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EXPERIMENTAL ARTICLES

Electron-Microscopic Studies of the Colonies of an Alkylsulfonate-utilizing Bacterial Consortium

O. A. Mogil'naya, A. P. Puzyr', Yu. L. Gurevich, and E. A. Babkina

Institute of Biophysics, Siberian Division, Russian Academy of Sciences, Krasnoyarsk, 660036 Russia

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Abstract—The light- and electron-microscopic analysis of the colonies of a bacterial consortium capable of utilizing alkylsulfonates, which are the main ingredients of waste from the synthetic rubber industry, revealed the presence of eight types of cells. All types of cells were gram-negative and differed in shape, size, and the presence of capsule and cytoplasmic inclusions. Three types of cells were present in all the colonies studied. The presence of the other types of cells depended on the inoculum used and on the composition of the growth medium.

Key words: electron microscopy, colonies, bacterial consortium.

The electron-microscopic examination of colonies without washing them off of the nutrient agar is one of the most gentle procedures that allows for the arrangement and ultrastructure of bacterial cells in the colonies to be studied. Using this method, we analyzed the colonies of pure cultures [1], a binary bacterial association [2], and a natural community [3]. The investigation of natural communities is of particular interest, since bacteria live in nature in the form of microcolonies, biofilms, and other associations. In spite of the fact that this method is relatively time- and labor-consuming, it adds well to various microbiological, biochemical, and genetic methods that are used for studying microcommunities. For instance, the electron-microscopic analysis of biofilms formed during the degradation of some organic substances [4, 5] and in the oral cavity [6] allowed the morphological diversity, the spatial arrangement, and other characteristics of component bacterial cells to be elucidated.

The bacterial consortium under study was formed by cultivating a mixture of various microorganisms deliberately introduced into sewage from the synthetic rubber industry. The major degradative bacteria found in the consortium are *Pseudomonas fluorescens*, *Pseudomonas putida*, *Pseudomonas* sp., and *Thiobacillus* sp. [7]. The taxonomic status of bacteria was determined based on their cultural, morphological, physiological, and biochemical characteristics by using the identification criteria described in the manuals [8, 9]. Depending on the cultivation conditions and the chemical composition of sewage, the consortium may also contain minor bacterial species up to 10 in number [7].

The aim of the present work is to characterize the consortium and its spatial organization during growth on agar medium without isolating the component bacteria in pure cultures.

MATERIALS AND METHODS

Consortium and cultivation conditions. The bacterial consortium used in this study was obtained at the Institute of Biophysics of the Siberian Division of the Russian Academy of Sciences for the purpose of the degradation of alkylsulfonates in sewage from the synthetic rubber industry [7]. Cultivation was performed under nonsterile conditions in a chemostat mode at a low oxygen content (Eh < 50 mV; the total content of $S_2O_3^{2-}$ and SO_3^{2-} is 210–260 mg/l) in sewage supplemented with (g/l) NH_4NO_3 – 1.0; KH_2PO_4 – 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; and NaCl, 30 (pH 6.4–6.8).

The consortium was grown on fish meal–peptone agar (FPA) or sewage solidified by the addition of 20 g/l agar. The agar media were stab-inoculated with the aid of a needle replicator. Material for inoculation was of two types: (1) cells grown in a chemostat culture (the so-called chemostat-culture inoculum) and (2) cells grown chemostatically and then subcultured aseptically in a batch mode (the so-called batch-culture inoculum). Subculturing was carried out using the same medium as was used for continuous cultivation.

Electron-microscopic studies were performed using 6- to 7-day-old colonies prepared as described earlier [1]. Colony sections were cut on a Reichert UM-03 ultratome. The semithin sections were stained with 1% toluidine blue. The ultrathin sections were additionally contrasted with 0.5% lead isocitrate. The native colonies were examined under a Jenavert light microscope (Karl Zeiss, Jena), the thin sections were examined under an MBI-15 light microscope (LOMO, Russia), and the ultrathin sections were examined in a JEM-100C electron microscope equipped with an EM-ASID 4 scanning device. The electron microscope was operated at an accelerating voltage of 80 kV. When being

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Table 1. The types of cells present in the colonies grown on different media from different inocula

Cell type	Continuous-culture inoculum		Batch-culture inoculum
	FPA	Sewage agar	FPA
I	+	+	+
II	+	+	+
III	+	+	+
IV	+	+	–
V	+	–	–
VI	+	–	–
VII	–	–	+
VIII	–	–	+

Note: "+" and "–" indicate, respectively, the presence and the absence of particular types of cells in colonies.

operated in a scanning transmission mode, the electron microscope provided digitally processed images.

