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# *Mycobacterium tuberculosis* Mutations Associated with Reduced Susceptibility to Linezolid

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**Linezolid (LZD) has become increasingly important for the treatment of multidrug-resistant tuberculosis (MDR-TB), but its mechanisms of resistance are not well characterized. We isolated 32 mutants of *Mycobacterium tuberculosis* with reduced susceptibility to LZD, which was accounted for by *rrl* and *rplC* mutations in almost equal proportions, causing lower and higher MICs, respectively. Our findings provide useful information for the rapid detection of LZD resistance for improved treatment of MDR-TB.**

Tuberculosis (TB) remains a major threat to global public health. Increasing emergence of multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) calls for urgent development of new drugs to combat drug-resistant TB. Although linezolid (LZD) has been used primarily to treat antibiotic-resistant Gram-positive bacterial infections (1), it has good activity against mycobacteria (2). LZD binds to the 50S ribosomal subunit and inhibits the translation process (3). This mechanism of action is different from that of first- and second-line TB drugs, which indicates no cross-resistance with current TB drugs. Several clinical studies have demonstrated the usefulness of LZD in the treatment of MDR-TB (4, 5), but resistance ranging from 1.9% to 10.8% has been reported in MDR *Mycobacterium tuberculosis* strains (6, 7). Previous studies have shown that mutations in the 23S rRNA gene *rrl* encoding the ribosomal L4 protein were present in 5 of 10 (50%) LZD-resistant mutants of *M. tuberculosis* isolated *in vitro*, with 4 having mutations at position 2061 and 1 having a mutation at position 2057 in the *rrl* gene (8). However, the other 5 mutants were not accounted for. A recent study showed that mutations in the *rplC* gene, encoding the L3 ribosomal protein, were found in 6 of 7 LZD-resistant mutants of *M. tuberculosis* (9). This was based on a study of *Staphylococcus aureus* isolates where *rplC* mutations were found to cause LZD resistance (10). Although the mechanisms of resistance to TB drugs have been well characterized for most drug resistances (11), there is limited information about the genetic analysis of mutations involved in LZD resistance in *M. tuberculosis*. So far, only 12 resistant mutants isolated *in vitro* have been studied (8, 9).

To further understand the mechanisms of resistance to LZD, we isolated 32 resistant mutants of *M. tuberculosis* H37Rv as a parental strain on 7H11 agar plates containing 0, 0.25, 0.5, 1, and 2 μg/ml LZD. We found that the H37Rv control strain did not grow at 0.25 μg/ml, indicating that its MIC (<0.25 μg/ml) is lower than previously reported MIC values of 0.5 μg/ml (12) and 1 μg/ml (6). This may be a reflection of the 7H11 agar solid medium used here compared to the MGIT liquid medium employed in the previous study (6) that reported higher MIC values for the susceptible control strain. The reduced susceptibility of the mutants was confirmed by growing them again on 7H11 agar plates containing LZD ranging from 0 to 2 μg/ml; the degree of growth of each mutant on LZD-containing plates is shown in Table 1. Based on the degree of mutant growth on plates containing LZD compared with that of the sensitive control strain, we chose 0.25 μg/ml LZD as the MIC breakpoint above which strains are considered to have reduced susceptibility. The DNA of these mutants and of the sensitive control strain H37Rv was extracted and used as the template for PCR amplification of known LZD resistance genes *rrl* and *rplC*. The primers used for PCR were *rrl* F1 (5′-CCT GAG GCA ACA CTC GGA CTT-3′) (156 bp before start codon), *rrl* R1 (5′-ACG GAT TTG CCT ATC GCT CT-3′), *rrl* F2 (5′-CCT AAG GCG AGG CCG ACA G-3′), *rrl* R2 (5′-GGC CGC CGT AAC TCT ATG C-3′) (114 bp after stop codon), *rplC* F (5′-TCCGCTCACCGCATAAGTACA-3′) (167 bp before start codon), and *rplC* R (5′-CGATGTTGGCCGGGACGT-3′) (112 bp after stop codon). The PCR conditions were initial denaturation at 95°C for 5 min and then 30 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 2 min, and a final extension at 72°C for 7 min. PCR products were purified and were used for Sanger DNA sequencing. Genomic DNA from the 7 mutants with higher LZD MIC values that did not show mutations in *rrl* or *rplC* by Sanger sequencing was subjected to whole-genome sequencing using Illumina MiSeq as described previously (13).

