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Patterns of protein synthesis in the moderately halophilic bacterium *Deleya halophila* in response to sudden changes in external salinity

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1. SUMMARY

Exposure of the moderately halophilic bacterium, *Deleya halophila*, to high NaCl concentrations (2 or 2.5 M) resulted in a transient cessation of cell division. The time taken for the cells to adapt and grow depended on the final salt concentrations. During the initial phases of adaption to high salt both the rate of protein synthesis and amino acid uptake were transiently inhibited. The extent and duration of the inhibition was dependent on the magnitude of the salt shock. Alterations in the patterns of pulse-labelled proteins were observed during adaption to high salt. The response of *Deleya halophila* cells to decreasing salinity (2.5 to 1 M NaCl) was also characterized by distinct changes in the protein profiles, whereas minor changes in the protein patterns were observed during adaptation from 1 M to 0.5 M NaCl. The labelled protein patterns of cells grown in 1 M or 2.5 M NaCl appear to be similar but not identical.

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2. INTRODUCTION

Moderately halophilic bacteria are characterized by their ability to grow over a broad range of NaCl concentrations (0.5 M–4.0 M) [1]. This ability may be attributed to an adaptation to the high salt environment rather than a selection of salt resistant populations [1]. The mechanism(s) of adaptation to elevated external salinity and restoration of the ionic balance or the turgor pressure of the cell is not fully understood. It has been suggested that biosynthesis and/or accumulation of osmotically active solutes (e.g. potassium, trehalose and glycine betaine) may play a significant role in the osmotic adaptation of halophilic bacteria [2,3].

Recently considerable effort has been concentrated on investigating the changes in gene expression during adaptation to osmotic stress in various non-halophilic bacteria [4,5]. Expression of the *pro*U locus in *Salmonella typhimurium* [6] and the *omp*F and *omp*C genes of *Escherichia coli*, which code for proteins that are located in the outer membrane [7], appears to be osmoregulated. The induction of three unidentified proteins by high osmolarity has also been described in *E.*

coli [8]. Furthermore, it has been reported that the protein composition of the outer membrane of the moderately halophilic bacterium, *Pseudomonas halosaccharolytica*, is influenced by the salt concentration of the growth medium [9].

Deleya halophila is a newly described species of moderately halophilic soil bacteria, which can grow between 0.3 M and 3.5 M NaCl [10,11]. Since variations in external salinity are likely to be a natural stress for this microorganism, it is conceivable that it may be suitable for studies on the mechanisms involved in haloadaptation. The present study has revealed some new aspects on protein synthesis of *D. halophila* in response to sudden variations in external salinity.

3. MATERIALS AND METHODS

3.1. Microorganisms and culture conditions

Deleya halophila (CCM 3662) has been obtained from Professor A. Ramos-Cormezana (University of Granada, Spain). Cultures were routinely maintained on slants containing (per liter): 2 g KCl, 0.2 g $MgSO_4 \cdot 7H_2O$, 1 g KNO_3, 1 g $(NH_4)_2HPO_4$, 0.5 g KH_2PO_4 (basal medium), 58 g NaCl (pH 7.2 adjusted with KOH), 5 g Peptone from soya (Serva), 10 g yeast extract (BBL), 5 g glucose and 20 g Bacto-agar (BBL) [12,13]. Bacteria were grown on a rotary shaker at 30 °C in basal medium containing the appropriate amounts of NaCl supplemented with 0.5% (w/v) glucose (minimal media). For the determination of growth rate, minimal media containing the different NaCl concentrations were inoculated using a 2% inoculum ($A_{540} = 0.4$) from a culture growing in minimal medium containing 1 M NaCl. To change the NaCl concentration of the culture medium during exponential growth (shift-up), basal medium containing 5 M NaCl was added to achieve the desired salt concentration. Appropriate volumes of minimal media containing the respective NaCl concentrations were also added in order to give similar cell densities in all samples examined. In the shift-down experiment, exponentially growing cultures in 1 M or 2.5 M NaCl were diluted with basal medium supplemented with 0.5% (w/v) glucose. Growth was monitored by measuring absorbance at A_{540}. The number of cells in the shift-up experiments was determined by serial dilutions and by spreading the cells on plates containing the same medium composition as the slants. Colonies were scored after incubation at 30 °C for 24 h. Duplicate plates were counted at each time point.

