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Characterization of the osmoregulated *Escherichia coli proU* promoter and identification of ProV as a membrane-associated protein

G. May, E. Faatz, J. M. Lucht, M. Haardt, M. Bolliger† and E. Bremer*
Department of Biology, University of Konstanz, PO Box 5560, D-7750 Konstanz, FRG.

Summary

The *Escherichia coli proU* operon encodes a high-affinity, binding-protein-dependent transport system for the osmoprotectant glycine betaine. Expression of *proU* is osmoregulated, and transcription of this operon is greatly increased in cells grown at high osmolarity. Characterization of the *proU* operon and its promoter provided results similar to those published elsewhere (Gowrishankar, 1989; Stirling *et al.*, 1989). The previously identified *proU601* mutation, which leads to increased *proU* expression both at low- and high osmolarity, is a G to A transition in the Pribnow box of the *proU* promoter, which increases the homology of the −10 region to the consensus sequence of *E. coli* promoters. Using an antiserum raised against a ProV-β-galactosidase hybrid protein, we have identified ProV as a protein associated with the cytoplasmic membrane. This cellular location is consistent with its proposed role as the energy-coupling component of the ProU transport system.

Introduction

The Gram-negative bacteria *Escherichia coli* and *Salmonella typhimurium* can adapt to environmental conditions of high osmolarity by a variety of mechanisms, one of which is the synthesis or uptake of osmoprotective organic compounds, the so-called compatible solutes (see Strom *et al.*, 1986; Epstein, 1986; Wood, 1988; Csonka, 1989). Glycine betaine is among the most effective compatible solutes (Le Rudulier *et al.*, 1984). In *E. coli* and *S. typhimurium*, there are two transport systems for this substrate: ProP and ProU (Perroud and Le Rudulier, 1985; Cairney *et al.*, 1985a, b; May *et al.*, 1986).

Received 9 May, 1989; revised 18 July, 1989. †Present address: Mikrobiologisches Institut, ETH Zürich, Schmelzbergstrasse 7, CH-8008 Zürich, Switzerland. *For correspondence. Tel. (7531) 882042.

The *proP*-encoded transport system has a low affinity for glycine betaine and is present in the cytoplasmic membrane (Milner *et al.*, 1988). The *proU*-encoded transport system has a high affinity for glycine betaine and is binding-protein-dependent (May *et al.*, 1986; Higgins *et al.*, 1987a; Barron *et al.*, 1987; for an overview, see Ames, 1986). Analyses of the cloned *proU* region from *E. coli* have demonstrated that this locus consists of at least three genes (*proV, proW*, and *proX*) that are organized in an operon (Gowrishankar *et al.*, 1986; Faatz *et al.*, 1988; Dattananda and Gowrishankar, 1989). From the recently determined *proU* DNA sequence (Gowrishankar, 1989), the products of these genes have been deduced. The *proV* gene encodes a hydrophilic protein (M_r = 44162) with homology to the energy-coupling component of binding-protein-dependent transport systems. A hydrophobic polypeptide (M_r = 37619) is encoded by *proW* and is thought to be located in the cytoplasmic membrane. The *proX* gene encodes the periplasmic glycine betaine-binding protein (GBBP; M_r = 33729).

Expression of the *proU* operon is strongly stimulated at the level of transcription by increases in medium osmolarity (Gowrishankar, 1985; Cairney *et al.*, 1985b; Dunlap and Csonka, 1985; Barron *et al.*, 1986; Gutierrez *et al.*, 1987). Uptake of K^+ and the concomitant synthesis of glutamic acid are the cell's primary adaptive response to an increase in medium osmolarity (Epstein, 1986). The strength of *proU* transcription appears to be linked to the intracellular accumulation of K^+-glutamate (Epstein 1986; Sutherland *et al.*, 1986; Higgins *et al.*, 1987b; Ramirez *et al.*, 1989). However, the mechanism by which K^+-glutamate influences *proU* expression is unclear. One possibility is that the effect of K^+-glutamate is mediated by a regulatory protein. Alternatively, accumulation of K^+-glutamate could influence the initiation of *proU* transcription directly by modifying RNA polymerase activity, its interaction with the *proU* promoter, or the DNA structure of the *proU* regulatory region. Recent evidence suggests an important role for DNA topology in *proU* transcription (Higgins *et al.*, 1988a), but there is no definitive proof that changes in DNA structure mediate the osmotic control of *proU* expression.

To analyse the ProU system further and determine the mechanism by which osmolarity regulates *proU* transcription, we have sequenced the *proU* regulatory region and

