The Gene-Enzyme Relationships of Proline Biosynthesis in *Escherichia coli*

By D. J. HAYZER AND TH. LEISINGER*

Mikrobiologisches Institut, Eidgenössische Technische Hochschule,
ETH-Zentrum, CH-8092 Zürich, Switzerland

(Received 23 November 1979)

A simple chromatographic procedure has been devised to separate γ -glutamyl phosphate reductase and 1-pyrroline-5-carboxylate reductase, allowing the measurement of the former in crude *Escherichia coli* extracts. Analysis of a number of strains of *E. coli* has demonstrated that gene *proA* codes for γ -glutamyl phosphate reductase and *proB* for γ -glutamyl kinase. Introduction of a ColE1 hybrid plasmid containing the *proA,B* region into a strain with a chromosomal deletion of *proA,B* led to 3- and 17-fold increases in the specific activities of γ -glutamyl kinase and γ -glutamyl phosphate reductase, respectively.

INTRODUCTION

In *Escherichia coli*, proline is synthesized from glutamate via a series of three enzymic steps with an intervening spontaneous conversion of intermediates:

- (1) L-Glutamate + ATP $\xrightarrow{\text{Mg}^{2+}}$ γ -Glutamyl phosphate + ADP
- (2) γ -Glutamyl phosphate + NADPH $\rightleftharpoons$ L-Glutamic acid 5-semialdehyde + NADP⁺ + P_i
L-Glutamic acid 5-semialdehyde $\rightleftharpoons$ L-1-Pyrroline-5-carboxylic acid
- (3) L-1-Pyrroline-5-carboxylic acid + NADPH $\rightleftharpoons$ L-Proline + NADP⁺

The reduction of glutamate to glutamic acid 5-semialdehyde is via the hypothesized intermediate γ -glutamyl phosphate, a compound of extreme lability when in a nucleophilic environment.

In reaction (1), the first enzyme in the sequence, γ -glutamyl kinase [ATP:L-glutamate 5-phosphotransferase; EC 2.7.2.11] catalyses the ATP-dependent γ -phosphorylation of glutamate (Baich, 1969). Regulatory control of the entire pathway by retro-inhibition by proline is exerted at this point (Baich, 1969). Reaction (2), the reduction of the activated intermediate to glutamic acid 5-semialdehyde, is mediated by an NADPH-dependent γ -glutamyl phosphate reductase (Baich, 1971). Spontaneous dehydration and cyclization results in the Schiff base formation of 1-pyrroline-5-carboxylate, which is reduced in reaction (3) by 1-pyrroline-5-carboxylate reductase [L-proline:NAD(P)⁺ 5-oxidoreductase; EC 1.5.1.2] to proline (Vogel & Davies, 1952; Rossi *et al.*, 1977). It has been suggested that the first and second enzymes of the pathway form a multimolecular complex to afford protection to the labile γ -glutamyl phosphate, direct transfer of the intermediate from one enzyme to the other avoiding equilibration with the surrounding medium (Baich, 1969; Gamper & Moses, 1974).

Proline biosynthesis is governed by three genetic loci. The loci *proA* and *proB* are associated with the conversion of glutamate to glutamic acid 5-semialdehyde. While it is controversial whether they are arranged contiguously (Condamine, 1971*a*) or at a distance of about 0.6 min from each other (Broda, 1974), they have been placed at 5.6 min on the linkage map of *E. coli* (Bachmann *et al.*, 1976). The *proC* locus is located at 8.7 min on the

