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Methods for Mutation and Selection of the Ergot Fungus

SUTHINEE SRIKRAI AND JAMES E. ROBBERS*

Department of Medicinal Chemistry and Pharmacognosy, School of Pharmacy and Pharmacal Sciences, Purdue University, West Lafayette, Indiana 47907

Received 12 October 1982/Accepted 17 January 1983

A new method is described in which the Salkowski reaction is used for the rapid selection of alkaloid-producing mutants of the ergot fungus. This method was used to investigate the influence of a second mutation with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) on various mutants selected by a preliminary NTG mutation of *Claviceps* sp. strain SD 58. Three groups of mutants were used: high alkaloid producers, low alkaloid producers, and auxotrophs. Results indicated that a second mutation of all three types of mutants could improve alkaloid yield and vegetative vigor. In addition, a second mutation increased the frequency of auxotroph production. The difficulty of producing stable mutants from ergot strains that have multinucleated cells and that do not readily produce conidia in culture, such as an ergotamine-producing strain, was overcome by first forming protoplasts of the fungus and then subjecting them to the mutagen. Stable auxotrophs were obtained in this manner.

Mutagenesis followed by subsequent selection of a strain producing superior yields is an important technique in increasing the fermentative yield of antibiotics. For example, penicillin yields have been increased by several hundredfold since the 1940s, when penicillin was first produced commercially (14). On the other hand, the application of this process to increasing the fermentative yield of ergot alkaloids has been rather limited. One of the first reports by Kobel et al. describing a mutant strain of *Claviceps purpurea* which produces an increased amount of alkaloids was published in 1962 (8), and subsequently, these workers have obtained an ergocristine-producing *C. purpurea* strain which shows a 180-fold increase in alkaloid production over the parent strain after an eight-step mutation-selection with different mutagens (9, 10). Their selection technique involves the isolation of 1,000 colonies after each mutation and the subsequent testing of each isolate in shake culture for alkaloid production. It is thus a time-consuming process.

We report here what we think is an improved and more rapid method for selecting mutants of the ergot fungus with increased alkaloid production abilities and techniques for obtaining auxotrophic mutants of two strains of the fungus, including a multinucleated, heterocaryotic strain.

MATERIALS AND METHODS

Culture procedures. The ergot strains used for this study were *Claviceps* sp. strain SD 58 (ATCC 26019), originally isolated from a sclerotium obtained from *Pennisetum typhoideum* Richard, and *Claviceps purpurea* (Fries) Tulasne (ATCC 20102), an ergotamine-producing strain isolated from a sclerotium formed on rye (*Secale cereale* L.).

Claviceps sp. strain SD 58 was maintained on Czapek-Dox or NL-406 agar slants (15) and grown in shake culture at 24°C and 220 rpm on a rotary shaker. Cultivation of the strain was achieved by inoculation of 500-ml Erlenmeyer flasks containing 100-ml portions of culture medium NL-406 with portions of the mycelium from an agar slant. After 10 days, the cultures were homogenized in a Waring blender at high speed for 1 min, and 2-ml portions of the cultures were transferred to fresh NL-406 medium. This step was repeated until the cultures were homogeneous in appearance. However, after the sixth transfer, the alkaloid titer gradually decreased. The culture procedure, starting from growth on slants, was repeated as necessary.

C. purpurea was maintained on agar slants containing T_2 medium (3) and grown in shake culture at 24°C and 220 rpm for 3 weeks on a rotary shaker. Cultivation was achieved by homogenizing the mycelium grown on slants with 10 ml of T_2 liquid medium. The homogenized mycelium was transferred to flasks containing 100-ml portions of T_2 liquid medium. After 8 to 10 days, 10-ml portions of these seed cultures (precultures) were transferred to production medium NL-833 (13).

Quantitation of alkaloids. The liquid portion of the *Claviceps* sp. strain SD 58 culture medium contained the major portion of the alkaloids. The mycelium was separated by vacuum filtration, washed, dried at room temperature, and weighed. A colorimetric assay with 2 ml of Van Urk reagent as modified by Allport and Cocking (2) was used to determine the quantity of alkaloids in the culture filtrate. The procedure for the assay has been previously described (16).

