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Osmotic Regulation of L-Proline Transport in *Salmonella typhimurium*

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The transport of proline is important for the adaptation of *Salmonella typhimurium* to osmotic stress because exogenous proline permits the growth of the organism in media of elevated osmotic strength that would otherwise be toxic. Measurements of the rate of [^{3}H]proline transport in *S. typhimurium* indicated that the organism has two distinct proline permeases, the ProU and the ProP systems, whose activities increase more than fivefold as a consequence of growth in media containing 0.3 M NaCl or 0.47 M sucrose. Transport via a third proline permease, the PutP system, is not affected by the osmotic strength of the medium. We constructed strains that carry fusions of *lacZ* to *proU* or *proP*, genes that are required for the two osmotically stimulated proline transport systems. Assays of β-galactosidase revealed that the transcription of the *proU* gene is increased more than 10-fold as a result of exposure of the cells to 0.3 M NaCl, 0.47 M sucrose, or equivalent concentrations of other solutes that are not freely diffusible across the cytoplasmic membrane. Increased transcription cannot be triggered by methanol, ethanol, and glycerol, substances that are freely diffusible across the membrane, suggesting that the signal for increased transcription might be an osmotic gradient across the cytoplasmic membrane. The *proP* gene does not show transcriptional regulation of sufficient magnitude to account for the stimulation of [^{3}H]proline transport. Thus, the osmotic stimulation of the ProP system might be mediated by some posttranscriptional event.

In addition to serving as a carbon or nitrogen source and a constituent of proteins, L-proline has a third, curious biological function. It is accumulated to high intracellular levels under conditions of osmotic stress in bacteria (6, 30), plants (19, 37, 38, 42), and animals (20). It has been suggested that the accumulation of proline may be beneficial for adaptation to osmotic stress because proline might be an osmotic balancer, acting to forestall the loss of water from the cells (19).

In *Salmonella typhimurium* and other enteric bacteria, the synthesis of proline is not stimulated by osmotic stress (13, 33), but these organisms can accumulate this imino acid to high levels during osmotic stress by transport from the medium (6, 9, 10, 14). The importance of the transport of proline for osmoregulation has been underscored by the observation that exogenous proline specifically stimulates the growth of some bacteria in media of inhibitory osmotic strength (9, 10, 13).

S. typhimurium possesses three proline transport systems. The first, the PutP system, is required for the utilization of proline as a carbon or nitrogen source, and it is subject to catabolite repression and induction by proline (36). This transport system probably does not play a major role in the accumulation of proline during osmotic stress because mutants that lack it show normal stimulation of growth by proline under conditions of osmotic stress (14). The second proline permease, the ProP system, is important for osmoregulation because mutations inactivating it cause approximately a twofold reduction in the stimulatory effect of proline in media of elevated osmotic strength (14). The residual stimulation by proline under osmotic stress in mutants lacking the ProP system is due to the third proline permease, the ProU system. This system does not function in cells grown in media of normal osmolarity, but only under conditions of osmotic stress (14).

The existence of the ProU system has been inferred from the patterns of sensitivity to proline analogs of mutants lacking the PutP and the ProP systems. Such double mutants are resistant to the proline analogs L-azetidine-2-carboxylate (Azt) and 3,4-dehydro-DL-proline (Dhp) in the normal media, but they become sensitive to both in media of elevated osmotic strength (14). It was suggested that this increased sensitivity is the result of the stimulation of the activity of the ProU system under conditions of osmotic stress (14). However, the presence of this transport system has not been demonstrated by direct assays of proline transport activity. In this publication, we present results showing that the rate of proline transport in *S. typhimurium* is stimulated by osmotic stress and that this stimulation is due to the enhancement of two proline permeases, the ProP and the ProU transport systems.

The stimulation of transport could result from increased transcription of the genes of the two permeases or from some posttranscriptional event. To distinguish between these two possibilities, we constructed strains carrying phage Mu dl(Amp^r *lac*) insertions (8) such that the *lacZ* gene within the Mu dl phage has been placed under the transcriptional control of the *proP* or *proU* promoters. We also present results of measurements of the β-galactosidase levels in these strains grown under various conditions of osmotic stress. Our results imply that although osmotic stress causes a rapid increase in the transcription of the *proU* gene, it does not greatly affect the transcription of the *proP* gene. Thus, the osmotic stimulation of the activity of the ProU and ProP transport systems may be brought about by two very different mechanisms.

MATERIALS AND METHODS

Bacterial media and culture conditions. The rich medium used was LB (17). The minimal medium was M63 (11). Culture conditions used were described in reference 14, except that strains lysogenic for phage Mu dl(Amp^r *lac*)

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were grown at 30°C. When used, the Azt concentration was 1 mM; the Dhp concentration was 0.3 mM; the 5-bromo-4-chloro-3-indolyl-β-D-galactosidase concentration was 40 mg/liter; the sodium ampicillin concentration was 25 mg/liter, the chloramphenicol concentration was 12.5 mg/liter; the tetracycline concentration was 15 mg/liter; and the kanamycin sulfate concentration was 75 mg/liter.

Bacterial strains. The bacterial strains used are presented in Table 1. Generalized transductions were carried out with phage P22 HT105/1 *int-201*, as described in reference 17. Phage Mu dl(Amp^r *lac*) lysogens were constructed by infecting phage P1-sensitive (P1^s) *S. typhimurium* strains with defective phage Mu dl that was grown with the helper phage Mu *c*ts62 *h*P1#1, having the host range of phage P1 (15). P1^s *S. typhimurium* mutants were obtained by plating 4×10^8 cells of the parental P1-resistant strains on a lawn of 10^9 PFU of phage F0 on galactose MacConkey (Difco Laboratories, Detroit, Mich.) plates containing 5 mM $CaCl_2$. Mutants resistant to phage F0 that are also unable to ferment galactose (*galE* or *galU*) are sensitive to phage P1 (35). These mutants were identified by their white colony color. Strains TL200 and T1212, thus obtained from strains TL145 and TL135, respectively, proved to be sensitive to phages P1 and Mu *c*ts62 *h*P1#1.

