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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 Δ^1-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 Δ^1-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 Δ^1-pyrroline-5-carboxylic acid (P5C). In the final step, P5C is converted to proline by the action of P5C 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 P5C 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 P5C reductase also functions in the pathway for arginine degradation (10). The ornithine that is formed from arginine is converted to glutamate semialdehyde and P5C in the cytoplasm. P5C is then converted to proline by P5C reductase, and the proline is transported into the mitochondria, where it is converted to glutamate (11). Therefore, P5C 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 P5C 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 P5C 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. Mating, 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

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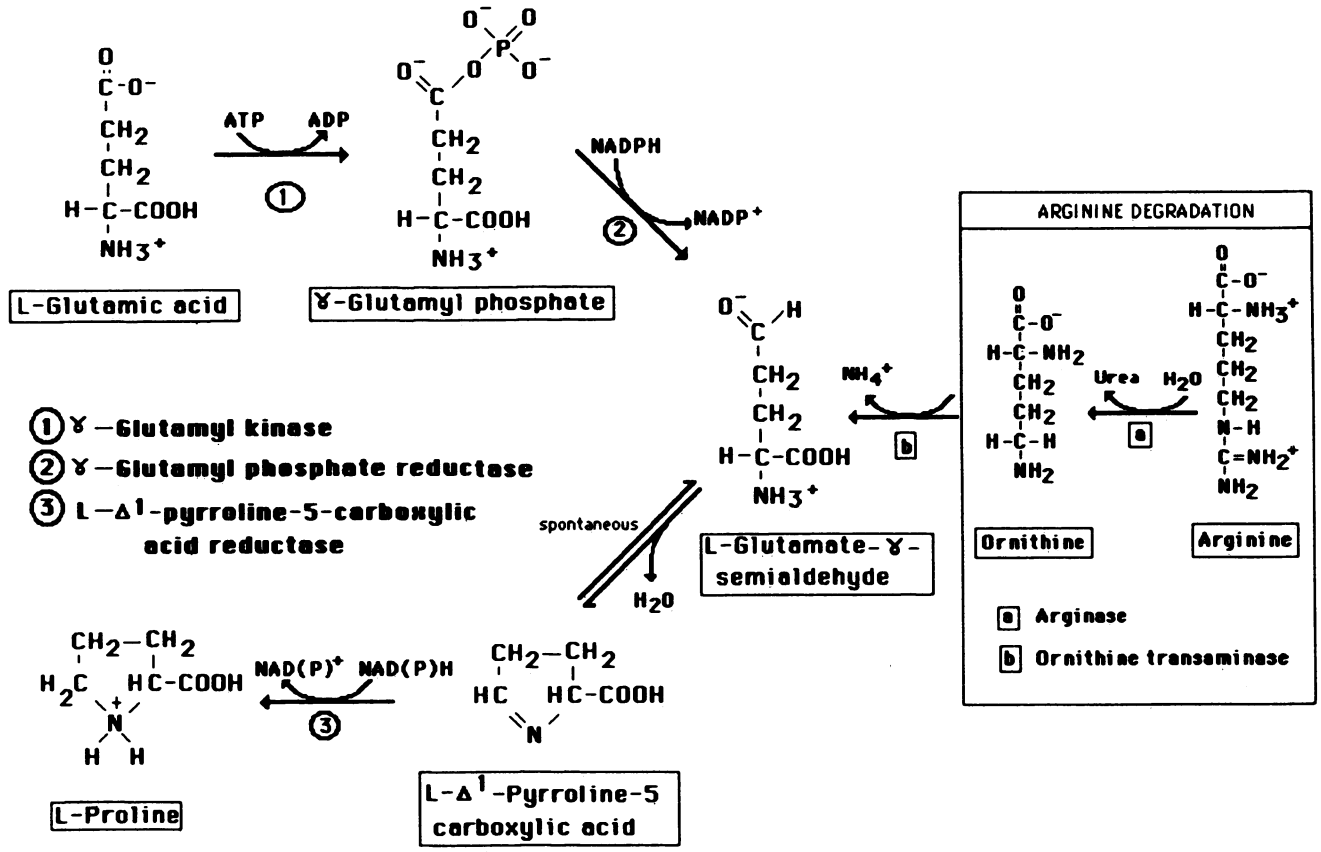

FIG. 1. Pathways leading to proline in the yeast *S. cerevisiae*.

