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EXPERIMENTAL ARTICLES

Electron Microscopic Detection and in situ Characterization of Bacterial Nanoforms in Extreme Biotopes

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Abstract—The morphology, ultrastructure, and quantity of bacterial nanoforms were studied in extreme biotopes: East Siberia permafrost soil (1–3 Ma old), petroleum-containing slimes (35 years old), and biofilms from subsurface oil pipelines. The morphology and ultrastructure of microbial cells in natural biotopes in situ were investigated by high-resolution transmission electron microscopy and various methods of sample preparation: ultrathin sectioning, cell replicas, and cryofractography. It was shown that the biotopes under study contained high numbers of bacterial nanoforms (29–43% of the total number of microorganisms) that could be assigned to ultramicrobacteria due to their size (diameter of ≤0.3 μm and volume of ≤0.014 μm^3) and structural characteristics (the presence of the outer and cytoplasmic membranes, nucleoid, and cell wall, as well as their division patterns). Seven different morphostructural types of nanoforms of vegetative cells, as well as nanospores and cyst-like cells were described, potentially representing new species of ultramicrobacteria. In petroleum-containing slimes, a peculiar type of nanocells was discovered, gram-negative cells mostly 0.18–0.20 × 0.20–0.30 μm in size, forming in situ spherical aggregates (microcolonies) of dividing cells. The data obtained promoted the isolation of pure cultures of ultramicrobacteria from petroleum-containing slimes; they resembled the ultramicrobacterium observed in situ in their morphology and ultrastructure.

Key words: nanoforms, ultramicrobacteria, microorganisms in situ, cell ultrastructure, extreme biotopes, fractionation of microorganisms.

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In recent years, microorganisms of ultrasmall size (volume of ca. 0.01–0.1 μm^3 or less and diameter of spherical forms <0.4 μm) have increasingly attracted the attention of researchers [1–7]. Many of them use the term ultramicrobacteria (UMB) to designate these microorganisms [1–5]. In the literature, they are sometimes termed nanobacteria, "dwarf" bacteria, filterable forms, etc. [2, 8]. The term nanobacteria, used as a synonym of ultramicrobacteria, is widely applied to denote ultrasmall bacteria, including those objects whose affiliation with a specific life form has not been reliably proved yet [2, 9–11]. This term is still disputable and, in our opinion, the term nanoforms is the most suitable for a primary designation of objects that can belong to ultramicrobacteria and nanobacteria. Direct observations in situ using transmission, scanning electron, and fluorescence microscopy showed that the microbial communities of soils, sludge, sediments, seawater, and other natural substrates contained a considerable share of ultrasmall cell forms 0.1–0.4 μm in diameter and 0.01–0.1 μm^3 in volume [1, 8, 12]. The study of more than 200 clones of 16S rRNA genes from bacteria obtained by ground water filtration through filters with a pore diameter of 0.2 μm showed that most of the gene sequences revealed belonged to unknown bacterial species [5]. The results of microscopic examinations and phylogenetic analyses suggest therefore that among the UMB inhabiting natural environments, there exist unknown microbial species.

Of particular interest is to examine nanoforms in extreme biotopes with an inadequately studied qualitative and quantitative abundance of ultrasmall microbial forms.

It should be noted that the microscopic examinations of microorganisms in situ in substrates like soil or sediment is complicated due to their solid mineral composition; in this case, it is necessary to use methods that enable cell separation from the mineral components and organic noncellular particles in order to obtain the concentrated microbial fractions. With this aim, different methods of fractionation of microorganisms have been developed and are effectively used at present

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Table 1. Comparative morphological and ultrastructural characteristics of the microorganisms revealed in situ in different extreme biotopes

Substrate sample	Gr+ (%)	Gr– (%)	Nano-cells, %	Cyst-like cells, %
Permafrost soil	49	51	43	98
Initial multiyear oil slime (MOS)	42	58	35	90
MOS incubated in aerotank (MOSI) for 1 month	10	90	20	3
MOSI, 5 months after incubation. Stationary condition	25	75	40	74
Corrosion film from oil pipeline walls	35	65	29	12

[8, 13–15]. However, they require further modification depending on the nature of target substrates and research goals. For example, to study microorganisms in permafrost soil, the authors developed a method for low-temperature fractionation of microorganisms at temperatures corresponding to those in nature [16, 17].

