Pipecolic acid is an osmoprotectant for *Escherichia coli* taken up by the general osmoporters ProU and ProP

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Exogenously supplied L-pipecolic acid was accumulated by *Escherichia coli* cells and protected them while growing at inhibitory osmolarity. Using specific uptake mutants and competitive assays, we established that the imino acid enters the cells through the ProP and ProU systems with K_m values of 225 and 53 μM , respectively. Surprisingly, in spite of the requirement for the wild-type *proX* gene for osmoprotective ability, no binding activity of labelled pipecolate with the periplasmic protein encoded by *proX* could be detected. In an attempt to demonstrate whether the two porters (ProP and ProU) are the only carriers involved in osmoregulation, a variety of molecules known for their intracellular osmolarity-dependent accumulation in various organisms were investigated. *N*-Dimethylproline (proline betaine), *N*-dimethylglycine, homobetaine (β -alanine betaine), γ -butyrobetaine and dimethylsulfoniopropionate were found to be capable of promoting the growth of osmotically stressed *E. coli*. All of these molecules enter bacterial cells via ProP and ProU porters. None of the osmoprotectants except *N*-dimethylproline was able to bind the periplasmic protein encoded by *proX*, while this protein was necessary for their uptake. Apparently, ProP and ProU are the sole osmoporters involved in osmolyte influx into *E. coli* cells.

Keywords: *Escherichia coli*, osmoprotection, pipecolic acid, ProP and ProU systems, periplasmic binding protein

INTRODUCTION

All micro-organisms have to cope with fluctuations in the osmolarity of their environment. The process of osmoregulation is similar in all living organisms. In response to elevated medium osmolarity, they first accumulate potassium and glutamate (Dinnbier *et al.*, 1988). In a second step, small organic compounds are accumulated by uptake from the medium or by *de novo* synthesis (Imhoff, 1986; Yancey *et al.*, 1982). These low-molecular-mass solutes increase internal osmolarity and protect macromolecules against denaturation; they are called osmoprotectants (Strom *et al.*, 1986; Csonka & Hanson, 1991).

The osmoprotective capacity of the well-known osmoprotectant glycine betaine (GB) is widespread among prokaryotic organisms. This onium compound is syn-

thesized *de novo* by only a few bacterial species including archaea, phototrophic bacteria and cyanobacteria (Lai *et al.*, 1991; Trüper & Galinski, 1990; Moore *et al.*, 1987) but it is available from most natural media. GB is commonly synthesized, together with the related compounds dimethylproline (DMP), trigonelline (TRG), γ -butyrobetaine (BB), homobetaine (HB) and dimethylsulfoniopropionate (DMSP) in seaweeds (Blunden & Gordon, 1986) and most higher organisms which have to cope with saline environments (Anthoni *et al.*, 1991; Rhodes & Hanson, 1993). Most of these molecules were found as osmoprotectants for bacteria in media of inhibitory osmolarity. The osmoprotection of *Rhizobium meliloti* was reported for DMP, BB (Bernard *et al.*, 1986), TRG and dimethylglycine (DMG) (Le Rudulier & Bernard, 1986). DMP was reported to be an osmoprotectant for *Escherichia coli* (Chambers & Kunin, 1987; Larsen *et al.*, 1987). In *Klebsiella pneumoniae* DMP, BB and DMSP have been described as compatible solutes (Mason & Blunden, 1989; Le Rudulier *et al.*, 1984). *E. coli* is able to import a variety of osmoprotectants from the medium;

Abbreviations: DMG, dimethylglycine; DMP, dimethylproline; TRG, trigonelline; BB, β -butyrobetaine; HB, homobetaine; DMSP, dimethylsulfoniopropionate; GB, glycine betaine; Pro, proline; PIP, pipecolic acid; GBBP, glycine-betaine-binding-protein.

