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Isozyme Patterns and Sexual Morphogenesis in *Schizophyllum**

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Abstract. Isozymes of several different classes of enzymes in partially purified protein extracts of five strains of *Schizophyllum commune*, isogenic except for genes controlling sexual morphogenesis, were separated on polyacrylamide gel by disc electrophoresis. After staining, isozyme patterns were compared on the bases of the presence or absence, electrophoretic mobility (R_f values), and relative activities of specific isozymes. Differences in isozyme patterns in 14 enzymes, i.e., NADH-dehydrogenase, NADPH-dehydrogenase, a number of NAD- and NADP-dependent dehydrogenases, acid phosphatases, leucine aminopeptidase, and esterases, were correlated with the operation or inactivity of the *A*- and *B*-sequences of sexual morphogenesis. In only a single instance, i.e., phenolases, no marked differences could be correlated with sexual morphogenesis.

In *Schizophyllum commune* and related higher fungi, mating of compatible, haploid, homokaryotic strains converts both mates into a fertile, specialized heterokaryon, the dikaryon, the genetic and physiological equivalent of the diploid. The morphological distinction between the homokaryon and the dikaryon is simple: the former consists of uninucleate cells, and its septa are simple; the latter is made up of binucleate cells (one nucleus from each original mate), and its septa have external appendages known as clamp connections. The establishment of the dikaryon has been termed *sexual morphogenesis*,[1,2] and it consists of a sequence of several steps that are precisely regulated by the *A* and *B* incompatibility factors. When both *A* and *B* factors of the two mates are different, the entire sequence of events ensues with the establishment of the dikaryon. When only the *A* factors are different, only certain of the stages occur. These are collectively termed the *A-sequence*, and the result is the common-*B* heterokaryon. Similarly, when only the *B* factors are different, only the initial step of the process, nuclear migration, occurs; this is the main event of the *B-sequence*, and the result is the common-*A* heterokaryon. Morphologically, the common factor heterokaryons are quite distinct from the homokaryon and the dikaryon, as well as from each other.[3]

It is a significant feature of this system that the *A*- and *B*-sequences can be "turned on" by means other than the interaction of pairs of different *A* and different *B* factors.[4,5] A mutation in either of the factors has the same effect in the homokaryon as a pair of compatible factors in the heterokaryon: a mutant-*B* strain is thus a close mimic of the common-*A* heterokaryon; a mutant-*A*

strain, of the common-*B* heterokaryon; a mutant-*A*-mutant-*B* strain, of the dikaryon.

The effects of such mutations in the *B* factor can be almost totally negated by additional mutations of two different types: (*a*) secondary mutations in the mutant-*B* incompatibility locus itself[5,6] and (*b*) certain types of modifying mutations that occur in loci outside the *B* incompatibility factor.[1] Both of these types of mutations restore normal morphology to the mutant-*B* homokaryon.

About a decade ago, it was demonstrated by serological means that there were major differences in the mycelial proteins of the dikaryon as compared with those of its two isogenic component homokaryotic strains, the proteins of which were virtually indistinguishable.[7] From this and certain other facts it was predicted that major specific differences in mycelial proteins would be correlated with the status of sexual morphogenesis, i.e., the operation or inactivity of the *A*- and *B*-sequences,[3] rather than with the specific incompatibility factors present. This prediction has been confirmed by a disc-electrophoretic comparison of total soluble-protein extracts of nine different types of mycelia—wild type and mutant, homokaryons, and heterokaryons.[8]

A closer examination of a number of specific enzyme activities might be expected to reveal differences between the various mycelial types in accordance with the involvement or lack of involvement of specific enzymes with sexual morphogenesis.

