Genetic Recombination in the Lignin-Degrading Basidiomycete

*Phanerochaete chrysosporium*

MARGARET ALIC AND MICHAEL H. GOLD*  

Department of Chemical, Biological, and Environmental Sciences, Oregon Graduate Center, Beaverton, Oregon 97006-1999

Received 19 November 1984/Accepted 12 April 1985

Heterokaryons made from auxotrophic strains of the lignin-degrading basidiomycete *Phanerochaete chrysosporium* were induced to fruit. The isolation of wild-type and double-mutant phenotypes from these crosses indicated that genetic recombination had occurred. Cytological studies demonstrated that more than 90% of the basidiospores from the wild-type and auxotrophic strains and from forced heterokaryons were binucleate. Colonies of the wild-type strain of *P. chrysosporium* arising from single, predominantly uninucleate conidia were all capable of producing fruiting bodies and basidiospores.

The basidiomycete *Phanerochaete chrysosporium* and other white-rot fungi have potential applications in a variety of schemes for the industrial processing of lignocellulose. These organisms have been already used in numerous studies concerned with lignin degradation (3), cellulose metabolism (4), and lignocellulose bioprocessing (13). The recent isolation of extracellular enzymes involved in lignin degradation (6, 11, 14, 22, 23) has increased our understanding of the biochemistry of this process. The development of genetic methods for producing strains with enhanced lignin-degrading capacities would be a significant step toward the utilization of these organisms for lignocellulose bioprocessing.

In earlier studies we described methods for inducing colonial growth and for replica plating with *P. chrysosporium* (7). We also determined the physiological conditions required for fruit body formation (8). Subsequently we described methods for the mutagenesis of *P. chrysosporium* conidia and for the isolation of auxotrophic marker strains (10). Most recently we reported on the preparation, regeneration, and fusion of *P. chrysosporium* protoplasts (9).

In this report we present cytological studies of basidiospore nuclei and recombination analysis of crosses between auxotrophic strains of this organism. Marker strains carrying multiple mutations can be readily recovered by fruiting heterokaryons of *P. chrysosporium*.

MATERIALS AND METHODS

Organism. Cultures of *P. chrysosporium* ME446 were maintained on slants of modified Vogel medium (19) supplemented with 3% malt extract and 0.15% yeast extract (7). Mutant strains were maintained on the same medium supplemented with a vitamin mixture (21) and 0.5% tryptone. Heterokaryons were forced on minimal medium consisting of modified Vogel medium supplemented with 1% glucose (10).

Fruit bodies were produced at 28°C as previously described (8), with a sixfold dilution of modified Vogel medium and 4.5% Walseth cellulose as the carbon source. For auxotrophic strains, the appropriate supplement was added to the fruiting medium (0.01 to 0.1% amino acids or 0.001% vitamins or adenine) (10). The basidiospores, which collected on the lids of inverted plates, were suspended in distilled water (8).

Wild-type fruiting. The wild-type strain was grown on MGPT slants (2% malt extract with 2% glucose, 0.1% Bacto-Peptone (Difco), 0.001% thiamine, and 1.5% agar) as previously described (10). Conidia were washed from the slants, diluted to 10<sup>6</sup> spores per ml, and sonicated for 3 min at approximately 60 W in a model W 185 F cell disruptor (Heat Systems-Ultrasonics) to unclump the conidia. The conidia were then plated on solid medium (50 to 100 spores per plate), and resultant individual colonies were transferred to fruiting medium.

Germination of basidiospores and conidia. Basidiospores were routinely plated in two types of solid media. Medium A consisted of 10% sorbose, 1% yeast nitrogen base, and 3% agar (pH 4.8) (9). Medium B consisted of 2% glucose, 0.1% K<sub>2</sub>HPO<sub>4</sub>, 0.05% KH<sub>2</sub>P<sub>2</sub>O<sub>7</sub>, 0.2% Bacto-Peptone, and 0.05% MgSO<sub>4</sub> (20) and was supplemented with 0.2% yeast extract. Agar was added to 1.5%, and the pH was adjusted to 5.8. Conidia were routinely plated on modified Vogel medium (19) with 10% sorbose and 2% agar (pH 4.8). The above media were supplemented with 0.01% amino acids, 0.001% vitamins, or adenine as appropriate.

Nuclear staining. Basidiospore nuclei from wild-type and auxotrophic strains and from heterokaryons were stained and counted as previously described for conidial nuclei (10).

Genetic analysis of recombination. Colonies derived from basidiospores or conidia and plated in or on supplemented medium were transferred to minimal and supplemented slants to determine phenotypes (10).

RESULTS

Wild-type fruiting. Conidia from MGPT slants, which have previously been shown to be about 62% uninucleate (10), were sonicated. Sonication of a conidial spore suspension, under the conditions used, resulted in the separation of all clumps of conidia into individual spores. The germination frequency of these sonicated spores equaled that of unsonicated conidia, indicating that the procedure did not markedly affect viability. All of the colonies thus obtained from single, predominantly uninucleate, wild-type conidia produced fruit bodies and basidiospores on fruiting medium. About the same number of basidiospores were released (approximately 10<sup>6</sup> spores per plate [60 by 15 mm]), regardless of whether the inoculum consisted of mycelial fragments or a colony.

