

Influence of Transcriptional Activator RamA on Expression of Multidrug Efflux Pump AcrAB and Tigecycline Susceptibility in *Klebsiella pneumoniae*

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Received 4 June 2004/Returned for modification 28 August 2004/Accepted 3 November 2004

Tigecycline is an expanded broad-spectrum antibacterial agent that is active against many clinically relevant species of bacterial pathogens, including *Klebsiella pneumoniae*. The majority of *K. pneumoniae* isolates are fully susceptible to tigecycline; however, a few strains that have decreased susceptibility have been isolated. One isolate, G340 (for which the tigecycline MIC is 4 µg/ml and which displays a multidrug resistance [MDR] phenotype), was selected for analysis of the mechanism for this decreased susceptibility by use of transposon mutagenesis with IS903φkan. A tigecycline-susceptible mutant of G340, GC7535, was obtained (tigecycline MIC, 0.25 µg/ml). Analysis of the transposon insertion mapped it to *ramA*, a gene that was previously identified to be involved in MDR in *K. pneumoniae*. For GC7535, the disruption of *ramA* led to a 16-fold decrease in the MIC of tigecycline and also a suppression of MDR. *Trans*-complementation with plasmid-borne *ramA* restored the original parental phenotype of decreased susceptibility to tigecycline. Northern blot analysis revealed a constitutive overexpression of *ramA* that correlated with an increased expression of the AcrAB transporter in G340 compared to that in tigecycline-susceptible strains. Laboratory mutants of *K. pneumoniae* with decreased susceptibility to tigecycline could be selected at a frequency of approximately 4×10^{-8} . These results suggest that *ramA* is associated with decreased tigecycline susceptibility in *K. pneumoniae* due to its role in the expression of the AcrAB multidrug efflux pump.

Tigecycline is an expanded broad-spectrum antibiotic representing a new class called the glycolcyclines. The glycolcyclines are semisynthetic derivatives of minocycline and have activity against many bacterial pathogens (2, 14, 15). It has been noted that a few species of gram-negative bacteria, including *Pseudomonas aeruginosa*, *Proteus* spp., *Providencia* spp., and *Morganella morganii*, are intrinsically less susceptible to tigecycline. Previous studies revealed the involvement of multidrug efflux systems such as MexXY and AcrAB in the decreased tigecycline susceptibility of *P. aeruginosa* and *Proteus mirabilis*, respectively (3, 22). These pumps belong to the resistance-nodulation-division (RND) family that combines bacterial transporters with a tripartite architecture and broad substrate specificity (9, 12). Due to the broad substrate specificity of RND pumps, their overexpression usually results in the multidrug resistance (MDR) phenotype.

Klebsiella pneumoniae causes infections of wounds, the urinary tract, and the respiratory system. This bacterial species is generally susceptible to tigecycline; however, a few clinical strains with decreased tigecycline susceptibility have been isolated. In this study, one such an isolate, G340, was investigated to determine the mechanism of decreased tigecycline susceptibility in *K. pneumoniae*.

(These results were reported, in part, previously [M. A.

Visalli, S. J. Projan, and P. A. Bradford, Abstr. 42nd Intersci. Conf. Antimicrob. Agents Chemother., abstr. C1-1600, 2002].)

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are shown in Table 1. Strains G595, G815, and G340 are clinical isolates from various infection types. Plasmid pVJT128 was kindly provided by David Figurski. The strains were propagated at 37°C in Luria-Bertani (LB) broth or agar.

Antibiotic susceptibility testing. Tigecycline and minocycline used in this study were from Wyeth Research, Pearl River, N.Y. Tetracycline, acriflavine, ethidium bromide, erythromycin, chloramphenicol, nalidixic acid, novobiocin, trimethoprim, norfloxacin, gentamicin, kanamycin, and IPTG (isopropyl-β-D-thiogalactopyranoside) were obtained from Sigma Chemical Co., St. Louis, Mo. The MICs of various antibacterial agents were determined by standard broth microdilution tests (11). Tests for tigecycline were performed using fresh Mueller-Hinton broth (<12 h old).

