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# Combined *ramR* Mutation and Presence of a Tn*1721*-Associated *tet*(A) Variant in a Clinical Isolate of *Salmonella enterica* Serovar Hadar Resistant to Tigecycline▽

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**A *Salmonella enterica* serovar Hadar strain resistant to tigecycline (MIC, 16 μg/ml) was isolated. Molecular characterization revealed the presence of a plasmid-borne *tet*(A) variant associated with Tn*1721* mediating a rise of the MIC for tigecycline when transferred to *Escherichia coli*. Additionally, a truncating mutation in *ramR* was detected. Transformation with wild-type *ramR* but not with the mutated *ramR* lowered the MIC for tigecycline. Characterization of this *Salmonella* isolate implicates *ramR* in resistance to tigecycline.**

Tigecycline is a novel broad-spectrum antibiotic belonging to the class of glycylcyclines and is chemically derived from the tetracycline minocycline (17). Tigecycline is notable for its antibacterial activity against an extraordinarily broad range of bacteria, with only few naturally resistant exceptions, namely, *Proteus* spp., *Morganella morganii*, *Providencia* spp., and *Pseudomonas aeruginosa*. In particular, the *in vitro* and *in vivo* activities of tigecycline against multidrug-resistant pathogens like methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus* (VRE), extended-spectrum beta-lactamase (ESBL)-expressing *Enterobacteriaceae*, and carbapenem-resistant strains make this drug a promising agent for difficult-to-treat infections (14, 18, 27). Resistance in isolates of usually susceptible pathogens has so far only rarely been described and has been limited to isolates of *Klebsiella pneumoniae* (3, 23), *Enterobacter cloacae* (12), *Acinetobacter baumannii* (21), *Escherichia coli* (13), *Enterococcus faecalis* (31), and *Staphylococcus aureus* (15). Although the exact mechanisms of resistance could not be definitely determined, a common finding in these sporadic isolates as well as in the naturally resistant species is overexpression of different efflux pumps: AcrAB RND-type efflux pumps in *Enterobacteriaceae*, certain other RND-type efflux pumps in *Pseudomonas aeruginosa* (MexXY) and *Acinetobacter* species (AdeABC and AdeIJK), and MATE family efflux pumps in *Staphylococcus aureus* (7, 8, 12, 13, 15, 24–26, 30). Although genes conferring resistance to tetracyclines do not seem to have an effect on susceptibility to tigecycline (9, 11), mutants of Tet(A) and Tet(B) with altered substrate specificities have been isolated that demonstrated low-level resistance against an early glycylcycline (10) and tigecycline (29). So far, tigecycline resistance due to Tet(A) or Tet(B) variants has not been described in clinical isolates.

MICs for tigecycline were determined in this study by broth microdilution (5, 6) using a commercially available tigecycline panel (MERLIN Diagnostika GmbH, Bornheim-Hersel, Germany) with freshly prepared (<12-h-old) Mueller-Hinton II broth (BBL, BD Bioscience, Sparks, MD). All MICs were interpreted according to EUCAST clinical breakpoints as susceptible, intermediate, or resistant.

