ISSN 0026-2617, Microbiology, 2011, Vol. 80, No. 4, pp. 535–548. © Pleiades Publishing, Ltd., 2011.
Original Russian Text © N.E. Suzina, V.I. Duda, T. Z. Esikova, A.P. Shorokhova, A.B. Gafarov, R.R. Oleinikov, V.N. Akimov, T.N. Abashina, V.N. Polivtseva, A.M. Boronin, 2011, published in Mikrobiologiya, 2011, Vol. 80, No. 4, pp. 529–542.

EXPERIMENTAL ARTICLES

Novel Ultramicrobacteria, Strains NF4 and NF5, of the Genus *Chryseobacterium*: Facultative Epibionts of *Bacillus subtilis*

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Received October 10, 2010

Abstract—Two strains, NF4 and NF5, of a yellow-colored gram-negative bacterium were isolated from sediments of Lake Baikal and from old oil sludge of the Nizhnekamsk oil-processing plant. The cells of the strains are ultrasmall coccoids or short rods, measuring 0.2–0.4 × 0.2–0.5 μm; the average cell volume ranges from 0.004 to 0.04 μm^3. A considerable proportion (30–60%) of cells have nanometer dimensions (180–300 nm in diameter and 0.004–0.02 μm^3 in volume). The new isolates are thus among the smallest representatives of presently known free-living ultramicrobacteria. The two studied isolates are gram-negative nonmotile cells possessing a pronounced outer membrane. The cells do not have flagella and are not capable of gliding motility. They divide by constriction, budding, and multiple septation. The multiplicity of reproduction mechanisms results in a high degree of cell polymorphism. The isolates are chemoorganotrophic, aerobic, psychrotolerant, oxidase- and catalase-positive. Their characteristic trait is the absence of extracellular hydrolytic enzymes, such as proteases, lipases, pectinases, and cellulases. Menaquinone MK-6 is the main respiratory quinone; the flexirubin pigment was not detected. The G + C contents of the DNA of strains NF4 and NF5 are 40.8 and 40.5 mol %, respectively. The DNA–DNA hybridization level of strains NF4 and NF5 was close to 100%. Analysis of the 16S rRNA gene sequences and the fatty acid compositions showed that the isolates are most closely related to certain representatives of the genus *Chryseobacterium* (*C. solincola, C. antarcticum*, and *C. jeonii*). However, the differences in the 16S rRNA gene sequences, as well as in the phenotypic properties, such as formation of ultrasmall cells, the absence of extracellular hydrolases, oligotrophy, and the capacity for epibiosis on bacterial cells, suggest that the studied strains belong to a new species of the genus *Chryseobacterium*. The capacity for epibiosis, i.e., the ability to exist in a tightly adhered state on the surfaces of host *Bacillus subtilis* cells, is a peculiar trait of the studied isolates. It is assumed that adhesion of the cells of strains NF4 and NF5 (members of the phylum *Bacteroidetes*) occurs via by the same unique mechanism as the mechanism that we previously described for representatives of *Alphaproteobacteria* (*Kaistia* sp., NF1, and NF3), which use polysaccharide chains equipped with sticky granules as trapping and constricting cords.

Keywords: epibiosis, bacterial parasitism, ultramicrobacteria, cell ultrastructure, cell cohesion, *Chryseobacterium* systematics.

DOI: 10.1134/S0026261711040187

The genus *Chryseobacterium* of the family *Flavobacteriaceae* comprised more than 20 bacterial species in 2009 widely occurring in nature and isolated from various habitats (soils, freshwater ecosystems, plants, insects, Antarctic permafrost) [1–3]. Four species have recently been added to this genus due to the reclassification of *Sejongia* [4] and *Kaistella* [5] species, as well as to the description of the species *Chryseobacterium solincola* [6]. All described *Chryseobacterium* species are gram-negative, aerobic, heterotrophic, mesophilic, and psychrotolerant bacteria. Only *Chryseobacterium meningosepticum* [7] is a pathogenic microorganism; all other species are typical saprotrophs, inhabitants of soils and aquatic ecosystems. The genus *Chryseobacterium* also includes strains of ultramicrobacteria isolated from a glacier in Greenland and described in [8].

Although *Chryseobacterium* species can be routinely differentiated on the basis of the 16S rRNA gene analysis, no clear-cut phenotypic or chemotaxonomic distinctions that would allow *Chryseobacterium* strains to be differentiated at the species level are currently known. When studying microorganisms isolated from the samples of Baikal sediment and old oil sludge from the Nizhnekamsk oil-processing plant, we found two strains, NF4 and NF5, to be phylogenetically close to members of the genus *Chryseobacterium* but to differ from them by such phenotypic properties as ultrasmall

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cell sizes and capacity for facultative epibiosis on *Bacillus subtilis* cells.

The aim of the present work was to analyze the ultrastructure, physiological and biochemical properties, and taxonomic position of the isolated ultrasmall bacteria, strains NF4 and NF5.

MATERIALS AND METHODS

The bacterial cultures were isolated from samples of Baikal sediment and from about 35-year-old oil sludge from the Nizhnekamsk oil-processing plant (Nizhnekamsk, Tatarstan, Russia), containing up to 40% of organic compounds (about 30% of them are oil products). The sediment suspension was filtered through a 0.4-μm membrane filter and plated onto agarized media. To obtain pure bacterial cultures, the following nutrient media were used: (à) the 5/5 tryptone–soy agar IBPM RAS containing the following (g/l): soy extract, 30; casein hydrolysate, 5; yeast extract, 1; and aminopeptide, 60 ml/l; pH 8.0; 20 m/l of agar was added to prepare solid medium; and (b) agarized oil sludge containing the following: crude oil sludge, 30 g; agar, 6 g; and tap water, 300 ml.

After plating of the samples of Baikal sediment and old oil sludge from the Nizhnekamsk oil-processing plant onto the 5/5 medium, growth of yellow colonies formed by ultrasmall coccoid cells was observed. Pure cultures of these bacteria were isolated by repeated plating of the obtained colonies onto tryptone–soy agar. The culture purity was assessed by the following criteria: (1) absence of colonies of xenologous microorganisms after inoculation on all media used, (2) absence of cells atypical of the isolates under study as inferred from microscopic examination under a light microscope with a phase-contrast device and a transmission electron microscope, and (3) absence of 16S rRNA gene sequences atypical of *Chryseobacterium solincola* in the DNA of the studied isolates. Two newly isolated microorganisms, designated as strains NF4 and NF5, satisfied these criteria.

Phenotypic properties of the isolates were studied using traditional methods [9] with taking into account the minimum list of standard properties recommended for the description of new taxa of the family *Flavobacteriaceae* [10].

Growth and physiological properties of the isolates. Bacterial growth in the liquid cultures was assessed by the optical density (OD) ($\lambda = 590$ nm, $l = 5$ mm, UV Specord, Carl Zeiss, Jena, Germany).

The cultures were grown on the following nutrient media: 5/5 tryptone–soy agar; Nutrient agar Difco (BBL), Brain heart infusion agar (Pronadisa, Spain), Zobell agar (agar, 15 g/l; peptone, 5 g/l; yeast extract, 1 g/l; and ferric citrate, 0.1 g/l), M-9 (Na_2HPO_4, 6 g/l; KH_2PO_4, 3 g/l; NaCl, 0.5 g/l; NH_4Cl, 1 g/l; $MgSO_4$, 0.25g/l; $CaCl_2$, 0.01 g/l; and agar, 20 g/l), and MacConkey agar (Difco). Inoculated media were incubated for 21 days.

Biochemical tests. The biochemical properties of the studied microorganisms and the spectrum of utilized carbon sources were determined using the API 20NE and API 20Å kits (bioMerieux) according to the manufacturer's instructions.

The flexirubin pigment was determined using the bathochromatic shift test with a 20% KOH solution [11]. Pigments were extracted from wet biomass of bacterial cells (5 days, tryptone–soy medium) with a mixture of chloroform and methanol (1 : 2) using traditional techniques. Absorption spectra were determined with a Pharma Spec UV-1700 UV–visible spectrophotometer (Shimadzu).

