Characterization of intact subcellular bodies in whole bacteria by cryo-electron tomography and spectroscopic imaging

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Key words. Cryo-electron microscopy, cryo-electron tomography, electron energy loss, spectroscopy difference imaging, tomographic reconstruction, volume rendering.

Summary

We illustrate the combined use of cryo-electron tomography and spectroscopic difference imaging in the study of subcellular structure and subcellular bodies in whole bacteria. We limited our goal and focus to bodies with a distinct elemental composition that was in a sufficiently high concentration to provide the necessary signal-to-noise level at the relatively large sample thicknesses of the intact cell. This combination proved very powerful, as demonstrated by the identification of a phosphorus-rich body in Caulobacter crescentus. We also confirmed the presence of a body rich in carbon, demonstrated that these two types of bodies are readily recognized and distinguished from each other, and provided, for the first time to our knowledge, structural information about them in their intact state. In addition, we also showed the presence of a similar type of phosphorus-rich body in Deinococcus grandis, a member of a completely unrelated bacteria genus. Cryo-electron microscopy and tomography allowed the study of the biogenesis and morphology of these bodies at resolutions better than 10 nm, whereas spectroscopic difference imaging provided a direct identification of their chemical composition.

Introduction

Electron tomography of plastic sections of eukaryotic cells and tissues is an established technique that has been used with great success in the three-dimensional reconstruction of subcellular organelles and macromolecular assemblies (McEwen & Marko, 2001; Marsh, 2005; and references therein). Previous work has made use of the combination of electron tomography with electron energy loss spectroscopy (EELS) to map elemental compositions in three dimensions for thin plastic sections of eukaryotic cells and tissues (Leapman et al., 2004, 2005; Midgley & Weyland, 2002). A technique for improving the resolution of electron tomograms using the energy loss spectrum has been proposed and successfully exploited (Bouwer et al., 2004). Electron spectroscopic imaging (ESI), the difference imaging technique based on electron loss spectroscopy whereby images are formed with electrons that have lost specific amounts of energy, has also been extensively used with great success (Leapman, 1982a,b; Egerton, 1996; Galembeck et al., 2001; references therein). Perhaps one of the best examples of a sophisticated use of ESI is its critical contribution to our understanding of chromatin organization within the nucleus. Accurate phosphorus and nitrogen levels obtained from element-specific maps can be used to delineate protein from nucleic acid-based structures in situ, within thin sections of plastic-embedded biological samples. Results based on this type of ESI data have led to the proposal of new models of chromatin structure (Bazett-Jones et al., 2005; Dellaire et al., 2004). Thin sections of plastic-embedded bacteria have also been studied by EELS and ESI, with very good results (Golecki & Heinrich, 1991; Daulton et al., 2002; Middleton et al., 2003). EELS and ESI of thin sections of eukaryotic and bacterial cells embedded in plastic are rich fields whose detailed review goes beyond the scope of the present work.

Cryo-electron tomography (cryo-ET) of whole cells is a relatively new field in full development (Koster & Klumperman, 2003; Koster et al., 1997; Baumeister, 2002; Medalia et al., 2002). The use of new instruments with Field Emission Gun (FEG) electron sources, operating at 300 kV and equipped with energy filters, is becoming routine. In cryo-ET of intact cells, it is critically important to operate at voltages of 300 kV or higher and to use an energy filter to record zero-loss images (Koster et al., 1997). The energy filters, however, can be readily used for the acquisition of energy loss spectra and electron spectroscopic images, giving us a straightforward way to compute chemical
maps for the same bacteria whose three-dimensional reconstructions we obtain by cryo-ET.

The study of whole bacteria by cryo-ET introduces new challenges. Fast freezing by cryo-plunging and the avoidance of stains assures a native or almost native preservation but the contrast is much lower than in stained, plastic-embedded samples. Furthermore, biological material embedded in amorphous ice is more radiation sensitive than the same material embedded in plastic. In addition, the diameters of most bacteria of interest are considerably larger than the thickness of typical plastic sections. As a result the signal-to-noise ratio in cryo-ET is much lower than in electron tomography of plastic-embedded samples. However, frozen-hydrated biological specimens scatter less strongly than plastic-embedded and stained specimens of the same thickness. This is an advantage for the tomography of thick specimens.

We are now discovering that the interior of bacterial cells is highly structured spatially, with the cellular location of many proteins as tightly controlled at each time in the cell cycle as is their concentration (McAdams & Shapiro, 2003). Light microscopic techniques have increased our understanding of the subcellular organization of microbial cells, and tomography presents a bridge between the analysis of individual macromolecules observed by very high resolution electron microscopy, such as single particle analysis (Frank, 1996), and gross structural analysis by light microscopy (Lewis, 2004).

In addition, the genomes of many important bacteria have been sequenced and the number of new sequencing projects keeps rising. With the sequenced genomes at hand, several projects aim at understanding complete metabolic pathways and regulatory networks, and should eventually provide the knowledge to manipulate bacteria for specific purposes. A particular example is the application of bacteria for remediation of the environment (Genomics: G7L Contractor-Grantee Workshop III, 2005). Metals and radio-nucleotides resulting from industrial activities and weapons production contaminate vast areas world-wide. The control of metal transformations in the environment by bacteria offers a great potential for the development of successful bioremediation strategies (Genomics: G7L Contractor-Grantee Workshop III, 2005; Barkay & Wiatrowski, 2005).

