Unique Thylakoid Membrane Architecture of a Unicellular N$_2$-Fixing Cyanobacterium Revealed by Electron Tomography[1][W][OA]

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Cyanobacteria, descendants of the endosymbiont that gave rise to modern-day chloroplasts, are vital contributors to global biological energy conversion processes. A thorough understanding of the physiology of cyanobacteria requires detailed knowledge of these organisms at the level of cellular architecture and organization. In these prokaryotes, the large membrane protein complexes of the photosynthetic and respiratory electron transport chains function in the intracellular thylakoid membranes. Like plants, the architecture of the thylakoid membranes in cyanobacteria has direct impact on cellular bioenergetics, protein transport, and molecular trafficking. However, whole-cell thylakoid organization in cyanobacteria is not well understood. Here we present, by using electron tomography, an in-depth analysis of the architecture of the thylakoid membranes in a unicellular cyanobacterium, *Cyanothece* sp. ATCC 51142. Based on the results of three-dimensional tomographic reconstructions of near-entire cells, we determined that the thylakoids in *Cyanothece* 51142 form a dense and complex network that extends throughout the entire cell. This thylakoid membrane network is formed from the branching and splitting of membranes and encloses a single lumenal space. The entire thylakoid network spirals as a peripheral ring of membranes around the cell, an organization that has not previously been described in a cyanobacterium. Within the thylakoid membrane network are areas of quasi-helical arrangement with similarities to the thylakoid membrane system in chloroplasts. This cyanobacterial thylakoid arrangement is an efficient means of packing a large volume of membranes in the cell while optimizing intracellular transport and trafficking.

Cyanobacteria are widely accepted as the evolutionary precursors of the chloroplasts of plants and algae. Modern-day cyanobacteria are an environmentally significant and diverse group of microbial phototrophs, and a number of strains are used as model systems to study fundamental processes including photosynthesis, nitrogen fixation, and carbon sequestration. However, a comprehensive understanding of cyanobacterial biology also requires detailed knowledge of cellular architecture, an area that has not been as thoroughly explored. Like chloroplasts, cyanobacteria are characterized by an internal complexity that includes differentiated membrane systems and compartmentation. Classified as gram-negative bacteria, cyanobacteria have an outer membrane and cytoplasmic membrane, and all cyanobacteria known to date, except *Gloeobacter violaceus* (Nakamura et al., 2003), have an internal system of thylakoid membranes in which the light reactions of photosynthesis and respiration occur. Besides the thylakoid membranes, the cyanobacterial cell interior contains components such as carboxysomes, glycogen granules, cyanophycin granules, polyphosphate bodies, lipid bodies, and polyhydroxybutyrate granules, depending on the strain and growth conditions (Allen, 1984). The arrangement, number, and associations of these components with each other and with the membrane systems remain largely uncharacterized at high resolution in many strains of cyanobacteria. However, recognition of this cellular complexity and interest in understanding the compartmentalization of enzymatic functions in cyanobacteria have led to studies such as those focusing on the components of the shell surrounding carboxysomes, a form of bacterial microcompartment in which the initial reactions of carbon fixation occur (Klein et al., 2009).

The architecture of thylakoid membranes in cyanobacteria is of particular interest because of their critical roles in housing the photosynthetic and respiratory complexes, maintaining a proton gradient for the production of ATP, and maximizing light capture. Regarding the organization of the thylakoid membranes, a number of characterizations have been made (for a recent review, see Nevo et al., 2009). Initial descriptions of thylakoid membrane organization in cyanobacteria were based on random thin-section electron micrographs and freeze-fracture studies, from which the overall thylakoid organization was extrapolated. In all cases, thylakoid membranes are found as pairs of bilayers that enclose the thylakoid lumenal space, where the pH is lower than in the surrounding cytoplasm during active photosynthetic and respiratory electron transport. In a number of strains of cyanobacteria, thylakoid membranes appear to follow the shape of the cell envelope, forming multiple concentric membrane layers interior to the plasma membrane (Mullineaux, 1999). This type of organization is particularly evident in widely studied strains such as *Synechocystis* sp. PCC 6803, *Synechococcus elongatus* sp. PCC 7942, and some *Prochlorococcus* strains (for review, see Liberton and Pakrasi, 2008). In contrast, other strains, such as some of those in the genus *Cyanothece*, have thylakoid membranes that appear to be arranged in a radial pattern within the cell, like spokes of a wheel, but still parallel to each other (Porta et al., 2000). Regardless of their overall arrangement, thylakoid membranes are typically separated from each other by a space sufficient for a double row of phycobilisomes, large light-harvesting antenna complexes, to fit between the membranes (Mullineaux, 1999), but have been reported with appressed regions in a few strains, including *Prochloron* (Giddings et al., 1980) and *Acaryochloris marina* (Marquardt et al., 2000; Chen et al., 2009).

The fact that thylakoids routinely appear in two-dimensional images as parallel membrane layers with no apparent connection between the layers is consistent with a model of arrangement in which the membranes enclose multiple independent lumenal spaces. However, such an organization raises numerous important questions regarding the maintenance of a proton gradient in individual compartments and the translocation of cellular components between isolated lumenal spaces. The application of high-resolution three-dimensional (3D) electron microscopy to examine cyanobacterial ultrastructure has significantly impacted our views about the cellular architecture of these organisms. Explorations of the ultrastructure of cyanobacterial cells in three dimensions began with computer-aided reconstructions of serial sections of *Synechococcus* sp. PCC 7002 (formerly *Agmenellum quadruplicatum*; Nierzwicki-Bauer et al., 1983), and progressed to using advanced techniques such as electron tomography and cryo-electron tomography. Recent analyses of tomographic data collected from ≤200-nm-thick sections through cyanobacterial cells

have shown that thylakoid membranes are connected by junctions and channels linking adjacent membranes in *Prochlorococcus* strains MIT9313 and MED4 (Ting et al., 2007) and by branching and fusion in *Microcoleus* sp. and *Synechococcus* sp. PCC 7942 (Nevo et al., 2007). Notably, tomographic data (van de Meene et al., 2006) and serial section reconstructions (Liberton et al., 2006) of larger cell volumes did not find clear continuity between thylakoid membrane layers in *Synechocystis* sp. PCC 6803. Furthermore, while these connections between thylakoid membrane layers were observed, their frequency in the cell and place in the overall thylakoid membrane organization remained unknown. The insights gained from these 3D analyses suggested the need for large tomographic reconstructions of cyanobacteria to understand their overall cellular architecture. While tomograms encompassing entire or near-entire cells have been described for *Schizosaccharomyces pombe* (Höög et al., 2007), archaea (Comolli et al., 2009), and a number of bacteria (Milne and Subramaniam, 2009), similar analysis of a cyanobacterium has not been described until now.

