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LETTER TO THE EDITOR

Decreased Susceptibility to Tigecycline Mediated by a Mutation in *mlaA* in *Escherichia coli* Strains

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Tigecycline, a type of glycylcycline, is a novel expanded-spectrum antibiotic that is active against most Gram-negative and Gram-positive bacteria, including antibiotic-resistant strains such as carbapenem-resistant members of the family *Enterobacteriaceae* (1–3). However, tigecycline-resistant strains have emerged since tigecycline was approved for clinical use (4, 5). It has been reported that overexpression of the AcrAB efflux pump results in decreased susceptibility to tigecycline in *Enterobacteriaceae* (6–8). Ribosomal protein S10 is also a general target of tigecycline adaptation (9–11). In addition to the efflux pumps and ribosomal S10 protein described previously, other possible mechanisms of tigecycline resistance require further investigation. In the present study, two pairs of *Escherichia coli* strains were studied by whole-genome sequencing to identify mutants under selective pressure from tigecycline.

E. coli ATCC 25922 was used as the initiating strain (12). The AcrAB efflux pump of *E. coli* ATCC 25922 was inactivated by deletion of the *acrAB* genes as previously described (13), with the hybrid primers listed in Table S1 in the supplemental material. The resulting strain was named 25922Δ*acrAB*. Tigecycline-resistant mutants were selected by successive passages through Luria-Bertani broth containing increasing concentrations of tigecycline in an induction experiment. *E. coli* ATCC 25922 and 25922Δ*acrAB* were used as parental strains. The selective tigecycline concentration began with 0.0625 μg/ml and doubled every 24 h until the mutants grew at a concentration of 32 μg/ml. The overnight cultures at each step were stored at −80°C in the presence of 20% glycerol. Two tigecycline-resistant mutants, namely, 25922-TGC8 (tigecycline MIC, 8 μg/ml) and 25922Δ*acrAB*-TGC8 (tigecycline MIC, 8 μg/ml), were obtained from ATCC 25922 (tigecycline MIC, 0.125 μg/ml) and 25922Δ*acrAB* (tigecycline MIC, 0.0625 μg/ml), respectively. Both mutants exhibited nonsusceptibility to tetracycline and minocycline (see Table S2 in the supplemental material). Genomic DNA from 25922-TGC8 and 25922Δ*acrAB*-TGC8 was sequenced with Illumina HiSeq 2000 (Illumina Inc., San Diego, CA) following a paired-end 2 × 100-bp protocol. The reads were mapped against the reference genome of *E. coli* ATCC 25922 (CP009072) with the CLC Genomics Workbench 9 software (Qiagen, Valencia, CA). The putative single nucleotide polymorphisms and deletion mutations in 25922-TGC8 and 25922Δ*acrAB*-TGC8 were also predicted (14). Twenty-three putative mutation sites in 25922Δ*acrAB*-TGC8 and 42 in 25922-TGC8 were found by bioinformatic analysis. Of these, two mutations in 25922Δ*acrAB*-TGC8 and six in 25922-TGC8 were confirmed by PCR and Sanger sequencing of the putative mutation sites (Table 1). One mutation in *mlaA* was found in both 25922-TGC8 and 25922Δ*acrAB*-TGC8. It was a deletion mutation of six nucleotides that resulted in the deletion of two amino acids and thus a truncated protein. Mutations in *rpsJ* (G169C) and *marR* (G311A) were also found in 25922-TGC8.

