactamase genes. It was previ-

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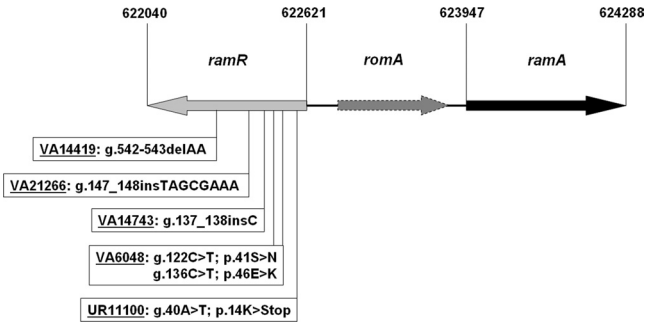

FIG. 1. Schematic diagram of the genomic region comprising *ramR* and *ramA* of *Klebsiella pneumoniae* subsp. *pneumoniae* MGH 78578 (CP000647). The mutations identified in the *ramR* genes of the nonsusceptible *Klebsiella* strains are indicated. g., gene (nucleotide position); p., protein (amino acid position); del, deletion; ins, insertion.

ously shown not to be involved in the *ramA*-mediated MDR phenotype (7), but if expressed, it may be coregulated by RamR due to its genomic localization, albeit with unknown significance. Interestingly, a putative palindromic binding element for RamR mutated in some fluoroquinolone-resistant *Salmonella* isolates (1, 13) is highly conserved in *Klebsiella pneumoniae* and located in the intergenic region between *ramR* and *ramA* (nucleotides 622742 to 622762). These similarities strongly suggest that the identified gene represents the *Klebsiella pneumoniae* homologue of *Salmonella ramR*.

We amplified the *ramR* gene and the surrounding genomic region from the tigecycline-resistant strains and the 12 randomly collected strains with MICs of 0.25 μg/ml and performed sequence analysis (forward [5′-CTGCAG-TGCCCGGTGAACCC TGGCGT] and reverse [5′-CTGCAG-ATTTGCTGATTCAGC AGCGAC] primers). In all five non-tigecycline-susceptible strains, mutations in *ramR* relative to the reference sequence *Klebsiella pneumoniae* subsp. *pneumoniae* MGH 78578 (CP000647), as depicted in Fig. 1, were detected. Four strains (UR11100, VA14419, VA14743, and VA21266) harbored deletions, insertions, or point mutations leading to a premature stop codon, which result in predicted truncated RamR proteins highly likely to be nonfunctional. VA6048 harbored two mutations leading to amino acid exchanges in the coding region of *ramR*. None of these mutations were found in the 12 tigecycline-susceptible strains. Instead, two different silent polymorphisms (594G→T and 150G→A [gene]) were detected and two strains harbored polymorphisms in the *ramR* gene, which resulted in amino acid exchanges (VA21490 harbored two exchanges, 437A→G [gene]/146I→T [protein] and 454A→T [gene]/152Y→N [protein], and VA21488 harbored one exchange identical to the first in VA24190, 437A→G [gene]/146I→T [protein]). We cloned the *ramR* genes of all the mutants, of a *Klebsiella pneumoniae* strain with a wild-type (WT) MIC to tigecycline, a wild-type sequence of *ramR* (from VA12262), and *ramR* sequences of the two strains (VA21488 and VA21490) harboring the coding polymorphisms together with the surrounding genomic regions into the PstI site of the pACYC177 vector using PCR products with the forward and reverse primers (see above). All constructs were verified by sequencing. Two of the mutant strains, VA6048 and VA14743, were amenable for transformation. Transformation of VA6048 and of VA14743 with wild-type $ramR_{VA12262}$ [*Klebsiella pneumoniae* VA6048 ($ramR_{VA12262\text{-}WT}$) and *Klebsiella pneumoniae* VA14743 ($ramR_{VA12262\text{-}WT}$), respectively] lowered the MIC for tigecycline in both strains from 2 μg/ml to 0.25 μg/ml, as shown in Table 1. Both $ramR_{VA21488}$ and $ramR_{VA21490}$ lowered the MICs for tigecycline of VA6048 and VA14743 to the same extent as $ramR_{VA12262\text{-}WT}$ (data not shown), indicating that the amino acid exchanges represent nonfunctional polymorphisms. In contrast, introduction of any of the mutated *ramR* genes