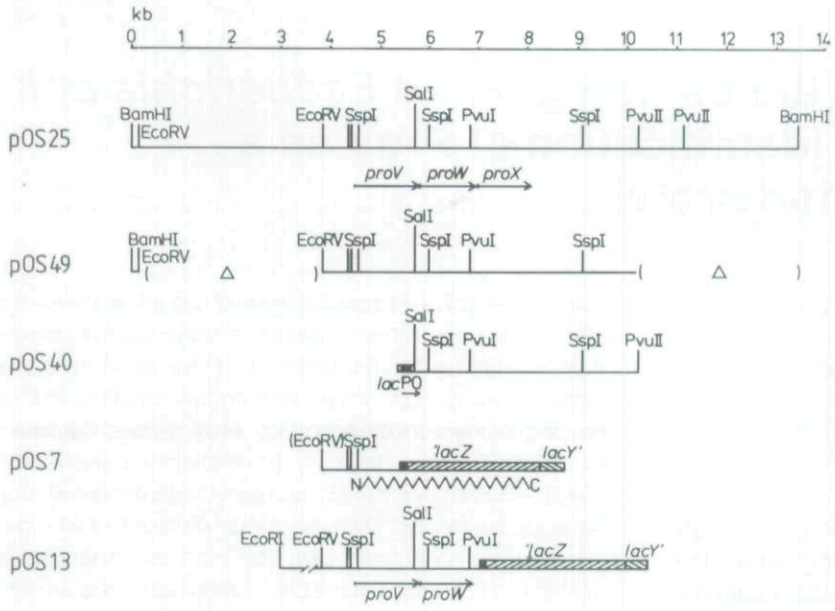

Fig. 1. Physical maps of plasmids used. Restriction maps of plasmids carrying the *proU* region or *proU-lacZ* fusions; only restriction sites relevant for this study are indicated. The chromosomal material carried by the various plasmids is shown. The extent of the *proU* operon and its direction of transcription are indicated by arrows. Chromosomal material 3′ to the *proU* operon was removed in plasmid pOS49 by *Bal*31 digestion, and the deletion endpoint was determined by restriction analysis with a margin of error of ±300 bp. A defined deletion using *Eco*RV was then introduced to remove the chromosomal material 5′ to *proU*. Plasmid pOS40 carries the 3′ part of the *proU* operon, allowing gene expression under *lacPO* (hatched box) control. Plasmids pOS7 and pOS13 carry *lacZ* fusions to different genes of the *proU* operon. Hatched bars represent the '*lacZ* and *lacY*' genes; solid bars represent the terminal 117 bp from the *S* end of phage Mu. The zig-zag line indicates the hybrid protein encoded by the Φ(*proU-lacZ*)hyb2 fusion; the amino- and carboxy-termini are indicated by *N* and *C*, respectively. For plasmid pOS13, the locations of the first two genes (*proV* and *proW*) of the *proU* operon are indicated. Plasmid pOS13 carries some material from phage λ to the left of the *Eco*RV site; the extent of this material is not known.

have defined the *proU* promoter by mutant analysis and mapping of the transcriptional initiation site. In addition, we have used an antiserum raised against a ProV-β-galactosidase hybrid protein to identify the cellular location of the *proV* gene product.

Results

Nucleotide sequence of the 5′-region of the proU *operon*

The DNA sequence of the *proU* regulatory region and the first two genes, *proV* and *proW*, of the *proU* operon was determined using DNA fragments from plasmids pOS7 (*proV-lacZ*) and pOS13(*proX-lacZ*) (Fig. 1). We have established the DNA sequence of a 3124 bp segment that begins at the *Eco*RV site proximal to the *proU* operon and extends to the *proX-lacZ* fusion joint in pOS13 (Fig. 1). This sequence (data not shown) is identical to that recently reported by Gowrishankar (1989) and is therefore not discussed here. We found only one minor difference: the G-C base pair at position 40 bp in Gowrishankar's sequence is not present in our sequence (see Fig. 4). As a result, the nucleotide positions we use here are one number lower than the corresponding positions in his sequence. We also determined the DNA sequence of the Φ (*proU-lacZ*) hyb2 and Φ (*proU-lacZ*) hyb11 fusion joints in plasmids pOS7 and pOS13, respectively. In pOS7, the G-C base pair at position 1582 bp within *proV* was fused to the 117 bp DNA segment from the Mu*S* region, which is present at the fusion joints of all λp*lac*Mu-generated *lacZ* protein fusions (Bremer *et al.*, 1984). The reading frames of the *proV*′ and the '*lacZ* genes are correctly aligned, permitting the synthesis of a large (150 kD) ProV-β-galactosidase hybrid protein (May *et al.*, 1986). These data provide experimental support for the *proV* reading frame proposed by Gowrishankar (1989). In plasmid pOS13, the Mu*S* region was fused to the A-T base pair at position 3124 bp in *proX*, the structural gene for GBBP. However, the reading frames of the *proX*′ and '*lacZ* genes are not properly aligned, resulting in an out-of-frame fusion. This explains why the expression of the Φ (*proU-lacZ*) hyb11 fusion is very low compared to that of *proX* (May *et al.*, 1986; Faatz *et al.*, 1988).

Immunological identification of the proV gene product

The ProV-β-galactosidase hybrid protein was used to identify the ProV protein and its cellular location. We purified this hybrid protein from the Δ(*proU*)*600* strain EF047(pOS7) and raised an antiserum against it. In Western blotting experiments, this antiserum reacted with the purified hybrid protein and with the hybrid protein present in whole-cell extracts of strain EF047(pOS7) (Fig. 2C, lanes 1 and 3). No specific reaction was seen with whole-cell extracts prepared from the Δ(*proU*)*600* strain

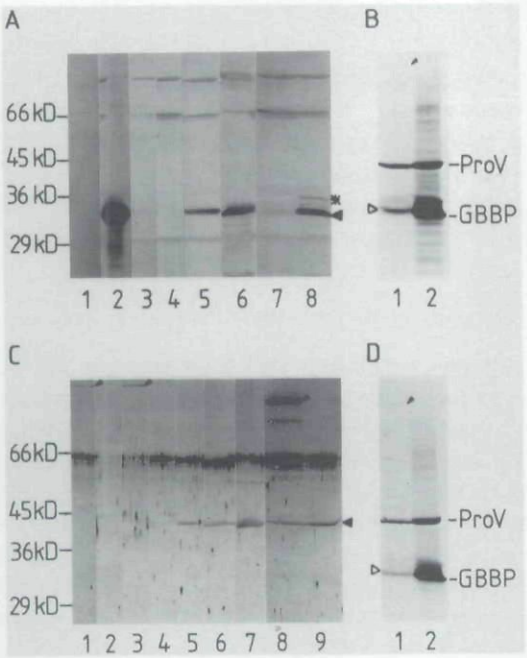

Fig. 2. Immunological detection of the GBBP and ProV proteins. Proteins were separated by SDS-PAGE, transferred to sheets of nitrocellulose and probed with antiserum against GBBP (A) or the ProV-β-galactosidase hybrid protein (C). Autoradiographs (B and D) were obtained from the Western blots of radiolabelled protein of minicells.

A. purified ProV-β-galactosidase hybrid protein (1), purified GBBP (2), whole-cell extracts from strains: EF047(pOS7) grown in MMA with 0.3 M NaCl (3), EF038 grown in MMA (4), EF038 grown in MMA with 0.3 M NaCl (5); periplasmic fraction from EF038 grown in MMA with 0.3 M NaCl (6); extracts from minicells radiolabelled in the presence of 0.3 M NaCl carrying plasmid pOS13 (7) or pOS49 (8). The solid triangle indicates the position of GBBP, and the asterisk marks the position of the presumed GBBP precursor.

B. Autoradiograph of lanes 7 and 8 from the Western blot shown in (A). The open triangle marks the position of the ProW protein; the arrowhead indicates the position of the pOS13-encoded hybrid protein.

