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Isolation and Preliminary Characterization of *Saccharomyces cerevisiae* Proline Auxotrophs

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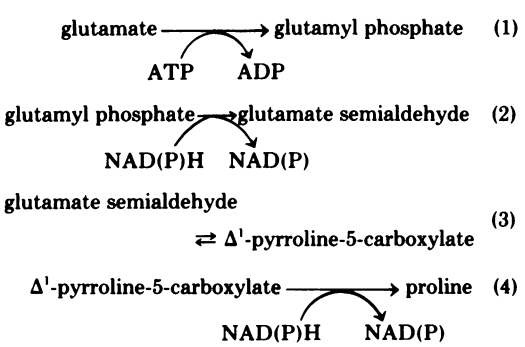
Proline-requiring mutants of *Saccharomyces cerevisiae* were isolated. Each mutation is recessive and is inherited as expected for a single nuclear gene. Three complementation groups could be defined which are believed to correspond to mutations in the three genes (*pro1*, *pro2*, and *pro3*) coding for the three enzymes of the pathway. Mutants defective in the *pro1* and *pro2* genes can be satisfied by arginine or ornithine as well as proline. This suggests that the blocks are in steps leading to glutamate semialdehyde, either in glutamyl kinase or glutamyl phosphate reductase. A *pro3* mutant has been shown by enzyme assay to be deficient in Δ^1-pyrroline-5-carboxylate reductase which converts pyrroline-5-carboxylate to proline. A unique feature of yeast proline auxotrophs is their failure to grow on the rich medium, yeast extract-peptone-glucose. This failure is not understood at present, although it accounts for the absence of proline auxotrophs in previous screening for amino acid auxotrophy.

The pathway of proline biosynthesis has been established by studies of the enzyme deficiencies in proline-requiring mutants of various microorganisms. In *Escherichia coli* (1, 2, 16), *Salmonella typhimurium* (12), and *Neurospora crassa* (15, 18), the pathway has been shown to be as follows:



Glutamyl kinase carries out step 1; the unstable intermediate, glutamyl phosphate, is believed to be enzyme-bound in *E. coli* (1, 8). Glutamate semialdehyde is formed by the action of glutamyl phosphate reductase on glutamyl phosphate (step 2). In the cell, glutamate semialdehyde spontaneously cyclizes to form Δ^1-pyrroline-5-carboxylate (P5C) (16). In the final reaction, P5C reductase converts P5C to proline.

Mutations leading to a growth requirement for proline occurred at frequencies comparable to those leading to other auxotrophies when large-scale mutant hunts were conducted in these microorganisms (3, 5, 12). Surprisingly no proline auxotrophs in yeast were ever described, although attempts were made to find them (6). In fact, mutations in other yeast biosynthetic pathways, in general, have been as easy to isolate and study as in the enteric bacteria or *Neurospora* (9, 10, 13).

The absence of this type of mutant in yeast raised questions as to the possible differences between yeast and other organisms, for example, in cellular physiology, the number of gene copies coding for the various enzymes involved, or the interactions of various biosynthetic pathways.

This paper describes the isolation and initial characterization of proline auxotrophs in *Saccharomyces cerevisiae* and a preliminary examination of the differences found in this system.

MATERIALS AND METHODS

Yeast strains. The strains used in this work were derived from isogenic strains Σ1278b(α) and 12079d(a) of Jean-Marie Wiame and are listed in Table 1.

Media. The minimal medium used was yeast nitrogen base without ammonium sulfate and amino acids (Difco) to which appropriate supplements were added.

For growth of cultures in liquid, the minimal medium contained 0.5% galactose, and 0.2% ammonium sulfate and/or 0.1% proline.

For the isolation and characterization of proline auxotrophs on plates, minimal medium contained 0.5% galactose and 0.1% glutamate with or without 0.1% proline. Where appropriate, 0.1% ornithine or 0.1% arginine substituted as sole nitrogen source, or in addition to ammonium sulfate.

