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Original papers

# Applying of bacteria *Cellulosimicrobium* sp. for cultivation protozoa of genus *Acanthamoeba*

**Volodymyr Shyrobokov[1], Vadym Poniatovskyi[1], Anastasiia Chobotar[1], Rusłan Sałamatin[2,3]**

[1]Department of Microbiology, Virology and Immunology, Bogomolets National Medical University, Peremogy av. 34, 03056 Kyiv, Ukraine
[2]Department of General Biology and Parasitology, Medical University of Warsaw, Chałubińskiego 5, 02-004 Warsaw, Poland
[3]Department of Parasitology and Vector-Borne Diseases, National Institute of Public Health – National Institute of Hygiene, Chocimska 24, 00-791 Warsaw, Poland

Corresponding Author: Vadym Poniatovskyi; e-mail: v.poniatovskyi@gmail.com

**ABSTRACT.** The aim of this study was identification features of cultivation representatives of genus *Acanthamoeba* isolated from bentonite using *Cellulosimicrobium* sp. as a bacteria-feeders. Identification of isolated bacteria was conducted by morphological, cultural and molecular-genetic methods. The cultivation of free-living „bentonite" amoeba on the lawn of *Cellulosimicrobium* sp. have gained significant advantages than using *Escherichia coli* as a bacteria-feeders was shown. "Bentonite" amoeba form crateroid plaques, which fit to the quantitative characteristic materials which contains amoeba, during deep co-cultivation *Acanthamoeba* sp. and *Cellulosimicrobium* sp. on 1% glucose meet-peptone agar.

**Keywords:** cultivation, *Cellulosimicrobium* sp., *Acanthamoeba* sp., bentonite

## Introduction

Bacterial consortia are one of the main components of soils ecosystems. These bacterial groups have complicated relationships with other ecosystems of soil. One of the factors which affects on quantitative microorganisms in soil are protozoa [1].

Protozoa of genus *Acanthamoeba* are spread almost ubiquitous. They were isolated from soil, water of different origin, air and other objects [2,3]. In some cases, *Acanthamoeba* sp. can cause diseases in humans and belong to group opportunistic microorganism. The cases of amoebic keratitis as a result of liquid, for saving lenses, contaminating [4], granulematous amoebic encephalitis, fatal disease of central nervous system [5], skin lesions an sinusitis in HIV-positive patients [6,7].

One of the ecological systems variants, which inhabiting eukaryoto-prokaryotic consortia, are sedimentary fine-grained rocks of silicate groups formed as a result of the destruction of rocks during weathering [8]. Clay minerals consist of layers containing silicon oxide tetrahedra and alumo-hydroxyl octahedrons. These layers are combined into elementary packages, the aggregate of which forms a mineral particle. One of the important groups of clay minerals, which has excellent sorption properties is a group of montmorillonite (smectite, nontronite, beidelite, etc.). This group contains three-layer packages of the type tetrahedron-octahedron tetrahedron. The connection between the packets is weak, as a result, they are easily dispersed, in the inter-package space penetrates the water and the mineral strongly swells. Minerals of this group are distinguished by high capacity of cation exchange [9].

## Materials and Methods

**Collection of samples and cultivation.** Amoebae were isolated from different bentonite clay deposits in Ukraine (Cherkasy, Transcarpathian and Crimean deposits). Bacteria *Cellulosimicrobium* sp. were isolated also from bentonite of Crimean deposit. Furthermore, amoebae were cultivated using nutrient agar (peptic digest of animal tissue – 5 g/l, meat extract – 1.5 g/l, yeast extract – 1.5 g/l, sodium chloride – 5.0 g/l; dextrose 10 g/l) with previously inoculated bacteria-feeders *Cellulosimicrobium* sp., strain bent-1. Cultivations were carried out in Petri dish during 5 days at 35ºC [10].

