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EXPERIMENTAL ARTICLES

Thermophilic Chitinolytic Microorganisms of Brown Semidesert Soil

N. A. Manucharova[a,1], A. N. Vlasenko[a], T. P. Tourova[b], A. N. Panteleeva[b], A. L. Stepanov[a], and G. M. Zenova[a]

[a] *Faculty of Soil Science, Moscow State University, Moscow, Russia*
[b] *Winogradsky Institute of Microbiology, Russian Academy of Sciences, pr. 60-letiya Oktyabrya 7, k. 2, Moscow, 117312 Russia*

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Abstract—In brown semidesert soil, thermophilic prokaryotic organisms identified as *Streptomyces roseolilacinus* and *Silanimonas lenta* were shown to play the main role in chitin transformation at 50°C. The phylogenetic positions of the isolated dominant chitinolytic microorganisms were determined on the basis of 16S rRNA gene sequencing. The consumption of chitin as a source of carbon and nitrogen by both the bacterium and the actinomycete was evident from considerable biomass accumulation, high emission of carbon dioxide, and presence in the medium of the chitinase exoenzyme.

Key words: chitinolytic microorganisms, thermophily, biopolymer transformation, ecology.

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Temperature is viewed by modern ecology as one of the most important autoecological factors affecting the growth and activity of microorganisms. In the present-day literature, it is noted that intense synthesis of chitin microfibrils and thickening of the cell wall, a shield protecting the cell, occur in fungi with a rise in temperature, upon irradiation, and in the presence of other negative factors [1]. On the other hand, the effect of temperature on microorganism growth is related to its immediate influence on the rate of chemical reactions. The highest activity of many enzymes is observed at elevated temperatures. In particular, endochitinase, an enzyme synthesized by both prokaryotes (the gram-negative bacterium *Serratia marcescens* [2]) and eukaryotes (fungi [3]), exhibits maximum activity at 55 and 40°C, respectively. As a result of anthropogenic impacts, e.g., development of hot water supply systems, extension of the zones of activity of thermotolerant and thermophilic microorganisms in soils occurs [4]. In addition, thermotolerant and thermophilic microorganisms attract increasing attention in connection with changes in the temperature regime of the biosphere due to increasing thermal contamination caused by the development of civilization. Given the ability of the microbial communities to switch from one trophic route to another, depending on the conditions, the question of the contribution of different groups of microorganisms to degradation of biopolymers (chitin, in particular) in soils influenced by high temperatures appears to be urgent.

[1] Corresponding author; e-mail: manucharova@mail.ru

The aim of this work was to study the thermophilic chitinolytic microbial complex of the brown semidesert soil.

MATERIALS AND METHODS

The subjects of this study were chitinolytic microorganisms isolated as dominants from the brown semidesert soil of Mongolia (C_{org} 0.64%; pH_{aqu} 8.1) by inoculating nutrient medium with dilutions of natural material enriched in advance with chitin (0.6% of the soil mass) and incubated at 50°C for 7 days. The agarized nutrient medium was of the following composition (g/l): colloid chitin, 4; KH_2PO_4, 0.3; K_2HPO_4, 0.7; $MgSO_4 \cdot 7H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.01; $ZnSO_4 \cdot 7H_2O$, 0.01; and $MnCl_2 \cdot 4H_2O$, 0.01. The purity of the microbial cultures was verified by microscopy. The pure cultures of the isolated microorganisms were grown at 50°C on medium of the same composition. A preparation of purified chitin (polyN-acetyl-1,4-β-D-glucosamine) (ICN Biomedicals Inc., Germany, catalogue no. 101334; molecular weight, 770.8) was used.

The phylogenetic position of the isolates (dominant chitinolytics) was determined by 16S rRNA gene sequencing. DNA was isolated from the bacteria according to the method described in [5]. The concentration of the DNA preparations obtained was 30–50 μg/ml; RNA was present in trace amounts (less than 1% according to the electrophoretic assay). Two independent DNA preparations were obtained. In order to perform PCR and further sequencing of the PCR frag-

ments of the 16S rRNA gene, a system of universal primers was used [6].

