Sub-cloning of the Wild-type *proAB* Region of the *Escherichia coli* Genome

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The genes *proA* and *proB* encoding the first two enzymes of the proline biosynthetic sequence in *Escherichia coli* were subcloned from a ColE1 hybrid plasmid containing 23.3 kilobases of genomic DNA. *proA* and *proB* are contiguous and constitute a single operon transcribed in the direction *proB-proA*. The *pro* operon is contiguous with the gene *phoE*. Hybridization experiments showed no homology between *proAB* of *E. coli* and the other regions of the *E. coli* genome or with the DNA of several other bacterial species.

INTRODUCTION

Proline biosynthesis in prokaryotes requires the reduction of the γ -carboxyl group of glutamate to yield glutamic acid 5-semialdehyde which then undergoes a non-enzymic cyclization and internal Schiff's base formation to give Δ^1 -pyrroline 5-carboxylate. This is subsequently reduced to proline (Vogel & Davies, 1952). The reduction of glutamate to the semialdehyde proceeds in two stages, probably by way of the γ -phosphorylation of glutamate, a sequence mediated by the enzymes γ -glutamyl kinase (ATP:L-glutamate 5-phosphotransferase; EC 2.7.2.11) (Baich, 1969) and glutamate semialdehyde dehydrogenase [L-glutamate-5-semialdehyde:NADP⁺ oxidoreductase (phosphorylating); EC 1.2.1.41] (Hayzer & Leisinger, 1982). Both enzymes from *Pseudomonas aeruginosa* have been partially purified (Krishna & Leisinger, 1979; Krishna *et al.*, 1979) while the semialdehyde dehydrogenase from *Escherichia coli* has been purified to homogeneity (Hayzer & Leisinger, 1982). There is also some evidence that a similar sequence of reactions may be found in certain animal tissues as part of the ornithine biosynthetic pathway (Menslee & Jones, 1982).

Regulatory control of the flow of carbon through the *E. coli* proline biosynthetic pathway is believed to reside in the feedback inhibition of the kinase by the final product L-proline (Baich & Pierson, 1965; Baich, 1969). Control at the nucleic acid level by any mechanism, however, has not been demonstrated as it has been with other amino acid biosynthetic pathways (Yanofsky, 1981); the measured levels of the proline enzymes in wild-type cells after growth on minimal medium with or without proline do not appear to differ (Hayzer & Leisinger, 1980).

Mutations of two genetic loci in *E. coli* (Bachmann & Low, 1980), *Salmonella typhimurium* (Sanderson & Hartman, 1978) and *Pseudomonas aeruginosa* (Pemberton & Holloway, 1972) result in a nutritional requirement satisfied by either proline or its immediate precursor Δ^1 -pyrroline-5-carboxylate. In *E. coli* the locus designated *proA* has been shown to code for the second enzyme in the pathway, glutamate semialdehyde dehydrogenase with *proB* coding for the γ -glutamyl kinase (Hayzer & Leisinger, 1980). Available mapping data are weighted towards the close proximity of *proA* to *proB* (Condamine, 1971) although there have also been reports that the two loci may be physically separated by a distance equivalent to 0.6 min of the genetic map (Broda, 1974) or 23.0 kb (Bachmann & Low, 1980) and even with an identifiable gene between them (Roberts & Reeve, 1970).

Genetical studies of the proline pathway would be greatly assisted by the cloning of the *proAB* region of the *E. coli* genome and would permit one to establish the physical relationship of the

