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Genetic Mapping in the Lignin-Degrading Basidiomycete *Phanerochaete chrysosporium*

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A method of meiotic segregation analysis based on recombinant selection in the homothallic basidiomycete *Phanerochaete chrysosporium* was developed. Using this method, we were able to reveal linkage relationships and to estimate recombination frequencies between seven mutations to auxotrophy. We detected two linkage groups, the first containing four and the second three of the seven mapped mutations.

The basidiomycete *Phanerochaete chrysosporium* is commonly used as a model organism in studies concerned with lignin biodegradation. In further investigations of the enzymology and regulation of ligninolytic activity as well as the construction of industrially applicable strains for biotechnological processing of lignin and lignocellulose, the genetics of this fungus is of great interest. Several reports dealing with the molecular genetics of *P. chrysosporium* have appeared recently (2, 8, 11, 17, 18). In the area of classical genetics, the first step was made by elucidating conditions for fruit body formation and basidiospore production (6). Subsequently, it was shown that basidiospores are binucleate and homokaryotic (3). Isolation of auxotrophic strains and the related complementation study were first published in 1982 (7). In 1985, a report describing crosses between auxotrophic strains appeared (1). After fruiting of balanced heterokaryons, recombinant sets of parental nutritional requirements were found in the progeny. Similar results were obtained in our laboratory (9), after which we started work on the construction of a genetic map of this organism.

As there is strong evidence that *P. chrysosporium* is a homothallic species (3), the methods of meiotic segregation analysis designed for fungi with homogeneic incompatibility systems cannot be used because an increased number of parental genotypes arising from selfing is expected, which mimics the linkage in the random spore analysis. Pontecorvo et al. (10) have suggested several approaches to solving this problem in their report concerned with *Aspergillus nidulans*. One of them, the method of recombinant selection, was used in this study, and the corresponding model of quadrifactorial crosses was developed.

MATERIALS AND METHODS

Organism. *P. chrysosporium* 284B was kindly supplied by E. C. Setliff (Forintek Corp., Vancouver, British Columbia, Canada). A single basidiospore isolate was derived from strain 284B. This isolate is referred to here as P-1. All strains were maintained on complete agar slants at 4°C.

Isolation of auxotrophic strains. Met21, Nic21, Ade21, and Sr21 strains were derived from strain 284B by UV mutagenesis (9). Strains Sr22, Ade22, and Nic22 were obtained by the same procedure, except that conidiospores of strain P-1 were used for mutagenesis and irradiation time was shortened from 20 to 12 min. The survival ratio was about 0.05% under these conditions, and 1 auxotrophic isolate was detected among about 500 surviving colonies. The auxotrophs used in this work are described in Table 1. Double auxotrophs were prepared by crossing single auxotrophic strains. In crosses Met21 × Sr21 and Met21 × Sr22, Met^- Sr^+ and Met^- Sr^- phenotypes in the progeny were distinguished by the forced heterokaryon test (4) with strains Nic21Sr21 and Nic21Sr22, respectively. Isolates that did not grow on thiosulfate but grew on methionine were forced to form heterokaryons with strain Nic21Sr21 (or Nic21Sr22) on minimal medium supplemented with thiosulfate, and small fragments of the resulting mycelia were then tested on minimal medium.

In the text, double auxotrophs are denoted by the names of the parental auxotrophs written together.

Media and culture conditions. The following media were used. Complete medium was a modified form of the medium described in reference 16 and consisted of the following (grams per liter): $MgSO_4 \cdot 7H_2O$, 0.5; KH_2PO_4, 0.46; K_2HPO_4, 0.76; Proteose Peptone (Difco), 2; yeast extract, 2; glucose, 20; agar, 20.

