Genetics and Physiology of Proline Utilization in *Saccharomyces cerevisiae*: Mutation Causing Constitutive Enzyme Expression

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A mutation resulting in inducer-independent expression of the proline-degradative enzymes was isolated in the yeast *Saccharomyces cerevisiae*. Strains carrying the mutation, *put3*, are partially constitutive for proline oxidase and Δ¹-pyrroline-5-carboxylate dehydrogenase when grown on a medium lacking proline and are hyperinducible for both enzyme activities when grown on a proline-containing medium. *put3* segregates as a single nuclear gene, is not linked to either of the presumed structural genes for proline oxidase and Δ¹-pyrroline-5-carboxylate dehydrogenase, and does not affect proline transport. When heterozygous in diploid strains, *put3* behaves neither fully dominant nor fully recessive. Endogenous induction by proline has been eliminated as a cause of the inducer-independent enzyme expression in the *put3* mutant and the mutation is believed to be in a regulatory component of the proline-degradative pathway.

In eucaryotic organisms, the genes coding for related functions are usually not clustered in operons as is typical in bacterial systems. The control of pathways with several enzymes encoded by spatially separated genes must, of necessity, involve trans-acting molecules, protein-protein regulatory interactions, or individual control regions for each isolated gene.

The proline utilization pathway in the yeast *Saccharomyces cerevisiae* is an example of a catabolic pathway involving two distinct enzymes whose structural genes are not closely linked on the yeast genome (2). We show in this paper that the two proline-degrading enzymes can be released from the wild-type control system by a mutation, *put3*, that results in inducer-independent enzyme expression.

MATERIALS AND METHODS

Strains. The strains of *S. cerevisiae* employed in this study are listed in Table 1. They are isogenic except for the specified genotype.

Media. The media used are described in an accompanying paper (2).

Mutagenesis. Cells of the wild-type strain MB1000 were spread on petri plates containing 2% glucose and 0.1% glutamate and allowed to dry. The plates were placed under a single General Electric germicidal UV lamp (model G15T8; 15 W) with an output measured at 11 ergs·s⁻¹·mm⁻² and were irradiated for 10 s. Killing was approximately 10%.

Isolation of proline oxidase-constitutive mutants. Sterile, precut filter paper was placed on top of each UV-irradiated petri plate. The plates were incubated at 30°C in the dark until colonies appeared to grow through the filter paper (2 to 3 days). The paper was then peeled off each plate and immersed in liquid nitrogen to render the cells permeable. A filter paper proline oxidase assay was done to identify those colonies containing proline oxidase at a high level (see below).

Genetic analysis. The techniques used have been described previously (2).

Enzyme assays. The chemicals, substrates, growth of cells, preparation of cell extracts, proline oxidase and Δ¹-pyrroline-5-carboxylate (PSC) dehydrogenase assays, and protein determinations have been described previously (2). A unit of enzyme activity was defined as 1 μmol of product formed per min. The data given are the average of at least two, and usually more, determinations. A filter paper proline oxidase assay was performed to screen large numbers of colonies for constitutive proline oxidase activity and is a modification of the method described by Dendiger and Brill (3). After the filter paper was removed from the petri plate and dipped in liquid nitrogen, it was allowed to come to room temperature. A 0.5-ml amount of proline oxidase assay mixture (per 1 ml: 0.1 M sodium cacodylate buffer [pH 6.6] with 3 mM MgCl₂, 0.4 ml of 10% proline, and 0.1 ml of 5-mg/ml o-aminobenzaldehyde in 20% ethanol) was placed in a petri plate lid, and the filter was allowed to absorb the mixture. The filters were incubated at 30°C until almost dry. The plate replicas corresponding to the colonies on the filter which were yellower than the background were examined further.

RESULTS

Isolation of strains with inducer-independent levels of proline oxidase. Colonies of yeast containing high levels of proline oxidase
activity were screened as described above. The detection scheme relied on the formation of a complex between P5C and the reagent o-aminobenzaldehyde, which turns a colony on a piece of filter paper yellow.