C. Lanes 1 to 5 as in (A); cytoplasmic fraction of EF038 grown in MMA with 0.3 M NaCl (6); inner membrane fraction of EF038 grown in MMA with 0.3 M NaCl (7); lanes 8 and 9 as lanes 7 and 8 in (A). The solid triangle marks the position of the ProV protein; hybrid proteins are indicated by arrowheads.

D. Autoradiograph of lanes 8 and 9 from the Western blot shown in (C). The symbols are as in (B).

grown at high osmolarity or from the *proU*$^+$ strain EF038 grown at low osmolarity, whereas a reacting band was detected in extracts of the latter strain when grown at high osmolarity. This band corresponds to a protein with an apparent molecular weight of 44 kD (Fig. 2C, lanes 3 to 5). When cells of strain EF038, grown at high osmolarity, were fractionated the 44 kD protein was detected in both the cytoplasmic and inner membrane fractions (Fig. 2C, lanes 6 and 7). This protein was also found in minicells carrying either the *proU*$^+$ plasmid pOS49 (see Fig. 1) or the *proV*$^+$ *proW*$^+$ plasmid pOS13 (Fig. 2C, lanes 8 and 9). The newly synthesized pOS49- and pOS13-encoded proteins in the minicells had been radiolabelled prior to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting; thus a direct correlation could be shown between the immunologically detected bands on the Western blot and the bands detected by autoradiography of this blot (compare Fig. 2C, lanes 8 and 9, with Fig. 2D). The apparent molecular weight of the immunologically reacting protein is in agreement with the molecular weight of ProV (M_r = 44 162) that was deduced from the *proV* DNA sequence (Gowrishankar, 1989; this study). Our results, therefore, characterize ProV as a protein associated with the cytoplasmic membrane.

We reinvestigated the immunological cross-reaction of the Φ(*proU-lacZ*)hyb2-encoded ProV-β-galactosidase hybrid protein with an antiserum raised against purified GBBP (Barron *et al.*, 1987). Our ProV-β-galactosidase antiserum did not react with purified GBBP (Fig. 2C, lane 2), and a rabbit antiserum raised against the purified GBBP (see the *Experimental procedures*) did not react with the purified ProV-β-galactosidase hybrid protein (Fig. 2A, lane 1). The anti-GBBP serum specifically detected the glycine betaine-binding protein in whole-cell extracts and periplasmic shock fluids of osmotically induced cultures of the *proU*$^+$ strain EF038 (Fig. 2A, lanes 2, 5, and 6). GBBP was absent from extracts of the Δ(*proU*)*600* strain EF047 (pOS7) and from uninduced cultures of strain EF038 (Fig. 2A, lanes 3 and 4). Thus, GBBP is not immunologically related to the ProV-β-galactosidase hybrid protein. We also tested the reaction of extracts from minicells carrying pOS49 (*proV*$^+$ *proW*$^+$ *proX*$^+$) or pOS13 (*proV*$^+$ *proW*$^+$ *proX-lacZ*) (Fig. 1A) with the anti-GBBP serum. GBBP could be detected in minicells carrying pOS49 but not in those harbouring pOS13 (Fig. 2A, lanes 7 and 8), as expected from the genetic organization of the *proU* operon (Faatz *et al.*, 1988; Dattananda and Gowrishankar, 1989; Gowrishankar, 1989). The product of *proW* did not react with the anti-GBBP serum (compare Fig. 2A, lane 7 with Fig. 2B, lane 1). The ProW protein migrates close to the position of GBBP in our gel system (Fig. 2B).

Plasmid pOS7 carries the regulatory sequences required for osmotic control of proU *expression*

Expression of the Φ(*proU-lacZ*)hyb2 protein fusion carried by plasmid pOS7 (Fig. 1) is osmotically controlled and results in the synthesis of a large amount of a 150 kD hybrid protein, which led to the suggestion that the sequences required in *cis* for the osmoresponsive expression of *proU* are present on pOS7 (May *et al.*, 1986). However, Ramirez *et al.* (1989) reported that the expression of the hybrid gene (monitored by β-galactosidase assays) carried on a multicopy plasmid increases strongly in cultures grown at moderate osmolarity and drops sharply when the osmolarity of the growth medium

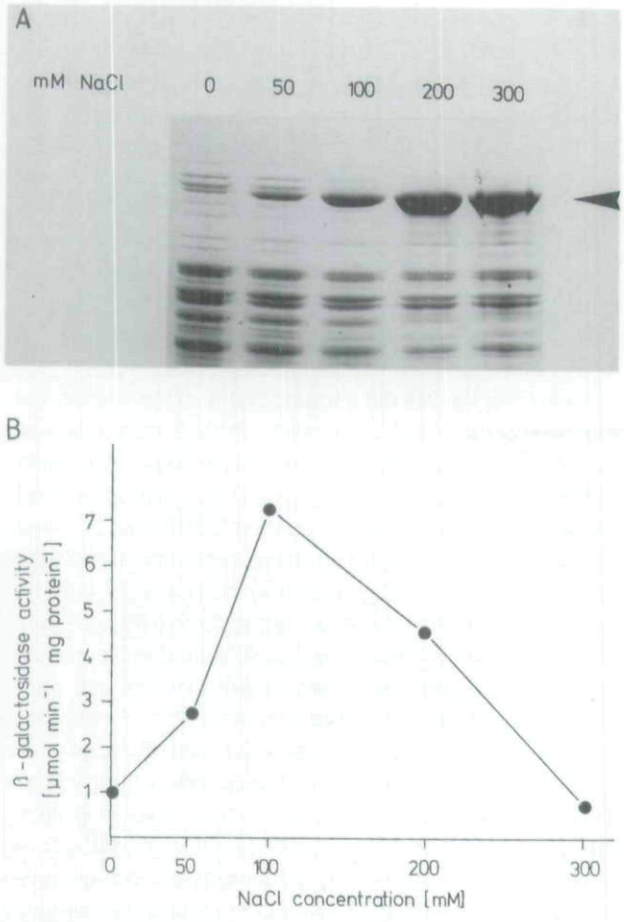

Fig. 3. Osmotically controlled synthesis and enzymatic activity of the ProV-β-galactosidase hybrid protein. Strain MC4100(pOS7) was grown overnight in glucose MMA with the indicated NaCl concentrations.
A. The cultures were used to prepare total cellular extracts to visualize the hybrid protein (indicated by an arrow) on a 7% polyacrylamide gel. Only the upper part of the Coomassie-Blue-stained gel is shown.
B. Specific β-galactosidase activity was determined in cells from the same cultures.

