ISSN 0026-2617, Microbiology, 2007, Vol. 76, No. 4, pp. 400–404. © Pleiades Publishing, Ltd., 2007.
Original Russian Text © V.P. Zhelifonova, A.G. Kozlovskii, 2007, published in Mikrobiologiya, 2007, Vol. 76, No. 4, pp. 456–461.

EXPERIMENTAL ARTICLES

Transport of Ergot Alkaloids and Quinocitrinins in the Producing Fungus *Penicillium citrinum*

V. P. Zhelifonova and A. G. Kozlovskii[1]

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

Received June 8, 2006

Abstract—The decrease in the concentration of alkaloids in the culture liquid of *Penicillium citrinum* grown to the early stationary phase was found to be due to the uptake of quinocitrinins and ergot alkaloids by fungal cells. The ability of the fungal mycelium to take up autogenous quinocitrinins does not depend on the mycelium age, whereas its ability to take up ergot alkaloids is higher in the young than in the 12-day-old mycelium. The uptake of exogenously added ergot alkaloids by the fungal mycelium is accompanied by excretion of intracellular quinocitrinins. The addition of quinocitrinins to the medium was found to exert different effects in different growth stages. Namely, the uptake of exogenously added quinocitrinins by the actively growing young mycelium inhibits the excretion of ergot alkaloids, but the excretion of ergot alkaloids by the 12- day-old mycelium occurs throughout the cultivation period. The excretion of both ergot alkaloids and quinocitrinins does not require energy.

Key words: microscopic fungi, *Penicillium*, secondary metabolites, transport.

DOI: 10.1134/S0026261707040030

Many alkaloid-producing fungi from the genus *Penicillium* accumulate alkaloids in the culture liquid and in the mycelium in a cyclic mode [1–8]. The phenomenon of the cyclic accumulation of alkaloids is poorly understood. Some authors believe that the culture age, the growth medium, and the cultivation conditions of alkaloid-producing fungi are related [5, 6]. Lovkova explained the cyclic accumulation of alkaloids by the fact that they serve as reserve depots of amino acids, pentoses, and other essential substances [9]. If this is the case, transport processes across the cell wall must be intense. In our earlier work [7], we suggested that the cyclic accumulation of alkaloids is due to the superposition of excretion and uptake processes. Our recent study showed that the transport of aurantioclavine in the fungus *Penicillium nalgiovense* depends on the mycelium age [8]. The transport of alkaloids is the fungus *Penicillium citrinum* VKM FW-800, which was isolated from ancient permafrost sediments, is of great interest because this fungus synthesizes alkaloids belonging to two different classes, ergot alkaloids and quinolines [10–12].

This work was undertaken to study some aspects of the transport of ergot alkaloids and quinocitrinins in different growth stages of the fungus *P. citrinum* FW-l800.

[1] Corresponding author's e-mail: kozlovski@ibpm.pushchino.ru.

MATERIALS AND METHODS

The strain *Penicillium citrinum* VKM FW-800 used in this work was isolated from ancient (1.8- to 3.0-million-year-old) Arctic permafrost sediments [13]. The strain was maintained on glucose–potato agar slants. The inoculum was prepared by suspending spores from a 14-day-old culture in water to a density of $(1–2 \times 10^7$ spores/ml. The basal medium contained (in g/l distilled water) mannitol, 50; succinic acid, 5.4; $MgSO_4 \cdot 7H_2O$, 0.3; KH_2PO_4, 1.0; and $ZnSO_4 \cdot 7H_2O$, 0.004. The pH of the medium was adjusted to 5.4 with an aqueous ammonium hydroxide solution. The fungus was cultivated at 24 ± 1°C on a shaker (220 rpm) in 750-ml Erlenmeyer flasks containing 150 ml of the medium. Samples for analysis were taken in triplicate 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 as described earlier [10, 11]. The extracts were analyzed by TLC on Silica gel 60 F_{254} plates (Merck, Germany) in a solvent mixture containing chloroform, methanol, and 25% NH_4OH in a proportion of 80 : 20 : 0.2. The separated substances were visualized by examining the developed plates under UV light and by spraying the plates with the Ehrlich (to detect ergot alkaloids) and Dragendorff (to detect quinocitrinins) reagents.