Table 1. *Bacteria and plasmids used in this study*

Strain or plasmid	Genotype or properties	Source or reference
<i>Escherichia coli</i> CSH52	F^- <i>thi ara</i> Δ (<i>lac pro</i>) <i>strA recA</i> ($\phi 80$ <i>dlac</i> ⁺)	Miller (1972)
CSH52/pLC44-11	as CSH52/pLC44-11	Hayzer & Leisinger (1980)
RR-1	F^- <i>hsdS20</i> ($r_b^- m_b^-$) <i>recA13 ara-14 proA2* lac</i> <i>Y1 galK2 rpsL20</i> (<i>Sm</i> ^r) <i>xyl-5 mtl-1 supE44</i> λ^-	This laboratory
RR-1/pE202	as RR-1/pE202	This study
RR-1/pEH21	as RR-1/pEH21	This study
RR-1/pHBE1	as RR-1/pHBE1	This study
RR-1/pPBB4	as RR-1/pPBB4	This study
RR-1/pHEP8	as RR-1/pHEP8	This study
$\chi 680$	$F^- \lambda^-$ <i>leu thr thi lac tsx strA proA</i>	R. Curtiss III
$\chi 278$	$F^- \lambda^-$ <i>leu thr thi lac tsx strA proB</i>	R. Curtiss III
$\chi 277$	$F^- \lambda^-$ <i>leu thr thi lac tsx strA proC</i>	R. Curtiss III
$\chi 210$	$F^- \lambda^-$ <i>leu thr thi lac tsx strA</i>	R. Curtiss III
$\chi 462$	F^- <i>leu lys met trp ara lac mal xyl strA proA</i>	R. Curtiss III
$\chi 474$	F^- <i>leu lys met trp ara lac mal xyl strA proB</i>	R. Curtiss III
$\chi 478$	F^- <i>leu lys met trp ara lac mal xyl strA proC</i>	R. Curtiss III
<i>Pseudomonas aeruginosa</i> PAO-1	Wild-type	Th. Leisinger
<i>Streptococcus faecalis</i> RU-6	Wild-type	This laboratory
<i>Staphylococcus epidermidis</i> RU-4	Wild-type	This laboratory
<i>Staphylococcus epidermidis</i> RU-7	Wild-type	This laboratory
Plasmid pBR322	Ap ^r Tc ^r	This laboratory
pLC44-11	ColE1 ^{Imm} Pro ⁺	Clarke & Carbon (1976)
pE202	Ap ^r Tc ^r Pro ⁺	This study
pEH21	Ap ^r Tc ^r Pro ⁺	This study
pHBE1	Ap ^r Tc ^r Pro ⁺	This study
pPBB4	Ap ^r Tc ^r Pro ⁺	This study
pHEP8	Ap ^r Tc ^r Pro ⁻	This study

* Extent of *proA2* deletion given by Tommassen *et al.* (1981).

genes, how many operons are present and any possible regulatory mechanisms. To this purpose a multi-copy ColE1 hybrid plasmid from the Clarke and Carbon colony bank (Clarke & Carbon, 1976), known to contain within it the desired genes (Hayzer & Leisinger, 1980) was used as the starting point for the subcloning and structural analysis described in this paper.

METHODS

Bacteria and culture conditions. The strains used in this study are listed in Table 1. Non-selective medium was LB-broth and resistance to antibiotics was determined using the same medium but it contained 50 μ g ampicillin (Ap) or tetracycline (Tc) ml⁻¹. Selection for the Pro⁺ phenotype was on minimal medium M63 (Pardee & Prestidge, 1961) containing 0.5% (w/v) glucose and an excess (200 μ g ml⁻¹) of each amino acid, other than proline, required by the strain in question. Media were solidified with 1.5% (w/v) agar (Difco). For DNA preparations bacteria were grown to mid-exponential phase ($A_{650} = 0.5$) on LB-broth unless indicated and where possible including ampicillin or tetracycline (50 μ g ml⁻¹). Staphylococci were grown on Trypticase Soy Broth (BBL). All incubations were at 37 °C.

Plasmid and total DNA preparations. The plasmid DNA of mid-exponential phase cells was amplified by the addition of chloramphenicol (200 μ g ml⁻¹) and prolonging the incubation for a further 16 h. The cells from 1 litre culture were harvested at 10000 *g* for 10 min and resuspended in 20 ml buffer A (Tris/HCl, 50 mM; EDTA, 20 mM; pH 8.0) containing 10 mg lysozyme and incubated at 37 °C for 30 min. Then 0.25 vol. SDS-EDTA (20%, w/v, and 20 mM, respectively, pH 8.0) was added to give a clear lysate followed by 0.25 vol. 5 M-NaCl and centrifugation at 27000 *g* for 60 min. Plasmid DNA was concentrated by polyethylene glycol precipitation (Humphreys *et al.*, 1975), collected by centrifugation and redissolved in 20 ml buffer A. Nucleic acid was precipitated overnight at -20 °C with 2 vol. ethanol, collected by centrifugation and resuspended in 2 ml buffer A and 6 ml water containing caesium chloride (8.0 g) and ethidium bromide (4.0 mg in 0.8 ml water). Centrifugation was in a Beckman Ti50 rotor at 3.8 $\times 10^4$ r.p.m. for 40 h. The lower, supercoiled DNA band was removed by bottom puncture of the tube, extracted three times with an equal volume of *n*-butanol equilibrated with TE buffer (Tris/HCl, 10 mM; EDTA 1.0 mM; pH 8.0) and dialysed against the same for 8 h. The DNA was precipitated overnight at -20 °C by 0.2 vol. 3 M-sodium acetate and 2 vol. ethanol, centrifuged, dried *in vacuo* and redissolved in TE buffer for storage at 4 °C. Total DNA, chromosomal and plasmid combined, was prepared by the procedure

of Marmur (Johnson, 1981). For screening transformants for plasmid content, the mini lysate method of Birnboim & Doly (1979) was used.

