Gene-Enzyme Relationships in the Proline Biosynthetic Pathway of Saccharomyces cerevisiae

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The PRO1, PRO2, and PRO3 genes were isolated by functional complementation of pro1, pro2, and pro3 (proline-requiring) strains of Saccharomyces cerevisiae. Independent clones with overlapping inserts were isolated from S. cerevisiae genomic libraries in YEp24 (2μm) and YCp50 (CEN) plasmids. The identity of each gene was determined by gene disruption, and Southern hybridization and genetic analyses confirmed that the bona fide genes had been cloned. Plasmids containing each gene were introduced into known bacterial proline auxotrophs, and the ability to restore proline prototrophy was assessed. Interspecies complementation demonstrated that the S. cerevisiae PRO1 gene encoded γ-glutamyl kinase, PRO2 encoded γ-glutamyl phosphate reductase, and PRO3 encoded Δ1-pyrroline-5-carboxylate reductase. The presence of the PRO3 gene on a high-copy-number plasmid in S. cerevisiae caused a 20-fold overproduction of Δ1-pyrroline-5-carboxylate reductase. The PRO2 gene mapped on chromosome XV tightly linked to cdc66, and the PRO3 gene was located on the right arm of chromosome V between HIS1 and the centromere.

The pathway of proline biosynthesis has been established in studies on several organisms (2, 3, 26, 36, 37, 40) and is shown in Fig. 1. Glutamate is activated by γ-glutamyl kinase (ATP:L-glutamate 5-phosphotransferase; EC 2.7.2.11) to form glutamyl phosphate. This unstable intermediate is subsequently converted to glutamate semialdehyde by γ-glutamyl phosphate reductase (L-glutamate 5-semialdehyde: NADP+ oxidoreductase [phosphorylating]; EC 1.2.1.41). This pathway has been extensively studied in Escherichia coli, and it is believed that the first two enzymes form a complex so that the glutamyl phosphate remains enzyme bound (17). Glutamate semialdehyde spontaneously cyclizes to form Δ1-pyrroline-5-carboxylate (PSC). In the final step, PSC is converted to proline by the action of PSC reductase [L-proline:NAD(P)+ 5-oxidoreductase; EC 1.5.1.2].

The isolation and preliminary characterization of mutations leading to proline auxotrophy in the yeast Saccharomyces cerevisiae have been reported (7). Three complementation groups (pro1, pro2, and pro3) were identified. The requirement for proline in the pro1 and pro2 strains could be satisfied by arginine or ornithine, since these compounds can be converted to glutamate semialdehyde by enzymes in the arginine degradative pathway, bypassing the early biosynthetic steps (Fig. 1). pro3 mutants can grow only when proline is added to the medium and are defective in PSC reductase enzyme activity. A unique property of these Pro− strains is that they cannot grow on the rich medium (yeast extract-peptone-glucose) standardly used in yeast laboratories.

In S. cerevisiae, the enzyme PSC reductase also functions in the pathway for arginine degradation (10). The ornithine that is formed from arginine is converted to glutamate semialdehyde and PSC in the cytoplasm. PSC is then converted to proline by PSC reductase, and the proline is transported into the mitochondria, where it is converted to glutamate (11). Therefore, PSC reductase functions in both the proline biosynthetic and the arginine degradative pathways.

Because the first two enzyme activities were never measured in the studies of S. cerevisiae, the deficiencies of the pro1 and pro2 strains were not determined. Although the PSC reductase activity was substantially reduced in the pro3 mutant strain, this did not constitute evidence that PRO3 was the structural gene for this enzyme.

In this report, we describe the cloning of the S. cerevisiae PRO1, PRO2, and PRO3 genes by functional complementation of the corresponding mutations in yeast cells. The genes were introduced into E. coli and Salmonella typhimurium strains carrying single proB, proA, and proC mutations to test for interspecies complementation. In all cases, complementation was obtained. These studies demonstrate that the PRO1, PRO2, and PRO3 genes encode the enzymes glutamyl kinase, glutamyl phosphate reductase, and PSC reductase, respectively.

