Invited Paper

Mineralization by Nanobacteria

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ABSTRACT

Nanobacteria are the smallest cell-walled bacteria, only recently discovered in human and cow blood and in commercial cell culture serum. In this study, we identified with energy-dispersive X-ray microanalysis and chemical analysis that all growth phases of nanobacteria produce biogenic apatite on their cell envelope. Fourier transform IR spectroscopy revealed the mineral as carbonate apatite. Previous models for stone formation have lead to a hypothesis that an elevated pH due to urease and/or alkaline phosphatase activity are important lithogenic factors. Our results indicate that carbonate apatite can be formed without these factors at pH 7.4 at physiological phosphate and calcium concentrations. Due to their specific macromolecules, nanobacteria can produce apatite very efficiently in media mimicking tissue fluids and glomerular filtrate and rapidly mineralizing most of available calcium and phosphate. This can be also monitored by ^{85}Sr incorporation and provides a unique model for in vitro studies on calcification.

Recently, bacteria have been implicated in the formation of carbonate (hydroxy) fluorapatite in marine sediments. Apatite grains are found so commonly in sedimentary rocks that apatite is omitted in naming the stone. To prove that apatite and other minerals are formed by bacteria would implicate that the bacteria could be observed and their actions followed in stones. We have started to approach this in two ways. Firstly, by the use of sensitive methods for detecting specific bacterial components, like antigens, muramic acid and nucleic acids, that allow for detecting the presence of bacteria and, secondly, by follow-up of volatile bacterial metabolites observed by continuous monitoring with ion mobility spectrometry , IMCELL, working like an artificial, educatable smelling nose. The latter method might allow for remote real time detection of bacterial metabolism, a signature of life, in rocks via fractures of drill holes with or without injected substrate solutions.

Nanobacteria may provide a model for primordial life-forms, such as replicating clay crystallites in a sandstone, where minerals and metal atoms associated to membranes, may play catalytic and structural roles reducing the number of enzymes and structural proteins needed for life. Such simple metabolic pathways may support the 10,OOO-fold slower growth rate of nanobacteria. as compared to the usual bacteria. They may also explain the endurability of this life-form in extreme environmental conditions. Altogether such properties do suggest that nanobacteria may have evolved from environmental sources, such as hot springs, to take advantage of the steady-state calcium and phosphate supply of the mammalian blood. Based upon our findings of nanobacteria, a novel theory for the early development of life, based on apatite-mediated chemistry on membranes selecting itself for its own catalytical machinery , is presented.

Keywords: nanobacteria, nannobacteria, mineralization, stone formation, detection of life, carbonate apatite, life-forms

1. INTRODUCTION

When E. Olavi Kajander, helped by several enthusiastic students, discovered 10 years ago carbonate/hydroxyapatite mineral-forming organisms in blood and sera for cell culture, such a discovery was unacceptable for the scientific (microbiological) community, because the results were against half a dozen basic 'truths' of microbiology. Most importantly, these autonomously replicating micro-organisms were approaching the theoretical limit of a self-replicating life with a size of only one hundredth of that of usual bacteria.[1,2,3] Their metabolic rate was at least 10,000-fold 'too slow'. They could not be detected with official sterility testing methods, but we were claiming that they caused chronic bacteremia in some humans, and commonly in cows, and that they were the most common cell culture contaminant around the world. At that time, it was also unacceptable that bacteria could cause mineral formation in humans, although mineral formation is present in some 20% of human diseases (see Ciftcioglu, Björklund and Kajander, in this issue) for unknown reasons. Furthermore, the organisms were shown to be resistant to heat and gamma-irradiation, unlike human microbial flora seen before. Their extraordinary nucleic acid properties further increased public rejection to their exsistence and decreased our grant funding to zero.

Things can change even in microbiology .A hidden biosphere has been discovered in recent years from rocks and water. The bacterial life may reach some 5 km deep, but its main part is in shaping the sedimentary rocks near to the surface. Much of this bacterial metabolism and function is very unlike that of previously known organisms, and is related to the extremely slow mineralization of inorganic and organic compounds available. Yet it is now accepted that the geochemical influence on Earth must have been enormous.[4] From such biota, morphologically similar organisms to our tiny nanobacteria had been discovered independently from us, at about the same time by Dr. Folk, in sedimentary rocks and in hot-springs in travertine. Those he had given the name nannobacteria.[5] Such bacteria seem to contribute to the formation of many kinds of carbonate minerals. These bacteria may secrete molecules acting as nucleation centers for depositing specific biogenic minerals in aquatic environments.[6] In a similar way, nanobacteria from blood surround themselves with apatite deposits.