The total amount of ergot alkaloids and quinocitrinins was determined in alkaline [10] and acid extracts, respectively. The optical density of the methanol solutions of ergot alkaloids and quinocitrinins was measured at 283 and 314 nm, respectively, using an SF-26 spectrophotometer (LOMO, Russia).

The degradation of alkaloids in the culture liquid was estimated as follows: the filtrate of the culture liquid prepared from a portion of an 11-day-old culture was sterilized by ultrafiltration through a 0.2-μm membrane filter (Schleicher & Schuell, Germany). The rest of the culture was cultivated for the next 24 h. The sterile filtrate (approximately 150 ml in volume) was analyzed for alkaloids before and after its incubation in a 750-ml flask under cultivation conditions for 24 h. The concentration of alkaloids in the filtrate before and after incubation was compared with their concentration in the 12-day-old culture.

The excretion of alkaloids was studied as follows: the mycelia of 6- and 12-day-old cultures were separated from the culture liquids by filtration and washed four times with 150 ml of cold distilled water (control experiments showed that such treatment was sufficient to remove all the alkaloids sorbed on the cell surface). The washed mycelia were suspended in 450 ml of 20 mM MOPS buffer (pH 5.4) and incubated at 24 ± 1°C on a shaker. After 5, 20, and 120 min of incubation, the mycelial suspensions were taken for analysis in 150-ml portions. The mycelia were collected by filtration, washed four times with cold distilled water, and dried to a constant weight. The concentration of alkaloids in the filtrates and the wash waters was determined as described above. The energy dependence of alkaloid excretion was studied by adding carbonyl cyanide &p- (trifluoromethoxy)phenylhydrazone (FCCP) to the incubation buffer at a concentration of 5 μM.

The uptake of exogenously added alkaloids by the mycelia was studied as follows: the wet mycelia (40 g) were suspended in 450 ml of 20 mM MOPS buffer (pH 5.4) containing ergot alkaloids at a concentration of 58 μg/ml (experiment 1), quinocitrinins at a concentration of 14 μg/ml (experiment 2), and a mixture of these alkaloids at the aforementioned concentrations (experiment 3). The suspensions were incubated at 24 ± 1°C on a shaker for 120 min. The alkaloids were analyzed as described above.

Tryptophan was analyzed using a T-339 amino acid analyzer (Czech Republic).

RESULTS AND DISCUSSION

As shown earlier [10, 12], the fungus *P. citrinum* VKM FW-800 grows in media containing two carbon sources (succinate and mannitol) with a diauxie and accumulates ergot alkaloids and quinocitrinins in the culture liquid in a cyclic mode. The concentration of ergot alkaloids and quinocitrinins increases almost concurrently with the biomass during the first stage of active fungal growth on succinate. During transition to the phase of mannitol consumption, fungal growth and alkaloid biosynthesis are suspended. During the phase of active growth on mannitol, the concentration of alkaloids in the medium again increases. In the early stationary phase (12 days of growth), the concentration of alkaloids decreases both in the mycelium and in the culture liquid, but then rises again (Table 1).

Table 1. The content of extracellular and intracellular alkaloids and free tryptophan in the *P. citrinum* mycelium of different age (in μg/g dry wt.)

