Rapid Response to Osmotic Upshift by Osmoregulated Genes in *Escherichia coli* and *Salmonella typhimurium*.

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The rapid *in vivo* response of both *Escherichia coli* and *Salmonella typhimurium* osmoregulated genes to an osmotic upshift was analyzed in detail by using chromosomal operon fusions. Within 10 min after the addition of 0.3 M NaCl to the culture medium, the differential rates of expression of both an *S. typhimurium proU-lac* fusion and a *proP-lac* fusion increased by 180- and 17-fold respectively, while an *E. coli ompC-lac* fusion increased by 3.4-fold. For all three stimulated promoters, the increased rate of expression was maintained until about 40 min after the osmotic upshift. Thereafter, *proU* expression continued at a steady-state rate that was 27-fold higher than that of the control, while *proP* and *ompC* expression fell to 1.4- and 2-fold of the control rates, respectively. In contrast, expression of an *E. coli ompF-lac* fusion decreased twofold within 2.5 min. For *proU*, the length of the lag phase, which preceded the onset of the rapid response, increased with the degree of osmotic upshift, above a threshold of 0.2 M NaCl; the onset of the rapid *proU* response also preceded the resumption of growth. The rapid response phase, which was first quantitated for *proU*, *proP*, *ompC*, and *ompF* in this study, is an important component of the osmoregulation of these promoters. The addition of the osmoprotectant glycine betaine at the time of osmotic upshift decreased both the length of the rapid response and the subsequent steady-state rate of expression of *proU*.

Osmotic upshift of growing cells of the enteric bacteria *Escherichia coli* and *Salmonella typhimurium* evokes a rapid adaptive response. To maintain an osmotic balance, the cell volume contracts in a rapid physiochemical response until an osmotic equilibrium is achieved. A longer-term rebalancing of internal osmolytes is also set in motion (17). While the complete picture of these adjustments is still unfolding, it is clear that the internal concentrations of potassium (8, 24), glutamate (19, 24), proline (19), and trehalose (30) all increase. When present in the medium, glycine betaine also increases (2). In concert with the decrease in cell volume and the rebalancing of internal osmolytes, cell division is delayed (25; S. B. Jovanovich, unpublished data), membrane-derived oligosaccharide biosynthesis is reduced (13), and the expression of a limited number of genes is altered (1), along with the activity of many transport systems (25). An increase is observed in the expression of the outer membrane porin gene *ompC* (11), the proline and glycine betaine transport genes *proU* (4, 5, 7) and *proP* (3, 7, 10), and the high-affinity potassium transport gene *kdp* (15), while the expression of the outer membrane porin gene *ompF* (11) is decreased.

When a collection of gene fusions was examined for *in vivo* effects of an osmotic upshift on gene expression, it was noted that only four osmoregulated genes, *proU*, *proP*, *ompC*, and *ompF*, displayed a rapid response. In this study we have focused on quantifying of the rapid change in the differential rate of expression of the four genes and on the time course of the response. The temporal similarity of the response of the osmostimulated genes suggests that a carefully timed osmotic stress signal(s) mediates the osmoregulation of these genes.

MATERIALS AND METHODS

Media, chemicals, and strains. TYG medium consisted of 10 g of tryptone, 5 g of yeast extract, and 0.4 g of D-glucose per liter.

o-Nitrophenyl-β-D-galactopyranoside and glycine betaine hydrochloride were obtained from the Sigma Chemical Co. (St. Louis, Mo.). Bicinchoninic reagent (BCA) was purchased from the Pierce Chemical Co. (Rockford, Ill.). Tryptone and yeast extract were obtained from Difco Laboratories (Detroit, Mich.).

Strains TL671 [*proU*1884::Mu d1674 (Lac⁺)] and TL425 [*proP*1905::Mu d1B::Tn9 (Lac⁺) Δ*putPA*557 *zcc*-68::Tn5] are *S. typhimurium* (7). Strains MH225 [MC4100 Φ(*ompC'*::*lac*)10-25 λp1(209)] and MH513 [MC4100 *ara*⁺ Φ(*ompF'*::*lac*)16-23 λp1(209)] are derivatives of *E. coli* MC4100 [F⁻ *araD*139 Δ(*argF-lac*)U169 *rpsL*150 *relA*1 *flbB*5301 *deoC*1 *ptsF*25 *rbsR*] (26). All of the fusions were present in a single copy on the chromosome in the same organism from which the fusion promoter was derived.

