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EXPERIMENTAL ARTICLES

Electron Microscopic Study of *Burkholderia cepacia* Biofilms

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Abstract—Using the methods of transmission electron microscopy, the structure of the biofilms formed by the bacterium *Burkholderia cepacia* (clinical isolate and mutants with an increased and decreased ability to produce biofilm) were investigated. The biofilms were obtained on a liquid nutrient medium or on an abiotic surface (polystyrene). It has been demonstrated that the cultures of the studied strains differ in some morphological and functional characteristics. In biofilms, changes in the size and submicroscopic organization of all the components of bacterial cells occur. Staining biofilms with ruthenium red revealed the presence of exopolysaccharides in the intercellular space. The differences in the ultrastructure of bacterial films formed on nutrient medium and abiotic surfaces were demonstrated.

Keywords: Burkholderia cepacia, biofilm, electron microscopy.

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Investigations carried out over the last decade have demonstrated that most bacteria are present in natural ecosystems in the form of specifically organized biofilms attached to substrates rather than as free-living planktonic cells [1–3]. Biofilm formation is a complexly regulated biological process. The process of biofilm formation is controlled by the regulatory systems of gene activity, including the quorum sensing system. The development of biofilm communities is one of the main strategies of bacterial survival both in the environment and inside infected host organisms. Biofilms are highly organized communities of one or several bacterial species; they contain both actively functioning and resting cells.

The ability of bacteria to form biofilms gives rise to great problems in various aspects of human life, including, most importantly, medicine. For instance, biofilms formed on the surfaces of medical equipment can result in infecting the patients and medical personnel. Biofilms developed on various endoprostheses, catheters, etc., can induce chronic infectious processes. All attempts to control biofilm formation are thwarted by their resistance to antimicrobial agents (including antibiotics), to protective immune mechanisms, and to unfavorable environmental factors (extreme temperatures, pH values, and osmotic pressure), which is higher than that of free-living bacteria [1, 2].

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At present, the range of pathogens that cause various human infectious diseases is extended significantly by inclusion of the microorganisms that have been previously considered nonpathogenic. The bacterium *Burkholderia cepacia*, which inhabits soil and water ecosystems and causes plant diseases [4], is an example of such a microorganism. In human pathology, this organism is associated with bronchopulmonary infections, septic complications in patients with various immunodeficiency disorders, and hospital infections. The widespread distribution of *B. cepacia* suggests that these bacteria have certain universal mechanisms that help them to survive in various environmental niches, including host organisms. It is quite possible that the ability of *B. cepacia* to form biofilms is one of these mechanisms.

The experimental method for obtaining biofilms is based on the ability of bacteria to colonize various surfaces. Bacterial cells are enclosed in an intercellular matrix which adheres to the surface, forming the biofilm.

To quantitatively assess the ability of bacteria to form a biofilm, the culture is grown on microtitration plates and stained. The strain capacity for biofilm formation is judged by the photometrically measured color intensity of the stained cells adhered to the plate surface [5]. We have previously used this method to assess the biofilm-forming capacities of various strains and mutants of *Salmonella typhimurium* [6]. It was demon-

strated that the biofilm obtained using this method could be examined by direct microscopy. Microbial biofilms are usually visualized by scanning electron microscopy, although this technique allows us to study only the surface structures. Recently, some attempts have been made to use the method of laser confocal scanning microscopy, which makes it possible to study almost intact biofilms [7]. A relatively small number of studies have applied transmission electron microscopy to investigate the ultrastructure of biofilms formed by various bacteria, although this approach is quite appropriate for comparative analysis of biofilms [3].

The goal of this work was to study the ultrastructure of biofilms formed by the clinical strain of *B. cepacia*, as well as by mutants of this organism with different biofilm-forming capacities, by transmission electron microscopy.

MATERIALS AND METHODS

Bacterial strains and cultivation conditions. In our study, we investigated strain *Burkholderia cepacia* 370, isolated from a patient and deposited in the strain collection of the Laboratory of Genetic Engineering of Pathogenic Microorganisms, Gamaleya Research Institute of Epidemiology and Microbiology, Russian Academy of Medical Sciences [8], and two mutants, Bcb+ and Bcb–, obtained by the insertion mutagenesis approach using mini-Tn5 transposon derivatives and characterized by high and low biofilm-forming capacities, respectively. The mutants were selected by undirected transposon mutagenesis after a total examination of a large number of insertion clones to reveal their capacity for biofilm formation determined by the quantitative method described in [6].

