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, 39169 and 44640, 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 1-pyroline-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-I** 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-I** 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 μg ml⁻¹; ampicillin (Ap), 200 μg 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 μg), prepared by CsCl density gradient centrifugation, was added to the reaction mixture (5 μl) containing L-[¹⁵N]methionine, according to the protocol of the manufacturer. After 60 min incubation at 37 °C, SDS-PAGE of ³⁵S-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

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*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

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or phenotype*</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli K12</td>
<td>thi</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td>JM109</td>
<td>proA2 leu thi</td>
<td>Boyer &amp; Roulland-Dussoix (1969)</td>
</tr>
<tr>
<td>HB101</td>
<td>Δ(lac-pro) leuB bio thi</td>
<td>National Institute of Genetics, Japan</td>
</tr>
<tr>
<td>M6E198</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. marcescens S41</td>
<td>Wild-type</td>
<td>Matsumoto et al. (1975)</td>
</tr>
<tr>
<td>8000</td>
<td>Nuc- r- Ap+ Km+</td>
<td>Takagi &amp; Kisumi (1985)</td>
</tr>
<tr>
<td>TTR92</td>
<td>putA</td>
<td>Sugiura et al. (1985a)</td>
</tr>
<tr>
<td>SP103</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBR322</td>
<td>Ap' Te'</td>
<td>Bolivar et al. (1977)</td>
</tr>
<tr>
<td>pLG339</td>
<td>Km' Te'</td>
<td>Stocket et al. (1982); derived from pSC101</td>
</tr>
<tr>
<td>pHS298</td>
<td>Km' lacZz</td>
<td>Takeshita et al. (1987)</td>
</tr>
<tr>
<td>pHS299</td>
<td>Tc' lacZ</td>
<td>Sharira et al. (1983)</td>
</tr>
<tr>
<td>pMC1871</td>
<td>Ap' Prac rrnBT, T2</td>
<td>Brosius &amp; Holy (1984); expression vector bearing a tac promoter and rrnBT, T2 terminator</td>
</tr>
<tr>
<td>pKK223-3</td>
<td></td>
<td></td>
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<tr>
<td>pUC18</td>
<td>Ap' lacZa</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td>pWP102</td>
<td>pBR322::proBA</td>
<td>This study</td>
</tr>
<tr>
<td>pWP142</td>
<td>pBR322::proBA</td>
<td>This study</td>
</tr>
<tr>
<td>pWP144</td>
<td>pBR322::proB</td>
<td>This study</td>
</tr>
<tr>
<td>pLGlacZ1</td>
<td>pLG339::lacZ</td>
<td>This study</td>
</tr>
<tr>
<td>pWP10</td>
<td>pLG339::proB-lacZ</td>
<td>This study</td>
</tr>
<tr>
<td>pUPE18</td>
<td>pUC18::rrnBT, T2</td>
<td>This study</td>
</tr>
<tr>
<td>pUTWP18</td>
<td>pUPE18::Ppro</td>
<td>This study</td>
</tr>
</tbody>
</table>

* 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 pHS298 and pHS299. Ordered deletion subclones were obtained by unidirectional digestion (Yanisch-Perron et al., 1985). The DNA chains were labelled with [α-32P]dCTP (40 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'-CAATTTCACAACCAATGTCT-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-Scal fragment, containing the rrnBT, T2 terminator and the 5' region of the ampicillin resistance gene from pKK223-3, into the PstI and Scal sites of pUC18, thus restoring resistance to ampicillin. A blunt-ended BamHI-SalI fragment, containing the proBA promoter region of pWP142, was cloned into the Smal and SalI sites of pUPE18 to give pUTWP18. RNA extracted from recombinant cells of S. marcescens TTT392 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., 1986) and 20 μg 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 mM-NaCl, 0 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. The DNA chains were labelled with [α-32P]dCTP (40 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).
**Analysis of Serratia marcescens proBA genes**

**Assay of enzyme activity.** Cells were grown at 30 °C in 500 ml flasks containing 200 ml of minimal medium. For the assay of GK activity the cells were harvested in the exponential growth phase, washed with 50 mM-Tris/HCl (pH 7.2) containing 1 mM-DTT and disrupted by sonication. The cell extracts were desalted with a Pharmacia PD-10 column and used for the hydroxamate assay of GK activity as described by Hayzer & Leisinger (1980). The standard assay, containing 50 mM-L-glutamate, was used. The difference between values with and without the addition of 100 mM-L-proline to the reaction mixture represented the proline-inhibitable GK activity. The proline-inhibitable GK of strain TT392 containing pWP142 was inhibited by 100 mM-L-proline to the level of the same strain without the plasmid.

