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EXPERIMENTAL ARTICLES

Ultrastructure of Resting Cells of Some Non-Spore-Forming Bacteria

N. E. Suzina*, A. L. Mulyukin, A. N. Kozlova**, A. P. Shorokhova*, V. V. Dmitriev*, E. S. Barinova*, O. N. Mokhova***, G. I. El'-Registan**, and V. I. Duda***

**Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, pr. Nauki 5, Pushchino, Moscow oblast, 142290 Russia*
***Winogradsky Institute of Microbiology, Russian Academy of Sciences, pr. 60-letiya Oktyabrya 7, k. 2, Moscow, 117312 Russia*
****Pushchino State University, Pushchino, Moscow oblast, 142290 Russia*

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Abstract—Using electron microscopy (ultrathin sections and freeze-fractures), we investigated the ultrastructure of the resting cells formed in cultures of *Micrococcus luteus, Arthrobacter globiformis*, and *Pseudomonas aurantiaca* under conditions of prolonged incubation (up to 9 months). These resting cells included cystlike forms that were characterized by a complex cell structure and the following ultrastructural properties: (i) a thickened or multiprofiled cell wall (CW), typically made up of a layer of the preexisting CW and one to three de novo synthesized murein layers; (ii) a thick, structurally differentiated capsule; (iii) the presence of large intramembrane particles (d = 180–270 Å), occurring both on the PF and EF faces of the membrane fractures of *M. luteus* and *A. globiformis*; (iv) a peculiar structure of the cytoplasm, which was either fine-grained or lumpy (coarse-grained) in different parts of the cell population; and (v) a condensed nucleoid. Intense formation of cystlike cells occurred in aged (2- to 9-month-old) bacterial cultures grown on diluted complex media or on nitrogen-, carbon-, and phosphorus-limited synthetic media, as well as in cell suspensions incubated in media with sodium silicate. The general morphological properties, ultrastructural organization, and physiological features of cystlike cells formed during the developmental cycle suggest that constitutive dormancy is characteristic of non-spore-forming bacteria.

Key words: cystlike cells, non-spore-forming bacteria, cell ultrastructure, membranes, cell walls, dormancy in microorganisms, permafrost.

The survival strategy of many bacteria under unfavorable environmental conditions is based on the formation of various types of specialized structurally complex resting cells, e.g., endo- and exospores, "conidia," cysts, and other forms. They result from cell differentiation at the terminal stages of a culture's developmental cycle and differ from metabolically active vegetative cells in terms of their morphogenesis, ultrastructure, physiology, and biochemical composition [1]. However, these specialized, structurally complex resting cells have not yet been revealed in a large number of prokaryotic species. A number of researchers, therefore, doubt that non-spore-forming bacteria ever exist in the constitutive dormancy state because they lack forms characterized by an appropriate level of metabolism [2]. However, it seems more feasible that prolonged dormancy and maintenance of viability in non-spore-forming bacteria under unfavorable conditions can be due to resting cells that differ from spores in terms of their structure and formation mechanism. Importantly, experimental cultivation on artificial media does not allow the bacteria involved to actualize their full phenotypic potential and to form the whole spectrum of resting cell types that are peculiar to microorganisms in nature. For instance, Zechman and Casida [3] concluded from their data that typical non-spore-forming gram-negative bacteria such as *Pseudomonas* form cystlike cells that can be stored for a long time. Our research on various types of dormancy revealed that a large number of microorganisms, including non-spore-forming bacteria and yeast, form highly refractory cystlike cells characterized by a number of important properties that are peculiar to resting cells [4–6]. In addition to other factors, their formation is conditional on the concentration and activity of extracellular factors d_1 [4, 5]. Factors d_1 belong to alkylhydroxybenzenes (AHBs) [7, 8], and their mechanism of action is considered in [9, 10]. We have briefly characterized the general morphology and ultrastructure of cystlike cells in several bacterial species [4–6]. However, the data available in the literature are insufficient for tackling the following important questions: (i) how serious the ultrastructural and molecular changes are in cystlike resting cells, in contrast to vegetative cells; (ii) what the

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degree of intraspecies heterogeneity is among various structural types of cystlike resting cells; and (iii) what similarities and differences can be revealed by comparing the ultrastructural organization of cystlike resting cells forms in nature and under experimental conditions. Obviously, we cannot understand the causes and mechanisms of long-term survival of microorganisms under unfavorable conditions in nature unless we elucidate the structural and functional peculiarities of resting cells.

This work presents data on the ultrastructural organization of cystlike cells of *Micrococcus luteus*, *Arthrobacter globiformis*, and *Pseudomonas aurantiaca* obtained under laboratory conditions during the developmental cycle of their cultures and resting forms isolated from natural soils by low-temperature fractionation.

MATERIALS AND METHODS

Microorganisms and cultivation conditions. Our studies used bacteria with different cell envelope structure, including the gram-positive bacteria *Micrococcus luteus*, strain NCIMB-13267, and *Arthrobacter globiformis*, strain B-1112 (All-Russia Collection of Microorganisms), and the gram-negative species *Pseudomonas aurantiaca*, strain B-1558 (All-Russia Collection of Microorganisms).

M. luteus cells were cultivated on a synthetic medium [5], and *A. globiformis* was grown on the medium described in [6]. *P. aurantiaca* was cultivated on 50% nutrient broth and on synthetic medium 23A with 0.2% glucose [11]. The bacteria were cultivated at 28°C in 250-ml flasks (containing 50 ml of the medium) using a shaker (140–160 rpm). Stationary phase cultures grown on nutrient broth served as inoculum. The inoculum was added at the concentration corresponding to an initial optical density of 0.1 (λ = 650 nm, l = 10 mm) of the resulting suspension.

Cystlike cells (CLC) were obtained (i) after long-term incubation (for 2–9 months), at room temperature, of nitrogen- or phosphorus-limited cultures; the nitrogen or phosphorus concentrations were ten times lower than those in standard media; (ii) in cultures incubated for 2 months after the addition (during the stationary growth phase) of 4-*n*-hexylresorcinol (C_6-AHB, M = 196) at concentrations of 10^{-4} to 5×10^{-4} M; and (iii) in cell suspensions incubated in a 0.09% solution of sodium silicate (pH 7.4) at room temperature for 3 months. The latter series of experiments used exponential phase (6-h) and stationary (3-day) cultures and also nitrogen-limited cultures stored for a long time (3–5 months). Cells in all these studies were precipitated by centrifugation (4000 *g*), resuspended in 2 ml of sterile sodium silicate solution (0.9 g/l SiO_2) and incubated at room temperature for 3 months. The viable cell number was estimated from the colony-forming units (CFU) counted after inoculating a cell suspension on meat–peptone agar.

Isolation of cell fractions from natural substrates. Microorganisms from natural substrates were isolated by the low-temperature cell fractionation method developed by us [12]. We used permafrost samples from East Siberia (the Kolyma River region) and oil-containing sludge samples that had been stored in the sludge collectors of the Nizhnekamskii oil refinery for 10 years. The permafrost samples used by us were taken at a depth of ~60 m, and their age was 1–3 million years. These samples were characterized by us earlier [13].

Light microscopy was performed using Amplival (German Democratic Republic) and MBI-15 microscopes equipped with phase-contrast devices.

Electron microscopy. *Ultrathin sections* were prepared as follows. Microbial cell sediments were fixed upon centrifugation in 1.5% glutaraldehyde solution in 0.05 M cacodylate buffer (pH 7.2) at 4°C for 1 h. They were washed three times 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. Upon desiccation, the material was encapsulated in Epon 812 epoxy resin. Ultrathin sections were contrasted for 30 min in 3% uranyl acetate solution in 70% ethanol and additionally stained with lead citrate (by the Reinolds method) at 20°C for 4–5 min.

