

ELEVATED LINEZOLID RESISTANCE IN CLINICAL *S. aureus cfr* ISOLATES IS ASSOCIATED WITH CO-OCCURRING MUTATIONS IN RIBOSOMAL PROTEIN L3

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ABSTRACT

Background: Linezolid (LZD) resistance is associated with mutations in genes encoding 23S rRNA and ribosomal proteins L3 and L4 or through methylation of 23S rRNA base A2503 via the horizontally-transferred Cfr methyltransferase. A 2008 outbreak of *cfr*-positive *Staphylococcus aureus* in Madrid, Spain resulted in 16 isolates with variable LZD MIC values (4-fold range). Here we investigated whether co-occurring ribosomal mutations could account for the variability in LZD resistance levels among some isolates.

Methods: The domain V region of all 23S rRNA alleles and the genes encoding L3 (*rplC*) and L4 (*rplD*) were sequenced in 6 of the 16 *S. aureus* clinical isolates. MICs of LZD, TR-700 (torezolid), tiamulin, chloramphenicol, and vancomycin were determined via broth microdilution (CLSI). Ribosomal mutations were analyzed using the LZD-bound *Deinococcus radiodurans* crystal structure (PDB code 3DLL). *S. aureus* ATCC 29213 and three isogenic L3 mutants thereof were transformed with the *cfr* plasmid isolated from clinical strain 42262.

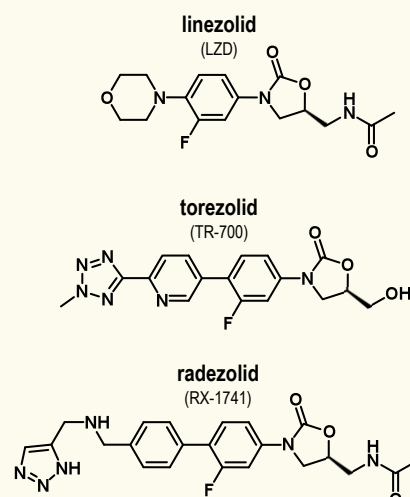
Results: Of the 6 *cfr*-positive isolates examined, 1 isolate possessed no ribosomal mutations (LZD MIC 16 µg/ml), 4 possessed the L3 mutation ΔSer145/His146Tyr (LZD MIC 32 µg/ml), and 1 possessed a 6 amino acid deletion in L3 ΔMet169-Gly174 (LZD MIC 64 µg/ml). Both of these novel L3 mutations occurred in close proximity to the LZD binding site in the PTC. TR-700 had greater potency than LZD against all strains, with MIC values ranging from 0.5 to 2 µg/ml. Identical elevated LZD MIC profiles were obtained in the 29213 L3 mutant *cfr*-transformed isogenic strain panel.

Conclusions: Mutations in ribosomal protein L3 were detected in each of the *S. aureus cfr* isolates with LZD MIC values >16 µg/ml. TR-700 maintained MIC values of ≤2 µg/ml against all isolates. This study is the first to document the co-occurrence of L3 mutations in clinical *S. aureus cfr* strains.

INTRODUCTION

- ❖ Oxazolidinone resistance is most often linked to mutations in 23S rRNA domain V (especially G2576T) and more recently to mutations in ribosomal proteins L3 and L4
- ❖ Methylation of 23S rRNA (A2503) by the horizontally-transmissible Cfr methyltransferase also confers resistance to linezolid (LZD) and other 50S-targeted antibiotics
- ❖ An outbreak of *cfr*-positive *S. aureus* in Madrid, Spain with variable levels of LZD resistance (MICs 16 to 64 µg/ml) prompted an investigation of other potential underlying resistance mechanisms