DNA techniques. Standard DNA manipulations such as restriction digestion and molecular cloning were performed as described previously (16). Chemically competent *Escherichia coli* strains TOP10 and INV110 (Invitrogen, Carlsbad, Calif.) were used for cloning experiments. Transformation was performed as specified by the manufacturer. DNA fragments were gel purified by using a Zymoclean Gel DNA recovery kit (Zymo Research, Orange, Calif.). *K. pneumoniae* genomic DNA was isolated by using a Puregene tissue kit (Gentra Systems, Inc., Minneapolis, Minn.) and used as a template for PCRs. A 575-bp *ramA*-containing DNA fragment was amplified by PCR using the primers listed in Table 2. The gel-purified PCR fragment was ligated into the pCR2.1-TOPO vector (Invitrogen). The resulting plasmid, pCLL3441, was modified by cloning a 878-bp XbaI fragment containing a gentamicin resistance cassette from pUCGm into the XbaI site of pCLL3441. The resulting plasmid, pCLL3442, was used in *trans*-complementation studies. DNA transformations of *K. pneumoniae* clinical isolates were performed by electroporation with a Gene Pulser II system (Bio-Rad, Hercules, Calif.), using the optimal electroporation settings of 2.5 kV, 25 µF, 200 Ω, and 5 ms.

Transposon mutagenesis. Transposon mutagenesis with IS903φkan was performed essentially as described previously (20). Briefly, the transposon carrier plasmid pVJT128 was electroporated into G340, and transformants were selected on LB plates containing 200 µg of chloramphenicol/ml. Seven individual

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
TOP10	<i>E. coli</i> cloning strain	Invitrogen
INV110	<i>dam</i> and <i>dcm</i> methylase-deficient <i>E. coli</i>	Invitrogen
G340	Clinical isolate	This study
G595	Clinical isolate	This study
G815	Clinical isolate	This study
GC7535	G340 insertion mutant, IS903 ϕ kan inserted in <i>ramA</i>	This study
GC7736	G595 transformed with pCLL3442	This study
GC7737	GC7535 transformed with pCLL3442	This study
Plasmids		
pCR2.1-TOPO	PCR cloning vector	Invitrogen
pUCGm	pUC19 derivative containing gentamicin resistance cassette	18
pVJT128	Plasmid carrier vector for IS903 ϕ kan transposon	20
pCLL3441	pCR2.1-TOPO with cloned <i>ramA</i> gene	This study
pCLL3442	pCLL3441 with cloned gentamicin cassette	This study

colonies were selected, inoculated into LB broth containing 1 mM IPTG and 200 μ g of chloramphenicol/ml, and then propagated overnight with shaking to induce transposition. Clones with transposon insertions were selected by plating aliquots of overnight culture onto LB plates containing 50 μ g of kanamycin/ml. Tigecycline-susceptible transposon mutants were isolated by replica plating with selection for colonies that grew on LB plates containing 50 μ g of kanamycin/ml but not on LB plates containing 2 μ g of tigecycline/ml. The carrier plasmid was cured by serial passage in chloramphenicol-free medium. Transposon insertions were mapped by an inverse PCR as described previously (21), using outward-facing primers (Table 2) (20). The products of inverse PCR were cloned into the pCR2.1-TOPO vector, and the nucleotide sequence was determined with an ABI 3700 automated sequencer (Applied Biosystems, Foster City, Calif.) using universal sequencing primers. The site of transposon insertion was determined by submitting sequence batches to the NCBI BLAST database (<http://www.ncbi.nlm.nih.gov/BLAST>).

Mutation frequency. The frequency of spontaneous mutations leading to decreased tigecycline susceptibility in two tigecycline-sensitive clinical isolates, G595 and G815, was determined essentially as described previously for the estimation of the frequency of MDR (4). Cells were grown overnight in LB broth and inoculated in triplicate on LB plates containing 4 μ g of tigecycline/ml using an inoculum size of 10^9 CFU per plate. After overnight incubation, the colonies were counted and the mutation frequency was calculated using the method of the median (8).

