Microbiology, Vol. 69, No. 3, 2000, pp 343–346. Translated from Mikrobiologiya, Vol. 69, No. 3, 2000, pp. 415–419.

EXPERIMENTAL ARTICLES

Taxonomic Position and Nitrogen-containing Secondary Metabolites of the Fungus *Penicillium vitale* Pidoplichko et Bilai apud Bilai

N. G. Vinokurova, S. M. Ozerskaya, V. P. Zhelifonova, and V. M. Adanin

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

Received June 30, 1999; in final form, November 4, 1999

Abstract—The type strain *Penicillium vitale* Pidoplichko et Bilai apud Bilai 1961 VKM F-3624 was found to considerably differ from a sibling species *P. janthinellium* (syn. *P. simplicissimum*) in some physiological and morphological features (growth rates at different temperatures, the size of philiades, and the shape of conidia), as well as in the pattern of the nitrogen-containing secondary metabolites produced (roquefortine, 3,12-dihydroroquefortine, meleagrin, aurantioclavine, indole-3-acetic acid, and *N*-acetyltryptamine). The data obtained suggest that *P. vitale* represents an independent species.

Key words: fungi, *Penicillium vitale*, secondary metabolites, taxonomy

The type strain *Penicillium vitale* Pidoplichko et Bilai apud Bilai 1961 VKM F-3624 is the only known representative of the species *P. vitale* [1]. Since 1950, this strain has been used for the production of Microcide (a crude antibiotic preparation), glucose oxidase [1], and catalase [2]. The industrial application of strain VKM F-36245 stimulated the investigation of its physiology and some problems related to the preparation and storage of material for inoculation [1]. To the best of our knowledge, no information is available in the literature on the secondary metabolites of this strain, including those which possess antibiotic activity and/or toxicity. Furthermore, the taxonomic position of *P. vitale* has not been conclusively determined.

The present work was undertaken to study the nitrogen-containing secondary metabolites of *P. vitale* VKM F-3624 cultivated in different media and to refine the taxonomic position of this strain.

MATERIALS AND METHODS

P. vitale VKM F-3624 was obtained from the All-Russia Collection of Microorganisms (VKM). The strain was grown either in a synthetic medium containing (g/l) 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 with 25% NH_4OH) or in the following complex media: (1) glucose–peptone medium containing (g/l) glucose, 50.0; peptone, 10.0; soybean meal, 5.0; KNO_3, 2.0; $MgSO_4 \cdot 7H_2O$, 0.5; and $CaCl_2$, 0.1 (the pH of the medium was adjusted to 6.2 with 25% NH_4OH) and (2) a modified Czapek medium containing (g/l) sucrose, 30.0; yeast extract, 5.1; $NaNO_3$, 2.0; KH_2PO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.3; and $FeSO_4 \cdot 7H_2O$, 0.01.

Submerged and surface cultures of the fungus were grown at 24 ± 1°C on a shaker (200–220 rpm) in 750-ml Erlenmeyer flasks containing 150 ml of media. The submerged fungal culture was analyzed for secondary metabolites on the 6th and 12th days of cultivation, whereas the surface culture was analyzed on the 12th and 20th days of cultivation (in both cases, these terms corresponded to the phases of active and stationary growth). In the case of submerged cultivation, metabolites for analysis were extracted from the culture liquid filtrate, whereas in the case of surface cultivation, both the culture liquid and mycelium were analyzed. The mycelium was homogenized in 2% tartaric acid by vigorous vortexing in an MPW-302 homogenizer (Mechanika precyzyjna, Poland) at 2500 rpm for 5 min. The homogenate was filtered, the pH of the filtrate was adjusted to 8–9 with 25% NH_4OH, and basic and neutral metabolites were trice extracted with chloroform. The aqueous phase that remained after chloroform extraction was acidified to pH 3 with 15% HCl, and acidic metabolites were also extracted with chloroform. Metabolites from the culture liquid filtrate were extracted in a similar way. Chloroform extracts were dehydrated with anhydrous Na_2SO_4, filtered, and dried by evaporation.

