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MarA-mediated overexpression of the AcrAB efflux pump results in decreased susceptibility to tigecycline in *Escherichia coli*

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***Objectives*: The purpose of this study was to characterize decreased susceptibility to tigecycline in clinical isolates of *Escherichia coli* obtained during Phase 3 clinical trials.**

***Methods*: Gene expression was analysed by transcriptional profile analysis and RT-PCR. Transposon mutagenesis with IS903φkan was used for selection of transposon mutants. Transposon insertions were mapped by DNA sequencing and PCR analyses. The MICs were determined by broth microdilution.**

***Results*: Both transcriptional profile analysis and Taqman RT-PCR demonstrated increased expression levels of MarA, a transcriptional activator, and AcrAB, an RND-type efflux pump, in the strains with elevated tigecycline MICs. Transposon mutagenesis generated nine mutants, the majority of which had either *marA* or *acrB* inactivated. Sequence analysis revealed a single nucleotide insertion in the open reading frame of the *marR* gene in less-susceptible strains of *E. coli*.**

***Conclusions*: This study suggested that a loss of MarR functionality due to a frameshift mutation resulted in constitutive overproduction of MarA and AcrAB and, consequently, in decreased susceptibility to tigecycline in clinical isolates of *E. coli*.**

Keywords: resistance nodulation cell division family, antibiotic resistance, multidrug efflux pump, RT-PCR, transcriptional profile analysis

Introduction

Tigecycline is a novel glycylcycline antibiotic, which has been approved worldwide to treat serious medical conditions such as complicated skin and skin structure infections and complicated intra-abdominal infections.[1] This compound is active against a broad range of Gram-negative, Gram-positive, anaerobic and atypical bacteria.[2–4] Tigecycline is also active against many antibiotic-resistant bacteria including methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant enterococci and extended-spectrum β-lactamase-producing Enterobacteriaceae. Tigecycline overcomes typical tetracycline resistance mechanisms such as the ribosomal protection determinant *tet*(M) and tetracycline-specific efflux pumps: *tet*(A), *tet*(B), *tet*(C) and *tet*(D).[5]

The AcrAB-TolC efflux pump is a tripartite complex containing AcrA, a fusion protein; AcrB, a cytoplasmic membrane transporter protein; and TolC, an outer membrane channel. Previously it was demonstrated that decreased susceptibility to tigecycline is due to an up-regulated expression of the AcrAB efflux pump in several species including *Proteus mirabilis*,[6] *Klebsiella pneumoniae*,[7] *Morganella morganii*[8] and *Enterobacter cloacae*.[9] Overexpression of this pump is often associated with a multidrug resistance (MDR) phenotype.[10]

Escherichia coli is usually susceptible to tigecycline, however a few less-susceptible clinical isolates were obtained during Phase 3 clinical trials. The purpose of this study was to characterize decreased susceptibility to tigecycline in these isolates.

Materials and methods

Bacterial strains and growth conditions

E. coli strains used in this study are shown in Table 1. Strains G4905, G4906, G4907, G5048, G5049 and G5050 were isogenic clinical isolates from a single patient treated with tigecycline in a clinical trial designed to study resistant Gram-negative pathogens. The strains were propagated at 37°C in Luria–Bertani (LB) broth or agar.