We isolated a *Salmonella enterica* serovar Hadar strain (VA5649) resistant to tigecycline with a MIC of 16 μg/ml. The patient had no known history of tigecycline exposure. Molecular analysis for the presence of known *tet* genes (2) revealed the presence of *tet*(A). Sequencing of the full open reading frame of the *tet*(A) gene revealed a previously described (29) double frameshift mutation compared to the RP1-linked *tet*(A) gene (gene id number gi:42508), leading to the substitution of amino acids 201, 202, and 203 (serine, phenylalanine, and valine to alanine, serine, and phenylalanine, respectively) in the interdomain loop. This variant was found to elevate the MIC for the glycylcycline GAR-936, now termed tigecycline (29). In order to analyze whether the *tet*(A) gene resides on a plasmid, we extracted plasmid DNA from *S. enterica* VA5649 and transformed competent tetracycline-susceptible *E. coli* (Top Ten; Invitrogen) with the DNA preparation. Tetracycline-resistant clones could be isolated that carried the same *tet*(A) gene as the *Salmonella* isolate, confirming that the *tet*(A) gene is localized on a plasmid. This transformed *E. coli* clone [*E. coli* DH10B with Tn*1721*-*tet*(A) plasmid] also exhibited an elevated MIC for tigecycline (MIC, 0.25 μg/ml), compared to the untransformed *E. coli* (strain DH10B) with a MIC of 0.065 μg/ml (Table 1). In order to exclude effects of other genes on the natural plasmid, the *tet*(A)/*tetR*(A) unit [54 nucleotides upstream of the *tet*(A) stop codon and 57 nucleotides downstream of the *tetR*(A) stop codon; primers AGGATCCTAGC TTGCCGGAAGTCGCCTTGA and AAAGCTTATGTTGT CTACATGGCTCTGC; reference sequence gi:48194] was amplified from the plasmid and cloned into the pSKII vector. *E. coli* carrying the pSKII-*tet*(A)/*tetR* construct {*E. coli* DH10B with pSKII[Tn*1721*-*tet*(A)]} showed the same MIC increase for tigecycline (0.25 μg/ml) as *E. coli* carrying the natural plasmid, compared to a strain carrying an empty pSKII vector (*E. coli* DH10B with pSKII; MIC, 0.065 μg/ml) (Table 1). Although *E. coli* DH10B with pSKII[Tn*1721*-*tet*(A)] remained

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TABLE 1. MICs and relative *ramA* and *acrB* expression levels of strains used in this study

| Isolate | MIC (μg/ml) (susceptibility)[a] | | | | Relative expression of[d]: | |
|---|---|---|---|---|---|---|
| | Tigecycline[b] | Ciprofloxacin[c] | Chloramphenicol[c] | Minocycline[c] | *ramA* | *acrB* |
| *S. enterica* serovar Hadar VA5649 | 16 (R) | 1.0 (I) | 8.0 (I) | 32 (R) | 1 | 1 |
| *E. coli* DH10B | 0.065 (S) | 0.002 (S) | ND[e] | 0.75 (S) | ND | ND |
| *E. coli* DH10B + Tn*1721-tet*(A) plasmid | 0.25 (S) | 0.002 (S) | ND | 8 (R) | ND | ND |
| *E. coli* DH10B + pSKII | 0.065 (S) | ND | ND | 0.75 (S) | ND | ND |
| *E. coli* DH10B + pSKII[Tn*1721-tet*(A)] | 0.25 (S) | ND | ND | 8.0 (R) | ND | ND |
| *S. enterica* TY2313-WT | 0.25 (S) | ND | 2.0 (S) | ND | 0.005 | 0.225 |
| *S. enterica* serovar Hadar VA5649(*ramR*-2313-WT) | 2 (I) | 0.25 (S) | 2.0 (S) | ND | 0.044 | 0.171 |
| *S. enterica* serovar Hadar VA5649(*ramR*-5649) | 16 (R) | 1.0 (I) | 8.0 (I) | ND | 1.932 | 1.569 |
| *S. enterica* serovar Hadar VA5649(pACYC177) | 16 (R) | 1.0 (I) | 8.0 (I) | ND | 1.189 | 1 |

[a] S, susceptible; I, intermediate; R, resistant (according to EUCAST clinical breakpoints [www.eucast.org]).
[b] Tested by broth microdilution.
[c] Tested by Etest.
[d] Measured by quantitative RT-PCR and normalized to expression levels of VA5649 (expression of 1).
[e] ND, not determined.