RESULTS

The morphology of colonies as observed by light microscopy. We examined three types of colonies: two types that were grown on FPA and agar-solidified sewage from the chemostat-culture inoculum and one type that was grown on FPA from the batch-culture inoculum (Table 1).

In spite of the fact that the material for inoculation was grown under different conditions, both types of colonies grown on FPA were similar in size (about 2 mm) and morphology. The colonies had an irregular shape, uneven edges, and a wrinkled surface with craters in the centers. The main body of the colonies was surrounded by a dull halo. The colonies of the consortium that was grown on solidified sewage were pinpoint, irregular, mucous, and umbonate. The halo was fan-shaped.

The microscopic examination of the semithin sections showed that the consortium cells grew both on the surface of agar (producing a normal colony in the site of the stab) and along the length of the stab (a hole in the agar medium produced by the replicator needle during inoculation) (Fig. 1). The colony sections stained nonuniformly. In particular, the central portion of the cell mass grown in the stab and some areas of the surface colony stained faintly.

Assuming that the section through the center of a colony is the central section of a solid of revolution, we calculated the total amount of cells grown on the agar surface to be about 8–10 times as high as the total amount of cells grown in the stab (Fig. 1).

The electron-microscopic examination of colonies allowed the causes of the nonuniform staining of the semithin sections of the colonies to be elucidated: (1) the location of bacterial cells of a particular type within particular regions of colonies; (2) the different packing densities of cells; and (3) the presence of lysed cells both in the surface colony and in the stab.

Based on the analysis of specific ultrastructural features, we classified the cells present in colonies into eight types. The particular set of the cell types present in a given colony depended on the inoculum and the composition of the agar medium (Figs. 2–4 and Tables 1 and 2). The analysis of the ultrastructure of cell walls showed that all the cells in the colonies were gram-negative.

The surface and below-surface parts of the colonies were characterized by their own sets of cell types. Three major types of cells (I, II, and III) were present in all three types of the colonies studied, type I cells being dominant (Fig. 2). In addition, the colonies grown on FPA from the chemostat-culture inoculum contained cells of types IV, V, and VI (Fig. 3 and Table 1). The colonies grown from the same inoculum on the solidified sewage additionally contained type IV cells.

The colonies grown from the batch-culture inoculum contained no cells of types IV, V, and VI, but instead they contained type VII and VIII cells (Fig. 4 and Table 1).

The structure of the mass of cells grown in the stab. The axial region of the biomass grown in the stab

Table 2. Some morphological characteristics of cells present in the consortium colonies

Cell type	Cell length, $\cong L$, μm	Cell diameter, $\cong D$, μm	Inclusions and their diameter, $\cong d$, μm	Microcapsule and its thickness, $\cong l$, μm	Other characteristics
I	3.0	0.5	Electron-dense, d = 0.082–0.08	–	–
II	2.2	0.9–1.2	Heterogeneous, d = 0.3	Fibrillar, l = 0.19	–
III	1.7	1.3	Electron-dense, d = 0.03–0.1 Electron-transparent, d = 0.1–0.2	–	–
IV	3.0	0.5	Electron-dense, d = 0.06	–	Prosthecae, $l \cong$ 0.14 μm
V	1.3	0.4	–	–	–
VI	2.5	0.3	Electron-dense, d = 0.02–0.03	–	Electron-dense nucleoid
VII	3.0	0.5	Electron-dense, d = 0.02–0.03	"Spiny", 0.06	–
VIII	1.4	0.6	–	Slimy, 0.5	–

