Elevated Linezolid Resistance in Clinical cfr-Positive Staphylococcus aureus Isolates Is Associated with Co-Occurring Mutations in Ribosomal Protein L3

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Linezolid (LZD) resistance was first associated with mutations in the domain V region of 23S rRNA genes (G2576T) (7, 30). Over time, a variety of 23S rRNA mutations have been identified, and these remain the most commonly reported class of mutation leading to LZD resistance (5, 9, 10). In rare cases, mutations in ribosomal protein L4 have also been associated with LZD resistance (8, 17, 32). More recently, a variety of mutations in ribosomal protein L3 have also been identified in both laboratory and clinically derived staphylococci associated with reduced susceptibility to oxazolidinones (15–17). Cfr-based LZD resistance, however, is potentially more worrisome than mutation-based chromosomally encoded resistance mechanisms (25, 29). The cfr gene encodes a methyltransferase which confers LZD resistance via methylation of carbon-8 on 23S rRNA base A2503 (6). Cfr is generally plasmid borne and transposon associated and therefore likely to be horizontally transmitted (11). Strains carrying cfr are resistant not only to LZD but also to phenicols, lincosamides, pleuromutilins, and streptogramin A class antibiotics (19), as well as 16-membered ring macrolides (28). Thus, selective pressure due to the use of any of these drug classes may lead to the spread of this resistance determinant.

The emergence of cfr and identification of additional LZD resistance mechanisms, including L3 mutations, raise the potential for multiple mechanisms to occur within a single strain. Our previous work identified coupled 23S rRNA and L3 mutations in both a laboratory LZD serially passaged Staphylococcus aureus strain (17) and a clinical Staphylococcus epidermidis isolate (15). Another recent report documented a Spanish outbreak of LZDr S. aureus strains typically cite LZD MIC values in the range of 8 to 16 μg/ml (13, 14, 21, 29). Analysis of cfr-positive LZDr MRSA from a 2008 hospital outbreak in Madrid, Spain, identified 18 isolates: 1 environmental and 12 patient intensive care unit (ICU) isolates, 3 patient isolates from other wards, and 2 additional patient isolates pre-dating the outbreak that were identified in a retrospective study (22). LZD MIC values for these strains were 8 (n = 4), 16 (n = 13), or 32 (n = 1) μg/ml (22), all of which are above the LZD breakpoint of 4 μg/ml. This study investigated whether additional oxazolidinone resistance mechanisms could account for the variability in LZD resistance levels among these clinical cfr isolates.

Finally, an outbreak of LZDr S. epidermidis in Ohio was comprised of isolates possessing an L4 mutation in conjunction with either the cfr gene or mutations in 23S rRNA (1). These reports are the first to demonstrate the co-occurrence of cfr with any other LZD resistance mechanism; however, given the low fitness cost of Cfr methylation (12), strains with resistance due to multiple mechanisms may not be unexpected. Characterization of such isolates and the evaluation of antibacterial activities of clinically relevant oxazolidinones is thus of high interest.

Recent reports of S. aureus cfr-positive clinical isolates and laboratory-generated cfr-transformed S. aureus strains typically cite LZD MIC values in the range of 8 to 16 μg/ml (13, 14, 21, 29). Analysis of cfr-positive LZDr MRSA from a 2008 hospital outbreak in Madrid, Spain, identified 18 isolates: 1 environmental and 12 patient intensive care unit (ICU) isolates, 3 patient isolates from other wards, and 2 additional patient isolates pre-dating the outbreak that were identified in a retrospective study (22). LZD MIC values for these strains were 8 (n = 4), 16 (n = 13), or 32 (n = 1) μg/ml (22), all of which are above the LZD breakpoint of 4 μg/ml. This study investigated whether additional oxazolidinone resistance mechanisms could account for the variability in LZD resistance levels among these clinical cfr isolates.

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MICs of clinically relevant oxazolidinones, LZD (ChemPacific, Inc., Baltimore, MD), TR-700 (torezolid, formerly known as DA-7157; Trius Therapeutics, Inc., San Diego, CA), and radezolid (RZD) (RX-1741; Medicilon, Chicago, IL), as well as tiamulin (TIA) (Wako Pure Chemical Industries, Ltd., Richmond, VA), chloramphenicol (CHL) (Sigma-Aldrich Corp., St. Louis, MO), and vancomycin (VAN) (Sigma), were determined via broth microdilution in accordance with CLSI guidelines as previously described (3, 17). Quality control of oxazolidinones was performed via nuclear magnetic resonance
Laboratory 29213 fell into three groups: group 1 (16 MIC values for a panel of the 6 representative strains included in this study) yielded identical results. LZD strain/drug combination MIC values were determined in at least three independent experiments. NMR, liquid chromatography-mass spectrometry (LC-MS), and biological activity assays. MIC values reported for each strain/drug combination were determined in at least three independent experiments, all yielding identical results. LZD strain/drug combination MIC values were determined in at least three independent experiments.

