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Binding protein dependent transport of glycine betaine and its osmotic regulation in *Escherichia coli* K12

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Summary. Glycine betaine, which functions as an osmoprotectant, is accumulated to high intracellular concentrations in *Escherichia coli* at high osmolarity. We demonstrate the presence of a high-affinity, binding protein dependent transport system for glycine betaine, which is encoded by the *proU* region. We show the osmotically regulated synthesis of a 32 kDa periplasmic protein that is a glycine betaine binding protein with a K_D of 1.4 μM. ProU-mediated glycine betaine transport is osmotically stimulated at the level of gene expression. The osmolarity of the medium also regulates the activity of the transport system, while binding of glycine betaine to its binding protein is independent of the osmolarity. We also find a second glycine betaine transport system that is dependent on *proP* and exhibits a lower substrate affinity. Like ProU, this system is regulated at two levels: both gene expression and the activity of the transport system are osmotically stimulated. Using λ*plac*Mu-generated *lacZ* operon and gene fusions, we find that expression of the *proU* region is osmotically regulated at the level of transcription. We cloned a part of the *proU* region together with the Φ(*proU-lacZ*)hyb2 gene fusion into a multicopy plasmid and show that the DNA sequences required in *cis* for osmotic regulation are present on the plasmid.

Key words: ProU – ProP – Binding protein – Osmoregulation – *lacZ* fusions

Introduction

Escherichia coli and other enteric bacteria employ common strategies to overcome the deleterious effects of elevated osmolarity in their environment. Among these are the rapid uptake of potassium ions into the cytoplasm to restore turgor pressure following osmotic upshock (Laimins et al. 1981; Epstein 1985), as well as the accumulation and synthesis of organic compounds like amino acids, sugars, polyols and betaines (for reviews see Yancey et al. 1982; Le Rudulier et al. 1984). Because these organic molecules are thought to increase the internal osmolarity without disturbing cellular metabolism, they have been termed "compatible solutes" (Yancey et al. 1982). One of the most ubiquitous of these seems to be glycine betaine, which is synthesized or accumulated not only by various bacteria but also by a wide range of higher plants, marine animals, and cyanobacteria.

In *E. coli*, osmotically stimulated synthesis of glycine betaine involves aerobic oxidation of exogenously added choline to glycine betaine aldehyde and glycine betaine (Landfald and Strøm 1986). This synthesis confers high levels of osmotic tolerance to cells grown in media of an otherwise inhibitory osmolarity (Styrvold et al. 1986). A similar osmoprotective effect can be achieved by the addition of low concentrations (1 mM) of glycine betaine to the growth medium. Increases in external osmolarity result in a strong stimulation of glycine betaine active transport; this substance is accumulated to high intracellular levels but is not metabolized (Perroud and Le Rudulier 1985). As a result, cell volume is restored and inhibition of growth and carbohydrate transport is reversed (Roth et al. 1985a, b). Glycine betaine also stimulates growth of *Salmonella typhimurium* in media of inhibitory osmotic strength (Le Rudulier and Bouillard 1983). In this organism, the characteristics of glycine betaine transport have been studied extensively, and two transport systems have been identified (Cairney et al. 1985a, b): a low-affinity transport system encoded by *proP* at min 92 on the *S. typhimurium* linkage map, and a high-affinity transport system encoded by the *proU* region at min 58 (Menzel and Roth 1980; Csonka 1982; Cairney et al. 1985a, b). Studies using *lac* operon fusions have demonstrated that an increase in external osmolarity leads to greater than 100-fold stimulation of expression of the *proU* region and to about 3-fold enhanced transcription of *proP* (Cairney et al. 1985a, b; Dunlap and Csonka 1985). Medium osmolarity not only regulates gene expression but also modulates the activity of both transport systems even in cells grown in media of elevated osmotic strength. Transport could be detected only when the uptake assay was performed at high osmolarity (Cairney et al. 1985a, b). The ProP and ProU systems were originally identified in *S. typhimurium* as permeases for proline, another major osmoprotective solute (Menzel and Roth 1980; Csonka 1982; Le Rudulier et al. 1984). However, recent data suggest that glycine betaine rather than proline is the main substrate for ProP (Cairney et al. 1985a). ProU-mediated proline transport was not detected by Cairney et al. (1985b), whereas other authors have provided evidence for a small contribution of ProU to the overall proline uptake in osmotically stressed cells (Dunlap and Csonka 1985). Both the ProP and ProU systems must have some affinity for proline, however, since mutations in *proP* and the *proU* region can be

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Table 1. Bacteria and bacteriophages

Strain	Description[a]	Origin/Reference
Strains derived from *Escherichia coli* K12		
MC4100	F⁻ *araD139Δ(argF-lac)U169 rpsL150 relA1 deoC1 ptsF25 rbsR flbB5301*	Casadaban (1976)
JWL152	F⁻ *mtlA2 srlA53D50 gatA50 upp thi1 xyl7 malA1 nutB1 rpsL*	J. Lengeler
GY3424	F⁻ *thi1 xyl7 malA1 relA1 rpsE argA21 cysC43 lysA22 thyA61*	R. Devoret, through J. Lengeler
SG477	MC4100 *envZ22*(am)	Garrett et al. (1983)
TK821	MC4100 *ompR-331*::Tn*10*	T. Palva
WG147	F⁻ *trp lacZ rpsl thi Δ(putPA)101 pyr-76*::Tn*10*	Stalmach et al. (1983)
821	F⁻ *thi thr leu proA his argB strA gshA*	Apontoweil and Berends (1975b), through B. Bachmann, CGSC5968
CS101B	*metB gabC*	Metzer et al. (1979), through Y. Halpern
S-5	*metB gabC gabP5*	Metzer et al. (1979), through Y. Halpern
GM37	MC4100 *Φ(proU-lacZ)*hyb2 (λ*plac*Mu15)	This work
GM40	MC4100 *Φ(proU-lacZ)*hyb5 (λ*plac*Mu15)	This work
GM41	MC4100 *Φ(proU-lacZ)*hyb6 (λ*plac*Mu15)	This work
GM50	MC4100 *Φ(proU-lacZ⁺)*3 (λ*plac*Mu55)	This work
GM114	MC4100 *zfi-551*::Tn*10*	This work
GM115	MC4100 *zfh-552*::Tn*10*	This work
GM119	MC4100 *srl*::Tn*10*	This work
GM124	GM37 *zfh-552*::Tn*10*	This work
EF001	MC4100 *Δ(proU)1*	This work
EF030	821 *Φ(proU-lacZ)*hyb2	This work
EF033	CS101B *met⁺ srl*::Tn*10*	This work
EF036	S-5 *met⁺ Φ(proU-lacZ)*hyb2	This work
EF037	MC4100 *Δ(putPA)101*	This work
EF038	MC4100 *Δ(putPA)101 proP*	This work
EF046	MC4100 *Δ(putPA)101 Δ(proU)1*	This work
EF047	MC4100 *Δ(putPA)101 proP Δ(proU)1*	This work
Bacteriophages		
λpMu507	MucIts62 *A⁺B⁺* Sam7 cIts857	Magazin et al. (1977)
λ*plac*Mu15	MucIts62 *ner⁺ A'*am1093 *'ara'* Mu*S' 'lacZ lacY⁺ lacA' kan imm λ*	E. Bremer
λ*plac*Mu55	MucIts62 *ner⁺ A'*am1093 *'uvrD'* Mu*S' 'trp' lacZ⁺ lacY⁺ lacA' kan imm λ*	E. Bremer

[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 symbols *lacZ⁺* and *lacZ* denote *lacZ* genes with or without translational initiation signals, respectively. Genes marked with a prime are incomplete. The genetic nomenclature is according to Bachmann (1983)

selected by using the toxic proline analogues L-acetidine-2-carboxylate (AC) and 3,4-dehydro-DL-proline (DHP) (Ratzkin et al. 1978; Csonka 1982; Stalmach et al. 1983; Grothe et al. 1986).