is increased further. This is in contrast to the steady increase in gene expression when the same fusion is present as a single copy in the *E. coli* chromosome (Barron *et al.*, 1986; May *et al.*, 1986) or when the copy number of the plasmid is lowered (Ramirez *et al.*, 1989). The data shown in Fig. 3 demonstrate that the aberrant pattern of β-galactosidase activity found in cells carrying pOS7 is an artefact. The amount of hybrid protein synthesized is not correlated with the measured β-galactosidase activity when larger amounts of the hybrid protein are made. This indicates that most of the overproduced hybrid protein is present in the cell in an enzymatically inactive form. Synthesis of the hybrid protein follows the osmotically modulated pattern of gene expression extensively documented for gene fusions to the *proU* locus (Gowrishankar, 1985; Cairney *et al.*, 1985b; Dunlap and Csonka, 1985; Barron *et al.*, 1986; Gutierrez *et al.*, 1987). Furthermore, when the pOS7-encoded *proU-lacZ* fusion was recombined into a λ specialized transducing phage and integrated as a single copy at *attB*, the osmotically controlled expression of this fusion was identical to that of the original fusion present in the *proU* operon (data not shown). We therefore conclude that pOS7 carries the regulatory sequences required in *cis* for the correct osmotic regulation of *proU*.

Determination of the proU *transcriptional start point*

A computer-aided search for DNA sequences with homology to the consensus sequence of *E. coli* promoters recognized by the RNA polymerase-δ_{70} holoenzyme (Harley and Reynolds, 1987) revealed several possible promoter sequences in the region 5′ to the translational initiation site of *proV* at the position 688 bp (Fig. 4). The sequence with the highest homology score (54%) according to the algorithm of Mulligan *et al.* (1984) is located between position 593 and 620 bp. Within this region, a possible −35 and −10 region, separated by 16 bp, can be recognized (Fig. 4). To determine whether this is the *proU* promoter, we mapped the transcriptional start site by the S1 nuclease protection assay as described by Aldea *et al.* (1988). As the template for mRNA synthesis, we used plasmid pJL19, a derivative of pUC18 into which a DNA fragment (position 3 to 833 bp) from the *proU* operon had been cloned. This DNA segment carries the complete *proU* regulatory region and the beginning of the *proV* structural gene. Total RNA was isolated from cultures of the Δ(*proU*)*600* strain EF027(pJL19), hybridized against a radiolabelled, single-stranded *proU* DNA probe, and digested with S1 nuclease. Only one set of DNA fragments, differing in size by one base, was protected by the *proU* mRNA (Fig. 5). This positions the transcriptional initiation site(s) between position 622 and 627 bp (Fig. 5), but the limited resolution of our S1 mapping experiment precludes pin-pointing the exact transcriptional start point within this region. The number and position of the protected fragments were the same when the mRNA was isolated from cells grown at low- or high osmolarity, demonstrating that no alternative promoter is used when the expression of *proU* is induced. As expected from the known regulatory pattern of *proU* expression, more *proU* mRNA is synthesized when the cells are grown at high osmolarity (Fig. 5; compare lanes 1 and 2). Synthesis of the ProU system is reduced when the high-osmolarity growth medium is supplemented with 1 mM glycine betaine (Barron *et al.*, 1986; May *et al.*, 1986), and this is reflected by the amount of *proU* mRNA made under these conditions (Fig. 5; compare lanes 2 and 3). Taken together, the results of these experiments provide good evidence that the −35 and −10 regions indicated in Fig. 4 define the *in vivo proU* promoter.

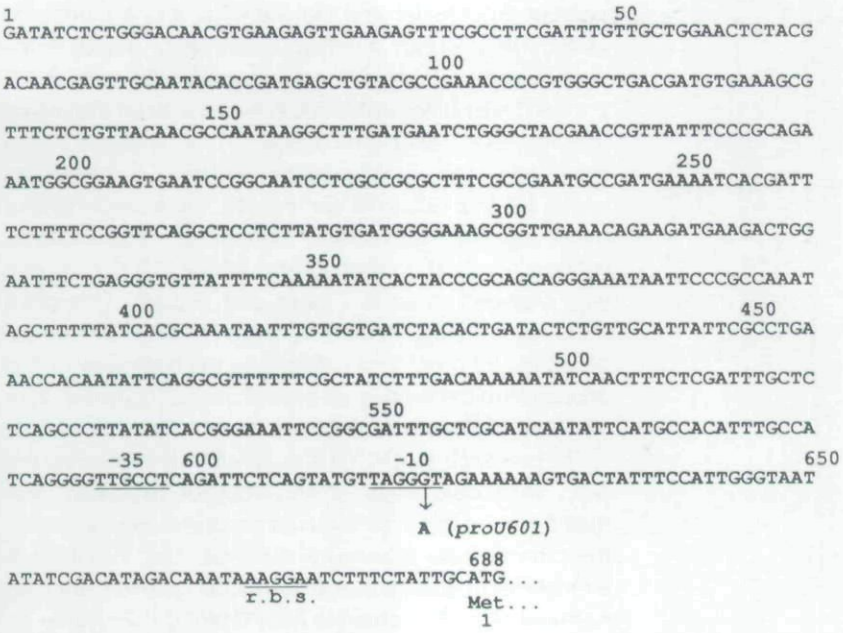

Fig. 4. Nucleotide sequence of the *proU* regulatory region. The −10 and −35 regions of the *proU* promoter are underlined, as well as the putative ribosome binding site (r.b.s.) for *proV*. The alteration in the −10 region caused by the *proU601* mutation is indicated by an arrow.