These samples were preliminarily separated by preparative thin-layer chromatography on Aluminium oxide F-254 glass plates (type E) from Merck (Germany) developed in a chloroform–methanol (95 : 5) mixture (system II). The metabolites that were stained with Ehrlich's reagent were eluted with methanol, and

0026-2617/00/6903-0343$25.00

Mass spectrometry of the secondary metabolites of *P. vitale* VKM F-3624 (relative abundances of ions are given in parentheses)

Metabolite	Characteristic ions, m/z
Meleagrin	41(40), 69(33), 107(27), 143(25), 277(62), 305(50), 318(38), 334(34), 364(63), 365(100), 402(17), 433(34, M)
Roquefortine	41(28), 69(28), 108(18), 130(57), 157(35), 192(10), 320(100), 389(29, M)
3,12-Dihydroroquefortine	41(19), 69(15), 110(52), 130(41), 157(28), 294(46), 322(100), 391(31, M)
Indole-3-acetic acid	77(15), 103(11), 130(100), 175(31, M)
Aurantioclavine	154(81), 167(57), 171(80), 182(61), 196(21), 225(57), 226(100, M)
N-Acetyltryptamine	44(32), 77(15), 103(12), 130(95), 143(100), 202(18, M)

the eluates were evaporated and analyzed by thin-layer chromatography on Silufol UV-254 plates (Kavalier, Czech Republic) developed in a chloroform–methanol–25% NH_4OH (90 : 10 : 1) mixture (system I).

The separated products were identified by cochromatography with the respective authentic compounds. The UV spectra of metabolites in methanol were recorded with a UV-160A spectrophotometer (Shimadzu, Japan), and their mass spectra were obtained with a high-resolution Finnigan MAT 8430 mass spectrometer (Germany). The concentration of metabolites in the culture liquid was determined spectrophotometrically, using calibration curves and the known coefficients of molar extinction.

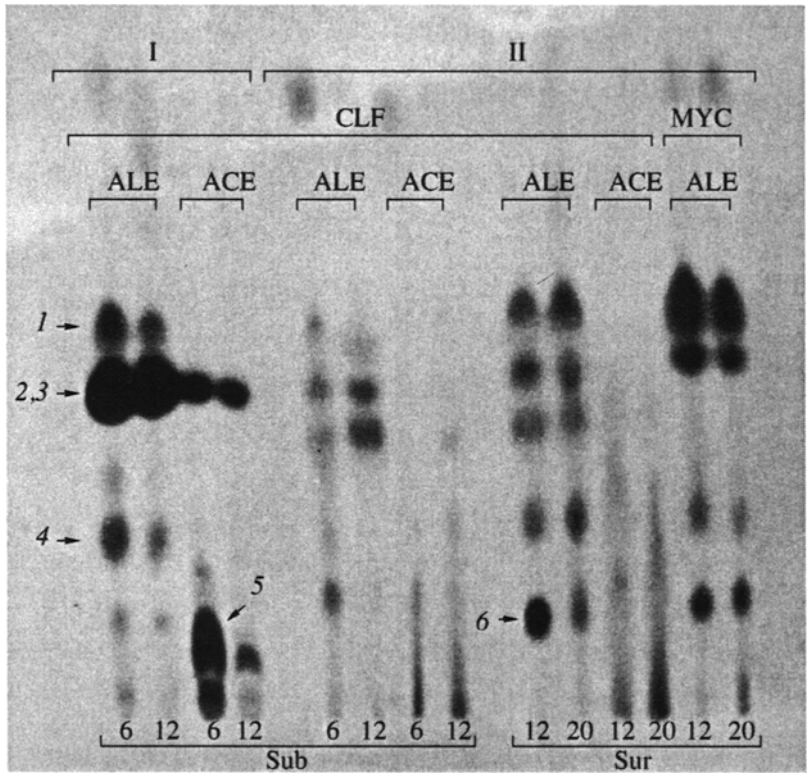
RESULTS AND DISCUSSION

Examination of the physiological and morphological characteristics of *P. vitale* VKM F-3624 showed that they correspond to the original description of N.M. Pidoplichko given in 1961 and published in 1972 [3]. Slow-growing colonies were first green–blue and then turned dirty-gray and fluffy, with a diameter of 17–20 mm after 7 days of growth; the colony reverse was at first pale yellow and then yellow–brown. Extracellular pigments were absent. Conidiophores were 250 to 300 by 2 to 3.5 μm in size and had whorled sterigmata 10 to 15 μm long and 2 to 3 μm thick; phialides had a size of 6 to 8 by 2 to 3.5 μm. Conidia were elliptical or egg-shaped (aged conidia were almost spherical) and had a size of 2.5 to 3 by 2 to 2.5 μm. The strain grew well at 25°C and failed to grow at 37°C.