Table 1. Bacterial strains

Strains	Genotypes	Origin
MC4100	F ⁻ <i>araD139</i> Δ (<i>argF-lac</i>)U169 <i>rpsL150 relA1 deoC1 ptsF25 flbB5301 rbsR</i>	Casadaban (1976)
BK23	MC4100 Δ (<i>putP.A</i>)101	E. Bremer
BK32	MC4100 Δ (<i>putP.A</i>)101 Δ (<i>proP</i>)2	E. Bremer
MHK13	MC4100 Δ (<i>putP.A</i>)101 Δ (<i>proP</i>)2 Δ (<i>proU</i>)608	E. Bremer
GM37	MC4100 ϕ (<i>proU-lacZ</i>) (hyb2) (λ plac Mu15)	Lucht & Bremer (1991)
GM50	MC4100 ϕ (<i>proU-lacZ</i>)3 (λ placMu55)	May <i>et al.</i> (1986)
GJ314	MC4100 Δ <i>putP.A</i> 101 <i>proP</i> 221 <i>proX</i> 224:: <i>lac</i> Δ (<i>pyr</i> 76:: <i>Tn</i> 10)462 <i>rec.A</i> Δ (<i>srl</i> :: <i>Tn</i> 10)461	Dattananda & Gowrishankar (1989)
GJ222	GJ134 <i>rec.A srl</i> :: <i>Tn</i> 10 <i>proW</i> 230:: λ placMu55 (Kan)	Dattananda & Gowrishankar (1989)
GJ229	GJ134 <i>rec.A srl</i> :: <i>Tn</i> 10 <i>proV</i> 229:: λ plac Mu55 (Kan)	Dattananda & Gowrishankar (1989)
GJ183	MC4100 Δ <i>putP.A</i> 101 <i>proP</i> 227:: <i>Mu d1</i> (Δ p. <i>lac</i>) Δ (<i>pyr</i> 76:: <i>Tn</i> 10)	Dattananda & Gowrishankar (1989)

proline (Pro), GB, 5-hydroxypipicolate, taurine, azetidine-2-carboxylate, 3,4-dehydro-DL-proline and ectoine are all accumulated via ProP and/or ProU transport systems (Cairney *et al.*, 1985; May *et al.*, 1986; McLaggan & Epstein, 1991; Gowrishankar, 1985, 1986; Grothe *et al.*, 1986; Csonka, 1989; Jebbar *et al.*, 1992). With the other accumulated compounds, the transport system involved in this influx has not yet been identified. Several authors have suggested that ProP and ProU are the only osmoprotectant transporters in *E. coli* but this has yet to be demonstrated.

In the present work, we determined first whether pipicolate acid (PIP), recently found in *Brevibacterium ammoniagenes* (renamed *Corynebacterium ammoniagenes*) (Gouesbet *et al.*, 1992), could act as an osmoprotectant for *E. coli* cells, and second if the transport systems involved in its uptake and that of other potential osmoprotectants are distinct from those described to date.

METHODS

Bacterial strains and growth media. The bacterial strains used in this study were derivatives of *E. coli* K12 (Table 1). They were grown aerobically at 37 °C with constant shaking (130 r.p.m.) on LB and defined M63 media (Miller, 1972). Elevation of osmotic strength was achieved by addition of NaCl at the indicated concentrations. The osmoprotectants GB, Pro, PIP, DMP, DMG, DMSP, HB, BB and TRG were added to a final concentration of 1 mM each. Bacterial growth was monitored as OD₅₇₀. For physiological studies, cells in the exponential growth phase were harvested by centrifugation, washed twice with an isotonic solution and stored at 4 °C until use.

Production of ¹⁴C-labelled osmoprotectants. [1,2-¹⁴C]Glycine betaine was prepared from [1,2-¹⁴C]choline (1.6 GBq mmol⁻¹) according to Ikuta *et al.* (1977) and purified as described by Perroud & Le Rudulier (1985). [U-¹⁴C]Pipicolate was synthesized by *B. ammoniagenes* ATCC 6872 from L-[U-¹⁴C]lysine monohydrochloride (11.1 GBq mmol⁻¹; Amersham-France). The labelled precursor and 1 M NaCl were added to a cell suspension growing in M63 medium. The mixture was incu-

bated for 2 h and pipicolate was purified as described by Gouesbet *et al.* (1992). The purified molecule was assayed by co-chromatography with unlabelled commercial pipicolate acid and HPLC analysis (Gouesbet *et al.*, 1992). The specific activity (1.83 GBq mmol⁻¹) was deduced from the HPLC analysis. The L-[U-¹⁴C]proline (9.84 GBq mmol⁻¹) was purchased from Amersham-France.

