Occurrence of a major protein associated with fruiting body development in *Neurospora* and related Ascomycetes (electrophoretic variants/serotype variants/Sordariaceae)

**JUNE BOWMAN NASRALLAH AND ADRIAN M. SRB**

Section of Genetics, Development, and Physiology, Cornell University, Ithaca, New York 14853

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**ABSTRACT** Electrophoretic and immunological analysis of fruiting body (perithecial) extracts demonstrates the occurrence of a major phase-specific perithecial protein in all *Neurospora* species and in the closely related *Gelasinospora cerealis* and *Sordaria fimicola*. The perithecial proteins from these different species fall into a number of groups with different electrophoretic mobilities. They appear to be immunologically closely related but not identical to one another even within the same genus, with only partial identity exhibited between the heterothallic and pseudohomothallic *Neurospora* on the one hand and the homothallic *Neurospora* on the other. In immunological analysis of fruiting body extracts of the other Ascomycetes, *Podospora anserina*, *Cochliobolus maydii*, and *Aspergillus nidulans*, and of ascus extracts of *Saccharomyces cerevisiae*, no crossreaction with the *Neurospora* perithecial protein was found.

Biochemical analysis of components of the *Neurospora* life cycle has revealed, in *N. crassa*, *N. sitophila*, *N. tetrasperma*, and *N. terricola*, a specific association between fruiting body (perithecial) development and a major protein species resolvable on polyacrylamide gels (1, 2). This protein can be detected in unfertilized fruiting bodies, but its concentration increases drastically in fertilized fruiting bodies until 4 or 5 days after fertilization, when it constitutes a major fraction of the total perithecial proteins. Later in maturation, as perithecial contents are lost in the perithecial exudate and subsequently in spore discharge, the levels of the major perithecial protein decrease (1, 2). Detailed reports of this type of perithecial protein have so far been limited to the usual laboratory species, *N. crassa* and *N. tetrasperma*. An important biological function in fruiting body maturation for such a phase-specific protein would be indicated if a comparable protein species were found to occur more generally among Ascomycetes. All the available *Neurospora* species and members of a number of other Ascomycetous genera have now been examined for the presence of such a protein, as summarized in this report.

**MATERIALS AND METHODS**

**Strains of Neurospora.** The strains of *Neurospora* used in this study were the following: (a) as representatives of the heterothallic species—the standard laboratory wild-type St. Lawrence strains of *N. crassa*, 74A and 77A; Honduras 3A and 1a, wild strains of *N. crassa* isolated in Honduras and obtained from R. H. Stover and S. R. Freiburg; *N. intermedia* P420 and P405, obtained from the Fungal Genetics Stock Center (FGSC); and *N. sitophila* S40-34A and 2a from J. R. S. Fincham; (b) as representatives of the pseudohomothallic species—homokaryotic strains of *N. tetrasperma* from Borneo, originally isolated as strain T-220 by J. H. Warcup, and designated *N. tetrasperma* (Warcup); *N. tetrasperma* strains 1270 (85A) and 1271 (85a), derived from an isolate by B. O. Dodge, obtained from the FGSC, and designated *N. tetrasperma* (Dodge); *N. toroi* (FGSC no. 688) obtained from the FGSC; (c) as representatives of the homothallic species—*N. terricola*, obtained from S. E. Gochnaur, *N. africana* (Africana N200), *N. dodegi* (ATCC 15509), *N. galapagosensis* (Galapagosensis C349), and *N. lineolata* (ATCC 18966), all obtained from the FGSC.

The *Neurospora* strains were selected to represent established species of the genus, especially as classified by Frederick et al. (3). Some named species were omitted, as warranted by more recent taxonomic work, namely, the exclusion of the stromatic *N. phoenix* from the genus (4), or by lack of live material, as is the case for *N. erythrea*. A strain of *N. toroi* was included in the serological study although recent work on the crossing behavior of strains assigned to this species suggests their identity with strains of *N. tetrasperma* (5, 6).

**Other Genera.** Members of the other genera tested were *Gelasinospora cerealis*, obtained from the FGSC; *Podospora anserina*, from D. Marcou; *Sordaria fimicola*, from L. S. Olive; *Aspergillus nidulans* (FGSC no. 4), from Ettta Käfer; *Saccharomyces cerevisiae*, from G. R. Fink; and *Cochliobolus maydii*, from O. C. Yoder.

**Culture Techniques.** Maintenance of stocks and of cultures for immunological work with vegetative mycelia of *Neurospora*, *Gelasinospora*, and *Sordaria* was at 25°C on the minimal medium of Beadle and Tatum (7) supplemented with 2% agar when solid medium was required. Vegetative mycelia of the homothallic strains were produced as submerged cultures in Erlenmeyer flasks containing liquid minimal medium and harvested before fruiting bodies formed. Production of protoperithecia and crosses involving *Neurospora* and *Gelasinospora* were carried out at 25°C on Difco cornmeal agar or on the liquid crossing medium of Westergaard and Mitchell (8) adjusted to pH 5.7, as described earlier (1), and supplemented with 2% agar where required. *Podospora* perithalia were produced on Difco cornmeal agar in the light; *Sordaria* crosses were made on enriched cornmeal agar (9); *Aspergillus* crosses were made according to Pontecorvo et al. (10), and sporulating cultures of *Saccharomyces* were produced according to Fowell (11). *Cochliobolus* perithecia were provided by O. C. Yoder.

