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EXPERIMENTAL ARTICLES

# Secondary Metabolite Profiles of the *Penicillium* Fungi Isolated from the Arctic and Antarctic Permafrost as Elements of Polyphase Taxonomy

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**Abstract**—The secondary metabolite profiles of the fungi of the subgenus *Penicillium* of the genus *Penicillium* isolated from permafrost were studied. Most of the tested strains synthesized biologically active alkaloids and polyketides. A novel producer of fumiquinazolines F and G was found. Species names of the strains were defined more exactly on the basis of their secondary metabolite profiles and micro- and macromorphological characteristics.

*Keywords*: microscopic fungi, subgenus *Penicillium*, secondary metabolites, taxonomy.

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The generally accepted identification of the *Penicillium* fungi by micro- and macromorphological characteristics seldom gives unambiguous results, particularly for the isolates from anthropogenically disturbed and poorly studied extreme habitats. In polyphasic taxonomy, the new system that has been used more often in recent years to identify the fungi of the subgenus *Penicillium*, secondary metabolite profiles are applied together with the micro- and macromorphological characteristics of the strains [1, 2]. Thus, it is supposed that the spectrum of secondary metabolites produced by the fungi isolated from insufficiently studied extreme habitats may contribute to more exact identification of their species affiliation.

The goal of this work was to study the profiles of secondary metabolites in the fungal strains of the subgenus *Penicillium* (genus *Penicillium*) isolated from permafrost and to clarify their taxonomic positions.

## MATERIALS AND METHODS

The subjects of research were 18 fungal strains of the genus *Penicillium* from the All-Russian Collection of Microorganisms (VKM), Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences (IBPM RAS). The strains were isolated from permafrost sediments of the Kolyma Lowland (VKM FW-809, VKM FW-1447, VKM FW-2251, VKM FW-2600, VKM FW-2604, VKM FW-2615, VKM FW-2648, VKM FW-2851, VKM FW-2876), from cryopegs of the Kolyma Lowland (VKM FW-869, VKM FW-2753), from permafrost soils of Antarctica (VKM FW-2881), from coastal waters of Antarctica (VKM FW-2921, VKM FW-2928), and from frozen volcanic ash of the Kamchatka Peninsula (VKM FW-2665, VKM FW-2852, VKM FW-2853, VKM FW-2854) [3, 4].

The strains were identified by macro- and micromorphological characteristics in 7-day cultures grown at 5, 15, and 37°C on the following agarized media: CYA (Czapek yeast autolysate), MEA (malt extract agar), G25N (25% glycerol nitrate agar), YEA (yeast extract sucrose agar), and CYAS (Czapek yeast autolysate with 5% NaCl) [1, 5]. Production of secondary metabolites was studied in the fungi grown in submerged culture in the medium containing the following (g/L distilled water): mannitol, 50.0; succinic acid, 5.4; $MgSO_4 \cdot 7H_2O$, 0.3; $KH_2PO_4$, 1.0; pH value was adjusted to 5.4 with 25% $NH_4OH$ solution. The fungi were grown in 150 mL of the medium in 750-mL flasks at 24 ± 1°C on a shaker (220 rpm). The medium was inoculated with aqueous suspensions of conidia ($1–2 \times 10^7$ spores/mL) from 14-day cultures grown on wort agar slants. The samples were taken on day 11 of cultivation.

Acidic, neutral, and alkaline extracellular metabolites were obtained from the filtered culture liquid by threefold extraction with chloroform according to the procedure described previously [6]. The extracts were analyzed by thin-layer chromatography (TLC) on silica gel plates (Silica gel $F_{254}$, Merck, Germany) in the chloroform–methanol–25% $NH_4OH$ systems I and

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II: 90 : 10 : 0.1 (I) and 80 : 20 : 0.2 (II). The substances were detected by absorption and fluorescence in ultraviolet light and after spraying the plates with the Dragendorff reagent (for nitrogen-containing metabolites), Ehrlich reagent (for indole alkaloids), and 5% $FeCl_3$ solution in methanol (for the phenolic group).