Extraction and identification of solutes. Solutes were identified after extraction and chromatographic analysis as described by Gouesbet *et al.* (1992).

Transport assays. Cells growing in M63 medium containing 0.3 M NaCl were transferred in mid-exponential phase to the same medium containing 0.5 M NaCl. After 30 min, the cells were centrifuged, washed twice and resuspended in M63 with 0.5 M NaCl to an OD₅₇₀ of 2. Transport assays and measurement of radioactivity were performed as described by Perroud & Le Rudulier (1985). The protein content was determined by the Lowry method using bovine serum albumin as a standard.

Preparation of periplasmic proteins and binding assays. Cells were grown to mid-exponential phase in M63 supplemented with 0.3 M NaCl, harvested by centrifugation, washed and resuspended in 10 mM Tris/HCl, pH 7.5, 0.3 M NaCl. Periplasmic proteins were released by the cold osmotic shock procedure of Neu & Heppel (1965). The shock fluids were filtered through a cellulose acetate filter (0.22 µm), centrifuged at 180 000 g (1 h), dialysed against 10 mM Tris/HCl, pH 7.5 and then freeze-dried. Proteins were resuspended in the same buffer and their concentration estimated by the Lowry method. The binding activity was detected by three different procedures: the ammonium sulfate precipitation technique (Richarme & Kepes, 1983) and the equilibrium dialysis technique (Argast & Boos, 1979) were used as described by Jebbar *et al.* (1992); direct polyacrylamide gel electrophoresis of the ligand-protein complex in non-denaturing conditions was as described by Le Rudulier *et al.* (1991).

RESULTS AND DISCUSSION

Osmoprotection of *E. coli* by pipicolate

Pipicolate (PIP) has been found recently to be a solute associated with osmotic adaptation in *B. ammoniagenes* (Gouesbet *et al.*, 1992). To test its osmoprotective ability

Fig. 1. Influence of PIP on growth of *E. coli* in medium of increasing osmolarity. MC4100 was grown in minimal M63 medium containing 0.2% glucose without or with 0.3 M (Δ , $\blacktriangle$), 0.5 M ($\circ$, $\bullet$) and 0.7 M ($\square$, $\blacksquare$) NaCl. PIP was added at 1 mM. +, No added osmoprotectant; filled symbols, cells grown without PIP; open symbols, in the presence of 1 mM DL-PIP.

it was added to *E. coli* cultures growing in media of increasing osmolarity. The addition of 1 mM DL-PIP had no effect when *E. coli* was grown in the absence of NaCl. In contrast, it enhanced both growth yield and growth rate of strain MC4100 in media containing 0.3–0.7 M NaCl (Fig. 1). Growth rate was reduced from 1.09 to 0.08 generations h^{-1} when the NaCl concentration was in-

creased from 0 to 0.7 M, respectively. The addition of 1 mM DL-PIP or GB stimulated the growth rate to 0.18 and 0.4 generations h^{-1} , respectively. PIP was less effective than GB in media of elevated osmolarity. Various L-PIP concentrations from 0.5 μM –20 mM were investigated; a concentration as low as 0.2 mM gave maximal stimulation of growth. When D-PIP was tested, no growth stimulation was observed; thus only L-PIP was able to promote *E. coli* growth in osmotic-stress conditions. L-PIP accumulation in response to osmotic stress has been already described in plants, human liver and kidney, *Ptelea* species and *B. ammoniagenes* (Steward & Larher, 1980; Zaar *et al.*, 1986; Chang *et al.*, 1990; Romeo & Prass, 1977; Gouesbet *et al.*, 1992).