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 Boehringer-Mannheim Biochemicals (Indianapolis, Ind.) and used as recommended by the manufacturer. DL-(+)-allo-δ-Hydroxylysine hydrochloride was purchased from Calbiochem (La Jolla, Calif.). NADH was purchased from Boehringer-Mannheim Biochemicals. *N*-Tris-(hydroxymethyl)methyl-3-aminopropanesulfonic acid buffer 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 (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) *Cla*I fragment of pTB7 into the *Cla*I site of plasmid YCp50. Plasmid pTB34 was constructed by cutting plasmid pTB7 with *Sma*I 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 *Sna*BI and *Pvu*II and inserting a 0.8-kb *Sma*I-*Stu*I fragment from plasmid pJH-W1 (a gift of J. Hill), which contains the *TRP1* gene. Plasmid pTB42 contains the *Pvu*II-*Sna*BI deletion without an insert. Plasmid pTB46 was constructed by inserting the 1.3-kb *Sac*I-*Pvu*II *URA3* fragment from plasmid pJH-U1 (a gift of J. Hill) into plasmid pTB34 cut with *Pvu*II and *Sac*I. Plasmid pTB47 was constructed by inserting a 1.3-kb *Xba*I-*Pvu*II *URA3* fragment from pJH-U1 into plasmid pTB34 cut with *Pvu*II and *Xba*I. Plasmid pTB45 was constructed by digesting plasmids pTB26 and YEp24 with *Ssp*I 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 *Bgl*II-*Pvu*II fragment from pTB6 to plasmid YEp24 cut with *Bam*HI and *Pvu*II. Plasmid pTB21 was constructed by inserting a 3-kb *Nco*I fragment (one *Nco*I site in the vector) from plasmid pTB6 into the *Nco*I site of plasmid YEp24. Plasmid pTB21 was digested with *Mlu*I, and the ends were filled in with Klenow fragment. A 1.2-kb *Sma*I-*Nae*I *TRP1* fragment was ligated to this fragment to form plasmid pTB27. Plasmid pTB41 was constructed in two steps. A 6-kb *Nru*I-*Pvu*II fragment from plasmid pTB6 (the *Nru*I site is in the vector) was ligated to plasmid YEp24 cut with *Pvu*II and *Sma*I, forming plasmid pTB39, with the fragment inserted so that a *Pvu*II site was not regenerated. A 3.5-kb *Pvu*II fragment from plasmid pTB4 (the second *Pvu*II site is outside the region shown, near the *Cla*I site) was ligated into the unique *Nru*I 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 *Pvu*II site of the *PRO2* gene.

(iii) ***PRO3.*** Plasmid pTB18 was constructed by cutting plasmid pTB1 with *Pvu*II, followed by ligation (Fig. 2C). Plasmid pTB19 contains the 3-kb *Bgl*II fragment of plasmid