TABLE 1. MICs and relative expressions of *ramA* and *acrB* of strains used in this study

Klebsiella pneumoniae isolate	MIC (μg/ml) and status[a]			Relative expression[d]		Origin[e]
	Tigecycline[b]	Ciprofloxacin[c]	Chloramphenicol[c]	*ramA*	*acrB*	
VA6048	2 I	0.125 S	24.0 R	28.9 ± 8.1	11.6 ± 1.3	Gallbladder
UR11100	2 I	4.0 R	32.0 R	25.1 ± 5.9	10.9 ± 0.4	Urine
VA14419	2 I	>32.0 R	>256.0 R	40.5 ± 11.4	14.9 ± 1.6	Wound
VA14743	2 I	4.0 R	24.0 R	48.9 ± 10.5	7.8 ± 1.0	Trachea
VA21266	2 I	0.25 S	32.0 R	38.9 ± 10.3	15.8 ± 2.8	Pharynx
VA12262-WT	0.25 S	0.064 S	8.0 I	1	1	Trachea
VA6048 ($ramR_{VA12262\text{-}WT}$)	0.25 S	0.012 S	1.0 S	0.9 ± 0.6	1.5 ± 0.2	
VA6048 ($ramR_{VA6048}$)	2 I	0.125 S	16.0 R	32.4 ± 7.2	10.9 ± 1.8	
VA6048(pACYC177)	2 I	0.125 S	24.0 R	46.5 ± 16.4	18.9 ± 6.6	
VA6048 ($ramR_{UR11100}$)	2 I	0.125 S	24.0 R	25.0 ± 11.6	5.8 ± 0.9	
VA6048 ($ramR_{VA14419}$)	2 I	0.125 S	16.0 R	21.2 ± 4.2	8.4 ± 3.0	
VA6048 ($ramR_{VA21266}$)	2 I	0.125 S	16.0 R	29.9 ± 10.7	14.5 ± 3.3	
VA14743 ($ramR_{VA12262\text{-}WT}$)	0.25 S	0.25 S	1.0 S	0.8 ± 0.1	1.5 ± 0.1	
VA14743 ($ramR_{VA14743}$)	2 I	4.0 R	32.0 R	50.4 ± 8.9	17.8 ± 9.3	
VA14743(pACYC177)	2 I	4.0 R	32.0 R	45.6 ± 5.4	5.1 ± 0.5	
VA14743 ($ramR_{UR11100}$)	2 I	4.0 R	16.0 R	43.8 ± 7.0	11.0 ± 2.3	
VA14743 ($ramR_{VA14419}$)	2 I	2.0 R	16.0 R	57.6 ± 15.3	22.0 ± 10.2	
VA14743 ($ramR_{VA21266}$)	2 I	4.0 R	32.0 R	66.7 ± 30.1	10.1 ± 4.7	

[a] S, susceptible; I, intermediate; R, resistant. Status determinations are according to EUCAST clinical breakpoints (www.eucast.org/).
[b] Tested by broth microdilution.
[c] Tested by Etest.
[d] Measured by quantitative RT-PCR, shown as *x*-fold expression of VA12262 (expression = 1). Results are means of 3 (*ramA*) or 2 (*acrB*) runs ± standard deviations.
[e] All isolates were obtained as a result of this study.

($ramR_{VA6048}$, $ramR_{UR11100}$, $ramR_{VA14419}$, $ramR_{VA14743}$, and $ramR_{VA21266}$) or the empty pACYC177 vector did not affect the MIC for tigecycline in VA6048 or VA14743. These findings suggest that the identified *ramR* homologue is involved in resistance to tigecycline and that the identified mutations in the nonsusceptible strains are functionally relevant. The AcrAB system is involved in resistance to antibiotics from multiple classes. Thus, we also tested MICs for ciprofloxacin and chloramphenicol in our strains, as both are known to be substrates of AcrAB. Consistent with the findings for tigecycline, MICs for ciprofloxacin and for chloramphenicol of VA6048 and VA14743 were lowered when wild-type *ramR* was introduced but remained unchanged when mutated *ramR* or empty pACYC177 vector was transformed (Table 1). These results strongly imply that *ramR* in *Klebsiella pneumoniae* is involved in the regulation of AcrAB in a manner similar to that in *Salmonella*.

