Microbiology, Vol. 72, No. 2, 2003, pp. 152–156. Translated from Mikrobiologiya, Vol. 72, No. 2, 2003, pp. 183–188.

EXPERIMENTAL ARTICLES

On the Mechanisms of the Excretion and Uptake of the Alkaloid Aurantioclavine during the Growth of the Fungus *Penicillium nalgiovense* VKM F-229

V. P. Zhelifonova, T. V. Kulakovskaya, and A. G. Kozlovskii

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

Received October 1, 2001; in final form, November 22, 2001

Abstract—The biphasic dynamics of the alkaloid aurantioclavine in the culture liquid of *P. nalgiovense* VKM F-229 is shown to be due to the diauxic growth of the fungus on two carbon sources, succinate and mannitol. In the phase of active growth on succinate, the fungus synthesizes aurantioclavine and excretes it into the medium in an energy-independent manner, as a result of which the concentration of the alkaloid in the culture liquid rises. During the phase of metabolic adaptation to the other carbon source, mannitol, the concentration of aurantioclavine in the culture liquid falls, probably due to the energy-dependent uptake of the alkaloid by fungal cells. The reversible excretion of aurantioclavine in *P. nalgiovense* indicates that this process is regulatory and depends on the growth parameters and the physiological state of the fungus.

Key words: filamentous fungi, *Penicillium*, biosynthesis, secondary metabolites, alkaloids, aurantioclavine, transport, excretion.

Aurantioclavine is an ergot alkaloid, whose molecular structure is characterized by a tetracyclic ergoline system with a seven-atom ring C and a modified ring D [1]. An efficient producer of this alkaloid is *Penicillium nalgiovense* Laxa (1932) VKM F-229, formerly known as *P. aurantiovirens* VKM F-229 [2]. Optimal media for the synthesis of aurantioclavine and other ergot alkaloids by *Penicillium* fungi contain succinate and mannitol as carbon sources and ammonium salts as nitrogen sources [3]. *P. nalgiovense* VKM F-229 synthesizes alkaloids (chanoclavine I, agroclavine, elimoclavine, and isopenniclavine) only under hypoxic conditions [3].

The concentration of alkaloids in the culture liquid and the mycelium of *Penicillium* fungi periodically changes in the course of cultivation [4–9]. This phenomenon is not understood in depth, although there is evidence that the culture age, the medium composition, and the cultivation mode are involved [8, 9] and that periodicity in the accumulation of alkaloids in the medium may be related to their alternating excretion and uptake by producing fungi [10]. Relevant investigations are scarce and limited largely to the study of the transport of the diketopiperazine alkaloid roquefortine in the fungus *P. crustosum*, which either secretes or takes up roquefortine from the medium [11–14]. These results, however, cannot be entirely extrapolated to cultivation conditions, since the experiments in those works were only performed in vitro with a young mycelium (two- to three-days-old).

The aim of this work was to study the mechanisms of the excretion and uptake of the alkaloid aurantioclavine during the growth of the fungus *P. nalgiovense* VKM F-229.

MATERIALS AND METHODS

The fungus *Penicillium nalgiovense* Laxa (1932) VKM F-229, formerly known as *P. aurantiovirens* VKM F-229, was obtained from the All-Russia Collection of Microorganisms (VKM). The fungus was maintained on glucose–potato agar slants and grown in a liquid medium containing (g/l distilled water) mannitol, 50; succinic acid, 5.4; $MgSO_4 \cdot 7H_2$, 0.3; and KH_2PO_4, 1.0. The pH of the medium was adjusted to 5.4 with NH_4OH. The fungus was cultivated at 24 ± 1°C in a 750-ml Erlenmeyer flask with 150 ml of the medium on a shaker (220 rpm).

Fungal growth was evaluated from the mass of the dry mycelium. The total nitrogen in the culture liquid was determined by the Kjeldahl method [15], protein was quantified by the method of Lowry *et al.* [16], and phosphates were measured as described by Weil-Mahlerbe and Green [17].

