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EXPERIMENTAL ARTICLES

Study of Ectoparasitism of Ultramicrobacteria of the Genus *Kaistia*, strains NF1 and NF3 by Electron and Fluorescence Microscopy

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Abstract—Transmission electron and fluorescence microscopy was used to study the character of the interaction of free-living ultramicrobacterial (UMB) strains NF1 and NF3, affiliated with the genus *Kaistia*, and seven species of gram-positive and gram-negative heterotrophic bacteria. Strains NF1 and NF3 were found to exhibit parasitic activity against gram-positive *Bacillus subtilis* and gram-negative *Acidovorax delafildii*. UMB cells are tightly attached to the envelopes of the victim cells and induce their lysis, thus demonstrating the features of typical ectoparasitism. The selectivity of parasitism of the studied UMB to the victim bacteria has been shown: only two soil microorganisms of the seven test objects, *B. subtilis* ATCC 6633 and an aerobic gram-negative bacterium *A. delafildii* 39, were found to be sensitive to UMB attack. Other bacteria (*Micrococcus luteus* VKM Ac-2230, *Staphylococcus aureus* 209-P, *Pseudomonas putida* BS394, *Escherichia coli* C 600, and *Pantoea agglomerans* ATCC 27155) were not attacked by UMB. It was established for the first time that free-living UMB may be facultative parasites not only of phototrophic bacteria, as we have previously demonstrated [1], but of heterotrophic bacteria as well. The UMB under study seem to play an important role in the regulation of the quantity of microorganisms and in the functioning of microbial communities in some natural ecotopes.

Key words: ultramicrobacteria, cell ultrastructure, bacterial parasitism, cell–cell contacts, electron and fluorescence microscopy, binary bacterial cultures.

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The facultative and obligate bacterial parasites known today exhibit a parasitic activity against various species of bacteria and algae. Obligate parasites include the members of the genera *Bdellovibrio*, *Vampirovibrio* [2], and *Micavibrio* [3]. Among them, only *Bdellovibrio* is an intracellular parasite of enterobacteria, although in the case of cyanobacteria *B. bacteriovorus* evidently behaves as an ectoparasite [4]. Nonobligate parasites include *Ensifer adhaerans* [5], *Cupriavidus necator* [6] and some species of myxobacteria and cytophagas [7] acting only as ectoparasites. However, the phenomenon of facultative parasitism in bacteria has been little studied.

As reported in [1], new gram-negative ultramicrobacteria (UMB) from a sample of oil slime from Nizhnekamsk refinery (Tatarsan) and the rhizosphere of *Pedilanthus tithymaloides* have been isolated and studied. These organisms form two types of cells in the cycle of their development: coccoid cells, 400–800 nm in diameter, and ultrasmall cells, ca. 200–300 nm in diameter. The strains designated as *Kaistia* sp. NF1 and NF3 belong to α-proteobacteria; according to the nucleotide sequence of the 16S rRNA gene and some phenotypic characters, they are closely related to the type strain of the recently described genus and species *Kaistia adipata* [1, 8]. However, the species status of strains NF1 and NF3 is still undefined. Both strains are heterotrophs and strict aerobes metabolizing a limited range of organic compounds: simple carbohydrates can be used as a carbon source; polymers (cellulose, pectin, proteins, or agar) are not degraded; and additional growth factors are required (yeast extract and vitamins) [1].

The typical property of UMB under study is their ability for parasitism on living cells of aerobic photosynthesizing prokaryotes, cyanobacteria. Joint cultivation of UMB and cyanobacteria (*Chlorogloeopsis fritschii* ATCC 27193, *Chlorogloeopsis* sp. S, *Anabaena variabilis* ATCC 29413, *Nostoc muscorum* 3, and *Spirulina* sp. 287) results in adsorption of UMB, their

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penetration into cyanobacterial cells, and lysis of the latter [1]. However, the character of interaction of ultramicrobacteria with heterotrophic bacteria has not been studied yet.

