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Probable protective mission of actinomycetes associated with black garden ants, *Lasius niger* (Linnaeus, 1758) (Hymenoptera: Formicidae)

Предполагаемая защитная роль актиномицетов, ассоциированных с муравьями *Lasius niger* (Linnaeus, 1758) (Hymenoptera: Formicidae)

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КЛЮЧЕВЫЕ СЛОВА: муравьи, *Lasius niger*, *Nocardia*, *Streptomyces*, антибиотики, «защитные симбиозы».

ABSTRACT. A collection of mycelial bacteria of the phylum Actinomycetota was isolated from adult working individuals of the ant *Lasius niger* (Linnaeus, 1758) selected on the territory of Moscow by plating on nutrient media. It is shown that cultured members of actinomycete communities are confined to the heads of insects, and belong to the genera *Nocardia* Trevisan, 1889 and *Streptomyces* Waksman and Henrici, 1943. Among the strains isolated from ants within the same family, producers of antibacterial compounds prevail over antifungal ones. Actinomycetes with antibacterial activity inhibit development of entomopathogenic bacteria *Bacillus thuringiensis* VKPM B-6650 and *Paenibacillus alvei* VKM B-502, as well as growth of the opportunistic bacterium *Staphylococcus aureus* ATCC 25923, representing one of the most important human pathogens worldwide. At the same time, ability of individual strains to restrain the growth of entomopathogenic fungi *Beauveria bassiana* VKPM F-1357, *Conidiobolus coronatus* VKPM F-1359, as well as clinically significant yeast *Candida albicans* ATCC 14053 was noted. Further studies are required to clarify the localization of possible symbionts and their ability to synthesize antimicrobial metabolites *in vivo*.

РЕЗЮМЕ. Из взрослых рабочих особей муравья *Lasius niger* (Linnaeus, 1978), отобранных на территории г. Москвы, методом посева на питательные среды выделена коллекция мицелиальных представителей филума Actinomycetota. Показано, что культивируемые участники актиномицетных сообществ приурочены к головному отделу насекомых, а в таксономическом отношении представлены родами *Nocardia* Trevisan, 1889 и *Streptomyces* Waksman et Henrici, 1943. Среди штаммов, выделенных из муравьев внутри одной семьи, превалируют продуценты антибактериальных, а не антигрибных соединений. Актиномицеты с антибактериальной активностью подавляют развитие энтомопатогенных бактерий *Bacillus thuringiensis* VKPM B-6650 и *Paenibacillus alvei* VKM B-502, а также рост оппортунистической бактерии *Staphylococcus aureus* ATCC 25923, одного из наиболее важных патогенов человека по всему миру. В то же время отмечена способность отдельных штаммов к сдерживанию роста энтомопатогенных грибов *Beauveria bassiana* VKPM F-1357, *Conidiobolus coronatus* VKPM F-1359, а также клинически значимых дрожжей *Candida albicans* ATCC 14053. Требуются дальнейшие исследования для уточнения локализации возможных симбионтов и способности их синтезировать антимикробные метаболиты *in vivo*.

Introduction

Scientific interest in the study of actinomycetes associated with insects originates from a series of publications about the fungus-growing ants (Formicidae: Attini), where the multilateral symbiosis between leaf-cutting ants and their symbiotic actinobacteria synthesizing metabolites to protect fungus cultivars from parasitic fungi was deeply investigated [Currie *et al*., 1999, 2003]. Then mutualistic actinomycetes were found in a solitary digger wasp, the European beewolf *Philanthus triangulum* (Fabricius, 1775), and their ability to synthesize an effective mixture of antibiotics to protect larvae in brood

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cells was shown [Kaltenpoth *et al*., 2005]. The term «defensive/protective symbioses» appeared [Kaltenpoth, 2009; Kaltenpoth, Engl, 2014] to describe mutually beneficial interactions between invertebrates and actinomycetes synthesizing active metabolites against host enemies. The list of similar examples has been expanded by publications about the presence of previously unknown actinomycetes in mycangia of southern pine beetles *Dendroctonus frontalis* (Zimmermann, 1868), producing the novel antifungal product mycangimycin [Oh *et al*., 2009]. The strain of *Streptomyces* sp. with an ability to synthesize a previously undescribed antibiotic sceliphrolactam has also been isolated from the mud wasp *Sceliphron caementarium* (Drury, 1773) [Oh *et al*., 2011]. There is an increasing number of recent reports about the isolation of mycelial prokaryote strains from various insects [Baranova *et al*., 2022], and ants remain the most popular object in this respect. Indeed, it was possible to show the presence of mycelial actinobacteria capable of synthesizing broad-spectrum antibiotics in adult individuals of carpenter and harvester ants [Zakalyukina *et al*., 2019, 2022a, b; Baranova *et al*., 2020]. Thus, it can be assumed that in the insect world, "protective symbioses" are not a random phenomenon, but an established relationship. However, there are still many unresolved questions: how specific these complexes are for particular ant species, where they are localized, and what metabolites they synthesize.