* Corresponding author.
arising from a single spore. Of these basidiospores, 20 to
40% germinated when plated in medium B. However, only
about 50% of colonies obtained from individual basidi-
spores produced fruit bodies when transferred to fruiting
medium. Of these, 74% released detectable numbers of
basidiospores.

Fruiting by auxotrophic strains. Under the conditions
used, 70% of the auxotrophic strains tested produced
basidiospores. However, with certain of the amino acid
auxotrophs, 10 times the usual amount of supplement
was required for fruiting. Several mutant strains produced fruit-
ing bodies but did not release detectable quantities of
basidiospores. Most auxotrophs produced between 10 and
100 times fewer basidiospores per plate as compared with
the wild type.

Basidiospore germination. Germination frequencies of 15
to 40% were obtained with basidiospores from wild-type and
auxotrophic strains plated in medium B. However, since
medium B does not induce colony formation, sorbose-
containing medium was used for recombination analysis.
The highest germination with a colony-inducing medium (5
to 15%) was obtained when basidiospores were plated in
medium A; this medium was therefore routinely used for
recombination analysis. The addition of supplements, in-
cluding yeast extract (0.01%), did not significantly increase
the germination frequency in medium A.

Nuclei per basidiospore. More than 90% of the basidi-
spores obtained from the wild-type and auxotrophic strains,
as well as those obtained from crosses between auxotrophs,
were uninucleate (Table 1). Some spores scored as uninu-
cleate may have been poorly stained or positioned in such a
way that only one nucleus was visible. It is most probable,
however, that a small percentage of basidiospores are
uninucleate. The Nic1 x His2 cross produced as many as 8%
uninucleate spores.

Recombination analysis. The results of a variety of crosses
between auxotrophic strains are shown in Table 2. Of 12
crosses, 11 resulted in the recovery of significant numbers of
double-mutant basidiospores, indicating that recombination
had indeed taken place. Basidiospores from a Nic1 x Rib1
cross were plated at concentrations of five spores per plate
(15 by 100 mm), thus ensuring that each resulting colony
arose from a single spore. The distribution of phenotypes
obtained in this way did not differ significantly from the
distribution obtained when spores were plated at substi-

dently higher concentrations, indicating that the presence of
ungerminated spores was not affecting the results. The
conidia from heterokaryons did not yield double-mutant
recombinants (Table 3), indicating that the double mutants
isolated from basidiospores did not arise through parasexual
recombination (5, 15).

DISCUSSION

In a previous report (8), we elucidated the physiological
conditions required for fruit body formation and subsequent
basidiospore production in P. chrysosporium. In a sub-
sequent study (10), we described the isolation and comple-
tation of auxotrophic mutants in this organism. In this
report, we describe the use of these mutants and the technol-
ogy for fruit body production for the study of genetic
recombination in P. chrysosporium.

Our cytological results demonstrate that the basidiospores
of P. chrysosporium are uninucleate (Table 1). Genetic re-
combination accompanies fruit body and basidiospore for-
nation (Table 2). The conidia of heterokaryons made from
two of our auxotrophic strains did not yield double-mutant

<table>
<thead>
<tr>
<th>Cross</th>
<th>Phenotypes and no. of colonies derived from basidiospores</th>
<th>No. of colonies examined</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prototrophs</td>
<td>Auxotrophs (parental types)</td>
</tr>
<tr>
<td>Ade1 x Rib1</td>
<td>26</td>
<td>19 Ade, 16 Rib</td>
</tr>
<tr>
<td>Nic1 x Rib1</td>
<td>19</td>
<td>18 Nic, 19 Rib</td>
</tr>
<tr>
<td>Nic1 x Rib1b</td>
<td>15</td>
<td>8 Nic, 5 Rib</td>
</tr>
<tr>
<td>Ade1 x Nic1</td>
<td>12</td>
<td>20 Ade, 31 Nic</td>
</tr>
<tr>
<td>Met1 x Arg1</td>
<td>21</td>
<td>10 Met, 34 Arg</td>
</tr>
<tr>
<td>Met1 x Ade1</td>
<td>25</td>
<td>18 Met, 21 Ade</td>
</tr>
<tr>
<td>Nic1 x His2</td>
<td>15</td>
<td>40 Nic, 9 His</td>
</tr>
<tr>
<td>Met1 x Ade2</td>
<td>19</td>
<td>14 Met, 21 Ade</td>
</tr>
<tr>
<td>Ade3 x Arg1</td>
<td>13</td>
<td>10 Ade, 42 Arg</td>
</tr>
<tr>
<td>Ade3 x His2</td>
<td>23</td>
<td>11 Ade, 17 His</td>
</tr>
<tr>
<td>Met1 x His1</td>
<td>18</td>
<td>19 Met, 19 His</td>
</tr>
<tr>
<td>Arg3 x Rib1</td>
<td>14</td>
<td>5 Arg, 9 Rib</td>
</tr>
<tr>
<td>His2 x Arg1</td>
<td>41</td>
<td>30 His, 63 Arg</td>
</tr>
</tbody>
</table>

* Parental strains were fruiting in pairs as described in the text. The phenotypes of resultant basidiospores were analyzed as described in the text. Abbreviations refer to the phenotype of the auxotroph (10).

