Analysis of the mutant proBA operon from a proline-producing strain of Serratia marcescens

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(Received 3 October 1991; revised 10 December 1991; accepted 30 December 1991)

The nucleotide sequence of the proBA operon from a proline-hyperproducing mutant of Serratia marcescens was determined. Two base substitutions were found: one in the proB structural gene, coding for γ-glutamyl kinase (GK), and a second one in the promoter region of the operon. The former base substitution led to a change of the predicted amino acid at position 117 from an alanine to a valine in GK. This mutation rendered GK 700-fold less sensitive to proline-mediated feedback inhibition than the wild-type enzyme. The other base substitution, a transversion from a G-C to an A-T, was located in the spacer region between the ‘–35’ and ‘–10’ sequences of the promoter, and it increased the transcriptional activity of this operon fourfold. Both these two base substitutions, which were acquired at the step of selecting mutants resistant to a toxic proline analogue, 3,4-dehydroproline, confer upon cells a high proline productivity and an increased osmotolerance.

Introduction

Proline is one of the important cellular osmolytes that are closely related to resistance to osmotic stress. The accumulation of proline decreases intracellular water potential and, therefore, makes various organisms osmotolerant (Aspinall & Paleg, 1981; Measures, 1975). Proline accumulation can be a result of proline uptake from the medium or increased proline biosynthesis.

In micro-organisms, proline is synthesized from glutamate via three enzymic reactions, catalysed by γ-glutamyl kinase (GK; proB product), γ-glutamyl phosphate reductase (GPR; proA product), and 1-pyrroline-5-carboxylate reductase (proC product). We have been working on proline metabolism in Serratia marcescens. Wild-type strains of this bacterium do not grow in minimal medium with increased osmolarity and produce only a small amount of proline (Sugiura & Kisumi, 1985a; Sugiura et al., 1985). Proline-analogue-resistant mutants have been isolated from S. marcescens and tested for proline productivity and osmotic stress tolerance (Sugiura & Kisumi, 1985a, b; Sugiura et al., 1985). Some of these mutants showed enhanced proline productivity and enhanced osmotolerance. One of them, SP187, produced more than 60 g of L-proline l−1 in a fermentation medium (Sugiura & Kisumi, 1985a).

We have recently determined the nucleotide sequence and the gene structure of the proBA operon from wild-type S. marcescens (Omori et al., 1991). S. marcescens is similar to Escherichia coli in the gene structure and the amino acid sequences of the two enzymes encoded by this operon. Proline synthesis from glutamate is regulated mainly through proline-mediated feedback inhibition of GK, the first enzyme in the pathway.

Strain SP187 described above was obtained by four rounds of mutagenesis and selection. This strain has the putA mutation, abolishing proline oxidase activity, and shows resistance to the proline analogues 3,4-DL-dehydroproline (DHP), L-thiazolidine-4-carboxylic acid (TAC) and L-azetidine-2-carboxylic acid (Sugiura & Kisumi, 1985a). Transductional analysis suggested that the DHP resistance mutation, designated the dpr-I genotype, might be closely linked to the proBA region but we had no further information on this mutation (Sugiura & Kisumi, 1985a).

In the work described here, we determined and analysed the nucleotide sequence of the mutant proBA...
overproduction, we constructed four hybrid (Takagi et al., 1985).

Methods

Strains, plasmids and media. The strains and plasmids are listed in Table 1. E. coli JM109 was used for the construction of plasmids. The rich medium used was LB (Davis et al., 1980); the minimal medium of Davis & Mingioli (1950) was modified by omitting the sodium citrate and supplemented with 0.5% glucose or 0.5% disodium succinate hexahydrate as a carbon source. Required L-amino acids were added at 1 mM. Antibiotics were added at the following concentrations: carbenicillin (Km), 200 µg ml⁻¹; ampicillin (Ap), 200 µg ml⁻¹.

General 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 & Kisman (1985).