For spore germination and tetrad analysis, minimal

TABLE 1. *Strains used*

Strain	Genotype	Comment
MB1000	α wild type	Σ1278b of J.-M. Wiame
MB1034	a wild type	12079d of J.-M. Wiame
MB1039	α *lys-23*	
MB1057	a *his4-42*	
AM269-6B	a *his4-42 lys-23*	
MB1135	α *pro1-59*	
MB272-3D	a *pro1-59 his4-42*	
MB1136	α *pro2-60*	
MB273-2A	a *pro2-60 his4-42*	
MB1137	α *pro2-63*	
MB276-4A	a *pro2-63*	
MB1138	α *pro2-64*	
MB277-1B	a *pro2-64 lys-23*	
MB1139	α *pro3-61*	
MB274-1B	a *pro3-61 lys-23*	
MB1140	α *pro3-62*	
MB275-2C	a *pro3-62 his4-42*	
MB1141	α *pro3-65*	
MB280-5C	a *pro3-65 his4-42 lys-23*	
MB1142	α *pro3-66*	
MB278-2C	a *pro3-66 lys-23*	
MB1143	α *pro3-67*	
MB279-6A	a *pro3-67*	
MB297	α *pro1-59* + *lys-23* + / a + *pro2-60* + *his4-42*	
MB298	α *pro1-59* + *his4-42* + / a + *pro3-66* + *lys-23*	
MB299	α *pro3-66* + *his4-42* + / a + *pro2-60* + *lys-23*	
MB303	α *pro2-63* *his4-42* + / a *pro2-64* + *lys-23*	
MB304	α *pro3-62* *lys-23* + / a *pro3-65* + *his4-42*	
MB305	α *pro3-67* *lys-23* + / a *pro3-65* + *his4-42*	

plates contained 2% glucose, 0.2% ammonium sulfate, and, where appropriate, histidine (20 mg/liter), lysine (30 mg/liter) and 0.1% proline. Yeast extract-peptone-dextrose (YPD) plates contained 2% glucose, 1% yeast extract (Difco), 2% peptone (Difco), and 2% agar (Difco). Yeast extract-peptone-galactose plates were identical to YPD plates, with 0.5% galactose instead of glucose. Sporulation medium contained 0.3% potassium acetate.

Mutagenesis. Mutants were induced by treatment with ethyl methane sulfonate by the method of Fink (7). The mutagenized cultures were grown out in liquid YPD before spreading onto minimal plates.

Isolation of proline auxotrophs. Mutagenized cells of MB1000(α) were spread on agar plates containing galactose, glutamate, and proline and were incubated at 30°C for 3 to 5 days. The colonies were then replica plated to galactose-glutamate and galactose-glutamate-proline plates. Colonies which failed to grow on the galactose-glutamate plates but did grow on the same medium supplemented with proline were purified by subcloning and characterized further.

Genetic analysis. Haploid strains of opposite mating type were mated on a permissive minimal medium, rather than YPD, whenever either haploid could not grow on YPD. The resulting diploids were isolated by selection for prototrophy and purified by subcloning on a selective minimal medium. Asci were dissected by micromanipulation after digestion with Glusulase (Endo Laboratories, Garden City, N.Y.).

Complementation analysis was performed by cross-streaking and replica plating the haploid strains on appropriately supplemented minimal medium. Complementation tests were scored after 3 to 5 days at 30°C.

Chemicals and substrates. *N*-Tris-(hydroxymethyl)methyl-3-aminopropane-sulfonic acid (TAPS) buffer and DL-plus-allo-δ-hydroxylysine-HCl were purchased from Calbiochem, La Jolla, Calif. NADH was purchased from P-L Biochemicals, Inc., Milwaukee, Wis. *o*-Aminobenzaldehyde was purchased from Sigma Chemical Co., St. Louis, Mo.

DL-P5C was synthesized by periodate oxidation of DL-δ-hydroxylysine as described by Williams and Frank (17). For use in enzyme assays, the P5C was neutralized with 10 N potassium hydroxide to a final pH of 7.0 immediately before use.