**Morphological identification.** Morphology of isolated from bentonite bacterium *Cellulosimicrobium* sp. was investigated using electronic (electronic microscope JEM-100CX), light and phase-contrast (microscope Carl Zeiss Axioplan) microscopy and different ways of staining.

**DNA extraction, amplification and sequencing.** Extraction of DNA was performed with pure cultures which were grown on 1% dextrose MPA for 24 hours. DNA was extracted by adsorption of silica by Boom et al. [11]. PCR amplification of 16S rRNA gene of bacteria-feeders was performed using universal primers: 27F 5'>AGAGTTTGATCMTGGCTCAG <3' and 1492R (s) 5'>GGTTACCTTGTTACGACT T<3'. PCR amplification program included a prolonged denaturation for 5 minutes at 95°C; 30 cycles at 95°C during 40s, 50°C during 40s, 72°C during 90s; final elongation at 72°C during 7 minutes. The total sample volume was 25 µl. The mixture contained 2 µl of isolated DNA, 1 unit of Taq DNA Polymerase, 0,2 mM of each dNTPs, 1× PCR buffer with 2,5 mM $MgCl_2$, 10 pm of each primer [12]. Analysis of amplified DNA fragments was performed by separation of DNA fragments in 1.5% agarose gel, with ethidium bromide as intercalating agent. DNA isolation from agarose gel was carried out using «Gel-Out izolacja DNA z żeli agarozywych» reagent package (©Kucharczyk Techniki Elektroforetyczne, Poland), according to the manufacturer's instructions. Amplified PCR products were sequenced using apparatus ABI3730 Genetic Analyzer (Institute of Biochemistry and Biophysics, Polish Academy of Sciences).

**Gene identification and phylogenetic analysis.** The analysis of the received sequence was carried out using the BLAST information system. The nucleotide sequences of the homologous gene fragments of the genus *Cellulosimicrobium* were obtained from GenBank for phylogenetic analysis. Multiple alignment of received sequences and sequences of the 16S rRNA gene from the data bank and construction of a phylogenetic tree were carried out using MrBayes 3.2.6 [13,14].

**Numbers in GenBank.** The resulting nucleotide sequences *Cellulosimicrobium* sp. are deposited in the GenBank under the number MH517543.

## Results and Discussion

We have isolated the original "bentonite" amoeba, when studied the bentonite clay of different fields. They were assigned to the genus *Acanthamoeba* by using a series of classical microbiological methods and sequencing of 18S RNA genes [10]. These amoebae were cultivated monoxenically on generally accepted systems with previously inoculated of *Escherichia coli*, *Enterobacter aerogenes* [15,16] but this technique showed poor reproducibility. Further detailed study of microbial inhabitants of bentonite allowed to isolate a strain of bacteria used by these amoeba as a food substrate. The growth of amoebae was accompanied by a creeping vitreous lysis of the lawn of microorganisms, starting from the second day of cultivation (Fig. 1).

This strain of microorganisms was named bent-1. Isolate of bacteria managed to select from the Kurtsivske field of bentonite clay of Ukraine by direct inoculation of mineral samples on the nutrient

Figure 1. Growth of *Acanthamoeba* sp. strain Cherkasy on glucose MPA with previous inoculation of bacteria *Cellulosimicrobium* sp. strain bent-1