3.2. Labelling of cellular proteins

Cells growing in minimal media containing 1 M or 2.5 M NaCl were shifted to the appropriate concentrations, and at the indicated time intervals aliquots (0.5 ml) were taken, pulse-labelled for 2 min with 10 μCi of [^{35}S]methionine (1000 Ci/mmol) and chased for 1.5 min with 250 μg unlabelled methionine (Sigma). Pulse-labelled cells were chilled on ice and mixed with an equal volume 10% (w/v) trichloroacetic acid (TCA). The precipitates were collected by centrifugation at $10\,000 \times g$ for 5 min at 4 °C, washed twice with ethanol and once with diethylether. The pellets were dissolved in an appropriate volume of sample buffer [15] containing phenylmethylsulphonyl fluoride (Sigma), heated at 100 °C for 3 min and centrifuged at $10\,000 \times g$ for 2 min. Incorporation into TCA-precipitable material was determined as described by Van den Bos et al. [14]. All the above-mentioned experiments were carried out three times.

Protein concentrations were measured using Biorad protein dye concentrate according to the manufacturer's protocol.

3.3. Polyacrylamide gel electrophoresis

Polypeptides were separated on 15% (w/v) denaturing polyacrylamide slab gels (SDS–PAGE) as described by Laemmli [15]. Proteins were transferred to nitrocellulose filters (Schleicher and Schuell) [23] and [^{35}S]methionine labelled proteins were visualized by autoradiography.

3.4. Uptake assay

Uptake of radioactive precursors was determined as described by Peleg et al. [16] with the following modification. Cells growing in minimal medium containing 1 M NaCl were subjected to a salt shift-up as described above. Cells were sampled at successive time intervals after the shift and

20 μCi/ml [^{35}S]methionine (1000 Ci/mmol) or 10 μCi/ml [^{3}H]uridine (50 Ci/mmol) or 10 μCi/ml L-[4,5-^{3}H]leucine (120 Ci/mmol) or 10 μCi/ml [^{3}H]thymidine (20 Ci/mmol) were added. All radiochemicals were purchased from New England Nuclear Corp. Boston. At the indicated time points, cells were labelled for 1 min and filtered through a 0.45 μm membrane filter (Sartorius). The filters were immediately washed with 5 ml of minimal medium containing the corresponding NaCl concentration, dried at 50°C for 30 min and radioactivity was determined [14].

4. RESULTS

Growth of *D. halophila* was determined in chemically defined minimal media containing different NaCl concentrations. Although the final cell densities attained were the same in media containing up to 3 M NaCl (data not shown), the rates of growth were reduced with increasing salt concentration (Fig. 1a). Similar salt requirements for optimal growth were observed when *D. halophila* cells were grown in rich media ([11] and our unpublished data). Cells growing in 1 M NaCl were subjected to sudden increases in salt concentrations (shift-up) and the rate of growth was determined. As shown in Fig. 1b, a lag phase in cell division was observed which appears to be dependent on the magnitude of the salt shift-up. The effect of a sudden decrease in salinity on the growth rate of *D. halophila* was also investigated. Cells growing exponentially in 1 M or 2.5 M NaCl were shifted to low salinity, 0.5 or 1 M, respectively (shift down). Immediately upon shift-down the cultures started growing with no apparent lag phase and with a growth rate determined by the final NaCl concentration (data not shown).

