Cryo-ET Characterization of Novel Cellular Extrusions in *Escherichia coli* Induced by the Major Subunit Protein of Type IV Pili, PilA, from *Pseudomonas aeruginosa*

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Extracellular membranous appendages are found in many prokaryotic species including archaea, Gram-positive bacteria, and Gram-negative bacteria and have been termed nanotubes, nanopods, or nanowires [1-4]. During our studies of type IV pili, we discovered nanotube like structures correlated with heterologous expression of the *Pseudomonas aeruginosa* major type IV pilin, PilA, in some *Escherichia coli* strains. We have coined these membranous tubular structures P-pods for pilin-induced pods based partly on their appearance in negative stain transmission electron microscopy images. Biochemical analysis showed that the major protein inside of P-pods is the inner membrane protein PilA. Because some membranous appendages serve as transport tunnels, we tested horizontal gene transfer in the P-pods; these studies revealed that P-pods are unable to transfer plasmid DNA.

To further characterize the structure of P-pods we carried out both negative stain TEM imaging and cryo-electron tomography. The cells were incubated on LB agar with inducers for 16 hours. Cells were resuspended in LB liquid medium to an $OD_{600} = 0.01$ and spotted on to glow-discharged, Quantifoil R2/1 mesh, gold grids (Quantifoil, Germany) with 5 nm carbon coating, in a humified chamber for 3.5 hours. Three μL of BSA-treated 10 nm colloidal gold (Electron Microscopy Sciences, USA) was applied onto the grids and then plunge-frozen in liquid ethane using a Leica EM GP (Leica Microsystems, Germany). Data collection was performed on a Titan Krios (Thermo Scientific, USA) FEG TEM operated at 300 kV and equipped with a Gatan K3 direct electron detector and a Gatan bioquantum energy filter, at the University of Wisconsin-Madison Cryo-EM Research Center. Images were acquired with a pixel size of 0.4603 nm on the specimen and a total electron does between 120 to 130 e$^-$/Å^2. Data were collected at 2° increments from -60° to +60° (61 images) with a nominal defocus range of -4.0 to -6.0 μm to enhance contrast of various cell components. Tilt series images were collected using SerialEM [5]. Tomographic reconstructions were generated using IMOD [6] following tilt-series image motion correction by motioncor2 [7] and data were binned two-fold during this process. Neural network based tomogram segmentation models of P-pods were produced with EMAN2 [8].

The resulting 3D reconstructions revealed that P-pods originate at the inner membrane and P-pod extrusion may occur in multiple ways (Figure 1). P-pods may exit the cells through breaks in the outer membrane or by blebbing of the outer membrane which eventually ruptures and releases P-pods (Figure 1). Also, our data indicates that P-pods are comprised of a single lipid-bilayer, do not transport other vesicles, and are typically observed as a string of segmented vesicles once outside of the cell (Figure 2).