The metabolites were isolated and purified by preparative TLC on silica gel plates and identified by cochromatography with the standard samples previously obtained at the Laboratory of Secondary Metabolites (IBPM RAS) and by other physicochemical methods. The UV spectra of compounds in methanol were recorded with a UV-160A spectrophotometer (Shimadzu, Japan). The mass spectra of compounds were recorded with an LCQ Advantage MAX mass spectrometer (Thermo Finnigan, Germany) using a single-channel syringe pump for injecting the samples directly into the chamber for chemical ionization under atmospheric pressure. The Xcalibur software package was used for collecting and processing the data of mass spectrometry. More complete information for detection of the metabolites was obtained by analyzing the positive and negative ions. MS/MS spectra were recorded at the normalized collision energy of 20–40%.

Epimerization of metabolite 10 (fumiquinazoline F) into metabolite 11 (fumiquinazoline G) was performed as follows. The sample of metabolite 10 (1 mL) was incubated in 100 μL of 40% KOH–MeOH solution (1 : 99) for 16 h. The volume of the solution was brought to 1 mL with distilled $H_2O$, acidified with 0.1 M HCl solution to ~pH 4, and extracted with chloroform (vol/vol). The chloroform extract was dried over $Na_2SO_4$ and evaporated in a rotor evaporator. The solid residual was analyzed by TLC in solvent systems I and II.

## RESULTS

Secondary metabolite profiling of fungal strains isolated from different permafrost regions showed that 17 out of 18 cultures were able to synthesize low-molecular compounds of various types (Table 1). The strains could be divided into groups depending on the range of produced secondary metabolites. The most abundant group including the strains VKM FW-1447, VKM FW-2753, VKM FW-2851, VKM FW-2853, VKM FW-2876, VKM FW-2881, VKM FW-2852, and VKM FW-2854 synthesized metabolite 1 with $R_f = 0.25$ (I), which was stained purple with the Ehrlich's reagent, indicating the presence of an indolic structure in this compound. Based on the physicochemical characteristics of the isolated metabolite, which almost coincided with the literature data [7], and direct comparison with the standard sample, this compound was identified as a clavine-type ergot alkaloid α-cyclopiazonic acid (CPA). The strains VKM FW-2852 and VKM FW-2854 were shown to have three more indole-containing metabolites. The UV spectra of the isolated metabolites were typical of clavine alkaloids (Table 1). The molecular mass of metabolite 2 (240 Da) and the pattern of fragmentation in its mass spectrum corresponded to the structure of clavine-type ergot alkaloids: festuclavine and its isomers pyroclavine, costaclavine, and epicostaclavine. The mass spectra and molecular masses of metabolites 3 and 4 (298 and 256 Da) were typical of fumigaclavine A and fumigaclavine B and their isomers: isofumigaclavine A and isofumigaclavine B, respectively (Table 1). The final and unambiguous evidence of the identity of metabolites 2, 3, and 4 to festuclavine, fumigaclavine A, and fumigaclavine B, respectively, but not to the respective isomers with similar properties, was obtained as a result of cochromatography with the standard samples of these compounds. Solvent systems I and II were used as developers.

The next group, represented by the strains VKM FW-2600, VKM FW-2665, VKM FW-2604, VKM FW-2615, VKM FW-2921, and VKM FW-2928, synthesized metabolites 5 and 6 identified as benzodiazepine alkaloids (cyclopenin and cyclopeptin) on the basis of physicochemical properties. The strain VKM FW-2615 was also shown to possess metabolite 7 identified as a cyclopenol (Table 1). The strains VKM FW-2604 and VKM FW-2921, in addition to these alkaloids, synthesized metabolite 8 corresponding to the quinoline alkaloid viridicatin according to its physicochemical properties. All these metabolites are connected by a single biosynthetic chain; their precursors are phenylalanine, anthranilic acid, and methionine. The first metabolite in this pathway is cyclopeptin; cyclopenin and cyclopenol synthesized from this metabolite are further transformed into the quinoline alkaloids viridicatin and viridicatol.

