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EXPERIMENTAL ARTICLES

The Fungus *Penicillium citrinum* Thom 1910 VKM FW-800 Isolated from Ancient Permafrost Sediments As a Producer of the Ergot Alkaloids Agroclavine-1 and Epoxyagroclavine-1

A. G. Kozlovskii*, V. P. Zhelifonova*, V. M. Adanin*, T. V. Antipova*, S. M. Ozerskaya*, G. A. Kochkina*, and U. Gräfe**

**Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, pr. Nauki 5, Pushchino, Moscow oblast, 142290 Russia*

***Hans Knöll Institute for Natural Products Research, Beutenbergstrasse 11a, D-07745 Jena, Germany*

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Abstract—The study of the secondary metabolites of the relict strain *Penicillium citrinum* VKM FW-800 isolated from ancient Arctic permafrost sediments showed that this fungus produces agroclavine-1 and epoxyagroclavine-1, which are rare ergot alkaloids with the 5R,10S configuration of the tetracyclic ergoline ring system. The production of the alkaloids by the fungus showed a biphasic behavior, being intense in the phase of active growth and slowing down in the adaptive lag phase and in the stationary growth phase. The addition of zinc ions to the incubation medium led to a fivefold increase in the yield of the alkaloids. The alkaloid-producing *Penicillium* fungi isolated from different regions exhibited the same tendencies of growth and alkaloid production.

Key words: microscopic fungi, permafrost, *Penicillium*, biosynthesis, secondary metabolites, ergot alkaloids, agroclavine-1, epoxyagroclavine-1.

Fungi of the genus *Penicillium* are promising candidates for the discovery of novel biologically active compounds [1]. Recent relevant studies have mainly been performed with the fungi isolated from extreme habitats, since such fungi are believed to synthesize active secondary metabolites in order to tolerate abnormal environmental condition. For this reason, of great interest is the fungus *P. citrinum* VKM FW-800, which was isolated from ancient permafrost sediments.

The aim of this work was to study the secondary metabolites of the above fungus and the particular characteristics of their biosynthesis.

MATERIALS AND METHODS

The relict strain *Penicillium citrinum* FW-800 used in this work was isolated from ancient (1.8- to 3.0-million-year-old) Arctic permafrost sediment (loamy silt) samples collected in the middle course of the Chukoch'ya River on the Kolyma Lowland [2]. The fungus was identified by the macro- and micromorphological characteristics of 7-day-old cultures grown on three different nutrient media at three different temperatures [3]. The strain was maintained on glucose–potato agar slants. Inoculum was prepared by suspending spores from 14-day-old cultures in water to a density of $(1–2) \times 10^7$ spores/ml. Basal medium contained (g/l distilled water) mannitol, 50; 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 5.4 with 25% NH_4OH. The fungus was cultivated at 24 ± 1°C in 750-ml Erlenmeyer flasks containing 150 ml of the medium on a shaker (220 rpm).

To study the effect of zinc ions on fungal growth and alkaloid production, the basal medium was supplemented with 1 mg/l zinc ions in the form of $ZnSO_4 \cdot 7H_2O$. Samples for analysis were taken at 1-day intervals. Growth was monitored by determining the dry weight of the fungal mycelium. Secondary metabolites were extracted from the culture liquid filtrate first at pH 4 and then at pH 8 as described earlier [4].

The extracts were analyzed by TLC on 60 F_{254} Silica gel plates (Merck, Germany) in three solvent systems containing chloroform, methanol, and 25% NH_4OH in proportions of 90 : 10 : 0.1 (system 1), 90 : 10 : 1 (system 2), and 80 : 20 : 0.2 (system 3). Separated substances were visualized by their absorbance of UV light and by spraying the developed plates with the Ehrlich reagent (to detect indole alkaloids) and the Dragendorff reagent (to detect nitrogen-containing secondary metabolites).