The goal of the present work was to investigate the character of the interrelations between soil ultramicrobacteria *Kaistia* sp. NF1 and NF3 and aerobic gram-positive and gram-negative heterotrophic bacteria.

MATERIALS AND METHODS

Bacterial cultures. The objects of research were ultramicrobacteria (UMB) *Kaistia* sp. NF1 and NF3 characterized in [1]. Test objects were aerobic heterotrophic bacteria: gram-positive *Bacillus subtilis* ATCC 6633, *Micrococcus luteus* VKM Ac-2230, and *Staphylococcus aureus* 209-P; and gram-negative *Pseudomonas putida* BS394, *Escherichia coli* C 600, *Pantoea agglomerans* ATCC 27155, and the soil bacterium *Acidovorax delafildii* 39, which we had isolated from the sludge of Lake Baikal. Identification of strain 39 as a representative of the species *A. delafildii* is based on the similarity of the phenotypic characteristics and the results of 16S rRNA gene nucleotide sequence. Strain 39 has a high degree of similarity (99%) of the 16S rRNA gene nucleotide sequence with the type strain *A. delafildii* ATCC 1705 (the 16S rRNA gene nucleotide sequence was determined as described in [1]). Some distinctive phenotypic characteristics of strain 39 are as follows: it is a chemoorganotroph, it grows well on peptone–soy medium, its cells are nonmotile, rod-shaped, and 0.5–0.6 × 1.8–2.2 μm in size, and the cell envelope contains an S-layer consisting of tetragonal-packed subunits.

The bacteria were cultured on a complete LB nutrient medium [9] at 24°C.

Antagonistic interrelations of UMB with other bacteria were revealed by inoculation of cultures onto agar surface as cross streaks and by the well diffusion method [10]. For this purpose, 10–15 μl of UMB culture was introduced into the wells, and after diffusion into the agar, covered an overlay of semisolid nutrient agar (0.7%) containing 5–7 × 10^7 cells/ml of a test culture. Plates were incubated at 24°C overnight. The antagonistic activity of UMB was estimated by the presence of zones of inhibition of test object growth around the wells and microscopically by UMB adsorption on the victim bacterial cells and lysis of the latter.

The lytic activity of ultramicrobacterial culture liquid (CL) cleared from cells by centrifugation (10000 *g*, 15 min) was also determined by the well diffusion method. Tenfold concentrated culture liquid was used in some experiments. For this purpose, it was cleared from cells by centrifugation, lyophilized, and dissolved in a small amount of water.

The presence of parasitic activity of UMB was determined by the intensity and strength of adsorption of UMB cells on victim bacteria and lysis of test organisms, using phase contrast, fluorescence, and electron microscopy.

The interaction of UMB and *B. subtilis* ATCC 6633 in a binary culture was studied as follows: bacterial cells were grown to the stationary phase in liquid LB medium, precipitated by centrifugation (10000 *g*, 10 min), washed with 0.01 M sodium phosphate buffer (pH 7.6), and resuspended in the same buffer. Initial cell concentrations of each bacterial strain in the control and experiments were equal: 7–8 × 10^9 cells/ml. The experiment was carried out for 20 days at 24°C. Samples for microscopy and viable count (CFU) by inoculation on agarized LB medium from respective dilutions were taken periodically.

Microscopy. The morphology and fine structure of bacterial cells in binary cultures were studied by phase contrast, fluorescence, and electron microscopy. The objects were studied in the phase contrast mode using light microscopes OPTON ICM 405 and LUMAM-2 (LOMO, Russia).

For fluorescence microscopy, cells in the control variants (monocultures) and in binary cultures were stained with Live/Dead reagent (Molecular Probs, Inc.) for 15 min at 28°C. The preparations were examined in a LUMAM microscope (LOMO, Russia) at an excitation wavelength within the 480–490 nm range, obtained by a combination of glass filters. Interference filters with the transmission peaks of 480 nm and 490 nm (Special Construction Department of Biological Instrument Making, Russian Academy of Sciences, Pushchino) were also used as the control.

