Roles of Salmonella multidrug efflux pumps in tigecycline resistance

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Objectives: Salmonella enterica strains exhibiting decreased susceptibility to tigecycline have been reported. In this study, we sought to elucidate the roles of Salmonella multidrug efflux pumps and AcrAB regulators in tigecycline resistance.

Methods: We examined the involvement of multidrug efflux pumps and AcrAB regulators in resistance to tigecycline and other glycylcyclines by determining the MICs of the drugs for Salmonella multidrug efflux pump and AcrAB regulator-overproducing or -deleted strains. Strains of S. enterica serovar Typhimurium derived from the wild-type strain ATCC 14028s were used in this study.

Results: A plasmid carrying the tet gene conferred resistance to 9-(N,N-dimethylglycylamido)-6-demethyl-6-deoxytetracycline (‘DMG-DMDOT’) minocycline, doxycycline and tetracycline, but does not affect tigecycline resistance. Deletion of acrB resulted in strains with significantly increased susceptibility to tigecycline and other glycylcyclines. Plasmids carrying the acrB or acrEF gene restored increased susceptibility of the acrB-deleted mutant to all tested compounds. Deletion of ramA, a positive regulator of acrB, slightly increased susceptibility to tigecycline. Overexpression of ramA and deletion of ramR, a repressor of ramA, resulted in decreased susceptibility to all tested compounds. This phenotype, modulated by ramA or ramR, was not observed in the acrB-deleted background.

Conclusions: AcrAB and AcrEF confer resistance to tigecycline and tetracycline derivatives in Salmonella. RamA and RamR are also involved in resistance to tigecycline in an AcrAB-dependent manner.

Keywords: glycylcyclines, multidrug resistance, resistance–nodulation–cell division family

Introduction

Salmonella causes a variety of diseases in humans, ranging from gastroenteritis to bacteraemia and typhoid fever.1 In the 1990s, the prevalence of multidrug-resistant Salmonella enterica increased dramatically in the UK, USA and Canada.2–5 Many other countries have also documented outbreaks associated with drug-resistant Salmonella in poultry, beef and pork.6–10 Emerging resistance in Salmonella has been observed in both humans and animals, and, thus, is a potentially serious public health problem.11,12 Drug resistance in bacteria is often associated with multidrug efflux pumps that decrease cellular drug accumulation.13,14 Drug resistance in Salmonella spp. is of grave concern, more so in quinolone-resistant and extended-spectrum β-lactamase-producing isolates that cause complicated infections. This has necessitated the search for newer classes of antimicrobials, such as glycylcyclines. Tigecycline (GAR-936), 9-((butylglycylamido)-minocycline, is a novel broad-spectrum antibiotic that is classified as a glycylcycline and is a derivative of minocycline.15 Modification at the 9 position enables tigecycline to overcome the two major mechanisms responsible for tetracycline resistance: tetracycline-specific efflux pump acquisition; and ribosomal protection. Tigecycline is a poor substrate for tetracycline-specific efflux pumps; it attaches to ribosomes that have been modified by the Tet(M) protein.15 It has strong antibacterial activity against many Gram-positive and Gram-negative organisms and lacks cross-resistance to other compounds. It is effective against multidrug-resistant pathogens such as methicillin-resistant Staphylococcus aureus, vancomycin-resistant enterococci, extended-spectrum β-lactamase-expressing Enterobacteriaceae and penicillin-resistant Streptococcus pneumoniae.16–18

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However, some bacterial strains are less susceptible to tigecycline. Tigecycline has been reported to have poor activity against *Pseudomonas aeruginosa*, in which there is active efflux by MexXY–OprM, a member of the resistance–nodulation–cell division (RND) family of efflux pumps. In *Enterobacter cloacae*, *Escherichia coli*, Morganella morgani and *Klebsiella pneumoniae*, it has also been reported that resistance to tigecycline results from overproduction of AcrAB. In *E. cloacae* and *K. pneumoniae*, it was reported that tigecycline resistance resulting from overproduction of AcrAB may be caused by overexpression of its positive regulator RamA.

