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REVIEW PAPERS

Nannobacteria

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Abstract—Bacterial nannocells 0.2–0.3 μm in size and hundredths of a cubic μm in volume have been revealed in natural habitats and obtained in pure cultures. The taxonomic analysis of naturally occurring nannobacteria showed that they belong to the known taxa of the kingdom *Eubacteria*. The results of the cytological investigation of nannocells suggest that they are universally formed in response to stress impacts.

Key words: nannobacteria, nanobacteria, nannocells, filterable forms

Microbiologists usually deal with bacterial cells 2–5 μm in size and several tenths of a cubic μm in volume, while smaller bacterial forms are much less frequently encountered. Some of the reasons for this are the low resolution of light microscopy (about 0.2 μm) [1] and the invisibility of the ultramicrocolonies produced by bacterial miniforms on rich nutrient media to the naked eye [2]. Thus, methodological difficulties in investigating bacterial miniforms have created the impression that they are rare in nature. To emphasize the scarcity of bacterial miniforms, researchers called them ultramicrobacteria or nannobacteria. The latter term implies that the dimensions of nannobacteria can be more conveniently expressed in nanometers than in micrometers (the prefix *nanno-* is common to biology, while *nano-*, to physics [3]). Tiny cells formed in laboratory cultures from normal cells about 1 μm in size are also called *elementary bodies, subunits*, or *filterable forms* (for details, see below). The older term *minicells* refers to small cells formed from normal cells due to their division or budding; such cells do not contain hereditary material in amounts sufficient for growth and/or reproduction [4, 5] and will not be considered here.

It is generally accepted that the minimum size of viable prokaryotic cells is close to 0.2 μm [1, 7], although some authors believe that the minimum size of bacterial cells is less than 0.1 μm [6]. Theoretically, viable bacterial cells may be 0.14 μm in diameter [8, 9].

The interest of researchers in bacterial miniforms grew as new important findings were reported, e.g., the discovery of nannocells inside Koch's bacilli [10, 11], elementary bodies in the bacterial cells treated with antibiotics [12–14], and filterable bacterial forms as causative agents of infectious diseases [15, 16]. Among recent findings, noteworthy is the detection of growing nannobacteria in the blood serum [17, 18] and of nannofossils in Martian meteorites [19–21]. In our recent publication [22], we described the formation of nannocells and nannoforms (0.1–0.6 μm) in pure bacterial cultures initially containing "normal" cells 2–5 μm in size.

The present review is an attempt to analyze relevant data available in the literature and our own experimental data on the nannoforms of bacterial cells.

PROBLEMS ASSOCIATED WITH COUNTING VIABLE BACTERIAL MINIFORMS

Exact enumeration of bacterial nannocells in mixtures of *L*-forms or in natural microflora by transmission electron microscopy presents considerable difficulties related to the process of specimen preparation. In particular, to prevent contamination of the specimen surface with organic compounds and salt crystals, excess liquid should be removed with a piece of filter paper. This may lead to the leakage of liquid, together with small suspended particles, from under a cover glass and, hence, to the removal of some of the nannobacteria from the specimen. For this reason, scanning electron microscopy reveals much more nannobacteria than transmission electron microscopy. After detecting a great number of nannobacteria on the surface of geological samples, some authors even claimed that bacteria with normal sizes comprise only a small part of the natural microflora [23–30].

This conclusion is in agreement with the data of 16S rRNA analysis showing that more than 99% of the bacterial species occurring in nature do not exist as laboratory cultures [35]. It should be noted, however, that the results of the investigation of geological samples by scanning and transmission electron microscopy have not been substantiated by microbiological analysis of these samples, although it is well known that minicells, which are close to nannobacteria in size, do not contain hereditary material and, hence, are unable to grow and reproduce. The presence of DNA and rRNA in the liq-

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uid passed through 0.2-μm-pore-size membrane filters cannot unequivocally prove that these nucleic acids belong to intact nannocells and not to broken cells of normal sizes. The calculation of the amount of DNA per number of intact cells is not an adequate control either.

