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MICROBIOLOGY

Phenotypic and Phylogenetic Characterization of Actinomycetes Isolated from *Lasius niger* and *Formica cunicularia* Ants

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Abstract—Multiple actinomycete strains were isolated from two ant species, *Lasius niger* and *Formica cunicularia*, and their phenotypic properties and phylogenetic position were studied. Partial sequencing of 16S rRNA assigned the greater part of them to the genus *Streptomyces*, but only one belonged to *Nocardia*. However, some isolates had significant color and morphological differences from their closest phylogenetic relatives. The abundance and biodiversity of actinomycete communities isolated from *L. niger* ants greatly exceeded those found for *F. cunicularia*. All of the actinomycetes associated with *F. cunicularia* ants demonstrated cellulolytic activity, but only one had such ability among the strains associated with black ants.

Keywords: actinomycetes, ants, ant-microbe symbiosis, *Lasius*, *Formica*, *Streptomyces*, 16S rRNA, resazurin.
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INTRODUCTION

Many authors have noted many times that mutualistic microorganisms can not only provide many insects, including ants, with nutrients for successful growth and reproduction [1, 2] but also protect the hosts and their food supply from pathogens, parasites, and even predators [3–5]. The ability of representatives of the order Actinomycetales to use a wide range of carbon and nitrogen sources and their extensive range of secondary metabolites are predisposing factors for participation in symbiotic relations with insects. It is shown that mutualistic actinobacteria are able to protect different species of ants, beetles, and wasps from pathogens by excretion of substances with antibiotic activity [6–9]. So-called defensive symbioses with the participation of actinomycetes can be a common and widespread phenomenon in insect ecology and evolution [3]. The study of secondary metabolites involved in these mutualistic interactions promises the discovery of new and promising compounds for biotechnology.

Especially promising for the detection of such symbioses are ants-pedobionts (for example, widespread species on the territory of Russia *Lasius niger* and *Formica cunicularia*), because their food resources and brood are constantly in contact with the soil and prone to fungal invasion [10]. Previously, the authors showed that, although communities of actinomycetes associated with *Lasius niger* and *Formica cunicularia* live ants are less varied than those found in the nests of these species, their species composition is specific to each species of ants [11]. Prokaryotic complexes associated with the life of *Lasius niger* and *Formica cunicularia* ants are dominated by representatives of the genus *Streptomyces* [11, 12].

In this paper, we aim to describe the taxonomic position and some physiological properties of filamentous prokaryotes isolated from *Lasius niger* and *Formica cunicularia* live ants.

MATERIALS AND METHODS

Sampling and Isolation of Actinomycetes

Lasius niger Linnaeus, 1758, (black garden ant) and *Formica cunicularia* Latreille, 1798, ants were collected in summer 2013 in Kasimovsky district of Ryazan oblast (55°5'30" N, 41°41'20" E) in a pilot area, where ant communities and their influence on soil biological activity have been studied for more than 10 years [11–13].

Optimal conditions and the planting scheme were selected in accordance with the previously obtained experimental data [11]. As the seeding material, we used *Lasius niger* and *Formica cunicularia* adult worker ants, 12 and 3 individuals, respectively. Since ants of these species considerably vary in size, to prepare suspensions of comparable concentrations of the substrate, one needs to know the average weight of individuals of both species. Weighing showed that the average *L. niger* ant has a mass of 1.6 ± 0.05 mg and *F. cunicularia* 6.3 ± 0.05 mg.