MATERIALS AND METHODS

Strains, media, and genetic methods. The S. cerevisiae and bacterial strains used in this work are described in Table 1. The media used for the growth of S. cerevisiae strains have been described previously (7), except that glucose was supplied instead of galactose. A minimal medium was used at all times instead of yeast extract-peptone-glucose medium. E. coli was grown in LB broth or agar supplemented with ampicillin (100 μg/ml) as required (24). M9 medium supplemented with Casamino Acids (25) was used in the bacterial proline auxotroph complementation studies. Matting, sporulation, and tetrad analyses were carried out by standard procedures (34).

Isolation of S. cerevisiae proline auxotrophs. Mutations were induced by treatment with ethyl methanesulfonate by the method of Fink (16). Isolation of proline-requiring strains was carried out by the method of Brandriss (7) in strain MB1433, which can be efficiently transformed with foreign DNA. Forty-seven independent recessive proline auxotrophic mutations were isolated and placed into complementation groups by crossing each Pro− strain with strains...
Plasmid pTB47 was constructed by inserting a 1.3-kb XbaI-PvuII URA3 fragment from pJH-U1 into plasmid pTB34 cut with PvuII and XbaI. Plasmid pTB45 was constructed by digesting plasmids pTB26 and YEp24 with SspI and recombining the fragments to form a 2μm vector carrying PRO1 DNA.

(ii) PRO2. The region of DNA that overlapped in plasmids pTB4, pTB5, and pTB6 is shown in Fig. 2B. Plasmid pTB20 was constructed by ligating the 3-kb BglII-PvuII fragment from pTB6 to plasmid YEp24 cut with BamHI and PvuII. Plasmid pTB21 was constructed by inserting a 3-kb NcoI fragment (one NcoI site in the vector) from plasmid pTB6 into the NcoI site of plasmid YEp24. Plasmid pTB21 was digested with MluI, and the ends were filled in with Klenow enzyme. A 1.2-kb Smal-NaeI TRP1 fragment was ligated to this fragment to form plasmid pTB27. Plasmid pTB41 was constructed in two steps. A 6-kb NruI-PvuII fragment from plasmid pTB6 (the NruI site is in the vector) was ligated to plasmid YEp24 cut with PvuII and SmaI, forming plasmid pTB39, with the fragment inserted so that a PvuII site was not regenerated. A 3.5-kb PvuII fragment from plasmid pTB4 (the second PvuII site is outside the region shown, near the ClaI site) was ligated into the unique NruI site (in pBR322 DNA) in plasmid pTB39 to form plasmid pTB41, so that the URA3 gene and 300 base pairs (bp) of plasmid pBR322 DNA were in effect inserted into the PvuII site of the PRO2 gene.

(iii) PRO3. Plasmid pTB18 was constructed by cutting plasmid pTB1 with PvuII, followed by ligation (Fig. 2C). Plasmid pTB19 contains the 3-kb BglII fragment of plasmid containing known proline biosynthetic mutations (MB1282, MB1291, and MB281-5A) (7). The mutagenesis yielded 16 pro1, 17 pro2, and 14 pro3 mutants.

Reagents. Restriction endonucleases were obtained from New England BioLabs (Beverly, Mass.) or BoehringerMannheim Biochemicals (Indianapolis, Ind.) and used as recommended by the manufacturer. DL-(+)-allo-8-Hydroxylysine hydrochloride was purchased from Calbiochem (La Jolla, Calif.). NADH was purchased from BoehringerMannheim Biochemicals. N-TRIS(hydroxymethyl)methyl-3aminopropanesulfonic acid buffer was purchased from Sigma Chemical Co. (St. Louis, Mo.). DL-P5C was synthesized by periodate oxidation of DL-6-hydroxylysine as described by Williams and Frank (38) and used as described previously (9).

