INVESTIGATION OF BACTERIAL FRAGMENTATION AS A POSSIBLE ORIGIN OF NANOBACTERIA

THESIS

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INTRODUCTION

Nanobacteria (also spelled nannobacteria[1,2]) are thought by some to be independent free-living organisms that range in size from 0.03 μm to 0.2 μm in diameter,[3] about one-tenth the diameter of normal bacteria (diameter 1.0 μm). In this thesis, I will call structures falling in the size range of 0.03 μm to 0.2 μm nanobacteria-like structures (NLS). These structures appear to be geologically abundant, present in soils, sediments and sedimentary minerals and rocks.[4] They have also been reported in blood and serum.[5]

Several hypothesis have been advanced to explain NLS. These include: stressed forms of normal bacteria (for example, normal bacteria that are reduced in size under starvation conditions), bacterial fragments, imaging artifacts or free-living-life forms.

History of NLS

Environment

While studying hot spring calcium carbonates in 1990, Folk, [1,2] discovered tiny ovoid shaped objects in calcite and aragonite, ranging in size from 0.025 μm – 0.2 μm. The tiny objects resembled normal bacteria in shape and arrangement. Based on his initial observations he proposed that the objects were "dwarf" bacteria, and named them nanobacteria.[1,2] Folk found NLS in many environments including water (tap, river, hot springs), clays and carbonate sedimentary rocks. Carbonate rocks include travertine, limestone, marine hardgrounds, ooids, ancient marine cement, micrite (1-5 μm size

crystals that are texturally analogous to siliceous muds), early dolomite and microspar.[6] Carbonate minerals include aragonite, calcite, dolomite and vaterite.

NLS have been observed by Folk in calcium carbonate samples that were formed by microorganisms. Travertine, a type of calcium carbonate, is formed by the precipitation of $CaCO_3$ from carbonate saturated waters[7,8] and is associated with plants, cyanophytes, and bacteria, particularly cyanobacteria.[7] Both cyanobacteria (*Lyngbya*[9] and *Synechococcus,*[10] for example) and non-photosynthetic bacteria such as sulfur-reducing bacteria[2] are involved in travertine deposition. The microbes become incorporated into the travertine and appear to act as templates for carbonate deposition.[10] Calcium carbonate deposition in the presence of *Synechococcus* typically corresponds with small increases in pH that are due to photosynthetic metabolism.[10] The photosynthetic metabolism of *Synechococcus* is directly responsible for the alkalinization of the microenvironment due to its ability to use HCO_3^- as its primary source of inorganic carbon.[11] As a result, this increases $CO_3^=$ levels, leading to an elevation of pH. Microorganisms can bind calcium to their cell surface,[12] and it is also possible that the calcium ions on the cell surface may act as nucleation sites for precipitation of calcium carbonate.[10] In travertine, the bacteria are found encased in calcite crystals that form layers. The layers are cemented together by inorganic sparry calcite.[13] Calcium carbonate can be deposited as either calcite or aragonite, depending on the temperature and chemical composition of the water.[14,15] Typically calcite is formed at cooler temperatures and aragonite is formed above 45-50°C.

NLS have been observed in at least two (aragonite and calcite) of the three mineral phases of calcium carbonate. Aragonite and vaterite are polymorphs with calcite,

meaning they all have the same chemical composition, but different crystal structures, symmetries and chemical properties. Aragonite is orthorhombic, vaterite is hexagonal, and calcite is trigonal. Calcite, the most stable mineral at most surface temperatures and pressures, is the most common of the three followed by aragonite. Vaterite on the other hand is rare. Over time aragonite is dissolved and replaced by calcite.[16]

NLS were found in aragonite at the Bullicame and Bagnaccio Springs in Viterbo, Italy. Aragonite is the main mineral phase precipitated because of the high temperature (50-52°C) of the springs, as well as the high calcium, carbonate and hydrogen sulfide content. Samples of the Viterbo travertines were observed using scanning electron microscopy (SEM), and normal-sized bacteria on the surfaces of crystals were seen at higher magnifications. Smooth spheres and some "jelly-bean" shaped structures (0.1 μm to 0.5 μm in size) were also observed. The tiny structures appeared to have the same distribution as normal bacteria, found in isolated clusters that resemble bacterial microcolonies.[2] Normal-sized bacteria, as well as the small structures (NLS), were found embedded within carbonate crystals. NLS were found in the centers of the aragonite spherulites, suggesting that the NLS may have nucleated the precipitation of the crystal. It was speculated that after the NLS catalyzed precipitation, inorganic precipitation formed the rest of the spherulite.[2]

Aragonite crystals from the Le Zitelle Hot Springs in Italy, when observed with the SEM, appeared to have NLS sitting on top of each aragonite "stalk". Each "stalk" diameter was the exact match of the diameter of the NLS, implying that the NLS may have precipitated the crystal. Fibers precipitated abiotically were "hexagonal columns with planar pinacoidal terminations" and NLS were not found on them.[17]

NLS have also been found on diagenetic clays. Clay flakes from Frio sandstone samples collected from South Texas, when observed with the SEM, appeared to have small bead-like structures (NLS) on their rims. The NLS appeared to be the same thickness as the flakes. Fibrous clay from Texas caliche (a form of diagenetic calcium carbonate widely distributed in desert soils) had the spherical structures on their tips and the filaments were the same diameter as the NLS that capped them. The bead-like structures resembled the structures found in calcium carbonate, suggesting that clays may also be precipitated by NLS.[17]

Dunn *et al.* found NLS as well as normal bacteria on sycamore leaves that were placed in the San Marcos River, Texas, for six days. Precipitated silica was found to be in the form of distinct clusters of 0.05 μm balls[18] after leaves with the nanobacteria-like-structures were placed in a silica solution following the period of time in the river. Leaves have hydrophobic surfaces and do not easily bind metal ions. In water (river), bacterial biofilms (surface-adherent communities of bacteria)[19] form on leaves. Electrostatic interactions between metal ions such as Fe (III), and the anionic biofilm cause the formation of the mineral ferric-hydrite which slows the decomposition of the leaf. The rapid mineral formation probably allows for the preservation (fossilization) of leaves found in riverbeds.[20] It is possible that the NLS found on the leaves caused the precipitation of the silica in a similar manner.

Martian Meteorite

Nanobacteria-like structures were observed on one of the Shergotty-Nakhla-Chassigny class of meteorites (ALH84001) from Mars, which was collected from

Antarctica. The meteorite, a crystalline orthopyroxenite, was made up of coarse-grained orthopyroxene and minor maskelynite, olivine, chromite, pyrite, and apatite. It crystallized about 4.5 billion years ago.[21-23] It contains globules 1 μm to 250 μm across, made up of secondary carbonate minerals.[22-25] The carbonate globules are found along fractures and pores spaces. Some of the fractures and pore spaces were shock-faulted,[21,22] an event that occurred either on Mars or in space 3.6 billion years ago.[24] Magnetite particles found in ALH84001 are similar to terrestrial magnetite particles known as magnetofossils,[26] which are fossilized bacterial magnetosomes.[27] Globules had iron-rich rims that typically consisted of aggregates of minute ovoids interspersed with small irregular, angular objects. The ovoids were about 100 nm in longest dimension and the irregular objects ranged from 20 μm to 80 μm across.[28] The surfaces of the center of the carbonate globules were rough in texture. Small, regularly shaped, ovoid and elongated forms 20 nm to 100 nm were found on the surfaces. The ovoid and elongated forms were similar to the NLS found on the surfaces of calcite precipitated from Pleistocene ground water in southern Italy.[29] The forms were thought to be nanobacteria that promoted calcite precipitation.[28]

There are several possibilities for the origin of the nanobacteria-like structures on ALH84001. One explanation is that they are the result of etching along fracture surfaces on earth. This possibility cannot be evaluated properly as no known terrestrial example has been found. A second possibility is that the structures are artifacts that were created while preparing the sample for microscopy or from laboratory contamination in the form of dust and cleaning and glue residues. Several control samples were treated identically to the meteorite chips, and it was concluded that the structures were not the result of

laboratory techniques. Another possibility is that the structures and the magnetite are products of terrestrial microbial activity. However, it is unlikely that the structures were formed in Antarctica by biogenic processes because of the lack of polycyclic aromatic hydrocarbons in other Antarctic meteorites, the sterility of the sample and the nearly unweathered nature of the meteorite.[28] The most intriguing possibility is that the structures may be independent life-forms (nanobacteria). The ovoid structures are similar to the nanobacteria-like-structures found in travertine and limestone.[2]

Serum

At about the same time Folk was making his discoveries in travertine, Kajander and others at the University of Kuopio, Finland, discovered autonomously replicating particles in serum. They made their discovery when they noticed that many of their 3T6 fibroblast cell cultures were dying without any signs of contamination. When observed with the transmission electron microscope (TEM) the dying cells appeared to have tiny vacuolized prokaryotic organisms (nanobacteria). The nanobacteria appeared in cellular vacuoles (endosomes and lysosomes). Most infected cells died within seventy-two hours by apoptosis.[30]

Kajander *et al.* named these autonomously replicating particles *Nanobacterium sanguinum.* [31]. These NLS, isolated from serum, were optically dense particles, a feature distinguishing them from *Mycoplasmas.* The particles had bacteria-like membranes and had thick cell walls surrounded by capsules and slime-like material.[31] Electron microscopy revealed that the novel agent had a thick cell wall and division septa, distinguishing it from an L-form of *Staphylococcus epidermidis.*[31]

Nanobacterium sanguinum are encapsulated, catalase negative, coccoidal microbes, 0.08 μm - 0.5 μm in size. They are covered with hydroxyapatite making them difficult to fix, to stain and resistant to antibiotics used in cell culture and heat. However, high doses of gamma-irradiation or aminoglycoside antibiotics stop replication.[32] They have a replication time about three days[33] in cell culture media, and can be found either on their own or in biofilms.[31,34,35] The source for the *Nanobacterium sanguinum* was found to be commercial fetal bovine serum (FBS).[34] They have been found in FBS, blood and blood products of some horses and from commercial human blood products in Finland.[36] Kajander and Ciftcioglu[34] have found that more than 80% of commercial ôsterileõ fetal and newborn bovine serum batches, each pooled from several thousand animals, have nanobacteria.[37]

16S rRNA genes were extracted from the isolated nanobacteria and sequenced. Kajander reports the phylogenetic position of *Nanobacterium sanguinum* has been found in the alpha-2 subgroup of proteobacteria.[5] The DNA sequence has been published in GenBank (accession numbers X98418 and X98419).

Under cell culture conditions, *N. sanguinum* replicated with and without mammalian cells. They did not grow on all common bacteriological media tested. In long-term culture, they formed biofilms and attached to surfaces. The formation of biofilms distinguished *N. sanguinum* from *Mycoplasmas* and other contaminants that are sometimes present in filter sterilized sera.[33] They are culturable in protein and lipid-free medium for more than three years with monthly passages. The organisms are mobile, coccoid and have diameters of 0.2 μm – 0.3 μm in serum. They can pass through 0.1 μm filters but not 0.05 μm filters.[38] TEM revealed the presence of cell walls, yet they can

pass through small-pore filters. During culture, the size of the nanobacteria increased due to the production of very thick apatite-containing cell envelopes.[34]

Microscopy of the nanobacteria showed a thick cell envelope surrounded by slime-like material. The surfaces of the envelopes were rough, and in old cultures they became extremely thick and had fibrils and hydroxyapatite crystals on them. As cultures aged, the nanobacteria biofilm slowly hardened and became bone-like.[33] The hardened biofilm composition was similar to that of bone, except carbonate apatite was formed rather than hydroxyapatite. Carbonate apatite is found in most extraskeletal tissue calcification and calculi.[32] *N. sanguinum* have a binding affinity for calcium and appear to be able to separate calcium from magnesium. They have specific oligopeptides that allow them to precipitate carbonate apatite under conditions of pH less than 8.5 and magnesium / calcium concentrations greater than 0.1 μm,[39] conditions that are present in the human body.[32]

Kidney Stones

Kidney stone disease is common, affecting 12% of males and 5% of females in the western world.[40] Struvite calculi and carbonate-apatite calculi are almost always associated with urinary tract infections and account for 15% - 20% of all urinary calculi. The bacteria that cause these stones are urease producers such as *Proteus* , *Klebsiella*, *Pseudomonas*, and *Corynebacterium* species, as well as some strains of urease positive *Escherichia coli*.[41] Struvite formation is due to the increase in urine pH by urease.[42] However, in most cases phosphate formations are not due to increased pH in the urine.[43] Most other stones are formed by metabolic disorders.[42]

NLS or *N. sanguinum* may be within the spherical deposits found in the kidneys of patients who suffer from kidney stones. When cultured, *N. sanguinum* first attach to the surface of the culture dish and then form apatite crusts with concave faces.[5,34] SEM images showed that *N. sanguinum* form their crusts by creating several thin mineral layers, in a manner similar to kidney stone formation.[43] Renal papillary stones are small, rounded concretions, with one smooth convex face and one concave face, features which suggest their implantation on the papilla.[44] It is possible that *N. sanguinum* act as nucleation sites for renal papillary stones.

Many types of mammalian cells (for example, fibroblasts) appear to internalize *N. sanguinum.* Once internalized, the *N. sanguinum* have a cytotoxic effect on the cells.[35] It was shown in rabbits that *N. sanguinum* have a tissue specific distribution with major accumulation in kidneys. The presence of live *N. sanguinum* in urine from rabbits is evidence that these organisms can be found when stones are present and allows a possible conclusion that they may be involved in kidney stone formation.[45] Katie Propst (personal communication) found that when mice were injected with nanobacteria (NLS) cultured from newborn calf serum, there was a decrease in particulate calcium in mouse urine.

It is believed that *N. sanguinum* may cause calcification and stone formation *in vivo* because they have been found in human blood.[34] They can be moved from blood to urine as living organisms[45] and nanobacterial antigens have been found in human kidney stones.[32] *N. sanguinum* have been shown to infect fibroblasts and cause calcification (both intra- and extracellular) with concentrically layered appearances similar to Michaelis-Gutmann calcospherules found in malacoplakia.[32]

Alternatives to Free-Living Nanobacteria

Small cells have been found in many environments. Electron microscopy[46] and flow cytometry[47] have shown that many of the bacterial cells found in soil are small, with diameters of less than 0.3 μm and cell volumes of less than 0.1 μm^3.[48] The dwarf cells[49] have intact genomes.[50]

Nanobacteria have been considered to be imaging artifacts, bacterial fragments, ultramicrobacteria and free-living life forms. Some of the tiny bacteria-like structures observed in rock samples may simply be artifacts due to laboratory techniques. It was found that some 0.05 μm - 0.1 μm granulation found on the rocks were artifacts produced when polishing the limestones or travertines.[2] Microgranulation on the polished or sawed rock surfaces, which looks like a head of broccoli, can extend up to ten millimeters into the rock. The granulation is probably due to vibration, which causes the calcium carbonate to part along cleavage planes or grain contacts to allow the dilute hydrochloric acid to enter and etch the stone into artifactual spheroidal forms.[2]

Nanobacteria found in Great Salt Lake stromatolites show that the spherical structures are not artifacts of sawing or polishing because neither method was used on the samples. The spheroids are not artifacts of gold sputter coating, because the bodies are only found in certain areas of the sample. They are only found in the equant microspar and not in the prismatic crystals. "If the spherical bodies are erratic blobs produced by sputter coating, their distribution should be similar in the two cement fabrics."[6]

The nano-sized objects found in calcite may be due to acid etching. Etching samples with hydrochloric, or some other acid (pH 4-5),[51] reveals and "creates" non-

biologic nano-sized objects that are not seen in unetched samples.[2] It can be argued, however, that nanobacteria are not artifacts of etching because they can be found densely packed in some places, whereas in others no nanobacteria can be found. They can also be found on both etched and non-etched aragonite needles, indicating that they are not artifacts of acid etching.[2]

Some bacteria respond to starvation by reducing cell size.[52] Some use their non-essential cell components.[53] They can produce specific starvation proteins,[54] or reduce their endogenous respiration rates.[55] Some bacteria (especially those found in sea water and soil) enter a state known as "viable but non-culturable" (VBNC).[56] These bacteria cannot grow on laboratory media; however, they have respiratory or metabolic responses.[57]