The isolated nanobacteria were named as *Nanobacterium sanquineum,* referring to their surprisingly small size and apparently vivid movements, and to their existence in blood. The organism was reposited in the German Collection of Micro-organisms (DSM No: 5819-5821). These organisms were isolated from 'sterile' commercial human and bovine sera. Fetal bovine serurn was found to be ubiquitously contaminated with nanobacteria so that about 80% of serum batches contained culturable organisms. Nanobacteria were found in about 5% of human serum samples.[7] Thus nanobacteria are the most common cell culture contaminant and a major cause for bacteremia. Nanobacteria could not be cultured in standard microbiological media, but could grow in commercial mammalian cell culture media at 37-45°C under 5-10% CO_2 -90- 95% air. Anaerobic cultures produced no growth. Based upon 1406 base long 16S rRNA gene sequences, deposited now in a gene bank (EMBL Entry X98418 and X98419), nanobacteria belong to the alpha-2 subgroup of Proteobacteria, distinct from their nearest relatives which are Phyllobacteria, Thiobacillus, Brucella, Bartonella, Rhizobia and Agrobacterium genera.[8] Furthermore, antibodies against nanobacteria cross-reacted with *Bartonella henselae* and *quintana,* and antibodies against *B. quintana* cross-reacted with nanobacteria in immunoassay. Thus, the known human pathogens Bartonel1a and Brucel1a are relatives of nanobacteria. Al1 share certain common properties: they are extremely difficult to culture, impermeable to stains and invade mammalian tissues and cel1s, sometimes with a preference for fetal tissues. Brucel1a is known to pass into urine, and the Kuopio research group has recently proven that this also happens with nanobacteria.[9] Al1 three can cause chronic bacteremia unlike that of any other known bacteria: huge nurnbers of bacteria are present in blood of patients having relatively little symptoms. Bartonella species have recently been shown to cause chronic bacteremia in almost half of the Earth's cats[10] and rodents[11]. Thus nanobacterial bacteremia has now first kinship by 'acceptable' bacterial species (however, in the past many bartonel1as were officially removed from the list of accepted bacteria and remained 'unacceptable' for several years). When this is understood by the microbiological society, these findings will change the microbiological 'truths'. Interestingly, 16S rRNA genes seem to indicate that mitochondria were derived from an ancestor of this bacterial group. Maybe it is possible to find or even develop more symbionts from this group? At least for tamed organisms of this group, a significant therapeutic potential can be seen: bartonel1as could be used for angiogenesis and nanobacteria for bone formation.

Nanobacteria are one of the most distinctive organisms ever found in humans. Their poor culturability and long doubling time, and cytotoxicity[12] can be compared only to some Mycobacteria, such as *M leprae.* The average diameter of nanobacteria measured with electron microscopic techniques, about 0.2 μm, is smaller than that of large viruses, e.g. *Vaccinia,* and the smallest nanobacteria capable for multiplication have sizes as small as 0.05 μm. The theoretical minimum diameter of a cell, based on size of those macromolecular components now considered to be necessary and sufficient for a living cell, has been calculated to be about 0.14 μm.[13, 14] Apparently, the minimum calculations need correction for reasons discussed later in this paper. Nanobacteria are highly resistant to heat and γ-irradiation, and thus are the first example of such organisms isolated from mammalian tissues. Bacteria with these properties are generally found in environments where these traits are essential. Nanobacteria must have emerged from a special environmental source, such as hot springs, perhaps only recently in evolutionary terms.

2. ALL FORMS OF NANOBACTERIA PRODUCE APATITE

Figure 1 (A and B) shows the several micrometer thick apatite formation, a castle, made and inhabited by a small community of nanobacteria. The cavity in the center of the apatite castle indicates its biogenic origin. Apparently, the openings of the cavities were facing the bottom of the culture vessel before scraping. Thus, the apatite shelters provided complete protection for the organisms. Organisms released from their castles also indicate apatite structures directly on their surfaces (Fig. 1 E-F). In addition, tiny vesicles about 50 nm in size can be seen outside the organism. The big organisms produce these small forms that we now call elementary particles, because they are the smallest forms still capable fir independent growth and mineralization. Such vesicles resemble those released by chondrocytes during mammalian bone formation.[15] Since our culture system has no mammalian cells, and such elementary particles can be observed also in serum- free conditions, any resemblance must be coincidental. Morphologically and chemically similar apatite mineral was found on the surface of the small nanobacteria cultured in serum containing medium (Fig. 1 C-D). Only the formations were smaller.