Phylogenetic analysis. Determination of the DNA G + C content and of the level of genomic similarity between the studied strains, as well as the analysis of the 16S rRNA gene sequences and phylogenetic analysis, were carried out using the techniques described in [12].

Genome sizes were determined using the techniques described in [13]. Native chromosomal DNA immobilized in agarose gel was isolated according to the *FIGE Mapper. Instruction Manual and Application Guide* (Bio-Rad). Restriction analysis of native chromosomal DNA was performed according to the manufacturer's recommendations and involved 29 enzymes (Fermentas) recognizing a sequence of six or eight base pairs. Two enzymes, *NheI* and *SpeI*, yielding the minimum number of restriction fragments, were chosen among these enzymes for further analysis. The sizes of small DNA fragments (5–150 kb) were determined by inversion gel electrophoresis (FIGE Mapper, BioRad). Electrophoresis was carried out according to the manufacturer's recommendations using program no. 8. Larger fragments were determined by pulsed field electrophoresis (Pulsaphor System, Pharmacia LKB). Electrophoresis was run at 120 V and 14°C for 24 h, at a pulse of 25–50 s.

Antagonistic relations between strains NF4 and NF5 and chemoorganotrophic bacteria were revealed by inoculation of interacting cultures onto the agar surface with cross streaks and by the well diffusion method. For latter purpose, 10–15 μl of the culture of ultramicrobacteria (UMBs) was introduced into the wells, and, after diffusion of UMB cells into the agar, it was covered with semisolid nutrient agar (0.7%) containing (5–7) × 10^7 cells/ml of the test bacterial culture. Inoculated media were incubated at 24°C overnight. The antagonistic activity of ultramicrobacteria was estimated from the presence of growth inhibition zones around the wells and by microscopic examination of UMB adsorption on the prey bacterial cells and lysis of the latter.

The lytic activity of the ultramicrobacterial culture liquid (CL) cleared from cells by centrifugation (10000 *g*, 15 min) was determined by the well diffusion method.

Lysis of bacterial cell walls. Test bacterial cells were bacteria were boiled for 5 min and added to melted agar; then, UMB cultures were streaked onto the agar surface. After a 7-day incubation, the presence and size of the zones of lysis of dead cells of tester bacteria around the streaks of grown UMB cultures were determined. The collection cultures of *Bacillus subtilis* ATCC 6633, *Micrococcus luteus* B-1813, *Escherichia coli* C2110, *Erwinia carotovora* 1, *Pseudomonas putida* BS 394, and *Alcaligenes ruhlandii* 1 were used as tester objects.

The capacity for epibiosis of strains NF4 and NF5 was assessed from the results of microscopic examinations, namely, from the extent and strength of adsorption of UMB cells on prey bacteria and from lysis of tester microorganisms; phase contrast, fluorescence, and electron microscopy was used. The tester bacteria were *B. subtilis* ATCC 6633 (the strain previously found to be sensitive and already used for determination of the capacity for epibiosis and ectoparasitism [13, 14]), *Staphylococcus aureus* 209-P, and *M. luteus* B1813 were used as tester bacteria.

The interaction between strains NF4 and NF5 and *B. subtilis* ATCC 6633 (*Bs*) in a binary culture was studied as follows. Bacterial cells were grown until 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. The initial cell concentrations of UMB and the tester cultures in the obtained suspensions varied in different experiments. In the first series of experiments, the concentrations of NF4 and *Bs* cells were 1.1×10^9 and 3.8×10^7 CFU/ml, respectively. In the second series of experiments, the concentrations of NF4 and *Bs* cells were 1.0×10^9 and 1.5×10^8 CFU/ml. In the third series, the concentrations of cells were 9.0×10^8 and 3.5×10^8 CFU/ml. The initial cell concentrations in the controls (monocultures of UMB and the tester strains) were the same as those above. The cultures were incubated at 24°C for 20–30 days or longer. Every 2 days, samples of mono- and mixed cultures were taken for microscopy and CFU count on agarized 5/5 medium. Differentiation of *Bs* and UMB colonies was carried out as described in [14]. The numbers of spores in mono- and mixed cultures were determined from the number of colonies grown on 5/5 medium after sample preheating at 80°C for 15 min.

Microscopy. The morphology and ultrastructure of bacterial cells were studied by phase contrast, epifluorescence, and electron microscopy. For epifluorescence microscopy, the cell preparations were fixed with 1.5% glutaraldehyde solution for 30 min and stained with DAPI (1 μg/ml) for 5 min. To distinguish viable and dead cells, a two-component *LIVE/DEAD* Baclight Bacterial Viability kit L-13152 (Molecular Probes, Inc.) was used; the final concentrations of Syto 9 and propidium iodide in the reactant were 6 and 30 μM, respectively. The specimens were examined under a Polyvar (Reichert) and LUMAM (LOMO, Russia) microscopes under UV excitation with a maximum at 360, 480, or 490 nm. For contrasting of the polysaccharides of capsules and cell walls, glutaraldehyde–osmium fixation in the presence of ruthenium red was applied [15]. After dehydration, the preparations were embedded into Epon 812 and ultrathin sections were obtained with an LKB III ultramicrotome (Uppsala, Sweden). The obtained ultrathin sections were mounted on copper grids covered with formvar support, stained with a 3% uranyl acetate solution in 70% ethanol for 30 min, and then stained with lead citrate [16] at 20°C for 4–5 min. Some specimens of intact bacterial cells were negatively stained with a 0.2% aqueous solution of uranyl acetate. The preparations were examined under a JEM-100B electron microscope at an accelerating voltage of 80 kV.

The cell sizes were determined using phase-contrast micrographs and electron micrographs of negatively stained cells and ultrathin sections cut through the central medial part of the cells. The cell volumes were calculated using the formula proposed in [17].

RESULTS

Cell morphology and development cycles of isolates NF4 and NF5. When grown on agarized media, strains NF4 and NF5 produce yellow (NF4) or brownish-yellow (NF5) slimy colonies 2–4 mm in diameter. The cells are spherical, ovoid, bean-shaped, or short rods (coccobacilli) (Figs. 1–4). They are $0.2–0.4 \times 0.2–0.5$ μm in size; their average volume ranges from 0.004 to 0.04 μm^3; a considerable portion (30–60%) of their cultures is represented by small cells (180–300 nm in diameter and with a volume of 0.004–0.02 μm^3). These cells may be considered nanoforms, i.e., extremely small (nanosized) cells (Fig. 1a). During the lag phase, coccoid cells become elongated and transform into irregularly shaped rods ($2–5 \times 0.2–0.4$ μm; 0.1–0.3 μm^3) (Fig. 1b). Binary division of these cells and formation of equally sized cells occurs rarely; more often, small spherical or ovoid cells or short rods (coccobacilli) are budded off at the cell poles (Fig. 1b); other elongated cells become undergo septation along their whole length with the resulting formation of coccoid cells (Fig. 1c). Coccoid cells reproduce by binary division and/or budding (Figs. 1 and 2); in the latter case, buds sometimes are localized laterally on the maternal cell, perpendicularly to its longitudinal axis (Fig. 2). The cell poles are rounded or cone-shaped. During division of rodlike cells, the daughter and maternal cells are connected angularly to each other, similarly to reproducing cells of arthrobacters and microbacteria.