We propose here the use of a combination of two technical capabilities of the electron microscope, the acquisition of tomographic tilt series and energy loss spectroscopic imaging, which in our view is ideally suited to the study of intact bacteria. We report the three-dimensional reconstructions of subcellular bodies in Caulobacter crescentus and Deinococcus grandis by cryo-ET, coupled with the elucidation of their primary chemical composition by EELS and ESI. We show that phosphate is, in both cases, stored in compact, spherical bodies. We also report the three-dimensional reconstruction of similar subcellular bodies in D. radiodurans, for which no EELS analysis was performed due to the great thickness of these intact bacteria. We also show the presence, in C. crescentus, of a different type of body, rich in carbon and depleted of phosphorus. This is the first time that such bodies have been clearly distinguished, structurally described and analysed in intact C. crescentus cells. We propose that this type of work can be further applied to the study of a large number of bacteria under a broad range of conditions, at different stages of their life cycles and as a way of monitoring how they adapt to different metabolic needs or environmental stresses. In addition, these technologies provide a very useful tool for the study of metal cycles in the environment and how they can be changed or engineered by bacteria.

We obtained optimal resolution in three-dimensional reconstructions of whole bacteria and subcellular bodies by zero-loss cryo-ET at 300 kV. ESI was then used to chemically identify the composition of subcellular bodies and three-dimensional features already reconstructed in their intact state. In cases where the specimen could not tolerate any dose in addition to that already received during the tomographic data acquisition, ESI had to be performed on different cells. In any case, a sufficiently large data set of cryo-electron tomograms, and a sophisticated characterization of the subcellular body under study, proved sufficient to reach unequivocal and general conclusions about them. Within a broader context we have occasionally referred to these subcellular structures as organelles because of their clear and distinct boundaries with the cytoplasm as well as their unique chemical composition. Additionally, the size and number of these bodies, and their presence in daughter cells before the completion of the cell division process, are presumably regulated by a control mechanism yet to be determined. However, we will refer to them as ‘bodies’ throughout the text because a discussion about whether the term ‘organelle’ should be reserved for membrane-bound and enclosed domains does not belong in the present work.

Caulobacter crescentus is a Gram-negative aquatic bacterium that divides asymmetrically during each cell division cycle, resulting in progeny cells that differ from the parent both structurally and functionally. At cell division, Caulobacter produces a motile swarmer cell and a stalked cell. The swarmer cell swims about for 30–45 min before shedding its flagellum and differentiating into a stalked cell. Caulobacter provides a simple model system for the study of both the bacterial cell cycle and the mechanisms of asymmetric cell division (McAdams & Shapiro, 2003). The molecular mechanisms that control Caulobacter’s developmental cycle are the subject of active research worldwide.

Gram-positive bacteria belonging to the family Deinococcaceae are quite unusual (Battista et al., 1999). Not only are they the most radiation-resistant organisms discovered but they are also vegetative, easily cultured and non-pathogenic.

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that had spoiled. It was soon found that this bacterium was extremely resistant to ionizing radiation, ultra-violet light, hydrogen peroxide and numerous other agents that damage DNA. This combination of factors has positioned D. radiodurans as a promising candidate for the study of mechanisms of DNA damage and repair, as well as its exploitation for practical purposes such as bioremediation of radioactive waste sites (Battista et al., 1999; Kobayashi et al., 2004). Six closely related radiation-resistant species of Deinococcaceae have been identified. D. grandis was the fifth one discovered and has an elongated shape, as opposed to the more rounded aspect of the others (Battista & Zimmerman, 2005).

Materials and methods

Specimen preparation

Caulobacter crescentus CB15N wild-type cells (courtesy of Harley McAdams, Stanford University) were grown in liquid PYE medium (0.4% bactopeptone, 0.2% yeast extract, 1 mM MgSO₄, 0.05 mM CaCl₂) for 6–8 h until an OD₆₅₀ of 0.4–0.7 was reached. Some cultures were kept for 3 days before preparing samples. Cells were also grown in minimal medium for a further control and handled in the same way.

For cryo-electron microscopy, aliquots of 5 µL were taken directly from the culture and placed onto lacy carbon grids (Ted Pella Redding, CA, cat.no. 01881) that were pre-treated by glow discharge. The Formvar support was not removed from the lacy carbon. The grids were manually blotted and plunged into liquid ethane by a compressed air piston, and then stored in liquid nitrogen. For electron microscopy at room temperature, 5-µL aliquots were placed on glow-discharged Formvar carbon-coated grids (Ted Pella 01811), and then the grids were blotted and air dried.

Deinococcus grandis and D. radiodurans (R1) cells (courtesy of John R. Battista, Louisiana State University, Baton Rouge) were grown in TGY medium (0.5% tryptone, 0.1% glucose, 0.5% yeast extract and 0.1% K₂HPO₄) at room temperature and samples were prepared as described for C. crescentus. These bacteria were observed during the log phase, as well as during the stationary phase. Lysis of D. radiodurans was performed as follows: 100 µL of cell culture with OD₆₅₀ between 0.4 and 0.6 was incubated at 37 °C for 15 min with 5 µL of the enzyme β-glucuronidase (Sigma, St Louis, MO, U.S.A.) and 2 µg mL⁻¹ of egg-white lysosome (Sigma), followed by sonicating and cryo-plunging.