Mycelium age, days	Ergot alkaloids		Quinocitrinins		Tryptophan
	extracellular	intracellular	extracellular	intracellular	
6	2470	130	–	–	–
11	12430	1770	680	140	11000
12	7670	970	580	50	0
13	13300	960	760	70	7100
14	12800	1310	700	50	27000

In fact, the cyclic accumulation of alkaloids in the medium and cells is typical of many alkaloid- synthesizing *Penicillium* fungi [1–8]. The number of cycles can be different. For example, the relict strain of *P. citrinum* and the agroclavine-I- and epoxyagroclavine-I-producing strains of *P. kapuscinskii* and *P. sizovae* show two cycles of alkaloid synthesis with minima in the diauxic shift period and in the early stationary phase [3, 4].

The concentration of alkaloids in the culture is determined by several processes: their biosynthesis in cells, excretion to the medium, degradation in the culture liquid, and uptake by the cells [7]. Presumably, biosynthesis and excretion prevail in the periods of the active growth of *P. citrinum* (from the second to the seventh and from the ninth to the tenth day of cultivation), whereas the degradation and/or uptake of alkaloids prevail during the diauxic shift period (the eighth day of cultivation) and in the early stationary phase (the eleventh to twelfth day of cultivation). The biphasic mode of accumulation of secondary metabolites in the culture liquid can be explained either by their conversion into other secondary metabolites [15] or by their uptake [10]. To choose between these possibilities, we analyzed the acidic and alkaline chloroform extracts of alkaloids prepared before and after drastic changes in their concentration in the culture liquid, including the changes observed on the twelfth day of cultivation. This analysis showed that the qualitative content of alkaloids does not change, thus suggesting that ergot alkaloids and quinocitrinins do not markedly convert into other alkaloids.

This finding does not, however, exclude the possibility that ergot alkaloids and quinocitrinins are con-

Table 2. The effect of FCCP on the excretion of alkaloids from the *P. citrinum* mycelium of different age (in μg/g dry wt.)

Alkaloids	Mycelium age, days	FCCP, μM	Incubation time, min		
			5	20	120
Ergot alkaloids	6	0	170	210	380
		5	210	210	250
	12	0	450	400	390
		5	390	340	450
Quinocitrinins	6	0	60	100	260
		5	100	110	160
	12	0	110	130	110
		5	70	60	70

verted (degraded) into simple metabolites by extracellular enzymes occurring in the culture liquid of *P. citrinum.* To verify this assumption, the sterile filtrate of the culture liquid of the 11-day-old fungus was incubated aseptically under the cultivation conditions. The filtrate initially contained ergot alkaloids and quinocitrinins in amounts of 100 and 25 mg/l, respectively. After 24 h of incubation, the concentration of alkaloids in the filtrate did not change, although their concentrations in the culture liquid of the growing fungus decreased to 58 and 11 mg/l, respectively, within the same incubation period. This experiment clearly shows that the decline in the concentration of alkaloids in the medium observed on the twelfth day of cultivation is due to their uptake rather than to their conversion by extracellular enzymes. In different growth stages, the *P. citrinum* mycelium may possess different ability to take up and to excrete ergot alkaloids and quinocitrinins. The uptake and the excretion of alkaloids were studied by using the mycelium washed with distilled water to remove the alkaloids adsorbed on the cell surface.

In the experiments on alkaloid excretion, the washed mycelium was incubated in the MOPS buffer (pH 5.4), and the alkaloid concentration in the buffer was measured after 5, 20, and 120 min of incubation. These experiments showed that the alkaloids were most extensively excreted from the 6- and 12- day-old mycelia within the first 5 min of incubation (Table 2, rows with a zero concentration of FCCP). The excretion of ergot alkaloids and quinocitrinins from the young mycelium took more time (at least 120 min) than from the stationary-phase 12-day-old mycelium. All ergot alkaloids were excreted from the 12-day-old mycelium within 5 min, whereas the excretion of all quinocitrinins took 20 min. During further incubation, the concentration of ergot alkaloids and quinocitrinins in the incubation buffer decreased, suggesting the uptake of the excreted alkaloids by the mycelia. Presumably, in the absence of carbon and nitrogen sources in the incubation buffer, the longer synthesis of ergot alkaloids and quinocitrinins by the young mycelium (compared to the stationary-phase mycelium) was due to internal energy sources. Indeed, the intracellular content of ergot alkaloids in the 6- and 12-day-old mycelia was found to be 130 and 970 μg/g dry wt., respectively. In this case, both mycelia excreted the same amount of ergot alkaloids (380 μg/g dry wt.) over the 120-min period of incubation. In contrast, over the same incubation period, the 6- day-old mycelium excreted two times more quinocitrinins than did the 12-day-old mycelium.