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Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Organism	Description	Tigecycline MIC (mg/L)	Reference
Strain				
G4905	*E. coli*	clinical isolate (isolated 14 December 2003 from skin infection)	0.5	this study
G4906	*E. coli*	clinical isolate (isolated 10 December 2003 from intra-abdominal site)	0.5	this study
G4907	*E. coli*	clinical isolate (isolated 10 December 2003 from intra-abdominal site)	0.5	this study
G5050	*E. coli*	clinical isolate (isolated 21 December 2003 from drain fluid)	0.5	this study
G5048	*E. coli*	clinical isolate (isolated 21 December 2003 from urine)	2	this study
G5049	*E. coli*	clinical isolate (isolated 30 December 2003 from drain fluid)	2	this study
GC7952	*E. coli*	G5049 insertion mutant, IS903ϕkan inserted in 21 bp of *marA*	0.125	this study
GC7953	*E. coli*	G5049 insertion mutant, IS903ϕkan inserted in 30 bp of *marA*	0.25	this study
GC7954	*E. coli*	G5049 insertion mutant, IS903ϕkan inserted in 87 bp of *marA*	0.25	this study
GC7955	*E. coli*	G5049 insertion mutant, IS903ϕkan inserted in 21 bp of *marA*	0.125	this study
GC7956	*E. coli*	G5049 insertion mutant, IS903ϕkan inserted in 3102 bp of *acrB*	0.25	this study
GC7957	*E. coli*	G5049 insertion mutant, IS903ϕkan inserted in 3141 bp of *acrB*	0.25	this study
GC7958	*E. coli*	G5049 insertion mutant, IS903ϕkan location not determined	0.25	this study
GC7959	*E. coli*	G5049 insertion mutant, IS903ϕkan location not determined	0.25	this study
GC7960	*E. coli*	G5049 insertion mutant, IS903ϕkan location not determined	0.5	this study
Plasmid				
pVJT128		plasmid carrier vector for IS903ϕkan transposon	NA	17

NA, not applicable.

DNA techniques

Standard DNA manipulations such as restriction digestion and molecular cloning were performed as described previously.[11] DNA transformations were performed by electroporation with the Gene Pulser II system (Bio-Rad, Hercules, CA, USA), using the optimal electroporation settings of 2.5 kV, 25 μF, 200 Ω and 5 ms. *E. coli* genomic DNA was isolated by using the Puregene tissue kit (Gentra Systems Inc., Minneapolis, MN, USA) and used as a template for PCRs. Primers used for PCR are listed in Table 2. The FailSafe PCR System (EpiCentre, Madison, WI, USA) was used to amplify *E. coli acrAB* and *marRAB* DNA sequences in accordance with the manufacturer's instructions. Oligonucleotide primers were obtained from Genelink (Hawthorne, NY, USA). PCR fragments were gel-purified by using Zymoclean Gel DNA Recovery kit (Zymo Research, Orange, CA, USA). The nucleotide sequence was determined with an automated sequencer ABI 3730 (Applied Biosystems, Foster City, CA, USA).

Transcriptional profile analysis

RNA was extracted from mid-log phase bacterial cultures using the RNAeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Reverse transcription, cDNA fragmentation and terminal labelling of cDNA fragments with biotin were carried out in accordance with the manufacturer's (Affymetrix Inc., Santa Clara, CA, USA) instructions for antisense prokaryotic arrays. In brief, hexamer random primers (Invitrogen, Carlsbad, CA, USA) were annealed to 10 μg of total denatured RNA at 25°C for 10 min. cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen) following the manufacturer's instructions. An RNAse inhibitor, SUPERase-In (Ambion, Inc., Austin, TX, USA) was included. Any remaining RNA was degraded by treatment with 1 M NaOH for 30 min at 65°C and neutralized by adding an equal volume of 1 M HCl. cDNA was purified using the DNA Clean and Concentrator kit (Zymo Research) and fragmented with DNase I in One-Phor-All buffer (Amersham Biosciences, Piscataway, NJ, USA) (0.6 U DNase I/μg cDNA). Fragmented cDNA was labelled with biotin on the 3' terminus using the Enzo BioArray terminal labelling kit with biotin ddUTP (Affymetrix). Labelled, fragmented cDNA (1.5 μg) was hybridized overnight to *E. coli* Antisense Genome GeneChips (Affymetrix). The 'antisense' oligonucleotide array is essentially the same as that described by Selinger *et al*.,[12] except that probe sequences were the same as the coding region sequences. GeneChips were stained, washed and scanned using the Agilent GeneArray laser scanner (Agilent Technologies, Palo Alto, CA, USA) as described previously.[13] Affymetrix algorithms calculated signal intensities (average difference values) and made present or absent calls for each gene as detailed by Lockhart *et al*.[14] Signal intensities for elements tiled onto each GeneChip were then normalized to account for loading errors and differences in labelling efficiencies by dividing each signal intensity value by the mean signal intensity for an individual GeneChip. Results were analysed using GeneSpring Version 6.1 software (Silicon Genetics, Redwood City, CA, USA). Changes in gene expression were only considered relevant if there was at least a 2-fold change between the relevant strains, that the genes in the up-regulated condition were considered to be present by Affymetrix algorithms and that differences in expression were significant (*t*-test with a *P* value cutoff of at least 0.05) as previously described.[15,16]