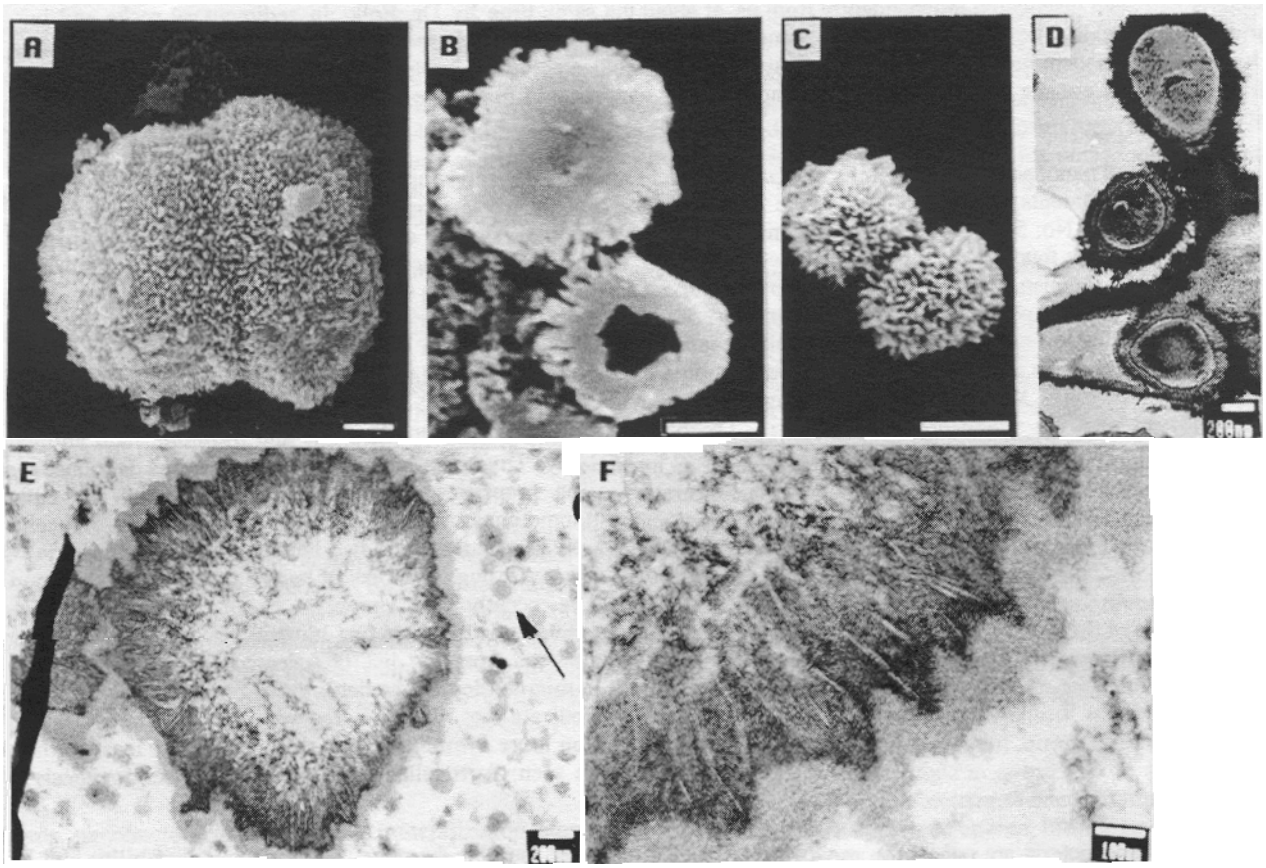

Figure 1. SEM (A-C) and TEM (D-F) micrographs of nanobacteria indicating how they produce apatite mineral on their surface. (A) Nanobacteria 'castle' from serum-free culture depicting the top mineral structures of a culture vessel-adhered group of organisms. (B) Similar castles with cavities as 'homes' for the organisms depicted from bottom. (C) Nanobacterial surface from culture with serum. (D) Acicular mineral crystals grow directly on the surface of serum cultured nanobacteria presented in (C). (E) Ultra-thin section of nanobacteria from serum-free culture showing 'hairy' mineral apparently associated with its cell membrane. The arrow shows 50-100 nm-sized coccoid elementary particles that may be responsible for the smallest self-replicating life on Earth. (F) Acicular mineral structure shown in (E) in detail. Bars: (A-B) 1 μm, (C, F) 0.1 μm, (D-E) 0.2 μm.

Chemical analysis using EDX (energy-dispersive X-ray microanalysis) gave Ca and P peaks similar to that detected for hydroxyapatite (Fig. 1 H and I). Cultures of the human isolate gave identical results (not shown). Chemical analysis of nanobacteria harvested after a 3-month culture period revealed a high content of inorganic material. The pellet dry weight varied from 23% to 39% and consisted of: N 1-1.3%, P 12.3-14.6%, Ca 23.4-23.5%, Mg 1.4-1.9%, K 0.1%, and Na 1.2- 1.4%, suitable for the presence of apatite in large proportions in the material. FTIR (Fourier transform IR spectroscopy) revealed that carbonate form of calcium apatite was present in samples from all cultural ages between 7-180 days in both human and bovine nanobacteria.[16] The analytical methods do not exclude the possible presence of minor quantities of other mineral phases. To exclude that possibility, crystallographic analysis are needed. Dr. H. Vali from McGill University is performing currently these studies, and he has found apatite lattice fringes indicative for apatite on our nanobacteria. Nanobacteria did not produce urease or alkaline phosphatase activity , and their culture medium remained at pH 7.4. This suggests that the mineral is formed on biogenic macromolecules on the surface of nanobacteria, not by simple physical precipitation due to a pH change.

