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EXPERIMENTAL ARTICLES

Secondary Metabolites of *Penicillium* Fungi Isolated from Permafrost Deposits as Chemotaxonomic Markers

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Abstract—Species identification of slow-growing fungi of the genus *Penicillium* isolated from ancient permafrost deposits was performed using micro- and macromorphological characteristics as well as the composition of secondary metabolites. The strains producing clavine ergot alkaloids fumigaclavines A and B and festuclavine were assigned to the species *P. palitans* Westling 1911, whereas the strains forming ochratoxins A and B were identified as *P. verrucosum* Dierckx 1901.

Key words: permafrost, *Penicillium*, taxonomy, fumigaclavines, festuclavine, ochratoxins.

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While the most widespread, fungi of the subgenus *Penicillium* are also the most difficult for identification by routine microbiological methods. Generally accepted identification of penicillia by micro- and macromorphological characteristics [1] is often unsuitable especially for the strains isolated from extreme habitats [2–6]. Correct species identification of these fungi is of importance due to the information on the species-specific production of various biologically active substances and to the capability of certain species for the synthesis of mycotoxins and other allergens. The new polyphasic taxonomy of the subgenus *Penicillium* has been recently suggested, in which the composition of secondary metabolites was used as a chemotaxonomic criterion together with micro- and macromorphological characteristics [7]. Information on the spectrum of secondary metabolites produced by the relict fungi could by helpful for more correct species identification of the strains isolated from ancient permafrost deposits.

The aim of the present work was to study the composition of the low-molecular secondary metabolites produced by slow-growing fungi of the genus *Penicillium* isolated from permafrost deposits and to use this information for species identification of the isolates.

MATERIALS AND METHODS

The study was carried out with nine fungal strains isolated from ancient permafrost deposits and obtained from the All-Russian Collection of Microorganisms (VKM): VKM FW-657, 667, 690, 747, 875, 877, 878, 907, and 908 [8]. The strains were identified using the macro- and micromorphological characteristics of 7-day cultures grown on Czapek agar with yeast extract (CYA), malt extract agar (MEA), and 25% nitrate agar with glycerol (G25N) at 5, 25, and 37°C [1]. To examine a possible adaptation of the cultures to the maintenance conditions, identification of the strains was repeated after their successive passages with 3-month intervals. The production of secondary metabolites was studied in freshly isolated cultures. Submerged cultivation of the fungi was carried out in 750-ml flasks with 150 ml of the medium containing the following (g/l of distilled water): mannitol, 50.0; succinic acid, 5.4; $MgSO_4 \cdot 7H_2O$, 0.3; KH_2PO_4, 1.0; pH was adjusted to 5.4 with 25% NH_4OH. The medium was inoculated with a water suspension of conidia ($1–2 \times 10^7$ spores/ml) of 14-day cultures grown on malt agar slants. Fungi were cultivated at 24 ± 1°C on a shaker (220 rpm).

The acid, neutral, and alkaline metabolites were isolated from the filtrate of the culture liquid by thrice-repeated extraction with chloroform as described earlier [2–6]. The alkaline extracts were analyzed by thin-layer chromatography (TLC) on Silica gel F_{254} plates (Merck, Germany) developed in the following solvent systems: chloroform : methanol : 25% NH_4OH in the following ratios: 90 : 10 : 0.1 (System I), 90 : 10 : 1 (System II), and 80 : 20 : 0.2 (System III). To examine the acid extracts, the plates were developed in Systems I–III and additionally in the following systems: chloroform : methanol 4 : 1 (System IV), 3 : 1 (System V), and benzene : acetic acid 4 : 1 (System VI).

The substances were detected by UV absorption and fluorescence under UV illumination after spraying the plates with the Dragendorff and Ehrlich reagents to reveal nitrogen-containing metabolites and indole alka-

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Table 1. Comparative growth parameters of the strains freshly isolated from permafrost deposits and the species *Penicillium verrucosum*

Parameters	Cultivation onditions		Type species	Strains VKM FW-								
	Temperature, °C	Medium		657	667	690	747	875	877	878	907	908
Colony diameter, mm (on the 7th day)	5	CYA	3.0	7.5	7.5	7.5	2.5	0.0	7.0	0.0	0.0	0.0
	25	CYA	20.0	13.0	13.0	13.0	23.5	16.6	12.5	16.6	16.0	17.0
		MEA	18.5	11.0	11.0	11.0	26.5	14.6	9.5	13.6	17.6	18.5
		G25N	18.0	6.0	6.0	6.0	18.5	18.6	20.5	19.0	16.0	19.0
	37	CYA	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Ratio of colony diameters (strain/species)	5	CYA	–	2.5	2.5	2.5	0.83	0	2.33	0.0	0.0	0.0
	25	CYA	–	0.65	0.65	0.65	1.18	0.83	0.63	0.83	0.8	0.85
		MEA	–	0.59	0.59	0.59	1.43	0.79	0.51	0.74	0.95	1.01
		G25N	–	0.33	0.33	0.33	1.03	1.03	1.14	1.06	0.89	1.06

loids, respectively, and with 5% iron chloride to visualize phenols and ochratoxins.