*S. enterica* strains exhibiting decreased susceptibility to tigecycline have recently emerged. A recent study has shown that *Salmonella* has at least nine multidrug efflux pumps. These pumps are classified into four families: RND; major facilitator; multidrug and toxic compound extrusion; and ATP-binding cassette. Among these pumps, AcrAB is effective in generating multidrug resistance and has wide substrate specificity. AcrAB is an RND family of efflux pumps. In *Pseudomonas aeruginosa*, multidrug efflux pumps and AcrAB regulator-mediated multidrug resistance in *Salmonella* acrAB have recently emerged. A recent study has shown that RamA is a master regulator of *Salmonella* acrAB. RamR is a local repressor of ramA, and RamA and RamR are involved in AcrAB-mediated multidrug resistance in *Salmonella*. In this study, we investigated the roles of *Salmonella* multidrug efflux pumps in resistance to tigecycline and other glyyclcyclines using *Salmonella* multidrug efflux pumps and AcrAB regulator-overproducing or -deleted strains.

### Materials and methods

#### Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. The strains of *S. enterica* serovar Typhimurium used in this study are derived from the wild-type strain ATCC 14028s. Bacterial strains were grown at 37°C in Luria–Bertani (LB) broth. Ampicillin was added to the growth medium at a final concentration of 100 mg/L for plasmid maintenance.

#### Construction of gene deletion mutants

To construct the ΔramR mutant, gene disruption was performed as described by Datsenko and Wanner. The following oligonucleotide primers were used for the construction of the mutants: ramR-P1 (TCCA ATCCCAAGCGGAAATTAGCCGATGGGGATCGCGGATGCTGTTAGCGGAC TGCTT); and ramR-P2 (AAGCTCTGAAAGCGCAACCCGATGATAGCG CAATCGCGTATCATGATATCTCCTTATG). The chloramphenicol resistance gene cat, flanked by Fip recognition sites, was amplified by PCR using the primers listed above. The resulting PCR products were used to transform the recipient ATCC 14028s strain that harbours plasmid pKD46.

### Table 1. Strains and plasmids used in this study

| Strain or plasmid | Original name | Characteristics | Source or reference |
|-------------------|---------------|-----------------|---------------------|
| wild-type         | ATCC 14028s   | *S. enterica* serovar Typhimurium wild-type | 29                  |
| wild-type/pBR322-tet | NKS1203       | ATCC 14028s/pBR322-tet | this study          |
| ΔacrB             | NKS1204       | ATCC 14028s/pBR322-Δtet | this study          |
| ΔacrB/pBR322-tet  | NKS148        | ΔacrB::KmR/pBR322-tet | 37                  |
| ΔacrB/pBR322-Δtet | NKS442        | ΔacrB::KmR/pBR322-Δtet | this study          |
| ΔacrB/Δacrb         | NKS1219       | ΔacrB::KmR/pUC118 | 37                  |
| ΔacrB/Δacrb/ΔacrEF | NKS576        | ΔacrB::KmR/pacrEF | 37                  |
| ΔacrB/Δacrb/ΔacrD  | NKS757        | ΔacrB::KmR/pacrD | 37                  |
| ΔacrB/Δacrb/ΔmdtABC | NKS758       | ΔacrB::KmR/pmdtABC | 37                  |
| ΔacrB/Δacrb/ΔmdsABC | NKS451       | ΔacrB::KmR/pmdsABC | 37                  |
| ΔacrB/Δacrb/ΔemrAB  | NKS443        | ΔacrB::KmR/emrAB | 37                  |
| ΔacrB/Δacrb/ΔmdfA   | NKS759        | ΔacrB::KmR/mdfA | 37                  |
| ΔacrB/Δacrb/ΔmdtK   | NKS447        | ΔacrB::KmR/mdtK | 37                  |
| ΔacrB/Δacrb/ΔmacAB  | NKS446        | ΔacrB::KmR/macAB | 37                  |
| ΔtolC              | NKS174        | ΔtolC/pBR322-tet | 37                  |
| ΔtolC/Δacrb         | NKS1220       | ΔtolC/pBR322-Δtet | this study          |
| ΔtolC/Δacrb/ΔtolC   | NKS745        | ΔtolC/pUC118 | 37                  |
| ΔtolC/Δacrb/ΔtolC   | NKS775        | ΔtolC/pacrAB | 37                  |
| ΔtolC/Δacrb/ΔtolC   | NKS747        | ΔtolC/pacrEF | 37                  |
| ΔtolC/Δacrb/ΔtolC   | NKS748        | ΔtolC/pacrD | 37                  |
| ΔtolC/Δacrb/ΔtolC   | NKS749        | ΔtolC/pmdtABC | 37                  |
| ΔtolC/Δacrb/ΔtolC   | NKS754        | ΔtolC/pmdsABC | 37                  |
| ΔtolC/Δacrb/ΔtolC   | NKS751        | ΔtolC/pemrAB | 37                  |
| ΔtolC/Δacrb/ΔtolC   | NKS750        | ΔtolC/mdfA | 37                  |