COMPOUNDS



METHODS

- ❖ All *S. aureus* strains were cultured at 37°C on Mueller-Hinton II agar (MHA) or in liquid broth (MHB)
- ❖ Compounds included: linezolid (LZD, ChemPacific), TR-700 (torezolid, Trius Therapeutics, Inc.), RX-1741 (radezolid, Medicilon), TIA (tiamulin, Wako Pure Chemicals), CHL (chloramphenicol, Sigma-Aldrich), and VAN (vancomycin, Sigma-Aldrich)
- ❖ MIC assays (broth microdilution, CLSI) were repeated independently at least 3 times per compound/strain pairing
- ❖ PCR was used to amplify individual *rm* alleles, *rplC*, *rplD*, and *rplV* genes encoding 23S rRNA and ribosomal proteins L3, L4, and L22, respectively
- ❖ An isogenic panel of ATCC 29213 *S. aureus* strains possessing L3 mutations and the *cfr* gene was generated through transformation of laboratory selected L3 mutants with the p42262 *cfr* plasmid isolated from a clinical strain (42262)
- ❖ Modeling of ribosomal mutations is based on the crystallographic structure of the *Deinococcus radiodurans* LZD-bound 50S subunit (Wilson *et al.*, 2008, PNAS, 109:13339-44; PDB accession code 3DLL)

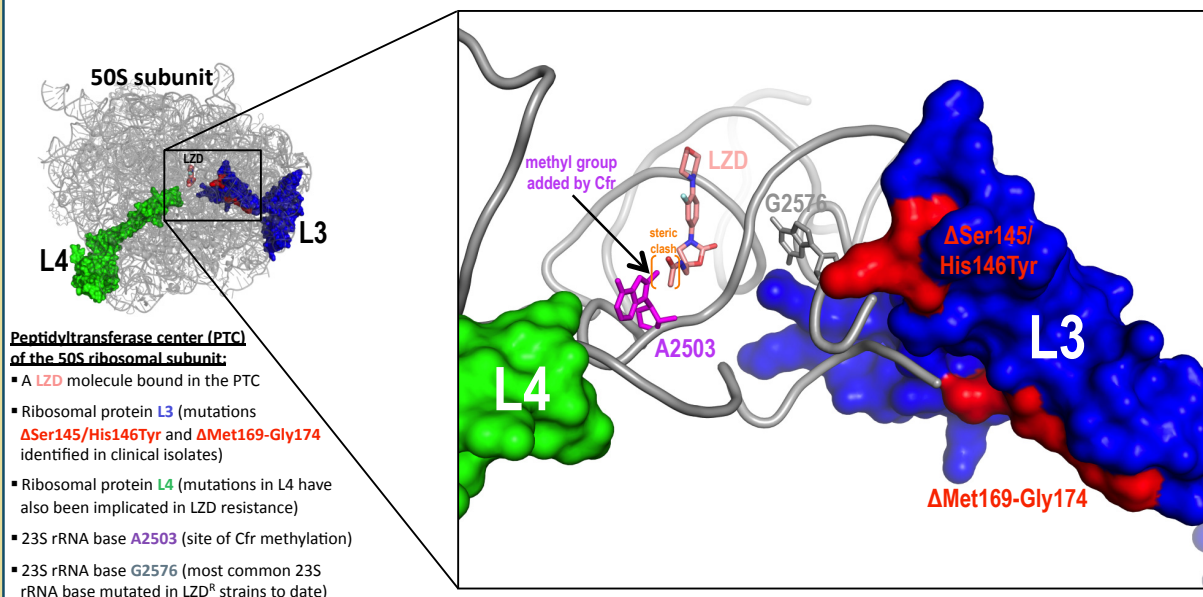
RESULTS

I. Characteristics of clinical and laboratory generated *S. aureus* strains possessing the *cfr* gene and mutations in ribosomal protein L3