Thus, reproduction of strains NF4 and NF5 occurs in the following ways: (1) separation of small coccoid cells from the poles of rodlike cells; (2) budding of coccoid and rodlike cells; (3) division of rodlike cells

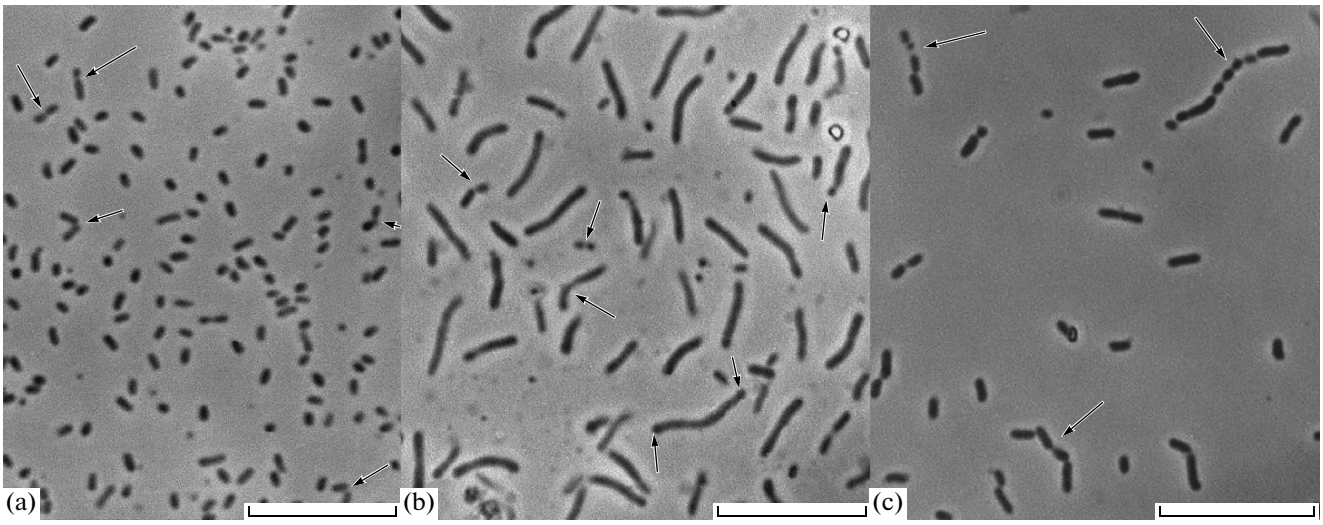

Fig. 1. Cells of strain NF4 in 4-day (a), 1-day (b), and 2-day (c) cultures grown in liquid 5/5 medium at 28°C. The morphology of strain NF5 cells under the same cultivation conditions was the same. Phase-contrast microscopy. The sites of cell division and splitting into coccoid cells are designated with arrows. Bar, 10 μm.

into numerous coccoid cells by septation; and, rarely, (4) binary division of rodlike cells. In exponential and stationary phase cultures, reproduction types 2 and 3 prevailed (Figs. 1a, 1c, 2, and 3). The multiplicity of reproduction types is responsible for the high level of cell polymorphism in the studied isolates. Figure 5 schematically presents the ways in which different cell morphotypes are formed in the course of cell reproduction at different stages of culture development.

Cell ultrastructure. Ultrathin sections revealed that the cell wall structure of the studied strains is of the gram-negative type; the cell walls consist of (a) the outer membrane (OM), (b) murein layer (M), and (c) periplasmic space (PS) (Fig. 3). The conclusion that the studied strains are of the gram-negative type of cell organization was confirmed by the positive results of the 3% KOH test, as well as by appearance of the surfaces of negatively stained intact cells: in this case, the cell surface had a moire (crinkled) structure (Fig. 2), which is typical of many gram-negative bacteria [18].

On the cell surface, a polysaccharide matrix was visualized, which appeared as a network of polysaccharide filaments (PF) carrying electron-dense nodules (Nd) shaped as granules (Fig. 4). The filaments and nodules were clearly visualized by staining with ruthenium red used to stain acidic polysaccharides (Figs. 3b and 9); they looked similar to the filaments and nodules of *Kaistia* strains NF1 and NF3 [14, 19]. The measurements performed using negatively stained specimens revealed that the thinnest filaments were ~5.5–6.0 nm thick, whereas bundles of filaments were 18–35 nm thick. The nodules (~25–35 nm in diameter) looked like rounded granules with an ultrastructure that was also granular. The granules were threaded (at a distance of ~50–100 nm) as beads along the filaments.

Nodule-free filaments are probably polysaccharide slime actively produced by both strains, as evidenced by the slimy consistency of colonies and the low sedimentation rate of bacterial cells (especially of NF4 cells) during centrifugation (10000 *g*, 20 min) due to the high viscosity of the culture liquid. The nucleoid was well-defined in thin sections and located in the central part of the cell in the form of large pale (electron-transparent) zone filled with DNA fibrils and surrounded by a thin layer of electron-dense cytoplasm (Figs. 3a–3c). The nucleoid of rodlike cells resembles a cord running from one pole to another along the longitudinal axis of the cell (Fig. 3c); its size is several times greater than that of the nucleoid of coccoid cells (Figs. 3a and 3b). The cells of both strains are nonmotile, which was confirmed by observations of the behavior of live cells in liquid media, as well as by the fact that no flagella or pili were detected in electron microphotographs. Neither did we reveal gliding motility of the cells.

Physiological and biochemical properties of the isolates. The isolates are aerobic, chemoorganotrophic, and psychrotolerant bacteria (Table 1). Strains NF4 and NF5 utilize a limited range of amino and organic acids as carbon and energy sources; other carbohydrates, mono- and disaccharides, pentoses and hexoses, and alcohols, as well as N-acetyl-glucosamine, do not support UMB growth (Table 1). Of the 18 amino acids tested, growth of strain NF5 was observed on D-L β-alanine, L-asparagine, glycine, D-L leucine, L-isoleucine, L-ornithine, and D-L threonine. Strain NF4 grew on D-L leucine, L-isoleucine, D-L β-phenyl-α-alanine, and D-L tryptophan.

Polymeric organic compounds—proteins, lipids, and polysaccharides (cellulose, murein, agar, pectin)—were not utilized; this indicates that the studied

isolates lack the extracellular hydrolases necessary for polymer utilization, which determines their ecological niche.

The studied microorganisms grew at temperatures ranging from 4 to 30°C, pH 6.0–8.0 (with an optimum at 7.0), and within a salinity range of 0–1.0%. The characteristic trait of the growth and development cycle of the studied isolates is prolonged lag phase (48 h) and a prolonged poststationary phase that does not result in a sharp decrease in the number of viable cells (Fig. 6). The following cellular fatty acids were predominant: *iso*- C15:0 (23.19%) and *anteiso*- C15:0 (38.41%), as well as *iso*- C17:1 (9.12 %) (Table 2). Menaquinone MK-6 is the main respiratory quinone; flexirubin pigment was not detected.

Interaction between strains NF4 and NF5 and *Bacillus subtilis* ATCC 6633. We have previously demonstrated that ultramicrobacteria of the genus *Kaistia* are capable of one of the types of epibiosis: contact-type parasitism on living cells of some autotrophic and heterotrophic bacteria, including *B. subtilis* ATCC 6633, the cell wall of which includes a surface layer consisting of regularly arranged subunits [12, 14]. In view of this, we tested strains NF4 and NF5 for their capacity for intercellular interaction with other bacteria. It was demonstrated that the cells of both UMB strains are able to firmly attach to *B. subtilis* ATCC 6633 (*Bs*) cells in binary cultures growing both in liquid and solid nutrient media, as well as in cell suspensions prepared using sterile tap water or phosphate buffer (Figs. 7a–7c, 3a, and 8). Adhered cells could not be separated either by water flow or by agitation and centrifugation of cell suspensions, which demonstrated the strength of cell cohesion. The number of UMB cells attached to a single *Bs* cell can vary from one–three to several tens.

Electron microscopic examinations revealed that UMB cells can attach to the surface subunit layers (S-layers) of *Bs* cells; in the sites of contact, piles consisting of several S-layers are formed (Figs. 8 and 9). Observation of mixed cell suspensions showed that adhesion occurs very rapidly: 10–15 min after the contact of test bacterial cells with UMBs, more than 50% of viable *Bs* cells had UMBs attached to their cell walls. The smallest UMB cells (nanocells) actively interact with *Bs* cells (Figs. 3a, 7, 8, and 9). Since, in this experiment, the medium was not supplemented with nutrient substrates, ultramicrobacteria could grow only at the expense of metabolites produced by *Bs* cells.