VBNCs are smaller than the cells from which they originate.[58] Small cells (<0.5 μm) or 'dwarfs' (<0.3 μm) are predominant in soils.[59] Many Gram negative bacteria *(E. coli* and *Vibrio cholerae*, for example) are thought to enter a (VBNC) state when starved.[56] The VBNC state has been defined as a cell that is metabolically active, but cannot undergo cellular division and grow on solid media in a laboratory.[60] *Micrococcus luteus*, a Gram positive coccus can survive in a dormant state when starved. The dormant cells cannot form colonies on solid media.[60]

The starvation / survival state is characterized by a drastic lowering of metabolic activity, which allows the organisms to survive for long periods without energy for growth and for reproduction. During the first stages of starvation metabolic activity increases.[61] Increased activity is expected since the cells are increasing in number and fragmenting into small cells. Lipids, poly-ß-hydroxybutryate, and carbohydrates

disappear from the cells early in starvation[62]. In *Vibrio cholerae*, protein, RNA, and DNA decline during a 30 day starvation period,[62] followed by a constant level of protein, a slight increase in DNA and an increase in RNA.[63] Starvation of bacteria causes the formation of ultramicrocells, which are sometimes called ultramicrobacteria,[64] dwarf-, mini-, pico-, oligo-, nano-bacteria[65] and minicells.[66] Minicells usually refers to small cells without nucleiods. Ultramicrocells may be found as vibrio, coccoid, spirilla and coccobacilli shapes[63] (cell length, less than 0.3 μm and volume of about 0.01 μm^3), [64] and usually appear after several cell divisions without growth under starvation conditions. Higher surface-to-volume ratios are created as the bacterium reduces in size, giving it a better ability to obtain necessary nutrients.[64]

L-forms of bacteria (cells without walls) can be isolated from septicemic patients, and can be cultured from serum along with *Mycoplasmas* and many viruses. It is commonly assumed that serum that is free of *Mycoplasmas* is free of osmotically sensitive L-forms.[67] They can pass through sterile filter, and may lack typical bacterial components.[31]

Mycoplasmas and *Ureaplasmas* form atypical colonies when grown under unfavorable conditions. Crystalline deposits can be found when the organism is in unfavorable conditions of pH and gaseous requirements.[68]

Many organisms respond to starvation and adverse environmental conditions by converting to spores, cysts, or fruiting bodies (all are structures much smaller than the vegetative cell). Many cells have the ability to reduce the activity of vegetative cells while remaining motile, without becoming dormant. When in a reduced metabolic state, there is no growth.[66] They are able convert to new resistant forms based on the energy

reserves that they had when the starvation started.[69] Sometimes part of the bacterial population dies. Cell lysis provides resources for the surviving cells to mature into resistant forms. Resources that are freed when the cells lyse include storage materials, such as polyhydroxybutryate, glycogen, or polyphosphate. When under starvation conditions *E. coli* can degrade and use ribosomes.[66]

Fick's diffusion law is the presumed reason that bacteria are small. Bacteria absorb nutrients through their external surface; therefore, it would seem that the surface-to-volume ratio is the standard for cell size. For environments providing high concentrations of nutrients, a smaller concentration of uptake transport assemblies per unit surface area might be enough for the cell, and the growth rate would not be limited, whereas the total surface area might limit growth.[66] Bacteria and Archaea have cell diameters that range from 0.17 μm - 4.57 μm (volumes 0.02 μm^3 - 400 μm^3). The lower size limit for a free-living prokaryotic cell is the requirement of being large enough to contain a genome, the ribosomes and the essential proteins. Non–free-living organisms (viruses and prions) can be smaller than the smallest prokaryote, because "they use the machinery and resources of their host."[66]

The surface-to-volume constraint is probably the reason that many bacteria are rod-shaped, filamentous vibrios or fusiforms. A nonspherical shape increases the surface-to-volume ratio because a sphere has the least surface for the volume enclosed. An organism would be the least limited by the diffusion[70] of resources into the cell if it is as small as possible in cross-section, even if that means it has to be longer. The cross-section should also be flattened in order to have a higher surface-to-volume ratio.[66] Theoretical estimates of the minimal diameter for a living cell assume a spherical shape

that contains nucleic acids, proteins, lipids, polysaccharides and water. *Mycoplasma*, one of the smallest living cells, has a diameter of about 0.12 μm. The water content of a cell is about 70% by weight on average.[71] In order for metabolism to occur, cellular components must have specific structural and dynamic properties.[71] If the water content of the cell is reduced from 70% to 20%, the fluidity of the system could be reduced, but basic cell functions could still occur. Many metabolic processes occur near membranes, at rates that are limited by diffusion. In phospholipid membranes, the rate of diffusion of membrane-associated water, membrane lipids, and small molecules can be restricted to the membrane space. The rapid enzymatic activity of certain frozen protein solutions, in which the quantity of unfrozen water is minimal, is evidence that cells can have a water content of less 20% or less and be viable. [71]

Hypothesis

My hypothesis is that nanobacteria are independent, free-living life forms when found in blood, or other biotic environments. When nanobacteria are found in mineral formations (abiotic environments) they may simply be fragments of normal bacteria. I tested this hypothesis by determining if NLS have the basic characteristics of free living cells, if they are stressed "normal" bacteria or if they are bacterial cell fragments.

Three questions should be asked about a particle that is smaller than a normal bacteria are: 1. Is the particle organic? 2. Is it alive (does it reproduce and metabolize independently)? and 3. Can it grow?[72]

RESEARCH DESIGN

Materials Used: Lysozyme, form chicken egg white #L 6876; Calcium acetate monohydrate ACS #C8570; Type XIV Bacterial Protease #P6141; Newborn calf serum N-4637, Lot # 106H4629; (Sigma-Aldrich, Inc); B-D tuberculin syringe, 3cc, #14-829-13B; Whatman syringe filter, 0.1 μm, 13 mm diameter; Millex-GV filter, 0.22 μm, 13 mm diameter, #SLGV013SL; Nalgene Filters 115 ml, 0.22μm #09-740-36K; Potassium phosphate, monobasic #P285-500; Potassium phosphate, dibasic #P288-500; NaCl crystalline #S271-500 (Fisher Scientific); R2A Agar #DF1826-07-3 (Burgoon Company); Mycoplasma enrichment W/O Penicillin lyophilized #BB12292; Mycoplasma agar base #BB11456*BT (VWR Scientific); LIVE/DEAD *Bac*Light Viabiliy Kit #L-7007 (Molecular Probes); Dulbecco's modified eagle medium, Lot# 1022316 (Gibco BRL); SeaKem LE agarose #50001 (FMC)

Can NLS be cultured from the environment?

Folk[1,2,4,6,17,18,20,73,74] observed NLS in many different environments. In order to determine whether the NLS that can be observed in aquatic environments are life forms, attempts to culture the organisms were made.

Samples of water from the Edward's Aquifer, San Marcos River, Jemez Springs, New Mexico, Le Zitelle Hot Springs in Viterbo, Italy, pond (from Southwest Texas State University), tap (from room 377 New Science Building, Southwest Texas State University, San Marcos, TX) and deionized water were filtered through 0.2 μm and 0.1 μm syringe filters. The filtrate from each sample was inoculated into various

microbiological media. Media tested were: 1) tryptic soy agar (TSA), a general nutrient media used to culture a wide range of bacteria; 2) R2A, used to grow oligotrophic organisms; 3) B4, used because it allows for the productions of calcium carbonate crystals;[75] and 4) BG11, used to grow cyanobacteria.[10]

Media plates sealed with parafilm to avoid dehydration, were incubated at 25°C, 37°C and 45°C for two weeks. If growth was observed, the filtration and plating process was repeated for the sample in triplicate to check for the possibility of contamination.

To eliminate the possibility of *Mycoplasma* being mistaken for nanobacteria, *Mycoplasma*-specific media, pleuro-pneumonia-like organism (PPLO) media supplemented with penicillin at 500 U/ml was also inoculated with each sample. Positive controls with *Mycoplasma* were not done.

Can NLS be cultured from serum?

To test if *N. sanguinum* exist in serum, 10% and 20% newborn calf serum was inoculated in Dulbecco's modified eagle medium. Tissue flasks were incubated at 37°C, 5% CO_2 for three months (with weekly checks for contamination). After incubation, samples were centrifuged at 15,600 x *g* onto 0.2% formvar-coated copper grids, stained with 5% uranyl acetate (a negative stain) and observed with the JEOL 1200 EX II transmission electron microscope (TEM).

Samples from the serum were also stained with LIVE / DEAD® *Bac*Light™ bacterial viability stain kit. The kit uses SYTO®9 green fluorescent nucleic acid stain and propidium iodide (red) nucleic acid stain. Cells with intact cell membranes

stain green and those with damaged membranes stain red. The stain was used as a preliminary check for nucleic acids.

An attempt to isolate DNA from the serum cultures using the freeze-thaw method was made. One ml samples of DMEM with the NLS were centrifuged and the supernatant was removed. The pellet was re-suspended in TE buffer and a freeze-thaw process[76] was conducted. The sample was subjected to electrophoresis in an 0.8% agarose gel for 1 1/2 hours at 100 volts. After electrophoresis, the gel was stained in 0.5 μg/ml ethidium bromide solution for 20 minutes and photographed using a Polaroid camera.

Can NLS be found in kidney stones?

N. sanguinum have been implicated in the formation of kidney stones.[32,43] It is thought that since *N. sanguinum* form thick apatite cell walls, they may act as nucleation centers for mineral precipitation, hence, urinary calculi formation. Calculi obtained form patients by percutaneous nephrolithotomy were observed using the JEOL T330A scanning electron microscope (SEM), as were laboratory-infected (sterile struvite stones were placed in tryptic soy broth with *Proteus mirabilis* and incubated for 96 hours) and laboratory-created stones (*Proteus mirabilis* was added to molten 10% agar and 1g CaCO3. Mixture was allowed to solidify in a microfuge tube). Calculi were fixed in 2.5% glutaraldehyde in HEPES buffer overnight, washed for ten minutes three times in HEPES, and dried overnight at room temperature. After drying, the stones were gold coated as described by Folk[17] and observed.

Are NLS stressed normal-sized bacteria or bacterial fragments?

To determine if the observed NLS are starved normal cells, I decided to use representative Gram positive (*Micrococcus luteus,* ATCC 4698) and Gram negative bacteria (*Pseudomonas aeruginosa,* ATCC 10145) to see if they would react to starvation conditions by reducing in size. The organisms were placed in nutrient-limited conditions (R2A broth) for a period of one month and observed with the TEM, after staining with 5% uranyl acetate. Both *P. aeruginosa* (Gram negative bacilli) and *M. luteus* (Gram positive cocci) are found in aquatic environments.

To determine if NLS are heat stressed (shocked) normal cells, I put 1 ml samples of fresh cultures of Gram positive, *Staphylococcus aureus* (ATCC 25923) and *Pseudomonas aeruginosa* (ATCC 10145) grown in tryptic soy broth were centrifuged to remove supernatant (broth) and washed with phosphate buffered saline, pH 7.2 (PBS). After the third wash, the samples, in 1 ml of PBS, were placed in 80°C water for 20 minutes. After 20 minutes, the samples were centrifuged onto formvar-coated grids, stained with 5% uranyl acetate and observed with the TEM.

NLS were sought in clinically-obtained, infectious kidney stones that were treated with the holmium: YAG lithotripter. Calculi were obtained through J.M.H. Teichman, an urologist at the University of Texas Health Science Center, San Antonio, TX. The holmium: YAG lithotripter is a clinical laser that uses photothermal energy to destroy kidney stones.

Sand-grain-like fragments of treated stones were sonicated in a bath sonicator for ten minutes and then observed with the TEM. Laser-treated stone fragments were observed with the SEM at laser blast sites and sites away from the laser-created crater.

To test if observed nano-sized particles may be bacterial fragments induced by osmotic pressures from the environment (since they have been found in mineral formations) *S. aureus* and *P. aeruginosa*, washed in phosphate buffered saline, pH 7.2, were subjected to excess calcium and magnesium. Washed bacteria (after centrifuging and discarding the supernatant) were added to 1.0 ml of ethanol, chloroform and acetone to determine if NLS were bacterial fragments induced by fixation processes for microscopy. NLS may have been bacterial fragments created by some other cause including an enzyme, mechanical lysis or bacteriophage-induced lysis. To determine this Lysozyme in PBS was added to tubes of bacteria. Both types of bacteria were also subjected to a freeze-thaw method[76] (Gillan, personal communication) and sonicated using a probe sonicator (washed cells were placed in 1 ml of PBS and sonicated twice for 2 seconds at 40Hz. All samples were centrifuged onto formvar-coated grids, stained with 5% uranyl acetate and observed with the TEM. A T4 bacteriophage-lysed *Escherichia coli* biofilm (from Brian Corbin) was observed with the TEM after staining with phosphotungsitc acid (PTA).

RESULTS

Can NLS be cultured form the environment?

Attempts to culture nanobacteria from the different aquatic environments proved unsuccessful. A few of the plates inoculated with filtered water did have some colonies growing on them, but after replication, no significant growth was found (see Tables 1-7).

R2A plates inoculated with non-filtered water from the Le Zitelle Hot Springs in Viterbo, Italy had abundant growth. All other agar plates inoculated with the hot springs water had no growth. However, 0.2 μm filtered water from site Ab1d and 0.1 μm filtered water from site Bh1d plated on R2A appeared to have tiny pockets of air in the agar. I have called these agar bubbles. This phenomenon was found on plates inoculated with 0.1 μm filtered water from the San Marcos River and from a pond (Southwest Texas State University) as well (see Tables 2, 4 and 5).

Non-filtered water samples from the Edward's Aquifer, the San Marcos River, the pond and deionized water all showed growth when plated on B4 agar (see Tables 1,2,5 and 7). Growth on the B4 agar plates inoculated with Edward's Aquifer water, San Marcos River water, and pond water had some calcium carbonate crystal formation (see Tables 1,2,5). Crystal production was determined by dropping dilute hydrochloric acid on suspected colonies. If effervescence was observed, it was determined that calcium carbonate had been formed. Only fungal growth was noted on BG11 plates inoculated with unfiltered water. No growth was found on all other plates. No growth was found on any of the pleuro-pneumonia-like organism agar plates.

Can NLS be cultured form serum?

After a prolonged incubation period (three months), transmission electron microscopy showed NLS in Dulbecco's modified eagle medium (DMEM) with 20% newborn calf serum (see Figures 1-2). The NLS appear to have membrane-like structures, and fall in the size range for purported nanobacteria. After a longer incubation period (four months) they also appear to form biofilm-like structures (Figure 2). Negative controls (only DMEM and only serum) did not have any NLS (see Figures 3-4). NLS from the DMEM-serum samples stained fluorescent green after staining with LIVE / DEAD® *Bac*Light™ bacterial viability stain kit (see Figure 5).

The attempt to isolate DNA from NLS isolated from the DMEM-serum samples using the freeze-thaw method proved unsuccessful. No bands were imaged after staining the agarose gel with ethidium bromide.

Can NLS be found in kidney stones?

Infectious renal calculi (*Proteus mirabilis* infected struvite) obtained by percutaneous nephrolithotomy were observed using the SEM. Nanobacteria-like-structures were seen (see Figure 6). Laboratory-infected struvite (*Proteus mirabilis*) also appeared to have nanobacteria-like-structures (see Figure 7).

Are NLS stressed normal sized bacteria or bacterial fragments?

Micrococcus luteus and *Pseudomonas aeruginosa* both showed reduction in size after one month incubation period in nutrient-limited conditions (see Figures 8-9).

Staphylococcus aureus and *P. aeruginosa* samples, when subjected to 80°C for twenty minutes, appeared to rupture, releasing cell debris. The cell debris were easily able to pass through a 0.1 μm filter (see Figures 10-11). After treating an infectious (*Proteus mirabilis*) kidney stone with the holmium:YAG lithotripter, nano-sized structures were observed with the TEM (see Figure 12). No NLS were observed using the SEM at laser blast sites, but at sites away from the laser produced crater, NLS were found (see Figures 13-15).

Subjecting *S. aureus* to excess calcium and magnesium did not appear to have any effect on them (they remained the same size and shape). However, when *P. aeruginosa* was exposed to excess calcium and magnesium they plasmolysed (Figure 16), and some membrane-like bound structures were observed after filtration through a 0.2 μm filter (Figure 17).