Cell *freeze-fractures* were obtained in a JEE-4X vacuum setup (JEOL, Japan) by means of devices that allowed cooling of microbial cells at a rate of about 10 grad/s. Specimens were frozen in liquid propane (overcooled with liquid nitrogen to a temperature of −196°C) without chemical prefixation or any other pretreatment. Cell freeze-fractures were obtained upon attaining a vacuum level of 3×10^{-4} Pa and a temperature of ~ −100°C. Replicas from the fracture surface were prepared by spraying a platinum–carbon mixture in vacuo at an angle of 30° and a strengthening pure carbon layer at an angle of 90°.

Ultrathin sections and fracture replicas were examined in a JEM-100B (JEOL, Japan) electron microscope at an accelerating voltage of 60 kV.

Lysozyme effect on resting cells. Egg yolk lysozyme (Biokhimreaktiv Research and Production Association) was added to 5- and 6-month cultures of *M. luteus* at concentrations of 1, 10, and 100 μg/ml and incubated at 29°C for 20 min. A part of each culture was diluted and plated onto the agarized synthetic medium used to cultivate micrococci; the other part of each culture was supplemented with glutaraldehyde to a final concentration of 1.5%. Subsequently, the cells were centrifuged, fixed, and embedded in resins using standard techniques. Ultrathin sections were prepared.

RESULTS

Phase-contrast light microscopy revealed that nitrogen- or phosphorus-limited *M. luteus, A. globiformis*, and *P. aurantiaca* cultures that were maintained for a

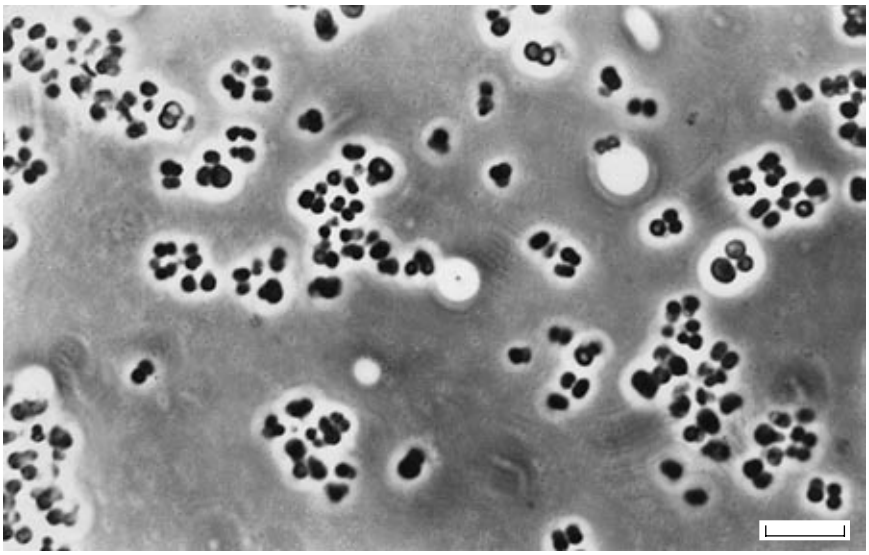


Fig. 1. Cells and their aggregates in a 9-month-old *M. luteus* culture under a phase-contrast microscope. Bar, 10 μm.

long time (for 2–9 months) at room temperature represented a morphologically intact cell population, analogously to cultures containing C_6-AHB ($3–5 \times 10^{-4}$ M). The viable cell percentage (based on CFU number) in old cultures was invariably below the vegetative cell number before dormancy and amounted to ~0.01–60%, depending on the experimental conditions and the bacterial species involved.

Structure of resting cells of *M. luteus*. The maximum percentage of viable cells was characteristic of aged, nitrogen-limited cultures of *M. luteus*.

Medium variant	Number and percentage of viable cells (CFU/ml) obtained under the specified conditions		
	at the onset of the stationary phase	after 2 months of storage	after 9 months of storage
Medium without nutrient limitation	3×10^9 (100%)	7×10^7 (2.3%)	2×10^6 (0.066%)
Nitrogen-limited medium	1.4×10^8 (100%)	8×10^7 (57%)	1.5×10^7 (11%)

Importantly, *M. luteus* colonies were predominantly formed by aggregates made up of two or three cells to several dozens of cells, not by solitary cells. Cells in the aggregates were tightly bound together by unseparated cell walls in the septum zone or surrounded by envelopes shared by several cells (Figs. 1–3). Even ultrasonic treatment of the suspension according to D.G. Zvyagintsev (15 kHz, 0.44 A, 1 min) yielded only several percent of solitary cells, whereas the rest of the suspension consisted of cell aggregates.

Electron microscopy of ultrathin sections and bacterial freeze-fracture replicas demonstrated the morphological heterogeneity of the cells after long-term storage or in suspensions containing C_6-AHB (3×10^{-4} M). A part of the cells was characterized by pronounced destructive changes in the organelles: the cytoplasmic membrane (CM) and cell wall (CW) were ruptured and the cytoplasm (C) and nucleoid (N) were lysed. The rest of the cells were morphologically intact, and no visible damage of their organelles was detectable at the ultrastructural level. Apparently, these cells represented colony-forming units. A comparative study of electron micrographs revealed the following distinctive features of the ultrastructural organization of resting cells:

(i) Formation of a continuous thick microcapsule layer (MC) that consists of an electron-dense fibrillar and granular substance (Figs. 2–6). The thickness of this layer varies from 10 to 60 nm in different cells, and the length of the fibrils arranged perpendicular to the cell surface reaches 0.3–0.5 μm. In contrast, fibrils on the surface of the thin capsule layer are rare in vegetative cells, and they represent short (10–50 nm) protrusions.

(ii) An increase in the cell wall (CW) thickness and the formation of multiple layers within the CW. The CW is 1.5–3 times thicker in resting cells than in vegetative cells from exponential or stationary phase cultures (Figs. 2–6). The CW thickness is 40–50 nm in