Restriction endonuclease digestions and ligation procedure. Endonuclease reactions were carried out in a buffer containing Tris/HCl, 50 mM; MgCl₂, 5 mM; DTT, 1 mM and NaCl, 50 mM, pH 7.4. With *Sal*I, *Ava*II and *Hinf*I the DTT was omitted and with *Pst*I (including double digests with *Sal*I) the buffer included Tris/HCl, 50 mM; MgCl₂, 10 mM and (NH₄)₂SO₄, 50 mM at pH 7.5. All digestions were overnight at 37 °C except with *Hpa*I which was for only 2 h. Before ligations the enzymes were inactivated at 80 °C for 10 min. A total of 4 µg DNA fragments were ligated overnight at 4 °C by 1.0 U of T₄ DNA ligase using the manufacturers protocol.

Electrophoresis. Intact plasmid DNA and fragments of 8.0–0.5 kb were analysed in 0.7% agarose gels with Tris/borate buffer (Tris base, 45 mM; boric acid, 45 mM and EDTA, 1.5 mM at pH 7.85) at 4–10 V cm⁻¹. Phage-λ DNA digested with *Eco*RI and *Eco*RI-*Hind*III were used as standards. Fragments of 1.5–0.05 kb were analysed in 5% acrylamide gels with the same buffer at 6 V cm⁻¹ with *Hinf*I and *Hae*III digested plasmid pBR322 as the standards.

Transformation. Mid-exponential phase cultures of 50 ml (*A*₆₅₀ = 0.5) were centrifuged and the cells resuspended in 20 ml cold 100 mM-CaCl₂ for 20 min. After centrifugation at 5000 *g* for 10 min, they were resuspended in 0.5 ml of the CaCl₂ and 0.1 ml samples were mixed with 1.0–4.0 µg DNA, cooled on ice for 30 min and heat-shocked at 37 °C for 6 min. After 45 min on ice the cell suspensions were diluted into 1.0 ml LB-broth and incubated at 37 °C with gentle shaking for 1 h prior to spreading 0.05–0.2 ml samples on to selective media. Plates were incubated for 24–48 h.

Nick-translation labelling of plasmid DNA. Plasmid pPBB4 (0.5 µg) cut with *Sal*I was incubated with dATP, dGTP and dTTP, 20 µM of each, ³²P-dCTP, 50 µCi (1.85 MBq) and 2 U DNA polymerase I in Tris/HCl, 50 mM, pH 7.5 containing MgSO₄, 10 mM and DTT, 1 mM. After 1 h at 15 °C the unincorporated nucleotides were removed by a Sephadex G-50 column (0.5 × 7.0 cm) and the DNA precipitated at –20 °C overnight with yeast tRNA (60 µg ml⁻¹), 0.5 M-potassium acetate and 2 vol. ethanol.

Filter hybridization. Samples of total DNA cut with restriction endonucleases were electrophoresed in 1.0% agarose gels and denatured and transferred to nitrocellulose filters as described by Southern (1975). Hybridizations were by the method of Thomas (1980) except that filters were washed at room temperature twice with 6 × standard saline citrate (SSC) buffer and four times with SSC 2 × (each wash containing 0.1% SDS). SSC buffer contained 0.15 M-NaCl and 0.015 M-sodium citrate at pH 7.0. Autoradiography used Kodak X-omat film with an enhancing screen at –70 °C for 3–48 h.

RESULTS

Restriction endonuclease cleavage mapping of plasmid pLC44-11

The first step in the subcloning of the *proAB* gene region was the determination of a restriction cleavage map of the source DNA, plasmid pLC44-11 (Fig. 1). The fragment sizes within the 5.0 kb *Sal*I region and the estimated size of pLC44-11 of 29.7 kb, the means of two independent experimental values, were in agreement with those reported by Tommassen *et al.* (1982). One gene, *phoE*, encoding the outer membrane porin PhoE was reported to lie within the 2.0 kb *Sal*I-*Eco*RI fragment and to contain the single *Pst*I site of this region. The exact locations of the two extremes of *phoE* were not established (Tommassen *et al.*, 1982). Earlier transductional analysis of the *phoE-argF* region of the chromosome gave the gene order of *phoE-proAB-argF* and showed that *phoE* and *proAB* are very closely linked at the 6 min position on the *E. coli* gene map (Tommassen & Lugtenberg, 1981). These published data, however, was not sufficient to locate *proAB* relative to *phoE* and the ColE1 vector DNA of pLC44-11 nor, indeed, to estimate how far apart the various regions are, in terms of kb pairs.