S. aureus samples that were exposed to solvents (ethanol and acetone) did not greatly differ from non-treated *S. aureus*. Samples of *P. aeruginosa* did appear different when treated with the solvents. In all three treatments, the cells appeared to be breaking down (see Figure 18). Some of the "debris" were able to pass through the 0.2 μm filter; however, they did not resemble NLS.

Lysozyme did not have an effect on *S. aureus*. It did have an effect on *P. aeruginosa*. Much like with the solvents, the cells appeared to lyse (see Figure 19). Some structures were able to pass through the 0.2 μm filter; but again, they did not resemble NLS.

The freeze-thaw method[76] did not lyse *S. aureus* (Figure 20). It did appear to disrupt *P. aeruginosa* (see Figure 21), but nothing was seen when the freeze-thawed suspension was filtered.

Both *S. aureus* and *P. aeruginosa* did show effects after treatment with the probe sonicator (Figures 22-23). The cell debris from each species of bacteria passed through both the 0.2 μm and 0.1 μm filter.

An *Escherichia coli* biofilm lysed with T4 bacteriophage produced cell debris similar to debris observed after other treatments (see Figure 24).

Edward's Aquifer Water

Media Type	25°C	37°C	45°C
TSA Control	TNTC	TNTC	TNTC
0.2 µm filter	NG	NG	NG
0.1 µm filter	NG	NG	NG
R2A Control	TNTC	TNTC	TNTC
0.2 µm filter	NG	Fungi*	NG
0.1 µm filter	NG	NG	NG
B4 Control	Little growth / some crystals	Little growth / no crystals	NG
0.2 µm filter	NG	NG	NG
0.1 µm filter	NG	NG	NG
BG11 Control	NG	NG	NG
0.2 µm filter	NG	NG	NG
0.1 µm filter	NG	NG	NG
PPLO Agar	NG	NG	NG

Table 1
Results from NLS culture attempts from Edward's Aquifer water, San Marcos, TX. TNTC indicates abundant growth. NG indicates no growth. * indicates growth on initial plate, but after replication, no growth was noted. (TSA—tryptic soy agar, PPLO—pleuro-pneumonia-like organism)

San Marcos River Water

Media Type	25°C	37°C	45°C
TSA Control	TNTC	TNTC	TNTC
0.2 µm filter	NG	NG	NG
0.1 µm filter	NG	NG	NG
R2A Control	TNTC	TNTC	TNTC
0.2 µm filter	Fungal growth*	NG	NG
0.1 µm filter	NG / agar bubbles	NG / agar bubbles	NG
B4 Control	TNTC / some crystals	TNTC / some crystals	NG
0.2 µm filter	Little growth and fungi* / no crystals	NG	NG
0.1 µm filter	NG	NG	NG
BG11 Control	Slime-like growth	NG	Fungi
0.2 µm filter	NG	NG	NG
0.1 µm filter	NG	NG	NG
PPLO Agar	NG	NG	NG

Table 2
Results from NLS culture attempts from San Marcos River Water, TX. TNTC indicates abundant growth. NG indicates no growth. * indicates growth on initial plate, but after replication, no growth was noted. (TSA—tryptic soy agar, PPLO—pleuro-pneumonia-like organism)

Jemez Springs Water

Media Type	25°C	37°C	45°C
TSA Control	NG	NG	NG
0.2 µm filter	NG	NG	NG
0.1 µm filter	NG	NG	NG
R2A Control	NG	NG	NG
0.2 µm filter	NG	NG	NG
0.1 µm filter	NG	NG	NG
B4 Control	NG	NG	NG
0.2 µm filter	NG	NG	NG
0.1 µm filter	NG	NG	NG
BG11 Control	NG	NG	NG
0.2 µm filter	NG	NG	NG
0.1 µm filter	NG	NG	NG
PPLO Agar	NG	NG	NG

Table 3
Results of NLS culture attempts from Jemez Springs water (New Mexico). TNTC indicates abundant growth. NG indicates no growth. (TSA—tryptic soy agar, PPLO—pleuro-pneumonia-like organism)

Le Zitelle Hot Springs Water

Media Type		An1d	Ab1d	Bh1d
TSA	Control	Fungi	NG	NG
	0.2 μm filter	Fungi*	NG	NG
	0.1 μm filter	Little growth*	NG	NG
R2A	Control	TNTC	TNTC	TNTC
	0.2 μm filter	NG	NG / agar bubbles	NG
	0.1 μm filter	NG	NG	NG / agar bubbles
B4	Control	NG	NG	NG
	0.2 μm filter	NG	NG	NG
	0.1 μm filter	NG	NG	NG
PPLO Agar		NG	NG	NG

Table 4
Results of NLS culture attempts from Le Zitelle Hot Springs water (Viterbo, Italy). TNTC indicates abundant growth. NG indicates no growth. * indicates growth on initial plate, but after replication, no growth was noted. (TSA—tryptic soy agar, PPLO—pleuro-pneumonia-like organism)

Pond Water

Media Type	25°C	37°C	45°C
TSA Control	TNTC	TNTC	TNTC
0.2 µm filter	Some growth*	NG	NG
0.1 µm filter	NG	NG	NG
R2A Control	TNTC	TNTC	TNTC
0.2 µm filter	NG	Fungi	NG
0.1 µm filter	NG / agar bubbles	NG / agar bubbles	NG
B4 Control	TNTC	TNTC / some crystals	NG
0.2 µm filter	NG	NG	NG
0.1 µm filter	NG	NG	NG
BG11 Control	Fungi	Fungi	NG
0.2 µm filter	NG	NG	NG
0.1 µm filter	NG	NG	NG
PPLO Agar	NG	NG	NG

Table 5
Results of NLS culture attempts from pond water (Southwest Texas State University, San Marcos, TX). TNTC indicates abundant growth. NG indicates no growth. * indicates growth on initial plate, but after replication, no growth was noted. (TSA—tryptic soy agar, PPLO—pleuro-pneumonia-like organism)

Tap Water

Media Type	25°C	37°C	45°C
TSA Control	TNTC	TNTC	TNTC
0.2 μm filter	NG	NG	NG
0.1 μm filter	NG	NG	NG
R2A Control	TNTC	TNTC	TNTC
0.2 μm filter	NG	NG	NG
0.1 μm filter	NG	NG	NG
PPLO Agar	NG	NG	NG

Table 6
Results of NLS culture attempts from tap water (New Science Building, Southwest Texas State University, San Marcos, TX). TNTC indicates abundant growth. NG indicates no growth. (TSA—tryptic soy agar, PPLO—pleuro-pneumonia-like organism)

Deionized Water

Media Type	25°C	37°C	45°C
TSA Control	TNTC	TNTC	TNTC
0.2 µm filter	155 CFUs†	110 CFUs†	Little growth*
0.1 µm filter	NG	NG	NG
R2A Control	TNTC	TNTC	TNTC
0.2 µm filter	NG	Fungi*	NG
0.1 µm filter	NG	NG	NG
B4 Control	TNTC	TNTC	NG
0.2 µm filter	NG	NG	NG
0.1 µm filter	NG	NG	NG
BG11 Control	Fungi	Fungi	NG
0.2 µm filter	NG	Fungi*	NG
0.1 µm filter	NG	NG	NG
PPLO Agar	NG	NG	NG

Table 7
Results from NLS culture attempts from deionized water (New Science Building, Southwest Texas State University, San Marcos, TX). TNTC indicates abundant growth. NG indicates no growth. * indicates growth on initial plate, but after replication, no growth was noted. † indicates average of three replications. (CFU—colony forming unit, TSA—tryptic soy agar, PPLO—pleuro-pneumonia-like organism)

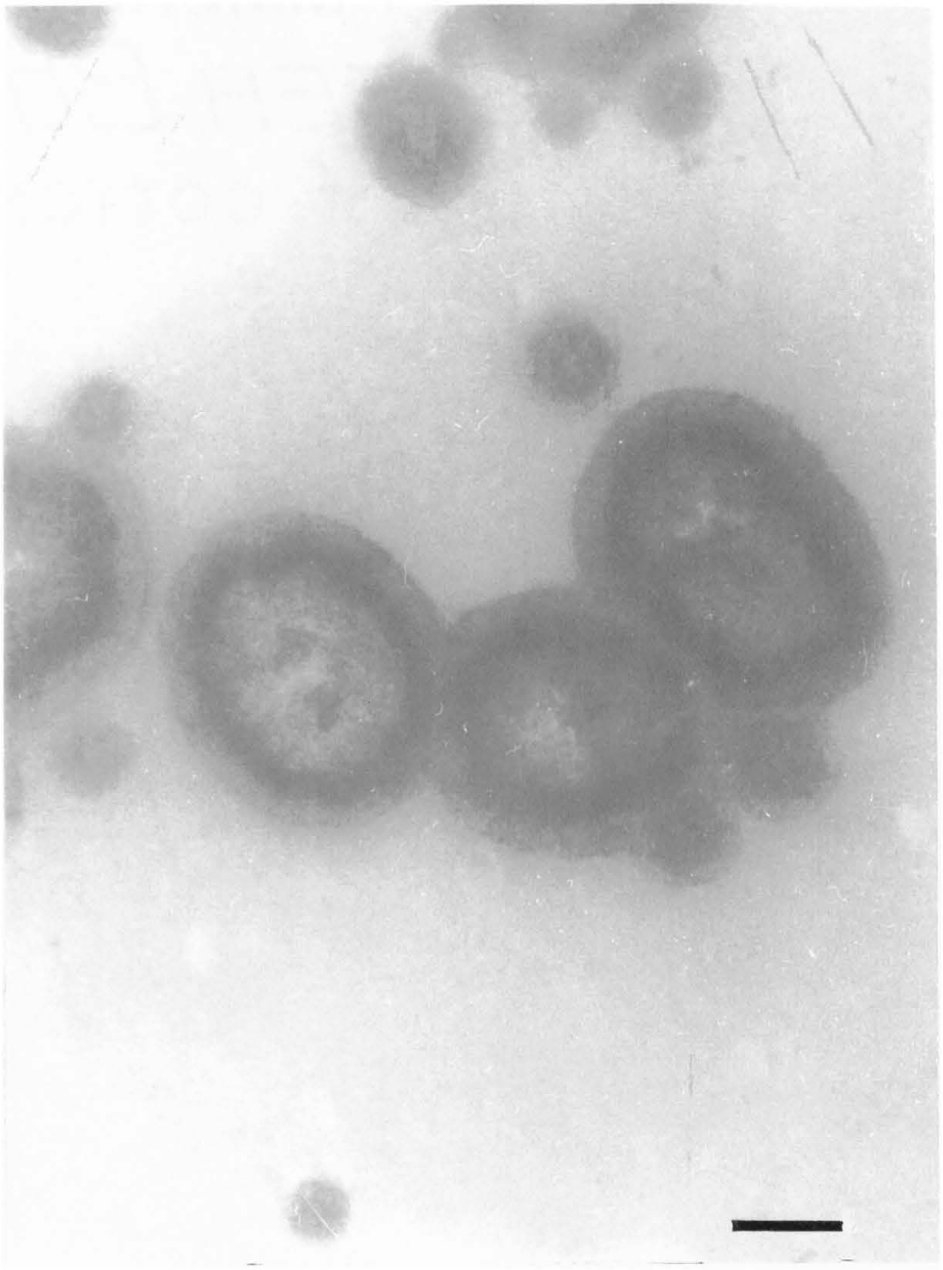

Figure 1
Nanobacteria-like-structures "grown" in 20% newborn calf serum in Dulbecco's modified eagle medium, observed with TEM. Bar = 100 nm.

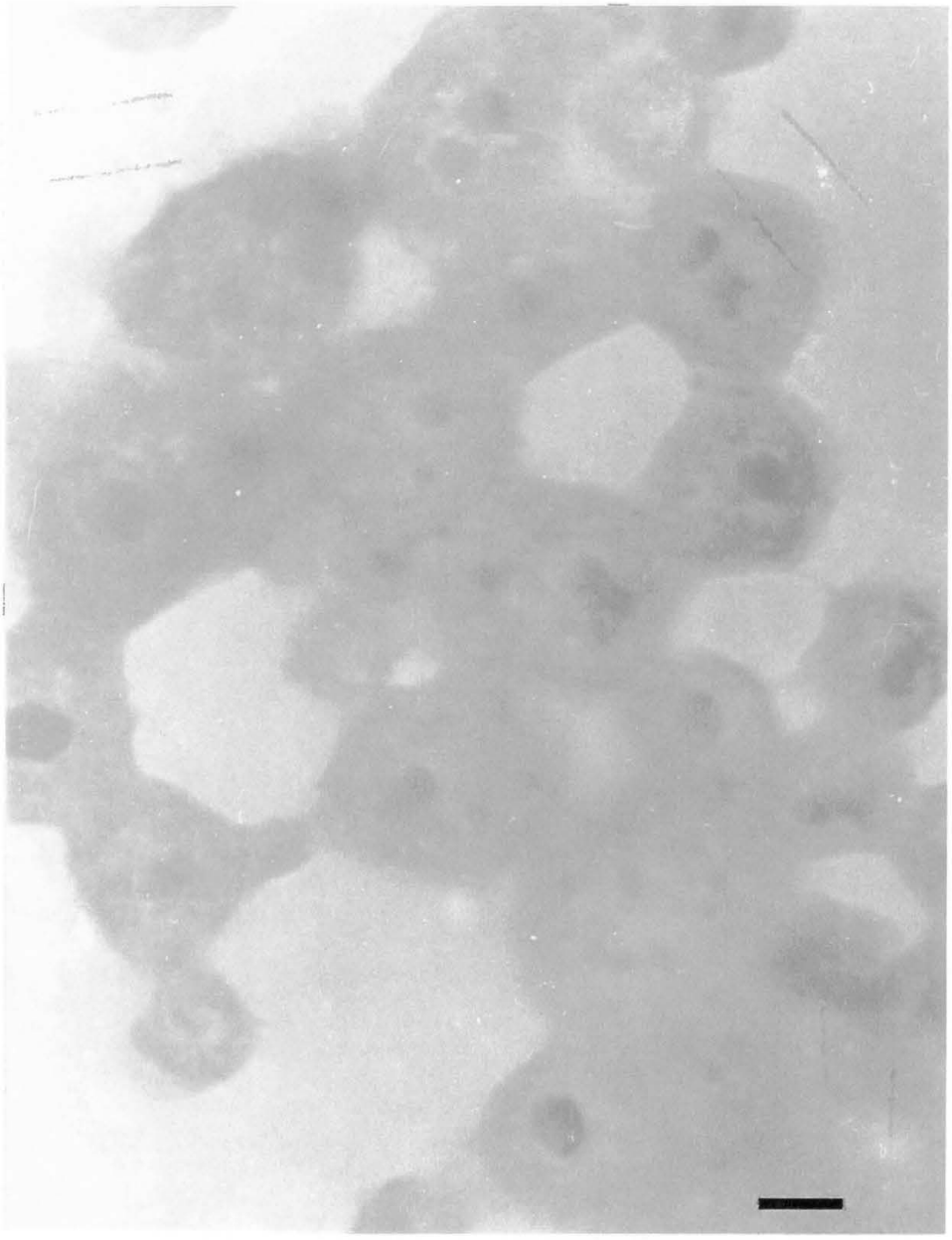

Figure 2
Nanobacteria-like-structures "grown" in 20% newborn calf serum in Dulbecco's modified eagle medium, observed with TEM, after 4 months incubation. Appears to be in a biofilm-like formation. Bar = 50 nm.

Figure 3
Dulbecco's modified eagle medium control. No serum added.