As for *L*-forms, also called elementary bodies, the routine method of obtaining them with the use of antibiotics leads to the formation of cells of different sizes, including cells which are the same or even greater than the original cells. The imperfect methods of *L*-form separation and antibiotic removal do not ensure that all the elementary bodies obtained are able to grow and reproduce.

ABUNDANCE OF BACTERIAL MINIFORMS IN NATURE

Bacterial miniforms 0.05–0.3 μm in size were revealed in natural habitats (soil, water, and blood serum) by direct transmission and scanning electron microscopic observations. In some cases, these observations were confirmed by obtaining miniforms in pure cultures using routine microbiological procedures, such as microfiltration, plating from serial dilutions, and subculturing.

The term *nannobacteria* was proposed by Folk, who studied geological samples by electron microscopy [23–30]. The major evidence for the existence of nannobacteria is the morphology of these "quasi-living" (Folk's term) particles [31]. Data on the abundance and distribution of nannobacteria in geological sources have led geologists to an inference about the prevalence of bacterial miniforms in nature, since more than 95% of the bacterial forms revealed in geological samples by them had small sizes. It should be noted that these observations need serious microbiological confirmation. In particular, miniforms should be separated from normal cells and cultivated in respective nutrient media to confirm their ability to grow and reproduce. Furthermore, it is necessary to show the presence of nucleic acids in nannocells. Analysis of geological samples showed that nannobacteria are readily mineralized, which, as geologists believe, is an indication of their involvement in the diagenesis of sedimentary rocks, the formation of apatite, and some other geochemical processes [32, 33]. The photographs of microobjects made by geologists are available not only in scientific journals but also at a website devoted to nannobacteria (http://www.geo.utexas.edu/illite). However, pertinent information obtained by geologists did not attract the attention of professional microbiologists in proper time and did not stimulate an investigation of microobjects at the necessary level.

The electron microscopic studies of Bae *et al.* [34] showed that 72% of bacterial cells detached from soil particles were dwarf (less than 0.3 μm in size). The majority of cells were coccobacilli 0.35 μm in diameter and 0.06 cubic μm in volume. The examination of sections prepared by routine methods (fixation with osmium tetroxide, treatment with uranyl acetate, etc.) revealed an ultrastructure typical of functional bacterial cells. The phylogenetic analysis of the eubacterial microflora of tundra soils by means of the amplification of the 16S rRNA genes using eubacterial primers showed that no less than 77% of this microflora differed phylogenetically from known bacterial taxa by more than 5% [35]. The number of the phylogenetic bacterial types determined by Torsvik *et al.* [36] reached 10000 and exceeded the number of bacterial cultures obtained from the same samples by about 170 times.

Small sizes of soil bacteria may be due to stressful environmental conditions. For instance, cells of the soil isolate *Pseudomonas fluorescens* R2f subjected to starvation became smaller and rounder in shape [37]; the effect was more pronounced with the early-log-phase cells. On the other hand, dwarf cells (0.03–0.04 cubic μm in volume) of the three strains isolated from the paddy soil of a rice field exhibited stable (and independent of substrate limitation) morphological and physiological properties [38]. Based on the results of 16S rRNA sequence analysis, these three strains were assigned to one taxonomic group, *Verrucomicrobiales*.

Aqueous samples are more appropriate for the isolation of bacterial miniforms than soil samples, since the primary isolation procedure used in hydrobiology and aquatic microbiology is filtration through membranes with different pore sizes [39]. The term *filterable bacteria* refers to the cells that pass through 0.45-μm-pore-size filters and do not pass through 0.22-μm-pore-size filters [40]. Small cells of aquatic bacteria do not necessarily have a small volume, since some cells may be as long as 30 μm. In aquatic microbiology, viable cells less than 0.3 μm in size are usually termed *ultramicrobacteria* [41–43], although Morita calls them *nannobacteria* [44]. In our opinion, these two terms can be considered to be synonyms. Despite technical difficulties, which considerably limit the number of relevant investigations, the electron microscopic studies of lake water samples confirmed that they contain functionally active nannocells with nucleic acids [45].

