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# Role of the Cys154Arg Substitution in Ribosomal Protein L3 in Oxazolidinone Resistance in *Mycobacterium tuberculosis*

**Gaëlle Guiewi Makafe,[a] Yuanyuan Cao,[a,b] Yaoju Tan,[c] Mugweru Julius,[a] Zhiyong Liu,[a] Changwei Wang,[a] Moses M. Njire,[a] Xingshan Cai,[c] Tianzhou Liu,[a,d] Bangxing Wang,[a,b] Wei Pang,[e,f] Shouyong Tan,[c] Buchang Zhang,[b] Wing Wai Yew,[g] Gyanu Lamichhane,[h] Jintao Guo,[a] Tianyu Zhang[a]**

State Key Laboratory of Respiratory Disease, Guangzhou Institutes of Biomedicine and Health (GIBH), Chinese Academy of Sciences (CAS), Guangzhou, China[a]; School of Life Sciences, Anhui University, Hefei, China[b]; State Key Laboratory of Respiratory Disease, Department of Clinical Laboratory, The Guangzhou Chest Hospital, Guangzhou, China[c]; School of Life Sciences, University of Science and Technology of China, Hefei, China[d]; Langfang Yanjing Vocational and Technical College, Langfang, China[e]; Forensic Medical Center, University of Maryland, Baltimore, Maryland, USA[f]; Stanley Ho Centre for Emerging Infectious Diseases, The Chinese University of Hong Kong, Hong Kong, China[g]; School of Medicine, Johns Hopkins University, Baltimore, Maryland, USA[h]

**We expressed the wild-type *rplC* and mutated *rplC* (Cys154Arg) genes, respectively, in *Mycobacterium tuberculosis* H37Ra and H37Rv in an attempt to delineate the role of *rplC* (Cys154Arg) regarding oxazolidinone resistance. An increase of the MICs of linezolid (LZD) and sutezolid (PNU-100480, PNU) against the recombinant mycobacteria with overexpressed *rplC* mutation (Cys154Arg) was found, suggesting the *rplC* gene is a determinant of bacillary susceptibilities to LZD and PNU.**

Currently, global control of tuberculosis (TB) is faced with the formidable challenge of worsening scenarios of drug-resistant disease, notably multidrug-resistant tuberculosis (MDR-TB), with bacillary resistance to at least rifampin and isoniazid, and extensively drug-resistant tuberculosis (XDR-TB), with additional resistance to fluoroquinolone(s) and second-line injectable agent(s) (1). Linezolid (LZD) has been widely used for the treatment of complicated MDR-TB and XDR-TB, improving the outcome of patients (2–5). Sutezolid (PNU-100480, PNU), a new congener of the same class, has also entered phase IIB TB treatment trials (http://www.newtbdrugs.org/project.php?id=135) after showing very good activity in the mouse TB model (6) and in the human whole-blood bactericidal system (7, 8).

Richter and colleagues (9) first reported on LZD-resistant clinical isolates of *Mycobacterium tuberculosis* with a 4- to 8-fold rise of the MICs from the susceptibility level of ≤1 μg/ml (10, 11). Whole-genome sequencing identified a point mutation, T460C (Cys154Arg), in the *rplC* gene that might constitute a putative marker for LZD resistance in *M. tuberculosis* (12). The *rplC* gene encodes the ribosomal protein L3 located in the S10 operon of the *Escherichia coli* chromosome, encoding 11 ribosomal proteins (13). The main part of L3 is positioned on the surface of the 50S ribosomal subunit, but a branched loop extends close to the peptidyl transferase center, the binding site for many different ribosomal antibiotics (14). Until now, the mechanisms of LZD resistance in *M. tuberculosis* were not yet fully unraveled because the association of *rplC* Cys154Arg mutation with LZD and PNU in *M. tuberculosis* was not confirmed by molecular genetic methods. Herein, we constructed recombinant strains to determine whether *rplC* Cys154Arg mutation would result in resistances to LZD and PNU in *M. tuberculosis*.

