

Novel nano-organisms from Australian sandstones

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ABSTRACT

We report the detection of living colonies of nano-organisms (nanobes) on Triassic and Jurassic sandstones and other substrates. Nanobes have cellular structures that are strikingly similar in morphology to *Actinomyces* and fungi (spores, filaments, and fruiting bodies) with the exception that they are up to 10 times smaller in diameter (20 nm to 1.0 μm). Nanobes are noncrystalline structures that are composed of C, O, and N. Ultra thin sections of nanobes show the existence of an outer layer or membrane that may represent a cell wall. This outer layer surrounds an electron dense region interpreted to be the cytoplasm and a less electron dense central region that may represent a nuclear area. Nanobes show a positive reaction to three DNA stains, [4',6-diamidino-2 phenylindole (DAPI), Acridine Orange, and Feulgen], which strongly suggests that nanobes contain DNA. Nanobes are communicable and grow in aerobic conditions at atmospheric pressure and ambient temperatures. While morphologically distinct, nanobes are in the same size range as the controversial fossil nanobacteria described by others in various rock types and in the Martian meteorite ALH84001.

INTRODUCTION

The identification and existence of nanobacteria have been proposed (Folk 1993; Sillitoe et al. 1996; Pedone and Folk 1996; McKay et al. 1996; Vasconcelos and Mckenzie 1997; Folk and Lynch 1997) but remain controversial (Clarke 1997; Volke 1997) and the subject of intense scientific debate (Maniloff 1997; Nealson 1997; Psenner and Loferer 1997; Harvey 1997). Until now, the evidence for the existence of sub-bacteria sized organisms has been based solely on the morphological similarity of fossil (mineralized) structures to living bacteria. The diameter of bacteria ranges from 150 nm to 50 μm , whereas the proposed “nanobacteria” are an order of magnitude smaller with diameters reported to range from 20 nm to 150 nm in diameter (Folk 1993, 1997a, 1997b). “Nanobacteria” are therefore considered by many to be too small to contain the enzymatic and genetic material essential for life (Maniloff 1997; Nealson 1997; Psenner and Loferer 1997; Harvey 1997). It remains unresolved whether “nanobacteria” are fossilized remnants of autonomous life-forms, artifacts of sample preparation, or unusual mineral deposits.

Although a biological origin of mineralized “nanobacteria” has not been confirmed, other micro-organisms termed “nanobacteria” have been isolated and cultured from fetal bovine serum (Ackerman et al. 1993; Ciftcioglu et al. 1997; Kajander et al. 1994; Kajander et al. 1994). These “nanobacteria” are generally larger than those described in rocks, ranging from 200 to 300 nm in diameter.

Molecular analysis indicated that the “nanobacteria” isolated from fetal bovine serum are members of the alpha-2 subgroup of the *Proteobacteriaceae* and are closely related to the genus *Brucella*.

This paper describes various organic features that were observed as unusual growths on sandstone samples and other substrates. These growths correspond in size to the mineralized structures referred to by Folk and others as “nanobacteria.” We do not follow Folk’s convention of spelling, nor do we relate the organisms we describe to bacteria until their phylogeny has been established. Instead, we refer to these features as nano-organisms or nanobes to indicate their significant difference in size to Eubacteria and Archaea, loosely following SI convention. This paper describes these organic features and documents their morphology, elemental composition, and structural detail by various electron optical, spectroscopic, and molecular analysis techniques. Based on this evidence, our thesis is that nanobes are biological organisms. Future DNA analyses will determine whether these organisms are related to bacteria or fungi or belong to a different phylogenetic tree altogether.

MATERIALS AND METHODS

Samples

Sandstone samples with observed in situ nanobe growths comprised both full diameter core and sidewall core taken from low permeability, extensively quartz overgrowth-cemented Triassic and Jurassic sandstones from petroleum exploration wells offshore western Australia. Sample depth ranged from 3400 to 5100 m below

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the sea bed with corresponding stabilized borehole temperatures of 117–170 °C.

Nanobe colonies were subsequently identified on copper SEM mounts and on the surfaces of storage containers (polystyrene and glass Petri dishes) that were used to store the sandstone samples.

Scanning electron microscopy

Freshly fractured sandstone samples for high-resolution scanning electron microscopy (SEM) were soaked in petroleum spirit for 12 h to remove residual hydrocarbons prior to mounting on copper SEM mounts with super glue and painted around the base with colloidal silver. The samples were placed in a vacuum dessicator for 24 h. Nanobe growths on the copper SEM mounts were examined *in situ* whereas those on polystyrene and glass substrates were micromanipulated off the Petri dishes using sterilized forceps and placed on double sided carbon tabs on copper SEM mounts.

All samples were coated with 10 nm of platinum and examined in a JEOL 890 field emission SEM operating at 25 or 40 kV. No other sample preparation for SEM was conducted. Samples were stored in a dessicator containing silica gel.

Transmission electron microscopy

Nanobe colonies that were observed to be growing on polystyrene Petri dishes were micromanipulated off the Petri dish using sterilized forceps and placed on Formvar-coated 200 mesh copper transmission electron microscopy (TEM) grids. These were examined in a JEOL JEM 1010 TEM operating at 80kV.

Nanobe colonies were processed *in situ* in the polystyrene Petri dish. Nanobe colonies were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer for 24 h at 4 °C. Colonies were subsequently post fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 2 h, then dehydrated in a graded ethanol series and embedded in Epon resin. Ultra thin sections (70–100 nm) were cut on a LEICA Ultracut T microtome, stained with 5% uranyl acetate in 50% methanol and Reynolds lead citrate, and placed on carbon-coated, Formvar-covered 200 mesh copper grids. The samples were examined in a JEOL JEM 1010 TEM operating at 80kV.

Energy dispersive X-ray spectroscopy

Nanobe filaments that were growing on the surface of polystyrene and glass Petri dishes were micromanipulated off the surface and placed on Formvar-coated 200 mesh copper TEM grids. Energy dispersive X-ray spectroscopy (EDXS) was conducted on individual (whole) unstained filaments and the background support film (Formvar) using a JEOL 2010 TEM fitted with a Link Si EDXS detector, operating at 200 kV with a probe size of 35 nm.