Only the largest of the *Eco*RI fragments could contain the ColE1 DNA (6.4 kb). *Hind*III and *Bam*HI sites within the other two *Eco*RI fragments are not present in ColE1. In addition, since approximately 5.3 kb of ColE1 is uncut by *Pst*I (Edlund *et al.*, 1979) if the vector was in the reverse orientation to that shown in Fig. 1 there would be a 6.7 *Sal*I-*Pst*I fragment, not the 1.45 kb fragment found.

Subcloning of the proAB gene region

Plasmid pLC44-11 was cleaved with the restriction endonucleases *Eco*RI, *Hind*III, *Bam*HI or *Sal*I and the resulting fragments were ligated into the vector pBR322 cut with the corresponding enzymes. Transformations were made into *E. coli* RR-1 carrying a deletion of the *pro* loci (Tommassen *et al.*, 1981) with the primary selection for the Pro⁺ phenotype. An incubation

Fig. 1. Circular restriction map of the ColE1 hybrid plasmid pLC44-11. Cleavage sites are given for restriction endonucleases *Eco*RI, E; *Hind*III, H; *Bam*HI, B; *Sal*I, S; *Pst*I, P; *Kpn*I, K; and *Bgl*II, C. The available data did not allow all of the *Pst*I sites to be located. *Pst*I fragments of 0.37, 0.58, 1.11, 1.46, 1.6, 1.6, 3.0, 5.95, and 7.25 kb pairs were found. ColE1 vector DNA is indicated by the heavy line and the approximate position of the *phoE* gene by --- (Tomassen *et al.*, 1982). The positions of the poly(dA.dT) connectors (Clarke & Carbon, 1976) are arrowed. The scale given by the figures inside the circle is in kb pairs.

period of 48 h was usually required to obtain such colonies which were replica-plated on to ampicillin or tetracycline-containing medium to show the presence of pBR322 DNA although they were routinely maintained on minimal medium lacking proline. Only *Eco*RI-cut pLC44-11 and pBR322 yielded any antibiotic-resistant (in this case Ap^r Tc^r) proline-independent isolates with mini lysate analysis showing the presence of plasmids greater than 4.3 kb in size. One isolate, carrying the smallest plasmid, was selected for further examination. The plasmid, designated pE202, was subjected to restriction endonuclease cleavage which showed that the 11.0 kb *Eco*RI fragment of pLC44-11 had been subcloned (Fig. 2) and therefore *proA* and *proB* were shown to be anticlockwise to *phoE* in the pLC44-11 cleavage map given in Fig. 1.

A further reduction in the size of the insert carrying *proA* and *proB* was achieved by a double digestion of pE202 with *Eco*RI and *Hind*III followed by ligation into *Eco*RI-*Hind*III-cut pBR322, thereby inactivating the tetracycline resistance gene of the vector. Again, the smallest plasmid found in a proline-independent transformant of *E. coli* RR-1 was selected, designated pEH21 and mapped by endonuclease cleavage (Fig. 2). An even greater reduction in the size of the insert was obtained by cutting pE202 with both *Hind*III and *Bgl*II with ligation into pBR322 cleaved with *Hind*III and *Bam*HI (*Bgl*II and *Bam*HI generate different 4-base 5'-extensions, the terminal two bases of each being complementary thus generating a sequence, upon the subsequent *in vivo* addition of the missing bases, which cannot be cut by either of the original enzymes but instead forms a novel *Sau*3a or *Mbo*I recognition site).

The resulting minimum sized plasmid selected, pHBE1, carried an insert of 4.44 kb and included most of the *phoE* gene (approximately 1.2 kb) in addition to *proA* and *proB* (Fig. 2). Restriction analysis also showed that while pHBE1 included the single *Hind*III end and the *Hpa*I site (marked with an asterisk in Fig. 2) of pE202 and pEH21 much of the region between including four *Pst*I sites (the precise positions of which could not be determined and therefore are not indicated in Fig. 2) and single *Pvu*II and *Bgl*II sites, had been deleted. One end of the deletion lay between *Hpa*I* and the *Pvu*II-*Bgl*II sites, separated by about 0.4 kb while the other end was approximately 0.6 kb from the *Hind*III site.