The black garden ant *Lasius niger* (Linnaeus, 1758) is the most typical and abundant member of ant communities of Moscow, since it is the most resistant to stress factors and anthropogenic transformations of the city's territories [Putyatina *et al*., 2017]. In the earlier studies, we noted an abundance of actinomycetes in the nests of black garden ants [Zakalyukina *et al*., 2014], and representatives of the phylum Actinomycetota could also be isolated from the ants [Zakalyukina *et al*., 2017], but their taxonomic position and antibiotic potential have not been characterized. In this paper, we sought to determine whether there were certain types of actinomycetes that were prone to forming associations with black ants, and what type of antibiotic activity they were endowed with.

Modern microbiome studies involve the use of metagenomics, since the number of cultivated microorganisms is limited by the laboratory conditions. However, for actinomycetes, this approach has significant limitations, since these bacteria are characterized by a high proportion of GC-pairs in the genome and are much more difficult to amplify than representatives of other phyla. Therefore, the isolation of actinomycetes by methods of classical microbiology remains the only opportunity to isolate and study these complex microorganisms.

Material and methods

Sampling of ants and isolation of actinomycetes

The collection of *Lasius niger* ants was carried out in September 2021 within the city of Moscow from three districts, where the urban communities of ants were previously studied [Putyatina *et al*., 2017]. The ants were collected from the nests "Krylatskoe" (55.770253° N, 37.412092° E), "Leninskie gory" (55.706206° N, 37.531215° E) and 'Filevsky park' (55.736023° N, 37.445172° E), with soil materials put in sterile boxes and then delivered to the laboratory.

Five workers from each nest were euthanized with ether, then intensively stirred in a vortex-shaker for 10 minutes, immersed in 75% ethanol for 60 seconds and then washed twice in sterile distilled water. The washed insects were divided into the head, chest and abdomen, which were separately crushed into 500 ml of sterile saline solution, and 10-fold dilutions were prepared. The aliquots from the obtained suspensions were spread on plates with Organic agar 79 supplemented by nalidixic acid and nystatin as described earlier [Zakalyukina *et al*., 2022a]. After 14-day incubation period at 28 °C mycelial colonies were accounted and transferred on International *Streptomyces* Project Medium 3 (ISP 3) slants, and stored as a suspension of spores in 20% glycerol at −80 °C.

Phenotypical characteristic of actinomycetes

Cultural characteristics of isolates were observed in ISP 2–ISP 7 media after cultivation for up to 21 days at 28 °C according to the ISP recommendation [Shirling, Gottlieb, 1966]. Morphological features (surface and shape of spore chains) were studied using scanning electron microscopy (JSM-6380LA, JEOL, Tokyo, Japan and Camscan S2, Cambridge Instruments). Carbon source utilization was assessed on basal medium ISP 9 [Shirling, Gottlieb, 1966] with the addition of 0.04% solution of bromocresol purple at 28 °C for 14 days. The degradation of casein, starch and cellulose was estimated from the clearing of insoluble compounds around areas of growth.

16S rRNA gene phylogeny of isolated strains

The extraction of the genomic DNA of isolates and PCR amplification were achieved using procedures described elsewhere [Baranova *et al*., 2020]. Both pairs of universal primers F27 (5'-AGAGTTTGATCMTGGCTCAG-3') and R1492 (5'-TACGGYTACCTTGTTACGACTT-3') and actinobacterial primers 243F (5'-GGATGAGCCCGCGGCCTA-30) and A3R (50-CCAGCCCCACCTTCGAC-30) were used. The amplicons were purified and sequenced using a commercial service (Genome Center, Moscow). All sequences were identified by searching close relatives with the BLAST service (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 31 October 2023). The comparative phylogenetic analysis with related strains of actinomycetes was conducted with the MEGA software Version X (https://www.megasoftware.net). To construct phylograms, neighbor joining (NJ) method was applied to 1000 alternative trees. Evolutionary distances between sequences were calculated using the Tamura–Nei model. Bootstrap analysis values above 60% are indicated. The scale corresponds to two substitutions for every 100 nucleotides. Strain *Bifidobacteruim longum* ATCC 15707 was selected as a reference organism not belonging to the genera *Nocardia* Trevisan, 1889 and *Streptomyces* Waksman et Henrici, 1943. Actual information on the nomenclature of bacteria and fungi was verified with the List of Prokaryotic names with Standing in Nomenclature (LPSN, https://lpsn.dsmz.de, accessed on 31 October 2023) and Mycobank databases (https://www.mycobank.org, accessed on 31 October 2023).