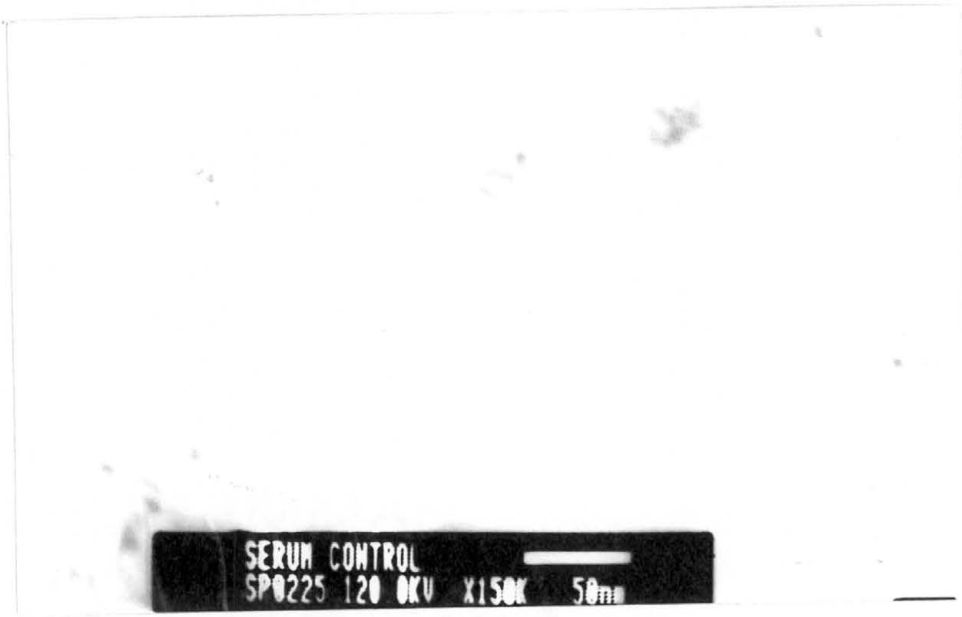

Figure 4
Newborn calf serum control. No Dulbecco's modified eagle medium added.

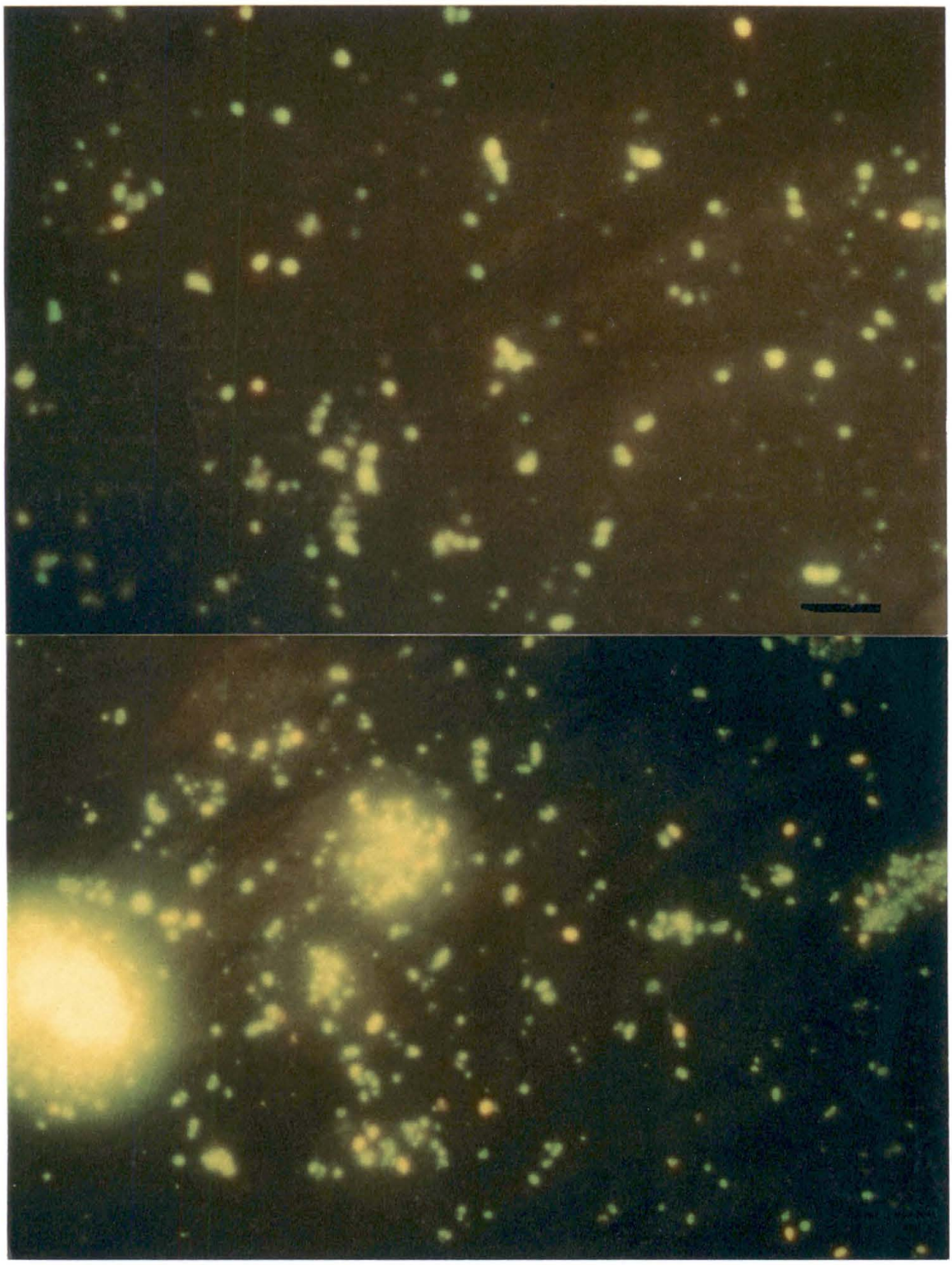

Figure 5
Nanobacteria-like-structures "grown" in 20% newborn calf serum in Dulbecco's modified eagle medium, stained with LIVE / DEAD® *Bac*Light™ bacterial viability stain. Bars = 10 μm.

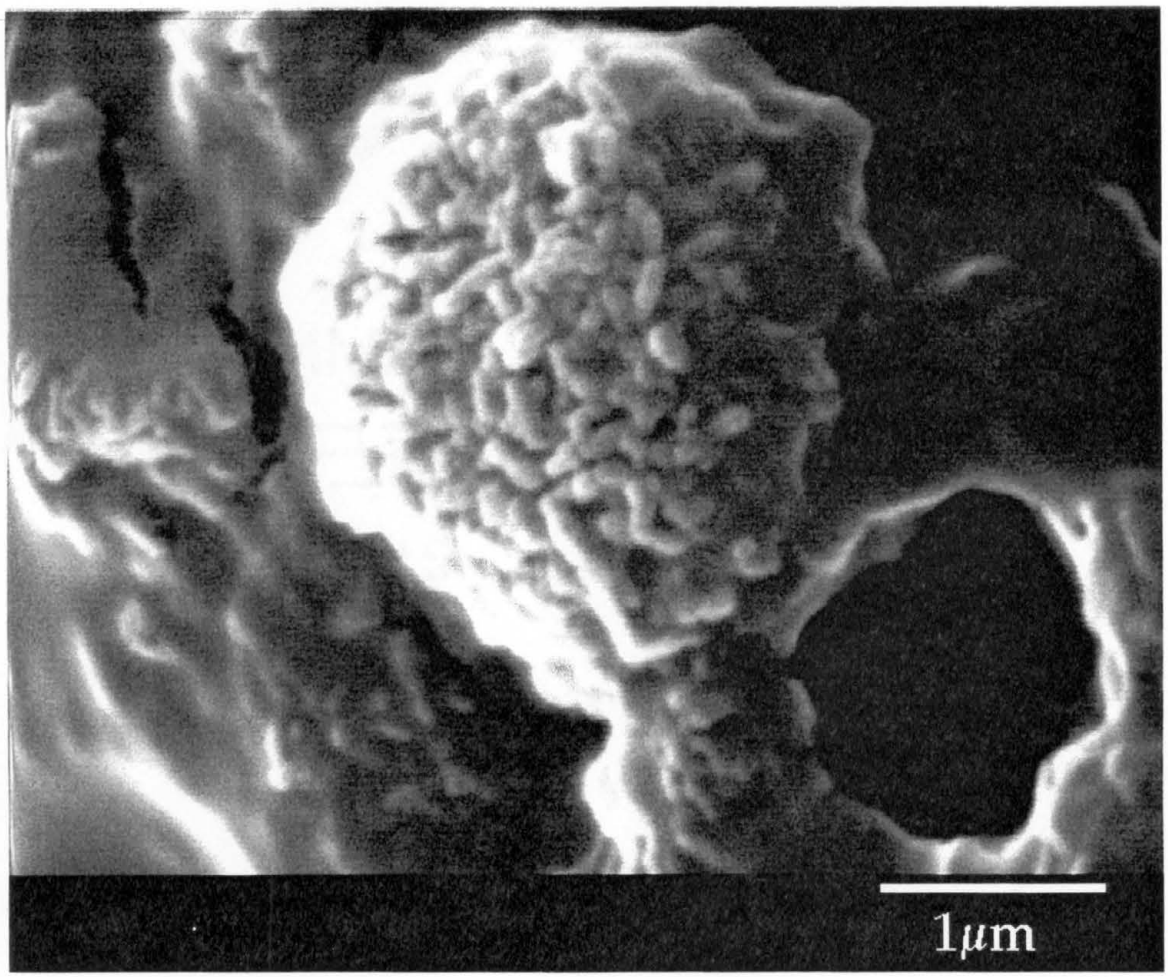

Figure 6
Nanobacteria-like-structures observed by SEM on infectious renal calculi obtained by percutaneous nephrolithotomy from a patient. Bar = 1 μm.

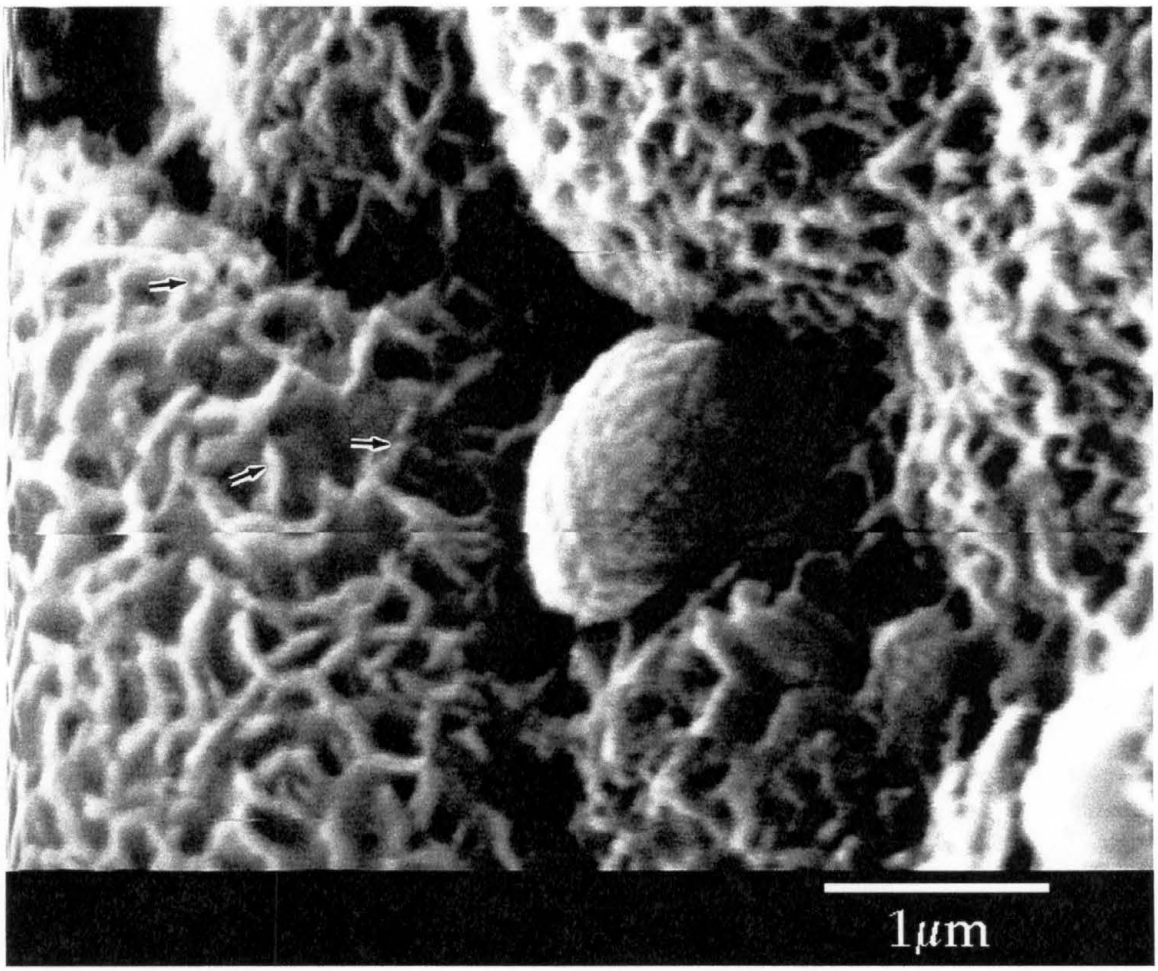

Figure 7
Arrows indicate nanobacteria-like-structures observed by SEM on laboratory-infected renal calculi. Bar = 1 μm

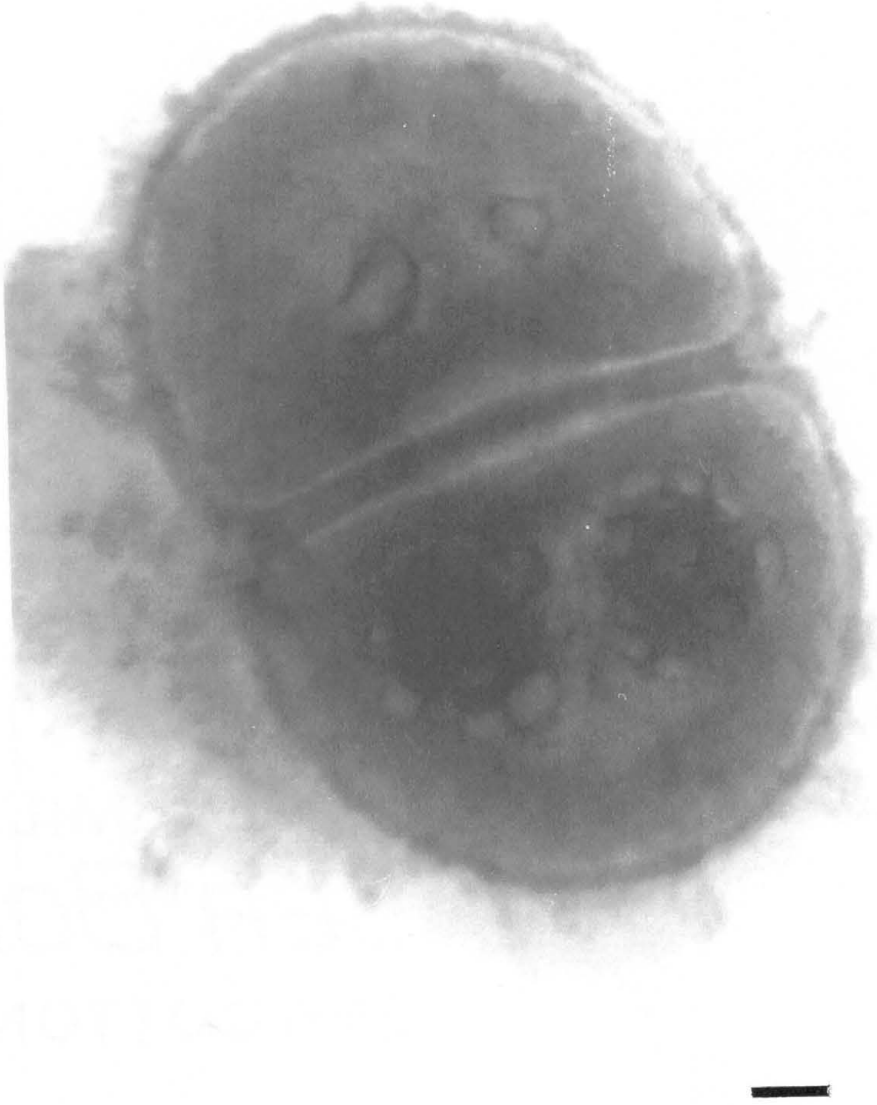

Figure 8
Nutrient-limited *Micrococcus luteus* observed with TEM. Bar = 100 nm.