DAPI, Acridine Orange, and Feulgen staining for DNA

Nanobes on copper substrates. DAPI is a DNA specific probe that forms a fluorescent complex by attaching

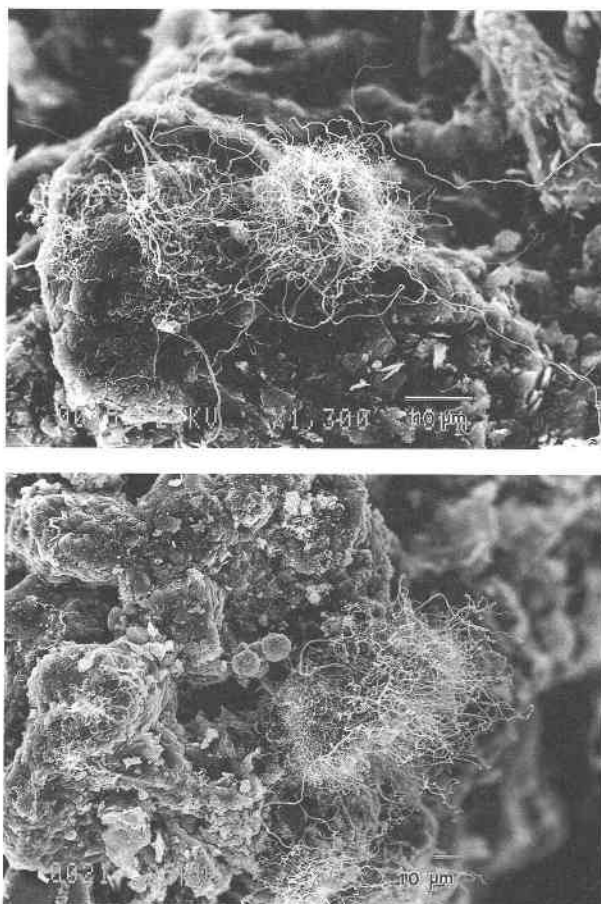


FIGURE 1. (top and bottom) Scanning electron micrographs of nanobe microcolonies composed of nano-filaments and micro-filaments protruding from the sandstone surface into the porespace.

in the minor groove of A–T (adenine–thymine) rich sequences of DNA (Kapuscinski 1995).

To determine whether nanobes contain DNA, nanobe colonies that were observed on copper SEM mounts were subjected to DAPI staining. DAPI stain was pipetted onto the nanobe colonies on the copper substrates and kept in the dark for up to three days. The DAPI stain was removed by gently rinsing the sample in ultra pure water. The nanobes were examined over a period of three days using an Olympus BX 60 optical microscope fitted with a UV filter. Following exposure to DAPI stain the nanobe colonies were examined uncoated in a Philips/Electroscan Environmental Scanning Electron Microscope (ESEM model E3) operating at 15kV, to confirm that the material that reacted with DAPI was nanobe colonies.

Nanobes on glass Petri dishes. A small sample was micromanipulated off the surface of the glass Petri dish and placed in a concave dimple of a sterilized glass slide. A small drop of 100% acetone was placed on the sample to partially break down the cell walls. The sample was dispersed in the acetone and subsequently precipitated on

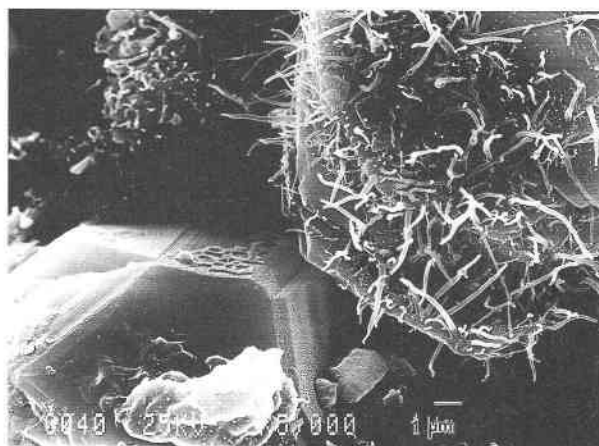
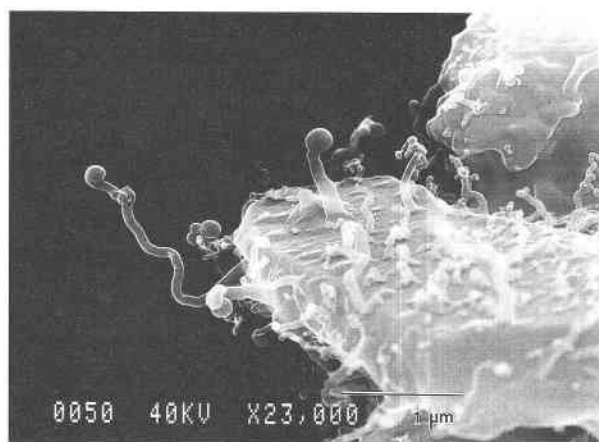


FIGURE 2. (top) Scanning electron micrograph of nano-filaments 30–135 nm (with platinum coating subtracted, 20–125 nm) projecting from the freshly fractured surface of the sandstone. (bottom) Scanning electron micrograph of nano-filaments and micro-filaments protruding from a quartz overgrowth into the porespace.