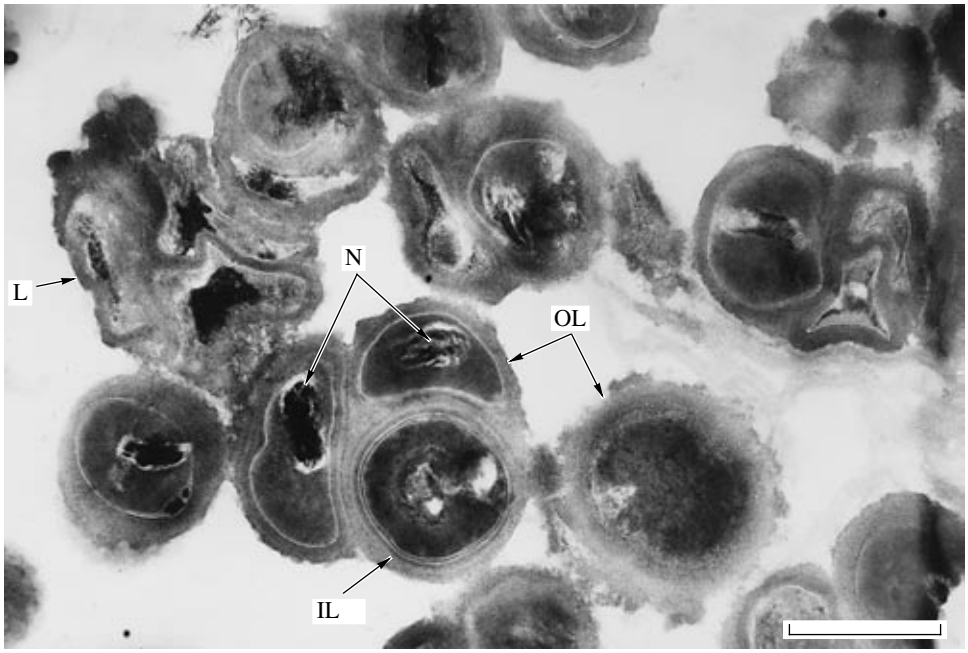
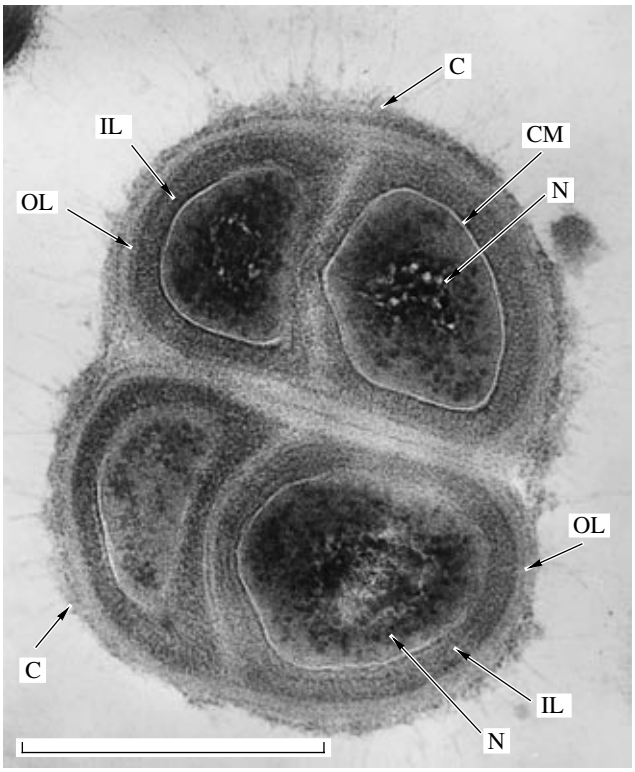


Fig. 2. Ultrastructure of the cells of a 9-month-old culture.

Figs. 2–9. Electron micrographs of ultrathin sections (Figs. 2–4 and 7–9) and freeze-fractures (Figs. 5, 6) of *M. luteus* cells. Bars in all of the further figures correspond to 0.5 μm. Designations: CP, cytoplasm; CW, cell wall; CM, cytoplasmic membrane; OM, outer membrane; C, capsule; N, nucleoid; OL, outer layer of the CW; OL-1 and OL-2, differentiated outer layers of the CW; IL, inner layer of the CW; L, lysed cells; Cr, crystallites; M, murein; R, ribosomes; PF, protoplasmic face of CM fractures.



Figs. 3 and 4. Ultrastructure of typical intact cells (with a characteristic ultrastructure of the capsule, cell wall, cytoplasmic membrane, nucleoid, and cytoplasm) from cultures incubated in a nitrogen-limited medium for 9 months.

3-day-old cultures (Fig. 5), 60–80 nm in resting 2-week-old cultures (nondividing, morphotype I cells), and 60–80 and 80–100 nm in resting 6- to 9-month-old cultures (cells of morphotypes II and III, respectively; Figs. 2–4, 6; Scheme 1). Cells with a thick CW (morphotypes II and III) prevail in the population and account for 70–80% of the total number of structurally intact cells in (i) 5- to 9-month-old nitrogen- or phosphorus-limited cultures, (ii) cultures grown on diluted (1 : 10) nutrient broth, and (iii) cell suspensions incubated in sodium silicate solution for 3 months. However, such cell types seldom (<1%) occur in cultures grown in undiluted nutrient broth. We denote cells of morphotypes I, II, and III as *cystlike cells* (CLC). The ultrastructural peculiarity of the thick CW of morphotype I and II CLC is that they consist of several (two to four) layers separated by very thin (10–20 nm) zones composed of a highly electron-dense substance (Figs. 2–4). Each monolayer is 30–40 nm thick. In a part of these CLC, the monolayers have identical internal structures and electron density (Fig. 4). As for the other part, the innermost layer is distinguished by a high electron density and filled with electron-dense granules (Figs. 2, 3). A comparative study of a large number of electron micrographs led us to believe that, during the development of CLC, the inner layers form after the outer layer. Hence, the outermost layer is the oldest. This layer is common to unseparated cells and encloses aggregates consisting of two or more cells (Figs. 2, 3). Thus, monolayers represent self-contained

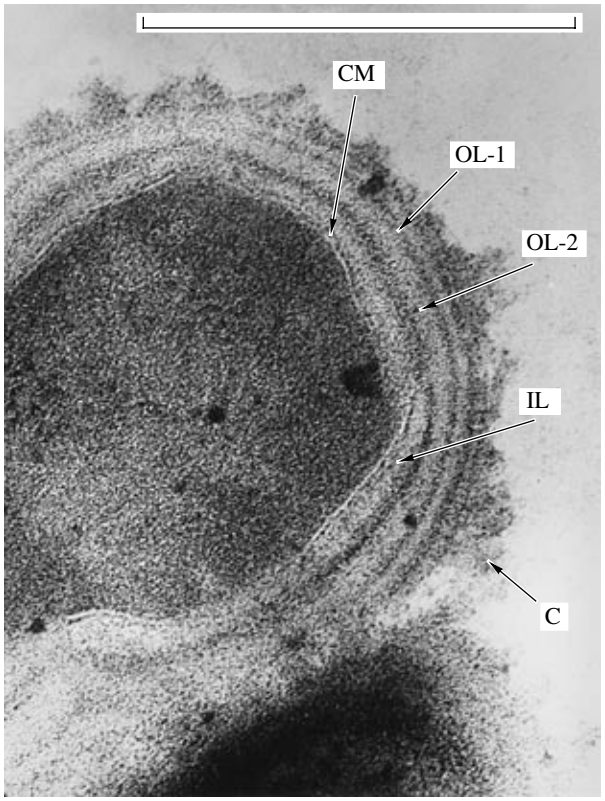
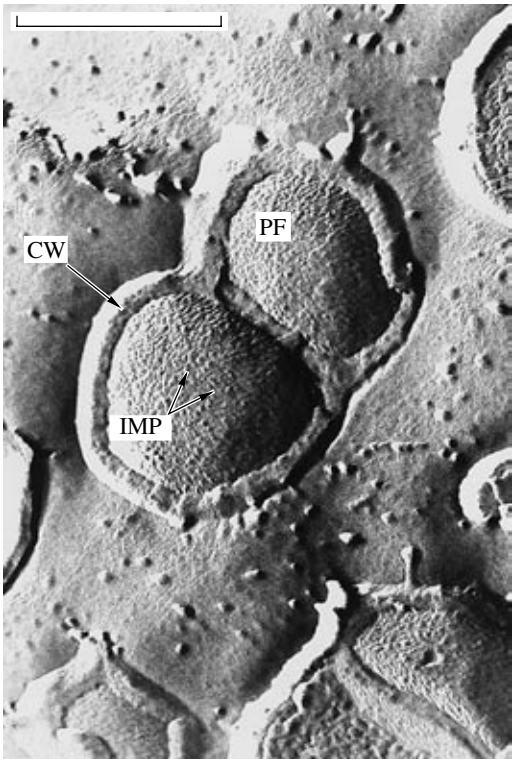


Fig. 4.



