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EXPERIMENTAL ARTICLES

Screening for Ergot Alkaloid Producers among Microscopic Fungi by Means of the Polymerase Chain Reaction

L. V. Boichenko, D. M. Boichenko, N. G. Vinokurova, T. A. Reshetilova, and M. U. Arinbasarov

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

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Abstract—The potential of the polymerase chain reaction for the detection of ergot alkaloid producers among microscopic fungi of the genera *Penicillium* and *Claviceps* was evaluated. Twenty-three strains of various species of fungi with a previously studied capacity for alkaloid production were used. The internal fragment of the gene encoding 4-dimethylallyltryptophan synthase, the enzyme catalyzing the first step in the biosynthesis of ergot alkaloids, was amplified using degenerate primers. This approach revealed an about 1.2-kb specific DNA fragment in micromycetes synthesizing ergot alkaloids with complete tetracyclic ergoline system. Microorganisms that produce alkaloids with modified C or D ergoline rings, as well as α-cyclopiazonic acid, did not yield the PCR fragment of the expected size. This fragment was also not found in fungi incapable of ergot alkaloid production.

Key words: alkaloids, microscopic fungi, PCR, screening.

Ergot alkaloids are indole compounds which were first isolated from *Claviceps purpurea* sclerotia. They represent one of the most interesting groups of secondary metabolites of microscopic fungi. Fungi of the genera *Acremonium*, *Balansia*, *Aspergillus*, *Penicillium*, and certain plants of the *Convolvulaceae* family were also found to synthesize ergot alkaloids. The genus *Claviceps* represents the major source of ergot alkaloids. These fungi produce clavines (agroclavine, elymoclavine, festuclavine) and peptide alkaloids. Some unique metabolites, such as isofumigaclavines A and B, agroclavine-I, epicostaclavine, etc., are encountered in the micromycetes of other genera [1].

Ergot alkaloids exhibit a wide spectrum of biological activities, which justifies the search for the novel producers. In medicine, their adrenoblocking, antiserotonin, and dopaminomimetic properties are used. Ergot alkaloids have a therapeutic effect on some forms of migraine, postpartum hemorrhages, mastopathy, and a sedative effect on the central nervous system [1, 2].

To reveal the producers of ergot alkaloids, submerged cultivation on synthetic medium is commonly used. Extraction of alkaloids from the culture liquid is followed by chromatographic and mass-spectrum analyses. High-performance liquid chromatography (HPLC) requires a set of markers. Thin-layer chromatography (TLC) is often incapable of differentiating between ergot alkaloids and other indole-containing metabolites. Expensive instrumentation is necessary for mass spectrometry. In addition, producers that are genetically capable of alkaloid synthesis but not of expressing the correspondent genes cannot be revealed by traditional methods.

Screening by means of the polymerase chain reaction (PCR) is a promising approach to the search for secondary-metabolite producers. This method has been successfully applied to reveal aflatoxin-synthesizing fungi [3]. The PCR method is simple, reproducible, relatively inexpensive, and it is not dependent on the cultivation conditions.

In this study, the PCR method was used to screen for ergot-alkaloid producers among the fungi belonging to the genera *Claviceps* and *Penicillium*.

MATERIALS AND METHODS

The following strains from the All-Russia Collection of Microorganisms were used in this study: *Claviceps* sp. VKM F-2609, *Penicillium aurantiogriseum* VKM F-329, F-1298; *P. canescens* VKM F-3108; *P. chrysogenum* VKM F-1987; *P. citrinum* VKM F-1079; *P. commune* VKM F-3088; *P. crustosum* VKM F-1746; *P. fellutanum* VKM F-1073; *P. fellutanum* VKM F-3020; *P. glandicola* VKM F-743; *P. janczewskii* VKM F-685; *P. nalgiovense* VKM F-229; *P. piscarium* VKM F-325; *P. roquefortii* VKM F-2389; *P. rugulosum* VKM F-352; *P. vitale* VKM F-3624; *P. vulpinum* VKM F-257. In addition, strains *P. viridicatum* C-47 and *Penicillium* sp. C-70 from the Institute of Regional Problems of Feeding (Almaty) and strains *P. chrysogenum* no. 4 and *P. vulpinum* no. 113 from the Depart-

