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Membrane-bounded nucleoid in the eubacterium *Gemmata obscuriglobus*

(prokaryote/eukaryote distinction/electron microscopy)

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ABSTRACT **The freshwater budding eubacterium *Gemmata obscuriglobus* possesses a DNA-containing nuclear region that is bounded by two nuclear membranes. The membrane-bounded nature of the nucleoid in this bacterium was shown by thin sectioning of chemically fixed cells, thin sectioning of freeze-substituted cells, and freeze-fracture/freeze-etch. The fibrillar nucleoid was surrounded by electron-dense granules that were in turn enveloped by two nuclear membranes separated by an electron-transparent space. Immunogold labeling of thin sections of conventionally fixed cells with anti-double-stranded DNA antibody demonstrated double-stranded DNA associated with fibrillar material within the membrane boundary. The occurrence of a membrane-bounded nucleoid in a eubacterial prokaryote is a significant exception to the evidence supporting the prokaryote/eukaryote dichotomous classification of cell structure.**

The membrane-bounded nucleus in eukaryotes has been a major distinguishing structural trait in support of the prokaryote/eukaryote distinction (1). In contrast, the nucleoid in bacterial cells is not known to be membrane-bounded (2), although associations of bacterial DNA with membranes have been reported (3–5). The appearance of the bacterial nucleoid is influenced dramatically by the preparative technique for electron microscopy employed (2, 6–8). Freeze-substitution (cryofixation–cryosubstitution) and freeze-fracture are examples of techniques for visualizing the nucleoid that are least subject to artifacts (6, 7, 9–11). Although some techniques are claimed to show a clear boundary between cytoplasm and nucleoid (11), a membrane enclosing the bacterial nucleoid has not been observed (2, 12).

Gemmata obscuriglobus is a budding, spherical bacterium isolated from a freshwater dam in Queensland, Australia (13). Like related genera (see below), it possesses features unusual for eubacteria, including crateriform structures (circular pits) on the cell surface and complete absence of peptidoglycan in its cell walls, which are predominantly proteinaceous. From 16S rRNA signature oligonucleotide analysis (14), this bacterium can be considered a member of the eubacterial *Planctomyces–Pirellula* group. This group, comprising the genera *Planctomyces*, *Pirellula*, and *Isosphaera* as well as *Gemmata*, is a distinct eubacterial phylum defined by 16S rRNA sequence analysis (16S rRNA sequence data or oligonucleotide catalogues exist for *Pirellula* and *Gemmata*) and so deeply branching within the phylogenetic tree of the eubacteria that it has been proposed as a new bacterial order, *Planctomycetales*, the validly published name for this group of bacteria (15–17). The close relations between all genera in the phylum have been confirmed by 5S rRNA sequencing (18). Although they possess unique molecular and phenotypic features, including the ultrastructural features mentioned above (18–21), conformation to eubacteria has been established by 16S rRNA oligonucleotide catalogues (22), full sequencing (23), and other criteria (22). *G. obscuriglobus* is the deepest-branching organism in the order *Planctomycetales* as derived from 16S rRNA oligonucleotide catalogues (14) or from 5S rRNA (18). A micrograph in the original description of *G. obscuriglobus* (figure 6 in ref. 13) showed "packaged" nuclear material, stimulating us to reexamine this bacterium. Here we present ultrastructural evidence that the DNA-containing nucleoid of *G. obscuriglobus* UQM 2246 is packaged within membranes.

In this paper, we use the term "nucleoid" for "confined" DNA-containing fibrillar regions in the sense used by Kellenberger (2). "Nucleus" is reserved here for the DNA-containing membrane-bounded organelle in eukaryotes. "Nuclear body" is used only for a membrane-bounded structure enclosing a bacterial DNA-containing nucleoid and nucleoplasm (material other than the fibrillar nucleoid but also confined by membrane). In this paper, "nuclear" in itself does not refer to, and is not intended to imply, a fully eukaryotic structure or evolutionarily homologous structure.