Osmotic upshift experiments. To quantitate the rapid response of the *lac* fusions to a 300 mM NaCl osmotic upshift, both the activity of the gene and a measure of the cell mass must be obtained. Since the optical density of a culture is grossly affected by an osmotic upshift (6; S. B. Jovanovich and M. Martinell, unpublished data), it is unsuitable for a quantitative measurement. Therefore, the total protein content was chosen as an index of cell mass.

A fresh overnight TYG inoculum of the strain was diluted 1:100 into 250 ml of TYG in a 1-liter Erlenmeyer flask. The culture was grown with rotation at 250 rpm in a 30°C water bath, and the optical density at 650 nm was followed. At an optical density of 0.25, 100-ml portions were placed into 1-liter Erlenmeyer flasks containing 100 ml of either TYG or TYG with 0.6 M NaCl.

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FIG. 1. The lag time for *proU* expression is dependent on the degree of osmotic upshift. TL671, ϕ (*proU-lac*), was shifted from TYG to TYG with various concentrations of NaCl, as indicated, and the β -galactosidase activity was measured. Symbols: ●, No NaCl; ○, 100 mM NaCl; ■, 200 mM NaCl; □, 300 mM NaCl; ▲, 400 mM NaCl; △, 500 mM NaCl; ×, 600 mM NaCl; *, 700 mM NaCl.

Samples were taken at various times. If the microtiter protein assay was to be used, 0.5 ml was withdrawn for an optical density measurement, 0.5 ml was saved on ice for a subsequent β -galactosidase assay, and 5 ml was placed in a 15-ml Corex tube on ice for a subsequent protein determination. The samples for the microtiter protein determination were centrifuged at 7,000 rpm in a SS-34 rotor for 10 min at 4°C. The pellet was washed once with 5 ml of 145 mM NaCl and recentrifuged. The pellet from the second spin was suspended in 1 ml of water and frozen for use in the protein assay.

If the macroscale protein assay was to be used, triplicate 1.2-ml samples for protein determination were placed in Eppendorf tubes on ice. The samples were centrifuged at 10,000 rpm for 5 min in a microfuge and washed once with either 145 mM NaCl for the control samples or with 400 mM NaCl for the osmotically upshifted samples. After the samples were pelleted, they were dried *in vacuo* for 2 min, suspended in 240 μ l of water, and frozen.

When the protein concentration was not determined, 200 ml of starter culture was grown in a 1-liter Erlenmeyer flask to an optical density of 0.25 at 650 nm. Portions of 15 ml were then used for the various shift experiments, with 1-ml samples taken for optical density measurement and β -galactosidase assays.

β -Galactosidase assays. β -Galactosidase assays were performed as described previously (12). Specific activity units per milliliter of cells assayed were calculated by the method described by Miller (20) except the optical density was measured at 650 nm; activity units were calculated in the same manner, but they were not normalized to the optical density of the culture. In general, the data are displayed in two basic representations. A plot of β -galactosidase activity versus the total cellular protein allows a determination of the differential rate of synthesis (29), which is the slope of the resultant curve. The differential rate of synthesis measures the level of expression of the fused promoter. The slopes were calculated by a least-squares fit. For the rapid-response phase, the approximate slopes that extended through the points used in the calculations were marked on the relevant figures (see Fig. 2A, 3A, 4, and 5). Some of the data are also displayed as plots of the specific activity of β -galactosidase versus the time of the sampling. This type of figure shows the result of the differential rate of the accumulation of β -galactosidase and explicitly displays temporal information.

Protein determination. Protein concentrations were determined by using the BCA method (27), with bovine albumin (Pierce) used as a standard. The BCA reaction is not affected by NaCl concentrations of up to 1 M (27). The macroscale assay was performed as described by the manufacturer, with incubation at room temperature, a 2-h reaction period, and triplicate samples.

To obtain accurate data on the initial time points by the microtiter assay (22, 28), typically, 48 individual assays were performed for each time point. A multichannel pipettor (Costar, Cambridge, Mass.) was used to transfer 25 μ l of washed and concentrated cells, as described above, into a row of a 96-well uncoated microtiter plate (Linbro; Titertek). The reaction was started by the simultaneous addition of 200 μ l of BCA reaction mix with a 96-well multichannel pipettor (Transtar-96; Costar). The plates were covered with plastic wrap and incubated at 43°C for 2 h. The reaction was quenched by placing the plates at room temperature. The optical density of the reaction was read at 540 nm on a microtiter plate reader (Multiskan MC; Titertek). Both the macroscale and microtiter assay methods yielded comparable results; however, the average standard deviation was about 8% by the microtiter method and only 2% by the macroscale method.

