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Galarion, LH, Clarke, JE, Schofield, H et al. (1 more author) (2023) Comment on: Identification of a novel tedizolid resistance mutation in rpoB of MRSA after in vitro serial passage. Journal of Antimicrobial Chemotherapy, 78 (1). pp. 317-318. ISSN 0305-7453

https://doi.org/10.1093/jac/dkac397

Comment on: Identification of a novel tedizolid resistance mutation in *rpoB* of MRSA after *in vitro* serial passage

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Sir,

A recent JAC article by Shen *et al.* [1] reported that a single amino acid substitution in RNA polymerase can mediate resistance to the oxazolidinone tedizolid (TZD) and other ribosome-targeting antibiotics in *Staphylococcus aureus*. Following 10 days' serial passage of *S. aureus* N315 in the presence of TZD, the authors of that study recovered a mutant (N315-TDZ4) that exhibited TZD resistance above the clinical breakpoint. N315-TDZ4 also displayed cross-resistance to several other classes of translation inhibitor that bind the ribosome in close proximity to TZD, a phenotype known as $PhLOPS_A$ (for resistance to Phenicols, Lincosamides, Oxazolidinones, Pleuromutilins and Streptogramins of group A). WGS of N315-TDZ4 identified a single nucleotide mutation relative to the parent strain; a $A_{1345}G$ change in the *rpoB* gene that encodes a $N_{449}D$ amino acid substitution in the β-subunit of RNA polymerase. The authors concluded that this substitution was responsible for the observed $PhLOPS_A$ phenotype.

We considered this a surprising finding for several reasons. Despite an extensive body of published work that has examined resistance to oxazolidinones and other $PhLOPS_A$ classes in laboratory strains and clinical isolates, there has to our knowledge been no previous suggestion that resistance can result from mutational change in RNA polymerase. Indeed, there is no obvious explanation for how genetic alteration of the transcription machinery might mediate resistance to a cross-section of structurally unrelated and mechanistically distinct antibacterial drug classes that act on the ribosome. Furthermore, it is not easy to reconcile the idea that resistance to TZD can result from a single point mutation with the observed difficulty of selecting mutants resistant to TZD *in vitro*. Typically, when *S. aureus* is challenged with an antibacterial drug against which resistance can arise via a single point mutation, resistant mutants are recovered at frequencies of $>10^{-9}$.[2] By contrast, we were unable to detect TZD-resistant mutants (frequency of $<10^{-11}$) upon plating concentrated cultures of *S. aureus* N315 onto agar containing 4X MIC of TZD (*data not shown*); and, indeed, Shen and colleagues had to resort to prolonged serial passage under escalating TZD selection to recover the resistant mutant described in their study.[1]

In view of the novel and surprising nature of the conclusion that mutation in RNA polymerase can mediate a $PhLOPS_A$ phenotype, we felt that experimental corroboration for such a causal link was warranted. Shen *et al.* had expressed the opinion that this was unnecessary because

only a single mutational change was detected in N315-TDZ4 relative to the parent strain, which they took to imply that this mutation must be responsible for the observed phenotype. However, this notion appears at odds with the authors' recognition that the short-read DNA sequencing they employed for analysis of N315-TDZ4 cannot be relied upon to detect all mutations present since it does not "…confidently recover variants occurring in repetitive or structurally complex genomic regions".[1]

With a view to establishing whether this mutation is responsible for the observed resistance, we examined whether introduction of *rpoB*$_{A1345G}$ into a 'clean', TZD-susceptible strain background would confer the PhLOPS$_A$ phenotype. Briefly, a DNA fragment corresponding to the entire *rpoB* gene and ribosome binding site (locus tag SAOUHSC_00524) – but carrying the $A_{1345}G$ mutation - was obtained by synthesis (GenScript) and ligated into a modified version of the pIMAY-Z plasmid[3] carrying the P$_{spac}$ promoter (the latter was included to ensure expression of genes downstream of *rpoB* in the same operon during the subsequent allelic exchange process, as transcription of these might otherwise have been disrupted upon integration of the plasmid). The resulting construct was established in *E. coli* IM08B[4] before recovery and electroporation[5] into two independent, TZD-susceptible hosts; *S. aureus* SH1000[6] and N315. Allelic exchange[7] was then performed to introduce the *rpoB*$_{A1345G}$ mutation into the chromosome of both strains, and successful replacement of the native nucleotide was verified initially by PCR amplification/ DNA sequencing and subsequently by WGS (MicrobesNG), with careful *in silico* interrogation of the sequence ~25Kb either side of the engineered mutation to exclude the possibility that other local mutations or genetic rearrangements had occurred during the allelic exchange process. Beyond the presence of the *rpoB*$_{A1345G}$ mutation, no additional genetic changes were detected in that region in the engineered SH1000 mutant. N315*rpoB*$_{A1345G}$ additionally carried four polymorphisms ($C_{1890}T$, $A_{1974}T$, $A_{2013}G$, $A_{2073}T$) in *rpoB* compared to the parent strain that were the result of inherent minor genetic differences between the *rpoB* sequence used for allelic replacement and that of N315; however, these represent synonymous polymorphisms and the encoded protein is therefore identical to that of N315-TDZ4.

Susceptibility testing of SH1000*rpoB*$_{A1345G}$ and N315*rpoB*$_{A1345G}$ revealed no difference in MIC for phenicols (chloramphenicol), oxazolidinones (TZD, linezolid), pleuromutilins (retapamulin) or group A streptogramins (virginiamycin M1) relative to the respective parent strains that

harbour wild-type *rpoB* sequences (*data not shown*). Thus, we have established that a $N_{449}D$ amino acid substitution in the β-subunit of RNA polymerase does not mediate a $PhLOPS_A$ phenotype, at least not by itself.

The genetic basis for resistance in N315-TDZ4 therefore remains to be defined. Our speculation is that it involves mutation in the 23S rRNA – not only are such mutations a well-recognised source of resistance to oxazolidinones and other $PhLOPS_A$ classes in *S. aureus*, [8,9] but they are also not readily detected by standard short-read WGS when present only in a minority subset of the 5-6 rRNA operons on the *S. aureus* genome.[10] We would therefore encourage Shen *et al.* to subject N315-TDZ4 to long-read WGS to either identify any 23S rRNA mutations present or to otherwise establish the true genetic basis for resistance in this strain. For the reason exemplified here, it will be necessary to definitively establish a causal relationship to the resistance phenotype for any such mutation(s) identified.

Funding

This work was supported by internal funding.

Transparency declarations

None to declare.

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