

# The *Escherichia coli proB* gene corrects the proline auxotrophy of *Saccharomyces cerevisiae pro1* mutants

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**Summary.** We constructed plasmids carrying the *Escherichia coli proB* gene that encodes  $\gamma$ -glutamyl kinase, under the control of the yeast *GAL1* promoter. This construction was carried out with both the wild-type *proB*<sup>+</sup> gene and a mutant allele, *proB74*, that specifies an enzyme resistant to feedback inhibition by proline. Yeast *pro1* mutants harboring these plasmids are proline prototrophs. We conclude that the *pro1* mutation results in a deficiency in the  $\gamma$ -glutamyl kinase activity in *Saccharomyces cerevisiae*. Expression of the *proB74* allele in yeast resulted in enhanced resistance to the proline analogue L-azetidine-2-carboxylate and in a 2.4-fold elevation of the intracellular free proline levels. This result suggests that  $\gamma$ -glutamyl kinase is the rate limiting step in proline biosynthesis in yeast.

**Key words:** L-azetidine-2-carboxylate resistance – *Escherichia coli* –  $\gamma$ -glutamyl kinase – Proline – *Saccharomyces cerevisiae*

## Introduction

In bacteria, proline is synthesized from glutamate via  $\gamma$ -glutamyl phosphate,  $\gamma$ -glutamyl semialdehyde, and  $\Delta^1$ -pyrroline-5-carboxylate. There are three enzymes involved in this pathway:  $\gamma$ -glutamyl kinase,  $\gamma$ -glutamyl phosphate reductase, and  $\Delta^1$ -pyrroline-5-carboxylate reductase, that are specified in Enterobacteriaceae by the *proB*<sup>+</sup>, *proA*<sup>+</sup>, and *proC*<sup>+</sup> genes, respectively (Csonka and Baich 1983; Leisinger 1987).

The proline biosynthetic pathway has not been completely characterized in eukaryotes. There is some evidence that in plants (Stewart 1981) and in *Saccharomyces cerevisiae* (Brandriss 1979; Brandriss and Magasanik 1980) proline is also synthesized from glutamate via  $\Delta^1$ -pyrroline-5-carboxylate, but the intermediates between glutamate and  $\Delta^1$ -pyrroline-5-carboxylate have not been identified in these organisms. Brandriss (1979) isolated mutations causing proline auxotrophy in yeast, and assigned them to three complementation groups: *PRO1*, *PRO2*, *PRO3*. She also demonstrated that the *pro3* mutations resulted in a loss of the  $\Delta^1$ -pyrroline-5-carboxylate reductase (Brandriss 1979). However, the enzymatic defects in the *pro1* and *pro2* mutants have not been established (Brandriss 1979). Although

the above results of Brandriss are consistent with the proline biosynthetic pathway being the same in yeast as in bacteria, other possible routes, such as the conversion of glutamate to  $\Delta^1$ -pyrroline-5-carboxylate via ornithine by the enzymes of the arginine biosynthetic pathway (Brandriss and Magasanik 1980), have not been ruled out. In order to gain a further insight into the proline biosynthetic pathway in yeast, we tested whether the *proB*<sup>+</sup> gene of *Escherichia coli* could repair the proline auxotrophy of yeast *pro1* or *pro2* mutants. In this paper, we report the results of these studies.

## Materials and methods

**Bacterial and yeast growth conditions.** Bacteria were grown aerobically at 37° C in LB medium (Davis et al. 1980), or in minimal medium 63 (Cohen and Rickenberg 1956) supplemented with 10 mM glucose, 0.2 mM of the required amino acids or nucleotides, and 0.05 mM of the required vitamins. Antibiotics when used were: ampicillin (Amp) at 100 mg/l, tetracycline (Tet) at 15 mg/l, and kanamycin sulfate (Kan) at 75 mg/l, unless otherwise indicated. L-azetidine-2-carboxylate (Azt) was at 1 mM, when used. The superscripts r or s denote resistance or sensitivity, respectively, to the indicated antibiotics. Yeast strains were grown aerobically at 30° C in yeast nitrogen base medium (Difco), without amino acids, supplemented with the indicated sugars at 20 g/l, proline at 1 g/l, and uracil and tryptophan at 20 mg/l. Unless otherwise stated, the nitrogen source for yeast was 20 g/l (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; urea when used was at 10 g/l.