Preparative TLC on silica gel plates was used for the isolation and purification of individual metabolites. Ochratoxins A and B were isolated, purified, and identified in all the studied cultures according to the following procedure (exemplified for strain VKM FW-877): the residue (51.9 mg) obtained after the evaporation of the acid chloroform extract obtained from 377 ml of a 14-day culture liquid was applied to the plates with Silica gel 60 (Merck, Germany); to compare, the standard sample of ochratoxin A was also applied to the plate. The plates were developed in System IV, air-dried, and visualized under UV light (366 nm). Two fluorescent zones were detected, of which one corresponded to the standard ochratoxin A (metabolite 1), whereas the R_f value and the characteristic color of fluorescence of another one corresponded to those of ochratoxin B (metabolite 2) [9]. Silica gel containing these zones was scraped off the plates and eluted with methanol. The total amounts of isolated metabolites 1 and 2 were 0.46 and 0.24 mg, respectively.

The metabolites were identified by physicochemical methods and by their cochromatography with the standard samples obtained previously in the Laboratory of Secondary Metabolites, Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences. The UV spectra of compounds dissolved in methanol were registered on an UV-160 A spectrophotometer (Shimadzu, Japan) and on a scanning spectrometer (Beckman, Germany). Electron impact mass spectra of the compounds were recorded on an AMD-402 mass spectrometer (AMD Intectra, Germany). Electrospray mass spectra of the samples were registered on a Quatro 400 quadrupole mass spectrometer (Fisions VG Biotech, United Kingdom).

RESULTS

Based on well-defined micromorphological characteristics, the slow-growing penicillia were assigned to the subgenus *Penicillium*. According to the accepted procedure for the identification of representatives of this genus [1], the growth rate of strains on various agar media at different temperatures was used as a taxonomic criterion. Based on the set of macro- and micromorphological characteristics, the cultures freshly isolated from the permafrost deposits could be assigned to one species *Penicillium verrucosum* Dierckx 1901. Table 1 demonstrates that for almost all the cultures grown at 25°C on the media applied, the colony diameter was less than the values cited in the literature for this species (the coefficient of correlation between the colony diameters was below 1). At the same time, the growth of some strains at 5°C was considerably more intensive compared to the literature data (the coefficient of correlation between the colony diameters was above 1) [10].

To examine a possible adaptation of the cultures to the maintenance conditions that can intensify their growth, identification of the chosen cultures was repeated after their successive passages on fresh media with intervals of several months. After successive passages, an increase in the growth rate was revealed in strains VKM FW-657, 667, 690, and 747, but not in strains VKM FW-875, 877, 878, 907, and 908. An increase in the colony diameters and the changes in some cultural characteristics were most marked at 25°C on all diagnostic media tested. The species names were therefore revised, since in the taxonomy of this part of the genus *Penicillium*, differentiation of morphologically close species is based mainly on the macromorphological characteristics determined on diagnostic media [1]. Species names obtained by repeated identification of these strains (after the second, third, and fourth passages) are presented in Table 2. Such difficul-

Table 2. Changes in the species affiliation of the cultures belonging to the subgenus *Penicillium* after repeated identification based on their macromorphological characters

Strains	First passage	Second passage	Third passage	Fourth passage
657	*P. verrucosum*	*P. granulatum*	*P. puberulum*	*P. commune*
667	*P. verrucosum*	*P. puberulum*	*P. aurantiogriseum*	*P. aurantiogriseum*
690	*P. verrucosum*	*P. granulatum*	*P. puberulum*	*P. aurantiogriseum*
747	*P. verrucosum*	*P. granulatum*	*P. puberulum*	*P. aurantiogriseum*
875	*P. verrucosum*	*P. verrucosum*	*P. verrucosum*	*P. verrucosum*
877	*P. verrucosum*	*P. verrucosum*	*P. verrucosum*	*P. verrucosum*
878	*P. verrucosum*	*P. verrucosum*	*P. verrucosum*	*P. verrucosum*
907	*P. verrucosum*	*P. verrucosum*	*P. verrucosum*	*P. verrucosum*
908	*P. verrucosum*	*P. verrucosum*	*P. verrucosum*	*P. verrucosum*

Table 3. Physicochemical properties of metabolites produced by the first group of strains