The *proP* and *proU* regions have also been identified in *E. coli* (Stalmach et al. 1983; Gowrishankar 1985) and the osmotically stimulated expression of *proP-lacZ* and *proU-lacZ* operon fusions (Gowrishankar 1985, 1986) parallels that of such fusions in *S. typhimurium* (Cairney et al. 1985a, b). As in *S. typhimurium*, ProP and ProU contribute to proline uptake (Stalmach et al. 1983; Grothe et al. 1986), but whether these uptake systems also mediate the glycine betaine transport detected in osmotically stressed *E. coli* cells (Perroud and Le Rudulier 1985) has not been tested. We show here that the ProU[1] system in *E. coli* is a binding protein dependent, high-affinity transport system for glycine betaine that is osmotically regulated at the level of gene expression and at the level of transport activity. In addition, we demonstrate the existence of a second low-affinity glycine betaine transport system that is *proP* dependent.

[1] We define "ProU" as a system that is composed of more than one polypeptide chain, mediating the transport of glycine betaine. Similarly, "the *proU* region" designates the genes for this transport system. The number of genes for this multi-component system is as yet unknown

Materials and methods

Bacterial strains and bacteriophages. Bacterial strains and bacteriophages used in this study are listed in Table 1.

Media and chemicals. Bacteria were grown aerobically in a shaking incubator at 37° C in LB medium (10 g/l Bacto tryptone, 5 g/l Bacto yeast extract, 5 g/l NaCl) or in minimal medium A (MMA; 10.5 g/l K_2HPO_4, 4.5 g/l KH_2PO_4, 1 g/l $(NH_4)_2SO_4$, 0.5 g/l sodium citrate·$2H_2O$, 0.1 g/l $MgSO_4 \cdot 7H_2O$) supplemented with 0.2% glucose. The osmolarity of liquid MMA was increased by the addition of 300 mM NaCl, 464 mM sucrose, or 516 mM glycerol. Lactose MacConkey and tetrazolium media were prepared as previously described (Miller 1972; Silhavy et al. 1984). Solid media with elevated osmolarity were prepared by adding 200 mM NaCl to MacConkey and MMA agar or 300 mM NaCl to tetrazolium agar. Agar plates spread with 0.1 ml of a 10 mg/ml 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), tetracycline (Tc), and ampicillin (Ap) were added to media at 30 μg/ml, 5 μg/ml, and 50 μg/ml, respectively. γ-Amino-n-butyric acid (GABA; Sigma) was used at a concentration of 0.2% in glucose MMA plates without $(NH_4)_2SO_4$ and sodium citrate (Metzer et al. 1979). Glycine

betaine (N,N,N-trimethylglycine), L-azetidine-2-carboxylic acid (AC) and 3,4-dehydro-DL-proline (DHP) were obtained from Sigma. Sodium nitroferricyanide dehydrate (sodium nitroprusside) was purchased from Aldrich Chemie (Steinheim, FRG).

Specific β-galactosidase activity was assayed as described (Miller 1972) and expressed as micromoles of substrate cleaved per minute per milligram of protein. Protein concentration was estimated from the optical density at 600 nm (OD_{600}) of the culture, assuming that an OD_{600} of 1.4 corresponds to approximately 150 µg protein/ml culture (Miller 1972).

Genetic procedures. Standard techniques were used for the growth of bacteria and bacteriophages and generalized transduction with phage P1 (Miller 1972; Silhavy et al. 1984). λ*plac*Mu15 and λ*plac*Mu55 were grown on strain MC4100, and λpMu507 was grown on strain MBM7014 (Berman and Beckwith 1979). Tn*10* insertions near the (*proU-lacZ*)hyb2 fusion were isolated by transduction of strain GM37 with a lysate of phage P1 grown on a pool of several thousand colonies of strain SH120 (Schweizer and Boos 1983) containing random insertions of Tn*10*. Tc-resistant transductants were screened for a $LacZ^-$ phenotype. Tc-sensitive clones from a strain carrying a Tn*10* insertion (GM124) were selected as described by Maloy and Nunn (1981). The inheritance of a *gshA* mutation (Apontoweil and Berends 1975b; Bachmann 1983) was tested by a staining procedure with sodium nitroprusside as described (Apontoweil and Berends 1975a). Strains with *gabC* ($GABA^+$) and *gabCP* ($GABA^-$) mutations were distinguished by streaking on glucose MMA plates with GABA as sole nitrogen source (Metzer et al. 1979).

Isolation of λplacMu15 and λplacMu55 insertions. The phages employed for the construction of *lac* gene and operon fusions differ from the previously described λ*plac*Mu phages (Bremer et al. 1984, 1985) because they are unable to transpose by themselves; thus, secondary transposition following a chromosomal insertion is prevented (E. Bremer, unpublished data). Transposition of these phages occurs when Mu transposition functions are provided in *trans* by coinfection with λpMu507 (MuA^+B^+). The *lacZ* gene fusions (λ*plac*Mu15) and *lacZ* operon fusions (λ*plac*Mu55) were isolated in strain MC4100 after coinfection with λpMu507 as described (Bremer et al. 1985). Several thousand Lac^+ colonies were selected on lactose minimal agar containing 200 mM NaCl. Fusions to genes the expression of which is stimulated by high osmolarity were identified by replica-plating; colonies showing a Lac^- phenotype (red) on lactose tetrazolium agar but a Lac^+ phenotype (white) on lactose tetrazolium agar with 300 mM NaCl were picked, purified, and tested for sensitivity to phage λ*vir*, resistance to phage λ*cI*h80, and Km resistance. To ensure that only one insertion of λ*plac*Mu was present, the fusions were transduced with phage P1 into strain MC4100. Km-resistant transductants were purified and retested for the λvir^s and $\lambda cIh80^r$ phenotypes. Expression of the *lac* genes was strongly increased in all strains when lactose MacConkey plates were supplemented with NaCl. These fusion strains also grew poorly or not at all on lactose minimal plates but formed normal size colonies on lactose minimal plates containing 200 mM NaCl.

Strain constructions. To construct a strain (EF001) deleted for the *proU* region, Tc-sensitive mutants of strain GM124 (*Φ(proU-lacZ)*hyb2 *zfh-552*::Tn*10*) were selected (Maloy and Nunn 1981). Removal of the entire λ*plac*Mu prophage in $Lac^-Tc^SKm^S$ colonies was confirmed by testing their immunity to superinfection with λ and Mu phages as previously described (Bremer et al. 1984, 1986). The deletion in strain EF001 (*Δ(proU)1*) does not extend into the *gshA* and *gabCP* genes.