The goal of the present work was to analyze the ultrastructure and morphometry, as well as to quantitatively characterize bacterial nanoforms in situ in three different extreme biotopes: old petroleum-containing slimes, East Siberian permafrost soil, and biofilms from the subsurface oil pipelines of Bashkiria.

MATERIALS AND METHODS

Subjects of research. The following subjects were used in the present studies: (1) 1.8–3.0 Ma old permafrost soil sampled aseptically at a depth of 50 to 70.8 m in Northeastern Siberia [16]; (2) original and activated slimes from the Nizhnekamsk Petrochemical Plant contaminated by petroleum products and heavy metals (over 35 years old); and (3) samples of biofilms taken from the subsurface oil pipelines in Bashkiria. The sediment in the slime contained 40% organic substances (30% of them were petroleum products) and 60% inorganic substances (silicates and aluminum silicates with an admixture of heavy metals: chrome, nickel, cadmium, zinc, cobalt, titanium, etc.) The chemical analyses were carried out at the Center of Instrumental Methods of Analyses, Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences.

Petroleum-containing slime was activated as follows: 5% suspension of slime in sterile tap water was supplemented with mineral compounds: KH_2PO_4 (1.85 g/l) and NH_4Cl (1.15 g/l). The suspension (28 l) was then placed into a microaerotank with the working volume of 28 l and aerated (0.7 l/min of air per 1 l of the medium) for a month. The cultivation was carried out at ca. ~20°C.

Methods for investigating microorganisms in situ. For microscopic examinations, cell fractions were obtained from the permafrost soil by low-temperature fractionation [16, 17]. The microbial cell fractions as well as the samples of oil slimes and biofilms were fixed and embedded in epoxy resin to obtain ultrathin sections [17].

Ultrathin sectioning. The pellets resulting from centrifugation of microbial fractions and the 3–5-mm samples of oil slime and biofilms were fixed in 1.5% glutaraldehyde solution in 0.05 M cacodylate buffer (pH 7.2) at 4°C for 1 h, washed thrice in the same buffer, and additionally fixed in 1% OsO_4 solution in 0.05 M cacodylate buffer (pH 7.2) for 3 h at 20°C. After dehydration, they were embedded in epoxy resin Epon 812. Ultrathin sections were contrasted for 30 min in 3% uranyl acetate solution in 70% alcohol and additionally stained with lead citrate according to Reynolds [18] at 20°C for 4–5 min.

Electron microscopic cryofractography. The samples were prepared on a JEE-4X vacuum evaporator (JEOL, Japan) equipped with devices for cooling microbial cells at a rate of about 10^4 deg/s. The material not exposed to preliminary chemical fixation or any other treatment was frozen in liquid propane supercooled with liquid nitrogen to –196°C. Fractures were obtained at a vacuum of 3×10^{-4} Pa and sample temperature of –100°C. The fracture surface replicas were obtained in vacuum by shadowing with platinum–carbon mixture applied at an angle of 30°; the strengthening carbon layer was obtained by application of carbon at an angle of 90°. Ultrathin sections and replicas from fracture surfaces were examined under a JEM-100B electron microscope (JEOL, Japan) at an accelerating voltage of 80 kV.

RESULTS

The electron microscopy of the fractions of native microbial cells isolated from permafrost soils revealed the presence of up to 43% ultrasmall (≤0.3 μm) coccoid nanocells (Tables 1, 2, Fig. 1a). In order to confirm that the studied nanoforms were true cells, we took into account, at the ultrastructural level, the presence of the cytoplasmic membrane (CM), nucleoid, ribosomes, and signs of cell division in cell-like particles. The sizes were determined on the sections crossing the central part of a cell and tangential sections were discarded. At least 200 cells were analyzed in each sample. Since the soils under study were ca. 1.8–3 Ma old and had a temperature of –12 to –14°C throughout this period, the cells in these soils were expected to be in a dormant state (anabiosis). The cytological analysis in situ confirmed this hypothesis: most of the cells in the fractions obtained by low-temperature fractionation exhibited no signs of cell division but had multilayered envelopes, capsules, and large intramembrane protein particles typical of bacterial cysts and cyst-like forms (Fig. 1). The ratio of gram-positive and gram-negative bacteria determined by the cell wall types was approximately 1 : 1 (Table 1).