Table 1. *Strains of Escherichia coli*

Strain(s)	Sex	Genotype	Origin/reference
χ 210	F ⁻	(λ^-) <i>leu thr thi lac tsx strA</i>	Curtiss (1964, 1965)
χ 680	F ⁻	(λ^-) <i>leu thr thi lac tsx strA proA</i>	Curtiss (1964, 1965)
χ 278	F ⁻	(λ^-) <i>leu thr thi lac tsx strA proB</i>	Curtiss (1964, 1965)
χ 277	F ⁻	(λ^-) <i>leu thr thi lac tsx strA proC</i>	Curtiss (1964, 1965)
χ 462	F ⁻	<i>leu lys met trp ara lac mal xyl strA proA</i>	Berg & Curtiss (1967)
χ 474	F ⁻	<i>leu lys met trp ara lac mal xyl strA proB</i>	Berg & Curtiss (1967)
χ 478	F ⁻	<i>leu lys met trp ara lac mal xyl strA proC</i>	Berg & Curtiss (1967)
G6, G8, G9, G10	F ⁻	(λ^-) <i>leu thr thi lac strA proA</i>	Condamine (1971 <i>a, b</i>)
G11, G12, G13, G14	F ⁻	(λ^-) <i>leu thr thi lac strA proB</i>	Condamine (1971 <i>a, b</i>)
CSH26	F ⁻	<i>thi ara Δ(lac pro)</i>	Miller (1972)
CSH52	F ⁻	<i>thi ara Δ(lac pro) strA recA</i> (ϕ 80d <i>lac</i> ⁺)	Miller (1972)
JA200/pLC44-11	F ⁺	<i>leu thr ΔtrpE5 lacY recA</i> (pLC44-11)*	Clarke & Carbon (1976)
CSH52/pLC44-11	F ⁺	<i>thi ara Δ(lac pro) strA recA</i> (ϕ 80d <i>lac</i> ⁺) (pLC44-11)*	F-mediated transfer of plasmid pLC44-11 to CSH52

* The ColE1 hybrid plasmid pLC44-11 constructed by Clarke & Carbon (1976) was tentatively identified as carrying the *proA* marker (Clarke & Carbon, 1976). The results of the present communication indicate that it carries the *proA* and the *proB* genes.

linkage map, on the distal side of *lac* (at 7.9 min) (Bachmann *et al.*, 1976). It has been identified as the gene coding for 1-pyrroline-5-carboxylate reductase (Baich & Pierson, 1965).

An unsolved problem has been to assign *proA* and *proB* to their respective enzymes, principally because of the difficulty of measuring γ -glutamyl phosphate reductase. The lability of γ -glutamyl phosphate precludes its use in the assay of this enzyme in the forward (biosynthetic) direction, and so the reverse reaction has to be measured, by the phosphate-dependent reduction of NADP⁺ to NADPH, with glutamic acid 5-semialdehyde (derived from equilibrium with 1-pyrroline-5-carboxylate) as the substrate. NADPH and 1-pyrroline-5-carboxylate, however, are also the substrates of 1-pyrroline-5-carboxylate reductase and the result is a cycle with the NADPH formed by the γ -glutamyl phosphate reductase being reoxidized. No net change in the A_{340} can be observed.

We have, therefore, devised a method for the removal of 1-pyrroline-5-carboxylate reductase from crude cell extracts, allowing a number of different *proA* and *proB* mutants to be screened for their γ -glutamyl phosphate reductase content. The proline-inhibitable activity of γ -glutamyl kinase could also be measured and consequently it was possible to establish the gene-enzyme relationships.

METHODS

Culture media. Nutrient yeast broth (NYB) was Difco no. 2 medium with additional yeast extract (0.5%, w/v). For testing strains for nutritional requirements and for the growth and storage of strains JA200/pLC44-11 and CSH52/pLC44-11, minimal medium M63 (Pardee & Prestidge, 1961) was used with glucose (0.5%, w/v) as the carbon source. Thiamin (0.001%, w/v) was always included. Amino acids, stored as stock solutions of 50 mM over chloroform, were each at a final concentration of 1.0 mM. Ability to metabolize lactose was determined using Difco EMB(lactose)-Levine medium.

Bacterial strains and plasmids. The strains used in this study are listed in Table 1. They were purified and examined for their amino acid and purine base requirements before overnight growth at 37 °C on NYB medium, addition of glycerol (final concentration 50%, v/v) and storage at -70 °C. For enzyme testing, cultures were taken from the -70 °C store and the purification and checking were repeated.