For the assay of β-galactosidase the cells were harvested in the exponential growth phase, washed with saline and resuspended in 100 mM-sodium phosphate buffer (pH 7:0) containing 10 mM-KCl, 1 mM-MgSO₄ and 50 mM-2-mercaptoethanol. After disruption by sonication, the resultant cell extracts were used for the assay of β-galactosidase. β-Galactosidase activities were measured using α-nitrophenyl β-D-galactoside as a substrate (Miller, 1972). 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.

**Results**

**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 BamHI 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 NcoI–MluI fragment carrying the proBA region, inserted into the BamHI and EcoRI sites of pBR322. This plasmid complemented the proBA mutations carried by strain ME6198 and the proA mutation carried by strain HB101. Plasmid pWP144, constructed by deleting a 0.7 kb EcoRV–MluI fragment from pWP142, did not complement the proA mutation, indicating that the EcoRV site is located in the proA gene.

**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*, 44000 and *M*, 39000 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*, 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*, values of 39169 and 44640,
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 35S-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 p-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. 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, 32P-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 32P-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

<table>
<thead>
<tr>
<th>Strain (plasmid)</th>
<th>L-Proline (mM)</th>
<th>NaCl (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>SP103(pLG339)</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>SP103(pLGlacZ1)</td>
<td>54</td>
<td>46</td>
</tr>
<tr>
<td>SP103(pWPL10)</td>
<td>470</td>
<td>460</td>
</tr>
</tbody>
</table>

* Units were expressed as nmol product formed min⁻¹ (mg protein)⁻¹.

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.

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.

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 the
numbers of amino acid residues of GK. However, GPR of \textit{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 \textit{S. marcescens} and \textit{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, \( \gamma \)-glutamyl phosphate and \( \beta \)-aspartyl phosphate, respectively. This sequence may, therefore, play an important structural role in the formation of carboxyl phosphates of amino acids. The \textit{S. marcescens} promoter sequence is very similar to the consensus sequence of the \textit{E. coli} promoter (Hawley & McClure, 1983). A typical sequence for the \( \rho \)-independent terminator was found downstream of the \textit{proA} gene.

The transcription initiation site of the \textit{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 \textit{proBA} operon very accurately. The position of the \textit{proBA} promoter, identified by the transcription initiation site, coincides with that predicted from the consensus sequence of the \textit{E. coli} promoter.

The feedback control mechanism for proline biosynthesis in \textit{S. marcescens} has been studied in this work. The promoter region of the \textit{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 \textit{E. coli} \textit{proBA} genes contains several inverted repeats, whereas that of \textit{S. marcescens} has a single inverted repeat structure, which might function as a regulatory locus for the expression of the \textit{proBA} genes. However, the transcription of the \textit{proBA} operon was not influenced by the addition of excess proline to the culture medium as judged from data on expression of the \textit{lacZ} gene fused with the \textit{proBA} promoter region. These results suggest that the \textit{proBA} operon from \textit{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 \textit{E. coli} (Deutch et al., 1984).

Amplification of the \textit{proB} gene in recombinant cells has enabled us to measure GK activity in \textit{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 \textit{E. coli} GK (Smith et al., 1984; Dandekar & Uratsu, 1988). Thus we concluded that, in \textit{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 \textit{putP}, \textit{proP} and \textit{proU} genes are responsible for proline uptake. However, there was a possibility that expression of the \textit{proBA} operon might be stimulated under conditions of high osmotic stress. Unexpectedly, a high concentration of NaCl did not

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**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 \textit{S. marcescens} (GK, this study; AK, our personal data), \textit{E. coli} (GK, Deutch et al., 1984; AKI, Katinka et al., 1980; AKII, Zakin et al., 1983; AKIII, Cassan et al., 1986), \textit{Bacillus subtilis} (AKII, Chen et al., 1987) and \textit{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).
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, 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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### References