Materials and Methods. Five isogenic strains (backcrossed for 10 or more generations to a single normal homokaryon) were used in this study:

Genotype	Mycelial type	Status of sexual morphogenesis[2,3]
A41 B41, *A43 B43*[8]	Wild type homokaryons	Neither A- nor B-sequence operates
A41 B41 + *A43 B43*	Wild type dikaryon	Both A- and B-sequences operate
Amut B41	Mutant-*A* homokaryon	Only A-sequence operates (mimic of common-*B* heterokaryon)
A43 Bmut	Mutant-*B* homokaryon	Only B-sequence operates (mimic of common-*A* heterokaryon)
A43 Bmut M11	Modified mutant-*B* homokaryon	A-sequence inoperative; B-sequence suppressed (mimic of wild-type homokaryon)

The maintenance of stock cultures, the growth and harvesting of mycelia, and the preparation of cell homogenates have been described in an earlier paper.[8] The cell homogenates were extracted for 60 min at 0°C with 0.1 *M* sodium phosphate (pH 7.0) buffer and centrifuged for 30 min at 0°C at 37,000 × *g*. The supernatant fluids were treated with 0.1 vol of 2% protamine sulfate at 0°C and again centrifuged. The clear supernatant fluids collected after centrifugation were dialyzed against three changes of large volumes of dilute (0.005 M sodium phosphate, pH 7.0) buffer at 4°C during a period of 24 hr. The dialyzed "total soluble protein" extracts were either used immediately for electrophoretic analysis or freeze dried and kept in a desiccator at 4°C. Dry extracts were dissolved in cold dilute buffer immediately before use. A quantitative colorimetric biuret method[9] was routinely used for protein determination.

Disc electrophoresis of soluble proteins was performed at pH 9.4 at 4°C on 7.5% standard gel columns in 0.8 × 14 cm glass tubes with a constant current of 4 mA/tube according to the procedures provided by Canal Industrial Corporation for the model 12 apparatus. Protein samples, 0.7 mg/tube, were incorporated into sample gels. After electrophoresis, gel columns were removed from the glass tubes, rinsed with cold distilled

TABLE 1. *Staining procedures.*

Enzyme activities	Incubation Time	Incubation Temperature (°C)
NADH-dehydrogenase: 0.1 M Tris-HCl (pH 8.3) buffer (50 ml), *p*-NBT (20 mg), NADH (25 mg)	1 hr	37
NAD-dependent dehydrogenases: 0.1 M Tris-HCl (pH 8.3) buffer (50 ml), *p*-NBT (20 mg), NAD (20 mg), substrate (500 μmoles)		
Substrates:		
Sodium L-malate	1$^1/_2$ hr	37
Sodium lactate	16 hr	37
Dihydro L-orotic acid	16 hr	37
L-Histidinol	1$^1/_2$ hr	37
Monosodium glutamate	16 hr	37
NADPH-dehydrogenase: 0.1 M Tris-HCl (pH 8.3) buffer (50 ml), *p*-NBT (20 mg), NADPH (25 mg)	2 hr	37
NADP-dependent dehydrogenases: 0.1 M Tris-HCl (pH 8.3) buffer (50 ml), *p*-NBT (20 mg), NADP (20 mg), substrate (500 μmols)		
Substrates:		
Sodium L-malate	16 hr	37
Glucose-6-phosphate	16 hr	37
DL-isocitrate trisodium	16 hr	37
Monosodium glutamate	16 hr	37
Esterases (cf. Mäkinen and Brewbaker[17])	40 min	25
Acid phosphatase (cf. Mäkinen and Brewbaker[17])	1$^1/_2$ hr	37
Leucine aminopeptidase (cf. Mäkinen and Brewbaker[17])	1 hr	37
Phenolases: 0.1 M Tris-maleate (pH 6.0) buffer (50 ml), dihydroxyphenylalanine (50 mg), L-proline (50 mg)	2 hr	37

water, and incubated with appropriate staining mixtures for the visualization of the various enzyme activities. The staining mixtures, incubation times, etc., are listed in Table 1.

After the staining, gel columns were thoroughly rinsed with distilled water, placed in glass tubes containing either 20% ethanol (for esterases) or 7.5% acetic acid (for all other enzymes), and photographed. The electrophoretic mobility (R_f values) of the isozymes were calculated from the positions of the stained bands on the photographs. Isozymes of the different types of mycelia that differed in R_f value by 0.01 or greater were considered to be distinct.[10] The number, R_f values, and relative intensities of the isozymes were used as the criteria of comparison.

Results. Several different classes of enzymes of *Schizophyllum commune* have been examined, and all, as in other organisms, occur in multiple molecular forms.

