

Invited Paper

Nanobacteria as extremophiles

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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. Nanobacteria do show several extreme properties, such as growth in over 5 % sodium chloride, small size, very slow growth rate and ability to survive under unfavorable conditions, including lack of nutrients, boiling temperature, high pressure and 1.5 Mrad dose of gamma irradiation. Environmental changes can cause drastic changes in their unit size: under unfavorable conditions they form very large multicellular units. Yet, they can release elementary particles, some of which are less than 100 nm in size, smaller than many viruses. Although metabolic rates of nanobacteria are very slow, they can produce carbonate apatite on their cell envelope mineralizing rapidly most of the available calcium and phosphate. Nanobacteria belong to, or may be ancestors of, alpha-2 subgroup of Proteobacteria. They may still partially rely on primordial life-strategies, where minerals and metal atoms associated to membranes, played catalytic and structural roles reducing the number of enzymes and structural proteins needed for life. Simple metabolic pathways apparently only compatible with life in very small cells, may support the 10.000-fold slower growth rate of nanobacteria, as compared to the common bacteria. Simplistic life-strategy may also explain the durability of this life-form in extreme environmental conditions.

A new hypothetical extremophile property may link nanobacteria from blood to the forms found in rocks: 'diminuthophilic' nature. This means that nanobacteria can produce extremely tiny units that allow for penetration through pores smaller than the diameter of intact replicating bacteria. This may enable them to colonize new areas in biological tissues, which essentially have compartments separated by microporous 'sieves'. Endothelial linings are essentially sieves of various porosity, the smallest ones being in kidney, and blood-brain and blood-testicle barriers. Similar life strategy may be advantageous in porous rocks: sedimentary rocks have extremely small pores allowing for water penetration. Nanobacteria may inhabit new territory in such rocks by releasing tiny units capable of penetrating through the pores. This hypothesis involves the revolutionary idea that (nano)bacteria may fragment into numerous tiny units that later can be reassembled, e.g. by their surface-mediated aggregation, to produce a fully competent replicant.

Keywords: nanobacteria, nannobacteria, extremophiles, diminuthophiles

1. EXTREMOPHILES

Extremophilic bacteria tolerate and thrive at extreme conditions, such as temperatures up to 117°C or below the freezing point, pH <2 or > 10, saturated saline or high pressure in the bottom of sea, or in sedimentary rocks. However, any extreme property can be considered as extremophilic as long as it is rare in nature. Nanobacteria from blood are apatite-forming organisms, that inhabit blood, a niche classically considered free of intruding organism, because of its powerful defense mechanisms. Nanobacteria do tolerate exposure to extreme conditions probably better than any other known organism. However, it is difficult to test their extremophilic nature in the classical sense, because nanobacteria are fastidious and require many nutrients ready in their medium. Nanobacteria do grow when their culture medium is supplemented with high concentrations of sodium chloride (5%), many antibiotics, disinfectants and even fixative materials, such as formalin. Nanobacteria tolerate exposures to 1.5 Mrad gamma irradiation, boiling for 30 min, repeated freeze-thawing, strong acids (IN HCL) and external pressures (in an X-press device) higher than those at the deepest ocean floor.

2. NANOBACTERIA

Nanobacteria are the smallest cell-walled bacteria, discovered only recently in human and bovine blood and commercial blood products. Nanobacterial cells are 100-500 nm in diameter and appear to produce elementary particles which, at 100 nm, are smaller than many viruses. These bacteria belong to Proteobacteria but are unique in that: they produce carbonate apatite on their cell envelope.¹ They are found in human kidney stones.² They are difficult to culture in microbial media and have low metabolic rates, which makes their detection difficult. Nanobacteria multiply under mammalian cell culture conditions and can be present in cell cultures.³ The sterility of cell culture supplements and related products such as vaccine materials, hormones and growth factors, depends largely on the filtration techniques that are used. Mycoplasmas are the smallest classical bacteria that can be grown under cell culture conditions, which also support the growth of cell wall-less bacteria (L-forms). All these bacteria can pass through sterile filters; filtration through 0.2 µm pore-size filters has resulted in 102-fold and 106-fold reductions in the number of mycoplasma and L-forms, respectively. In the case of nanobacteria, the reduction was less than 10-fold and they passed through even 0.1 µm pore-size filters.^{4,5} This novel organism was named

Nanobacterium sanguineum, referring to its surprisingly small size and its presence in blood, and it was deposited in the German Collection of Microorganisms (DSM No: 5819-5821). This organism was isolated from 'sterile' human and bovine sera obtained from commercial sources. Fetal bovine serum was found to be contaminated to the extent that about 80% of serum batches contained culturable nanobacteria. About 5% of human serum samples were found to contain nanobacteria.⁴