To construct a set of isogenic strains with mutations in *proP* and the *proU* region, the *Δ(putPA)101* deletion from strain WG147 was transferred into strain MC4100 by cotransduction with a linked *pyr-76*::Tn*10* insertion (Stalmach et al. 1983). The *pyr-76*::Tn*10* insertion was removed by phage P1 transduction to Pyr^+. The resulting strain (EF037) was resistant to AC and sensitive to DHP (Stalmach et al. 1983). Spontaneous *proP* mutants were isolated from *Δ(putPA)101* strains by selecting for resistance to 0.4 mM DHP on glucose minimal plates; such mutations have previously been mapped to min 93 on the *E. coli* linkage map (Bachmann 1983; Stalmach et al. 1983; Gowrishankar 1985). These *Δ(putPA)101 proP* mutants were still DHP^S on glucose MMA agar in the presence of 300 mM NaCl due to expression of the ProU system (Stalmach et al. 1983; Grothe et al. 1986). The mutation conferring DHP resistance at low osmolarity in one of these strains (EF038) was mapped by phage P1 transduction. It showed a cotransduction frequency of 49% with a Tn*10* insertion (*zje*::Tn*10*) located between 94 and 95 min on the *E. coli* linkage map. We conclude that the mutation is in *proP*.

Using a P1 lysate grown on strain GM37, the (*proU-lacZ*)hyb2 fusion was introduced into strain EF038 (*Δ(putPA)101 proP*), selecting for Km resistance. As expected (Grothe et al. 1986), the resulting strain was AC^r and DHP^r on glucose MMA agar containing 300 mM NaCl. To introduce the *Δ(proU)1* deletion, an *srl*::Tn*10* insertion was transferred into this strain using phage P1 and the *Δ(proU)1* mutation from strain EF001 was then introduced by selecting for growth on sorbitol MMA agar and screening for loss of the Lac^+ and Km^r phenotypes. Resistance of one of these strains (EF047) to AC and DHP at high osmolarity was tested to confirm the presence of the *Δ(proU)1* deletion. An analogous strategy was used to introduce the *Δ(proU)1* mutation from strain EF001 into strain EF037 (*Δ(putPA)101 proP*$^+$ *proU*$^+$), yielding strain EF046 (*Δ(putPA)101 proP*$^+$ *Δ(proU)1*).

Fine-structure mapping of the proU region. A P1 lysate grown on strain GM114 (*zfi-551*::Tn*10*) was used to transduce strains JWL152 (*srlA53*), GY3424 (*cysC43*), and GM37 (*Φ(proU-lacZ)*hyb2) to Tc resistance. The transductants were tested for loss of the Srl^-, Cys^- and Km^r and Lac^+ phenotypes, respectively. Similarly, a P1 lysate grown on strain GM119 (*srl*::Tn*10*) was used to transduce strain GY3424 to Tc resistance, and the resulting transductants were tested for their Cys phenotype. Strains GM115 (*gshA*$^+$ *proU*$^+$ *zfh-552*::Tn*10*) and GM119 (*gshA*$^+$ *proU*$^+$ *srl*::Tn*10*) were used as donors in three-factor crosses with strain EF030 (*srl*$^+$ *gshA Φ(proU-lacZ)*hyb2). Inheritance of the Lac^+ and Km^r phenotypes and the *gshA* mutation was scored in Tc^r transductants. In an analogous three-factor cross between strain EF033 (*srl*::Tn*10 proU*$^+$ *gabC*) and strain EF036 (*srl*$^+$ *Φ(proU-lacZ)*hyb2 *gabCP*), the Km^r and $GABA^-$ phenotypes of Tc^r transductants were tested.

In each cross, between 100 and 300 transductants were tested for the relevant phenotypes.

Methods used with nucleic acids. Isolation and transformation of plasmid DNA, digestion with restriction enzymes, DNA ligation with T4 DNA ligase, and recovery of restriction fragments from agarose gels were all performed as previously described (Maniatis et al. 1982; Silhavy et al. 1984).

Preparation of proteins and gel electrophoresis. Total cell proteins were prepared and analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described (Silhavy et al. 1984). To obtain periplasmic proteins, cells were grown overnight in 2 l MMA with or without 300 mM NaCl, washed in 20 ml 10 mM Tris-HCl, pH 7.3 with 300 mM or 30 mM NaCl, respectively, and resuspended in 20 ml 30 mM or 10 mM Tris-HCl, pH 7.3. Periplasmic proteins were released by cold osmotic shock according to the method of Neu and Heppel (1965), dialysed against 10 mM Tris-HCl, pH 7.3, freeze dried, and resuspended in 10 mM Tris-HCl, pH 7.3. Protein concentrations were determined as described by Lowry et al. (1951). The proteins were analysed by SDS-PAGE, using a 12% gel (Laemmli 1970). Protein bands were visualized by staining with Coomassie blue. To determine the relative amount of the glycine betaine binding protein, the gel was scanned with a laser densitometer (2202 Ultrascan, LKB Bromma).

Transport assay. Cells were grown overnight in glucose MMA with or without 300 mM NaCl and were washed with their growth medium without carbon source. They were diluted in MMA with or without 300 mM NaCl to an OD_{578} of 0.1 or 1.0 to measure transport at 7 or 70 μM substrate, respectively. The cell suspension was equilibrated at room temperature for 10 min. Transport was initiated by mixing 2 ml of cells with [*methyl*-^{14}C] betaine (7.1 mCi/mmol; Amersham Buchler, Braunschweig). Samples (200 μl) were removed at the indicated times, the cells were collected by filtration through Millipore filters (pore size 0.45 μm) and washed with 10 ml MMA with or without 300 mM NaCl; the filters were dried and the radioactivity measured by scintillation counting.

Binding assay. The K_D of the glycine betaine binding protein was measured by a previously described dialysis technique (Argast and Boos 1979). A small Visking tubing with one open end was attached at its open end onto a bluntly cut plastic pipette tip. After 250 μl of shock fluid (1.3 mg protein/ml) had been introduced, the tubing was immersed in 1 l of 10 mM Tris-HCl, pH 7.3, so that both surface levels were equal. The external buffer was gently stirred. After equilibrating for 15 min at room temperature, the reaction was started by adding 2.8 nmol [*methyl*-^{14}C] betaine (7.1 mCi/mmol) to the shock fluid. Aliquots of 20 μl were removed at the indicated times, and the amount of glycine betaine remaining was determined by scintillation counting.

As an alternative, the method of Richarme and Kepes (1983) was used. Eighty microlitres of shock fluid (1.3 mg protein/ml) in 10 mM Tris-HCl, pH 7.3 was mixed with 10 μl of 56 μM [*methyl*-^{14}C] betaine (7.1 mCi/mmol) and the mixture was incubated at room temperature for 5 min. Proteins were precipitated by adding 1 ml of an ice-cold saturated ammonium sulphate solution, collected by filtration onto Millipore filters (pore size 0.45 μm), and washed with 10 ml of ammonium sulphate solution. Filters were dried, and their radioactivity was determined in a scintillation counter. To perform the assay at high osmolarity, NaCl was added to a concentration of 300 mM. The final concentration of [*methyl*-^{14}C] betaine in the assay was 5.8 μM.

