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NANOBACTERIA: METHODS FOR GROWTH AND IDENTIFICATION OF THIS RECENTLY DISCOVERED CALCIFEROUS AGENT

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"*Nanobacterium sanguineum*"[1] might be viewed as the "poster microbe" for the convergence of three areas of microbiology: microbes in chronic diseases, "filter-passing" bacteria, and microbes in geology and astrobiology (Boyce 1999; Lorber 1999; Wainwright 1999). Nanobacteria are yet to be completely characterized, but the information available indicates they have novel properties of relevance to these areas of microbiology (Table 1). Unlike any currently known blood-borne microbe, nanobacteria produce a calcium apatite coat at physiologic levels of calcium and phosphate (Ciftcioglu, Bjorklund, and Kajander 1998; Kajander, Bjorklund, and Ciftcioglu 1998; Kajander and Ciftcioglu 1998). Nanobacteria are present in nearly all human kidney stones (Ciftcioglu et al. 1999a) and are reported to induce calculi in rodent kidneys

[1]"*Nanobacterium sanguineum*" is the type culture designate for nanobacteria. It has been deposited in the German Collection of Microogranisms (DSM No. 5819; Braunschweig, Germany) and is described in U.S. patent No. 5,135,851,1992. For a microbe to be accepted into microbiology nomenclature, it must be described in the *International Journal of Systematic Bacteriology* (IJSB) or be accepted onto their Validation List. Microbes not yet submitted to and approved by IJSB are cited in quotation marks.

Table 1. General Characteristics and Behavior of Nanobacteria

- Nanobacteria are gram-negative, sterile-filterable bacteria with varying amounts of a carbonate apatite coat.
- The size of an individual nanobacterium ranges from 80 to 500 nm.
- By light and electron microscopy, apatite "igloos" have a central chamber occupied by one or more nanobacteria.
- Under low-nutrient conditions (e.g., serum-free), nanobacteria tend to form microscopic colonies in liquid media surrounded by a thick coat of calcium apatite; calcified colonies can approach 1 mm in size.
- Nanobacteria show budding and fragmentation, social behavior, and communities, including biofilms.
- Unique characteristics are consistent with that of extremophiles.
- Serum forms have a generation time of 3 days.
- Serum-free forms double every 6 days or more in RPMI-1640 or Dulbecco's Modified Eagles Medium.
- In artificial urine, serum-free forms double every 1.5–2 days (Burton and Lappin-Scott 2000).
- They can be passaged indefinitely in serum.
- Their metabolism is 10 000 times slower than *Escherichia*.
- They incorporate uridine and methionine acids into DNA and protein, respectively.
- They grow best under aerobic conditions: 5% CO_2:95% air.
- Inhibitors of nucleic acid synthesis; 5-fluoruracil and cytosine arabinoside, inhibit nanobacterial growth.
- Tetracycline, an apatite-binding protein synthesis inhibitor, is the only class of those antibiotics tested to date that inhibits nanobacterial growth at therapeutically achievable blood levels (0.3 μg/mL); at suprapharmacologic levels, gentamycin will also inhibit growth.
- Calcium chelators, such as EGTA and citrate, inhibit growth in vitro.
- Nanobacteria biomass contains novel proteins and "tough" polysaccharides.
- Over 30 proteins have been found by sodium dodecylsulfate–polyacrylamide gel electrophoresis.
- One of these proteins is a bacterial porin protein.
- Muramic acid, a major component of bacterial peptidoglycans, was identified.
- Monoclonal antibodies to the nanobacterial porin protein and peptidoglycan recognize intact nanobacteria as shown by immunogold labeling.
- Demineralization of nanobacteria enhances their endotoxin positivity in the Limulus amebocyte lysate assay.
- Monoclonal antibodies to *Chlamydial* lipopolysaccharide (i.e., endotoxin) react with nanobacteria.
- Hoechst DNA fluorochrome stains nanobacteria.
- The 16S rDNA of nanobacteria places it in the alpha-2 subgroup of proteobacteria.

Table 1 continued on the next page

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- Enzymes alone (i.e., lysozyme, proteinase K) are ineffective in extraction of nucleic acids from nanobacteria; demineralization with ethylenediaminetetraacetic acid or hydrochloric acid is required.
- DNA (nucleic acid fraction) from nanobacteria does not stain with ethidium bromide, is resistant to formic acid hydrolysis, has an inhibitory effect in polymerase chain reaction assays, masks staining of normal DNA, and exhibits an absorbance maximum of 270 nm.
- Some, but not all, isolates of nanobacteria show cytotoxicity to mammalian cells in vitro.
- Nanobacteria can bind to mammalian cells in vitro and be internalized by endocytosis.
- In human and animal tissues, transmission electron microscopy has revealed intracellular putative nanobacteria.
- When injected into rabbits, nanobacteria localize to the kidney, where they cause apoptosis and sloughing of renal tubule epithelium.