Determination of antagonistic activity of isolates

All actinomycete isolates were cultivated on a set of nutrient media: mineral agar Gauze 1 (G1), organic medium 79 (Org79), glucose-asparagine agar (GA), soy flour mannitol

agar (SFM) and oatmeal agar (ISP3) for 10 days at 28 °C. Antifungal and antibacterial activities of actinomycete strains were tested by Petri plate bioassay experiments against test microorganisms, including *Candida albicans* ATCC 14053, *Staphylococcus aureus* ATCC 25923, as well as against entomopathogenic microorganisms: *Bacillus thuringiensis* VKPM B-6650, *Paenibacillus alvei* VKM B-502, *Beauveria bassiana* VKPM F-1357, *Conidiobolus coronatus* VKPM F-1359, as described earlier [Baranova *et al*., 2020]. The thickness of the agar plates was kept constant in all experiments by using a gel levelling table and fixed volume of agar.

Preliminary identification of antibiotics

Actinobacteria isolates were cultivated in different liquid nutrient media at 28°C with constant shaking: optimal conditions for the antibiotic production were selected separately for each strain. Culture liquids were separated from biomass by centrifugation and the supernatants were subjected to solid-phase extraction and primary fractionation on LPS-500-H sorbent (LLC "Technosorbent", Moscow, Russia) using water-acetonitrile mixtures as eluents. HPLC fractionation and mass spectrometry analysis were performed according to the scheme earlier described in detail [Volynkina *et al*., 2022].

Results

Three nests of black garden ants were selected for the study, located in different districts of Moscow in biotopes characterized as a lawn (Krylatskoe), boulevard (Leninskie gory) and a small depressed artificial park (Filevsky park), previously used as objects of research [Putyatina *et al*., 2017]. Five adult workers from each nest were prepared and examined individually, divided into the head, thoracic and abdominal sections on rich nutrient medium with the addition of antibiotics to limit the growth of gram-negative bacteria and fungi.

The maximum amount of mycelial bacterial colonies was isolated from the heads. It was one or two orders of magnitude higher than that from the thoracic sections, meanwhile actinomycetes were not detected in the abdominal sections. The number of colonies in the head sections varied significantly between individuals within each nest, from 50 to 700 CFU/specimen. It had been observed that larger individuals had a greater number of colonies with a greater species diversity. The taxonomic diversity of mycelial bacteria identified in the head sections was represented by the genera *Nocardia* and *Streptomyces* (Fig. 1).

All isolated *Nocardia* strains from studied individuals were characterized by high similarity of their 16S rRNA gene fragments and formed well-supported clade with the type strain *Nocardia fluminea* DSM 44489 with bootstrap value 100% (Fig. 2). However, BLAST-calculated percent identities with previously described types of *Nocardia* strains, isolated from ants, *Nocardia lasii* 3C-HV12^{T} [Liu *et al*., 2016a] and *Nocardia camponoti* 1H-HV4^{T} [Liu *et al*., 2016b], were 99.46% and 97.54% respectively (Fig.2).

Actinomycetes isolated from the head sections were characterized by high species diversity. Within one nest, the set of detected morphotypes was limited to 4-5, however, these sets did not coincide in the three families studied (Fig. 2). Although the nucleotide sequence of the 16S rRNA gene does not have sufficient resolution to differentiate streptomycetes at the species level, each family had its own dominant *Streptomyces* species, which also differed in phenotypic characteristics (Table 1, Fig. 3).

A study of the antagonistic activity against both clinically significant test organisms and entomopathogenic microorganisms showed that streptomycete isolates considerably inhibited gram-positive bacteria, entomopathogenic bacilli (*Bacillus thuringiensis* Berliner, 1915 and *Paenibacillus alvei* (Cheshire et Cheyne, 1885)) as well as *Staphylococcus aureus* Rosenbach, 1884. Some strains of streptomycetes showed little activity against fungi infecting many species of insects, *Conidiobolus coronatus* (Costantin, 1897) and *Beauveria bassiana* (Balsamo-Crivelli, 1835) (Fig. 3). However, the strains of *Nocardia*, representing about half of the actinomyc-