The isolation of strains with *proP*::Mu dl insertions was based on the fact that in M63, *proP*$^+$ Δ*putPA* strains are sensitive to the proline analog Dhp, but *proP* derivatives are resistant (14). Strain TL212 (Δ*putPA galE* or *galU*) was infected with a Mu dl/Mu *c*ts62 *h*P1#1 lysate, and ampicillin-resistant (Amp^r) transductants were replica plated to M63 glucose plates containing Dhp and to M63 glucose plates. After 16 to 24 h, colonies resistant to Dhp were detectable at the approximate frequency of 2 per 10^4 Amp^r colonies. Twenty independent colonies (strains TL356 to TL375), picked from the M63 glucose master plates, were saved. As a preliminary test of whether these strains carried a Mu dl-induced *proP* mutation, we checked whether the mutations causing Dhp^r were linked to the *melA361*::Tn*10* insertion, which has been shown to be 20 to 40% cotransducible with previously characterized *proP* mutations (31). Strains TL356 to TL375 were transduced to Tet^r by phage P22 grown on strain TT16721 (*melA361*::Tn*10*), and 20 transductants obtained with each recipient were tested for Dhp^s and Amp^s. Depending on the recipient, 25 to 70% of the transductants became Dhp^s Amp^s, indicating that strains TL356 to TL375 carry a single Mu dl insertion in the *proP* gene or in a nearby gene of associated function.

The basis of the construction of *proU*::Mu dl insertions was the observation that *proU*$^+$ *proP* Δ*putPA* strains, which are resistant to the proline analogs Dhp and Azt in M63, are sensitive to both in M63 containing ≥0.3 M NaCl, but *proU* derivatives are resistant to both analogs even in media of elevated osmolarity (14). For the selection, strain TL200 (*proU* Δ*putPA galE* or *galU*) was infected with Mu dl, and Amp^r colonies were replica plated to M63 glucose containing Azt and 0.3 M NaCl and to M63 glucose. Colonies resistant to Azt in the presence of 0.3 M NaCl appeared after 3 days at the approximate frequency of 2 per 10^4 lysogens. Eighteen independent mutants that were Azt^r with 0.3 M NaCl (strains TL333 to TL350) were picked from the M63 glucose master plates and saved. The location of the Mu dl insertion was determined by mapping against the *proU1655*::Tn*10* insertion, but the resident Tn*10* in the strains had to be removed first (4). A Tet^s derivative obtained from each strain was transduced to Tet^r by P22 grown on strain TL187 (*proU1655*::Tn*10*). With each recipient, 10 transductants that were tested proved to have become Amp^s, indicating that the Mu dl insertions in strains TL333 to TL350 are probably in the *proU* gene or in a nearby gene of related function.

Because the Mu dl carries a temperature-sensitive mutation (*c*ts62) in the repressor gene, lysogens have a growth defect above 37°C, and the phage transposes at a high frequency to new sites, even at 30°C. Both of these problems can be ameliorated by the introduction of a Tn*9* insertion into the phage *B* gene (3; P. Blum, L. Blaha, R. Shand, D. Holzschu, and S. Artz, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, H 128, p. 134). Strains TL384, TL386, and TL388 (*proP*::Mu dl *B*::Tn*9*) and strains TL378, TL380, and TL382 (*proU*::Mu dl *B*::Tn*9*) are Cml^r transductants of strains TL361, TL370, TL372, TL333, TL343, and TL346, respectively, by P22 grown on strain AZ1430 [F::Mu dl *B*::Tn*9* (Amp^r Cml^r *lac*)]. The *B*::Tn*9* insertion restored normal growth to the lysogens at 42°C, and as was noted by Baker et al. (3), it reduced but did not completely eliminate the ability of the phage to transpose to new sites (see below).

To confirm further that Mu dl *B*::Tn*9* insertions in the above transductants were in fact in the *proP* or *proU* genes, the fusions were backcrossed into new *proP*$^+$ or *proU*$^+$ strains, respectively. Phage P22 grown on strains TL384, TL386, and TL388 (*proP*::Mu dl *B*::Tn*9*) was used to transduce strain TL135 (Δ*putPA*) to Amp^r. In each case, >99% of the transductants were also Cml^r and able to grow at 42°C. However, faithful inheritance of the *proP*::Mu dl *B*::Tn*9* insertion was rare. Only 0.3 to 1% of the transductants also became Dhp^r. When tested on lactose MacConkey plates, the Amp^r Dhp^s transductants exhibited a range of phenotypes from Lac$^-$ to fully Lac$^+$, indicating that the phage had transposed to new loci during the transduction. Nevertheless, with each of the donor strains TL384, TL386, and TL388, we obtained Dhp^r transductants, designated as strains TL423 to TL425, respectively. In P22 transduction, the Dhp^r, Amp^r, and Cml^r characters in strains TL423, TL424, and TL425 were lost simultaneously at the approximate frequency of 80% upon the inheritance of the *melA361*::Tn*10* allele.

The *proU*::Mu dl *B*::Tn*9* insertions in strains TL378, TL380, and TL382 were transduced with similar complications into strain TL179 (*proP* Δ*putPA*). The desired *proU*::Mu dl *B*::Tn*9* *proP* Δ*putPA* transductants, strains TL390, TL391, and TL393, were identified by their Azt^r phenotype in the presence of 0.3 M NaCl. The Amp^r and Cml^r phenotypes in the latter three strains were lost at >98% frequency upon the inheritance of the *proU1655*::Tn*10* allele in P22 transduction.

Since faithful transduction of the *proP* or *proU*::Mu dl *B*::Tn*9* insertions proved to be a rare event, we were concerned whether a deletion or other rearrangement fusing the *lacZ* gene to an unknown promoter might not have occurred during the construction of the few *proP* or *proU* transductants that were obtained. This is an important consideration, especially in the case of the presumed *proU*::Mu dl *B*::Tn*9* mutants, because the synthesis of β-galactosidase in these strains appears to be nearly constitutive (see below). To ascertain that the mutation in these strains was due to a simple insertion of phage Mu dl *B*::Tn*9* into the *proP* gene, we made use of the fact that the *B*::Tn*9* insertion enables the reversion of Mu dl insertions by a perfect excision of the phage (P. Blum, L. Blaha, R. Shand, D. Holzchu, and S. Artz, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, H 128, p. 134).