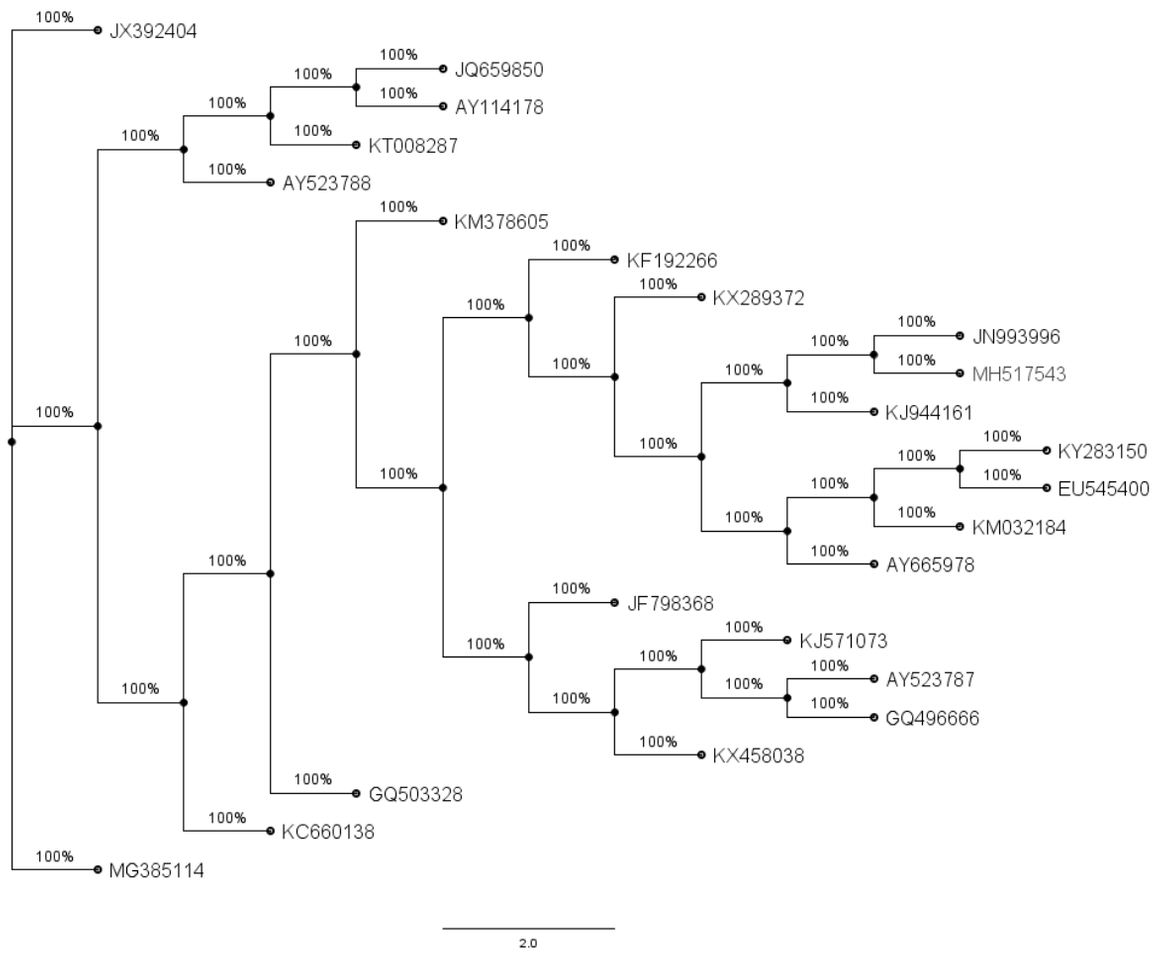


Figure 2. Bayesian inference tree based on fragment of sequences obtained at the small-subunit rRNA gene (SSU rDNA) of *Cellulosimicrobium* sp. isolates, performed using MrBayes 3.2.6.

medium for the cultivation of microorganisms with the addition of glucose (HiMedia). A number of morphological, cultural and biochemical characteristics, along with sequencing of 16S RNA gene were used to characterize the strain bent-1.

The determination of the genetic nature of isolated bacteria was conducted using sequencing in the first stage of the experiments. It has been shown that the experimental microorganisms belong to the genus *Cellulosimicrobium* using BLAST (Basic Local Alignment Search Tool). In addition, the EMBL database was also used. When comparing the obtained nucleotide sequence and the database sequences were shown investigated bacteria have genetic similarities to 99% with bacteria: *Cellulosimicrobium cellulans* and *Cellulosimicrobium funkei* (Fig. 2). Bacteria, were isolated from the bentonite, are located in the vicinity of the strain *Cellulosimicrobium* sp. *L403* (GenBank: KJ944161) on a phylogenetic tree that reflect evolutionary history. Strain *Cellulosimicrobium* sp. *L403* was isolated by Guo X. (Nanjing Agricultural University, China) from biotite, which is a mineral of a silicates class. This is another indication that *Cellulosimicrobium* sp. strain bent-1 refers to so-called autochthonous bentonite microflora.

