Genetics of Ergoline Alkaloid Formation in Penicillium roquefortii

SOOBOK L. HONG AND JAMES E. ROBBERS*

Department of Medicinal Chemistry and Pharmacognosy, School of Pharmacy and Pharmaca1 Sciences, Purdue University, West Lafayette, Indiana 47907

Received 25 October 1984/Accepted 4 February 1985

Auxotrophic, spore color, and alkaloid biosynthetic mutants of Penicillium roquefortii were selected after N-methyl-N'-nitro-N-nitrosoguanidine treatment. Diploids were obtained via protoplasting fusion techniques, and the segregants from a diploid were genetically analyzed. The data demonstrated the potential of parasexual recombination in this organism. Evidence was obtained which suggests that the his and stS (sensitivity to Sulfatase) genes may be linked. The genetic information obtained in this study can serve as a starting point for further mapping of genes in P. roquefortii, and indications are that this organism may serve as a promising vehicle for the genetic study of the formation of ergoline alkaloids.

Ergot alkaloids are typically obtained from different species of Claviceps (6). Although there have been many biosynthetic and physiological studies on the formation of the ergot alkaloids in Claviceps species, only a limited number of genetic studies have been performed with this organism (9, 15, 16, 18).

Conidiospores are produced by some strains of the fungus in saprophytic culture; however, the sexual cycle with the production of ascospores can only be completed by passage through a host plant. To achieve genetic recombination for genetic studies in Claviceps spp., either the laborious and time-consuming processes involving parasitic field cultivation on rye would be required or the parasexual cycle could be utilized. Unfortunately, the parasexual cycle has never been demonstrated in Claviceps spp.

One of the fungal species producing ergot-type alkaloids is Penicillium roquefortii (11), commonly used for the production of blue cheeses. This fungus has advantages over Claviceps spp. for performing genetic studies. It produces unincultated conidiospores on solid media within 1 week of incubation, thus it is easy to obtain uninucleate entities for analysis. Also, spore color mutants can be recovered so that visual genetic markers can be used in addition to biochemical markers in genetic analysis.

Although the parasexual cycle has not yet been demonstrated in P. roquefortii, there are many species of Penicillium in which the parasexual cycle has been demonstrated (5, 12, 17). Also, heterokaryon formation, an important first step in the parasexual cycle, has been obtained through the protoplasting fusion technique in P. roquefortii (4). In this report, parasexual recombination is demonstrated in P. roquefortii, and genetic aspects of production of alkaloids in this organism are discussed.

MATERIALS AND METHODS

Strains and culture conditions. P. roquefortii Thom (NRRL 849) was used for this study. All mutant strains used in the experiments were obtained after treatment of conidiospores with N-methyl-N'-nitro-N-nitrosoguanidine (Aldrich Chemical Co., Milwaukee, Wis.). For the protoplasting fusion experiment, a green-sporingulate histidine auxotroph (1-5 grn+ his) and a yellow-sporingulate nicotinic acid auxotroph (45-3 ylo nic) were used.

Potato dextrose-yeast agar (yeast extract [Difco Labora-
tories, Detroit, Mich.], 1.5 g; K2HPO4, 0.5 g; MgSO4·7H2O, 0.5 g; Difco potato dextrose agar, 39.0 g in 1 liter) was used for maintenance of the wild-type strain or nonauxotrophic spore color mutants. Minimum medium (MM) consisted of the following, for 1 liter: sucrose, 30.0 g; NaNO3, 3.0 g; K2HPO4, 1.0 g; MgSO4·7H2O, 0.5 g; FeSO4·7H2O, 0.01 g; proper amount of HCl to adjust the pH to 5.5; and agar, 15.0 g. Complete medium (CM) was prepared by adding 0.2% Difco yeast extract to MM. Hypertonic MM and hypertonic CM were prepared by adding 0.7 M NaCl to MM and CM, respectively. Diagnostic medium for characterization of auxotrophs was prepared according to Holliday (7) by adding 0.1 ml of stock solution of the required nutrient pool to 25 ml of MM.
Spalla and Marnati (15); i.e., the protoplasts of the nutritionally complementary auxotrophs (10⁶ to 10⁷ each) were mixed and centrifuged (6,000 × g, 10 min). Pelleted protoplasts were suspended in 1 ml of prewarmed (30°C) solution of PEG and incubated (30°C) for 15 min. The PEG-treated protoplasts were washed twice with 1 M MgSO₄ in 2 M sodium succinate (pH 5.0) and finally resuspended in 0.7 M KCl solution. Serial dilutions of the treated protoplasts were plated onto hypertonic MM and hypertonic CM to select fusion products and to count the total number of viable protoplasts. The fusion frequency was expressed as percent, which was determined by the ratio of the number of colonies developed on MM/number of colonies developed on CM after 3 to 4 days of incubation at room temperature.

