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Cloned structural genes for the osmotically regulated binding-protein-dependent glycine betaine transport system (ProU) of *Escherichia coli* K-12

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Summary

The *proU* locus of *Escherichia coli* encodes a high-affinity, binding-protein-dependent transport system (ProU) for the osmoprotectant glycine betaine. We cloned this locus into both low-copy-number lambda vectors and multicopy plasmids and demonstrated that these clones restore osmotically controlled synthesis of the periplasmic glycine betaine binding protein (GBBP) and the transport of glycine betaine in a Δ(*proU*) strain. These clones allowed us to investigate the influence of osmolarity on ProU transport activity independent of the osmotically controlled expression of *proU*. ProU activity was strongly stimulated by a moderate increase in osmolarity and was partially inhibited by high osmolarity. This activity profile differs from the profile of the osmotically regulated *proU* expression. The *proU* locus is organized in an operon and the position of the structural gene (*proV*) for GBBP is defined using a minicell system. We determined that at least three proteins (in addition to GBBP) are encoded by the *proU* locus. We also investigated the permeation of glycine betaine across the outer membrane. At low substrate concentration (0.7 μM), permeation of glycine betaine was entirely dependent on the OmpF and OmpC porins.

Introduction

Many prokaryotes subjected to the stress of high osmolarity in their environment respond by accumulating or synthesizing glycine betaine (Yancey *et al.*, 1982; Le Rudulier *et al.*, 1984). This substance is among the most important group of organic compounds known as 'compatible solutes'. Unlike inorganic ions, these solutes can be accumulated to high levels in the cytoplasm without disturbing the functioning of cellular proteins (Arakawa and Timasheff, 1985). They are, therefore, used by the cell as an osmotic balance to prevent dehydration of the cytoplasm when the level of external solutes is high (Somero, 1986).

In *Escherichia coli*, these osmoprotective effects can be achieved by either the synthesis or the uptake of glycine betaine. The osmotically stimulated synthesis of glycine betaine confers high levels of osmotic tolerance to cells grown in media of an otherwise inhibitory osmolarity (Styrvold *et al.*, 1986; Landfald and Strøm, 1986). Uptake of glycine betaine is also stimulated by high osmolarity and, likewise, results in osmoprotection (Perroud and Le Rudulier, 1985). The mechanisms of glycine betaine uptake have been intensively studied in *Salmonella typhimurium* and *E. coli*, and two distinct transport systems (ProP and ProU) have been identified (Cairney *et al.*, 1985a; 1985b; May *et al.*, 1986). Both systems were originally found to be minor permeases for L-proline, another important osmoprotectant (Anderson *et al.*, 1980; Menzel and Roth, 1980; Csonka, 1982; Stalmach *et al.*, 1983; Dunlap and Csonka, 1985; Grothe *et al.*, 1986). Subsequent studies with mutant strains deficient in either ProP or ProU established their role in glycine betaine uptake. ProP was found to have a low affinity for glycine betaine, whereas ProU has high affinity for this substrate (Cairney *et al.*, 1985a; 1985b; May *et al.*, 1986). Both systems appear to be controlled at two levels. Increased uptake of glycine betaine at high osmolarity results largely from *de novo* synthesis of the ProP and ProU transport components, since the transcription of *proP-lacZ* and *proU-lacZ* fusions is enhanced (approximately 3-fold and 100-fold, respectively) when the fusion strains are grown at high osmolarity or subjected to a sudden osmotic upshock (Dunlap and Csonka, 1985; Cairney *et al.*, 1985a; 1985b; Gowrishankar, 1985; 1986; Barron *et al.*, 1986; May *et al.*, 1986). In addition, the activity of the transport systems is osmotically regulated, since full transport activity was seen only when glycine betaine uptake was measured at high osmolarity (Cairney *et al.*, 1985a; 1985b; May *et al.*, 1986).

These two levels of osmotic control, and the osmotolerance conferred by ProU-mediated glycine betaine uptake, make ProU an interesting model for the study of bacterial adaptation to changes in environmental osmolarity. Certain features of the osmotically controlled expression of *proU* have already been elucidated: at low osmolarity *proU* is very weakly expressed; increases in the external osmolarity are closely reflected by increases in its expression,

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and these increases are sustained at elevated osmolarity. Glycine betaine acts as an osmoregulatory signal, since its addition to medium of high osmolarity substantially reduces the expression of *proU*.

Osmoregulation of *proU* differs in several important aspects from that of other osmoresponsive genes (Higgins *et al.*, 1987b). Unlike the expression of the *ompC* and *ompF* porin genes (Hall and Silhavy, 1979), *proU* expression is independent of *ompB* (Cairney *et al.*, 1985b; May *et al.*, 1986). The *kdp* operon encodes a potassium transport system that enables the cell to restore turgor after osmotic upshock (Epstein, 1985). Its expression, unlike that of *proU*, is transient and returns to basal level when turgor is restored (Laimins *et al.*, 1981). In addition, this high-affinity transport system is expressed specifically when potassium levels are low, whereas expression of *proU* is dependent on an increase in the intracellular concentration of potassium (Laimins *et al.*, 1981; Sutherland *et al.*, 1986). The pattern of *proU* expression argues that a decrease in turgor is not a signal that triggers ProU synthesis. It has been suggested that the internal concentration of potassium or the ionic strength within the cell determines the level of *proU* expression (Sutherland *et al.*, 1986). Recently, in *E. coli* and *S. typhimurium*, DNA supercoiling was found to be an important factor in *proU* regulation (Higgins *et al.*, 1988). However, the molecular basis for the osmoresponsive transcription of *proU* is still not fully understood. In both *E. coli* and *S. typhimurium*, it has been shown that ProU, which mediated the high-affinity glycine betaine transport, is binding-protein dependent (May *et al.*, 1986; Higgins *et al.*, 1987a). This type of transport system usually comprises a periplasmic substrate-binding protein and three to four polypeptides integrated in (or associated with) the cytoplasmic membrane, and their structural genes are often encoded by an operon (Ames, 1986; Hiles *et al.*, 1987). Thus it is likely that '*proU*' is not a single gene (May *et al.*, 1986; Higgins *et al.*, 1987a). In a recent study, two distinct regions required for ProU function were reported to be present at the *proU* locus in *E. coli* (Gowrishankar *et al.*, 1986), but the total number of polypeptide components required for ProU function, the organization of their structural genes, and the location of the DNA sequences governing their osmoresponsive expression remain unknown. We have cloned the *E. coli proU* locus and used these clones to investigate its genetic organization, its protein components, and its functional properties.

Results

Isolation of recombinant phage carrying the proU *region*

In cloning *the proU* region of *E. coli*, we took advantage of plasmid pOS7, which carries the Φ(*proU-lacZ*)hyb2 fusion. Cells harbouring pOS7 respond to high osmolarity of the growth medium by synthesizing a large amount of a 150 000-Dalton hybrid protein (May *et al.*, 1986) that cross-reacts with a polyclonal antiserum directed against purified GBBP (Barron *et al.*, 1987). Hence, plasmid pOS7 must carry a large segment of the structural gene for GBBP. Radiolabelled DNA from pOS7 was used to probe a library of recombinant phage containing chromosomal *Eco*RI restriction fragments ligated into the lambda replacement vector EMBL3 (Frischauf *et al.*, 1983). Of approximately 10 000 phage tested, eight phage reacted with the probe, and three were chosen for further study. Restriction analysis with *Eco*RI showed that all three recombinant phage contained a single 13.5-kb restriction fragment in addition to the 19.3-kb left arm and the 9.2-kb right arm of the parental EMBL3 phage. Subsequent hybridization with radiolabelled DNA of pOS7 confirmed that the cloned *Eco*RI fragment carries homology to our probe. This is documented for one of the phage (λpOS4, Fig. 1) analysed (Fig. 2A). These data demonstrate that the cloned chromosomal *Eco*RI fragment contains at least part of the structural gene for GBBP.

The cloned region restores GBBP synthesis and glycine betaine transport

To determine the extent of the genetic information encoded by the cloned *Eco*RI fragment, we wanted to test its ability to restore GBBP synthesis and glycine betaine uptake in a strain lacking the ProU and ProP transport systems. Lysogens of recombinant derivatives of the phage EMBL3 cannot be selected readily (Frischauf *et al.*, 1983), so we recloned the chromosomal fragment from λpOS4, using phage λSE6 as the vector. When lysogenized, phage λSE6 and its recombinant derivatives replicate as low-copy-number plasmids (Elledge and Walker, 1985), thus avoiding the potential complications that can arise when components of binding-protein-dependent transport systems are strongly overproduced. A 12.5-kb *Bam*HI fragment from λpOS4 (Fig. 1) was inserted into the *Bam*HI cloning sites of λSE6, and the resulting recombinant phage, λpOS5, was lysogenized into strain EF061 carrying the Δ(*proU*)600 and the *proP*1 mutations. In combination, these mutations render a strain entirely deficient in GBBP synthesis and glycine betaine uptake (May *et al.*, 1986). Restoration of ProU activity (i.e., GBBP synthesis and high-affinity glycine betaine transport) in strain EF061 lysogenic for λpOS5 indicates that these functions are encoded by the recombinant phage.