Plasmid constructions. (i) PRO1. Plasmids pTB22 and pTB26 were constructed by restriction endonuclease digestion of pTB7 and subsequent ligation (Fig. 2A). Plasmid pTB23 was constructed by insertion of the 6-kilobase (kb) ClaI fragment of pTB7 into the ClaI site of plasmid YCP50. Plasmid pTB34 was constructed by cutting plasmid pTB7 with SmaI and deleting the URA3 gene on the vector as well as the region depicted in Fig. 2A. Plasmid pTB33 was constructed by cutting plasmid pTB26 with SnaBI and PvuII and inserting a 0.8-kb Smal-StuI fragment from plasmid pJH-W1 (a gift of J. Hill), which contains the TRP1 gene. Plasmid pTB42 contains the PvuII-SnaBI deletion without an insert. Plasmid pTB46 was constructed by inserting the 1.3-kb SacI-PvuII URA3 fragment from plasmid pJH-U1 (a gift of J. Hill) into plasmid pTB34 cut with PvuII and SacI.
pTB1 inserted into the BamH1 site of plasmid YEp24. Plasmid pTB14 was constructed by cutting plasmid pTB1 with KpnI, removing the 3' overhang by using DNA polymerase, and inserting the TRPI gene (a 0.8-kb Smal-Stul fragment from plasmid pHJ-W1). Plasmid pTB13 was constructed by cutting plasmid pTB1 with KpnI and BamH1 and inserting a KpnI-BgII fragment from plasmid pHJ-W1 containing the TRPI gene.

**TABLE 1. Strains used**

| Strain    | Genotype  | Source or reference |
|-----------|-----------|---------------------|
| S. cerevisiae |           |                     |
| MB1433    | MATa trp1 ura3-52 | M. C. Brandriss |
| DT1000    | MATa trp1 ura3-52 pro1-4 | This work |
| DT1019    | MATa trp1 ura3-52 pro1-6 | This work |
| DT1103    | MATa trp1 ura3-52 pro1-36 | This work |
| MB1282    | MATa hist4-42 lys2-1 pro1-189 | M. C. Brandriss |
| DT1104    | MATa trp1 ura3-52 pro2-5 | This work |
| DT1105    | MATa trp1 ura3-52 pro2-8 | This work |
| DT1112    | MATa trp1 ura3-52 pro2-198 | M. C. Brandriss |
| DT1125    | MATa trp1 ura3-52 pro3-6 | This work |
| DT1102    | MATa trp1 ura3-52 pro3-8 | This work |
| MB281-5A  | MATa hist4-42 pro3-66 | M. C. Brandriss |
| DT1100    | MATa trp1 ura3-52 pro3::TRPI | This work |
| DMT23-8A  | MATa trp1 hist4-42 pro3::TRPI | This work |
| K393-27C  | MATa ura3-1 his2 leu1-12 lys1-1 met4 pet8 | 23 |
| K396-27B  | MATa ura3-1 ade1 his1 leu2 lys7 met3 trp5-d | 23 |
| MB681-2C  | MATa ura3-52 ade6 arg4 aro7-1 met14 lys2-1 trp1 | M. C. Brandriss |
| MB681-1C  | MATa ura3-52 ade6 arg4 asp5 met14 lys2-1 pet17 | M. C. Brandriss |
| DMT30     | MATa trp1 + + + + + + | This work |
| DMT35     | MATa + ura3 pro3-6 his1 leu2 | This work |
|           | MATa + + + + + + cdc6 met7 gal2 ura1 |         |

| E. coli |           |                     |
|---------|-----------|---------------------|
| HB101   | hisD  hisM proA2 lacZ24 leuB thi-1 rpsL20 supE44 recA13 | 24 |
| G8      | F' = proB leu thr thi lac rpsL | L. Csonka |
| G14     | F' = proB leu thr thi lac rpsL | L. Csonka |
| KC201   | F'128 proB::T5 Φ(lac-lacZ) lacY' Φ + recA (proBA-gpt) hisD20 leu | L. Csonka |
| BW5560  | Δ(recA-argF-lac205 Φ(proH-proC44-1 rpsL) | L. Csonka |

| S. typhimurium |           |                     |
|----------------|-----------|---------------------|
| TL312          | proA39 galE496 metA22 metE55 rpsL20 xyl-404 ilv H1-b nml H2-enx | L. Csonka |
|                | hsdL6 hsdSA29 |                      |
| TL313          | proB9 galE496 metA22 metE55 rpsL20 xyl-404 ilv H1-b nml H2-enx | L. Csonka |
|                | hsdL6 hsdSA29 |                      |
| ProC137        | proC137    | L. Csonka |

Preparation of cell extracts and enzyme assays. The preparation of cell extracts and the P5C reductase enzyme assay have been described previously (7).

Protein determination. Protein concentrations were determined by the method of Bradford (6) with crystalline bovine serum albumin used as the standard.