RESULTS

The timing of the *proU* response is related to the degree of osmotic upshift. When cultures of *E. coli* or *S. typhimurium* are osmotically upshifted, there is an instantaneous increase in the optical density of the culture which is caused by a decrease in the cytoplasmic volume. The optical density then gradually decreases to a minimum, which is followed by a gradual increase until balanced logarithmic growth is resumed under the new environmental conditions. Concomitant with the changes in the optical properties of the cell, a series of adaptive changes occur, including changes in the expression of osmoregulated genes.

In preliminary studies it was noted that the differential rate of synthesis of an *S. typhimurium proU-lac* fusion dramatically increased within 15 min after the addition of NaCl. A time course of the effect of a range of NaCl additions on total β -galactosidase activity produced from the *proU* promoter is shown qualitatively in Fig. 1. As a result of the osmotic upshift, the β -galactosidase activity of the culture was stimulated. The lag time before the activity started to increase varied, depending on the concentration of NaCl added. For cultures with NaCl added to 100 or 200 mM, the lag was less than 2.5 min. As the NaCl concentration was increased from 300 to 700 mM NaCl, the lag was successively increased: 5 min for 300 mM, 20 min for 400 mM, 35 min for 500 mM, 45 min for 600 mM, and 90 min for 700 mM. Thus, the timing of the onset of increased *proU* expression is variable and dependent on the degree of the osmotic shock.

The lag times of *proU* expression (Fig. 1) coincided in all cases with the time of the optical density minimum of the culture (data not shown). A detailed time course of the viable cell count of a culture that was osmotically upshifted to 500 mM NaCl indicated that the minimum of the optical density significantly preceded the resumption of growth (data not shown), as has been observed previously in *E. coli* (6). This suggests that the lag time for *proU* expression may be related to the completion of an internal event(s) in the adaptation to the osmotic upshift.

***proU* expression is rapidly stimulated by osmotic upshift.** A quantitative and more detailed investigation of the rapid

response of *proU* expression to the addition of NaCl to 300 mM was undertaken. Two complementary representations of the data, a differential rate plot and a plot of the specific activity as a function of time, are displayed in Fig. 2A and B, respectively. Initially, the osmotically upshifted culture continued to express *proU* at the control rate (Fig. 2A). By 10 min (at about 18 μ g of protein per ml), however, a rapid transient response occurred and the differential rate of synthesis increased 180-fold. After this transient burst of synthesis, the differential rate of synthesis decreased to a new steady-state rate which was still 27-fold higher than that of the control (derived by using samples from 35 to 259 μ g of protein per ml; the data for greater than 90 μ g of protein per ml are not shown). An essentially identical result was obtained when the osmotic upshift was elicited by the addition of 0.47 M sucrose. No effect was seen when either 0.52 M glycerol or 0.52 M ethanol was added. The difference was presumably that glycerol and ethanol are readily equilibrated across the membrane and do not produce an osmotic gradient.

The effect of the osmotically induced changes in the differential rate of synthesis of *proU* on the specific activity is shown in Fig. 2B. The results shown in Fig. 2B confirm and extend previous results obtained with minimal media (4, 7). The specific activity rose very sharply after the 7.5-min sample because of the 180-fold increase in the differential

FIG. 2. *proU* transcription is stimulated by an osmotic upshift. An osmotic upshift of TL671 Φ (*proU-lac*) from TYG to TYG with 0.3 M NaCl was performed as described in the text. The points in panel A correspond to samples taken at 2.5, 5, 7.5, 10, 12.5, 15, 20, 25, 30, 40, 50, 60, 75, and 90 min after the upshift. (A) Differential rate plot of the effect of the osmotic upshift on *proU* expression. (B) Effect of the change in differential rate on the specific activity as a function of time after the osmotic upshift. Symbols: ●, Control culture; ○, 0.3 M NaCl culture.