The patterns of isozymes of NADH-dehydrogenase activity of the five mycelial types on stained gel columns (Fig. 1*A*) may serve as a model for the analyses of other enzymes studied. Because individual gel columns often differ in length after electrophoresis and staining, minor differences in banding patterns among mycelial types are not readily recognized. When the same isozymes are plotted in a diagram according to their electrophoretic mobility, these differences are readily visualized (Fig. 1*B*). This plot also serves to relate the isozymes of this enzyme in the various mycelial types to sexual morphogenesis and to its control by the incompatibility factors. The isozymes of the wild type homokaryon are given roman numbers in order from the origin; isozymes of other mycelial types that differ in R_f value by 0.01 or more from those of the homokaryon are correspondingly given arabic numbers. The isozymes in the various mycelial types may be further differentiated with reference to the two major components of the

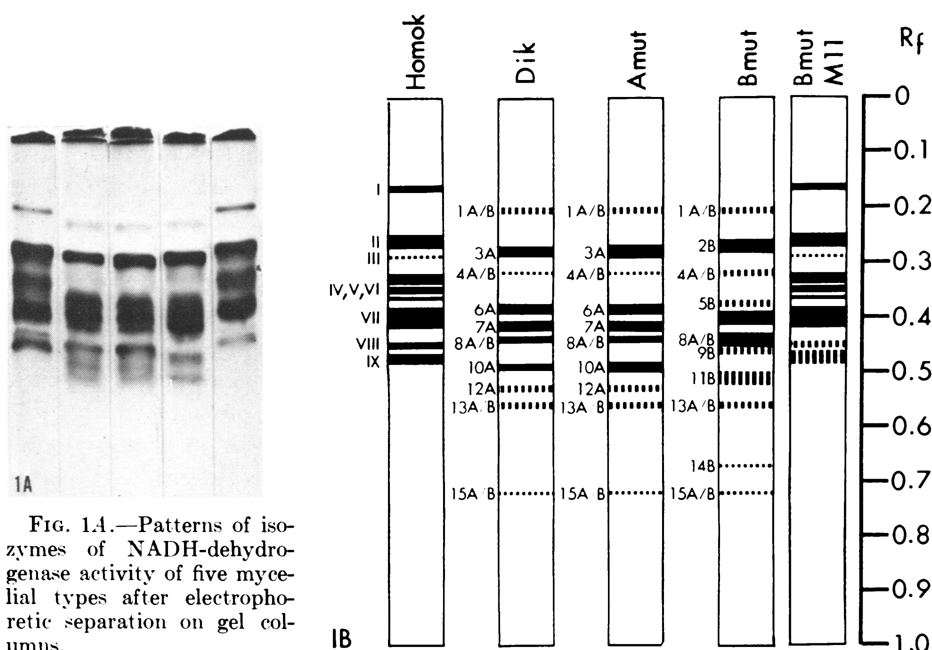
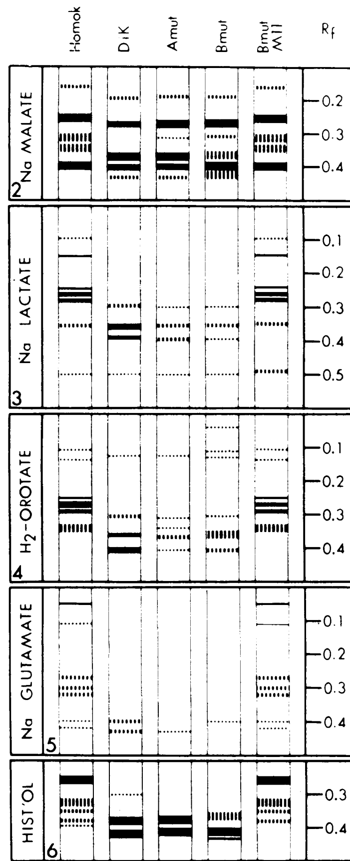


FIG. 1*A*.—Patterns of isozymes of NADH-dehydrogenase activity of five mycelial types after electrophoretic separation on gel columns.