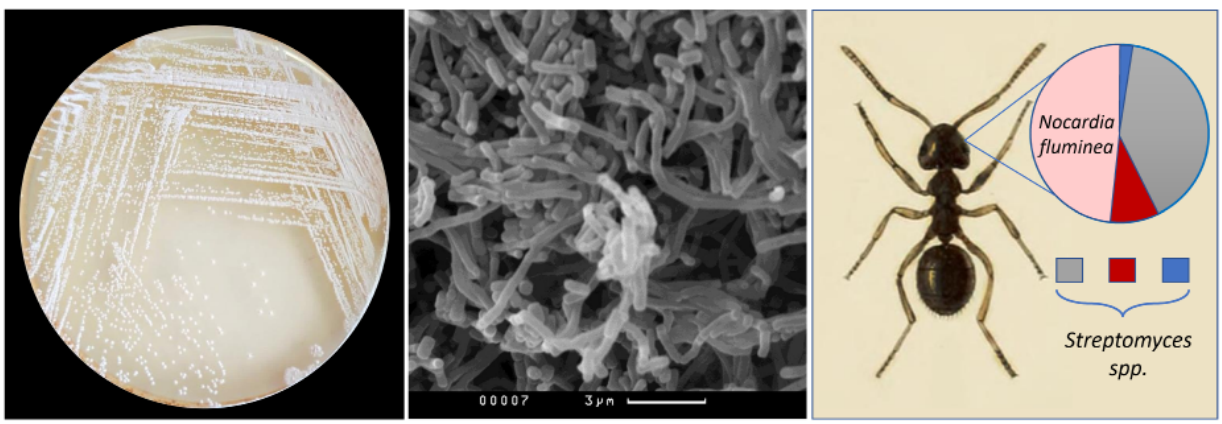

Fig. 1. *Nocardia* strains in heads of *Lasius niger* are most related to *Nocardia fluminea* DSM 44489: cultural features on ISP 2 (left), morphological characteristics studied by SEM (center), the structure of actinomycete communities on ants (right).
Рис. 1. Представители рода *Nocardia* в головном отделе *Lasius niger* представлены штаммами, наиболее близкими к *Nocardia fluminea* DSM 44489: культуральные признаки на среде ISP2 (слева), морфологические признаки, изученные с помощью сканирующей электронной микроскопии (в центре), структура актиномицетных комплексов (справа).