General imaging

Images were acquired on JEOL-3100-FEF and JEOL-2010 electron microscopes (JEOL Ltd., Tokyo, Japan). The JEOL-3100 is equipped with an FEG electron source operating at 300 kV, an Omega energy filter, a Gatan 795 2kx2k CCD camera (Gatan Inc., Pleasanton, CA) and cryo-transfer stage. The stage can be cooled with either liquid nitrogen to 80 K or liquid helium to 16.4 K.

In the course of our work on the dose tolerance of bacterial specimens at liquid nitrogen and helium temperatures (Comolli & Downing, 2005), image series were acquired with an exposure of 15.6 e⁻ Å⁻² per image and total exposure up to 300 e⁻ Å⁻². We observed the onset of differential radiation damage in all series.

In the course of this work a data set of over 400 zero-loss cryo-images of C. crescentus was also acquired at liquid nitrogen temperature, capturing cells at different stages of the cell cycle. Exposures were between 50 and 80 e⁻ Å⁻², with nominal magnification values of 25 000, 20 000 and 15 000 x, corresponding to an actual magnification of 32 000, 27 000 and 20 000 x, respectively, at the detector plane. The pixel size corresponded to 1, 1.2 and 1.5 nm on the specimen, respectively. The slit width of the energy filter was 32 eV, centred on the zero-loss peak, and the defocus between 3.6 and 4.9 nm, respectively. For a few dozen cells, additional images were subsequently acquired to corroborate the onset of differential radiation damage, which in our observations has been absolutely consistent.

Data sets of over 50 cryo-images of D. grandis and D. radiodurans cells each were acquired at liquid nitrogen temperature, with the same range of illumination conditions described in the previous paragraph for C. crescentus.

Electron tomography

A total of 18 tomographic data sets were acquired for C. crescentus, nine for D. grandis and seven for D. radiodurans. Tomographic tilt series were acquired under low dose conditions, typically over an angular range between +65° and −65° (±5° with increments of 1.5° or 2°). Between 70 and 91 images were recorded for each series. All data sets were acquired using DigitalMicrograph (Gatan, Inc.) operated ‘by hand’. No automated acquisition software was used.

For the C. crescentus data sets all images were recorded using a magnification of 32 000 or 27 000 x at the detector giving a pixel size of 0.94 or 1.2 nm at the specimen, respectively. Underfocus values ranged between 6.4 ± 0.5 and 12 ± 0.5 µm, and energy filter widths ranged between 32 and 50 eV.

For D. grandis data sets images were recorded using magnifications of 32 000, 27 000 and 20 000 x. For D. radiodurans data sets the magnification was 20 000 x, resulting in a pixel size of 1.5 nm at the specimen. For all data sets the maximum dose used per complete tilt series was 80 e⁻ Å⁻², with typical values of approximately 70 e⁻ Å⁻².

Cryo-electron spectroscopic imaging

Electron spectroscopic imaging of cryo samples was performed in the JEOL-3100-FEF electron microscope using the Omega
filter. Control experiments were performed on cryo samples of microtubules and on λDNA toroids blotted at room temperature (but observed at liquid nitrogen temperature), both prepared as described under Specimen Preparation. For cryo-EM ESI only the L edge of phosphorus and the K edge of carbon were used. Three-window carbon maps (Egerton, 1996; DigitalMicrograph EELS Analysis User’s Guide from Gatan, Inc. and references therein) were acquired using an energy filter slit width of 20 eV, centring the slit at 294 eV for the post-edge image and at 269 and 259 eV for pre-edge images. Three-window phosphorus maps were similarly acquired by positioning the slit at 152, 117 and 97 eV for post-edge, first and second pre-edge images, respectively. The background on-edge was modelled using the pre-edge images by means of the R law, smooth R law and exponential fit (Egerton, 1996; DigitalMicrograph EELS Analysis User’s Guide from Gatan, Inc. and references therein). The best fit was provided by the smooth R law and constant R law models. DigitalMicrograph was used for all image acquisition, background subtraction and elemental map computation. The high level of noise from these types of cryo samples means that each energy loss image must be acquired with a relatively high dose of 20 e− Å−2 or greater and thus only three or four images can be acquired before serious specimen damage occurs. Collecting more images, such as to encompass a wider energy range, results in the onset of bubbling, which makes background subtraction meaningless. Hence, spectra were acquired for calibration purposes only (correct position of elemental edges in the energy loss spectrum) with cryo samples. A total of 12 carbon and 12 phosphorus maps, all using three-window acquisition, were computed from cryo-preserved samples of C. crescentus.

Room-temperature electron energy loss spectroscopy

Electron energy loss spectra of air-dried bacteria and EELS-based difference maps were acquired at room temperature in a JEOL-2010 electron microscope, equipped with a LaB6 filament electron source, a GIF Tridierm post-column energy filter (Gatan, Inc.), and an Ultrascan 1000 CCD camera (Gatan, Inc.). A total of 12 energy loss spectra were acquired, eight for C. crescentus and four for D. grandis. The energy loss spectra were acquired with a slit width of 12 eV, with 10-eV steps over a range of up to 500 eV using 30-s acquisition. Elemental maps were computed from the spectra for carbon, phosphorus and, as a control, nitrogen. Three-window phosphorus maps for the K edge of phosphorus were also acquired, with a slit width of 50 eV centred at 2176, 2051 and 2111 eV for post-edge, first pre-edge and second pre-edge images, respectively.