Crude extract preparation. Starter cultures were grown overnight in 25 ml NYB medium or minimal medium M63, suitably supplemented for strains JA200/pLC44-11 and CSH52/pLC44-11. Cells were transferred to fresh NYB medium or to medium M63 (1 l per 2 l Fernbach flask) and grown with rotary shaking at 37 °C for 7 h (at which point they were in late-exponential phase), then centrifuged at 10000 g for 20 min, washed once with 50 mM-Tris/HCl buffer, pH 7.2, and stored as a paste at -20 °C. Frozen cell paste (2 g) was suspended in 7 ml 50 mM-Tris/HCl buffer, pH 7.2, containing 1 mM-dithiothreitol (buffer A). Cellular disruption was effected by a double passage through a French press at 38.5 MPa and the cell debris was removed by centrifugation at 40000 g for 30 min. All operations were at 0 °C.

Assays for the enzymes of proline biosynthesis in crude extracts. γ -Glutamyl kinase. Crude extract (2.5 ml) was desalted with a Pharmacia PD-10 (Sephadex G-25M) column equilibrated with buffer A. The enzyme assay mixture contained, in a final volume of 0.25 ml: L-glutamate, 50 mM; ATP, 10 mM; MgCl₂, 20 mM; hydroxylamine.HCl, 100 mM; Tris base, 50 mM, adjusted to pH 7.0; and 100 μ l of desalted extract containing approximately 3.0 mg protein. The reaction was started by the addition of extract. Parallel tubes contained 100 mM-L-proline in addition. After 30 min at 37 °C the reaction was stopped by the addition of 1.0 ml of a solution containing FeCl₃.3H₂O (2.5%, w/v) and trichloroacetic acid (6%, w/v) in 2.5 M-HCl. Precipitated protein was removed by centrifugation and the A_{535} was recorded against a water blank. All tubes were in quadruplicate and the absorbance values were corrected for the values obtained at zero incubation time. The amount of γ -glutamyl hydroxamate produced was estimated from the molar extinction coefficient of 250 l mol⁻¹ cm⁻¹ for the Fe³⁺-hydroxamate complex obtained with authentic γ -glutamyl hydroxamate. The difference, after 30 min incubation, between tubes with and without proline represented the activity of the proline-inhibitable γ -glutamyl kinase.

γ -Glutamyl phosphate reductase. To 5.0 ml crude extract was added 0.55 ml streptomycin sulphate (20%, w/v, in buffer A at pH 7.2) and the mixture was stirred for 30 min. Precipitated nucleic acids were removed by centrifugation at 40000 g for 30 min. The supernatant was applied to the drained surface of a DEAE-cellulose (Whatman DE 52) column (1.4 $\times$ 6.0 cm) equilibrated with buffer A and the column was washed with the same buffer at a flow rate of 44 ml h⁻¹. Fractions of 2.0 ml were collected. Eluted proteins were detected by their absorbance at 280 nm. The enzyme was assayed with a reaction mixture containing, in a final volume of 1.0 ml: DL-1-pyrroline-5-carboxylate, 2 mM; NADP⁺, 1 mM; potassium phosphate, 100 mM; imidazole base, 50 mM; and 100 μ l of DEAE-cellulose-treated extract at pH 7.0. The rate of increase in A_{340} at 30 °C was recorded against a parallel cuvette identical to the above but lacking the phosphate.

1-Pyrroline-5-carboxylate reductase. The assay mixture contained, in a final volume of 1.0 ml: DL-1-pyrroline-5-carboxylate, 0.5 mM; NADPH, 0.15 mM; Tris base, 50 mM; and crude extract at pH 7.0. The rate of decrease in A_{340} was recorded at 30 °C.

All enzyme assays were checked for linearity of product formation with time and protein concentration. One enzyme unit (U) was defined as the synthesis of 1.0 μ mol product min⁻¹ under standard assay conditions.