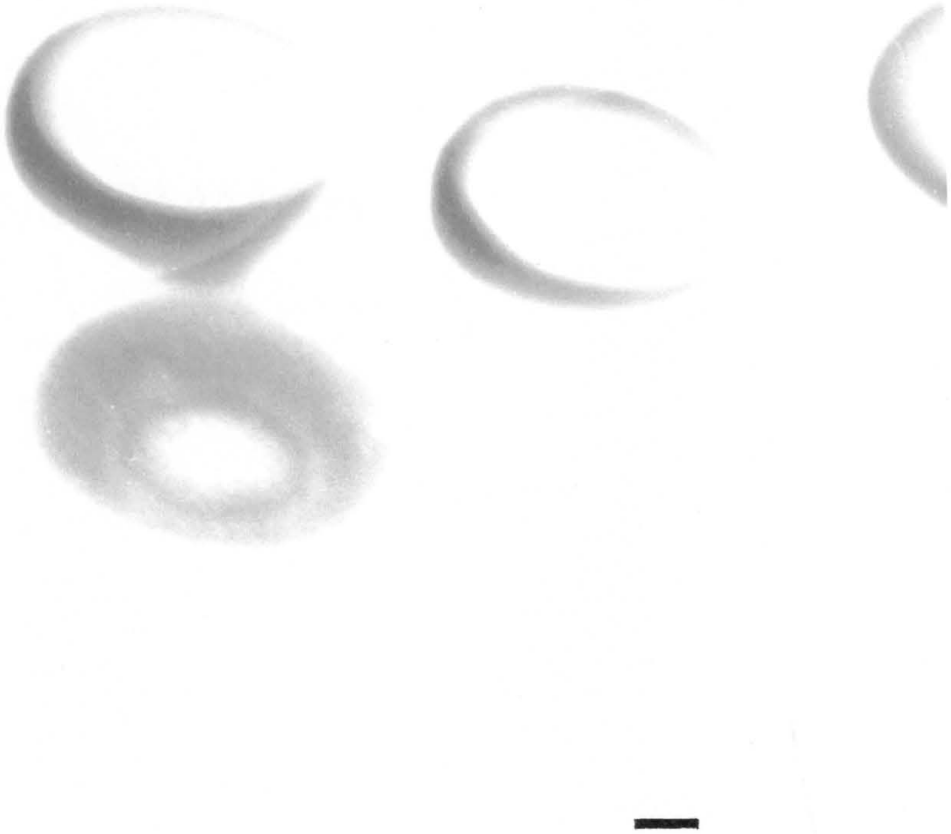

Figure 9
Nutrient-limited *Pseudomonas aeruginosa* observed with TEM. Bar = 200 nm.

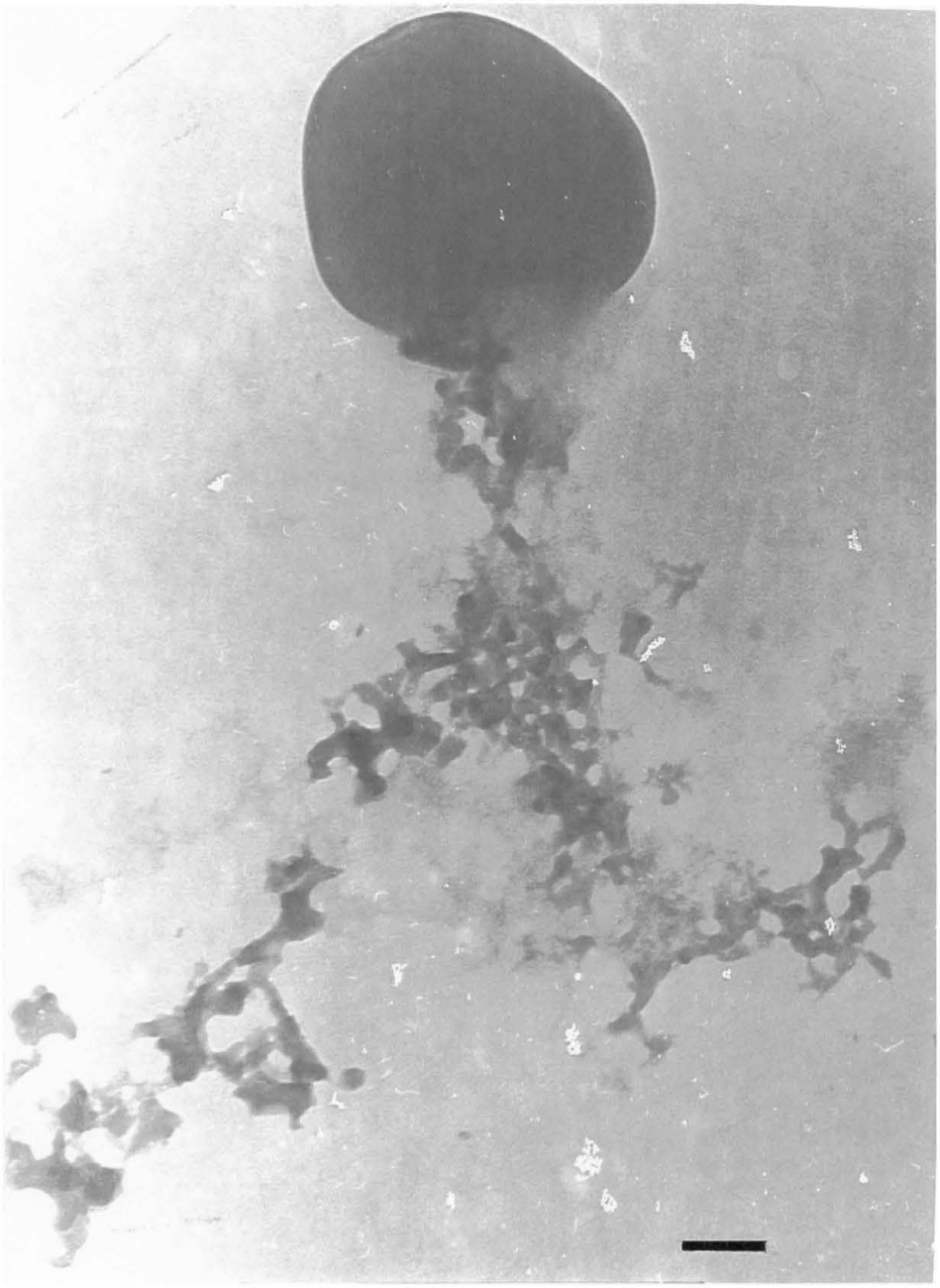

Figure 10
Staphylococcus aureus subjected to 80°C, observed with TEM. Bar = 100 nm.

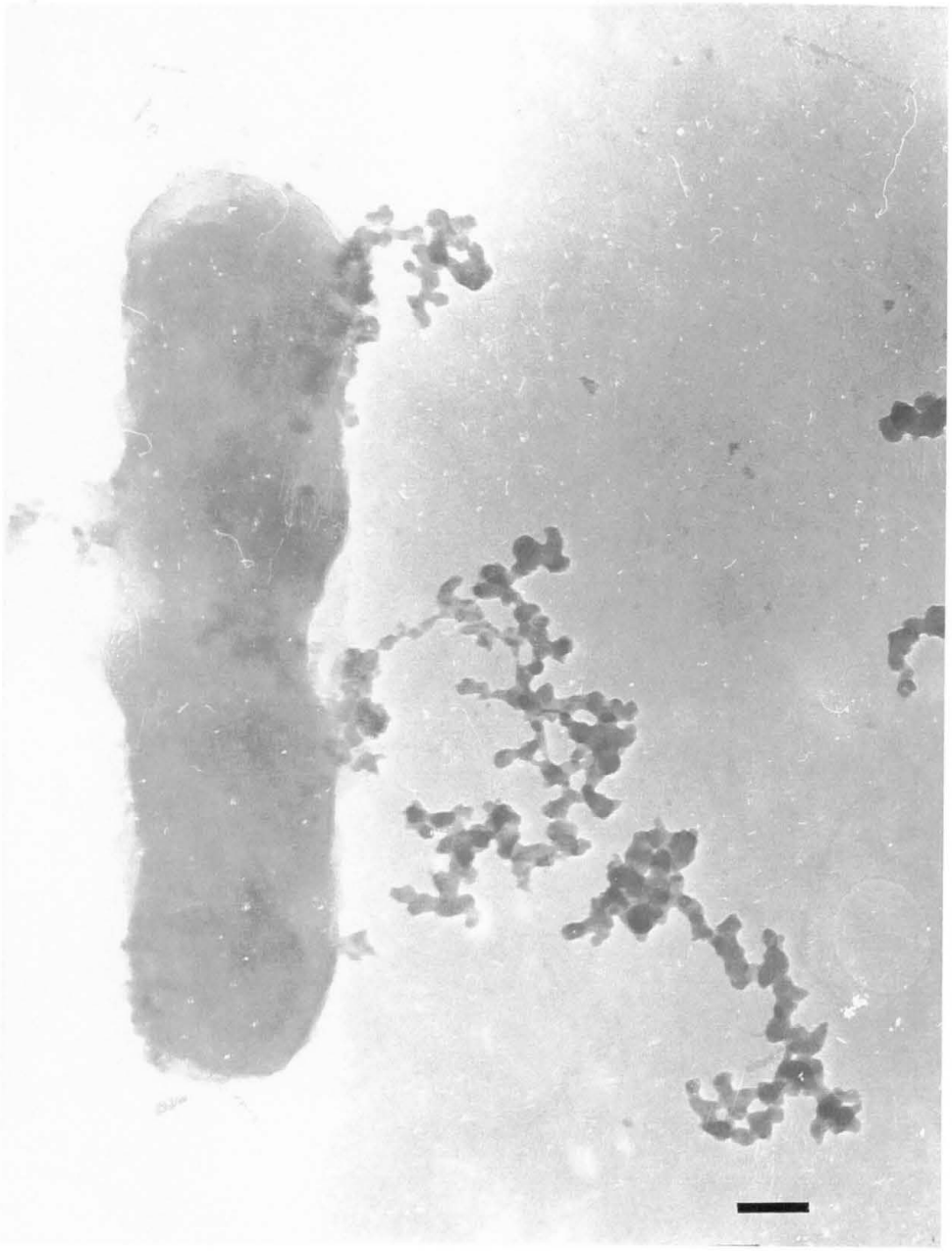

Figure 11
Pseudomonas aeruginosa filtered through 0.1 μm filter after being subjected to 80°C. Observed with TEM. Bar = 100 nm.

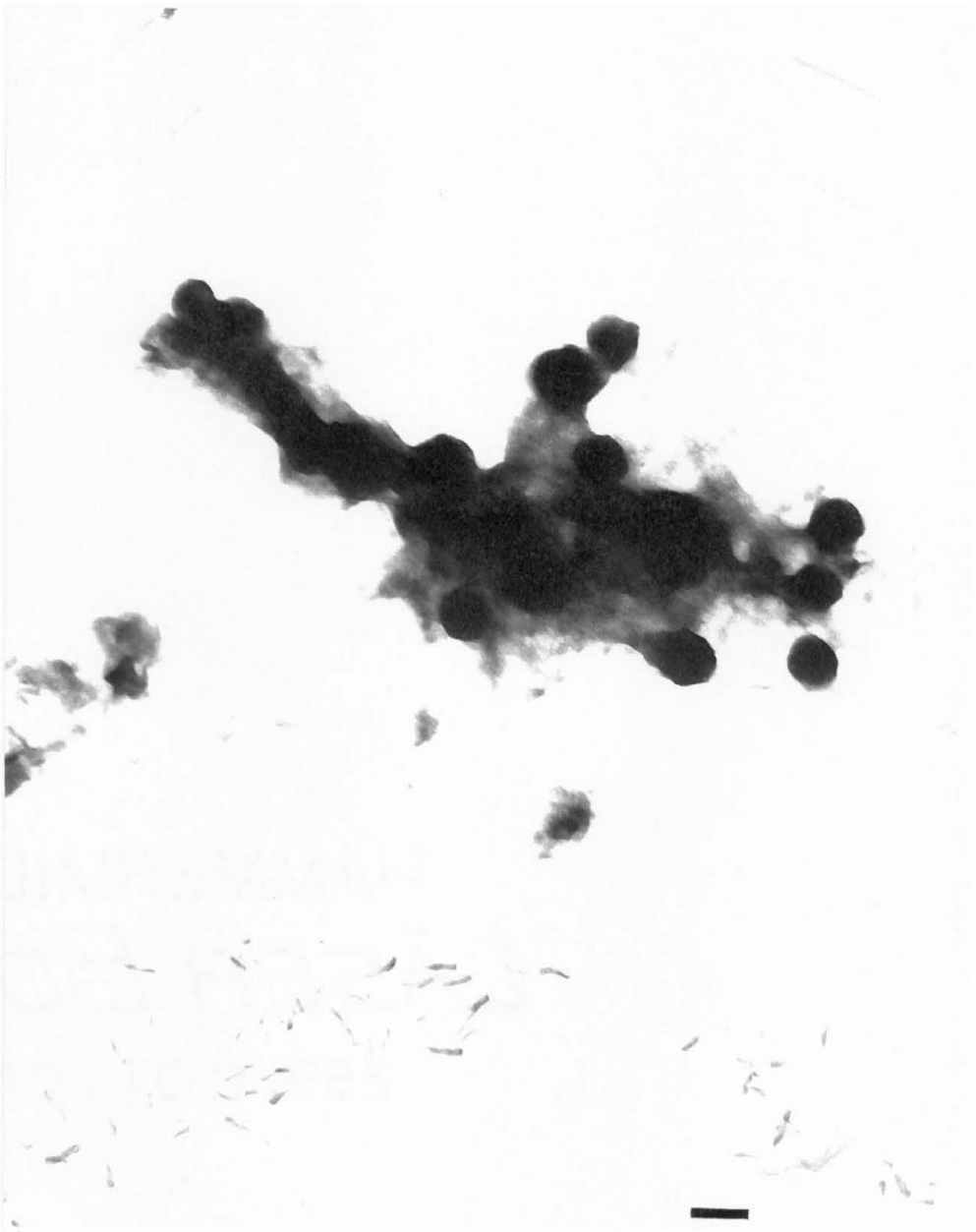

Figure 12
Nanobacteria-like-structures seen in holmium: YAG lithotripter treated renal calculi.

Observed with TEM. Bar = 200 nm.

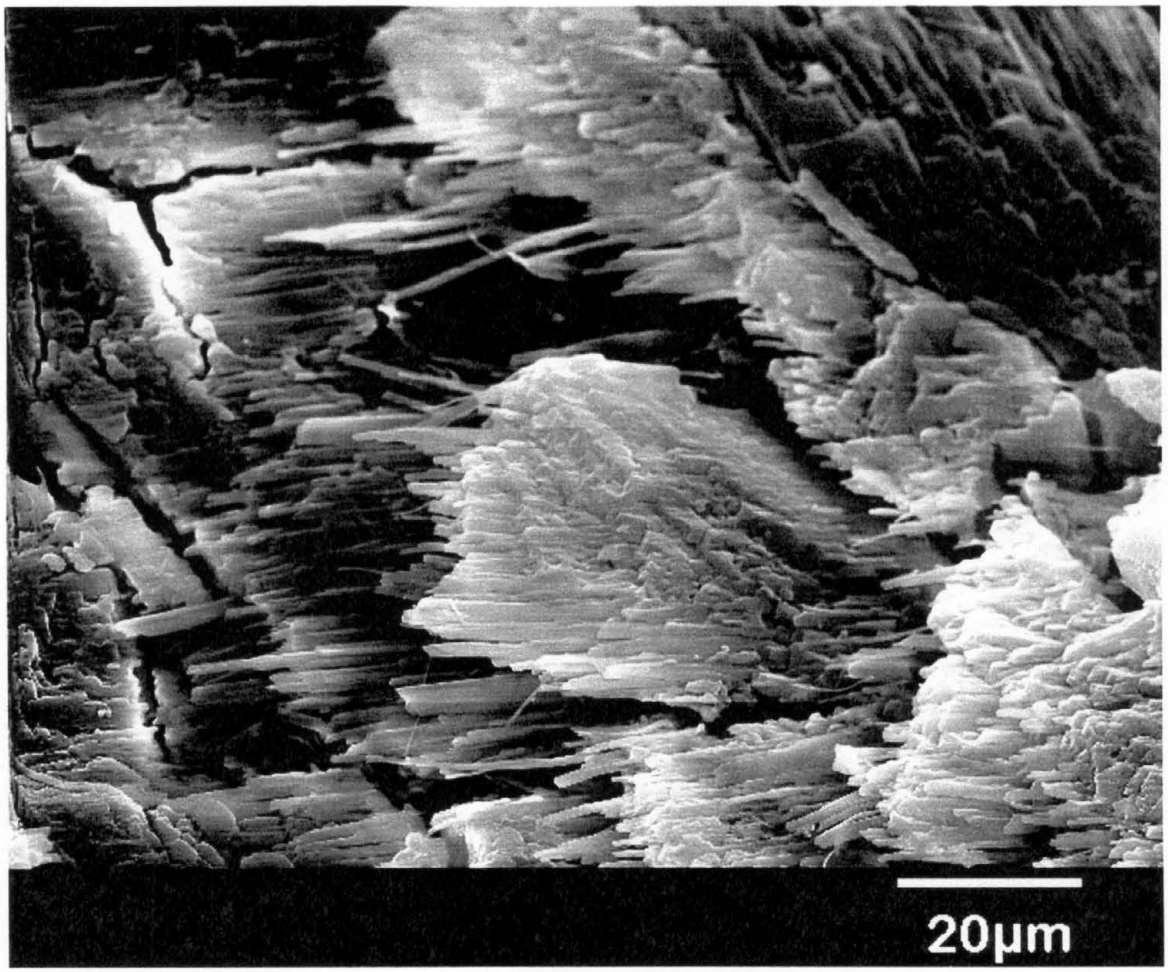

Figure 13
Overview of laser blast site on infectious kidney stone after treatment with holmium: YAG lithotripter. Observed with SEM. Bar = 20 µm.