TABLE 1. Strains used

Strain	Genotype	Source or reference
S. cerevisiae		
MB1433	*MATα trp1 ura3-52*	M. C. Brandriss
DT1000	*MATα trp1 ura3-52 pro1-4*	This work
DT1019	*MATα trp1 ura3-52 pro1-6*	This work
DT1103	*MATα trp1 ura3-52 pro1::URA3*	This work
MB1282	*MATa his4-42 lys2-1 pro1-189*	M. C. Brandriss
DT1043	*MATα trp1 ura3-52 pro2-5*	This work
DT1051	*MATα trp1 ura3-52 pro2-8*	This work
DT1102	*MATα trp1 ura3-52 pro2::URA3*	This work
MB1291	*MATa his4-42 lys2-1 pro2-198*	M. C. Brandriss
DT1025	*MATα trp1 ura3-52 pro3-6*	This work
DT1012	*MATα trp1 ura3-52 pro3-8*	This work
MB281-5A	*MATa his4-42 pro3-66*	M. C. Brandriss
DT1100	*MATα trp1 ura3-52 pro3::TRP1*	This work
DMT23-8A	*MATa trp1 his4-42 pro3::TRP1*	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	*MATα trp1* + + + + *MATa* + *ura3 pro3-6 his1 leu2*	This work
DMT35	*MATα trp1 ade2 his3 pro2-8* + + + + *MATa* + + + + *cdc66 met7 gal2 ura1*	This work
E. coli		
HB101	*hsdR hsdM proA2 lacZ24 leuB thi-1 rpsL20 supE44 recA13*	24
G8	F⁻ *proA leu thr thi lac rpsL*	L. Csonka
G14	F⁻ *proB leu thr thi lac rpsL*	L. Csonka
KC201	F'128 *proB*::Tn*5* Φ(*lacI-lacZ*) *lacY*⁺*A*⁺ *recA* Δ(*rpoBA-gpt*) *hsdS20 leu thi ara-14 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44*	L. Csonka
BW5560	Δ(*argF-lac*)*205* Δ(*phoA-proC*)*44-1 rpsL*	L. Csonka
S. typhimurium		
TL312	*proA39 galE496 metA22 metE55 rpsL120 xyl-404 ilv H1-b nml H2-enx hsdL6 hsdSA29*	L. Csonka
TL313	*proB9 galE496 metA22 metE55 rpsL120 xyl-404 ilv H1-b nml H2-enx hsdL6 hsdSA29*	L. Csonka
ProC137	*proC137*	L. Csonka

pTB1 inserted into the *Bam*HI site of plasmid YEp24. Plasmid pTB14 was constructed by cutting plasmid pTB1 with *Kpn*I, removing the 3′ overhang by digestion with DNA polymerase, and inserting the *TRP1* gene (a 0.8-kb *Sma*I-*Stu*I fragment from plasmid pJH-W1). Plasmid pTB13 was constructed by cutting plasmid pTB1 with *Kpn*I and *Bam*HI and inserting a *Kpn*I-*Bgl*II fragment from plasmid pJH-W1 containing the *TRP1* gene.

DNA preparation and transformation. DNA from YEp24 (5) and YCp50 (30) libraries (gifts from the laboratory of D. Botstein) was prepared from *E. coli* by the CsCl gradient method (24). Rapid isolation of plasmid DNA from *E. coli* was performed by the alkaline denaturation method of Birnboim and Doly (4). Isolation of plasmid DNA from *S. cerevisiae* was performed as described by Lorincz (A. Lorincz, Focus **6**:11, 1984). *S. cerevisiae* genomic DNA was isolated by the method of Holm et al. (20).

E. coli transformation was carried out by the $CaCl_2$ method described by Cohen et al. (12). *S. cerevisiae* transformation was performed by the spheroplasting method of Hinnen et al. (19) or the lithium acetate method of Ito et al. (21).

Electrophoresis of DNA, transfer to nitrocellulose, and hybridization. The methods used for electrophoresis, transfer to nitrocellulose, and hybridization (35) have been described previously (8).

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 *Mlu*I, and the plasmid pTB1 (*PRO3*) was linearized by digestion with *Kpn*I (Fig. 2). The linearized plasmids were used to transform strain MB1433 (*MATα 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