Visualization

The program IMAGEJ (NIH, http://rsb.info.nih.gov/ij/) was used for analysis of the two-dimensional image projections. Volume rendering and image analysis of tomographic reconstructions were performed using the program VOLUME (KitWare). All movies were made with the package AVS EXPRESS (AVS Advanced Visual Systems, Clifton Park, New York, NY, http://www.avs.com).

Results

Caulobacter crescentus

A dividing C. crescentus cell is shown in Fig. 1(A). The cells are shaped as an arc or ‘crescent’ and measure between 1.2 and 1.5 µm in length and 0.5 and 0.65 µm in diameter. In our observations at least 90% of the cells contained round bodies in the cytoplasm, clearly visible due to their contrast or higher density relative to the cytoplasm. The dividing cell shown in Fig. 1(A), acquired from a frozen-hydrated specimen, contains six round bodies. Figure 1(B) shows a 1-pixel-thick slice through a tomographic reconstruction of a frozen-hydrated Caulobacter cell in the early stages of cell division. Two discs are clearly visible, one with much higher contrast than the other. It is immediately apparent that this marked different contrast must be due to a considerably different mass density, chemical composition, or both. One of these bodies seems to be considerably more ‘compact’, or less transparent to the electron beam, whereas the other is distinguished from the cytoplasm not so much because of a large contrast difference but rather because of the uniformity of its compact texture within well-defined boundaries. Figure 1(C and D) shows slices from three-dimensional volume-rendered tomographic reconstructions of Caulobacter cells. High intensities, corresponding to thin areas of the grid and pure ice, have been coloured red, very low intensities, corresponding to high mass distributions, have been coloured blue, and an intermediate range of intensities has been coloured green. The type of body that shows high contrast in Fig. 1(B) is mainly coloured blue in Fig. 1(C and D). They have an almost perfect spherically symmetric shape, are highly compact and contain no internal details. There are no changes in their mass density as a function of the radius. The low contrast type of body in Fig. 1(B) is shown in green in Fig. 1(C). This type of body also has an ellipsoidal shape, and is far less uniform and symmetric. As with the other type of body, there are no visible internal details within our resolution limit but their mass density is less constant. Three-dimensional reconstructions of both types of bodies are shown in Supplementary material movies S1 and S2.

In the course of previous work, we noticed that the amount of bubbling, caused by electron beam radiation damage in the frozen-hydrated cells, was very different for different components of the cell (Comolli & Downing, 2005). Figure 2 shows an area of a Caulobacter cell, with two subcellular bodies, after the acquisition of a first image (Fig. 2A) and after the acquisition of six consecutive images at the same dose per image (Fig. 2B). The dose per image was 15.6 e− Å−2 and hence the total dose accumulated by the sample in Fig. 2(B) was 94 e− Å−2. The first cell component to show bubbling is the low contrast or low
density body. At higher doses, there is additional visible damage at the cell membranes and finally within the cytoplasm. The dense body, however, does not show signs of large-scale radiation damage until a very high dose, of the order of $300 \text{ e}^{-\text{Å}^2}$ and above. The combination of clear-cut differences in both electron density and dose tolerance is sufficient to classify these bodies into two distinct types or classes. However, we were motivated to try to better understand the origin of these differences by carrying out EELS and ESI, both with cryo samples in our 300-kV instrument and with samples dried at room temperature using a JEOL-2010 at Gatan, Inc.

Figure 3 shows ESI obtained from ice-embedded bacteria, kept at liquid nitrogen temperature, with a 300-kV instrument.
Fig. 3. Spectroscopic difference imaging of *Caulobacter crescentus* subcellular bodies by cryo-EM. (A) A carbon difference map. The strongest signal comes from the lacy carbon film and the body of the cell. The circular area shown by the blue arrow gives rise to a higher signal than the cytoplasm. It corresponds to the low contrast body shown in Fig. 2 bubbling at $94 \text{ e}^{-}\text{Å}^{-2}$. (B) A carbon difference map of a different cell. The body shown by the red arrow has a lower signal and appears as a dark circular area. This body corresponds to the high contrast type shown in Fig. 2, not bubbling at $94 \text{ e}^{-}\text{Å}^{-2}$. (C) A phosphorus difference map showing the two types of bodies adjacent to each other: red arrow, high phosphorus content and below background level of carbon; blue arrow, a body depleted of phosphorus with a high content of carbon. (D) Cryo-EM image of a cell before acquisition of a three-window map. (E) A carbon map and (F) a cryo-EM image of the same cell after acquisition of the carbon map and an additional exposure of $12 \text{ e}^{-}\text{Å}^{-2}$. The carbon-rich body indicated by a blue arrow has started to bubble, whereas the higher contrast body right above it remains intact at this resolution (red arrow). (G) Cryo-EM image of a cell before acquisition of a three-window map. (H) A phosphorus map and (I) a cryo-EM image of the same cell after the phosphorus map and an additional exposure of $12 \text{ e}^{-}\text{Å}^{-2}$. The high contrast phosphorus-rich body indicated by a red arrow remains intact at this resolution whereas the carbon-rich body adjacent to it, below and to the left with much lower contrast shows radiation damage in (I).
Figure 3(A and B) shows three-window carbon difference maps, using the carbon K edge at 284 ev, and Fig. 3(C) shows a three-window phosphorus difference map using the phosphorus low energy L edge at 132 ev. The highest carbon signal comes, of course, from the lacy-carbon film. As expected, the whole cells give a substantial carbon signal that clearly distinguishes them from the frozen growth medium. In Fig. 3(A) we see a circular area with a higher carbon signal than the rest of the cell, indicated by a blue arrow. Imaging the cells after the acquisition of spectroscopic difference images consistently shows that this signal comes from the low contrast bodies. Continued imaging causes them to bubble before the rest of the cell. In Fig. 3(B) we see a circular region of lower carbon signal than the rest of the cell, indicated by a red arrow. Imaging after ESI consistently shows that this area depleted of carbon corresponds to the high contrast subcellular body type. Continued imaging verifies the appearance of radiation damage in the rest of the cell, starting with the low contrast body, whereas no damage is seen in this type of body until considerably higher doses. Phosphorus maps, as in Fig. 3(C), consistently show the high contrast type to be extremely rich in phosphorus (red arrow).