The final stage in the reduction of the insert length was accomplished by the removal of most of the remaining *phoE* DNA. pEH21 was cut by *Pst*I and *Bgl*II, ligated into *Pst*I-*Bam*HI-cut pBR322 and transformed into strain RR-1. Selection was for the Pro⁺ phenotype. Plasmid

Fig. 2. Subcloning of the *proAB* region of the *E. coli* genome from the *Eco*RI insert of plasmid pE202. Vector DNAs are given by the heavy lines and the approximate position of the *phoE* gene by . The double vertical line between the *Hind*III and *Hpa*I* sites of pHBE1 indicate where a region of pE202 was deleted. Restriction sites are designated as in Fig. 1 with the additions of *Pvu*II, D and *Hpa*I, F.

pPBB4 thus obtained contained a 2.92 kb insert and a 3.23 kb vector DNA region. One end of the remaining *phoE* DNA lay between the *Pst*I junction and the *Pvu*II/*Hinc*II-*Sal*I site. These latter three could not be distinguished. The extent of the residual *phoE* DNA could be estimated from the coding capacity required for γ -glutamyl kinase with a subunit approximately 4.3×10^4 Dal in size (unpublished data) and glutamate semialdehyde dehydrogenase with a subunit of 4.7×10^4 Dal (Hayzer & Leisinger, 1982) each equivalent to 1.2–1.3 kb of DNA; i.e. about 2.5 kb in total. The remaining 400–500 bases of the pPBB4 insert represents the maximum *phoE* region carried by pPBB4. A detailed restriction cleavage map of pPBB4 is shown in Fig. 3 with individual fragment sizes given in Table 2.

Localization of the deletion which occurred during the formation of plasmid pHBE1

Endonuclease *Hpa*I was used to confirm the positioning of certain fragments within the map shown in Fig. 3 and to localize one of the ends of the deletion that occurred during the formation of pHBE1. Thus, the 500 base *Alu* fragment was cleaved by *Hpa*I to 280 and 200 base lengths, the 288 base *Sau*96 fragment was cleaved to 170 and 88 base lengths, the 600 base *Dde* fragment to 320 and 280 bases, the 205 base *Hinf*I fragment to 190 and 15 bases and the *Hae*III 235 base fragment to 175 and 60 bases. Furthermore, these same fragments were present and similarly cleaved when either pHBE1 or pPBB4 was the source DNA, demonstrating that the deletion from pE202 which occurred during the construction of pHBE1 was limited at one end to a point between the *Dde*I site (marked with +) and the *Bgl*II/*Bam*HI insert-vector junction of pPBB4 (Fig. 3).

The approximate position of the opposite end of the deletion from pE202 was determined from the *Pst*I-*Hpa*I distance of pHBE1 of 1.52 kb of which 0.78 kb was contained within the

Fig. 3. Detailed restriction cleavage map of plasmid pPBB4. pBR322 vector DNA is given by the heavy line. Only those cuts within the pBR322 region which result in a fragment containing part of the insert are shown. Those *HinfI* and *HaeIII* fragments which could not be positioned are marked with an asterisk in Table 2. Restriction sites are designated as in Figs 1 and 2 with the additions of *AluI*, A; *AvalI*, G; *DdeI*, I; *HaeIII*, J; *HincII*, L; *HinfI*, M; and *Sau96*, N.

Table 2. Restriction endonuclease cleavage products of plasmid pPBB4

All values are the means of two determinations.