3. MINERALIZATION IS RAPID UNDER 8ERUM FREE CONDITIONS

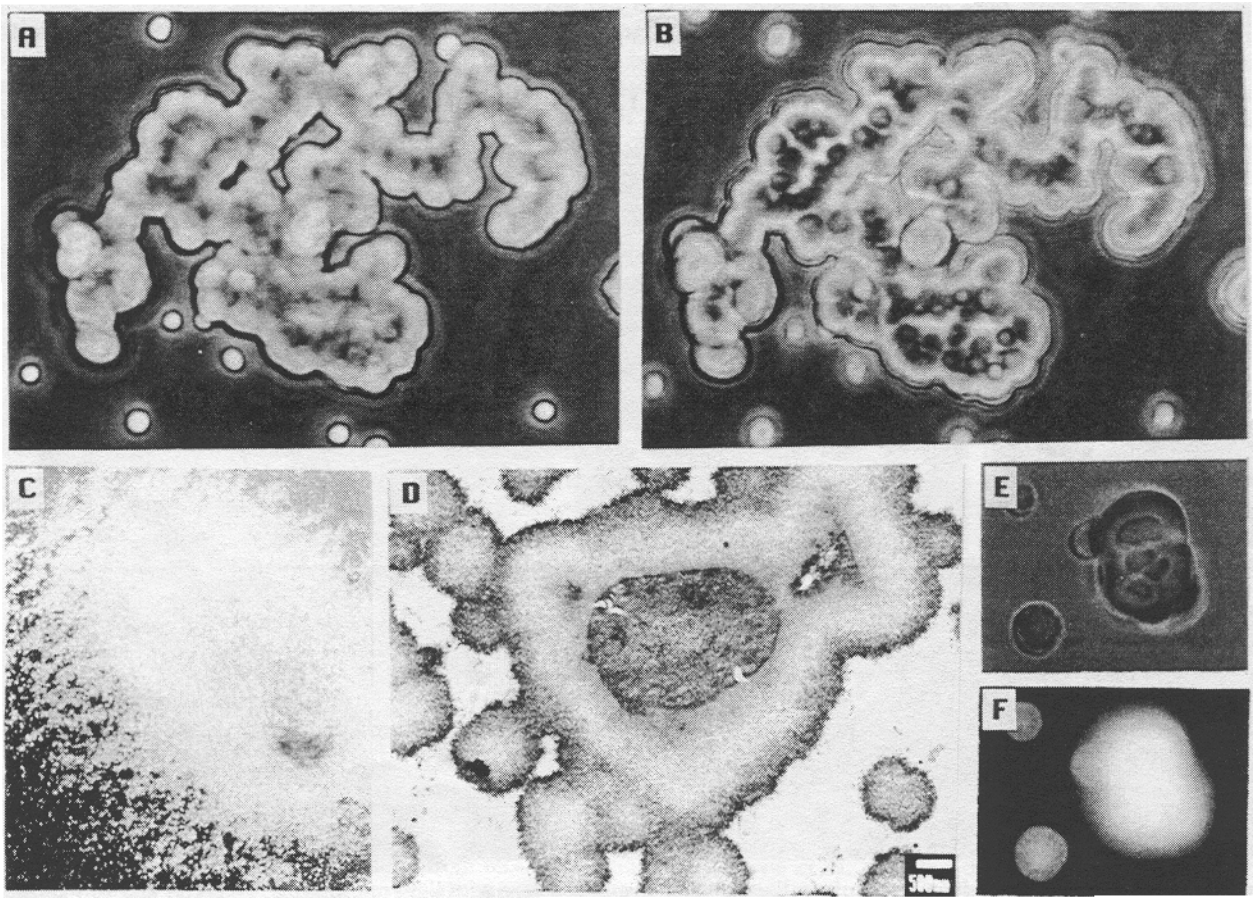

Figure 2. Light and electron microscopic images of serum-free nanobacteria and their immunostaining localization. (A) and (B) show light microscopy of a massive multicellular nanobacterial formation at two different focus planes. The interior of the formation harbors numerous large nanobacteria with mineral on them. (C) A colony-like formation of optically dense nanobacteria from a serum-free condition. (D) TEM micrograph of serum-free nanobacteria showing that extensive mineral formations are budding with a unique mechanism. (E) A light microscopy image of 2-month cultured nanobacterial castle showing interior compartment with the organisms. (F) Nanobacteria from the previous sample as stained with FITC-anti-nanobacterial monoclonal antibody. Control experiments with nonrelevant antibodies remained negative. Magnifications: (A-B) 800x, (C) 80x, (E-F) 1000x, (D) bar 500 nm.