**Harvesting of Material and Biochemical and Immunological Analysis.** Harvesting and extraction of samples on 0.1M phosphate buffer (pH 7.0) and electrophoresis on 7.5% polyacrylamide gels were as described earlier (1). The protein antigen for immunological studies was obtained from perithecial extracts of *N. crassa* or *N. tetrasperma* (Dodge) which were subjected to electrophoresis. The protein was eluted in 0.1 M phosphate buffer (pH 7.0) from acrylamide gel slices corresponding to the protein band of interest. The gel
eluate was dialyzed against the same buffer, and tested for electrophoretic purity by polyacrylamide gel electrophoresis. Antiserum against the antigen were produced in New Zealand White rabbits by subcutaneous and intramuscular injection of a 1:1 mixture of antigen and Freund's complete adjuvant (Difco).

Two injection protocols were followed: (i) The rabbits were injected weekly for 4 weeks and bled 5 weeks after the start of the injections. (ii) Two weekly injections were done, and sera were collected 6 weeks after the first injection. Identical experimental results were obtained with sera produced by either protocol. To the sera, sodium azide to a final concentration of 0.1% (wt/vol) was added as a preservative, and the sera were frozen and stored at −10°C in small aliquots. Immunological reactivity was tested by the double diffusion method of Ouchterlony (12), on slides coated with 1% (wt/vol) Difco purified agar containing 0.1% sodium azide as a preservative. The purity of the sera was demonstrated by the formation of a single precipitin arc when whole unstained polyacrylamide gels on which the peritheial proteins had been electrophoresed were placed in agar troughs and analyzed by double diffusion. Antigen was quantitated by the single radial immunodiffusion method of Mancini et al. (13), in which the antigen is allowed to diffuse in agar containing undiluted specific antiserum in a ratio of 7:1 (vol/vol). The area, or (diameter)², of the precipitin circles that develop around the antigen wells is proportional to the antigen concentration.

RESULTS

The agar gel diffusion test of Ouchterlony with antisera raised to the N. crassa or N. tetrasperma (Dodge) major perithecial protein was used to investigate the possible association of a similar protein with sexual morphogenesis in all the available Neurospora species and in members of a number of other Ascomyceteous genera. As shown in Fig. 1, precipitin reactions were observed for perithecial extracts of all Neurospora species, of Gelasinospora cerealis, and of Sordaria fimicola, but not for perithecial extracts of Podospora anserina and Cochliobolus maydis or for cleistothecial extracts of Aspergillus nidulans or for ascus extracts of Saccharomyces cerevisiae. Furthermore, on the basis of the immunological reactions obtained with two different antisera, the crossreacting strains can be divided into three groups, with members of each group exhibiting reactions of complete identity (end-to-end fusion) with one another and reactions of only partial identity (indicated by spur formation) with members of other groups. Thus, N. crassa, N. crassa (Honduras), N. sitophila, N. intermedia, N. tetrasperma (Dodge and Warcup), and N. toro fall in one group; N. africana, N. dodgii, N. galapagensis, N. lineolata, N. terricola, and Gelasinospora cerealis in another; and Sordaria fimicola falls in still another group.

Polyacrylamide gel electrophoresis of perithecial extracts of the various Neurospora species and Gelasinospora cerealis reveals a major proteinaceous band in each case, with a number of electrophoretic variants of the protein occurring (Fig. 2). The correlation of this major protein with the crossreacting antigen for each of the strains tested was demonstrated by immunological analysis of gel slices taken along the length of unstained gels, inasmuch as only slices corresponding to the major band reacted with the immune sera. Polyacrylamide gels of perithecial extracts from Sordaria fimicola did not reveal such a major protein upon staining. However, immunological analysis of gel slices showed that reactivity was confined to a region very close to the tracking dye, indicating that, under the electrophoretic conditions used, the Sordaria major perithecial protein was not resolved from the front.

The functional homology of the crossreacting antigens in the different species was tested by following the course of development of the antigens with maturation of the perithecia. First, vegetative mycelia extracts from some of the strains were assayed immunochemically (Fig. 3). As was reported earlier for N. crassa and N. tetrasperma (1, 2), all species tested lacked the perithecial antigen in vegetative mycelia. Then, by the technique of single radial immunodiffusion, the changes in concentration of the antigen in perithecia were measured over a period of 8 days after fertilization for the heterothallic and pseudohomothallic species and 8 days after the first appearance of recognizable fruiting bodies for homothallic species (Fig. 4). The curve obtained for N. crassa by this method was similar to that obtained previously by electrophoretic protein analysis (1). All species providing a crossreacting protein showed an increase in antigen concentration from initially undetectable levels to high levels, followed by a decrease, as illustrated in Fig. 5 for four of the strains tested.
DISCUSSION

The results presented in this paper demonstrate the general occurrence of a protein species associated with sexual morphogenesis in the genus *Neurospora* and in members of certain closely related genera of the Sordariaceae. The homology of the various perithecial specific protein species identified in *Neurospora*, *Gelasinospora*, and *Sordaria* is clearly indicated (a) by their similar behavior on polyacrylamide gels as rapidly migrating acidic proteinaceous molecules, (b) by their absence in vegetative mycelia, (c) by the observed increase in their concentration during fruiting body development, and (d) by their close immunological relatedness.