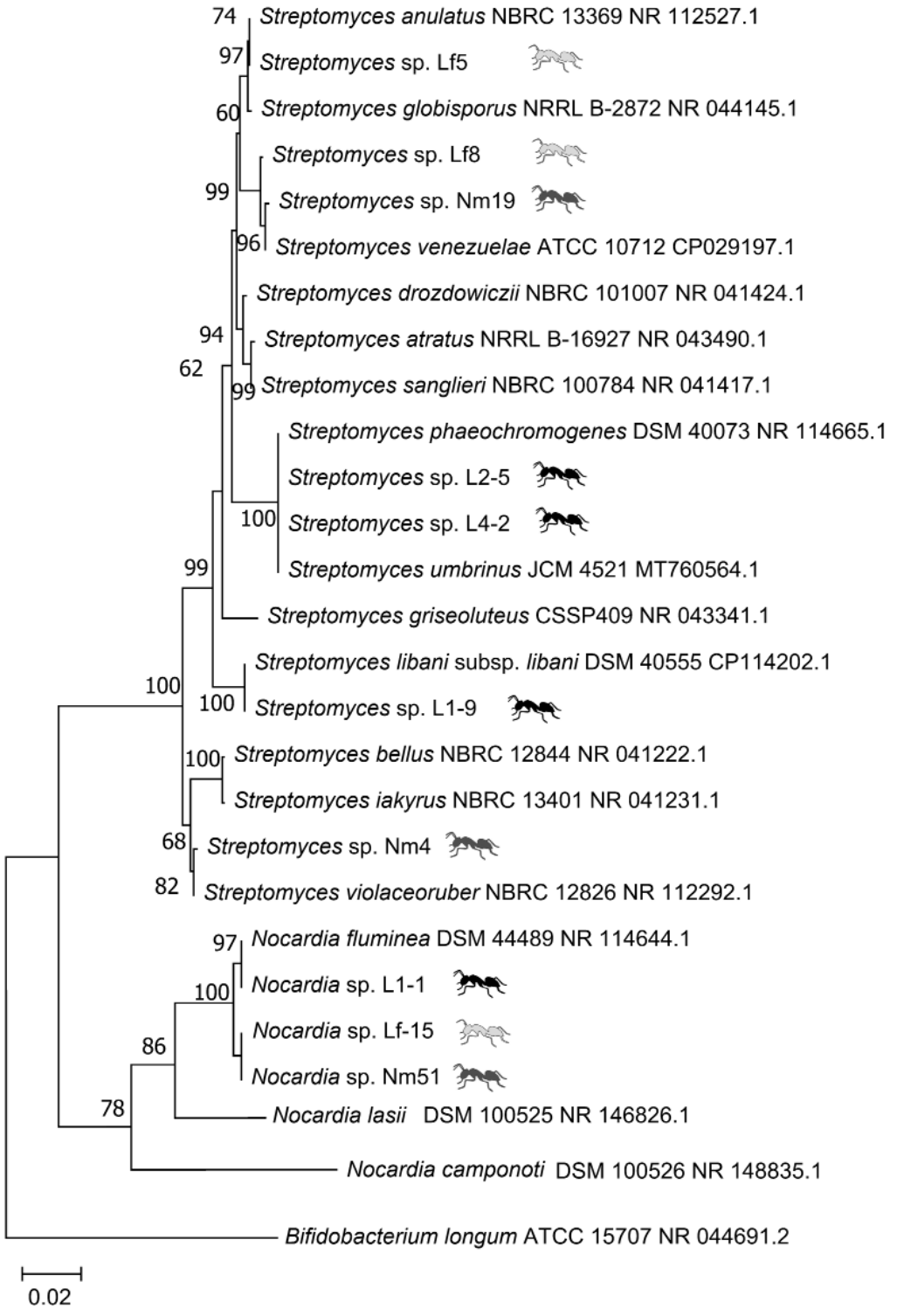

Fig. 2. Neighbor-joining tree based on 16S rRNA gene sequences showing the relationship between actinomycetes associated with *Lasius niger* and related type strains. Strains from different nests are marked with pictograms of different colors: Krylatskoye (black), Leninskie gory (gray), Filevsky park (white).

Рис. 2. Филогенетическое положение штаммов актиномицетов, ассоциированных с *Lasius niger* и близких им генетически типовых штаммов стрептомицетов, на основании NJ-анализа нуклеотидных последовательностей фрагмента гена 16S рРНК. Штаммы из разных гнезд отмечены пиктограммами разных цветов: Крылатское (черные), Ленинские горы (серые), Филевский парк (белые).