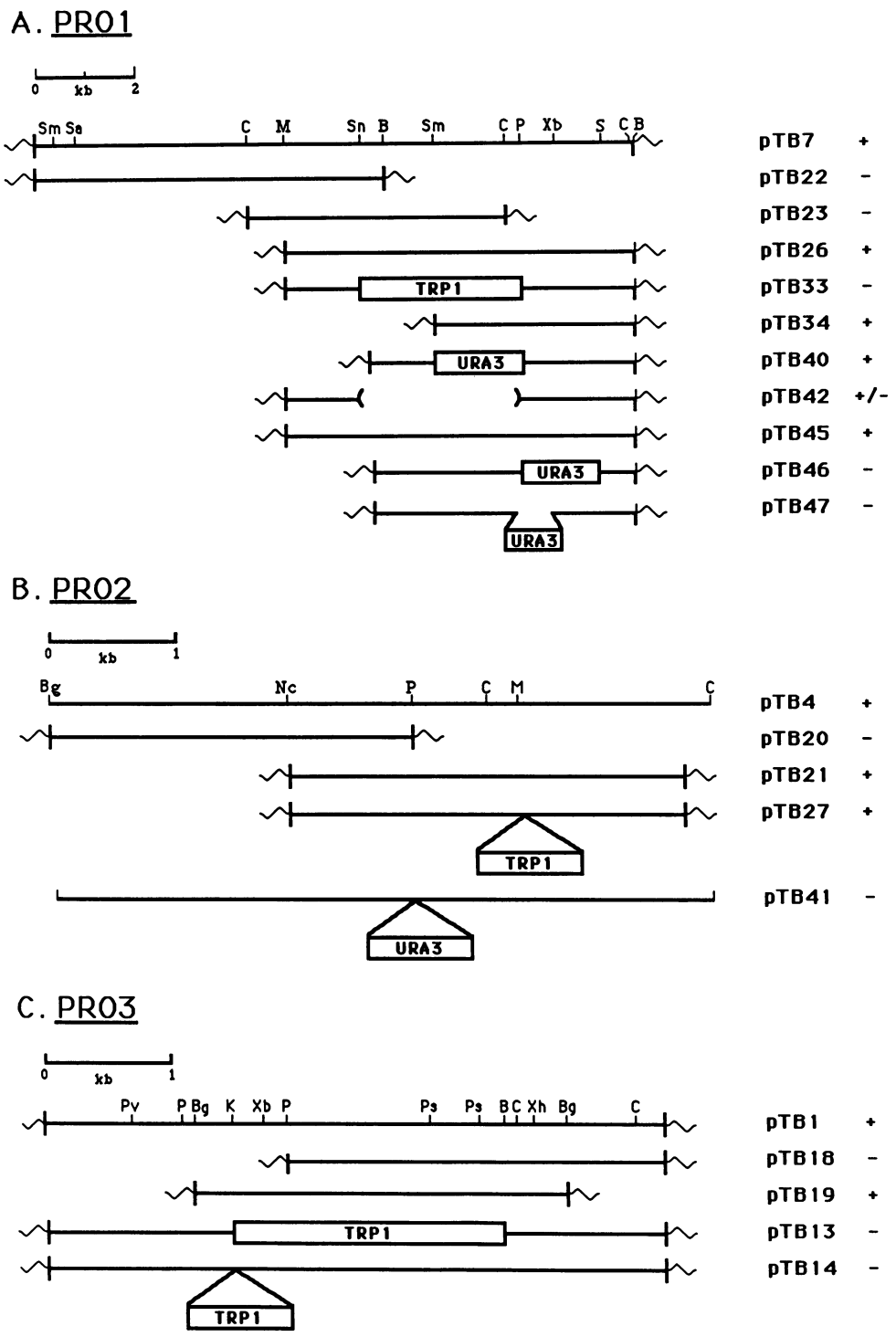

FIG. 2. Restriction maps of subclones and gene disruptions of plasmids containing (A) *PRO1*, (B) *PRO2*, or (C) *PRO3*. Abbreviations: B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; K, *Kpn*I; M, *Mlu*I; Nc, *Nco*I; P, *Pvu*II; Ps, *Pst*I; Pv, *Pvu*I; S, *Sac*I; Sa, *Sal*I; Sm, *Sma*I; Sn, *Sna*BI; Xb, *Xba*I; Xh, *Xho*I. Plasmid designations and the ability of strains carrying each plasmid to grow in the absence of are shown on the right.

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 that 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.