Table 2. The effect of various solutes on the expression of β-galactosidase in *lacZ* fusion strains

Strain	Specific β-galactosidase activity [μmol/min per mg protein] in				
	MMA	MMA + 300 mM NaCl	MMA + 300 mM NaCl + 1 mM glycine betaine	MMA + 464 mM sucrose	MMA + 516 mM glycerol
MC4100	0	0	0	0	0
GM37	0.071	5.02	1.67	5.98	0.064
GM50	0.071	1.76	n.d.	2.71	0.111

Strains MC4100 (*Δlac*), GM37 (*lacZ* gene fusion), and GM50 (*lacZ* operon fusion) were grown in glucose MMA to an OD_{600} of 0.2 to 0.25. Samples were withdrawn and the osmolarity of the remaining culture was suddenly increased by addition of the indicated solutes. After further growth for 100 min, β-galactosidase activity was assayed. The data show the mean values of two independent experiments; n.d.: not determined

Results

Characterization of proU-lac fusions

Derivatives of the previously described λp*lac*Mu phages (Bremer et al. 1985) were used to construct *lacZ* gene (ten strains) and operon (five strains) fusions that exhibited enhanced gene expression at high osmolarity. To facilitate the genetic mapping of the *lac* fusions, we isolated two Tn*10* insertions (later named *zfi*::Tn*10* and *zfh*::Tn*10*) linked to the λp*lac*Mu insertion in strain GM37. All of the originally isolated *lac* fusions showed very similar cotransduction frequencies with these two Tn*10* insertions: 1%–4% for *zfi*::Tn*10* and 85%–95% for *zfh*::Tn*10* (data not shown). Thus, all 15 independently isolated fusions had occurred in the same gene or cluster of genes. Expression of these fusions was analysed by measuring β-galactosidase activity in cells grown in minimal medium A, the osmolarity of which had been increased by the addition of osmotically equivalent concentrations of NaCl, sucrose or glycerol. Addition of either NaCl or sucrose to the growth medium resulted in a 20- to 70-fold increase in *lac* expression, whereas the addition of glycerol produced no change in the β-galactosidase activity. The presence of 1 mM glycine betaine in MMA with 300 mM NaCl strongly reduced the expression of the *lacZ* fusions; representative data are shown in Table 2. Such an osmotically modulated pattern of gene expression has recently been described for *proU-lacZ* operon fusions in *S. typhimurium* and *E. coli* (Cairney et al. 1985b; Gowrishankar 1985). To test whether the fusions we had isolated were also in the *proU* region, cotransduction frequencies between the gene fusion in strain GM37 and several genes in the 57 to 59 min region were determined by P1-mediated two- and three-factor crosses. The results located the *lac* fusion between the *gshA* and *gabCP* genes (Fig. 1) at min 57.5 on the *E. coli* linkage map (Bach-

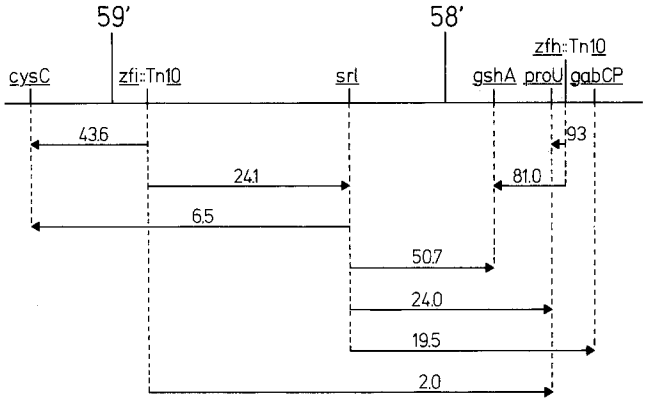

Fig. 1. Chromosomal location of the *proU* region. The 58 to 59 min region of the *Escherichia coli* chromosome is shown together with the position of relevant genes and Tn*10* insertions. The *arrows* originate from the selected markers and point towards the scored genes in P1 transduction experiments. The *numbers* above the arrows indicate cotransduction frequencies expressed as percentages. In the recipient strains the *proU* region was always defined by the (*proU-lacZ*)hyb2 gene fusion and loss of the Lac$^+$ and Km^r phenotypes was scored. When cotransduction frequencies between *srl* and *cysC* or *srl* and the *lac* fusion were determined, an *srl*::Tn*10* insertion was used as the selected marker

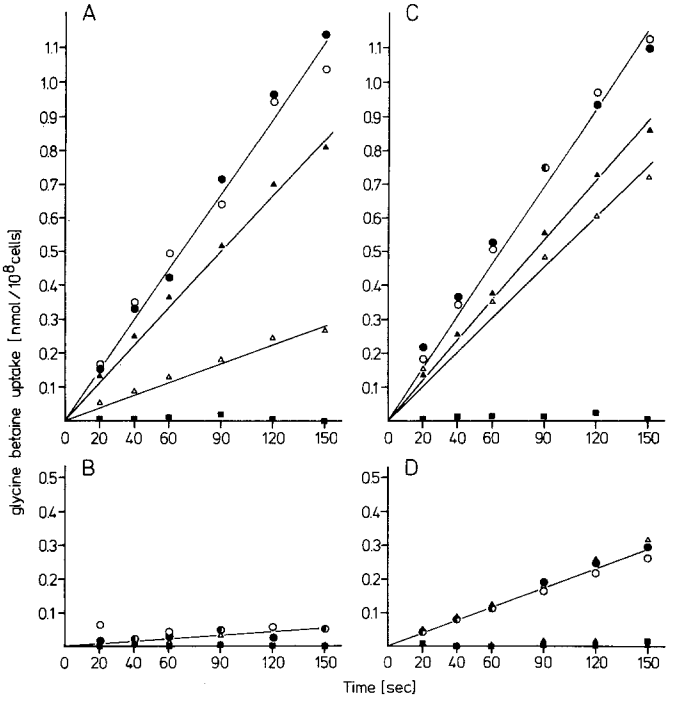

Fig. 2A–D. Osmotically stimulated transport of glycine betaine. Transport of [^{14}C]-glycine betaine was measured in cells of strains MC4100 (*putPA*$^+$ *proP*$^+$ *proU*$^+$; ●, EF037 (*Δ(putPA)101 proP*$^+$ *proU*$^+$; ○, EF038 (*Δ(putPA)101 proP proU*$^+$; ▲, EF046 (*Δ(putPA)101* proP$^+$ *Δ(proU)1*; △: and EF047 (*Δ(putPA)101 proP Δ(proU)1*; ■, grown in MMA with 300 mM NaCl (**A, C**) or without additional NaCl (**B, D**). Glycine betaine transport at 7 μM (**A, B**) and 70 μM (**C, D**) substrate was assayed in the presence of 300 mM NaCl

mann 1983). A similar location has been reported for *proU-lacZ* operon fusions in *S. typhimurium* (Dunlap and Csonka 1985; Cairney et al. 1985b) and in *E. coli* (Gowrishankar 1985).

A strain lacking the two major proline permeases PutP and ProP is still sensitive to the toxic proline analogues AC and DHP at high osmolarity whereas an isogenic strain carrying a *proU* mutation is resistant (Csonka 1982; Grothe et al. 1986). We introduced, with phage P1, the Lac$^+$ λ*plac*Mu insertion from strain GM37 into strain EF038 (*Δ(putPA)101 proP*) and found that the resulting transductants were resistant to both AC and DHP at high osmolarity. This provides further evidence that the *lac* fusions described here are to the *proU* region.