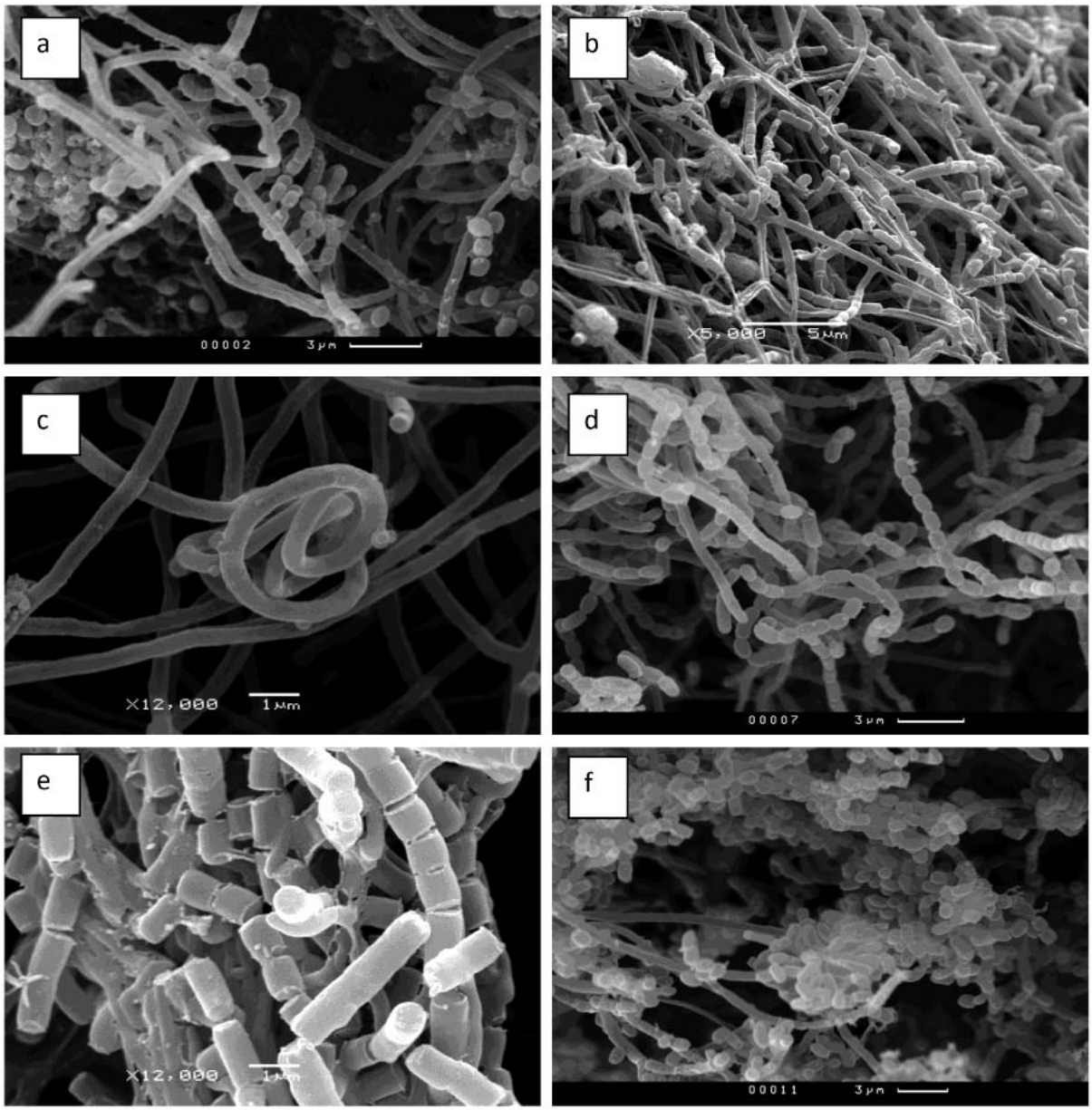

Fig. 3. Morphology of spore chains of the dominated streptomycete strains after incubation on ISP 3 agar at 28°C for 14 days: *Streptomyces* sp. L1-9 (a), *Streptomyces* sp. L2-5 (b), *Streptomyces* sp. L4-2 (c), *Streptomyces* sp. Lf5 (d), *Streptomyces* sp. Nm19 (e), *Streptomyces* sp. Nm4 (f).
Рис. 3. Морфология цепочек спор доминирующих штаммов стрептомицетов, выделенных из муравьев *Lasius niger*, после инкубации в течении 14 дней на овсяном агаре при 28°C: *Streptomyces* sp. L1-9 (a), *Streptomyces* sp. L2-5 (b), *Streptomyces* sp. L4-2 (c), *Streptomyces* sp. Lf8 (d), *Streptomyces* sp. Nm19 (e), *Streptomyces* sp. Nm4 (f).

ete complexes, showed no *in vitro* antagonism against selected test-microorganisms.

For the identification of active metabolites, streptomycete strains were cultured on preferred media and extracted as described earlier [Volynkina *et al*., 2022]. Since the most important problem was establishing the chemical nature of antimicrobial secondary metabolites, we performed solid-phase extraction of the supernatants, focusing mainly on the ability of the fractions to inhibit the growth of bacteria (*B. thuringiensis*) and yeast (*C. albicans* (Robin, 1923)).

High-performance liquid chromatography (HPLC) analysis revealed the fractions with antimicrobial activity, and their spectra of UV absorption at 274 nm were studied. The compounds were analyzed by liquid chromatography–mass spectrometry (LCMS) with mass-fragmentation using the Global Natural Product Social Molecular Networking (GNPS) MS/MS spectra library, revealing the antimicrobial compounds that can be preliminarily assigned to several groups of antibiotics – pikromycins, mangromycins, thiazostatins, aquayamycins (Fig.4). More precise identification requires further research.

Table 1. Phenotypic features of streptomycetes revealed in the studied families of *L. niger*.
Таблица 1. Фенотипические признаки стрептомицетов, выявленных в семьях *L. niger.*

Location	Krylatskoe			Leninskie gory		Filevsky park	
Streptomycete strains	L1-9	L2-5	L4-2	Nm4	Nm19	Lf5	Lf8
Aerial mycelium (ISP4)	light grey	pale pink	ivory	grey	grey	beige	ivory
Substrate mycelium (ISP4)	colorless	brown	brown	blue	colorless	colorless	light brown
Diffusible pigment (ISP3, ISP4)	none	brown	brown	blue, violet	none	none	light brown
Melanoid pigment	–	+	+	–	+	–	–
Spore chains	spiral	right	spiral	spiral	right	right	right
Acid from*	glucose, ribose, trehalose, arabinose, mannose, maltose	glucose, ribose, arabinose, xylose, inositol	glucose, ribose, galactose, sucrose, rhamnose maltose, xylose, inositol	glucose, ribose, arabinose, mannose, maltose, xylose, inositol	glucose, lactose, trehalose, mannose, sucrose, rhamnose, maltose, xylose, inositol, sorbitol, mannitol	n/d	glucose, fructose, sucrose, xylose, rhamnose, raffinose