Figure 3(D–I) shows images of cells before acquisition of spectroscopic data, carbon and phosphorus difference maps, and images of the cells after data acquisition. The differential radiation damage of each type of subcellular body is clearly seen after additional exposure.

Figure 4 shows ESI performed on air-dried bacteria at room temperature with a 200-kV instrument, reproducing the results from frozen-hydrated cells shown in Fig. 3. Figure 4(A and B) shows carbon and phosphorus elemental maps, respectively, for the same bacterium. The carbon-rich bodies give a much lower phosphorus signal than the rest of the bacteria as well as, of course, a significantly higher carbon signal. Figure 4(C and D) shows carbon and phosphorus elemental maps, respectively, acquired for another bacterium. In this case, the bacterium had one body of each type, namely carbon-rich and phosphorus-rich. Notice the complementary signals of carbon and phosphorus; the carbon-rich body gives rise to a background level phosphorus signal and the phosphorus-rich body to a substantial signal. The elemental maps acquired at room temperature were all computed as described in Materials and methods from full energy loss spectra. The phosphorus elemental maps were acquired from the phosphorus low energy L edge as described for carbon and the high energy loss K edge by three-window map computation with identical results.

In the 18 tomographic reconstructions of *C. crescentus* cells that we have completed to date, we have a total of 30 reconstructed phosphorus-rich and 25 reconstructed carbon-rich bodies. Phosphorus-rich bodies always show a high degree of spherical symmetry. In these reconstructions their diameters range between 30 and 180 nm. The carbon-rich bodies are not always spherical, often adopting a more irregular but always quite ellipsoidal shape such as the one shown in
Fig. 1(C). In our reconstructions we saw them with diameters as small as 40 nm and as large as 200 nm. We generally saw one or two large carbon-rich bodies per cell, with a diameter in the range 120 ± 20 nm, and between one and three phosphorus-rich bodies with a smaller diameter, generally between 50 and 120 nm. Very often we found a phosphorus-rich body adjacent to a carbon-rich body, as also seen in Figs 1(B and C), 2, 3(C) and 4(C and D). Sometimes they partially overlapped each other in two-dimensional projection views. However, all of our tomographic reconstructions show that they are always distinctly separated and never connected to each other. Supplementary material movie S1 shows two carbon-rich as well as two phosphorus-rich bodies. Supplementary material movie S2 shows one of the largest phosphorus-rich bodies that we have observed, near the cell division plane. This is not the most common location, although it is frequently observed. As is quite clear in the Supplementary material movies, these bodies have a very uniform texture at this resolution, resulting in a high contrast to the electron beam. The phosphorus-rich bodies are delineated by a sharp boundary with no visible membranes. The change in electron density between the body and the surrounding cytoplasm is such that we would clearly resolve any boundary structure between the two, such as a membrane, with a composition that would result in a different density from both the cytoplasm and interior of the body. However, in the case of the carbon-rich bodies, we do not discard the possibility that a thin laminar structure, such as a lipid bilayer, could provide the boundary. Such structures have a similar mass density, and hence a similar contrast. For this reason, we simply cannot be certain to resolve such a structure at the resolution and signal-to-noise ratio reported here.

