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In vitro reconstitution of osmoregulated expression of *proU* of *Escherichia coli*

(glycine betaine/*lac* fusion/osmotic stress)

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ABSTRACT **Osmoregulated expression of *proU* has been reconstituted in a cell-free system. *proU* encodes an osmotically inducible, high-affinity transport system for the osmoprotectant glycine betaine in *Escherichia coli*. Previously, a *proU–lacZ* fusion gene had been cloned, resulting in plasmid pOS3. *In vivo* osmoregulation of this extrachromosomal *proU–lacZ* fusion gene at low copy number showed that the plasmid-encoded fusion contained all the necessary sequences in cis for correctly receiving osmoregulatory signals during induction by osmotic stress and repression by glycine betaine. Using a cell-free (S-30) extract, plasmid pOS3 was then used to program protein synthesis *in vitro*. The ionic compound potassium glutamate specifically stimulated *proU–lacZ* expression in a concentration-dependent manner. Potassium acetate also induced some *proU* expression, but other salts were ineffective, thereby ruling out ionic strength as the stimulatory signal. High concentrations of sucrose, trehalose, or glycine betaine did not induce *proU* expression *in vitro* either, eliminating osmolarity *per se* as the stimulus. Reconstitution in a cell-free system rules out osmoregulatory mechanisms that depend on turgor, transmembrane signaling, or trans-acting regulators synthesized after osmotic upshock.**

In *Escherichia coli*, as in many other bacteria (1), the primary response to increased medium osmolarity is the influx and accumulation of K^+. To a first approximation, intracellular potassium concentration increases in proportion to external osmolarity (2). K^+ transport in *E. coli* is mediated by two genetically distinct transport systems, Trk and Kdp (3). The high-affinity Kdp system is induced by a reduction in cellular turgor pressure measured by a transmembrane osmosensor, KdpD and -E (4, 5). Stressed cells also accumulate large amounts of glutamate, which may act as a counterion to K^+ for maintaining electroneutrality (6, 7). The net result is that K^+ glutamate is the predominant ionic species within the osmotically stressed cell. Elevated K^+ concentration apparently affects *E. coli* adversely, since cells shocked with salt concentrations greater than 0.2 M NaCl exhibit decreased growth rates (8). Osmoprotective compounds, such as choline (9), proline (10), or glycine betaine (11) restore the cell's ability to grow in osmotically stressful environments. Glycine betaine uptake in *E. coli* and *Salmonella typhimurium* is mediated by two transport systems, ProP and ProU (12–14). *proP* encodes a constitutive, low-affinity transport system whose expression is stimulated severalfold during osmotic upshock (12, 14). *proU* encodes a high-affinity system that is strongly induced at the transcriptional level by elevated osmolarity (8, 12–14). Both systems are also regulated at the level of transport activity (12–15). In *E. coli*, one gene product of the ProU system has been purified and characterized. It is a periplasmic polypeptide that specifically binds glycine betaine (16).

Most studies of *proU* regulation have utilized genetic fusions with *lacZ* (14, 17). β-Galactosidase activity from Φ*proU–lacZ* fusions is induced 60- to 100-fold by osmotic upshock (8, 13). The mechanism of *proU* osmoregulation is unknown, although intracellular K^+ concentration has been proposed as the signal stimulating gene expression (17). Addition of exogenous glycine betaine represses *proU* expression (8, 14). Glycine betaine is accumulated to high intracellular concentrations under osmotic stress, replacing K^+ as the major osmolyte (11, 18). The mechanism of glycine betaine repression of *proU* is also undefined.

Osmotic stress stimulates the expression of a number of genes (19, 20), one of which specifies the outer membrane porin OmpC (21). Expression of another porin, OmpF, is affected in a reciprocal manner (21); both porins respond to a two-protein regulatory system encoded by the unlinked *ompB* operon composed of *envZ* and *ompR* (22). EnvZ is an inner membrane protein sensor whereas OmpR, a cytoplasmic protein, is a positive regulator of *ompC* and *ompF* transcription (23).

