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AcrAB Efflux Pump Plays a Role in Decreased Susceptibility to Tigecycline in *Morganella morganii*

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Transposon mutagenesis of a clinical isolate of *Morganella morganii*, G1492 (tigecycline MIC of 4 μg/ml), yielded two insertion knockout mutants for which tigecycline MICs were 0.03 μg/ml. Transposon insertions mapped to *acrA*, which is constitutively overexpressed in G1492, suggesting a role of the AcrAB efflux pump in decreased susceptibility to tigecycline in *M. morganii*.

Tigecycline is a novel expanded broad-spectrum glycylcycline antibiotic (1, 8, 9). Tigecycline circumvents classical tetracycline resistance mechanisms such as ribosomal protection and efflux by tetracycline-specific pumps (3, 9). Nevertheless, several species of gram-negative bacteria are intrinsically less susceptible to tigecycline; among them are *Pseudomonas aeruginosa*, *Proteus* spp., *Providencia* spp., and *Morganella morganii*. In addition, a few strains of *Klebsiella pneumoniae* have been isolated with acquired decreased susceptibility to tigecycline.

Previous studies showed that decreased tigecycline susceptibility is associated with multidrug efflux systems such as MexXY in *P. aeruginosa* and AcrAB in *Proteus mirabilis* and *K. pneumoniae* (4, 10, 14). These pumps belong to the resistance/nodulation/division family and are often associated with multidrug resistance (MDR). As determined previously, the constitutive overexpression of the pump components correlated with reduced susceptibility to tigecycline in *P. aeruginosa* and *K. pneumoniae* (4, 10).

This study was performed to identify the mechanism of decreased susceptibility to tigecycline in many strains of *M. morganii*. For the majority of *M. morganii* strains, the tigecycline MIC was 1 to 4 μg/ml. Two clinical isolates of *M. morganii*, G858 and G1492, which represent the lower and higher ends of the range of tigecycline MICs, were selected for this study.

Bacterial strains and plasmids used in this study are shown in Table 1. The strains were propagated at 37°C in Luria-Bertani broth or agar. Standard DNA manipulations such as restriction digestion and molecular cloning were performed as described previously (11). Chemically competent *Escherichia coli* strains TOP10 and INV110 (Invitrogen, Carlsbad, Calif.) were used for cloning experiments. DNA transformations were performed by electroporation with the 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 was done with the EZ::TN <R6γori/KAN-2> transposome kit (Epicentre, Madison, Wis.) according to the manufacturer's protocol. The selection of tigecycline-susceptible transposon mutants, rescue cloning, and mapping of transposon insertions were performed as described previously (14). The nucleotide sequence was determined with an ABI 3700 automated sequencer (Applied Biosystems, Foster City, Calif.). The MICs of various antibacterial agents were determined by standard broth microdilution tests (6). MICs of tigecycline were determined with fresh Mueller-Hinton broth (<12 h old).

Two tigecycline-susceptible transposon mutants, GC7676 and GC7677, were obtained upon transposon mutagenesis of a clinical isolate of *M. morganii* G1492. The sites of transposon insertion were mapped by rescue cloning and sequencing to a homolog of the *acrA* gene. GC7676 had an insertion at nucleotide 453, and GC7677 had an insertion at nucleotide 946. The nucleotide sequence of a 6,104-bp genomic DNA fragment containing the entire *acrRAB* locus of G1492 was determined from the rescue clones. The *acrRAB* genes of *M. morganii* were

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
E. coli		
TOP10	Cloning strain	Invitrogen
INV110	*dam* and *dcm* methylase deficient	Invitrogen
M. morganii		
G858	Clinical isolate, tigecycline MIC = 1 μg/ml	This study
G1492	Clinical isolate, tigecycline MIC = 4 μg/ml	This study
GC7676	G1492 insertion mutant, EZ::TN inserted in 453 bp of *acrA*	This study
GC7677	G1492 insertion mutant, EZ::TN inserted in 946 bp of *acrA*	This study
GC7743	GC7676 transformed with pCLL3444	This study
GC7744	GC7677 transformed with pCLL3444	This study
Plasmids		
pCR2.1-TOPO	PCR cloning vector	Invitrogen
pUCGm	pUC19 derivative containing gentamicin resistance cassette	13
pCLL3443	pCR2.1-TOPO with cloned *acrAB* genes (4,859-bp PCR fragment)	This study
pCLL3444	pCLL3443 with cloned gentamicin cassette (878-bp XbaI fragment)	This study

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TABLE 2. Antibiotic susceptibility for *M. morganii* strains

Strain	AcrA overexpression	MIC (μg/ml)[a]										
		TGC	MIN	TET	ACR	EtBr	SDS	ERY	CHL	NA	NOV	TRM
G858	−	1	2	1	128	512	>2,048	128	4	2	8	2
G1492	+	4	8	32	32	1,024	>2,048	>256	64	4	256	2
GC7676	−	0.03	0.5	16	2	2	64	16	8	1	2	0.25
GC7677	−	0.03	0.5	16	4	2	128	16	8	1	2	0.25
GC7743	+	4	8	64	32	1,024	>2,048	256	128	4	256	2
GC7744	+	8	16	64	32	1,024	>2,048	>256	128	4	256	4

[a] Abbreviations: TGC, tigecycline; MIN, minocycline; TET, tetracycline; ACR, acriflavine; EtBr, ethidium bromide; SDS, sodium dodecyl sulfate; ERY, erythromycin; CHL, chloramphenicol; NA, nalidixic acid; NOV, novobiocin; TRM, trimethoprim.

