Analysis of the *Serratia marcescens* *proBA* operon and feedback control of proline biosynthesis

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The *proBA* operon, coding for γ -glutamyl kinase (GK) and γ -glutamyl phosphate reductase (GPR), was cloned from the chromosome of the wild-type strain of *Serratia marcescens* Sr41. From our sequence data the *proB* (1101 bp) and *proA* (1472 bp) genes were shown to code for two proteins of M_r 39 169 and 44 640, respectively. Analysis of expression of the *lacZ* structural gene fused with the *proBA* promoter showed that the *proBA* operon is not subject to proline-mediated feedback repression. Amplification of the *proBA* operon enabled us to determine GK activity, which was inhibited in the presence of a low concentration of L-proline. Comparison of the amino acid sequences of the *S. marcescens* GK and GPR proteins with those of the GK and GPR proteins from *E. coli* revealed extensive similarities.

Introduction

In micro-organisms, proline is synthesized from glutamate via three enzymic reactions catalysed by γ -glutamyl kinase (GK; *proB* product, EC 2.7.2.11), γ -glutamyl phosphate reductase (GPR; *proA* product, EC 1.2.1.41) and L-pyrroline-5-carboxylate reductase (*proC* product, EC 1.5.1.2) (Adams & Frank, 1980). Feedback control mechanisms and genetic backgrounds for proline biosynthesis have been extensively studied in *Escherichia coli* K12. GK, the first enzyme in this pathway, is subject to feedback inhibition by proline (Baich, 1969). The *proB* and *proA* genes constitute the *pro* operon, which is distant from the *proC* gene on the chromosome (Bachmann & Low, 1980). Proline-hyperproducing strains of *Serratia marcescens* Sr41 have been constructed by selection of mutants resistant to proline analogues and cloning of the relevant genes (Sugiura *et al.*, 1985a; Takagi *et al.*, 1985). One of these strains, carrying the *dpr-1* mutation, was selected for resistance to dehydroproline and has been reported to produce more than 60 g of L-proline l^{-1} in a fermentation medium containing sucrose and urea (Sugiura *et al.*, 1985b). Transductional analysis indicated that the *dpr-1* mutation is located in the *proBA* region. However, there have been few

findings on genetic and enzymic profiles of the regulation of proline biosynthesis in *S. marcescens*.

This paper deals with the analysis of the gene structure of the wild-type *proBA* operon of *S. marcescens* and, in addition, the study of the feedback control of proline biosynthesis in this bacterium.

Methods

Strains, plasmids and media. The strains and plasmids used are listed in Table 1. *E. coli* JM109 was used as a host for the construction of plasmids. The rich medium used was LB (Davis *et al.*, 1980) and the minimal medium of Davis & Mingioli (1950) was modified by omitting sodium citrate and increasing the glucose concentration to 0.5%. Required L-amino acids were used at a concentration of 1 mM. Antibiotics were added at the specified concentrations: kanamycin (Km), 200 μ g ml^{-1} ; ampicillin (Ap), 200 μ g ml^{-1} .

Genetic methods. DNA manipulations were carried out according to standard procedures (Maniatis *et al.*, 1982). Transformation of *E. coli* or *S. marcescens* was done as described by Takagi & Kisumi (1985).

In vitro transcription-translation analysis. The prokaryotic cell-free coupled transcription-translation system from Amersham (DeVries & Zubay, 1967) was employed for labelling proteins encoded by plasmids. Plasmid DNA (0.4 μ g), prepared by CsCl density gradient centrifugation, was added to the reaction mixture (5 μ l) containing L-[35 S]methionine, according to the protocol of the manufacturer. After 60 min incubation at 37 °C, SDS-PAGE of 35 S-labelled proteins was carried out as described by Laemmli (1970). Labelled polypeptide bands were visualized by autoradiography.

DNA sequence analysis. The DNA sequence of the plasmid containing the cloned *S. marcescens* *proBA* genes was determined by the

Abbreviations: AK, aspartokinase; GK, γ -glutamyl kinase; GPR, γ -glutamyl phosphate reductase; ORF, open reading frame.

The nucleotide sequence data reported in this paper have been submitted to GenBank, and have been assigned the accession number X53086.

Table 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or phenotype*	Source or reference
<i>E. coli</i> K12		
JM109	<i>thi</i>	Yanisch-Perron <i>et al.</i> (1985)
HB101	<i>proA2 leu thi</i>	Boyer & Roulland-Dussoix (1969)
ME6198	$\Delta(lac-pro) leuB bio thi$	National Institute of Genetics, Japan
<i>S. marcescens</i> Sr41		
8000	Wild-type	Matsumoto <i>et al.</i> (1975)
TT392	Nuc ⁻ r ⁻ Ap ^s Km ^s	Takagi & Kisumi (1985)
SP103	<i>putA</i>	Sugiura <i>et al.</i> (1985a)
Plasmids		
pBR322	Ap ^r Tc ^r	Bolivar <i>et al.</i> (1977)
pLG339	Km ^r Tc ^r	Stocker <i>et al.</i> (1982); derived from pSC101
pHSG298	Km ^r <i>lacZ</i> α	Takeshita <i>et al.</i> (1987)
pHSG299		
pMC1871	Tc ^r <i>lacZ</i>	Shapira <i>et al.</i> (1983)
pKK223-3	Ap ^r <i>Ptac rrnBT₁T₂</i>	Brosius & Holy (1984); expression vector bearing a <i>tac</i> promoter and <i>rrnBT₁T₂</i> terminator
pUC18	Ap ^r <i>lacZ</i> α	Yanisch-Perron <i>et al.</i> (1985)
pWP102	pBR322:: <i>proBA</i>	This study
pWP142	pBR322:: <i>proBA</i>	This study
pWP144	pBR322:: <i>proB</i>	This study
pLGlacZ1	pLG339:: <i>lacZ</i>	This study
pWPL10	pLG339:: <i>proB-lacZ</i>	This study
pUPE18	pUC18:: <i>rrnBT₁T₂</i>	This study
pUTWP18	pUPE18:: <i>Ppro</i>	This study