FIG. 3. *proP* expression is stimulated by an osmotic upshift. TL425, Φ (*proP-lac*), was grown in TYG and was osmotically upshifted by the addition of NaCl to 0.3 M, as described in the legend to Fig. 2. (A) Differential rate plot, with points sampled as described in the legend to Fig. 2, except an additional sample at 105 min is shown. (B) Specific activity as a function of the time after the osmotic upshift. Symbols: ●, Control culture; ○, 0.3 M NaCl culture.

rate. The maximal increase in specific activity was 11-fold. From 30 to 180 min, the new steady-state differential rate, while still 27-fold higher than the control rate, was only sufficient to approximately maintain the increase in specific activity that was achieved during the rapid response. Therefore, after an osmotic upshift of the addition of NaCl to 300 mM, the majority of the response of the osmoregulated gene *proU* occurred rapidly in the time period between 10 and 30 min.

***proP* expression is rapidly and transiently stimulated by osmotic upshift.** When the effect of an osmotic upshift on the expression of a *S. typhimurium proP-lac* fusion was examined, a rapid response was again observed (Fig. 3A and B). Similarly to *proU*, after the osmotic upshift there was a lag before the induction of *proP* expression. Then, a rapid response occurred, with the differential rate of expression increasing 17-fold (Fig. 3A). After about 25 min of expression at the increased rate, the differential rate of the osmotically upshifted culture dropped significantly to a rate that was only 1.4-fold greater than that of the control unshocked culture (derived by using samples from 38 to 296 μ g of protein per ml; the data for greater than 110 μ g of protein per ml are not shown). This new steady-state rate was maintained for the duration of the experiment.

The effects of the differential rate changes on the accumulation of β -galactosidase specific activity, which was directed by the *proP* promoter, are shown in Fig. 3B. A lag period of about 15 min preceded the rapid response, which

FIG. 4. The differential rate of *ompC* is transiently stimulated by an osmotic upshift. An *E. coli ompC-lac* fusion in strain MH225 was osmotically upshifted as described in the legend to Fig. 2, and the differential rate of synthesis was measured. Symbols: ●, TYG control culture; ○, TYG plus 0.3 M NaCl culture.

then lasted for about 25 min. For *proP* the rapid response resulted in about a fourfold increase in the specific activity within 40 min of the osmotic upshift. Thereafter, the lower steady-state differential rate of *proP* expression was insufficient to maintain the increased specific activity, and the specific activity dropped to 1.6-fold higher than that of the control.

A comparison of the data in Fig. 3B with those in Fig. 2B accentuates both the similarities and differences in the osmoregulation of *proU* and *proP*. The lag time before the specific activity rises was similar, as was the duration of the rapid response. After the rapid response, however, the regulation of *proU* and *proP* was distinct, with the specific activity of *proU* being maintained at a new high level and the specific activity of *proP* returning to only slightly higher than the control level.

***ompC* expression is rapidly and transiently stimulated by osmotic upshift.** The *ompC* gene of *E. coli* has been shown to be stimulated by osmotic upshift (11). The effect of an osmotic upshift was therefore explored to determine whether the rapid response would be found in *E. coli* with an *ompC-lac* operon fusion. The differential rate of the osmotically upshifted culture was rapidly stimulated by 3.4-fold (Fig. 4). This stimulation occurred after a lag of about 10 min and lasted for about 30 min, which was similar to that for both *proU* and *proP*. Like *proP*, after the transient stimulation during the rapid response, the differential rate fell, to about twofold the initial control rate. Similarly, in steady-state experiments, the differential rate of a culture growing in TYG with 0.3 M NaCl was twofold higher than the differential rate of a culture growing in TYG (data not shown).

Transcription of *ompF* is rapidly inhibited by osmotic upshift. Since three osmostimulated genes exhibited the rapid response, the response of the *E. coli ompF* gene, whose transcription is inhibited by osmotic upshift (11), was studied. The addition of NaCl to 300 mM caused the differential rate of synthesis of an *E. coli ompF-lac* fusion to decrease by twofold by the first sample, which was taken at 2.5 min (Fig. 5). For the osmotically upshifted culture, *ompF* expression continued at a lowered differential rate for up to 150 min.