Differences among these perithecial proteins do, however, exist. The perithecial proteins from different genera, from different species within the same genus, and from different geographically isolates of the same species have different electrophoretic mobilities, with a total of seven variants observed (Table 1). In addition, the patterns of increase of the perithecial antigen during maturation are not identical for all strains tested; i.e., the maximum concentration attained and the times at which that concentration is reached differ. The significance of the observed differences in pattern of increase is difficult to evaluate, inasmuch as variability is also observed in separate assays of the same strain. Because of asynchronous perithecial development, absolute values may not be reliable. In any case, the trend in all strains is clearly an increase in antigen concentration early in fruiting body maturation.

Immunologically, differences among the proteins were also observed. While the serological differences do not correlate with the electrophoretic differences, they occur in a pattern of possible taxonomic significance; that is, by our tests, perithecial proteins from heterothallic and pseudohomothallic *Neurospora*

![Fig. 3. Immunodiffusion pattern of mycelial extracts. Central well: antiserum produced against *N. crassa* perithecial antigen. Peripheral wells: 1 and 2, *N. crassa* perithecial extract; 3, *Gelasinospora mycelial extract; 4, *Sordaria* mycelial extract; 5, *N. dodgei* mycelial extract; 6, *N. africana* mycelial extract.](image)

![Fig. 4. Single radial immunodiffusion plate showing changes in antigen concentration associated with fruiting body development of *N. terricola*. Numbers indicate days after first appearance of fruiting bodies. Extracts of 20 perithecia were used per well.](image)

![Fig. 5. Changes in the levels of the perithecial antigens in perithecia collected at different times after the first appearance of fruiting bodies. Maximal reactivity for each strain corresponds to the highest absolute amount of antigen obtained in the time sequence, with all other values expressed as percentages of that number. (a) *N. dodgei*; (b) *N. lineolata*; (c) *Gelasinospora cerealis*; (d) *Sordaria fimicola*.](image)

### Table 1. Immunological relationships and electrophoretic mobilities of the crossreacting perithecial protein from different species and genera

<table>
<thead>
<tr>
<th>Cross-reaction groups</th>
<th>Strains</th>
<th>Electro-phenotypic variant, $R_f$</th>
</tr>
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<tbody>
<tr>
<td>I</td>
<td><em>Honduras, N. intermedia</em></td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td><em>N. tetrasperma</em> (Warcup)</td>
<td>0.77</td>
</tr>
<tr>
<td></td>
<td><em>N. crassa, N. sitophila, N. tetrasperma</em></td>
<td>0.77</td>
</tr>
<tr>
<td></td>
<td><em>(Dodge)</em></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td><em>N. galapagosensis</em></td>
<td>0.77</td>
</tr>
<tr>
<td></td>
<td><em>N. lineolata</em></td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td><em>N. terricola</em></td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td><em>N. africana</em></td>
<td>0.87</td>
</tr>
<tr>
<td></td>
<td><em>N. dodgei, Gelasinospora cerealis</em></td>
<td>0.98</td>
</tr>
<tr>
<td>III</td>
<td><em>Sordaria fimicola</em></td>
<td>0.99</td>
</tr>
</tbody>
</table>

* Perithecial proteins listed as having the same $R_f$ values were shown by electrophoresis of pairwise mixtures of the respective perithecial extracts to exhibit one major band. Perithecial proteins with different $R_f$ values were shown in the same way to exhibit two major bands, each of which was contributed by one of the strains.
species are serologically indistinguishable but show only partial serological identity with the proteins from homothallic *Neurospora* species and *Gelasinospora*. The serological criterion may thus be added to a number of other properties differentiating the heterothallic and pseudohomothallic from the homothallic *Neurospora*, which, in addition to lacking a mating type system, generally lack conidiation and have poor mycelial growth. The array of distinctions may warrant exclusion of the homothallics from the genus.

Before a final decision on the taxonomic status of the homothallic Neurosporas is reached, however, the development of suitable hybridization techniques (6) is desirable, as are more extensive serological analyses of varied antigenic components in a large number of strains and with a wider range of antisera raised against the *N. crassa* and *N. tetrasperma* perithecial antigen or against perithecial antigens from other relevant species.

Our failure to detect any crossreacting antigen in the Ascomycetous genera less closely related to *Neurospora* may simply indicate phylogenetic divergence and does not exclude the existence of a similar phase-specific protein of analogous function, at least in genera that produce fruiting bodies. Electrophoretic analysis of the appropriate fruiting body extracts should indicate whether such a major low molecular weight acidic protein is present.

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