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Antibiotic activity of a *Byssochlamys nivea* fungus associated with larva of black soldier fly *Hermetia illucens*

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Abstract: Investigation of microorganisms associated with larva of black soldier fly *Hermetia illucens* led to isolation of a fungal strain named OPG. Phylogenetic analysis revealed that the strain is close to *Byssochlamus nivea* on the basis of genetic and morphological characteristics. The strain exhibited pronounced antibacterial properties and moderate antifungal activity. Moreover, the OPG strain was capable of inhibiting growth of 6 entomopathogenic strains. Isolation of the individual active component led to an unstable compound with tendency to oligomerization into inactive form.

Keywords: Antimicrobial activity; antibiotic, fungi; larva; Hermetia.

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1. Introduction

The search for biologically active substances that overcome antibiotic-resistance is being actively carried out not only among extremophilic fungi [1] but also among inhabitants of unique ecological niches with special habitat conditions. These include the intestines of invertebrates: earthworms, millipedes, ants, termites, etc. [2]. Many actinobacteria (*Streptomyces* spp., *Nocardia* spp., *Pseudonocardia* spp., *Amycolatopsis* spp. etc.) were found to form strong ecological association with insects (ants, wasps, bugs, etc.) and defend their hosts, offspring or food supplies against fungal or bacterial invasions [3–6]. Fungal spores are found in the stomachs and intestinal tracts of invertebrates. The interaction between fungi and invertebrates contributes to the emergence of new compounds that are valuable for further development of antibiotic substances. In this work, we studied microorganisms associated with larva of black soldier fly *Hermetia illucens*. It was possible to isolate a strain of a fungus with a pronounced antibacterial activity, including against gram-negative bacteria.

2. Materials and Methods

2.1. Isolation of microorganisms

Isolation of microorganisms was carried out from the contents of the intestine of a larva of black soldier fly *H. illucens*. Larvae of black soldier fly *H. illucens* were grown on compound feed for laying hens. Larva bodies were refrigerated, soaked in 95% alcohol and dried. Autopsy of the body was performed with sterile scissors and the intestines were removed. The intestines were placed in a sterile plastic tube with a sterile saline

solution and shaken on a vortex. The resulting suspension was triturated with a spatula on a petri plate. Isolation of microorganisms was carried out on potato dextrose agar (PDA) (HiMediaLab).

2.2. Morphology studies

Measurements and descriptions of macromorphological characteristics were made from isolate OPG grown on potato-dextrose agar (PDA, HiMediaLab) and Czapek's agar (CZA, g/L: saccharose – 30.0, $NaNO_3$ – 3.0, K_2HPO_4 – 1.0, KCI – 0.5, $MgSO_4 \times 7H_2O$ – 0.5, $FeSO_4 \times 7H_2O$ – 0.01, agar – 20.0) at 25°C for 7–15 days. Micromorphological observation of sexual (asci, ascospores) and asexual (conidia, chlamydospores) structures were made using the sellotape technique [7]. Slide preparations were stained by lacto-fuchsin (dissolve 0.1 g acid fuchsin in 100 mL 85% anhydrous lactic acid) [8] and observed by light microscopy (Fisherbrand AX-502, Thermo Fisher Scientific). The shape of conidia and ornamentation of surfaces were examined by SEM (Quattro S, Thermo Fisher Scientific). Samples for SEM were prepared according to known procedure [9].

2.3. Gene amplification and phylogenetic analysis

The OPG isolate was grown on PDA for 7 d at 25°C, mycelia were scraped from the surfaces of the media and ground with glass beads and lysing buffer (TrisBase, 50 mM; NaCl, 250 mM; EDTA, 50 mM; SDS, 0.3%; pH 8). Genomic DNA was extracted using a protocol described by Zhang and Li (2009). Primers ITS1f (5′-CTTGGTCATTTAGAGAAGTA) and NL4 (5′-GGTCCGTGTTTCAAGACGG) were used for amplification the D1/D2 region of the large subunit (LSU) rDNA gene. The thermal cycling program was performed with 35 cycles after an initial denaturation at 95°C for 6 min. Each cycle included a denaturation step at 95°C for 30 s, annealing at 59°C for 40 s, an extension step at 72°C for 40 s and a final elongation step of 7 min at 72°C. PCR products were separated by electrophoresis on a 1% agarose gel. The amplicons were purified and sequenced using a commercial service (EvroGen, Moscow). For identification, data of the GenBank (ncbi.nlm.nih.gov) and MycoID (www.mycobank.org) databases were used. Phylogenetic analyses were conducted using MEGA version X [10].