To determine extracellular aurantioclavine, the pH of the culture liquid filtrate was adjusted to 8.0 with aqueous ammonia and extracted thrice with chloroform (1 : 1, v/v). The chloroform extracts were pooled, dehydrated with anhydrous sodium sulfate, and then completely dried using a vacuum rotary evaporator. To

0026-2617/03/7202-0152$25.00

assay intracellular aurantioclavine, the mycelium was washed thrice with distilled water, suspended in 2% tartaric acid, and disrupted using an MPW-302 homogenizer (Mechanika precyzyjna, Poland) at 3000 rpm for 3 min. The homogenate was filtered, the filter cake was washed thrice with 2% tartaric acid, and the four filtrates were pooled. Then the pooled filtrates were treated in the same manner as described above for extracellular aurantioclavine.

Aurantioclavine was analyzed by thin-layer chromatography (TLC) and UV spectrophotometry. To this end, the dry extracts were dissolved in aliquots of chloroform and applied onto Silufol UV-254 plates (Czech Republic). The plates were developed in a chloroform–methanol–saturated ammonia solution (80 : 20 : 0.2) mixture. Zones with aurantioclavine spots, which were visualized under UV light, were cut and eluted with methanol. The aurantioclavine-containing eluate was clarified by passing through glass filter no. 4. The concentration of aurantioclavine in the filtrate was determined from its optical density measured at 287 nm in an SF-26 spectrophotometer (Russia) using a calibration curve constructed with an authentic sample of aurantioclavine. The standard error of these measurements was ±5%.

The possibility of aurantioclavine degradation in the culture liquid was tested in the following experiment. The culture liquid filtrate (300 ml) was sterilized by passing through a 0.2-μm membrane filter (Schleicher & Schüll, Germany). Half of this filtrate (150 ml) was placed in a sterile Erlenmeyer flask (750 ml), incubated for 24 h under the cultivation conditions of the fungus, and then analyzed for the concentration of aurantioclavine. The other half of the filtrate was analyzed for its concentration of aurantioclavine immediately after filtration.

The concentration of aurantioclavine in the culture liquid of the growing fungus was determined three times per day between the 3rd and 13th days of cultivation. To elucidate the mechanism by which fungal cells take up aurantioclavine from the medium, a nine-day fungal culture was supplemented with the protonophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) to a concentration of 5 μM, and cultivation was continued for 5 h. Then the culture liquid was analyzed for the aurantioclavine concentration.

The excretion of aurantioclavine was studied in vitro using a young (three-day-old) mycelium. The mycelium was harvested by filtration and washed thrice with distilled water. Aliquots of the washed mycelium (5 g by wet wt or 0.42 g by dry wt) were placed in flasks with 150 ml of 20 mM MOPS-Na buffer (pH 5.4) and incubated at 24 ± 1°C on a shaker. The content of aurantioclavine in the buffer was determined after 3, 5, 15, 20, 30, 60, and 240 min of incubation. The effect of certain compounds on the excretion of aurantioclavine was studied by adding 22 mM sodium succinate, 20 mM L-tryptophan, or 5 μM FCCP to the buffer before incubation. The concentration of aurantioclavine in these experiments was measured after 20 and 240 min of incubation.

RESULTS AND DISCUSSION

When cultivated in the mineral medium with two carbon sources, succinate and mannitol, and NH_4OH as the nitrogen source, *P. nalgiovense* VKM F-229 showed typical diauxic growth with two values of the maximal specific growth rate and a transient lag phase, which began on the ninth day of cultivation (Fig. 1). On the tenth day of cultivation, the fungus began growing again. As was shown earlier, during the transient lag phase, the fungus adapts to growing on the second carbon source, mannitol [6].

The dynamics of aurantioclavine in the culture liquid of the fungus also displayed a biphasic pattern. The extracellular concentration of the alkaloid increased concurrently with the biomass during the first phase of active growth on succinate, decreased almost twofold during the transient lag phase, and increased again during the second phase of active growth on mannitol to reach a maximum by the beginning of the stationary growth phase (Fig. 1).