DNA sequencing. The nucleotide sequence of the plasmid containing the mutant-type proBA genes from S. marcescens SP187 was determined by dyeoxy chain-termination method (Sanger et al., 1977) with the aid of a sequencing system purchased from Takara Shuzo (Kyoto, Japan). After subcloning DNA fragments into pUC18, pUC19, 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). Nucleotide sequence data were analysed by using the computer program GENETYX (Software Development, Japan).

Proline excretion. To determine the mutation responsible for proline overproduction, we constructed four hybrid proBA plasmids containing mutations in the proBA operon. The 2.9 kb BamHI-EcoRI proBA fragments of pWP142 and pY1720 were subcloned into the corresponding sites of pLG339 to give pWPK342 (wild-type) and pYIK920 (mutant), respectively. Plasmid pWPK100, carrying the proP2 mutation, was constructed by replacing the 0.4 kb Ncol–AsuI fragment containing the promoter region of pWPK342 with one isolated from pYIK920 (see Fig. 1). Plasmid pWPK010, carrying the proB1 mutation, was obtained by replacing the 0.9 kb AsuI–SphI fragment containing the proB gene of pWPK342 with one isolated from pYIK920 (see Fig. 1). S. marcescens TT392 was used as a host for these plasmids.

Proline excretion was determined by an auxanographic cross-feeding test using a proline auxotroph, S. marcescens SP139 (Sugarui et al., 1985). A minimal agar plate seeded with this strain at 5 x 10⁶ cells ml⁻¹ was spotted with cells of the strain to be tested and incubated at 30 °C for 24–48 h. Excretion was scored positive if a halo of satellite growth of the strain to be tested 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.

Analysis of proB–lacZ expression. The transcriptional activity of the proBA promoter was measured by using the plasmid pLGlacZ1 (Omori et al., 1991). We constructed a gene fusion by ligating the beginning of the lacZ structural gene in-frame to the region containing the proP2 promoter and the 5' terminus of the proB gene of pWPK342 with one isolated from pYIK920 (see Fig. 1). S. marcescens TT392 was used as a host for these plasmids.

For the assay of β-galactosidase, 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 o-nitrophenyl β-D-galactoside as a substrate (Miller, 1972).

The units of GK and β-galactosidase activities are nmol product 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.

Analysis of proB–lacZ expression. The transcriptional activity of the proBA promoter was measured by using the plasmid pLGlacZ1 (Omori et al., 1991). We constructed a gene fusion by ligating the beginning of the lacZ structural gene in-frame to the region containing the proBA promoter and the 5' terminus of the proB gene. The proB–lacZ gene fusion plasmid, pYL10, was constructed by cloning the 0.3 kb BamHI–SacII fragment of pY1720 into the filled-in BamHI site of pLGlacZ1 (see Fig. 4). The proline-oxidase-deficient mutant of S. marcescens, SP103, was used as a host to prevent the degradation of the proline-expressing plasmid. E. coli JM109 was used for the construction of plasmids.

In vitro transcription-translation analysis of plasmid DNAs. The prokaryotic cell-free coupled transcription-translation system from Amersham was employed for labelling proteins encoded by plasmids.