Minimal medium consisted of the following (grams per liter): $MgSO_4 \cdot 7H_2O$, 0.5; KH_2PO_4, 0.6; K_2HPO_4, 0.4; $(NH_4)_2SO_4$, 0.5; glucose, 20; agar, 20. Concentrations of nutritional factors in the test media were as follows (milligrams per liter): adenine, 2.5; methionine, 50; histidine, 50; nicotinic acid, 5; $Na_2S_2O_3 \cdot 5H_2O$, 100. For selective plating of basidiospores, minimal medium with growth-restricting agents (10 g of L-sorbose per liter and 0.1 g of sodium deoxycholate per liter instead of glucose) and thiamine (1 mg/liter) was used (5). Before pouring, various supplements were added as indicated in the text.

Modified Gold's fructification medium (6) of the following composition (grams per liter) was used for fruit body and basidiospore production: $MgSO_4 \cdot 7H_2O$, 0.5; KH_2PO_4, 0.6; K_2HPO_4, 0.4; thiamine, 0.01; ammonium tartrate, 0.18; microcrystalline cellulose, 10; agar, 20.

The following complete medium was used for control plating of basidiospores (grams per liter): $MgSO_4 \cdot 7H_2O$, 0.5; KH_2PO_4, 0.6; K_2HPO_4, 0.4; ammonium tartrate, 0.5; sodium deoxycholate, 0.1; yeast extract (Oxoid), 2; casein hydrolysate, 1; L-sorbose, 10; agar, 20.

All incubations were performed at 28°C unless stated otherwise.

Crossing and isolation of basidiospore progeny. Crossed strains were inoculated in close proximity onto the complete medium and incubated for 1 day to increase the contact of

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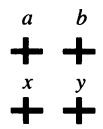
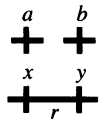
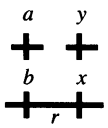
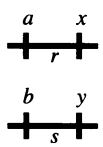
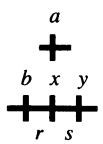
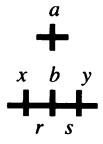
TABLE 1. List of auxotrophs

Strain	ATCC no.	Mutation	Growth requirement
Met21	66144	*met21-1*	Methionine
Nic21	66143	*nic21-1*	Nicotinic acid
Nic22		*nic22-1*	Nicotinic acid
Sr21	66142	*s21-1*	Methionine or cysteine or thiosulfate
Sr22		*s21-2*	Methionine or cysteine or thiosulfate
Ade21	66145	*ade21-1*	Adenine
Ade22		*ade22-1*	Adenine or histidine

hyphae and the probability of plasmogamy. The center of the resulting colony was then transferred onto minimal medium supplemented with thiamine (1 mg/liter) and incubated until the colony reached the edge of the plate (9-cm dishes). An inoculum from the edge of the colony was then transferred onto the fructification medium and incubated for 5 days. The dishes were then taken out of the incubator and incubated upside down at room temperature. The lids were changed when a basidiospore coat appeared, and the 1-day yield of basidiospores was washed from the lids with 0.9% NaCl solution. The basidiospores were counted in a hemacytometer and plated onto the appropriate media at a concentration of about 100 per plate. After 6 days of incubation, growing colonies were isolated and their growth requirements were tested.

Mapping crosses. The following quadrifactorial mapping crosses were performed. Two double auxotrophs were crossed, each carrying one of two independently segregating marker mutations (*a*, *b*) and one of two mapped mutations (*x*, *y*). Basidiospores from such crosses were plated onto selective media which did not permit germination of basidiospores carrying one or both marker mutations and onto complete sorbose medium as a control. Selective plating removed parental genotypes, and only 4 of 16 possible genotypes germinated (++++, ++*x*+, +++*y*, and ++*xy*). Expected frequencies of these genotypes in the progeny of the mapping cross recovered after selective plating depend on the actual linkage relationship between the four involved loci and can be expressed as functions of frequencies of recombination between these loci. If marker mutations *a* and *b* are unlinked, there are 14 possible linkage relationships and the expected frequencies mentioned above equal, as can easily be derived, four times the frequencies of the respective genotypes in the total progeny arising from meiotic events after karyogamy of unlike nuclei. Corresponding equations are given in Table 2. Only 6 of 14 possible linkage