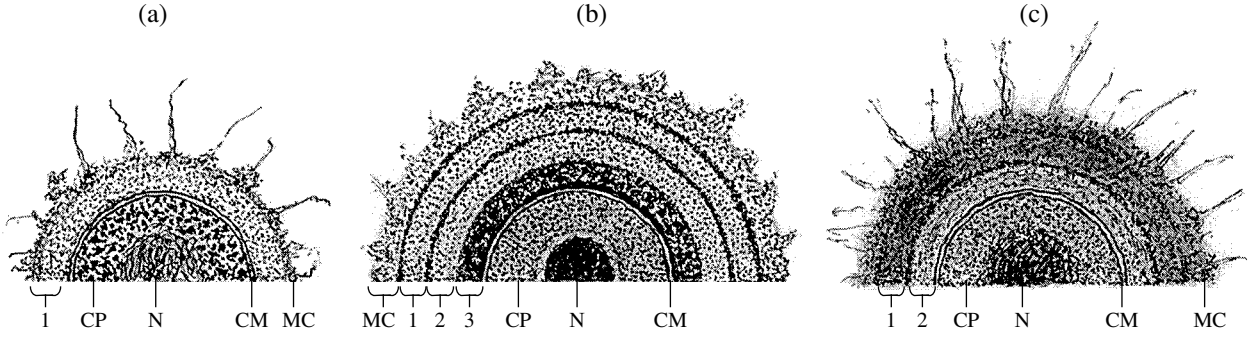
Figs. 5 and 6. PF side of the cytoplasmic membrane and cell wall in the cells of 3-day-old (Fig. 5) and 9-month-old cultures (Fig. 6). Note the granular intramembrane particles (IMP).

(elementary) CW of different age and origin, and, collectively, they constitute a multicell wall envelope structured on the encapsulation principle. A similar process was revealed in actinomycete systems characterized by intrahyphal growth of new hyphae and the formation of special "endospores" [14]. Mono- and bilayer CW are typical of the CLC forming in C_6-AHB-containing cultures.

(iii) All CLC types display changes in the molecular structure of the cytoplasmic membrane (CM): smooth, intramembrane particle (IMP)–lacking zones occur in the PF fractures of the CM (Fig. 6), while IMP are evenly distributed on the PF face of the CM in vegetative cells (Fig. 5). The IMP size is 80–100 and 230–270 Å in vegetative cells and CLC, respectively (Fig. 6). Mesosomes occur rarely.

(iv) The cytoplasm undergoes structural changes. In some of the cells, it is characterized by a fine granular structure, while the rest of them display large electron-dense lumps. On the whole, the cytoplasm is characterized by a high electron density in many cells.

(v) The nucleoid represents a discrete compact structure. DNA fibrils aggregate with the formation of lumps (occasionally strands) of an electron-dense substance that is surrounded by an electron-transparent zone, which also contains thin DNA fibrils (Figs. 2, 3).



Scheme 1. Surface structures of *M. luteus* CLC. MC, microcapsule; 1, 2, and 3, different CW layers. For other designations, see Figs. 2–15.

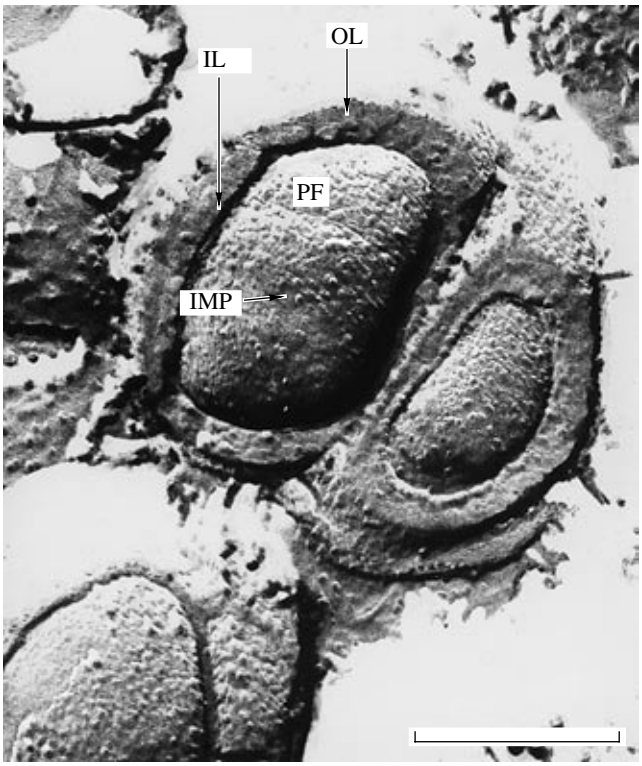
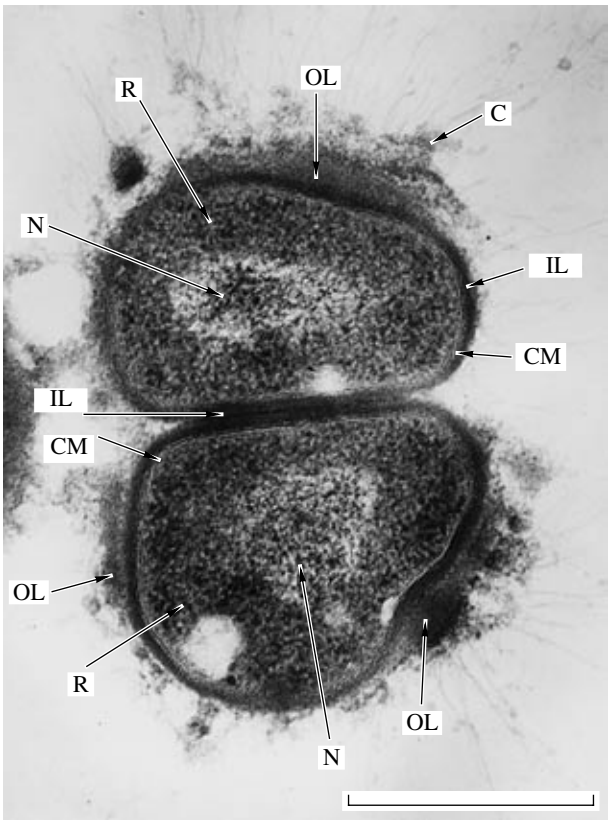


Fig. 6.



Figs. 7–9. Ultrastructure of the typical cells dominating the microbial population in a 6-month-old culture treated with lysozyme (1 μg/ml, 15 min). Note the cells retaining fragments of the bilayer OL and an intact IL (Fig. 7), the cells with an almost completely destroyed OL (the CW surface representing the former IL is covered with capsule fibrils, Fig. 8), and the remnants of a lysed cell surrounded by a capsule layer (Fig. 9).

The nucleoid is unclear (Fig. 4) in cells with the fine granular structure and structurally resembles the nucleoid of *Bacillus* endospores.

An electron microscope study of lysozyme-treated CLC revealed that pronounced changes in cell structure occurred starting from a lysozyme concentration of 1 μg/ml. In 6-month-old cultures, the CFU number decreased from $5.4 \times 10^7 \pm 0.1$ to $2.6 \times 10^7 \pm 0.3$, $2.6 \times 10^6 \pm 0.1$, and $8.5 \times 10^4 \pm 0.1$ at lysozyme concentrations of 1, 10, and 100 μg/ml, respectively. These data attest to the presence of highly lysozyme-resistant cells in old cultures of the micrococcus. In the control experiment, lysozyme at the minimum concentration (1 μg/ml) caused complete lysis of vegetative cells. By examining the ultrathin sections, we established that lysozyme (1 μg/ml) destroys cells with thin CW (morphotype I) and the suspensions contain only CLC with thick and multilayer envelopes (morphotypes II and III). However, most of them display pronounced ultrastructural changes.