In binary culture, with the initial cell concentrations of strains NF4 and *Bs* of 1.1×10^9 and 3.8×10^7 CFU/ml, respectively, the number of CFU of prey bacteria decreased by five to six times after 3–5 days of interaction (Fig. 10). However, at the initial cell concentrations of strains NF4 and *Bs* of 1.0×10^9 and 1.5×10^8 CFU/ml, as well as of 9.0×10^8 and 3.5×10^8 CFU/ml, no significant decrease in the number of *Bs* cells during long-term (up to 12 days) incubation of the mixtures was observed (data not presented). Under these conditions, the CFU number of the ultramicrobacteria increased (Fig. 10).

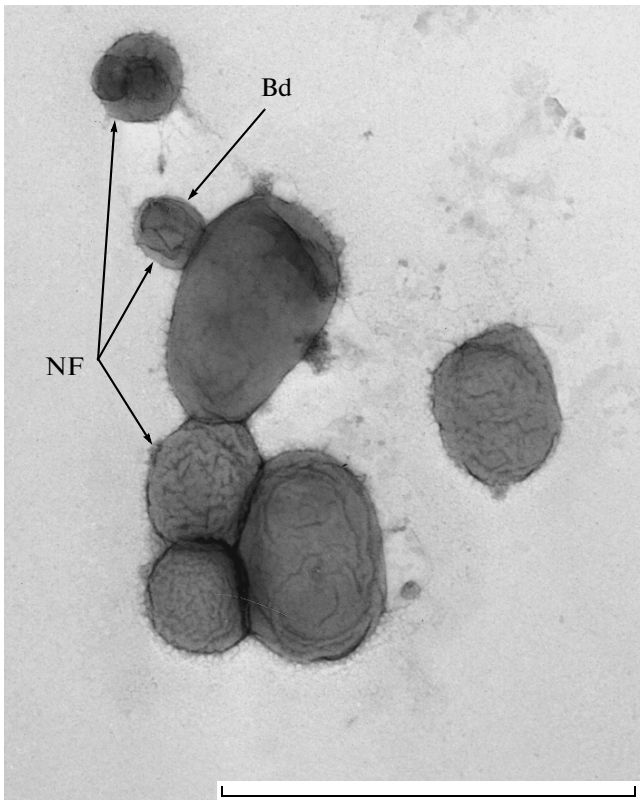

Fig. 2. Negatively stained cells from the 4-day culture of strain NF4 grown on 5/5 agar medium. Transmission electron microscopy. Ultrasmall coccoid cells (nanoforms) are indicated with arrows. Designations: NF, nanoforms; Bd, buds. Bar, 1 μm.

The results of phase contrast microscopy, as well as of fluorescence microscopy carried out using the two-component *LIVE/DEAD* reactant, demonstrated that, in binary suspensions, *Bs* cells (carrying attached UMB cells) remained viable for a long time; only some of them were destroyed (Fig. 7b). It should be noted that strains NF4 and NF5 were unable to lyse bacterial cell walls, which was determined in our experiments with dead cells of *Bs* and five other bacterial species. Under the same conditions, the bacteriolytic activity of the reference microorganisms *Bacillus* sp. XI and *Micrococcus luteus* B1813 was clearly demonstrated: the diameter of growth inhibition zones on agar with live *Pseudomonas putida* and *M. luteus* cells reached 9–15 mm. No lytic activity could be detected in cell-free UMB culture liquid. However, in binary cultures, pronounced inhibition of spore formation was observed (Fig. 11).

Noteworthy is that UMBs affected the process of *Bs* spore formation: about 60% of spores were found to be immature: they looked dark under the phase con-

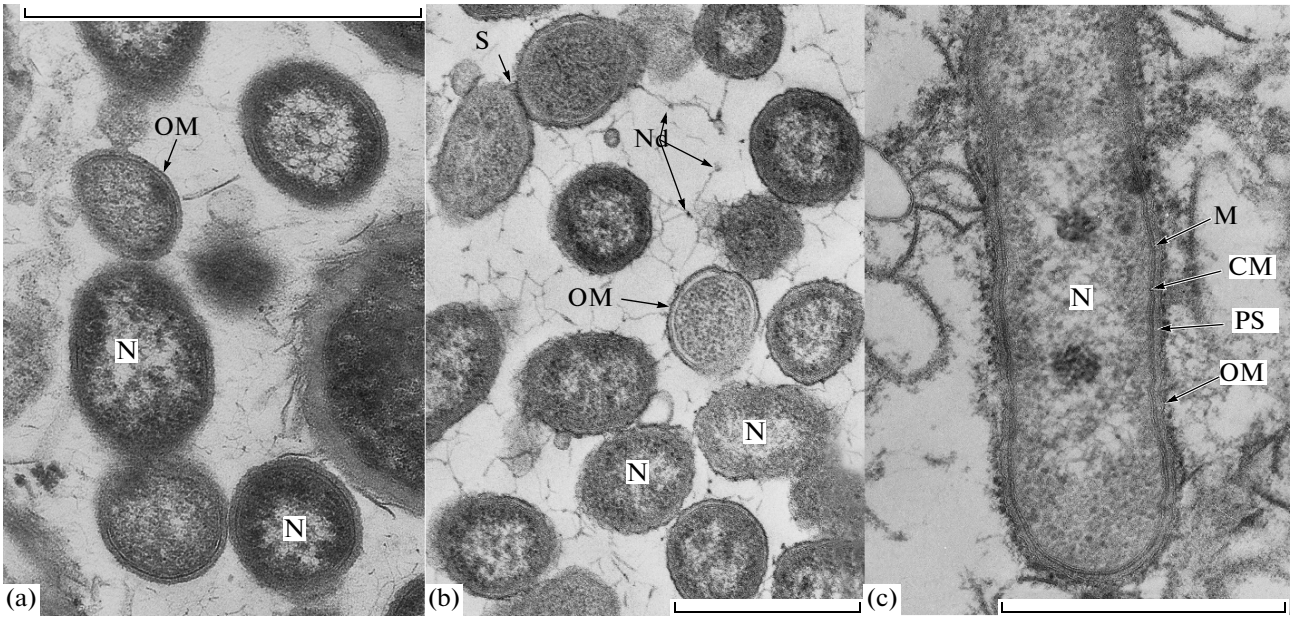

Fig. 3. Ultrathin sections of the strain NF4 cells, standard fixation. The microphotographs show (a) budding and constriction in a 24-h binary culture with *B. subtilis*, (b) polysaccharide filaments with nodules in a 24-h binary culture with *B. subtilis*, and (c) a rodlike cell in a 24-h culture. Designations: N, nucleoid; OM, outer membrane; Nd, nodules on the exocellular polysaccharide filaments; PS, periplasmic space; C, constriction; CM, cytoplasmic membrane; M, murein layer. The scale bars are 1 (a, b) and 0.13 μm (c).

trast microscope (Fig. 7c) and had a cortex and coats that were rudimentary (Fig. 8) compared to spores in the control *Bs* monoculture.

Experiments with other test cultures demonstrated that such a mandatory characteristic of epibiosis as adsorption of UMB cells on host cells was much less pronounced in these cases compared to the variant with *Bs* cells used as host cells: only a few cells of *B. sphaericus* and *S. aureus* had UMBs attached to their cell walls. Other bacterial species are probably more sensitive to the studied ultramicrobacteria; to check this, further studies are required. Thus, strains NF4 and NF5 can be considered facultative epibionts.