Environmental studies suggest that very small bacteria, possibly nanobacteria, are widely distributed on our planet. Particles resembling the tiniest nanobacteria were discovered in sedimentary rocks by Dr. Folk, who named them nannobacteria and suggested that they may contribute to the formation of carbonate minerals.⁶ This raises the possibility for environmental sources of nanobacterial contamination. Ultramicrobacteria, which are able to pass through sterile filters, were found in soil and natural water sources about 20 years ago. They are difficult to culture and therefore have remained largely uncharacterized and their possible connection to nanobacteria is not known. Normal bacteria may acquire a dormant state and will not grow on subsequent culture.⁷ The size of such starved cells can be only a fraction of the size of actively growing cells. Dormant state can not be detected easily in nanobacteria, since their metabolic rates are always very slow, at least at the tested physiological temperatures.

3. EVIDENCE SUPPORTING THE EXISTENCE OF NANOBACTERIA

1. Nanobacteria can be cultured with a doubling time of about 3 days, and they can be passaged, apparently indefinitely. At present, they have been passaged monthly for over seven years.
2. They produce biomass at a rate of about 0.0001 times that of *E. coli*.
3. Their biomass contains novel proteins and 'tough' polysaccharides.
4. SDS-PAGE of nanobacterial samples shows over 30 prominent protein bands. Amino terminal sequences are available from six different proteins and one of them has been tentatively shown to be a functional porin (unpublished work in collaboration with Dr. James Coulton, McGill University). Porins, a hallmark of gram-negative bacteria, are located in the outer membrane where they function in the transport of small molecules. In nanobacteria, the porin(s) seem to be located in the mineral layers. Muramic acid, a major component of peptidoglycan, has also been detected. Thus, nanobacterial cell walls have typical gram-negative components, although the ultrastructure of is unique and varies as a function of the growth phase.
5. Nanobacteria contain modified nucleic acids that have been detected with specific stains and spectroscopy and their components have been analyzed by mass-spectroscopy.⁸ The 16S rRNA gene has been amplified by PCR and its sequence (EMBL Entries X98418 and X98419) suggests an affiliation of nanobacteria with the alpha-2 subgroup of Proteobacteria.
6. Nanobacterial growth can be inhibited with small concentrations of tetracycline antibiotics or with high concentrations of aminoglycoside antibiotics. Both antibiotics stop bacterial protein synthesis at the ribosomal level.
7. Nanobacterial growth can be inhibited with small concentrations of cytosine arabinoside or fluoro-uracil, both of which are antimetabolites that prevent nucleic acid synthesis in all types of cells.
8. Nanobacteria can be detected with metabolic labeling using methionine or uridine.
9. Nanobacteria have unique strategies for social behavior and multiplication, including communities, budding and fragmentation.

4. EXTREMOPHILIC PROPERTIES OF NANOBACTERIA

Nanobacteria are highly resistant to heat and gamma-irradiation and thus provide the first example of such organisms isolated from mammalian tissues. Bacteria with these properties are found in environments where these traits are essential. Nanobacteria must have emerged from a special environment, perhaps only recently in evolutionary terms. Currently we cannot state that nanobacteria would be hyperthermophiles simply because, due to their culture medium, we cannot test growth at very high temperatures. But they do withstand exposures to boiling temperature. Interestingly nanobacterial structure can resist also high physical forces. So repeated freeze-thawing do not break them. Even X-press treatment creating huge pressures (about 1 ton per mm²) does not break down nanobacteria although pulverizes ice.

High doses of aminoglycoside antibiotics (1 mg/ml) effectively inhibit the replication of nanobacteria for about a week, which indicates the presence of a bacterial type of protein synthesis. Thereafter, the growth is re-established (see Ciftcioglu and Kajander, in this issue). Somehow nanobacteria overcome the inhibition thereafter and start to grow, although the antibiotic concentration remains the same. Before that nanobacteria have aggregated and formed slimy connections. This is nicely depicted in Figure 1. Some of the connections between the nanobacterial cells appear to be tube-like and may indicate metabolic exchange directly between cells. The resistance to these antibiotics may be due to the impermeable cell wall. The impermeability of the cell wall is further increased by slime and mineral deposits, which are composed of biogenic carbonate apatite. Figure 2A and B show apatite formation, several micrometers in thickness, made and inhabited by small communities of nanobacteria. The caves in the apatite indicates its biogenic origin. Under the protection of such 'castles' nanobacteria are almost indestructible. Thus, in some experiments, they have survived exposure to 4% formalin and even mixture of formalin and glutaraldehyde. However, treatment with such fixatives cross-links molecules obviously mediating apatite formation and this results in a rapid calcification. 'Normal' calcification process results in a slow accumulation of

organized apatite crystals, whereas in the fixative treated samples, the mineralization is fast and appears to be poorly organized (Fig.2C,D). It must be pointed out that ability to grow in the presence of fixative compounds is extremophilic in nature.