The *mlaA* gene was deleted from ATCC 25922 and 25922Δ*acrAB* with the λ Red recombinase system (for the primers used, see Table S1 in the supplemental material). The isolates obtained were named 25922Δ*mlaA* and 25922Δ*acrAB*Δ*mlaA*. DNA fragments carrying the wild-type *mlaA* gene and a mutated *mlaA* gene were amplified from ATCC 25922 and 25922Δ*acrAB*-TGC8, respectively. After amplification, the amplimer was cloned into plasmid pCR2.1. *mlaA* deletion-carrying strains 25922Δ*mlaA* and 25922Δ*acrAB*Δ*mlaA* were used for transformation. The tigecycline MICs for 25922Δ*mlaA* and 25922Δ*acrAB*Δ*mlaA* were the same as those for their parental strains. However, when 25922Δ*mlaA* and 25922Δ*acrAB*Δ*mlaA* were complemented with mutational *mlaA* (named *mlaA*+), the MICs of tigecycline were 8-fold higher than those for the parental strains (see Tables S3 and S4 in the supplemental material), while no change was noted when the bacteria were transformed with the empty pCR2.1 vector and wild-type *mlaA*. In addition, we detected three mutated loci (*mlaA*, *marR*, and *rpsJ*) in the genomes of the series of isolates of 25922-TGC8 recovered at successive steps of the induction experiment (tigecycline MICs of 0.25 to 8 μg/ml). *mlaA* was the first mutated gene that appeared in the successive-passage experiment, and the MIC of tigecycline increased to 1 μg/ml. The *marR* mutation was the second mutation detected, and the MIC increased to 4 μg/ml. *rpsJ* was the last mutation that appeared, and the MIC increased to 8 μg/ml (see Table S5 in the supplemental material). Interestingly, it seems that multiple mechanisms (Mla system, efflux pump, and ribosomal S10 protein) can accumulate gradually in the development of tigecycline resistance.

In this study, we constructed an AcrAB efflux pump deletion strain (25922Δ*acrAB*) and induced resistance to tigecycline. According to the whole-genome sequencing data, only two mutations could be verified in 25922Δ*acrAB*-TGC8: *mlaA* and *infB*. It seems that tigecycline resistance can occur without the AcrAB efflux pump, and a mutation in ribosomal protein S10 was also not mandatory. Because the *mlaA* mutation was found in both 25922Δ*acrAB*-TGC8 and 25922-TGC8, it is reasonable to postulate that this *mlaA* mutation may play an important role in tigecycline resistance. This hypothesis was confirmed by the deletion and complementation experiments, in

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TABLE 1 Mutations in 25922Δ*acrAB*-TGC8 and 25922-TGC8 compared with parental strains

Strain and reference position	Gene and product	Change	
		Nucleotide sequence	Amino acid sequence
25922Δ*acrAB*-TGC8			
286573	*mlaA*, putative phospholipid-binding lipoprotein	CTTCAA deletion at 129–134	NF deletion at 43–44
1435949	*infB*, translation initiation factor IF-2	G793C	G265R
25922-TGC8			
286573	*mlaA*, putative phospholipid-binding lipoprotein	CTTCAA deletion at 129–134	NF deletion at 43–44
1300420	*rpsJ*, ribosomal protein S10	G169C	G57L
1534008	*tsaD*, tRNA threonylcarbamoyladenosine modification protein	TTG insertion at 346–347	L insertion at 116
3541486	*marR*, multiple antibiotic resistance protein	G311A	G104D
3865514	*fadR*, fatty acid metabolism transcriptional regulator	G397T	133 stop
4621400	*entC*, isochorismate synthase	C915A	S305R

which the *mlaA* mutation led to an 8-fold increase in the tigecycline MIC (see Table S4 in the supplemental material). The Mla system is an ABC transport system that can transfer phospholipids from the outer membrane (OM) to the inner membrane to maintain OM lipid asymmetry (15). We propose that mutation of *mlaA* may increase the efficiency of this transfer and thus enhance the barrier function of the OM. Because of the widely distributed Mla system in Gram-negative bacteria, it may be easy to induce *mlaA* mutations under the stress of tigecycline. Our study contributes to the comprehensive understanding of tigecycline resistance mechanisms in *Enterobacteriaceae*.

Accession number(s). The genome sequences of 25922-TGC8 and 25922Δ*acrAB*-TGC8 have been deposited in the NCBI SRA database and assigned accession numbers SRR3744959 and SRR3744956.

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We have no competing interests to declare.

