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Adaptation of *Escherichia coli* to high osmolarity environments: Osmoregulation of the high-affinity glycine betaine transport system ProU

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Abstract: A sudden increase in the osmolarity of the environment is highly detrimental to the growth and survival of *Escherichia coli* and *Salmonella typhimurium* since it triggers a rapid efflux of water from the cell, resulting in a decreased turgor. Changes in the external osmolarity must therefore be sensed by the microorganisms and this information must be converted into an adaptation process that aims at the restoration of turgor. The physiological reaction of the cell to the changing environmental condition is a highly coordinated process. Loss of turgor triggers a rapid influx of K^+ ions into the cell via specific transporters and the concomitant synthesis of counterions, such as glutamate. The increased intracellular concentration of K^+-glutamate allows the adaptation of the cell to environments of moderately high osmolarities. At high osmolarity, K^+-glutamate is insufficient to ensure cell growth, and the bacteria therefore replace the accumulated K^+ ions with compounds that are less deleterious for the cell's physiology. These compatible solutes include polyoles such as trehalose, amino acids such as proline, and methyl-amines such as glycine betaine. One of the most important compatible solutes for bacteria is glycine betaine. This potent osmoprotectant is widespread in nature, and its intracellular accumulation is achieved through uptake from the environment or synthesis from its precursor choline. In this overview, we discuss the properties of the high-affinity glycine betaine transport system ProU and the osmotic regulation of its structural genes.

Key words: Transcriptional regulation; Osmoprotectants; Trehalose; Proline; Choline; Nucleoid-associated protein H-NS

Introduction

Bacteria have evolved a remarkable repertoire of mechanisms to adapt to environmental changes. The osmotic strength of the environment is an important physical parameter that influences the ability of organisms to grow in and successfully compete for a given habitat. In recent years, the mechanisms of osmoadaptation have been studied intensively in *Escherichia coli* and *Salmonella typhimurium* by biochemical and molecular approaches (for overviews see [19,20,31,55,56,60,68, 107,120,121]). Work performed in our laboratory has focused on high-osmolarity growth conditions and uses the *E. coli proU* operon as a model system to study the mechanism by which a change

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in the environmental osmolarity is sensed and converted to a signal that finally results in altered gene expression. The *proU* operon encodes an osmotically inducible, binding-protein-dependent transport system for the osmoprotectant glycine betaine. The intracellular accumulation of glycine betaine permits the growth of *E. coli* in high-osmolarity media, which are otherwise inhibitory for its proliferation.

E. coli maintains an osmotic pressure in the cytoplasm that is higher than that of the surrounding environment, resulting in a outward directed pressure, the turgor, which has been estimated at 3–5 atm [106]. Maintenance of turgor is essential for the division and growth of the cells [79]. Since the cell envelope is permeable to water, a sudden increase in the osmolarity of the growth medium results in the rapid efflux of water from the cytoplasm. The resultant loss of turgor, increase in the concentrations of ions and macromolecules in the cytoplasm, and plasmolysis severely inhibit DNA replication, protein synthesis and, finally, cell growth. Consequently, *E. coli* has evolved mechanisms to sense changes in the environmental osmolarity and to adapt rapidly and efficiently to such adverse growth conditions. Osmoadaptation entails a two-step process. First, high intracellular concentrations of K^+-glutamate are accumulated by increased K^+ uptake and concomitant de novo synthesis of counterions such as glutamate. Subsequently, osmoprotective compounds such as trehalose, proline, glycine betaine and proline betaine (Fig. 1) are accumulated by either synthesis or uptake from the environment. Accumulation of these osmoprotectants then triggers an efflux of K^+ from the cytoplasm. Through this series of events, turgor is restored, and the cell can resume growth in high osmolarity environments [9,19,25,31,112].

The primary response to high-osmolarity stress: uptake of K^+

K^+ is the most abundant cation in the cytoplasm of *E. coli* and *S. typhimurium*. The central role of K^+ for osmoadaptation and proper maintenance of cell turgor has been firmly established [31,32]. The steady-state intracellular concentration of K^+ increases from 0.15 M to 0.55 M when the osmolarity of the medium is increased from 100 mOsm to 1200 mOsm with solutes unable to permeate across the cytoplasmic membrane [32,56]. *E. coli* has several active transport systems for this ubiquitous ion: Kdp, TrkA and TrkD (for a recent summary, see [26]). The trans-

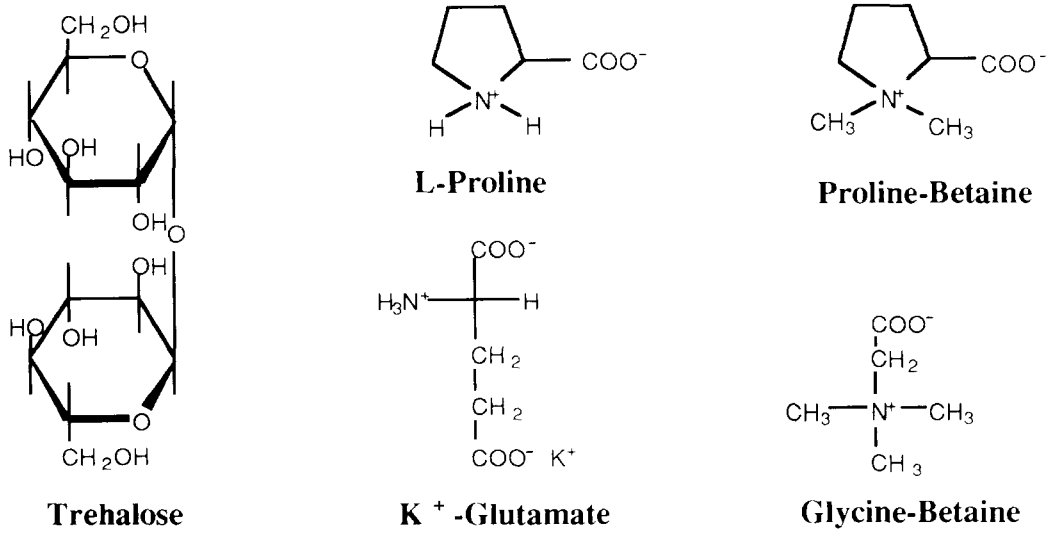

Fig. 1. Structures of osmoprotectants of *E. coli* and other enteric bacteria.

port activity of each of these systems is increased in response to hyperosmotic shock. The structural genes for the Trk K^+ uptake systems are constitutively expressed, whereas the expression of the Kdp structural genes responds to environmental signals [3,63,101]. In addition, there are at least three distinct K^+ efflux systems (Kef) in *E. coli* [27].

The Trk systems have a low affinity for K^+ but a very high transport rate. Under normal growth conditions, activity of the Trk systems is sufficient to satisfy the cell's need for K^+ and to maintain proper turgor. Under conditions of K^+ limitation and after a severe osmotic upshock, which result in loss of turgor, the high-affinity Kdp transport system becomes active. Increased activity of Kdp results primarily from increased gene expression. An interesting mechanism for the regulation of the *kdp* structural genes has been proposed, in which a mechanical signal, turgor reduction, is converted into a genetic response that results in increased *kdp* transcription [31,63]. Expression of the *kdp* operon is under the control of a two-component regulatory system. A inner membrane-embedded sensor protein, KdpD, relates the signal to a soluble response regulator protein, KdpE, via a phosphorylation cascade. KdpE then interacts with the regulatory region of the *kdpABC* operon encoding the components of the high-affinity Kdp K^+ transporter [83,101,111,118].

In concert, the cell's K^+ uptake systems can rapidly increase the intracellular K^+ pool after exposure to a high-osmolarity environment. In order to maintain the membrane potential, increased K^+ concentration must be balanced by the accumulation of anions or the expulsion of cations. The major accumulated counterion in *E. coli* is glutamate, the concentration of which is strongly increased by de novo synthesis during growth at high osmolarity [12,78]. Thus, the cell's primary response to a high osmolarity environment is the re-establishment of the osmotic pressure gradient across the cell membrane by strongly increasing the intracellular K^+-glutamate concentration. Other compounds, such as glutamine and the γ-glutamyl peptides γ-glutamylglutamine and glutathione, also contribute to the balance of the intracellular K^+ pool [77].

Compatible solutes

Cytoplasmic K^+ concentrations of up to 0.9 M have been measured during the initial phase of osmoadaptation [96], but high K^+ concentrations have deleterious effects on cell physiology and can inhibit the functioning of key enzymes [2,121]. Therefore, as a secondary response to high environmental osmolarity, the cell replaces much of the intracellular K^+ by so-called compatible solutes. This class of compounds is operationally defined as osmotically active solutes that can be accumulated to high intracellular concentrations without disturbing essential metabolic functions of the cell [9,19,55,107]. In the osmotically stressed cell, the intracellular accumulation of compatible solutes counterbalances high extracellular concentrations of osmolytes and, consequently, helps to maintain turgor. Some of these compounds are also known to stabilize the integrity of cell components and the function of proteins in solutions of high ionic strength [2,19,20,70,92,99,121].

The accumulation of compatible solutes in response to high-osmolarity stress is a universal phenomenon observed across the microbial, plant and animal kingdoms [19,121]. A limited group of low-molecular-mass compounds have been adopted as compatible solutes: polyols (e.g. glycerol, trehalose), amino acids and amino acid derivatives (e.g. proline, taurine, β-alanine), urea and methylamines (e.g. glycine betaine, proline betaine) [121]. Many of these compounds are found widely in nature. For instance, the physiologically important osmolyte glycine betaine, a trimethylated derivative of the amino acid glycine (Fig. 1), is synthesized in species as distantly related as enterobacteria, vascular plants and humans, and it is found in sources as diverse as human urine [13] and yeast extract [29]. Therefore, osmoprotective compatible solutes are found in many complex media used to grow bacteria in the laboratory. The structural formulas of several osmoprotectants important for *E. coli* are shown in Fig. 1, and their synthesis, transport and function are discussed below.