Localization 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 *Pvu*II and *Bam*HI sites of plasmid pTB34 (Fig. 2A). The fact that the *TRP1* insertion/deletion of plasmid pTB33 destroyed complementing activity while the *URA3* insertion/deletion of pTB40 did not presented a paradox. Further investigation demonstrated that plasmid pTB42 (deleted for the DNA between the *Sna*BI and *Pvu*II sites) was able to restore partial growth to a *pro1* auxotroph. Both the *TRP1* and the *URA3* genes are situated so that transcription is from right to left in the diagram. However, the promoter region of the *TRP1* 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 *Pvu*II site is located within the promoter region of the *PRO1* gene and that the *TRP1* 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 *Nco*I to the insert-pBR322 DNA junction left of the *Cla*I site on plasmid pTB21 retained the *PRO2*-complementing activity. The insertion of the *TRP1* gene into the *Mlu*I site of plasmid pTB21 (plasmid pTB27) did not interfere with the ability to complement. The insertion of the *URA3* gene into the *Pvu*II site (plasmid pTB41) destroyed *PRO2*-complementing activity and localized the gene to a 2-kb region between the *Nco*I and *Mlu*I restriction sites.

Figure 2C shows complementation results with the *PRO3*-bearing plasmids. A 3-kb *Bgl*II fragment of plasmid pTB1 was inserted into the *Bam*HI 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 *TRP1* gene into the *Kpn*I site (plasmids pTB13 and pTB14) also destroyed 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*::*TRP1*) (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 *TRP1* gene. It should also be noted that *trp1* suppressors and revertants occur at a relatively high frequency and cause significant background.

One Ura$^+$ Pro$^-$ or 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 *Bam*HI and subjected to Southern analysis as described in Materials and Methods. The resulting blot was probed with the 5-kb *Bam*HI fragment from plasmid pTB26 (Fig. 2A). The 5-kb *Bam*HI 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*::*TRP1*) were digested with *Bgl*II and subjected to Southern analysis. Blots containing wild-type and *pro2* strains were probed with the 5.9-kb *Bgl*II 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 *Bgl*II 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 *Bgl*II 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 *TRP1*-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 disrupted, 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 tetrads analyzed in each cross. Strain DT1100 was