To investigate the effect of NaCl shift-up on the rate of bulk protein synthesis, exponentially growing cells were subjected to sudden increases of NaCl concentrations and at successive time intervals the rate of protein synthesis was monitored by determining the incorporation of [^{35}S] methionine into TCA-precipitable material. Results (Fig. 2) are expressed as percentage relative to the level of [^{35}S]methionine incorporation into

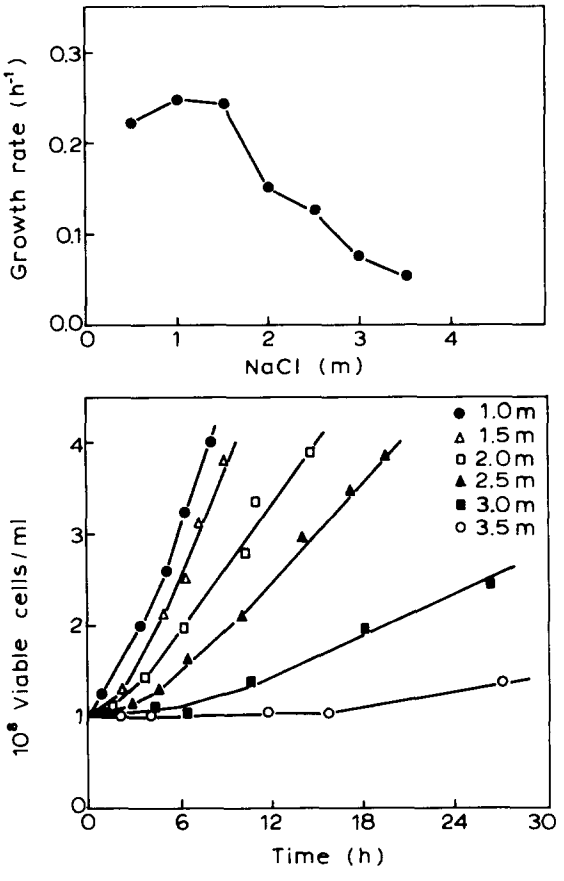

Fig. 1. Effect of NaCl concentrations on the growth rate and viability of *D. halophila* cells. Cultures of *D. halophila* were grown at the indicated salt concentrations, as detailed in MATERIALS AND METHODS, and the growth rate was determined (A). Cells grown to exponential phase (OD_{540} = 0.4, approximately 1×10^8 cells/ml) were subjected (at zero time) to sudden increases in NaCl concentrations as detailed in MATERIALS AND METHODS. Aliquots were taken from each culture at successive time intervals and the numbers of cells were estimated (B).

the control, that is cells grown in medium containing 1 M NaCl and represented at 100 on the ordinate. Immediately after shift-up to 1.5 M NaCl the rate of protein synthesis was slightly reduced and subsequently rose again. An abrupt increase to high salt concentrations (2 or 2.5 M) provoked a quite different response; within 5 min the rate of protein synthesis was significantly reduced to 3–5% or 1–3%, respectively, of that of the controls, followed by a temporal cessation of protein synthesis the duration of which appears to be