Trehalose is an osmoprotectant

The disaccharide trehalose (Fig. 1) is accumulated in many organisms in response to various

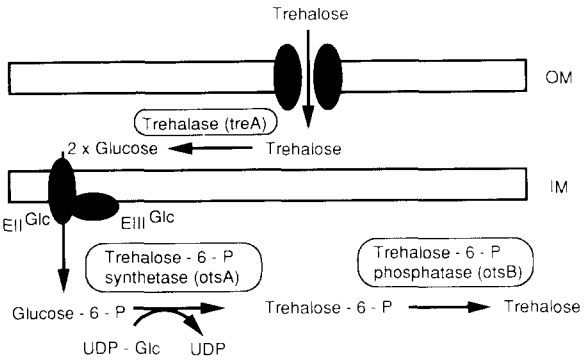

Fig. 2. Synthesis of trehalose in *E. coli*.

types of stress. When *E. coli* is grown at high osmolarity, it synthesizes trehalose in large amounts [25,67,108]. This process involves the condensation of glucose-6-phosphate and UDP-glucose to trehalose-6-phosphate via a trehalose-6-phosphate synthetase (OtsA). Trehalose-6-phosphate is then enzymatically converted to free trehalose by a trehalose-6-phosphate phosphatase (OtsB) (Fig. 2). High osmolarity stimulates the transcription of the *otsA* and *otsB* structural genes, and the enzymatic activity of the trehalose-6-phosphate synthetase is activated by K^+-glutamate [38]. Together, gene induction and enzyme activation result in the accumulation of trehalose under high-osmolarity growth conditions. Mutants impaired in the synthesis of trehalose are osmotically sensitive [38]. Uptake of exogenous trehalose does not contribute to its cellular accumulation under high-osmolarity growth conditions. This is surprising since *E. coli* possesses an efficient transport system for trehalose. It consists of a specific enzyme II^{Tre} (TreB) which acts together with enzyme III^{Glc} of the phosphotransferase carbohydrate uptake system (PTS) [8,108]. The activity of this PTS trehalose uptake system is inhibited by high osmolarity. In addition, high-level synthesis of the components of the trehalose transport system and the trehalose catabolic enzymes at high osmolarity is prevented by removal of the inducer (trehalose-6-phosphate) for their structural genes, by the osmoregulated trehalose-6-phosphate phosphatase (OtsB) [61].

Under high-osmolarity growth conditions or after a osmotic downshock, some of the internally synthesized trehalose leaks into the periplasm. A highly active trehalase (TreA) is present in this cell compartment that can split this disaccharide into two units of glucose [7,47,110]. The monosaccharides can then be taken up by a high-affinity glucose-specific PTS transport system, thus preventing loss of trehalose into the medium (Fig. 2). In addition, the presence of this trehalase permits the growth of *E. coli* at high osmolarity with trehalose as the sole carbon source. Expression of the structural gene for the periplasmic trehalase, *treA*, is stimulated by high osmolarity [7,47]. Another factor contributing to the transcriptional regulation of the *treA*, *otsA* and *otsB* genes is the growth phase of the bacterial culture. Expression of these genes is stimulated during the entry of the cells into stationary phase, and an alternative sigma factor, RpoS, is involved in the stationary phase and osmotic induction of these genes [48,59].

The osmoregulatory choline-glycine betaine pathway

The intracellular accumulation of glycine betaine is of central importance for the adaptation of many microorganisms to high-osmolarity environments [19,20,107,121]. *E. coli* cannot synthesize this osmoprotectant de novo but is able to accumulate glycine betaine to high intracellular levels when its precursor molecules, choline and glycine betaine aldehyde, are present in the growth medium [66]. *E. coli* can scavenge these biosynthetic precursors from the environment through an efficient transport system (Fig. 3), and their enzymatic conversion to glycine betaine allows the cells to grow in high-osmolarity environments that are otherwise inhibitory for their proliferation [64]. Strains impaired in the uptake of the biosynthetic building blocks or in the enzymatic synthesis of glycine betaine are sensitive against high-osmolarity growth conditions [109]. Neither glycine betaine nor its precursors can be utilized as substrate for the de novo synthesis of high-molecular mass cell components [66]. Hence, the accumulation of glycine betaine in *E. coli* serves only an osmoprotective function and is

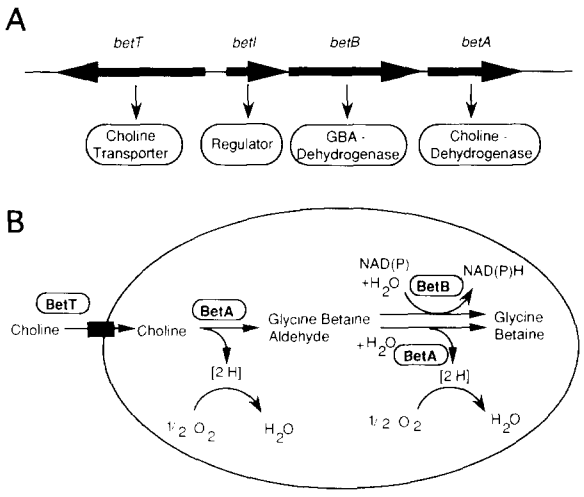

Fig. 3. The osmoregulatory choline-glycine betaine pathway. (**A**) Genetic organization of the *bet* gene cluster. (**B**) Uptake of external choline via the BetT transporter and its enzymatic conversion into glycine betaine.

observed only in osmotically stressed cells [68,90]. In other microorganisms, such as *Rhizobium meliloti*, glycine betaine can function as a carbon, nitrogen and energy source as well as an osmoprotectant [114]. Whether glycine betaine is used as a nutrient or as a osmoprotectant in *R. meliloti* is determined by the osmolarity of the growth medium: the activities of the enzymes involved in the degradation of glycine betaine are inhibited at high osmolarity, whereas those involved in its synthesis from choline are either stimulated or not affected [114].

The steps involved in the uptake of choline and its enzymatic conversion into glycine betaine in *E. coli* have been intensively studied. A low-affinity (K_m 0.5 mM) and a high-affinity (K_m 8 μM) uptake system for choline have been detected [109]. The well-characterized high-affinity choline transporter, BetT, is an integral inner membrane protein and is energized by the proton motive force. BetT is activated at the level of transport by high osmolarity [64]. Its structural gene, *betT*, maps at 7.5 min on the *E. coli* linkage map and is tightly linked to the genes encoding the enzymes for glycine betaine synthesis (Fig. 3A). Two enzymes are involved in the formation of glycine betaine from choline: a choline dehydrogenase (BetA) and a glycine betaine aldehyde dehydrogenase (BetB). BetA is a membrane-bound, oxygen-dependent enzyme that can catalyze the oxidation both of choline to glycine betaine aldehyde and of glycine betaine aldehyde to glycine betaine. The BetB protein is a soluble NAD-dependent glycine betaine aldehyde dehydrogenase with a marked substrate specificity. The BetB protein exhibits a marked salt tolerance, an important feature of an enzyme involved in the biosynthesis of an osmoprotecting compound [37]. In concert, the choline transporter and the BetAB enzymes can convert very low (1 mM) extracellular concentrations of choline into high (several hundred millimolar) intracellular concentrations of the osmoprotectant glycine betaine [66]. Consequently, the choline–glycine betaine pathway serves an important function in the adaptation process of *E. coli* to a high-osmolarity environment. However, many *E. coli* laboratory strains do not possess the *bet* genes, since they are covered by the commonly used Δ(*argF-lac*)U169 deletion [66]. The choline–glycine betaine pathway is not essential for osmoadaptation, since *S. typhimurium*, which is evolutionarily closely related to *E. coli*, lacks the *bet* gene cluster entirely, due to a large chromosomal deletion [98]. The value of the *bet* genes for osmoadaptation is manifested by the fact that the transfer of these genes from *E. coli* to *S. typhimurium* results in increased osmotolerance when external choline is provided [1].

The level of *bet* gene expression is subjected to regulation by several environmental stimuli. Transcription of the *bet* gene cluster is induced under high-osmolarity conditions and is further stimulated by the presence of choline in the growth medium. Full expression requires an aerobic environment and high growth temperature [33]. Choline regulation of *bet* expression is probably mediated by the BetI protein. Its structural gene, *betI*, is part of the *bet* gene cluster (Fig. 3A), and it encodes a polypeptide with homologies to several repressor proteins [64]. Presently, it is unclear whether BetI also mediates the regulation of *bet* expression by medium osmolarity and other environmental stimuli.

ProU and ProP: uptake systems for osmoprotectants

Early work by Christian in 1955 showed that, in *Salmonella oranienburg*, exogenously provided proline could function as an osmoprotectant [14,15]. A large increase in the intracellular concentration of proline was demonstrated by Measures [78] in the enteric bacteria *E. coli* and *S. typhimurium* when these were subjected to osmotic stress. This accumulation cannot be accomplished by de novo synthesis, since the proline biosynthetic enzymes are subjected to feedback inhibition by proline [19]. However, proline-overproducing mutants with altered feedback inhibition show enhanced osmotolerance [16,22]. In wild-type cells, the accumulation of proline observed under high-osmolarity growth conditions is achieved by increased uptake from the environment. Both organisms possess three distinct proline transport systems: PutP, ProP and ProU. The major proline permease, PutP, functions when proline is used as a nutrient, but plays no role in uptake of this amino acid at high osmolarity. Activity of PutP is inhibited by osmotic stress [120]. In contrast, the ProP and ProU transporters function under conditions of osmotic stress and they constitute the proline uptake systems which allow the intracellular accumulation of proline as an osmoprotectant [17,18,30,40,44, 104]. They were also shown to be responsible for the uptake of other osmoprotectants, including glycine betaine [10,11,74], whose uptake in *E. coli* is strongly stimulated by high-osmolarity growth conditions [90]. Consequently, these transporters play a central role in the efficient scavenging of osmoprotectants from the environment.

