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Highly Efficient Mutagenesis of *Claviceps purpurea* by Using Protoplasts

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Claviceps purpurea ATCC 20102, which is aconidial under laboratory conditions, was grown in submerged culture in the presence of mutagens and various nutritional additives. Protoplasts from such cultures were prepared and regenerated on solid medium to obtain colonies from single cell units. Frequencies of auxotrophs and high alkaloid producers were on the order of 1 to 2%. Some of the auxotrophic mutants derived from strain ATCC 20102 were constantly segregating prototrophs. High-alkaloid-producing derivatives showed sclerotia-like morphology and violet-brown pigmentation, in contrast to the parent strain; some of them also showed segregation sectors when grown as giant colonies. Mutagenesis of strain 1029, isolated during this study and having an increased level of alkaloid synthesis and sclerotia-like cell morphology, was done in the same fashion as with the original parent strain, ATCC 20102. Mutants obtained from this strain were all stable with respect to their genotypes. However, a large proportion of colonies derived from regenerated protoplasts, even in the mutagen-free controls, showed a lowered level of alkaloid production and were morphologically more similar to the original wild type, ATCC 20102. The influence of protoplast preparation or regeneration or both on the stability of genes involved in differentiation is discussed.

The ergot fungus *Claviceps purpurea* is the source of the therapeutically important ergot alkaloids. Most strains of *C. purpurea* used in research and industrial production apparently were selected for high yields of alkaloids with or without previous treatment with a mutagen. For those fungi producing conidia, these are generally used for mutagenesis. This was also applied for *C. purpurea* (9), whereas in the case of strains not producing conidia under laboratory conditions, treatment of fragmented mycelia has been reported to be successful (8). On the other hand, recent work revealed that efficient mutagenesis of *C. purpurea* is rather difficult with respect to both yields and stability of the mutants obtained (10). In this paper, I describe a mutagenic procedure for *C. purpurea* ATCC 20102, a strain that does not produce conidia under laboratory cultures. The method is based on cultivation of mycelia in the presence of a mutagen, with subsequent preparation of protoplasts and protoplast regeneration to obtain single colonies. It will be shown that this method is most suitable for the production of various stable mutants of *C. purpurea*.

(Preliminary results of this work were presented at the Symposium for Enzymatic Synthesis of Peptides, Berlin, 1980 [6].)

MATERIALS AND METHODS

Strains and cultures. *C. purpurea* ATCC 20102 and a mutant designated 1029 were used in this study. The organisms were maintained on medium T 2 (2). The wild type produces 10 to 15 mg of alkaloids (ergotamine plus ergocryptine) per liter after 7 to 10 days of growth in the inoculum medium of Amici et al. (1), whereas 1029 produces 450 to 700 mg/liter^{-1} during the same period. Liquid media were inoculated with 1-cm^2 pieces of mycelial mat from T 2 agar plates or, in the case of mutagenic treatment, with samples from 5-day-old liquid cultures. Liquid media were inoculum medium and medium T 25 (2).

Protoplasts. Protoplasts were prepared as described previously (5), with the exception that both sedimenting and floating protoplasts were collected for subsequent plating on regeneration agar.

Protoplast suspensions (5×10^8 ml^{-1}) were diluted from 10^0 to 10^{-8}, and 0.1-ml portions were spread in four parallels each on regeneration plates containing medium T 2 with 0.6 M sucrose supplemented with appropriate requirements. After incubation for 10 days at 27°C, single colonies derived from regenerated protoplasts were isolated for successive characterization.

