Characterization of Leucine Auxotrophs of the White Rot Basidiomycete *Phanerochaete chrysosporium*

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Six leucine auxotrophic strains of the white rot basidiomycete *Phanerochaete chrysosporium* were characterized genetically and biochemically. Complementation studies involving the use of heterokaryons identified three leucine complementation groups. Since all of the leucine auxotrophs grew on minimal medium supplemented with α-ketoisocaproate as well as with leucine, the transaminase catalyzing the last step in the leucine pathway was apparently normal in all strains. Therefore, the wild-type, auxotrophic, and several heterokaryotic strains were assayed for the activities of the other enzymes specific to leucine biosynthesis. Leu2 and Leu4 strains (complementation group I) lacked only α-isopropylmalate synthase activity; Leu3 and Leu6 strains (group III) lacked isopropylmalate isomerase activity; and Leu1 and Leu5 strains (group II) lacked β-isopropylmalate dehydrogenase. Heterokaryons formed from leucine auxotrophs of different complementation groups had levels of activity for all three enzymes similar to those found in the wild-type strain.

The basidiomycete *Phanerochaete chrysosporium* and other white rot fungi have potential applications in a variety of schemes for the bioprocessing of lignocellulose. Numerous studies have focused on lignin degradation (3) and cellulose metabolism (4) in *P. chrysosporium*, as well as on the bioprocessing of lignocellulose (13). The purification and characterization of two extracellular enzymes involved in lignin degradation (6, 11, 17, 24) have increased our understanding of the biochemistry of this process. The elucidation of the genetic system in this fungus and comparison with analogous systems that have been thoroughly studied in other organisms could lead to the development of genetic methods for producing strains with enhanced lignin-degrading capacities. Such methods would represent a significant step toward the utilization of this organism in bioprocessing applications.

In earlier studies we described methods for inducing colonial growth and for replica plating with *P. chrysosporium* (7) and determined the physiological conditions required for fruiting body formation (8). Subsequently we described the mutagenesis of conidia, the isolation of auxotrophic marker strains, and complementation studies with these various auxotrophs (10). We also reported on the preparation, fusion, and regeneration of *P. chrysosporium* protoplasts (9) from auxotrophic strains. Most recently, we reported on the use of these auxotrophs for studies of genetic recombination (1).

The enzymes and the gene loci and regulation involved in the biosynthesis of leucine have been studied in detail in several procaryotic and eucaryotic organisms, including *Escherichia coli* (23), *Neurospora crassa* (2, 12), and *Saccharomyces cerevisiae* (14, 19, 25). In this report we present the results of biochemical genetic studies on various enzymes involved in the biosynthesis of leucine in *P. chrysosporium*. Our wild-type strain, the five leucine auxotrophs described previously (10), and a newly isolated leucine auxotroph were used in the studies. Heterokaryons made from leucine auxotrophs of different complementation groups were also used. We demonstrate that our six *P. chrysosporium* leucine auxotrophs correspond to mutations in three different genes, each leading to a deficiency of one of the three enzymes specific to leucine biosynthesis. These mutations are analogous to those identified in other microorganisms (2, 12, 14, 19, 25).

**MATERIALS AND METHODS**

**Organism and growth media.** Cultures of *P. chrysosporium* ME446 were maintained on slants of Vogel medium N (26), with thiamine replacing biotin (modified Vogel medium) (21), supplemented with 2% glucose. Auxotrophic strains were maintained on slants of double-strength modified Vogel medium supplemented with 3% malt extract, 0.5% tryptone, 0.5% yeast extract, and a vitamin mixture (22). Heterokaryons were forced on minimal medium consisting of modified Vogel medium supplemented with 1% glucose (10).

Fresh cultures for dry-weight determination were grown in liquid modified Vogel medium containing 2% glucose, with or without 3 mM leucine. Fresh cultures for crude cell extracts were grown in the above medium supplemented with 1.5 mM leucine for growth of the auxotrophic strains. The auxotrophs were also grown in the above medium supplemented with 1.5 mM α-ketoisocaproate.

