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EXPERIMENTAL ARTICLES

An Electron Microscopic Study of the Ultrastructure of Microbial Cells in Extreme Biotopes In Situ

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Abstract—The ultrastructure of microbial cells was studied in situ in natural biotopes by high-resolution transmission electron microscopy using the known methods of cryofractography, thin sectioning, and the negative staining of total cell specimens, as well as the new methods of the low-temperature fractionation of microbial cells (providing for the recovery of cells from natural sources and their concentration), the preparation of micromonoliths, and aimed electron microscopy. Among the natural biotopes studied were permafrost ground and oil sludge. Most of the microorganisms found in the 1- to 3-million-year-old permafrost ground were represented by resting forms (spores, cysts, and cystlike cells with specific organomineral envelopes). Oil sludge older than 35 years contained bacteria of atypical morphology and ultrastructure, including various resting forms and ultramicrobacteria. The data obtained is indicative of considerable promise of high-resolution electron microscopy for studying microbial communities in situ.

Key words: cell ultrastructure in situ, electron microscopy, low-temperature cell fractionation, resting microbial forms.

Modern microbial ecology is paying ever-increasing attention to the study of the composition, structure, and function of microbial communities in situ [1]. Culturable microorganisms comprise only 1–10% of the total amount of microbial genomes detected in natural biotopes by advanced molecular biological methods [2, 3]. Consequently, only a small fraction of microflora has presently been studied. In particular, little is known about the size, composition, and diversity of microorganisms in natural habitats (in situ). Of great interest is how microorganisms that are unable to produce spores, cysts, or other special resting forms can survive unfavorable environmental conditions and remain viable over periods of millions and even hundreds of millions of years [4]. The in situ study of microbial cells that remain viable in extreme biotopes (such as permafrost, glaciers, ancient halites, and anthropogenically polluted biotopes) for a long time may answer this question, whereas laboratory studies with the use of artificial nutrient media cannot disclose the entire phenotypic potential of microorganisms, including the diversity of vegetative cells and resting forms, their interactions, etc.

Of great promise in this regard are electron microscopic studies with the use of new methodological approaches to the recovery of microbial cells from natural sources and their concentration, fractionation, and preparation without artifacts. The conventional methods of light and scanning electron microscopy cannot provide insight into the fine structure, morphology, diversity, or physiological state of microbial cells occurring in natural habitats in situ. As an approach to this, Bae *et al.* [5] proposed a new method for the recovery and fractionation of native soil microorganisms. A disadvantage of this method is that samples should be treated at temperatures above zero for a long time. Recently, we modified this method for a low temperature (–12°C) and described it in short [6]. In this paper, we describe the method and its variants in detail and present experimental data on the ultrastructure of native microbial cells recovered from permafrost ground and old oil sludge that were obtained by the proposed method.

MATERIALS AND METHODS

Samples. Permafrost grounds 1.8–3 million years old were sampled aseptically in northeastern Siberia from depths of 50–70.8 m [7]. The sampled oil sludge of OAO Nizhnekamskneftekhim was more than 35 years old and contained 40% organic substances (about 30% were oil products) and 60% inorganic substances (predominantly silicates and alumosilicates) contaminated with the heavy metals chromium, nickel, cadmium, zinc, cobalt, titanium, and some others [8].