(i) The superficial zone of the CW of some of the cells become less dense, while the outer and middle murein layers of other cells are partially (Fig. 7) or completely (Fig. 8) destroyed, and the cells only retain the innermost layer, which is in contact with the cytoplasmic membrane. CW lysis proceeds to a different extent in different parts of the CW surface. If this process is incomplete, multilayer portions ("sandwiches") of the outer and the middle layer still remain on the innermost CW layer. The capsule is retained. It occurs even in almost completely destroyed cells (Fig. 9). Hence, the lysozyme effect confirms that all CLC envelope layers (except the capsule) are composed of murein.

(ii) The nucleoid is decompacted, and it assumes a structure similar to the nucleoid of actively growing vegetative cells (Figs. 7, 8).

(iii) The cytoplasm has a structural pattern that is peculiar to vegetative cells. It is characterized by a normal electron density and clearly visible ribosomes. The sequence of these structural changes in CLC in the presence of lysozyme is analogous to those occurring during the initial stages of cyst germination. The data obtained provide direct evidence that CLC are viable and lysozyme-resistant (a detailed analysis of the effect of lysozyme on resting cells will be performed in a special work). Plausibly, a lysozyme-treated CLC population contains "superdormant" forms that do not germinate, but this issue calls for additional studies.

Structure of resting cells of *Arthrobacter globiformis*. Four-month-old, nitrogen-limited *A. globiformis*

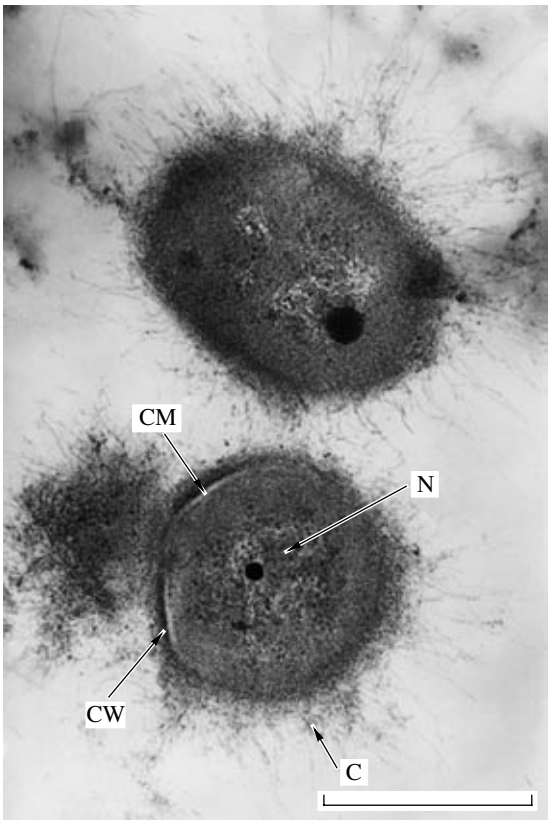


Fig. 8.

cultures (the CFU number was 3.6×10^8 ml^{-1}, i.e., 60% of the initial number) contain numerous cells with thickened, multilayer envelopes, in contrast to the vegetative forms (Figs. 10–12); compact nucleoids; and a cytoplasm containing large granules. In general, their ultrastructural pattern resembles that of the type I CLC of the micrococci. The outer envelope layer is particularly prominent, and it is characterized by a granular structure (Fig. 12). From the sections, it is evident that numerous electron-dense granules are attached to this layer (Fig. 10). The inner homogeneous layer is structurally similar to the cell wall of a vegetative cell (Fig. 10). The changes in the CM substructure of resting forms are analogous to those in *M. luteus*: extensive smooth particle-free areas appear on the PF face, and the IMP occurring in other CM parts are larger in size (~230–270 Å) than the IMP of the vegetative cells (~140–160 Å). The undulate, "hilly" pattern of CM fractures is apparently due to the compression of cocoonlike cells (Fig. 12). Cells of this type are to be regarded as CLC, based not only on their ultrastructural peculiarities but also on the data on their survival during prolonged storage and high temperature resistance; these properties were described earlier [6].

Structure of resting cells of *Pseudomonas aurantiaca*. The number of viable cells drastically decreased in *P. aurantiaca* cultures during long-term storage (for 5 months); the final CFU number was 3×10^4 ml^{-1}, i.e., 0.001% of the initial number. Electron microscopy revealed that most cells in these cultures were lysed and the remaining structurally intact cells were shortened and rounded. They were characterized by the following ultrastructural properties (Figs. 13, 14; Scheme 2): (i) an enlarged periplasmic space that was up to 80–90 nm in thickness (in contrast to 40–50 nm in the vegetative cells of exponentially growing cultures); (ii) a fine granular structure of the cytoplasm that lacked any clearly visible ribosomes; and (iii) condensed chromatin detectable in some portions of the nucleoid. Nitrogen-limited cell suspensions that were resuspended and incubated in sodium silicate solution for 3 months were characterized by a high percentage of viable cells (the CFU number was 1.5×10^9 ml^{-1}, i.e., 50% of the initial number) and contained CLC. In addition to the above properties, they were also distinguished by a thickened

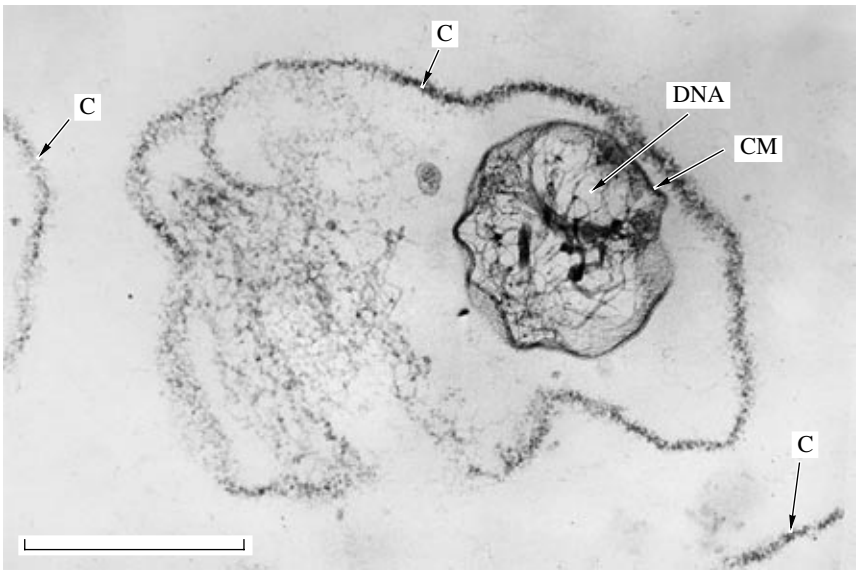
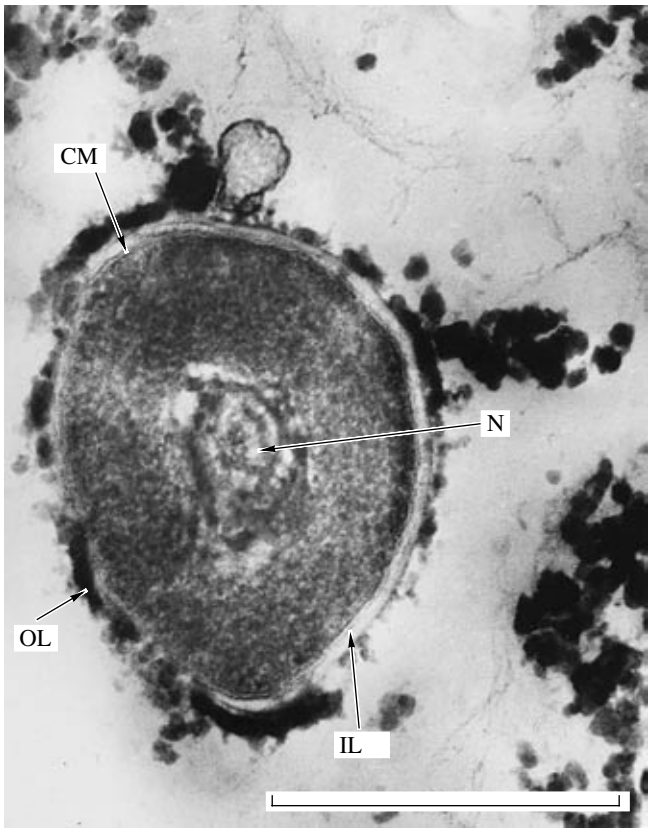


Fig. 9.