The cyclic character of accumulation was also observed for many other alkaloids of clavine, diketopiperazine, benzodiazepine, and quinoline nature [4–9], the number of cycles ranging from one in the case of the fungus *P. farinosum* [5] to two in the case of *P. kapuscinskii* [6] and *P. sizovae* [7] and to four in the case of *P. verricosum* [8]. The complex character of alkaloid accumulation is not perfectly understood, although there is evidence that it depends on the physiological and biochemical properties of growing strains and is related to the culture age, the composition of the medium, and the cultivation mode [4, 8].

The similar dynamics of the specific growth rate (μ) and the specific rate of aurantioclavine accumulation in the medium (q_a) (Fig. 2) indicate that the processes of growth and alkaloid synthesis are closely related and that the biphasic pattern of aurantioclavine accumulation in the medium may be due to the diauxic growth of the fungus on two carbon sources, succinate and mannitol.

In general, the content of an alkaloid in a culture liquid is determined by the relative rates of its synthesis, excretion from and uptake by cells, and degradation in the culture liquid [8]. During the active growth phase (the second to sixth days of cultivation), the processes of aurantioclavine synthesis and excretion are obviously prevalent, whereas when μ decreases (the eighth to ninth days of cultivation), the uptake of the alkaloid from the culture liquid and/or its degradation there are dominant.

Changes in the content of a secondary fungal metabolite in the medium are usually explained either by its conversion into other products [18] or by its uptake by producing cells [10]. To clarify which of these possible

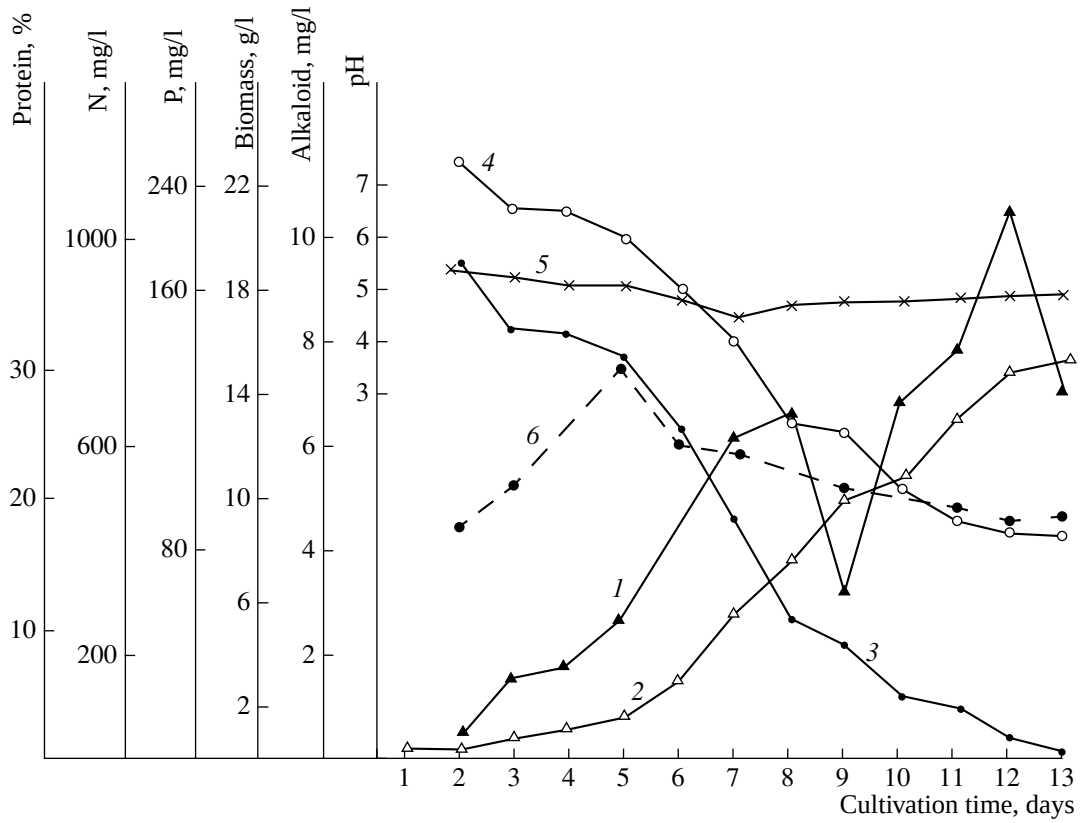

Fig. 1. The dynamics of the biomass, aurantioclavine concentration, and some other parameters during the cultivation of *P. nalgiovense* VKM F-229: (*1*) alkaloid; (*2*) biomass; (*3*) total nitrogen; (*4*) phosphates; (*5*) pH; (*6*) protein.