Continued
Table 1. Continued

| Strain or plasmid | Original name | Characteristics | Source or reference |
|-------------------|---------------|-----------------|---------------------|
| ΔtolC::pmdtK      | NKS753        | ΔtolC::pmdtK    | 37                  |
| ΔtolC::pmacAB     | NKS752        | ΔtolC::pmacAB   | 37                  |
| ΔacrAB            | NKS145        | ΔacrAB::CmR     | 25                  |
| ΔacrEF            | NKS176        | ΔacrEF          | 25                  |
| ΔacrD             | NKS177        | ΔacrD           | 25                  |
| ΔmdtABC           | NKS151        | ΔmdtABC::CmR    | 25                  |
| ΔmdsABC           | NKS168        | ΔmdsABC::CmR    | 25                  |
| ΔemrAB            | NKS133        | ΔemrAB::CmR     | 25                  |
| ΔmdfA             | NKS135        | ΔmdfA::CmR      | 25                  |
| ΔmdtK             | NKS137        | ΔmdtK::CmR      | 25                  |
| ΔmacAB            | NKS136        | ΔmacAB::CmR     | 25                  |
| ΔacrAB            | NKS175        | ΔacrAB::CmR     | 25                  |
| ΔacrAB acrEF      | NKS181        | ΔacrABΔacrEF    | 25                  |
| ΔacrAB acrEF acrD | NKS183        | ΔacrABΔacrEFΔacrD | 25                  |
| ΔacrAB acrEF acrD mdtABC | NKS185 | ΔacrABΔacrEFΔacrDΔmdtABC | 25 |
| ΔacrAB acrEF acrD mdtABC mdsABC | NKS186 | ΔacrABΔacrEFΔacrDΔmdtABCΔmdsABC::CmR | 25 |
| ΔacrAB acrEF acrD mdtABC mdsABC emrAB | NKS188 | ΔacrABΔacrEFΔacrDΔmdtABCΔmdsABCΔemrAB::CmR | 25 |
| ΔacrAB acrEF acrD mdtABC mdsABC emrAB mdfA | NKS190 | ΔacrABΔacrEFΔacrDΔmdtABCΔmdsABCΔemrABΔmdfAΔmdfK::KmR | 25 |
| ΔacrAB acrEF acrD mdtABC mdsABC emrAB mdfA mdtK | NKS195 | ΔacrABΔacrEFΔacrDΔmdtABCΔmdsABCΔemrABΔmdfAΔmdfK::KmR | 25 |
| ΔacrAB acrEF acrD mdtABC mdsABC emrAB mdfA mdtK macAB | NKS196 | ΔacrABΔacrEFΔacrDΔmdtABCΔmdsABCΔemrABΔmdfAΔmdfK::KmRΔmacAB::CmR | 25 |
| ΔmarA             | NES15         | ΔmarA::CmR      | 27                  |
| Δrob              | NES23         | Δrob::CmR       | 27                  |
| ΔsoxS             | NES21         | ΔsoxS::CmR      | 27                  |
| ΔsdiA             | NES34         | ΔsdiA::CmR      | 27                  |
| ΔramA             | NES57         | ΔramA::CmR      | 27                  |
| ΔramR             | NKS910        | ΔramR::CmR      | 27                  |
| ΔacrB ramR        | NKS1211       | ΔacrB::KmRΔramA::CmR | 25 |
| wild-type/vector (pMAL) | NES79 | ATCC 14028s/pMALc2X | this study |
| wild-type/pramA   | NES80         | ATCC 14028s/pramA | this study |
| ΔacrB/vector (pMAL) | NKS1209 | ΔacrB::KmR/pMALc2X | this study |
| ΔacrB/pramA       | NKS1210       | ΔacrB::KmR/pramA | this study |