To address these open issues in cyanobacterial cell biology, we began a study of the whole-cell architecture of the unicellular nitrogen-fixing cyanobacterium *Cyanothece* sp. ATCC 51142 (hereafter *Cyanothece* 51142). By using serial electron tomography combined with montaging, we were able to reconstruct large volumes of cyanobacterial cells in three dimensions, to our knowledge the first report of such extensive reconstructions in an oxygenic photosynthetic prokaryote. In *Cyanothece* 51142, thylakoid membranes are radially arranged around a central cytoplasmic area. Models constructed from tomographic data showed that this band of radial thylakoids spirals around the cell periphery, an organization not previously seen in cyanobacteria. Our data show that this peripheral band of thylakoid membranes is a dense and complex interconnected network derived from the continuous branching and splitting of membranes. Importantly, we show that the thylakoid membranes form one extensive system enclosing a single space, the thylakoid lumen. The extensive nature of the thylakoid membrane network and the intricate detail of its organization were not apparent in random thin-section electron micrographs, or even in small-volume tomograms, but were readily observable in the larger-volume tomographic data presented here.

RESULTS

Intracellular Arrangement and Compartmentation in *Cyanothece* 51142 Cells

Cyanothece 51142 is a unicellular diazotrophic cyanobacterium that is routinely grown under nitrogen-fixing conditions in a light regime of 12-h of light/12-h of dark, so that the cells perform photosynthesis during the light period and nitrogen fixation in the

dark (Sherman et al., 1998). The periodic alterations in storage granule accumulation and degradation have been described: Carbon is stored as glycogen during the light period and utilized during the dark, while nitrogen is stored as cyanophycin during the dark and utilized during the light period (Schneegurt et al., 1994; Li et al., 2001). To determine if these diurnal changes included alterations to the thylakoid membranes, we examined cells collected at time points throughout the diurnal cycle. We prepared *Cyanothece* 51142 cells for electron microscopy by high-pressure freezing followed by freeze substitution and embedding in resin. Whole cells and details of intracellular features of cells harvested at time points L10 and D10 (after growth in 10 h of light or dark, respectively) are shown in Figure 1. For this analysis, we examined thin sections of more than 250 cells from each time point, including cells that were harvested from independently grown cultures. From this in-depth analysis, we were able to thoroughly document intracellular features and changes that occurred during the diurnal cycle.

We found that, regardless of the time point during the diurnal cycle, *Cyanothece* 51142 cells display an intracellular arrangement that is fairly consistent between cells and cultures: a central cytoplasmic region containing ribosomes, carboxysomes, and polyphosphate bodies surrounded by a peripheral ring of thylakoid membranes (Fig. 1, A and D). In the thylakoid membrane region, the accumulation and distri-

bution of the numerous glycogen granules at time point L10 were observed (Fig. 1, A–C), whereas fewer glycogen granules remained at D10 (Fig. 1, D and E). Glycogen granules can vary in electron density between preparations, and were found between thylakoid membrane layers (Fig. 1, B and C). Individual granules are relatively uniform in size, can appear to have a coat-like outer covering, and can be connected to each other (Fig. 1, B and C). During the dark period, cyanophycin is usually found near the plasma membrane as large round inclusions (Fig. 1E) approximately 200 nm in diameter. Cyanophycin granules are larger and more electron dense than the more numerous and smaller lipid bodies (Fig. 1E). Cyanophycin granules have a homogenous interior and can have a delimiting boundary in some preparations (Fig. 1E). Although glycogen granules, cyanophycin granules, and lipid bodies were all observed in close proximity with thylakoid membranes and the plasma membrane, direct continuity or connectivity between the thylakoid membranes or the plasma membrane and these inclusions could not be conclusively determined.

Two types of cellular components, carboxysomes and polyphosphate bodies, occupy a considerable portion of the central cytoplasmic region. Carboxysomes are the sites of concentrated Rubisco and carbonic anhydrase, and function to enhance carbon fixation by elevating the levels of CO_2 in the vicinity of Rubisco. It has become apparent that carboxysomes are one type of bacterial microcompartment that func-

Figure 1. *Cyanothece* 51142. Standard transmission electron micrographs of cells and cellular structures from cultures grown under nitrogen-fixing conditions and harvested at the L10 (A–C) and D10 (D–E) time points. Thylakoid membranes (T) are oriented in a roughly radial pattern around the cell periphery at both time points. Lipid bodies (L), polyphosphate bodies (P), and glycogen granules (G) are also found in the peripheral area of the cells. The central cytoplasmic region is typically devoid of thylakoid membranes but can contain carboxysomes (C) and polyphosphate bodies. B and C, Glycogen granules at the L10 time point, showing the coat-like covering (arrow in C), and junction with neighboring granules (arrowhead in C). E, Detail of a cyanophycin granule (Cy) showing the delimiting border (arrow in E). Bar = 500 nm in A and D, bar = 100 nm in B, C, and E.
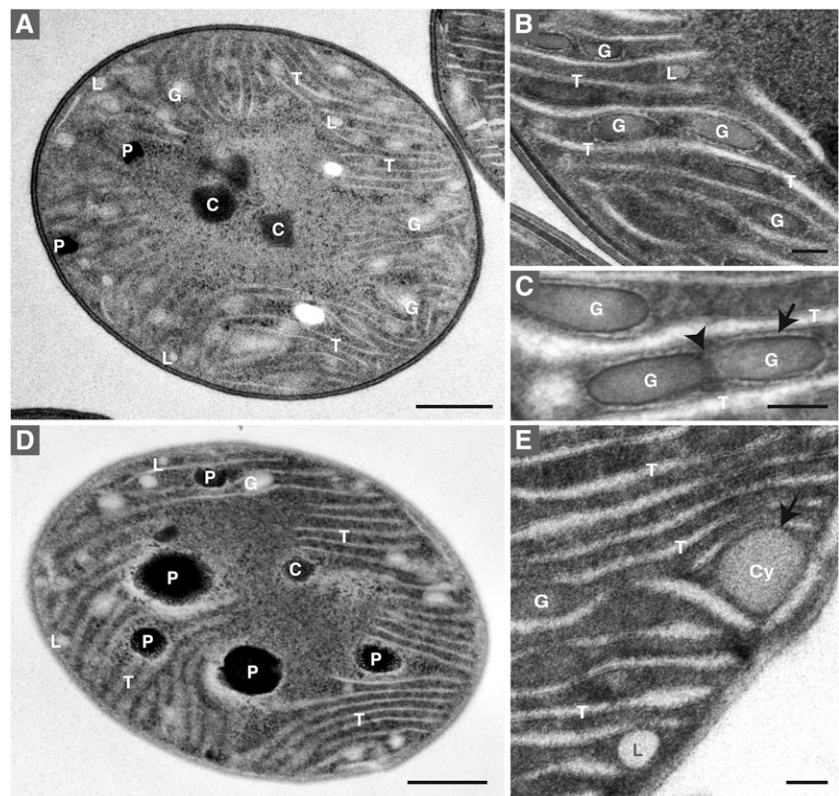
tions like an intracellular organelle by sequestering and concentrating specific enzymes, and studies are identifying the protein components of the carboxysome and describing the structure and composition of the shell (Yeates et al., 2008). The role of polyphosphate bodies in cyanobacteria is likely as a store of phosphate for ATP, phospholipid, and nucleic acid biosynthesis, but many details concerning the function of polyphosphate bodies in cyanobacteria are unknown. While polyphosphate bodies could be found in between the thylakoid membranes, carboxysomes were found exclusively in the cell interior (Fig. 1, A and D). Polyphosphate bodies and carboxysomes did not appear to have a circadian-related accumulation and degradation pattern like glycogen and cyanophycin, but remained relatively constant during the 24-h period.