* Symbols used for relevant genotypes and phenotypes are as follows: Nuc⁻, no production of extracellular nuclease; r⁻, defect of a host restriction enzyme; *Ppro*, promoter of the *proBA* genes.

dideoxy chain termination method (Sanger *et al.*, 1977) with templates prepared from DNA subcloned in pHSG298 and pHSG299. Ordered deletion subclones were obtained by unidirectional digestion (Yanisch-Perron *et al.*, 1985). The DNA chains were labelled with [α -³²P]dCTP (400 Ci mmol⁻¹; 15 TBq mmol⁻¹). Analysis of sequence data was performed using the computer program GENETYX (Software Development, Japan), and the protein sequence databases NBRF-PDB (National Biomedical Research Foundation) and SWISS-PROT (European Molecular Biology Laboratory).

RNA analysis by primer extension. *pro* operon mRNA was analysed by reverse-transcriptase-directed primer extension using a synthetic 20-mer oligonucleotide, 5'-CAATTTCAACAACCAATGTCT-3', complementary to a region from position +54 to +73 of the mRNA (De la Peña & Zasloff, 1987). To facilitate the detection of extended products, a pUC-derived, high-copy-number plasmid (pUPE18), which contains the transcription terminator of the *rrnB* gene, was used. Unless otherwise stated, blunt-ending was achieved by digestion with mung bean nuclease. pUPE18 was constructed by cloning a blunt-ended 0.9 kb *EcoRI*-*ScaI* fragment, containing the *rrnBT₁T₂* terminator and the 5' region of the ampicillin resistance gene from pKK223-3, into the *PvuII* and *ScaI* sites of pUC18, thus restoring resistance to ampicillin. A blunt-ended *Bam*HI-*SacI* fragment, containing the *proBA* promoter region of pWP142, was cloned into the *SmaI* and *SacI* sites of pUPE18 to give pUTWP18. RNA extracted from recombinant cells of *S. marcescens* TT392 containing pUTWP18 was thus enriched for the transcript lacking the 3' region of the *proBA* coding sequence. RNA was purified using the glass-beads/hot-phenol extraction procedure (Hinnebusch & Fink, 1983; Peoples *et al.*, 1988) and 20 μ g of RNA was mixed with 20 pmol of 5' end α -³²P-labelled primer. The mixture was

denatured at 80 °C in 50 mM-PIPES/KOH (pH 6.4), 0.4 M-NaCl, 1 mM-EDTA and 50% formamide for 15 min and chilled on ice. Primer-RNA hybridizations were carried out at 20 °C for 16 h. After ethanol precipitation, DNA-RNA hybrids were extended at 42 °C for 60 min in 50 mM-Tris/HCl (pH 8.3), 100 mM-KCl, 10 mM-MgCl₂, 1 mM-DTT, 1 mM each of dGTP, dATP, dCTP, dTTP, 0.5 units of human placental ribonuclease inhibitor (Promega) and 22 units of RAV reverse transcriptase (Takara Shuzo). The products were fractionated on an 8% polyacrylamide sequencing gel. A dideoxy sequencing ladder was run in parallel for size comparison.

Analysis of *proB-lacZ* expression. The regulation of the *proBA* promoter was examined by using a promoter analysis plasmid. Amplification of the regulatory site of the gene in a high-copy-number plasmid might cause abnormal expression of the gene because of the absence of its repressor or activator. For this reason we constructed a low-copy-number promoter analysis plasmid pLGlacZ1, by cloning a blunt-ended (filled in) 3.0 kb *Bam*HI fragment from pMC1871, carrying the promoterless *lacZ* structural gene, into the filled in *EcoRI* and nuclease treated *Bam*HI sites of the low-copy-number plasmid pLG339. The plasmid that carried the *lacZ* gene in the opposite orientation to the *tet* gene of pLG339 was selected as pLGlacZ1. We constructed a gene fusion by ligating the beginning of the *lacZ* structural gene in frame with the region containing the *proBA* promoter and the N-terminus of the *proB* gene product. The *proB-lacZ* gene fusion plasmid pWPL10 was constructed by cloning the blunt-ended 0.3 kb *Bam*HI-*SacI* fragment of pWP142 into the filled in *Bam*HI site of pLGlacZ1. The proline-oxidase-deficient mutant of *S. marcescens*, SP103, was used as a host to prevent the degradation of L-proline added to the medium (Sugiura *et al.*, 1985a).