Growth of cells for enzyme assays. Two-liter

flasks containing 250 ml of galactose-ammonia-proline medium were inoculated to a density of 5 to 10 Klett units, using a Klett-Summerson colorimeter (blue filter), with a stationary-phase culture grown in the same medium. Cultures were incubated at 30°C with shaking until the density reached approximately 100 to 125 Klett units. Cells were recovered by centrifugation and washed with 5 ml of 0.05 M TAPS buffer (pH 7.7).

Preparation of cell extracts. The washed cells were disrupted by intermittent sonic oscillation for 4 min by use of a Branson sonifier (Heat Systems, Melville, N.Y.) with an output of 70 to 100 W. Cell debris was removed by centrifugation at 20,200 × *g* for 20 min at 4°C. A 2.5-ml volume of supernatant was eluted through a G-25 Sephadex column (PD-10, Pharmacia, Uppsala, Sweden) with 3.5 ml of TAPS buffer and assayed immediately for P5C reductase activity.

Enzyme assays. All assays of P5C reductase [L-proline: $NAD(P)^+$ 5-oxidoreductase, EC 1.5.1.2] were carried out at 30°C in 0.05 M TAPS buffer (pH 7.0). Reactions were initiated by the addition of substrate. Enzymatic activity was monitored by following the decrease in absorbance at 340 nm using a Zeiss recording spectrophotometer. The assay mixture contained (in 1 ml): 0.05 M TAPS buffer (pH 7.0), 0.14 mM NADH, 1 mM P5C (pH 7.0), and 5 to 15 μl of extract.

NADH oxidase activity was assayed in the absence of P5C and was subtracted from the activity found in the presence of P5C for the net P5C reductase activity. This background activity was always less than 10% of the P5C reductase activity.

Protein determination. Protein was determined by the method of Lowry et al. (11) using bovine serum albumin as standard.

RESULTS

Isolation of proline auxotrophs. After ethyl methane sulfonate mutagenesis, cells of the prototrophic wild-type strain MB1000 were spread on minimal plates containing galactose as sole carbon source, with glutamate and proline as nitrogen sources. Colonies which appeared on these plates after 3 to 5 days of incubation at 30°C were replica plated to galactose-glutamate and galactose-glutamate-proline plates. Those colonies which failed to grow on the galactose-glutamate plates but did grow on the same medium supplemented with proline were purified by subcloning and tested further.

The isolation procedure described above failed to yield any proline auxotrophs when the mutagenized culture was spread on YPD plates before replica plating to the minimal medium.

Galactose was used here as the carbon source to counterselect respiratory-deficient mutants which often appear after mutagenesis. An ethidium bromide-induced petite strain derived from MB1000 will not grow on galactose-containing medium. This characteristic varies with strain background and is not true for all laboratory yeast strains.

The nine Pro^- mutants characterized in this study are independent of one another as determined by their behavior in complementation tests or by their derivation in separate mutagenic treatments.

Growth characteristics of proline auxotrophs. The most striking characteristic of all the proline auxotrophs isolated was their inability to grow on YPD medium. This was true even when YPD was supplemented with 0.1% proline. This finding explains the inability to isolate these mutants when growth on YPD plates was required before replica plating. Although the EMS mutagenesis procedure included outgrowth in liquid YPD medium, the proline auxotrophs remained viable in the culture, and appeared on the minimal proline-containing plates.

Table 2 summarizes the growth characteristics of nine proline auxotrophs. In four of the strains, ornithine, arginine, or proline satisfied the requirement; in the remaining strains, only the presence of proline permitted growth on the minimal medium.

When 0.5% galactose was substituted for 2% glucose in the YPD medium, the first class of mutants showed slight growth, whereas the second group showed none.

The presence of 2% glucose in the plates is not in itself inhibitory to growth. The proline auxotrophs grow well on 2% glucose-ammonia-proline medium, which was used as spore germination medium and for tetrad analysis.

Inheritance of proline auxotrophy. Each α strain bearing a proline requirement was crossed to an isogenic a strain, AM269-6B, carrying histidine and lysine markers. All the resulting diploids were proline prototrophs, indicating that the mutation in each case was recessive.