Northern blot hybridization. DNA fragments containing *ramA*, *acrA*, and *rseE* were amplified by PCR (Table 2). 32 P-labeled probes were generated by random prime labeling of gel-purified PCR fragments by using a High Prime kit (Roche Diagnostics, Mannheim, Germany) with Redivue 5'-[α - 32 P]dCTP (Amersham, Piscataway, N.J.) as the source of the 32 P isotope. Total RNA was isolated from mid-log-phase bacterial cultures by using an RNAeasy kit (QIAGEN, Valencia, Calif.). RNA samples were separated on a 1.2% agarose-0.66 M formaldehyde gel and transferred to a Hybond-ND⁺ nylon membrane (Amersham). The membrane was hybridized with labeled probes, washed, and exposed to BioMax MS X-ray film (Kodak, Rochester, N.Y.) according to standard molecular procedures (1). X-ray images were scanned on a ScanJet ADF scanner (Hewlett-Packard, Palo Alto, Calif.), and densitometry analysis was performed by using Quantity One 4.1.1 software (Bio-Rad).

RT-PCR. Oligonucleotide primers and probes used for real-time (RT)-PCR (Table 2) were designed with Primer Express Software version 2.0 (Applied Biosystems) and purchased from QIAGEN. The probes were labeled by the manufacturer with the reporter dye 6-carboxyfluorescein (6'-FAM) at the 5' end and with the quencher dye 6-carboxytetramethylrodamine (TAMRA) at the 3' end. DNase-treated RNA template was prepared from mid-log-phase bacterial cultures by using an RNAeasy kit (QIAGEN). RT-PCR was performed by using a Taqman One-Step RT-PCR master mix reagents kit (Applied Biosystems) on an ABI Prism 7900HT sequence detection system (Applied Biosystems). A typical RT-PCR sample (20 μ l) contained 5 μ l of a serial dilution of RNA template (range, 0.04 ng/ml to 400 μ g/ml), 4.38 μ l of nuclease-free water (Ambion, Austin, Tex.), 10 μ l of AmpliTaq Gold DNA polymerase mix (2 \times), 0.5 μ l of RT enzyme mix (40 \times), 0.04 μ l of 100 μ M solutions of both forward and reverse gene-specific primers, and 0.04 μ l of a 100 μ M solution of gene-specific

probe. Each sample was run in duplicate. The critical threshold cycle (C_T) numbers were determined by the detection system software. Relative quantification of target (*ramA*) gene expression was performed by normalization to an endogenous reference (*rseE*) as recommended by the manufacturer. Briefly, the amount of target is given as $2^{-\Delta\Delta T}$, where $\Delta\Delta T$ is the difference between the C_T values for a target and a reference.

Western blot analysis. Bacterial cells were grown in LB broth to an A_{600} of 0.7 to 1.0. Approximately 1 μ g of the total cell lysate proteins was loaded onto a 10% Tris-HCl gel (Bio-Rad) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described previously (7). The proteins were transferred from the polyacrylamide gel to a nitrocellulose filter (Bio-Rad) by use of a Multiphor II system (Amersham). The membrane was blocked for 1 h at 4°C with shaking in TBST (20 mM Tris-HCl [pH 7.6], 137 mM NaCl, 0.1% Tween 20) supplemented with 5% nonfat dry milk (Bio-Rad). The membrane was probed with an anti-AcrA polyclonal antibody (1:10,000; gift from H. Zgurskaya, University of Oklahoma, Norman) at room temperature while being shaken. The membrane was washed in TBST buffer three times at room temperature and probed for 1 h with antirabbit antibodies conjugated to horseradish peroxidase (1:50,000; Pierce, Rockford, Ill.). The blots were developed using SuperSignal West Pico solutions (Pierce) and exposed to X-ray film (BioMax MR; Kodak). X-ray images were scanned on a ScanJet ADF scanner (Hewlett-Packard), and densitometry analysis was performed by using Quantity One 4.1.1 software (Bio-Rad).