Table 1. Cultural and morphological characteristics of isolated actinomycetes

Strain*	Color groups by Gauze et al., 1983		MP**	Spore chain shape***	Identified as
	Section	Series			
Ln1	*Cinereus*	*Chromgenes*	+	RF	*Streptomyces sp.*
Ln3	*Roseus*	*Roseoviolaceus*	+	S	*Streptomycessp.*
Ln5	*Albus*	*Albus*	—	RF	*Streptomyces sp.*
Ln6	*Cinereus*	*Chromgenes*	—	RF	*Streptomyces sp.*
Ln7	*Cinereus*	*Achromogenes*	—	RF	*Streptomyces sp.*
Ln8	*Helvolo-Flavus*	*Helvolus*	—	RF	*Streptomyces sp.*
Ln9	*Cinereus*	*Aureus*	+	RF	*Streptomyces sp.*
Ln11	*Cinereus*	*Chromgenes*	+	RF	*Streptomyces sp.*
Ln13	*Cinereus*	*Achromogenes*	—	RF	*Streptomyces sp.*
Ln15					*Nocardia sp.*
Fu1	*Cinereus*	*Chromgenes*	—	RF	*Streptomyces sp.*
Fu2	*Cinereus*	*Achromogenes*	+	RA	*Streptomyces sp.*
Fu4	*Helvolo-Flavus*	*Helvolus*	—	RF	*Streptomyces sp.*
Fu5	*Cinereus*	*Violaceus*	—	RA	*Streptomyces sp.*
Fu6	*Cinereus*	*Achromogenes*	—	RF	*Streptomyces sp.*

* strains isolated from *L. niger* (Ln) and *F. cunicularia* (Fu) ants, ** formation of melanoid pigment (MP) was evaluated in ISP-6 medium [14], *** studied in ISP-3 medium: RF, flexuous chains of spores; RA, spore chains in open loops; S, spiral spore chains [15].

Euthanized insects were manually triturated for 2 min in a mortar with 3 mL of sterile tap water; then, the resulting suspensions were vortexed in a Multi Reax shaker (Heidolph, Germany) for 10 min at 2000 rpm. The resulting suspensions were diluted tenfold with sterile water for inoculation in a nutrient medium of the following composition: 20 g/L soluble starch, 0.5 g/L K_2HPO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 1 g/L KNO_3, 0.5 g/L NaCl, 0.01 g/L $FeSO_4$, 20 g/L agar. In order to limit the growth of filamentous fungi and Gram-negative bacteria, we added nystatin (250 μg/mL) and nalidixic acid (7 μg/mL) to the medium before inoculation. Inoculated Petri dishes were incubated at 27°C for a week, followed by counting the colonies and isolating the clones for further work. Isolated cultures of filamentous prokaryotes were stored in tubes with the beveled medium ISP 3 [14] at 4°C.

Determination of Phenotypic and Physiological Properties of the Isolated Cultures

Diagnostic features of the isolated cultures were studied on the range of nutrient media accepted under the International Streptomyces Project [14]: IPS 3, IPS 6, and IPS 9 as well as mineral agar 1, organic medium 79, and glycerol-nitrate medium [15]. Morphological features were studied using an Axiostar light microscope (Carl Zeiss, Germany).

The ability of strains to assimilate various substrates as a sole carbon source was studied in a 96-well microplate (Greiner Bio-One, Austria). As a complete mineral base, we introduced into each well 200 μL of liquid medium ISP-9 supplemented with 1% carbon substrate: arabinose, sucrose, xylose, mannose, fructose, rhamnose, raffinose, soluble starch, and filter paper. The plate was inoculated with pure cultures of actinomycetes and incubated for 5 days at 28°C. To determine metabolic activity, into each well of the plate, we added 25 μg/mL acid-base indicator resazurin (Sigma-Aldrich, United States), which transformed to resorufin in the presence of metabolically active cells. Incubation with resazurin was carried out for 5 h at 28°C. Quantitative determination of resulting resorufin was carried out using an Infinite F200 microtiter fluorescence scanner (Tecan, Austria) at excitation and emission wavelengths of 560 nm and 590 nm, respectively. Wells with the base medium ISP-9 without addition of the carbon source was a negative control and the same medium with 1% glucose was a positive control.

The efficiency of utilization of various carbon sources was assessed using the acid-base indicator resazurin. This method is based on the ability of living metabolically active cells to restore blue resazurin to bright pink resorufin and is very convenient to determine the growth of microorganisms in minimal volumes of the media in microplates [16].