**Chemicals.** β-Isopropylmalate (β-IPM) for the isomerase and dehydrogenase assays was kindly provided by G. B. Kohlhafl, Purdue University. All other chemicals were purchased from Sigma Chemical Co.

**Isolation of the leucine auxotroph.** In addition to the five previously isolated leucine auxotrophs, a newly isolated strain (Leu6) was obtained through X-ray mutagenesis and tested for complementation with the other leucine auxotrophs as previously described (10).

**Dry-weight determinations.** Spore suspensions (0.25 ml of suspensions of approximately 10⁶ spores per ml) were inoculated into 250-ml flasks containing 20 ml of liquid medium as described above. Stationary cultures were incubated at 37°C. After 3 days, the mycelial mats were harvested by
TABLE 1. Growth of wild-type, leucine auxotrophic, and heterokaryotic strains

<table>
<thead>
<tr>
<th>Strains</th>
<th>Dry wt (g) when grown in:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2% glucose + Vogel medium</td>
</tr>
<tr>
<td>Wild type</td>
<td>0.141</td>
</tr>
<tr>
<td>Leu1</td>
<td>—</td>
</tr>
<tr>
<td>Leu2</td>
<td>—</td>
</tr>
<tr>
<td>Leu3</td>
<td>—</td>
</tr>
<tr>
<td>Leu4</td>
<td>—</td>
</tr>
<tr>
<td>Leu5</td>
<td>—</td>
</tr>
<tr>
<td>Leu6</td>
<td>—</td>
</tr>
<tr>
<td>Leu1 + Leu4</td>
<td>0.106</td>
</tr>
<tr>
<td>Leu3 + Leu6</td>
<td>0.130</td>
</tr>
<tr>
<td>Leu2 + Leu3</td>
<td>0.107</td>
</tr>
</tbody>
</table>

* Cultures were grown, broken, and extracted, and enzymes were assayed as described in the text.
* Average of two cultures.
* —, Negligible.

These six auxotrophs form three complementation groups consisting of (i) Leu1 and Leu5; (ii) Leu2 and Leu4; and (iii) Leu3 and Leu6.

The dry-weight determinations for 3-day-old stationary cultures of the *Phanerochaete chrysosporium* wild type, leucine auxotrophs, and forced heterokaryons of leucine auxotrophs are shown in Table 1. When medium lacking leucine was inoculated with conidia from the leucine auxotrophs, there was no detectable growth. When the leucine auxotrophs were grown in media containing 3 mM leucine, their dry weights were similar to the dry weights of the wild type and the heterokaryons. All leucine auxotrophs grew well when the medium was supplemented with α-ketoisocaproate rather than leucine.

The specific activities of the three leucine biosynthetic enzymes in each of the strains are shown in Table 2. The crude extract of Leu2 lacked detectable α-IPM synthase activity, while that of Leu4 exhibited low levels of synthase activity in one assay and no detectable activity in a second assay. The crude extracts of Leu3 and Leu6 lacked detectable isomerase activity. The extracts of Leu1 and Leu5 lacked detectable β-IPM dehydrogenase activity. In contrast, all three enzyme activities were present in the wild type and in the heterokaryotic strains.

In several cases the isomerase and dehydrogenase activities of the auxotrophs were significantly higher than in the wild-type strain (Table 2). For example, the specific activity of the isomerase in Leu1 and Leu5 were 10.4- and 9.5-fold higher, respectively, than those of the wild-type strain. In Leu2 and Leu4, the isomerase specific activity was approximately threefold higher. Likewise, the dehydrogenase activities of Leu2 and Leu4 were 4.0- and 3.4-fold higher, respectively, than those of the wild type.

Each of the heterokaryons exhibited all three enzyme activities. In the case of the Leu5-Leu6 heterokaryon, each component had α-IPM synthase activity when grown alone, and synthase activity in the heterokaryon was approximately equal to that in the wild type. However, with heterokaryons in which one component lacked synthase activity, the heterokaryotic strain exhibited higher synthase activity than did the wild type. All three heterokaryons exhibited slightly higher IPM isomerase activity than did the wild type, but in each case the values were lower than the enzyme activity found in the individual auxotrophs that possessed IPM synthase activity.

