J. Basic Microbiol. 30 (1990) 1, 51—56

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Isolation and characterization of an alkaloid-blocked mutant of *Claviceps paspali*

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(Received 24 April 1989/Accepted 3 May 1989)

After UV-irradiation an alkaloid-blocked mutant of *Claviceps paspali* was isolated from an ergometrine and lysergic acid amides (1 : 1) producing strain. The formation of ergometrine is blocked in this particular mutant strain which accumulates only lysergic acid amides. A significant morphological differentation of colonies of mutant as well as of mycelium in submerged cultures could be observed and total alkaloid yields are rather influenced.

The fungus *Claviceps* may be divided into numerous chemical races with different alkaloid spectra. *Claviceps paspali* strains in submerged cultures can produce: α-hydroxyethylamide of lysergic acid (ARCAMONE *et al.* 1961), ergometrine (GRÖGER and TYLER 1963) and $\Delta^{8,9}$-lysergic acid (KOBEL *et al.* 1964), whereas several strains of *C. purpurea* produce mainly peptide alkaloids (GRÖGER and ERGE 1970, SPALLA 1980, UDVARDY 1980, BIANCHI *et al.* 1982). From the results of several investigators (KLEINEROVA and KYBAL 1969, KOBEL *et al.* 1962, VOIGT and KEIPERT 1967) it can be concluded that the composition of the alkaloid complex is genetically determined and that in nature biosynthetic pathway can proceed up to the peptide alkaloids, or be blocked more or less completely at some intermediate point.

The spectra of saprophytically synthesized ergot alkaloids may be changed either by mutation (STRNADOVA 1964, 1967, KOBEL and SANGLIER 1973, GRÖGER 1979) or by changing the culture conditions (KREN *et al.* 1987, BREUEL *et al.* 1982). The literature concerning the mutational biosynthesis in *Claviceps* is very sparse. An alkaloid-blocked mutant was isolated from an ergotoxine-producing *C. purpurea* strain, which accumulates chanoclavine-I and the corresponding chanoclavine-I aldehyde (MAIER *et al.* 1980). Another interesting mutant, obtained by multiple mutagenesis with UV light, was described by KREN *et al.* (1986). The block in the biosynthetic pathway of this mutant was only partial, since beside chanoclavine-I and chanoclavine-I aldehyde also small amounts of tetracyclic clavines elymoclavine and agroclavine were produced. From a *C. paspali* lysergic acid α-hydroxyethylcarbinolamide producing strain a mutant capable of accumulating ergometrine was attained (MARNATI *et al.* 1975).

In the present communication we describe another alkaloid-blocked mutant of *C. paspali* obtained after UV-irradiation. The mutant accumulates lysergic acid amides, while the formation of ergometrine is blocked.

Materials and methods

Microorganism: A *Claviceps paspali* (STEVENS and HALL) selected strain L-52, capable of producing simple lysergic acid derivatives, and from it derived the stable UV mutant strain designated as CP2, were employed in this study.

Mutagenic treatment: A four-day old vegetative mycelium of *C. paspali* was homogenized in a WARING blendor for 10 sec and mycelial fragments in 0.9% NaCl solution were exposed to UV irradiation (300 J/m^2)

for time sufficient to kill 90—99% of the cells. Treated mycelial fragments were plate — cultured on potato-glucose agar. The resulting morphologically changed colonies were screened for alkaloid production and qualitative changes of alkaloid spectrum.

Culture media: Seed-stage medium was modified after ARCAMONE *et al.* (1961) and composed of 40 g/l mannitol, 10 g/l glucose, 10 g/l succinic acid, 3 g/l KH_2PO_4, 0,3 g/l $MgSO_4 \cdot 7\ H_2O$ and 2 g/l chick pea-meal in tap water; the pH of 5.2 was adjusted with ammonia before sterilization.

Production-stage media: Three different media were used for the production cultures: Medium P-2: 150 g/l mannitol and 50 g/l peptone "Torlak" in distilled water; pH was about 6.8 without regulation (SOČIČ *et al.* 1982).