Plasmids

| Plasmid | Source or reference |
|---------|---------------------|
| pKD3    | rep_resize ApR FRT CmR FRT Takara Bio, Inc. |
| pBR322-tet | CoE1-type plasmid carrying tet gene Takara Bio, Inc. |
| pBR322-Δtet | pBR322, but tet gene disrupted this study |
| pUC118  | rep_UC118 ApR Takara Bio, Inc. |
| pacrAB  | acrAB genes cloned into pUC118, ApR |
| pacrEF  | acrEF genes cloned into pUC118, ApR |
| pacrD   | acrD genes cloned into pUC118, ApR |
| pmdtABC | mdtABC genes cloned into pUC118, ApR |
| pmdsABC | mdsABC genes cloned into pUC118, ApR |
| pemrAB  | emrAB genes cloned into pUC118, ApR |
| pmdfA   | mdfA genes cloned into pUC118, ApR |
| pmdtK   | mdtK genes cloned into pUC118, ApR |
| pmacAB  | macAB genes cloned into pUC118, ApR |
| pMALc2X | vector, ApR New England Biolabs |
| pramA   | ramA-His6 gene cloned into pMALc2X, ApR |

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which expresses Red recombinase. The chromosomal structure of the mutated loci was verified by PCR.30

**Chemicals**

Tigecycline, 9-(N,N-dimethylglycylamido)-6-demethyl-6-deoxytetracycline (DMG-DMDOT), minocycline, doxycycline and tetracycline were obtained from Wyeth-Lederle Japan (Tokyo, Japan).

**Determination of MICs of toxic compounds**

The MICs of various agents were determined on LB agar plates containing tigecycline, DMG-DMDOT, minocycline, doxycycline and tetracycline at various concentrations. Agar plates were prepared by the 2-fold agar dilution technique as described previously.31 To determine the MICs, bacteria were grown in LB broth at 37°C overnight, diluted into the same medium and then tested at a final inoculum size of 10^5 cfu/μL using a multipoint inoculator (Sakuma Seisakusyo, Tokyo, Japan) after incubation at 37°C for 20 h. The MIC was the lowest concentration of the compound that inhibited cell growth.

**Results and discussion**

**Involvement of the tet gene in tigecycline resistance**

Previous studies suggest that the tetracycline resistance determinant gene tet does not affect susceptibility to tigecycline in Enterobacteriaceae, S. aureus and S. pneumoniae isolates.32,33 In order to confirm whether tet is involved in resistance to

| Table 2. Susceptibilities of Salmonella multidrug efflux pump and AcrAB regulator-overproducing or -deleted strains to tigecycline and other glyyclcyclines