Electron microscopy (ultrathin sections). Cell pellets were fixed in 1.5% glutaraldehyde solution in 0.05 M cacodylate buffer (pH 7.2) at 4°C for 1 h, washed three times with 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. For contrasting of polysaccharides, the cells were fixed in the presence of ruthenium red according to [11]. After dehydration in a series of alcohols, the material was embedded in epoxy resin Epon 812. Ultrathin sections were mounted on supporting grids, contrasted for 30 min with 3% uranyl acetate solution in 70% alcohol, and additionally contrasted by led citrate according to Reynolds [12]. Ultrathin sections were examined in a JEM-100B electron microscope (JEOL, Japan) at an 80-kV accelerating voltage.

RESULTS AND DISCUSSION

At the first stage of the work, the well diffusion method was used to test the antagonistic activity of UMB under study against aerobic heterotrophic bacteria, both gram-negative (*P. putida* BS394, *E. coli* C 600, *P. agglomerans* ATCC 27155, *A. delafildii* 39) and gram-positive (*B. subtilis* ATCC 6633, *M. luteus* VKM Ac-2230, *S. aureus* 209-P) (table). Formation of small zones of test object growth inhibition around the wells

with the UMB cultures was observed only on the lawn of *B. subtilis* cells (*A. delafildii* was not studied by this method). Formation of small zones of growth inhibition is evidence of UMB antagonistic activity; such zones possibly resulted from the action of the UMB extracellular enzymes, which have a lytic effect, or from the low-molecular antibiotics secreted into the culture liquid. It was therefore necessary to find out what particular factor induced the lysis of *B. subtilis* culture under a UMB attack. For this purpose, the lytic effect of the filtrates of UMB CL was studied. However, UMB CL was not found to induce the formation of lytic zones on the lawn of *B. subtilis* cells. The zones of absence of the test bacterium growth did not appear even at higher amounts of CL introduced into the wells or more concentrated (tenfold) CL samples.

Preliminary heating of test bacterial cells at 60°C for 30 min usually is known to promote a more effective action of the extracellular hydrolytic enzymes involved in the degradation of cell wall components [13]. Moreover, a number of works demonstrated that the enzymes with a lytic effect against a certain cell wall component exhibit even higher activity against the killed bacterial cells or the cell walls isolated from them [14]. In our experiments, the testing of UMB culture liquid on agarized nutrient media containing autoclaved *B. subtilis* cells showed no formation of lysis zones. Thus, the antagonistic activity of UMB against bacilli is not induced by antibiotics or lytic enzymes secreted into the culture liquid.

The method of inoculation of UMB and test objects on complete nutrient media as cross streaks revealed that both UMB strains inhibited the growth of *B. subtilis* and *A. delafildii* 39. In the area of streaks crossing, the density of mixed bacterial cultures decreased and the lysis of test object cells was observed. The microscopic study of the interaction of these bacteria showed that UMB were tightly attached to the surface of larger cells of test objects (Fig. 1). Thus, it is quite obvious that the lytic activity of UMB against *B. subtilis* and *A. delafildii* occurs only in close contact with living cells.

The dynamics of interaction of strains *Kaistia* sp. NF1 and NF3 with *B. subtilis* cells in the absence of nutrients was studied using binary bacterial cultures. As Fig. 2 shows, in the course of joint incubation with bacilli in the absence of carbon sources and additional growth factors (vitamins) required for UMB growth, the quantity of *Kaistia* sp. NF1 cells increased with time. The increase in CFU numbers of UMB was accompanied by a considerable decrease in the number of bacilli cells. At the same time, the quantity of *B. subtilis* CFU in a monoculture decreased insignificantly. Similar results were also obtained for *Kaistia* sp. NF3. These results demonstrate that UMB exist in binary cultures due to the nutrients obtained at the lysis of *B. subtilis* cells which they induce. Such changes of bacterial numbers in binary cultures are typical of a "parasite–victim" system and may be evidence of the parasitic character of the interrelations between UMB and bacilli [15].