Medium MCK was modified after PERTOT *et al.* (1984) and composed of 50 g/l mannitol, 10 g/l citric acid, 5 g/l yeast extract "Torlak", 1 g/l KH_2PO_4, 0.3 g/l $MgSO_4 \cdot 7\ H_2O$ and 20 g/l NaCl in tap water; the pH of 5.2 was adjusted with ammonia.

Medium D (KOBEL *et al.* 1964): 50 g/l sorbitol. 36 g/l succinic acid, 2 g/l KH_2PO_4, 0.3 g/l $MgSO_4 \cdot 7\ H_2O$, 1 mg $FeSO_4 \cdot 7\ H_2O$ and 10 mg/l $ZnSO_4 \cdot 7\ H_2O$ in distilled water and was adjusted to a pH of 5.4 with NH_4OH.

All culture media were sterilized for 25 min at 121 °C, except medium D which was sterilized for 20 minutes at 108 °C.

Culture conditions: *C. paspali* strains, both the parent and the mutant strain, were maintained on potato-glucose agar at 24 °C and 14—21-day colonies were used for inoculating the seed medium.

Screening experiments were carried out in two stages: seed-stage fermentation and production-stage fermentation. The 6-day old cultures were homogenized in a WARING blendor for 10 sec and 10—15% (V/V) were used to inoculate the production medium. The production-stage cultures were harvested after 15 days. The alkaloid content as well as the composition of the alkaloid complex were then determined. Submerged cultures were incubated on a rotary shaker (frequency 4 Hz, 50 mm stroke, 24 °C).

Analytical methods: Mycelial dry weight: Culture broth (50 ml) was filtered, washed twice with water and dried at 85 °C to constant weight.

Total alkaloids: Culture filtrate suitably diluted (2 ml) was mixed with VAN URK reagent (4 ml), which was prepared as described by AGURELL (1966), and the blue color determined spectrophotometrically with reference to a standard solution of ergometrine (free base).

The alkaloid composition was evaluated by thin-layer chromatography according to GRÖGER and ERGE (1963) and scanned on a CAMAG TLC scanner II.

Results and discussion

The *Claviceps paspali* CP-2 mutant isolated after UV irradiation significantly differs from the original strain. Differences can be observed in morphological characteristics of colonies and differentiation of submerged mycelium as well as in the composition of the alkaloid spectrum.

Colonies of the mutant, grown on potato-glucose agar have a smooth surface and white fluffy aerial mycelium, covered with yellow or greenish droplets of the exudate ("honeydew"), while colonies of the parent strain are compact with an even border, without fluffy aerial hyphae and without honeydew droplets (Fig. 1). These morphological differences facilitated the selection.

No conidia or arthrospores could be observed in the mutant's honeydew droplets as it was noticed in some other *C. paspali* strains (RYLKO *et al.* 1988). We have found that honeydew droplets contain polysaccharides as also reported for other *Claviceps* strains (RIČICOVA *et al.* 1986). Further we have found that polysaccharides are composed mainly of arabinose, galactose and xylose. The composition of sugars in the honeydew depends very probably on the composition of the medium used.

In submerged cultures the mycelium of the mutant differentiated more or less into swollen, arthrosporoid-like cells and showed a tendency to fragmentize (Fig. 2). Such a morphological differentiation has never been observed with the wild strain of *C. paspali* which retains filamentous structure throughout the fermentation.