It has been reported that the osmotically modulated expression of *proU* is independent of *ompR* in *S. typhimurium* (Cairney et al. 1985b). To test whether this was also the case for the *proU* region of *E. coli*, we used phage P1 to transduce the (*proU-lacZ*$^+$)3 operon fusion from strain GM50 into strains TK821 (*ompR-331*::Tn*10*) and SG477 (*ompR*$^+$ *envZ 22*(am)). Expression of the fusion in the resulting transductants was not altered at high or at low osmolarity in comparison with the parent strain GM50 (data not shown).

Osmotically stimulated transport of glycine betaine

In *S. typhimurium*, *proU* and *proP* have been shown to encode transport systems for glycine betaine with high and low affinity, respectively (Cairney et al. 1985a, b). Although osmotically stimulated glycine betaine transport has been demonstrated in *E. coli* (Perroud and Le Rudulier 1985), it is not known whether this uptake is also ProU and ProP mediated. We measured glycine betaine uptake at a substrate concentration of 7 μM in cultures of strain MC4100 (*proP*$^+$ *proU*$^+$) and strain GM37 (*proP*$^+$ *Φ(proU-lacZ)* hyb2) grown in MMA with 300 mM NaCl. The presence of the (*proU-lacZ*)hyb2 fusion reduced transport from 0.46 nmol/10^8 cells per min to 0.13 nmol/10^8 cells per min (data not shown). Thus, ProU must also be involved in glycine betaine transport in *E. coli*.

To analyse uptake in more detail, we constructed a set of isogenic strains with mutations in the *proU* region or in *proP* (Materials and methods) and measured transport at glycine betaine concentrations of 7 μM and 70 μM in cells grown in MMA, or in MMA with 300 mM NaCl (Fig. 2). No transport could be detected in cells defective in both ProU and ProP. When either ProU or ProP was functional, glycine betaine transport occurred, but each of these transport systems contributed differently to the net uptake found in strain EF037 (*proU*$^+$ *proP*$^+$) (Fig. 2A, C). ProU-mediated transport was the major component of this net uptake at low substrate concentration (7 μM). Increasing the substrate concentration tenfold only slightly enhanced the ProU-mediated transport, whereas it strongly enhanced transport mediated by *ProP* (Fig. 2A, C). Thus, as in *S. typhimurium* (Cairney et al. 1985a, b), glycine betaine uptake in *E. coli* (Perroud and Le Rudulier 1985) is mediated by two distinct systems: the high-affinity transport system ProU and the low-affinity transport system ProP. Both systems can be osmotically stimulated (Fig. 2). Even at a high substrate concentration (70 μM) no ProU-mediated transport was detected in cells grown in MMA (Fig. 2D), consistent with the low levels of β-galactosidase activity in *proU-lacZ* fusion strains under these conditions (Table 2). This suggests that the osmotic stimulation of ProU-mediated transport is the result of enhanced gene expression. In contrast, *proP*$^+$ strains grown in MMA still showed considerable glycine betaine transport at a high

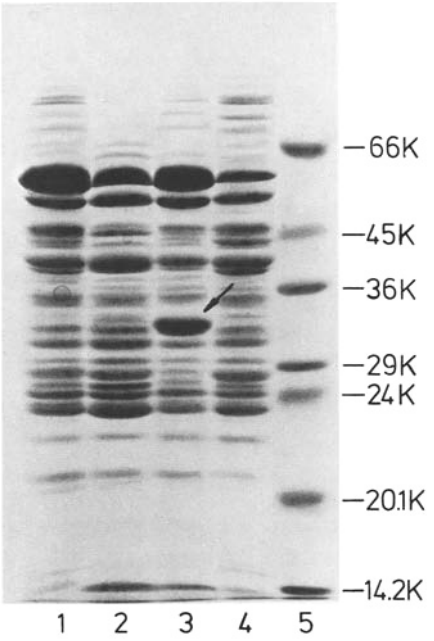

Fig. 3. Osmotically modulated synthesis of a glycine betaine binding protein. Periplasmic proteins of strain GM37 (*Φ(proU-lacZ)*hyb2; lane 1) and strain MC4100 (*proU*$^+$; lanes 2–4) were analysed by SDS-PAGE (12% acrylamide). The gel was stained with Coomassie blue. Proteins were released by osmotic shock from cells grown in glucose MMA (lane 4), glucose MMA with 300 mM NaCl (lanes 1 and 3), and glucose MMA with 300 mM NaCl and 1 mM glycine betaine (lane 2). The position of the glycine betaine binding protein is indicated by the *arrow*; a molecular weight standard is shown in lane 5

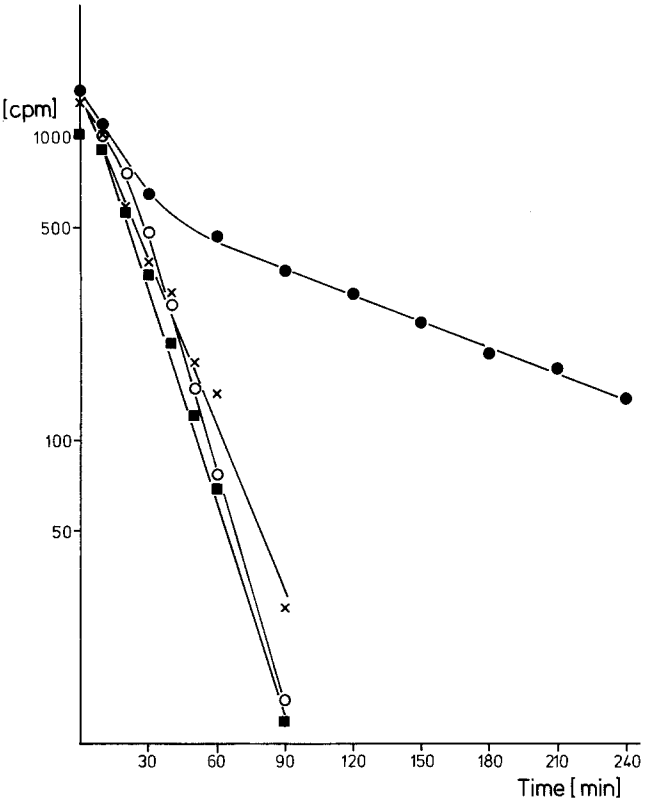

Fig. 4. Binding of glycine betaine to periplasmic proteins. Binding of [^{14}C]-glycine betaine at 10.8 μM to periplasmic proteins of strain GM37 (*Φ(proU-lacZ)*hyb2; ■) and strain MC4100 (*proU*$^+$; ○, ●) was measured. Cells were grown in glucose MMA (○) or in glucose MMA with 300 mM NaCl (■, ●). Binding was assayed in 10 mM Tris-HCl, pH 7.3. The same preparations of periplasmic protein as displayed in Fig. 3 were used. The symbol x indicates a control experiment without periplasmic proteins in the assay. The K_D of binding was calculated as described in the text

(70 μM) substrate concentration (Fig. 2D). This ProP-mediated transport could be osmotically stimulated two- to threefold (Fig. 2C, D) in cells grown in MMA with 300 mM NaCl. This result is in excellent agreement with the finding that the transcription of *proP-lacZ* operon fusions in *S. typhimurium* and *E. coli* (Cairney et al. 1985a; Gowrishankar 1986) is osmotically stimulated approximately three fold.