Bacterial cultures were grown in liquid Luria–Bertani (LB) medium at 37°C in a shaker (for 24 h and 48 h) and under static conditions (for 48 h and 120 h). In addition, bacteria were grown on agarized LB medium at 37°C for 24 h and 48 h.

Microscopic investigations. The ultrastructure of bacterial cells grown on liquid and solid media was studied by electron microscopy of negatively stained preparations and ultrathin sections. The biofilm formed on the plastic surface was investigated by electron microscopy of ultrathin sections.

The specimen samples were obtained by centrifugation (3.3 *g* for 5 min) of broth suspensions and cells washed off from the agar surface. The pellets were then fixed with 2% glutaraldehyde in cacodylate buffer (pH 7.4) for negative staining; to obtain ultrathin sections, the specimens were fixed according to Ito and Karnovsky [9].

Whole-cell preparations of bacteria grown on the agarized medium and in broth were negatively stained and examined by electron microscopy. The obtained bacterial suspensions were deposited on copper grids covered with a carbon-stabilized formvar support. The excessive liquid was removed with filter paper. Ammonium molybdate (1% wt/vol) aqueous solution was used as a contrasting agent.

To detect acid mucopolysaccharides in the intercellular matrix, the biofilm produced by the culture grown on broth was stained with ruthenium red as described in [10].

The biofilms formed in polystyrene plate cells were carefully detached from the walls and bottoms of the wells, fixed according to Ito and Karnovsky, and centrifuged at 2.3 *g* for 5 min.

All the specimens for the ultrathin sectioning were prepared as described in [11]. Ultrathin sections were obtained with an LRB-3 ultratome, stained according to Reynolds [12], and analyzed in a JEM-100B transmission electron microscope at an accelerating voltage of 80 kV.

RESULTS AND DISCUSSION

Ultrastructure of the *B. cepacia* cells grown in liquid medium. No biofilms were produced by cultures grown in the liquid medium in a shaker. Biofilms were formed at the air–liquid interface after 48 to 120 h of incubation under static conditions.

Negative staining revealed that the planktonic cells of all the studied 24-h cultures had flagella and thin long pili (Fig. 1a, b). The giant Cbl pili that bind to the mucin of the respiratory tract and are often found in the epidemically significant *B. cepacia* strains [13] have not been detected in the studied strains under the above cultivation conditions.

In the suspension, planktonic bacteria grew as individual cells; their morphology was typical of gram-negative bacteria (Fig. 1c). The bacteria have a layered cell wall; their periplasmic space is not expanded; the cytoplasmic membrane is clearly seen around the whole cell perimeter. In the cytoplasm of medium electron density, ribosomes were detected. The nucleoid zone with DNA chains and condensed material is located in the central part of the cell. Electron-transparent spherical poly-β-hydroxybutyrate inclusions were detected in the cells. The presence of similar inclusions in *B. cepacia* cells has been previously confirmed by the results of chemical analysis [14]. The cells divide by constriction, which is typical of gram-negative bacteria. Lysed cells were not detected in the preparation.

The cells grown statically, i.e., under conditions similar to natural ones, have been studied as well. Comparative study of the structure of bacterial cells within the biofilm formed at the air–liquid interface and of the cells under this biofilm was carried out. Lysed individual cells were predominant in the planktonic culture (Fig. 1d).

Although the biofilm grown under static conditions was observed at the early stages of cultivation, it was loose, porous, and easily precipitated. A thicker biofilm formed after 120 h of incubation. The cells in the bio-