Proline auxotrophic strains that are also deficient in the

TABLE 1. Bacterial strains used[a]

Strain	Genotype				Source
	proP	*proU*	*putPA*	Others	
AZ1430	+	+	+	*pyrF146* Δ(*trp*)-43 *leu-500 recA1 srlC2*::Tn*10*/ F′::Mu d1 *B*^{x}::Tn*9*(Amp^{r} Cml^{r} *lac*)	P. H. Blum
TL1	+	+	+	+	Wild-type *S. typhimurium* LT2 Ames, from J. L. Ingraham
TL135	+	+	Δ(*putPA*)*557*	*zcc-628*::Tn*5*	This laboratory (14)
TL145	*proP673*	+	Δ(*putPA*)557	*zcc-628*::Tn*5 zjd-27*::Tn*10*	This laboratory (14)
TL154	+	+	+	*recA1 srlC2*::Tn*10 galE496 metA22 metE55 ilv(?) xyl-404 rpsL120* H1-*bnml* H2-*enx hsdL6 hsdSA29* Fels^{-} [Mu d1 (Amp^{r} *lac*) Mu cts62 *h*P1]	This laboratory (15)
TL179	*proP1654*	+	Δ(*putPA*)*557*	*zcc-628*::Tn*5*	This laboratory (14)
TL187	*proP1652*	*proU1655*::Tn*10*	Δ(*putPA*)*557*	*zcc-628*::Tn*5*	This laboratory (14)
TL195	*proP1654*	*proU1655*::Tn*10*	Δ(*putPA*)*557*	*zcc-628*::Tn*5*	This laboratory (14)
TL197	*proP1654*	+	+	+	This laboratory (14)
TL199	*proP1654*	*proU1655*::Tn*10*	*putP*$^{+}$*A842*::Tn*5*	+	This laboratory (14)
TL200	*proP675*	+	Δ(*putPA*)*557*	*galE* or *galU zcc-628*::Tn*5 zjd-27*::Tn*10*	Phage F0^{r} phage P1^{s} derivative of TL145; see text
TL201	*proP1654*	*proU1655*::Tn*10*	+	+	P22 · TL187 → TL197 = Tet^{r}
TL212	+	+	Δ(*putPA*)*557*	*galE* or *galU zcc-628*::Tn*5*	Phage F0^{r} phage P1^{s} derivative of TL135; see text
TL333–TL350	*proP675*	*proU1871-proU1888*::Mu d1	Δ(*putPA*)*557*	*galE* or *galU zcc-628*::Tn*5 zjd-27*::Tn*10*	Mu d1 · TL154 → TL200 = Amp^{r} Azt^{r} on M63 + 0.3 M NaCl; see text
TL356–TL375	*proP1889-proP1908*::Mu d1	+	Δ(*putPA*)*557*	*galE* or *galU zcc-628*::Tn*5*	Mu d1 · TL154 → TL212 = Amp^{r} Dhp^{r}; see text
TL378, TL380, TL382	*proP675*	*proU1871*, *proU1881*, *proU1884*::Mu d1 *B*::Tn*9* (Amp^{r} Cml^{r} *lac*)	Δ(*putPA*)*557*	*galE* or *galU zcc-628*::Tn*5 zjd-27*::Tn*10*	P22 · AZ1430 → TL333, TL343, TL346 = Cml^{r}, 42°C^{r}
TL384, TL386, TL388	*proP1894*, *proP1903*, *proP1905*::Mu d1 *B*::Tn*9*	+	Δ(*putPA*)*557*	*galE* or *galU zcc-628*::Tn*5*	P22 · AZ1430 → TL361, TL370, TL372 = Cml^{r} 42°C^{r}; see text
TL390, TL391, TL393,	*proP1654*	*proU1871*, *proU1881*, *proU1884*::Mu d1 *B*::Tn*9*	Δ(*putPA*)*557*	*zcc-628*::Tn*5*	P22 · TL378, TL380, TL382 → TL179 = Amp^{r} Cml^{r} Azt^{r} on M63 + 0.3 M NaCl 42°C^{r}; see text
TL423, TL424, TL425	*proP1894*, *proP1903*, *proP1905*::Mu d1 *B*::Tn*9*	+	Δ(*putPA*)*557*	*zcc-68*::Tn*5*	P22 · TL384, TL386, TL388 → TL135 = Amp^{r} Cml^{r} Dhp^{r} 42°C^{r}; see text
TL472, TL474, TL476	*proP1894*, *proP1903*, *proP1905*::Mu d1 *B*::Tn*9*	*proU1655*::Tn*10*	Δ(*putPA*)*557*	*zcc-628*::Tn*5*	P22 · TL187 → TL423, TL424, TL425 = Tet^{r}
TL478, TL480, TL482	*proP*$^{+}$/*proP1894*, *proP1903*, *proP1905*::Mu d1 *B*::Tn*9*	+	Δ(*putPA*)*557*	*zcc-628*::Tn*5 mel*$^{+}$/ *melA361*::Tn*10*	P22 · TT1672 → TL423, TL424, TL425 = Tet^{r} Mel^{+} Dhp^{s} Amp^{r} Cml^{r} Lac^{+}; strains carry duplications of the *melA-proP* region; see text (1).
TT1672	+	+	+	*melA361*::Tn*10*	J. R. Roth (31)

[a] All strains are derived from *S. typhimurium* LT2. The abbreviations P22 · $x \rightarrow y = a, b, c$ denotes that a phage P22 lysate grown on strain x was used to transduce strain y, selecting phenotype a, and screening phenotypes b, c.

PutP and the ProP systems require abnormally high concentrations (≥0.5 mM) of proline for rapid growth, whereas derivatives with a functional proline transport system grow well with 0.1 mM proline (14, 36). We introduced a *proB*::Tn*10* mutation into strains TL423 to TL425 (*proP*::Mu d1 *B*::Tn*9* Δ*putP*) and used the resultant transductants to select derivatives that were able to grow on M63 5-bromo-4-chloro-3-indolyl-β-D-galactosidase tetracycline plates containing 0.1 mM proline. Such colonies appeared at the frequency of 10^{-6}. Of these, only 0.1 to 1% were white (Lac^-), but we obtained Lac^- derivatives with each strain tested. These were restreaked on the selective medium and tested for growth on LB ampicillin and LB chloramphenicol with the result that each was Amp^s and Cml^s. These results show that it is possible to revert the *proP1894*::Mu d1 *B*::Tn*9*, *proP1903*::Mu d1 *B*::Tn*9*, and *proP1905*::Mu d1 *B*::Tn*9* insertions by perfect excision of the phage. Thus, these *proP* alleles are simple Mu d1 *B*::Tn*9* insertions, and therefore, the *lacZ* gene in strains TL423 to TL425 is transcribed from the *proP* promoter. (We did not analyze the majority class of strains that formed blue colonies in the above selection. These strains were still Amp^r Cml^r, so presumably they were still *proP*::Mu d1. They might be strains that express the ProU system constitutively at a high level [18; J. Druger-Liotta and L. N. Csonka, unpublished data], strains with alterations in the specificity of some permease enabling the rapid uptake of proline, or strains carrying suppressors of the proline auxotrophy [22]).