The mycelium portion of the *C. purpurea* culture

medium contained approximately 90% of the alkaloids. The quantitation of alkaloids from this strain was a modification of the original procedure described by Amici et al. (3). The mycelium was separated by vacuum filtration, washed, and then suspended in an equal mixture of an aqueous solution of 4% (wt/vol) tartaric acid and acetone. The mixture was homogenized at high speed for 1 min, and suction filtration was used to separate the mycelium. The mycelium was washed, dried at room temperature, and weighed. Acetone in the filtrate was removed with a Büchi Rotavapor. The filtrate was made alkaline with an ammonium hydroxide solution, and the alkaloids were extracted with chloroform three times. Portions of the chloroform extract were dried, and each was treated with 1 ml of 4% (wt/vol) tartaric acid and 2 ml of Van Urk reagent. After 20 min, the optical density at 545 nm was determined for the assay mixture. This result was multiplied by 115.0, which is the reciprocal of the slope of a standard curve based on ergotamine, the major product of this strain.

Identification of clavine alkaloids. Alkaloid extracts of strain SD 58 mutant culture filtrates were prepared as previously described (15) and chromatographed on precoated silica gel glass plates (E. Merck AG, Darmstadt, Germany) with reference alkaloids. Chloroform-methanol-NH_4OH (80:20:0.2) was used as the developing solvent.

Mutation technique. *Claviceps* sp. strain SD 58 was grown in submerged culture for 1 month. The culture was filtered through sterile sintered glass (coarse) to collect the mycelium and conidia, which were dissolved in 40 ml of sterile distilled water and filtered through a piece of cheesecloth. We transferred 9 ml of the filtrate to a sterile centrifuge tube and added 1 ml of mutagen solution. The mutagen solution was freshly prepared by dissolving 5 mg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG; Aldrich Chemical Co., Milwaukee, Wis.) in 20 ml of sterile distilled water. The final concentration of the mutagen solution was 25 μg/ml of solution in the centrifuge tube. The reaction tube was incubated at 24°C and 220 rpm for 55 min and then centrifuged at 4,000 rpm for 5 min. The cells were collected and suspended in 40 ml of sterile distilled water. Using a pin device, we transferred the mutagenized cells to NL-406 agar plates, which we incubated at 24°C until colonies appeared. The pin device (12 by 12 by 2 cm) is made of clear Lucite, with 20 pins symmetrically distributed within a diameter of 9 cm.

Protoplasts were formed from the mycelium of the *C. purpurea* strain, which is thought to be a heterocaryote (17), and these were subjected to the same mutation procedures used for strain SD 58.

Protoplast formation. Protoplasts were prepared by a modification of the method of Keller et al. (7). The *C. purpurea* mycelium from 8-day-old production medium (NL-833) was harvested on a funnel of coarse sintered glass and washed three times with a small volume of 0.4 M sucrose solution. We suspended 2.5 g of mycelium in 30 ml of protoplast medium consisting of 0.8 M sucrose, 10 mM sodium citrate (pH 4.7), 10 mM $MgCl_2$, and 10 mM $CaCl_2$. After careful homogenization, 1.2 ml of β-glucuronidase type H-3 crude solution from *Helix pomatia* (Sigma Chemical Co., St. Louis, Mo.) was added, and the mixture was incubated with shaking (120 rpm) at 26°C for 12 h, at which time protoplasts were observed under a microscope. The protoplast solution was diluted threefold with protoplast medium and filtered through glass wool to remove large mycelial fragments. The filtered solution was centrifuged at 5,000 × *g* for 20 min, and the top layer of protoplasts was collected in a test tube. The supernatant was discarded, and the protoplast residue was resuspended in 20% of the original volume of protoplast medium. This procedure was repeated, and the combined layers of floating protoplasts were collected and resuspended in 10 ml of protoplast medium, 9 ml of which was added to 1 ml of the NTG solution. After mutagenesis, the protoplasts were plated on NL-406 agar with the pin device. The regeneration of intact cells took place within 5 days.