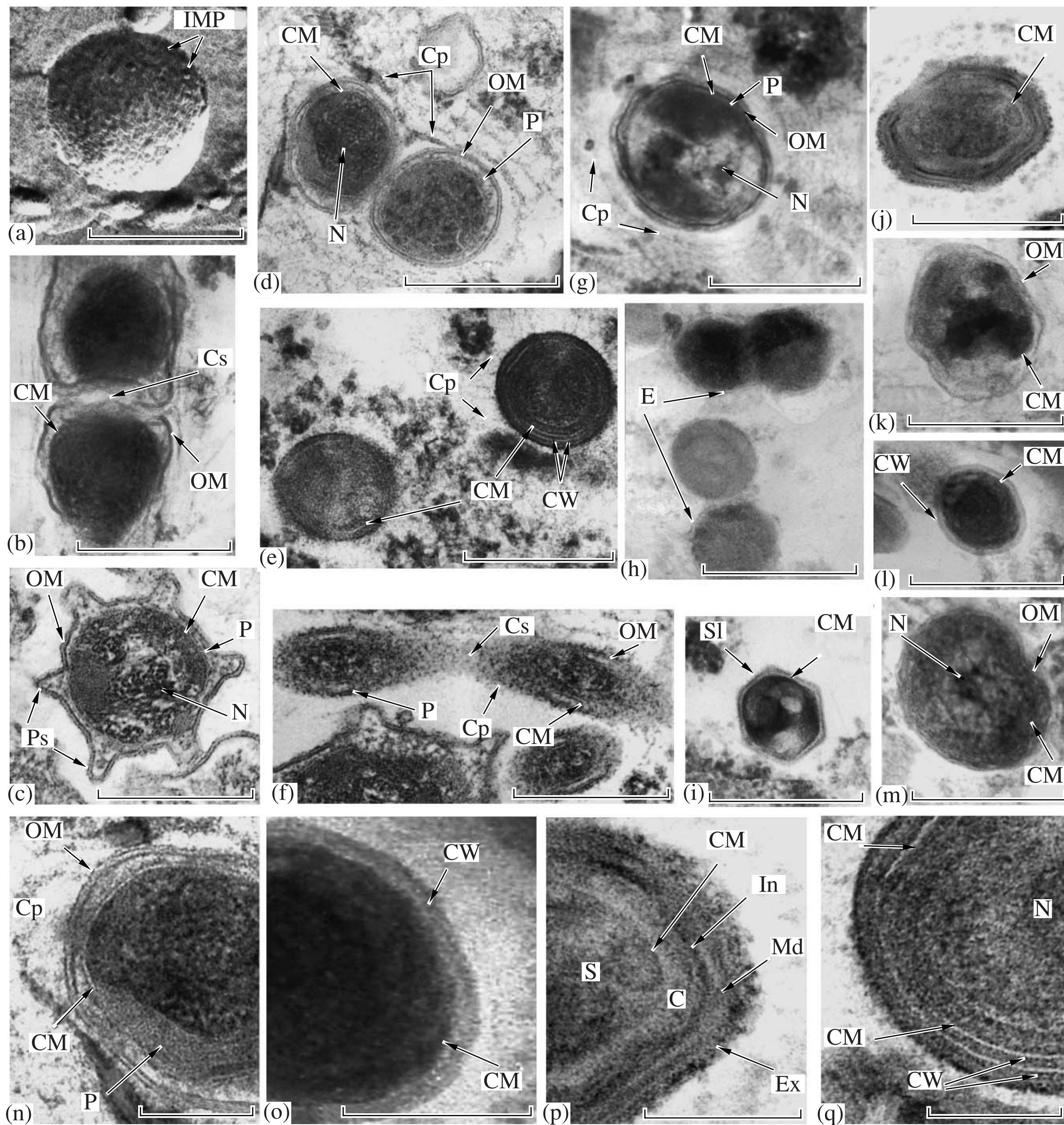

Fig. 1. Ultrastructure of bacterial nanoforms in extreme biotopes: (a) cryofracture of CM (PF surface) of a bacterial cell obtained by the method of low-temperature fractionation from permafrost soil; (b–q) ultrathin sections of bacterial nanoforms revealed in situ in oil-containing slime: (b) gram-negative bacterial dividing cell; (c) gram-negative bacterial cell with protrusions (prosthecae type) formed by the outer membrane and periplasm; (d) gram-negative dividing bacterial cell (late division phase); (e) gram-positive cyst-like nanocell with a multilayered cell wall; (f) gram-negative dividing rod-shaped bacteria; (g) gram-negative bacterial cell; (h) diplococci of unknown nature (the envelope is visible; CM and OM are not revealed); (i) a hexagonal nanoform of unknown nature; the surface structure is probably an S-layer; (j) a resting bacterial form, an ultrasmall spore (nanospore) with ultrastructural features peculiar of a typical bacterial spore; (k) gram-negative bacterial cell; (l) gram-positive bacterial cell; (m) gram-negative bacterial cell; (n) a fragment of the cell shown in Fig. 1d; (o) a fragment of the cell shown in Fig. 1l; (p) a fragment of the nanospore shown in Fig. 1j; (q) a fragment of the cyst-like nanocell with a multilayered cell wall shown in Fig. 1e.

Symbols for Figs. 1, 2, 3: IMP, intramembrane particles; In, internal layer of coats; Ex, external layer of coats; C, cortex; Cp, capsule; CW, cell wall; N, nucleoid; OM, outer membrane; E, envelope; P, periplasm; PC, peripheral cell; Cs, constriction; Ps, prosthecae; Bd, bud; S, spore core; Md, middle layer of coats; Sl, S-layer of the envelope; CC, central cell; CM, cytoplasmic membrane. Scale bar is 0.3 μm in Fig. 1a–m and 0.1 μm in Fig. 1n–q.

Table 2. Morphometric characteristics of cell nanoforms shown in Figs. 1–4

Figure No	Source of isolation	Cell diameter, μm	Cell volume, μm^3
1a	Permafrost soil	0.3	0.014
1b	Oil slimes	0.28	0.012
1c	″	0.32	0.017
1d	″	0.25	0.008
1e	″	0.23	0.007
1f	″	0.13	0.004
1g	″	0.31	0.015
1h	″	0.17	0.002
1i	″	0.19	0.003
1j	″	0.28	0.011
1k	″	0.3	0.014
1l	″	0.17	0.003
1m	″	0.32	0.017
2b	″	0.25–0.3	0.009
2c	Oil slime. Pure culture	0.25–0.3	0.009