Chromosomal location of the PRO2 and PRO3 genes. The 2μm mapping method of Falco et al. (15) was used to determine the chromosomal location of the PRO2 and PRO3 genes. The plasmid pTB21 (PRO2) was linearized by digestion with MluI, and the plasmid pTB1 (PRO3) was linearized by digestion with KpnI (Fig. 2). The linearized plasmids were used to transform strain MB1433 (MATa trp1 ura3-52) to Ura+. Stable (0 Ura− in 300 colonies) Ura− transformants were screened and subsequently mated to the 4 Ura− mapping strains (K393-27C, K396-27B, MB681-2C, and MB681-1C) listed in Table 1. After growth in yeast extract-peptone-glucose medium, the diploids showed instability for the Ura− character, segregating Ura− cells at a frequency of 1 to 5%. The Ura− segregants were tested for the appearance of additional genetic traits by replica plating to the appropriate medium.

RESULTS

Cloning the proline biosynthetic genes. The PRO1, PRO2, and PRO3 genes were identified on recombinant DNA.
plasmids by the functional complementation of mutations causing proline auxotrophy in *S. cerevisiae*.

New *pro1*, *pro2*, and *pro3* alleles were isolated by the method of Brandriss (7) (see Materials and Methods) in strain MB1433, which can take up DNA efficiently. Strains DT1000 (*pro1-4*), DT1051 (*pro2-8*), and DT1025 (*pro3-6*) were used in a two-step process to identify plasmids carrying the appropriate gene. DNA libraries based on plasmids YEp24 (high copy number and unstable) and YCp50 (low copy number and stable) were used to transform strains to uracil prototrophy. The Ura*+* transformants were pooled and screened for single colonies that were capable of growth
in the absence of proline. No transformants carrying pro1-complementing DNA were recovered after screening more than 60,000 Ura+ transformants from the YEp24 plasmid library. However, they were found after screening fewer than 8,000 Ura+ transformants from the YCp50 library. Colonies were obtained that carried pro2- or pro3-complementing DNAs on 2μm plasmids after screening fewer than 8,000 Ura+ colonies. Growth on a nonselective medium resulted in simultaneous loss of both markers at frequencies expected for genes on 2μm or CEN vectors.

Plasmid DNAs isolated from these transformants were used to transform E. coli to ampicillin resistance, from which large-scale plasmid preparations were obtained. Each plasmid carrying the complementing DNA was checked by reintroduction into the original auxotroph from which it was isolated as well as a second auxotroph of the same group (e.g., DT1019, pro1-6; DT1043, pro2-5; and DT1012, pro3-8). In each instance, all of the Ura+ transformants obtained were also Pro+. These plasmids were then analyzed by restriction endonuclease digestion to generate the maps shown in Fig. 2.

Localize of the PRO genes. A series of subclones of each of the PRO genes were constructed (see Materials and Methods), and the plasmids were tested for their ability to complement the corresponding auxotrophic mutation. A summary of the subclones and the results of the complementation tests are shown in Fig. 2.

The pro1 gene was located on approximately 2 kb of DNA bordered by the PvuII and BamHI sites of plasmid pTB34 (Fig. 2A). The fact that the TRPI insertion/deletion of plasmid pTB33 destroyed complementing activity while the URA3 insertion/deletion of plasmid pTB40 did not presented a paradox. Further investigation demonstrated that plasmid pTB42 (deleted for the DNA between the SnaBI and PvuII sites) was able to restore partial growth to a pro1 auxotroph. Both the TRPI and the URA3 genes are situated so that transcription is from right to left in the diagram. However, the promoter region of the TRPI gene was contiguous with the PRO1 DNA, while the URA3 promoter region was separated by approximately 300 bp of pBR322 DNA. It is possible that the PvuII site is located within the promoter region of the PRO1 gene and that the TRPI promoter interferes with PRO1 transcription, resulting in the Pro- phenotype. Alternatively, a fortuitous juxtaposition of DNA sequences may have caused the differences in gene expression.

A region common to three independent clones that could complement a pro2 auxotroph is shown in Fig. 2B. Several subclones were constructed in order to localize the gene within this region. A 3-kb region from NcoI to the insert pBR322 DNA junction left of the ClaI site on plasmid pTB21 retained the PRO2-complementing activity. The insertion of the TRPI gene into the MluI site of plasmid pTB21 (plasmid pTB27) did not interfere with the ability to complement. The insertion of the URA3 gene into the PvuII site (plasmid pTB41) destroyed PRO2-complementing activity and localized the gene to a 2-kb region between the NcoI and MluI restriction sites.

