Inactivation of the Indigenous Methyltransferase RlmN in *Staphylococcus aureus* Increases Linezolid Resistance

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The indigenous methyltransferase RlmN modifies A2503 in 23S rRNA. A recently described *rlmN* mutation in a clinical *Staphylococcus aureus* isolate decreases susceptibility to linezolid and was thought to increase the extent of A2503 modification. However, we show that the mutation in fact abolishes RlmN activity, resulting in a lack of A2503 modification. Since many mutations could inactivate the *rlmN* gene, our findings unveil a potential mechanism for future linezolid resistance in clinical strains.

The oxazolidinone antibiotic linezolid (LZD), introduced in 2000, is one of the newest antibacterials (4). This drug targets the bacterial large ribosomal subunit and is used for the treatment of serious infections, including those caused by methicillin-resistant *Staphylococcus aureus* (MRSA) (5, 6, 10, 12, 15). During the early years of the drug’s use, clinically relevant resistance occurred only rarely and was mostly limited to target site mutations in rRNA and ribosomal proteins (16, 18, 22, 27). The first clinical linezolid-resistant MRSA isolate with an acquired linezolid resistance gene was described in 2007 (24). The resistance in this strain was conferred by methylation of the C8 position of A2503 in 23S rRNA by the Cfr methyltransferase (8, 13, 24). Many new clinical isolates exhibit linezolid resistance due to acquisition of the *cfr* gene (1–3, 19, 20). Besides being the target for the Cfr resistance enzyme, A2503 is also naturally methylated at C2 by the action of an indigenous methyltransferase, RlmN, which is highly homologous to Cfr and utilizes the same radical S-adenosylmethionine (SAM) mechanism (25, 28). The *rlmN* gene is widespread and is found in the genomes of most bacterial pathogens. Although *rlmN* is thought to play a role in interaction of the ribosome with the nascent peptide (26), its inactivation in *Escherichia coli* or *S. aureus* has little effect upon cell growth. *S. aureus* (but not *E. coli*) cells lacking the *rlmN* gene showed slight (1 dilution or less) elevation in susceptibility to linezolid (23, 25).

A new mechanism of decreased susceptibility to linezolid was described in a recent study (7). Upon prolonged treatment of a MRSA-infected patient with linezolid, a mutation (an insertion of an additional Glu codon after codon 353) arose in the *rlmN* gene. This mutation increased the linezolid MIC from 0.75 μg/ml observed in the original MRSA strain (JKD6210) to 2 μg/ml in the mutant (JKD6229) when a high-level inoculum (2 McFarland units) was used in the Etest. Engineered into the original *S. aureus* strain JKD6210, this mutation also increased the linezolid MIC to 2 μg/ml in the resulting strain (JKD6300), revealing the causative relationship of the reduced susceptibility with the genetic change in *rlmN*.

In view of the previous report that *rlmN* inactivation causes a slight increase in linezolid susceptibility, it was suspected that the resistance mutation in *rlmN* increases the degree of A2503 methylation (7). For example, the mutation could change the enzyme specificity to allow RlmN to methylate not only C2 but also C8 of A2503, as Cfr does. To test this hypothesis and to provide a molecular explanation for the *rlmN*-associated resistance mechanism, we investigated the status of A2503 modification in the mutant strains. Primer extension analysis can distinguish between C2-monomethylated adenine, C2- and C8-dimethylated adenine, or the completely nonmethylated nucleotide. Methylation at C2 results in a moderate reverse transcriptase stop which generates an extra band of a moderate intensity on a sequencing gel, C2- and C8-dimethylated A2503 produces a strong stop, and the complete lack of nucleotide modification allows for unimpeded progression of reverse transcriptase (13, 24, 25). To our surprise, the *rlmN* mutation found in the selected (JKD6229) or engineered (JKD6300) *S. aureus* strain which decreased susceptibility to linezolid did not cause an increase in the methylation at 2503, as originally suspected, but rather the complete abatement of modification (Fig. 1). Thus, the insertion of an additional Glu codon at position 353 in the *rlmN* gene results in an inactive form of the RlmN enzyme.

This result seemingly contradicted our previous report where an *rlmN* knockout led to a slight decrease in linezolid resistance (25). Therefore, we revisited those results. Despite multiple attempts, direct MIC testing in which we compared the linezolid susceptibility of the *rlmN* knockout mutant of the *S. aureus* Newman strain (SAV1218) with that of the strain with a knockout of the neutral gene *xylA* (SAV1986) did not provide a clear-cut answer. The MIC difference was equal to or less than 1 dilution, so that even small variations between experiments would provide inconclusive results. Therefore, we decided to mimic the clinical “natural selection” setting where the spontaneous mutant needs to compete with the wild type under antibiotic pressure.

A mixture of the *rlmN* knockout (SAV1218) cells and neu-
tural knockout control (SAV1986) cells was cocultured in brain heart infusion (BHI) medium in the presence of 0.5 μg/ml LZD, and the ratio of the cells was monitored over ca. 30 cell generations. In the control cells, A2503 is fully C-2 modified, whereas in the mutant that lacks modification, no stop is observed (Fig. 1). Therefore, primer extension on 23S rRNA isolated from the mixed population can be used as an efficient way to monitor the ratio of RlmN+ and RlmN− cells. As Fig. 2 indicates, the strain lacking A2503 methylation was more prevalent by the end of the experiment, indicating that under selective pressure, cells lacking functional RlmN do indeed outcompete those with active RlmN. We observed the same results when growth of the original MRSA cells (JKD6210) and of the engineered codon-insertion mutant (JKD6300) was monitored in the same experimental setting (data not shown). These data are in a complete agreement with previous findings (9, 11, 14, 21) and call for monitoring the sequence of the rlmN gene and its regulatory regions in clinical isolates subjected to linezolid exposure.

In the originally described clinical strain JKD6229, the elevated resistance to linezolid was associated with a codon insertion in the rlmN gene which, as our findings demonstrate, inactivates the encoded enzyme. However, as our studies of the rlmN knockout show, the same effect could be expected from any mutation that prevents expression of functional RlmN. Any nonsense mutation in the gene, missense mutation at functionally critical positions of the protein, or promoter mutation is expected to decrease susceptibility to linezolid. Given that the lack of the RlmN-mediated modification has little effect on cell growth in the absence of antibiotic (25), such resistance mechanisms can be easily maintained and rapidly spread among clinical pathogens treated with linezolid. Our findings expand the list of resistance mechanisms based on the lack of natural rRNA modification (9, 11, 14, 21) and call for a more in-depth sequencing of the rlmN gene and its regulatory regions in clinical isolates subjected to linezolid exposure.

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