**TABLE 1** DNA primers and fragments used in this study

| Primer | Nucleotide sequence (5′–3′) |
|---|---|
| *Kan*-f | ATGAGCCATATTCAACGGGA |
| *Kan*-r | TTAGAAAAACTCATCGAGCA |
| *rplC*f | GTGAATTCCTGACGGACGAGACCA |
| *rplC*r | CTTCTAGACGCAGCCATCACTTCT |
| *tsr*F | GGTGGTACCATGGAAGACGCCAAAAAC |
| *tsr*R | GCTCTAGATTACAATTTGGACTTTCC |
| *rplC*-F | GGGAATTCCATATGGCACGAAAGGGCATTC |
| *rplC*-R | CCCAAGCTT TCACTTCTCACCTCGTTTG |
| *hyg*-f | GTGACACAAGAATCCCTG |
| *hyg*-r | TCAGGCGCCGGGGGCGGTG |

The oxazolidinone susceptibilities were evaluated on Middlebrook 7H11 medium (Difco). The *rplC* gene is preceded by an upstream *rpsJ* gene, and a putative promoter was identified (Fig. 1A); hence, primers *rplC*f and *rplC*r (Table 1) for amplification of the promoter-*rpsJ-rplC* fragment (Fig. 1A) were designed with EcoRI and XbaI restriction sites at the 5′ terminals, respectively, and the fragment was cloned into the integrative plasmid

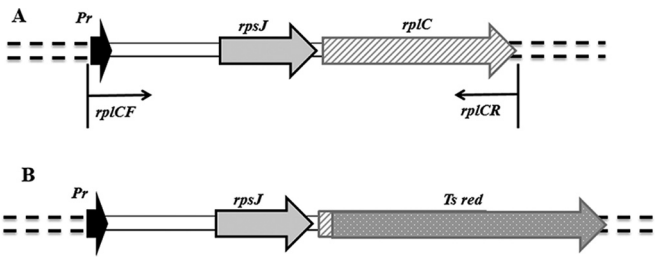


**FIG 1** Diagrammatic illustration of the position of *rplC* gene (A) and its predicted promoter and the position of *Ts red* in pLZD-luc (B).

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Address correspondence to Tianyu Zhang, zhang_tianyu@gibh.ac.cn.
G.G.M., Y.C., and Y.T. contributed equally to this article.

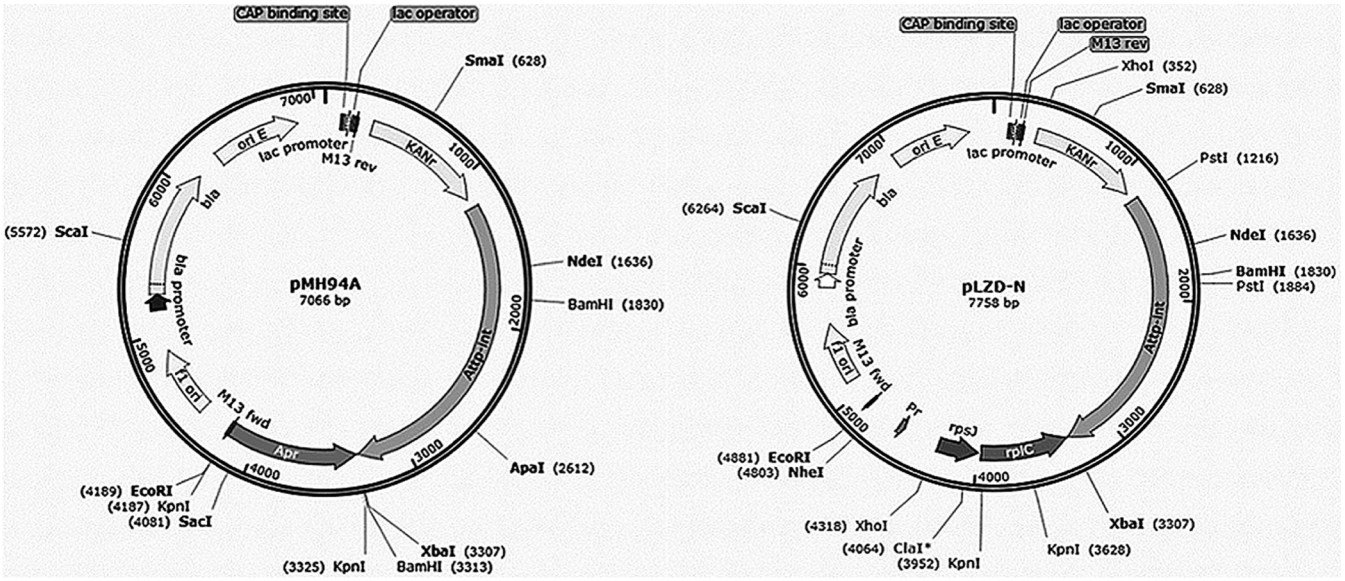


**FIG 2** Integrative plasmid pMH94A and pLZD-N. *oriE*, origin region in *E. coli*; KANr, kanamycin-resistant gene from Tn*9O3*; *bla*, ampicillin-resistant gene; *rplC*, gene from *M. tuberculosis* H37Rv.