Fate of pipecolate and accumulation level in *E. coli*

To test the ability of L-PIP to be used as a carbon or nitrogen source, it was added to M63 medium without any other carbon or nitrogen source. No growth was observed when strain MC4100 was inoculated into these media without or with 0.3 M NaCl. It could be that PIP is converted intracellularly into another compound, as is choline, which is metabolized to GB. Strain MC4100 was grown in M63 medium containing 1 mM [^{14}C]PIP (2.65 MBq mmol^{-1}) in the presence of 0.3, 0.5 and 0.7 M NaCl. Cells were grown for four generations and extracted with 80% (v/v) ethanol; soluble fractions were analysed by chromatography and electrophoresis. All the radioactivity was recovered in the soluble fraction and was associated with authentic pipecolate whatever the osmotic strength of the medium. PIP was, therefore, accumulated in *E. coli* without further metabolism.

To estimate this accumulation, MC4100 was grown in M63 medium with various concentrations of NaCl in the presence of 1 mM [^{14}C]PIP (2.65 MBq mmol^{-1}) or 1 mM [^{14}C]GB (3.3 MBq mmol^{-1}). All the incorporated radioactivity was recovered as PIP or GB in the soluble fraction. As shown in Table 2, the amount of PIP or GB increased with medium osmolarity. The rates of GB

Table 2. Effect of growth medium osmolarity on GB and PIP accumulation in strain MC4100 and its derivatives GM50, BK32 and MHK13

Cells were grown in minimal M63 medium for three generations with NaCl at the indicated concentration. [^{14}C]Pipecolic acid (2.65 MBq mmol^{-1}) (PIP) or [^{14}C]glycine betaine (3.3 MBq mmol^{-1}) (GB) were added at 1 mM. After alcohol extraction, the labelled compounds were recovered and radioactivity was measured by scintillation counting. The results are the means of three independent experiments. Standard error was less than 10%.

NaCl added (M)	Accumulated GB or PIP [nmol (mg dry wt) $^{-1}$]					
	MC4100		GM50 (<i>proU</i>)		BK32 (Δ <i>proP</i>)	
	[^{14}C]GB	[^{14}C]PIP	[^{14}C]GB	[^{14}C]PIP	[^{14}C]GB	[^{14}C]PIP
–	30	40	20	20	20	20
0.3	420	570	320	460	470	360
0.5	1090	1300	670	740	810	830
0.7	1600	1580	1410	1150	1580	1300

Table 3. Effect of competitors on GB, Pro and PIP uptake in strain MC4100 and its derivatives

Cells were grown in minimal M63 medium with 0.3 M NaCl. [^{14}C]Glycine betaine, [^{14}C]proline and [^{14}C]pipecolate were used at 45 μM , 300 μM and 450 μM , respectively. The competitors, glycine betaine (GB), pipecolic acid (PIP) and proline (Pro) were added at 10- and 100-fold excess. Results are given as a percentage of the control uptake rate in the absence of competitor. The absolute rates of transport in strains MC4100, GM50 and BK32, respectively, were, for [^{14}C]GB, 2.5, 0.75 and 0.7 $\text{nmol min}^{-1} (\text{mg protein})^{-1}$, for [^{14}C]Pro, 3.1, 1.7 and 2.58 $\text{nmol min}^{-1} (\text{mg protein})^{-1}$, and for [^{14}C]PIP, 1.3, 0.46 and 0.62 $\text{nmol min}^{-1} (\text{mg protein})^{-1}$. Values are means of duplicate determinations; the standard error did not exceed 8%.