We first investigated whether λpOS5 would restore GBBP synthesis. Strains EF038 (*proP1, proU*$^+$), EF047 (*proP1,* Δ(*proU*) 600) and EF061 (λpOS5) were grown for 4 h in MMA without or with 0.3 M NaCl, and their periplasmic proteins were then isolated and electrophoretically

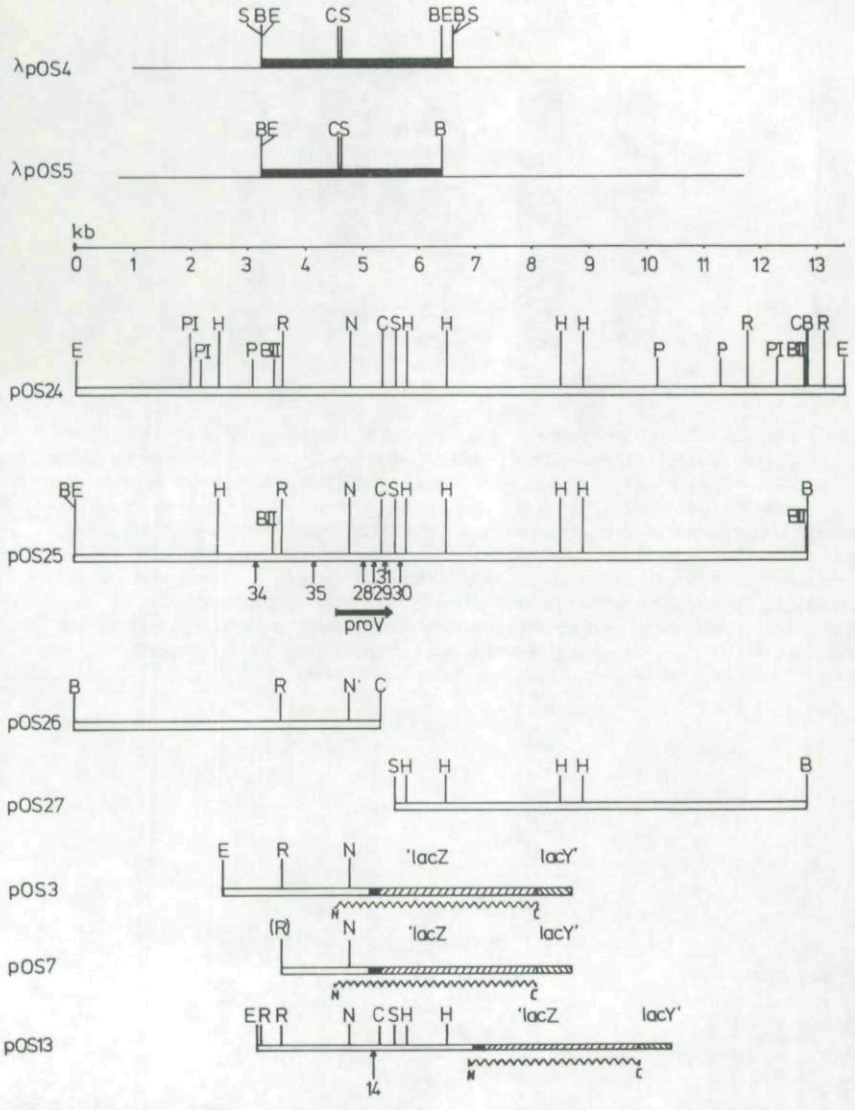

Fig. 1. Restriction maps of lambda-phage and plasmids carrying the *proU* region or *proU-lacZ* fusions. The restriction endonucleases are abbreviated as follows: *Bam*HI (B); *Bgl*II (BII); *Eco*RI (E); *Eco*RV (R); *Hpa*I (H); *Nde*I (N); *Pst*I (PI); *Pvu*II (P); *Sal*I (S). The lambda phages λpOS4 and λpOS5 are not drawn to scale; the cloned chromosomal material is represented by solid bars and only the relevant restriction sites are shown. A complete restriction map of the chromosomal material (open bars) cloned into pOS24 for the indicated restriction endonucleases is shown, but between position 0 and 3 kb there are additional restriction sites for *Eco*RV (3 sites) and *Pvu*II (2 sites) that were not localized precisely. In the restriction maps of pOS25, 26, 27, 3, 7 and 13, only the relevant restriction sites are indicated. The open bars represent chromosomal DNA, hatched bars represent the '*lacZ* and *lacY*' genes, solid bars represent the terminal 117 bp from the *S* end of phage Mu. The zigzag lines indicate the hybrid proteins; their amino- and carboxy-terminal ends are indicated by N and C, respectively. The positions of Tn*5* insertions in plasmids pOS25 and pOS13 are indicated by a vertical arrow subscribed with the number of the pOS25- or pOS13-derivative carrying that insertion. The location of *proV* and its direction of transcription is indicated by a horizontal bold arrow. Plasmids pOS3 and pOS7 have been described (May *et al.*, 1986); The *Eco*RV site in the chromosomal DNA of pOS7 was destroyed during the construction of this plasmid and its position is therefore shown in parentheses. Plasmids pOS3 and pOS13 carry some material from phage lambda to the left of the *Eco*RV site common to both plasmids; the extent of the material from phage lambda is not known.

separated on a 12% SDS-polyacrylamide gel (Fig. 3A). In response to high osmolarity, the *proU*$^+$ strain EF038 synthesized a 31-kD protein, which is the GBBP-binding protein (May *et al.*, 1986; Barron *et al.*, 1987). This protein was absent from the shock fluids of the Δ(*proU*)600 strain EF047. The strain lysogenic for the recombinant phage λpOS5 synthesized a large amount of GBBP in response to high osmolarity. It is evident from Fig. 3A that the strain carrying λpOS5 overproduces GBBP in comparison to the chromosomal *proU*$^+$ strain by approximately 3-fold (data not shown).

We then analysed the ability of λpOS5 to restore high-affinity glycine betaine transport. The three strains used above were grown in MMA with or without 0.3 M NaCl and their initial rates of glycine betaine uptake at a substrate concentration of 7 μM were measured (Fig. 4). In the chromosomally *proU*$^+$ strain, glycine betaine transport increased strongly in cells grown at high osmolarity, while in the control strain EF047 (*proP1*, Δ(*proU*)600) there was no transport. In the lysogen carrying λpOS5, glycine betaine uptake in cells grown at high osmolarity was restored and exceeded that of the *proU*$^+$ strain EF038 approximately 3-fold. This increased transport activity is in excellent agreement with the increased amount of GBBP present in this strain (Fig. 3A). Taken together, these data provide strong evidence that the chromosomal DNA carried by λpOS5 encodes all the genes necessary for ProU-mediated glycine betaine uptake.

ProU transport activity is osmotically modulated

Previous data suggesting that ProU transport activity is osmotically regulated are inconclusive because the cells were grown at high osmolarity and subjected to osmotic downshock (Cairney *et al.*, 1985b; May *et al.*, 1986). Such

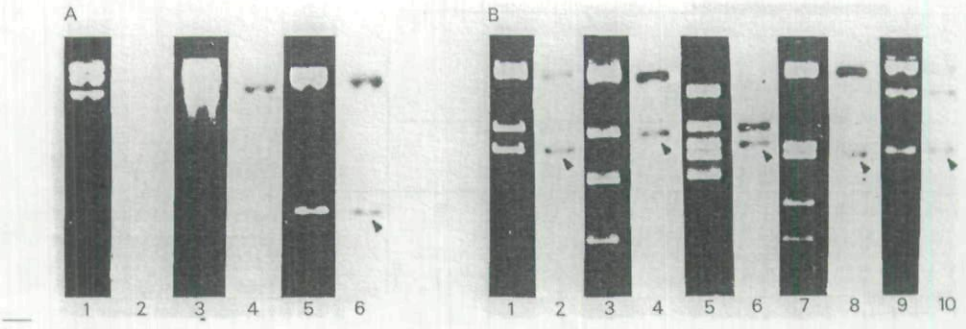

Fig. 2. Southern hybridization of the cloned *proU* region with plasmid pOS7. (A) DNA from the cloning vector EMBL3 (lanes 1 and 2), from its recombinant *proU*$^+$ derivative λpOS4 (Lanes 3 and 4), and from the *proU*$^+$ plasmid pOS24 (lanes 5 and 6) was cleaved with *Eco*RI. Restriction fragments were separated on a 0.7% agarose gel (the ethidium-bromide-stained gel is shown in lanes 1, 3 and 5), transferred onto a sheet of nitrocellulose, hybridized with radiolabelled pOS7 DNA, and autoradiographed (lanes 2, 4 and 6). Phage λpOS4 and plasmid pOS24 carry the same 13.5-kb *Eco*RI fragment; the second hybridizing fragment in lane 6 corresponds to the pUC18 vector part (2.3 kb) of plasmid pOS24 that has homology to the pBR322-based vector segment of plasmid pOS7 (Yanisch-Perron *et al.*, 1985; May *et al.*, 1986).
(B) DNA from plasmid pOS24 was digested with several restriction endonucleases and the resulting DNA fragments were electrophoretically separated and hybridized to pOS7 DNA as described above: lanes 1 and 2, *Bgl*II and *Eco*RI; lanes 3 and 4, *Eco*RV; lanes 5 and 6, *Hpa*I and *Eco*RI; lanes 7 and 8; *Pvu*II; lanes 9 and 10, *Nde*I. These digests liberated the pUC18 vector segment from the cloned chromosomal DNA in pOS24 either precisely (lanes 1 and 2; 5 and 6) or nearly precisely (lanes 3 and 4; 7 and 8; 9 and 10; see Fig. 1). The restriction fragment containing the pUC18 vector DNA is indicated by an arrow. The other hybridizing chromosomal DNA fragments carry a region homologous to the *proU* region on plasmid pOS7.