Selection of diploid conidiospores and haploidization. Diploid conidiospores were selected on the basis of their larger size as compared with haploid conidia and prototrophic colonies developed on MM from the PEG-treated protoplasts transferred onto MM on day 4. After 10 days of incubation, green-pigmented conidiospores from a colony exhibiting mostly green spores were picked with a needle and suspended in distilled water. The spore diameter was determined with an ocular micrometer, using a microscope (×1,000).

Haploidization was carried out by the method of Lhoas (10), using p-fluorophenylalanine (PFA). The conidiospores from a 5-day-old colony on MM originating from a single diploid conidium were collected, plated onto PFA-CM, and incubated for 7 days; the resulting colonies were transferred onto CM.

The success of haploidization was determined by collecting both yellow and green conidiospores (segregants), testing segregants for their nutritional requirement(s), and measuring spore size.

Isolation, quantitation, and identification of alkaloids. Duplicate submerged cultures of parental and segregant strains were grown in NL-406 medium, filtered through Whatman no. 1 filter paper under vacuum, and washed with distilled water. An alkaloid extract was obtained by using standard partitioning techniques involving chloroform and 2% succinic acid solution. The final dried extract was dissolved in a known amount of chloroform, and a sample was transferred to a small test tube and evaporated to dryness; the residue was redissolved in 1 ml of 2% succinic acid solution. A colorimetric assay with Van Urk reagent (1) was used to quantitate the alkaloids. To the test tube 2 ml of reagent was added; the contents were mixed with a mechanical mixer and placed in the dark for 20 min. The total alkaloid content was calculated by multiplying absorbance at 590 nm by 59.0, a correction factor corresponding to the reciprocal slope of a calibration curve based on isoumigalaclavine A (J. L. Yeager, M.S. thesis, Purdue University, West Lafayette, Ind., 1978).

Ergoline alkaloids on chromatograms were visualized immediately after spraying with Ehrlich reagent (1 g of p-dimethylaminobenzaldehyde dissolved in 10 ml of water and 20 ml of HCl). A nonergoline indole alkaloid, roquefortine, was visualized by heating the chromatogram at 110°C for 10 min after Ehrlich reagent spray or by standing under ordinary laboratory conditions for 1 to 2 days after Ehrlich reagent spray. Roquefortine can also be detected under UV light (long and short wavelength). The intensity of the color reaction with Ehrlich reagent was utilized to quantitatively estimate the relative production of isoumigalaclavine A to roquefortine. Isoumigalaclavine A develops a blue-purple color and roquefortine develops a gray-green color.

The isolation and purification of some of the individual alkaloids were performed by preparative thin-layer chromatography, using the following solvent systems: methanol-chloroform-NH₄OH (20:80:0.4), methanol-chloroform-NH₄OH (15:85:1), ethanol-chloroform (10:90), diethylamine-chloroform (10:90), and diethylamine-chloroform (20:80).

Identification of the alkaloids was accomplished by comparison of Rf values in various solvent systems, color reaction with Ehrlich reagent, and mass spectra.

**RESULTS AND DISCUSSION**

Heterokaryon formation was achieved in *P. roquefortii* via the technique of protoplast fusion. Complementation of the auxotrophic requirements for histidine and nicotinic acid on MM diagnosed heterokaryon formation in a frequency of 0.08%. Subsequent microscopic examination of green conidiospores produced by colonies of heterokaryons provided the direct method of distinguishing diploid spores from haploid segregants and parental strains. It has been demonstrated for a number of filamentous fungi that the size of conidiospores increases with ploidy. The first report of this phenomenon was with *Aspergillus nidulans* in which diploid conidia had a diameter of approximately 1.3 times that of parent (haploid) strains (14). The same observation has been made with diploid spores obtained from interspecific somatic hybrids of *Penicillium* species (2, 3). In our case the average diameter for diploid conidiospores of *P. roquefortii* was 5.67 μm and that for haploid conidiospores was 4.16 μm, giving the diploid spores an average diameter 1.36 times greater than the haploid conidia (Table 1). The segregants developed from a single diploid conidiospore on PFA-CM were categorized into nine types on the basis of spore color, nutritional status, and ploidy (Table 1). Most of the spores recovered were the same phenotype as the original diploid. It has been reported (16) that all of the segregants were haploid when *A. niger* diploids were treated with PFA. However, PFA does not seem to work as a haploidization agent in *P. roquefortii* as efficiently as in *A. niger*, although there were enough haploid segre-
TABLE 2. Relative ergoline alkaloid production and sensitivity to the cell wall lytic enzyme Sulfatase type H 1 (Sigma) in various strains of P. roquefortii