Selection technique. Two types of selection criteria were employed: the amount of alkaloid produced by the mutant and nutritional deficiency.

Selection based on alkaloid production was accomplished by utilizing the Salkowski reaction (11). In this method, the mutant colony is replicated before the experiment, and the reverse side of the original colony is then scraped until no traces of agar are present. After the reverse side is sprayed with a reagent consisting of 1 ml of 1.5 M $FeCl_3$ solution and 100 ml of 60% sulfuric acid, the colony is incubated at 60°C for 5 min. The intensity of the blue color which develops corresponds to the amount of alkaloid present.

To select auxotrophic mutants, colonies were replicated on plates with unsupplemented NL-406 medium without yeast extract. If the mutant failed to grow on this minimal medium, the original mutant colony was transferred into 30 ml of liquid NL-406 medium and incubated at 24°C and 220 rpm for 5 to 7 days or until mycelial growth was sufficient for subsequent transfer to fresh medium. The culture was filtered under aseptic conditions, and the mycelium was washed with sterile distilled water several times. The mycelium was then suspended in 30 ml of sterile distilled water. One loopful of suspension was spread on a fresh minimal medium agar plate, which was incubated at 24°C for 14 days. Leaky mutants were discarded or submitted to the selection procedure a second time. Nonleaky mutants were grown in a submerged culture of complete medium and then tested for specific nutritional deficiencies as described by Holliday (6).

RESULTS AND DISCUSSION

In our laboratory, wild-type strain SD 58 normally produces clavine-type alkaloids in the range of 500 to 800 μg of total alkaloids per ml of culture filtrate or about 35 to 60 μg of total alkaloids per mg of mycelial dry weight. After subjecting the wild-type strain to mutation and examining 11,500 resultant colonies, we selected three groups of mutants, which were designated first-stage mutants.

The Salkowski reaction was used to differentiate two groups of alkaloid production mutants. High-alkaloid-producing mutants became dark blue to black and, upon submerged culturing, produced alkaloid in titers of >1,200 μg/ml of culture filtrate. We isolated 28 of these mutants, 3 of which produced alkaloid in titers of >2,000 μg/ml of culture filtrate. The 13 low-alkaloid-producing mutants isolated were light blue to

TABLE 1. Auxotrophic mutants of strain SD 58 and an ergotamine-producing *C. purpurea* strain

Auxotrophic mutant	Deficiency
Strain SD 58	
A_{118}	Threonine
S_{34}	Methionine
S_{37}	Cysteine
W_4	Homoserine
1-36	Leucine
2-29	Cysteine or methionine
10-10	Lysine
10-11	Adenine
C. purpurea	
Tam A_{37}	Cysteine
Tam A_{95}	Cysteine
Tam 8-14	Lysine
Tam 12-15	Leucine
Tam 17-2	Homoserine or lysine

colorless in the Salkowski reaction and produced <300 μg of total alkaloids per ml of culture filtrate. Of the high producers, two superior mutants, designated M-26 and M-71, were used in a second mutation experiment. They were relatively stable in ability to produce high levels and retained this property after 12 transfers over a 16-month period. The low producers used for a second mutation were mutants M-64 and A-176, which produced consistently low levels after eight transfers over 16 months. Auxotrophic mutants, which constituted a third group, are listed in Table 1.

The highest alkaloid titers obtained from mutants M-26 and M-71 were in the ranges of 1,250 to 1,350 and 1,150 to 1,300 μg/ml, respectively. The alkaloid titers of different fermentations of the same mutant can vary slightly, depending on the age of the inoculum and the number of preceding transfers. The alkaloid titers of mutants were determined between days 9 and 12, the optimum age for alkaloid production, and after the cultures had been transferred two or three times at 10-day intervals.

Before a second mutation treatment, a cell count was obtained by plating various concentrations of M-26 and M-71 cell suspensions onto NL-406 agar plates. The numbers of colonies appearing on these plates after a 2-week incubation were determined. A cell count was again determined after exposure to the mutagen in order to determine the frequency of survival, which was 2.7% for M-26 and 3.9% for M-71.