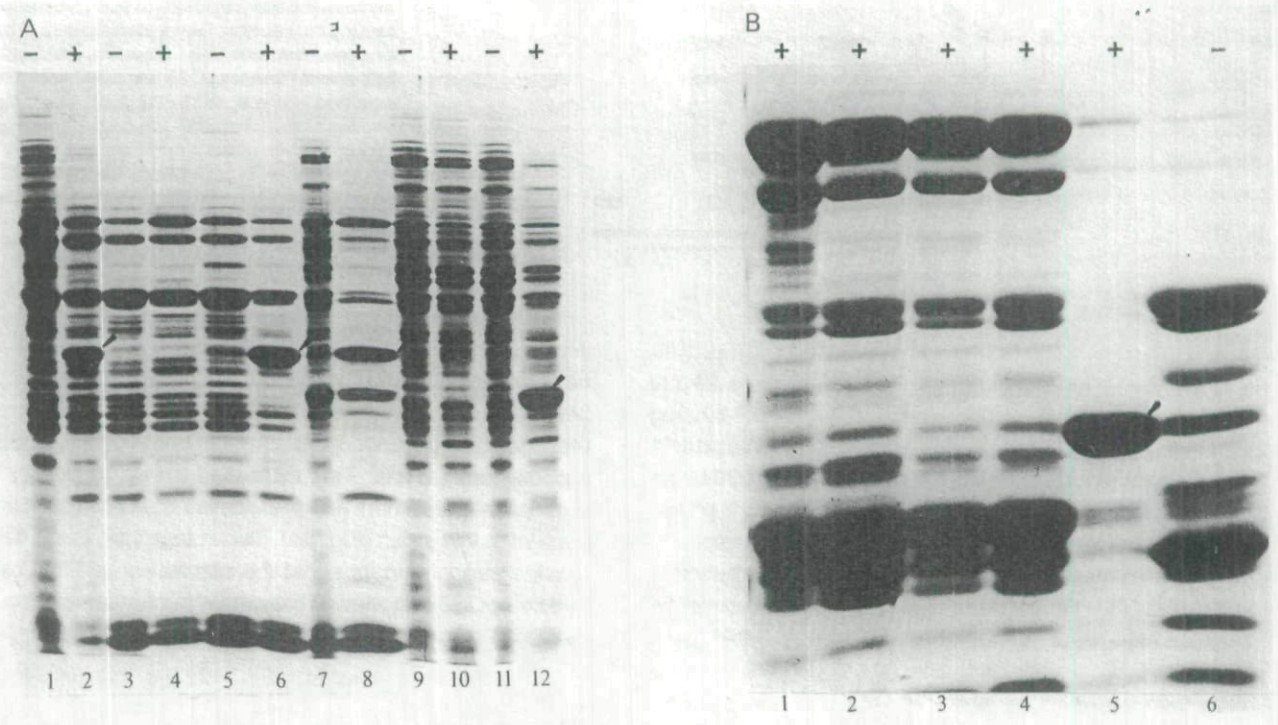

Fig. 3. Osmotically controlled synthesis of the glycine betaine binding protein. Shock fluids from the various strains were prepared from cultures grown in glucose MMA without (−) or with 0.3 M NaCl (+) and analysed by SDS-PAGE (12% acrylamide). The position of GBBP and that of the truncated GBBP is indicated by a small arrow.
(A) Strain EF038 (*proU*$^+$), lanes 1 and 2; EF061 (*proU*$^-$), lanes 3 and 4; EF061 (λpOS5; *proU*$^+$), lanes 5 and 6; strain EF047 (pOS25; *proU*$^+$), lanes 7 and 8; EF047 (pOS27; *proU*$^-$), lanes 9 and 10; EF047 (pOS26; *proU*$^-$), lanes 11 and 12.
(B) Strain EF047 carrying: pOS31 (*proV*::Tn*5*), lane 1; pOS30 (pOS25::Tn*5*), lane 2; pOS29 (*proV*::Tn*5*), lane 3; pOS28 (*proV*::Tn*5*), lane 4; pOS34 (pOS25::Tn*5*; *proU*$^+$), lanes 5 and 6.

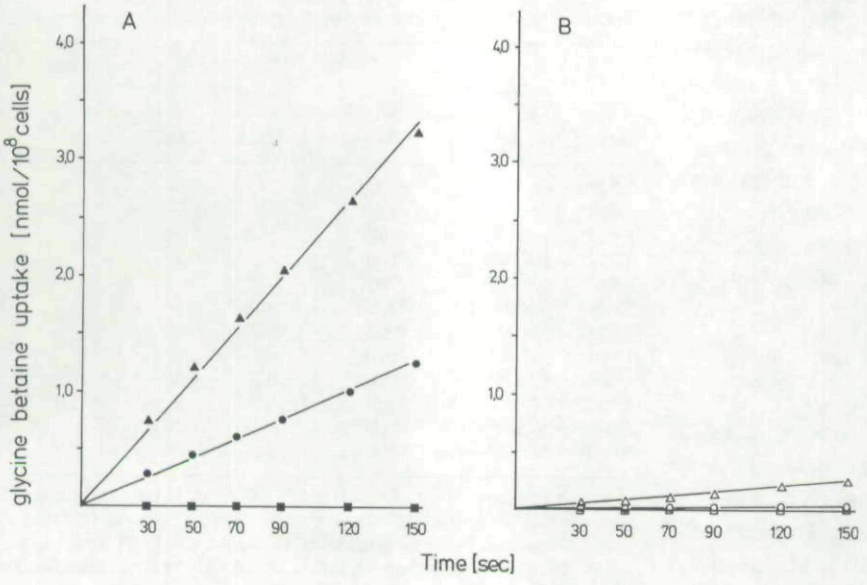

Fig. 4. Osmotically induced glycine betaine transport in cells lysogenic for λpOS5 (*proU*$^+$). Cells of EF038 (*proP*1, *proU*$^+$) (○, ●), EF061 (*proP*1, Δ(*proU*)600) (□, ■) and EF061 (λpOS5) (△, ▲) were pregrown overnight in glucose MMA at 28°C. Cultures (5 ml) were centrifuged and resuspended in the same volume of glucose MMA (B, open symbols) or glucose MMA containing 0.3 M NaCl (A, closed symbols) and then grown for 3 h at 28°C. To measure the initial rate of glycine betaine uptake, the cells were diluted 1:10 in MMA containing 0.3 M NaCl, incubated for 5 min at room temperature, and assayed at a final concentration of 7 μM glycine betaine.

osmotic downshocks have been shown to cause a nonspecific reduction in the uptake of several amino acids (Milner *et al.*, 1987). To determine the influence of high osmolarity on ProU activity more accurately, it was necessary to devise experimental conditions in which the osmotically controlled transcription of *proU* was uncoupled from the transport assay. The strain carrying the cloned *proU* locus overproduces the ProU system and expresses *proU* to some extent when grown at low osmolarity (Fig. 4B). The weak but reproducibly measurable glycine betaine uptake by this strain grown at low osmolarity allowed us to investigate the influence of high osmolarity on ProU activity.

Strain EF061 (λpOS5) was grown overnight in glucose MMA, and the initial rates of glycine betaine uptake were measured at various times after the exposure to 200 mM NaCl. These cells showed strong stimulation of glycine betaine uptake 30 sec after exposure to the high-osmolarity assay conditions, unlike the cells that were assayed in MMA alone (Fig. 5A). Transport activity increased further when the cells were incubated in MMA with 0.2 M NaCl for 3 min and 5 min prior to the assay (Fig. 5A) but could not be stimulated above this level by pre-incubation of the cells for 10 min (data not shown). The observed stimulation of transport activity in the presence of 200 mM NaCl was also found when 300 mM sucrose was used as the osmoticum (data not shown). ProU-mediated transport was found to have a distinct activity profile (Fig. 5B). Cells of strain EF061 (λpOS5) were grown overnight in glucose MMA. Cells were then exposed to increasing concentrations of NaCl, and after 5 min they were assayed for glycine betaine uptake. There was a

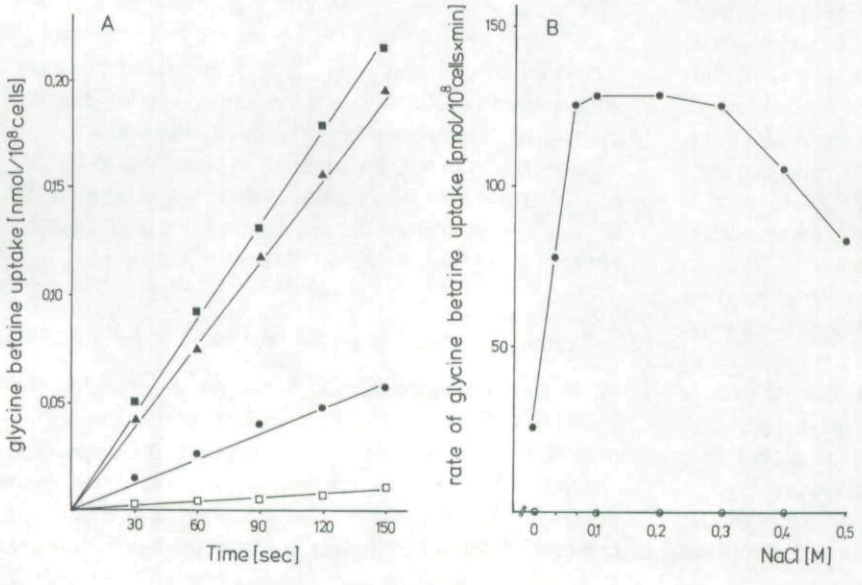

Fig. 5. Osmotic control of ProU activity. Transport of [^{14}C]-glycine betaine was measured in cells of strains EF061 (*proP*1, Δ(*proU*)600) and EF061 lysogenic for λpOS5 (*proU*$^+$) at a final substrate concentration of 7 μM. Cells were grown in glucose MMA overnight at 28°C, diluted 1:10 in MMA without a carbon source but supplemented with various concentrations of NaCl. (A) Cells of strain EF061 (λpOS5) were preincubated in MMA for 1 min (□) or in MMA containing 0.2 M NaCl for 0.5 min (●), 3 min (▲) and 5 min (■) and then immediately assayed for glycine betaine uptake using the same media as for preincubation. (B) Cells of strains EF061 (○) and EF061 (λpOS5) (●) were preincubated for 5 min in MMA containing the indicated NaCl concentrations and subsequently assayed for glycine betaine uptake at the same NaCl concentration used for preincubation.

strong stimulation in the uptake rates up to 100 mM NaCl, a broad maximum of ProU activity being between 100 mM and 300 mM NaCl, and a decline in glycine betaine transport at higher osmolarities. Throughout the experiment, the ProP$^-$ ProU$^-$ strain EF061 showed no glycine betaine uptake (Fig. 5B), demonstrating that the uptake we observed in strain EF061 (λpOS5) was mediated by ProU. These two sets of experiments provide strong evidence that osmolarity regulates the activity of the ProU system.