Figure 2C shows complementation results with the PRO3-bearing plasmids. A 3-kb BglII fragment of plasmid pTB1 was inserted into the BamHI site of plasmid YEp24 to construct plasmid pTB19, which maintained PRO3-complementing activity. Plasmid pTB18 lacked the 0.8 kb on the left and failed to complement the pro3 mutation. This localized the PRO3 gene to this region. Insertion of the TRPI gene into the KpnI site (plasmids pTB13 and pTB14) also demonstrated complementation and further supported this conclusion.

Disruptions of the PRO genes. To eliminate the possibility that suppressors of the various proline mutations had been cloned, it was necessary to prove that the complementing activity carried on the plasmids was due to the bona fide PRO1, PRO2, or PRO3 genes. The PRO-complementing DNA inserts were disrupted on plasmids and reintroduced into the wild-type genome to replace the resident wild-type copy by the method of Rothstein (33). The disrupted genes present on plasmids pTB47 (pro1::URA3), pTB41 (pro2::URA3), and pTB13 (pro3::TRPI) (see Materials and Methods for their construction) were released by using appropriate restriction digestion, and the resulting fragment was used to transform the wild-type strain MB1433 to either tryptophan or uracil prototrophy. Several prototrophs from each transformation were tested for their ability to grow on minimal medium lacking proline. Of 16 Ura+ transformants that resulted from integration of plasmid pTB47, 15 were also Pro-. Of 16 Ura+ transformants from integration of the pTB39 fragment, 14 were Pro-. Only 1 of 32 of the pTB13 transformants tested was Pro-. The low frequency of Trp+ Pro- transformants from the pTB13 fragment may have resulted from the limited region of PRO3 homology (less than 600 bp) on either side of the TRPI gene. It should also be noted that trp1 suppressors and revertants occur at a relatively high frequency and cause significant background.

One Ura+ Pro- Trp+ Pro+ transformant of each type was analyzed further by molecular and genetic tests. Genomic DNAs from strains MB1433 (wild-type parent), DT1000 (pro1-4), and DT1103 (pro1::URA3) were digested with BamHI and subjected to Southern analysis as described in Materials and Methods. The resulting blot was probed with the 5-kb BamHI fragment from plasmid pTB26 (Fig. 2A). The 5-kb BamHI fragment in MB1433 and DT1000 was replaced with a 5.7-kb fragment in strain DT1103 (Fig. 3A). These results are in agreement with the expected fragment sizes of the wild-type (pTB7) and disrupted (pTB47) plasmids.

Genomic DNAs from strains MB1433, DT1051 (pro2-8), DT1102 (pro2::URA3), DT1025 (pro3-6), and DT1100 (pro3::TRPI) were digested with BglII and subjected to Southern analysis. Blots containing wild-type and pro2 strains were probed with the 5.9-kb BglII fragment of plasmid pTB4 (Fig. 2B). The 5.9-kb fragment in MB1433 and DT1051 was replaced with a 7.2-kb fragment in strain DT1102 (Fig. 3B). Blots containing wild-type and pro3 strains were probed with the 3-kb BglII fragment of plasmid pTB1 (Fig. 2C). The 3-kb fragment in MB1433 and DT1025 was replaced by a 1.4-kb fragment in strain DT1100 (Fig. 3C). The fragment sizes obtained corresponded to the BglII fragment sizes on the wild-type plasmids pTB4 and pTB1 and the gene-disrupted plasmids pTB39 and pTB13. Therefore we conclude that homologous recombination between the fragment and the genome replaced the wild-type genes with the URA3- or TRPI-disrupted copies.

To confirm that the PRO1-, PRO2-, and PRO3-complementing DNAs were indeed the bona fide PRO genes and that the genetic region had been cloned, the following genetic analyses were performed. To prove genetic linkage of the marker gene and the PRO gene, strains DT1103 and DT1102 were each crossed to Pro+ ura3 strains to study cosegregation of the URA3 and pro1 or pro2 markers, respectively. The URA3 and Pro- markers cosegregated in all 21 tetrad analyzed in each cross. Strain DT1100 was
crossed to a Pro + trp1 strain to study the cosegregation of the disrupted gene and the TRPI marker. In all 21 tetrads analyzed, the Trp + and Pro + markers cosegregated.