To decide whether the excretion of ergot alkaloids and quinocitrinins is an energy-dependent process or not, we used FCCP, a protonophore that, at a concentration of 5 μM, eliminates the electrochemical gradient of H^+ ions on plasma membranes and, hence, inhibits energy-dependent transport processes. The addition of this substance to the incubation medium virtually did not influence the excretion of alkaloids (Table 2), except for some inhibition of the excretion of quinocitrinins from the 12-day-old mycelium. These results suggest that the excretion of alkaloids is an energy-independent process.

To study the ability of the fungus to take up excreted alkaloids, the 6- and 12-day-old mycelia were incubated in the MOPS buffer supplemented with ergot alkaloids and quinocitrinins at physiological concentrations (Table 3). This table shows that the concentration of alkaloids in the medium gradually decreased over the course of incubation, suggesting that the alkaloids were consumed by the mycelia. The decrease in the alkaloid concentration was most pronounced within 5 min of incubation. Such rapid process suggests that the alkaloids are sorbed on the cell surface. The uptake of ergot alkaloids by the mycelium almost did not depend on its age (experiment 1), whereas the 12-day-old mycelium took up quinocitrinins at a 40% lower rate than did the 6-day-old mycelium (experiment 2). The uptake of ergot alkaloids by the young mycelium took more time (at least 120 min) than by the stationary-phase 12-day-old mycelium (5 min). The uptake of extracellular quinocitrinins by the 6- and 12-day-old mycelia persisted to the end of the experiment, when half of these alkaloids remained in the medium. The uptake of ergot alkaloids by the 6- and 12-day-old mycelia throughout the incubation period were equal 60 and 40%, respectively. Thus, the ability of *P. citrinum* to take up extracellular quinocitrinins does not depend on the fungal age. In contrast, the ability to take up ergot alkaloids is higher in the young mycelium.

Table 3 also shows that the uptake of added ergot alkaloids was accompanied by the excretion of intracellular quinocitrinins throughout the incubation period (experiment 1). The excretion dynamics of quinocitrinins did not depend on the fungal age and was most intensive within the first 5 min of incubation.

The uptake of added quinocitrinins stopped the excretion of ergot alkaloids by the 6-day-old mycelium, whereas the excretion of ergot alkaloids from the 12-day-old mycelium was observed throughout the observation period (experiment 2). In the control experiment, all ergot alkaloids were excreted from the 12-day-old mycelium within 5 min.

Theoretically, fungal cells can take up alkaloids through their sorption on the cell surface and through their transport into the cell interior across the cell membrane. The rapid consumption of extracellular alkaloids within the first 5 min of incubation suggests that they are consumed due to sorption on the cell surface.

Accordingly, the decrease in the extracellular concentration of alkaloids is due to their uptake by the mycelium rather than by their conversion into other metabolites. The experiments show that the ability of the 12-day-old mycelium to take up the excreted alkaloids is not higher than that of the young 6-day-old mycelium. The excretion of ergot alkaloids and quinocitrinins from the mycelia of different age is energy-independent and occurs along the concentration gradient. In other words, the amount of excreted alkaloids is determined by the intensity of their synthesis. In the absence of carbon and nitrogen sources in the medium, ergot alkaloids and quinocitrinins are synthesized in the young mycelium over a longer period of time (compared to the stationary-phase mycelium) at the expense of internal energy sources.