Location of the structural gene for GBBP within the cloned fragment

The coding capacity of the chromosomal *Eco*RI restriction fragment present in λpOS4 (approximately 13.5 kb) exceeds that usually required to encode a binding-protein-dependent transport system (Ames, 1986). To facilitate further physical analysis of this fragment, we subcloned it into the unique *Eco*RI site of plasmid pUC18 (Yanisch-Perron *et al.*, 1985) and established a restriction map of the resulting plasmid, pOS24 (Fig. 1) that expands the data reported by Gowrishankar *et al.* (1986). Because plasmid pOS7 carries most of the structural gene for GBBP, we used this plasmid as a hybridization probe to determine the position of this gene on plasmid pOS24. Restriction fragments of pOS24 were electrophoretically separated on 0.7% agarose gels, transferred onto nitrocellulose sheets, and hybridized with radiolabelled pOS7 DNA. Representative examples of this analysis are shown in Fig. 2B. The restriction fragments from the chromosomal DNA of pOS24 that were able to hybridize with the pOS7 probe have a common region located between the *Eco*RV site at position 3.6 kb and the *Hpa*I site at position 5.8 kb (Fig. 1). This 2.2-kb region must, therefore, contain the structural gene for GBBP. By comparing the restriction maps of pOS7 and pOS24 and correlating this information with our Southern hybridization data and with the known genetic structure of the Φ(*proU-lacZ*)hyb2 in pOS7 (May *et al.*, 1986), we were able to determine the coding region for GBBP fairly accurately and determine its direction of transcription (Fig. 1). This region coincides with the *proV* cistron identified by Gowrishankar *et al.* (1986); thus, our data show that *proV* is the structural gene for GBBP.

Subsequent analyses of two different plasmids (pOS26 and pOS27; Fig. 1) support this assignment. Plasmid pOS27 carries the chromosomal region distal to *proV* (Fig. 1); it does not complement the Δ(*proU*)600 mutation for glycine betaine transport (Table 1) or for synthesis of GBBP (Fig. 3A). Plasmid pOS26 carries almost exactly (with the exception of a 200-bp to 300-bp *Cla*I-*Sal*I fragment; Fig. 1) the chromosomal material absent from plasmid pOS27; it was also unable to restore glycine betaine uptake (Table 1); and no intact GBBP was detected in shock fluids (Fig. 3A). It did, however, direct the

Table 1. Osmotically induced transport of glycine betaine.

Strain	Plasmid or lysogenic phage carried	Phenotype	Rates of glycine betaine uptake (nmol Bet min^{-1} 10^{-8} cells)	
			MMA	MMA+0.3 M NaCl
EF038	–[a]	ProU$^+$	0.0	0.06
EF061	–[a]	ProU$^-$	0.0	0.0
EF061	λpOS5	ProU$^+$	0.04	0.36
EF047	pBR322[a]	ProU$^-$	0.0	0.0
EF047	pOS25	ProU$^+$	0.13	0.65
EF047	pOS26	ProU$^-$	N.D.	0.0
EF047	pOS27	ProU$^-$	N.D.	0.0
EF047	pOS28[b]	ProU$^-$	N.D.	0.0
EF047	pOS29[b]	ProU$^-$	N.D.	0.0
EF047	pOS30[c]	ProU$^-$	N.D.	0.0
EF047	pOS34[d]	ProU$^+$	0.17	1.20
EF047	pOS35[d]	ProU$^+$	0.24	1.50

Cells were pregrown overnight in LB at 28°C. Cultures (5 ml) were washed with MMA, split into two 2.5-ml portions, which were then recentrifuged and resuspended in 2.5 ml glucose MMA or glucose MMA containing 0.3 M NaCl. These cultures were grown at 28°C for 4 h. The cells were then diluted 1:25 in MMA containing 0.3 M NaCl, incubated for 5 min at room temperature, and then assayed for glycine betaine uptake at a final substrate concentration of 7 μM.

N.D.: not determined.

a. These strains carry no recombinant plasmid or phage and are chromosomal *proU*$^+$ (EF038) or *proU*$^-$ (EF061, EF047) controls.

b. These plasmids are derivatives of pOS25 and carry Tn*5* insertions in *proV*.

c. This plasmid is a derivative of pOS25 and carries a Tn*5* insertion downstream of *proV*.

d. These plasmids are derivatives of pOS25 and carry Tn*5* insertions upstream of *proV*.

osmotically inducible synthesis of a protein somewhat smaller than GBBP, which is likely to be a truncated GBBP. Other investigators have demonstrated that similar truncated proteins can be secreted into the periplasm (Ito and Beckwith, 1981; Koshland and Botstein, 1982; Hiles and Higgins, 1986). The truncated GBBP appears to be nonfunctional, since crude shock fluids containing this protein exhibited no glycine betaine binding activity (May *et al.*, 1986): this is in contrast to crude shock fluids from the *proU*$^+$ strain EF038 (data not shown). Thus the coding region for GBBP must extend into the *Cla*I-*Sal*I fragment absent from pOS26 (Fig. 1). We previously located the osmotically controlled promoter region within an approximately 800-bp to 900-bp segment of plasmid pOS7 (May *et al.*, 1986): from this we calculate that the osmotically controlled promoter for the *proV* gene is located between position 3.6 kb and 4.5 kb on plasmid pOS24 (Fig. 1).

Tn5 mutagenesis of the proU$^+$ *plasmid pOS25*

Although it was necessary to define more accurately the extent of the *proU* locus using Tn*5* mutagenesis, plasmid pOS24 was not suitable for such experiments because it yielded highly variable (and in some cases, no) glycine betaine uptake rates and strains containing this plasmid grew poorly (data not shown). We attribute this to the very

high copy number of pOS24 and the concomitant overproduction of the ProU system. We therefore constructed a *proU*$^+$ plasmid (pOS25, Fig. 1) using pBR322, since this vector has a lower copy number (Balbas *et al.*, 1986) than the plasmid pUC18 used to construct pOS24. When pOS25 was introduced into the Δ(*proU*)600 strain EF047, there was osmotically controlled synthesis of a very large amount of GBBP (Fig. 3B; lanes 5 and 6) and strong, osmotically regulated glycine betaine uptake (Table 1). We isolated a series of Tn*5* insertions into this *proU*$^+$ plasmid and correlated the position of these insertions (Fig. 1) with the cells' ability to transport glycine betaine (Table 1).

Out of eleven independently isolated insertions, nine were located in the 3.5 kb *Hpa*I restriction fragment of pOS25 (Fig. 1 and additional data not shown) and all these insertions either abolished or altered glycine betaine transport (Table 1 and additional data not shown). This is surprising, since we did not directly select for Tn*5* insertions affecting the ProU system or expose the cells to osmotic stress that might indirectly select for Tn*5* insertions in the *proU* locus. The other two isolated Tn*5* insertions were located in the pBR322 vector part of the 17.1-kb pOS25 plasmid. There was a clustering of Tn*5* insertions (6/9) in *proV* (Fig. 1) that totally abolished glycine betaine uptake (Table 1) and GBBP synthesis at high osmolarity (Fig. 3). Only a single Tn*5* insertion (pOS30) was found that mapped downstream from *proV* (Fig. 1); as expected, strain EF047 (pOS30) was entirely deficient in glycine betaine uptake (Table 1). Interestingly, we did not detect GBBP or any truncated form of this binding protein in shock fluids from strain EF047 (pOS30) (Fig. 3B). The remaining two Tn*5* insertions (plasmids pOS34 and pOS35) were located in different positions upstream of *proV* (Fig. 1). These plasmids restored the osmotically inducible GBBP synthesis and glycine betaine transport in strain EF047. Glycine betaine uptake in these strains was partially constitutive (Table 1). This could be explained by transcription directed by a Tn*5*-encoded promoter, Tn*5* insertion into a regulatory gene, or Tn*5*-induced topological changes in the *proU* regulatory region. The strong bias for Tn*5* insertions in *proV* prevented more extensive mapping of the *proU* locus by this approach.

Osmotically induced expression of proU-lacZ *fusions and the* proU *locus in minicells*

Plasmid pOS3 (Fig. 1) encodes the Φ(*proU-lacZ*)hyb2 protein fusion, and in whole cells, the osmotically controlled synthesis of a 150-kD hybrid protein can be detected easily (May *et al.*, 1986). As shown in Fig. 6A, a similar regulatory pattern was also detected in minicells when the radiolabelling of newly synthesized proteins was carried out in the absence or presence of 0.3 M NaCl (Fig. 6A). These results show that the minicell system can be used to analyse components of the ProU system by taking advantage of their strongly stimulated gene expression at high osmolarity.