In addition to the tomographic reconstructions and the series of observations of differential radiation damage, we carried out a systematic measurement of the diameter of both subcellular bodies in 110 C. crescentus cells. We chose those images that contained entire cells grown in non-restrictive growth medium (PYE), in order to also provide a statistical inventory of their numbers within our sample size. The tomographic data sets were acquired using nominal magnifications of 25 000 and 20 000 x (Materials and methods), and focused almost entirely on dividing cells. Mother and daughter cells were larger than the field of view and therefore we do not include these data in our statistics. We observed a total of 132 carbon-rich and 123 phosphorus-rich bodies, which gives an average of 1.2 carbon-rich bodies and 1.1 phosphorus-rich bodies per cell. We therefore observed an average of one of each type of body per cell, with the occasional presence of two or three bodies of each type per cell. We observed no carbon-rich bodies in three cells from the set of 110, i.e. they were present in 97% of the cells examined. Phosphorus-rich bodies were also absent in only three cells within our sample, these being different individuals than those without visible carbon-rich bodies. Tables 1 and 2 summarize the sizes and statistics for both types of body. Finally, although we systematically observed both types of body in bacteria grown under either minimal or rich medium, the size of the phosphorus-rich bodies appears considerably larger in the former case. This is not surprising as in other bacteria the accumulation of phosphate is known to dramatically increase in the absence of amino acids in the growth medium (Poindexter & Eley, 1983; Qi & Rehm, 2001). The range of sizes of the carbon-rich bodies seems about the same for both media. We also observed them either during the log or stationary phase. With respect to the cell division process, at the stage of the cell cycle in which cells show an evident constriction both types of bodies are already found in approximately equal numbers and sizes in what will become mother and daughter cytoplasmic compartments. It is clearly not necessary that they undergo an active partition process between mother and daughter cells. Rather, our observations suggest that it is more likely that their localized biogenesis ensures their distribution.

**Deinococcaceae**

*Deinococcus grandis* are elongated, rod-like cells that measure approximately 2 µm in length and 1 µm across. As seen in cryo-EM whole mounts, they have a cross-section of ellipsoidal rather than cylindrical symmetry, with a thickness in the direction orthogonal to the plane of the grid of the order of 0.75 µm. *D. radiodurans* usually forms tetrads with a diameter between 2.5 and 3 µm, made of four similar cells with a round

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**Table 1.** Phosphorus bodies.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Mean diameter (nm)</th>
<th>Minimum diameter (nm)</th>
<th>Maximum diameter (nm)</th>
<th>SD (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Caulobacter crescentus</em></td>
<td>116 ± 2</td>
<td>51 ± 2</td>
<td>249 ± 2</td>
<td>27</td>
</tr>
<tr>
<td><em>Deinococcus grandis</em></td>
<td>158 ± 2</td>
<td>45 ± 2</td>
<td>264 ± 2</td>
<td>87</td>
</tr>
</tbody>
</table>

**Table 2.** Carbon bodies.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Mean diameter (nm)</th>
<th>Minimum diameter (nm)</th>
<th>Maximum diameter (nm)</th>
<th>SD (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Caulobacter crescentus</em></td>
<td>167 ± 2</td>
<td>52 ± 2</td>
<td>290 ± 2</td>
<td>53</td>
</tr>
</tbody>
</table>
shape. These tetrads have a thickness of approximately 1 µm. *D. grandis* cells form dyads although they can also be found as individuals. Figure 5(A) shows an image of a cell-to-cell interface of a *D. grandis* dyad. Each cell contains two electron-dense, spherical bodies, with an aspect strikingly similar to the phosphorus-rich bodies observed in *C. crescentus*. Figure 5(B) shows a slice from a volume-rendered tomographic reconstruction of a *D. grandis* cell, which shows the spherical symmetry of the electron-dense bodies. Figure 5(C and D) shows carbon and phosphorus elemental maps, respectively, of two cells forming a dyad, confirming that these bodies are depleted of carbon but rich in phosphorus. In our nine tomographic reconstructions of *D. grandis* and five tomographic reconstructions of *D. radiodurans* we observed one or two of these bodies per individual cell, with diameters between 30 and 180 nm. When two were present, one was large and the other much smaller, as in Fig. 1(A). We also observed, in both organisms, smaller electron-dense objects with sizes in the order of 10–15 nm that could correspond to the early stages of their formation. Supplementary material movie S3 shows a phosphorus subcellular body in a *D. grandis* cell. EELS and ESI were performed only for *D. grandis*, as the thickness of *D. radiodurans* tetrads was too large for reliable interpretation of elemental maps. Intracellular bodies in *D. radiodurans* look very similar to those observed in *D. grandis* (Fig. 5E). In Fig. 5(F) we show a spherical body extracted from *D. radiodurans* by rapid cell lysis followed by plunge-freezing, a methodology we propose to apply in the near future to further study subcellular structure.

Fig. 5. Phosphorus intracellular bodies in *Deinococcus grandis*. (A) A cryo-EM image of two *D. grandis* cells forming a dyad. Clearly visible are two circular, high contrast bodies in each cell, the inner membrane (IM), outer membrane (OM) and the cell wall surrounding the IM (not labelled). Ice frost can be observed as indicated by the arrow (f). (B) A 110-voxel-thick slice from a volume-rendered tomographic reconstruction of a cell showing the spherical, high contrast body. (C and D) Carbon and phosphorus maps, respectively, from air-dried cells acquired at room temperature. The red arrow indicates a carbon-depleted body (C), which corresponds to a very high phosphorus signal (D). (E) A slice from a volume-rendered tomographic reconstruction of *D. radiodurans* showing similar high density bodies as those seen in *D. grandis*. (F) *D. radiodurans* cell lysate with a high contrast spherical body.
In this case at least, the subcellular body remained intact but embedded in a much thinner cryo sample, hence suitable for higher resolution work and reliable energy loss spectroscopy.