(Garcia Cuerpo et al. 2000). The finding of cytotoxic nanobacteria in kidney and liver cysts, blood, and urine from patients with human polycystic kidney disease (PKD) has raised the possibility that nanobacteria may contribute to cyst formation and the other tissue lesions of PKD (Akerman et al. 1997; Hjelle et al. 2000a, 2000b; Miller-Hjelle et al. 1997). The slow growth rate, protective calcium coat, cytoxicity to mammalian cells, and mineral composition similar to known pathologic calcifications are consistent with a role for nanobacteria in chronic diseases (Carson 1998; Ciftcioglu and Kajander 1998; Ciftcioglu et al. 1999b).

"Filter-passing" microbes have been known but have been largely unappreciated in medicine and ecology for over a century (Wainwright 1999). Molecular techniques (i.e., deoxyribonucleic acid [DNA] and immunology-based methods) have established the existence of difficult-to-culture organisms and implied the existence of yet-to-be cultured or isolated microbes (Cassell 1998; Fredericks and Relman 1996). Many bacteria show diverse biochemistries depending on their environment (e.g., biofilms). Finally, astrobiology undertakes to detect "life-forms" that survive under the extreme conditions encountered on planets and space objects. Such "extremophiles" must be able to withstand radiation, low nutrient levels, and wide fluctuations in temperature. In their calcified forms, nanobacteria show characteristics of an extremophile (Bjorklund, Cifteioglu, and Kajander 1998; Ciftcioglu and Kajander 1999). The relationship between nanobacteria in medicine and those described in earth geology as nannobacteria (Folk 1993) and nanobes (Uwins, Webb, and Taylor 1998) is unknown. Interestingly, Burton and Lappin-Scott (2000) cultured nanobacteria from marine, pond, and potable water using both published

techniques (Kajander et al. 1997) developed for biological fluids and previously unpublished methods.

NANOBACTERIA NOMENCLATURE

Folk (1997) credits Richard Morita (1988) with coining the term *nanobacteria.* In the context of small (200 nm), autonomously replicating particles, Kajander used this same term to describe calciferous material cultured from calf sera, and more recently in kidney stones and other biological fluids. Although nanobacteria may ultimately be proved to be novel bacteria or infective agents and contribute to tissue calcification (Carson 1998), caution must be used in attributing all biological calcifications to nanobacteria. Other sources of nonskeletal, small, calciferous particles may include fragments of classical bacteria and fungi seen under metastable in vitro conditions (Streckfuss, Vogel, and Brown 1981; Ennever and Summers 1975; Ennever and Creamer 1967) and membranes derived from mammalian cells (Anderson 1988); inorganic polyphosphate from microbial and mammalian sources may influence the formation of concretions (Ennever and Creamer 1967; Kornberg and Fraley 2000). The more inclusive term *nanoforms* (H. Vali, McGill University) describes nanobacteria-sized biomineralizations of unknown biological origin (i.e., living and nonliving entities, including proteins). Here the operational term *nanobacteria* is used to mean a small, calciferous agent of unknown origin found in biological materials that is able to yield increasing particle numbers in vitro, where the context of nanobacteria research is infectious disease and related pathology.

LABORATORY METHODS FOR NANOBACTERIA

In the absence of standardized methods for the growth and identification of nanobacteria, the authors describe current research findings that have been found to yield nanobacteria. Nanobacteria or nanoforms (a biomineralization-based definition) from cattle and humans have been cultured by the authors' groups, as Dr. H. Vali (in press), McGill University, has done (Barr 1999). Dr. Stephen Barr (1999), Cornell University, has grown nanobacteria from nearly all (>98% of 217) cattle sera tested; sera from cats, goats, and dogs were also culture positive. Drs. Burton and Lappin-Scott (2000) also cultured nanobacteria from oral swabs, horse serum, and fetal bovine serum, as well as nanobacteria isolates provided by Drs. Kajander and Ciftcioglu.

Nanobacteria Culture Methods

Because nanobacteria pass through 0.22 μm pore size filters, which exclude most common microbes, filtration is often used to clean up fluid specimens before culture for nanobacteria (Kajander et al. 1997). Organisms that pass 0.2 μm

filters are of serious concern to the biopharmaceutical industry, which relies on filtration to sterilize its products. Table 2 gives culture conditions found to yield propagation of nanobacteria. Replication can be measured by particle counting and optical density at 650 nm. In the absence of serum or growth factors, propagation can take several weeks to months, depending on inoculum size.