Characteristics of the ProP system

The ProP transporter is a component of the cytoplasmic membrane and consists of a single polypeptide [21]. The energy for substrate transport through ProP is provided by the proton motive force. High osmolarity strongly stimulates the activity of the ProP system [80,81]. This modulation of transport activity might be induced by a conformational change in the ProP protein. Its structural gene, *proP*, is expressed at a significant level in media of low osmolarity. However, high-osmolarity growth conditions can further increase *proP* expression [11,41]. The ProP system has a very low affinity ($K_m = 0.3$ mM) for proline, in comparison to PutP ($K_m = 2$ μM) [120]. In addition to proline, the ProP transporter accepts taurine (an osmolyte in eukaryotes), ectoine (an osmoprotective cyclic amino acid found in several moderately halophilic and halotolerant bacteria), glycine betaine and structural analogues of glycine betaine as its substrate [11,17,41,44,57,74,76,90, 100]. Despite the rather low affinity of the ProP system for these osmoprotectants, it serves an important physiological function for osmoadaptation, since osmotically stimulated transport activity allows the cell to react rapidly to a sudden increase in medium osmolarity [11].

ProU: an efficient uptake system for glycine betaine

Studies using strains lacking the PutP and ProP transporters revealed a third proline permease (ProU), which functions preferentially at high osmolarity growth conditions [17]. ProU contributes little to total proline uptake. Subsequent work showed that the ProU system from *E. coli* and *S. typhimurium* has a very high affinity for glycine betaine ($K_m = 1.3$ μM) [10,74]. ProU is a multicomponent, binding-protein-dependent transport system [4,53,74]. Analysis of the *proU* locus at 57.5 min on the *E. coli* linkage map showed that it is an operon in which three structural genes (*proV*, *proW* and *proX*) are coordinately expressed under the control of one major osmoregulated promoter (Fig. 4) [42,73,87,89,105]. The *proV* gene encodes a hydrophilic polypeptide (44.1 kDa), which is associated with the cytoplasmic membrane. The ProV protein is the energy-coupling component of the ProU system and has been shown to hydrolyze ATP [82]. It shows extensive homologies to the energy-transducing components of other binding-protein-dependent transport systems. The second gene in the operon, *proW*, encodes a hydrophobic protein (37.6 kDa) that is integrated into the inner membrane. In analogy to other binding-protein-dependent transporters, the ProV and ProW proteins are likely to exist in the cell as homodimers [49]. The *proX* gene encodes the periplasmic glycine-be-

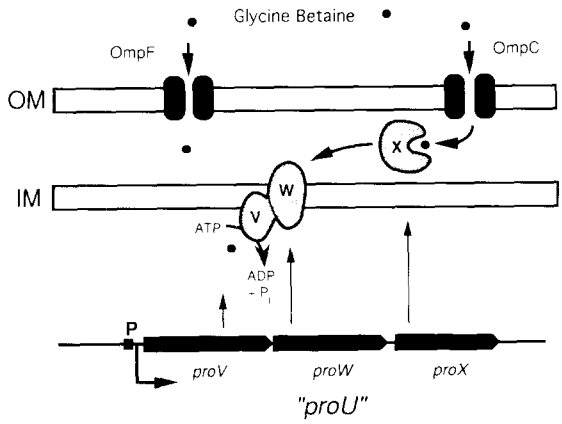

Fig. 4. The binding-protein-dependent ProU transport system.

taine-binding protein (Fig. 4). This substrate-binding protein (33.8 kDa) binds glycine betaine with high affinity (K_D = 1 μM) [4,53,74] and delivers it to a complex consisting of the ProV and ProW proteins. Together, these three proteins are responsible for the efficient translocation of glycine betaine across the cytoplasmic membrane.

Proline betaine (Fig. 1), like glycine betaine, is accumulated to high intracellular concentrations under high-osmolarity growth conditions in *E. coli* [67]. Work in our laboratory has established that this osmoprotectant is transported through both the ProP and ProU systems. Strains with defective ProU and ProP transporters are no longer protected against the detrimental effects of high osmolarity by externally provided proline betaine, showing that no additional transporter exists for this osmoprotectant (unpublished results). Like ProP, the ProU system also permits the uptake of osmoprotectants other than glycine betaine. Proline, taurine, ectoine and structural analogues of glycine betaine are all transported through the ProU system, albeit with rather low affinity [11,17,44,57,74,76,90]. In addition, the low-affinity uptake activity for choline detected previously in *E. coli* [109] was recently shown to be mediated by the ProU transport system [65]. Hence, the ProU system has a rather broad substrate specificity, and it is apparent that it serves an important physiological function for the efficient scavenging of a wide variety of osmoprotectants from the environment when *E. coli* is confronted with high-osmolarity growth conditions.

Access of glycine betaine to the periplasmic glycine betaine-binding protein (ProX) is provided by the integral outer membrane proteins OmpC and OmpF (Fig. 4) [34]. These proteins form water-filled pores within the outer membrane and allow the passive diffusion of a great variety of compounds with a molecular mass of less than 600 Da [85]. The relative amounts of the OmpC and OmpF porins vary in a reciprocal fashion in response to medium osmolarity [117]. At high osmolarity, OmpC predominates and consequently the OmpC pores constitute the major pathway for the permeation of glycine betaine across the outer membrane [34].

Glycine betaine can be accumulated to very high concentrations (exceeding 800 mM) in the cytoplasm of osmotically stressed cells [67]. If the osmolarity of the growth medium is lowered, the intracellular solute content of such cells has to be reduced to preserve the normal osmotic pressure gradient across the cell membrane. Recent data suggest that both *E. coli* and *S. typhimurium* possess efflux systems for glycine betaine separate from the ProU and ProP uptake systems that permit a rapid reduction of the intracellular concentration of the osmoprotectant [62,65]. However, the molecular details of these efflux systems and their mode of control remain to be elucidated. The intracellular concentration of glycine betaine is therefore determined by the relative rates of uptake and excretion, permitting a rapid and sensitive adjustment of the osmotic strength of the cytoplasm in osmoregulating cells. Thus, the ProU and ProP uptake systems not only function in the efficient scavenging of osmoprotectants from the environment, but also play an important role in preventing loss of these compounds from the cell into the medium by their re-uptake from the periplasm.

Regulation of the ProU system at the biochemical and genetic level

Osmoregulation of ProU transport activity

At low osmolarity, the components of the ProU transport system are present only in very small

amounts; uptake of glycine betaine through the ProU system under these environmental conditions is barely detectable. Glycine betaine transport is strongly stimulated when the cells are subjected to a sudden osmotic upshift or are grown in high-osmolarity media [10,74,90]. Increased transport is largely due to increased synthesis of the ProU components. In addition, the osmolarity of the environment also influences the activity of the ProU transport system (Fig. 5). A stimulation in ProU activity is observed shortly after a sudden exposure of cells grown at low osmolarity to increased osmotic pressure [34]. This stimulation of ProU transport activity is independent of de novo protein synthesis. The mechanism resulting in osmotic control of ProU activity is not well understood [20]. High osmolarity does not increase the affinity of the glycine betaine-binding protein for its substrate [53,74]. It is conceivable that the osmotic activation of the ProU system is mediated by its inner membrane components. Plasmolysis caused by osmotic upshock could produce conformational changes that would permit the optimal functioning of these components or their productive interaction with the substrate-loaded glycine betaine-binding protein.

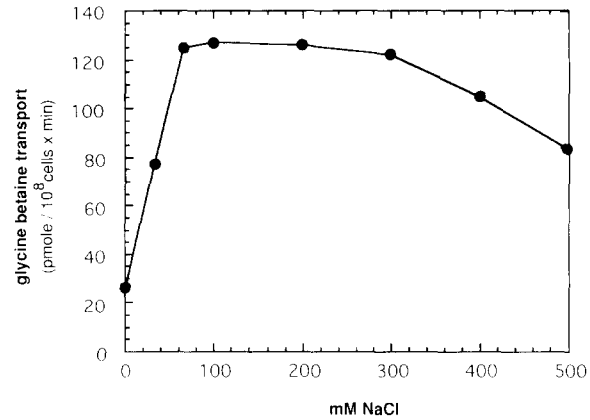

Fig. 5. Osmotically modulated ProU transport activity. The strain used for the transport assays carries the cloned *proU* operon on a low copy number phasmid. The cells were grown in minimal medium (MMA), preincubated for 5 min in MMA containing the indicated NaCl concentrations, and were subsequently assayed for glycine betaine uptake at the same NaCl concentration used for preincubation [34].