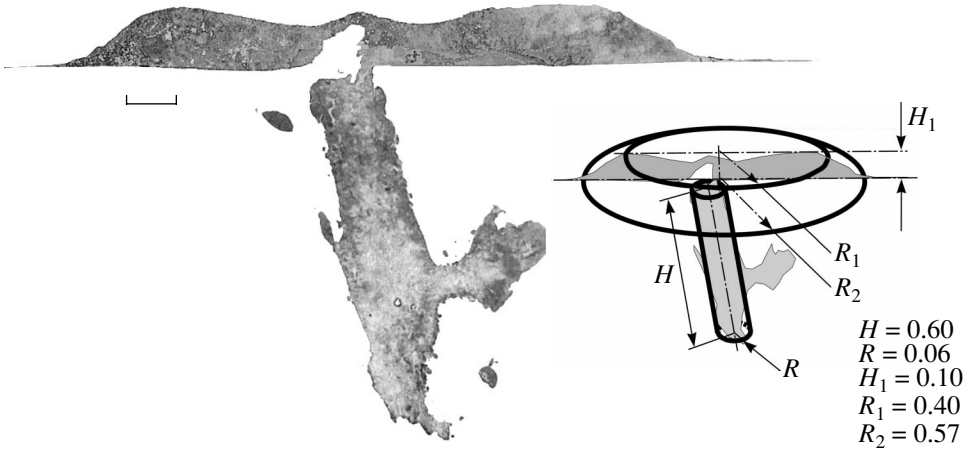

Fig. 1. Micrograph of the semithin section of a colony of the alkylsulfonate-utilizing bacterial consortium and the schematic representation of this colony. The cylinder with height H and radius R represents the part of the colony grown in the stab made in the agar by the replicator needle. The truncated cone with height H_1, the base radius R_2, and the plane section radius R_1 represents the part of the colony grown on the agar surface. Bar represents 0.1 mm.

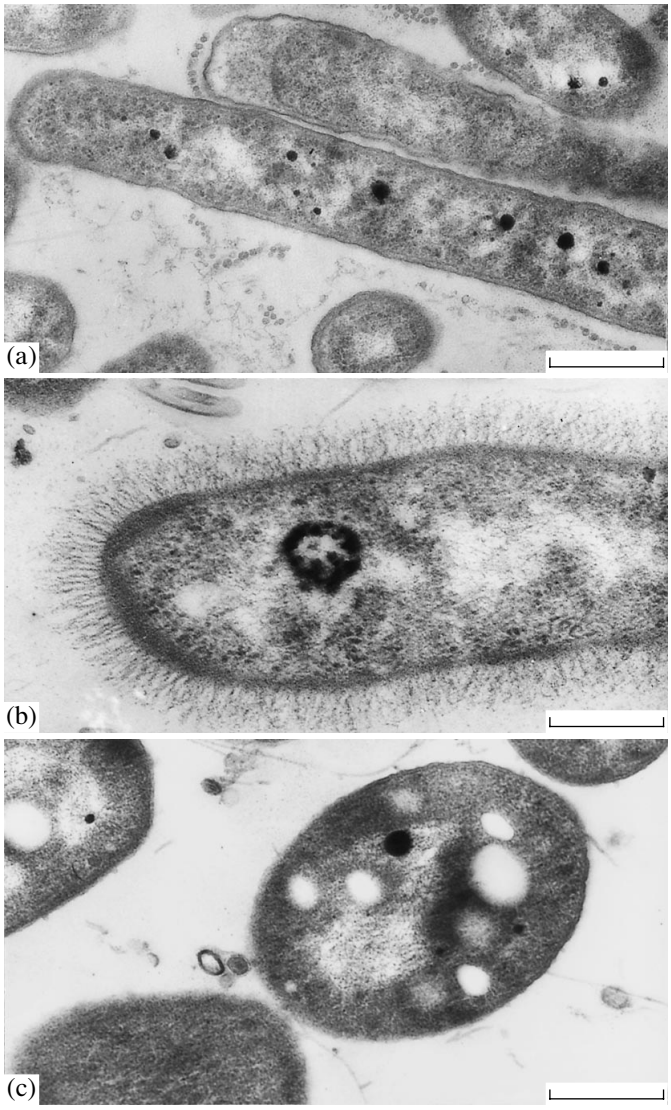

Fig. 2. The types of cells revealed in the colonies grown on different media from different inocula: (a) type I cell; (b) type II cell; and (c) type III cell. The bars represent 0.5 μm.