Because the amount of calcium apatite per nanobacterium can vary substantially, establishing a standardized inoculum based on nanobacteria pellet volume or weight is problematic when applied across serum and serum-free growth conditions (Ciftcioglu, Peltarri, and Kajander 1997; Ciftcioglu et al. 1999b; Kajander, Bjorklund, and Ciftcioglu 1998). For a standardized inoculum within a single growth condition, the authors use nanobacteria harvested by centrifugation (14 000 × g for 45 minutes), washed in phosphate-buffered saline (PBS), and resuspended in media or PBS with density adjusted to 0.5 McFarland standard unit (McF. = 1.5×10^8 colony forming units [CFUs]).

Bovine serum is a common source of nanobacteria (Ciftcioglu and Kajander 1998; Kajander et al. 1997). Attempts to kill the nanobacteria or nanoforms in bovine serum using high gamma radiation have yielded mixed results. Although 10% high gamma-irradiated (30 kGy) serum in Dulbecco's Modified Eagles Medium (DMEM) or RPMI-1640 does not yield growth for up to 4 weeks of incubation, longer times have yielded replicative growth in some batches of gamma-irradiated serum. Results from incubations longer than 4 weeks are sometimes difficult to interpret because protein precipitation may take place. Subculturing in a ratio of 1 part culture to 9 parts new medium (1/10 dilution) is recommended if longer incubation periods are desired. Nanobacteria have been cultured in serum-free cell culture media, DMEM, and RPMI-1640. Their culturability has been shown by monthly subculture in fresh medium for over 6 years without any major changes in their general properties, as observed initially under serum-free conditions (i.e., they calcify and grow as bottom attached biofilm) (Kajander and Ciftcioglu 1999) (Figure 1).

In the authors' experience, some patient sera relatively quickly yield protein precipitates during culture that are difficult to differentiate by light microscopy from nanobacteria. The authors provide a fast method for the quality control of cultured nanobacteria. At room temperature, a 5 μL aliquot of culture material is placed on a carbon-coated copper electron microscopy grid and allowed to stand for 5 minutes. The grid is then washed three times (2 seconds per wash) with pyrogen-free, polymerase chain reaction–grade water. The washed grid is dried with the use of Whatman paper and then viewed directly by transmission electron microscopy (TEM) without any staining. Because of the electron-dense nature of their apatite coat, nanobacteria/nanoforms are visualized, whereas protein precipitates do not appear. Visualization by light microscopy but not TEM suggests the presence of serum precipitates. The dried grid can also be stained for 2 seconds with 2% uranyl acetate to reveal the presence of both nanobacteria and protein (Figure 2).

Table 2. Effect of Culture Media on Nanobacteria Growth and Calcification When Incubated at 37 °C under a Humidified 5% CO_2/95% Air Atmosphere

Culture Media	Replication	Size of Particle[a]	Apatite and Structure
Dulbecco's Modified Eagles Medium (DMEM)	YES	1 μ to 1 mm	3+ (highly mineralized; sandlike in form)
RPMI-1640[b]	YES	1 μ to 1 mm	3+ (sandlike in form)
50% DMEM[b]/50% urine	YES	1 μ to 1 mm	3+ (sandlike in form)
100% urine	Marginal		
Artificial urine	YES	600–1000 nm	2+ (moderately mineralized)
10% to 50% bovine serum in DMEM or RPMI-1640	YES	200–400 nm	2+
100% bovine or human serum	YES	200–400 nm	1+ (modestly mineralized)
Modified Loeffler medium	YES	1 μ to 1 mm	3+ (tumorlike in form)
DMEM with *Bacillus*-derived growth factors	YES		
10% human kidney cyst fluid/95% RPMI-1640	YES	200–400 nm	2+