The presence of parasitic activity of ultramicrobacteria *Kaistia* sp. NF1 and NF3 against some gram-positive and gram-negative heterotrophic bacteria*

Test object	Ultramicrobacteria	
	strain NF1	strain NF3
Gram-negative bacteria		
P. putida BS394	–	–
E. coli C 600	–	–
P. agglomerans ATCC 27155	–	–
A. delafildii 39	+**	+
Gram-positive bacteria		
B. subtilis ATCC 6633	+	+
M. luteus VKM Ac-2230	–	–
S. aureus 209-P	–	–

Notes: * Interaction was detected by formation of lysis zones on agar plates and by adhesion of ultramicrobacteria on host cells determined by electron and fluorescence microscopy.

** Symbol "+" means the presence of an interaction.

The character of UMB interaction with *B. subtilis* and *A. delafildii* was studied further by the methods of electron and fluorescence microscopy. The percentage of live and dead cells in the preparations stained with the Live/Dead reagent was assessed in the control variants (prior to mixing cultures). The ratio of dead cells was found to be $1.36 \pm 0.14\%$ in the initial suspension of *B. subtilis* and $4.41 \pm 1.09\%$ in the suspension of NF1.

At the first stages of interaction of the cultures in binary systems, UMB cells are attached to the cells of both *B. subtilis* and *A. delafildii* 39. This process is well detected by the methods of phase contrast and fluorescence microscopy (Fig. 1). In liquid media, UMB attachment to the surface of bacilli cells proceeds very quickly, in the first few minutes after suspensions have been mixed. Up to ten or more UMB cells can be attached to a single bacillus cell, often covering almost the whole cell surface (Fig. 1). The character of interaction in the binary system of strain NF1 + strain 39 during the first minutes of interaction is different; UMB cells are attached to both poles of strain 39 cells, forming a dumbbell shaped association.

Since the cells of the analyzed UMB strains have neither flagella nor active motility, the processes of chemotaxis are not involved in the interaction of UMB and the victim cells.

Observation of the process of adsorption of bacteria in wet mount preparations showed that liquid currents did not remove the UMB cells adsorbed on *B. subtilis* and *A. delafildii*, which indicated a high strength of their attachment. Some UMB cells make local fluctuating