The units of GK and β -galactosidase activity were expressed as nmol products formed min^{-1} (mg protein) $^{-1}$. Protein concentration was determined by the use of a Bio-Rad protein assay kit with bovine serum albumin as the standard protein.

Cloning of the wild-type *proBA* operon of *S. marcescens*

The *proBA* operon of *S. marcescens* 8000, a wild-type strain of Sr41, was cloned into the *Bam*HI site of pBR322 by selecting Pro⁺ clones derived from *E. coli* HB101 (*proA*) and the resultant plasmid was denoted pWP102 (Fig. 1). This plasmid complemented the *proBA* mutation carried by *E. coli* ME6198. Southern analysis demonstrated that the insert DNA came from the *S. marcescens* chromosome and that there was no rearrangement of DNA (data not shown). The restriction map of the inserted DNA fragment agreed with that of the insert of pYI333, which was previously reported to carry the mutant-type *pro* genes from a proline-producing strain (Takagi *et al.*, 1985). Plasmid pWP142 contains a 2.9 kb

Analysis of the pWP142-encoded proteins

In order to determine the sizes of the *proA* gene products encoded by pWP142, we used an *in vitro* expression system (DeVries & Zubay, 1967) and SDS-PAGE (Fig. 2). Unique ³⁵S-methionine-labelled proteins of *M*_r 44 000 and *M*_r 39 000 were detected for pWP142, whereas similar proteins were not detected for pBR322.

Nucleotide sequence of the 2.9 kb DNA fragment carrying the proBA operon

Figs 1 and 3 show the strategy for the DNA sequencing and the complete nucleotide sequence of the 2.9 kb *NcoI*–*MluI* fragment of pWP102. Analysis of this nucleotide sequence for potential protein coding regions revealed two tandem open reading frames (ORFs). The first ORF extends from an ATG initiation codon at position 182 to a TAA termination codon at position 1283, and consists of 1101 nucleotides. The second ORF starts 10 nucleotides downstream of the termination codon of the first ORF and extends for 1472 nucleotides to a termination codon at position 2546. Putative Shine–Dalgarno (SD) sequences, AGAA and AGGA, have been identified upstream of the two initiation codons, respectively. M_r values of the two gene products were calculated on the basis of the predicted amino acid sequences. The first and second ORFs code for polypeptides of 367 and 417 amino acids with deduced M_r values of 39 169 and 44 640,

Fig. 1. Restriction maps of the *proBA* plasmids pWP102 and pWP142, and sequencing strategy for the *proBA* region. *S. marcescens* DNA was digested with *Bam*HI and a 5.8 kb DNA fragment was cloned into *Bam*HI-digested pBR322, resulting in pWP102. For the construction of pWP142, a 2.9 kb *Nco*I–*Mlu*I fragment (filled in) from pWP102 was cloned into the filled in *Bam*HI and *Eco*RI sites of pBR322. The thin arrows indicate the strategy used to obtain the complete nucleotide sequence of the *Nco*I–*Mlu*I fragment. Open arrows show the ORFs of the *proBA* operon.

Fig. 2. Analysis of polypeptides encoded by recombinant plasmid pWP142 carrying the *proBA* genes. Plasmid-specified polypeptides were produced in S-30 cell-free extract from *E. coli*. The ^{35}S -labelled polypeptides synthesized *in vitro* were analysed by SDS-PAGE and visualized by autoradiography. Lane 1, no plasmid; lane 2, pBR322; lane 3, pWP142. The migration of protein standards is indicated on the left-hand side. *bla* indicates β -lactamase encoded by the plasmids used.

respectively. These values are consistent with those obtained from the *in vitro* expression system analysis above. Based on these data, together with the complementation results described above for pWP144, in which a part of the second ORF was deleted, we concluded that the first and second ORFs represent the *proB* and *proA* genes, respectively. The dyad symmetry of the T-rich cluster which follows the *proA* gene suggests a strong stem-loop structure with a 10 bp stem and a 6 base loop. This sequence is similar to the ρ -independent terminators from *E. coli* (Rosenberg & Court, 1979). These observations confirmed that the *proBA* genes of *S. marcescens* form a single operon. The short distance between the two genes is supplementary evidence for a single operon.

Transcription initiation site of the *proBA* promoter region

To locate the transcription initiation site of the *proBA* operon, we used primer extension analysis. We subcloned the promoter region of the *proBA* operon into a pUC-derived vector and obtained pUTWP18, which produces the 5' region of the *proBA* mRNA but does not affect cell growth because of lack of the structural genes. A synthetic oligonucleotide complementary to the 5' region of the mRNA of the *proB* gene was synthesized as a primer. Three distinct extended products were observed after the reverse transcriptase reaction using the RNA from *S. marcescens* TT392 containing pUTWP18 (Fig. 4). The *proBA* gene is transcribed from the first start site located at the T residue at position 142. The two sequences, TTGGCA and TACAAA, are located 35 and 10 bp upstream of this position, respectively. These sequences were thus confirmed to constitute the promoter of the *pro* operon (Fig. 3). The putative -35 region (TTGGCA) and its flanking sequences contain a dyad symmetry centred on positions 107 and 108.