Synthesis of DL-1-pyrroline-5-carboxylate. The method of Williams & Frank (1975) was employed.

Protein estimation. Protein was determined by the biuret method as described by Cooper (1977).

Plasmid transfer. *Escherichia coli* strains CSH52 and JA200/pLC44-11 were grown overnight with aeration at 37 °C in NYB medium, and then 10 ml of each culture were mixed and very gently shaken at 37 °C for 1 h. Samples of the mixture were subsequently plated on to minimal medium and incubated for 48 h. Colonies were purified on the same medium and one was selected for enzymic analysis.

RESULTS

Chromatographic separation of γ -glutamyl phosphate reductase and 1-pyrroline-5-carboxylate reductase

γ -Glutamyl phosphate reductase could not be detected in the crude extracts of any *E. coli* strain which had an active 1-pyrroline-5-carboxylate reductase. However, the passage of crude extract containing streptomycin sulphate through a column of DEAE-cellulose separated the two enzymes, permitting the γ -glutamyl phosphate reductase to be measured (Fig. 1). It was essential that the streptomycin sulphate addition was made and the excess not removed by dialysis, otherwise both enzymes bound to the matrix and their individual elutions required salt washes to be applied, reducing the convenience of the method. For routine operation, only the first 10 fractions of eluate, containing the γ -glutamyl phosphate reductase and about one-third of the applied protein, were collected. 1-Pyrroline-5-carboxylate reductase was most readily assayed with non-chromatographed crude extract, although 0.3 M-NaCl was effective in eluting this enzyme from the column.

Fig. 1. DEAE-cellulose chromatography of crude cell-free extract of *E. coli* strain χ 210 following treatment with streptomycin sulphate. The procedure is described in Methods. NaCl (0.3 M) was included in the buffer A after fraction 8. Protein elution was determined by the absorbance at 280 nm (○). Enzyme activities: γ -glutamyl phosphate reductase (●); 1-pyrroline-5-carboxylate reductase (□).

γ -Glutamyl phosphate reductase could be directly measured in the crude extract of strain χ 277 (*proC*) from which 1-pyrroline-5-carboxylate reductase was absent. By this means it was possible to estimate that the percentage yield of the enzyme, after the DEAE-cellulose step, was approximately 75%, the loss of activity being predominantly due to an effect of the streptomycin sulphate.

Enzyme analysis of proline auxotrophic mutants of E. coli

The measured activities of the three enzymes of proline biosynthesis in *E. coli* are given in Table 2. The *pro*⁺ strains χ 210, JA200/pLC44-11 (the latter containing a ColE1-*proA,B* hybrid plasmid) and a strain with a chromosomal *lac pro* deletion but also possessing the hybrid plasmid (CSH52/pLC44-11) had detectable amounts of all three enzymes. Mutants designated *proA* lacked an active γ -glutamyl phosphate reductase whereas *proB* mutants were without γ -glutamyl kinase activity. Apparent double mutants (χ 278 and χ 474) were identified. 1-Pyrroline-5-carboxylate reductase was inactive when *E. coli* carried a *proC* mutation, as previously observed (Baich & Pierson, 1965). Two strains, CSH26 and CSH52, with the *lac pro* region of the genome deleted lacked both the γ -glutamyl kinase and γ -glutamyl phosphate reductase.

All strains except JA200/pLC44-11 and CSH52/pLC44-11 were grown on NYB medium and it was possible that repression of the proline biosynthetic enzymes had occurred. This was tested by growing strain χ 277 on minimal medium with limiting proline (120 μ g ml⁻¹) and harvesting the cells 1 h after exhaustion of proline, as established by the cessation of growth. Enzyme activities were insignificantly different from those obtained with cells grown on NYB medium.