When washed nanobacteria were subcu1tured in DMEM, bottom-attached coccoid organisms were observed within one day. DIC microscopy revealed a several-micrometer-thick mineral layer around each nanobacteria reaching the size of a yeast cell within one week and that of an erythrocyte in 2-3 weeks (large dots in the background of Fig. 2A). Fig. 2 A and B show large multicellular communities of such nanobacteria. Mineral formation makes them easily visible without any staining or phase contrast, as shown with a low magnification in Fig 2 C. The apatite formation was rapid: approx. half of the medium calcium was bound in one week, as measured by AAS. ^{85}Sr was incorporated to the mineral, but at a lower efficiency than calcium. Incorporation decreased after one week, obviously because of the very small free calcium and phosphate levels in the medium. Accordingly, when cultures were refed, mineralization continued at higher extent than without refeeding, see Fig. 3 E and D, respectively.

Ultrastructure of nanobacteria under serum-free conditions (Fig. 2 D) differed extensively from the coccoid nanobacteria found in serum culture, but similar DNA stainability was observed and the same surface antigens could be detected with monoclonal antibodies,12 as shown in Fig 2 E and F for the serum-free ones. They produced biomass at about half of the rate observed in serum containing cultures. The metabolic incorporation of ^{35}S methionine and 5-^{3}H uridine is proof that they were replicating.[8] The cultures could be passaged monthly for over 5 years and always followed a similar growth pattern. The human isolate produced similar formations.

4. NANOBACTERIA CAN PRODUCE SMALL AND LARGE APATITE FORMATIONS

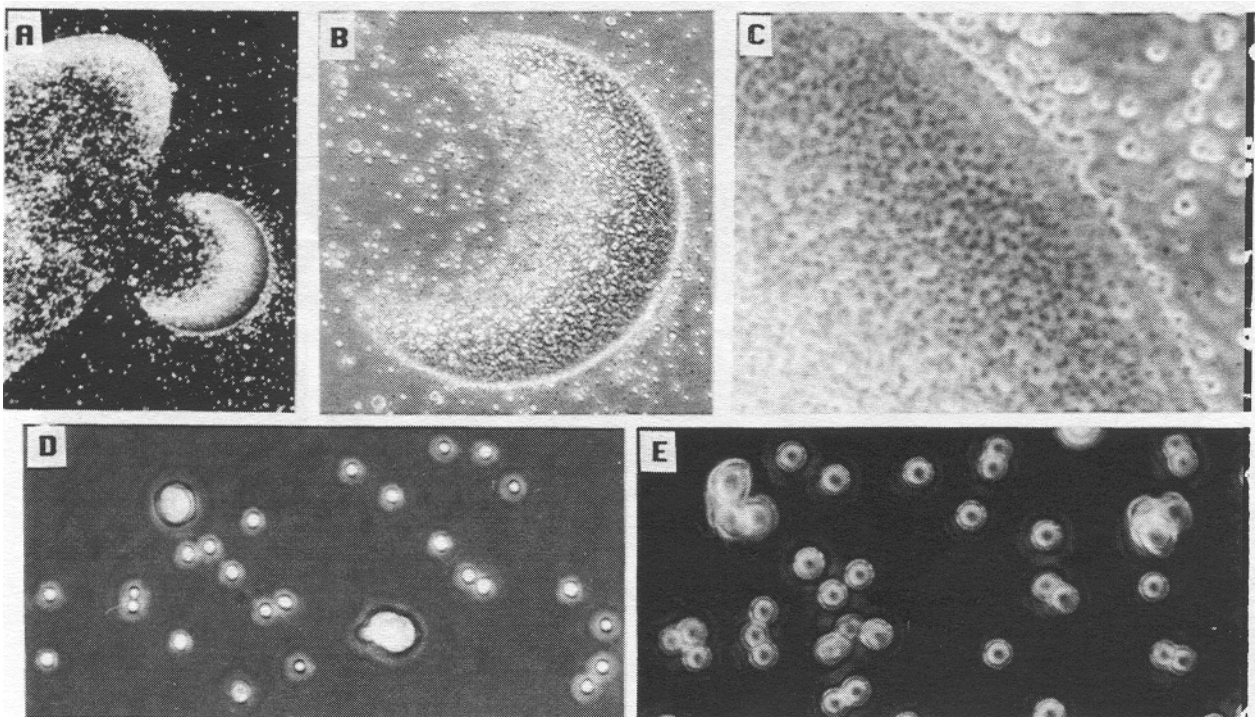

Figure 3. Nanobacteria can produce large mineralized objects visualized here with light microscopy. (A), (B) and (C) show details of mineralized colony-like formations. (D) and (E) show the effect of refeeding on the mineralization, (D), l-month cultured organisms without refeeding, (E), with weekly refeeding by replenishing all their culture medium. Magnifications: (A) 80x, (B) 200x, (C-E) 800x.