3	Corrosion film	0.3	0.014

A high density of nanocells was observed also in oil-containing slimes: depending on the state of oil slimes, it varied from 20 to 40% of the total cells (Tables 1, 2). According to their size (≤0.3 μm) and volume (≤0.017 μm^3), they could be assigned to UMB. The results of this part of the research are of particular importance: they have been obtained in studies of microorganisms in situ *sensu stricto*, i.e. by direct total analysis of microorganisms in oil slimes without fractionation of microbial cells. These procedures can evidently affect both the completeness of extraction of different microorganisms from soils and the state and size of fractionated cells. Among UMB, coccoid nanocells of ultrasmall size (0.2 to 0.3 μm or less) often occurred in situ (Figs. 1b, d, e, k, l, and m). As with permafrost bacteria, a considerable portion of nanoforms (up to 90%) in oil-containing slimes were dormant forms represented by cyst-like forms, cysts, and endospores (Table 1). The ratio of dormant cells decreased drastically (to 3%) on activation of samples of oil slimes and their incubation in a microaerotank for 1 month.

It is noteworthy that the most of the individual cells or cell aggregates consisting of the cells of one or several morphotypes are enclosed in thick capsular layers and envelopes. This is evidently an indication of the fact that the cells exposed to high concentrations of hydrocarbons, toxic organic substances, heavy metal compounds, and other adverse factors need to synthesize protective multilayered surface structures. The study of microbial fractions obtained from oil slimes and activated in a microaerotank revealed the decline in the number of capsular and cyst-like cells (Table 1).

On the basis of the data on the general morphology and cell ultrastructure, nine major morphotypes of the smallest (<0.3 μm in diameter) nanoforms were differentiated:

Type 1: gram-negative (by the cell wall type) spherical cells 0.25 to 0.32 μm in diameter and 0.008 to 0.017 μm^3 in volume. Distinctive features are division by constriction, enlarged periplasmic space, and presence of a capsule and of a sheath uniting the cells (Figs. 1b, d, g, m, Table 2).