Since only a brief description [3] of the type strain of *P. vitale* was available to foreign mycologists, they assigned this species to a sibling species, *P. janthinellium* Biourge [4]. However, according to our data on growth rates, the size of phialides, and the shape of conidia, *P. vitale* VKM F-3624 differs from *P. janthinellium*. Furthermore, as mentioned above, *P. vitale* VKM F-3624 fails to grow at 37°C, whereas *P. janthinellium* can [5].

It is known that the toxigenic potential of microscopic fungi depends on growth conditions, the composition of the nutrient medium, the temperature, humidity, etc. [6]. Cultivation conditions can drastically influence the synthesis of secondary metabolites, mycotoxins in particular, as well as the proportions between these metabolites and their distribution between the mycelium and medium [7]. For this reason, to analyze the range of the secondary metabolites of *P. vitale* VKM F-3624, it was grown in both surface and submerged modes either in a modified synthetic Abe medium, which is optimal for the synthesis of clavine alkaloids [8], or in nutritionally complex media. Metabolites were extracted from the mycelium and culture liquid filtrate under alkaline (pH 8–9) and acidic (pH 3) conditions.

The four metabolites isolated from the alkaline extract of the culture liquid of the submerged culture of *P. vitale* VKM F-3624 grown in synthetic medium were stained with Ehrlich's reagent. The major metabolite, produced in an amount of about 8 mg/l, was stained to give an intense orange color typical of meleagrin [9]. The data obtained by cochromatography with the authentic sample of meleagrin, mass spectrometry, and UV spectroscopy (λ_{max} = 207, 229, 286 (shoulder), and 345 nm) also indicated that this metabolite was meleagrin. The other two metabolites, produced in amounts of ~3 and 0.2 mg/l, were identified as roquefortine and 3,12-dihydroroquefortine, respectively, since they were stained blue with Ehrlich's reagent and had UV spectra typical of these alkaloids (λ_{max} = 207, 240, and 324 nm for roquefortine; 207, 243, and 304 nm for 3,12-dihydroroquefortine). Their structure was confirmed by mass spectrometry (see table) and cochromatography with the authentic samples of these alkaloids. All three compounds belong to the group of diketopiperazine alkaloids and have the common precursors histidine, tryptophan, and mevalonic acid. These alkaloids are synthesized by a great number of species belonging to the subgenus *Penicillium* of the section *Penicillium* and to some other subgenera [9–12]. The production of roquefortine is a specific characteristic of some fungal species [11, 12]; however, the strains of *P. janthinellium* that would be able to synthesize roquefortine and/or other diketopiperazine alkaloids are unknown. The fourth metabolite, produced in an amount of about 2 mg/l, gave an intense lilac color with Ehrlich's reagent and had a UV spectrum typical of an indole chromophore (λ_{max} = 221, 271 (shoulder), 282, and 290 nm).



Thin-layer chromatography of the secondary metabolites of *P. vitale* VKM F-3624 in system I: I, synthetic medium; II, glucose–peptone medium; Sub, submerged cultivation; Sur, surface cultivation; CLF, culture liquid filtrate; MYC, mycelium; ALE, alkaline extract; ACE, acidic extract; *1*, meleagrin; *2*, roquefortine; *3*, *N*-acetyltryptamine; *4*, 3,12-dihydroroquefortine; *5*, indole-3-acetic acid; *6*, aurantioclavine.

With allowance for the mass spectroscopy data (see table), this metabolite was identified as *N*-acetyltryptamine. The presence of abundant fragments with *m/z* 130 and 143 in the mass spectrum of this compound suggests that the acetyl group is bound to an indole nitrogen atom rather than to a side chain. Earlier, the products of the partial degradation of tryptophan and their derivatives were revealed in the fungus *P. fellutanum* VKM F-3020 grown in a synthetic medium containing phenylalanine or tryptophan [13].