The strain VKM FW-2251 synthesized metabolite 9 identical by its physicochemical properties to griseofulvin, a polyketide metabolite (Table 1).

Metabolites 10 and 11 with $R_f = 0.35$(I) and $R_f = 0.27$(I), staining lilac with the Ehrlich's reagent and giving a positive reaction with the Dragendorff's reagent, were found in the strain VKM FW-869. The UV spectra of these compounds had the same absorption bands, corresponding to an indolic chromophore at 271, 284, 292 nm, and additional shoulders in the regions of 306 and 322 nm. The mass spectra and molecular masses of metabolites 10 and 11 (358 Da), according to the literature data [8], corresponded to the values for quinazoline alkaloids (fumiquinazolines F and G) differing in the *cis*- and *trans*-positions of the $CH_3$ group at position 3 (Figure). These metabolites are epimers and have different thermodynamic characteristics [8]. An experiment on epimerization of metabolite 10 by the above-described method was performed to confirm the fact that metabolites 10 and 11 were stereoisomers. It was shown that epimerization reaction resulted in formation of a mixture of two compounds at a ratio of 3 : 2, which corresponded to

**Table 1.** Physicochemical properties of isolated metabolites

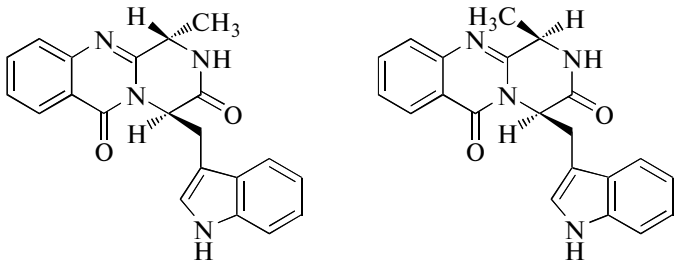
| Metabolite no. | Standard sample | Staining with reagents | | | Chromatographic mobility in systems | | UV spectrum, $\lambda_{max}$, nm | Molecular ion and characteristic peaks in MS/MS spectra | |
|---|---|---|---|---|---|---|---|---|---|
| | | Ehrlich | $FeCl_3$ | Dragendorff | I | II | | $[M-H]^-$ | $[M+H]^+$ |
| 1 | CPA | Purple | Brown | Orange | 0.25 | 0.55 | 223, 251(pl.), 281, 290 | 335, 180, 140 | 337, 182, 196 |
| 2 | Festuclavine | ″ | –* | ″ | 0.08 | 0.22 | 224, 275, 280, 292 | 239, 238, 223, 208 | 241, 210 |
| 3 | Fumigaclavine A | ″ | – | ″ | 0.20 | 0.47 | 224, 275, 282, 292 | 297, 237 | 299, 24 |
| 4 | Fumigaclavine B | ″ | – | ″ | 0.05 | 0.10 | 222, 275, 281, 293 | 255, 185, 154 | 257, 239 |
| 5 | Cyclopenin | Yellow-green | Dark green | ″ | 0.50 | 0.70 | 213, 290 | 293, 250, 236, 222, 159 | 295, 264, 251, 239, 177 |
| 6 | Cyclopeptin | ″ | ″ | ″ | 0.50 | 0.70 | 213, 290 | 279, 226 | 281, 253, 134, 120 |
| 7 | Cyclopenol | ″ | ″ | ″ | 0.45 | 0.65 | 211, 285 | 309, 252, 238, 175 | 311, 177 |
| 8 | Viridicatin | – | ″ | ″ | 0.40 | 0.60 | 225, 240, 287, 309, 330 | 236, 208 | 238, 223, 192, 132 |
| 9 | Griseofulvin | – | Gray | – | 0.30 | 0.45 | 236, 252, 291, 324 | 351, 319, 307, 137 | 353, 321, 285, 215, 165 |
| 10 | Fumiquinazoline F | Lilac | – | Orange | 0.35 | 0.55 | 207, 219, 270, 278, 289, 306, 322 | 357, 228 | 359, 230 |
| 11 | Fumiquinazoline G | ″ | – | ″ | 0.27 | 0.46 | ″ | ″ | ″ |
| 12 | PC-2 | Pale pink | – | – | 0.55 | 0.75 | 207, 230, 280 | – | 213, 195, 167, 139, 125, 111 |