ProU must function under extreme osmotic stress in order to allow the intracellular accumulation of osmoprotectants. These growth condi-

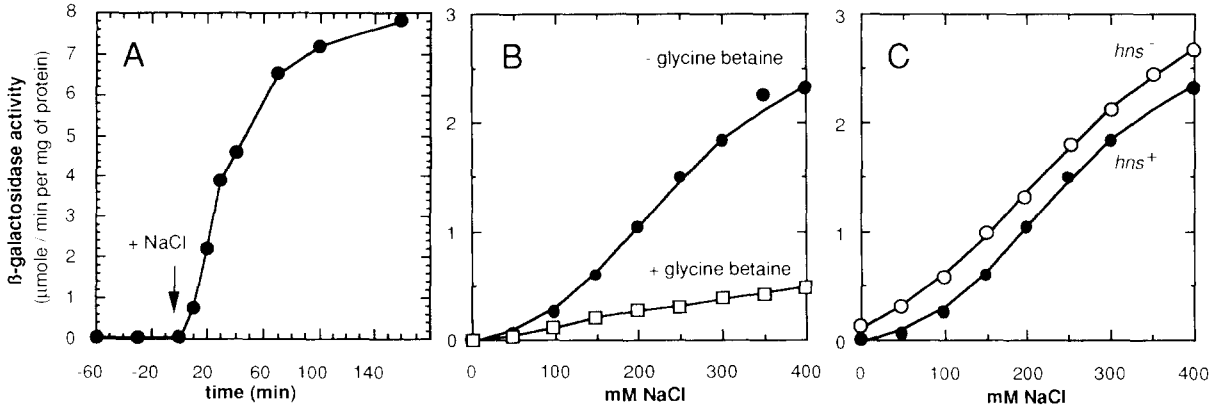

Fig. 6. Osmotically stimulated *proU* expression. (**A**) Time course of *proU* induction after sudden osmotic upshift. A strain carrying a chromosomal *proU–lac* fusion was grown in minimal medium. At the time indicated by an arrow, medium osmolarity was increased by the addition of 0.3 M NaCl. The data shown are from reference [5]. (**B**) Expression of *proU* in response to high osmolarity growth media. A *proU-lac* fusion strain was grown overnight in minimal medium whose osmolarity had been increased by the indicated concentration of NaCl. The cultures were grown overnight in the absence (closed symbols) or in the presence (open symbols) of 1 mM glycine betaine. (**C**) Influence of an *hns* mutation on *proU* expression. A pair of isogenic strains carrying either the *hns*$^+$ gene (closed symbols) or the *hns200* mutation (open symbols) were grown overnight in media with increasing osmolarities. The data for (**B**) and (**C**) were taken from reference [50].

tions are known to strongly inhibit the activity of many transport systems present in *E. coli* [97]. Consequently, the ability of the ProU system to operate efficiently at high osmolarity serves an important physiological function. In addition, activation of ProU activity by high osmolarity allows the cell to utilize pre-existing ProU components to adjust rapidly to increases in external osmolarity. The osmotically modulated profiles of ProU activity and of *proU* gene expression are distinct (Figs. 5 and 6C). Very small increases in osmolarity (addition of up to 0.1 M NaCl to a minimal medium with approx. 330 mOsm) result in a substantial increase in glycine betaine uptake, but have little effect on the transcription of *proU*. The activity of the ProU transport system remains at a constant high level in the range between 0.1 M and 0.3 M added NaCl. A decline in ProU activity is observed when the osmolarity is increased further (Fig. 5) [34]. This osmoresponsive profile of ProU activity is very similar to that found for the ProP transporter [80]. We note that the inhibition of ProU activity by high osmolarity begins before expression of its structural genes has reached its maximum (Figs. 5 and 6C). Consequently, at high osmolarity, total glycine betaine uptake through the ProU system reflects both stimulation of *proU* expression and a transport activity that is beginning to decline.

Osmotic control of ProU transcription

The genetic regulation of *proU* expression in *E. coli* and *S. typhimurium* has been extensively studied with the aid of *lacZ* and *phoA* gene fusions [5,10,30,40,45]. These studies have revealed that the level of *proU* expression is sensitively determined by the osmolarity of the growth medium. The basal transcription of *proU* is very low, and it is rapidly and strongly stimulated upon a sudden osmotic upshock (Fig. 6A). Osmotic induction of *proU* expression can be triggered by a great variety of osmolytes that cannot permeate the cytoplasmic membrane, and the increased level of *proU* expression is maintained

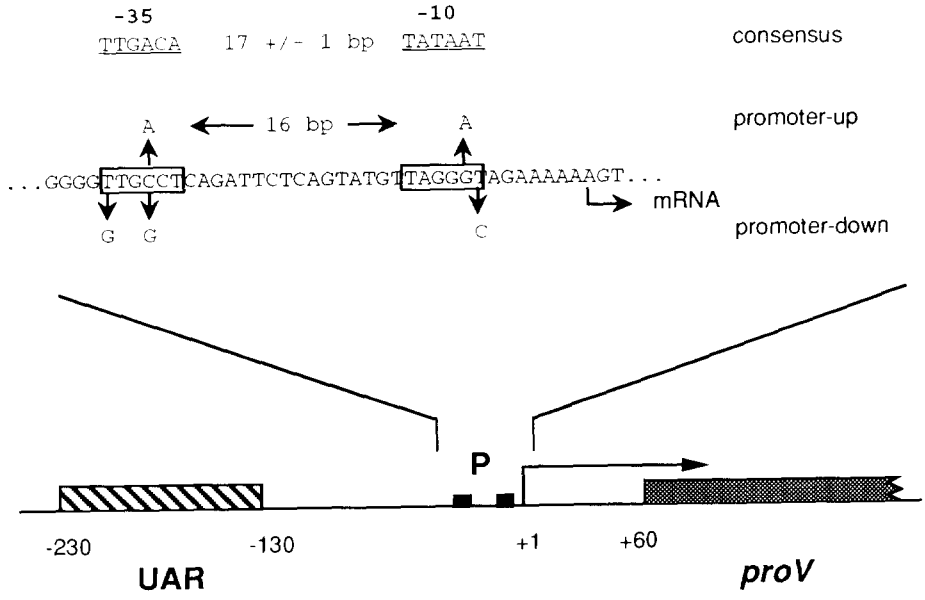

Fig. 7. Structure of the *proU* regulatory region. The scheme indicates the relative positions of the *proU* promoter (P), the 5' 'upstream activating region' (UAR) and the beginning of the first structural gene, *proV*. Above, the DNA sequence of the *proU* promoter is shown in comparison to the 'consensus sequence' for *E. coli* promoters. The positions of some promoter point mutations that affect *proU* expression is marked by arrows.

as long as the osmotic stimulus persists. The level of *proU* transcription during growth at elevated osmolarity is proportionally correlated with the osmolarity of the growth medium (Fig. 6B). This pattern of *proU* expression suggests the presence of a finely tuned sensing and regulatory mechanism.

The *proU* promoter has been identified by mapping the start site for the *proU* mRNA. The same promoter is used for the low-level basal transcription detected in cells grown at low osmolarity and the efficient transcription occurring in cells grown at high osmolarity [42,71,73,87,89,105]. The −35 and −10 sequences of the *proU* promoter show homology to the consensus sequence of σ^{70}-dependent *E. coli* promoters (Fig. 7). However, both the −35 and −10 regions deviate at a number of positions from this consensus sequence. The poor match of the *proU* promoter sequence to the consensus sequence and the suboptimal spacing of 16 bp between the −10 and −35 regions probably contribute to the very low basal *proU* transcription level detected at low osmolarity. We have characterized a number of *cis*-acting mutations that result in increased *proU* expression both at low and high osmolarity [71]. These point mutations map in either the −35 or the −10 region and increase the homology of the *proU* promoter to the consensus sequence of σ^{70}-dependent *E. coli* promoters (Fig. 7). In addition, a number of mutations affecting base pairs known to be important for promoter function were found in both the −10 and the −35 regions (Fig. 7). Taken together, these data suggest that no alternative sigma factor is involved in the regulation of *proU* expression. Indeed, *proU* expression in vitro is inhibited by antibodies directed against σ^{70} [58]. Since alterations in the *proU* −10 and −35 regions do not abolish osmotic regulation, the particular DNA sequences present at the *proU* promoter cannot be the prime determinants for the osmotic regulation of *proU* transcription. This view is supported by the regulatory features of an IS*1* insertion into the spacer sequence between the −35 and −10 region. This IS*1* insertion creates a hybrid promoter consisting of a −35 region contributed by the insertion element and the authentic *proU* −10 sequence. This novel promoter functions only inefficiently but still allows osmoregulated expression of *proU* [71].

Subcloning experiments aimed at the definition of the minimal sequences required for osmotically regulated *proU* expression have been carried out by several groups [23,71,86,88,89]. The results from these studies suggest that relatively small DNA segments can mediate osmoregulated transcription. We recently found that a 60-bp fragment that carries just the *proU* promoter and small segments of upstream and downstream material is capable of driving osmotically regulated transcription (Lucht et al., in preparation). However, the induction factor of these minimal constructs is much lower than that of the wild-type *proU* operon. It is thus clear that, while DNA segments in close proximity to or overlapping the *proU* promoter can mediate some degree of osmoregulation, additional sequences surrounding the *proU* promoter are required to achieve the strong induction of *proU* after an osmotic upshock.

Deletion analysis of chromosomal sequences 5′ of *proU* revealed that its maximal expression requires the presence of an 'upstream activating region' (UAR) extending approximately 200 bp 5′ to the −35 region (Fig. 7). The successive shortening of these sequences results in a gradual decline of *proU* expression but does not affect osmoregulation of *proU* transcription [71]. The position of the sequences required for the full expression of *proU* matches that of a DNA segment displaying features of 'curved' DNA [42,113]. Sequence distributions associated with DNA curvature upstream of a number of prokaryotic promoters with upstream activation of transcription have been described [91]. However, it has not yet been clearly established whether there is a causal relationship between DNA curvature and activation of *proU* transcription. Within the UAR segment, Dattananda et al. [23] found a weak second osmoregulated promoter. The importance of this minor promoter for the expression of *proU* is not clear. It is unlikely that this promoter plays a decisive role in *proU* expression, since – in contrast to the main *proU* promoter – no mutations affecting *proU*

expression have been found in this sequence. Furthermore, deletion of this minor promoter does not affect the osmotic induction of *proU* [71].

Deletion analysis of the region 3′ to the *proU* promoter revealed the presence of DNA sequences early in *proV*, the first gene in the *proU* operon (Figs. 4 and 7), that are important for the proper expression of *proU*. Removal of these sequences results in a strong increase of the basal *proU* transcription level at low medium osmolarity, but the expression in these constructs is still further induced upon an osmotic upshift ([23,86,88]; Lucht et al., in press). Therefore, the DNA segment at the 5′ end of the *proV* gene is not essential for osmoregulated gene expression, but is required to shut down *proU* expression under low-osmolarity growth conditions. The presence of this 'silencer' thus serves an important function for the high induction ratio and the finely tuned expression of *proU* in response to changes in the osmolarity of the environment.