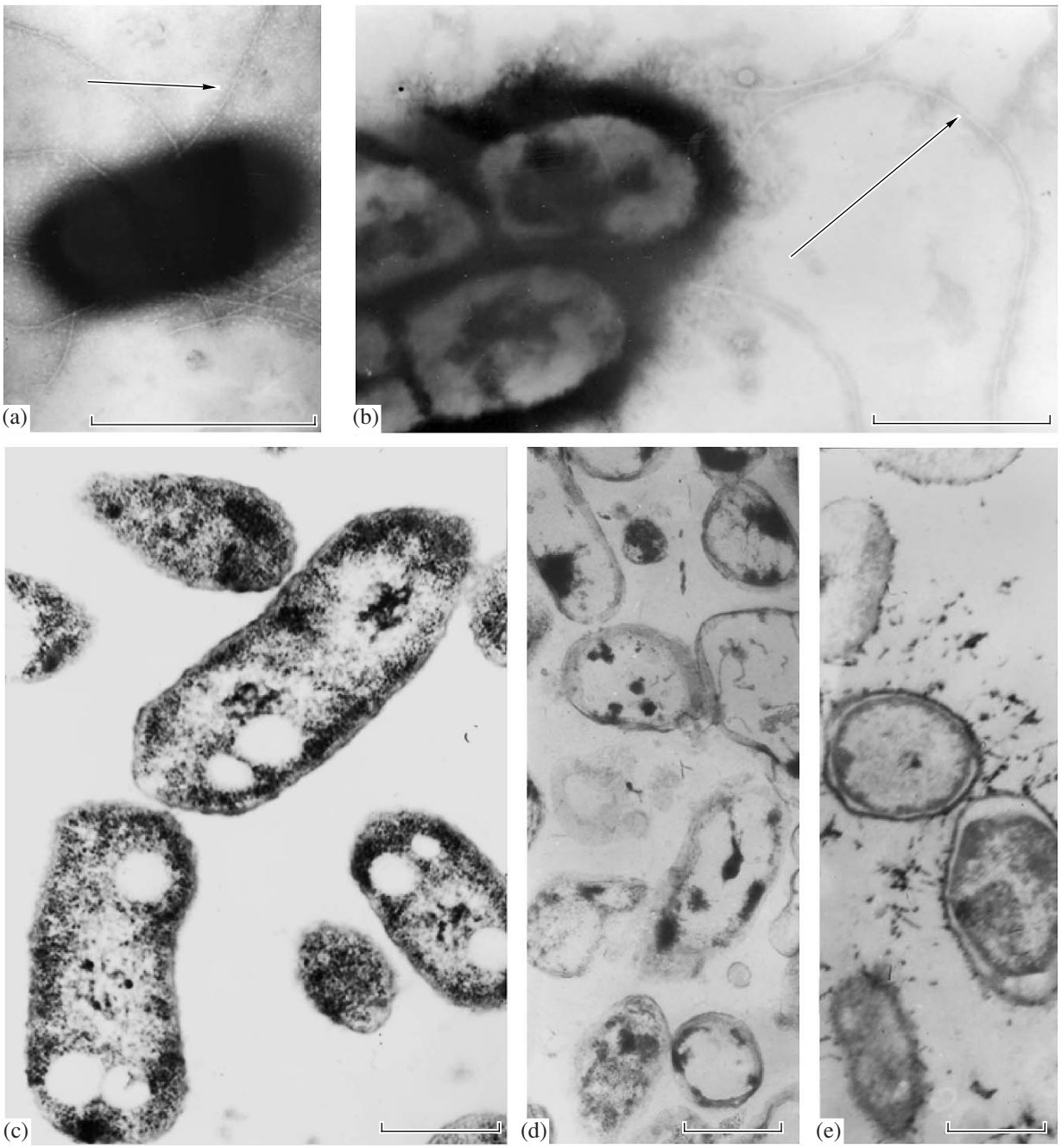

Fig. 1. Morphology of the cells grown in nutrient broth: (a) negative staining; the arrow points to pili; (b) negative staining; the arrow points to flagella; (c) ultrathin section of planktonic cells (24 h); (d) ultrathin section of planktonic cells (120 h); (e) ultrathin section of the biofilm grown on nutrient broth (120 h); ruthenium red staining. In all the figures, the scale bars are 1 μm.

film did not form an uninterrupted layer and were partially lysed. The cells were not as densely packed as *Salmonella* cells under similar cultivation conditions [3]. The intercellular matrix in the biofilm formed by *Burkholderia* cells could not be revealed without special staining. On the ultrathin sections stained with ruthenium red (specific for acidic mucopolysaccharides), electron-dense fibrous structures that extend from the cell wall to adjacent bacterial cells were detected in the intercellular matrix (Fig. 1e). It seems likely that, apart from maintaining the biofilm integrity, these structures facilitate intercellular contacts and communication.