MATERIALS AND METHODS

Bacteria and Culture Conditions. *G. obscuriglobus* UQM 2246 (University of Queensland Department of Microbiology Culture Collection strain 2246) was grown on medium consisting of $CaCO_3$-treated (10 g of $CaCO_3$ per liter of autoclaved soil extract), filtered soil extract (1 kg of soil per liter of distilled water, autoclaved at 121°C, 20 min), 100 ml; 67 mM phosphate buffer (pH 7.0), 50 ml; glucose, 1 g; agar, 15 g; and distilled water, 850 ml. The agar medium without glucose was autoclaved (108°C, 25 min) and 10 ml of filter-sterilized 10% glucose solution was then added. Cells were grown on this medium incubated aerobically at 28°C for 7, 14, 17, or 19 days.

Chemical Fixation and Thin Sectioning. Cells from a 17-day culture were fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer, enrobed in agarose, postfixed in 1% osmium tetroxide in 0.1 M cacodylate, dehydrated through a graded ethanol series, and embedded in LR White resin. Sections were stained with aqueous uranyl acetate and lead citrate and were viewed on a Hitachi H-800 transmission electron microscope at 100 kV.

Freeze-Substitution and Thin Sectioning. Cells in colonies of a 19-day culture on agar blocks were cryofixed using a Reichert-Jung KF80 cryofixation system fitted with an MM80 metal mirror. Cryosubstitution by the technique of Steinbrecht (24) was carried out with 2% osmium tetroxide in molecular-sieve-dried acetone at −79°C (dry ice/acetone bath) for 50 hr. The temperature was increased to −20°C over 14 hr. Specimens were brought to room temperature, then washed in acetone followed by ethanol; they were then embedded, sectioned,

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Abbreviation: dsDNA, double-stranded DNA.

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stained, and viewed by transmission electron microscopy as described above.

Freeze–Fracture. Cells from a 14-day culture were directly harvested, without chemical fixation, in 20% (vol/vol) aqueous glycerol as cryoprotectant prior to freezing in liquid Freon 22. Fracturing was performed using a Balzers BAE 120 apparatus fitted with a complementary fracturing device, at −115°C and 10^{-7} torr (1 torr = 133 Pa). Replicas were produced using platinum/carbon and stabilized with a layer of carbon. In some experiments (using 7-day cultures), cells for freeze–fracture were first fixed by the Ryter–Kellenberger procedure (8) as described by Nanninga (25).

Immunogold Labeling. Cells were prepared as described under *Chemical Fixation and Thin Sectioning*. Sections were immunolabeled using an anti-double-stranded (ds) DNA monoclonal IgM antibody (Sera-Lab, Crawley Down, Sussex, U.K.) and goat anti-mouse IgM antibody coupled to 10-nm colloidal gold (Janssen Life Sciences, Olen, Belgium). A control using only goat anti-mouse IgM/colloidal gold gave no specific labeling. In another control, the anti-dsDNA monoclonal antibody was shown to specifically label DNA in human testis nuclei. Thin sections of freeze–substituted cells were immunolabeled in the same manner.

RESULTS

Chemical Fixation and Thin Sectioning. Glutaraldehyde- and osmium tetroxide-fixed *G. obscuriglobus* cells display a well-defined membranous boundary surrounding the nucleoid (Fig. 1). This boundary consists of two membranes separated by a relatively electron-transparent space. Due to the plane of section and the appression of membrane to electron-dense material, only one of these membranes can be seen clearly in some regions of the boundary in Fig. 1. The compartment thus formed by the membranes consists of a classical fibrillar region, similar in appearance to conventional bacterial DNA-containing nucleoids, and an outer electron-dense granular region (Fig. 1). For this complete membrane-bounded compartment including both fibrillar and granular areas, we use the term nuclear body. The electron density of the outer granular region is markedly greater than that of ribosome-containing cytoplasm outside the nuclear body (Fig. 1; see also Fig. 5). Membrane-bounded nucleoids were also seen after fixation by the Ryter–Kellenberger protocol. When cells are grown on a medium richer than soil extract (e.g., 0.1% glucose/0.1% casein hydrolysate/agar), the membrane-bound nuclear body may appear to occupy a much greater fraction of the sectioned cell and can consequently be difficult to distinguish from surrounding cytoplasm.