Osmoregulation of *proU* expression is not dependent on the sensor/regulator protein pair EnvZ and OmpR, which mediate the reciprocal osmoregulation of the *ompC* and *ompF* porin genes [10,45,74]. Intensive searches have been made in several laboratories for *trans*-acting regulatory proteins that might control osmoregulation of *proU* expression [28,50,71]. These genetic experiments have so far not revealed any classical negative- or positive-acting regulator. Such studies cannot rigorously rule out the involvement of such a factor in *proU* regulation, since the regulatory protein might be essential for other processes as well, and mutations in its structural gene might consequently be lethal. However, the inability so far to detect any *cis*- or *trans*-acting mutations that completely abolish osmotic regulation of *proU* make it highly unlikely that *proU* expression is regulated by a single regulatory protein.

We have recovered many mutants with altered *proU* expression that carry lesions in a gene, *hns*, (previously designated *osmZ*), which is unlinked to the *proU* operon and which maps at 27.4 min on the *E. coli* linkage maps [50,71,72]. Mutations in *hns* alter the pattern of osmoregulated *proU* expression (Fig. 6C). They increase *proU* transcription both at low and high osmolarity and shift the 'response curve' as if the cell were sensing a higher osmolarity than is actually present (Fig. 6C). The properties of *hns* mutations might at first suggest that the process of osmosensing is affected in such strains. Indeed, mutations in *hns* affect the expression of a number of osmoregulated genes [43,46,52]; however, the *hns* gene product does not constitute a general osmoregulatory protein. Analysis of *hns* demonstrated that it is the structural gene for the non-specific DNA-binding protein H-NS [39,54, 72] that is tightly associated with the chromosomal DNA [35]. The H-NS protein has profound effects on DNA structure, on the stability of the genetic material and on the expression of a large number of genes with diverse functions (for an overview, see [51]). The wild-type *hns* gene cannot be cloned into multicopy plasmids due to detrimental effects of the overproduction of H-NS on cell morphology and viability [54,72,103]. We devised a rapid and efficient purification procedure for this nonspecific DNA-binding protein by utilizing a newly constructed low-copy-number T7 expression vector and by exploiting the property of H-NS to tightly bind DNA [24]. The purified protein was then used to investigate its interaction with the *proU* regulatory region in competitive band shift assays. At low protein concentration, H-NS interacts preferentially with a 680-bp restriction fragment carrying the *proU* promoter. Sequences located upstream [113] and downstream ([88, Lucht et al., in preparation) of this promoter are targets for H-NS binding. In DNaseI footprinting experiments we found a segment at the 5′ end of the *proV* structural gene where several H-NS molecules bind tightly to an extended H-NS binding region. This binding region coincides with the *cis*-acting 'silencer' region ([23,86,88, Lucht et al., in press) that is required to keep *proU* expression low at low osmolarity.

Several molecular mechanisms can be envisioned by which H-NS can influence the level of *proU* expression. H-NS might function indirectly, by altering the DNA structure of the *proU* regulatory region [88]. It is well established that the H-NS protein can compact DNA both in vitro

and in vivo [102,103]. A second model for indirect action of H-NS is that this protein might act via a second, specific, *proU* regulatory protein. H-NS is known to influence the interaction of specific DNA-binding proteins with their operator sites [36]. However, as discussed above, there is no evidence so far for such a specific regulatory protein for *proU*. Alternatively, H-NS might act directly by influencing the productive interaction of RNA-polymerase with the *proU* promoter or by blocking transcript elongation. Our finding of an extended H-NS binding region at the 5′ end of the *proV* gene (Lucht et al., in preparation) that coincides with a functionally defined 'silencer' region suggests that H-NS might exert its effect on *proU* expression by direct, physical interaction with a sequence downstream of the *proU* promoter, either by acting as a transcriptional roadblock or by hindering early steps of transcription initiation. In vitro studies with the *lac* and *gal* promoters have shown that the kinetics of open complex formation at these promoters is slowed down in the presence of H-NS [102]. In the case of *proU*, H-NS molecules bound to a high-affinity site downstream of the promoter might act as a 'nucleation site' for the cooperative binding of more H-NS molecules, thereby extending the DNA segment bound by H-NS into the promoter region. The model of direct action of H-NS on *proU* expression is strongly supported by a recent report showing that H-NS can act as specific repressor of *proU* transcription in a purified in vitro system [116].

Osmosensing and osmotic control of gene expression

How does the cell sense environmental osmolarity and transform this extracellular physical parameter into a signal that finally results in altered *proU* transcription? The flux of K^+ across the cytoplasmic membrane is considered as the key event for the process of osmotic adaptation [9,31,112]. An osmotic upshift results in immediate reduction in turgor. To restore turgor, K^+ is rapidly transported into the cell via several uptake systems, stimulated either at the level of transport activity or gene expression. Counterions, such as glutamate, are concomitantly synthesized to balance the increased intracellular K^+ pool. Subsequently, a number of compatible solutes are accumulated that can replace K^+. Thus, loss of turgor is sensed by the K^+ transporters and the intracellularly accumulated K^+-glutamate then functions as a 'second messenger' that elicits the secondary responses (e.g. increased glycine betaine synthesis and uptake and accumulation of trehalose) of the cell to adapt to a high-osmolarity environment.

The regulatory pattern of *proU* expression is consistent with such a model. Clear differences are observed when the pattern of the osmoregulated transcription of *proU* is compared with that of the turgor-controlled *kdp* operon [63,112]. A sudden upshift in the osmolarity of the growth medium results in loss of turgor and a concomitant increase in *kdp* expression. Elevated *kdp* transcription occurs only transiently and ceases when turgor is restored by the uptake of K^+ [63]. In contrast, *proU* expression is maintained at high levels as long as the osmotic stimulus persists (Fig. 6). Therefore, the loss of turgor initially observed after an osmotic upshift cannot be the signal that triggers the increase of *proU* expression. Furthermore, in contrast to *kdp*, *proU* expression cannot be induced in high-osmolarity media containing limiting amounts of K^+ [112]. Hence, the presence of K^+ is a prerequisite for *proU* induction. Uptake of glycine betaine triggers an efflux of K^+ and consequently lowers the intracellular concentration of the 'secondary messenger', K^+-glutamate [9,112]. Consequently, if the intracellular concentration of K^+-glutamate is the critical determinant for regulating the level of *proU* transcription, one would predict that *proU* expression is reduced in the presence of glycine betaine in the growth medium. This is exactly what is observed (Fig. 6B).

How might the intracellular K^+-glutamate concentration influence *proU* expression? K^+-glutamate might stimulate *proU* transcription directly by facilitating a productive interaction between RNA polymerase and the *proU* promoter. K^+-glutamate is known to enhance the stability of protein–DNA interaction and to generally stimu-

late transcription in in vitro systems [69]. Increased *proU* expression in vitro in the presence of elevated concentrations of K^+-glutamate in a coupled transcription/translation (S-30) extract has been observed [58,94]. The reconstitution of K^+-glutamate-stimulated transcription from the *proU* promoter in a purified system containing only RNA polymerase holoenzyme, nucleotides, and a circular template DNA has also been described [93,115]. However, the increase in *proU* transcription achieved by the addition of K^+-glutamate to these in vitro systems is low compared with the strong increase in *proU* expression observed in vivo after an osmotic upshock, making it unlikely that this mechanism is the sole determinant for *proU* regulation in vivo. An alternative model for osmoregulation of *proU* expression has been suggested by Higgins and coworkers [50,52,84,88]. In this model, *proU* transcription is regulated mainly by changes in the DNA supercoiling that directly affect the strength of the *proU* promoter, possibly by influencing the DNA twist at the *proU* promoter [119]. These changes might be caused by an influence of the intracellular K^+-glutamate concentration directly on DNA structure, on the activity of DNA topoisomerases, or on the binding of histone-like proteins to the chromosomal DNA. This model is based on the observation that mutations and drugs that result in altered DNA supercoiling lead to altered *proU* expression. In addition, an increase in the supercoiling of reporter plasmids was observed in cells grown at high osmolarity [50,75]. Interactions of the H-NS protein with sequences downstream of the *proU* promoter might be required to form a 'scaffold', holding the DNA in an appropriate conformation to allow transcriptional regulation via topological changes at the promoter sequence [88]. However, the causal relationship between the increase in DNA supercoiling in cells grown at high osmolarity and the induction of *proU* expression has been questioned [95]. Furthermore, the moderate increase of *proU* in vitro transcription caused by elevated supercoiling of the DNA template [116] is insufficient by itself to account for the strong in vivo induction of *proU* expression observed at high osmolarity growth conditions.

Both models, the direct stimulation of *proU* transcription by K^+-glutamate and regulation by changes in the supercoiling of the *proU* promoter, are not mutually exclusive, and it is likely that both mechanisms contribute in a synergistic manner to mediate osmotic regulation of *proU* transcription. However, it is now clear that this promoter-mediated osmoregulation is further modulated by a mechanism that involves 'silencer'-like sequences located downstream of the promoter that are required to repress the basal transcription driven by the *proU* promoter in low-osmolarity media ([23,86,88]; Lucht et al., in press). To ensure a very high induction ratio of *proU* expression after an osmotic upshock, this repression mechanism should only function at low medium osmolarity and should be relieved at higher osmolarity, and should therefore also be osmotically regulated. How can such a mechanism act? We recently found that the histone-like H-NS protein binds to an extended binding site coinciding with these 'silencer' sequences (Lucht et al., in press), and Ueguchi and Mizuno [116] described that H-NS can act as a specific repressor for *proU* transcription in vitro. This repressing effect is abolished by increased K^+-glutamate concentrations that mimic the conditions found in osmotically stressed cells. Taken together, these results suggest that the 'silencer' sequences located downstream of the *proU* promoter can be bound by the H-NS protein, which represses *proU* expression only at low medium osmolarities but not if the extracellular osmolarity – and thus the cytoplasmatic K^+-glutamate concentration – is increased. Several molecular mechanisms might therefore act in a concerted fashion to mediate the finely tuned osmotic regulation of *proU* expression, without the requirement for a specific regulatory protein. Further studies are needed to test this model, and to define the relative contributions of the mechanisms involved in this regulatory network.