**Bacterial and yeast strains.** The microbial strains used are listed in Table 1. The wild-type *proB*<sup>+</sup> gene of *E. coli* and the mutant allele derived from it that confers Azt<sup>r</sup> (Csonka 1981) were cloned into pBR322, resulting in plasmids pLA1 and pLA101, respectively (Mahan and Csonka 1983). In the wild-type strain,  $\gamma$ -glutamyl kinase is subject to allosteric feedback inhibition by proline (Baich 1969). Smith (1985) carried out a partial purification of  $\gamma$ -glutamyl kinase from a strain carrying pLA101 and found that the enzyme from this strain was 300-fold less sensitive to feedback inhibition by proline than the wild-type enzyme.

**Recombinant DNA procedures.** All DNA modification enzymes were purchased from Bethesda Research Laboratories (BRL), except *EcoRV* (Boehringer Mannheim), and T4 DNA ligase (New England Biolabs). The *Bam*HI linkers were from BRL. The enzymes and linkers were used according to the recommendations of the suppliers.

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**Table 1.** Microbial strains usedA. Bacteria<sup>a</sup>

Strain	Chromosomal genotype	Plasmid (markers on plasmid)	Source
TL249	$\Delta(proBA)-47$	pLA101 ( <i>Escherichia coli</i> K12 <i>proB74 proA<sup>+</sup> phoE<sup>+</sup></i> )	Mahan and Csonka (1983)
TL250	$\Delta(proBA)-47$	pLA1 ( <i>Escherichia coli</i> K12 <i>proB<sup>+</sup> proA<sup>+</sup> phoE<sup>+</sup></i> )	Mahan and Csonka (1983)

B. *Saccharomyces cerevisiae* host strains

Strain	Genotype	Source or derivation
DT1000	$\alpha pro1-4 trp-1 ura3-52$	D. Tomenchock and M. Brandriss
DT1043	$\alpha pro2-5 trp-1 ura3-52$	D. Tomenchock and M. Brandriss
DT1051	$\alpha pro2-8 trp-1 ura3-52$	D. Tomenchock and M. Brandriss
MB1282	$\alpha pro1-189 his4-42 lys2-1$	Brandriss (1979)
MB1291	$\alpha pro2-198 his4-42 lys2-1$	Brandriss (1979)
MB1433	$\alpha trp1 ura3-52$	Brandriss (1979)
YM1296	$\alpha ura3-52 ade2-101 lys2-801$	H.M. Johnston
YM1384	$\alpha pro2-198 ura3-52 lys2-1$	YM1296 × MB1291
YM1388	$\alpha pro1-189 ura3-52 lys2-1$	YM1296 × MB1282

C. *Saccharomyces cerevisiae* transformants carrying plasmids

Strain	Host	Plasmid	Plasmid markers
HW1014	MB1433	pBM150	<i>URA3</i>
HW1015	DT1000	pCO228	$\Phi(P_{GAL1-proB^+}) URA3$
HW1016	DT1000	pCO138	$\Phi(P_{GAL1-proB74}) URA3$
HW1017	DT1000	pBM150	<i>URA3</i>
HW1018	DT1043	pCO228	$\Phi(P_{GAL1-proB^+}) URA3$
HW1019	DT1043	pCO138	$\Phi(P_{GAL1-proB74}) URA3$
HW1020	DT1043	pBM150	<i>URA3</i>

<sup>a</sup> Strains TL249 and TL250 are derived from *Salmonella typhimurium* LT-2; they carry the *proBA* region of *E. coli* K12 cloned into pBR322

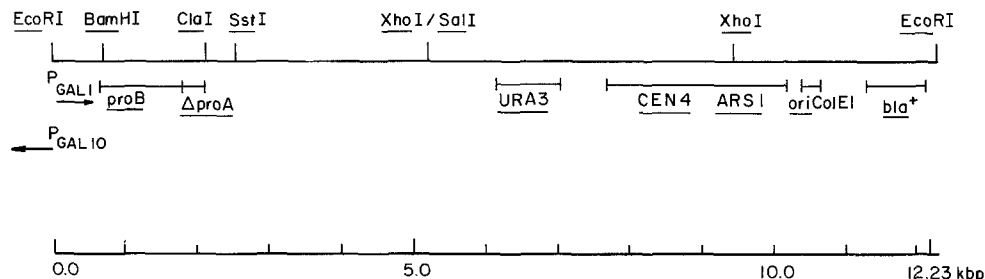
Small quantities of plasmid DNA were isolated by the rapid mini-prep. procedure of Holmes and Quigley (1981) and larger, purer quantities by the procedure of Birnboim and Doly (1979). Transformations of *E. coli* were done according to the procedure described by Maniatis et al.

(1982). Yeast strains were transformed according to the procedure of Ito et al. (1983).