Enzyme activities in E. coli bearing plasmid pLC44-11

Escherichia coli strains carrying the hybrid plasmid pLC44-11 had elevated specific activities of both γ -glutamyl kinase and γ -glutamyl phosphate reductase when compared with strains in which the genes *proA* and *proB* were only chromosomal (Table 2). Maximal activity of γ -glutamyl phosphate reductase was found under conditions which applied

Table 2. *Specific activities of the enzymes of proline biosynthesis in Escherichia coli*

Figures in parentheses indicate the number of times each strain was tested; where this was three or more the mean deviation is given, except when enzyme activities were not detectable.

Strain	Relevant genotype	$10^3 \times$ Enzyme activity [U (mg protein) ⁻¹]				Phenotype		
		γ -Glutamyl kinase	γ -Glutamyl phosphate reductase	1-Pyrroline-5-carboxylate reductase		EI	EII	EIII
$\chi 210$	<i>pro</i> ⁺	0.66 $\pm$ 0.05 (5)	1.14 (2)	657 $\pm$ 80 (3)		+	+	+
JA200/pLC44-11	<i>pro</i> ⁺ (<i>proA</i> ⁺ <i>B</i> ⁻)	1.87 (1)	4.97 (2)	261 (1)		+	+	+
CSH52/pLC44-11	Δ <i>proA,B</i> (<i>proA</i> ⁺ <i>B</i> ⁻)	1.71 $\pm$ 0.28 (3)	19.90 (2)	454 (2)		+	+	+
$\chi 680$	<i>proA</i>	0.62 (2)	< 0.03 (2)	417 (2)		+	-	+
$\chi 462$	<i>proA</i>	0.51 (2)	< 0.03 (2)	570 (1)		+	-	+
G6	<i>proA</i>	0.50 (1)	< 0.03 (1)	540 (1)		+	-	+
G8	<i>proA</i>	0.92 (1)	< 0.03 (1)	597 (1)		+	-	+
G9	<i>proA</i>	0.48 (1)	< 0.03 (1)	609 (1)		+	-	+
G10	<i>proA</i>	0.56 (1)	< 0.03 (1)	652 (1)		+	-	+
$\chi 278$	<i>proB</i>	0.09 (2)	< 0.03 (2)	574 (1)		-	-	+
$\chi 474$	<i>proB</i>	0.12 $\pm$ 0.16 (3)	< 0.03 (3)	570 (1)		-	-	+
G11	<i>proB</i>	< 0.06 (2)	1.00 (2)	495 (1)		-	+	+
G12	<i>proB</i>	< 0.06 (2)	0.60 (2)	462 (1)		-	+	+
G13	<i>proB</i>	< 0.06 (2)	0.75 (2)	1219 (1)		-	+	+
G14	<i>proB</i>	< 0.06 (2)	0.57 (2)	717 (1)		-	+	+
$\chi 277$	<i>proC</i>	0.51 $\pm$ 0.16 (4)	1.02 $\pm$ 0.35 (10)	0.0 (3)		+	+	-
$\chi 478$	<i>proC</i>	0.44 (1)	0.62 (2)	0.0 (2)		+	+	-
CSH26	Δ <i>proA,B</i>	< 0.06 (1)	< 0.03 (1)	470 (1)		-	-	+
CSH52	Δ <i>proA,B</i>	< 0.06 (1)	< 0.03 (1)	440 (1)		-	-	+

Table 3. *Effect of growth conditions on the specific activities of γ -glutamyl kinase and γ -glutamyl phosphate reductase in E. coli CSH52/pLC44-11*

Figures in parentheses indicate the number of determinations made; average values of the specific activities are given.

Strain	$10^3 \times$ Enzyme activity [U (mg protein) ⁻¹]			
	γ -Glutamyl kinase		γ -Glutamyl phosphate reductase	
	Minimal medium	NYB medium	Minimal medium	NYB medium
CSH52/pLC44-11	1.71 (3)	1.70 (2)	19.90 (2)	7.23 (2)

selective pressure for the retention of the plasmid, i.e. with a *pro* host (CSH52) growing in minimal medium without proline (Table 3).