Conclusions

The ProU system has been intensively characterized in recent years by genetic, molecular, and

biochemical approaches both in *E. coli* and *S. typhimurium*. Much has already been learned through these studies: however, many questions remain with respect to the structure and function of the ProU transport system and its fascinating regulation. The complex control of the ProU system by medium osmolarity at the levels of both transport activity and gene expression makes ProU an attractive model system for unravelling the process by which a bacterial cell adapts to a high-osmolarity environment.

The results derived from studies on bacterial osmoregulation not only provide interesting insights into the way microorganisms cope with environmental stress, but they might also find useful biotechnological applications. It has already been firmly established that many of the compounds employed by microorganisms as osmoprotectants can stabilize isolated proteins from denaturation [2]. In addition, it has recently been shown that the yield of an active recombinant protein from *Agrobacterium* can be greatly increased by growing the producing *E. coli* cultures under high osmotic stress conditions in the presence of glycine betaine [6]. The ProP- and ProU-mediated accumulation of glycine betaine at high osmolarity might therefore be an important part of a general strategy for the overproduction of soluble recombinant proteins.

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References

1 Andresen, P.A., Kaasen, I., Styrvold, O.B., Boulonis, G. and Strøm, A.R. (1988) Molecular cloning, physical mapping and expression of the *bet* genes governing the osmoregulatory choline-glycine betaine pathway of *Escherichia coli*. J. Gen. Microbiol. 134, 1737–1746.

2 Arakawa, T. and Timasheff, S.N. (1985) The stabilization of proteins by osmolytes. Biophys. J. 47, 411–414.

3 Asha, H. and Gowrishankar, J. (1993) Regulation of *kdp* operon expression in *Escherichia coli*: evidence against turgor as signal for transcriptional control. J. Bacteriol 175, 4528–4537.

4 Barron, A., Jung, J.U. and Villarejo, M. (1987) Purification and characterization of a glycine betaine binding protein from *Escherichia coli*. J. Biol. Chem. 262, 11841–11846.

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

6 Blackwell, J.R. and Horgan, R. (1991) A novel strategy for production of a highly expressed recombinant protein in an active form. FEBS Lett. 295, 10–12.

7 Boos, W., Ehmann, U., Bremer, E., Middendorf, A. and Postma, P. (1987) Trehalase in *Escherichia coli*. J. Biol. Chem. 262, 13212–13218.

8 Boos, W., Ehmann, U., Forkl, H., Klein, W., Rimmele, M. and Postma, P. (1990) Trehalose transport and metabolism in *Escherichia coli*. J. Bacteriol. 172, 3450–3461.

9 Booth, I.R. and Higgins, C.F. (1990) Enteric bacteria and osmotic stress: intracellular potassium glutamate as a secondary signal of osmotic stress? FEMS Microbiol. Rev. 6, 239–246.

10 Cairney, J., Booth, I.R. and Higgins, C.F. (1985) Osmoregulation of gene expression in *Salmonella typhimurium*: *proU* encodes an osmotically induced betaine transport system. J. Bacteriol. 164, 1224–1232.

11 Cairney, J., Booth, I.R. and Higgins, C.F. (1985) *Salmonella typhimurium proP* gene encodes a transport system for the osmoprotectant betaine. J. Bacteriol. 164, 1218–1223.

12 Cayley, S., Lewis, B.A., Guttman, H.J. and Record, M.T. (1991) Characterization of the cytoplasm of *Escherichia coli* K-12 as a function of external osmolarity: implications for protein–DNA interactions in vivo. J. Mol. Biol. 222, 281–300.

13 Chambers, S.T. and Kunin, C.M. (1987) Isolation of glycine betaine and proline betaine from human urine. J. Clin. Invest. 79, 731–737.

14 Christian, J.H.B. (1955) The influence of nutrition on the water relations of *Salmonella oranienburg*. Aust. J. Biol. Sci. 8, 75–82.

15 Christian, J.H.B. (1955) The water relations of growth and respiration of *Salmonella oranienburg* at 30°C. Aust. J. Biol. Sci. 8, 490–497.

16 Csonka, L.N. (1981) Proline over-production results in enhanced osmotolerance in *Salmonella typhimurium*. Mol. Gen. Genet. 182, 82–86.

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

18 Csonka, L.N. (1988) Regulation of cytoplasmic proline levels in *Salmonella typhimurium*: effect of osmotic stress on synthesis, degradation, and cellular retention of proline. J. Bacteriol. 170, 2374–2378.
19 Csonka, L.N. (1989) Physiological and genetic responses of bacteria to osmotic stress. Microbiol. Rev. 53, 121–147.
20 Csonka, L.N. and Hanson, A.D. (1991) Prokaryotic osmoregulation: genetics and physiology. Ann. Rev. Microbiol. 45, 569–606.
21 Culham, D.E., Lasby, B., Marangoni, A.G., Milner, J.L., Steer, B.A., van Nues, R.W. and Wood, J.M. (1993) Isolation and sequencing of *Escherichia coli* gene *proP* reveals unusual structural features of the osmoregulatory proline/betaine transporter, ProP. J. Mol. Biol. 229, 268–276.
22 Dandekar, A.M. and Uratsu, S.L. (1988) A single base pair change in proline biosynthesis gene causes osmotic stress tolerance. J. Bacteriol. 170, 5943–5945.
23 Dattananda, C.S., Rajkumari, K. and Gowrishankar, J. (1991) Multiple mechanisms contribute to osmotic inducibility of *proU* operon expression in *Escherichia coli*: demonstration of two osmoresponsive promoters and of a negative regulatory element within the first structural gene. J. Bacteriol. 173, 7481–7490.
24 Dersch, P., Schmidt, K. and Bremer, E. (1993) Synthesis of the *Escherichia coli* K-12 nucleoid-associated DNA-binding protein H-NS is subjected to growth-phase control and autoregulation. Mol. Microbiol. 8, 875–889.
25 Dinnbier, U., Limpinsel, E., Schmid, R. and Bakker, E.P. (1988) Transient accumulation of potassium glutamate and its replacement by trehalose during adaptation of growing cells of *Escherichia coli* K-12 to elevated sodium chloride concentrations. Arch. Microbiol. 150, 348–357.
26 Dosch, D.C., Helmer, G.L., Sutton, S.H., Salvacion, F.F. and Epstein, W. (1991) Genetic analysis of potassium transport loci in *Escherichia coli*: Evidence for 3 constitutive systems mediating uptake of potassium. J. Bacteriol. 173, 687–696.
27 Douglas, R.M., Roberts, J.A., Munro, A.W., Ritchie, G.Y., Lamb, A.J. and Booth, I.R. (1991) The distribution of homologues of the *Escherichia coli* KefC K^+-efflux system in other bacterial species. J. Gen. Microbiol. 137, 1999–2005.
28 Druger-Liotta, J., Prange, V.J., Overdier, D.G. and Csonka, L.N. (1987) Selection of mutants that alter the osmotic control of transcription of the *Salmonella typhimurium proU* operon. J. Bacteriol. 169, 2449–2459.
29 Dulaney, E.L., Dulaney, D.D. and Rickes, E.L. (1968) Factors in yeast extract which relieve growth inhibition of bacteria in defined medium of high osmolarity. Dev. Ind. Microbiol. 9, 260–269.
30 Dunlap, V.J. and Csonka, L.N. (1985) Osmotic regulation of L-proline transport in *Salmonella typhimurium*. J. Bacteriol. 163, 296–304.
31 Epstein, W. (1986) Osmoregulation by potassium transport in *Escherichia coli*. FEMS Microbiol. Rev. 39, 73–78.
32 Epstein, W. and Schultz, S.G. (1965) Cation transport in *Escherichia coli*. V. Regulation of cation content. J. Gen. Physiol. 49, 221–234.
33 Eshoo, M.W. (1988) *lac* fusion analysis of the *bet* genes of *Escherichia coli*: regulation by osmolarity, temperature, oxygen, choline, and glycinebetaine. J. Bacteriol. 170, 5208–5215.
34 Faatz, E., Middendorf, A. and Bremer, E. (1988) Cloned structural genes for the osmotically regulated binding-protein dependent glycine betaine transport system (ProU) of *Escherichia coli* K-12. Mol. Microbiol. 2, 265–279.
35 Falconi, M., Gualtieri, M.T., La Teana, A., Losso, M.A. and Pon, C.L. (1988) Proteins from the prokaryotic nucleoid: primary and quaternary structure of the 15-kD *Escherichia coli* DNA binding protein H-NS. Mol. Microbiol. 2, 323–329.
36 Falconi, M., McGovern, V., Gualerzi, C., Hillyard, D. and Higgins, N.P. (1991) Mutations altering chromosomal protein H-NS induce mini-Mu transposition. New Biol. 3, 615–625.
37 Falkenberg, P. and Strøm, A.R. (1990) Purification and characterization of osmoregulatory betaine aldehyde dehydrogenase of *Escherichia coli*. Biochim. Biophys. Acta 1034, 253–259.
38 Giæver, H.M., Styrvold, O.B., Kaasen, I. and Strom, A.R. (1988) Biochemical and genetic characterization of osmoregulatory trehalose synthesis in *Escherichia coli*. J. Bacteriol. 170, 2841–2849.
39 Göransson, M., Sondén, B., Nilsson, P., Dagberg, B., Forsman, K., Emanuelsson, K. and Uhlin, B.E. (1990) Transcriptional silencing and thermoregulation of gene expression in *Escherichia coli*. Nature 344, 682–685.
40 Gowrishankar, J. (1985) Identification of osmoresponsive genes in *Escherichia coli*: evidence for the participation of potassium and proline transport systems in osmoregulation. J. Bacteriol. 164, 434–445.
41 Gowrishankar, J. (1986) *proP*-mediated proline transport also plays a role in *Escherichia coli* osmoregulation. J. Bacteriol. 166, 331–333.
42 Gowrishankar, J. (1989) Nucleotide sequence of the osmoregulatory *proU* operon of *Escherichia coli*. J. Bacteriol. 171, 1923–1931.
43 Graeme-Cook, K.A., May, G., Bremer, E. and Higgins, C.F. (1989) Osmotic regulation of porin expression: A role for DNA supercoiling. Mol. Microbiol. 3, 1287–1294.
44 Grothe, S., Krogsrud, R.L., McClellan, D.J., Milner, J.L. and Wood, J.M. (1986) Proline transport and osmotic stress response in *Escherichia coli* K-12. J. Bacteriol. 166, 253–259.
45 Gutierrez, C., Barondess, J., Manoil, C. and Beckwith, J. (1987) The use of transposon Tn*phoA* to detect genes for cell envelope proteins subject to a common regulatory stimulus. J. Mol. Biol. 195, 289–297.
46 Gutierrez, C. and Devedjian, J.C. (1991) Osmotic induction of gene *osmC* expression in *Escherichia coli* K12. J. Mol. Biol. 220, 959–973.
47 Gutierrez, J.A., Ardourel, M., Bremer, E., Middendorf,