Transcriptional control of the *proBA* operon

We constructed a *proB-lacZ* fusion plasmid, pLGlacZ1, and analysed the activity of β -galactosidase in *S. marcescens* SP103 containing this plasmid to examine the transcriptional regulation of the *proBA* operon. β -Galactosidase activity in this strain was not influenced by the addition of L-proline to the culture medium (Table 2). In addition, osmotic stress caused by the addition of NaCl to the culture medium did not affect *proB-lacZ* expression.

Feedback inhibition of GK

There have been few reports on feedback control of the level of inhibition of enzyme activities involved in proline biosynthesis in *S. marcescens*. This has probably been due to the failure to detect GK activity in strains previously examined. We used *S. marcescens* TT392 containing the high-copy-number *proBA* recombinant plasmid, pWP142, to measure the proline-inhibitable GK activity. L-Proline completely inhibited the activity at concentrations greater than 0.04 mM and inhibited it by 50% at 0.016 mM (the absolute value corresponding to

Fig. 3 (on facing page). Nucleotide sequence of the *S. marcescens proBA* operon and its flanking regions. The deduced amino acid sequences of the *proB*- and *proA*-encoded proteins are given under the nucleotide sequence. Only the restriction sites used for subsequent experiments are shown. The possible ribosome binding sites (SD) and putative -10 and -35 sequences are underlined. The transcription start site is indicated by stars and the direction of transcription by an arrow. A region of dyad symmetry, centred on nucleotide positions 107 and 108, and a potential transcription termination sequence (positions 2573-2598) are indicated by arrows. The ORF upstream of the *proBA* promoter, discussed in the text, is boxed.