Total alkaloids were extracted from 10 l of the culture liquid filtrate of a 12-day-old culture. The filtrate was alkalinized to pH 8.0–8.5 with an aqueous solution of ammonia and extracted with chloroform. The completeness of the extraction was controlled with the van Urk reagent. If necessary, the extraction procedure was repeated. The extracts were pooled, dehydrated with

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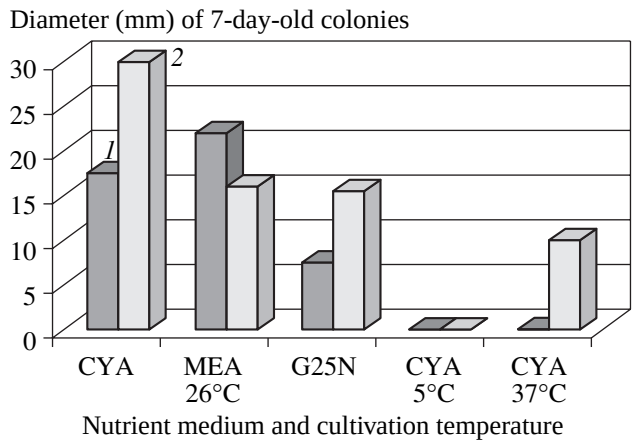

Fig. 1. The effect of cultivation conditions on the growth of (*1*) *P. citrinum* VKM FW-800 and (*2*) reference strain [3]. CYA, Czapek medium with yeast autolysate; MEA, malt extract agar; G25N, 25% nitrate agar with glycerol.

anhydrous sodium sulfate, and dried using a vacuum rotary evaporator.

The dry residue (873.9 mg) was analyzed by chromatography on a 20-mm-diameter column packed with 60 g of Silica gel (0.04–0.063 mm, Merck, Germany). The column was eluted with solvent system 1. The eluate collected in 5-ml fractions was analyzed by TLC on the aforementioned Silica gel plates developed in system 1. Fractions 28 through 36 contained 304.4 g of a substance with R_f = 0.28, which was designated as metabolite 1. Fractions 40 through 60 contained 71.3 g of another substance with R_f = 0.18, which was designated as metabolite 2.

The metabolites were identified by chromatography in different solvent systems using the standard samples of substances obtained from the Laboratory of Secondary Metabolites, as well as by UV spectroscopy, mass spectrometry, and NMR spectroscopy.

The content of epoxyagroclavine-1 (EAC-1) and agroclavine-1 (AC-1) in samples was determined as follows: A sample was dissolved in a minimal volume of chloroform and applied onto a Silufol plate. The plate was developed in system 1. The spots that corresponded to the alkaloids were marked under UV light, and then these regions of the plate were cut out and eluted with methanol. The eluates were passed through paper filters. The content of EAC-1 and AC-1 in the respective filtrates was determined, using a calibration curve, from the optical density of the filtrates measured at 283 nm using an SF-26 spectrophotometer (Russia).

To obtain intracellular alkaloids, washed fungal mycelium was suspended in 2% tartaric acid and disintegrated in an MPW-302 homogenizer (Mechanika precyzyjna, Poland) run at 3000 rpm. The homogenate was filtered and washed thrice with 2% tartaric acid. The pooled filtrate was alkalinized to pH 8.0 and extracted thrice with chloroform. The pooled extract was dehydrated with anhydrous sodium sulfate and dried using the vacuum rotary evaporator.

The UV spectra of compounds dissolved in methanol were recorded on a Shimadzu UV-160A spectrophotometer (Japan). The mass spectra of the compounds were recorded using a Finnigan MAT-8430 mass spectrometer (Germany). The ^1H and ^{13}C NMR spectra of the compounds were recorded on a DRX 500 spectrometer (Bruker Avance, Germany) at 500 and 150 MHz, respectively. Chemical shifts δ (expressed in ppm) were determined relative to tetramethylsilane as the internal standard.

RESULTS AND DISCUSSION

Investigations showed that the strain *P. citrinum* VKM FW-800 isolated from permafrost sediments slightly differs from the type and reference strains of this species in such parameter as the optimum growth temperature (Fig. 1), the micro- and macromorphology of the strain being typical of the species *P. citrinum* Thom 1910 [3]. The latter belongs to the subgenus *Furcatum* of the section *Furcatum* [3] and holds an invariable position in the variable *Penicillium* taxonomy [5]. The representatives of *P. citrinum* are ubiquitous in soils and are known as the fungal contaminants of food- and feedstuffs and the active biodegraders of various polymeric materials [3].

Some *P. citrinum* strains produce citrinin, an O-heterocyclic mycotoxin [5, 6], and some produce the clavine ergot alkaloids pyroclavine, cividiclavine [6], chanoclavine-I, isochanoclavine-I, costaclavine, and epicostaclavine [7].

