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EXPERIMENTAL ARTICLES

The Biosynthesis of Low-Molecular-Weight Nitrogen-Containing Secondary Metabolites—Alkaloids—by the Resident Strains of *Penicillium chrysogenum* and *Penicillium expansum* Isolated on Board the *Mir* Space Station

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Abstract—The analysis of the absorption spectra of the low-molecular-weight nitrogen-containing secondary metabolites—alkaloids—of four *Penicillium chrysogenum* strains and six *P. expansum* strains isolated on board the *Mir* space station showed that all these strains synthesize metabolites of alkaloid origin (roquefortine, 3,12-dihydroroquefortine, meleagrin, viridicatin, viridicatol, isorugulosuvin, rugulosuvin B, *N* acetyltryptamine, and a "yellow metabolite" containing the benzoquinone chromophore).

Key words: astromicrobiology, fungi, *Penicillium chrysogenum, Penicillium expansum*, secondary metabolites, alkaloids.

Both manned and unmanned spacecraft launched into outer space are usually contaminated by various microorganisms, which are very adaptable to extreme environments. The analysis of the microflora present on board the *Mir* space station showed that it comprised more than 100 species of microscopic fungi, some of which have been inhabiting indoor construction materials and on-board equipment for many years [1]. The fungal strains that were isolated in 1997–1998 turned out to be descendants of the strains that had been isolated from *Mir*'s living quarters more than 8 years ago. Those strains were found to belong to two species of the genus *Penicillium*, *P. expansum* Link and *P. chrysogenum* Thom [2], which are known as producers of various secondary metabolites characterized by a wide range of biological activities [3, 4]. In particular, they may be toxic to plants and animals and may possess antibiotic, cytostatic, and anticancer activities.

Much research effort is presently being directed toward the study of microorganisms isolated from extreme environments, since it is these microorganisms that are expected to synthesize hitherto unknown secondary metabolites, among which new efficient biologically active compounds may be found.

The *Mir* space station can be considered a unique ecosystem, whose microflora has long been subject to the action of specific technogenic and cosmic factors.

The present work was undertaken to study the low-molecular-weight nitrogen-containing secondary metabolites—alkaloids—produced by the resident strains of *P. chrysogenum* and *P. expansum* isolated on board the *Mir* space station.

MATERIALS AND METHODS

Experiments were carried out with four strains (1-3 through 1-6) of *Penicillium chrysogenum* and six strains (2-2 through 2-7) of *P. expansum*, obtained from the Institute of Medical and Biological Problems. The strains were isolated in 1995–1996 from *Mir*'s living quarters and identified at the Department of Mycology and Algology, Faculty of Biology, Moscow State University, by the Pitt method [5].

The strains were stored on Czapek slants immersed in mineral oil. To study their secondary metabolites, the strains were grown at 24 ± 1°C in 750-ml flasks with 150 ml of a medium on a shaker (220–240 rpm). The medium contained (g/l distilled water) mannitol, 50.0; succinic acid, 5.4; $MgSO_4 \cdot 7H_2O$, 0.3; and KH_2PO_4, 1.0. The pH of the medium was adjusted to 5.2 by adding 25% NH_4OH. The medium was inoculated with 14-day-old spores in an amount of $1–2 \times 10^7$ spores/ml. The culture liquid filtrate was analyzed for the presence and composition of low-molecular-weight nitrogen-

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Table 1. Characterization of the secondary metabolites produced by *P. chrysogenum* and *P. expansum* strains

Secondary metabolite	Color in reaction with		Mobility ($R_f \times 100$) in systems					λ_{max} in UV spectra, nm
	Ehrlich's reagent	$FeCl_3$	I	II	III	IV	V	
Roquefortine	Yellow turning blue		31	45	61			207, 240, 324
3,12-Dihydroroquefortine	Yellow turning blue		15	28	43			207, 242, 301
Meleagrin	Orange		42	52				207, 226, 283, 343
Isorugulosuvin	Violet		35	45	47			221, 272, 281, 289
Rugulosuvin B	Gray-violet		71	78	46		28	218, 244, 275, 284
Viridicatin		Muddy green	42		50	56		222, 280, 318, 330
Viridicatol		Muddy green	21		30	50		226, 284, 304, 316, 329
N-Acetyltryptamine	Violet		40	48	56			221, 271 sh, 282, 290
Yellow metabolite	Violet-red		67	72			15	207, 220, 417

Note: All of the secondary metabolites positively react with the Dragendorff reagent.

containing secondary metabolites after 5, 7, 10, 13, and 17 days of growth.