RESULTS

Estimation of mutation frequency. The MIC of tigecycline for G340, a clinical strain of *K. pneumoniae*, was 4 μ g/ml, which represents a 16-fold increase over the MIC for the tigecycline-susceptible isolate G595 (Table 3). In addition, G340 displayed the MDR phenotype in that the MICs of a number of other antibiotics for this strain were also elevated (Table 3). The discovery of such a strain prompted an investigation to determine a frequency of spontaneous mutations that results in decreased tigecycline susceptibility in *K. pneumoniae*. Tigecycline-susceptible clinical isolates G595 and G815 were plated on plates containing tigecycline at a concentration 16 times the MIC for each strain, after which tigecycline-resistant colonies emerged at a frequency of 3.5×10^{-8} for G595 and 4.4×10^{-8} for G815. This result is consistent with the mutation frequency of 2.2×10^{-8} reported previously for the emergence of MDR in *K. pneumoniae* strain ECL8 (4).

Transposon mutagenesis and mapping. To elucidate the mechanism of the decreased tigecycline susceptibility in *K. pneumoniae*, strain G340 was subjected to transposon mu-

tagenesis using IS903 ϕ kan, which is carried on a plasmid and therefore can be maintained in the host prior to the induction of transposition (20). Mutagenesis of G340 with IS903 ϕ kan resulted in the selection of a tigecycline-susceptible transposon insertion mutant, GC7535. The site of IS903 ϕ kan insertion in GC7535 was mapped by inverse PCR to a 102-bp position within the *ramA* gene (Fig. 1). This gene is a part of a previously characterized genetic locus that includes *romA* and *orf82* (Fig. 1), as well as a putative cation transporter (not shown) (4).

Susceptibility testing and trans-complementation studies.

The transposon insertion in the *ramA* gene of G340 resulted in suppression of the MDR phenotype, as shown by the decrease in the MICs of minocycline, tetracycline, ethidium bromide, nalidixic acid, novobiocin, trimethoprim, and norfloxacin (Table 3). In addition, the MIC of tigecycline decreased substantially (16-fold; from 4 to 0.25 μ g/ml) (Table 3). This result indicated that the *ramA* locus was linked to the decreased tigecycline susceptibility in G340.

To assess whether inactivation of the *ramA* gene is the primary factor responsible for the observed phenotype of a transposon insertion mutant, a typical *trans*-complementation experiment was performed. pCLL3442, a multicopy plasmid that carries full-length *ramA*, was introduced into transposon mutant GC7535. In the resulting strain, GC7737, the MDR phenotype was restored in that the MICs were either equal to or higher than those observed for the original parental strain, G340 (Table 3). Such an increase over the original MICs might be due to the multicopy nature of the carrier plasmid as well as to the elevated level of constitutive *ramA* transcription from the plasmid-encoded promoter.

Analysis of *ramA* and *acrA* expression. The *ramA*-encoded protein, RamA, is related to the DNA-binding proteins MarA and SoxS from the AraC family of transcriptional activators (4). Recent studies showed that transcription of *ramA* was increased in fluoroquinolone-resistant clinical isolates of *K. pneumoniae* (17). To test whether a similar scenario existed for the decreased tigecycline susceptibility, Northern blot analysis of *ramA* expression was performed. As shown in Fig. 2, the amount of *ramA* transcript was significantly larger in G340 than in the tigecycline-susceptible strain G595. According to densitometry analysis, there was an approximate 12-fold increase in *ramA* expression in G340 over that of G595. An additional quantitative analysis of *ramA* expression was performed by RT-PCR. By use of this technique, the level of *ramA* expression was determined to be about 40-fold higher in G340 than in G595 (Table 4). These results indicated that decreased tigecycline susceptibility was linked to *ramA* overexpression in *K. pneumoniae*. In addition, the MICs of the various substrates were elevated for GC7736, which contains pCLL3442 expressing *ramA* (Table 3).