P. citrinum VKM FW-800 grown in the basal medium produced two secondary metabolites, metabolite 1 with R_f = 0.28 (system 1) and metabolite 2 with R_f = 0.18 (system 1), which were isolated from the alkaline fraction of the culture liquid filtrate and turned violet in reaction with the Ehrlich reagent. Metabolites 1 and 2 exhibited similar characteristics: both were fairly soluble in the polar organic solvents chloroform, acetone, and alcohols and were poorly soluble in hexane and water. After precipitation from chloroform solutions, the metabolites could be obtained in the form of white amorphous powders. The UV spectra of the metabolites with maxima at 223, 276, 282, and 292 nm (metabolite 1) and at 223, 276, 282, and 293 nm (metabolite 2) were typical of the clavine ergot alkaloids lacking a conjugated double bond at the 9,10 position of the tetracyclic ergoline ring system [8].

The mass spectrum of metabolite 1 was characterized by an intense peak of a fragment molecular ion with *m/z* = 254. High-resolution mass spectrometry showed that this molecular ion had the formula $C_{16}H_{18}N_2O$ (the measured and calculated values of *m/z* were found to be 254.1415 and 254.1419, respectively). The intense peaks of fragment molecular ions with *m/z* = 154, 168, and 182 were typical of ergoline deriv-

Table 1. Biomass and alkaloid production in the relict *P. citrinum* VKM FW-800 strain and modern EAC-1 and AC-1 producers isolated from different habitats

Strain	Origin	Biomass, g/l	Concentration of alkaloids, mg/l			Alkaloid yield with respect to biomass (Y_p/x), mg/g
			AC-1	EAC-1	total alkaloids	
P. citrinum VKM FW-800	Arctic permafrost sediments	6.5	0.6	2.7	3.3	0.5
P. fellutanum VKM FW-1073	Syrian soil	12.0	1.4	73.4	73.4	6.1
P. kapuscinskii IBPM F-152	No data available	12.0	4.8	16.4	21.2	1.8
P. implicatum F-114	Turkmenistan serozem	–	–	Traces	–	–

atives [9, 10]. The occurrence of these peaks was indicative of the preservation of the ergoline rings A, B, and C and the lack of additional functional groups. The mass spectrum of metabolite 1 greatly differed from that of elymoclavine, setoclavine, and lysergol [9] (alkaloids with a molecular mass of 254) and was almost identical to the mass spectrum of the standard epoxyagroclavine-1 obtained as described earlier [11]. The ^{1}H and ^{13}C NMR spectra of metabolite 1 were also very similar to those of EAC-1 [11]. The chromatographic mobilities of metabolite 1 in solvent systems 1, 2, and 3 (R_f = 0.28, 0.29, and 0.48, respectively) also corresponded to EAC-1 [11]. Based on these experimental data, metabolite 1 was identified as epoxyagroclavine-1.

The mass spectrum of metabolite 2 was characterized by an intense peak of a fragment molecular ion with m/z = 238. The fragmentation of this metabolite, which yielded intense molecular ions with m/z = 237, 223, 207, 192, 180, 167, 154, 127, and 108, was typical of both agroclavine and agroclavine-1. However, the chromatographic mobilities of metabolite 2 in solvent systems 1, 2, and 3 (R_f = 0.18, 0.24, and 0.30, respectively) corresponded to agroclavine-1 and greatly differed from the respective chromatographic mobilities of agroclavine (R_f = 0.22, 0.30, and 0.42) [11, 12]. Based on these experimental data, metabolite 2 was identified as agroclavine-1.

It should be noted that the acidic fraction of the culture liquid filtrate of *P. citrinum* also contained a secondary metabolite, which exhibited the chromatographic mobility R_f = 0.25 in solvent system 2 and reacted with the Dragendorff reagent. The identification of this metabolite is in progress in our laboratory. On the other hand, citrinin (a frequent metabolite of various *P. citrinum* strains) was not detected in the culture liquid filtrate of *P. citrinum* VKM FW-800.