Secondary metabolites were extracted from the culture liquid filtrate with chloroform under alkaline (pH ≈ 8) and acidic (pH ≈ 4) conditions and analyzed by thin-layer chromatography on Silica Gel 60 F_{254} plates (Merck, Germany) using 5 different solvent systems: I, chloroform–methanol–25% NH_4OH (90 : 10 : 0.1); II, chloroform–methanol–25% NH_4OH (90 : 10 : 1); III, chloroform–methanol–25% NH_4OH (85 : 15 : 10); IV, toluene–ethylacetate–formic acid (5 : 4 : 1), and V, chloroform–acetone (93 : 7).

Chromatographic spots were visualized either through light absorption or through fluorescence in UV light after spraying of the developed plates with Ehrlich's reagent (to detect indole, benzodiazepine, and diketopiperazine alkaloids), a 3% solution of $FeCl_3$ in ethanol (to detect quinoline and phenolic compounds), and the Dragendorff reagent (to detect nitrogen-containing metabolites).

The plate regions with spots were cut out and eluted with a chloroform–methanol (1 : 1) mixture, after which the eluate was dried using a vacuum rotary evaporator. Purified secondary metabolites were identified by their cochromatography with the authentic samples obtained earlier [6], as well as by UV spectrophotometry and mass spectrometry. UV spectra were recorded using a UV-160A spectrophotometer (Shimadzu, Japan). Mass spectra were recorded using a Finnigan MAT 8430 mass spectrometer (Germany).

RESULTS AND DISCUSSION

The species *P. chrysogenum* Thom and *P. expansum* Link belong to the subgenus *Penicillium*, section *Penicillium*, series *Expansa* [5]. These species are ubiquitous in soils of different climatic zones and often contaminate food and feedstuffs [4, 5]. They are known to produce a wide range of secondary metabolites [3, 4].

Most *P. chrysogenum* strains synthesize the alkaloids roquefortine and meleagrin in comparable amounts [4]. Some strains of this species also produce isorugulosuvin [7] and some, isolated from extreme environments, are characterized by a special set of synthesized alkaloids. For instance, strain 4KPB MGU, isolated from an anthropogenically impacted ecosystem, synthesizes the clavine alkaloids fumigaclavine A, fumigaclavine B, and pyroclavine [8]. Based on the analysis of secondary metabolites, Frisvad and Filtenborg divided the species *P. chrysogenum* into two varieties: *P. chrysogenum* Thom var. *chrysogenum*, which synthesizes roquefortine and meleagrin, and *P. chrysogenum* var. *dupodomyis*, which synthesizes a metabolite of an unknown structure with blue fluorescence but does not synthesize roquefortine [4].

Roquefortine is a cyclic dipeptide with the diketopiperazine structure, which is synthesized from tryptophan, histidine, and dimethylallyl pyrophosphate. Among the secondary metabolites of roquefortine-producing strains, the products of roquefortine transformation (glandicolin, meleagrin, and oxalin) are usually detected [3, 9].

Two *P. chrysogenum* strains studied in this work (1-3 and 1-4) were found to produce roquefortine and meleagrin (Table 1), which allows them to be ascribed to *P. chrysogenum* Thom var. *chrysogenum*.

Strains 1-4 and 1-5 synthesized a yellow metabolite with a high chromatographic mobility ($R_f = 0.67$ in system I), which turned violet-red in reaction with Ehrlich's reagent and positively reacted with the Dragendorff reagent. The UV spectrum of this compound had absorption peaks with maxima at 207, 219, and 417 nm. Preliminarily, the yellow metabolite contains the benzoquinone chromophore. The structure of this compound is being established.

The species *P. expansum* synthesizes the quinoline, benzodiazepine, and diketopiperazine alkaloids viridicatin, cyclopenin, cyclopeptin, dehydrocyclopeptin,

Table 2. The secondary metabolites produced by particular *P. chrysogenum* and *P. expansum* strains

Strain	Secondary metabolites
P. chrysogenum	
1-3, 1-4	Roquefortine, meleagrin, N-acetyltryptamine
1-5, 1-6	Yellow metabolite*
P. expansum	
2-2	Isorugulosuvin*, rugulosuvin B*, viridicatin, viridicatol
2-3	Isorugulosuvin*, rugulosuvin B
2-4	Roquefortine, 3,12-dihydroroquefortine, meleagrin*
2-5	Roquefortine, meleagrin*
2-6	Isorugulosuvin*, N-acetyltryptamine*
2-7	Yellow metabolite*

* The asterisk marks the secondary metabolites that are detected for the first time in particular strains.

and roquefortine [9]. Some strains of this species also synthesize, in minor amounts, the ergot alkaloid aurantioclavine [10], which was first described as a specific metabolite of alkaloid origin in the fungus *P. aurantiovirens* VKM F-229 [11].

