SUPPLEMENTARY INFORMATION

We previously submitted a proposal in October 2019 where we proposed three separate projects. Two of the projects were well-received with both reviewers acknowledging that high-resolution structures would be attained if awarded time, while a third project was thought to be too preliminary (overall score of 1.9). For this application, the projects have now been separated as requested, allowing us to expand on how samples were prepared EM analysis, including expected structural details that will be derived from high-resolution structures. Additionally, we have updated the time requested based reviewers' suggestions. We thank the reviewers for their suggestions.

Francisella tularensis 70S ribosome rescue by a novel stalling factor.

Background. Ribosome stalling occurs frequently during protein synthesis and the inability to relieve this stalling to release ribosomes results in cell death[1-3]. Importantly, the factors involved in rescuing stalled ribosomes are different in bacteria and eukaryotes thereby establishing bacterial rescue systems as novel antimicrobial targets[4-9]. Sequence analyses of diverse pathogens reveal significant differences in the factors that rescue ribosomes[10]. In particular, *F. tularensis (Ft)* has a novel rescue factor called ArfT that interacts with both release factor 1 (RF1) and release factor 2 (RF2) to terminate translation during stalling, allowing ribosomes to be recycled. Since this stalling factor interacts with both release factors in *Ft* (unlike the *E. coli* ArfA), defining the molecular details of its action will provide important insights into potential mechanisms to inhibit *Francisella* (a bioterrorism agent). We propose to solve two *Ft* 70S structures: 1) *Ft* 70S-ArfT-RF1-mRNA and 2) *Ft* 70S-ArfT-mRNA-RF2.

Preliminary data. In order to assemble this ribosomal complex, *Ft* 70S ribosomes and recombinant *Ft* ArfT, *Ft* RF1 and *Ft* RF2 were purified as previously published[11]. The *Ft* non-stop complex was formed using a procedure similar to what we have previously published[6]. We biochemically confirmed that our ribosomes contain all ligands. Non-stop ribosome complexes containing ArfT-RF1-mRNA were placed on glow-discharged grids (Quantifoil 1.2/1.3 300 mesh Cu) and blotted using a FEI Vitrobot. Grids were screened using a JEOL JEM-1400 120 kV and a 2700 micrograph dataset was collected on an FEI Talos Arctica transmission electron microscope operating at 200 keV with BioQuantum/Gatan K2 direct electron detector. Micrographs were collected using a defocus range of -0.5 to -3.5 µm and a dose of 54.54 e^- per pixel and were acquired as 40-frame movies with 15s exposure time. Motion correction and dose weighting was performed with MotionCorr2 and contrast transfer function parameters determined with Gctf. Two-dimensional and three-dimensional classification was conducted in Relion-3.0. Semi-autonomous particle picking was used to select ribosomes and incorrectly selected particles discarded after reference-free two-dimensional classification. Three-dimensional refinement was conducted with a 45-Å low-pass filtered cryo-EM *E. coli* 70S reference map (PDB: 5mgp). Three-dimensional classification without alignment was conducted to discard 50S ribosome particles and poorly aligned particles.

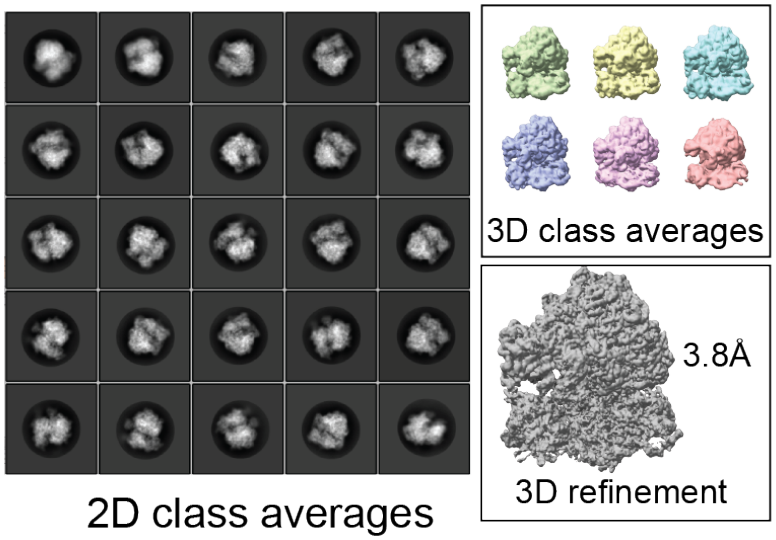

Figure 1. Preliminary cryo-EM analysis of 70S bound to a novel stalling factor. 2D class averages and 3D class averages of the 70S are shown. 3D refinement of the dataset to ~3.8Å.

Proposed studies. We have confirmed our sample contains ligands. However, we are limited by resolution and need to collect higher resolution to confidently identify the molecular details of these interactions. **We request one day for two datasets.**

Expected structural details. We anticipate our structure will provide molecular insights into how a novel rescue factor interacts with both release factors to rescue ribosomes that are stalled. Additionally, biochemical assays to determine the specificity and kinetics of ArfT interactions with different stalled *Ft* 70S complex will provide complementary data to identify the molecular basis for this action.

REFERENCES

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