DISCUSSION

Previous attempts to establish the gene-enzyme relationships of proline biosynthesis in *E. coli* have relied upon indirect methods to determine whether γ -glutamyl kinase and γ -glutamyl phosphate reductase were present in a particular mutant. Incubation of resting *proA* cells in the presence of glutamate and hydroxylamine gave γ -glutamyl hydroxamate (Inuzuka *et al.*, 1976). It was concluded that, since *proB* strains showed no such activity, inactivation of γ -glutamyl phosphate reductase had resulted in the accumulation of γ -glutamyl phosphate which reacted with hydroxylamine. It was not demonstrated whether proline, the retro-inhibitor of the γ -glutamyl kinase (Baich, 1969), could inhibit the hydroxamate synthesis. Condamine (1971*b*) demonstrated that mutations in the *proB* locus resulted in proline excretion, presumably due to the loss of feedback regulation of the glutamyl kinase.

The conclusion to be drawn from these reports is that *proB* is the gene for the γ -glutamyl kinase and that *proA* codes for γ -glutamyl phosphate reductase. The results presented in this paper confirm, by direct measurement of the enzyme activities, these gene-enzyme relationships. The relationship of gene *proC* to 1-pyrroline-5-carboxylate reductase is in agreement with established results (Baich & Pierson, 1965).

Strains χ 278 and χ 474 lack both the γ -glutamyl kinase and γ -glutamyl phosphate reductase (Table 2). Genetic analysis (Curtiss, 1965) and the rate of spontaneous reversion (Condamine, 1971*a*) support the notion that strain χ 278 is a point, *proB*, mutant. However, F-prime factors carrying the *proA* or *proB* locus were unable to complement the deficiency of strain χ 278, suggesting that both gene products were absent (Condamine, 1971*a*) as, indeed, was the situation shown by the enzyme analysis reported in this paper. The absence of enzymically active products of the *proA* and *proB* genes in strain χ 278 would also explain why it proved impossible to obtain *in vitro* complementation between extracts of this mutant and extracts of strain χ 680 (*proA*) (Hayzer & Moses, 1978). Conjugational and transductional data from strain χ 474 have suggested that this strain, in addition to the *proB* mutation, carries a second mutation very close to *proA* independently conferring a proline requirement (Broda, 1974). While our enzyme measurements are in agreement with such a view, the mutational events leading to the absence of the *proB* as well as the *proA* gene products in strains χ 278 and χ 474 await further clarification. The residual γ -glutamyl kinase activities observed in extracts of these strains (Table 2) argue in favour of polar mutations in *proB* (Condamine, 1971*a*) rather than independent mutations in *proA* and *proB* (Broda, 1974).

The original host of the ColE1-*proA,B* plasmid was a *pro*⁺ strain necessitating the inclusion of colicin E1 in the growth medium to selectively maintain the plasmid-bearing cells (Clarke & Carbon, 1976). To facilitate the maintenance of the plasmid and thus to obtain high levels of the γ -glutamyl kinase and γ -glutamyl phosphate reductase for their subsequent purification, the plasmid was transferred to a *pro* deletion strain permitting selection for the complementation of the host *proA,B* deletion. The new strain, CSH52/pLC44-11, with a 20-fold increase in the specific activity of γ -glutamyl phosphate reductase, is now being used as the source of this enzyme for purification.

The decrease in the γ -glutamyl phosphate reductase activity following growth of plasmid-bearing strain CSH52/pLC44-11 under non-selective conditions could be accounted for by loss of the plasmid-borne *proA,B* genes. The specific activity of γ -glutamyl kinase was not affected by the growth medium. Either a feature of the experimental procedure prevented a higher maximum activity from being measured or the enzyme itself is, perhaps, unstable or fails to fold at synthesis at high concentrations. The differential increase of the two enzyme activities in both plasmid-bearing strains compared with the wild-type activity could likewise be explained, but awaits further investigation.

This work was supported by a Royal Society European Science Exchange Programme fellowship to D.J.H. and by the Swiss National Foundation for Scientific Research (project no. 3.263.78).