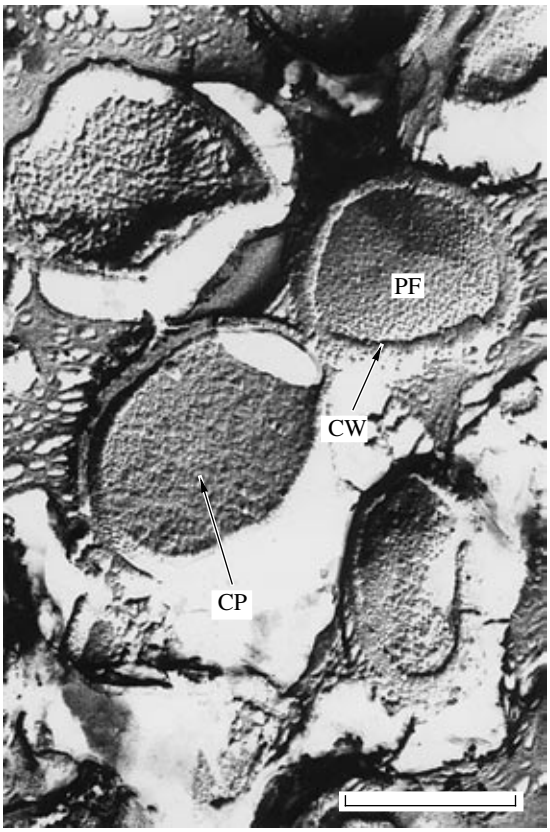


Fig. 11.

Figs. 10–12. Electron micrographs of sections (Fig. 10) and freeze-fractures (Figs. 11 and 12) of *A. globiformis* cells from cultures incubated for 2 months on a nitrogen-limited medium. Note the bilayer envelope structure (Fig. 10) and the CW and CM structure in the cells of 3-day-old (control, Fig. 11) and 6-month-old (Fig. 12) cultures.

CW murein layer and a large capsule. In some of the cells, the capsule had a bilayer structure consisting of an inner, electron-transparent layer with a fibrillar structure (60–70 nm thick) and an outer, electron-dense layer (~40–50 nm thick) characterized by a granular–fibrillar ultrastructure (Fig. 15, Scheme 2). The CLC structure of *P. aurantiaca* bears a significant similarity to *Ramlibacter tataouinensis* cysts [15].

Novitsky and Morita [16] earlier described the enlargement of the periplasmic space in the cells of another gram-negative bacterium, a marine vibrio, in cultures incubated for a long time (3 weeks). Frenkiel-Krispin *et al.* [17] showed compactization of nucleoid in the cells of aged, starving *Escherichia coli* cultures.

Studies of resting cells in natural habitats in situ. In order to detect CLC in natural habitats in situ, we investigated "populations" of microbial cells isolated from natural substrates (Figs. 16–18). Of particular interest in this respect are permafrost soil and subsoil sediments that have been frozen (at –10 to –12°C) for 1–3 million years because it is accepted that microorganisms exist in the anabiotic state under these conditions [13]. A study of the ultrastructural organization of bacterial cells isolated from permafrost revealed that most of them represent cystlike forms, or CLC (Fig. 17). Among them, we often observed bacteria that were morphologically and ultrastructurally similar to the CLC of coryneform bacteria. They were characterized by thick multilayer envelopes (0.15–0.4 μm thick), which were structurally similar to the envelopes of bacterial cysts or CLC obtained under experimental conditions. The layers of the envelope differed in their ultrastructure. The inner, weakly electron-dense layer (IL) was located between the outer membrane and the outer layer (OL) with a granular–lamellar structure (Fig. 18). Particles (electron-dense needlelike crystallites, granules, and plate- and leaf-shaped structures) were embedded in the fibrillar–reticulate OL matrix or located on its surface. The particles apparently were composed of mineral substances, including clayey structures and compounds containing iron and other metals occurring in the soils. These particles were tightly bound to the envelope OL, and they were not removed by ultrasonic treatment (15 kHz, 0.44 A, 1 min) with subsequent repeated centrifugation. Such CLC also occur in oil-containing sludge samples.

A characteristic ultrastructural property of CLC isolated from natural samples is that their CM contains large intramembrane particles with a diameter of

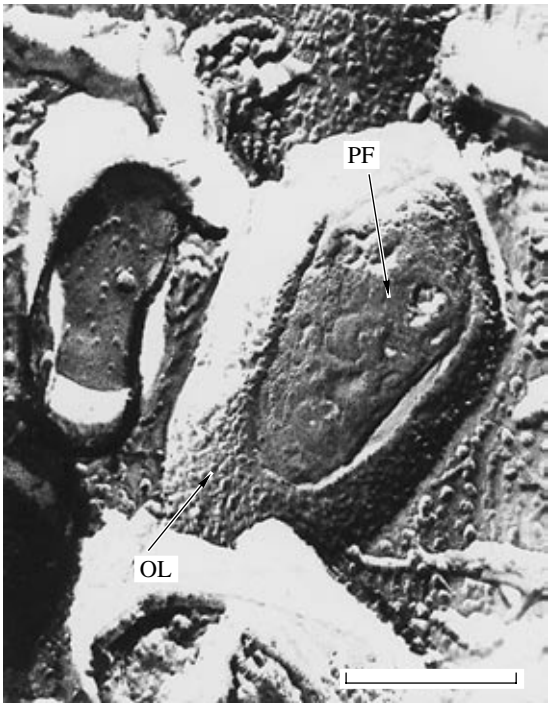
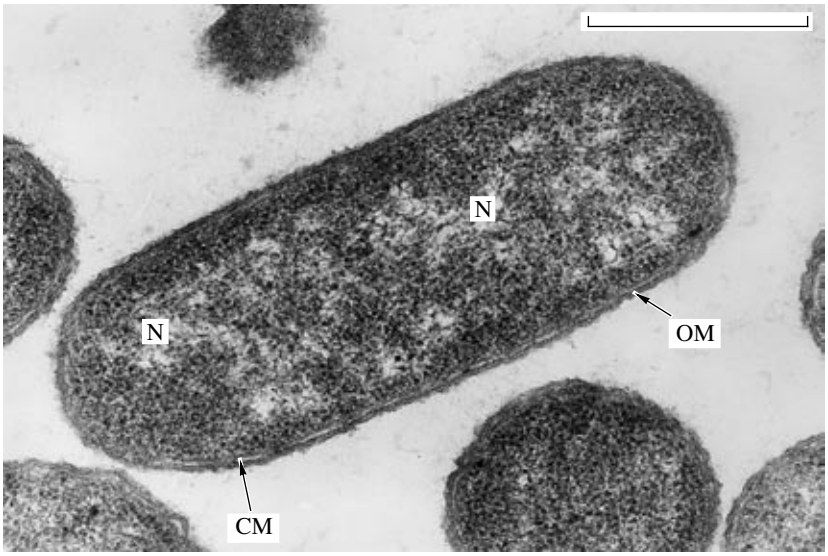


Fig. 12.