Although the hybrid protein encoded by the Φ(*proU-lacZ*)hyb2 protein fusion can be visualized easily

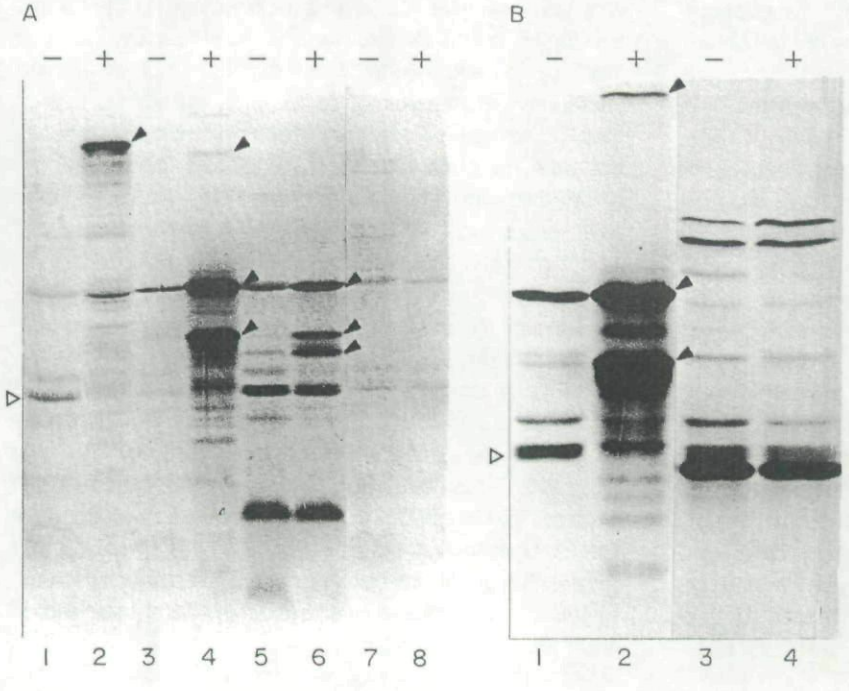

Fig. 6. Osmotically stimulated expression of *proU* in minicells. Radiolabelling of minicells prepared from strain DL410T carrying various plasmids, was carried out in glucose MMA wihout (−) or with 0.3 M NaCl (+). The proteins were separated by SDS-PAGE (15% acrylamide). The dried gel was autoradiographed. Proteins which show osmotically stimulated expression are indicated with small arrows; the position of β-lactamase is marked by open arrows.
(A) Lanes 1 and 2, proteins encoded by pOS3 (Φ(*proU-lacZ*)hyb2); lanes 3 and 4, proteins encoded by pOS13 (Φ(*proU-lacZ*)hyb11. The hybrid protein, as well as the 42-kD and 33-kD osmotically inducible proteins (lane 4) are indicated by arrows. Lanes 5 and 6 show proteins encoded by pOS24 (*proU*$^+$). The 42-kD, 33-kD and 31-kD (GBBP) inducible proteins (lane 6) are indicated by arrows. Proteins encoded by pUC18 are shown in lanes 7 and 8.
(B) Lanes 1 and 2 show proteins encoded by pOS13, and lanes 3 and 4 show proteins encoded by pOS14 (pOS13; *proV*::Tn*5*).

among total cellular proteins by means of SDS-PAGE, the gene product of the weakly expressed Φ(*proU-lacZ*)hyb11 protein fusion could not be detected. We have previously speculated that in this latter strain the *'lacZ* gene is fused to a gene encoding a membrane component of the ProU system (May *et al.*, 1986). We cloned this osmotically regulated Φ(*proU-lacZ*)hyb11 fusion into plasmid pMLB524. Restriction analysis of the resulting plasmid, pOS13, demonstrated that this fusion had occurred in a region downstream of *proV* (Fig. 1). When the proteins encoded by pOS13 were analysed in minicells, we detected the osmotically controlled synthesis of a 120-kD protein (Fig. 6A). Using a polyclonal antiserum directed against β-galactosidase, we showed that this polypeptide is the product of the Φ(*proU-lacZ*)hyb11 hybrid gene (data not shown). The amount of this hybrid protein synthesized at high osmolarity is very low compared with that found in minicells carrying pOS3 (Fig. 6A), reflecting the different β-galactosidase activities found in the corresponding fusion strains (G. May, personal communication). In addition to the hybrid protein, plasmid pOS13 encodes two other polypeptides whose synthesis is strongly stimulated at high osmolarity (Fig. 6A). We estimate their sizes to be 42 kD and 33 kD; two osmotically inducible proteins of identical size are present in minicells carrying the *proU*$^+$ plasmid pOS24 (Fig. 6A), strongly suggesting that these proteins are components of the ProU system. Plasmid pOS24 encodes a third protein whose synthesis follows the same regulatory pattern. Its molecular weight (approx. 31 kD) suggests that it might be the glycine betaine binding protein (May *et al.*, 1986; Higgins *et al.*, 1987a; Barron *et al.*, 1987). The absence of this protein in minicells carrying pOS13 (Fig. 6B) is consistent with the absence of GBBP in shock fluids of GM46 carrying the chromosomal Φ(*proU-lacZ*)hyb11 fusion (data not shown). Thus the ProU transport system is composed of at least four polypeptides: the glycine betaine binding protein (31 kD), two proteins (42 kD and 33 kD), and a fourth, very weakly expressed polypeptide, that is defined by the Φ(*proU-lacZ*)hyb11-encoded hybrid protein. We were unable to detect this fourth protein in minicells carrying the *proU*$^+$ plasmid pOS24.

The absence of GBBP in strain GM46 is puzzling since our analysis indicates that the fusion joint of the Φ(*proU-lacZ*)hyb11 fusion is distal to the 3'-end of *proV* (Fig. 1) and the DNA segment cloned from the specialized transducing phage carrying this fusion includes *proV* sequences (as shown by Southern hybridization; data not shown) as well as the region required for the osmotically controlled expression of the protein fusion. For reasons discussed later, we believe that strain GM46 has acquired a mutation which abolishes GBBP synthesis but which does not prevent the osmotically regulated expression of several other genes of the *proU* locus.

The proU *locus is genetically organized into an operon*

The osmotically modulated Lac phenotype of a strain carrying the cloned Φ(*proU-lacZ*)hyb11 fusion (pOS13) allowed us to identify Tn*5* insertions into pOS13 that altered the osmotically controlled expression of this fusion. Two groups of Tn*5* insertions were found. In the first group were strains that had a LacZ$^-$ phenotype on LB plates with XG, but restriction analysis showed that the transposons were inserted into the *'lacZ* gene (data not shown). In the second group were strains that, like the parent, showed a weak LacZ$^+$ phenotype on LB plates with XG, but, unlike cells carrying pOS13, were Lac$^-$ on lactose MacConkey plates supplemented with 0.2 M NaCl. One of these Tn*5* insertions (pOS14) was mapped and analysed in minicells for the encoded polypeptides. The Tn*5* insertion in pOS14 had occurred 1.7 kb upstream of the *'lacZ* gene in a region that we have identified as the GBBP structural gene (Fig. 1). Comparison of the polypeptide pattern of minicells carrying pOS13 or pOS14 revealed a striking difference (Fig. 6B): the three osmotically inducible proteins encoded by pOS13 were absent in minicells carrying pOS14. The simultaneous disappearance of these three polypeptides strongly suggests that their structural genes belong to a transcriptional unit.

Permeation of glycine betaine across the outer membrane

To interact with its periplasmic protein, glycine betaine must first cross the outer membrane. Many small hydrophilic molecules like glycine betaine penetrate this permeability barrier by diffusion through water-filled channels with little substrate specifity, such as the OmpC and OmpF porins (Nikaido and Vara, 1985). Synthesis of these porins is known to be osmotically regulated: at high osmolarity, expression of *ompC* is favoured and that of *ompF* is reduced. To test whether glycine betaine crosses the outer membrane through OmpC and OmpF we constructed strains lacking these porins (Fig. 7A). We induced *proU* expression by growing the cells at high osmolarity and then measured the uptake of glycine betaine at very low substrate concentrations (0.7 μM) so that its permeation through the outer membrane would be the rate-limiting factor (Nikaido and Vara, 1985). In strain EF038 (OmpC$^+$, OmpF$^+$), glycine betaine was taken up at a rate of 0.23 nmol 10^{-8} cells min^{-1}, whereas no transport was detected in the OmpC$^-$, OmpF$^-$ strain BRE2105 (*proU*$^+$) (Fig. 7B). Thus the *ompC ompF* double mutant strain behaved like strain EF047 (OmpC$^+$, OmpF$^+$), which lacks the ProU transport system (Fig. 7C). We conclude that permeation of glycine betaine across the outer membrane at this low substrate concentration is entirely dependent upon the OmpC and OmpF porins.