| Strain                          | TGC | DMG-DMDOT | MIN | DOX | TET |
|--------------------------------|-----|-----------|-----|-----|-----|
| Wild-type                      | 1   | 2         | 4   | 4   | 4   |
| Wild-type/pBR322-tet           | 1   | 16        | 16  | 64  | 256 |
| ΔacrB                          | 0.25| 0.5       | 0.25| 0.5 | 1   |
| ΔacrB/pBR322-tet               | 0.25| 4         | 0.5 | 4   | 32  |
| ΔacrB/Δtet                     | 0.25| 0.5       | 0.25| 0.5 | 1   |
| ΔacrB/vector                   | 0.25| 0.5       | 0.25| 0.5 | 1   |
| ΔacrB/pacrAB                   | 2   | 2         | 4   | 4   | 4   |
| ΔacrB/pacrEF                   | 2   | 2         | 4   | 2   | 2   |
| ΔacrB/pmdfA                    | 0.25| 0.5       | 0.25| 0.5 | 1   |
| ΔtolC                          | 0.25| 0.5       | 0.25| 0.5 | 1   |
| ΔtolC/pBR322-tet               | 0.25| 4         | 0.5 | 4   | 32  |
| ΔtolC/Δtet                     | 0.25| 0.5       | 0.25| 0.5 | 1   |
| ΔtolC/Vector                   | 0.25| 0.5       | 0.25| 0.5 | 1   |
| ΔtolC/pacrAB                   | 0.25| 0.5       | 0.25| 0.5 | 1   |
| ΔtolC/pacrEF                   | 0.25| 0.5       | 0.25| 0.5 | 1   |
| ΔtolC/pmdfA                    | 0.25| 0.5       | 0.25| 0.5 | 1   |
| ΔacrAB                         | 0.25| 0.5       | 0.25| 0.5 | 1   |
| ΔacrEF                         | 1   | 2         | 4   | 4   | 4   |
| ΔacrAB acrEF                   | 0.25| 0.5       | 0.25| 0.5 | 1   |
| ΔacrAB acrEF acrD mdtABC mdsABC emrAB mdfA mdtK macAB | 0.25| 0.5 | 0.25 | 0.5 | 1 |
| ΔramA                          | 0.5 | 2         | 4   | 4   | 4   |
| ΔramR                          | 4   | 4         | 16  | 16  | 8   |
| ΔacrB ramR                     | 0.25| 0.5       | 0.25| 0.5 | 1   |
| Wild-type/vector(pMAL)         | 1   | 2         | 4   | 4   | 4   |
| Wild-type/promA                | 4   | 4         | 16  | 16  | 8   |
| ΔacrB/vector(pMAL)             | 0.25| 0.5       | 0.25| 0.5 | 1   |
| ΔacrB/promA                    | 0.25| 0.5       | 0.25| 0.5 | 1   |

TGC, tigecycline; MIN, minocycline; DOX, doxycycline; TET, tetracycline. Values in bold type are larger than those for parental control strains. MICs of all tested compounds for ΔacrB harbouring pacrD, pmdtABC, pmdsABC, pemrAB, pemrK or pmacAB were the same as those for ΔacrB/vector. MICs of all tested compounds for ΔtolC harbouring pacrD, pmdtABC, pmdsABC, pemrAB, pemrK or pmacAB were the same as those for ΔtolC/vector. MICs of all tested compounds for ΔacrD, ΔmdtABC, ΔmdsABC, ΔemrAB, ΔmdfA, ΔmdtK and ΔmacAB were the same as those for wild-type. MICs of all tested compounds for ΔacrAB acrEF acrD mdtABC mdsABC emrAB mdfA mdtK were the same as those for ΔacrAB. MICs of all tested compounds for ΔmarA, Δrob, ΔsoxS and ΔsdiA were the same as those for wild-type.
Tigecycline resistance conferred by efflux pumps

