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# *rplC* T460C Identified as a Dominant Mutation in Linezolid-Resistant *Mycobacterium tuberculosis* Strains

**Patrick Beckert,[a] Doris Hillemann,[b] Thomas A. Kohl,[c] Jörn Kalinowski,[c] Elvira Richter,[b] Stefan Niemann,[a] and Silke Feuerriegel[a]**

Molecular Mycobacteriology, Research Center Borstel, Borstel, Germany[a]; National Reference Center for Mycobacteria, Research Center Borstel, Borstel, Germany[b]; and Institute for Genome Research and Systems Biology, Center for Biotechnology, Bielefeld University, Bielefeld, Germany[c]

**The ribosomal L3 protein was identified as a novel target in linezolid (LZD)-resistant *Mycobacterium tuberculosis* strains. Next-generation sequencing confirmed *rplC* T460C as the sole mutation in an LZD-resistant *M. tuberculosis* H37Rv strain selected *in vitro*. Sequencing analysis revealed the *rplC* T460C mutation in eight further LZD-resistant isolates (three *in vitro*-selected mutants and five patient isolates, including isolates from three different patients that developed LZD resistance during treatment) but in none of the susceptible control strains ($n = 84$).**

*Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB), still represents a huge challenge for infectious disease control worldwide. Since the appearance of multidrug-resistant (MDR; resistant to at least isoniazid and rifampin) (8) and extensively drug-resistant (XDR; additionally resistant to one fluoroquinolone and one injectable drug) (4) *M. tuberculosis* strains, the application of reserve antibiotics has become more important. Linezolid (LZD), which belongs to the oxazolidinone group (1), has been used primarily for the treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococcus infections (10) and has been used off-label to treat MDR and XDR TB patients (9, 12). LZD acts on the 50S ribosomal subunit, specifically, the peptidyl-transferase center (PTC), by blocking the binding of tRNA and thus inhibiting bacterial cell growth (5).

The first occurrence of LZD-resistant *M. tuberculosis* clinical isolates was described by Richter et al. (11) As potential target genes, the 23S rRNA gene, *rplV* and *rplD* (which encode ribosomal proteins L4 and L22), *erm-37* (which encodes methyltransferase), and *whiB7* (which encodes a putative regulator) were sequenced. However, since no mutations were detected in these genes, the mechanism of LZD resistance in clinical *M. tuberculosis* isolates has remained unclear. To gain further insight into the mechanisms involved, LZD-resistant *M. tuberculosis* strains were selected *in vitro* (2). Sequence analysis of the 23S rRNA gene revealed mutations in 50% of the selected mutants, primarily at nucleotide position 2061. However, no further polymorphisms were found in the remaining selected clones.

Recently, mutations in *rplC* were described as a novel LZD resistance mechanism in MRSA (7). To determine if *rplC* is also involved in LZD resistance in *M. tuberculosis*, we sequenced *rplC* in (i) LZD-resistant, *in vitro*-selected mutants; (ii) LZD-resistant clinical isolates; (iii) follow-up isolates from three different patients; and (iv) a reference collection comprising all of the major phylogenetic lineages. The primers used for amplification were *rplC*_5′(+249) (5′-GCTGCGGCTGGACGACTC-3′) and *rplC*_3′(+668) (5′-CTCTTGCGCAGCCATCACTTC-3′). The conditions used were (i) initial denaturation at 95°C for 15 min, (ii) denaturation at 94°C for 30 s, (iii) annealing at 65°C for 30 s, (iv) elongation for 30 s at 72°C, and (v) terminal elongation for 10 min at 72°C. Steps ii to iv were performed 35 times. The PCR products thus obtained were sequenced using an ABI 3130xl genetic analyzer (Applied Biosystems) and an ABI BigDye Terminator cycle sequencing kit (version 3.1) according to the manufacturer's instructions. Analysis of sequence data was performed using the DNAStar Lasergene (version 8.0) software suite.

First, the *rplC* genes of strains from the aforementioned selection experiments were sequenced (2) (selected on 4 μg/ml LZD; MICs for LZD of 4 to 16 μg/ml). As references, the genes of the parental strains used for *in vitro* selection were sequenced. All but one of the five strains sequenced carried a mutation at nucleotide 460 (T/C) compared to the respective reference strain, resulting in an amino acid exchange from cysteine to arginine at codon 154 (Table 1).