By an analogous procedure, we confirmed the structure of the presumed *proU*::Mu d1 *B*::Tn*9* insertions in strains TL390, TL391, and TL393. We transduced the strains to *proB*::Tn*10* and selected derivatives able to grow on M63 5-bromo-4-chloro-3-indolyl-β-D-galactoside tetracycline medium containing 0.1 mM proline and 0.3 M NaCl. Such derivatives arose at the approximate frequency of 10^{-6}. About 0.1% of the colonies that appeared were $proU^+$ strains that lost the Mu d1 phage by proper excision, as judged by the fact that they were Lac^- Amp^s Cml^s. (The remainder of the colonies that were Lac^+ might be strains with suppressors of the proline biosynthetic on transport defects or revertants of the *proP* mutation.)

Strains heterozygous for $proP^+$ and *proP*::Mu d1 insertions were obtained as derivatives with a chromosomal duplication in the *melA-proP* region. A small fraction of cells in cultures of *S. typhimurium* carry spontaneous duplications of various segments of the chromosome (1). Strains TL423 to TL425 (*proP*::Mu d1) were transduced to Tet^r on LB tetracycline plates by phage P22 grown on strain TT1672 (*melA*::Tn*10*), and the transductants obtained were replica plated to M63 melibiose plates. About 1% of the progeny proved to be Mel^+. These may be *melA*::Tn*10*/mel^+ heterozygotes that carry a duplication, or they may be transductants in which the Tn*10* has moved to a new site (1). Strains which carried a duplication that included the *proP* gene and which coinherited the $proP^+$ allele with the *melA*::Tn*10* were recognized by being simultaneously Dhp^s Amp^r Lac^+. The frequency of such strains was ca. 5% of that of the Mel^+ Tet^r transductants. One characteristic of chromosomal duplications is their instability due to homologous recombination (1). At a frequency of 0.1 to 1%, the presumed *melA*::Tn*10* $proP^+$/mel^+ *proP*::Mu d1 heterozygotes segregated derivatives that were Lac^- Amp^s Dhp^s ($proP^+$ haploid), Dhp^r Amp^r Lac^+ (*proP*::Mu d1 haploid), or Tet^s Mel^+ (mel^+ haploid). Because of this instability, the cells were grown before assays in M63 tetracycline and ampicillin with melibiose as the carbon source.

Assays of β-galactosidase. Strains were grown overnight in M63 glucose, diluted to the density of 5×10^7 cells per ml into the various media to be tested, and grown to the density of 4×10^8 cells per ml. The cells were sedimented by centrifugation at room temperature and taken up to the original volume in Z buffer (32), and β-galactosidase was assayed at 24°C as described by Miller (32), except that the results were expressed as nanomoles of product formed per minute per milligram of protein, with the protein concentration determined by the method of Lowry et al. (28).

Proline transport assays. We used flow dialysis (12) to measure the rate of proline uptake. We carried out the assays as described by Dankert et al. (16) with some minor modifications. The cells were grown overnight in M63 plus 20 mM glucose and the indicated concentrations of NaCl or sucrose, and then they were subcultured at a 10-fold dilution into fresh medium of the same composition and allowed to double 1.5 times to an approximate cell density of 10^9 cells per ml. For the assay, 0.96 ml of the cultures was introduced into the upper chamber of the flow dialysis apparatus, and unless stated otherwise, chloramphenicol was added to a final concentration of 100 μg/ml. After 5 min, 5 μCi of [^{3}H]proline (ICN Radiochemicals, Inc., Irvine, Calif.) together with carrier proline at a final concentration of 20 μM was added, bringing the final volume to 1.00 ml. The assays were done at 24°C, with the cells aerated by vigorous stirring. The volume of the lower chamber of the flow dialysis apparatus was 2.8 ml. Medium, which was always identical to that in which the cells were grown and assayed in, was pumped through the lower chamber at the rate of 4.8 ml/min. Effluent fractions were collected at 0.5- or 1-min intervals. A 0.4-ml sample from each fraction was mixed with scintillation fluid, and the radioactivity was measured by a scintillation counter. The radioactivity in each sample was plotted against time, generating a curve that rises sharply to a peak during the first 4 to 5 min, due to the lag time required to pump the sample from the flow dialysis apparatus into the fraction collector tubes. After the peak, the curve decreases linearly for at least the next 5 min, due to the uptake of substrate from the medium by the cells (see Fig. 1). The transport rate is proportional to the slope of the curve during the linear portion of the decay, with the constant of proportionality being dependent on the specific activity of the substrate and the permeability properties of the particular dialysis membrane used. To take the latter into account, the slope of the linear portion of the curve was divided by the height of the curve at the peak, which is a measure of the initial rate of diffusion of proline across the membrane. The transport rate was obtained by multiplying the value thus obtained by the initial amount of proline in the upper chamber, 20 nmol. The results were expressed as nanomoles of substrate taken up from the medium per minute per milligram of protein, with the protein measured by the method of Lowry et al. (28).

RESULTS

Osmotic stress stimulates [^{3}H]proline uptake via the ProP and ProU transport systems. We measured the rate of [^{3}H]proline transport by cells grown in M63 or M63 containing 0.3 M NaCl (Table 2). In strains that are proficient for all three proline permeases (TL1 and TL106), the rate of proline transport increases four- to sixfold as a consequence of growth in the presence of 0.3 M NaCl. Osmotic stimulation of proline transport occurs in mutants that possess the ProP system (strain TL192), the ProU system (strain TL179), or

both (strain TL135). Proline transport is reduced to background levels in the mutant that lacks all three transport systems (strain TL195) both in M63 and in M63 plus 0.3 M NaCl. These results imply that *S. typhimurium* has two distinct proline permeases, the ProP and the ProU, whose activity is enhanced by osmotic stress. The third proline permease, the PutP system, is not regulated by osmotic stress; the transport activity of mutants possessing only that system (strains TL199 and TL201) is unaffected by the presence of 0.3 M NaCl (Table 2). The *putA* gene product, which has been proposed to be the regulatory protein for the transcription of the *putP* gene (29), is not involved in the osmotic control of the proline transport systems, because the rate of proline transport in the presence of 0.3 M NaCl is not affected substantially by a *putA* mutation.