NcoI	
1	CCATGGAACTGGGGCTGGAACCGCCGACGATTTCAAAGAAAACTGATCAAACCTCTCCGCTGACCCCGTTGACCACGTATTGTGCTGATTTCGCGTT
	--MetGluLeuGlyLeuGluProAlaAspAspPheLysGluLysLeuIleLysLeuSerAla***
MluI	
101	GTGCTGTGGCATAACGCGTCTCTCTGTACAAAGACCTTGCACCTCTTCTTCATCATTACACACGGCAGAAATCTATGAACGGCAGCCAGACAT
	-35 -10 -1+1 mRNA SD
	MetAsnGlySerGlnThrL
201	TGGTTGTGAATTGGGCACCGCTGTGACCGCGGATCGCTGCGTCTGAACCGCGCCACATCGTCGAATTGGTGCGCCAGTGCGCGCAGCAACACGC
	euValValLysLeuGlyThrSerValLeuThrGlyGlySerLeuArgLeuAsnArgAlaHisIleValGluLeuValArgGlnCysAlaGlnGlnHisAl
301	CGCGGGCCACCGCATTTGTCATCGTCACCTCCGGCGCCATCGCCGCGGGCGCAACACCTGGGCTACCCCGAGCTGCCCGCCACCATCGCTTCCAAGCAG
	aAlaGlyHisArgIleValIleValThrSerGlyAlaIleAlaAlaGlyArgGluHisLeuGlyTyrProGluLeuProAlaThrIleAlaSerLysGln
401	CTGCTGGCAGCGGTGGGCGAGACCGCTGATCCAGCTGTGGAAACAGCTGTTCTCCATTTACGGTATCCACGTCGGGCAGATGCTGCTGACGCGCGCG
	LeuLeuAlaAlaValGlyGlnSerArgLeuIleGlnLeuTrpGluGlnLeuPheSerIleTyrGlyIleHisValGlyGlnMetLeuLeuThrArgAlaA
501	ATCTGGAAGACCGCAGCGCTTCTCAACGCGCGCATACCATGACGCGCTGTGGACAACCGCATCGTGCCGGTAATCAATGAGAACGACGCGCTCGC
	spLeuGluAspArgGluArgPheLeuAsnAlaArgAspThrMetThrAlaLeuLeuAspAsnArgIleValProValIleAsnGluAsnAspAlaValAl
601	CACCGCCGAATCAAGTGGGCGACAACGATAACCTGTCGGCACTGGCGGCCATTTGCGCGGGCGCAAACTGCTGCTGCTGACCGATCAGCAGGGC
	aThrAlaGluIleLysValGlyAspAsnAspAsnLeuSerAlaLeuAlaAlaIleLeuAlaGlyAlaAspLysLeuLeuLeuLeuThrAspGlnGlnGly
701	CTCTACACCGCCGATCCGCGCAACAAATCCGCGAGCGCAACTGATCCGTGAAGTGCACGGCATCGACGACGCGCTGCGCGCCATCGCGCGGACAGCGTCT
	LeuTyrThrAlaAspProArgAsnAsnProGlnAlaGluLeuIleArgGluValHisGlyIleAspAspAlaLeuArgAlaIleAlaGlyAspSerValS
801	CCGGTCTGGGCACCGCGGCATGGGCACCAAGCTGCGAGCGCGCGACGTGGCTGCCGCGCGGCATCGACGTGGTATCGCCGCGGCGAGCAAGCCGG
	erGlyLeuGlyThrGlyMetGlyThrLysLeuGlnAlaAlaAspValAlaCysArgAlaGlyIleAspValValIleAlaAlaGlySerLysProG
901	CGTGGTGGCGACGTGATCGAAGGCAACCGGTGGCGACCGCTTCCACGCGTGGAAACCCGCTGGAAAACCGAAGCGCTGGATCTTTGGCGCGCCA
	yValValAlaAspValIleGluGlyLysProValGlyThrArgPheHisAlaLeuGluThrProLeuGluAsnArgLysArgTrpIlePheGlyAlaPro
1001	CCGGCCGGTGGATCACCGTCCGACGCGCGCGTGAAGCGATGATGGCGCGCGCAGCTCGCTGTTGCCAAAGGCGATCCGCGAAGTGAAGGCGGACT
	ProAlaGlyGluIleThrValAspAspGlyAlaValGluAlaMetMetAlaArgGlySerSerLeuLeuProLysGlyIleArgGluValLysGlyAspP
1101	TCTCGCGCGCGAAGTATCCGTATCCGCAACCTGACGGGCGCGATCTGGCGCACGGCTGAGCCGCTACAACAGCGACGCGATGCGCATGATCGCCGG
	heSerArgGlyGluValIleArgIleArgAsnLeuThrGlyArgAspLeuAlaHisGlyValSerArgTyrAsnSerAspAlaMetArgMetIleAlaGl
1201	GCACCACTCGCAGAAATCAGCGAGATCCTCGGTTATGAATACGGTCCGGTGGCAGTGCACCGCAGCATATGATTGTCTAGTTAAGGAGTGAGCTGCTG
	yHisHisSerGlnGluIleSerGluIleLeuGlyTyrGluTyrGlyProValAlaValHisArgAspAspMetIleValSer***SD
1301	GAGCAGATGGGGAAGCCGCAAGCAGGCTCCTGGCAGCTGGCGGTGCTGAGCACGGCGAAGAAGATCAGGTGCTGTCGGTGATGGCCGACAGGCTGG
	GluGlnMetGlyLysAlaAlaLysGlnAlaSerTrpGlnLeuAlaValLeuSerThrAlaLysLysAsnGlnValLeuSerValMetAlaAspArgLeuG
1401	AAGCCAACAGTGAAGCGATCCTGCTGGCGAATGAACAAGACATGGCGCAGGCGCGGCCACCGGCATGAGCGAAGCGCTGCTCGATCGCTGTTGCTGAC
	luAlaAsnSerGluAlaIleLeuLeuAlaAsnGluGlnAspMetAlaGlnAlaArgAlaThrGlyMetSerGluAlaLeuLeuAspArgLeuLeuTh
1501	CCCGCGCGGCTGGCGCGATCGCCAACGACGTGCGCAGGTGTCGCCCTGAACGATCCGGTCCGCCACGTGCTGGACGGCAACCTGCTGGACAGCGGG
	rProAlaArgLeuAlaAlaIleAlaAsnAspValArgGlnValCysArgLeuAsnAspProValGlyHisValLeuAspGlyAsnLeuLeuAspSerGly
1601	CTGAAGCTGGAGCGCGCGGGTCCGCTCGCGGTGATTCGAGGCGCGCGCAACGTCACCATCGACGTCGCCAGCCTGTGCTGAAAA
	LeuLysLeuGluArgArgArgValProLeuGlyValIleGlyValIleTyrGluAlaArgProAsnValThrIleAspValAlaSerLeuCysLeuLysT
1701	CCGGCAACGAGTATCCTGCGCGCGGTAAAGAGACGCACAACCAACACGAGCGGCGGTAAGTATCCAGCAGGCGTTGGAACAGTGTGGCCTGCC
	hrGlyAsnAlaValIleLeuArgGlyGlyLysGluThrHisAsnThrAsnGlnAlaThrValLysValIleGlnGlnAlaLeuGluGlnCysGlyLeuPr
1801	GGCGCGCGCGGTGACGCGATCGACAGCCCGGATCGCGCGCTGGTGAACGAACTGCTGCGCTGGATCGCTACGTCGACATGCTGATCCCGCGCGCGGC
	oAlaAlaAlaValGlnAlaIleAspSerProAspArgAlaLeuValAsnGluLeuLeuArgLeuAspArgTyrValAspMetLeuIleProArgGlyGly
1901	GCCGGCTACACAAGCTGTGCCGCGAGCAATCGACCATCCCGGTGATCACCGCGGCATCGCGGTGCCATACCTATGTGGATGCCGACGTTGATTTCG
	AlaGlyLeuHisLysLeuGlyGlnSerThrIleProValIleThrGlyGlyIleGlyValCysHisThrTyrValAspAlaAspValAspPheA
2001	ACAAGCGCTGACGGTATTGAAAACGCCAAGATCCAGCGCCGAGCGCTGTAACCTCGTGAGACGCTGCTGGTGAACCGCAGCATCGCCCGGAGTT
	spLysAlaLeuThrValIleGluAsnAlaLysIleGlnArgProSerAlaCysAsnSerLeuGluThrLeuLeuValAsnArgSerIleAlaAlaGluPh
2101	CCTGCCGCGCTGAGCGCCAAAATGGCGGCGCGGGCTGACGCTGCACGCGCGGAAAACGCGCTGCCGCTGCTGCAGGCGCGCGCGGACGGTGGTG
	eLeuProAlaLeuSerAlaLysMetAlaAlaAlaGlyValThrLeuHisAlaAlaGluAsnAlaLeuProLeuLeuGlnGlyGlyProAlaThrValVal
2201	CCGGTGAACGCGGAGGATTACGACGACGAATGGCTGTCGCTGGATCTGAACGTCGCTGGTGGACGATATCGATCAGGCCATCGATCACATCCGACCC
	ProValAsnAlaGluAspTyrAspAspGluTrpLeuSerLeuAspLeuAsnValLeuLeuValAspAspIleAspGlnAlaIleAspHisIleArgThrH
2301	ACGGCACTAACCATTCCGACGCGATCCTACCCGTTCCGCTCAGCAGCGCGGAGCACTTCGTGCGCGGGTGGACTCCTCGCGGTGTACGTCAACGCCAG
	isGlyThrAsnHisSerAspAlaIleLeuThrArgSerLeuSerSerAlaGluHisPheValArgAlaValAspSerSerAlaValTyrValAsnAlaSe
2401	CACCCGCTTACCGACGCGCGGAGTTCGGCTTGGCGCTGAAGTGGCGGTGAGCACCCAGAACTGCACGCGCGCGCGGCGGATGGCGCTGGACGCGCTG
	rThrArgPheThrAspGlyGlyGlnPheGlyLeuGlyAlaGluValAlaValSerThrGlnLysLeuHisAlaArgGlyProMetGlyLeuAspAlaLeu
2501	ACCACCTACAAGTGGATTGGCTACGGCAGCATCTGGTGGTAGCTAAGGCCGATTACGGTCATAAAGAGGTCAGGGCGCACCATGCTGCGCCCTGATT
	ThrThrTyrLysTrpIleGlyTyrGlyAspAspLeuValArgSer***
2601	TTTTTTGACGCTAGCTGCAGTGCGGCTGTTTGCAAAACAACCAACGCCCCGCGGCTATTGACTCATTTCGCGCGCAGGTCTACCTTGTCAACGCAC
2701	TTCCGGCTTGCCCCGACGGCCCGGCTCGATGCCGATATAGCTCAGTTGGTAGACAGCGCATTCGTAATGCGAAGTCGTAGGTCGACTCCTATTATCG
2801	GCACCAATTTCAAGTTTCACTGCCTTCTTAGTAAAAATACGACGACTGGCGTCTGCAACGCTTGCGGTTGATATTTCTGCTTATCGCTATGTGGCAATA
2901	TGCTGCAAGGTATAATACAAACGCGT
	MluI
	2926