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REFERENCES

1. **Pankey GA.** 2005. Tigecycline. J Antimicrob Chemother **56:**470–480. http://dx.doi.org/10.1093/jac/dki248.
2. **Jenner L, Starosta AL, Terry DS, Mikolajka A, Filonava L, Yusupov M, Blanchard SC, Wilson DN, Yusupova G.** 2013. Structural basis for potent inhibitory activity of the antibiotic tigecycline during protein synthesis. Proc Natl Acad Sci U S A **110:**3812–3816. http://dx.doi.org/10.1073/pnas.1216691110.
3. **Schedlbauer A, Kaminishi T, Ochoa-Lizarralde B, Dhimole N, Zhou S, Lopez-Alonso JP, Connell SR, Fucini P.** 2015. Structural characterization of an alternative mode of tigecycline binding to the bacterial ribosome. Antimicrob Agents Chemother **59:**2849–2854. http://dx.doi.org/10.1128/AAC.04895-14.
4. **Spanu T, De Angelis G, Cipriani M, Pedruzzi B, D'Inzeo T, Cataldo MA, Sganga G, Tacconelli E.** 2012. *In vivo* emergence of tigecycline resistance in multidrug-resistant *Klebsiella pneumoniae* and *Escherichia coli*. Antimicrob Agents Chemother **56:**4516–4518. http://dx.doi.org/10.1128/AAC.00234-12.
5. **van Duin D, Cober ED, Richter SS, Perez F, Cline M, Kaye KS, Kalayjian RC, Salata RA, Evans SR, Fowler VG, Jr, Bonomo RA.** 2014. Tigecycline therapy for carbapenem-resistant Klebsiella pneumoniae (CRKP) bacteriuria leads to tigecycline resistance. Clin Microbiol Infect **20:**O1117–O11120. http://dx.doi.org/10.1111/1469-0691.12714.
6. **Keeney D, Ruzin A, McAleese F, Murphy E, Bradford PA.** 2008. MarA-mediated overexpression of the AcrAB efflux pump results in decreased susceptibility to tigecycline in Escherichia coli. J Antimicrob Chemother **61:**46–53.
7. **He F, Fu Y, Chen Q, Ruan Z, Hua X, Zhou H, Yu Y.** 2015. Tigecycline susceptibility and the role of efflux pumps in tigecycline resistance in KPC-producing Klebsiella pneumoniae. PLoS One **10:**e0119064. http://dx.doi.org/10.1371/journal.pone.0119064.
8. **Keeney D, Ruzin A, Bradford PA.** 2007. RamA, a transcriptional regulator, and AcrAB, an RND-type efflux pump, are associated with decreased susceptibility to tigecycline in Enterobacter cloacae. Microb Drug Resist **13:**1–6. http://dx.doi.org/10.1089/mdr.2006.9990.
9. **Beabout K, Hammerstrom TG, Perez AM, Magalhaes BF, Prater AG, Clements TP, Arias CA, Saxer G, Shamoo Y.** 2015. The ribosomal S10 protein is a general target for decreased tigecycline susceptibility. Antimicrob Agents Chemother **59:**5561–5566. http://dx.doi.org/10.1128/AAC.00547-15.
10. **Lupien A, Gingras H, Leprohon P, Ouellette M.** 2015. Induced tigecycline resistance in Streptococcus pneumoniae mutants reveals mutations in ribosomal proteins and rRNA. J Antimicrob Chemother **70:**2973–2980. http://dx.doi.org/10.1093/jac/dkv211.
11. **Villa L, Feudi C, Fortini D, Garcia-Fernandez A, Carattoli A.** 2014. Genomics of KPC-producing *Klebsiella pneumoniae* sequence type 512 clone highlights the role of RamR and ribosomal S10 protein mutations in conferring tigecycline resistance. Antimicrob Agents Chemother **58:**1707–1712. http://dx.doi.org/10.1128/AAC.01803-13.
12. **Minogue TD, Daligault HA, Davenport KW, Bishop-Lilly KA, Broomall SM, Bruce DC, Chain PS, Chertkov O, Coyne SR, Freitas T, Frey KG, Gibbons HS, Jaissle J, Redden CL, Rosenzweig CN, Xu Y, Johnson SL.** 2014. Complete genome assembly of *Escherichia coli* ATCC 25922, a serotype O6 reference strain. Genome Announc **2:**e00969-14. http://dx.doi.org/10.1128/genomeA.00969-14.
13. **Datsenko KA, Wanner BL.** 2000. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc Natl Acad Sci U S A **97:**6640–6645. http://dx.doi.org/10.1073/pnas.120163297.
14. **Ruan Z, Feng Y.** 2016. BacWGSTdb, a database for genotyping and source tracking bacterial pathogens. Nucleic Acids Res **44:**D682–D687. http://dx.doi.org/10.1093/nar/gkv1004.
15. **Malinverni JC, Silhavy TJ.** 2009. An ABC transport system that maintains lipid asymmetry in the Gram-negative outer membrane. Proc Natl Acad Sci U S A **106:**8009–8014. http://dx.doi.org/10.1073/pnas.0903229106.