Expression of *proU*, *kdp*, and *ompC* are all stimulated by high osmolarity, yet they fundamentally differ with respect to their regulatory mechanisms. Whereas *kdp* expression is controlled by changes in turgor pressure (5) and is repressed by K^+, elevated *proU* expression correlates with high intracellular levels of K^+ (17). Unlike *ompC*, *proU* expression is not dependent on *envZ* or *ompR* (13, 14). Furthermore, extensive mutant searches have not revealed the involvement of any unlinked specific regulatory loci controlling *proU* expression (24, 25). Thus, the precise nature of the signal and mechanism by which *proU* expression is induced by high osmolarity remains unknown. In this study, we report the *in vitro* reconstitution of osmoregulated *proU* expression in a cell-free S-30 system. These results faithfully reproduce effects seen *in vivo* and further define the signals and mechanism controlling *proU* expression during osmotic stress.

MATERIALS AND METHODS

Strains, Plasmids, and Growth Conditions. Bacterial strains and plasmids used in this study are listed in Table 1. Standard synthetic medium contained M9 salts (28), 0.4% glycerol, and thiamine (1 μg/ml). Typically, a stationary-phase culture, grown in LB medium (28) containing an appropriate antibiotic, would be used to inoculate an overnight M9/glycerol culture, grown aerobically at 37°C with agitation. Cells from the latter were then used to inoculate fresh M9/glycerol medium. After resuming growth and upon reaching midlogarithmic phase (≈45 Klett units; red filter), cells were osmotically upshocked either by the addition of 5 M NaCl to

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Table 1. Bacterial strains and plasmids

	Pertinent genotype*	Ref(s).
Strains		
MC4100	Δ*lac proU*$^+$	14
MRi93	*pcnB80 proU*$^+$	26
GM37	Φ(*proU–lacZ*)hyb2 (λplacMu15)	14
JJ715	Φ(*proU*::Tn*phoA*)11, *proC*$^+$ transductant of CLG7	This study and 20
Plasmids		
pOS3	Φ(*proU–lacZ*) Amp^R	14
pBP1	*proU'* Amp^R	This study
pAT153	Amp^R Tet^R	27

*All strains are derived from MC4100. Amp^R, ampicillin resistance; Tet^R, tetracycline resistance.

the culture or by concentrating the cells and adding a small volume of the concentrate to medium of the appropriate osmolarity. Antibiotic selection was maintained throughout growth in M9 medium. Increasing cell density was monitored with a Klett–Summerson colorimeter.

Antibiotics were used in the following concentrations: ampicillin at 200 μg/ml, kanamycin at 30 μg/ml, and tetracycline at 10 μg/ml.

Genetic Procedures. Strain MRi93 (26) was transformed with the plasmid pOS3 (14) according to the procedure of Silhavy *et al.* (29). All other strains were transformed by the procedure of Maniatis *et al.* (30) in 0.1 M $CaCl_2$.

Assays. For β-galactosidase assays, 0.5 ml of cells were harvested by centrifugation at 4°C and frozen at −70°C overnight. Thawed pellets were resuspended in PM2 buffer (28) without 2-mercaptoethanol. Cells were lysed by vortex mixing with 5% (vol/vol) chloroform and 0.01% $NaDodSO_4$, then placed on ice for 30 min. β-Galactosidase activity was assayed colorimetrically (420 nm) in PM2 buffer at 37°C with 2-mercaptoethanol, by measuring the hydrolysis of *o*-nitrophenyl galactoside after centrifugation. Extinction coefficient and definition of units of activity are as cited (31).

To measure alkaline phosphatase activity in strain JJ715, washed 1-ml cell samples were also frozen overnight at −70°C, thawed, and sonicated. Alkaline phosphatase activity was assayed colorimetrically (410 nm) by measuring the hydrolysis of *p*-nitrophenyl phosphate after centrifugation.

Protein concentration was determined by the method of Lowry *et al.* (32) with bovine serum albumin as a standard.

DNA-Directed Cell-Free Protein Synthesis. Cell-free, coupled transcription/translation was performed using a commercially available kit (Amersham), according to the instructions supplied. Proteins synthesized *in vitro* were labeled with 30 μCi of Tran^{35}S-label (ICN; 1 Ci = 37 GBq) for 1 hr at 37°C and separated by $NaDodSO_4$/PAGE. Coomassie blue-stained gels were dried under vacuum on Whatman paper and placed on Kodak XAR-5 film for autoradiography with an intensifying screen.