Freeze–Substitution and Thin Sectioning. Thin sections of cells fixed by cryofixation and processed by cryosubstitution display a membrane-bounded nuclear body similar to that observed with chemical fixation and room-temperature processing, with a dense fibrillar nucleoid core surrounded by a coarsely granular region (Fig. 2*a*). The organization of the nuclear compartment after freeze–substitution appears analogous to that after chemical fixation as used for Fig. 1, except that the former technique produces a more coarsely structured outer granular region more similar in structure to the surrounding cytoplasm and that the cryofixed membrane-bounded nuclear body appears confined less within the area of the cell than the chemically fixed membrane-bounded nuclear body. The envelope surrounding the nuclear body consists of two double-track membranes separated by an electron-transparent region (Fig. 2*b*). Each membrane consists of a relatively dense and relatively light layer separated by an electron-transparent region. The outer nuclear membrane often appears to be connected to

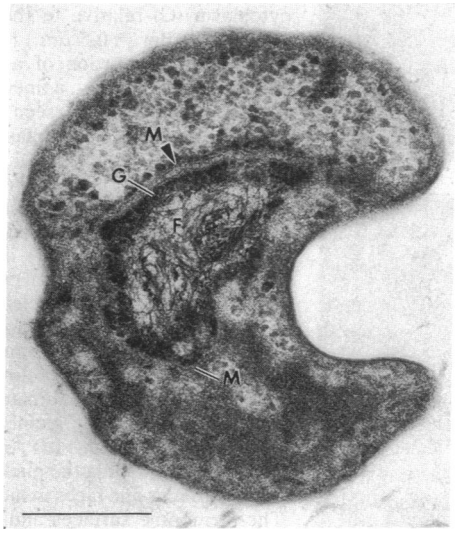

FIG. 1. Electron micrograph of thin section of a chemically fixed *G. obscuriglobus* cell showing a membrane-bounded nuclear region. The nuclear region is divided into a central fibrillar core (F) and an outer granular area (G) and is bordered by bounding membranes (M), the double nature of which can clearly be discerned in some areas (arrowhead). (Bar = 0.5 μm.)

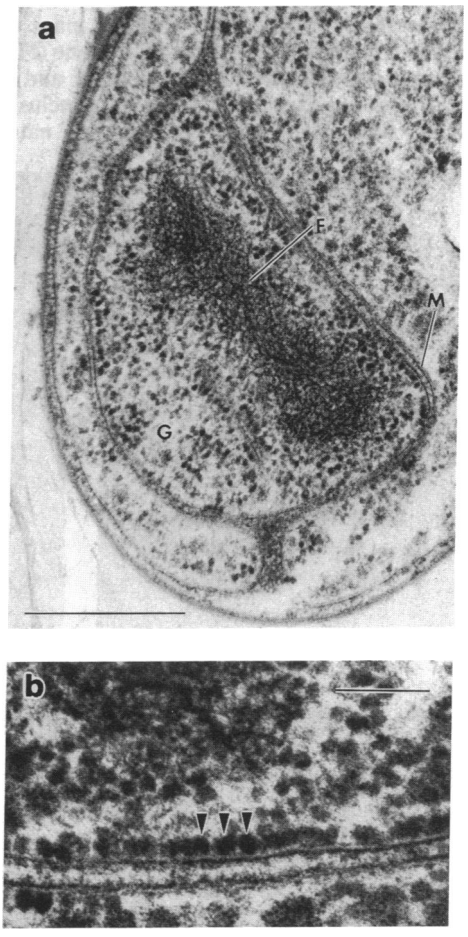

FIG. 2. Electron micrographs of thin sections of cryosubstituted *G. obscuriglobus* cells displaying the nuclear body. (*a*) A membrane-bounded nuclear region with central fibrillar area (F) and outer granular area (G). The double-membrane structure of the boundary (M) is apparent. (Bar = 0.5 μm.) (*b*) Membranes enclosing a nuclear region of another cell displaying the double-track layers in each of the two nuclear membranes separated by an electron-transparent layer. Note the close apposition of linearly arranged ribosome-like particles (arrowheads) to the inner nuclear membrane. (Bar = 0.1 μm.)

other intracellular membranes and possibly to the cytoplasmic membrane at the cell periphery (Fig. 2*a*). Dense particles with an appearance consistent with ribosomes often appear to be closely appressed to the nuclear membrane, including the inner nuclear membrane (Fig. 2*b*). These are lined up in a similar manner to the way in which ribosomes of eukaryotes are arranged in rough endoplasmic reticulum and when attached to the outer nuclear membrane of interphase cells (26, 27).