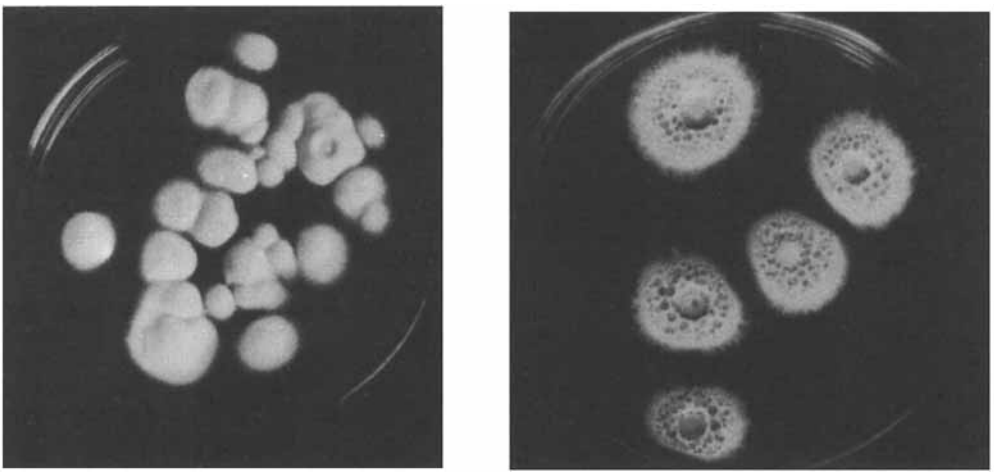

Fig. 1
Colonies of *C. paspali*: parent strain (left) and mutant strain (right)

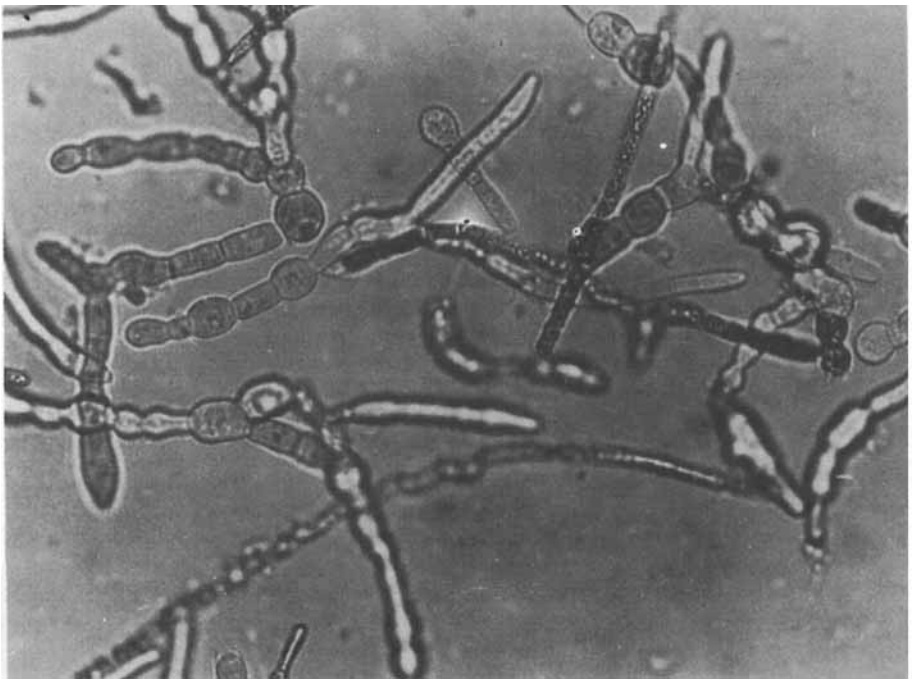

Fig. 2
Differentiated hyphae of a *C. paspali* mutant strain in submerged culture

Table 1
Percent content of individual alkaloids comprising the total alkaloid mixture of the parent and mutant strain

Alkaloid composition	Media					
	Parent strain			Blocked-mutant		
	P-2	D	MCK	P-2	D	MCK
Lysergole	1.8	0.59	0.71	2.16	2.21	6.65
Lysergic acid amide	14.75	10.64	23.72	31.56	9.47	36.65
α-hydroxyethyl amide of lysergic acid	28.55	22.5	29.87	41.55	18.60	44.97
Ergometrine	32.51	2.82	24.59	—	—	—
Ergometrinine	6.06	2.7	11.43	—	—	—
Isolysergic acid amide	8.31	33.7	4.5	14.08	38.06	6.36
α-hydroxyethyl amide of isolysergic acid	5.22	26.29	3.45	10.32	27.71	5.09
Total alkaloids mg/l	2646	1028	1644	1552	1147	1257

Table 2
Biosynthetic activity of the parent strain and isolated mutant in submerged cultures in different media

	Medium P-2		Medium D		Medium MCK	
	Biomass g/l	Alkaloids mg/l	Biomass g/l	Alkaloids mg/l	Biomass g/l	Alkaloids mg/l
Parent strain	20.17	2647	9.63	1028	12.97	1644
Mutant strain	21.52	1552	14.43	1147	15.30	1257