Investigation of the morphology, physiology, and biochemistry of ultramicrobacterial isolates showed that they most likely belong to known bacterial genera, such as *Aeromonas*, *Alcaligenes*, *Pseudomonas*, and *Vibrio* [42]. The growth of aquatic nannobacteria can be stimulated by decreasing the organic matter content of cultivation media to a level close to that of their natural habitats. At the same time, the size of cells of marine isolates decreased under stressful conditions, in particular, under nutrient starvation [46–48]. For instance, depending on the growth rate, the volume of unstressed cells comprised from 0.48 to 5.94 cubic μm, whereas the minimum volume of starved cells was only 0.05 cubic μm [49]. It should be noted that the volume fraction of hereditary material ranged from 0.06 to 0.48

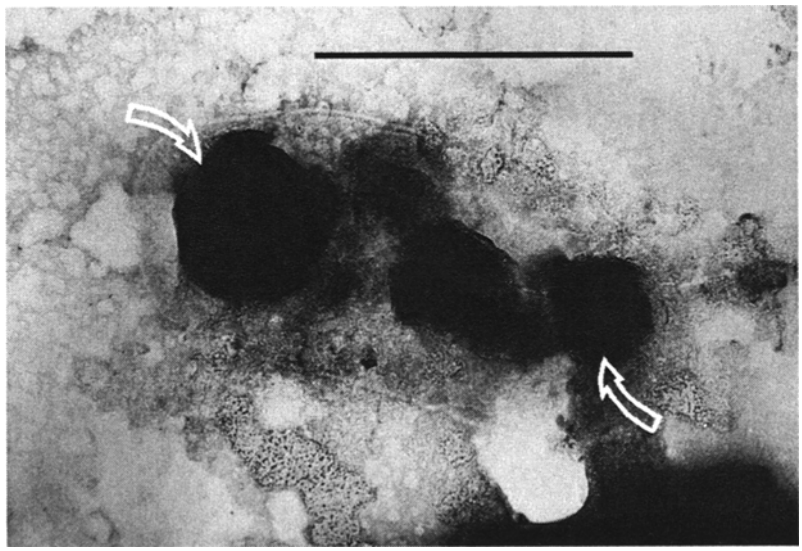

Fig. 1. Subunits (nannocells) surrounded by their own membrane inside a normal bacterial cell. *E. coli* VKM B-125 cells were grown on agar media in an electric current gradient. Bar represents 1 μm.

in unstressed cells and from 0.06 to 0.40 in starved cells; i.e., it was virtually the same, although the cell volume decreased by 10 to 100 times [49]. These results suggest that nannocells complete the reproduction of their genomes before attaining normal sizes.

Recent studies confirmed the observation that the DNA content of cells may greatly vary. Indeed, 90% of cells of heterotrophic bacteria isolated from the mesotrophic Lake Zurich had volumes ranging from 0.06 to 0.66 cubic μm. A comparison of the DNA content estimated with DAPI and the cell volume estimated by flow cytometry showed that some cells contain normal amounts of cytoplasm and very low amounts of DNA. For instance, Button *et al.* [50] found that the mean size of the genome of viable nannobacteria is 200 kb. Taking into account that the minimum size of the genome of culturable bacteria is about 500 kb, the viability of the nannobacteria described by Button *et al.* is questionable. One of the marine ultramicrobacteria, which was identified as *Sphingomonas* sp., was characterized by a low DNA content, a high protein content, and the presence of only one copy of the rRNA operon [51].