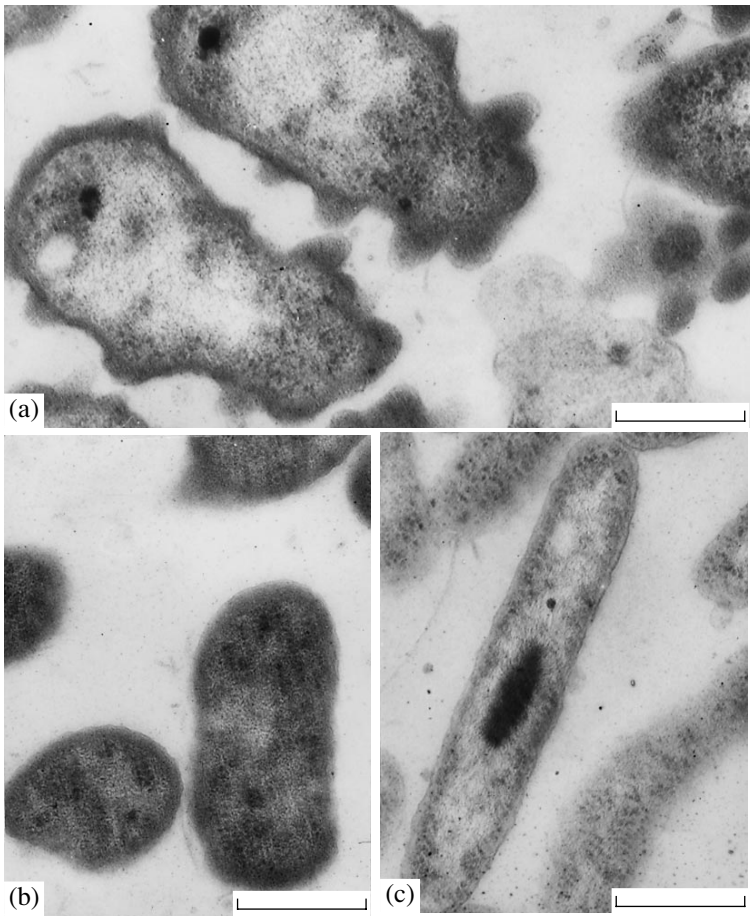

Fig. 3. Minor types of cells revealed in the colonies grown on agar media from the continuous-culture inoculum: (a) type IV cell; (b) type V cell; and (c) type VI cell. The bars represent 0.5 μm.

stained faintly, since this region represented closely packed, more or less lysed type I cells with distinct groups of type II cells enclosed in microfibrillar microcapsules and placed in an intercellular matrix. The cells in the stab that contacted the nutrient agar were viable and were arranged in the form of a sheath composed of closely packed type I cells with inclusions of type III cells (Fig. 5). Type IV, V, VI, and VII cells were observed on the sections of the respective parts of the colonies as solitary cells or as small clusters of cells. In the colonies grown on FPA from the batch-culture inoculum, type VIII cells formed relatively large aggregates (especially in the lower part of the stab) enclosed in thick, slimy microcapsules.

The structure of colonies grown on the agar surface. The cell community grown on the agar surface primarily contained type I cells (Fig. 6). The upper part of the surface colonies, which came into contact with the air, contained the alternating regions of closely and loosely packed type I cells. The packing density of the cells increased further into the colony. The bottom part of the colony represented closely packed type I cells. In the central area of the colony, these cells were arranged perpendicular to the agar surface. When observed under the light microscope, the colonies were surrounded by dull halos. The electron-microscopic examination of the ultrathin sections of the halo region showed that it is formed by a monolayer of type I cells lying on the agar surface.

Bacterial cells with distinct microcapsules (type II, VII, and VIII cells) formed local regions several tens of microns in size throughout the colony (Fig. 6). Of interest is the fact that type VII and VIII cells were usually observed together.

Bacterial cells with electron-transparent cytoplasmic inclusions (type III cells) formed clusters of several cells located close to the colony center. Type IV and VI cells also tended to be located near colony center. The groups of small and compact type V cells were observed close to the agar surface (at relatively small magnification values, these groups looked as electron-opaque regions).