TABLE 2. Expected frequencies of genotypes in the progeny of the quadrifactorial mapping cross recovered after selective plating

No. of linkage groups involved	Linkage relationships[a]	Expected class frequency in cross *a*+*x*+ × +*b*+*y* of the following genotypic class:				Expected class frequency in cross *a*++*y* × +*bx*+ of the following genotypic class:			
		++++	++*x*+	+++*y*	++*xy*	++*xy*	+++*y*	++*x*+	++++
4		0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
3		*r*/2	(1 − *r*)/2	(1 − *r*)/2	*r*/2	*r*/2	(1 − *r*)/2	(1 − *r*)/2	*r*/2
3		*r*/2	(1 − *r*)/2	*r*/2	(1 − *r*)/2	*r*/2	(1 − *r*)/2	*r*/2	(1 − *r*)/2
2		(1 − *r*) (1 − *s*)	*r*(1 − *s*)	*s*(1 − *r*)	*rs*	(1 − *r*) (1 − *s*)	*r*(1 − *s*)	*s*(1 − *r*)	*rs*
2		*rs*	(1 − *r*) (1 − *s*)	*r*(1 − *s*)	*s* (1 − *r*)	*rs*	(1 − *r*) (1 − *s*)	*r*(1 − *s*)	*s*(1 − r)
2		*r*(1 − *s*)	(1 − *r*) (1 − *s*)	*rs*	*s*(1 − *r*)	*r*(1 − *s*)	(1 − *r*) (1 − *s*)	*rs*	*s*(1 − *r*)

[a] *r* and *s* represent recombination frequencies; *a* and *b* are unlinked marker mutations; *x* and *y* are mapped mutations (see Materials and Methods).

TABLE 3. Test of independent segregation of mutations *met21-1* and *nic21-1*

Cross	No. of isolates in the following phenotypic class:	
	Prototroph	Nic$^-$ auxotroph
Met21Nic21 × Sr21	351	330
Met21 × Nic21Sr21	433	413

relationships are included in Table 2. All others can be obtained by exchanging the symbols *x* and *y* and/or the symbols *a* and *b* throughout Table 2.

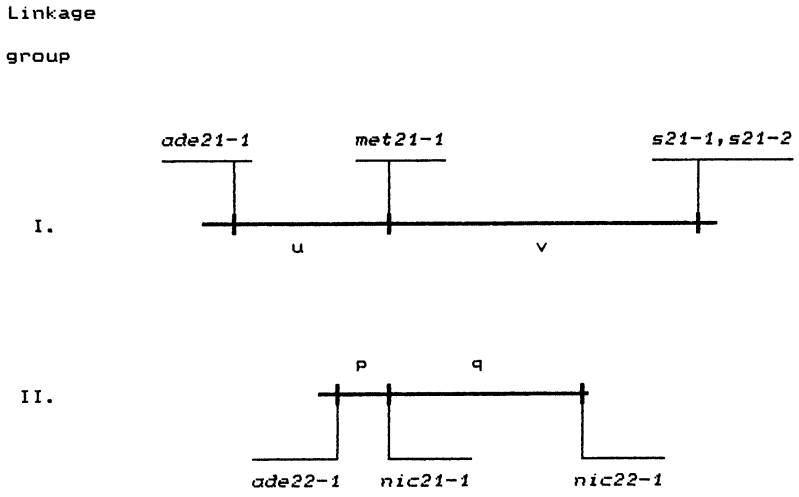
RESULTS AND DISCUSSION

Strain P-1. When strain 284B was fruited, basidiospore progeny fell into two distinct groups with respect to the growth rate. Approximately 50% of isolates grew very slowly (about 3 mm/day on complete medium); the other half of the progeny exhibited a normal growth rate (about 40 mm/day). Although the nature of slowly growing isolates was not studied in detail, this heterogeneity indicated that strain 284B is a heterokaryon. In fact, heterokaryosis in wild-type strains of *P. chrysosporium* appears to be of frequent occurrence (3). To avoid difficulties in further genetical analyses, a basidiospore isolate with a normal growth rate was taken as a wild type and named P-1. Basidiospore progeny of strain P-1 are homogeneous with respect to the growth rate and are fast growing. Four auxotrophic strains derived from strain 284B as well as strain 284B itself grew normally.