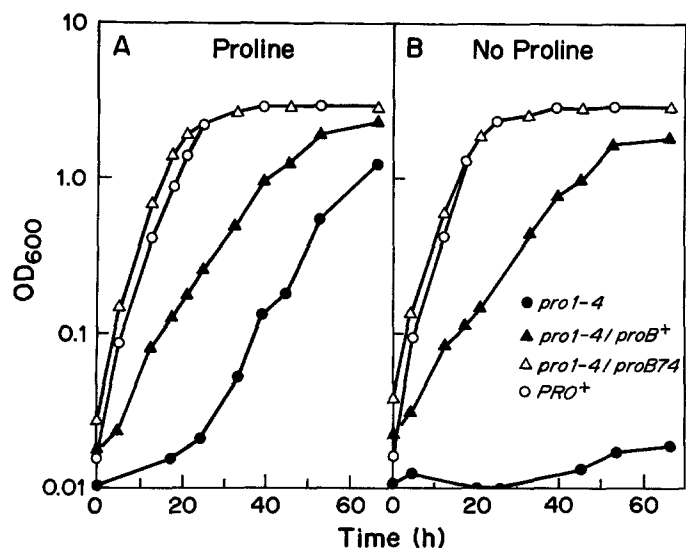
**Determination of proline levels.** Yeast strains were grown to an OD<sub>600</sub> of 1 (ca. 10<sup>7</sup> cells/ml), and cells from 5 ml samples were harvested by centrifugation. The cells were resuspended in 5 ml of methanol, and the proline content determined by gas chromatography, according to the method of Rhodes et al. (1981). The results are expressed as nmol proline per mg cell protein, with the latter determined by the method of Lowry et al. (1951).

**Insertion of the *proB* alleles under the control of the yeast *GAL1* promoter.** There is an *EcoRV* site 33 nucleotides upstream of the translation initiation site of the *E. coli proB* gene, and a *ClaI* site 1430 nucleotides from the *EcoRV* site within the downstream *proA* gene, such that the entire *proB* gene, plus the first 293 N-terminal residues of the *proA* gene are contained on this ca. 1.4 kbp *EcoRV-ClaI* fragment (Deutch et al. 1984). We isolated the respective 1.4 kbp *EcoRV-ClaI* fragments from pLA1 and pLA101 and converted the *EcoRV* ends to *BamHI* by the addition of a linker. Plasmids pCO22 and pCO13 were generated by inserting the above *BamHI-ClaI* fragments carrying the *proB<sup>+</sup>* or *proB74* alleles, respectively, into *BamHI* and *ClaI* sites of a plant expression vector, pBG8 (C.S. Orser, L.N. Csonka, B.W. Goodner and S.B. Gelvin, manuscript in preparation). Plasmids pCO22 and pCO13 carry an *XhoI* site 2.85 kbp downstream from the *ClaI* site, and the *proB<sup>+</sup>* and *proB74* alleles were isolated from pCO22 and pCO13 on a 4.25 kbp *BamHI-XhoI* fragment.

The yeast shuttle vector pMB150 (Johnston and Davis 1984) contains: (1) a bacterial replicon derived from pBR322 and the *bla<sup>+</sup>* gene (conferring Amp<sup>r</sup>) for maintenance and selection in *E. coli*, (2) a centromere and origin of replication (*CEN4 ARS1*) for maintenance in yeast, (3) the *URA3* gene, providing a selectable marker for the plasmid in yeast *ura3* mutants, and (4) the *GAL1/GAL10* promoters, suitable for the expression of foreign genes in yeast. There is a unique *BamHI* site in pMB150, 56 nucleotides downstream from the *GAL1* transcription start site (4 nucleotides upstream of the translation initiation site of the *GAL1* gene), and a unique *SalI* site 275 nucleotides downstream from the *BamHI* site. The *proB<sup>+</sup>* and *proB74* alleles were placed under control of the *GAL1* promoter by inserting the 4.25 kbp *BamHI-XhoI* fragments carrying these alleles into the *BamHI* and *SalI* sites of pMB150. The structures of the resultant plasmids pCO228 (*proB<sup>+</sup>*) and pCO138 (*proB74*) are shown in Fig. 1).