For each test strain, a variant with the highest resultant resorufin was taken as 100%; for most strains, such a substrate was glucose, which was selected as a positive control. Accordingly, the remaining results were normalized from 0 to 100% and ranked (Table 2): less than 10%, the substrate is not utilized (–); 10–40%, weak growth and metabolism

(±); 40–70%, good growth and substrate utilization (+); more than 70%, intensive growth, rapid metabolism (++).

16S rRNA Gene Sequencing

The phylogenetic position of the isolated strains was determined based on the analysis of 16S rRNA fragments and comparing them with similar fragments from the GenBank database.

Genomic DNA was isolated using the QIAamp DNA Mini Kit (QIAgen, Germany) according to the manufacturer's instructions. To amplify the DNA region containing the 16S rRNA gene, we used a pair of primers specific to the order Actinomycetales: direct 243F 5'-GGATGAGCCCGCGGCCTA-3' and reverse A3R 5'-CCAGCCCCACCTTCGAC-3' [17], as well as a mix for the polymerase chain reaction (PCR) ScreenMix (Evrogen, Russia). PCR was carried out in a T100 thermocycler (BioRad, United States) according to the following program: initial denaturation for 5 min at 95°C; then 30 cycles: denaturation for 94 s at 4°C, annealing of primers for 68 s at 120°C, synthesis of DNA for 90 s at 72°C, and final elongation for 10 min at 72°C.

The resulting PCR products were checked for the presence of the target DNA fragment by electrophoresis in 1% agarose gel and transferred together with the primer samples to ZAO Synthol (Moscow, Russia) for cleaning and sequencing. The obtained nucleotide sequences were compared with the material deposited in Genbank NCBI (http://blast.ncbi.nlm.nih.gov/) using the GeneStudio Pro v. 2.2.0.0 software package (http://genestudio.com). Multiple alignment of the nucleotide sequences and the construction of a phylogenetic tree was carried out using MEGA v. 7 software (http://www.megasoftware.net/).

RESULTS AND DISCUSSION

Previously, the authors carried out comparative analysis of the biodiversity of actinomycetes associated with nests of *Lasius niger* and *Formica cunicularia* ants. It was shown that actinomycetic communities found in nests of these ants are similar to the community of intact soil in terms of the α-biodiversity. However, actinomycetic complexes associated with live insects, first, are significantly different from the communities of the nests, and, second, are unique to each species of ants [11]. Therefore, for a more detailed study of communities of filamentous prokaryotes associated with living ants, we selected worker individuals of *Lasius niger* and *Formica cunicularia* from the same nests that we studied 4 years earlier.