The compound isolated from the acidic extract of the fungus was identified as indole-3-acetic acid (IAA), based on the data of UV spectroscopy (λ_m = 221, 269, 281, and 290 nm), mass spectrometry (see table), and cochromatography with the authentic sample of IAA.

The fungus grew poorly in synthetic medium in unshaken flasks. The secondary metabolite patterns of the fungus grown in complex media (glucose–peptone medium and Czapek medium with yeast extract) were similar but considerably differed from the secondary metabolite pattern observed in the case of growth in the synthetic medium. Submerged fungal cultures grew well in complex media but showed a poor synthesis of indole alkaloids (see figure): alkaline extracts contained small amounts of meleagrin and *N*-acetyltryptamine, and acidic extracts contained no IAA. In the case of surface growth, meleagrin was the major secondary metabolite of the fungus, localized predominantly in the mycelium. Another indole-containing metabolite, which was stained lilac with Ehrlich's reagent, was identified as aurantioclavine: the mass spectrum of this compound was characterized by the presence of an abundant molecular ion with *m/z* = 226 (see table), and its UV spectrum had a peak at λ_{max} = 286 nm. Aurantioclavine, an ergot alkaloid with a modified C ring [14], is known to be synthesized by a number of fungi belonging to different *Penicillium* subgenera: *P. aurantiovirens* Biourge (syn. *P. aurantiogriseum* Dierckx) VKM F-229 [14], *P. janczewskii* Zaleski VKM F-685 [15], *P. vulpinum* (Cooke et Massee) Seifert et Samson N16 [16], and *P. expansum* Link [17]. The latter two fungi can also synthesize roquefortine. Unlike the aforementioned fungi, which produce aurantioclavine when grown in synthetic medium in a submerged culture, *P. vitale* VKM F-3624 synthesized this alkaloid when grown in complex media in the surface culture. In this case, the alkaloid was already synthesized in high amounts at the early growth stages (on day 6 of growth).

Thus, *P. vitale* VKM F-3624 synthesizes six indole-containing secondary metabolites, three of which (meleagrin, roquefortine, and 3,12-dihydroroquefortine) are diketopiperazine alkaloids, one of which (aurantioclavine) is an ergot alkaloid with a modified C

ring, and two of which (IAA and *N*-acetyltryptamine) may result from the partial transformation or degradation of tryptophan. The pattern of the fungal secondary metabolites produced depends on the cultivation conditions.

As noted above, *P. vitale* has the synonyms *P. janthinellium* Biourge (subgenus *Furcatum*, section *Furcatum*) [4] and *P. simplicissimum* (Oudemans) Thom [18]. The identity of *P. janthinellium* and *P. simplicissimum* has been confirmed by the identity of their secondary metabolite patterns, which included the polyketide metabolites xanthomegnin, semiviolaxanthin, viomellin, and their derivatives [12]. Some strains of these fungi are known to produce a great number of other mycotoxins [5, 12, 19]; however, the indole alkaloids, revealed by us in *P. vitale* VKM F-3624, have not yet been detected in the fungi *P. janthinellium* or *P. simplicissimum*.

To conclude, the type strain *P. vitale* VKM F-3624 differs from *P. janthinellium* in some physiological and morphological characteristics and in the pattern of the secondary metabolites produced and, hence, may represent an independent species.