Complementation tests. Strains Met21Sr21 and Nic21Sr22 were forced to form a heterokaryon on minimal medium supplemented with thiosulfate. When the resulting heterokaryon was tested on minimal medium, no complementation occurred. The same result was obtained with strains Met21Sr22 and Nic21Sr21. Mutations in strains Sr21 and Sr22 are therefore allelic. Mutations in strains Nic21 and Nic22 and in strains Ade21 and Ade22 were shown to be complementary.

Independent segregation of mutations *met21-1* and *nic21-1*. In preliminary experiments, the relative frequency of double auxotrophs from the cross Met21 × Nic21 was nearly as high as the relative frequency of parental type Nic$^-$ (9). This led to the hypothesis that corresponding mutations segregate independently. To test this, we performed crosses Met21Nic21 × Sr21 and Met21 × Nic21Sr21. Basidiospores from these crosses were plated onto minimal sorbose medium supplemented with nicotinic acid (10 mg/liter) to remove meiotic products arising from selfing. Results of these crosses are given in Table 3.

Assuming independent segregation in both pairs *met21-1 nic21-1* and *s21-1 nic21-1*, the ratio of prototrophs to Nic$^-$ isolates in the progeny of both crosses should not differ significantly from 1:1. The corresponding χ^2 test with 2 degrees of freedom equals 1.12, which is insignificant. Using the law of noncentral chi-square distribution, it can be found



Recombination frequency	Estimated value	Standard deviation of the estimate
u	0.0837	0.01043
v	0.1573	0.00969
p	0.0112	0.00334
q	0.1016	0.01299

FIG. 1. Linkage map.

TABLE 4. Results of mapping crosses

Phenotypic class	No. of isolates	Expected class frequency[a]	Theoretical no. of isolates	Total
Cross 1 (Met21Sr21 × Nic21Ade21)				
Prototroph	16	$u(1 - v)$	18.124	257
Sr^-	1	uv	3.384	
Ade^-	201	$(1 - u)(1 - v)$	198.441	
Sr^-Ade^-	39	$v(1 - u)$	37.051	
Cross 2 (Met21Ade21 × Nic21Sr21)				
Prototroph	62	$v(1 - u)$	45.701	317
Sr^-	226	$(1 - u)(1 - v)$	244.770	
Ade^-	4	uv	4.174	
Sr^-Ade^-	25	$u(1 - v)$	22.365	
Cross 3 (Met21Sr22 × Nic21Ade21)				
Prototroph	13	$u(1 - v)$	9.238	131
Sr^-	0	uv	1.725	
Ade^-	102	$(1 - u)(1 - v)$	101.151	
Sr^-Ade^-	16	$v(1 - u)$	18.886	
Cross 4 (Met21Sr22 × Nic21Ade22)				
Prototroph	431	$(1 - p)(1 - v)$	416.629	500
Sr^-	64	$v(1 - p)$	77.789	
Ade^-	3	$p(1 - v)$	4.704	
Sr^-Ade^-	2	pv	0.878	
Cross 5 (Met21Ade22 × Nic21Sr22)				
Prototroph	1	pv	0.362	206
Sr^-	1	$p(1 - v)$	1.938	
Ade^-	33	$v(1 - p)$	32.049	
$Sr^- Ade^-$	171	$(1 - v)(1 - p)$	171.651	
Cross 6 (Met21Nic21 × Ade22Nic22)				
Prototroph	0	pq	0.151	133
$Nic21^-$	118	$(1 - p)(1 - q)$	118.155	
$Nic22^-$	2	$p(1 - q)$	1.334	
$Nic21^- Nic22^-$	13	$q(1 - p)$	13.360	
Cross 7 (Met21Nic22 × Ade22Nic21)				
Prototroph	20	$q(1 - p)$	13.762	137
$Nic21^-$	2	$p(1 - q)$	1.374	
$Nic22^-$	115	$(1 - p)(1 - q)$	121.709	
$Nic21^- Nic22^-$	0	pq	0.155	
Cross 6a (Met21Nic21 × Ade22Nic22)				
Prototroph	0	pq	0.172	152
Nic^-	152	$1 - pq$	151.828	
Cross 7a (Met21Nic22 × Ade22Nic21)				
Prototroph	22	$q(1 - p)$	27.423	273
Nic^-	251	$1 - q + pq$	245.577	