We assessed the independent contribution of these

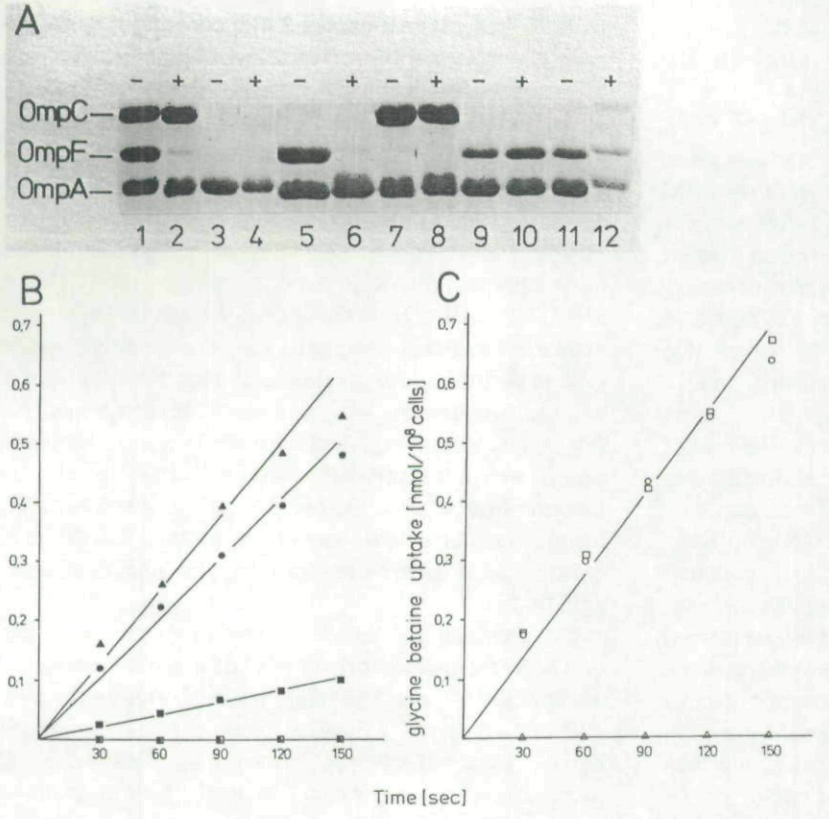

Fig. 7. Influence of the OmpC and OmpF proteins on glycine betaine uptake. Cultures of the various strains were grown at 37°C overnight in 0.4% glycerol MMA without or with 0.3 M NaCl. These cells were then used to isolate outer membrane proteins (A). To measure the initial rate of glycine betaine uptake (B, C), we used the cultures grown at high osmolarity. (A) The outer membrane proteins were electrophoretically separated on a 9% SDS-polyacrylamide gel containing 8 M urea; only the relevant part of the gel is shown. The lanes show the outer membrane profile of a given strain grown in MMA (−) or MMA containing 0.3 M NaCl (+). Lanes 1 and 2, EF038 (*ompC*$^+$, *ompF*$^+$, *ompR*$^+$); lanes 3 and 4, BRE2105 (*ompC*$^-$, *ompF*$^-$, *ompR*$^+$); lanes 5 and 6; BRE2103 (*ompC*$^-$, *ompF*$^+$, *ompR*$^+$); lanes 7 and 8, BRE2104 (*ompC*$^+$, *ompF*$^-$, *ompR*$^+$); lanes 9 and 10, BRE2106 (*ompC*$^+$, *ompF*$^+$, *ompR*20); lanes 11 and 12, BRE2107 (*ompC*$^-$, *ompF*$^+$, *ompR*20). (B and C) The overnight cultures were diluted with MMA containing 0.3 M NaCl to an optical density (OD_{578}) of approximately 0.04 and incubated for 5 min at room temperature. Glycine betaine uptake was measured at a final substrate concentration of 0.7 μM in MMA with 0.3 M NaCl. In (B), glycine betaine uptake of the *ompR*$^+$ strains EF038 (●), BRE2105 (◨), BRE2103 (■) and BRE2104 (▲) and in (C) that of the *ompR*20 mutant strains BRE2106 (○) and BRE2107 (□) are shown. As a control, the Δ(*proU*)600 strain EF047 (*ompC*$^+$, *ompF*$^+$, *ompR*$^+$) (△) is included in (C) but its outer membrane protein profile is not shown in (A).

porins by performing the same experiments with *ompC* (BRE2103) and *ompF* (BRE2104) single mutant strains (Fig. 7A) and found that glycine betaine can diffuse through both porins. In the *ompC*$^+$ strain, the rate of glycine betaine uptake slightly exceeded that of the wild-type strain EF038 (Fig. 7B). It is likely that this higher rate can be explained by compensatory overproduction of OmpC by the cells (Fig. 7A). In the strain producing only the OmpF porin, the rate of glycine betaine uptake was strongly reduced (to approximately 15% of the uptake rate of the wild type) (Fig. 7B). This low uptake rate is caused by the weak expression of the *ompF* gene at high osmolarity (Fig. 7A) and not by a greatly reduced ability of glycine betaine to diffuse through the OmpF pore. This was shown by comparing glycine betaine uptake in the *ompC*$^-$ *ompF*$^+$, *ompR*$^+$ strain BRE2103 with its derivative (strain BRE2107) carrying the *ompR*20 mutation, which allows OmpF synthesis at high osmolarity (Fig. 7A). The *ompR*20 mutant exhibited a much greater ability to transport glycine betaine (Fig. 7B and 7C). The *ompR*20 mutation also reduces OmpC synthesis at both high and low osmolarity (Nara *et al.*, 1984). By introducing this mutation into the *ompC*$^+$, *ompF*$^+$ strain EF038 (Fig. 7A), we were able to evaluate the efficiency of both porins for glycine betaine uptake. In the *ompR*20 mutant strain BRE2106, glycine betaine uptake exceeded that in the *ompR*$^+$ parent EF038 (Fig. 7B and 7C), although the amount of OmpF in strain BRE2106 was less than the combined amounts of OmpC and OmpF in the wild-type strain EF038 (Fig. 7A). This suggests that OmpF is a more efficient pore for glycine betaine than OmpC at low (0.7 μM) substrate concentration, which is consistent with the larger pore diameter demonstrated for OmpF (Nikaido and Vara, 1985).

Discussion

The *proU* locus encodes a high-affinity, binding-protein-dependent transport system for glycine betaine (May *et al.*, 1986; Higgins *et al.*, 1987a). ProU-mediated uptake of this osmoprotectant confers a high level of osmotic tolerance, and thus the ProU system enables the cell to adapt to osmotically varying habitats. We have cloned the *proU* locus into both low-copy-number vectors and multicopy plasmids and have demonstrated that these clones provide the known functions of an intact *proU* locus: they complement a Δ(*proU*) strain both for the osmotically controlled synthesis of GBBP and for the osmotically inducible transport of glycine betaine. Recently, in an independent study, Gowrishankar *et al.* (1986) reported the cloning of the *proU* locus from *E. coli* and demonstrated that multiple copies of this locus enhance osmotolerance in the presence of glycine betaine and L-proline. However, they did not establish the basis of this ProU-mediated osmoprotection since neither GBBP synthesis nor glycine

betaine transport were investigated. These workers established that two distinct regions (*proV* and *proU*) are necessary for osmoprotection.

Southern hybridization between our *proU*$^+$ clone and a plasmid known to carry part of the GBBP structural gene, together with a comparison of restriction data revealed that *proV* is the structural gene for GBBP. Furthermore, our data provide good evidence that the region located downstream from *proV*, and called *proU* by Gowrishankar *et al.* (1986), is not a single gene but rather encodes at least three polypeptides and is cotranscribed with *proV* in an operon. This genetic organization is similar to that of other binding-protein-dependent transport systems (Ames, 1986; Hiles *et al.*, 1987). By analogy, we predict that genes located downstream from *proV* encode the inner membrane components of the ProU transport system. In *S. typhimurium*, the *proU* locus has also been shown to be an operon (C.F. Higgins, personal communication). The existence of several incompletely characterized genes in the *proU* region raised the question of their nomenclature. We feel that for historic reasons the established term '*proU*' should be reserved for the locus encoding the entire binding-protein-dependent transport system for glycine betaine (ProU) and that individual genes within the locus should be designated *proV, proW,* etc. As mentioned above, we define *proV* as the structural gene for GBBP; further assignment of gene symbols awaits determination of the gene order within the *proU* locus.

The minicell system has been widely used to demonstrate the synthesis of proteins encoded by cloned genes (Reeve, 1979). By using osmotically regulated *proU-lacZ* fusions and *proU*$^+$ clones, we have shown that this system reflects osmotically mediated changes in *proU* gene expression. The specificity of this system was assessed with the cloned Φ(*proU-lacZ*)hyb2 fusion (pOS3). In whole cells, plasmid pOS3 directs the osmotically controlled synthesis of a large GBBP-β-galactosidase hybrid protein (May *et al.*, 1986) and the same pattern of gene expression was found in minicells when newly synthesized proteins were radiolabelled at low- and at high osmolarity. When we analysed the expression of our *proU*$^+$ clone (plasmid pOS24) in minicells, we detected the osmotically stimulated synthesis of three polypeptides with estimated molecular weights of 42 kD, 33 kD and 31 kD. This relatively large plasmid (16.8 kb) directs the synthesis of several other polypeptides whose synthesis is not osmotically stimulated; thus, these proteins are apparently unrelated to the ProU system. The 31-kD protein we observed in minicells is probably GBBP, which is known to have a molecular weight of 31 kD (32 kD) (May *et al.*, 1986; Higgins *et al.*, 1987a; Barron *et al.*, 1987). The relationship of the 42-kD and 33-kD proteins to the ProU system is evident not only from their osmotically stimulated synthesis but also from their presence in minicells carrying pOS13. This plasmid carries 3.4 kb contiguous to the osmotically regulated Φ(*proU-lacZ*)hyb11 protein fusion, and the osmotically stimulated synthesis of both these proteins, as well as the hybrid protein, is abolished by a Tn*5* insertion 1.7 kb upstream of the fusion joint. We speculate that these *proU*-encoded polypeptides are inner membrane components of the ProU transport system. Because binding-protein-dependent transport systems usually contain at least three inner membrane components (Ames, 1986; Hiles *et al.*, 1987), it is likely that the Φ(*proU-lacZ*)hyb11 fusion defines a fourth gene in the *proU* locus. The amount of hybrid protein synthesized was very low and the 120-kD polypeptide could be detected only when radiolabelled in minicells (Fig. 6A) or when western blots of whole cell extracts were probed with an antiserum directed against β-galactosidase (unpublished results). Correspondingly low expression of the unfused gene would account for our failure to detect its gene product in minicells carrying the *proU*$^+$ plasmid, pOS24.