Nanobacterial culture systems allow for the production of apatite ca1cifications *in vitro.* Depending on culture conditions, tiny nanocolloid-sized particles covered with apatite, or biofi1m, sand, stones and tumor-like growths of apatite could be produced. The precondition for mineralization was low levels of intact serum in the culture medium. Serum contains powerful proteinaceous inhibitors of apatite crystal formation, osteopontin, osteocalcin[15] and fetuin,[17] which may account for the observed inhibition of mineral formation in the presence of serum. Mineralization increased in parallel with the dilution of the serum in cell culture medium. Under serum-free conditions, the apatite formation was extensive and rapid. Although modified Loemer medium contains 75% serum, the serum proteins were denatured during the sterilization steps. Thus, apatite formation was not inhibited resulting in solid apatite colonies about 1-5 mm in diameter in 6 weeks.18 Living nanobacteria are needed to produce apatite in the nanobacterial model. γ-Irradiated nanobacteria did not multiply and produced no real calcifications even after 6-month long incubations.

5. NANOBACTERIA AS A MODEL FOR PRIMITIVE LIFE-FORMS

All biologists now agree that bacterial cells cannot fonn from non-living chemicals in one step. If life arises from non-living chemicals, there must be intennediate fonns, precellular life. Of the various theories of precellular life, the most popular has been that ofthe RNA world. RNA has the ability to function as both genes and enzymes, and can be transcribed into DNA. Because synthesizing nucleotides and achieving replication of RNA under plausible prebiotic conditions have proved so challenging, chemists are increasingly considering the possibility that RNA was not the first self-replicating molecule. There is a proteins first school, but it has not solved the replication problem. A.G. Cairns-Smith[19] has proposed that clay crystals could have served as the scaffolding upon which the first short DNA or RNA genome was constructed. Initially, colloidal clays would have provided the main materials out of which organisms of a first kind were made, and organic molecules had little if any part to play right at the beginning. These mineral organisms evolved modes of survival and propagation that would have seemed highly engineered. They became a form of life. The minerals would have been forming on the primitive Earth as they do on the Earth now. Some evolved primary organisms started to make organic molecules through photosynthesis. This led to organisms that had both inorganic and organic genes. Eventually the control of their own synthesis passed entirely to the organic genes composed of nucleic acids which by now operated through the synthesis of protein. [19]

Nanobacteria are colloidal-sized and have apatite as a part of their cell wall. They are thus the first examples of mineral organisms which could be similar to the more-developed primitive life-forms proposed by Cairns-Smith.[19] We see apatiteas an important addendum to the clay theory for solving several very important problems in a primordial life in its phase of genetic overtake. Current life on the Earth is based on phosphate-mediated biochemistry using mainly C, N, O and H. Organic phosphate compounds are a fingerprint of life, and this fact must have far-reaching reasons, since other ways fir high energy bonding and storage could be available. We are

putting forward an apatite theory of early chemical life. Our apatite theory is saying membrane-associated surface chemistry was first. The metabolic pathways with phosphorylated metabolites selected their own catalysts that later developed into more efficient enzyme proteins. Enzymes were made initially probably as random peptides in an analogous way to DNA expression libraries used as a tool in modem biotechnology .The nucleic acid machinery may have originated in apatite-mediated chemistry or came from extraterrestrial sources.

We are claiming that tiny cell-like vesicles with membrane-associated apatite mineral, initially developed the chemistry of life, the metabolic pathways for synthetic reactions necessary for their own function and replication. Apatite is unique in this respect since it is associated also nowadays with membranes, e.g. in bone formation, and apatite can co- crystallize with membrane components, such as cholesterol or phospholipids, in *in vitro* and apparently also in ossification process *in vivo*.[15, 20] We think that apatite may have formed parts of the membranes even in the very beginning of life. The primordial lipids might have come from hydrocarbons found nowadays even in the interstellar material.