RESULTS

Chromosomal and Plasmid Encoded Φ(*proU–lacZ*) Fusion Genes Respond Identically to Osmotic Stimuli *in Vivo*. To develop a physiologically relevant *in vitro* expression system for *proU* it was first necessary to determine whether plasmid pOS3 (Fig. 1) carried all the necessary control regions in cis for properly receiving osmoregulatory signals. Previous studies have shown that a chromosomal Φ(*proU–lacZ*)hyb2 fusion gene in strain GM37 responds to increasing external osmolarity with increasing levels of expression (8). This *proU–lacZ* fusion gene was cloned into a pBR322 derivative resulting in plasmid pOS3 (ref. 14, Fig. 1, and Table 1) that is maintained at 25–40 copies per chromosome. Preliminary data presented elsewhere (14) and data below showed that

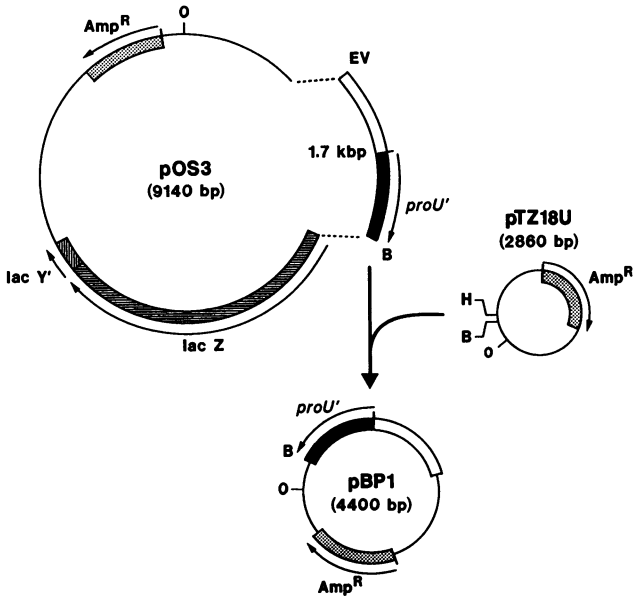

FIG. 1. Structure of pOS3 and construction of pBP1. Plasmid pOS3 was described (14). It carries the Φ(*proU–lacZ*)hyb2 fusion gene and the β-lactamase gene conferring ampicillin resistance (Amp^R). pOS3 was digested with *Bam*HI and *Eco*RV to yield a 1.7-kilobase fragment carrying the *proU'* portion of the fusion and <600 base pairs of upstream DNA. This fragment was ligated into the multiple cloning site of the vector pTZ18U, previously digested with *Bam*HI and *Hin*cII, resulting in plasmid pBP1. Standard techniques (30) were used for cloning and screening. EV, *Eco*RV; B, *Bam*HI; H, *Hin*cII.

osmoregulation of pOS3 at this high copy number was abnormal (Fig. 2). To eliminate regulatory artifacts due to high copy number, pOS3 was transformed into strain MRi93, which carries the *pcnB80* mutation (26). Assuming that the uninduced β-galactosidase activities are proportional to gene dosage, we found that the *pcnB80* mutation reduced the copy number of pOS3 to the expected level of ≈6 copies per chromosome (Fig. 2) as reported (26). Thus, this strain allowed copy number to be lowered without altering the plasmid construction. To correct for growth rate inhibition due to high medium osmolarity and multicopy plasmids, we compared the differential rates of expression of Φ(*proU–lacZ*) in GM37 and MRi93(pOS3) in medium of increasing osmolarities (Fig. 2). In this low copy state, the response of the pOS3-encoded fusion to salt induction closely resembled that observed with GM37, the strain carrying a single chromosomal copy of Φ(*proU–lacZ*) (Fig. 2 *A* and *B*). In both strains, high rates of synthesis occurred in standard medium supplemented with 0.35 M and 0.5 M NaCl during the first generation after salt addition. In medium supplemented with 0.1 M NaCl, the initial differential rate of synthesis was lower.