The reaction mixture for PCR included the following: primers, 25 pmol of each; 10× buffer, 2.5 μl; 2 mM dNTP, 2.5 μl; Bio*Taq* DNA polymerase (Dialat, Moscow, 5 U/μl), 0.2 μl; template DNA, 50 ng; H_2O, to 25 μl. The reaction scheme included 30 cycles of 94°C for 0.5 min, 45°C for 1 min, and 72°C for 1 min and final polymerization for 7 min.

The PCR products were assayed electrophoretically in 2% agarose gel at an electric field voltage of 6 V/cm. Electrophoretogram photography was carried out using the BioDocII video documentation system (Biometra, Germany).

The primary analysis of nucleotide sequences of 16S rRNA genes of the strains studied was carried out by means of the BLAST server. The sequences were aligned with 16S rRNA gene sequences of the closest bacterial strains using the CLUSTAL.W program [6]. Phylogenetic trees were constructed using the methods implemented in the TREECON software package [7].

The rate of chitin consumption by the isolates was determined by gas chromatography (CO_2 emission rate) and spectrophotometry (biomass accumulation and chitinase production) [1].

The experiments were carried out in the following way: pure microbial cultures were placed into penicillin vials (10^7 cells/ml) with sterile liquid medium in which chitin was the only source of carbon and nitrogen (the medium composition was specified above). The vials were hermetically closed with sterile rubber stoppers and incubated in a thermostat at 50°C for 20 days. Pure cultures incubated in the same medium devoid of chitin served as controls. During the experimental process, daily carbon dioxide accumulation in the gas phase was determined [8]; biomass accumulation was determined in a parallel series of experiments from accumulation of protein measured by the Bradford method [8]. Chitinase production was determined using chitin-azure (C3020 Sigma) as the substrate [9].

RESULTS AND DISCUSSION

When the composition of the thermophilic chitinolytic microbial complex from the brown semidesert soil was determined, only prokaryotes (bacteria and actinomycetes) were isolated on solid nutrient media with chitin; no fungi were revealed.

The taxonomic position of the thermophilic chitinolytic isolates was determined on the basis of both phenotypic features, which were revealed using differentiating nutritive media, and 16S rRNA gene sequencing.

Based on the phenotypic features, the mycelial prokaryotic strain was assigned to the genus *Streptomyces* and identified as *St. roseolilacinus* [10]. The preliminary BLAST analysis of the sequenced 16S rRNA gene fragment of strain Man1 (1397 nucleotides corresponding to *E. coli* positions from 36 to 1450) confirmed the affiliation of this strain with the genus *Streptomyces* of the actinomycete lineage of gram-positive bacteria. The 16S rRNA gene sequences of the type strain of the species *St. roseolilacinus* were the closest to the sequence of strain Man1 (Fig. 1). At present, public databases contain two identical 16S rRNA gene sequences of the type strain of *St. roseolilacinus*, determined in independent studies.

Determination of the phylogenetic position of the bacterial strain Man2, isolated at 50°C from the brown semidesert soil, on the basis of its 16S rRNA gene sequence (1452 nucleotides corresponding to *E. coli* positions from 12 to 1466) showed that this strain belonged to the family *Xanthomonadaceae* of gammaproteobacteria. The closest relative (99.2% 16S rRNA gene homology) was the type strain of *Silanimonas – Silanimonas lenta*, the only representative of the recently described genus *Silanimonas* [11]. Sequences of the representatives of other genera of the family *Xanthomonadaceae* available from the GenBank database were also used for determining the exact phylogenetic position of the strain within the family *Xanthomonadaceae*. In the constructed phylogenetic tree, the 16S rRNA sequences of the strain studied and the type strain of *Sl. lenta* formed a single cluster (Fig. 2) standing apart from other closely related sequences with a 100% bootstrap support.

Thus, among the dominant mycelial and unicellular prokaryotes of the brown semidesert soil isolated on medium with chitin at 50°C, the actinomycete strain was identified as *St. roseolilacinus* and the bacterial strain was defined as *Sl. lenta*, a representative of the recently discovered genus *Silanimonas*, belonging to the family *Xanthomonadaceae* of gammaproteobacteria.

