EUROPEAN SYNCHROTRON RADIATION FACILITY

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Experiment Report Form

	Experiment title: *Structural study of the tmRNA- SmpB complex with the stalled ribosome of Pseudomonas aeruginosa and Enterococcus faecium*	**Experiment number**: **MX-2574**
Beamline: **CM1**	**Date of experiment**: from: **27-09-2023** to: **29-09-2023**	**Date of report**: **14-11-2023** *Received at ESRF:*
Shifts: **6**	**Local contact(s)**: MCGREGOR Lindsay	

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Here we are submitting the first preliminary report for the project titled MX-2574

The proposed project was designed to study the structure of the ribosomes from six ESKAPE bacteria ongoing *trans*-translation to near-atomic resolution using Single Particle Analysis by cryo-EM. The World Health Organization designated seven "ESKAPEE" pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, *Enterobacter spp*., and *Escherichia coli*) as critical targets for drug discovery. Since *trans*–translation is important for pathogen virulence and does not exist in eukaryotic cells, it represents a very attractive target for the development of new narrow-spectrum antibiotics. Therefore, current ongoing project aims at studying the molecular interactions between tmRNA-SmpB complexes and stalled ribosomes during *trans*-translation in six ESKAPE pathogens and compare them with the *E.coli* one already obtained in our lab, and within each other.

Following acceptance of our application, we shipped the first sample consisting of reconstituted *E. faecium trans*-translating complex for acquisition on a Titan Krios at the ESRF. Eight grids (four 4x2 and four 2x1 c-flat grids) were shipped to ESRF and some minor problems occurred during the clipping of the grids (upon removing the lid, the grids stuck to the plastic and only the grid in position 3 remained safely in the box). The four 4x2 c-flat grids were used for the acquisition while the four 2x1 grids were kept at the ESRF.

Image data collection parameters:
Camera type: Gatan K3; Acquisition mode: counting; Compression: TIFF LZW; Gain normalized: yes; Magnification 81k; Pixel size 1.06 Ang/pixel; Electron dose 19.4 electrons/pixel/s; Exposure time 2s; Total dose per movie 34.5 electron/Ang2; Number of frames per movie 40; Defocus spread -0.6 to -2.4 um in 0.2 um step.; Energy filter: Gatan Quantum LS; Slit width: 20 eV: Phase plate: no

During the allocated six shifts, we acquired up to 17480 movies (27-28-29 September 2023). While the ice/grid quality was far from perfect, *trans*-translating ribosome were observed in the "on-the-fly" 2D classification. We downloaded the data and completed our analysis (**see box 1**). Our results are very promising but as shown **Fig. 1** however the tmRNA-SmpB complex is somehow under resolved. This is partly due to the intrinsic flexibility of the tmRNA pseudoknot ring but, for the TLD-SmpB (which is deeply inserted into the ribosome A-site and therefore much more stable), it is mostly due to the small number of particles (43,870) retained for the final reconstruction.