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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>(Δlac-proBA) thi</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td>JM109</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT392</td>
<td>Nuc r Ap Km</td>
<td>Takagi &amp; Kisman (1985)</td>
</tr>
<tr>
<td>SP103</td>
<td>putA</td>
<td>Sugiuira et al. (1985)</td>
</tr>
<tr>
<td>SP139</td>
<td>putA proB/A</td>
<td>Sugiuira et al. (1985)</td>
</tr>
<tr>
<td>Plasmids</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>Stocker et al. (1982); derived from pSC101</td>
</tr>
<tr>
<td>pUC18</td>
<td>Ap lacZ</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td>pUC19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pHSG298</td>
<td>Km lacZ</td>
<td>Takeshita et al. (1987)</td>
</tr>
<tr>
<td>pHSG299</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pLGlacZ1</td>
<td>pLG339: lacZ</td>
<td>Omori et al. (1991)</td>
</tr>
<tr>
<td>pY1333</td>
<td>pKP1154: :proP2 proBA*</td>
<td>Takagi et al. (1985)</td>
</tr>
<tr>
<td>pY1720</td>
<td>pBR322: :proP2 proBA*</td>
<td>This study</td>
</tr>
<tr>
<td>pWPK342</td>
<td>pLG339: :proB:A*</td>
<td>This study</td>
</tr>
<tr>
<td>pYIK920</td>
<td>pLG339: :proP2 proBA*</td>
<td>This study</td>
</tr>
<tr>
<td>pWPK100</td>
<td>pLG339: :proP2 proB:A*</td>
<td>This study</td>
</tr>
<tr>
<td>pWPK010</td>
<td>pLG339: :proB*A</td>
<td>This study</td>
</tr>
<tr>
<td>pWPL10</td>
<td>pLGlacZ1: :proP2 proB'</td>
<td>Omori et al. (1991)</td>
</tr>
<tr>
<td>pYIL10</td>
<td>pLGlacZ1: :proP2 proB'</td>
<td>This study</td>
</tr>
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</table>

* Symbols used for relevant genotypes and phenotypes are as follows: Nuc-, no production of extracellular nuclease; r-, defect of a host restriction enzyme; putA, destruction of proline oxidase; proBA, proline auxotrophy; proP2, promoter of the proBA genes; proP2, up-mutation in the promoter enhancing transcription of the proline oxidase deficient mutant of S. marcescens, SP103, was used as a host to prevent the degradation of the proline-expressing plasmid. E. coli JM109 was used for the construction of plasmids.
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Fig. 1. Restriction maps of the proBA plasmids pYI333 and pYI720, and the base substitutions found in the mutant proBA operon. The restriction sites indicated on the map are defined. Open arrows show the ORFs and the promoter region (P) of the proBA operon. The positions of two base substitutions, Ppro2 and proBI, and the resultant amino acid substitution in the proBA operon, are indicated.

Fig. 2. Base substitution found in the promoter region of the proBA operon. The nucleotide sequence of the promoter region of the mutant is shown. The distance in base pairs from the first transcription start site, which is designated +1, is given above the sequence. Potential -10' and -35' sequences are underlined. A region of dyad symmetry, centred at position 107–108 (-34–35), is shown by inverted arrows. The base change from a C to a T at nucleotide 127 (-16) is indicated by an arrow.

Plasmid DNA (0.4 µg), prepared by CsCl density-gradient centrifugation, was added to the reaction mixture (5 µl) containing L-[35S]-methionine, according to the manufacturer's protocol. SDS-PAGE of 35S-labelled proteins was carried out as described by Laemmli (1970). Labelled polypeptide bands were visualized by autoradiography.

Sensitivities to osmotic stress and to proline analogues. Osmotolerance and proline analogue resistance were measured as described by Sugiura et al. (1985). To determine the sensitivity to osmotic stress, we used a putA+ strain, S. marcescens TT392, as host. Cells harbouring pWPK342, pWPK100 or pWPK101 were grown at 30°C in the minimal medium containing 0.5% glucose as carbon source with or without the addition of 0.6 M-NaCl to increase osmolarity. To examine proline analogue sensitivity, the purA strain S. marcescens SP103 was used, to prevent the degradation of L-proline and proline analogues. To determine the sensitivities to DHP and TAC, cells carrying the above plasmids were grown in the minimal medium containing 0.5% glucose or 0.5% sodium succinate, respectively. Growth was estimated turbidimetrically at 660 nm with a Hitachi electric photometer (EPO-B type).