It was previously reported that *ramA* overexpression was observed in conjunction with an elevated expression of the multidrug efflux pump AcrAB in *K. pneumoniae*, suggesting that RamA might be involved in the regulation of AcrAB production (17). To investigate this hypothesis, Northern blot analysis of *acrA* expression was performed with G595, G340, and the *ramA* knockout mutant GC7535. As shown in Fig. 2, the level of *acrA* transcription in G340 exceeded that seen in either G595 or GC7535. Since *acrA* is located upstream of *acrB*

TABLE 2. Primers and fluorescent probes used for PCR

Gene	Product size (bp)	Purpose	Forward primer (5'-3')	Reverse primer (5'-3')	Fluorescent probe ^a (5'-3')
NA ^b	NA	Inverse PCR primer 1	GTTTCCCGTTGAATATATGGCTGGG	NA	NA
NA	NA	Inverse PCR primer 2	GCAGTTTCATTGTGATGCTCGA	NA	NA
<i>ramA</i>	575	TOPO cloning	GGATGAACCCGTATCAACG	CCATTGAGTATCTGGTGC	NA
<i>ramA</i>	335	Probe for Northern analysis	GCATATGACCGATTCCGCTCAG	ACTGTGGTTCCTTTGCG	NA
<i>acrA</i>	557	Probe for Northern analysis	TTCTGATGCTCTCAGGC	TGACCATTTCTGTACCAGC	NA
<i>rseE</i>	525	Probe for Northern analysis	GTAAGCTAATACCGCATTAACGTCGC	GCTACACCTGGAAATTTACC	NA
<i>ramA</i>	66	RT-PCR	GCATCAACCGCTGGGTATT	CGTTGCAGATGCCATTTCG	NA
<i>rseE</i>	71	RT-PCR	TTGACCGTTAACCCTCAGGAAGAA	GCTTGACACCCTCCGTATTACC	ATCGCTCGCCATGCCCCGGGTAT TAACTCCGTGCCAGCAGCCCG

^a Labeled with 6'-FAM at the 5' end and with TAMRA at the 3' end.
^b NA, not applicable.

TABLE 3. Antibiotic susceptibility

Strain	RamA overexpression	MIC ($\mu\text{g/ml}$)										
		TGC ^a	MIN	TET	ACR	EtBr	ERY	CHL	NA	NOV	TRM	NOR
G595	—	0.25	2	1	128	1,024	128	4	4	128	0.5	0.125
GC7736	+	8	64	32	512	2,048	256	256	>64	>512	16	2
G340	+	4	32	16	256	2,048	256	128	64	>512	2	1
GC7535	—	0.25	1	1	128	512	128	256	4	128	0.25	0.06
GC7737	+	16	>128	64	512	2,048	256	>512	>64	>512	16	4

^a Abbreviations: TGC, tigecycline; MIN, minocycline; TET, tetracycline; ACR, acriflavine; EtBr, ethidium bromide; ERY, erythromycin; CHL, chloramphenicol; NA, nalidixic acid; NOV, novobiocin; TRM, trimethoprim; NOR, norfloxacin.

and both genes share a common promoter (5), the size of the *acrA* transcript (~ 4 kb) was consistent with the size of a bicistronic *acrAB* mRNA, implying that both of the components of the AcrAB pump were overexpressed in G340. Densitometry analysis showed that the level of *acrA* expression was about 3.4-fold higher in G340 than in either G595 or GC7535. The amount of AcrA protein was elevated in G340, as revealed by Western blot analysis of cell lysates (Fig. 3). Densitometry analysis showed about a fourfold excess of AcrA in G340 than in G595 or GC7535. These results suggested that RamA was indeed a part of the regulatory pathway that governs the production of AcrAB in *K. pneumoniae*.

DISCUSSION

Following selection with tigecycline in the laboratory, spontaneous mutants of *K. pneumoniae* with decreased tigecycline susceptibility emerged at a frequency of about 10^{-8} . Such an order of magnitude suggests that less-susceptible colonies can arise as a result of single-step mutations. Despite this fact, the majority of *K. pneumoniae* clinical isolates are susceptible to tigecycline at typical MICs of 0.25 to 1 $\mu\text{g/ml}$. To date, only a few isolates that result in tigecycline MICs exceeding 1 $\mu\text{g/ml}$ have been collected.