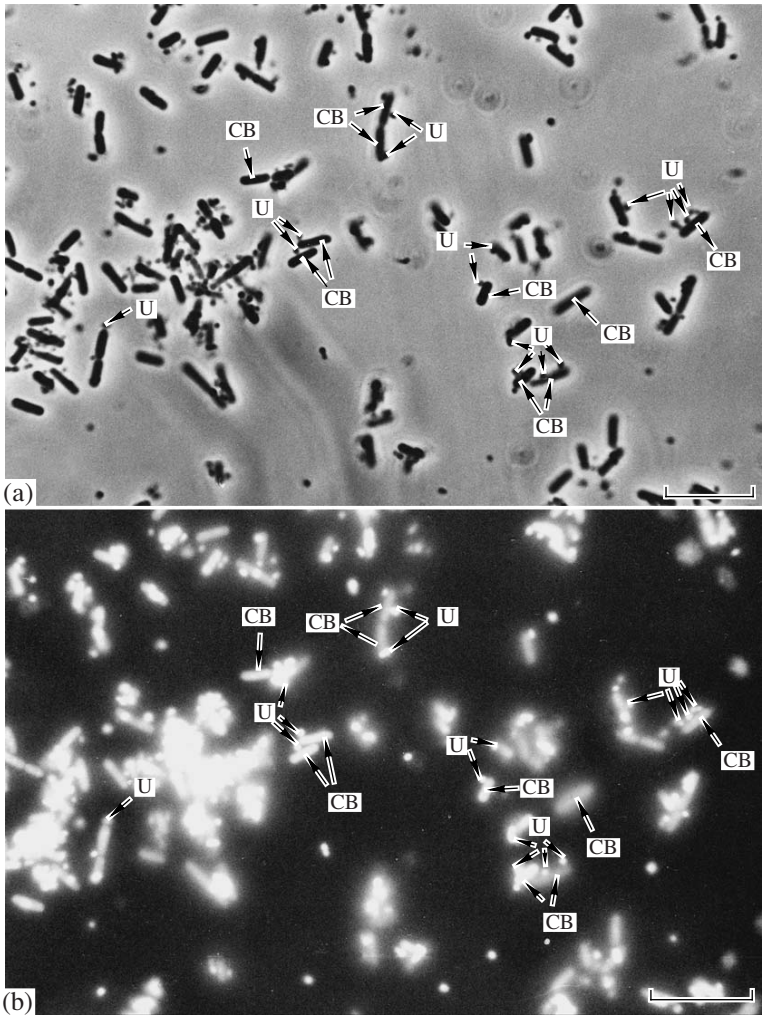

Fig. 1. Phase contrast (a) and fluorescence (b) microphotographs of cells in the binary culture of *Bacillus subtilis* + UMB after cultivation for 1 h. CB, cells of bacilli; U, cells of ultramicrobacteria. Scale bar: 10 μm.

movements on the surface of victim cells, which possibly means that they are still at the initial stage of attachment to the surface by means of the polysaccharide filaments of their microcapsules. This assumption was confirmed by the results of electron microscopic analysis, which revealed the presence of numerous polysaccharide filaments binding the cells, especially in the preparations stained with ruthenium red (a contraster for polysaccharides) (Fig. 3). Meanwhile, the UMB cells remain immobile when they are more tightly attached to the victim cell surface by tight contacts of the surface layers of the pair of cell envelopes (Figs. 3, 4).

The analysis of preparations stained with Live/Dead reagent showed that NF1 cells were attached only to the live cells of bacilli (green fluorescence) (Fig. 1b). The phase contrast and fluorescence microscopy showed that the interaction in the binary culture with UMB is accompanied by decrease in cytoplasm density followed by death of the victim cells (bacilli and acidovorax). A fraction of the victim cells, however, remains alive for a long time (5–7 days), in spite of the numerous UMB cells attached to their surface. It should be particularly mentioned that UMB in binary cultures have a strong inhibiting effect on sporulation of bacilli cells, delaying the start of this process for 3 days and reducing the quantity of formed spores.

The electron microscopic analysis of ultrathin sections of the cells in binary cultures of UMB + *B. subtilis* and UMB + *A. delafildii* revealed the ultrastructural details of attachment of UMB cells to the envelopes of the victim cells. Characteristics of the structure and supramolecular organization of bacterial cell walls and outer layers are known to be the determining factors in interacting bacterial cell systems. The bacillus strain used in this work possesses characteristic thin lamellar formations in its outer cell wall layers; they are oriented in parallel to the cell surface (Fig. 4a, b). Although these formations resemble the surface paracrystal S-layers (orderly layered glycoprotein) widespread in the bacterial world, unlike S-layers they do not have a peri-