2.4. Fermentation and Antimicrobial Assay

For antibiotic production, the *Byssochlamus nivea* strain OPG was cultivated in organic potato dextrose broth medium (200 mL in a 750-mL Erlenmeyer flask) at 28°C with shaking (200 rpm) for 14 days. Antifungal and antibacterial activities of the *B. nivea* strain OPG were monitored by agar-diffusion assay using a number of test microorganisms: *Candida albicans* ATCC 14053, *Aspergillus niger* ATCC 16404, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853.

Antagonistic activity of the *B. nivea* strain OPG against entomopathogenic fungi was determined by dual-culture assay [11] and cross streak assay [12] (Fig. 1). The following entomopathogenic strains were used: *Beauveria bassiana* VKPM F-1357, *Conidiobolus coronatus* (*Entomophthora coronate*) VKPM F-1359, *Metarhizium rileyi* (*Nomuraea rileyi*) VKPM F-1360, *Ophiocordyceps sinensis* (*Cordyceps sinensis*) VKPM F-1479, *Conidiobolus coronatus* VKPM F-442, and *Lecanicillium lecanii* (*Verticillium lecanii*) VKPM F-837.

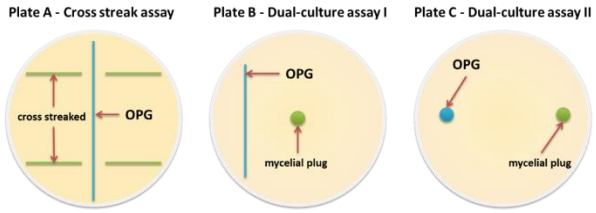

Figure 1. Design of the antagonistic activity testing experiments: plate A, Cross streak assay [12], plate B, Dual-culture assay I [11] and II.

In vitro antimicrobial activity of the isolated compound was determined using the broth microdilution method, as described in the CLSI guidelines document M100-S25 [13–15].

2.5. Isolation of antibiotics

Culture liquid of *B. nivea* strain OPG was passed through a filter on a Buchner funnel under vacuum to separate it from the mycelium. The filtrate was extracted with ethyl acetate (thrice-repeated extraction with 20% of culture liquid volume). Combined organic layers were concentrated to dryness *in vacuo* (at 42°C), taken up in 50% EtOH and separated on silica gel (40–63 µm, column size 40×60 mm, CH_2Cl_2/MeOH step elution, v/v 50:1, 20:1, 10:1, 0:100, 100 mL each). Solvent removal from fractions with solvent ratio from 20:1 to 10:1 yielded an antibiotic concentrate. Further analysis was performed with reverse phase HPLC on a 4.6×250 mm column (C18, Beckman, Ultrasphere, 5 µm). Eluent A was deionized water, eluent B was acetonitrile. The column was eluted at a flow rate of 1 mL/min: 0–20 min 95:5 → 85:15 (A:B, v/v). Detection wavelength was 205 nm. Isolation of compounds was carried out using preparative HPLC on a 250×21.2 mm column (ZORBAX SB-C18, 7 µm). The column was eluted at a flow rate of 20 mL/min: 0–14 min 94:6 → 94:6; 14–15 min 94:6 → 5:95; 15–24 min 5:95 → 5:95 (A:B, v/v), fractions containing active compound were collected and solvent was removed.

3. Results

3.1. Isolation and Characterization of B. nivea strain OPG

Figure 2. The objects of study: (A) – larvae of black soldier fly *H. illucens*; (B) – adult black soldier fly.

We studied microorganisms associated with larva of black soldier fly *H. illucens* (Fig. 2A, 2B). The contents of the intestines of two larvae were applied on potato-dextrose agar, yielding only one cultured microorganism, showing antagonistic activity against fungi, yeast, gram-positive and gram-negative bacteria.

3.2. Morphological characterization

After one week at 30°C, colonies of OPG exhibited moderate growth on CZA (Fig. 3A, 3B).