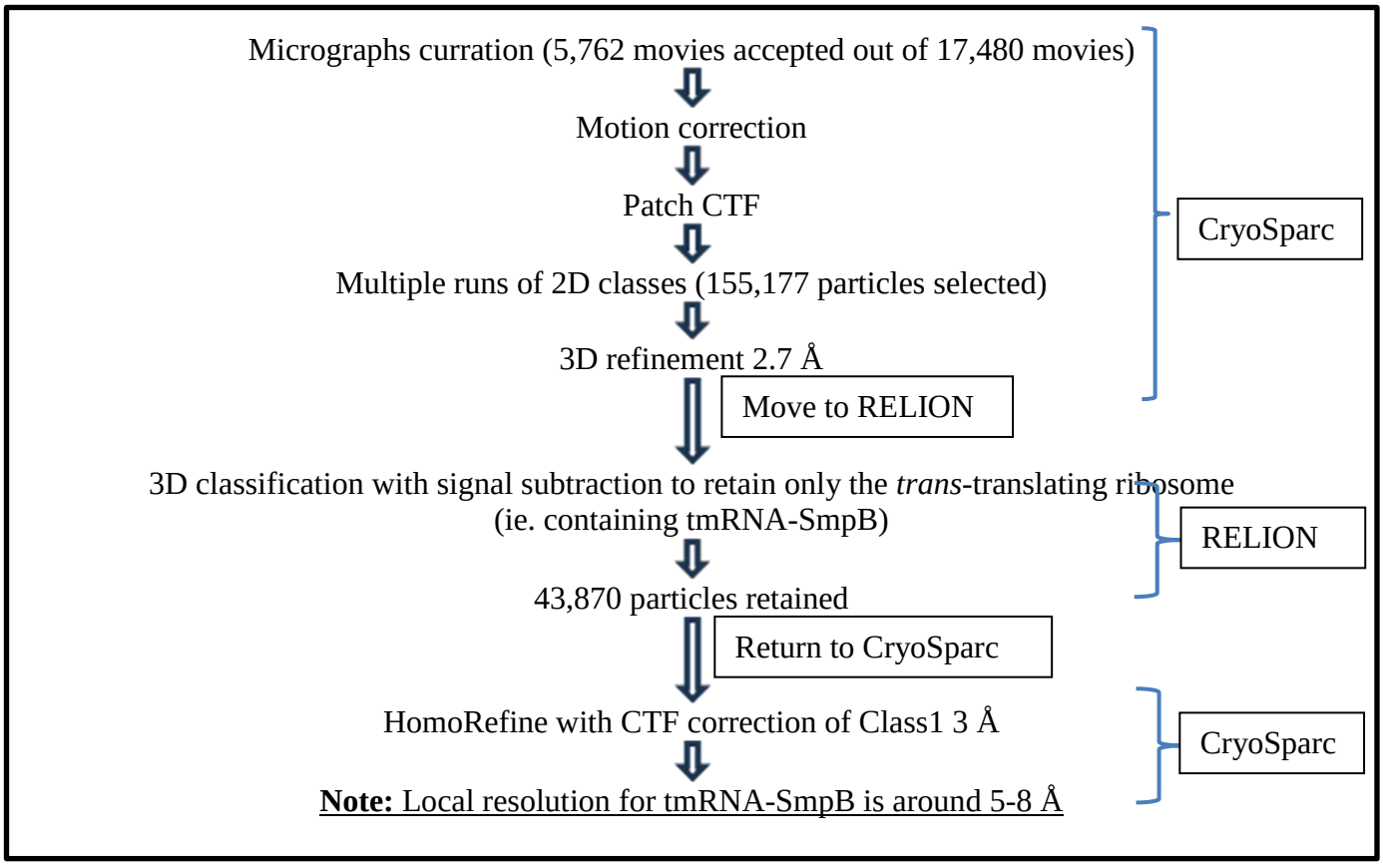
Indeed while starting from 17,480 movies we had to remove two third of the movies (empty ice) and only 5,762 movies (corresponding to the thicker ice where ribosome were present) were kept for further analysis. This

leaved us with 155,177 initial particles, which were further separated in: 27,498 blurry particles, 83,809 stalled ribosomes and 43,870 *trans*-translating ribosomes with a visible pseudoknot ring. While our final global resolution is around 3 Å the local resolution of the tmRNA-SmpB complex range between 5 - 8 Å which is too low to be able to analyses the contact within the three partner at the atomic or even at the residue level.

Concerned by the quality of our grids we also tested another sample frozen at the same time and using the same condition using 2x1 c-flat grids and the ice quality was indeed much better, which let us hope for the best with the 4 remaining grids.

Taken all together, we strongly believe that we need more images to improve the local resolution of the tmRNA-smpB complex and to understand how they interact with each other and with the ribosome. Considering that another set of 4 grids are already clipped and stored at the ESRF, that there was no transportation/clipping issue with those grids and that the ice quality should be better, we would like to request one more session of acquisition on a Titan Krios with the same acquisition parameter as for the previous session (27-29th September 2023).

Box 1: For our image analysis, we use both CryoSparc (v4.2.1) and RELION (4.0).