Glycine betaine affects the rapid response. Glycine betaine is an osmoprotectant (17, 30) which can be transported by both the *proU* (4, 18) and *proP* (3, 18) transport systems. The presence of glycine betaine during an osmotic shock dimin-

FIG. 5. The differential rate of *ompF* is decreased by an osmotic upshift. The effect of an osmotic upshift, as described in the legend to Fig. 2, on the differential rate of an *E. coli ompF-lac* fusion is shown. Symbols: ●, TYG control culture; ○, TYG plus 0.3 M NaCl culture.

ishes the osmotic response of *proU* (18). Therefore, the effects of glycine betaine on the rapid response of *proU* were analyzed.

When glycine betaine was added simultaneously with 300 mM NaCl, the growth of the culture was affected. The addition of 0.1 or 1 mM glycine betaine slightly stimulated growth of the culture, while 5 mM glycine betaine was slightly inhibitory (data not shown). At 10 mM glycine betaine, the growth rate was slowed by about 50%, and at 20 mM glycine betaine there was no growth within 2 h (data not shown).

Glycine betaine could affect any part of the *proU* rapid response, i.e., the length of the lag, the differential rate during the rapid response, the length of the rapid response, or the new steady-state differential rate. No effect of glycine betaine on the length of the lag was found (data not shown). A differential rate plot of *proU* expression after an osmotic upshift with a range of glycine betaine concentrations is shown in Fig. 6. At concentrations of glycine betaine from 1 to 10 mM, the initial rapid response component was very similar to that of the control. However, as the concentration of glycine betaine increased, the cultures reached the new

FIG. 6. Glycine betaine reduces the stimulation of *proU* expression by osmotic upshift. The differential rate of TL671, ϕ (*proU-lac*), was measured after a shift from TYG to TYG with 300 mM NaCl and various concentrations of glycine betaine. Samples were taken at 2, 5, 7.5, 10, 12.5, 15, 20, 25, 30, 35, 40, 50, 60, 75, and 90 min after the osmotic upshift. Symbols: ●, 300 mM NaCl, no glycine betaine; ○, 300 mM NaCl, 1 mM glycine betaine; ■, 300 mM NaCl, 5 mM glycine betaine; □, 300 mM NaCl, 10 mM glycine betaine.

steady-state rates at successively earlier times. Therefore, glycine betaine diminished the length of the rapid response. The steady-state differential rates were also lowered as the glycine betaine concentration increased (data not shown). At 20 mM glycine betaine, there was neither growth nor any increase in activity (data not shown). Thus, for *proU* glycine betaine affects both the time of transition from the rapid response to the steady state and the differential rate of the steady state.

DISCUSSION

Enteric bacteria possess a number of regulatory systems to respond to environmental changes. A rapid response component, which is necessary to adapt quickly to changed growth conditions, is found in the response systems for heat shock (16, 32), amino acid starvation (9), nitrogen limitation (14, 23), and carbon source utilization (33). Therefore, it is not surprising that the response to an osmotic upshift also contains a quick adaptive response of osmoregulated genes.

In this study, four osmoregulated genes, *ompC*, *ompF*, *proP*, and *proU*, were observed to respond rapidly to osmotic stress. For all four genes, the response started within 10 min. The differential rate of *ompF* was decreased by the osmotic shock and continued at a reduced rate for the duration of the experiment. For the stimulated genes, the initial rapid response lasted until about 40 min after the osmotic upshift. For *proU*, after the rapid response subsided, the differential rate of synthesis continued at a much higher rate than did that of the control unshifted culture, while for *ompC* and *proP* the differential rates fell to 2- and 1.4-fold of the control rates, respectively. For the stimulated promoters, the increased differential rate of expression during the rapid response caused the increase in specific activity: when the rapid response ended, the specific activity no longer increased, and for *proP* and *ompC*, it actually decreased when compared with the peak value. This suggests that while expression of the osmostimulated genes is increased during the steady state, the increased expression is only at most sufficient to maintain the increased accumulation of the gene product which occurred during the rapid response. Therefore, the majority of the osmostimulation of the expression of these genes occurred during the initial rapid response, in a series of as yet undefined early events.

In previous studies, the steady-state effects of osmotic strength on *proU*, *proP*, *ompC*, and *ompF* have been observed. The findings of this study are in agreement with those results; i.e., the expression of *proU*, *proP*, and *ompC* all increased after an osmotic upshift, and *ompF* expression decreased. Study of the initial response, however, has revealed additional information. First, the osmoregulation of *proP*, which is described in more detail below, is clearly observed. Second, it is evident that there is a phase from 10 to 40 min after the upshift during which all three stimulated genes behave very similarly. Finally, it is clear that a secondary response occurs at 40 min under these conditions. This secondary response differentiates between *proU*, which continues to be expressed at a high rate relative to that of the control, and *proP* and *ompC*, which are expressed at rates that are only slightly higher than those of the controls. An analysis of the nature of the events which causes the stages of the response may help to illuminate the mechanisms of the adaptive response.