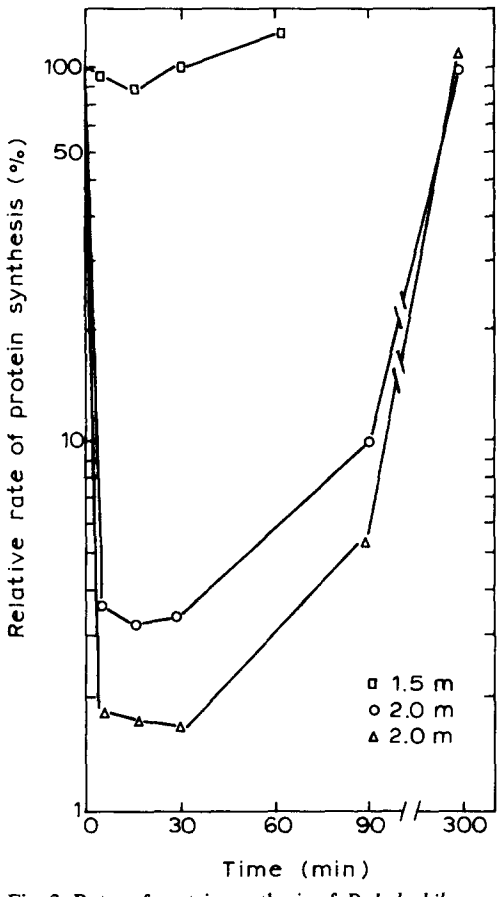

Fig. 2. Rates of protein synthesis of *D. halophila* upon sudden shift-up in different NaCl concentrations. A culture growing in medium containing 1 M NaCl was shifted either in 1.5 M or 2 M or 2.5 M, as detailed in Fig. 1. Aliquots of cells were sampled and pulsed-labelled with [^{35}S]methionine at the indicated intervals after the salt shift. Labelled cells were acid-precipitated as described in MATERIALS AND METHODS and radioactivity in each sample was determined. For convenience of representation, the results are plotted with the level of [^{35}S]methionine incorporation into cells grown in 1 M NaCl (at zero time) taken as 100%.

dependent on the severity of the imposed salt shock. The rate of protein synthesis returned to control levels after 300 minutes of salt shock. These studies were complemented by determining the rate of protein synthesis during shift-down experiments (see above). The rate of protein synthesis in both cases was virtually unaffected.

It has been reported that exposure of moderately halophilic bacteria to high salt concentrations inhibited the uptake of radio-labelled aminoacids [16]. To assess to what extent this occurs in *D. halophila* cells, the levels of uptake of [^{35}S]methionine and [^{3}H]leucine were determined. In addition the uptake of [^{3}H]uridine and [^{3}H] thymidine was monitored after a salt shift-up. The uptake of all radiolabelled compounds examined was dramatically reduced depending on the magnitude of the salt shift, which was subsequently restored following kinetics similar to those seen in Fig. 2.

In an attempt to determine the effect of sudden elevation of salt concentration on the relative rates of synthesis of individual proteins equal numbers of pulse-labelled cells from an experiment identical to that described in Fig. 2 were analyzed by SDS–PAGE and autoradiographed. As shown in Fig. 3 (lanes 2 and 3), during the initial stages (5 to 15 min) of adaptation to 1.5 M NaCl no significant alterations in the protein patterns were observed. At later stages (60 min) the protein patterns contained several induced polypeptides (indicated by the arrows) (Fig. 3, lane 5). Immediately after exposure to 2 or 2.5 M NaCl, on the other hand, only one protein of 64 kDa (indicated by the arrows) appears to be synthesized after overloading of the gels (see Legend in Fig. 3). The synthesis of this protein remained at low but detectable levels throughout the adaptational period, suggesting that a small fraction of the protein synthesizing machinery is still functional (Fig. 3, panel A, lanes 6–8 and 11–13). Overexposure of the autoradiographs was necessary to allow better visualization of the 64 kDa protein (Fig. 3, panel B, lanes 6–8 and 11–13). After 90 minutes of exposure to high salt, normal protein synthesis resumed (Figs. 2 and 3). At 300 minutes after the salt shift-up, several induced proteins (indicated by the arrows) are clearly visible when compared to the control (Fig. 3, lanes 10 and 15). It is to be noted that the latter protein profiles are almost identical to those seen at 60 minutes after shift-up to 1.5 M NaCl (e.g. Fig. 3, lanes 5, 10 and 15).