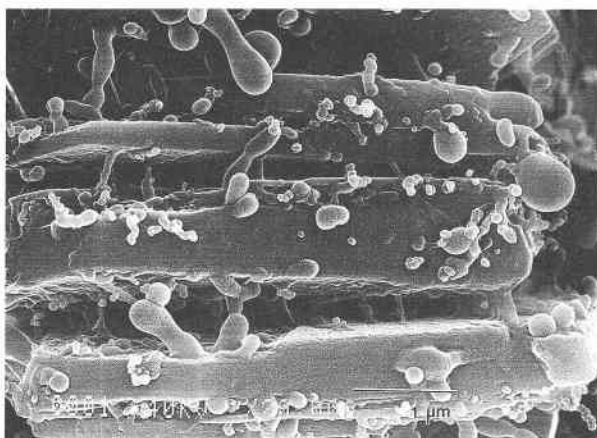
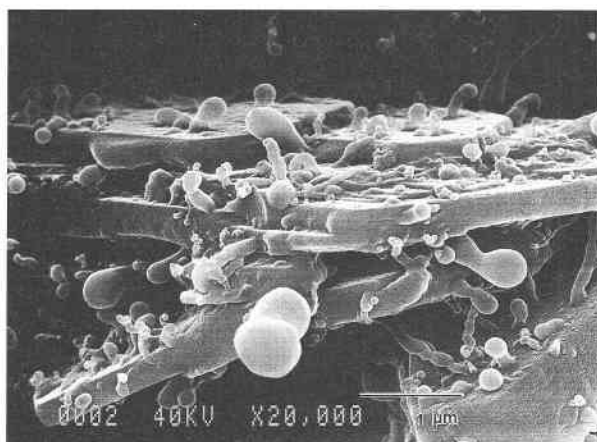


FIGURE 3. (top and bottom) Scanning electron micrographs of abundant pleomorphic nano-filaments growing on and between individual kaolinite platelets. Nanobes range in diameter from 50 to 500 nm (with platinum coating subtracted, 40–490 nm). Isolated spores range in diameter from 45 to 100 nm (with platinum coating subtracted 35–90 nm). Note the swollen structures that may represent a reproductive structure.

the glass slide as small discrete round aggregates. The acetone-treated material was reacted with DAPI stain for 15 min and then rinsed in ultra pure water to remove the DAPI.

Acridine Orange is a molecular probe that interacts with DNA and RNA. Acridine Orange shows a characteristic green fluorescence when bound to DNA and red fluorescence when bound to RNA under blue excitation. A small sample was prepared for staining with Acridine Orange following the same procedures outlined above (dispersion in acetone prior to treatment). The acetone treated material was reacted with Acridine Orange for 2–5 min, then rinsed thoroughly in ultra pure water and examined using an Olympus BX 60 optical microscope under blue excitation.

Feulgen is a specific method for measuring the deoxyribose sugars of DNA (Kiernan 1991; Bancroft et al. 1996) and shows a characteristic red fluorescence when bound to DNA under green excitation. A small sample was dispersed in acetone in the same manner as outlined

above for DAPI and Acridine Orange staining. The acetone-treated material was rinsed in cold 1 M HCl and incubated in an oven at 60 °C in 1 M HCl for 10 min. The sample was then rinsed in cold 1 M HCl, followed by distilled water, and reacted with Schiff's reagent for 30 min. The sample was rinsed in water for 5 min and examined using an Olympus BX60 optical microscope under green excitation.

RESULTS AND DISCUSSION

SEM

Nanobe growths on sandstones. Examination of quartzose sandstone samples from petroleum exploration wells revealed the presence of nanobe colonies on recently fractured surfaces (Figs. 1 and 2) including quartz overgrowths, and on and between clay platelets (kaolinite) (Fig. 3).

Features identified in SEM as nanobe colonies have filamentous, coryneform (club shaped), and swollen

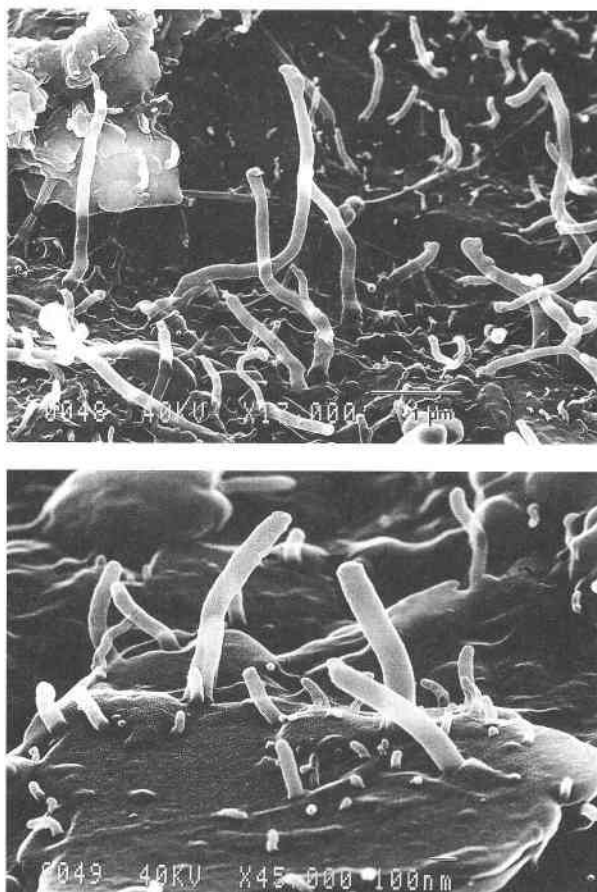


FIGURE 4. (top and bottom) Scanning electron micrographs of sandstone surfaces showing abundant unbranched nano-filaments 30–138 nm (with platinum coating subtracted 20–128 nm) in various stages of germination.

structures that are interpreted to represent fruiting bodies (Fig. 3). Individual filaments (Fig. 4) are 20–128 nm in diameter with variable lengths. Throughout this paper we refer to nanobe filaments with diameters <200 nm as nano-filaments and filaments >200 nm as micro-filaments. Terminations of nano-filaments are rounded. Larger micro-filaments (200 nm to 1.0 μm diameter) are often branched.

Colonies appeared to grow spontaneously on freshly fractured rock becoming visible to the unaided eye within 2–3 weeks. Because nanobes appear to germinate from the freshly fractured sandstone surfaces, nanobe spores may be endogenous to the sandstones. However, whereas we consider it unlikely given the low permeability of the sandstones, we cannot exclude the possibility of contamination from formation fluids, drilling fluids, or other airborne contaminants.