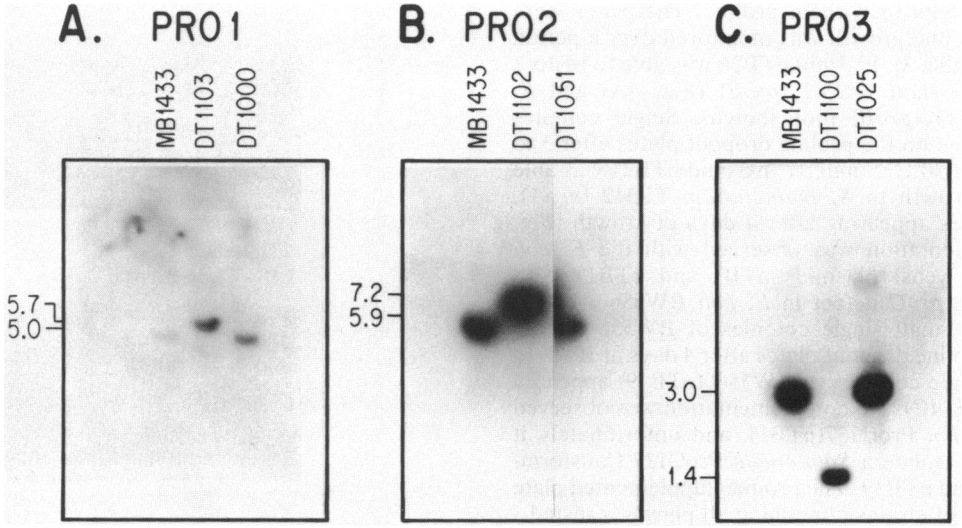

FIG. 3. Southern analysis of wild-type and mutant Pro$^-$ strains. (A) DNAs from strains MB1433 (wild type), DT1103 (*pro1*::*URA3*), and DT1000 (*pro1-4*) were digested with *Bam*HI. The DNAs were probed with the nick-translated 5-kb *Bam*HI fragment from plasmid pTB26. The insertion of the *URA3* gene increased the *Bam*HI fragment size from 5.0 to 5.7 kb. (B) DNAs from strains MB1433, DT1102 (*pro2*::*URA3*), and DT1051 (*pro2-8*) were digested with *Bgl*II. The DNAs were probed with the nick-translated 5.9-kb *Bgl*II fragment from plasmid pTB4. Insertion of the *URA3* gene increased the *Bgl*II fragment size from 5.9 to 7.2 kb. (C) DNAs from MB1433, DT1100 (*pro3*::*TRP1*), and DT1025 (*pro3-6*) were digested with *Bgl*II. The DNAs were probed with the nick-translated 3-kb *Bgl*II fragment from plasmid pTB1. Insertion of the *TRP1* gene decreased the *Bgl*II fragment size from 3 to 1.4 kb.

crossed to a Pro$^+$ *trp1* strain to study the cosegregation of the disrupted gene and the *TRP1* 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 P5C 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 digestions 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. P5C reductase levels in wild-type, mutant, and *PRO3*-transformed strains

Strain	Relevant genotype	P5C reductase sp act[a]
MB1433	*PRO3*	315 ± 16
DT1025	*pro3-6*	4 ± 4
DT1025(pTB1)[b]	*PRO3* on 2μm plasmid	5,899 ± 332

[a] 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.

[b] 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* TL313 (*proB*) (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 P5C 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 4). The *pro2* and *cdc66* mutations were not allelic, since a *pro2/cdc66* diploid was neither temperature sensitive nor proline-requiring at 35°C. Furthermore, addition of proline to the medium did not relieve the temperature sensitivity of the *cdc66* strain, as would be predicted if *cdc66* were a temperature-sensitive proline auxotroph.

Tetrad analysis was performed on diploid DMT30, which carried *pro3*, *ura3*, and *his1* mutations. The results (Table 4)

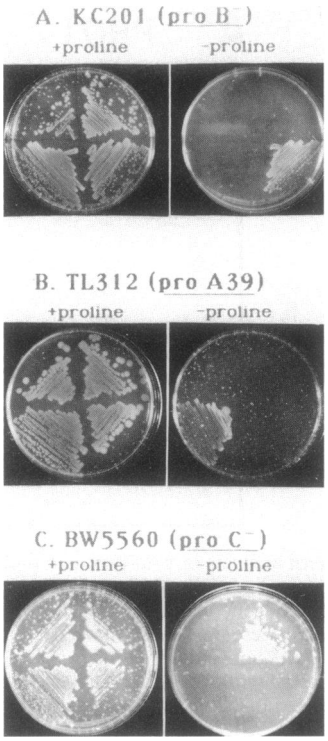

FIG. 4. Interspecies complementation of *E. coli* and *S. typhimurium* strains. Each plate pictured contained strains with the following plasmids: upper left, YEp24; upper right, pTB19; lower right, pTB26; lower left, pTB21. (A) Strain KC201 (*proB*) is pictured after 4 days of growth. The presence of 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.

TABLE 3. Interspecies complementation of the *S. cerevisiae PRO1*, *PRO2*, and *PRO3* genes

Strain[a]	Growth of strain carrying plasmid[b]:				
	YEp24	pTB26 (*PRO1*)	pTB21 (*PRO2*)	pTB1 (*PRO3*)	pTB19 (*PRO3*)
E. coli					
G8 (*proA*)	−	−	−	−	−
G14 (*proB*)	−	−	−	−	−
KC201 (*proB*)	−	+	−	−	−
BW5560 (*proC*)	−	−	−	+/−	+
S. typhimurium					
TL312 (*proA39*)	−	−	+	−	−
TL313 (*proB9*)	−	+	−	−	−
ProC137 (*proC*)	−	−	−	−	ND[c]

[a] Strain BW5560 carries a deletion of the *proC* gene. Strain KC201 carries a Tn*5* insertion in the *proB* gene.
[b] M9 medium without proline at 30°C on plates.
[c] ND, Not determined.