Mutagenic procedure. Mutagenesis was done by the use of either ethyl methane sulfonate (EMS) or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG). Erlenmeyer flasks (300 ml) containing 60 ml of either inoculum medium or medium T 25 were inoculated with 2 ml of a 5-day-old culture of *C. purpurea* grown

in the inoculum medium. In parallel, 0.033 to 0.2 mM NTG (as a solution in methanol containing 50 mg ml^{-1}) or 8 to 24 mM EMS was added. Controls contained no mutagen. Cultures containing the mutagen always showed a growth lag of up to 2 to 6 days until biomass formation started. After having reached 2.5 to 4 g (wet weight) per flask, mycelia were harvested by centrifugation, and protoplasts were prepared. A value of 2.5 to 4 g corresponds to about half the maximum biomass of the control cultures after 8 days (about 6 g). Control protoplasts were prepared from a comparably old culture. To obtain auxotrophic mutants, liquid and regeneration media were supplemented with cystine, methionine, alanine, proline, phenylalanine, tyrosine, tryptophan, histidine, valine, isoleucine, and leucine (20 μg ml^{-1} each), pyridoxine, nicotinic acid, *p*-amino benzoic acid, riboflavine, and thiamine (1 μg ml^{-1} each), and yeast extract (100 μg ml^{-1}). Media for mutagenesis with the aim of obtaining alkaloid mutants contained no additional requirements.

Isolation of mutants. Single colonies on regeneration plates derived from regenerated protoplasts were transferred with sterile toothpicks onto identically supplemented medium T 2 (20 per plate). These plates were incubated for 8 days at 27 to 28°C. In the case of screening for high-alkaloid-producing mutants, pieces of mycelia from each colony were transferred aseptically into Erlenmeyer flasks (300 ml) containing 60 ml of inoculum medium. These flasks were incubated with shaking at 180 rpm for 8 days at 24°C. (Pilot Shake; System Kühner; Braun-Melsungen). After this period, 1- to 10-ml samples were withdrawn from the flasks, and alkaloid was extracted as described previously (2). Quantitative determination was based on the spectrophotometric assay by the van Urk reaction (3).

In the case of screening for auxotrophic mutants, plates were replica plated with velvet onto medium T 2 without any supplement (minimal medium). Colonies failing to grow on the replicas after 8 days of incubation at 27°C were combined on master plates and analyzed with respect to their requirements. Genotypes were considered to be stable if, after five successive transfers on appropriately supplemented minimal medium, no loss of markers was observed.

Chemicals. NTG was obtained from Sigma Chemical Co., and EMS was purchased from Merck. All other chemicals were of the highest purity commercially available.

RESULTS

Induction of high-alkaloid-yielding mutants from *C. purpurea* ATCC 20102. Flasks containing either inoculum medium or medium T 25 and various NTG or EMS concentrations were inoculated with mycelia of *C. purpurea* ATCC 20102 as described above. In contrast to the controls, cultures containing mutagen started growth after a lag period of up to 6 days. The length of this period depended on the concentration of mutagen used. Once the inhibitory effect of the mutagen was overcome, cells grew as quickly as in the controls. After harvest of mutagenized and control mycelia, protoplasts were prepared and plated on regeneration agar. Regeneration efficiencies were between 5 and 15%, estimated by comparison of microscopical counts versus counts of colonies on the various dilution plates. Control experiments that involved diluting protoplast suspensions with water instead of protoplast medium (5) revealed the almost complete absence of nonprotoplasted cells (<0.01%). Colonies obtained from regenerated protoplasts (single colonies) were tested for alkaloid production in submerged culture (Table 1). Not one colony derived from protoplasts regenerated without previous mutagenic treatment of growing mycelia showed increased ergot peptide synthesis. Frequencies of mutants with increased levels ranged between 1 and 3%, with no significant preference for either of the two mutagens or media. All high-yielding mutants showed a violet-brown pigmentation and an increased tendency toward sclerotia-like morphology compared to the parent strain, which is white with slender vegetative hyphae and relatively low fat content (data not shown). None of the high-yielding strains listed in Table 1 has lost its increased capacity of alkaloid formation within the last 2 years. Several strains (e.g., 1013) showed sectoring when grown as giant colonies, whereas others (e.g., 1029) never did. Strains with sclerotia-like morphology were kept on T 2 under liquid paraffin at −32°C. No loss of viability was observed under these conditions after storage for at least 1.25 years.