Enzyme/s	Fragment sizes (kb pairs)
<i>PstI</i>	6.15
<i>SalI</i>	3.57, 1.45, 1.02
<i>PvuII</i>	2.27, 2.13, 1.64
<i>AvalI</i> †	3.45
<i>HincII</i>	3.45, 1.45, 0.645, 0.34, 0.096
<i>PstI-SalI</i>	3.02, 1.49, 1.06, 0.59
<i>PstI-PvuII</i>	2.31, 1.75, 1.54, 0.62
<i>SalI-PvuII</i>	2.14, 1.47, 1.36, 0.84, 0.32
<i>AluI</i> †	1.0, 0.77, 0.63, 0.505, 0.403
<i>AluI-PstI</i>	1.075, 0.715, 0.65, 0.515, 0.403
<i>AluI-SalI</i>	0.82, 0.735, 0.63, 0.505, 0.335, 0.151
<i>AluI-PvuII</i>	1.025, 0.80, 0.645, 0.505, 0.403
<i>AluI-HincII</i>	0.85, 0.75, 0.665, 0.6, 0.365, 0.290, 0.200, 0.092, 0.074
<i>Sau96</i> †	1.6, 0.905, 0.285, 0.249, 0.249
<i>Sau96-PstI</i>	1.587, 0.915, 0.287, 0.237, 0.237, 0.105
<i>Sau96-SalI</i>	0.865, 0.815, 0.660, 0.285, 0.255, 0.249
<i>Sau96-PvuII</i>	0.865, 0.815, 0.287, 0.245, 0.245
<i>Sau96-HincII</i>	0.79, 0.710, 0.610, 0.249, 0.185, 0.170, 0.153, 0.142, 0.126, 0.092, 0.087, 0.079
<i>DdeI</i> †	1.675, 1.200, 0.620, 0.546, 0.409
<i>DdeI-PstI</i>	1.675, 1.200, 0.620, 0.546, 0.284, 0.182
<i>DdeI-SalI</i>	1.100, 0.885, 0.620, 0.490, 0.43, 0.300, 0.116, 0.409
<i>DdeI-PvuII</i>	1.250, 1.050, 0.520, 0.49, 0.409, 0.126
<i>DdeI-HincII</i>	1.100, 0.850, 0.500, 0.445, 0.409, 0.360, 0.325, 0.280, 0.094
<i>HinfI</i> †	0.754*, 0.593, 0.344, 0.310*, 0.298*, 0.253*, 0.243*, 0.220*, 0.220*, 0.089*, 0.054*
<i>HinfI-PstI</i>	0.754, 0.596, 0.310, 0.298, 0.255, 0.243, 0.243, 0.220, 0.220, 0.112, 0.089, 0.054
<i>HinfI-SalI</i>	0.743, 0.436, 0.344, 0.307, 0.298, 0.248, 0.240, 0.220, 0.201, 0.160, 0.090, 0.053
<i>HinfI-HincII</i>	0.730, 0.344, 0.335, 0.310, 0.298, 0.245, 0.238, 0.198, 0.193, 0.165, 0.092, 0.055
<i>HaeIII</i> †	1.120, 0.565, 0.300*, 0.234*, 0.215*, 0.145*, 0.114*, 0.104*, 0.073*, 0.057*
<i>PstI</i>	1.000, 0.57, 0.302, 0.234, 0.215, 0.135, 0.118, 0.118, 0.104, 0.072, 0.057
<i>HaeIII-SalI</i>	0.740, 0.365, 0.320, 0.300, 0.251, 0.234, 0.213, 0.125, 0.114, 0.104, 0.072, 0.057
<i>HaeIII-PvuII</i>	0.770, 0.557, 0.434, 0.345, 0.295, 0.231, 0.213, 0.132, 0.115, 0.104, 0.072, 0.057

* *HinfI* and *HaeIII* fragments marked thus could not be positioned on the cleavage map shown in Fig. 3.

† Only those fragments specific to the insert of pPBB4 are given.

Fig. 4. Formation and endonuclease cleavage map of plasmid pHEP8. Plasmid pPBB4 was cut with *Hpa*I. pBR322 was cut with *Eco*RI and *Pvu*II. The DNA (50 µg) was precipitated with ethanol, resuspended in water (10 µl) and incubated with nuclease *Bal*-31 (2 U) and reaction buffer according to manufacturers instructions for 30 min at 37 °C. The DNA was phenol extracted and ethanol precipitated, dissolved in 10 µl water and ligated with the cleaved pPBB4 and transformed as described in Methods. —, pBR322 DNA.

pBR322 vector (Fig. 2). Since one end of the deletion was 0.3–0.4 kb from the *Hpa*I site, within the *Dde*I-*Bgl*/II section the other must be about 0.5 kb from the *Hind*III site of pHEB1. Thus the region lost from pE202 was approximately 3.8 kb in length.

Insertional inactivation of the proline biosynthetic gene system

Inactivation of either *proA* or *proB* in pPBB4 would also be expected to eliminate the only selective markers present on the plasmid since both the ampicillin and tetracycline resistant determinants of pBR322 were no longer intact. To allow one of the *pro* genes to be inactivated and at the same time provide a means of selecting such a mutant the *Eco*RI-*Pvu*II 2.07 kb fragment of pBR322, carrying the entire region coding for tetracycline resistance (Sutcliffe, 1978) was inserted into the single *Hpa*I site of pPBB4. The *Eco*RI-*Pvu*II fragment was pre-treated with nuclease *Bal*-31 to remove the cohesive *Eco*RI 5' strand prior to the blunt-end ligation into the recipient and transformation into *E. coli* RR-1. Those transformants with the Tc^rAp^rPro⁻ phenotype were examined for their plasmid content, in particular for a plasmid approximating 8.0 kb (6.1 kb from pPBB4 plus 2.07 kb from pBR322). The cleavage map of the plasmid from one such isolate, pHEP8, is given in Fig. 4 and shows the orientation of the 2.07 kb insert which resulted in *Sal*I fragments of 2.08 and 1.1 kb; the alternative orientation would have given fragments of 1.86 and 1.31 kb. No *Eco*RI site was found but the *Hind*III site was present indicating the limit of *Bal*-31 digestion.