When pOS3 was present at high copy number, the response of Φ(*proU–lacZ*) to salt stimulation was altered. Basal β-galactosidase activity, in the absence of added salt, was 20- to 50-fold higher than in the single copy strain GM37 (data not shown). Furthermore, β-galactosidase activity peaked at 0.1 M NaCl added to M9 medium and decreased at higher osmolarities (Fig. 2*C*). This abnormal regulation was apparently not due to titration of a limiting repressor, since pOS3 at high copy number did not derepress a chromosomal *proU–phoA* fusion gene (strain JJ715) when present in trans (data not shown). Rather, we suspect that the reduced β-galactosidase activity at higher osmolarities is an artifact due to inactivation of the over-produced chimeric enzyme, since the amount of fusion protein increases while activity decreases (data not shown). Most important, the data from the low copy

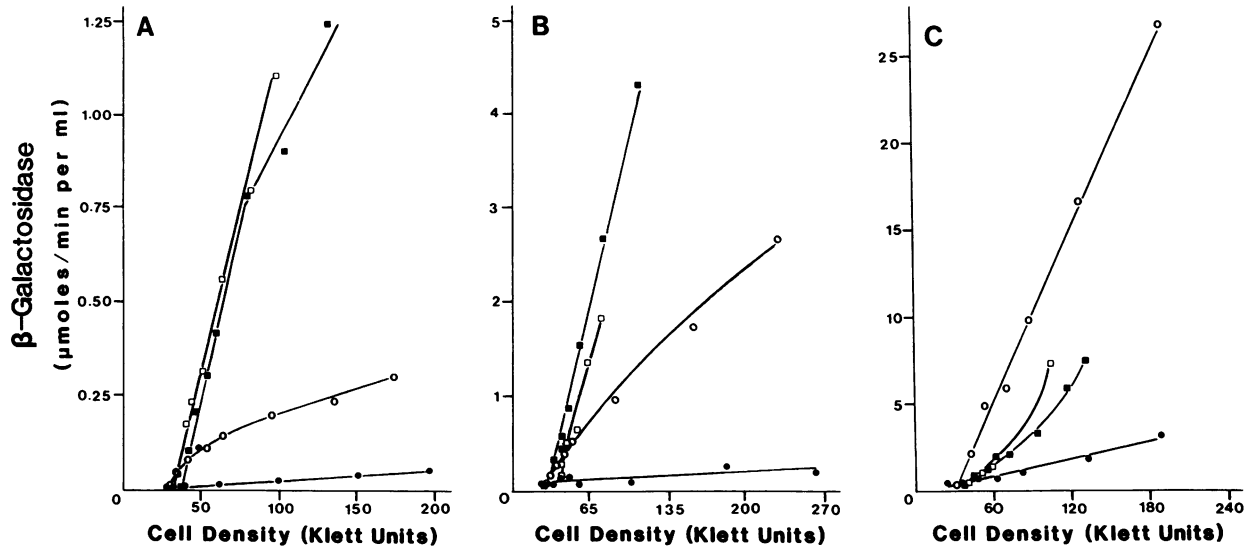

FIG. 2. Differential rates of synthesis of Φ(*proU–lacZ*)-dependent β-galactosidase activity. Cultures of strains GM37 [Φ(*proU–lacZ*)] (*A*), MRi93(pOS3) [*pcnB80 proU*$^+$//Φ(*proU–lacZ*)] (*B*), and MC4100(pOS3) [*proU*$^+$//Φ(*proU–lacZ*)] (*C*), were grown and resuspended in M9/glycerol medium (●), or in medium supplemented with 0.1 M NaCl (○), 0.35 M NaCl (■), or 0.5 M NaCl (□). Ampicillin (200 μg/ml) was included for plasmid-containing strains, while kanamycin (30 μg/ml) was added for GM37. Aliquots were removed as indicated and assayed for β-galactosidase activity.

state indicate that all the cis regulatory sites needed for normal osmoregulated induction of *proU* are located on plasmid pOS3, despite the distorted regulatory pattern associated with the high copy state.

The osmotic induction of *proU* is normally subject to feedback repression by internally accumulated glycine betaine (8). The response of the cloned Φ(*proU–lacZ*) gene in MRi93(pOS3) to glycine betaine was virtually identical to that of the chromosomal fusion in strain GM37, differing only in enzyme levels due to gene dosage (data not shown). Therefore, any sites needed for repression of *proU* expression by glycine betaine are also present on plasmid pOS3.