TABLE 2. Specific activity of leucine biosynthetic enzymes in various strains of *Phanerochaete chrysosporium*.

<table>
<thead>
<tr>
<th>Strains</th>
<th>α-IPM synthase (10³ U/mg of protein)</th>
<th>β-IPM dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>0.50</td>
<td>17.5</td>
</tr>
<tr>
<td>Leu1</td>
<td>0.61</td>
<td>182.0</td>
</tr>
<tr>
<td>Leu2</td>
<td>&lt;0.001</td>
<td>51.0</td>
</tr>
<tr>
<td>Leu3</td>
<td>1.12</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Leu4</td>
<td>0.16, &lt;0.001</td>
<td>63.7</td>
</tr>
<tr>
<td>Leu5</td>
<td>0.62</td>
<td>166.5</td>
</tr>
<tr>
<td>Leu6</td>
<td>1.07</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Leu1 + Leu4</td>
<td>1.22</td>
<td>48.9</td>
</tr>
<tr>
<td>Leu5 + Leu6</td>
<td>0.40</td>
<td>50.7</td>
</tr>
<tr>
<td>Leu2 + Leu3</td>
<td>0.79</td>
<td>25.5</td>
</tr>
</tbody>
</table>

* Cells were grown, broken, and extracted, and enzymes were assayed as described in the text.
* 10⁴ micromoles per minute per milligram of protein or nanomoles per minute per milligram of protein.
* Values from two separate experiments.

suction filtration, dried at 45°C for 48 h in tared dishes, and weighed.

**Preparation of crude cell extracts.** Stationary cultures were inoculated as described above and incubated at 37°C for 3 to 4 days. The mycelial mats from two such cultures were homogenized in a Waring blender, inoculated into a 2-liter flask containing 1 liter of medium, and incubated at 28°C at 150 rpm on a rotary shaker for 3 days. The mycelia were then harvested, washed by suction filtration, and ground with sand in approximately 25 ml of cold 50 mM Tris hydrochloride (pH 7.1) in a chilled mortar. The resultant suspension was centrifuged at 11,000 × g (4°C), and the supernatant was assayed for enzyme activity.

**Enzyme assays.** α-Isopropylmalate (α-IPM) synthase activity was determined by the method of Ulm et al. (25), with 5,5'-dithiobis-(2-nitrobenzoate) to measure the liberation of coenzyme A from acetyl coenzyme A. Reactions were run for 20 min at 30°C and stopped with 95% ethanol. A₄₁₂ was measured with a Shimadzu UV-260 spectrophotometer; cuvettes with a 1-cm light path were used. The value was corrected with a blank lacking the substrate α-ketoisovalerate.

β-IPM isomerase activity was determined by monitoring the rate of formation of dimethylcitrate from β-IPM at 234 nm by the method of Gross (12).

β-IPM dehydrogenase activity was determined by monitoring the reduction of NAD at 340 nm in the presence of β-IPM (16). The initial rate of NAD reduction was determined by using a reaction mixture containing 100 mM potassium phosphate (pH 8.0), 0.7 mM MgCl₂, 66 mM KCl, 1.1 mM NAD, 4.2 mM pyrazole, and 30 µl of cell extract. β-IPM was then added to a final concentration of 1 mM in a total volume of 0.2 ml, and the new rate of NAD reduction was measured.

Protein concentration in the crude extracts was determined by the method of Lowry et al. (15), with bovine serum albumin as the standard.

**RESULTS**

In an earlier report we determined the complementation groups for our five leucine auxotrophs (10). The newly isolated leucine auxotroph (Leu6; OGC 1121-3) formed a heterokaryon on minimal medium with all other leucine auxotrophs except Leu3. Taken with our previous results (10), which we recently confirmed, these results show that the...
isomerase. The levels of α-IPM dehydrogenase activity in all three heterokaryons were similar to that of the wild type.