Nanobe growths on copper, polystyrene and glass substrates. Nanobes also appeared to grow spontaneously on copper SEM sample mounts, polystyrene (Fig. 5), and glass Petri dishes in aerobic conditions at 22 °C. Inoculation of substrates appears to have been from the

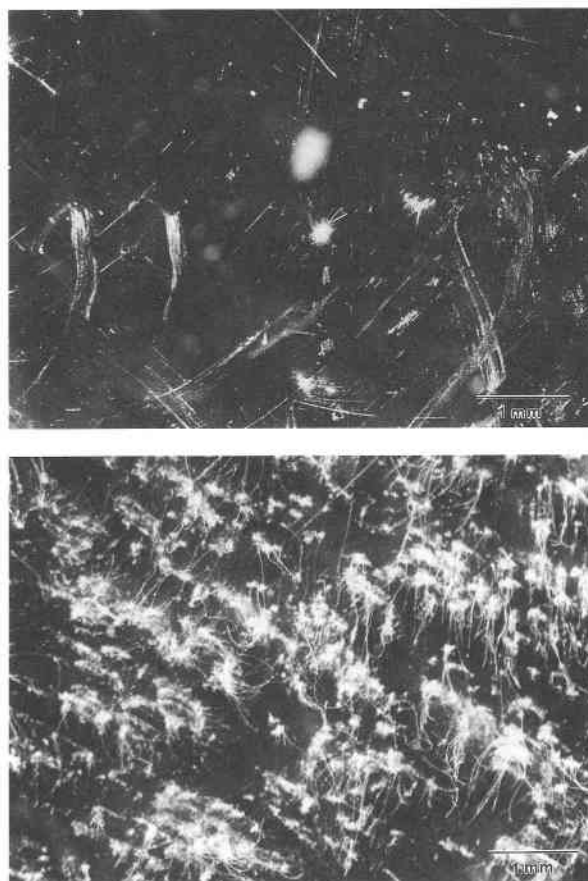


FIGURE 5. Optical photomicrograph of the surface of a polystyrene Petri dish showing a range of nanobe colonies (white markings). (top) A small radial colony (in center). This exact colony was processed for TEM (see Fig. 8). (bottom) A substantial area of the Petri dish with a dense covering of nanobe colonies.

sandstones that were adjacent to the substrates. Nanobe colonies were observed on these other substrates over a period of several days to several months. Once established these colonies became visible to the unaided eye (0.1–0.2 mm; Fig. 5, top) as opaque, white, brown, or gray filamentous colonies.

Of particular interest was the observation that contamination with organic compounds appears to have promoted the growth of nanobes because some microcolonies were arranged to form replicas of fingerprints on the surfaces of the polystyrene Petri dishes. This growth form may indicate that nanobes have a heterotrophic metabolism. Although the origin of the nanobes on the polystyrene Petri dishes is uncertain, they are similar in appearance and in size to those found on the fresh fractured sandstone surfaces shown in Figure 4. It follows that because the Petri dishes were exposed to sandstones rich in nanobes, it is likely that the source of the nanobes on the fingerprints was by inoculation from spores in the sandstones.

High-resolution SEM examination of the nanobes from

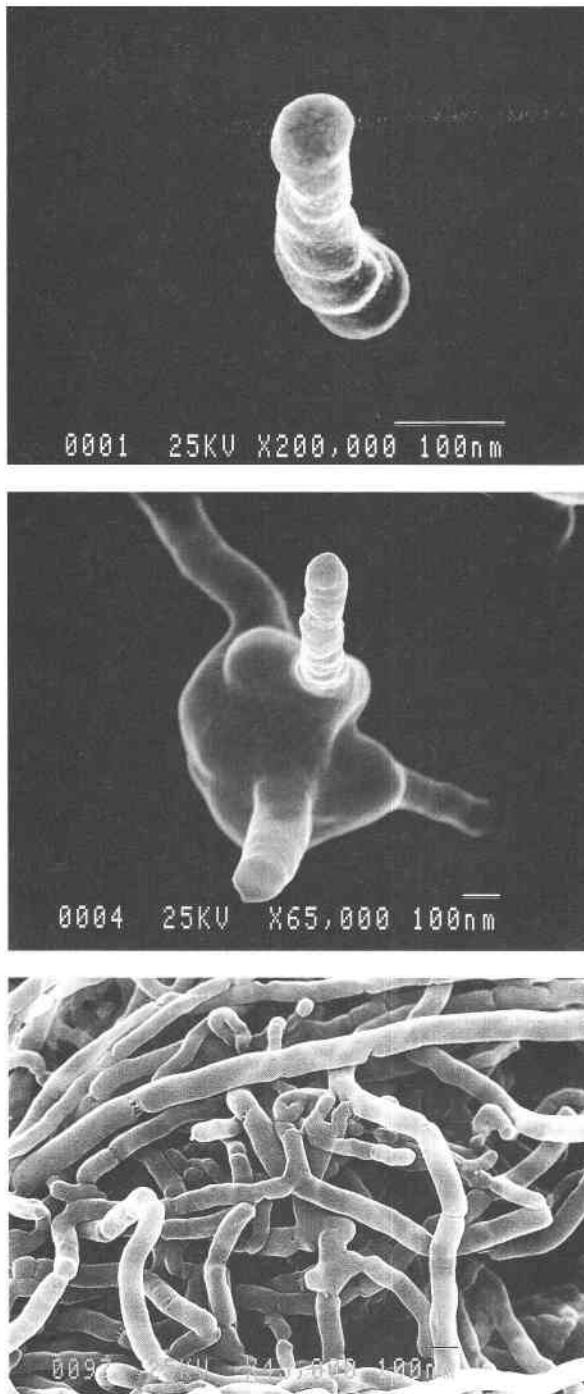


FIGURE 6. Scanning electron micrographs of nanobes that were micromanipulated off polystyrene Petri dishes. **(top)** shows a young nano-filament and **(middle)** shows a swollen structure with protruding nano-filaments. Scanning electron micrographs of nanobes micromanipulated off glass Petri dishes **(bottom)** show filamentous material strikingly similar to fungal hyphae but with most filaments 100 nm or below. Constrictions along the filaments most likely represent septa in the filaments.