Strain (genotype)	Percentage inhibition											
	[^{14}C]PIP				[^{14}C]GB				[^{14}C]Pro			
	GB		PRO		PRO		PIP		GB		PIP	
	$\times 10$	$\times 100$	$\times 10$	$\times 100$	$\times 10$	$\times 100$	$\times 10$	$\times 100$	$\times 10$	$\times 100$	$\times 10$	$\times 100$
MC4100 (WT)	86	92	84	89	63	80	42	62	75	90	33	74
GM50 (<i>proU</i>)	52	58	40	45	67	90	16	55	78	89	26	56
BK32 (Δ <i>proP</i>)	80	92	85	87	0	0	2	5	89	94	65	78

accumulation were similar to those previously reported (Larsen *et al.*, 1987; Jebbar *et al.*, 1992). The osmotic pressure developed by PIP dissolved in water is less than that developed by GB [1.2 and 1.5 $\text{osmol (kg water)}^{-1}$ at 1 M, respectively]. Hence the concentration of PIP necessary to maintain the osmotic balance of the cytoplasm may be greater than that of GB. Accumulation data were in agreement with this observation; indeed, the PIP level was similar to, or slightly greater than, that of GB. Converted into osmotic pressure, PIP would allow a similar turgor pressure to GB. Nevertheless, the growth rates observed with GB were faster than those obtained with PIP in medium containing 0.7 M NaCl. This could be explained by the lower rate of PIP accumulation compared to that of GB at this osmolarity. The PIP level at this osmolarity may give a lower osmotic pressure than that developed by GB and thus may not give the full cell turgor pressure.

Pipecolate enters the cell via ProP and ProU

PIP is structurally analogous to proline. GB, Pro, and various structural analogues of Pro such as azetidine-2-carboxylate, 3,4-dehydro-DL-proline and 5-hydroxy-L-pipecolate enter the cell through ProP or ProU transport systems (Gowrishankar, 1986; Csonka, 1989). To investigate whether PIP uses the same transporters, *E. coli* strains lacking one or more of the transporters PutPA, ProU, ProP were used for growth, uptake and competition experiments.

The characteristics of pipecolate uptake in the wild-type strain were studied. Competition with unlabelled compounds was performed with [^{14}C]GB, [^{14}C]Pro and [^{14}C]PIP in strain MC4100 (Table 3). The uptake of radioactive osmoprotectants was measured by introducing unlabelled competitors into the assay mixtures at 10 and 100 times the concentration of the labelled

compound (Table 3). [^{14}C]GB uptake was inhibited by Pro and, to a lesser degree, by PIP (80 and 62%, inhibition, respectively) when competitors were added at 100-fold excess. Uptake of [^{14}C]Pro was reduced to a greater extent by GB or PIP added in 10- or 100-fold excess. GB and Pro had a strong inhibitory effect on [^{14}C]PIP uptake.

If PIP was competitive for GB, and more especially for Pro, they may have a common uptake route. Initial rates of [^{14}C]PIP uptake were examined at 30, 60, 90 and 120 s at substrate concentrations of 20–500 μM . For the wild-type strain, Eadie-Hofstee plots of uptake data revealed a K_m of 71 μM and a $V_{\max}$ of 0.64 $\text{nmol min}^{-1} (\text{mg protein})^{-1}$.

In strain BK23 (Δ *putPA*) GB and PIP showed the same osmoprotective ability as in the parental strain MC4100. Values for PIP rate and uptake data were identical to those for MC4100 (data not shown); thus the *putPA*-encoded transporter is not involved in PIP uptake. In strain MHK13 (Δ *putPA*, Δ *proP*, *proU*) neither GB nor PIP was able to promote growth in M63 minimal medium containing 0.7 M NaCl (Fig. 2a). The amount of PIP detected in cells growing in medium of increasing osmolarity was negligible and no [^{14}C]GB or [^{14}C]PIP uptake was observed in the strain defective in *putPA*, *proP* and *proU*. Thus ProP and ProU were necessary for PIP uptake.