Characterization of a cis*-acting* proU *regulatory mutation*

Strain BRE2074 carries a mutation, *proU601*, that increases the expression of the Φ(*proU-lacZ*)hyb2 fusion both at high- and low osmolarity relative to the parental strain GM37 (Table 1). This mutation is closely linked to, but separable from, the *lac* fusion present in strain BRE2074 when the hybrid gene is transduced with phage P1 into strain MC4100 (Higgins *et al.*, 1988a). To test the effect of the *proU601* mutation on the expression of the *proU* operon, we isolated from strain BRE2074 a specialized λ transducing phage that carried the entire *proU-lacZ* fusion and the *proU601* mutation (see the *Experimental procedures*). This phage, λpGM22, was then used to isolate lysogens in the *proP proU*$^+$ strain, EF038. Integration of λpGM22 into the chromosome can only occur by homologous recombination between the *proU* sequences present in the chromosome of EF038 and on the λ phage, and this recombination yields a *proU*$^+$/Φ(*proU-lacZ*)hyb2 merodiploid. Depending on the point of recombination, the *proU601* mutation will either be crossed into the *proU*$^+$ locus or will remain linked to the *proU-lacZ* fusion. Two classes of λpGM22 lysogens with different patterns of osmoregulated expression of the *lac* fusion were found: one class, represented by strain GM238, showed a pattern of expression of the fusion similar to that of the wild-type strain, GM37; the second class, represented by strain GM239, showed the regulatory pattern of the *proU601* strain, BRE2074 (Table 1). The effect of the *proU601* mutation on the expression of the *proU* operon in strains GM238 and GM239 was tested by measuring the uptake of glycine betaine in cells grown in glucose MMA with 75 mM NaCl. The control strains EF047 (*proP proU*) and EF038(*proP proU*$^+$) showed no (EF047), or only weak (EF038), uptake of radiolabelled substrate under these conditions (Fig. 6). Strain GM238, which showed wild-type regulation of the *proU-lacZ* fusion, displayed a much higher rate of glycine betaine uptake than the *proU*$^+$ strain EF038 (Fig. 6). In contrast, in strain GM239, which showed increased expression of the *proU-lacZ* fusion, the rate of uptake was similar to that of the wild-type strain, EF038 (Fig. 6). From the data shown in Table 1 and Fig. 6 it is clear that the *proU601* mutation is *cis*-acting and affects the *proU*$^+$ operon as well as the hybrid gene used to identify the mutation.

To characterize this mutation further, we cloned it together with the Φ(*proU-lacZ*)hyb2 fusion from λpGM22 into the *lacZ* fusion vector, pMLB524 (see the *Experimental procedures*) and obtained plasmid pOS101. When grown at low osmolarity, strain MC4100(pOS101) expressed the fusion more strongly than strain MC4100 (pOS7), which carries the same Φ(*proU-lacZ*)hyb2 fusion but not the *proU601* mutation (Table 1); thus pOS101 confers the ProU601 phenotype. At high osmolarity, the β-galactosidase activities of the plasmid-carrying strains (Table 1) were not reliable indicators of expression of the Φ(*proU-lacZ*)hyb2 fusion (see Fig. 3). These growth conditions led to much larger amounts of the hybrid protein in total cellular extracts of MC4100(pOS101) than in those of MC4100(pOS7), as determined by SDS-PAGE (data not shown). Restriction analysis revealed no structural differences between the mutated Φ(*proU-lacZ*)hyb2 fusion

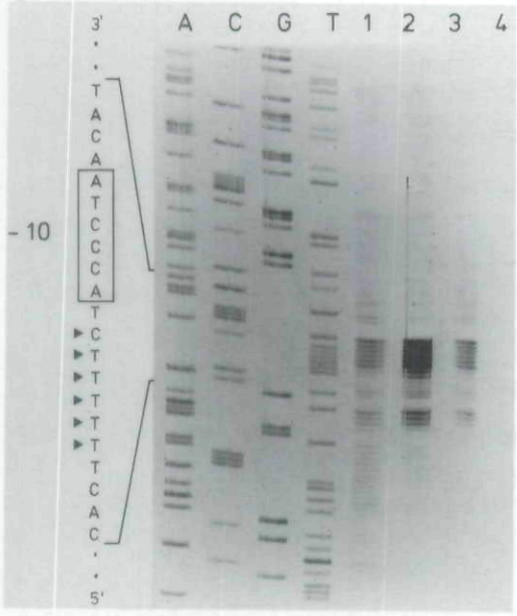

Fig. 5. Determination of the *proU* transcriptional start point by S1 nuclease mapping. Total RNA was extracted from the Δ(*proU*)*600* strain EF027(pJL19) (lanes 1, 2, 3) and EF027(pUC18) (lane 4). Strains were grown in glucose MMA (lane 1), glucose MMA with 464 mM sucrose (lanes 2 and 4), and glucose MMA with 464 mM sucrose and 1 mM glycine betaine (lane 3). The M13mp18 recombinant phage used as a template for the synthesis of the radiolabelled *proU* DNA probe was sequenced according to Sanger *et al.* (1977) and the sequencing reactions (A, C, G, T) were electrophoresed on the same gel. The sizes of the DNA fragments protected from S1 digestion can be determined directly by comparing them with the sequencing ladder (Aldea *et al.*, 1988). A partial sequence of the coding strand, including the −10 promoter region, is shown on the left; the 3′ ends of the protected fragments are indicated by solid triangles.

present on pOS101 and the wild-type fusion carried by pOS7. The *proU601* mutation presumably affects transcription or translation of the *proU* operon. To localize the *proU601* alteration, we cloned two *Xmn*I-*Xmn*I fragments (position 33 to 376 bp, and position 377 to 785 bp) carrying the 5′ end of the *proV* gene and the *proU* promoter into vector M13mp19 and determined the DNA sequences of these regions. The mutant differed from the wild-type sequence only at position 619 bp, where a G-C base pair was changed to an A-T base pair (Fig. 4). This point mutation is located in the −10 region of the promoter we proposed for *proU*, and it increases the homology of this sequence to the consensus sequence of *E. coli* promoters (Harley and Reynolds, 1987).

The presence of pOS101 is apparently deleterious to the cell, since derivatives of MC4100(pOS101) were frequently found that had acquired secondary mutations on the plasmid and expressed the *proU-lacZ* fusion at a strongly reduced level both at low- and high osmolarity. An example of such a strain is MC4100(pOS102) (Table 1). Restriction analysis of pOS102 revealed no difference in the structure between this plasmid and its parental plasmid, pOS101 (data not shown).