Taxonomic position of strains NF4 and NF5. Strains NF4 and NF5 are closely related to each other according to the DNA G + C base content (40.8 and 40.5 mol %, respectively), DNA–DNA homology (almost 100% DNA–DNA hybridization), and their 16S rRNA gene sequences. Most phenotypic properties of these isolates are similar as well. Therefore, these strains

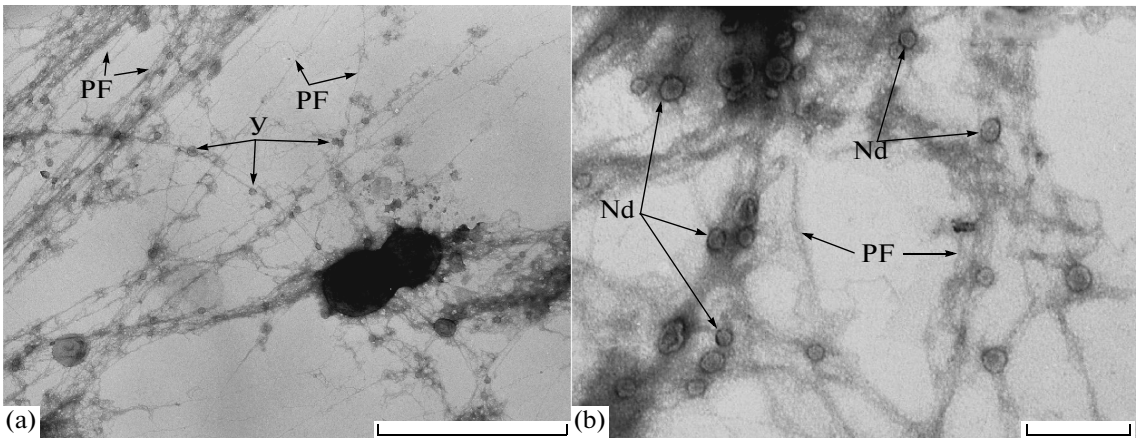

Fig. 4. Polysaccharide filaments with nodules. Intact cells from a 3-day culture of strain NF4. Whole-cell specimen, negative staining. Transmission electron microscopy. Designations: PF, polysaccharide filaments; Nd, nodules on polysaccharide filaments. The scale bars are 1 (a) and 0.2 μm (b).

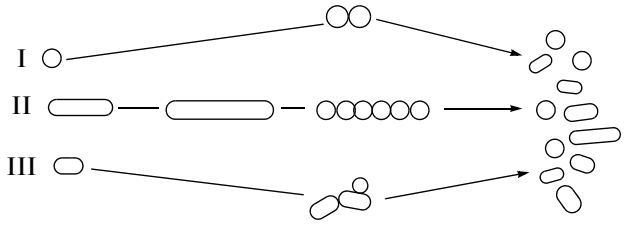

Fig. 5. Schematic depiction of the cell cycle of strains NF4 and NF5 and of the formation of coccoid and rodlike cells: I, division of spherical cells resulting in the formation of coccoid cells; II, septation of long rods and formation of coccoid cells; III, division of rodlike cells resulting in the formation of short rods.

undoubtedly belong to the same species. However, some differences were observed in the properties of the studied UMB isolates. For example, NF5 colonies have a more intense yellow-brown color and, unlike strain NF4 and excrete less polysaccharide slime; the strains differ in the range of utilized amino acids as well. According to their phenotypic properties, fatty acid profiles (Table 2), the DNA G + C contents, and 16S rRNA gene sequences, strains NF4 and NF5 are close to representatives of the genus *Chryseobacterium*: *C. solincola, C. antarcticum*, and *C. jeonii* (Fig. 12). Strains NF4 and NF5 differ significantly from the described representatives of the genus *Chryseobacterium* by their oligotrophy (they are able to utilize only a limited range of amino acids), ultrasmall cell size, a peculiar development cycle, and their capacity for epibiotic growth. The species affiliation of strains NF4 and NF5 requires further studies, including determination of the DNA–DNA hybridization level with the closest relative *Chryseobacterium solincola* CCUG 55604^T (soil inhabitant) [6]. The genome size of strains NF4 and NF5 is small (~1.7 Mb). However, it is so far impossible to compare the studied strains with other *Chryseobacterium* species in terms of this property because of lack of published data on the genome sizes of representatives of the genus *Chryseobacterium*.

DISCUSSION

Two strains, NF4 and NF5, of ultrasmall gram-negative bacteria were isolated from sediment of Lake Baikal and from oil sludge of the Nizhnekamsk oil-processing plant. According to their phenotypic and genotypic properties, these strains can be assigned to the genus *Chryseobacterium*.

The isolates exhibit the following unique properties.

1. They are capable of producing ultrasmall coccoid cells and short rods 0.2–0.4 μm × 0.2–0.5 μm); the cell volume ranges from 0.004 to 0.04 μm^3. A considerable proportion (30–60%) of cells are nanoforms (180–300 nm in diameter and 0.004–0.02 μm^3 in volume). On the basis of these data, the new isolates can be regarded as the smallest representatives of presently known ultramicrobacteria (with the exception of the pathogenic *Mycoplasma genitalium* and *Pelagibacter ubique*). Massive formation of these ultrasmall cells occurs in exponential-phase cultures. The physiologi-

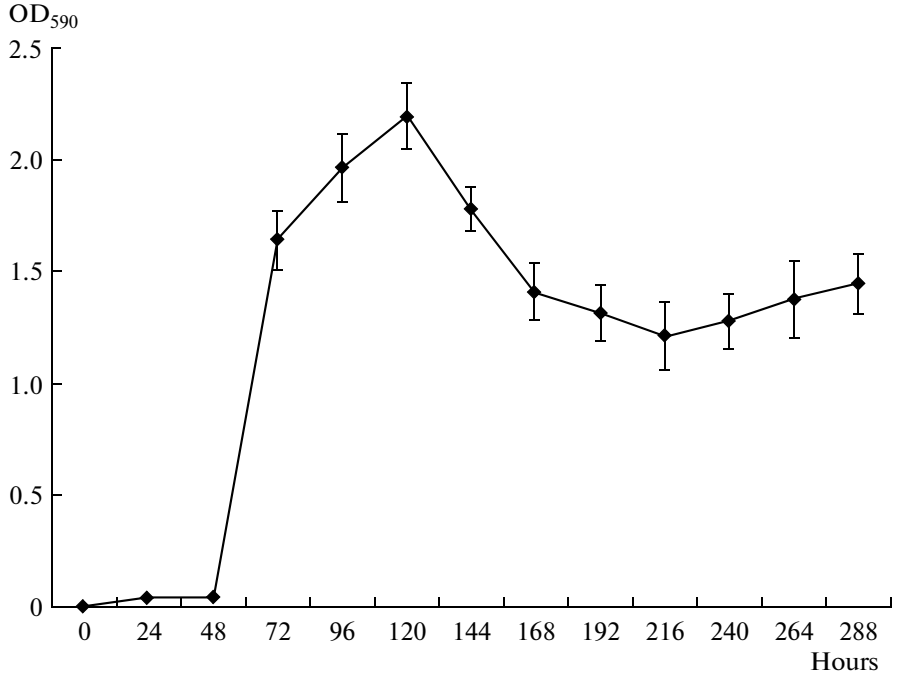

Fig. 6. Dynamics of growth (OD_{590}) of strain NF4 in liquid 5/5 medium with a complete set of nutrients at 28°C.

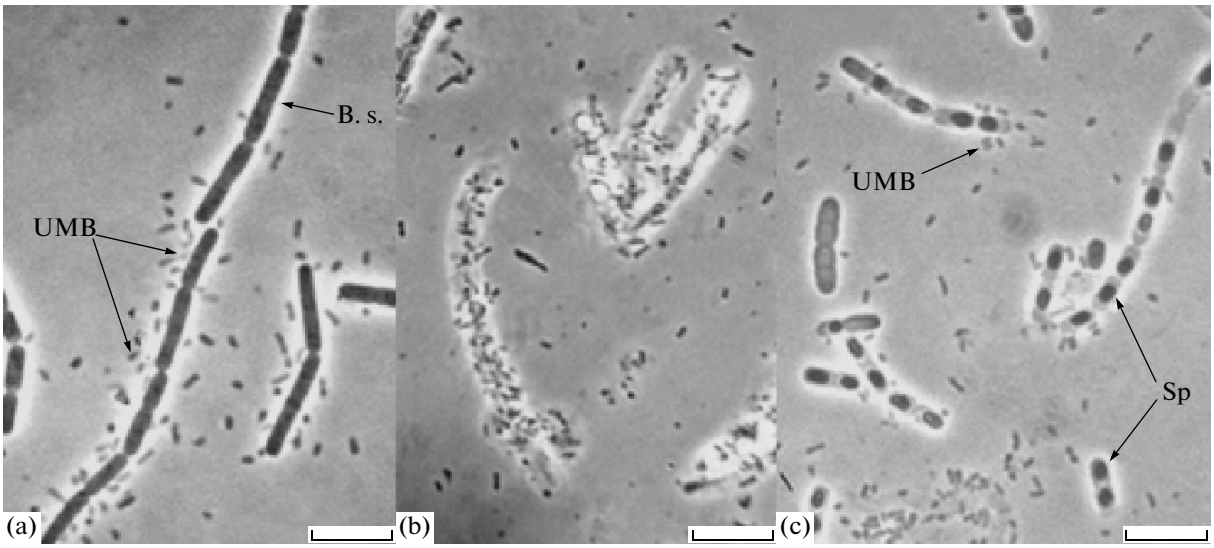

Fig. 7. Interacting cells in a binary culture of *B. subtilis* and NF4 incubated for (a) 20 min, (b) 7 days, and (c) 20 days. Phase-contrast microscopy. Designations: UMB, ultramicrobacteria of strain NF4; B. s., *B. subtilis*; Sp, *B. subtilis* spores. Bar, 10 μm.