Each of the disrupted strains was crossed to an appropriate proline auxotroph, forming diploid strains DMT37 (DT1103 × MB1282), DMT38 (DT1102 × MB1291), and DMT39 (DT1025 × DMT23-8A). The ability of the diploid to grow on a medium without proline was assessed. In each case the disrupted gene failed to complement the gene with the Pro + point mutation. The diploid strains DMT37, DMT38, and DMT39 were then sporulated, and the tetrads were analyzed. All pro2 and pro3 tetrads and 15 of 16 pro1 tetrads analyzed showed 0+/4+ segregation for proline prototrophy. One pro1 tetrad segregated 1+ : 3+ for proline prototrophy and is believed to reflect a gene conversion event. Since the parent diploid strain formed papillae on plates lacking proline, the disrupted PRO1 gene fragment contains information that can rescue the pro1 point mutation. These results confirm that the disruption mutations and the corresponding proline point mutations are allelic and not noncomplementing unlinked genes (1).

These results prove that the complementing activities carried on the cloned DNA represent the bona fide PRO1, PRO2, and PRO3 genes and not suppressors of the various mutations.

Levels of P5C reductase in the transformants. P5C reductase levels were measured in the wild-type (MB1433), mutant (DT1025), and PRO3 plasmid-bearing [DT1025 (pTB1)] strains. This plasmid contained PRO3-complementing activity located on a high-copy-number vector. The strain harboring this plasmid showed a 20-fold increase in enzyme levels compared with the wild-type strain (Table 2). This suggests that PRO3 is the structural gene for P5C reductase.

Measurement of glutamyl kinase and glutamyl phosphate reductase levels must await adaptation of existing bacterial enzyme assays to the S. cerevisiae system.

Complementation of bacterial proline auxotrophs. Although the Southern analysis and the genetic data confirmed that the bona fide PRO genes had been cloned, this did not prove that the cloned genes were the structural genes for the enzymes of proline biosynthesis. To assign the various PRO genes as the structural genes, we attempted interspecies complementation with strains of E. coli and S. typhimurium carrying proline biosynthetic mutations, since the gene-enzyme relationships of the bacterial proline biosynthetic enzymes have been firmly established (13, 14). The bacterial proB gene encodes the enzyme glutamyl kinase, the proA gene encodes the enzyme glutamyl phosphate reductase, and the proC gene encodes the enzyme PSC reductase.

Plasmids pTB26, pTB21, pTB19 (or pTB1), and YEp24 carrying the cloned yeast genes PRO1, PRO2, and PRO3 or no yeast insert were used to transform several E. coli and S. typhimurium proline auxotrophs to ampicillin resistance. Rapid isolation of plasmid DNAs and subsequent restriction digests were performed to establish that the ampicillin resistance was plasmid borne and due to the specified plasmid.

Strains containing each of the plasmids were then streaked

**TABLE 2.** 5SC reductase levels in wild-type, mutant, and PRO3-transformed strains

| Strain       | Relevant genotype | P5C reductase (sp act) |
|--------------|-------------------|------------------------|
| MB1433       | PRO3              | 315 ± 16               |
| DT1025       | pro2-6            | 4 ± 4                  |
| DT1025(pTB1) | PRO3 on 2µm plasmid | 5,899 ± 332           |

* The units are nanomoles of NADH oxidized per minute per milligram of protein. The values represent the average of at least three determinations. The growth medium contained 0.5% glucose and 0.2% ammonium sulfate, with proline (0.1%), uracil (20 mg/liter), and tryptophan (20 mg/liter) as supplements.