Interestingly, in both light and dark time points, thylakoid membranes were similar in number and distribution within the cells. Apparently, the periodic changes in inclusion body accumulation and utilization occur within the context of the relatively stable thylakoid membranes in these cells. Furthermore, in these thin sections, thylakoid membranes appeared as individual membrane layers with no obvious continuity between the layers.

Cellular Architecture of *Cyanothece* 51142 Examined by Electron Tomography

Our tomographic analysis of the cellular ultrastructure of *Cyanothece* 51142 cells focused on the L10 and D10 time points because of the accumulation of the storage granules glycogen and cyanophycin peaked around these time points, respectively (Sherman et al., 1998). We collected dual-axis tomograms from 200- to 250-nm-thick sections of four cells from each time point. In each of these tomograms, we found a similar cellular arrangement to that observed in thin sections and described above. However, these tomograms encompassed only approximately 10% of the total cell volume. For a more complete 3D analysis, we prepared serial thick sections of approximately 300 nm from L10 time point cells. We collected montaged (2 × 2) dual-axis tilt series from seven serial sections and constructed tomograms of approximately 80% of the volume of one cell and approximately half the volume of two other cells, an analysis that included a total of approximately 2,600 tomographic slices. These tomograms are the most complete reconstructions of an oxygenic photosynthetic cell to date, and provide a comprehensive insight into the whole cell organization of a cyanobacterium.

One cell was examined in detail and models were constructed using the IMOD software (Fig. 2). This cell was oriented along the long axis, and the tomographic reconstruction included the entire cell except for approximately 0.3 μm and approximately 0.6 μm at the beginning and end of the volume, respectively. *Cyanothece* 51142 cells are typically oblong, measuring approximately 3.5 to 4.5 μm long and approximately 2.5 μm wide; this cell measured 4.5 × 2.4 μm at its widest points. Figure 2, A to D shows four slice images at different locations in the cell volume. When sectioning through the cell, we observed that the thylakoid membranes were densely packed at the cell periphery (Fig. 2A), began to open to expose the central cytoplasmic region in the interior of the cell (Fig. 2, B and C), and again filled the intracellular space on the opposite side of the cell (Fig. 2D). The peripheral band of thylakoid membranes was arranged in a generally radial pattern, extending from the vicinity of the plasma membrane for approximately 500 to 600 nm into the cell interior. Membranes were also observed that deviated from the radial arrangement by forming loops (asterisk in Fig. 2B), membranes concentric to the plasma membrane, or membranes that extended into the cell interior (asterisks in Fig. 2C). The tomographic data confirmed that the cell was essentially divided into two regions: the thylakoid membrane region that contained glycogen granules, lipid bodies, some ribosomes, some polyphosphate bodies, and the central cytoplasmic region that included carboxysomes, the majority of the polyphosphate bodies, ribosomes, and presumably the nuclear material. We generated models that included many of these components to show their arrangements within the cell (Fig. 2, E–G; Supplemental Video S1).

We expanded upon the analyses of thin sections and small-scale tomograms with a detailed examination of the organization of cellular components in their whole-cell context. Slice images of representative regions of the tomogram upon which our analyses were based are shown in Figure 2, H to M. Ribosomes were very numerous, and located largely within the central cytoplasmic region, although many were also found between thylakoid membranes and near the plasma membrane. Glycogen granules were numerous and found between thylakoid membranes; their electron density varied throughout the depth of each serial thick section, appearing more electron transparent in optical sections near the surface of a serial section (Fig. 2, A, I, and M) and more electron dense deeper within the section (Fig. 2, B–D, H, K, and L). As expected at the L10 time point, cyanophycin granules were not observed. Phycobilisomes were not resolved clearly enough to determine their distribution. The association of thylakoid membranes with the plasma membrane and with other cellular components was also examined. While there were many examples of thylakoid membranes in close proximity with the plasma membrane (Fig. 2, H–M), no clear continuity between these membrane systems was observed.

Carboxysomes and polyphosphate bodies are very prevalent in *Cyanothece* 51142, and we modeled all carboxysomes and many of the polyphosphate bodies in the cell (Fig. 2, E–G). Carboxysomes were found only in the central cytoplasmic region, where they often closely grouped with polyphosphate bodies, as was previously reported in *Synechococcus* 7002 (Nierzwicki-Bauer et al., 1983). Carboxysomes in groups could be