cal activity of these cells, their active interaction with *B. subtilis* cells, should be noted. Rodlike cells developing during the lag phase of the NF4 and NF5 cultures are larger (~0.4–0.5 × 0.3–0.4 μm; the average cell volume is from 0.02 to 0.04 μm^3). Three morphotypes of strain NF4 and NF5 cells can be distinguished (Figs. 1 and 2). Elongated multinucleate rodlike cells developing during the lag phase may be regarded as multicellular forms, due to the fact that most of them split into several ultrasmall cells at the beginning of the logarithmic growth phase (Figs. 1b and 1c).

2. Strains NF4 and NF5 are highly oligotrophic: they are able to utilize only a limited range of amino acids as carbon and energy sources; no growth was detected on other organic compounds (with the exception of sodium citrate) (Table 1). In this connection, it should be noted that the studied isolates do not have extracellular depolymerases, judging from the fact that they are incapable of utilizing such polymeric organic compounds as proteins, lipids, and polysaccharides (cellulose, murein, agar, pectin). This determines their ecological niche. In this respect they are similar to oligotrophic microorganisms belonging to a specific ecologic–trophic group, dissipotrophs [20], the metabolism of which in nature depends on low-molecular-weight compounds formed owing to the extracellular hydrolases and lyases of hydrolytic microorganisms and dispersed in natural substrates. Among the described *Chryseobacterium* species, microorganisms exhibiting such properties are unknown. The range of organic compounds utilized by strains NF4 and NF5 is much narrower that used by strains NF and NF3 (facultatively parasitic ultramicrobacteria belonging to the genus *Kaistia* [12], whose genome size is ~2.4 Mb according to our data). The genomes of strains NF4 and NF5 are smaller (~1.7 Mb). Thus, among the compared facultatively parasitic ultramicrobacteria, there is a correlation between the ranges of substrates utilized, cell size, and genome size.

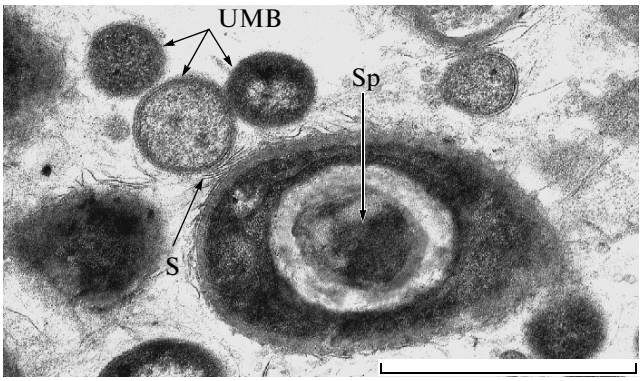

Fig. 8. An ultrathin section of cells from a binary culture of NF4 and *B. subtilis*: shown is a contact between the cell wall of an NF4 cell with the subunit layer of the *B. subtilis* cell wall. Standard fixation. Designations: UMB, ultramicrobacteria; S, subunit layer of the cell wall of *B. subtilis*; Sp, a spore of the *B. subtilis* cell. Bar, 0.5 μm.

3. The studied isolates have demonstrated the capacity for epibiosis, i.e., the ability to grow firmly attached to *B. subtilis* cells (and, probably, other bacteria). Epibiosis, a form of symbiosis that involves direct intercellular contacts, does not always result in dramatic inhibition of growth of host cells or their

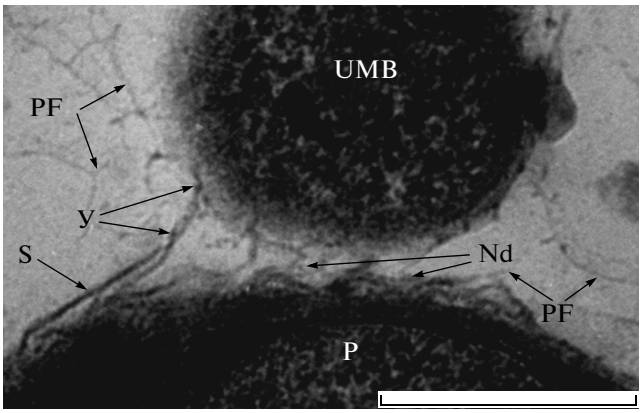

Fig. 9. An ultrathin section of interacting cells in a binary culture of NF4 and *B. subtilis* culture. The specimen was stained with ruthenium red. Polysaccharide filaments with nodules adsorbed on the S-layers of the *B. subtilis* cell wall are shown. Designations: UMB, ultramicrobacteria (NF4); PF, polysaccharide filaments; Nd, nodules on polysaccharide filaments; P, prey cell (*B. subtilis*). Bar, 3 μm.

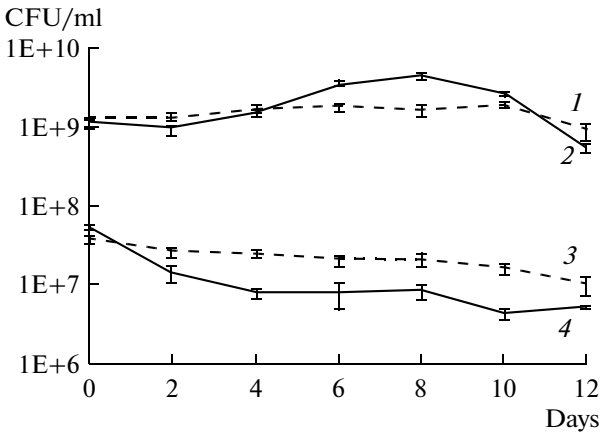

Fig. 10. Growth dynamics of the ultramicrobacterium NF4 (*1, 2*) and *B. subtilis* (*3, 4*) in mono- (*1, 3*) and binary (*2, 4*) cultures in phosphate buffer.

death, which is only characteristic of typical bacterial predators, periplasmic parasites from the families *Bacteriovoracaceae* [21–23] and *Vampirovibrio* [24, 25]. For example, the archaeon *Nanoarchaeum equitans*, the genomic characteristics of which are similar to those of parasites [26], produces no inhibitory effect on host cultures [27]. Our studies demonstrated that, under certain conditions, our UMB isolates, strains NF4 and NF5, exert an inhibitory effect on *Bs* cells in binary cultures, which manifests itself in a decrease in the number of host *Bs* cells and considerable (by 50%) inhibition of spore formation (Figs. 7c, 10, and 11). It seems likely that the patterns of interaction between the studied UMB and *Bs* cells depend on the cell density and ratio in binary cultures. For example, as was mentioned above, in binary culture with the initial cell concentrations of *Bs* and NF4 cells of 3.8×10^7 and 1.2×10^9 CFU/ml, respectively, the *Bs* CFU number decreased by five to six times after 3–5 days of interaction (Fig. 10), whereas the CFU number of the studied ultramicrobacteria (NF4) increased by three to four times under these conditions. However, in binary cultures with the initial concentrations NF4 and *Bs* cells of 1.0×10^9 and 1.5×10^8 CFU/ml, respectively, as well as of 9.0×10^8 and 3.5×10^8 CFU/ml, no significant decrease in the *Bs* CFU number was observed over long-term (up to 12 days) incubation of the mixture.