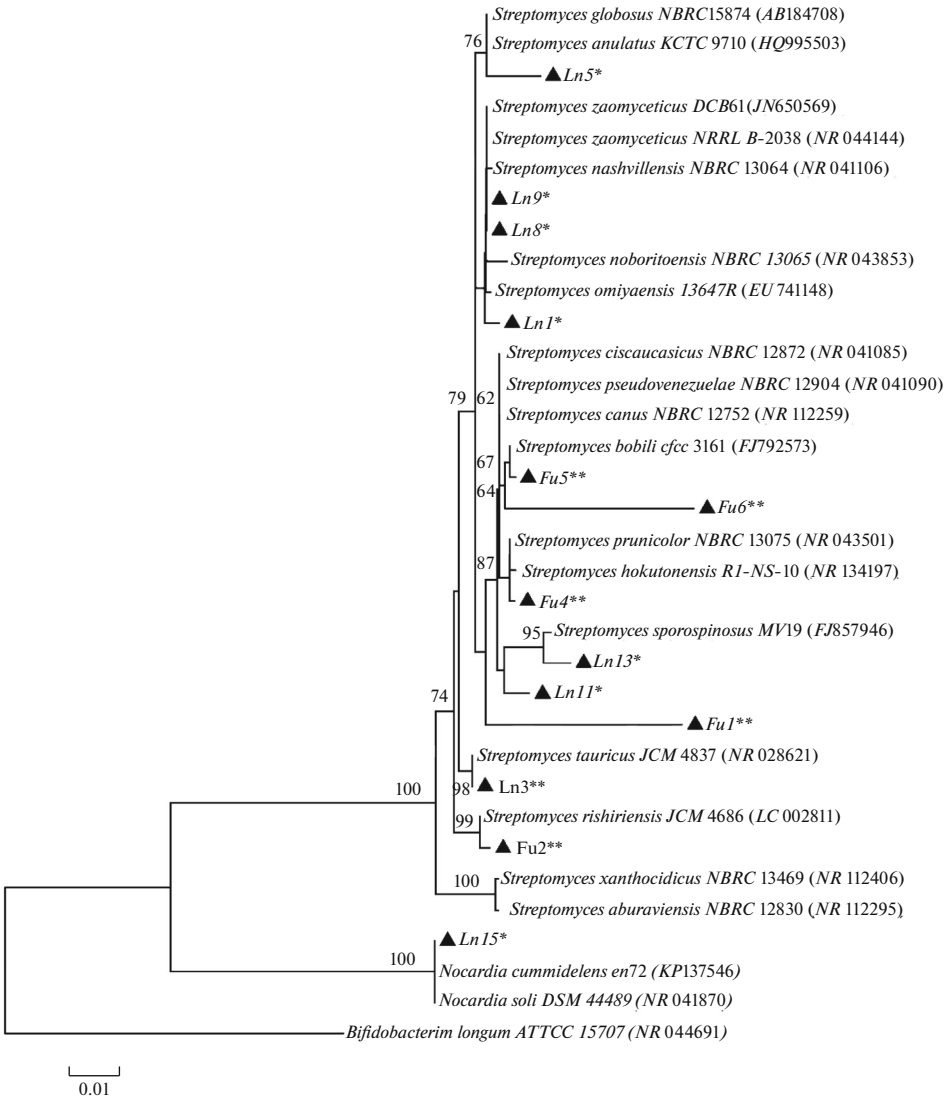
It was found that, in spite of the smaller sizes of specimens, the actinomycetic complex associated with black ants is more varied and numerous than that of the ants of the genus *Formica*. Thus, the specific number of filamentous prokaryotes isolated from *Lasius niger* ants is $(2.6 \pm 0.39) \times 10^6$ CFU/g of ant biomass, which is an order of magnitude higher than that identified for the *Formica cunicularia* ants: $(1.9 \pm 0.28) \times 10^6$ CFU/g of ant biomass. The number of morphotypes isolated from the homogenate of *L. niger* was twice as high as in the inoculation from *Formica cunicularia*: ten and five strains, respectively. Based on analysis of 16S rRNA gene fragments, taxonomic identification of the isolated strains showed that almost all of them are representatives of the genus *Streptomyces* (Table 1).

Table 2. Utilization of various carbon sources in isolated actinomycetes. NC, negative control; GL, glucose; AR, arabinose; SC, sucrose; XY, xylose; MN, mannose; FR, fructose; RM, rhamnose; RF, raffinose; SS, soluble starch; FP, filter paper

Strain	NC	GL	AP	SC	XY	MN	FR	RM	RF	SS	FP
Ln1	—	++	±	++	++	±	++	++	++	++	±
Ln3	—	++	++	++	++	++	++	++	++	++	—
Ln5	—	++	++	++	++	++	++	++	++	++	±
Ln6	—	++	+	++	++	++	++	+	++	++	±
Ln7	—	++	+	++	++	+	++	+	++	++	±
Ln8	—	±	—	—	++	—	±	+	+	++	±
Ln9	—	++	+	++	++	±	++	++	++	++	±
Ln11	—	++	++	++	++	++	++	++	++	++	++
Ln13	—	++	++	++	++	++	++	++	++	+	±
Ln15	—	±	±	±	++	±	±	—	±	+	—
Fu1	—	++	++	++	++	+	+	+	++	++	++
Fu2	—	++	++	++	+	+	++	+	+	++	++
Fu4	—	++	++	++	++	+	++	+	++	++	++
Fu5	—	++	++	++	++	+	++	+	++	++	++
Fu6	-	++	++	++	+	±	+	±	±	++	+

The results of evaluation of the total number and diversity of actinomycetes detected by culturing from homogenates of live insects are fully consistent with the data obtained previously for the same ant species [11]. Other patterns marked earlier were also confirmed: the lack of common species of filamentous prokaryotes in *L. niger* and *F. cunicularia* ants; greater abundance and species richness of actinomycetes associated with black ants despite their smaller sizes.