***In Vitro* Expression of *proU*.** The signals responsible for triggering expression of *proU* in response to osmotic stress are unknown. To identify these signals at the molecular level, we sought to reconstitute osmoregulated expression of the cloned gene in a cell-free system. *proU* is a good candidate for *in vitro* reconstitution since pOS3 carries all the necessary regulatory sequences in cis, and there is no evidence for additional required regulatory proteins in trans. To reconstitute expression *in vitro*, we used an S-30 coupled transcription/translation system and modified the osmolarity by the addition of several different osmolytes. Three plasmids were used to direct protein synthesis *in vitro*: pOS3, pBP1, or the control plasmid pAT153 (see Fig. 1 and Table 1). Operational proof that the S-30 extract was functional was provided by monitoring the simultaneous expression of the β-lactamase gene product, encoded by all three plasmids. K^+ glutamate was chosen as an ionic osmolyte to simulate the physiological milieu that is found in salt-stressed *E. coli* cells (3, 6, 7, 33).

The Φ(*proU–lacZ*) fusion protein of plasmid pOS3 was not synthesized under standard assay conditions, although β-lactamase encoded by the same plasmid was strongly expressed (Fig. 3, lane 2). Addition of K^+ glutamate, to a final concentration of 0.3 M, stimulated the synthesis of the ProU-β-galactosidase hybrid protein encoded by the Φ(*proU–lacZ*) fusion gene (lane 3). The array of lower molecular mass bands that appeared beneath the fusion protein could represent partially synthesized polypeptides or degradation products. Conversely, the added K^+ glutamate significantly inhibited β-lactamase expression, when either pOS3 or pAT153 were used as templates (lanes 3 and 8). The non-ionic compound sucrose and zwitterionic glycine betaine (0.3 M and 0.2 M, final concentrations, in lanes 4 and 5, respectively) did not stimulate Φ(*proU–lacZ*) expression.

K^+ glutamate stimulation of *proU* was concentration-dependent: the amount of fusion protein expressed from the pOS3 template increased as the concentration of added K^+ glutamate was increased up to 0.3 M (Fig. 4, lanes 1–5). Inhibition of β-lactamase expression was also a function of added salt concentration. All protein synthesis was inhibited when 0.4 M K^+ glutamate was added.

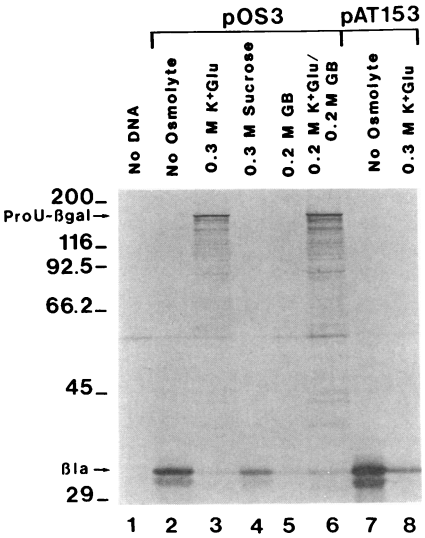

FIG. 3. Effect of osmolytes on *in vitro* expression of β-lactamase (βla) and Φ(*proU–lacZ*). Plasmid DNAs were incubated in an S-30 protein synthesizing system for 1 hr at 37°C. Samples (6 μl) were removed, subjected to $NaDodSO_4$/PAGE and autoradiography. The following treatments and templates were used. Lanes: 1, S-30 untreated, no DNA; 2, S-30 untreated, pOS3; 3, S-30 plus 0.3 M K^+ glutamate (K^+Glu), pOS3; 4, S-30 plus 0.3 M sucrose, pOS3; 5, S-30 plus 0.2 M glycine betaine (GB), pOS3; 6, S-30 plus K^+ glutamate and glycine betaine, pOS3; 7, S-30 untreated, pAT153; 8, S-30 plus K^+ glutamate, pAT153. Molecular mass markers (in kDa) and products specified by the plasmids are indicated on the left. βgal, β-galactosidase.