in the susceptible range, the elevation of the MIC suggested that the *tet*(A) variant contributes to tigecycline resistance in the *Salmonella* isolate. Furthermore, we confirmed the observation that this variant also confers resistance against minocycline (Table 1), a property commonly attributed only to Tet(B) among the tetracycline efflux pumps (4). Sequencing of the upstream and downstream regions of the plasmid starting from the *tet*(A) locus showed that it is situated in a highly conserved Tn*1721* element, which has 3,163 nucleotides deleted at the 5′ end, compared with the complete Tn*1721* element (gi:48194). No mutations in the *tetR*(A) regulator or the intergenic region between *tet*(A) and *tetR*(A), which harbors regulatory sequences, were found on comparison with the Tn*1721* reference sequence (gi:48194). Subsequent database searches revealed that Tn*1721*-associated *tet*(A) commonly harbors the above-described amino acid exchanges in the interdomain loop, which may suggest that bacteria carrying the Tn*1721* element may generally be less susceptible to tigecycline.

The high MIC for tigecycline in *S. enterica* VA5649 suggested additional resistance mechanisms. Resistance to tigecycline in *Enterobacter cloacae* and *Klebsiella pneumoniae* has been linked to overexpression of *ramA*, a positive regulator of the AcrAB efflux system (12, 26). The AcrAB system is an efflux pump that confers low-level resistance to a wide range of antibiotics (19, 20). Recently, mutations in *ramR*, a putative negative regulator of *ramA*, were shown to result in overexpression of *ramA* and resistance to ciprofloxacin in *Salmonella* (1, 16, 22). We reasoned that *ramR* may be involved in resistance to tigecycline in *Salmonella* and sequenced the coding region of *ramR* and the intergenic region between *ramA* and *ramR*, as previously described (1). Sequencing revealed, besides two silent nucleotide exchanges (207T>C and 330G>A), deletion of cytosine 515, resulting in a frameshift after amino acid 171 with a divergent C terminus and truncation of 22 amino acids. *ramR* was cloned from an *S. enterica* isolate (TY2313) with a wild-type MIC for tigecycline (MIC, 0.25 μg/ml) and from the resistant strain *S. enterica* VA5649 and inserted into the pACYC177 low-copy-number vector. Transformation of *S. enterica* VA5649 with *ramR*-2313 [*Salmonella enterica* serovar Hadar VA5649(*ramR*-2313-WT)] lowered the MIC for tigecycline from 16 μg/ml to 2 μg/ml (Table 1). In contrast, transformation of VA5649 with either the mutated *ramR* [*Salmonella enterica* serovar Hadar VA5649(*ramR*-5649)] or the empty pACYC177 vector [*Salmonella enterica* serovar Hadar VA5649(pACYC177)] did not lower the tigecycline MIC (Table 1). Furthermore, MICs for two other known substrates of AcrAB, ciprofloxacin and chloramphenicol (determined by Etest; AB Biodisk, Solna, Sweden), were affected in a similar manner. *S. enterica* serovar Hadar VA5649, *S. enterica* serovar Hadar VA5649(*ramR*-5649), and *S. enterica* serovar Hadar VA5649(pACYC177) exhibited MICs of 1.0 μg/ml for ciprofloxacin and 8.0 μg/ml for chloramphenicol, which were both interpreted as intermediate according to EUCAST clinical breakpoints, yet the introduction of wild-type *ramR* in *S. enterica* serovar Hadar VA5649(*ramR*-2313) lowered the MIC for ciprofloxacin to 0.25 μg/ml and the MIC for chloramphenicol to 2.0 μg/ml, which were both interpreted as susceptible. As RamR had been suggested to be a negative regulator of *ramA*, we analyzed expression of *ramA* in the different strains by Northern blotting (Fig. 1) (hybridization probes for *ramA* were generated with primers ATG ACCATTTCCGCTCAGGTTA and TCAATGCGTACGA CCATG and for *acrB* and 16S rRNA we used the reverse transcription-PCR [RT-PCR] primers described in reference 16) and by quantitative RT-PCR as described previously (16) (Table 1). While *ramA* expression was virtually absent in the wild-type strain *S. enterica* TY2313, *ramA* was massively overexpressed in the tigecycline-resistant strain *S. enterica* serovar Hadar VA5649. Introduction of wild-type *ramR* into VA5649 [*S. enterica* serovar Hadar VA5649(*ramR*-2313-WT)] effectively repressed *ramA*. In contrast, transformation with the mutated allele in *S. enterica* serovar Hadar VA5649(*ramR*-5649) or the empty vector in *S. enterica* serovar Hadar VA5649(pACYC177) did not repress *ramA* expression. Furthermore, upregulation of *ramA* in strains carrying only the mutated *ramR* gene, *S. enterica* serovar Hadar VA5649, *S. enterica* serovar Hadar VA5649(*ramR*-5649), and *S. enterica* serovar Hadar VA5649(pACYC177), was paralleled by up-