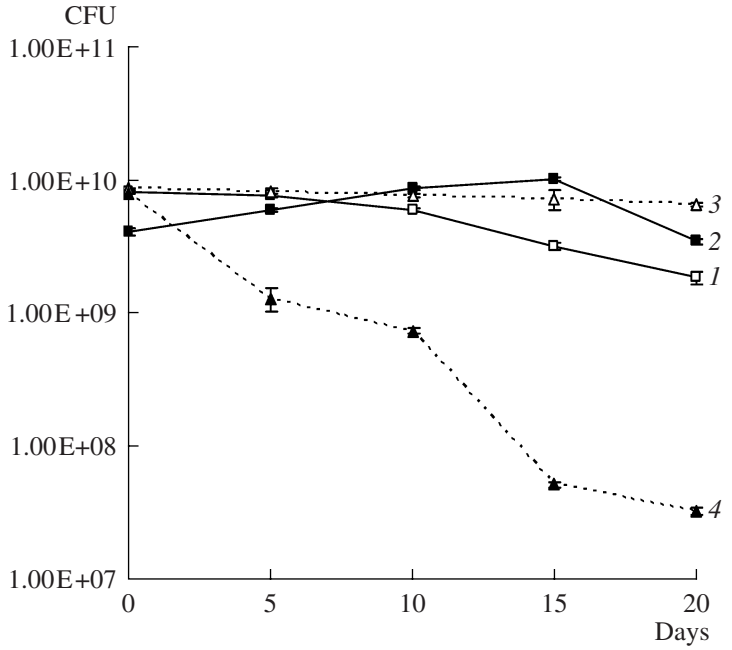

Fig. 2. The dynamics of development of UMB and *B. subtilis* ATCC 6633 in monocultures and binary cultures: *1*, NF1, monoculture; *2*, NF1, binary culture; *3*, *B. subtilis,* monoculture; *4, B. subtilis*, binary culture.

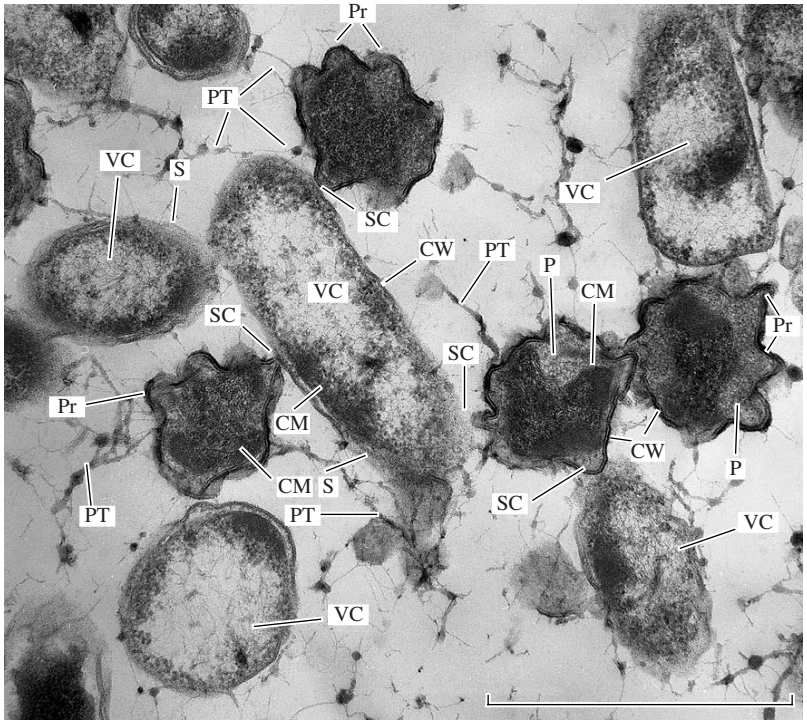

Fig. 3. Ultrathin section of the cells from the binary culture of strains 39 + NF1, contrasted with ruthenium red, after three days of interaction. Fibrils of UMB microcapsule are bound with the surface of the victim cell CW (shown by arrow). The zones of contacts of UMB prosthecae (NF1) with the S-layer of *A. delafildii* are visible. PT, polysaccharide threads; Pr, prosthecae; SC, sites of contacts of UMB prosthecae with victim cell; CW, cell wall; VC, victim cell; P, periplasm; CM, cytoplasmic membrane; S, subunits on the S-layer tangential section. Scale bar: 1 μm.

