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1 **Identification of a novel tedizolid resistance mutation in *rpoB* of methicillin-resistant**
2 **Staphylococcus aureus**
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24 **Abstract**

25 A tedizolid-resistant isolate of MRSA was selected by serial passage. Whole genome
26 sequencing revealed only a single nucleotide variant in *rpoB*. Cross-resistance to linezolid,
27 chloramphenicol, and quinupristin-dalfopristin was observed but susceptibility to other drugs
28 including rifampin was unchanged. Models of the RNA-polymerase-ribosomal complex revealed
29 that the mutated residue was unlikely to interact directly with the oxazolidinone binding site. This
30 is the first time that *rpoB* mutation has been associated with resistance to the PhLOPSa
31 antimicrobials.

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32 Tedizolid is an oxazolidinone antimicrobial with broad spectrum activity against Gram-
33 positive bacteria including methicillin-resistant *S. aureus* (MRSA).[1] Like other oxazolidinones
34 tedizolid exerts its antibacterial activity by binding the 23s rRNA component of the 50s-
35 ribosomal subunit and thus inhibiting protein synthesis. Resistance to tedizolid is uncommon but
36 mutations in the ribosomal proteins L3, L4, and L22 (encoded by *rplC*, *rplD*, and *rplV*
37 respectively), and the 23S rRNA target, which also mediate the so-called PhLOPSa (phenicol,
38 lincosamide, oxazolidinone, pleuromutilin, and streptogramin A) resistance phenotype have
39 been implicated.[2, 3] Acquisition of the transferable rRNA methyltransferase gene, *cfr*, may also
40 cause resistance to linezolid and other PhLOPSa antimicrobials but is generally believed to be
41 insufficient to produce tedizolid resistance on its own even though it may increase the tedizolid
42 MIC. The plasmid-carried *optrA* gene, which encodes an ABC transporter, has been implicated
43 in oxazolidinone and phenicol resistance in enterococci and streptococci but has not been
44 identified in *S. aureus.*[4] Previous serial passage studies with tedizolid have seen limited success
45 in selecting for tedizolid resistance and only mutations in the 23S rRNA have been recovered
46 following tedizolid exposure.[5] Genes affecting quinolone efflux such as *norA* and *mepA,* or
47 lincosamide efflux such as *mdeA* have not been reported to affect oxazolidinone activity.
48 In this study, we observed the emergence of a mutant exhibiting a novel mechanism of
49 PhLOPSa resistance from a well characterized MRSA strain after serial passage in escalating
50 concentrations of tedizolid.
51 Using the well characterized MRSA strain, N315, we selected for tedizolid resistance by
52 serial passage in escalating concentrations of tedizolid in Mueller Hinton II broth (MHB) starting
53 with 0.5x the MIC. Once visible growth was observed a sample of the broth was diluted 1:1000
54 into fresh MHB with twice the previous concentration of tedizolid until an isolate with an MIC of
55 ≥4 mg/mL was recovered. This MIC was selected since it is 1 $\log_2$ dilution above the breakpoint
56 for resistance (MIC ≥ 2mg/L). After 10 passages, we recovered an isolate (N315-TDZ4) with a
57 stable tedizolid MIC of 4mg/L or 16x the MIC of the parent strain, N315. To explore the cross

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58 resistance associated with this evolved strain we reevaluated susceptibility to a panel of other
59 antimicrobial agents (Table 1) by broth microdilution in accordance with Clinical Laboratory
60 Standards Institute (CLSI) guidelines[6] or by gradient strip in the case of quinupristin-dalfopristin
61 (Liofilchem®). Cross-resistance to chloramphenicol, linezolid, and quinupristin-dalfopristin was
62 observed but susceptibility to other drugs tested was relatively unchanged (Table 1). N315 is
63 resistant to clindamycin so lincosamide cross-resistance was unevaluable in this study and we
64 did not have access to pleuromutilins, which are not yet a clinically important class of
65 antimicrobials. While macrolide susceptibility is not considered part of the PhLOPSa group
66 macrolides also target the 50s ribosome, however, erythromycin susceptibility was also
67 unevaluable since N315 is resistant to erythromycin at baseline.
68 N315-TDZ4 was subjected to whole genome sequencing (WGS) using the MiSeq
69 platform (Illumina, San Diego, CA, USA) as previously described[7] to an average read depth of at
70 least 50X per isolate and the. Sequence data from this study is freely available through the
71 NCBI Sequence Read Archive (PRJNA578164). WGS of the N315-TDZ4 and comparison to the
72 parent strain revealed a single nucleotide variant (SNV) in the *rpoB* gene (1345 A>G)
73 corresponding to the amino acid substitution Asn449Asp. This mutation lies outside of the
74 rifampin resistance-determining regions, which span from nucleotides 1384 – 1464 (AA 462–
75 488) and 1543 – 1590 (AA 462–488) [8, 9], and we correspondingly did not observe any change in
76 rifampin susceptibly (Table 1). Previous studies have reported an association between
77 vancomycin and daptomycin susceptibility and certain rpoB mutations including Ala621Glu, and
78 Ala477Asp, and His481Tyr.[10-12] We did not observe any change in daptomycin MIC for our
79 mutant but we did see a 1 $\log_2$-dilution increase in vancomycin MIC. To further assess the
80 potential significance of this increase in vancomycin MIC we tested the N315-TDZ4 isolate and
81 N315 parent strains for the heterogeneous vancomycin intermediate *S. aureus* (hVISA)
82 phenotype by the gold standard population analysis profile (PAP) as previously described.[13]
83 Interestingly, we observed that PAP-AUC ratio with Mu3 of N315-TDZ4 was 0.85, up from 0.44

