Supplementary Information for

Insights into the improved macrolide inhibitory activity from the high-resolution cryo-EM structure of dirithromycin bound to the E. coli 70S ribosome.

Running title: Structure of dirithromycin bound to E.coli ribosome.

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This file includes:

I. Supplementary Tables S1 and S2;
II. Supplementary Figures S1 to S4 with legends;
III. Supplementary Movie S1;
IV. Supplementary References.
I. SUPPLEMENTARY TABLES

Table S1. Cryo-EM data collection and 50S model refinement statistics.

| Complexes                  | 70S complex with A- and P-tRNAs and Dirithromycin |
|----------------------------|--------------------------------------------------|
| **Cryo-EM data**           |                                                  |
| Microscope                 | FEI Titan Krios                                  |
| Accelerating Voltage, kV   | 300                                              |
| Detector                   | Falcon II                                        |
| Spherical aberration, mm   | <0.1                                             |
| Magnification              | 75,000x                                          |
| Defocus range, μm          | -0.3 to -2.2                                    |
| Micrographs                | 4,986                                            |
| **Cryo-EM reconstruction** |                                                  |
| Particles picked           | 973,000                                          |
| Particles refined          | 401,905                                          |
| Resolution achieved, Å      | 2.1Å                                             |
| **Refinement**             |                                                  |
| No. of Non-Hydrogen Atoms  |                                                  |
| All atoms                  | 94,209                                           |
| Protein residues           | 3,352                                            |
| Nucleotides                | 3,169                                            |
| Waters                     | 0                                                |
| Ramachandran Plot          |                                                  |
| Favored regions, %         | 96.68                                            |
| Allowed regions, %         | 2.86                                             |
| Outliers, %                | 0.46                                             |
| Deviations from ideal values (RMSD) |                        |
| Bond, Å                    | 0.007                                            |
| Angle, degrees             | 0.683                                            |
| Chirality                  | 0.042                                            |
| Planarity                  | 0.004                                            |
| Dihedral, degrees          | 18.603                                           |
| Average B-factor (overall), Å² | 92.3                                              |
Table S2. Lower resolution maps obtained from the dataset after focused 3D classification.

| Structural Class      | I               | II              |
|-----------------------|-----------------|-----------------|
| tRNA occupancy        | A- and P-site tRNAs | P-site tRNA    |
| Number of particles   | 117,919         | 65,269          |
| Resolution achieved, Å | 2.54            | 2.66            |
II. SUPPLEMENTARY FIGURES

Figure S1. Fourier shell correlation (FSC) curve for the 70S-DIR cryo-EM structure. (A) Based on the “gold-standard FSC” cutoff value 0.143, the overall resolution for the large ribosomal subunit in the 50S ribosomal subunit is 2.1 Å. FSC values for plotting were calculated in Relion 3.1beta using half-maps. (B) Slice through the 50S subunit showing local resolution range. Note that the region of DIR binding (located at the heart of the large ribosomal subunit) is characterized by the highest local resolution. The color scale bar is in Ångstroms. 30S ribosomal subunit is not shown and was not included in the final model because the local resolution and connectivity of the map for the 30S subunit were poor after the focused refinement with a mask covering 50S subunit. (C) High-resolution features of the cryo-EM map.
Figure S2. Flowchart showing the steps of cryo-EM data processing.
Figure S3. Binding of BODIPY-ERY to *T. thermophilus* 70S ribosomes measured by fluorescence anisotropy. Non-linear regression analysis of obtained data yielded apparent dissociation constant of BODIPY-ERY equal to 43 ± 2 nM.
Figure S4. Multiple sequence alignment of the ribosomal protein L4 from several bacterial species.

Blue box highlights the amino acid residue of uL4, which contacts the side chain of DIR in our previous X-ray crystal structure of DIR in complex with \textit{Tth} 70S ribosome (Khabibullina et al. 2019). Note that \textit{E. coli} and many other bacteria have glycine residue in the same position.
III. SUPPLEMENTARY MOVIES

Movie S1. DIR functional site in the bacterial 70S ribosome from E. coli. The movie shows (1) zoom-out and (2) close-up views of the DIR binding site in the large subunit of the E. coli ribosome; (3) details of DIR interactions with the 23S rRNA in the PTC of the ribosome.
IV. SUPPLEMENTARY REFERENCES

Khabibullina NF, Tereshchenkov AG, Komarova ES, Syroegin EA, Shiriaev DI, Paleskava A, Kartsev VG, Bogdanov AA, Konevega AL, Dontsova OA et al. 2019. Structure of dirithromycin bound to the bacterial ribosome suggests new ways for rational improvement of macrolides. *Antimicrob Agents Chemother* **63**: e02266-02218.