The name *Cellulosimicrobium* was firstly proposed by Schumann et al. [17]. This genus of microorganisms still remains taxonomically unstable and was repeatedly reclassified from genera *Cellulomonas*, *Oerskovia*, *Brevibacterium* and *Arthrobacter* [17,18]. Today it contains three species of microorganisms: *Cellulosimicrobium funkei*, *Cellulosimicrobium cellulans* and *Cellulosimicrobium terreum* [19]. The closest phylogenetic similarities to the strain bent-1 are strains *Cellulosimicrobium cellulans* and *Cellulosimicrobium funkei*, similarity of their nucleotide sequence of 16S rRNA gene is 99.5–99.8% [20]. Therefore, for establish the accurate taxonomic position of the isolated strain by us, the genetic analysis supplemented the definitions of a number of biochemical properties.

*Cellulosimicrobium* sp. strain bent-1 is gram-

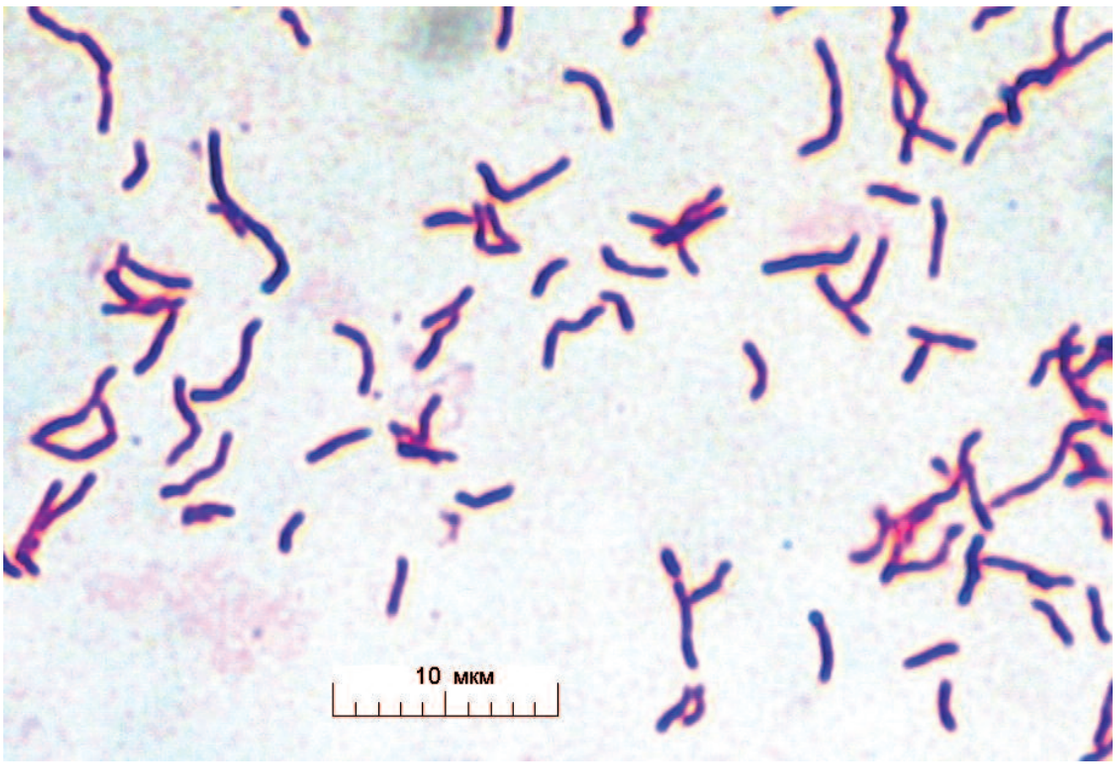


Figure 3. Bacteria *Cellulosimicrobium* sp. strain bent-1, which are used for the cultivation of amoebae