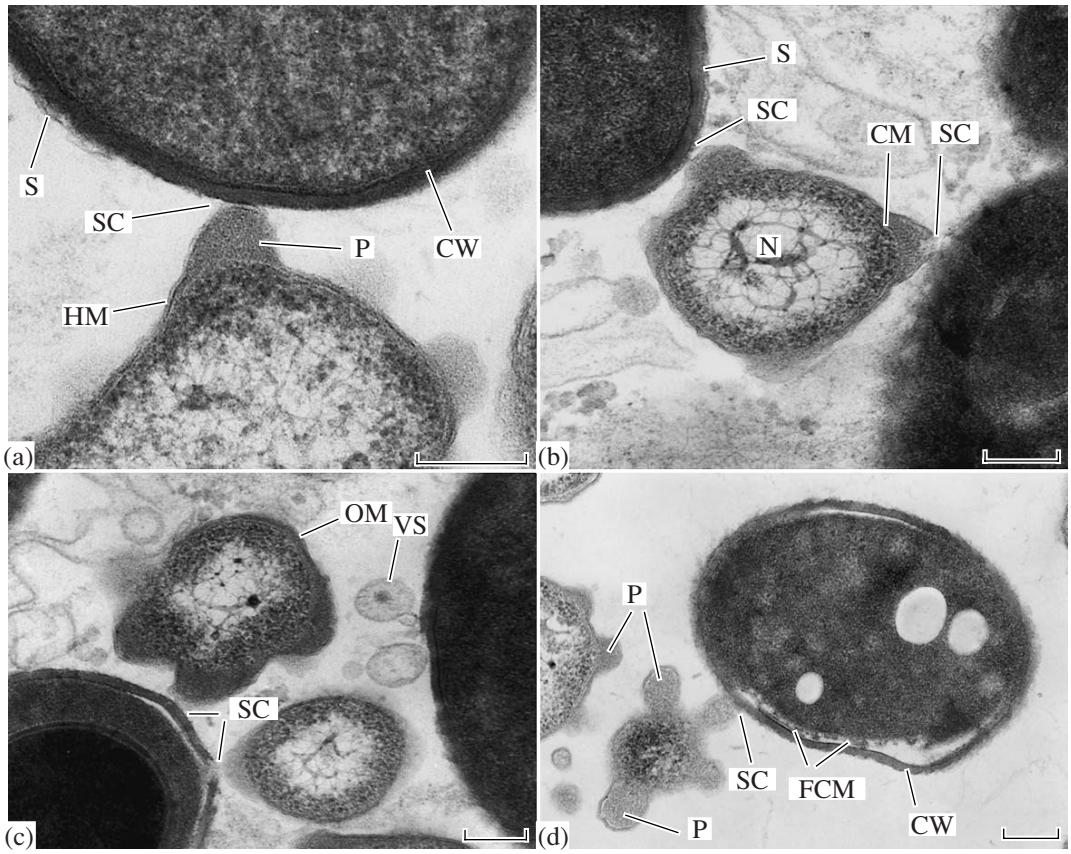

Fig. 4. Fragments of ultrathin sections of cells from the binary culture of *Bacillus subtilis* + UMB: (a) the contact of UMB prosthecae envelope with the external surface of *Bacillus subtilis* CW; (b) the transfer of prosthecae contents (SC) into *B. subtilis* cell (shown by arrow); (c) the absence of CW region in the site of contact with UMB prosthecae envelope and disrupted integrity of *B. subtilis* envelope in the site of contact; (d) initial stages of lysis of *B. subtilis* cell accompanied by destruction of vast areas of CW, cytoplasmic membrane, and cytoplasm. FCM, fragments of destroyed cytoplasmic membrane; SC, sites of contact of UMB prosthecae with victim cell; P, periplasm; N, nucleoid; CW, cell wall; S, S-layer of bacillus cells; OM, outer membrane; VS, vesicular membrane structures; CM, cytoplasmic membrane. Scale bar: 200 nm.

odic structure. In the rod-shaped cells of *A. delafildii*, however, a distinct S-layer is present; it is located on the surface of the cell wall consisting of the murein layer and the outer membrane (Figs. 3, 5). Probably, these peculiarities of organization of cell wall surface layers are responsible for the sensitivity of the studied bacillus strain and *A. delafildii* 39 to UMB attack.