Figure 2. Tomographic reconstruction of a *Cyanothece* 51142 cell. A to D, Tomographic slice images (constructed from three superimposed serial 2-nm slices) through a cyanobacterial cell from a large-scale tomogram. Slice position (0, 340, 735, 900) in the tomogram is labeled. Asterisks denote variations to the radial thylakoid membrane arrangement. T, Thylakoid membrane; C, carboxysome; G, glycogen granule; P, polyphosphate body. Bar = 1,000 nm. E to G, Model of the cell constructed from the tomogram shown in A to D, showing cellular components and organization. The model in E was rotated 90° to derive the side view in F, and F was rotated 90° to derive the view shown in G. Thylakoids in the lower approximately one-half of the cell are shown modeled. Blue gray, Plasma membrane (rendered partially transparent for clarity); white, lipid bodies; blue, carboxysomes; green, thylakoid membranes; gray, polyphosphate bodies. H to J and K to M, Tomographic slice images (constructed from two superimposed serial 2-nm slices) through two different regions of the tomogram showing the arrangement of cellular structures. Arrowheads, Ribosomes; L, lipid bodies. Bar = 200 nm.
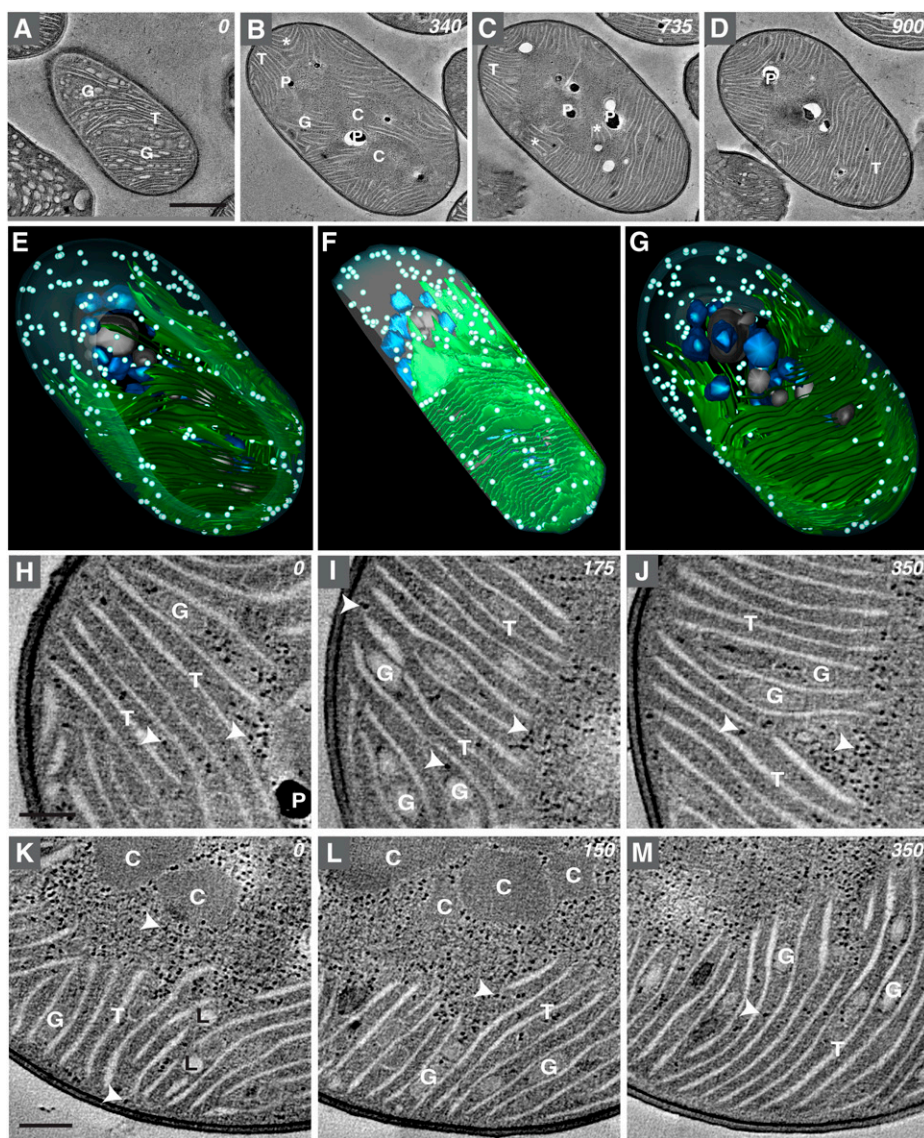
tightly packed, with the shapes of individual carboxysomes conforming to the shape of neighbors (Fig. 2, K and L). The number of carboxysomes in *Synechococcus* 7002 and *Synechocystis* 6803 averaged six and seven per cell (Nierzwicki-Bauer et al., 1983; van de Meene et al., 2006), respectively, and approximately the same in *Prochlorococcus* (Ting et al., 2007), compared to our finding of 21 in this *Cyanothece* 51142 cell. The size of *Cyanothece* 51142 carboxysomes ranged between 250 to 600 nm, also larger and more variable when compared to approximately 90 nm for *Prochlorococcus* MED4 and 130 nm for *Prochlorococcus* MIT9313 (Ting et al., 2007). In *Synechocystis* 6803, polyphosphate bodies were reported in one in every 10 cells (van de Meene et al., 2006), but with an average of five per cell in *Synechococcus* 7002 (Nierzwicki-Bauer et al., 1983). Polyphosphate bodies were much more numerous in *Cyanothece* 51142, with approximately 60 in the cell volume examined. The appearance of polyphosphate bodies was

greatly variable, from very small to large (50–700 nm), and the shape varied from uniformly spherical to highly irregular.

We identified the large number of small, relatively uniform spherical inclusions as lipid bodies in *Cyanothece* 51142 cells based on their similar appearance to previously identified lipid bodies in other cyanobacterial strains (van de Meene et al., 2006). In *Synechocystis* 6803, lipid bodies averaged 20 per cell (van de Meene et al., 2006), and 17 per cell in *Synechococcus* 7002 (Nierzwicki-Bauer et al., 1983). Lipid bodies were much more numerous in *Cyanothece*, at approximately 250 in the cell volume examined (Fig. 2, E–G). Lipid bodies varied slightly in size and shape, from approximately 60 nm to approximately 100 nm, and were almost always found in the near vicinity of thylakoid membranes, specifically often found near the tips or edges of the membranes (Fig. 2K). However, direct association or continuity between lipid bodies and

thylakoids or the plasma membrane could not be conclusively determined.

Thylakoid Membranes in *Cyanothece* 51142 Form a Dense Network That Spirals around the Cell Periphery

Our large-volume tomographic reconstructions showed nearly the entire thylakoid membrane system in *Cyanothece* 51142 cells, so that the whole-cell membrane organization could be visualized in this organism. We modeled the thylakoid membranes in approximately half the cell to understand the underlying architecture, and thylakoid membranes in the portion of the cell that was not modeled appeared to be arranged in a comparable manner (Fig. 2). The thylakoid membranes occupied a considerable portion of the cell volume: Using IMOD software, we calculated the volume of the modeled portion of the thylakoids to be 0.79 μm^3, or about 15% of the volume of that half of the cell.

In slice images of the cell, we noted that the thylakoid membranes were oriented at different angles, depending on the position in the volume, and analysis of the tomogram showed that the angle of the membranes gradually changed when progressing through the volume (Fig. 3, A–C). Models showed that the thylakoids formed a dense band of membranes that spiraled around the cell (Fig. 3, D–G), which accounted for the continually changing angle of the membranes

seen in the tomogram. This organization is particularly evident when a portion of the thylakoids is rendered in a contrasting color and the model is rotated (yellow versus green, Fig. 3, D–G). We examined tomograms generated from portions of other cells and found that this spiral ring of thylakoids was a general pattern in all cells examined. Evidently, this spiral organization represents the typical architecture of thylakoid membranes in *Cyanothece* 51142, one that has not been previously observed in any cyanobacterium.