Type 2: gram-negative star-shaped bacteria, 0.32 μm in diameter and 0.017 μm^3 in volume. Cells have short periplasmic prosthecae and a broad periplasmic space (Fig. 1c, Table 2).

Type 3: gram-negative bacteria, spherical cells 0.17 μm in diameter and 0.002 μm^3 in volume (Fig. 1k, Table 2).

Type 4: gram-negative coccoid and egg-shaped cells with a size of 0.18–0.25 × 0.20–0.30 μm and an average volume of 0.011 μm^3. Distinctive features were reproduction by budding, formation of spherical multicellular aggregates (SA) whose central cells are of ca. 0.4–0.5 μm in diameter, and large capsules around individual SAs and their complexes (Figs. 2a, 2b). Some of these bacteria (Fig. 2c) were isolated as pure culture and one of them has been described in detail [19].

Type 5: ultrasmall gram-negative rods with diameter 0.13 × 0.30 μm and volume 0.004 μm^3 having a thin microcapsule layer (Fig. 1f, Table 2).

Type 6: ultrasmall coccoid cell-like forms, 0.17 μm in diameter and 0.002 μm^3 in volume. Cell wall and cytoplasmic membrane are not definitely discernable. Sometimes occur in the form of diplococci (Fig. 1h, Table 2).

Type 7: hexagonal forms, 0.19 μm in diameter and 0.003 μm^3 in volume; on their surface, there are envelopes similar to bacterial S-layers and a layer that is probably a cytoplasmic membrane (Fig. 1i, Table 2).

Type 8: spherical cyst-like cells with cell walls of a gram-positive type, 0.23 μm in diameter and 0.007 μm^3 in volume (Fig. 1o, Table 2). Their cell wall consists of three broad layers (Fig. 1e) typical of cyst-like cells of nonsporeforming bacteria [20, 21].

Type 9: ultrasmall oval endospores (nanospores), 0.32 × 0.28 μm in size and 0.011 μm^3 in volume. These