The initial catalysts were metals and minerals and associated organic molecules. Apatite may act as the scaffold in several ways: (1) Apatite could immobilize many reaction intermediates like growing polymers, and to a lesser degree the necessary substrates. Binding would be enhanced by charged metals and minerals associated with apatite. Phosphorylated intermediates would be bound on cationic groups. Binding increases with the length of the polymer. The tremendous importance of immobilization is obvious from recent experiments by Ferris *et al.* showing that protein and nucleic acid polymers can be produced without enzymatic catalysis at room temperature, only if the polymers are bound to a solid phase.[21] The minerals do not necessarily have to be catalytic, but substrate-mineral surface catalysis would be advantageous. In solution, hydrolytic reactions surpassed the synthetic ones and no large polymers could be obtained. Apatite was successfully used for the nonenzymic formation of aspartic acid polypeptides. Importantly, apatite is also known to bind some nucleic acids. (2) Apatite is a storage form of phosphate providing the high local phosphate concentration necessary to drive uncatalyzed phosphorylation reactions that probably took place, e.g., driven by very high temperatures. The phosphate-activated reactants could thus be concentrated around the apatite-immobilized polymers resulting in favorable conditions for synthetic reactions. Amorphous apatite has easily dissolvable phosphate, and functions as a phosphate storage providing ways for a homeostatic control of phosphate blood levels even in higher life-forms nowadays.[15] Amorphous apatite is plentifully present in biological systems and co-exists with crystalline hydroxyl and carbonate apatite. Amorphous apatite may not be found in geologica1 processes since, over time, it will spontaneously form the crystal line and insoluble forms of apatite, so no signs of it may remain in sedimentary rocks. Processes resulting in formation of amorphous apatite may offer a solution for the enigmatic source of phosphate for the primordial life that is easier to understand than the suggested extraterrestrial sources, such as meteorites.[22] (3) Crystalline apatite provided a highly organized platform where metals and other mineral catalysts could be bound in a reproducible way. This made metabolic pathways possible. Apatite-bound cata1ysts are protected from heat denaturation. This may explain the exceptional ability of nanobacteria to withstand high temperatures. It would permit life to be formed near boiling temperatures. This may offer also explanations to the question why apatite should act as a safe-deposit vault[23] for the 3.8 billion years old Earth's oldest bacterial fossils discovered by Mojzsis and others[6] in banded iron formations. We propose that the hypothetical inorganic catalytic apparatus would function optimally if reactants and/or temperatures would vary cyclically, analogous to a thermal cycler used for PCR, creating conditions for the synthetic cycles to take place.

We think that such a system would have been automatically selected for improved catalysts, since primordial cells equipped with such would have multiplied faster. The vesicles could have passed the nongenetically inherited metabolic pathways to daughter vesicles, simply by budding and transferring the metabolic pathways on membrane-associated apatite crystals. The apatite units were self-replicating in that meaning that they guided the self-assembly of a catalyst system. Increasing the ability to bud daughter cells might have been a major driving force for early evolution. Membrane synthesis involves complex metabolism, but only those cells capable for that, could have fulfilled the Earth. Vesicles capable for different metabolic reactions could also have fused, allowing for a more advanced metabolism. Such evolution would have become very fast when nucleic acids overtook the system, providing unlimited variation in catalyst design.

The proposed theory can be put to the test. Present data suggest that nanobacteria may partially continue the primordial type of metabolism. Since their metabolic rate is 10,000-fold less than that of usual bacteria and their metabolic capacity is limited, e.g., they need amino acids from culture medium, they may depend largely on nonenzyrnic catalysis in performing their functions. Their slow metabolism means that they cannot compete with usual bacteria and need their own ecological niche that is hostile for the usual forms of bacteria. Such conditions can be present in hot springs, interiors of stones or mammalian tissues. Such a strategy would reduce considerably the need for genes necessary for life. This has not been considered when genes necessary for smallest bacterial life have been calculated as being about 256.[14] Nanobacteria that withstand repeated exposures up to boiling point temperatures (see Björklund *et al.,* in this issue) may prove to be the best available model for a relatively primitive life in primordial soups, simply because they have preserved some of the primordial tactics for life.

6. NANOBACTERIA AND METHODS FOR DETECTING LIFE-FORMS

Under the protection of apatite castles nanobacteria are almost indestructible, but they are limited to a very slow metabolic activity .It is noteworthy that nanobacteria cannot be detected by any of the standard sterility testing methods. Sensitivity and severe other obstacles are hindering the use of standard methods, and even the most sensitive methods, like PCR, suffer from the matrix problems caused by the stone material.

What kind of biomarkers should be looked from apatite mineral inclusions in stones? Firstly, apatite itself, since carbonate apatite in aqueous environments can be a bacterial product.[6] Bacterial colony-sized apatite grains are so commonly found in sedimentary rocks that apatite is omitted in naming the stone. Phosphate and calcium are consumed by living organisms very rapidly. Formation of apatite crystals on bacteria specifically binding these elements may be a possible explanation fir the presence of apatite. According to our hypothesis similar mechanism also produces the human kidney stones.[16, 24] Secondly, apatite preserves exceptionally well biomolecules, e.g., bacterial fossils for 3850 million years[6], and protein[25] and nucleic acid macromolecules for at least 25,000 years under normal Earthly conditions.