Since *D. halophila* cells demonstrated some visible alterations in protein synthesis patterns

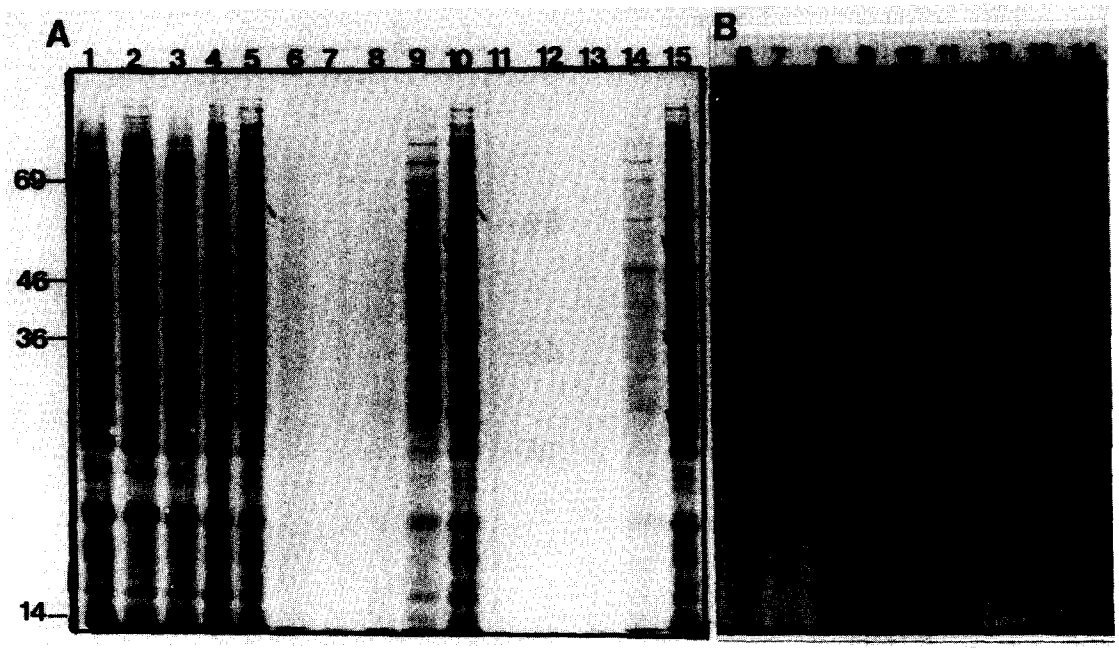

Fig. 3. Proteins synthesized during adaptation to elevated NaCl concentrations. The acid-precipitated cells obtained from the experiment in Fig. 2 were analyzed on SDS–PAGE. Gels were blotted to nitrocellulose and autoradiographed. Lane 1, proteins synthesized in 1 M NaCl. Lanes 2, 3, 4 and 5, proteins synthesized 5, 15, 30 and 60 min, respectively, following the shift to 1.5 M NaCl. Lanes 6, 7, 8, 9 and 10, proteins synthesized 5, 15, 30, 90 and 300 min, respectively, following the shift to 2 M NaCl. Lanes 11, 12, 13, 14 and 15 proteins synthesized 5, 15, 30, 90 and 300 min, respectively, following the shift to 2.5 M NaCl. In each lane equal amounts of protein (1.5 μg) were loaded with the exception of lanes 6, 7, 8, 11, 12 and 13 where 6 μg of protein were loaded in order to enable the visualization of the 64 kDa protein (panel A). The autoradiograph was exposed for longer time (5×) in order to allow better detection of the 64 kDa protein (panel B). Arrows indicate the positions of the induced proteins. Arrowheads denote the position of the 64 kDa protein. Molecular weights (kDa) of standard proteins are indicated on the left.

during adaptation to increasing salt concentration, it was of interest to determine whether a shift-down in salinity elicited a comparable response. To test this, *D. halophila* cells grown in 1 M or 2.5 M NaCl were shifted to 0.5 M or 1 M NaCl, respectively, and at the indicated time intervals aliquots of cells were pulse-labelled. The data in Figs. 4 and 5 depict the alterations of [^{35}S]methionine-labelled polypeptides detectable during such shifts. During adaptation from 2.5 M to 1 M NaCl (Fig. 5) several proteins (denoted by the arrows) were induced, while others were repressed (denoted by arrowhead). The alterations in the protein patterns during the shift from 2.5 M to 1 NaCl (Fig. 5) are more pronounced compared to those seen during the shift from 1 M to 0.5 M NaCl (Fig. 4). In the latter, some minor proteins (indicated by the arrows) appear to be induced.