**Fig. 1.** The structures of plasmids pCO138 and pCO228. Plasmids pCO138 and pCO228 carry the *proB74* and the *proB<sup>+</sup>* alleles of *Escherichia coli* respectively, under control of the yeast *GAL1* promoter in plasmid pMB150. The plasmids are shown in a linearized form, cut at the *EcoRI* site



**Fig. 2.** The growth in liquid medium of yeast *pro1-4* mutants carrying the *Escherichia coli proB<sup>+</sup>* or *proB74* alleles. The optical density at 600 nm ( $OD_{600}$ ) of the cultures is plotted as a function of time of growth in yeast nitrogen base medium (Difco) without amino acids, containing 20 g/l galactose, 20 g/l raffinose, 20 g/l ammonium sulfate, 20 mg/l tryptophan, and 1 g/l proline as indicated. Yeast strains: ○, HW1014 (*PRO<sup>+</sup>*); ▲, HW1015 (*pro1-4/proB<sup>+</sup>*); △, HW1016 (*pro1-4/proB74*); and ●, HW1017 (*pro1-4*)

## Results and discussion

### *The proB<sup>+</sup> gene of E. coli complements yeast pro1 mutations*

Having placed the wild-type and proline over-producing mutant *proB* alleles under the control of the yeast *GAL1* promoter in plasmids pCO228 and pCO138, we transformed them, selecting *URA<sup>+</sup>* progeny, into *S. cerevisiae pro1 ura3* mutant strains YM1388 and DT1000, and into *pro2 ura3* mutant strains YM1384, DT1043, and DT1051. We found that both the *proB<sup>+</sup>* and the *proB74* alleles of *E. coli* conferred proline prototrophy on the *pro1* mutants but not on the *pro2* mutants, on solid media containing galactose plus raffinose (data not shown). Restoration of proline prototrophy of the *pro1* mutants did not occur in media lacking galactose or containing glucose (data not shown), in accordance with the regulatory characteristics of the *GAL1* promoter (Adams 1972; Johnston and Davis 1984).

In Fig. 2, we present the growth characteristics of the

transformants of the *pro1-4* mutant strain DT1000 in liquid culture. Both the wild-type (*proB<sup>+</sup>*) and the over-producing mutant (*proB74*) alleles of *E. coli* corrected the proline auxotrophy of this strain. These observations indicate that the *pro1* mutation of *S. cerevisiae* results in an impairment of either the synthesis or the in vivo activity of  $\gamma$ -glutamyl kinase. We can also conclude from these results that proline biosynthesis in yeast proceeds via the same intermediates as in bacteria, and a pathway involving ornithine is not likely to be of significance. Recently, M. Brandriss and D. Tomenchock (personal communication) found that the cloned *PRO1* gene of yeast can complement *proB* mutations in *Salmonella typhimurium*. This result is consistent with our observations, and moreover it conclusively establishes that the *PRO1* gene is the structural gene for  $\gamma$ -glutamyl kinase in *S. cerevisiae*.

Strain HW1015, which carries the wild-type *proB<sup>+</sup>* gene, grew more slowly in the absence of proline than the isogenic strain HW1016, which carries the proline over-producing *proB74* allele, or the control *PRO<sup>+</sup>* yeast strain HW1014 (Fig. 2). Thus, either the *proB<sup>+</sup>* allele is not expressed optimally, or its protein product does not have sufficient enzymatic activity or stability in yeast to support normal rates of proline formation. The feedback insensitive  $\gamma$ -glutamyl kinase encoded by the *proB74* allele apparently has greater in vivo activity, so that it can catalyze proline production at a nearly normal rate.

It was suggested on the basis of in vitro studies that in *E. coli*,  $\gamma$ -glutamyl kinase forms a complex with  $\gamma$ -glutamyl phosphate reductase (Hayzer and Moses 1978; Smith et al. 1984). Our finding that the *E. coli proB<sup>+</sup>* or *proB74* alleles can correct yeast *pro1* lesions suggests either that the *E. coli*  $\gamma$ -glutamyl kinase can complex adequately with the yeast  $\gamma$ -glutamyl phosphate reductase, or that it is not necessary to form such a complex.

Brandriss and Magasanik (1981) found that in yeast  $\Delta^1$ -pyrroline-5-carboxylate reductase is cytoplasmic. Our observation that the *E. coli proB* gene can correct yeast *pro1* mutations suggests that the  $\gamma$ -glutamyl kinase reaction probably occurs in the cytoplasm of yeast, since we did not attach a transit sequence to the cloned bacterial gene to direct the protein product to the mitochondria.