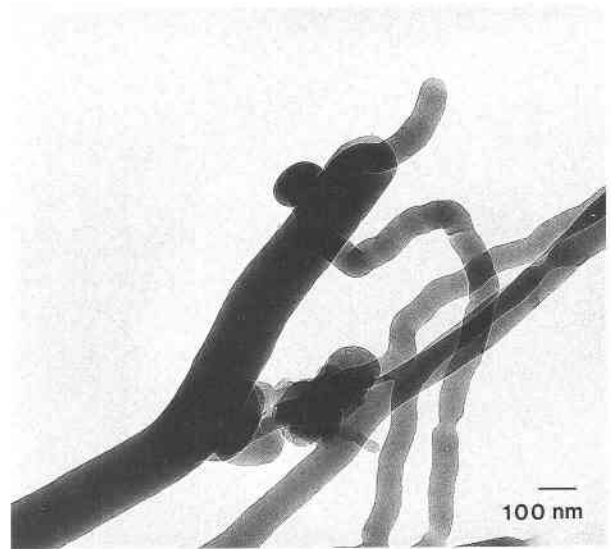


FIGURE 7. Transmission electron micrographs of whole cell nano-filaments (25–150 nm) micromanipulated off polystyrene Petri dishes. Note the distinctly rounded ends.

polystyrene Petri dishes revealed similar morphologies, coccoid bodies, and nano-filaments (Fig. 6, top) observed in colonies growing on the sandstones (compare with lower magnification micrographs, Fig. 4). Figure 6 (middle) shows an unusual swollen structure that may represent a reproductive structure. Nanobes from glass Petri dishes revealed a dense network of filamentous material with diameters of 100 nm or less (Fig. 6, bottom) with clearly defined constrictions “septa” and exhibiting coarsely granular structures between “septa.” Individual filaments range in diameter from 50 to 100 nm. The morphologies of these nanobes closely resemble fungal hyphae.

TEM

Nanobes from polystyrene Petri dishes (whole cells). Transmission electron micrographs of whole cell preparations of nanobes micromanipulated off polystyrene Petri dishes show coccoid bodies and nano-filaments (Fig. 7) that are consistent with the morphology of the spores and nano-filaments identified by SEM. Nano-filaments and micro-filaments have a constant diameter within individual filaments with invaginations possibly representing septa and have rounded ends. These features are characteristic of biological structures.

Selected-area electron diffraction data demonstrate that nanobes have an amorphous membrane structure, which is consistent with biological material and excludes the presence of crystalline mineral compounds.

Nanobes from polystyrene Petri dishes (ultra thin sections). Sample preparation for TEM proved difficult because these organisms were found to have a highly hydrophobic wall structure. Nanobes do not behave like “normal” tissue in that they do not fix easily in glutar-

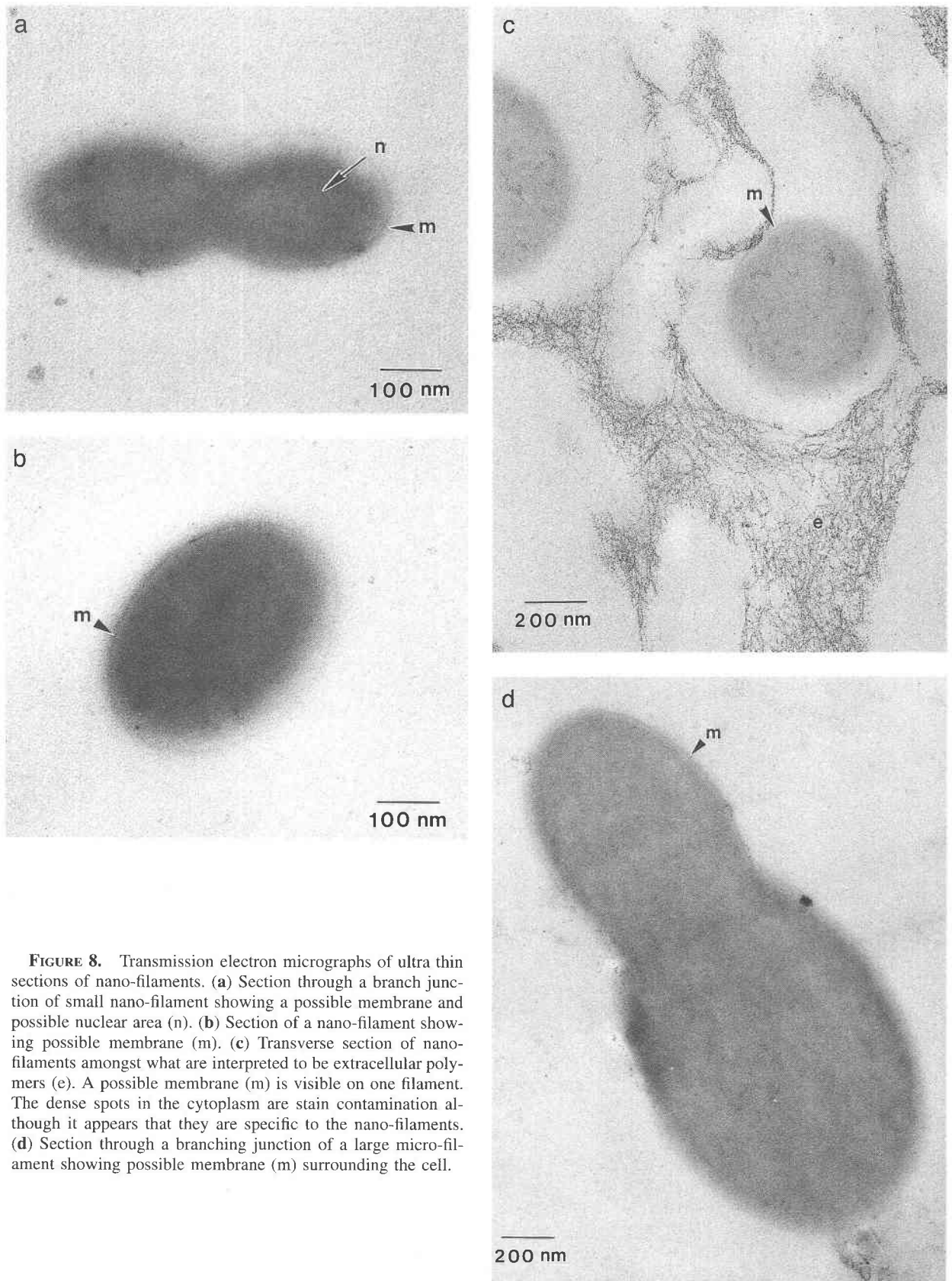


FIGURE 8. Transmission electron micrographs of ultra thin sections of nano-filaments. (a) Section through a branch junction of small nano-filament showing a possible membrane and possible nuclear area (n). (b) Section of a nano-filament showing possible membrane (m). (c) Transverse section of nano-filaments amongst what are interpreted to be extracellular polymers (e). A possible membrane (m) is visible on one filament. The dense spots in the cytoplasm are stain contamination although it appears that they are specific to the nano-filaments. (d) Section through a branching junction of a large micro-filament showing possible membrane (m) surrounding the cell.

aldehyde and react adversely to dehydrating agents such as acetone, ethanol, and methanol. Furthermore they do not embed well in resin and can fall out during sectioning.