Fig. 4. 5'-End mapping of mRNA from the *proBA* locus by primer extension analysis. The extended products are shown alongside the dideoxy sequencing products primed by the same primer on the coding strand for size comparison. Lane 1, C; lane 2, T; lane 3, A; lane 4, G; lane 5, 32 P-labelled primer; lane 6, extended products. The three most likely endpoints of the extension reaction are indicated by stars on the non-coding strand shown on the right-hand side. The 32 P-labelled primer cannot be seen in the part of the gel shown (lane 5).

Table 2. Levels of β -galactosidase activity of *proB-lacZ* fusions in *S. marcescens* grown in modified minimal medium supplemented with L-proline or NaCl

Strain (plasmid)	Specific activity of β -galactosidase (units)*				
	L-Proline (mM)				NaCl (mM)
	0	1	10	100	
SP103(pLG339)	<10	<10	<10	<10	<10
SP103(pLGlacZ1)	54	46	45	44	120
SP103(pWPL10)	470	460	490	500	400

* Units were expressed as nmol product formed min^{-1} (mg protein) $^{-1}$.

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1
SM  INGSQTLIVKLGTSVLTGGSLRLNRAHIVELVRQCAQHAAGHRIVIVTSGAIAAGREHL
EC  MSQSQTLIVKLGTSVLTGGSLRLNRAHIVELVRQCAQHAAGHRIVIVTSGAIAAGREHL
1
SM  GYPPLPATIASKQLLAAYGQSRLIQLWEQLFSIYGIHVGMQLLTLADLEDRERFLNARDT
EC  GYPPLPATIASKQLLAAYGQSRLIQLWEQLFSIYGIHVGMQLLTLADLEDRERFLNARDT
SM  MTALLDNITVPVINENDAVATAEIKVGDNDNLSALAAIILAGADKLLLLTDQGLYTADPR
EC  LRALLDNITVPVINENDAVATAEIKVGDNDNLSALAAIILAGADKLLLLTDQGLYTADPR
SM  NNPAELITRETHGIDDALRAIAGDSVSLGTGGMSTKLQAADVACRAGIDVYLAAGSKPG
EC  NNPAELITRETHGIDDALRAIAGDSVSLGTGGMSTKLQAADVACRAGIDVYLAAGSKPG
SM  VVADVIEGKPVGTIRFHALETPLENRKRITFGAPPAGEITVDGAVEMARGSSLLPKGT
EC  VVADVIEGKPVGTIRFHALETPLENRKRITFGAPPAGEITVDGAVEMARGSSLLPKGT
SM  REVKQDFSRGEVIRIRNLTGRDLAHGVSRYNSDAMMITAGHSQETSEILGYEYGPVAVH
EC  KSVTGNFSRGEVIRIRNLTGRDLAHGVSRYNSDAMMITAGHSQETSEILGYEYGPVAVH
SM  RDDNITVS
EC  RDDNITVS
367

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Fig. 5. Comparison of the predicted amino acid sequence of the *S. marcescens* GK with that of the *E. coli* GK. The entire amino acid sequences of *S. marcescens* GK (SM; this work) and *E. coli* GK (EC; Deutch *et al.*, 1984; Csonka *et al.*, 1988) are presented in the one-letter code. Identical residues are indicated by boxed regions.