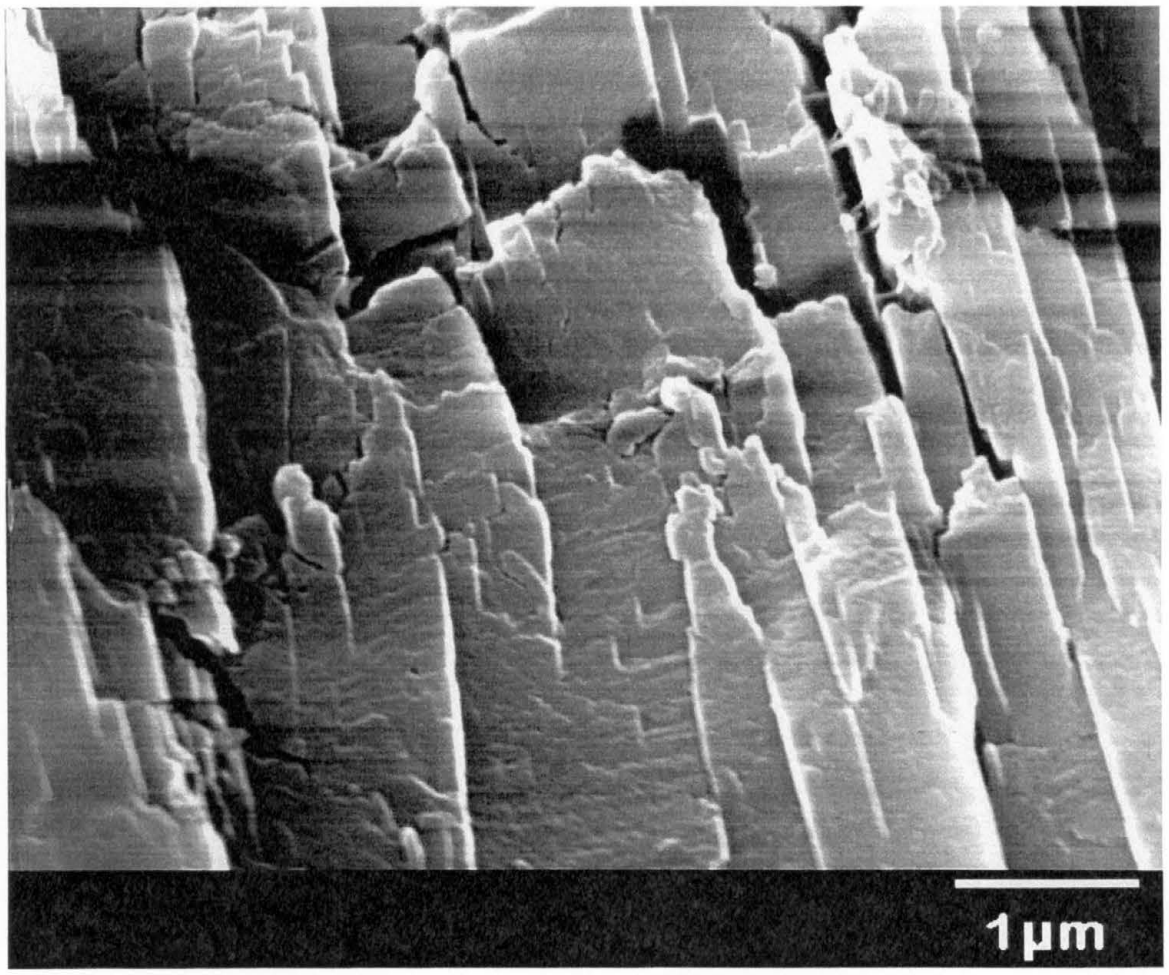

Figure 14
Laser blast site on infectious kidney stone after treatment with holmium: YAG lithotripter. No nanobacteria-like-structures can be seen. Observed with SEM. Bar = 1µm.

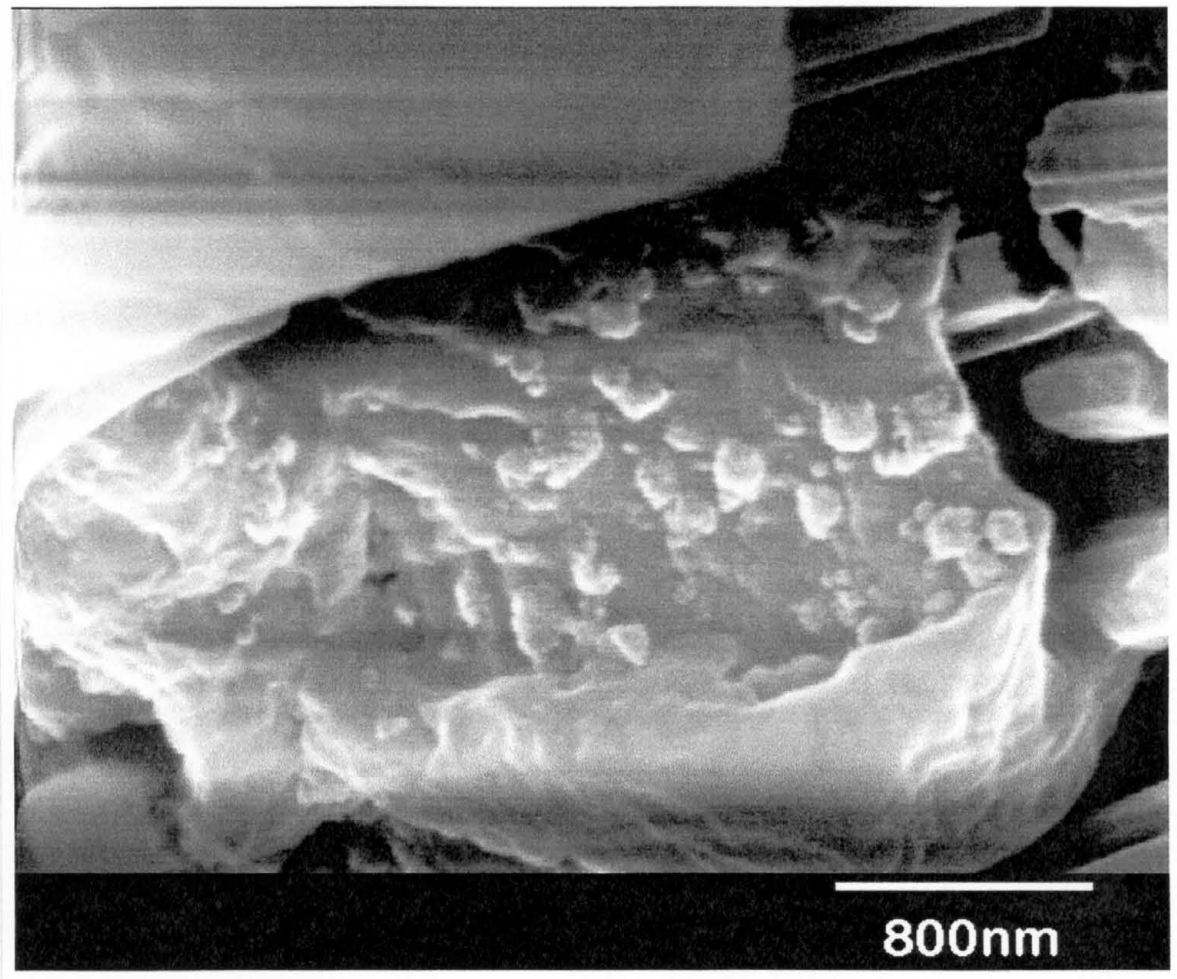

Figure 15
Area away from laser blast site on infectious kidney stone after treatment with holmium: YAG lithotripter. Observed with SEM. Bar = 800 nm.

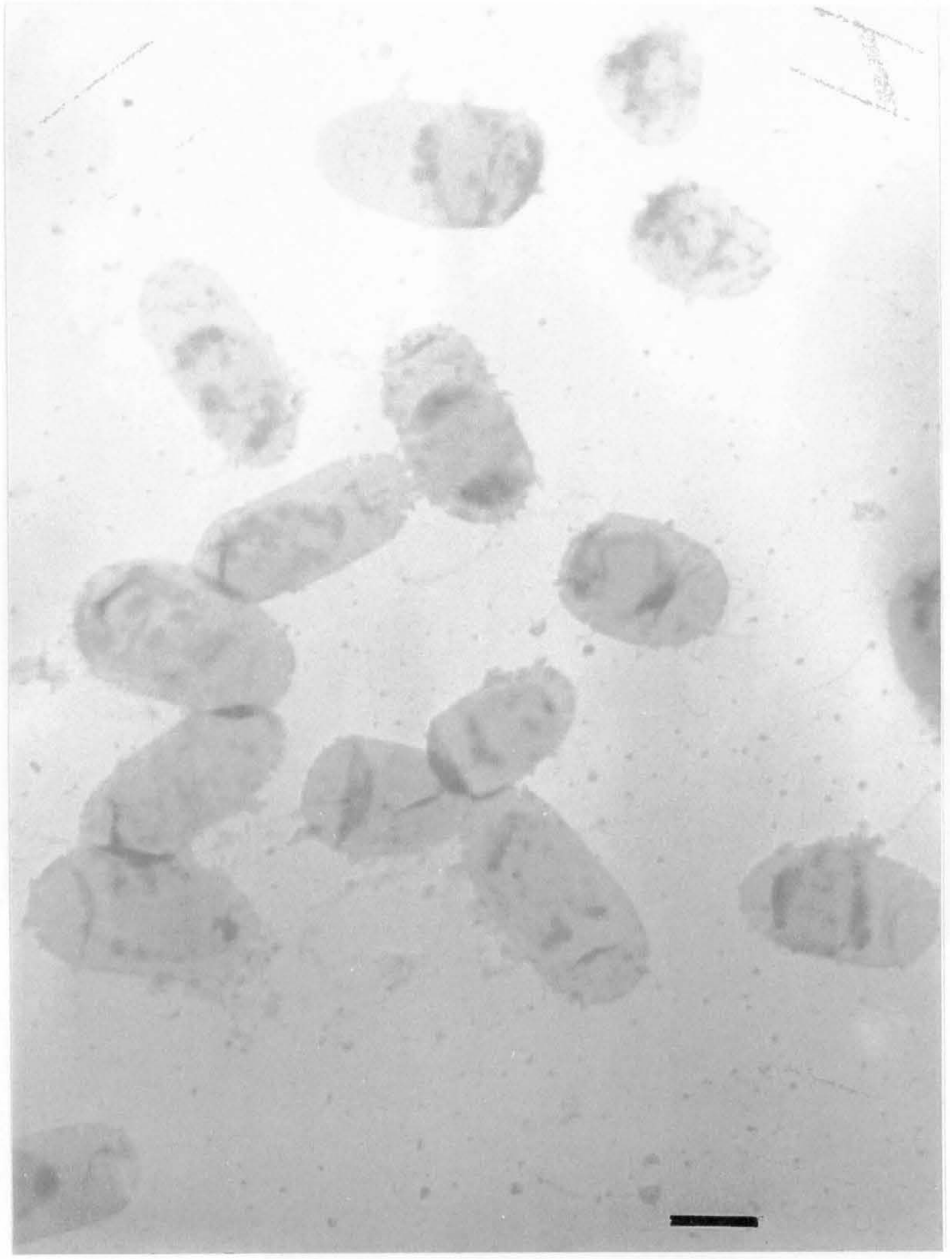

Figure 16
Pseudomonas aeruginosa after exposure to excess calcium and magnesium. Observed with TEM. Bar = 500 nm.

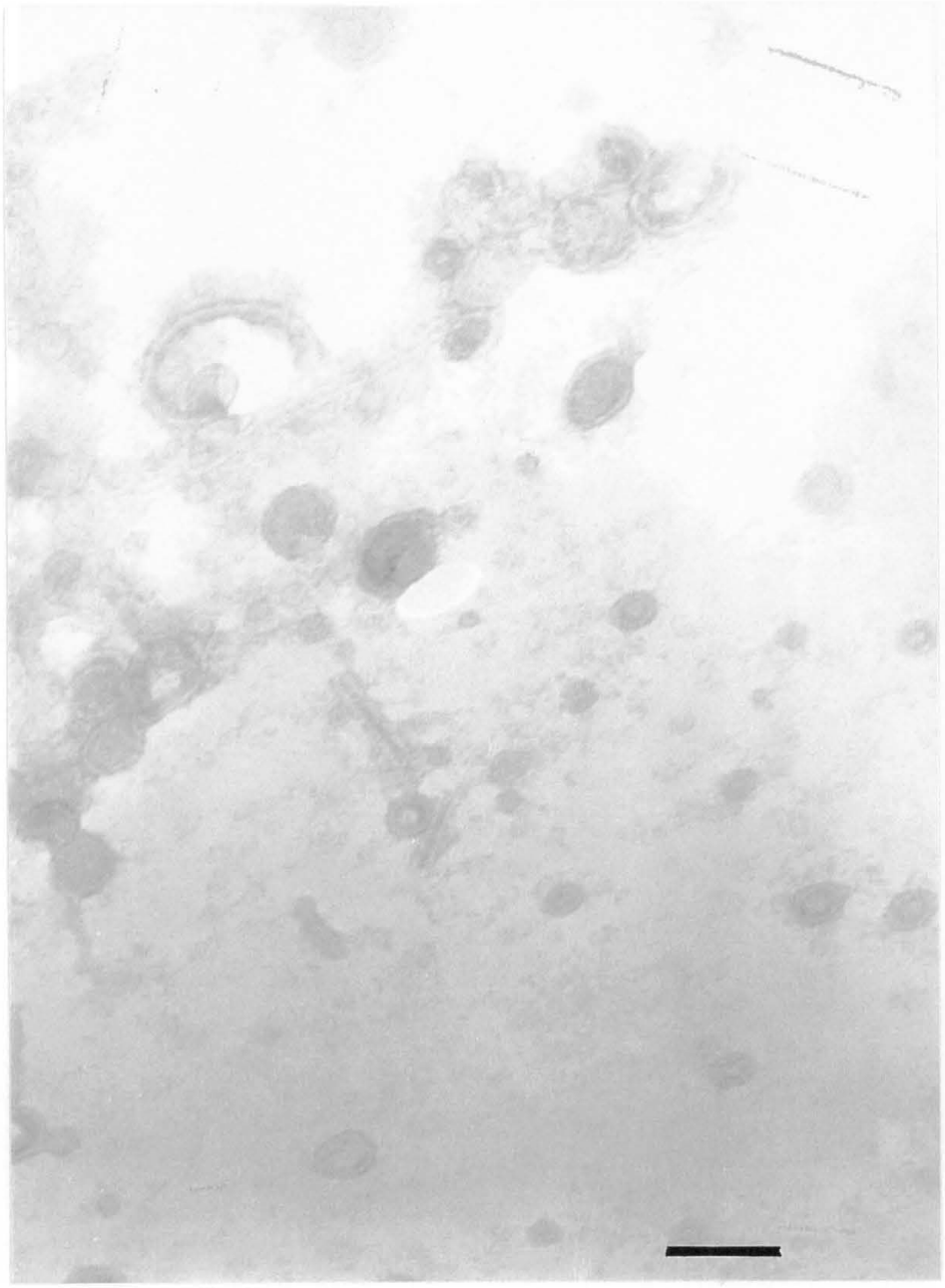

Figure 17
Pseudomonas aeruginosa filtered through 0.2 μm filter after exposure to excess calcium and magnesium. Membrane-bound-like structures may be membrane blebs. Observed with TEM. Bar = 100 nm.