Freeze–Fracture. Replicas of freeze–fractured cells not fixed prior to freezing also display membrane-defined nuclear bodies (Fig. 3 *a* and *b*). Fibrillar regions can be seen (Fig. 3 *a* and *b*) that are similar in appearance to conventional bacterial nucleoids in freeze–fracture replicas after osmium tetroxide fixation (7). The organization of the nuclear body in freeze–fractured material is similar to that in freeze–substituted thin-sectioned material, in that the fibrillar region is surrounded by a granular area, both regions being surrounded by a membrane boundary that we interpret as consisting of two membranes (Fig. 3 *a* and *b*). The granular area is more coarse in texture than the cytoplasmic region outside the nuclear membrane boundary (Fig. 3 *a* and *b*). This is consistent with corresponding differences in electron density observed between these regions in thin-sectioned chemically fixed material (Fig. 1). In favorable freeze–fracture replicas of some cells, a large intracellular inclusion, which we presume is the nuclear body, displays membrane surfaces that can be seen where fracture has taken place along the surface or through the middle of each membrane (Fig. 3 *c* and *d*). The membrane surfaces of both the inner and the outer nuclear membrane are exposed, confirming conclusions from thin sectioning regarding the double-membrane nature of the nuclear envelope and the absence of nuclear pores. We have interpreted the fracture surfaces and faces by using the conventions of Branton *et al.* (28): PF_i is the convex fracture face of the nucleoplasmic half of the inner nuclear membrane, EF_o is the convex fracture face of the half of the outer membrane nearest the perinuclear space, and PS_o is the outer surface (not a membrane fracture face) of the outer membrane, nearest to the non-nuclear cytoplasm (Fig. 3*d*). The PS_o surface has been revealed by etching after the fracture process and before replica preparation. Faces analogous to the PF_i and EF_o identified here are commonly revealed when nuclei of eukaryotes are freeze–fractured so as to reveal the convex fracture faces of the nuclear membranes, as in a study of *Zea mays* root cells (29). This interpretation is consistent with the observation of inner and outer nuclear membranes in the nuclear body of thin sections of *G. obscuriglobus*. The coherence of the nuclear-body membranes in the freeze–fracture image of Fig. 3 *c* and *d* constitutes stronger evidence for the continuity of the nuclear membranes around the nuclear body than can be deduced only from the two dimensions of thin sections. The shape of the nuclear body cannot be deduced from these freeze–fracture replicas (only part of the complete nuclear-body surface is visible), and in particular no evidence in support of a spherical shape can be deduced from them. Freeze–fracture of chemically fixed cells also shows nuclear bodies, but with more distinct nucleoids (Fig. 4), consistent with results obtained when chemically fixed cells of other bacteria are freeze–fractured (30).

Immunogold Labeling. Immunogold labeling using an anti-dsDNA antibody indicates the presence of dsDNA in the fibrillar nucleoid region of the nuclear body but not over the