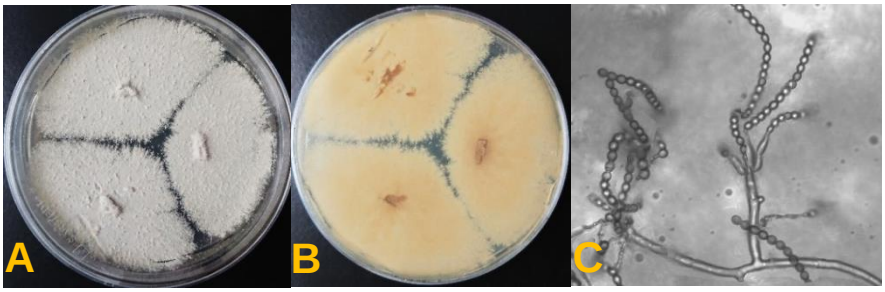

Figure 3. Growth of isolate OPG on CZA 14 d at 30C (A, B): conidiophores, phialides and conidia in chains (optical microscope)(C).

Microscopic imaging (Fig. 4, 3C) showed the morphology of the strain. Chlamydospores are present, finely roughened (Fig. 4A). Ascospores are ellipsoidal, smooth, 4.0–4.5×2.7–3.2 μm size (Fig. 4B). Conidiophores are irregularly branched and asexual conidia are predominantly globose to subglobose, with a flattened base, 2.4–2.6×2.7–2.9 μm size (Fig. 4C).

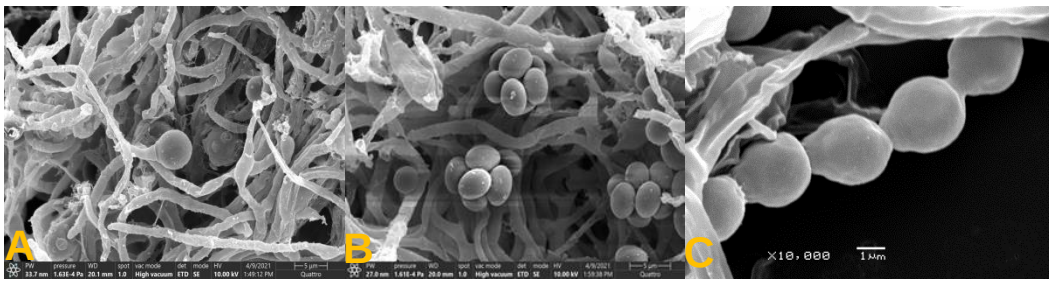

Figure 4. Scanning electron microscopy of strain OPG grown on PDA media for 14 days at 28°C: (A) - mycelium with chlamydospore, (B) - mycelium with ascospores, (C) - asexual spore.

3.3. DNA sequencing

The phylogenetic tree was constructed by the Neighbor-Joining method on the ITS region (Fig. 5). It convincingly shows that the OPG strain is a member of genus *Byssochlamys* and is clustered very closely with *B. nivea* (Fig. 5), with bootstrap value of more than 60%.

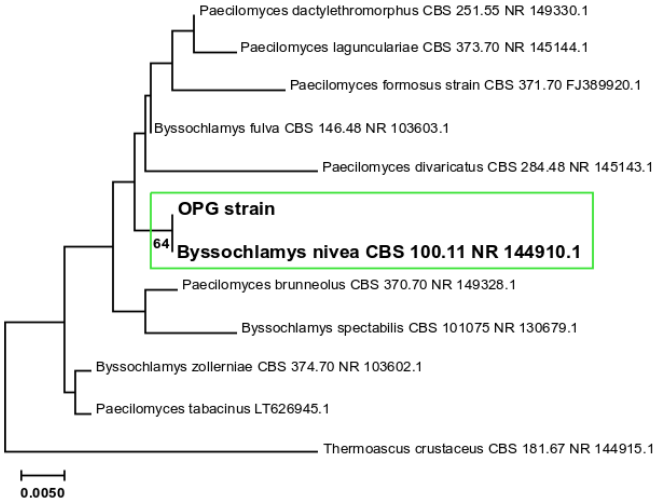

Figure 5. Neighbor-joining phylogenetic tree based on 18S rRNA gene sequence analysis showing the position of *B. nivea* strain OPG and related species.

On the basis of the genetic and morphological characteristics, isolate OPG was identified as a member of genus *Byssochlamys*, with *B. nivea* as the closest taxon.

3.4. Antimicrobial properties of B. nivea strain OPG

The OPG strain was cultured and extracted as described above. Culture fluid extracts exhibited pronounced antibacterial properties and moderate antifungal activity (Table 1).