Nanobacterial cultures should be good models for finding biomarkers for present and ancient colonization of stones by bacteria. The sensitivity of methods for detecting specific bacterial components and follow-up of volatile bacteria1 metabolites could be tested with this challanging target. Ion mobility spectrometry with novel IMCELL, working like an artificial educatable smelling nose, might allow for remote real time detection of bacterial metabolism, a signature of life, in rocks via fractures of drill holes with or without injected substrate solutions. Fig. 4 shows its principle and the preliminary results obtained from nanobacterial cultures. Two channels out of 6 indicated significant signal difference between nanobacteria and their culture medium. This is a promising method for monitoring living organisms, since it is mobile and gives real-time results. We have already shown the efficiency and sensitivity of immunoassay for detecting specific 1ifeforms, e.g., in kidney stones. Muramic acid is a major component of bacterial cell walls, it is bacteria-specific, and extremely sensitive new methods allow detection of only 10 pg quantities from samples like 1 mg house dust. [26] Muramic acid can be detected from stones extracted with strong HCI at over boiling temperatures. Thus, these components may be detectable even in ancient samples subjected to metamorphosis. PCR and fluorescent stains for nucleic acids allow for detection of bacteria and their typing. At least in nanobacterial case, they may be exploited with limitations, as also culture, even for stones subjected to HCI etching or extraction, opening thus a new dimension in the testing possibilities.

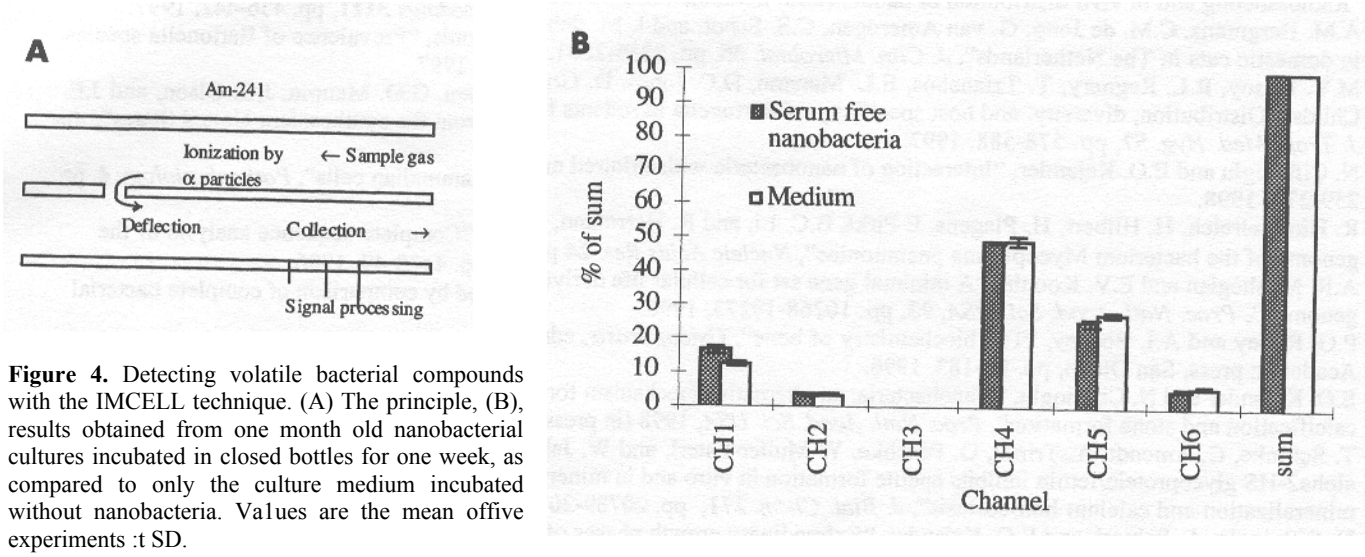

Figure 4. Detecting volatile bacterial compounds with the IMCELL technique. (A) The principle, (B), results obtained from one month old nanobacterial cultures incubated in closed bottles for one week, as compared to only the culture medium incubated without nanobacteria. Va1ues are the mean offive experiments :t SD.

7. CONCLUSIONS

Nanobacteria are slowly growing organisms capable of independent life with only a very limited metabolic capacity .They are the first mineral forming organisms with carbonate apatite directly attached to their cell membrane. Their mineral formation is controlled and provides a model for mineralization. Nanobacteria have adapted, apparently recently to living in mammalian blood and tissues. They provide a model for pathological calcifications. Their special properties suggest that they may serve as a model for primordial life-forms. Nanobacteria with their apatite membranes may open important clues for how life started in rocks and sediments, and how life might have spread in the solar system. As the discoverers of nanobacteria, we have tried to launch research on such paramount themes, but while doing so, have only obtained disbelieving rejection. Primordial funding policies should become extinct.

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