A., Boos, W. and Ehmann, U. (1989) Analysis and DNA sequence of the osmoregulated *treA* gene encoding the periplasmic trehalase of *Escherichia coli* K12. Mol. Gen. Genet. 217, 347–354.

48 Hengge-Aronis, R., Klein, W., Lange, R., Rimmele, M. and Boos, W. (1991) Trehalose synthesis genes are controlled by the putative sigma factor encoded by *rpoS* and are involved in stationary-phase thermotolerance in *Escherichia coli*. J. Bacteriol. 173, 7918–7924.

49 Higgins, C.F. (1992) ABC transporters: from microorganisms to man. Annu. Rev. Cell Biol. 8, 67–113.

50 Higgins, C.F., Dorman, C.J., Stirling, D.A., Waddell, L., Booth, I.R., May, G. and Bremer, E. (1988) A physiological role for DNA supercoiling in the osmotic regulation of gene expression in *Salmonella typhimurium* and *Escherichia coli*. Cell 52, 569–584.

51 Higgins, C.F., Dorman, C.J. and Ni Bhriain, N. (1990) Environmental influences on DNA supercoiling: a novel mechanism for the regulation of gene expression. In: The Bacterial Chromosome (Drlica, K. and Riley, M., Eds.), pp. 421–432. American Society for Microbiology, Washington, D.C.

52 Higgins, C.F., Hinton, J.C.D., Hulton, C.S.J., Owen-Hughes, T., Pavitt, G.D. and Seirafi, A. (1990) Protein H1: A role for chromatin structure in the regulation of bacterial gene expression and virulence? Mol. Microbiol. 4, 2007–2012.

53 Higgins, C.F., Sutherland, L., Cairney, J. and Booth, I.R. (1987) The osmotically regulated *proU* locus of *Salmonella typhimurium* encodes a periplasmic betaine-binding protein. J. Gen. Microbiol. 133, 305–310.

54 Hulton, C.S.J., Seirafi, A., Hinton, J.C., Sidebotham, J.M., Waddell, L., Pavitt, G.D., Owen-Hughes, T., Spassky, A., Buc, H. and Higgins, C.F. (1990) Histone-like protein H1 (H-NS), DNA supercoiling, and gene expression in bacteria. Cell 63, 631–642.

55 Imhoff, J.F. (1986) Osmoregulation and compatible solutes in eubacteria. FEMS Microbiol. Rev. 39, 57–66.

56 Ingraham, J. (1987) Effect of temperature, pH, water activity and pressure on growth. In: *Escherichia coli* and *Salmonella typhimurium* (Neidhard, F.C., Ed.), pp. 1543–1554.American Society for Microbiology, Washington, D.C.

57 Jebbar, M., Talibart, R., Gloux, K., Bernard, T. and Blanco, C. (1992) Osmoprotection of *Escherichia coli* by ectoine: uptake and accumulation characteristics. J. Bacteriol. 174, 5027–5035.

58 Jovanovich, S.B., Record, M.T.J. and Burgess, R.R. (1989) In an *Escherichia coli* coupled transcription-translation system, expression of the osmoregulated gene *proU* is stimulated at elevated potassium concentrations and by an extract from cells grown at high osmolarity. J. Biol. Chem. 264, 7821–7825.

59 Kaasen, I., Falkenberg, P., Styrvold, O.B. and Strøm, A.R. (1992) Molecular cloning and physical mapping of the *otsBA* genes, which encode the osmoregulatory trehalose pathway of *Escherichia coli*: Evidence that transcription is activated by KatF (AppR). J. Bacteriol. 174, 889–898.

60 Kennedy, E.P. (1987) Membrane-derived oligosaccharides. In: *Escherichia coli* and *Salmonella typhimurium* (Neidhard, F.C., Ed.), pp. 672–679. American Society for Microbiology, Washington, D.C.

61 Klein, W., Ehmann, U. and Boos, W. (1991) The repression of trehalose transport and metabolism in *Escherichia coli* by high osmolarity is mediated by trehalose-6-phosphatase. Res. Microbiol. 142, 359–371.

62 Koo, S.P., Higgins, C.F. and Booth, I.R. (1991) Regulation of compatible solute accumulation in *Salmonella typhimurium* – Evidence for a glycine betaine efflux system. J. Gen. Microbiol. 137, 2617–2625.

63 Laimins, L.A., Rhoads, D.B. and Epstein, W. (1981) Osmotic control of *kdp* operon expression in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 78, 464–468.

64 Lamark, T., Kaasen, I., Eshoo, M.W., Falkenberg, P., McDougall, J. and Strøm, A.R. (1991) DNA sequence and analysis of the *bet* genes encoding the osmoregulatory choline-glycine betaine pathway of *Escherichia coli*. Mol. Microbiol. 5, 1049–1064.

65 Lamark, T., Styrvold, O.B. and Strøm, A.R. (1992) Efflux of choline and glycine betaine from osmoregulating cells of *Escherichia coli*. FEMS Microbiol. Lett. 96, 149–154.

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

67 Larsen, P.I., Sydnes, L.K., Landfald, B. and Strøm, A.R. (1987) Osmoregulation in *Escherichia coli* by accumulation of organic osmolytes: betaines, glutamic acid, and trehalose. Arch. Microbiol. 147, 1–7.

68 Le Rudulier, D., Strøm, A.R., Dandekar, A.M., Smith, L.T. and Valentine, R.C. (1984) Molecular biology of osmoregulation. Science 224, 1064–1068.

69 Leirmo, S., Harrison, C., Cayley, D.S., Burgess, R.R. and Record, M.T.J. (1987) Replacement of potassium chloride by potassium glutamate dramatically enhances protein–DNA interactions in vitro. Biochemistry 26, 2095–2101.

70 Lippert, K. and Galinski, E.A. (1992) Enzyme stabilization by ectoine-type compatible solutes: protection against heating, freezing and drying. Appl. Microbiol. Biotechnol. 37, 61–65.

71 Lucht, J.M. and Bremer, E. (1991) Characterization of mutations affecting the osmoregulated *proU* promoter of *Escherichia coli* and identification of 5′ sequences required for high-level expression. J. Bacteriol. 173, 801–809.

72 May, G., Dersch, P., Haardt, M., Middendorf, A. and Bremer, E. (1990) The *osmZ* (*bglY*) gene encodes the DNA binding protein H-NS (H1a), a component of *Escherichia coli* K12 nucleoid. Mol. Gen. Genet. 224, 81–90.

73 May, G., Faatz, E., Lucht, J.M., Haardt, M., Bolliger, M. and Bremer, E. (1989) Characterization of the osmoregu-

lated *Escherichia coli proU* promoter and identification of ProV as a membrane-associated protein. Mol. Microbiol. 3, 1521–1531.

74 May, G., Faatz, E., Villarejo, M. and Bremer, E. (1986) Binding protein-dependent transport of glycine betaine and its osmotic regulation in *Escherichia coli* K-12. Mol. Gen. Genet. 205, 225–233.

75 McClellan, J.A., Boublikova, P., Palecek, E. and Lilley, D.M.J. (1990) Superhelical tension in cellular DNA responds directly to environmental and genetic factors. Proc. Natl. Acad. Sci. USA 87, 8373–8377.

76 McLaggan, D. and Epstein, W. (1991) *Escherichia coli* accumulates the eukaryotic osmolyte taurine at high osmolarity. FEMS Microbiol. Lett. 65, 209–213.

77 McLaggan, D., Logan, T.M., Lynn, D.G. and Epstein, W. (1990) Involvement of γ-glutamyl peptides in osmoadaptation of *Escherichia coli*. J. Bacteriol. 172, 3631–3636.

78 Measures, J.C. (1975) Role of amino acids in osmoregulation of non-halophilic bacteria. Nature 257, 398–400.

79 Meury, J. (1988) Glycine betaine reverses the effects of osmotic stress on DNA replication and cellular division in *Escherichia coli*. Arch. Microbiol. 149, 232–239.

80 Milner, J.L., Grothe, S. and Wood, J.M. (1988) Proline porter II is activated by a hyperosmotic shift in both whole cells and membrane vesicles of *Escherichia coli* K12. J. Biol. Chem. 263, 14900–14905.