TABLE 2. *Growth characteristics of proline auxotrophs*[a]

Isolate	Growth on nitrogen source[b]:				
	NH_3	NH_3 + pro	NH_3 + orn, orn, arg	YPgal[c]	YPD[c]
59, 60, 63, 64	−	+	+	±	−
61, 62, 65, 66, 67	−	+	−	−	−

[a] Growth is scored after 2 days at 30°C on agar plates with 0.5% galactose as sole carbon source, except for 2% glucose in YPD plates. Symbols: +, heavy growth; ±, slight growth; −, no growth.

[b] $(NH_4)_2SO_4$ is supplied at 0.2%, all others at 0.1%. Abbreviations: pro, proline; orn, ornithine; arg, arginine; YP, yeast extract-peptone.

[c] Identical results found when 0.1% proline was added to the medium. YPgal, Yeast extract–peptone–galactose plates.

The diploids also grew on YPD medium.

When the diploids were allowed to sporulate, the proline auxotrophy segregated 2:2 in each tetrad, as expected for a single, nuclear gene. In addition, the inability to grow on YPD cosegregated with the proline auxotrophy in every tetrad (data not shown).

Complementation analysis. From each of the above diploids, **a** and α spore progeny bearing each of the mutations were mated and complementations were carried out. Table 3 gives the results of the tests.

The mutants divided into three classes on the basis of complementation. The first class (I), containing only one member, *pro-59*, complemented all other mutations. The second and third classes were defined by at least one mutation which failed to complement all members of that class. In class II, *pro-60* failed to complement *pro-63* and *pro-64*, although these two mutations complemented each other weakly. Similarly, in class III, *pro-61* and *pro-66* failed to complement each other and all others in this group, although some class III members showed complementation, namely, *pro-65* with *pro-67* and *pro-62*.

Diploids from these intraclass complementations were isolated and allowed to sporulate, and the tetrads were analyzed. If the *pro* markers are allelic or tightly linked, all tetrads will be parental ditypes yielding four proline-requiring spores. Two of the spores will have a complementation pattern like one parent, and two will have a complementation pattern like the other parent. If the two *pro* mutations are not closely linked, prototrophic spores will segregate in tetratype and nonparental ditype tetrads.

All three intraclass diploids failed to yield prototrophic spores in seven tetrads analyzed (Table 4), indicating that the two mutations are either allelic or closely linked.

The three complementation groups were called *pro1*, *pro2*, and *pro3*, corresponding to class I, class II, and class III, respectively. To determine linkage relationships, one member of each class was crossed to the others, the diploids were allowed to sporulate, and the tetrads were analyzed. Although only a small number of tet-

TABLE 3. *Complementation analysis of proline-requiring strains*[a]

Class	α strain containing mutation no.:	**a** strain containing mutation no.:								
		59	*60*	*63*	*64*	*61*	*62*	*65*	*66*	*67*
I	*59*	−	+	+	+	+	+	+	+	+
II	*60*	+	−	−	−	+	+	+	+	+
	63	+	−	−	±	+	+	+	+	+
	64	+	−	±	−	+	+	+	+	+
III	*61*	+	+	+	+	−	−	−	−	−
	62	+	+	+	+	−	−	+	−	−
	65	+	+	+	+	−	+	−	−	+
	66	+	+	+	+	−	−	−	−	−
	67	+	+	+	+	−	−	+	−	−

[a] **a** and α strains were streaked on permissive minimal plates, allowed to grow for 2 days at 30°C, and replica plated in a cross-hatched pattern to minimal plates lacking proline. Growth at intersections scored after 2 days. Symbols: +, heavy growth; ±, slight growth; −, no growth.