The osmoprotective effects of PIP and GB were studied in strain GM50 (Δ *proU*). A stimulation of growth rate was observed from 0.08 generations h^{-1} in 0.7 M NaCl medium to 0.375 and 0.363 generations h^{-1} in presence of GB and PIP, respectively (Fig. 2b). The PIP content of strain GM50 was less than in strain MC4100 but remained proportional to the osmolarity of the medium and higher than that of GB (Table 2). When Pro and PIP were used as competitors for [^{14}C]GB uptake, the results were

Fig. 2. Influence of medium osmolarity and osmoprotectants on the growth of strain MC4100 derivatives. Cells were grown in minimal M63 medium containing 0.2% glucose without or with 0.7 M NaCl. Osmoprotectants were added at 1 mM. (a) Strain MHK13, (b) strain GM50 and (c) strain BK32. $\square$, 0 M NaCl; $\bullet$, 0.7 M NaCl; $\blacksquare$, 0.7 M NaCl and 1 mM GB; $\circ$, 0.7 M NaCl and 1 mM L-PIP.

similar to those obtained in the wild-type strain. PIP showed a reduced competitive ability for [14 C]Pro uptake in strain GM50 ($\Delta proU$). GB and Pro only inhibited [14 C]PIP uptake by 50 and 45 %, respectively (Table 3). Kinetic parameters of [14 C]PIP uptake through ProP were determined in strain GM50 ($\Delta proU$). They showed a K_m of 225 μ M and a V_{max} of 0.85 nmol min $^{-1}$ (mg protein) $^{-1}$.

The presence of GB and PIP enhanced the growth rate of strain BK32 ($\Delta putP A, proP$) from 0.06 generations h $^{-1}$ in 0.7 M NaCl medium to 0.4 and 0.33 generations h $^{-1}$, respectively (Fig. 2c). In contrast to strains MC4100 and GM50, strain BK32 accumulated GB at a concentration greater than that of PIP whatever the osmolarity of the medium (Table 2). No competition was observed in strain BK32 when Pro or PIP was added as competitor of [14 C]GB uptake. This could be explained by the high affinity of *proU* for GB (1 μ M) (May *et al.*, 1986) (Table 3). The inhibition of [14 C]Pro and [14 C]PIP uptake was as strong as that found in the wild-type strain MC4100 when unlabelled competitors were added in 10- or 100-fold excess (Table 3). The ProU transport system had a higher affinity for PIP than ProP with a K_m of 53 μ M and a lower V_{max} of 0.57 nmol min $^{-1}$ (mg of protein) $^{-1}$.

These results showed that ProU was the main system for both accumulation and uptake. PIP and Pro had a similar affinity for ProP (K_m 300 μ M), lower than that of GB (K_m 44 μ M). The affinity of PIP for ProU porter (K_m 53 μ M) was greater than that of Pro (K_m 200 μ M) but significantly less than that of GB (1 μ M) (May *et al.*, 1986). This difference could explain the longer lag phase of growth observed with PIP, the steady state of accumulation needing a longer time to be reached. Nevertheless, in exponential-phase cells the accumulation levels of each molecule were similar.

Role of *proU* components in pipecolate uptake

As ProU was involved in PIP uptake, we analysed the influence of various Mu insertion mutations in the *proU* operon on PIP uptake in *proP*-defective strains. Strains GJ314 (defective for ProP and ProX proteins), GJ229 (defective for ProP, ProW and ProX products) and GJ222 (defective for ProP and all ProU components) were all unable to transport [14 C]PIP, whereas their parental strain GJ183 (*proP*) gave uptake parameters identical to those of strain BK32 (data not shown). No restoration of growth was observed when PIP was added to a culture of strain GJ314 in 0.7 M NaCl medium. Thus all ProU components, including the periplasmic binding protein, are necessary for PIP uptake via ProU.

We tried to detect the binding of [14 C]PIP to periplasmic proteins of strain MC4100 by the ammonium sulfate precipitation technique. No labelled PIP was bound to the precipitate, whereas a [14 C]GB-protein complex was formed. The binding of [14 C]GB could be reversed by competition with excess unlabelled GB, but not by PIP even in 1000-fold excess (Fig. 3).