Insertion of the foreign DNA into the single *Hpa*I site of pPBB4 resulted in the loss of proline independence for the *E. coli* RR-1 transformant. This endonuclease site must therefore lie within either *proA* or *proB*. To establish which gene was affected, pHEP8 and the parental pPBB4 were each transformed into a series of different proline requiring mutants. Selection, in the case of pPBB4 was for proline independence and with pHEP8, initially for tetracycline resistance and subsequently for proline independence. Both plasmids failed to complement *proC* mutants.

Table 3. Transformations of proline-auxotrophic strains of *Escherichia coli* by plasmids pPBB4 and pHEP8

Transforming plasmid	Phenotype selected	Recipient strain and relevant genotype							
		RR-1 $\Delta proA$	CSH52 $\Delta proAB$	$\chi 277$ <i>proC</i>	$\chi 278$ <i>proB</i>	$\chi 680$ <i>proA</i>	$\chi 462$ <i>proA</i>	$\chi 474$ <i>proB</i>	$\chi 478$ <i>proC</i>
pPBB4	Pro ⁺	+	+	—	+	+	+	+	—
pHEP8	Tc ⁺	+	+	+	+	+	+	+	+
	Pro ⁺	—	—	—	—	—	—	—	—

+, indicates growth of recipient.

Fig. 5. Gel electrophoretic and hybridization analysis of total bacterial DNA preparations cleaved with restriction endonucleases. Tracks 1–5, *E. coli* $\chi 210$ cleaved with *EcoRI*, *HindIII*, *BamHI*, *PstI* or *SalI*, respectively; Tracks 6–10, *E. coli* CSH52/pLC44-11 cleaved with *EcoRI*, *HindIII*, *BamHI*, *PstI* or *SalI*, respectively; tracks 11 and 12, *E. coli* RR-1/pPBB4 cleaved with *PstI* or *SalI*, respectively. Tracks 1–5 contained 3.3 μ g DNA, tracks 6–10 2.1 μ g, and 11–12 0.8 μ g. All digestions were overnight at 37 °C with approximately 5 U of an enzyme used. The kb values shown were determined from λ DNA digested with *EcoRI* and *EcoRI* plus *HindIII*.

pPBB4 conferred proline independence upon all the other strains as expected. pHEP8, however, failed to give any Pro⁺ transformants even though the Tc^r phenotype showed that the plasmid had entered the cells (Table 3). Mini lysate analysis of randomly selected transformants confirmed the plasmids used were indeed present

Hybridization of plasmid pPBB4 with total bacterial DNAs

³²P-labelled pPBB4 was used as a probe to detect homologous regions in bacterial total DNA preparations following digestion with restriction endonucleases and the separation of the fragments by agarose electrophoresis.

Single bands of homologous DNA with sizes greater than 19.0 kb were found when the DNA from *E. coli* χ 210, a proline prototroph, was digested with *Eco*RI, *Hind*III or *Bam*HI. *Pst*I gave a strong band of 6.0 kb and two weaker bands at 3.4 and 11.0 kb. *Sal*I gave three bands, two faint at about 20 and 14 kb and a stronger one at 1.45 kb (Fig. 5). This latter was also present, as expected, in DNA from strain CSH52 carrying the plasmid pLC44-11 since this fragment overlapped *proA* and *proB* and was present in all the subclones.

The DNA from strain CSH52/pLC44-11 when cut with *Eco*RI, *Hind*III or *Bam*HI gave single hybridizable fragments, all greater than 15 kb each (other experiments, not shown, give an *Eco*RI band at about 10 kb). Inspection of the restriction cleavage map for pLC44-11 (Fig. 1) shows similar fragments carrying the entire *Pst*I-*Bgl*II region subsequently subcloned into pPBB4. There was a prominent 3.4 kb *Pst*I band, as expected and one or two larger pieces, presumably due to homology between the ColE1 region of pLC44-11 and the pBR322 vector DNA of pPBB4. All three *Sal*I fragments of pLC44-11 had regions homologous with pPBB4.