Since the technique of flow dialysis measures transport indirectly, the question might arise whether the apparent increase in proline uptake in the *proP*$^+$ or *proU*$^+$ strains in the presence of 0.3 M NaCl is truly due to the stimulation of two active transport systems or whether it could be explained by some experimental artifact, such as a change in the diffusion properties of the dialysis membrane. To address this point, we performed transport assays in the presence of 0.3 M NaCl and 20 μM carbonyl-cyanide *m*-chlorophenylhydrazone added 10 or 13 min after the start of the assay (Fig. 1). The rapid exit of [^{3}H]proline from the cells could be detected within 3 min after the addition of carbonyl-cyanide *m*-chlorophenylhydrazone. The proP and ProU transport systems are also sensitive to KCN. At 2 mM, this poison inhibited transport via the former system by ca. 50% and via the latter system by ca. 95% (data not shown). Thus, the uptake of proline via both systems is an active transport process driven by the proton motive force.

The results in Table 2 were obtained in assays in which the proline concentration was 20 μM. A limited number of measurements were carried out with the substrate concentration at 200 μM. At this higher substrate concentration, the transport rate in the wild-type strain TL1 was 3.5 nmol/min per mg in M63 and 27.8 nmol/min per mg in M63 containing 0.3 M NaCl. In strain TL179 (*proU*$^+$ *proP putP*) it was 15.2 nmol/min per mg in M63 plus 0.3 M NaCl. Since in the flow dialysis technique the transport rate is calculated from the relative rate of disappearance of the substrate, and since small changes in the diffusion rate are more difficult to measure when the diffusion rates are high, the scatter in the data points tended to be greater at higher substrate concentrations. For that reason, most of the assays were performed with 20 μM proline, even though this concentration could be below the K_m of the proline transport systems (2).

TABLE 2. The stimulation of proline transport in *S. typhimurium* by 0.3 M NaCl

Strain	Genotype			Mean proline transport activity (nmol/min per mg of protein) ± SD with[a]:	
	putP	*proP*	*proU*	M63	M63 + 0.3 M NaCl
putA					
TL106	+	+	+	0.78 ± 0.08	4.78 ± 0.25
TL135	−	+	+	0.57 ± 0.06	4.80 ± 0.20
TL179	−	−	+	0.40 ± 0.15 (5)	2.14 ± 0.49 (9)
TL192	−	+	−	0.66 ± 0.03 (3)	3.68 ± 0.59 (5)
TL199	+	−	−	0.62 ± 0.03	0.57 ± 0.16
TL195	−	−	−	0.27 ± 0.05	0.28 ± 0.01
putA$^+$					
TL1	+	+	+	1.14 ± 0.04	4.54 ± 0.36
TL201	+	−	−	0.76 ± 0.01	0.63 ± 0.11

[a] Transport assays were done as described in the text. The results are the averages of two independent determinations, except in cases where indicated by the numbers in parentheses.

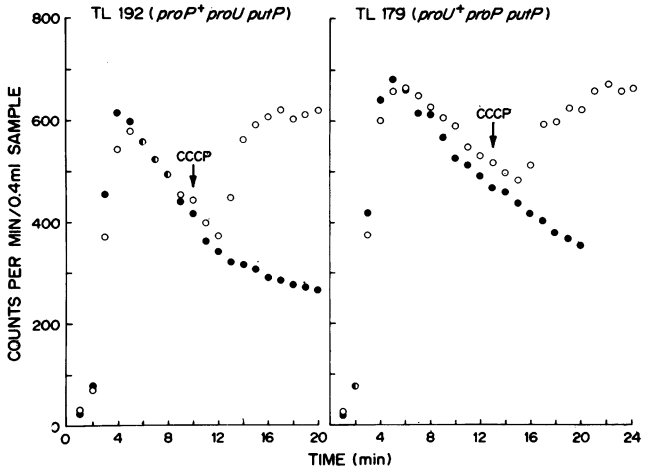

FIG. 1. The sensitivity of the ProP and ProU transport systems to the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). The proline transport activity of strains TL192 (*proP*$^+$ *proU putP*) and TL179 (*proU*$^+$ *proP putP*) was determined in the presence of 0.3 M NaCl as described in the text. Symbols: (○) results obtained in the presence of 20 μM CCCP added at 10 min in the case of strain TL192 and at 13 min in the case of strain TL179; (●) results in the control experiment without CCCP.

In the experiments described above, only NaCl was used to increase osmolarity of the growth medium. The transport of some metabolites, including proline, is known to be dependent on Na^+ or other alkali ions (7, 24). Therefore, the stimulation of proline transport might be due to the presence of Na^+ (or Cl^-) ions that are not normally in M63. We dismiss this possibility on the basis of an experiment in which we used 0.47 M sucrose (whose osmotic strength is approximately equal to that of 0.3 M NaCl) to increase the osmolarity of M63. The transport activity of strain TL192 (*proP*$^+$ *proU putP*) in M63 plus 0.47 M sucrose was 3.06 ± 0.32 nmol/min per mg, and that of TL179 (*proU*$^+$ *proP putP*) was 2.60 ± 0.15 nmol/min per mg. Within experimental error, these values agree with those obtained with 0.3 M NaCl (Table 2).

Strains carrying *proP*::Mu dl or *proU*::Mu dl insertions. To detect possible transcriptional regulation of the *proP* and *proU* genes, we constructed strains that carry phage Mu dl insertions in these loci. We had obtained a total of 11 strains in which the *lacZ* gene is transcribed from the *proP* promoter and 12 strains in which the *lacZ* gene is transcribed from the *proP* promoter, as judged by their blue colony color on M63 containing 0.3 M NaCl and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (see above). The effect of osmotic stress on the β-galactosidase levels of three representative *proP-lacZ* fusion strains and three representative *proU-lacZ* fusion strains is shown in Table 3. The three *proP-lacZ* strains (and eight similar others; data not shown) exhibited some increase in the β-galactosidase levels upon exposure to 0.3 M NaCl, but since these increases were at most two- to threefold and not observed consistently, they are not likely to account for the stimulation of [^{3}H]proline transport re-

TABLE 3. The effect of osmotic stress on the β-galactosidase activity in *proP*::Mu dl and *proU*::Mu dl strains

Strain[a]	β-Galactosidase activity (nmol/min per mg of protein) with[b]:		
	M63	M63 + 0.3 M NaCl	Ratio of activity (with NaCl/ without NaCl)
TL361 (*proP1894*::Mu dl)	107	214	2.0
TL370 (*proP1903*::Mu dl)	128	287	2.2
TL372 (*proP1905*::Mu dl)	137	174	1.3
TL333 (*proU1871*::Mu dl)	29	483	17
TL343 (*proU1188*::Mu dl)	37	258	7
T1346 (*proU1884*::Mu dl)	7	545	78

[a] The strains used are representatives of all *proP-lacZ* or *proU-lacZ* fusion strains we obtained which express β-galactosidase, i.e, form blue colonies on M63 containing 0.3 M NaCl and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (see text). In eight other similar *proP-lacZ* fusion strains, the ratio of β-galactosidase (nanomoles per minute per milligram) in the presence of 0.3 M NaCl/absence of NaCl ranged from 257:160 to 158:54. In nine other similar *proU-lacZ* strains the ratio of β-galactosidase in the presence/absence of NaCl ranged from 318:34 to 181:4. In addition, we obtained a number of *proP*::Mu dl or *proU*::Mu dl strains that formed white colonies on M63 containing 0.3 M NaCl and 5-bromo-4-chloro-3-indolyl-β-D-galactoside. These strains probably carry Mu dl in the orientation that is incorrect for the transcription of the *lacZ* gene from the *proP* and *proU* promoters. The β-galactosidase levels in these strains were low (<7 nmol/min per mg) and were not influenced by the presence of 0.3 or 0.65 M NaCl.