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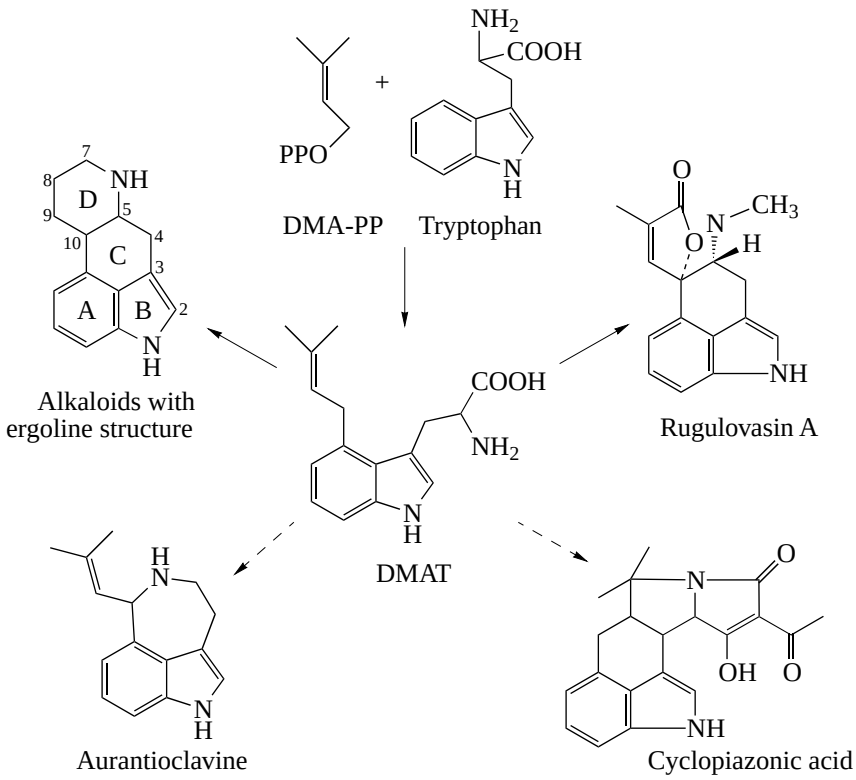

Fig. 1. Scheme of clavine alkaloid biosynthesis in fungi of the genera *Claviceps* and *Penicillium.*

ment of Soil Biology of the Moscow State University were used.

Fungal DNA was isolated by the method described by Cenis [4].

The polymerase chain reaction was carried out on a Perkin-Elmer GeneAmp 2400 cycler (United States). The reaction mixture contained 100 ng of DNA template, 2.5 μl of a buffer (100 mM Tris–HCl, pH 8.8; 500 mM KCl; 0.8% Nonidet P40), 2.5 μl of a dNTP mixture (2 mM each); 1.5 μl of a solution of 25 mM $MgCl_2$; 20 pmol primers, and 2 units of Taq-polymerase (Fermentas). The temperature cycling consisted of 30 cycles: denaturation for 30 s at 94°C, annealing for 20 s at 56°C, and polymerization for 60 s at 72°C. Amplification products were analyzed by electrophoresis in 0.8% agarose gel.

The isolation of plasmid DNA, cloning, and Southern hybridization were performed using conventional techniques [5]. Nucleotide sequences were analyzed on a ABI PRIZM 310 analyzer (Perkin-Elmer, United States) with fluorescent terminators.

The cultivation of microorganisms and the isolation and analysis of alkaloid fractions were performed as described earlier [6].

RESULTS AND DISCUSSION

In fungi of the genus *Claviceps*, the ergot alkaloid biosynthetic pathway has been adequately studied [2]. The first step in their biosynthesis is the alkylation of L-tryptophan with dimethylallyldiphosphate, which results in the formation of 4-dimethylallyltryptophan (DMAT) (Fig. 1). DMAT synthase catalyzes the reaction in which the aromatic ring of tryptophan serves as the acceptor of a prenyl group. The first stage is followed by a series of DMAT modifications, resulting in the formation of various ergot alkaloids. Among these are clavine alkaloids: the derivatives of 6-methylergoline (festuclavine, pyroclavine, fumigaclavines A and B, and others), 6-methylergolenes with a double bond at position 8,9 (agroclavine, agroclavine-I and others) and at position 9,10 (penniclavine, setoclavine and others), alkaloids with open ring D (chanoclavines), modified rings C and D (aurantioclavine and rugulovasins, respectively), and α-cyclopiazonic acid (α-CPA). DMAT synthase is involved in one of the first stages of their biosynthesis [1, 2] (Fig. 1).