To verify the identified *rplC* T460C mutation as the only polymorphism in the clones selected, and thus responsible for the resistance phenotype, whole-genome sequencing of one of the *in vitro*-selected LZD-resistant mutants (9679/00 1.1.2) was carried out. Isolated genomic DNA was sequenced by GATC Biotech in a paired end run on the Illumina platform. Derived reads had a length of 46 bp for strain 9679/00 1.1.2. Reads were mapped to the *M. tuberculosis* H37Rv genome (GenBank accession no. NC_000962.2) using the CLC Genomics Workbench software (CLC bio) with default settings. For strain 9679/00 1.1.2, 11,431,954 of the 12,400,138 reads were matched to the reference genome, resulting in an average coverage of 119 reads. CLC Genomics Workbench was also employed for single nucleotide polymorphism (SNP) detection using default settings with a minimum coverage of four reads and a minimum variant frequency of 75%. A total of 30 SNPs were detected compared to the H37Rv reference sequence (see Table S1 in the supplemental material). Of these SNPs, the only ones consid-

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Address correspondence to Silke Feuerriegel, sfeuerriegel@fz-borstel.de.
S.N. and S.F. contributed equally to this work.
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**TABLE 1** LZD susceptibility test and *rplC* sequencing[d] results of *in vitro*-selected mutants and resistant clinical isolates

| Sample | LZD resistance[a] | MIC ($\mu$g/ml) | *rplC* | L3 protein |
|---|---|---|---|---|
| *In vitro*-selected mutants | | | | |
| 9679/00[c] | S | <1 | WT | |
| 9679/00 1.1. 2 | R | 4–8 | T460C | Cys154Arg |
| 9679/00 1.4.1 | R | 16 | T460C | Cys154Arg |
| 9679/00 4.4.1 | R | 4–8 | WT | |
| 6446/02[c] | S | <1 | WT | |
| 6446.2/02 | R | 4–8 | T460C | Cys154Arg |
| 4414/06[c] | S | <1 | WT | |
| 4414.1/06 | R | 4–8 | T460C | Cys154Arg |
| Resistant clinical isolates | | | | |
| 2807/08 | R | ND[b] | T460C | Cys154Arg |
| 11932/10 | R | ND | T460C | Cys154Arg |

[a] BACTEC MGIT 960 (critical concentration, 1 $\mu$g/ml.). S, susceptible; R, resistant.
[b] ND, not determined.
[c] Parental strain used for *in vitro* selection of LZD-resistant mutant strains. 9679/00, *M. tuberculosis* H37Rv; 6446/02, *M. tuberculosis* red Euro-American; 4414/06, *M. tuberculosis* Delhi/CAS.
[d] The WT *rplD* sequence of each strain was determined.

**TABLE 2** LZD resistance and *rplC* sequencing results for follow-up isolates from three different patients

| Patient no. and sample[a] | LZD resistance[b] | *rplC* gene | Protein L3 |
|---|---|---|---|
| 1 | | | |
| 4177/97 | ND | WT | |
| 1185/98 | ND | WT | |
| 2698/00 | ND | WT | |
| 1633/02 | S | WT | |
| 9512/03 | S | WT | |
| 6995/04 | S | WT | |
| 10202/04 | S | WT | |
| 3444/05 | R | T460C | Cys154Arg |
| 2010/06 | R | T460C | Cys154Arg |
| 1126/07 | R | T460C | Cys154Arg |
| 3082/08 | R | T460C | Cys154Arg |
| 2952/09 | R | T460C | Cys154Arg |
| 2 | | | |
| 10781/00 | ND | WT | |
| 3971/01 | S | WT | |
| 5740/02 | S | WT | |
| 1499/03 | ND | T460C | Cys154Arg |
| 4600/03 | R | T460C | Cys154Arg |
| 7321/03 | R | T460C | Cys154Arg |
| 310/06 | R | T460C | Cys154Arg |
| 3 | | | |
| 8528/02 | ND | WT | |
| 5391/03 | S | WT | |
| 7450/03 | S | WT | |
| 10987/03 | R | T460C | Cys154Arg |
| 11672/03 | R | T460C | Cys154Arg |

[a] The last two digits after the slash indicate the year of sample collection. The *rplD* sequences in all of the strains were analyzed. All of the strains collected from patients 1 and 2 showed the WT *rplD* sequence. Follow-up isolates taken from patient 3 all displayed a synonymous SNP (Asn214Asn [AAC/AAT]) in *rplD*, irrespective of resistance or susceptibility to LZD.
[b] BACTEC MGIT 960 (critical concentration, 1 $\mu$g/ml). ND, not determined; S, susceptible; R, resistant.

ered were those not located in repetitive elements (possible mapping artifacts) and absent from the genomes of three further H37Rv variants selected *in vitro* for resistance to other antibiotics (possible failures in the H37Rv reference sequence). The *rplC* T460C mutation was the only SNP detected in the *in vitro*-selected LZD-resistant strain (9679/00 1.1.2) under these criteria.