At the next stage of this work, the activity of chitin degradation by the microbial strains studied was determined. The rates of respiration, biomass accumulation, and chitinase production by the microbial cultures were the parameters studied. The emission of carbon dioxide by the cultures growing on medium with chitin was higher than in the control for both microbial strains studied (Fig. 3). For the mycelial and unicellular culture, the carbon dioxide emission on medium with chitin exceeded that in the control variant two- and fourfold, respectively. Similar regularities were also noted for biomass accumulation: the biomass of the bacterial strain increased to 80 μg/ml, which exceeded the biomass in the control by a factor of 4, and the actinomycete biomass on medium with chitin was twice as large as in the control (Fig. 4).

In order to evaluate the rate of chitin degradation by pure microbial cultures, the specific rate of their respiration (B) was determined, understood as the ratio of the difference between the carbon dioxide emission rates on medium with chitin (a) and on medium without

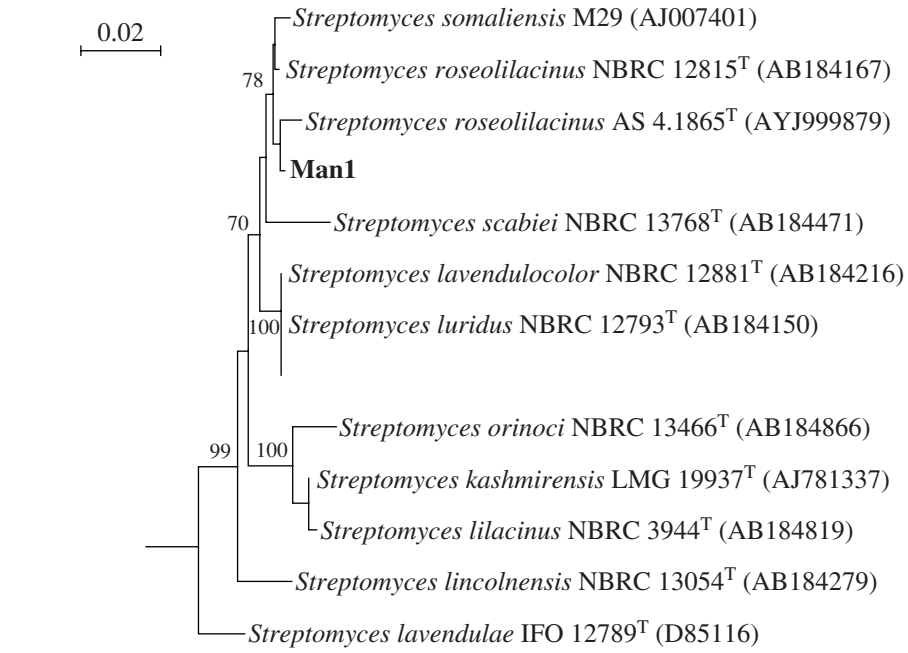

Fig. 1. Phylogenetic position of the actinomycete strain isolated at 50°C from brown semidesert soil. The scale bar shows the evolutionary distance corresponding to two nucleotide substitutions per every 100 nucleotides. The numerals at the branching points show the significance of the branching order as determined by bootstrap analysis of 100 alternative trees (only values higher than 70 are shown).