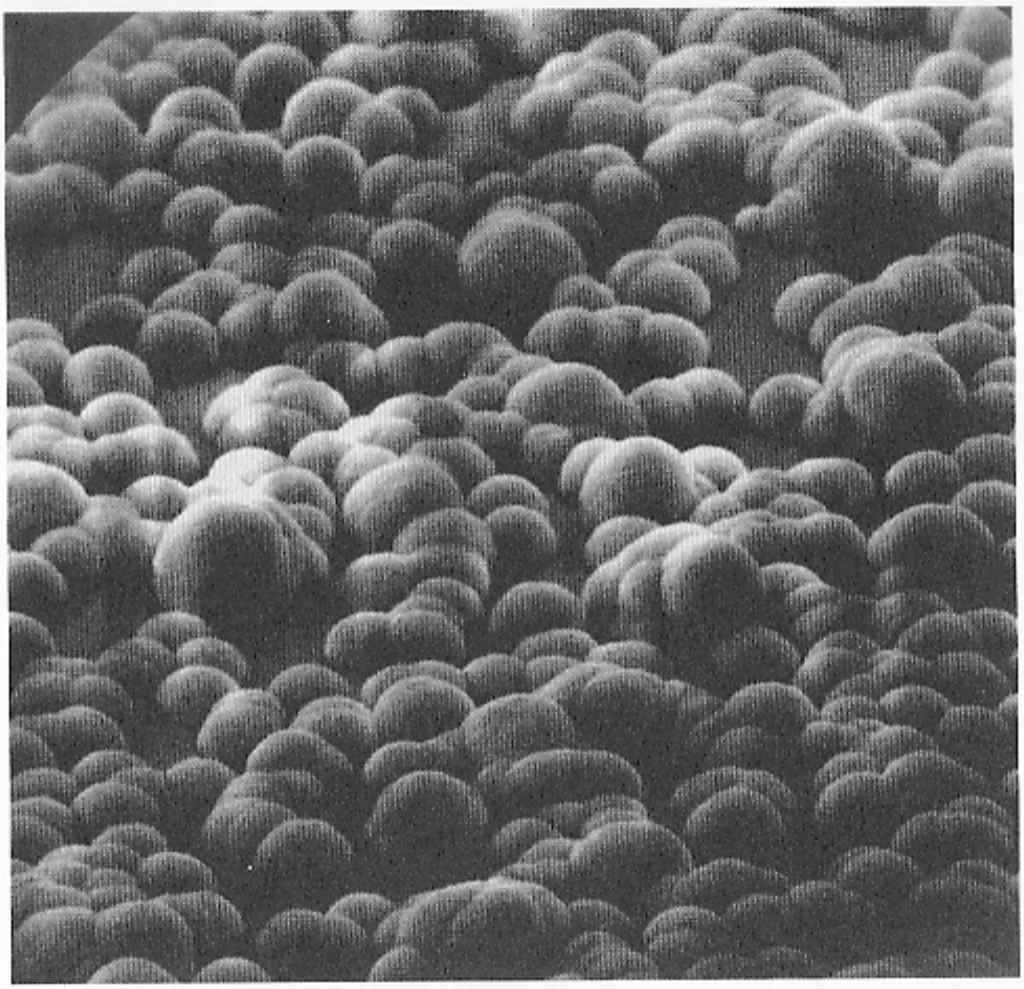
Note: Yes indicates replicative growth. 1+ to 3+ indicates increasing degree of calcium apatite formation on the surface of the nanobacterium or the nanobacterial colony.

[a]Particles can be individual nanobacteria (80–500 nm); nanobacterial colonies within a shared calcified "igloo" can approach 1 mm in diameter.

[b]DMEM and RPMI-1640 were supplemented with 1–2 mM glutamine. Only high gamma-irradiated bovine serum was used (Kajander and Ciftciogu 1998). Artificial urine (Brooks and Keevil 1997) was as utilized by Burton and Lappin-Scott (2000). Modified Loeffler medium was prepared by DMEM replacement of water in the formula and supplementation with 10% conditioned medium from a 1–3 month nanobacteria culture. Supernate from centrifugation of an as yet incompletely characterized *Bacillus* culture at 100,000 × g for 1 h was the source of *Bacillus*-derived nanobacteria growth fraction (Kajander and Ciftcioglu 1999).

Nanobacteria can be major contaminants in mammalian cell culture and are fully resistant to penicillin and streptomycin at concentrations applied to tissue culture (Kajander et al. 1997; Ciftcioglu et al. 1999b; Burton and Lappin-Scott 2000). The most effective antibiotics against nanobacteria in vitro are tetracyclines; aminoglycoside antibiotics were only active at high concentrations that were not clinically achievable in serum. Nothing is known of the effectiveness of any antibiotic in treating nanobacterial infection in humans.

Figure 1. Scanning Electron Micrograph of Bovine-Derived Nanobacteria Cultured for 3 Months in Dulbecco's Modified Eagles Medium with Monthly Refeeding