Physiological studies of the put3 mutation. One colony with the highest levels of proline oxidase was chosen for further study. The mutation in this strain was labeled put3. Table 2 lists the specific activities of the enzymes in the proline-degradative pathway, as well as the proline uptake system in the wild-type and put3 strains. It is clear that the put3 mutation has no effect on the proline permease. However, the activities of the put3 strain have become inducer independent for both the proline oxidase and P5C dehydrogenase, although the isolation screened detected elevated proline oxidase activity only.

Several other enzyme activities were examined to determine whether the put3 mutation affected any other pathways. Arginase, ornithine transaminase, P5C reductase, and glutamine synthetase had wild-type enzyme levels and were all regulated in the put3 strain as in the wild-type strain (data not shown).

The results in Table 2 show that the put3 mutation has not rendered the two enzymes fully constitutive. Cultivation of the put3 strain in a medium containing proline, rather than ammonia or urea as the sole source of nitrogen, brought about a considerable increase in the levels of both enzymes. It can also be seen that the constitutive expression of the enzymes was not reduced by the presence of ammonia; the levels of the enzymes were essentially the same whether ammonia or urea served as the sole source of nitrogen. On the other hand, hyperinduction of the enzymes by proline was largely prevented by the presence of ammonia. Thus, the hyperinduction of the enzymes by proline in the put3 strain is just as sensitive to the presence of ammonia as is the induction of the enzymes by proline in the wild-type strain. Because of the insensitivity of the constitutive synthesis in the put3 strain to ammonia, the levels of both enzymes when the strain is grown in a medium containing proline as well as ammonia are considerably higher than those of the wild-type strain.

Inheritance of the put3 mutation. The put3 mutation behaves as a single, nuclear gene in crosses, segregating two spores with high levels of proline oxidase on galactose-ammonia medium and two spores with low proline oxidase activity.

Table 1. Strain list

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>MB1000</td>
<td>a Wild type</td>
<td>Σ1278b of J.-M. Wiame</td>
</tr>
<tr>
<td>MB1121</td>
<td>a put3</td>
<td></td>
</tr>
<tr>
<td>MB1125</td>
<td>a put2-58 put3</td>
<td></td>
</tr>
<tr>
<td>MB214-6A</td>
<td>a his4-42 put3</td>
<td></td>
</tr>
<tr>
<td>MB218-2C</td>
<td>a lys-33 pro3-66</td>
<td></td>
</tr>
<tr>
<td>MB313-2A</td>
<td>a lys-23</td>
<td></td>
</tr>
<tr>
<td>MB313-2B</td>
<td>a his4-42 put3</td>
<td></td>
</tr>
<tr>
<td>MB313-2C</td>
<td>a pro3-66 lys-23</td>
<td></td>
</tr>
<tr>
<td>MB313-2D</td>
<td>a pro3-66 put3 his4-42</td>
<td></td>
</tr>
<tr>
<td>MB313-19A</td>
<td>a pro3-66 put3 his4-42</td>
<td></td>
</tr>
<tr>
<td>MB313-19B</td>
<td>a lys-23</td>
<td></td>
</tr>
<tr>
<td>MB313-19C</td>
<td>a</td>
<td></td>
</tr>
<tr>
<td>MB313-19D</td>
<td>a pro3-66 put3 his4-42</td>
<td>lys-23</td>
</tr>
<tr>
<td>MB313-20A</td>
<td>a pro3-66 his4-42</td>
<td></td>
</tr>
<tr>
<td>MB313-20B</td>
<td>a lys-33 put3</td>
<td></td>
</tr>
<tr>
<td>MB313-20C</td>
<td>a lys-33 put3</td>
<td></td>
</tr>
<tr>
<td>MB313-20D</td>
<td>a pro3-66 his4-42</td>
<td></td>
</tr>
<tr>
<td>MB214</td>
<td>a his4-42</td>
<td>+</td>
</tr>
<tr>
<td>MB215</td>
<td>a his4-42 put1-54</td>
<td>+</td>
</tr>
<tr>
<td>MB217</td>
<td>a his4-42 put2-57</td>
<td>+</td>
</tr>
<tr>
<td>MB220</td>
<td>a his4-42 put2-58 put3</td>
<td>+</td>
</tr>
<tr>
<td>MB228</td>
<td>a his4-42 put3</td>
<td>+</td>
</tr>
<tr>
<td>MB271</td>
<td>a his4-42</td>
<td>+ put3</td>
</tr>
<tr>
<td>MB313</td>
<td>a lys-23</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>a + his4-42</td>
<td>+ put3</td>
</tr>
<tr>
<td></td>
<td>pro3-66</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>+ put3</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 2. Specific activities of proline-degradative enzymes in the wild-type and put3 strains