Correlative light and electron microscopy (CLEM) could provide additional evidence for the localization of PilA within the cell and P-pods and may be the focus of follow up experiments. The biochemical and structural studies presented here reveal that, though similar to nanotubes, P-pods are novel membranous extracellular appendages that spontaneously assemble as the result of PilA over-expression in *E. coli* and may be a result of protein crowding that leads to positive membrane curvature of the inner membrane [9].
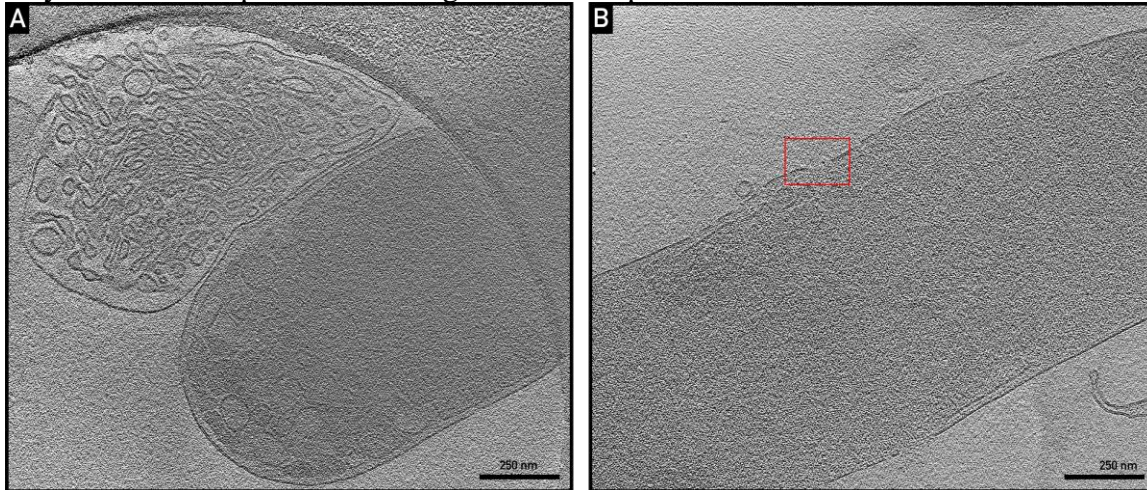
Figure 1. Characterization of P-pod extrusion via blebbing or membrane breach. A) Tomogram reconstruction of an *E. coli* cell expressing *P. aeruginosa* PilA protein resulting in blebbing of the outer membrane and localization of p-pods to the bleb. B) Tomogram reconstruction of the PilA expression system resulting in breaks along the outer membrane which allow for extrusion of p-pods. Figures represent a central slice with a thickness of 4.6 nm.
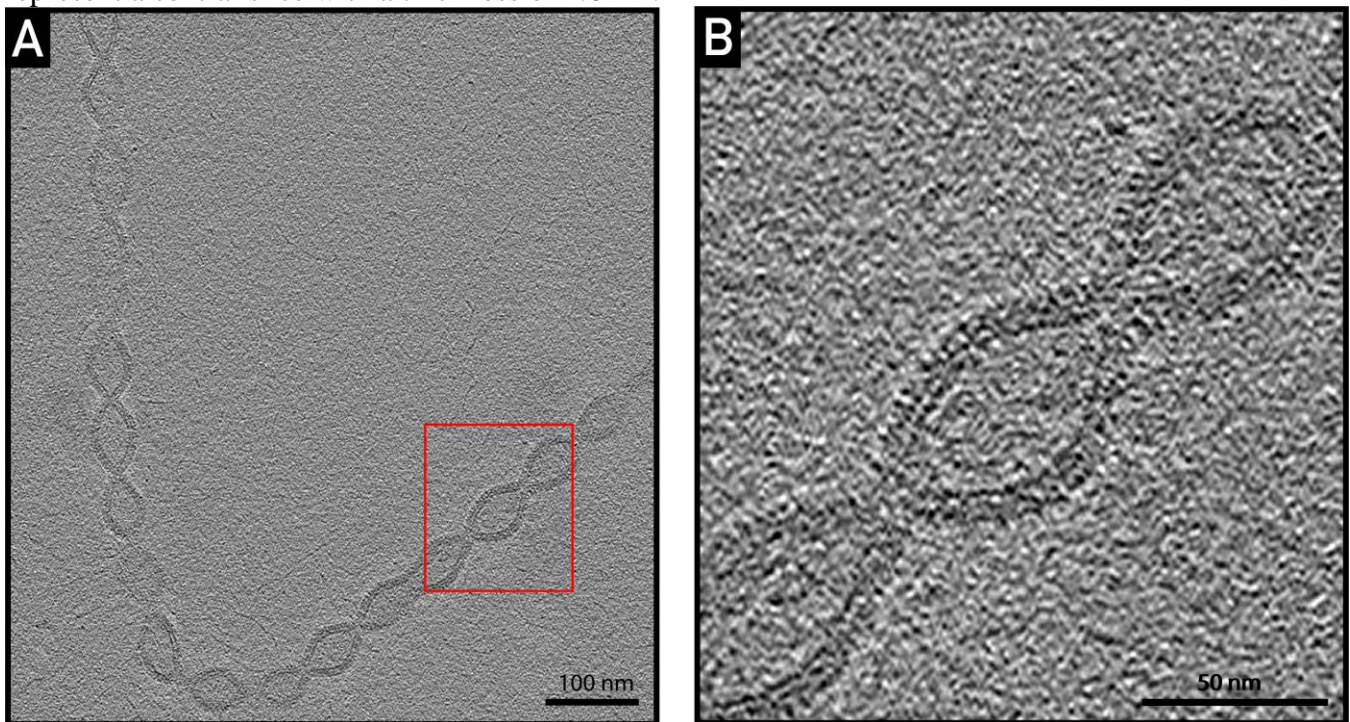
Figure 2. Extracellular P-pod structures. A) Tomogram reconstruction of extracellular p-pods as a string of segmented vesicles and B) a magnified view of a p-pod segment. Figures represent a central slice with a thickness of 4.6 nm.

References

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