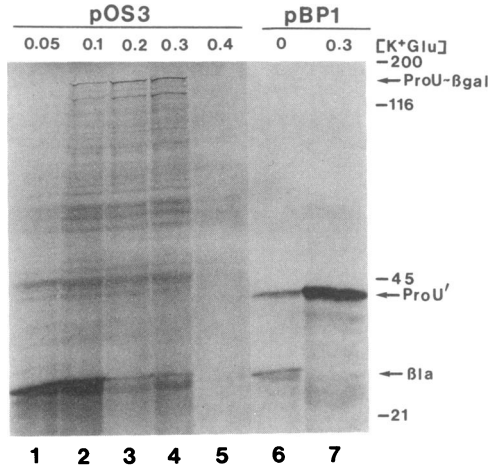

FIG. 4. Effects of K^+ glutamate concentration on *in vitro* expression from pOS3 and pBP1. Plasmid DNAs were incubated in a cell-free extract as in Fig. 3. Samples (15 μl) were removed, and labeled proteins synthesized *in vitro* were resolved by $NaDodSO_4$/PAGE and autoradiography. The following K^+ glutamate concentrations were used. Lanes: 1, 0.05 M; 2, 0.1 M; 3, 0.2 M; 4 and 7, 0.3 M; 5, 0.4 M; 6, no salt. Molecular mass markers (in kDa) and products specified by plasmids are indicated on the right. βgal, β-galactosidase; βla, β-lactamase.

The template plasmid pOS3 carries about 2.5 kilobase pairs (kbp) of *E. coli* chromosomal DNA. To determine whether this region of the DNA has a role in osmoregulation, we subcloned a 1.7-kbp fragment containing the *proU* structural gene and <600 bp of upstream DNA into the pUC-derived vector pTZ18U (Fig. 1). The construct pBP1 also lacks the β-galactosidase portion of the fusion protein. *In vitro*, pBP1 programmed the synthesis of a 42-kDa protein that was strongly induced by K^+ glutamate (Fig. 4, lanes 6 and 7), showing that this smaller insert contains all necessary information for osmoregulation.

These results show that *proU* expression is stimulated by an ionic compound, K^+ glutamate. To determine whether the signal might be the cation, the anion, or a simple increase in ionic strength, a variety of salts at 0.1 and 0.3 M were tested for their ability to stimulate synthesis of ProU *in vitro* (Fig. 5). Using pBP1 as our template, we found that K^+ acetate was stimulatory at 0.1 M. At 0.3 M, both K^+ acetate and KCl

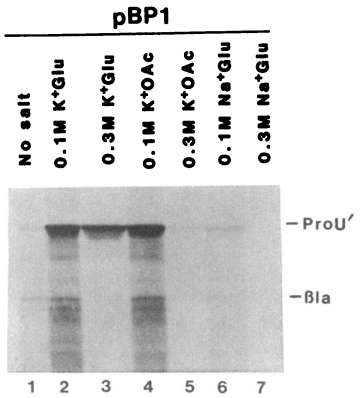

FIG. 5. Effects of various salts on *in vitro* expression from pBP1. Plasmid DNA was incubated in a cell-free extract as in Fig. 3. Samples (15 μl) were removed, and labeled proteins synthesized *in vitro* were resolved by $NaDodSO_4$/PAGE and autoradiography. The following salts and concentrations were used. Lanes: 1, no salt; 2, 0.1 M K^+ glutamate; 3, 0.3 M K^+ glutamate; 4, 0.1 M K^+ acetate; 5, 0.3 M K^+ acetate; 6, 0.1 M Na^+ glutamate; 7, 0.3 M Na^+ glutamate. Products specified by pBP1 are indicated on the right. βla, β-lactamase.

completely inhibited protein synthesis. No stimulation was observed with Na^+ glutamate (lanes 6 and 7), Na^+ acetate, Tris glutamate, and Tris acetate (data not shown). All salts had the same inhibitory effect on β-lactamase as K^+ glutamate, that is, reducing its expression at 0.1 M and totally inhibiting it at 0.3 M. Mg^{2+} acetate at 0.06 and 0.2 M completely inhibited protein synthesis. The nonionic sugar trehalose was tested since this dissacharide accumulates in salt-stressed cells (34). Trehalose behaved identically with sucrose, neither stimulating *proU* nor inhibiting β-lactamase expression. These results support the model of internal K^+ concentration as the molecular signal for *proU* osmoregulation, although the counterion appears important for maintaining the integrity of the transcription/translation apparatus.