* does not form.

metabolites 10 and 11 by their chromatographic characteristics. It unambiguously indicates that the isolated metabolites were fumiquinazolines F and G. In addition to fumiquinazolines, this strain was also shown to possess metabolite 12, which stained pale pink with the Ehrlich's reagent. The UV spectrum, molecular mass (212 Da) and mass spectrum of the metabolite (Table 1) corresponded to the polyketide metabolite PC-2 [7]. Previously, the strain VKM FW-2648 was shown to synthesize two indole-containing metabolites [9] also identical to fumiquinazolines F and G in their physicochemical characteristics.

## DISCUSSION

Novel producers of biologically active compounds structurally related to ergot alkaloids, benzodiazepine, quinoline, quinazoline alkaloids, and polyketides were found (Table 2). Most of the strains synthesized CPA, cyclopenin, and cyclopeptin—the ergot alkaloids usually referred to as mycotoxins based on their physiological activity. CPA is one of the most dangerous mycotoxins. Its toxic effect is characterized by rapid development of the symptoms affecting the central nervous system and necrotic changes in the internal organs [2]. It should be noted that CPA, being the most widespread metabolite produced by permafrost isolates, has already been found earlier in four strains isolated from the same habitats [9]. One of the strains synthesized griseofulvin, which is known as a fungicidal antibiotic but rarely used today because of its high toxicity. Two strains synthesized fumiquinazolines F and G possessing antitumor activity [8]. The strains were isolated from different regions of the Arctic and Antarctic; therefore, with due regard to the global warming predictions, our data on the toxicogenic potential of these fungi are certainly interesting to the experts in human and animal health protection.



Fumiquinazoline F Fumiquinazoline G

Structures of fumiquinazolines.

The *Penicillium* strains freshly isolated from permafrost sediments were identified on the basis of distinct micromorphological features and growth rates on diagnostic nutrient media at different temperatures and assigned to the subgenus *Penicillium* (the species *P. griseofulvum* (section *Penicillium*, series *Urticicolae*), *P. viridicatum* (section *Viridicata*, series *Viridicata*), *P. verrucosum* (section *Viridicata*, series *Verrucosa*) [1, 5] and to the subgenus *Furcatum* (*P. canescens*) (Table 2). The earlier studies have shown that it is difficult to exactly diagnose the species of psychrotolerant fungi at the moment of their isolation from natural substrates because of the temperature optimum shift towards low temperatures [10–12]. Moreover, the species *P. canescens* is known to be closely related to terverticillate *Penicillium* fungi of the subgenus *Penicillium* [13]. Hence, more exact diagnoses of the species were made on the basis of secondary metabolite profiles used in the diagnostics of the *Penicillium* fungi [1]. Their application is based on empirical observations of the common physiological and biochemical characteristics in phylogenetically related organisms. The potential and actual production of secondary metabolites is a part of the physiological/biochemical identification. For example, the chemotaxonomic markers of the species are as follows: griseofulvin, CPA, patuline, and roquefortine C for *P. griseofulvum*; ochratoxin A, citrinine, verrucolone, and verrucines for *P. verrucosum*; and penicillic acid, brevianamide A, xanthomegnins, and viridicatinic