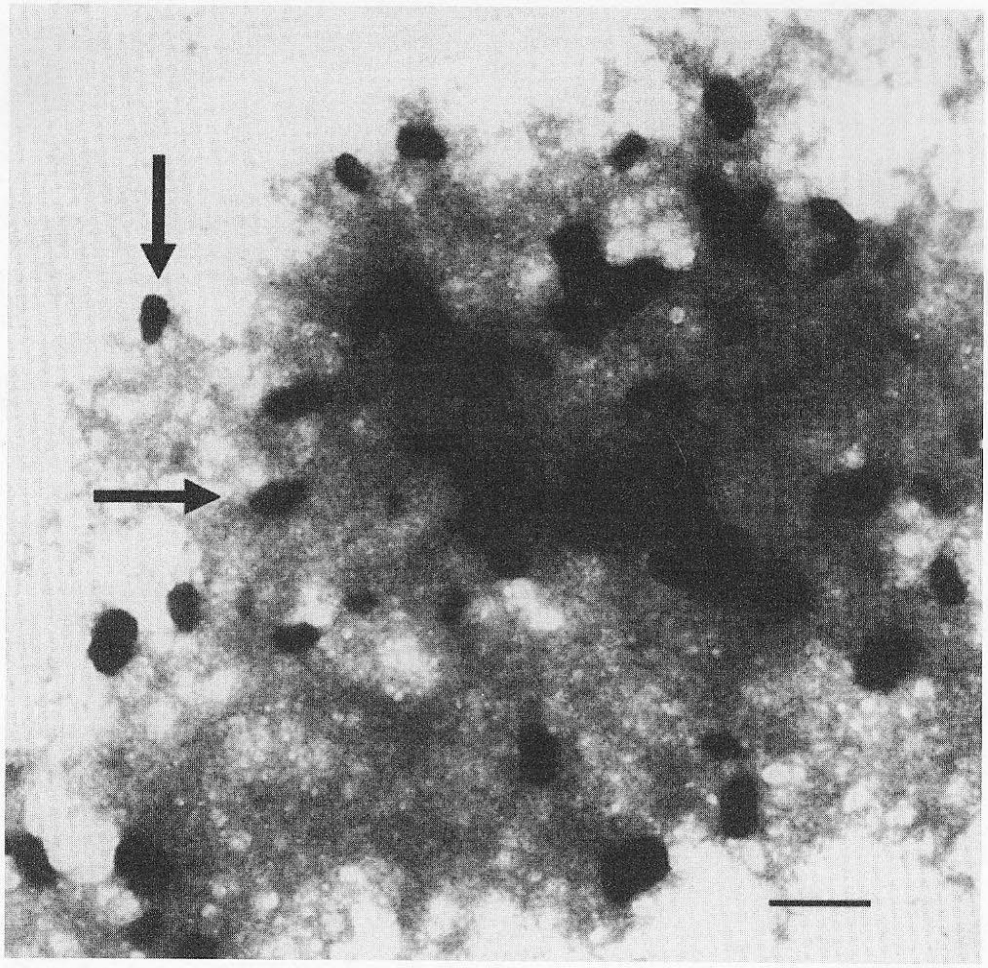


Note the similarity of size and apparent multiplication by budding. Bar = 10 μm.

HANDLING AND DECONTAMINATION PROCEDURES FOR NANOBACTERIA

Because human and some animal isolates of nanobacteria show cytotoxicity to mammalian cells in vitro (Ciftcioglu and Kajander 1998) and nanobacteria are present in human diseases (Ciftgioglu et al. 1999a; Hjelle et al. 2000b), caution must be used in the handling and disposal of nanobacteria. Unfortunately, nanobacteria are resistant to many commonly used disinfectant

Figure 2. Monitoring of Nanobacterial Growth in Vitro by Transmission Electron Microscopy



Nanobacteria were cultured for 4 weeks in Dulbecco's Modified Eagles Medium fortified with 10% high gamma-irradiated fetal bovine serum. A 5 μL aliquot from the culture vessel was placed directly on a carbon-coated copper grid, then negatively stained for transmission electron microscopy, as described in the text. Nanobacteria (*arrows*) appear as black particles, even in the absence of uranyl acetate; protein precipitates only appear when stained with uranyl acetate, as shown in this micrograph. Bar = 500 nm.

procedures (e.g., 70% ethanol, 2% glutaraldehyde,4% formaldehyde, 0.5% hypochlorite, 3% hydrogen peroxide, 1 M hydrochloric acid, 1 M sodium hydroxide, 1% sodium dodecylsulfate, 1% Tween 80 or Triton X-100, 3 M urea, autoclaving, ultraviolet irradiation overnight, microwaves, drying at room temperature) (Bjorklund, Ciftcioglu, and Kajander 1998). Only 1% Virkon

(which in concentrated form contains 50% potassium persulfate, 5% sulfaminoic acid) (Antec International Ltd., Suffolk, United Kingdom) killed both serum and serum-free forms of nanobacteria. Nanobacteria grown in serum could also be killed by dry heating at 100°C for 1 h.

For handling of nanobacteria, the authors use disposable plastic tools and absorbant lab mats in a microbiological safety hood. Standard barrier techniques (i.e., gloves, coats, and eyewear) are used throughout the handling procedures. All plasticware and disposable materials are incinerated by a commercial service. Aerosol-contained centrifuges are used. Waste cultures are sent directly for incineration.