[b] The β-galactosidase activity was determined as described in the text, except that the cells were grown overnight in the indicated media to the exhaustion of glucose.

ported in Table 2. In contrast, the *proU-lacZ* fusion strains displayed a consistent increase in the β-galactosidase levels in response to 0.3 M NaCl. Although there was some variation in the magnitude of the increase (ranging from 7-fold in strain TL343 to more than 70-fold in strain TL346), these results indicate that osmotic stress stimulates the transcription of the *proU* gene.

The data in Table 3 were obtained with cells grown overnight to exhaustion of glucose in the indicated media, but similar results were observed in exponentially growing cells (data not shown). The phage Mu dl insertions in the strains were made heat resistant and more stable against transposition by the introduction of a Tn*9* insertion in the phage *B* gene, and the resultant *proP*::Mu dl *B*::Tn*9* or the *proU*::Mu dl *B*::Tn*9* alleles were backcrossed into strains TL135 (Δ*putP*) or TL179 (*proP* Δ*putP*), respectively (see above). The β-galactosidase levels in the resultant transductants were, within experimental error, identical to those in the respective parental strains under various conditions of osmotic stress (data not shown). A detailed analysis of the regulation of the *proP* or *proU* genes was carried out with these transductants.

Assays of [^{3}H]proline transport activities of strains carrying *proP*::Mu dl::Tn*9* or *proU*::Mu dl::Tn*9* insertions. To verify that the presumed *proP*::Mu dl *B*::Tn*9* and *proU*::Mu dl *B*::Tn*9* insertions inactivated the relevant proline transport systems, we measured the proline transport activities of strains carrying these mutations. In accordance with the expected results, the proline transport activity of these strains in the presence of 0.3 M NaCl is 5- to 10-fold lower than in the appropriate *proP*$^+$ or *proU*$^+$ control strains (Table 4).

Expression of β-galactosidase in *proP-lacZ* fusion strains. Since the β-galactosidase levels in strains carrying *proP-lacZ* fusions were not greatly affected by growth in 0.3 M NaCl (Table 3), we tested whether exposure to more extreme osmotic stress might uncover the possible transcriptional regulation of the *proP* gene. The result was again negative. The β-galactosidase levels in strains TL423 to TL425 (*proP*::Mu dl *B*::Tn*9*) ranged from 29 to 64 nmol/min per mg of protein in cells grown in M63 containing 0.65 M NaCl, as compared with a range of 41 to 47 nmol/min per mg of protein in cells grown in M63. One possible reason for our inability to detect transcriptional regulation of the *proP* gene could be either that the *proP*$^+$ gene product might be required for normal regulation or that the regulatory protein might be part of the operon that contains the *proP* gene, and the Mu dl phage might have polar effects on its transcription. To investigate this point, we constructed strains carrying chromosomal duplications, such that they were heterozygous for *proP*$^+$ and the *proP1894*::Mu dl *B*::Tn*9*, *proP18903*::Mu dl *B*::Tn*9*, or *proP1905*::Mu dl *B*::Tn*9* insertions (see above). The β-galactosidase activities in these strains displayed similar nearly constitutive expression (61 to 75 nmol/min per mg in M63 and 66 to 94 nmol/min per mg in M63 containing 0.3 M NaCl) as was seen in the corresponding strains carrying only the *proP*::Mu dl insertions (Table 3), indicating that the lack of transcriptional regulation is not due to loss of *proP*$^+$ gene product or a regulatory protein. The above results suggest that the stimulation of transport activity via the ProP system is probably the result of some posttranscriptional regulation, such as translational control, or modification of the transport protein brought about by osmotic stress.

Anderson et al. reported that the rate of proline transport via the ProP system is stimulated as a result of starvation for proline or other amino acids (2). To test whether this stimulation is due to increased transcription of the *proP* gene, we introduced into strain T1425 (*proP*::Mu dl) mutations blocking the proline, histidine, or leucine biosynthetic pathways and attempted to grow the resultant auxotrophs under the respective amino-acid-limiting conditions that Anderson et al. found to enhance the activity of the ProP system (2). We did not find any significant changes in the β-galactosidase levels of these strains (data not shown), indicating that the enhancement of the ProP activity upon amino acid starvation is not due to a transcriptional control.

Regulation of *proU* gene expression by osmotic stress. The most important result presented in this paper is that the

TABLE 4. Proline transport activity in strains carrying *proP*::Mu dl or *proU*::Mu dl insertions

Strain[a]	Mean proline transport activity (nmol/min per mg of protein) ± SD[b]
TL192 (*proP*$^+$ *proU*::Tn*10* Δ*putP*)	4.90 ± 0.21
TL472, TL474, TL476 (*proP*::Mu dl *proU*::Tn*10* Δ*putP*)	0.34 ± 0.13
TL179 (*proU*$^+$ *proP* Δ*putP*)	2.26 ± 0.25
TL390, TL391, TL393 (*proU*::Mu dl *proP* Δ*proP*)	0.26 ± 0.14
TL450 (*proP*$^+$ *proU*::Mu dl Δ*putP*)	4.28 ± 0.05
TL423 (*proU*$^+$ *proP*::Mu dl Δ*putP*)	1.70 ± 0.24

[a] The Mu dl insertions used in the strains in this experiment all carry Tn*9* in the phage *B* gene. The *proP* allele in strains TL179, TL390, TL391, and TL393 is the spontaneous mutation *proP1654*.