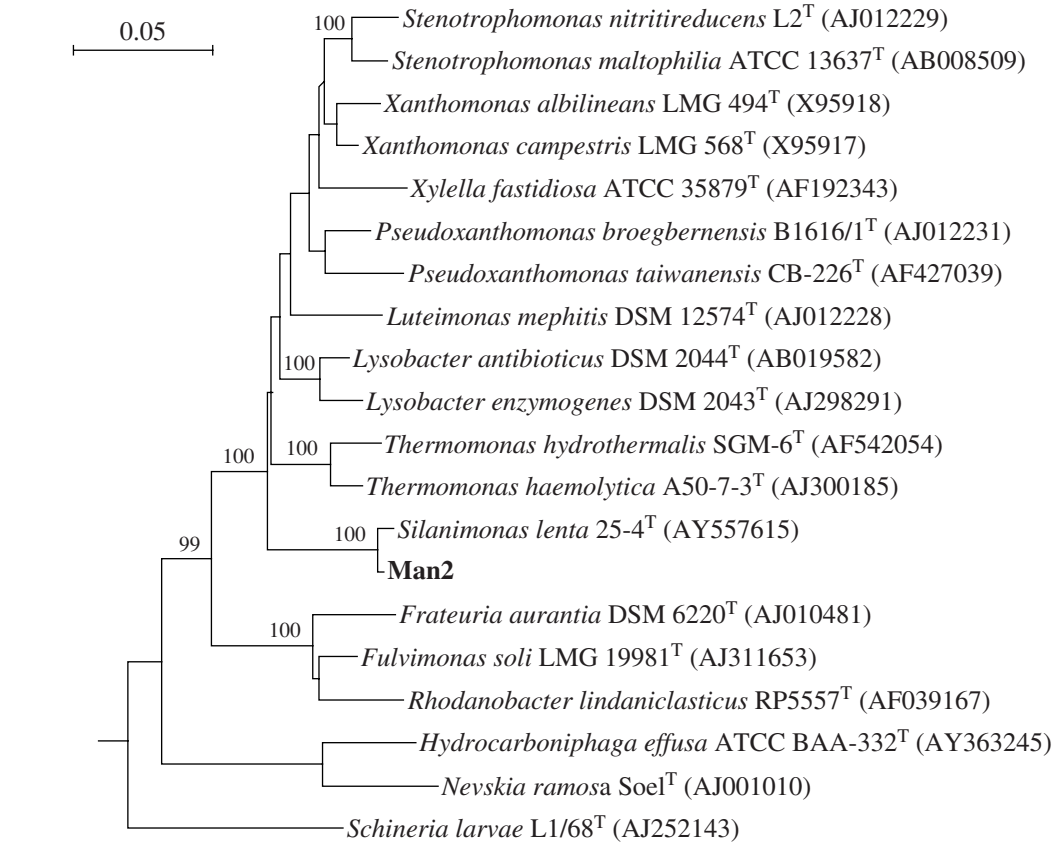

Fig. 2. Phylogenetic position of the bacterial strain isolated at 50°C from brown semidesert soil. The scale bar shows the evolutionary distance corresponding to five nucleotide substitutions per every 100 nucleotides. The numerals at the branching points show the significance of the branching order as determined by bootstrap analysis of 100 alternative trees (only values higher than 95 are shown).

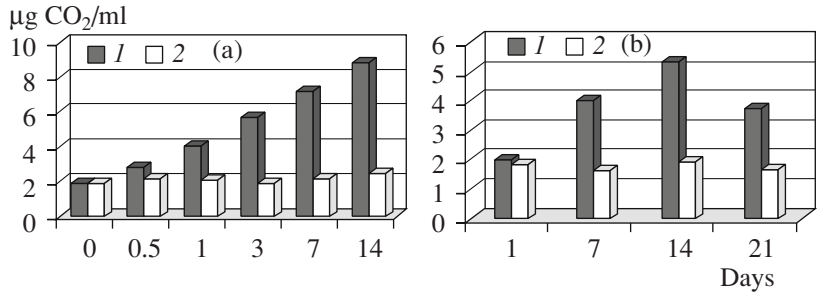

Fig. 3. Dynamics of carbon dioxide emission by pure cultures of (a) *Sl. lenta* and (b) *St. roseolilacinus* incubated at 50°C on (*1*) medium with chitin and (*2*) chitin-free medium.

it (a_0) to the difference between the biomasses accumulated on the same media (Fig. 5):

$$B = (a - a_0)/(b - b_0),$$

where B is the specific rate of respiration of pure cultures on medium with chitin; a and a_0 are CO_2 emissions by the cultures (μg CO_2–C/ml medium) on medium with chitin and without chitin, respectively; and b and b_0 are the biomasses on medium with chitin and without chitin, respectively.

Over the first 24 h of the experiment, the specific rates of respiration of the bacterium and actinomycete were similar. Beginning with the third day and through the end of the experiment, the respiration rate was higher in the *Sl. lenta* strain.