pMH94A between EcoRI and XbaI sites to create pLZD-N (Fig. 2; Table 2). The *rplC* in the construct pLZD-N was confirmed by DNA sequencing (BGI, Shenzhen, China). We further induced a point mutation of the *rlpC* gene at T460C (Cys154Arg) in pLZD-N creating the plasmid pLZD-NM (Generay Biotech, Shanghai, China) (Table 2). The *rplC* and *rplC* Cys154Arg genes were amplified by PCR from pLZD-N and PLZD-NM plasmids, respectively, using the oligonucleotide primers (*rplC*-F and *rplC*-R; Table 2) of the *rplC* open reading frame only, and were cloned into the NdeI-HindIII sites of an extrachromosomal plasmid p60luxN (15) under the control of a strong promoter, *Hsp60*, to yield p60-rplCN (Fig. 3) and p60-rplcNM. The four plasmids pLZD-N, pLZD-NM, p60-rplCN, and p60-rplcNM were transformed into wild-type *M. tuberculosis* (H37Rv and H37Ra) through electroporation as described previously (16). Positive selection was confirmed by PCR amplification of the kanamycin

**TABLE 2** Bacterial strains and plasmids used in this study

| Strain/plasmid | Relevant characteristic(s) | Source or reference |
|---|---|---|
| pMH94 | pUC119 carrying KAN$^r$ from Tn*9O3* and *attp-int* cassette from L5 mycobacteriophage at SalI-SalI | 17 |
| pMH94A | pMH94 containing apramycin-resistant gene (*Apr*) gene at KpnI sites | This study |
| pLZD-N | pMH94A containing *M. tuberculosis* natural promoter-*rpsJ-rplC* gene at EcoRI-XbaI sites | This study |
| pLZD-NM | Mutated *rplC* CYS154ARG gene in pLZD-N | This study |
| pN60-RIK | Plasmid containing the *Ts red* | Unpublished |
| pLZD-luc | pLZD-N and pLZD-NM plasmids containing the *Ts red* firefly luciferase gene | This study |
| p60luxN | Containing the strong promoter *Hsp60*, origins of replication for *E. coli* and mycobacteria and hygromycin-resistant gene (*hyg*) | 15 |
| p60-rplcN | p60luxN inserted the *rplC* wild-type gene at NdeI-HindIII sites | This study |
| p60-rplcNM | p60luxN containing the mutated *rplC* CYS154ARG gene at NdeI-HindIII sites. | This study |
| *E. coli* DH5α | General-purpose cloning strain; F$^-$ [ϕ80d *lacZ*ΔM15] Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17 glnV44 thi-1 gyrA96 relA* | 17 |
| *M. tuberculosis* H37Rv | A widely used virulent laboratory *M. tuberculosis* strain, ATCC 27294 | 16 |
| *M. tuberculosis* H37Ra | A widely used avirulent laboratory *M. tuberculosis* strain | This study |
| *M. tuberculosis* H37Rv::pLZD-N | *M. tuberculosis* H37Rv containing pLZD-N | This study |
| *M. tuberculosis* H37Rv::pLZD-NM | *M. tuberculosis* H37Rv containing pLZD-NM | This study |
| *M. tuberculosis* H37Ra::pLZD-N | *M. tuberculosis* H37Ra containing pLZD-N | This study |
| *M. tuberculosis* H37Ra::pLZD-NM | *M. tuberculosis* H37Ra containing pLZD-NM | This study |
| *M. tuberculosis* H37Rv::p60-rplCN | *M. tuberculosis* H37Rv containing p60-rplCN | This study |
| *M. tuberculosis* H37Rv::p60-rplCNM | *M. tuberculosis* H37Rv containing p60-rplCNM | This study |
| *M. tuberculosis* H37Ra::p60-rplCN | *M. tuberculosis* H37Ra containing p60-rplCN | This study |
| *M. tuberculosis* H37Ra::p60-rplCNM | *M. tuberculosis* H37Ra containing p60-rplCNM | This study |
| *M. tuberculosis* H37Ra::pN60-RIK | *M. tuberculosis* H37Ra containing pN60-RIK | This study |
| *M. tuberculosis* H37Ra::pLZD-luc | *M. tuberculosis* H37Ra containing pLZD-luc | This study |