[a] Expected class frequencies are functions of recombination frequencies shown in Fig. 1.

that in experiments of such size (1,527 isolates), the critical value of the 5% level test would be exceeded with more than 95% probability if either distance in pairs *met21-1 nic21-1* and *s21-1 nic21-1* is less than 45 centimorgans. The only disturbing situation is the arrangement *met21-1 nic21-1 s21-1* in one linkage group, with both recombination frequencies being equal. Under this condition, the ratio of prototrophs to Nic^- auxotrophs would be 1:1 also. This, however, is excluded due to the results of crosses 1 and 2 (see Table 4); with this arrangement, the number of Sr^- isolates in cross 1 should not be lower than the number of prototrophs. Similarly, the number of Ade^- isolates in cross 2 should not be lower than the number of double-mutant strains.

Mapping crosses. Seven quadrifactorial mapping crosses were performed. Results of these are given in Table 4. Corresponding plating conditions are shown in Table 5. The number of colonies on the selective plating media varied from 5 to 25% of control plating. Final density of the colonies on selective media varied from 4 to 20 per plate. For each cross, with the exception of cross 3 (which is essentially identical to cross 1 due to the allelism of mutations *s21-1* and *s21-2*), phase permutation was performed, i.e., both crosses $a+x+ \times +b+y$ and $a++y \times +bx+$ were analyzed. From Table 2 it follows that expected frequencies are then exchanged within pairs of genotypic classes $++$, xy and $x+$, $+y$, irrespective of the actual linkage relationship. This fact was used in a partial test of equal viability of genotypes by means of the chi-square homogeneity test. Results of these

TABLE 5. Plating conditions of mapping crosses

Crosses	Supplement(s) in the selective plating medium (mg/liter)	Marker mutations
1, 2, 3	Adenine (10), $Na_2S_2O_3 \cdot 5H_2O$ (100)	*met21-1*, *nic21-1*
4, 5	Adenine (2.5), histidine (50), $Na_2S_2O_3 \cdot 5H_2O$ (100)	*met21-1*, *nic21-1*
6, 7, 6a, 7a	Nicotinic acid (5)	*met21-1*, *ade22-1*

tests are presented in Table 6. In crosses 6 and 7, three nicotinic acid-dependent genotypes were distinguished by the complementation test with strains Nic21 and Nic22. Because this procedure is laborious, another set of basidiospore progeny was classified only into two groups (prototroph and Nic$^-$ auxotroph). Corresponding results are presented as crosses 6a and 7a, respectively.