This study demonstrated that one of the formerly described MDR determinants, *ramA*, is involved in reduced tigecycline susceptibility in *K. pneumoniae*. The *ramA*-encoded protein, RamA, belongs to the family of transcriptional activators that

also includes MarA, SoxS, and Rob, which are known for their role in promoting antibiotic resistance due to the up-regulation of the multidrug efflux pump AcrAB-TolC (10, 13, 19, 23). As shown recently, the AcrAB efflux system is associated with reduced susceptibility to tigecycline in *P. mirabilis* (22). The present study revealed that the increased tigecycline MIC for *K. pneumoniae* strain G340 correlated with the constitutive overexpression of *ramA*, suggesting that RamA might act as an activator of expression of Acr transporter. A similar hypothesis was proposed recently when elevated levels of *ramA* transcription coincided with increased expression of AcrAB in *K. pneumoniae* strains resistant to fluoroquinolones (17). In contrast, expression levels of known Acr regulators such as *marA* and

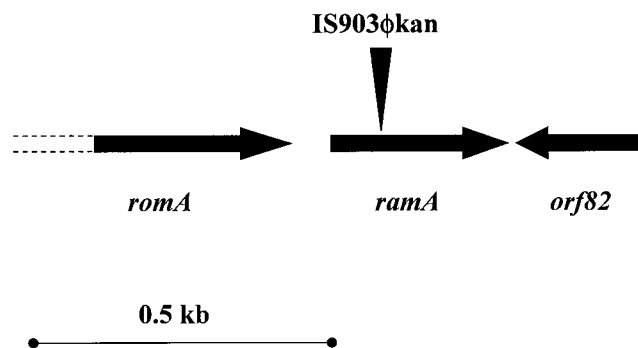


FIG. 1. Map of the chromosomal region around the IS903 ϕ kan insertion site in GC7535. The genetic organization of the *ramA* locus of *K. pneumoniae* was described previously (4). Open reading frames are shown by arrows; the unsequenced portions of *romA* are shown by dashed lines. The site of the IS903 ϕ kan insertion in *ramA* is shown by a vertical triangle.

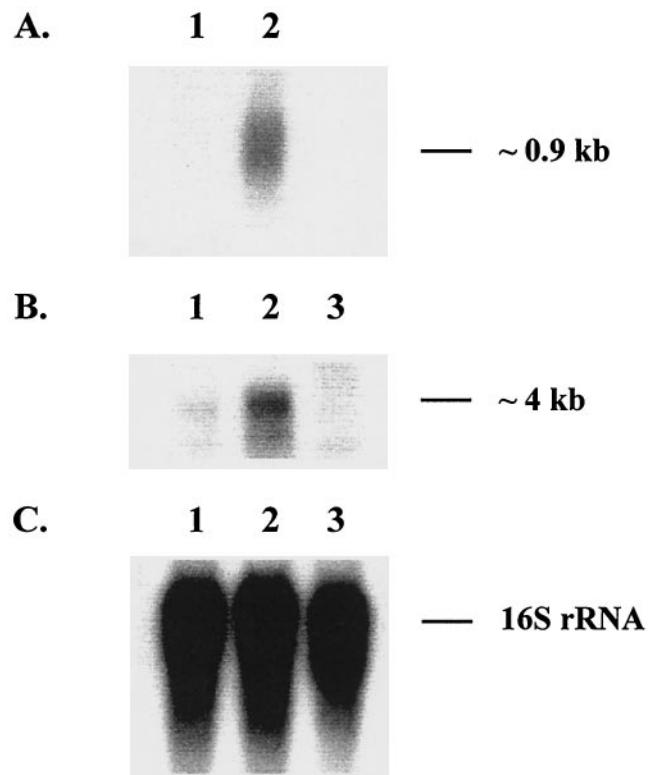


FIG. 2. Northern blot analysis of *ramA* and *acrA* expression. Total bacterial RNA was isolated from mid-log-phase cultures of G595 (lane 1), G340 (lane 2), and GC7535 (lane 3) and hybridized with ³²P-labeled DNA probes specific to either *ramA* (A), *acrA* (B), or *rrsE* (C). Hybridization with *rrsE* that encodes 16S rRNA served as an RNA loading control.