REFERENCES

- BACHMANN, B. J., LOW, K. B. & TAYLOR, A. L. (1976). Recalibrated linkage map of *Escherichia coli* K-12. *Bacteriological Reviews* **40**, 116-167.
- BAICH, A. (1969). Proline synthesis in *Escherichia coli*. A proline-inhibitable glutamic acid kinase. *Biochimica et biophysica acta* **192**, 462-467.
- BAICH, A. (1971). The biosynthesis of proline in *Escherichia coli*. Phosphate-dependent glutamate γ -semialdehyde dehydrogenase (NADP), the second enzyme in the pathway. *Biochimica et biophysica acta* **244**, 129-134.
- BAICH, A. & PIERSON, D. J. (1965). Control of proline synthesis in *Escherichia coli*. *Biochimica et biophysica acta* **104**, 397-404.
- BERG, C. M. & CURTISS III, R. (1967). Transpositions of an Hfr strain of *Escherichia coli* K-12. *Genetics* **56**, 503-525.
- BRODA, P. (1974). Modified map positions for *lac* and *pro* markers in *Escherichia coli* K-12. *Journal of Bacteriology* **117**, 741-746.
- CLARKE, L. & CARBON, J. (1976). A colony bank containing synthetic ColE1 hybrid plasmids representative of the entire *E. coli* genome. *Cell* **9**, 91-99.
- CONDAMINE, H. (1971*a*). Mutants des voies de biosynthèse et de dégradation de la proline chez *E. coli* K(12). *Annales de l'Institut Pasteur* **120**, 9-22.
- CONDAMINE, H. (1971*b*). Sur la régulation de la production de proline chez *E. coli* K(12). *Annales de l'Institut Pasteur* **120**, 126-143.
- COOPER, T. G. (1977). *The Tools of Biochemistry*, pp. 51-52. New York: John Wiley.
- CURTISS III, R. (1964). A stable partial diploid strain of *Escherichia coli*. *Genetics* **50**, 679-694.
- CURTISS III, R. (1965). Chromosomal aberrations associated with mutations to bacteriophage resistance in *Escherichia coli*. *Journal of Bacteriology* **89**, 28-40.
- GAMPER, H. & MOSES, V. (1974). Enzyme organization in the proline biosynthetic pathway of *Escherichia coli*. *Biochimica et biophysica acta* **354**, 75-87.
- HAYZER, D. J. & MOSES, V. (1978). Proline biosynthesis by cell-free extracts of *Escherichia coli* and potential errors arising from the use of a bioradiological assay procedure. *Biochemical Journal* **173**, 207-217.
- INUZUKA, M., MIYANO, H. & TOMOEDA, M. (1976). Specific action of 4-nitropyridine 1-oxide on *Escherichia coli* K-12 *pro*⁺ strains leading to the isolation of proline-requiring mutants: isolation and characterization of *pro*⁻ mutants. *Antimicrobial Agents and Chemotherapy* **10**, 325-332.
- MILLER, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor: Cold Spring Harbor Laboratory.
- PARDEE, A. B. & PRESTIDGE, L. S. (1961). The initial kinetics of enzyme induction. *Biochimica et biophysica acta* **49**, 77-88.
- ROSSI, J. J., VENDER, J., BERG, C. M. & COLEMAN, W. H. (1977). Partial purification and some properties of Δ^1 -pyrroline-5-carboxylate reductase from *Escherichia coli*. *Journal of Bacteriology* **129**, 108-114.
- VOGEL, H. J. & DAVIES, B. D. (1952). Glutamic γ -semialdehyde and Δ^1 -pyrroline-5-carboxylic acid, intermediates in the biosynthesis of proline. *Journal of the American Chemical Society* **74**, 109-112.
- WILLIAMS, I. & FRANK, L. (1975). Improved chemical synthesis and enzymatic assay of Δ^1 -pyrroline-5-carboxylic acid. *Analytical Biochemistry* **64**, 85-97.