Based on the model experiments, we may suggest that the biosynthesis of alkaloids and their energy-independent excretion from the fungus *P. citrinum* prevail in the phase of active growth. In the early stationary phase, the biosynthesis of alkaloids stops because of the absence of free tryptophan in the cells (Table 1). As a result, the uptake of alkaloids from the medium begins to prevail over their excretion from the cells.

Investigations into the *Claviceps* fungi allowed Robbers et al. to propose that ergot alkaloids bind to excessive primary metabolites, the alkaloid precursor tryptophan in particular, thus preventing a feedback inhibition of fungal metabolism by this amino acid [15]. The data presented in this article allow the suggestion that alkaloids are consumed in the early stationary phase of *P. citrinum* due to a low content of primary metabolites, including tryptophan, which are necessary for fungal growth.

The study of the transport and excretion of alkaloids is very important in elucidating their role in fungal metabolism and the regulatory mechanisms of alkaloid synthesis. Despite the scarcity of relevant studies, it is clear that alkaloids are not physiologically inert compounds and may play a certain physiological role in the producing organism, being involved in its growth and development.

To conclude, the low concentration of alkaloids in the culture liquid of *P. citrinum* grown to the early stationary phase is due to the uptake of quinocitrinins and ergot alkaloids by fungal cells. No conversion of these alkaloids by extracellular enzymes is observed. The ability of the fungal mycelium to take up autogenous quinocitrinins does not depend on the mycelium age, whereas its ability to take up ergot alkaloids is higher in the young 6-day-old mycelium than in the stationary-phase 12-day-old mycelium. The uptake of the exogenously added ergot alkaloids by the fungal mycelium is accompanied by excretion of intracellular quinocitrinins. Quinocitrinins added to the incubation medium exert different effects on the mycelium of different age. Namely, the uptake of exogenous quinocitrinins by the actively growing mycelium arrests the excretion of ergot alkaloids, whereas ergot alkaloids are excreted from the 12-day-old mycelium throughout the period of observation. The excretion of ergot alkaloids and quinocitrinins does not require energy.

Table 3. The dynamics of alkaloids in the incubation medium of the *P. citrinum* mycelium of different age (in mg/l)

Experiment	Mycelium age, days	Alkaloids	Incubation time, min			
			0	5	20	120
Control	6	EAs	0	2.2	2.8	5.1
		QAs	0	0.7	1.4	3.5
	12	EAs	0	4.3	4.2	4.0
		QAs	0	1.1	1.3	1.2
1	6	EAs	58.0	27.6	26.6	28.7
		QAs	0	1.5	1.4	1.9
	12	EAs	58.0	25.0	29.0	29.0
		QAs	0	1.6	1.4	2.2
2	6	EAs	0	0	0	0
		QAs	14.0	6.8	7.5	7.6
	12	EAs	0	2.6	3.9	5.0
		QAs	14.4	9.1	7.0	5.8
3	6	EAs	57.6	31.9	30.5	35.6
		QAs	13.4	8.0	7.1	8.0
	12	EAs	58.0	21.3	30.7	22.9
		QAs	13.6	7.8	8.3	7.5

REFERENCES

1. Reshetilova, T.A. and Kozlovskii, A.G., Biosynthesis of Alkaloids in Mycelial Fungi, *Prikl. Biokhim. Mikrobiol.*, 1990, vol. 26, no. 3, pp. 291–306.
2. Kozlovsky, A.G., Solovieva, T.F., Reshetilova, T.A., and Skryabin, G.K., Biosynthesis of Roquefortine and 3,12-Dihydroroquefortine by the Culture *Penicillium farinosum, Experientia*, 1981, no. 37, pp. 472–473.
3. Kozlovskii, A.G. and Solov'eva, T.F., The Effect of Cultivation Conditions on Alkaloid Biosynthesis in *Penicil-*

lium kapuscinskii, Mikrobiologiya, 1986, vol. 55, no. 1, pp. 34–40.