Differences in the alkaloid composition between the CP-2 mutant and the parent strain are also significant and are presented in Table 1 where each value represents the arithmetic mean value of at least 12 individual cultures. The alkaloid composition varies somehow with the medium composition; as can be seen from Table 1, in the P-2 and MCK media the l-series simple derivatives of lysergic acid prevail while the medium D favours formation of d-forms, isolysergic acid derivatives, which are less interesting. As known, many compounds in the lysergic acid series exhibit pronounced pharmacological effects but the corresponding isolysergic acid derivatives are almost completely devoid of pharmacological activity.

The main alkaloid components produced by the parent *Claviceps paspali* strain are the l- and d-forms of ergometrine, lysergic acid α-hydroxyethylamide and lysergic acid amide. The CP-2 mutant synthesizes neither ergometrine nor ergometrinine in all three different nutrient media used, though the parent strain produced a significant quantity of both, particularly in the P-2 and MCK media. These results clearly indicate a block in the mutant's biosynthetic pathway.

The amide components of ergometrine and lysergic acid α-hydroxyethylamide are derived from L-alanine (GRÖGER *et al.* 1968), the presumable intermediate being lysergylalanine (REHAČEK, 1983). It seems that an enzyme responsible for reduction of lysergylalanine to ergometrine is blocked in the UV mutant, and only the oxydation step leading to the lysergic acid amides takes place (Fig. 3).

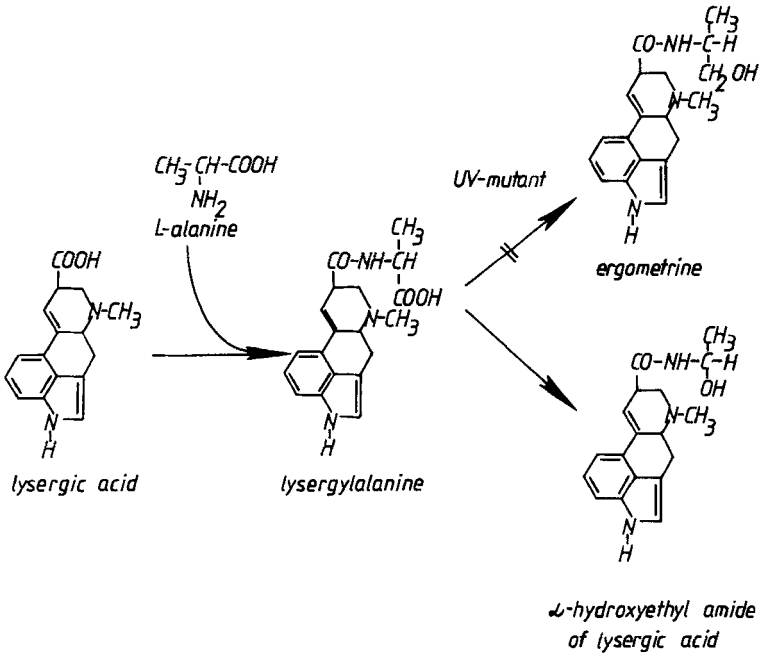

Fig. 3
Biosynthetic pathway of simple lysergic acid derivatives. Bar indicates the position of the block

These results are just contrary to the modification of the biosynthetic pathway reported by MARNATI *et al.* (1975) who, by means of mutation with NTG, obtained a *C. paspali* strain capable of producing only ergometrine.

Total alkaloid yields of the CP-2 mutant were found to be lower than yields exhibited by the original strain, irrespective of the medium used. On the contrary the mycelial dry weight of the mutant was always somewhat higher.

The isolated mutant has proved to be perfectly stable. After several passages through agar and submerged cultures the morphological appearances and the composition of alkaloid spectra have remained fairly constant.

In any case, the mutant described, accumulating only lysergic acid amides, is interesting not only from the research point of view but also as a useful tool for the semisynthetic preparation of lysergic acid and its other pharmacologically active derivatives.

Acknowledgement

The financial support of Research Community of Slovenia is gratefully acknowledged.

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