Results

Base substitutions in the mutant proBA operon

The mutant proBA operon from strain SP187 had been previously cloned into pKP1154, resulting in plasmid pYI333 (Takagi et al., 1985). The 2.9 kb Ncol–Muli fragment of this plasmid was subcloned in the BamH1–EcoRI interval of pBR322, creating pYI720, and was confirmed to contain the entire proBA genes by a complementation test. The structure of pYI720 carrying the mutant proBA operon was shown to be identical by restriction analysis to that of pWP142 carrying the wild-type proBA (Omori et al., 1991). The complete nucleotide sequence of the insert carried by pYI720 was determined. Subsequently, this insert was confirmed to
I, include the two open reading frames coding for GK and GPR, a promoter region, and a terminator region. Nucleotide sequence comparisons with the wild-type proBA operon revealed two base substitutions in the mutant-type operon (Fig. 1). One, designated Ppro2, was a G-C to A-T transversion at nucleotide 127, in the spacer between the ‘−35’ and ‘−10’ sequences (Fig. 2). The other, designated proB1, was a G-C to A-T transversion at nucleotide 531 in the proB structural gene. This substitution predicts an amino acid substitution from an alanine to a valine at amino acid residue 117 in the mutant GK. Although the proline-overproducing mutation in the pro operon of strain SP187 was previously designated dpr-I (Sugiura & Kisumi, 1985), here we have found that this mutation consists of two base substitutions in the operon. In this paper, we use Ppro2 and proB1 as genotypes of the mutations.

**Effects of the individual base substitutions on proline production**

We subcloned the wild-type and the mutant proBA fragments into a low-copy-number vector, pLG339, for stable expression of the proBA operon, generating pWPK342 and pYIK920, respectively. To elucidate the
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Lack of feedback inhibition of GK encoded by the proB1 gene

We have previously reported that the wild-type GK produced by the plasmid carrying the wild-type proBA operon is very sensitive to proline-mediated feedback inhibition (Omori et al., 1991). The activity of GK produced by the proB1 plasmid, pWPK010, was examined for feedback inhibition. The concentration of L-proline causing 50% inhibition was 11 mM for the mutant GK and 0.016 mM for the wild-type (results not shown).

Elevated expression of the proBA operon caused by the Ppro2 mutation

To examine the effect of the Ppro2 mutation on gene expression, we constructed the proB–lacZ gene fusion plasmid pYL1.0, carrying the Ppro2 mutation, as described for pWPL10 by Omori et al. (1991). β-Galactosidase activities were examined using cells harbouring each of the two gene fusion plasmids (Fig. 4). The promoter carrying the Ppro2 mutation showed about fourfold greater transcriptional activity than the wild-type promoter. SDS-PAGE analysis of in vitro protein synthesis confirmed the overproduction of the GPR and GK proteins, of 44 and 39 kDa, respectively, directed by the plasmid pY1720, carrying the Ppro2 mutation (Fig. 5).

The role of the two regulatory mutations in osmotolerance and in proline analogue resistance

We determined the sensitivities to osmotic stress and to proline analogues of S. marcescens SP103 harbouring the Ppro2 and proB1 plasmids (Table 2). Cells carrying the plasmid with the wild-type proB1A+ operon, pWPK342, showed high sensitivities to an increased osmolarity and to the proline analogue DHP. The Ppro2 plasmid, pWPK100, was similar to the wild-type plasmid in the sensitivities it conferred to osmolarity and DHP. Although the proB1 plasmid, pWPK010, impaired the growth rate of its host in media of normal osmolarity, it conferred an enhanced tolerance to osmotic stress and a high resistance to DHP. However, this plasmid did not give TAC resistance to its host.

Cells harbouring pYL920, which carries both Ppro2 and proB1 mutations, grew more slowly than those harbouring the proB1 plasmid, pWPK010. Plasmid pYL920 was unstable in S. marcescens and cells carrying this plasmid segregated mutants that no longer excreted proline. Plasmids isolated from these mutants could complement the proBA mutation. Nucleotide sequence analysis of these plasmid DNAs revealed a
base substitution at nucleotide 531 that predicted a change of the amino acid 117 from a valine (mutant) to an alanine (wild-type) in the GK protein.