The average diameter of nanobacteria, measured with electron microscopic techniques, is 0.2-0.3 μm , smaller than that of any known cell-walled organism. Several viruses (e.g. Vaccinia) are larger than nanobacteria, which can be as small as 0.1 μm . Ultrafiltration methods produce even smaller estimates of size, as nanobacteria readily pass through 0.1 μm pore size filters. Apparently, nanobacteria can pass through pores smaller than their own size and must therefore have flexible cell walls. We have also shown that during filtration nanobacteria may fragment and lose part of their cell wall.⁸ The theoretical minimum diameter of a cell, based on the size of those macromolecular components now considered to be necessary and sufficient for a living cell, is about 0.14 μm ,⁹ although many experts regard the appropriate smallest range for independent life-forms to be much higher, around 0.3 μm . The measured size of nanobacteria approaches or is even smaller than the theoretical lower limit for the size of a living organism. The very slow growth rate and requirement of a very rich medium may be adaptations to the very small size of the cell.¹⁰

Cell wall-less bacteria, L-forms, have small and large forms. Conventional culture methods do not support the growth of L-form microbes. L-forms can pass through sterile filters after which they can be easily lysed and their nucleic acids and proteins extracted. *Mycoplasma*, *Chlamydia* and *Rickettsia* are smallest 'classically known' bacteria with sizes approaching 200 nm and they can be cultured with mammalian cells under cell culture conditions, but only mycoplasma can grow autonomously. All three bacteria can be a problem in sterile filtration as they can be present in biological material and have been shown to pass through sterile filters. Filtration through 0.2 μm pore-size filters results in over 100-fold reduction in the numbers of these bacteria, bacterial L-forms are reduced by a factor of 106, whereas with nanobacteria the reduction is typically less than 10-fold.^{8,11} When nanobacteria were subjected to filtration through 0.2 μm pore-size filters, the temperature and back-pressure were found to influence the number of cells that were able to pass through. Only 2% went through the filter at 4°C and at low back-pressure, whereas at 56°C the numbers reached up to 50%. Nanobacteria could pass through 0.1 but not 0.05 μm nominal pore size filters. In filtration, cell fragmentation may result in very tiny forms that can pass through filters. This has been observed with mycoplasma and may take place with nanobacteria as well.

Although cell size is considered to be a characteristic of a given bacterial species, examples where the size, shape or morphology change in response to the environmental and social status of the organism, have been described. *Myxococcus xanthus* has a life cycle, carefully controlled by cell density and nutrient levels, which includes tiny forms, actively moving large forms and huge social formations that produce mushroom-like fruiting bodies. Rapidly growing mycoplasma 'forget'