**Table 2.** Species diagnoses of the strains after repeated identification based on the morphological characteristics and secondary metabolite production

| VKM FW- | Initial species diagnosis | Identified metabolites | Final species diagnosis |
|---|---|---|---|
| 1447, 2753, 2851, 2853, 2876, 2881 | *P. viridicatum* Westling | CPA | *P. commune* Thom |
| 2852, 2854 | " | CPA, fumigaclavine A, festuclavine, fumigaclavine B | *P. palitans* Westling |
| 2604, 2921 | " | Cyclopenin, cyclopeptin, viridicatin | *P. solitum* Westling |
| 2615 | " | Cyclopenin, cyclopeptin, cyclopenol | |
| 2928 | " | Cyclopenin, cyclopeptin | |
| 2600, 2665 | *P. verrucosum* Dierckx | Cyclopenin, cyclopeptin | *P. solitum* Westling |
| 809 | " | – | *P. viridicatum* Westling |
| 869 | *P. griseofulvum* Dierckx | Fumiquinazoline F and G, PC-2 | *P. thymicola* Frisvad et Samson |
| 2648 | *P. canescens* Sopp | Fumiquinazoline F and G | |
| 2251 | *P. griseofulvum* Dierckx | Griseofulvin | *P. griseofulvum* Dierckx |

acid for *P. viridicatum* [1]. Among the cultures studied, only the strain VKM FW-2251 showed the correspondence of its chemotaxonomic marker to the certain species *P. griseofulvum*. Other cultures divided into several groups depending on the range of synthesized metabolites. Each group of the strains adapted to maintenance under laboratory conditions was characterized by an identical set of macro- and micromorphological characteristics.

CPA only was identified in the strains VKM FW-1447, VKM FW-2753, VKM FW-2851, VKM FW-2853, VKM FW-2876, and VKM FW-2881. Production of CPA alone is characteristic of a single species: *P. camemberti* Thom. Chemotaxonomic markers of other CPA-producing *Penicillium* species of the series *Camemberti* may be other compounds as well: rugulovasines for *P. commune* and fumigaclavines for *P. palitans*. Taking into account the pronounced cultural and morphological characteristics, and the fact that the ability to synthesize rugulovasines could have been lost during long-term survival (for more than 100 thousand years) in permafrost sediments, all of the above strains were assigned to the species *P. commune* (Table 2).

Production of CPA, fumigaclavines A and B, and festuclavine, as well as the morphological characteristics of the strains VKM FW-2852 and VKM FW-2854 isolated from permafrost volcanic ashes of the Kamchatka Peninsula unambiguously indicated their affiliation with the species *P. palitans*. It is known that the production of such set of metabolites is characteristic of this species only [1].

The strains VKM FW-2600, VKM FW-2604, VKM FW-2615, VKM FW-2665, VKM FW-2921, and VKM FW-2928 isolated from the Arctic and Antarctic sediments of different age and type were assigned to the section *Viridicata* based on their morphological characteristics. Species identification within this section is often problematic due to the presence of the common interspecies morphological characteristics and, hence, it is of prime importance to determine the ability of these strains to produce secondary metabolites of diagnostic value. The substances of the same metabolic family (cyclopenins and viridicatines) were identified in the tested strains (Table 2). These compounds are chemotaxonomic markers of the species *P. cyclopium* Westling, *P. freii* Frisvad et Samson, *P. neoechinulatum* (Frisvad, Filtenborg et Wicklow) Frisvad et Samson, *P. polonicum* K.M. Zalessky in the series *Viridicata*, and of the species *P. discolor* Frisvad et Samson, *P. echinulatum* Fassatiova, and *P. solitum* Westling in the series *Solita*. The morphological characteristics displayed on the diagnostic media adapted to maintenance of the cultures under laboratory conditions, as well as the biosynthesis of cyclopenins and viridicatins, were a basis for assigning the strains VKM FW-2600, VKM FW-2604, VKM FW-2615, VKM FW-2665, VKM FW-2921, and VKM FW-2928 to the species *P. solitum*. The marker metabolites of other cyclopenin- and viridicatin-producing species may be other compounds as well. For example, biosynthesis of the following compounds is obligatory for the species: chaetoglobosin for *P. discolor*, penicillic acid and aurantiamine for *P. neoechinulatum*, penicillic acid, verrucofortines (=puberulins), viridicatols and anacin for *P. polonicum*, penicillic acid and verrucofortines for *P. cyclopium*, and penicillic acid and auranthiamine for *P. freii*.