The rapid and transient increase in the specific activity of *proP* after the osmotic upshift and the maintenance at a twofold higher steady-state level suggests that *proP* is osmo-

regulated. The osmoregulation of gene expression of *proP* is consistent with the observation that *ProP* can transport the osmoprotectants proline and glycine betaine (3) and may explain the two- to threefold increase in the *proP* steady-state specific activity which was previously found many hours after salt addition (3, 7, 10a). The increase in the steady-state rate also partially explains the observed 5.6-fold increase in *ProP*-mediated proline transport after osmotic upshift (7). The data from this study, however, do not preclude an additional posttranscriptional increase in the activity of the *ProP* system.

The rapid response is not a general effect of osmotic upshift on gene expression, but rather, it is a specific feature of the response of osmoregulated genes. A survey of the response of 36 genetically defined operon fusions to osmotic upshift revealed that only the genes noted herein produced a rapid response (S. Jovanovich, M. Martinell, and R. Burgess, manuscript in preparation). It is noteworthy that the screening of random gene fusions for osmoregulated genes, which has detected only *proU* (4, 10), *kdp*, and *lamB* (10), would not detect genes which only transiently respond to osmotic upshift or genes in which the insertion of *Mu d* would diminish the viability at a high osmolarity. This, coupled with the finding that *ompC* and *proP* have only large transient responses after an osmotic upshift, suggests that other undescribed, transiently osmoregulated genes may exist. In particular, the genes for trehalose synthesis (*tre*) and betaine synthesis (*bet*) might be expected to be transiently expressed after an osmotic upshift. As other osmoregulated gene fusions become available, it will be of interest to examine if and when the rapid response occurs.

Glycine betaine did not affect the time of the onset of the rapid response of *proU* or the differential rate of the response. Instead, it affected the length of the response and the subsequent steady-state rate. Since the rich medium used in this study contains proline and perhaps glycine betaine, the steady-state differential rates may underestimate the values in a minimal medium. Furthermore, since the strain used has a *proU* insertion, enough glycine betaine may be transported in the absence of a functional *proU* system to both affect the expression of *proU* and to inhibit growth at high concentrations.

Results of this study have shown that the time course of the rapid response of three stimulated genes is quite similar. A comparison of the time course for the three stimulated genes with the stimulation of *kdp* expression in *E. coli* (15) and glycine betaine transport by osmotic upshift (21) has revealed that they are virtually identical. In addition, the effect of the degree of the osmotic upshift on the timing of the *proU* response (Fig. 1) is equivalent to results obtained with *kdp* (15).

The similarity of the time course of the rapid responses of *ompC*, *proU*, *proP*, and *kdp* to an osmotic upshift raises the question of whether a common primary signal exists for all the stimulated genes. The rapid inhibition of *ompF* may suggest that the same putative common signal functions for genes that are inhibited by an osmotic upshift. Since the insertion of Tn5 in *ompR* had no effect on *proU* expression (4), it appears unlikely that the common signal is transduced by the *ompR-envZ* regulatory system. Sutherland et al. (31) have concluded that *kdp* and *proU* are regulated by different mechanisms because of their different responses to potassium levels. In their study, however, they did not examine the period of the rapid response, during which the stimulated genes are most similar. One interpretation of the lag before the stimulatory response is that the signal may be simply the

change in turgor pressure (15) and that the stimulated genes are prevented from responding until the turgor pressure and internal ionic environment are partially adapted by physicochemical means. As alternative interpretation is that some compound(s) or product(s) must be accumulated to a threshold level before the adaptive response of the osmostimulated genes can begin. Moreover, since the new steady-state rate of *proU* expression is dependent on the new osmotic strength of the medium, while the new steady-state rate of *proP* and *ompC* expression is less affected by osmotic strength, there may be a secondary signal which discriminates between these osmostimulated genes. A detailed temporal dissection of the cascade of events initiated by an osmotic upshift may allow an ordering of the events and thereby lead to an understanding of the signals for each step.

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