Next, we directly analyzed the influence of *Klebsiella pneumoniae ramR* on the transcriptional expression level of *ramA* by Northern blot hybridization (hybridization probes for *ramA* were generated with primers 5′-ATGACGATTTCCGCTCAG GTGA and 5′-CAGTGGGCGCGACTGTGGTTC, and those for 16S rRNA [*rrsE*] were generated with primers 5′-TTGAC GTTACCCGCAGAAGAA and 5′-TCTACAAGACTCTAG CCTGCCA; these were labeled with [α-^{32}P]dCTP [Hartmann-Analytic, Braunschweig, Germany] by using the Megaprime DNA labeling system from GE Healthcare). We also used SYBR green quantitative reverse transcription-PCR (qRT-PCR) using the qPCR Core SYBR green I kit from Eurogentec, Seraing, Belgium (primers used for qRT-PCR are the same as for the generation of the Northern blot hybridization probes except ramA-rev-qPCR [5′-CAGCCGTTGCAGATG CCATTTC]). RNA was isolated with the RNeasy kit (Qiagen, Hilden, Germany). First, expression levels of *ramA* in the 12 randomly selected *Klebsiella pneumoniae* strains with MICs of 0.25 μg/ml were compared to those in the five nonsusceptible strains by Northern blot hybridization. Three micrograms of the isolated total RNA was separated by electrophoresis in a gel containing 1% agarose and 1.2% formaldehyde and was subsequently transferred to a nylon membrane (Macherey-Nagel, Dueren, Germany) by neutral capillary elution in 20× SSC (3 M NaCl, 0.3 M trisodium-citrate dehydrate). Hybridization was carried out at 65°C in 10 ml hybmix (7% SDS, 10% PEG 20000, 0.22 M NaCl, 1.5 mM EDTA, 15 mM sodium phosphate, 5 μg/ml sonicated salmon sperm DNA, 500,000 cpm specific probe) overnight, washed three times at 65°C with 2× SSC–0.1% SDS, and then exposed to Kodak MS autoradiograph films. While expression of *ramA* was uniformly low in strains with wild-type MICs to tigecycline, *ramA* expression was very prominent in the nonsusceptible strains (VA6048, UR11100, VA14419, VA14743, and VA21266), suggesting massive upregulation (Fig. 2A).

For qRT-PCR, RNA was pretreated with DNase I (Roche, Mannheim, Germany) and then reverse transcribed with the SuperScript kit (Invitrogen, Karlsruhe, Germany). qRT-PCRs were run on a Rotor Gene Q cycler (Qiagen, Hilden, Germany) with 45 cycles of 20 s at 95°C, 20 s at 60°C, and 30 s at 72°C. Data were analyzed by using the $2^{-\Delta\Delta CT}$ method (15). Quantification by qRT-PCR demonstrated 25-fold to nearly 50-fold upregulation in the nonsusceptible strains compared to