FIG. 1*B*.—Diagrammatic representation of the same isozymes shown in Fig. 1*A*. Dashed lines represent faint bands. Bands I–IX, isozymes observed in the wild type homokaryon. Bands 1–15, isozymes observed in other mycelial types but absent in the wild type homokaryon; *A*, *B*, and *A*/*B* indicate association of isozymes with *A*- and *B*-sequences of the morphogenetic process. See text for details.

morphogenetic process that are under the control of the *A* and *B* incompatibility factors respectively. *A*/*B*-isozymes 1, 4, 8, 13, and 15 of Figure 1*B* occur in mycelia in which either the *A*-sequence or the *B*-sequence is operative and in mycelia in which both sequences operate. By contrast, *B*-isozymes 2, 5, 11, and 14 are present when the *B*-sequence is operative (also see *B*-isozymes in acid phosphatase, Fig. 13), whereas *A*-isozymes 3, 6, 7, and 12 occur when the *A*-sequence is functioning. Another class of isozymes, *AB*, which appears only when both sequences are operating simultaneously (i.e., in the dikaryon), is absent from the NADH-dehydrogenase; isozymes of this type, however, occur in acid phosphatase (Fig. 13) and esterases (Fig. 12).

Differences in isozyme patterns correlated with sexual morphogenesis and comparable to those shown in Figure 1*B* were observed in most of the enzyme activities examined. The enzyme activities studied included five NAD-dependent dehydrogenases (Figs. 2–6), NADPH-dehydrogenase and four NADP-dependent dehydrogenases (Figs. 7–11), esterases (Fig. 12), acid phosphatases (Fig. 13), leucine aminopeptidase (Fig. 14), and phenolases (Fig. 15). Individual isozymes are not labeled in the diagrams of these enzymes, but marked differences in isozyme patterns occur in practically all the cases. Variations of three kinds account for the differences in patterns: (*a*) certain isozymes of the wild type homokaryon are absent in the dikaryon, the *Amut* homokaryon, or *Bmut* homo-



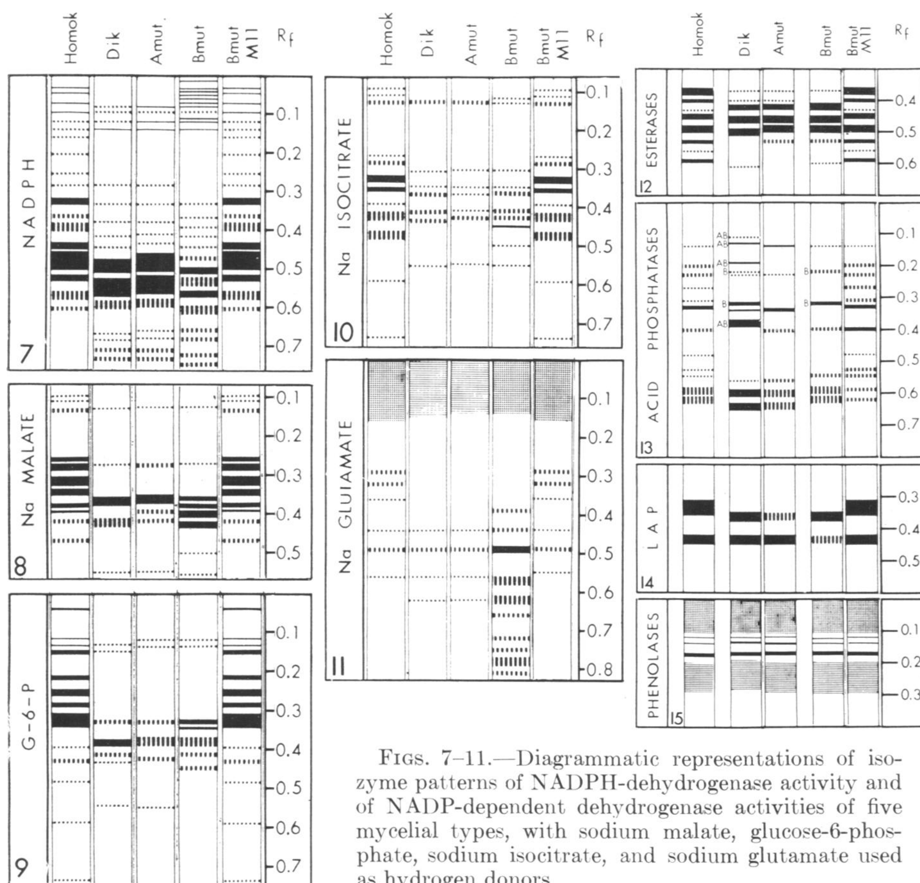
FIGS. 2–6.—Diagrammatic representations of isozyme patterns of NAD-dependent dehydrogenase activities of five mycelial types, with sodium malate, sodium lactate, dihydro-orotic acid, sodium glutamate, and L-histidinol used as hydrogen donors.