High osmolarity not only stimulates transcription of *proP* and the *proU* region; it also regulates the activity of the transport components. Even when grown in MMA with 300 mM NaCl, ProU- and ProP-mediated transport of glycine betaine was strongly reduced when the assay was performed in MMA without additional NaCl: from 0.34 nmol/10^8 cells per min to 0.04 nmol/10^8 cells per min in strain EF038 (*proP*$^-$ *proU*$^+$) and from 0.29 nmol/10^8 cells per min to 0.05 nmol/10^8 cells per min in strain EF046 (*proP*$^+$ *Δ(proU)1*) at a substrate concentration of 70 μM. Osmotic modulation of ProU- and ProP-mediated glycine betaine transport activity has also been demonstrated in *S. typhimurium* (Cairney et al. 1985a, b). Finally, it should be noted that the construction of *proP* and *proU* strains necessitated the introduction of the *Δ(putPA)101* mutation into strain MC4100 (Stalmach et al. 1983; Grothe et al. 1986). The presence of this mutation did not influence glycine betaine transport (Fig. 2).

Binding protein dependent transport of glycine betaine

When *E. coli* is grown in media of high osmolarity, synthesis of several proteins is increased (Clark and Parker 1984). Among the periplasmic proteins the most striking effect is the appearance of an abundant protein with an apparent molecular weight of 32,000 (Barron et al. 1986). Regulation of its synthesis parallels that of the *proU-lacZ* fusions; synthesis is stimulated by high osmolarity (Fig. 3, lanes 3 and 4) and can be reduced by the addition of 1 mM glycine betaine to MMA with 300 mM NaCl (Fig. 3, lane 2). This protein is absent in a strain with a (*proU-lacZ*)hyb2 fusion grown in MMA with 300 mM NaCl (Fig. 3, lane 1), suggesting that its synthesis is dependent on the *proU* region. To determine whether it might function as a binding protein for glycine betaine, we measured the binding of [^{14}C]-glycine betaine to periplasmic proteins using a dialysis technique (Argast and Boos 1979) based on the retention phenomenon of binding proteins (Silhavy et al. 1975). Figure 4 shows a rapid, linear exit of free glycine betaine from a dialysis bag that contained only buffer or shock fluids lacking the 32 kDa protein. When this protein was present in large amounts, there was an initial rapid efflux of excess glycine betaine followed by a slow dissociation of bound substrate. The first-order rate constant of decrease of substrate in the dialysis bag in the absence of binding protein (v_1) compared to the first-order rate constant of decrease in its presence (v_2) is a function of binding protein concentration [P] and its K_D: $v_1/v_2 = 1 + [P]/K_D$ (Silhavy et al. 1975). Total protein concentration in the shock fluid pre-

pared from strain MC4100 grown in MMA with 300 mM NaCl was 1.3 mg/ml, and the relative amount of glycine betaine binding protein was calculated (Materials and methods) to be approximately 20% of the total protein. Assuming one high-affinity binding site per molecular weight of 32,000, we calculated a K_D of 1.4 μM for glycine betaine binding.

Since the activity of the transport system was dependent on the osmolarity, we determined whether this osmotic regulation occurs at the level of substrate binding to the binding protein. The binding of [^{14}C]-glycine betaine to periplasmic proteins of strain MC4100 grown in MMA with 300 mM NaCl was assayed at low and high osmolarity using the method of Richarme and Kepes (1983). It was found to be 1.7 nmol betaine/mg protein at both low and high osmolarity with a free ligand concentration of 4 μM. Thus, binding was independent of osmolarity and, consequently, the osmotic modulation of glycine betaine transport activity must occur at the level of the cytoplasmic membrane components or energization. Glycine betaine binding to periplasmic proteins from cells of strain MC4100 grown in MMA was less than 35 pmol betaine/mg protein at a free ligand concentration of 5.8 μM.

Analysis of hybrid proteins synthesized in response to elevated osmolarity

Lac$^+$ gene fusions created by insertions of λp*lac*Mu15 can contain a variable 5′-segment from the target gene and, thus, can encode hybrid proteins of variable length (Bremer et al. 1984). To demonstrate the synthesis of hybrid proteins in response to elevated osmolarity, we analysed total cellular proteins from ten *proU-lacZ* gene fusion strains by SDS-PAGE. In eight of these strains, we observed increased synthesis of a hybrid protein after the addition of NaCl to the growth medium. These hybrid proteins had apparent molecular weights of 121,000 (four strains), 141,000 (two strains), and 150,000 (two strains); representative examples are shown in Fig. 5 (lanes 4, 6, and 8, respectively). The amount of hybrid protein synthesized was very similar in all eight strains, suggesting that these strains contain λp*lac*Mu15 insertions at different positions within the same gene. In the two remaining strains, no hybrid protein could be identified on polyacrylamide gels stained with Coomassie blue, although these fusions showed the same map position as the others and the strains were also deficient in high-affinity uptake of glycine betaine. Binding protein dependent transport systems in *E. coli* are usually encoded by a cluster of genes. Therefore, it seems possible that these two *lac* fusions within the *proU* region are to a different gene, which is expressed at a low level. Alternatively, these hybrid proteins could be rapidly digested proteolytically and, therefore, might not be readily detected.

Cloning of the proU regulatory region

We made use of the lambda prophage adjacent to the (*proU-lacZ*)hyb2 gene fusion in strain GM37 to isolate a Lac$^+$ specialized transducing phage (λpGM1). This phage was shown to carry the entire gene fusion and the *proU* regulatory region (Barron et al. 1986). DNA from λpGM1 was digested with *Eco*RI, and the restriction fragments were ligated into the unique *Eco*RI site in plasmid pMLB524 (Silhavy et al. 1984). After transformation into strain MC4100, plasmids conferring a LacZ$^+$ phenotype were

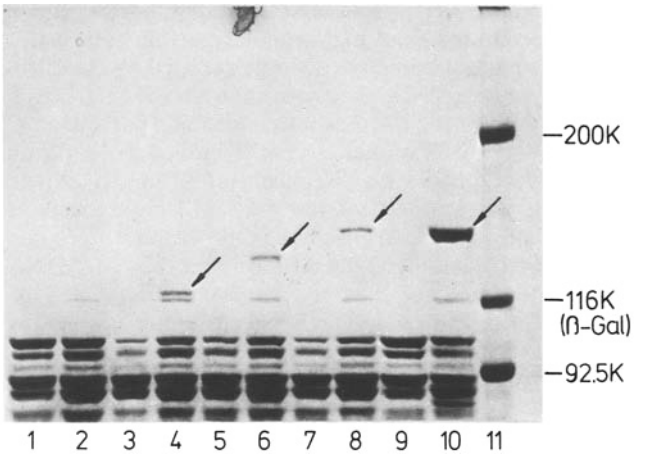

Fig. 5. Synthesis of hybrid proteins in response to high osmolarity. Total cellular proteins from strains MC4100 (*Δlac*; lanes 1 and 2), GM40 (*Φ(proU-lacZ)*hyb5; lanes 3 and 4), GM41 (*Φ(proU-lacZ)*hyb6; lanes 5 and 6) and GM37 (*Φ(proU-lacZ)*hyb2; lanes 7 and 8) were separated by SDS-PAGE (7% acrylamide) and stained with Coomassie blue. Lanes 9 and 10 show proteins from strain MC4100 containing plasmid pOS3, which carries the cloned (*proU-lacZ*)hyb2 gene fusion. Strains were grown in glucose MMA (lanes 1, 3, 5, 7, and 9) or in glucose MMA with 300 mM NaCl (lanes 2, 4, 6, 8, and 10). Hybrid proteins are indicated by *arrows* and a molecular weight standard is shown in lane 11. Only the upper part of the gel is shown