Compared to other cyanobacteria in which thylakoid membrane organization has been examined in detail, *Cyanothece* 51142 cells contain comparatively more thylakoid membrane layers in addition to this spiral architecture. We sought to understand how this dense spiral band of membranes is constructed from its individual membrane components. To show how individual thylakoid membranes fit into this spiral pattern, we modeled a single thylakoid and its neighboring membranes (Fig. 3, H–J). This membrane (designated by the yellow arrowhead in Fig. 3, D and H) formed a long ribbon that extended for approximately 1,500 nm before branching (Fig. 3I). Another branch occurred within about 600 nm (Fig. 3J). In the small region modeled in Figure 3, H to J, four branch points were present within this group of membranes. The thylakoids in *Cyanothece* 51142 are thus not separate membranes that spiral around the cell, but rather components that form a larger system.
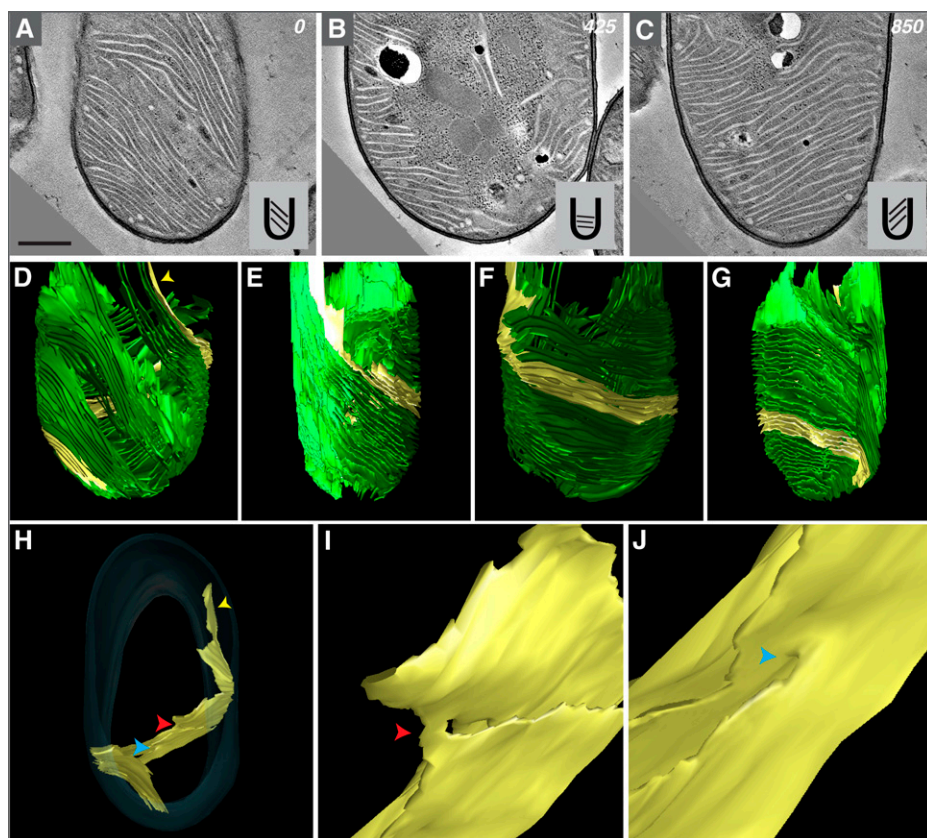
Figure 3. Thylakoid membranes form a spiral network around the cell periphery. A to C, Tomographic slice images (constructed from three superimposed serial 2-nm slices) showing the thylakoid membranes in the lower approximately one-half of the cell. The images have been rotated so that the long axis of the cell is vertical. The change in orientation of the thylakoid membranes when progressing through the tomogram is evident in the inset diagrams. Bar = 500 nm. D to G, model of thylakoid membranes in the lower portion of the cell rotated approximately 90° in each section to show how the membranes spiral around the cell. One region of membranes is modeled in yellow to highlight the spiral architecture. H to J, Model showing an individual thylakoid membrane (designated by the yellow arrowhead in D and H). This membrane extends as a narrow ribbon until an initial branch point (red arrowhead, and modeled in I), which is followed closely by another branch point (blue arrowhead, and modeled in J).

The *Cyanothece* 51142 Thylakoid Membrane Network Is Extensively Branched and Contains Areas with a Quasi-Helical Organization

In thin-section electron micrographs of *Cyanothece* 51142 cells, the thylakoid membranes appeared as separate sacs enclosing a lumenal space (Fig. 1; Schneegurt et al., 1994, 2000; Li et al., 2001). In our tomograms, most of the thylakoid membranes in *Cyanothece* 51142 were parallel to each other, separated by approximately 50 to 60 nm, and did not form stacks or appressed areas. Tomograms showed that, rather than forming individual sheets, the membranes were in fact interconnected, and the areas where the membranes coalesced could be clearly visualized (Figs. 3 and 4). We examined the distribution of branching, splitting, or fusing membranes in our large-scale tomogram to determine their number and overall locations. We found a total of 115 such points distributed throughout the cell, with additional events likely existing in the area of the cell not included in the tomogram. These points were mapped within the cell and overlaid onto the modeled portion of the thylakoid membranes (Fig. 4, A and B); note that some points fall outside the modeled portion of the thylakoid membranes but still represent branch points identified in the tomogram. This map showed that these points had a somewhat unequal distribution, with more branching and fusion points located in the lower half of the cell compared to the upper half. In general, such points were located more toward the cell interior versus the periphery of the thylakoid membrane band. The membrane topology at these locations took a number of forms: Membranes branched and split extensively, forming new membrane sheets (Fig. 4, C and D) or ribbons of membranes extended to connect neighboring membrane layers (Fig. 4, E and F). However, sheet-like membranes routinely continued for long distances throughout the cell without contacting other membranes (as described in Fig. 3), perhaps accounting for why the branching architecture was not previously observed in electron micrographs. Nonetheless, the number and distribution of the interconnections were sufficient to show that the thylakoid membrane network forms an extensive system enclosing a single lumenal space.

While branching and splitting of membranes could be widely spaced, in some regions such interconnections appeared close together, or in clusters (Fig. 4, A and B). We examined these regions in detail to determine if these membranes formed a specific organization, as has been observed in the plant chloroplast thylakoid membrane system. The details of the 3D arrangement of grana and stroma thylakoids in chloroplasts have been debated for many years, and different models have been proposed (for review, see Mustárdy and Garab, 2003; Nevo et al., 2009). The foremost of these, the helical model, was originally based on serial-section electron microscopy data collected over decades, and describes stroma thylakoids as joining grana at numerous junctures or frets, forming a stair-step arrangement in which multiple right-handed helices
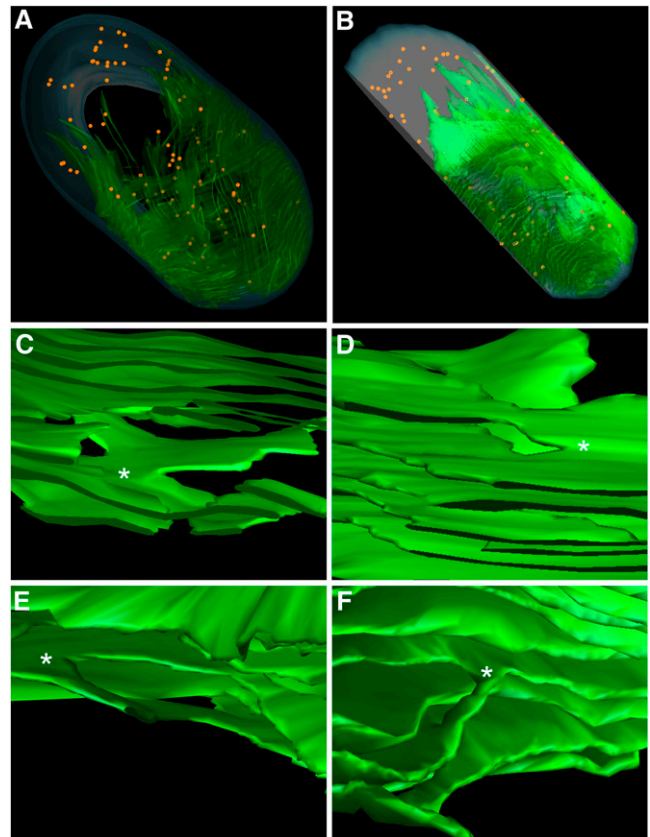
Figure 4. Thylakoid membranes in *Cyanothece* 51142 form an extensive network from the branching and splitting of membranes. A and B, Sites of membrane branching, splitting, and fusion mapped within the cell; B is rotated 90° relative to A. Sites (orange) are overlaid onto the modeled thylakoid membranes (green), which are rendered partially transparent for clarity. Additional sites found within the thylakoid network in the tomogram but outside the modeled portion of the membranes are also shown in the top region of the cell. C to F, Four examples of sites are shown. C, The membrane sheet labeled with the asterisk branches to form a new membrane sheet. D, The labeled membrane sheet branches into two parallel sheets. E and F, The labeled membrane sheets form connecting tubules that join the membrane sheets below.

of stroma thylakoids wind around a granum. Together, the grana and stroma thylakoids enclose a single lumenal space in chloroplasts (Paolillo, 1970; Brangeon and Mustárdy, 1979; Mustárdy and Garab, 2003). Recent studies and discussions have focused on the precise details of the architecture of this system, with refinements (Mustárdy et al., 2008) as well as alternative models proposed (Shimoni et al., 2005). New work using data generated by cryo-electron tomography has also described stroma thylakoids as connecting to grana in a step-wise manner (Daum et al., 2010).