Ultra thin sections of nanobes from the colony shown in Figure 5 (top) revealed that the stained nano-filaments contain an electron dense central area interpreted to be a cytoplasm surrounded by an outer layer interpreted to represent a cell membrane or cell wall (Figs. 8a–8d). The enclosed less dense regions within the cytoplasm may represent a nuclear area. A filamentous network of fine material is associated with some nanobe filaments (Fig. 8c) and may represent an extracellular polymer matrix. Although the nano-filaments did not appear to stain densely with uranyl acetate and Reynolds lead citrate, the extracellular polymers reacted strongly with these stains.

EDXS

Comparison of spectra obtained from nanobes from both polystyrene (Fig. 9 top) and glass substrates and the background support film (Formvar, Fig. 9, bottom) indicates that the nanobes are most probably composed of carbon, oxygen, nitrogen, and minor silicon. EDXS spectra of the background support film showed the presence of carbon, oxygen, and silicon but in considerably lower amounts when compared with the nanobes. No nitrogen was detected in the Formvar support film. The presence of carbon in the nanobes, however, is not conclusive as samples can be contaminated with carbon in the microscope deposited by the electron beam. Contamination by carbon is unlikely, though, as no carbon deposition was seen on the nano-filaments and so does not explain the magnitude of the carbon peak obtained. The nitrogen peak, however, is conclusive because there are no other nitrogen sources in the microscope that could account for the nitrogen detected in the nanobes.

A small silicon peak was detected in both the background film and the nano-filaments. Silicates are often found in eukaryotes and some Eubacteria, and so the presence of silicon in such small quantities would not exclude nanobes from being biological structures. However, as Formvar films are cast on glass surfaces, this small silicon peak could be accounted for by silicon in the support film. No other high energy peaks were observed.

EDXS results are consistent with the nanobes being composed of biological materials given that >78% of a typical cell (Rosenberg and Cohen 1983) is composed of C (50%), O (18%), N (13%), and H (10% undetectable by EDXS). If nanobes are not composed of organic material, but are an inorganic compound, the range of possible candidates is very limited. In particular, silicates and metallic oxides that previously have been proposed as possible explanations for "nannobacterial" morphologies (Harvey 1997) can be excluded.

DAPI, Acridine Orange, and Feulgen staining for DNA

Nanobes on copper substrates. Nanobe colonies showed a positive reaction to DAPI developing a char-

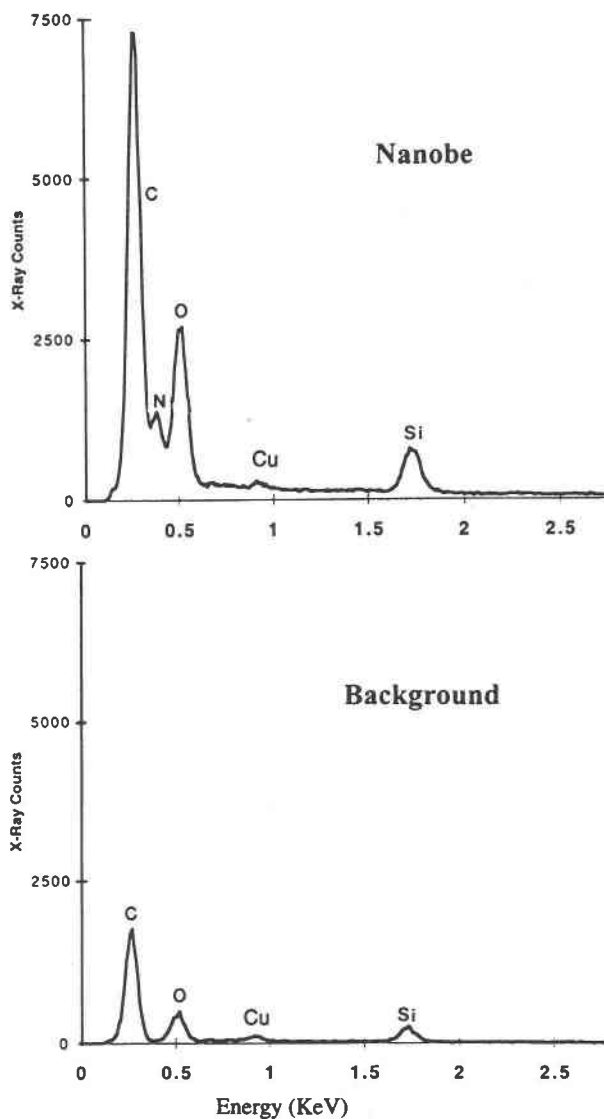


FIGURE 9. Representative TEM EDXS spectra of (a) nanobes, and (b) background film (Formvar). Spectra were obtained on a JEOL 2010 TEM with Link EDXS detector at 20kV for 300 s with a probe size of 35 nm.

acteristic blue fluorescence over a period of three days (Fig. 10a). This result is not surprising given the hydrophobic nature of the nanobe walls.

The limits of resolution of optical microscopy prevent detection of nanobes in their smallest forms. To ensure that the nanobe colonies that reacted positively to DAPI stain were nano-organisms, the exact same colonies that were examined in the optical microscope were examined uncoated immediately after DAPI staining in the ESEM (Figs. 10b and 10c). Figure 10c shows the typical form of the nanobe colonies comprising long filamentous organisms and therefore confirms that the material that reacted positively to DAPI was nanobes.