Similarly we detected the binding of [14 C]GB to concentrated shock fluid from MC4100 cells grown at high osmolarity by the equilibrium dialysis technique and by PAGE of the ligand-protein complex in non-denaturing conditions. As previously, no binding of labelled PIP was detected, and the binding of [14 C]GB was reversed only by an excess of unlabelled GB. Thus, in spite of the lack of PIP uptake in *proX* strains, it seems that PIP is unable to bind to the periplasmic binding protein encoded by *proX*. Its behaviour is, in this respect, similar to that of Pro and ectoine (Barron *et al.*, 1987; Jebbar *et al.*, 1992). The exact role of the binding protein remains unclear.

Fig. 3. Effect of PIP and other osmoprotectants on [^{14}C]GB binding to shock fluid proteins. Shock fluid periplasmic proteins (100 μg) obtained from cells grown in medium of high osmolarity were incubated with 150 pmol [^{14}C]GB (1.6 GBq mmol $^{-1}$) in 10 mM Tris/HCl (pH 7.5). The assay mixture was 30 μl . The competitors were added in 1-, 10-, 100- and 1000-fold excess. □, GB; ●, DMP; △, L-PIP. After 5 min, proteins were precipitated by adding 1 ml ice-cold saturated ammonium sulfate solution.

Role of ProP and ProU in the uptake of other osmoprotectants

PIP is taken up by *E. coli* via ProP and ProU porters like GB, Pro, azetidine-2-carboxylate, 3,4-dehydro-DL-proline, ectoine and taurine (Cairney *et al.*, 1985; May *et al.*, 1986; Gowrishankar, 1985, 1986; Grothe *et al.*, 1986; Csonka, 1989; McLaggan & Epstein, 1991; Jebbar *et al.*, 1992). To check if ProP and ProU are the only means of entry of osmoprotective molecules, we investigated the

uptake of DMP, BB, TRG, DMSP, DMG and HB, which are known to be accumulated in response to osmotic stress in *E. coli* or in other organisms and for which the uptake system remains unknown. We tested the ability of these compounds to restore growth of *E. coli* in M63 medium supplemented with 0.7 M NaCl. Except for TRG, which allowed only a weak elevation of growth rate from 0.08 to 0.15 generations h $^{-1}$, the other molecules behaved as powerful osmoprotectants for the wild-type strain MC4100 (Table 4). This stimulatory effect was studied in strains with deletions of *proP* and/or *proU*. In the *proU* strain, GM50, we noted the same osmoprotective effects as found in strain MC4100 (Table 4). In strain BK32 (*proP*) only DMP, HB and BB allowed a good restoration of growth (Table 4), whereas DMG and DMSP were less efficient at improving growth. No osmoprotective effect was observed in the *proU*, *proP* strain, MHK13. Thus the ability of these compounds to restore growth of *E. coli* in media of high osmolarity is dependent on the ProU and ProP systems. In the absence of available ^{14}C -labelled molecules, the relative affinity of ProP and ProU for each compound was determined by competitive experiments with [^{14}C]GB and [^{14}C]Pro. As shown in Table 5, only DMP, DMG, DMSP, and, to a lesser degree, HB were efficient competitors for [^{14}C]GB uptake. In the *proU* strain, GM50, a similar effect of the various compounds was observed. In contrast, in the *proP* strain, BK32, only DMP was able to significantly inhibit [^{14}C]GB uptake.

The effect of osmoprotectants on [^{14}C]Pro uptake was studied (Table 5). In strain MC4100, all compounds decreased uptake, with a weak effect for HB, BB and TRG. The same effects were found in the *proU* strain, GM50. In strain BK32 (*proP*), a strong inhibition was observed with all the molecules tested. This result showed that among the assayed compounds DMP was the better substrate for the ProP and ProU systems, and the most effective osmoprotectant for *E. coli*. Its affinities for ProP and ProU were close to those of GB. HB, another efficient osmoprotectant, had comparatively lower affinities for ProP and ProU. According to the growth rates and data from the competitive experiments, DMG and DMSP

Table 4. Effect of osmoprotectants on growth rate of *E. coli*

Growth rates (generations h $^{-1}$) of strains MC4100, GM50 (*proU-lacZ*) and BK32 (*putP A proP*) were determined in M63 medium without or with 0.7 M NaCl. Osmoprotectants were added at a final concentration of 1 mM. Results are the means of duplicate experiments; the standard deviation did not exceed 5%.