REFERENCES

1. Nikol'skaya, E.A., Effect of Inoculum on the Synthesis of Glucose Oxidase and Catalase by *Penicillium vitale* Pidoplichko et Bilai, in *Metabolity pochvennykh mikromitsetov* (Metabolites of Soil Micromycetes), Kiev: Naukova Dumka, 1971, pp. 82–102.
2. Gudkova, L.V., Latyshko, I.V., Degtyar', R.G., and Gulyi, M.F., Biosynthesis of Enzymes in Microorganisms, *Tez. dokl. 4 Vses. konf.* (Proc. 4th All-Union Conf.), Tashkent, September 19–22, 1988, pp. 8–9; Refer. Zhurn. Fizikokhim. Biol. Biotekhnol., 1989, 5E272.
3. Pidoplichko, N.M., *Penitsillii* (Penicillia), Kiev: Naukova Dumka, 1972, p. 71.
4. Pitt, J.J., *The Genus Penicillium* and Its Teleomorphic States *Eupenicillium* and *Talaromyces*, New York: Academic, 1979, p. 577.
5. *Penicillium* and *Acremonium*, Peberdy, J.F., Ed., New York: Plenum, 1987.
6. Rzhekhachek, Z., New Achievements in the Investigation of Ergot Alkaloids, *Izv. Akad. Nauk SSSR, Ser. Biol.*, 1976, no. 2, pp. 208–220.
7. Vinokurova, N.G., Reshetilova, T.A., and Kozlovskii, A.G., Biosynthesis of Alkaloids by *Penicillium palitans* under Different Growth Conditions, *Prikl. Biokhim. Mikrobiol.*, 1992, vol. 28, no. 6, pp. 875–879.
8. Rzhekhachek, Z., Physiological Aspects of Synthesis of Ergot Alkaloids, *Prikl. Biokhim. Mikrobiol.*, 1983, vol. 19, no. 2, pp. 267–277.
9. Kozlovskii, A.G., Vinokurova, N.G., and Ozerskaya, S.M., Production of Alkaloids by *Penicillium chrysogenum* Strains Isolated from Soils of Different Climatic Zones, *Mikrobiologiya*, 1998, vol. 67, no. 4, pp. 483–487.
10. Reshetilova, T.A. and Kozlovskii, A.G., Biosynthesis of Alkaloids by Mycelial Fungi, *Prikl. Biokhim. Mikrobiol.*, 1990, vol. 26, no. 3, pp. 291–306.
11. Frisvad, J.C. and Filtenborg, O., Terverticillate Penicillia: Chemotaxonomy and Mycotoxin Production, *Mycologia*, 1989, vol. 81, no. 6, pp. 837–861.
12. Frisvad, J.C. and Filtenborg, O., Revision of the *Penicillium* Subgenus *Furcatum* Based on Secondary Metabolites and Conventional Characters, *Modern Concepts* in *Penicillium* and *Aspergillus* Classification, Samson, R.A. and Pitt, J.J., Eds., New York: Plenum, 1990, pp. 159–170.
13. Kozlovskii, A.G., Zhelifonova, V.P., Vinokurova, N.G., and Adanin, V.M., Some Aspects of Biosynthesis of Diketopiperazine Alkaloids by *Penicillium fellutanum* Biourge VKM F-3020, *Mikrobiologiya*, 1997, vol. 66, no. 5, pp. 605–610.
14. Kozlovskii, A.G., Solov'eva, T.F., Sakharovskii, V.G., and Adanin, V.M., Biosynthesis of Unusual Ergot Alkaloids by *Penicillium aurantiovirens, Dokl. Akad. Nauk SSSR*, 1981, vol. 260, no. 1, pp. 230–233.
15. Kozlovskii, A.G., Vinokurova, N.G., Zhelifonova, V.P., and Adanin, V.M., Secondary Metabolites of the Fungus *Penicillium janczewskii, Prikl. Biokhim. Mikrobiol.*, 1997, vol. 33, no. 1, pp. 70–74.
16. Kozlovsky, A.G., Marfenina, O.E., Vinokurova, N.G., Zhelifonova, V.P., and Adanin, V.M., Mycotoxin Production Profiles of the Genus Penicillium Fungi Isolated from Natural and Polluted Ecosystems, *Mycotoxins*, 1998, no. 48, pp. 37–43.
17. Reshetilova, T.A., Solov'eva, T.F., Fadeeva, L.M., and Kozlovskii, A.G., Biosynthesis of Alkaloids by Some *Penicillium expansum* Strains, *Mikrobiologiya*, 1992, vol. 61, no. 3, pp. 391–394.
18. Stolk, A.C. and Samson, R.A., The Ascomycete Genus *Eupenicillium* and Related *Penicillium* Anamorphs, *Stud. Mycol.*, 1983, vol. 23, pp. 1–149.
19. Bilai, V.I. and Kurbatskaya, O.L., *Opredelitel' toksinobrazuyushchikh mikromitsetov* (A Handbook on the Identification of Toxin-producing Micromycetes), Kiev: Naukova Dumka, 1990, pp. 78–81.