190–220 Å (Fig. 17). They are similar in size to the IMP of the CLC obtained under experimental conditions (Fig. 6). These particles were easily detectable on the PF and EF faces of CM freeze-fractures; the structure and the properties of the CLC occurring in these soils are considered in more detail in the work by Dmitriev *et al.* [12]. A comparative study of the ultrastructure of the CLC of *M. luteus*, *A. globiformis*, and *P. aurantiaca* revealed significant similarities with CLC-1 and CLC-2 occurring in natural substrates (permafrost and oil-containing sludge samples).

DISCUSSION

The data available in the literature do not allow us to provide an adequate explanation for the causes and mechanisms of long-term survival of non-spore-forming microorganisms under extreme conditions in nature. Based on the data on the long-term survival of non-spore-forming bacteria in soils and their resistance to unfavorable factors, Zechman and Casida [3] concluded that the bacteria form cystlike cells under natural conditions. We conducted electron microscope studies on cell populations isolated from permafrost soil using the low-temperature fractionation method. Our data support the conclusion that microbes persist as resting (anabiotic) forms in permafrost soils. In the first place, such cells are characterized by thickened envelopes composed of several differentiated layers (Figs. 16–18). Similar forms were also detected in native and desiccated soil samples. We cultivated *M. luteus*, *A. globiformis*, and *P. aurantiaca* under quasi-natural conditions (on N-, P-, and C-limited media containing significant amounts of soluble Si forms) and revealed that these bacterial cultures contained resting cells that were structurally similar to natural CLC forms. A detailed electron microscope study conducted by us demonstrated that the structural transformation associated with the transition from vegetative cells to resting forms (CLC) has a systemic, complex pattern, involving changes in the envelope (the CW and



Figs. 13–15. Electron micrographs of ultrathin sections of various types of *P. aurantiaca* cells. Fig. 13 shows cells of a 2-day-old culture; Fig. 14, resting cells of a 4-month-old nitrogen-limited culture; Fig. 15, CLC incubated in a medium with sodium silicate for 3 months. P, periplasmic space.

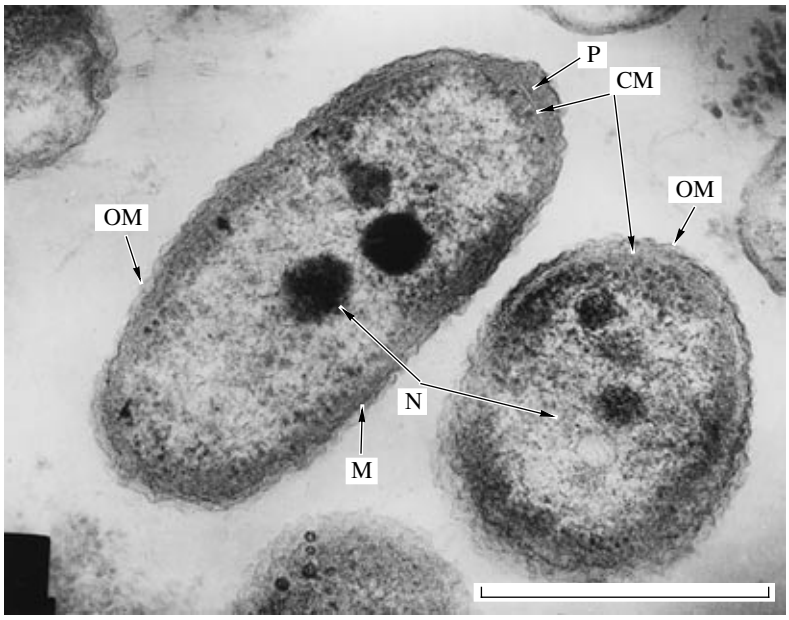


Fig. 14.

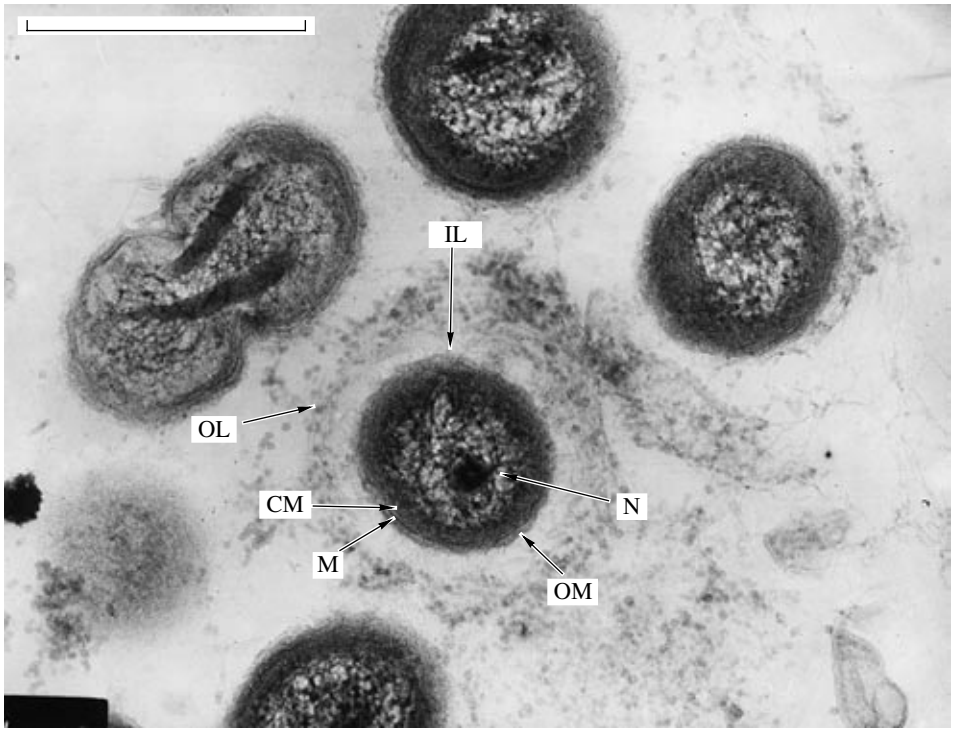
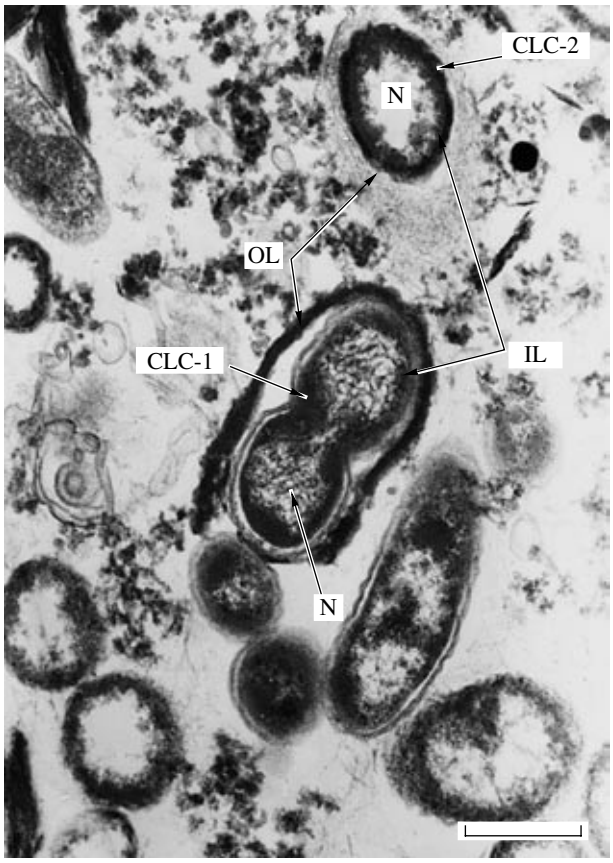