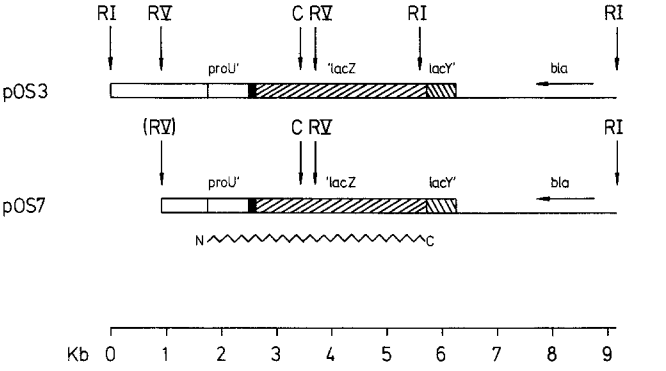

Fig. 6. Structure of plasmids carrying the (*proU-lacZ*)hyb2 gene fusion. The *open bar* represents chromosomal DNA, *hatched bars* represent the *'lacZ* and *lacY'* genes, and the *solid bar* represents the terminal 117 bp from the *S* end of phage Mu. Sequences from plasmid pBR322 are depicted by the *thin line* and the *horizontal arrow* shows the position of the *bla* gene encoding β-lactamase. The *zigzag line* represents the hybrid protein; its amino- and carboxy-terminal ends are indicated by N and C, respectively. The *vertical arrows* indicate restriction sites for *Eco*RI (RI), *Eco*RV (RV), and *Cla*I (C). Plasmid pMLB1034, which was used to construct plasmid pOS7, lacks the *Eco*RI site normally found in the *lacZ* gene. When the *Eco*RV-*Cla*I fragment from pOS3 was ligated into the *Sma*I-*Cla*I fragment from plasmid pMLB1034, the *Eco*RV site of the chromosomal DNA was destroyed; its position is therefore shown in *parentheses*

identified on selective medium containing XG. One of these plasmids (pOS3) was chosen for further analysis and shown to contain an inserted *Eco*RI fragment of 5.6 kb. This fragment carries part of the *lacZ* gene, 117 bp from the Mu*S* end, and 2.5 kb of chromosomal DNA (Fig. 6). To determine whether pOS3 contained the *proU* regulatory region, we analysed total cellular proteins from strain MC4100

(pOS3) by SDS-PAGE. A large amount of a hybrid protein was produced in media of high osmolarity (Fig. 5, lane 10); this protein had the same size as that encoded by the chromosomal (*proU-lacZ*)hyb2 fusion in strain GM37 (Fig. 5, lane 8). As with the chromosomal fusion (Barron et al. 1986), the level of expression of the cloned gene fusion was dependent on the NaCl concentration in the growth medium and was strongly reduced by 1 mM glycine betaine (data not shown). Thus, plasmid pOS3 seems to contain both the (*proU-lacZ*)hyb2 gene fusion and the *proU* DNA sequences required for osmotic regulation. However, expression of the (*proU-lacZ*)hyb2 gene fusion carried by pOS3 differed from that of the chromosomal fusion in that no further increase in the amount of hybrid protein was detected when the NaCl concentration exceeded 200 mM (data not shown).

To locate the *proU* regulatory region more precisely, we constructed a derivative of pOS3. A 2.5 kb *Eco*RV-*Cla*I restriction fragment from pOS3 was cloned into plasmid pMLB1034 (Silhavy et al. 1984) that had been cleaved with *Sma*I and *Cla*I. Expression of the (*proU-lacZ*)hyb2 gene fusion on the resulting plasmid (pOS7; Fig. 6) was also osmotically stimulated. From the size of the hybrid protein we estimate that 750 bp are required to encode its ProU portion. Therefore, the remaining 830 bp of chromosomal DNA in pOS7 must contain the *proU* regulatory sequences.

Discussion

We have shown that uptake of glycine betaine in *E. coli* occurs via two distinct transport systems. This situation is analogous to that in *S. typhimurium*, where the *proU* and *proP* regions specify high- and low-affinity glycine betaine transport systems, respectively (Cairney et al. 1985a, b). The osmotically responsive *lac* fusions we have isolated in the *proU* region abolish high-affinity glycine betaine transport. We also found an abundant periplasmic protein of 32 kDa the synthesis of which was stimulated by elevated osmolarity. Since this protein was absent in strains with λp*lac*Mu insertions in the *proU* region, and since shock fluids containing the protein bind glycine betaine avidly, we conclude that the high-affinity transport system encoded by the *proU* region is binding protein dependent.

The K_D of 1.4 μM for dissociation of glycine betaine from the periplasmic protein is comparable to the K_m of 1.3 μM determined for ProU-mediated transport in *S. typhimurium* (Cairney et al. 1985b). An earlier study in *E. coli* reported a K_m of 35 μM for glycine betaine transport (Perroud and Le Rudulier 1985). Since *E. coli* also contains the low-affinity ProP system, it is likely that the measurement of 35 μM represents the sum of both ProU and ProP activities. Unlike other transport systems in *E. coli*, which are inhibited at high osmotic strength (Roth et al. 1985a), glycine betaine uptake in both *E. coli* and *S. typhimurium* (Cairney et al. 1985a, b) is active only in high osmolarity medium. We have demonstrated here that interaction of glycine betaine with the binding protein is independent of medium osmolarity. Hence, transport through the inner membrane and/or energization of transport must be the osmoresponsive component of ProU activity.

The *proU* region has previously been mapped to min 57.5 on the *E. coli* and *S. typhimurium* linkage maps (Csonka 1982; Gowrishankar 1985). Our studies extend the fine structure mapping to several flanking markers. All of our osmoresponsive *lac* fusions map in this region, but they may not all be in the same gene. We would expect several structural genes to encode glycine betaine transport components because binding protein dependent transport systems typically comprise multiple protein components whose structural genes are organized in operons. Indeed, we have recently obtained evidence that the *proU* region contains at least three genes in an operon, whose expression is osmotically stimulated (E. Bremer, unpublished data). Although all of the *proU-lacZ* fusion strains we have analysed lack the glycine betaine binding protein, the λp*lac*Mu insertions may not be in its structural gene. The absence of this protein could be due to polar effects of λp*lac*Mu on expression of distal genes in an operon (Bremer et al. 1984) or to disruption of a regulatory gene.

We have studied regulation of expression of the *proU* region with both *lacZ* operon and gene fusions. Since both types of *lac* fusions display the same increase in gene expression with elevated osmolarity, we conclude that the *proU* region is regulated at the transcriptional level. Thus, both synthesis and activity of the glycine betaine transport system ProU is subject to osmotic control. The steady state expression of the *proU* region during growth at high osmolarity differs from the transient expression of the *kdp* operon following osmotic upshock (Laimins et al. 1981). The temporary decrease in cellular turgor pressure thought to control *kdp* is therefore not likely to be the signal that triggers sustained transcription of the *proU* region. Osmotic regulation of the *proU* region also differs from that of the porin genes *ompF* and *ompC* (Hall and Silhavy 1981) in that it is not influenced by *ompB*. The cloning of the (*proU-lacZ*)hyb2 fusion reported here provides an important tool to study further the osmotic control of expression of the *proU* region at the molecular level.