karyon, (*b*) other isozymes in some or all of these latter mycelial types are absent in the wild type homokaryon, and (*c*) there are differences in the relative activities of isozymes common to two or more of the five mycelial types. In addition, two equally striking features are evident in the isozyme patterns of practically all of these enzyme activities: (*d*) marked differences between the isozyme patterns of the wild type homokaryon and those of the dikaryon, the *Amut* homokaryon, and the *Bmut* homokaryon on the one hand, and (*e*) essential identity of the isozyme patterns of the wild type homokaryon with those of the double mutant *Bmut M11* homokaryon on the other hand. A few exceptions to the two latter generalities should be noted.

Only very slight differences, not clearly correlated with sexual morphogenesis, were seen in one case: the isozyme patterns of phenolases of the five mycelial types (Fig. 15) were essentially identical when DOPA was used as the substrate. In a second case, that of leucine aminopeptidase (Fig. 14), only a single difference could be correlated with sexual activity.

There were also a few cases in which the isozyme patterns of the double mutant *Bmut M11* strain differed noticeably from those of the wild type homokaryon: (*a*) differences in relative activity of one or two isozymes of NADH-dehydrogenase (Fig. 1*B*) and of the NAD-dependent dehydrogenase for dihydro-orotic acid (Fig. 4), (*b*) the absence in the *Bmut M11* homokaryon of an isozyme of NAD-dependent L-histidinol dehydrogenase (Fig. 6), and (*c*) some very minor differences in electrophoretic mobility and relative activities of a few isozymes of acid phosphatases in the two homokaryons (Fig. 13).

In addition to these comparative details of the isozyme patterns characteristic of the different types of mycelia, several other features were observed. (*a*) In some cases, the *Amut* homokaryon resembled the dikaryon, e.g., NADH-dehydrogenase (Fig. 1*B*), NADPH-dehydrogenase (Fig. 7), NADP-dependent isocitrate dehydrogenase (Fig. 10), and NADP-dependent glutamate dehydrogenase (Fig. 11). (*b*) In other cases, the *Amut* homokaryon closely resembled the *Bmut* homokaryon, e.g., NAD-dependent malate dehydrogenase (Fig. 2) and NAD-dependent lactate dehydrogenase (Fig. 3). (*c*) Except for these in-



Figs. 7–11.—Diagrammatic representations of isozyme patterns of NADPH-dehydrogenase activity and of NADP-dependent dehydrogenase activities of five mycelial types, with sodium malate, glucose-6-phosphate, sodium isocitrate, and sodium glutamate used as hydrogen donors.

Figs. 12–15.—Diagrammatic representations of isozyme patterns of esterases, acid phosphatases, leucine amino, peptidase and phenolases of five mycelial types. *AB*-isozymes, observed exclusively in the dikaryon; *B*-isozymes, observed in the *Bmut* homokaryon and the dikaryon. See text for details.

stances, the isozyme patterns of the *Bmut* homokaryon differed in detail from those of all other mycelial types examined (Figs. 1*B*, 4–11).

Discussion. It is now generally recognized that most enzymes, even in the highly purified crystalline state, exist in multiple molecular forms, or isozymes. Comparative isozyme patterns are commonly used in taxonomic, developmental, physiological, biochemical, pathological, and clinical studies, and many intensive investigations have been made in recent years on the genetic control of isozymes in humans, higher animals, plants, insects, protozoa, and bacteria.[11] The fungi have not been extensively examined in this respect, but differences in isozymes of certain enzymes between wild type and mutant strains of *Aspergillus* have been reported.[12]

The work reported here goes a step beyond a simple demonstration of a genetic basis for variations in isozymes; it relates such variations to the expression of morphogenetic processes known to involve large numbers of genetic factors

(recognized only in the mutant or modifying state) that are in turn under the control of the four genes of the *A* and *B* incompatibility factors.[1] The final isozyme patterns are thus not determined solely or even primarily by the identities of the genes present. The basis of the differences in isozymes as related to the morphogenetic processes cannot be specified from present data and can only be established in further studies. Pending the opportunity to complete such studies, however, some assessment of the significance of present results seems worthwhile.