The NF1 and NF3 cells in binary cultures are characterized by the presence of a vast periplasmic space (P) and a great number of periplasmic protrusions filled with granular substance of high electron density. These protrusions can be designated as periplasmic prosthecae (Pr). In the interacting binary cultures, Pr are often hemispherical, spherical, or conical (Figs. 3, 4). The intercellular space of the binary cultures of strains NF1 and NF3 reveals the presence of numerous separate vesicles surrounded by the membrane and filled with a substance similar to that of the periplasm. Apparently, a significant part of Pr is released into the growth medium as outer membrane vesicles through their separation from the cell wall. The cells of strains NF1 and NF3 start their interaction with the victim cells by establishing close contacts between the UMB outer membrane and the external surface of a *Bacillus* CW or with the outer S-layer of the *Acidovorax* cell envelope. First, the contact is established in the sites where UMB prosthecae join the CW surface of victim cells by the tips of their spherical or conical protrusions (Figs. 3, 4). Then, the peripheral part of Pr in the sites of contact slightly flattens; as a result, the length of contacting surfaces on ultrathin sections reaches 15 nm or more (Fig. 4a, c, d). In some cases, the transfer of Pr content into a bacillus cells may be observed in the site of contact (Fig. 4b). The following local changes in the *Bacillus* CW structure are observed in the sites of contacts: lysis of small CW regions (comparable with the size of contacting surfaces) (Fig. 4a, b, c, d) and clarification of CW regions throughout the whole depth (Fig. 4c). Sometimes, significant complex destructive changes were observed in the sites of cell–cell contacts. These

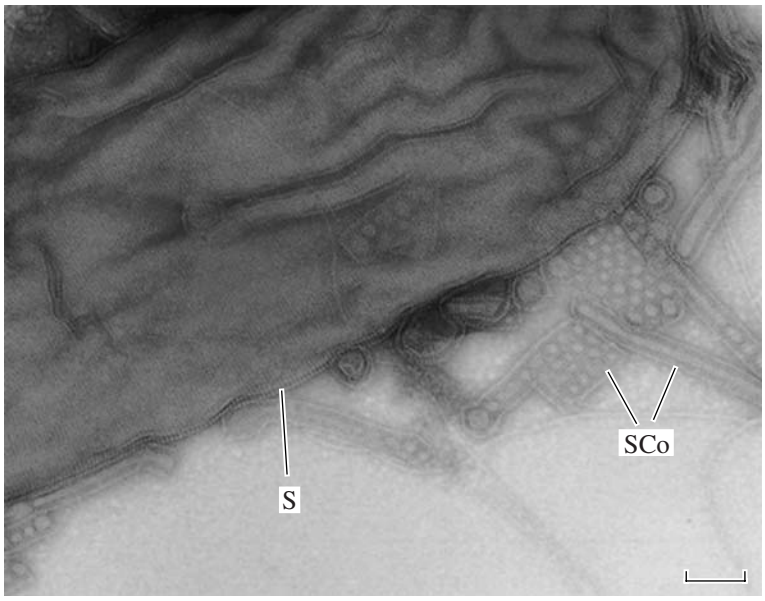

Fig. 5. S-layer on the surface of *A. delafildii* cell with tetragonal symmetry of packing of protein subunits (negative contrasting with uranylacetate). S, subunits of S-layer; SCo, sites of S-layer cast-off. Scale bar: 100 nm.

changes affected all peripheral cell structures: CW, the cytoplasmic membrane, and the cytoplasm (Fig. 4d).

In 7–10 days, the described processes resulted in the lysis of a considerable part of *Bacillus* and *Acidovorax* populations. This was confirmed by experiments on the dynamics of CFU decrease (the results are presented in Fig. 2).

Thus, it has been shown for the first time that free-living ultramicrobacteria can be parasites of both phototrophic and heterotrophic bacteria. Analysis by electron and fluorescence microscopy made it possible to identify the type of parasitism of the UMB under study and refer them to ectoparasites. Apparently, the studied ultramicrobacteria can participate in the regulation of the quantity of microorganisms in microbial communities of some natural ecotopes.

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