Auxotrophic mutants from *C. purpurea* ATCC 20102. Mutagenesis to obtain auxotrophic mutants from wild-type strain ATCC 20102 was done exclusively in inoculum medium supple-

TABLE 1. Induction of high-alkaloid-yielding mutants by mutagenizing submerged cultures of *C. purpurea* ATCC 20102[a]

Expt	Mutagen	Concn of mutagen (mM)	Medium	No. of colonies tested	No. of high-yielding colonies
1	NTG	0.067	T 25	64	2 (1013, 1029)
2	NTG	0.135	T 25	78	1 (2021)
3	NTG	0.135	T 25	76	1 (2028)
4	None		T 25	112	0
5	NTG	0.271	Inoculum	79	0
6	NTG	0.067	Inoculum	90	1 (1022)
7	None		Inoculum	126	0
8	EMS	26.5	Inoculum	96	1 (E 1)
9	EMS	16.1	Inoculum	85	1 (E 2)
10	EMS	26.5	T 25	92	1 (E 3)
11	None		T 25	104	0
12	None		Inoculum	61	0

[a] Titers of alkaloids after 8 days of growth in the inoculum medium: parent, 15 to 20 mg $liter^{-1}$; 1013, 70 to 100 mg $liter^{-1}$; 1029, 300 to 500 mg $liter^{-1}$; 2021, 100 to 150 mg $liter^{-1}$; 2028, 80 to 120 mg $liter^{-1}$; 1022, 50 to 100 mg $liter^{-1}$; E 1, 60 to 100 mg $liter^{-1}$; E 2, 150 to 200 mg $liter^{-1}$; E 3, 50 to 90 mg $liter^{-1}$.

TABLE 2. Induction of auxotrophic mutants by mutagenizing submerged cultures of *C. purpurea* ATCC 20102[a]

Expt	Mutagen	Concn of mutagen (mM)	No. of colonies tested	No. of auxotrophs	Requirements[b] and frequency of occurrence
1	None		930	0	
2	NTG	0.135	200	3	Cys or Met, Met, Arg
3	NTG	0.202	80	3	Lys, Met, Ile
4	NTG	0.217	517	5	Cys or Met 2, Met, Ade, Nic
5	EMS	16.1	100	2	Cys or Met, Met
6	EMS	26.5	1040	21	Cys or Met 7, Met 3, Aro 2, Pab 2, Nic 4, Leu, Ade, His + Ade

[a] Mutagenesis was done by using inoculum medium containing all requirements as indicated in the text. Of the mutants above, 11 were unstable.

[b] Ade, Adenine; Nic, nicotinic acid; Aro, requirement for aromatic amino acids; Pab, *p*-amino benzoic acid.

mented with various requirements. After regeneration of protoplasts, single colonies were analyzed with respect to their requirements. Frequencies of auxotrophs after treatment with mutagens (NTG and EMS) are shown in Table 2. The data clearly reveal the usefulness of the method to obtain auxotrophs from *C. purpurea*. Interestingly, a considerable portion of mutants had requirements for methionine or for cystine or methionine. This may be explained merely by the fact that after mutagenic events, subsequent mycelial growth took place, giving rise to a mutated clone. However, Strnadová (9) obtained a similar predominance for these two markers with another *C. purpurea* strain by directly plating UV-treated condidia. Therefore, the method described here does not differ from conventional methods with respect to the spectrum of mutants.

About 30% of the mutants listed in Table 2 were unstable, i.e., they constantly segregated prototrophs. All of the auxotrophic mutants were white and had the same morphology as the parent strain ATCC 20102. Freezing and keeping under liquid paraffin were possible with only a limited number of these mutants. The viability of the vegetative growth type was lower than that of sclerotia-like cells.