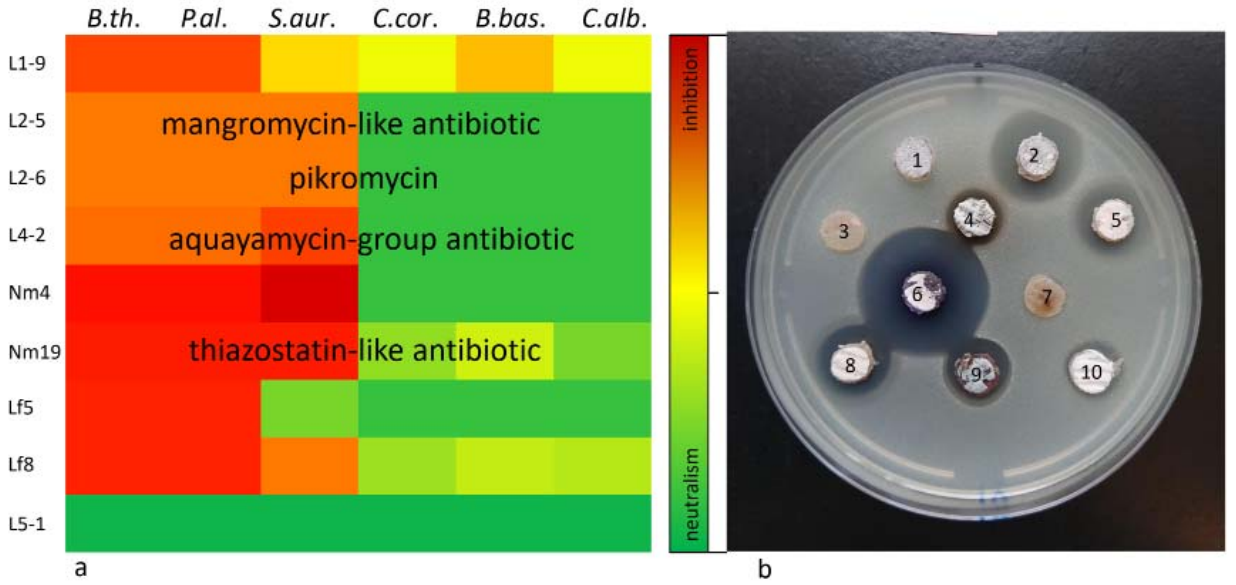

Fig. 4. Antagonistic activity of actinomycete strains, associated with *L. niger*: a — heatmap of antagonism to *Bacillus thuringiensis* (*B.th.*), *Paenibacillus alvei* (*P.al.*), *Staphylococcus aureus* (*S.aur.*), *Conidiobolus coronatus* (*C.cor.*), *Beauveria bassiana* (*B.bas.*), *Candida albicans* (*C.alb*): from the absence of inhibition zones of test organisms (neutralism) to pronounced suppression zones with a width of more than 15 mm (antagonism); b — agar diffusion method for *in vitro* determination of antimicrobial activity against *Staphylococcus aureus* ATCC 25923. The numbers indicate strains: 1 — Lf5; 2 — Nm19; 3 — L5-1; 4 — L2-5; 5 — L4-2; 6 — Nm4; 7 — Lf15; 8 — Lf8; 9 — L2-6; 10 — L1-9.
Рис. 4. Антагонистическая активность актиномицетов, ассоциированных с *L. niger*: а — тепловая карта подавления роста *Bacillus thuringiensis* (*B.th.*), *Paenibacillus alvei* (*P.al.*), *Staphylococcus aureus* (*S.aur.*), *Conidiobolus coronatus* (*C.cor.*), *Beauveria bassiana* (*B.bas.*), *Candida albicans* (*C.alb*): от отсутствия зон ингибирования тест-организмов (нейтрализм) до выраженных зон угнетения шириной более 15 мм (антагонизм); b — определение антимикробной активности *in vitro* в отношении *Staphylococcus aureus* ATCC 25923 методом диффузии в агар. Цифрами помечены штаммы: 1 — Lf5, 2 — Nm19, 3 — L5-1, 4 — L2-5, 5 — L4-2, 6 — Nm4, 7 — Lf15, 8 — Lf8, 9 — L2-6, 10 — L1-9.

Discussion

Symbiotic bacteria strongly associated with insects, such as *Wolbachia* Hertig, 1936, have a long history of study and a great potential for the regulation of insect populations. Coexistence of the macro- and microorganisms is possible through close interactions and an intensive exchange of chemical signals [Baranova *et al*., 2022]. However, it is difficult to prove connections and mutually beneficial ecological relationships between insects and actinobacteria. Unlike bacterial symbionts involved in trophic associations, producers of biologically active substances can be localized not in the digestive tract, but in other places, i.e., in the antennae [Kaltenpoth *et al*., 2005] or on the cuticle [Currie *et al*., 1999], which makes it difficult to detect them. Secondly, it is not easy to prove the continuity of such symbioses, since they may be optional. And, finally, the spectrum of parasite protection of a symbiotic bacterium is often unclear.