The nannobacteria revealed in blood and the blood serum by electron microscopy were culturable coccoid cells 0.2 μm in diameter [17, 52–54]. The authors of these papers succeeded in obtaining nannocells in amounts sufficient for genetic analysis, but they failed to induce the growth of nannocells to "normal" sizes. Nor were they able to obtain visible colonies of these cells on microbiological media. According to 16S rRNA sequence data, these nannobacteria were assigned to the α-2 subgroup of proteobacteria. Three strains of these nannobacteria, which have been deposited in the German Collection of Microorganisms with designations DSM 5819, DSM 5820, and DSM 5821, were proposed to belong to a novel species of a novel genus, *"Nanobacterium sanguineum."* Of interest is the fact that the nannobacteria under discussion can cause the formation of salt deposits in human tissues [55, 56]; i.e, in this case, they serve the function that was described by geologists [31]. The deposition of insoluble salts on the surface of minicells is a separate and interesting problem. Indeed, the thickness of mineral deposits may exceed the diameter of the minicells, which excludes the possibility of the active metabolism of such minicells and suggests that they serve only as centers of salt deposition irrespective of their viability.

Thus, there is ample evidence for the existence of tiny (less than 0.35 μm in diameter) bacterial cells. Under stressful conditions, they can be formed from normal cells. Many identified bacterial miniforms turned out to belong to known bacterial taxa. The reproduction of bacterial miniforms in natural habitats has been demonstrated only for nannobacteria living in blood.

FORMATION OF BACTERIAL NANNOFORMS IN LABORATORY CULTURES

The formation of nannoforms in bacterial cultures was first observed during the investigation of *L*-forms and bacterial heteromorphism. The latter can be of two types: forced heteromorphism, which is induced by physicochemical factors, and natural, or spontaneous, heteromorphism [57, 58]. Inasmuch as *L*-forms can easily be obtained, they are described in more detail than the phenomenon of heteromorphism.

When exposed to the antibiotics repressing the synthesis of the bacterial cell wall, bacteria produce so-called *L*-forms, i.e., spheroplasts from 0.1 to 50 μm (!) in size. The smallest reproductive cells (so-called elemen-