To confirm the clinical significance of the *rplC* T460C mutation, the *rplC* DNA sequence was analyzed in two XDR TB patient isolates also showing LZD resistance (Table 1). Both of the strains analyzed showed the *rplC* T460C mutation as the only variation in the sequence analyzed.

To further investigate the significance of *rplC* T460C for LZD resistance development in clinical isolates, follow-up isolates from three different patients were sequenced (Table 2). The isolates were described in detail in a previous report (11). All patients were infected with *M. tuberculosis* strains which acquired LZD resistance during treatment. Samples were taken over a time span of several years. IS*6110* fingerprint genotyping was used to confirm that the isolates obtained from one patient were the same over the entire treatment period (no reinfection). Furthermore, IS*6110* fingerprinting confirmed that the three patients do not belong to one cluster, as the patterns of the strains showed marked differences (data not shown).

*rplC* sequence analysis of the follow-up isolates showed a clear correlation between the occurrence of the LZD resistance phenotype and the emergence of the *rplC* T460C mutation (Table 2). All of the susceptible isolates obtained before the emergence of LZD resistance had no mutation in *rplC* ($n = 13$).

Besides mutations in *rplC*, alterations in the *rplD* gene, which codes for ribosomal protein L4, have been described as mediating LZD resistance in *Staphylococcus aureus* (7), *Clostridium perfringens* (3), and pneumococci (13). To further elucidate previously unknown resistance mechanisms in the strains analyzed in this study, the *rplD* genes of all of the strains were sequenced by using primers *rplD*_5′(−153) (5′-CCGGG CGGATGGGCAATGACC-3′) and *rplD*_3′(+782) (5′-GGAATC CGGGCGCACCAAAAAC-3′).

The wild-type (WT) *rplD* sequence was determined for all of the *in vitro*-selected mutant strains, all of the resistant clinical isolates (Table 1), and the follow-up isolates collected from patients 1 and 2 (Table 2). A synonymous SNP (Asn214Asn [AAC/AAT]) was detected in all of the follow-up isolates from patient 3 (Table 2). However, as this polymorphism occurs in both susceptible and resistant isolates, it obviously does not play a role in mediating LZD resistance. Whole-genome sequencing might be interesting to determine putative previously unknown mutations responsible for the resistance phenotype of *in vitro*-selected, LZD-resistant mutant 9679/00 4.4.1 and the elevated MIC for 9679/00 1.4.1 (16 $\mu$g/ml).

To investigate if mutations in *rplC*, especially T460C, occur in a phylogenetic-lineage-dependent manner, we sequenced the *rplC* genes of 71 LZD-susceptible strains of the *M. tuberculosis* complex, comprising all of the major phylogenetic lineages (see Table S2 in the supplemental material). The collection comprises 14 different *M. tuberculosis* genotypes, as well as 3 *M. africanum* genotypes, *M. bovis*, *M. canettii*, *M. caprae*, *M. microti*, and *M. pinnipedii*. For the majority of the genotypes, three different strains were included. One

phylogenetic SNP was detected in all of the *M. canettii* strains (A459G). Although the polymorphism detected is directly adjacent to the T460C SNP, it is highly unlikely to play a role in LZD resistance development, as the A459G mutation is a synonymous SNP which does not lead to an amino acid exchange. The *rplC* T460C mutation has not been found in any of the strains analyzed and can therefore definitely be excluded as a phylogenetic SNP.

In conclusion, this study reveals a novel mechanism of LZD resistance in *M. tuberculosis* complex strains. The same mutation in the *rplC* gene (T460C) was identified in *in vitro*-selected mutants and in clinical isolates. Mutations in *rplC* have previously been identified in LZD-resistant *Staphylococcus* strains at positions Gly152Asp, Gly155Arg, Ala157Arg, and Met169Leu, located in the PTC region (6, 7). An amino acid alignment of the L3 proteins of *M. tuberculosis* and *S. aureus* illustrates that the *rplC* T460C mutation (Cys154Arg) corresponds to the Ala157Arg position and is therefore probably also located directly in the ribosome and drug binding region. However, to definitely prove the influence of the *rplC* T460C mutation on LZD resistance, the generation of an isogenic mutant strain via homologous recombination is mandatory.

The MICs for the *in vitro*-selected mutants analyzed ranged from 4 to 8 μg/ml (16 μg/ml for one strain); higher MICs (32 μg/ml) were detected for strains displaying mutations in the 23S rRNA gene (2). As was observed in *S. aureus* (7), mutations in *rplC* could be responsible for lower-level LZD resistance, whereas mutations in the 23S rRNA gene might, alone or in combination with *rplC* mutations, mediate higher levels of resistance in *M. tuberculosis*.

This study highlights the clinical relevance of the *rplC* T460C mutation for LZD resistance, which should be incorporated into molecular assays for the detection of second-line drug resistance in *M. tuberculosis*.

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