Our proposal that the *proU* locus is an operon is supported by the polar effects of the Tn*5* insertion mentioned above. Furthermore, Tn*5* insertions in *proV* carried by plasmid pOS25 (*proU*$^+$) have a strong polar effect on the osmotically stimulated synthesis of the 42-kD and 33-kD proteins (unpublished results). In addition, we tested plasmid pOS27, which contains *proU* material downstream of *proV*, in minicells and found a low level of synthesis of these proteins. However, this synthesis was not influenced by osmotic changes (unpublished results). Taken together, these findings indicate that the major, osmotically controlled promoter of the *proU* locus lies within the 800–900-bp DNA segment preceding *proV*. Gowrishankar *et al.* (1986) argued against the existence of a *proU* operon in *E. coli* and found that when *proV* and the downstream '*proU*' sequences were carried on separate and compatible plasmids (the latter being similar to our plasmid pOS27), ProU function (osmoprotection) was restored in *proU* cells. We believe that the complementation they observed is due to expression from a weak promoter within the *proU* operon or from plasmid-encoded transcription elements. The low-level, non-osmotically controlled synthesis of the 42-kD and 33-kD proteins in minicells carrying pOS27 is consistent with either explanation.

There is evidence that the synthesis of GBBP in the absence of the other components of the ProU transport system is harmful to the cell (Gowrishankar *et al.*, 1986). Certain observations made in the course of our work may be relevant to this phenomenon. Strain GM46, which carries the Φ(*proU-lacZ*)hyb11 protein fusion, was isolated under conditions that favoured high-level synthesis of GBBP and was subsequently chosen by screening for osmotically controlled β-galactosidase activity (May *et al.*, 1986). We found that this fusion was located downstream from *proV*, most likely within the coding region for an inner

membrane component of the ProU transport system, and yet the strain produced no GBBP. It may be that in the absence of the inner membrane protein, cell survival requires a mutation that prevents GBBP synthesis. Consistent with this suggestion, we found that P1 transduction of this fusion (in contrast to other *proU-lacZ* protein fusions) into other strains was very difficult (unpublished results).

A similar situation occurred when we isolated Tn*5* insertions in the *proU* locus in plasmid pOS25. There was a strong preference for insertions in *proV*. Only one insertion downstream from *proV* was obtained, and the strain carrying this insertion did not produce GBBP. Despite repeated attempts we were unable to construct a multicopy plasmid carrying an intact, functional *proV* gene but lacking the genes for the ProU membrane components; the converse construct was readily obtained. Gowrishankar *et al.* (1986) isolated a plasmid containing the *proV* region but did not establish that it directs the osmotically controlled synthesis of GBBP. The *proU* locus when carried on a multicopy plasmid is only weakly expressed at low osmolarity. However, cell growth was poor on minimal medium, and derivatives with no or greatly reduced transport occurred frequently. Tn*5* insertions in a large plasmid occurred most frequently in the *proV* gene, although no direct pressure for ProU$^-$ colonies was applied. Thus the presence of the cloned *proU* locus appears to be detrimental to the cell.

To probe the outer membrane for components that might influence glycine betaine uptake, we used a set of strains lacking either OmpC or OmpF or both. We found that the permeation of glycine betaine across this membrane was entirely dependent on the OmpC and OmpF porins at very low substrate concentration. This strongly suggests that *E. coli* contains no outer membrane protein (e.g., a substrate-specific pore or receptor protein) connected directly with the ProU system. The binding-protein-dependent transport system for maltose and maltodextrins uses a substrate-specific pore (LamB) that is particularly important when substrate concentration is very low (Nikaido and Vara, 1985; Schwartz, 1987), for example, like the glycine betaine concentration we used. Glycine betaine diffused through both the OmpC and the OmpF pores. The OmpC channel is of greater physiological importance at high osmolarity simply because much more OmpC protein is present in the outer membrane than OmpF.

Previous reports that the transport activity of the ProU system is osmotically regulated were based on experiments involving osmotic downshock (May *et al.*, 1986; Cairney *et al.*, 1985b). Such downshocks have since been shown to cause nonspecific reduction in the uptake of several amino acids (Milner *et al.*, 1987) necessitating a reinvestigation of the osmotic modulation of ProU activity. For this work we used cells carrying λpOS5, which accumulate betaine in measurable quantities at low osmolarity. Consequently, this eliminated the need to downshock cells. We found that sudden exposure of cells grown at low osmolarity to increased osmotic pressure (by addition of either NaCl or sucrose) resulted in stimulation of ProU-mediated glycine betaine transport within seconds. Such osmotic activation is not typical of transport systems; in contrast, many carbohydrate uptake systems are severely inhibited by osmotic upshock (Roth *et al.*, 1985). It is known that high osmolarity does not increase the affinity of GBBP for its substrate (May *et al.*, 1986; Higgins *et al.*, 1987a). We speculate that the osmotic activation of the ProU system is mediated by its inner membrane components: it is conceivable that plasmolysis caused by osmotic upshock could produce conformational changes that would permit the optimal functioning of these components. The osmotically modulated profiles of ProU activity and of *proU-lacZ* expression are distinct. First, very small increases in osmolarity (up to 0.1 M NaCl) result in a dramatic increase in glycine betaine uptake but have little effect on the expression of *proU-lacZ* fusions. This suggests that the cell can utilize existing ProU components very efficiently and sensitively to adjust to even small increases in external osmolarity. Second, inhibition of ProU activity by high osmolarity begins before expression of its structural genes has reached its maximum. Consequently, at high osmolarity, total glycine betaine uptake through the ProU system reflects both stimulation of the *proU* expression and a transport activity that is beginning to decline.

Experimental procedures

Bacterial strains, bacteriophage and plasmids

The bacterial strains, phage and plasmids used in this study are listed in Table 2. All strains were derivatives of *E. coli* K-12.

Growth conditions, media and chemicals

Bacteria were grown aerobically at 37°C (unless otherwise indicated) in LB medium or minimal medium A (MMA) supplemented with 0.2% glucose or 0.4% glycerol (Miller, 1972, Silhavy *et al.*, 1984). The osmolarity of liquid MMA was elevated by addition of 0.3 M NaCl. Lactose MacConkey medium was prepared as described (Silhavy *et al.*, 1984), and with elevated osmolarity achieved by adding 0.2 M NaCl. 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), tetracycline (Tc) and ampicillin (Ap) were added to media at 30 μg ml^{-1}, 5 μg ml^{-1} and 50 μg ml^{-1}, respectively.

Genetic procedures and construction of bacterial strains

Standard techniques were used for the growth of bacteriophage and for generalized transduction with phage P1 (Miller, 1972; Silhavy *et al.*, 1984). The Φ(*ompC-lacZ*$^+$)10–25 and the Φ(*ompF-*

Table 2. Bacteria, bacteriophage and plasmids.

Strain	Description[a]	Reference/origin
Strains derived from *E. coli* K-12		
MC4100	*F*⁻ Δ(*argF-lac*) *U*169 *araD*139 *rpsL*150 *deoC*1 *relA*1 *ptsF*25 *flbB*5501 *rbsR*	Casadaban (1976)
TST3	MC4100 *malT*::Tn *10*	T. J. Silhavy
RM41	Q358, F⁻ r⁻ m⁺ *supE*Φ80ʳ	R. Maurer; through P. Gött
RM42	RM41, P2 lysogen	R. Maurer; through P. Gött
MH225	MC4100 *malQ*7 Φ(*ompC-lacZ*⁺) 10-25 (λp1(209))	Hall and Silhavy (1979)
MH513	MC4100 *ara*⁺ Φ(*ompF-lacZ*⁺) 16-23 (λp1(209))	Hall and Silhavy (1981)
FN101	*F*⁻ *purE pheA trp lac*85 *gal*2 *mtl xyl*2 *ara rpsL* (λ) *ompR*20 *malA*⁺	Nara *et al.* (1984)
DST410T	*minB ara lacY malA mtl xyl rpsL thi fhuA azi* Δ(*glpT-glpA*)593	Larson *et al.* (1982)
EF038	MC4100 Δ(*putPA*)101 *proP*1	May *et al.* (1986)
EF047[b]	MC4100 Δ(*putPA*)101 *proP*1 Δ(*proU*)600	May *et al.* (1986)
EF061	EF047 (λgt4-*lac*5)	This study
BRE2103	EF038 Φ(*ompC-lacZ*⁺)10-25 (λp1(209))	This study
BRE2104	EF038 Φ(*ompF-lacZ*⁺)16-23 (λp1(209))	This study
BRE2105	BRE2104 *ompC*	This study
BRE2106	EF038 *ompR*20	This study
BRE2107	BRE2103 *ompR*20	This study
Bacteriophage		
EMBL3	replacement vector	Frischauf *et al.* (1983)
λSE6	replacement vector	Elledge and Walker (1985)
λpOS4	*proU*⁺ derivative of EMBL3	This study
λpOS5	*proU*⁺ derivative of λSE6	This study
λpGM14	*Lac*⁺ specialized transducing phage carrying the Φ(*proU-lacZ*)hyb11 fusion	G. May
λ::Tn*5*	λ*b*221 *rex*::Tn*5* c*I*857 *O*am29 *P*am80	De Bruijn and Lupski (1984)
Plasmids		
pBR322	cloning vector	Bolivar *et al.* (1977)
pUC18	cloning vector	Yanisch-Perron *et al.* (1985)
pMLB524	cloning vector for *lacZ* fusions	Silhavy *et al.* (1984)
pOS3	Φ(*proU-lacZ*)hyb2 in pMLB524	May *et al.* (1986)
pOS7	Φ(*proU-lacZ*)hyb2 in pMLB1034	May *et al.* (1986)
pOS13	Φ(*proU-lacZ*)hyb11 in pMLB524	This study
pOS14	pOS13::Tn*5*	This study
pOS24	*proU*⁺ derivative of pUC18 carrying a 13.5-kb *Eco*RI restriction fragment	This study
pOS25	*proU*⁺ derivative of pBR322 carrying a 12.8-kb *Bam*HI restriction fragment	This study
pOS26	pOS25 deleted for a 7.9-kb *Cla*I restriction fragment	This study
pOS27	pOS25 deleted for a 5.5-kb *Sal*I restriction fragment	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 symbols *lacZ*⁺ and *lacZ* denote *lacZ* genes with or without translational initiation signals, respectively. The genetic nomenclature is according to Bachmann (1983).