RT-PCR

Oligonucleotide primers and probes used for real-time RT-PCR were designed with Primer Express Software version 2.0 (Applied Biosystems) and purchased from Operon Biotechnologies (Huntsville, AL, USA). The probes were labelled 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 templates were

Table 2. Primers used in this study

Gene	Product size (bp)	Purpose	Forward primer (5′–3′)	Reverse primer (5′–3′)	Fluorescent probe[a] (5′–3′)
marRAB	955	DNA sequence analysis	CCTGTGTATCTGGGTTATCAGC	CCTGCATATTGGTCATCC	NA
marA	320	transposon mapping	ATGACGATGTCCAGACG	CCTGCATATTGGTCATCC	NA
acrAB	4300	transposon mapping	GTATGAGATCCTGAGTTGGTGG	AACCTCTGCAGATCACAACC	NA
acrAB	1990	transposon mapping	TGATAATGGCGATCACCCAC	TCTCCAGTAAGATGGCAACG	NA
acrB	900	transposon mapping	GTATGAGATCCTGAGTTGGTGG	CGAAATACCGTATGCTGCC	NA
acrB	1950	transposon mapping	GTATGAGATCCTGAGTTGGTGG	ACGTTGAGCGTGTTATGGC	NA
acrB	3325	transposon mapping	GTATGAGATCCTGAGTTGGTGG	GCACAAGAAGTTACCGCTG	NA
acrA	67	RT-PCR	CTATCACCCTACGCGCTATCTTC	GCGCGCACGAACATACC	CGAACCCGGATCACACTCT
marA	65	RT-PCR	TTGGACTGGATCGAGGACAAC	CCCGAACGCTCTGACACTTT	TGGAATCGCCACTGTCACTGGA
rrsE	71	RT-PCR	TTGACGTTACCCGCAGAAGAA	GCTTGCACCCTCCGTATTACC	TAACTCCGTGCCAGCAGCCG

NA, not applicable.
[a]Labelled with 6′-FAM at the 5′ end and with TAMRA at the 3′ end.

prepared from mid-log phase bacterial cultures by using RNAeasy kit (Qiagen). RT-PCR was performed by using iScript One-Step RT-PCR Kit for Probes (Bio-Rad) on iCycler iQ5™ Real-Time PCR Detection System (Bio-Rad). A typical RT-PCR sample (25 μL) contained: 5 μL of a serial dilution of RNA template (range, 2 ng/mL to 200 mg/L), 6.85 μL of nuclease-free water (Ambion, Austin, Tex.), 12.5 μL of RT-PCR mixture (2×), 0.5 μL of iScript RT enzyme mix (50×), 0.05 μL of 100 μM solutions of both forward and reverse gene-specific primers and 0.05 μL of a 100 μM solution of gene-specific probe. Relative quantification of the target gene expression (*acrA* or *marA*) was performed by iCycler iQ5™ software using normalized expression analysis method. The 16S rRNA gene served as a reference gene and G4907 served as a reference condition. Each sample was run in triplicate.