Fig. 15.

the capsule), cytoplasmic membrane, cytoplasm, and nucleoid. CW thickening was earlier described in the resting cells of *M. luteus* by Mukamolova *et al.* [18], Votyakova *et al.* [19], and Mulyukin *et al.* [5] and in the CLC of *A. globiformis* by Demkina *et al.* [6]. This phenomenon was demonstrated in this work using ultrathin sections and artifact-free cryofractography (freeze-fractures). It should be stressed that, in their ultrastructural characteristics, the CLC of gram-negative bacteria, described for *P. aurantiaca* in the present work and for *E. coli* in earlier studies [20], are similar to cystlike forms of *Legionella pneumophila* [21].



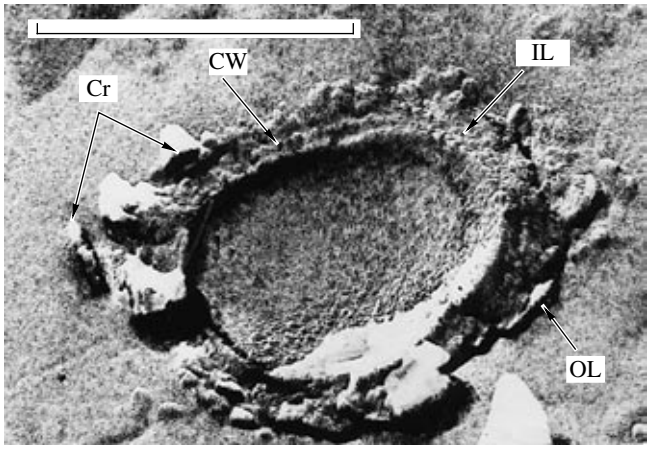
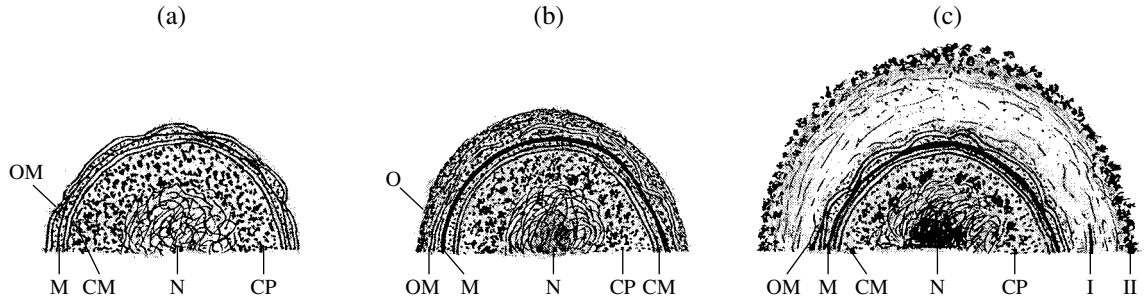


Fig. 17.

Figs. 16–18. Electron micrographs of sections (Figs. 16 and 18) and freeze-fractures (Fig. 17) of cells isolated from oil-containing sludge (Figs. 16 and 18) and permafrost samples (Fig. 17) by low-temperature fractionation. Designations: CLC-1, resting cells resembling *M. luteus* and *A. globiformis* CLC; CLC-2, resting cells resembling *P. aurantiaca* CLC; for other designations, see Figs. 2–15.

We found that, apart from CW thickening, CLC are characterized by CW differentiation into several (two to four) layers and each layer represents an elementary CW (CW "polymerization"). A cytological analysis revealed that the outer CW layer represents a preexisting structure that is shared by two or three cells. The inner CW layer forms de novo. All the layers consist of murein, based on the data on their destruction by lysozyme. The percentage of the cells with multilayer CW may amount to 70–75% of the total number of structurally intact cells in CLC populations. Apart from the data that the cells are ultrastructurally intact, the retention of the colony-forming capacity after thermal treatment or the addition of lysozyme attests to the viability of these cells [5, 6]. Direct evidence that *M. luteus* CLC are viable was provided by electron microscopy studies on the structural transformation of CLC to vegetative cells under the influence of lysozyme (Figs. 7, 8). The complex structure of the cells, their resistance to high temperatures and lytic factors, and their long-term viability allow us to regard the tested types of resting forms as cystlike cells (CLC). Taking into account that these forms are highly heterogeneous in morphological and ultrastructural terms and no dominant type can be singled out, it seems expedient to consider them cystlike forms and not to regard the cells as typical cysts. Structurally similar CLC were detected by us in natural habitats. These data are of special importance in terms of the mechanisms and microbial forms involved in long-term survival of non-spore-forming bacteria under natural conditions, particularly in 2- to 3-million-year-old permafrost.



Scheme 2. Surface structures of *P. aurantiaca.* I and II, the outer and the inner layers of the capsule envelope. For other designations, see Figs. 2–15.

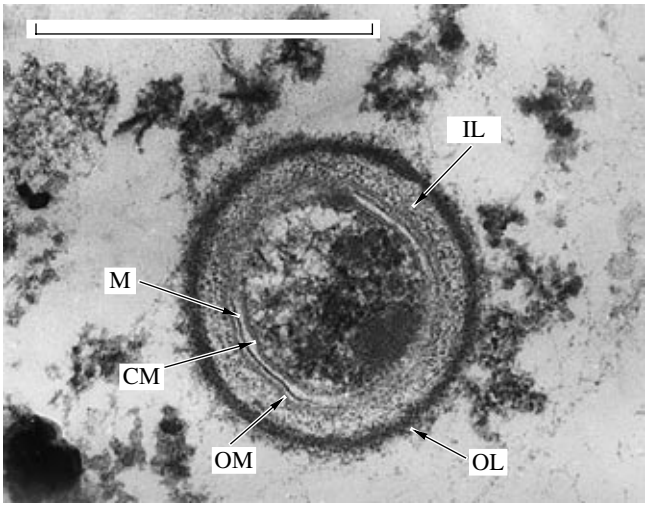


Fig. 18.

Special attention should be given to the increase in the complexity and thickness of the polysaccharide-containing envelopes (cell walls and capsules) of cystlike cells. Apparently, these surface structures perform, apart from the protective function, a function similar to that of the cortex of *Bacillus* endospores, which contains an osmotically active negatively charged polymer (peptidoglycan) and accounts for partial dehydration of endospore protoplasts [22]. The suggestion concerning the possible osmoregulatory function of the "thick-walled structures" containing negatively charged polymers in endospores and, apparently, cysts and other resting microbial forms was made by Gould and Dring for the first time several decades ago [22]. However, we currently still lack detailed data on the chemical properties of the polymers forming part of the thick and multilayer surface structures of cystlike cells. Such data could support the above suggestion. We have not yet clarified the question of the intensity and the pattern of metabolism in maturating and mature CLC. Although the oxygen uptake by aged cultures cannot be determined polarographically, our data on the synthesis of new large surface structures and the inhibition of CLC formation in the micrococcus under anaerobic conditions (data not shown) suggest that metabolic processes do occur in the resting forms described by us.

From the data reported here we conclude that the constitutive form of dormancy (anabiosis) is characteristic of non-spore-forming bacteria.

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