Epibionts with a such a lifestyle are to be considered parasites if we use definitions of classical parasitology, according to which one organism (parasite) can use another organism (host) as a habitat (first-order habitat) or a food source, making it responsible for the parasite's interactions with the external environment (second-order habitat) [28, 29].

The results of our study suggest that strains NF4 and NF5 can use *Bs* cells as first- and second-order habitats. The advantages of existence in the state of epibiosis (attachment to a host cell) are as follows: (1) NF4 and NF5 cells attached to *Bs* cells exist in a different microenvironment; (2) as nonmotile microorganisms, they are able to move around using the host cell motility (bacterial "free riders"); (3) adsorbed NF4 and NF5 cells are at the epicenter of the hydrolytic activity of host cells and of the excretion of metabolites produced by living *Bs* cells. This fact is especially important for strains NF4 and NF5, since they are able to utilize a limited range of amino acids as growth substrates. These amino acids arise in the

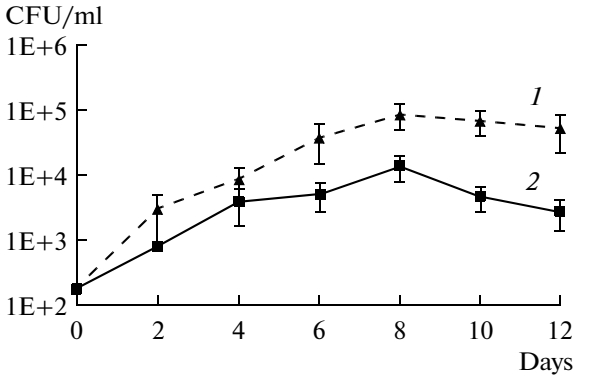

Fig. 11. Dynamics of spore formation by *B. subtilis* in (*1*) mono- and (*2*) binary cultures (in the presence of strain NF4) in phosphate buffer.

Table 1. Comparison of the characteristics of strains NF4 and NF5 and of close *Chryseobacterium* species

Characteristics	NF4	NF5	*C. antarcticum*	*C. jeonii*	*C. solincola*
Isolation source	Sediment (Lake Baikal)	Oil sludge (Nizhnekamsk)	Soil (Antarctica)	Moss (Antarctica)	Hydrocarbon-contaminated soil (eastern Algeria)
Morphology and cell sizes (μm)	Short rods and coccoid cells, 0.2–0.4 × 0.2–0.5*	Short rods and coccoid cells, 0.2–0.4 × 0.2–0.5*	Rods, 1.0–3.1 × 0.4–0.5	Rods, 1.0–3.1 × 0.4–0.5	Filaments, 12–80 × 0.5–1; rods, 2–4 × 0.5–1; short rods, 0.5–0.7 × 0.9–1.5
Growth:					
on cetrimide agar	–	–	–	–	ND
on MacConkey agar	–	–	–	–	–
Temperature range for growth	5–30°C optimum at 28°C	5–30°C optimum at 28°C	4–28°C optimum at 18.9°C	5–31°C, optimum at 21.5°C	4–37°C optimum at 28–30°C
pH range for growth	6–9 optimum at 7–8	6–9 optimum at 7–8	6–11 optimum at 7–8	6–11 optimum at 7–8	5–11 optimum at 7.3–8
At different NaCl concentrations in the media	0–1%, optimum at – 0%	0–1%, optimum at – 0%	0–3%, optimum at – 0%	0–3%, optimum at – 0%	0–6%, optimum at – 4%
Hydrolysis:					
Twin-80	±	–	+	+	–
Twin-40	–	–	ND	ND	ND
cellulose	–	–	–	–	–
esculin	±	±	+	+	–
casein	–	–	+	+	–
starch	+	+	+	+	–
gelatin	–	–	+	+	–
Destruction of bacterial cell walls	–	–	ND	ND	ND
Enzymatic activities:					
catalase	+	+	+	+	–
oxidase	+	+	+	+	–
β-galactosidase	–	–	–	–	–
arginine dihydrolase	–	–	–	–	+
urease	–	–	–	–	+
gelatinase	–	–	+	+	+
DNase	+	+	+	+	–
nitrate reductase	–	–	–	–	+
Indole production	+	+	+	+	–
H_2S production	–	–	–	–	–
Acetoin production	±	–	ND	ND	–
Production of acids from:					
D-glucose	+	+	+	+	+
D-mannitol	–	–	–	–	–
inositol	–	–	–	–	–
D-sorbitol	–	–	–	–	–
L-arabinose	–	–	–	–	–
D-maltose	+	+	+	+	–
esculin	±	±	+	+	–
λ_{nm} of absorption of carotenoid pigments	459 (major), 487	459.4 (major), 488	452 (major), 480	452 (major), 480	ND
Reaction to oxygen	Aerobes, capable of growth under microaerobic conditions (5–15% O_2 and 5–12% CO_2)	Aerobes, capable of growth under microaerobic conditions (5–15% O_2 and 5–12% CO_2)	Aerobes, capable of growth under microaerobic conditions (5–15% O_2 and 5–12% CO_2)	Aerobes, capable of growth under microaerobic conditions (5–15% O_2 and 5–12% CO_2)	Strict aerobes
G + C, mol %	40.8	40.5	34	36	40.9

Note: ND, no data;
±, weak reaction;
* volume: 0.004–0.04 μm^3;
–, negative results.

medium due to the activity of *Bs* proteases. It should also be noted that *Bs* cells are able to produce various amino acids on media without organic nitrogen. Utilization of these amino acids may be of vital importance to the studied ultramicrobacteria developing in natural environments (soils, sediments, and water) where concentrations of amino acids are extremely low. However, the character of relationships between these pairs of microorganisms in natural environments requires further study. It is probable that, in natural habitats, such bacteria as NF4 and NF5 are most active in biofilms, microcolonies, and other aggregates consisting of microbial cells susceptible to parasites. For example, it was shown that the bacterial predators *Bdellovibrio bacteriovorus* and *Micavibrio aeruginosavorus* are able to actively destroy biofilms consisting of pathogenic bacteria susceptible to these parasites [30, 31].

The strength of attachment of NF4 and NF5 cells to host cells can obviously be attributed to the specific adhesive apparatus of these ultramicrobacteria, consisting of polysaccharide nets with sticky nodules (granules) on the polysaccharide filaments (Figs. 4a, 4b, and 9). The appearance, structure, and size of the constituents of this apparatus are similar to those of the apparatus that we previously described in *Kaistia* strains, NF1 and NF3, which are members of the class *Alphaproteobacteria* [14, 19]. The difference is that NF4 and NF5 cells lack cone-shaped protrusions typical of NF1 and NF3 cells and involved in cell adhesion to host cells as described in [12, 14, 32]. A schematic depiction of interactions between strains NF4 and NF5 cells and host cells is shown in Fig. 13. The discovery of this apparatus and of the epibiosis phenomenon in representatives of another phylum of gram-negative bacteria, *Bacteroidetes*, put forward several questions concerning the evolution. The results that we present broadens the knowledge on the biodiversity of bacterial epibionts/parasites, as well as on bacteria of the genus *Chryseobacterium*.

It should be noted that the isolates that we describe in the present work are not the first ultramicrobacteria described as members of the genus *Chryseobacterium*. Several UMB strains belonging to the genus *Chryseobacterium* have recently been isolated from a glacier in Greenland and described in [8]. However, comparison of these strains with strains NF4 and NF5 is hindered due to the fact that the paper on the Greenland glacier isolates does not provide a detailed description of their phenotypic and genotypic properties.

ACKNOWLEDGMENTS

We thank E.N. Detkova for determination of the DNA G + C content, E.N. Kaparullina for determination of the pigment composition, and B.P. Baskunov for help in determination of the menaquinones of the studied bacteria.