b The Δ(*proU*) mutation present in this strain has previously been called Δ(*proU*)1 (May *et al.*, 1986).

lacZ⁺)16–23 fusions were transduced with phage P1 into strain EF038 using P1 lysates prepared on strains MH225 and MH13, respectively (Table 2). Transductants were selected on lactose-minimal plates. Several Lac⁺ colonies from each transduction were tested for loss of the OmpC and OmpF proteins by cross-streaking against the OmpC-specific phage hy2 and TuIb and the OmpF-specific phage K20 and Tula, respectively (Hancock and Reeves, 1975; Bassford *et al.*, 1977; Datta *et al.*, 1977). An *ompC-ompF* double mutant was isolated from strain BRE2105 (*ompC*⁺, Φ(*ompF-lacZ*⁺)16–26) by selecting for resistance against a mixture of the OmpC-specific phage hy2 and TuIb. The resulting strain (BRE2105) was re-tested for absence of the OmpF protein by cross-streaking against phage K20 and Tula. The *ompR*20 mutation present in strain FN101 was transduced with phage P1 into strains EF038 and BRE2103 by taking advantage of the linkage between the *malT* and *ompR* genes. First, *malT*::Tn*10* derivatives of EF038 and BRE2103 were isolated using a P1-lysate prepared on strain TST3 (MC4100 *malT*::Tn*10*). These strains were then transduced to Mal⁺ using a P1-lysate grown on strain FN101. Inheritance of the *ompR*20 mutation was determined by cross-streaking the Mal⁺ transductants against the OmpF-specific phage Tula on glycerol-minimal plates supplemented with 20% sucrose. On these plates, *ompR*⁺ strains are resistant against Tula, whereas *ompR*20 strains are sensitive. To lysogenize phage λgt4-*lac*5 into strain EF047, serial dilutions of the phage lysate were spotted onto a lawn of strain EF047; after overnight growth at 32°C, cells from the infected areas were streaked onto lactose MacConkey plates and lysogens of λgt4-*lac*5 were identified by their Lac⁺ phenotype (EF061).

Construction of a gene bank in phage EMBL3 and detection of proU⁺ *recombinant phage*

Phage EMBL3 (Frischauf *et al.*, 1983) is a replacement cloning vector in which a stuffer fragment flanked by *Sal*I, *Bam*HI and *Eco*RI restriction sites can be replaced by 9-kb to 22-kb segments of heterologous DNA. Recombinant phage can be selected by their Spi⁻ phenotype. Vector DNA was digested with *Eco*RI and extracted with phenol, and the DNA fragments were precipitated with ethanol. Chromosomal DNA from strain MC4100 was prepared as described by Silhavy *et al.* (1984) and cut to completion with *Eco*RI. The resulting restriction fragments were separated on a 0.7% agarose gel, and fragments with a size between 9 kb and 22 kb were eluted from the agarose gel and ligated into the *Eco*RI-digested vector DNA. The ligated DNA was packaged *in vitro* using packaging extracts purchased from Boehringer (Mannheim), and recombinant Spi⁻ phage were selected by plating on strain RM42, a P2-lysogen (Table 2). The library we obtained (approximately 8×10^4 independent clones) was amplified once by preparation of a plate lysate on strain RM42.

Recombinant bacteriophage carrying the *proU* region were detected by plaque hybridization with radiolabelled pOS7 DNA, essentially as described by Maniatis *et al.* (1982). Phage from positive plaques were purified by plating onto the Δ(*proU*)600 strain EF047, and individual plaques were then rehybridized to the pOS7 probe. High-titre plate lysates were prepared from three independent phage showing a positive signal, and their DNA was analysed with restriction enzymes.

A 12.8-kb *Bam*HI restriction fragment was isolated from one of these phage (λpOS4) and ligated into the *Bam*HI cloning sites of phage λSE6 (Elledge and Walker, 1985); recombinant Spi⁻

phage were selected after *in vitro* packaging of the ligation mixture, as described above. One such phage (λpOS5) was purified and lysogenized into strain EF061 by selecting for kanamycin-resistant colonies. DNA from λpOS5 was used to clone the 12.8-kb *Bam*HI fragment into plasmid pBR322. The resulting plasmid, pOS25, was then used for further physical and genetic analysis of the *proU* locus.

*Cloning of the Φ(*proU-lacZ*)hyb11 fusion*

λpGM14 is a Lac^+-specialized transducing phage carrying the osmotically inducible Φ(*proU-lacZ*)hyb11 protein fusion (May *et al.* 1986; G. May personal communication). DNA from this phage was used to clone the Φ(*proU-lacZ*)hyb11 fusion into plasmid pMLB524 (Silhavy *et al.*, 1984), as described previously (May *et al.*, 1986). The resulting plasmid (pOS13) was characterized by restriction analysis and the osmotically controlled expression of the *proU-lacZ* fusion was verified by assaying β-galactosidase activity in cultures grown at low or high osmolarity.

Isolation of Tn5 insertions in plasmids pOS25 and pOS13

Tn*5* mutagenesis of plasmid pOS25 was carried out essentially as described by De Bruijn and Lupski (1984) using λ::Tn*5* as the donor of the transposon. Insertions of Tn*5* into plasmid pOS25 were selected on LB plates supplemented with ampicillin (50 μg ml^{-1}) and kanamycin (300 μg ml^{-1}). Colonies appearing after 36 h of incubation at 32°C were pooled; their plasmid DNA was extracted and used to transform strain EF047, and Ap^r and Km^r transformants were selected as described above. Individual transformants were purified on the same medium, their plasmid DNA was prepared, and the position of the Tn*5* insertion in pOS25 was determined by suitable digests with the restriction enzymes *Hpa*I, *Bgl*II, and *Sal*I. Seven separate experiments were carried out, and in each experiment only Tn*5* insertions in pOS25 that showed clearly different map positions were analysed further for their ability to synthesize the glycine betaine binding protein (GBBP) and to transport glycine betaine. Tn*5* insertions in plasmid pOS13 were isolated as described above using strain MC4100(Δ*lac*) for transformation. The Km^r, Ap^r transformants were screened for altered or abolished expression of the Φ(*proU-lacZ*)hyb11 fusion on LB plates with XG and on lactose MacConkey plates supplemented with 0.2 M NaCl.

SDS-polyacrylamide gel electrophoresis

Proteins were analysed by SDS-PAGE as described by Laemmli (1970) and the separated proteins were visualized by staining with Coomassie blue or by autoradiography.

Transport assay

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

Methods used with nucleic acids

Plasmid DNA was prepared by CsCl density gradient centrifugation or by the alkaline extraction procedure of Birnboim and Doly (1979). DNA from phage was isolated according to Silhavy *et al.* (1984). Digestion with restriction enzymes, DNA ligation with T4 ligase, transformation of plasmid DNA, and recovery of restriction fragments from agarose gels were all performed as described previously (Maniatis *et al.*, 1982, Silhavy *et al.*, 1984). Restriction fragments were separated by agarose gel electrophoresis using Tris-borate-EDTA buffer. Transfer of restriction fragments from agarose gels onto nitrocellulose sheets (BA85, 0.45 μm; Schleicher & Schuell), Southern hybridization with radiolabelled plasmid DNA and radiolabelling of the probe with [α-^{32}P]-ATP were performed as described by Maniatis *et al.* (1982).

Radiolabelling of proteins in minicells

Minicells were isolated from strain DS410T carrying the various plasmids we used (Reeve, 1979). Labelling of minicells (0.5×10^9 cells) was performed in 500 μl 0.4% glucose MMA with or without 0.3 M NaCl using 12 μCi of [^{35}S]-methionine (1000 mCi $mmol^{-1}$, Amersham) for 1 h at 37°C. Minicells were then pelleted by centrifugation, washed once with 1 ml of TNE buffer (50 mM Tris-HCl at pH 7.5, 100 mM NaCl, 1 mM EDTA), resuspended in 25 μl of sample buffer, and solubilized for 20 min at 50°C before loading onto an SDS-polyacrylamide gel. The radiolabelled proteins were visualized by autoradiography (Kodak X-omatic 100) at −70°C.

Preparation of periplasmic proteins

To obtain periplasmic proteins, cells were grown overnight in 50 ml of 0.2% glucose MMA, pelleted by centrifugation and resuspended in 2 ml of glucose MMA. In each case, 1 ml was used to inoculate 0.5 l of glucose MMA without or with 0.3 M NaCl. These cultures were grown with aeration for 3–5 h. Periplasmic proteins were then isolated as described (Neu and Heppel, 1965; May *et al.*, 1986). Since strains carrying $proU^+$ multicopy plasmids grew poorly in glucose MMA, we grew 100-ml precultures in LB medium to obtain cultures of sufficient density. These were then centrifuged, and the cells were resuspended in 2 ml of glucose MMA and used as described above.

Preparation of outer membrane proteins

Strains were grown overnight in 0.4% glycerol MMA (100 ml) with or without 0.3 M NaCl. The cells were collected by centrifugation, resuspended in 4 ml of MMA containing 4 mg of DNase I, and disrupted by passing them three times through a French pressure cell at 1500 p.s.i. and unbroken cells were removed by centrifugation (1200×g for 10 min). The supernatant was layered onto a two-step sucrose gradient (70% sucrose (w/v), 54% sucrose (w/v) in 10 mM Tris-HCl(pH 8.0)) and centrifuged at 8°C for 16 h at 80 000×g. The outer membrane fraction was collected, diluted with 5 ml of water, and centrifuged for 1 h at 140 000×g. The pellet, primarily containing material from the outer membrane, was resuspended in 500 μl of sample buffer and the outer membrane proteins were solubilized at 100°C for 10 min. Aliquots were then electrophoresed on a 9% SDS-polyacrylamide gel containing 8 M urea.

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