Discussion

In *E. coli* and *S. typhimurium*, the efficient uptake of the osmoprotectant glycine betaine is mediated by the high-affinity binding-protein-dependent transport system encoded by the *proU* locus (Cairney *et al.*, 1985b; May *et al.*, 1986; Higgins *et al.*, 1987a). Our DNA sequence analysis of part of this operon confirms the findings of Gowrishankar (1989) regarding the genetic organization of *proU*.

Table 1. The effect of the *proU601* mutation on the osmoregulated expression of the Φ(*proU-lacZ*)hyb2 fusion.

Strain	Description[a]	Specific β-galactosidase activity of cells grown in MMA	Specific β-galactosidase activity of cells grown in MMA + NaCl
Chromosomal fusions			
GM37	Φ(*proU-lacZ*)hyb2	0.03	4.94
BRE2074	Φ(*proU-lacZ*)hyb2 *proU601*	0.37	6.04
GM238	*proU601*/Φ(*proU-lacZ*)hyb2	0.05	5.52
GM239	*proU*$^+$/Φ(*proU-lacZ*)hyb2 *proU601*	0.36	8.23
Plasmid-encoded fusions			
MC4100	pMLB524	0	0
MC4100	pOS7	1.56	15.12
MC4100	pOS101	5.27	11.41
MC4100	pOS102	0.25	0.35

The strains were grown in glucose MMA without or with 0.3 M NaCl (for chromosomal fusions), or with 0.1 M NaCl (for plasmid-enceded fusions). The β-galactosidase activities shown are the mean values of two independent experiments and are expressed as μmol min^{-1} mg protein^{-1}.

a. Strains GM37 and BRE2074 carry no functional *proU* operon since the integration of the λ*plac*Mu15 phage used to isolate the *proU-lacZ* protein fusion destroys the integrity of this operon (May *et al.*, 1986). The merodiploid strains GM238 and GM239 are derivatives of EF038 (*proP proU*$^+$) in which the λpGM22 transducing phage was integrated by homologous recombination at the *proU*$^+$ locus. The plasmids pOS7, pOS101 and pOS102 carry the Φ(*proU-lacZ*)hyb2 fusion.

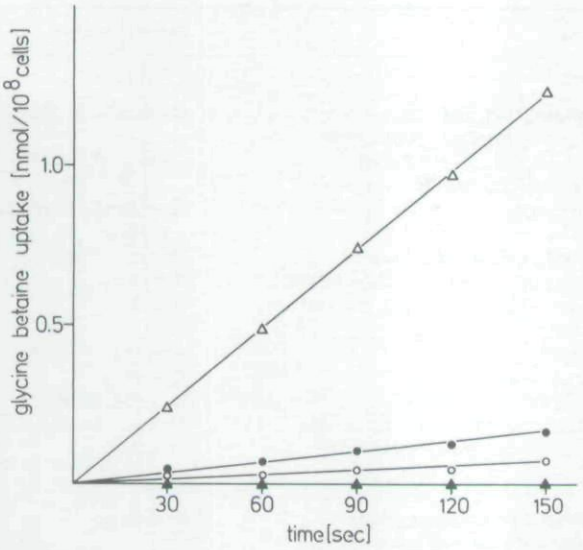

Fig. 6. Influence of the *proU601* mutation on glycine betaine uptake. The initial rate of glycine betaine uptake was measured in cells from exponentially growing cultures in glucose MMA with 75 mM NaCl. The assay medium contained glycine betaine at a final substrate concentration of 7 μM and 300 mM NaCl to ensure the optimal functioning of the ProU system (Faatz *et al.*, 1988). Strains assayed for glycine betaine uptake were: EF047(*proP proU*) (▲); EF038(*proP proU*$^+$) (●); GM239(*proP proU*$^+$/Φ(*proU-lacZ*)hyb2 *proU601*) (○) and GM238 (*proP proU601*/Φ(*proU-lacZ*)hyb2) (△).

Our earlier suggestion (Faatz *et al.*, 1988) that the first gene, *proV*, of this operon encodes GBBP is no longer tenable. The immunological data we present here show that the *proV* gene product is a 44 kD protein partly associated with the cytoplasmic membrane. Furthermore, ProV shows strong homologies (Gowrishankar, 1989) to the presumed energy-coupling components of other binding-protein-dependent transport systems, which are typically also associated with the cytoplasmic membrane (Ames, 1986; Higgins *et al.*, 1988b; Gallagher *et al.*, 1989). These data suggest that ProV serves the energy-coupling function in the ProU transport system. In contrast to the results presented by Barron *et al.* (1987), our experiments using antisera raised against GBBP and the Φ(*proU-lacZ*) hyb2-encoded ProV-β-galactosidase hybrid protein show clearly that these two proteins are not immunologically related.

Transcription of the *proU* operon is tightly controlled by the osmolarity of the medium. Both the intracellular accumulation of K^+-glutamate and an increase in the negative DNA supercoiling upon an increase in medium osmolarity have been implicated in the osmotic control of *proU* expression (Epstein, 1986; Sutherland *et al.*, 1986; Higgins *et al.*, 1987b; 1988a; Ramirez *et al.*, 1989). However, the exact mechanism by which these factors contribute to the regulation of *proU* has not been elucidated. By mapping the transcriptional start site(s) and by analysis of a *cis*-acting mutation, we have determined that the *proU* promoter is located 67 bp upstream of the transcriptional initiation codon for *proV*. The −35 (TTGCCT) and −10 (TAGGGT) sequences, which are separated by 16 bp, show homologies to the consensus sequences of *E. coli* promoters recognized by the RNA-polymerase-δ_{70} holoenzyme (Harley and Reynolds, 1987; Mulligan *et al.*, 1984). However, the Pribnow box is unusual since it contains three consecutive G-C base pairs. Among the 263 promoter sequences compiled by Harley and Reynolds (1987) there is no −10 region with such a string of G-C base pairs. At low osmolarity, *proU* is only weakly expressed, but high osmolarity leads to a strong increase in *proU* transcription. It seems likely that the unusual DNA sequence of the *proU* −10 region contributes to the low basal level of expression, possibly because it requires a higher energy for strand separation and open complex formation. Consistent with this view, we found that the *proU601* mutation, which increases *proU* expression but does not affect its osmotic regulation, is a G-C to A-T transition in the −10 (TAGGAT) region of the promoter. This mutation increases the homology of the *proU* −10 region to the consensus Pribnow box (TATAAT), raising the homology score for the *proU* promoter region from 54% to 59% (Mulligan *et al.*, 1984). It should also facilitate strand separation because the free energy required for the transition from double helix to single strands is increased ($\Delta G = +1.3$ kcal mol^{-1}) for the mutated −10 region. This is consistent with the findings of Margalit *et al.* (1988), who have shown that 80% of 'up' mutations in the −10 region are correlated with increased free energy.