Two strains of *P. expansum*, 2-4 and 2-5, were found to synthesize roquefortine together with meleagrin. It should be noted that the combined synthesis of these two alkaloids is not characteristic of this species [9]. Strain 2-4 also synthesized 3,12-dihydroroquefortine in minor amounts. Three strains of *P. expansum*, 2-2, 2-3, and 2-6, produced a metabolite (metabolite 1) that absorbed UV light, turned violet in reaction with Ehrlich's reagent, and was characterized by a low chromatographic mobility (R_f = 0.35 in system I). Strains 2-2 and 2-3 also produced a second metabolite (metabolite 2), which turned gray-violet and had a high mobility in system II (R_f = 0.71). The UV spectrum of metabolite 1 had absorption peaks with maxima at 209, 272, 281, and 289 nm. The mass spectrum of this compound had the low- and high-intensity peaks of molecular ions, with *m*/*z* = 333 and 130, respectively, as well as peaks belonging to the 3-alkyl indole fragments of these ions, with *m*/*z* = 103 and 77. The molecular formula of metabolite 1 derived from the mass spectral data was $C_{27}H_{19}N_3O_2$ (measured 333, 1491; calculated 333, 1477). According to its physicochemical characteristics, metabolite 1 is isorugulosuvin, which was isolated earlier from *P. piscarium* VKM F-325 [12]. The molecular formula of metabolite 2, derived from mass spectral data was $C_{27}H_{29}N_3O_3$ (measured 443, 2209; calculated 443, 2208). The UV spectrum of metabolite 2, with absorption peaks at 218, 244, 275, and 284 nm, as well as its chromatographic mobility in different solvent systems corresponded to rugulosuvin B, which was isolated earlier from *P. puberulum* VKM F-329 [13, 14]. This alkaloid is also known as puberulin [13] and fructigenine A [15].

Isorugulosuvin and rugulosuvin B are structurally close diketopiperazine alkaloids, representing the cyclic dipeptides of tryptophan and phenylalanine. Rugulosuvin B differs from isorugulosuvin in that it has a 1',1'-dimethyl-2-propenyl radical at position 3 and an acetylated indole nitrogen atom. These two alkaloids are known to be produced by the species *P. piscarium*, *P. canescens*, and *P. melinii* of the subgenus *Furcatum* [12, 16] and by the species *P. aurantiogriseum* of the subgenus *Penicillium* [17]. The synthesis of isorugulosuvin and rugulosuvin B by the species *P. expansum* is described for the first time.

P. expansum strain 2-2 also synthesized two other metabolites, which turned muddy green in reaction with a 3% solution of $FeCl_3$ in ethanol. These metabolites were identified as viridicatin and viridicatol by cochromatography with the authentic samples (Table 1). Viridicatin and viridicatol belong to the group of quinoline alkaloids, which are synthesized from anthranilic acid and phenylalanine with benzodiazepine derivatives (cyclopeptin, dehydrocyclopeptin, cyclopenin, and cyclopenol) as intermediates [18].

Quinoline alkaloids are known to be synthesized by species of the subgenus *Penicillium*: *P. aurantiogriseum, P. crustosum, P. commune, P. chrysogenum, P. expansum*, and *P. viridicatum* [3, 4]. Some strains of these species produce all of the aforementioned quinoline alkaloids [9, 13], whereas others (*P. expansum* 2-2 and strains described by Luckner [18]) produce only the end secondary metabolites viridicatin and viridicatol.

P. expansum strain 2-7 synthesized a yellow metabolite analogous to the yellow metabolite of *P. chrysogenum* strains 1-5 and 1-6.

In the phase of active growth (5–7 days after inoculation), *P. chrysogenum* strains 1-3 and 1-4 and *P. expansum* strain 2-6 produced a metabolite that turned violet in reaction with Ehrlich's reagent. This metabolite was identified by mass spectrometry as *N*-acetyltryptamine (*m*/*z* 202(M^+), $C_{12}H_{14}N_2O$), which is likely a product of the partial transformation of tryptophan.

Thus, the resident strains of *P. chrysogenum* and *P. expansum* isolated from materials on board the *Mir* space station synthesize a number of secondary metabolites of alkaloid origin (Table 2). According to the structure of the alkaloids synthesized, these strains can be divided into three groups. Group I comprises two *P. chrysogenum* strains (1-3 and 1-4) and two *P. expansum* strains (2-4 and 2-5) that synthesize secondary metabolites of roquefortine origin. Group II comprises three *P. expansum* strains (2-2, 2-3, and 2-6) that synthesize the diketopiperazine alkaloids isorugulosuvin and rugulosuvin B. One of these strains (2-2) also synthesizes the quinoline alkaloids viridicatin and viridicatol. It should be noted that the synthesis of the dike-

topiperazine alkaloids by strains of the species *P. expansum* is reported here for the first time. Group III includes two *P. chrysogenum* strains (1-5 and 1-6) and one *P. expansum* strain (2-7) that synthesize a single yellow metabolite containing the benzoquinone chromophore.

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