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1
SM  MLEQMGAAKQASWOLAVLSTAKKIQVLSVMDRLANSBATTLANEODMAQARATQMS
EC  MLEQMGAAKQASYKLAQLSSREKIRVLEKIADELEAQSBITLANAODMADARANGISE
1
SM  ALLDRILLTPARIAAATANDVRQVORDNDPVGVLDGNDLDSGLKLERRRVPLGVIGVIYE
EC  ALLDRILLTPARIKGLADDVRQVORDNDPVGVLDGNDLDSGLKLERRRVPLGVIGVIYE
SM  ARPNTVIDVASLCKTGNVILRGGKETHTNFKATVKVIOQALDEQGLPAAAVQOAITSPD
EC  ARPNTVIDVASLCKTGNVILRGGKETHTNFKATVKVIOQALDEQGLPAAAVQOAITSPD
SM  RALVMEIRLIDRMVMDLIPRGGAGLHKLCREQSTIPVITGGIGVCHINVDVDFDKALT
EC  RALVMEIRLIDRMVMDLIPRGGAGLHKLCREQSTIPVITGGIGVCHINVDVDFDKALT
SM  VTNNAKICORPSACNSLETLLVNRSTAAEFPLPALSAMMAAGVTLHAENADPLCOGPAT
EC  VTNNAKICORPSTCNTVETLLVNKNLADSLPALSQMAESCVTLHADAAALAOAGPAK
SM  VVHNAEDYDDEMLSLDLNVLLMDIDCATDHTIRHGTNHSDAITLRSLSAAEHFVRAVD
EC  VVHNAEDYDDEMLSLDLNVLLMDIDCATDHTIRHGTNHSDAITLRSLSAAEHFVRAVD
SM  SSAYVYNASTRFTDGGQFGLGAEVAVSTQKLHARGPMGLDALTYYKWIGYEDDLVRS
EC  ----VNASTRFTDGGQFGLGAEVAVSTQKLHARGPMGLDALTYYKWIGYEDDLVRS
417

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Fig. 6. Comparison of the predicted amino acid sequence of the *S. marcescens* GPR with that of the *E. coli* GPR. The entire amino acid sequences of *S. marcescens* GPR (SM; this work) and *E. coli* GPR (EC; Deutch *et al.*, 1984; Csonka *et al.*, 1988) are presented in the one-letter code. Identical residues are indicated by boxed regions. To maximize homology, 10 gaps were introduced into the *E. coli* GPR sequence.

100% was 8.3 units of specific activity). Thus, feedback inhibition of the first enzyme in proline biosynthesis was demonstrated in *S. marcescens*.

Discussion

The *proB* and *proA* genes, encoding GK and GPR, respectively, constitute a single operon in *S. marcescens* as they do in *E. coli*. Nucleotide sequence analysis has revealed that the structure of this operon in *S. marcescens* is similar to that in *E. coli*. Comparison of the *proB* and *proA* products of *S. marcescens* with those of *E. coli* demonstrated 88% and 74% homology, respectively, based on the predicted amino acid sequences (Figs 5 and 6). There is no difference between the two bacteria in

GK	<i>E. coli</i>	159	L	A	G	A	D	K	L	L	L	T	D	Q	K	G	L	Y	T	A	D	P	R	S	N	P	Q	A	E	L	I	K	D	190	
GK	<i>S. marcescens</i>	159	L	A	G	A	D	K	L	L	L	T	D	Q	K	G	L	Y	T	A	D	P	R	S	N	P	Q	A	E	L	I	K	D	190	
AKI	<i>S. marcescens</i>	217	C	L	R	A	D	C	C	E	I	W	T	D	V	D	G	V	Y	T	C	D	P	R	T	V	P	D	A	R	L	L	K	S	248
AKI	<i>E. coli</i>	217	C	L	R	A	D	C	C	E	I	W	T	D	V	D	G	V	Y	T	C	D	P	R	T	V	P	D	A	R	L	L	K	S	248
AK	<i>S. cerevisiae</i>	245	A	V	N	A	D	E	I	Q	V	W	K	E	V	D	G	I	F	T	A	D	P	R	K	V	P	E	A	R	L	L	D	S	276
AKII	<i>E. coli</i>	219	L	A	G	V	S	R	V	T	I	W	S	D	V	A	G	V	Y	S	A	D	P	R	K	V	K	D	A	C	L	L	P	L	250
AKIII	<i>E. coli</i>	211	A	L	H	A	S	R	V	D	I	W	T	D	V	P	G	L	Y	T	T	D	P	R	V	V	S	A	A	K	R	T	D	E	242
AKII	<i>B. subtilis</i>	160	L	A	A	A	L	K	V	D	I	T	D	V	P	G	L	F	T	T	D	P	R	Y	V	K	S	A	R	K	T	E	G	194	