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<table>
<thead>
<tr>
<th>Origin</th>
<th>Strain(s)</th>
<th>Reference or source</th>
<th>PFGE type</th>
<th>Presence of cfr</th>
<th>L3 mutation(s)$^a$</th>
<th>MIC (µg/ml)$^b$</th>
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<tr>
<td>Clinical</td>
<td>Group 1$^c$</td>
<td>22</td>
<td>C</td>
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<td>∆Ser145/His146Tyr</td>
<td>16 0.5 4 &gt;64 &gt;64 2</td>
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<tr>
<td>Group 2$^d$</td>
<td>22</td>
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<tr>
<td>Group 3$^e$</td>
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<td>D</td>
<td>+</td>
<td>∆Met169-Gly174</td>
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<tr>
<td>Laboratory</td>
<td>29213$^f$</td>
<td>ATCC</td>
<td>NA’</td>
<td>–</td>
<td>2 0.5 1 0.5 8 1</td>
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<tr>
<td>29213-1$^g$</td>
<td>17</td>
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<td>–</td>
<td>Gly155Arg</td>
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<td>64 2 8 &gt;64 &gt;64 2</td>
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</table>

$^a$ Ribosomal protein L3 mutations are given using staphylococcal numbering.
$^b$ MICs were determined via broth microdilution (CLSI) (3).
$^c$ Group 1 contained isolate 42262.
$^d$ Group 2 included 32289, P-978 (environmental), 42292, and 56351.
$^e$ Group 3 contained isolate 51312.
$^f$ ATCC 29213 is included as an LZD control strain and is not isogenic to any of the clinical cfr strains in this study.
$^g$ 29213-1, -2, and -3 L3 mutants were selected in vitro with LZD and/or TR-700 in a previous study.
$^h$ p42262 is a cfr-containing plasmid isolated from group 1 strain 42262 and is used to transform ATCC 29213 wild type and the three isogenic L3 mutant strains.

$^i$ NA, not analyzed.

Strains possessing mutations in the domain V region of all 23S rRNA alleles and the genes encoding L3 were selected with LZD and/or TR-700 in a previous study. Reduced susceptibility to oxazolidinones has been documented in S. aureus strains possessing mutations in some of the residues involved in these L3 mutations, including ∆Ser145 (15, 16), Met169Leu/Gly155Arg (17), and ∆Phe127-His146 (17). The presence of additional ribosomal mutations was assessed by sequencing the domain V region of all 23S rRNA alleles and the genes encoding L3. Reduced susceptibility to oxazolidinones has been documented in S. aureus strains possessing mutations in some of the residues involved in these L3 mutations, including ∆Ser145 (15, 16), Met169Leu/Gly155Arg (17), and ∆Phe127-His146 (17). The presence of additional ribosomal mutations was assessed by sequencing the domain V region of all 23S rRNA alleles and the genes encoding L3. Reduced susceptibility to oxazolidinones has been documented in S. aureus strains possessing mutations in some of the residues involved in these L3 mutations, including ∆Ser145 (15, 16), Met169Leu/Gly155Arg (17), and ∆Phe127-His146 (17). The presence of additional ribosomal mutations was assessed by sequencing the domain V region of all 23S rRNA alleles and the genes encoding L3.

In an effort to recapitulate and validate the coupled Cfr plus L3 resistance trends observed in these clinical isolates, we generated a panel of isogenic comparator strains in the S. aureus ATCC 29213 background. The wild-type ATCC 29213 parent strain and three isogenic, laboratory-selected L3 mutants (17) having two different oxazolidinone susceptibility profiles were transformed with the p42262 cfr plasmid (isolated from clinical strain 42262) (23) via electroporation as previously described (24). Transformant MICs mirrored the oxazolidinone MICs observed in the clinical strains, suggesting that the Cfr and Cfr plus L3-based resistance mechanisms are the primary contributing factors to the observed LZD resistance levels in these clinical isolates. Based on the structural analyses and MIC trends from cfr-transformed 29213 L3 mu-
Novel mutations in ribosomal protein L3 were detected in each of the *S. aureus cfr* isolates examined with LZD MIC values of >16 μg/ml. TR-700 was the only oxazolidinone tested with MIC values of ≤2 μg/ml against all strains. Because the breakpoints for TR-700 and radezolid have not been determined, the clinical relevance of MIC values against these strains cannot yet be assessed. Enhanced activity of TR-700 and radezolid have not been determined, the clinical relevance of MIC values against these strains cannot yet be assessed. Enhanced activity of TR-700 is primarily due to reduced steric hindrance of the TR-700 C-5 hydroxymethyl substituent compared to the acetamide of LZD or radezolid in the presence of Cfr methylation (14, 26). This study is the first to document the co-occurrence of cfr in clinical *S. aureus* isolates possessing L3 mutations, following reports of cfr coupled with 23S rRNA (2) or L4 (1) mutation-based resistance mechanisms. Cfr methylation has now been shown to be compatible with each of the three documented classes of mutation-based staphylococcal resistance to LZD, thus highlighting the need for next-generation oxazolidinones to maintain activity against a variety of resistance mechanisms.

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**ADDENDUM IN PROOF**

During the review process of this manuscript, work documenting the co-occurrence of the cfr gene in clinical *Staphylococcus aureus* isolates possessing L3 mutations was published (R. E. Mendes, L. Deshpande, E. Rodriguez-Noriega, J. E. Ross, R. N. Jones, and R. Morfin-Otero, *J. Clin. Microbiol.* 48:3041–3043).

**REFERENCES**