The clavine alkaloids AC-1 and EAC-1 with the uncommon 5R,10S configuration of the ergoline ring system are produced by a limited number of *Penicillium* strains, namely, *P. fellutanum* VKM F-1073 (*P. sizovae*) isolated in 1969 from the soils of Syria [13], *P. corylophilum* IBPM F-152 [11] (reidentified later as *P. kapuscinskii* (*P. janczewskii*) IBPM F-152), and *P. implicatum* F-114 isolated from the serozems of Turkmenistan [14]. The search for other AC-1 and EAC-1 producers among three *P. fellutanum* strains and seven *P. janczewskii* strains obtained from the All-Russia Collection of Microorganisms was unsuccessful [15–17].

A comparison of the growth and alkaloid production parameters of the relict strain VKM FW-800 and other EAC-1 and AC-1 producers isolated from different habitats showed that VKM FW-800 is the lest efficient producer of epoxyagroclavine-1 and agroclavine-1 (Table 1). It should be noted that the low biomass (6.2 g/l) and the early formation of conidia in the relict strain grown in Abe medium (which contains sufficient amounts of carbon and nitrogen sources and macroelements) can be due to a deficiency of, for instance, some microelements.

Our recent investigation showed that zinc ions stimulate the growth of *P. citrinum* VKM F-1079 and alkaloid production by this strain [4]. Furthermore, there is evidence that zinc ions are involved in the regulation of the synthesis of tryptophan-containing alkaloids through the regulation of tryptophan metabolism [18]. As can be seen from Table 2, zinc ions stimulated the growth of *P. citrinum* VKM FW-800 by 3.2 times and augmented the content of AC-1 and EAC-1 in the culture liquid of this strain by 52 and 11 times, respectively. The enhanced production of the alkaloids cannot be explained merely by the increased biomass of the strain, since the relative yield of AC-1 and EAC-1 with respect to the biomass ($Y_{\text{AC-I}}/x$ and $Y_{\text{EAC-I}}/x$) increased by 15 and 9 times, respectively, after the addition of zinc ions. Consequently, zinc ions stimulated both the primary metabolism of VKM FW-800 (as is evident from the increased biomass of this strain) and the biosynthesis of clavine alkaloids. Rosazza *et al.* [19] explained the stimulating effect of zinc ions on alkaloid production by the activation of tryptophan synthetase by these ions. The possibility cannot also be excluded that zinc ions exert their action on the excretion of alkaloids from the mycelium, as is evident from the fact that the content of EAC-1 and AC-1 in the mycelium grown in the presence of zinc ions was 35% lower than it was

Table 2. The effect of zinc ions on the biomass and alkaloid production in the relict *P. citrinum* VKM FW-800 strain

Parameter	Basal medium	Basal medium supplemented with zinc ions
Biomass, g/l	6.2	19.8
Extracellular alkaloids, mg/l:		
AC-1	0.6	72.2
EAC-1	2.7	29.3
Total	3.3	101.5
Yield of extracellular alkaloids with respect to biomass, mg/g:		
AC-1	0.1	1.5
EAC-1	0.4	3.6
Total	0.5	5.1
Total intracellular alkaloids, mg/g	0.2	0.13

in the mycelium grown in the basal medium lacking zinc ions (Table 2).

The study of the growth dynamics of VKM FW-800 in the basal medium showed the absence of any noticeable diauxie in spite of the presence of two carbon sources (succinic acid and mannitol) in this medium (Fig. 2a). In contrast, this strain showed diauxic growth with two μ_{max} values in the medium with zinc ions (Fig. 2b). In response to the addition of zinc ions, the first maximum specific growth rate increased by 4.4 times (Fig. 3), indicating a deficiency of these ions in the basal medium.

The accumulation of alkaloids in the culture liquid of *P. citrinum* VKM FW-800 was biphasic irrespective of whether zinc ions were added to the medium or not (Fig. 2). The biphasic pattern of alkaloid accumulation was also found in the fungi *P. fellutanum* [13] and *P. kapuscinskii* [11].

A relationship between growth processes and alkaloid production was most evident when the relict strain was cultivated in the medium with zinc ions (Fig. 2b). In the first phase of active growth, the total concentration of alkaloids in the culture liquid increased together with the biomass. In the adaptive lag phase (6–7 days of cultivation) the concentration of alkaloids in the medium decreased by about 30%. After the growth resumption, the content of the alkaloids in the medium began to increase and reached a maximum by the 11th day of cultivation (in the growth retardation phase). In the stationary growth phase (12 days of cultivation), the total concentration of the alkaloids decreased by 25%. In the case of *P. fellutanum* VKM F-1073, another known producer of AC-1 and EAC-1, the concentration of these alkaloids in the medium fell in the adaptive lag phase [13].