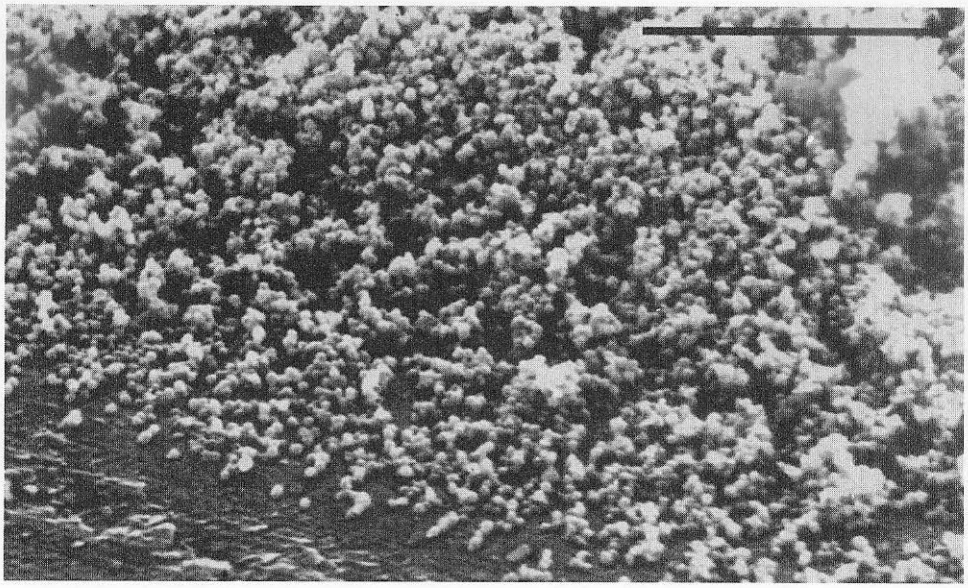
IDENTIFICATION OF NANOBACTERIA

Nanobacteria change their structure depending on the nutritional and microbial environment and can adopt characteristics of a biofilm (Figures 1 and 3) (Ciftcioglu, Peltarri, and Kajander 1997; Kajander and Ciftcioglu 1999; Burton and Lappin-Scott 2000). Light and electron micrographs of nanobacteria under diverse conditions are available (Ciftcioglu et al. 1999a; Kajander and Ciftcioglu 1998; Hjelle et al. 2000b). When cultured in 10% serum, nanobacteria show less calcification than when grown under serum-free conditions, where they form colonies protected by a remodelable calcium apatite layer. Much is yet to be learned of the process by which nanobacteria and its fragments mediate biomineralization.

In liquid culture, nanobacteria can be viewed by phase-contrast microscopy using an inverted microscope (Kajander and Ciftcioglu 1998; Hjelle et al. 2000b). During the first weeks in culture, nanobacteria exhibit Brownian-like movement, but as the culture ages nanobacteria attach to the plastic surface and begin the formation of "igloo-like" calcium shelters having a central chamber occupied by multiple nanobacteria: a calcified nanobacterial colony (Kajander and Ciftcioglu 1998) (Figure 4). Growth of the colony into multichambered structures appears by scanning electron microscopy (SEM) to occur by budding (Figure 1). Such calcified nanobacteria can be visualized with von Kossa stain and Jones' methenamine silver stain (Kajander and Ciftcioglu 1998). The presence of putative amyloid material stainable with Congo Red is apparent in 4- to 6-week-old cultures (Kajander, Liesi, and Ciftcioglu 1993, 41). Visual inspection of the culture reveals a white granular layer of material at the bottom of the flask.

For one to visualize nanobacteria on glass slides, the nanobacteria must first be fixed to the slide by heating at 70°C for 15 minutes (Kajander et al. 1997). In the absence of firm attachment, nanobacteria will be removed during the washing steps of most staining procedures.

Figure 3. Scanning Electron Micrograph of Nanobacteria Biofilm after 13 Days of Growth in Artificial Urine