On the basis of micrograms of alkaloid per milliliter of culture filtrate, we determined that second-stage M-26 mutants showed an alkaloid production level ≤1.3 times that of first-stage mutants and ≤2.8 times that of the wild type. Second-stage M-71 mutants showed an alkaloid production level ≤1.5 times that of first-stage mutants and ≤2.8 times that of the wild type strain, as determined on the basis of micrograms of alkaloid per milliliter of culture filtrate (1.1 times that of first-stage mutants and 1.9 times that of the wild type, as determined on the basis of micrograms of alkaloid per mg of dry mycelium). Compared with levels produced by first-stage M-71 mutants, those produced by two second-stage mutants were higher in the culture filtrates but lower in the dry mycelia, indicating an increase in growth rate.

Mutants M-26 and M-71 were also tested for auxotrophs. The frequencies of M-26 and M-71 auxotrophs were 1.3 and 2.8%, respectively, whereas the frequency of auxotrophs resulting from mutation of strain SD 58 was approximately 1/1,500 (0.07%). Thus, a second mutation with previously mutated strains produced a higher frequency of auxotrophs in the second-stage mutant population.

Thin-layer chromatography of alkaloid extracts from second-stage mutant cultures showed alkaloid patterns similar to those found for extracts from first-stage M-26 and M-71 mutants as well as strain SD 58. However, the chanoclavine-agroclavine-elymoclavine proportion differed from those of strain SD 58 and the first-stage mutants. No mutants blocked in the ability to synthesize the full complement of clavine-type alkaloids were found.

In studies utilizing low-alkaloid-producing mutants, it was found that the first-stage mutants fell into two groups. The first group was characterized by a rate of growth similar to that of strain SD 58. When these mutants were subjected to a second mutation, some cells survived. A second group grew very slowly, possibly indicating impaired metabolism. This group of mutants did not survive a second mutation.

One mutant of the first group, M-64, had an alkaloid titer of 4.5 μg/mg of dry mycelium (44 μg/ml of culture filtrate). The frequency of cell survival was 0.1% after a second mutation. None of the six second-stage mutants isolated were able to produce an increased level of alkaloids, and four were auxotrophs. Another mutant of the first group, A-176, possessed morphology and growth rate similar to those of strain SD 58. The frequency of cell survival was 2.9% after a second mutation, and of the 162 mutants obtained, 11.1% were auxotrophs.

None of the second-stage mutants produced alkaloid titers as high as those produced by high-producing strains (1,200 μg/ml of culture filtrate); however, four A-176 mutants produced an alkaloid titer in culture filtrate similar to that produced by strain SD 58. On the other hand, the alkaloid titers in dry mycelium were about one-half that of the wild type (Table 2), indicat-

ing an increased growth of the mutants that resulted in a greater mycelial mass. Generally, mutant A-176 grew slowly and did not produce much mycelium. An improvement in vegetative development, as indicated by an increase in mycelial growth, may be a distinct advantage, since strains which have vigorous vegetative growth have a better chance for surviving, especially during long-term preservation, and for providing enhanced vegetative development in tank fermentation.

Auxotrophic mutation generally leads to lower productivity of organisms producing secondary metabolites (1, 12). This was true for auxotrophic SD 58 mutants, even though they were grown in a nutritionally supplemented medium. There was a similarity between the low-alkaloid-producing mutants and the auxotrophic mutants in growth patterns. Several auxotrophs were subjected to a second mutation, and many of them did not survive. The surviving second-stage mutants were classified into two groups: the first group retained the auxotrophic properties: inability to survive in unsupplemented medium and low alkaloid production, and the second group, designated "auxotrophic revertants," were prototrophic, as characterized by the ability to grow in unsupplemented medium and the regaining of alkaloid production levels.

Mutants M 2-29 (Cys^-/Met^-) and M 10-10 (Lys^-) were subjected to a second mutation, and the frequencies of cell survival were 0.6 and 3.6%, respectively. Of 92 mutant M 2-29 colonies isolated, 13% were high alkaloid producers and 48% were auxotrophs. Of 110 mutant M 10-10 colonies, 15% were high alkaloid producers and 45% were auxotrophs. Total alkaloid production of representative high-alkaloid-producing mutants selected from the second mutation is shown in Table 2.