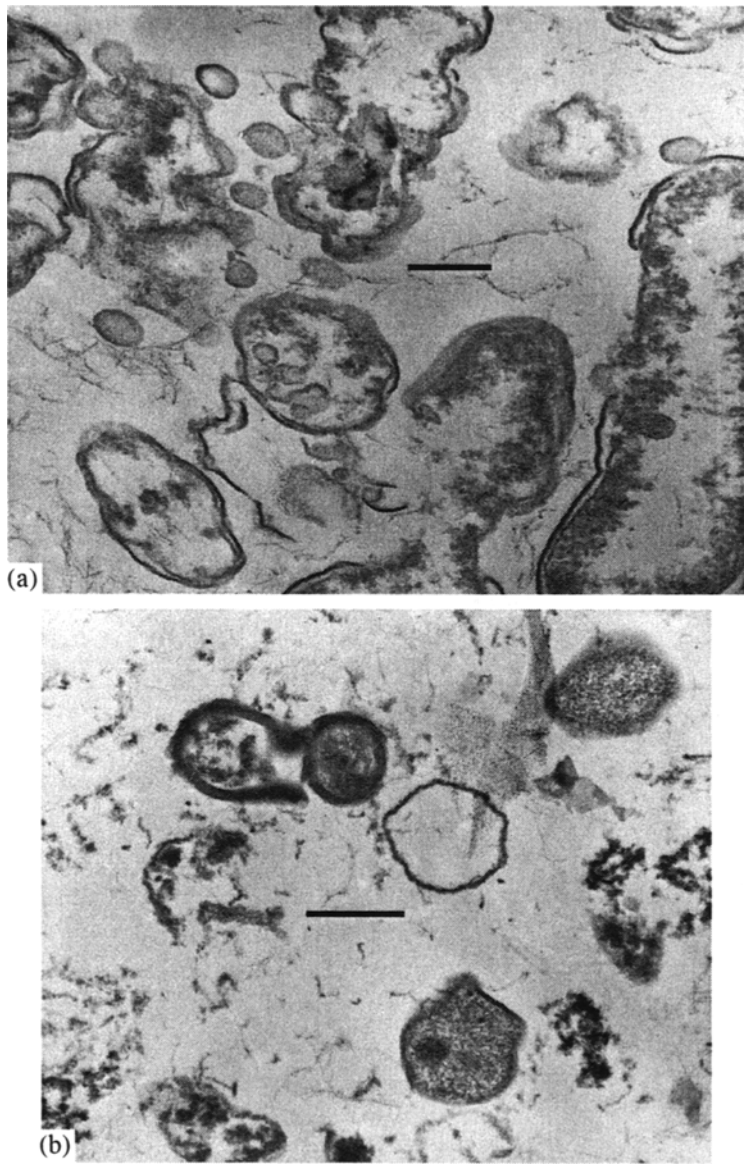

Fig. 2. Nannocells (nannoforms) in intercellular space. (a) *P. aeruginosa* VKM B-889 cells containing noncrystalline magnetic inclusions were lysed by exposing them to a magnetic field with an intensity of 2 T. Round nannocells are seen to leave the original cells. (b) Thin sections of *B. cereus* VKM B-504 cells exposed to 600-MHz radiation for 10 min. One of the nannocells retains a fragment of the cell envelope. Bars represent 0.3 μm.

tary bodies) have linear sizes of 0.2–0.3 μm [12–16, 59, 60]. Under beneficial conditions, small cells can restore their initial size and form. Presumably, elementary bodies are produced in the regions of dense cytoplasm and can be liberated into the environment through the lysis of large bodies or the impairment of their membranes. The investigation of nannocells in cultures presents difficulties, since the presence of chemical agents that caused their formation suppresses the growth and reproduction of nannocells, whereas the problem of removing such agents has yet to be solved. It should be noted that, in early works, the size of the reproductive cells was determined by their filtration through membrane filters with 0.22- to 0.3-μm pore sizes. Since *L*-forms lack a rigid cell wall, their passage through filter pores might lead to cell deformation and to mixing of the cellular content. As a result, *L*-forms became nonviable.

Generally, bacterial heteromorphism is a change of cell morphology under stressful conditions, although some bacteria, for instance, *Desulfosarcina variabilis*, can change their morphology in response to the change of the growth substrate [60]. The phenomenon of nannoforms is well described by Timakov and Kagan: "Heteromorphism is one of the survival-promoting responses of bacteria exposed to physical, chemical, or biological stressful factors. A long-term exposure to or high doses of these factors can cause the death of heter-

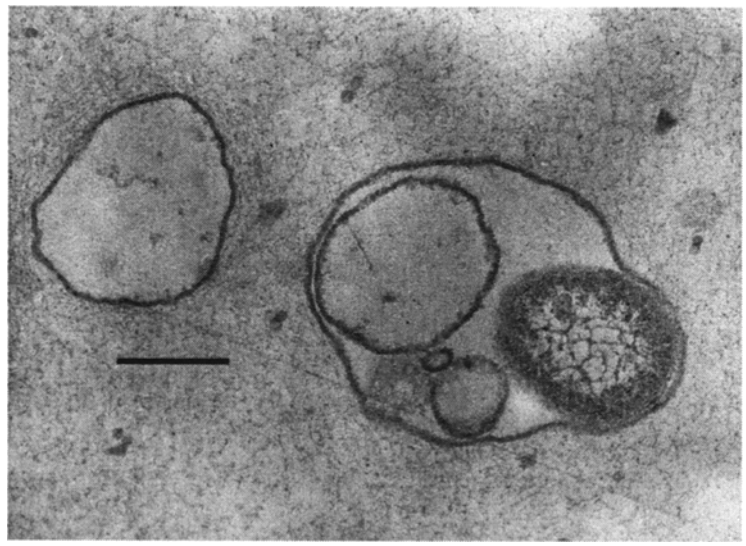

Fig. 3. Thin sections of *Genus* sp. strain 22 cells. Nannocells inside one cell envelope are separated by membranes. At least one nannocell contains the nucleoid. Sections are contrasted with uranyl acetate. Bar represents 0.3 μm.

omorphic forms or their breakdown into submicroscopic filterable forms. After the removal of stressful factors, heteromorphic forms can easily revert to the initial bacterial forms" [16].