* Approximately 70% of the cells in the culture carried the plasmid under these growth conditions.
onto M9 medium with or without proline. The plates were incubated at 30°C, and growth was monitored over a period of several days (Table 3). Plasmid pTB26 was able to restore slow growth to E. coli KC201 (proB) (Fig. 4A) and S. typhimurium TL312 (proA) (not shown). Single colonies were distinguishable on the proline dropout plates after 3 to 4 days of growth at 30°C. Similarly, plasmid pTB21 was able to restore slow growth to S. typhimurium TL312 (proA), with single colonies appearing after 4 days of growth (Fig. 4B). No complementation was observed with the E. coli proA strain. The yeast plasmids pTB1 and pTB19 both complemented the proC defect in E. coli BW5560, but to different extents. Small single colonies of BW5560(pTB1) were visible on proline dropout plates after 4 days at 30°C. In contrast, large single colonies of BW5560(pTB19) appeared in only 2 days (Fig. 4C). No complementation was observed with S. typhimurium ProC137(pTB1), and unfortunately it was impossible to isolate a Salmonella ProC137 transformant carrying plasmid pTB19. The proline-supplemented plate supported growth of strains containing all plasmids tested.

The ability of the S. cerevisiae genes to complement the various bacterial Pro + strains establishes the gene-enzyme relationships of the yeast PRO1, PRO2, and PRO3 genes. Therefore, it can be concluded that the yeast PRO1 gene encodes glutamyl kinase, the PRO2 gene encodes glutamyl phosphate reductase, and the PRO3 gene encodes PSC reductase.

Chromosomal mapping of the PRO2 and PRO3 genes. The cloned PRO2 and PRO3 genes were assigned to chromosomes XV and V, respectively, by the 2μm mapping method of Falco et al. (15) as described in Materials and Methods. The PRO1 gene was refractory to mapping by this method.

In the diploid that carried the integrated PRO2 2μm plasmid, 3 of 100 Ura - colonies exhibited a Gly - phenotype, revealing the pet17 marker. This result indicates that PRO2 was located on chromosome XV. In the diploid that carried the integrated PRO3 2μm plasmid, 20 of 20 Ura - colonies were simultaneously His -, uncovering the his1 marker. This result indicates that the PRO3 gene is located on chromosome V.

These chromosome assignments were confirmed by tetrad analysis. The diploid strain DMT35 was formed and carried the markers pro2, his3, ade2, met7, and cdc66. pro2 was tightly linked to cdc66 and was located approximately 47 centimorgans (cM) distal to met7 on chromosome XV (Table 3).

![FIG. 4. Interspecies complementation of E. coli and S. typhimurium strains. Each plate pictured contained strains with the following plasmids: upper left, YEp24; upper right, pTB19; lower right, pTB26; lower left, pTB21. (A) Strain KC201 (proB) is pictured after 4 days of growth. The presence of plasmid pTB26 containing the PRO1 gene allowed growth in the absence of proline. (B) Strain TL312 (proA) is pictured after 4 days of growth. The presence of plasmid pTB21 containing the PRO2 gene allowed growth in the absence of proline. (C) Strain BW5560 (proC) is pictured after 2 days of growth. The presence of plasmid pTB19 containing the PRO3 gene allowed growth in the absence of proline.](http://www.jbact.net/41/2354.html)

| Strain* | Growth of strain carrying plasmid** |
|---------|-----------------------------------|
|         | YEp24 | pTB26 | pTB21 | pTB1 | pTB19 |
| E. coli |       |       |       |      |       |
| G8 (proA) | - | - | - | - | - |
| G14 (proB) | - | - | - | - | - |
| KC201 (proB) | - | + | - | - | - |
| BW5560 (proC) | - | - | +/− | - | + |
| S. typhimurium |       |       |       |      |       |
| TL312 (proA39) | - | + | - | - | - |
| TL313 (proB9) | - | + | - | - | - |
| ProC137 (proC) | - | + | - | - | ND |

* Strain BW5560 carries a deletion of the proC gene. Strain KC201 carries a Trc insertion in the proB gene.

** M9 medium without proline at 30°C on plates.

### TABLE 4. Chromosomal mapping of PRO2 and PRO3

| Gene (strain) | Gene pair* | No. of asc of type** | Map distance (cM)* |
|---------------|------------|----------------------|-------------------|
| PRO2 (DMT35)  | ade2-his3  | 5 0 17               | 38 (49.9)         |
|               | his3-met7  | 10 0 12              | 27 (23.5)         |
|               | met7-cdc66 | 5 1 16               | 47                |
|               | pro2-cdc66 | 22 0 2               | 0                 |
| PRO3 (DMT30)  | ura3-pro3  | 17 1 27              | 36.6              |
|               | his3-pro3  | 7 6 32               | Unlinked          |

* See Table 1 for genotypes of strains DMT35 and DMT30.