Data detailing the helical grana-stroma arrangement in chloroplasts have traditionally shown slice images through the grana stack and adjoining stroma thylakoids with the membranes labeled to show the progression of membrane connections when comparing sections or slices (Paolillo, 1970; Brangeon and Mustárdy, 1979). In

this way, the ascent and descent of the stroma thylakoids relative to the thylakoids of the granum were evident and demonstrated the helical architecture. Using the same strategy for analysis, we labeled membrane regions in the thylakoid system of *Cyanothece* 51142 with numbers and letters (Fig. 5). By following the rearrangements to the letter-number pairs, it was apparent that the membrane regions to the right (lettered) advanced downward relative to the region on the left (numbered), when moving through the tomogram. This organization is similar to the progression seen in the analyses of chloroplasts. Two examples of this type of architecture found in *Cyanothece* 51142 are shown (Fig. 5, A–D and G–J) of the approximately 15 examples found in this cell. We observed evidence of the same simplified helical architecture in tomograms of other cells from the L10 time point, as well as in those from other time points such as D10. We interpret these data as indicating that *Cyanothece* 51142 membranes have a limited helical architecture that is a general aspect of the membrane organization in this cyanobacterium. While connections between thylakoid membranes in cyanobacteria have previously been observed (Nevo et al., 2007; Ting et al., 2007), a specific orientation or pattern has not been described. This combination of a rudimentary helical membrane organization and a single thylakoid system in this cyanobacterial cell is comparable to the architecture of thylakoid membranes in plant chloroplasts.

DISCUSSION

Cyanobacteria related to *Cyanothece* 51142 have recently been recognized for their contributions to nitrogen fixation in the open oceans (Zehr et al., 2001). We have examined this organism at the level of genomics (Welsh et al., 2008), transcriptomics (Stöckel et al., 2008; Toepel et al., 2008), and proteomics (J. Stöckel, J.M. Jacobs, T. Elvitigala, M. Liberton, E.A. Welsh, A.D. Polpitiya, M.A. Gritsenko, C.D. Nicora, D.W. Koppenaal, R.D. Smith, and H.B. Pakrasi, unpublished data) to obtain a systems level model of a cyanobacterium. To complement these analyses, we undertook a study of *Cyanothece* 51142 at the level of cell organization and architecture, to understand more completely the relationship between cellular structure and function in these cyanobacteria. Our data provide a comprehensive analysis of the cell composition and organization of this cyanobacterium. Importantly, our analyses examined much larger tomographic volumes than have been previously studied in any oxygenic photosynthetic prokaryote, resulting in a more complete interpretation of the cellular ultrastructure. An advantage of electron tomography of resin-embedded samples is that serial thick sections can be used to examine entire cells that are much too big to be contained in single sections of a thickness suitable for cryotomography. The larger size of *Cyanothece* 51142 cells, combined with our goal to examine close to complete cell volumes, made the use of plastic embedding and serial sectioning the preferred approach.

We have reconstructed in 3D the overall organization of this cyanobacterial cell, which may serve as a baseline for further comparison between this and other strains. These data, complemented with conventional thin-section electron micrographs, show the complexity of the *Cyanothece* 51142 cell, but also the level of regulation and control exercised by the cell to maintain a
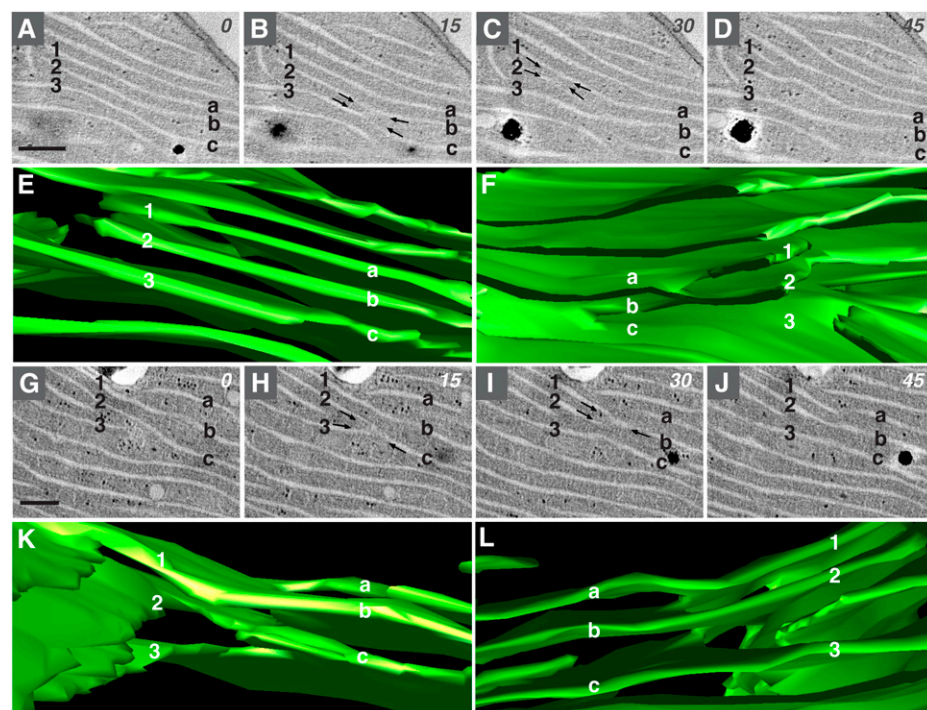
Figure 5. The thylakoid membrane network in *Cyanothece* 51142 has regions with rudimentary helical organization. Two examples are shown. A to D and G to J, Tomographic slice images (constructed from superimposed serial 2-nm slices) with relative positions in the tomogram labeled. Arrows show sites of membrane coalescence. Regions of the membranes are numbered or lettered to show how the pairing of membranes changes through the volume shown. E and F and K and L, Models of the regions shown in A to D and G to J, respectively. F and L are rotated 180° relative to E and K. Bar = 200 nm.

consistent pattern of cellular organization. This is demonstrated by our finding that the thylakoid membrane system remains consistent throughout the diurnal cycle despite significant changes in surrounding inclusion bodies.