DKC

Fig. 7. Comparison of the amino acid sequences of homologous regions in GK and AK enzymes (AKI, AKII and AKIII). Partial amino acid sequences of GK and AK enzymes from *S. marcescens* (GK, this study; AK, our personal data), *E. coli* (GK, Deutch *et al.*, 1984; AKI, Katinka *et al.*, 1980; AKII, Zakin *et al.*, 1983; AKIII, Cassan *et al.*, 1986), *Bacillus subtilis* (AKII, Chen *et al.*, 1987) and *Saccharomyces cerevisiae* (AK; Rafalski & Falco, 1988) were compiled. Residues are presented in the one-letter code and are numbered at both ends of line. Identical and similar residues are enclosed by solid and dotted lines, respectively (accepted alternatives are I-L-V-M-F, D-E, N-Q, R-K, S-T).

numbers of amino acid residues of GK. However, GPR of *S. marcescens* has an additional 10 residues in the C-terminal portion. To examine the structural features of GK, the amino acid sequence was compared with other GK and aspartokinase (AK) sequences from the database. We found that specific short regions of GK from *S. marcescens* and *E. coli* (Fig. 7) demonstrate considerable sequence similarity with regions of AK isoenzymes (AK, AKI, AKII and AKIII) from four micro-organisms although GK does not show any overall amino acid homology with AK isoenzymes. GK and AK produce acylphosphates, that is, γ -glutamyl phosphate and β -aspartyl phosphate, respectively. This sequence may, therefore, play an important structural role in the formation of carboxyl phosphates of amino acids. The *S. marcescens* promoter sequence is very similar to the consensus sequence of the *E. coli* promoter (Hawley & McClure, 1983). A typical sequence for the *p*-independent terminator was found downstream of the *proA* gene.

The transcription initiation site of the *proBA* operon has been identified by primer extension analysis. Previously, when wild-type cells were used, it was difficult to detect extended DNA products because of the low levels of mRNA templates. The use of cells containing a high-copy-number recombinant plasmid with the cloned gene solves this problem. However, amplification of the entire gene, including the promoter and structural regions, may disturb growth of the cells and, hence, we subcloned only the promoter region into a high-copy-number plasmid. This enrichment of the mRNA enabled us to locate the transcription initiation site of the *proBA* operon very accurately. The position of the *proBA* promoter, identified by the transcription initiation site, coincides with that predicted from the consensus sequence of the *E. coli* promoter.

The feedback control mechanism for proline biosynthesis in *S. marcescens* has been studied in this work. The

promoter region of the *proBA* operon has no attenuator-like structure, but rather a dyad symmetrical structure which might be related to gene expression. The promoter region of the *E. coli proBA* genes contains several inverted repeats, whereas that of *S. marcescens* has a single inverted repeat structure, which might function as a regulatory locus for the expression of the *proBA* genes. However, the transcription of the *proBA* operon was not influenced by the addition of excess proline to the culture medium as judged from data on expression of the *lacZ* gene fused with the *proBA* promoter region. These results suggest that the *proBA* operon from *S. marcescens* is not subject to proline-mediated feedback repression, although a previous paper reported the possibility that proline biosynthesis might be repressed by proline (Sugiura *et al.*, 1985b). However, the findings described above coincide with those observed for *E. coli* (Deutch *et al.*, 1984).

Amplification of the *proB* gene in recombinant cells has enabled us to measure GK activity in *S. marcescens*. This enzyme was found to be very sensitive to inhibition by proline. The proline concentration required to decrease the activity by 50% was 0.016 mM and was similar to that observed for *E. coli* GK (Smith *et al.*, 1984; Dandekar & Uratsu, 1988). Thus we concluded that, in *S. marcescens*, feedback inhibition of the first enzyme in the proline biosynthetic pathway represents the only means of regulation.

The intracellular content of proline is elevated by osmotic stress (Grothe *et al.*, 1986). Proline is well known as an effective osmoprotectant and it has been reported that wild-type bacteria accumulate proline in response to osmotic stress. The *putP*, *proP* and *proU* genes are responsible for proline uptake. However, there was a possibility that expression of the *proBA* operon might be stimulated under conditions of high osmotic stress. Unexpectedly, a high concentration of NaCl did not

affect the transcription level and, therefore, it was concluded that the *proBA* operon is not subject to osmoregulation.

S. marcescens differs from *E. coli* in the gene structure upstream of the promoter region. In *E. coli* the *phoE* gene lies in close proximity to the *proBA* promoter region on the opposite strand. In *S. marcescens*, however, an ORF was identified immediately upstream of the promoter region in the same orientation as *proBA*. The sequence of this ORF shows a high degree of similarity with that of the *E. coli* protein (M_r 15000) identified downstream of the *phoE* gene (Overbeeke *et al.*, 1983; Nüesch & Schümperli, 1984). This finding indicates that in *S. marcescens* the *phoE* gene may be located at a separate site on the chromosome.

The final goal of our research is a better understanding of the mechanisms of proline hyperproduction in *S. marcescens* mutants and we are currently determining base changes in the *proB* gene cloned from one of these mutants.

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