Thus, the biofilms formed by *B. cepacia* on liquid media are different from those formed by *Salmonella* species. Most probably, this is the result of the chemical properties of the intercellular matrix specific for these bacteria. It was demonstrated that cellulose is the main

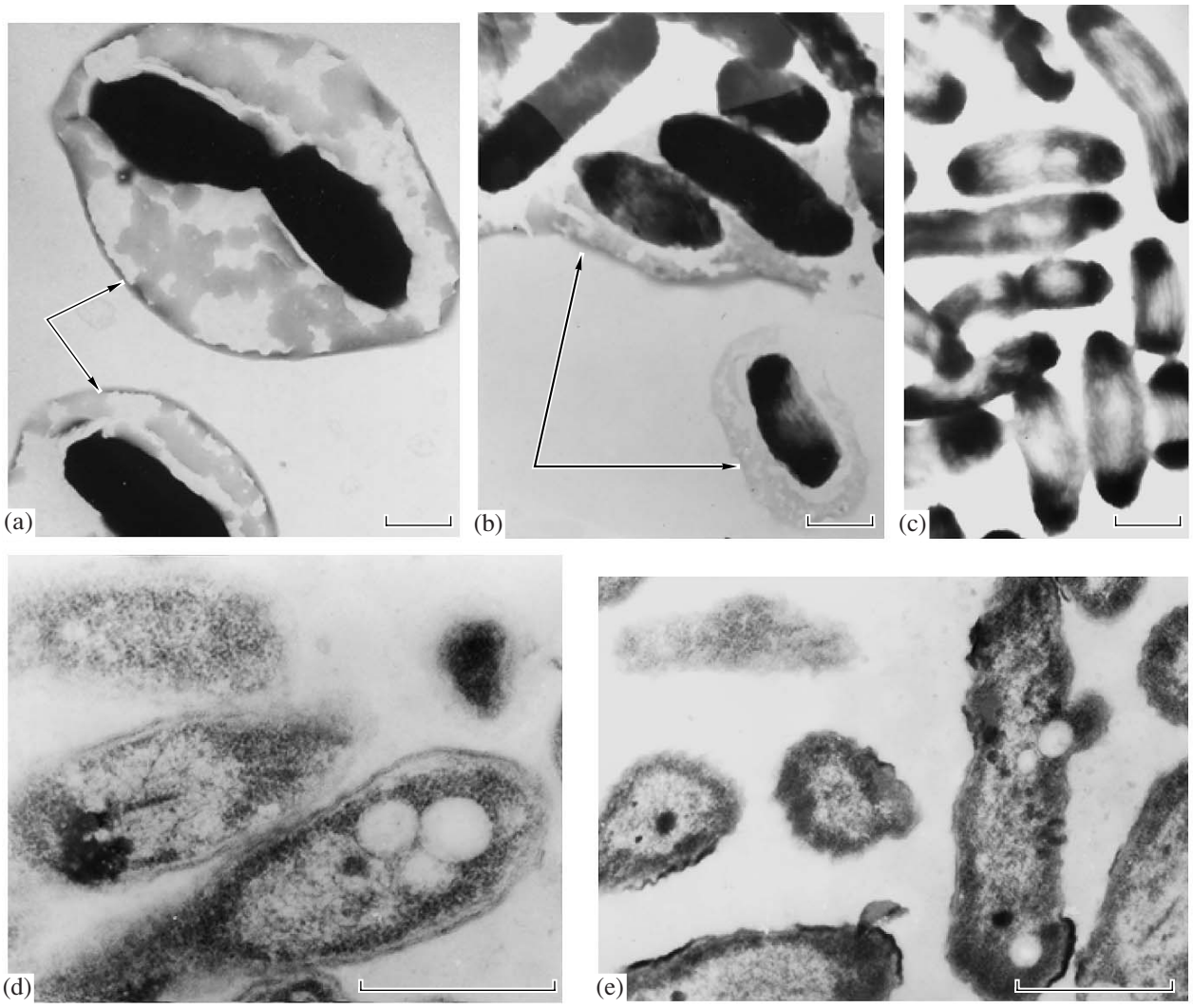

Fig. 2. Morphology of the cells grown on agarized medium: (a) negative staining of the clinical isolate; the arrow points to a capsule; (b) negative staining of the mutant strain Bcb+ cells; the arrow points to a capsule; (c) negative staining of the mutant strain Bcb– cells; (d) ultrathin section of the clinical isolate; (e) ultrathin section of the mutant strain Bcb+ cells.

constituent of the biofilm matrix produced by *S. typhimurium*, whereas, in the case of *B. cepacia*, the main component of the matrix is the exopolysaccharide cepacian named in accordance with the specific name of these bacteria [15].