TABLE 4. *Tetrad analysis of complementing proline auxotrophs*

Diploid strain	Genotype	No. of ascus type[a]:		
		PD (4 *pro*⁻:0 *pro*⁺)	NPD (2 *pro*⁻:2 *pro*⁺)	T (3 *pro*⁻:1 *pro*⁺)
MB303	$\frac{\alpha\ pro2\text{-}63\ his4\text{-}42\ +}{\mathbf{a}\ pro2\text{-}64\ +\ lys\text{-}23}$	7	0	0
MB304	$\frac{\alpha\ pro3\text{-}62\ lys\text{-}23\ +}{\mathbf{a}\ pro3\text{-}65\ +\ his4\text{-}42}$	7	0	0
MB305	$\frac{\alpha\ pro3\text{-}67\ lys\text{-}23\ +}{\mathbf{a}\ pro3\text{-}65\ +\ his4\text{-}42}$	7	0	0

[a] PD, Parental ditype ascus; NPD, nonparental ditype ascus; T, tetratype ascus. Close linkage of two genes is indicated by an excess of PD to NPD tetrads. Random assortment is indicated by a PD:NPD:T ratio of 1:1:4 (14).

rads was analyzed, it is clear that no tight linkage was observed among *pro1*, *pro2*, and *pro3* (Table 5).

Enzyme deficiencies of the proline auxotrophs. Currently, there is no direct assay to measure the first two enzymes in this pathway. Baich (1) has assayed the first step in *E. coli* by measuring proline-inhibitable phosphate release, and the second step by running the reaction from P5C to glutamyl phosphate in the reverse direction (2). In *S. cerevisiae*, the first reaction is identical to that performed by glutamine synthetase in the formation of glutamine so that partial purification of the enzyme away from glutamine synthetase or use of a glutamine auxotroph is required for unambiguous measurements of glutamyl kinase. The second reaction, run in reverse, risks contamination from the proline degradative enzyme, P5C dehydrogenase, which converts P5C to glutamate with NAD as electron acceptor.

Complete analysis of the enzyme deficiencies of *pro1* and *pro2* mutants awaits the development of purification methods for the glutamyl kinase and glutamyl phosphate reductase.

The third enzyme, P5C reductase, can be measured unambiguously in yeast. Table 6 lists the P5C reductase levels in the wild-type and proline-requiring strains. The difference between the highest and lowest levels in the wild type is believed to be insignificant. *pro3* mutants have about one-sixth the level of the wild-type enzyme and are thus clearly deficient in P5C reductase activity. This level is twice the background level and probably represents a small amount of enzyme activity.

The *pro3* mutations are believed to be in the structural gene coding for P5C reductase although, as yet, no evidence exists to rule out mutations in a regulatory component.

Since arginine and ornithine feed into the proline pathway at P5C (see Fig. 1) and can satisfy the proline requirement of *pro1* and *pro2* mutants, the finding that these mutants have P5C reductase is not surprising. The lack of P5C reductase in *pro3* mutants explains their inability to use ornithine or arginine as substitute for proline.

The P5C reductase appears to be a constitutive enzyme, insensitive to repression by both the end product of the pathway, proline, and by ammonia, a preferred nitrogen source. This result confirms a previous finding of Bechet and Wiame (4).

DISCUSSION

The study of mutations leading to proline auxotrophy in yeast suggests that the pathway from glutamate to proline is the same as that found in other organisms (1, 2, 12, 15). Arginine and ornithine can satisfy the proline requirement in *pro1* and *pro2* mutants since they are converted to glutamate semialdehyde by enzymes in the arginine degradative pathway. P5C reductase converts the cyclized form of glutamate semialdehyde, P5C, to proline in the wild-type *pro1* and *pro2* strains, but not in the P5C reductase-deficient *pro3* strain. Figure 1 shows the interrelations of the pathways involved, the enzymes, and the mutational blocks.

Each mutation causes a recessive defect in a single nuclear gene and complements mutations in the other two genes. All are unlinked to one

TABLE 6. *P5C reductase activities*

Strain	Genotype	Reductase activity[a] with nitrogen source[b]:		
		NH_3	NH_3 + pro	pro
MB1000	Wild type	269 ± 50	310 ± 50	359 ± 50
MB1135	*α pro1-59*	NG[c]	349 ± 81	ND[d]
MB1136	*α pro2-60*	NG	372 ± 8	ND
MB1142	*α pro3-66*	NG	56 ± 9	45 ± 3

[a] Expressed as nanomoles of NAD formed per minute per milligram of protein.
[b] $(NH_4)_2SO_4$ is supplied at 0.2%; proline (pro) at 0.1%. 0.5% Galactose is sole carbon source.
[c] NG, No growth.
[d] ND, Not determined.