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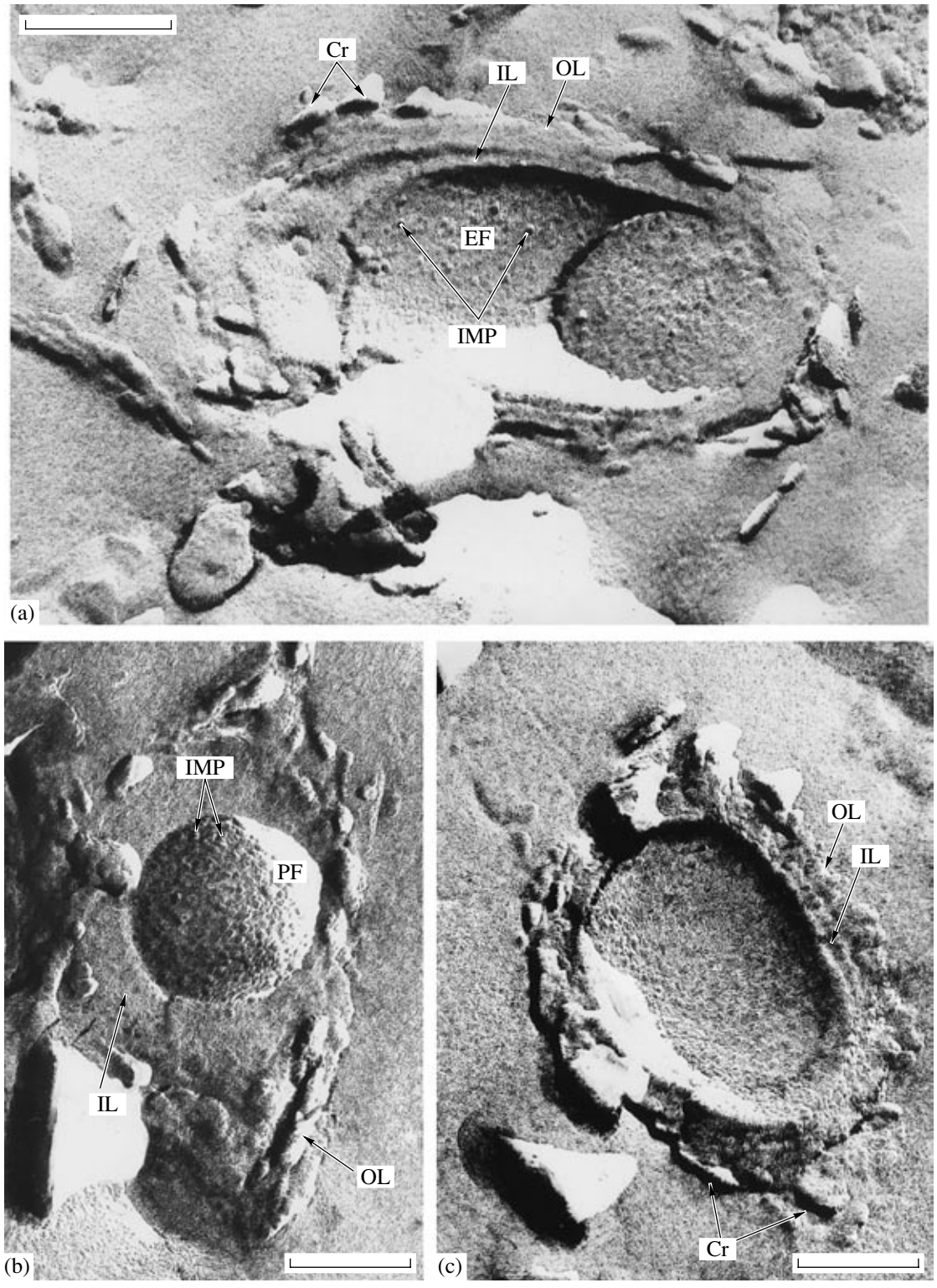
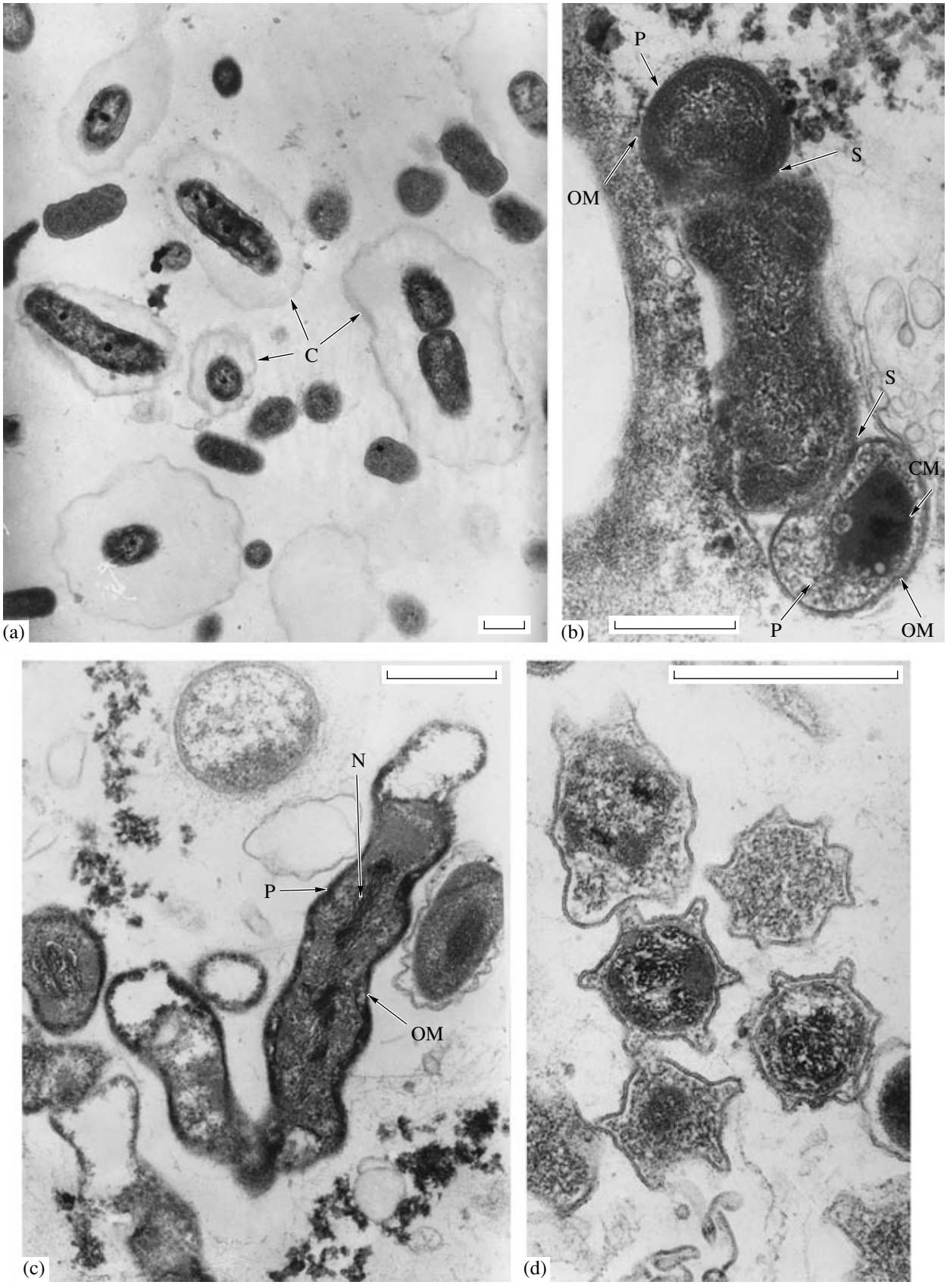