Strains	M63 medium									
	Without NaCl	With NaCl								
		None	GB	Pro	DMP	DMG	DMSP	HB	BB	TRG
MC4100	1.09	0.08	0.40	0.35	0.33	0.29	0.28	0.32	0.26	0.15
GM50	0.70	0.08	0.37	0.34	0.44	0.29	0.26	0.28	0.20	0.16
BK32	1.00	0.06	0.40	0.27	0.30	0.12	0.10	0.28	0.23	0.14

Table 5. Effect of unlabelled competitors on [14 C]GB and [14 C]Pro uptake

The uptake of radioactive substrate was measured after 30, 60, 90 and 120 s by a filtration method. GB and Pro were present at a concentration of 45 μ M (specific activity 15000 c.p.m. nmol $^{-1}$) and 300 μ M (specific activity 900 c.p.m. nmol $^{-1}$), respectively. Inhibitors were added at concentrations of 450 μ M and 3 mM for GB and Pro uptake, respectively. The data are given as the percentage reduction in uptake rates, which were: for GB, 4.1 nmol min $^{-1}$ (mg protein) $^{-1}$ in MC4100, 0.76 in GM50 (*proU-lacZ*) and 4.2 in BK32 (*putP A proP*); and for proline, 2.1 nmol min $^{-1}$ (mg protein) $^{-1}$ for MC4100, 1.3 for GM50 and 2.9 for BK32. The standard error did not exceed 10%.

Inhibitor	Percentage inhibition of [14 C]GB uptake			Percentage inhibition of [14 C]Pro uptake		
	Strain MC4100	Strain GM50	Strain BK32	Strain MC4100	Strain GM50	Strain BK32
GB	82	70	84	75	74	73
Pro	63	62	10	77	75	71
DMP	80	73	79	65	61	95
DMG	28	17	3	47	47	91
DMSP	25	15	12	32	23	91
HB	21	8	23	12	13	92
BB	20	10	9	20	15	58
TRG	10	11	10	19	18	86

acted with a higher affinity for ProP. The other compounds did not show any preference for one or other transport system.

Since uptake of the above compounds was mediated by the *proU* transport system, we analysed the involvement of the binding protein, GBBP, encoded by *proX* in the *proU* operon. The osmoprotective effect of the compounds was studied in the *proP*, *proX* strain, GJ314. None of the osmoprotectants assayed was efficient in media of high osmolality. Therefore, the *proX*-encoded binding protein was essential for osmoprotective ability. Moreover, we tested, by the ammonium sulfate precipitation technique, the ability of these molecules to compete with the binding of [14 C]GB to the periplasmic protein, GBBP. The non-radioactive GB, at onefold excess, inhibited 40% of the [14 C]GB binding to the precipitate (Fig. 3). When several compounds were used as non-radioactive competitors, only DMP allowed 20% binding inhibition at onefold excess and 63% at tenfold excess. The other molecules had no detectable affinity for the periplasmic protein.

True exogenous osmoprotectants are compounds accumulated from the medium and not metabolized (Csonka, 1989); thus choline and trehalose should be not considered as genuine osmoprotectants when given to *E. coli*. The ProP and ProU systems are specifically involved in the uptake of exogenously provided osmoprotectants. They were first identified as proline porters; they should now be considered as osmoprotectant porters, able to transport a large diversity of molecules such as quaternary onium compounds (like GB), sulfonium molecules (DMSP) and imino acids (PIP). ProU is involved in the uptake of various compounds, but of those studied here, only GB and DMP were shown to bind the ProX protein;

this last is necessary for the uptake of the other molecules. The role of ProX in uptake remains unclear and needs further biochemical investigation.

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