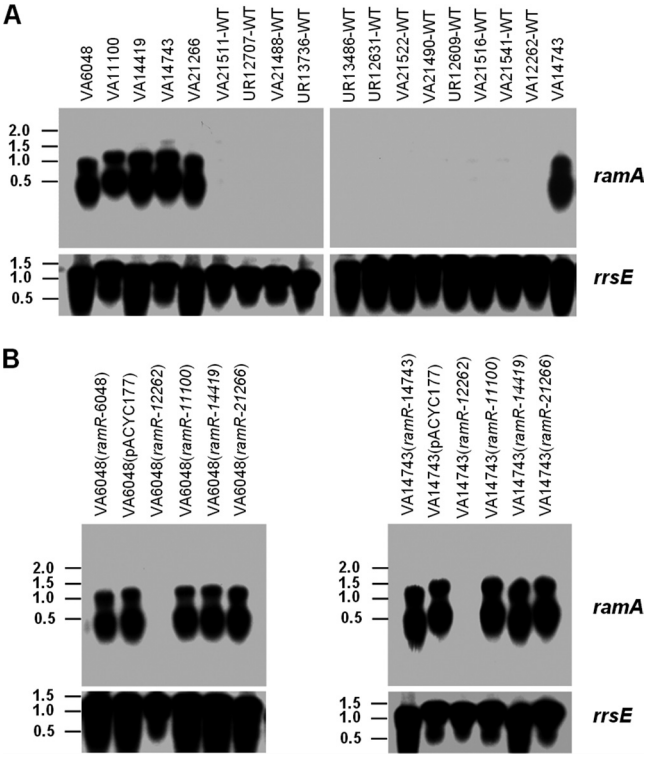

FIG. 2. RamR represses expression of *ramA* in *Klebsiella pneumoniae*. Expression levels of *ramA* were analyzed by Northern blot hybridization. Total RNAs of the strains indicated were isolated from mid-log-phase cultures. Three micrograms of total RNA was loaded into each lane, and the filters were hybridized with [^{32}P]dCTP-labeled probes of *ramA* and subsequently 16S rRNA (*rrsE*) as a loading control. Values on the left of the panels are band sizes in kbp. (A) Comparison of *ramA* expression levels in non-tigecyline-susceptible strains and susceptible strains. VA14743 is included in the Northern blot on the right side as a positive control. (B) *ramA* expression in nonsusceptible strains transformed with mutated or wild-type *ramR*.

VA12262-WT, which served as the reference strain (Table 1). Furthermore, qRT-PCR of *acrB* (forward primer, 5′-TTAAT ACCCAGACCGGATGC; reverse primer, 5′-TGGCCGCGG GCCAGTTAGGCGGTA), a target gene of RamA, revealed concomitant 8-fold-to-15-fold upregulation in the mutants compared to the level for the wild-type strain VA12262 (Table 1). Transformation of wild-type *ramR* (from VA12262-WT) into VA6048 [*Klebsiella pneumoniae* VA6048 ($ramR_{VA12262\text{-}WT}$)] and into VA14743 [*Klebsiella pneumoniae* VA14743 ($ramR_{VA12262\text{-}WT}$)] resulted in strongly repressed *ramA* expression, while no change in *ramA* expression was noted in strains transformed with any of the mutated *ramR* genes from the nonsusceptible strains or the empty pACYC177 vector as analyzed by Northen blot hybridization (Fig. 2B) and by qRT-PCR (Table 1). Again, changes in the expression levels of *acrB* accompanied those observed for *ramA* (Table 1). All strains harboring mutated *ramR* overexpressed *acrB* in comparison to the wild-type strain VA12262 or the mutants VA6048 and VA14743 complemented with wild-type *ramR* [*Klebsiella pneumoniae* VA6048 ($ramR_{VA12262\text{-}WT}$) and *Klebsiella pneumoniae* VA14743 ($ramR_{VA12262\text{-}WT}$), respectively]. These

experiments establish *ramR* in *Klebsiella pneumoniae* as a repressor of *ramA*.

In summary, we identified a gene in *Klebsiella pneumoniae* with homology to *ramR*, a repressor of *ramA* in *Salmonella enterica*, which is mutated in strains resistant to tigecycline (9) and to ciprofloxacin (1, 13, 17, 24). Our results imply that *ramA* in *Klebsiella pneumoniae* is regulated in a manner similar to its regulation in *Salmonella* and provide a molecular mechanism for tigecycline resistance in *Klebsiella pneumoniae*. All of our non-tigecycline-susceptible *Klebsiella pneumoniae* strains harbored mutations in the *ramR* gene, suggesting that this is a major molecular mechanism for tigecycline resistance in *Klebsiella pneumoniae*. Though the susceptibilities of all of our strains were clearly reduced compared to those of wild-type strains, none of our strains exhibited full resistance to tigecycline by definition (MIC $>$ 2 μg/ml). However, fully resistant *Klebsiella* strains have been described previously (6, 28), suggesting that several mechanisms might contribute to tigecycline resistance. Acquisition of mutations in *ramR* may represent one step in the development of full resistance; however, in certain body compartments like the bloodstream, where only low concentrations of tigecycline can be achieved, an intermediate phenotype may be sufficient to result in therapeutic failure of tigecycline (3, 18). Other resistance mechanisms, like Tn*1721*-associated *tet*(A) (9, 31), may be additive and upon acquisition successively result in full resistance. It is particularly worrisome that AcrAB-mediated multidrug resistance can be induced by prior treatment with a multitude of antibiotics. Furthermore, it cannot be unambiguously inferred from the resistance phenotype exhibited by an individual isolate *in vitro* during routine diagnostic resistance testing. Thus, susceptibility testing to tigecycline of all relevant isolates would be beneficial when tigecyline treatment is an option.

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