mechanisms is responsible for the decrease in the concentration of aurantioclavine in the culture liquid of *P. nalgiovense* VKM F-229 between the eighth and ninth days of cultivation, we analyzed the chloroform extracts of the filtrates of the three- and nine-day-old cultures and found that the alkaloid composition of these extracts was the same. Accordingly, the decrease in the concentration of extracellular aurantioclavine cannot be explained by its conversion into other alkaloids. On the other hand, the possibility cannot be excluded that some extracellular enzymes are able to degrade aurantioclavine into simple products undetectable by the method employed.

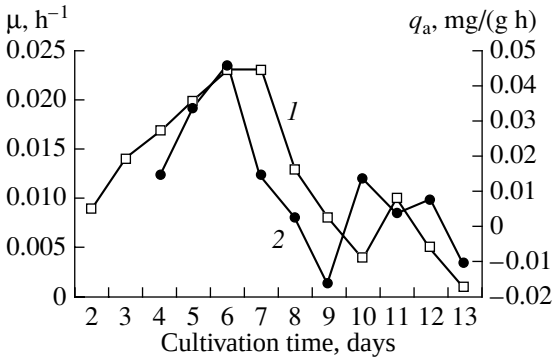

Fig. 2. Changes in (*1*) the specific growth rate (μ) and (*2*) the specific rate of aurantioclavine accumulation in the culture liquid (q_a) during the growth of *P. nalgiovense* VKM F-229.

To verify this supposition, the culture liquid filtrate of the nine-day-old fungus was sterilized by ultrafiltration (to prevent the inactivation of enzymes possibly present in the culture liquid) and incubated as described in the *Materials and Methods* section. The initial concentration of aurantioclavine in the culture liquid filtrate was 260 μg/l. After the incubation of the filtrate for 24 h, the concentration of aurantioclavine virtually did not change, whereas the concentration of the alkaloid in the growing culture decreased almost twofold (to 133 μg/l) over the same time period.

Similar results were obtained when the culture liquid for sterile incubation was taken from other growth phases of the fungus. These data strongly suggest that the decrease in the extracellular concentration of aurantioclavine observed on the ninth day of cultivation is due to the uptake of the alkaloid by fungal cells rather than to its conversion by extracellular enzymes. Earlier, the uptake of the diketopiperazine alkaloid roquefortine in an unmodified form by *P. farinosum* cells was shown directly using the [^{14}C]-labeled alkaloid [11].

To answer the question of whether the transport of aurantioclavine is energy-dependent or not, we employed the uncoupler FCCP at a concentration of 5 μM, at which this protonophore eliminates the electrochemical gradient of protons across the plasma membrane and thereby suppresses energy-dependent transport processes. These experiments were carried out with a nine-day-old fungal culture, which underwent metabolic adaptation to the second substrate mannitol and took up aurantioclavine from the medium (Fig. 1). FCCP was added directly to the culture on the ninth day of cultivation (zero time in the table). After 5 h of incubation, the fungal biomasses in the experimental and the control (into which FCCP was not added) flasks were the same. In contrast, after such incubation, the concentration of aurantioclavine in the experimental flask (into which FCCP was added) slightly increased (by 22%), whereas it decreased more than twofold in the control flask (table).

The effect of the addition (on the ninth day of growth) of 5 μM FCCP on the content of aurantioclavine in the culture liquid and on the biomass of *P. nalgiovense* VKM F-229 cells

Incubation time, h	Concentration of aurantioclavine in the culture liquid, μg/l		Content of aurantioclavine in the biomass, μg/g	
	Control	FCCP	Control	FCCP
0	230	230	16	16
5	80	280	16.9	17.7

These data show that FCCP inhibits the uptake of aurantioclavine by *P. nalgiovense* cells, which suggests that this uptake is energy-dependent, like the uptake of roquefortine by the nitrogen-starved *P. crustosum* cells [12]. It should be noted that the latter fungus also has another, energy-independent, transport system for roquefortine, which operates by the mechanism of facilitated diffusion [12].