<table>
<thead>
<tr>
<th>Nitrogen source</th>
<th>Strain</th>
<th>Proline permease</th>
<th>P5C dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₃</td>
<td>Wild type</td>
<td>1.5</td>
<td>11.6</td>
</tr>
<tr>
<td>Urea</td>
<td>Wild type</td>
<td>22.9</td>
<td>67.3</td>
</tr>
<tr>
<td>NH₃ + proline</td>
<td>Wild type</td>
<td>2.5</td>
<td>23.8</td>
</tr>
<tr>
<td>Urea + proline</td>
<td>Wild type</td>
<td>7.6</td>
<td>31.6</td>
</tr>
<tr>
<td>Proline</td>
<td>Wild type</td>
<td>23.2</td>
<td>96.9</td>
</tr>
</tbody>
</table>

The values are expressed as nanomoles of [¹⁴C]-proline taken up per minute per milligram of protein. The carbon source was 0.5% galactose. PD, expressed as initial velocity (nanomoles of [¹⁴C]-proline taken up per minute per milligram of protein). OC, expressed as nanomoles of P5C formed per minute per milligram of protein. OC, expressed as nanomoles of NADH formed per minute per milligram of protein. *ND, Not determined.

a (NH₄)₂SO₄ was supplied at 0.2%; urea and proline were supplied at 0.1%. The carbon source was 0.5% galactose.

b Expressed as initial velocity (nanomoles of [¹⁴C]-proline taken up per minute per milligram of protein).

c Expressed as nanomoles of P5C formed per minute per milligram of protein.

d Expressed as nanomoles of NADH formed per minute per milligram of protein.

MB1000.
MB1121.
ND, Not determined.
activity. In the small number of tetrads analyzed to date, put3 is not closely linked to put1 and put2 (which are believed to code for proline oxidase and P5C dehydrogenase, respectively [2]), and its chromosome map location is not known.

Is endogenous induction responsible for the put3 enzyme levels? Inducer-independent expression of the proline-degradative enzymes could result from a mutation in a regulatory component, e.g. a repressor or an activator. A more trivial cause of high levels of enzyme activity could be the increase in the pool of intracellular proline, a consequence, for instance, of increased biosynthesis of proline. To examine the latter possibility, a haploid strain was constructed which contained the put3 mutation and a mutation (pro3) in the last enzyme of the proline biosynthetic pathway, P5C reductase (1). We took advantage of the observation that in a medium containing proline as well as ammonia, the proline oxidase levels of the put3 mutant are threefold higher than the level in the wild-type or pro3 strain (Table 2; see also Table 4 in the accompanying paper [2]). The proline oxidase level in this double mutant strain was measured and compared with the levels in the pro3 and put3 parent strains. If the proline oxidase level remained high, as in the put3 parent strain, the proline biosynthetic pathway could be eliminated as a cause of the inducer-independent enzyme activity. If the double mutant had low enzyme activity, then one could conclude that endogenous induction was responsible for the high levels of proline-degradative enzymes seen in the absence of added inducer.

Table 3 shows the proline oxidase levels of the parent strains and three tetrads resulting from the diploid formed by crossing the pro3 strain with the put3 strain. It was necessary to follow the inheritance of both the Pro− and the Put3 phenotypes to be able to determine which of the spore progeny were pro3 put3 in genotype. It can be seen that the put3 marker segregated 2:2 and that even when the strain contained a mutation in the proline biosynthetic pathway as well (e.g., MB313-2D, MB313-19A, MB313-19D), the proline oxidase level was still high. Therefore, the inducer-independent level of proline oxidase in the put3 mutant cannot be attributed to endogenous induction from enhanced biosynthesis of proline.