Fig. 1. Cryofractured replicas of cystlike microbial cells isolated from Siberian permafrost grounds by the LTFM method: (a, b) multilayer envelopes; (a, c) mineral particles (crystallites) in the outer envelope layer. IL, inner envelope layer; OL, outer (organomineral) envelope layer; Cr, crystallites; PF, P-face of the freeze-fractured cytoplasmic membrane (CM); EF, E-face of the freeze-fractured CM; IMP, intramembrane particle. The scale bars represent 0.2 μm.


(a)
C
(b)
P
S
OM
S
CM
P
OM
(c)
N
P
OM
(d)

Electron microscopic methods. Thin sections and specimens for cryofractography were prepared as described earlier [9]. The preparations were examined in a JEM-100B electron microscope (JEOL, Japan) operated at a voltage of 80 kV.

RESULTS AND DISCUSSION

The ultrastructure of microorganisms in extreme habitats in situ was studied by three methods.

1. Low-temperature fractionation of microorganisms (LTFM). This method provides for the recovery of intact microorganisms from natural sources and their concentration and fractionation.

2. Direct electron microscopy. The relatively high concentration of microbial cells in oil sludge makes unnecessary their fractionation. For this reason, the distribution of microorganisms in oil sludge was studied with the aid of micromonoliths (~3 mm^3 in volume), which were fixed in liquid propane at –196°C or in a 1.5% solution of glutaraldehyde in 0.05 M cacodylate buffer at 4°C, followed by the preparation of freeze-fractured replicas and thin sections.

3. Aimed electron microscopy. In this method, specimens are first examined by conventional light or electron microscopy for the most specific microloci, which are then studied in detail by high-resolution electron microscopy with the use of thin sectioning and cryofractography.

I. Model Experiments on the Fractionation of Microorganisms

LTFM was developed by using two test cultures. *Cryptococcus albidus* strain 2561 was isolated from a permafrost ground [10]. Strain KH1 (unidentified pigmented micrococci with a cell size of ~0.4 μm) was isolated from the oil-contaminated soil samples collected on the territory of OAO Nizhnekamskneftekhim. These strains were chosen for their specific features, which allowed their colonies to be easily identified on solid nutrient media. Strain KH1 produced red colonies, which influenced the color of the surrounding medium as well. The yeast *Cr. albidus* strain 2561 produced slimy colonies. The LTFM experiments were carried out with humic gley soil collected from horizon A_1 on the territory of the Oka Terrace State Nature Reserve. This soil virtually did not contain indigenous yeasts. The soil sample was mixed with a suspension of either bacteria (strain KH1) or yeasts (strain 2561) containing 1.0×10^9 cells/ml and incubated for 15 min to allow cell sorption on soil particles. It should be noted that the sorption/desorption parameters of microbial cells depend on the properties of sorbing substrates. For this reason, all procedures associated with the fractionation of microbial cells (ultrasonic treatment, centrifugation, and others) must be optimized. This was controlled by plating the fractions onto nutrient media, followed by cell counting. In addition, the fractions were examined by light and electron microscopy. These model experiments showed that the LTFM method allows 80–85% of microbial cells present in samples to be recovered.

II. LTFM Steps

Step 1. An aliquot (1 g) of soil or ground was suspended in 5 ml of a cold (–12°C) aqueous solution containing 35% glycerol, 0.1% sodium pyrophosphate, and 0.1% sodium pyruvate. The suspension was homogenized with a mechanical stirrer. To maintain the required temperature (–12°C), all procedures were carried out in an ice bath containing a mixture of ice (100%), KCl (19%), and NH_4Cl (14%). Sodium pyruvate was added to the recovery solution by virtue of its ability to reactivate inactive and damaged microbial cells [11]. The beneficial effect of pyruvate was particularly profound in the case of oil sludge (an increase in CFU by 70%). This can be explained by the fact that oil sludge contains heavy metals, for instance, Fe^{2+}, which initiate the breakdown of hydrogen peroxide with the formation of cell-damaging reactive oxygen species [12].

Step 2. This step involved the shaking of the soil suspension and its ultrasonic treatment (0.44 A; 15 kHz; 1 min) to desorb cells from soil particles and to disintegrate cell aggregates.

Step 3. The sonicated suspension was centrifuged at 800 *g* for 45 s to give precipitate 1 and supernatant 1.