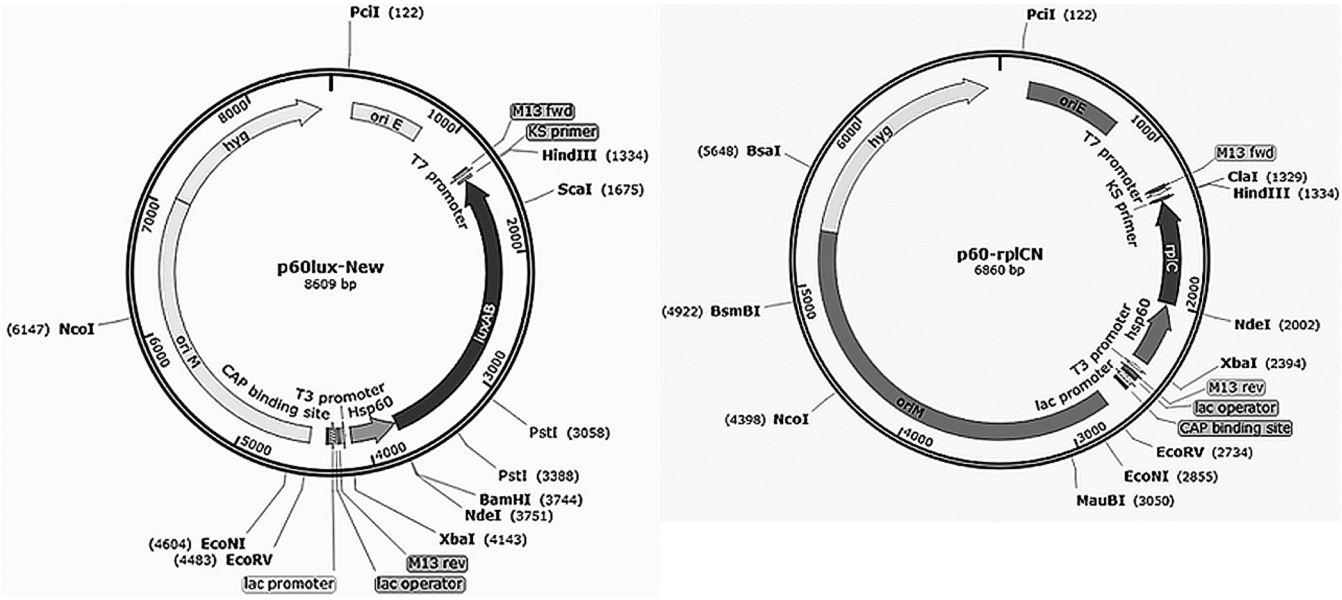


**FIG 3** Plasmids p60luxN and p60-rplcN. *oriE*, origin region in *E. coli*; *oriM*, origin region in mycobacteria; *hyg*, hygromycin-resistant gene; *rplC* and *rplC* (CYS154ARG), genes from pLZD-N and PLZD-NM plasmids, respectively.

(KAN)-resistant marker gene (KAN$^r$) in pLZD-N and pLZD-NM using primers *kan*-r and *kan*-f or PCR of the hygromycin-resistant marker gene (*hyg*) in p60-rplCN and p60-rplCNM using primers *hyg*-r and *hyg*-f (Table 1). The MICs of recombinant and wild-type bacteria against LZD and PNU were determined by the classical agar plate method (17). We observed no changes in the MICs of LZD and PNU against the two recombinant strains containing integrative pLZD-N and pLZD-NM, comparing to their parental strains (Table 3). However, 4- and 2-fold increments in LZD and PNU MICs, respectively, were observed in *M. tuberculosis* H37Ra and *M. tuberculosis* H37Rv containing p60-rplcNM, but no MIC changes were observed in the strain containing p60-rplCN.