The intracellular content of aurantioclavine in *P. nalgiovense* VKM F-229 cells was the same both in the presence and absence of FCCP, indicating that the consumed alkaloid is rapidly metabolized in the cells. Earlier studies showed that the [^{14}C]roquefortine consumed by *P. crustosum* cells was also rapidly metabolized intracellularly, so that after 1 h of incubation with the labeled alkaloid, the radioactive label was detected in the PF1–PF4 precursors of roquefortine, proteins, and in other cellular constituents [12].

Thus, it can be inferred that nine-day-old *P. nalgiovense* VKM F-229 cells, occurring in the transient lag phase, take up the aurantioclavine synthesized and excreted during earlier growth phases and that the alkaloid uptake is an energy-dependent process.

Of interest was to study the effect of FCCP on the excretion of aurantioclavine from cells. These experiments were carried out with three-day-old *P. nalgiovense* VKM F-229 cells, which actively synthesized the alkaloid (Fig. 1). To prevent the possible inhibitory effect of FCCP on the actively growing cells, they were washed free of the nutrient medium and placed in 20-mM MOPS-Na buffer (pH 5.4). After 30 min of incubation, the extracellular concentration of aurantioclavine reached 50 μg/l and then did not change for at least 4 h (the total time of observations), indicating that the alkaloid was not consumed by fungal cells. The excretion of aurantioclavine under these conditions did not depend on the presence of succinate (as a source of energy) and was not inhibited by FCCP, suggesting that the excretion is an energy-independent process and presumably occurs along the concentration gradient. The termination of the excretion of aurantioclavine after 30 min of incubation can be explained by the suppressed synthesis of the alkaloid because of a nitrogen deficiency in cells and the equalization of the intra- and extracellular concentrations of the alkaloid. The validity of this explanation can be confirmed by the fact that the addition of L-tryptophan to the incubation medium raised the concentration of excreted aurantioclavine almost twofold.

Knowledge of the alkaloid transport mechanisms is important in elucidating the general role of alkaloids in the metabolism of fungal cells and the regulatory mechanisms of alkaloid biosynthesis. Relevant studies are scarce and limited to the study of the transport of roquefortine in the fungus *P. crustosum* [11–14]. Like the excretion of aurantioclavine by the fungus *P. nalgiovense*, the excretion of roquefortine by *P. crustosum* is energy-independent [13]. The uptake of these alkaloids by the growing fungi is energy-dependent, the difference between these fungi lying in the fact that the uptake of roquefortine by *P. crustosum* cells is stimulated by nitrogen starvation [12], whereas the uptake of aurantioclavine by *P. nalgiovense* VKM F-229 cells is related to the metabolic adaptation of the fungus from growth on succinate to growth on mannitol (Figs. 1, 2).

It should be noted that roquefortine can be consumed by the young, three-day-old, nutrient-starved *P. crustosum* mycelium, whereas the fungus *P. nalgiovense* acquires the ability to take up aurantioclavine after only eight to nine days of cultivation. In both fungi, the alkaloids consumed probably undergo rapid catabolic conversions [11].

Thus, it can be concluded that, as a rule, the excretion of clavine and diketopiperazine alkaloids by the growing fungi is an energy-independent process, whereas the alkaloid uptake is an energy-dependent process. Relevant regulatory systems have yet to be studied in depth.

ACKNOWLEDGMENT

This work was supported from the Russian Foundation for Basic Research (grant no. 00-04-48713).