All auxotrophic strain SD 58 mutants that produced high alkaloid levels after a second mutation reverted to prototrophs. Reversion resulted in an alkaloid production level in the mycelium that was ≤1.6 times that of the original prototroph. Mutants which retained their auxotrophic character were poor alkaloid producers.

A possible explanation for the increase in the production of a secondary metabolite by auxotrophic revertants is that the revertants are feedback-resistant mutants in which production of a primary metabolite precursor is no longer regulated, resulting in its increase. If the amount of this precursor is rate limiting in biosynthesis, there would be an increased production of the secondary metabolite. Reversion of a *Streptomyces viridifaciens* methionine auxotroph has been shown to increase chlortetracycline production threefold (4), and since methionine is a precursor of the antibiotic, perhaps the increase is due to this mechanism. In another case, reversion of cysteine, leucine, and isoleucine auxotrophic *Streptomyces lipmanii* mutants has been shown to result in an increase in the production of methoxycephalosporin C (5). The same study also showed that the revertants excrete these amino acids into the culture medium, indicating an increase in production. Because the amino acids can be used as precursors of the antibiotic by some of the revertants, there is improved antibiotic production. Similar mechanisms may be operating in our *Claviceps* system.

TABLE 2. Total alkaloid production of representative second-stage mutants isolated from a second mutation of mutants A-176, M 2-29 (Cys^-/Met^-), and M 10-10 (Lys^-), as compared with production by first-stage mutants and wild-type strain SD 58

Strain	Total alkaloids	
	μg/ml of filtrate	μg/mg of dry mycelium
SD 58	602	49
A-176 (first stage)	69	3
A-176 DM 1	693	22
A-176 DM 2	704	22
A-176 DM 3	716	25
A-176 DM 4	762	26
M 2-29 (first stage)	96	8
M 2-29 DM 2	1,400	76
M 2-29 DM 6	866	51
M 10-10 (first-stage)	450	35
M 10-10 DM 1	1,455	80
M 10-10 DM 4	1,409	67
M 10-10 DM 9	1,317	56

The yield improvements of strain SD 58 after a second mutation are shown in Table 3. We think that our results indicate that a series of stepwise mutations and selections conducted with strain SD 58 would improve both alkaloid yield and vegetative vigor.

C. purpurea, an ergotamine-producing strain, presented special problems in mutation and selection studies. This strain produces few, if any, conidia in culture, and the mycelial cells are multinucleated. Spalla and Marnati (18) have made the general observation that alkaloid-producing strains of *C. purpurea* are heterocaryotes and do not produce conidia under normal conditions. The difficulty of obtaining mononucleated cells for mutagenic treatment was circumvented by preparing protoplasts of the fungus before treatment with the mutagen. This procedure increased the chances for separating out mononucleated entities when plating with the pin

TABLE 3. Alkaloid yield improvement of strain SD 58 mutants after a second mutation[a]

Second-stage mutant	Alkaloid yield	
	μg/ml of filtrate	μg/mg of dry mycelium
High producers[b]		
M-26 DM 6-3	2.8	1.8
M-71 DM 8	2.8	1.9
Low producers[b]		
M-64	ND[c]	ND
M-176 DM 4	1.3	0.5
Auxotrophs[b]		
M2-29 DM 2	2.3	1.6
M10-10 DM 1	2.4	1.6

[a] Yields are expressed in arbitrary units; SD 58 yield = 1.
[b] First-stage mutant grouping.
[c] ND, Not determined.

device on cell regeneration medium. Before the use of protoplasts, auxotrophic *C. purpurea* mutants reverted to prototrophs after only a few transfers. Table 1 lists the auxotrophic mutants obtained after mutagenic treatment of *C. purpurea* protoplasts. All *C. purpurea* mutants tested were low alkaloid producers. This finding is not surprising, since Spalla et al. (17) have provided evidence that peptide alkaloid-producing strains of the fungus must be in the multinucleated, heterocaryotic state for alkaloid production.

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