DNA from strain RR-1/pPBB4 cleaved with *Sal*I gave the three bands expected from the cleavage map of pPBB4. No others were seen. *Pst*I unexpectedly gave a band, in addition to that at 6.0 kb due to the uniquely cleaved plasmid, in a position corresponding to a size greater than 23 kb, possibly as a result of concatameric plasmid forms which prevent *Pst*I digestion. This is supported by finding no such anomaly with *Sal*I digestions.

No homologous bands were found with DNA from *E. coli* strains CSH52 and RR-1, which carry deletions of *proA* and *proB*, when pLC44-11 or pPBB4 were absent or with *Pseudomonas aeruginosa*, *Streptococcus faecalis* or *Staphylococcus epidermidis* DNA, even with the low stringency washing of the filters, high loadings of the agarose gels with digested DNA (up to 50 μ g DNA per track) and with exposures of the hybridized filters to the autoradiographic films for periods five to ten times as long as the sample shown in Fig. 5.

DISCUSSION

Escherichia coli genomic DNA contained within the hybrid plasmid pLC44-11 includes both the *proA* and *proB* genes (Hayzer & Leisinger, 1980). The *phoE* gene is also present and all three loci are closely linked at the 6 min genomic position (Tommassen *et al.*, 1981). Subcloning of the genes conferring proline independence into an *E. coli* strain with a deletion of *proAB*, as described in this paper, has allowed the isolation of a 2.9 kb fragment of DNA at least 85% of which consists of the two genes. Thus there is little possibility of intermediate genes; *proA* and *proB* are contiguous with one another and with *phoE*, at least in the strain of *E. coli*, CS520 (Clarke & Carbon, 1976), used as the source of DNA in constructing the original ColE1 hybrid plasmid.

The insertion of foreign DNA, in this case a 2.07 kb fragment of pBR322 carrying the intact gene for tetracycline resistance, into pPBB4 inactivated both *pro* genes. The novel plasmid failed to complement the mutations carried by *proA* or *proB* strains and strongly supported the existence of a single *pro* operon with polarity of transcription in the direction of *proB-proA*. Such a possibility has previously been proposed as the basis of certain *proB* mutants which lack both enzyme activities. The likelihood of double mutations was not supported by the observed rate of spontaneous reversion of proline auxotrophy (Hayzer & Leisinger, 1980). The results described in this paper clearly show that a mutation at a single defined site produced a similar *ProA*⁻*B*⁻ phenotype although the actual enzyme levels were not investigated. The insertional inactivation does fail, however, to distinguish between the location of the *Hpa*I site within the *proB* structural gene or a preceding regulatory region.

It would be interesting to examine in greater detail those *E. coli* strains reported to have a gene between *proA* and *proB* (Roberts & Reeve, 1970). If such a situation does exist, it would imply either the addition of a new promotion site before *proA* or the loss of such in the creation of the strain, or its ancestor, from which the *proBA* operon cloned in this present series of experiments originated. The existence of the 1.45 kb *Sal*I fragment could be used as an indicator of contiguous *proA* and *proB* genes.

The construction of plasmid pHBE1 was possible because of an apparently spontaneous

deletion of approximately 3·8 kb between the *Hind*III and *Hpa*I sites of pE202. No obvious mechanism for this occurrence is evident, such as the existence of insertion elements in the regions at the termini of the deleted segment and a suitable explanation, if possible, might have to await a much more detailed analysis of this region of the *E. coli* genome.

The isolation of the entire *proBA* operon should now facilitate studies into the question of whether there is coregulation of the synthesis of the two enzymes encoded, perhaps from the determination of the nucleotide sequence of the region preceding the structural genes and thus to detect an attenuator region similar to that associated with other amino acid biosynthetic pathways (Yanofsky, 1981).

Sequencing of the subcloned *proAB* region will also permit a comparison with the *pro* genes from other sources and even with genes encoding enzymes of related functions found in other metabolic pathways, e.g. the aspartokinases of threonine-methionine-lysine synthesis, glutamine synthetase, and *N*-acetylglutamyl kinase and *N*-acetylglutamic acid semialdehyde dehydrogenase of arginine biosynthesis. In *P. aeruginosa* the *pro* genes are not closely linked on the genome (Royle *et al.*, 1981) and have no apparent homology with the corresponding *E. coli* genes reflecting their evolutionary divergence.

Recently the *proBA* region of the *S. typhimurium* genome has been isolated (LeRudulier & Valentine, 1982). The restriction map of a 10·3 kb *Eco*RI fragment carrying the two genes appear to be very similar to that found for the corresponding *E. coli* region (Fig. 1) indicative of the very close sequence homology between the two species in this part of their respective genomes.

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