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

Gene (strain)	Gene pair[a]	No. of asci of type[b]:			Map distance (cM)[c]
		PD	NPD	T	
PRO2 (DMT35)	*ade2-his3*	5	0	17	38 (49.9)
	his3-met7	10	0	12	27 (23.5)
	met7-cdc66	5	1	16	47
	met7-pro2	5	1	16	47
	pro2-cdc66	22	0	0	2
PRO3 (DMT30)	*ura3-pro3*	17	1	27	36.6
	ura3-his1	7	6	32	Unlinked
	his1-pro3	19	0	26	28.8

[a] See Table 1 for genotypes of strains DMT35 and DMT30.
[b] PD, Parental ditype; NPD, nonparental ditype; T, tetratype.
[c] Distance in centimorgans equals [100(T + 6NPD)]/[2(PD + 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 *his1*. 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-*his1*.

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 Tn*5* 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 Tn*5* insertion strain may allow the yeast protein to form a complex with the glutamyl phosphate reductase to form a functional enzyme.

The *E. coli* P5C 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* P5C 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 P5C 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 P5C 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 on the basis of the response of *gcn* (*aas*) and *gcd* (*tra*) mutations to the presence of a proline analog. Using a different series of tests, Niederberger et al. (29) found no evidence that 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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LITERATURE CITED

1. **Atkinson, D. K.** 1985. Two recessive suppressors of *Saccharomyces cerevisiae CHO1* that are unlinked but fall in the same complementation group. Genetics **111**:1–6.
2. **Baich, A.** 1969. Proline synthesis in *Escherichia coli*. A proline inhibitable glutamic acid kinase. Biochim. Biophys. Acta **192**:462–467.
3. **Baich, A.** 1971. The biosynthesis of proline in *Escherichia coli*. Phosphate dependent glutamic γ-semialdehyde dehydrogenase (NADP), the second enzyme in the pathway. Biochim. Biophys. Acta **244**:129–134.
4. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
5. **Botstein, D., S. C. Falco, S. E. Stewart, M. Brennan, S. Scherer, D. T. Stinchcomb, K. Struhl, and R. W. Davis.** 1979. Sterile host yeast (SHY): a eucaryotic system of biological containment for recombinant DNA experiments. Gene **8**:17–24.
6. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
7. **Brandriss, M. C.** 1979. Isolation and preliminary characterization of *Saccharomyces cerevisiae* proline auxotrophs. J. Bacteriol. **138**:816–822.
8. **Brandriss, M. C.** 1983. Proline utilization in *Saccharomyces cerevisiae*: analysis of the cloned *PUT2* gene. Mol. Cell. Biol. **3**:1846–1856.
9. **Brandriss, M. C., and B. Magasanik.** 1979. Genetics and physiology of proline utilization in *Saccharomyces cerevisiae*: enzyme induction by proline. J. Bacteriol. **140**:498–503.
10. **Brandriss, M. C., and B. Magasanik.** 1980. Proline: an essential