We also carried out the systematic observation of 56 high-dose images of D. grandis cells in order to have a first statistical distribution of the number per cell and diameter of these phosphorus-rich bodies. We were able to observe them in 53 of the 56 cells examined (94.6%). We observed 84 of these bodies in 56 cells (an average of 1.5 per cell). Table 1 summarizes the measured sizes for these bodies.

Discussion

It has been known for several decades that bacteria and protozoa accumulate phosphate in their cytoplasm (Kornberg et al., 1956; Kornberg, 1995). Early work characterized the presence of inorganic polyphosphate bodies in bacteria and protozoa by electron microscopy and biochemistry (Friedberg & Avigad, 1968; Gezelius, 1974; Stewart, 1978; Mayer, 1986; references therein). More than three decades ago, two enzymes of polyphosphate metabolism were identified and studied in cell-free extracts of Dictyostelium discoideum: a polyphosphatase and a polyphosphate-ADP-phosphotransferase (Gezelius, 1974). More recently, it has been shown that in most known cases phosphate is stored in bacteria in the form of polyphosphate bodies (Kornberg, 1995; Rashid et al., 2000; Kornberg & Brown, 2004). The importance of understanding in detail the function, mechanisms of biogenesis and storage of these polyphosphate bodies has become widely appreciated mainly due to work by Kornberg and associates (Kornberg, 1995; Kornberg et al., 2002; Kornberg & Brown, 2004). Ahn & Kornberg (1990) defined polyphosphate kinase 1 (PPK1), the enzyme that produces polyphosphate in Escherichia coli. The enzyme carries out the reversible synthesis of inorganic phosphates from the terminal phosphate of ATP. It has since become clear that this enzyme is widely conserved in bacteria and protozoa. The normal activity of this enzyme is essential in several bacteria for the production of virulence factors, biofilm development, quorum-sensing communication and flagella-initiated movement (Kornberg, 1995; Kornberg et al., 2002; Kornberg & Brown, 2004).

Phosphate deposits appear, when viewed by the electron microscope, as electron-dense granules. Quantitative and semiquantitative analysis of the elemental composition of bacterial and protozoal polyphosphate bodies has been performed before by electron spectroscopy and X-ray analysis (Goldberg et al., 2001; Ogawa & Amano, 1987; LeFurgey et al., 1990; Hensgens et al., 1996). These first organisms whose polyphosphate bodies have been studied in more detail by microscopy and spectroscopy include, quite naturally, human and animal pathogens such as Plesiomonas shigelloides, Staphylococcus aureus and Leishmania major. The easy identification of phosphate-rich bodies was originally used as an additional diagnostic tool for certain pathogens but attention has now turned to the possibility of directly eliminating these bacteria and protozoa from their hosts through the control of their polyphosphate metabolism. A more detailed review of this subject is well beyond the scope of this work. All of this previous electron microscopy and electron spectroscopy work was carried out either on thin or ultra-thin sections of plastic-embedded specimens.

The presence in C. crescentus of intracellular poly-β-hydroxybutyric acid and inorganic polyphosphate polymer granules was postulated by Poindexter more than two decades ago (Poindexter & Eley, 1983). These researchers developed a procedure capable of the sequential quantitative assay of both polymers and observed that they were present in amounts consistent with electron microscopic observations of intracellular granules. More recently, C. crescentus has been reported to accumulate polyhydroxybutyrate (Qi & Rehm, 2001). These authors characterized the enzyme polyhydroxybutyrate synthase and the production of polyhydroxyalkanoates in C. crescentus cultivated in the presence of excess carbon sources. They interpreted the metabolic storage of these compounds as responsible for the appearance of intracellular granules observed by phase-contrast microscopy. Finally, Poindexter et al. (2001) have also recently reported the observation of two types of granules in whole C. crescentus cells by electron microscopy of air-dried samples. They interpreted the lower contrast bodies as carbon reserves, possibly poly-β-hydroxybutyrate, and the high contrast bodies as polyphosphate bodies. We present here direct proof that there are indeed carbon-rich intracellular bodies present in C. crescentus. The intracellular bodies of higher contrast, are indeed rich in phosphate. The existence of these phosphate-rich bodies is consistent with the existence of active mechanisms for polyphosphate accumulation. The complete genome of C. crescentus has been sequenced (Nierman et al., 2001). The Institute for Genomic Research annotated locus CC3416 corresponds to a conserved hypothetical protein with high homology to PPK1 (http://www.tigr.org/). Further work should elucidate if this gene product is directly responsible for the subcellular bodies described here. It is also worth pointing out that the mass density of butyric acid would be too low to explain the density observed in the carbon-rich bodies described in the present work. It is very likely that the carbon-rich bodies are composed of a salt of butyric acid, which has a pK_a of 4.81, as the cytoplasm has a pH close to 7. Counter-ions would help to provide a charge balance and could account for the observed contrast.