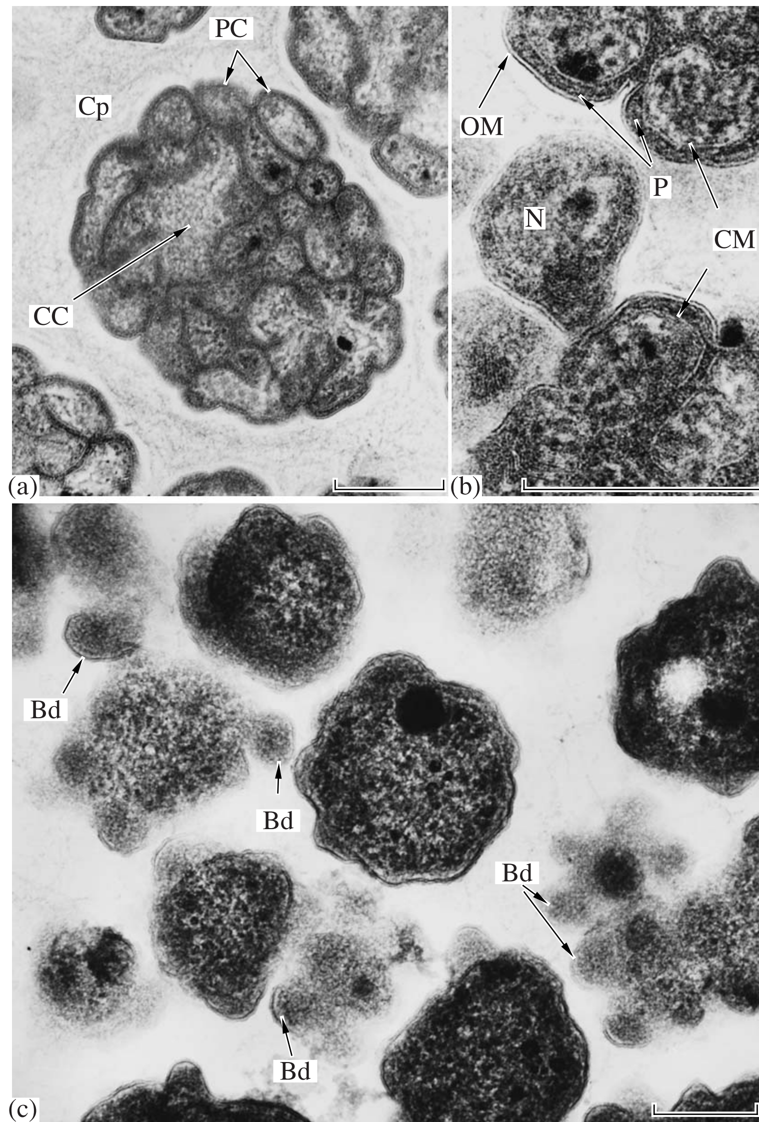

Fig. 2. Spherical structures (in situ in oil slime): (a) aggregates of ultrasmall dividing cells, inside each aggregate there is a big central cell; (b) a small nanocell separating from a spherical aggregate (budding); (c) pure culture of *Kaistia* sp. NF1, large cells and banch-like clusters of ultrasmall cells (buds) are evident. Scale bar in Fig. 1a–c is 0.3 μm. Designations as on Fig. 1.

nanoforms have all the structures typical of endospores: a core; a cortex; inner, middle, and outer covers (Fig. 1p, Table 2).

Of the above nanoforms, only types 6 and 7 could not be unambiguously identified as true cells because of the unclear nature of their surface structures. Type 7 can therefore be classified as either archaea or viral particles. A protozoan virus has been described recently, with a similar morphology and very large size, 0.4 μm in diameter [22]. The other seven types of nanoforms all have the features typical of bacteria (listed above) and can be described as UMB.

The viability of cell nanoforms is confirmed by their reproduction in natural substrates, evidenced by the images of dividing nanocells with typical constrictions (s) (Figs. 1b, d, f) and budding cells in the above spherical multicellular aggregates.

The comparative study of microorganisms in situ in samples taken from oil-containing slimes under varying conditions (Table 1) showed that a one-month activation and incubation of oil slime decreased the quantity of gram-positive and dormant forms of microorganisms as well as of nanocells. However, the quantity of nanocells still remained rather high in this variant (ca. 20% of the total cells in the sample). The decrease in their numbers can be explained by the fact that in the initial oil slime sample some of the nanocells (ca. 15%) were in fact transient forms that later had developed into larger cells. The five-month post-incubation period was characterized by a reverse process: the recovery of the percent ratio of nanocells (Table 1).

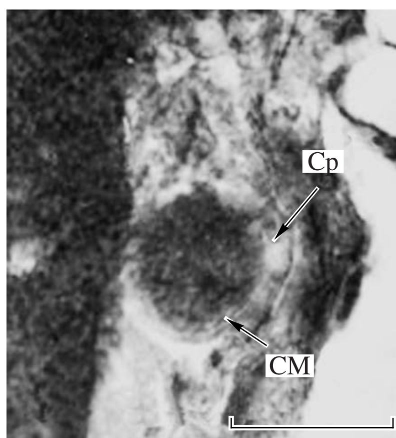

Fig. 3. Coccoid bacterial cell in the biofilm (biocorrosion layer).

The ultrastructure of microorganisms in the biofilm (biocorrosion layer) sampled from local defects of subsurface oil pipelines was studied. Of the total microorganisms in the biofilms, about 30% turned out to be nanocells, coccoid bacteria of less than 0.3 μm in diameter. The absence of signs of division in 12–14% of such cells and the structure of their CM and cell envelope are indicative of their dormant state (Table 1). Most of the cells in the biofilm samples had a large electron-dense capsule (Fig. 3).

DISCUSSION

The data obtained showed that to study bacterial nanoforms in natural substrates, examination of ultrathin sections by transmission electron microscopy (TEM) is necessary, since differentiation between the cells and cell-like particles requires information on the presence of cell components and results of their identification. In this regard, characterization of ultramicrobacteria in situ obtained by fluorescence and scanning electron microscopy is insufficient. Although the reliability of the data on the percent ratio of nano- and "usual" size cells in situ obtained by TEM is beyond doubt [8], determination of the absolute number of nanocells in substrates using ultrathin sectioning involves big problems. In particular, when studying microbial cell fractions separated from soil and sludge, the fact that complete desorption of all the cells adsorbed on soil particles is impossible should be taken into account. Examination of microbial communities in situ using TEM and ultrathin sectioning without desorption and fractionation of microbial cells (in situ *sensu stricto*) is therefore the most accurate approach. However, at present, this is only possible in the case of "solid state" natural substrates with sufficiently high plasticity, reduced content of mineral particles, and a high content of organic substances and microorganisms. The old oil-containing slime, one of the biotypes studied in this work [17], satisfies these requirements to the utmost.