4. Kozlovskii, A.G., Vepritskaya, I.G., and Gayazova, N.B., Biosynthesis of Alkaloids in the Fungus *Penicillium sizovae, Prikl. Biokhim. Mikrobiol.*, 1986, vol. 22, no. 2, pp. 205–210.
5. Reshetilova, T.A. and Kozlovsky, A.G., Synthesis and Metabolism of Roquefortine in *Penicillium* Species, *J. Basic Microbiol.*, 1990, vol. 30, no. 2, pp. 109–114.
6. Reshetilova, T.A., Yarchuk, N.I., Shurukhin, Yu.V., and Kozlovskii, A.G., Alkaloids in the Fungus *Penicillium expansum, Prikl. Biokhim. Mikrobiol.*, 1991, vol. 27, no. 5, pp. 725–730.
7. Kozlovskii, A.G. and Reshetilova, T.A., Biosynthesis and Metabolism of Roquefortine Alkaloids in Microscopic Fungi, *Biotekhnol.*, 1987, vol. 3, no. 5, pp. 565–571.
8. Zhelifonova, V.P., Kulakovskaya, T.V., and Kozlovskii, A.G., On the Mechanisms of the Excretion and Uptake of the Alkaloid Aurantioclavine during the Growth of the Fungus *Penicillium nalgiovense* VKM F-229, *Mikrobiologiya*, 2003, vol. 72, no. 2, pp. 183–188.
9. Lovkova, M.Ya., *Biosintez i metabolizm alkaloidov v rasteniyakh* (Biosynthesis and Metabolism of Alkaloids in Plants), Moscow: Nauka, 1981.
10. Kozlovskii, A.G., Zhelifonova, V.P., Adanin, V.M., Antipova, T.V., Ozerskaya, S.M., Kochkina, G.A., and Grefe, U. 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, *Mikrobiologiya*, 2003, vol. 72, no. 6, pp. 816–821.
11. Kozlovsky, A.G., Zhelifonova, V.P., Antipova, T.V., Adanin, V.M., Ozerskaya, S.M., Kochkina, G.A., Schlegel, B., Dahse, H.M., Gollmick, F.A., and Grefe, U., Quinocitrinines A and B, New Quinoline Alkaloids from *Penicillium citrinum* VKM FW-800, a Permafrost Fungus, *J. Antibiot.*, 2003, vol. 56, no. 5, pp. 488–491.
12. Kozlovskii, A.G., Zhelifonova, V.P., and Antipova, T.V., The Fungus *Penicillium citrinum* Thom 1910 VKM FW-800 Isolated from Ancient Permafrost Sediments as a Producer of Ergot Alkaloids and New Quinoline Alkaloids Quinocitrinins, *Prikl. Biokhim. Mikrobiol.*, 2005, vol. 45, no. 5, pp. 568–572.
13. Kochkina, G.A., Ivanushkina, N.E., Karasev, S.G., Gavrish, K.Yu., Gurina, L.I., Evtushenko, L.I., Spirina, E.V., Vorob'eva, E.A., Gilichinskii, D.A., and Ozerskaya, S.M. Survival of Micromycetes and Actinobacteria under Conditions of Long-Term Natural Cryopreservation, *Mikrobiologiya*, 2001, vol. 70, no. 3, pp. 412–420.
14. Betina, V., Differentiation and Secondary Metabolism in Some Prokaryotes and Fungi, *Folia Microbiol.*, 1995, vol. 40, no. 1, pp. 51–67.
15. Robbers, J.E., Eggert, W.W., and Floss, H.G., Physiological Studies on Ergot: Time Factor Influence on the Inhibitory Effect of Phosphate and the Induction Effect of Tryptophan on Alkaloid Production, *Lloydia*, 1978, vol. 41, no. 2, pp. 120–129.