648, 1,194, and 3,177 bp long, respectively, and had 63, 57, and 81% amino acid sequence identity to *acrRAB* genes of *P. mirabilis* (14). The orientation of genes was identical to that of *P. mirabilis*, with the *acrR* and *acrAB* genes being divergently transcribed from a common intergenic region.

Transposon insertion into the *acrA* gene of G1492 resulted in a substantial decrease in the MIC of tigecycline (128-fold reduction from 4 to 0.03 μg/ml; Table 2). In addition, the MDR phenotype was suppressed, as demonstrated by the decrease in the MICs of minocycline, acriflavine, ethidium bromide, sodium dodecyl sulfate, erythromycin, chloramphenicol, nalidixic acid, novobiocin, and trimethoprim for GC7676 and GC7677 (Table 2).

For transcomplementation studies, a DNA fragment containing the full-length *acrAB* of G1492 was amplified by PCR with the primers listed in Table 3. Genomic DNA of G1492 was isolated by using the Puregene tissue kit (Gentra Systems, Inc., Minneapolis, Minn.) and served as a template for PCR. The gel-purified PCR fragment was cloned into the pCR2.1-TOPO vector (Invitrogen) as specified by the manufacturer. The resulting plasmid, pCLL3443, was modified by cloning an 878-bp XbaI fragment containing the gentamicin resistance cassette from pUCGm into the XbaI site of pCLL3443. The resulting plasmid, pCLL3444, was used in transcomplementation studies. Introduction of pCLL3444 into transposon mutants resulted in tigecycline MICs of 4 to 8 μg/ml and in the restoration of the MDR phenotype. It should be noted that both *acrA* and *acrB* were required for transcomplementation because these genes are cotranscribed; therefore, both were inactivated by transposon insertions in *acrA*. These results indicated that the *acrAB* locus was linked to the decreased tigecycline susceptibility and MDR phenotype of G1492.

To determine whether the decreased tigecycline susceptibility of G1492 was associated with *acrAB* overexpression, the transcriptional levels of *acrA* were analyzed by Taqman quantitative real-time PCR (RT-PCR) on the iCycler iQ real-time PCR detection system (Bio-Rad). DNase-treated RNA templates were prepared from mid-log-phase bacterial cultures by using the RNAeasy kit (Qiagen, Valencia, Calif.). Oligonucleotide primers and probes used for RT-PCR are shown in Table 3. RT-PCR and the quantification of the target gene expression (*acrA*) relative to an endogenous reference (*rrsE*) were performed as described previously (10). As shown in Table 4, the level of *acrA* expression was approximately 48.5-fold higher in G1492 (tigecycline MIC = 4 μg/ml) than in G858 (tigecycline MIC = 1 μg/ml). In addition, the elevated level of *acrA* expression in G1492 in comparison to that in G858 was confirmed by Northern blot hybridization (data not shown), further suggesting that reduced susceptibility to tigecycline was linked to *acrA* overexpression in *M. morganii*. It should be noted that strains G858 and G1492 are nonisogenic, and, therefore, an additional analysis of *acrAB* expression levels in the same-strain background would be beneficial to confirm the correlation between increased levels of *acrAB* expression and decreased susceptibility to tigecycline in *M. morganii*.

In conclusion, the mechanism of efflux-mediated decreased susceptibility to tigecycline in *M. morganii* appears to be similar to that identified earlier in *P. aeruginosa*, *P. mirabilis*, and *K. pneumoniae* (4, 10, 14). Future studies will be directed toward understanding the molecular mechanisms of constitutive pump overexpression and the regulation of pump production in *M. morganii*. Based on previously published studies, the overexpression of the pump components is commonly caused by inactivation of the pump repressor or overproduction of transcriptional activator(s) (2, 4, 5, 7, 12). The possibility that any of these mechanisms caused the increased production of AcrAB in *M. morganii* will be an area for future study.

Only limited data on genetic characterization of *M. morganii*

TABLE 3. Primers and fluorescent probes used for PCR and RT-PCR

Gene(s)	Product size (bp)	Purpose	Sequence (5′→3′) of:		
			Forward primer	Reverse primer	Fluorescent probe[a]
acrAB	4,859	TOPO cloning	ATCCACTAATGACGTCGC	GCAGAGTAAATGCCTGAACG	NA[b]
acrA	72	RT-PCR	ACCTGCGTCTGAAACAAGAAATC	CCAGGCTGACAGCAACTTTACC	CCAGCGGTGCCGTGGATAAAGAAC
rrsE	71	RT-PCR	TTGACGTTACCCGCAGAAGAA	GCTTGCACCCTCCGTATTACC	TAACTCCGTGCCAGCAGCCG

[a] Labeled with 6-carboxyfluorescein (6′-FAM) at the 5′ end and with 6-carboxytetramethylrodamine (TAMRA) at the 3′ end.
[b] NA, not applicable.

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

Gene	Strain	Ct[a]	Relative expression vs that of *rrsE*[b]	Overexpression ratio[c]
acrA	G858	30.9	3.32e–6	48.5
	G1492	25.6	1.61e–4	
rrsE	G858	12.7		
	G1492	13.0		

[a] Ct (critical threshold cycle) numbers represent an average for duplicate samples with a final RNA concentration of 40 ng/ml.

[b] Relative expression is calculated as $2^{-\Delta\Delta T}$, where $\Delta\Delta T = Ct_{target} - Ct_{reference}$. The target is *acrA*, and the reference is *rrsE*.

[c] Ratio between relative expression level of the target gene in G1492 versus that in G858.

are available. The present study provides the first sequence and description of *acrRAB* in this species.

Nucleotide sequence accession number. The nucleotide and protein sequences of the *acrRAB* genes of *M. morganii* have been registered in GenBank under accession no. AY669147.

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