Transposon mutagenesis

Transposon mutagenesis with IS903φkan was performed essentially as described previously.[7,17] Briefly, the transposon carrier plasmid, pVJT128, was electroporated into G5049 and transformants were selected on LB plates containing 200 mg/L chloramphenicol. Individual colonies were selected, inoculated into LB broth containing 1 mM IPTG and 200 mg/L chloramphenicol and propagated overnight with shaking to induce transposition. Clones with transposon insertions were selected by plating aliquots of overnight culture onto LB plates containing 50 mg/L kanamycin. Tigecycline-susceptible transposon mutants were isolated by replica plating and selecting for colonies that grew on LB plates containing 50 mg/L kanamycin but not on LB plates containing 2 mg/L tigecycline. The carrier plasmid was cured by serial passage in chloramphenicol-free medium. Transposon insertions were mapped by PCR and sequence analysis of the *marRAB* and *acrAB* operons.

Antibiotic susceptibility testing

Tigecycline used in this study was obtained from Wyeth Research (Pearl River, NY, USA). Tetracycline, minocycline, acriflavine, ethidium bromide, erythromycin, chloramphenicol, nalidixic acid, novobiocin, trimethoprim, norfloxacin and kanamycin were obtained from Sigma Chemical Co. (St Louis, MO, USA). The MICs of various antibacterial agents were determined by standard broth microdilution tests.[18] Tests for tigecycline susceptibility were performed using fresh Mueller–Hinton broth (<12 h old).[19,20]

Results

Transcriptional profile analysis of total RNA

Transcriptional profile analysis of total RNA isolated from four isogenic strains, G4907, G5048, G5049 and G5050, was performed to identify genes that were either up- or down-regulated in strains with an increased tigecycline MIC. Two other isolates, G4905 and G4906, were omitted from expression studies because these strains had tigecycline MIC values identical to those for G4907 and G5050. The results are shown in Tables 3 and 4. Genes of the *mar* operon displayed the greatest changes in expression. Other genes that increased in expression between 2- and 8-fold included *acrA*, *acrB* and *tolC*, which encode components of the AcrAB multidrug efflux pump. Other up-regulated genes are shown in Table 3.

Several genes were also down-regulated in G5048 and G5049, among them were *ompF*, *fimA* and genes from the *ymc*

Table 3. Up-regulation of genes in GAR5048 and GAR5049 compared with basal expression levels

Gene name[a]	Product	Fold change compared with strain G4907		
		G5050	G5048	G5049
htrB	heat shock protein	1.03	2.13	1.92
ychA	orf, hypothetical protein	1.13	2.39	2.62
yadG	putative ATP-binding component of a transport system	1.61	3.73	4.03
yadH	orf, hypothetical protein	1.16	3.6	3.52
marR	**multiple antibiotic resistance protein; repressor of mar operon**	**0.64**	**26.94**	**32.14**
marA	**multiple antibiotic resistance; transcriptional activator of defence systems**	**1.02**	**38.75**	**45.2**
marB	**multiple antibiotic resistance protein**	**0.99**	**10.29**	**11.21**
yadR	orf, hypothetical protein	0.95	2.23	1.35
yedO	putative 1-aminocyclopropane-1-carboxylate deaminase	0.94	2.41	2.8
yeiR	orf, hypothetical protein	1.42	4.35	4.41
inaA	pH-inducible protein involved in stress response	0.92	6.81	8.48
cysK	cysteine synthase A, *O*-acetylserine sulfhydrolase A	1.89	1.19	3.92
gshA	γ-glutamate-cysteine ligase	1.16	2.34	1.93
yggJ	orf, hypothetical protein	1.16	5.87	4.03
gshB	glutathione synthetase	1.1	3.67	3.36
tolC	**outer membrane channel**	**1.01**	**2.79**	**1.91**
ygiA	orf, hypothetical protein	1.6	3.52	2.29
yrbL	orf, hypothetical protein	1.18	3.32	2.68
yhcN	orf, hypothetical protein	1.82	4.08	4.15
hemB	5-aminolevulinate dehydratase = porphobilinogen synthase	0.71	2.41	2.89
acrB	**acridine efflux pump**	**1.1**	**2.37**	**2.39**
acrA	**acridine efflux pump**	**1.13**	**3**	**3.16**
nfnB	oxygen-insensitive NAD(P)H nitroreductase	0.79	6.65	6.34
bioD	dethiobiotin synthetase	0.83	1.29	3.06
ompX	outer membrane protein X	1.21	2.57	2.09
ybjC	orf, hypothetical protein	0.83	7.57	6.87
nfsA (mdaA)	oxygen-insensitive nitroreductase	1.05	8.4	7.31
rimK	ribosomal protein S6 modification protein	0.98	11.19	11.35
ybjN	putative sensory transduction regulator	1.3	3.72	2.89