Step 4. Steps 2 and 3 were repeated ten times, and the ten supernatants (1.1–1.10) were mixed with supernatant 1 and centrifuged at 10000 *g* for 60 min.

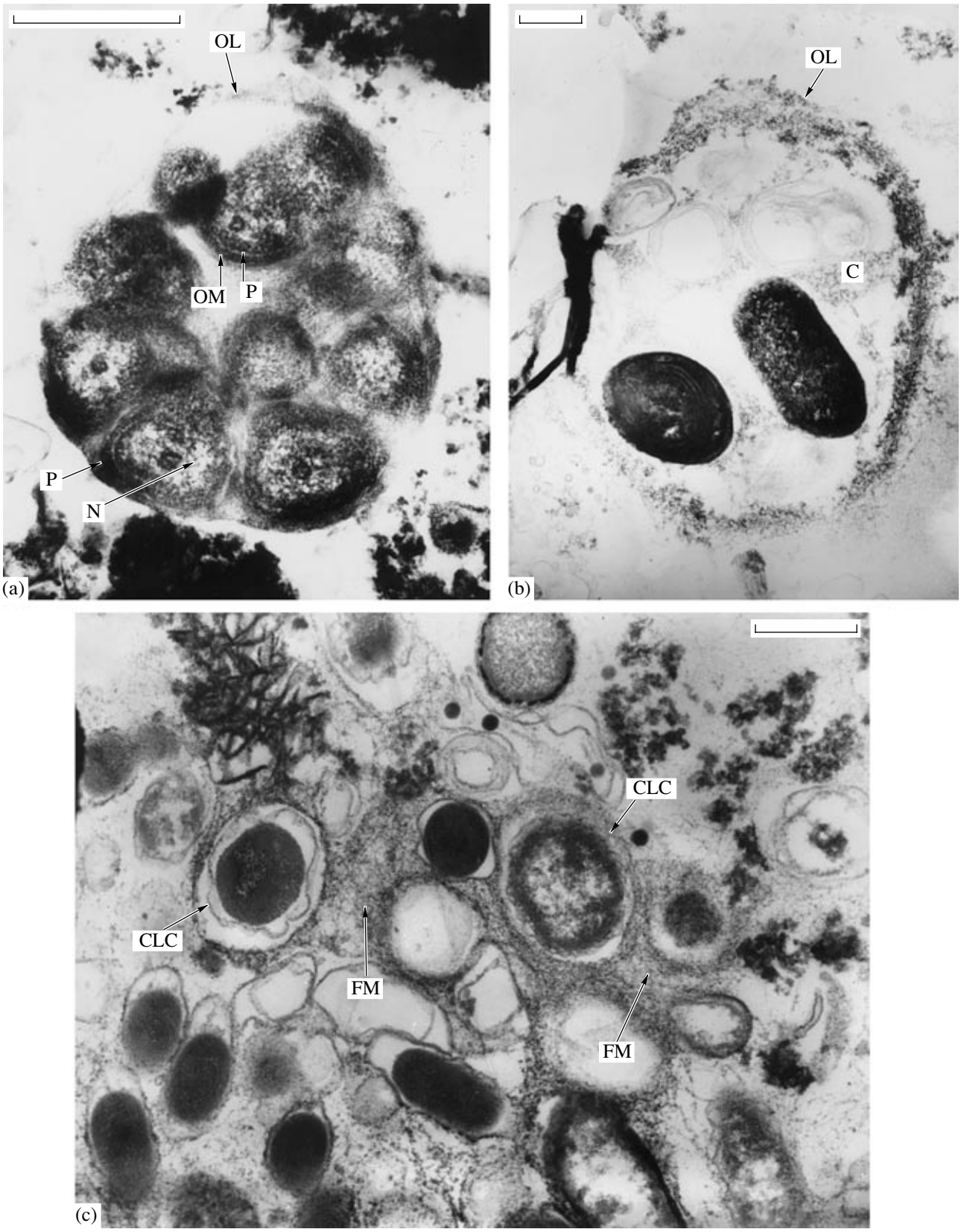
Step 5. The pooled supernatants were centrifuged at 10000 *g* for 60 min.