The colonies grown from the continuous-culture inoculum contained a great number of lysed cells concentrated in regions 30 μm or more in size. Such

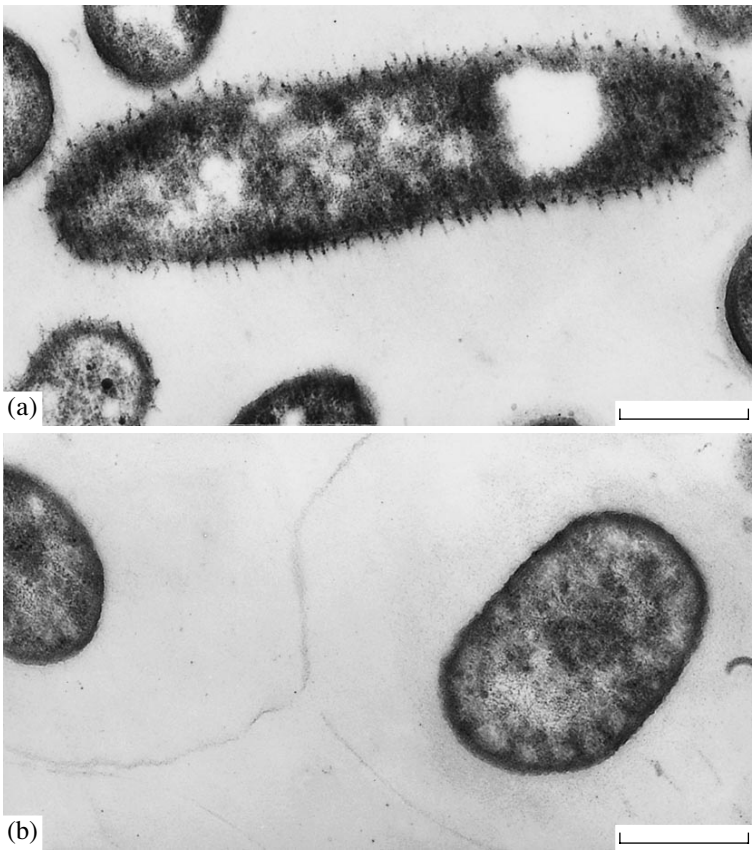

Fig. 4. Minor types of cells revealed in the colonies grown on agar media from the batch-culture inoculum: (a) type VII cell and (b) type VIII cell. The bars represent 0.5 μm.

regions were not observed in the colonies grown from the batch-culture inoculum.

DISCUSSION

The present work is a continuation of the electron-microscopic studies of the structural organization of bacterial colonies [1–3]. The alkylsulfonate-utilizing bacterial consortium studied in this work primarily contains *Pseudomonas fluorescens*, *P. putida*, *Pseudomonas* sp., and *Thiobacillus* sp., as well as up to 6–10 satellite bacterial species [7].

Such electron-microscopic studies allow the colonies of the consortium to be characterized without isolating the component bacterial species in pure cultures. Based on the results of the electron-microscopic analysis of the colonies, we differentiated eight types of cells constituting the consortium (Table 1 and Figs. 2–4).

Three major types of cells, I, II, and III, were present in all three types of the colonies studied, with the type I cells being dominant. In addition, the colonies grown on FPA from the chemostat-culture inoculum contained some amounts of IV, V, and VI type cells. The colonies grown from the same inoculum on solidified sewage contained in addition type IV cells. It should be noted that type I and IV cells are similar except that the latter have oval outgrowths of the cell wall and that their membranes resemble prosthecae typical of many gram-negative cells. It is known that prosthecate bacteria retain their specific morphology only during growth at low concentrations of organic substrates. At high substrate concentrations, prosthecae decrease in size or even disappear [4, 10, 11].

The colonies of the alkylsulfonate-utilizing consortium contain small amounts of prosthecate cells located close to the colony center. The prosthecae of these cells are small in size (below 0.14 μm). In our opinion, the prosthecate cells represent either type I cells trying to adapt to the unfavorable conditions of growth in the colony interior or the cells of satellite bacterial species, which appear after the long-term incubation of the consortium in the presence of certain organic substances. The latter suggestion is more likely, since the cells of this type are not observed if the consortium is grown aseptically on the selective medium (as sewage). The same is probably true for type V and VI cells.