[a]Genes of the *mar* and *acr* operons are shown in bold.

Table 4. Down-regulation of genes in GAR5048 and GAR5049 compared with basal expression levels

Gene name	Product	Fold change compared with strain G4907		
		G5050	G5048	G5049
etk (*yccC*)	protein tyrosine kinase	−0.75	−5.27	−3.28
etp (*yccY*)	putative phosphatase	−0.74	−68.89	−65.59
yccZ	putative function in exopolysaccharide production	−0.84	−17.83	−39.00
ymcA	orf, hypothetical protein	−0.54	−39.86	−45.60
ymcB	orf, hypothetical protein	−0.58	−24.57	−33.17
ymcC	putative regulator	−0.86	−14.39	−17.68
ymcD	orf, hypothetical protein	−0.76	−16.52	−14.29
manZ	PTS enzyme IID, mannose-specific	−1.03	−2.16	−1.46
frdD	fumarate reductase, anaerobic, membrane anchor polypeptide	−1.52	−2.03	−1.33
fimA	major type 1 subunit fimbrin (pilin)	−1.53	−4.49	−6.21
ompF	outer membrane protein 1a (Ia;b;F)	−1.17	−4.05	−3.39

and *ycc* clusters. Other down-regulated genes are shown in Table 4.

Analysis of acrA expression by RT-PCR

Transcriptional profile analysis indicated that a decrease in susceptibility to tigecycline might correlate with elevated expression of the AcrAB efflux pump. To confirm the results, RT-PCR analysis of *acrA* expression was performed. As shown in Figure 1, increased expression of *acrA* was observed in the less-susceptible strains. Quantitative analysis revealed that expression of *acrA* increased 2.75- and 3.5-fold in G5048 and G5049, respectively, as compared with tigecycline-susceptible strain, G4907, whereas the expression level in another tigecycline-susceptible strain, G5050, was not elevated relative to G4907. Because *acrA* and *acrB* are co-transcribed, this result implied that both genes were overexpressed in the less-susceptible strains and further suggested that overexpression of the AcrAB pump is involved in decreased tigecycline susceptibility in *E. coli*.

Analysis of marA expression by RT-PCR

The transcriptional activator MarA was previously shown to up-regulate the production of the AcrAB efflux pump in *E. coli*.[21] To test the hypothesis that MarA could be involved in the overexpression of AcrA in the strains in this study, RT-PCR analysis of *marA* expression was performed. As shown in Figure 2, *marA* expression was increased over 30-fold in two strains, G5048 and G5049, compared with both G4907 and G5050. As mentioned above, G5048 and G5049 also displayed elevated levels of *acrAB* expression as compared with strains G4907 and G5050, suggesting that increased production of the AcrAB pump in G5048 and G5049 is the result of *marA* overexpression.