### *The proB74 allele confers increased proline production in yeast*

We tested whether the *proB74* allele might impart resistance to Azt in yeast. Because in the presence of ammonia the

**Table 2.** The doubling times and intracellular proline levels of the *Saccharomyces cerevisiae* strains

Additives to growth medium	Doubling time (h)			Intracellular free proline (n mole/mg cellular protein)		
	HW1014 ( <i>PRO1</i> )	HW1016 ( <i>pro1-4/proB74</i> )	HW1015 ( <i>pro1-4/proB<sup>+</sup></i> )	HW1014 ( <i>PRO1</i> )	HW1016 ( <i>pro1-4/proB74</i> )	HW1015 ( <i>pro1-4/proB<sup>+</sup></i> )
None	3.2	3.9	6.7	1.7	4.0	0.45
0.5 M NaCl	3.9	4.3	No growth	1.5	6.2	Not determined
1.0 M NaCl	6.1	7.5	No growth	3.5	7.0	Not determined
1 mM L-azetidine-2-carboxylate	8.6	3.0	27	0.5	3.7	0.15

The growth medium was yeast nitrogen base medium without amino acids, 20 mg/l tryptophan, 1 g/l urea, 20 g/l galactose, 20 g/l raffinose and the indicated additives

transport system for proline and Azt is repressed (Lasko and Brandriss 1981), we carried out these tests in a medium in which the  $(\text{NH}_4)_2\text{SO}_4$  was replaced with urea. The growth, on solid media, of both the *PRO*<sup>+</sup> strain HW1014 and strain HW1015 (*pro1-4/proB*<sup>+</sup>) was completely inhibited by 1.5 mM Azt, whereas strain HW1016 (*pro1-4/proB74*) was not affected, indicating that the proline over-producing bacterial allele confers Azt<sup>r</sup> on yeast. Growth tests in liquid medium also demonstrated that the *proB74* allele was able to confer increased Azt resistance on yeast (Table 2). Strain HW1015 (*pro1-4/proB*<sup>+</sup>) was much more sensitive to Azt than the control strain HW1014 (*PRO*<sup>+</sup>), in accordance with our above conclusion that the wild-type  $\gamma$ -glutamyl kinase synthesized under the control of the *GAL1* promoter cannot keep pace with the normal rates of proline synthesis in yeast.

We also determined the free proline levels in the three yeast strains of interest under various conditions, and presented the results in Table 2. The *proB74* transformant (strain HW1016) grown in galactose-raffinose-urea medium had slightly greater than a twofold higher proline level than the control *PRO*<sup>+</sup> strain HW1014. This result, together with the observation that the *proB74* allele imparts Azt<sup>r</sup> on yeast, suggests that  $\gamma$ -glutamyl kinase is the rate limiting step of proline biosynthesis in yeast, as in bacteria. The proline level found in the *proB*<sup>+</sup> transformant (strain HW1015) was lower than in either of the above two strains (Table 2), confirming our conclusion that the wild-type  $\gamma$ -glutamyl kinase of *E. coli* is unable to produce proline at the optimal rate in yeast. We also determined the free proline content of strains grown in the presence of 1.0 mM Azt (Table 2), and found that exposure to this proline analogue resulted in an approximately threefold decrease in the proline levels of strain HW1014 (*PRO*<sup>+</sup>) and strain HW1015 (*pro1/proB*<sup>+</sup>), whereas it caused less than a 10% decrease in the proline level of the Azt resistant strain HW1016 (*pro1/proB74*). The increased Azt resistance of the latter strain is probably the result of the increased production of proline, but we cannot rule out the possibility that Azt itself might be an allosteric inhibitor of both the bacterial and the yeast  $\gamma$ -glutamyl kinases, with the mutant enzyme encoded by the *proB74* allele having lost sensitivity to both feedback inhibitors.

In enteric bacteria, high intracellular proline levels bring about enhanced osmotic stress tolerance (Csonka 1981). The doubling times of the three yeast strains of interest grown in the presence of an additional 0.5 M or 1.0 M NaCl are also shown in Table 2. As can be seen, the *proB74* allele did not result in enhanced tolerance of osmotic stress in yeast. There are several possible reasons for this lack of increased NaCl tolerance. The increase in proline levels in the yeast strain carrying the *proB74* allele was only modest, and thus may not be sufficient to cause increased osmotic stress tolerance. In *S. typhimurium* (Csonka 1981) and in cultured tobacco cells (Handa et al. 1986), increased osmotic stress tolerance was seen in cells where proline levels were at least 100-fold greater than in the parental cell lines. Yeast accumulates high concentrations of glycerol in response to osmotic stress (Brown and Edgley 1980), and it may be that this organism cannot use proline as an osmoprotectant because it lacks transport systems that are able to accumulate or retain proline at the high concentrations that are required for this compound to function as an osmoprotectant.

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