** PD, Parental ditype; NPD, nonparental ditype; T, tetratype.

* Distance in centimorgans equals [100(T + NPD) + NPD + T] (31). Previously published values are shown in parentheses (27, 28).
placed pro3 on the right arm of chromosome V between the centromere and hisl. By using trp1 as a centromere-linked marker, the distance between pro3 and its centromere was determined to be approximately 28 cM. The order and distances between these genes were ura3-10 cM-CEN5-28 cM-pro3-28 cM-hisl.

DISCUSSION

The PRO1, PRO2, and PRO3 genes were isolated from yeast genomic libraries by their ability to complement the respective proline auxotrophic mutations in S. cerevisiae. The apparent absence of PRO1-complementing activity in the 2μm libraries led us to wonder whether the PRO1 gene might be toxic in high copy. However, a subclone carried on the 2μm plasmid pTB45 (Fig. 2A) was maintained in high copy and allowed strain DT1000 to grow in the absence of proline. It is possible that a nearby gene outside the region carried on plasmid pTB45 is toxic in high copy or that the PRO1 gene is fortuitously absent from the YEp24 libraries screened.

When the plasmid-borne PRO genes were interrupted by the URA3 or TRP1 gene, they lost their complementing ability. Independent integration of the disrupted genes into the yeast genome resulted in replacement of the genomic copy with the URA3- or TRP1-disrupted gene. Subsequent genetic and Southern analysis demonstrated that the bona fide PRO1, PRO2, and PRO3 genes had been isolated and eliminated the possibility that a suppressor gene had been cloned.

Since it is not uncommon for S. cerevisiae genes to be efficiently transcribed and translated in E. coli (32), the yeast PRO genes were introduced into bacteria without the addition of specific bacterial promoters. The results in Fig. 4 and Table 3 show that the yeast genes varied in their ability to complement the bacterial mutations. This variation may result from differences in strain background or from interference by the remaining bacterial enzyme subunits.

The first two enzymes of proline biosynthesis are believed to function in a complex comprising six subunits of each polypeptide (13). In addition, purified glutamyl kinase activity is detectable only in assays containing purified glutamyl phosphate reductase (2, 17, 18). It is interesting that the PRO1 gene was able to complement a proB transposon Tn5 insertion (KC201), but was unable to complement the proB point mutation (G14). This result may be explained if the inactive E. coli subunit is bound to the enzyme complex, preventing interaction with the yeast enzyme, or if there is substantial subunit mixing. The absence of the bacterial protein in the Tn5 insertion strain may allow the yeast protein to form a complex with the glutamyl phosphate reductase to form a functional enzyme.

The E. coli PSC reductase (proC gene product) functions as a multimeric protein as well (14). The fact that intragenic complementation is frequent among various pro3 mutations (7; data not shown) suggests that the S. cerevisiae PSC reductase may also function as a multimer. The S. cerevisiae PRO3 gene was able to complement the proC deletion strain E. coli BW5560 but failed to complement an S. typhimurium point mutation, proC137. This result may again be attributed to interference from a nonfunctional subunit in the Salmonella strain which is not present in the proC deletion strain.

The ability of the yeast genes to complement the various bacterial PRO- strains establishes that the yeast PRO1, PRO2, and PRO3 genes encode glutamyl kinase, glutamyl phosphate reductase, and PSC reductase, respectively. Our observations support those of L. Csonka (personal communication), who found that the E. coli proB gene was able to complement the pro1 mutation in strain DT1000. This further suggests that the first two steps of proline biosynthesis occur in the cytoplasm, since it is unlikely that the E. coli protein possesses a mitochondrial signal sequence. The third step involving PSC reductase was shown to be cytoplasmic (11). This complete separation of the pathways of proline biosynthesis and degradation protects the cell from futile cycling and competition for common intermediates.

Many of the genes involved in amino acid biosynthetic pathways in S. cerevisiae are coregulated by a system known as the general amino acid control system (reviewed in reference 22). In response to starvation for any single amino acid, the expression of many biosynthetic enzymes is turned up. Wolfner et al. (39) suggested that at least one enzyme in the proline biosynthetic pathway was under general control. Now that the proline biosynthetic genes have been cloned, it will be possible to examine directly whether this pathway is regulated by general control or proline-specific mechanisms.

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