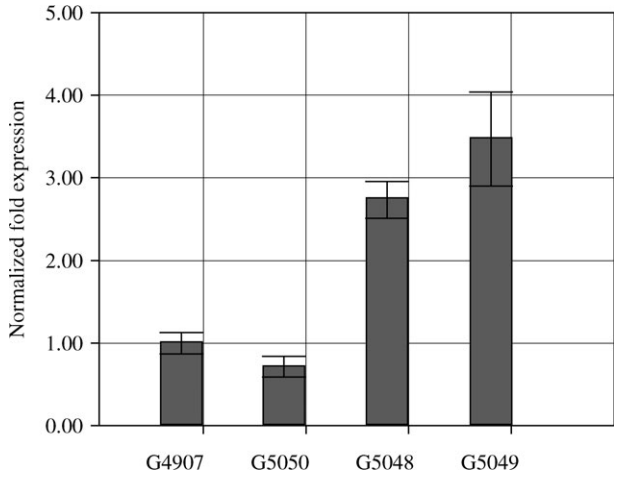

Figure 1. RT-PCR analysis of *acrA* expression in *E. coli*. Total bacterial RNA was isolated from mid-log phase cultures of G4907 (tigecycline MIC 0.5 mg/L), G5050 (tigecycline MIC 0.5 mg/L), G5048 (tigecycline MIC 2 mg/L) and G5049 (tigecycline MIC 2 mg/L). Expression of *acrA* was analysed by Taqman RT-PCR. The normalized *acrA* expression ratios are shown. The error bars represent the standard deviation for the mean of triplicate samples.

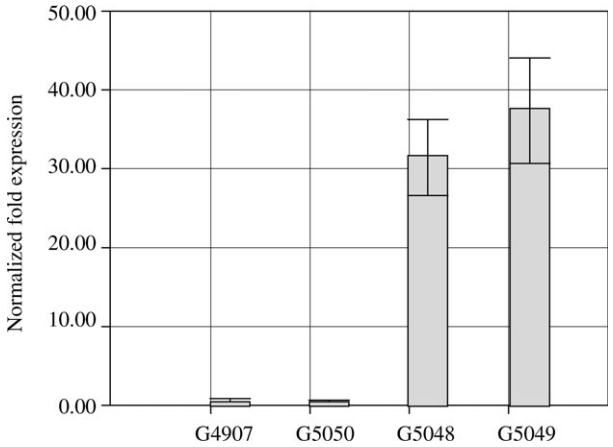

Figure 2. RT-PCR analysis of *marA* expression in *E. coli*. Total bacterial RNA was isolated from mid-log phase cultures of G4907 (tigecycline MIC 0.5 mg/L), G5050 (tigecycline MIC 0.5 mg/L), G5048 (tigecycline MIC 2 mg/L) and G5049 (tigecycline MIC 2 mg/L). Expression of *marA* was analysed by Taqman RT-PCR. The normalized *marA* expression ratios are shown. The error bars represent the standard deviation for the mean of triplicate samples.

Sequence analysis of marR gene

MarA activates the *acr* operon by binding to the intergenic region between *acrR* and *acrA* thereby lifting the repression caused by the AcrR repressor protein.[21] Regulation of the *mar* operon is in turn, accomplished by the MarR repressor, a dimeric protein that binds to the *mar* operator region. The nucleotide sequence of *marR* gene was analysed to determine whether this might contribute to the constitutive overexpression of both *mar* and *acr* operons in G5048 and G5049. Both G5048 and G5049 had an insertion of a cytosine residue at position 355 in *marR*, whereas tigecycline-susceptible strains lacked this mutation. The addition of a cytosine residue at this position causes a frameshift that is likely to result in MarR losing its repressor function, which would lead to constitutive overproduction of MarA and AcrAB and, consequently, to decreased susceptibility to tigecycline in G5048 and G5049.

Transposon mutagenesis and mapping of transposon insertions

To gain an additional insight into the mechanism of the decreased tigecycline susceptibility in *E. coli*, strain G5049 was subjected to transposon mutagenesis. Mutagenesis of G5049 with IS903ϕkan resulted in the selection of nine tigecycline-susceptible transposon insertion mutants (Table 1). The sites of transposon insertions were mapped by PCR and sequence analyses of the *marRAB* and *acrAB* regions. In four of the mutants, transposon was mapped to three different positions within the *marA* gene: nucleotide 21 in GC7952 and GC7955; nucleotide 30 in GC7953; and nucleotide 87 in GC7954. In the other two mutants, transposon inserted into *acrB* gene: nucleotide 3102 in GC7956 and nucleotide 3141 in GC7957. In the remaining three mutants, neither *marRAB* nor *acrAB* regions contained the transposon.