Figure 18
Pseudomonas aeruginosa after treatment with chloroform. Observed with TEM. Bar = 500 nm.

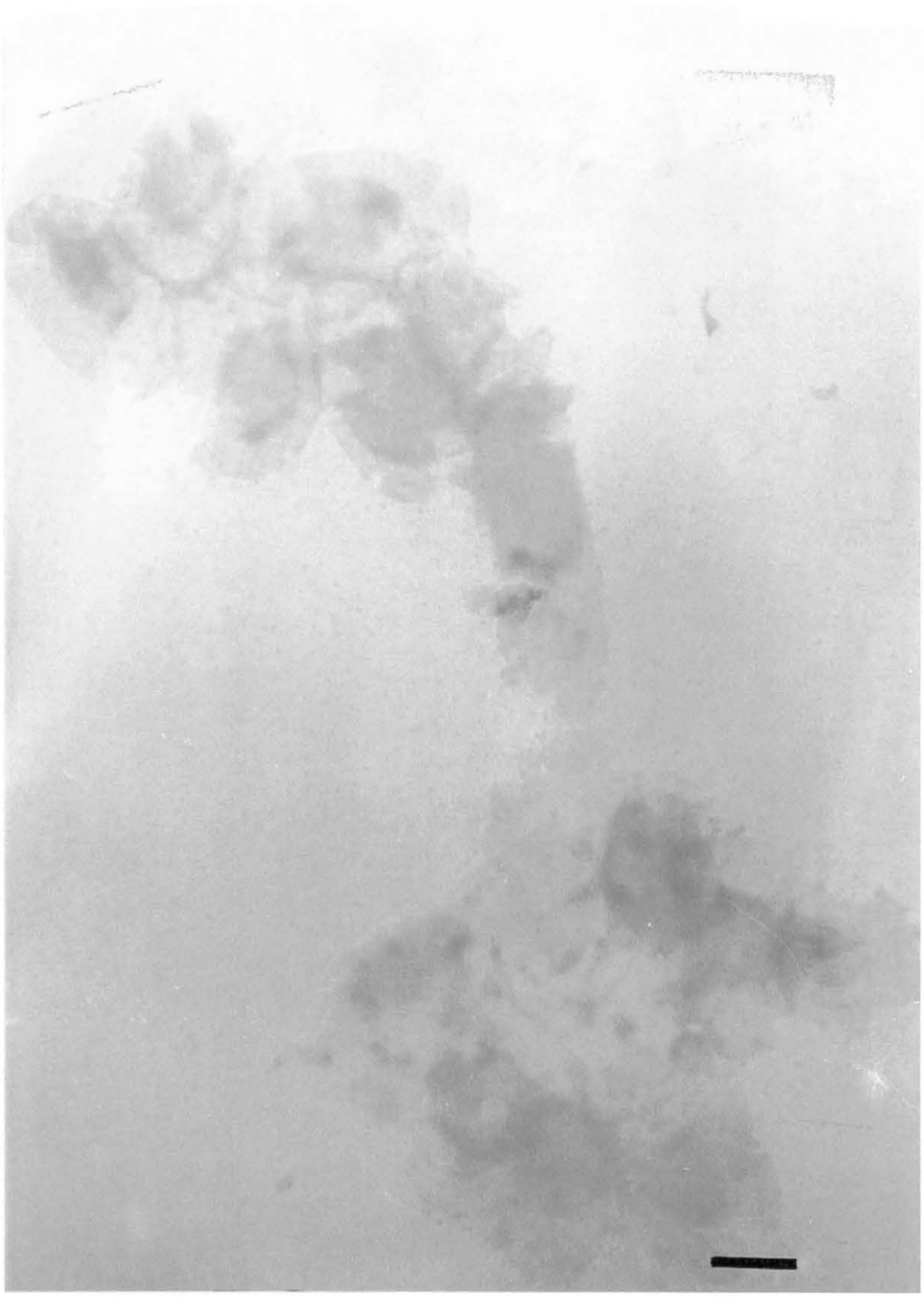

Figure 19
Pseudomonas aeruginosa after treatment with lysozyme. Observed with TEM. Bar = 500 nm.

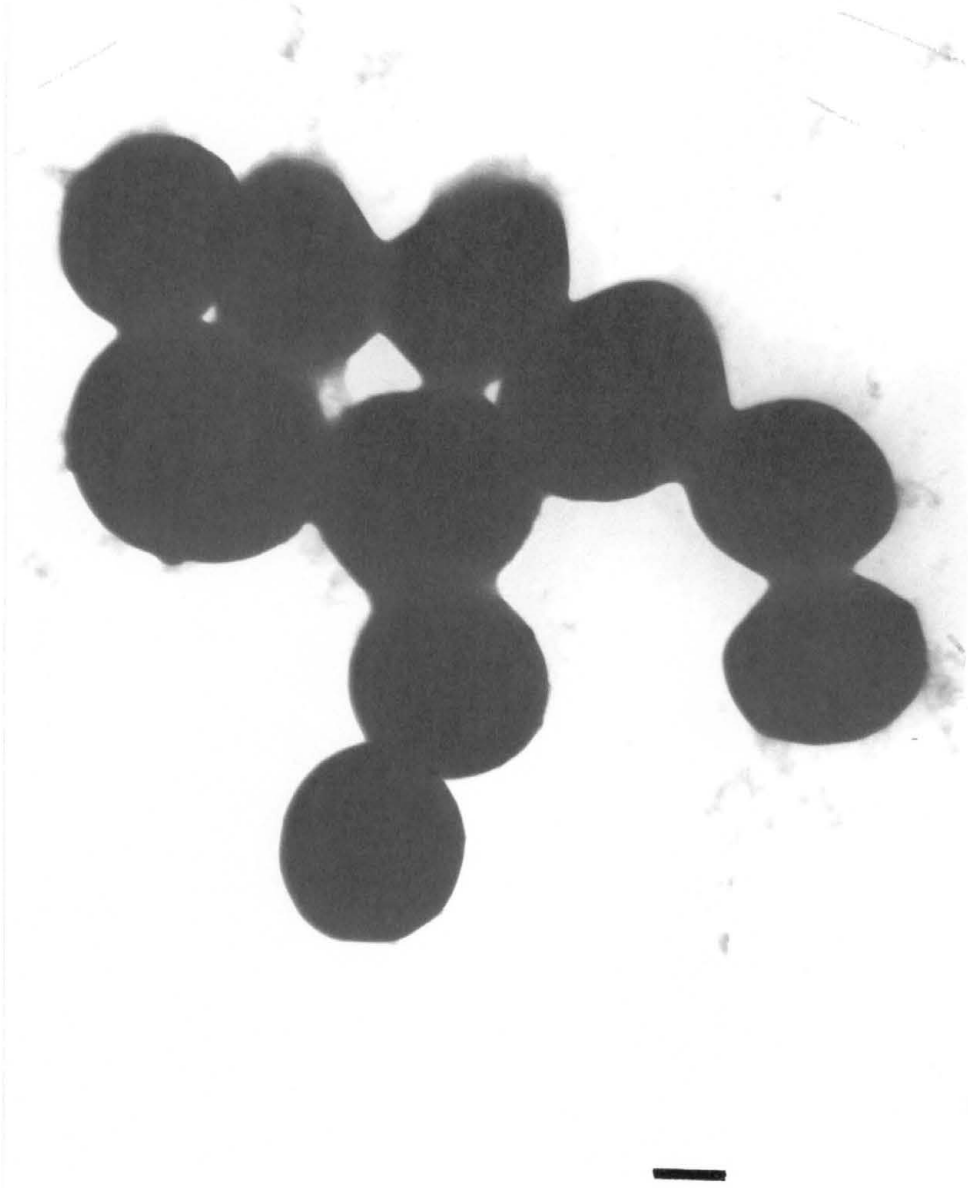

Figure 20
Staphylococcus aureus after freeze-thaw treatment. Observed with TEM. Bar = 200 nm.

Figure 21
Pseudomonas aeruginosa after freeze-thaw treatment. Observed with TEM. Bar = 500 nm.

Figure 22
Staphylococcus aureus filtered through 0.1 μm filter after treatment with probe sonicator. Observed with TEM. Bar = 200 nm.

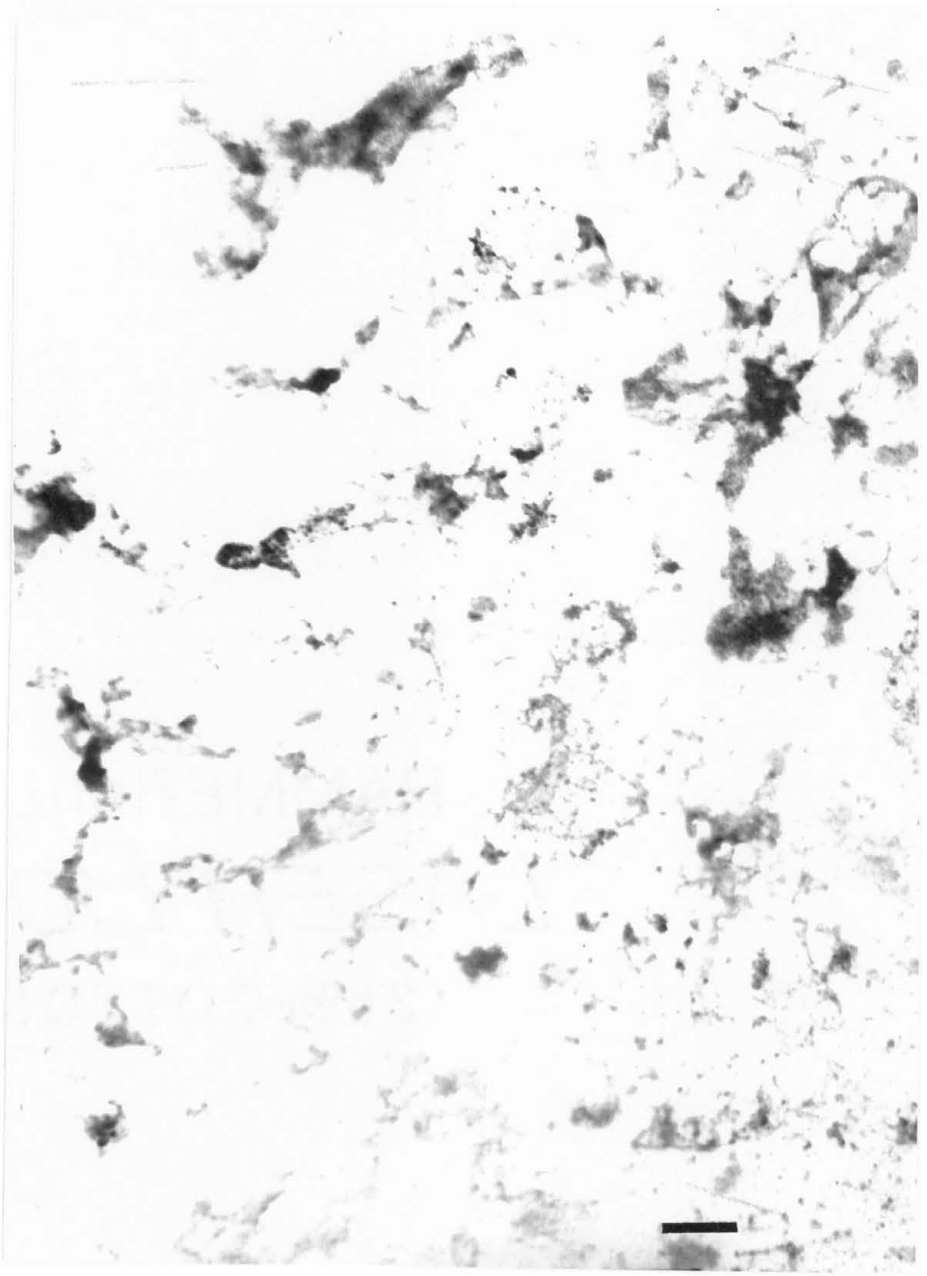

Figure 23
Pseudomonas aeruginosa filtered through 0.1 μm filter after treatment with probe sonicator. Observed with TEM. Bar = 500 nm.

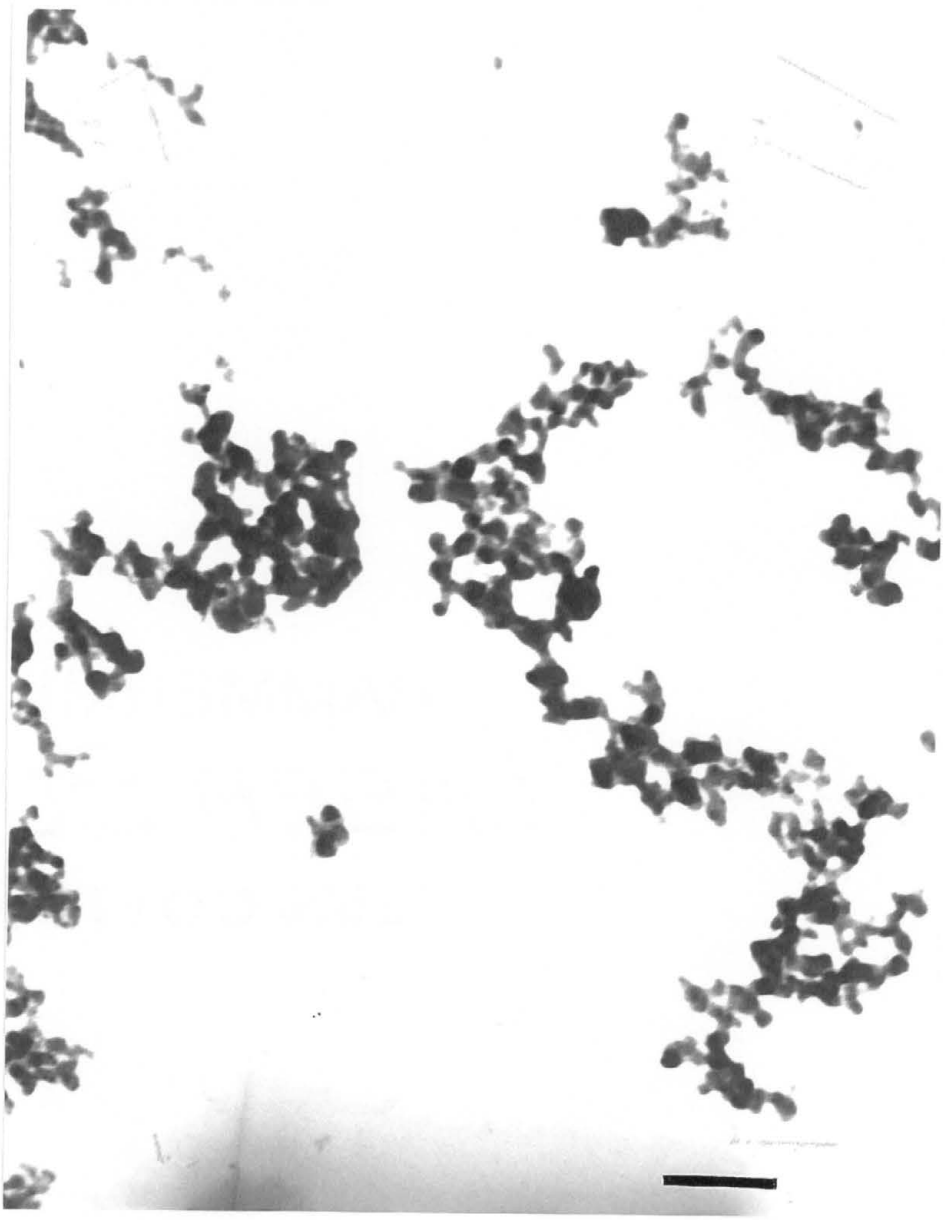

Figure 24
Escherichia coli cellular debris treated with T4 bacteriophage. Observed with TEM. Bar = 100 nm.