Step 6. The final fraction (1.1–1.10 + 1) and the supernatant were examined by electron microscopy and analyzed by plating onto solid nutrient media.

III. Electron Microscopic Studies

The study of cell fractions isolated from permafrost grounds. Fractions of microbial cells were obtained from permafrost grounds that were at –10 to

Fig. 2. Thin sections of various bacterial morphotypes found in oil sludge: (a) association of gram-negative rodlike cells enclosed in large slimy cocoons; (b) gram-negative bacterium with atypical division (daughter cells are formed on both poles of a rodlike mother cell) and thick periplasm filled with an electron-opaque granular substance; (c) gram-negative bacterium with a wavy envelope margin and thick periplasm filled with an electron-opaque granular substance; (d) small gram-negative cells with six periplasmic prosthecae. C, capsule; P, periplasm; S, septum; CM, cytoplasmic membrane; OM, outer membrane; N, nucleoid. The scale bars represent 0.5 μm.


OL
OM
P
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(a)
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CLC
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–12°C for 1–3 million years. The electron microscopic study of freeze-fractured replicas and thin sections of these cells showed that they represented coccoid, rodlike, coryneform, and budding bacterial cells with a gram-negative or gram-positive cell wall. Most of these cells had large laminar capsulelike envelopes 0.15–0.4 μm in thickness (Figs. 1a–1c). The fibrillar–reticular matrix of the outer capsular layer contained, either inside or on the surface, mineral particles in the form of plates, leaflets, electron-opaque crystallites, and granules, which were likely to represent clay minerals and compounds of iron and other elements of ground origin. The particles were tightly bound to the cell envelope and could not be detached by ultrasonic treatment (0.44 A; 15 kHz; 1 min) or repeated centrifugation. The cytoplasmic membrane of these cells contained large intramembrane particles (IMPs) 200–250 Å in diameter, which were close in size to the IMPs of resting and cystlike cells (CLCs) [13]. IMPs were detected on both the P-face and the E-face of freeze-fractured replicas (Figs. 1a, 1b). The cytoplasm of many cells had a fine-grained structure (Fig. 1a). Ribosomes and inclusions were not detected. All these features are typical of model CLCs [13]. Dividing cells were very rare. Cells lacking capsulelike envelopes were very few in number and contained degraded cell walls, membranes, cytoplasm, and nucleoids. Thus, most of the structurally intact cells recovered from permafrost grounds (a) did not show obvious signs of division, (b) contained large laminar capsular layers (envelopes), and (c) were characterized by the state of membranes typical of resting bacterial cells. The endospores and typical cysts found in the recovered fractions were not numerous. All this confirms the suggestion that microorganisms in permafrost grounds occur in an anabiotic state [14].