Table 2. Fatty acid composition of cells of strain NF4 and closely related species of the genus *Chryseobacterium* (% of total FA)

Fatty acids	Strain NF4	*C. solincola*	*C. antarcticum*	*C. jeonii*
$C_{10:0}$	0	*ND	ND	ND
$C_{12:0}$	0	ND	ND	ND
iso-$C_{12:0}$	ND	ND	Tr	1.0
iso-$C_{13:0}$	ND	ND	2.5	2.9
anteiso-$C_{13:0}$	ND	ND	3.2	3.6
*ECL 13.566	ND	ND	*Tr	Tr
$C_{14:0}$	Tr	ND	ND	ND
$C_{14:0}$ 3-OH	Tr	ND	ND	ND
iso-$C_{14:0}$	1.1	2.0	1.5	5.0
iso-$C_{14:0}$ 3-OH	Tr	ND	ND	ND
$C_{15:0}$	0	ND	2.6	1.5
$C_{15:0}$ 2-OH	ND	1.4	1.9	1.9
iso-$C_{15:0}$	23.2	14.0	13.6	12.2
iso-$C_{15:0}$ 2-OH	0	ND	ND	ND
iso-$C_{15:0}$ 3-OH	4.3	ND	1.0	1.3
anteiso-$C_{15:0}$	38.4	41.4	15.2	24.2
anteiso-$C_{15:0}$ 3OH	5.2	ND	ND	ND
iso-$C_{15:1}$	1.1	ND	ND	ND
anteiso-$C_{15:1}$ A	ND	2.4	6.6	0
$C_{16:0}$	2.3	ND	0	0
$C_{16:0}$ 3-OH	ND	ND	0	0
iso-$C_{16:0}$	3.0	7.2	2.8	5.7
iso-$C_{16:0}$ 3-OH	2.5	4.2	5.1	9.0
$C_{16:1}$ ω5	0	ND	ND	ND
$C_{16:1}$ ω7	0	ND	ND	ND
$C_{16:1}$ ω9c	Ñë	ND	ND	ND
iso-$C_{16:1}$ H	1.1	2.8	3.6	9.1
ECL 16.580	ND	ND	Tr	Tr
iso-$C_{16:1}$ ω3c	ND	1.3	ND	ND
$C_{17:0}$ 2-OH	ND	5.7	3.3	2.3
iso-$C_{17:0}$	ND	ND	0	0
iso-$C_{17:0}$ 3-OH	2.5	5.2	5.6	4.4
anteiso-$C_{17:0}$ 3OH	2.2	ND	ND	ND
iso-$C_{17:1}$	9.1	ND	ND	ND
iso-$C_{17:1}$ ω9c	ND	9.0	21.3	8.6
anteiso-$C_{17:1}$ ω9c	ND	1.8	2.5	1.9
$C_{18:0}$	2.5	ND	ND	ND
$C_{18:1}$ ω5c	ND	ND	1.5	Tr
$C_{18:1}$ ω9	0	ND	ND	ND
$C_{18:1}$ ω7c	0	ND	ND	ND

Note: ND, no data; Tr, trace amounts, less than 1%; ECL, unknown acid

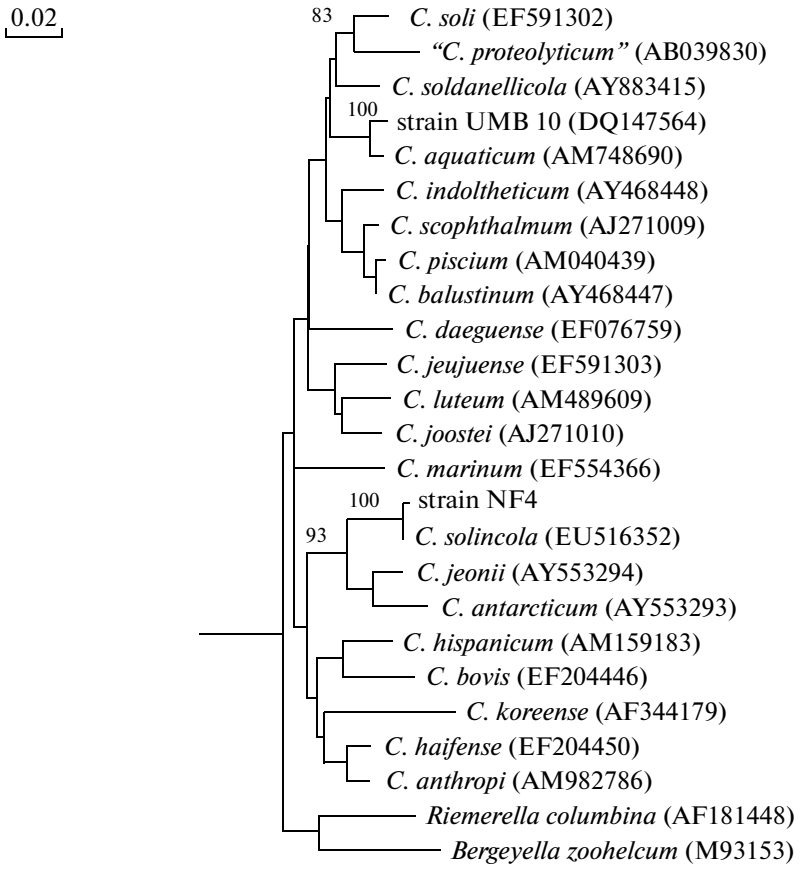

Fig. 12. Phylogenetic tree showing the position of strain NF4 among the close species of the genus *Chryseobacterium*.

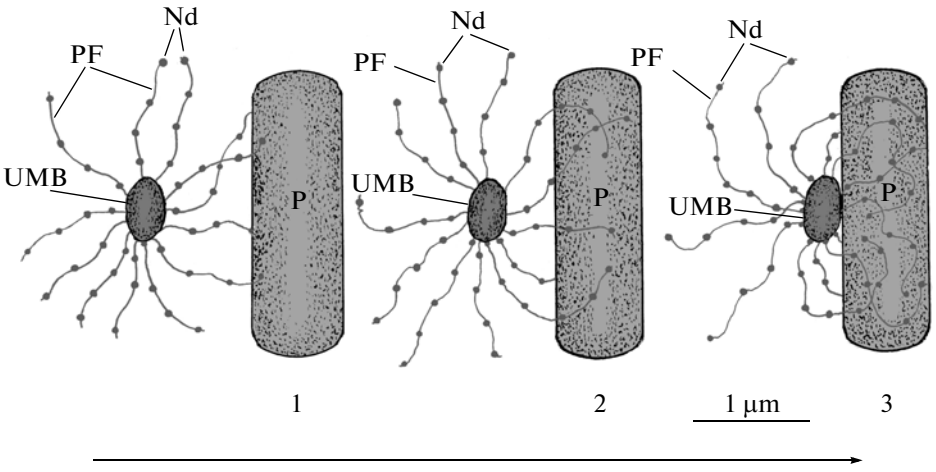

Fig. 13. Schematic depiction of the mechanism responsible for cohesion of NF4 and *Bacillus subtilis* cells. The mechanism is used by predator ultramicrobacteria and based on the use of a net of polysaccharide filaments (PF) with sticky granules (nodes) (Nd). (1) first stage of cohesion: sticky Nd capture the prey; (2) second stage of cohesion: NF4 cell approaches the *Bs* cell by consecutive use of many sticky nodules; polysaccharide filaments act as cords that pull the predator cell (UMB) to the prey cell (P); (3) tight coupling of the cells via adhesion of the cell wall of the predator to that of the prey cell. Designations: UMB, a cell of the ultramicrobacterium NF4; PF, polysaccharide filaments; Nd, nodules on polysaccharide filaments; P, prey cell (*B. subtilis*).

This work was supported by the Analytical Departmental Targeted Program "Development of the Scientific Potential of Higher Education" of the Ministry of Education and Science of the Russian Federation, project no. 2.1.1/10937, and Federal Targeted Program "Scientific and Pedagogic Personnel of Innovative Russia," GC no. 16.740.11.0481.

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