TABLE 5. *Linkage relationships among Pro⁻ mutations*

Diploid	Relevant genotype	No. of ascus type[a]: PD (4 *pro⁻*:0 *pro⁺*)	NPD (2 *pro⁻*:2 *pro⁺*)	T (3 *pro⁻*:1 *pro⁺*)	Conclusion
MB297	$\frac{\alpha\ pro1\text{-}59\ \ +}{a\ \ +\ \ pro2\text{-}60}$	3	1	6	Unlinked
MB298	$\frac{\alpha\ pro1\text{-}59\ \ +}{a\ \ +\ \ pro3\text{-}66}$	2	2	5	Unlinked
MB299	$\frac{\alpha\ pro3\text{-}66\ \ +}{a\ \ +\ \ pro2\text{-}60}$	1	3	6	Unlinked

[a] See footnote *a*, Table 4.

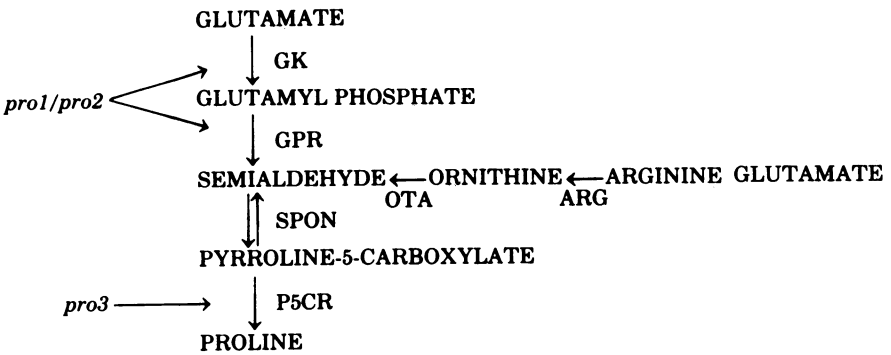


FIG. 1. *Interaction between pathways of proline biosynthesis and arginine degradation. Abbreviations for the enzymes: arginase (ARG), glutamyl kinase (GK), glutamyl phosphate reductase (GPR), ornithine transaminase (OTA), pyrroline-5-carboxylate reductase (P5CR), spontaneous (SPON).*

another. At the present time, the enzymatic deficiencies of *pro1* and *pro2* mutants have not been distinguished. This determination awaits partial purification of the yeast enzymes, glutamyl kinase and glutamyl phosphate reductase, and the development of reliable assays.

Mutants in *pro2* and *pro3* each display the following complementation pattern: in each group, at least one mutant fails to complement all the others in that group and at least two of the latter complement each other. This finding suggests that the enzymes encoded by these genes may be multimeric.

The inability of the proline auxotrophs to grow on YPD medium is not understood. It is possible to imagine a nitrogen- or carbon-catabolite-sensitive uptake system which prevents the uptake of proline and/or arginine and ornithine in a medium where multiple preferred carbon and nitrogen sources are present. Alternatively, the growth on YPD medium by the mutants could cause a toxic accumulation of intermediates which does not occur in the wild-type strain. Uptake studies and the characterization of revertants capable of YPD growth may clarify the situation.

The results of this work emphasize, once again, that the "standard conditions" considered fully permissive for the isolation of mutants may, in reality, select against a certain subset. It may be necessary, as in this case, to vary the isolation conditions if the desired mutant fails to appear. The classical procedure for the isolation of nutritional, temperature-sensitive, and UV-sensitive mutants, among others (14), starts with growth on YPD medium after mutagenesis. Since YPD is not a permissive medium for proline auxotrophs, it is not surprising that such mutants have not been isolated previously.

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