The details of intracellular transport of proteins and lipids in cyanobacterial cells have long been a topic of interest. Ideas of thylakoid membranes as concentric shells that present barriers to transfer of materials between the cell envelope and the central cytoplasmic region have been gradually replaced by data showing that thylakoid membranes in some strains are discontinuous, with holes, gaps, and bridges to allow circulation around the cell to occur. *Cyanothece* 51142 cells present another solution to this problem: the enclosure of the thylakoid lumenal space into a single compartment, coupled with the radial arrangement of membranes around the cell periphery. This type of membrane arrangement allows for largely unobstructed movement of materials from the central cytoplasmic region through the radial thylakoid membranes to the cell envelope. Furthermore, our results show that thylakoid membranes form an interconnected network throughout the entire *Cyanothece* 51142 cell. A cyanobacterial cell in which the thylakoid membranes enclose one continuous lumenal space would enable communication throughout the cell, as has been noted (Nevo et al., 2007). Importantly, a single thylakoid lumen would eliminate the problem of needing to coordinate the regulation of pH levels in multiple intercellular compartments, and thus greatly simplify regulation. We also determined that the thylakoid membranes occupy a significant part of the cell volume, approximately 15%. Radial arrangement, interconnectivity, and spiraling may provide an efficient way of packing a large amount of membranes in a limited space while still allowing circulation of materials throughout the cell.

An outstanding question regarding thylakoid membranes concerns the means by which the membranes are formed, modified, and repaired in cyanobacteria. It has been proposed that thylakoid membranes arise from the plasma membrane, but analyses of thylakoid membrane/plasma membrane associations have shown that these two membrane systems are discontinuous (Liberton et al., 2006), or have rare connections that would be insufficient for the flux of material necessary to form the thylakoid membrane needed during cell growth and division (van de Meene et al., 2006). Likewise, the existence of vesicles has been described as a means of transporting lipid and protein to the thylakoids (Nevo et al., 2007), but this does not appear to be a universal mechanism observed widely in cyanobacteria. Thus, the mechanism for thylakoid membrane biogenesis remains an unresolved issue in these organisms. Our tomograms showed that the thylakoid membrane system in *Cyanothece* 51142 is constructed from the continuous branching and splitting of membranes. In our tomographic data, we observed a number of examples of structures that could represent sites of thylakoid membrane biogenesis by a branching and splitting mechanism (Figs. 3 and 4). Since we could find no evidence for physical continuity with the plasma membrane, and no structures resembling transport vesicles were observed, a possibility for thylakoid membrane biogenesis is the formation or growth of additional membrane from existing thylakoids. Such occurrences have been proposed for chloroplast thylakoids (Brangeon and Mustárdy, 1979). The means of lipid delivery to the thylakoid membrane to facilitate this expansion remains unknown, however, but might involve the abundant lipid bodies observed in this study (Fig. 2).

In higher plant chloroplasts, thylakoid membranes have a distinct architecture, forming an intricate network of stacks of flattened or appressed lamellae, the grana, that are connected by unstacked stroma thylakoids that traverse the chloroplast stroma matrix. The foremost model of grana-stroma organization describes stroma thylakoids as right-handed helices that wind around grana stacks (Mustárdy and Garab, 2003). In contrast, cyanobacterial thylakoid membranes typically do not form grana stacks and stroma thylakoids. A cyanobacterium or cyanobacterial ancestor is widely recognized as the progenitor of chloroplasts, and it is logical that some aspects of the modern-day chloroplast morphology evolved from the endosymbiont and may be maintained in present-day cyanobacteria. However, evidence of a morphological link between chloroplasts and cyanobacteria has previously been elusive. Our finding of rudimentary areas of helical arrangement within the thylakoid membrane system in *Cyanothece* 51142 may be such a link, which furthermore suggests that some aspects of chloroplast morphology originated in the cyanobacterial ancestor and were not a new invention in chloroplasts. This cyanobacteria-chloroplast similarity implies that homologous machinery to form and maintain helical arrangement was present in the prokaryotic ancestor and persists to some degree in at least one present-day cyanobacterium.

The model of thylakoid membrane architecture in cyanobacteria that emerges from our work is one that is much more similar to the thylakoid system in plant chloroplasts than has been previously described. Rather than simple layers bridged by connecting membranes, we describe a complex system that, as in plants, has a highly interconnected nature, encloses a single lumen, and has aspects of helical organization. The architecture of thylakoid membranes that we describe here has so far not been detected in other cyanobacteria. However, few strains have been analyzed using extensive tomographic reconstructions, which may be necessary for the detection of complex and large-scale 3D arrangements. Future analyses of thylakoid membranes in other cyanobacteria will likely yield answers to further important questions concerning the architecture of thylakoid membrane systems.

MATERIALS AND METHODS

Bacterial Cell Growth Conditions

Cyanothece sp. ATCC 51142 cells were grown in liquid ASP2 medium without added nitrogen under 12-h light/12-h dark conditions under 30 μmol photons m^{-2}s^{-1} white light and at 30°C for 5 to 6 d (Stöckel et al., 2008).

Sample Preparation for Electron Microscopy

Cells for transmission electron microscopy were ultra-rapidly frozen by high-pressure freezing. One-hundred-milliliter culture aliquots were centrifuged, the cell pellet was resuspended in a small volume, pipetted into planchettes with 100- to 200-mm-deep wells, and frozen in a Baltec high-pressure freezer (Bal-Tec). Samples were freeze substituted in 2% osmium/acetone or 1% glutaraldehyde plus 0.1% tannic acid (3 d at −80°C, 15 h at −60°C, slow thaw to room temperature), and embedded in Epon/Araldite or Spurrs resin. Thin sections were stained with uranyl acetate and lead citrate.

Electron Microscopy and Tilt Series Acquisition

For conventional electron microscopy, thin sections (approximately 80 nm) were cut from high-pressure-frozen cells and digital images were viewed and collected using a LEO 912 transmission electron microscope operating at 120 kV and a ProScan digital camera. For intermediate voltage electron microscopy, thick sections, or serial thick sections (250–350 nm), were cut and tilt series were acquired using a FEI Tecnai TF30 microscope operating at 300 kV. Images were collected at ×15,000 from +60° to −60° at 1° intervals about two orthogonal axes using a Gatan digital camera.

Tomogram Construction and Modeling

Images were aligned using gold fiducial markers (15 nm). Single-axis tomograms were constructed (from montaged or single images) using eTomo software (a component of 3DMOD), and the single-axis tomograms were combined into a dual-axis tomogram. For serial-section tomograms, individual dual-axis tomograms were aligned and combined using eTomo. Contours of cellular features were modeled using the 3dmod software, and meshed models were created. Sizes of cellular components and volume measurements were made using IMOD.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Video S1. Model of *Cyanothece* 51142 showing major cell components.