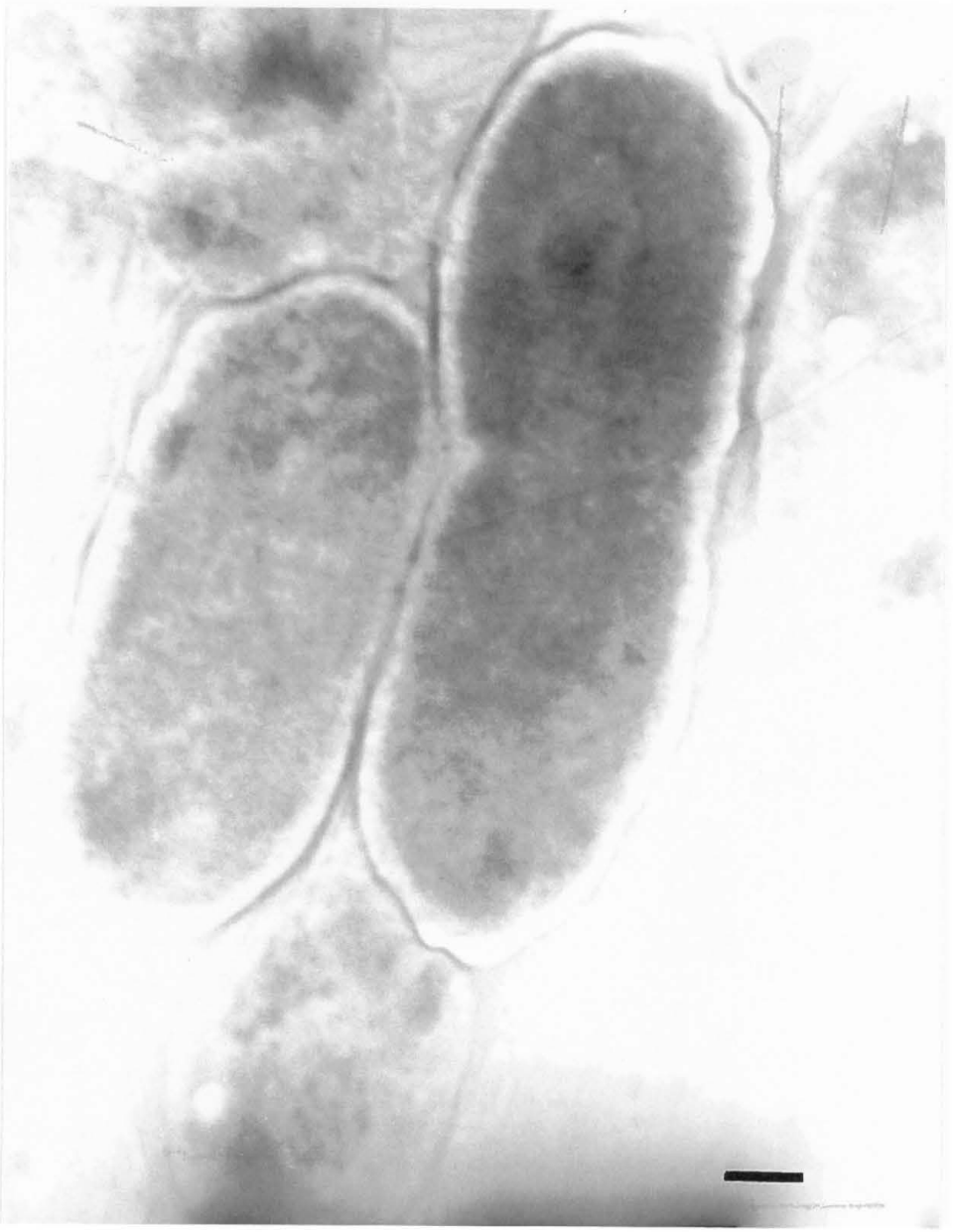

Figure 25
Non-treated *Pseudomonas aeruginosa*. Observed with TEM. Bar = 200 nm.

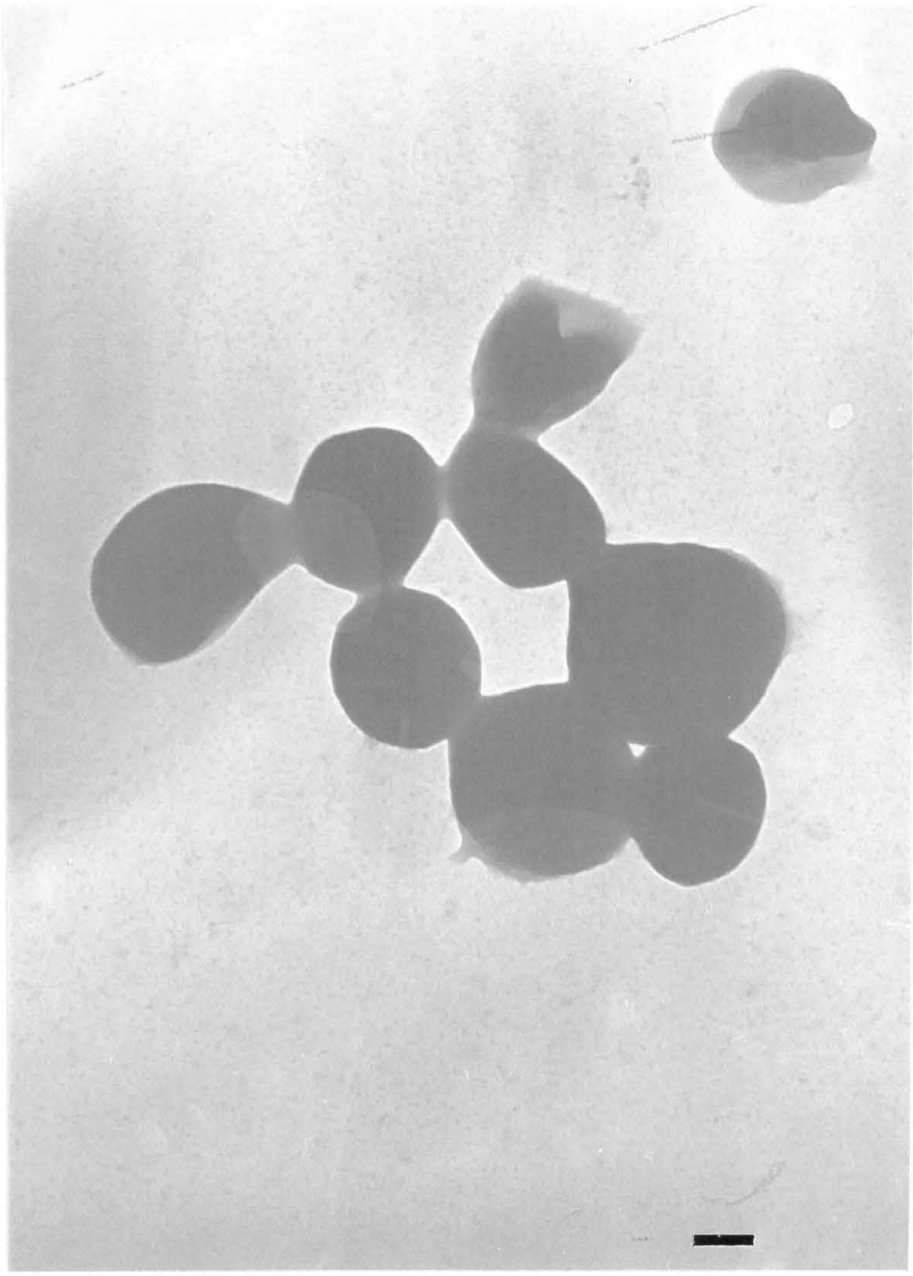

Figure 26
Non-treated *Staphylococcus aureus*. Observed with TEM. Bar = 200 nm.

DISCUSSION

Can NLS be cultured from the environment?

When I started working on this project, I hoped I would successfully be able to culture some of the NLS from the environment, that were observed by SEM. My attempts to grow NLS from different aquatic environments on several common microbiological media were unsuccessful. Although no significant growth was noted on plates with filtered samples, abundant growth was found on plates inoculated with non-filtered samples, indicating that the lack of growth was not due to improper plating techniques.

An interesting phenomena was noted on some of the plates. Agar bubbles appeared in R2A agar plates inoculated with 0.1 μm filtered San Marcos River water, 0.1 μm filtered water from the Le Zitelle Hot Springs from site Bh1d, 0.1 μm filtered pond water, and 0.2 μm filtered water from the Le Zitelle Hot Springs collected from site Ab1d. The bubbles were found on plates after replication, but attempts to transfer the bubbles proved unsuccessful. Several possible causes for the bubbles were considered. They could have been caused by microaerophillic organisms, they could have resulted from nanobacteria, or the bubbles could have been caused by extracellular enzymes found in the water. After the addition of pronase (protease) to the water samples, the bubbles disappeared showing that they were probably caused by extracellular enzymes.

Based on the results from the culture attempts, the tiny structures observed by Folk *et al.* using the scanning electron microscope could have been a number of things including inorganic artifacts, living organisms that were unable to pass through the filters, viable but non-culturable cells (only 2-4% of soil bacteria can be cultured in the

laboratory).[59] A possible reason for the lack of growth may have been that the correct media for growth of the nano-sized structures were not tested. To determine whether the observed NLS were viable but non-culturable cells, metabolic activity studies should be conducted on water that has been filtered to remove "big" bacteria and the presence of nucleic acids should be resolved.

Can NLS be cultured from serum?

At the outset of the serum-culture project, nothing could be found in the filtered tissue culture medium (DMEM), nor could anything be found in the serum. After a very long incubation period, structures that resembled bacteria were observed by transmission electron microscopy. The structures appeared to have membranes and walls, and they fell in the size range for nanobacteria. After checking all the samples and observing the same results, I decided to do a preliminary check for nucleic acids by staining with a bacterial viability stain kit, which uses propidium iodide and SYTO®9. The clumps of cells stained green, suggesting the presence of nucleic acids. My attempt to culture NLS from newborn calf serum yielded positive results.

I then tried to isolate DNA from the cultured NLS by using the freeze-thaw method. Perhaps because *N. sanguinum* have been said to have hydroxyapatite crusts, this attempt was unsuccessful, perhaps due to the hydroxyapatite coat *N. sanguinum* are supposed to have.[32] Further attempts to isolate DNA should be made, perhaps by removing the hydroxyapatite crust with EDTA and subjecting the cells to intense heat (using a microwave oven) to lyse them. (Ciftcioglu, personal communication).

Metabolic activity studies should also be conducted with the serum-isolated NLS. Perhaps this could be performed with radioactive amino acid uptake studies.

Can NLS be found in kidney stones?

SEM imaging of struvite (magnesium ammonium phosphate hexahydrate) kidney stones showed the presence of both *Proteus mirabilis* (which caused the formation of the stones) and NLS. The observed NLS could have been nanobacteria (*N. sanguinum*), *P. mirabilis* fragments (artifacts), or imaging artifacts. It is unlikely that the NLS were artifacts due to microscopy sample preparation because no etching was performed and samples were gold coated for only 30 seconds as recommended by Folk and Lynch.[17] It is possible, however, that the NLS could have been produced by subjection of the *P. mirabilis* to desiccation and to the high vacuum in the SEM. Further studies into the effects of desiccation and vacuum pressure are warranted to rule out this possibility.

Are NLS stressed normal-sized bacteria or bacterial fragments?

When placed under nutrient limited conditions for an extended period of time, laboratory strains of *Micrococcus luteus* (Gram positive cocci) and *Pseudomonas aeruginosa* (Gram negative bacilli) appeared to change morphology and reduce in size. They were not, however, small enough to be nanobacteria. If they were "starved" for a longer period they may have continued to reduce in size. Most of the *M. luteus* appeared to be dividing and the *P. aeruginosa* looked more coccoid than bacillar (see Figures 9 and 25).

When *Staphylococcus aureus* (Gram positive cocci) and *P. aeruginosa* were subjected to 80°C for 20 minutes they both lysed, releasing cell debris. The cell debris

were able to pass through both the 0.2 μm and 0.1 μm filters. It is possible that if the cell debris were treated with enzymes like DNAse, proteases, *etc.*, NLS could be seen.

Treatment of struvite calculi with the holmium: YAG laser produced interesting results. When observed with the TEM, NLS were seen, as were tiny "clothes-peg" looking structures. I was not able to identify these structures were; perhaps they were fragments of the mineral. When the stone fragments were observed with the SEM, NLS were seen at sites away from the laser-produced craters. But, at the blast sites there was an absence of organic matter. The mineral itself appeared altered. This is probably due to the heat produced. Perhaps the heat caused a phenomenon similar to contact metamorphism in which there is a breakdown of organic molecules and a change in the enthalpy of the mineral (for example CO_2 is driven off in contact metamorphism).

Excess calcium and magnesium did not appear to have an effect on the Gram positive organism (*S. aureus*). However, it caused plasmolysis cell shrinkage due to osmosis in the *P. aeruginosa*. When the suspension of cells and calcium / magnesium was filtered through the 0.2 μm filter, tiny membrane-like bound structures were observed that were less than 100 nm in diameter. It is possible that these tiny structures are contaminants, although this is not likely as the structures were not seen in any of the controls. Another possibility for the tiny structures could be membrane blebs or vesicles. Gram negative organisms can produce membrane vesicles of the correct "nanobacteria-size," (Beveridge, personal communication) and plasmids, linear DNA and RNA can be found in membrane blebs. In *Neisseria gonorrhoeae*, wild-type strains have been shown to incorporate the nucleic acids from the membrane blebs.[77]

Solvents also appeared to have no effect on *S. aureus*. They did appear to have lytic effects on *P. aeruginosa*. In all cases, cells appeared to be damaged. Some cell debris were able to pass through the filters, but they did not resemble nanobacteria when observed with the TEM. It would be interesting to observe the fragments with the SEM and compare the images to purported nanobacteria.

P. aeruginosa appeared to react to lysozyme in a similar manner as they did to the solvents tested. The lysozyme-caused fragments resembled images seen after solvent treatment. Lysozyme did not appear to have an effect on *S. aureus*. A different type of lysozyme (one specific to *S. aureus*) should be used.

The freeze-thaw method and the use of a probe sonicator are commonly used to disrupt cells to isolate DNA. The freeze-thaw method did not appear to have an effect on the more resistant *S. aureus*, although, the probe sonicator completely destroyed the Gram positive cells. The cell debris produced were able to pass with ease through a 0.1 μm filter and resembled the cell debris produced after subjecting both organisms to 80°C.

The freeze-thaw method had an effect on *P. aeruginosa*, but the cell debris were not able to pass through the filters. The probe sonicator completely destroyed the cells, and the cell debris passed through both filters. The cell debris from both bacteria could easily be mistaken for NLS when examined with a SEM.

Treatment of a biofilm with a bacteriophage produced images that resembled the cell debris that was produced when *S. aureus* and *P. aeruginosa* were exposed to extreme heat and to the probe sonicator. The presence of the cell debris-looking structures in the biofilm after exposure to the bacteriophage gives validity to my calling the structures observed after treatment with heat and the probe sonicator, cell debris.

CONCLUSION

I was not able to culture nanobacteria from aquatic environments where previous SEM images showed the presence of NLS. Alternatives for the observed NLS may be imaging artifacts, bacterial fragments (such as those produced by altering the osmotic pressure in the environment of *P. aeruginosa*), and viable but non-culturable bacterial cells. The NLS may also be nanobacteria. It is possible that the "correct" media to grow NLS was not tested.

Further culture studies should be conducted using a wider variety of media (both liquid and solid) and a wider variety of incubation temperatures. NLS have been observed in waters from hot springs with temperatures above 50° Celsius.

The newborn calf serum study results suggest that the NLS observed from serum and in kidney stones may exist as independent life-forms (such as *Nanobacterium sanguinum* and possibly others as of yet unidentified). Cell-like structures were observed in cultures where nothing was initially present. They were probably not precipitates as the structures could not be found in the controls and when stained with a fluorescent DNA stain, the structures fluoresced. I replicated, in part, the results from some of the experiments conducted by Kajander, *et al.*[5,30,35,36]

To provide further proof for the existence of *N. sanguinum*, it will be necessary to conduct metabolic activity studies (such as nutrient uptake and nutrient utilization studies) and to determine the presence of nucleic acids. To determine if *N. sanguinum* cause kidney stones it will be necessary to infect an animal model with cultured *N. sanguinum* and look for the presence of calculi formation, following Koch's postulates.

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