TABLE 4. RT-PCR analysis of *ramA* expression

Gene	Strain	C_T^a	Relative expression level versus <i>rrsE</i> ^b	Overexpression ratio ^c
<i>ramA</i>	G340	26.5	2.09e-3	36.7
	G595	31.3	5.69e-5	
<i>rrsE</i>	G340	17.6		
	G595	17.2		

^a C_T values represent averages for duplicate samples with a final RNA concentration of 1 ng/ml.

^b Relative expression is calculated as $2^{-\Delta\Delta T}$. $\Delta\Delta T = C_T(\text{target}) - C_T(\text{reference})$, where the target is *ramA* and the reference is *rrsE*.

^c The overexpression ratio is the ratio between the relative expression level of the target gene in G340 versus that in G595.

soxS were unchanged (17). The results presented here further support the role of RamA in the regulation of the Acr pump by linking the overexpression of *ramA* to up-regulation of AcrAB and the inactivation of *ramA* to the down-regulation of AcrAB expression. Similar to fluoroquinolone-resistant isolates, no increase in *marA* expression was detected (data not shown), indicating that induction of AcrAB expression by RamA is not mediated through overexpression of MarA. Due to the broad substrate specificity of the Acr pump, these data might explain the increased efflux of tetracycline and chloramphenicol that was previously observed in strains that overexpressed *ramA* and provide a mechanism for the previously established role of *ramA* as a determinant of MDR in *K. pneumoniae* (4).

The study presented here leaves several questions open. First, the exact mechanism of *ramA* overexpression in *K. pneumoniae* is unknown, and it is possible that RamA production is inducible. Because *K. pneumoniae* is generally susceptible to tigecycline, it is unlikely that tigecycline serves as an inducer of either RamA or AcrAB production in this organism. It is possible that RamA plays some physiological role and perhaps is produced in response to certain environmental stimuli. In addition to *K. pneumoniae*, the close homologs of RamA are identified in *Enterobacter cloacae* (6), *Enterobacter aerogenes* (GenBank accession number AJ404625) and *Salmonella en-*

terica (24), whereas other bacteria, e.g., *E. coli*, lack a RamA equivalent, suggesting that RamA might function in certain species-specific environmental niches. It is also possible that overexpression of *ramA* results in the overproduction of MDR pumps other than AcrAB. Further experiments, e.g., involving disruption of the *acrAB* locus in GC7535 and G340, are required to establish whether overexpression of *acrAB* is the only factor contributing to decreased tigecycline susceptibility in *K. pneumoniae*.

ACKNOWLEDGMENTS

We thank David Figurski for providing plasmid pVJT128 and Helen Zgurskaya for a kind gift of anti-AcrA antibodies.

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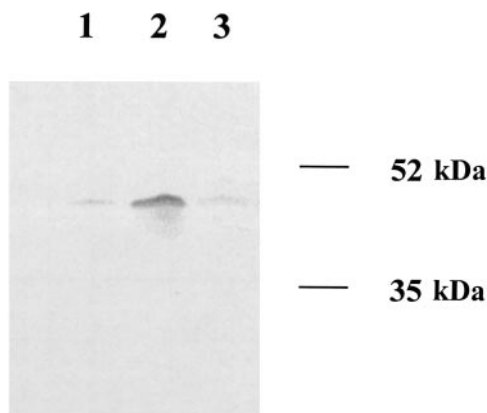


FIG. 3. Western blot analysis of AcrA expression. Total cell lysate proteins of G595 (lane 1), G340 (lane 2), and GC7535 (lane 3) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and probed with polyclonal anti-AcrA antibodies (gift from H. Zgurskaya).

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