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Cryo-electron tomography of plunge-frozen whole bacteria and vitreous sections to analyze the recently described bacterial cytoplasmic structure, the Stack

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ABSTRACT

Cryo-electron tomography (CET) of plunge-frozen whole bacteria and vitreous sections (CETOVIS) were used to revise and expand the structural knowledge of the "Stack", a recently described cytoplasmic structure in the Antarctic bacterium *Pseudomonas deceptionensis* M1^T. The advantages of both techniques can be complementarily combined to obtain more reliable insights into cells and their components with three-dimensional imaging at different resolutions. Cryo-electron microscopy (Cryo-EM) and CET of frozen-hydrated *P. deceptionensis* M1^T cells confirmed that Stacks are found at different locations within the cell cytoplasm, in variable number, separately or grouped together, very close to the plasma membrane (PM) and oriented at different angles (from 35° to 90°) to the PM, thus establishing that they were not artifacts of the previous sample preparation methods. CET of plunge-frozen whole bacteria and vitreous sections verified that each Stack consisted of a pile of oval disc-like subunits, each disc being surrounded by a lipid bilayer membrane and separated from each other by a constant distance with a mean value of 5.2 ± 1.3 nm. FM4-64 staining and confocal microscopy corroborated the lipid nature of the membrane of the Stacked discs. Stacks did not appear to be invaginations of the PM because no continuity between both membranes was visible when whole bacteria were analyzed. We are still far from deciphering the function of these new structures, but a first experimental attempt links the Stacks with a given phase of the cell replication process.

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1. Introduction

In the last years, advances in transmission electron microscopy (TEM) have enhanced our knowledge of bacterial ultrastructure (Chang et al., 2014; Hoenger, 2014; Comolli et al., 2011, 2013; Milne et al., 2013; Basler et al., 2012; Gan and Jensen, 2012; Chen et al., 2010; Sani et al., 2010; Tocheva et al., 2010; Li and Jensen, 2009; Milne and Subramaniam, 2009; Jensen and Briegel, 2007). Cryo-electron microscopy (Cryo-EM) combined with tomography has allowed already known structures to be visualized in a close-to-native state, providing the highest resolution available for the imaging of biological specimens. These "pure" cryo-techniques have revealed cellular organelles and macromolecular assemblies in a frozen-hydrated state, avoiding contrast-enhancing staining solutions, chemical fixatives and resins, which may contaminate the sample with artifacts. One way to obtain the cooling rates required for water vitrification is plunge freezing, after which 'whole-mount' plunge-frozen specimens can be imaged directly when their thickness is below 0.5 μm, a range that includes many bacteria and archaea (Beeby et al., 2012; Toso et al., 2011; Kudryashev et al., 2010; Comolli et al., 2006, 2008; Khursigara et al., 2008; Murphy et al., 2008; Li et al., 2007; Zhang et al., 2007; Briegel et al., 2006; Komeili et al., 2006; Scheffel et al., 2006). However, the resolution of Cryo-EM of plunge-frozen whole bacteria is conditioned by the thickness of the sample, limiting the

Abbreviations: CET, cryo-electron tomography; CEMOVIS, cryo-electron microscopy of vitreous sections; CETOVIS, cryo-electron tomography of vitreous sections; Cryo-EM, cryo-electron microscopy; HPF, high-pressure freezing; FS, freeze substitution; LN_2, liquid nitrogen; PM, plasma membrane; TEM, transmission electron microscopy; VIS, vitreous sections.

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observation of molecular details. Cryo-electron microscopy of vitreous sections (CEMOVIS) is an alternative technique to study frozen-hydrated bacteria, consisting of obtaining thin sections from cryoimmobilized whole bacteria, which provide a better resolution than plunge-frozen whole bacteria. To achieve thin sections, high-pressure freezing (HPF) provides vitrification of the samples up to 200 μm by increasing the pressure to 2048 bars during the cooling (Studer et al., 2008). Ultrathin sections of 50 nm can be directly obtained from high-pressure frozen bacteria and imaged in the microscope, disclosing molecular details such as the lipid bilayer membrane (Hoffmann et al., 2008; Zuber et al., 2008). However, it is important to bear in mind that the mechanical action of cutting can add conspicuous artifacts to the sample (Al-Amoudi et al., 2005).

Frozen-hydrated specimens can be processed by cryo-electron tomography (CET), performed by incrementally tilting the sample in the Cryo-EM through a range up to ±70 and imaged at each step. Afterwards, the tilt series of images is aligned and processed to generate a 3D reconstruction or tomogram of the specimen. CET has been applied to already known structural assemblies such as layers of the bacterial cell envelope (Kishimoto-Okada et al., 2010; Hoffmann et al., 2008; Zuber et al., 2008; Al-Amoudi et al., 2004), chemoreceptors (Briegel et al., 2008; Khursigara et al., 2008; Zhang et al., 2007), cytoskeleton filaments (Pilhofer et al., 2011; Ingerson-Mahar et al., 2010; Salje et al., 2009; Li et al., 2007; Briegel et al., 2006; Komeili et al., 2006; Scheffel et al., 2006; Kürner et al., 2005; Erickson, 1997), flagella (Chen et al., 2011; Kudryashev et al., 2010; Liu et al., 2009; Murphy et al., 2008), magnetosomes (Komeili et al., 2006; Scheffel et al., 2006), storage granules (Beeby et al., 2012; Hoenger and McIntosh, 2009; Comolli et al., 2006), carboxysomes (Iancu et al., 2010) and the Type VI secretion system (Chang et al., 2014), providing new information and a greater understanding of those structures in their natural context. Furthermore, the use of Cryo-EM and CET has led to the discovery of new bacterial structures, thanks to studies of new bacterial species, improvements in sample preparation that avoid the addition of artifacts, and gains in resolution. This is the case of the recently described structures "nanopods" and "hami" (Shetty et al., 2011; Moissl et al., 2005).