The colonies grown on FPA additionally contained type VII and VIII cells. Type VII cells are similar to type I cells but have spiny microcapsules. Generally, almost all bacteria living in nature develop glycocalyx,

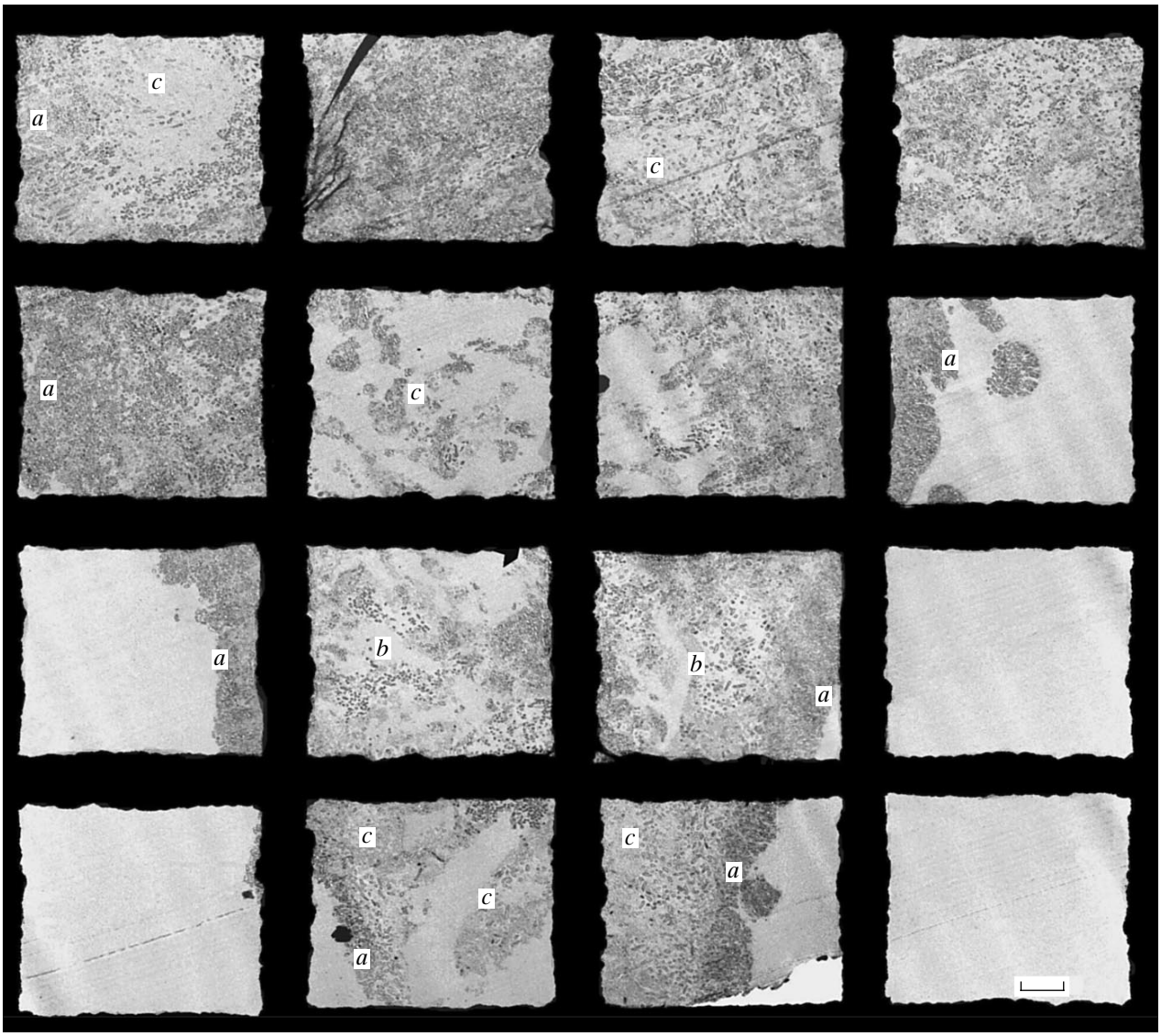

Fig. 5. Micrographs of the mass of cells grown in the stab made by the replicator needle in the agar medium: (*a*) the areas occupied by type I cells; (*b*) the areas occupied by other types of cells; and (*c*) the areas occupied by lysed cells. The bar represents 10 μm.

a complex polysaccharide-containing structure lying outside the outer membrane of gram-negative cells and the peptidoglycan of gram-positive cells. In the absence of bacterial antagonists (this is typical of, for instance, pure cultures), the glycocalyx is often lost [12]. In the colonies studied, type VII cells were mainly observed in border areas between the colony regions occupied by type I cells and encapsulated type II and VIII cells. It is possible that the last two types of cells are antagonistic to type I cells, which develop the exopolysaccharide glycocalyx to defend against type II and VIII cells.