* Single spores were isolated before germination.

<table>
<thead>
<tr>
<th>Heterokaryon</th>
<th>Wild-type recombinants + heterokaryons</th>
<th>Auxotrophs (parental types)</th>
<th>Double auxotrophs (recombinants)</th>
<th>No. of colonies examined</th>
</tr>
</thead>
<tbody>
<tr>
<td>Met1 + Ade2</td>
<td>Basidiospores 19</td>
<td>14 Met, 21 Ade</td>
<td>18</td>
<td>72</td>
</tr>
<tr>
<td>Conidia</td>
<td>58</td>
<td>2 Met, 8 Ade</td>
<td>0</td>
<td>68</td>
</tr>
<tr>
<td>Nic1 + Rib1</td>
<td>Basidiospores 19</td>
<td>18 Nic, 19 Rib</td>
<td>16</td>
<td>72</td>
</tr>
<tr>
<td>Conidia</td>
<td>36</td>
<td>0 Nic, 0 Rib</td>
<td>0</td>
<td>36</td>
</tr>
</tbody>
</table>

* Heterokaryons were forced and basidiospores and conidia were produced, collected, and analyzed for phenotype as described in the text.
Aspergillus nidulans (15). The early observation that P. chrysosporium does not produce clamp connections (1) suggests that this organism is homothallic. Although the absence of clamp connections was confirmed in the present study, some heterothallic basidiomycetes do not produce clamp connections or do so only under particular conditions (24). That more than 200 isolates of unclumped and predominantly uninucleate wild-type basidiospores are homokaryotic and that the two heterothallism, even in a homothallic organism. Results with auxotroph arose from a single viable nucleus. The remaining mutant recombinants, and prototrophs. At least four of the existence of homothallism in this organism is further supported by the fact that 70% of the auxotrophic strains produce basidiospores, since it is almost certain that each auxotroph arose from a single viable nucleus. The remaining auxotrophs may be prevented from fruiting by a physiological block(s). Further experiments are currently under way to determine whether the infertility exhibited by more than half of the colonies arising from individual basidiospores is due to a mutation(s) or some physiological or environmental factor.

The recombination analyses shown in Table 2 vary somewhat among the crosses between different auxotrophs. However, every cross produced single auxotrophs, double-mutant recombinants, and prototrophs. At least four of the crosses resulted in progeny with phenotypes approaching a ratio of 1:1:1:1. Although homothallism might be expected to give rise to significantly more parental types (single auxotrophs) than wild-type or double-mutant recombinants, there are several possible explanations for the observed ratios. Although preferential fusion of genetically dissimilar nuclei has been described (2), it is more likely that karyogamy is random but that heterokaryotic hyphae have a great advantage in a nutritionally forced heterokaryon. The nuclei of complementary auxotrophs would thus be in close proximity and more likely to fuse and recombine during basidiospore formation. As a result, recombination analysis of nutritionally forced heterokaryons would tend to mimic heterothallism, even in a homothallic organism. Results with conidia support this hypothesis: conidia from heterokaryons are predominately (>90%) binucleate (10), and >80% of the conidia from heterokaryons were themselves heterokaryotic (Table 3), suggesting that the hyphae from which the conidia arose contain nuclei of complementary genotypes in close proximity. Similar results have been found in the self-fertile Aspergillus nidulans (15).

The ratio of recombinants to parental types of approximately 1:1:1:1 also suggests that the binucleate basidiospores are homokaryotic and that the two nuclei arise from a postmeiotic mitotic event. This is a common occurrence among basidiomycetes (16, 17). Furthermore, the existence of postmeiotic mitotic division has been confirmed in P. chrysosporium by electron microscopy (E. C. Settliff, personal communication).

Several of the crosses shown in Table 2 show a preponderance of wild-type over double-mutant recombinants, probably owing to contamination by heterokaryotic conidia. It is also possible that particular phenotypes were being selected for, either at the level of basidiospore formation or during germination in supplemented media. Additional cytological and genetic studies are being planned to further elucidate the morphogenesis of fruit body and basidiospore formation as well as the mating system of P. chrysosporium.

The use of genetic recombination for obtaining multiple-mutant strains should have application to the construction of complex strains with superior capacities for lignocellulose bioprocessing. In this regard, the construction of mutant strains containing markers for both auxotrophy and ligninolytic defects (12) will be of particular interest.

ACKNOWLEDGMENTS

This work was supported by grant DMB 8311441 from the National Science Foundation.

LITERATURE CITED