The number of linkage groups involved and the ordering of mutations can be deduced by using the model presented in Table 2. For example, in cross 1 the ratio of four selected genotypes is clearly distinct from 1:1:1:1, which eliminates the linkage relationship shown in the first line of Table 2 (four unlinked loci). Moreover, four classes in cross 1 cannot be divided into two groups each consisting of two classes with approximately equal frequencies, which would suggest the presence of three linkage groups. We thus inferred that there are only two linkage groups involved. If we consider mutations *nic21-1*, *met21-1*, *ade21-1*, and *s21-1* to be the mutations *a*, *b*, *x*, and *y*, respectively, then cross 1 is the cross $a+x+ \times +b+y$. The most frequent class in the progeny is class $++x+$ (Ade$^-$ isolates), and the least frequent one is class $+++y$ (Sr$^-$ isolates). This situation corresponds to the last linkage relationship shown in Table 2, and we thus inferred that loci *ade21*, *met21*, and *s21* are linked and that locus *met21* is located between the other two.

Recombination frequencies u, v, p, and q (Fig. 1) were estimated by the maximum likelihood method (15) while taking into account allelism of mutations *s21-1* and *s21-2*. The variation matrix of these estimates was obtained by the inversion of the corresponding Fisher information matrix. Due to the grouping of Nic$^-$ classes in crosses 6a and 7a, estimates of recombination frequencies p and q are mutually dependent, their correlation coefficient being equal to 6.56×10^{-3}. The other pairs of estimates are independent.

The corresponding linkage map is presented in Fig. 1. The suitability of the whole model can be tested by the chi-square test, which in this case has 19 degrees of freedom (32 groups minus 9 crosses minus 4 estimated parameters). The value obtained is 25.4 ($P = 0.15$).

The model presented in Table 2 is based on the following three assumptions. (i) Marker mutations are unlinked. The approach used in this work does not give a strict proof that marker mutations *met21-1* and *nic21-1* are located on different chromosomes. More direct evidence can be obtained, for example, by using tetrad analysis or analysis based on mitosis. At present, relevant techniques are not developed for *P. chrysosporium*.

(ii) The viability of selected genotypes in one cross is equal under selective plating conditions. This assumption is usually tested by means of control platings. There are, however, strains which produce neither conidiospores nor fruit. In some cases, it could be difficult to obtain a double-mutant strain. If, however, phase permutation is performed, it is possible to test equal viability in a pair consisting of a wild type and a double mutant as well as in a pair consisting of $x+$ and $+y$ mutant strains by using the chi-square homogeneity test. In our experiments, results of mapping crosses and their phase permutations were found to be homogeneous; we therefore consider the viability of prototrophs and double auxotrophs as well as of both types of single auxotrophs equal in each cross. In addition, we compared the viability of basidiospores of strains Sr21, Sr22, and P-1 under plating conditions of cross 1, basidiospores of strains Sr22 and P-1 under plating conditions of cross 4, and basidiospores of strains Nic21 and P-1 under plating conditions of cross 6. We did not detect differences in viability in any of these comparisons. Obtained phenotypic ratios therefore reflect linkage rather than differential viability of genotypes.

(iii) All recombinations are independent events. This condition would not be satisfied if both mapped loci were closely linked to one of the two marker loci and interference occurred. This would be demonstrated by rejection of model adequacy in the final chi-square test for the corresponding cross. If this takes place, an additional parameter of coefficient of coincidence should be included in the model.

As far as we know, the only other reports dealing with genetic mapping in *P. chrysosporium* were presented by Raeder and Broda (12) and Raeder et al. (13, 14). Their approach depends on the presence of interallelic restriction site polymorphism. Using a set of 38 cloned sequences as probes and DNA preparations from 53 basidiospore isolates of heterokaryotic strain ME446, they demonstrated a high percentage of segregational congruence between some pairs of cloned sequences exhibiting this polymorphism. Interestingly, there was about 50% congruence between some pairs of sequences, which indicates that selfing did not influence the results significantly.

TABLE 6. Tests of homogeneity of mapping crosses

Crosses	χ^2	df	P
1, 2, 3	8.834	6	0.183
4, 5	1.334	3	0.721
6, 7	1.465	2[a]	0.481
Total	11.633	11	0.392

[a] A zero column is excluded from the corresponding contingency table.

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