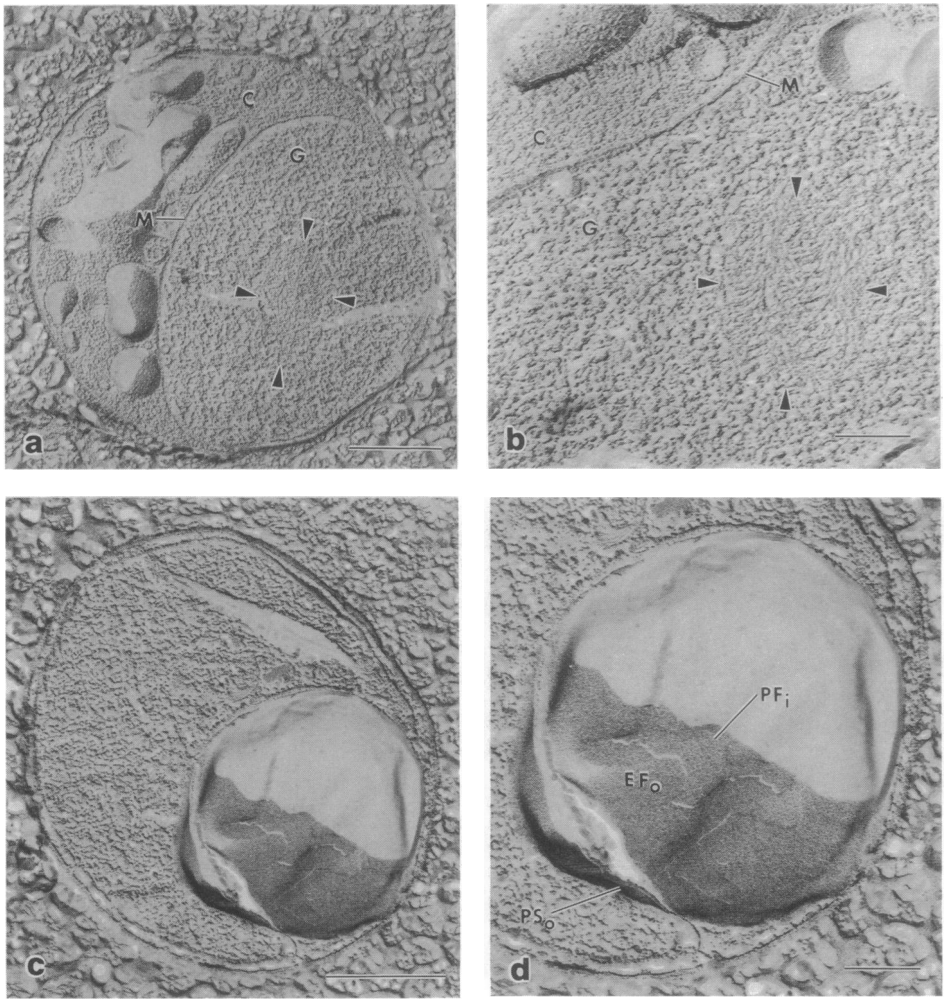

FIG. 3. Electron micrographs of replicas of freeze–fractured unfixed *G. obscuriglobus* cells demonstrating membrane-bound nuclear bodies. (*a*) In this cross-fractured whole cell, a membrane (M)-bound nuclear body occupies a large proportion of the cell; a fibrillar nucleoid region (arrowheads) occupies the centre of the nuclear body. Note the vesicles occupying the cytoplasm outside the nuclear boundary, and the difference in texture of the cytoplasm (C) relative to the nucleoplasm (G). (Bar = 0.5 μm.) (*b*) In this cross-fractured portion of a cell displaying a nuclear body, a finely fibrillar region (arrowheads) is seen within a coarsely granular nucleoplasmic area (G) of the nuclear body, which is bounded by membrane layers (M). Note that the cytoplasm (C) outside the nuclear region is less coarse in texture than the nucleoplasm (G) inside the membrane. (Bar = 0.2 μm.) (*c*) In the whole cell displayed, a nuclear body is present in which fracture has occurred along bilayers of the nuclear membranes, revealing the outline of the whole nuclear body demarcated by several different nuclear membrane surfaces. (Bar = 0.5 μm.) (*d*) An enlargement of *c*, displaying the nuclear membrane surfaces and faces in more detail. The membrane surfaces and faces are labeled according to the convention of Branton *et al.* (28): PS_o, external surface of outer nuclear membrane; EF_o, face of outer nuclear membrane bilayer half closest to perinuclear space; PF_i, face of inner nuclear membrane nearest to nucleoplasm. (Bar = 0.2 μm.)