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References

Apontoweil P, Berends W (1975a) Isolation and initial characterization of glutathione-deficient mutations of *Escherichia coli* K-12. Biochim Biophys Acta 399:10–22

Apontoweil P, Berends W (1975b) Mapping of *gshA*, a gene for the biosynthesis of glutathione in *Escherichia coli* K-12. Mol Gen Genet 141:91–95

Argast M, Boos W (1979) Purification and properties of the *sn*-glycerol-3-phosphate-binding protein of *Escherichia coli*. J Biol Chem 254:10931–10935

Bachmann BJ (1983) Linkage map of *Escherichia coli* K-12, ed. 7. Microbiol Rev 47:180–230

Barron A, May G, Bremer E, Villarejo M (1986) Regulation of envelope composition during adaptation to osmotic stress in *Escherichia coli*. J Bacteriol 167:433–438

Berman ML, Beckwith J (1979) Fusions of the *lac* operon to the transfer RNA gene *tyrT* of *Escherichia coli*. J Mol Biol 130:285–301

Bremer E, Silhavy TJ, Weisemann JM, Weinstock GM (1984) λ*plac*Mu: a transposable derivative of bacteriophage lambda for creating *lacZ* protein fusions in a single step. J Bacteriol 158:1084–1093

Bremer E, Silhavy TJ, Weinstock GM (1985) Transposable λ*plac*Mu bacteriophages for creating *lacZ* operon fusions and kanamycin resistance insertions in *Escherichia coli*. J Bacteriol 162:1092–1099

Bremer E, Silhavy TJ, Maldener M, Cole ST (1986) Isolation and characterization of mutants deleted for the *sulA-ompA* region of the *Escherichia coli* K-12 chromosome. FEMS Microbiol Lett 33:173–178

Cairney J, Booth IR, Higgins CF (1985a) *Salmonella typhimurium proP* gene encodes a transport system for the osmoprotectant betaine. J Bacteriol 164:1218–1223

Cairney J, Booth IR, Higgins CF (1985b) Osmoregulation of gene expression in *Salmonella typhimurium: proU* encodes an osmotically induced betaine transport system. J Bacteriol 164:1224–1232

Casadaban MJ (1976) Transposition and fusion of the *lac* genes to selected promoters in *Escherichia coli* using bacteriophage lambda and Mu. J Mol Biol 104:541–555

Clark D, Parker J (1984) Proteins induced by high osmotic pressure in *Escherichia coli*. FEMS Microbiol Lett 25:81–83

Csonka LN (1982) A third L-proline permease in *Salmonella typhimurium* which functions in media of elevated osmotic strength. J Bacteriol 151:1433–1443

Dunlap VJ, Csonka LN (1985) Osmotic regulation of L-proline transport in *Salmonella typhimurium*. J Bacteriol 163:296–304

Epstein W (1985) The Kdp system: a bacterial K^+ transport ATPase. Curr Top Membr Transp 23:153–175

Garrett S, Taylor RK, Silhavy TJ (1983) Isolation and characterization of chain-terminating nonsense mutations in a porin regulator gene, *envZ*. J Bacteriol 156:62–69

Gowrishankar J (1985) Identification of osmoresponsive genes in *Escherichia coli*: Evidence for participation of potassium and proline transport systems in osmoregulation. J Bacteriol 164:434–445

Gowrishankar J (1986) *proP*-mediated proline transport also plays a role in *Escherichia coli* osmoregulation. J Bacteriol 166:331–333

Grothe S, Krogsrund RL, McClellan DJ, Milner JL, Wood JM (1986) Proline transport and osmotic stress response in *Escherichia coli* K-12. J Bacteriol 166:253–259

Hall MN, Silhavy TJ (1981) Genetic analysis of the major outer membrane proteins of *Escherichia coli*. Annu Rev Genet 15:91–142

Laemmli UK (1970) Cleavage of structural proteins during assembly of bacteriophage T4. Nature 227:680–685

Laimins LA, Rhoads DB, Epstein W (1981) Osmotic control of *kdp* operon expression in *Escherichia coli*. Proc Natl Acad Sci USA 78:464–468

Landfald B, Strøm AR (1986) Choline-glycine betaine pathway confers a high level of osmotic tolerance in *Escherichia coli*. J Bacteriol 165:849–855

Le Rudulier D, Bouillard L (1983) Glycine betaine, an osmotic effector in *Klebsiella pneumoniae* and other members of the *Enterobacteriaceae*. Appl Environ Microbiol 46:152–159

Le Rudulier D, Strøm AR, Dandekar AM, Smith LT, Valentine RC (1984) Molecular biology of osmoregulation. Science 224:1064–1068

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275

Magazin M, Howe M, Allet B (1977) Partial correlation of the genetic and physical maps of bacteriophage Mu. Virology 77:677–688

Maloy RM, Nunn WD (1981) Selection for loss of tetracycline resistance by *Escherichia coli*. J Bacteriol 145:1110–1112

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Menzel R, Roth J (1980) Identification and mapping of a second proline permease in *Salmonella typhimurium*. J Bacteriol 141:1064–1070

Metzer E, Levitz R, Halpern YS (1979) Isolation and properties of *Escherichia coli* K-12 mutants impaired in the utilization of γ-aminobutyrate. J Bacteriol 137:1111–1118

Miller JH (1972) Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Neu HC, Heppel LA (1965) The release of enzymes from *Escherichia coli* by osmotic shock and during the formation of spheroplasts. J Biol Chem 240:3685–3692

Perroud B, Le Rudulier D (1985) Glycine betaine transport in *Escherichia coli*: osmotic modulation. J Bacteriol 161:393–401

Ratzkin B, Grabnar M, Roth J (1978) Regulation of major proline permease gene of *Salmonella typhimurium*. J Bacteriol 133:737–743

Richarme G, Kepes A (1983) Study of binding protein-ligand interaction by ammonium sulfate-assisted adsorption on cellulose esters filters. Biochim Biophys Acta 742:16–24

Roth WG, Leckie MP, Dietzler DN (1985a) Osmotic stress drastically inhibits active transport of carbohydrates by *Escherichia coli*. Biochem Biophys Res Commun 126:434–441

Roth WG, Porter SE, Leckie MP, Porter BE, Dietzler DN (1985b) Restoration of cell volume and the reversal of carbohydrate transport and growth inhibition of osmotically upshocked *Escherichia coli*. Biochem Biophys Res Commun 126:442–449

Schweizer H, Boos W (1983) Cloning of the *ugp* region containing the structural genes for the *pho* regulon-dependent *sn*-glycerol-3-phosphate transport system of *Escherichia coli*. Mol Gen Genet 192:177–186

Silhavy TJ, Szmelcman S, Boos W, Schwartz M (1975) On the significance of the retention of ligand by protein. Proc Natl Acad Sci USA 72:2120–2124

Silhavy TJ, Berman ML, Enquist LW (1984) Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Stalmach ME, Grothe S, Wood JM (1983) Two proline porters in *Escherichia coli* K-12. J Bacteriol 156:481–486

Styrvold OB, Falkenberg P, Landfald B, Eshoo MW, Bjørnsen T, Strøm AR (1986) Selection, mapping and characterization of osmoregulatory mutants of *Escherichia coli* blocked in the choline-glycine betaine pathway. J Bacteriol 165:856–863

Yancey PH, Clark ME, Hand SC, Bowlus RD, Somero GN (1982) Living with water stress: evolution of osmolyte systems. Science 217:1214–1222

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