In addition to the protective function, the fibrillar microcapsules of type VII cells may perform other functions. For instance, they may implement trophic relations between the cells of the consortium.

It can be suggested that alkylsulfonates are utilized by type I, II, III, and VIII cells, whereas type V and VI cells represent satellite bacterial species, and type IV and VII cells are morphological variants of type I cells.

The inoculation of agar medium by stabbing gave rise to normal colonies on the agar surface and led to the growth of bacterial cells in the stab produced in the agar by the replicator needle. Both parts of the colonies (Fig. 1) contained identical sets of cell morphotypes, among which type I cells were dominant. These cells form a matrix in which other types of cells grow. Type I cells probably play an important role in the colonization of agar medium, as is evident from the formation of halos around colonies, which are made up of these cells. The lysis of the cells in the axial region of the stab was most likely caused by the prevention of the penetration of oxygen into the stab by the growing heap of cells at the stab opening.

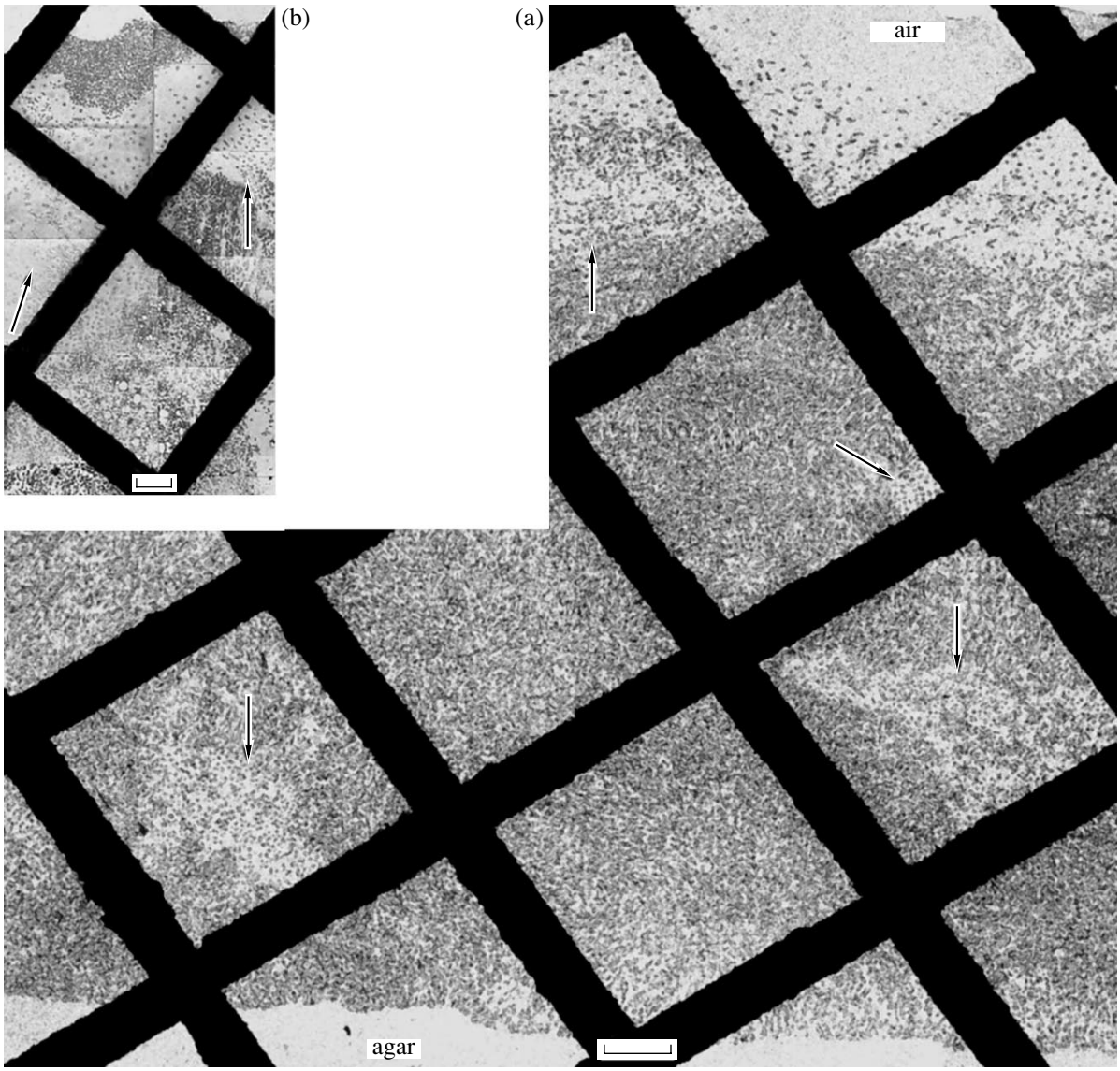

Fig. 6. Micrographs of a colony grown on the agar surface: (a) the region of the colony with a loose surface contacting the air and its dense base contacting the agar; (b) the region of the colony with a dense surface. The arrows point to the regions occupied by cells other than type I cells. The bar represents 10 μm.

The low quantity of the biomass grown in the stab can be explained by the fact that bacterial cells grow only in the free space of the stab and do not penetrate into its agar walls.

If our suppositions are valid, the morphological diversity of the consortium cells and their spatial arrangement in the colony may provide some information on relations between the members of the consortium.

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REFERENCES

1. Puzyr', A.P., Mogil'naya, O.A., and Tirranen, L.S., Architectonics of the *Flavobacterium* sp. 56 and *Flavobacterium* sp. 22 Colonies According to Transmission Electron Microscopy Data, *Mikrobiologiya*, 1998, vol. 67, no. 5, pp. 672–679.