Given that the samples were not axenic cultures, it

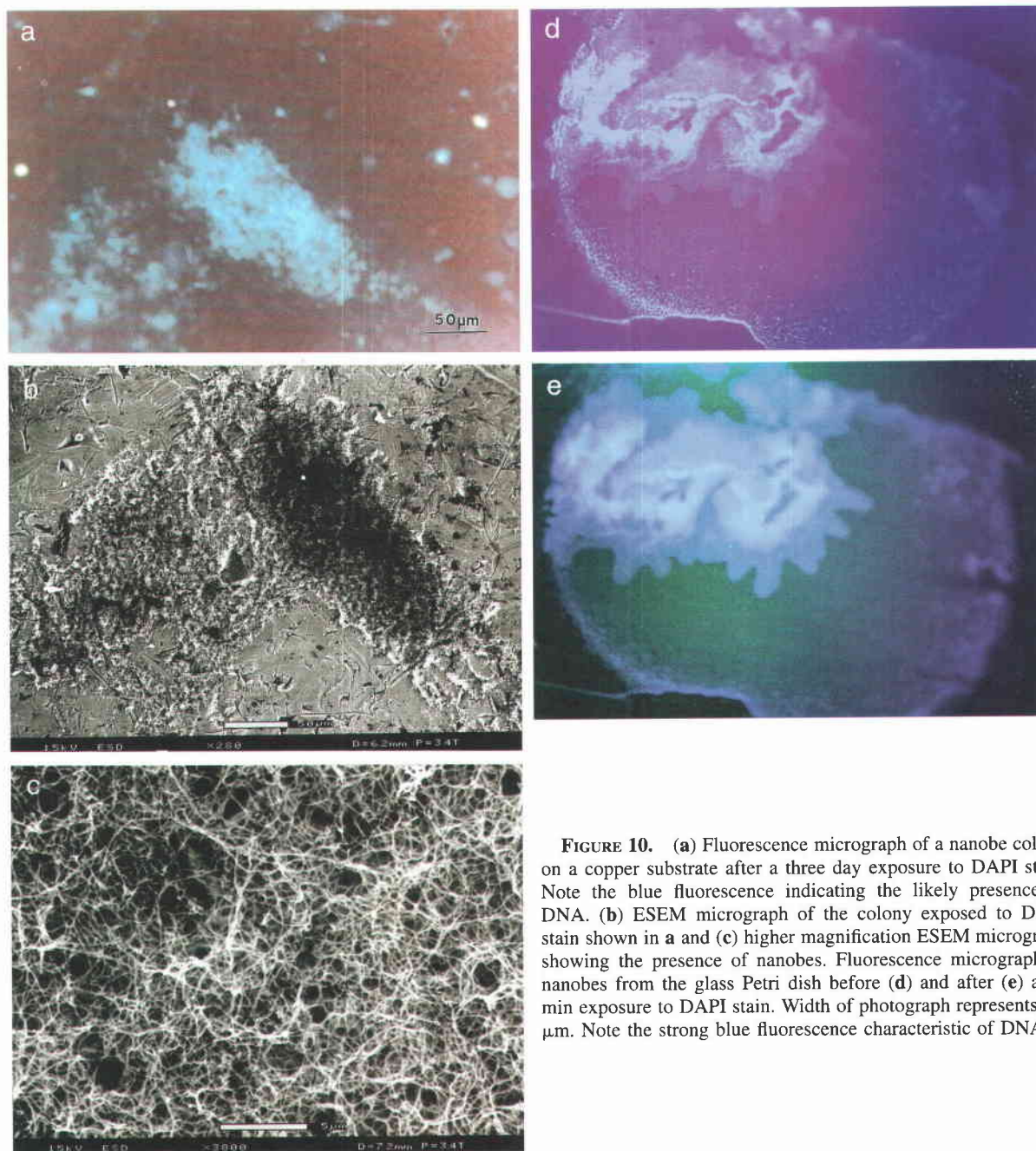


FIGURE 10. (a) Fluorescence micrograph of a nanobe colony on a copper substrate after a three day exposure to DAPI stain. Note the blue fluorescence indicating the likely presence of DNA. (b) ESEM micrograph of the colony exposed to DAPI stain shown in a and (c) higher magnification ESEM micrograph showing the presence of nanobes. Fluorescence micrograph of nanobes from the glass Petri dish before (d) and after (e) a 15 min exposure to DAPI stain. Width of photograph represents 2.4 μm . Note the strong blue fluorescence characteristic of DNA.

could be argued that the positive reaction to DAPI staining could be from contamination by other micro-organisms present in the samples. Contamination by other microbes is unlikely to be significant because (1) the filamentous structures were typical of nanobes (Fig. 10c) and (2) no other microbes were identified in both the optical microscope or ESEM.

Nanobes on glass Petri dishes. Fluorescence micrographs taken before (Fig. 10d) and after DAPI treatment (Fig. 10e) showed a rapid and very strong reaction to DAPI indicating the likely presence of DNA in the ma-

terial. It follows that treatment of the sample with acetone assisted in partially breaking down the hydrophobic cell walls allowing for the rapid penetration of DAPI.

Fluorescence micrographs before staining with Acridine Orange showed no fluorescence and hence are not illustrated. However, fluorescence micrographs after staining with Acridine Orange for 2–5 min (Fig. 11, top, middle, bottom) showed a rapid and very strong reaction to the stain producing the characteristic green fluorescence (illustrated in black and white) for DNA. The characteristic red fluorescence indicative of RNA was not

CONCLUSIONS

There are many properties of the nano-organisms documented in this study that support our thesis that nanobes are biological structures such as the following. (1) Nanobes are communicable and grow spontaneously in aerobic conditions at atmospheric pressure and ambient temperatures (22 °C) on various substrates. (2) Nanobes appear to have a heterotrophic metabolism as shown by their preference to grow on fingerprints on polystyrene substrates. (3) Nanobes show striking morphological similarities to *Actinomycetes* and fungi, but are much smaller. The characteristic radial and axial symmetries governing the morphology of the spores, filaments, and branched hyphae are typical of membrane bound structures. (4) Nanobes are composed of C, O, and N. These results are consistent with biological materials. (5) Nanobes appear to be membrane-bound structures surrounding a possible cytoplasm and nuclear area. (6) Nanobes have amorphous wall structures as determined by selected area electron diffraction. (7) Nanobes contain DNA as indicated by DAPI, Acridine Orange, and Feulgen staining. This is consistent with life.