Antibiotic susceptibility

As shown in Table 5, in addition to decreased susceptibility to tigecycline, G5049 displayed an MDR phenotype, which is

Table 5. Antibiotic susceptibility

Strain	IS903φkan insertion site	AcrAB overexpression	MIC (mg/L)										
			TGC	MIN	TET	TMP	EtBr	ERY	CHL	NAL	NOV	Acr	NOR
G5049	none	+	2	16	8	2	512	>128	128	>32	>256	64	>8
GC7952	*marA*	−	0.125	2	4	0.25	256	128	>256	>32	128	32	>8
GC7953	*marA*	−	0.25	2	4	0.5	256	128	>256	>32	128	32	>8
GC7954	*marA*	−	0.25	2	2	0.25	256	128	>256	>32	128	32	>8
GC7955	*marA*	−	0.125	2	4	0.25	256	128	>256	>32	128	32	>8
GC7956	*acrB*	−	0.25	2	2	0.5	64	8	>256	>32	128	16	>8
GC7957	*acrB*	−	0.25	2	2	0.25	128	8	>256	>32	64	16	>8
GC7958	ND	ND	0.25	2	2	2	512	128	>256	>32	>256	64	>8
GC7959	ND	ND	0.25	2	2	2	512	64	>256	>32	>256	64	>8
GC7960	ND	ND	0.5	2	2	1	512	64	>256	>32	>256	64	>8

TGC, tigecycline; MIN, minocycline; TET, tetracycline; Acr, acriflavine; NOR, norfloxacin; ERY, erythromycin; EtBr, ethidium bromide; CHL, chloramphenicol; NAL, nalidixic acid; NOV, novobiocin; TMP, trimethoprim; ND, not determined.

consistent with the overexpression of the AcrAB multidrug efflux pump in this strain. All the transposon insertions resulted in a reduction in the MIC of tigecycline; however, they differed in their effect on the MICs of other antibiotics. Insertional inactivation of *acrB* (strains GC7956 and GC7957) resulted in the most profound effect: resistance decreased to every antibiotic in the panel with the exception of chloramphenicol, nalidixic acid and norfloxacin. Inactivation of *marA* (strains GC7952 through GC7955) produced less of an effect on the MDR phenotype, which might indicate that the presence of an intact AcrAB pump, although not overexpressed, is sufficient to result in the elevated MICs of erythromycin, ethidium bromide and acriflavine in these strains. Unmapped transposon insertions in strains GC7958 through GC7960 only affected the MICs of tigecycline, tetracycline and minocycline.

None of the transposon insertions affected the MICs of chloramphenicol, nalidixic acid and norfloxacin, which indicated that strain G5049 might possess specific determinants of resistance to these drugs.

Discussion

Tigecycline is a novel glycylcycline antibiotic developed to overcome bacterial resistance to tetracycline and minocycline. Recently however, our laboratory has reported that increased expression of the multidrug efflux pump AcrAB correlated with decreased susceptibility to tigecycline in *P. mirabilis*, *K. pneumoniae*, *M. morganii* and *E. cloacae*.[6–9]

The results of the present study indicate that genes of the *mar* and *acr* operons are associated with decreased susceptibility to tigecycline in *E. coli*. This was suggested by results from transcriptional profile analysis, RT-PCR and by transposon mutagenesis. These results are in agreement with those of Hirata *et al*.,[22] who constructed AcrAB-expressing *E. coli* strains that showed a 4-fold decrease in susceptibility to tigecycline compared with wild-type strains.