DMAT synthase has been previously isolated from ergot, and its amino acid sequence was partially determined. The gene coding for DMAT synthases in some fungi of the genera *Claviceps* and *Balansia* have been cloned [7]. Upon a comparison of their nucleotide sequences in several organisms, degenerate primers were designed to be used for the amplification of an

Screening for alkaloid-producing strains by the traditional method and by polymerase chain reaction

Producers	Alkaloids synthesized	Amplification of the 1.2-kb fragment
1. *Claviceps* sp. VKM F-2609 (=*Claviceps* sp. IBPM F-401)	Clavine alkaloids (chanoclavine I, agroclavine, elymoclavine, setoclavine, isosetoclavine, and penniclavine) [6]	+
2. *Penicillium aurantiogriseum* VKM F-329 *(=P. puberulum)*	Diketopiperazine and benzodiazepine alkaloids [8]	–
3. *P. aurantiogriseum* VKM F-1298	Diketopiperazine alkaloids	–
4. *P. canescens* VKM F-3108	Diketopiperazine alkaloids [9]	–
5. *P. chrysogenum* VKM F-1987	Diketopiperazine alkaloids and alkaloids of the roquefortine group [10]	–
6. *P. chrysogenum* no. 4	Clavine alkaloids (fumigaclavines A and B, pyroclavine, chanoclavine I), α-CPA [10]	+
7. *P. citrinum* VKM F-1079 (=*P. gorlenkoanum)*	Clavine alkaloids (costaclavine, epicostaclavine, chanoclavines) [11]	+
8. *P. commune* VKM F-3088 *(P. palitans)*	Clavine alkaloids (fumigaclavines A and B, festuclavine, pyroclavine, agroclavine, chanoclavine-I), α-CPA [12]	+
9. *P. crustosum* VKM F-1746 (=*P. farinosum)*	Diketopiperazine alkaloids [13]	–
10. *P. fellutanum* VKM F-1073 *(=P. sizovae* F-209)	Clavine alkaloids (agroclavine-I, epoxyagroclavine-I, chanoclavines) [9]	+
11. *P. fellutanum* VKM F-3020	Diketopiperazine alkaloids [9]	–
12. *P. glandicola* VKM F-743	Diketopiperazine alkaloids and alkaloids of the roquefortine group [14]	–
13. *P. janczewskii* VKM F-685	Aurantioclavine [9]	–
14. *P. nalgiovense* VKM F-229 *(=P. aurantiovirens)*	Aurantioclavine [15]	–
15. *P. piscarium* VKM F-325	Diketopiperazine alkaloids [16]	–
16. *P. purpurescens* 359	Diketopiperazine alkaloids [17]	–
17. *P. roquefortii* VKM F-2389 *(=P. roquefortii* IBPM F-141)	Clavine alkaloids (isofumigaclavines A and B, festuclavine, chanoclavine-I), diketopiperazine alkaloids [18]	+
18. *P. rugulosum* VKM F-352	Diketopiperazine alkaloids [19]	–
19. *P. viridicatum* C-47	Benzodiazepine and quinoline alkaloids, rugulovasins A and B [20]	–
20. *P. vitale* VKM F-3624	Diketopiperazine alkaloids, aurantioclavine [21]	–
21. *P. vulpinum* VKM F-257	Patulin, griseofulvin [22]	–
22. *P. vulpinum* no. 113	α-CPA, α-CPA imine [22]	–
23. *Penicillium* sp. C-70	Diketopiperazine alkaloids [20].	–

internal fragment of this gene. These were the P1-dmat primer (5'-CAYCTGGGYATYTTCAAGAAGCAYAT-3') and the P2-dmat primer (5'-TGGAGGTADTTNARN-GAYTCRAARTCVTG-3').

This study used 23 strains of various fungal species from the genera *Penicillium* and *Claviceps*, whose capacity for alkaloid production had been previously studied. With these strains, the results obtained by PCR screening and by screening with the use of traditional methods could be compared (table).

PCR with total fungal DNA isolated from strains synthesizing clavine alkaloids yielded fragments of approximately the expected size, although size variations within a range of 100 bp were observed for some cultures (1.1 kb for F-1073 and 1.2 kb for no. 4, F-2389, F-3088, F-1079, and F-2609) (Fig. 2).

Some additional bands were also observed after electrophoresis, which was probably a result of nonspecific primer annealing. The PCR products obtained with DNA from the same strains hybridized to a radioactively labeled fragment of the DMAT-synthase gene from *Claviceps purpurea*, which confirms that these PCR products indeed were fragments of the DMAT-synthase gene (Fig. 3). The partial sequencing of fragments obtained by amplification of DNA from *Claviceps* sp. and *P. chrysogenum* was used for in the final identification of the PCR products. Alignment of protein products showed that they were 50 to 99% similar