5. DISCUSSION

Although considerable effort has been concentrated on the investigation of cellular phospholipid metabolism during adaptation to increasing NaCl concentrations or the fatty acid and phospholipid composition at the adapted state (i.e. steady state growth under high salt conditions) of moderately halophilic [17–19] and halotolerant [20] eubacteria, little is known about the genes involved in haloadaptation. Our studies demonstrate that adaptation of *D. halophila* cells to sudden changes

of the external salinity activated the induction of several proteins. Analysis of the protein patterns of *E. coli* by gel electrophoresis showed only a few discernible changes in response to elevated medium osmolarity [4,8].

It has been proposed that the adaptation process of moderately halophilic bacteria to elevated salinity can be distinguished in three phases [3]. The data shown in Figs. 2 and 3 are consistent with this hypothesis. The initial response (phase I) is characterized by an inhibition and/or slow down of most cellular functions (i.e. transport of amino-acids, protein synthesis, RNA synthesis, etc.). This is followed by phase II which encompasses the process of adjustment and adaptation. In this phase, rates of protein synthesis are gradually increased to levels comparable to those seen before the salt shift-up (Fig. 2). In addition several inducible proteins become visible (Fig. 3, lanes 5, 10 and 15). The extent of the inhibition as well as

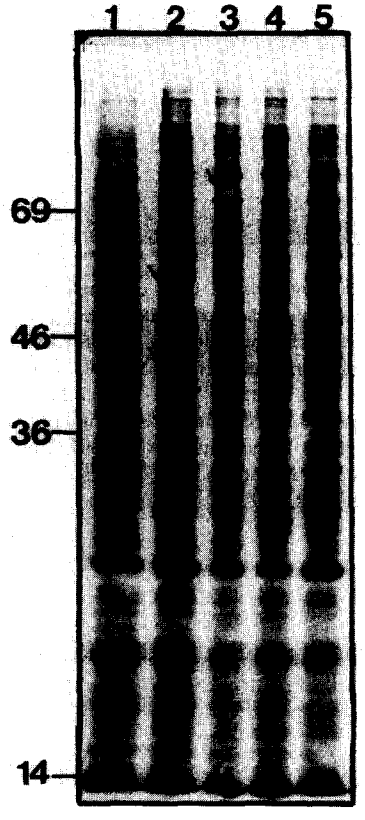

Fig. 4. Proteins synthesized during adaptation from 1 M to 0.5 M NaCl. *D. halophila* cells grown in media containing 1 M NaCl were shifted-down to 0.5 M, as detailed in MATERIALS AND METHODS. Lane 1, proteins synthesized in 1 M NaCl. Aliquots of cells sampled 5 min (lane 2), 15 min (lane 3), 30 min (lane 4) and 60 min (lane 5) after the salt shift, were taken and labelled. The acid-precipitated cells were analyzed on SDS–PAGE. An equal number of TCA-precipitable counts was applied in each lane. Gels were blotted to nitrocellulose and autoradiographed. Arrows indicate the position of induced proteins. Molecular weights (kDa) of standard proteins are indicated on the left.