If internal K^+ concentration is the signal, combining glycine betaine and K^+ glutamate would be predicted to stimulate pOS3-dependent Φ(*proU–lacZ*) expression to the same extent as K^+ glutamate alone. This is the result observed (Fig. 3, lane 6), suggesting that the *in vivo* repression of *proU* expression by glycine betaine is due to substitution of glycine betaine for internal K^+ and hence a reduced level of signal. In summary, the ionic compound K^+ glutamate provides an effective, specific signal to activate plasmid-encoded *proU* expression, utilizing presynthesized components found in an S-30 extract obtained from cells grown under unstressed conditions.

DISCUSSION

Reconstitution of *proU* osmoregulation *in vitro* indicates that neither turgor pressure nor transmembrane signaling are required for osmotic control of this operon. In this respect, osmoregulation of *proU* apparently differs from that of *kdp* or the OmpC/F porins (4, 5, 22). These systems are also osmoregulated *in vivo*, but their expression is mediated by sensory, dual-component regulatory systems associated with the cell membrane (4, 22, 35). The lack of a role for turgor pressure in *proU* regulation had been inferred from *in vivo* studies (14, 17).

The molecular signal that stimulates *proU* expression is not simply an increase in osmolarity since high concentrations of sucrose, trehalose, or glycine betaine did not trigger *proU* expression *in vitro*. This is consistent with the *in vivo* observation that osmotically stressed cells accumulate glycine betaine and trehalose to high internal concentrations yet have very low levels of *proU* expression. Rather, *proU* responded strongly to the ionic compound K^+ glutamate and to a lesser extent to K^+ acetate. Since other salts give no response at all, the osmostimulatory signal cannot be ionic strength. The simplest model is that internal K^+ concentration is the signal, but its effect is modulated by the anions present, probably through their effect on the efficiency of the transcription/translation machinery. K^+ glutamate was chosen as the osmolyte for our initial *in vitro* studies since K^+ and glutamate are the predominant cation and anion in osmotically stressed *E. coli* (2, 6, 7), and intracellular K^+ concentration correlates with the level of *proU* expression *in vivo* (17). When glycine betaine is present in the external medium, it is accumulated in preference to K^+ glutamate and would reduce *proU* expression by substituting for the signal ion. The *in vitro* response to K^+ glutamate and the lack of stimulation by glycine betaine faithfully reproduce the *in vivo* regulatory pattern. This contrasts with studies in which dramatic *in vitro* ion effects on *lac* repressor–operator interactions and RNA polymerase–promoter interactions virtually disappeared in the intact cell (7, 36).

Further evidence for the physiological relevance of K^+ glutamate stimulation comes from its specificity. Increasing ionic strength above the isotonic optimum is known to inhibit

transcription (36). In the cell-free system, β-lactamase expression represents the general phenomenon; synthesis of the β-lactamase gene product is inhibited by addition of all salts tested, whether the gene is located on pOS3 or is alone on the control plasmid pAT153. The response of *proU* is specific and in the opposite direction: increasing ionic strength with only K^+ glutamate or K^+ acetate turns on gene expression. For final verification of the *in vitro* regulation, we would like to isolate a constitutive promoter mutant and to demonstrate the same altered regulation *in vitro*. Unfortunately, no truly constitutive mutants of *proU* have been identified despite an extensive search.

How might an increase in internal K^+ glutamate specifically alter transcription? By analogy to the heat shock response (37), we might postulate an inducible osmotic stress σ factor. However, our studies eliminate this model since all components required in trans to transmit osmostimulatory signals were present in an S-30 extract derived from cells grown in standard medium. Other reasonable hypotheses involve a constitutive alternate σ factor that becomes activated by K^+ glutamate addition, alteration of the DNA template, synthesis of a small molecule activator, or modification of RNA polymerase specificity. Although some evidence has been obtained for an effect of intracellular osmotic strength on plasmid supercoiling, no direct relationship with osmoregulation has been demonstrated (25). We have carried out preliminary experiments to test the effect of plasmid conformation on *proU* expression. Linearizing the template, by treatment of pBP1 with *Bam*HI, reduced expression of both plasmid genes in the S-30 *in vitro* system. However, *proU* expression was more severely reduced than β-lactamase. While this result may be significant, we believe that many other parameters must be thoroughly explored before any conclusions can be reached and will be the topic of future research. Therefore, although the molecular signal stimulating *proU* expression is now identified as K^+ glutamate, its detailed mechanism of action remains unknown.

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