The strains VKM FW-869 and VKM FW-2648 were characterized by low growth rate and psychrotolerance (the former strain was also halotolerant). According to the data of [1], these ecophysiological properties are typical of the four *Penicillium* species of the section *Viridicata*: *P. verrucosum, P. thymicola* Frisvad et Samson (series *Verrucosa*), *P. camemberti* Thom (series *Camemberti*), and *P. radicicola* Overy et Frisvad (series *Corymbifera*). Production of fumiquinazolines and micromorphological characteristics of the strains VKM FW-869 and VKM FW-2648 confirm their affiliation with the species *P. thymicola*. *P. thymicola* is a novel species assigned by Frisvad and Samson to the section *Viridicata* of the series *Verrucosa* [1]. It differs from the related *P. verrucosum* and *P. nordicum* Dragoni et Marino in the composition of produced metabolites, rough conidia, and yellow reverse of CYA-grown colonies Chemotaxonomic markers of the species *P. thymicola*, in addition to fumiquinazolines, are verrucolone and alantrypinone; the synthesis of serantrypinone, aurantianine, verrucolone and daldinin D has been also shown for representatives of this species. Only a few strains of the species *P. thymicola* are presently known; they have been isolated in different geographical regions from the air, herbarium material, and soil [5]. The strain VKM FW-869 was isolated in the Arctic from cryopeg water aged 100–120 thousand years and the strain VKM FW-2648 was isolated from the modern permafrost of the Kolyma river bank. It should be noted that previously the strain VKM FW-2648 was assigned to the species *P. canescens* (subgenus *Fucatum*, section *Divaricatum*) based on some micromorphological characteristics displayed by a fresh isolate on diagnostic media [9].

No biosynthesis of nitrogen-containing metabolites and polyketides was found in the strain VKM FW-809. The morphological characteristics of the strain VKM FW-809 corresponded to those of the species *P. verrucosum*. However, it is known that the synthesis of ochratoxin A is obligatory for representatives of the species *P. verrucosum* and *P. nordicum* [5]. Therefore, the above strain cannot be assigned to *P. verrucosum*.

Thus, secondary metabolites have been studied in the *Penicillium* which are components of the ancient microbial communities of permafrost sediments of different genesis and age. Most of the strains under study produced secondary metabolites related to ergot alkaloids (CPA, festuclavine, fumigaclavines A and B), benzodiazepine (cyclopenins), quinoline (viridi-

catin), and quinazoline (fumiquinazolines F and G) alkaloids. Polyketides (griseofulvin and PC-2) were also found. Many of them are mycotoxins. The novel producers of physiologically active quinazoline substances, fumiquinazolines F and G possessing antitumor activity, were found. These fungi were usually characterized by the biosynthesis of metabolites of a single biosynthetic family, while the *Penicillium* fungi from modern habitats produce a broader set of chemical compounds. In spite of certain limitations, determination of the chemotaxonomic markers contributes to more exact diagnosing of fungal strains of the genus *Penicillium* in the cases when cultural and morphological characteristics of the strains do not correspond to diagnostic descriptions of the species after their long-term survival in permafrost sediments.

Based on the above, it may be concluded that comprehensive study of the cultural and morphological properties and biochemical characteristics makes it possible to assess the taxonomic diversity of fungal complexes preserved under the conditions of prolonged natural cryoconservation. These results encourage creating a collection of living cultures of paleofungi, which are important for development of biotechnology and have been shown to produce new promising chemical compounds.

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