Ultrastructure of the *B. cepacia* cells grown on solid media. Using the technique of negative staining, we have revealed that both the initial strain 370 and the mutant Bcb+ with high ability to form biofilms develop massive electron-transparent capsules of a distinct shape when grown on agarized media (Fig. 2a, b). The presence of capsules in the cultures grown on agarized media indicates that the released biopolymers accumulate around the cell. The biopolymer layer in the form of a capsule was clearly visualized by negative staining. The capsules contained densely packed cells and did not disintegrate during centrifugation, which indicates that they are firmly attached to the cell surfaces.

The cells of the mutant Bcb– strain, which did not form biofilms, also did not produce capsules (Fig. 2c).

Analysis of the ultrathin sections of *B. cepacia* has shown that the cell morphology of the initial strain 370 grown on liquid or solid nutrient media remained practically unchanged (Fig. 2d).

The mutant Bcb+ cells were smaller than those of the initial strain, with supple thin cell walls and unusual protrusions filled with cytoplasm. Electron transparent poly-β-hydroxybutyrate inclusions were detected immediately under the cell wall (Fig. 2e).

Ultrastructure of the biofilm formed by *B. cepacia* on the polystyrene surface. Electron microscopic observations revealed that bacterial communities which produce biofilms on polystyrene surfaces possess a pronounced morphological diversity. The ultrathin sections of the biofilm formed by the initial strain 370 and