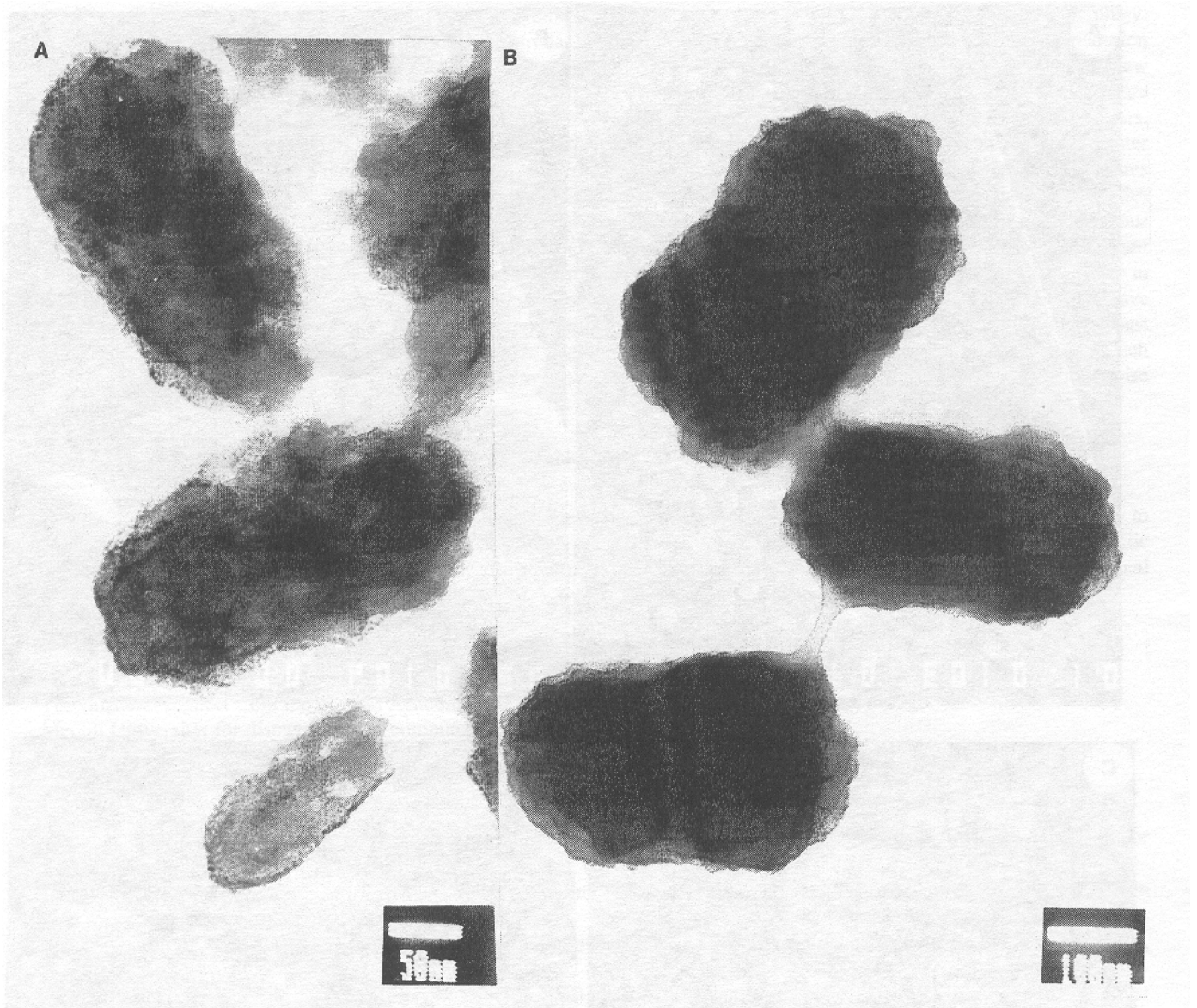


Figure 1. TEM images of nanobacteria before (A), and after (B) gentamycin exposure. Negative staining was applied to the both samples. Notice in (B) the cytoplasmic connections between nanobacteria, apparently as a defense mechanism.

cell division, forming very long multicellular structures. Nanobacteria can have several growth forms, sizes and social formations depending on culture conditions. A small size is not directly linked to the size of the genome. The *Myxococcus xanthus* genome, at 9.4 Mb, is among the largest, whereas mycoplasmas have the smallest known genomes, at 0.58-1.6 Mb. *Chlamydia* and *Rickettsia* have genomes of 1Mb. The size of the nanobacterial genome is unknown but quantitative Hoechst staining suggests it to be smaller than that of mycoplasmas.