We have been unable to find previous work addressing in higher structural detail the presence of polyphosphate bodies in intact D. radiodurans cells. The complete genome of this bacterium has been sequenced (White et al., 1999) and it also encodes for a polyphosphate kinase (http://www.tigr.org/). Further work addressing the possible use of this bacterium in bioremediation should characterize the gene products involved in polyphosphate metabolism and their relationship with the subcellular bodies described here.
To the best of our knowledge, we report here the first study of intact phosphate-rich and carbon-rich bodies in whole bacteria. Although the resolution and quantitative scope of spectroscopic information are limited in cryo-electron microscopy, we achieved tomographic reconstructions at relatively high resolution of the intact bodies and cells at different stages of their life cycles. Cryo-ET samples of whole _C. crescentus_ cells have thicknesses between one and three inelastic mean free paths for 300-kV electrons. With thicker bacteria, such as _D. grandis_ or _D. radiodurans_, multiple inelastic scattering events make ESI more unreliable. These samples, however, can still be studied by a combination of whole-cell cryo-ET and EELS of air-dried samples. The goal is to obtain the maximum resolution for whole bacteria from cryo-ET and to obtain chemical identification by EELS. We also show that the rapid lysis of cells, immediately followed by plunge-freezing of the lysate on a lacey-carbon EM film, is a suitable approach that allows the pursuit of higher resolution structural characterization of subcellular elements and the obtaining of spectroscopic information about them. Although the relative localization of subcellular bodies and macromolecular complexes within the cell is lost, their identity and tightly bound interacting partners will in most cases be preserved, yielding higher resolution information by cryo-EM that would otherwise be inaccessible. Further work along these lines is in progress and belongs to projects within a new framework.

The phosphate-rich bodies have a remarkably similar aspect in the bacteria studied in this work. We did not observe any biological membrane acting as a boundary between their interior and the cytoplasm, which precludes the presence of a trans-membrane active transport mechanism responsible for the phosphorus accumulation. An essential part of their biogenesis must be a local accumulation or nucleation site and a rather isotropic distribution of the enzyme(s) that synthesizes its constitutive compound around this nucleation site to account for their spherical symmetry. The fact that we observed very similar bodies in completely unrelated bacteria lends support to the hypothesis that mechanisms for phosphate accumulation are universally necessary from bacteria to higher order life forms. New work should address the presence of mono- or divalent counter-ions, such as Mg$^{2+}$, K$^+$, etc., in both types of bodies described here. Future work, using a combination of analytical spectroscopy and molecular biology tools in combination with the technology reported here, can address the question of their biogenesis as well as the most basic aspects of their roles.

Our ability to directly observe these bodies within a wide range of sizes, and at different stages of their biogenesis, leads us to propose the use of this technology in the study of how bacteria can affect metal cycles in the environment. Our methodology can capture structural aspects of the full biogenesis and anatomical evolution of these types of bodies, inclusion bodies or vesicles with metals. We can also study changes in bacterial morphology as a function of metal oxidation/reduction and metal deposition/precipitation outside the bacterial cell bodies. The biotransformation of metals is an exciting, developing strategy to treat metal contamination, especially in environments that are not accessible to other remediation technologies. The ability to use these strategies depends on the ability to monitor these transformations as they take place (Barkay & Wiatrowski, 2005). Work by Criddle and associates (Middleton et al., 2003) elucidating the kinetics and physiology of Cr(VI) reduction by _Shewanella oneidensis_ is among the first to apply transmission electron microscopy and EELS to the study of how microbes can affect metal cycles. Their methodology is based on thin sections of plastic-embedded samples. This approach has the advantage of obtaining high resolution elemental maps, which can contain detailed quantitative spectroscopic information on various elements. At the same time, however, this methodology lacks the power of observing the whole, intact bacteria throughout their life cycles and physiological changes, with the certainty that no structural changes are introduced or lost during sample preparation. We propose that a combination of such an approach with the type of cryo-ET and whole cell EELS shown in the present work can add significant insight to these problems.

**Acknowledgements**

The Director, Office of Science, Office of Basic Energy Sciences, U.S. Department of Energy provided support under Contract No. DE-AC02-05CH11231 (LRC and KHD). VOLVIEW was used under a personal free licence (LRC) from KitWare. The movies shown in the Supplementary material were made by Cristina E. Siegerist at the Scientific Visualization Group, Lawrence Berkeley National Laboratory. We thank Robert Glaeser (University of California, Berkeley) and Harley McAdams (Stanford) for constant encouragement and extensive discussions. We also thank Adam Arkin (University of California, Berkeley and Lawrence Berkeley National Laboratory) for discussing the material.

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Supplementary material

The following material is available for this paper online: Movies S1, S2 and S3.

Movie S1. A slice from a volume-rendered three-dimensional reconstruction of a dividing Caulobacter crescentus cell. Low intensities were coloured in blue and intermediate intensities in green. The high intensities corresponding to the ice have been thresholded-out. Carbon-rich bodies are seen in uniform and more intense green than the cytoplasm, in the upper part the cell body. They are less compact than the phosphorus-rich bodies and not always spherical. A number of smaller phosphorus-rich bodies with higher contrast can be observed in blue.

Movie S2. A slice from a volume-rendered three-dimensional reconstruction of a Caulobacter crescentus dividing cell with a phosphorus-rich body. The highly compact and uniform texture is quite apparent, as well as the almost perfectly spherical symmetry. This body measures approximately 120 nm in diameter and is one of the densest that we have observed. These cells were grown in minimal medium (Materials and methods).

Movie S3. A slice from a volume-rendered three-dimensional reconstruction of a Deinococcus grandis cell. A phosphorus-rich body, in blue, is in the centre of the cell. As with Caulobacter crescentus it has a highly compact texture and almost perfectly spherical symmetry.