We investigated whether the *rplC* gene expression level influenced oxazolidinone resistance. A reporter thermostable red firefly luciferase gene (*Ts red*) (18, 19) was introduced into pLZD-N by exploiting the KpnI and XbaI restriction sites inside the *rplC* gene (primers *tsr*F and *tsr*R; Table 1) to obtain plasmid pLZD-luc. The *Ts red* was fused with 9 amino acids of RplC at its N terminal (Fig. 1B). In the presence of the D-Luciferin (BBI Life Sciences), the *E. coli* strains containing pN60-RIK and pLZD-Luc (Table 2) produced strong light, which meant the fused luciferase was functional (Table 4). The 2 plasmids were transformed into *M. tuberculosis* H37Ra as previously described (16), and the KAN-resistant colonies were selected after confirmation of the resistant marker genes by PCR. The luciferase activities of the recombinant bacteria were measured using D-Luciferin bioluminescent assays (Table 4). *M. tuberculosis* H37Ra::pN60-RIK produced at least a 25-fold stronger light than *M. tuberculosis* H37Ra::pLZD-luc (Table 4). The light produced by *M. tuberculosis* H37Ra::pLZD-luc was weak, only about 2-fold greater than the background reading (Table 4). Our data suggest that the *rplC-rplC* (Cys154Arg) integrated into *M. tuberculosis* can be expressed but at a low level. Given that the mutations of 50S ribosomal protein L3 (*rplC* encoded) may alter the structure of the adjacent LZD binding site in the peptidyl transferase center (14), an excessive amount of the mutated L3 protein will be required to titrate out the drug-binding sites. It has also been reported that integration of a single-copy target gene (with mutation) in mycobacteria failed to induce resistance that was otherwise conferred with the multicopy plasmid (20). Therefore, the low level of expression may explain why integration of the *rplC* (Cys154Arg) gene in *M. tuberculosis* H37Ra::pLZD-NM failed to induce resistance to LZD and PNU, as the number of gene copies and/or the level of gene expression are important determinants of the resistance phenotype.

**TABLE 3** MICs of LZD and PNU for wild-type and recombinant *M. tuberculosis* strains

| *M. tuberculosis* strain | MIC (μg/ml) | |
|---|---|---|
| | LZD | PNU |
| H37Rv | 1.0 | 0.25 |
| H37Rv::pLZD-N | 1.0 | 0.25 |
| H37Rv::pLZD-NM | 1.0 | 0.25 |
| H37Rv::p60-rplCN | 1.0 | 0.25 |
| H37Rv::p60-rplCNM | 4.0 | 0.5 |
| H37Ra | 1.0 | 0.25 |
| H37Ra::pLZD-N | 1.0 | 0.25 |
| H37Ra::pLZD-NM | 1.0 | 0.25 |
| H37Ra::p60-rplCN | 1.0 | 0.25 |
| H37Ra::p60-rplCNM | 4.0 | 0.5 |

Zhang et al. (21) recently found 3 LZD-resistant *M. tuberculosis* strains with the TGC460CGC mutation and one strain with the CAC463GAC mutation, indicating Cys154Arg and His155Asp amino acid substitutions, respectively. However, we found that the sequence of 457- to 468-bp of *rplC* gene was GGATGTGC CACG (460T was underlined and 462T and 465C were boxed) and the TGC460CGC and CAC463GAC mutations in the *rplC* gene in their report (21) may be TGT(Cys)462TGC(Cys) and GCC (Ala)465GCA(Ala). If so, they do not result in any amino acid substitution. Until now, *rplC* (Cys154Arg) has been shown to

**TABLE 4** Detection of the firefly luciferase activity

| Detection | *E. coli* containing pN60-RIK | *E. coli* containing pLZD-luc | *M. tuberculosis* H37Ra containing pN60-RIK | Control: *M. tuberculosis* H37Ra containing pLZD-luc without luciferin | *M. tuberculosis* H37Ra containing pLZD-luc with luciferin |
|---|---|---|---|---|---|
| RLU[a] | >1,700,000 | >1,000,000 | >5,000 | 52.50 ± 7.41 | 145.3 ± 30.42[b] |

[a] Mean relative light unit ± standard error of mean from 4 independent measurements.
[b] $P = 0.025 < 0.05$ compared with luciferin-free control.

cause resistance to all the congeners of the oxazolidinone class, and the mutated sequence is the only one found in this gene (22).

In conclusion, we have verified by molecular genetics that the mutation Cys154Arg in *rplC* can cause resistance to LZD and PNU oxazolidinone. Our findings, therefore, concur with Beckert et al. (12) that the Cys154Arg mutation in the *rplC* gene possibly plays a major role in LZD resistance in *M. tuberculosis* in clinical isolates. As shown elsewhere, the equivalent mutation N149R in *E. coli* L3 protein also increased the MIC from 8 to 32 μg/ml (14). We hence propose further work to establish a novel, rapid, and high-throughput drug-screening model for undertaking such oxazolidinone resistance using the autoluminescent *M. tuberculosis* (16, 23) overexpressing *rplC* (Cys154Arg). Our data support that the Cys154Arg mutation in *rplC* may be considered one of the markers for oxazolidinone resistance in clinical *M. tuberculosis* isolates.

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We declare no conflicts of interest.

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