The electron microscopic examinations of microorganisms in extreme biotopes carried out in the present work revealed a great number of bacterial nanoforms, some of them being extremely small: <0.2 μm in diameter (spherical forms) and 0.008 μm^3 in volume. The diameters of most of the spherical nanocells studied varied from 0.32 to 0.17 μm. However, some of the nanocells revealed in situ by TEM might be so-called filterable forms and other forms originating from the "usual" size bacteria rather than representatives of ultramicrobacteria. The comparative study of the nanocell density in oil-containing slimes (initial, activated, and in the stationary post-incubation state) indicates that a considerable number of microbial nanoforms are not transient structures and permanently exist in natural environments as a regular component of microbiocenoses. Among these nanoforms, there are evidently both bacteria forming nanocells only in the course of their development, and those permanently existing as nanocells. The latter can be exemplified by the recently described free-living UMB *Sphyngopyxis alaskensis* (previously *Sphyngomonas*) [23], *Pelagibacter ubigue* [24], and soil anaerobic bacterium of the genus *Verrucomicrobium* [4].

The cellular (morphology) and ultrastructure analyses revealed a broad diversity of nanoforms in the substrates studied in situ: spherical, oval and rod-like forms, cell aggregates (microcolonies), bacteria with gram-negative and gram-positive types of cell wall, spores, and cyst-like cells. The classification of particular nanoforms with bacteria was based on the presence of cell components typical of bacteria: nucleoid, a cell wall of gram-negative or gram-positive type, periplasm, prosthecae, and capsules (Figs. 1a–g, j–q, Figs. 2a–c, Figs. 3, 4).

There were also unidentified hexagonal cell-like particles, ca. 0.19 μm in diameter and 0.003 μm^3 in volume, with an envelope similar to a cell wall of the S-layer of some archaea (Fig. 1i). A similar structure is peculiar to a giant mimivirus [22], but that structure is 1.5 to 2 times larger than the cell-like particles. Elucidation of the nature of these nanoforms requires further research. We classified with nanocells neither the vesicles surrounded by a membrane and having a granular content (they can possibly be considered as the outer membrane vesicles) nor ultrasmall spherical granules (0.17 μm in diameter) with no evident cytoplasmic membrane and no nucleoid (Fig. 1h); such a pattern is acquired usually by granules of storage compounds released by bacteria.

The viability of nanocells from samples under study is evidenced by pronounced indications of cell division observed in many of them (Figs. 1b, d, f).

The seven isolated and characterized structural types of nanocells are probably representatives of various not yet described bacterial species. Pure cultures of

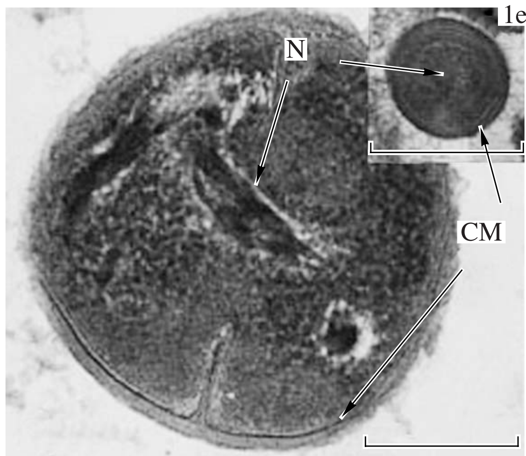

Fig. 4. Comparison of the sizes of bacterium *Micrococcus luteus* and a bacterial nanoform shown in Figure 1e at the same magnification (the difference in volume is 50-fold). Scale bar in Figs. 3, 4 are 0.3 μm.

ultramicrobacteria have been isolated from oil-containing slime and permafrost soil. One of them bears significant similarities to the nanobacterium observed in situ in morphology and ultrastructure (Fig. 2). It was thoroughly described in [19].

On the strength of the presented results obtained by electron microscopic examinations of microorganisms in situ and the complex of cytological criteria, one can conclude that a considerable share of nanoforms observed in the three different extreme biotopes are UMB which we have divided into seven morphological and ultrastructural types.

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