As shown in Table 1, 17 of 32 mutants had a mutation in the *rrl* gene, and these mutations can be divided into 5 different mutation types, C2848A, A2810T, G2270C, G2270T, and G2746A; mutations at G2746A and G2270 were the most dominant at 47.1% (8/17) and 41.2% (7/17), respectively (Fig. 1). Fifteen mutants without *rrl* mutations all had the same T460C mutation, which caused substitution of Cys to Arg at amino acid position 154 of the *rplC* gene. The LZD-susceptible parent strain H37Rv had no mutations in *rrl* or in *rplC*.

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**TABLE 1** Mutations associated with reduced susceptibility to LZD in 32 mutants of *M. tuberculosis*

| Strain[a] | *rrl*[b] | *rplC*[b] | RplC amino acid change | Mutation percentage | Growth on various concentrations of LZD (μg/ml)[c] | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | 0 | 0.25 | 0.5 | 1 | 2 |
| H37Rv | (-) | (-) | | | 4+ | - | - | - | - |
| L1T5-3 | G2270C | (-) | | | 4+ | 4+ | 3+ | 2+ | - |
| L1T1-5 | G2270T | (-) | | | 4+ | 4+ | 4+ | 3+ | - |
| L1T3-1 | G2270T | (-) | | | 4+ | 4+ | 3+ | 2+ | - |
| *L1T3-4 | G2270C | (-) | | 20 | 4+ | 4+ | 2+ | 1+ | - |
| L1T5-4 | G2270T | (-) | | | 4+ | 3+ | 3+ | 2+ | - |
| *L1T5-5 | G2270T | (-) | | 6 | 4+ | 3+ | 2+ | 1+ | - |
| L1T4-3 | G2270T | (-) | | | ND | ND | ND | ND | ND |
| L1T4-6 | G2746A | (-) | | | ND | ND | ND | ND | ND |
| L1T4-7 | G2746A | (-) | | | ND | ND | ND | ND | ND |
| L1T13-2 | G2746A | (-) | | | ND | ND | ND | ND | ND |
| L1T6-3 | G2746A | (-) | | | 4+ | 3+ | 3+ | 1+ | - |
| L1T4-4 | G2746A | (-) | | | 4+ | 4+ | 3+ | 2+ | - |
| L1T4-5 | G2746A | (-) | | | 4+ | 4+ | 3+ | 2+ | - |
| L1T4-8 | G2746A | (-) | | | 4+ | 4+ | 2+ | 2+ | - |
| L1T4-9 | G2746A | (-) | | | 4+ | 4+ | 2+ | 1+ | - |
| L1T1-6 | A2810T | (-) | | | 4+ | 3+ | 2+ | 1+ | - |
| *L1T3-6 | C2848A | (-) | | 13 | 4+ | 2+ | 2+ | 1+ | - |
| L1T3-2 | (-) | T460C | Cys154Arg | | 4+ | 4+ | 4+ | 4+ | 4+ |
| L1T4-2 | (-) | T460C | Cys154Arg | | 4+ | 4+ | 4+ | 4+ | 3+ |
| L1T5-2 | (-) | T460C | Cys154Arg | | 4+ | 4+ | 4+ | 4+ | 4+ |
| L2T14-1 | (-) | T460C | Cys154Arg | | 4+ | 4+ | 4+ | 4+ | 4+ |
| L2T15-1 | (-) | T460C | Cys154Arg | | 4+ | 4+ | 4+ | 4+ | 4+ |
| L2T15-3 | (-) | T460C | Cys154Arg | | 4+ | 4+ | 4+ | 4+ | 3+ |
| L2T15-4 | (-) | T460C | Cys154Arg | | 4+ | 4+ | 4+ | 4+ | 3+ |
| L2T15-5 | (-) | T460C | Cys154Arg | | 4+ | 4+ | 4+ | 4+ | 3+ |
| L2T15-6 | (-) | T460C | Cys154Arg | | 4+ | 4+ | 4+ | 4+ | 3+ |
| L2T22-1 | (-) | T460C | Cys154Arg | | 3+ | 3+ | 3+ | 2+ | 2+ |
| L2T22-2 | (-) | T460C | Cys154Arg | | 4+ | 4+ | 4+ | 4+ | 4+ |
| *L1T3-3 | (-) | T460C | Cys154Arg | 17 | 4+ | 4+ | 4+ | 4+ | 3+ |
| *L1T5-1 | (-) | T460C | Cys154Arg | 60 | 4+ | 4+ | 4+ | 4+ | 4+ |
| *L1T3-5 | (-) | T460C | Cys154Arg | 27 | 4+ | 4+ | 4+ | 4+ | 3+ |
| *L1T3-7 | (-) | T460C | Cys154Arg | 17 | 4+ | 4+ | 4+ | 4+ | 3+ |