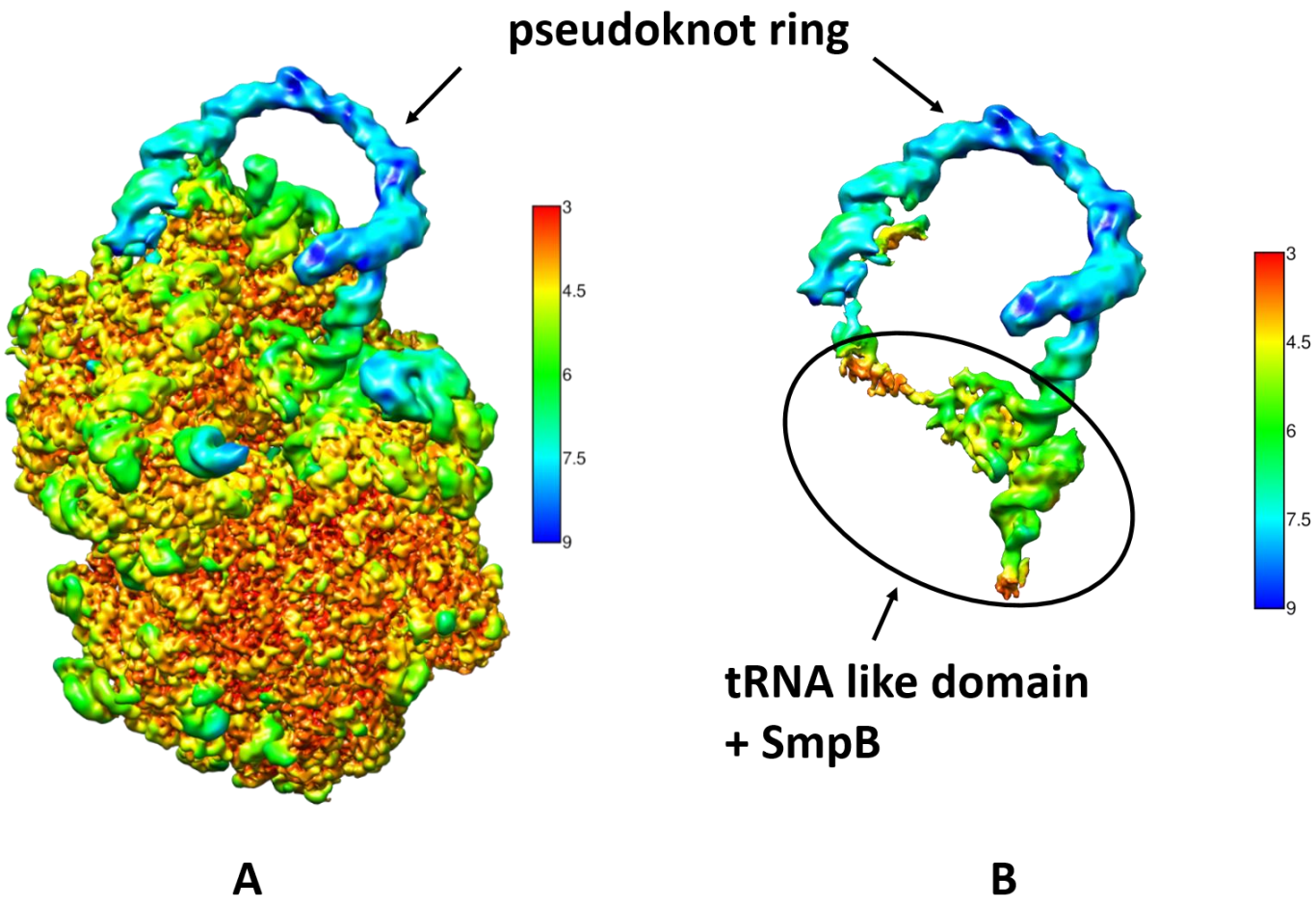


Fig. 1 Initial electron density maps of *E faecium* in *trans*-translation state (A) 70s Ribosome with tmRNA and smpB (average resolution of 3 Å); (B) Local resolution for the tmRNA-smpB part only (average resolution of 5 - 8 Å)