During the growth of both alkaloid producers (*P. citrinum* VKM FW-800 and *P. fellutanum* VKM F-1073), the proportion between EAC-1 and AC-1 gradually changed [13], so that AC-1 prevailed before the adap-

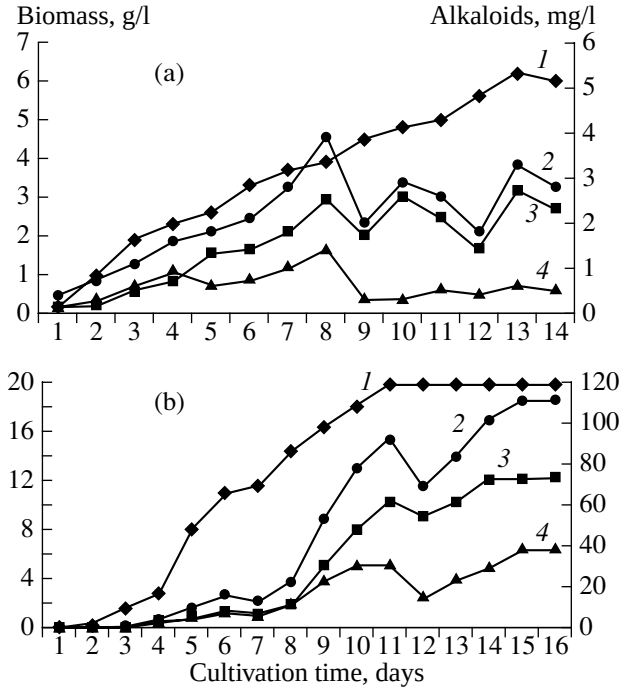

Fig. 2. Growth dynamics and the content of alkaloids in the culture liquid of *P. citrinum* VKM FW-800 grown in (a) basal medium and (b) basal medium supplemented with zinc ions: (*1*) biomass; (*2*) total alkaloids; (*3*) EAC-1; and (*4*) AC-1.

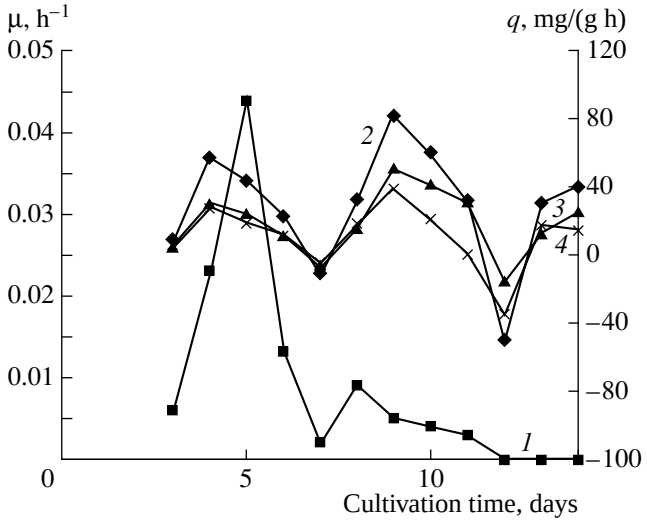

Fig. 3. Dynamics of (*1*) the specific growth rate μ and the specific rates *q* of the accumulation of (*2*) total alkaloids, (*3*) EAC-1, and (*4*) AC-1 in the culture liquid of *P. citrinum* VKM FW-800 grown in basal medium supplemented with zinc ions.

tive lag phase, and EAC-1 prevailed after this phase. In the phase of active growth, the concentrations of EAC-1 and AC-1 in the culture liquid of the relict strain were about the same, whereas in the stationary growth phase, the relative content of AC-1 decreased to 14–25% of the total content of the alkaloids (Figs. 2a, 2b).

Thus, there is a direct relationship between alkaloid synthesis in the relict strain VKM FW-800 and its growth, which is also typical of other known producers of secondary metabolites. The biphasic pattern of aurantioclavine accumulation in the culture liquid of *P. nalgiovense* VKM F-229 was found to be related to the reversible processes of its excretion and consumption regulated by growth processes and the physiological state of the culture [20].

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