If nanobes are not biological organisms it is difficult to propose a known nonbiological material that could account for the observed structures. (1) A crystalline mineral origin is excluded by the absence of electron diffraction patterns, demonstrating the amorphous nature of nanobe walls. If these growths are amorphous mineral deposits, it is difficult to propose a mechanism for growth given the environment of growth (22 °C at atmospheric pressure), and the highly restricted elemental composition lacking significant quantities of silicon, sulfur, and metals. Consequently any inorganic compound mimicking nanobes could not be a silicate, sulfide, or metal oxide in either crystalline or amorphous form. (2) Carbonates also can be excluded because no element was detected in sufficient abundance to represent the cation in any carbonate material. Furthermore the oxygen peak is too small for the nanobes to be composed of any carbonate mineral. (3) Nanobes are amorphous structures as shown by electron diffraction. These data rule out the chance that the nanobes could be fullerenes or carbon nanotubes. Carbon nanotubes would show clear lattice structures in TEM that are not seen in nanobes. Furthermore, carbon nanotubes are produced at very high temperatures (>1200 °C) by depositing carbon from a carbon arc in an argon atmosphere and do not form in ambient conditions. Carbon nanotubes are black in color whereas nanobes are white, gray, or brown in color. (4) Nanobes have extremely robust wall structures being able to withstand vacuum and the electron beam in both the SEM and TEM. This rules out the possibility that nanobes are beam-sensitive crystalline minerals that may yield amorphous electron diffraction patterns in the TEM. (5) It could be argued that the structures that we have identified as nanobes are non-living polymers with unusual growth habits. If this were the case it is difficult to propose a mechanism for growth

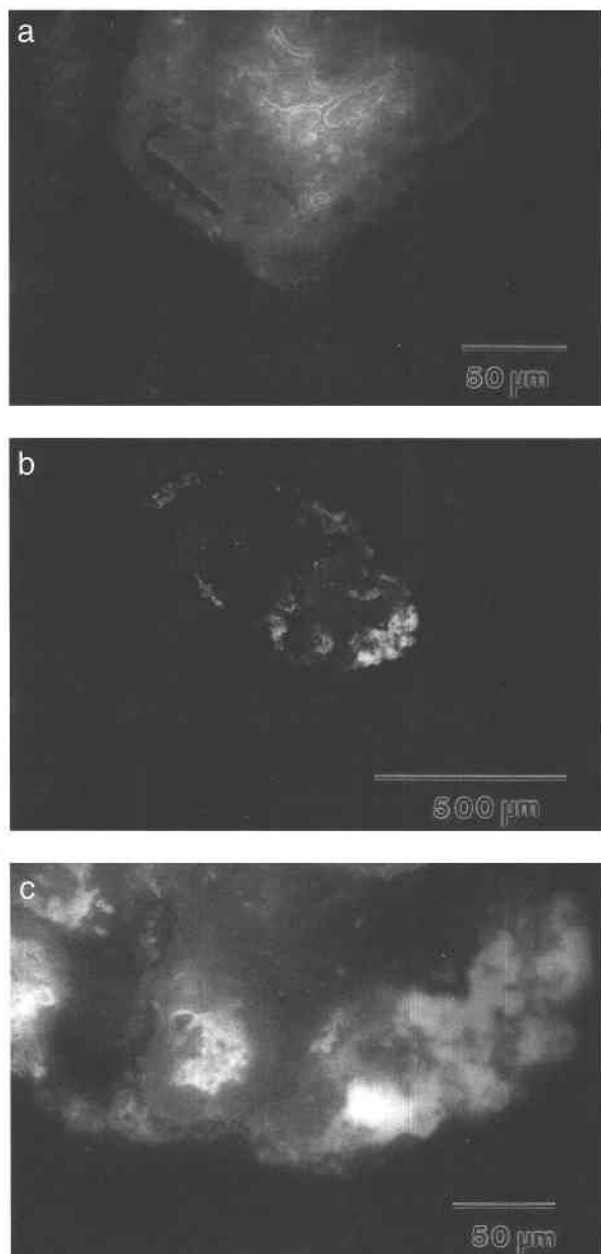


FIGURE 11. Fluorescence micrograph of nanobes from the glass Petri dish following <5 min exposure to Acridine Orange (top, middle, bottom). Note the strong fluorescent signal (which was green) characteristic of DNA following Acridine Orange staining.

seen. This result is not surprising given the very strong response to DNA that can swamp the RNA signal resulting in a dull even black fluorescence (Sigma Products information sheet, product number A6014).

The nanobes that were reacted with Feulgen stain yielded a characteristic red fluorescence under green excitation, which is a positive and specific result that the nanobes contain DNA.

given the environment of growth and absence of reactive ingredients. In addition and perhaps the strongest argument for nanobes being biological life forms is their positive reaction to DAPI. Because DAPI is a DNA specific probe that forms a fluorescent complex by attaching in the minor groove of A-T rich sequences of DNA, any non-living polymer or other nonbiological material would need to contain DNA to fluoresce following DAPI staining.

We do not present conclusive evidence for reproduction and metabolism, nor have we established the phylogeny of these organisms to date, but our evidence strongly suggests the existence of nanobes as biological organisms. Our samples were retrieved from 3–5 km below the sea bed where the temperature and pressure is approximately 115–170 °C (Baker and Uwins 1997) and 2000 atm, respectively, and the nanobes grew under aerobic conditions at 22 °C and 1 atm pressure. We have contemplated the chance of contamination of pore space within the rock samples by drilling fluid or other substances and regard it as highly unlikely due to the low permeability and porosity of the rocks. It appears that the nanobes were not actively growing in the rock from which they were retrieved, but thrive when exposed to aerobic conditions.

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