<table>
<thead>
<tr>
<th>Strain</th>
<th>pro3 genotype</th>
<th>Proline oxidase sp act</th>
<th>Deduced put3 genotype</th>
<th>Ascus type</th>
</tr>
</thead>
<tbody>
<tr>
<td>MB278-2C (parent)</td>
<td>−</td>
<td>6.9</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>MB214-6A (parent)</td>
<td>+</td>
<td>14.8</td>
<td>c</td>
<td></td>
</tr>
<tr>
<td>MB1000 (wild type)</td>
<td>+</td>
<td>7.1</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>MB313-2A</td>
<td>+</td>
<td>6.9</td>
<td>+</td>
<td>T</td>
</tr>
<tr>
<td>MB313-2B</td>
<td>+</td>
<td>33.5</td>
<td>c</td>
<td></td>
</tr>
<tr>
<td>MB313-2C</td>
<td>−</td>
<td>8.2</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>MB313-2D</td>
<td>−</td>
<td>19.0</td>
<td>c</td>
<td></td>
</tr>
<tr>
<td>MB313-19A</td>
<td>−</td>
<td>20.9</td>
<td>c</td>
<td>N</td>
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<tr>
<td>MB313-19B</td>
<td>+</td>
<td>5.8</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>MB313-19C</td>
<td>+</td>
<td>8.4</td>
<td>+</td>
<td></td>
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<tr>
<td>MB313-19D</td>
<td>−</td>
<td>17.5</td>
<td>c</td>
<td></td>
</tr>
<tr>
<td>MB313-20A</td>
<td>−</td>
<td>4.6</td>
<td>+</td>
<td>P</td>
</tr>
<tr>
<td>MB313-20B</td>
<td>+</td>
<td>15.1</td>
<td>c</td>
<td></td>
</tr>
<tr>
<td>MB313-20C</td>
<td>+</td>
<td>15.8</td>
<td>c</td>
<td></td>
</tr>
<tr>
<td>MB313-20D</td>
<td>−</td>
<td>7.3</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

* +, Wild-type allele; −, mutant allele resulting in P5C reductase deficiency.

The cells were grown on medium containing 0.5% galactose, 0.2% ammonium sulfate, 0.1% proline, 20 mg of histidine hydrochloride per liter, and 30 mg of lysine hydrochloride per liter. The results are expressed as nanomoles of P5C formed per minute per milligram of protein.

* c, Constitutive, put3 phenotype.

* d, T, Tetraplicate; N, nonparental ditocyte; P, parental ditocyte with respect to pro3 and put3.

mU/mg of protein (data not shown). The lack of a clear relationship between gene dosage and the induced enzyme level may be a reflection of our assay, which may be limited by the capacity of the mitochondrial electron transport system.

When the cells were cultured in the absence of proline in a medium containing galactose with ammonia as the source of nitrogen, the levels of proline oxidase were between 1 and 2 mU/mg of protein, unless they were of strains carrying at least one copy of the mutant put3 allele responsible for constitutive synthesis of proline oxidase (data not shown). The proline oxidase levels in cells of strains carrying the put3 mutation are summarized in Table 4. It can be seen that the cells of the haploid and diploid put3 mutant strains have the same level of proline oxidase (Table 4, experiments 1 and 2). In cells of the diploid strain carrying a single functional put1 allele, the level is reduced to approximately one-half (experiments 2 and 3), demonstrating a gene
### DISCUSSION

The *put3* mutation resulted in constitutive, but hyperinducible formation of proline oxidase and P5C dehydrogenase. Enzyme levels in the absence of proline were about 10-fold higher than in the wild-type strain for proline oxidase and 4-fold higher for P5C dehydrogenase. In the presence of proline, the levels of both enzymes in the mutant were two- to threefold higher than in the wild-type strain. In contrast to the hyper-inducible enzyme activity, the constitutive enzyme activity was not sensitive to the presence of ammonia. The proline permease was unaffected by the *put3* mutation, as were arginine and ornithine transaminase, enzymes of arginine catabolism responsible for the production of P5C, an intermediate common to both proline and arginine degradation.