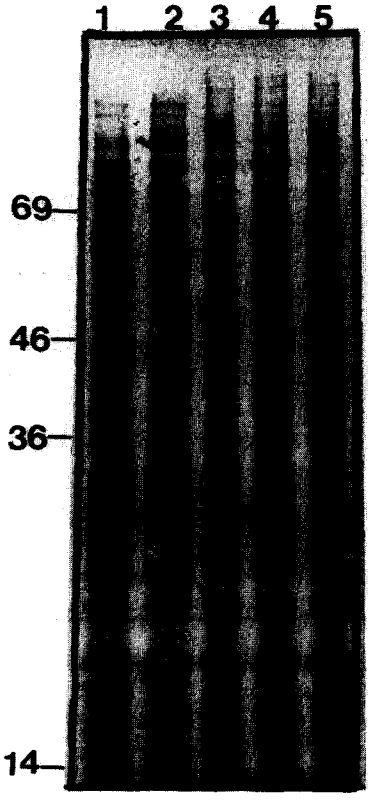

Fig. 5. Proteins synthesized during adaptation from 2.5 M to 1 M NaCl. *D. halophila* cells grown in media containing 2.5 M NaCl were shifted-down to 1 M, as detailed in MATERIALS AND METHODS. Lane 1, proteins synthesized in 2.5 M NaCl. Aliquots of cells sampled 5 min (lane 2), 15 min (lane 3), 30 min (lane 4) and 60 min (lane 5) after the salt shift, were taken and labelled. The acid-precipitated cells were analyzed on SDS–PAGE. An equal number of TCA-precipitable counts was applied in each lane. Gels were blotted to nitrocellulose and autoradiographed. Arrows indicate the position of induced proteins. Arrowheads denote the position of repressed proteins. Molecular weights (kDa) of standard proteins are indicated on the left.

the time taken before protein synthesis resumed depends on the magnitude of the salt shift-up (Fig. 2). One noteworthy aspect of these studies is that during the initial stages of adaptation to elevated NaCl concentrations (2 or 2.5 M), the cell is brought to a stage of starvation (uptake of radiolabelled compounds is inhibited) for a definite period at which the synthesis of a major 64 kDa protein and some other minor polypeptides are detectable (Fig. 3, panel B, lanes 6–8 and 11–13). The selective synthesis of the 64 kDa protein implies its possible involvement in the protective mechanisms, which enable the cell to adapt either by extruding the deleterious excessive concentrations of K^+ or other counterions, or by restoring the turgor pressure through the production of compatible solutes. A basal level of continued protein synthesis appears to play a significant role in the survival of mesophilic [22] and marine bacteria [23] under starvation conditions. In addition to the 64 kDa protein, a group of low molecular weight polypeptides (3–5 kDa) was detected after the shift to high salt (gels with high concentrations (20–25%) of polyacrylamide were used). Whether these polypeptides represent normal protein synthesis or products of premature termination of translation is not known (data not shown). However, their synthesis may account for the low but detectable levels of TCA-precipitable material (Fig. 2). Phase III is characterized by exponential growth at a rate which is determined by the final salt concentration. However, the most striking aspect of phase III is that most of the proteins which appear to be overproduced during the haloadaptation process are not detected when cells are growing at steady state in 2.5 M NaCl (Fig. 3, lane 1 and Fig. 4, lane 1 vs Fig. 5, lane 1). Comparison of the labelled protein patterns of cells grown in 1 M or 2.5 M NaCl resemble each other but they are not identical (Figs. 3 and 5).

The data shown in Fig. 5 indicate that during adaptation from 2.5 M to 1 M NaCl several proteins appear to be induced or repressed. Interestingly, these 'salt-responsive' proteins show striking differences in their relative rate of synthesis, in terms of speed, duration and levels to which they are synthesized suggesting that independent control of at least some of these genes is probable.

From our data (Figs. 3, 4 and 5) it is apparent that in response to changes in external salinity several pulse-labelled polypeptides are induced and/or repressed. Employment of two-dimensional gel electrophoresis may allow better resolution of these proteins. Whether these changes in protein profiles are due to changes at the transcriptional and/or translational level is not clear, since alterations in the functional stability of several mRNAs during adaptation to changes in salt concentration complicates the interpretation of the data. Recent studies in mesophilic bacteria have demonstrated that environmental conditions or the growth rate determine the stability of some specific mRNAs [24,25].

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