Properties	Metabolite 1 (festuclavine)	Metabolite 2 (fumigaclavine A)	Metabolite 3 (fumigaclavine B)
Overall appearance	white microcrystals	white microcrystals	white microcrystals
Molecular mass, Da	240	298	256
M^+, *m/z*			
detected:	240.1629	298.1687	256.1585
calculated:	240.3471	298.1681	256.1575
Molecular formula	$C_{16}H_{20}N_2$	$C_{16}H_{22}O_2N_2$	$C_{16}H_{20}ON_2$
Melting temperature, °C	239–240 (MeOH)	84–85 (water : MeOH)	244–245 (water : MeOH)
UV absorption (λ_{max}) in methanol, nm	224, 275, 280, 292	222, 275, 282, 292	222, 275, 281, 293
R_f in TLC systems:			
I	0.08	0.20	0.05
II	0.13	0.27	0.05
III	0.22	0.47	0.10

ties in the species identification of the fungi belonging to this group and isolated from extreme habitats are probably not rare.

The composition of secondary metabolites produced by fungi of the genus *Penicillium* is known to correlate well with the species identification especially for the group of terverticillate penicillia to which all the studied cultures belonged [8, 12]. To confirm species identification of the relict cultures carried out on the basis of macro- and micromorphological characteristics, the study of their low-molecular secondary metabolites was performed. According to the spectra of secondary metabolites produced, the tested strains were divided into two groups characterized by identical sets of metabolites.

The first group included strains VKM FW-657, 667, 690, and 747 producing three metabolites, which were identified by physicochemical methods as clavine ergot alkaloids fumigaclavine A, fumigaclavine B, and festuclavine (Table 3). The isolated metabolites were of alkaline character, readily soluble in water solutions of acids and could be extracted with such organic solvents as chloroform at alkaline pH values. These compounds were nearly insoluble in hexane, light petroleum, and water, they were fairly soluble in alcohols, chloroform, and acetone, and moderately soluble in benzene and ether. On chromatograms treated with the Ehrlich and Dragendorff reagents, these metabolites were colored purple-blue and orange, respectively. The metabolites were visualized as UV-absorbing spots. The UV spectra of the isolated metabolites were typical of clavine alkaloids containing no double bond at the 9, 10 position (Table 3). The character of mass-spectrometric fragmentation and the gross-formulas determined by high-resolution mass-spectrometry completely corresponded to the structures of clavine ergot alkaloids festuclavine (metabolite 1), fumigaclavine A (metabolite 2), and fumigaclavine B (metabolite 3) [13]. The mass spectrum of metabolite 1 was characterized by the presence of molecular ion M^+ with *m/z* 240 and a set of characteristic peaks with *m/z* 225, 197, 167, 154, and

144 that corresponded to mass spectra of festuclavine and its isomers, pyroclavine, costaclavine, and epicostaclavine. The mass spectrum of metabolite 2 contained an intense peak of molecular ion M^+ with *m/z* 298 and a set of characteristic fragments with *m/z* 239, 223, 154, and 144, which are typical of fumigaclavine A and its isomer isofumigaclavine A. The mass spectrum of metabolite 3 contained a peak of molecular ion M^+ with *m/z* 256 and characteristic peaks with *m/z* 239, 213, 197, 154, and 144, which are typical of fumigaclavine B and its isomer isofumigaclavine B. Among clavine alkaloids, chanoclavines have the same molecular mass as metabolite 3, but their mass spectra contains a set of characteristic peaks with *m/z* 237, 183, 182, 168, and 167, which were absent in the mass spectrum of metabolite 3. The cochromatography of the isolated metabolites with the corresponding standards ensured the final and unequivocal identification of metabolites 1, 2, and 3 as festuclavine, fumigaclavine A, and fumigaclavine B, respectively, rather than the corresponding isomers, such as pyroclavine, costaclavine, and epicostaclavine (similar in their properties to festuclavine) or isofumigaclavines A and B (similar to the corresponding fumigaclavines). Thus, based on physicochemical analyses, the metabolites produced by strains VKM FW-657, 667, 690, and 747 were identified as clavine ergot alkaloids fumigaclavine A, fumigaclavine B, and festuclavine.

In the acid extracts of the second group of the relict strains, VKM FW-875, 877, 878, 907, and 908, two metabolites were revealed, which exhibited pale blue and dark blue fluorescence under UV illumination (366 nm). Both metabolites gave a positive reaction with iron chloride that is indicative of the presence of a phenol group. No nitrogen-containing and indole metabolites were found in this group of strains.