It was shown in another paper that ammonia strongly represses the proline permease (2). We have shown in this paper that ammonia has no effect on the inducer-independent formation of the enzymes in the *put3* mutant but interferes with hyperinduction by proline. Our present observations are therefore in good agreement with the view that, in our medium which contains galactose as the source of energy, ammonia does not directly inhibit the formation of the proline-degrading enzymes, but rather prevents their induction by proline through its repression of the proline permease.

Since the data in Table 3 show that the constitutive synthesis is not dependent on the ability of the cell to produce proline, it is unlikely that the high enzyme levels found in the *put3* mutant in the absence of the exogenously supplied proline are due to high internal concentrations of proline. Therefore, we conclude that the *put3* mutation alters the function of a regulatory component specific for the proline catabolic pathway.

The *put3* mutation behaves neither fully dominant nor fully recessive when in the heterozygous state in a diploid. In a medium lacking proline, the level of proline oxidase observed in such a diploid is four times the level of the homozygous wild type (*put3*/*put3*) diploid but only one-third the level of the homozygous constitutive (*put3*/*put3*) diploid. Too few data are available to distinguish between a model of negative regulation involving a repressor and one of positive regulation involving an activator. Further study of additional regulatory mutants is required before any conclusions concerning the mechanism of regulation of proline utilization can be drawn.

**ACKNOWLEDGMENTS**

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**LITERATURE CITED**


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### TABLE 4. Gene dosage effects of the put mutations

<table>
<thead>
<tr>
<th>Expt</th>
<th>Strain</th>
<th>Genotype*</th>
<th>Sp act of proline oxidase³</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MB1121</td>
<td>+</td>
<td>c</td>
</tr>
<tr>
<td>2</td>
<td>MB229</td>
<td>+/+</td>
<td>+/+</td>
</tr>
<tr>
<td>3</td>
<td>MB271</td>
<td>+/-</td>
<td>+/+</td>
</tr>
<tr>
<td>4</td>
<td>MB214</td>
<td>+/+</td>
<td>+/+</td>
</tr>
<tr>
<td>5</td>
<td>MB215</td>
<td>+/+</td>
<td>+/+</td>
</tr>
<tr>
<td>6</td>
<td>MB1125</td>
<td>+</td>
<td>c</td>
</tr>
<tr>
<td>7</td>
<td>MB220</td>
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<td>+/+</td>
</tr>
<tr>
<td>8</td>
<td>MB217</td>
<td>+/+</td>
<td>+/+</td>
</tr>
</tbody>
</table>

* +, Wild-type allele; -, mutant allele resulting in enzyme deficiency; c, mutant allele resulting in constitutive enzyme activity. Two characters (e.g., +/c) indicate a diploid genotype.

*² Expressed as described in Table 2, footnote c. The medium contained 0.5% galactose and 0.2% (NH₄)₂SO₄.

³ dosage of *put1*, the gene for proline oxidase. Replacement of one mutant *put3* allele by the corresponding gene of the wild type reduces the proline oxidase level by more than one-half, but does not lower it to the level characteristic for cells carrying only the wild-type *put3* allele, that is, between 1 and 2 μU/mg of protein (experiments 2 and 4). The phenotype determined by the *put3* mutation is clearly not dominant, nor is it fully recessive. A similar reduction in enzyme level results from replacement of the *put3* gene in a diploid cell containing only one functional *put1* gene (experiments 3 and 5). We have previously reported that in cells lacking a functional *put2* gene, proline oxidase is more sensitive to induction by proline than it is in normal cells (2). We now find that in haploid or diploid *put3* mutant strains the loss of one functional *put2* gene does not significantly affect the uninduced level of proline oxidase (experiments 6 and 7). However, in diploid cells carrying a wild-type allele as well as a *put3* allele, the level of proline oxidase is higher with one than with two functional *put2* genes (experiments 4 and 8). We believe that this is due to an increased concentration of endogenous proline, which results from the block in *put2* function and the action of P5C reductase on the accumulating P5C.