FIG. 4. Electron micrograph of a replica of the nuclear body within a freeze–fractured *G. obscuriglobus* cell that was chemically fixed before freezing and fracturing; a coarse, roughly textured central region (arrowheads) is apparent within the membrane (M)-bound nuclear body. (Bar = 0.2 μm.)

dense granular outer region of the nuclear body (Fig. 5). Immunolabeling of freeze–substituted cells confirmed these results.

DISCUSSION

We conclude that the DNA of the bacterial genophore in *G. obscuriglobus* is packaged in a distinct nuclear body that is bounded by a double membrane. This conclusion is supported by results obtained when cells are prepared by a variety of different techniques, including those reported to minimize possible artifacts due to conventional fixation and dehydration, such as freeze–substitution and freeze–fracturing. The organization of the nuclear membranes of this bacterium bears some resemblance to the organization of those in the nuclear envelope of eukaryotes, in which an inner and an outer nuclear membrane enclose a perinuclear space, an electron-transparent lumen (27). Measurements from the nuclear membranes and "perinuclear space" between these membranes in

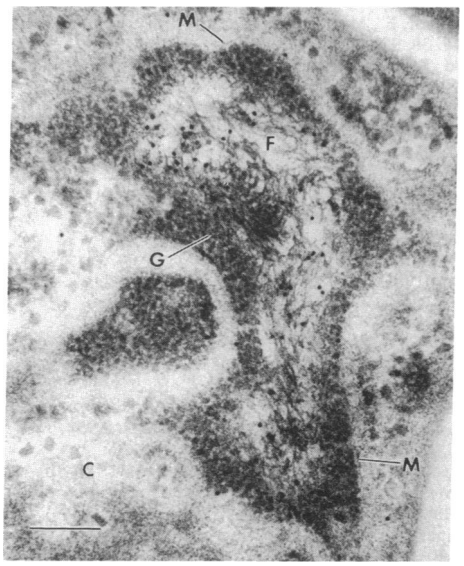

FIG. 5. Electron micrograph of the nuclear body portion of a thin-sectioned chemically fixed *G. obscuriglobus* cell that was immunogold-labeled to localize dsDNA. Colloidal gold particles are visible only in association with the central fibrillar nucleoid region (F) of the nuclear body, and not with the granular peripheral area (G) of the nuclear body. The membranes (M) bounding the nuclear body separate it from the cytoplasm (C). (Bar = 0.2 μm.)

sections of *G. obscuriglobus* prepared by cryosubstitution are within the range reported for the corresponding structures in the eukaryotic nuclear envelope (31). However, there is no evidence to support the presence of a standard eukaryote nucleus in this bacterium. Some features characteristic of many eukaryotic nuclei, such as the nucleolus and peripheral nuclear lamina bordering the inner nuclear membrane (27, 32, 33), do not appear to be associated with the nuclear body of *G. obscuriglobus*. Nuclear pores do not appear to be present as judged from thin sections and freeze–fracture.

Of particular interest is the demonstration of the membrane-bounded structure of the nuclear body in material prepared by freeze–substitution and freeze–fracture, two methods that are relatively artifact-free. This supports our view that the membrane-bounded nuclear body occurs in the living cell. Freeze–substitution employing cryofixation (ultra-rapid freezing) and cryosubstitution, in which fixative penetrates cells at low temperature before fixation at higher temperatures (34), has been claimed to provide the best preservation of cell ultrastructure for eubacteria (10) and in particular of the ultrastructure of bacterial nucleoids (2). Artifactual membrane structures such as mesosomes do not appear in cells prepared by this method (6).

Although freeze–fracture micrographs suggest that the nuclear body is an entirely rather than partially bounded structure, definitive evidence of this will require extensive serial sectioning.