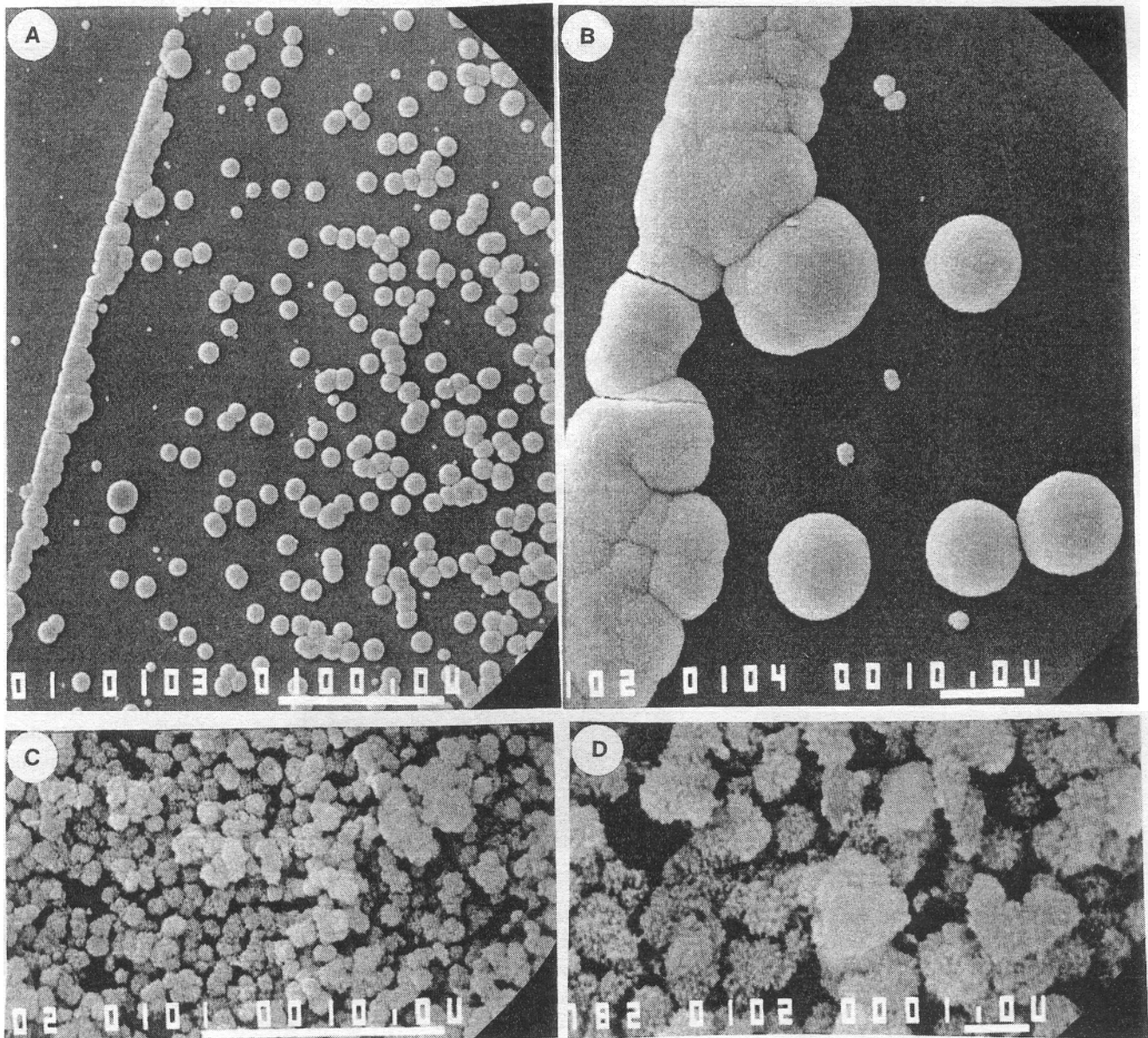


Figure 2. SEM images of highly mineralized nanobacterial social communities, fortresses. The fortresses appear having a coccoid morphology (A and B), when nanobacteria are cultivated in serum-free cell culture medium. The organized morphology was lost and nanobacterial biofilm produced more apatite mineral after exposure to 2% formaldehyde -4% glutaraldehyde before the cultivation period (C and D).

5. NEW EXTREMOPHILIC PROPERTY: DIMINUTHOPHILIA

As discussed above, nanobacteria do have a novel extreme property, which seems to be fundamental for their life strategy: nanobacteria are 'diminuthophilic'. This means that nanobacteria can produce extremely tiny units that allow for penetration through pores smaller than the diameter of intact replicating bacteria. This may enable them to colonize and inhabit new areas in biological tissues, which essentially have compartments separated by microporous 'sieves'. Endothelial linings are essentially sieves of various porosity, the smallest ones being in kidney tissue, and blood-brain and blood-testicle barriers. Similar life strategy may be advantageous in porous rocks: sedimentary rocks have extremely small pores allowing for water penetration. Nanobacteria may inhabit new territory in such rocks by release of tiny units capable of penetrating the pores with the slow water currents. Considering such a life strategy, all of the previously mentioned extremophilic properties would be advantageous. This hypothesis involves the revolutionary idea that (nano) bacteria may fragment into numerous tiny units that later can be reassembled, e.g. by their surface-mediated aggregation, to produce a fully competent

replicant form. How might such tiny units retain their macromolecular components? The answer may be that they are packed either in membrane vesicles, or that the particles would be mineralized thus immobilizing the macromolecular components. We have shown that nanobacteria form apatite particles via apparently suicidal fragmentation and that the particles contain at least protein antigens, and that nanobacteria or their units do aggregate and form multicellular type of social communities. Such communities may obtain enough elementary units to reconstitute whole genomes and start new life cycle in an otherwise inaccessible location.

6. CONCLUSIONS

Nanobacteria are extremophilic bacteria showing a wide spectrum of extreme properties. The most striking may be ability to colonize microporous materials via release of diminute units. Nanobacteria are examples of extreme diminuthophilic organisms. Diminuthophilic bacteria should be regarded as a new class of extremophiles that can inhabit new ecological niches not accessible for common bacteria.

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