Table 1. Biochemical characteristic of *Cellulosimicrobium* sp. strain bent-1

| Characteristic | *Cellulosimicrobium* sp. bent-1 |
|---|---|
| Haemolytic properties | + (α- hemolysis) |
| Pigmentation | + |
| Nitrate recovery | – |
| Utilization of carbohydrates | |
| Glucose | + |
| D-mannit | – |
| Saccharose | + |
| L-arabinose | – |
| Lactose | + |
| Maltose | – |
| Dulcit | – |
| D-Sorbitol | – |
| Salicin | + |
| Raffinose | – |
| Mannose | – |
| Catalase activity | + |
| Hydrolysis: | |
| Starch | + |
| Casein | + |
| Gelatin | + |
| Growth within pH | 6.0–10.0 |
| Temperature range of growth | 18–40°C |
| Formation of indole | – |
| Formation of hydrogen sulfide | – |

positive, rod-shaped, can also be coccoid, not acid-resistant microorganisms that don't form spores (Fig. 3). In young cultures, they can form a primitive mycelium. Frequently these microorganisms are isolated from the soil. They are not pathogenic to humans, although they can sometimes cause disease in people with immunodeficiency [21,22].

The bacteria strain bent-1 grows well on organic media with the formation of S-shaped colonies (round, convex, smooth, shiny). They are optional anaerobes in relation to oxygen. Catalase-positive, have a number of hydrolytic and proteolytic properties (Table 1). Growth within the pH range from 6.0 to 10.0. It forms a pigment of yellow color on a nutrient agar. It shows growth in the temperature range of 18–40°C (range of observation). It does not form indole, hydrogen sulfide.

The phenomenon of plaque formation with crater-like depressions and growth in agar were observed at simultaneous inoculation of amoeba and bacteria-feeders in the layer of agar (Fig. 4).

Brook et al. [23] described the social behavior of amoebae in the form of primitive farming.

This phenomenon was also observed in our case with prolonged cultivation of representatives of the genus *Acanthamoeba*. „Bentonite" amoebae exhibited a primitive variant of the farm symbiosis, which was expressed in the following: in the