intermediate in arginine degradation in *Saccharomyces cerevisiae*. J. Bacteriol. **143**:1403–1410.
11. **Brandriss, M. C., and B. Magasanik.** 1981. Subcellular compartmentation in control of converging pathways for proline and arginine metabolism in *Saccharomyces cerevisiae*. J. Bacteriol. **145**:1359–1364.
12. **Cohen, S. N., A. C. Y. Chang, and L. Hsu.** 1973. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. Proc. Natl. Acad. Sci. USA **69**:2110–2114.
13. **Deutch, A. H., K. E. Rushlow, and C. J. Smith.** 1984. Analysis of the *Escherichia coli proBA* locus by DNA and protein sequencing. Nucleic Acids Res. **12**:6337–6355.
14. **Deutch, A. H., C. J. Smith, K. E. Rushlow, and P. J. Kretschmer.** 1982. *Escherichia coli* Δ^1-pyrroline-5-carboxylate reductase: gene sequence, protein overproduction and purification. Nucleic Acids Res. **10**:7701–7714.
15. **Falco, S. C., L. Yuyang, J. R. Broach, and D. Botstein.** 1982. Genetic properties of chromosomally integrated 2μ plasmid DNA in yeast. Cell **29**:573–584.
16. **Fink, G. R.** 1970. The biochemical genetics of yeast. Methods Enzymol. **17A**:59–78.
17. **Hayzer, D. J., and T. Leisinger.** 1981. Proline biosynthesis in *Escherichia coli*. Biochem. J. **197**:269–274.
18. **Hayzer, D. J., and V. Moses.** 1978. Proline biosynthesis by cell-free extracts of *Escherichia coli* and potential errors arising from the use of a bioradiological assay procedure. Biochem. J. **173**:207–217.
19. **Hinnen, A., J. B. Hicks, and G. R. Fink.** 1978. Transformation of yeast. Genetics **75**:1929–1933.
20. **Holm, C., D. W. Meeks-Wagnor, W. L. Fangman, and D. Botstein.** 1986. A rapid, efficient method for isolating DNA from yeast. Gene **42**:169–173.
21. **Ito, H., Y. Fukoda, K. Murata, and A. Kimura.** 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153**:163–168.
22. **Jones, E. W., and G. R. Fink.** 1982. Regulation of amino acid biosynthesis and nucleotide biosynthesis in yeast, p. 181–229. *In* J. N. Strathern, E. W. Jones, and J. R. Broach (ed.), The molecular biology of the yeast *Saccharomyces*: metabolism and gene expression. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **Klapholz, S., and R. E. Esposito.** 1982. A new mapping method employing a meiotic Rec$^-$ mutant of yeast. Genetics **100**: 387–412.
24. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
25. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 431. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Miyake, T., and M. Demerec.** 1960. Proline mutants of *Salmonella typhimurium*. Genetics **45**:755–762.
27. **Mortimer, R. K., and D. Schild.** 1985. Genetic map of *Saccharomyces cerevisiae*, edition 9. Microbiol. Rev. **49**:181–212.
28. **Mortimer, R. K., and D. Schild.** 1980. Genetic map of *Saccharomyces cerevisiae*. Microbiol. Rev. **44**:519–571.
29. **Niederberger, P., G. Miozzari, and R. Hutter.** 1981. Biological role of the general control of amino acid biosynthesis in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **1**:584–593.
30. **Parent, S. A., C. M. Fenimore, and K. A. Bostian.** 1985. Vector systems for the expression, analysis and cloning of DNA sequences in *S. cerevisiae*. Yeast **1**:83–138.
31. **Perkins, D. D.** 1949. Biochemical mutants in the smut fungus *Ustilago maydis*. Genetics **34**:607–626.
32. **Ratzkin, B., and J. Carbon.** 1977. Functional expression of clone yeast DNA in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **74**:487–491.
33. **Rothstein, R. J.** 1983. One-step gene disruption in yeast. Methods Enzymol. **101**:202–211.
34. **Sherman, F., G. R. Fink, and C. W. Lawrence.** 1978. Methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
35. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
36. **Vogel, H. J., and D. M. Bonner.** 1954. On the glutamate-proline-ornithine interrelation in *Neurospora crassa*. Proc. Natl. Acad. Sci. USA **40**:688–694.
37. **Vogel, H. J., and B. D. Davis.** 1952. Glutamic γ-semialdehyde and Δ^1-pyrroline-5-carboxylic acid intermediates in the biosynthesis of proline. J. Am. Chem. Soc. **74**:109–112.
38. **Williams, I., and L. Frank.** 1975. Improved chemical synthesis and enzymatic assay of Δ^1-pyrroline-5-carboxylic acid. Anal. Biochem. **64**:85–97.
39. **Wolfner, M., D. Yep, F. Messenguy, and G. R. Fink.** 1975. Integration of amino acid biosynthesis into the cell cycle of *Saccharomyces cerevisiae*. J. Mol. Biol. **96**:273–290.
40. **Yura, T.** 1959. Genetic alteration of pyrroline-5-carboxylate reductase in *Neurospora crassa*. Proc. Natl. Acad. Sci. USA **45**:197–204.