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Recent progress in structure and dynamics of dual-membrane-spanning bacterial nanomachines

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Advances in hard-ware and soft-ware for electron cryo-microscopy and tomography have provided unprecedented structural insights into large protein complexes in bacterial membranes. Tomographic volumes of native complexes *in situ*, combined with other structural and functional data, reveal functionally important conformational changes. Here, we review recent progress in elucidating the structure and mechanism of dual-membrane-spanning nanomachines involved in bacterial motility, adhesion, pathogenesis and biofilm formation, including the type IV pilus assembly machinery and the type III and VI secretions systems. We highlight how these new structural data shed light on the assembly and action of such machines and discuss future directions for more detailed mechanistic understanding of these massive, fascinating complexes.

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Introduction

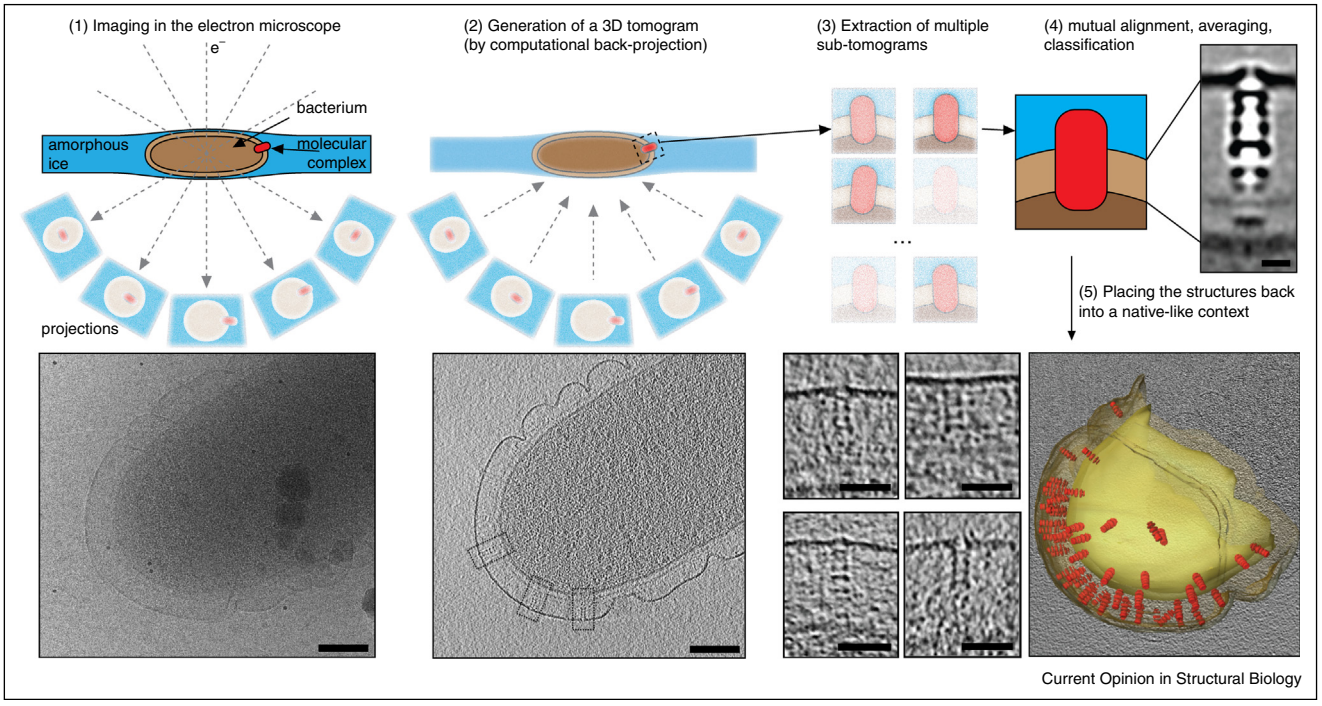
A distinctive feature of all Gram-negative bacteria is that they have two membranes — a relatively porous outer membrane and an impermeable cytoplasmic membrane. Sandwiched between them is the densely crowded periplasm and shape-determining peptidoglycan layer. Bacteria have evolved highly specialized protein machineries that span both membranes, enabling complex functions, such as cell movement [1] and molecular targeting, including protein secretion and DNA uptake [2]. Pathogenic and symbiotic Gram-negative bacteria interact with other cells by secreting effector proteins across both bacterial membranes, into the extracellular matrix or directly into host cells [3]. The mechanistic complexity of these nanomachines is achieved by assembling multiple copies of more than 20 different proteins, including both soluble and membrane-anchored subunits as structural and regulatory elements [4]. Understanding their molecular mechanism requires a combination of structural biology with genetic tools and functional studies. Some of the most pertinent questions are: how do dual-membrane spanning nanomachines assemble in order to perform their biological function? What is their mode of action? How is communication between different subunits regulated and controlled?