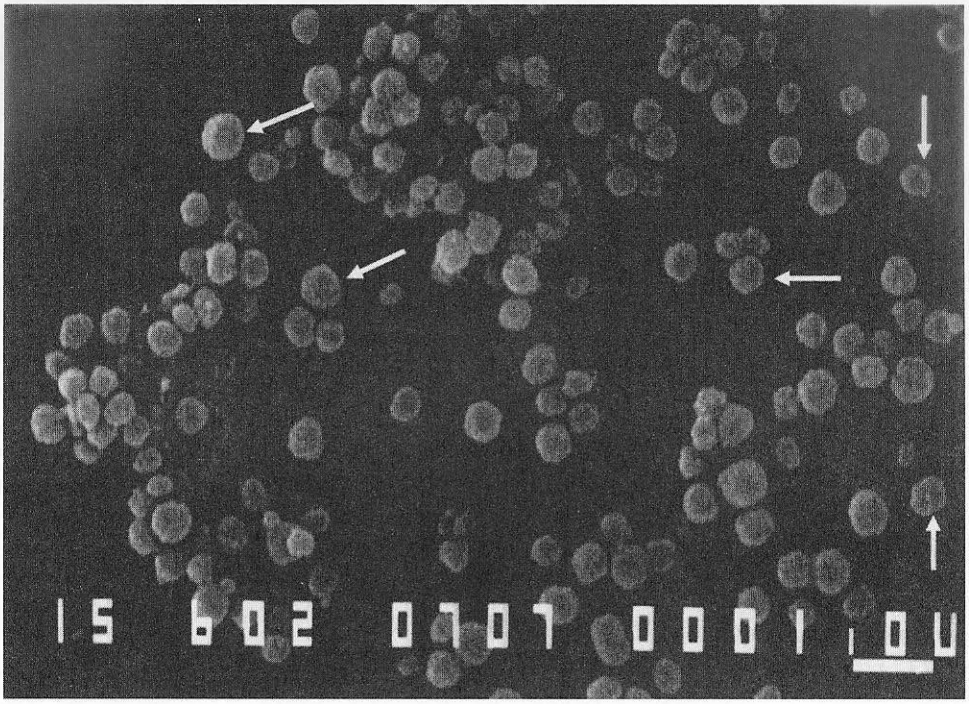


Note the uniformity of particle size. Bar = 20 μm. (Courtesy of Drs. Sara Burton and Hilary Lappin-Scott, Exeter University, United Kingdom.)

Kajander et al. (1997) and Kajander and Ciftcioglu (1998b) reported that nanobacteria can be visualized by the use of a commercially available, modified Hoechst fluorochrome 33258 DNA-staining procedure (Hoechst Stain Kit, Flow Laboratories, Ayrshire, Scotland). At 0.5 μg fluorochrome/mL for 5 minutes, common bacteria are stained; at 5.0 μg/mL for 30–45 minutes, nanobacteria also become stained and visible by fluorescence microscopy. Staining of parallel samples at low and high dye concentrations will differentiate nanobacteria from common microbes present in the sample. When one is staining nanobacteria associated with mammalian cells, the mitochondria will also be stained by the higher concentration of dye. Immunodetection, as described below, and TEM can accomplish identification of nanobacteria within cells.

Using a monoclonal antibody specific for a nanobacterial porin protein epitope, indirect immunofluorescence can be used to visualize nanobacteria (Kajander et al. 1997) and probe cultures or biological specimens for nanobacterial antigen (Ciftcioglu and Kajander 1998; Hjelle et al. 2000b). An enzyme-linked immunosorbent assay kit is available (NANOBAC OY, Neulaniementie 2 L14, Bioteknia Science Park, FIN 70210 Kuopio, Finland; e-mail:

Figure 4. Scanning Electron Micrograph of Nanobacteria Harvested after a 6-Week Culture in Dulbecco's Modified Eagles Medium and 10% High Gamma-Irradiated Fetal Bovine Serum



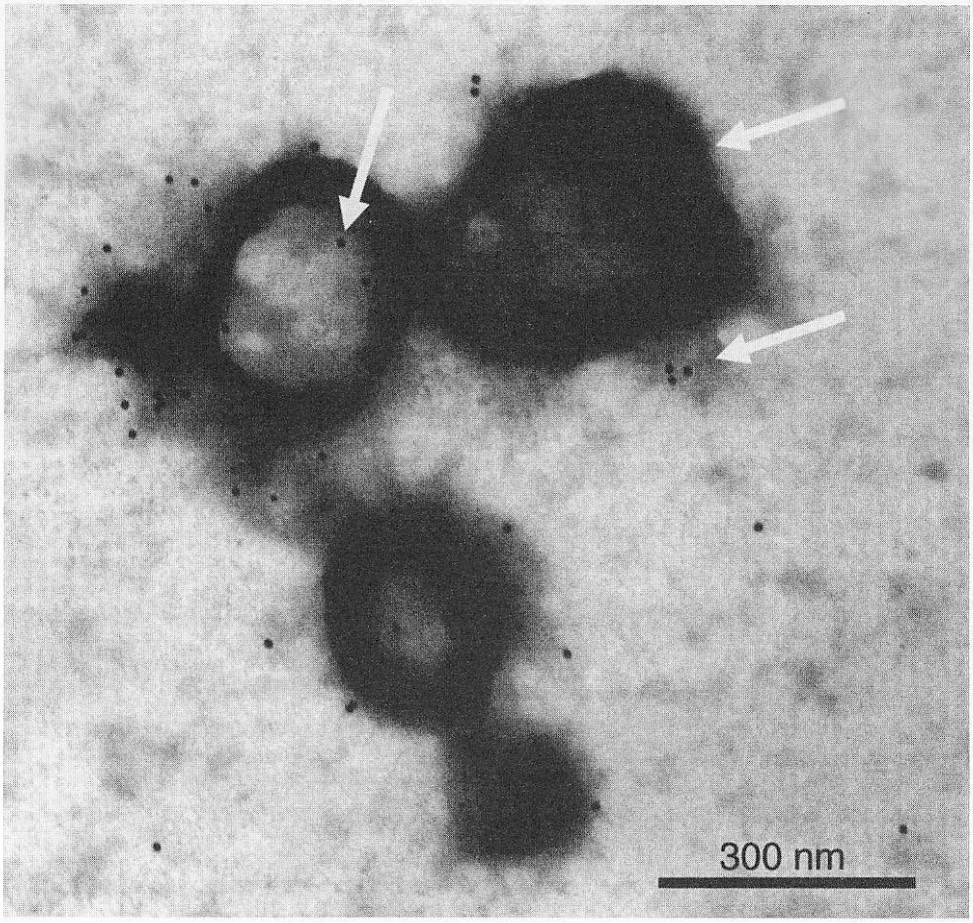
Scraping of the nanobacteria exposes the chambers, as noted by *arrows*, of these so-called nano-igloos. Bar = 1 μm.