Effects of drug efflux pumps on MICs of tigecycline and other glycylcyclines

The plasmids carrying the acrAB, acrEF and mdfA genes conferred resistance to tetracycline in the ΔacrB mutant (Table 2). This is in agreement with the results of a previous study. The plasmids carrying acrAB and acrEF conferred resistance to all tested compounds in the ΔacrB mutant (2- to 16-fold increase in MICs compared with ΔacrB/vector) (Table 2). However, the plasmids carrying acrAB and acrEF did not confer resistance to the ΔtolC mutant. These results indicate that the AcrAB–TolC and AcrEF–TolC systems are involved in resistance to tigecycline and other glycylcyclines. Deletion of the acrA gene resulted in strains with significantly increased (4- to 16-fold compared with wild-type) susceptibility to all tested compounds, although deletion of the acrEF gene did not affect susceptibility to any of the tested compounds (Table 2). These results suggest that AcrAB makes a principal contribution to resistance to tigecycline and other glycylcyclines. Stepwise deletion of the nine efflux genes from the ΔacrB mutant did not affect susceptibility to any of the tested compounds (Table 2). These data support the claim discussed above that AcrAB plays a critical role in resistance to tigecycline and other glycylcyclines.

Involvement of AcrAB regulators in resistance to tigecycline and other glycylcyclines

Deletion of the ramA gene resulted in slightly increased (2-fold decrease in MIC compared with wild-type) susceptibility to tigecycline (Table 2) and overexpression of ramA in the wild-type strain resulted in decreased (2- to 4-fold increase in MICs compared with wild-type) susceptibility to all tested compounds (Table 2). Deletion of the ramR gene resulted in significantly decreased (2- to 4-fold increase in MICs compared with wild-type) susceptibility to all tested compounds. Deletion of other AcrAB regulator genes, such as marA, rob, soxS and sdiA, did not affect susceptibility to any of the tested compounds. These results suggest that RamA and RamR are involved in resistance to tigecycline and other glycylcyclines. To investigate the involvement of the AcrB efflux pump in glycylcycline resistance modulated by RamA and RamR, we determined the MICs for the RamA-overproducing ΔacrB strain and the ΔacrB ramR double mutant. Neither overproduction of RamA nor RamR deletion in the ΔacrB mutant affected susceptibility to the tested compounds. These data indicate that RamA and RamR are involved in resistance to tigecycline and other glycylcyclines in an AcrAB-dependent manner.

Conclusions

Our results suggest that AcrAB and its close homologue AcrEF confer tigecycline resistance in S. enterica serovar Typhimurium ATCC 14028s. Results for the ΔacrB mutant showed that the AcrAB efflux pump made a significant contribution to resistance to all the compounds that were tested, but that deletion of the acrEF gene did not affect susceptibility to these compounds. These findings may be attributable to the difference in expression level between the acrAB and acrEF genes. A recent study has shown that acrEF is repressed by the histone-like nucleoid structuring protein (H-NS), whereas acrAB is not repressed and is constitutively expressed in S. enterica. AcrAB therefore makes a principal contribution to resistance to tigecycline and other glycylcyclines.

Deletion of the ramA gene, one of several regulators of Salmonella multidrug efflux pumps, resulted in strains with slightly increased susceptibility to tigecycline, and overexpression of the ramA gene resulted in resistance to all tested compounds. Deletion of the ramR gene also resulted in significant resistance to all tested compounds. This resistance, modulated by RamA and RamR, was dependent on the presence of the AcrB efflux pump. Our data suggest that RamA and RamR are effective in exhibiting resistance and that marA, rob, soxS and sdiA do not affect susceptibility to tigecycline and other glycylcyclines.

In this study, we elucidated the mechanism of tigecycline resistance in Salmonella. Our results suggest that the AcrAB–TolC and AcrEF–TolC systems play a role in resistance to tigecycline and other glycylcyclines, and that AcrAB makes a major contribution. In addition, overexpression of ramA and inactivation of ramR conferred increased (4-fold) resistance to tigecycline in an AcrAB-dependent manner.

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Transparency declarations

None to declare.

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