Improvement of alkaloid production of strain 1029. To obtain mutants with even higher levels of alkaloid production, strain 1029 (450 to 700 mg of ergot peptides per liter after 10 days in inoculum medium) was treated with mutagens in the manner described above. The results of several experiments are shown in Table 3. Data clearly indicate that mutants with higher levels of alkaloid synthesis rarely occurred. On the other hand, a high number of mutants with lower yields was obtained. Most of them had a phenotype very similar to that of the wild type. Surprisingly, the same observation was made in the case of the mutagen-free control. However, colonies with dark pigmentation and sclerotia-like growth but low levels of alkaloid production were also obtained (1 to 3%). In this series of experiments, mutagenesis in medium T 25 seems to be somewhat more efficient for obtaining mutants with an increased capacity of alkaloid formation. Thus, I obtained two mutants, 1029–10 and 1029–13, producing about 900 to 1,100 mg liter^{-1} after 12 days of growth in the inoculum medium.

Auxotrophic mutants of strain 1029. Auxotrophic mutants of strain 1029 were induced as in the case of wild type. The results are summarized in Table 4. Most mutants had requirements for methionine or methionine or cystine. The frequencies also were similar, ranging between 1 and 2%. In the untreated controls, no auxo-

TABLE 3. Influence of mutagens on the induction of mutations affecting alkaloid synthesis of strain 1029[a]

Alkaloid[b] (mg liter^{-1})	No. of mutants in expt:					
	1	2	3	4	5	6
<5	30	23	37	26	32	21
5–75	21	16	14	25	32	14
75–150	18	7	3	5	3	2
150–225	10	2	9	8	8	7
225–300	4	2	6	7	3	6
300–400[c]	12	10	12	11	9	9
400–500	4	1	1	2	2	4
500–600	0	0	0	1	1	1
600–700	0	0	0	1	1	0

[a] Conditions and number of isolates tested (in parentheses): experiment 1, 10 mM EMS in inoculum medium (99); experiment 2, 0.135 mM NTG in inoculum medium (61); experiment 3, no mutagen in inoculum medium (82); experiment 4, 10 mM EMS in T 25 (86); experiment 5, 0.135 mM NTG in T 25 (91); experiment 6, no mutagen in T 25 (64).

[b] Measured after 8 days of growth in the inoculum medium.

[c] Level of the parent strain grown under the same conditions (inoculation with a piece of mycelial mat).

TABLE 4. Induction of auxotrophic mutants of strain 1029[a]

Expt	Mutagen	Concn of mutagen (mM)	No. of colonies tested	No. of auxotrophs	Requirements[b] and frequency of occurrence
1	NTG	0.033	522	6	Cys or Met 2, Met 2, Nic, Pdx
2	NTG	0.135	619	6	Cys or Met 2, Met 2, Nic 2
3	EMS	10	500	8	Cys or Met 2, Met 2, Nic 2, His, Leu
4	None		440	0	
5 (directed for Trp)	EMS	10	620	1	Trp

[a] Mutagenesis was done by using inoculum medium containing all requirements as indicated in the text. There were no unstable mutants.

[b] Nic, Nicotinic acid; Pdx, pyridoxine.

trophic mutant was found. Interestingly, all of the mutants with requirements for amino acids showed lowered alkaloid production (<25 mg liter^{-1}) regardless of dark pigmentation, whereas mutants with requirements for vitamins produced the same titer as the parent strain. Once isolated as auxotrophic mutants, none of them showed instability similar to that observed in several mutants of the wild type. Introduction of further markers into auxotrophic mutants of the wild type and 1029 proved to be effective. In three experiments, frequencies of 1.25 to 6.5% were observed with respect to the occurrence of further requirements in mutagenesis experiments. All the genotypes obtained were stable, and no revertants (in a sample size of 400 per experiment) were observed (results not shown).

The high frequencies of auxotrophic mutants stimulated further experiments with the aim of isolating mutants with desired properties. To obtain a tryptophan-defective mutant, strain 1029 was grown in the presence of EMS in inoculum medium supplemented with 50 μg of tryptophan per ml. The result of this experiment is included in Table 4. One tryptophan-defective mutant of 620 colonies was isolated. As stated above, this mutant was a low-alkaloid-producing strain (<25 mg liter^{-1}). However, the principal possibility of obtaining mutants with requirements for the precursors of ergot peptides is of particular interest for a directed feeding of *Claviceps* strains in relation to studies on alkaloid biosynthesis and the search for new compounds. The data clearly illustrate the suitability of the method described here for obtaining other desired mutants of *C. purpurea* ATCC 20102.