Several alternative interpretations of our data are possible, including that membrane envelopment of DNA occurs by an artifactual mechanism similar to mesosome induction, that membrane-bounded nuclei in *G. obscuriglobus* represent pre-spore stages of endospores, that they represent nonspecific compartmentalization of cytoplasm and nucleoplasm such as that occurring in the chemolithotroph *Nitrosolobus* (35), and that the phenomenon is similar to that of the DNA-containing membrane vesicles reported from many Gram-negative bacteria. The first of these we have rejected above. The second we have excluded because even 97-day cultures are killed by exposure to 100°C for 10 min, cells from such cultures do not display refractile intracellular bodies by phase-contrast light microscopy, and electron microscopy of thin sections of such cells reveals no immature or mature endospores. In addition, endospores are not known to be produced by any member of the order *Planctomycetales*. The third requires reexamination of this chemolithotroph, since partial compartmentalization of cytoplasm by intracellular membrane invagination in such bacteria might involve nucleoid (35). However, in *Nitrosolobus*, phylogenetically distant from *Gemmata* (36), such compartmentalization involves intrusion of the inner layers of the cell wall. The packaging of DNA within membrane-derived vesicles has been reported to occur in a wide range of Gram-negative bacteria (37). However, these are outer-membrane-derived extracellular membrane vesicles harboring plasmid DNA. These vesicles do not represent internal intracellular membrane-bound DNA-containing structures and are not comparable with the nuclear bodies of *G. obscuriglobus*.

Although the nuclear body of *G. obscuriglobus* appears similar in some respects to the eukaryotic nucleus, it is not necessarily a homologous structure phylogenetically. *G. obscuriglobus* is a member of the order *Planctomycetales*, a phylogenetically distinct phylum within the eubacterial kingdom. Pending detailed rRNA sequence comparisons, the only molecular similarity between planctomycetes and eukaryotes is the occurrence of several unusual lipid components also known to occur in eukaryotic genera—for example, oleic acid in *Acanthamoeba, Euglena, Cryptomonas*, and *Pythium*, among many others (38, 39). Superficial similarity to a eukaryotic nucleus could merely be the result of convergent evolution or of envelopment of DNA without accompanying functional

correlates found in eukaryotes. If true homology is present, structures such as nuclear pores and lamina and such functional correlates as RNA splicing, introns, absence of protein synthesis in the nucleus, and a role for centriole-like microtubule-containing bodies in nuclear division might be found (unless association of nuclear bodies with cytoplasmic membrane accounts for nuclear-body distribution during division). There is no evidence so far for nuclear pores, nucleosomes, nuclear lamina, microtubules, or centrioles in *G. obscuriglobus*, but it should be noted that nuclear pores, at least, are not universal features of eukaryotic nuclei; they are not detectable in protists such as the microsporidian *Pleistophora* and, with respect to its micronucleus, the ciliate *Blepharisma*, or in certain cell types of some non-protist eukaryotes (40–43).

The presence of a membrane-bounded nucleoid in a well-defined member of the eubacteria (that is, the "domain" Bacteria in a recently proposed scheme) could prove to be consistent with Woese's view that the prokaryote/eukaryote kingdom dichotomy is no longer appropriate as a way of classifying living organisms phylogenetically (44, 45), a view already well-established through discovery of the archaebacteria, the "domain" Archaea (46, 47). It appears that there is at least one ultrastructural exception to the prokaryote/eukaryote dichotomy, even if viewed simply as a distinction in types of cell organization. However, the nuclear body of *G. obscuriglobus*, while membrane-bounded, lacks many structural features of eukaryotic nuclei. The occurrence of a major feature superficially similar to eukaryote cell organization in a eubacterium may of course be found to have deeper phylogenetic significance upon more extensive molecular investigation. The evidence from small-subunit rRNA sequencing of protists that lack mitochondria, such as *Vairimorpha necatrix*, a microsporidian, and *Giardia lamblia*, a diplomonad, suggests the existence of a distinct group of extremely ancient eukaryotes, sometimes called the "Archezoa" (48–50). If more detailed comparisons of the Archezoan small-subunit rRNAs with *G. obscuriglobus* 16S rRNAs do not support any deep relationship, then the data presented here will be evidence for the polyphyly of membranes bounding genophores.

Regardless of the phylogenetic implications of the membrane-bounded nuclear body in *G. obscuriglobus*, the presence of a membrane-bounded nuclear body may imply molecular and cytological features analogous to those correlated with or necessitated by the presence of a membrane-bounded nucleus in eukaryotes—for example, cytoskeletal components, introns, and spliceosomes (33, 51, 52).

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