Recent advances in single-particle electron cryo-microscopy (cryo-EM) have made it possible to determine structures of isolated proteins or complexes at near-atomic resolution [5]. However, a major technical challenge associated with studies of large multi-component membrane protein complexes is the difficulty of purifying them in an intact state. Electron cryo-tomography (cryo-ET) enables large protein complexes inside cells to be observed in 3D at a resolution of several nanometers, by tilting a sample through a series of increments [6,7]. The resolution may be further improved by sub-tomogram averaging (StA), whereby multiple copies of the same protein or protein complex are mutually aligned and added together (Figure 1). This can yield sub-nanometer resolution, sufficient to distinguish protein domains [8] or even secondary structure [9•]. Integration of higher-resolution information obtained by single particle cryo-EM or X-ray crystallography into the StA density maps provides detailed mechanistic understanding at the level of the entire complex [8,10,11]. In addition, trapping of specific physiological states or computational classification of different protein subsets can yield intermediates of assembly and function. Here, we review recent exciting insights into structure and dynamics of the type IV pili (T4P) assembly machinery, and of the type III and VI secretion systems (T3SS/T6SS) in bacteria [3], obtained by cryo-EM and cryo-ET.

Requirements for *in situ* structural analysis

Several technical aspects need to be considered for structural determination of bacterial membrane protein complexes *in situ*. Firstly, sample thickness places a strict limit on the quality of tomographic volumes. In practice this means a maximum thickness of 400–500 nm. It is possible to reduce cell thickness by optimizing culture conditions [12], genetically engineering thin cells or small minicells

Figure 1



Workflow for structure determination of protein complexes *in situ* by cryo-ET and StA. **(1)** Top panel: bacterial cells embedded in a layer of amorphous ice are imaged in the electron microscope. Incremental tilts of the sample yield a series of projections from different viewing angles. The mutual orientations of the bacterium (brown) and the macromolecular complex of interest (red) vary depending on the projection angle. Bottom panel: a 0° tilt projection image of a *T. thermophilus* cell pole. Scale bar, 100 nm. **(2)** Top panel: a three-dimensional tomogram is reconstructed from the two-dimensional image series by computational back-projection. Multiple sub-tomograms containing the molecule of interest are identified and computationally cropped out of the tomogram for StA. Bottom panel: a slice through a reconstructed tomogram of the cell shown in step (1). Closed T4P (pilus retracted) complexes seen in the periplasm are boxed. Scale bar, 100 nm. **(3)** Top panel: noisy sub-tomograms with anisotropic resolution are extracted from the full volume. Bottom panel: a series of extracted sub-tomograms containing the closed state of the T4P machinery. Scale bar, 50 nm. **(4)** Left panel: particles are aligned, averaged and classified to recover the structure of the initial object. Right panel: sub-tomogram average of the T4P machinery in the closed state [41]. Scale bar, 10 nm. **(5)** Structures are placed back into three-dimensional space in order to visualize their distribution in the native-like context. The closed state of the T4P machinery (red) is shown at the cell pole, localized between the inner membrane (yellow) and outer membrane (transparent brown).

[13], or by physical sectioning of the frozen bacteria with a diamond knife [14] or a focused ion beam [15]. Secondly, cryo-EM has benefitted greatly from direct electron detector cameras [16] and phase plates [17], generating high quality data in a high-throughput and automated manner. Thirdly, an inherent problem with cryo-ET is the 'missing wedge' of information, resulting in resolution anisotropy in the direction of the electron beam [18]. The missing wedge results from the current physical inability to rotate the sample in the microscope by a full 180°. Effects of uneven sampling are alleviated or eliminated by averaging particles in different orientations by StA [19]. Fourthly, large membrane protein complexes are often flexible, resulting in variable low-resolution maps. Conformational flexibility may be reduced biochemically, for example by cross-linking [20], or genetically, by introduction of stabilizing mutations or truncations, which carry the disadvantage of compromising the native state of the complex. Alternatively, computational methods of classification can be used to quantify and account for flexibility [21]. Finally, a prerequisite for both classification and high resolution is the collection of very large particles data sets. Parallel and GPU-enhanced software, and thorough image analysis helps to obtain unbiased structures and structural intermediates [21–24].

Bacterial type IV pilus assembly machinery

The type IV pilus is a surface-exposed filamentous protein polymer that is several μm long, anchored to cells of evolutionarily divergent Gram-negative and Gram-positive bacteria [25]. In Gram-negative bacteria, the assembly machinery forms a multimeric dual-membrane-spanning protein supercomplex [26]. T4P play an important role in cell motility, enabling cells to adhere to and move along surfaces, form colonies and biofilms [1], and can even act as nanowires carrying electrical current [27]. Pathogenic proteobacteria, such as *Pseudomonas aeruginosa* and *Neisseria meningitidis*, use T4P to mediate adhesion to host cells prior to infection [28,29]. The machinery that assembles T4P is also implicated in DNA uptake, which is critical for lateral