It is very difficult to carry out species identification of streptomycetes based solely on 16s rRNA gene sequence analysis, because nucleotide sequences of this gene have a high affinity for representatives of all taxa within the family Streptomycetaceae [18]. To compare information on phenotypic traits with the genetic data, we studied cultural (color of the aerial and substrate mycelia, presence and color of the soluble pigment), morphological (shape of spore chains), and physiological characteristics (utilization of various substrates) of the isolated strains (Tables 1, 2).



Phylogenetic tree of 16S rRNA sequences belonging to actinomycetes (904 bp) isolated from (*) *L. niger* and (**) *F. cunicularia* ants and collection strains genetically closest to them (brackets indicate GenBank accession numbers). The phylogram is built by neighbor-joining algorithm. Bootstrap values for nodes characterized by values above 60% are shown. The evolutionary distance is calculated by using the maximum likelihood algorithm, and the segment shows 0.01 nucleotide substitutions per site. Strain *Bifidobacterium longum* ATCC 15707 was chosen as the reference organism that does not belong to the genera *Streptomyces* and *Nocardia*. Marker ▲ indicates strains isolated in our study.

Despite the fact that the taxonomic significance of such a feature as consumption of carbohydrate substrates by actinomycetes has recently been questioned, this information may be useful in the study of ecological features of the organisms in question.

It was found that glucose, which was selected as a positive control, was not a universal substrate; it was xylose that was actively metabolized by all the studied strains. Most isolated actinomycetes actively consumed all available pentose and hexose, except for strains Ln8 and Ln15, preferring only xylose.

The ability to hydrolyze polymers, starch and cellulose, is characteristic of filamentous prokaryotes. Amylolytic activity was observed in all the examined strains, but the studied cultures divided in terms of the ability to hydrolyze cellulose. All the strains isolated from *Formica cunicularia* ants showed a pronounced ability to utilize cellulose, while this activity was observed only for strain Ln11 among the strains isolated from *L. niger* ants (Table 2).

According to the authors who have carried out a major project on phylogenetic analysis of species within the family Streptomycetaceae [18], the value of

Table 3. Phylogenetic analysis of actinomycetes isolated from *Lasius niger* and *Formica cunicularia* ants based on comparison of 16s rRNA gene fragments

Strain	Isolation source	Nearest related organisms in GenBank	Access no.	Similarity, %
Ln1	*Lasius niger* ants	*Streptomyces zaomyceticus DCB61*	JN650569	99
Ln3		*Streptomyces tauricus JCM 4837*	NR_028621	99
Ln5		*Streptomyces anulatus KCTC 9710*	HQ995503	100
		**Streptomyces globosus NBRC 15874*	AB184708	99
Ln6		*Streptomyces xanthocidicus NBRC 13469*	NR_112406	99
Ln7		*Streptomyces prunicolor NBRC 13075*	NR_043501	99
Ln8		*Streptomyces omiyaensis 13647R*	EU741148	98
		**Streptomyces aburaviensis NBRC 12830*	NR_112295	96
Ln9		*Streptomyces zaomyceticus NRRL B-2038*	NR_044144	99
Ln11		*Streptomyces ciscaucasicus NBRC 12872*	NR_041085	99
		Streptomyces pseudovenezuelae NBRC 12904	NR_041090	99
		Streptomyces canus NBRC 12752	NR_112259	99
		**Streptomyces nashvillensis NBRC 13064*	NR_041106	97
Ln13		*StreptomycessporospinosusMV19*	FJ857946	100
Ln15		*Nocardia soli DSM 44489*	NR_041870	100
		**Nocardia cummidelens en47*	KP137546	99
Fu1	*Formica cunicularia* ants	*Streptomyces noboritoensis NBRC 13065*	NR_043853	96
Fu2		*Streptomyces rishiriensis JCM 4686.*	LC002811	99
Fu4		*Streptomyces prunicolor NBRC 13075*	NR_043501	99
Fu5		*Streptomyces bobili cfcc3161*	FJ792573	99
Fu6		*Streptomyces hokutonensis R1-NS-10*	NR_134197	98