The study of chromatographic mobility of metabolite 1 on silica gel plates in various solvent systems showed that its R_f value completely coincided with that of the standard ochratoxin A (Table 4). Moreover, after development of the plates in the acidic System VI, fluorescence of both metabolite 1 and ochratoxin A became bright green. In the UV-spectrum of metabolite 1, two absorption zones were revealed with the λ_{max} at 218 and 333 nm that almost coincided with the UV-spectrum of ochratoxin A [9, 13]. The chromatographic mobility of metabolite 2 (Table 4), change of its fluorescence to bluish green under acidic conditions (System VI), and its UV-spectrum (λ_{max} in methanol at 218 and 320 nm) corresponded to the published data for ochratoxin B [9, 13]. Thus, metabolites 1 and 2 were identified as ochratoxins A and B, respectively. Therefore, it can be stated that the relict strains VKM FW-875, 877, 878, 907, and 908 synthesized ochratoxins A and B.

Table 4. Chromatographic mobility (R_f) of metabolites isolated from the second group of strains and ochratoxin A in different solvent systems

Systems	Metabolite 1	Metabolite 2	Ochratoxin A
I	0.07	0	0.07
III	0.08	0.08	0.08
IV	0.28	0.10	0.28
V	0.40	0.15	0.40
VI	0.50	0.35	0.50

DISCUSSION

According to the recently proposed scheme of taxonomy of the subgenus *Penicillium* fumigaclavines A and B serve as chemotaxonomic markers only for the species *P. palitans* Westling 1911 [7]. These metabolites revealed in one of the *P. clavigerum* strains were not the markers for this species [7]. The affiliation of the relict strains producing fumigaclavines A and B to the species *P. commune, P. aurantiogriseum* (=*P. puberulum*), or to *P. glandicola* (=*P. granulatum*) can be doubtful since the chemotaxonomic markers for these species were not found in the studied strains. The diagnostic metabolites include anacine, aurantiamine, aurantin, penicillic acid, verrucosidin, and pseurotin for the species *P. aurantiogriseum* (=*P. puberulum*); roquefortine C, meleagrin, patulin, and penitrem A for the species *P. glandicola* (=*P. granulatum*); rugulovasines A and B, cyclopiazonic acid, and palitantin for the species *P. commune* [7]. It should be noted that *P. palitans*, which was known for a long time as an independent species, was assigned by J. Pitt to the species *P. commune* based on their morphological similarity [1]. However, the differences in the spectra of secondary metabolites produced by these species allowed the authors of the new polyphasic taxonomy of the subgenus *Penicillium* to designate *P. palitans* as an independent species [7].

Thus, the relict strains VKM FW-657, 667, 690, and 747 were designated as representatives of the species *P. palitans* based mainly on the production of fumigaclavines A and B in spite of the vague identification based on their morphological characteristics (Table 5).

Ochratoxin A is used as a chemotaxonomic marker for identification of only two species, *P. verrucosum* Diercks 1901 and *P. nordicum* Dragoli, Cantoni ex Ramires 1985 [7]. The authors of the new polyphasic taxonomy of the penicillia of the subgenus *Penicillium* were doubtful about identification of such known producers of ochratoxin A as *P. atramentosum, P. aurantiogriseum, P. brevicompactum, P. camemberti, P. chrysogenum, P. commune, P. corymbiferum, P. cyclopium, P. expansum, P. glandicola, P. griseofulvum, P. palitans, P. solitum*, and *P. viridicatum* [7]. The species *P. verrucosum* and *P. nordicum*, while similar in micromorphological and most of the macromorphological

Table 5. Species identification of the cultures of the subgenus *Penicillium* based on the production of secondary metabolites

Strains	Metabolites	Species
657	Fumigaclavines A and B and festuclavine	*P. palitans*
667	Fumigaclavines A and B and festuclavine	*P. palitans*
690	Fumigaclavines A and B and festuclavine	*P. palitans*
747	Fumigaclavines A and B and festuclavine	*P. palitans*
875	Ochratoxins A and B	*P. verrucosum*
877	Ochratoxins A and B	*P. verrucosum*
878	Ochratoxins A and B	*P. verrucosum*
907	Ochratoxins A and B	*P. verrucosum*
908	Ochratoxins A and B	*P. verrucosum*

characteristics, differ in the reverse color on the yeast extract–sucrose agar medium (YES) and in the spectra of their secondary metabolites. Some isolates of *P. verrucosum* are able to synthesize citrinin and verrucolone in addition to ochratoxin A [7], whereas in the case of *P. nordicum*, chemotaxonomic markers, in addition to ochratoxin A and verrucolone, included anacine. The relict strains VKM FW-875, 877, 878, 907, and 908 had a red-brown reverse color on the YES agar [7] and produced no indole-containing metabolites; this undoubtedly indicated their affiliation with species *P. verrucosum* (Table 5).

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