Origin	Strain(s)	PFGE	L3 mutation ^a	<i>cfr</i>	MIC (µg/ml)						Reference/Source
					LZD	TR-700	RX-1741	TIA	CHL	VAN	
Clinical	42262	C	-	+	16	0.5	4	>64	>64	2	Morales <i>et al.</i> , 2010, Clin. Infect. Dis., 50:821-825
	32289, P-978, 42292, 56351	A, B	ΔSer145/His146Tyr	+	32	1	4	>64	>64	1	Morales <i>et al.</i> , 2010, Clin. Infect. Dis., 50:821-825
	51312	D	ΔMet169-Gly174	+	64	2	8	>64	>64	2	Morales <i>et al.</i> , 2010, Clin. Infect. Dis., 50:821-825
Laboratory	29213 ^b	n/a	-	-	2	0.5	1	0.5	8	1	ATCC
	29213-1 ^c	n/a	Gly155Arg	-	4	1	1	8	8	1	Locke <i>et al.</i> , 2009, AAC, 53(12): 5265-5274
	29213-2 ^c	n/a	Gly155Arg/Met169Leu	-	8	2	2	4	8	2	Locke <i>et al.</i> , 2009, AAC, 53(12): 5265-5274
	29213-3 ^c	n/a	ΔPhe127-His146	-	8	2	2	4	8	2	Locke <i>et al.</i> , 2009, AAC, 53(12): 5265-5274
	29213 + p42262 ^d	n/a	-	+	16	0.5	2	>64	>64	1	This study
	29213-1 + p42262 ^d	n/a	Gly155Arg	+	32	1	4	>64	>64	2	This study
	29213-2 + p42262 ^d	n/a	Gly155Arg/Met169Leu	+	64	2	8	>64	>64	2	This study
29213-3 + p42262 ^d	n/a	ΔPhe127-His146	+	64	2	8	>64	>64	2	This study	

^aRibosomal protein L3 mutations are stated using staphylococcal numbering; ^bATCC 29213 is included as a LZD^r control strain and is not isogenic to any of the clinical *cfr* strains included here; ^c29213-1, -2, -3 L3 mutants were selected in vitro with LZD or TR-700; ^dp42262 is a *cfr*-containing plasmid isolated from strain 42262 and used to transform ATCC 29213 wild-type and the three isogenic L3 mutant strains. TIA (tiamulin); CHL (chloramphenicol); VAN (vancomycin).

II. Structural analysis of L3 mutations identified in clinical Cfr isolates



RESULTS

- ❖ Novel mutations in ribosomal protein were identified in clinical *cfr* strains with LZD MIC values of 32 µg/ml (ΔSer145/His146Tyr) or 64 µg/ml (ΔMet169-Gly174)
- ❖ Both mutations mapped to a region of L3 in close proximity to the LZD binding site in the 50S peptidyl transferase center
- ❖ The severity of the L3 mutation correlated with the level of LZD resistance
- ❖ LZD resistance levels were recapitulated through transformation of laboratory derived *S. aureus* ATCC 29213 L3 mutant strains with the clinical p42262 *cfr* bearing plasmid
- ❖ Decreased susceptibility among TR-700, LZD, and RX-1741 was observed against the 29213 L3 mutant strains
- ❖ The addition of *cfr* to the 29213 L3 mutants did not lead to further MIC increases for TR-700, but increased MICs 8X for LZD and 4X for RX-1741

CONCLUSIONS

- ❖ This is the first report of co-occurring L3 mutations in clinical *S. aureus* isolates possessing *cfr*
- ❖ *Cfr* has now been documented in conjunction with mutations in 23S rRNA and ribosomal proteins L3 and L4, supporting earlier working demonstrating a low fitness cost of *cfr*
- ❖ The smaller C-5 hydroxymethyl group of TR-700 is compatible with binding in the presence of Cfr methylation, whereas the more bulky C-5 acetamide group in LZD and RX-1741 experiences steric hindrance and a reduction in activity
- ❖ Increasing reports of LZD^r clinical isolates possessing both ribosomal mutations (23S rRNA, L3, or L4) and the *cfr* gene underscore the need for 2nd generation oxazolidinones with activity against a variety of resistance mechanisms
- ❖ TR-700 maintains a 4-fold potency advantage over RX-1741 and a 32-fold potency advantage over LZD against strains with L3 mutations and *cfr*

ACKNOWLEDGEMENTS

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