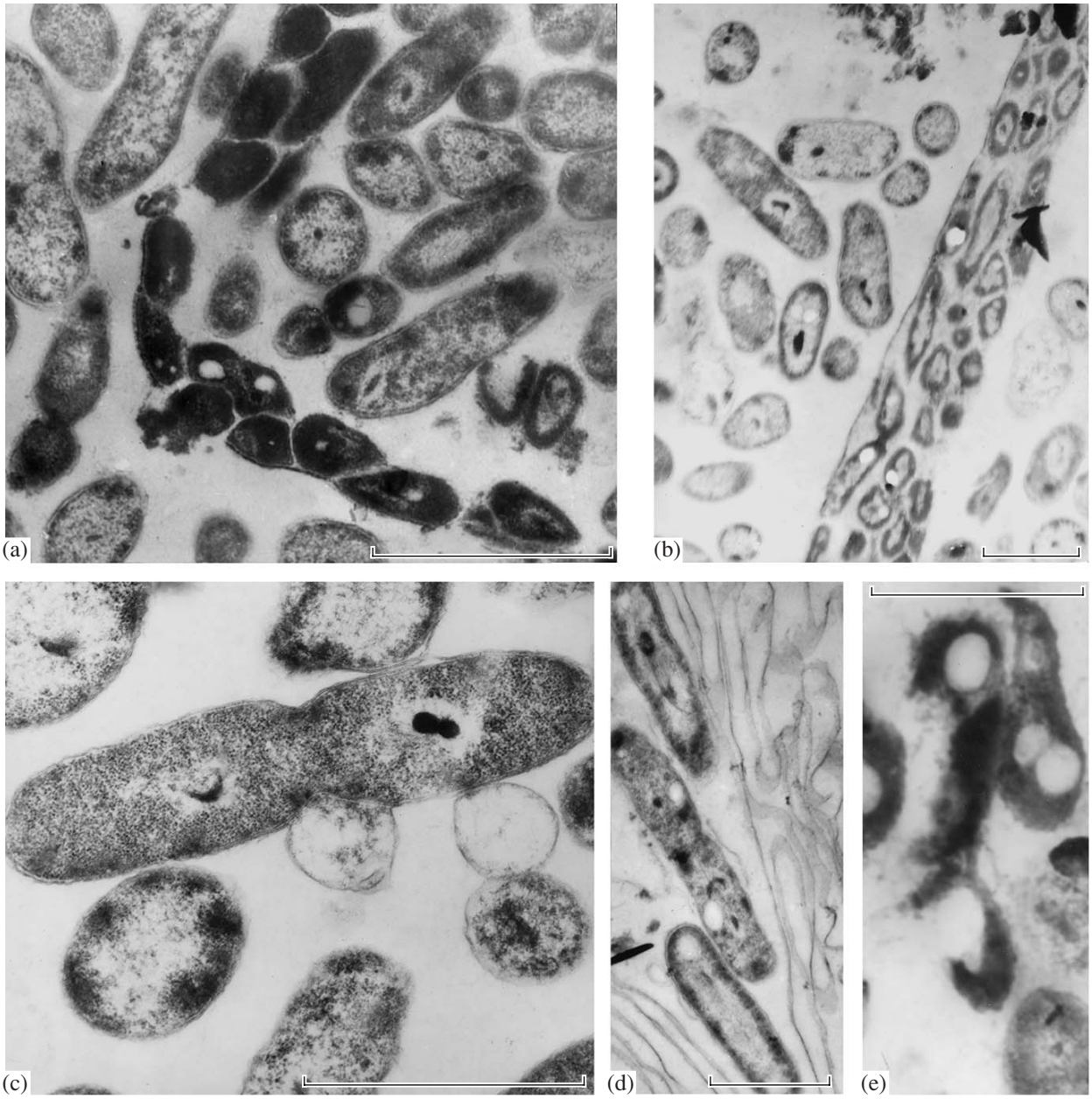

Fig. 3. Ultrathin sections of the biofilm formed on the polystyrene surface: (a) cells of the first and second types; (b) cells enclosed in a sheath; (c) membrane vesicles on the surface of the cell wall; (d) membrane structures, components of the biofilm; (e) release of poly-β-hydroxyoxybutyrate granules.

the mutant Bcb+ strain demonstrate two different types of cells (Fig. 3a, b). The morphology of the first type of cells irregularly distributed in the biofilm was similar to that of planktonic cells from the culture grown in nutrient broth. It is probable that these cells were the remnants of the bacterial suspension retained in the well after the biofilm was detached from its walls and bottom. However, it is possible that these cells represent some stage of the biofilm formation. The second type was represented by bacterial cells enclosed in sheaths; the form, size, and electron density of these cells differed significantly from those of unsheathed cells. Cells of both types contained electron-transparent inclusions.

Cells of the first type contained vesicles on the cell wall surface (Fig. 3c). The cell wall of gram-negative bacteria, including *Burkholderia* species, is characterized by outer membrane vesicles involved in the transport of exoenzymes and exotoxins [16, 17].

Unusual extensive membrane structures were also observed in the biofilm preparations (Fig. 3d). They permeated through the biofilm, forming compartments and separating cell groups from each other. Similar

membrane structures have been found in biofilms produced by *Neisseria gonorrhoae* [18]. These structures, in addition to their barrier function, possibly make the biofilm more solid and thus ensure its structural integrity.

Cells of the second type were of irregular shape and of varying size; they were enclosed in sheaths and thus more separated from other cells. The tightly packed groups of these cells formed a unique mosaic pattern. Their cytoplasm was of high electron density; the ribosomes and DNA chains were not observed, although the nucleoid zone was well-defined. Poly-β-hydroxybutyrate inclusions were clearly seen in the cells. The morphology of these cells is comparable with the submicroscopic structure of the uncultivable forms of *S. typhimurium*, which we have previously observed in a laboratory model simulating the nonculturable state of *Salmonella* species [11]. It has been demonstrated that each biofilm community includes uncultivable cells resistant to various environmental factors and therefore providing the biofilm community with enhanced resistance to these factors [19]. The strains grown on polystyrene surfaces included cells which were able to release poly-β-hydroxybutyrate (Fig. 3e). The presence of these storage inclusions, which occupy a major part of the cell volume, has been previously reported for bacteria in biofilms [20]. These inclusions are known to form in cells grown in the presence of carbon-containing energy sources and are consumed during starvation. Poly-β-hydroxybutyrate inclusions released during lysis and accumulated in the intercellular matrix are an additional source of nutrients for the biofilm bacteria.

Comparative analysis of the ultrastructure of the clinical *B. cepacia* isolate and its mutants with an altered biofilm-forming capacity has shown that the initial strain and its mutant with high biofilm-forming capacity differ from the mutant with the low biofilm-forming capacity in the presence of capsules in the former strains. The genetic nature of the obtained mutants is currently being studied.

Thus, it was established that *B. cepacia* exhibits a high ability to form biofilms under the conditions of in vitro cultivation at air–solid, air–liquid, or liquid–solid interfaces. The formation of biofilms is associated with some changes in the submicroscopic organization of bacterial cells and the formation of a sheath which encompasses the cells. The properties of both bacteria and substrates are particularly important for colonization of various substrates. On nutrient agar substrate, the vital functions of bacteria (even encapsulated ones) are maintained from the outside, whereas microbial communities grown on abiotic polystyrene substrates are probably able to survive due to the mobilization of internal resources. The structure of biofilms grown on a solid substrate is specific. The heterogeneity of the biofilm community largely depends on the variety of conditions within the biofilm that result from the presence of internal barriers which, most probably, possess selective permeability. These conditions contribute to the specialization and division of functions between the community members, which eventually results in the survival of the community.

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