nanobac@nanobac.com). Figure 5 shows the use of antinanobacterial porin protein with immunogold detection in cultured nanobacteria.

Nanobacteria are positive for bacterial endotoxin in the Limulus amebocyte lysate assay (Hjelle et al. 2000b), the classical test for endotoxin in biological and pharmaceutical fluids. Note that antibodies that recognize lipopolysaccharide (LPS; endotoxin) from *Chlamydia* sp. and hyperimmune sera (mice) to *Bartonella henselae* were found to react with nanobacteria cultured from human kidney and *Nanobacterium sanguineum* (Hjelle et al. 2000b). The limited data available suggest that nanobacteria are related by their 16S ribosomal DNA (rDNA) sequence to *Bartonella* and *Brucella* in the alpha-2 group of proteobacteria.

Kajander and Ciftcioglu (1999) have reported 16S rDNA sequences for two separate isolates of nanobacteria (GENBANK accession No. X98418 and 98419); in unpublished findings, Dr. S. Barr (1999) has independently con-

Figure 5. Immunogold Labeling



By transmission electron microscopy, immunogold labeling (Nb8/0 primary antibody) of nanobacteria in culture for 2 weeks in Dulbecco's Modified Eagles Medium fortified with 10% high gamma-irradiated fetal bovine serum. Gold particles (*arrows*) linked to secondary antibody appear as uniform black dots over the nanobacteria.

firmed a very similar sequence in nanobacteria cultured from a bovine serum sample obtained in the United States. However, much is yet to be learned of the nanobacterial DNA extraction methods from routine cultures and biological specimens. At this time, use of solely DNA methods to detect nanobacteria is not recommended.

FUTURE RESEARCH

Nanobacteria are far from being characterized. Are viable nanobacteria or fragments of nanobacteria initiators or contributors—or both—to the calcium apatite depositions observed in many human diseases (Carson 1998)? Because isolates of nanobacteria range from benign to highly cytotoxic, are there virulent strains of nanobacteria? Are such strains specific to human diseases or tissues? What are the sources of nanobacteria, and how are nanobacteria transmitted into and through humans? What is the nature of nanobacterial biology and how is it related, if at all, to geologic and astrobiological findings or concepts of nannobacteria/nanobes? The methods to study nanobacteria/nanoforms will continue to develop, as will concepts of microbes and their parts as provocateurs of chronic diseases in animals and humans.

ADDENDUM

The initial finding of nanobacterial antigens in human brain tissue (Miller-Hjelle et al. 2000) has been followed by a report that unidentified small particles were found in cerebral spinal fluid (CSF) of schizophrenia patients but not normal volunteers (Wetterberg et al. 2002). The first complete report of nanobacteria susceptibility in vitro to diverse drugs has appeared (Ciftcioglu et al. 2002). A review of potential nanobacterial mechanisms of pathogenecity was recently published (Kajander et al. 2001), as was a finding of nanobacterial antigens in cattle (Breitschwerdt et al. 2001). Finally, in a gracious recognition of the early work of Laszlo Puskas, L. Tiszlavicz, L. Torday, and J. Papp of the University of Szeged, Hungrey, Rasmussen et al. (2002) confirmed the finding of nanobacteria in human athersclerotic vessels but not normal vessels.

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