LITERATURE CITED

Allen MM (1984) Cyanobacterial cell inclusions. Annu Rev Microbiol **38**: 1–25

Brangeon J, Mustárdy L (1979) The ontogenetic assembly of intra-chloroplastic lamellae viewed in 3-dimension. Biol Cell **36**: 71–80

Chen M, Floetenmeyer M, Bibby TS (2009) Supramolecular organization of phycobiliproteins in the chlorophyll d-containing cyanobacterium *Acaryochloris marina*. FEBS Lett **583**: 2535–2539

Comolli LR, Baker BJ, Downing KH, Siegerist CE, Banfield JF (2009) Three-dimensional analysis of the structure and ecology of a novel, ultra-small archaeon. ISME J **3**: 159–167

Daum B, Nicastro D, Austin J II, McIntosh JR, Kühlbrandt W (2010) Arrangement of photosystem II and ATP synthase in chloroplast membranes of spinach and pea. Plant Cell **22**: 1299–1312

Giddings TH, Withers NW, Staehelin LA (1980) Supramolecular structure of stacked and unstacked regions of the photosynthetic membranes of *Prochloron* sp., a prokaryote. Proc Natl Acad Sci USA **77**: 352–356

Höög JL, Schwartz C, Noon AT, O'Toole ET, Mastronarde DN, McIntosh JR, Antony C (2007) Organization of interphase microtubules in fission yeast analyzed by electron tomography. Dev Cell **12**: 349–361

Klein MG, Zwart P, Bagby SC, Cai F, Chisholm SW, Heinhorst S, Cannon GC, Kerfeld CA (2009) Identification and structural analysis of a novel carboxysome shell protein with implications for metabolite transport. J Mol Biol **392**: 319–333

Li H, Sherman DM, Bao S, Sherman LA (2001) Pattern of cyanophycin accumulation in nitrogen-fixing and non-nitrogen-fixing cyanobacteria. Arch Microbiol **176**: 9–18

Liberton M, Howard Berg R, Heuser J, Roth R, Pakrasi HB (2006) Ultrastructure of the membrane systems in the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803. Protoplasma **227**: 129–138

Liberton M, Pakrasi HB (2008) Membrane systems in cyanobacteria. *In* A Herrero, E Flores, eds, The Cyanobacteria: Molecular Biology, Genomics, and Evolution. Horizon Scientific Press, Norwich, UK, pp 217–287

Marquardt J, Mörschel E, Rhiel E, Westermann M (2000) Ultrastructure of *Acaryochloris marina*, an oxyphotobacterium containing mainly chlorophyll *d*. Arch Microbiol **174**: 181–188

Milne JL, Subramaniam S (2009) Cryo-electron tomography of bacteria: progress, challenges and future prospects. Nat Rev Microbiol **7**: 666–675

Mullineaux CW (1999) The thylakoid membranes of cyanobacteria: structure, dynamics and function. Aust J Plant Physiol **26**: 671–677

Mustárdy L, Buttle K, Steinbach G, Garab G (2008) The three-dimensional network of the thylakoid membranes in plants: quasihelical model of the granum-stroma assembly. Plant Cell **20**: 2552–2557

Mustárdy L, Garab G (2003) Granum revisited: a three-dimensional model—where things fall into place. Trends Plant Sci **8**: 117–122

Nakamura Y, Kaneko T, Sato S, Mimuro M, Miyashita H, Tsuchiya T, Sasamoto S, Watanabe A, Kawashima K, Kishida Y, et al (2003) Complete genome structure of *Gloeobacter violaceus* PCC 7421, a cyanobacterium that lacks thylakoids. DNA Res **10**: 137–145

Nevo R, Charuvi D, Shimoni E, Schwarz R, Kaplan A, Ohad I, Reich Z (2007) Thylakoid membrane perforations and connectivity enable intracellular traffic in cyanobacteria. EMBO J **26**: 1467–1473

Nevo R, Chuartzman SG, Tsabari O, Reich Z, Charuvi D, Shimoni E (2009) Architecture of Thylakoid Membrane Networks. *In* H Wada, N Murata, eds, Lipids in Photosynthesis: Essential and Regulatory Functions. Springer Science and Business Media, Dordrecht, The Netherlands, pp 295–328

Nierzwicki-Bauer SA, Balkwill DL, Stevens SE Jr (1983) Three-dimensional ultrastructure of a unicellular cyanobacterium. J Cell Biol **97**: 713–722

Paolillo DJ Jr (1970) The three-dimensional arrangement of intergranal lamellae in chloroplasts. J Cell Sci **6**: 243–255

Porta D, Rippka R, Hernández-Mariné M (2000) Unusual ultrastructural features in three strains of *Cyanothece* (cyanobacteria). Arch Microbiol **173**: 154–163

Schneegurt MA, Sherman DM, Nayar S, Sherman LA (1994) Oscillating behavior of carbohydrate granule formation and dinitrogen fixation in the cyanobacterium *Cyanothece* sp. strain ATCC 51142. J Bacteriol **176**: 1586–1597

Schneegurt MA, Tucker DL, Ondr JK (2000) Metabolic rhythms of a diazotrophic cyanobacterium, *Cyanothece* sp. strain ATCC 51142, heterotrophically grown in continuous dark. J Phycol **36**: 107–117

Sherman LA, Meunier P, Colon-Lopez MS (1998) Diurnal rhythms in metabolism: a day in the life of a unicellular, diazotrophic cyanobacterium. Photosynth Res **58**: 25–42

Shimoni E, Rav-Hon O, Ohad I, Brumfeld V, Reich Z (2005) Three-dimensional organization of higher-plant chloroplast thylakoid membranes revealed by electron tomography. Plant Cell **17**: 2580–2586

Stöckel J, Welsh EA, Liberton M, Kunnvakkam R, Aurora R, Pakrasi HB (2008) Global transcriptomic analysis of *Cyanothece* 51142 reveals robust diurnal oscillation of central metabolic processes. Proc Natl Acad Sci USA **105**: 6156–6161

Ting CS, Hsieh C, Sundararaman S, Mannella C, Marko M (2007) Cryo-electron tomography reveals the comparative three-dimensional archi-

tecture of *Prochlorococcus*, a globally important marine cyanobacterium. J Bacteriol **189:** 4485–4493

Toepel J, Welsh E, Summerfield TC, Pakrasi HB, Sherman LA (2008) Differential transcriptional analysis of the cyanobacterium *Cyanothece* sp. strain ATCC 51142 during light-dark and continuous-light growth. J Bacteriol **190:** 3904–3913

van de Meene AM, Hohmann-Marriott MF, Vermaas WF, Roberson RW (2006) The three-dimensional structure of the cyanobacterium *Synechocystis* sp. PCC 6803. Arch Microbiol **184:** 259–270

Welsh EA, Liberton M, Stöckel J, Loh T, Elvitigala T, Wang C, Wollam A,

Fulton RS, Clifton SW, Jacobs JM, et al (2008) The genome of *Cyanothece* 51142, a unicellular diazotrophic cyanobacterium important in the marine nitrogen cycle. Proc Natl Acad Sci USA **105:** 15094–15099

Yeates TO, Kerfeld CA, Heinhorst S, Cannon GC, Shively JM (2008) Protein-based organelles in bacteria: carboxysomes and related microcompartments. Nat Rev Microbiol **6:** 681–691

Zehr JP, Waterbury JB, Turner PJ, Montoya JP, Omoregie E, Steward GF, Hansen A, Karl DM (2001) Unicellular cyanobacteria fix N_2 in the subtropical North Pacific Ocean. Nature **412:** 635–638