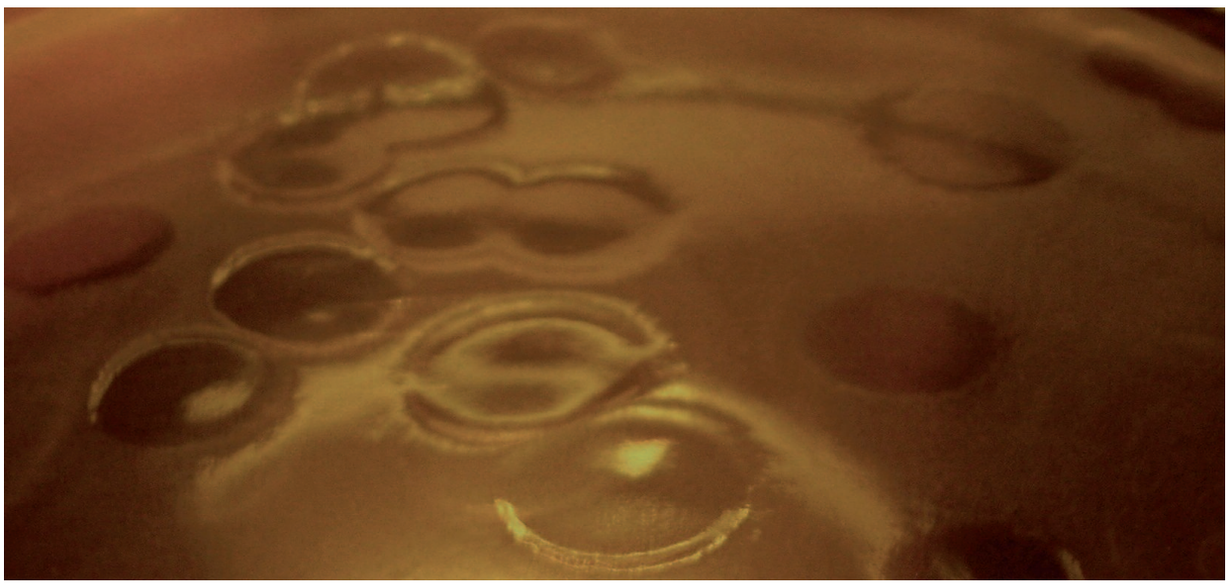

Figure 4. Plaque formation by „bentonite" amoebae

presence of a large number of bacteria *Cellulosimicrobium* sp. in the nutrient medium, amoeba forms plaques that are able to grow over time (Fig. 5a and b). After almost complete disappearance of bacteria from the media, amoeba is planted from its fetal bodies bacteria *Cellulosimicrobium* sp. and stimulate their growth. When enough bacteria accumulate, amoebae are used them again as a food ingredient (Fig. 5 c).

This phenomenon has been repeatedly confirmed in the long-term cultivation of amoebae.

The presence of close symbiotic relationships between bacteria *Cellulosimicrobium* sp. strain bent-1 and the protozoa genus of *Acanthamoeba* shows to their joint evolutionary development. As known, the protozoa are able to interact intensively with bacteria, if they are in the same ecosystem [24]. For example, Bjørnlund et al. [25] was shown that the presence of the protozoa influences the amount of bacteria *Arthrobacter sp.* in the ecosystem.

Thus, the experimental studies carried out for the first time showed the presence of symbiotic relationships between representatives of the bacterial world *Cellulosimicrobium* sp. and amoebae of the genus *Acanthamoeba* by the type of commensalism. Furthermore, this allows us to make conclusion about using these bacteria for the cultivation of free-living amoebae in laboratory conditions.

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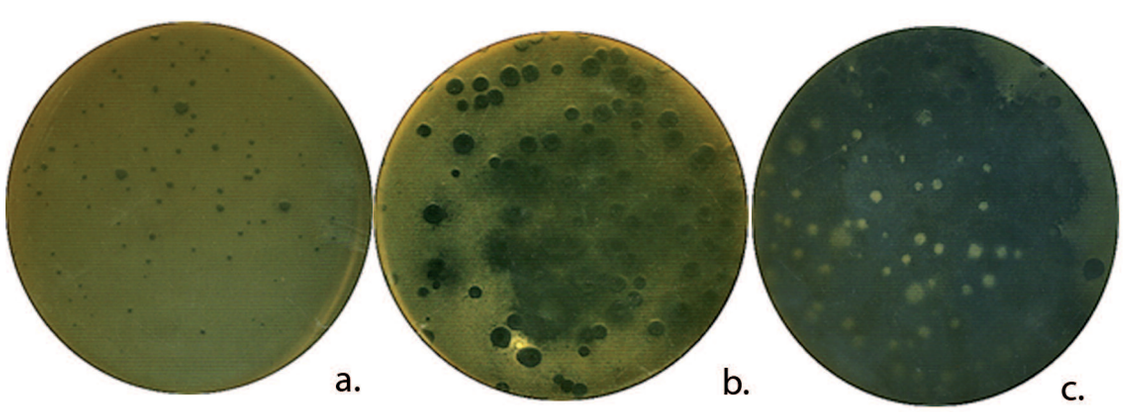


Figure 5. Plaques „growth" on nutrient agar for cultivation of bacteria: a. – 3rd day of cultivation; b. – 7th day of cultivation; c. – 14th day of cultivation

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