[a] Strains beginning with asterisks were subjected to whole-genome sequencing.
[b] (-), no mutation.
[c] 4+, robust growth; 3+, good growth; 2+, moderate growth; 1+, poor growth; -, no growth; ND, not determined.

An interesting observation of this study is that mutations in *rplC* are associated with higher LZD MIC values (MICs of >2 μg/ml), whereas mutations in *rrl* are correlated with lower MIC values (MICs of 0.5 to 1 μg/ml) (Table 1). It will be of interest to determine if different levels of reduced susceptibility mediated by *rplC* or *rrl* have any impact on clinical treatment of drug-resistant TB using LZD in the future.

It has been reported that the G2061T or G2576T mutation in the *rrl* gene may cause LZD resistance in mutants of *M. tuberculosis* isolated *in vitro* (8). In this work, it is noteworthy that we found 5 new mutations (C2848A, A2810T, G2270C, G2270T, and G2746A) in the *rrl* gene that are associated with reduced susceptibility to LZD (Table 1). The reason why the mutations we identified in *rrl* differ from those reported in the previous study is unclear but may be due to differences in the mutant selection conditions or to a reflection of different levels of susceptibility. Future structural studies are needed to shed light on how these mutations at different locations can cause LZD resistance. The *rplC* T460C substitution was found to be a dominant mutation in 6 of 7 LZD-resistant strains isolated from patients *in vitro* and *in vivo* (9), and this mutation accounted for all of the remaining 15 mutants with higher MIC values without *rrl* mutations in this study (Table 1). Thus, the T460C mutation is likely an important mutation for *in vitro* and *in vivo* conditions.

An intriguing observation is that 7 mutants with reduced susceptibility initially failed to show any mutation in *rrl* or *rplC* by Sanger sequencing (Table 1, strains with an asterisk). However, subsequent Illumina whole-genome sequencing was able to iden-

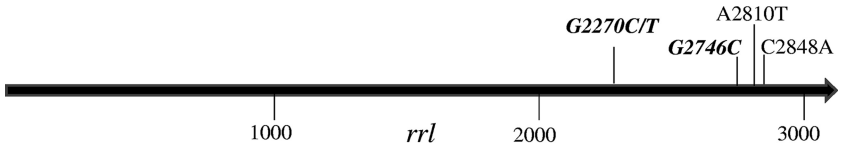


**FIG 1** Distribution of mutations in the *rrl* gene of *M. tuberculosis* mutants with reduced susceptibility to LZD. Bold and italic fonts at G2270 and G2746 indicate dominant mutation sites.

tify a mixed population of mutants having *rrl* or *rplC* mutations with sensitive organisms with wild-type *rrl* or *rplC* sequences in various proportions (Table 1). Nevertheless, it is of interest to note that the minor proportion of the mutants was able to confer a phenotype of reduced susceptibility (Table 1). This seems unusual, as it is not seen for other drug-resistant mutants conferring resistance to other TB drugs such as pyrazinamide (PZA) (13). This may be due to the nature of LZD in producing a mixed population during mutant selection. Further studies are needed to determine the mechanism and whether this also occurs *in vivo* during treatment. Alternatively, since efflux has been shown to play a role in LZD resistance in *M. tuberculosis* (6), it is possible that the reduced susceptibility to LZD in the 7 mutants with only a minor portion of mutants containing *rrl* or *rplC* mutations (Table 1) may be due to elevated efflux, which works together with resistance mutations to confer a high level of resistance (14). Future studies are needed to confirm this.

In conclusion, we found that mutants with reduced bacillary susceptibility to LZD harbor *rrl* and *rplC* mutations in about equal proportions. In addition, we found new mutations located at the C-terminal part of *rrl* that have not been previously reported. Furthermore, mutations in *rplC* are associated with higher MIC values than *rrl* mutations. These findings provide useful new information on the relative frequency of mutations and should help to develop molecular tests for rapid detection of LZD resistance for more effective treatment of MDR/XDR-TB.

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