81 Milner, J.L., McClellan, D.J. and Wood, J.M. (1987) Factors reducing and promoting the effectiveness of proline as an osmoprotectant in *Escherichia coli* K12. J. Gen. Microbiol. 133, 1851–1860.

82 Mimmack, M.L., Gallagher, M.P., Pearce, S.R., Hyde, S.C., Booth, I.R. and Higgins, C.F. (1989) Energy coupling to periplasmic binding protein-dependent transport systems: stoichiometry of ATP hydrolysis during transport in vivo. Proc. Natl. Acad. Sci. USA 86, 8257–8261.

83 Nakashima, K., Sugiura, A., Kanamaru, K. and Mizuno, T. (1993) Signal transduction between the two regulatory components involved in the regulation of the *kdpABC* operon in *Escherichia coli*: phosphorylation-dependent functioning of the positive reulator, KdpE. Mol. Microbiol. 7, 109–116.

84 Ni Bhriain, N., Dorman, C.J. and Higgins, C.F. (1989) An overlap between osmotic and anaerobic stress responses: a potential role for DNA supercoiling in the coordinate regulation of gene expression. Mol. Microbiol. 3, 933–942.

85 Nikaido, H. and Vaara, M. (1987) Outer membrane. In: *Escherichia coli* and *Salmonella typhimurium* (Neidhard, F.C., Ed.), pp. 7–22. American Society for Microbiology, Washington, D.C.

86 Overdier, D.G. and Csonka, L.N. (1992) A transcriptional silencer downstream of the promoter in the osmotically controlled *proU* operon of *Salmonella typhimurium*. Proc. Natl. Acad. Sci. USA 89, 3140–3144.

87 Overdier, D.G., Olson, E.R., Erickson, B.D., Ederer, M.M. and Csonka, L.N. (1989) Nucleotide sequence of the transcriptional control region of the osmotically regulated *proU* operon of *Salmonella typhimurium* and identification of the 5′ endpoint of the *proU* mRNA.J. Bacteriol. 171, 4694–4706.

88 Owen-Hughes, T.A., Pavitt, G.D., Santos, D.S., Sidebotham, J.M., Hulton, C.S.J., Hinton, J.C.D. and Higgins, C.F. (1992) The chromatin-associated protein H-NS interacts with curved DNA to influence DNA topology and gene expression. Cell 71, 255–265.

89 Park, S.F., Stirling, D.A., Hulton, C.S.J., Booth, I.R., Higgins, C.F. and Stewart, G.S.A.B. (1989) A novel, non-invasive promoter probe vector: Cloning of the osmoregulated *proU* promoter of *Escherichia coli* K12. Mol. Microbiol. 3, 1011–1023.

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

91 Plaskon, R.R. and Wartell, R.M. (1987) Sequence distributions associated with DNA curvature are found upstream of strong *Escherichia coli* promoters. Nucleic Acids Res. 15, 785–796.

92 Pollard, A. and Wyn Jones, R.G. (1979) Enzyme activities in concentrated solutions of glycine betaine and other solutes. Planta 144, 291–298.

93 Prince, W.S. and Villarejo, M.R. (1990) Osmotic control of *proU* transcription is mediated through direct action of potassium glutamate on the transcription complex. J. Biol. Chem. 265, 17673–17679.

94 Ramirez, R.M., Prince, W.S., Bremer, E. and Villarejo, M. (1989) In vitro reconstitution of osmoregulated expression of *proU* of *Escherichia coli*. Proc. Natl. Acad. Sci. USA 86, 1153–1157.

95 Ramirez, R.M. and Villarejo, M. (1991) Osmotic signal transduction to *proU* is independent of DNA supercoiling in *Escherichia coli*. J. Bacteriol. 173, 879–885.

96 Richey, B., Cayley, D.S., Mossing, M.C., Kolka, C., Anderson, C.F., Farrar, T.C. and Record, M.T.J. (1987) Variability of the intracellular ionic environment of *Escherichia coli*. J. Biol. Chem. 262, 7157–7164.

97 Roth, W.G., Leckie, M.P. and Dietzler, D.N. (1985) Osmotic stress drastically inhibits active transport of carbohydrates by *Escherichia coli*. Biochem. Biophys. Res. Commun. 126, 434–441.

98 Sanderson, K.E. and Roth, J.R. (1988) Linkage map of *Salmonella typhimurium*. Edition VII. Microbiol. Rev. 52, 485–532.

99 Santoro, M.M., Liu, Y., Khan, S.M.A., Hou, L.-H. and Bolen, D.W. (1992) Increased thermal stability of proteins in the presence of naturally occurring osmolytes. Biochemistry 31, 5278–5283.

100 Severin, J., Wohlfarth, A. and Galinski, E.A. (1992) The predominant role of recently discovered tetrahydropyrimidines for the osmoadaptation of halophilic eubacteria. J. Gen. Microbiol. 138, 1629–1638.

101 Siebers, A. and Altendorf, K. (1993) K^+-translocating Kdp-ATPases and other bacterial P-type ATPases. In: Alkali Cation Transport Systems in Prokaryotes (Bakker, E.P., Ed.), pp. 225–251. CRC Press, Boca Raton, FL.

102 Spassky, A., Rimsky, S., Garreau, H. and Buc, H. (1984)

H1a, an *Escherichia coli* DNA-binding protein which accumulates in stationary phase, strongly compacts DNA in vitro. Nucleic Acids Res. 12, 5321–5340.

103 Spurio, R., Durrenberger, M., Falconi, M., La, T.A., Pon, C.L. and Gualerzi, C.O. (1992) Lethal overproduction of the *Escherichia coli* nucleoid protein H-NS: ultramicroscopic and molecular autopsy. Mol. Gen. Genet. 231, 201–211.

104 Stalmach, M.E., Grothe, S. and Wood, J.M. (1983) Two proline porters in *Escherichia coli* K-12. J. Bacteriol. 156, 481–486.

105 Stirling, D.A., Hulton, C.S.J., Waddell, L., Park, S.F., Stewart, G.S.A.B., Booth, I.R. and Higgins, C.F. (1989) Molecular characterization of the *proU* loci of *Salmonella typhimurium* and *Escherichia coli*: the osmoregulated glycin betaine transport systems. Mol. Microbiol. 3, 1025–1038.

106 Stock, J.B., Rauch, B. and Roseman, S. (1977) Periplasmic space in *Salmonella typhimurium* and *Escherichia coli*. J. Biol. Chem. 252, 7850–7861.

107 Strøm, A.R., Falkenberg, P. and Landfald, B. (1986) Genetics of osmoregulation in *Escherichia coli*: uptake and biosynthesis of organic osmolytes. FEMS Microbiol. Rev. 39, 79–86.

108 Strøm, A.R. and Kaasen, I. (1993) Trehalose metabolism in *Escherichia coli*: stress protection and stress regulation of gene expression. Mol. Microbiol. 8, 205–210.

109 Styrvold, O.B., Falkenberg, P., Landfald, B., Eshoo, M.W., Bjørnsen, T. and Strøm, A.R. (1986) Selection, mapping and characterization of osmoregulatory mutants of *Escherichia coli* blocked in the choline-glycine betaine pathway. J. Bacteriol. 165, 856–863.

110 Styrvold, O.B. and Strøm, A.R. (1991) Synthesis, accumulation, and excretion of trehalose in osmotically stressed *Escherichia coli* K-12 strains: influence of amber suppressors and function of the periplasmic trehalase. J. Bacteriol. 173, 1187–1192.

111 Sugiura, A., Nakashima, K., Tanaka, K. and Mizuno, T. (1992) Clarification of the structural and functional features of the osmoregulated *kdp* operon of *Escherichia coli*. Mol. Microbiol. 6, 1769–1776.

112 Sutherland, L., Cairney, J., Elmore, M.J., Booth, I.R. and Higgins, C.F. (1986) Osmotic regulation of transcription: induction of the *proU* betaine transport gene is dependent on accumulation of intracellular potassium. J. Bacteriol. 168, 805–814.

113 Tanaka, K., Muramatsu, S., Yamada, H. and Mizuno, T. (1991) Systematic characterization of curved DNA segments randomly cloned from *Escherichia coli* and their functional significance. Mol. Gen. Genet. 226, 367–376.

114 Tombras Smith, L., Pocard, J.-A., Bernard, T. and Le Rudulier, D. (1988) Osmotic control of glycine betaine biosynthesis and degradation in *Rhizobium meliloti*. J. Bacteriol. 170, 3142–3149.

115 Ueguchi, C. and Ito, K. (1992) Multicopy suppression: an approach to understanding intracellular functioning of the protein export system. J. Bacteriol. 174, 1454–1461.

116 Ueguchi, C. and Mizuno, T. (1993) The *Escherichia coli* nucleoid protein H-NS functions directly as a transcriptional repressor. EMBO J. 12, 1039–1046.

117 Van Alphen, W. and Lugtenberg, B. (1977) Influence of osmolarity of the growth medium on the outer membrane protein pattern of *Escherichia coli*. J. Bacteriol. 131, 623–630.

118 Walderhaug, M.O., Polarek, J.W., Voelkner, P., Daniel, J.M., Hesse, J.E., Altendorf, K. and Epstein, W. (1992) KdpD and KdpE, proteins that control expression of the *kdpABC* operon, are members of the two-component sensor–effector class of regulators. J. Bacteriol. 174, 2152–2159.

119 Wang, J.Y. and Syvanen, M. (1992) DNA twist as a transcriptional sensor for environmental changes. Mol. Microbiol. 6, 1861–1866.

120 Wood, J.M. (1988) Proline porters effect the utilization of proline as nutrient or osmoprotectant for bacteria. J. Membrane Biol. 106, 183–202.

121 Yancey, P.H., Clark, M.E., Hand, S.C., Bowlus, R.D. and Somero, B.N. (1982) Living with water stress: evolution of osmolyte systems. Science 217, 1214–1222.