2. Puzyr', A.P., Mogil'naya, O.A., and Pavlyuchenko, E.L., Architectonics of the Colonies of a Mixed *Flavobacterium* sp. 56 and *Bacillus cereus* 60 Culture, *Izv. Akad. Nauk, Ser. Biol.*, 1999, no. 3, pp. 378–384.
3. Puzyr', A.P., Mogil'naya, O.A., Gurevich, Yu.L., and Babkina, E.A., The Structure of the Colonies of a Nitri-

fying Bacterial Consortium, *Mikrobiologiya*, 2001, vol. 70, no. 1, pp. 98–105.

4. Kinner, J.A., Maratea, D., and Bishop, P.L., An Electron Microscopic Evaluation of Bacteria Inhabiting Rotating Biological Contactor Biofilms during Various Loading Conditions, *Sci. Technol. Lett.*, 1985, vol. 6, pp. 455–466.
5. Wolfaardt, G.M., Lawrence, J.R., Robarts, R.D., Caldwell, S.J., and Caldwell, D.E., Multicellular Organization in a Degradative Biofilm Community, *Appl. Environ. Microbiol.*, 1994, vol. 60, pp. 434–446.
6. Kinniment, S.L., Wimpenny, J.W., Adams, D., and Marsh, P.D., Development of Steady-State Oral Microbial Biofilm Community Using the Constant-Depth Film Fermentor, *Microbiology*, 1996, vol. 142, pp. 631–638.
7. Manukovskii, N.S., Gurevich, Yu.L., Kovalev, A.S., and Petrov, A.I, Consortium of the Bacteria *Pseudomonas* sp., *Pseudomonas fluorescens*, *Pseudomonas putida*, and *Thiobacillus* sp. Used To Remove Alkylsulfonates from Waste Water, RF Patent no. 2103356, 1998.
8. *Bergey's Manual of Determinative Bacteriology*, Buchanan and Gibbons, Eds., Baltimore: Williams & Wilkins Co., 1974.
9. *The Prokaryotes: A Handbook on Habitats, Isolation, and Identification of Bacteria*, Starr, M.P. *et al.*, Eds., Berlin: Springer, 1981, vol. 1.
10. Stanier, R.Y., Adelberg, E.A., and Ingraham, J.L., *The Microbial World*. Translated under the title *Mir mikrobov*, Moscow: Mir, 1979, vol. 1.
11. Gromov, B.V. and Pavlenko, G.V., *Ekologiya bakterii* (Bacterial Ecology), Leningrad: Len. Gos. Univ., 1989.
12. Costerton, J.W., Structure and Plasticity at Various Organization Levels in the Bacterial Cell, *Can. J. Microbiol.*, 1988, vol. 34, pp. 513–521.