REFERENCES

1. Kozlovskii, A.G., Solov'eva, T.F., Sakharovskii, V.G., and Adanin, V.M., The Biosynthesis of Uncommon Ergot Alkaloids by the Fungus *Penicillium aurantiovirens*, *Dokl. Akad. Nauk SSSR*, 1981, vol. 260, no. 1, pp. 230–233.
2. *Katalog kul'tur mikroorganizmov* (The Catalogue of Microbial Cultures), Pushchino, 1992, p. 362.
3. Solov'eva, T.F., Kuvichkina, T.N., Baskunov, B.P., and Kozlovskii, A.G., The Alkaloids of the Fungus *Penicillium aurantiovirens* Biourge and Some Aspects of Their Biosynthesis, *Mikrobiologiya*, 1995, vol. 64, no. 5, pp. 645–650.
4. Reshetilova, T.A. and Kozlovskii, A.G., The Biosynthesis of Alkaloids by Mycelial Fungi, *Prikl. Biokhim. Mikrobiol.*, 1990, vol. 26, no. 3, pp. 291–306.
5. 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.
6. Kozlovskii, A.G. and Solov'eva, T.F., The Effect of Cultivation Conditions on the Biosynthesis of Alkaloids by *Penicillium kapuscinskii*, *Mikrobiologiya*, 1986, vol. 55, no. 1, pp. 34–40.
7. Kozlovskii, A.G., Vepritskaya, I.G., and Gayazova, N.B., Synthesis of Alkaloids by the Fungus *Penicillium sizovae*, *Prikl. Biokhim. Mikrobiol.*, 1986, vol. 22, no. 2, pp. 205–210.
8. 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.
9. Reshetilova, T.A., Yarchuk, N.I., Shurukhin, Yu.V., and Kozlovskii, A.G., The Alkaloids of *Penicillium expansum*, *Prikl. Biokhim. Mikrobiol.*, 1991, vol. 27, no. 5, pp. 725–730.
10. Kozlovskii, A.G. and Reshetilova, T.A., The Biosynthesis and Metabolism of Roquefortine Alkaloids in Microscopic Fungi, *Biotekhnologiya*, 1987, vol. 3, no. 5, pp. 565–571.
11. Reshetilova, T.A., Kulakovskaya, T.V., Kuvichkina, T.N., and Kozlovskii, A.G., The Study of Roquefortine Transport into *Penicillium farinosum* Cells, *Mikrobiologiya*, 1994, vol. 63, no. 3, pp. 411–416.
12. Kulakovskaya, T.V., Kuvichkina, T.N., and Reshetilova, T.A., About the Mechanism of Roquefortine Transport into Cells of the Producing Fungus *Penicillium crustosum* Thom F-1076, *Prikl. Biokhim. Mikrobiol.*, 1995, vol. 31, no. 2, pp. 224–228.
13. Kulakovskaya, T.V., Reshetilova, T.A., Kuvichkina, T.N., and Vinokurova, N.G., Roquefortine Excretion and Uptake by *Penicillium crustosum* Thom VKM F-1746, *Process Biochemistry*, 1996, vol. 32, no. 1, pp. 29–33.
14. Reshetilova, T.A., Kulakovskaya, T.V., Vinokurova, N.G., and Kozlovskii, A.G., The Study of the Excretion of Roquefortine from Cells of the Producing Fungus *Penicillium crustosum* Thom VKM F-1746, *Prikl. Biokhim. Mikrobiol.*, 1997, vol. 33, no. 2, pp. 206–208.
15. Houben-Weyl, *Methoden der organischen Chemie: Analytische Methoden*, Stuttgart: Georg Tieme, 1953, Band II. Translated under the title *Metody organicheskoi khimii: Metody analiza*, Moscow: Khimiya, 1967, vol. 2, pp. 192–197.
16. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., Protein Measurement with the Folin Phenol Reagent, *J. Biol. Chem.*, 1951, vol. 193, pp. 265–275.
17. Weil-Mahlerbe, H. and Green, R.H., The Catalytic Effect of Molybdate on the Hydrolysis of Organic Phosphate Bonds, *Biochem. J.*, 1951, vol. 49, pp. 286–291.
18. Betina, V., Mycotoxins as Secondary Metabolites, *Mycotoxins, Production, Isolation, Separation, and Purification*, Betina, V., Ed., Amsterdam: Elsevier, 1984, p. 20.