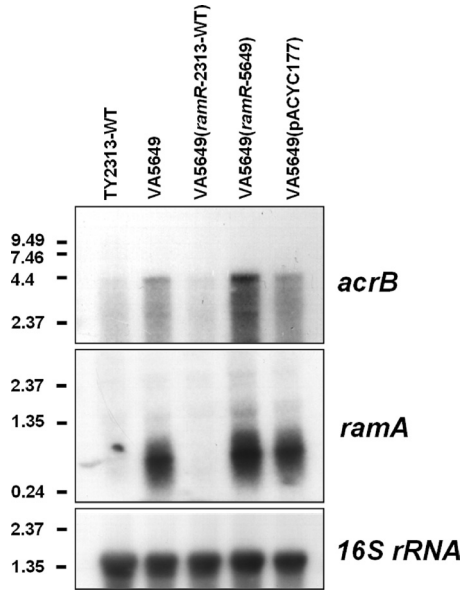


FIG. 1. RamR is a repressor of *ramA*. Expression levels of *ramA* and *acrB* were analyzed in different strains by Northern blotting. Total RNA was extracted from mid-log-phase cultures of the indicated strains. Three micrograms of total RNA was loaded onto each lane. The filter was hybridized consecutively in the following order with [$^{32}$P]dCTP-labeled probes of *ramA*, *acrB*, and 16S rRNA. Expected sizes of bands: *ramA*, ~350 bp; *acrB*, ~4,000 bp; 16S rRNA, ~1,500 bp. Values on the left margin are band sizes in kbp.

regulated *acrB* expression, while *acrB* expression was low in the wild-type strain, *S. enterica* TY2313, and the complemented strain *S. enterica* serovar Hadar VA5649(*ramR*-2313-WT). These findings support the concept of RamR being a repressor of *ramA* and of RamA being an activator of the AcrAB system.

In conclusion, we report here a clinical *Salmonella* isolate highly resistant to tigecycline and have characterized the underlying molecular mechanisms. Our results imply that the combination of the two low-level resistance mechanisms, Tn*1721-tet*(A) and inactivation of *ramR*, results in complete resistance to tigecycline. The prevalence of Tn*1721-tet*(A) in clinical isolates is currently unknown but may be low, and its clinical significance might be limited. Recent studies did not find full resistance to tigecycline in *tet*(A)-carrying *E. coli* isolates, yet the presence of the Tn*1721*-associated *tet*(A) was not specifically investigated (28). None of the tetracycline-resistant *Salmonella* strains from our collection ($n = 17$) carried this gene (data not shown). It will be important to systematically address this issue for a broader range of bacteria in future studies. Mutations in *ramR* have so far only been described in *Salmonella* resistant to ciprofloxacin. We have shown for the first time that mutations in *ramR* also mediate resistance to tigecycline, presumably by upregulation of *ramA*. Thus, a preceding therapy with ciprofloxacin (and presumably other antibiotics) may affect susceptibility to tigecycline, even though the patient has never been treated with this drug.

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