The formation of filterable bacterial forms in response to starvation, senescence, and other factors has been described by many researchers (for the literature, see, e.g., [16]). Unfortunately, the low reproducibility of experiments and the possibility of the passage of large flexible cells through membrane pores call into question the results of these experiments. Filterable heteromorphic forms resulting from the breakdown of normal cells and filterable *L*-forms generated from the antibiotic-treated streptococci have much in common. In particular, these types of filterable forms are characterized by similar sizes (correspondingly, they pass through the same filters and precipitate at the same centrifugation speeds), similar morphology, and slow regeneration processes [16].

Under laboratory conditions, bacterial miniforms result from damage inflicted on normal cells and/or their growth under stressful conditions. The application of such difficult-to-remove stressful factors as starvation or antibiotics impeded relevant investigations. The finding that physical factors can also induce the formation of *L*-forms and similar structures (for instance, X-rays cause the formation of filterable forms from protists, and UV light, from *Agrobacterium tumefaciens* [16]) has considerably facilitated investigations.

Aquatic nannobacteria (ultramicrobacteria) exhibited a higher resistance to stress factors (heat shock and treatment with hydrogen peroxide and ethanol) than bacteria of normal sizes [51]. Nannobacteria from the blood serum also showed increased resistance to stress factors [62].

In relevant investigations, the authors of the present review used other physical factors, such as electromagnetic radiation within a frequency range of 150–5000 MHz (power, 300–1400 W; exposure time, 5–15 min) and the gradient of a constant electric current of 0.1 mA on the surface of agar media [22]. Pure cultures of bacteria and archaebacteria were obtained from the All-Russia Collection of Microorganisms (VKM): *Bacillus cereus* VKM B-504, *Escherichia coli* VKM B-125, *Halococcus morrhuae* VKM B-1772, *Haloferax mediterranei* VKM B-1748, *Janthinobacterium lividum* strain Gr, *Natronobacterium magadii* VKM B-1751, *Pseudomonas aeruginosa* VKM B-889, *Rhodospirillum rubrum* VKM B-1621, and *Staphylococcus aureus* VKM B-128. The ease with which irradiation can be started and terminated allowed the population dynamics of bacterial miniforms to be followed. In experiments, two types of bacterial miniforms were found. Hereafter, we distinguish nannocells with sizes from 0.2 to 0.6 μm and nannoforms with sizes of less than 0.2 μm.

Different physical agents (high-frequency radiation, magnetic field, and electric current) produced similar changes in different bacterial species: redistribution and local solidification of the cytoplasm, formation of small vesicles inside the original bacterial cells (Fig. 1), and the appearance of nannocells and/or nannoforms on the surface of the original cells and in intercellular space (Fig. 2). In these experiments, both nutrient media and physiological saline solution were used.

It was found that low radiation power (300 W) and exposure time (5 min) caused the formation of nannocells from 0.2 to 0.6 μm in size. The examination of sections contrasted with uranyl acetate showed that, unlike minicells, they contained hereditary material (Fig. 3). After the cessation of irradiation, nannocells incubated in nutrient medium at an optimal temperature