The study of microbial cells in oil sludge in situ. The electron microscopic study of the thin sections of micromonoliths taken from oil sludge revealed the presence of gram-negative and gram-positive bacterial cells very diverse in morphology (Figs. 2–4): straight and curved rods, ovoid and coccoid forms, budding bacteria, cells with different prosthecae, hexagonal cells, cysts, and spores (some of these morphotypes are shown in Fig. 2). The cell size varied from 0.15 to 0.9 μm, the size 0.4–0.6 μm being dominant. Some morphotypes have not yet been described in the literature, among which are gram-negative cells with six symmetrical periplasmic prosthecae (Fig. 2d), coryneform (irregularly curved) gram-negative rods (Fig. 2c), and ultramicrobacteria 0.2–0.3 μm in size. Most of the gram-negative bacteria contained a hypertrophic periplasm 0.01–0.2 μm in thickness, which was filled with a granular electron-opaque substance (Figs. 2b, 2c, 3a). Most of the bacterial cells (Fig. 2a) or their associations (microcolonies) (Fig. 3) were enclosed in large capsules. Resting (anabiotic) forms were represented by spores, cysts, and CLCs (Fig. 4). The latter could easily be identified according to the three aforementioned criteria. In contrast to the envelopes of vegetative cells, the envelopes of CLCs had a complex structure with a thickened cell wall and two or three layers presumably of polysaccharide nature. The outer layer was organomineral; i.e., its polysaccharide matrix included mineral particles, which resembled ground particles in structure and electron density. Many vegetative cells contained capsules (Figs. 2a, 3a, 3b, 4b–4d), which, like the polysaccharide envelopes of CLCs, probably protect the cells from detrimental factors typical of oil sludge (oil hydrocarbons at high concentrations; toxic metal compounds; detergents; and, possibly, microbial lytic enzymes and metabolites).

High-resolution electron microscopy is a very effective tool to study the ultrastructure of microbial cells in natural biotopes in situ, to detect new microorganisms, and to characterize submicroscopic microbial forms. However, electron microscopy is not so efficient for studying soils, sludges, and grounds, which contain few microbial cells but a great amount of mineral particles hindering the preparation of microscopic specimens. For this reason, Bae *et al.* [5] devised a method for the recovery and fractional concentration of microbial cells from natural substrates that allows a wider range of microorganisms to be detected and statistical data processing to be performed. The disadvantage of the method proposed by Bae *et al.* is that it requires lengthy procedures to be performed at temperatures above zero, which can considerably alter the cell ultrastructure during preparation. The LTFM method which we developed, allows the ultrastructure of microbial cells recovered from permafrost grounds to be studied and, in some cases, the physiological status of these cells to be determined from their ultrastructural and electron cytochemical characteristics. Concentrates of microbial cells obtained by this method were successfully used for the inoculation of nutrient media and isolation of nonculturable microorganisms [15]. Another approach used in this study (direct electron microscopy of microorganisms in situ) turned out to be efficient in studying oil sludge. The high concentration of microorganisms and the plasticity of this substrate make unnecessary cell fractionation and allow specimens for microscopic studies to be prepared in the form of micromonoliths and then thin sections. This approach made it possible to study microbial associations in unaltered natural environments, to characterize the morphological and ultrastructural diversity of

Fig. 3. Thin sections of associations of microbial cells found in oil sludge: (a) coccoid gram-negative cells with an extended periplasm filled with a dense matrix, which form spherical cell aggregates enclosed in a common envelope; (b) a spherical cell aggregate enclosed in a common electron-opaque fibrillar envelope (most cells are lysed); (c) a conglomerate of various bacterial cells embedded in a common fibrillar matrix. OL, outer layer; P, periplasm; OM, outer membrane; C, capsule; CLC, cystlike cell; FM, fibrillar matrix, N, nucleoid. The scale bars represent 0.5 μm.