Nevertheless, based on the published data and the results of our research, we assume the possibility of the presence of "protective symbioses" in black garden ants similarly to other individual hymenopterans [Kaltenpoth, Engl, 2014]. First, this is corroborated by the stable isolation of mycelial prokaryotes from adult *L. niger*, regardless of the location of the nest. Second, the cultivated actinomycetes are localized in the heads of these insects, similarly to *Lasius fuliginosus* (Latreille, 1798) [Liu *et al*., 2016a; Ye *et al*., 2017] and *Camponotus japonicus* Mayr, 1866 [Li *et al*., 2016; Liu *et al*., 2016b; Cao *et al*., 2017; Jiang *et al*., 2018]. Third, the isolated streptomycetes have a broad-spectrum antibiotic activity and are able to inhibit the growth of widespread entomopathogenic microbes.

L. niger is the most numerous, widespread and robust species of Moscow ant communities. The increased ecological plasticity of this species, which allows it to adapt to changes in the environment, has been repeatedly noted [Putyatina *et al*., 2017]; however, the exact factors determining its outstanding viability are still the subject of research. Diversification of cytochromes and DNA repair systems, along with reduced odorant communication, may be the basis of the polyphagy and resistance to pollutants in *L. niger*, while non-territorial and mobilization strategies allows more efficient exploitation of large but patchy food sources [Konorov *et al*., 2017]. It seems a good working hypothesis that resistance to external stress factors is also associated with the protective or stimulating influence of bacterial symbionts.

As other social insects, *L. niger* possesses a massive and very complex system of exocrine glands producing a great variety of pheromones for the defense systems, alarm communication, nestmate recognition and sex pheromones for males and queens [Niculita *et al*., 2007]. Various species of fungus-growing ants rear antibiotic-producing bacteria in elaborate cuticular crypts, supported by unique exocrine glands, on propleural plates [Currie *et al*., 2006]. In our study, the isolation of actinomycetes from separated body sections of *L. niger* shows that the bacteria are confined to glands located in the head, and not on thorax or abdomen, which seems to be true for *Lasius fuliginosus* and *Camponotus japonicus* as well.

Black garden ants have three types of cephalic glands: the mandibular, postpharyngeal and propharyngeal ones, that may harbor actinomycetes. Trophallaxis is known to play a vital role in colony physiology, nutrient distribution, and communication in these ants [Meurville, Leboeuf, 2021], but the risk of alimentary transmission of infections is very high under these circumstances. The production of antimicrobial substances directly in the oral cavity of ants can protect the family from epizootics, although this assumption requires experimental confirmation.

Bacterial diseases of ants are still undescribed, unlike those caused by various entomopathogenic fungi, probably due to the difficulty of detecting such cases. However, ants are likely to have various mechanisms of protection against bacterial pathogens, from their own immunity to the protective action of symbiotic bacteria. In our study, all streptomyces strains had the ability to produce substances with an antibacterial action. Nevertheless, the role of the genus *Nocardia* seems to be unclear, since phylogenetically similar strains were found in all individuals in the three families located f ar from each other.

Conclusion

The cultivated actinomycete communities were isolated from black garden ants *Lasius niger* collected in the different biotopes of Moscow. It is shown that actinomycete communities are confined to the head sections of adult workers. For all studied ants from three different nests, about 50% of the actinomycete colonies belonged to *Nocardia fluminea*, and the other half was represented by various species of the genus *Streptomyces*.

The dominant strains of streptomycetes had high antagonistic activity against gram-positive bacteria, in particular entomopathogenic bacilli. Some streptomycete strains were also able to limit the growth of fungi, such as *Conidiobolus coronatus* and *Beauveria bassiana*, infecting different species of insects. Despite their abundance, no *Nocardia* strain showed antagonistic action in the tests against the selected microorganisms. Some antimicrobial substances were isolated and preliminarily assigned to the previously described groups of antibiotics (mangromycins, pikromycins, thiazostatins et al.).

Further work in this area will focus on establishing the exact molecular structure of all active metabolites of the actinomycetes, as well as clarifying the localization of possible symbionts and their ability to synthesize antimicrobial metabolites *in vivo*.

Competing interests. The authors declare that they have no conflict of interest.

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"Three-dimensional electron microscopy and spectroscopy") and at Electron Microscopy Laboratory of the Faculty of Biology, Moscow State University. The authors are grateful to R.R. Kharsiev for his help in sampling and testing.

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