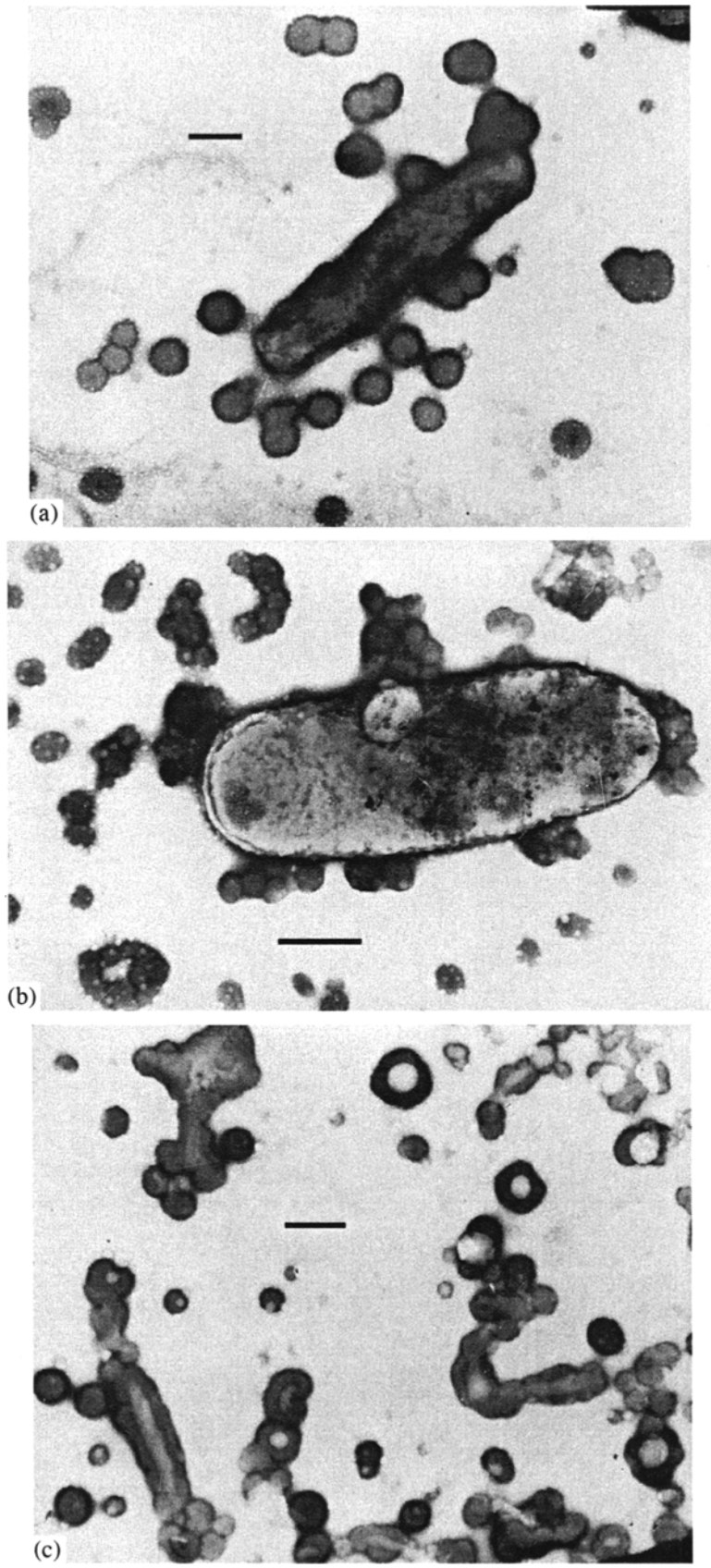

Fig. 4. Fusion of nannocells. (a, b) *Rhodospirillum rubrum* VKM B-1621 cell and nannoforms 0.5 hour after exposure to 600-MHz radiation for 10 min and (c) nannocells 1 day after such exposure. Bars represent 0.3 μm.

gradually reverted into normal cells (the complete reversion took a little more than 1 day).

The number of nannoforms produced increased with the radiation power and exposure time. Thirty minutes after formation, some nannoforms fused to form round particles 0.3 μm and more in size (Fig. 4). It remains unclear whether such particles can revert into the original bacterial form. The ability of nannoforms to fuse and attach to various objects supports the idea that they can serve as centers of salt deposits.

These data are in agreement with the findings of other authors concerning elementary bodies. Analysis of these data allows the following inferences to be made:

(1) Naturally occurring nannocells are probably formed from normal bacterial cells.

(2) Data on the *"N. sanguineum"* cells subcultured in sterile blood serum indicate that a bacterial size of 0.2–0.3 μm is sufficient for active cell reproduction.