Table 1. Antimicrobial properties of *B. nivea* strain OPG

Test strain	Growth inhibition*		
	Culture liquid before extraction	Extract	Culture liquid after extraction
A. niger INA 00760	-	+	-
C. albicans CBS 8836	-	+	-
B. subtilis ATCC 6633	+	+++	-

E. coli ATCC 25922	++	+++	+

* Growth inhibition data: −—no inhibition; +—weak inhibition; ++—moderate inhibition; +++—high inhibition.

Since the *B. nivea* strain OPG was isolated from the intestine of a larva of the black soldier fly *H. illucens*, we decided to test it for antagonistic activity against entomopathogenic strains. The OPG strain significantly inhibited growth of the majority of the tested entopomathogens (Table 2).

Table 2. Antagonism of *B. nivea* strain OPG

Test strain	Growth inhibition rate (%)* from		Growth inhibition from cross streak assay
	dual-culture assay I	dual-culture assay II	
O. sinensis VKPM F-1479	59±4	55±9	+++
C. coronatus VKPM F-1359	41±6	37±8	+++
B. bassiana VKPM F-1357	71±4	71±4	++
C. coronatus VKPM F-442	48±2	48±2	+++
M. rileyi VKPM F-1360	35±9	44±17	+++
L. lecanii VKPM F-837	6±5	7±5	+

* Growth inhibition rate (%) determined at 14 days after inoculation with entomopathogens on *B. nivea* strain OPG. Data are means ± standard error over three replicates. −—no inhibition; +—weak inhibition; ++—moderate inhibition; +++—high inhibition.

We isolated the individual active component of OPG extracts using a combination of chromatographic approaches with preparative HPLC as the final step. The resulting compound exhibited relatively low *in vitro* antimicrobial activity: 16–32 μg/mL against gram-negative bacteria, 32–128 μg/mL against gram-positive bacteria and 128 μg/ml against yeast and fungi.

4. Discussion

Known for its presence in pasteurised fruit, *B. nivea* is able to produce various biologically active compounds, in particular mycotoxin patulin, a heat-stable lactonethat is active against Gram-positive and Gram-negative bacteria. Moreover, *B. nivea* forms toxic extrolites, such as byssotoxin A and byssochlamic acid [16]. Besides mycotoxins, an antitumor metabolite, byssochlamysol, a steroid against IGF-1 dependent cancer cells, is also produced by *B. nivea* [17].

The study of microorganisms associated with larva of black soldier fly *H. illucens* led to the isolation of a micromycete morphologically and genetically close to *B. nivea*. This strain has a pronounced antagonistic activity against entomopathogens, suggesting possible involvement of the strain in protective symbiosis with the studied insects.

The *B. nivea* strain OPG isolated in this work also exhibits pronounced antimicrobial activity. High and selective antagonistic activity against gram-negative bacteria makes the OPG strain promising for further study. We managed to isolate the individual active compound with preparative HPLC separation. Nevertheless, the activity of the isolated component, determined using the serial dilutions method, turned out to be significantly lower than could be expected based on the data of disk-diffusion analysis of the culture fluid and enriched fractions. We assume that this is due to rapid oligomerization of the compound into an inactive form in acidic conditions or in DMSO. In combination with the low solubility of the compound in most solvents, this significantly complicates its chemical study and determination of its structure. Therefore, to further clarify the physicochemical and biological properties of this antibiotic, it is necessary to work out separation conditions that would prevent the oligomerization or inactivation of the substance.