Transcriptional activators such as MarA have been shown to regulate the expression of the AcrAB efflux pump. RamA is a homologue of MarA, which was previously described in *K. pneumoniae* and *Enterobacter aerogenes*.[23,24] In earlier studies, a correlation between increased overexpression of *ramA* and *acrAB* and decreased susceptibility to tigecycline was demonstrated in *K. pneumoniae*.[7] In this study, transcriptional profile analysis and RT-PCR both demonstrated that increased expression of *marA* in G5048 and G5049 correlated with the overexpression of the *acrAB* transcript.

MarR has been established as one of the central regulators of the *mar* operon. DNA sequencing of the *marR* gene in this study revealed a frameshift mutation in the less-susceptible strains G5048 and G5049, which serves as a likely explanation for the overexpression of both *marA* and *acrAB* genes observed in these strains. This is in agreement with previous studies, which described the effect of mutations in *marR* that increased the MICs of chloramphenicol, tetracycline and cefuroxime.[25,26]

Transcriptional profile analysis was undertaken in order to determine genes that might play a role in decreased susceptibility to tigecycline in *E. coli*. Many of the genes that were up-regulated in G5048 and G5049 corresponded to the genes that responded to either constitutive overexpression of *marA* or stimulation by paraquat as previously reported.[27,28] In addition to the *mar* and *acr* loci, other up-regulated genes included *nfsA* (*mdaA*), *rimK* and *ybjC*, genes that have been reported to comprise a single operon whose function is related to oxidative stress.[29] The activator of this operon is SoxS, a transcriptional activator belonging to the same XylS/AraC family of activators as MarA. It may not be surprising that genes activated by SoxS can also be up-regulated by MarA, since the two activators share a 41% protein identity.[29] It should be noted that, the data presented here did not show an up-regulation of the ORF *b0853*, which is another putative member of the *nfsA*, *rimK* and *ybjC* operon.

Among the genes that were down-regulated in G5048 and G5049 were *ompF*, encoding an outer membrane porin, and *fimA*, a gene associated with adherence to host tissue. A correlation between decreased expression level of *ompF* and antibiotic resistance has been described previously in *E. coli*.[5,27] The down-regulation of these two genes in response to either the constitutive overexpression of *marA* or stimulation by paraquat has been described previously.[27,28] The altered expression level of several other genes, however, including those of the *ymc* and

ycc gene clusters, has not been noted earlier. These genes may potentially have a role in the modification of the carbohydrate exterior of the cell. A recent study by Peleg *et al.*[30] grouped several of the *ymc* and *ycc* genes into the G4C operon and proposed that they have a role in the formation of the O-antigen capsule. In addition, Paulson *et al*. described a similarity between *etk*, a tyrosine kinase member of this operon, to other members of the MPA1 family, which may function in the export of complex carbohydrates in bacteria.[31,32] Whether the G4C operon is down-regulated in response to an up-regulation of *marA* remains to be determined. Other studies have noted that MarA may repress the transcription of genes such as *purA* and *hdeA* and that modification of the exopolysaccharide layer can occur in association with the efflux mechanism.[33,34]

It should be noted that because expression analyses of G5048 and G5049 demonstrated an overexpression of genes of the *mar* and *acr* operons, targeted mapping of these sites in the IS903ϕkan transposon mutants was accomplished using PCR and DNA sequencing. The majority of transposon insertions mapped to either *marA* or *acrB* confirming the involvement of MarA and AcrAB in the decreased tigecycline susceptibility in *E. coli*. However, three of the transposon insertions did not map to either the *marRAB* or the *acrAB* operon. Further studies are in progress to map these mutations and elucidate their effect on tigecycline susceptibility.

In summary, the results suggested that *marA* overexpression is a key factor that leads to overproduction of the AcrAB multidrug efflux pump and, consequently, to decreased tigecycline susceptibility in *E. coli*.

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Transparency declarations

All authors are employees of Wyeth and own shares in the company.

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