<table>
<thead>
<tr>
<th>Strains</th>
<th>Ergoline alkaloid production (µg/ml of culture filtrate)</th>
<th>Production pattern</th>
<th>Sensitivity (iss)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>102 ± 10</td>
<td>C</td>
<td>No</td>
</tr>
</tbody>
</table>

Parental strains
45-3 ylo nic | 76 ± 3 | A | Yes |
1-5 grn+ his | 39 ± 2 | B | No |
Diploid | 102 ± 9 | C | No |
Segregants
S1-1 ylo nic his | 11 ± 1 | B | No |
S4-4 ylo nic + | 121 ± 2 | A | Yes |
S9-4 ylo + his | 12' | B | No |
S3-2 ylo + + | 159 ± 59 | C | Yes |
S10-6 grn+ nic + | 123 ± 32 | C | Yes |
S2-9 grn+ + his | 101 ± 3 | A | No |
S5-2 grn+ + | 107 ± 23 | C | Yes |

diploid strain was very similar to that of the wild-type strain, indicating that complementation has occurred in physiological characters in addition to auxotrophy and spore color. Segregants of different colony types were arbitrarily chosen and their growth and alkaloid productivity were tested. The segregants could be categorized into two groups, one group resembling the mutant 1-5 grn+ his and the other resembling the mutant 45-3 ylo nic as to alkaloid production (Table 2). Generally, strains with good growth had good ergoline alkaloid productivity.

Possibly the correlation between good growth and good production in P. roquefortii might be just the consequence of growth. In other words, strains which did not grow well could produce only small quantities of ergoline alkaloid because there was less of the enzymatic machinery for alkaloid production. In this case, the parental strains used for the fusion may have been affected by mutagen only at the cell growth level and not at the ergoline alkaloid production level.

Since the parental haploid strain 1-5 grn+ his was a poor producing strain, it was considered that the histidine gene might have something to do with ergoline alkaloid production. Even though segregants S4-1 ylo nic his and S9-4 ylo + his support this hypothesis, segregant S2-9 grn+ + his showed good alkaloid production, indicating that the histidine gene is not a determinant in production.

Concerning alkaloid production, it was interesting to note that the wild type and the diploid strains produced both isofumigaclavine A and roquefortine as major alkaloids. In the case of the haploid mutant parenteral strains, 45-3 ylo nic produced isofumigaclavine A as the major alkaloid and 1-5 grn+ his produced little isofumigaclavine A as compared with roquefortine. This is reflected also in the quantitative measurement for alkaloid since the assay is designed to detect ergoline alkaloids such as isofumigaclavine A rather than roquefortine. In the segregants the pattern of alkaloid production could not be linked to any one of the three genetic markers used in our studies.

In protoplast formation, cell wall lytic enzymes from Helix pomatia (Sulfatase [Sigma] or β-glucoronidase/arylsulfatase [Boehringer Mannheim]) were not effective on the wild-type and mutant strains of P. roquefortii with the exception of a yellow-sporulating mutant, the parent strain of mutant 45-3 ylo nic. Novozyme alone or in combination with strepzyme worked successfully on the cell wall of the wild-type strain and some mutants of P. roquefortii, with a yield of one-quarter of that obtained with Sulfatase on the yellow-sporulating mutant.

Since strain 45-3 ylo nic was the only strain of those investigated which was sensitive to the action of Sulfatase, it was considered that the cell wall composition might be different from the wild type, which in turn would be under genetic control. To demonstrate the genetic recombination of the sensitivity of cell wall to the enzyme, the ability of the segregants to yield protoplasts with Sulfatase was tested. All of the conditions were identical to those used for the fusion experiment. All segregants prototrophic for histidine showed sensitivity (designated as sts) to the enzyme and histidine auxotrophs showed resistance to the enzyme (Table 2). Since any strain which showed sensitivity to the enzyme was directly related to the parent strain 45-3 ylo nic, and there is no case of recombination between sts and his, it is possible that these genes are linked.

In the case of the diploid prototroph which showed resistance to Sulfatase, it can be explained that the sensitivity to Sulfatase (sts) inherited from strain 45-3 ylo nic could...
be compensated with resistance to Sulfatase \((sts^+\)) inherited from the 1-5 \(grn^+\) his parent, resistance being dominant and inherent in the wild-type strain.

The genetic information obtained in this study can serve as a starting point for further mapping of genes in \(P.\ roquefortii\), and indications are that this organism may serve as a promising vehicle for the genetic study of the formation of ergoline alkaloid.

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**LITERATURE CITED**