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References

1. Baranova, A.A.; Alferova, V.A.; Korshun, V.A.; Tyurin, A.P. Antibiotics from extremophilic micromycetes. *Russ. J. Bioorg. Chem.* **2020**, *46*, 903–971, doi:10.1134/S1068162020060023.
2. Baranova, A.A.; Chistov, A.A.; Tyurin, A.P.; Prokhorenko, I.A.; Korshun, V.A.; Biryukov, M.V.; Alferova, V.A.; Zakalyukina, Y.V. Chemical ecology of *Streptomyces albidoflavus* strain A10 associated with carpenter ant *Camponotus vagus*. *Microorganisms* **2020**, *8*, 1948, doi:10.3390/microorganisms8121948.
3. Seipke, R.F.; Barke, J.; Brearley, C.; Hill, L.; Yu, D.W.; Goss, R.J.M.; Hutchings, M.I. A single *Streptomyces* symbiont makes multiple antifungals to support the fungus farming ant *Acromyrmex octospinosus*. *PLoS One* **2011**, *6*, e22028, doi:10.1371/journal.pone.0022028.
4. Kaltenpoth, M.; Engl, T. Defensive microbial symbionts in Hymenoptera. *Funct. Ecol.* **2014**, *28*, 315–327, doi:10.1111/1365-2435.12089.
5. Haeder, S.; Wirth, R.; Herz, H.; Spiteller, D. Candicidin-producing *Streptomyces* support leaf-cutting ants to protect their fungus garden against the pathogenic fungus *Escovopsis*. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 4742–4746, doi:10.1073/pnas.0812082106.
6. Kroiss, J.; Kaltenpoth, M.; Schneider, B.; Schwinger, M.-G.; Hertweck, C.; Maddula, R.K.; Strohm, E.; Svatoš, A. Symbiotic streptomycetes provide antibiotic combination prophylaxis for wasp offspring. *Nat. Chem. Biol.* **2010**, *6*, 261–263, doi:10.1038/nchembio.331.
7. Schubert, K.; Groenewald, J.Z.; Braun, U.; Dijksterhuis, J.; Starink, M.; Hill, C.F.; Zalar, P.; de Hoog, G.S.; Crous, P.W. Biodiversity in the *Cladosporium herbarum* complex (*Davidiellaceae*, *Capnodiales*), with standardisation of methods for *Cladosporium* taxonomy and diagnostics. *Stud. Mycol.* **2007**, *58*, 105–156, doi:10.3114/sim.2007.58.05.
8. *Laboratory Protocols in Fungal Biology: Current Methods in Fungal Biology*; Gupta, V.K., Tuohy, M.G., Ayyachamy, M., Turner, K.M., O'Donovan, A., Eds.; Springer New York: New York, NY, 2013; ISBN 978-1-4614-2355-3.
9. Glauert, A.M. *Fixation, Dehydration and Embedding of Biological Specimens. Practical Methods in Electron Microscopy*; 1st ed.; 1974; Vol. 3.
10. Tamura, K.; Peterson, D.; Peterson, N.; Stecher, G.; Nei, M.; Kumar, S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **2011**, *28*, 2731–2739, doi:10.1093/molbev/msr121.
11. Gao, Y.; Zeng, X.D.; Ren, B.; Zeng, J.R.; Xu, T.; Yang, Y.Z.; Hu, X.C.; Zhu, Z.Y.; Shi, L.M.; Zhou, G.Y.; et al. Antagonistic activity against rice blast disease and elicitation of host-defence response capability of an endophytic *Streptomyces albidoflavus* Osilf-2. *Plant Pathol.* **2020**, *69*, 259–271, doi:10.1111/ppa.13118.
12. Muthukrishnan, P.; Chithra Devi, D.; Mostafa, A.A.; Alsamhary, K.I.; Abdel-Raouf, N.; Nageh Sholkamy, E. Antimicrobial efficacy of *Nocardiopsis* sp. MK_MSt033 against selected multidrug resistant clinical microbial pathogens. *J. Inf. Publ. Health* **2020**, *13*, 1522–1532, doi:10.1016/j.jiph.2020.06.025.
13. Clinical and Laboratory Standards Institute (CLSI). Performance Standards for Antimicrobial Susceptibility Testing: Twenty Fifth Informational Supplement. CLSI Document M100-S25. Wayne, PA: CLSI; 2015.
14. CLSI Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard, 10th Ed. CLSI Document M07-A10, Wayne, PA, 2015.
15. CLSI. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard, 3rd Ed, CLSI Document M27–A3, Clinical and Laboratory Standards Institute, Wayne, PA. 2008.
16. Samson, R.A.; Houbraken, J.; Varga, J.; Frisvad, J.C. Polyphasic taxonomy of the heat resistant ascomycete genus *Byssochlamys* and its *Paecilomyces* anamorphs. *Persoonia* **2009**, *22*, 14–27, doi:10.3767/003158509X418925.
17. Mori, T.; Shin-Ya, K.; Takatori, K.; Aihara, M.; Hayakawa, Y. Byssochlamysol, a new antitumor steroid against IGF-1-dependent cells from *Byssochlamys nivea*. II. Physico-chemical properties and structure elucidation. *J. Antibiot.* **2003**, *56*, 6–8, doi:10.7164/antibiotics.56.6.