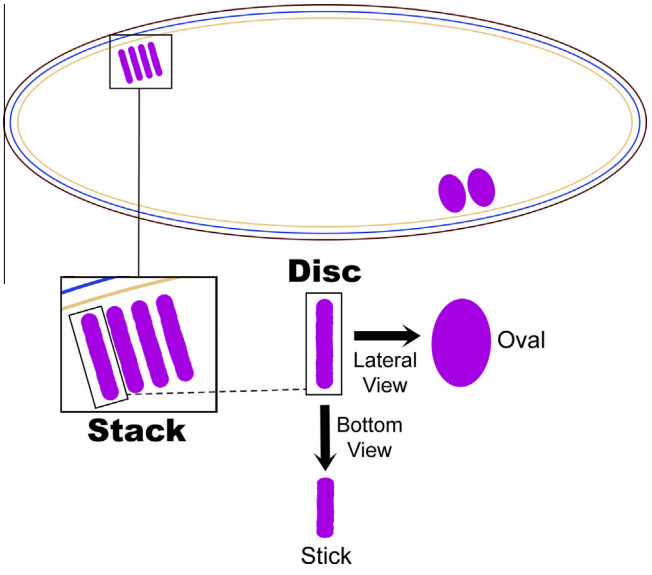

Fig.1. Model of a *P. deceptionensis* M1^T cell showing Stacks in the cytoplasm. A pile of four subunits integrates the Stack in the squared area. The lateral and bottom view of the squared subunit are shown. In 2D, the Stack can be observed as an oval structure or as a pile of sticks. Pink: Stacks; red: outer membrane; blue: peptidoglycan layer; cream-color: PM. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

In our previous work, we described a new bacterial structure termed a "Stack", which was revealed by different electron microscopy techniques and electron tomography and CEMOVIS (Delgado et al., 2013). Stacks, which were observed in bacteria of the *Pseudomonas* genus, were defined as piles of oval disc subunits surrounded by a membrane-like structure and localized in the cell cytoplasm. Stacks can be present in variable number within each cell, separately or grouped together, frequently very close to the plasma membrane (PM). In three-dimensional views (3D), each subunit appears as a flat oval disc, while in two-dimensional views (2D) the Stack is seen either as an oval structure or a pile of sticks (Fig. 1). The function of Stacks was not elucidated, but they were mostly observed very close to DNA fibers, suggesting they could be somehow related to the bacterial chromosome dynamics and/or the cell replication process.

Our goal in the current work was to refine the structural characterization of Stacks using methods known to preserve bacterial cell structures very close to their native state, and to rule out their possible origin as artifacts derived from the sample preparation methods. We imaged the architecture of Stacks within the whole bacterial cell by CET of plunge-frozen whole bacteria. We also applied cryo-electron tomography of vitreous sections (CETOVIS) to high-pressure frozen bacteria in an attempt to achieve molecular details not provided by previously used techniques.

2. Materials and methods

2.1. Cell growth

Studies were performed in *Pseudomonas deceptionensis* M1^T (LMG 25555) isolated from marine sediments collected in Deception Island (Antarctica) and characterized by our group as a new species (Carrión et al., 2011). *P. deceptionensis* M1^T was grown for 12 days at 0°C on tryptone soy agar (TSA, Oxoid) according to the manufacturer's specifications, unless otherwise specified.

2.2. Plunge freezing

Colonies of bacteria were suspended in milliQ water, centrifuged at 3000 rpm for 8 min and the supernatant was discarded. The process was repeated twice. Then, bacteria cells were resuspended in milliQ water. One drop of the suspension was applied on the carbon surface of a glow-discharged Quantifoil®200 mesh copper grid (Quantifoil Micro Tools, Jena, Germany). The sample was maintained at 100% humidity and the excess of liquid was blotted with filter paper, a thin film of suspension remaining on the grid. The sample was cryo-immobilized by plunging the grid into liquefied ethane using the Vitrobot Mark III (FEI Company, Eindhoven, Netherlands). The vitrified samples were stored in liquid nitrogen (LN_2) until their observation in the cryo-electron microscope.

2.3. High-pressure freezing (HPF)

HPF of samples was performed on colonies resuspended in 30% dextran (Fluka) in 0.01 M phosphate buffer saline (PBS) (Delgado et al., 2013). The suspensions were introduced into 350 μm inner diameter copper tubes and ultrarapidly frozen using a Leica EMPACT High-Pressure Freezer (Leica Microsystems, Vienna, Austria). The copper tubes were stored in LN_2 until further use for cryo-sectioning.

2.4. Vitreous cryo-sectioning

Quantifoil® carbon-coated 200 mesh copper grids were used to pick up the vitreous sections (VIS). Firstly, fiducial markers were

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