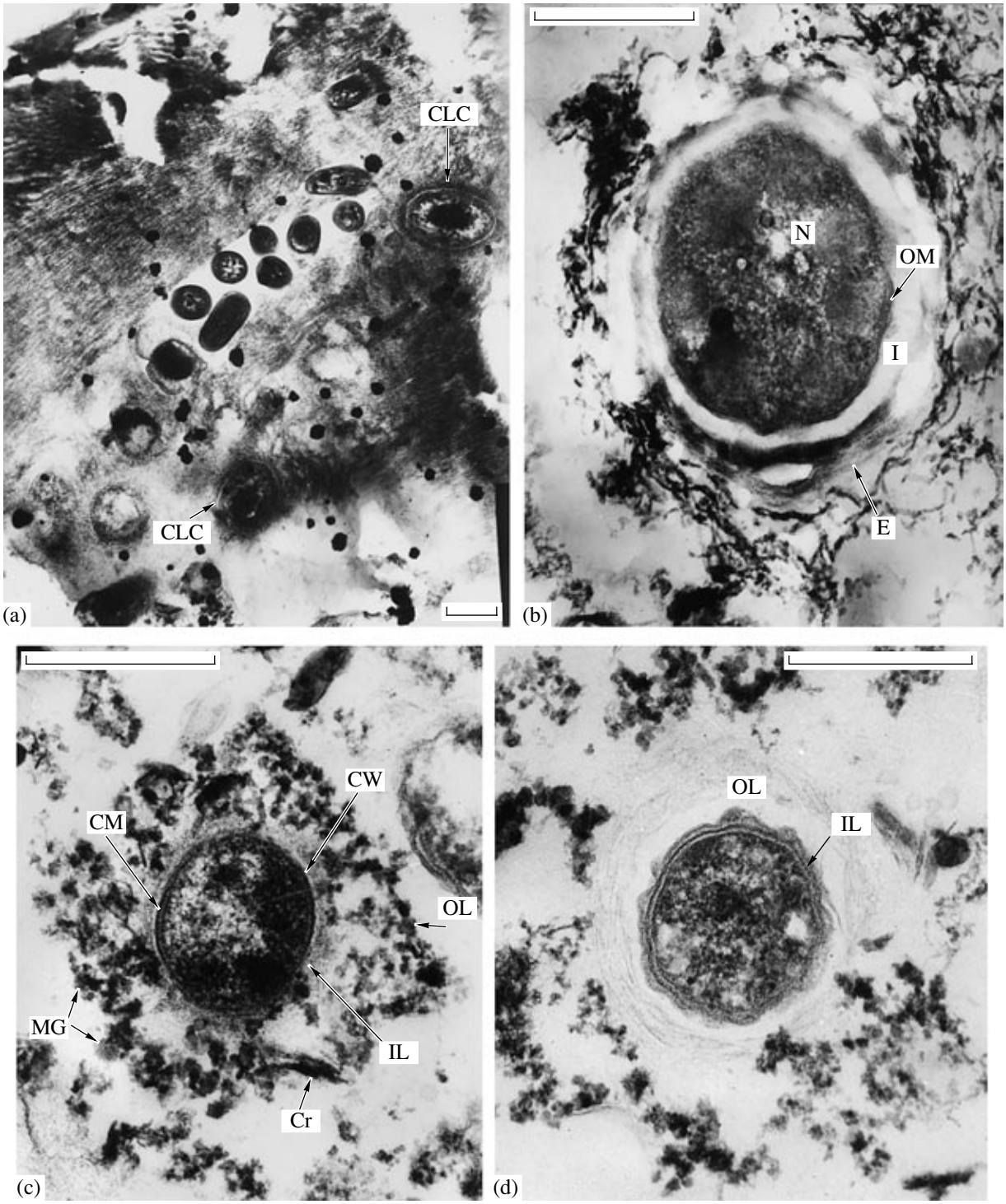

Fig. 4. Thin sections of various resting bacterial forms found in oil sludge: (a) cells inside a large organomineral particle; (b) cyst; (c, d) other resting forms. OM, outer membrane; CLC, cystlike cell; I, intine; E, extine; N, nucleoid; CM, cytoplasmic membrane; OL, outer layer; IL, inner layer; Cr, crystallite; MG, microgranule; CW, cell wall. The scale bars represent 0.5 μm.

microorganisms in oil sludge, to reveal atypical cell morphotypes, and to detect structurally differentiated resting cystlike bacterial forms.

The efficiency of the electron microscopic study of microorganisms in situ should further increase if the methods of electron cytochemistry, particularly, immunocytochemistry, are applied. In this case, not only can the metabolic activity of microbial cells be evaluated, but their identification to the species level can also be achieved.

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