[b] Proline transport activity was determined as described in the text. The results are the averages of two independent measurements, with the deviation from the mean indicated, except for the set of isogenic strains TL472, TL474, and TL476 and strains TL390, TL391, and TL393, for which the result reported is the average of the results for the set of the three strains, two independent measurements with each, with the error being the largest deviation from the average.

synthesis of β-galactosidase in the *proU*::Mu dl strains is elevated in response to osmotic stress (Table 3), implying that the ProU transport system is regulated at the level of transcription. Since the regulation of synthesis of β-galactosidase in all *proU-lacZ* fusion strains was very similar, we carried out further characterization of the regulation of the *proU* gene with strain TL393, whose Mu dl prophage has been stabilized with the *B*::Tn*9* insertion.

First, we determined the relationship between the osmotic strength of the medium and the expression of the *proU* gene (Fig. 2). The steady-state transcription of this gene increased linearly over a 14-fold range, as the NaCl concentration was varied from 0 to 0.5 M. No further increase in the expression of the gene was brought about by NaCl concentrations above 0.5 M (data not shown). Increased transcription of the *proU* gene occurred very rapidly upon osmotic shock. There was a detectable increase in the synthesis of β-galactosidase in the *proU*::Mu dl strain within 9 to 12 min after exposure to 0.3 M NaCl (Fig. 3). These experiments were carried out in the absence of proline, indicating that exogenous proline is not required for the osmotic regulation of the *proU* gene.

The question might arise whether increased transcription of the *proU* gene is triggered specifically by NaCl or whether it is the result of a generalized osmotic stress. Therefore, we grew strain TL393 (*proU*::Mu dl) in media whose osmotic strength has been increased by solutes other than NaCl and measured the levels of β-galactosidase (Table 5). Several of these solutes, both ionic [$(NH_4)_2SO_4$ and KH_2PO_4] and nonpolar (sucrose and polyethylene glycol 6000), are able to induce the *proU* gene. As was the case with NaCl, the level of expression of the *proU* gene, measured with various concentrations of polyethylene glycol 6000 ranging from 0 to 200 g/liter, was proportional to the osmotic strength of the medium (data not shown). However, there were a number of

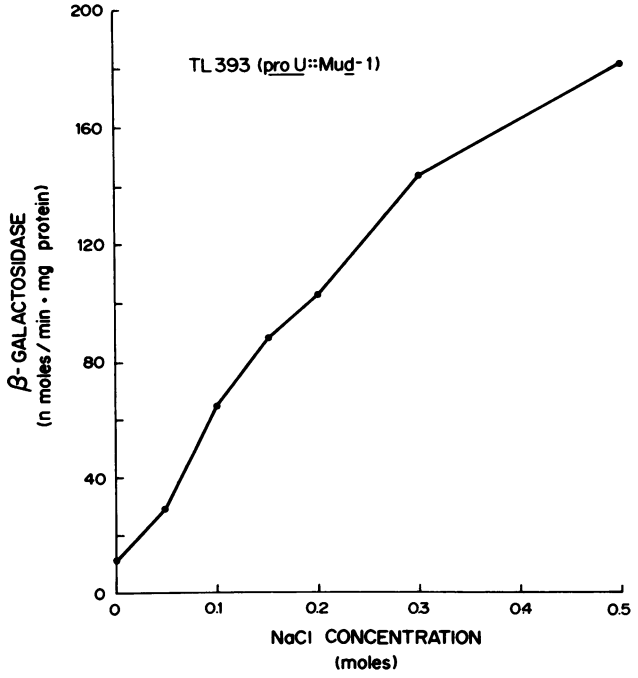

FIG. 2. The expression of the *proU* gene as a function of the concentration of NaCl in the growth medium. Strain TL393 (*proU*::Mu dl) was grown in the media with the indicated NaCl concentration and assayed as described in the text.

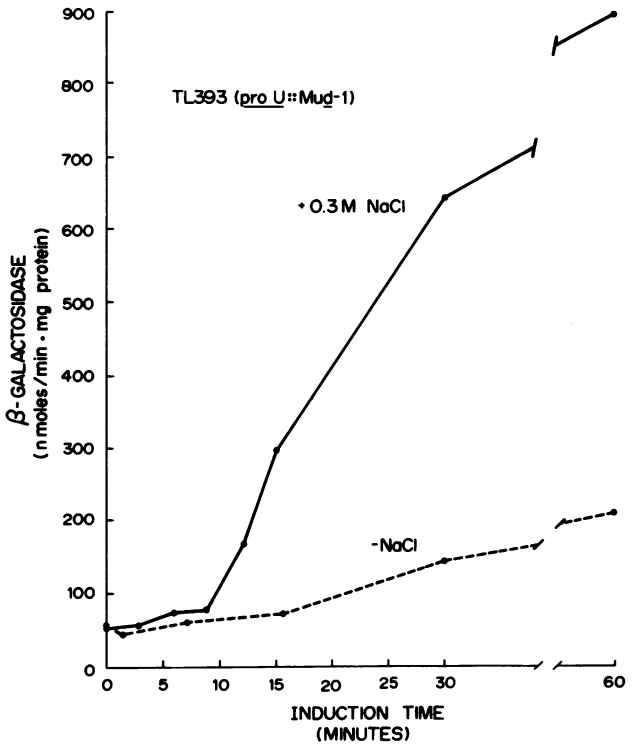

FIG. 3. The kinetics of induction of the *proU* gene. Strain TL393 (*proP*::Mu dl) grown exponentially in M63 glucose was subcultured into M63 glucose (– – –) or M63 glucose containing 0.3 M NaCl (——), and the β-galactosidase activity was assayed in aliquots removed at the indicated times.

solutes (methanol, ethanol, and glycerol) that did not cause an induction of the *proU* gene, even though they were present at osmolarities that were approximately equal to those of the other solutes. It could be argued that the solutes that do not cause an induction of the *proU* gene may interfere with the induction rather than being merely unable to elicit it. This possibility can be dismissed because normal

TABLE 5. The effect of various solutes on the expression of β-galactosidase in a *proU*::Mu dl *B*::Tn*9* strain

Addition to M63[a]	Concn (M)	Medium osmolarity[b]	β-galactosidase activity (nmol/min per mg)
None		0.22	19
NaCl	0.30	0.83	451
$(NH_4)_2SO_4$	0.26	1.06	383
KH_2PO_4	0.33	0.75	455
Polyethylene glycol 6000	0.034	0.72	205
Sucrose	0.47	1.05	316
Methanol	0.56	0.95	7
Ethanol	0.54	0.93	4
Glycerol	0.52	0.97	11

[a] Strain TL393 (*proU1884*::Mu dl *B*::Tn*9*) was grown in M63 with the indicated additions, and β-galactosidase activity was assayed as described in the text.