* reference species with greater phenotypic rather than genotypic similarity with the test strain.

similarity of 16s rRNA gene sequences above 98.5%, as adopted in the literature for species identification of organisms, is not reliable for streptomycetes. The taxonomic value of the sequence of this genetic locus consists in generic identification or confirmation of novelty of unknown strains of streptomycetes, especially when there is a correlation with morphological and physiological characteristics [18]. The same authors in phylogenetic hierarchy analysis propose that the significant bootstrap value of clustering the studied species should be more than 60%.

For the following strains, it was possible to find nucleotide sequences belonging to cultures with complete phenotypic and genotypic correspondence: Ln3 and *S. tauricus* JCM 4837 as well as Fu2 and *Streptomyces rishiriensis* JCM 4686, which is evidenced by the very high reliability of clustering (98% and 99%, respectively, see figure 1).

For strains Fu4 and Fu5, among those closest in terms of sequences and offered by the search service BLAST, we selected phenotypically similar *S. prunicolor* NBRC 13075 and *S. bobili* cfcc3161, the accuracy of clustering with which accounts for 87 and 67%, respectively (figure).

For the nucleotide sequence of strain Ln5, we found a corresponding sequence in the phenotypically identical culture *Streptomyces anulatus* KCTC 9710 with the level of similarity of 100% (Table 3). The reliability of combining these data nucleotide sequences together with *Streptomyces globosus* NBRC 15874 into one cluster is 76% (figure), suggesting their phylogenetic relatedness.

For strain Ln13 isolated from *L. niger* ants, we detected 100% genotypic compliance with the strain *Streptomyces* sporospinosus MV19 (Table 3) isolated from the hindgut of the South African termite *Microhodotermes viator* [19]. However, in spite of the maximum genetic matching, these strains differed in terms of the shape of spore chains. Ln13 formed straight chains of spores on all nutrient media, in contrast to *Streptomyces* sporospinosus MV19, which was characterized by the presence of spiral spore chains. The reliability of associating these organisms into one cluster is 95%, which should be seen as an indicator of high phylogenetic relatedness (see figure).

Of all streptomycetic sequences with 99% similarity for strain Ln1 returned by GenBank, none of the collection strains match it for all phenotypic traits. For phylogenetic analysis, we selected a sequence belonging to the culture *Streptomyces* zaomyceticus DCB61 with the least significant phenotypic differences from strain Ln1: color of the substrate mycelium and the

soluble pigment of strain Ln1 on the starch and oat media were reddish-brown, in contrast to the bright yellow ones, characteristic of *S. zaomyceticus* [15]. Phylogenetic analysis did not confirm any similarity between these strains (figure).

For streptomycete Ln9, among sequences with 99% similarity returned by the search algorithm, we selected a sequence belonging to strain *S. zaomyceticus* NRRL B-2038 as the most phenotypically relevant. However, the accuracy of clustering the strain Ln9 with the group of species closely related to *S. zaomyceticus* NRRL B-2038 was below the threshold in the constructed phylogenetic tree. It should be noted that nucleotide sequences of the *S. zaomyceticus* strains stored in different collections (NRRL, United States and DCB, China) also did not make a single cluster with a high reliability value.