The study of the rate of chitinase accumulation in the culture fluid of the organisms studied showed the amount of the exoenzyme to increase by the sixth day of the experiment from 0.02 to 0.12 U/ml medium for the bacterium and to 0.15 U/ml medium for the actinomycete (Fig. 6). Thus, an increase in the specific activity of chitinase was observed for both the bacterium and the actonimycete grown on medium with chitin at 50°C.

It should be mentioned that published data exist indicating that representatives of gammaproteobacteria are capable of chitinase production; however, for the genus *Silanimonas*, assigned to this group, chitinolytic activity was revealed for the first time.

Thus, the main role in chitin transformation at 50°C in brown semidesert soil was played by the prokaryotes *St. roseolilacinus* and *Sl. lenta*. The consumption of chitin as a source of carbon and nitrogen by both the bacterium and the actinomycete was evidenced by considerable biomass accumulation, high emission of carbon dioxide, and presence in the medium of the chitinase exoenzyme.

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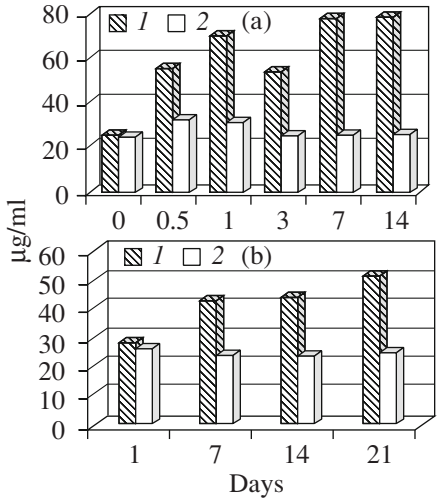

Fig. 4. Dynamics of biomass accumulation by pure cultures of (a) *Sl. lenta* and (b) *St. roseolilacinus* incubated at 50°C on (*1*) medium with chitin and (*2*) chitin-free medium.

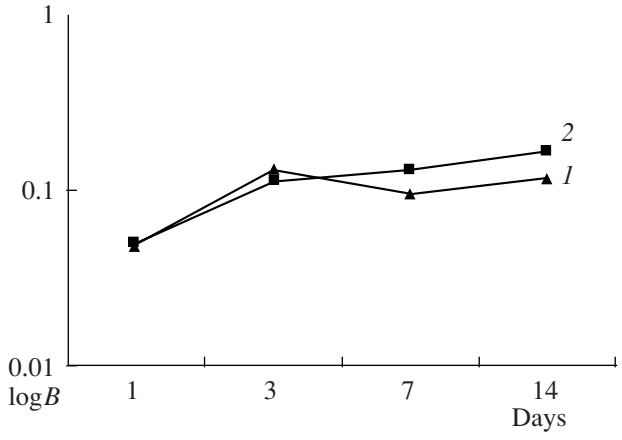

Fig. 5. Dynamics of the specific respiratory activity (B) of pure cultures of (*1*) *Sl. lenta* and (*2*) *St. roseolilacinus* incubated at 50°C.

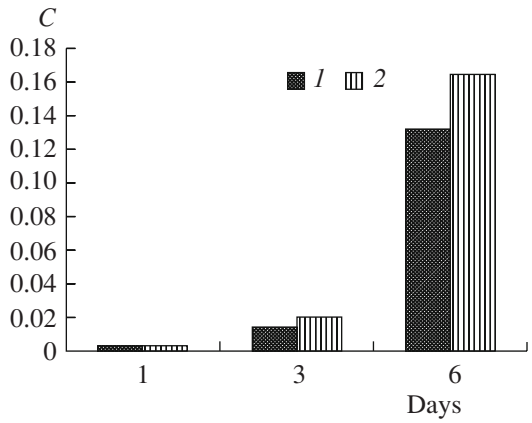

Fig. 6. Dynamics of the specific rate of chitinase production (*C*, chitinase unit/μg biomass) by pure cultures of (*1*) *Sl. lenta* and (*2*) *St. roseolilacinus* incubated at 50°C.

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