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84 of the parent strain N315 (Table 1). While this increase is substantial it falls below the
85 categorical criterion to declare this isolate an hVISA (PAP AUC ratio with Mu3 of ≥0.9).
86 In an effort to evaluate the potential impact of the rpoB Asn449Asp mutation on target
87 protein function, we constructed a homology model of *S. aureus* rpoB using I-TASSER,[14] and
88 modeled the relative orientations of the RNA polymerase (RNAP) and the ribosome (Figure 1).
89 This model suggests that the RNAP mutation would be unlikely to interact directly with binding
90 sites for the oxazolidinones and other PhLOPSa drugs located in the 50S ribosomal subunit
91 (Figure 1). This finding suggests an indirect mechanism of resistance, possibly involving one of
92 the many factors that interact with the beta-subunit of RNAP.[8]
93 In this study, we report for the first time a novel mechanism of resistance to
94 oxazolidinones, phenicols, and streptogramins involving mutation in *rpoB*, which encodes the β-
95 subunit of RNAP. The precise molecular mechanism by which this mutation mediates this
96 resistance phenotype is unclear but may involve transcriptional modulation by altered sigma-
97 factor binding. Previous studies have demonstrated that the 30s-ribosomal protein S10 encoded
98 by rpsJ links directly with the β-subunit of RNAP facilitating the tight linkage between
99 transcription and translation in bacteria but the RNAP only interacts with the leading 30s-subunit
100 and no known interaction between the 50s subunits and the RNAP exist. The fact that cross
101 resistance was isolated to 50s-ribosomally active agents and not 30s active agents
102 (doxycycline) suggests a relatively specific interaction with the 50s-ribosome and not a broader
103 modulation of translation or protein synthesis. Based on the absence of altered susceptibility to
104 other intracellularly active agents including moxifloxacin and doxycycline it seems unlikely that
105 this variant facilitates multidrug efflux. For now, tedizolid resistance remains uncommon
106 clinically and it is unknown whether this unique mutation could be selected for by other
107 PhLOPSa drugs or would be likely to emerge after clinical exposure to tedizolid.
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Table 1	**MIC (mg/L)**			**Target**
Drug	**N315**	**N315-TDZ4**	**Log_2 Fold Change**	--
Chloramphenicol	**8**	**128**	**4**	50s ribosome
Daptomycin	0.25	0.25	0	Cell membrane
Doxycycline	0.125	0.125	0	30s ribosome
Linezolid	**2**	**8**	**2**	50s ribosome
Moxifloxacin	0.0625	0.0625	0	DNA gyrase/topoisomerase
Quinupristin-dalfopristin	0.38	2	**2**	50s ribosome
Rifampin	0.001	0.001	0	RNA polymerase
Tedizolid	**0.25**	**4**	**4**	50s ribosome
Vancomycin	**0.5**	**1**	**1**	Cell wall synthesis
-PAP AUC ratio with Mu3	**0.44**	**0.85**	**---**	

Table 1. Minimum inhibitory concentrations (MIC) of parent strain, N315, and tedizolid passaged *rpoB* mutant, N315-TDZ4 to various antimicrobials, including fold change in MIC and antimicrobial target. PAP, population analysis profile; AUC, area under the cure

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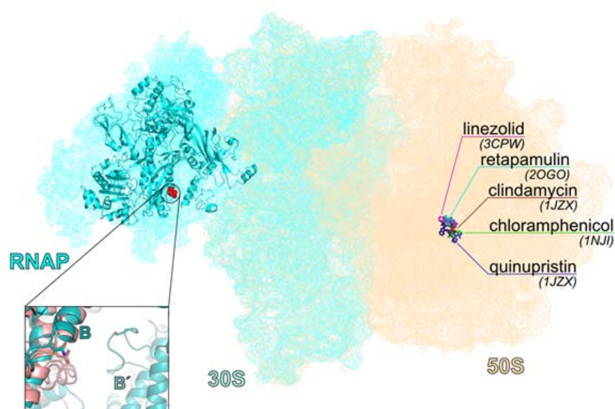

180

181 **Figure 1. Model of *S. aureus* rpoB N449D docked into the RNA polymerase (RNAP)-
182 ribosome transcriptional complex (protein data bank, IDs 5MY1 and 5U9F), illustrating
183 that the mutated residue in RNAP lies ~170Å from the binding site of PHLOPSa drugs on
184 the 50S ribosomal subunit. Drug binding sites are drawn from the PDB IDs indicated in
185 parentheses. The great distance between the mutated residue and linezolid binding site
186 suggests an indirect mechanism; notably, the susceptibility of doxycycline, which acts
187 on the 30S ribosomal subunit, was not affected. One CryoEM study of the structure of the
188 RNAP-ribosomal complex indicates an S1 protein crosslink between the 30S and RNAP
189 at the helix-turn-helix affected by this mutation.**

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