**DISCUSSION**

The leucine biosynthetic pathway has been elucidated in *E. coli*, *S. cerevisiae*, and *N. crassa* (2, 12, 14, 19, 23, 25) (Table 3). The steps in this pathway are catalyzed by the specific enzymes α-IPM synthase, IPM isomerase, and β-IPM dehydrogenase (Fig. 1). No mutants completely blocked in the final step of the pathway have been isolated. Therefore, it has been inferred that more than one enzyme can catalyze the transamination of α-ketoisocaprate to leucine (20). We showed previously (10) that our *P. chrysosporium* leucine auxotrophs form three different complementation groups: group I (Leu2 and Leu4), group II (Leu1 and Leu5), and group III (Leu3). Complementation studies with our newly isolated leucine auxotroph, Leu6, indicate that it belongs with Leu3 in group III.

Although in the absence of leucine, none of the leucine auxotrophs grew in liquid culture, in the presence of leucine they grew approximately as well as the wild type and heterokaryons made from leucine auxotrophs. The poor growth exhibited by Leu1 may be due to poor uptake of leucine or inhibition of growth by leucine in the medium (Table 1). Since all of the auxotrophs grew well in liquid culture supplemented with α-ketoisocaprate, none of these strains are deficient in glutamate-α-ketoisocaprate transaminase, the final enzyme in the pathway (18). The results of assays for the other three enzymes in the pathway (Table 2) confirm our complementation studies with heterokaryons. Fungal mutations in a single complementation group probably reflect different lesions in the same gene (5). While it is not certain that each of these mutations is in a structural gene, this is the most probable explanation, since each mutation negatively affects only one of the enzymes.

Although Leu4 does not grow in liquid minimal medium, it does occasionally grow at a very slow rate on minimal slants and plates. The low level of α-IPM synthase activity detected in one assay (Table 2) suggests that Leu4 is leaky. In several instances, the specific activities of each of the other two leucine biosynthetic enzymes are considerably higher in the auxotrophs than in the wild type (Table 2). Similar results have been obtained with *N. crassa* (14). The high levels of IPM isomerase activity in Leu1 and Leu5 may reflect different levels of α-IPM. α-IPM induces the IPM isomerase in *N. crassa* (13). Although enzyme levels varied among the three groups of auxotrophs, those within a group exhibited remarkably similar levels of enzyme activity. This suggests that the high enzyme levels in the auxotrophs as compared with those in the wild type, as well as the variation in activities among the three groups, probably reflect regulation of the pathway.

Heterokaryons made from leucine auxotrophs in different complementation groups exhibited all three enzyme activities. The specific activity of IPM isomerase was higher in the heterokaryons than in the wild type, but lower than in the individual auxotrophs that possess the isomerase activity (Table 2).

The leucine genes in yeasts, *E. coli*, and *N. crassa* that are analogous to the leucine auxotrophs isolated from *P. chrysosporium* are shown in Table 3. The *P. chrysosporium* genes have been classified according to the nomenclature system for *Saccharomyces cerevisiae*. Thus, *P. chrysosporium* Leu2 and Leu4 lack α-IPM synthase, corresponding to the yeast leu3 gene; Leu3 and Leu6 lack IPM isomerase, corresponding to the yeast leu1 gene; and Leu1 and Leu5 lack β-IPM dehydrogenase, corresponding to the yeast leu2 gene. With this biochemical characterization of the leucine auxotrophs of *P. chrysosporium*, we are now prepared to utilize these mutants in additional studies designed to further elucidate the genetics and molecular genetics of this organism.

**TABLE 3.** Biochemical genetic characterization of the leucine pathway in *P. chrysosporium*, *S. cerevisiae*, *E. coli*, and *N. crassa*

<table>
<thead>
<tr>
<th>Organism</th>
<th>Mutant genes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. chrysosporium</em></td>
<td>leu3 (Leu2, Leu4)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td>leu3</td>
<td>14</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>leuA</td>
<td>23</td>
</tr>
<tr>
<td><em>N. crassa</em></td>
<td>leu4</td>
<td>12</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mutants were characterized as described in Materials and Methods.
<sup>b</sup> (α-IPM synthase).
<sup>c</sup> (IPM isomerase).
<sup>d</sup> (β-IPM dehydrogenase).

![FIG. 1. Leucine biosynthetic pathway in microorganisms (see reference 14).](http://aem.asm.org/)

**ACKNOWLEDGMENTS**

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**LITERATURE CITED**

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