DISCUSSION

The main difficulty in mutagenesis of filamentous fungi that do not form conidia under laboratory conditions is to obtain single mutagenized colonies. The results presented here illustrate the advantage of using protoplast regeneration of aconidial strains of *C. purpurea* to obtain colonies mostly derived from single cell units. In this study such colonies were obtained by regeneration of protoplasts derived from mycelia of a mutagenized culture. Results suggest that this may be advantageous compared to mutagenizing protoplasts or fragmented mycelia directly because, through postmutational growth, segregation of mutated nuclei can take place before protoplasts are made, thus increasing the potential number of mutants. Despite the fact that, in most samples derived from the same mutagenic treatment, several mutants with the same nutritional requirements appeared (Tables 2 and 4), the yields of different genotypes are comparable or even better than those obtained with mutagenized conidia in another *C. purpurea* strain (19). In addition to the introduction of auxotrophic markers, mutagenic treatments had a significant effect on the appearance of mutants of wild-type strain ATCC 20102, producing higher levels of peptide alkaloid (Table 1). The observation that several of the latter mutants showed segregation (sectored colonies) indicates that they were unstable. Increased alkaloid production of these mutants was not seriously affected by segregation if selective transfer of mycelia from individual sectors was avoided. A similar segregation was also observed with the parent, wild-type strain ATCC 20102, which frequently produced sectors when grown as giant colonies (for comparison, see reference 7). Furthermore, several of the auxotrophic mutants of the wild type constantly segregated prototrophs. On the other hand, all auxotrophic mutants derived from strain 1029 proved to be stable during successive transfers on the same medium. Also, strain 1029 itself is stable in respect to alkaloid production and never showed visible sectors in single colony platings or when grown as a giant colony. Apparently, the state of karyosis in the parent strains is of importance

for the stability of the mutants derived from them. This suggestion is further substantiated by the finding that mutagenic treatment of stable auxotrophic mutants derived from both strains gave exclusively stable doubly auxotrophic mutants and no revertants.

The appearance of a high portion of colonies with altered morphology after protoplasting and protoplast regeneration of strain 1029, even in the controls without mutagenic treatment, is surprising. Since this behavior was never observed when fragments of sclerotia-like hyphae were streaked on solid medium, it must be assumed that it is a consequence of the process of protoplasting and/or protoplast regeneration. In the case of the wild type, a comparable phenotypic variation was difficult to detect because, besides a few sclerotia-like colonies (mutagenized culture), most of the population appeared to be of the vegetative growth type macroscopically. Detailed microscopic examinations were not performed. What kind of influence protoplasting and/or protoplast regeneration may have on the observed genetic instability in *C. purpurea* with respect to morphological and physiological differentiation is unknown. It is remarkable, in this context, that a comparable labilization of some genetic traits in procaryotic organisms like *Streptomyces* has also been described. Spontaneous loss of plasmids after protoplast regeneration has been shown in several cases (4).

The loss of high alkaloid production in most of the morphological derivatives of strain 1029 (reduced pigmentation and increased tendency to vegetative growth-like morphology) strengthens the close relationship between the ability to produce high levels of ergot peptides and sclerotia-like cell morphology. It may be speculated that both groups of genes determining physiological and morphological differentiation become particularly labilized when the organism is subjected to an abnormal situation of cellular restoration like protoplast regeneration.

The data also show that to obtain mutants with blocks in alkaloid synthesis, higher numbers of isolates must be analyzed. Several colonies with sclerotia-like cell morphology and lowered alkaloid production were observed after mutagenic treatment of strain 1029, but no isolate was really alkaloid negative. Mutants of the latter type would be particularly interesting for studies on ergot peptide synthesis.

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