Discussion

Our results indicate that S. marcescens SP187, a proline-hyperproducing mutant, possesses two mutations in the proBA operon. The proline overproduction and the resultant resistance to both osmotic stress and a proline analogue are due to one of these mutations, proB1. This mutation is a single base substitution in the proB gene which directs an amino acid substitution of the GK protein. The GK enzyme specified by this mutant-type proB gene is 700-fold less sensitive to proline-mediated feedback inhibition than the wild-type. Another mutation, Ppro2, is a base substitution located in the spacer region of the promoter (Hawley et al., 1983; Harley & Reynolds, 1987).

The Ppro2 mutation stimulates expression of proBA fourfold over that of the wild-type. Expression of proBA is unlikely to be subject to any transcriptional regulation (Omori et al., 1991). Hence, enhanced proBA expression caused by the Ppro2 mutation is not likely to be due to a loss of repression but rather to the up-mutation of the promoter. Up-mutations in the spacer region have been reported in the lac (Dickson et al., 1975) and gal (Busby et al., 1984) promoters of E. coli. Stefano & Gralla (1982) have proposed that the spacer region is responsible for the local conformation of the ‘−10’ and ‘−35’ regions which make contact with RNA polymerase. The Ppro2 mutation may cause a subtle change in the conformation of the DNA of the promoter region. Although the Ppro2 mutation alone confers neither proline excretion nor resistance to osmotic stress and proline analogues, it results in the overproduction of the GK and GPR proteins, and consequently enhances proline production. This indicates that in S. marcescens, the regulation of proline synthesis depends mainly on the feedback inhibition of GK and that the proB1 mutation which alters the allosteric properties of GK is a key mutation leading to proline overproduction. Transductional analysis of strain SP187 showed that the two mutations in the proBA region were acquired at the step of selecting DHP-resistant mutant SP105, the grandparent of strain SP187. Strain SP105 was selected as the best proline producer among DHP-resistant mutants (Sugiura & Kisumi, 1985a).

Nucleotide sequence analysis of the proBA genes of mutants segregated from cells carrying the mutant proBA plasmids revealed that the proBA genes carry a reverse mutation in proB, a change of amino acid 117 from a valine (mutant) to an alanine (wild-type) in the GK protein. This mutation is the cause of the loss of proline productivity reported by Takagi et al. (1985).

S. marcescens is similar to E. coli in the structure of the proBA operon (Omori et al., 1991). The GK proteins produced by both bacteria consist of 367 amino acid residues and homology between them is 88%. In E. coli, proB mutants resistant to a proline analogue and overproducing proline have been isolated (Csonka, 1981; Mahan & Csonka, 1983; Smith et al., 1984). The proB mutations, DHP8 and proB74, result in a loss of allosteric inhibition of GK and depend on single amino acid substitutions: at amino acid residues 143 and 107 in the DHP8 GK and proB74 GK, respectively (Csonka et al., 1988; Dandekar & Uratsu, 1988; Rushlow et al., 1984). The S. marcescens proB mutation reported here, designated proB1, predicts an amino acid substitution at amino acid residue 117 in the GK protein. These three amino acid substitutions are all located in a region (amino acid residues 107–143) which lies apart from the sequence (amino acid residues 160–190) homologous to aspartokinase (Omori et al., 1991) (Fig. 6). The S. marcescens proB1 and E. coli proB74 mutations confer osmotolerance on the cells, but the DHP8 mutation does not. This region of GK is probably important for binding of proline to the allosteric site of the wild-type GK. Further kinetic studies of the mutant GKs, analysis of the other DHP resistance mutations, and X-ray crystallographic analysis of mutant-type GKS, should clarify this.
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We thank Dr. I. Chibata, Dr. T. Tosa and Dr. T. Takagi for helpful discussion.

References