(3) It remains unclear whether all of the nannocells formed from the original bacterial cells are functionally active, i.e., able to grow and reproduce.

In the final analysis, the problem of nannobacteria existence is reduced to the problem of the amount of hereditary material in a cell and the possibility of its distribution among the nannocells produced with the reproduction ability retained.

THE PROBLEM OF THE HEREDITARY MATERIAL PARTITIONING UNDER THE ACTION OF FACTORS INDUCING THE FORMATION OF NANNOFORMS

The adaptation of bacteria to varying environmental factors (temperature, pH, atmospheric and osmotic pressure, availability of oxygen and growth substrates, the presence of toxic compounds, etc.) may require a rearrangement of hereditary material in cells. The discovery of the *proU* operon responsible for the rapid adaptation of bacteria to stressful conditions implies that the responses of cells to different physical and chemical stresses have a common mechanism [63].

Some researchers believe that one bacterial cell can contain hereditary material in amounts corresponding to about 40 whole genomes [1] and that such an excess of hereditary material promotes cell reproduction under optimum conditions. It remains still unknown whether these genomes are independent or not, although the possibility of formation of up to five endospores inside one bacterial cell [64] is indicative of genome independence.

Traditionally, however, hereditary material in a cell is considered to be a single genome (chromosome) comprising millions of base pairs: from 4.5 to 5.5 Mbp in different strains of *E. coli* [65]; from 3.75 to 4.64 Mbp in different strains of *P. stutzeri* [66]; 3.1, 3.6, and 3.7 Mbp in *Desulfovibrio desulfuricans, D. vulgaris,* and *Desulfobulbus propionicus,* respectively [67]; and from 1.8 to 3.4 Mbp in different species of lactic acid bacteria belonging to the genera *Lactococcus, Streptococcus, Lactobacillus, Leuconostoc, Pediococcus,* and *Carnobacterium* [68].

Therefore, the sizes of chromosomes can differ severalfold in different species and by 1 Mbp in different strains of a given species. Genetic mapping has demonstrated a considerable plasticity of the *L. lactis* subsp. *cremoris* chromosome manifested in multiple inversions and translocations. The reason for these and similar genetic rearrangements remains unknown [68, 69].

It should be noted that all of the aforementioned data refer to cells of normal sizes (several μm) grown in rich nutrient media. As for the genome of nannobacteria, which comprises only about 0.2 Mbp [50], its functional activity remains unknown. The minimum size of the bacterial chromosome whose functional activity has been proved is 0.5 Mbp (these data refer to mycoplasmas cultivated in rich nutrient media) [70]. In *Mycoplasma genitalicum*, the 580-kbp genome codes for 468 proteins. There is, however, evidence that part of the chromosome material is not informative. In particular, *M. genitalicum* may lose half of its genes (256) without any adverse effect on the cells [9]. In any event, there are grounds to believe that the formation of several nannocells from one original cell of normal size is associated not only with the partitioning of hereditary material but also with its replication.

CONCLUSION

Bacterial nannocells 0.2–0.3 μm in size and hundredths of a cubic μm in volume were revealed in natural habitats and obtained in pure cultures. The taxonomic analysis of naturally occurring nannobacteria showed that they belong to known taxa of the kingdom *Eubacteria.* The results of the cytological investigation of artificially obtained nannocells suggest that they are universally formed in response to stressful impacts.

The reversion and the full functional activity of nannobacteria have not been unequivocally proved, except for *"Nanobacterium sanguineum"* nannocells with a diameter of 0.2–0.3 μm, which were shown to be capable of reproduction. The genome of naturally occurring nannobacteria contains 0.2 Mbp, and that of nannobacteria cultivated under laboratory conditions is several times greater.

The formation of numerous nannocells from normal cells exposed to stressful conditions suggests that the biological role of nannocells is to provide for bacterial survival. The results obtained in this work show that the resistance of nannocells to various stress factors is higher than that of normal cells.

In both human organisms and geological ecosystems, nannobacteria are involved in the processes of mineralization, where these bacteria should not necessarily be viable.

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