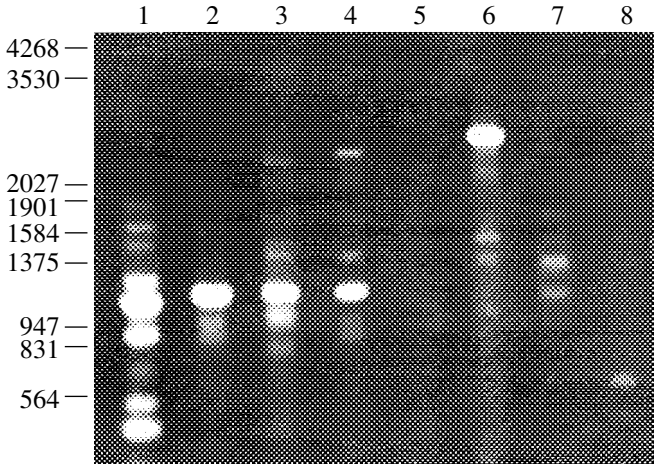

Fig. 2. Analysis of products obtained by amplification of DNA from strains (1) *P. fellutanum* F-1073; (2) *P. chrysogenum* no. 4; (3) *P. roquefortii* F-2389; (4) *P. commune* F-3088; (5) *P. nalgiovense* F-229; (6) *P. janczewskii* F-685; (7) *P. canescens* F-3108; and (8) *P. fellutanum* F-3020.

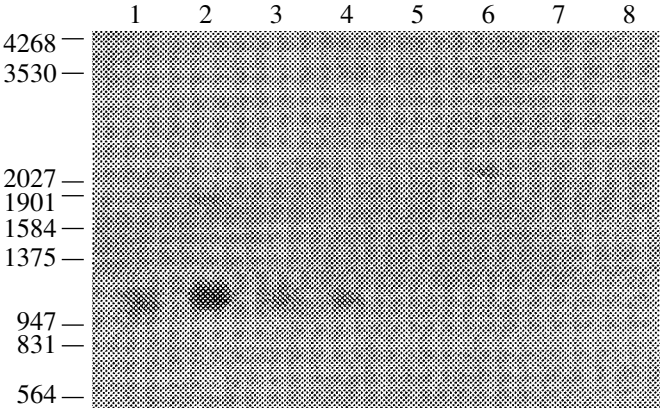

Fig. 3. Blot-hybridization of the amplification products with a fragment of the DMAT-synthase gene from *C. purpurea*. 1, *P. fellutanum* F-1073; 2, *P. chrysogenum* no. 4; 3, *P. roquefortii* F-2389; 4, *P. commune* F-3088; 5, *P. nalgiovense* F-229; 6, *P. janczewskii* F-685; 7, *P. canescens* F-3108; 8, *P. fellutanum* F-3020.

to the products of DMAT-synthase genes from *C. purpurea* and *C. fusiformis.*

The strains incapable of ergot alkaloid production either yielded no bands or the band size differed from the expected one, (e.g., in strain F-3020). Southern hybridization revealed no similarity between these fragments and the DMAT-synthase gene from the *Claviceps* sp. Thus, the PCR products obtained in this case are likely unrelated to ergot alkaloid synthesis.

PCR performed with DNA from strain F-3108 yielded a weak band of an expected size. Nevertheless, chromatographic analysis revealed no clavine alkaloids in this case. Since other strains of the same species do synthesize clavine alkaloids [17], strain F-3108 is probably also capable of producing these compounds under alternative cultivation conditions.

In the strains synthesizing aurantioclavine (F-229, F-685, and F-3624), PCR did not yield specific fragment, although a 2.3-kb fragment was obtained with the DNA from strain F-229. Since DMAT formation is absolutely required for aurantioclavine synthesis, these results most probably mean that the genes encoding DMAT synthases differ in aurantioclavine-producing strains and in strains producing classical ergot alkaloids.

No marker fragment was obtained with DNA from strain C-47, which synthesizes rugulovasins, and with DNA from strain no. 113, an active producer of α-CPA. Note that dymethylallyldiphosphate alkylates modified tryptophan (Fig. 1), and, therefore, the genes encoding DMAT synthase in strains producing α-CPA may differ in their structure from the genes of ergot alkaloid producers.

Thus, in ergot alkaloid–synthesizing micromycetes, a specific 1.2-kb DNA fragment can be revealed by means of PCR. In strains producing either alkaloids with modified C and D ergoline rings or α-CPA, no marker fragment of the expected size was obtained by the PCR method. This fragment was also not obtained with DNA from fungi synthesizing no ergot alkaloids (as determined using traditional screening methods).

The results of this study demonstrate that the PCR technique may serve as a useful tool to screen for strains producing ergot alkaloids with a complete tetracyclic ergoline system.

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