One important result of our work is the finding that the transcriptional start site(s) of *proU* is not shifted when expression of the operon is induced by high medium osmolarity. This demonstrates that no alternative promoter is activated under inducing conditions. For these experiments, we used a multicopy plasmid carrying the *proU* regulatory region. We found that the osmotically modulated expression of the Φ(*proU-lacZ*)hyb2 protein fusion was the same, regardless of whether the fusion was carried by a plasmid (pOS7) or located in the chromosome. Earlier indications that the expression of this plasmid-encoded fusion differed from that of the same fusion in the chromosome (May *et al.*, 1986; Ramirez *et al.*, 1989) are explained by our finding that much of the overproduced ProV-β-galactosidase hybrid protein is present in the cell in an insoluble, enzymatically inactive form. Our data show, therefore, that the sequences necessary in *cis* for correct osmoregulation of *proU* are present on pOS7, but they do not permit definition of the limits of the sequences required. Since the position of the *proU* promoter is now known, further mutational analysis should reveal the sequences essential for the osmoregulated transcription of the *proU* operon.

Table 2. Bacteria, bacteriophages and plasmids.

Strain	Description[a]	Reference/origin
Strains derived from *E. coli* K12		
MC4100	F⁻ Δ(*argF-lac*)*U169, araD139, rpsL150, deoC1, relA1, ptsF25, flbB5501, rbsR*	Casadaban (1976)
TG1	Δ(*lac-pro*), *supE, thi, hsd5*/F′*traD36, proA*⁺*B*⁺ *lacI*ᴿ *lacZ*ΔM15	Carter *et al.* (1985)
HB290	*minB, rpsL, mgl*	G. Hazelbauer through K. Heller
GM37	MC4100 Φ(*proU-lacZ*)hyb2, (λp*lac*Mu15)	May *et al.* (1986)
GM238	EF038 *proU601*/Φ(*proU-lacZ*)hyb2 (λpGM22)	This study
GM239	EF038 *proU*⁺/Φ(*proU-lacZ*)hyb2, *proU601* (λpGM22)	This study
BRE2074	GM37 *proU601*	Higgins *et al.* (1988a)
EF027	MC4100 Δ(*proU*)*600*	This study
EF038	MC4100 Δ(*putPA*)*101, proP1*	May *et al.* (1986)
EF047	MC4100 Δ(*putPA*)*101, proP1*, Δ(*proU*)*600*	May *et al.* (1986)
EF086	HB290 Φ(*proU-lacZ*)hyb2, (λp*lac*Mu15)	This study
Bacteriophage		
λpGM22	Lac⁺ specialized transducing phage carrying the Φ(*proU-lacZ*)hyb2 and the *proU601* mutation	This study
Plasmids		
pMLB524	Cloning vector for *lacZ* fusions; *bla*⁺	Silhavy *et al.* (1984)
pUC18	Cloning vector, *bla*⁺	Norrander *et al.* (1983)
pOS7	Φ(*proU-lacZ*)hyb2; *bla*⁺	May *et al.* (1986)
pHG329	Expression vector with *lacPO*; *bla*⁺	Stewart *et al.* (1986)
pOS13	Φ(*proU-lacZ*)hyb11; *bla*⁺	Faatz *et al.* (1988)
pOS25	*proU*⁺ derivative of pBR322; *bla*⁺	Faatz *et al.* (1988)
pOS40	Derivative of pHG329 carrying the 3′ part of the *proU* operon under *lacPO* control; *bla*⁺	This study
pOS49	*proU*⁺ deletion derivative of pOS25	This study
pOS101	Φ(*proU-lacZ*)hyb2 *proU601*; *bla*⁺	This study
pOS102	pOS101 *proU602*; *bla*⁺	This study

a. The symbol Φ indicates the presence of a *lacZ* fusion, and the abbreviation hyb indicates that the gene fusion encodes a hybrid protein. The λp*lac*Mu15 prophage in strains GM37, BRE2074, and EF086 carries the kanamycin resistance gene *kan*. This gene is also present on phage λpGM22, which is derived from the prophage in strain BRE2074.

Experimental procedures

Bacterial strains, bacteriophages and plasmids

The bacterial strains, phages and plasmids used in this study are listed in Table 2. All strains are derivatives of *E. coli* K12.

Growth conditions, media and chemicals

Bacteria were grown aerobically at 37 °C in LB medium or minimal medium A (MMA) with 0.2% glucose as the carbon source (Miller, 1972; Silhavy *et al.*, 1984). The osmolarity of liquid MMA was elevated by the addition of NaCl or sucrose as required. Agar plates spread with 0.1 ml of a 10 mg ml^{-1} solution of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (XG) in dimethylformamide were used to distinguish between LacZ⁻ and LacZ⁺ phenotypes. Kanamycin (Km) and ampicillin (Ap) were added to media at 30 μg ml^{-1} and 50 μg ml^{-1}, respectively.

Genetic procedures and construction of bacterial strains

Standard techniques were used for the purification and growth of bacteriophage (Silhavy *et al.*, 1984). A LacZ⁺ specialized transducing phage, λpGM22, carrying the Φ(*proU-lacZ*)hyb2 fusion with the tightly linked *proU601* mutation present in strain BRE2074 (Table 2), was isolated after u.v. induction of the λp*lac*Mu15 prophage, as described by Silhavy *et al.* (1984). Lysogens carrying λpGM22 were isolated in strain EF038 by selecting for LacZ⁺ Km^R colonies on LB plates supplemented with Km and XG. The osmoregulated expression of the Φ(*proU-lacZ*) hyb2 fusion in these strains was verified by streaking the lysogens on MacConkey-lactose plates without or with added NaCl (May *et al.*, 1986). The minicell-producing strain, HB290 (Table 2) was made *proU* by transduction with a P1 lysate grown on GM37 (Φ(*proU-lacZ*)hyb2; λp*lac*Mu15) and subsequent selection for Km^R transductants; this resulted in strain EF086.