Collection strains whose nucleotide sequences were returned by BLAST for strain Ln11 as those similar by 99% (Table 3) were completely inconsistent with this strain in terms of phenotypic characteristics. However, even in the phylogenetic tree, they did not fall in the same significantly isolated cluster (figure).

All nucleotide fragments returned by BLAST for strains Ln8, Fu1, and Fu6 were consistent with the inputted sequences for not more than 98%, which, as noted above, is insufficient for species identification. Not surprisingly, those inputted sequences belong to species whose description does not correspond to the phenotypic traits observed in the studied strains. However, for phylogenetic analysis, we selected sequences with the maximum nucleotide similarity, although no significant associations were found. The value of similarity of nucleotide sequences, which is low for streptomycetes, can be the starting point to test the hypothesis that these strains might belong to new species.

All the above-mentioned strains, without a doubt, belong to the genus *Streptomyces*, because, together with all collection cultures of *Streptomyces*, they can be combined into a single cluster with maximum reliability, 100% (figure).

Strain Ln15, which is the only isolated actinomycete that does not belong to the genus *Streptomyces*, has phenotypic and genotypic similarity with the collection strain *Nocardia soli* DSM 44489, forming a single cluster with it with maximum reliability (figure).

The study of actinomycete communities associated with ants remains a still little explored area of zoomicrobe interactions. Besides a textbook example of mutualism between fungus-growing *Attini* ants and actinobacteria *Pseudonocardia* [4–6, 20], the literature contains very scarce data on the distribution and nature of relationships between actinomycetes and other species of ants. Meanwhile, in recent years, there have been opinions that the presence of exo- and endosymbionts in insects is not a unique phenomenon but a general rule and that the functional roles of microbial participants in such unions may be much broader and more varied than just defensive or nutritive [3, 5].

As part of this work, we were able to confirm previous findings that the actinomycete complexes associated with *L. niger* ants are more numerous and varied compared to those isolated from *F. cunicularia* ants. In addition, each species of ants possesses a unique set of associated actinomycetes, the vast majority of which belongs to the genus *Streptomyces* [11, 12].

The literature contains only single cases of data about research of actinomycete communities associated with species not belonging to the ecological group of fungus-growing ants [8, 12]. The authors of this paper were the first to select *L. niger* and *F. cunicularia* ants as objects of a comparative study of ant-actinomycete relationships.

We were the first to evaluate the effectiveness of utilization of various carbon sources by *Streptomyces* cultures in the format of a 96-well microplate using the acid-base indicator resazurin. This technique has a number of fundamental advantages over traditional inoculation in the range of solid nutrient media: it requires small volumes of media, makes it possible to simultaneously study a large number of objects, and is characterized by rapid and objective assessment of the results. Although the ability of *Streptomyces* to assimilate certain carbon sources is currently not a significant taxonomic trait, it could serve as a basis for the study of the functional role of actinomycetes in the community.

It was found that all streptomycetes associated with *F. cunicularia* ants exhibit significant cellulolytic activity, while such activity was revealed only for one among the strains associated with black ants. Equally noticeable differentiation among actinomycetic communities in respect of utilization of the plant-origin polymer may indicate the presence of plant residues in the habitat of *F. cunicularia*. However, this assumption requires experimental verification, since the ecology of these species of ants is very similar theoretically: both of those build their nests in the soil of the same site and the food resources of both the species consist in honeydew of aphids and insects. As for the possible differences, it is known that *F. cunicularia* is a relatively more thermophilic species and its nests lie at a greater depth than those of *L. niger* [10]. Perhaps the different depth of nests is reflected at the floristic composition, which corresponds to nests of different species of ants. On the other hand, it is possible that the feeding sites are associated with different plant species. Undoubtedly, this issue requires further study.

Determination of nucleotide sequences of 16S rRNA gene fragments in all the isolated strains showed that part of the cultures have significant phenotypic differences from the most closely related species returned by the BLAST search algorithm. For strains Ln8, Fu1, and Fu6, the similarity to sequences from

the GenBank database was not above 98%, which can serve as a basis for testing the hypothesis that these strains might belong to a new species.

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