[b] The osmolarity of the media was measured with a dew point psychrometer, except in the case of methanol, ethanol, and glycerol, in which it was determined from freezing point depression. The osmolarity is expressed as the moles per liter of an ideal solute that would produce the water potential that was determined.

induction of the gene by 0.3 M NaCl took place even in the presence of 0.52 M glycerol (data not shown). The difference between the compounds that can induce the *proU* gene and those that cannot may reside in the fact that the latter compounds are lipid-soluble substances that are freely diffusible across the cell membrane (5, 21, 39), whereas the former compounds are either nondiffusible or are taken up by active transport systems. These observations suggest that the signal for the increased transcription of the *proU* gene might be an osmotic gradient across the cell membrane that cannot be produced by freely diffusible substances.

We tested whether the *proU* gene might be subject to regulation by catabolite repression or the utilization of proline as a nitrogen source. These experiments were carried out with a *putP*$^+$*A*$^+$ *proP*$^+$ derivative of strain TL393 (*proU*::Mu d1). Growth with citrate instead of glucose as the carbon source or with proline instead of NH_4^+ as the nitrogen source affected neither the basal nor the increased level of expression of the *proU* gene obtained in the presence of 0.3 M NaCl (data not shown). We also investigated whether proline starvation affected the expression of the *proU* gene. A mutation conferring proline auxotrophy was transduced into strain TL393 (*proU*::Mu d1 *proP* Δ*putP*). Because of the defects in proline transport, the growth rate of this strain can be controlled by varying the external proline concentration (14, 36). We found that the expression of the *proU* gene was not altered by proline limitation or excess in either M63 or M63 containing 0.3 M NaCl (data not shown). Thus, the only environmental factor we found to control the expression of the *proU* gene is the osmotic strength of the medium.

DISCUSSION

We have presented evidence that the transport of [^{3}H]proline by *S. typhimurium* is stimulated by growth in media of elevated osmotic strength. This enhanced proline uptake is due to the stimulation of two independent permeases, which we designated as the ProP and the ProU systems. By constructing *lacZ* fusions to genes *proP* and *proU* that are required for the functioning of the two systems, we carried out experiments to investigate the transcriptional regulation of the two systems. We found that exposure of the cells to osmotic stress causes a rapid, 10-fold increase in the transcription of the *proU* gene. We were unable to detect a comparable transcriptional regulation of the *proP* gene. It may be that the transcription of the *proP* gene itself is not regulated by osmotic stress, but there are other unknown genes encoding components of the ProP system whose transcription is regulated. Alternatively, the component(s) of the ProP system may be synthesized constitutively, and the stimulation by osmotic stress is the consequence of some posttranscriptional event. Thus, our results suggest that there may be interesting differences between the mechanisms of osmoregulation of the ProP and the ProU systems.

Proline transport via the ProP and the ProU systems is sensitive to the inhibitors of oxidative phosphorylation, carbonyl cyanide *m*-chlorophenylhydrazone or KCN, indicating that both systems accumulate proline actively against a concentration gradient. In the case of the ProP system, the initial rate of uptake in the presence of 0.3 M NaCl was 3.68 nmol/min per mg (Table 2; strain TL192) and was linear for at least the first 5 min (Fig. 1). The cell concentration used in the assay (~10^9/ml) corresponds to a protein concentration of 0.35 mg/ml. So, in 5 min, a total of 3.68 × 5 × 0.35 or ~6 nmol of substrate was taken up by the cells. The total internal volume of 10^9 cells is ca. 10^{-3} ml (39), so the internal concentration of proline after 5 min is 6 mM. Since after 5 min the external proline concentration is 20 − 6 μM, or 14 μM, the internal concentration of proline that is accumulated by the ProP system in only the first 5 min was ca. 400-fold greater than the external concentration. By a similar calculation, we can arrive at the result that after 5 min the ProU system could maintain a 200-fold difference between the internal and external concentrations of proline. It has been proposed that proline, accumulated under conditions of osmotic stress, functions as an osmotic balancer and thus forestalls the dehydration of the cytoplasm (19). Normally, 0.5 mM external proline is required to elicit the optimal protection against osmotic stress (14). If the two transport systems together could maintain a 500- to 1,000-fold concentration gradient in the presence of 0.5 mM proline, then the internal concentration of proline would be of sufficient magnitude to function as an osmotic balancer.

Stimulation of proline transport by osmotic stress has been observed in *Escherichia coli* by Britten and McClure (6) and C. A. Weaver (Ph.D. thesis, University of Illinois, Champaign-Urbana, 1981) and in *Streptomyces griseus* by Killham and Firestone (26). However, these experiments did not address the question of how many transport systems are involved or how they are regulated. Kaback and Deuel reported that the uptake of proline by cell-free vesicles of *E. coli* membranes was stimulated by their exposure to buffers of elevated osmotic strength (23). Since protein synthesis could not occur in the vesicles, it is likely that the stimulation of transport was the result of some posttranslational modification activating the ProP system of *E. coli*.

Fusions of *lacZ* to an uncharacterized gene whose transcription is regulated by osmotic stress have been isolated in *E. coli* K-12 (R. T. Vinopal, personal communication). This gene is in the analogous region of the chromosome where the *proU* gene has been located in *S. typhimurium*, and thus it may be the *proU* gene of *E. coli*. Laimins et al. (27) have constructed *E. coli* strains carrying *lacZ* fusions to the *kdp* operon that encodes components of a potassium transport system. These researchers found that the synthesis of β-galactosidase in these strains was induced by osmotic stress. However, in contrast with our results that osmotic stress causes an increase in the steady state transcription of the *proU* gene, Laimins et al. (27) found that osmotic stress caused only a burst of increased transcription of the *kdp* operon lasting for ~30 min, after which the transcription returned to basal levels.

Besides the transport systems for proline and potassium, there are a number of other functions that respond to osmoregulation in bacteria. These include the synthesis of glutamate and glutamine (41), the intracellular content of polyamines (33), the synthesis of a membrane-derived oligosaccharide found in the periplasmic space (25), and the synthesis of two outer membrane porin proteins encoded by the *ompC* and *ompF* genes (34, 40). It is not clear to what extent these cellular responses are regulated by a common mechanism. Laimins et al. proposed a model to account for the osmotic control of the transcription of the *kdp* operon (27). According to this model, the regulatory protein is at least partially membrane bound, and its conformation, which determines its capacity to bind to the *kdp* operator, is influenced by the physical force of the turgor pressure exerted on the cytoplasmic membrane. Since our results suggest that an osmotic gradient across the membrane might be the signal for the regulation of the *proU* gene, it is possible that the sensory protein regulating the transcription

of the *proU* gene may also be located in the cytoplasmic membrane.

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