Transport assays

Glycine betaine uptake was measured as described by May *et al.*, (1986) using methyl-[^{14}C]-glycine betaine (7.1 mCi mmol^{-1}; Amersham).

Methods used with nucleic acids and construction of plasmids

Isolation of plasmid and phage λ DNA and routine manipulations of nucleic acids were all as described (Maniatis *et al.*, 1982; Silhavy *et al.*, 1984). DNA sequencing was performed using a modification of the dideoxy nucleotide chain-termination method (Sanger *et al.*, 1977; Biggin *et al.*, 1983).

Plasmid pOS101 was constructed by cloning an *Eco*RI-*Eco*RI restriction fragment from λpGM22 into the *lacZ* fusion vector, pMLB524 (Silhavy *et al.*, 1984), as described by May *et al.* (1986). The structure of one of the resulting LacZ$^+$ plasmids, pOS101, was characterized by restriction analysis. Plasmid pOS40 (Fig. 1) was constructed by cloning a *Sal*I-*Pvu*II fragment from the *proU*$^+$ plasmid pOS25 (Faatz *et al.*, 1988; Fig. 1) into the *Sal*I and *Sma*I sites in the polylinker region of pHG329 (Stewart *et al.*, 1986), allowing the expression of the 3′ end of the *proU* operon under the control of the *lac* promoter. To reduce the size of the chromosomal material present on pOS25, DNA 3′ to the *proU* operon was removed by *Bal*31 digestion (Silhavy *et al.*, 1984) and DNA 5′ to the *proU* regulatory region was removed by digestion with *Eco*RV and religation. One of the *proU*$^+$ plasmids obtained is pOS49 (Fig. 1).

Mapping of the proU *transcription initiation site*

The transcriptional start point of the *proU* operon was determined according to the protocol of Aldea *et al.* (1988). We constructed a derivative of M13mp18 with an 839 bp *Eco*RI-*Bal*I insert carrying the *proU* regulatory sequences and the 5′ end of *proV* (Fig. 1). The *proU* fragment is inserted into the M13 phage such that the universal priming site within the *lac*-α region is adjacent to the *Bal*I end of the cloned fragment. This *Eco*RI-*Bal*I fragment was cloned in the same way into the polylinker region of pUC18, resulting in plasmid pJL19. RNA was extracted from cells carrying pJL19 or the control plasmid pUC18, grown under different conditions. A single-stranded, radiolabelled *proU* DNA probe was prepared by primer extension using the universal *lac* primer and the recombinant M13mp18 phage, described above, as the template. The RNA was hybridized to this probe, DNA-RNA hybrids were digested with S1 nuclease, and the sizes of the protected fragments were determined on a sequencing gel.

Purification of GBBP and the ProV-β-galactosidase hybrid protein

Periplasmic proteins were isolated from strain EF047(pOS40) grown in glucose MMA using the cold osmotic-shock procedure of Neu and Heppel (1965). As the first step in the purification of GBBP, we employed ion-exchange chromatography on a DEAE-Sephacel column as described by Barron *et al.* (1987). Fractions containing GBBB were pooled, and the proteins were concentrated with Aquacide (Serva) and dialysed against a large volume of 16 mM Tris-HCl (pH 8.3). The proteins were then loaded onto an FPLC ion-exchange column (Mono-Q; Pharmacia), and the bound polypeptides were eluted using a linear NaCl gradient (0–250 mM NaCl in 16 mM Tris-HCl, pH 8.3). This yielded 99% pure and functional GBBP, as judged by SDS-PAGE and glycine betaine-binding assays (May *et al.*, 1986). The osmotically induced expression of the Φ(*proU-lacZ*)hyb2 fusion carried by pOS7 resulted in strong overproduction of the ProV-β-galactosidase hybrid protein. This hybrid protein sedimented in large amounts with the membrane fraction when French-pressed cells were centrifuged for 30 min in a Sorvall SS34 rotor at 15000 r.p.m. The pellet was solubilized using a 2% SDS solution, the proteins were electrophoretically separated by SDS-PAGE, and the hybrid protein was electroeluted from the gel. The protein-containing solution was concentrated with Aquacide and dialysed against water containing 0.2% SDS. The purity of the protein was assessed by SDS-PAGE.

Preparation of GBBP and ProV-β-galactosidase antisera and immunological detection of the antigens

Rabbits were given injections of GBBP or ProV-β-galactosidase proteins (1 mg and 0.5 mg of protein, respectively) in 1 ml of BBS-buffer (140 mM NaCl, 166 mM Tris-borat, pH 8.0) and 1 ml of complete Freund's adjuvant. After 14 days, the animals were given booster injections of the same doses of protein suspended in BBS buffer; 14 days after this, the blood of the animals was collected. For the immunological detection of the antigens, *E. coli* proteins were separated by SDS-PAGE and electrophoretically transferred (Towbin *et al.*, 1979) to a sheet of nitrocellulose (pore size, 0.45 μm; Schleicher & Schuell). The bound proteins were then probed with a crude rabbit antiserum. The antigen-antibody complexes formed were visualized with a second goat anti-rabbit immunoglobulin G peroxidase-coupled antibody using 3-3′-dimethoxybenzidine (Sigma) as a substrate.

Radiolabelling of proteins in minicells, fractionation of cellular proteins, and SDS-PAGE

Minicells were isolated from strain EF086 carrying various plasmids. Radiolabelling of the plasmid-encoded proteins with [^{35}S]-methionine (1000 mCi mmol^{-1}; Amersham) in the presence of 0.3 M NaCl was performed as described by Faatz *et al.* (1988). The proteins were separated by SDS-PAGE (Laemmli, 1970), and the radiolabelled polypeptides were visualized by autoradiography using Kodak X-omatic 100 film. Periplasmic proteins were isolated using the cold osmotic-shock procedure (Neu and Heppel, 1965; May *et al.*, 1986), and proteins from the inner and outer membrane of *E. coli* were separated by sucrose gradient density centrifugation, as described by Hengge *et al.* (1983).

β-galactosidase assays

Specific β-galactosidase activity, expressed as micromoles of substrate cleaved per min per mg of protein, was assayed as described by May *et al.* (1986).

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