There can be no question of the reality of significant differences in isozyme patterns in the various mycelial types that represent different morphogenetic states. The significance of the demonstrated differences in isozyme patterns, however, is far more difficult to assess in the absence of specific information that can relate isozyme differentiation to new or to previously formed proteins.

The critical determination of final electrophoretic mobility of the many isozymes may be epigenetic, the result of slight differences in pH, concentration of some critical substance, or some other biochemical or physiological condition associated with different states of differentiation.

We prefer the explanation that the incompatibility genes control the transition from one morphogenetic state to another by activating or inactivating different combinations of genes (such as the "batteries of producer genes" postulated by Britten and Davidson[13]). This relationship is suggested by a comparison of the isozyme patterns of the five mycelial types. The isozymes of the wild type homokaryon, in which neither the *A*-sequence nor the *B*-sequence is operating, are quite different from those of the dikaryon, in which both of the two sequences are active. Yet, the dikaryon was formed by the interaction of two highly isogenic strains of wild type homokaryons having almost identical genomes except for the alleles of the incompatibility loci.[8] That the responsibility for differences in isozymes between the homokaryon and the dikaryon is not dependent upon these allelic differences, however, is shown by the many similarities between the isozyme patterns of the dikaryon and those of the *Amut* and *Bmut* homokaryons, in each of which one or the other sequence is active but differs from the wild type homokaryon by a single gene. Finally, an isozyme pattern that is usually indistinguishable from that of the wild type homokaryon results from the association with the mutant-*B* factor of a type IV modifying mutation, which appears to "turn off" the *B*-sequence by suppressing the action of the mutant-*B* factor. The differences in isozyme patterns among the five types of mycelia can thus be strictly correlated to the morphogenetic progression and to its two components, the *A*- and *B*-sequences.

Nothing is known of the role that the enzymes studied here might play in the morphogenetic process. Most of the systems examined represent common metabolic enzymes. The wild type homokaryon, the dikaryon, the *Amut* homokaryon, and the *Bmut M11* homokaryon all display normal vegetative vigor, and on this basis, little or no differences in isozyme patterns could have been expected for these metabolic enzymes. In comparison with these mycelial types, the *Bmut* homokaryon is decidedly subnormal in vegetative vigor. In no case, however, are differences in isozyme patterns correlated with normal versus sub-

normal growth—hence, presumably, the isozyme patterns do not reflect differences in purely metabolic functions.

Previous work with *S. commune* has shown significant differences in the levels of enzymatic activity of a number of dehydrogenases in different stages of development, i.e., spores, germlings, the homokaryon, and the dikaryon.[14] In only a single case, however, has a specific enzyme been identified as probably critically involved in an event of the morphogenetic progression.[15,16] High specific activity of R-glucanase, which hydrolyzes a major component of the cell wall (R-glucan), occurs under conditions of septal disruption and nuclear migration, i.e., in compatible matings at the time of nuclear migration, in the common-*A* heterokaryon, and in the *Bmut* homokaryon.

One objective of the work reported here was the identification of additional enzymes that might be critically involved in one or more stages of the morphogenetic progression. This objective was not achieved, but the actual results may well have more significance than the anticipated dissection of the morphogenetic process. The indication that practically all of the organism's array of enzymes are functionally altered by events controlled by the four genes of the *A* and *B* factors—admittedly a long extrapolation from the 15 enzyme activities examined here—gives some intimation of the ramifications and corollary effects of regulatory systems in eukoryotic forms.

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Abbreviations used: NAD, nicotinamide-adenine dinucleotide; NADP, nicotinamide-adenine dinucleotide phosphate; *p*-NBT, *p*-Nitro Blue Tetrazolium; and Tris, tris(hydroxymethyl)aminomethane.

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